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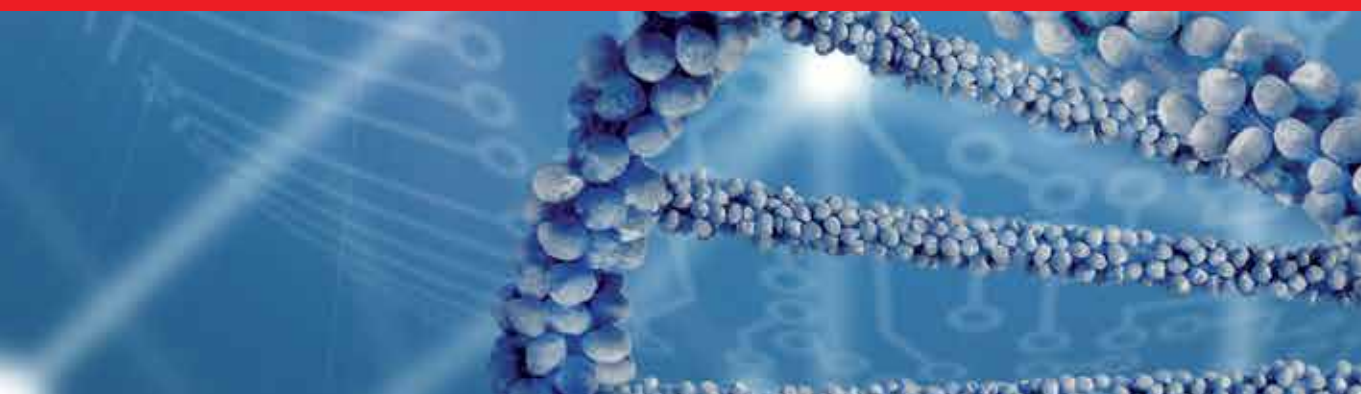
DNA Repair

On the Pathways to Fixing DNA Damage and Errors

Edited by Francesca Storici



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Contributors

M. Cristina Vega, Francisco J. Fernandez, Miguel Lopez-Estepa, Yeon-Soo Seo, Tuan Anh Nguyen, Chul-Hwan Lee, Shisheng Li, Danielle Tatum, Christina Emmanouil, Hiroshi Iwasaki, Yasuhiro Tsutsui, Akihito Kawasaki, Maria Salas, Modesto Redrejo Rodriguez, Javier Rodriguez, Maria Rosaria Faraone Mennella, Sarah Martin, Ghislaine Henneke, Rosane Lestini, Christophe Creze, Didier Flament, Hannu Myllykallio, Christine Blattner, Volker Middel, Denis S.F. Biard, Kenji Fukui, Atsuhiro Shimada, Hitoshi Iino, Ryoji Masui, Seiki Kuramitsu, Maria Zannis-Hadjopoulos, Emmanouil Rampakakis, Mikhail Alexeyev, Inna Shokolenko, Glenn Wilson, Susan LeDoux, Christine Falaschetti, Tatjana Paunesku, Gayle Woloschak, Emily Mirkin, Sumita Raha, Jack Tuszynski, Khaled Hasaan Barakat, George Iliakis

Notice

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Meet the editor

Francesca Storici was born in Triestre (Italy) in 1968. She received a Biology degree in 1993 from the University of Trieste and a Ph.D. in Molecular Genetics from the International School of Advanced Studies (ISAS), working at the International Center for Genetic Engineering and Biotechnology of Trieste in 1998. From 1999 to 2007, she was a Visiting and then a Research Fellow at the National Institute of Environmental and Health Sciences (NIEHS, NIH) in North Carolina (USA). In 2007, she was a Research Assistant Professor at the Gene Therapy Center of the University of North Carolina at Chapel Hill. In August 2007 she joined the faculty at the School of Biology of the Georgia Institute of Technology in Atlanta, Georgia as Assistant Professor and soon after she received the title of Distinguished Cancer Scholar from the Georgia Cancer Coalition. Her research is on genome stability, DNA repair and gene targeting.

Contents

Preface

DNA repair is a central component of DNA transactions. Every day living cells battle to offset DNA damage and errors that lead to aging and could cause cancer or other genetic diseases. DNA repair is an important mechanism of defense against the potential dangers for the integrity of genes and genomes, damage to which derives from environmental genotoxic stress, like chemicals, tobacco smoke and radiation or simply from endogenous sources. There could be mistakes in DNA synthesis or the threat from reactive oxygen species that are produced by normal cellular metabolism. The genetic information is thus always at risk to change and mutate, due to the occurrence of continuous errors and distortions.

DNA can be damaged in many different ways. Defects could arise because a wrong nucleotide is introduced, or because nucleotides are modified, or because the DNA has been broken or degraded. How are defects in DNA identified, how do cells recognize the different types of damage, how is the wrong information discarded and how does repair occur? I have been in the field of DNA repair since the beginning of my postdoctoral research work and I have had the opportunity to see how much this field has grown in the last decade. Although we certainly have gained important understanding on the numerous mechanisms of DNA repair, still a lot is unknown. The area is vast, there is much more to discover. We can imagine the DNA repair system to be like a particular cosmos. What is its limit, what its potential? How is DNA repair coordinated? Is it perfect or are there flaws, can it be manipulated?

When asked to edit a book on DNA repair, I thought this could be an exciting chance to further navigate into the 'DNA repair cosmos'. This book was not intended to provide the whole picture (besides impossible task) of DNA repair. Rather, this book was conceived to be like a voyager vessel and its goals are:

I) To cover aspects of DNA repair processes that target nucleotide, base or sugar modifications in DNA, starting with basic principles of DNA replication, recombination and the cell cycle to the multiplicity of DNA repair mechanisms and their biological importance, emphasizing recent major advances and future directions in this rapidly expanding field.

II) To convey the fact that DNA repair is not merely a system in which a few factors detect and correct damage in DNA, but instead a complex of dynamic, intricate and interconnected mechanisms.

III) Furthermore, to stimulate the readers to read more and especially to explore more into the fascinating field of DNA repair.

Putting together the chapters of this book has been a great pleasure and exciting experience. The credit belongs to all the authors of the chapters, who took their effort in sharing their knowledge, expertise and ideas for this book. I wish to express my gratitude to Prof. Ana Nikolic, InTech Editorial Team Manager, who first contacted me to initiate this project under the working title DNA Repair. I would like to thank very much Ms. Alenka Urbancic, InTech Publishing Process Manager, for her constant assistance and support during all phases of preparation of this book. I am also indebted to InTech and its staff for the accomplishment of this book project.

Francesca Storici
School of Biology
Georgia Institute of Technology
Atlanta, GA
USA

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Lagging Strand Synthesis and Genomic Stability

Tuan Anh Nguyen, Chul-Hwan Lee and Yeon-Soo Seo
Korea Advanced Institute of Science and Technology, South Korea

1. Introduction

In eukaryotic cells, DNA replication starts at many origins in each chromosome during S phase of cell cycle. Each origin is activated at different time points in S phase, which takes place once and only once per cell cycle. In yeast and most likely higher eukaryotes, the origin-recognition complex (ORC) and several other initiation factors play a pivotal role in activation and regulation of replication origins. Briefly, the ORC-bound origins are sequentially activated and deactivated along the progression of cell cycle. The prereplicative complex (pre-RC) is formed by loading the replicative helicase MCM complex onto the ORC-bound origins with the aid of Cdc6 and Cdt1. This complex is activated by S-phase cyclin dependent kinases (Cdks) when cells enter S phase. The elevated levels of Cdk activities lead to removal of some initiation proteins such as Cdc6 by proteolysis, allowing the pre-RC to be further activated for subsequent DNA synthesis. The irreversible removal of initiation factors is a major mechanism to ensure DNA to be replicated once and only once per cell cycle. The assembly of replication initiation complex and its activation are well reviewed in many literatures (Sclafani & Holzen, 2007; Remus & Diffley, 2009; Araki, 2010). Activation of origins leads to the establishment of bidirectional replication forks for the DNA synthesis of leading and lagging strands.

2. Overview of lagging strand synthesis

Leading strand synthesis, once initiated, occurs in a highly processive and continuous manner by a proofreading DNA polymerase. Unlike leading strands, lagging strands are synthesized as discrete short DNA fragments, termed 'Okazaki fragments' which are later joined to form continuous duplex DNA. Synthesis of an Okazaki fragment begins with a primer RNA-DNA made by polymerase (Pol) α-primase. The synthesis of RNA portion (~ 10 to 15 ribonucleotides) and subsequent extension of short (~20 to 30 nucleotides, nt) DNA are coupled. The recognition of a primer RNA-DNA by the Replication-Factor C (RFC) complex leads to dissociation of Pol α-primase and loading of proliferating cell nuclear antigen (PCNA), resulting in recruitment of Pol δ to the primer-template junction, a process called 'polymerase switching.' Then the primer RNA-DNA is elongated by Pol δ. When Pol δ encounters a downstream Okazaki fragment, it displaces the 5′ end region of the Okazaki fragment, generating a single-stranded (ss) nucleic acid flap. The flaps formed can be efficiently processed by the combined action of Flap endonuclease 1 (Fen1) and Dna2 to

eventually create nicks. The nicks are finally sealed by DNA ligase 1 to complete Okazaki fragment processing. The current model is summarized in Fig. 1.

3. Potential risks associated with lagging strand synthesis in eukaryotes

Lagging strand maturation appears to be intrinsically at high risks of suffering DNA alterations for several reasons. First, a substantial part (up to 20%) of short Okazaki fragments (~150-nt in average) is synthesized by Pol α which does not contain a proofreading function (Conaway and Lehman, 1982; Bullock et al., 1991). Thus, the high-incidence errors in Okazaki fragments, if not effectively removed, could become a source of genome instability. Second, the *modus operandi* of Okazaki fragment processing could put eukaryotic chromosomes at risks of DNA alteration. It involves the formation and subsequent removal of a flap structure (Bae & Seo, 2000; Bae et al., 2001a); flaps could be a source of a potential risk because they can take a variety of structures according to their sizes and sequences. Third, since the size of Okazaki fragments is very small, cells require a great number (for example, 2 x 10^7 in humans) of Okazaki fragments to be synthesized, processed, and ligated per cell cycle. This bewilderingly great number of events would make infallible processing of all Okazaki fragments dependent on multiple back-up or redundant pathways. Forth, lagging strand synthesis is mechanistically more complicated than leading strand synthesis, implying that the sophisticated machinery for this process may come across accidents in many different ways. Therefore, failsafe synthesis of lagging strand is highly challenging by virtue of the complex multi-step process and the sophisticated machinery for Okazaki fragment processing.

4. 'Core' factors for synthesis and maturation of lagging strands

The protein factors required for synthesis of lagging strands include Pol α-primase, Pol δ, PCNA, RFC, RPA, Fen1 (5′ to 3′ exonuclease or MF1, maturation factor 1), RNase H, and DNA ligase 1. In essence, a combined action of these factors was sufficient and necessary for completion of lagging strand synthesis in vitro in simian virus 40 DNA replication (Ishimi et al., 1988; Waga & Stillman, 1994). Among them, the two nucleases Fen1 and RNase H were shown to have roles in the removal of primer RNA of Okazaki fragments. In yeasts, however, the deletion of genes encoding Fen1 (RAD27) or RNase H (RNH35) was not lethal, indicating the presence of redundant pathways in eukaryotes (Tishkoff et al., 1997a; Qui et al, 1999). In addition, Dna2, which was originally reported as a helicase (Budd & Campbell 1995; Budd et al., 1995), was shown to play a critical role in the processing of Okazaki fragments using its endonuclease activity (Bae et al., 1998; Bae & Seo, 2000; Bae et al., 2001a; MacNeill, 2001; Kang et al., 2010). Displacement DNA synthesis by Pol δ generates flap structures, which can be substrates for Dna2 and Fen1 endonuclease activities (Bae & Seo, 2000). For the convenience sake, all enzymes (Pol δ, PCNA, RFC, RPA, Fen1, RNase H, Dna2, and DNA ligase 1) described early from yeast and human studies are referred to as 'core' factors for synthesis of lagging strands in this chapter. We refer to all the others as 'auxiliary' factors which may not be needed normally, but become critical under specific circumstances (Fig. 1 and see also Fig. 3). These factors have been screened for their abilities to suppress the crippled function of Dna2 or Fen1. It is believed that (i) the 'auxiliary' factors come to assist the 'core' machinery that does not function appropriately, (ii) they provide additional enzymatic activities to resolve hairpin or higher-ordered structures in flaps, or

(iii) they are needed to resolve toxic recombination intermediates arising during lagging strand metabolism. Thus, it is the multiplicity of 'auxiliary' factors that allows the 'core' machinery to be fine-tuned in response to diverse situations with regard to Okazaki fragment processing.

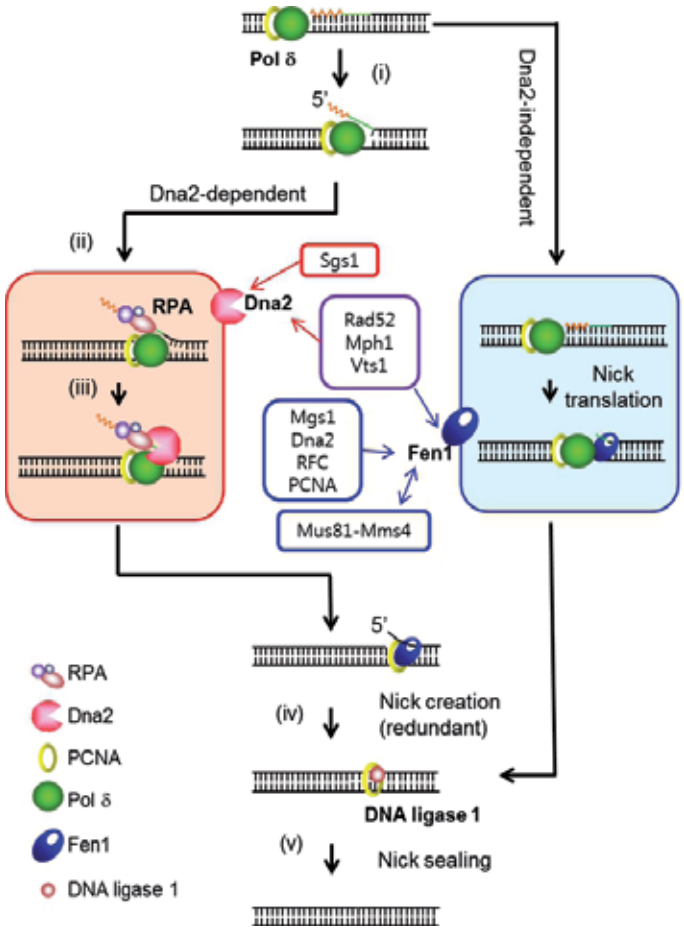

Fig. 1. A current model for processing of Okazaki fragments in eukaryotes. Dna2-dependent pathway includes: (i) The 5' terminus of an Okazaki fragment containing the primer RNA-DNA is rendered single-stranded by displacement DNA synthesis catalyzed by Pol δ. (ii) RPA rapidly forms an initial complex with the nascent flap structure and (iii) then recruits Dna2 to form a ternary complex. This leads to the initial cleavage of RNA-containing segments by Dna2, (iv) leaving a short flap DNA that can be further processed either by Fen1 (Fen1-dependent) or by other nucleases, possibly Exo1 or 3' exonuclease of Pol δ (Fen1-independent) (not shown; see the text for details). (v) Finally, the resulting nick is sealed by DNA ligase 1. Short flaps can be processed directly by Fen1 (Dna2-independent pathway) that involves the 'idling' (not shown) or 'nick translation' (see the text for details). Nicks generated by this mechanism are directly channelled into the nick sealing step. 'Auxiliary' factors that stimulate Dna2 or Fen1 or both are boxed and their targets are indicated by arrowheads. A double arrowhead indicates mutual stimulation.

4.1 Multiple pathways in parallel with Fen1

Fen1 is a major, but not the only enzyme that can create ligatable nicks directly from flap structures (Harrington & Lieber, 1994; Murante et al., 1995; Liu et al., 2004; Garg & Burgers 2005). In vivo studies demonstrated that double-strand break(DSB)-induced DNA repair, which requires replication of both leading and lagging strands, still occurred 50% in Fen1-deficient strains compared to wild type (Holmes & Haber, 1999), indicating that the 50% of the repair events were carried out with nicks created by nuclease(s) other than Fen1. The ability of Pol δ to switch from displacement DNA synthesis to its 3′ exonuclease could constitute a pathway to create nicks; the retrograde 3′ exonucleolytic degradation of a newly elongated end, followed by annealing of the displaced flap to the lagging strand template, can be a mechanism for nick formation (Jin et al., 2001). The overexpression of Exo1 in rad27Δ restored growth of the mutant cells at the nonpermissive temperature (Tishkoff et al., 1997b). Single mutant cells with either rad27Δ or exo1Δ were viable, whereas rad27Δ exo1Δ double mutants were not (Budd et al., 2000; Tishkoff et al., 1997b). Yeast Exo1 has 5′ exonuclease activity acting on double stranded (ds) DNA and an associated 5′-flap endonuclease activity (Tran et al., 2001). In addition, yeast rad27Δ cells (lacking yeast Fen1) were not lethal, but temperature-sensitive (ts) in growth, consistent with existence of multiple pathways for nick generation in yeasts. It was shown that Pol δ has a unique ability to maintain dynamically the nick position in conjunction with Fen1, via a process called 'idling'. In addition, Pol δ cooperates with Fen1 and PCNA to carry out 'nick translation' to progressively remove primer RNA-DNAs (Garg et al., 2004). The endonuclease activity of Fen1 can keep cleaving a flap while it is being displaced by Pol δ, allowing nicks to be changed in their positions along with Pol δ movement.

4.2 Structured flaps are special types of DNA damage that could cause genome instability

Failure to create nicks by Fen1 in a timely manner could cause genome instability. The importance of Fen1 in this regard was clearly demonstrated by the dramatic increase of small (5- to 108-bp) duplications flanked by 3- to 12-bp repeats in rad27Δ mutants (Tishkoff et al., 1997a). This unusual type of duplication mutations is in keeping with the current model of Okazaki fragment processing; unprocessed flaps, rapidly accumulated in the absence of Fen1, are ligated with the 3′-end of the downstream Okazaki fragment, resulting in duplication mutations. In the absence of Fen1, many types of repeat DNA sequences in eukaryotic chromosomes are not stably maintained. These include dinucleotide, trinucleotide, micro- or mini-satellite DNA, and telomeric DNA (Johnson et al., 1995; Kokoska et al., 1998; Xie et al., 2001; Freudenreich et al., 1998; Spiro et al., 1999; White et al., 1999; Maleki et al., 2002; Lopes et al., 2002; Lopes et al., 2006). Most notably, expansion of trinucleotide repeats such as CTG/CAG or CGG/CCG has been extensively studied using yeasts as model system (Schweitzer & Livingston, 1998; Freudenreich et al., 1998; Shen et al., 2005), because of their clinical relevance to many human neurodegenerative diseases such as Fragile X Syndrome, Huntington's Disease, and Myotonic Dystrophy (Pearson et al., 2005; Kovtun & McMurray, 2008). All of the disease-causing trinucleotide repeats are able to form secondary or higher-ordered structures in solution, such as hairpins (CAG, CTG, CGG, and CCG repeats), G quartets (CGG repeats), and triplexes (GAA and CTT) (Fig. 2).

Trinucleotide repeats, once displaced by Pol δ, could reanneal to the template in a misaligned manner. If they are joined to the 3′ end of the new Okazaki fragment, followed by a subsequent round of DNA replication, the repeats could be expanded. In yeast, stability

of trinucleotide repeats is greatly affected by their orientation with respect to nearby replication origins (Freudenreich et al., 1997; Miret et al., 1998). The orientation-dependent and sequence-specific instability of trinucleotide repeats support the model that expansions of CTG and CAG tracts result from aberrant DNA replication via hairpin-containing Okazaki fragments. In addition, telomere repeats are not stably maintained in the absence of functional Fen1 in yeasts (Parenteau & Wellinger, 1999 and 2002). Although Fen1 is critical for repeat stability in yeasts, it remains unclear in mice or humans (Spiro & McMurray, 2003; Moe et al., 2008; van den Broek et al., 2006). One explanation is that unlike yeasts, mammals may have more diverse pathways to remove or prevent formation of long flaps, since instability of the trinucleotide repeats occurs through formation of long flaps. Alternatively, Fen1 is responsible for formation of most nicks in mammals because deletion of Fen1 caused embryonic lethality in mice (Kucherlapati et al., 2002). The human minisatellite DNA became unstable in rad27 or dna2 mutant cells when it was inserted into one of the yeast chromosomes (Lopes et al., 2002; Cederberg & Rannug, 2006). These data also are in keeping with the idea that improperly processed 5′ flap instigates minisatellite destabilization. DNA instability associated with secondary or higher-ordered structures in the flap indicates that structures formed during DNA metabolisms can be regarded as special forms of DNA damage that need to be immediately removed (Fig. 2). The role of Fen1 in safeguarding the genome integrity has qualified Fen1 as a tumor suppressor in mammals and its physiological importance was recently reviewed with an emphasis on studies of human mutations and mouse models (Zheng et al., 2011).

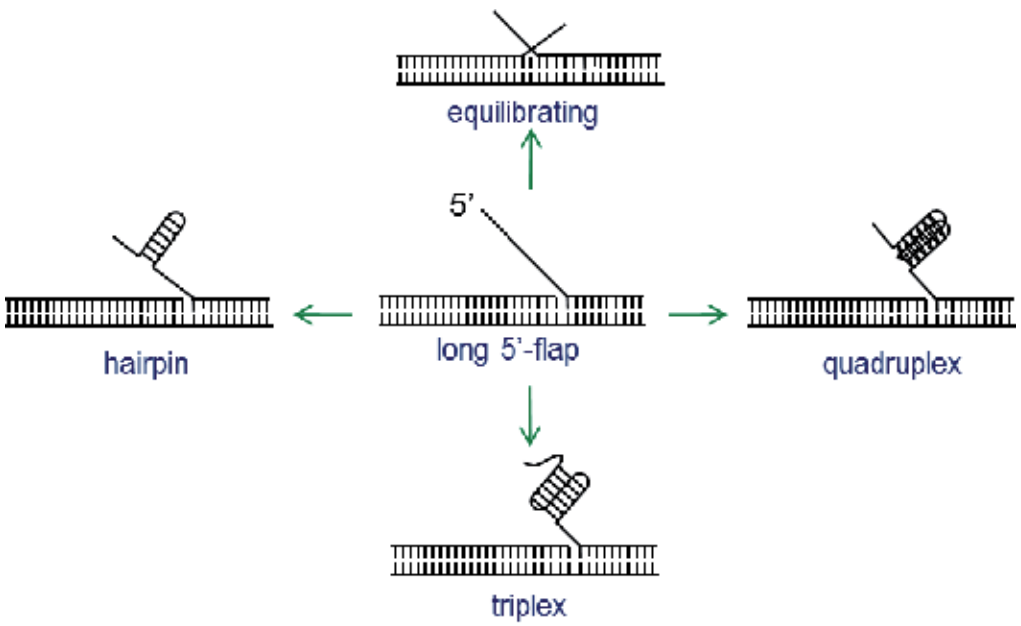

Fig. 2. A variety of structures are possible in unprocessed 5′-ssDNA flaps. If an excessively long 5′ flap is not processed in a timely manner, the flap can reanneal back to the template DNA, generating an 'equilibrating' flap which is more difficult to process by Fen1 alone. Alternatively, it could form hairpin or higher-order structures such as triplex or quadruplex according to the sequence context.

4.3 Dna2 as a preemptive means to prevent formation of long flaps

4.3.1 Long flaps are in vivo substrates preferred by Dna2

Dna2 is highly conserved throughout eukaryotes and contains at least two catalytic domains for helicase and endonuclease activities (Budd & Campbell, 1995; Budd et al., 1995; Bae et al., 1998; Bae et al., 2001b). Genetic data from fission and budding yeasts indicate that the endonuclease activity of Dna2 is essential, playing an essential role in vivo in Okazaki fragment processing (Kang et al., 2000; Lee et al., 2000; Budd et al., 2000; Kang et al., 2010). There are several lines of evidence that long flaps can be formed in vivo that need the action of Dna2. Long flaps, once formed, could impose formidable burdens to cells, most likely due to their tendency to bind proteins nonspecifically or to form hairpin or higher-ordered structure that is difficult to be processed. In this sense, any structural intermediates formed in flaps can be regarded as a special type of DNA damage. The requirement of Dna2 endonuclease and helicase activities for a complete removal of long or hairpin flaps supports the idea that the major role of Dna2 is to prevent formation of excessively long flaps by cleaving them into shorter ones as soon as they occur. The flaps shortened by Dna2 are not able to form secondary or higher-ordered structure. Thus, Dna2 functions to maintain flaps as short as possible during replication. The marked increase of unusual duplications or trinucleotide expansions in the absence of Fen1 (Tishkoff et al., 1997a) provide strong evidence that long flaps are produced in vivo. It was shown that calf thymus Pol δ was able to displace downstream duplex DNA longer than 200 bps in vitro, revealing its intrinsic ability to form extensive flaps (Podust & Hubscher, 1993; Podust et al., 1995; Maga et al., 2001). In vitro reconstitution experiments using yeast enzymes showed that a portion of flaps grows long up to 20- to 30-nt, although flaps formed in vitro are primarily short, up to 8-nt in length (Rossi & Bambara, 2006). The frequency of long flaps can be affected by sequence in the lagging strand template or by interactions of Pol δ/Dna2 with other proteins. For example, Pol δ lacking PCNA-interaction tends to preferentially generate short flaps (Jin et al., 2003; Garg et al., 2004; Tanaka et al., 2004). In contrast, Pif1 helps to create long flaps through its helicase activity in vitro (Rossi et al., 2008) and in vivo (Ryu et al., 2004). Several other elaborate genetic experiments are in keeping with involvement of Dna2 in the cleavage of long flaps. First, dna2-1 was lethal in combination with a mutation in Pol δ (pol3-01) which increased strand displacement synthesis. Meanwhile, deletions of Pol32 subunit, which reduces strand displacement activity of Pol δ in vitro, suppressed the growth defects of dna2-1 and dna2-2 (Burgers & Gerik, 1998; Garg et al., 2004; Johansson et al., 2004). Similar results were also obtained in S. pombe (Reynolds et al., 2000; Zuo et al., 2000; Tanaka et al., 2004). The observation that overexpression of RPA alleviates the requirement of Dna2 helicase activity (Bae et al., 2002) is also consistent with formation of long flaps in vivo. In order for dsDNA-destabilizing activity of RPA to substitute for the helicase activity of Dna2, flaps should be at least long enough to form hairpin structure.

4.3.2 RPA acts as a molecular switch between Dna2 and Fen1

Several independent observations indicate that RPA plays a critical role in Okazaki fragment processing in conjunction with Dna2; (i) a mutation in DNA2 was identified during a synthetic lethal screen with rfa1Y29H, a ts mutant allele of RFA1. Furthermore, Dna2 and Rpa1 (a large subunit of RPA encoded by RFA1) physically interacted with each other both in vivo and in vitro (Bae et al., 2003). (ii) The 32 kDa subunit of RPA was crosslinked to primer RNA–DNA in the lagging strand of replicating SV40 chromosomes (Mass et al., 1998). (iii) The genetic interaction between RPA and Dna2 was discovered from

screening of suppressors that rescued ts growth defects of dna2Δ405N mutant when expressed in a multicopy plasmid (Bae et al., 2001a). The fact that RPA binds most efficiently ssDNA longer than 20-nt and interacts genetically with Dna2 is consistent with the idea that the in vivo substrates of Dna2 are long ssDNA flaps. In vitro, RPA markedly stimulated Dna2-catalyzed cleavage of 5′ flap at physiological salt concentration (Bae et al., 2001a), which was further confirmed by others (Ayyagari et al., 2003; Kao et al., 2004). However, RPA inhibited Fen1-catalyzed cleavage of 5′ flaps. This inhibition was readily relieved by the addition of Dna2 (Bae et al., 2001a). Thus, a 5′ flap longer than 20-nt first binds RPA, and then rapidly recruits Dna2 to form a ternary complex. Dna2-catalyzed cleavage of the flap releases free RPA-bound ssDNA and a shortened flap (mostly 6-nt). The short flap produced is no longer resistant to and can be completely removed by Fen1 to produce ligatable nicks. Therefore, RPA acts as a molecular switch between Dna2 and Fen1 for the sequential action in cleavage of long flaps, Dna2 followed by Fen1, of the two endonucleases (Bae et al., 2001a).

4.3.3 A concerted action of helicase and endonuclease activities for removal of hairpin flaps

The presence of both endonuclease and helicase activities in one polypeptide of Dna2 implies that both activities act in a collaborative manner. The lethality of dna2 mutation lacking helicase activity (Budd et al., 1995) suggests that DNA unwinding activity is critical for its physiological function in vivo. The addition of ATP not only activates helicase activity, but also alters the cleavage pattern of flap DNA by Dna2. The average size of cleaved flaps is expanded in the presence of ATP (Bae et al., 2002). Furthermore, the addition of ATP allowed wild type Dna2, not helicase-negative Dna2K1080E mutant, to cleave secondary-structured flap via its combined action of helicase and nuclease activities (Bae et al., 2002). The mixture of helicase-negative Dna2K1080E and nuclease-negative Dna2D657A mutant enzymes failed to recover wild type action on these structured flaps. Therefore, it is critical essential that these two essential activities should be concerted. In keeping with this, simultaneous expression of both mutant proteins in dna2Δ cell did not allow cells to grow. Dna2 is also capable of unwinding G-quadruplex DNA structures, suggesting another critical role of Dna2 helicase in resolving the structural intermediates arising during DNA metabolisms (Masuda-Sasa et al., 2008). It was also shown that concerted action of exonuclease and gap-dependent endonuclease activities of Fen1 could contribute to the resolution of trinucleotide-derived secondary structures formed during maturation of Okazaki fragments (Singh et al., 2007).

4.3.4 Dna2 as an alternative means to remove mismatches

Since the Pol α-synthesized DNA in Okazaki fragments is highly mutagenic, eukaryotic cells need to eliminate this mutagenic DNA to prevent accumulation of errors. Recently, it was shown that in yeast Pol α incorporates ribonucleotides more frequently than Pol δ or Pol ε (Nick McElhinny et al., 2010b). The unrepaired ribonucleotides in DNA could inflict a potential problem on DNA replication because Pol ε has difficulty bypassing a single ribonucleotide present within a DNA template in yeasts. This again emphasizes that processing of Okazaki fragments is associated with high risks of DNA alterations. It has been puzzling that eukaryotic cells maintain a low mutation rate, despite the fact that a substantial portion (~10%) of total DNA is synthesized by Pol α, a flawed DNA polymerase. To account for this enigma, it was proposed that in mammals Pol α is associated with a 3′

exonuclease that may confer a proofreading function on Pol α (Bialec and Grosse, 1993). In yeasts, an intermolecular proofreading mechanism was proposed in which Pol δ could play a role in proofreading errors made by Pol α during initiation of Okazaki fragments (Pavlov et al., 2006). Mismatch repair (MMR) can correct mismatches in the Pol α-synthesized DNA (Modrich & Lahue 1996; Kolodner & Marsischky,1999; Kunkel & Erie, 2005). One unsolved fundamental problem in eukaryotic MMR, however, is the strand discrimination signal, although a strand-specific nick is generally believed to be the signal (Holmes et al., 1990; Thomas et al., 1991; Modrich, 1997). Equally possible is that the presence of flaps, which may be as abundant as nicks in lagging strand, could act as the strand discrimination signal. At any rate, the accuracy of MMR would depend on the rate at which nicks or flaps (the strand discrimination signals) are being removed. Thus, MMR could be unreliable if MMR is kinetically slower than sealing nicks. The ability of Dna2 to efficiently remove the RPA-bound flap containing the whole RNA-DNA primer could offer an alternative mechanism to remove mismatches present in the primer DNA of Okazaki fragments.

5. Multi-factorial interplays as a means to ensure high-fidelity replication of lagging strand

If one of the 'core' factors is crippled, a redundant factor(s) that works in parallel can reveal itself. In our laboratory, we have focused on isolating genetic suppressors that can rescue dna2 mutations in order to identify redundant pathways for Okazaki fragment processing. Most suppressors isolated turned out to have roles in maintenance of genome integrity, in keeping with the notion that faulty processing of Okazaki fragment could lead to genome instability. The in vivo and in vitro interactions of the suppressors with Dna2 or Fen1 suggest that Okazaki fragment processing is a converging place for DNA replication, repair, and recombination proteins to ensure removal of flaps in an accurate and timely manner in eukaryotes.

5.1 RNase H2 as an enzyme to clean up ribonucleotides in lagging strands

Both type I and type II RNase H play a role in the removal of ribonucleotides present in duplex DNA (Ohtani et al., 1999; Cerritelli & Crouch, 2009). The S. cerevisiae RNase H2 enzyme is active as a heterotrimeric complex that consists of Rnh201, Rnh202, and Rnh203, which are encoded by RNH201 (formerly known as RNH35), RNH202, and RNH203, respectively (Jeong et al., 2004). Expression analyses and other results suggest that RNase H2 plays roles in DNA replication and/or repair (Frank et al., 1998; Qiu et al., 1999; Arudchandran et al., 2000). Since rnh201Δ and rnh202Δ displayed synthetic lethal interactions with dna2-1 and rad27Δ, yeast RNase H2 has been implicated in Okazaki fragment processing (Budd et al., 2005). The unique ability of eukaryotic RNase H2 (type II) to cleave the 5′ side of a single ribonucleotide embedded within duplex DNA suggests an additional role, that is, the removal of ribonucleotides misincorporated into DNA (Rydberg & Game, 2002). The catalytic activity of RNase H2 was critical for a pathway requiring the function of RAD27 since all rnh201 mutant alleles failed to complement the growth defect of rad27Δrnh201Δ. Moreover, the addition of 20 mM hydroxyurea to growth media rescued the ts phenotype of dna2Δ405N, but failed to suppress the double mutants, dna2Δ405N rnh201Δ, dna2Δ405N rnh202Δ and dna2Δ405N rnh203Δ (Nguyen et al., 2011). Thus, the suppression of dna2 mutation also depends on a functional RNase H2, suggesting that RNase H2 plays a critical role in the removal of primer RNAs if cells have impaired Dna2.

An alternative explanation, which is not mutually exclusive from the above possibility, is that the addition of 20 mM HU might have led to a decreased ratio of deoxyribonucleotides to ribonucleotides, causing a dramatic increase in ribonucleotide incorporation. This might render cells more dependent on the clean-up function of RNase H2 to remove misincorporated ribonucleotides present in newly synthesized DNA strands by replicative polymerases (Nick McElhinny et al., 2010a). The fact that Pol α misincorporates ribonucleotides more frequently than Pol δ or Pol ε is consistent with a more critical role of RNase H2 in lagging strand synthesis than in leading strand (Nick McElhinny et al., 2010b). It was shown that in humans, Rnh202-PCNA interaction is important to recruit RNase H2 to replication foci (Bubeck et al., 2011). Since the biochemical activity of RNase H2 is dedicated to the removal of ribonucleotide incorporated into DNA, the interaction between PCNA and RNase H2 may function to recruit RNase H2 to lagging strands for Okazaki fragment processing. It was also shown that elevated levels of misincorporated ribonucleotides during DNA replication cause genomic instability (Nick McElhinny et al., 2010a). Mutations in the human homologs of the three yeast RNase H2 subunits are related to the development of Aicardi-Goutieres syndrome (Crow et al., 2006).

5.2 Many stimulators of Dna2 and Fen1 to prevent formation of structural intermediates

5.2.1 Mgs1

MGS1 (Maintenance of Genome Stability 1) of S. cerevisiae was found to act as a multicopy suppressor of the ts growth defect of dna2Δ405N mutation (Kim et al., 2005). Mgs1 stimulated the structure-specific nuclease activity of yeast Fen1 in an ATP-dependent manner. ATP binding but not hydrolysis was sufficient for the stimulatory effect of Mgs1. Suppression of dna2Δ405N required the presence of a functional copy of RAD27. MGS1 is a highly conserved enzyme containing both DNA-dependent ATPase and DNA annealing activities, playing a role in post-replicational repair processes (Hishida et al., 2001 and 2002).

5.2.2 Vts1

VTS1 (*vt*i1–2 *s*uppressor) of S. cerevisiae was originally identified as a multicopy (and lowcopy) suppressor of vti1-2 mutant cells that displayed defects in growth and vacuole transport (Dilcher et al., 2001). The Vts1 protein is also highly conserved in eukaryotes and encodes a sequence- and structure-specific RNA binding protein that has a role in posttranscriptional regulation of a specific set of mRNAs with cognate binding sites at their 3′-untranslated region (Aviv et al., 2003). VTS1 was identified as a multi-copy suppressor of helicase-negative dna2K1080E. The suppression was allele-specific since overexpression of Vts1 did not suppress the ts growth defects of dna2Δ405N (Lee et al., 2010). Purified recombinant Vts1 stimulated the endonuclease activity of wild type Dna2, but not of Dna2Δ405N devoid of the N-terminal domain, indicating that the activation requires the N-terminal domain of Dna2. Stimulation of Dna2 endonuclease activity by Vts1 appeared to be the direct cause of suppression, although it also stimulated Fen1 activity.

5.2.3 PCNA and RFC

RFC and PCNA are processivity factors for Pol δ and Pol ε. RFC, a clamp loader of PCNA, consists of five subunits (Rfc1 to 5) which share significant homology in seven regions referred to as RFC boxes (box II-VIII) (Cullman et al., 1995; Majka & Burgers, 2004). Although PCNA has been well known for its ability to stimulate Fen1 (Li et al., 1995; Tom et

al., 2000; Frank et al., 2001; Gary et al., 1999; Gomes & Burgers, 2000), human RFC complex was recently found to markedly stimulate Fen1 activity via multiple stimulatory motifs per molecule (Cho et al., 2009). Fen1 stimulation by RFC is a separable function from ATP-dependent PCNA loading to primer ends. Analysis of stimulatory domain of RFC4 revealed that only a small part ($RFC4_{170\text{-}194}$; subscripts indicate positions of amino acids) of it was sufficient to stimulate Fen1 activity and among them, the four amino acid residues were critical for Fen1 stimulation (Cho et al., 2009). The multiple stimulatory motifs present in the RFC complex could contribute to more rapid formation of ligatable nicks as an integral part of replication machinery while it moves along with replication forks (Masuda et al., 2007).

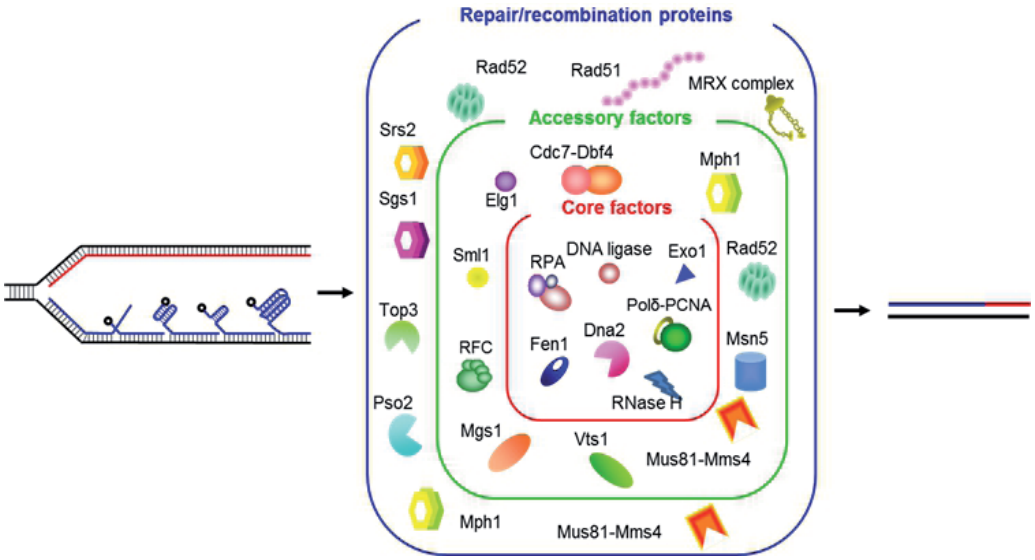

Fig. 3. Multiple layers of redundant pathways for failsafe processing of Okazaki fragments. Various flap structures, exemplified by four types only, can be generated during lagging strand synthesis. In most cases, it is believed that they can be processed by the combined action of 'core' factors in the first layer (indicated in the red box), the basic machinery for Okazaki fragment synthesis. 'Accessory factors' that constitute the second layer (indicated in the green box) function mostly to strengthen enzymatic activities of Dna2 and/or Fen1. When the 'core' proteins fail to function, unprocessed flaps can be removed by proteins in the third layer (indicated in the blue box) that contains factors for DNA repair and recombination (see text for details). Msn5 or Sml1 may not be directly related to Dna2 or Fen1 and thus need to be tested in this regard. Note that some proteins can belong to more than one layer. Pol α-primase is not shown for simplicity.

5.2.4 Mus81-Mms4

Mus81-Mms4 is a structure-specific endonuclease that can cleave nicked Holliday junctions, D-loop, replication forks, and 3'-flaps that could arise in vivo during the repair of damaged replication forks (Boddy et al., 2001; Kaliraman et al., 2001; Bastin-Shanower et al., 2003; Ciccia et al., 2003; Whitby et al., 2003). Overexpression of Mus81 suppressed the lethality of helicase-negative dna2K1080E (Kang et al., 2010) as well as dna2-2 and dna2-4, the two other dna2 mutant alleles isolated by others (Formosa & Nittis, 1999). In addition, Mus81-Mms4

and Fen1 stimulated each other in a manner requiring a specific protein-protein interaction. This indicates that the three endonucleases, Rad27, Mus81-Mms4, and Dna2, collaborate to remove a variety of structural intermediates in vivo.

5.2.5 Mph1 and Rad52

MPH1 was first identified as a mutator phenotype 1 gene (Entian et al., 1999), and the mph1Δ mutant displayed increased mutation rates and sensitivity to a variety of DNA damaging agents (Scheller et al., 2000). Based on this and other genetic studies, MPH1 is proposed to function in an error-free DNA damage bypass pathway that requires homologous recombination (Schürer et al., 2004). It was shown that Mph1 has DNA-dependent ATPase and 3′ to 5′ helicase activities (Prakash et al., 2005). Overexpression of Mph1 increased gross chromosomal rearrangements (GCR) by partially inhibiting homologous recombination through its interaction with RPA (Banerjee et al., 2008). These data suggest that Mph1 is important in maintaining the integrity of genome. MPH1 was isolated as a multicopy suppressor of dna2Δ405N and dna2K1080E. Purified Mph1 markedly stimulated the endonuclease activities of both Dna2 and Fen1 in vitro in an ATP-independent manner (Kang et al., 2009). Stimulation depends on the specific protein-protein interaction between the N-terminal domain of Dna2 and Mph1. Since overexpression of Mph1 also suppressed the dna2Δ405N mutant, the suppression of the Dna2 defect by Mph1 is due to the stimulation of Fen1 activity, and not of Dna2. Rad52 that mediates exchanging RPA with Rad51 in ssDNA is a multi-copy suppressor of dna2K1080E. Purified Rad52 is able to stimulate both Fen1 and Dna2 in vitro (Lee et al., 2011). The stimulation is independent of the recombination activity of Rad52.

5.3 Speculations on the presence of numerous stimulators of Dna2 and Fen1

In addition to the proteins mentioned above, the list of proteins that stimulate Fen1 and Dna2 is growing, which are most likely involved in maintenance of genome integrity. In humans, WRN, BLM, and RecQ5, the human homologues of yeast RecQ are an example of Fen1 stimulator (Brosh et al., 2001; Wang et al., 2005; Speina et al., 2010). Recently, it was shown that Dna2 and Pif1 can contribute to rapid nick formation by stimulating FEN1 (Henry et al., 2008). In addition, low levels of RPA also stimulated Fen1 activity particularly when short flaps were used as substrates. The acquisition of the ability of Fen1 or Dna2 to be stimulated by many proteins that work in close proximity may have conferred evolutionary benefits, because such an ability may permit faster generation and sealing of DNA nicks. Rapid generation and sealing of ligatable nicks may be more favorable in the preservation of genome integrity by converting unstable nicked DNA into stable duplex DNA.

5.4 Repair of faulty processing of Okazaki fragments

5.4.1 Homologous recombination as a last resort to repair faulty Okazaki fragments

When rad27-p (impaired interactions with PCNA) was combined with pol3-5DV (a mutant allele of a Pol δ subunit, defective in 3′ exonuclease and increased in displacement DNA synthesis), the double mutant cells were lethal in the absence of RAD51 that is essential for DSB repair (Jin et al., 2003). The lethal phenotype of rad27-p pol3-5DV rad51Δ was suppressed by overexpression of Dna2, suggesting that increased levels of long flaps resulting from mutant Pol δ required elevated levels of Dna2 for appropriate processing. In addition, the result above raises the possibility that excess levels of long flaps produced in

rad27-p pol3-5DV cells could undergo DSB that can be harmlessly repaired by RAD51-dependent repair pathway. This idea is further supported by a number of genetic data. First, dna2-C2 mutant cells displayed extensive chromosomal fragmentation like cdc9 (DNA ligase 1) mutation in S. pombe (Kang et al., 2000). Second, rad27Δ rad52Δ, dna2-1 rad27Δ, dna2-1 rad52Δ, dna2-2 rad52Δ double mutants are synthetic lethal (Jin et al., 2003; Budd et al., 2005). Third, ts dna2-22 mutant displayed increase in the rates of recombination and chromosome loss at non-permissive temperature (Fiorentino and Crabtree, 1997). Forth, the dna2-2 mutant cells showed hyperrecombination of rDNA, causing reduced life span of S. cerevisiae (Hoopes et al., 2002). In S. pombe, it was shown that functions of $rhp51^+$ (recombination gene RAD51 homolog) were required for viability of dna2 mutants (Tsutsui et al., 2005). Moreover, Rad52 was isolated as a multi-copy suppressor of helicase-negative dna2K1080E. Rad52 plays a role in the formation of Rad51-ssDNA filament by exchanging RPA with Rad51 (Song and Sung, 2000). Thus, the mediator function of Rad52 is crucial to initiate strand invasion. The rad52-QDDD-308-311-AAAA (rad52-QDDD/AAAA) mutant cells failed to form MMS-induced DNA repair foci and were not able to repair MMS-induced damage (Plate et al., 2008). Moreover, the mutant Rad52-QDDD/AAAA protein barely interacted with RPA and showed inefficient recombination mediator activity while retaining wild type levels of DNA binding activity (Plate et al., 2008). The suppression of dna2 mutation by Rad52 required the mediator activity of Rad52; rad52QDDD/AAAA mutant was not able to suppress dna2K1080E (Lee et al., 2011). This suggests that faulty Okazaki fragment could lead to elevated levels of homologous recombination. In support of this, we discovered that dna2Δ405N showed increases in the rates of inter- and intra-chromosomal recombination and unequal sister chromatid recombination (Lee et al., 2011). Our results suggest that incomplete replication of lagging strand synthesis due to faulty processing of Okazaki fragments could be efficiently repaired via Rad52-dependent homologous recombination pathway (Fig. 4) (Reagan et al., 1995; Tishkoff et al., 1997b; Budd and Campbell, 2000). Recently, it was found that Dna2 itself is a critical player in DSB repair by directly participating in long-range resection of DSB ends in cooperation with Sgs1 in a redundant fashion with Exo1 (Mimitou and Symington, 2008; Zhu et al., 2008). Both helicase activity of Sgs1 and nuclease activity of Dna2 were essential for this resection, whereas the helicase activity of Dna2 was dispensable (Mimitou and Symington, 2008 and 2009; Zhu et al., 2008; Niu et al., 2010; Shim et al., 2010).

5.4.2 Roles of Mph1 and Mus81-Mms4 as structure managers

The involvement of Mph1, Mus81-Mms4, and Rad52 in Okazaki fragment processing is particularly interesting, not only because of their abilities to stimulate the endonuclease activity of Dna2 and/or Fen1, but also because of their roles in recombinational repair of lagging strand replication defect as suggested previously (Ii & Brill, 2008). We found that Mph1 is a multipurpose helicase that can unwind a variety of DNA structures such as junction structures containing three or four DNA strands. Mph1 is able to unwind fixed double-flap DNA (an intermediate form of equilibrating flaps) in such a way that among the two flaps the displacement of 5′ flap occurs first (Kang et al., 2011). Thus, the helicase activity of Mph1 could contribute to Okazaki fragment processing by facilitating conversion of equilibrating flaps into 5′ flaps, which are readily cleaved by Fen1. In addition, Mph1 was able to efficiently displace hairpin-containing oligonucleotides, as long as short (~5-nt) ssDNA regions were present at the ssDNA/dsDNA junction. The ability of Mph1 to

displace 5′ secondary-structure flaps may allow cells to strip off the chronically problematic Okazaki fragments from the template, resulting in a gap equivalent in size to an Okazaki fragment, which can be filled in by Pol δ. Fen1 and Mus81-Mms4 appear to function in two separate processes because of their different substrate specificity (5′- and 3′-flap specific, respectively), the mutual stimulation observed in yeasts suggests a more direct inter-functional role between the two structure-specific endonucleases. The joint role of Fen1 and Mus81-Mms4 could come into effect via the interconversion between the substrates specific for each endonuclease. The 5′ or 3′ flap can be converted into a 3′ or 5′ flap, respectively, in a manner similar to that seen in Holliday junction migration. The equilibrating flaps (see Fig. 1. for structure) could be processed more rapidly if 5′ and 3′ flap specific enzymes could stimulate each other's activity.

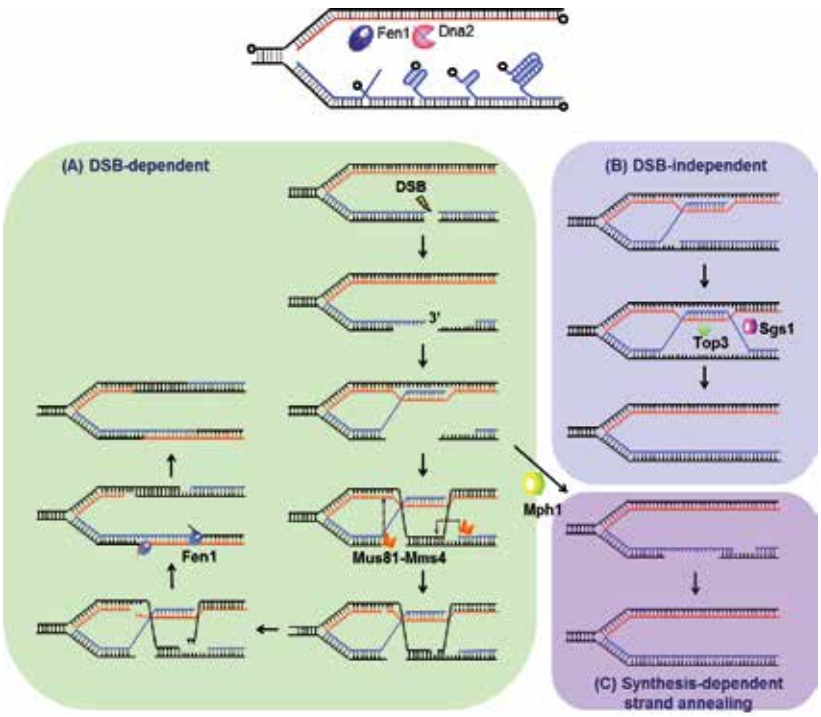

Fig. 4. Possible repair pathways for unprocessed flaps due to malfunction of Fen1 and/or Dna2. The unprocessed flap can be repaired via either DSB-dependent or -independent pathway. (A) In DSB-dependent pathway, replicated lagging strand containing unprocessed flap undergoes a DSB, followed by resection by the MRX complex (not shown). The resulting 3′ overhang starts homologous recombination by invading leading strand DNA. (B) If DSB is not involved, the 3′ flap, which could result from a 5′ unprocessed flap via 'equilibration,' can initiate recombination by invading leading strand DNA. If nicks are available, the resulting recombination intermediate can be resolved by Mus81-Mms4 catalyzed nick-directed cleavage (not shown in *B*). Alternatively, the intermediate can be converted into substrates for the Sgs1-Top3 pathway by forming pseudo double Holliday junctions (not shown in *A*). (C) Mph1 can remove the D-loop formed, facilitating synthesis-dependent strand annealing.

A helicase such as Mph1 could facilitate the interconversion process by virtue of its ability to displace the downstream strand. The product formed by this reaction would contain either 5′ or 3′ ssDNA flap depending on the polarity of the helicase involved, generating the structures suitable for cleavage by either Mus81-Mms4 or Fen1. Most likely candidates for such a function would include the helicases with branch migration activities such as WRN, RecQ1, and Mph1 (Prakash et al., 2009; Opresko et al., 2009; Burgreev et al., 2008). The human BLM helicase was shown to stimulate nuclease activity of the Mus81-Eme1 complex (Zhang et al., 2005). In addition, Rad54 was found to strongly stimulate Mus81-Mms4 in an ATP-independent manner in humans and yeasts (Matulova et al., 2009). Alternatively, a nuclease(s) that can simultaneously process both 5′ and 3′ double flaps could reduce the length of both flaps. This could more rapidly generate a DNA substrate that can be processed by either Fen1 or Mus81-Mms4. It was shown that endonuclease activity of human Dna2 is stimulated in the presence of double flaps (Kim et al., 2006).

6. Concluding remarks

Processing of Okazaki fragments is a complicated process at high risks of various types of DNA alterations such as base change, repeat expansion, and small duplications due to the involvement of anomalous structural DNA– a special type of DNA damages which, if left unrepaired, can promote genome instability. Examples of anomalous structure include nicks, unprocessed flaps, DSBs, and recombination intermediates. Formation of anomalous structures can be prevented by preemptive actions of Dna2 and/or by numerous 'auxiliary' factors that enhance endonuclease activities of Fen1 or Dna2. Alternatively, anomalous structures can be repaired by first forming DSBs, a key event that activates recombination. DSB-mediated recombination is regarded as the basis of genetic instability in eukaryotes since it can be a source of illegitimate recombination in higher organisms. A diverse array of auxiliary factors identified up to date may be a mirror image of a variety of structural intermediates present in vivo. The highly dynamic and capricious nature of structural intermediates renders correct processing of Okazaki fragments a formidable task which has to rely on a number of factors important for genome maintenance. Thus, Okazaki fragment processing is a platform where a number of proteins with roles in DNA replication and repair/recombination act together to minimize the hazardous outcome associated with its mechanisms in eukaryotes. In the future, the biggest challenge would be complete understanding of how each of the factors involved is regulated to fit into the complicated and dynamic network of protein-protein interactions required for failsafe processing of Okazaki fragments.

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8. References

Araki, H. (2010). Cyclin-dependent kinase-dependent initiation of chromosomal DNA replication. *Curr. Opin. Cell. Biol*. 22(6):766-771.

Arudchandran, A., Cerritelli, SM., Narimatsu, SK., Itaya, M., Shin, DY., Shimada, Y. & Crouch, RH. (2000). The absence of ribonuclease H1 or H2 alters the sensitivity of

Saccharomyces cerevisiae to hydroxyurea, caffeine and ethyl methanesulphonate: implications for roles of RNases H in DNA replication and repair, *Genes Cells* 5(10):789–802.

Ayyagari, R., Gomes, XV., Gordenin, DA. & Burgers, PM. (2003). Okazaki fragment maturation in yeast. I. Distribution of fucntions between Fen1 and Dna2, *J. Biol. Chem.* 278(3):1618–1625.

Aviv, T., Lin, Z., Lau, S., Rendl, LM., Sicheri, F. & Smibert, CA. (2003). The RNA-binding SAM domain of Smaug defines a new family of post-transcriptional regulators, *Nat. Struct. Biol.* 10(8):614-621.

Bae, SH., Choi, E., Lee, KH., Park, JS., Lee, SH. & Seo, YS. (1998). Dna2 of Saccharomyces cerevisiae possesses a single-stranded DNA-specific endonuclease activity that is able to act on double-stranded DNA in the presence of ATP, *J. Biol. Chem.* 273(41):26880-26890.

Bae, SH. & Seo, YS. (2000). Characterization of the enzymatic properties of the yeast dna2 Helicase/endonuclease suggests a new model for Okazaki fragment processing, *J. Biol. Chem.* 275(48):38022-35031.

Bae, SH., Bae, KH., Kim, JA. & Seo, YS. (2001a). RPA governs endonuclease switching during processing of Okazaki fragments in eukaryotes, *Nature* 412(6845):456–461.

Bae, SH., Kim, JA., Choi, E., Lee, KH., Kang, HY., Kim, HD., Kim, JH., Bae, KH., Cho, Y., Park, C. & Seo, YS. (2001b). Tripartite structure of Saccharomyces cerevisiae Dna2 helicase/endonuclease, *Nucleic Acids Res*. 29(14):3069-3079.

Bae, SH., Kim, DW., Kim, J., Kim, JH., Kim, DH., Kim., HD., Kang. HY. & Seo, YS. (2002). Coupling of DNA helicase and endonuclease activities of yeast Dna2 facilitates Okazaki fragment processing, *J. Biol. Chem.* 277(29):26632-26641.

Bae, KH., Kim, HS., Bae, SH., Kang, HY., Brill, S. & Seo, YS. (2003). Bimodal interaction between replication-protein A and Dna2 is critical for Dna2 function both in vivo and in vitro, *Nucleic Acids Res.* 31(12):3006-3015.

Banerjee, S., Smith, S., Oum, JH., Liaw, HJ., Hwang, JY., Sikdar, N., Motegi, A., Lee, SE. & Myung, K. (2008). Mph1p promotes gross chromosomal rearrangement through partial inhibition of homologous recombination, *J. Cell. Biol.* 181(7):1083-1093.

Bastin-Shanower, SA., Fricke, WM., Mullen, JR. & Brill, SJ. (2003). The mechanism of Mus81-Mms4 cleavage site selection distinguishes it from the homologous endonuclease Rad1-Rad10, *Mol. Cell. Biol.* 23(10):3487-3496.

Bialek, G. & Grosse, F. (1993). An error-correcting proofreading exonuclease-polymerase that copurifies with DNA-polymerase-alpha-primase, *J. Biol. Chem.* 268(8):6024-6033.

Boddy, MN., Gaillard, PH., McDonald, WH., Shanahan, P., Yates, JR. & Russell, P. (2001). Mus81-Eme1 are essential components of a Holliday junction resolvase, *Cell* 107(4):537-548.

Brosh, RMJr., Von Kobbe, C., Sommers, JA., Karmakar, P., Opresko, PL., Piotrowski, J., Dianova, I., Dianov, GL. & Bohr, VA. (2001). Werner syndrome protein interacts with human flap endonuclease 1 and stimulates its cleavage activity, *EMBO J.* 20(20):5791-5801.

Bubeck, D., Reijns, MA., Graham, SC., Astell, KR., Jones, EY. & Jackson, AP. (2011). PCNA directs type 2 RNase H activity on DNA replication and repair substrates, *Nucleic Acids Res.* 39(9):3652-3666.

Budd, ME. & Campbell, JL. (1995). A yeast gene required for DNA replication encodes a protein with homology to DNA helicases, *Proc. Natl. Acad. Sci. USA* 92(17):7642-7646.

Budd, ME., Choe, WC. & Campbell, JL. (1995). DNA2 encodes a DNA helicase essential for replication of eukaryotic chromosomes, *J. Biol. Chem.* 270(45):26766-26769.

Budd, ME., Choe, WC. & Campbell, J. (2000). The nuclease activity of the yeast Dna2 protein, which is related to the RecB-like nucleases, is essential in vivo, *J. Biol. Chem.* 275 (22):16518-16529.

Budd, ME., Tong, A., Peng, X., Polaczek, A., Boone, A. & Campbell, JL. (2005). A network of multi-tasking proteins at the DNA replication fork preserves genome stability, *PLoS Genetics* 1(6):634-650.

Bugreev, DV., Brosh, RMJr. & Mazin, AV. (2008). RECQ1 possesses DNA branch migration activity, *J. Biol. Chem.* 283(29):20231-20242.

Burgers, PM. & Gerik, KJ. (1998). Structure and processivity of two forms of Saccharomyces cerevisiae DNA polymerase delta, *J. Biol. Chem.* 273(31):19756-19762.

Bullock, PA., Seo, YS. & Hurwitz, J. (1991). Initiation of simian virus 40 DNA synthesis in vitro, *Mol. Cell. Biol.* 11(5):2350–2361.

Cederberg, H. & Rannug, U. (2006). Mechanisms of human minisatellite mutation in yeast, *Mutat Res.* 598(1-2):132-143.

Ciccia, A., Constantinou, A. & West, SC. (2003). Identification and characterization of the human Mus81-Eme1 endonuclease, *J. Biol. Chem.* 278(27):25172-25178.

Cerritelli, SM. & Crouch, RJ. (2009). Ribonuclease H: the enzymes in eukaryotes, *FEBS J.* 276(6):494-505.

Cho, IT., Kim, DH., Kang, YH., Lee, CH., Amangyelid, T., Nguyen, TA., Hurwitz, J. & Seo, YS. (2009). Human replication factor C stimulates flap endonuclease 1, *J. Biol. Chem.* 284(16):10387-10399.

Conaway, RC. & Lehman, IR. (1982). A DNA primase activity associated with DNA polymerase alpha from Drosophila melanogaster embryos, *Proc. Natl. Acad. Sci. USA* 79(8):2523-2527.

Crow, YJ., Leitch, A., Hayward, BE., Garner, A., Parmar, R., Griffith, E., Ali, M., Semple, C., Aicardi, J., Babul-Hirji, R., Baumann, C., Baxter, P., Bertini, E., Chandler, KE., Chitayat, D., Cau, D., Dery, C., Fazzi, E., Goizet, C., King, MD., Klepper, J., Lacombe, D., Lanzi, G., Lyall, H., Martinez-Frias, ML., Mathieu, M., McKeown, C., Monier, A., Oade, Y., Quarrell, OW., Rittey, CD., Rogers, RC., Sanchis, A., Stephenson, JB., Tacke, U., Till, M., Tolmie, JL., Tomlin, P., Voit, T., Weschke, B., Woods, CG., Lebon, P., Bonthron, DT., Ponting, CP. & Jackson, AP. (2006). Mutations in genes enconding ribonulease H2 subunits cause Aicardi-Goutieres syndrome and mimic congenital viral brain infection, *Nat. Genet.* 38(8):910-916.

Cullmann, G., Fien, K., Kobayashi, R. & Stillman, B. (1995). Characterization of the five replication factor C genes of Saccharomyces cerevisiae, *Mol. Cell. Biol.* 15(9):4661-4671.

Dilcher, M., Kohler, B. & von Mollard, GF. (2001). Genetic interactions with the yeast Q-SNARE VTI1 reveal novel functions for the R-SNARE YKT6, *J. Biol. Chem.* 276(37):34537-34544.

Entian, KD., Schuster, T., Hegemann, JH., Becher, D., Feldmann, H., Güldener, U., Götz, R., Hansen, M., Hollenberg, CP., Jansen, G., Kramer, W., Klein, S., Kötter, P., Kricke, J.,

Launhardt, H., Mannhaupt, G., Maierl, A., Meyer, P., Mewes, W., Munder, T., Niedenthal, RK., Ramezani Rad, M., Röhmer, A., Römer, A., Hinnen A, et al. (1999). Functional analysis of 150 deletion mutants in Saccharomyces cerevisiae by a systematic approach, *Mol. Gen. Genet*. 262(4-5):683-702.

Fiorentino, DF. & Crabtree, GR. (1997). Characterization of Saccharomyces cerevisiae dna2 mutants suggests a role for the helicase late in S phase, *Mol. Biol. Cell.* 8(12):2519-2537.

Formosa, T. & Nittis, T. (1999). Dna2 mutants reveal interactions with Dna polymerase alpha and Ctf4, a Pol alpha accessory factor, and show that full Dna2 helicase activity is not essential for growth, *Genetics* 151(4):1459-1470.

Frank, P., Braunshofer-Reiter, C. & Wintersberger, U. (1998). Yeast RNase H(35) is the counterpart of the mammalian RNase HI, and is evolutionarily related to prokaryotic RNase HII, *FEBS Lett.* 421(1):23–26.

Frank, G., Qiu, J., Zheng, L. & Shen B. (2001). Stimulation of eukaryotic flap endonuclease-1 activities by proliferating cell nuclear antigen (PCNA) is independent of its in vitro interaction via a consensus PCNA binding region, *J. Biol. Chem.* 276(39):36295-36302.

Freudenreich, CH., Stavenhagen, JB. & Zakian, VA. (1997). Stability of a CTG/CAG trinucleotide repeat in yeast is dependent on its orientation in the genome, *Mol. Cell. Biol.* 17(4):2090-2098.

Freudenreich, CH., Kantrow, SM. & Zakian, VA. (1998). Expansion and length-dependent fragility of CTG repeats in yeast, *Science* 279(5352):853-856.

Garg, P., Stith, CM., Sabouri, N., Johansson, E. & Burgers, PM. (2004). Idling by DNA polymerase delta maintains a ligatable nick during lagging-strand DNA replication, *Genes Dev.* 18(22):2764–2773.

Garg, P. & Burgers, PM. (2005). How the cell deals with DNA nicks, *Cell Cycle* 4(2):221-224.

Gary, R., Park, MS., Nolan, JP., Cornelius, HL., Kozyreva, OG., Tran, HT., Lobachev, KS., Resnick, MA. & Gordenin, DA. (1999). A novel role in DNA metabolism for the binding of Fen1/Rad27 to PCNA and implications for genetic risk, *Mol. Cell. Biol* 19(8):5373-5382.

Gomes, XV. & Burgers, PM. (2000). Two modes of FEN1 binding to PCNA regulated by DNA, *EMBO J.* 19(14):3811-21.

Harrington, JJ. & Lieber, MR. (1994). The characterization of a mammalian DNA structure-specific endonuclease, *EMBO J.* 13(5):1235-1246.

Henry, RA., Balakrishnan, L., Ying-Lin, ST., Campbell, JL. & Bambara, RA. (2010). Components of the secondary pathway stimulate the primary pathway of eukaryotic Okazaki fragment processing. *J. Biol. Chem*. 285(37):28496-28505.

Hishida, T., Iwasaki, H., Ohno, T., Morishita, T. & Shinagawa, H. (2001). A yeast gene, MGS1, encoding a DNA-dependent AAA(+) ATPase is required to maintain genome stability, *Proc. Natl. Acad. Sci. USA* 98(15):8283-8289.

Hishida, T., Ohno, T., Iwasaki, H. & Shinagawa, H. (2002). Saccharomyces cerevisiae MGS1 is essential in strains deficient in the RAD6-dependent DNA damage tolerance pathway *EMBO J.* 21(8):2019–2029.

Holmes, J., Clark, S. & Modrich, P. (1990). Strand-specific mismatch correction in nuclear extracts of human and Drosophila melanogaster cell lines, *Proc. Natl. Acad. Sci. USA* 87(15):5837–5841.

Holmes, AM. & Haber, J. (1999). Double-strand break repair in yeast requires both leading and lagging strand DNA polymerases, *Cell* 96(3):415-424.

Hoopes, LL., Budd, M., Choe, W., Weitao, T. & Campbell, JL. (2002). Mutations in DNA replication genes reduce yeast life span, *Mol. Cell. Biol.* 22(12):4136-4146.

Ishimi, Y., Claude, A., Bullock, P. & Hurwitz, J. (1988). Complete enzymatic synthesis of DNA containing the SV40 origin of replication, *J. Biol. Chem.* 263(36):19723-19733.

Jeong, HS., Backlund, PS., Chen, HC., Karavanov, AA. & Crouch, RJ. (2004). RNase H2 of Saccharomyces cerevisiae is a complex of three proteins, *Nucleic Acids Res.* 32(2): 407-414.

Jin, YH., Obert, R., Burgers, PM., Kunkel, TA., Resnick, MA. & Gordenin, DA. (2001). The 3'→5' exonuclease of DNA polymerase delta can substitute for the 5' flap endonuclease Rad27/Fen1 in processing Okazaki fragments and preventing genome instability, *Proc. Natl. Acad. Sci. USA* 98(9):5122-5127.

Jin, YH., Ayyagari, R., Resnick, MA., Gordenin, DA. & Burgers, PM. (2003). Okazaki fragment maturation in yeast. II. Cooperation between the polymerase and 3'-5'-exonuclease activities of Pol delta in the creation of a ligatable nick, *J. Biol. Chem.* 2003 278(3):1626-1633.

Johansson, E., Garg, P. & Burgers, PM. (2004). The Pol32 subunit of DNA polymerase delta contains separable domains for processive replication and proliferating cell nuclear antigen (PCNA) binding, *J. Biol. Chem.* 279(3):1907-1915.

Johnson, RE., Kovvali, GK., Prakash, L. & Prakash, S. (1995). Requirement of the yeast RTH1 5' to 3' exonuclease for the stability of simple repetitive DNA, *Science* 269(5221):238-240.

Kaliraman, V., Mullen, JR., Fricke, WM., Bastin-Shanower, S. A. & Brill, SJ. (2001). Functional overlap between Sgs1-Top3 and the Mms4-Mus81 endonuclease, *Genes Dev.* 15(20):2730-2740.

Kang, HY., Choi, E., Bae, SH., Lee, KH., Gim, BS., Kim, HD., Park, C., MacNeill, SA. & Seo, YS. (2000). Genetic analyses of Schizosaccharomyces pombe dna2(+) reveal that dna2 plays an essential role in Okazaki fragment metabolism, *Genetics* 155(3):1055-1067.

Kang, MJ., Lee, CH., Kang, YH., Cho, IT., Nguyen, TA. & Seo, YS. (2010). Genetic and functional interactions between Mus81-Mms4 and Rad27, *Nucleic Acids Res.* 38(21):7611-7625.

Kang, YH., Kang, MJ., Kim, JH., Lee, CH., Cho, IT., Hurwitz, J. & Seo, YS. (2009). The MPH1 gene of Saccharomyces cerevisiae functions in Okazaki fragment processing, *J. Biol. Chem.* 284(16):10376-10386.

Kang, YH., Lee, CH. & Seo, YS. (2010). Dna2 on the road to Okazaki fragment processing and genome stability in eukaryotes, *Crit. Rev. Biochem. Mol. Biol.* 45(2):71-96.

Kang, YH., Munashingha, PR., Lee, CH., Nguyen, TA. & Seo, YS. (2011). The DNA helicase activity of Mph1 has diverse roles in genome maintenance (in submission)

Kao, HI., Veeraraghavan, J., Polaczek, P., Campbell, JL. & Bambara, RA. (2004). On the roles of Saccharomyces cerevisiae Dna2p and Flap endonuclease 1 in Okazaki fragment processing, *J. Biol. Chem.* 279(15):15014-15024.

Kim, JH., Kang, YH., Kang, HJ., Kim, DH., Ryu, GH., Kang, MJ. & Seo, YS. (2005). In vivo and in vitro studies of Mgs1 suggest a link between genome instability and Okazaki fragment processing, *Nucleic Acids Res.* 33(19):6137-6150.

Kim, JH., Kim, HD., Ryu, GH., Kim, DH., Hurwitz, J. & Seo, YS. (2006). Isolation of human Dna2 endonuclease and characterization of its enzymatic properties, *Nucleic Acids Res.* 34(6):1854-1864.

Kokoska, RJ., Stefanovic, L., Tran, HT., Resnick, MA., Gordenin, DA. & Petes, TD. (1998). Destabilization of yeast micro- and minisatellite DNA sequences by mutations affecting a nuclease involved in Okazaki fragment processing (rad27) and DNA polymerase delta (pol3-t), *Mol. Cell. Biol.* 18(5):2779-2788.

Kolodner, RD. & Marsischky GT. (1999). Eukaryotic DNA mismatch repair, *Curr. Opin. Genet. Dev*. 9:89–96.

Kovtun, IV. & McMurray, CT. (2008). Features of trinucleotide repeat instability in vivo, *Cell Res.* 18(1):198-213.

Kucherlapati, M., Yang, K., Kuraguchi, M., Zhao, J., Lia, M., Heyer, J., Kane, MF., Fan, K., Russell, R., Brown, AM., Kneitz, B., Edelmann, W., Kolodner, RD., Lipkin, M. & Kucherlapati, R. (2001). Haploinsufficiency of Flap endonuclease (Fen1) leads to rapid tumor progression, *Proc. Natl. Acad. Sci. USA* 99(15):9924-9929.

Kunkel, TA. & Erie, DA. (2005). DNA mismatch repair, *Annu. Rev. Biochem.* 74:681–710.

Kunkel, TA. (2009). Evolving views of DNA replication (in)fidelity, *Cold Spring Harb. Symp. Quant. Biol.* 74:91-101.

Lee, CH., Shin, YK., Phung, TT., Bae, JS., Kang, YH., Nguyen, TA., Kim, JH., Kim, DH., Kang, MJ., Bae, SH. & Seo, YS. (2010). Involvement of Vts1, a structure-specific RNA-binding protein, in Okazaki fragment processing in yeast, *Nucleic Acids Res.* 38(5):1583-1595.

Lee, CH., Munasingha, PR., Lee, MJ., Huong, PTT. & Seo, YS. (2011). Recombination-mediated repair of faulty Okazaki fragment processing (manuscript in preparation)

Lee, KH., Kim, DW., Bae, SH., Kim, JA., Ryu, GH., Kwon, YN., Kim, KA., Koo, HS. & Seo YS. (2000). The endonuclease activity of the yeast Dna2 enzyme is essential in vivo, *Nucleic acids Res.* 28(15):2873-2881.

Ii, M. & Brill, SJ. (2005). Roles of SGS1, MUS81, and RAD51 in the repair of lagging-strand replication defects in Saccharomyces cerevisiae, *Curr. Genet.* 48(4):213-225.

Li, X., Li, J., Harrington, J., Lieber, MR. & Burgers, PM. (1995). Lagging strand DNA synthesis at the eukaryotic replication fork involves binding and stimulation of FEN-1 by proliferating cell nuclear antigen, *J. Biol. Chem.* 270(38):22109-12.

Liu, Y., Kao, H. I. & Bambara, R. A. (2004). Flap endonuclease 1: a central component of DNA metabolism, *Annu. Rev. Biochem.* 73:589–615.

Lopes, J., Debrauwère, H., Buard, J. & Alain Nicolas, A. (2002). Genome Stability and DynamicsInstability of the human minisatellite CEB1 in rad27 and dna2-1 replication-deficient yeast cells, *EMBO J.* 21(12):3201–3211.

Lopes, J., Ribeyre, C. & Nicolas, A. (2006). Complex minisatellite rearrangements generated in the total or partial absence of Rad27/hFEN1 activity occur in a single generation and are Rad51 and Rad52 dependent, *Mol. Cell. Biol.* 26(17):6675-6689.

MacNeill, SA. (2001). DNA replication: partners in the Okazaki two-step, *Curr. Biol.* 11(20):R842-844.

Maga, G., Villani, G., Tillement, V., Stucki, M., Locatelli, GA., Frouin, I., Spadari, S. & Hübscher, U. (2001). Okazaki fragment processing: modulation of the strand displacement activity of DNA polymerase delta by the concerted action of

replication protein A, proliferating cell nuclear antigen, and flap endonuclease-1, *Proc. Natl. Acad. Sci. USA* 98(25):1298-1303.

Majka J. & Burgers, PM. (2004). The PCNA-RFC families of DNA clamps and clamp loaders, *Prog. Nucleic Acid. Res. Mol. Biol.* 78:227-260.

Maleki, S., Cederberg, H. & Rannug, U. (2002). The human minisatellites MS1, MS32, MS205 and CEB1 integrated into the yeast genome exhibit different degrees of mitotic instability but are all stabilised by RAD27, *Curr. Genet.* 41(5):333-341.

Mass, G., Nethanel, T. & Kaufmann, G. (1998). The middle subunit of replication protein A contacts growing RNA-DNA primers in replicating simian virus 40 chromosomes, *Mol. Cell. Biol.* 18(11):6399-6407.

Masuda, Y., Suzuki, M., Piao, J., Gu, Y., Tsurimoto, T. & Kamiya, K. (2007). Dynamics of human replication factors in the elongation phase of DNA replication, *Nucleic Acids Res.* 35(20):6904-6916.

Masuda-Sasa, T., Polaczek, P., Peng, XP., Chen, L. & Campbell, JL. (2008). Processing of G4 DNA by Dna2 helicase/nuclease and replication protein A (RPA) provides insights into the mechanism of Dna2/RPA substrate recognition, *J. Biol. Chem.* 283(36):24359-24373.

Matulova, P., Marini, V., Burgess, R. C., Sisakova, A., Kwon, Y., Rothstein, R., Sung, P. & Krejci, L. (2009). Cooperativity of Mus81•Mms4 with Rad54 in the Resolution of Recombination and Replication Intermediates, *J. Biol. Chem.* 284(12): 7733–7745.

Mimitou, EP. & Symington, LS. (2008). Sae2, Exo1 and Sgs1 collaborate in DNA double-strand break processing, *Nature* 455(7214):770-774.

Mimitou, EP. & Symington, LS. (2009). Nucleases and helicases take center stage in homologous recombination, *Trends Biochem. Sci.* 34(5):264-272.

Miret, JJ., Pessoa-Brandão, L. & Lahue, RS. (1998). Orientation-dependent and sequence-specific expansions of CTG/CAG trinucleotide repeats in Saccharomyces cerevisiae, *Proc. Natl. Acad. Sci. USA* 95(21):12438-12443.

Modrich, P. & Lahue, R. (1996). Mismatch repair in replication fidelity, genetic recombination, and cancer biology, *Annu. Rev. Biochem.* 65:101–133.

Modrich, P. (1997). Strand-specific mismatch repair in mammalian cells, *J. Biol. Chem.* 272(40):24727-24730.

Moe, SE., Sorbo, JG. & Holen, T. (2008). Huntingtin triplet-repeat locus is stable under long-term Fen1 knockdown in human cells, *J. Neurosci. Methods* 171(2):233-238.

Murante, RS., Rust, L. & Bambara, RA. (1995). Calf 5' to 3' exo/endonuclease must slide from a 5' end of the substrate to perform structure-specific cleavage, *J. Biol. Chem.* 270(51):30377-30383.

Nguyen, TA., Tak, YS., Lee, CH., Kang, YH., Cho, IT. & Seo, YS. (2011). Functional reconstitution of Saccharomyces cerevisiae RNase H2 complex (manuscript in submission)

Nick McElhinny, SA., Kumar, D., Clark, AB., Watt, DL., Watts, BE., Lundström, EB., Johansson, E., Chabes, A. & Kunkel, TA. (2010a). Genome instability due to ribonucleotide incorporation into DNA, *Nat. Chem. Biol.* 6(10):774-781.

Nick McElhinny, SA., Watts, BE., Kumar, D., Watt, DL., Lundström, EB., Burgers, PM., Johansson, E., Chabes, A. & Kunkel, TA. (2010b). Abundant ribonucleotide incorporation into DNA by yeast replicative polymerases, *Proc. Natl. Acad. Sci. USA* 107(11):4949-4954.

Niu, H., Chung, WH., Zhu, Z., Kwon, Y., Zhao, W., Chi, P., Prakash, R., Seong, C., Liu, D., Lu L., Ira, G. & Sung, P. (2010). Mechanism of the ATP-dependent DNA end-resection machinery from Saccharomyces cerevisiae, *Nature* 467(7311):108-1011.

Ohtani, N., Haruki, M., Morikawa, M., Crouch, RJ., Itaya, M. & Kanaya, S. (1999). Identification of the genes encoding Mn^{2+}-dependent RNase I-III and Mg^{2+}-dependent RNase HIII from Bacillus subtilis: classification of RNases H into three families, *Biochemistry* 38(2):605-618.

Opresko, PL., Sowd, G. & Wang, H. (2009). The Werner syndrome helicase/exonuclease processes mobile D-loops through branch migration and degradation, *PLoS One* 4(3):e4825.

Parenteau, J. & Wellinger, RJ. (1999). Accumulation of single-stranded DNA and destabilization of telomeric repeats in yeast mutant strains carrying a deletion of RAD27, *Mol. Cell. Biol.* 9(6):4143-4152.

Parenteau, J. & Wellinger, RJ. (2002). Differential processing of leading- and lagging-strand ends at Saccharomyces cerevisiae telomeres revealed by the absence of Rad27p nuclease, *Genetics* 162(4):1583-1594.

Pavlov, YI., Frahm, C., Nick McElhinny, SA., Niimi, A., Suzuki, M. & Kunkel, TA. (2006). Evidence that errors made by DNA polymerase alpha are corrected by DNA polymerase delta, *Curr. Biol.* 16(2):202-207.

Pearson, CE., Nichol Edamura, K. & Cleary, JD. (2005). Repeat instability: mechanisms of dynamic mutations, *Nat. Rev. Genet.* 6(10):729-742.

Plate, I., Hallwyl, SC., Shi, I., Krejci, L., Müller, C., Albertsen, L., Sung, P. & Mortensen, UH. (2008). Interaction with RPA is necessary for Rad52 repair center formation and for its mediator activity, *J. Biol. Chem.* 283(43):29077-29085.

Podust, VN. & Hübscher, U. (1993). Lagging strand DNA synthesis by calf thymus DNA polymerases alpha, beta, delta and epsilon in the presence of auxiliary proteins, *Nucleic Acids Res.* 21(4):841–846.

Podust, VN., Podust, LM., Müller, F. & Hübscher, U. (1995). DNA polymerase delta holoenzyme: action on single-stranded DNA and on double-stranded DNA in the presence of replicative DNA helicases, *Biochemistry* 34(15):5003-5010.

Prakash, R., Krejci, L., Van Komen, S., Anke Schürer, K., Kramer, W. & Sung, P. (2005). Saccharomyces cerevisiae MPH1 gene, required for homologous recombination-mediated mutation avoidance, encodes a 3' to 5' DNA helicase, *J. Biol. Chem.* 280(9):7854-7860.

Prakash, R., Satory, D., Dray, E., Papusha, A., Scheller, J., Kramer, W., Krejci, L., Klein, H., Haber, J. E., Sung, P. & Ira, G. (2009). Yeast Mph1 helicase dissociates Rad51-made D-loops: implication for crossover control in mitotic recombination, *Genes Dev.* 23(1):67-79

Qiu, JZ., Qian, Y., Frank, P., Wintersberger, U. & Shen, BH. (1999). Saccharomyces cerevisiae RNase H(35) functions in RNA primer removal during lagging-strand DNA synthesis, most efficiently in cooperation with Rad27 nuclease, *Mol. Cell. Biol.* 19(12):8361-8371.

Reagan, MS., Pittenge, C., Siede, W. & Friedberg, EC. (1995). Characterization of a mutant strain of Saccharomyces cerevisiae with a deletion of the RAD27 gene, a structural homolog of the RAD2 nucleotide excision-repair gene, *J. Bacteriol.* 177(2):364–371.

Remus, D. & Diffley, JFX. (2009). Eukaryotic DNA replication control: Lock and load, then fire, *Curr. Opin. Cell. Biol.* 21(6):771–777

Reynolds, N., Warbrick, E., Fantes, PA. & MacNeill, SA. (2000). Essential interaction between the fission yeast DNA polymerase delta subunit Cdc27 and Pcn1 (PCNA) mediated through a C-terminal p21(Cip1)-like PCNA binding motif, *EMBO J.* 19(5):1108-1118.

Rossi, ML., Pike, JE., Wang, W., Burgers, PM., Campbell, JL. & Bambara, RA. (2008). Pif1 helicase directs eukaryotic Okazaki fragments toward the two-nuclease cleavage pathway for primer removal, *J. Biol. Chem.* 283(41):27483-27493.

Rossi, ML. & Bambara, RA. (2006). Reconstituted Okazaki fragment processing indicates two pathways of primer removal, *J. Biol. Chem.* 281(36):26051-26061.

Rossi, ML., Ghosh, AK. & Bohr, VA. (2010). Roles of Werner syndrome protein in protection of genome integrity, *DNA Repair* 9(3):331-344.

Rydberg, B. & Game, J. (2002). Excision of misincorporated ribonucleotides in DNA by RNase H (type 2) and FEN-1 in cell-free extracts, *Proc. Natl. Acad. Sci. USA* 99(26):16654-16659.

Ryu, GH., Tanaka, H., Kim, DH., Kim, JH., Bae, SH., Kwon, YN., Rhee, JS., MacNeill, SA. & Seo, YS. (2004). Genetic and biochemical analyses of Pfh1 DNA helicase function in fission yeast, *Nucleic Acids Res.* 32(14):4205-4216.

Scheller, J., Schürer, A., Rudolph, C., Hettwer, S. & Kramer, W. (2000). MPH1, a yeast gene encoding a DEAH protein, plays a role in protection of the genome from spontaneous and chemically induced damage, *Genetics* 155(3):1069-1081.

Schweitzer, JK. & Livingston, DM. (1998). Expansions of CAG repeat tracts are frequent in a yeast mutant defective in Okazaki fragment maturation, *Hum. Mol. Genet.* 7(1):69-74.

Schürer, KA., Rudonph, C., Ulrich, HD. & Kramer, W. (2004). Yeast MPH1 gene functions in an error-free DNA damage bypass pathway that requires genes from Homologous recombination, but not from postreplicative repair, *Genetics* 166(4):1673-1686.

Sclafani, RA. & Holzen, TM. (2007). Cell Cycle Regulation of DNA Replication, *Annu. Rev. Genet.* 41:237–280.

Shen, B., Singh, P., Liu, R., Qiu, J., Zheng, L., Finger, LD. & Alas, S. (2005). Multiple but dissectible functions of FEN-1 nucleases in nucleic acid processing, genome stability and diseases, *Bioessays* 27(7):717-29.

Shim, EY., Chung, WH., Nicolette, ML., Zhang, Y., Davis, M., Zhu, Z., Paull, TT., Ira, G. & Lee, SE. (2010). Saccharomyces cerevisiae Mre11/Rad50/Xrs2 and Ku proteins regulate association of Exo1 and Dna2 with DNA breaks, *EMBO J.* 29(19):3370-3380.

Singh, P., Zheng, L., Chavez, V., Qiu, J. & Shen, B. (2007). Concerted action of exonuclease and Gap-dependent endonuclease activities of FEN-1 contributes to the resolution of triplet repeat sequences (CTG)n- and (GAA)n-derived secondary structures formed during maturation of Okazaki fragments, *J. Biol. Chem.* 282(6):3465-3477.

Song, B. & Sung, P. (2000). Functional interactions among yeast Rad51 recombinase, Rad52 mediator, and replication protein A in DNA strand exchange, *J. Biol. Chem.* 275(21):15895-15904.

Speina, E., Dawut, L., Hedayati, M., Wang, Z., May, A., Schwendener, S., Janscak, P., Croteau, DL. & Bohr, VA. (2010). Human RECQL5beta stimulates flap endonuclease 1, *Nucleic Acids Res.* 38(9):2904-2916.

Spiro, C., Pelletier, R., Rolfsmeier, ML., Dixon, MJ., Lahue, RS., Gupta, G., Park, MS., Chen, X., Mariappan, SV. & McMurray, CT. (1999). Inhibition of FEN-1 processing by DNA secondary structure at trinucleotide repeats, *Mol. Cell* 4(6):1079-1085.

Spiro, C. & McMurray, CT. (2003). Nuclease-deficient FEN-1 blocks Rad51/BRCA1-mediated repair and causes trinucleotide repeat instability, *Mol. Cell. Biol.* 23(17):6063–6074.

Tanaka, H., Ryu, GH., Seo, YS. & MacNeill, SA. (2004). Genetics of lagging strand DNA synthesis and maturation in fission yeast: suppression analysis links the Dna2-Cdc24 complex to DNA polymerase delta, *Nucleic Acids Res.* 32(21):6367-6377.

Thomas, DC., Roberts, JD. & Kunkel TA. (1991). Heteroduplex repair in extracts of human HeLa cells, *J. Biol. Chem.* 266(6):3744–3751.

Tishkoff, DX., Filosi, N., Gaida, GM. & Kolodner, RD. (1997a). A novel mutation avoidance mechanism dependent on S. cerevisiae RAD27 is distinct from DNA mismatch repair, *Cell* 88(2):253-263.

Tishkoff, DX., Boerger, AL., Bertrand, P., Filosi, N., Gaida, GM., Kane, MF. & Kolodner, RD. (1997b). Identification and characterization of Saccharomyces cerevisiae EXO1, a gene encoding an exonuclease that interacts with MSH2, *Proc. Natl. Acad. Sci. USA* 94(14):7487-7492.

Tom, S., Henricksen, LA. & Bambara, RA. (2000). Mechanism whereby proliferating cell nuclear antigen stimulates flap endonuclease 1, *J. Biol. Chem.* 275(14):10498-10505.

Tran, PT., Erdeniz, N., Dudley, S. & Liskay, RM. (2001). Characterization of nuclease-dependent functions of Exo1p in Saccharomyces cerevisiae, *DNA Repair* 1(11):895-912.

Tsutsui, Y., Morishita, T., Natsume, T., Yamashita, K., Iwasaki, H., Yamao, F. & Shinagawa, H. (2005). Genetic and physical interactions between Schizosaccharomyces pombe Mcl1 and Rad2, Dna2 and DNA polymerase alpha: evidence for a multifunctional role of Mcl1 in DNA replication and repair, *Curr. Genet.* 48(1):34–43.

van den Broek, WJ., Nelen, MR., Van der Heijden, GW., Wansink, DG. & Wieringa, B. (2006). Fen1 does not control somatic hypermutability of the (CTG)(n)*(CAG)(n) repeat in a knock-in mouse model for DM1, *FEBS Lett.* 580(22):5208-5214.

Waga, S. & Stillman, B. (1994). Anatomy of a DNA replication fork revealed by reconstitution of SV40 DNA replication in vitro, *Nature* 369(6477):207-212

Wang, W. & Bambara, RA. (2005). Human Bloom protein stimulates flap endonuclease 1 activity by resolving DNA secondary structure, *J. Biol. Chem.* 280(7):5391-5399.

Whitby, MC., Osman, F. & Dixon, J. (2003). Cleavage of model replication forks by fission yeast Mus81-Eme1 and budding yeast Mus81-Mms4, *J. Biol. Chem.* 278(9):6928-6935.

White, PJ., Borts, RH. & Hirst, MC. (1999). Stability of the human fragile X (CGG)(n) triplet repeat array in Saccharomyces cerevisiae deficient in aspects of DNA metabolism, *Mol. Cell. Biol.* 19(8):5675-5684.

Xie, Y., Liu, Y., Argueso, JL., Henricksen, LA., Kao, HI., Bambara, RA. & Alani, E. (2001). Identification of rad27 mutations that confer differential defects in mutation avoidance, repeat tract instability, and flap cleavage, *Mol. Cell. Biol.* 21(15):4889-4899.

Zhang, R., Sengupta, S., Yang, Q., Linke, SP., Yanaihara, N., Bradsher, J., Blais, V., McGowan, CH. & Harris, CC. (2005). BLM helicase facilitates Mus81 endonuclease activity in human cells, *Cancer Res.* 65(7):2526-2531.

Zheng, L., Jia, J., Finger, LD., Gua, ZG., Zer, C. & Shen, BH. (2011). Functional regulation of FEN1 nuclease and its link to cancer, *Nucleic Acids Res.* 39(3):781-794.

Zuo, S., Bermudez, V., Zhang, G., Kelman, Z. & Hurwitz, J. (2000). Structure and activity associated with multiple forms of Schizosaccharomyces pombe DNA polymerase delta, *J. Biol. Chem.* 275(7):5153-5162.

Zhu, Z., Chung, WH., Shim, EY., Lee, SE. & Ira, G. (2008). Sgs1 helicase and two nucleases Dna2 and Exo1 resect DNA double-strand break ends, *Cell* 134(6):981-994.

2

Synergy Between DNA Replication and Repair Mechanisms

Maria Zannis-Hadjopoulos and Emmanouil Rampakakis
Goodman Cancer Research Centre and Department of Biochemistry, McGill University, Montreal, Canada

1. Introduction

DNA replication is a fundamental and stringently regulated cellular process that ensures the accurate propagation of the cell's genetic material. An accurate duplication of the genome and segregation to the daughter cells is essential, as any unreplicated genomic regions will result in breaks and deletions during mitosis, including regions containing tumor suppressor genes, while local DNA over-replication will result in gene, and possibly oncogene, amplification (Gonzalez et al., 2005). Several DNA replication proteins, both initiator and replication fork (reviewed in (Hubscher, 2009)) proteins, have been shown to also play an essential role in several DNA repair pathways, such as base excision repair, nucleotide excision repair, and double-strand (ds) break and mismatch repair. Recent work from prokaryotes and eukaryotes has indicated that replication initiator proteins are also directly involved in multiple cellular processes (reviewed in (Scholefield et al., 2011)), coordinating the initiation of DNA replication with other cell cycle-related activities, including DNA repair (Moldovan et al., 2007; Oakley and Patrick, 2010). DNA repair, like all major cellular functions, including transcription and DNA replication, is a tightly regulated process. This review deals with the apparent synergy between the DNA replication and repair mechanisms.

2. Mammalian DNA replication

2.1 Replication origins

Mammalian DNA replication is initiated at multiple sites (estimated to be about 10^4-10^6), termed replication origins, and proceeds bidirectionally (reviewed in (Aladjem, 2007; Arias and Walter, 2007; Rampakakis et al., 2009a; Sclafani and Holzen, 2007; Zannis-Hadjopoulos, 2005)). Clusters of adjacent origins are activated at different times throughout S phase and are replicated in a defined spatial and temporal order. Replication origins are marked by the presence of a mammalian consensus sequence (Di Paola et al., 2006) throughout the genome and the binding of initiator proteins (IPs), which unwind the DNA and recruit additional downstream proteins. Origin activation starts with the binding of an IP to specific recognition sequences, triggering melting at the origin, leading to the formation of a stable pre-replication complex (pre-RC) that contains locally unwound DNA (Bell and Dutta, 2002; Dutta and Bell, 1997) and promoting the assembly of the multienzyme complexes required

for replication. The timing and frequency of initiation may be regulated by the availability of the IP or by topological changes in the DNA that affect the IP's ability to interact with the origin (Kornberg and Baker, 1992), reviewed in (Rampakakis et al., 2010) .

2.2 Pre-replication complex (Pre-RC)

The first initiator protein to bind to the origin and the best characterized is the hexameric origin recognition complex (Orc1-6; reviewed in (Sasaki and Gilbert, 2007)). All ORC subunits, except for ORC6, belong to the superfamily of AAA^+ ATPases (*A*TPases *A*ssociated with various cellular *A*ctivities) with conserved Walker A, B, C and D motifs (Bell and Dutta, 2002; Koonin, 1993). ORC acts as landing pad for the binding of additional replication proteins during G_1-phase, such as Cdc6, another AAA^+-ATPase. ATP binding of Cdc6 leads to a conformational change that promotes its association with chromatin (Tatsumi et al., 2000). Binding of Cdc6 to DNA-bound ORC leads to the activation of the ORC1 ATPase activity (Bell and Dutta, 2002) as well as to a conformational change, which increases the stability and specificity of the ORC-Cdc6-DNA complex [Mizushima, 2000 #5780; Speck, 2005 #9948; Speck, 2007 #9958]. Origin-bound Cdc6 facilitates the recruitment of Cdt1, which physically interacts with components of the putative DNA helicase, the minichromosome maintenance protein complex (MCM2-7), participating in their nuclear translocation and chromatin loading (Cook et al., 2004; Nishitani et al., 2000; Tanaka and Diffley, 2002b; Yanagi et al., 2002) as well as with Cdc6 (Dhar et al., 2001; Nishitani et al., 2000).

Following MCM loading onto ORC-Cdc6, Cdc6 and Cdt1 dissociate from the origins and, finally, ATP hydrolysis by ORC completes the MCM helicase loading reaction (Randell et al., 2006; Speck et al., 2005; Speck and Stillman, 2007). At this stage, origins are primed and awaiting the activity of the cyclin-dependent kinases (CDKs) in order to be activated and S-phase to begin. Activation of the pre-RC to an active initiation complex is regulated by CDKs and other signaling proteins, which promote further protein assembly that eventually leads to the loading of the polymerases and the activation of the MCM helicase.

Upon entry into S phase, multiple mechanisms ensure that the replication initiation machinery is inactivated so as to avoid re-replication of chromosomal regions and genome instability (Blow and Dutta, 2005; Dorn et al., 2009; Hook et al., 2007; Krasinska et al., 2008; Rampakakis et al., 2009a) and references therein.

2.3 The replisome

Entry into S phase is accompanied by the activation of the replisome, a multiprotein complex that unzips the parental helix and duplicates the separated strands. The core components of the eukaryotic replisome include the putative replicative helicase MCM2-7 complex, which encircles the leading DNA strand, the primase/polymerase α complex, the single-strand DNA (ssDNA) binding protein RPA, the clamp loader replication factor C (RFC; or replication protein C, RPC), the proliferating cell nuclear antigen (PCNA) sliding clamp, and the replicative DNA polymerases δ and ε, as well as the more recently identified Cdc45 and GINS proteins (Sheu and Stillman, 2006; Yabuuchi et al., 2006) (Figure 1).

The structure of the eukaryotic putative MCM helicase has been deduced by using as models the atomic structure of the N-terminus of the MCM protein from *Methanobacterium thermoautotrophicum* (Mth-MCM) and the SV40 T antigen (Fletcher et al., 2003; Gomez-Llorente et al., 2005; Li et al., 2003; Pape et al., 2003; Sclafani et al., 2004). Mth-MCM is a true homologue of the eukaryotic MCM, while the SV40 T antigen is an analogue, resulting from

convergent evolution. Using this reconstructive method the MCM helicase is believed to be a planar, double hexamer in head-to-head conformation. The N-terminal domain is believed to be responsible for oligomerization and DNA binding, while the C-terminal contains the catalytic ATPase and helicase domains. In agreement with this model, using electron microscopy (EM), the eukaryotic MCM complex was shown to have a central large opening between the two hexamers (34 Å), which is thought to accommodate dsDNA participating in its unwinding (Yabuta et al., 2003).

Purification of *in vivo* MCM complexes in human cells led to the identification of a MCM4/6/7 subcomplex with ATPase, ssDNA-binding, dsDNA-binding and helicase activities. This subcomplex is believed to be the catalytic core of the MCM hexamer, while MCM2/3/5 represent the regulatory subunits (Ishimi, 1997; Ishimi et al., 1996). This model was further confirmed by *in vitro* reconstitution experiments using recombinant MCM subunits from yeast, frog and mouse cells (Schwacha and Bell, 2001; Ying and Gautier, 2005; You et al., 2002). However, the helicase activity of the MCM complex was shown to be very weak and not as processive as one would expect from the replicative helicase (Patel and Picha, 2000). This was later explained by the fact that the MCM helicase activity is greatly enhanced by the Cdc45 and GINS co-factors in both *X.laevis* (Masuda et al., 2003; Pacek and Walter, 2004) and *D.melanogaster* (Moyer et al., 2006).

Cdc45 binds onto origins after MCM recruitment, but prior to DNA unwinding and polymerase recruitment [Walter, 2000 #9377;Mimura, 2000 #10138] as well as travels with the replication fork (Aparicio et al., 1999), thus being important for both replication initiation and fork progression [Tercero, 2000 #6212;Zou, 2000 #6499].

GINS is a recently identified member of the replisome composed of the Sld5, Psf1, Psf2 and Psf3 proteins. It has a ring-like structure in the electron microscope and functions interdependently with Cdc45 in the loading of the replisome, including the DNA polymerases and RPA (Aparicio et al., 1999; Kubota et al., 2003; Takayama et al., 2003) and, possibly, the coupling of MCM with other factors at DNA replication forks (Labib and Gambus, 2007).

Upon synthesis of the initial RNA primer by the DNA primase, RFC, an arc-shaped complex of five essential AAA+ type ATPases, recognizes the 3′ ends of the template-primer and loads the proliferating cell nuclear antigen (PCNA) in an ATP-binding dependent manner. PCNA is a homotrimeric ring-shaped complex, which encircles DNA and acts as a sliding clamp able to slide freely in both directions. The PCNA ring tethers polymerases δ and ε firmly to DNA, increasing their processivity from 10-15bp to thousands of nucleotides (Ayyagari et al., 1995), and functions as a moving platform for factors involved in replication-linked processes such as DNA repair, chromatin remodelling and epigenetic inheritance (Moldovan et al., 2007).

3. Interplay between DNA replication and repair proteins

Several proteins that are part of the multi-protein replication complex, but are not a member of the pre-RC, have a dual role in DNA replication and repair, such as PCNA (Dimitrova et al., 1999; Moldovan et al., 2007), the Replication Protein A (RPA)(Chesnokov, 2007) and the multifunctional Ku protein (reviewed in (Rampakakis et al., 2009a).

3.1 Proliferating cell nuclear antigen (PCNA)

PCNA, the DNA polymerase processivity factor, associates with replication foci at the onset of S-phase, co-localizes with early-replicating chromatin and is present at initiating

replication forks (Moldovan et al., 2007; O'Keefe et al., 1992). In addition to tethering polymerases δ and ε to DNA, it acts as a landing pad for a large number of factors related to DNA metabolism. Together with its loader RFC (Replication Factor C) they are essential players for processive replication and coordinated DNA repair (Bylund et al., 2006).
Encounter of the replication machinery with DNA lesions can be deleterious as it may result in fork stalling and possibly chromosomal rearrangements or even cell death, if it is prolonged. In response to this, a PCNA-mediated bypass mechanism is activated, named translesion synthesis (TLS). TLS involves the temporary switch from the replicative polymerases δ and ε to error-prone polymerases, such as pol η, with large enough active sites which can accommodate DNA lesions, thus allowing their bypass (Moldovan et al., 2007). Error-free TLS has also been found but its mechanism is still unknown. Hoege et al. showed that post-translational modification of PCNA with ubiquitin is an important process during TLS (Hoege et al., 2002); in fact, a "switch" mechanism was described according to which PCNA mono-ubiquitilation activates the error-prone TLS, whereas PCNA poly-ubiquitilation triggers the error-free TLS. In agreement, human Polη was found to interact specifically with monoubiquitylated PCNA upon UV-induced photodamage (Kannouche et al., 2004).
A role for PCNA in the mismatch repair (MMR) of complementary base mismatches or insertion/deletion loops through direct interaction with the MSH3, MSH6 and MLH1 sensor proteins and exonuclease I (EXOI) has also been shown. The current MMR model involves the recognition of the error-containing newly synthesized DNA strand through the presence of a gap, such as the end of the Okazaki fragment, and the directional orientation of PCNA followed by the excision of the defective strand in the 5′ to 3′ direction by EXOI (Modrich, 2006). A different mode of function of the MMR machinery was also proposed by Kadyrov et al., who showed that MutLα (MLH1/PMS2) is a latent endonuclease activated by MutSα, RFC and PCNA in a mismatch- and ATP-dependent manner. Consequently, a mismatch-containing DNA segment flanked by two strand breaks is removed by EXOI and replaced upon targeting of the DNA synthesis machinery (Kadyrov et al., 2006).
Finally, PCNA functions as a scaffold for factors functioning in base excision repair (BER). More specifically, PCNA has been shown to interact with the UNG2, MPG, and NTH1 DNA glycosylases, as well as the APE2 AP endonuclease, stimulating their ability to generate abasic sites and cleave them in order for repair to take place (Ko and Bennett, 2005; Oyama et al., 2004; Tsuchimoto et al., 2001; Xia et al., 2005). An interaction between PCNA and the structure-specific repair endonuclease xeroderma pigmentosum (XP) G was also found, suggesting a function in nucleotide excision repair (NER) (Gary et al., 1997), but in this case PCNA is recruited by XPG upon nucleotide excision by ERCC1, resulting in the gap filling by polymerase δ (Mocquet et al., 2008).

3.2 Replication protein A (RPA)

RPA is the major eukaryotic single-stranded (ss) DNA binding protein and it is required for DNA replication, recombination and repair. RPA helps recruit DNA primase/polymerase α to the origins, stabilizing ssDNA in the proper extended conformation so that it can be copied by DNA primase, and stimulates its polymerase activity and processivity (Maga et al., 2001). Furthermore, during replication fork progression, RPA stimulates the replicative polymerases δ and ε, possibly through its interaction with PCNA (Dianov et al., 1999; Loor et al., 1997).

Parallel to its function in DNA replication RPA participates in a variety of nuclear metabolism repair processes, involving single-stranded DNA through a complex network of protein-protein interactions. RPA has been shown to play a role in nucleotide excision repair (NER) through its interaction with the XPF-ERCC1 and XPG endonucleases, positioning them at the 5′ and 3′ of the lesions, respectively (Bessho et al., 1997; De Laat et al., 1998; He et al., 1995; Stigger et al., 1998). Furthermore, RPA has been shown to stimulate the base excision repair (BER) of abasic sites in DNA as well as the excision process during mismatch repair (MMR), by binding the human DNA glycosylases UNG2 and hMYH, or the hExoI, respectively (Dianov et al., 1999; Genschel and Modrich, 2003; Nagelhus et al., 1997; Parker et al., 2001). Finally, a role for RPA has also been suggested in the repair of double-strand DNA breaks (DSBs) at stalled replication forks through homologous recombination. More specifically, RPA was shown to protect the ssDNA after DNA strand resection and 3′ DNA overhang generation at DSBs upon hydroxyurea-induced replication stalling, recruit RAD52 through direct interaction and act as a nucleation point for the RAD51 and RAD52 proteins (Sleeth et al., 2007).

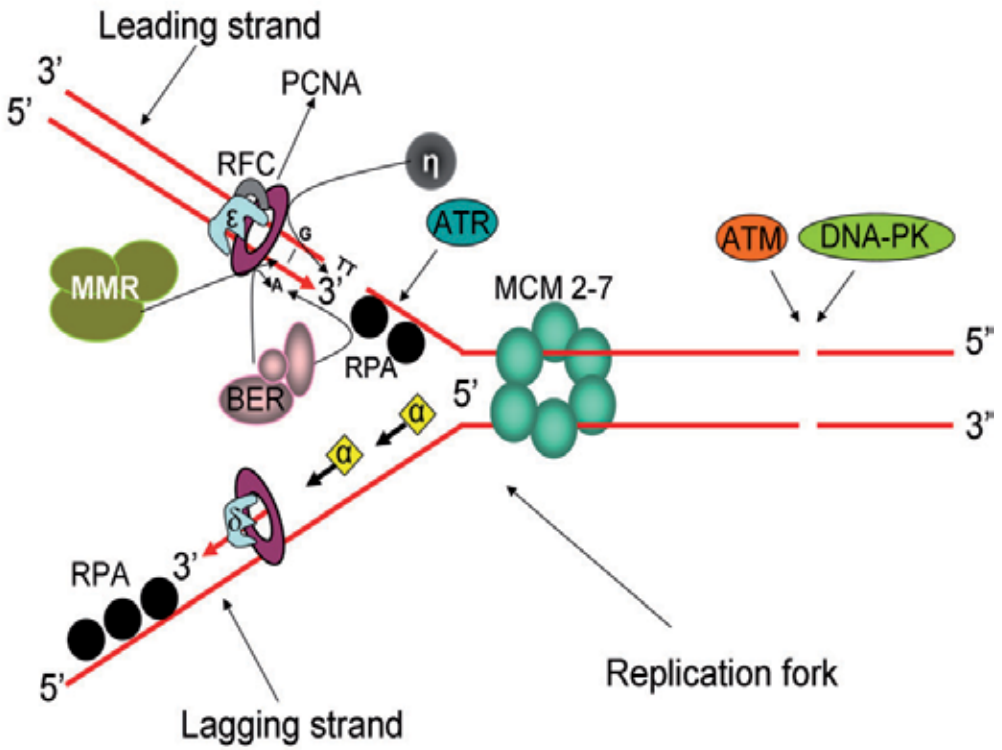

Fig. 1. Interplay between the DNA replication and DNA repair machineries. Encounter of the replication fork with various types of damaged DNA results in the recruitment of DNA repair enzymes and triggers the activation of cell cycle checkpoints, cell cycle arrest or apoptosis.

3.3 The Ku protein

The heterodimeric Ku protein (Ku70/Ku80; reviewed in (Tuteja and Tuteja, 2000)) is a multifunctional guard of the genome, participating in DNA replication and repair, recombination, telomeric maintenance, and the suppression of chromosomal rearrangements (Downs and Jackson, 2004; Zannis-Hadjopoulos et al., 2004). Ku is a member of the non-homologous end-joining (NHEJ) machinery, participating in the repair of double-strand DNA breaks (DSBs) by recruiting and allosterically activating the DNA-dependent protein kinase (DNA-PK) (Collis et al., 2005), as well as of the DNA replication licensing machinery, binding onto mammalian DNA replication origins at the end of G1-phase (Novac et al., 2001) and recruiting the DNA replication machinery (Rampakakis et al., 2009a; Rampakakis et al., 2008; Sibani et al., 2005b).

3.3.1 Ku and mammalian DNA replication

There has been a lot of accumulated evidence implicating the Ku protein in the initiation of mammalian DNA replication. Ku was initially identified as the DNA-dependent ATPase purified from HeLa cells (Cao et al., 1994), which co-fractionated with a 21S multiprotein complex that is able to support SV40 *in vitro* DNA replication (Vishwanatha and Baril, 1990). It was subsequently shown to co-immunoprecipitate with well characterized DNA replication proteins involved in either the initiation or the elongation phase, such as DNA polymerases α, δ and ε, PCNA, topoisomerase II, RF-C, RP-A, and ORC-2 (Matheos et al., 2002). In agreement with and corroborating the previous studies, a proteomic analysis using a TAP affinity purification procedure, identified Ku as part of a complex with MCM2-7 proteins, the putative replicative DNA helicase (Burckstummer et al., 2006). Furthermore, Ku was identified as part of a human protein initiation complex, important for the replication of Kaposi's sarcoma associated HSV (KHSV) (Wang et al., 2008).

Ku is an origin binding protein, binding to several replication origins, among them the adenovirus type 2 origin (de Vries et al., 1989), the Herpes Simplex Virus Type 1 (HSV1) origin (Murata et al., 2004), the B48 human origin (Toth et al., 1993), the mammalian replication origin consensus sequence, A3/4 (Price et al., 2003; Ruiz et al., 1999), the Chinese hamster dihydrofolate reductase (DHFR) replication origin, oriβ, and the monkey replication origins ors8 and ors12 (Novac et al., 2001), as well as the human origins lamin B2, β-globin, c-myc (Sibani et al., 2005a, b) and dnmt1 (DNA-methyltransferase) (Araujo et al., 1998). Ku was shown to associate *in vivo* with replication origins in a cell cycle dependent manner (Novac et al., 2001; Ruiz et al., 1999; Sibani et al., 2005a) and its differential binding to DNA is a determining factor in its involvement in DNA replication, exhibiting distinct origin DNA binding properties from its association with DNA ends or other internal DNA sequences (Schild-Poulter et al., 2003).

The role of Ku in DNA replication is believed to be two-fold. First, with regard to the initiation of DNA replication, Sibani et al. showed that Ku binds to human replication origins prior to the ORC assembly and Ku-deficiency results in decreased origin usage and initiation of DNA replication (Sibani et al., 2005a, b). A possible mechanism for this was recently proposed, involving the DNA topology machinery. Topoisomerases I and II, the major constituents of the DNA topology machinery, were previously found to interact with the lamin B2 origin and participate in their activation (Abdurashidova et al., 2007). Recently, Rampakakis et al. showed that the binding of Ku and Topo IIβ to the human replication origins lamin B2 and hOrs8 (in a complex also containing DNA-PK and PARP-1) is

associated with a transient, site-specific dsDNA break at these origins, which leads to local topological changes and recruitment of the replication initiator machinery (Rampakakis et al., 2009a). As the DNA topology and NHEJ machineries have reverse enzymatic activities, generating and repairing DNA DSBs, respectively, their functional synergy in replication origin activation is striking. A possible scenario is that Ku functions in tethering Topo IIβ onto replication origins, thus increasing the sequence specificity of its cleaving enzymatic activity (Figure 2), in a manner similar to that shown for RAG recombinases, which have similar enzymatic properties to DNA topoisomerases (Sawchuk et al., 2004). Alternatively, recruitment of DNA-PK by Ku and repair of the DSBs through NHEJ may function as a backup mechanism, ensuring chromosomal stability in cases of Topo II malfunction.

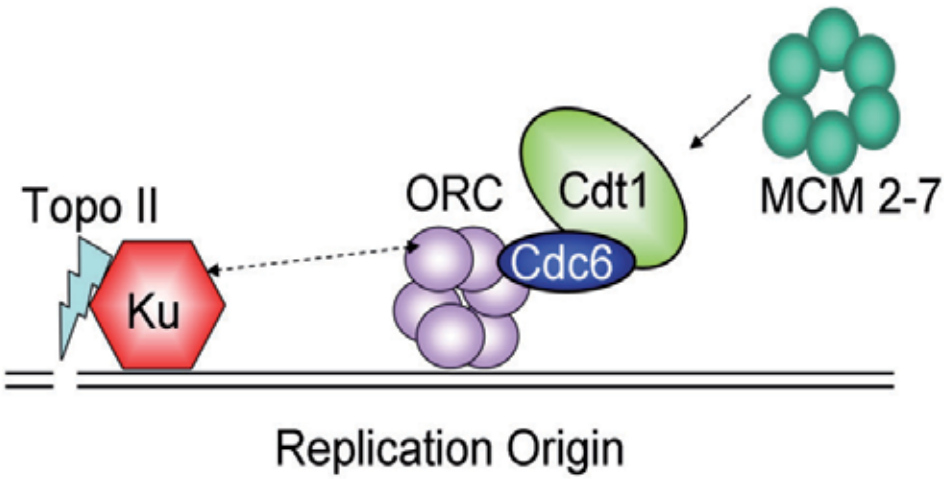

Fig. 2. Model for the role of Ku in pre-RC assembly. Targeting of Ku and Topoisomerase II onto chromatin during G1 phase leads to topologic changes in the chromosomal regions that correspond to replication origins, facilitating the assembly/stability of the ORC hexamer.

Second, at the replication fork progression level, Park et al. showed that upon IR-induced DNA damage, Ku-, but not DNA-PKcs-, deficient cells exhibited significantly slow S phase progression due to collapse of PCNA from the replication fork (Park et al., 2004). These results led the authors to suggest a role for Ku in maintaining the sliding clamp on chromatin at chromosomal breaks, thus facilitating efficient resumption of DNA replication. In agreement with a role for Ku in the replication fork progression, Hoek et al. showed that Ku directly associates with the chromatin assembly factor 1 (CAF-1) (Hoek et al., 2011), the primary DNA replication-coupled histone deposition factor, which is attached to the replication fork through PCNA (Shibahara and Stillman, 1999). Although no functional evidence was shown, the authors suggested that the significance of this interaction may involve the recruitment of CAF-1 to sites of DSBs in order to establish the appropriate local chromatin structure, which would allow cell cycle progression. Finally, a DNA-PKcs dependent role for Ku was also shown during DNA replication (Shimura et al., 2007). Using the DNA replication inhibitor aphidicolin to transiently perturb DNA replication, Shimura et al. showed that persistent DNA breaks accumulated in DNA-PKcs deficient cells, resulting in the activation of an ATR-mediated S-phase checkpoint and blockage of cell cycle

progression. In contrast, their wild-type cells continued to synthesize DNA and were able to promptly repair the DNA breaks, suggesting a role of DNA-PK in immediately repairing DNA breaks following deceleration of DNA replication.
Altogether these results suggest that, in addition to its role in repairing dsDNA breaks that occur during replication fork progression (Shimura et al., 2007), Ku is also involved in the prevention of DNA breaks caused by replication fork collapse by: i) binding onto DNA replication origins at G1 phase (Novac et al., 2001; Ruiz et al., 1999), recruiting the DNA replication machinery (Rampakakis et al., 2008; Rampakakis and Zannis-Hadjopoulos, 2009; Sibani et al., 2005b) and ensuring genomic duplication and maintenance (Toth et al., 1993) (progression into S phase without the appropriate number of activated replication origins would lead to an increase of the average replicon size, resulting in stalled replication forks and chromosomal instability (Ekholm-Reed et al., 2004; Tanaka and Diffley, 2002a)); and ii) maintaining the DNA polymerase processivity factor PCNA on chromatin following ionizing radiation (Park et al., 2004).

3.4 DNA damage checkpoints

Accurate and precise genome duplication and segregation to the daughter cells is essential, as small unreplicated regions will result in breaks and deletions during mitosis, including in tumor suppressor genes, while local over-replication would result in gene, and possibly oncogene, amplification (Gonzalez et al., 2005). Thus, the cell has evolved surveillance mechanisms (cell cycle checkpoints) to monitor the proper succession of events throughout the cell cycle. The checkpoint proteins are activated following DNA lesions (Branzei and Foiani, 2008; Hakem, 2008) or insufficient replication initiator proteins (Lau and Jiang, 2006; Machida and Dutta, 2005) and arrest cells in the cell cycle in order for DNA-repair to take place.

3.4.1 Initiation of DNA replication and checkpoint activation

Low levels of replication initiator proteins, were shown to induce a blockage of cells to late G_1 phase, due to Cyclin E/Cdk2 inactivation (Machida and Dutta, 2005; Rampakakis et al., 2008), or apoptosis (Feng et al., 2003). Blockage of pre-RC assembly by overexpressing a stable form of geminin in primary fibroblasts resulted in G1 arrest with reduced Cyclin E levels and hypophosphorylated pRB (Shreeram et al., 2002). Altogether, these results suggest the existence of a G1/S checkpoint overseeing the efficient pre-RC formation. Although the significance of this checkpoint is still obscure, it is thought to protect cells from DNA replication crisis and possible aberrant genome duplication, since premature progression into S phase without the appropriate number of activated replication origins would lead to an increase of the average replicon size, resulting in stalled replication forks and chromosomal instability (Ekholm-Reed et al., 2004; Tanaka and Diffley, 2002b). In agreement with this scenario, deregulation of Cyclin E was shown to impair pre-RC formation and cause chromosome instability in human cancer cells (Ekholm-Reed et al., 2004).
Origin re-replication due to erroneous pre-RC inactivation upon S-phase entry activates a different type of cell cycle checkpoint acting at the G2/M border. Overexpression of Cdt1 or Cdc6 induces an ATM/ATR– and p53-dependent checkpoint pathway preventing re-replication (Vaziri et al., 2003). Similarly, re-replication induced by geminin depletion resulted in the activation of a G2/M checkpoint which, however, was p53-independent, but Chk1-dependent (Melixetian et al., 2004; Zhu et al., 2004). Blow et al. showed that the underlying mechanism behind this checkpoint involves the generation of short re-replicated

dsDNA strands due to head-to-tail collision of replication forks (Davidson et al., 2006). As a result, cell cycle arrest prevents cells from entry into M phase and mitotic catastrophe.

3.4.2 Replication fork progression and checkpoint activation

Replication errors in S-phase trigger changes in the cdk cycle, either blocking the cells in specific stages or causing them to succumb to apoptosis, in case of extensive damage. Inhibition of fork progression by topoisomerase inhibitors (Clifford et al., 2003; Downes et al., 1994; Mikhailov et al., 2004) or by double-strand breaks (Kastan and Bartek, 2004) leads to the activation of a G_2/M checkpoint before mitotic entry.

Due to its complexity, DNA replication during S phase is often accompanied by various types of DNA damage (Branzei and Foiani, 2008). In most cases this damage is detected by cellular surveillance mechanisms, resulting in the activation of cell cycle checkpoints and DNA repair mechanisms. Unrepaired dsDNA breaks (DSBs) or DNA lesions during G1 phase may result in the collapse of replication forks, whereas DNA lesions or gaps may induce fork stalling. ATM and DNA-PK are the main effectors of the dsDNA break-induced checkpoints, whereas ATR is mainly activated by ssDNA and stalled replication forks. DSB resection, also leads to the ATR activation due to the generation of intermediate RPA-covered ssDNA (Jazayeri et al., 2006). Recruitment of DNA-PK, ATM and ATR at damaged DNA sites induces the activation of a complex network of downstream effectors, including checkpoint kinases 1 and 2 (Chk1 and Chk2, respectively), and resulting in DNA repair (Matsuoka et al., 2007; Shrivastav et al., 2008).

3.4.3 DNA damage checkpoints and cancer

A number of studies have shown that the DNA damage and DNA replication checkpoints represent a tumorigenesis barrier and that deregulation of their constituents occurs during transformation to the malignant phenotype, allowing genomic instability and progression towards uncontrolled cellular proliferation (Bartkova et al., 2005; Bartkova et al., 2006; Holland and Cleveland, 2009; Lau et al., 2007). DSBs are considered to be among the most detrimental forms of DNA damage and can arise both from exogenous stimuli (i.e., DNA damaging agents, ionizing radiation) and endogenous processes (i.e., base oxidation due to reactive oxygen species, DNA depurination due to hydrolysis, and replication fork collapse (Branzei and Foiani, 2008). In such cases, cells elicit a DNA damage response (DDR), which consists of a biochemical cascade leading to p53 activation (Halazonetis et al., 2008). The nature of the DDR response depends on the extent of damage and can either involve repair of the damage, or cell growth arrest in the form of senescence or apoptosis (Bartkova et al., 2006; Gorgoulis and Halazonetis, 2010; Gorgoulis et al., 2005). The DDR represents an early inducible barrier in carcinogenesis that can be activated by compromised DNA replication (Halazonetis et al., 2008), which commonly coincides with oncogenic factor overexpression. Such factors include a variety of oncogenes, such as traditional ones that promote cellular growth as well as replication licensing ones (Bartkova et al., 2006; Liontos et al., 2007). Sustained production of DSBs can eventually lead to increased activation of the DDR pathway and a selective pressure for p53 inactivation. Eventually, a loss of the anti-tumor barriers takes place, leading to the emergence of genomic instability. Normal cells, on the other hand, maintain these checkpoints intact, being able to arrest in the cell cycle in response to genotoxic stress, and this disparity is an obvious target for therapeutic exploit (Lau and Jiang, 2006). Thus: i) DNA repair inhibitors represent a promising therapeutic target, either as single agents or in combination with DNA-damaging agents, depending on

the tumor genetic background with regard to the DNA repair machinery status (Antoni et al., 2007), and ii) the status of the various constituents of the DNA repair machinery could be used as a prognostic factor in many cases.

4. The role of chromatin structure

The architecture of chromatin is of central importance in cellular processes such as DNA replication, DNA repair and gene expression (reviewed in (Winkler and Luger, 2011)). Chromatin reconfiguration that occurs during embryonic DNA replication has a direct effect on reactivation of gene expression (Forlani et al., 1998), while remodeling of chromatin structure is necessary for enabling eukaryotic cell DNA repair (Groth et al., 2007). Furthermore, chromatin structure affects the selection, activation and temporal program of replication origins (Rampakakis et al., 2009b). Chromatin dynamics are directly influenced by histone modifications, affecting the association of various chromatin modifying, DNA replication, repair and transcription factors to chromatin. It was also recently shown that PCNA affects the epigenetic landscape by influencing the composition of histone modifications on chromatin (Miller et al., 2010). PCNA also recruits a large number of chromatin-modifying enzymes to DNA replication sites, including the maintenance DNA methyltranseferase DNMT1, the chromatin assembly factor CAF-1, histone deacetylases (HDACs), and WSTF-SNF2h (reviewed in (Groth et al., 2007)), thus connecting DNA replication with epigenetic inheritance (Zhang et al., 2000). Recent studies indicate that the ubiquitination and SUMOylation of PCNA regulate the manner by which eukaryotic cells respond to different types of DNA damage as well as the selection of the appropriate repair pathways (reviewed in (Chen et al., 2011)).
In view of the fact that the chromatin dynamics during DNA repair are distinct from those seen during DNA replication (Groth et al., 2007), it is very likely that high order chromatin structure also influences the activity of those proteins with a dual role in DNA replication and repair. Thus, the temporal regulation of both the expression and proper targeting of chromatin modifiers to specific DNA loci may be responsible for directing these proteins toward one or the other of their dual functions (i.e., DNA replication or repair), depending on the cellular requirements of the moment.

5. Conclusion

Accumulated evidence points to a synergy between the DNA replication and repair machineries, as several proteins are involved in both pathways. The functional significance of the synergy between DNA replication and repair proteins lies in the fact that several proteins are strategically located on the DNA and poised to carry both replication and repair functions, depending on the local environment and cellular requirements for normal functioning and survival. The existence of proteins with a dual role in DNA replication and repair is logical, economical and beneficial for the cell, allowing it to coordinate the two important processes of replication and repair, thus optimizing its likelihood of accurate genome duplication and survival.

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7. References

Abdurashidova, G., Radulescu, S., Sandoval, O., Zahariev, S., Danailov, M.B., Demidovich, A., Santamaria, L., Biamonti, G., Riva, S., & Falaschi, A. (2007). Functional interactions of DNA topoisomerases with a human replication origin. Embo J *26*, 998-1009.

Aladjem, M.I. (2007). Replication in context: dynamic regulation of DNA replication patterns in metazoans. Nat Rev Genet *8*, 588-600.

Antoni, L., Sodha, N., Collins, I., & Garrett, M.D. (2007). CHK2 kinase: cancer susceptibility and cancer therapy - two sides of the same coin? Nat Rev Cancer *7*, 925-936.

Aparicio, O.M., Stout, A.M., & Bell, S.P. (1999). Differential assembly of Cdc45p and DNA polymerases at early and late origins of DNA replication. ProcNatlAcadSciUSA *96*, 9130-9135.

Araujo, F.D., Knox, J.D., Szyf, M., Price, G.B., & Zannis-Hadjopoulos, M. (1998). Concurrent replication and methylation at mammalian origins of replication. Mol Cell Biol *18*, 3475-3482.

Arias, E.E., & Walter, J.C. (2007). Strength in numbers: preventing rereplication via multiple mechanisms in eukaryotic cells. Genes Dev *21*, 497-518.

Ayyagari, R., Impellizzeri, K.J., Yoder, B.L., Gary, S.L., & Burgers, P.M.J. (1995). A mutational analysis of the yeast proliferating cell nuclear antigen indicates distinct roles in DNA replication and DNA repair. MolCellBiol *15*, 4420-4429.

Bartkova, J., Horejsi, Z., Koed, K., Kramer, A., Tort, F., Zieger, K., Guldberg, P., Sehested, M., Nesland, J.M., Lukas, C., *et al.* (2005). DNA damage response as a candidate anti-cancer barrier in early human tumorigenesis. Nature *434*, 864-870.

Bartkova, J., Rezaei, N., Liontos, M., Karakaidos, P., Kletsas, D., Issaeva, N., Vassiliou, L.V., Kolettas, E., Niforou, K., Zoumpourlis, V.C., *et al.* (2006). Oncogene-induced senescence is part of the tumorigenesis barrier imposed by DNA damage checkpoints. Nature *444*, 633-637.

Bell, S.P., & Dutta, A. (2002). DNA replication in eukaryotic cells. Annu Rev Biochem *71*, 333-374.

Bessho, T., Sancar, A., Thompson, L.H., & Thelen, M.P. (1997). Reconstitution of human excision nuclease with recombinant XPF-ERCC1 complex. J Biol Chem *272*, 3833-3837.

Blow, J.J., & Dutta, A. (2005). Preventing re-replication of chromosomal DNA. Nat Rev Mol Cell Biol *6*, 476-486.

Branzei, D., & Foiani, M. (2008). Regulation of DNA repair throughout the cell cycle. Nat Rev Mol Cell Biol *9*, 297-308.

Burckstummer, T., Bennett, K.L., Preradovic, A., Schutze, G., Hantschel, O., Superti-Furga, G., & Bauch, A. (2006). An efficient tandem affinity purification procedure for interaction proteomics in mammalian cells. Nat Methods *3*, 1013-1019.

Bylund, G.O., Majka, J., & Burgers, P.M. (2006). Overproduction and purification of RFC-related clamp loaders and PCNA-related clamps from Saccharomyces cerevisiae. Methods Enzymol *409*, 1-11.

Cao, Q.P., Pitt, S., Leszyk, J., & Baril, E.F. (1994). DNA-dependent ATPase from HeLa cells is related to human Ku autoantigen. Biochemistry *33*, 8548-8557.

Chen, J., Bozza, W., & Zhuang, Z. (2011). Ubiquitination of PCNA and Its Essential Role in Eukaryotic Translesion Synthesis. Cell Biochem Biophys.

Chesnokov, I.N. (2007). Multiple functions of the origin recognition complex. Int Rev Cytol *256*, 69-109.

Clifford, B., Beljin, M., Stark, G.R., & Taylor, W.R. (2003). G2 arrest in response to topoisomerase II inhibitors: the role of p53. Cancer Res *63*, 4074-4081.

Collis, S.J., DeWeese, T.L., Jeggo, P.A., & Parker, A.R. (2005). The life and death of DNA-PK. Oncogene *24*, 949-961.

Cook, J.G., Chasse, D.A., & Nevins, J.R. (2004). The regulated association of Cdt1 with minichromosome maintenance proteins and Cdc6 in mammalian cells. J Biol Chem *279*, 9625-9633.

Davidson, I.F., Li, A., & Blow, J.J. (2006). Deregulated replication licensing causes DNA fragmentation consistent with head-to-tail fork collision. Mol Cell *24*, 433-443.

De Laat, W.L., Appeldoorn, E., Sugasawa, K., Weterings, E., Jaspers, N.G.J., & Hoeijmakers, J.H.J. (1998). DNA-binding polarity of human replication protein A positions nucleases in nucleotide excision repair. Genes Dev *12*, 2598-2609.

de Vries, E., van Driel, W., Bergsma, W., Arnberg, A., & van der Vliet, P. (1989). HeLa nuclear protein recognizing DNA termini and translocating on DNA forming a regular DNA-multimeric protein complex. J Mol Biol *208*, 65-78.

Dhar, S.K., Yoshida, K., Machida, Y., Khaira, P., Chaudhuri, B., Wohlschlegel, J.A., Leffak, M., Yates, J., & Dutta, A. (2001). Replication from oriP of Epstein-Barr virus requires human ORC and is inhibited by geminin. Cell *106*, 287-296.

Di Paola, D., Price, G.B., & Zannis-Hadjopoulos, M. (2006). Differentially active origins of DNA replication in tumor versus normal cells. Cancer Res *66*, 5094-5103.

Dianov, G.L., Jensen, B.R., Kenny, M.K., & Bohr, V.A. (1999). Replication protein A stimulates proliferating cell nuclear antigen- dependent repair of abasic sites in DNA by human cell extracts. Biochemistry *38*, 11021-11025.

Dimitrova, D.S., Todorov, I.T., Melendy, T., & Gilbert, D.M. (1999). Mcm2, but not RPA, is a component of the mammalian early G1-phase prereplication complex. J Cell Biol *146*, 709-722.

Dorn, E.S., Chastain, P.D., 2nd, Hall, J.R., & Cook, J.G. (2009). Analysis of re-replication from deregulated origin licensing by DNA fiber spreading. Nucleic Acids Res *37*, 60-69.

Downes, C.S., Clarke, D.J., Mullinger, A.M., Gimenez-Abian, J.F., Creighton, A.M., & Johnson, R.T. (1994). A topoisomerase II-dependent G2 cycle checkpoint in mammalian cells. Nature *372*, 467-470.

Downs, J.A., & Jackson, S.P. (2004). A means to a DNA end: the many roles of Ku. Nat Rev Mol Cell Biol *5*, 367-378.

Dutta, A., & Bell, S.P. (1997). Initiation of DNA replication in eukaryotic cells. Annual Review Cell and Developmental Biology *13*, 293-332.

Ekholm-Reed, S., Mendez, J., Tedesco, D., Zetterberg, A., Stillman, B., & Reed, S.I. (2004). Deregulation of cyclin E in human cells interferes with prereplication complex assembly. J Cell Biol *165*, 789-800.

Feng, D., Tu, Z., Wu, W., & Liang, C. (2003). Inhibiting the expression of DNA replication-initiation proteins induces apoptosis in human cancer cells. Cancer Res *63*, 7356-7364.

Fletcher, R.J., Bishop, B.E., Leon, R.P., Sclafani, R.A., Ogata, C.M., & Chen, X.S. (2003). The structure and function of MCM from archaeal M. Thermoautotrophicum. Nat Struct Biol.

Forlani, S., Bonnerot, C., Capgras, S., & Nicolas, J.F. (1998). Relief of a repressed gene expression state in the mouse 1-cell embryo requires DNA replication. Development *125*, 3153-3166.

Gary, R., Ludwig, D.L., Cornelius, H.L., MacInnes, M.A., & Park, M.S. (1997). The DNA repair endonuclease XPG binds to proliferating cell nuclear antigen (PCNA) and shares sequence elements with the PCNA-binding regions of FEN-1 and cyclin-dependent kinase inhibitor p21. J Biol Chem *272*, 24522-24529.

Genschel, J., & Modrich, P. (2003). Mechanism of 5'-directed excision in human mismatch repair. Mol Cell *12*, 1077-1086.

Gomez-Llorente, Y., Fletcher, R.J., Chen, X.S., Carazo, J.M., & San Martin, C. (2005). Polymorphism and double hexamer structure in the archaeal minichromosome maintenance (MCM) helicase from Methanobacterium thermoautotrophicum. J Biol Chem *280*, 40909-40915.

Gonzalez, M.A., Tachibana, K.E., Laskey, R.A., & Coleman, N. (2005). Control of DNA replication and its potential clinical exploitation. Nat Rev Cancer *5*, 135-141.

Gorgoulis, V.G., & Halazonetis, T.D. (2010). Oncogene-induced senescence: the bright and dark side of the response. Curr Opin Cell Biol *22*, 816-827.

Gorgoulis, V.G., Vassiliou, L.V., Karakaidos, P., Zacharatos, P., Kotsinas, A., Liloglou, T., Venere, M., Ditullio, R.A., Jr., Kastrinakis, N.G., Levy, B.*, et al.* (2005). Activation of the DNA damage checkpoint and genomic instability in human precancerous lesions. Nature *434*, 907-913.

Groth, A., Rocha, W., Verreault, A., & Almouzni, G. (2007). Chromatin challenges during DNA replication and repair. Cell *128*, 721-733.

Hakem, R. (2008). DNA-damage repair; the good, the bad, and the ugly. Embo J *27*, 589-605.

Halazonetis, T.D., Gorgoulis, V.G., & Bartek, J. (2008). An oncogene-induced DNA damage model for cancer development. Science *319*, 1352-1355.

He, Z., Henricksen, L.A., Wold, M.S., & Ingles, C.J. (1995). RPA involvement in the damage-recognition and incision steps of nucleotide excision repair. Nature *374*, 566-569.

Hoege, C., Pfander, B., Moldovan, G.L., Pyrowolakis, G., & Jentsch, S. (2002). RAD6-dependent DNA repair is linked to modification of PCNA by ubiquitin and SUMO. Nature *419*, 135-141.

Hoek, M., Myers, M.P., & Stillman, B. (2011). An Analysis of CAF-1-interacting Proteins Reveals Dynamic and Direct Interactions with the KU Complex and 14-3-3 Proteins. J Biol Chem *286*, 10876-10887.

Holland, A.J., & Cleveland, D.W. (2009). Boveri revisited: chromosomal instability, aneuploidy and tumorigenesis. Nat Rev Mol Cell Biol *10*, 478-487.

Hook, S.S., Lin, J.J., & Dutta, A. (2007). Mechanisms to control rereplication and implications for cancer. Curr Opin Cell Biol *19*, 663-671.

Hubscher, U. (2009). DNA replication fork proteins. Methods Mol Biol *521*, 19-33.

Ishimi, Y. (1997). A DNA helicase activity is associated with an MCM4, -6, and -7 protein complex. J Biol Chem *272*, 24508-24513.

Ishimi, Y., Ichinose, S., Omori, A., Sato, K., & Kimura, H. (1996). Binding of human minichromosome maintenance proteins with histone H3. J Biol Chem *271*, 24115-24122.

Jazayeri, A., Falck, J., Lukas, C., Bartek, J., Smith, G.C., Lukas, J., & Jackson, S.P. (2006). ATM- and cell cycle-dependent regulation of ATR in response to DNA double-strand breaks. Nat Cell Biol *8*, 37-45.

Kadyrov, F.A., Dzantiev, L., Constantin, N., & Modrich, P. (2006). Endonucleolytic function of MutLalpha in human mismatch repair. Cell *126*, 297-308.

Kannouche, P.L., Wing, J., & Lehmann, A.R. (2004). Interaction of human DNA polymerase eta with monoubiquitinated PCNA: a possible mechanism for the polymerase switch in response to DNA damage. Mol Cell *14*, 491-500.

Kastan, M.B., & Bartek, J. (2004). Cell-cycle checkpoints and cancer. Nature *432*, 316-323.

Ko, R., & Bennett, S.E. (2005). Physical and functional interaction of human nuclear uracil-DNA glycosylase with proliferating cell nuclear antigen. DNA Repair (Amst) *4*, 1421-1431.

Koonin, E.V. (1993). A common set of conserved motifs in a vast variety of putative nucleic acid-dependent ATPases including MCM proteins involved in the initiation of eukaryotic DNA replication. Nucleic Acids Res *21*, 2541-2547.

Kornberg, A., & Baker, T.A. (1992). DNA replication (New York, W.H. Freeman and Company).

Krasinska, L., Besnard, E., Cot, E., Dohet, C., Mechali, M., Lemaitre, J.M., & Fisher, D. (2008). Cdk1 and Cdk2 activity levels determine the efficiency of replication origin firing in Xenopus. Embo J *27*, 758-769.

Kubota, Y., Takase, Y., Komori, Y., Hashimoto, Y., Arata, T., Kamimura, Y., Araki, H., & Takisawa, H. (2003). A novel ring-like complex of Xenopus proteins essential for the initiation of DNA replication. Genes Dev *17*, 1141-1152.

Labib, K., & Gambus, A. (2007). A key role for the GINS complex at DNA replication forks. Trends Cell Biol *17*, 271-278.

Lau, E., & Jiang, W. (2006). Is there a pre-RC checkpoint that cancer cells lack? Cell Cycle *5*, 1602-1606.

Lau, E., Tsuji, T., Guo, L., Lu, S.H., & Jiang, W. (2007). The role of pre-replicative complex (pre-RC) components in oncogenesis. Faseb J *21*, 3786-3794.

Li, D., Zhao, R., Lilyestrom, W., Gai, D., Zhang, R., DeCaprio, J.A., Fanning, E., Jochimiak, A., Szakonyi, G., & Chen, X.S. (2003). Structure of the replicative helicase of the oncoprotein SV40 large tumour antigen. Nature *423*, 512-518.

Liontos, M., Koutsami, M., Sideridou, M., Evangelou, K., Kletsas, D., Levy, B., Kotsinas, A., Nahum, O., Zoumpourlis, V., Kouloukoussa, M., *et al.* (2007). Deregulated overexpression of hCdt1 and hCdc6 promotes malignant behavior. Cancer Res *67*, 10899-10909.

Loor, G., Zhang, S.J., Zhang, P., Toomey, N.L., & Lee, M.Y.W.T. (1997). Identification of DNA replication and cell cycle proteins that interact with PCNA. Nucleic Acids Res *25*, 5041-5046.

Machida, Y.J., & Dutta, A. (2005). Cellular checkpoint mechanisms monitoring proper initiation of DNA replication. J Biol Chem *280*, 6253-6256.

Maga, G., Frouin, I., Spadari, S., & Hubscher, U. (2001). Replication protein A as a fidelity clamp for DNA polymerase alpha. J Biol Chem *8*, 8.

Masuda, T., Mimura, S., & Takisawa, H. (2003). CDK- and Cdc45-dependent priming of the MCM complex on chromatin during S-phase in Xenopus egg extracts: possible activation of MCM helicase by association with Cdc45. Genes Cells *8*, 145-161.

Matheos, D., Ruiz, M.T., Price, G.B., & Zannis-Hadjopoulos, M. (2002). Ku antigen, an origin-specific binding protein that associates with replication proteins, is required for mammalian DNA replication. Biochim Biophys Acta *1578*, 59-72.

Matsuoka, S., Ballif, B.A., Smogorzewska, A., McDonald, E.R., 3rd, Hurov, K.E., Luo, J., Bakalarski, C.E., Zhao, Z., Solimini, N., Lerenthal, Y., *et al.* (2007). ATM and ATR

substrate analysis reveals extensive protein networks responsive to DNA damage. Science *316*, 1160-1166.

Melixetian, M., Ballabeni, A., Masiero, L., Gasparini, P., Zamponi, R., Bartek, J., Lukas, J., & Helin, K. (2004). Loss of Geminin induces rereplication in the presence of functional p53. J Cell Biol *165*, 473-482.

Mikhailov, A., Shinohara, M., & Rieder, C.L. (2004). Topoisomerase II and histone deacetylase inhibitors delay the G2/M transition by triggering the p38 MAPK checkpoint pathway. J Cell Biol *166*, 517-526.

Miller, A., Chen, J., Takasuka, T.E., Jacobi, J.L., Kaufman, P.D., Irudayaraj, J.M., &d Kirchmaier, A.L. (2010). Proliferating cell nuclear antigen (PCNA) is required for cell cycle-regulated silent chromatin on replicated and nonreplicated genes. J Biol Chem *285*, 35142-35154.

Mocquet, V., Laine, J.P., Riedl, T., Yajin, Z., Lee, M.Y., & Egly, J.M. (2008). Sequential recruitment of the repair factors during NER: the role of XPG in initiating the resynthesis step. Embo J *27*, 155-167.

Modrich, P. (2006). Mechanisms in eukaryotic mismatch repair. J Biol Chem *281*, 30305-30309.

Moldovan, G.L., Pfander, B., & Jentsch, S. (2007). PCNA, the maestro of the replication fork. Cell *129*, 665-679.

Moyer, S.E., Lewis, P.W., & Botchan, M.R. (2006). Isolation of the Cdc45/Mcm2-7/GINS (CMG) complex, a candidate for the eukaryotic DNA replication fork helicase. Proc Natl Acad Sci U S A *103*, 10236-10241.

Murata, L.B., Dodson, M.S., & Hall, J.D. (2004). A human cellular protein activity (OF-1), which binds herpes simplex virus type 1 origin, contains the Ku70/Ku80 heterodimer. J Virol *78*, 7839-7842.

Nagelhus, T.A., Haug, T., Singh, K.K., Keshav, K.F., Skorpen, F., Otterlei, M., Bharati, S., Lindmo, T., Benichou, S., Benarous, R., *et al.* (1997). A sequence in the N-terminal region of human uracil-DNA glycosylase with homology to XPA interacts with the c-terminal part of the 34-kDa subunit of replication protein A. JBiolChem *272*, 6561-6566.

Nishitani, H., Lygerou, Z., Nishimoto, T., & Nurse, P. (2000). The Cdt1 protein is required to license DNA for replication in fission yeast [see comments]. Nature *404*, 625-628.

Novac, O., Matheos, D., Araujo, F.D., Price, G.B., & Zannis-Hadjopoulos, M. (2001). In vivo association of Ku with mammalian origins of DNA replication. Mol Biol Cell *12*, 3386-3401.

O'Keefe, R.T., Henderson, S.C., & Spector, D.L. (1992). Dynamic organization of DNA replication in mammalian cell nuclei: spatially and temporally defined replication of chromosome-specific alpha-satellite DNA sequences. J Cell Biol *116*, 1095-1110.

Oakley, G.G., & Patrick, S.M. (2010). Replication protein A: directing traffic at the intersection of replication and repair. Front Biosci *15*, 883-900.

Oyama, M., Wakasugi, M., Hama, T., Hashidume, H., Iwakami, Y., Imai, R., Hoshino, S., Morioka, H., Ishigaki, Y., Nikaido, O., *et al.* (2004). Human NTH1 physically interacts with p53 and proliferating cell nuclear antigen. Biochem Biophys Res Commun *321*, 183-191.

Pacek, M., & Walter, J.C. (2004). A requirement for MCM7 and Cdc45 in chromosome unwinding during eukaryotic DNA replication. Embo J *23*, 3667-3676.

Pape, T., Meka, H., Chen, S., Vicentini, G., van Heel, M., & Onesti, S. (2003). Hexameric ring structure of the full-length archaeal MCM protein complex. EMBO Rep *4*, 1079-1083.

Park, S.J., Ciccone, S.L., Freie, B., Kurimasa, A., Chen, D.J., Li, G.C., Clapp, D.W., & Lee, S.H. (2004). A positive role for the Ku complex in DNA replication following strand break damage in mammals. J Biol Chem *279*, 6046-6055.

Parker, A., Gu, Y., Mahoney, W., Lee, S.H., Singh, K.K., & Lu, A.L. (2001). Human homolog of the MutY repair protein (hMYH) physically interacts with proteins involved in long patch DNA base excision repair. J Biol Chem *276*, 5547-5555.

Patel, S.S., & Picha, K.M. (2000). Structure and function of hexameric helicases. Annu Rev Biochem *69*, 651-697.

Price, G.B., Allarakhia, M., Cossons, N., Nielsen, T., Diaz-Perez, M., Friedlander, P., Tao, L., & Zannis-Hadjopoulos, M. (2003). Identification of a cis-element that determines autonomous DNA replication in eukaryotic cells. J Biol Chem *278*, 19649-19659.

Rampakakis, E., Arvanitis, D.N., Di Paola, D., & Zannis-Hadjopoulos, M. (2009a). Metazoan origins of DNA replication: regulation through dynamic chromatin structure. J Cell Biochem *106*, 512-520.

Rampakakis, E., Di Paola, D., Chan, M.K., & Zannis-Hadjopoulos, M. (2009b). Dynamic changes in chromatin structure through post-translational modifications of histone H3 during replication origin activation. J Cell Biochem *108*, 400-407.

Rampakakis, E., Di Paola, D., & Zannis-Hadjopoulos, M. (2008). Ku is involved in cell growth, DNA replication and G1-S transition. J Cell Sci *121*, 590-600.

Rampakakis, E., Gkogkas, C., Di Paola, D., & Zannis-Hadjopoulos, M. (2010). Replication initiation and DNA topology: The twisted life of the origin. J Cell Biochem *110*, 35-43.

Rampakakis, E., & Zannis-Hadjopoulos, M. (2009). Transient dsDNA breaks during pre-replication complex assembly. Nucleic Acids Res *37*, 5714-5724.

Randell, J.C., Bowers, J.L., Rodriguez, H.K., & Bell, S.P. (2006). Sequential ATP hydrolysis by Cdc6 and ORC directs loading of the Mcm2-7 helicase. Mol Cell *21*, 29-39.

Ruiz, M.T., Matheos, D., Price, G.B., & Zannis-Hadjopoulos, M. (1999). OBA/Ku86: DNA binding specificity and involvement in mammalian DNA replication. Mol Biol Cell *10*, 567-580.

Sasaki, T., & Gilbert, D.M. (2007). The many faces of the origin recognition complex. Curr Opin Cell Biol *19*, 337-343.

Sawchuk, D.J., Mansilla-Soto, J., Alarcon, C., Singha, N.C., Langen, H., Bianchi, M.E., Lees-Miller, S.P., Nussenzweig, M.C., & Cortes, P. (2004). Ku70/Ku80 and DNA-dependent protein kinase catalytic subunit modulate RAG-mediated cleavage: implications for the enforcement of the 12/23 rule. J Biol Chem *279*, 29821-29831.

Schild-Poulter, C., Matheos, D., Novac, O., Cui, B., Giffin, W., Ruiz, M.T., Price, G.B., Zannis-Hadjopoulos, M., & Hache, R.J. (2003). Differential DNA binding of ku antigen determines its involvement in DNA replication. DNA Cell Biol *22*, 65-78.

Scholefield, G., Veening, J.W., & Murray, H. (2011). DnaA and ORC: more than DNA replication initiators. Trends Cell Biol *21*, 188-194.

Schwacha, A., & Bell, S.P. (2001). Interactions between two catalytically distinct MCM subgroups are essential for coordinated ATP hydrolysis and DNA replication. Mol Cell *8*, 1093-1104.

Sclafani, R.A., Fletcher, R.J., & Chen, X.S. (2004). Two heads are better than one: regulation of DNA replication by hexameric helicases. Genes Dev *18*, 2039-2045.

Sclafani, R.A., & Holzen, T.M. (2007). Cell cycle regulation of DNA replication. Annu Rev Genet *41*, 237-280.

Sheu, Y.J., & Stillman, B. (2006). Cdc7-Dbf4 phosphorylates MCM proteins via a docking site-mediated mechanism to promote S phase progression. Mol Cell *24*, 101-113.

Shibahara, K., & Stillman, B. (1999). Replication-dependent marking of DNA by PCNA facilitates CAF-1-coupled inheritance of chromatin. Cell *96*, 575-585.

Shimura, T., Martin, M.M., Torres, M.J., Gu, C., Pluth, J.M., Dibernardi, M.A., McDonald, J.S., & Aladjem, M.I. (2007). DNA-PK Is Involved in Repairing a Transient Surge of DNA Breaks Induced by Deceleration of DNA Replication. J Mol Biol *367*, 665-680.

Shreeram, S., Sparks, A., Lane, D.P., & Blow, J.J. (2002). Cell type-specific responses of human cells to inhibition of replication licensing. Oncogene *21*, 6624-6632.

Shrivastav, M., De Haro, L.P., & Nickoloff, J.A. (2008). Regulation of DNA double-strand break repair pathway choice. Cell Res *18*, 134-147.

Sibani, S., Price, G.B., & Zannis-Hadjopoulos, M. (2005a). Decreased origin usage and initiation of DNA replication in haploinsufficient HCT116 Ku80+/- cells. J Cell Sci *118*, 3247-3261.

Sibani, S., Price, G.B., & Zannis-Hadjopoulos, M. (2005b). Ku80 binds to human replication origins prior to the assembly of the ORC complex. Biochemistry *44*, 7885-7896.

Sleeth, K.M., Sorensen, C.S., Issaeva, N., Dziegielewski, J., Bartek, J., & Helleday, T. (2007). RPA mediates recombination repair during replication stress and is displaced from DNA by checkpoint signalling in human cells. J Mol Biol *373*, 38-47.

Speck, C., Chen, Z., Li, H., & Stillman, B. (2005). ATPase-dependent cooperative binding of ORC and Cdc6 to origin DNA. Nat Struct Mol Biol *12*, 965-971.

Speck, C., & Stillman, B. (2007). Cdc6 ATPase activity regulates ORC x Cdc6 stability and the selection of specific DNA sequences as origins of DNA replication. J Biol Chem *282*, 11705-11714.

Stigger, E., Drissi, R., & Lee, S.H. (1998). Functional analysis of human replication protein a in nucleotide excision repair. JBiolChem *273*, 9337-9343.

Takayama, Y., Kamimura, Y., Okawa, M., Muramatsu, S., Sugino, A., & Araki, H. (2003). GINS, a novel multiprotein complex required for chromosomal DNA replication in budding yeast. Genes Dev *17*, 1153-1165.

Tanaka, S., & Diffley, J.F. (2002a). Deregulated G1-cyclin expression induces genomic instability by preventing efficient pre-RC formation. Genes Dev *16*, 2639-2649.

Tanaka, S., & Diffley, J.F. (2002b). Interdependent nuclear accumulation of budding yeast Cdt1 and Mcm2-7 during G1 phase. Nat Cell Biol *4*, 198-207.

Tatsumi, Y., Tsurimoto, T., Shirahige, K., Yoshikawa, H., & Obuse, C. (2000). Association of human origin recognition complex 1 with chromatin DNA and nuclease-resistant nuclear structures. J Biol Chem *275*, 5904-5910.

Toth, E.C., Marusic, L., Ochem, A., Patthy, A., Pongor, S., Giacca, M., & Falaschi, A. (1993). Interactions of USF and Ku antigen with a human DNA region containing a replication origin. Nucl Acids Res *21*, 3257-3263.

Tsuchimoto, D., Sakai, Y., Sakumi, K., Nishioka, K., Sasaki, M., Fujiwara, T., & Nakabeppu, Y. (2001). Human APE2 protein is mostly localized in the nuclei and to some extent in the mitochondria, while nuclear APE2 is partly associated with proliferating cell nuclear antigen. Nucleic Acids Res *29*, 2349-2360.

Tuteja, R., & Tuteja, N. (2000). Ku autoantigen: a multifunctional DNA binding protein. Crit Rev Biochem Mol Biol *35*, 1-33.

Vaziri, C., Saxena, S., Jeon, Y., Lee, C., Murata, K., Machida, Y., Wagle, N., Hwang, D.S., & Dutta, A. (2003). A p53-Dependent Checkpoint Pathway Prevents Rereplication. Mol Cell *11*, 997-1008.

Vishwanatha, J.K., & Baril, E.F. (1990). Single-stranded DNA-dependent ATPase from HeLa cells that stimulates DNA polymerase □-primase activity: purification and characterization of the ATPase. Biochemistry *29*, 8753-8759.

Wang, Y., Li, H., Tang, Q., Maul, G.G., & Yuan, Y. (2008). Kaposi's Sarcoma-Associated Herpesvirus ori-Lyt-dependent DNA Replication: Involvement of Host Cellular Factors in the Replication. J Virol.

Winkler, D.D., & Luger, K. (2011). The histone chaperone Fact: Structural insights and mechanisms for nucleosome reorganization. J Biol Chem.

Xia, L., Zheng, L., Lee, H.W., Bates, S.E., Federico, L., Shen, B., & O'Connor, T.R. (2005). Human 3-methyladenine-DNA glycosylase: effect of sequence context on excision, association with PCNA, and stimulation by AP endonuclease. J Mol Biol *346*, 1259-1274.

Yabuta, N., Kajimura, N., Mayanagi, K., Sato, M., Gotow, T., Uchiyama, Y., Ishimi, Y., & Nojima, H. (2003). Mammalian Mcm2/4/6/7 complex forms a toroidal structure. Genes Cells *8*, 413-421.

Yabuuchi, H., Yamada, Y., Uchida, T., Sunathvanichkul, T., Nakagawa, T., & Masukata, H. (2006). Ordered assembly of Sld3, GINS and Cdc45 is distinctly regulated by DDK and CDK for activation of replication origins. Embo J *25*, 4663-4674.

Yanagi, K., Mizuno, T., You, Z., & Hanaoka, F. (2002). Mouse geminin inhibits not only Cdt1-MCM6 interactions but also a novel intrinsic Cdt1 DNA binding activity. J Biol Chem *277*, 40871-40880.

Ying, C.Y., & Gautier, J. (2005). The ATPase activity of MCM2-7 is dispensable for pre-RC assembly but is required for DNA unwinding. Embo J *24*, 4334-4344.

You, Z., Ishimi, Y., Masai, H., & Hanaoka, F. (2002). Roles of Mcm7 and Mcm4 subunits in DNA helicase activity of mouse Mcm4/6/7 complex. J Biol Chem *30*, 30.

Zannis-Hadjopoulos, M., Sibani, S., & Price, G.B. (2004). Eukaryotic Replication Origin Binding Proteins. Frontiers in Bioscience *9*, 2133-2143.

Zannis-Hadjopoulos, M. & Price, G.B. (2005). DNA replication. Encyclopedia of Life Sciences.

Zhang, Z., Shibahara, K., & Stillman, B. (2000). PCNA connects DNA replication to epigenetic inheritance in yeast [In Process Citation]. Nature *408*, 221-225.

Zhu, W., Chen, Y., & Dutta, A. (2004). Rereplication by depletion of geminin is seen regardless of p53 status and activates a G2/M checkpoint. Mol Cell Biol *24*, 7140-7150.

3

New Insight on Entangled DNA Repair Pathways: Stable Silenced Human Cells for Unraveling the DDR Jigsaw

Biard Denis S.F.
Commissariat à l'Energie Atomique,
DSV-iRCM / INSERM U935, Villejuif
France

1. Introduction

1.1 Need for outstanding cell models for studying interwoven DNA repair pathways

During normal human cell growth, each cell is exposed to numerous DNA-damaging events. DNA lesions are mainly inflicted by endogenous insults, such as normal biochemical activities, by-product synthesis and the *in situ* production of reactive oxygen species (ROS). DNA is also subject to genotoxic injuries resulting from diverse exogenous sources. It is not surprising that living organisms have evolved numerous intricate strategies to counteract these environmental pressures and to allow living cells to thrive in aerobic conditions. Through evolution various highly sophisticated pathways for protecting the genetic information have been retained. The first lines of defense include detoxification metabolisms and defense against oxidative stress. When these caretaker processes fail to insure a correct protection of biological molecules, such as genomic and mitochondrial DNA, DNA repair pathways become the ultimate bulwark against DNA damage. However, when DNA damage is not dealt with properly, it can adversely threaten the fidelity of the genetic information and ultimately lead to hereditary diseases or neoplastic processes.

Amongst DNA lesions, DNA double-strand breaks (DSBs) are likely to be the ultimate lethal ones because unrepaired they can lead to chromosomal rearrangements, malignant transformation or apoptosis (Roos & Kaina, 2006). Endogenous DSBs mainly arise from the processing of single-strand breaks (SSBs) when they are converted to DSBs by DNA replication and/or transcription mechanisms (Mladenov & Iliakis, 2011). Given the chemical variety of DNA lesions encountered, evolution has retained a **large diversity** of DNA repair pathways and a tight interplay between DNA replication and DNA repair. While numerous DNA repair mechanisms exist, the major pathways include mismatch, excision and recombinational repair (Hoeijmakers, 2001) and some factors can participate in divergent processes. This is the case of the structure-specific endonuclease ERCC1 / XPF, which is required in two distinct mechanisms: excision (nucleotide excision repair or NER) and recombinational (single-strand annealing or SSA) repair pathways. ERCC1 / XPF endonuclease plays a critical role in NER by being recruited at the site of damaged DNA in order to cleave one strand of the damaged DNA. It is also involved in SSA, which appears to

be an alternative pathway to homologous recombination (HR) or nonhomologous end joining (NHEJ) (Al-Minawi et al., 2008). Moreover, albeit belonging to the same complex, it is hypothesized that ERCC1 and XPF have distinct functions *in vivo* because their deficiency can lead to different phenotypes in humans. For instance, the only patient carrying a mutated *ERCC1* gene exhibits a cerebro-oculo-facio-skeletal syndrome with severe neurological defects but a moderate sensitivity to UV light and mitomycin C, the hallmark of XPF patients (Jaspers et al., 2007). Furthermore, there is a large body of evidence that raises the notion that the failure of one DNA repair pathway could modify the efficiency and/or fidelity of another one. An interesting example is the **cross-talk** between the mismatch repair (MMR) and recombinational pathways. MMR appears to be an essential mechanism for guaranteeing the fidelity of DNA replication because misincorporated nucleotides have to be excised immediately after DNA synthesis. Inherited defects in the MMR trigger a spontaneous mutation rate 50- to 1000-fold higher than that observed in MMR proficient cells, with a tremendous increase of spontaneous base substitution and frameshift mutations (for review (Iyer et al., 2006)). These mutational events could facilitate illegitimate recombination between nearly-homologous sequences, contributing to the onset of hereditary nonpolyposis colon cancer (HNPCC) (for review (Iyer et al., 2006)). Human MutSα ((Msh2-Msh6 heterodimer), and MutLα (Mlh1-Pms2 heterodimer) participate in the fidelity of genetic recombination and the suppression of gene amplification (Chen et al., 2001).

Another recurring theme in the DNA repair of complex genomes, such as the mammalian genome, is the existence of proteins with partly overlapping activities. This **genetic redundancy** appears essential for maintaining the stability of a complex genome but this represents a major drawback for experimental approaches designed to unravel the specific functions of a particular DNA repair protein. A classic example arises from the repair of uracil by BER. BER includes a recognition step which is performed by specific uracil-DNA glycosylases, following by a synthesis step conducted by the DNA polymerase beta (Pol β). Different DNA glycosylases travel down the DNA molecule scanning for potential lesions (Sartori et al., 2002), and gene redundancy might make it difficult to generate uracil glycosylase–deficient cells because there are several genes in the mammalian genome that encode enzymes able to excise uracil from DNA (Pearl, 2000). Another example is the PARP family where PARP1 and PARP2 possess partially redundant functions as well as divergent activities (Menissier de Murcia et al., 2003, Schreiber et al., 2002). This functional partial overlap explains the survival of human cells when either the *PARP1* or *PARP2* gene is silenced (see below); in contrast double *PARP1* and *PARP2* knockdown leads to cell death (unpublished data). Fortunately, the genetic redundancy and the overlap between DNA repair pathways support the notion that **compensating repair activities** can take place over time. This is essential to understanding of DNA damage response (DDR)–deficient human cells, but also cells handled *in vitro,* such as knockdown cells.

An additional point in the complex study of DNA repair factors is that several of them are involved in **other physiological pathways**, even in the absence of DNA damage. This is the case for certain of the NER factors and their tight relationship with the transcriptional machinery (Le May et al., 2010a, Le May et al., 2010b). This is also observed when different DNA repair pathways are key building blocks in the primary and secondary antibody diversification processes taking place in B lymphocytes (for review (Durandy, 2009)).

Actually, BER, D-NHEJ (classic DNA PKcs-dependent NHEJ), b-NHEJ (backup NHEJ), MMR and DNA damage signaling factors actively contribute to immunoglobulin diversification.

These compelling data explain why a mutation of one DNA repair gene could trigger fetal or embryonic death or lead to a dramatic hereditary disorder. Human syndromes where one DNA repair gene is mutated have been collectively classified as "*DDR-defective syndromes*". The range of clinical features associated with these disorders attests to the complexity of the DDR, its redundancy and its connection with other essential processes. That explains the diversity of the phenotypes observed in patients with DNA repair disorders (**Table 1**). It turned out that growing evidence demonstrates that ubiquitylation of key proteins is critically involved in the emergence of DDR-defective syndromes as observed for NER (DDB2), HR (FancD2) or TLS (PCNA). (for review (Huang & D'Andrea, 2006)). The pivotal role of DNA repair pathways during normal human development explains one hallmark of numerous DDR-defective syndromes. Actually, the main clinical features observed in numerous DDR-defective syndromes are hematopoietic defects (e.g. anemia or immunodeficiency) and neurological deficits (e.g. microcephaly), in parallel with genomic instability and specific DNA damage-induced sensitivities. This highlights crosslinks between DNA repair mechanisms and either neuronal development (O'Driscoll & Jeggo, 2008) or immunoglobulin diversification processes (for review (Durandy, 2009)). Hence, numerous DDR-defective disorders exhibit microcephaly, such as LIG4 syndrome (*DNA ligase IV* gene) but also XLF-Cernunnos-SCID (*XLF-Cernunnos* gene), Seckel syndrome (*ATR* gene), Nijmegen breakage syndrome (*NBS1* gene), Fanconi anemia (*FancD1/BRCA2* gene), Bloom syndrome (*BLM* gene), Cockayne syndrome (*CSA, CSB, XPB, XPD* and *XPG* genes), Xeroderma pigmentosum (*XPA* to *XPG* genes), and cerebro-oculo-facio-skeletal syndrome (*ERCC1* gene) ((Jaspers et al., 2007); for review (McKinnon, 2009, O'Driscoll & Jeggo, 2008)). LIG4 syndrome and XLF-Cernunnos-SCID also exhibit multiple immune abnormalities because both LigIV and XLF/Cernunnos are involved in the V(D)J and NHEJ pathways which are required during the primary repertoire of antibodies and the secondary diversification processes (Yan et al., 2007).

1.2 Long term silenced human cells

A better understanding of these hereditary disorders requires detailed insight into each DNA repair pathway that can operate on the damaged genome. Furthermore, the importance of the DDR during the multistage process leading to tumorigenesis emphasizes the need for outstanding biological tools to study DNA repair genes. Altogether this compelling evidence points to the need of outstanding cell models for unraveling the DDR jigsaw both for fundamental research and for the development of novel therapeutic strategies.

Over the last seven years we have developed a rational strategy to silence the main DNA repair factors so as to unveil their functions. Since the emergence of the RNA interference technology, many studies have developed transient or middle-term gene silencing experiments targeting DDR genes, but few of them have characterized stable clones. Our project is based on the exceptional efficiency of pEBVsiRNA vectors in ensuring stable gene silencing. Our approach has been extensively described previously (Biard, 2007, Biard & Angulo, 2007).

Pathways	Diseases or syndromes	Mutated genes (targeted genes*)	Main symptoms and/or remarks
Cell cycle control	Li-Fraumeni syndrome	**p53**	Sarcoma, breast, brain, leukemia
	familial retinoblastoma	**Rb**	Retinoblastoma, osteosarcoma
	familia melanoma	**p16**	Melanoma, pancreas cancers
	Sporadic cancers	**Chk1**	Colorectal, stomach, lung, endometrial, melanoma, mesothelioma cancers (for review (Solyom et al., 2010))
	Li-Fraumeni syndrome	**Chk2**	Breast, lung, colon, urinary, bladder, testis cancers, melanoma
Signaling pathways	Ataxia telangiectasia	**ATM**	Neurodegeneration, sterility, telangiectasia, dysarthria, immunological defects, sensitivity to IR, lymphomas
	ATR-Seckel syndrome	**ATR**	Microcephaly and mental retardation, growth defects
	Ataxia telangiectasia-like disorder	**MRE11**	Ataxia, neurodegeneration, dysarthria and oculomotor apraxia, mild immunological defects, lymphomas
	Familial breast cancer 1 & 2	**BRCA1, BRCA2**	Chromosome instability, sensitivity to DNA damage, HR deficiency, cancer
	Nijmegen breakage syndrome	**NBS1**	Microcephaly, immunological defects and lymphoid malignacy, lymphomas
	NBS-like disorder (NBSlD)	**Rad50**	
DNA DSB repair	LIG4 syndrome	**LIG4**	Microcephaly, developmental/growth delay, immunodeficiency and lymphomas
	Human immunodeficiency with microcephaly	**XLF/Cernunnos**	Microcephaly, immunodeficiency
	glioblastoma (M059J cells)	**DNAPKcs**	(Allalunis-Turner et al., 1993)
	Fanconi anaemia	**BCRA2**	Microcephaly and medulloblastoma, Bone marrow and congenital defects
DNA SSB repair	Spinocerebellar ataxia with axonal neuropathy	**TDP1**	Ataxia, neurodegeneration, peripheral axonal motor, and sensory neuropathy, and muscle weakness…
	Ataxia with oculomotor apraxia 1	**APTX**	Ataxia, neurodegeneration, oculomotor apraxia and peripheral neuropathy, hypercholesterolaemia and hypoalbuminaemia
	cerebro-oculo-facio-skeletal syndrome	**ERCC1**	Microcephaly, moderate sensitivity to UV and mitomycin C (Jaspers et al., 2007)
NER	Xeroderma pigmentosum (XP)	**XPA to XPG**	Neurodegeneration and microcephaly, UV sensitivity and skin cancer
	Cockayne syndrome (CS)	**CSA, CSB, XPB, XPD, XPG**	Microcephaly and dysmyelination , TCR-specific disorder. Segmental progeria, no increase in cancer incidence
	Trichothiodystrophy (TDD)	**XPD, XPB, TTD-A**	Neurodevelopmental defects and dysmyelination, brittle hair, nails and scaly skin. Segmental progeria without an increase in cancer incidence (Giglia-Mari et al., 2004)

Pathways	Diseases or syndromes	Mutated genes (targeted genes*)	Main symptoms and/or remarks
TLS	XP variant (XP-V)	**XPV (pol iota)**	UV-induced skin cancer
DNA cross link repair & Ubiquitin ligase	Fanconi anaemia	**FancA, B, C, D1 (BRCA2), D2, E, F, G, I, J, L, M, N**	Microcephaly and medulloblastoma, cervical cancer, brain tumours i(FANCD2 and FANCN), anaemia, developmental defects, ovarian carcinomas, head and neck squamous cell carcinomas, bone marrow failure, and myeloid leukemias.
BER	Multiple colorectal adenoma and carcinoma	**MutYH**	DNA glycosylase involved in the repair of oxidative damage (Al-Tassan et al., 2002)
	Ligase I	**Ligase I**	Iimmunodeficiencies and cellular hypersensitivity to DNA-damaging agents (Barnes et al., 1992)
MMR	HNPCC	**MLH1, MLH3, MSH2, MSH6, PMS2**	Hereditary nonpolyposis colon cancer, rectum, gastric, endometrium, ovarian, urinary organ cancers (Peltomaki, 2003)
Helicase	Werner syndrome	**WRN**	Severe progeria, various cancers
	Rothmund Thomson syndrome	**RTS**	Osteosarcomas
	Bloom syndrome	**BLM**	Proportional dwarfism, leukemias, lymphomas and others cancer
	Ataxia with oculomotor apraxia 2	**SETX**	Ataxia, neurodegeneration and oculomotor apraxia

(*: genes targeted with pEBVsiRNA plasmids)

Table 1. Main DDR defective Syndromes. (Adaptated from (Hoeijmakers, 2001) and (McKinnon, 2009)).

Briefly, for each gene, three pEBVsiRNA vectors are constructed and validated through both short-term (several days) and long-term (several weeks) experiments. Afterwards, we used only one "validated" vector to establish stable clones (**Fig. 1**). Four years ago we adopted the DSIR program developed by Vandenbrouck and collaborators (Vert et al., 2006) to design shRNA sequences. This program includes an exact similarity search algorithm for potential off-target detection. In a recent comparison of methods for a rational siRNA design, DSIR is among the three best predictive programs (Matveeva et al., 2007). Our siRNA sequences mainly target the open reading frame of the targeted genes, but when necessary we also use siRNA sequences stretching to the 3'-UTR (e.g. for rescue experiments). Among the targeted genes and in using our approach we have always obtained at least one vector able to impose long-term shut down greater than 80% as compared with control cells (as evidenced by real-time RT-PCR).

Using this technology, more than 160 human genes in different human cell models such as HeLa (Ame et al., 2009, Amine et al., 2009, Aressy et al., 2008, Betous et al., 2009, Biard, 2007, Biard et al., 2005, Biard & Angulo, 2007, Boehler et al., 2011, Bouley et al., 2010, Britton et al., 2009a, Despras et al., 2007, Godon et al., 2008, Le May et al., 2010b, Ousset et al., 2010, Pennarun et al., 2008, Pennarun et al., 2010, Wu et al., 2007), U2OS (Betous et al., 2009, Rey et al., 2009) and MRC5-V1 (Bouquet et al., 2011, Britton et al., 2009b, Schmutz et al., 2010) cells have been silenced. Our approach has also been successfully tested in other human tumor-derived cell lines, such as RKO (Biard & Angulo, 2007), HCT-116 (Aressy et al., 2008), Caco2 (Coant et al., 2010), SH-SY5Y cells (Schulte et al., 2008), MCF7, MDA-MB 231, K562, UT7

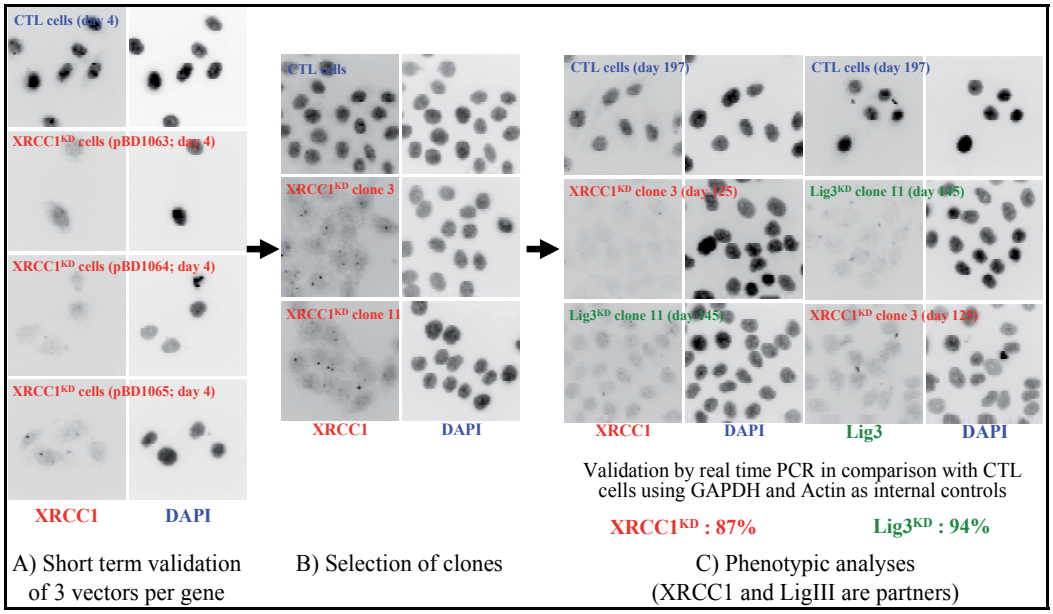

Fig. 1. Establishment of stable clones.

(papers in preparation), and even in mouse NIH-3T3 cells (Meulle et al., 2008). Some authors have previously suggested the importance of "position-specific" criteria for efficient gene silencing. With the benefit of hindsight, we have never observed such a positioning effect in either short-term (*few days*) or long term (*several months*) experiments. In **Figure 2** we show the position of different siRNA sequences able to impose a very efficient long-term gene silencing along a representative mRNA and we demonstrate no positioning effect.

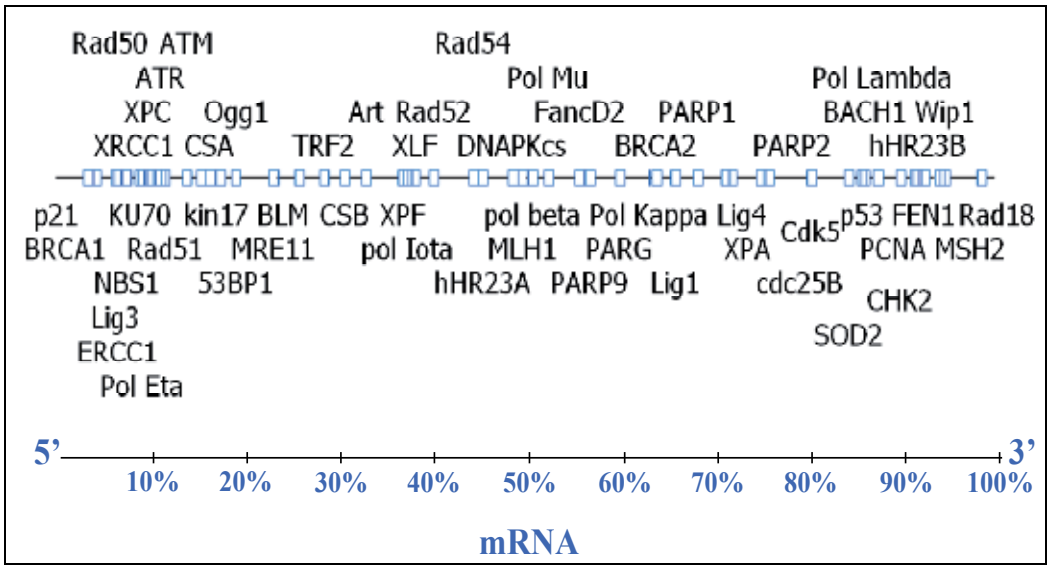

Fig. 2. Position of validated siRNA sequences along a representative mRNA.

The maintenance of stable gene silencing for several months affords the opportunity to validate different siRNA sequences for an unfailing and specific gene silencing. Importantly, transient assays may mask the real effects of gene silencing, due to the saturation of the RNAi (and miRNA) machinery and by side-effects resulting from the high siRNA concentrations currently used. In the long-term experiments, we do not exclude the possibility of skews, and the suppression of gene expression over a long period may provoke compensatory cellular responses during an "adaptive period". During this period, cellular metabolism may compensate for the decrease in protein concentration, particularly if the protein plays an important role in the cell. These compensating activities are also observed during the multistage process leading to tumorigenesis, where a normal cell undergoes serial genetic changes, including initiation, clonal expansion, pre-malignant lesions, and malignant progression, before acquiring a tumor phenotype. These properties acquired by cells to escape DDR defects are essential to our understanding of tumor cell behavior following chemo- or radiotherapy. We can now assess the usability of the numerous stable clones affecting all branches of the DDR that have been created. This unique cell model appears relevant for studying DNA repair, DNA replication, DNA recombination and cross-talk between them.

To date, we have established numerous clones, creating a library of stable isogenic cells which no longer express a specific DNA repair gene. This approach has helped us to untangle the interwoven DNA repair pathways and represents a powerful tool for research, drug screening and for preclinical testing of new therapies. This review will concentrate on two fields of research investigated using these knockdown clones.

2. Example of stable DNA repair gene silencing studies

2.1 Dual roles of some NER factors

NER is one of the more versatile DNA repair processes and removes diverse bulky lesions located on one DNA strand, including UV-induced photoproducts. In mammals, more than 30 proteins are required for this process, which comprises first a DNA damage recognition and structure distortion step involving XPC-hHR23B-centrin 2 and XPE in the global genome (GG)-NER or RNA polymerase II in the transcription-coupled (TC)-NER. NER also includes the verification of lesions (XPA-RPA), strand-separating helicases (TFIIH containing XPB and XPD DNA helicases), structure-specific endonucleases (ERCC1-XPF and XPG), and the enzymes needed for gap filling (DNA polymerase δ/ε, PCNA, RFC, and RPA). For example, ERCC1^{KD} and XPF^{KD} cells exhibited a tremendous and stable decrease of both targeted mRNA and protein, as evidenced by real time PCR and immunofluorescence staining (**fig. 3**). Beside, as documented in the literature, the loss of one of these proteins induces the disappearance of the other partner.

In GG-NER, the XPC-hHR23B-centrin 2 complex is responsible for the detection of damaged DNA. In TC-NER the displacement of stalled RNA polymerase complexes with the CSA and CSB proteins allows coordination of transcription and DNA repair. In order to unravel new roles for some of these gene products in this wide DNA repair network, we have established stable XPA^{KD}, XPC^{KD}, hHR23A^{KD}, hHR23B^{KD}, ERCC1^{KD} and XPF ^{KD} HeLa cells. In the **figure 4** established clones are represented for the NER and SSBR pathways.

Several clones displaying undetectable protein levels of XPA or XPC were established and grown for more than 300 days in culture with a tremendous stability of the gene-silenced

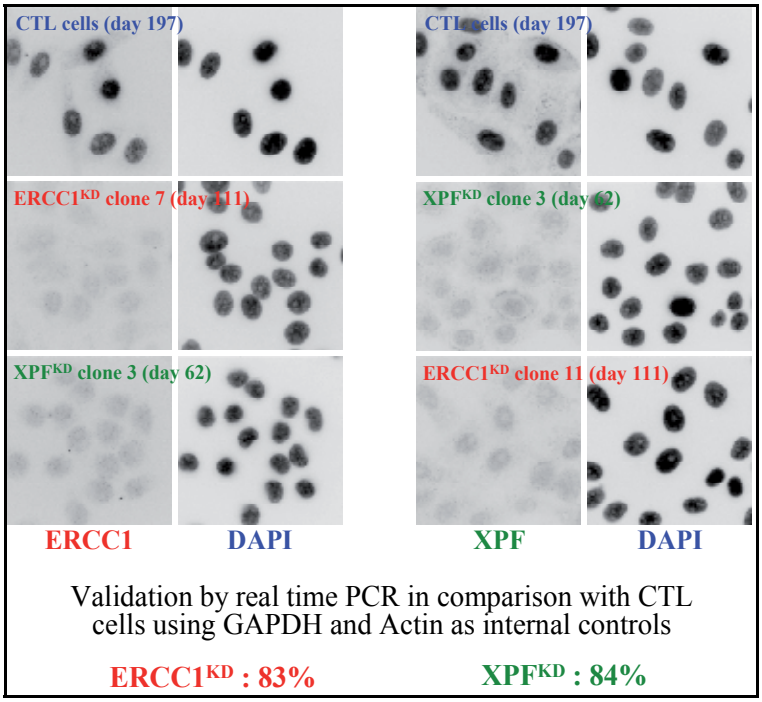

Fig. 3. Analysis of ERCC1^{KD} and XPF^{KD} cells by immunofluorescence.

and expected phenotypes (Biard et al., 2005). As expected, XPA^{KD} and XPC^{KD} HeLa cells were highly UVC sensitive and exhibited cell cycle arrest in early and middle S phase after UVC irradiation, showing that the persistence of UVC lesions blocks DNA replication. Both clones also show an impaired unscheduled DNA synthesis (UDS) after UVC irradiation. However, unlike *XPA*, the silencing of the *XPC* gene dramatically impeded HeLa cell growth. Furthermore, XPC^{KD} HeLa clones were more sensitive to UVC than their XPA^{KD} counterparts. In parallel we have analyzed the behavior of our hHR23B^{KD} and hHR23A^{KD} cells. hHR23B^{KD} cells displayed a significant sensitivity to UVC, in contrast to their hHR23A^{KD} counterparts which strongly tolerated UVC irradiation (Biard, 2007). While hHR23A^{KD} cells were not blocked in S phase after UVC irradiation, the exit from the S-phase of hHR23B^{KD} cells was hindered, suggesting the presence of unrepaired (or unrepairable) UVC-induced DNA damage. These data clearly demonstrate that hHR23A and hHR23B have diverse biological functions in human cells and that hHR23B^{KD} cells have a phenotype closely resembling that of XPC^{KD} cells. To understand why the silencing of the *XPC* gene can trigger major changes in cell behavior, we have performed hygromycin B withdrawal experiments.

After about 200 days of culture, hygromycin B was removed from the culture medium in order to reverse the gene-silencing phenotype by the slow and progressive disappearance of pEBV episomes. Under these experimental conditions, XPA or XPC protein levels returned to "control" levels after 15 to 20 days in culture. Unexpectedly, reverted XPC^{KD} cells (*XPC re-expressing cells*) did not recover a normal resistance to UVC, unlike XPA^{KD} cells. This striking result suggests that irreversible genetic changes have been fixed in the genome during the long-term *XPC* gene silencing and that, beside their canonical roles, some NER

factors such as XPC function in other essential pathways. Whilst this can be considered to be a limitation of this experimental system, it allows the possibility of determining what "back-up" systems or adaptive pathways are activated in the absence of key repair proteins.

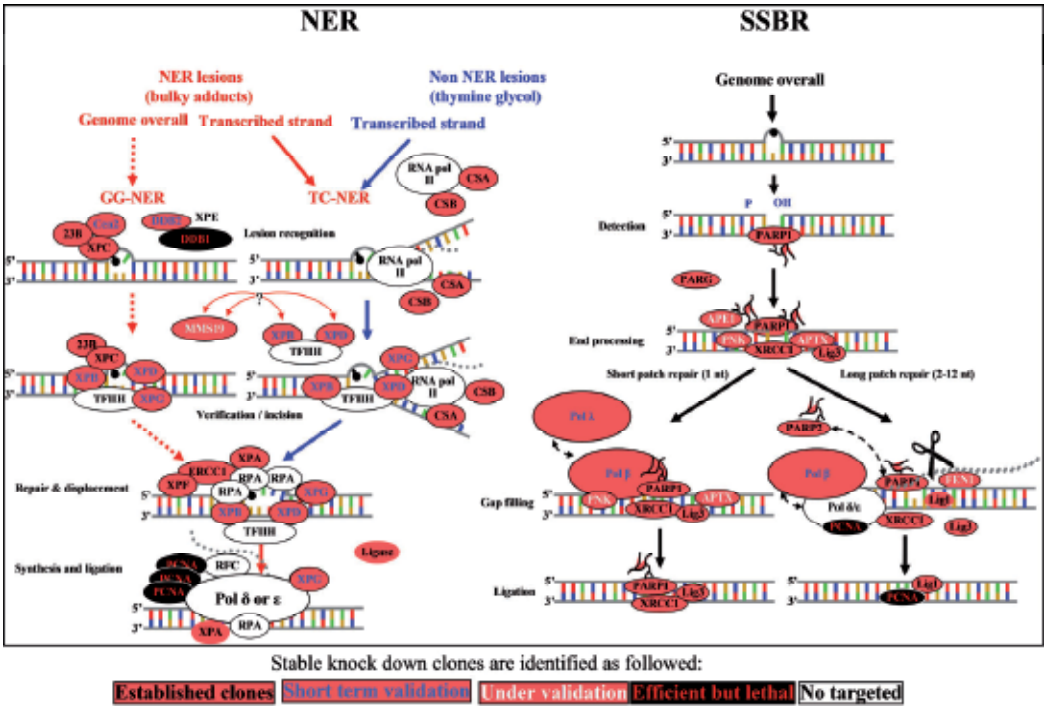

Fig. 4. NER versus SSBR/BER pathways.

In the literature it is reported that in certain XP cells (e.g. XP-V), a prolonged replication arrest due to unrepaired UV photoproduct could trigger an early commitment to recombination repair pathways (Limoli et al., 2005). This alternative pathway could be reinforced with a deregulated p53 pathway, as observed in HeLa cells, where the HPV18 E6 protein may degrade a part of the newly synthesized p53 protein. These data raise a question: are recombinational pathways altered in XPC^{KD} cells after UVC-induced stalled replication forks in HeLa cells? Various reports have suggested that XPC defects elicit impaired cellular responses to ionizing radiation (IR), indicating a possible role of XPC in the cellular response to DSBs. We have sought to determine the sensitivity of NER-deficient cells to DSB-generating agents.

We used our DNA PKcs^{KD}, XRCC4^{KD}, ligase IV^{KD}, Rad54^{KD}, ligase III^{KD} MRE11^{KD}, Rad50^{KD}, Nbs1^{KD}, ATM^{KD} and ATR^{KD} cells as controls for screening the DDR. NER-deficient HeLa cells were treated with either IR or etoposide (VP16), a topoisomerase II inhibitor that creates DSBs partly through the progression of DNA replication forks (Biard, 2007, Despras et al., 2007). Strikingly, XPC^{KD} and hHR23B^{KD} cells displayed intolerance to acute γ ray irradiation, in contrast to their XPA^{KD} and hHR23A^{KD} counterparts. 24 h after high-dose irradiation (6 Gy) XPC^{KD} cells, and to a lower extent hHR23B^{KD} cells, exhibited a strong arrest in G2 phase as did NHEJ- and HR-deficient cells. However, using clonogenic survival both XPC^{KD} and hHR23B^{KD} cells showed a moderate sensitivity to IR (1 Gy). These data

suggest that beside its canonical function in the early steps of the NER, the XPC protein could be essential in the coordination of other recovery pathways, such as those involved in the repair of IR- and etoposide-induced DNA damage.

In mock treated cells, the persistence of XPC on chromatin structures was shown by experiments in which the XPC protein remained tightly anchored to detergent-insoluble nuclear structures (Despras et al., 2007). Interestingly, XPC was released from these structures after induction of DSBs by calicheamicin or neocarzinostatin, two potent specific DSB inducers. The reduction of chromatin-fixed XPC correlated with the increase of H2AX phosphorylation and presumably with the recruitment of DNA repair factors at sites of damaged DNA. This sequence of events was partly confirmed by the subsequent recruitment of phosphorylated-XRCC4 and LigIV into the less extractable nuclear fraction after DSB induction, as previously described (Drouet et al., 2005). Therefore, XPC should be considered as a genome caretaker protein, which is (i) recruited for initiating the GG-NER in the presence of bulky DNA damage, but which (ii) also displays other functions in the presence of DSBs.

Using the HeLa isogenic KD model we have also focused our attention on the efficiency of NER-deficient cells in performing NHEJ, using an *in vitro* assay making use of DNA PKcs^{KD} and XRCC4^{KD} cells. The DNA PKcs^{KD} cells used displayed an undetectable protein level and a nearly total loss of the endogenous kinase activity (Despras et al., 2007), and the isolated XRCC4^{KD} clones all displayed a residual XRCC4 protein level corresponding to about 15% of the control (CTL); this residual level might reflect the essential role played by XRCC4 in cell survival. These XRCC4^{KD} cells are particularly interesting experimentally too as there are no human cell lines lacking the XRCC4 protein. In ligase IV^{KD}, DNA PKcs^{KD} and XRCC4^{KD} cells, NHEJ efficiencies dropped to 50, 30 and 20%, respectively, as compared with control (personal data and (Despras et al., 2007)). This also correlated with a markedly increased sensitivity towards IR. Our results also argue for XRCC4 being a limiting factor in the NHEJ process, at least *in vitro*. Strikingly, while the expression of NHEJ factors was not altered in XPC^{KD} cells, XPC deficiency led to a decrease of *in vitro* NHEJ efficiency. In both XPC^{KD} and DNA PKcs^{KD} cells, XRCC4 and ligase IV proteins were mobilized to damaged nuclear structures at lower doses of chemical DSB inducer in comparison with proficient cells. In contrast, *XPA* gene silencing did not modify HeLa cell response to DSBs. Our results reinforce the notion that XPC^{KD} cells display an unexpected behavior towards DSBs, presumably due to an intrinsic characteristic of XPC, rather than being a consequence of NHEJ deficiency. We can also rule out a direct role of XPC in the NHEJ process *per se*. Presumably XPC deficiency could locally change the chromatin structure and interfere with other pathways.

It is notable that in our experiments we have always observed that *XPA* gene silencing could lead to an enhanced cell growth several weeks after transfection of HeLa cells and in the absence of genotoxic injuries. In contrast, knocking down of XPC triggered major growth defects and tremendous cellular stress as well as elevated sensitivity to genotoxic agents. Presumably XPA and XPC can participate in major pathways required for normal growth, but with opposite effects. Because relationships between some NER factors and transcription have been extensively related in the literature (for review (Le May et al., 2010a)), we have questioned whether XPA and XPC factors could be involved in the regulation of transcription in the absence of exogenous DNA damage. The transcription / repair factor TFIIH is organized into a core complex (XPB, XPD, p62, p52, p44, p34, and p8/TTDA) that associates with the Cdk-activating kinase (CAK) complex (Cdk7, cyclin H,

and MAT1). In response to DNA damage, XPA catalyzes the detachment of the CAK from the core TFIIH, changing this transcription factor into a repair factor (Coin et al., 2008). Thereafter, new NER proteins are recruited around the TFIIH factor such as XPC / hHR23B. After repair, resumption of CAK activity is required for continuation of transcription.

By using our XPA^{KD}, XPC^{KD} and ERCC1^{KD} clones, we have determined the role of these NER proteins during the transcriptional regulation of active promoters. Interestingly, we observed that the recruitment of NER factors at promoters of inducible nuclear receptor genes (including the retinoic acid receptors α and γ) occurred in a sequential order and required XPC, CSB, XPA / RPA, the two endonucleases, XPG and ERCC1 / XPF and XPE with the RNA pol II machinery (Le May et al., 2010b). This transcriptional complex containing NER factors is formed in the absence of any genotoxic injury, at the site of the promoter. Contrary to the coordinated recruitment observed in control cells, none of the NER factors were recruited to the promoter in XPC^{KD} HeLa cells. XPC association is thus a pre-requisite step and abnormal XPC protein levels could affect normal transcription. This XPC-dependent transcriptional complex is distinct from a repair complex. In contrast, in XPA^{KD} cells, only XPC and CSB were detected at the promoter, and in ERCC1^{KD} cells we detected XPC, XPA, and XPG together with RAR, RXR, RNA pol II, and TFIIH. Furthermore, during the transcriptional initiation step, XPC is required to achieve optimal DNA demethylation and histone posttranscriptional modifications. In control cells, transcription initiation and recruitment of NER factors are accompanied by a global DNA demethylation. A local DNA demethylation at sites of 5′-CpG-3′ islands was also detected around the proximal RARβ2 promoter region. In contrast, in XPC^{KD}, XPA^{KD}, and ERCC1^{KD} HeLa cells the global methylation levels were lowered as compared with control cells. More importantly, XPC^{KD} and XPA^{KD} cells, but not ERCC1^{KD} cells, failed to demethylate the RARβ2 promoter. Afterwards, during the transcription elongation in distal regions of the gene, NER factors escort the RNA-Pol and form a complex which now excludes XPC but needs CSB. This latter complex could appear as a pre-TC-NER complex. In all of these studies, the phenotype of the knockdown HeLa cells was compared with that of deficient XP and CS fibroblasts from patients.

Altogether these data demonstrate that NER factors could actively contribute to transcription of particular promoters in the absence of DNA damage and then interfere with cellular homeostasis. These results help us to explain the striking phenotype observed in our XPC^{KD} and hHR23B^{KD} cells in comparison with control cells or their XPA^{KD} counterparts. Recently, in an effort to silence other genes belonging to the NER, we have observed that *DDB1* gene silencing strongly disrupts HeLa cell growth a few weeks after transfection (unpublished data). This raises the question whether XPE (DDB2-DDB1 heterodimer) also participates in transcription regulation in the absence of exogenous DNA damage, as has been seen for XPC.

2.2 Parp1, between inhibition and gene silencing

We have also employed our cell model to shed light on the poly(ADP-ribose) polymerase (PARP) family. New developments of mono- and combined therapeutic approaches based on PARP inhibitors reinforce the crucial role played by these proteins in the DDR. The PARP family contains 17 members and its founding member, PARP1, carries out the majority of poly(ADP-ribose) (PAR) synthesis in mammalian cells (Ame et al., 2004, D'Amours et al., 2001). Poly(ADP-ribosyl)ation is an immediate DNA damage–dependent posttranslational modification of numerous nuclear proteins indispensable for an accurate

DDR. In contrast to what is frequently stated in the literature, PARP1 is not a DNA repair protein in *stricto sensu* but rather a signaling and scaffold protein which binds to DNA nicks and breaks in order to facilitate DNA repair by attracting other factors to damaged sites (e.g. XRCC1). Hence, PARP1 participates in numerous DNA repair pathways. It is a key building block in the SSBR, more precisely in the SPR (short patch repair) pathway, but probably in the first steps of the LPR (long patch repair) pathway, but not after SSB generating agents (see below; **fig. 4**). In addition, PARP1 is also involved in NER, b-NHEJ (**fig. 5**), transcription, cellular bioenergetics, telomere cohesion and mitotic segregation, centromere and/or kinetochore function and energy metabolism (Schreiber et al., 2006). A recent study shows that loss of PARP1 leads to spontaneous hyper-recombinogenic phenotype in mice, suggesting a balance between SSBR and HR (Claybon et al., 2010). Moreover, Patel et al. have observed that transient chemical inhibition of PARP1 and gene silencing interfered with NHEJ activities, emphasizing an interplay between the error-prone NHEJ and the error-free HR (Patel et al., 2011).

We have addressed this issue by creating PARP1^{KD}, PARP2^{KD}, PARP3^{KD} and PARG^{KD} silenced cells (**fig. 6**). Our aim was to analyze spontaneous and genotoxic-induced genetic instability (Ame et al., 2009, Boehler et al., 2011, Godon et al., 2008). In a preliminary approach, we focused our attention on the requirement of PARP1 in the two SSBR pathways (SPR *versus* LPR). This approach requires the establishment of additional clones such as XRCC1^{KD}, ligase III^{KD} and ligase I^{KD} cells, together with other knockdown cells which are presently under evaluation (Fen1^{KD}; PNK^{KD}, APTX^{KD}, polβ^{KD}).

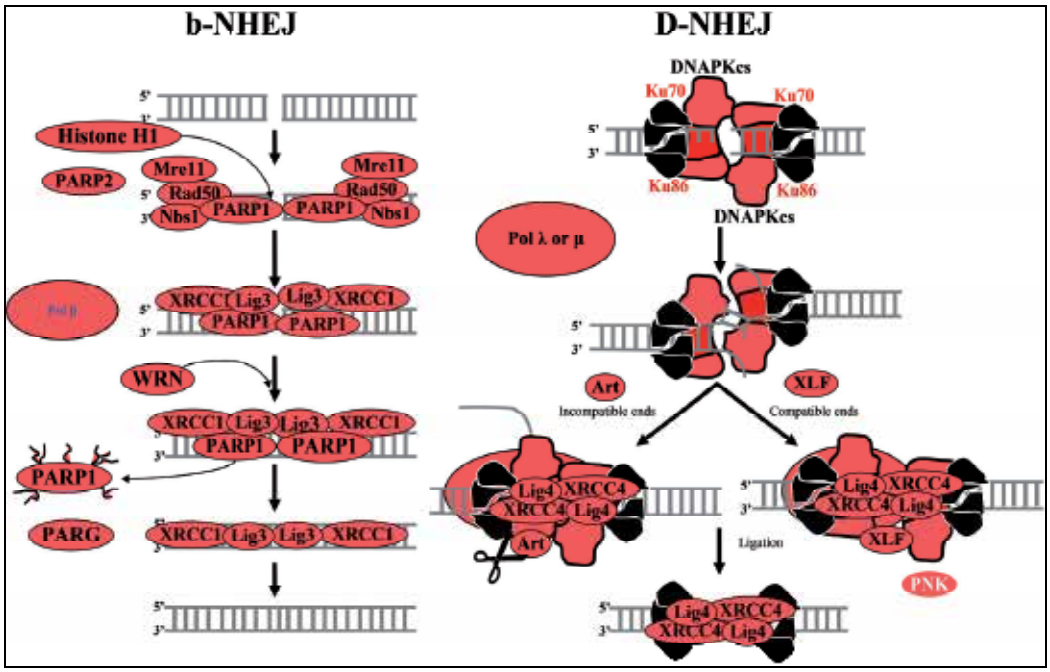

Fig. 5. b-NHEJ and D-NHEJ pathways. Stable knock down clones are identified as indicated in the legend of fig. 2.

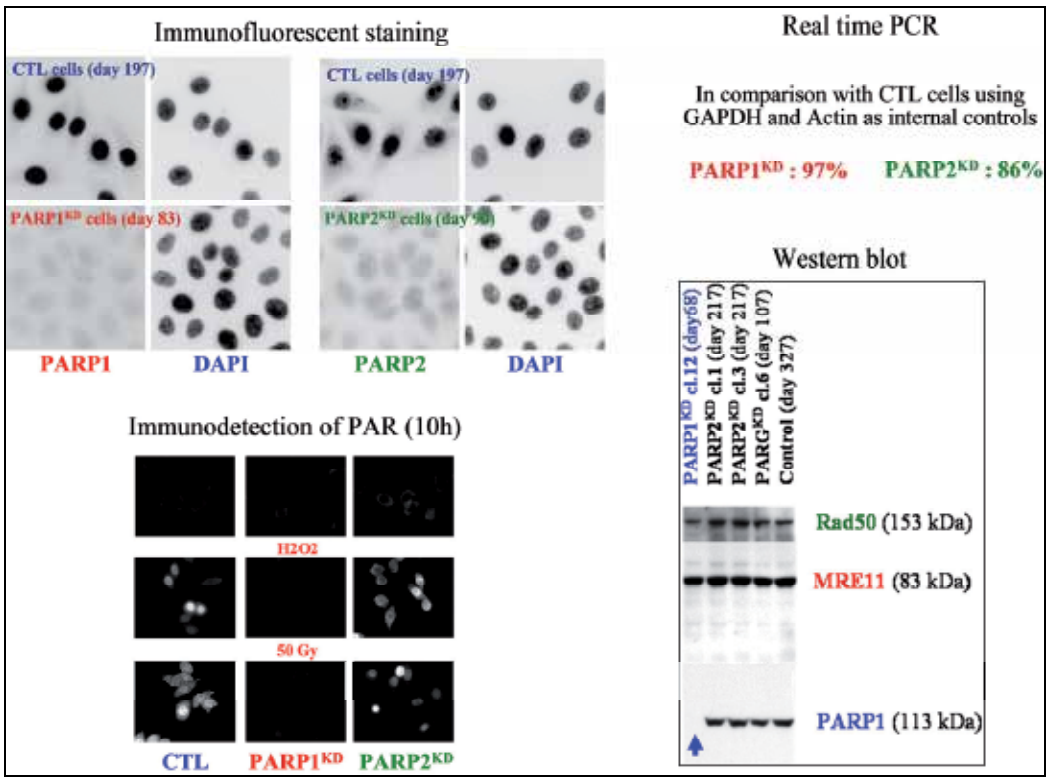

Fig. 6. Characterization of PARP1^{KD} and PARP2^{KD} stable clones.

This work has also been carried out to point pitfalls arising from conflicting data obtained after gene silencing *versus* chemical inhibition. Interestingly, PARP1 inhibition and gene silencing triggered different outcomes in terms of SSBR and radiosensitivity. Our PARP1^{KD} HeLa cells display a substantial reduction in both protein and mRNA levels, with undetectable poly(ADP-ribose) (PAR) synthesis following exposure to H_2O_2 (1 mM, 10 min) or even after exposure to 50 Gy γ rays (Godon et al., 2008). PARP1^{KD} cells are 2.5-fold more radiosensitive than both controls and XRCC1^{KD} cells, and XRCC1^{KD} cells are 5-fold more sensitive to methyl methane sulfonate than their PARP1^{KD} counterparts. *PARP1* gene silencing prevents XRCC1-YFP recruitment at sites of local laser irradiation (405 nm), but does not affect the lifetime of PCNA-GFP foci, suggesting that impaired SPR (PARP1- and XRCC1-dependent) could be efficiently replaced by LPR (PCNA- and ligase I–dependent). However, we can not rule out the partial resolution of SSB by way of HR, as suggested elsewhere (Claybon et al., 2010). S phase–irradiated PARP1^{KD} (and XRCC1^{KD}) cells complete SSBR as rapidly as controls, while SSBR is slower in G1 cells but reaches completion. In contrast, PARP1 inhibition with 4-amino-1,8-naphthalimide (ANI) enhances radiosensitivity in highly proliferating cells (e.g. tumor cells), presumably due to the collision of unrepaired DNA lesions with replication forks (Noel et al., 2006). This also prevents XRCC1-YFP recruitment at sites of damaged DNA (laser micro-irradiation) and cells displayed a 10-fold slower SSBR. We also observe accumulation of huge PARP1-GFP and PCNA-GFP foci. These results suggest that the chemically inhibited PARP1 protein remains tethered to nuclear structures and that this steric hindrance impedes the recruitment of further DNA

repair proteins. These data emphasize that the need for careful interpretation of results from the use of chemical inhibitors which could be riddled with pitfalls. Moreover, it is noteworthy that PARP inhibitors not only inhibit PARP1, but also PARP2 and PARP3 (Loseva et al., 2010).

After a genotoxic injury, PARP1 activation leads to a tremendous but transient synthesis of PAR, in order to label DNA-damaged sites, open the chromatin structure and recruit repair factors, such as the scaffold protein XRCC1 (Dantzer et al., 2006). Because this reaction is transient, PAR polymers have to be rapidly degraded by PARG. PARP1 and PARG display opposite enzymatic activities which govern the balance between life and death after DNA injuries. Our knockdown clones clearly demonstrate that PARP1, PARP2, PARP3 and PARG activities contribute to this homeostasis, even in the absence of exogenous genotoxic attack (Ame et al., 2009, Boehler et al., 2011). PARG^{KD} HeLa cells exhibit a stable loss of the three PARG isoforms (nuclear, cytoplasmic and mitochondrial) and a spectacular loss of function. Surprisingly, constitutive PARG depletion and subsequent PAR accumulation are rather beneficial in that they protect cells from spontaneous SSBs and telomeric abnormalities. In contrast, irradiation of PARG^{KD} cells triggers PAR accumulation, delayed SSB and DSB repair, centrosome amplification and mitotic defects, all of which contribute to cell death by mitotic catastrophe (Ame et al., 2009).

The complexity and the redundancy of the PARP family members toward the DDR are reinforced by our recent data demonstrating that PARP3 is a newcomer in the cellular response to DNA damage and mitotic progression (Boehler et al., 2011). PARP3 is closely related to PARP1 and PARP2, but unlike these two counterparts PARP3 is a mono(ADP-ribose) polymerase. It has been proposed that PARP3 could be involved in transcriptional silencing in association with Polycomb group proteins. Moreover, PARP3 could also be a component of the DDR because it is found in complexes mainly containing Ku70 and Ku80, but also PARP1, DNA ligase III, DNA PKcs and DNA ligase IV (Rouleau et al., 2007). This raises the question whether PARP3 participates in SSBR (when PARP1 is deficient?), D-NHEJ (with DNA PKcs, DNA ligase IV, Ku70, and Ku80), b-NHEJ (with DNA ligase III) and telomere maintenance (with Ku70 and Ku80). This was partly confirmed by a recent study which shows that PARP3 might be a novel DSB sensor which functions in the same pathway as APLF (aprataxin- and PNK-like factor) in order to accelerate chromosomal DSB repair (Rulten et al., 2011). APLF is a poly(ADP-ribose)-binding protein which interacts directly with Ku80 and XRCC4 at sites of DSBs (Macrae et al., 2008). To gain further insight into PARP3 function in the DDR we have validated pEBVsiPARP3 plasmids targeting the two known PARP3 isoforms. Stable clones exhibiting an almost complete depletion of PARP3 were carefully characterized (Boehler et al., 2011). PARP3^{KD} cells displayed spontaneous DSBs and genome instability, delayed repair after irradiation, but no significant radiosensitivity as compared with control cells. Our results reinforce recent data showing that PARP3-deficient cells were as sensitive to a topoisomerase I poison (camptothecin) as control cells (Loseva et al., 2010). These unexpected results could be explained by partly compensating activities between PARP3 and PARP1. These data strongly suggest a functional synergistic cross-talk between PARP1 and PARP3. Interestingly, PARP3 interacts directly and strongly with PARP1 and PARP3 is able to activate PARP1 in the absence of DNA (Loseva et al., 2010). Another significant event observed in PARP3^{KD} cells is an elevated frequency of sister telomere fusions and sister telomere loss. This is explained by the functional association between PARP3, tankyrase I and NuMa (microtubule-associated protein involved in spindle dynamics). Altogether, these three proteins appear to be key

regulators of mitotic progression. This study will now continue by establishing new cell lines silenced for other members of the PARP family such as PARP9, PARP14, tankyrase 1 (PARP5a) and tankyrase 2 (PARP5b).

3. Conclusions

In the field of cancer research, numerous questions remain unanswered, such as how do different pathways cooperate to repair DNA damage in tumor cells? How can we explain the chemo- and radioresistance of tumor cells? Can we target DDR to enhance chemotherapy? How do genetic compensation events take place? How can we detect the combinations of genes leading to **synthetic lethality**? Are DNA repair factors involved in other processes? All of these questions have to be carefully analyzed in order to design specific and less toxic therapies for cancer. Currently, chemotherapeutic approaches are based on the fact that highly proliferating (*tumor and unfortunately hair, bone marrow and colon*) cells are more sensitive to DNA damage than their slowly proliferating (*normal*) counterparts. Alterations in DNA repair pathways in tumor cells can make some cancer cells dependent on a reduced set of DNA repair pathways for their survival. These adaptive but potentially error-prone bypasses could render DNA damage–based cancer therapies less efficient and allow tumor cells to escape specific treatments. Recently substantial progress has been made through studies of genes involved in the DDR in order to circumvent rescue pathways. A breakthrough has emerged with the concept of synthetic lethality, which is defined as a genetic interaction where the minimal combination of two nonlethal mutations leads to cell death. Because naturally occurring synthetic lethal mutants are unviable we have to develop outstanding cell models in order to unravel the DDR and subsequently to detect these combinations that give rise to synthetic lethality. In light of these concerns, an emerging strategy has been to use PARP inhibitors (e.g. iniparib, olaparib or veliparib) combined or not with DNA-damaging chemotherapeutic agents in the treatment of breast and ovarian cancers exhibiting germ-line mutations in *BRCA* genes (Bryant et al., 2005, Farmer et al., 2005, Mullan et al., 2006). Because of the partial **redundancy** between BRCA functions, PARP inhibitors have to be administered to patients displaying loss of copies of both the *BRCA1* and *BRCA2* (*FancD1*) genes. This approach is based on compelling evidence demonstrating why BRCA1 and 2 act as molecular determinants in the response to chemotherapeutic agents (Quinn et al., 2003). Amongst prominent defects observed in BRCA1/2-deficient tumor cells, aberrant G2/M checkpoint control and impaired DNA repair (HR) modulate sensitivity to genotoxic agents (Hartman & Ford, 2002, Moynahan et al., 1999). Interestingly, BRCA1 also participates in GG-NER (but not TC-NER) in a p53-independent manner by inducing the expression of XPC, DDB2 (XPE), and GADD45 (Hartman & Ford, 2003). In tumor cells, **compensating repair activities** taking place during clonal expansion could compensate HR (and GG-NER) deficiencies with other DNA repair pathways, such as those dependent on PARP1 (SSBR or b-NHEJ). In these conditions, PARP inhibition might lead to the persistence of DNA lesions normally repaired by HR and trigger tumor cell death without affecting normal cells (Farmer et al., 2005). Other genetic defects could lead to synthetic lethality associated with PARP inhibition, such as impaired PTEN (phosphatase and tensin homolog) (Mendes-Pereira et al., 2009), Fanconi anemia genes (D'Andrea, 2010) or ATM (Williamson et al., 2010) genes. Now, this approach has been enlarged to metastatic triple-negative breast cancers having inherent defects in DNA repair (O'Shaughnessy et al., 2011). Interestingly, a recent paper shows that PARP inhibition could

also interfere with the NHEJ pathways in that PARP inactivation in HR-deficient cells enhances NHEJ activities (Patel et al., 2011). We have to keep in mind that, in mammalian cells, the high-speed 'classic' DNA-PKcs–dependent NHEJ (D-NHEJ) pathway repairs general DSBs. While some DNA ends may be rapidly joined through the D-NHEJ, other breaks are processed for homology searches. These ends may be substrates for the alternative low-speed *backup* NHEJ (b-NHEJ, also termed microhomology-based end-joining pathway) involving ligase III, XRCC1 and PARP1 (Audebert et al., 2006, Iliakis et al., 2004, Verkaik et al., 2002, Wang et al., 2006). Hence, this raises the question whether PARP1 inactivation induces NHEJ compensation due to impaired HR function or b-NHEJ function or both. To strengthen this notion we have observed that NHEJ activities were enhanced in ligase III^{KD} HeLa cells when the b-NHEJ was expected to be hampered (as well as BER) (unpublished data). Altogether this striking example clearly highlights the requirement to study the interwoven DNA repair pathways in tumor cells using a relevant cell model. Now we are seeking to evaluate the compensating activities between different pathways, such as D-NHEJ *versus* b-NHEJ or HR *versus* D-NHEJ. We have also established TLS-deficient clones in order to determine the role of specialized (TLS) DNA polymerases in the absence of DNA injuries (**fig. 7**). Our published results show that these polymerases facilitate the progression of the replication fork through external replication barriers (e.g. bulky adducts) and also through naturally occurring DNA structures (G4 structures, H-DNA or Z-DNA). More precisely, Pol η and Pol κ help to prevent genomic instability occurring at such natural DNA sequences (Betous et al., 2009). Pol η also maintains chromosomal stability and prevents common fragile site breakage during unperturbed S phase (Rey et al., 2009).

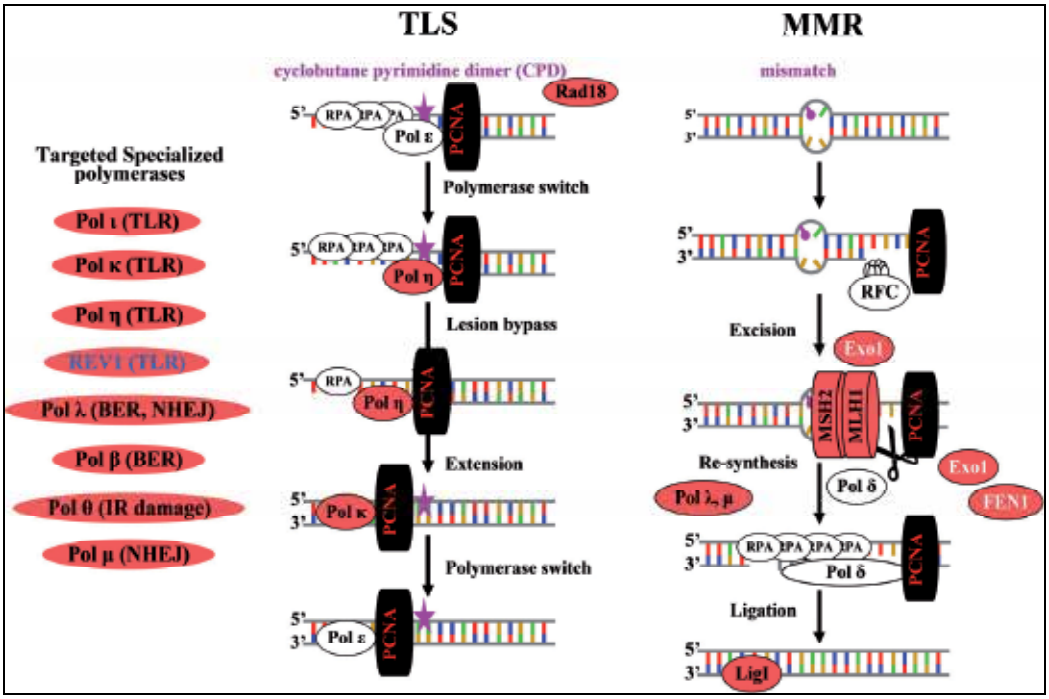

Fig. 7. TLS and MMR pathways. Stable knock down clones are identified as indicated in the legend of fig. 3.

To conclude, the major advantage of this strategy is the rapid establishment of new stable knockdown clones in various tumor-derived cells, which display stable gene silencing. A recent development has been to create dual pEBVsiRNA plasmids allowing efficient knockdown of two or more genes. For instance, double knockdown cells have been created where both DNA PKcs and ligase III were efficiently silenced with a single pEBVsiRNA vector. These cells, which grow normally, are expected to be deficient for both D-NHEJ and b-NHEJ. We have also developed plasmids targeting an endogenous gene and re-expressing an exogenous transcript carrying functional mutations. The latter approach allows mutant cells to be generated when the loss of the targeted gene is lethal. Hence, because we can easily and efficiently create DDR-deficient cells where one or more genes are silenced, we are now able to unravel the spectacular network of DNA repair pathways.

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5. References

Allalunis-Turner, M. J.; Barron, G. M.; Day, R. S., 3rd; Dobler, K. D. & Mirzayans, R. (1993). Isolation of two cell lines from a human malignant glioma specimen differing in sensitivity to radiation and chemotherapeutic drugs. Radiat Res, Vol. 134, pp 349-54.

Al-Minawi, A. Z.; Saleh-Gohari, N. & Helleday, T. (2008). The ERCC1/XPF endonuclease is required for efficient single-strand annealing and gene conversion in mammalian cells. Nucleic Acids Res, Vol. 36, pp 1-9.

Al-Tassan, N.; Chmiel, N. H.; Maynard, J.; Fleming, N.; Livingston, A. L.; Williams, G. T.; Hodges, A. K.; Davies, D. R.; David, S. S.; Sampson, J. R. & Cheadle, J. P. (2002). Inherited variants of MYH associated with somatic G:C-->T:A mutations in colorectal tumors. Nat Genet, Vol. 30, pp 227-32.

Ame, J. C.; Fouquerel, E.; Gauthier, L. R.; Biard, D.; Boussin, F. D.; Dantzer, F.; de Murcia, G. & Schreiber, V. (2009). Radiation-induced mitotic catastrophe in PARG-deficient cells. J Cell Sci, Vol. 122, pp 1990-2002.

Ame, J. C.; Spenlehauer, C. & de Murcia, G. (2004). The PARP superfamily. Bioessays, Vol. 26, pp 882-93.

Amine, A.; Rivera, S.; Opolon, P.; Dekkal, M.; Biard, D. S.; Bouamar, H.; Louache, F.; McKay, M. J.; Bourhis, J.; Deutsch, E. & Vozenin-Brotons, M. C. (2009). Novel anti-metastatic action of cidofovir mediated by inhibition of E6/E7, CXCR4 and Rho/ROCK signaling in HPV tumor cells. PLoS One, Vol. 4, pp e5018.

Aressy, B.; Bugler, B.; Valette, A.; Biard, D. & Ducommun, B. (2008). Moderate variations in CDC25B protein levels modulate the response to DNA damaging agents. Cell Cycle, Vol. 7, pp 2234-40.

Audebert, M.; Salles, B.; Weinfeld, M. & Calsou, P. (2006). Involvement of polynucleotide kinase in a poly(ADP-ribose) polymerase-1-dependent DNA double-strand breaks rejoining pathway. J Mol Biol, Vol. 356, pp 257-65.

Barnes, D. E.; Tomkinson, A. E.; Lehmann, A. R.; Webster, A. D. & Lindahl, T. (1992). Mutations in the DNA ligase I gene of an individual with immunodeficiencies and cellular hypersensitivity to DNA-damaging agents. Cell, Vol. 69, pp 495-503.

Betous, R.; Rey, L.; Wang, G.; Pillaire, M. J.; Puget, N.; Selves, J.; Biard, D. S.; Shin-ya, K.; Vasquez, K. M.; Cazaux, C. & Hoffmann, J. S. (2009). Role of TLS DNA polymerases eta and kappa in processing naturally occurring structured DNA in human cells. Mol Carcinog, Vol. 48, pp 369-78.

Biard, D. S. (2007). Untangling the relationships between DNA repair pathways by silencing more than 20 DNA repair genes in human stable clones. Nucleic Acids Res, Vol. 35, pp 3535-50.

Biard, D. S.; Despras, E.; Sarasin, A. & Angulo, J. F. (2005). Development of new EBV-based vectors for stable expression of small interfering RNA to mimick human syndromes: application to NER gene silencing. Mol Cancer Res, Vol. 3, pp 519-29.

Boehler, C.; Gauthier, L. R.; Mortusewicz, O.; Biard, D. S.; Saliou, J. M.; Bresson, A.; Sanglier-Cianferani, S.; Smith, S.; Schreiber, V.; Boussin, F. & Dantzer, F. (2011). Poly(ADP-ribose) polymerase 3 (PARP3), a newcomer in cellular response to DNA damage and mitotic progression. Proc Natl Acad Sci U S A, Vol., pp

Bouley, J.; Pionneau, C.; Varinot, J.; Biard, D.; Genestie, C.; Antoine, M.; Coulet, F.; Henri Stern, M.; Stoppa-Lyonnet, D. & Soubrier F. (2010). Proteomic analysis of BRCA1-depleted cell line reveals a putative role for RPA2 up-regulation in BRCA1 breast tumor development. Proteomics Clin. Appl, Vol. 4, pp 1-10.

Bouquet, F.; Ousset, M.; Biard, D.; Salles, B. & Muller, C. (2011). A DNA-dependent stress response involving DNA-PK occurs in hypoxic cells and contributes to cellular adaptation to hypoxia. J Cell Sci, Vol. In press, pp in press.

Britton, S.; Frit, P.; Biard, D.; Salles, B. & Calsou, P. (2009a). ARTEMIS nuclease facilitates apoptotic chromatin cleavage. Cancer Res, Vol. 69, pp 8120-6.

Britton, S.; Froment, C.; Frit, P.; Monsarrat, B.; Salles, B. & Calsou, P. (2009b). Cell nonhomologous end joining capacity controls SAF-A phosphorylation by DNA-PK in response to DNA double-strand breaks inducers. Cell Cycle, Vol. 8, pp 3717-22.

Bryant, H. E.; Schultz, N.; Thomas, H. D.; Parker, K. M.; Flower, D.; Lopez, E.; Kyle, S.; Meuth, M.; Curtin, N. J. & Helleday, T. (2005). Specific killing of BRCA2-deficient tumours with inhibitors of poly(ADP-ribose) polymerase. Nature, Vol. 434, pp 913-7.

Chen, S.; Bigner, S. H. & Modrich, P. (2001). High rate of CAD gene amplification in human cells deficient in MLH1 or MSH6. Proc Natl Acad Sci U S A, Vol. 98, pp 13802-7.

Claybon, A.; Karia, B.; Bruce, C. & Bishop, A. J. (2010). PARP1 suppresses homologous recombination events in mice in vivo. Nucleic Acids Res, Vol. 38, pp 7538-45.

Coant, N.; Ben Mkaddem, S.; Pedruzzi, E.; Guichard, C.; Treton, X.; Ducroc, R.; Freund, J. N.; Cazals-Hatem, D.; Bouhnik, Y.; Woerther, P. L.; Skurnik, D.; Grodet, A.; Fay, M.; Biard, D.; Lesuffleur, T.; Deffert, C.; Moreau, R.; Groyer, A.; Krause, K. H.; Daniel, F. & Ogier-Denis, E. (2010). NADPH oxidase 1 modulates WNT and NOTCH1 signaling to control the fate of proliferative progenitor cells in the colon. Mol Cell Biol, Vol. 30, pp 2636-50.

Coin, F.; Oksenych, V.; Mocquet, V.; Groh, S.; Blattner, C. & Egly, J. M. (2008). Nucleotide excision repair driven by the dissociation of CAK from TFIIH. Mol Cell, Vol. 31, pp 9-20.

D'Amours, D.; Sallmann, F. R.; Dixit, V. M. & Poirier, G. G. (2001). Gain-of-function of poly(ADP-ribose) polymerase-1 upon cleavage by apoptotic proteases: implications for apoptosis. J Cell Sci, Vol. 114, pp 3771-8.

D'Andrea, A. D. (2010). Susceptibility pathways in Fanconi's anemia and breast cancer. N Engl J Med, Vol. 362, pp 1909-19.

Dantzer, F.; Ame, J. C.; Schreiber, V.; Nakamura, J.; Menissier-de Murcia, J. & de Murcia, G. (2006). Poly(ADP-ribose) polymerase-1 activation during DNA damage and repair. Methods Enzymol, Vol. 409, pp 493-510.

Despras, E.; Pfeiffer, P.; Salles, B.; Calsou, P.; Kuhfittig-Kulle, S.; Angulo, J. F. & Biard, D. S. (2007). Long-term XPC silencing reduces DNA double-strand break repair. Cancer Res, Vol. 67, pp 2526-34.

Drouet, J.; Delteil, C.; Lefrancois, J.; Concannon, P.; Salles, B. & Calsou, P. (2005). DNA-dependent protein kinase and XRCC4-DNA ligase IV mobilization in the cell in response to DNA double strand breaks. J Biol Chem, Vol. 280, pp 7060-9.

Durandy, A. (2009). Immunoglobulin class switch recombination: study through human natural mutants. Philos Trans R Soc Lond B Biol Sci, Vol. 364, pp 577-82.

Farmer, H.; McCabe, N.; Lord, C. J.; Tutt, A. N.; Johnson, D. A.; Richardson, T. B.; Santarosa, M.; Dillon, K. J.; Hickson, I.; Knights, C.; Martin, N. M.; Jackson, S. P.; Smith, G. C. & Ashworth, A. (2005). Targeting the DNA repair defect in BRCA mutant cells as a therapeutic strategy. Nature, Vol. 434, pp 917-21.

Giglia-Mari, G.; Coin, F.; Ranish, J. A.; Hoogstraten, D.; Theil, A.; Wijgers, N.; Jaspers, N. G.; Raams, A.; Argentini, M.; van der Spek, P. J.; Botta, E.; Stefanini, M.; Egly, J. M.; Aebersold, R.; Hoeijmakers, J. H. & Vermeulen, W. (2004). A new, tenth subunit of TFIIH is responsible for the DNA repair syndrome trichothiodystrophy group A. Nat Genet, Vol. 36, pp 714-9.

Godon, C.; Cordelieres, F. P.; Biard, D.; Giocanti, N.; Megnin-Chanet, F.; Hall, J. & Favaudon, V. (2008). PARP inhibition versus PARP-1 silencing: different outcomes in terms of single-strand break repair and radiation susceptibility. Nucleic Acids Res, Vol. 36, pp 4454-64.

Hartman, A. R. & Ford, J. M. (2002). BRCA1 induces DNA damage recognition factors and enhances nucleotide excision repair. Nat Genet, Vol. 32, pp 180-4.

Hartman, A. R. & Ford, J. M. (2003). BRCA1 and p53: compensatory roles in DNA repair. J Mol Med, Vol. 81, pp 700-7.

Hoeijmakers, J. H. (2001). Genome maintenance mechanisms for preventing cancer. Nature, Vol. 411, pp 366-74.

Huang, T. T. & D'Andrea, A. D. (2006). Regulation of DNA repair by ubiquitylation. Nat Rev Mol Cell Biol, Vol. 7, pp 323-34.

Iliakis, G.; Wang, H.; Perrault, A. R.; Boecker, W.; Rosidi, B.; Windhofer, F.; Wu, W.; Guan, J.; Terzoudi, G. & Pantelias, G. (2004). Mechanisms of DNA double strand break repair and chromosome aberration formation. Cytogenet Genome Res, Vol. 104, pp 14-20.

Iyer, R. R.; Pluciennik, A.; Burdett, V. & Modrich, P. L. (2006). DNA mismatch repair: functions and mechanisms. Chem Rev, Vol. 106, pp 302-23.

Jaspers, N. G.; Raams, A.; Silengo, M. C.; Wijgers, N.; Niedernhofer, L. J.; Robinson, A. R.; Giglia-Mari, G.; Hoogstraten, D.; Kleijer, W. J.; Hoeijmakers, J. H. & Vermeulen, W. (2007). First reported patient with human ERCC1 deficiency has cerebro-oculo-facio-skeletal syndrome with a mild defect in nucleotide excision repair and severe developmental failure. Am J Hum Genet, Vol. 80, pp 457-66.

Le May, N.; Egly, J. M. & Coin, F. (2010a). True lies: the double life of the nucleotide excision repair factors in transcription and DNA repair. J Nucleic Acids, Vol. 2010, pp

Le May, N.; Mota-Fernandes, D.; Velez-Cruz, R.; Iltis, I.; Biard, D. & Egly, J. M. (2010b). NER factors are recruited to active promoters and facilitate chromatin modification for transcription in the absence of exogenous genotoxic attack. Mol Cell, Vol. 38, pp 54-66.

Limoli, C. L.; Giedzinski, E. & Cleaver, J. E. (2005). Alternative recombination pathways in UV-irradiated XP variant cells. Oncogene, Vol. 24, pp 3708-14.

Loseva, O.; Jemth, A. S.; Bryant, H. E.; Schuler, H.; Lehtio, L.; Karlberg, T. & Helleday, T. (2010). PARP-3 is a mono-ADP-ribosylase that activates PARP-1 in the absence of DNA. J Biol Chem, Vol. 285, pp 8054-60.

Macrae, C. J.; McCulloch, R. D.; Ylanko, J.; Durocher, D. & Koch, C. A. (2008). APLF (C2orf13) facilitates nonhomologous end-joining and undergoes ATM-dependent hyperphosphorylation following ionizing radiation. DNA Repair (Amst), Vol. 7, pp 292-302.

Matveeva, O.; Nechipurenko, Y.; Rossi, L.; Moore, B.; Saetrom, P.; Ogurtsov, A. Y.; Atkins, J. F. & Shabalina, S. A. (2007). Comparison of approaches for rational siRNA design leading to a new efficient and transparent method. Nucleic Acids Res, Vol. 35, pp e63.

McKinnon, P. J. (2009). DNA repair deficiency and neurological disease. Nat Rev Neurosci, Vol. 10, pp 100-12.

Mendes-Pereira, A. M.; Martin, S. A.; Brough, R.; McCarthy, A.; Taylor, J. R.; Kim, J. S.; Waldman, T.; Lord, C. J. & Ashworth, A. (2009). Synthetic lethal targeting of PTEN mutant cells with PARP inhibitors. EMBO Mol Med, Vol. 1, pp 315-22.

Menissier de Murcia, J.; Ricoul, M.; Tartier, L.; Niedergang, C.; Huber, A.; Dantzer, F.; Schreiber, V.; Ame, J. C.; Dierich, A.; LeMeur, M.; Sabatier, L.; Chambon, P. & de Murcia, G. (2003). Functional interaction between PARP-1 and PARP-2 in chromosome stability and embryonic development in mouse. Embo J, Vol. 22, pp 2255-63.

Meulle, A.; Salles, B.; Daviaud, D.; Valet, P. & Muller, C. (2008). Positive regulation of DNA double strand break repair activity during differentiation of long life span cells: the example of adipogenesis. PLoS One, Vol. 3, pp e3345.

Mladenov, E. & Iliakis, G. (2011). Induction and repair of DNA double strand breaks: The increasing spectrum of non-homologous end joining pathways. Mutat Res, Vol., pp in press.

Moynahan, M. E.; Chiu, J. W.; Koller, B. H. & Jasin, M. (1999). Brca1 controls homology-directed DNA repair. Mol Cell, Vol. 4, pp 511-8.

Mullan, P. B.; Gorski, J. J. & Harkin, D. P. (2006). BRCA1--a good predictive marker of drug sensitivity in breast cancer treatment? Biochim Biophys Acta, Vol. 1766, pp 205-16.

Noel, G.; Godon, C.; Fernet, M.; Giocanti, N.; Megnin-Chanet, F. & Favaudon, V. (2006). Radiosensitization by the poly(ADP-ribose) polymerase inhibitor 4-amino-1,8-

naphthalimide is specific of the S phase of the cell cycle and involves arrest of DNA synthesis. Mol Cancer Ther, Vol. 5, pp 564-74.

O'Driscoll, M. & Jeggo, P. A. (2008). The role of the DNA damage response pathways in brain development and microcephaly: insight from human disorders. DNA Repair (Amst), Vol. 7, pp 1039-50.

O'Shaughnessy, J.; Osborne, C.; Pippen, J. E.; Yoffe, M.; Patt, D.; Rocha, C.; Koo, I. C.; Sherman, B. M. & Bradley, C. (2011). Iniparib plus chemotherapy in metastatic triple-negative breast cancer. N Engl J Med, Vol. 364, pp 205-14.

Ousset, M.; Bouquet, F.; Fallone, F.; Biard, D.; Dray, C.; Valet, P.; Salles, B. & Muller, C. (2010). Loss of ATM positively regulates the expression of hypoxia inducible factor 1 (HIF-1) through oxidative stress: Role in the physiopathology of the disease. Cell Cycle, Vol. 9, pp 2814-22.

Patel, A. G.; Sarkaria, J. N. & Kaufmann, S. H. (2011). Nonhomologous end joining drives poly(ADP-ribose) polymerase (PARP) inhibitor lethality in homologous recombination-deficient cells. Proc Natl Acad Sci U S A, Vol. 108, pp 3406-11.

Pearl, L. H. (2000). Structure and function in the uracil-DNA glycosylase superfamily. Mutat Res, Vol. 460, pp 165-81.

Peltomaki, P. (2003). Role of DNA mismatch repair defects in the pathogenesis of human cancer. J Clin Oncol, Vol. 21, pp 1174-9.

Pennarun, G.; Granotier, C.; Hoffschir, F.; Mandine, E.; Biard, D.; Gauthier, L. R. & Boussin, F. D. (2008). Role of ATM in the telomere response to the G-quadruplex ligand 360A. Nucleic Acids Res, Vol., pp 1741-54.

Pennarun, G.; Hoffschir, F.; Revaud, D.; Granotier, C.; Gauthier, L. R.; Mailliet, P.; Biard, D. S. & Boussin, F. D. (2010). ATR contributes to telomere maintenance in human cells. Nucleic Acids Res, Vol. 38, pp 2955-63.

Quinn, J. E.; Kennedy, R. D.; Mullan, P. B.; Gilmore, P. M.; Carty, M.; Johnston, P. G. & Harkin, D. P. (2003). BRCA1 functions as a differential modulator of chemotherapy-induced apoptosis. Cancer Res, Vol. 63, pp 6221-8.

Rey, L.; Sidorova, J. M.; Puget, N.; Boudsocq, F.; Biard, D. S.; Monnat, R. J., Jr.; Cazaux, C. & Hoffmann, J. S. (2009). Human DNA polymerase eta is required for common fragile site stability during unperturbed DNA replication. Mol Cell Biol, Vol. 29, pp 3344-54.

Roos, W. P. & Kaina, B. (2006). DNA damage-induced cell death by apoptosis. Trends Mol Med, Vol. 12, pp 440-50.

Rouleau, M.; McDonald, D.; Gagne, P.; Ouellet, M. E.; Droit, A.; Hunter, J. M.; Dutertre, S.; Prigent, C.; Hendzel, M. J. & Poirier, G. G. (2007). PARP-3 associates with polycomb group bodies and with components of the DNA damage repair machinery. J Cell Biochem, Vol. 100, pp 385-401.

Rulten, S. L.; Fisher, A. E.; Robert, I.; Zuma, M. C.; Rouleau, M.; Ju, L.; Poirier, G.; Reina-San-Martin, B. & Caldecott, K. W. (2011). PARP-3 and APLF function together to accelerate nonhomologous end-joining. Mol Cell, Vol. 41, pp 33-45.

Sartori, A. A.; Fitz-Gibbon, S.; Yang, H.; Miller, J. H. & Jiricny, J. (2002). A novel uracil-DNA glycosylase with broad substrate specificity and an unusual active site. Embo J, Vol. 21, pp 3182-91.

Schmutz, V.; Janel-Bintz, R.; Wagner, J.; Biard, D.; Shiomi, N.; Fuchs, R. P. & Cordonnier, A. M. (2010). Role of the ubiquitin-binding domain of Pol{eta} in Rad18-independent

translesion DNA synthesis in human cell extracts. Nucleic Acids Res, Vol. 38, pp 6456-65.

Schreiber, V.; Ame, J. C.; Dolle, P.; Schultz, I.; Rinaldi, B.; Fraulob, V.; Menissier-de Murcia, J. & de Murcia, G. (2002). Poly(ADP-ribose) polymerase-2 (PARP-2) is required for efficient base excision DNA repair in association with PARP-1 and XRCC1. J Biol Chem, Vol. 277, pp 23028-36.

Schreiber, V.; Dantzer, F.; Ame, J. C. & de Murcia, G. (2006). Poly(ADP-ribose): novel functions for an old molecule. Nat Rev Mol Cell Biol, Vol. 7, pp 517-28.

Schulte, J. H.; Kuhfittig-Kulle, S.; Klein-Hitpass, L.; Schramm, A.; Biard, D. S.; Pfeiffer, P. & Eggert, A. (2008). Expression of the TrkA or TrkB receptor tyrosine kinase alters the double-strand break (DSB) repair capacity of SY5Y neuroblastoma cells. DNA Repair (Amst), Vol., pp

Solyom, S.; Pylkas, K. & Winqvist, R. (2010). Screening for large genomic rearrangements of the BRIP1 and CHK1 genes in Finnish breast cancer families. Fam Cancer, Vol. 9, pp 537-40.

Verkaik, N. S.; Esveldt-van Lange, R. E.; van Heemst, D.; Bruggenwirth, H. T.; Hoeijmakers, J. H.; Zdzienicka, M. Z. & van Gent, D. C. (2002). Different types of V(D)J recombination and end-joining defects in DNA double-strand break repair mutant mammalian cells. Eur J Immunol, Vol. 32, pp 701-9.

Vert, J. P.; Foveau, N.; Lajaunie, C. & Vandenbrouck, Y. (2006). An accurate and interpretable model for siRNA efficacy prediction. BMC Bioinformatics, Vol. 7, pp 520.

Wang, M.; Wu, W.; Rosidi, B.; Zhang, L.; Wang, H. & Iliakis, G. (2006). PARP-1 and Ku compete for repair of DNA double strand breaks by distinct NHEJ pathways. Nucleic Acids Res, Vol. 34, pp 6170-82.

Williamson, C. T.; Muzik, H.; Turhan, A. G.; Zamo, A.; O'Connor, M. J.; Bebb, D. G. & Lees-Miller, S. P. (2010). ATM deficiency sensitizes mantle cell lymphoma cells to poly(ADP-ribose) polymerase-1 inhibitors. Mol Cancer Ther, Vol. 9, pp 347-57.

Wu, P. Y.; Frit, P.; Malivert, L.; Revy, P.; Biard, D.; Salles, B. & Calsou, P. (2007). Interplay between Cernunnos-XLF and Nonhomologous End-joining Proteins at DNA Ends in the Cell. J Biol Chem, Vol. 282, pp 31937-31943.

Yan, C. T.; Boboila, C.; Souza, E. K.; Franco, S.; Hickernell, T. R.; Murphy, M.; Gumaste, S.; Geyer, M.; Zarrin, A. A.; Manis, J. P.; Rajewsky, K. & Alt, F. W. (2007). IgH class switching and translocations use a robust non-classical end-joining pathway. Nature, Vol. 449, pp 478-82.

4

Base Excision Repair Pathways

Christina Emmanouil
Benaki Phytopathological Institute
Athens,
Greece

1. Introduction

DNA repair is a process vital to the cell since the genetic material is the target of a multitude of daily attacks. Cells have evolved into possessing distinct but simultaneously intercalating ways of dealing with repair of DNA lesions. These repair pathways may include practices dealing with single strand damage (Base Excision Repair, Nucleotide Excision Repair, Mismatch Repair) or with double strand damage (Non-homologous End Joining and Homologous Recombination) as well as Direct Reversal Repair and Translesion Synthesis. The present chapter deals with one of these pathways (Base Excision Repair), which rectifies damage at the point of the single nucleotide.

2. Overview of base excision repair

Small non-helix distorting DNA alterations are very common in living organisms and they are due both to exogenous and endogenous sources. Endogenous damage can be summarised into the following categories: a) misincorporation of uracil in the genome or spontaneous deamination of cytosine (Sung and Demple, 2006) b) hydrolysis of all four bases or oxidation by reactive oxygen species (ROS), hormones, reactive nitrogen species, heme precursors and amino acids (Nilsen and Krokan, 2001; Wood et al., 2001) c) alkylation of purines and pyrimidines by lipid end-products (Sung and Demple, 2000) or other parameters (e.g. S-adenyl-methionine). Spontaneous abasic sites are also common lesions and 10000 purines are detached from DNA per human genome per day (Wilson and Kunkel, 2000; Nilsen and Krokan, 2001). Besides all these endogenous reactions, exogenous agents as xenobiotics and radiation are also able to cause similar damage. All these small, point lesions are rectified by Base Excision Repair (BER) (Krokan et al., 2000; Cabelof et al., 2002). BER was discovered by Tomas Lindahl in 1974 (Krokan et al., 2000), it is a tightly conserved pathway from bacteria to mammals (Izumi et al., 2003; Didzaroglu, 2005) and it must be preserved in a highly coordinated way to be effective (Moustacchi, 2000; Allinson et al., 2004).
BER is initiated by the cleaving of the damaged base by a specialized enzyme: a DNA *N*-glycosylase. The glycosylases implicated in BER fall into two main groups regarding their mechanisms of action: monofunctional and bifunctional glycosylases (Fortini et al., 1999; Krokan et al., 2000; Cabelof et al., 2002). In the case of the monofunctional glycosylases, an aspartic acid (Asp) residue activates a water molecule, which in its turn performs a nucleophilic attack on the *N*-glycosidic bond. In bifunctional glycosylases, the Asp residue

activates an amino group of a lysine (Lys) residue. The amino group forms a Schiff base ($\overset{R_2}{\underset{R_1}{}}\!\!>\!\!=\!\!N\!-\!R_3$) with C1' followed by β-elimination at the 3' side of the deoxyribose (Bailly et al., 1989; Nilsen and Krokan, 2001). In the case of a monofunctional glycosylase, the net result is an apurinic or apyrimidinic site (AP site) and in the case of a bifunctional glycosylase the net result are two single strand breaks: one strand with a 3'-α,β unsaturated aldehyde end (3'PUA) and another strand with a 5'-phosphate end. However, some of the bifunctional glycosylases (namely the bacterial Fpg and Nei and the mammalian NEIL1) are able to further process 3'PUA via δ-elimination bearing a 3'phosphate end (Nilsen and Krokan, 2001; Gros et al., 2002; Wiederhold et al., 2004).

The AP site created (as well as the SSB) must be quickly processed further since they are highly cytotoxic (Allinson et al., 2004) and mutagenic (Nilsen and Krokan, 2001). This is done by an AP endonuclease (APE1 for mammalian organisms) resulting in the formation of a 3'-hydroxyl end (3'OH) and a 5' abasic sugar phosphate end (5'dRP) (Memisoglu and Samson, 2000). AP endonuclease APE1 is also involved in the "trimming" of the blocked 3' end created by bifunctional glycosylases (Mitra et al., 2001; Cabelof et al., 2002; Izumi et al., 2003). However some researchers argue that the phosphatase activity of APE1 is low and that polynucleotide kinase (PNK) is the only enzyme that cleaves successfully the products of βδ-elimination (Mitra et al., 2002; Wiederhold et al., 2004).

The formation of SSB by APE1 is a critical point in the BER process since two sub-pathways may follow: the short-patch or the long-patch pathway (Christmann et al., 2003; Sung and Demple, 2006). The short patch may be initiated by *N*-glycosylases whereas the long-patch may be the pathway of choice for spontaneous hydrolysis of bases (Didzaroglu, 2005). Cell cycle stage may also affect the choice of sub-pathway (Krokan et al., 2000): bifunctional glycosylases point to a short-patch mode of action whereas damage rectified by monofunctional glycosylases may follow either pathway (Fortini et al., 1999). The long-patch process may have evolved as a more efficient or as a redundant mechanism for abasic moieties (Wilson and Thompson, 1997). In some cases these moieties are refractory to 5'phosphodiesterase activity. Indeed oxidized abasic sites do not give rise to deoxyribose phosphate (dRP) under physiological conditions. In this case the cleaving of the sugar-phosphate backbone must be done downstream towards the 3'end (Sung and Demple, 2006) and the same happens with reduced abasic sites (Zhang and Dianov, 2005).

No matter what the underlying reason for differentiation is, the two sub-pathways are substantially different. In the short-patch polymerase β attaches a single nucleotide to the trimmed 3'OH end, displacing the dRP at the 5'end (Wilson and Thompson, 1997; Schärer and Jiricny, 2001) and it also cleaves 5'dRP by its intrinsic lyase activity through a covalent Schiff intermediate (Sung and Demple, 2006). Ligase III/XRCC1 seals the gap and DNA integrity is restored (Wilson and Thompson, 1997; Cabelof et al., 2002). Polymerase β does not have proofreading abilities and sometimes it incorporates an incorrect nucleotide which is subsequently re-excised by APE1 (Noble, 2002). In the long-patch, polymerase β or polymerase δ/ε with the proliferating cell nuclear antigen (PCNA), add a few more nucleotides at the 3'OH end (Christmann et al., 2003). The number of extra nucleotides is according to researchers up to six (Schärer and Jiricny, 2001), up to ten (Christmann et al., 2003) or up to thirteen (Suttler et al., 2003). This action creates a flap at the 5'dRP end. This flap is then excised by flap endonuclease (FEN1) and afterwards PCNA/ligase I seals the gap (Christmann et al., 2003). A representation of BER pathways is depicted in Figure 1.

3. Common polymorphisms in BER and cancer risk

Given the crucial role of BER in DNA repair, it is expected that polymorphisms which alter enzyme activity may be linked with increased cancer risk. *In vitro* modified (increased or decreased) activity of mutated alleles is not always corroborated by similar *in vivo* activity and human studies are sometimes few and/or inconclusive. In other cases, however, a strong link between mutation and development or propagation of cancer has been verified.
The substitution of serine by cysteine in codon 326 (Ser326Cys) in OGG1 *N*-glycosylase (see subchapter 4) is one of the cases of questionable involvement in cancer; the imperative need for comprehensive human studies regarding this polymorphism which has been shown to be less efficient in oxidative lesion removal *in vitro* (Ishida et al., 1999; Yamane et al., 2004; Hill and Evans, 2006) has already been highlighted (Loft and Moller, 2006). Epidemiological studies of this polymorphism in relation to lung cancer have yielded mixed results showing a weak association between homozygous Ser326Cys and cancer development (Hatt et al., 2008). Two recent meta-analyses (statistical re-evaluations of separate but related studies) of 1925 and 3253 lung cancer patients showed indeed an increased risk in homozygous populations (Kohno et al., 2006; Hung et al., 2005 respectively). Furthermore, a meta-analysis of 6375 cases showed implications of Ser326Cys homozygous genotype in cancer development in non-smokers only (Li et al., 2008). Incrimination of this OGG1 polymorphism in lung cancer development is thus possible. Colorectal cancer is less strongly associated with this polymorphism; the Ser326Cys homozygous populations were at increased risk for colorectal cancer in certain studies (Moreno et al., 2006). The Ser326Cys homozygous populations were at increased risk for colorectal cancer, only when other incriminating factors (increased meat intake, cigarette smoking) were co-present (Kim et al., 2003). Other studies however have not found a correlation between this polymorphism and colorectal adenomas or carcinomas (Hansen et al., 2005).
On the contrary, mutations in the MYH gene (see also subchapter 4) have been proven without doubt to be able to cause an autosomal recessive form of familial adenomatous polyposis (Lindor, 2009). Individuals with biallelic inherited mutations of the MYH gene run a high risk of colorectal cancer that approaches 100% (Dolwani et al., 2007). Transversion mutations in MYH disrupt the normal base excision repair of adenines misincorporated opposite 7,8-dihydro-8-oxoguanine, a prevalent and stable product of oxidative damage to DNA, leading to hereditary colorectal neoplasms (Sampson et al, 2005). Patients with MYH-associated polyposis (MAP) present with clinical features similar to classic FAP (familial adenomatous polyposis). Patients typically present between the ages of 40–60 years with a variable number of colorectal adenomatous polyps, however, MYH mutation carriers do not usually present with multiple polyps before the age of 30 years (Kastrinos and Syngal, 2007).
Given the critical role of APE1 in BER a total of 18 polymorphisms in APE1 gene have been reported. The most extensively studied is the Asp148Glu; A meta-analysis of 12432 cases showed an increased risk of cancers, especially of colorectal cancer for this allele. Functional studies have shown that this variant may have altered endonuclease and DNA-binding activity and reduced ability to interact with other BER proteins (Gu et al., 2009) in order to form critical complexes for nucleotide excision/incorporation during the rectifying process. Besides its endonuclease activity, APE1 has been shown to stimulate the DNA binding activity of numerous transcription factors that are involved in cancer promotion and progression such as Fos, Jun, nuclear factor-κB and p53, thus is actively involved in redox regulation of oncoproteins (Kelley et al., 2010).

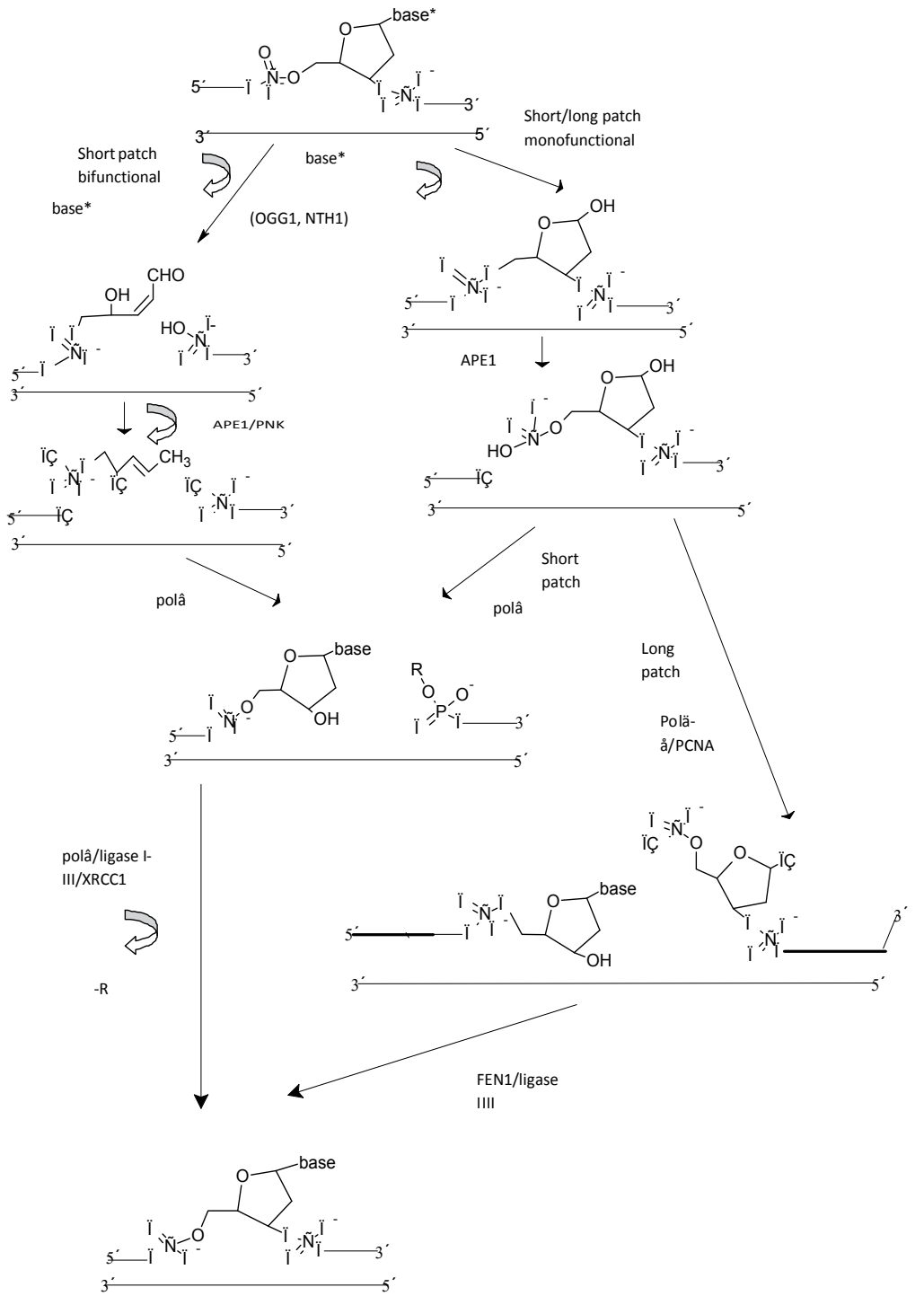

Fig. 1. Schematic representation of mammalian Base Excision Repair pathway (adapted from Wiederhold et al., 2004).

The XRCC1 protein plays a major role in facilitating the repair of single-strand breaks in mammalian cells, via an ability to interact with multiple enzymatic components of repair reactions (Caldecott, 2003). In BER, XRCC1 acts as a scaffold for DNA ligase III, polymerases and PAPR. Some of the most common XRCC1 polymorphisms are Arg194Trp, Arg280His and Arg399Gln (Xue et al., 2011). A meta-analysis of 40 studies regarding these three polymorphisms and breast cancer showed a recessive effect of Arg280His and Arg399Gln variants in Asians only (Li et al., 2009). However, no increase in gastric cancer risk for Arg194Trp, Arg280His and Arg399Gln has been noted (Geng et al., 2008; Xue et al., 2011). According to other studies, the Arg194Trp variant contributes to a reduced risk of various types of cancer (Goode et al., 2002; Hu et al., 2005). Indeed, the XRCC1 Arg194Trp and Arg280His variants were each associated with a reduced risk of lung cancer compared with common allele homozygotes (Hung et al., 2005). These last results are somewhat surprising since a common perception is that a change in amino-acid structure would be deleterious to function and would result in an increased risk of cancer. In the specific case of XRCC1, it is possible that a change to tryptophan would cause a transition from the positively charged arginine of the wild type to a hydrophobic tryptophan, which could positively affect binding to DNA and increase efficiency (Ladiges, 2006).
PCNA, another important component for BER polymerases scaffolding is also characterised as "the ringmaster of the genome". It interacts with p53-controlled proteins Gadd45, MyD118, CR6 and p21, in the process of deciding cell fate: proliferation, repair or apoptosis (Paunesku et al., 2001). A novel form of PCNA has been described in malignant breast cells. This unique form is not the result of a genetic alteration, as demonstrated by DNA sequence analysis but it is the product of post-translational modification. This example shows the diverse and multifaceted ways that BER enzyme variations may affect cancer frequency (Bechtel et al., 1988).

4. Specific BER N-glycosylases implicated in oxidised base removal

4.1 Prokaryotic organisms (*E.coli*)

i) Fpg (MutM): Formamidopyrimidine glycosylase (Fpg) is a glycosylase which excises 8-oxo-deoxyguanosine (8-oxo-dG) from 8-oxo-dG: C pairs (Beckman and Ames, 1997; Gros et al., 2002; Russo et al., 2006), but not from 8-oxo-dG: A mispairs because that would lead to a stable G-T transition (Wang et al., 1998). One of the most extensively studied glycosylases, Fpg has been also found in *Deinococcus radiodurans* (Gros et al., 2002), in yeast *Candida albicans* (Wallace, 2002) and in the plant *Arabidopsis thaliana* (Rosenquist et al., 2003). It is a globular monomer of 269 aminoacids and 30.2 kDa, which cleaves 8-oxo-dG, its opened ring form 2,6-diamino-4-hydroxy-5-formamidopyrimidine (Fapy) and a variety of pyrimidines (5-hydroxycytosine, 5-hydroxyuracil and thymine glycol) (Gros et al., 2002). Fpg possesses a C-terminal Zn finger motif, which stabilises the bound DNA and contributes to substrate interaction (Rosenquist et al., 2003). Fpg has an AP lyase activity performing βδ elimination of the abasic site (Gros et al., 2002; Russo et al., 2006).
ii) Nei (endo VII): Nei exhibits an overlapping substrate-specificity with Fpg. It comprises 263 amino acids and a C-terminal Zn finger motif (Gros et al., 2002; Rosenquist et al., 2003).
iii) MutT: MutT is a GTPase which sanitises nucleotide pools from 8-oxo-GTP by hydrolysing it to monophosphate, thus preventing its misincorporation in DNA (Beckman and Ames, 1997; Fortini et al., 2003). MutT deficient strains exhibit a strong mutator phenotype (Fortini et al., 2003).

iv) MutY: MutY excises A which is wrongly incorporated opposite 8-oxo-dG (Beckman and Ames, 1997; Vidal et al., 2001). MutY is a 39 kDa protein which can also excise A opposite to G, C and 8-oxo-dA (Le Page et al., 1999; Gros et al., 2002). Fpg/MutY double null strains exhibit a strong mutator phenotype (Wang et al., 1998; Russo et al., 2006).
v) Nth: Nth also excises Fpg substrates and it has a strong activity against thymine glycol and other oxidised pyrimidines (Gros et al., 2002; Izumi et al., 2003; Rosenquist et al., 2003). Nth possesses a β-lyase activity besides its *N*-glycosylase activity (bifunctional glycosylase) (Izumi et al., 2003). Nth mutants are not sensitive towards oxidative insult, however the double mutants *nth/nei* exhibit a mutator phenotype after exposure to ionising radiation or H_2O_2 (Gros et al., 2002).
The triad MutT, MutY and MutM (Fpg) comprise the so called GO system which suppresses effectively Guanine Oxidation via the concerted actions of sanitising of the nucleotide pool, excising misincorporated A and excising 8-oxo-dG from duplex DNA respectively (Beckman and Ames, 1997).

4.2 Eykaryotic organisms (mammals)

i) OGG1: hOGG1 is the functional analogue of Fpg in humans. Besides 8-oxo-dG, OGG1 has activity against Fapy (Nohmi et al., 2005) and very weak activity against 4,6-diamino-5-formamidopyrimidine (FapyA) (Wallace, 2002). The mammalian OGG1 contains a helix-hairpin-helix motif (HhH) with an Asp-activated Lys residue as an active site. The residue forms a Schiff base with the substrate and subsequently creates an AP site (Izumi et al., 2003). OGG1 is a bifunctional glycosylase, however it acts as a monofunctional *in vivo* since APE1 precludes the lyase activity of OGG1 (Vidal et al., 2001; Fortini et al., 2003). Given the crucial role of OGG1 in 8-oxo-dG control, it is rather surprising that *ogg1*-/- mice are viable and do not show malignant phenotype (Klungland et al., 1999; Gros et al., 2002). Implications of other glycosylases or even other pathways are probably some of the reasons for this incident (Izumi et al., 2003). However it has been shown in the same mice that the incidents of spontaneous lung carcinoma/adenoma and UV-induced skin tumours are elevated 1.5 years after birth (Sakumi et al., 2003). In humans OGG1 polymorphisms have been incriminated for certain cancer types, especially the mutation Ser326Cys as discussed earlier in this chapter. It is postulated that Cys mutants have lower 8-oxo-dG excision capacity than wild type cells (Nohmi et al., 2005).
ii) NEIL: 3 human and 3 mouse homologues of the bacterial Nei have been cloned, namely NEIL1, NEIL2 and NEIL3. They contain a helix-two turn-helix motif and NEIL 2 and 3 may also contain Zn finger motifs (Rosenquist et al., 2003). NEIL1 and NEIL2 perform a βδ elimination on their substrate with a 3' and a 5' phosphate strand as end products (Izumi et al., 2003). NEIL excises thymine glycol (TG), FapyG and FapyA but shows only nominal activity against 8-oxo-dG (Rosenquist et al., 2003). According to other researchers NEIL activity against 8-oxo-dG is significant and the tissue-specific high expression of NEIL3 may be a back up mechanism for removal of oxidised guanine (Slupphaug et al., 2003).
iii) MTH1: the mammalian homologue of MutT is MTH1. MTH1 sanitises the nucleotide pool from 8-oxo-GTP and it also degrades 2OH-dATP and 2OH-ATP (Slupphaug et al., 2003; Nohmi et al., 2005). *Mth*-/- mice showed an increased rate of point mutations (Nakabeppu et al., 2006). Some polymorphisms of MTH1 have been found in cancer patients but a correlation between cancer and MTH1 variations has not been established (Nohmi et al., 2005). However the polymorphism Val83Met was dominant in female Japanese patients with Type I diabetes mellitus (Miyako et al., 2004).

iv) MYH: the mammalian homologue of MutY is MYH. MYH removes A from 8-oxo-dG:A mispairs (Nagashima et al., 1997; Fortini et al., 2003). It also recognises A:G and A:C mispairs (Fortini et al., 2003; Izumi et al., 2003). It is mainly a monofunctional glycosylase with a weak AP lyase activity (Russo et al., 2006). Important variants of MYH were found in siblings afflicted by multiple colon adenoma and carcinoma and further studies proved its role in colorectal adenoma and carcinoma predisposition (Gros et al., 2002; Nohmi et al., 2005). Double mutants mice for MYH and OGG1 had increased tumours in lung, small intestine and ovaries (Russo et al., 2006). The unique action of MYH does not seem to have any back up from other glycosylases, which explain the importance of its mutations (Izumi et al., 2003).

v) NTH1: The mammalian homologue of nth is NTH1. NTH1 has similar substrate specificity with nth (Gros et al., 2002). It possesses both an *N*-glycosylase and an endonuclease activity but a product inhibition dissociates the two activities (Izumi et al., 2003; Marenstein et al., 2003). Double mutant embryonic cells still showed TG repair because of the accessory enzymes TGG1 and TGG2 (Gros et al., 2002). Furthermore, *nth1*-/- mice stayed healthy but exhibited a slower excision activity for NTH1 substrates (Izumi et al., 2003).

vi) OGG2: the novel glycosylase OGG2 has been found in human (Wang et al., 1998; Bohr and Dianov, 1999) and yeast (Nash et al., 1996) cells. OGG2 probably excises wrongly incorporated 8-oxo-dG opposite A, in a nascent strand (Izumi et al., 2003).

The triad MTH1, MYH and OGG1 together with other accessory proteins like OGG2 and NEIL comprise the mammalian GO system which suppresses effectively Guanine Oxidation. A representation of the GO system is given in Figure 2.

5. Specific BER N-glycosylases implicated in alkylated base removal

It has been estimated that 20,000 DNA lesions are produced per cell per day but the contribution of alkylation damage is not well established (Drabløs et al., 2004). However alkylation damage occurs frequently as part of the normal metabolism of the cell. It has been shown that the methyl donor *S*-adenyl-methionine can methylate spontaneously DNA to 3-methyl-adenine (3meA) (Seeberg et al., 1995). Also alkylation occurs as a consequence of lipid peroxidation and of nitrosocompounds in the gastrointestinal tract. Furthermore, there are naturally occurring 7-methylguanine (7meG) residues in the genome (Xiao and Samson, 1993). Exogenous sources of alkylation include chloromethane gas by plants, fungi and industrial uses, *N*-nitrosocompounds in tobacco smoke and alkylating anticancer drugs like temozolomide, carmustin and lomustine (Drabløs et al., 2004). Almost all the main mechanisms of DNA repair (direct damage reversal, BER, NER and recombination repair) are implicated in alkylation damage repair and almost all alkylating agents can form *O* and *N*-adducts in all bases and *O*-adducts in phosphodiesters (Drabløs et al., 2004). Regarding BER, a series of *N*-glycosylases have evolved especially for excision of alkylated bases.

5.1 Prokaryotic organisms (*E.coli*)

i) Tag: The bacterial Tag excises 3meA and 3meG from dsDNA only (Bjeeland and Seeberg, 1996) and it is not inducible (Seeberg et al., 1995). *E.coli* strains, which had enhanced 3meA glycosylase activity however, were more susceptible to mutations if they were simultaneously deficient in AP endonuclease activity (Taverna and Sedwick, 1996). The same happened with the functional homologue of *S. cerevisiae* when it was overexpressed in AP endonuclease

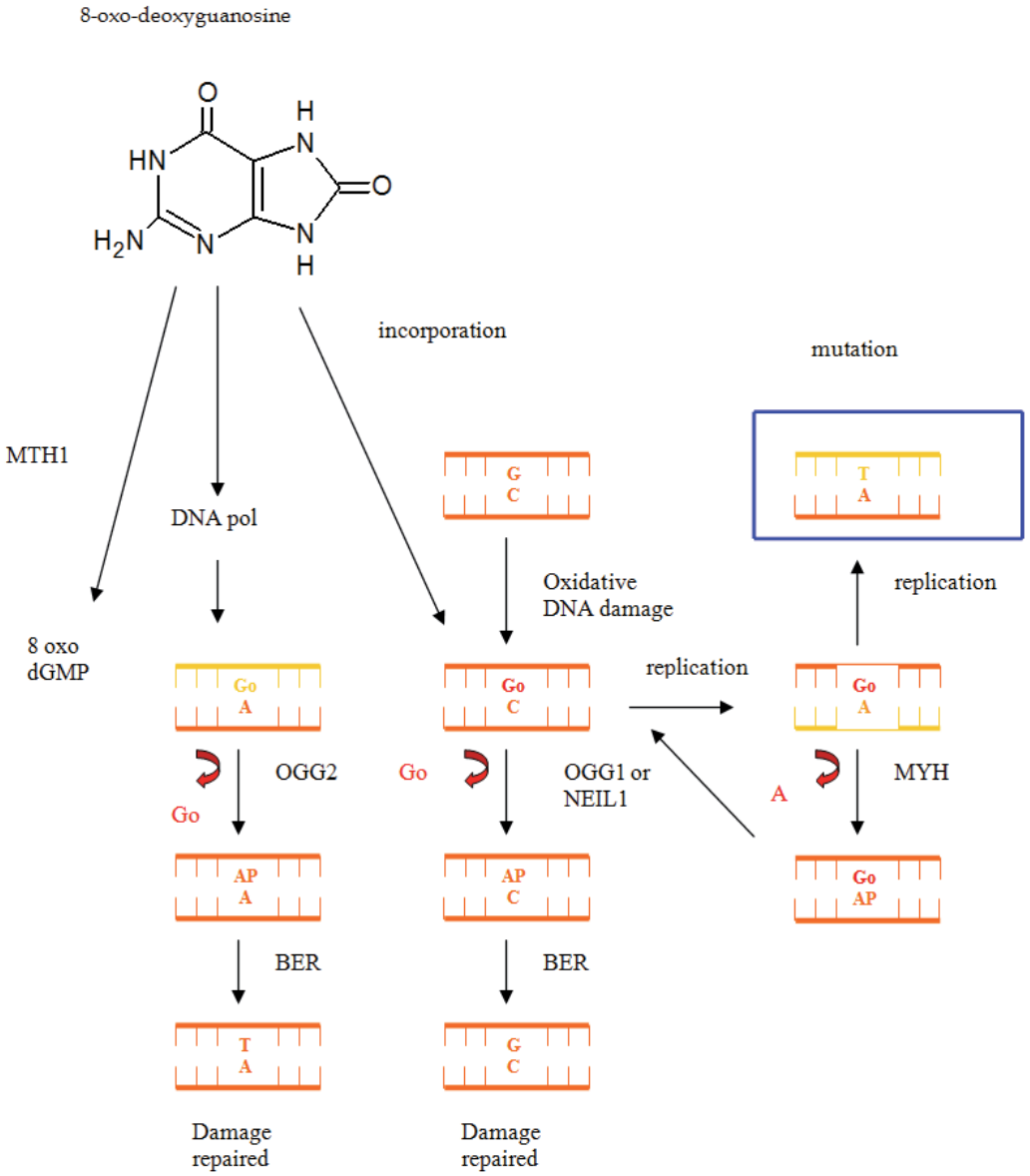

Fig. 2. Schematic representation of the "GO-system" in mammalian cells (adapted from Slupphaug et al., 2003).

deficient organisms (Xiao and Samson, 1993). These data suggest that the expression of Tag should be carefully controlled for achievement of optimal activity.

ii) AlkA: The bacterial AlkA excises 3meA, 7meA, 7meG, O^2 methylcytosine, O^2 methylthymine and hypoxanthine (Seeberg et al., 1995; Hollis et al., 2000; Drabløs et al., 2004). It is a monofunctional glycosylase (Labahn et al., 1996) with a HhH motif and an Asp328 as an active site. Also the active pocket of AlkA is rich in aromatic residues, which interact with the alkylated bases (Cunningham et al., 1997; Lau et al., 1998). AlkA is using a

base-flipping mechanism which projects the modified base into the active pocket of the enzyme. The charged, deficient bases may stack more tightly against the aromatic residues of AlkA than the uncharged bases (Hollis et al., 2000). Double mutant strains of *E. coli* for Tag and AlkA are extremely sensitive to alkylating DNA damage (Seeberg et al., 1995).

5.2 Eukaryotic organisms (mammals)

i) AAG (MPG, ANPG): The mammalian AAG shares the same broad specificity with the bacterial AlkA. It also removes intact guanines at very low frequencies but it cannot remove O^2-alkylated pyrimidines (Drabløs et al., 2004). However, AlkA and AAG bear little or no sequence similarity between them: the yeast MAG and AlkA possess some common sequence characteristics but there is no sequence analogy between AlkA and the plant or mammalian AAG (Labahn et al., 1996). The broad specificity of AAG is an interesting phenomenon. It is probable that AAG outstacks completely or partially nucleotides and scans along DNA searching for alkylation damage (Lau et al., 1998). Base flipping of the modified base is accompanied by its intercalation with Tyr162, its stabilisation and a nucleophilic attack by a water molecule deprotonated by Glu125 (Lau et al., 1998; Hollis et al., 2000).

As already mentioned. AAG is able to rectify exocyclic etheno adducts like εdA, 3,N^4-ethenodeoxycytidine (εdC), 1,N^2-ethenodeoxyguanosine (1,N^2εdG) and N^2, 3-ethenodeoxyguanosine (N2-3εdG) which are formed during lipid peroxidation or by vinyl chloride, vinyl fluoride, vinyl carbamate, urethanes and other carcinogens (Ham et al., 2004). *In vitro* AAG was the only enzyme able to repair these kinds of adducts, however experiments with *Aag* -/- mice showed that there is still a weak activity against etheno-adducts via other unknown enzymes or via other pathways (Ham et al., 2004). Overexpression of AAG may enhance the cytotoxicity of alkylating agents thus, protection from AAG or sensitization by AAG may be tissue-specific and its levels should be carefully controlled in order to achieve optimal activity (Drabløs et al., 2004). It is probable that enhancement of its glycosylase activity creates a surplus of abasic sites which are not properly processed by endonuclases leading to a repair imbalance.

6. Conclusion

Base Excision Repair pathway is a tightly conserved pathway, from prokaryotic organism to higher mammals. At the same time it is an adapting and flexible mechanism, which covers repair of a variety of small DNA lesions as evidenced by its diverse *N*-glycosylases. BER works both under normal conditions and during stressful incidents. The importance of BER in cell survival is highlighted by the fact that the most common genetic damages due to oxidative stress are rectified mainly through this pathway. Furthermore, polymorphisms of BER enzymes which compromise their activity may lead or contribute to neoplasias to a certain extent.

7. References

Allinson S.L., Sleeth K.M., Matthewman G.E., and Dianov G.L., (2004) Orchestration of base excision repair by controlling the rates of enzymatic activities. DNA Repair (Amst) 3(1): 23-31.

Bailly V., Derydt M., and Verly W.G., (1989) Delta-elimination in the repair of AP (apurinic/apyrimidinic) sites in DNA. Biochem J 261(3): 707-713.

Beckman K.B., and Ames B.N., (1997) Oxidative decay of DNA. J Biol Chem 272(32): 19633-19636.

Bechtel PE, Hickey RJ, Schnaper L, Sekowski JW, Long BJ, Freund R, Liu N, Rodriguez-Valenzuela C and Malkas LH. (1998) A unique form of proliferating cell nuclear antigen is present in malignant breast cells. Cancer Res 58(15):3264-3269

Bjelland S., and Seeberg E., (1996) Different efficiencies of the Tag and AlkA DNA glycosylases from Escherichia coli in the removal of 3-methyldenine from single-stranded DNA. FEBS Lett 397: 127-129.

Bohr V.A., and Dianov G.L., (1999) Oxidative DNA damage processing in nuclear and mitochondrial DNA. Biochimie 81(1-2): 155-160.

Cabelof D.C., Raffoul J.J., Yanamadala S., Guo Z., and Heydari A.R., (2002) Induction of DNA polymerase beta-dependent base excision repair in response to oxidative stress in vivo. Carcinogenesis 23(9): 1419-1425.

Caldecott K.W. (2003) XRCC1 and DNA strand break repair. DNA Repair 9: 955-969

Christmann M., Tomicic M.T., Roos W.P., and Kaina B., (2003) Mechanisms of human DNA repair: an update. Toxicology 193(1-2): 3-34.

Cunningham R.P., (1997) DNA glycosylases. Mutat Res 383:189-196.

de Boer J.G., (2002) Polymorphisms in DNA repair and environmental interactions. Mutat Res 509(1-2): 201-210.

Dizdaroglu M., (2005) Base-excision repair of oxidative DNA damage by DNA glycosylases. Mutat Res 591(1-2): 45-59.

Dolwani S., Williams G.T., West K.P., Newman J., Stock D., Griffiths A.P., Best J., Cheadle J.P., and Sampson J.R. (2007) Analysis of inherited MYH/(MutYH) mutations in British Asian patients with colorectal cancer. Gut 56(4): 593

Drablos F., Feyzi E., Aas P.A., Vaagbo C.B., Kavli B., Bratlie M.S., Pena-Diaz J., Otterlei M., Slupphaug G., and Krokan H.E., (2004) Alkylation damage in DNA and RNA-repair mechanisms and medical significance. DNA Repair 3:1389-1407.

Fortini P., Parlanti E., Sidorkina O.M., Laval J., and Dogliotti E., (1999) The type of DNA glycosylase determines the base excision repair pathway in mammalian cells. J Biol Chem 274(21): 15230-15236.

Geng J, Zhang YW, Huang GC, Chen LB. (2008) XRCC1 genetic polymorphism Arg399Gln and gastric cancer risk: A meta-analysis. World J Gastroenterol 14(43):6733-6737.

Goode EL, Ulrich CM, Potter JD. (2002). Polymorphisms in DNA repair genes and associations with cancer risk Epidemiol Biomarkers Prev 11(12): 1513–1530.

Gros L., Saparbaev M.K., and Laval J., (2002) Enzymology of the repair of free radicals-induced DNA damage. Oncogene 21(58): 8905-8925.

Gu D, Wang M, Wang M, Zhang Z and Chen J. (2009) The DNA repair gene APE1 T1349G polymorphism and cancer risk: a meta-analysis of 27 case-control studies. Mutagenesis. 24(6):507-512.

Ham A.-J.L., Engelward B.P., Koc H., Sangaiah R., Meira L.B., Samson L.D., and Swenberg J.A., (2004) New immunoaffinity-LC-MS/MS methodology reveals that Aag null mice are deficient in their ability to clear 1,N6-etheno-deoxyadenosine DNA lesions from lung and liver in vivo. DNA Repair 3:257-265.

Hansen R, Saebø M, Skjelbred CF, Nexø BA, Hagen PC, Bock G, Bowitz Lothe IM, Johnson E, Aase S, Hansteen IL, Vogel U, Kure EH. (2005) GPX Pro198Leu and OGG1 Ser326Cys polymorphisms and risk of development of colorectal adenomas and colorectal cancer. Cancer Lett 229(1):85-91

Hatt L, Loft S, Risom L, Møller P, Sørensen M, Raaschou-Nielsen O, Overvad K, Tjønneland A and Vogel U. (2008) OGG1 expression and OGG1 Ser326Cys polymorphism and risk of lung cancer in a prospective study. Mutat Res 639(1-2):45-54.

Hill JW, and Evans M.K. (2006) Dimerization and opposite base-dependent catalytic impairment of polymorphic S326C OGG1 glycosylase. Nucleic Acids Res 34(5): 1620–1632

Hollis T., Lau A., and Ellenberger T., (2000) Structural studies of human alkyladenine glycosylase and E.coli 3-methyladenine glycosylase. Mutat Res 460: 201-210

Hu Z, Ma H, Chen F, Wei Q, Shen H. (2005).XRCC1 polymorphisms and cancer risk: A meta-analysis of 38 case-control studies Cancer Epidemiol Biomarkers Prev 14(7): 1810–1818

Lissowska J., Rudnai P. , Fabianova E., Mates D., Foretova L., Janout V., Bencko V., Chabrier A., Borel S., Hall J., and Boffetta P. (2005). Large-Scale Investigation of Base Excision Repair Genetic Polymorphisms and Lung Cancer Risk in a Multicenter Study Natl Cancer Inst 97(8): 567–576

Hung J., Hall J., Brennan P., and Boffetta P. (2005) Genetic Polymorphisms in the Base Excision Repair Pathway and Cancer Risk: A HuGE Review. Am J Epidem 162:925–942

Ishida T, Takashima R, Fukayama M, Hamada C, Hippo Y, Fujii T, Moriyama S, Matsuba C, Nakahori Y, Morita H, Yazaki Y, Kodama T, Nishimura S and Aburatani H.(1999). New DNA polymorphisms of human MMH/OGG1 gene: prevalence of one polymorphism among lung-adenocarcinoma patients in Japanese. Int J Cancer: 80, 18–21

Izumi T., Wiederhold L.R., Roy G., Roy R., Jaiswal A., Bhakat K.K., Mitra S., and Hazra T.K., (2003) Mammalian DNA base excision repair proteins: their interactions and role in repair of oxidative DNA damage. Toxicology 193(1-2): 43-65.

Kastrinos F and Syngal S (2007) Recently identified colon cancer predispositions: MYH and MSH6 mutations. Semin Oncol. 34(5):418-24.

Kelley MR, Cheng L, Foster R, Tritt R, Jiang J, Broshears J and Koch M. (2001) Elevated and altered expression of the multifunctional DNA base excision repair and redox enzyme Ape1/ref-1 in prostate cancer. Clin Canc Res 7(4):824-830.

Kim JI, Park YJ, Kim KH, Kim JI, Song BJ, Lee MS, Kim CN and Chang SH. (2003) hOGG1 Ser326Cys polymorphism modifies the significance of the environmental risk factor for colon cancer. World J Gastroenterol 9(5):956-60.

Klungland A., Rosewell I., Hollenbach S., Larsen E., Daly G., Epe B., Seeberg E., Lindahl T., and Barnes D.E., (1999) Accumulation of premutagenic DNA lesions in mice defective in removal of oxidative base damage. Proc Natl Acad Sci U S A 96(23): 13300-13305.

Kohno T, Kunitoh H, Toyama K, Yamamoto S, Kuchiba A, Saito D, Yanagitani N, Ishihara S, Saito R and Yokota J. (2006) Association of the OGG1-Ser326Cys polymorphism with lung adenocarcinoma risk. Cancer Sci 97: 724-728

Krokan H.E., Nilsen H., Skorpen F., Otterlei M., and Slupphaug G., (2000) Base excision repair of DNA in mammalian cells. FEBS Lett 476(1-2): 73-77.

Labahn J., Scharer O.D., Long A., Ezaz-Nikpay K., Verdine G.L., and Ellenberger T.E., (1996) structural basis for the excision repair of alkylation-damaged DNA. Cell 86:321-329.

Lau A.Y., Scharer O.D., Samson L., Verdine G.L., and Ellenberger T., (1998) Crystal structure of a human alkylbase-DNA repair enzyme complexed to DNA: mechanisms for nucleotide flipping and base excision. Cell 95:249-258.

Le Page F., Gentil A., and Sarasin A., (1999) Repair and mutagenesis survey of 8-hydroxyguanine in bacteria and human cells. Biochimie 81(1-2): 147-153.

Marenstein D.R., Chan M.K., Altamirano A., Basu A.K., Boorstein R.J., Cunningham R.P., and Teebor G.W., (2003) Substrate specificity of human endonuclease III (hNTH1). Effects of human APE1 on hNTH1 activity. J Biol Chem 278(11): 9005-9012.

Memisoglu A., and Samson L., (2000) Base excision repair in yeast and mammals. Mutat Res 451(1-2): 39-51.

Ladiges W C (2006) Mouse models of XRCC1 DNA repair polymorphisms and cancer Oncogene 25: 1612–1619

Li H, Ha TC, Tai BC (2009) XRCC1 gene polymorphisms and breast cancer risk in different populations: a meta-analysis. Breast. 18(3):183-191.

Li H, Hao X, Zhang W, Wei Q, Chen K. (2008) The hOGG1 Ser326Cys polymorphism and lung cancer risk: a meta-analysis. Cancer Epidemiol Biomarkers Prev. 17(7):1739-45

Lindor NM.(2009) Hereditary colorectal cancer: MYH-associated polyposis and other newly identified disorders. Best Pract Res Clin Gastroenterol 23(1):75-87.

Loft S and Møller P. Oxidative DNA damage and human cancer: need for cohort studies. 2006 Antioxid Redox Signal. 8(5-6):1021-31.

Mitra S., Izumi T., Boldogh I., Bhakat K.K., Hill J.W., and Hazra T.K., (2002) Choreography of oxidative damage repair in mammalian genomes. Free Radic Biol Med 33(1): 15-28.

Miyako K., Kohno H., Ihara K., Kuromaru R., Matsuura N., and Hara T., (2004) Association study of human MTH1 gene polymorphisms with type I diabetes mellitus. Endocr J 51(5): 493-498.

Moreno V., Gemignani F., Landi S., Patricola L., Chabrier A., Blanco I.,Gonzalez S.,Guino E., Capella G., Canzian F., and Bellvitge Colorectal Cancer Study Group (2006) Polymorphisms in Genes of Nucleotide and Base Excision Repair: Risk and Prognosis of Colorectal Cancer. Clin Cancer Res 12:2101-2108

Moustacchi E., (2000) DNA damage and repair: consequences on dose-responses. Mutat Res 464(1): 35-40.

Nagashima M., Sasaki A., Morishita K., Takenoshita S., Nagamachi Y., Kasai H., and Yokota J., (1997) Presence of human cellular protein(s) that specifically binds and cleaves 8-hydroxyguanine containing DNA. Mutat Res 383: 49-59.

Nakabeppu Y., Sakumi K., Sakamoto K., Tsuchimoto D., Tsuzuki T., and Nakatsu Y., (2006) Mutagenesis and carcinogenesis caused by the oxidation of nucleic acids. Biol Chem 387(4): 373-379.

Nash H.M., Bruner S.D., Scharer O.D., Kawate T., Addona T.A., Spooner E., Lane W.S., and Verdine G.L., (1996) Cloning of a yeast 8-oxoguanine DNA glycosylase reveals the existence of a base-excision DNA-repair protein superfamily. Curr Biol 6(8): 968-980.

Nilsen H., and Krokan H.E., (2001) Base excision repair in a network of defence and tolerance. Carcinogenesis 22(7): 987-998

Nohmi T., Kim S.R., and Yamada M., (2005) Modulation of oxidative mutagenesis and carcinogenesis by polymorphic forms of human DNA repair enzymes. Mutat Res 591(1-2): 60-73.

Paunesku T, Mittal S, Protić M, Oryhon J, Korolev SV, Joachimiak A and Woloschak GE. (2001). Proliferating cell nuclear antigen (PCNA): ringmaster of the genome. Int J Radiat Biol 77(10):1007-21.

Rosenquist T.A., Zaika E., Fernandes A.S., Zharkov D.O., Miller H., and Grollman A.P., (2003) The novel DNA glycosylase, NEIL1, protects mammalian cells from radiation-mediated cell death. DNA Repair (Amst) 2(5): 581-591.

Russo M.T., De Luca G., Degan P., and Bignami M., (2006) Different DNA repair strategies to combat the threat from 8-oxoguanine. Mutat Res 614(1-2): 69-76.

Sakumi K., Tominaga Y., Furuichi M., Xu P., Tsuzuki T., Sekiguchi M., and Nakabeppu Y., (2003) Ogg1 knockout-associated lung tumorigenesis and its suppression by mth1 gene disruption. Cancer Res 63: 902-905.

Sampson JR, Jones S, Dolwani S, Cheadle JP. (2005)MutYH (MYH) and colorectal cancer. Biochem Soc Trans. 33:679-83.

Sattler U., Frit P., Salles B., and Calsou P., (2003) Long-patch DNA repair synthesis during base excision repair in mammalian cells. EMBO Rep 4(4): 363-367

Scharer O.D., and Jiricny J., (2001) Recent progress in the biology, chemistry and structural biology of DNA glycosylases. Bioessays 23(3): 270-81, 2001.

Seeberg E., Eide L., and Bjoras M., The base excision repair pathway. TIBS 20:391-398, 1995.

Slupphaug G., Kavli B., and Krokan H.E., (2003) The interacting pathways for prevention and repair of oxidative DNA damage. Mutat Res 531(1-2): 231-251.

Sung J.S., and Demple B., (2006) Roles of base excision repair subpathways in correcting oxidized abasic sites in DNA. Febs J 273(8): 1620-1629.

Taverna P., and Sedgwick B., (1996) Generation of an endogenous DNA-methylating agent by nitrosation in Escherichia coli. J Bacteriol 178(17): 5105-5111

Yamane A, Kohno T, Ito K., Sunaga N., Aoki K., Yoshimura K., Murakami H., Nojima Y. and Yokota J. (2004) Differential ability of polymorphic OGG1 proteins to suppress mutagenesis induced by 8-hydroxyguanine in human cell in vivo Carcinogenesis 25:1689-1694

Vidal A.E., Hickson I.D., Boiteux S., and Radicella J.P., (2001) Mechanism of stimulation of the DNA glycosylase activity of hOGG1 by the major human AP endonuclease: bypass of the AP lyase activity step. Nucleic Acids Res 29(6): 1285-192

Wallace S.S., (2002) Biological consequences of free radical-damaged DNA bases. Free Radic Biol Med 33(1): 1-14

Wang D., Kreutzer D.A., and Essigmann J.M., (1998) Mutagenicity and repair of oxidative DNA damage: insights from studies using defined lesions. Mutat Res 400(1-2): 99-115.

Wiederhold L., Leppard J.B., Kedar P., Karimi-Busheri F., Rasouli-Nia A., Weinfeld M., Tomkinson A.E., Izumi T., Prasad R., Wilson S.H., Mitra S., and Hazra T.K., (2004) AP endonuclease-independent DNA base excision repair in human cells. Mol Cell 15(2): 209-220.

Wilson III D.M., and Thompson L.H., (1997) Life without DNA repair. Proc Natl Acad Sci USA 94:12754-12757.

Wilson S.H., and Kunkel T.A., (2000) Passing the baton in base excision repair. Nat Struct Biol 7(3): 176-178.

Wood R.D., Mitchell M., Sgouros J., and Lindahl T., (2001) Human DNA repair genes. Science 291(5507): 1284-1289.

Xiao W., and Samson L., (1993) In vivo evidence for endogenous DNA alkylation damage as a source of spontaneous mutation in eukaryotic cells. Proc Natl Acad Sci USA 90:2117-2121.

Xue H, Ni P, Lin B, Xu H and Huang G. (2011) X-ray repair cross-complementing group 1 (XRCC1) genetic polymorphisms and gastric cancer risk: A HuGE review and meta-analysis. Am J Epidemiol 173(4):363-375.

5

Repair of Viral Genomes by Base Excision Pathways: African Swine Fever Virus as a Paradigm

Modesto Redrejo-Rodríguez[1], Javier M. Rodríguez[2], José Salas[1] and María L. Salas[1]

[1]Centro de Biología Molecular "Severo Ochoa", Consejo Superior de Investigaciones Científicas-Universidad Autónoma de Madrid, Universidad Autónoma de Madrid, Cantoblanco, Madrid (Spain) and [2]Centro Nacional de Microbiología, Instituto Nacional de Salud Carlos III, Majadahonda, Madrid Spain

1. Introduction

African swine fever virus (ASFV) is an enveloped deoxyvirus that infects suids and causes a fatal disease in domestic pigs. ASFV also propagates in ticks of the genus *Ornithodoros*, being the only known DNA arbovirus. Because of its unique features, ASFV is the sole member of the *Asfaviridae* family (Salas 1999; Dixon and Chapman 2008), although comparative genome analyses suggest that ASFV shares a common origin with the members of the proposed nucleocytoplasmic large DNA viruses (NCLDVs), along with poxviruses, iridoviruses and mimivirus, among others (Iyer et al. 2001; Iyer et al. 2006).

The disease, African swine fever (ASF), was reported for the first time in Kenya in the 1920s, as an acute hemorrhagic syndrome of domestic pigs (Montgomery 1921). The infection spread outside Africa to the Iberian Peninsula, initially to Portugal in 1957 and 1960, and subsequently to Spain and several other countries in Europe and Latin America. The virus has been eradicated from all of these regions, apart from sub-Saharan Africa countries and the Mediterranean island Sardinia, where the disease remains enzootic (Gómez-Tejedor Ortíz 1993). In 2007, a new transcontinental spread of ASF occurred with the introduction of ASF to Georgia in the Caucasus region (Beltrán-Alcrudo et al. 2008; Chapman et al. 2008; Rowlands et al. 2008), followed by widespread distribution to neighboring countries, including Armenia, Azerbaijan and several territories in Russia. Currently there is no vaccine available for ASF and the disease is controlled only by animal quarantine and slaughter. Therefore, ASF has potentially devastating effects on the commercial and subsistence pig production sectors, particularly in developing countries (Costard et al. 2009).

The virus particle has an overall icosahedral shape and an average diameter of 200 nm. The ASFV genome is a double-stranded DNA molecule of 170 to 190 kbp, structured in a central constant region of about 125 kbp and two variable regions at the ends (Blasco et al. 1989). The two strands are covalently closed, at both ends, by a 37 nucleotide-long hairpin loops, followed by a perfect terminal inverted repeat (TIR). A comparison of restriction maps of different ASFV isolates has shown that the two variable regions show deletion or additions

up to 8.6 kbp and contain five multigene families that comprise different number of members in different isolates (Blasco et al. 1989). Several complete sequences of the ASFV genome have been published, showing that it encodes more than 150 polypeptides, including a variety of enzymes involved in gene transcription and DNA replication and also in DNA repair (Yáñez et al. 1995; Chapman et al. 2008; de Villiers et al. 2010) (Table 1). The replication cycle occurs mainly in the cytoplasm of the infected cell, but an initial phase of viral DNA replication in the nucleus has been described (García-Beato et al. 1992). Analysis of ASFV replicating DNA molecules has shown the synthesis of DNA fragments of small size in the nucleus and the existence of head-to-head linked molecules that may be replicative intermediates and full length genome molecules in the cytoplasm (Rojo et al. 1999), confined to a specific area termed as viral factory. These factories contain also high amounts of viral structural proteins and ER-derived membranous material needed for particle assembly (Rouiller et al. 1998). A reducing environment in the virus factory is critical for the particle assembly (Cobbold et al. 2007). However, the virus codes for a sulfhydryl oxidase, which might be involved in the formation of the disulfide bonds found in viral proteins (Rodríguez et al. 2006).

The maintenance of genomic integrity is essential not only for the survival of cellular organisms but also viruses. Endogenous aerobic metabolism and a variety of exogenous factors generate reactive oxygen species (ROS), which can damage macromolecules including lipids, proteins and nucleic acids. Macrophages and other immune cells, including monocytes and neutrophils, where ASFV replication mainly occurs (Fernández et al. 1992), have been reported to produce ROS in response to viral infection (Klebanoff and Coombs 1992; Suzuki et al. 1997). Therefore, viral genomes may undergo a highly oxidative stress during its replication, which could generate lesions such as oxidized bases and single-strand breaks bearing 3′-blocking termini in the viral DNA.

Base Excision Repair systems (BER, see Figure 2) are the main pathways that surgically locate and remove damaged bases from DNA and are ubiquitous in Archaea, Bacteria and Eukarya. In the classical BER pathway, a DNA glycosylase recognizes and excises the damaged base. A uracil DNA glycosylase (UNG) or other monofunctional DNA glycosylase liberate the damaged base (typically uracil) and leaves an apurinic/apyrimidinic (AP) site in the DNA. Subsequently, AP endonuclease cleaves the sugar-phosphate backbone at the 5′-side of the AP site resulting in 3′-OH and 5′- deoxyribose phosphate (dRP) groups at the margins of a single nucleotide gap in DNA (Hegde et al. 2008). DNA polymerase β (pol β) inserts a nucleotide into the gap and removes the 5′-dRP group through its associated lyase activity, resulting in nicked DNA that will be sealed by a DNA ligase (Robertson et al. 2009). This sub-pathway is designated as short-patch or single-nucleotide base excision repair (SN-BER). However, if the 5′-sugar group is oxidized or reduced it is not recognized by the pol β dRP lyase and the DNA ligase cannot seal the nick. In this case, the repair occurs through an alternate long patch base excision repair (LP-BER) sub-pathway, involving removal of several nucleotides by a 5′→3′ exonuclease or a flap endonuclease activity prior to their replacement by a DNA polymerase (Sung and Demple 2006; Robertson et al. 2009).

A second group of DNA glycosylases, the bifunctional glycosylases also incise the AP site after the base removal, generating a single-stranded DNA break with 3′-sugar phosphate groups that must be removed prior to the gap-filling synthesis step. This cleansing step can be performed either by an AP endonuclease (for 3′-phosphate or 3′-phosphoaldehyde moieties) or by a polynucleotide kinase (only for 3′-phosphate group) (Hegde et al. 2008). The majority of oxidized DNA bases are removed in the BER pathway initiated by

redundant bifunctional DNA glycosylases (Fromme et al. 2004; Zharkov 2008). However, certain types of oxidative DNA damage such as the alpha-anomeric 2′-deoxynucleosides (αdA, αdT and αdC) cannot be repaired by DNA glycosylases but rather by the AP endonucleases in the alternative nucleotide incision repair (NIR) pathway (Ischenko and Saparbaev 2002; Ishchenko et al. 2006). NIR is a DNA glycosylase-independent conserved BER mechanism that is initiated by an AP endonuclease that makes an incision 5′ next to a damaged base, providing a proper 3′-OH group for DNA polymerization and a 5′-dangling damaged nucleotide. Oxidatively damaged pyrimidines including 5,6-dihydrothymine (DHT), 5,6-dihydrouracil (DHU), 5-hydroxyuracil (5OHU) and 5-hydroxycytosine (5OHC) are substrates for both BER and NIR pathways suggesting that the latter pathway can serve as a back-up system to counteract oxidative stress (Couvé-Privat et al. 2007).

Protein function	ORF(s) name(s)	Ref.
DNA polymerase α	G1207R	(Rodríguez et al. 1993)
Thymidine kinase	K196R	(Blasco et al. 1990)
Thymidylate kinase	A240L	(Yáñez et al. 1993)
Ribonucleotide reductase	F778R and F334L	(Boursnell et al. 1991)
DNA primase	C962R	(Yáñez et al. 1995)
DNA helicase	D1133L, Q706L, A859L, B962L, F1055L, QP509L	(Yáñez et al. 1993; Yáñez et al. 1995)
DNA ligase	NP419L	(Yáñez and Viñuela 1993; Lamarche et al. 2005)
dUTPase	E165L	(Oliveros et al. 1999)
DNA polymerase X	O174L	(Yáñez et al. 1995; Oliveros et al. 1997)
AP endonuclease	E296R	(Lamarche and Tsai 2006; Redrejo-Rodríguez et al. 2006)
5′→3′ exonuclease	D345L	(Iyer et al. 2001; de Villiers et al. 2010)
ERCC4-type endonuclease	EP364R	(Yáñez et al. 1995)
PCNA-like	E301R	(Yáñez et al. 1995)

Table 1. ASFV genes involved in DNA replication and repair.

Many DNA viruses, like herpesvirus, poxvirus or mimivirus encode one or more DNA glycosylases that may initiate a putative viral BER pathway (Caradonna et al. 1987; Upton et al. 1993; Raoult et al. 2004). However, only mimivirus, entomopoxvirus (the poxvirus subgroup that infects insects) and the recently described *Cafeteria roenbergensis* virus (CroV), contain ORFs that may code for reparative pol β-like DNA polymerase or AP endonuclease proteins (Afonso et al. 1999; Raoult et al. 2004; Fischer et al. 2010). On the contrary, the ASFV BER system includes a pol X family DNA polymerase, a class II AP endonuclease, a DNA ligase and other factors (Table 1), but lacks a DNA glycosylase homolog. This chapter aims to review the properties of ASFV BER pathway elements, and to provide new data to further characterize the viral BER mechanism(s). The major host cell preference of ASFV for macrophages and other immune cells constitute an important hallmark in ASFV replication cycle environment and might result in a specific variety of oxidative DNA damage, which

may explain the differences with other viruses. The role of DNA repair mechanisms in the viral replication, pathogenesis and evolution of ASFV and other viruses is also discussed.

2. African swine fever virus DNA repair

2.1 DNA damage prevention: dUTPase avoids uridine misincorporation into the viral DNA

dUTPases are enzymes that catalyze the conversion of dUTP to dUMP and PPi. This activity is critical to cell survival because excess dUTP is incorporated into DNA, leading to futile excision repair cycles, DNA breakage, and death. Therefore, dUTPases function is not a DNA repair mechanism itself, but a prophylactic strategy. It is highly conserved in biological kingdoms and has been shown to be essential for DNA replication and consequently for survival (revised in McClure 2001).

Recombinant purified ASFV dUTPase (pE165L, Table 1) is a trimeric enzyme, highly specific for dUTP and with an elevated affinity for its substrate (K_m= 1 μM). The protein is expressed at early and late times of infection and is localized in the cytoplasm of the infected cells, which is consistent with a role in maintaining a high dTTP/dUTP ratio to minimize the introduction of uracil into the viral DNA during the whole replication process (Oliveros et al. 1999). A recombinant virus with a deletion of the dUTPase gene was generated in the Vero cell adapted BA71V ASFV strain (vΔE165R). This mutant virus was successfully purified from cultured Vero cells and further analysis demonstrated that it replicates with the same kinetics and to the same extent than the parental virus. However, the growth of vΔE165R virus was strongly impaired in cultured porcine macrophages, the main target in natural ASFV infections (Oliveros et al. 1999). The differences in virus replication observed between these two cell types could be due to the levels of cellular dUTPase. The differentiated macrophages are quiescent cells, thus they may have very low levels of host cell dUTPase activity, revealing the required biological role of the viral protein, as found for other viruses (Baldo and McClure 1999). Moreover, as already mentioned, sequence analyses have not identified any protein with clear similarity to UNG that might repair incorporated or cytosine deamination-generated uracil bases. Therefore, a proficient dUTPase activity might be especially important to prevent the introduction of deoxyuridine during the replication of the large ASFV genome.

2.2 An early step in base excision repair catalyzed by AP Endonuclease

As previously indicated, the enzymatic activity that cleaves the sugar-phosphate bond in the BER pathways is named AP endonuclease and it generates 3′-OH and 5′-dRP ends. In human cells, AP sites are processed by APE1, whereas in yeast the primary AP endonuclease is termed APN1. These enzymes are the major constitutively expressed AP endonucleases in these organisms and are homologous to the *Escherichia coli* enzymes Exonuclease III (Xth) and Endonuclease IV (Nfo) respectively, which represent the two conserved archetypes of AP endonuclease enzymes.

ASFV protein pE296R is an Nfo-like AP endonuclease, named after the viral gene E296R. It is expressed since early times during the infection and progressively accumulates at later times. The early enzyme is localized in the nucleus and the cytoplasm, while the late protein is detected only in the cytoplasm, supporting a role in BER of viral genomes. The blockage of viral DNA replication results in the accumulation of pE296R in the cell nucleus,

suggesting a function during the nuclear stage of DNA replication, more likely in DNA repair (Redrejo-Rodríguez et al. 2006).

Purified recombinant pE296R protein contains AP endonuclease and 3′→5′ exonuclease activities (Lamarche and Tsai 2006; Redrejo-Rodríguez et al. 2006), as well as 3′-phosphodiesterase, 3′-phosphatase and weak NIR activities against 5ohC and dihydropyrimidines (Lamarche and Tsai 2006; Redrejo-Rodríguez et al. 2009). The 3′→5′ exonuclease activity of pE296R is more efficient against 3′-mismatched substrates (Redrejo-Rodríguez et al. 2006), 3′-damaged nucleotides and pyrimidines over purines (Redrejo-Rodríguez et al. 2009). Strikingly, all DNA repair functions of pE296R protein (AP endonucleolytic, 3′→5′ exonuclease, 3′-diesterase and nucleotide incision repair (NIR) activities) as well as its DNA binding capacity are reversibly inhibited by reducing agents. Furthermore, cysteine residues alkylation experiments showed the presence of bound cysteines in the recombinant protein (Redrejo-Rodríguez et al. 2009). The results suggest that the native protein has one disulfide bond and that the break-up of this cysteine-cysteine bridge by reducing agents may lead to the loss of DNA binding and enzymatic activities of pE296R. Although the in vivo significance of these observations is not well known at present, we propose that the presence of a disulfide bond in the viral AP endonuclease may provide a mechanism for regulation of the enzyme activity in the infected cells by inducing or breaking this bond. In relation to this possibility, it is interesting to mention again that ASFV codes for a sulfhydryl oxidase (Rodríguez et al. 2006), which may be involved in the formation of the disulfide bond.

The biological role of protein pE296R has been studied using two different strategies. A first approach was based in complementation assays of an AP endonuclease deficient *E. coli* xth nfo strain exposed to various genotoxic agents by the expression of the pE296R protein. This is a well-characterized model for the study of the genetic requirements to counteract specific DNA damages that can be repaired by BER mechanisms (Cunningham et al. 1986; Ishchenko et al. 2006). Methylmethanesulfonate (MMS) is an agent that induces alkylation of DNA bases, which can be removed by means of BER, requiring an AP endonuclease activity (Weinberger and Sperling 1986). Among the oxidizing agents, H_2O_2 is produced by the host immune cells infected with certain viruses and therefore might generate oxidative lesions in the ASFV DNA (Israel and Gougerot-Pocidalo 1997; Suzuki et al. 1997). Expression of pE296R protein in the mutant bacteria strain conferred resistance against MMS and H_2O_2 (Lamarche and Tsai 2006; Redrejo-Rodríguez et al. 2009), which strongly suggests that the viral AP endonuclease can repair 3′-blocking groups, 3′-oxidized bases and AP sites *in vivo*. Importantly, the protection against H_2O_2 and MMS provided by both pE296R and Nfo endonucleases is very similar (Redrejo-Rodríguez et al. 2009), suggesting highly efficient properties of the viral AP endonuclease to neutralize DNA damage. Nfo protein NIR activity has been shown to be involved in the repair of oxidizing damage that is produced in the presence of *tert*-butylhydroperoxyde (*t*-BuO_2H) (Ishchenko et al. 2006). Expression of pE296R is also able to complement AP endonucleases in *E. coli* against *t*-BuO_2H-induced DNA damage (Redrejo-Rodríguez et al. 2009). Thereby, although *in vitro* the ASFV AP endonuclease-catalyzed NIR activity is much weaker compared to other DNA repair functions, a role of pE296R in the repair of oxidative DNA base lesions via the DNA glycosylase-independent NIR pathway *in vivo* can be suggested.

A virus mutant lacking the E296R gene allowed additional characterization of the biological role of protein pE296R in the context of the infected macrophage. The viral endonuclease is required for virus growth in swine cultured macrophages but not in Vero cells, supporting

the existence of a viral reparative system to maintain virus viability in macrophages, the ASFV major host cell. Furthermore, the presence of H_2O_2, *t*-BuO_2H and MMS during the infection in Vero cells decreased viral production in a dose-dependent manner (Redrejo-Rodríguez et al. 2009). This corroborates the role of ASFV AP endonuclease in the repair of AP sites and DNA strand breaks in the viral genome, and suggests the involvement of a viral NIR pathway in the maintenance of genome integrity *in vivo*.

2.3 A minimalist but proficient DNA polymerase X

ASFV gene O174L codes for a highly distributive X-family DNA polymerase, named pol X, that is the smallest naturally occurring DNA-dependent DNA polymerase. Sequence alignment shows that this small protein (20 kDa) contains most of the conserved critical residues involved in DNA binding, nucleotide binding, and catalysis of the polymerization reaction, but lacks the N-terminal 8-kDa domain of pol β that contains the dRP lyase active site. Therefore, ASFV pol X most likely represents the minimal functional version of an evolutionarily conserved pol β-type DNA polymerase core, constituted by only the "palm" and "thumb" subdomains (Oliveros et al. 1997; Showalter et al. 2001).

Pol X is able to efficiently repair single-nucleotide gapped DNA substrates, which is consistent with its participation in a BER process during ASFV infection (Oliveros et al. 1997; Showalter and Tsai 2001; García-Escudero et al. 2003). In agreement with sequence analysis predictions, the recombinant purified enzyme lacks the 5′-deoxyribose phosphate (dRP) lyase activity characteristic of cellular pol β that eliminates the 5′-dRP blocking group generated during the BER process by the action of the AP endonuclease on the abasic site in the DNA. However, pol X, as well as pol β, exhibits lyase activity on unincised AP sites. Taking this into account, the existence of an alternative viral short patch BER pathway has been proposed (García-Escudero et al. 2003) in which the AP lyase activity of pol X would act on AP sites in the viral DNA. Following this, the 3′-phosphodiesterase and 3′-phosphatase activities of the pE296R (Lamarche and Tsai 2006; Redrejo-Rodríguez et al. 2009) protein would excise the 3′-terminal unsaturated aldehyde, allowing the pol X to fill the gap. Nevertheless, a long patch pathway or the existence of a dRP lyase activity in another viral protein that would excise the dRP moiety should be considered.

Pol X binds to single and double stranded DNA (ssDNA and dsDNA). The total site-size of the pol X-ssDNA complex is 16 ± 2 nucleotides, surprisingly large for such a small protein (Jezewska et al. 2007). Regarding BER intermediates, the enzyme forms two different complexes with gapped DNAs, with dramatically different affinities. The high affinity complex is formed preferably with 1-2 nucleotide gaps and engages the total DNA binding site, while in the low affinity complex the enzyme binds to the dsDNA parts of the gapped DNA, using only one of the DNA-binding subdomains (Jezewska et al. 2007). Pol X binds gapped DNAs with cooperative interactions, which increase with the decreasing gap size. Surprisingly, the specific structure necessary to recognize the short gaps is induced by the binding of magnesium to the protein.

The three-dimensional structure of ASFV pol X determined by multidimensional NMR spectroscopy (Maciejewski et al. 2001; Showalter et al. 2001) has confirmed that pol X is formed by only a palm domain (105 amino acids) with the catalytic site and a C-terminal subdomain (69 residues) involved in dNTP selection. The two independently determined pol X structures differ in the presence of a disulfide bond between Cys-81 and Cys-86, the only cysteines present in the protein, located in the catalytic subdomain of the structure

obtained by Showalter et al. (Showalter et al. 2001) and its absence in that described by Maciejewski et al. (Maciejewski et al. 2001).

Controversial results have been obtained regarding the fidelity of ASFV pol X. Table 2 summarizes the fidelity parameters of pol X in a single nucleotide gap BER intermediate with the 5′ end phosphorylated, found by different authors in different experimental conditions. The misinsertion frequency found by Tsai and coworkers (Showalter and Tsai 2001; Lamarche et al. 2006) was much higher than the values reported in our laboratory (García-Escudero et al. 2003). We found values that resemble those described for pol β (Chagovetz et al. 1997; García-Escudero et al. 2003), except for the G:G misinsertion that was higher (7.1×10^{-4} in pol X vs. 2×10^{-6} for pol β), but much less frequent than the reported by Tsai's laboratory (Table 2). Differences in experimental conditions between those reports might somewhat account for the contradictory results. The variances include different salt and pH conditions, and, more importantly, different kinetic analysis and a great difference in enzyme concentration for incorrect nucleotide insertion assays (steady-state and 2 μM pol X in García-Escudero et al. (2003) vs. presteady-state and 50 or 450 nM pol X in Showalter et al. (2001) and Lamarche et al. (2006)). This latter difference may also modify the dNTP insertion fidelity, since, as mentioned above, protein concentration is critical for pol X binding to the gapped DNA substrate (Jezewska et al. 2007). The extremely low fidelity rates reported by Tsai and coworkers prompted these authors to propose a mutagenic role of the pol X, suggesting that an error prone BER system may increase the variability in viral genomes. Furthermore, they speculate that a higher genomic divergence would raise the adaptability of the viral populations. However, genomic variability in ASFV isolates is concentrated in the terminal variable end regions, whereas most of the ORFs in the central segment of the genome are highly conserved (Yáñez et al. 1995; Chapman et al. 2008). Besides, the alterations found in the multigenic families of the variable regions are deletions, duplications and translocations of large fragments of DNA that can be more likely explained by recombination mechanisms rather than due to a mutagenic BER pathway (Blasco et al. 1989; Agüero et al. 1990).

A recent study on the relevance of the disulfide bridge for the modulation of the catalytic activity and fidelity of ASFV pol X provides further explanations to the discrepancies reported (Voehler et al. 2009). These authors showed that the oxidized form of pol X containing a disulfide bond between Cys-81 and Cys-86 has about 10-fold lower fidelity than the reduced pol X, when assayed with gapped DNA substrates during dNTP insertion opposite a template G (Table 2). The disulfide linkage is located between two β-strands in the palm domain, nearby the dNTP binding site. Furthermore, even the presence of a reducing agent will not prevent oxidation over time, which may be the reason why Showalter et al. (Showalter et al. 2001) found the disulfide bond in the presence of 1 mM DTT and Maciejewski et al. (Maciejewski et al. 2001) found the reducing form in the presence of 10 mM DTT.

Structural alignment of pol X with a pol β ternary structure suggests that the disulfide bond formation and breakage might modulate fidelity by altering the ability of the palm domain to properly place and stabilize the primer terminus and catalytic metal ion for phosphoryl transfer. Therefore, the structural changes that occur in the palm domain provide molecular basis for the distinct fidelities observed with the oxidized and reduced forms of pol X. Hence, the DNA polymerase fidelity can be modulated by the redox state of the enzyme and its associated conformational changes (Voehler et al. 2009).

Base Pair	Redox conditions (stored protein)	Redox conditions (reaction)	Misinsertion frequency	Reference
G:G	7 mM β-ME	1 mM DTT	7.1×10^{-4}	(García-Escudero et al. 2003)
G:A	7 mM β-ME	1 mM DTT	6.1×10^{-5}	(García-Escudero et al. 2003)
G:T	7 mM β-ME	1 mM DTT	2.4×10^{-4}	(García-Escudero et al. 2003)
G:A	0.5 mM DTT	1 mM DTT	7.1×10^{-3}	(Showalter and Tsai 2001)
G:T	0.5 mM DTT	1 mM DTT	6.2×10^{-2}	(Showalter and Tsai 2001)
G:G	0.5 mM DTT	1 mM DTT	0.52	(Showalter and Tsai 2001)
G:G	0.5 mM DTT	1 mM DTT	5.3×10^{-2}	(Lamarche et al. 2006)
G:G	100 mM DTT	10 mM DTT	9.3×10^{-3}	(Voehler et al. 2009)
G:A	100 mM DTT	10 mM DTT	4.5×10^{-4}	(Voehler et al. 2009)
G:T	100 mM DTT	10 mM DTT	1×10^{-4}	(Voehler et al. 2009)
G:G			7.6×10^{-2}	(Voehler et al. 2009)
G:A			7.6×10^{-2}	(Voehler et al. 2009)
G:T			6.8×10^{-4}	(Voehler et al. 2009)

Table 2. Comparison of pol X fidelity rates reported in a BER intermediate single nucleotide gap. Data from references (García-Escudero et al. 2003; Lamarche et al. 2006; Voehler et al. 2009) were determined by steady state experiments and in ref. (Showalter and Tsai 2001) kinetic parameters were analyzed under pre-steady state conditions. Frequency of misincorporation is calculated as $[k_{cat}(\text{incorrect})/K_m(\text{incorrect})]/[k_{cat}(\text{correct})/K_m(\text{correct})]$.

The redox states of the ASFV AP endonuclease and pol X proteins during viral infection are not known and the biological significance of these findings are currently under investigation in our laboratory. The fact that AP endonuclease is inhibited under reducing agents may provide a fine-tune regulation system in which variations of reducing conditions may increase or reduce nuclease activities and modulate pol X fidelity. It is also tempting to consider a scenario in which low rate but accurate DNA repair activity works in a reducing environment but removal of damaged nucleotide and DNA integrity restoration prevail under oxidized conditions, with lower DNA sequence fidelity.

2.4 DNA ligase and other factors that may play multiple functions in DNA replication, recombination and repair

DNA ligases are found in all free-living organisms and are essential for the maintenance of cellular genome integrity. They are responsible for joining Okazaki fragments on the DNA replication fork and restoring the continuity of the DNA backbone subsequent to nucleotide excision and base excision repair (Timson et al. 2000). DNA viruses, as extrachromosomal replicons, also rely on ligases to accomplish DNA replication and to guard their genomes against breaks introduced during recombination or DNA damage.

ASFV protein pNP419L is an ATP-dependent DNA ligase (Hammond et al. 1992; Yáñez and Viñuela 1993). ATP-dependent ligases are common within NCLDV, they are present in the genomes of many chordopoxviruses and phycodnaviruses, whereas NAD-dependent ligases appear to be more scattered, and are found only in entomopoxvirus and mimivirus genomes. Interestingly, many viruses belonging to NCLDVs lack any ligase gene (Yutin and Koonin 2009). It is likely that ligase loss is counteracted by the host protein, as has been demonstrated for vaccinia virus and cellular DNA ligase I (Paran et al. 2009).

Recombinant purified protein pNP419L has been shown to be a proficient ligase for DNA nicks (Lamarche et al. 2005). It can be detected at early and late times post infection (Yáñez and Viñuela 1993) and seems to be essential for viral replication, since no deletion mutant can be constructed (García-Escudero, R., Salas ML. and Salas J., unpublished results). Altogether, the available data strongly suggest that ASFV ligase function in Okazaki fragment sealing is essential for viral DNA replication process. Under the experimental conditions of Lamarche et al. (2005), the purified protein was found to ligate efficiently 3' mismatched substrates, which was proposed to be a viral strategy where genome integrity prevails over the sequence fidelity in DNA repair mechanisms (Showalter and Tsai 2001; Lamarche et al. 2006).

Consistent with their large genome size and relative replication autonomy, most NCLDVs possess also multiple recombination enzymes. RuvC-like Holliday junction resolvases (HJR) encoded by poxviruses, iridoviruses, phycodnaviruses and the mimivirus could participate in resolution of concatemer replication intermediates and recombination. However, in ASFV, this resolvase is replaced by a ERCC4/Mus81-like nuclease (EP364R, Table 1), which is related to the principal Holliday junction resolvase of the eukaryotes, Mus81 (Iyer et al. 2006). A predicted Fen-1/FLAP-like endonuclease has been reported in poxviruses (G5R), iridoviruses and the mimivirus, and might participate in DNA replication and repair (Da Silva et al. 2006; Iyer et al. 2006). A lambda-like exonuclease, which might be involved in processing DNA ends for strand exchange or single-strand annealing during recombination, has been predicted in ASFV (pD345L, Table 1), phycodnaviruses, CroV and other viruses (Iyer et al. 2006; Fischer et al. 2010; Moreau et al. 2010; Weynberg et al. 2011). Unfortunately, none of those annotated protein sequences have been characterized functionally or biochemically.

Lambda-like viral recombinase paradigm is SPP1 Chu exonuclease (Vellani and Myers 2003). Chu protein, as well as lambda exonuclease, forms an oligomer and functions as a highly processive alkaline exonuclease that digests linear double-stranded DNA in a Mg^{2+}-dependent reaction, showing a preference for 5'-phosphorylated DNA ends. In SPP1 and other phages it forms part of the synaptase/exonuclease two-component viral recombinase functional unit. The other component is a single strand binding protein, named synaptase, that protects the single stranded DNA and favors the strand invasion required for the recombination process. However, this element has not been identified in any ASFV genome.

A structural model of ASFV pD345L protein, based upon the lambda exonuclease protein (Kovall and Matthews 1997) is shown in Figure 1. The model corresponds to two thirds of the N-terminus of the pD345L protein sequence and displays a strong correlation in the position of the catalytic center residues Asp119, Lys131 and Glu129 (Kovall and Matthews 1997), supporting the existence of common features and roles in DNA recombination and repair processes.

Failed attempts to purify a virus deletion mutant by plaque isolation, led us to conclude that ASFV mutants lacking the D345L gene cannot be isolated, even though the protein should be present for the first recombination process. This indicates that the virus mutants are not

viable or enough competitive when compared with the parental virus, suggesting that the protein may be essential for successful viral genome replication.
A biochemical characterization of protein pD345L using a recombinant purified histidine-tagged protein shows that it has a 5′→3′ exonuclease activity on a single stranded substrate and a much weaker activity on double stranded or BER intermediate with a flap structure. The exonucleolytic activity is strongly stimulated by 5′-phosphate ends. Moreover, mismatched nick and gaps are also substrates for protein pD345L-catalyzed 5′→3′ exonuclease activity (Redrejo-Rodríguez, M., Rodríguez J.M., Salas, J. and Salas M.L., unpublished results). The preference for single stranded substrates suggests also a possible function in the degradation of the flap structure in LP-BER and NIR, that may be removed by a FEN specific endonucleolytic activity or by sequential 5′→3′ exonuclease steps.

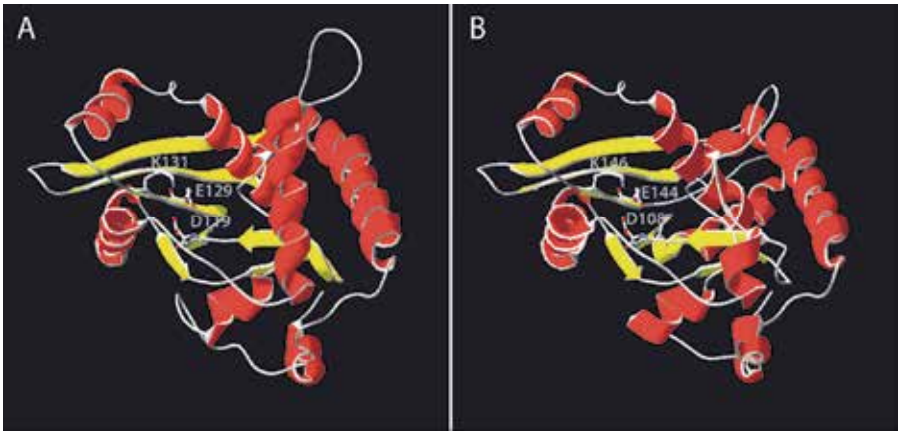

Fig. 1. Three-dimensional structure prediction for ASFV pD345L protein (Schwede et al. 2003). ASFV pD345L structure model (B) was inferred from the structure of lambda phage exonuclease (A, accession no. 1AVQ in the RCSB Protein Data Bank). The model was obtained with the SwissModel server (*Schwede et al. 2003*) and rendered with Swiss-PDB Viewer sotfware. Catalytic center residue disposition in the 1AVQ template (A) and the model obtained for the ASFV pD345L protein is also represented (B).

The ASFV genome also contains a gene, named E301R (Table 1), that has sequence similarity to the proliferating cell nuclear antigen (PCNA) and thus could be a processivity factor of the viral replicative DNA polymerase holoenzyme (Yáñez et al. 1995). Although most of DNA polymerases holoenzymes of NCLDVs contain a processivity factor, they are very divergent, which may be related with the additional functions performed by some of those proteins, like the role of poxvirus G8R in transcription (Iyer et al. 2001; Da Silva and Upton 2009).
Protein pE301R is a non-structural late protein (Redrejo-Rodríguez 2009); therefore it cannot be involved in the early nuclear DNA replication stage. It should be noted that at early times post infection only small DNA fragments are detected, whereas genome size DNA fragments are synthesized during the cytoplasmatic late DNA replication stage, thus DNA polymerase processivity may be more important in the late stage. E301R gene deletion-mutants are reluctant to purification (García-Escudero, R., Salas ML. and Salas J., unpublished results), suggesting an essential requirement for successful genome replication. At late times post-infection pE301R protein signal is detected in the cytoplasm and

accumulates in the viral factories, in agreement with a role in the stimulation of the replicative DNA polymerase processivity. Besides, it may also act as coordination factor for polymerase switching at repair processes.

3. Conclusion and perspectives

BER pathways in mammalian cells and the ASFV-encoded proteins that can be responsible of each step in a viral pathway(s) are summarized in Figure 2. Early steps of a viral BER mechanism might require the pE296R protein to remove AP sites and a number of oxidatively modified bases, through BER and NIR pathways. To date, the required 5′-dRP lyase or hydrolase activity necessary for SN-BER has not been identified in ASFV. Though the 5′- dRP group can be lost spontaneously via β-elimination, the half-life of this reaction under physiological conditions is rather long (on the order of 30 h), suggesting that LP-BER and NIR pathways are more likely to happen after an endonucleolytic cleavage on 5′-side of the lesion. ASFV Pol X AP lyase activity could initiate an alternative pathway for the reparation of abasic sites (García-Escudero et al. 2003) and besides, since there is no viral DNA glycosylase, the participation of host monofunctional or bifunctional glycosylase(s) cannot be ruled out. The 3′-phosphatase and 3′-phosphodiesterase activities of pE296R protein are able to cleanse 3′-moieties derived from pol X or bifunctional glycosylases AP-lyase activities, providing the proper 3′-OH ends that pol X needs to fill the gap. The viral 5′→3′ exonuclease pD345L and the putative processivity factor pE301R might participate in LP-BER or NIR pathways and the ATP-dependent ligase pNP419L would seal the nick. Moreover, the 3′→5′ exonuclease activity of the pE296R protein might act as editing activity that would increase the repair fidelity, as proposed for *E. coli* and human AP endonucleases (Chou and Cheng 2002; Kerins et al. 2003).

ROS can induce also single and double strand breaks. BER pathway is involved in single strand breaks repair but double strand breaks must be repaired by homologous recombination (HR) or non-homologous end joining (NHEJ) pathways. Oxidatively induced DNA breaks usually contain damaged bases and/or 3′-phosphate ends that can be removed by the 3′-activities of pE296R protein. The 5′→3′ exonuclease activity of pD345L could generate the single stranded homologous end for the strand invasion in HR. On the other hand, a viral NHEJ mechanism might require also the pNP419L ligase and pol X. An oxidative environment that might induce double strand breaks may also be compatible with the pol X Cys-81 - Cys-86 disulfide bond. Therefore, it is tempting to speculate that a putative viral NHEJ pathway may favor genome structural stability over fidelity.

The existence of a viral BER pathway involved in ASFV genome maintenance was proposed as a result of the analysis of the first complete genome sequence (Yáñez et al. 1995), based upon two main reasons. First, the presence of ORFs with homology to several DNA repair genes, particularly a class II AP endonuclease and a pol β-like DNA polymerase; second, the fact that ASFV mainly infects macrophages and other immune cells suggests that the viral enzymes may be required to cope with a potentially highly oxidative environment of the infected cells. Subsequent biochemical and genetic evidences further support that model. Still, the specific DNA repair mechanisms that constitute the viral BER pathway(s) must be confirmed. Current and future work on ASFV genome repair mechanisms should pursue a double objective. First, a deeper knowledge of the biochemical and genetic mechanisms of BER pathways, and second, a study on the fidelity and biological role of the pol X in the context of the potentially genotoxic environment of the infected macrophage.

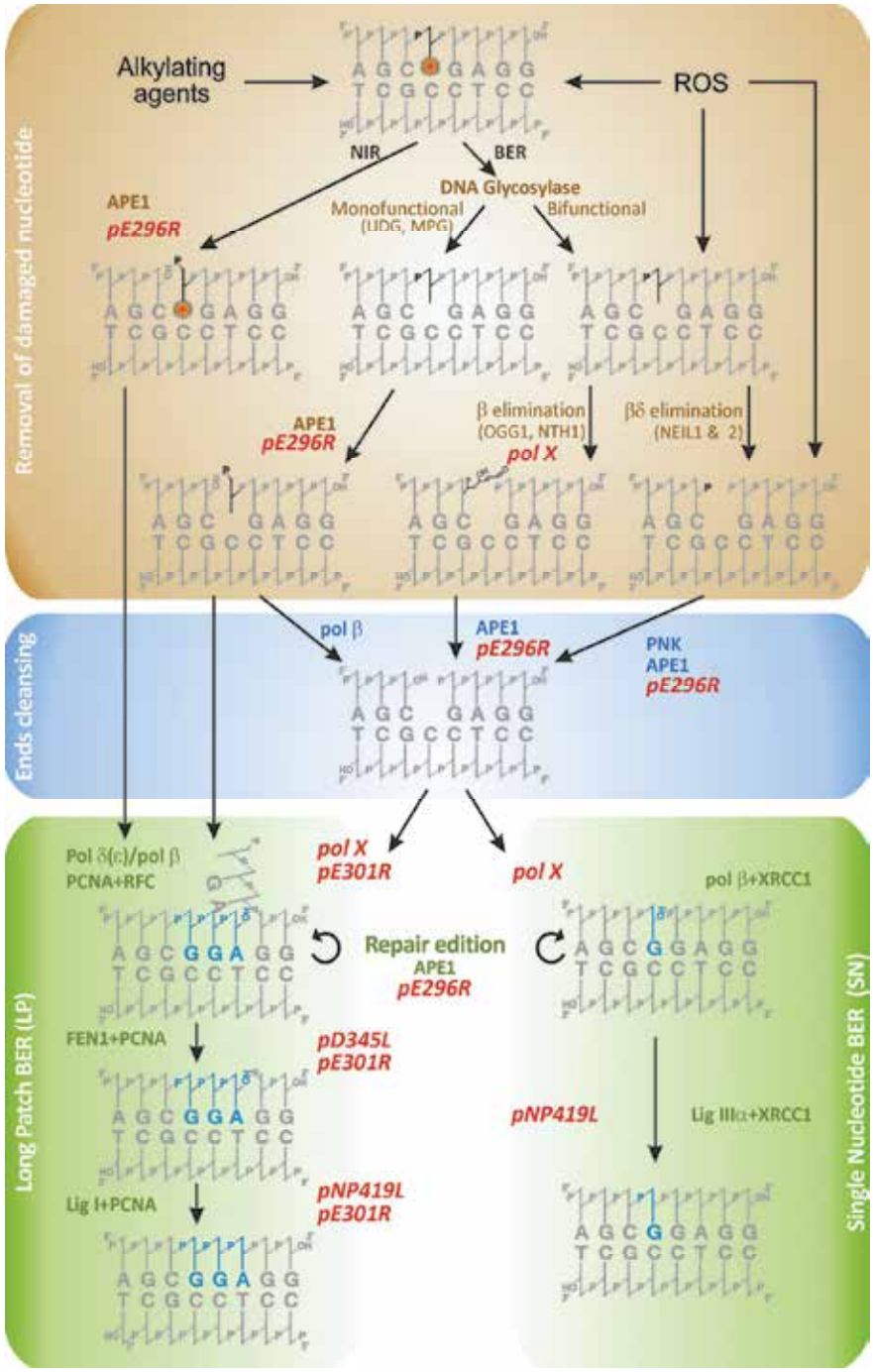

Fig. 2. African swine fever virus possible repair pathways in the framework of mammalian BER mechanism. Damaged nucleotide stands out in black. Different repair pathways or even some DNA damaging agents −like ROS− can induce breaks in the DNA backbone (brown upper zone). These processes converge to a key step in which a 3′-OH end should be

generated (blue zone) and subsequently extended by a reparative DNA polymerase that inserts the correct nucleotide (blue). The 3′→5′ exonucleolytic activity of the AP endonuclease might remove misincorporated nucleotides (circular arrow), thus increasing the repair fidelity. SN-BER consists in a surgical removal of a single damaged nucleotide (right green zone at the bottom), whereas NIR and LP-BER require additional factors that collaborate to replace a few nucleotides (left green zone). The viral proteins that might play a role in each step are indicated in red. As reference, some of the major human proteins involved in each stage are also indicated.

Different viruses present alternative strategies that might aim to control the stability of genetic information. Some retroviruses, including HIV, incorporate a host DNA glycosylase (Willetts et al. 1999) to avoid uridine misincorporation in the retrotranscribed DNA (Priet et al. 2005). On the other hand, flexivirus, a RNA virus, encodes a AlkB-like glycosylase that removes methylated bases from genomic RNA (van den Born et al. 2008).

Alternative or complementary hypothesis may be argued to justify the constant presence of DNA repair systems in different viruses. Several host nucleic acid modification proteins can destabilize viral genomes through deamination or direct degradation. This strategy has been described as a host "intrinsic immunity" to impair replication of some retroviruses (Bieniasz 2006; Lloyd et al. 2006). It would be extremely interesting to evaluate its role in a wider range of viral infections and whether it can be counteracted by viral DNA repair mechanisms. This strategy reminds the bacterial restriction enzymes and it may have played a striking role during evolution, as recently reported for SUKH protein superfamily that includes a number of nucleases and nucleic acid deaminases, that have evolved to different functions in various eukaryotic and DNA viral systems (Zhang et al. 2011).

Finally, we wish to share some hypothesis about the practical lessons that can be learned from a detailed understanding of mechanisms to maintain viral genome stability. First, an engineered virus with highly stable genome would increase the biosecurity of viruses for multiple applications (vaccines, gene therapy or other biotechnology purposes). On the other hand, a controlled or predictable deterioration of genetic information may be useful in vaccine development, since it would allow generating virus mutants able to accomplish only one or a few rounds of replication and therefore producing abortive infections that may be enough to immunize the organism but not enough to trigger the disease.

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5. References

Afonso, C. L., E. R. Tulman, et al. (1999). "The genome of Melanoplus sanguinipes entomopoxvirus." J Virol 73(1): 533-552.

Agüero, M., R. Blasco, et al. (1990). "Analysis of naturally occurring deletion variants of African swine fever virus: multigene family 110 is not essential for infectivity or virulence in pigs." Virology 176(1): 195-204.

Baldo, A. M. and M. A. McClure (1999). "Evolution and horizontal transfer of dUTPase-encoding genes in viruses and their hosts." Journal of Virology 73(9): 7710-7721.

Beltrán-Alcrudo, D., J. Lubroth, et al. (2008). African Swine Fever in the Caucasus. FAO Empres Watch. ftp://ftp.fao.org/docrep/fao/011/aj214e/aj214e00.pdf

Bieniasz, P. D. (2006). "Late budding domains and host proteins in enveloped virus release." Virology 344(1): 55-63.

Blasco, R., M. Agüero, et al. (1989). "Variable and constant regions in African swine fever virus DNA." Virology 168(2): 330-338.

Blasco, R., I. de la Vega, et al. (1989). "Genetic variation of African swine fever virus: variable regions near the ends of the viral DNA." Virology 173(1): 251-257.

Blasco, R., C. López-Otín, et al. (1990). "Sequence and evolutionary relationships of African swine fever virus thymidine kinase." Virology 178(1): 301-304.

Boursnell, M., K. Shaw, et al. (1991). "The sequences of the ribonucleotide reductase genes from African swine fever virus show considerable homology with those of the orthopoxvirus, vaccinia virus." Virology 184(1): 411-416.

Caradonna, S., D. Worrad, et al. (1987). "Isolation of a herpes simplex virus cDNA encoding the DNA repair enzyme uracil-DNA glycosylase." Journal of Virology 61(10): 3040-3047.

Chagovetz, A. M., J. B. Sweasy, et al. (1997). "Increased activity and fidelity of DNA polymerase beta on single-nucleotide gapped DNA." J. Biol. Chem. 272(44): 27501-27504.

Chapman, D. A., V. Tcherepanov, et al. (2008). "Comparison of the genome sequences of non-pathogenic and pathogenic African swine fever virus isolates." J Gen Virol 89(Pt 2): 397-408.

Chou, K. M. and Y. C. Cheng (2002). "An exonucleolytic activity of human apurinic/apyrimidinic endonuclease on 3' mispaired DNA." Nature 415(6872): 655-659.

Cobbold, C., M. Windsor, et al. (2007). "Reduced redox potential of the cytosol is important for African swine fever virus capsid assembly and maturation." Journal of General Virology 88(Pt 1): 77-85.

Costard, S., B. Wieland, et al. (2009). "African swine fever: how can global spread be prevented?" Philosophical Transactions of the Royal Society of London B Biological Sciences 364(1530): 2683-2696.

Couvé-Privat, S., A. A. Ischenko, et al. (2007). Nucleotide Incision Repair: An Alternative and Ubiquitous Pathway to Handle Oxidative DNA Damage. Oxidative Damage to Nucleic Acids. M. D. Evans and M. S. Cooke, Landes Bioscience.

Cunningham, R. P., S. M. Saporito, et al. (1986). "Endonuclease IV (nfo) mutant of Escherichia coli." J Bacteriol 168(3): 1120-1127.

Da Silva, M., L. Shen, et al. (2006). "Predicted function of the vaccinia virus G5R protein." Bioinformatics 22(23): 2846-2850.

Da Silva, M. and C. Upton (2009). "Vaccinia virus G8R protein: a structural ortholog of proliferating cell nuclear antigen (PCNA)." PLoS ONE 4(5): e5479.

de Villiers, E. P., C. Gallardo, et al. (2010). "Phylogenomic analysis of 11 complete African swine fever virus genome sequences." Virology 400(1): 128-136.

Dixon, L. K. and D. Chapman (2008). African Swine Fever Virus. Encyclopedia of Virology. B. W. J. Mahy and M. H. V. v. Regenmortel. Oxford, Academic Press: 43-51.

Fernández, A., J. Pérez, et al. (1992). "Distribution of ASFV antigens in pig tissues experimentally infected with two different Spanish virus isolates." Zentralbl. Veterinarmed. B. 39(6): 393-402.

Fischer, M. G., M. J. Allen, et al. (2010). "Giant virus with a remarkable complement of genes infects marine zooplankton." Proceedings of the National Academy of Sciences of the United States of America 107(45): 19508-19513.

Fromme, J. C., A. Banerjee, et al. (2004). "DNA glycosylase recognition and catalysis." Current Opinion in Structural Biology 14(1): 43-49.

García-Beato, R., M. L. Salas, et al. (1992). "Role of the host cell nucleus in the replication of African swine fever virus DNA." Virology 188(2): 637-649.

García-Escudero, R., M. García-Díaz, et al. (2003). "DNA polymerase X of African swine fever virus: insertion fidelity on gapped DNA substrates and AP lyase activity support a role in base excision repair of viral DNA." J Mol Biol 326(5): 1403-1412.

Gómez-Tejedor Ortíz, C. (1993). "Peste Porcina Africana: Epizootiología. Patogenia." Porci Aula Veterinaria 17: 19-23.

Hammond, J. M., S. M. Kerr, et al. (1992). "An African swine fever virus gene with homology to DNA ligases." Nucleic Acids Research 20(11): 2667-2671.

Hegde, M. L., T. K. Hazra, et al. (2008). "Early steps in the DNA base excision/single-strand interruption repair pathway in mammalian cells." Cell Res 18(1): 27-47.

Ischenko, A. A. and M. K. Saparbaev (2002). "Alternative nucleotide incision repair pathway for oxidative DNA damage." Nature 415(6868): 183-187.

Ishchenko, A. A., E. Deprez, et al. (2006). "Uncoupling of the base excision and nucleotide incision repair pathways reveals their respective biological roles." Proc Natl Acad Sci U S A 103(8): 2564-2569.

Israel, N. and M. A. Gougerot-Pocidalo (1997). "Oxidative stress in human immunodeficiency virus infection." Cell Molecular Life Science 53(11-12): 864-870.

Iyer, L. M., L. Aravind, et al. (2001). "Common origin of four diverse families of large eukaryotic DNA viruses." Journal of Virology 75(23): 11720-11734.

Iyer, L. M., S. Balaji, et al. (2006). "Evolutionary genomics of nucleo-cytoplasmic large DNA viruses." Virus Research 117(1): 156-184.

Jezewska, M. J., P. J. Bujalowski, et al. (2007). "Interactions of the DNA polymerase X from African swine fever virus with gapped DNA substrates. Quantitative analysis of functional structures of the formed complexes." Biochemistry 46(45): 12909-12924.

Jezewska, M. J., P. J. Bujalowski, et al. (2007). "Interactions of the DNA polymerase X of African swine fever virus with double-stranded DNA. Functional structure of the complex." Journal of Molecular Biology 373(1): 75-95.

Kerins, S. M., R. Collins, et al. (2003). "Characterization of an endonuclease IV 3'-5' exonuclease activity." J Biol Chem 278(5): 3048-3054.

Klebanoff, S. J. and R. W. Coombs (1992). "Viricidal effect of polymorphonuclear leukocytes on human immunodeficiency virus-1. Role of the myeloperoxidase system." Journal of Clinical Investigation 89(6): 2014-2017.

Kovall, R. and B. W. Matthews (1997). "Toroidal structure of lambda-exonuclease." Science 277(5333): 1824-1827.

Lamarche, B. J., S. Kumar, et al. (2006). "ASFV DNA polymerase X is extremely error-prone under diverse assay conditions and within multiple DNA sequence contexts." Biochemistry 45(49): 14826-14833.

Lamarche, B. J., A. K. Showalter, et al. (2005). "An error-prone viral DNA ligase." Biochemistry 44(23): 8408-8417.

Lamarche, B. J. and M. D. Tsai (2006). "Contributions of an endonuclease IV homologue to DNA repair in the African swine fever virus." Biochemistry 45(9): 2790-2803.

Lloyd, A. G., S. Tateishi, et al. (2006). "Effect of DNA repair protein Rad18 on viral infection." PLoS Pathog 2(5): e40.

Maciejewski, M. W., R. Shin, et al. (2001). "Solution structure of a viral DNA repair polymerase." Nat Struct Biol 8(11): 936-941.

McClure, M. A. (2001). "Evolution of the DUT gene: horizontal transfer between host and pathogen in all three domains of life." Curr Protein Pept Sci 2(4): 313-324.

Montgomery, R. E. (1921). "On a form of swine fever ocurring in British East Africa (Kenya colony)." J Comp Pathol 34: 159-191.

Moreau, H., G. Piganeau, et al. (2010). "Marine prasinovirus genomes show low evolutionary divergence and acquisition of protein metabolism genes by horizontal gene transfer." Journal of Virology 84(24): 12555-12563.

Oliveros, M., R. García-Escudero, et al. (1999). "African swine fever virus dUTPase is a highly specific enzyme required for efficient replication in swine macrophages." J Virol 73(11): 8934-8943.

Oliveros, M., R. J. Yáñez, et al. (1997). "Characterization of an African swine fever virus 20-kDa DNA polymerase involved in DNA repair." J Biol Chem 272(49): 30899-30910.

Paran, N., F. S. De Silva, et al. (2009). "Cellular DNA ligase I is recruited to cytoplasmic vaccinia virus factories and masks the role of the vaccinia ligase in viral DNA replication." Cell Host Microbe 6(6): 563-569.

Priet, S., N. Gros, et al. (2005). "HIV-1-associated uracil DNA glycosylase activity controls dUTP misincorporation in viral DNA and is essential to the HIV-1 life cycle." Mol Cell 17(4): 479-490.

Raoult, D., S. Audic, et al. (2004). "The 1.2-megabase genome sequence of Mimivirus." Science 306(5700): 1344-1350.

Redrejo-Rodríguez, M. (2009), Doctoral Thesis. Universidad Autónoma de Madrid.

Redrejo-Rodríguez, M., R. Garcia-Escudero, et al. (2006). "African swine fever virus protein pE296R is a DNA repair apurinic/apyrimidinic endonuclease required for virus growth in swine macrophages." J Virol 80(10): 4847-4857.

Redrejo-Rodríguez, M., A. A. Ishchenko, et al. (2009). "African swine fever virus AP endonuclease is a redox-sensitive enzyme that repairs alkylating and oxidative damage to DNA." Virology.

Robertson, A. B., A. Klungland, et al. (2009). "Base excision repair: the long and short of it." Cellular and Molecular Life Sciences.

Rodríguez, I., M. Redrejo-Rodríguez, et al. (2006). "African swine fever virus pB119L protein is a flavin adenine dinucleotide-linked sulfhydryl oxidase." J Virol 80(7): 3157-3166.

Rodríguez, J. M., R. J. Yáñez, et al. (1993). "The DNA polymerase-encoding gene of African swine fever virus: sequence and transcriptional analysis." Gene 136(1-2): 103-110.

Rojo, G., R. García-Beato, et al. (1999). "Replication of African swine fever virus DNA in infected cells." Virology 257(2): 524-536.

Rouiller, I., S. M. Brookes, et al. (1998). "African swine fever virus is wrapped by the endoplasmic reticulum." J Virol 72(3): 2373-2387.

Rowlands, R. J., V. Michaud, et al. (2008). "African Swine Fever Virus Isolate, Georgia, 2007." Emerging Infectious Diseases 14(12): 1870-1874.

Salas, M. L. (1999). African Swine Fever Virus (Asfarviridae). Encyclopedia of Virology. R. Webster, Granof, A. London, Academic Press: 30-38.

Schwede, T., J. Kopp, et al. (2003). "SWISS-MODEL: An automated protein homology-modeling server." Nucleic Acids Res. 31(13): 3381-3385.

Showalter, A. K., I. J. Byeon, et al. (2001). "Solution structure of a viral DNA polymerase X and evidence for a mutagenic function." Nat Struct Biol 8(11): 942-946.

Showalter, A. K. and M. D. Tsai (2001). "A DNA polymerase with specificity for five base pairs." J. Am. Chem. Soc. 123(8): 1776-1777.

Sung, J. S. and B. Demple (2006). "Roles of base excision repair subpathways in correcting oxidized abasic sites in DNA." FEBS Journal 273(8): 1620-1629.

Suzuki, S., M. Kameoka, et al. (1997). "Superoxide generation by monocytes following infection with human cytomegalovirus." Immunopharmacology 37(2-3): 185-190.

Timson, D. J., M. R. Singleton, et al. (2000). "DNA ligases in the repair and replication of DNA." Mutation Research 460(3-4): 301-318.

Upton, C., D. T. Stuart, et al. (1993). "Identification of a poxvirus gene encoding a uracil DNA glycosylase." Proceedings of the National Academy of Sciences of the United States of America 90(10): 4518-4522.

van den Born, E., M. V. Omelchenko, et al. (2008). "Viral AlkB proteins repair RNA damage by oxidative demethylation." Nucleic Acids Res.

Vellani, T. S. and R. S. Myers (2003). "Bacteriophage SPP1 Chu is an alkaline exonuclease in the SynExo family of viral two-component recombinases." Journal of Bacteriology 185(8): 2465-2474.

Voehler, M. W., R. L. Eoff, et al. (2009). "Modulation of the structure, catalytic activity, and fidelity of African swine fever virus DNA polymerase X by a reversible disulfide switch." Journal of Biological Chemistry 284(27): 18434-18444.

Weinberger, S. and J. Sperling (1986). "Characterization of Escherichia coli mutant strains deficient in AP DNA-repair synthesis." Mutation Research 166(2): 123-134.

Weynberg, K. D., M. J. Allen, et al. (2011). "Genome sequence of Ostreococcus tauri virus OtV-2 enlightens the role of picoeukaryote niche separation in the ocean." Journal of Virology.

Willetts, K. E., F. Rey, et al. (1999). "DNA repair enzyme uracil DNA glycosylase is specifically incorporated into human immunodeficiency virus type 1 viral particles through a Vpr-independent mechanism." J Virol 73(2): 1682-1688.

Yáñez, R. J., J. M. Rodríguez, et al. (1993). "Two putative African swine fever virus helicases similar to yeast 'DEAH' pre-mRNA processing proteins and vaccinia virus ATPases D11L and D6R." Gene 134(2): 161-174.

Yáñez, R. J., J. M. Rodríguez, et al. (1995). "Analysis of the complete nucleotide sequence of African swine fever virus." Virology 208(1): 249-278.

Yáñez, R. J., J. M. Rodríguez, et al. (1993). "African swine fever virus thymidylate kinase gene: sequence and transcriptional mapping." J Gen Virol 74 (Pt 8): 1633-1638.

Yáñez, R. J. and E. Viñuela (1993). "African swine fever virus encodes a DNA ligase." Virology 193(1): 531-536.

Yutin, N. and E. V. Koonin (2009). "Evolution of DNA ligases of nucleo-cytoplasmic large DNA viruses of eukaryotes: a case of hidden complexity." Biol Direct 4: 51.

Zhang, D., L. M. Iyer, et al. (2011). "A novel immunity system for bacterial nucleic acid degrading toxins and its recruitment in various eukaryotic and DNA viral systems." Nucleic Acids Research.

Zharkov, D. O. (2008). "Base excision DNA repair." Cellular and Molecular Life Sciences 65(10): 1544-1565.

6

Nucleotide Excision Repair in *S. cerevisiae*

Tatum, Danielle and Li, Shisheng
Louisiana State University
United States

1. Introduction

Each day organisms are faced with a barrage of genomic insults which damage and jeopardize the integrity of DNA (Lindahl and Wood 1999). DNA damage stems from both endogenous sources such as water and reactive oxygen species generated by regular cellular metabolism and exogenous sources such as sunlight, chemicals, and tobacco smoke. These DNA damaging agents can cause various types of genomic damage including base losses and modifications, strand breaks, crosslinks, bulky chemical adducts, and other DNA alterations. These genomic insults alter the chemistry of DNA and can accumulate and become mutagenic and/or cytotoxic. At the cellular level, DNA damage that is undetected or left unrepaired can result in genomic instability, apoptosis, or senescence, which can greatly affect the aging and development processes. At the level of the organism, genetic instability can predispose the organism to immunodeficiency, neurological disorders, and cancer, illustrating the need to understand the molecular basis of mutagenesis and the mechanisms of DNA repair.

In an effort to maintain the integrity of the genome, evolution has led cells to develop an elaborate DNA damage response system to counteract potentially mutagenic and cytotoxic genomic insults. This highly evolutionarily conserved system is made up of multiple DNA repair pathways, each focusing on a specific category of lesion, as well as multiple checkpoint, signal transduction, and effector systems which crosstalk with replication, transcription, recombination, and chromatin remodeling in order to control DNA damage (Harper and Elledge 2007; Hoeijmakers 2009). The complexity of and the energetic expense dedicated by cells to this process underscores the importance of preserving genomic integrity (Hoeijmakers 2009).

One of the various DNA repair pathways cells have at their employ is the highly conserved nucleotide excision repair (NER), which is the most versatile repair mechanism in terms of lesion recognition [for a recent review, see (Nouspikel 2009)]. NER deals with a wide class of bulky, helix-distorting lesions that generally obstruct transcription and normal replication, such as UV-induced cyclobutane pyrimidine dimers (CPDs) and 6-4 photoproducts [(6-4)PP], as well as adducts and crosslinks induced by chemical agents (e.g. benzo[a]pyrene and cisplatin). NER was first discovered in bacteria in the mid-1960s by Philip Hanawalt and David Pettijohn with the observation of non-semiconservative DNA synthesis during the excision of CPDs (Pettijohn and Hanawalt 1964). Almost simultaneously, excision repair of UV-induced DNA damage was identified in mammalian cells (Rasmussen and Painter 1964).

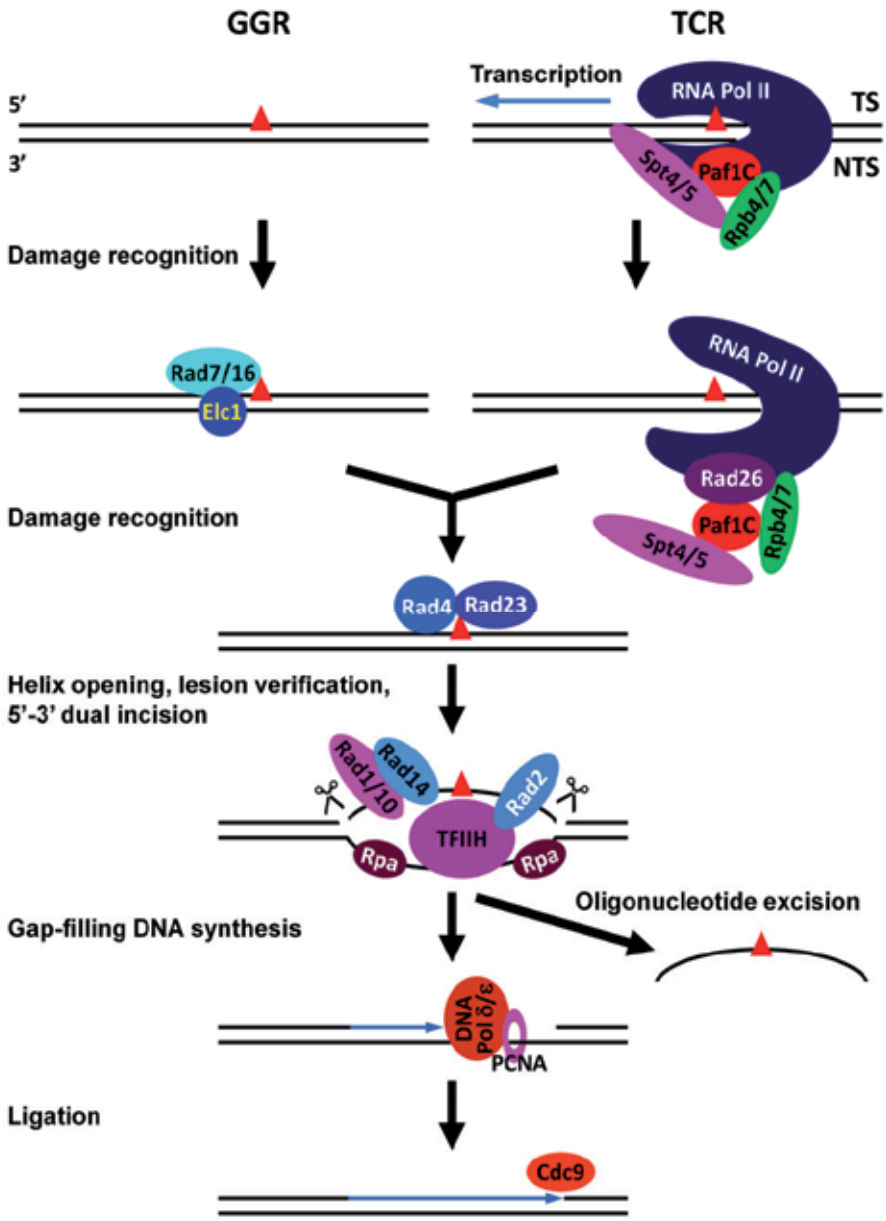

Fig. 1. The process of NER in *S. cerevisiae*. Red triangle denotes a DNA lesion. TS, transcribed strand; NTS, nontranscribed strand.

NER is a multistep reaction which includes damage recognition, helix opening, lesion verification, dual incision of the damaged strand bracketing the lesion, excision of an oligonucleotide containing the lesion, gap-filling DNA synthesis and ligation (Figure 1) (Prakash and Prakash 2000). The distinguishing characteristic of NER is that the damaged bases are enzymatically excised from the genome as an oligonucleotide fragment of about 24-32 nucleotides in length in mammalian cells and 24-27 nucleotides in length in yeast

(Prakash and Prakash 2000). The biological importance of NER for human health is illustrated by the existence of rare autosomal recessive human disorders which result from defects of genes involved in NER, namely xeroderma pigmentosum (XP), Cockayne syndrome (CS), and trichothiodystrophy (TTD), all of which are associated with increased sensitivity to sunlight (Cleaver, Lam, and Revet 2009).

Although the process of NER in eukaryotes and prokaryotes share many similar features, such as damage recognition, excision, repair synthesis and ligation, the molecular mechanisms in eukaryotic cells seem much more complicated. In both prokaryotes and eukaryotes, the core NER factors, defined as the proteins which are necessary and sufficient to carry out the NER reaction, have been identified. The NER process in *Escherichia coli* is relatively well understood and requires only six proteins, whereas the NER process in eukaryotes displays a considerably higher degree of genetic complexity, requiring more than 30 proteins to reconstitute the reaction *in vitro* (Aboussekhra et al. 1995; Guzder et al. 1995).

NER is a heterogeneous process which repairs lesions in the transcribed strands of transcriptionally active genes faster than it repairs lesions in the nontranscribed strands or transcriptionally silent regions. Based on this heterogeneity, NER is traditionally divided into two pathways: global genomic repair (GGR) and transcription coupled repair (TCR). Damage in transcriptionally silent regions and in the nontranscribed strand (NTS) of active genes is repaired by GGR, while TCR is dedicated to repairing lesions in the transcribed strand (TS) of active genes. Though TCR and GGR are generally differentiated as distinct pathways of NER, they only differ in the initial steps of DNA damage recognition. Therefore, after lesion recognition and verification, a general outline of the GGR process becomes applicable to TCR as well. A defining characteristic of NER substrates is that they cause local distortion of the DNA double helix, and in GGR, this local distortion appears to be the first structure recognized.

2. NER in *S. cerevisiae*

Studies using the budding yeast *Saccharomyces cerevisiae* as a model organism have made major contributions in elucidating the core NER mechanism in eukaryotes and have yielded important insights into the functions of a multitude of NER proteins (Prakash and Prakash 2000). Many cellular processes such as replication, repair, cell division, and recombination are highly conserved from lower to higher eukaryotes. Indeed, most NER factors are conserved proteins and have orthologs in humans, yeast, and other eukaryotes (Table 1). In addition to having many homologs to humans, yeast offers many advantageous features to researchers, such as having a well-defined genetic system, the ease of growth and maintenance in the laboratory, and the ability to be maintained in either a haploid or diploid state. Taken together, these features provide researchers with a powerful genetic tool to study NER.

2.1 GGR in *S. cerevisiae*

In *S. cerevisiae*, Rad7, Rad16, and Elc1 are specifically required for GGR (Lejeune et al. 2009; Verhage et al. 1994). Rad4 is the homolog of human XPC (Legerski and Peterson 1992). However, unlike XPC which is specifically required for GGR but dispensable for TCR in human cells, Rad4 is essential for both TCR and GGR in yeast (Prakash and Prakash 2000).

S. cerevisiae	Human homolog or counterpart	Function(s)	Reference
Rad4	XPC	DNA damage recognition and binding	(Guzder et al. 1998)
Rad23	hRAD23B	Interacts with and stimulates Rad4	(Guzder et al. 1998)
TFIIH	TFIIH	DNA helicase activity mediates helix opening	(Egly and Coin 2011)
Mms19	MMS19L	Stabilizes XPD subunit of TFIIH	(Kou et al. 2008)
Rad14	XPA	Stabilizes preincision complex; lesion recognition	(Guzder et al. 2006)
Rpa	RPA	Stabilizes open single stranded DNA; damage recognition	(Guzder et al. 1995)
Rad2	XPG	Catalyzes 3′ incision; stabilizes open complex	(Habraken et al. 1993)
Rad10	ERCC1	Catalyzes 5′ incision; forms complex with Rad1	(Sung et al. 1993; Tomkinson et al. 1994)
Rad1	XPF	Catalyzes 5′ incision	(Sung et al. 1993; Tomkinson et al. 1994)
Rad26	CSB	TCR-specific factor; DNA-dependent ATPase	(van Gool et al. 1994)
Rpb9	Rpb9	TCR-specific factor; subunit of RNA polymerase II	(Li and Smerdon 2002)
Rad7-Rad16	DDB1-DDB2	GGR-specific factor; damage recognition; ubiquitinates Rad4	(Gillette et al. 2006; Ramsey et al. 2004; Reed 2005; Verhage et al. 1994)
Elc1	Elongin C	GGR-specific factor; forms complex with Rad7-Rad16	(Lejeune et al. 2009; Ramsey et al. 2004)
DNA polymerase δ	DNA polymerase δ	Gap-filling repair synthesis	(Wu et al. 2001)
DNA polymerase ε	DNA polymerase ε	Gap-filling repair synthesis	(Wu et al. 2001)
PCNA	PCNA	Sliding clamp for DNA polymerase δ	(Huang et al. 1998)
Cdc9	DNA ligase I	Ligation	(Wu, Braithwaite, and Wang 1999)

Table 1. Core yeast NER factors, their human homologs or counterparts, and their functions in NER.

The exact roles of Rad7, Rad16, and Elc1 in GGR are not yet clear. Rad7 and Rad16 form a complex that binds specifically to UV-damaged DNA in an ATP-dependent manner (Guzder et al. 1997). Although no structural homologs of Rad7 and Rad16 have been identified in human cells, some striking functional similarities exist between Rad7-Rad16 and DDB1-DDB2 (XPE) complexes [for a review see, (Reed 2005)]. Mutations in both the

yeast and human genes result in defective GGR. Both exhibit physical interactions and can bind damaged DNA. Both form components of a class of cullin based E3 ubiquitin ligase whose substrate includes Rad4/XPC, that are homologues of the same repair factor in yeast and humans, respectively. The ATP dependence of the Rad7-Rad16 complex for damage binding distinguishes this complex from Rad14, RPA, and the Rad4–Rad23 complex, which do not exhibit such dependence on ATP for damage binding (Prakash and Prakash 2000). Rad16 shares marked homology with Snf2, the catalytic subunit of the SWI/SNF chromatin remodeling complex (Bang et al. 1992), and Snf2 contains conserved motifs found in a superfamily of ATPases thought to be involved in chromatin remodeling activities (Eisen, Sweder, and Hanawalt 1995). Accordingly, the Rad7–Rad16 complex displays a DNA-dependent ATPase activity. It has been shown that this ATPase activity is inhibited when the complex comes across DNA damage (Guzder et al. 1998). This finding led to the formation of a model which suggested that the Rad7-Rad16 complex may act as an ATP-dependent motor which translocates along the DNA in search of damage, and upon encountering a lesion, the complex is stalled, which may remodel and open damaged chromatin, thereby facilitating recruitment of other NER factors (Guzder et al. 1998) According to this model, the Rad7–Rad16 complex would arrive first on the scene of a damage site in nontranscribed regions of the genome and serve as the nucleation site for the recruitment of the other NER factors. It was also previously suggested that the ATPase activity of Rad16 generates superhelical torsion in DNA that has an altered structure due to UV-induced damage, and that this torsion is necessary for the excision of damaged bases in GGR (Yu et al. 2004), suggesting a role for Rad16-Rad7 in the later steps of GGR.

Yeast Elc1 is a homolog of mammalian elongin C which forms a heterotrimeric complex with elongins A and B (Aso et al. 1995; Bradsher et al. 1993). The *elc1*Δ mutation was shown to be epistatic to *rad7*Δ and *rad16*Δ mutations, but resulted in a synergistic enhancement of UV sensitivity when combined with *rad26*Δ (Ribar, Prakash, and Prakash 2006). A study utilizing a technique which measures NER at the nucleotide level revealed that Elc1 plays an important role in GGR, as *elc1*Δ cells showed no detectable repair of CPDs in the NTS of the constitutively expressed *RPB2* gene, but no role in TCR (Lejeune et al. 2009). The role of Elc1 is not via stabilizing Rad7 or Rad16, as levels of either do not change in *elc1*Δ cells. Furthermore, the role of Elc1 does not seem to be subsidiary to that of Rad7 or Rad16, as overexpression of either or both in the absence of Elc1 did not restore GGR (Lejeune et al. 2009). The precise nature of the role of Elc1 in GGR remains unknown. Genetic studies have revealed multiple roles for this gene in separate cellular processes (Ribar, Prakash, and Prakash 2006). In one of these, Elc1 is a component of a ubiquitin ligase (E3) that contains Rad7 and Rad16 and is responsible for regulating the levels of Rad4 protein in response to UV damage (Gillette et al. 2006; Ramsey et al. 2004). It has also been suggested that Elc1 is a component of another ubiquitin ligase complex, which contains Ela1, Cul3, and Roc1 and is responsible for the polyubiquitylation and subsequent degradation of RNA polymerase II (Pol II) in response to DNA damage (Ribar, Prakash, and Prakash 2006, 2007).

The TFIIH multiprotein complex, which is organized into a 7-subunit core associated with a 3-subunit CDK-activating kinase module (CAK), is involved in both Pol II-mediated transcription and NER (Egly and Coin 2011). Tfb5, the homolog of human TTD-A, is a subunit of the core TFIIH. Unlike other subunits of the core TFIIH which are required for both GGR and TCR, Tfb5 has been shown to be essential for GGR but not absolutely required for TCR, as no apparent repair can be detected in the NTS, but a certain extent of

repair can be seen in the TS of either the *RPB2* or *GAL1* genes (Ding et al. 2007). The effect is unlikely due to changes in the steady state levels of other TFIIH subunits, as Tfb5 does not seem to affect the stability of other TFIIH components (Ranish et al. 2004). This may be different from human cells, where TTD-A (the homolog of yeast Tfb5) has been shown to stabilize other subunits of TFIIH (Vermeulen et al. 2000). Yeast Tfb5 interacts with Tfb2, another subunit of the TFIIH core (Zhou, Kou, and Wang 2007), and it was proposed that yeast Tfb5 acts as an architechtural stabilizer giving structural rigidity to the core TFIIH so that the complex is maintained in its functional architecture (Zhou, Kou, and Wang 2007). Another possibility is that the Rad25 ATPase activity of TFIIH needs to be stimulated by Tfb5 (Coin et al. 2006) in order to efficiently unwind the double helix around a lesion in the chromatin environment *in vivo* (Ding et al. 2007).

2.1.1 GGR in the context of chromatin

Although the core biochemical mechanism of NER is known, much remains unanswered. One of the looming questions currently being addressed is the issue of NER, especially GGR, in chromatin. As with all DNA-related processes, the NER machinery must deal with the presence of organized chromatin and the physical obstacles that it presents. How cells detect and repair lesions in diverse chromatin environments is a question that remains unanswered. Rearrangement of chromatin structure during NER was discovered more than two decades ago, however the molecular basis of chromatin dynamics during NER in eukaryotic cells is still not well understood (Gong, Kwon, and Smerdon 2005; Nag and Smerdon 2009; Waters et al. 2009).

The basic repeating unit of chromatin is the nucleosome, which is comprised of 146 base pairs of DNA wrapped around an octamer of the four core histone proteins H2A, H2B, H3, and H4 (Luger et al. 1997). Most of this DNA is tightly wrapped in about 1.6 left-handed superhelical turns around the histone octamer, with linker DNA (ranging from 20 to 90 base pairs in length) separating nucleosome cores, and giving the "beads-on-a-string" appearance familiar from electron microscopy. DNA is then further compacted by the organization of nucleosomes into higher order structures, such as 30 nm fibers and the highly condensed state of chromosomes (Wolffe 1999). Adjacent nucleosomes can be arranged in various configurations which affect the accessibility of DNA, thus the DNA-nucleosome polymer must be flexible in order to allow various cellular processes such as replication, transcription, recombination, and repair (Zhang, Jones, and Gong 2009). The two primary mechanisms which are involved in this flexibility are histone modifications and chromatin remodeling (Palomera-Sanchez and Zurita 2011). Below we summarize new findings regarding NER and the roles of histone modifications and chromatin remodeling activities. The results of previous studies focused on elucidating these mechanisms have been summarized in several very good recent reviews (Altaf, Saksouk, and Cote 2007; Ataian and Krebs 2006; Palomera-Sanchez and Zurita 2011; Zhang, Jones, and Gong 2009).

2.1.1.1 Chromatin remodeling and GGR

The complexity of NER and the size of the repair machinery can make it difficult to imagine how DNA distorting lesions can be recognized and processed without temporary rearrangement of chromatin (Thoma 1999). Instead of utilizing specific enzymes that each recognize a specific type of lesion, NER's damage recognition factors recognize helix distortion and bind to DNA to test its local conformation (which explains the wide versatility of NER). The excision step in NER requires considerable space. About 25-30 bp of

DNA are unwound in the open complex during NER (Evans et al. 1997), and the human excision complex requires about 100 bp of DNA to excise the lesion *in vitro* (Huang and Sancar 1994). Such a complex is incompatible with the structure of the nucleosome, and the linker DNA between nucleosomes is too short to accommodate a repair complex (Thoma 1999). Thus it is obvious that *in vivo* alterations of chromatin either by the lesion itself, by the action of chromatin remodelers or histone modifications, or by DNA metabolizing processes such as transcription, are required to facilitate damage recognition and repair (Fousteri et al. 2005).

Although chromatin structures can restrict the NER machinery from accessing sites of DNA damage, limited pieces of evidence have emerged recently that chromatin metabolism may also play an active role in the repair process (Waters et al. 2009). The SWI/SNF superfamily of ATP-dependent chromatin remodeling enzymes all possess an ATPase subunit which can disrupt or alter DNA-histone associations. SWI/SNF is the prototypical chromatin remodeling factor and is present in all eukaryotes (Martens and Winston 2003). Previous studies have demonstrated that these chromatin remodeling enzymes play an important role in enabling access of the NER machinery to nucleosomal DNA [for a review, see (Osley, Tsukuda, and Nickoloff 2007)]. As transcription disturbs chromatin structure, only the GGR pathway of NER is modulated by chromatin. As DNA damage recognition is a slow and rate-limiting step in NER (Mone et al. 2004) and it has been thought that this step required chromatin remodeling activities, a *in vivo* study examined the possible association between SWI/SNF and the DNA damage recognition complex Rad4-Rad23 in yeast (Gong, Fahy, and Smerdon 2006). Using His-tag pulldown and coimmunoprecipitation assays, this study provided evidence linking a chromatin remodeling complex with NER by demonstrating that Snf6 and Snf5, two subunits of the SWI/SNF complex in yeast, co-purify with the Rad4-Rad23 heterodimer (Gong, Fahy, and Smerdon 2006). It was further shown that this association was stimulated by UV irradiation, indicating that SWI/SNF facilitates chromatin remodeling during NER and that it has a role in facilitating GGR. Based on these findings, it was postulated that Rad4-Rad23 may recruit the SWI/SNF complex to facilitate NER at damage sites *in vivo*, or that SWI/SNF may recognize and bind to another feature of damaged chromatin and aid in recruiting Rad4-Rad23 (Gong, Fahy, and Smerdon 2006). The GGR-specific factor Rad16 is also a member of the SWI/SNF family of DNA-dependent ATPases and is thought to have a role in DNA damage recognition (Prakash and Prakash 2000). Interestingly, no association was found between Snf6 and Rad16, which is surprising given that Rad16 is required for GGR. This suggests that SWI/SNF and Rad16 may operate at different stages in the repair process (Gong, Fahy, and Smerdon 2006).

Another example of chromatin remodeling in NER comes from a recently published report which showed that the Ino80 chromatin remodeling complex promotes removal of UV lesions in regions with high nucleosome occupancy (Sarkar, Kiely, and McHugh 2010). More specifically, the study showed that Ino80 interacts with the early damage recognition complex of Rad4-Rad23 and was recruited to Rad4 in a UV-dependent manner. *ino80*Δ mutants were shown to be defective in both recruitment of repair factors to the damage site and restoration of nucleosome structure after repair. This suggests that Ino80 is recruited to sites of UV DNA damage through interactions with the NER machinery and is required for restoration of chromatin structure after repair (Sarkar, Kiely, and McHugh 2010). The role of Ino80 in NER appears to be conserved in eukaryotic cells. In mammalian cells deletion of two core components of the Ino80 complex, INO80 and ARP5, significantly hampered cellular removal of UV-induced photo lesions but had no significant impact on the

transcription of NER factors (Jiang et al. 2010). Loss of INO80 abolished the assembly of NER factors, suggesting that prior chromatin relaxation is important for the NER incision process.

Because transcription disturbs chromatin, only GGR is modulated by chromatin structure. Indeed, there is no correlation between the heterogeneity in NER and chromatin structure in TCR. However, chromatin remodeling activities associated with the transcription process are likely to play a role in damage recognition during TCR (Zhang, Jones, and Gong 2009). As mentioned previously, Rad26 is a DNA-dependent ATPase of the SWI/SNF superfamily (Guzder et al. 1996). CSB, the human homolog of yeast Rad26, has been shown to interact with XPG (Sarker et al. 2005) and attracts repair factors and a histone acetyltransferase to the site of a damage-stalled Pol II (Newman, Bailey, and Weiner 2006). Similar to its human homolog, Rad26 has also been found to play a role in repairing apparently transcriptionally inactive genes, a role possibly exacted through its putative chromatin remodeling activities (Bucheli, Lommel, and Sweder 2001). However, caution needs to be exercised regarding the explanation of role of Rad26 in repairing transcriptionally repressed genes, which can be through TCR initiated by noise transcription that commonly occurs in both strands of supposedly repressed genes (Li et al. 2007; Tatum and Li 2011).

2.1.1.2 Histone modifications and GGR

From recent evidence, it is clear that GGR requires different mechanisms to relax chromatin and ultimately removes lesions (Palomera-Sanchez and Zurita 2011). In addition to chromatin remodelers, histone modifications have been implicated in various mechanisms of DNA repair. Histones are subject to a multitude of post-translational modifications including acetylation, methylation, phosphorylation, sumoylation, and ubiquitination (Kouzarides 2007). Some of these modifications may modulate the NER process (Gong, Kwon, and Smerdon 2005; Nag and Smerdon 2009; Waters et al. 2008). However, the effects of histone modifications on NER in living cells documented previously are generally quite modest and are most likely due to the alteration of chromatin compaction and/or stability. Some recent studies implicating histone modifications in the facilitation of NER are discussed below.

The functional correlation between histone hyperacetylation and efficient NER has been known for some time (Ramanathan and Smerdon 1989; Smerdon et al. 1982). More recent studies have confirmed this correlation, demonstrating reduced CPD removal in yeast cells lacking the histone acetyltransferase (HAT) Gcn5, which acetylates histone H3 on lysines 9 and 14 (H3K4 and H3K19). Furthermore, the acetylation of H3K9 and H3K14 was shown to increase throughout the genome after irradiation with UV light and resulted in more efficient GGR (Teng et al. 2008; Yu et al. 2005). This modification seems to be conserved in mammalian cells, as a recent report showed that the E2F1 transcriptional factor in human cells is recruited in the chromatin at sites of UV damage and associates with GCN5 to acetylate H3K9 (Guo et al. 2011). Histone H3 in yeast has also been shown to be hyperacetylated in strains lacking the damage recognition factors Rad4 or Rad14, indicating that H3 acetylation occurs before the repair process and is not stimulated by NER (Yu et al. 2005). However, reinstating the acetylation level to a pre-UV state was shown to be dependent on NER (Yu et al. 2005). Additionally, UV-induced hyperacetylation of H3K9 and K14 was shown to be mediated by the GGR-specific factor Rad16 (Teng et al. 2008). Interestingly, it was demonstrated that pre-hyperacetylated regions could undergo efficient repair even in the absence of Rad16 (Teng et al. 2008), thus providing a direct link between

GGR and histone acetylation. However, it remains to be elucidated if the Rad16-independent repair is indeed GGR or TCR initiated by noise transcription that may not occur at normal conditions but takes place when the chromatin is pre-hyperacetylated. The noise transcripts can be hard to detect by traditional techniques as they are rapidly degraded after being produced (Struhl 2007). It has been postulated that histone hyperacetylation could regulate NER either directly through generating a suitable binding surface for repair proteins or indirectly through altering the compaction of nucleosomes (Irizar et al. 2010).

Much like the trend observed for gene expression, the effect of histone acetylation on repair varies according to chromatin status. In yeast, heavily compacted and suppressive regions of chromatin (i.e. heterochromatin) such as telomeres, silenced mating loci, and rDNA repeats, show reduced levels of histone H3 and H4 acetylation after UV. A recent study examined GGR of the *URA3* gene in subtelomeric regions (Irizar et al. 2010). These regions are hypoacetylated and bound by Sir proteins, which are involved in establishing silenced and heterochromatic regions in the genome. One particular Sir protein, Sir2, a NAD^+-dependent histone deacetylase (HDAC), has been shown to have a preference for removing the acetyl group from H3K9 and K14 as well as H4K16 (Imai et al. 2000; Landry et al. 2000). Repair of CPDs in these regions was shown to be slow, likely a result of the reduced histone acetylation. Furthermore, a significant increase in histone H3 and H4 acetylation after UV was shown in *sir2*Δ deletion mutants, indicating an important role for Sir2 in regulating histone acetylation in response to UV. This increase in histone acetylation resulted in improved NER efficiency, suggesting that the action of the different mechanisms that modify histones to facilitate NER may be influenced by the type of chromatin environment and the prevalence of specific factors like Sir2 in subtelomeric chromatin (Palomera-Sanchez and Zurita 2011).

In addition to histone acetylation, histone methylation has also been shown to play a role in the GGR process. Dot1 is a histone methyltransferase required for methylation of histone H3 lysine 79 (H3K79). *dot1*Δ mutants are sensitive to UV (Bostelman et al. 2007) and have a defect in activation of DNA damage checkpoints (Giannattasio et al. 2005). Indeed, H3K79 methylation was shown to be required for efficient NER in a silenced locus of yeast (Chaudhuri, Wyrick, and Smerdon 2009). An even more recent study demonstrated Dot1 to be a novel GGR-specific factor which mediates GGR by methylating its sole known substrate, H3K79 (Tatum and Li 2011). Using a nucleotide resolution method which uses streptavidin magnetic beads and biotinylated oligonucleotides to facilitate isolation and strand-specific end-labeling of DNA fragments of interest to measure GGR, the study showed that Dot1 and H3K79 methylation are required for GGR in both nucleosomal core regions and inter-nucleosomal linker regions, but play no role in TCR (Tatum and Li 2011). It was previously suggested that the role of H3K79 methylation in GGR may be via affecting expression of repair factors, such as Rad16 (Chaudhuri, Wyrick, and Smerdon 2009). However, it was shown that overexpression of Rad16 in cells whose genomic H3 genes (*HHT1* and *HHT*2) were deleted and complemented with a plasmid encoding the K79A mutant histone H3 (H3K79A) cells did not affect GGR, suggesting that the effect of H3K79 methylation on GGR is not through regulating the expression of Rad16 (Tatum and Li 2011). It was proposed that the addition of methyl moieties to H3K79 may serve as a docking site for repair factors on the chromatin. In the absence of the methyl groups, the repair machinery may be excluded from the chromatin, including the vicinities of inter-nucleosomal linker regions (Tatum and Li 2011).

We have recently found additional evidence for the involvement of histone modifications in GGR in studies involving the yeast Pol II-associated factor 1 complex (Paf1C). Paf1C is comprised of 5 subunits, namely Paf1, Rtf1, Cdc73, Leo1, and Ctr9 and interacts with Pol II and chromatin at both promoters and throughout the coding regions of genes [for a recent review, see (Jaehning 2010)]. Loss of Rtf1 or Cdc73 causes the dissociation of Paf1C from Pol II and chromatin. Paf1C has been shown to be required for monoubiquitination of histone H2B at lysine 123 (H2BK123) by Bre1 (an E3 ubiquitin ligase) in complex with Rad6 (an E2 ubiquitin conjugase) (Krogan et al. 2003; Ng, Dole, and Struhl 2003; Wood et al. 2003). The Bre1-Rad6-mediated monoubiquitination of H2BK123 is, in turn, partially required for dimethylation and fully required for trimethylation of H3K79 by Dot1 (Levesque et al. 2010; Nakanishi et al. 2009; Shahbazian, Zhang, and Grunstein 2005). Dot1 can add one methyl group to H3K79 by itself, meaning that Paf1C indirectly enables di- and trimethylation of H3K79. Although it can be associated with Pol II, Paf1C may function in enabling these histone modifications independent of Pol II, as both monoubiquitination of H2BK123 (Schulze et al. 2009) and methylation of H3K79 (Ng et al. 2003; Pokholok et al. 2005; van Leeuwen, Gafken, and Gottschling 2002) do not seem to be correlated with the transcriptional activity of a gene. Elimination of one of the PAF components (Rtf1) resulted in significantly compromised GGR, especially in inter-nucleosomal linker regions (Tatum et al. 2011). Genetic analysis revealed an epistatic relationship between *RTF1* and *BRE1* and *DOT1*, indicating that these proteins function in the same pathway in response to UV damage. It was further demonstrated that elimination of Rtf1 in *bre1*Δ or *dot1*Δ cells did not affect GGR speed, confirming the presence of an epistatic relationship as well as indicating that the function of Paf1C in GGR is accomplished through enabling monoubiquitination of H2BK123 by Bre1, which in turn permits di- and tri-methylation of H3K79 by Dot1 (Tatum et al. 2011).

In addition to acetylation and methylation, studies have also provided evidence for multiple roles of histone ubiquitination in NER (Nouspikel 2011). Nucleosome stability is controlled mainly by acetylation, but also to some degree by ubiquitination. Histone H2A is constitutively ubiquitinated even in the absence of DNA damage, especially in condensed chromatin. This ubiquitination was shown to disappear rapidly after UV-induced DNA damage and reappear within 30 minutes to 2 hours (Kapetanaki et al. 2006). Histones H2B, H3, and H4 are also constitutively ubiquitinated but to a much lower level (Nouspikel 2011). It was shown that ubiquitination of H3 and H4 increased within 1 hour of UV irradiation, decreased by 4 hours, and returned to original levels at 8 hours (Wang et al. 2006). It was postulated that this may act as a means of destabilizing nucleosomes, permitting better access of the repair machinery to the site of the lesion. However, there is a lack of experimental support for this idea. In fact, *in vitro* experiments showed that ubiquitination of H3 and H4 does not cause dissociation from DNA, and *in vivo*, only about half of H3 ubiquitinated after UV-induced damage dissociated from chromatin (Bergink et al. 2006; Wang et al. 2006).

Evidence for UV-induced ubiquitination came from a study which used cells expressing GFP-tagged ubiquitin (Bergink et al. 2006). These cells were UV-irradiated through a micropore filter to induce localized spots of DNA damage in the nucleus. Interestingly, after induction of DNA damage by UV, ubiquitinated histone H2A was found to accumulate at damage sites. This ubiquitination of histone H2A was shown to be dependent on NER and occurred after incision of the damaged strand, indicating a role in the later steps of NER. Indeed, a subsequent study demonstrated that UV-induced accumulation of ubiquitinated H2A at damage sites is a part of the chromatin restoration process (Zhou et al. 2008).

2.2 TCR in *S. cerevisiae*

Lesions that arrest or stall transcription by Pol II on the transcribed strand (TS) are repaired 5-10 times faster than the nontranscribed strand (NTS) by TCR (Hanawalt 1994). TCR has been shown to function in *E. coli* (Mellon and Hanawalt 1989), *S. cerevisiae* (Smerdon and Thoma 1990), and mammalian cells (Mellon, Spivak, and Hanawalt 1987). While the mechanistic details of TCR in *E. coli* are relatively well understood, the mechanisms of TCR in eukaryotes appear to be extremely complicated [for reviews, see (Fousteri and Mullenders 2008; Hanawalt and Spivak 2008)].

TCR in eukaryotic cells is believed to be triggered by stalled Pol II at a lesion in the transcribed strand of a gene (Fousteri and Mullenders 2008; Hanawalt and Spivak 2008). Rad26, the yeast homolog of human CSB and a putative transcription repair coupling factor, is important for TCR but dispensable for GGR (van Gool et al. 1994). However, TCR in yeast is not solely dependent on Rad26, as a significant amount of repair still occurs in cells lacking Rad26 (Li and Smerdon 2002, 2004; Verhage et al. 1996). Rpb9, a nonessential subunit of Pol II, has also been shown to play a role in mediating TCR (Li and Smerdon 2004, 2002; Li et al. 2006; Li et al. 2007).

Rad26- and Rpb9- mediated TCR subpathways have been shown to have different efficiencies in different regions of a gene (Li et al. 2006). Rpb9-mediated TCR operates more effectively in the coding region than in the region upstream of the transcription start site; whereas the Rad26-mediated subpathway operates equally well in both regions (Li and Smerdon 2002, 2004). Additionally, in log phase wild type cells, the relative contributions of these two subpathways of TCR may be different from gene to gene. For the *URA3* gene, Rad26 seems to be absolutely required, except for a short region close to the transcription start site (Tijsterman et al. 1997), indicating that TCR is accomplished primarily by the Rad26 subpathway. Rad26 is partially required for TCR in the *RPB2* gene (Bhatia et al. 1996; Gregory and Sweder 2001; Li and Smerdon 2002; Verhage et al. 1996), indicating that both subpathways contribute to TCR in this gene. For the *GAL1* gene, Rad26 is almost dispensable, especially in the coding region, indicating that TCR in this gene of log-phase cultures is fulfilled primarily by the Rpb9 subpathway. The different contributions of the two subpathways of TCR in different genes may be caused by different levels of transcription. Rad26- and Rpb9-mediated repair are also differently modulated by different promoter elements (Li et al. 2006). In the yeast *GAL1* gene, the efficiency of TCR mediated by Rad26 is determined by the upstream activating sequence (UAS), but not by the TATA or local sequences. However, both the UAS and TATA are necessary to confine Rad26-mediated repair to the transcribed strand of the gene. Abrogating or abolishing transcription by mutation or deletion of the TATA sequence or mutation of the UAS results in Rad26-mediated repair in both the TS and NTS of the *GAL1* gene (Li et al. 2006). This suggests that Rad26-mediated repair can be either transcription-coupled, provided that a substantial level of transcription is present, or transcription-independent, if transcription is too low or absent. However, as mentioned above, noise transcription, which cannot be easily detected by traditional techniques, may occur in both strands upon the mutation or deletion of the UAS or TATA. This unexpected noise transcription may cause Rad26-mediated repair (which is TCR) to occur in both strands. Conversely, Rpb9-mediated TCR only occurs in the transcribed strand and is efficient only if the TATA and UAS sequences are present, suggesting that TCR mediated by Rpb9 is strictly transcription coupled and is only efficient when the level of transcription is high (Li et al. 2006).

Rpb9 also plays an important role in promoting ubiquitylation and degradation of Rpb1, the largest subunit of Pol II, in response to UV damage (Chen, Ruggiero, and Li 2007). Rpb9 is

composed of three distinct domains: the N-terminal Zn1, the C-terminal Zn2, and the central linker. The Zn1 and linker domains are essential for both transcription elongation and TCR functions, but the Zn2 domain is almost dispensable (Li et al. 2006). However, the Zn2 domain is essential for Rpb9 to promote degradation of Rpb1, whereas the Zn1 and linker domains play a subsidiary role in the degradation. This function of Rpb9 seems to be unrelated to any pathways of NER, including both subpathways of TCR, and it remains to be determined how Rpb9 promotes ubiquitination and degradation of Rpb1 (Chen, Ruggiero, and Li 2007).

2.2.1 The role of Rad26 in TCR

Like its human homolog CSB, Rad26 is a DNA-stimulated ATPase and functions in transcription elongation (Lee et al. 2001; Selby and Sancar 1997). Due to its ATPase activity, Rad26 is the most promising yeast transcription repair coupling factor (Svejstrup 2002). However, how Rad26 functions in TCR remains to be elucidated. Several models have been proposed based on its DNA-dependent ATPase activity (Svejstrup 2002). Because other members of the Swi/Snf family are able to alter contacts between DNA and DNA-binding proteins, one possibility is that Rad26, through its Swi/Snf-like activity, may displace a stalled Pol II complex at a damage site (Svejstrup 2002). This is the case in *E. coli* where the transcription repair coupling factor Mfd, an ATP-dependent translocase, moves stalled Pol II forward from the damage site, allowing it to continue transcription (Selby and Sancar 1994; Park, Marr, and Roberts 2002). However, other than ATPase domains, there is little structural homology between Rad26 and Mfd. Furthermore, an *in vitro* study demonstrated that CSB cannot displace Pol II stalled at a damage site (Selby and Sancar 1997).

A second model postulates that a Pol II complex stalled at a lesion may be pushed back by the general transcription factor TFIIS, which facilitates Pol II elongation through transcriptional arrest sites and stimulates transcript cleavage, allowing resumed forward translocation during normal transcription elongation (Kettenberger, Armache, and Cramer 2003; Saeki and Svejstrup 2009). Yeast strains lacking Rad26 exhibit a synergistic increase in sensitivity to the DNA-damaging agent methyl methanesulfonate (MMS) when combined with inactivating mutations in NER, suggesting a role for Rad26 in promoting Pol II transcription elongation through damage sites in DNA (Lee et al. 2002). However, TFIIS does not seem to play any role in TCR in both yeast (Verhage et al. 1997) and mammalian cells (Jensen and Mullenders 2010).

Alternative models addressing the fate of a damage-stalled Pol II, such as accessory-factor-mediated lesion bypass and keeping Pol II at a distance through damage-binding factors, might also be relevant in certain situations (Svejstrup 2002). The finding that Rpb1, the largest subunit of Pol II, is ubiquitinated and subsequently degraded in the CSA- and CSB-dependent manner in response to DNA damage that blocks transcription prompted researchers to propose a model whereby Pol II degradation facilitates lesion access and repair (Bregman et al. 1996; Ratner et al. 1998). However, a more recent report showed that CSA and CSB are not directly involved in Rpb1 ubiquitylation. The defects in Rpb1 ubiquitylation observed in CS cells are caused by an indirect mechanism: these cells shut down transcription in response to DNA damage, effectively depleting the substrate for ubiquitylation, namely elongating Pol II (Anindya, Aygun, and Svejstrup 2007). Also, evidence has shown that the ubiquitination and degradation of Rpb1 do not seem to be necessary for TCR in yeast. Rsp5, the only yeast ubiquitin-protein ligase that modifies Pol II,

is not required for TCR (Lommel, Bucheli, and Sweder 2000). Def1, which forms a complex with Rad26 in chromatin, is required for Pol II degradation in response to DNA damage but is not required for TCR (Woudstra et al. 2002). Furthermore, as mentioned above, Rpb9 plays an important role in ubiquitination and degradation of Rpb1 (Chen, Ruggiero, and Li 2007). However, this function of Rpb9 is unrelated to TCR mediated by Rpb9 itself and that mediated by Rad26 (Chen, Ruggiero, and Li 2007).
As will be discussed below, recent evidence indicates that the role of Rad26 in TCR may be entirely through indirect mechanisms, by antagonizing the actions of TCR suppressors (Figure 1).

2.2.2 Suppressors of Rad26-independent TCR

Recently, a number of TCR suppressors have been identified. Interestingly, in each case, the release of suppression (i.e. reinstatement of TCR) is present only in cells lacking Rad26. Below is a discussion of each of the known suppressors of Rad26-independent TCR and their possible interactions.
Yeast Spt4 and Spt5 form a complex which has been shown to physically interact with Pol II (Hartzog et al. 1998). The *SPT4* gene is dispensable (Malone, Fassler, and Winston 1993), whereas *SPT5* is essential (Swanson, Malone, and Winston 1991), for cell viability. These proteins are conserved transcription elongation factors and are generally required for normal development and viral gene expression in multicellular eukaryotes (Winston 2001). It was previously shown that deletion of *SPT4* negates the requirement of Rad26 for TCR in yeast, suggesting that Spt4 suppresses Rad26-independent TCR (Jansen et al. 2000). It has been further demonstrated that the suppression effect of Spt4 is indirect via protecting its interacting partner, Spt5, from degradation and by stabilizing the interaction of Spt5 with Pol II (Ding, LeJeune, and Li 2010). Indeed, overexpression of Spt5 in the absence of Spt4 suppresses Rad26-independent TCR (Ding, LeJeune, and Li 2010), supporting the notion that Spt4 plays an indirect role in this suppression.
Spt5 possesses a C-terminal repeat (CTR) domain, which is dispensable for cell viability and is not involved in interactions with Spt4 and Pol II (Ding, LeJeune, and Li 2010). Repair analysis of *rad26Δ* cells whose genomic *SPT5* gene had been deleted and complemented with a plasmid encoding either the full length or CTR-deleted Spt5 revealed that TCR in these cells expressing the CTR-deleted Spt5 was significantly faster than in those expressing full length Spt5, indicating that the Spt5 CTR is involved in suppressing Rad26-independent TCR (Ding, LeJeune, and Li 2010). Additional evidence for the role of the CTR in this suppression came from analyzing the phosphorylation state of the CTR. The CTR domain contains 15 6-amino acid repeats with the consensus sequence S(A/T)WGG(A/Q) (Swanson, Malone, and Winston 1991), with the serine and threonine residues being potential phosphorylation sites. It has been shown that the Spt5 CTR is phosphorylated by the Bur kinase (Ding, LeJeune, and Li 2010; Liu et al. 2009; Zhou et al. 2009). The kinase activity of Bur1 is dependent upon its cyclin partner Bur2. Deletion of Bur1 is lethal to cells, but deletion of Bur2 is not. Additionally, *bur1*Δ and *bur2*Δ mutations result in nearly identical phenotypes (Yao, Neiman, and Prelich 2000). Interestingly, it was shown that deletion of Bur2 also partially alleviates the necessity of Rad26 for TCR, suggesting that the phosphorylation of the Spt5 CTR may be partially responsible for suppressing TCR in the absence of Rad26 (Ding, LeJeune, and Li 2010).
It is not yet clear how the CTR of Spt5 is acting to suppress Rad26-independent TCR. It was recently reported that the Spt5 CTR is a platform for the association of proteins that promote

both transcription elongation and histone modifications (Zhou et al. 2009). One such protein complex recruited by the Spt5 CTR is Paf1C (Zhou et al. 2009). Indeed, the Rtf1 subunit of Paf1C has been shown to have extensive physical and physical connections with Spt5 (Squazzo et al. 2002). Additionally, optimal association of Paf1C with Pol II is dependent upon Spt4 (Qiu et al. 2006) and the Spt5 CTR (Tatum et al. 2011). Furthermore, recruitment of Paf1C requires the Bur-mediated phosphorylation of the CTR of Spt5 (Liu et al. 2009). Results from our lab showed that deletion of any of Paf1C's 5 subunits in *rad26*Δ cells causes increased TCR, indicating that Paf1C too is a suppressor of Rad26-independent TCR. Furthermore, simultaneous deletion of Spt4 along with a Paf1C component in *rad26*Δ cells resulted in similar degrees of repair restoration, suggesting that these suppressors are acting through a common pathway to suppress Rad26-indepedent TCR. However, unlike Spt4, Paf1C appears to be indispensable for suppressing Rad26-independent TCR, as overexpression of Spt5 in cells lacking a Paf1C component did not affect the overall TCR rate in these cells. This suggests that both Paf1C and Spt5 are required for suppressing TCR in the absence of Rad26 and that the role of Paf1C in this suppression is not subsidiary to that of Spt5.

Rpb4 is another nonessential subunit of Pol II (Woychik and Young 1989) and forms a subcomplex with Rpb7 (Armache, Kettenberger, and Cramer 2003; Bushnell and Kornberg 2003), a small but essential subunit of Pol II. This subcomplex can dissociate from Pol II, and deletion of Rpb4 abolishes the association of Rpb7 with Pol II. Interestingly, it was shown that, like Spt4/Spt5, deletion of Rpb4 reinstates TCR in *rad26*Δ cells, indicating that Rpb4 is also a suppressor of Rad26-independent TCR (Li and Smerdon 2002).

Pol II is a globular protein with a deep central cleft (Armache, Kettenberger, and Cramer 2003; Bushnell and Kornberg 2003). The DNA template enters and travels along this cleft to the active site. On one side of the cleft is a flexible clamp structure, which can switch between an open or closed position. The Rpb4-Rpb7 subcomplex is located downstream of the catalytic site in the center of this cleft, and its binding to the 10-subunit core Pol II pushes the clamp to the closed position (Armache, Kettenberger, and Cramer 2003; Bushnell and Kornberg 2003).

RNA polymerases (Cramer 2002) and Spt4/Spt5 (Ponting 2002) are conserved in all three kingdoms of life: bacteria, archaea, and eukaryotes. The archaeal Spt4/Spt5 has recently been co-crystallized with the clamp domain of an archaeal polymerase (Martinez-Rucobo et al. 2011). Based on this co-crystal structure, a model of the complete yeast Pol II-Spt4/Spt5 elongation complex has been proposed. This model posits that the NGN domain of Spt5 binds to the clamp of Pol II and closes the central cleft to lock nucleic acids and render the elongation complex processive and stable. The KOW1 domain of Spt5 may contact DNA and/or exiting RNA, which could possibly contribute to stability of the elongating Pol II complex and may also involve the Rpb4/Rpb7 subcomplex. The locations of the other domains of Spt5, including the CTR, are currently unpredictable (Martinez-Rucobo et al. 2011). Spt4, which does not directly contact Pol II, binds to the other side of the Spt5 NGN domain and points away from the surface of Pol II. How Paf1C interacts with Pol II is currently unknown, but one point of contact between Paf1C and Pol II is thought to be an indirect one via the Rtf1 subunit of Paf1C and Spt5, an idea supported by the extensive interactions of Rtf1 and Spt5.

Structure-function analyses of Pol II elongation complexes containing a thymine-thymine CPD in the TS showed that the CPD slowly passes a translocation barrier and enters into the active site of Pol II. The 5′ thymine of the CPD directs misincorporation of uridine into the

elongating mRNA, which stalls the translocation of Pol II (Brueckner and Cramer 2007). All of the above findings regarding suppression of Rad26-independed repair suggest that Rpb4/Rpb7, Spt4/Spt5, and Paf1C act cooperatively and through the same pathway to exert this suppression effect. It is possible that when Rad26 is absent, a lesion becomes "locked" into the active site of a Pol II elongation complex, which is stabilized by the coordinated interactions of these suppressors with each other and with the core Pol II complex. Deletion or mutation of any of these suppressors may result in the destabilization of elongating Pol II, making it possible for TCR to occur (Tatum et al. 2011). How Rad26 affects the association of these factors with Pol II is unknown. A possible role for Rad26 in TCR may be to destabilize the Pol II elongation complex (Figure 1). This is supported by the evidence that indicates that Rad26 is dispensable for TCR in the absence of any of these suppression factors. This may explain why this 'megasuppressor' complex only suppresses TCR in the absence of Rad26.

As an interesting aside, it has been demonstrated that Rpb4 (Li and Smerdon 2002) and Paf1C (Tatum et al. 2011) have dual roles in TCR. Not only do they suppress Rad26-independent TCR, but they have also been shown to facilitate Rad26-dependent TCR to a certain extent. However, how each serves to facilitate this subpathway of TCR remains unknown. Rad26 has been shown to play a role in transcription elongation (Lee et al. 2001), leading to the possibility that Paf1C may play a positive role in TCR by cooperating with Rad26 to promote transcription elongation. The interaction of Rpb4 with other subunits of Pol II may change the conformation of the polymerase complex, and this may, in turn, improve the interactions with Rad26 (Figure 1) (Li and Smerdon 2002).

3. Concluding remarks and future direction

Although most, if not all, core NER factors have been identified and extensively characterized, new accessory factors which modulate GGR and/or TCR are continuously being identified. It is not only important to identify these factors, but also to uncover the role they play (i.e. facilitation or suppression), how they exact their functions, and the interactions they have with other repair proteins in order to gain a more holistic understanding of the repair process. Furthermore, current understanding of NER in living yeast cells is limited to either genome-overall-level or to certain very limited regions of the genome. The detailed DNA damage distribution and NER kinetics in the vast majority of the genomes are still virtually unknown. This illustrates the need for a high-resolution, genome-wide assessment of damage, repair, and repair kinetics. Only then will we be able to paint a complete picture and have full understanding of this repair mechanism that has thus far proven elusive.

In bacteria, most NER enzymes are induced by the SOS response after DNA damage (Janion 2008), but this does not seem to be the case in higher organisms. By and large, NER in eukaryotes seems to be modulated by posttranslational modifications and protein-protein interactions, not transcriptional induction of genes encoding repair factors (Nouspikel 2011). This seems intuitive, as DNA damage (such as CPDs) can impede transcription, making it a safer choice to rely on the activation of present enzymes rather than on their damage-induced synthesis. Many posttranslational modifications of histones, including ones not discussed here (as this review is not exhaustive), have been shown to have important functions in NER. These modifications operate in a concerted manner to coordinate a plethora of tasks such as damage signaling, opening/relaxing chromatin to allow repair

factors access to damage sites, activating the DNA damage cell cycle checkpoint, facilitating lesion identification, and restoring the chromatin to its original state once the repair process is complete. The discovery that H3K79 methylation is required for GGR (Tatum and Li 2011) unveiled a critical link between chromatin modification and the repair process. However, it remains to be understood as to whether the methylation indeed serves as a docking site for the NER machinery or the modification is indirectly involved in GGR.

Though progress has been made in recent years regarding chromatin dynamics in NER, many questions remain unanswered. Many studies attempting to elucidate the roles of histone modifications during NER have focused only on specific histone tail residues or single modifications, yet there may be many other modifications involved in the NER process (Palomera-Sanchez and Zurita 2011). While informative, this provides us with only a narrow glimpse into the cellular response to genomic insult and lacks the broader scope of examining the changes to histones throughout the entire genome in response to DNA damage. This underscores the need for a genome-wide analysis to monitor the responses of the DNA damage-induced histone modifications that occur in all of the chromosomes and how these different modifications crosstalk. Until then, continued efforts to decipher the encrypted code of these modifications will provide a much clearer understanding of the tightly regulated mechanisms of NER and its crosstalk with other processes such as DNA damage-induced checkpoint activation. These future findings could prove to be valuable clinically, as they may be advantageous targets for chemotherapeutics or treatment of other diseases related to genomic instability.

The TCR mechanism in eukaryotic cells remains largely mysterious. The interactions among Pol II, Rad26 and the various known and possibly unknown TCR suppressors remain to be elucidated and are the major key to unlocking this mystery. Determining the exact binding site of Rad26 on Pol II would provide valuable insight into the antagonistic effect of Rad26 on the suppression of Rad26-independent TCR. Furthermore, Rad26 does not seem to be a true transcription-repair-coupling factor and may facilitate TCR indirectly rather than by directly recruiting NER factors, as in the absence of a suppressor, Rad26 can be entirely dispensable. It is therefore likely that either Pol II itself is intrinsically proficient in mediating TCR or a true transcription-repair-coupling factor has not been discovered. These different possibilities remain to be addressed.

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5. References

Aboussekhra, A., M. Biggerstaff, M. K. Shivji, J. A. Vilpo, V. Moncollin, V. N. Podust, M. Protic, U. Hubscher, J. M. Egly, and R. D. Wood. 1995. Mammalian DNA nucleotide excision repair reconstituted with purified protein components. *Cell* 80 (6):859-68.

Altaf, M., N. Saksouk, and J. Cote. 2007. Histone modifications in response to DNA damage. *Mutat Res* 618 (1-2):81-90.

Anindya, R., O. Aygun, and J. Q. Svejstrup. 2007. Damage-induced ubiquitylation of human RNA polymerase II by the ubiquitin ligase Nedd4, but not Cockayne syndrome proteins or BRCA1. *Mol Cell* 28 (3):386-97.

Armache, K. J., H. Kettenberger, and P. Cramer. 2003. Architecture of initiation-competent 12-subunit RNA polymerase II. *Proc Natl Acad Sci U S A* 100 (12):6964-8.

Aso, T., W. S. Lane, J. W. Conaway, and R. C. Conaway. 1995. Elongin (SIII): a multisubunit regulator of elongation by RNA polymerase II. *Science* 269 (5229):1439-43.

Ataian, Y., and J. E. Krebs. 2006. Five repair pathways in one context: chromatin modification during DNA repair. *Biochem Cell Biol* 84 (4):490-504.

Bang, D. D., R. Verhage, N. Goosen, J. Brouwer, and P. van de Putte. 1992. Molecular cloning of RAD16, a gene involved in differential repair in Saccharomyces cerevisiae. *Nucleic Acids Res* 20 (15):3925-31.

Bergink, S., F. A. Salomons, D. Hoogstraten, T. A. Groothuis, H. de Waard, J. Wu, L. Yuan, E. Citterio, A. B. Houtsmuller, J. Neefjes, J. H. Hoeijmakers, W. Vermeulen, and N. P. Dantuma. 2006. DNA damage triggers nucleotide excision repair-dependent monoubiquitylation of histone H2A. *Genes Dev* 20 (10):1343-52.

Bhatia, P. K., R. A. Verhage, J. Brouwer, and E. C. Friedberg. 1996. Molecular cloning and characterization of Saccharomyces cerevisiae RAD28, the yeast homolog of the human Cockayne syndrome A (CSA) gene. *J Bacteriol* 178 (20):5977-88.

Bostelman, L. J., A. M. Keller, A. M. Albrecht, A. Arat, and J. S. Thompson. 2007. Methylation of histone H3 lysine-79 by Dot1p plays multiple roles in the response to UV damage in Saccharomyces cerevisiae. *DNA Repair (Amst)* 6 (3):383-95.

Bradsher, J. N., K. W. Jackson, R. C. Conaway, and J. W. Conaway. 1993. RNA polymerase II transcription factor SIII. I. Identification, purification, and properties. *J Biol Chem* 268 (34):25587-93.

Bregman, D. B., R. Halaban, A. J. van Gool, K. A. Henning, E. C. Friedberg, and S. L. Warren. 1996. UV-induced ubiquitination of RNA polymerase II: a novel modification deficient in Cockayne syndrome cells. *Proc Natl Acad Sci U S A* 93 (21):11586-90.

Brueckner, F., and P. Cramer. 2007. DNA photodamage recognition by RNA polymerase II. *FEBS Lett* 581 (15):2757-2760.

Bucheli, M., L. Lommel, and K. Sweder. 2001. The defect in transcription-coupled repair displayed by a Saccharomyces cerevisiae rad26 mutant is dependent on carbon source and is not associated with a lack of transcription. *Genetics* 158 (3):989-97.

Bushnell, D. A., and R. D. Kornberg. 2003. Complete, 12-subunit RNA polymerase II at 4.1-A resolution: implications for the initiation of transcription. *Proc Natl Acad Sci U S A* 100 (12):6969-73.

Chaudhuri, S., J. J. Wyrick, and M. J. Smerdon. 2009. Histone H3 Lys79 methylation is required for efficient nucleotide excision repair in a silenced locus of Saccharomyces cerevisiae. *Nucleic Acids Res* 37 (5):1690-700.

Chen, X., C. Ruggiero, and S. Li. 2007. Yeast Rpb9 plays an important role in ubiquitylation and degradation of Rpb1 in response to UV-induced DNA damage. *Mol Cell Biol* 27 (13):4617-25.

Cleaver, J. E., E. T. Lam, and I. Revet. 2009. Disorders of nucleotide excision repair: the genetic and molecular basis of heterogeneity. *Nat Rev Genet* 10 (11):756-68.

Coin, F., L. Proietti De Santis, T. Nardo, O. Zlobinskaya, M. Stefanini, and J. M. Egly. 2006. p8/TTD-A as a repair-specific TFIIH subunit. *Mol Cell* 21 (2):215-26.

Cramer, P. 2002. Multisubunit RNA polymerases. *Curr Opin Struct Biol* 12 (1):89-97.

Ding, B., D. LeJeune, and S. Li. 2010. The C-terminal repeat domain of Spt5 plays an important role in suppression of Rad26-independent transcription coupled repair. *J Biol Chem* 285 (8):5317-26.

Ding, B., C. Ruggiero, X. Chen, and S. Li. 2007. Tfb5 is partially dispensable for Rad26 mediated transcription coupled nucleotide excision repair in yeast. *DNA Repair (Amst)* 6 (11):1661-9.

Egly, J. M., and F. Coin. 2011. A history of TFIIH: Two decades of molecular biology on a pivotal transcription/repair factor. *DNA Repair (Amst)*.

Eisen, J. A., K. S. Sweder, and P. C. Hanawalt. 1995. Evolution of the SNF2 family of proteins: subfamilies with distinct sequences and functions. *Nucleic Acids Res* 23 (14):2715-23.

Evans, E., J. G. Moggs, J. R. Hwang, J. M. Egly, and R. D. Wood. 1997. Mechanism of open complex and dual incision formation by human nucleotide excision repair factors. *EMBO J* 16 (21):6559-73.

Fousteri, M., and L. H. Mullenders. 2008. Transcription-coupled nucleotide excision repair in mammalian cells: molecular mechanisms and biological effects. *Cell Res* 18 (1):73-84.

Fousteri, M., A. van Hoffen, H. Vargova, and L. H. Mullenders. 2005. Repair of DNA lesions in chromosomal DNA impact of chromatin structure and Cockayne syndrome proteins. *DNA Repair (Amst)* 4 (8):919-25.

Giannattasio, M., F. Lazzaro, P. Plevani, and M. Muzi-Falconi. 2005. The DNA damage checkpoint response requires histone H2B ubiquitination by Rad6-Bre1 and H3 methylation by Dot1. *J Biol Chem* 280 (11):9879-86.

Gillette, T. G., S. Yu, Z. Zhou, R. Waters, S. A. Johnston, and S. H. Reed. 2006. Distinct functions of the ubiquitin-proteasome pathway influence nucleotide excision repair. *EMBO J* 25 (11):2529-38.

Gong, F., D. Fahy, and M. J. Smerdon. 2006. Rad4-Rad23 interaction with SWI/SNF links ATP-dependent chromatin remodeling with nucleotide excision repair. *Nat Struct Mol Biol* 13 (10):902-7.

Gong, F., Y. Kwon, and M. J. Smerdon. 2005. Nucleotide excision repair in chromatin and the right of entry. *DNA Repair (Amst)* 4 (8):884-96.

Gregory, S. M., and K. S. Sweder. 2001. Deletion of the CSB homolog, RAD26, yields Spt(-) strains with proficient transcription-coupled repair. *Nucleic Acids Res* 29 (14):3080-6.

Guo, R., J. Chen, D. L. Mitchell, and D. G. Johnson. 2011. GCN5 and E2F1 stimulate nucleotide excision repair by promoting H3K9 acetylation at sites of damage. *Nucleic Acids Res* 39 (4):1390-7.

Guzder, S. N., Y. Habraken, P. Sung, L. Prakash, and S. Prakash. 1995. Reconstitution of yeast nucleotide excision repair with purified Rad proteins, replication protein A, and transcription factor TFIIH. *J Biol Chem* 270 (22):12973-6.

Guzder, S. N., Y. Habraken, P. Sung, L. Prakash, and S. Prakash. 1996. RAD26, the yeast homolog of human Cockayne's syndrome group B gene, encodes a DNA-dependent ATPase. *J Biol Chem* 271 (31):18314-7.

Guzder, S. N., C. H. Sommers, L. Prakash, and S. Prakash. 2006. Complex formation with damage recognition protein Rad14 is essential for Saccharomyces cerevisiae Rad1-

Rad10 nuclease to perform its function in nucleotide excision repair in vivo. *Mol Cell Biol* 26 (3):1135-41.

Guzder, S. N., P. Sung, L. Prakash, and S. Prakash. 1997. Yeast Rad7-Rad16 complex, specific for the nucleotide excision repair of the nontranscribed DNA strand, is an ATP-dependent DNA damage sensor. *J Biol Chem* 272 (35):21665-8.

Guzder, S. N., P. Sung, L. Prakash, and S. Prakash. 1998. Affinity of yeast nucleotide excision repair factor 2, consisting of the Rad4 and Rad23 proteins, for ultraviolet damaged DNA. *J Biol Chem* 273 (47):31541-6.

Guzder, S. N., P. Sung, L. Prakash, and S. Prakash. 1998. The DNA-dependent ATPase activity of yeast nucleotide excision repair factor 4 and its role in DNA damage recognition. *J Biol Chem* 273 (11):6292-6.

Habraken, Y., P. Sung, L. Prakash, and S. Prakash. 1993. Yeast excision repair gene RAD2 encodes a single-stranded DNA endonuclease. *Nature* 366 (6453):365-8.

Hanawalt, P. C. 1994. Transcription-coupled repair and human disease. *Science* 266 (5193):1957-8.

Hanawalt, P. C., and G. Spivak. 2008. Transcription-coupled DNA repair: two decades of progress and surprises. *Nat Rev Mol Cell Biol* 9 (12):958-70.

Harper, J. W., and S. J. Elledge. 2007. The DNA damage response: ten years after. *Mol Cell* 28 (5):739-45.

Hartzog, G. A., T. Wada, H. Handa, and F. Winston. 1998. Evidence that Spt4, Spt5, and Spt6 control transcription elongation by RNA polymerase II in Saccharomyces cerevisiae. *Genes Dev* 12 (3):357-69.

Hoeijmakers, J. H. 2009. DNA damage, aging, and cancer. *N Engl J Med* 361 (15):1475-85.

Huang, J. C., and A. Sancar. 1994. Determination of minimum substrate size for human excinuclease. *J Biol Chem* 269 (29):19034-40.

Huang, W., W. J. Feaver, A. E. Tomkinson, and E. C. Friedberg. 1998. The N-degron protein degradation strategy for investigating the function of essential genes: requirement for replication protein A and proliferating cell nuclear antigen proteins for nucleotide excision repair in yeast extracts. *Mutat Res* 408 (3):183-94.

Imai, S., C. M. Armstrong, M. Kaeberlein, and L. Guarente. 2000. Transcriptional silencing and longevity protein Sir2 is an NAD-dependent histone deacetylase. *Nature* 403 (6771):795-800.

Irizar, A., Y. Yu, S. H. Reed, E. J. Louis, and R. Waters. 2010. Silenced yeast chromatin is maintained by Sir2 in preference to permitting histone acetylations for efficient NER. *Nucleic Acids Res* 38 (14):4675-86.

Jaehning, J. A. 2010. The Paf1 complex: platform or player in RNA polymerase II transcription? *Biochim Biophys Acta* 1799 (5-6):379-88.

Janion, C. 2008. Inducible SOS response system of DNA repair and mutagenesis in Escherichia coli. *Int J Biol Sci* 4 (6):338-44.

Jansen, L. E., H. den Dulk, R. M. Brouns, M. de Ruijter, J. A. Brandsma, and J. Brouwer. 2000. Spt4 modulates Rad26 requirement in transcription-coupled nucleotide excision repair. *EMBO J* 19 (23):6498-507.

Jensen, A., and L. H. Mullenders. 2010. Transcription factor IIS impacts UV-inhibited transcription. *DNA Repair (Amst)* 9 (11):1142-50.

Jiang, Y., X. Wang, S. Bao, R. Guo, D. G. Johnson, X. Shen, and L. Li. 2010. INO80 chromatin remodeling complex promotes the removal of UV lesions by the nucleotide excision repair pathway. *Proc Natl Acad Sci U S A* 107 (40):17274-9.

Kapetanaki, M. G., J. Guerrero-Santoro, D. C. Bisi, C. L. Hsieh, V. Rapic-Otrin, and A. S. Levine. 2006. The DDB1-CUL4ADDB2 ubiquitin ligase is deficient in xeroderma pigmentosum group E and targets histone H2A at UV-damaged DNA sites. *Proc Natl Acad Sci U S A* 103 (8):2588-93.

Kettenberger, H., K. J. Armache, and P. Cramer. 2003. Architecture of the RNA polymerase II-TFIIS complex and implications for mRNA cleavage. *Cell* 114 (3):347-57.

Kou, H., Y. Zhou, R. M. Gorospe, and Z. Wang. 2008. Mms19 protein functions in nucleotide excision repair by sustaining an adequate cellular concentration of the TFIIH component Rad3. *Proc Natl Acad Sci U S A* 105 (41):15714-9.

Kouzarides, T. 2007. Chromatin modifications and their function. *Cell* 128 (4):693-705.

Krogan, N. J., J. Dover, A. Wood, J. Schneider, J. Heidt, M. A. Boateng, K. Dean, O. W. Ryan, A. Golshani, M. Johnston, J. F. Greenblatt, and A. Shilatifard. 2003. The Paf1 complex is required for histone H3 methylation by COMPASS and Dot1p: linking transcriptional elongation to histone methylation. *Mol Cell* 11 (3):721-9.

Landry, J., A. Sutton, S. T. Tafrov, R. C. Heller, J. Stebbins, L. Pillus, and R. Sternglanz. 2000. The silencing protein SIR2 and its homologs are NAD-dependent protein deacetylases. *Proc Natl Acad Sci U S A* 97 (11):5807-11.

Lee, K. B., D. Wang, S. J. Lippard, and P. A. Sharp. 2002. Transcription-coupled and DNA damage-dependent ubiquitination of RNA polymerase II in vitro. *Proc Natl Acad Sci U S A* 99 (7):4239-44.

Lee, S. K., S. L. Yu, L. Prakash, and S. Prakash. 2001. Requirement for yeast RAD26, a homolog of the human CSB gene, in elongation by RNA polymerase II. *Mol Cell Biol* 21 (24):8651-6.

Legerski, R., and C. Peterson. 1992. Expression cloning of a human DNA repair gene involved in xeroderma pigmentosum group C. *Nature* 359 (6390):70-3.

Lejeune, D., X. Chen, C. Ruggiero, S. Berryhill, B. Ding, and S. Li. 2009. Yeast Elc1 plays an important role in global genomic repair but not in transcription coupled repair. *DNA Repair (Amst)* 8 (1):40-50.

Levesque, N., G. P. Leung, A. K. Fok, T. I. Schmidt, and M. S. Kobor. 2010. Loss of H3 K79 trimethylation leads to suppression of Rtt107-dependent DNA damage sensitivity through the translesion synthesis pathway. *J Biol Chem* 285 (45):35113-22.

Li, S., X. Chen, C. Ruggiero, B. Ding, and M. J. Smerdon. 2006. Modulation of Rad26- and Rpb9-mediated DNA repair by different promoter elements. *J Biol Chem* 281 (48):36643-51.

Li, S., B. Ding, D. Lejeune, C. Ruggiero, X. Chen, and M. J. Smerdon. 2007. The roles of Rad16 and Rad26 in repairing repressed and actively transcribed genes in yeast. *DNA Repair (Amst)* 6 (11):1596-606.

Li, S., and M. J. Smerdon. 2002. Rpb4 and Rpb9 mediate subpathways of transcription-coupled DNA repair in Saccharomyces cerevisiae. *EMBO J* 21 (21):5921-9.

Li, S., and M. J. Smerdon. 2004. Dissecting transcription-coupled and global genomic repair in the chromatin of yeast GAL1-10 genes. *J Biol Chem* 279 (14):14418-26.

Lindahl, T., and R. D. Wood. 1999. Quality control by DNA repair. *Science* 286 (5446):1897-905.

Liu, Y., L. Warfield, C. Zhang, J. Luo, J. Allen, W. H. Lang, J. Ranish, K. M. Shokat, and S. Hahn. 2009. Phosphorylation of the transcription elongation factor Spt5 by yeast Bur1 kinase stimulates recruitment of the PAF complex. *Mol Cell Biol* 29 (17):4852-63.

Lommel, L., M. E. Bucheli, and K. S. Sweder. 2000. Transcription-coupled repair in yeast is independent from ubiquitylation of RNA pol II: implications for Cockayne's syndrome. *Proc Natl Acad Sci U S A* 97 (16):9088-92.

Luger, K., A. W. Mader, R. K. Richmond, D. F. Sargent, and T. J. Richmond. 1997. Crystal structure of the nucleosome core particle at 2.8 A resolution. *Nature* 389 (6648):251-60.

Malone, E. A., J. S. Fassler, and F. Winston. 1993. Molecular and genetic characterization of SPT4, a gene important for transcription initiation in Saccharomyces cerevisiae. *Mol Gen Genet* 237 (3):449-59.

Martens, J. A., and F. Winston. 2003. Recent advances in understanding chromatin remodeling by Swi/Snf complexes. *Curr Opin Genet Dev* 13 (2):136-42.

Martinez-Rucobo, F. W., S. Sainsbury, A. C. Cheung, and P. Cramer. 2011. Architecture of the RNA polymerase-Spt4/5 complex and basis of universal transcription processivity. *EMBO J* 30 (7):1302-10.

Mellon, I., and P. C. Hanawalt. 1989. Induction of the Escherichia coli lactose operon selectively increases repair of its transcribed DNA strand. *Nature* 342 (6245): 95-8.

Mellon, I., G. Spivak, and P. C. Hanawalt. 1987. Selective removal of transcription-blocking DNA damage from the transcribed strand of the mammalian DHFR gene. *Cell* 51 (2):241-9.

Mone, M. J., T. Bernas, C. Dinant, F. A. Goedvree, E. M. Manders, M. Volker, A. B. Houtsmuller, J. H. Hoeijmakers, W. Vermeulen, and R. van Driel. 2004. In vivo dynamics of chromatin-associated complex formation in mammalian nucleotide excision repair. *Proc Natl Acad Sci U S A* 101 (45):15933-7.

Nag, R., and M. J. Smerdon. 2009. Altering the chromatin landscape for nucleotide excision repair. *Mutat Res* 682 (1):13-20.

Nakanishi, S., J. S. Lee, K. E. Gardner, J. M. Gardner, Y. H. Takahashi, M. B. Chandrasekharan, Z. W. Sun, M. A. Osley, B. D. Strahl, S. L. Jaspersen, and A. Shilatifard. 2009. Histone H2BK123 monoubiquitination is the critical determinant for H3K4 and H3K79 trimethylation by COMPASS and Dot1. *J Cell Biol* 186 (3):371-7.

Newman, J. C., A. D. Bailey, and A. M. Weiner. 2006. Cockayne syndrome group B protein (CSB) plays a general role in chromatin maintenance and remodeling. *Proc Natl Acad Sci U S A* 103 (25):9613-8.

Ng, H. H., D. N. Ciccone, K. B. Morshead, M. A. Oettinger, and K. Struhl. 2003. Lysine-79 of histone H3 is hypomethylated at silenced loci in yeast and mammalian cells: a potential mechanism for position-effect variegation. *Proc Natl Acad Sci U S A* 100 (4):1820-5.

Ng, H. H., S. Dole, and K. Struhl. 2003. The Rtf1 component of the Paf1 transcriptional elongation complex is required for ubiquitination of histone H2B. *J Biol Chem* 278 (36):33625-8.

Nouspikel, T. 2009. DNA repair in mammalian cells : Nucleotide excision repair: variations on versatility. *Cell Mol Life Sci* 66 (6):994-1009.

Nouspikel, T. 2011. Multiple roles of ubiquitination in the control of nucleotide excision repair. *Mech Ageing Dev*.

Osley, M. A., T. Tsukuda, and J. A. Nickoloff. 2007. ATP-dependent chromatin remodeling factors and DNA damage repair. *Mutat Res* 618 (1-2):65-80.

Palomera-Sanchez, Z., and M. Zurita. 2011. Open, repair and close again: chromatin dynamics and the response to UV-induced DNA damage. *DNA Repair (Amst)* 10 (2):119-25.

Park, J. S., M. T. Marr, and J. W. Roberts. 2002. E. coli Transcription repair coupling factor (Mfd protein) rescues arrested complexes by promoting forward translocation. *Cell* 109 (6):757-67.

Pettijohn, D., and P. Hanawalt. 1964. Evidence for Repair-Replication of Ultraviolet Damaged DNA in Bacteria. *J Mol Biol* 9:395-410.

Pokholok, D. K., C. T. Harbison, S. Levine, M. Cole, N. M. Hannett, T. I. Lee, G. W. Bell, K. Walker, P. A. Rolfe, E. Herbolsheimer, J. Zeitlinger, F. Lewitter, D. K. Gifford, and R. A. Young. 2005. Genome-wide map of nucleosome acetylation and methylation in yeast. *Cell* 122 (4):517-27.

Ponting, C. P. 2002. Novel domains and orthologues of eukaryotic transcription elongation factors. *Nucleic Acids Res* 30 (17):3643-52.

Prakash, S., and L. Prakash. 2000. Nucleotide excision repair in yeast. *Mutat Res* 451 (1-2):13-24.

Qiu, H., C. Hu, C. M. Wong, and A. G. Hinnebusch. 2006. The Spt4p subunit of yeast DSIF stimulates association of the Paf1 complex with elongating RNA polymerase II. *Mol Cell Biol* 26 (8):3135-48.

Ramanathan, B., and M. J. Smerdon. 1989. Enhanced DNA repair synthesis in hyperacetylated nucleosomes. *J Biol Chem* 264 (19):11026-34.

Ramsey, K. L., J. J. Smith, A. Dasgupta, N. Maqani, P. Grant, and D. T. Auble. 2004. The NEF4 complex regulates Rad4 levels and utilizes Snf2/Swi2-related ATPase activity for nucleotide excision repair. *Mol Cell Biol* 24 (14):6362-78.

Ranish, J. A., S. Hahn, Y. Lu, E. C. Yi, X. J. Li, J. Eng, and R. Aebersold. 2004. Identification of TFB5, a new component of general transcription and DNA repair factor IIH. *Nat Genet* 36 (7):707-13.

Rasmussen, R. E., and R. B. Painter. 1964. Evidence for Repair of Ultra-Violet Damaged Deoxyribonucleic Acid in Cultured Mammalian Cells. *Nature* 203:1360-2.

Ratner, J. N., B. Balasubramanian, J. Corden, S. L. Warren, and D. B. Bregman. 1998. Ultraviolet radiation-induced ubiquitination and proteasomal degradation of the large subunit of RNA polymerase II. Implications for transcription-coupled DNA repair. *J Biol Chem* 273 (9):5184-9.

Reed, S. H. 2005. Nucleotide excision repair in chromatin: the shape of things to come. *DNA Repair (Amst)* 4 (8):909-18.

Ribar, B., L. Prakash, and S. Prakash. 2006. Requirement of ELC1 for RNA polymerase II polyubiquitylation and degradation in response to DNA damage in Saccharomyces cerevisiae. *Mol Cell Biol* 26 (11):3999-4005.

Ribar, B., L. Prakash, and S. Prakash. 2007. ELA1 and CUL3 are required along with ELC1 for RNA polymerase II polyubiquitylation and degradation in DNA-damaged yeast cells. *Mol Cell Biol* 27 (8):3211-6.

Saeki, H., and J. Q. Svejstrup. 2009. Stability, flexibility, and dynamic interactions of colliding RNA polymerase II elongation complexes. *Mol Cell* 35 (2):191-205.

Sarkar, S., R. Kiely, and P. J. McHugh. 2010. The Ino80 chromatin-remodeling complex restores chromatin structure during UV DNA damage repair. *J Cell Biol* 191 (6):1061-8.

Sarker, A. H., S. E. Tsutakawa, S. Kostek, C. Ng, D. S. Shin, M. Peris, E. Campeau, J. A. Tainer, E. Nogales, and P. K. Cooper. 2005. Recognition of RNA polymerase II and transcription bubbles by XPG, CSB, and TFIIH: insights for transcription-coupled repair and Cockayne Syndrome. *Mol Cell* 20 (2):187-98.

Schulze, J. M., J. Jackson, S. Nakanishi, J. M. Gardner, T. Hentrich, J. Haug, M. Johnston, S. L. Jaspersen, M. S. Kobor, and A. Shilatifard. 2009. Linking cell cycle to histone modifications: SBF and H2B monoubiquitination machinery and cell-cycle regulation of H3K79 dimethylation. *Mol Cell* 35 (5):626-41.

Selby, C. P., and A. Sancar. 1994. Mechanisms of transcription-repair coupling and mutation frequency decline. *Microbiol Rev* 58 (3):317-29.

Selby, C. P., and A. Sancar. 1997. Human transcription-repair coupling factor CSB/ERCC6 is a DNA-stimulated ATPase but is not a helicase and does not disrupt the ternary transcription complex of stalled RNA polymerase II. *J Biol Chem* 272 (3):1885-90.

Shahbazian, M. D., K. Zhang, and M. Grunstein. 2005. Histone H2B ubiquitylation controls processive methylation but not monomethylation by Dot1 and Set1. *Mol Cell* 19 (2):271-7.

Smerdon, M. J., S. Y. Lan, R. E. Calza, and R. Reeves. 1982. Sodium butyrate stimulates DNA repair in UV-irradiated normal and xeroderma pigmentosum human fibroblasts. *J Biol Chem* 257 (22):13441-7.

Smerdon, M. J., and F. Thoma. 1990. Site-specific DNA repair at the nucleosome level in a yeast minichromosome. *Cell* 61 (4):675-84.

Squazzo, S. L., P. J. Costa, D. L. Lindstrom, K. E. Kumer, R. Simic, J. L. Jennings, A. J. Link, K. M. Arndt, and G. A. Hartzog. 2002. The Paf1 complex physically and functionally associates with transcription elongation factors in vivo. *EMBO J* 21 (7):1764-74.

Struhl, K. 2007. Transcriptional noise and the fidelity of initiation by RNA polymerase II. *Nat Struct Mol Biol* 14 (2):103-5.

Sung, P., P. Reynolds, L. Prakash, and S. Prakash. 1993. Purification and characterization of the Saccharomyces cerevisiae RAD1/RAD10 endonuclease. *J Biol Chem* 268 (35):26391-9.

Svejstrup, J. Q. 2002. Mechanisms of transcription-coupled DNA repair. *Nat Rev Mol Cell Biol* 3 (1):21-9.

Swanson, M. S., E. A. Malone, and F. Winston. 1991. SPT5, an essential gene important for normal transcription in Saccharomyces cerevisiae, encodes an acidic nuclear protein with a carboxy-terminal repeat. *Mol Cell Biol* 11 (6):3009-19.

Tatum, D., and S. Li. 2011. Evidence that the histone methyltransferase dot1 mediates global genomic repair by methylating histone h3 on lysine 79. *J Biol Chem* 286 (20):17530-5.

Tatum, D., Li, W., M. Placer, and S. Li. 2011. The roles of RNA polymerase II-associated factor 1 complex in different subpathways of nucleotide excision repair. *J Biol Chem.* in press.

Teng, Y., H. Liu, H. W. Gill, Y. Yu, R. Waters, and S. H. Reed. 2008. Saccharomyces cerevisiae Rad16 mediates ultraviolet-dependent histone H3 acetylation required for efficient global genome nucleotide-excision repair. *EMBO Rep* 9 (1):97-102.

Thoma, F. 1999. Light and dark in chromatin repair: repair of UV-induced DNA lesions by photolyase and nucleotide excision repair. *EMBO J* 18 (23):6585-98.

Tijsterman, M., R. A. Verhage, P. van de Putte, J. G. Tasseron-de Jong, and J. Brouwer. 1997. Transitions in the coupling of transcription and nucleotide excision repair within RNA polymerase II-transcribed genes of Saccharomyces cerevisiae. *Proc Natl Acad Sci U S A* 94 (15):8027-32.

Tomkinson, A. E., A. J. Bardwell, N. Tappe, W. Ramos, and E. C. Friedberg. 1994. Purification of Rad1 protein from Saccharomyces cerevisiae and further characterization of the Rad1/Rad10 endonuclease complex. *Biochemistry* 33 (17):5305-11.

van Gool, A. J., R. Verhage, S. M. Swagemakers, P. van de Putte, J. Brouwer, C. Troelstra, D. Bootsma, and J. H. Hoeijmakers. 1994. RAD26, the functional S. cerevisiae homolog of the Cockayne syndrome B gene ERCC6. *EMBO J* 13 (22):5361-9.

van Leeuwen, F., P. R. Gafken, and D. E. Gottschling. 2002. Dot1p modulates silencing in yeast by methylation of the nucleosome core. *Cell* 109 (6):745-56.

Verhage, R. A., J. Heyn, P. van de Putte, and J. Brouwer. 1997. Transcription elongation factor S-II is not required for transcription-coupled repair in yeast. *Mol Gen Genet* 254 (3):284-90.

Verhage, R. A., A. J. van Gool, N. de Groot, J. H. Hoeijmakers, P. van de Putte, and J. Brouwer. 1996. Double mutants of Saccharomyces cerevisiae with alterations in global genome and transcription-coupled repair. *Mol Cell Biol* 16 (2):496-502.

Verhage, R., A. M. Zeeman, N. de Groot, F. Gleig, D. D. Bang, P. van de Putte, and J. Brouwer. 1994. The RAD7 and RAD16 genes, which are essential for pyrimidine dimer removal from the silent mating type loci, are also required for repair of the nontranscribed strand of an active gene in Saccharomyces cerevisiae. *Mol Cell Biol* 14 (9):6135-42.

Vermeulen, W., E. Bergmann, J. Auriol, S. Rademakers, P. Frit, E. Appeldoorn, J. H. Hoeijmakers, and J. M. Egly. 2000. Sublimiting concentration of TFIIH transcription/DNA repair factor causes TTD-A trichothiodystrophy disorder. *Nat Genet* 26 (3):307-13.

Wang, H., L. Zhai, J. Xu, H. Y. Joo, S. Jackson, H. Erdjument-Bromage, P. Tempst, Y. Xiong, and Y. Zhang. 2006. Histone H3 and H4 ubiquitylation by the CUL4-DDB-ROC1 ubiquitin ligase facilitates cellular response to DNA damage. *Mol Cell* 22 (3):383-94.

Waters, R., S. H. Reed, Y. Yu, and Y. Teng. 2008. Chromatin modifications and nucleotide excision repair. *SEB Exp Biol Ser* 59:189-201.

Waters, R., Y. Teng, Y. Yu, S. Yu, and S. H. Reed. 2009. Tilting at windmills? The nucleotide excision repair of chromosomal DNA. *DNA Repair (Amst)* 8 (2):146-52.

Winston, F. 2001. Control of eukaryotic transcription elongation. *Genome Biol* 2 (2):REVIEWS1006.

Wolffe, A. P. 1999. *Chromatin: Structure and Function*. 3rd ed. London and New York: Academic Press.

Wood, A., N. J. Krogan, J. Dover, J. Schneider, J. Heidt, M. A. Boateng, K. Dean, A. Golshani, Y. Zhang, J. F. Greenblatt, M. Johnston, and A. Shilatifard. 2003. Bre1, an E3 ubiquitin ligase required for recruitment and substrate selection of Rad6 at a promoter. *Mol Cell* 11 (1):267-74.

Woudstra, E. C., C. Gilbert, J. Fellows, L. Jansen, J. Brouwer, H. Erdjument-Bromage, P. Tempst, and J. Q. Svejstrup. 2002. A Rad26-Def1 complex coordinates repair and RNA pol II proteolysis in response to DNA damage. *Nature* 415 (6874):929-33.

Woychik, N. A., and R. A. Young. 1989. RNA polymerase II subunit RPB4 is essential for high- and low-temperature yeast cell growth. *Mol Cell Biol* 9 (7):2854-9.

Wu, X., E. Braithwaite, and Z. Wang. 1999. DNA ligation during excision repair in yeast cell-free extracts is specifically catalyzed by the CDC9 gene product. *Biochemistry* 38 (9):2628-35.

Wu, X., D. Guo, F. Yuan, and Z. Wang. 2001. Accessibility of DNA polymerases to repair synthesis during nucleotide excision repair in yeast cell-free extracts. *Nucleic Acids Res* 29 (14):3123-30.

Yao, S., A. Neiman, and G. Prelich. 2000. BUR1 and BUR2 encode a divergent cyclin-dependent kinase-cyclin complex important for transcription in vivo. *Mol Cell Biol* 20 (19):7080-7.

Yu, S., T. Owen-Hughes, E. C. Friedberg, R. Waters, and S. H. Reed. 2004. The yeast Rad7/Rad16/Abf1 complex generates superhelical torsion in DNA that is required for nucleotide excision repair. *DNA Repair (Amst)* 3 (3):277-87.

Yu, Y., Y. Teng, H. Liu, S. H. Reed, and R. Waters. 2005. UV irradiation stimulates histone acetylation and chromatin remodeling at a repressed yeast locus. *Proc Natl Acad Sci U S A* 102 (24):8650-5.

Zhang, L., K. Jones, and F. Gong. 2009. The molecular basis of chromatin dynamics during nucleotide excision repair. *Biochem Cell Biol* 87 (1):265-72.

Zhou, K., W. H. Kuo, J. Fillingham, and J. F. Greenblatt. 2009. Control of transcriptional elongation and cotranscriptional histone modification by the yeast BUR kinase substrate Spt5. *Proc Natl Acad Sci U S A* 106 (17):6956-61.

Zhou, W., P. Zhu, J. Wang, G. Pascual, K. A. Ohgi, J. Lozach, C. K. Glass, and M. G. Rosenfeld. 2008. Histone H2A monoubiquitination represses transcription by inhibiting RNA polymerase II transcriptional elongation. *Mol Cell* 29 (1):69-80.

Zhou, Y., H. Kou, and Z. Wang. 2007. Tfb5 interacts with Tfb2 and facilitates nucleotide excision repair in yeast. *Nucleic Acids Res* 35 (3):861-71.

7

Biochemical Properties of MutL, a DNA Mismatch Repair Endonuclease

Kenji Fukui[1], Atsuhiro Shimada[2], Hitoshi Iino[1],
Ryoji Masui[1,2] and Seiki Kuramitsu[1,2]
[1]*RIKEN SPring-8 Center and*
[2]*Graduate School of Science,*
Osaka University
Japan

1. Introduction

DNA mismatch repair (MMR) is one of the most widely conserved DNA repair systems, which repairs mismatched bases generated mainly by the error of DNA polymerases during replication (Friedberg, et al., 2006, Iyer, et al., 2006, Kunkel, et al., 2005, Morita, et al., 2010). MMR increases the replication fidelity by 20 to 400-fold (Schaaper, 1993). Mutations and epigenetic silencing in MMR genes cause human hereditary nonpolyposis colon cancers as well as sporadic tumors (Fishel, et al., 1995, Fishel, et al., 1994, Kane, et al., 1997, Leach, et al., 1993, Modrich, et al., 1996, Suter, et al., 2004), indicating the significance of this repair system.

To date, two types of MMR mechanisms have been clarified: one is employed by eukaryotes and most bacteria (Fig. 1A and B) (Modrich, 2006) and the other is specific to *Escherichia coli* and other γ-proteobacteria (Fig. 1C) (Modrich, et al., 1996). The fundamental mechanism and the required proteins in the two types of MMRs are relatively similar to each other. A mismatch is recognized by the bacterial MutS homodimer, eukaryotic MutSα (MSH2-MSH6 heterodimer), or MutSβ (MSH2-MSH3 heterodimer) (Acharya, et al., 2003, Drotschmann, et al., 2002, Gradia, et al., 1997, Gradia, et al., 1999, Lamers, et al., 2000, McCulloch, et al., 2003, Obmolova, et al., 2000, Tachiki, et al., 2000). Subsequently, the bacterial MutL homodimer or eukaryotic MutLα (MLH1-PMS2 and MLH1-PMS1 heterodimers in humans and yeast, respectively) is recruited to the mismatched DNA to stimulate downstream events (Acharya, et al., 2003, Kadyrov, et al., 2006). The largest difference between the two types of MMR mechanisms is in the "strand discrimination" system. Although both bases constituting the mismatch are canonical, MMR needs to identify which base is to be repaired. In eukaryotes and most bacteria, MMR directs the repair to the error-containing strand of the mismatched duplex by recognizing the strand discontinuities in the newly synthesized strand (Kadyrov, et al., 2006, Kadyrov, et al., 2007, Larrea, et al., 2010, Modrich, 2006). The termini of leading and lagging strands are thought to serve as discrimination signals. On the other hand, *E. coli* MMR reads the absence of methylation at the restriction site in the newly synthesized strand (Iyer, et al., 2006, Kunkel, et al., 2005, Larrea, et al., 2010). Before the site-specific DNA methylase (e.g., *E. coli* Dam methylase (Schlagman, et

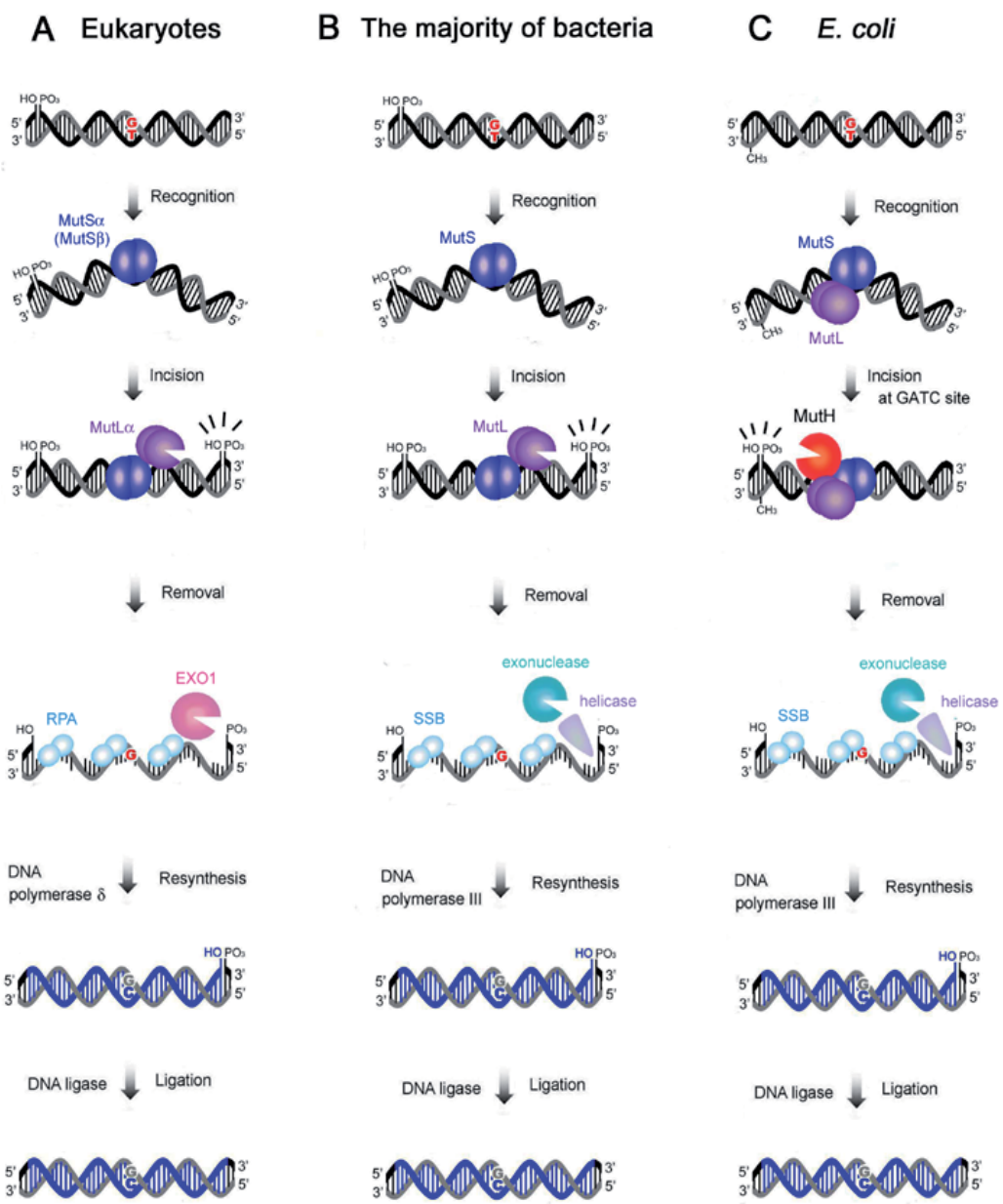

Fig. 1. A schematic representation of MMR pathway models. (A) Eukaryotic MMR. A mismatch is recognized by MutSα, and MutLα nicks the 3'- or 5'-side of the mismatched base on the discontinuous strand. The effective incision by MutLα requires MutSα, replication factor C, proliferating cell nuclear antigen (PCNA), and ATP. The resulting DNA segment is excised by a 5'-3' exonuclease, EXO1, in cooperation with a single-stranded DNA-binding protein, replication protein A (RPA). The DNA strand is resynthesized by DNA polymerase δ and DNA ligase 1. No DNA helicase has been identified to participate in eukaryotic MMR. This mechanism is elucidated by using an *in vitro* reconstituted system.

The pre-existing strand discontinuity can be located on both 5'- and 3'-sides of the mismatch; therefore, there should be 5'- and 3'-nick directed MMR mechanisms. The detail has been described elsewhere (Constantin, et al., 2005, Dzantiev, et al., 2004, Fukui, 2010, Genschel, et al., 2002, Kadyrov, et al., 2009). (B) A speculative model for MMR in *mutH*-less bacteria. The mismatch is recognized by a MutS homodimer. After incision of the discontinuous strand by MutL, the error-containing DNA strand is removed by the cooperative functions of DNA helicases, such as UvrD, the exonucleases RecJ and ExoI, and the single-stranded DNA-binding protein (SSB). DNA polymerase III and DNA ligase fill the gap to complete the repair. Although, no studies have reported the *in vitro* reconstituted system of bacterial nick-directed MMR, it has been elucidated that the endonuclease activity of MutL is required for *in vivo* MMR activity (Fukui, et al., 2008). The involvement of RecJ and ExoI in this MMR system has been implicated experimentally (Shimada et al., 2010). (C) *E. coli* MMR. MutS recognizes the mismatch, and MutL interacts with MutS. Subsequently, the MutH endonuclease is activated to incise the unmethylated strand at the GATC site to create an entry point for the excision reaction. DNA helicase, SSB, and several exonucleases are involved in the excision reaction. At least three models have been proposed for the mechanism by which a MutS homologue stimulates downstream events. They are the "Molecular switch", "Stationary", and "Translocation" models. The major difference between these models is whether a MutS homologue dissociates from the mismatch after recognizing it. Details have been provided in other publications (Kunkel, et al., 2005, Li, 2008).

al., 1986)) completes the modification of the newly synthesized strand, hemi-methylated sites exist and serve as strand-discrimination signals. In both MMR systems, a nicking endonuclease plays a central role in the strand discrimination mechanism. In eukaryotes and most bacteria, MutL homologues are thought to incise the discontinuous strand to introduce the entry or termination point of the excision reaction. In *E. coli*, MutH nicks the unmethylated strand of the duplex to generate the excision entry point. After incision by MutL homologues or MutH, the error-containing strand is removed by helicases and exonucleases. DNA polymerases then resynthesize the strand, and DNA ligases seal the nick to complete the repair reaction.

Although *in vivo* MMR achieves mismatch- and daughter strand-specific incision, eukaryotic MutLα and bacterial MutL show an apparently non-specific endonuclease activity against lesion-less DNA *in vitro* (Duppatla, et al., 2009, Fukui, et al., 2008, Kadyrov, et al., 2006, Kadyrov, et al., 2007, Mauris, et al., 2009), indicating that MMR requires a sequence- or structure-non-specific endonuclease activity to introduce an excision entry point wherever it is needed. The regulatory mechanism underlying this apparently non-specific endonuclease activity has been argued (Yang, 2007). Both eukaryotic and bacterial MutL consist of N-terminal ATPase and C-terminal dimerization (also endonuclease) domains (Fig. 2) (Fukui, et al., 2008, Kadyrov, et al., 2006). The two domains are usually separated by a long, flexible linker region. This domain organization is characteristic of the GHKL ATPase/kinase superfamily that undergoes a large conformational change upon ATP binding and/or hydrolysis (Ban, et al., 1999, Dutta, et al., 2000). Generally, ATP binding and/or hydrolysis control the molecular functions of these superfamily proteins. ATP binding- and/or hydrolysis-induced conformational change is expected to be involved in the regulation of MutL endonuclease activity. Recent biochemical characterizations have demonstrated the effects of ATP binding and hydrolysis on the function of MutL (Duppatla, et al., 2009, Fukui,

et al., 2008, Kim, et al., 2009, Mauris, et al., 2009, Sacho, et al., 2008). In addition to ensuring mismatch-specific incision, cells also need to direct the MutL-dependent nicking reaction to the newly synthesized strand of the mismatched duplex. Interactions of MutL with other MMR proteins have been reported to participate in this regulatory mechanism. In this chapter, we review the biochemical properties of MutL endonucleases that are related to those regulatory mechanisms.

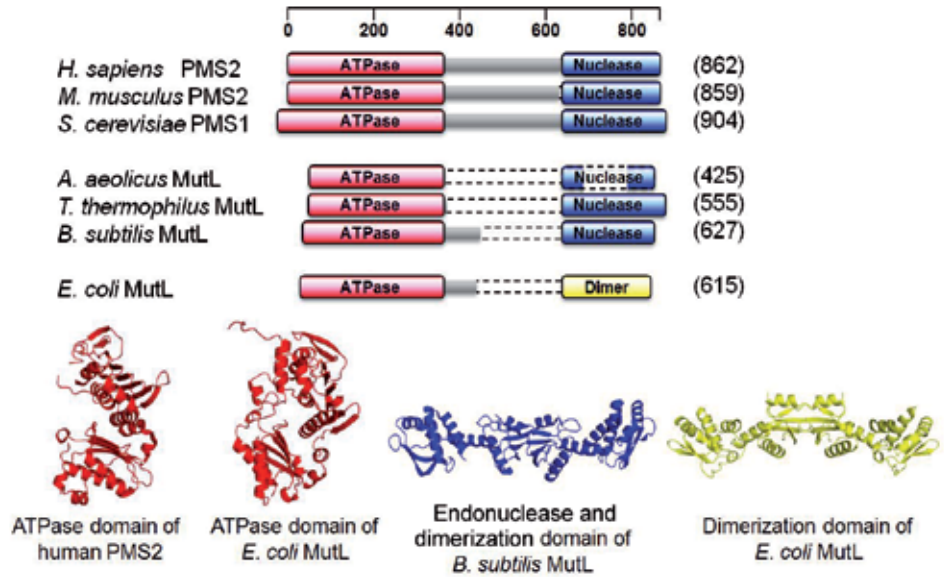

Fig. 2. A schematic representation of the domain structure of MutL homologues. ATPase, endonuclease, and dimerization domains are represented by *red*, *blue*, and *yellow* boxes, respectively. Numbers in parentheses indicate the length of each protein. The interdomain linker regions are shown as *gray bars*. The crystal structures of the human PMS2 N-terminal ATPase domain (PDB ID: 1EA6) (Guarné, et al., 2001), *E. coli* MutL ATPase domain (PDB ID: 1B63) (Ban, et al., 1999), *Bacillus subtilis* MutL C-terminal endonuclease domain (PDB ID: 3KDK) (Pillon, et al., 2010), and *E. coli* MutL C-terminal dimerization domain (PDB ID: 1X9Z) (Guarné, et al., 2004) are shown.

2. Structure of the C-terminal endonuclease and N-terminal ATPase domains of MutL

The C-terminal domain of MutL endonucleases contains two highly conserved sequence motifs (Fig. 3). One of them is the DQHA$(x)_2$E$(x)_4$E motif, which is essential for the nicking endonuclease activity (Fukui, et al., 2008, Kadyrov, et al., 2006). Aspartic acid and histidine residues in this motif are expected to coordinate one or two metal ions to catalyze the nicking reaction (Kosinski, et al., 2008, Pillon, et al., 2010, Yang, 2008). The other is the zinc-binding motif CPHGRP (Kosinski, et al., 2008), which is not essential for the nicking endonuclease activity but is required for the *in vivo* MMR activity (Fukui, et al., 2008, Kosinski, et al., 2008). Crystal structures of *Bacillus subtilis* and *Neisseria gonorrhoeae* MutL C-terminal domains (Namadurai, et al., 2010, Pillon, et al., 2010) revealed that their overall

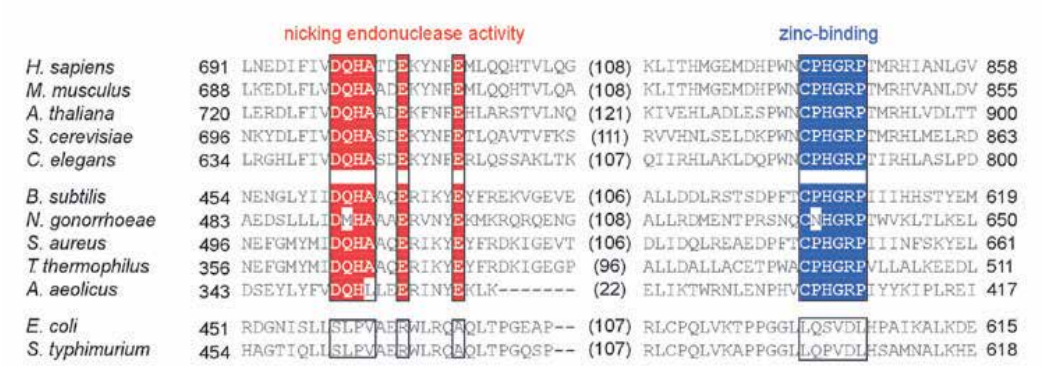

Fig. 3. Amino acid sequence alignment of the C-terminal regions of MutL homologues. Red and blue boxes indicate the conserved sequence motifs, DQHA(x)$_2$E(x)$_4$E and CPHGRP, respectively. The numbers on the left and right show the distances from the N-termini for each protein. The numbers in parentheses show the number of residues that are omitted for the sake of clarity. The NCBI Entrez GI numbers of the sequences are as follows: 4505913 (*Homo sapiens* PMS2), 121583910 (*Mus musculus* PMS2), 18411951 (*Arabidopsis thaliana* PMS1), 46562124 (*Saccharomyces cerevisiae* PMS1), 17562796 (*Caenorhabditis elegans* PMS2), 16078768 (*Bacillus subtilis* MutL), 59801161 (*Neisseria gonorrhoeae* MutL), 15926879 (*Staphylococcus aureus* MutL), 55981292 (*Thermus thermophilus* MutL), 15606703 (*Aquifex aeolicus* MutL), 16131992 (*Escherichia coli* MutL), and 16767605 (*Salmonella typhimurium* MutL).

structures, which are dimeric molecules, resemble that of the *E. coli* MutL C-terminal domain (Fig. 4A) (Guarné, et al., 2004, Kosinski, et al., 2005), although the *E. coli* MutL C-terminal domain lacks the DQHA(x)$_2$E(x)$_4$E and CPHGRP motifs. In those crystal structures, the CPHGRP motif is located adjacent to the DQHA(x)$_2$E(x)$_4$E motif to form a catalytic site in the subunit (Fig. 4B). In the crystal structure of the *B. subtilis* MutL C-terminal domain, two zinc ions are coordinated near the catalytic site by residues including the histidine and cysteine of the DQHA(x)$_2$E(x)$_4$E and CPHGRP motifs, respectively (Fig. 4C) (Pillon, et al., 2010). Although the function of these zinc ions has not been precisely explained, the difference between the zinc-bound and zinc-unbound forms of the *B. subtilis* MutL C-terminal domain demonstrated that binding of zinc ions brings about a local structural rearrangement in the catalytic site (Fig. 4C) (Pillon, et al., 2010). Since the addition of zinc ions to the reaction mixture slightly stimulates the nicking endonuclease activity of MutL (Pillon, et al., 2010), the local structural change would be a prerequisite for the formation of the active form of the catalytic site.

The N-terminal ATPase domain of MutL contains a single ATP-binding motif per subunit just like other GHKL superfamily proteins (Ban, et al., 1998, Guarné, et al., 2001). Unlike the C-terminal domain, the amino acid sequence of the N-terminal ATPase domain of the MutL endonuclease is highly homologous to that of *E. coli* MutL (Iino, et al., 2010). Therefore, the crystal structure of the *E. coli* MutL N-terminal domain can be utilized when considering the structure and function of the N-terminal domain of the MutL endonuclease. Ban and Yang described the apo and AMPPNP-bound forms of the *E. coli* MutL N-terminal domain (Ban, et al., 1999, Ban, et al., 1998), which clearly demonstrated the ATP binding-induced conformational change of this domain (Fig. 5A). Upon AMPPNP binding, the disordered region found in the apo structure formed ordered structures, which led to the dimerization

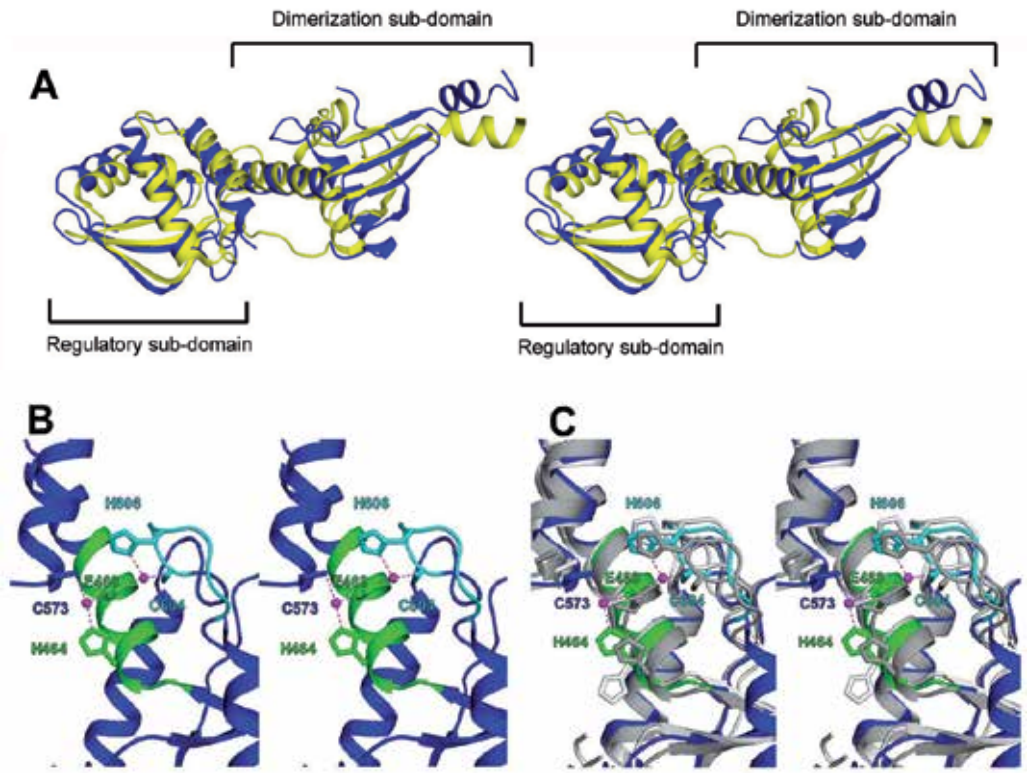

Fig. 4. Crystal structure of the nicking endonuclease domain of *B. subtilis* MutL (One subunit of the dimer is shown). (A) The overall structure of the endonuclease domain of *B. subtilis* MutL (*blue*) (zinc-bound form, PDB ID: 3KDK) (Pillon, et al., 2010) is superposed onto the dimerization domain of *E. coli* MutL (*yellow*) (PDB ID: 1X9Z) (Guarné, et al., 2004). The endonuclease domain is comprised of regulatory and dimerization sub-domains. The $DQHA(x)_2E(x)_4E$ and CPHGRP motifs are included in the dimerization sub-domain. (B) The $DQHA(x)_2E(x)_4E$ motif (*green*) is located near the CPHGRP motif (*cyan*). Two zinc ions (*pink spheres*) are coordinated by several residues including the histidine (*a green stick*) of the $DQHA(x)_2E(x)_4E$ motif and the cysteine and histidine (*cyan sticks*) of the CPHGRP motif. (C) The zinc ion binding induces a structural rearrangement of the catalytic site in the endonuclease domain. The zinc-bound form (*colored*) is superposed onto the unbound crystal forms I (*white*) (PDB ID: 3GAB) and II (*gray*) (PDB ID: 3KDG) (Pillon, et al., 2010). All structures are shown in a stereo view.

of the N-terminal domain. As with the MutL endonuclease, the crystal structure of the N-terminal domain of human PMS2 has been reported (Fig. 5B) (Guarné, et al., 2001). Intriguingly, the N-terminal domain of PMS2 bound to ATPγS even in the absence of the N-terminal domain of MLH1, which is the only report concerning ATP binding by a monomeric GHKL superfamily protein. However, it is expected that in the presence of the MLH1 subunit, ATP binding induces dimerization of the N-terminal domains. In line with this notion, a direct observation using atomic force microscopy suggested that ATP binding causes dimerization of the N-terminal domain in yeast MutLα (Sacho, et al., 2008).

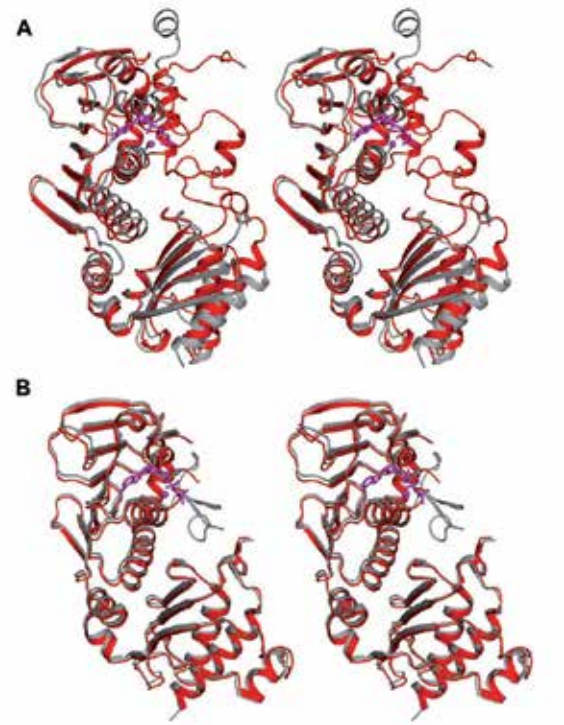

Fig. 5. Crystal structures of the N-terminal ATPase domains of *E. coli* MutL and human PMS2. (A) Stereo view of the *E. coli* MutL N-terminal ATPase domain in the apo form (*gray*) (PDB ID: 1BKN) and AMPPNP-bound form (*red*) (PDB ID: 1B63) (Ban, et al., 1999, Ban, et al., 1998). AMPPNP and a magnesium ion are shown as a *pink stick* and *sphere*, respectively. (B) Stereo view of human PMS2 N-terminal ATPase domain in the apo form (*gray*) (PDB ID: 1H7S) and ATPγS-bound form (*red*) (PDB ID: 1H7U) (Guarné, et al., 2001). ATPγS and a magnesium ion are shown as a *pink stick* and *sphere*, respectively.

3. ATP modulates the nicking endonuclease activity of MutL

The effect of ATP on the biochemical properties of the MutL endonuclease has been examined using the bacterial MutL endonuclease as a model molecule. *Thermus thermophilus* MutL stably bound one ATP molecule per subunit at a physiological concentration (2 mM) of ATP without any detectable hydrolysis activity in the absence of MutS and mismatch (Fukui, et al., 2008). Limited proteolysis indicated the ATP- or AMPPNP-dependent conformational change of *T. thermophilus* MutL (Fukui, et al., 2008).

In order to detect a nicking endonuclease activity, the covalently closed circular form of plasmid DNA is often used as a substrate (Fukui, et al., 2007). A nicking endonuclease activity converts the closed circular form into an open circular form of the plasmid DNA that can be easily separated from the closed circular form and the linearized form by agarose gel electrophoresis. Mn^{2+} facilitates the mismatch-, MutS-, clamp-, and clamp loader-independent incision of the closed circular form by non-sequence-specific MutL endonuclease activity (Duppatla, et al., 2009, Fukui, et al., 2008, Kadyrov, et al., 2006, Mauris, et al., 2009).

When *T. thermophilus* MutL was preincubated with physiological concentrations of ATP or AMPPNP before to the addition of substrate DNA, the initial rate of the nicking activity was significantly reduced (Fukui, et al., 2008). This was also supported by the result of a gel electrophoretic mobility shift assay, which indicates that ATP or AMPPNP prevents the non-specific DNA binding of *T. thermophilus* MutL (Fukui, et al., 2008). The endonuclease activities of *Aquifex aeolicus* and *N. gonorrhoeae* MutL were also suppressed by the addition of ATP (Duppatla, et al., 2009, Fukui, et al., 2008). One may speculate that the observed suppressing effect is due to the chelating ability of ATP to deprive the manganese ion from MutL. However, this possibility is ruled out by the following two experimental evidences: ATP has no inhibitory effect on the endonuclease activity of the C-terminal domain of MutL (Duppatla, et al., 2009); alteration of the cysteine residue in the CPHGRP motif to an alanine results in perturbation of the suppressing effect of ATP (Fukui, et al., 2008). These results suggest that ATP-dependent suppression requires the binding of ATP to the N-terminal domain and that the zinc ion in the C-terminal domain is required for sensing ATP binding. Interestingly, AMPPNP and a mismatch facilitated the stable interaction between *T. thermophilus* MutL and MutS (Fukui, et al., 2008). The ATP-bound form of MutL would specifically interact with the MutS-DNA complex in the presence of a mismatch. Because the ATPase activity of MutL is expected to be stimulated by its interaction with MutS, the formation of the MutS-MutL complex may promote the endonuclease activity of MutL by unlocking the ATP binding-dependent suppression (Fig. 6).

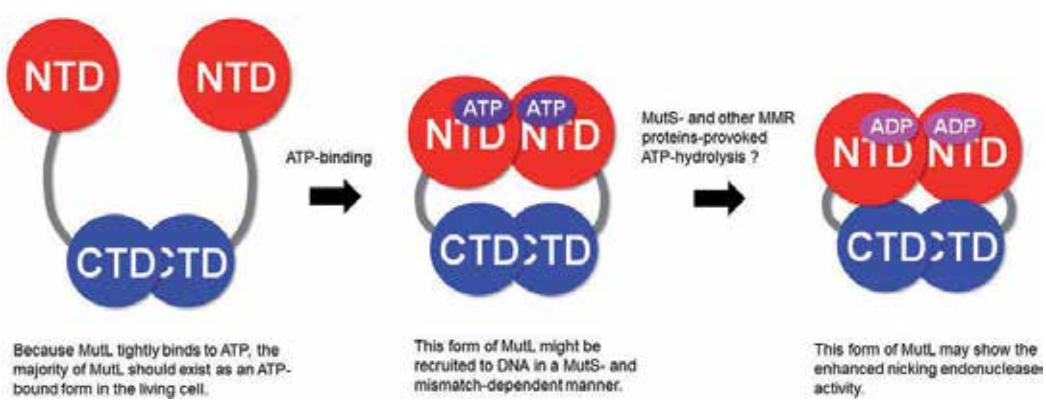

Fig. 6. A speculative model of the regulatory mechanism for the mismatch-specific enhancement of MutL nicking endonuclease activity. *NTD* and *CTD* represent the N- and C-terminal domains, respectively. ATP binding induces the dimerization of NTD and the approach of NTD to CTD. DNA-unbound MutL exists as an ATP-bound form whose endonuclease activity is inactive, but preferably binds to the MutS-DNA complex. The interaction with the MutS-DNA complex and other MMR proteins induces the ATP hydrolysis of MutL. This ATP hydrolysis induces the tight contact between NTD and CTD, resulting in the stimulation of endonuclease activity.

Mauris and Evans reported the detailed biochemical experiment on *A. aeolicus* MutL, in which they demonstrated that ATP stimulates the nicking endonuclease activity of relatively high concentrations of *A. aeolicus* MutL in the absence of MutS and mismatch (Mauris, et al., 2009). This result suggests that the effect of ATP on the MutL endonuclease activity depends

on the concentration of MutL, which is consistent with the previous report describing the promoting effect of ATP on the nicking endonuclease activity of eukaryotic MutLα (Kadyrov et al., 2006, Kadyrov et al., 2007). These evidences clearly indicate that ATP is utilized not only to suppress the non-specific endonuclease activity of MutL but also to actively enhance its activity. It would therefore be necessary to clarify whether ATP hydrolysis is required for enhancing the endonuclease activity. On one hand, it was reported that AMPPNP can stimulate the endonuclease activity of relatively high concentrations of *A. aeolicus* MutL (Mauris, et al., 2009). On the other hand, the endonuclease activity of *B. subtilis* MutL was not stimulated by AMPPNP even under conditions where ATP could stimulate the activity (Pillon, et al., 2010).

4. The N-terminal ATPase domain stimulates the endonuclease activity of the C-terminal domain

As described in the previous section, the endonuclease activity of MutL is modulated by ATP binding and/or hydrolysis. Because the ATP binding and endonuclease active sites are located in the N- and C-terminal domains, respectively, the interdomain interaction between them had been expected. This prediction was verified by the recent experiment using recombinant N- and C-terminal domains from *A. aeolicus* MutL. The N-terminal domain stimulated the endonuclease activity of the C-terminal domain by at least a 4-fold magnitude in the absence of ATP (Iino, et al., 2010). Interestingly, this promoting effect was abolished by the depletion of zinc ions from the reaction mixture or by the substitution of cysteine in the CPHGRP motif by alanine (Iino, et al., 2010). These results indicate that zinc ions are required for the N-terminal domain-dependent stimulation of the C-terminal domain. It remains to be investigated whether the zinc ions are directly involved in the interdomain interaction or whether they indirectly influence the interaction through rearrangement of the local structure.

It is expected that this interdomain interaction is involved in the ATPase cycle-dependent regulatory mechanism of MutL. Direct observation using atomic force microscopy has suggested the possible ATP binding-induced association of the N-terminal domain to the C-terminal domain (Fig. 6, middle) (Sacho, et al., 2008). Such an approach may reflect the interdomain interaction that is required for stimulating the nicking endonuclease activity. However, as mentioned above, ATP binding suppresses and ATP hydrolysis promotes the nicking endonuclease activity (Duppatla, et al., 2009, Fukui, et al., 2008, Pillon, et al., 2010). Therefore, ATP hydrolysis may create a tighter contact of the N-terminal domain with the C-terminal domain than that created by ATP binding (Fig. 6, right). Such a tight contact may stimulate the nicking endonuclease activity. Further studies are necessary to clarify whether and how ATP hydrolysis affects the structure and function of MutL endonuclease.

5. Interaction with a sliding clamp directs the MutL-dependent incision to the discontinuous strand

In the above sections, we reviewed the possible regulatory mechanism that assures the mismatch-specific nicking endonuclease activity of MutL. We also have to consider a regulatory mechanism that directs the nicking endonuclease activity of MutL to the error-containing strand of the mismatched duplex. Mismatch itself has no signal to discriminate which base is incorrect (Friedberg, et al., 2006). *In vitro* characterization of MMR activity in the

eukaryotic nuclear extracts has shown that discontinuities in the substrate mismatched DNA can serve as the signal to direct the MutL-dependent incision to the discontinuous strand (Kadyrov, et al., 2006, Kadyrov, et al., 2007, Modrich, 2006). In the cell, the 5'- and 3'-termini of the newly synthesized strand are expected to be utilized as the discrimination signal.

Additionally, another question has arisen: how does MutL sense the strand discontinuity that is remote from the MutL incision site? In an *in vitro* reconstituted system of eukaryotic MMR, the discontinuous strand is distinguished by the cooperative function of MutLα with PCNA and replication factor C (Kadyrov, et al., 2006, Modrich, 2006). Recently, it has also been clarified that PCNA directs the incision reaction at the terminus-containing strand through direct interaction with MutLα and that replication factor C is required only for loading PCNA to the DNA (Pluciennik, et al., 2010). The MLH1 subunit of MutLα contains the PCNA-interacting motif QxxLxxFF in its C-terminal domain (Fig. 7A) (Lee, et al., 2006). The PCNA-dependent activation of MutLα was hindered by a peptide containing the PCNA-interacting motif (Pluciennik, et al., 2010). PCNA recognizes the 3'-terminus of the primed sites in DNA (Yao, et al., 2000) and tightly binds to the plasmid DNA containing a pre-existing strand break (Pluciennik, et al., 2010). PCNA has two nonequivalent faces (Gulbis, et al., 1996) and binds to the strand break with a specific orientation (Bowman, et al., 2004, Georgescu, et al., 2008). Because the interface to MutLα is on one side of the clamp (Pluciennik, et al., 2010), the interaction between PCNA and the MLH1 subunit of MutLα is expected to facilitate the asymmetric binding of the mismatched duplex with the discontinuous strand bound in the catalytic site of the PMS2 subunit (Pluciennik, et al., 2010). This may assure the daughter strand-specific incision.

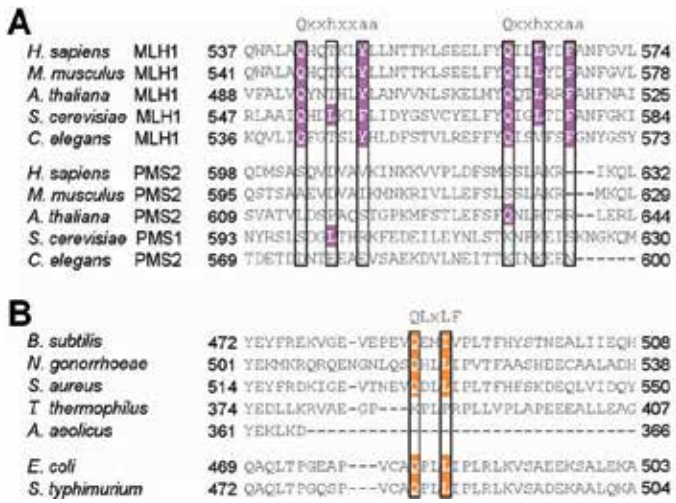

Fig. 7. Amino acid sequence alignment of the PCNA- or β-clamp-interacting motifs in the C-terminal domains of MutL homologues. The consensus sequence motif is shown above the alignments. (A) The conserved residues of the PCNA-interacting motif are shown in purple. In the consensus motif, *h* and *a* represent residues with hydrophobic side-chains and aromatic side-chains, respectively. The NCBI Entrez GI numbers of the sequences are as follows: 4557757 (*H. sapiens* MLH1), 255958238 (*M. musculus* MLH1), 30680985 (*A. thaliana* MLH1), 6323819 (*S. cerevisiae* MLH1), 71991825 (*C. elegans* MLH1). (B) The conserved residues of the β-clamp-interacting motif in bacterial MutL are shown in orange.

Interestingly, *B. subtilis* MutL endonuclease also interacts with a β-sliding clamp (Pillon, et al., 2011), a bacterial counterpart to eukaryotic PCNA, which also has two distinguishable faces (Kong, et al., 1992). Most bacterial MutL endonucleases have the β clamp-interacting motif QLxLF at the regulatory sub-domain of the C-terminal domain (Fig. 7B) (Pillon, et al., 2010, Pillon, et al., 2011). Mutation of this sequence motif results in defects in the *in vivo* MMR activity (Pillon, et al., 2011), implying that the β-clamp-dependent activation of MutL is necessary in the cell and that bacterial MMR also adopts a strand discrimination mechanism similar to that of eukaryotic MMR. However, MutL endonucleases from the Aquificae phylum lack the regulatory sub-domain (Iino, et al., 2010). In addition, MutL endonucleases from the Thermus-Deinococcus phylum have no obvious β-clamp-interacting motifs (Fig. 7B), although they retain the regulatory sub-domain. Therefore, it should be carefully investigated whether this discrimination mechanism is universally present among all nick-directed MMRs.

6. Bacterial MutL is a homodimeric nicking endonuclease

Crystal structures of *B. subtilis* and *N. gonorrhoeae* MutL C-terminal domains, and other biochemical studies, have revealed that bacterial MutL C-terminal domains are homo-dimeric (Duppatla, et al., 2009, Iino, et al., 2010, Namadurai, et al., 2010, Pillon, et al., 2010). Generally, linear double-stranded DNA-specific dimeric endonucleases incise both strands of the duplex. Type II (and Type IIs) restriction endonucleases and Type II DNA topoisomerases are representative of double-strand incising dimeric endonucleases. On the other hand, double-stranded DNA-specific nicking endonucleases are usually monomeric, with the exception of several structure-specific nicking endonucleases (Fukui, et al., 2008, Komori, et al., 2002). For example, the following linear double-stranded DNA-specific nicking endonucleases are all monomeric proteins (Table 1): N-type nicking endonucleases (e.g, N. *Bsp*QI), sequence-specific nicking endonucleases naturally or artificially created by mutating restriction enzymes to lose their dimerization ability (Higgins, et al., 2001, Roberts, et al., 2003, Xu, et al., 2001, Yunusova, et al., 2006, Zheleznaya, et al., 2009); V-type nicking endonucleases (e.g., *E. coli* Vsr), a short patch MMR nicking endonuclease (Tsutakawa, et al., 1999, Tsutakawa, et al., 1999); Type I DNA topoisomerases (e.g., *E. coli* Topo I), an enzyme with a supercoil-relaxing activity (Kirkegaard, et al., 1978); retrotransposon-targeting endonucleases (e.g., L1 endonuclease), a site-specific nicking endonuclease that directs the invasion of the retrotransposon (Feng, et al., 1996, Feng, et al., 1998, Maita, et al., 2007, Weichenrieder, et al., 2004); bovine DNase I, a non-specific nicking endonuclease that functions in the host defense (Suck, et al., 1988); *E. coli* MutH (Ban, et al., 1998), the MMR nicking endonuclease; bacterial UvrC (Nazimiec, et al., 2001), a nucleotide excision repair nicking endonuclease; bacterial endonuclease V (Dalhus, et al., 2009), a deaminated DNA-specific nicking endonuclease; and bacterial and eukaryotic AP endonucleases (Hosfield, et al., 1999, Mol, et al., 2000), an abasic site-specific nicking endonuclease. Known DNA repair systems other than MMR all adopt a monomeric nicking endonuclease to introduce the entry point for the excision reaction. Therefore, the dimerization ability of the MutL C-terminal domain might be related to the strand-discrimination mechanism of bacterial MMR.

Enzyme	Cellular function	Substrate	Biological unit	References
N-type nicking endonucleases (e.g., N. *Bsp*QI)	Host defense (Artificial)	Asymmetric sequence	Monomer	(Higgins, et al., 2001, Roberts, et al., 2003, Xu, et al., 2001, Yunusova, et al., 2006, Zheleznaya, et al., 2009)
V-type nicking endonucleases (e.g., *E. coli* Vsr)	DNA repair[4] and other	Methylated DNA	Monomer	(Tsutakawa, et al., 1999, Tsutakawa, et al., 1999)
Type I DNA topoisomerases (e.g., *E. coli* Topo I)	Various DNA transactions	Supercoiled DNA	Monomer	(Kirkegaard, et al., 1978)
Retrotransposon-targeting endonucleases[1] (e.g., L1 endonuclease)	Targeting of retrotranspo son	Target sequence	Monomer	(Feng, et al., 1996, Feng, et al., 1998, Maita, et al., 2007, Weichenrieder, et al., 2004)
Bovine DNase I[1]	Host defense	Non-specific	Monomer	(Suck, et al., 1988)
E. coli MutH	DNA repair[4]	GATC site	Monomer	(Ban, et al., 1998)
E. coli UvrC	DNA repair[4]	DNA strand with bulky adducts	Monomer	(Nazimiec, et al., 2001)
E. coli endonuclease V	DNA repair[4]	Deaminated DNA	Monomer	(Dalhus, et al., 2009)
AP endonucleases[1] (e.g., human APE1)	DNA repair[4]	DNA with abasic sites	Monomer	(Hosfield, et al., 1999, Mol, et al., 2000)
Serratia nuclease[2]	Host defense	Non-specific	Dimer	(Franke, et al., 1998, Franke, et al., 1999)
Bacterial MutL[3] (e.g., *B. subtilis* MutL)	DNA repair[4]	DNA strand with mismatched bases	Dimer	(Namadurai, et al., 2010, Pillon, et al., 2010)

Table 1. Linear double-stranded DNA-specific nicking endonucleases. [1]Structural analyses have revealed that AP endonucleases, retrotransposon-targeting endonucleases, and DNase I are closely related to each other. [2]*Serratia* nuclease can convert the covalently closed circular form of plasmid DNA not only to the linear form but also to the open circular form; however, the major product of this nuclease is the double-strand break. *Serratia* nuclease also incises single-stranded DNA, and dimerization is not essential for the nuclease activity. [3]MutL shows no structural similarity to other known endonucleases. [4]These nicking endonucleases introduce the starting point for the excision reaction in damaged or error-containing single-stranded DNA. Among these DNA repair nicking endonucleases, only MutL forms a dimer.

The homodimeric structure of bacterial MutL prompts the question of how the symmetric homodimer generates asymmetric nicking products. As with eukaryotic MutLα, the asymmetry would be derived from the nature of the heterodimer. Eukaryotic MutLα has a single catalytic site for the endonuclease activity. On the other hand, bacterial MutL contains two catalytic sites that are apparently equivalent to each other. It may be possible that bacterial MutL dissociates from the substrate DNA before the catalysis of the second strand incision because of its low velocity, or that the binding of the product to the one subunit induces a non-productive binding mode of the substrate to the other subunit. Alternatively, as proposed by Namadurai *et al*., the inverted arrangement of the bacterial MutL C-terminal domain dimer may enable interactions with other MMR proteins to interfere with one of the two active sites during the reaction (Namadurai, et al., 2010).

7. Conclusion

In this chapter, the biochemical properties of MutL endonucleases are reviewed, with an emphasis on their regulatory mechanisms. The regulatory mechanism needs to ensure both mismatch- and daughter-strand-specific incisions. The ATPase cycle-dependent conformational and functional changes of the MutL endonucleases are expected to play a central role in these mechanisms. Since the ATPase cycle-dependent conformational change would involve the rearrangement of the interaction between the N- and C-terminal domains, the structural analysis of full-length MutL is urgently required. For the structural analysis, MutL homologues from some thermophilic bacterium may be suitable because of the lack of flexible interdomain linker region as well as their extreme thermostability. However, the interdomain linker region plays a significant role in the *in vitro* function of eukaryotic MutLα (Gorman, et al., 2010). Therefore, it is necessary to carefully judge whether the obtained information is universal among all MutL endonucleases.

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9. References

Acharya, S., Foster, P. L., Brooks, P. & Fishel, R., The coordinated functions of the *E. coli* MutS and MutL proteins in mismatch repair, *Molecular Cell,* Vol. 12, No. 1, (July 2003), pp. 233-246, ISSN 1097-2765

Ban, C., Junop, M. & Yang, W., Transformation of MutL by ATP binding and hydrolysis: a switch in DNA mismatch repair, *Cell,* Vol. 97, No. 1, (April 1999), pp. 85-97, ISSN 0092-8674

Ban, C. &Yang, W., Crystal structure and ATPase activity of MutL: implications for DNA repair and mutagenesis, *Cell,* Vol. 95, No. 4, (November 1998), pp. 541-552, ISSN 0092-8674

Ban, C. &Yang, W., Structural basis for MutH activation in *E.coli* mismatch repair and relationship of MutH to restriction endonucleases, *EMBO Journal,* Vol. 17, No. 5, (March 1998), pp. 1526-1534, ISSN 0261-4189

Bowman, G. D., O'Donnell, M. & Kuriyan, J., Structural analysis of a eukaryotic sliding DNA clamp-clamp loader complex, *Nature,* Vol. 429, No. 6993, (June 2004), pp. 724-730, ISSN 1476-4687

Constantin, N., Dzantiev, L., Kadyrov, F. A. & Modrich, P., Human mismatch repair: reconstitution of a nick-directed bidirectional reaction, *Journal of Biological Chemistry,* Vol. 280, No. 48, (December 2005), pp. 39752-39761, ISSN 0021-9258

Dalhus, B., Arvai, A. S., Rosnes, I., Olsen, O. E., Backe, P. H., Alseth, I., Gao, H., Cao, W., Tainer, J. A. & Bjoras, M., Structures of endonuclease V with DNA reveal initiation of deaminated adenine repair, *Nature Structural and Molecular Biology,* Vol. 16, No. 2, (February 2009), pp. 138-143, ISSN 1545-9985

Drotschmann, K., Hall, M. C., Shcherbakova, P. V., Wang, H., Erie, D. A., Brownewell, F. R., Kool, E. T. & Kunkel, T. A., DNA binding properties of the yeast Msh2-Msh6 and Mlh1-Pms1 heterodimers, *Biological Chemistry,* Vol. 383, No. 6, (June 2002), pp. 969-975, ISSN 1431-6730

Duppatla, V., Bodda, C., Urbanke, C., Friedhoff, P. & Rao, D. N., The C-terminal domain is sufficient for endonuclease activity of *Neisseria gonorrhoeae* MutL, *Biochemical Journal,* Vol. 423, No. 2, (September 2009), pp. 265-277, ISSN 1470-8728

Dutta, R. &Inouye, M., GHKL, an emergent ATPase/kinase superfamily, *Trends in Biochemical Sciences,* Vol. 25, No. 1, (January 2000), pp. 24-28, ISSN 0968-0004

Dzantiev, L., Constantin, N., Genschel, J., Iyer, R. R., Burgers, P. M. & Modrich, P., A defined human system that supports bidirectional mismatch-provoked excision, *Molecular Cell,* Vol. 15, No. 1, (July 2004), pp. 31-41, ISSN 1097-2765

Feng, Q., Moran, J. V., Kazazian, H. H., Jr. & Boeke, J. D., Human L1 retrotransposon encodes a conserved endonuclease required for retrotransposition, *Cell,* Vol. 87, No. 5, (November 1996), pp. 905-916, ISSN 0092-8674

Feng, Q., Schumann, G. & Boeke, J. D., Retrotransposon R1Bm endonuclease cleaves the target sequence, *Proceedings of the National Academy of Sciences of the United States of America,* Vol. 95, No. 5, (March 1998), pp. 2083-2088, ISSN 0027-8424

Fishel, R. &Kolodner, R. D., Identification of mismatch repair genes and their role in the development of cancer, *Current Opinion in Genetics & Development,* Vol. 5, No. 3, (June 1995), pp. 382-395, ISSN 0959-437X

Fishel, R., Lescoe, M. K., Rao, M. R., Copeland, N. G., Jenkins, N. A., Garber, J., Kane, M. & Kolodner, R., The human mutator gene homolog MSH2 and its association with hereditary nonpolyposis colon cancer, *Cell,* Vol. 77, No. 1, (April 1994), p. 1 p following 166, ISSN 0092-8674

Franke, I., Meiss, G., Blecher, D., Gimadutdinow, O., Urbanke, C. & Pingoud, A., Genetic engineering, production and characterisation of monomeric variants of the dimeric *Serratia* marcescens endonuclease, *FEBS Letters,* Vol. 425, No. 3, (April 1998), pp. 517-522, ISSN 0014-5793

Franke, I., Meiss, G. & Pingoud, A., On the advantage of being a dimer, a case study using the dimeric *Serratia* nuclease and the monomeric nuclease from *Anabaena* sp. strain PCC 7120, *Journal of Biological Chemistry,* Vol. 274, No. 2, (January 1999), pp. 825-832, ISSN 0021-9258

Friedberg, E. C., Walker, G. C., Siede, W., Wood, R. D., Schultz, R. A., & Ellenberger, T. (2006) DNA repair and mutagenesis, 2nd edition, Washington, DC: American Society for Microbiology, ISBN 978-1555813194

Fukui, K., DNA mismatch repair in eukaryotes and bacteria, *Journal of Nucleic Acids,* Vol. 2010, (July 2010), p. 260512, ISSN 2090-021X

Fukui, K., Kosaka, H., Kuramitsu, S. & Masui, R., Nuclease activity of the MutS homologue MutS2 from *Thermus thermophilus* is confined to the Smr domain, *Nucleic Acids Research,* Vol. 35, No. 3, (January 2007), pp. 850-860, ISSN 1362-4962

Fukui, K., Nakagawa, N., Kitamura, Y., Nishida, Y., Masui, R. & Kuramitsu, S., Crystal structure of MutS2 endonuclease domain and the mechanism of homologous recombination suppression, *Journal of Biological Chemistry,* Vol. 283, No. 48, (November 2008), pp. 33417-33427, ISSN 0021-9258

Fukui, K., Nishida, M., Nakagawa, N., Masui, R. & Kuramitsu, S., Bound nucleotide controls the endonuclease activity of mismatch repair enzyme MutL, *Jorunal of Biological Chemistry,* Vol. 283, No. 18, (May 2008), pp. 12136-12145, ISSN 0021-9258

Genschel, J., Bazemore, L. R. & Modrich, P., Human exonuclease I is required for 5' and 3' mismatch repair, *Journal of Biological Chemistry,* Vol. 277, No. 15, (April 2002), pp. 13302-13311, ISSN 0021-9258

Georgescu, R. E., Kim, S. S., Yurieva, O., Kuriyan, J., Kong, X. P. & O'Donnell, M., Structure of a sliding clamp on DNA, *Cell,* Vol. 132, No. 1, (January 2008), pp. 43-54, ISSN 0092-8674

Gorman, J., Plys, A. J., Visnapuu, M. L., Alani, E. & Greene, E. C., Visualizing one-dimensional diffusion of eukaryotic DNA repair factors along a chromatin lattice, *Nature Structural and Molecular Biology,* Vol. 17, No. 8, (August 2010), pp. 932-938, ISSN 1545-9985

Gradia, S., Acharya, S. & Fishel, R., The human mismatch recognition complex hMSH2-hMSH6 functions as a novel molecular switch, *Cell,* Vol. 91, No. 7, (December 1997), pp. 995-1005, ISSN 0092-8674

Gradia, S., Subramanian, D., Wilson, T., Acharya, S., Makhov, A., Griffith, J. & Fishel, R., hMSH2-hMSH6 forms a hydrolysis-independent sliding clamp on mismatched DNA, *Molecular Cell,* Vol. 3, No. 2, (February 1999), pp. 255-261, ISSN 1097-2765

Guarné, A., Junop, M. S. & Yang, W., Structure and function of the N-terminal 40 kDa fragment of human PMS2: a monomeric GHL ATPase, *EMBO Journal,* Vol. 20, No. 19, (October 2001), pp. 5521-5531, ISSN 0261-4189

Guarné, A., Ramon-Maiques, S., Wolff, E. M., Ghirlando, R., Hu, X., Miller, J. H. & Yang, W., Structure of the MutL C-terminal domain: a model of intact MutL and its roles in mismatch repair, *EMBO Journal,* Vol. 23, No. 21, (October 2004), pp. 4134-4145, ISSN 0261-4189

Gulbis, J. M., Kelman, Z., Hurwitz, J., O'Donnell, M. & Kuriyan, J., Structure of the C-terminal region of p21(WAF1/CIP1) complexed with human PCNA, *Cell,* Vol. 87, No. 2, (October 1996), pp. 297-306, ISSN 0092-8674

Higgins, L. S., Besnier, C. & Kong, H., The nicking endonuclease N.*Bst*NBI is closely related to type IIs restriction endonucleases *Mly*I and *Ple*I, *Nucleic Acids Research,* Vol. 29, No. 12, (June 2001), pp. 2492-2501, ISSN 1362-4962

Hosfield, D. J., Guan, Y., Haas, B. J., Cunningham, R. P. & Tainer, J. A., Structure of the DNA repair enzyme endonuclease IV and its DNA complex: double-nucleotide flipping at abasic sites and three-metal-ion catalysis, *Cell,* Vol. 98, No. 3, (August 1999), pp. 397-408, ISSN 0092-8674

Iino, H., Kim, K., Shimada, A., Masui, R., Kuramitsu, S. & Fukui, K., Characterization of C- and N-terminal domains of *Aquifex aeolicus* MutL endonuclease: N-terminal domain stimulates the endonuclease activity of C-terminal domain in a zinc-dependent manner, *Bioscience Reports,* Vol. 31, No. 5, (October 2011), pp. 309-322, ISSN 0144-8463

Iyer, R. R., Pluciennik, A., Burdett, V. & Modrich, P. L., DNA mismatch repair: functions and mechanisms, *Chemical Review,* Vol. 106, No. 2, (February 2006), pp. 302-323, ISSN 0009-2665

Kadyrov, F. A., Dzantiev, L., Constantin, N. & Modrich, P., Endonucleolytic function of MutLα in human mismatch repair, *Cell,* Vol. 126, No. 2, (July 2006), pp. 297-308, ISSN 0092-8674

Kadyrov, F. A., Genschel, J., Fang, Y., Penland, E., Edelmann, W. & Modrich, P., A possible mechanism for exonuclease 1-independent eukaryotic mismatch repair, *Proceedings of the National Academy of Sciences of the United States of America,* Vol. 106, No. 21, (May 2009), pp. 8495-8500, ISSN 1091-6490

Kadyrov, F. A., Holmes, S. F., Arana, M. E., Lukianova, O. A., O'Donnell, M., Kunkel, T. A. & Modrich, P., *Saccharomyces cerevisiae* MutLα is a mismatch repair endonuclease, *Journal of Biological Chemistry,* Vol. 282, No. 51, (December 2007), pp. 37181-37190, ISSN 0021-9258

Kane, M. F., Loda, M., Gaida, G. M., Lipman, J., Mishra, R., Goldman, H., Jessup, J. M. & Kolodner, R., Methylation of the hMLH1 promoter correlates with lack of expression of hMLH1 in sporadic colon tumors and mismatch repair-defective human tumor cell lines, *Cancer Research,* Vol. 57, No. 5, (March 1997), pp. 808-811, ISSN 0008-5472

Kim, T. G., Cha, H. J., Lee, H. J., Heo, S. D., Choi, K. Y., Ku, J. K. & Ban, C., Structural insights of the nucleotide-dependent conformational changes of *Thermotoga maritima* MutL using small-angle X-ray scattering analysis, *Journal of Biochemistry,* Vol. 145, No. 2, (February 2009), pp. 199-206, ISSN 1756-2651

Kirkegaard, K. &Wang, J. C., *Escherichia coli* DNA topoisomerase I catalyzed linking of single-stranded rings of complementary base sequences, *Nucleic Acids Research,* Vol. 5, No. 10, (October 1978), pp. 3811-3820, ISSN 0305-1048

Komori, K., Fujikane, R., Shinagawa, H. & Ishino, Y., Novel endonuclease in Archaea cleaving DNA with various branched structure, *Genes & Genetic Systems,* Vol. 77, No. 4, (August 2002), pp. 227-241, ISSN 1341-7568

Kong, X. P., Onrust, R., O'Donnell, M. & Kuriyan, J., Three-dimensional structure of the β subunit of *E. coli* DNA polymerase III holoenzyme: a sliding DNA clamp, *Cell,* Vol. 69, No. 3, (May 1992), pp. 425-437, ISSN 0092-8674

Kosinski, J., Plotz, G., Guarné, A., Bujnicki, J. M. & Friedhoff, P., The PMS2 subunit of human MutLα contains a metal ion binding domain of the iron-dependent repressor protein family, *Journal of Molecular Biology,* Vol. 382, No. 3, (Month 2008), pp. 610-627, ISSN 1089-8638

Kosinski, J., Steindorf, I., Bujnicki, J. M., Giron-Monzon, L. & Friedhoff, P., Analysis of the quaternary structure of the MutL C-terminal domain, *Journal of Molecular Biology,* Vol. 351, No. 4, (October 2005), pp. 895-909, ISSN 0022-2836

Kunkel, T. A. &Erie, D. A., DNA mismatch repair, *Annual Review of Biochemistry,* Vol. 74, March 2005), pp. 681-710, ISSN 0066-4154

Lamers, M. H., Perrakis, A., Enzlin, J. H., Winterwerp, H. H., de Wind, N. & Sixma, T. K., The crystal structure of DNA mismatch repair protein MutS binding to a G x T mismatch, *Nature,* Vol. 407, No. 6805, (October 2000), pp. 711-717, ISSN 0028-0836

Larrea, A. A., Lujan, S. A. & Kunkel, T. A., SnapShot: DNA mismatch repair, *Cell,* Vol. 141, No. 4, (May 2010), p. 730 e731, ISSN 1097-4172

Leach, F. S., Nicolaides, N. C., Papadopoulos, N., Liu, B., Jen, J., Parsons, R., Peltomaki, P., Sistonen, P., Aaltonen, L. A., Nystrom-Lahti, M., Guan, E. Y., Zhang, J., Meltzer, P. S., Yu, J. W., Kao, F. T., Chen, D. J., Cerosaletti, K. M., Fournier, R. E. K., Todd, S., Lewis, T., Leach, R. J., Naylor, S. L., Weissenbach, J., Mecklin, J. P., Jarvinen, H., Petersen, G. M., Hamilton, S. R., Green, J., Jass, J., Watson, P., Lynch, H. T., Trent, J. M., Chapelle, A., Kinzler, K. W. & Vogelstein, B., Mutations of a *mutS* homolog in hereditary nonpolyposis colorectal cancer, *Cell,* Vol. 75, No. 6, (December 1993), pp. 1215-1225, ISSN 0092-8674

Lee, S. D. &Alani, E., Analysis of interactions between mismatch repair initiation factors and the replication processivity factor PCNA, *Journal of Molecular Biology,* Vol. 355, No. 2, (January 2006), pp. 175-184, ISSN 0022-2836

Li, G. M., Mechanisms and functions of DNA mismatch repair, *Cell Research,* Vol. 18, No. 1, (January 2008), pp. 85-98, ISSN 1748-7838

Maita, N., Aoyagi, H., Osanai, M., Shirakawa, M. & Fujiwara, H., Characterization of the sequence specificity of the R1Bm endonuclease domain by structural and biochemical studies, *Nucleic Acids Research,* Vol. 35, No. 12, (May 2007), pp. 3918-3927, ISSN 1362-4962

Mauris, J. &Evans, T. C., Adenosine triphosphate stimulates *Aquifex aeolicus* MutL endonuclease activity, *PLoS One,* Vol. 4, No. 9, (September 2009), p. e7175, ISSN 1932-6203

McCulloch, S. D., Gu, L. & Li, G. M., Nick-dependent and -independent processing of large DNA loops in human cells, *Journal of Biological Chemistry,* Vol. 278, No. 50, (December 2003), pp. 50803-50809, ISSN 0021-9258

Modrich, P., Mechanisms in eukaryotic mismatch repair, *Journal of Biological Chemistry,* Vol. 281, No. 41, (October 2006), pp. 30305-30309, ISSN 0021-9258

Modrich, P. &Lahue, R., Mismatch repair in replication fidelity, genetic recombination, and cancer biology, *Annual Review of Biochemistry,* Vol. 65, (March 1996), pp. 101-133, 0066-4154

Mol, C. D., Izumi, T., Mitra, S. & Tainer, J. A., DNA-bound structures and mutants reveal abasic DNA binding by APE1 and DNA repair coordination [corrected], *Nature,* Vol. 403, No. 6768, (January 2000), pp. 451-456, ISSN 0028-0836

Morita, R., Nakane, S., Shimada, A., Inoue, M., Iino, H., Wakamatsu, T., Fukui, K., Nakagawa, N., Masui, R. & Kuramitsu, S., Molecular mechanisms of the whole DNA repair system: a comparison of bacterial and eukaryotic systems, *Journal of Nucleic Acids,* Vol. 2010, (October 2010), p. 179594, ISSN 2090-021X

Namadurai, S., Jain, D., Kulkarni, D. S., Tabib, C. R., Friedhoff, P., Rao, D. N. & Nair, D. T., The C-Terminal Domain of the MutL Homolog from *Neisseria gonorrhoeae* Forms an Inverted Homodimer, *PLoS One,* Vol. 5, No. 10, (October 2010), p. e13726, ISSN 1932-6203

Nazimiec, M., Lee, C. S., Tang, Y. L., Ye, X., Case, R. & Tang, M., Sequence-dependent interactions of two forms of UvrC with DNA helix-stabilizing CC-1065-N3-adenine adducts, *Biochemistry,* Vol. 40, No. 37, (September 2001), pp. 11073-11081, ISSN 0006-2960

Obmolova, G., Ban, C., Hsieh, P. & Yang, W., Crystal structures of mismatch repair protein MutS and its complex with a substrate DNA, *Nature,* Vol. 407, No. 6805, (October 2000), pp. 703-710, ISSN 0028-0836

Pillon, M. C., Lorenowicz, J. J., Uckelmann, M., Klocko, A. D., Mitchell, R. R., Chung, Y. S., Modrich, P., Walker, G. C., Simmons, L. A., Friedhoff, P. & Guarné, A., Structure of the endonuclease domain of MutL: unlicensed to cut, *Molecular Cell,* Vol. 39, No. 1, (July 2010), pp. 145-151, ISSN 1097-4164

Pillon, M. C., Miller, J. H. & Guarné, A., The endonuclease domain of MutL interacts with the β sliding clamp, *DNA Repair (Amst),* Vol. 10, No. 1, (January 2011), pp. 87-93, ISSN 1568-7856

Pluciennik, A., Dzantiev, L., Iyer, R. R., Constantin, N., Kadyrov, F. A. & Modrich, P., PCNA function in the activation and strand direction of MutLα endonuclease in mismatch repair, *Proceedings of the National Academy of Sciences of United States of America,* Vol. 107, No. 37, (September 2010), pp. 16066-16071, ISSN 1091-6490

Roberts, R. J., Belfort, M., Bestor, T., Bhagwat, A. S., Bickle, T. A., Bitinaite, J., Blumenthal, R. M., Degtyarev, SKh, Dryden, D. T., Dybvig, K., Firman, K., Gromova, E. S., Gumport, R. I., Halford, S. E., Hattman, S., Heitman, J., Hornby, D. P., Janulaitis, A., Jeltsch, A., Josephsen, J., Kiss, A., Klaenhammer, T. R., Kobayashi, I., Kong, H., Kruger, D. H., Lacks, S., Marinus, M. G., Miyahara, M., Morgan, R. D., Murray, N. E., Nagaraja, V., Piekarowicz, A., Pingoud, A., Raleigh, E., Rao, D. N., Reich, N., Repin, V. E., Selker, E. U., Shaw, P. C., Stein, D. C., Stoddard, B. L., Szybalski, W., Trautner, T. A., Van Etten, J. L., Vitor, J. M., Wilson, G. G. & Xu, S. Y., A nomenclature for restriction enzymes, DNA methyltransferases, homing endonucleases and their genes, *Nucleic Acids Research,* Vol. 31, No. 7, (April 2003), pp. 1805-1812, ISSN 1362-4962

Sacho, E. J., Kadyrov, F. A., Modrich, P., Kunkel, T. A. & Erie, D. A., Direct visualization of asymmetric adenine-nucleotide-induced conformational changes in MutLα, *Molecular Cell,* Vol. 29, No. 1, (January 2008), pp. 112-121, ISSN 1097-2765

Schaaper, R. M., Base selection, proofreading, and mismatch repair during DNA replication in *Escherichia coli*, *Journal of Biological Chemistry,* Vol. 268, No. 32, (November 1993), pp. 23762-23765, ISSN 0021-9258

Schlagman, S. L., Hattman, S. & Marinus, M. G., Direct role of the *Escherichia coli* Dam DNA methyltransferase in methylation-directed mismatch repair, *Journal of Bacteriology,* Vol. 165, No. 3, (March 1986), pp. 896-900, ISSN 0021-9193

Shimada, A., Masui, R., Nakagawa, N., Takahata, Y., Kim, K., Kuramitsu, S. & Fukui, K., A novel single-stranded DNA-specific 3'-5' exonuclease, *Thermus thermophilus* exonuclease I, is involved in several DNA repair pathways, *Nucleic Acids Research,* Vol. 38, No. 17, (September 2010), pp. 5692-5705, ISSN 1362-4962

Suck, D., Lahm, A. & Oefner, C., Structure refined to 2A of a nicked DNA octanucleotide complex with DNase I, *Nature,* Vol. 332, No. 6163, (March 1988), pp. 464-468, ISSN 0028-0836

Suter, C. M., Martin, D. I. & Ward, R. L., Germline epimutation of MLH1 in individuals with multiple cancers, *Nature Genetics,* Vol. 36, No. 5, (May 2004), pp. 497-501, ISSN 1061-4036

Tachiki, H., Kato, R. & Kuramitsu, S., DNA binding and protein-protein interaction sites in MutS, a mismatched DNA recognition protein from *Thermus thermophilus* HB8, *Journal of Biological Chemistry,* Vol. 275, No. 52, (December 2000), pp. 40703-40709, ISSN 0021-9258

Tsutakawa, S. E., Jingami, H. & Morikawa, K., Recognition of a TG mismatch: the crystal structure of very short patch repair endonuclease in complex with a DNA duplex, *Cell,* Vol. 99, No. 6, (December 1999), pp. 615-623, ISSN 0092-867

Tsutakawa, S. E., Muto, T., Kawate, T., Jingami, H., Kunishima, N., Ariyoshi, M., Kohda, D., Nakagawa, M. & Morikawa, K., Crystallographic and functional studies of very short patch repair endonuclease, *Molecular Cell,* Vol. 3, No. 5, (May 1999), pp. 621-628, ISSN 1097-2765

Weichenrieder, O., Repanas, K. & Perrakis, A., Crystal structure of the targeting endonuclease of the human LINE-1 retrotransposon, *Structure,* Vol. 12, No. 6, (June 2004), pp. 975-986, ISSN 0969-2126

Xu, Y., Lunnen, K. D. & Kong, H., Engineering a nicking endonuclease N.*Alw*I by domain swapping, *Proceedings of the National Academy of Sciences of United States of America,* Vol. 98, No. 23, (November 2001), pp. 12990-12995, ISSN 0027-8424

Yang, W., Human MutLα: the jack of all trades in MMR is also an endonuclease, *DNA Repair (Amst),* Vol. 6, No. 1, (January 2007), pp. 135-139, ISSN 1568-7864

Yang, W., An equivalent metal ion in one- and two-metal-ion catalysis, *Nature Structural & Molecular Biology,* Vol. 15, No. 11, (November 2008), pp. 1228-1231, ISSN 1545-9985

Yao, N., Hurwitz, J. & O'Donnell, M., Dynamics of beta and proliferating cell nuclear antigen sliding clamps in traversing DNA secondary structure, *Journal of Biological Chemistry,* Vol. 275, No. 2, (January 2000), pp. 1421-1432, ISSN 0021-9258

Yunusova, A. K., Rogulin, E. A., Artyukh, R. I., Zheleznaya, L. A. & Matvienko, N. I., Nickase and a protein encoded by an open reading frame downstream from the nickase *Bsp*D6I gene form a restriction endonuclease complex, *Biochemistry (Mosc),* Vol. 71, No. 7, (July 2006), pp. 815-820, ISSN 0006-2979

Zheleznaya, L. A., Kachalova, G. S., Artyukh, R. I., Yunusova, A. K., Perevyazova, T. A. & Matvienko, N. I., Nicking endonucleases, *Biochemistry (Mosc),* Vol. 74, No. 13, (December 2009), pp. 1457-1466, ISSN 1608-3040

8

The Pathways of Double-Strand Break Repair

Emil Mladenov and George Iliakis
Institute of Medical Radiation Biology,
University of Duisburg-Essen Medical school, Essen
Germany

1. Introduction

1.1 Induction and repair of double-strand breaks (DSBs) in the DNA

DNA encodes and transmits genetic information into the progeny of cells and organisms. As a result, processes associated with DNA replication, repair and recombination are at the center of biological research. Although the double-stranded nature of the DNA molecule is not a requirement for its replication or transcription, it is essential for the repair of practically all forms of damage that are limited to one DNA strand. Thus, in the base excision repair (BER) pathway, the damaged base is excised by the appropriate DNA glycosylase and the resulting apurinic/apyrimidinic site is recognized by the AP-endonuclease (APE1) which opens the sugar phosphate backbone and removes the sugar residue (Fig. 1A). The resulting single nucleotide gap is filled-in using information from the complementary strand with the help of DNA polymerase β (polβ) and the sugar phosphate backbone is resealed with the help of XRCC1/DNA LigaseIII (LigIII) complex (Sancar et al., 2004). In an alternative form of this repair pathway, more nucleotides are removed from the vicinity of the damaged base and are subsequently replaced by the DNA polymerase, hence the name long-patch base excision repair.

A further example of a repair pathway relying exclusively on the complementarity of the DNA strands for the faithful restoration of the DNA molecule is the nucleotide excision repair pathway (NER) (Fig. 1B). In this repair pathway gross structural distortions are recognized in the DNA rather than altered bases. Such structural alterations can be generated by pyrimidine dimers forming after exposure to UV light, as well as by several other forms of DNA lesions, including DNA crosslinks and DNA "bulky" adducts. Upon their recognition by a multi-protein complex (see Fig. 1B for details) and with the help of two independently acting 3′- and 5′- excision nucleases XPG and XPF/ERCC1 respectively, an incision is placed 6 ±3 and 20 ±5 nucleotides upstream and downstream of the pyrimidine dimer ultimately removing an approximately 24-32 nt single stranded DNA segment including the lesion. The resulting gap is filled in, again using information available on the complementary strand with the help of a polymerase. The continuity of the DNA is finally restored by ligation with DNA Ligase I (LigI) (Sancar et al., 2004).

This fundamental concept of DNA repair, i.e. the use of a complementary strand to restore sequence information in the damaged strand, fails when complex damage is generated in the DNA consisting of multiple lesions distributed on both strands of the DNA in close proximity (Fig. 1C). Such forms of DNA damage are generated after exposure of cells to

ionizing radiation (IR) and are the direct consequence of the energy deposition patterns of this physical cytotoxic agent (Fig. 1C). The most widely investigated, complex form of damage is the DNA double-strand break (DSB). DSBs are highly dangerous DNA lesions that have been implicated not only in cell death but also in the induction of mutations and in carcinogenesis.

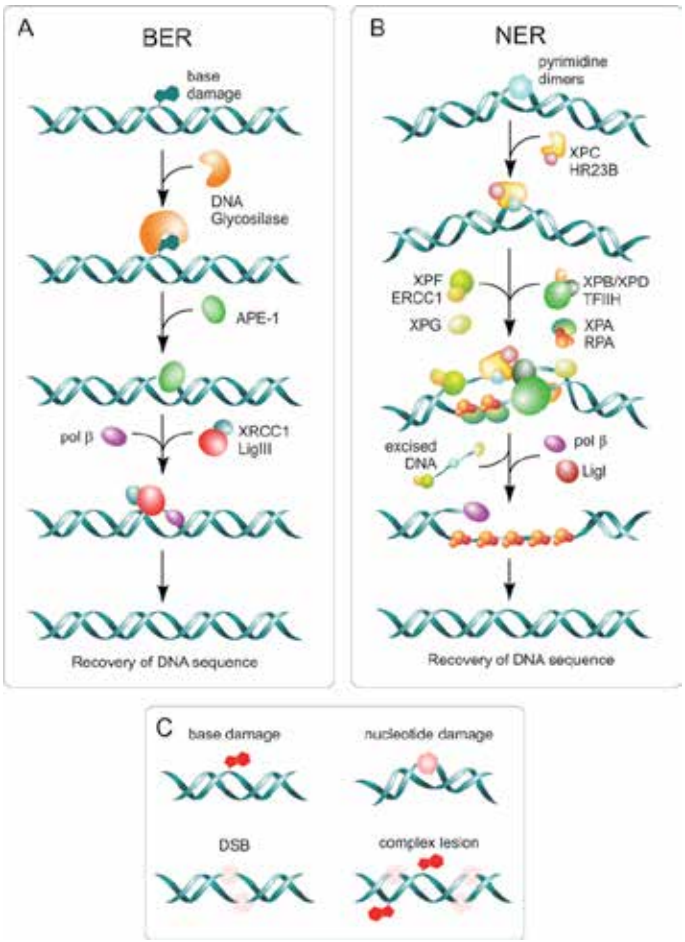

Fig. 1. Base excision repair (BER) and nucleotide excision repair (NER) pathways. Both BER and NER repair pathways utilize the complementary DNA strand to restore sequence information lost in the damaged DNA strand. A) Schematic representation of the basic steps followed during short-patch BER (see text for details). B) Main sequence of events and enzymatic activities implicated in NER. C) Forms of lesions generated in the DNA by IR.

The difficulties that cell faces in its attempt to faithfully process DSBs have two distinct aspects. First, the resulting disruption of the DNA molecule (note that disruption of the DNA molecule does not occur in case of lesions processed by BER or NER) physically interrupts its continuity and destabilizes the surrounding chromatin. Second, since both DNA strands carry damage closely juxtaposed, an intact template is lacking for ensuring the faithful restoration of the sequence in the vicinity of the break. The selective pressure for

evolving mechanisms for the detection and processing of DSBs would have been low had this type of lesion been extremely rare. However, in addition of IR, a number of chemical and physical cytotoxic agents generate DSBs. Most importantly, however, DSBs are also generated during the normal life cycle of the cell, particularly during DNA replication or in meiosis (Baudat & de Massy, 2007; Longhese et al., 2009), as well as during V(D)J and class switch recombination required for the differentiation of B and T cells of the hematopoietic system (Franco et al., 2006; Maizels, 2005). To cope with these multiple sources of DSBs, cells have evolved sophisticated mechanisms for detecting and repairing this form of DNA lesion. Notably, these repair mechanisms have been intimately coupled to the cell cycle, transcription and apoptosis machineries, suggesting a close coordination with the overall cellular metabolism.

Two conceptually different mechanisms can in principle remove DSBs from the genome in cells of higher eukaryotes. Homologous recombination repair (HRR) is equipped to maintain fidelity in the sequence of the DNA molecule, but because the damage affects both DNA strands it retrieves information from a homologous DNA molecule that is used as a template. There are two sources of homology in mammalian cells. The homologous chromosome that is present throughout the life cycle of the cell and the sister chromatid that is generated after DNA replication and which therefore exists only during the S and G2 phases of the cell cycle. Existing evidence supports the view that HRR requires the sister chromatid as a source of template, a requirement that automatically restricts the function of this repair pathway to the S and G2 phases of the cell cycle. This requirement probably derives from the fact that in a eukaryotic cell nucleus the homologous chromosomes are accommodated in distinct and frequently distantly located domains that renders search for homology (a key step of HRR, see below) difficult, if not impossible (Cremer & Cremer, 2001; Folle, 2008).

Non-homologous end joining (NHEJ), on the other hand, simply restores integrity in the DNA by joining the two ends without necessarily preserving the original sequence. As a result, it is error prone. Because a second DNA molecule is not required for the function of this repair pathway, it remains active throughout the cell cycle, but has a limited contribution to the rejoining of DNA lesions generating a single DNA terminus.

The fact that at least two genetically and conceptually distinct repair pathways are involved in the elimination of DSBs, poses questions regarding their coordination. If these pathways operate independently of each other it is possible that they compete against each other. If they collaborate, the question arises as to how their functions are coordinated. In this regard, it appears puzzling that cells of higher eukaryotes appear programmed to utilize preferentially NHEJ.

In the following sections we summarize the salient features of HRR and NHEJ and explain the concepts underlying their operation. Further, we describe a "dormant" pathway of DSB repair that unfolds its activity mostly when D-NHEJ for some reason fails and which therefore is considered to have a backup nature. Finally, we cover connections between DSB repair and cell cycle progression and discuss potential sources of errors during DSB repair that affect genomic stability and may lead to cancer development.

2. Homologous recombination repair – the only genuine repair process

DSB repair based on homology is frequently termed homologous recombination repair (HRR). A breakthrough in our understanding of the process of homologous recombination

(HR) in general and of HRR in particular was the model proposed in 1964 by Robin Holliday to explain meiotic recombination. The model introduced several key concepts including a mechanism for the exchange of genetic material between homologous chromosomes through the formation of what is now known as Holliday junction (HJ) (Liu & West, 2004) (see below for description). The Holliday model described some of the basic steps of the recombination process, but was unable to explain all sets of available genetic data. This was later achieved by a model proposed by Jack Szostak, now known as the double-strand break repair model (DSBR) (Szostak et al., 1983). Furthermore, analysis of genetic experiments in Drosophila revealed that DNA recombination may not require the formation of a Holliday junction and may instead depend on what is now known as synthesis-dependent strand annealing (SDSA) (Ferguson & Holloman, 1996; San Filippo et al., 2008). Recombination events carried out by this mechanism in mitotic cells lack crossover products (exchange of chromosome arms).

All current recombination models are formulated on the basis of genetic data and emphasize the role of HR during meiosis or mitosis. The meiotic function of HR mediates the exchanges of genetic material between the homologous chromosomes of the gamete precursor cells and ensures genetic diversity in the progeny (San Filippo et al., 2008).

Genetic and biochemical data provide strong evidence for the involvement of mitotic HR in the repair of DSBs. Moreover, HR is required for the restart of blocked or collapsed replication forks, as well as during the repair of inter-strand crosslinks (ICLs) (Ide et al., 2008; Nikolova et al., 2010; Petermann et al., 2010). The ultimate goal of HRR is to assist a DNA molecule that has suffered sequence information loss as a result of damage to both strands, to retrieve this information from an undamaged homologous DNA sequence. To this end, damaged and undamaged DNA molecules will need to directly interact, i.e. to undergo synapsis. In particular, the damaged DNA molecule will need processing to generate DNA forms capable of "reading-off" sequence information. Also the chromatin structure on both molecules will need to be modified to facilitate the search for homologous regions in neighboring DNA molecules. Once homology has been found sequence information will need to be copied by appropriately directed DNA synthesis, and finally the synapsed molecules will need to be separated.

Because DSBs are frequently generated in the genome accidently, the cell needs to be prepared for their repair by maintaining sufficient pools of repair factors. Indeed, there is evidence that in eukaryotic cells the level of the repair proteins is higher compared to the level of other proteins of the cellular metabolism (Shrivastav et al., 2008). These pools may have a cell cycle component for repair pathways such as HRR that show preferential function in certain phases of the cell cycle.

In addition to the random induction of DSBs after accidental or intentional exposure to physical or chemical agents, cells also induce DSBs in their genome in a programmed fashion as part of certain differentiation programs. The differentiation of germ cells and of the cells of the hematopoietic system is a good example along these lines. Programmed DSBs for such functions are generated through the action of specific enzymes (Spo11 during meiosis and Rag1/Rag2 during V(D)J recombination). In general, these DSB inducing nucleases interact with components of the repair pathways that are associated with the proper recognition and processing of the generated DSBs (Keeney et al., 1997; McBlane et al., 1995; Oettinger, 1992). Although there is evidence that HR events may be initiated by a single-strand break (Metzger et al., 2011), it is widely accepted that the ultimate initiating event of homologous recombination is the DSB. This recognition implicates the DSBR and

SDSA repair models in the mechanistic foundation of DSB repair through homologous recombination (Brugmans et al., 2007; Pardo et al., 2009; Wyman & Kanaar, 2006).

To accommodate the specific requirements of DSB repair as mediated by DNA homology, HRR starts with the resection of DNA ends around the DSB, causing the formation of 3′-single stranded DNA (ssDNA) regions (West, 2003; Wyman & Kanaar, 2006). This form of DNA can invade and pair to homologous sequences present in an intact molecule and be directly extended by polymerization to copy missing sequence information (see below). Therefore, the effectiveness of HRR may be dictated by the ability of cells to execute end resection in a proper orientation, immediately after the generation of the DSB. In cells of higher eukaryotes the initial DNA end processing is orchestrated by the Mre11/Rad50/Nbs1 (MRN complex) (Fig. 2) (D'Amours & Jackson, 2002; Rupnik et al., 2010), assisted by the functions of recently identified resection promoting factor CtIP (Fig. 3) (Farah et al., 2009; Sartori et al., 2007). The MRN complex exhibits multiple activities many of which are implicated in HRR. Surprisingly, despite its nuclease activity, many reports pointed out that MRN may not be directly involved in the extensive resection of DNA ends to generate the 3′ ssDNA, suggesting that other enzymes with nuclease functions should fulfill this requirement (Longhese et al., 2010; Mimitou & Symington, 2009; Stewart et al., 2010). Thus MRN may have a regulatory role in the coordination between different DSB repair pathways (Borde & Cobb, 2009; Rupnik et al., 2010; Stracker & Petrini, 2011).

The MRN complex is one of the first proteins recruited to DSBs. It consists of the Mre11 nuclease, the Rad50 protein, an ATP-binding polypeptide with bridging functions through a coiled-coil motif and the Nbs1 protein, a polypeptide rich in protein-protein interaction domains (Fig. 2) (Stracker & Petrini, 2011).

The significance of the MRN complex in DSB repair and meiotic recombination was first shown in yeasts by genetic screening of mutants hypersensitive to DNA damaging agents (D'Amours & Jackson, 2002). After the cloning of the yeast *MRE11* and *RAD50* genes, homologues were identified in all model organisms (Ajimura et al., 1993; Chin & Villeneuve, 2001; Dolganov et al., 1996). In addition, it was shown that in higher eukaryotes dysfunction of *MRE11* underlies the ataxia-telangiectasia-like disorder (ATLD), implicating thus Mre11 in ATM dependent DSB repair and signaling pathways (Stewart et al., 1999). However, the identification of the human Xrs2 homolog (the third subunits of the yeast MRX complex) was hampered owing to its high sequence diversity between species.

Ultimately, it was shown that the gene mutated in the Nijmegen breakage syndrome, *NBS1,* is the human *XRS2* homolog, and that its product physically interacts with Mre11. Deficiency in Nbs1 causes the clinical phenotype characterized by hypersensitivity to DNA damaging agents generating DSBs, through defective repair and checkpoint activation (Digweed et al., 1999; Matsuura et al., 2004; Tauchi et al., 2002; Varon et al., 1998).

Mre11 is an 80 kDa protein that harbors three constitutive phosphoesterase N-terminal motifs and one phosphoesterase motif similar to the SbcD subunit of the SbcCD nuclease (Fig. 2). It acts as an endonuclease that cleaves hairpin structures, as well as an exonuclease that degrades linear double-stranded (ds) DNA molecules (Biroccio et al., 2011; Sachs et al., 2011). The Mre11 phosphoesterase motifs are folded into a nuclease domain that exhibits 3′-5′ dsDNA dependent exonuclease activity and single-stranded (ss) and dsDNA dependent endonuclease activity on a variety of DNA substrates (Paull & Gellert, 1998; Trujillo et al., 1998). Curiously, the nuclease activities of Mre11 are not appropriate for the end-resection step required during HRR and recent observations suggest that the exonuclease activity of Mre11 is not involved in extensive DSB end-processing (Llorente & Symington, 2004). Krogh

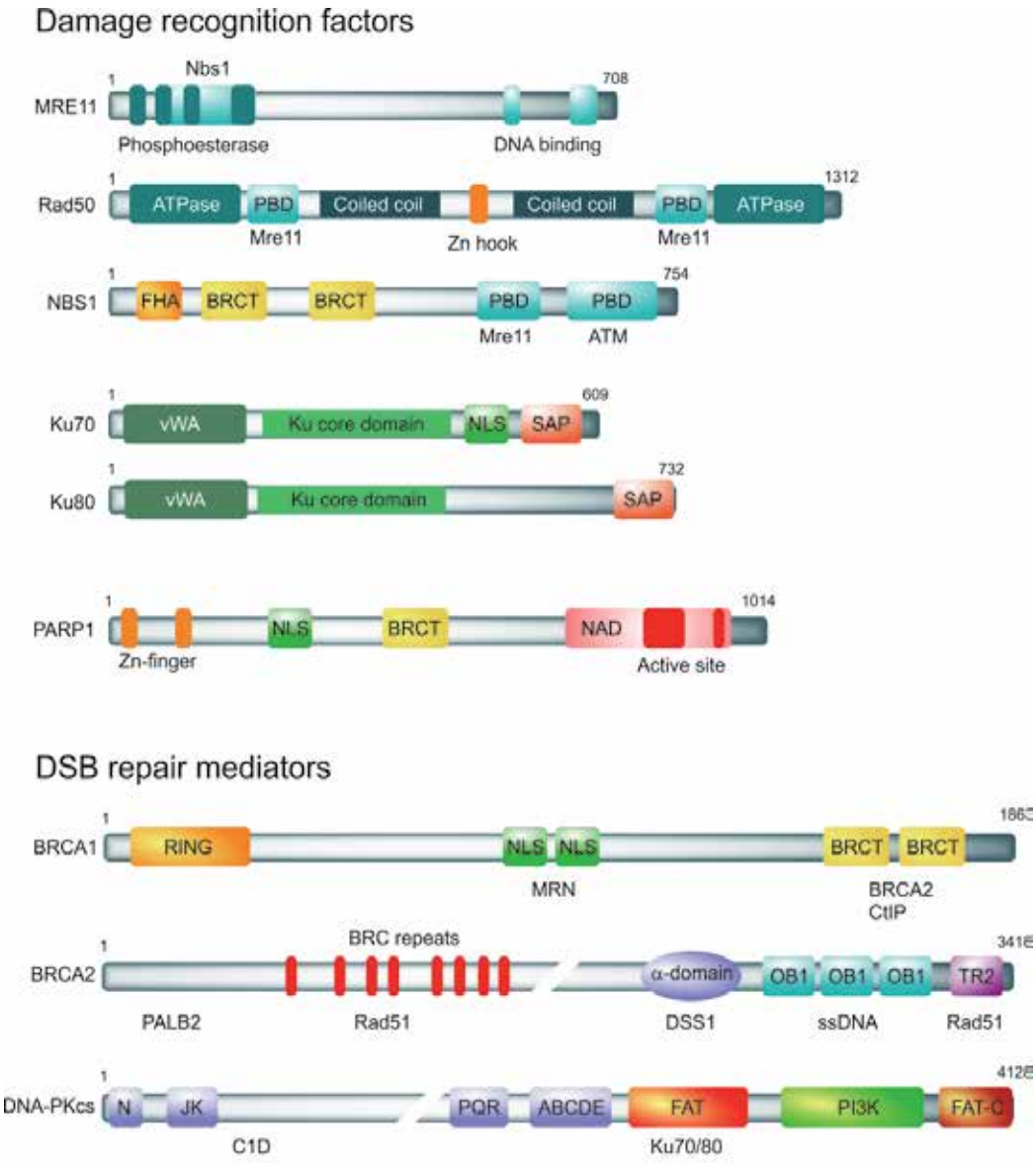

Fig. 2. Schematic representations of identified consensus domains in DSB repair proteins. Proteins participating in the initial steps of DSB repair and those considered to play a mediating role during signaling and repair are presented.

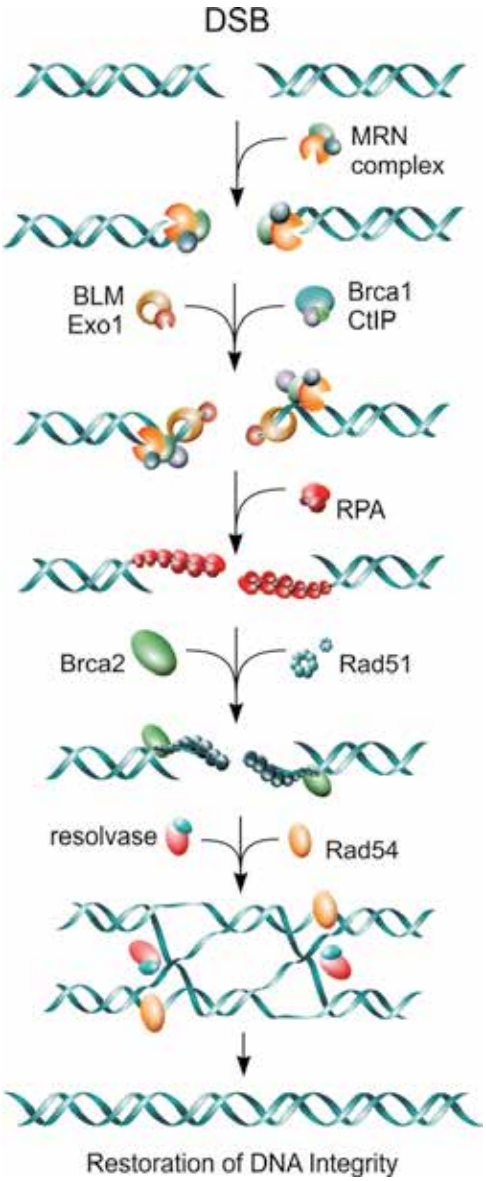

Fig. 3. Homology mediated repair of DSBs. The repair of DSBs by HRR is initiated by the resection of the DNA ends through the combined action of the MRN complex, the CtIP, ExoI and the BLM helicase, that catalyze the generation of 3' ssDNA regions and the formation of a Rad51 nucleoprotein filament - the structure involved in homology search. In subsequent steps and after localization of, and invasion into the homologous DNA region, repair synthesis is initiated and a HJ is generated from each DNA end, which in the end of the process is resolved by the resolvase complex.

and Symington have proposed a model, explaining the MRX (MRN) role in processing of DSB ends during meiosis or mitosis (Krogh & Symington, 2004). According to this model, Mre11 can execute its function through the cooperative action of an enzyme(s) with helicase and/or exonuclease activity. Through the action of such putative helicase the initial unwinding of DNA occurs, resulting in a formation of secondary DNA structures. In addition, Mre11 can process the 5' strand using its endonuclease activity by trimming the secondary DNA structures. Another enzyme with exonuclease activity might then catalyse the extensive resection of the 5′-end resulting in the generation of 3′-overhangs.

IR breaks the DNA molecule by damaging its sugar moiety, thus generating ends that are not amenable to ligation before processing. Mre11 may participate in such initial end processing but not in the final processing of the 5′ DNA strand. In agreement with this putative function, unmodified DSB ends generated by HO nuclease are substrates for nucleolytic enzymes even in the absence of active Mre11, suggesting that the nuclease function of MRN is only needed during the initial steps of the end-resection reaction. Studies in yeast have suggested that at least two nucleases (Exo1 and Dna2) in complex with a helicase (Sgs1) are involved in the end-resection step during HRR (Mimitou & Symington, 2009; Mimitou & Symington, 2008), but the functions of their mammalian homologs are incompletely characterized. However, it has been reported that the human homologue of the yeast resection factor Exo1, is important for the recruitment of RPA and Rad51 proteins through the generation of ssDNA regions. The potential role of Exo1 in HRR was also shown in experiments with Exo1 depleted cells, which develop hypersensitivity to ionizing radiation and show increased chromosomal instability (Bolderson et al., 2010). Moreover, the localization of Exo1 to DSBs depends on CtIP and MRN and its exonuclease activity is controlled by CtIP, supporting the idea that MRN is much more involved in the mediation of end-resection rather than in the direct digestion of 5′-DNA strand of the DSB (Eid et al., 2010).

After processing of the DNA ends, the generated single-stranded 3′-overhangs are covered by the RPA heterotrimer, the major mammalian ssDNA binding protein. During HRR, one of the functions of this protein is to protect ssDNA and to prevent the formation of secondary DNA structures (Fig. 3) (Fanning et al., 2006). However, RPA also mediates the recruitment of the ATR/ATRIP complex to the single stranded regions and initiates in this way the DDR signaling cascades, which among others inhibit cell cycle progression through activation of the corresponding checkpoints (Cimprich & Cortez, 2008; Zegerman & Diffley, 2009). Indeed, there is evidence that RPA functions as a checkpoint activator (Stephan et al., 2009), as well as a regulator of the repair process, possibly through shifts in its function by DNA damage-mediated post-translational modifications (Anantha et al., 2007; Vassin et al., 2009). RPA also facilitates indirectly Rad51 filament formation by mediating DNA-Rad52 or DNA-BRCA2 interactions (see below) (Mortensen et al., 2009; Thorslund & West, 2007). Genetic and biochemical data support the notion that HRR is driven by the proteins of *RAD52* epistasis group of genes, including Rad52, Rad54 and Rad51 with its paralogs (XRCC2, XRCC3, Rad51B, Rad51C and Rad51D) (Fig. 3) (Krogh & Symington, 2004; West, 2003). Along with its ability to promote the synapsis between the homologous DNA sequences, Rad51 arises as a central recombination protein facilitating in general the formation of hybrid DNA duplexes (Heyer et al., 2010). Rad51 interacts with RPA-coated single stranded 3′-overhangs to form a right-handed nucleoprotein filament. The nucleoprotein filament represents the active state of Rad51 recombinase and plays a pivotal role in the homology search reaction (Raderschall et al., 1999; West, 2003). The importance of

the *RAD51* gene in HRR was first shown in yeasts, where Rad51 deficiency is tolerated but Rad51 null cells exhibit an increased sensitivity to IR and to a variety of DNA damaging agents producing DSBs. These mutants also show defects in mitotic and meiotic recombination (Ofir et al., 2011; Shinohara et al., 1992).

The human *RAD51* gene was identified in 1993 by Morita et al., which have described a gene encoding a product sharing significant homology with bacterial RecA and ScRad51 recombinase (Morita et al., 1993). Despite extensive sequence similarity between human and yeast Rad51, the vertebrate Rad51 recombinase fails to complement the HR defects of yeast Rad51 mutants, suggesting evolutionary divergent properties for the two proteins (Shinohara et al., 1993). In contrast to lower eukaryotes, Rad51 is essential in vertebrates and *RAD51*$^{-/-}$ knockout mice die early during embryogenesis (Tsuzuki et al., 1996).

Rad51 exhibits ssDNA and dsDNA-stimulated ATPase activity, which drives the nucleation and extension of the Rad51 nucleoprotein filament. There is clear evidence that only Rad51-ssDNA nucleoprotein filament is able to catalyze DNA joint formation, supporting the assessment that Rad51 is recruited to ssDNA generated by nucleolytic processing of DNA termini (Ristic et al., 2005). Formation of Rad51 nucleoprotein filament also depends on a large number of factors controlling the effectiveness of HRR. One of the most important players mediating nucleation of Rad51 over DNA is Rad52 and BRCA2. While the Rad52 function is essential for yeast viability after IR (West, 2003), in mammalian cells BRCA2 substitutes Rad52 activity and plays more important role in the regulation of HRR than Rad52 (Davies et al., 2001; Thorslund & West, 2007; West, 2003). Nevertheless, both proteins are involved in the delivery of Rad51 monomers to the ssDNA overhangs in combination with the elimination of a negative effect of RPA on Rad51 filament formation (Thorslund & West, 2007). Notably, a BRCA2 homolog has not been identified in yeast, suggesting evolutionarily distinct requirements for HRR in yeast and in higher eukaryotes. The structural studies of BRCA2 and its orthologous proteins revealed a protein domain allowing DNA binding (Marmorstein et al., 2001; Zhou et al., 2009) and degenerative conservative motifs called the BRC repeats, physically interacting with Rad51 (Fig. 2) (Carreira et al., 2009; Carreira & Kowalczykowski, 2009; Davies et al., 2001). The structurally undisclosed TR2 motif in the extreme C-terminus of BRCA2 was found to play an important role in the regulation of Rad51 nucleoprotein filament formation and dissociation, and the binding of Rad51 to this motif is controlled by its phosphorylation at S3291 in a cell cycle dependent manner (Fig. 2) (Esashi et al., 2005; Esashi et al., 2007; Thorslund & West, 2007).

The formation of a Rad51 nucleoprotein filament marks the initiation of a pre-synaptic step of HRR, while strand invasion and the search for homology characterize the synaptic reaction (Fig. 3). After alignment of the invading DNA strand with the homologous DNA duplex, the chromatin remodeling functions of Rad54 and its homolog Rad54B operate to facilitate DNA synthesis and branch migration resulting in formation of double Holliday junctions when both processed 3′-overhangs invade the undamaged DNA molecule (Fig. 3) (Mazin et al., 2010). During the DNA repair synthesis step, sequence information is copied from the undamaged DNA molecule to the damaged one assisting thus its restoration. At the final stages of HRR the HJs are resolved by protein complexes identified as resolvases (Figure 3) (Liu et al., 2007; Mazina & Mazin, 2008; Symington & Holloman, 2008; West, 2009). While the resolution of the HJs by resolvases can lead to either gene conversion or crossing over, there is evidence that during repair of DSBs by HRR in higher eukaryotes gene conversion dominates.

3. When effectiveness is chosen over accuracy: DNA-PKcs dependent non-homologous end joining (D-NHEJ)

Perhaps surprisingly the above described high fidelity pathway of DSB repair, HRR, is utilized preferentially by prokaryotes and lower eukaryotes. Higher eukaryotes appear to preferentially utilize end joining to remove DSBs from their genome despite its error-prone nature. However, the reason for this preference remain hypothetical. Moreover, it is notable that vertebrates have extended their arsenal of end joining activities with a unique protein, the catalytic subunit of DNA-dependent protein kinase (DNA-PKcs) (Fig. 2), which is likely to play a role in this shift from HRR to NHEJ (Chen et al., 2011; Shibata et al., 2011). For this reason and in order to discriminate this pathway of NHEJ from other pathways of NHEJ that have recently surfaced (see below), we will refer to it in the remainder of this chapter as D-NHEJ. Other designations that can be found in the literature include "classical" or "canonical" NHEJ.

In principle, rejoining of DNA ends by NHEJ can be accomplished by standard ligation reactions, when ligatable DNA ends are present (Fig. 4). As pointed out above, simple end joining is not possible for IR induced DSBs, which therefore required end processing before ligation. In general, DSB repair by NHEJ is associated with limited or extensive additions or deletions of nucleotides at the generated junction, which alters the original DNA sequence at the damaged site (Marshall, 2011; van Gent & van der Burg, 2007). As a result, NHEJ is an error-prone repair pathway, which may be considered disadvantageous for higher eukaryotes, frequently opt for this repair mechanism. It is frequently reasoned that this risk may be mitigated by the excess of non-coding DNA in these organisms, which allows flexibility in terms of nucleotide substitutions, deletions or additions.

However, since DSBs are also generated randomly throughout the genome, they will also be induced in coding regions where changes in the nucleotide sequence are bound to have serious consequences.

Notably, there are also instances where the error prone nature of NHEJ is exploited to generate a specific biological result that requires sequence modification. Thus, addition or deletion of nucleotides during NHEJ associated with V(D)J recombination, increases the diversity of the antibodies generated (Lieber et al., 2006).

The importance of D-NHEJ factors in higher eukaryotes is indicated by diseases resulting from mutations in *Artemis*, *LIGIV* and *Cernunnos/XLF* genes. Thus, hypomorphic mutations in the *LIGIV* gene lead to severe immunodeficiency, radiosensitivity and developmental delay and account for the development of the LigIV syndrome (Chistiakov et al., 2009; Girard et al., 2004). Mutations in *Artemis* are associated with progressive radiosensitive severe combined immunodeficiency (RS-SCID) and patients with dysfunctional *Artemis* are characterized by increased radiosensitivity and impaired V(D)J recombination. DNA-PKcs deficient patients develop a classical SCID syndrome and show slight differences in their symptoms from *RAG* or *Artemis* deficient individuals (Hendrickson et al., 1991; Schuler & Bosma, 1989; van der Burg et al., 2009). The fact that most mutations in D-NHEJ genes are hypomorphic suggests that complete deletion of their activities is not compatible with human survival.

At the biochemical level, it is well documented that one of the most abundant cellular proteins, Ku, initiates NHEJ by binding to the ends generated at the DSBs (Lieber, 2010) (Fig. 4). Ku consists of two subunits, Ku70 and Ku86 (Ku80), which form a toroidal shaped

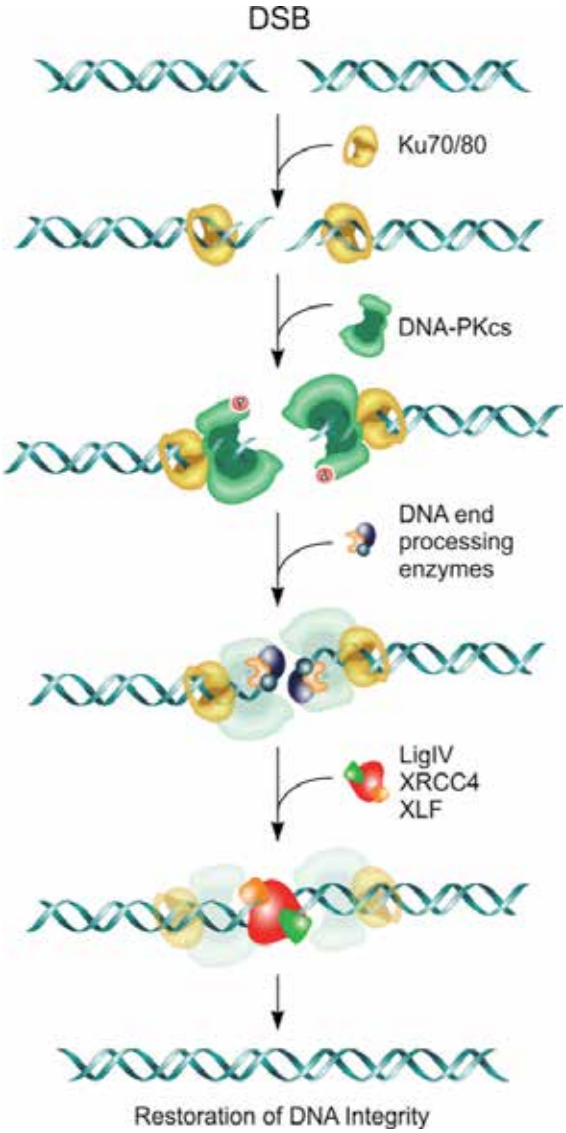

Fig. 4. Repair of DSBs by DNA-PKcs-dependent non homologous end joining. Major enzymatic activities involved in the repair of DSBs by the simple joining of the free DNA ends are depicted.

structure binding dsDNA with a variety of configurations at the ends. Once bound to DNA ends, Ku heterodimer changes its conformation and slides inward the DNA, thus attracting the catalytic subunit of DNA-PKcs to form an active DNA-PK holoenzyme (Meek, 2009; Meek et al., 2004). The activity of DNA-PKcs increases at least 10 fold upon interaction with the Ku-DNA complex (Lees-Miller & Meek, 2003). Therefore, D-NHEJ is greatly compromised in the absence of DNA-PKcs and, interestingly, under such conditions HRR is enhanced (Delacote et al., 2002; Shrivastav et al., 2009). However, more recent work points to inhibition of HRR in cells with altered or inhibited DNA-PKcs (Neal et al., 2011).

With its molecular weight of about 470 kDa, DNA-PKcs is by far the largest enzyme found to operate in D-NHEJ (Hill & Lee, 2010; Kirwan et al., 2011; Weterings & Chen, 2007). The enormous size of DNA-PKcs accommodates many important domains that may be involved in the regulation of its enzymatic activity and the interaction with other proteins (Fig. 2). DNA-PKcs is a serine/threonine kinase with specificity for S/TQ sites (Marshall, 2011) that regulates its activity through autophosphorylation. It targets, RPA2, WRN, Cernunnos/XLF, LigIV, and XRCC4 (Chen et al., 2000; Cruet-Hennequart et al., 2008; Otsuki et al., 2007; Soubeyrand et al., 2006; Yu et al., 2008). In addition to its catalytic function at the DSB ends, DNA-PKcs may also tether the broken DNA ends to facilitate rejoining (Meek et al., 2004). Although the DNA-PKcs kinase activity catalyzes the phosphorylation of many NHEJ related substrates, the phosphorylation of the DNA-PKcs itself is the only physiologically relevant event identified so far (Chen et al., 2000; Cruet-Hennequart et al., 2008; Otsuki et al., 2007; Soubeyrand et al., 2006; Yu et al., 2008). DNA-PKcs autophosphorylation appears to be important for DSB repair as DNA-PKcs mutated at key phosphorylation sites (T2609 and S2056 at ABCDE and PQR clusters respectively) is impaired in its function in D-NHEJ (Cui et al., 2005; Meek et al., 2007).

Elegant experiments demonstrate that DNA-PKcs autophosphorylation facilitates structural shifts, which allow other D-NHEJ end processing or ligation factors (polynucleotide kinase phosphatase, PNKP, terminal deoxynucleotidyl transferase, TDT, DNA polymerases λ and μ, LigIV/XRCC4/XLF complex) to be recruited to DNA ends (Kirwan et al., 2011). After end processing, two locally available DNA ends are joined through the coordinated action of the LigIV/XRCC4/XLF and the DNA-PK complexes and if the two sealed DNA ends originate from one DSB the integrity of the DNA molecule is restored (Ahnesorg et al., 2006; Wu et al., 2007; Yano et al., 2009) (Fig. 4). At present, it is not known whether as of yet uncharacterized functions incorporated into the NHEJ machinery have means of ensuring rejoining of the original ends. Available evidence is compatible with efficiently D-NHEJ joining any DNA ends, irrespectively of whether they belong to the same or to different DSBs. Such DNA end-promiscuity is considered the main cause of chromosomal translocations in repair proficient cells and may also contribute to the formation of chromosome aberrations in irradiated repair proficient cells (Iliakis et al., 2007).

4. An alternative pathway of non-homologous end joining with putative backup function (B-NHEJ)

Until relatively recently, D-NHEJ and HRR were considered as the only available pathways for removing DSBs from the genome. This raised the question of their coordination and labor separation under circumstances where both were active, as well as the function of each of them when the other was chemically or genetically compromised. The rationale was that when components of the one pathway were compromised residual DSB repair activity could be attributed to the function of the remaining active pathway. However, experiments testing this hypothesis failed to yield the expected results. Thus, although cells with mutations in genes encoding proteins involved in D-NHEJ exhibit a severe DSB repair defect, substantial residual rejoining is still detectable. Intriguingly, this rejoining activity does not rely on HRR, since cells with defects in this repair pathway show normal DSB repair as assayed by pulsed-field gel electrophoresis (PFGE) and HRR defects in D-NHEJ mutants fail to exacerbate their DSB repair phenotype. We speculated, therefore, the function of an additional DSB repair pathway based on end joining and functioning as backup (Fig. 5)

(DiBiase et al., 2000; Wang et al., 2003; Wang et al., 2001). This pathway is considered distinct and normally suppressed by D-NHEJ (Perrault et al., 2004), only coming to the fore whenever D-NHEJ is inactivated. We have proposed to term this form of end joining backup-NHEJ (B-NHEJ) in order to differentiate it from D-NHEJ and to indicate its putative backup function (Fig. 5) (Iliakis et al., 2004).

Extensive biochemical studies have provided evidence for the operation of B-NHEJ activities in *in vitro* end joining reactions. Thus, extracts of cells lacking DNA-PKcs showed normal end joining activity. However, the possible function of B-NHEJ to the repair of IR-induced DSBs received only limited attention until the demonstration that such pathways robustly substitute for D-NHEJ in class switch recombination in *LIG4* deficient mice (Soulas-Sprauel et al., 2007; Yan et al., 2007). Alternative pathways were also found to operate in V(D)J recombination in D-NHEJ deficient cells when mutations in *RAG1* and *RAG2* generate proteins forming DSBs without holding the DNA ends, which could then be processed by alternative repair pathways (Corneo et al., 2007; Jones & Simkus, 2009; Lee et al., 2004). Other reports subsequently showed near wild type CSR activity in *XRCC4* and *LIG4*-deficient mice associated with chromosome abnormalities at the *IgH* locus that hinted to the error prone nature of B-NHEJ (Soulas-Sprauel et al., 2007; Yan et al., 2007) (see below).

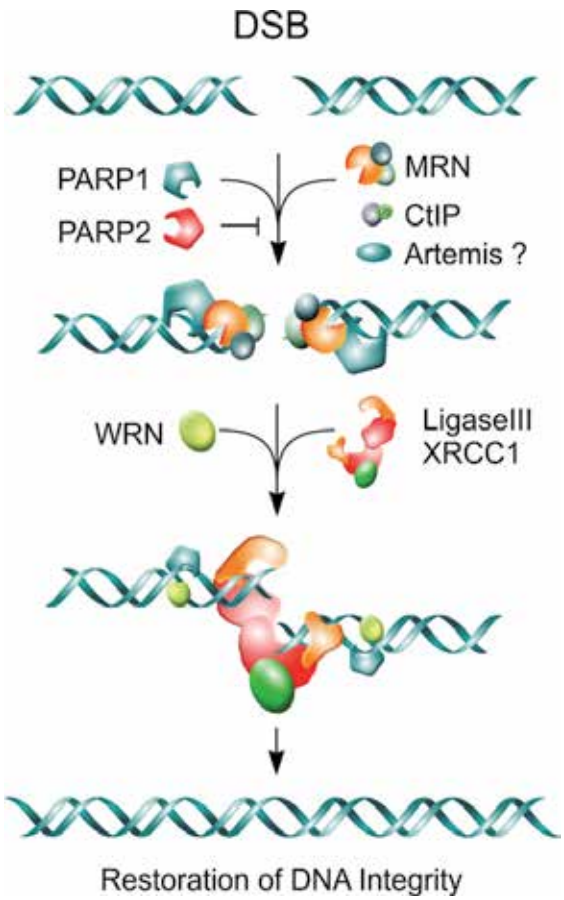

Fig. 5. Backup pathway of non homologous end joining.

These observations placed B-NHEJ not only at the forefront of DSB repair research but also at the center of carcinogenesis and led to an avalanche of studies describing its various characteristics. Various names were also proposed by different investigators including alternative NHEJ (A-NHEJ, or alt-NHEJ), microhomology-mediated end-joining (MMEJ), KU-independent end-joining, or LigIV-independent NHEJ (Corneo et al., 2007; Haber, 2008; Iliakis, 2009; Liang et al., 2008; Ma et al., 2003). We prefer to use the term B-NHEJ to emphasize its putative backup function. We anticipate that as the characterization of B-NHEJ proceeds and mechanistic information becomes available, better terms will develop reflecting important mechanistic properties of this repair pathway rather than arbitrarily selected phenomenological manifestations of the same.

A breakthrough in the characterization of factors involved in B-NHEJ was the identification of LigIII/XRCC1 complex as a key component (Wang et al., 2001a; Wang et al., 2001b). It is interesting that LigIII is only presented in vertebrates, where it also functions in the mitochondria (Ellenberger & Tomkinson, 2008; Tomkinson et al., 2006). Recent reports demonstrate that the essential functions of LigIII derive exclusively from its role in this organelle (Gao et al., 2011; Simsek et al., 2011). However, it remains open what contribution LigI might have on B-NHEJ and what kind of hierarchy exists between LigI and LigIII regarding to their functionality. LigIII has a broad substrate specificity and could participate in B-NHEJ via enzymatic activities outlined in the recently proposed "jackknife model" (Ellenberger & Tomkinson, 2008). According to this model the Zn-finger domain and the DNA-binding domain of LigIII act in a cooperative way to facilitate the ligation of DNA substrates with discontinuities in the sugar-phosphate backbone of the DNA.

As primary function of LigIII is considered the involvement in the repair of single-strand DNA breaks and base damages, where it operates with other proteins partners, it is therefore possible that the same interacting partners contribute to B-NHEJ. Principal candidate for promoting LigIII action during B-NHEJ, together with XRCC1, is Poly (ADP-ribose) polymerase 1 (PARP1). Indeed, we and others demonstrated that PARP1 operates in B-NHEJ (Audebert & Calsou, 2008; Audebert et al., 2006; Wang et al., 2006). It has also been demonstrated that PARP1 binds to DNA ends in direct competition with Ku heterodimer and may serve as a loading platform and coordinator of a subsequent steps of B-NHEJ (Wang et al., 2006). It is interesting in this regard that PARP1 and PARP2 are utilized in an AID-dependent manner during CSR in D-NHEJ deficient cells, suggesting that these proteins play a role in the processing of switch regions. However, it is not clear whether this function occurs as part of B-NHEJ (Robert et al., 2009). It is also notable that only PARP1 facilitates alternative end-joining mechanisms, while PARP2 actually suppresses translocations between *IgH* and *c-Myc* loci in D-NHEJ deficient B-lymphocytes (Robert et al., 2009).

Recently, histone H1 surfaced as an interesting factor involved in B-NHEJ (Rosidi et al., 2008). Although, histone H1 enhances the DNA end joining activities of both LigIV and LigIII, the enhancement of LigIII activity is significantly stronger. Further putative factors of B-NHEJ include the BCR/ABL protein. This protein is mutated in chronic myeloid leukaemia (CML), which results in stimulation of cellular proliferation, inhibition of apoptosis and altered cell adhesion. BCR/ABL may down-regulate D-NHEJ allowing thus the function of B-NHEJ. The latter would explain the genomic instability of leukemic cells (Poplawski & Blasiak, 2009). Other studies report a decrease in the level of key D-NHEJ proteins, Artemis and DNA LigIV and up-regulation of LigIII and Werner's syndrome protein (WRN) in CML cells (Sallmyr et al., 2008).

The frequent generation of non-ligatable DNA ends after IR suggests that resection might be involved in B-NHEJ. This function may be provided by end-processing factors like Mre11 and CtIP. Indeed, silencing of MRE11 in human fibroblasts decreases the frequency of global end-joining (Rass et al., 2009). Also, inhibition of MRE11 by mirin in XRCC4 and KU-deficient cells compromises B-NHEJ (Rass et al., 2009). Furthermore, mice lacking the entire MRN complex in their B cells are compromised in CSR mediated by both D-NHEJ and B-NHEJ (Dinkelmann et al., 2009). Recent reports also show that depletion of CtIP decreases the frequency of chromosomal translocations (Zhang & Jasin, 2011). Despite the above insights, the molecular mechanisms underpinning B-NHEJ are not fully understood and are at present under intensive investigation. Models have been proposed invoking microhomology (McVey & Lee, 2008). However, it is likely that the presence of microhomology at DNA ends is not a prerequisite for B-NHEJ; rather microhomology use may be a random event, determined by the nucleotide distribution along the dissected ends of the DSB (Simsek & Jasin, 2010). The level of dissection achieved is also likely to play a decisive role and the mechanisms regulating this dissection step deserve intensive investigation.

The accumulated data in the field allow the conclusion that deficiencies in the error-prone D-NHEJ pathway are associated with an increase in chromosomal translocations. Thus, B-NHEJ surfaces as a major determinant of chromosomal translocation formation in mammalian cells with potential contributions to carcinogenesis (Iliakis et al., 2007). Thus, B-cells deficient in D-NHEJ show frequent translocations between *IgH* and *c-Myc* loci (Boboila et al., 2010; Yan et al., 2007; Wang et al., 2009).

Notably, B-NHEJ appears to also be involved in telomere maintenance. Thus, depletion of TRF2, a known component of the shelterin complex, results in end-to-end chromosome fusions mediated by D-NHEJ, whereas depletion of TPP1–POT1a/b, another member of the shelterin complex, initiates robust chromosome fusions that are mediated by B-NHEJ (Rai et al., 2010).

In summary, B-NHEJ surfaces as an important pathway for the maintenance of the genome in higher eukaryotes. However, it exercises this function at the expense of fidelity. If modification of the sequence in the vicinity of the DSB is a weakness of D-NHEJ, hyper-recombination might be added as weakness of B-NHEJ.

5. Sharing the responsibility: coordination of DNA DSBs repair pathways and their cell cycle control and growth state dependencies

The fundamental differences between HRR and NHEJ and the error prone nature of the latter raise questions regarding their relative utilization and the principles applied to select one of them to repair a given DSB - beyond the obvious cell cycle specificity. This is because although HRR deficiency is not associated with a detectable defect in DSB repair as measured by PFGE, it is associated with increased radiosensitivity to killing, in many cases at magnitude similar to that observed with D-NHEJ mutants. This implies that HRR is a significant contributor to DSB repair. The following questions arise from the synthesis of these observations:

1. Why do cells opt for D-NHEJ for removing DSBs from their genome and how do they cope with induced changes in DNA sequence?
2. If HRR is involved in DSB repair, why is PFGE unable to detect a defect? Is it because it only processes a very small fraction (less than 10%) of the induced DSBs? Or is it

because unknown factors limit its contribution to low doses? Indeed, HRR mutants show a small defect in DSB repair when analyzed by scoring γ-H2AX foci (Rothkamm et al., 2003).

3. A frequently formulated hypothesis in the field is that D-NHEJ and HRR compete for DSBs and that pathway choice reflects the outcome of this competition. In line with this postulate, inactivation of NHEJ through mutations in the participating factors enhances some of the functions of HRR (Shrivastav et al., 2009). However competition between two so different repair mechanisms is difficult to rationalize. Are we missing something here? Notably, a situation reflecting competition between HRR and NHEJ is not detectable when the repair of IR induced DSBs is followed in different mutants (DiBiase et al., 2000; Wang et al., 2001)
4. The situation is now more complicated with the identification of B-NHEJ. How is B-NHEJ fitting in this overall picture and what does it really backs-up. Aspects of regulation of pathway selection and coordination are essential for our understanding of DSB repair and further investigations are warranted.

6. Conclusions

The importance of processing of DSBs generated in a programmed or accidental manner has been clearly demonstrated. Recent evidence emphasizes the role of a less known repair pathway, mechanistically and genetically distinct from the dominant DSB repair pathways, HRR and NHEJ. This pathway is capable of substituting almost entirely for D-NHEJ, but exhibits an increased propensity in rejoining wrong ends and in causing thus chromosome translocations. The characterization of factors involved in B-NHEJ and its integration to other pathways of DSB repair, as well as with DNA damage signaling and checkpoint activation, is expected to be vigorously pursued in the coming years. The fact that the function of B-NHEJ is associated with increased genomic instability leading to carcinogenesis is certain to provoke further investigations on the mechanisms of cancer development.

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8. References

Ahnesorg, P., Smith, P. & Jackson, S. P. (2006). XLF Interacts with the XRCC4-DNA Ligase IV Complex to Promote DNA Nonhomologous End-Joining. *Cell,* Vol.124, No.2, pp. 301-313

Ajimura, M., Leem, S.-H. & Ogawa, H. (1993). Identification of new genes required for meiotic recombination in *Saccharomyces cerevisiae. Genetics,* Vol.133, pp. 51-66

Anantha, R. W., Vassin, V. M. & Borowiec, J. A. (2007). Sequential and Synergistic Modification of Human RPA Stimulates Chromosomal DNA Repair. *Journal of Biological Chemistry,* Vol.282, No.49, pp. 35910-35923

Audebert, M. & Calsou, P. (2008). Effect of double-strand break DNA sequence on the PARP-1 NHEJ pathway. *Biochemical and Biophysical Research Communications,* Vol.369, pp. 982-988

Audebert, M., Salles, B., Weinfeld, M. & Calsou, P. (2006). Involvement of Polynucleotide Kinase in a Poly(ADP-ribose) Polymerase-1-dependent DNA Double-strand Breaks Rejoining Pathway. *Journal of Molecular Biology,* Vol.356, pp. 257-265

Baudat, F. & de Massy, B. (2007). Regulating double-stranded DNA break repair towards crossover or non-crossover during mammalian meiosis. *Chromosome Research,* Vol.15, No.5, pp. 565-577

Biroccio, A., Porru, M., Rizzo, A., Salvati, E., D'Angelo, C., Orlandi, A., Passeri, D., Franceschin, M., Stevens, M. F. G., Gilson, E., Beretta, G., Zupi, G., Pisano, C., Zunino, F. & Leonetti, C. (2011). DNA Damage Persistence as Determinant of Tumor Sensitivity to the Combination of Topo I Inhibitors and Telomere-Targeting Agents. *Clinical Cancer Research,* Vol.17, No.8, pp. 2227-2236

Boboila, C., Jankovic, M., Yan, C. T., Wang, J. H., Wesemann, D. R., Zhang, T., Fazeli, A., Feldman, L., Nussenzweig, A., Nussenzweig, M. & Alt, F. W. (2010). Alternative end-joining catalyzes robust IgH locus deletions and translocations in the combined absence of ligase 4 and Ku70. *Proceedings of the National Academy of Sciences of the United States of America,* Vol.107, No.7, pp. 3034-3039

Bolderson, E., Tomimatsu, N., Richard, D. J., Boucher, D., Kumar, R., Pandita, T. K., Burma, S. & Khanna, K. K. (2010). Phosphorylation of Exo1 modulates homologous recombination repair of DNA double-strand breaks. *Nucleic Acids Research,* Vol.38, No.6, pp. 1821-1831

Borde, V. & Cobb, J. (2009). Double functions for the Mre11 complex during DNA double-strand break repair and replication. *International Journal of Biochemistry & Cell Biology,* Vol.41, No.6, pp. 1249-1253

Brugmans, L., Kanaar, R. & Essers, J. (2007). Analysis of DNA double-strand break repair pathways in mice. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis,* Vol.614, No.1-2, pp. 95-108

Carreira, A., Hilario, J., Amitani, I., Baskin, R. J., Shivji, M. K. K., Venkitaraman, A. R. & Kowalczykowski, S. C. (2009). The BRC Repeats of BRCA2 Modulate the DNA-Binding Selectivity of RAD51. *Cell,* Vol.136, pp. 1032-1043

Carreira, A. & Kowalczykowski, S. C. (2009). BRCA2 Shining light on the regulation of DNA-binding selectivity by RAD51. *Cell Cycle,* Vol.8, No.21, pp. 3445-3447

Chen, L., Trujillo, K., Sung, P. & Tomkinson, A. E. (2000). Interactions of the DNA Ligase IV-XRCC4 Complex with DNA Ends and the DNA-dependent Protein Kinase. *Journal of Biological Chemistry,* Vol.275, No.34, pp. 26196-26205

Chen, X., Wong, J. Y. C., Wong, P. & Radany, E. H. (2011). Low-Dose Valproic Acid Enhances Radiosensitivity of Prostate Cancer through Acetylated p53-Dependent Modulation of Mitochondrial Membrane Potential and Apoptosis. *Molecular Cancer Research,* Vol.9, No.4, pp. 448-461

Chin, G. M. & Villeneuve, A. M. (2001). C. elegans mre-11 is required for meiotic recombination and DNA repair but is dispensable for the meiotic G(2) DNA damage checkpoint. *Genes & Development,* Vol.15, No.5, pp. 522-34.

Chistiakov, D. A., Voronova, N. V. & Chistiakov, A. P. (2009). Ligase IV syndrome. *European Journal of Medical Genetics,* Vol.52, No.6, pp. 373-378

Cimprich, K. A. & Cortez, D. (2008). ATR: an essential regulator of genome integrity. *Nature Reviews. Molecular Cell Biology,* Vol.9, No.8, pp. 616-627

Corneo, B., Wendland, R. L., Deriano, L., Cui, X., Klein, I. A., Wong, S.-Y., Arnal, S., Holub, A. J., Weller, G. R., Pancake, B. A., Shah, S., Brandt, V. L., Meek, K. & Roth, D. B. (2007). Rag mutations reveal robust alternative end joining. *Nature,* Vol.449, pp. 483-486

Cremer, T. & Cremer, C. (2001). Chromosome territories, nuclear architecture and gene regulation in mammalian cells. *Nature Reviews. Genetics,* Vol.2, pp. 292-301

Cruet-Hennequart, S., Glynn, M. T., Murillo, L. S., Coyne, S. & Carty, M. P. (2008). Enhanced DNA-PK-mediated RPA2 hyperphosphorylation in DNA polymerase [eta]-deficient human cells treated with cisplatin and oxaliplatin. *DNA Repair,* Vol.7, No.4, pp. 582-596

Cui, X., Yu, Y., Gupta, S., Cho, Y.-M., Lees-Miller, S. P. & Meek, K. (2005). Autophosphorylation of DNA-Dependent Protein Kinase Regulates DNA End Processing and May Also Alter Double-Strand Break Repair Pathway Choice. *Molecular and Cellular Biology,* Vol.25, No.24, pp. 10842-10852

D'Amours, D. & Jackson, S. P. (2002). The Mre11 Complex: At the Crossroads of DNA Repair and Checkpoint Signalling. *Nature Reviews,* Vol.3, pp. 317-327,

Davies, A. A., Masson, J. Y., McIlwraith, M. J., Stasiak, A. Z., Stasiak, A., Venkitaraman, A. R. & West, S. C. (2001). Role of BRCA2 in control of the RAD51 recombination and DNA repair protein. *Molecular Cell,* Vol.7, No.2, pp. 273-282

Delacote, F., Han, M., Stamato, T. D., Jasin, M. & Lopez, B. S. (2002). An xrcc4 defect or Wortmannin stimulates homologous recombination specifically induced by double-strand breaks in mammalian cells. *Nucleic Acids Research,* Vol.30, pp. 3454-3463,

DiBiase, S. J., Zeng, Z.-C., Chen, R., Hyslop, T., Curran, W. J., Jr. & Iliakis, G. (2000). DNA-dependent protein kinase stimulates an independently active, nonhomologous, end-joining apparatus. *Cancer Research,* Vol.60, pp. 1245-1253

Digweed, M., Reis, A. & Sperling, K. (1999). Nijmegen breakage syndrome: consequences of defective DNA double strand break repair. *BioEssays,* Vol.21, pp. 649-656

Dinkelmann, M., Spehalski, E., Stoneham, T., Buis, J., Wu, Y., Sekiguchi, J. M. & Ferguson, D. O. (2009). Multiple functions of MRN in end-joining pathways during isotype class switching. *Nature Structural & Molecular Biology,* Vol.16, No.8, pp. 808-813

Dolganov, G. M., Maser, R. S., Novikov, A., Tosto, L., Chong, S., Bressan, D. A. & Petrini, J. H. J. (1996). Human Rad50 Is Physically Associated with Human Mre11: Identification of a Conserved Multiprotein Complex Implicated in Recombinational DNA Repair. *Molecular and Cellular Biology,* Vol.16, pp. 4832-4841

Eid, W., Steger, M., El-Shemerly, M., Ferretti, L. P., Pena-Diaz, J., Konig, C., Valtorta, E., Sartori, A. A. & Ferrari, S. (2010). DNA end resection by CtIP and exonuclease 1 prevents genomic instability. *EMBO Reports,* Vol.11, No.12, pp. 897-984

Ellenberger, T. & Tomkinson, A. E. (2008). Eukaryotic DNA Ligases: Structural and Functional Insights. *Annual Review of Biochemistry,* Vol.77, pp. 313-338

Esashi, F., Christ, N., Gannon, J., Liu, Y., Hunt, T., Jasin, M. & West, S. C. (2005). CDK-dependent phosphorylation of BRCA2 as a regulatory mechanism for recombinational repair. *Nature,* Vol.434, pp. 598-604

Esashi, F., Galkin, V. E., Yu, X., Egelman, E. H. & West, S. C. (2007). Stabilization of RAD51 nucleoprotein filaments by the C-terminal region of BRCA2. *Nature Structural & Molecular Biology,* Vol.14, No.6, pp. 468-474

Fanning, E., Klimovich, V. & Nager, A. R. (2006). A dynamic model for replication protein A (RPA) function in DNA processing pathways. *Nucleic Acids Research,* Vol.34, No.15, pp. 4126-4137

Farah, J. A., Cromie, G. A. & Smith, G. R. (2009). Ctp1 and Exonuclease 1, alternative nucleases regulated by the MRN complex, are required for efficient meiotic recombination. *Proceedings of the National Academy of Sciences of the United States of America,* Vol.106, No.23, pp. 9356-9361

Ferguson, D. O. & Holloman, W. K. (1996). Recombinational repair of gaps in DNA is asymmetric in *Ustilago maydis* and can be explained by a migrating D-loop model. *Proceedings of the National Academy of Sciences of the United States of America,* Vol.93, pp. 5419-5424

Folle, G. A. (2008). Nuclear architecture, chromosome domains and genetic damage. *Mutation Research/Reviews in Mutation Research,* Vol.658, No.3, pp. 172-183

Franco, S., Alt, F. W. & Manis, J. P. (2006). Pathways that suppress programmed DNA breaks from progressing to chromosomal breaks and translocations. *DNA Repair,* Vol.5, No.9-10, pp. 1030-1041

Gao, Y., Katyal, S., Lee, Y., Zhao, J., Rehg, J. E., Russell, H. R. & McKinnon, P. J. (2011). DNA ligase III is critical for mtDNA integrity but not Xrcc1-mediated nuclear DNA repair. *Nature,* Vol.471, No.7337, pp. 240-244

Girard, P.-M., Kysela, B., Härer, C. J., Doherty, A. J. & Jeggo, P. A. (2004). Analysis of DNA ligase IV mutations found in LIG4 syndrome patients: the impact of two linked polymorphisms. *Human Molecular Genetics,* Vol.13, pp. 2369-2376

Haber, J. E. (2008). Alternative endings. *Proceedings of the National Academy of Sciences of the United States of America,* Vol.105, No.2, pp. 405-406

Hendrickson, E. A., Qin, X.-Q., Bump, E. A., Schatz, D. G., Oettinger, M. & Weaver, D. T. (1991). A link between double-strand break-related repair and V(D)J recombination: The *scid* mutation. *Proceedings of the National Academy of Sciences of the United States of America,* Vol.88, pp. 4061-4065

Heyer, W. D., Ehmsen, K. T. & Liu, J. (2010). Regulation of Homologous Recombination in Eukaryotes. *Annual Review of Genetics,* pp. 1545-2948 (Electronic)

Hill, R. & Lee, P. W. K. (2010). The DNA-dependent protein kinase (DNA-PK). *Cell Cycle,* Vol.9, No.17, pp. 3460-3469

Ide, H., Nakano, T., Salem, A. M. H., Terato, H., Pack, S. P. & Makino, K. (2008). Repair of DNA-protein crosslink damage: Coordinated actions of nucleotide excision repair and homologous recombination. *Nucleic Acids Symposium Series,* Vol.52, No.1, pp. 57-58

Iliakis, G. (2009). Backup pathways of NHEJ in cells of higher eukaryotes: Cell cycle dependence. *Radiotherapy and Oncology,* Vol.92, pp. 310-315

Iliakis, G., Wu, W., Wang, M., Terzoudi, G. I. & Pantelias, G. E. (2007). Backup Pathways of Nonhomologous End Joining May Have a Dominant Role in the Formation of Chromosome Aberrations. *Journal,* Vol. No. Issue, pp. 67-85

Jones, J. & Simkus, C. (2009). The roles of the RAG1 and RAG2 "non-core" regions in V(D)J recombination and lymphocyte development. *Archivum Immunologiae et Therapiae Experimentalis,* Vol.57, No.2, pp. 105-116

Keeney, S., Giroux, C. N. & Kleckner, N. (1997). Meiosis-specific DNA double-strand breaks are catalyzed by Spo11, a member of a widely conserved protein family. *Cell,* Vol.88, pp. 375-384

Kirwan, M., Beswick, R., Walne, A. J., Hossain, U., Casimir, C., Vulliamy, T. & Dokal, I. (2011). Dyskeratosis congenita and the DNA damage response. *British Journal of Haematology,* Vol.153, No.5, pp. 634-643,

Krogh, B. O. & Symington, L. S. (2004). Recombination Proteins in Yeast. *Annual Review of Genetics,* Vol.38, pp. 233-271

Lee, G. S., Neiditch, M. B., Salus, S. S. & Roth, D. B. (2004). RAG Proteins Shepherd Double-Strand Breaks to a Specific Pathway, Suppressing Error-Prone Repair, but RAG Nicking Initiates Homologous Recombination. *Cell,* Vol.117, pp. 171-184

Lees-Miller, S. P. & Meek, K. (2003). Repair of DNA double strand breaks by non-homologous end joining. *Biochimie,* Vol.85, pp. 1161-1173

Liang, L., Deng, L., Nguyen, S. C., Zhao, X., Maulion, C. D., Shao, C. & Tischfield, J. A. (2008). Human DNA ligases I and III, but not ligase IV, are required for microhomology-mediated end joining of DNA double-strand breaks. *Nucleic Acids Research,* Vol.36, No.10, pp. 3297-3310

Lieber, M. R. (2010). The Mechanism of Double-Strand DNA Break Repair by the Nonhomologous DNA End-Joining Pathway. *Annual Review of Biochemistry,* Vol.79, pp. 1.1-1.31

Lieber, M. R., Yu, K. & Raghavan, S. C. (2006). Roles of nonhomologous DNA end joining, V(D)J recombination, and class switch recombination in chromosomal translocations. *DNA Repair,* Vol.5, No.9-10, pp. 1234-1245

Liu, Y., Tarsounas, M., O'Regan, P. & West, S. C. (2007). Role of RAD51C and XRCC3 in Genetic Recombination and DNA Repair. *Journal of Biological Chemistry,* Vol.282, No.3, pp. 1973-1979

Liu, Y. & West, S. C. (2004). Happy Hollidays: 40th anniversary of the Holliday junction. *Nature Reviews. Molecular Cell Biology,* Vol.5, No.11, pp. 937-44, 1471-0072

Llorente, B. & Symington, L. S. (2004). The Mre11 Nuclease Is Not Required for 5' to 3' Resection at Multiple HO-Induced Double-Strand Breaks. *Molecular and Cellular Biology,* Vol.24, No.21, pp. 9682-9694

Longhese, M. P., Bonetti, D., Guerini, I., Manfrini, N. & Clerici, M. (2009). DNA double-strand breaks in meiosis: Checking their formation, processing and repair. *DNA Repair,* Vol.8, No.9, pp. 1127-1138

Longhese, M. P., Bonetti, D., Manfrini, N. & Clerici, M. (2010). Mechanisms and regulation of DNA end resection. *EMBO Journal,* Vol.29, No.17, pp. 2864-2874

Ma, J.-L., Kim, E. M., Haber, J. E. & Lee, S. E. (2003). Yeast Mre11 and Rad1 Proteins Define a Ku-Independent Mechanism To Repair Double-Strand Breaks Lacking Overlapping End Sequences. *Molecular and Cellular Biology,* Vol.23, pp. 8820-8828

Maizels, N. (2005). Immunoglobulin Gene Diversification. *Annual Review of Genetics,* Vol.39, No.1, pp. 23-46

Marmorstein, L. Y., Kinev, A. V., Chan, G. K., Bochar, D. A., Beniya, H., Epstein, J. A., Yen, T. J. & Shiekhattar, R. (2001). A human BRCA2 complex containing a structural

DNA binding component influences cell cycle progression. *Cell,* Vol.104, No.2, pp. 247-257,

Marshall, E. (2011). Cancer Research and The $90 Billion Metaphor. *Science,* Vol.331, pp. 1540-1541

Matsuura, S., Kobayashi, J., Tauchi, H. & Komatsu, K. (2004). Nijmegen Breakage Syndrome and DNA Double Strand Break Repair by NBS1 Complex. *Advances in Biophysics,* Vol.38, pp. 65-80

Mazin, A. V., Mazina, O. M., Bugreev, D. V. & Rossi, M. J. (2010). Rad54, the motor of homologous recombination. *DNA Repair,* Vol.9, No.3, pp. 286-302

Mazina, O. M. & Mazin, A. V. (2008). Human Rad54 protein stimulates human Mus81-Eme1 endonuclease. *Proceedings of the National Academy of Sciences of the United States of America,* Vol.105, No.47, pp. 18249-18254

McBlane, J. F., van Gent, D. C., Ramsden, D. A., Romeo, C., Cuomo, C. A., Gellert, M. & Oettinger, M. A. (1995). Cleavage at a V(D)J recombination signal requires only RAG1 and RAG2 proteins and occurs in two steps. *Cell,* Vol.83, pp. 387-395

McVey, M. & Lee, S. E. (2008). MMEJ repair of double-strand breaks (director's cut): deleted sequences and alternative endings. *Trends in Genetics,* Vol.24, No.11, pp. 529-538

Meek, K. (2009). New targets to translate DNA-PK signals. *Cell Cycle,* Vol.8, No.23, pp. 3809,

Meek, K., Douglas, P., Cui, X., Ding, Q. & Lees-Miller, S. P. (2007). trans Autophosphorylation at DNA-Dependent Protein Kinase's Two Major Autophosphorylation Site Clusters Facilitates End Processing but Not End Joining. *Molecular and Cellular Biology,* Vol.27, No.10, pp. 3881-3890

Meek, K., Gupta, S., Ramsden, D. A. & Lees-Miller, S. P. (2004). The DNA-dependent protein kinase: the director at the end. *Immunological Reviews,* Vol.200, No.1, pp. 132-141, 1600-065X

Metzger, M. J., McConnell-Smith, A., Stoddard, B. L. & Miller, A. D. (2011). Single-strand nicks induce homologous recombination with less toxicity than double-strand breaks using an AAV vector template. *Nucleic Acids Research,* Vol.39, No.3, pp. 426-435

Mimitou, E. P. & Symington, L. S. (2009). DNA end resection: Many nucleases make light work. *DNA Repair,* Vol.8, No.9, pp. 983-995

Mimitou, E. P. & Symington, L. S. (2008). Sae2, Exo1 and Sgs1 collaborate in DNA double-strand break processing. *Nature,* Vol.455, No.7214, pp. 770-774

Morita, T., Yoshimura, Y., Yamamoto, A., Murata, K., Mori, M., Yamamoto, H. & Matsushiro, A. (1993). A mouse homolog of the escherichia coli recA and saccharomyces cerevisiae RAD51 genes. *Proceedings of the National Academy of Sciences of the United States of America,* Vol.90, pp. 6577-6580

Mortensen, U. H., Lisby, M. & Rothstein, R. (2009). Rad52. *Current Biology,* Vol.19, No.16, pp. R676-R677

Neal, J. A., Dang, V., Douglas, P., Wold, M. S., Lees-Miller, S. P. & Meek, K. (2011). Inhibition of Homologous Recombination by DNA-Dependent Protein Kinase Requires Kinase Activity, Is Titratable, and Is Modulated by Autophosphorylation. *Molecular and Cellular Biology,* Vol.31, No.8, pp. 1719-1733

Nikolova, T., Ensminger, M., Löbrich, M. & Kaina, B. (2010). Homologous recombination protects mammalian cells from replication-associated DNA double-strand breaks

arising in response to methyl methanesulfonate. *DNA Repair,* Vol.9, No.10, pp. 1050-1063

Oettinger, M. A. (1992). Activation of V(D)J recombination by *RAG1* and *RAG2*. *Trends in Genetics,* Vol.8, pp. 413-416

Ofir, M., Hacohen, D. & Ginsberg, D. (2011). miR-15 and miR-16 Are Direct Transcriptional Targets of E2F1 that Limit E2F-Induced Proliferation by Targeting Cyclin E. *Molecular Cancer Research,* Vol.9, No.4, pp. 440-447

Otsuki, M., Seki, M., Kawabe, Y.-i., Inoue, E., Dong, Y. P., Abe, T., Kato, G., Yoshimura, A., Tada, S. & Enomoto, T. (2007). WRN counteracts the NHEJ pathway upon camptothecin exposure. *Biochemical and Biophysical Research Communications,* Vol.355, No.2, pp. 477-482

Pardo, B., Gómez-González, B. & Aguilera, A. (2009). DNA double-strand break repair: how to fix a broken relationship. *Cellular and Molecular Life Sciences,* Vol.66, No.6, pp. 1039-1056

Paull, T. T. & Gellert, M. (1998). The 3' to 5' exonuclease activity of Mre11 facilitates repair of DNA double-strand breaks. *Molecular Cell,* Vol.1, pp. 969-979

Petermann, E., Orta, M. L., Issaeva, N., Schultz, N. & Helleday, T. (2010). Hydroxyurea-Stalled Replication Forks Become Progressively Inactivated and Require Two Different RAD51-Mediated Pathways for Restart and Repair. *Molecular Cell,* Vol.37, No.4, pp. 492-502

Poplawski, T. & Blasiak, J. (2009). BCR/ABL downregulates DNA-PKCS-dependent and upregulates backup non-homologous end joining in leukemic cells. *Molecular Biology Reports,* Vol.in press, pp. doi:10.1007/s11033-009-9730-0

Raderschall, E., Golub, E. I. & Haaf, T. (1999). Nuclear foci of mammalian recombination proteins are located at single-stranded DNA regions formed after DNA damage. *Proceedings of the National Academy of Sciences, (USA),* Vol.96, pp. 1921-1926

Rai, R., Zheng, H., He, H., Luo, Y., Multani, A., Carpenter, P. B. & Chang, S. (2010). The function of classical and alternative non-homologous end-joining pathways in the fusion of dysfunctional telomeres. *EMBO Journal,* Vol.29, No.15, pp. 2598-610, 1460-2075, 0261-4189

Rass, E., Grabarz, A., Plo, I., Gautier, J., Bertrand, P. & Lopez, B. S. (2009). Role of Mre11 in chromosomal nonhomologous end joining in mammalian cells. *Nature Structural & Molecular Biology,* Vol.16, No.8, pp. 819-24, 1545-9985, 1545-9985

Ristic, D., Modesti, M., van der Heijden, T., van Noort, J., Dekker, C., Kanaar, R. & Wyman, C. (2005). Human Rad51 filaments on double- and single-stranded DNA: correlating regular and irregular forms with recombination function. *Nucleic Acids Research,* Vol.33, No.10, pp. 3292-3302

Robert, I., Dantzer, F. & Reina-San-Martin, B. (2009). Parp1 facilitates alternative NHEJ, whereas Parp2 suppresses IgH/c-myc translocations during immunoglobulin class switch recombination. *Journal of Experimental Medicine,* Vol.206, No.5, pp. 1047-1056,

Rosidi, B., Wang, M., Wu, W., Sharma, A., Wang, H. & Iliakis, G. (2008). Histone H1 functions as a stimulatory factor in backup pathways of NHEJ. *Nucleic Acids Research,* Vol.36, No.5, pp. 1610-1623

Rothkamm, K., Krüger, I., Thompson, L. H. & Löbrich, M. (2003). Pathways of DNA Double-Strand Break Repair during the Mammalian Cell Cycle. *Molecular and Cellular Biology,* Vol.23, pp. 5706-5715

Rupnik, A., Lowndes, N. & Grenon, M. (2010). MRN and the race to the break. *Chromosoma,* Vol.119, No.2, pp. 115-135

Sachs, R. K., Johnsson, K., Hahnfeldt, P., Luo, J., Chen, A. & Hlatky, L. (2011). A Multicellular Basis for the Origination of Blast Crisis in Chronic Myeloid Leukemia. *Cancer Research,* Vol.71, No.8, pp. 2838-2847

Sallmyr, A., Tomkinson, A. E. & Rassool, F. V. (2008). Up-regulation of WRN and DNA ligase IIIα in chronic myeloid leukemia: consequences for the repair of DNA double-strand breaks. *Blood,* Vol.112, No.4, pp. 1413-1423

San Filippo, J., Sung, P. & Klein, H. (2008). Mechanism of Eukaryotic Homologous Recombination. *Annual Review of Biochemistry,* Vol.77, pp. 229-257

Sancar, A., Lindsey-Boltz, L. A., Ünsal-Kacmaz, K. & Linn, S. (2004). Molecular Mechanisms of Mammalian DNA Repair and the DNA Damage Checkpoints. *Annual Review of Biochemistry,* Vol.73, pp. 39-85

Sartori, A. A., Lukas, C., Coates, J., Mistrik, M., Fu, S., Bartek, J., Baer, R., Lukas, J. & Jackson, S. P. (2007). Human CtIP promotes DNA end resection. *Nature,* Vol.450, pp. 509-514,

Schuler, W. & Bosma, M. J. (1989). Nature of the *scid* defect: a defective VDJ recombinase system. *Current Topics in Microbiology and Immunology,* Vol.152, pp. 55-62

Shibata, A., Conrad, S., Birraux, J., Geuting, V., Barton, O., Ismail, A., Kakarougkas, A., Meek, K., Taucher-Scholz, G., Lobrich, M. & Jeggo, P. A. (2011). Factors determining DNA double-strand break repair pathway choice in G2 phase. *EMBO Journal,* Vol.30, No.6, pp. 1079-1092

Shinohara, A., Ogawa, H., Matsuda, Y., Ushio, N., Ikeo, K. & Ogawa, T. (1993). Cloning of human, mouse and fission yeast recombination genes homologous to RAD51 and recA. *Nature Genetics,* Vol.4, pp. 239-243

Shinohara, A., Ogawa, H. & Ogawa, T. (1992). Rad51 protein involved in repair and recombination in S. cerevisiae is a RecA-like protein. *Cell,* Vol.69, pp. 457-470

Shrivastav, M., De Haro, L. P. & Nickoloff, J. A. (2008). Regulation of DNA double-strand break repair pathway choice. *Cell Research,* Vol.18, No.1, pp. 134-147

Shrivastav, M., Miller, C. A., De Haro, L. P., Durant, S. T., Chen, B. P. C., Chen, D. J. & Nickoloff, J. A. (2009). DNA-PKcs and ATM co-regulate DNA double-strand break repair. *DNA Repair,* Vol.8, No.8, pp. 920-929

Simsek, D., Furda, A., Gao, Y., Artus, J., Brunet, E., Hadjantonakis, A.-K., Van Houten, B., Shuman, S., McKinnon, P. J. & Jasin, M. (2011). Crucial role for DNA ligase III in mitochondria but not in Xrcc1-dependent repair. *Nature,* Vol.471, No.7337, pp. 245-248

Simsek, D. & Jasin, M. (2010). Alternative end-joining is suppressed by the canonical NHEJ component Xrcc4-ligase IV during chromosomal translocation formation. *Nature Structural & Molecular Biology,* Vol.17, No.4, pp. 410-416

Soubeyrand, S., Pope, L., De Chasseval, R., Gosselin, D., Dong, F., de Villartay, J.-P. & Hache, R. J. G. (2006). Artemis Phosphorylated by DNA-dependent Protein Kinase Associates Preferentially with Discrete Regions of Chromatin. *Journal of Molecular Biology,* Vol.358, No.5, pp. 1200-1211

Soulas-Sprauel, P., Le Guyader, G., Rivera-Munoz, P., Abramowski, V., Olivier-Martin, C., Goujet-Zalc, C., Charneau, P. & de Villartay, J.-P. (2007). Role for DNA repair factor

XRCC4 in immunoglobulin class switch recombination. *Journal of Experimental Medicine,* Vol.204, No.7, pp. 1717-1727

Stephan, H., Concannon, C., Kremmer, E., Carty, M. P. & Nasheuer, H.-P. (2009). Ionizing radiation-dependent and independent phosphorylation of the 32-kDa subunit of replication protein A during mitosis. *Nucleic Acids Research,* Vol.37, No.18, pp. 6028-6041

Stewart, G. S., Maser, R. S., Stankovic, T., Bressan, D. A., Kaplan, M. I., Jaspers, N. G., Raams, A., Byrd, P. J., Petrini, J. H. & Taylor, A. M. (1999). The DNA double-strand break repair gene hMRE11 is mutated in individuals with an ataxia-telangiectasia-like disorder. *Cell,* Vol.99, No.6, pp. 577-587

Stewart, J. A., Campbell, J. L. & Bambara, R. A. (2010). Dna2 is a structure-specific nuclease, with affinity for 5'-flap intermediates. *Nucleic Acids Research,* Vol.38, No.3, pp. 920-930

Stracker, T. H. & Petrini, J. H. J. (2011). The MRE11 complex: starting from the ends. *Nature Reviews. Molecular Cell Biology,* Vol.12, No.2, pp. 90-103

Symington, L. S. & Holloman, W. K. (2008). Resolving Resolvases: The Final Act? *Molecular Cell,* Vol.32, pp. 603-604

Szostak, J. W., Orr-Weaver, T. L., Rothstein, R. J. & Stahl, F. W. (1983). The double-strand-break repair model for recombination. *Cell,* Vol.33, pp. 25-35

Tauchi, H., Matsuura, S., Kobayashi, J., Sakamoto, S. & Komatsu, K. (2002). Nijmegen breakage syndrome gene, NBS1, and molecular links to factors for genome stability. *Oncogene,* Vol.21, pp. 8967-8980

Thorslund, T. & West, S. C. (2007). BRCA2: a universal recombinase regulator. *Oncogene,* Vol.26, No.56, pp. 7720-7730

Tomkinson, A. E., Vijayakumar, S., Pascal, J. M. & Ellenberger, T. (2006). DNA Ligases: Structure, Reaction Mechanism, and Function. *Chemical Reviews,* Vol.106, No.2, pp. 687-699, 0009-2665

Trujillo, K. M., Yuan, S.-S., Lee, E. Y.-H. & Sung, P. (1998). Nuclease activities in a complex of human recombination and DNA repair factors Rad50, Mre11, and p95. *Journal of Biological Chemistry,* Vol.273, pp. 21447-21450

Tsuzuki, T., Fujii, Y., Sakumi, K., Tominaga, Y., Nakao, K., Sekiguchi, M., Matsushiro, A., Yoshimura, Y. & Morita, T. (1996). Targeted disruption of the Rad51 gene leads to leathality in embryonic mice. *Proceedings of the National Academy of Sciences of the United States of America,* Vol.93, pp. 6236-6240

van der Burg, M., IJspeert, H., Verkaik, N. S., Turul, T., Wiegant, W. W., Morotomi-Yano, K., Mari, P.-O., Tezcan, I., Chen, D. J., Zdienicka, M. Z., van Dongen, J. J. M. & van Gent, D. C. (2009). A DNA-PKcs mutation in a radiosensitive T^-B^- SCID patient inhibits Artemis activation and nonhomologous end-joining. *Journal of Clinical Investigation,* Vol.119, No.1, pp. 91-98

van Gent, D. C. & van der Burg, M. (2007). Non-homologous end-joining, a sticky affair. *Oncogene,* Vol.26, No.56, pp. 7731-7740

Varon, R., Vissinga, C., Platzer, M., Cerosaletti, K. M., Chrzanowska, K. H., Saar, K., Beckmann, G., Seemanova, E., Cooper, P. R., Nowak, N. J., Stumm, M., Weemaes, C. M. R., Gatti, R. A., Wilson, R. K., Digweed, M., Rosenthal, A., Sperling, K., Concannon, P. & Reis, A. (1998). Nibrin, a novel DNA double-strand break repair protein, is mutated in Nijmegen breakage syndrome. *Cell,* Vol.93, pp. 467-476

Vassin, V. M., Anantha, R. W., Sokolova, E., Kanner, S. & Borowiec, J. A. (2009). Human RPA phosphorylation by ATR stimulates DNA synthesis and prevents ssDNA accumulation during DNA-replication stress. *Journal of Cell Science,* Vol.122, No.22, pp. 4070-4080

Wang, H., Perrault, A. R., Takeda, Y., Qin, W., Wang, H. & Iliakis, G. (2003). Biochemical evidence for Ku-independent backup pathways of NHEJ. *Nucleic Acids Research,* Vol.31, pp. 5377-5388

Wang, H., Zeng, Z.-C., Bui, T.-A., Sonoda, E., Takata, M., Takeda, S. & Iliakis, G. (2001). Efficient rejoining of radiation-induced DNA double-strand breaks in vertebrate cells deficient in genes of the RAD52 epistasis group. *Oncogene,* Vol.20, pp. 2212-2224

Wang, H., Zhao-Chong, Z., Perrault, A. R., Cheng, X., Qin, W. & Iliakis, G. (2001). Genetic evidence for the involvement of DNA ligase IV in the DNA-PK-dependent pathway of non-homologous end joining in mammalian cells. *Nucleic Acids Research,* Vol.29, pp. 1653-1660

Wang, J. H., Gostissa, M., Yan, C. T., Goff, P., Hickernell, T., Hansen, E., Difilippantonio, S., Wesemann, D. R., Zarrin, A. A., Rajewsky, K., Nussenzweig, A. & Alt, F. W. (2009). Mechanisms promoting translocations in editing and switching peripheral B cells. *Nature,* Vol.460, No.7252, pp. 231-236

Wang, M., Wu, W., Wu, W., Rosidi, B., Zhang, L., Wang, H. & Iliakis, G. (2006). PARP-1 and Ku compete for repair of DNA double strand breaks by distinct NHEJ pathways. *Nucleic Acids Research,* Vol.34, No.21, pp. 6170-6182

West, S. C. (2003). Molecular views of recombination proteins and their control. *Nature Reviews. Molecular Cell Biology,* Vol.4, pp. 1-11

West, S. C. (2009). The search for a human Holliday junction resolvase. *Biochemical Society Transactions,* Vol.37, pp. 519-526

Weterings, E. & Chen, D. J. (2007). DNA-dependent protein kinase in nonhomologous end joining: a lock with multiple keys? *Journal of Cell Biology,* Vol.179, No.2, pp. 183-186

Wu, P.-Y., Frit, P., Malivert, L., Revy, P., Biard, D., Salles, B. & Calsou, P. (2007). Interplay between Cernunnos-XLF and Nonhomologous End-joining Proteins at DNA Ends in the Cell. *Journal of Biological Chemistry,* Vol.282, No.44, pp. 31937-31943

Wyman, C. & Kanaar, R. (2006). DNA Double-Strand Break Repair: All's Well that Ends Well. *Annual Review of Genetics,* Vol.40, No.1, pp. 363-383

Yan, C. T., Boboila, C., Souza, E. K., Franco, S., Hickernell, T. R., Murphy, M., Gumaste, S., Geyer, M., Zarrin, A. A., Manis, J. P., Rajewsky, K. & Alt, F. W. (2007). IgH class switching and translocations use a robust non-classical end-joining pathway. *Nature,* Vol.449, pp. 478-482

Yano, K.-i., Morotomi-Yano, K. & Akiyama, H. (2009). Cernunnos/XLF: A new player in DNA double-strand break repair. *International Journal of Biochemistry & Cell Biology,* Vol.41, No.6, pp. 1237-1240

Yu, Y., Mahaney, B. L., Yano, K.-I., Ye, R., Fang, S., Douglas, P., Chen, D. J. & Lees-Miller, S. P. (2008). DNA-PK and ATM phosphorylation sites in XLF/Cernunnos are not required for repair of DNA double strand breaks. *DNA Repair,* Vol.7, No.10, pp. 1680-1692

Zegerman, P. & Diffley, J. F. X. (2009). DNA replication as a target of the DNA damage checkpoint. *DNA Repair,* Vol.8, No.9, pp. 1077-1088,

Zhang, Y. & Jasin, M. (2011). An essential role for CtIP in chromosomal translocation formation through an alternative end-joining pathway. *Nature Structural & Molecular Biology,* Vol.18, No.1, pp. 80-84

Zhou, Q., Kojic, M. & Holloman, W. K. (2009). DNA-binding Domain within the Brh2 N Terminus Is the Primary Interaction Site for Association with DNA. *Journal of Biological Chemistry,* Vol.284, No.13, pp. 8265-8273

9

Human CtIP and Its Homologs: Team Players in DSB Resection Games

Yasuhiro Tsutsui, Akihito Kawasaki and Hiroshi Iwasaki
Department of Life Science, School and Graduate School of Bioscience and Biotechnology, Tokyo Institute of Technology
Japan

1. Introduction

Double-strand breaks (DSBs) are among the most serious forms of DNA damage and thus must be efficiently repaired. In order for effective repair to take place, cells must employ the following steps: 1) recognition of the DSBs, 2) cell-cycle arrest via checkpoint activation, and 3) repair of the breaks. In the repair step, DSB ends are nucleolytically processed, which leads to the subsequent recruitment of appropriate repair proteins. Several proteins, including the Mre11 nuclease, are known to be involved in the processing of DSB ends. Additionally, recent studies have identified human CtIP and its orthologs as novel components required for DNA end processing among eukaryotes. This protein is involved not only in repair via homologous recombination (HR) but also in several important biological processes, such as transcriptional regulation and checkpoint control. Importantly, CtIP acts as a tumor suppressor in mammals. In this chapter, we will summarize the existing knowledge on this multi-functional molecule.

2. Identification of CtIP

2.1 DSB repair pathways

DSBs could lead to chromosomal aberrations, the disruption of genome integrity, and tumorigenesis in higher eukaryotes. DSBs are generated either by exogenous sources such as gamma-irradiation or by endogenous factors such as replication fork collapse. In addition, programmed DSBs are induced during meiosis at several loci, known as recombination hot spots. While such programmed DSBs are repaired by error-free HR, incidentally-generated DSBs are repaired by one of two major DNA repair pathways: HR or error-prone non-homologous end-joining (NHEJ), both of which are highly conserved from yeast to humans. NHEJ and HR function predominantly in cell cycle phases G_1 and S/G_2, respectively. DSBs are repaired using intact homologous sequences (sister-chromatids or homologs) as a template in HR, whereas in NHEJ, the broken ends are directly rejoined.

When DSBs occur, a protein complex called MRN(X) (see below for details) is recruited at the DSB ends and activates the DNA damage checkpoint. The Ku70-Ku80 heterodimer, involved in the NHEJ pathway, is also recruited. The ends are then nucleolytically processed by the MRN(X) complex in a process called “DNA end resection” or simply “resection”, leading to the conversion of “dirty ends” to repairable “clean ends”. Human

CtIP and its orthologs, together with the MRN(X) complex, play a critical role in this DNA end processing.

2.2 Several Y2H screenings with different 'baits' identified CtIP

Human CtIP was first identified by a yeast two-hybrid (Y2H) assay as one of the interacting proteins of CtBP (**C** **t**erminus-**b**inding **p**rotein), which is a transcriptional corepressor (Schaeper et al., 1998). CtBP binds to the C-terminal PLDLS motif of adenovirus E1A, resulting in anti-tumorigenic activity (Schaeper et al., 1995; Boyd et al., 1993). CtIP was also identified as RBBP8 in another Y2H screen in which the retinoblastoma (Rb) protein, a tumor suppressor with a protein-binding "pocket" domain, was applied as a bait (Fusco et al., 1998). CtIP/RBBP8 contains the LECEE sequence, known as an Rb-binding domain, which is required for interaction with Rb in a Y2H system (Fusco et al., 1998). CtIP was also found to associate with Rb-related protein p130 in a different Y2H screening (Meloni et al., 1999).

The breast and ovarian tumor suppressor BRCA1 has important functions in cell cycle checkpoint control and DNA repair. Two tandem BRCA1 C-terminal (BRCT) motifs are essential for the tumor suppression activity of BRCA1. The BRCT motifs of BRCA1 have also been shown to interact with CtIP both *in vivo* and *in vitro* (Li et al., 1999; Wong et al., 1998; Yu et al., 1998).

CtIP has also been isolated in two independent Y2H assays with Ikaros and LMO4 used as bait proteins (Koipally and Georgopoulos, 2002; Sum et al., 2002). Ikaros is a zinc finger protein that plays key roles in hemolymphoid development and homeostasis (Koipally and Georgopoulos, 2002). LMO4 belongs to the LIM-only (LMO) group of transcriptional regulators (Sum et al., 2002).

2.3 Identification of CtIP homologs

Sae2/Com1 was identified in the budding yeast *Saccharomyces cerevisiae* (Sc) from two independent genetic screens for mutants showing sporulation in the absence of Spo11 (McKee and Kleckner, 1997b; 1997a; Prinz et al., 1997). However, in ten years, no structurally or functionally similar Sae2/Com1 homologs have been reported in any organism other than *Saccharomyces*. In 2007, Russell and colleagues identified the *ctp1*$^{+}$ gene when investigating a subclass of cell-cycle-regulated genes in *Schizosaccharomyces pombe* (Sp) (Limbo et al., 2007). A database search revealed that SpCtp1 is homologous to proteins that have previously been characterized in several species such as ScSae2/Com1, COM-1 in *Caenorhabditis elegans* (Ce), GR1 from *Arabidopsis thaliana* (At), and human CtIP (Limbo et al., 2007) (Fig. 1A).

SpCtp1 was also found to be coded by an *slr* (synthetically lethal with *rad2*Δ) gene and interacts genetically with the Nbs1 protein. Thus, it was originally termed as Nip1 (Nbs1 interacting protein 1) (Akamatsu et al., 2008).

CeCom-1 was originally identified from a mutagenesis screen for mutants causing maternal-effect embryonic lethality (Penkner et al., 2007; Gönczy et al., 1999). AtGR1 was isolated from a screen for mRNAs that accumulate after DNA damage induced by ionizing radiation (Deveaux et al., 2000).

2.4 CtIP is conserved protein from yeast to humans

As previously mentioned, *S. cerevisiae* Sae2/Com1 was first identified as a CtIP homolog (McKee and Kleckner, 1997b; 1997a; Prinz et al., 1997), but clear Sae2/Com1 homologs have

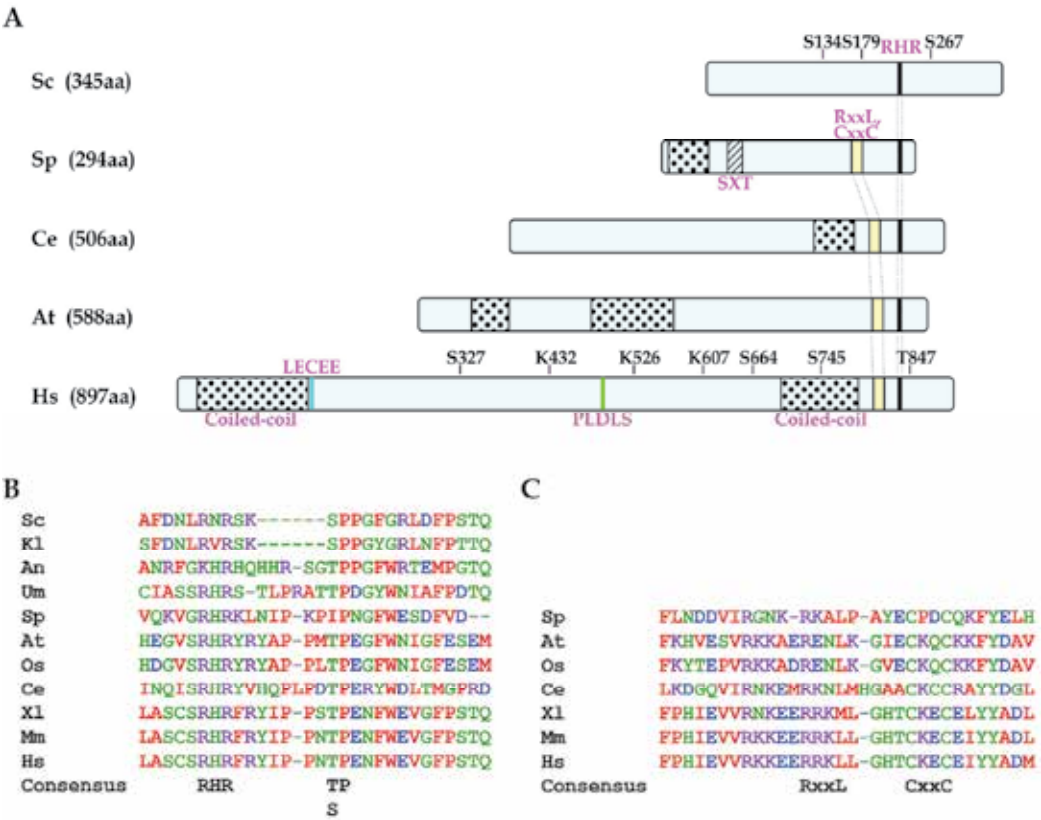

Fig. 1. (A) Schematic diagram of CtIP homologs. (B) Sequence alignment of the RHR motif and conserved CDK phosphorylation site. (C) Sequence alignment of the RxxL (D-box) and CxxC motifs. Kl, *Kluyveromyces lactis*; An, *Aspergillus nidulans*; Um, *Ustilago maydis*; Os, *Oryza sativa*; Xl, *Xenopus laevis*; Mm, *Mus musculus*; Hs, *Homo sapiens*.

not since been reported in any organism due to limited sequence similarity. The recent identification of SpCtp1 has led to the insight that it is homologous to proteins that have previously been characterized in several species such as ScSae2/Com1, CeCOM-1, AtGR1, and human CtIP (Limbo et al., 2007). In parallel, Jackson's group suggested that human CtIP shares some sequence homology with ScSae2 (Sartori et al., 2007). CtIP family proteins share several domains with highly conserved sequences but also demonstrate highly diverse sequences in other regions and show variety in protein length. These features might clarify why the orthologs were not recognized for a long time.

2.5 Domain structure of CtIP and its homologs

CtIP homologs retain C-terminal core domains, including RHR and CxxC motifs (Fig. 1A). The RHR motif is proposed to be the representative signature of Sae2/Com1 homologs (Limbo et al., 2007) and the limited 30 aa-region containing the RHR motif is well-conserved from ScSae2/Com1 to human CtIP (Fig. 1B). The CxxC motif is generally found in proteins with a D-box (RxxL) motif, which is representative among APC/C substrates, and the CxxC motif in some proteins is known to be involved in zinc chelation (Hopfner et al., 2002). The

CxxC and D-box motifs are conserved among CtIP homologs from fission yeast to humans with the exception of some other fungi, including *Saccharomyces* and *Aspergillus* (Fig. 1C). One or two coiled-coil motifs are also found in human CtIP and homologs from *S. pombe* and *A. thaliana*, but not in homologs from *C. elegans* or *S. cerevisiae* (Akamatsu et al., 2008; Limbo et al., 2007). The coiled-coil, LECEE and PLDLS motifs in human CtIP are required for dimerization (Dubin et al., 2004), Rb binding (Fusco et al., 1998) and CtBP binding (Schaeper et al., 1998), respectively. However, neither LECEE nor PLDLS motifs are found in the other homologs.

2.6 Posttranslational modifications of CtIP and its homologs

Several post-translational modification sites have been identified in CtIP homologs. Human CtIP has two CDK-dependent and two ATM-dependent phosphorylation sites. Phosphorylation at Ser-327 of CtIP by CDK increases around S/G_2 phases in unperturbed cells and might be responsible for CtIP-BRCA1 complex formation, which occurs in G_2 phase (Yu and Chen, 2004). Although this phosphorylation site does not seem to be conserved among species, another CDK-dependent site, Thr-847, which is likely to be conserved from yeast to humans, has been identified (Huertas and Jackson, 2009). Two ATM-target sites, Ser-664 and Ser-745, are phosphorylated in response to DNA damage, leading to Chk1 phosphorylation and the G_2/M transition checkpoint (Li et al., 2000). In addition to these phosphorylation events, CtIP is ubiquitinylated by the BRCA1-BARD1 heterodimer *in vivo* and *in vitro*. This ubiquitination is dependent on the RING domain of BRCA1 and phosphorylated Ser-327 of CtIP. Interestingly, the BRCA1-mediated ubiquitination does not target CtIP for degradation but for damage-induced foci formation (Yu et al., 2006). On the other hand, ubiquitin E3 ligase family protein, SIAH-1, is shown to interact with CtIP and promote its degradation (Germani et al., 2003). Therefore, ubiquitination of CtIP by SIAH-1 might be functionally different from that by BRCA1. Furthermore, it has been reported that CtIP is acetylated at Lys-432, Lys-526 and Lys-604 *in vivo*, and these acetylations are proposed to be important for the regulation of CtIP activity (Kaidi et al., 2010).

Similar to what has been observed in human CtIP, ScSae2 is phosphorylated periodically during the unperturbed cell cycle and in response to DNA damage (Baroni et al., 2004). Both cell cycle- and DNA damage-dependent Sae2 phosphorylation require the checkpoint kinase Mec1. Another pathway, involving Tel1 and MRX complex, is also required for full DNA damage-induced Sae2 phosphorylation (Baroni et al., 2004). Sae2 contains three potential CDK phosphorylation sites, Ser-134, Ser-179 and Ser-267, the last of which is a well-conserved residue that maps to the C-terminal region most highly conserved among organisms (Fig. 1C). Mutation of Ser-267 to an Ala residue causes phenotypes comparable to those observed in the *sae2*Δ null mutant (Huertas et al., 2008). Sae2 phosphorylation also occurs at the onset of premeiotic S phase, is maximal at the time of meiotic DSB generation and decreases when DSBs are repaired by homologous recombination, and is shown to be important to support the protein's meiotic recombination functions (Cartagena-Lirola et al., 2006). Recently, Sae2 was found to be acetylated and deacetylated, as seen in human CtIP (Robert et al., 2011).

In *S. pombe*, the CDK phosphorylation site corresponding to Ser-267 of ScSae2 has not been found, but Ctp1 contains two putative CDK-dependent phosphorylation sites and two putative Rad3/Tel1-dependent phosphorylation sites. Some of these sites have been suggested to be phosphorylated *in vivo*, whereas cells containing mutations in all of these sites show no obvious phenotype (Akamatsu et al., 2008). In addition, two putative Casein kinase 2 (CK2) phosphorylation sites (SXT motifs) are found in Ctp1 (Fig. 1C). It is still

unknown whether or not CK2 directly phosphorylates SXT motifs of Ctp1, though the phosphorylation of these motifs is essential for DNA damage repair *in vivo* and for binding with Nbs1 (Williams et al., 2009; Lloyd et al., 2009; Dodson et al., 2010) (See later). Phosphorylation by CK2 has not reported in other CtIP homologs to date.

3. CtIP and its homologs are involved in several biological processes

3.1 CtIP is involved in transcriptional regulation

As mentioned above, CtIP interacts with several proteins involved in transcriptional regulation, one of which is CtBP. CtBP acts as a transcriptional corepressor of several tumor suppressors such as E-cadherin, p16Ink4a, p15Ink4b, and PTEN, indicating a strong association with tumorigenesis and tumor progression (Chinnadurai, 2009). Complete transcriptional repression by CtIP requires binding to CtBP through its PLDLS domain (Meloni et al., 1999). The PLDLS motif of adenovirus E1A disrupts the CtBP-CtIP complex *in vitro*, which might potentiate the tumorigenesis-restraining activity of E1A exon 2 (Schaeper et al., 1998).

CtIP is also suggested to be a corepressor with Rb and p130 (Meloni et al., 1999). In contrast, CtIP has also been shown to bind Rb, allowing CtIP to bind its own promoter and an E2F target such as cyclin D1 during the G_1/S transition (Liu and Lee, 2006). This releases Rb-mediated transcriptional repression and increases the expression of genes required for S-phase entry. Furthermore, other groups have shown that CtIP can interact with the general transcription factors, TATA binding protein (TBP) and transcription factor IIB (Koipally and Georgopoulos, 2002). However, the functions of CtIP in transcriptional regulation might be limited to vertebrates and its orthologs might not play an important role in transcription.

3.2 Meiotic recombination and HR repair

In meiosis of *S. cerevisiae*, programmed DSBs are formed by a topoisomerase-like protein Spo11. Spo11 covalently attaches to the 5′ ends of the break, and a subsequent endonucleolytic step, dependent on the MRX complex, releases Spo11 bound to a short oligonucleotide (Neale et al., 2005). The MRX protein complex consists of Mre11, Rad50 and Xrs2 (Table 1) and is required for the formation of meiotic DSB and the processing of the DNA ends. Mre11 contains the phosphodiesterase motif responsible for nuclease activity. Rad50 contains Walker A and B motifs separated by a coiled-coil region and belongs to the SMC family proteins. The amino acid sequences of Mre11 and Rad50 are conserved among eukaryotes, while the amino acid sequence of Xrs2 is much less conserved. Its functional counterpart is called as Nbs1, exists among other eukaryotes, such as vertebrates, plants, nematodes and fission yeast (Rupnik et al., 2010). The degree of overall sequence similarity between Xrs2 and Nbs proteins is generally poor and homology is limited to an N-terminal forkhead-associated (FHA) domain and a small C-terminal region. Nbs1, but not Xrs2, contains a BRCT domain in the N-terminal region. It also forms a protein complex, MRN, similar to the MRX complex, which will henceforth be referred to as MRX(N).

S. cerevisiae rad50S mutations, separation-of-function mutations of *RAD50*, are defective in the processing of Spo11-induced DSBs and cause the accumulation of unprocessed DSBs with covalently attached Spo11 (Alani et al., 1990). The deletion mutants of the *sae2/com1* gene exhibit a meiotic-defective phenotype very similar to that of *rad50S* mutants, and Spo11-oligonucleotide complexes are not produced in either *rad50S* or *sae2*Δ/*com1*Δ mutants (Neale et al., 2002; Keeney and Kleckner, 1995). Similar observations were also made in *S.*

*pombe ctp1*Δ/*nip1*Δ cells (Hartsuiker et al., 2009a; Milman et al., 2009; Rothenberg et al., 2009; Akamatsu et al., 2008). Taken together with other results (Farah et al., 2009; Neale et al., 2002), yeast CtIP homologs are thought to be involved in DSB end resection in cooperation with the MRX(N) complex in meiosis.

During mitosis, *S. cerevisiae* mutants lacking either a component of the MRX complex or Sae2 exhibit sensitivity toward DNA-damaging agents and are defective in strand resection of DSB ends (McKee and Kleckner, 1997b; Clerici et al., 2005; Neale et al., 2005). In *S. pombe*, Ctp1 has been shown to function in an MRN-dependent HR repair pathway, but not in NHEJ (Akamatsu et al., 2008; Limbo et al., 2007). Chromatin immunoprecipitation (ChIP) assay of RPA at an HO-induced DSB site revealed that Ctp1, as well as Mre11, is required for DSB end resection (Limbo et al., 2007). These results are consistent with those from the Sae2 analysis. Additionally, ChIP assay showed that Ctp1 localizes to an HO-induced DSB site in a Mre11-dependent manner (Limbo et al., 2007). Thus, at least in fungi, the MRX(N) complex and CtIP homologs are implicated as cooperating in DSB end-processing during both mitotic and meiotic cell cycles. Furthermore, Ctp1 is required for the resection of the Top2-DNA complex, whereas Rad50 resects the Top1-DNA complex (Hartsuiker et al., 2009b). Recent analyses in *S. cerevisiae* revealed the requirement of Exo1, Sgs1, Top3, Rmi1 and Dna2 for processive DSB end resection (Mimitou and Symington, 2008; Zhu et al., 2008). The proteins involved in DSB end resection are listed in Table 1. One of these, Exo1, is a 5′-3′ exonuclease/flap endonuclease and another, Dna2, is an endonuclease with 5′-3′ helicase activity.

Sc	Sp	Hs	Note
Mre11	Rad32	MRE11	• single-strand endonuclease • 3′-5′ double-strand exonuclease • weak hairpin-opening activity
Rad50	Rad50	RAD50	• split ABC-type ATPase containing two heptad repeats • stimulates the 3′-5′ exonuclease and hairpin-opening activities of Mre11
Xrs2	Nbs1	NBS1	• contains an N-terminal FHA domain and a small C-terminal conserved domain • Nbs1, but not Xrs2, contains a BRCT domain in the N-terminal region • overall sequence similarity between Xrs2 and Nbs1 is weak and limited to an N-terminal FHA domain and a small C-terminal conserved domain
Sae2	Ctp1	CtIP	• endonuclease activity on single stranded DNA (Sae2) • stimulates nuclease activity of MR complex (Sae2 and CtIP)
Exo1	Exo1	EXO1	• 5′-3′ exonuclease, flap endonuclease
Sgs1	Rqh1	BLM	• RecQ family DNA helicase
Rmi1	Rmi1	RMI1 (BLAP75)	• RecQ-mediated genome instability protein • forms a complex with Sgs1 and Top3
Top3	Top3	TOPOIIIα	• type 1A topoisomerase
Dna2	Dna2	DNA2	• 5′-3′ helicase/endonuclease

Table 1. Proteins involved in DSB end resection.

Sgs1, a RecQ family helicase, forms a protein complex called RTR with Top3 and Rmi1. The RTR complex has multiple functions in DSB repair, including double Holliday junction dissolution (for review see (Ashton and Hickson, 2010)). The MRX complex and Sae2 in *S. cerevisiae* initiate 5′ degradation, leading to a subsequent step in which Exo1 and/or the RTR complex with Dna2 extensively degrade 5′ strands to generate long 3′ strands (Mimitou and Symington, 2008; Zhu et al., 2008). SpExo1 can substitute for Ctp1 on a *pku80*Δ background, suggesting that a similar mechanism may exist in *S. pombe* (Limbo et al., 2007).

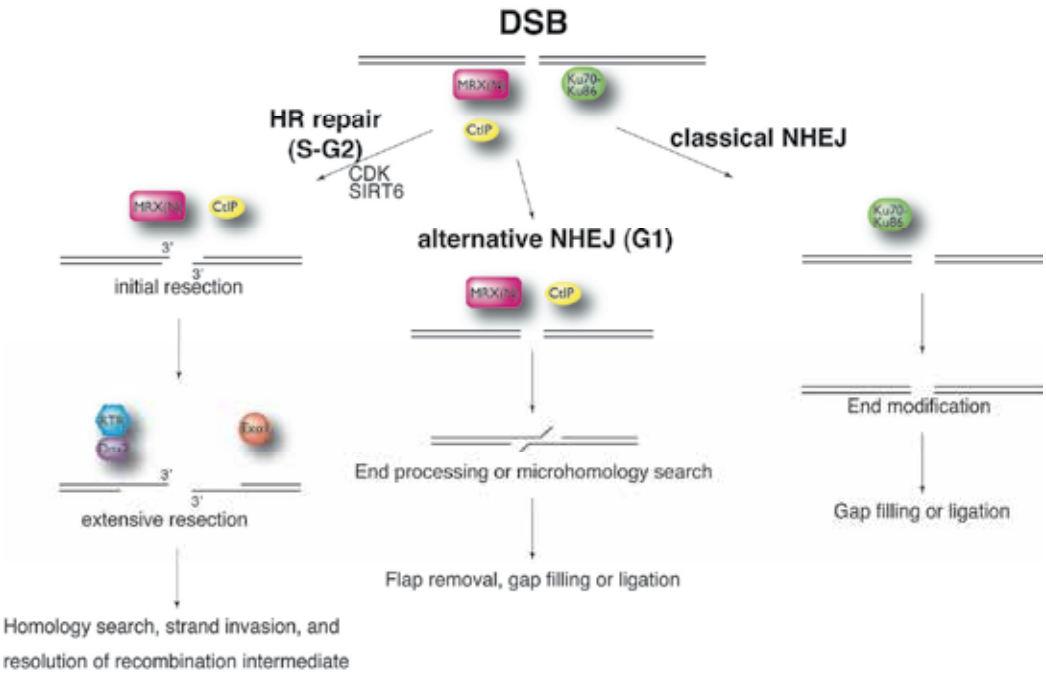

Fig. 2. Roles of CtIP/Sae2/Ctp1 in DSB repair.

Biochemically, Mre11 retains 3′->5′ exonuclease and ssDNA endonuclease and hairpin opening activities, all of which require Mn^{2+} as a metal cofactor (Trujillo and Sung, 2001; Sigurdsson et al., 2001). Both ATP and Rad50 stimulate the 3′->5′ exonuclease and hairpin opening activities of Mre11, where ATP is thought to regulate the DNA binding of the Mre11 complex via Rad50 (Trujillo and Sung, 2001). Remarkably, the ATP-dependent DNA end-resection reaction including the MRX complex, the RTR complex, Dna2 and the heterotrimeric ssDNA-binding protein RPA has been reconstituted (Cejka et al., 2010; Niu et al., 2010). Sae2 itself is shown to stimulate the nuclease activity of Mre11 and, interestingly, can cleave hairpin DNA, even in the absence of the MRX complex, at a cleavage site on the 3′ overhang adjacent to the hairpin (Lengsfeld et al., 2007). Recently, Paull and colleagues showed that MRX and Sae2 cooperatively promote Exo1-mediated 5′ strand degradation at DNA ends *in vitro*, but mutations in *RAD50*, *EXO1* or *MRE11* abrogate this end processing. Furthermore, *sae2* mutations reduce the efficiency of Exo1-mediated DSB resection both *in vitro* and *in vivo* (Nicolette et al., 2010). However, as neither the MRX complex or Sae2 have been shown to exhibit 5′->3′ exonuclease activity, it remains unclear how the generation of protruding 3′- ssDNA involves these proteins.

3.3 CtIP is involved in alternative NHEJ (A-NHEJ)

There are two varieties of NHEJ: classical-NHEJ (C-NHEJ) and alternative-NHEJ (A-NHEJ) (Zha et al., 2009). CtIP is required not only for HR repair in S/G_2 phase but also for A-NHEJ in G_1 upon generation of DSBs in human cells. The function of CtIP in A-NHEJ is independent of the phosphorylation of Ser-327 and recruitment of BRCA1. Cells expressing CtIP protein carrying mutations at Ser-327 are specifically defective in homologous recombination and show decreased levels of ssDNA after DNA damage, whereas A-NHEJ remains unaffected. Therefore, the phosphorylation of Ser-327 of CtIP is proposed to be a molecular switch to shift the balance of DSB repair from error-prone DNA end-joining to error-free homologous recombination in humans (Yun and Hiom, 2009).

3.4 Checkpoint control by CtIP-BRCA1 interaction

CtIP interacts with the BRCT motifs of BRCA1 in a manner dependent on the phosphorylation of Ser-327. The knockdown experiment shows that this interaction is required for DNA-damage-induced Chk1 phosphorylation and the G2/M transition checkpoint but not the damage-induced G2 accumulation checkpoint, for which BRCA1-BACH1 interaction is required. Therefore, the BRCA1 checkpoint pathway is divided into the BACH1-dependent pathway and the CtIP pathway (Yu and Chen, 2004). A crystal structure of the BRCT repeats in BRCA1 with a phosphopeptide corresponding to 322–333 residues of human CtIP has been solved (Varma et al., 2005). The BRCA1-CtIP interaction is ablated by several tumor-associated mutations affecting the BRCT motifs, suggesting that the interaction may be required for tumor suppression by BRCA1 (Liu et al., 1999; Wong et al., 1998; Yu et al., 1998). On the other hand, a sequence-based screen for mutations in the CtIP coding region in a panel of 89 tumor cell line cDNAs identified five missense variants (Wong et al., 1998). Therefore, CtIP itself may act as a tumor suppressor in human cells.

The MRN complex senses DSBs and activates the ataxia-telangiectasia mutated (ATM) kinase, resulting in a DNA damage response in human cells (Lee and Paull, 2004; Uziel et al., 2003). Similarly, the MRN(X) complex is required for the activation of the ATM ortholog, Tel1, in both *S. cerevisiae* and *S. pombe* (You et al., 2005; D'Amours and Jackson, 2001). Upon activation, ATM and its orthologs phosphorylate downstream substrates such as Mre11, Nbs1/Xrs2 and CtIP/Sae2 (Rupnik et al., 2010) (and see above). In contrast to the MRN(X) complex, CtIP and its homologs seem not to be involved in checkpoint activation (Limbo et al., 2007).

Interestingly, Sae2 has been shown to be required for proper recovery from checkpoint-mediated cell cycle arrest after DNA damage in *S. cerevisiae* (Clerici et al., 2006). However, the phenomenon of recovery from cell cycle arrest or the involvement of Sae2 homologs in this phenomenon has not been reported in organisms other than *S. cerevisiae*.

4. Functional regulation of CtIP and its homologs

4.1 Interaction with Nbs1 regulates CtIP

The N-terminal region of Nbs1 contains FHA and BRCT motifs, both of which are known to be phosphopeptide-binding protein modules. Recently, the crystal structure of SpNbs1 has revealed that the FHA domain of Nbs1 is fused directly to the tandem BRCT domain, leading to consideration of the functional interactions of CtIP with Mre11-Rad50 through Nbs1 (Lloyd et al., 2009; Williams et al., 2008).

SpCtp1 was shown to genetically interact with SpNbs1 in *S. pombe* and the FHA domain of SpNbs1 was implicated to play an important role in this interaction (Akamatsu et al., 2008). CtIP was also reported to interact with the MRN complex (Sartori et al., 2007) and, subsequently, it was demonstrated that recombinant CtIP prepared from insect cells binds directly to hNBS1 (Chen et al., 2008).

The FHA domain of hNBS1 interacts with phosphorylated SDT sites on hMDC1, whose sequence is a recognition motif for CK2 (Chapman and Jackson, 2008; Melander et al., 2008; Spycher et al., 2008). The FHA domain of SpNbs1, which is also important for cellular survival upon treatment with DNA damaging agents, is essential for Ctp1-binding *in vivo* (Lloyd et al., 2009; Williams et al., 2008). Interestingly, mutations in the SXT sites in Ctp1 sensitize cells to DNA damage and disrupt interactions with Nbs1, indicating that the interaction of Nbs1 and the phosphorylated SDT sites of Ctp1 through FHA is essential for DNA damage repair (Dodson et al., 2010). Although it has yet to be addressed whether or not CK2 directly phosphorylates the SXT sites of Ctp1, the crystal structure of the SpNbs1 bound to the phosphopeptide at a SXT site on Ctp1 has shown that the phosphorylation of the SXT sites is a prerequisite for the complex formation (Lloyd et al., 2009; Williams et al., 2008).

The interface of the association of Nbs1 with Mre11 maps to the C-terminal region in Nbs1 (You et al., 2005; Falck et al., 2005) and the interface of Mre11 dimerization is located away from the DNA-binding cleft (Williams et al., 2008).

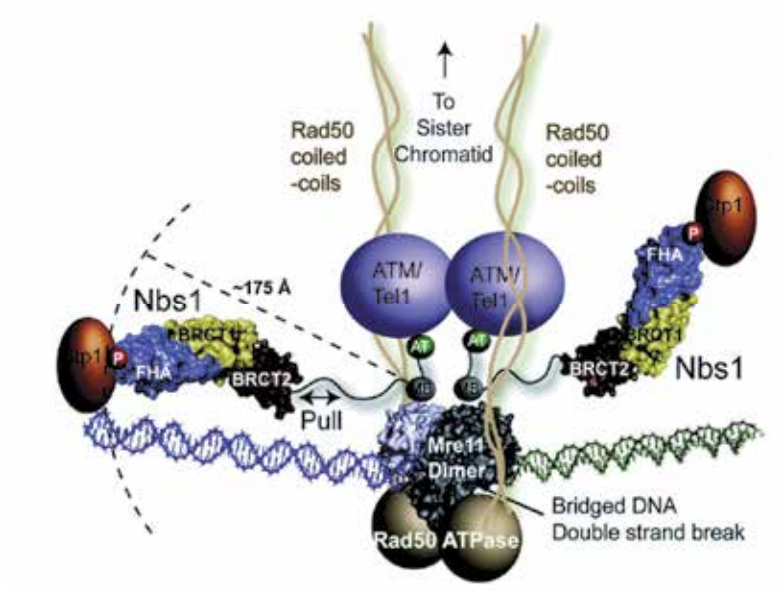

Fig. 3. Model of the MRN-Ctp1 complex bound at a bridging DNA DSB as proposed by (Williams et al., 2008). The flexible Nbs1 C-terminus links FHA-bound Ctp1 to an Mre11-Rad50 heterotetrameric core complex bridging a DSB.

The distance from the Mre11 binding domain to the N-terminal FHA domain was determined to be ~175 Å, as assessed by SAXS and X-ray crystallography (Lloyd et al., 2009; Williams et al., 2008). The MRN complex and Ctp1 colocalize to within ~200 bp (<700 Å) of a

single DSB site cleaved by a defined endonuclease *in vivo*. Based on this geometry and other evidence, a model has been proposed in which the flexible Nbs1 C-terminus links the FHA-bound Ctp1 to an Mre11-Rad50 heterotetrameric core complex, bridging two ends of a DSB (Williams et al., 2008) (Fig. 3). The model effectively accounts for recent insights into the roles of the Mre11-Rad50 heterotetrameric core at a DSB end (Williams et al., 2008; Chen et al., 2001; Hopfner et al., 2002; 2001; Moreno-Herrero et al., 2005).

4.2 CtIP homologs are highly regulated in the cell cycle

Gene expression of human and mouse CtIP proteins increases during G_1 to S phase (Liu and Lee, 2006). SpCtp1 is periodically transcribed in S phase and is regarded as one of the putative MBF-regulated genes (Limbo et al., 2007). This role in transcriptional regulation and the other functions of CtIP and its homologs seem to be regulated posttranslationally. As previously mentioned, the mechanism regulating homologous recombination by CDK is conserved between human and budding yeast cells; however, the corresponding CDK phosphorylation site has not been found in SpCtp1. The regulation of Ctp1 by CDK might not be absolutely necessary as G_1 phase is relatively short in *S. pombe*.

Recently, it has been found that CtIP and Sae2 are also modified by acetylation. Jackson's group found that CtIP is constitutively acetylated but is deacetylated by SIRT6 upon treatment with a DNA damaging agent (Kaidi et al., 2010). SIRT6 is an NAD^+ (nicotinamide adenine dinucleotide)-dependent sirtuin (class III), a member of the family of protein lysin deacetylases (KDACs). CtIP was identified as a target protein of SIRT6 and it was shown that deacetylation of CtIP by SIRT6 is an important regulatory event in DSB end processing.

On the other hand, Foiani's group revealed that treatment with the HDAC inhibitor, valproic acid (VPA), causes DSB processing defects in *S. cerevisiae* (Robert et al., 2011). Upon VPA treatment, acetylation levels of Sae2 increased and Sae2 was degraded. Rapamycin treatment induced Sae2 degradation through autophagy, and mutations in the genes involved in autophagy rescued Sae2 levels. Furthermore, two HDACs (Rpd3 and Hda1) and one HAT (Gcn5) influenced Sae2 turnover. It is not known whether Sae2 is directly acetylated/deacetylated by these HAT and HDACs. However, it is indisputable that the acetylation of CtIP homologs plays an important role in the regulation of DSB end resection in both human and yeast cells.

5. Conclusion

As DSBs can ultimately have toxic effects on cells, such as chromosome translocation, deletion, or duplication, they must be repaired appropriately. Cells utilize several damage responses depending on the cell-cycle phase. CtIP (and its orthologs) is one of the most important key players in the initial steps of DSB repair, in which cells determine the appropriate repair pathway and process the DSB ends. In addition, the importance of posttranslational modifications of CtIP is now being elucidated. However, the precise molecular mechanism of the generation of the recombinogenic 3′ ssDNA overhang by the combined actions of CtIP/Ctp1/Sae2 and the MRN(X) complex still remains unclear.

CtIP and many of its binding partners, such as BRCA1, CtBP and Rb, are classified as tumor suppressors. Furthermore, NBS1 and MRE11 are associated with Nijmegen breakage syndrome (NBS) and ataxia-telangiectasia-like disorder (ATLD), respectively, both of which

are chromosome instability syndromes. Cells carrying mutations in either of these genes show DNA damage hyper-sensitivity (Carney et al., 1998; Stewart et al., 1999). Taken together, these facts illustrate not only the scientific interest but also the clinical importance of understanding the molecular mechanism of DSB end resection mediated by MRN(X) and CtIP (or its orthologs).

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7. References

Akamatsu, Y., Murayama, Y., Yamada, T., Nakazaki, T., Tsutsui, Y., Ohta, K., and Iwasaki, H. (2008). Molecular characterization of the role of the *Schizosaccharomyces pombe nip1+/ctp1+* gene in DNA double-strand break repair in association with the Mre11-Rad50-Nbs1 complex. Mol Cell Biol *28*, 3639-3651.

Alani, E., Padmore, R., and Kleckner, N. (1990). Analysis of wild-type and *rad50* mutants of yeast suggests an intimate relationship between meiotic chromosome synapsis and recombination. Cell *61*, 419-436.

Ashton, T. M., and Hickson, I. D. (2010). Yeast as a model system to study RecQ helicase function. DNA Repair *9*, 303-314.

Baroni, E., Viscardi, V., Cartagena-Lirola, H., Lucchini, G., and Longhese, M. P. (2004). The functions of budding yeast Sae2 in the DNA damage response require Mec1- and Tel1-dependent phosphorylation. Mol Cell Biol *24*, 4151-4165.

Boyd, J. M., Subramanian, T., Schaeper, U., La Regina, M., Bayley, S., and Chinnadurai, G. (1993). A region in the C-terminus of adenovirus 2/5 E1a protein is required for association with a cellular phosphoprotein and important for the negative modulation of T24-ras mediated transformation, tumorigenesis and metastasis. EMBO J *12*, 469-478.

Carney, J. P., Maser, R. S., Olivares, H., Davis, E. M., Le Beau, M., Yates, J. R., Hays, L., Morgan, W. F., and Petrini, J. H. (1998). The hMre11/hRad50 protein complex and Nijmegen breakage syndrome: linkage of double-strand break repair to the cellular DNA damage response. Cell *93*, 477-486.

Cartagena-Lirola, H., Guerini, I., Viscardi, V., Lucchini, G., and Longhese, M. P. (2006). Budding Yeast Sae2 is an In Vivo Target of the Mec1 and Tel1 Checkpoint Kinases During Meiosis. Cell Cycle *5*, 1549-1559.

Cejka, P., Cannavo, E., Polaczek, P., Masuda-Sasa, T., Pokharel, S., Campbell, J. L., and Kowalczykowski, S. C. (2010). DNA end resection by Dna2-Sgs1-RPA and its stimulation by Top3-Rmi1 and Mre11-Rad50-Xrs2. Nature *467*, 112-116.

Chapman, J. R., and Jackson, S. P. (2008). Phospho-dependent interactions between NBS1 and MDC1 mediate chromatin retention of the MRN complex at sites of DNA damage. EMBO Rep *9*, 795-801.

Chen, L., Nievera, C. J., Lee, A. Y., and Wu, X. (2008). Cell cycle-dependent complex formation of BRCA1.CtIP.MRN is important for DNA double-strand break repair. J Biol Chem *283*, 7713-7720.

Chen, L., Trujillo, K., Ramos, W., Sung, P., and Tomkinson, A. E. (2001). Promotion of Dnl4-catalyzed DNA end-joining by the Rad50/Mre11/Xrs2 and Hdf1/Hdf2 complexes. Mol Cell *8*, 1105-1115.

Chinnadurai, G. (2009). The transcriptional corepressor CtBP: a foe of multiple tumor suppressors. Cancer Res *69*, 731-734.

Clerici, M., Mantiero, D., Lucchini, G., and Longhese, M. P. (2006). The *Saccharomyces cerevisiae* Sae2 protein negatively regulates DNA damage checkpoint signalling. EMBO Rep *7*, 212-218.

Clerici, M., Mantiero, D., Lucchini, G., and Longhese, M. P. (2005). The *Saccharomyces cerevisiae* Sae2 protein promotes resection and bridging of double strand break ends. J Biol Chem *280*, 38631-38638.

D'Amours, D., and Jackson, S. P. (2001). The yeast Xrs2 complex functions in S phase checkpoint regulation. Genes Dev *15*, 2238-2249.

Deveaux, Y., Alonso, B., Pierrugues, O., Godon, C., and Kazmaier, M. (2000). Molecular cloning and developmental expression of AtGR1, a new growth-related Arabidopsis gene strongly induced by ionizing radiation. Radiat Res *154*, 355-364.

Dodson, G. E., Limbo, O., Nieto, D., and Russell, P. (2010). Phosphorylation-regulated binding of Ctp1 to Nbs1 is critical for repair of DNA double-strand breaks. Cell Cycle *9*.

Dubin, M. J., Stokes, P. H., Sum, E. Y. M., Williams, R. S., Valova, V. A., Robinson, P. J., Lindeman, G. J., Glover, J. N. M., Visvader, J. E., and Matthews, J. M. (2004). Dimerization of CtIP, a BRCA1- and CtBP-interacting protein, is mediated by an N-terminal coiled-coil motif. J Biol Chem *279*, 26932-26938.

Falck, J., Coates, J., and Jackson, S. P. (2005). Conserved modes of recruitment of ATM, ATR and DNA-PKcs to sites of DNA damage. Nature *434*, 605-611.

Farah, J. A., Cromie, G. A., and Smith, G. R. (2009). Ctp1 and Exonuclease 1, alternative nucleases regulated by the MRN complex, are required for efficient meiotic recombination. Proceedings of the National Academy of Sciences *106*, 9356-9361.

Fusco, C., Reymond, A., and Zervos, A. S. (1998). Molecular cloning and characterization of a novel retinoblastoma-binding protein. Genomics *51*, 351-358.

Germani, A., Prabel, A., Mourah, S., Podgorniak, M., Di Carlo, A., Ehrlich, R., Gisselbrecht, S., Varin-Blank, N., Calvo, F., and Bruzzoni-Giovanelli, H. (2003). SIAH-1 interacts with CtIP and promotes its degradation by the proteasome pathway. Oncogene *22*, 8845-8851.

Gönczy, P., Schnabel, H., Kaletta, T., Amores, A. D., Hyman, T., and Schnabel, R. (1999). Dissection of cell division processes in the one cell stage *Caenorhabditis elegans* embryo by mutational analysis. J Cell Biol *144*, 927-946.

Hartsuiker, E., Mizuno, K., Molnar, M., Kohli, J., Ohta, K., and Carr, A. M. (2009a). $Ctp1^{CtIP}$ and $Rad32^{Mre11}$ nuclease activity are required for $Rec12^{Spo11}$ removal, but $Rec12^{Spo11}$ removal is dispensable for other MRN-dependent meiotic functions. Mol Cell Biol *29*, 1671-1681.

Hartsuiker, E., Neale, M. J., and Carr, A. M. (2009b). Distinct requirements for the $Rad32^{Mre11}$ nuclease and $Ctp1^{CtIP}$ in the removal of covalently bound topoisomerase I and II from DNA. Mol Cell *33*, 117-123.

Hopfner, K., Craig, L., Moncalian, G., Zinkel, R. A., Usui, T., Owen, B. A. L., Karcher, A., Henderson, B., Bodmer, J., McMurray, C. T., et al. (2002). The Rad50 zinc-hook is a

structure joining Mre11 complexes in DNA recombination and repair. Nature *418*, 562-566.

Hopfner, K. P., Karcher, A., Craig, L., Woo, T. T., Carney, J. P., and Tainer, J. A. (2001). Structural biochemistry and interaction architecture of the DNA double-strand break repair Mre11 nuclease and Rad50-ATPase. Cell *105*, 473-485.

Huertas, P., Cortés-Ledesma, F., Sartori, A. A., Aguilera, A., and Jackson, S. P. (2008). CDK targets Sae2 to control DNA-end resection and homologous recombination. Nature *455*, 689-692.

Huertas, P., and Jackson, S. P. (2009). Human CtIP mediates cell cycle control of DNA end resection and double strand break repair. J Biol Chem *284*, 9558-9565.

Kaidi, A., Weinert, B. T., Choudhary, C., and Jackson, S. P. (2010). Human SIRT6 promotes DNA end resection through CtIP deacetylation. Science *329*, 1348-1353.

Keeney, S., and Kleckner, N. (1995). Covalent protein-DNA complexes at the 5' strand termini of meiosis-specific double-strand breaks in yeast. Proc Natl Acad Sci USA *92*, 11274-11278.

Koipally, J., and Georgopoulos, K. (2002). Ikaros-CtIP interactions do not require C-terminal binding protein and participate in a deacetylase-independent mode of repression. J Biol Chem *277*, 23143-23149.

Lee, J., and Paull, T. T. (2004). Direct activation of the ATM protein kinase by the Mre11/Rad50/Nbs1 complex. Science *304*, 93-96.

Lengsfeld, B. M., Rattray, A. J., Bhaskara, V., Ghirlando, R., and Paull, T. T. (2007). Sae2 is an endonuclease that processes hairpin DNA cooperatively with the Mre11/Rad50/Xrs2 complex. Mol Cell *28*, 638-651.

Li, S., Chen, P. L., Subramanian, T., Chinnadurai, G., Tomlinson, G., Osborne, C. K., Sharp, Z. D., and Lee, W. H. (1999). Binding of CtIP to the BRCT repeats of BRCA1 involved in the transcription regulation of p21 is disrupted upon DNA damage. J Biol Chem *274*, 11334-11338.

Li, S., Ting, N. S., Zheng, L., Chen, P. L., Ziv, Y., Shiloh, Y., Lee, E. Y., and Lee, W. H. (2000). Functional link of BRCA1 and ataxia telangiectasia gene product in DNA damage response. Nature *406*, 210-215.

Limbo, O., Chahwan, C., Yamada, Y., de Bruin, R. A. M., Wittenberg, C., and Russell, P. (2007). Ctp1 is a cell-cycle-regulated protein that functions with Mre11 complex to control double-strand break repair by homologous recombination. Mol Cell *28*, 134-146.

Liu, F., and Lee, W. (2006). CtIP activates its own and cyclin D1 promoters via the E2F/RB pathway during G1/S progression. Mol Cell Biol *26*, 3124-3134.

Liu, Z., Macias, M., Bottomley, M., Stier, G., Linge, J., Nilges, M., Bork, P., and Sattler, M. (1999). The three-dimensional structure of the HRDC domain and implications for the Werner and Bloom syndrome proteins. Structure *7*, 1557-1566.

Lloyd, J., Chapman, J. R., Clapperton, J. A., Haire, L. F., Hartsuiker, E., Li, J., Carr, A. M., Jackson, S. P., and Smerdon, S. J. (2009). A supramodular FHA/BRCT-repeat architecture mediates Nbs1 adaptor function in response to DNA damage. Cell *139*, 100-111.

McKee, A. H., and Kleckner, N. (1997a). A general method for identifying recessive diploid-specific mutations in *Saccharomyces cerevisiae*, its application to the isolation of

mutants blocked at intermediate stages of meiotic prophase and characterization of a new gene *SAE2*. Genetics *146*, 797-816.

McKee, A. H., and Kleckner, N. (1997b). Mutations in *Saccharomyces cerevisiae* that block meiotic prophase chromosome metabolism and confer cell cycle arrest at pachytene identify two new meiosis-specific genes *SAE1* and *SAE3*. Genetics *146*, 817-834.

Melander, F., Bekker-Jensen, S., Falck, J., Bartek, J., Mailand, N., and Lukas, J. (2008). Phosphorylation of SDT repeats in the MDC1 N terminus triggers retention of NBS1 at the DNA damage-modified chromatin. J Cell Biol *181*, 213-226.

Meloni, A. R., Smith, E. J., and Nevins, J. R. (1999). A mechanism for Rb/p130-mediated transcription repression involving recruitment of the CtBP corepressor. Proc Natl Acad Sci USA *96*, 9574-9579.

Milman, N., Higuchi, E., and Smith, G. R. (2009). Meiotic DNA double-strand break repair requires two nucleases, MRN and Ctp1, to produce a single size class of Rec12 (Spo11)-oligonucleotide complexes. Mol Cell Biol *29*, 5998-6005.

Mimitou, E. P., and Symington, L. S. (2008). Sae2, Exo1 and Sgs1 collaborate in DNA double-strand break processing. Nature *455*, 770-774.

Moreno-Herrero, F., de Jager, M., Dekker, N. H., Kanaar, R., Wyman, C., and Dekker, C. (2005). Mesoscale conformational changes in the DNA-repair complex Rad50/Mre11/Nbs1 upon binding DNA. Nature *437*, 440-443.

Neale, M. J., Pan, J., and Keeney, S. (2005). Endonucleolytic processing of covalent protein-linked DNA double-strand breaks. Nature *436*, 1053-1057.

Neale, M. J., Ramachandran, M., Trelles-Sticken, E., Scherthan, H., and Goldman, A. S. H. (2002). Wild-type levels of Spo11-induced DSBs are required for normal single-strand resection during meiosis. Mol Cell *9*, 835-846.

Nicolette, M. L., Lee, K., Guo, Z., Rani, M., Chow, J. M., Lee, S. E., and Paull, T. T. (2010). Mre11-Rad50-Xrs2 and Sae2 promote 5' strand resection of DNA double-strand breaks. Nat Struct Mol Biol *17*, 1478-1485.

Niu, H., Chung, W., Zhu, Z., Kwon, Y., Zhao, W., Chi, P., Prakash, R., Seong, C., Liu, D., Lu, L., et al. (2010). Mechanism of the ATP-dependent DNA end-resection machinery from Saccharomyces cerevisiae. Nature *467*, 108-111.

Penkner, A., Portik-Dobos, Z., Tang, L., Schnabel, R., Novatchkova, M., Jantsch, V., and Loidl, J. (2007). A conserved function for a *Caenorhabditis elegans* Com1/Sae2/CtIP protein homolog in meiotic recombination. EMBO J *26*, 5071-5082.

Prinz, S., Amon, A., and Klein, F. (1997). Isolation of *COM1*, a new gene required to complete meiotic double-strand break-induced recombination in *Saccharomyces cerevisiae*. Genetics *146*, 781-795.

Robert, T., Vanoli, F., Chiolo, I., Shubassi, G., Bernstein, K. A., Rothstein, R., Botrugno, O. A., Parazzoli, D., Oldani, A., Minucci, S., et al. (2011). HDACs link the DNA damage response, processing of double-strand breaks and autophagy. Nature *471*, 74-79.

Rothenberg, M., Kohli, J., and Ludin, K. (2009). Ctp1 and the MRN-complex are required for endonucleolytic Rec12 removal with release of a single class of oligonucleotides in fission yeast. PLoS Genet *5*, e1000722.

Rupnik, A., Lowndes, N. F., and Grenon, M. (2010). MRN and the race to the break. Chromosoma *119*, 115-135.

Sartori, A. A., Lukas, C., Coates, J., Mistrik, M., Fu, S., Bartek, J., Baer, R., Lukas, J., and Jackson, S. P. (2007). Human CtIP promotes DNA end resection. Nature *450*, 509-514.

Schaeper, U., Subramanian, T., Lim, L., Boyd, J. M., and Chinnadurai, G. (1998). Interaction between a cellular protein that binds to the C-terminal region of adenovirus E1A (CtBP) and a novel cellular protein is disrupted by E1A through a conserved PLDLS motif. J Biol Chem *273*, 8549-8552.

Schaeper, U., Boyd, J. M., Verma, S., Uhlmann, E., Subramanian, T., and Chinnadurai, G. (1995). Molecular cloning and characterization of a cellular phosphoprotein that interacts with a conserved C-terminal domain of adenovirus E1A involved in negative modulation of oncogenic transformation. Proc Natl Acad Sci USA *92*, 10467-10471.

Sigurdsson, S., Trujillo, K., Song, B., Stratton, S., and Sung, P. (2001). Basis for avid homologous DNA strand exchange by human Rad51 and RPA. J Biol Chem *276*, 8798-8806.

Spycher, C., Miller, E. S., Townsend, K., Pavic, L., Morrice, N. A., Janscak, P., Stewart, G. S., and Stucki, M. (2008). Constitutive phosphorylation of MDC1 physically links the MRE11-RAD50-NBS1 complex to damaged chromatin. J Cell Biol *181*, 227-240.

Stewart, G. S., Maser, R. S., Stankovic, T., Bressan, D. A., Kaplan, M. I., Jaspers, N. G., Raams, A., Byrd, P. J., Petrini, J. H., and Taylor, A. M. (1999). The DNA double-strand break repair gene hMRE11 is mutated in individuals with an ataxia-telangiectasia-like disorder. Cell *99*, 577-587.

Sum, E. Y. M., Peng, B., Yu, X., Chen, J., Byrne, J., Lindeman, G. J., and Visvader, J. E. (2002). The LIM domain protein LMO4 interacts with the cofactor CtIP and the tumor suppressor BRCA1 and inhibits BRCA1 activity. J Biol Chem *277*, 7849-7856.

Trujillo, K. M., and Sung, P. (2001). DNA structure-specific nuclease activities in the *Saccharomyces cerevisiae* Rad50*Mre11 complex. J Biol Chem *276*, 35458-35464.

Uziel, T., Lerenthal, Y., Moyal, L., Andegeko, Y., Mittelman, L., and Shiloh, Y. (2003). Requirement of the MRN complex for ATM activation by DNA damage. EMBO J *22*, 5612-5621.

Varma, A. K., Brown, R. S., Birrane, G., and Ladias, J. A. A. (2005). Structural basis for cell cycle checkpoint control by the BRCA1-CtIP complex. Biochemistry *44*, 10941-10946.

Williams, R. S., Moncalian, G., Williams, J. S., Yamada, Y., Limbo, O., Shin, D. S., Groocock, L. M., Cahill, D., Hitomi, C., Guenther, G., et al. (2008). Mre11 dimers coordinate DNA end bridging and nuclease processing in double-strand-break repair. Cell *135*, 97-109.

Williams, R. S., Dodson, G. E., Limbo, O., Yamada, Y., Williams, J. S., Guenther, G., Classen, S., Glover, J. N. M., Iwasaki, H., Russell, P., et al. (2009). Nbs1 flexibly tethers Ctp1 and Mre11-Rad50 to coordinate DNA double-strand break processing and repair. Cell *139*, 87-99.

Wong, A. K., Ormonde, P. A., Pero, R., Chen, Y., Lian, L., Salada, G., Berry, S., Lawrence, Q., Dayananth, P., Ha, P., et al. (1998). Characterization of a carboxy-terminal BRCA1 interacting protein. Oncogene *17*, 2279-2285.

You, Z., Chahwan, C., Bailis, J., Hunter, T., and Russell, P. (2005). ATM activation and its recruitment to damaged DNA require binding to the C terminus of Nbs1. Mol Cell Biol *25*, 5363-5379.

Yu, X., Fu, S., Lai, M., Baer, R., and Chen, J. (2006). BRCA1 ubiquitinates its phosphorylation-dependent binding partner CtIP. Genes Dev *20*, 1721-1726.

Yu, X., Wu, L. C., Bowcock, A. M., Aronheim, A., and Baer, R. (1998). The C-terminal (BRCT) domains of BRCA1 interact in vivo with CtIP, a protein implicated in the CtBP pathway of transcriptional repression. J Biol Chem *273*, 25388-25392.

Yu, X., and Chen, J. (2004). DNA damage-induced cell cycle checkpoint control requires CtIP, a phosphorylation-dependent binding partner of BRCA1 C-terminal domains. Mol Cell Biol *24*, 9478-9486.

Yun, M. H., and Hiom, K. (2009). CtIP-BRCA1 modulates the choice of DNA double-strand-break repair pathway throughout the cell cycle. Nature *459*, 460-463.

Zha, S., Boboila, C., and Alt, F. W. (2009). Mre11: roles in DNA repair beyond homologous recombination. Nat Struct Mol Biol *16*, 798-800.

Zhu, Z., Chung, W., Shim, E. Y., Lee, S. E., and Ira, G. (2008). Sgs1 helicase and two nucleases Dna2 and Exo1 resect DNA double-strand break ends. Cell *134*, 981-994.

10

Archaeal DNA Repair Nucleases

Roxanne Lestini[2], Christophe Creze[1], Didier Flament[1],
Hannu Myllykallio[2] and Ghislaine Henneke[1]
[1]*Ifremer, Centre National de la Recherche Scientifique, Université de Bretagne Occidentale, Laboratoire de Microbiologie des Environnements Extrêmes*
[2]*Centre National de la Recherche Scientifique, Laboratoire d'Optique et Biosciences*
France

1. Introduction

DNA is the carrier of genetic information, but is constantly assaulted by endogenous and exogenous genotoxic attacks in all living organisms. If left unrepaired damaged or structurally altered DNA can impede pathways of DNA metabolism and maintenance of genomic stability and lead to cell death or uncontrolled proliferation. Archaea comprise diverse microorganisms that can thrive in harsh environments like hydrothermal vents and acidic hot springs. They can live without sunlight or organic carbon as food, and instead survive on sulfur hydrogen, and other materials that most bacteria and eukaryotes can not metabolize. Considering the extreme environmental niches inhabited by archaeal species, DNA lesions could be massively induced by exposure to hazardous environmental factors, (e.g., ultraviolet, X- and γ-rays, elevated temperatures and endogenous mutagens, e.g., reactive oxygen/nitrogen species, alkylating agents and toxic metals), and very high rates of potentially mutagenic DNA lesions (deamination, depurination, oxidation by hydrolytic mechanisms, alkylations and subsequent strand breakage) are expected to arise. However, and interestingly, it was demonstrated that the hyperthermophilic crenarchaeota *Sulfolobus Acidocaldarius* exhibited a modest rate of spontaneous mutations nearly close to that of the mesophilic bacteria, *Escherichia Coli* (*E. coli*) (Grogan et al., 2001). Similarly, the euryarchaeota *Pyrococcus abyssi* can survive high doses of ionizing gamma irradiation (Jolivet et al., 2003b) and abasic sites formation in the hyperthermophilic chromosome was found to occur at a rate moderately higher than in *E. coli* (Palud et al., 2008). Thus, archaeal organisms seem to evolve efficient strategies for repairing DNA damage and thus avoiding mutations.

Like bacteria and eukaryotes, archaeal repair mechanisms seem to include nucleolytic processing of DNA. Consequently, this article sets out to review archaeal DNA nucleases based on current knowledge of sequence, structure and mechanism. We have focused on recent work on several DNA repair nucleases, with a detailed description of substrate preference and cleavage specificity of these archaeal enzymes. Crystal structures, when available, are discussed in the context of biochemical data to outline mechanistic features, such as enzymatic DNA cleavage, DNA binding, and sometimes, although not always, functions. This review stresses the molecular mechanisms which have been conserved throughout evolution with reference to eukaryotic DNA nucleases and, in some cases, to

bacterial counterparts. On the other hand, DNA nucleases which appears unique to archaea are emphasized with the aim to describe novel aspects of repair mechanisms.

2. Type 2 Ribonuclease H, a structure-specific DNA repair nuclease

2.1 RNase HII/2: a ubiquitous enzyme

Ribonucleases H (RNases H) catalyse the cleavage of the RNA portion of RNA/DNA hybrid molecules that are ubiquitously present in cells (Stein and Hausen, 1969). RNases H are classified into two major families, type 1 and type 2, based on amino acid sequence identities and distinct biochemical properties. Genes encoding RNases H are found in viruses, archaea, bacteria and eukaryotes and, at least, one RNase H is present within a single cell. Furthermore, type 2 RNases H are more widely distributed than type 1 RNases H in prokaryotic and eukaryotic genomes (Ohtani et al., 1999b). Biological roles, including DNA replication, DNA repair, and transcription have been assigned for these RNases H, as recently reviewed (Cerritelli and Crouch, 2009; Tadokoro and Kanaya, 2009). Here, we report recent progress in the structural and functional characterization of type 2 ribonucleases H (RNase HII/2) presumed to be involved in an excision repair system for the removal of ribose residues with a particular accent on archaeal enzymes.

2.2 RNases HII/2 orthologs

2.2.1 Distribution and amino acid sequence identities

In the process of analysing the 95 sequenced archaeal genomes, type 2 RNases H (RNases HII) have been detected among the five archaeal phyla: Euryarchaeota, Crenarchaeota, Korarchaeota, Thaumarchaeota and Nanoarchaeota. In contrast to the type 1 enzymes, archaeal RNases HII appear universally distributed, and most organisms only contain RNase HII, with the exception of few archaea, such as *Sulfolobus tokodaii, Haloferax volcanii*, *Halobacterium* sp. NRC-1 and *Pyrobaculum aerophilum* which possess both types. Despite the multiplicity of *rnh* genes within a single cell, the ubiquitous occurrence of RNase HII suggests that type 2 may provide the major RNase H activity in archaea cells, as recently proposed for eukaryotes (Bubeck et al., 2011; Frank et al., 1998b).

Sequence comparison within archaeal RNases HII has revealed a high degree of amino acid sequence identity (Chai et al., 2001; Haruki et al., 1998; Le Laz et al. 2010; Muroya et al., 2001). For instance, *Pyrococcus abyssi* (*Pab*RNase HII) shows amino acid sequence identities of 64% to *Thermococcus kodakaraensis* RNase HII (*Tko*RNase HII), 49% to *Archaeoglobus fulgidus* RNase HII (*Afu*RNase HII) and 40% to *Methanocaldococcus jannaschii* RNase HII (*Mja*RNase HII). Likewise, archaeal RNases HII have highly sequence similar orthologs in bacteria and eukaryotes. *Afu*RNase HII shows amino acid sequence identities of 31% to *Thermotoga maritima* RNase HII (*Tma*RNase HII) and 30% to the catalytical subunit of *Mus musculus* RNase H2A (*Mmu*RNase H2A), the latter composed of three distinct subunits (Shaban et al., 2010).

2.2.2 Biochemical characterization

The apparent sequence conservation among RNases HII/2 orthologs would indicate that these enzymes have biochemical properties in common. Interestingly, archaeal RNases HII display activity at alkaline pH (Chai et al., 2001; Haruki et al., 1998; Le Laz et al., 2010), and this property seems to be a hallmark of type 2 RNases H (Chon et al., 2009; Rohman et al., 2008; Rychlik et al., 2010). As first reported by Haruki, *et al*., the archaeal *Tko*RNase HII

activity was not salt-dependent, but was greatly attenuated by salt-concentrations higher than 100 mM (Haruki et al., 1998). More recent biochemical characterizations, however, seem to indicate that most of type 2 RNase H activities are salt-dependent (Chon et al., 2009; Ohtani et al., 2000; Rohman et al., 2008; Rychlik et al., 2010). All archaeal RNases HII studied to date have been shown to be strictly metal-dependent nucleases. *Pab*RNase HII prefers Mg^{2+} to Mn^{2+} or Co^{2+} for activity, *Tko*RNase HII shows preference for Co^{2+} over Mg^{2+}, Mn^{2+} or Ni^{2+}, and *Afu*RNase HII mostly prefers Mg^{2+} and Mn^{2+} to other metals (Chai et al., 2001; Haruki et al., 1998; Le Laz et al., 2010). Thus, metal ion usage by archaeal RNases HII may be a consequence of the environmental conditions under which they thrive. It may also dictate the substrate requirement for hydrolysis and confer a specialized function on the enzyme in the maintenance of genome integrity. This concern is nicely exemplified by bacterial RNases HII, for which Mg^{2+}- or Mn^{2+}- dependent activities are imposed by the nature of the substrate. This is in contrast to the eukaryotic RNases HII which appear more active in the presence of Mg^{2+} (Chon et al., 2009; Frank et al., 1994; Rohman et al., 2008).

Cleavage specificities for substrates containing single or few ribonucleotides embedded in double-stranded DNA (dsDNA) of type 2 RNases H are now well documented. Such structural substrates can arise *in vivo* during Okazaki fragment processing from intrinsic RNA ligation activity (Rumbaugh et al., 1997) or erroneous nucleotide incorporation by DNA polymerases (Nick McElhinny et al., 2010a; Nick McElhinny et al., 2010b), and during exposure to external damaging agents (Von Sonntag and Schulte-Frohlinde, 1978). Initial studies revealed that the archaeal *Tko*RNase HII was active on four ribonucleotides embedded in dsDNA (DNA-RNA_4-DNA/DNA). In the presence of Co^{2+} and 50 mM NaCl, the enzyme specifically cleaves at the phosphodiester bond between the third and fourth ribonucleotides, which is one ribonucleotide upstream of the RNA.DNA junction (Haruki et al., 1998). This cleavage specificity is consistent with what has been recently reported using the same substrate, but Mg^{2+} instead of Co^{2+} (Rohman et al., 2008). Similarly, *Tko*RNase HII exhibited a unique cleavage site on single ribonucleotides embedded in dsDNA (DNA-RNA_1-DNA/DNA) and specifically cut at the 5′ side of the ribonucleotide. When kinetic parameters of *Tko*RNase HII were determined in the presence of both DNA-RNA_1-DNA/DNA and DNA-RNA_4-DNA/DNA, substrate binding and turnover number of proteins were found to be comparable. Thus, one or few ribonucleotides embedded in dsDNA must be uniformly recognised and hydrolysed with similar efficiency in this archaeal organism. *Afu*RNase HII also possesses such catalytic specificities. In the presence of Mg^{2+} and 50 mM KCl, *Afu*RNase HII is shown to be active on DNA-RNA_1-DNA/DNA, unless the ribonucleotide is positioned<4 bases from the 5′ end or <2 bases from the 3′ end, and cleavage occurs at the phosphodiester bond 5′ of the junctional ribonucleotide (Bubeck et al., 2011). In addition, PCNA (Proliferating Cell Nuclear Antigen), described as a scaffold for DNA repair and replication enzymes (Maga and Hubscher, 2003; Meslet-Cladiere et al., 2007), enhances cleavage activity of *Afu*RNase HII on DNA-RNA_1-DNA/DNA, with the exception of ribonucleotide located within the first ten 5′-bases of the strand containing it. Interestingly, this result is consistent with that observed previously for *Pab*RNase HII (Meslet-Cladiere et al., 2007). *Afu*RNase HII also shows cleavage specificity on DNA-RNA_4-DNA/DNA, cutting 5′ of the last ribonucleotide of the junction in the presence of Mg^{2+} (Chai et al., 2001). Similarly, the archaeal *Pab*RNase HII acts as a specific nuclease on single embedded ribonucleotides, exhibiting cleavage activity in the presence of Mg^{2+} (Le Laz et al., 2010). Bacterial and eukaryotic type 2 RNase H enzymes share comparable substrate specificity for single or few ribonucleotides embedded in dsDNA. Substrate and cleavage

specificities for bacterial RNases HII, e.g. *Escherichia coli* RNase HII (*Eco*RNase HII) and *Tma*RNase HII, on DNA-RNA_1-DNA/DNA were found to be identical in the presence of Mg^{2+} and 50 mM NaCl (Chon et al., 2009; Ohtani et al., 2008; Rychlik et al., 2010). All bacterial enzymes specifically cleaved at the 5′ side of the ribonucleotide of the RNA.DNA junction. Similar cleavage specificities of few ribonucleotides embedded in dsDNA were also observed, leaving a mono-ribonucleotide at the 5′ terminus of the RNA.DNA junction (Ohtani et al., 2008; Rychlik et al., 2010). Thus, bacterial RNases HII share common features on junction substrates, in which Mg-dependent cleavage likely dominates over Mn-dependent hydrolysis. This statement can also be applicable to the eukaryotic RNases H2. Indeed, both mammalian and yeast enzymes displayed a unique cleavage site on DNA-RNA_1-DNA/DNA, cutting at the 5′-deoxyribonucleotide–ribonucleotide bond at the RNA.DNA junction in the presence of Mg^{2+} (Bubeck et al., 2011; Chon et al., 2009; Jeong et al., 2004; Rohman et al., 2008; Shaban et al., 2010). In contrast to *Afu*RNase HII, cleavage efficiency of *Human sapiens* RNase H2 (*Hsa*RNase H2) was not stimulated by its cognate PCNA, although they have been shown to co-localize and to interact *in vivo* (Bubeck et al., 2011; Chon et al., 2009). Under the same reaction conditions than those described for hydrolysis of single embedded ribonucleotides, cleavage specificity of *Saccharomyces cerevisiae* RNase H2 (*Sce*RNase H2) on DNA-RNA_4-DNA/DNA took place at the phosphodiester bond between the third and fourth ribonucleotides (Chai et al., 2001; Jeong et al., 2004; Rohman et al., 2008). Moreover, kinetic parameters indicated that substrate binding and turnover number of proteins were found equivalent for both DNA-RNA_1-DNA/DNA and DNA-RNA_4-DNA/DNA, as also observed for *Tko*RNase HII (Rohman et al., 2008).

Overall, it appears that Mg-dependent hydrolysis of single or few ribonucleotides embedded in dsDNA along with the unique substrate specificity are a hallmark of type 2 RNases H, implying that key structural elements necessary for activity must be conserved among eukaryotes and prokaryotes.

2.3 Structure and catalysis by RNases HII/2

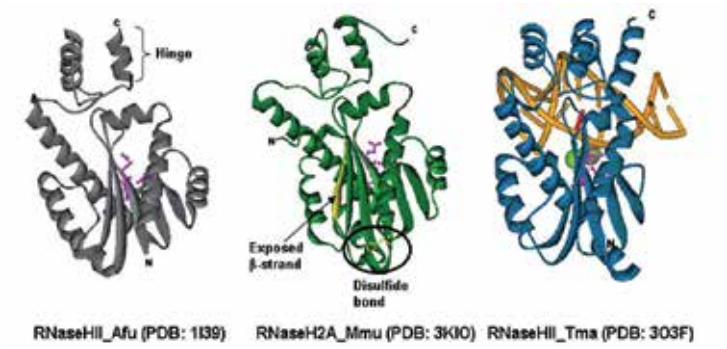
2.3.1 Overall topology

Structural comparison of three type 2 RNases H from archaea (*Afu*RNase HII: PDB code 1I39), bacteria (*Tma*RNase HII: PDB code 3O3F) and eukaryotes (*Mmu*RNase H2A: PDB code 3KIO) identifies a conserved catalytic core, termed RNase H fold, consisting of a five-stranded β sheet with three antiparallel and two parallel strands (54123, ↑↑↑↓↑) surrounded by α-helices (Fig. 1a)(Nowotny and Yang, 2009; Yang and Steitz, 1995). Concomitant with high sequence similarity, the three-dimensional structures of single polypeptide archaeal RNases HII from *A. fulgidus*, *M. jannaschii* and *T. kodakaraensis* share analogous topology and fold (Chapados et al., 2001; Lai et al., 2000; Muroya et al., 2001). They are composed of two distinct domains. The N-terminal domain comprises the central catalytic core flanked by seven α-helices (α1- α7). The C-terminal domain mainly consists of two parallel α-helices (α8- α9). Unlike *Tko*RNase HII, the helix α9 is incomplete in *Afu*RNase HII, *Mja*RNase HII and *Pab*RNase HII, as recently reported (Le Laz et al., 2010). This secondary structure element is important for *Tko*RNase HII to bind substrate (Muroya et al., 2001). On the other hand, a flexible hinge region (residues $_{195}$SNLR$_{198}$ in helix α9) in *Afu*RNase HII allows a wide range of motion when bound to its cognate PCNA (Bubeck et al., 2011)(Fig. 1a). Likewise, the structures of archaeal RNases HII are very similar to the bacterial ortholog, *Tma*RNase HII. For instance, the structures of *Afu*RNase HII and *Tma*RNase HII are superimposable

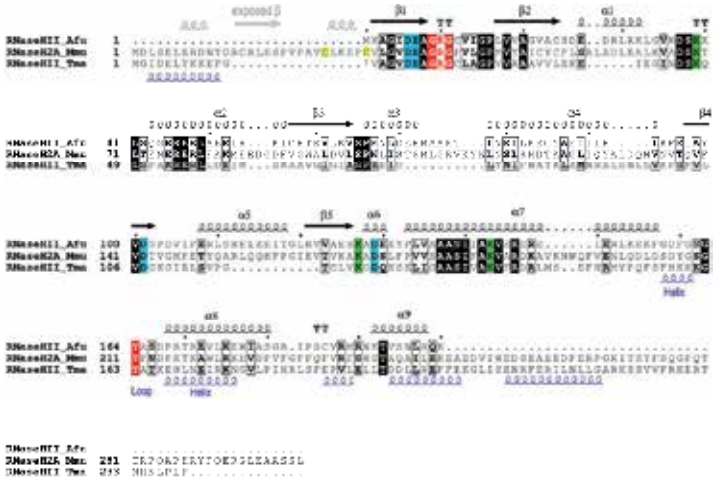
with root-mean-square deviations (RMSD) of 2.6 Å over 128 Cα atoms (SuperPose V1.0 server (Maiti et al., 2004)). *Tma*RNase HII consists of the N-terminal domain with the five-stranded β sheet flanked by two helices on one side and three helices on the other side. The C-terminal domain contains two helices constituting a helix-loop-helix motif (Rychlik et al., 2010)(Fig. 1b). Additionally, *Tma*RNase HII possesses approximate 39-amino acids C-terminal and 12-amino acids N-terminal extensions that are not found in the archaeal RNase HII (Fig. 1b). Recently, the crystal structure of the heterotrimeric mouse RNase H2 (*Mmu*RNase H2) has been solved, consisting of the RNase H2B-RNase H2C subcomplex that interfaces with the catalytic RNase H2A protein (Shaban et al., 2010). The RNase H2B-RNase H2C dimer is reported to provide a structural support for RNase H2A to become active and also acts as a platform for interactions with other proteins, such as PCNA (Bubeck et al., 2011; Chon et al., 2009). *Mmu*RNase H2A shares significant structural similarity to the archaeal *Afu*RNase HII and the bacterial *Tma*RNase HII. For example, the structures of *Afu*RNase HII and *Mmu*RNase H2A are superimposable with RMSD of 3.6 Å over 196 Cα atoms (SuperPose V1.0 server (Maiti et al., 2004)). *Mmu*RNase H2A contains the conserved RNase H fold surrounded by α-helices (Shaban et al., 2010). Moreover, it comprises approximate 30-amino acids N-terminal and 50-amino acids C-terminal extensions that are absent in *Afu*RNase HII (Fig. 1b). Unlike *Tma*RNase HII that contains a helix-loop-helix motif at its extended C-terminal domain (Rychlik et al., 2010), this region appears disordered in the *Mmu*RNase H2A structure (Fig. 1b) (Shaban et al., 2010). *Mmu*RNase H2A N-terminal extension is structurally organised and forms a β-strand (called β1 or exposed β-strand) held rigidly by a disulfide bond (Fig. 1). This exposed β-strand is thought to act as an additional protein-protein interface (Shaban et al., 2010).

2.3.2 Active site, substrate binding and catalytic mechanism

Because the structures of type 2 RNases H contain a common RNase H fold, the catalytic center may be similar among archaea, bacteria and eukaryotes. Comparison of secondary structure among *Afu*RNase HII, *Mmu*RNase H2A and *Tma*RNase HII for which crystal structures have been solved (Bubeck et al., 2011; Chapados et al., 2001; Shaban et al., 2010) points out that these three enzymes have remarkably similar active sites, consisting of four highly conserved carboxylates (DEDD motif) (Fig. 1b). The spatial clustering of these carboxylates is preserved among archaeal enzymes (Chapados et al., 2001; Lai et al., 2000; Muroya et al., 2001), and forms a small catalytic pocket composed of strands β1, β4 and helix α6. This active site geometry has been functionally analysed in *Afu*RNase HII and *Tko*RNase HII (Chapados et al., 2001; Muroya et al., 2001). Mutations of D6, E7, and D101 abolished the nucleolytic activity, while mutation of D129 showed 50% reduced activity in *Afu*RNase HII. Corresponding residues (D34, E35, D142, and D170) have been mutated in *Mmu*RNase H2A, and the resulting heterotrimeric mutant RNase H2 exhibited no detectable activity (Shaban et al., 2010). Similarly, conservative mutations and subsequent functional analyses of three potential active site carboxylates in yeast RNase H2A led to a loss of substrate cleavage (Jeong et al., 2004). The four carboxylates are positionally conserved in *Mmu*RNase H2A, and form a catalytic pocket composed of strands β2 and β5, and helices α4 and α5 (Shaban et al., 2010). The active site of *Tma*RNase HII is also composed of four conserved carboxylates that are structurally similar to *Afu*RNase HII, *Mmu*RNase H2A. These residues (D18, E19, D107, and D124) are located in the catalytic cleft that is lined by strands β1 and β4, and a loop before the last helix of the RNase H fold (Rychlik et al., 2010). Thus, the similar active site geometry observed in eukaryotes and prokaryotes suggests a conserved two-metal ion catalytic mechanism in type 2 RNases H.



(a)



(b)

Fig. 1. Structure and structure-based sequence alignment of *Archaeoglobus fulgidus* (Afu), *Mus Musculus* (Mmu) and *Thermotoga maritima* (Tma) type 2 RNases H. (a) Ribbon diagrams of RNaseHII_Afu (PDB ID: 1I39), RNaseH2A_Mmu (PDB: 3KIO) and RNaseHII_Tma (PDB: 3O3F). The active-site carboxylates are shown as magenta ball-and-stick models. Exposed β-sheet and disulfide bonds are shown in *yellow* in RNaseH2A_Mmu. The flexible hinge is highlighting ($_{195}$SNLR$_{198}$). The two magnesium ions are shown as *green* spheres in RNaseHII_Tma complexed with the DNA-RNA$_1$-DNA/DNA substrate, (b) Sequence alignment of the three type 2 RNases H based on the three-dimensional structure of RNaseHII_Afu. Conserved active site residues are highlighted in *blue*. Conserved residues that contact the nucleic acid backbone in the co-crystal structure of RNaseHII_Tma are highlighted in *green*. Conserved GRG motif and tyrosine residue involved in junction sensing coupled to catalysis in RNaseHII_Tma are highlighted in *red*. Cysteine residues forming a disulfide bond in RNaseH2A_Mmu are highlighted in *yellow*.

In the crystal structure of *Tma*RNase HII in complex with single embedded ribonucleotide, the substrate is bound to the protein, such that the noncleaved strand fits in a groove on the protein surface at the C-terminal domain, and the cleaved strand containing the single ribonucleotide interacts with the catalytic site (Rychlik et al., 2010). Several basic residues, e.g., K47, K122, and K138, are involved in substrate binding, and contact with the phosphate

groups of the nucleic acid backbone in *Tma*RNase HII. These three lysines are strictly conserved in *Afu*RNase HII and *Mmu*RNase H2A (Fig. 1b), which strongly supports a putative role for these residues in substrate binding. In fact, mutational studies indicated that K138 equivalent in *Afu*RNase HII is important for nucleic acid binding (Chapados et al., 2001). Furthermore, a substrate recognition motif (G21, R22, and G23) and Y163 have been identified as key structural elements responsible for the detection and cleavage of single embedded ribonucleotides in *Tma*RNase HII (Rychlik et al., 2010). Accordingly, mutational analyses of Y163 equivalent in *Afu*RNase HII suggested that this residue may intercalate into the duplex to stabilize a bent conformation required for substrate recognition and catalysis (Chapados et al., 2001). The highly conserved GRG motif and tyrosine residue (Fig. 1b) convincingly suggests that the specific recognition mechanism for single embedded ribonucleotides must be similar among type 2 RNases H. Besides, it has been recently described that the four conserved carboxylates of the DEDD motif were directly involved in coordinating the metal ions in *Tma*RNase HII, the distance between the two metal ions (A and B) imposed by the geometry of the active site carboxylates and substrate (Rychlik et al., 2010). The influence of nucleic acid on the metal-ion coordination ensures the catalytic specificity of *Tma*RNase HII. One of the most important features observed in the co-crystal structure of *Tma*RNase HII is that the tyrosine required for 2'-OH detection of single embedded ribonucleotides also participates in metal ion positioning, because it induces a slight deformation of the nucleic backbone at the RNA.DNA junction, and renders possible the coordination of metal ion A by the phosphate group. Moreover, a conserved DSK motif (D45, S46, and K47) located in the vicinity of the catalytic pocket has been proposed to participate in the active site formation of *Tma*RNase HII (Rychlik et al., 2010). Based on strictly conserved key structural elements responsible for substrate binding, metal ion coordination, and formation of the catalytic center in *Tma*RNase HII, the cleavage mechanism of single ribonucleotides embedded in dsDNA likely proceeds in a similar fashion in archaeal, bacterial and eukaryotic type 2 RNases H.

2.4 Physiological roles for RNases HII/2

Type 2 RNases H are represented in organisms across domains and exhibit a conserved core structure. Moreover, they have been identified as the sole enzymes able to recognise and cleave a single ribonucleotide embedded in dsDNA (Eder and Walder, 1991), thereby contrasting to type 1 RNases H that requires at least four ribonucleotides for cleavage (Ohtani et al., 1999a). As mentioned earlier, single ribonucleotides embedded in dsDNA can arise from external damaging agents (Von Sonntag and Schulte-Frohlinde, 1978), and can occur by intrinsic RNA ligation (Rumbaugh et al., 1997) or erroneous nucleotide incorporation during DNA replication (Nick McElhinny et al., 2010a; Nick McElhinny et al., 2010b). The presence of riboses in DNA has been shown to induce a helical alteration, promoting a B- to A-form transition in DNA (Horton and Finzel, 1996). This result is consistent with the local DNA backbone distortion recently observed in the structure of *Tma*RNase HII bound to single embedded ribonucleotide substrates (Rychlik et al., 2010). If left unrepaired, such structural alterations could be mutagenic for the cells, given that accurate DNA synthesis by replicative DNA polymerases depends on DNA helix geometry. Since type 2 RNases H specifically cleave single embedded ribonucleotides, they can be considered to be involved in DNA repair. However, as the enzymes cut the phosphodiester bond 5' of the junctional ribonucleotide, other components are required to eliminate the remaining ribonucleotide. To clarify the physiological role of type 2 RNases H in bacteria, archaea and eukaryotes, mutant strains containing one or two RNases H-encoding genes

have been constructed. In single-celled species, deletions of all RNase H genes were not lethal, but showed modest sensitivity to DNA-damaging agents, indicating their requirement in DNA repair (Arudchandran et al., 2000; Fukushima et al., 2007; Itaya et al., 1999; Meslet-Cladiere et al., 2007). Conversely, in a multicellular organism, both type 1 and type 2 RNases H were shown to be essential. Deletion of type 1 RNases H causes embryonic lethality in mouse and *Drosophila Melanogaster* (Cerritelli et al., 2003; Filippov et al., 2001), the former impairing mitochondrial DNA replication (Cerritelli et al., 2003). On the other hand, type 2 RNases H have been described as the major source of RNase H activity in eukaryotes (Eder and Walder, 1991; Frank et al., 1998a), and mutations in human RNase H2 can cause the neurological disorder, Aicardi-Goutières Syndrome (AGS). The pathogenesis of AGS is linked to the activation of innate immune system, likely because of accumulation of normally degraded RNA/DNA nucleic acids (Crow et al., 2006). Likewise, defect of the catalytic mutant *Mmu*RNase H2 to hydrolyse single embedded ribonucleotides pointed toward a role for eukaryotic RNase H2 in DNA repair (Shaban et al., 2010). Furthermore, based on genetic and biochemical results, removal of single embedded ribonucleotides seems to involve at least type 2 RNases H, Fen1 (Flap Endonuclease 1) and PCNA (Bubeck et al., 2011; Meslet-Cladiere et al., 2007; Nick McElhinny et al., 2010a; Rumbaugh et al., 1997; Rydberg and Game, 2002), with PCNA:RNase HII/2 complex acting as a sensor of erroneous ribonucleotides. The association of such protein components likely suggests a role of type 2 RNases H in base excision repair (BER) to accomplish the removal of mutagenic ribonucleotides. In this pathway, type 2 RNase H would act initially by recognising and incising the damaged strand at the 5′ side of the ribonucleotide. However, further studies are now required to identify and reconstitute the sequential enzymatic steps involved in this repair process.

3. Endonucleases of the XPF/MUS81 family

DNA repair and replication restart pathways generate a variety of branched structures such as four-way DNA junctions (Holliday junctions, HJs), fork structures and 5'- or 3'-flaps, all of which are substrates for structure-specific endonucleases. Many nucleases that act upon 3'-flap structures belong to the XPF/MUS81 family of proteins, which are present throughout eukarya and archaea but are not found in bacteria. Defects in XPF/MUS81-family members are associated with human disease such as Xeroderma pigmentosum (XPF-ERCC1) (Sijbers et al., 1996) or Fanconi anemia (FANCM) (Meetei et al., 2005).

3.1 Eucaryal members of the XPF/MUS81 family of endonucleases

The human XPF-ERCC1 complex and its counterpart RAD1-RAD10 in *Saccharomyces cerevisiae* have been thoroughly characterized for their role in nucleotide excision repair (NER), cleaving damaged DNA 5′ to the lesion (Bardwell et al., 1994; Sijbers et al., 1996). Moreover RAD1-RAD10 acts in DNA double-strand break repair (Ma et al., 2003; Schiestl and Prakash, 1988), a role also conserved in mammalian cells (Ahmad et al., 2008; Al-Minawi et al., 2008). Notably, the XPF-ERCC complex has been directly observed in living cells using fluorescence resonance energy and spectral imaging techniques (Dinant et al., 2008).

MUS81 complexes are distinct to XPF, and initial work in *Schizosaccharomyces pombe* led to the proposal that Mus81-Eme1 is a Holliday junction resolvase (Boddy et al., 2001). However, *in vitro* work has shown that recombination intermediates such as D-loops and nicked HJs are cleaved by Mus81-Eme1, and are likely to represent its main substrates *in*

vivo (Gaillard et al., 2003; Gaskell et al., 2007; Osman et al., 2003). By contrast the *S. cerevisiae* ortholog Mus81-Mms4, which displays similar substrate specificity *in vitro* (Ehmsen and Heyer, 2008; Gaskell et al., 2007; Whitby et al., 2003), is not the principal HJ resolvase (de los Santos et al., 2003) but instead resolves aberrant joint molecules in meiosis (Jessop and Lichten, 2008; Oh et al., 2008). The recent discovery in humans and *S. cerevisiae* of the Hen1/Yen1 Holliday junction resolvase, which is absent from *S. pombe*, suggests that this enzyme is a meiotic HJ resolvase, and that in its absence Mus81 can fulfill that role (Ip et al., 2008). Nevertheless, in both *S. cerevisiae* and *S. pombe*, the primary function of Mus81 appears to be the restart of collapsed-replication forks by homologous recombination (Doe et al., 2004; Froget et al., 2008; Kai et al., 2005; Matulova et al., 2009; Roseaulin et al., 2008), a role that is functionally redundant with the Sgs1-Top3 and the Rqh1-Top3 complexes, respectively (Doe et al., 2004; Kaliraman et al., 2001). The MUS81 complex is also found in humans (Chen et al., 2001), and appears to promote replication fork restart by homologous recombination (Ciccia et al., 2003; Franchitto et al., 2008; Hanada et al., 2007; Shimura et al., 2008).

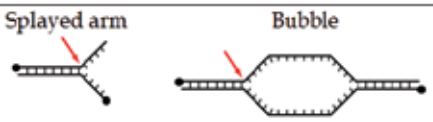
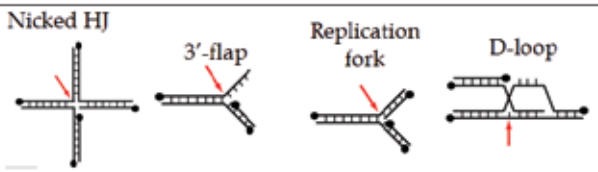
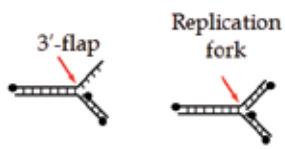
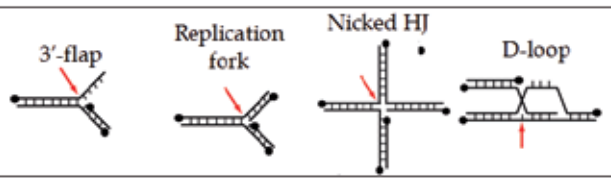
Human FANCM was identified thanks to the archaeal ortholog Hef, an XPF/MUS81 family protein featuring a helicase:nuclease fusion (Meetei et al., 2005; Mosedale et al., 2005). The FANCM-FAAP24 complex is a XPF/MUS81 member found in humans (Ciccia et al., 2007) that possesses two separate functions: (i) to recruit the Fanconi anemia core complex to the repair of DNA interstrand crosslinks (Ciccia et al., 2007; Kim et al., 2008; Meetei et al., 2005; Mosedale et al., 2005), and (ii) to facilitate the response to replication stress by the ATR pathway, *via* its ATP-dependent translocase activity (Collis et al., 2008; Gari et al., 2008; Xue et al., 2008). Since FANCM promotes fork reversal *in vitro*, it has been proposed that FANCM ATP-dependent activity at stalled forks is needed to allow processing for replication restart (Gari et al., 2008; Xue et al., 2008). The recently-identified *S. pombe* FANCM ortholog Fml1 appears to promote homologous recombination at stalled forks, suggesting that the ATP-dependent helicase activity of FANCM at DNA replication forks is conserved in *S. pombe* (Sun et al., 2008).

3.2 Archaeal members of the XPF/MUS81 family of endonucleases

All archaea encode a protein of the XPF/MUS81/FANCM family of endonucleases except the *Thermoplasmatales*. Archaeal XPF exists in two forms: the long form consisting of an N-terminal helicase domain fused to a C-terminal nuclease domain, specific to the euryarchaea, and a short form lacking the helicase domain found in the crenarchaea and thaumarchaea.

3.2.1 Hef

Hef (helicase-associated endonuclease fork-structure DNA) is present only in euryarchaeota and was identified in *Pyrococcus furiosus* due to its activity on branched DNA structures (Komori et al., 2002). Similarly to for instance eukaryotic FANCM proteins, Hef has both an active helicase domain and an active nuclease domain. The C-terminal fragment of *Pyrococcus furiosus* Hef adopts a similar domain organization to those in the XPF/MUS81 proteins, corresponding to the nuclease domain containing the $ERKX_3D$ signature sequence involved in nuclease activity and the helix-hairpin-helix motifs of the HhH domain (Nishino et al., 2003). Mutational analyses showed that residues in the $ERKX_3D$ motif are indeed involved in the cleavage of Hef endonuclease. The Hef nuclease domain adopts a type II

	Substrate specificity *in vitro* (nuclease activity)	Function
S. cerevisae Rad1/Rad10 *H. sapiens* XPF/ERCC1		NER, ICL repair
Yeast Mus81/Eme1 and Mus81/mms4		Stalled fork repair, meiotic recombination
P. furiosus Hef		Stalled fork repair, NER (?)
S. solfataricus XPF		Stalled fork repair (?), NER (?)

Abbreviations: HJ, Holliday junction; NER, Nucleotide excision repair; *S. cerevisae, Saccharomyces cerevisae; H. sapiens, Homo sapiens; P. furiosus, Pyrococcus furiosus; S. solfataricus, Sulfolobus solfataricus.*

Table 1. Substrate specificities and functions of members of the XPF/MUS81 family. Schematic representation of the various substrates that are cleaved *in vitro* by various XPF/MUS81 family proteins. A red arrow indicates the approximate sites of cleavage within each DNA structure. Black circle indicates 5′ termini.

restriction endonuclease fold, indicating that Hef nuclease belongs to this restriction endonuclease family (Nishino et al., 2003). Accordingly, the Hef nuclease activity is strictly dependent on Mg^{2+} or Mn^{2+} whereas Ca^{2+} cannot substitute.

The C-terminal fragment of *Pyrococcus furiosus* Hef and the entire Hef protein form dimers through a combination of two interfaces, one in the nuclease domain and one in the HhH domain, which function independently with each other. It appears that simultaneous dimer formation in both the nuclease and the HhH domains is crucial to substrate recognition specificity (Nishino et al., 2003). In the homodimer, both HhH domains are equally important for substrate recognition while at least one of the nuclease active site is required for cleavage of the fork-structured DNA. The active site of the catalytic domain is positioned near the cleavage site, two to three bases away from the junction, and has the potential to introduce unpairing near the junction center. The HhH region is bound to duplex regions and is not directly involved in the recognition of the fork structure but dramatically enhanced the catalytic site unpairing.

The substrate specificity for the cleavage activity of the Hef protein is contained in the C-terminal domain as both the C-terminal fragment and the entire Hef protein recognize and

cleave nicked, flapped and fork-structured DNAs at the 5′ side of the nicked position. *P. furiosus* Hef thus displays XPF/Mus81-like specificity, suggesting that Hef is involved in NER (Nishino et al., 2005a; Nishino et al., 2005b).

The N-terminal fragment of *P. furiosus* Hef containing all the conserved helicase motifs consist of three structural subdomains. Domains 1 and 3 are each folded into the RecA-like architectures with the conserved helicase motifs. Domain 2 is a relatively mobile domain with a positively charged surface inserted into domain 3 (Nishino et al., 2005b). Hef domain 2 exhibits architectural similarity with the thumb domain of *Taq* DNA polymerase being involved in double-stranded DNA binding. Mutational analyses show that the domain 2 dictates the recognition of specific DNA structures, especially fork-structured DNA, while domains 1 and 3 are crucial for the structure-specific helicase activity. Interestingly, two other SF2 helicase members recognizing branched structures, RecQ and RecG, contain an insertion, after and before the helicase core respectively. It remains unclear how Hef domain 2 participates in branched structure recognition.

The N-terminal domain of Hef displays a DNA structure-specific helicase activity as the most prominent enhancement of the ATPase activity is observed with fork-structured DNAs. Interestingly *in vitro* experiments suggest that the N-terminal domain binds to the fork-structured DNA and process the DNA to increase cleavage of the substrate by the endonuclease domain. These observations have led to the proposal that Hef also acts at stalled replication forks, both the helicase and the nuclease activities being required for the rearrangement of forked-structure DNA (Komori et al., 2004).

The genetic study of Hef mutant in the euryarchaea *Haloferax volcanii* suggest that Hef is involved in the restart of arrested replication forks as an alternative pathway to homologous recombination-dependent pathway (Lestini et al., 2010). In this organism Hef is not involved in nucleotide excision repair but *Haloferax volcanii* possesses bacterial homologs of NER proteins which may have displaced the original archaeal NER proteins. Therefore the absence of an NER function of Hef cannot be generalized to all euryarchaea. In support to this, a recent genetic analysis of *hef* mutants in the hyperthermophilic archaeon, *Thermococcus Kodakaraensis*, has demonstrated that Hef is involved in the repair of a wide variety of DNA damages. In addition, the higher sensitivity of Δ*hef* mutants to methyl sulfonate and mitomycin C, suggests a central role for Hef protein in the archaeal NER and/or ICL repair pathways (Fujikane et al., 2010).

3.2.2 XPF in Crenarcheota

By contrast, the XPF ortholog found in crenarchaeota contains only the C-terminal nuclease domain. The structure of XPF from the crenarchaea *Aeropyrum pernix* reveals two domains, a N-terminal nuclease domain and a $(HhH)_2$ domain (Newman et al., 2005). As expected by analogy with Hef, dimers are formed by interaction of the two nuclease domains and by the interaction of the two $(HhH)_2$ domains from each monomer. Comparaison of the structure of the protein in the presence and absence of dsDNA reveals that the $(HhH)_2$ domain plays a major role in interacting with DNA. Upon binding to DNA a domain movement allow the coupling between the $(HhH)_2$ domain and the nuclease domain to recognize and cleave the DNA thanks to the flexibility of the connecting linker sequence between the two domains. It seems that dimer interaction with DNA involves two binding sites that can only both interact with DNA if the substrate is bent by around 90°, suggesting that XPF may recognize ds/ssDNA junctions by their susceptibility to distorsion. The structural data suggest that only one monomer is catalytically active at a time in a dimer bound to DNA.

The studies of XPF from *Sulfolobus solfataricus* have shown that XPF strickly requires interaction with PCNA to show any nuclease activity and it has been suggested that PCNA directly stimulates the nuclease activity without changing the binding affinity of XPF for its substrate (Hutton et al., 2008). XPF preferentially cleaves 3′-flap but the presence of downstream duplex influences the choice of position cleavage, *Sso*XPF appearing to act as a processive nuclease *in vitro* by processing 3′-flap into gapped duplex products. In this respect, it is noteworthy that recent fluorescence quenching and FRET studies have indicated that PCNA and XPF cooperate to distort DNA substrates (Hutton et al., 2009). It has also been observed that SsoXPF can act on substrates containing a variety of types of DNA damage or modification, suggesting a role in the removal of these lesions *in vivo* (Roberts and White, 2005). Altogether these data suggest that crenarchaeal XPF is recruited by PCNA to act in NER and replication fork restart, but to date this scenario has not yet been addressed using *in vivo* data.

4. The Mre11-Rad50 complex in Archaea

The processing of DNA double strand breaks (DSBs) is a crucial mechanism for genomic integrity. DNA breaks can arise during replication as intermediates in programmed DNA rearrangements including meiosis and immune system development or can be caused by oxidative damages and exposure to ionizing radiations. Double strand break repair (DSBR) is an essential repair pathway in the three domains of life, and plays a major role in the rescue of stalled or collapsed replication forks. In bacteria, DSBs are processed *via* homologous recombination, whereas, in eukarya, they are repaired by homologous or non-homologous recombination (Kowalczykowski et al., 1994; Sonoda et al., 2006). In archaea, the picture is not clear. Homologous recombination (HR) is presumably the preferred process of DSBR as proteins involved in HR have been identified whereas Ku homologs have not (White, 2011); yet a recent genetic study on *Haloferax volcanii* demonstrate that DSBR by HR is restrained, likely because this species is highly polyploid (Delmas et al., 2009). Nevertheless, the study of such processes in hyperthermophilic archaea is of importance, given that they are exposed to DNA damaging temperatures and are among the most radioresistant organisms, repairing fragmented chromosomes efficiently (DiRuggiero et al., 1997; Gerard et al., 2001; Jolivet et al., 2003a; Jolivet et al., 2003b). The core component in charge of the early steps of this critical event in eukaryotic cells is the Mre11-Rad50 (MR) complex, associated with a third component, Xrs2 in yeast and Nbs1 in higher eukaryotes. Homologs of the Mre11-Rad50 complex have been found in the three domains of life; however, Xrs2/Nbs1 additional component has not been found in bacteria and archaea, it appears thus that Mre11 and Rad50 comprise the core enzymatic members of this conserved multiprotein machine.

4.1 Catalytic activities and DSB ends processing

The archaeal homologs of Mre11 and Rad50 were initially identified in *Pyrococcus furiosus* (Hopfner et al., 2000a). *Pfu*Mre11 shares significant similarities with eukaryotic counterparts, particularly in the N-terminal region, which contains five phosphoesterase motifs that form the nuclease domain of the protein (Williams et al., 2008). The enzyme is endowed with ssDNA endonuclease and manganese-dependent 3′-5′exonuclease activities (Hopfner et al., 2000a). However, at temperature closer to physiological conditions (55°C), *Pfu*Mre11 displays an ATP dependent nuclease activity in magnesium. This activity consists

of both a weak 3′-5′ exonuclease activity as well as endonucleolytic cleavage activity on the 5′ strand at a break (Hopkins and Paull, 2008). *Pfu*Rad50 is related to the Structural Maintenance of Chromosomes (SMC) family and displays the classical modular structure composed of N- and C- terminal head domains, each bearing the Walker A and B motifs, respectively, separated by a coiled-coil region with a zinc hook. Rad50 has ATPase and adenylate kinase activities (Bhaskara et al., 2007; Hopfner et al., 2000b). Consistent with the model based on the crystallographic structure of *Pfu*Rad50 catalytic domain, the MR complex of *P. furiosus* likely exhibits ATP-dependent DNA binding activity. Thus, Rad50 may regulate DNA binding and release after proper DNA end processing in conjunction with Mre11 (Hopfner et al., 2000b). All the archaeal genomes sequenced to date contain clear homologs of eukaryal Mre11 and Rad50 and the initial biochemical characterization of the *P. furiosus* homologs indicate that the archaeal MR complex is functionally similar to those from Bacteria and Eukarya. However, the 3′-5′ exonuclease activity described for the complex is opposite to the polarity required for the 5′-3′ resection of DSB ends necessary for the initiation of HR, suggesting the involvement of additional components to catalyse efficient DSB resection. As in the other thermophilic archaea, Mre11 and Rad50 are commonly found in an operon that frequently includes the *herA* and *nurA* genes and the four genes are co-transribed in the crenarchaeon *Sulfolobus acidocaldarius* (Constantinesco et al., 2002; Constantinesco et al., 2004). HerA is an ATP-dependent helicase, which is strikingly bidirectional and can thus unwind DNA from both 3′ and 5′ single-stranded overhangs. NurA defines a new nuclease family exhibiting both a single strand endonuclease activity and a 5'-3' exonuclease activity on single and double stranded DNA (Constantinesco et al., 2002; Constantinesco et al., 2004; Manzan et al., 2004). The cooperation of the four proteins for the 5′ strand resection at DSB has been demonstrated by Hopkins and Paull (Hopkins and Paull, 2008). This process depends on the enzymatic activities of HerA, NurA and Rad50, Mre11 activity being partially dispensable. The *P. furiosus* MR complex generates short 3′ single stranded overhangs through limited degradation of the 5′ strand at a DSB. This specific structure allows the entry of the complex HerA-NurA and the NurA nuclease together with HerA helicase activities generate the long 3′ single strand suitable for RadA-catalysed strand exchange. The role of Mre11 nuclease activity may be confined to the removal of short 5′ terminal oligonucleotides, which could be essential for the clearance of covalently attached proteins at the 5′ strand. HerA and NurA have not been detected in eukaryotes, however, recent studies in budding yeast demonstrate that while MRX complex is involved in DSBs processing initiation, the functional homologs Sgs1 and Exo1 nucleases and Dna2 nuclease/helicase, are necessary for the extensive 5′ strand resection (Mimitou and Symington, 2008; Zhu et al., 2008). In bacteria, the DSB recognition and 5′strand resection to produce the 3′-OH overhang for RecA mediated strand exchange is performed by a single RecBCD complex (Kowalczykowski et al., 1994). SbcCD, the bacterial homolog of MR complex, has been shown to cleave hairpin DNA, which can block stalled replication fork, prior to homologous recombination rescue of the fork (Connelly et al., 1998) and to be implicated in the removal of covalently attached protein to promote repair (Connelly et al., 2003).

4.2 Structural insight into the Mre11-Rad50 complex

The archaeal MR complex is structurally very similar to their eukaryal counterparts, and has proven very useful for crystallographic and biophysical studies (Arthur et al., 2004; Hopfner et al., 2002a; Hopfner et al., 2001; Hopfner et al., 2000a; Hopfner et al., 2000b; Hopfner et al.,

2002b; Williams et al., 2008). Indeed, with the exception of the recent description of the first eubacterial Mre11 nuclease, the bulk of structural data have been obtained from analysis of *P. furiosus* Mre11-Rad50 complex (Das et al., 2010).

The core Mre11-Rad50 complex forms a large globular complex at the root of an elongated coiled-coil structure. The complex exists as a heterotetrameric assembly (M_2R_2) and the globular head is composed of two Mre11 and two Rad50 ATPase domains, both of which bind DNA (Hopfner et al., 2001). This bipolar structure of the MR complex is consistent with both the enzymatic role in DNA end processing and structural function in DNA end joining. Indeed the M_2R_2 heterotetramer contains two DNA binding/processing active sites, which could be important in the alignment of DNA ends in NHEJ or of DNA ends and sister chromatids in HR (Hopfner et al., 2002a). X-ray crystallographic data from the *P. furiosus* Rad50 coiled-coil region reveals a hook structure that caps the distal end of the coiled-coils with a conserved Cys-X-X-Cys motifs that mediates Rad50-Rad50 dimerization through this motif by coordinated binding of a zinc atom (Hopfner et al., 2002b). The ability to interact through coiled coil ends in supra-molecular complexes is proposed to be necessary for the mechanistic role of MR complex (de Jager et al., 2004). The crystallographic structure of *Pfu*Mre11 reveals a two-domain architecture consisting of a protein-phosphatase-like domain and a small capping domain that interact at the active site (Fig.2).

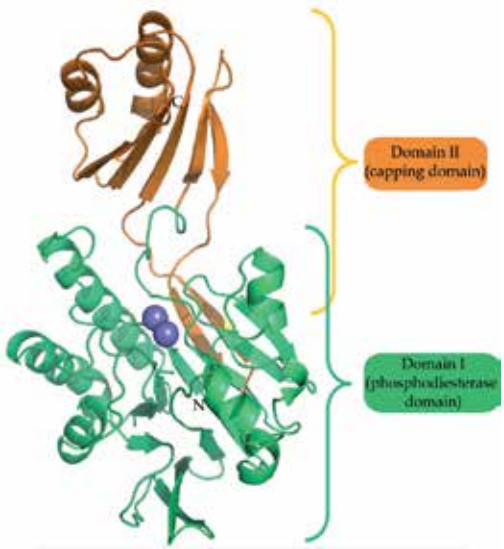


Fig. 2. Crystal structure of *Pfu*Mre11. Ribbon diagram of the two domain fold of *P. furiosus* Mre11 (1-342, PDB_1II7). Two Mn^{2+} ions (purple spheres) are coordinated by seven conserved residues of the phosphodiesterase motifs (Hopfner et al., 2001).

The first domain contains the five phosphoesterase motifs which form the nuclease active site. This domain is composed of two parallel mixed β sheets that are flanked by seven α helices. The capping domain consists of a 5-stranded β sheet and two helices and partially caps the nuclease catalytic motifs of the N-terminal domain, suggesting that the capping domain might be involved in DNA-binding specificity (Hopfner et al., 2001). X-ray structure of *Pfu*Mre11 bound to DNA reveals that Mre11 dimerization is critical for efficient DNA binding and is mediated by N-terminal conserved domains (Williams et al., 2008). Six DNA

recognition loops promote DNA binding and assemble into a contiguous DNA interaction surface. The interaction with DNA is mediated by contacts to the sugar-phosphate backbone, which is consistent with the lack of sequence specificity for Mre11 in DNA binding. The structure of the archaeal Mre11 was used to map eukaryotic Mre11 mutations linked to human disease and yeast DSBR defect and helped define a large surface area outside the nuclease motif, which may be an important protein-protein or protein-DNA interface (Hopfner et al., 2001).

4.3 Physiological roles of Mre11-Rad50 complex

Genetic studies in eukaryotes indicate that the MR complex is required for genomic stability and is involved in a large variety of different functions in response to DSBs (Stracker and Petrini, 2011). *In vivo* studies, in budding and fission yeast, have demonstrated a critical role for Mre11 and the other subunits of the MRX complex, for survival of DSBs caused by ionizing radiations and genotoxins (Chahwan et al., 2003; D'Amours and Jackson, 2002). In addition, hypomorphic mutations in the human *mre11* and *nsb1* genes lead to ataxia telangiectasia-like disorder and Nijmegen Breakage syndrome, respectively. The cellular features resulting from these mutations include hypersensitivity to ionizing radiation, radioresistant DNA synthesis, and abrogation of ATM-dependent events (Stewart et al., 1999; Williams et al., 2007). These phenotypes emphasize the importance of the eucaryotic Mre11 complex in a large variety of DNA metabolic pathways. The bacterial homologue of Mre11-Rad50 is SbcCD, and it has been shown that *sbcCD* mutants of *Deinococcus radiodurans* display reduced survival and present a delay in kinetics of DSB repair (Bentchikou et al., 2007). In *Bacillus subtilis* and *D. radiodurans*, the deletion of *sbcC* results in an increased sensitivity of the cells to ionizing radiation (Mascarenhas et al., 2006).

In archaea, the first evidence of the involvement of Mre11 in DNA repair process was demonstrated by Quaiser et al. (Quaiser et al., 2008), using an immunodetection approach to determine the roles of Mre11, Rad50, NurA and HerA proteins, in post-irradiation DNA repair in *S. acidocaldarius*. They observe that a complex of the three proteins HerA, Mre11 and Rad50 is formed constitutively *in vivo*. Rad50 is constitutively associated with DNA and upon chromosome fragmentation, Mre11 proteins is recruited to the DNA or to Rad50 DNA-bound proteins, suggesting that Mre11 is actively involved in DNA repair processes and/or acts as an inducible damage sensor. The analyses of *mre11 rad50* deletion mutants of the halophilic *Halobacterium sp.* strain NRC1 also suggest that Mre11 and Rad50 may have independent functions outside the MR complex in archaea, since the absence of Rad50 has no effect on the repair of DSBs, whereas the loss of Mre11 results in a decrease rate of DSBR, due to the loss of either nuclease activity or the DNA damage-sensing activity of Mre11 (Kish and DiRuggiero, 2008). Surprinsingly, the *mre11 rad50* deletion mutant of *Haloferax volcanii* displays an enhanced resistance to DNA damage that correlates with a higher level of homologous recombination in the mutant, suggesting that Mre11-Rad50 restrains the use of HR for repair (Delmas et al., 2009). The unrestrained use of HR in *mre11 red50* mutant enhances cells survival but leads to a slower recovery presumably due to difficulties in the resolution of repair intermediates. Two non-exclusive hypotheses are proposed to account for that increased resistance observed in *mre11 rad50* mutants: (i) Mre11-Rad50 binds to double-strand breaks and prevents HR, and/or (ii) Mre11-Rad50 stimulates an alternative pathway of double-strand breaks repair. To add some complexity, a recent genetic analysis in the hyperthermophilic archaeon *Thermococcus kodakaraensis*, has shown that the *mre11*, *rad50*, *herA*, *nurA* and *radA* genes are essential for *T. kodakaraensis*, which is in contradiction

with the previous genetic analyses in archaea, yeast and bacteria. This result could reflect the importance of HR to repair DNA damage caused by the high temperature required for *T. kodakaraensis* for viability (Fujikane et al., 2010).
Contrasting with the wealth of structural and biochemical data gained from the study of archaeal MR complex, the paucity and the conflicting nature of the genetic analyses underscore the importance to develop more effective genetic tools, for hyperthermophilic archaea in particular, to improve our knowledge on the functions of the complex in response to DSBs. Biochemical and *in vivo* investigations of the functions of HerA, NurA and the single stranded DNA binding proteins, RadA and SSB/RPA, required for strand exchange, should also improve our knowledge of the HR/DSBR in archaea and potentially shed light on the eukaryal pathway.

5. The Pab2263-NucS protein

5.1 Identification of a novel nuclease

Many nucleases are highly regulated by the sliding clamp PCNA (Proliferating Cell Nuclear Antigen). For instance, PCNA increases the affinity of Fen-1 for its substrate (Hutton et al., 2008) as well as catalytic rate of *Sso*XPF (Hutton et al., 2009). Interaction of Fen-1 and other proteins with PCNA is mediated by the so-called PIP-motif (PCNA Interacting Motif), a relatively short peptide motif found in a large number of PCNA-interacting proteins (Vivona and Kelman, 2003; Warbrick, 1998). The sequence motif in *Pyrococcus abyssi* species has been experimentally defined and corresponds to the peptide motif QX_2LX_2[WLFT][LFT] (Meslet-Cladiere et al., 2007). Among others, previously uncharacterized protein encoded by the *pab2263* gene was shown to carry this peptide motif at its carboxy-terminus.
Pab2263 belongs to the DUF91 family (Domain of Unknown Function 91) and, as many members of this family, contains the C-terminal domain that carries the characteristic residues forming the active site of the RecB family nucleases. This nuclease domain is found in many enzymes with potential functions in DNA replication and/or repair (Aravind et al., 2000). For instance, the DUF91 family members are found in euryarchaeota (59 homologues annotated in 2011), crenarchaeota (33 homologues), actinobacteria (259 homologues), and proteobacteria (41 homologues). Up to date, no eukaryotic member of the DUF91 family has been identified.

5.2 Structure of Pab2263-NucS

Crystallographic structure of Pab2263 has been solved (Ren et al., 2009; Ren et al., 2007) and is the first representative of the DUF91 family. Pab2263 is composed of two independent domains, separated by a long and flexible linker (~28Å). This multi-domain organisation is common for many nuclease domains which are often associated with helicase domains (Rouillon and White, 2010).
The C-terminal domain of this protein family displays the minimal endonuclease fold (Pingoud et al., 2005): an α/β structure composed of a five-stranded β-sheet and four flanking α helices. Active site is represented by a sequence motif conserved in the RecB-like nucleases (Aravind et al., 2000): E----[Gh]xxD----hxhh[ED]hK---QhxxY, where 'h' refers to hydrophobic residues (YFWLIVMA) and '---' indicates that the characteristic residues are not consecutive in the sequence. A conserved patch of four basic residues flanks one side of the cleft of the active site and might be involved in the binding of nucleic acids.
Strikingly, the N-terminal domain of Pab2263 displays a half-closed β-barrel, composed of height β-strands arranged in two antiparallel β-sheets. This fold was never previously

described, but can remotely be seen as a structural homologue of the OB- or the Sm-folds, two folds that are involved in the binding of nucleic acid (Kambach et al., 1999; Theobald et al., 2003). In Pab2263, the potential binding site involves two patches of three consecutive basic residues, two conserved aromatic residues and a conserved arginine. High affinity ssDNA binding activity of the N-terminal domain was desmonstrated using site-directed mutagenesis and EMSA experiments (Ren et al., 2009).
The N-terminal domain displays a large hydrophobic patch exposed to the C-terminal domain, and is involved in the dimerisation of the protein. Dimer formation brings one extra residue of one monomer to the active site of the second monomer and the flexible linker cap the active site. As a result, the active site becomes a 'closed' channel, which indicates that the substrate for the enzyme must have a free end.

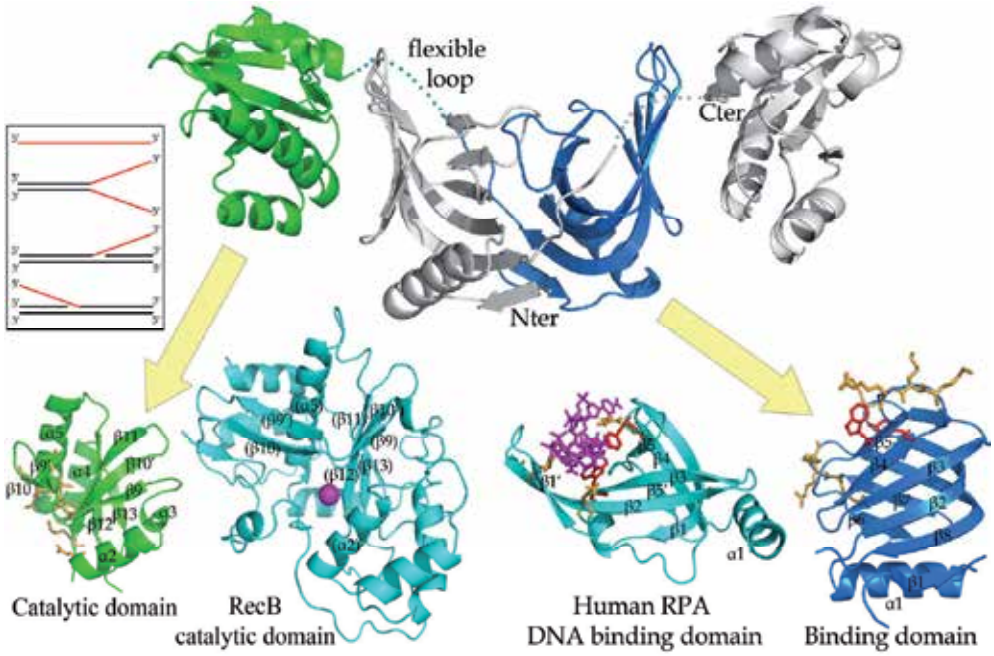


Fig. 3. The structure of Pab2263-NucS. Up: overall organisation of the NucS dimer, one protein is depicted in grey, the other in shades of blue. Down: comparison of the fold of each domain with the RecB one and the RPA one. Structural features of RecB are numbered in the same scheme as NucS. Residues of RPA and NucS involved in the binding of the nucleic acids are detailed; orange and red are respectively basic and aromatic residues. Inlet: range of substrates processed by NucS, cleaved part is indicated in red.

5.3 Activity of the Pab2263-NucS protein

Tests of various substrates on Pab2263 reveal its surprisingly broad range of substrate specificity. In agreement with the structural data indicating that the active site of the Pab2263 is located in the closed channel, this protein preferentially cleaves single stranded DNA, and was thus renamed 'NucS', for 'NUClease specific for single-stranded DNA'. Under stoechiometric binding conditions, single-stranded regions of splayed arms, 3' flaps and 5' flaps are all cleaved by NucS, leaving only double-stranded products. Long single

stranded DNA substrates are cleaved to regularly spaced products, which suggests that the protein could somehow measure its distance to the DNA end.
In high concentration of NucS, nuclease activity can invade to the double stranded DNA regions, suggesting that NucS proteins carry a weak helicase and/or unmelting activity of dsDNA. Important observation is that addition of PCNA directs the cleavage activity of *P. abyssi* NucS towards the ss/ds DNA junctions, thus increasing the cleavage specificity (Ren et al., 2009).
Pab2263-NucS is a founding member of a new family of structure-specific DNA endonuclease. The discovery of this novel nuclease family thus further indicates that archea contain many more nucleases than previously expected on the basis of search of homologous of 'conventional' eukaryotic or bacterial nucleases. For example, the Bax1 and GAN (GINS Associated Nuclease) nucleases from the DUF790 and Phosphoesterase RecJ-like families, respectively, have been identified in a similar manner (Li et al., 2011; Richards et al., 2008; Roth et al., 2009; Rouillon and White, 2010).

6. Conclusion

DNA repair pathways require the function of nucleases to ensure the removal of damages in DNA and thus integrity of the genetic information. The molecular mechanisms of the archaeal DNA nucleases reviewed in this work clearly underscore the conservation of the genetic information processing in archaea and eukarya. Whereas considerable amount of biochemical and structural data are available for archaeal DNA repair nucleases, their physiological roles remain less understood. More biochemical and genetic studies to investigate physiological functions of classical and recently discovered archaeal nucleases have clearly a strong potential to contribute to understanding complexity of eukaryotic DNA repair in the cellular context.

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8. References

Ahmad, A., Robinson, A.R., Duensing, A., van Drunen, E., Beverloo, H.B., Weisberg, D.B., Hasty, P., Hoeijmakers, J.H., and Niedernhofer, L.J. (2008). ERCC1-XPF endonuclease facilitates DNA double-strand break repair. *Mol Cell Biol* 28, 5082-5092.

Al-Minawi, A.Z., Saleh-Gohari, N., and Helleday, T. (2008). The ERCC1/XPF endonuclease is required for efficient single-strand annealing and gene conversion in mammalian cells. *Nucleic Acids Res* 36, 1-9.

Aravind, L., Makarova, K.S., and Koonin, E.V. (2000). SURVEY AND SUMMARY: holliday junction resolvases and related nucleases: identification of new families, phyletic distribution and evolutionary trajectories. *Nucleic Acids Res* 28, 3417-3432.

Arthur, L.M., Gustausson, K., Hopfner, K.P., Carson, C.T., Stracker, T.H., Karcher, A., Felton, D., Weitzman, M.D., Tainer, J., and Carney, J.P. (2004). Structural and functional analysis of Mre11-3. *Nucleic Acids Res* 32, 1886-1893.

Arudchandran, A., Cerritelli, S., Narimatsu, S., Itaya, M., Shin, D.Y., Shimada, Y., and Crouch, R.J. (2000). The absence of ribonuclease H1 or H2 alters the sensitivity of Saccharomyces cerevisiae to hydroxyurea, caffeine and ethyl methanesulphonate: implications for roles of RNases H in DNA replication and repair. *Genes Cells* 5, 789-802.

Bardwell, A.J., Bardwell, L., Tomkinson, A.E., and Friedberg, E.C. (1994). Specific cleavage of model recombination and repair intermediates by the yeast Rad1-Rad10 DNA endonuclease. *Science* 265, 2082-2085.

Bentchikou, E., Servant, P., Coste, G., and Sommer, S. (2007). Additive effects of SbcCD and PolX deficiencies in the in vivo repair of DNA double-strand breaks in Deinococcus radiodurans. *J Bacteriol* 189, 4784-4790.

Bhaskara, V., Dupre, A., Lengsfeld, B., Hopkins, B.B., Chan, A., Lee, J.H., Zhang, X., Gautier, J., Zakian, V., and Paull, T.T. (2007). Rad50 adenylate kinase activity regulates DNA tethering by Mre11/Rad50 complexes. *Mol Cell* 25, 647-661.

Boddy, M.N., Gaillard, P.H., McDonald, W.H., Shanahan, P., Yates, J.R., 3rd, and Russell, P. (2001). Mus81-Eme1 are essential components of a Holliday junction resolvase. *Cell* 107, 537-548.

Bubeck, D., Reijns, M.A., Graham, S.C., Astell, K.R., Jones, E.Y., and Jackson, A.P. (2011). PCNA directs type 2 RNase H activity on DNA replication and repair substrates. *Nucleic Acids Res*. 39, 3652-3666

Cerritelli, S.M., and Crouch, R.J. (2009). Ribonuclease H: the enzymes in eukaryotes. *Febs J* 276, 1494-1505.

Cerritelli, S.M., Frolova, E.G., Feng, C., Grinberg, A., Love, P.E., and Crouch, R.J. (2003). Failure to produce mitochondrial DNA results in embryonic lethality in Rnaseh1 null mice. *Mol Cell* 11, 807-815.

Chahwan, C., Nakamura, T.M., Sivakumar, S., Russell, P., and Rhind, N. (2003). The fission yeast Rad32 (Mre11)-Rad50-Nbs1 complex is required for the S-phase DNA damage checkpoint. *Mol Cell Biol* 23, 6564-6573.

Chai, Q., Qiu, J., Chapados, B.R., and Shen, B. (2001). Archaeoglobus fulgidus RNase HII in DNA replication: enzymological functions and activity regulation via metal cofactors. *Biochem Biophys Res Commun* 286, 1073-1081.

Chapados, B.R., Chai, Q., Hosfield, D.J., Qiu, J., Shen, B., and Tainer, J.A. (2001). Structural biochemistry of a type 2 RNase H: RNA primer recognition and removal during DNA replication. *J Mol Biol* 307, 541-556.

Chen, X.B., Melchionna, R., Denis, C.M., Gaillard, P.H., Blasina, A., Van de Weyer, I., Boddy, M.N., Russell, P., Vialard, J., and McGowan, C.H. (2001). Human Mus81-associated endonuclease cleaves Holliday junctions in vitro. *Mol Cell* 8, 1117-1127.

Chon, H., Vassilev, A., DePamphilis, M.L., Zhao, Y., Zhang, J., Burgers, P.M., Crouch, R.J., and Cerritelli, S.M. (2009). Contributions of the two accessory subunits, RNASEH2B and RNASEH2C, to the activity and properties of the human RNase H2 complex. *Nucleic Acids Res* 37, 96-110.

Ciccia, A., Constantinou, A., and West, S.C. (2003). Identification and characterization of the human mus81-eme1 endonuclease. *J Biol Chem* 278, 25172-25178.

Ciccia, A., Ling, C., Coulthard, R., Yan, Z., Xue, Y., Meetei, A.R., Laghmani el, H., Joenje, H., McDonald, N., de Winter, J.P., *et al.* (2007). Identification of FAAP24, a Fanconi anemia core complex protein that interacts with FANCM. *Mol Cell* 25, 331-343.

Collis, S.J., Ciccia, A., Deans, A.J., Horejsi, Z., Martin, J.S., Maslen, S.L., Skehel, J.M., Elledge, S.J., West, S.C., and Boulton, S.J. (2008). FANCM and FAAP24 function in ATR-

mediated checkpoint signaling independently of the Fanconi anemia core complex. *Mol Cell* 32, 313-324.

Connelly, J.C., de Leau, E.S., and Leach, D.R. (2003). Nucleolytic processing of a protein-bound DNA end by the E. coli SbcCD (MR) complex. *DNA Repair (Amst)* 2, 795-807.

Connelly, J.C., Kirkham, L.A., and Leach, D.R. (1998). The SbcCD nuclease of Escherichia coli is a structural maintenance of chromosomes (SMC) family protein that cleaves hairpin DNA. *Proc Natl Acad Sci U S A* 95, 7969-7974.

Constantinesco, F., Forterre, P., and Elie, C. (2002). NurA, a novel 5'-3' nuclease gene linked to rad50 and mre11 homologs of thermophilic Archaea. *EMBO Rep* 3, 537-542.

Constantinesco, F., Forterre, P., Koonin, E.V., Aravind, L., and Elie, C. (2004). A bipolar DNA helicase gene, herA, clusters with rad50, mre11 and nurA genes in thermophilic archaea. *Nucleic Acids Res* 32, 1439-1447.

Crow, Y.J., Leitch, A., Hayward, B.E., Garner, A., Parmar, R., Griffith, E., Ali, M., Semple, C., Aicardi, J., Babul-Hirji, R., *et al.* (2006). Mutations in genes encoding ribonuclease H2 subunits cause Aicardi-Goutieres syndrome and mimic congenital viral brain infection. *Nat Genet* 38, 910-916.

D'Amours, D., and Jackson, S.P. (2002). The Mre11 complex: at the crossroads of dna repair and checkpoint signalling. *Nat Rev Mol Cell Biol* 3, 317-327.

Das, D., Moiani, D., Axelrod, H.L., Miller, M.D., McMullan, D., Jin, K.K., Abdubek, P., Astakhova, T., Burra, P., Carlton, D., *et al.* (2010). Crystal structure of the first eubacterial Mre11 nuclease reveals novel features that may discriminate substrates during DNA repair. *J Mol Biol* 397, 647-663.

de Jager, M., Trujillo, K.M., Sung, P., Hopfner, K.P., Carney, J.P., Tainer, J.A., Connelly, J.C., Leach, D.R., Kanaar, R., and Wyman, C. (2004). Differential arrangements of conserved building blocks among homologs of the Rad50/Mre11 DNA repair protein complex. *J Mol Biol* 339, 937-949.

de los Santos, T., Hunter, N., Lee, C., Larkin, B., Loidl, J., and Hollingsworth, N.M. (2003). The Mus81/Mms4 endonuclease acts independently of double-Holliday junction resolution to promote a distinct subset of crossovers during meiosis in budding yeast. *Genetics* 164, 81-94.

Delmas, S., Shunburne, L., Ngo, H.P., and Allers, T. (2009). Mre11-Rad50 promotes rapid repair of DNA damage in the polyploid archaeon Haloferax volcanii by restraining homologous recombination. *PLoS Genet* 5, e1000552.

Dinant, C., van Royen, M.E., Vermeulen, W., and Houtsmuller, A.B. (2008). Fluorescence resonance energy transfer of GFP and YFP by spectral imaging and quantitative acceptor photobleaching. *J Microsc* 231, 97-104.

DiRuggiero, J., Santangelo, N., Nackerdien, Z., Ravel, J., and Robb, F.T. (1997). Repair of extensive ionizing-radiation DNA damage at 95 degrees C in the hyperthermophilic archaeon Pyrococcus furiosus. *J Bacteriol* 179, 4643-4645.

Doe, C.L., Osman, F., Dixon, J., and Whitby, M.C. (2004). DNA repair by a Rad22-Mus81-dependent pathway that is independent of Rhp51. *Nucleic Acids Res* 32, 5570-5581.

Eder, P.S., and Walder, J.A. (1991). Ribonuclease H from K562 human erythroleukemia cells. Purification, characterization, and substrate specificity. *J Biol Chem* 266, 6472-6479.

Ehmsen, K.T., and Heyer, W.D. (2008). Saccharomyces cerevisiae Mus81-Mms4 is a catalytic, DNA structure-selective endonuclease. *Nucleic Acids Res* 36, 2182-2195.

Filippov, V., Filippov, M., and Gill, S.S. (2001). Drosophila RNase H1 is essential for development but not for proliferation. *Mol Genet Genomics* 265, 771-777.

Franchitto, A., Pirzio, L.M., Prosperi, E., Sapora, O., Bignami, M., and Pichierri, P. (2008). Replication fork stalling in WRN-deficient cells is overcome by prompt activation of a MUS81-dependent pathway. *J Cell Biol* 183, 241-252.

Frank, P., Albert, S., Cazenave, C., and Toulme, J.J. (1994). Purification and characterization of human ribonuclease HII. *Nucleic Acids Res* 22, 5247-5254.

Frank, P., Braunshofer-Reiter, C., and Wintersberger, U. (1998a). Yeast RNase H(35) is the counterpart of the mammalian RNase HI, and is evolutionarily related to prokaryotic RNase HII. *FEBS Lett* 421, 23-26.

Frank, P., Braunshofer-Reiter, C., Wintersberger, U., Grimm, R., and Busen, W. (1998b). Cloning of the cDNA encoding the large subunit of human RNase HI, a homologue of the prokaryotic RNase HII. *Proc Natl Acad Sci U S A* 95, 12872-12877.

Froget, B., Blaisonneau, J., Lambert, S., and Baldacci, G. (2008). Cleavage of stalled forks by fission yeast Mus81/Eme1 in absence of DNA replication checkpoint. *Mol Biol Cell* 19, 445-456.

Fujikane, R., Ishino, S., Ishino, Y., and Forterre, P. (2010). Genetic analysis of DNA repair in the hyperthermophilic archaeon, Thermococcus kodakaraensis. *Genes Genet Syst* 85, 243-257.

Fukushima, S., Itaya, M., Kato, H., Ogasawara, N., and Yoshikawa, H. (2007). Reassessment of the in vivo functions of DNA polymerase I and RNase H in bacterial cell growth. *J Bacteriol* 189, 8575-8583.

Gaillard, P.H., Noguchi, E., Shanahan, P., and Russell, P. (2003). The endogenous Mus81-Eme1 complex resolves Holliday junctions by a nick and counternick mechanism. *Mol Cell* 12, 747-759.

Gari, K., Decaillet, C., Delannoy, M., Wu, L., and Constantinou, A. (2008). Remodeling of DNA replication structures by the branch point translocase FANCM. *Proc Natl Acad Sci U S A* 105, 16107-16112.

Gaskell, L.J., Osman, F., Gilbert, R.J., and Whitby, M.C. (2007). Mus81 cleavage of Holliday junctions: a failsafe for processing meiotic recombination intermediates? *EMBO J* 26, 1891-1901.

Gerard, E., Jolivet, E., Prieur, D., and Forterre, P. (2001). DNA protection mechanisms are not involved in the radioresistance of the hyperthermophilic archaea Pyrococcus abyssi and P. furiosus. *Mol Genet Genomics* 266, 72-78.

Grogan, D.W., Carver, G.T., and Drake, J.W. (2001). Genetic fidelity under harsh conditions: analysis of spontaneous mutation in the thermoacidophilic archaeon Sulfolobus acidocaldarius. *Proc Natl Acad Sci U S A* 98, 7928-7933.

Hanada, K., Budzowska, M., Davies, S.L., van Drunen, E., Onizawa, H., Beverloo, H.B., Maas, A., Essers, J., Hickson, I.D., and Kanaar, R. (2007). The structure-specific endonuclease Mus81 contributes to replication restart by generating double-strand DNA breaks. *Nat Struct Mol Biol* 14, 1096-1104.

Haruki, M., Hayashi, K., Kochi, T., Muroya, A., Koga, Y., Morikawa, M., Imanaka, T., and Kanaya, S. (1998). Gene cloning and characterization of recombinant RNase HII from a hyperthermophilic archaeon. *J Bacteriol* 180, 6207-6214.

Hopfner, K.P., Craig, L., Moncalian, G., Zinkel, R.A., Usui, T., Owen, B.A., Karcher, A., Henderson, B., Bodmer, J.L., McMurray, C.T., *et al.* (2002a). The Rad50 zinc-hook is a structure joining Mre11 complexes in DNA recombination and repair. *Nature* 418, 562-566.

Hopfner, K.P., Karcher, A., Craig, L., Woo, T.T., Carney, J.P., and Tainer, J.A. (2001). Structural biochemistry and interaction architecture of the DNA double-strand break repair Mre11 nuclease and Rad50-ATPase. *Cell* 105, 473-485.

Hopfner, K.P., Karcher, A., Shin, D., Fairley, C., Tainer, J.A., and Carney, J.P. (2000a). Mre11 and Rad50 from Pyrococcus furiosus: cloning and biochemical characterization reveal an evolutionarily conserved multiprotein machine. *J Bacteriol* 182, 6036-6041.

Hopfner, K.P., Karcher, A., Shin, D.S., Craig, L., Arthur, L.M., Carney, J.P., and Tainer, J.A. (2000b). Structural biology of Rad50 ATPase: ATP-driven conformational control in DNA double-strand break repair and the ABC-ATPase superfamily. *Cell* 101, 789-800.

Hopfner, K.P., Putnam, C.D., and Tainer, J.A. (2002b). DNA double-strand break repair from head to tail. *Curr Opin Struct Biol* 12, 115-122.

Hopkins, B.B., and Paull, T.T. (2008). The P. furiosus mre11/rad50 complex promotes 5' strand resection at a DNA double-strand break. *Cell* 135, 250-260.

Horton, N.C., and Finzel, B.C. (1996). The structure of an RNA/DNA hybrid: a substrate of the ribonuclease activity of HIV-1 reverse transcriptase. *J Mol Biol* 264, 521-533.

Hutton, R.D., Craggs, T.D., White, M.F., and Penedo, J.C. (2009). PCNA and XPF cooperate to distort DNA substrates. *Nucleic Acids Res* 38, 1664-1675.

Hutton, R.D., Roberts, J.A., Penedo, J.C., and White, M.F. (2008). PCNA stimulates catalysis by structure-specific nucleases using two distinct mechanisms: substrate targeting and catalytic step. *Nucleic Acids Res* 36, 6720-6727.

Ip, S.C., Rass, U., Blanco, M.G., Flynn, H.R., Skehel, J.M., and West, S.C. (2008). Identification of Holliday junction resolvases from humans and yeast. *Nature* 456, 357-361.

Itaya, M., Omori, A., Kanaya, S., Crouch, R.J., Tanaka, T., and Kondo, K. (1999). Isolation of RNase H genes that are essential for growth of Bacillus subtilis 168. *J Bacteriol* 181, 2118-2123.

Jeong, H.S., Backlund, P.S., Chen, H.C., Karavanov, A.A., and Crouch, R.J. (2004). RNase H2 of Saccharomyces cerevisiae is a complex of three proteins. *Nucleic Acids Res* 32, 407-414.

Jessop, L., and Lichten, M. (2008). Mus81/Mms4 endonuclease and Sgs1 helicase collaborate to ensure proper recombination intermediate metabolism during meiosis. *Mol Cell* 31, 313-323.

Jolivet, E., L'Haridon, S., Corre, E., Forterre, P., and Prieur, D. (2003a). Thermococcus gammatolerans sp. nov., a hyperthermophilic archaeon from a deep-sea hydrothermal vent that resists ionizing radiation. *Int J Syst Evol Microbiol* 53, 847-851.

Jolivet, E., Matsunaga, F., Ishino, Y., Forterre, P., Prieur, D., and Myllykallio, H. (2003b). Physiological responses of the hyperthermophilic archaeon "Pyrococcus abyssi" to DNA damage caused by ionizing radiation. *J Bacteriol* 185, 3958-3961.

Kai, M., Boddy, M.N., Russell, P., and Wang, T.S. (2005). Replication checkpoint kinase Cds1 regulates Mus81 to preserve genome integrity during replication stress. *Genes Dev* 19, 919-932.

Kaliraman, V., Mullen, J.R., Fricke, W.M., Bastin-Shanower, S.A., and Brill, S.J. (2001). Functional overlap between Sgs1-Top3 and the Mms4-Mus81 endonuclease. *Genes Dev* 15, 2730-2740.

Kambach, C., Walke, S., Young, R., Avis, J.M., de la Fortelle, E., Raker, V.A., Luhrmann, R., Li, J., and Nagai, K. (1999). Crystal structures of two Sm protein complexes and their implications for the assembly of the spliceosomal snRNPs. *Cell* 96, 375-387.

Kim, J.M., Kee, Y., Gurtan, A., and D'Andrea, A.D. (2008). Cell cycle-dependent chromatin loading of the Fanconi anemia core complex by FANCM/FAAP24. *Blood* 111, 5215-5222.

Kish, A., and DiRuggiero, J. (2008). Rad50 is not essential for the Mre11-dependent repair of DNA double-strand breaks in Halobacterium sp. strain NRC-1. *J Bacteriol* 190, 5210-5216.

Komori, K., Fujikane, R., Shinagawa, H., and Ishino, Y. (2002). Novel endonuclease in Archaea cleaving DNA with various branched structure. *Genes Genet Syst* 77, 227-241.

Komori, K., Hidaka, M., Horiuchi, T., Fujikane, R., Shinagawa, H., and Ishino, Y. (2004). Cooperation of the N-terminal Helicase and C-terminal endonuclease activities of Archaeal Hef protein in processing stalled replication forks. *J Biol Chem* 279, 53175-53185.

Kowalczykowski, S.C., Dixon, D.A., Eggleston, A.K., Lauder, S.D., and Rehrauer, W.M. (1994). Biochemistry of homologous recombination in Escherichia coli. *Microbiol Rev* 58, 401-465.

Lai, L., Yokota, H., Hung, L.W., Kim, R., and Kim, S.H. (2000). Crystal structure of archaeal RNase HII: a homologue of human major RNase H. *Structure* 8, 897-904.

Le Laz, S., Le Goaziou, A., and Henneke, G. (2010). Structure-specific nuclease activities of Pyrococcus abyssi RNase HII. *J Bacteriol* 192, 3689-3698.

Lestini, R., Duan, Z., and Allers, T. (2010). The archaeal Xpf/Mus81/FANCM homolog Hef and the Holliday junction resolvase Hjc define alternative pathways that are essential for cell viability in Haloferax volcanii. *DNA Repair (Amst)* 9, 994-1002.

Li, Z., Pan, M., Santangelo, T.J., Chemnitz, W., Yuan, W., Edwards, J.L., Hurwitz, J., Reeve, J.N., and Kelman, Z. (2011). A novel DNA nuclease is stimulated by association with the GINS complex. *Nucleic Acids Res*.

Ma, J.L., Kim, E.M., Haber, J.E., and Lee, S.E. (2003). Yeast Mre11 and Rad1 proteins define a Ku-independent mechanism to repair double-strand breaks lacking overlapping end sequences. *Mol Cell Biol* 23, 8820-8828.

Maga, G., and Hubscher, U. (2003). Proliferating cell nuclear antigen (PCNA): a dancer with many partners. *J Cell Sci* 116, 3051-3060.

Maiti, R., Van Domselaar, G.H., Zhang, H., and Wishart, D.S. (2004). SuperPose: a simple server for sophisticated structural superposition. *Nucleic Acids Res* 32, W590-594.

Manzan, A., Pfeiffer, G., Hefferin, M.L., Lang, C.E., Carney, J.P., and Hopfner, K.P. (2004). MlaA, a hexameric ATPase linked to the Mre11 complex in archaeal genomes. *EMBO Rep* 5, 54-59.

Mascarenhas, J., Sanchez, H., Tadesse, S., Kidane, D., Krisnamurthy, M., Alonso, J.C., and Graumann, P.L. (2006). Bacillus subtilis SbcC protein plays an important role in DNA inter-strand cross-link repair. *BMC Mol Biol* 7, 20.

Matulova, P., Marini, V., Burgess, R.C., Sisakova, A., Kwon, Y., Rothstein, R., Sung, P., and Krejci, L. (2009). Cooperativity of Mus81.Mms4 with Rad54 in the resolution of recombination and replication intermediates. *J Biol Chem* 284, 7733-7745.

Meetei, A.R., Medhurst, A.L., Ling, C., Xue, Y., Singh, T.R., Bier, P., Steltenpool, J., Stone, S., Dokal, I., Mathew, C.G., *et al*. (2005). A human ortholog of archaeal DNA repair protein Hef is defective in Fanconi anemia complementation group M. *Nat Genet* 37, 958-963.

Meslet-Cladiere, L., Norais, C., Kuhn, J., Briffotaux, J., Sloostra, J.W., Ferrari, E., Hubscher, U., Flament, D., and Myllykallio, H. (2007). A novel proteomic approach identifies new interaction partners for proliferating cell nuclear antigen. *J Mol Biol* 372, 1137-1148.

Mimitou, E.P., and Symington, L.S. (2008). Sae2, Exo1 and Sgs1 collaborate in DNA double-strand break processing. *Nature* 455, 770-774.

Mosedale, G., Niedzwiedz, W., Alpi, A., Perrina, F., Pereira-Leal, J.B., Johnson, M., Langevin, F., Pace, P., and Patel, K.J. (2005). The vertebrate Hef ortholog is a component of the Fanconi anemia tumor-suppressor pathway. *Nat Struct Mol Biol* 12, 763-771.

Muroya, A., Tsuchiya, D., Ishikawa, M., Haruki, M., Morikawa, M., Kanaya, S., and Morikawa, K. (2001). Catalytic center of an archaeal type 2 ribonuclease H as revealed by X-ray crystallographic and mutational analyses. *Protein Sci* 10, 707-714.

Newman, M., Murray-Rust, J., Lally, J., Rudolf, J., Fadden, A., Knowles, P.P., White, M.F., and McDonald, N.Q. (2005). Structure of an XPF endonuclease with and without DNA suggests a model for substrate recognition. *EMBO J* 24, 895-905.

Nick McElhinny, S.A., Kumar, D., Clark, A.B., Watt, D.L., Watts, B.E., Lundstrom, E.B., Johansson, E., Chabes, A., and Kunkel, T.A. (2010a). Genome instability due to ribonucleotide incorporation into DNA. *Nat Chem Biol* 6, 774-781.

Nick McElhinny, S.A., Watts, B.E., Kumar, D., Watt, D.L., Lundstrom, E.B., Burgers, P.M., Johansson, E., Chabes, A., and Kunkel, T.A. (2010b). Abundant ribonucleotide incorporation into DNA by yeast replicative polymerases. *Proc Natl Acad Sci U S A* 107, 4949-4954.

Nishino, T., Komori, K., Ishino, Y., and Morikawa, K. (2003). X-ray and biochemical anatomy of an archaeal XPF/Rad1/Mus81 family nuclease: similarity between its endonuclease domain and restriction enzymes. *Structure* 11, 445-457.

Nishino, T., Komori, K., Ishino, Y., and Morikawa, K. (2005a). Structural and functional analyses of an archaeal XPF/Rad1/Mus81 nuclease: asymmetric DNA binding and cleavage mechanisms. *Structure* 13, 1183-1192.

Nishino, T., Komori, K., Tsuchiya, D., Ishino, Y., and Morikawa, K. (2005b). Crystal structure and functional implications of Pyrococcus furiosus hef helicase domain involved in branched DNA processing. *Structure* 13, 143-153.

Nowotny, M., and Yang, W. (2009). Structural and functional modules in RNA interference. *Curr Opin Struct Biol* 19, 286-293.

Oh, S.D., Lao, J.P., Taylor, A.F., Smith, G.R., and Hunter, N. (2008). RecQ helicase, Sgs1, and XPF family endonuclease, Mus81-Mms4, resolve aberrant joint molecules during meiotic recombination. *Mol Cell* 31, 324-336.

Ohtani, N., Haruki, M., Morikawa, M., Crouch, R.J., Itaya, M., and Kanaya, S. (1999a). Identification of the genes encoding Mn2+-dependent RNase HII and Mg2+-dependent RNase HIII from Bacillus subtilis: classification of RNases H into three families. *Biochemistry* 38, 605-618.

Ohtani, N., Haruki, M., Morikawa, M., and Kanaya, S. (1999b). Molecular diversities of RNases H. *J Biosci Bioeng* 88, 12-19.

Ohtani, N., Haruki, M., Muroya, A., Morikawa, M., and Kanaya, S. (2000). Characterization of ribonuclease HII from Escherichia coli overproduced in a soluble form. *J Biochem* 127, 895-899.

Ohtani, N., Tomita, M., and Itaya, M. (2008). Junction ribonuclease: a ribonuclease HII orthologue from Thermus thermophilus HB8 prefers the RNA-DNA junction to the RNA/DNA heteroduplex. *Biochem J* 412, 517-526.

Osman, F., Dixon, J., Doe, C.L., and Whitby, M.C. (2003). Generating crossovers by resolution of nicked Holliday junctions: a role for Mus81-Eme1 in meiosis. *Mol Cell* 12, 761-774.

Palud, A., Villani, G., L'Haridon, S., Querellou, J., Raffin, J.P., and Henneke, G. (2008). Intrinsic properties of the two replicative DNA polymerases of Pyrococcus abyssi

in replicating abasic sites: possible role in DNA damage tolerance? *Mol Microbiol* 70, 746-761.

Pingoud, A., Fuxreiter, M., Pingoud, V., and Wende, W. (2005). Type II restriction endonucleases: structure and mechanism. *Cell Mol Life Sci* 62, 685-707.

Quaiser, A., Constantinesco, F., White, M.F., Forterre, P., and Elie, C. (2008). The Mre11 protein interacts with both Rad50 and the HerA bipolar helicase and is recruited to DNA following gamma irradiation in the archaeon Sulfolobus acidocaldarius. *BMC Mol Biol* 9, 25.

Ren, B., Kuhn, J., Meslet-Cladiere, L., Briffotaux, J., Norais, C., Lavigne, R., Flament, D., Ladenstein, R., and Myllykallio, H. (2009). Structure and function of a novel endonuclease acting on branched DNA substrates. *EMBO J* 28, 2479-2489.

Ren, B., Kuhn, J., Meslet-Cladiere, L., Myllykallio, H., and Ladenstein, R. (2007). Crystallization and preliminary X-ray analysis of a RecB-family nuclease from the archaeon Pyrococcus abyssi. *Acta Crystallogr Sect F Struct Biol Cryst Commun* 63, 406-408.

Richards, J.D., Cubeddu, L., Roberts, J., Liu, H., and White, M.F. (2008). The archaeal XPB protein is a ssDNA-dependent ATPase with a novel partner. *J Mol Biol* 376, 634-644.

Roberts, J.A., and White, M.F. (2005). An archaeal endonuclease displays key properties of both eukaryal XPF-ERCC1 and Mus81. *J Biol Chem* 280, 5924-5928.

Rohman, M.S., Koga, Y., Takano, K., Chon, H., Crouch, R.J., and Kanaya, S. (2008). Effect of the disease-causing mutations identified in human ribonuclease (RNase) H2 on the activities and stabilities of yeast RNase H2 and archaeal RNase HII. *Febs J* 275, 4836-4849.

Roseaulin, L., Yamada, Y., Tsutsui, Y., Russell, P., Iwasaki, H., and Arcangioli, B. (2008). Mus81 is essential for sister chromatid recombination at broken replication forks. *EMBO J* 27, 1378-1387.

Roth, H.M., Tessmer, I., Van Houten, B., and Kisker, C. (2009). Bax1 is a novel endonuclease: implications for archaeal nucleotide excision repair. *J Biol Chem* 284, 32272-32278.

Rouillon, C., and White, M.F. (2010). The evolution and mechanisms of nucleotide excision repair proteins. *Res Microbiol* 162, 19-26.

Rumbaugh, J.A., Murante, R.S., Shi, S., and Bambara, R.A. (1997). Creation and removal of embedded ribonucleotides in chromosomal DNA during mammalian Okazaki fragment processing. *J Biol Chem* 272, 22591-22599.

Rychlik, M.P., Chon, H., Cerritelli, S.M., Klimek, P., Crouch, R.J., and Nowotny, M. (2010). Crystal structures of RNase H2 in complex with nucleic acid reveal the mechanism of RNA-DNA junction recognition and cleavage. *Mol Cell* 40, 658-670.

Rydberg, B., and Game, J. (2002). Excision of misincorporated ribonucleotides in DNA by RNase H (type 2) and FEN-1 in cell-free extracts. *Proc Natl Acad Sci U S A* 99, 16654-16659.

Schiestl, R.H., and Prakash, S. (1988). RAD1, an excision repair gene of Saccharomyces cerevisiae, is also involved in recombination. *Mol Cell Biol* 8, 3619-3626.

Shaban, N.M., Harvey, S., Perrino, F.W., and Hollis, T. (2010). The structure of the mammalian RNase H2 complex provides insight into RNA.NA hybrid processing to prevent immune dysfunction. *J Biol Chem* 285, 3617-3624.

Shimura, T., Torres, M.J., Martin, M.M., Rao, V.A., Pommier, Y., Katsura, M., Miyagawa, K., and Aladjem, M.I. (2008). Bloom's syndrome helicase and Mus81 are required to induce transient double-strand DNA breaks in response to DNA replication stress. *J Mol Biol* 375, 1152-1164.

Sijbers, A.M., de Laat, W.L., Ariza, R.R., Biggerstaff, M., Wei, Y.F., Moggs, J.G., Carter, K.C., Shell, B.K., Evans, E., de Jong, M.C., *et al.* (1996). Xeroderma pigmentosum group F caused by a defect in a structure-specific DNA repair endonuclease. *Cell* 86, 811-822.

Sonoda, E., Hochegger, H., Saberi, A., Taniguchi, Y., and Takeda, S. (2006). Differential usage of non-homologous end-joining and homologous recombination in double strand break repair. *DNA Repair (Amst)* 5, 1021-1029.

Stein, H., and Hausen, P. (1969). Enzyme from calf thymus degrading the RNA moiety of DNA-RNA Hybrids: effect on DNA-dependent RNA polymerase. *Science* 166, 393-395.

Stewart, G.S., Maser, R.S., Stankovic, T., Bressan, D.A., Kaplan, M.I., Jaspers, N.G., Raams, A., Byrd, P.J., Petrini, J.H., and Taylor, A.M. (1999). The DNA double-strand break repair gene hMRE11 is mutated in individuals with an ataxia-telangiectasia-like disorder. *Cell* 99, 577-587.

Stracker, T.H., and Petrini, J.H. (2011). The MRE11 complex: starting from the ends. *Nat Rev Mol Cell Biol* 12, 90-103.

Sun, W., Nandi, S., Osman, F., Ahn, J.S., Jakovleska, J., Lorenz, A., and Whitby, M.C. (2008). The FANCM ortholog Fml1 promotes recombination at stalled replication forks and limits crossing over during DNA double-strand break repair. *Mol Cell* 32, 118-128.

Tadokoro, T., and Kanaya, S. (2009). Ribonuclease H: molecular diversities, substrate binding domains, and catalytic mechanism of the prokaryotic enzymes. *Febs J* 276, 1482-1493.

Theobald, D.L., Mitton-Fry, R.M., and Wuttke, D.S. (2003). Nucleic acid recognition by OB-fold proteins. *Annu Rev Biophys Biomol Struct* 32, 115-133.

Vivona, J.B., and Kelman, Z. (2003). The diverse spectrum of sliding clamp interacting proteins. *FEBS Lett* 546, 167-172.

Von Sonntag, C., and Schulte-Frohlinde, D. (1978). Radiation-induced degradation of the sugar in model compounds and in DNA. *Mol Biol Biochem Biophys* 27, 204-226.

Warbrick, E. (1998). PCNA binding through a conserved motif. *Bioessays* 20, 195-199.

Whitby, M.C., Osman, F., and Dixon, J. (2003). Cleavage of model replication forks by fission yeast Mus81-Eme1 and budding yeast Mus81-Mms4. *J Biol Chem* 278, 6928-6935.

White, M.F. (2011). Homologous recombination in the archaea: the means justify the ends. *Biochem Soc Trans*. 39, 5-9.

Williams, R.S., Moncalian, G., Williams, J.S., Yamada, Y., Limbo, O., Shin, D.S., Groocock, L.M., Cahill, D., Hitomi, C., Guenther, G., *et al.* (2008). Mre11 dimers coordinate DNA end bridging and nuclease processing in double-strand-break repair. *Cell* 135, 97-109.

Williams, R.S., Williams, J.S., and Tainer, J.A. (2007). Mre11-Rad50-Nbs1 is a keystone complex connecting DNA repair machinery, double-strand break signaling, and the chromatin template. *Biochem Cell Biol* 85, 509-520.

Xue, Y., Li, Y., Guo, R., Ling, C., and Wang, W. (2008). FANCM of the Fanconi anemia core complex is required for both monoubiquitination and DNA repair. *Hum Mol Genet* 17, 1641-1652.

Yang, W., and Steitz, T.A. (1995). Recombining the structures of HIV integrase, RuvC and RNase H. *Structure* 3, 131-134.

Zhu, Z., Chung, W.H., Shim, E.Y., Lee, S.E., and Ira, G. (2008). Sgs1 helicase and two nucleases Dna2 and Exo1 resect DNA double-strand break ends. *Cell* 134, 981-994.

11

Nucleases of Metallo-β-Lactamase and Protein Phosphatase Families in DNA Repair

Francisco J Fernandez, Miguel Lopez-Estepa and M. Cristina Vega
Center for Biological Research, Spanish National Research Council (CIB-CSIC)
Spain

1. Introduction

All living organisms must struggle to maintain genomic integrity and long-term stability in the face of the lesions that are constantly inflicted upon the genome by environmental factors, e.g., genotoxic chemicals, UV light, ionizing radiation (IR), and endogenous factors, e.g., during DNA replication. These various DNA lesions (or injuries) encompass a bewildering array of chemical and physical modifications to the DNA structure that must be repaired to preserve the faithful maintenance of the genome. A prevalent class of DNA lesion consists of a break across both DNA strands, termed double strand break (DSB) (Fig. 1 and Table 1). Only of endogenous origin, about 50 DSBs have been calculated to occur per human cell division (Vilenchik and Knudson 2003). Many of these DSBs are generated by IR, reactive oxygen species, and DNA replication across a nick (Ma, J.L. et al. 2003). If left unrepaired, DSBs can cause dire effects such as gene loss during cell division, chromosomal translocations, increased mutation rates, and carcinogenesis (Khanna and Jackson 2001). The various cellular mechanisms that are collectively referred to as DNA repair include DNA damage detection (or sensing), binding and recruitment of specialized protein complex machinery to the site of damage, signaling, initiation of repair, repair, and resolution of the lesion (Fig. 1).

Central to all DNA repair processes are nucleases, enzymes and enzyme complexes that can cleave DNA either in a sugar specific fashion (e.g., DNA and RNA nucleases) or in a sugar unspecific fashion (Marti and Fleck 2004). Nucleases can be further divided into exonucleases, which remove nucleotides from a free 5′ or 3′ end, and endonucleases, which hydrolyze internal phosphodiester bonds without the requirement for a free end. DNA nucleases, which can cleave single stranded (ss) or double stranded (ds) DNA, cleave a phosphodiester bond between a deoxyribose and a phosphate group, thus producing one cleavage product with a 5′ terminal phosphate group and another product with a 3′ terminal hydroxyl group.

Two kinds of DNA lesions, double strand breaks (DSBs) and interstrand crosslinks (ICLs) (Fig. 1), are significantly dependent on the timely action of DNA nucleases, since the initiating step in the repair pathways of DSBs and ICLs often consists of an exonucleolytic or endonucleolytic cleavage that exposes the substrate for the next DNA repair activity. Without the action of a nuclease, the DNA lesion would stay unrepaired because of chemically inaccessible or sterically blocked DNA intermediates. Therefore, nucleases are an integral part of the cellular mechanisms that have evolved to handle DNA damage. Indeed,

quality repair mechanisms that strive to reconstitute the undamaged, original DNA structure imply that DNA lesion repair, after the initial nucleolytic processing, requires additional factors, minimally DNA synthesis and ligation, but it also can involve a complex sequence of molecular events.

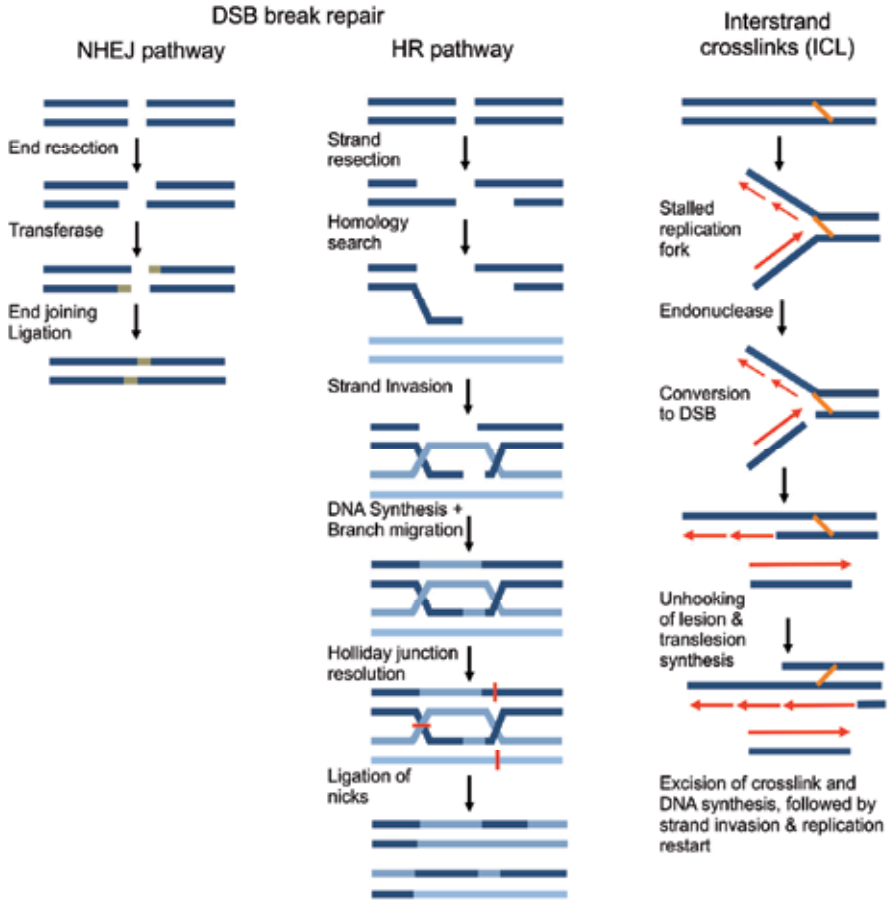

Fig. 1. Schematic of the DNA repair pathways wherein nucleases of the metallo-β-lactamase and protein phosphatase 2B families have been shown to participate.

DNA lesion	DNA Repair Pathway	Nucleases	Fold
DSB	Non-homologous end joining (NHEJ)	Mre11	PP2B
		Artemis	MBL
DSB	Homologous recombination (HR)	Mre11	PP2B
DSB	Microhomology-mediated end joining	Mre11	PP2B
ICL	Repair of interstrand crosslinks (ICL)	SNM1A/B	MBL

Table 1. Nucleases in DNA repair span a growing number of prokaryotic, archeal and eukaryotic exo- and endonucleolytic enzymes with specialized roles in different repair pathways. DSB, double strand break; ICL, interstrand crosslink; MBL, metallo-β-lactamase; PP2B, protein phosphatase 2B.

DSBs are repaired in human cells mainly by two alternative mechanisms, non-homologous end joining (NHEJ) and homologous recombination (HR) (Fig. 1). While HR occurs mostly in S/G2 phase (Takata et al. 1998), when a sister chromatid is available to provide a template to replace the damaged nucleotides, NHEJ, which does not require a template (Ma, J.L. et al. 2003), is prevalent during G1/early S phase (Takata et al. 1998). The sequential steps necessary for NHEJ comprise synapsis (the protein-mediated structure whereby the two ends of a DSBs are tethered, or held together in close proximity, to allow successful repair), end resection (catalyzed by nucleases), DNA synthesis, and ligation (Fig 1). HR requires too an initial exonucleolytic step that consists of the resection of both strands at the DSB end, thus preparing them for the invasion of the neighboring, intact chromatid DNA (Fig. 1). DNA synthesis, branch migration, Holliday junction resolution, and ligation of remaining nicks, are the next steps needed to complete DNA repair by HR. Regardless of the repair pathway used, an exonucleolytic step is always required to provide the DNA substrates for the subsequent repair processes.

A second class of DNA lesions, ICL, can be generated exogenously by mono- or bifunctional alkylating agents (crosslinkers), IR, and endogenously by the collapse of replication forks. A crosslink at a replication fork leads to stalling, since the individual DNA strands can no longer be unwound for DNA synthesis. Repair of an ICL-induced stalled replication fork can be achieved by the series of steps outlined in Fig. 1. Here again, the initial, critical step is an endonucleolytic cleavage catalyzed by DNA nucleases, which helps convert the stalled fork to a DSB-like lesion that is susceptible to repair.

In the following we will survey two families of DNA nucleases that play significant roles in one or various processes involved in the repair of DSB and ICL lesions, from the indispensable initiation of end resection of broken DNA ends to other specialized DNA repair processes such as those in ICL repair. These families, which are structurally and functionally related, are the metallo-β-lactamase (MBL) and the protein phosphatase 2B (PP2B) families. Members of those two families, such as Artemis (MBL) and Mre11 (PP2B), provide essential activities for DNA repair (Table 1). Here, we will discuss the structural and functional properties of the MBL and PP2B nucleases with roles in DNA repair, and will draw a comprehensive portrait of the structural, biochemical, cellular, and evolutionary framework wherein they function.

2. Nucleases of the MBL and PP2B families in DNA repair

Here we summarize current knowledge on two related families of DNA repair nucleases that share significant similarity at the topology, fold structure, active site composition and metal-ion binding: the metallo-β-lactamase (MBL) and the protein phosphatase 2B (PP2B) families (Tables 1, 2 & 3). Despite some degree of functional redundancy, the two families are distinguished by the exact composition and location of the catalytic residues, which explain their distinct functional roles. Likewise, differences in the insertion of accessory domains have allowed the incorporation of substrate-binding domains that widen, or restrict, the range of potential substrates that can be recognized and processed by either class of nucleases.

Both the MBL and PP2B families of nucleases belong to the two-metal-ion-dependent nucleases (Yang 2010), an operational class that encompasses the largest variety of tertiary folds and the broadest range of biological outcomes among the nucleases. The defining feature of these enzymes is the absolute dependence for catalytic competence of an active

center with two metal ions, one of which acts by polarizing the substrate phosphoester whereas the second is more commonly associated with the stabilization of the nascent negative charge on the leaving group. Given the enormous variety of folds and substrate structures, this simple principle (that this class of nucleases are unified by their two-metal-ion dependence) provides an appropriate framework for discussing their structural and mechanistic properties (Yang 2010). Nucleases from the MBL and PP2B families have been implicated in two specific repair pathways of DNA lesions, the repair of DSBs by non-homologous end joining (NHEJ) and the repair of interstrand crosslinks (ICL) (Fig. 1 and Table 1).

2.1 Metallo-β-lactamase fold nucleases

The metallo-β-lactamase (MBL) fold is characterized by a four-layered $\alpha\beta/\beta\alpha$ fold with a wide, shallow active site that is always located on the same side of the fold and that ligands one or, usually, two catalytic metal ions (Aravind 1999; Daiyasu et al. 2001; Callebaut et al. 2002; Dominski 2007). The core of the MBL domain is formed by a β-sandwich of eight β-strands with α-helices packing on both sides. The degree of sequence conservation between MBLs tends to be so low that novel proteins are often only confidently assigned as an MBL once the crystal structure is solved. Despite the low sequence identity between its members, the MBL family presents five sequence motifs that define the active site and provide a signature for the metal binding center (Fig. 2). Motifs 1 and 2 are located in the first β-sheet, and motif 2 comprises the HXHXDH sequence motif that is nearly absolutely conserved among all of the MBLs, and where the first His and Asp residues are completely invariable (Fig. 2). The histidine residues belonging to motifs 3 and 4 are located in the second β-strand; and motif 5 is a cysteine or an acidic residue located at the C terminus.

The MBL family encompasses a large number of enzymes with hydrolytic activities toward a variety of different substrates and, less frequently, oxidorreductases. The best-known hydrolytic MBLs include the zinc-dependent β-lactamases (class B β-lactamases) that hydrolyze β-lactam and non-β-lactam antibiotics (Wang, Z. et al. 1999). Examples of other hydrolytic MBL subfamilies are glyoxylase II, methyl parathion hydrolase, Pce-catalytic domain-like, alkylsulfatase, PqsE-like (all of them zinc-dependent enzymes) and L-ascorbate-6-phosphate lactonase (UlaG) (which is manganese-dependent) (Garces et al. 2010). The ROO N-terminal domain family represents an MBL with oxidorreductase activity. Some MBL families with hydrolase activity act on nucleic acid substrates, predominantly on RNA, such as the β-CASP, arylsulfatase (or RNAse Z-like) and YhfI-like families. Of those, only certain members of the β-CASP family have been demonstrated to use DNA as a substrate, whereas the remaining enzymes display RNase activity. A common trait among the β-CASP MBLs is the presence of an additional domain inserted into the MBL canonical fold, which itself contains unique sequence and structural features (Fig. 2).

The β-CASP motif was first defined by Callebuet et al. (2002), and the name was inspired by the common features that displayed the C termini of certain MBLs that were capable of binding nucleic acid substrates [CPSF-73 (Cleavage and Polyadenylation Specificity Factor-73), Artemis, SNM1 and Pso2]. Interestingly, of the four β-CASP founder sequences three (Artemis, SNM1, and Pso2) are known to bind DNA and to possess nuclease activity that is relevant in DNA repair processes. The β-CASP domain is characterized by three motifs (Fig. 2): (1) motif A, consisting of an acidic residue (D or E) preceded by hydrophobic residues (ϕ) in a pattern typical of β-strands (ϕ-ϕ-ϕ-(D,E)-(T,S)-T); (2) motif B (His); and (3) motif C,

which is a His residue in all the proteins with exception of Artemis/SNM1/Pso2 where it is a Val (Fig. 2). Since the three motifs are located near the active center, the functions of β-CASP are associated with the specific recognition and binding of the substrate. Other proteins from the β-CASP family are CPSF-100, RC-47, RC-68, Snm1B (Apollo) and the ribonucleases RNases J1 and J2 from *Bacillus* spp. (Callebaut et al. 2002; Dominski et al. 2005; Even et al. 2005; Dominski 2007). Several recently published crystal structures of members of the β-CASP family have further confirmed the insertion of the β-CASP domain inside the MBL fold as a α/β domain, which resembles a helicase-like α/β domain without the P-loop motif (Table 2 and Fig. 3).

		Metallo-β-lactamase			
		I	II	III	IV
Human	SNM1A	FTV**D**AF	LT**HF**H**SDH**YA	AN**H**CP	ILHTG**D**FR
Yeast	SNM1/Pso2	IVV**D**GF	LS**HF**H**SDH**YI	AN**H**CP	ILHTG**D**FR
Human	SNM1B/Apollo	IAV**D**FW	LS**HF**H**SDH**YT	AN**H**CP	ILYTG**D**FR
Human	SNM1C/Artemis	IS**ID**RF	LS**HC**H**KDH**MK	AG**H**CP	VLYTG**D**FR
Human	CPSF-73	IML**D**CG	I**SHF**H**LDH**CG	AG**H**VL	LLYTG**D**FR
E. coli	UlaG	VCV**D**FW	AT**HD**H**ND**HID	AF**D**RT	LYHSG**D**SH
B. cereus	BLM	VLV**DS**S	IT**HA**H**AD**RIG	KG**H**TE	ILVCG**C**LV

		β-CASP domain		
		A	B	C
Human	SNM1A	LYL**D**TT	E**H**SSY	IPT**V**N
Yeast	SNM1/Pso2	LYL**D**TT	E**H**SSF	IPT**V**N
Human	SNM1B/Apollo	LYL**D**NT	D**H**SSY	VPI**V**S
Human	SNM1C/Artemis	VYL**D**TT	F**H**SSY	YPN**V**I
Human	CPSF-73	LII**E**ST	A**H**TDY	ILV**H**G

Fig. 2. Characteristic sequence motifs of DNA nucleases of the MBL/β-CASP family.

	β-CASP protein	**Organism**	**PDB ID (Reference)**
EUKARYA	CPSF-73	Human	2I7T (Mandel et al. 2006)
	CPSF-100 (Ydh1p)	Yeast	2I7X (Mandel et al. 2006)
ARCHEA	CPSF subunit PH1404	*P. horikoshii*	3AF5 (Nishida et al. 2010)
	CPSF homolog	*M. mazei*	2XR1 (Mir-Montazeri et al. 2011)
EUBACTERIA	TTHA0252	*Th. termophilus*	2DKF (Ishikawa et al. 2006)
	EF2904	*E. faecalis*	2AZ4 (MSDG)

Table 2. Nucleases of the β-CASP family with known crystal structure. PDB ID, Protein Data Bank Identification code.

β-CASP nucleases, in contrast to conventional MBL amidohydrolases, which degrade the β-lactam moiety of many antibiotics that inhibit cell wall biosynthesis (Wang, Z. et al. 1999),

are nucleases that hydrolyze the phosphodiester bond in RNA or DNA. The nuclease activity can be either endonuclease or 5′- to 3′-exonuclease. Despite the fact that the overall β-CASP fold is reminiscent of the DNase I fold (Callebaut et al. 2002), the number of β-strands and the relative ordering of them in the central β-sheet is different between the β-CASP and the DNase I folds. In keeping with conventional MBLs, β-CASP nucleases appear to have a strong preference for the tight binding of two Zn^{2+} ions regardless of substrate, a selectivity enhanced by the presence of multiple conserved His residues in the catalytic center (Baldwin et al. 1979). As of recent, however, a new MBL has been discovered whose preferred catalytic metal ion is Mn^{2+} rather than Zn^{2+} and that shows some limited phosphodiesterase activity toward cyclic nucleotides (Garces et al. 2010). This raises the interesting possibility that other nucleases with the MBL fold may tolerate (or even prefer) Mn^{2+} in their active sites, a well-known metal catalyst in DNA-dependent nucleases such as Mre11 (a nuclease with the PP2B fold; Table 1).

Even though there is no known crystal structure for DNA nucleases of the β-CASP family, sequence conservation patterns and the accumulated knowledge on structure/function of diverse MBL enzymes lend support to the notion that the overall fold and domain arrangements of β-CASP DNA nucleases will be similar to those of β-CASP RNases (Callebaut et al. 2002). These shared features would include the presence of a β-CASP domain inserted into the two-metal-ion MBL fold scaffold, with motifs A and B of the β-CASP domain lying near the canonical motifs I-IV of MBLs, which configure the active site (Figs. 2-3 and Table 2).

In Archea, homologs of the β-CASP-containing MBL protein CPSF-73 contain a specific N-terminal domain that precedes the MBL domain. This extra N-terminal domain, termed CPSF-KH domain (Nishida et al. 2010; Mir-Montazeri et al. 2011), is composed of two type-II KH-domains (N-KH and C-KH domains) linked by α-helices. As in other proteins where the type-II KH domains are found, the CPSF-KH domain is involved in RNA binding. In *M. mazei* CPSF-KH domain, the N-KH domain is not canonical and therefore shows very low sequence identity to other KH domains (Nishida et al. 2010; Mir-Montazeri et al. 2011); in particular, the signature motif in type-II KH domains (VIGXXG) is only fully conserved in the C-KH domain but not in N-KH domain (Fig. 3).

The first β-CASP/MBL that was found to be involved in DNA repair pathways was the yeast protein Pso2/Snm1 (heretofore Pso2), which was identified using genetic screenings designed to specifically isolate mutants hypersensitive to interstrand crosslinking (ICL) (Henriques and Moustacchi 1980, 1981; Ruhland et al. 1981a; Ruhland et al. 1981b). The two mutant strains found, *SNM1* (Sensitivity to Nitrogen Mustard) and *PSO2* (sensitivity to PSOralen), turned out to be allelic (Cassier-Chauvat and Moustacchi 1988). Several homologous proteins to yeast Pso2 have subsequently been found in higher eukaryotes, all of which constitute the SNM1 family. Two of these proteins, CPSF-73 and ELAC2, are involved in RNA maturation, whereas three additional proteins are known to participate in DNA repair pathways and are DNA nucleases: SNM1A, SNM1B (also known as Apollo), and SNM1C (also known as Artemis) (Demuth and Digweed 1998; Aravind 1999; Dronkert et al. 2000). As evidence accumulates, it points to a functional compartmentalization of SNM1A and SNM1B (being involved in ICL resolution), like yeast Snm1/Pso2, whereas SNM1C has been involved in certain steps of DSB repair (Cattell et al. 2010).

2.1.1 SNM1A/Pso2

In yeast, Pso2 levels are strictly conserved with less than one mRNA molecule per cell; however, upon induction of interstrand crosslinks (ICLs) the amount of *PSO2* message

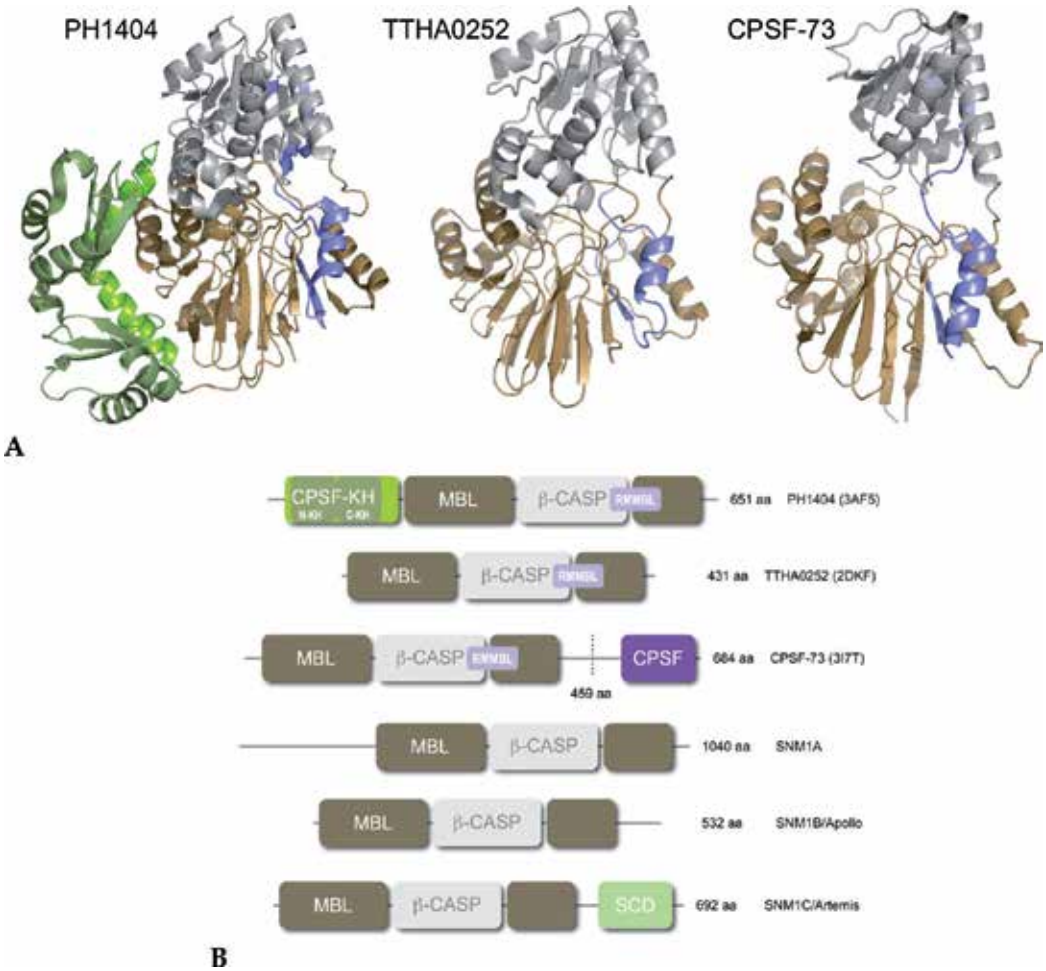

Fig. 3. *A*, Crystal structures of representative nucleases of the β-CASP family (human CPSF-73 and two hypothetical proteins, PH1404 and TTHA0252, from Archea and Bacteria, respectively), shown in ribbons. Different colors highlight the domains and motifs characteristic of the β-CASP nucleases. *B*, Domain organization, including β-CASP DNA nucleases involved in DNA repair, e.g., SNM1A, Apollo, and Artemis. Color coding as in *A*.

becomes dramatically upregulated (Wolter et al. 1996; Lambert et al. 2003). Accordingly, *pso2* mutants exhibit elevated sensitivity to a wide spectrum of crosslinking agents. The nuclease activity most convincingly shown for Pso2 is a 5′- to 3′-exonuclease acting on ssDNA and dsDNA with little preference (Li et al. 2005). The MBL domain of Pso2 is crucial for this activity, since mutation of D252 in motif II (Fig. 2) completely ablates Pso2 function in a manner indistinguishable from the null mutant (Li and Moses 2003). Interestingly, Pso2 appears to play a role in the repair of DSBs associated with ICLs, which result, e.g., from the collapse of stalled replication forks, since *pso2* mutants are greatly incapacitated to repair ICL-associated DSBs (Li and Moses 2003; Barber et al. 2005; Dudas et al. 2007). Furthermore, Pso2, together with two other nucleases, Exo1 and Mre11, have been involved in the processing of IR-induced DSBs (Lam et al. 2008), a function that is provided for partially by

all three nucleases. Since attempts to find potential Pso2 binding partners by two-hybrid screen have thus far failed (Dudas et al. 2007), the functional context where Pso2 acts remains speculative (Cattell et al. 2010).
Of the higher eukaryotic homologs of Pso2, the slightly greater sequence identity and comparatively longer N terminus of SNM1A makes it the closest in terms of sequence and domain structure (Fig. 3). This similarity suggested that SNM1A could be, too, the closest vertebrate ortholog to yeast Pso2, and therefore exhibit similar functions in ICL repair. Even though current evidence partly supports that proposal, one must caution that ICL processing is significantly more complex in vertebrates than in yeast, in part because of the concourse of two complexes [XPF-ERCC1 (De Silva et al. 2000, 2002) and Fanconi anemia proteins (Niedernhofer et al. 2005; Taniguchi and D'Andrea 2006)] that are lacking in yeast. Like yeast Pso2, SNM1A shows 5′- to 3′-exonuclease activity on ssDNA (slightly preferred) and dsDNA and importantly can complement *pso2* mutants in yeast (Hejna et al. 2007; Hazrati et al. 2008). In mammals, the precise role of SNM1A in ICL repair appears to restrict to a specific kind of ICL that originates from mytomicin C (MMC) treatment but not other ICL-inducing agents. Although currently unknown, this selectivity might arise from the fact that these ICLs do not induce large structural distortions of the DNA and could therefore be better detected by transcriptional or replicative stalling, which would render SNM1A's function cell cycle phase and checkpoint arrest specific (Cattell et al. 2010).

2.1.2 SNM1B/APOLLO

Apollo is termed after Artemis (SNM1C; see section 2.1.3) because of their structural and gene sequence similarities (Demuth et al. 2004) (Fig. 2-3). There are two splice variants of *APOLLO*, with the longer Apollo protein sharing 33% sequence identity with yeast Pso2; the second, shorter splice variant has so far resisted functional assignment. Full-length Apollo has 5′- to 3′-exonuclease activity. Aside from, but connected with, Apollo's role in DNA repair, its best-studied role is in telomere maintenance, mainly by interacting with the telomere-associated factor TRF2 (Freibaum and Counter 2006; Lenain et al. 2006; van Overbeek and de Lange 2006). TRF2 is responsible for the recruitment of several protective factors to the telomeres, and forms part of the six-subunit complex Shelterin (consisting of TRF1, TRF2, Rap1, TIN2, TPP1, and POT1), which protects the telomeres from the DNA damage response and therefore maintains their length (van Overbeek and de Lange 2006). In DNA repair, cells depleted of Apollo show various defects upon exposure to ionizing radiation and in the subsequent cellular response to DSBs, including attenuation in the autophorphorylation of ATM and in the phosphorylation of downstream ATM target proteins (Demuth et al. 2008). Part of this effect has an interesting connection with telomere maintenance, since cells depleted of both Apollo and TRF2 suffer increased DNA damage response and growth abnormalities than any of the single mutants (Lenain et al. 2006). Defects in Apollo are felt mainly during S phase, thereby suggesting a role for Apollo principally linked to DNA replication (van Overbeek and de Lange 2006) and associated with the repair of fork-stalling ICLs, much as Pso2 and SNM1A.

2.1.3 SNM1C/ARTEMIS

Artemis is a third vertebrate β-CASP DNA nuclease with roles in DNA repair by the NHEJ pathway (Ma, Y. et al. 2002; Rooney et al. 2003) and, in addition, in V(D)J recombination, a process indispensable for acquired immunity (Rooney et al. 2002). In contrast to Pso2,

SNM1A, and Apollo, Artemis does not appear to be involved in the repair of ICL lesions. As Apollo and SNM1A, Artemis has been described as a 5′- to 3′-exonuclease toward ssDNA and dsDNA (Ma, Y. et al. 2002) in a DNA PKcs independent manner, as well as an endonuclease whose activity is directed toward DNA hairpins and as a 3′- to 5′-exonuclease on DNA overhangs (Ma, Y. et al. 2002; Niewolik et al. 2006). The endonuclease activity on ssDNA appears to be intrinsic to Artemis (Gu et al. 2010; Pawelczak and Turchi 2010) and regulatable by DNA PKcs (Huang et al. 2009; Gu et al. 2010; Pawelczak and Turchi 2010).
An area that is hotly debated concerns the activation of Artemis upon DNA damage. Early studies suggested that the activation of Artemis depended on its phosphorylation by DNA PKcs, on the basis that Artemis has eleven Ser and Thr residues that are phosphorylatable in vitro (Ma, Y. et al. 2002; Niewolik et al. 2006). More recently, it has been shown that DNA cleavage by Artemis could be facilitated by a hypothetical DNA conformational change upon DNA PKcs autophosphorylation (Goodarzi et al. 2006; Yannone et al. 2008; Gu et al. 2010). Another element of discussion is whether Artemis has one single active site that is responsible for both the exonucleolytic and the endonucleolytic activity, or there are two separate, though partially overlapping, active sites for each of these activities. This question is based on the fact that mutants of Artemis impair only the endonuclease activity but have no consequences for Artemis exonuclease activity (Ma, Y. et al. 2002; Pannicke et al. 2004); strikingly, not even an Asp736 mutant of Artemis, a mutant that losses activity in all other SNM1 family members, compromises the 5′- to 3′-exonuclease activity. The latter, and the fact that two (even partially) separated active sites could coexist in a β-CASP nuclease, would make Artemis completely unique in the MBL superfamily. Even more recently, the assignment of 5′- to 3′-exonuclease activity has been called into question, as this activity could not be detected in further purified samples of Artemis (Pawelczak and Turchi 2010).
Efforts to clarify which roles does Artemis play in DNA repair have provided two sound answers. First, failure of Artemis deficient cells to show defects upon exposure to ICL inducing chemicals dispels a potential role for Artemis in the repair of ICL lesions. Instead, Artemis nuclease activity has been shown to be involved in the repair of a subgroup of DSBs (10–15%) produced by IR that contain covalently modified ends refractory to direct repair by other nucleases (Riballo et al. 2004; Wang, J. et al. 2005; Darroudi et al. 2007). The processing by Artemis of those 'blocked' DSBs so that they become accessible to downstream DNA repair machinery would be fitting with the known ability of Artemis-DNA PKcs to process 5′ or 3′ overhangs, hairpins, loops, gaps, or flaps, within DNA (Ma, Y. et al. 2005), as well as oxidation lesions at DNA ends (Povirk et al. 2007). In fact, DNA PKcs has been demonstrated to recruit Artemis to DSB sites, especially in heterochromatin, where DNA PKcs could modify the DNA structure so as to facilitate cleavage by Artemis (Goodarzi et al. 2006). At DSBs, Artemis collaborates with ATM to promote DSB repair by two different pathways, NHEJ at G0/G1 phase and HR at G2 phase (Goodarzi et al. 2006).

2.2 Protein phosphatase fold nucleases (Mre11)

The most prominent DNA nuclease of the protein phosphatase 2B (PP2B) fold is Mre11 (Meiotic recombination 11) (Gueguen et al. 2001), which is one of the central nucleases for the repair of DSBs by the non-homologous end joining (NHEJ) and homologous recombination (HR) repair pathways. Phylogenetic analyses show that Mre11 is conserved across the tree of life, likely because of its vital functionality in DNA repair. Mre11 contains five conserved motifs (shared with some structurally related polymerase small subunits), including a two-metal-ion-binding site that has a strong preference for manganese (Gueguen et al. 2001) and is

essential for catalysis in the archeal, yeast, and human enzymes. At least in vitro, Mre11 exhibits the following enzymatic activities: ssDNA endonuclease, dsDNA 3′- to 5′-exonuclase, DNA hairpin cleaving (Hopfner et al. 2001), and activation of DNA checkpoint kinase (ATM in humans, Tel1 in yeast) (Williams, R.S. et al. 2008). Mn^{2+} is required for all these activities, and interaction of Mre11 with Rad50 is necessary for dsDNA 3′- to 5′-exonuclease and cleaving DNA hairpins (Hopfner et al. 2001; Ghosal and Muniyappa 2005; Williams, R.S. et al. 2008). Besides, Mre11 has been observed to participate in 5′ to 3′ end resection of DSBs in vivo (Williams, R.S. et al. 2007), although the precise mechanism remains to be completely elucidated. A current working model involves other enzymes with nuclease or helicase activity in addition to Mre11, like Sae2, Exo1, Dna2, or Sgs1 (Zhu et al. 2008). The cooperation between these enzymes is supported by the observation of a reduced 5′- to 3′-exonucleolytic activity in cells lacking Exo1 and a complete ablation of this activity when Exo1, Sae2, and the MRX complex are all absent (Zhu et al. 2008). It appears that Mre11, together with Sae2, initiates DSB resection by facilitating trimming of 5′ ends, which can then be degraded by Exo1 or Dna2, in collaboration with the Sgs1 helicase, thus generating long single-stranded overhangs (Mimitou and Symington 2008).

As many other DNA processing enzymes, Mre11 is part of a multisubunit complex whose core is composed of four subunits, two subunits of Mre11 and two of Rad50 (Table 1). In this four-subunit MR complex, Mre11 acts as the nuclease engine, whereas Rad50 contributes localization and tethering specific functions. In eukaryotic organisms, as opposed to the simpler archeal and bacterial MR complex, there is a third subunit associated with Mre11 and Rad50, Nbs1 (or Xrs2 in yeast). Nbs1 is an integral part of the eukaryotic complex, which is named MRN (in yeast, MRX) (Hopfner et al. 2001). The MRN complex participates in various DNA repair processes such as in DNA damage detection, HR (Williams, R.S. et al. 2008), telomere maintenance, or checkpoint signaling, meiotic recombination, NHEJ and MMEJ (Lammens et al. 2011). Through its capacity to activate the ATM kinase, the MRN complex participates in the cell cycle (Lammens et al. 2011).

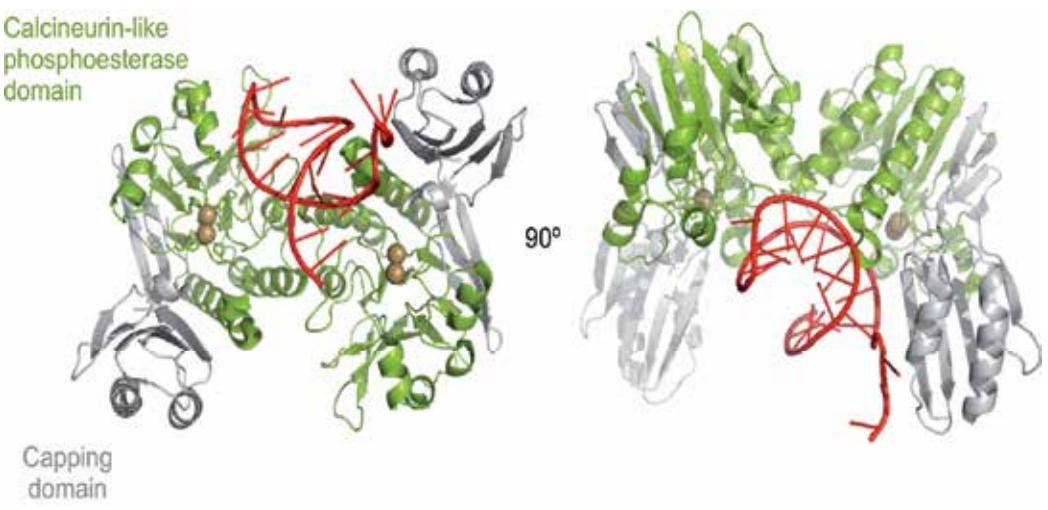

Fig. 4. Crystal structure of *Pf*Mre11 (two views 90° apart) in complex with branched DNA (Williams, R.S. et al. 2008), an intermediate during DSB repair.

	PP2B protein	Organism	PDB ID (Reference)
ARCHEA	Mre11:Rad50	*P furiosus*	3QKR (Williams, G.J. et al. 2011)
	Mre11	*P. furiosus*	3DSD, 3DSC (Williams, R.S. et al. 2008)
	Mre11	*P. furiosus*	1II7 (Hopfner et al. 2001)
	Mre11-3	*P. furiosus*	1S8E (Arthur et al. 2004)
EUBACTERIA	Mre11:Rad50	*T. maritima*	3QF7, 3QG5 (Lammens et al. 2011)
	Mre11	*T. maritima*	2Q8U (Das et al. 2010)

Table 3. X-ray crystal structures of archeal and eubacterial Mre11.

Several crystal structures of Mre11 in several functionally relevant complexes have been solved in two extremophilic microorganisms: the Archea *Pyrococcus furiosus* (Hopfner et al. 2001) (Fig. 4 and Table 3) and the Eubacteria *Thermotoga maritima* (Das et al. 2010) (Table 3). *Pf*Mre11 is composed of two domains, domain I (or nuclease domain) and domain II (or capping domain). Domain I is formed by two parallel β-sheets surrounded by seven α-helices, and is characterized by five conserved phosphodiesterase motifs that confer nuclease activity. These conserved motifs are the blueprint of the PP2B superfamily to which Mre11 belongs. In addition, the conserved five motifs are very similar, at the structural level, to those found in Ser/Thr phosphatases (although the latter phosphatases show preference for different metal ions, such as Zn^{2+} and Fe^{2+}). The capping domain is composed of five β-strands and two α-helices, and its function is to specifically bind DNA (Hopfner et al. 2001). Like in many two-domain proteins, there is flexibility in the relative orientation around the linker that connects the two domains, and it has been suggested that this flexibility enables Mre11 to efficiently bind widely different substrates (dsDNA, ssDNA, or hairpin DNA). In addition, this flexibility allows the capping domain to rotate while bound to DNA thus facilitating the opening of the DNA substrate (Hopfner et al. 2001)

Similarly to other nucleases of the MBL fold, archeal and human Mre11 are homodimeric enzymes. Two chains of Mre11 assemble in a homodimer with an interface composed of α-helices that are stabilized by apolar interactions between hydrophobic and aromatic residues, as Leu61, Ile65, Pro92, Leu97, and Phe101 (Williams, R.S. et al. 2008). This dimerization interface appears to be conserved at the sequence level in Bacteria, Archea, fungi, and humans (Das et al. 2010), and sequence alignments with *Trypanosoma brucei* or *Arabidopsis thaliana* lend support to the notion that Mre11 may share its quaternary structure even in paramecia and plants (Tan et al. 2002). Despite its evolutionary conservation, the integrity of the dimer structure does not appear to be required for the nuclease activity, at least in *P. furiosus* Mre11 (*Pf*Mre11). Instead, the dimer structure seems to be essential for binding the DNA substrate in the correct orientation. The dimeric architecture of Mre11 enables it to simultaneously bind to the two ends of a DSB in a synaptic complex whereby the two ends of a DSB are drawn near one another (or 'tethered') (Hopfner et al. 2001; Williams, R.S. et al. 2008). This tethering represents an essential prerequisite for the successful Mre11-mediated processing of a DSB lesion, since close proximity of the two DSB ends is necessary for ligation.

As explained above, the presence of two metal ions is an absolute requirement for the nuclease activity of Mre11. In *Pf*Mre11, residues Asn84 and His85 have been shown to be essential for catalysis. In fact, the residues implicated in metal coordination are conserved, at least in *P. furiosus*, yeast and humans (Hopfner et al. 2001). This feature has not been proven in *T. maritima* Mre11 (*Tm*Mre11) because of the absence of any bound metals in the crystal structure (Das et al. 2010); this does not necessarily imply that *Tm*Mre11 has no requirement

for Mn^{2+}, since metalloenzymes can become depleted of the metal they need for catalysis during purification. Mutations in *Pf*Mre11 of two catalytic His residues of the nuclease domain, His85 and His52, eliminate the 3′-5′ exonuclease activity (Williams, R.S. et al. 2008). Mutations in His85 abolish endonuclease activity, while a H52S mutant affects only weakly to the endonuclease activity (Williams, R.S. et al. 2008), therefore indicating that His85 is the most crucial residue since it is required to maintain both the exonuclease and the endonuclease activities. Some authors propose that the major function of Mre11 is endonucleolytic, as needed for homologous recombination (Williams, R.S. et al. 2008). His85 is believed to act as a proton donor to the 3′-OH group of the substrate (Hopfner et al. 2001), while His52 facilitates the rotation of the phosphate group required for 3′-5′ exonucleolytic activity (Williams, R.S. et al. 2008). Given the conservation of these two His residues, this mechanism assumes that the nuclease activity is highly conserved among Mre11 orthologs, including *T. maritima* and yeast (Das et al. 2010). Assays with dAMP (deoxyadenosine monophosphate) proved that the interaction of Mre11 with the substrate occurs via the phosphate group of dAMP, without a specific recognition of the adenine base (i.e., non-specifically) (Hopfner et al. 2001). This observation is in agreement with the sequence non-specificity of Mre11, which has been shown to interact mostly with the minor groove of DNA (Williams, R.S. et al. 2008). This property may further enhance Mre11's capacity to recognize widely different DNA sequences and to accomplish diverse activities.

*Pf*Mre11 interacts with its DNA substrate in two different ways, both of which are biologically relevant (Williams, R.S. et al. 2008): A synaptic DNA complex, which simulates dsDNA end joining of two DSB products, a fundamental process in DSB repair; and a branched DNA complex (Fig. 4), wherein the joined ssDNA-dsDNA structure sits at the interface between the nuclease and the capping domains of Mre11. The branched DNA complex, as opposed to the synaptic complex, is an asymmetric complex (Fig. 4).

In *T. maritima*, *Tm*Mre11 forms a crystallographic homodimer of slightly smaller subunits, and the nuclease domain is composed of 12 β-strands and five α-helices (Das et al. 2010). The C-terminal, DNA binding domain contains three β-strands and two α-helices, and is equivalent to the capping domain of *Pf*Mre11. Unlike *Pf*Mre11, solution analyses (size-exclusion chromatography and static light scattering) indicate that *Tm*Mre11 can be monomeric. In support of a physiologic homodimeric structure, conserved residues at the dimerization interfaces of *Pf*Mre11, yeast, and human Mre11, are also conserved in *Tm*Mre11, including Leu75, Leu78, Lys79, and Ile113. Phe102 and Phe105, two hydrophobic residues at the putative dimerization interface of *Tm*Mre11 that would be exposed in the monomeric structure, have too equivalent residues in *Pf*Mre11, yeast, and human Mre11. Although there are many shared features in the DNA binding domain of *Tm*Mre11, the specific configuration of this domain may explain the differential functionality among these species. In keeping with the conservation of the nuclease domain, enzymatic studies have shown that *Tm*Mre11 possesses both exonuclease and endonuclease activities. Furthermore, His94 in *Tm*Mre11 seems to carry out the same function as His85 in *Pf*Mre11, and His61 (*Tm*Mre11) is functionally equivalent to His52 (*Pf*Mre11) (Das et al. 2010).

The MR complex from *T. maritima*, like that of *P. furiosus*, comprises four subunits, two each of Mre11 and Rad50, the core of the complex being an Mre11 homodimer that is stabilized by a core of hydrophobic interactions. Each Mre11 subunit in the core dimer contacts one Rad50 subunit (Lammens et al. 2011), with an overall organization of the bacterial MR complex that is identical to that of the archeal MR complex. This notion that not only the constituent subunits but also their mode of interaction inside a seemingly isofunctional

complex is a fundamental aspect of protein complex evolution, which has been elegantly shown too for other DNA and chromatin binding complexes, e.g. the histone acetylation and deacetylation complexes (Doyon et al. 2006). The human MR complex has been studied by SFM (scanning force microscopy), revealing a large central globular domain whence two long, flexible projections emerge (de Jager et al. 2001), which has led to the proposal of an evolutionarily conserved architecture for the MR complex. In contrast to the prokaryotic MR complexes, the human MR complex seems to bind DNA preferably at the end of linear dsDNA, whereas in circular DNA the MR complex binds almost exclusively as monomers. The yeast MR complex has been studied as well, with the result that mutations that perturb the complex lead to genome aberrations, loss of cell viability, problems in recombination, and mistakes in telomere maintenance; many of these defects stem from a reduced DSB repair (Ghosal and Muniyappa 2005). In yeast, as in humans, a third subunit (Xrs2) is associated with Mre11 and Rad50 in a so-called MRX complex, which has roles mostly in DSB repair by the HR pathway (Yamaguchi-Iwai et al. 1999).

Telomere ends are a special class of DSBs, and in this context it has been shown that yeast Mre11 (*Sc*Mre11) is implicated in the removal of Spo11p, a protein that generates DSB during meiotic homologous recombination (Diaz et al. 2002). Apparently, *Sc*Mre11 has high affinity for parallel G-quadruplex (G4) DNA, a feature of yeast telomeres. Indeed, *Sc*Mre11 can cleave G4 DNA as well as G-rich ssDNA. This activity of *Sc*Mre11 toward G4 DNA and related DNAs is thought to facilitate the action of telomerase and the binding of other proteins to the telomeres. *Sc*Mre11 can also bind ssDNA and dsDNA, though with lower affinity (Ghosal and Muniyappa 2005). More recently, some studies have shown a relationship between *Sc*Mre11 and topoisomerases (Hamilton and Maizels 2010), whereby two known topoisomerase inhibitors (camptothecin and etoposide) show differential effects in an *mre11Δ* knockout. In turn, an H59A mutant is affected in the resistance to hydroxyurea and IR, whereas it is not affected by camptothecin (Hamilton and Maizels 2010). Thus, these results suggest an interaction, whether direct or indirect, between Mre11 and topoisomerases, both of which carry out functions on a DNA substrate. Like *Sc*Mre11, *Tb*Mre11 is thought to participate in telomere maintenance (Tan et al. 2002), an observation that raises the possibility that the conservation of telomeric repeat structure from yeast to vertebrates may be linked to the conservation of Mre11.

3. Structural and catalytic parallels

The MBL and PP2B families have a number of significant similarities at different, functionally relevant levels, and therefore many parallels can be drawn between the two nuclease families, structurally and catalytically. First, the core of either fold consists of β-sheets with analogous topology between the β-CASP nucleases and Mre11. Second, their tertiary structures are similar. Third, both nuclease folds use a two-metal-ion binding site for catalysis (Zn^{2+} in the β-CASP nucleases, and Mn^{2+} by Mre11), and have similar active-site residues to perform catalysis. The differences in substrate specificity and reactivity can be rationalized, very preliminary in the absence of additional structural information for the β-CASP DNA nucleases, as stemming from the different localization of the active-site residues in either fold, and in the prominent role of the accessory or inserted domains in modulating catalysis by the nuclease domain.

Several crystal structures of archeal Mre11 alone and in complex with DNA reveal a conserved homodimer with a tertiary structure and active sites that are reminiscent to those

of β-CASP nucleases (Hopfner et al. 2001; Williams, R.S. et al. 2008). In both fold families, the core of each β-sheet is composed of seven strands ordered as (↑↓↑↑↑↑↑), and the active site is surrounded by many conserved His residues and carboxylates that bind the catalytic metal ions. The two Mn^{2+} ions in Mre11 are located in equivalent positions to the Zn^{2+} ions in the β-CASP nucleases. Are the catalytic roles of Zn^{2+} in β-CASP nucleases transferable to Mre11 Mn^{2+} ions? Even though the DNA co-crystal structures of Mre11 have not captured the DNA substrate within coordination distance of the two Mn^{2+} ions, there is some experimental evidence from structural and mutagenic analyses that indicate that one conserved His residue is active in orienting DNA substrate for the exonuclease cleavage, while a second His residue could be involved in either metal-ion binding or catalysis (Williams, R.S. et al. 2008). This behavior might indeed bear some resemblance to the classical catalytic mechanism of MBLs, whereby one Zn^{2+} ion binds and orientates the substrate, whereas a second Zn^{2+} ion fulfills a distinct, but essential role, in, e.g., shielding the negative charge that develops in the leaving group during the transition state.

This array of similarities between Mre11 and the β-CASP family, spanning the overall structure, active site configuration, and the presence of two tightly bound metal ions, has been suggested as the underlying cause of the partially overlapping functions of Mre11 and the MBL nucleases Snm1 and Pso2 (Lam et al. 2008; Yang 2010). Indeed, having more than one enzyme to fulfill a vital function is a well-established cellular strategy to preserve viability in the event that one of the enzymes is lost due to mutation or deletion, and the fact that Mre11 and Snm1/Pso2 exhibit partial functional redundancy illustrates how important these functions are.

4. Nucleases at the heart of DNA repair complexes

Protein complexes, rather than isolated proteins, carry out the immense majority of cellular functions, and the intricate processes of DNA repair are no exception. Even though there are nucleases that perform catalysis in the absence of physically associated protein partners, the highly regulated and exquisitely orchestrated process of DNA repair requires protein multisubunit complexes able to sense inputs and effect biological outcomes via the nucleosome engine subunit. A conspicuous example is the MRN complex, which has been described as an analog computer molecular machine.

All of the β-CASP DNA nucleases establish stable or transient interactions with other proteins or protein complexes. Even though no stable interaction partners for Pso2/SNM1A have thus far been found, both yeast Pso2 and mammalian SNM1A participate in complex processes that require the intervention of other proteins, in particular other nucleases, and it is then plausible that transient interactions play a major role in the correct orchestration of, e.g., repair of ICLs. In contrast, SNM1B/Apollo has been demonstrated to form several stable complexes. For example, Apollo can associate with the six-subunit Shelterin complex that protects the telomeres likely through an interaction between its C terminus and TRF2, an interaction that has been shown in a co-crystal structure of TRF2 and a C-terminal Apollo peptide (Chen et al. 2008). Another functionally important interaction of Apollo, this time via the MBL domain, involves Astrin; the disruption of this interaction by mutations in the MBL domain of Apollo causes deficient prophase checkpoint (Liu et al. 2009).

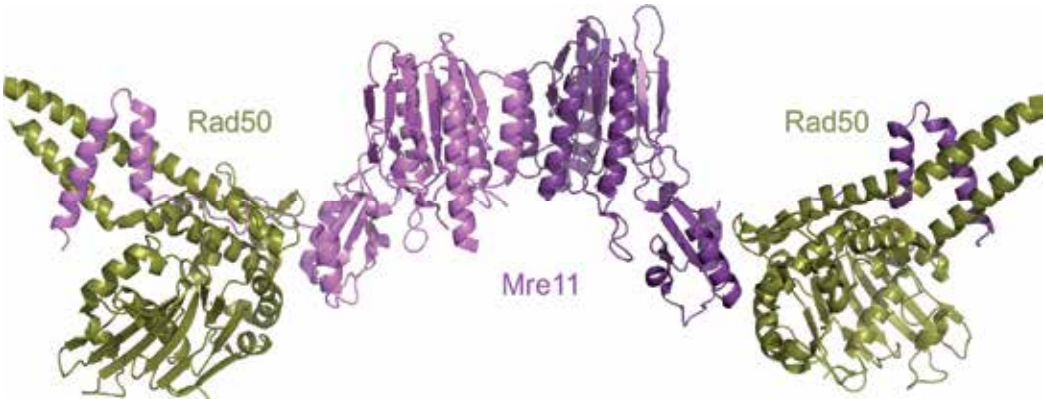

Fig. 5. Crystal structure of the bacterial MR complex (Mre11-Rad50) (Lammens et al. 2011) (PDB ID 3QG5).

As has been pointed out above (Section 2), Mre11 acts in the context of multisubunit complexes with Rad50 and/or Nbs1/Xrs2 (MR and MRN/MRX complexes, respectively) that provide expanded functionality in the recognition and tethering of DSBs and the sensing of cellular stress signals via its non-nuclease subunits. These extra capabilities are essential to target DSBs and avoid wasteful scanning and/or enzymatic processing by Mre11. Small-angle x-ray scattering (SAXS) and analytical ultracentrifuge (AUC) experiments have provided compelling evidence that the MR complex is a heterotetramer formed by two subunits each of Mre11 and Rad50; corroborative evidence of the subunit composition of the MR complex has been obtained by electron microscopy (EM) (Hopfner et al. 2001). Furthermore, the tethering of DNA by the MRX complex has been shown by atomic force microscopy (AFM) (Williams, R.S. et al. 2008). Perhaps the most convincing evidence is the direct observation of the interaction surfaces between Mre11 with the nucleotide-binding domain of Rad50 (Fig. 5), and of the coiled coil segment of Rad50 and an Mre11-derived peptide, both captured by x-ray crystallography from the archeal and the eubacterial MR complexes (Williams, G.J. et al. 2011).

Apart from its role as the nucleolytic engine of the MRN complex, Mre11 acts jointly with other nucleases in what may be described as a functional cooperation. A chief example of this comes from the observation made in yeast that both Mre11 and another exonuclease, Exo1, are both required for the efficient initiation and processivity of resection at specific DSBs generated during meiosis (Hodgson et al. 2011). Loss of function of either Mre11 or Exo1 causes severe delay in resection, therefore suggesting that Mre11 and Exo1 are the major nucleases involved in creating the resection tracts typical of meiotic recombination (Hodgson et al. 2011).

5. Evolution of DNA repair nucleases

MBL fold nucleases involved in DNA repair have most likely evolved from precursor enzymes with the capacity to act upon RNA substrates, which are widespread across the tree of life and include enzymes that can recognize either sequence features, structure, or combined sequence-structure signatures on RNA substrates. Changing the substrate specificity from RNA to DNA should have been easily achieved during evolution, as the same fold scaffolds have been proven to catalyze either reaction. Many of these MBL RNases

utilize inserted domains to assist recognition and binding of RNA molecules, which are large and very densely charged molecules. One outstanding example concerns RNases from the MBL superfamily, which often possess a β-CASP domain for recognition and binding of the nucleic acid. Although no structure of a DNA nuclease of the MBL fold is available to date, it is conceivable that an RNA binding MBL enzyme might support sequence modifications that allow it to bind DNA, either ssDNA or dsDNA. Actually, binding ssDNA in the context of melted dsDNA molecules is not only conceivable but also most likely true, since many of the MBL enzymes have been convincingly shown to act upon ssDNA segments. Artemis, for example, is activated by DNA-PKcs, and one way this could happen is by the melting action of DNA PKcs upon a dsDNA substrate, which would be sufficient to provide ssDNA substrate to Artemis. However, a complete clarification of the involved processes will have to wait until more careful experiments are conducted.

PP2B nucleases, like Mre11, are also widespread across the tree of life and have been identified in Bacteria, Archea, and Eukarya. Crystal structures of the eubacterial and archeal enzymes are available in the Protein Data Bank for comparison, and they have been shown to be of different length while maintaining all of the conserved key residues for catalysis, as well as the identity of the catalytic metal ion (manganese). Therefore, it is quite plausible that there existed an Mre11-like enzyme in the last universal common ancestor (LUCA) of all extinct life forms with, potentially, similar roles in DSB repair and maintenance of genome integrity. Further support for this idea is derived from the clear assumption that the selection pressure for sophisticated and efficient DNA repair machinery for LUCA must have been even stronger than at present.

6. Disease states and mutations in nucleases

There is a plethora of mutation studies in MBL and PP2B nucleases carried out in model organisms that can be related to human pathophysiology linked to DNA repair and genome stability. These disease-associated mutations provide a wealth of information on function, specificity, and redundancy of the DNA repair nucleases.

Among the nucleases from the MBL family, a well-known syndrome is radiation sensitive severe combined immunodeficiency (RS-SCID), a disease condition that arises from defects in Artemis and is truly the result of impaired function of Artemis in DNA repair and in V(D)J recombination (Dominski 2007). Another striking example comes from patients with Hoyeraal-Hreidarsson (HH) syndrome, a severe form of *dyskeratosis congenita* caused by impaired telomere protection. Patients with HH syndrome suffer of premature aging and are immunodeficient. At the molecular level, the HH syndrome is characterized by a unique *APOLLO* splice variant that lacks the (TRFH)-binding motif (TBM) to TRF2 (Touzot et al. 2010). In addition to the roles involved in DNA repair and telomeric protection, Apollo deficient cells present a deficient prophase checkpoint increased when the interaction of Astrin and the MBL domain of Apollo is disrupted by mutations in such domain (Liu et al. 2009). Fanconi anemia (FA) is another example. Mutations in up to 14 different FANC genes have been associated with FA, a DNA repair disorder that dramatically enhances predisposition to cancer and is characterized by progressive bone marrow failure, congenital development defects, chromosomal abnormalities, and cellular hypersensitivity to ICL agents. Although none of the FANC genes are MBLs or PP2B fold nucleases, functional associations with MBL nucleases have been described, therefore FA has interconnections

with MBL nucleases of DNA repair that underlie the complex network of functions that is disrupted by FA.

The PP2B family protein Mre11 has a vital role across phylogenetically diverse organisms ranging from Bacteria to vertebrates, on the basis of its crucial role in DNA repair. Well-established links between *MRE11* mutations and disease states exist, e.g., in yeast, where the N113S mutation (in the nuclease domain) causes an enhanced sensitivity to ionizing radiation (IR). In humans, the same mutation (N117S) has been implicated in the onset of ataxia-telangiectasia disorder (ATLD) (Hopfner et al. 2001). Another yeast mutation, P162S, affects the repair of DSBs carried out by Mre11 (Hopfner et al. 2001), which in metazoans would likely affect genome stability and increase the chances of neoplastic transformation. Other mutations in Mre11, like H129N, confer early embryonic lethality in homozygosis in mice (Buis et al. 2008), and depletion of chicken Mre11 appears critical in the survival of animal cells through its participation in homologous recombination repair, leading to the accumulation of DSBs and increased of radiosensitivity (Yamaguchi-Iwai et al. 1999). Other studies show that animal cells deficient in MRE11 seem to be non-viable, whereas in *Trypanosoma brucei Tb*Mre11 is not so critical for cell maintenance, but this discrepancy could be reconciled with most of the current knowledge on Mre11 if other, functionally redundant mechanisms were found to repair DSBs in *T. brucei* (Tan et al. 2002). In agreement with the important role of multisubunit complexes in DNA repair, mutations in another component of the human MRN complex, Nbs1, give rise to Nijmegen breakage syndrome, a paradigm of a disease linked to DNA repair defects (de Jager et al. 2001) that has been associated with an enhanced predisposition to colorectal cancer.

7. Conclusions and future outlook

There are many standing issues in the field of DNA repair nucleases whose elucidation awaits further research. Some of these issues include the complete biochemical and structural characterization of DNA nucleases of the MBL fold family, which are known to play key roles in DNA repair but which have thus far proved hard to reveal their substrate specificities (e.g., ssDNA versus dsDNA), activities (e.g., controversies over the 3′-5′ and 5′-3′ exonuclease activities of Artemis), or even the requirement for post-translational modifications (such as DNA PKcs mediated phosphorylation of Artemis). In protein phosphatase nucleases, the best-known example is archeal and eukaryotic Mre11 and the architecture of the MRN complex. There, one crucial aspect is to decipher how the MRN complex processes all its inputs and delivers a comprehensive functional outcome. In more applied science, there is always the wide-ranging and crucial question of how can the tremendous amount of basic science results be put to clinical use. In DNA repair, the identification of mutations that cause, or predispose, to acquire certain diseases must advance to the point that early diagnosis becomes feasible for many. Cures to these diseases may be far into the future, but the current and near future research is providing sure steps toward this much-longed end.

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9. References

Aravind, L. (1999). An evolutionary classification of the metallo-beta-lactamase fold proteins. *In silico biology,* Vol.1, No.2, (July 2001), pp. 69-91, ISSN 1386-6338.

Arthur, L. M., K. Gustausson, K. P. Hopfner, C. T. Carson, T. H. Stracker, A. Karcher, D. Felton, M. D. Weitzman, J. Tainer & J. P. Carney (2004). Structural and functional analysis of Mre11-3. *Nucleic acids research,* Vol.32, No.6, (March 2004), pp. 1886-1893, ISSN 1362-4962.

Baldwin, G. S., S. G. Waley & E. P. Abraham (1979). Identification of histidine residues that act as zinc ligands in beta-lactamase II by differential tritium exchange. *The Biochemical journal,* Vol.179, No.3, (June 1979), pp. 459-463, ISSN 0264-6021.

Barber, L. J., T. A. Ward, J. A. Hartley & P. J. McHugh (2005). DNA interstrand cross-link repair in the Saccharomyces cerevisiae cell cycle: overlapping roles for PSO2 (SNM1) with MutS factors and EXO1 during S phase. *Molecular and cellular biology,* Vol.25, No.6, (March 2005), pp. 2297-2309, ISSN 0270-7306.

Buis, J., Y. Wu, Y. Deng, J. Leddon, G. Westfield, M. Eckersdorff, J. M. Sekiguchi, S. Chang & D. O. Ferguson (2008). Mre11 nuclease activity has essential roles in DNA repair and genomic stability distinct from ATM activation. *Cell,* Vol.135, No.1, (October 2008), pp. 85-96, ISSN 1097-4172.

Callebaut, I., D. Moshous, J. P. Mornon & J. P. de Villartay (2002). Metallo-beta-lactamase fold within nucleic acids processing enzymes: the beta-CASP family. *Nucleic acids research,* Vol.30, No.16, (August 2002), pp. 3592-3601, ISSN 1362-4962.

Cassier-Chauvat, C. & E. Moustacchi (1988). Allelism between pso1-1 and rev3-1 mutants and between pso2-1 and snm1 mutants in Saccharomyces cerevisiae. *Current genetics,* Vol.13, No.1, (January 1988), pp. 37-40, ISSN 0172-8083.

Cattell, E., B. Sengerova & P. J. McHugh (2010). The SNM1/Pso2 family of ICL repair nucleases: from yeast to man. *Environmental and molecular mutagenesis,* Vol.51, No.6, (February 2010), pp. 635-645, ISSN 1098-2280.

Chen, Y., Y. Yang, M. van Overbeek, J. R. Donigian, P. Baciu, T. de Lange & M. Lei (2008). A shared docking motif in TRF1 and TRF2 used for differential recruitment of telomeric proteins. *Science,* Vol.319, No.5866, (January 2008), pp. 1092-1096, ISSN 1095-9203.

Daiyasu, H., K. Osaka, Y. Ishino & H. Toh (2001). Expansion of the zinc metallo-hydrolase family of the beta-lactamase fold. *FEBS letters,* Vol.503, No.1, (August 2001), pp. 1-6, ISSN 0014-5793.

Darroudi, F., W. Wiegant, M. Meijers, A. A. Friedl, M. van der Burg, J. Fomina, J. J. van Dongen, D. C. van Gent & M. Z. Zdzienicka (2007). Role of Artemis in DSB repair and guarding chromosomal stability following exposure to ionizing radiation at different stages of cell cycle. *Mutation research,* Vol.615, No.1-2, (December 2006), pp. 111-124, ISSN 0027-5107.

Das, D., D. Moiani, H. L. Axelrod, M. D. Miller, D. McMullan, K. K. Jin, P. Abdubek, T. Astakhova, P. Burra, D. Carlton, H. J. Chiu, T. Clayton, M. C. Deller, L. Duan, D. Ernst, J. Feuerhelm, J. C. Grant, A. Grzechnik, S. K. Grzechnik, G. W. Han, L.

Jaroszewski, H. E. Klock, M. W. Knuth, P. Kozbial, S. S. Krishna, A. Kumar, D. Marciano, A. T. Morse, E. Nigoghossian, L. Okach, J. Paulsen, R. Reyes, C. L. Rife, N. Sefcovic, H. J. Tien, C. B. Trame, H. van den Bedem, D. Weekes, Q. Xu, K. O. Hodgson, J. Wooley, M. A. Elsliger, A. M. Deacon, A. Godzik, S. A. Lesley, J. A. Tainer & I. A. Wilson (2010). Crystal structure of the first eubacterial Mre11 nuclease reveals novel features that may discriminate substrates during DNA repair. *J Mol Biol,* Vol.397, No.3, (February 2010), pp. 647-663, ISSN 1089-8638.

de Jager, M., J. van Noort, D. C. van Gent, C. Dekker, R. Kanaar & C. Wyman (2001). Human Rad50/Mre11 is a flexible complex that can tether DNA ends. *Molecular cell,* Vol.8, No.5, (December 2001), pp. 1129-1135, ISSN 1097-2765.

De Silva, I. U., P. J. McHugh, P. H. Clingen & J. A. Hartley (2000). Defining the roles of nucleotide excision repair and recombination in the repair of DNA interstrand cross-links in mammalian cells. *Molecular and cellular biology,* Vol.20, No.21, (October 2000), pp. 7980-7990, ISSN 0270-7306.

De Silva, I. U., P. J. McHugh, P. H. Clingen & J. A. Hartley (2002). Defects in interstrand cross-link uncoupling do not account for the extreme sensitivity of ERCC1 and XPF cells to cisplatin. *Nucleic acids research,* Vol.30, No.17, (August 2002), pp. 3848-3856, ISSN 1362-4962.

Demuth, I., P. S. Bradshaw, A. Lindner, M. Anders, S. Heinrich, J. Kallenbach, K. Schmelz, M. Digweed, M. S. Meyn & P. Concannon (2008). Endogenous hSNM1B/Apollo interacts with TRF2 and stimulates ATM in response to ionizing radiation. *DNA repair,* Vol.7, No.8, (May 2008), pp. 1192-1201, ISSN 1568-7864.

Demuth, I. & M. Digweed (1998). Genomic organization of a potential human DNA-crosslink repair gene, KIAA0086. *Mutation research,* Vol.409, No.1, (November 1998), pp. 11-16, ISSN 0027-5107.

Demuth, I., M. Digweed & P. Concannon (2004). Human SNM1B is required for normal cellular response to both DNA interstrand crosslink-inducing agents and ionizing radiation. *Oncogene,* Vol.23, No.53, (October 2004), pp. 8611-8618, ISSN 0950-9232.

Diaz, R. L., A. D. Alcid, J. M. Berger & S. Keeney (2002). Identification of residues in yeast Spo11p critical for meiotic DNA double-strand break formation. *Molecular and cellular biology,* Vol.22, No.4, (January 2002), pp. 1106-1115, ISSN 0270-7306.

Dominski, Z. (2007). Nucleases of the metallo-beta-lactamase family and their role in DNA and RNA metabolism. *Critical reviews in biochemistry and molecular biology,* Vol.42, No.2, (April 2007), pp. 67-93, ISSN 1040-9238.

Dominski, Z., X. C. Yang, M. Purdy, E. J. Wagner & W. F. Marzluff (2005). A CPSF-73 homologue is required for cell cycle progression but not cell growth and interacts with a protein having features of CPSF-100. *Molecular and cellular biology,* Vol.25, No.4, (February 2005), pp. 1489-1500, ISSN 0270-7306.

Doyon, Y., C. Cayrou, M. Ullah, A. J. Landry, V. Cote, W. Selleck, W. S. Lane, S. Tan, X. J. Yang & J. Cote (2006). ING tumor suppressor proteins are critical regulators of chromatin acetylation required for genome expression and perpetuation. *Molecular cell,* Vol.21, No.1, (January 2006), pp. 51-64, ISSN 1097-2765.

Dronkert, M. L., J. de Wit, M. Boeve, M. L. Vasconcelos, H. van Steeg, T. L. Tan, J. H. Hoeijmakers & R. Kanaar (2000). Disruption of mouse SNM1 causes increased sensitivity to the DNA interstrand cross-linking agent mitomycin C. *Molecular and cellular biology,* Vol.20, No.13, (January 2000), pp. 4553-4561, ISSN 0270-7306.

Dudas, A., D. Vlasakova, Z. Dudasova, D. Gabcova, J. Brozmanova & M. Chovanec (2007). Further characterization of the role of Pso2 in the repair of DNA interstrand cross-link-associated double-strand breaks in Saccharomyces cerevisiae. *Neoplasma,* Vol.54, No.3, (April 2007), pp. 189-194, ISSN 0028-2685.

Even, S., O. Pellegrini, L. Zig, V. Labas, J. Vinh, D. Brechemmier-Baey & H. Putzer (2005). Ribonucleases J1 and J2: two novel endoribonucleases in B.subtilis with functional homology to E.coli RNase E. *Nucleic acids research,* Vol.33, No.7, (April 2005), pp. 2141-2152, ISSN 1362-4962.

Freibaum, B. D. & C. M. Counter (2006). hSnm1B is a novel telomere-associated protein. *The Journal of biological chemistry,* Vol.281, No.22, (April 2006), pp. 15033-15036, ISSN 0021-9258.

Garces, F., F. J. Fernandez, C. Montella, E. Penya-Soler, R. Prohens, J. Aguilar, L. Baldoma, M. Coll, J. Badia & M. C. Vega (2010). Molecular architecture of the Mn2+-dependent lactonase UlaG reveals an RNase-like metallo-beta-lactamase fold and a novel quaternary structure. *Journal of molecular biology,* Vol.398, No.5, (April 2010), pp. 715-729, ISSN 1089-8638.

Ghosal, G. & K. Muniyappa (2005). Saccharomyces cerevisiae Mre11 is a high-affinity G4 DNA-binding protein and a G-rich DNA-specific endonuclease: implications for replication of telomeric DNA. *Nucleic acids research,* Vol.33, No.15, (August 2005), pp. 4692-4703, ISSN 1362-4962.

Goodarzi, A. A., Y. Yu, E. Riballo, P. Douglas, S. A. Walker, R. Ye, C. Harer, C. Marchetti, N. Morrice, P. A. Jeggo & S. P. Lees-Miller (2006). DNA-PK autophosphorylation facilitates Artemis endonuclease activity. *The EMBO journal,* Vol.25, No.16, (July 2006), pp. 3880-3889, ISSN 0261-4189.

Gu, J., S. Li, X. Zhang, L. C. Wang, D. Niewolik, K. Schwarz, R. J. Legerski, E. Zandi & M. R. Lieber (2010). DNA-PKcs regulates a single-stranded DNA endonuclease activity of Artemis. *DNA repair,* Vol.9, No.4, (February 2010), pp. 429-437, ISSN 1568-7856.

Gueguen, Y., J. L. Rolland, O. Lecompte, P. Azam, G. Le Romancer, D. Flament, J. P. Raffin & J. Dietrich (2001). Characterization of two DNA polymerases from the hyperthermophilic euryarchaeon Pyrococcus abyssi. *European journal of biochemistry / FEBS,* Vol.268, No.22, (November 2001), pp. 5961-5969, ISSN 0014-2956.

Hamilton, N. K. & N. Maizels (2010). MRE11 function in response to topoisomerase poisons is independent of its function in double-strand break repair in Saccharomyces cerevisiae. *PLoS One,* Vol.5, No.10, (November 2010), pp. e15387, ISSN 1932-6203.

Hazrati, A., M. Ramis-Castelltort, S. Sarkar, L. J. Barber, C. J. Schofield, J. A. Hartley & P. J. McHugh (2008). Human SNM1A suppresses the DNA repair defects of yeast pso2 mutants. *DNA repair,* Vol.7, No.2, (November 2007), pp. 230-238, ISSN 1568-7864.

Hejna, J., S. Philip, J. Ott, C. Faulkner & R. Moses (2007). The hSNM1 protein is a DNA 5'-exonuclease. *Nucleic acids research,* Vol.35, No.18, (September 2007), pp. 6115-6123, ISSN 1362-4962.

Henriques, J. A. & E. Moustacchi (1980). Isolation and characterization of pso mutants sensitive to photo-addition of psoralen derivatives in Saccharomyces cerevisiae. *Genetics,* Vol.95, No.2, (June 1980), pp. 273-288, ISSN 0016-6731.

Henriques, J. A. & E. Moustacchi (1981). Interactions between mutations for sensitivity to psoralen photoaddition (pso) and to radiation (rad) in Saccharomyces cerevisiae. *Journal of bacteriology,* Vol.148, No.1, (October 1981), pp. 248-256, ISSN 0021-9193.

Hodgson, A., Y. Terentyev, R. A. Johnson, A. Bishop-Bailey, T. Angevin, A. Croucher & A. S. Goldman (2011). Mre11 and Exo1 contribute to the initiation and processivity of resection at meiotic double-strand breaks made independently of Spo11. *DNA repair,* Vol.10, No.2, (December 2010), pp. 138-148, ISSN 1568-7856.

Hopfner, K. P., A. Karcher, L. Craig, T. T. Woo, J. P. Carney & J. A. Tainer (2001). Structural biochemistry and interaction architecture of the DNA double-strand break repair Mre11 nuclease and Rad50-ATPase. *Cell,* Vol.105, No.4, (May 2001), pp. 473-485, ISSN 0092-8674.

Huang, Y., W. Giblin, M. Kubec, G. Westfield, J. St Charles, L. Chadde, S. Kraftson & J. Sekiguchi (2009). Impact of a hypomorphic Artemis disease allele on lymphocyte development, DNA end processing, and genome stability. *The Journal of experimental medicine,* Vol.206, No.4, (April 2009), pp. 893-908, ISSN 1540-9538.

Ishikawa, H., N. Nakagawa, S. Kuramitsu & R. Masui (2006). Crystal structure of TTHA0252 from Thermus thermophilus HB8, a RNA degradation protein of the metallo-beta-lactamase superfamily. *Journal of biochemistry,* Vol.140, No.4, (September 2006), pp. 535-542, ISSN 0021-924X.

Khanna, K. K. & S. P. Jackson (2001). DNA double-strand breaks: signaling, repair and the cancer connection. *Nature genetics,* Vol.27, No.3, (March 2001), pp. 247-254, ISSN 1061-4036.

Lam, A. F., B. O. Krogh & L. S. Symington (2008). Unique and overlapping functions of the Exo1, Mre11 and Pso2 nucleases in DNA repair. *DNA Repair (Amst),* Vol.7, No.4, (February 2008), pp. 655-662, ISSN 1568-7864.

Lambert, S., S. J. Mason, L. J. Barber, J. A. Hartley, J. A. Pearce, A. M. Carr & P. J. McHugh (2003). Schizosaccharomyces pombe checkpoint response to DNA interstrand cross-links. *Molecular and cellular biology,* Vol.23, No.13, (June 2003), pp. 4728-4737, ISSN 0270-7306.

Lammens, K., D. J. Bemeleit, C. Mockel, E. Clausing, A. Schele, S. Hartung, C. B. Schiller, M. Lucas, C. Angermuller, J. Soding, K. Strasser & K. P. Hopfner (2011). The Mre11:Rad50 Structure Shows an ATP-Dependent Molecular Clamp in DNA Double-Strand Break Repair. *Cell,* Vol.145, No.1, (April 2011), pp. 54-66, ISSN 1097-4172.

Lenain, C., S. Bauwens, S. Amiard, M. Brunori, M. J. Giraud-Panis & E. Gilson (2006). The Apollo 5' exonuclease functions together with TRF2 to protect telomeres from DNA repair. *Current biology,* Vol.16, No.13, (May 2006), pp. 1303-1310, ISSN 0960-9822.

Li, X., J. Hejna & R. E. Moses (2005). The yeast Snm1 protein is a DNA 5'-exonuclease. *DNA repair,* Vol.4, No.2, (December 2004), pp. 163-170, ISSN 1568-7864.

Li, X. & R. E. Moses (2003). The beta-lactamase motif in Snm1 is required for repair of DNA double-strand breaks caused by interstrand crosslinks in S. cerevisiae. *DNA repair,* Vol.2, No.1, (January 2003), pp. 121-129, ISSN 1568-7864.

Liu, L., S. Akhter, J. B. Bae, S. S. Mukhopadhyay, C. T. Richie, X. Liu & R. Legerski (2009). SNM1B/Apollo interacts with astrin and is required for the prophase cell cycle checkpoint. *Cell Cycle,* Vol.8, No.4, (February 2009), pp. 628-638, ISSN 1551-4005.

Ma, J. L., E. M. Kim, J. E. Haber & S. E. Lee (2003). Yeast Mre11 and Rad1 proteins define a Ku-independent mechanism to repair double-strand breaks lacking overlapping end sequences. *Molecular and cellular biology,* Vol.23, No.23, (November 2003), pp. 8820-8828, ISSN 0270-7306.

Ma, Y., U. Pannicke, K. Schwarz & M. R. Lieber (2002). Hairpin opening and overhang processing by an Artemis/DNA-dependent protein kinase complex in nonhomologous end joining and V(D)J recombination. *Cell,* Vol.108, No.6, (April 2002), pp. 781-794, ISSN 0092-8674.

Ma, Y., K. Schwarz & M. R. Lieber (2005). The Artemis:DNA-PKcs endonuclease cleaves DNA loops, flaps, and gaps. *DNA repair,* Vol.4, No.7, (June 2005), pp. 845-851, ISSN 1568-7864.

Mandel, C. R., S. Kaneko, H. Zhang, D. Gebauer, V. Vethantham, J. L. Manley & L. Tong (2006). Polyadenylation factor CPSF-73 is the pre-mRNA 3'-end-processing endonuclease. *Nature,* Vol.444, No.7121, (November 2006/11/28), pp. 953-956, ISSN 1476-4687.

Marti, T. M. & O. Fleck (2004). DNA repair nucleases. *Cellular and molecular life sciences : CMLS,* Vol.61, No.3, (February 2004), pp. 336-354, ISSN 1420-682X.

Mir-Montazeri, B., M. Ammelburg, D. Forouzan, A. N. Lupas & M. D. Hartmann (2011). Crystal structure of a dimeric archaeal cleavage and polyadenylation specificity factor. *Journal of structural biology,* Vol.173, No.1, (September 2010), pp. 191-195, ISSN 1095-8657.

Niedernhofer, L. J., A. S. Lalai & J. H. Hoeijmakers (2005). Fanconi anemia (cross)linked to DNA repair. *Cell,* Vol.123, No.7, (December 2005), pp. 1191-1198, ISSN 0092-8674.

Niewolik, D., U. Pannicke, H. Lu, Y. Ma, L. C. Wang, P. Kulesza, E. Zandi, M. R. Lieber & K. Schwarz (2006). DNA-PKcs dependence of Artemis endonucleolytic activity, differences between hairpins and 5' or 3' overhangs. *The Journal of biological chemistry,* Vol.281, No.45, (August 2006), pp. 33900-33909, ISSN 0021-9258.

Nishida, Y., H. Ishikawa, S. Baba, N. Nakagawa, S. Kuramitsu & R. Masui (2010). Crystal structure of an archaeal cleavage and polyadenylation specificity factor subunit from Pyrococcus horikoshii. *Proteins,* Vol.78, No.10, (June 2010), pp. 2395-2398, ISSN 1097-0134.

Pannicke, U., Y. Ma, K. P. Hopfner, D. Niewolik, M. R. Lieber & K. Schwarz (2004). Functional and biochemical dissection of the structure-specific nuclease ARTEMIS. *The EMBO journal,* Vol.23, No.9, (April 2004), pp. 1987-1997, ISSN 0261-4189.

Pawelczak, K. S. & J. J. Turchi (2010). Purification and characterization of exonuclease-free Artemis: Implications for DNA-PK-dependent processing of DNA termini in NHEJ-catalyzed DSB repair. *DNA repair,* Vol.9, No.6, (March 2010), pp. 670-677, ISSN 1568-7856.

Povirk, L. F., T. Zhou, R. Zhou, M. J. Cowan & S. M. Yannone (2007). Processing of 3'-phosphoglycolate-terminated DNA double strand breaks by Artemis nuclease. *The Journal of biological chemistry,* Vol.282, No.6, (November 2006), pp. 3547-3558, ISSN 0021-9258.

Riballo, E., M. Kuhne, N. Rief, A. Doherty, G. C. Smith, M. J. Recio, C. Reis, K. Dahm, A. Fricke, A. Krempler, A. R. Parker, S. P. Jackson, A. Gennery, P. A. Jeggo & M. Lobrich (2004). A pathway of double-strand break rejoining dependent upon ATM, Artemis, and proteins locating to gamma-H2AX foci. *Molecular cell,* Vol.16, No.5, (December 2004), pp. 715-724, ISSN 1097-2765.

Rooney, S., F. W. Alt, D. Lombard, S. Whitlow, M. Eckersdorff, J. Fleming, S. Fugmann, D. O. Ferguson, D. G. Schatz & J. Sekiguchi (2003). Defective DNA repair and

increased genomic instability in Artemis-deficient murine cells. *The Journal of experimental medicine,* Vol.197, No.5, (March 2003), pp. 553-565, ISSN 0022-1007.

Rooney, S., J. Sekiguchi, C. Zhu, H. L. Cheng, J. Manis, S. Whitlow, J. DeVido, D. Foy, J. Chaudhuri, D. Lombard & F. W. Alt (2002). Leaky Scid phenotype associated with defective V(D)J coding end processing in Artemis-deficient mice. *Molecular cell,* Vol.10, No.6, (December 2002), pp. 1379-1390, ISSN 1097-2765.

Ruhland, A., E. Haase, W. Siede & M. Brendel (1981a). Isolation of yeast mutants sensitive to the bifunctional alkylating agent nitrogen mustard. *Molecular & general genetics : MGG,* Vol.181, No.3, (January 1981), pp. 346-351, ISSN 0026-8925.

Ruhland, A., M. Kircher, F. Wilborn & M. Brendel (1981b). A yeast mutant specifically sensitive to bifunctional alkylation. *Mutation research,* Vol.91, No.6, (January 1981), pp. 457-462, ISSN 0027-5107.

Takata, M., M. S. Sasaki, E. Sonoda, C. Morrison, M. Hashimoto, H. Utsumi, Y. Yamaguchi-Iwai, A. Shinohara & S. Takeda (1998). Homologous recombination and non-homologous end-joining pathways of DNA double-strand break repair have overlapping roles in the maintenance of chromosomal integrity in vertebrate cells. *The EMBO journal,* Vol.17, No.18, (September 1998), pp. 5497-5508, ISSN 0261-4189.

Tan, K. S., S. T. Leal & G. A. Cross (2002). Trypanosoma brucei MRE11 is non-essential but influences growth, homologous recombination and DNA double-strand break repair. *Molecular and biochemical parasitology,* Vol.125, No.1-2, (December 2002), pp. 11-21, ISSN 0166-6851.

Taniguchi, T. & A. D. D'Andrea (2006). Molecular pathogenesis of Fanconi anemia: recent progress. *Blood,* Vol.107, No.11, (February 2006), pp. 4223-4233, ISSN 0006-4971.

Touzot, F., I. Callebaut, J. Soulier, L. Gaillard, C. Azerrad, A. Durandy, A. Fischer, J. P. de Villartay & P. Revy (2010). Function of Apollo (SNM1B) at telomere highlighted by a splice variant identified in a patient with Hoyeraal-Hreidarsson syndrome. *Proceedings of the National Academy of Sciences of the United States of America,* Vol.107, No.22, (May 2010), pp. 10097-10102, ISSN 1091-6490.

van Overbeek, M. & T. de Lange (2006). Apollo, an Artemis-related nuclease, interacts with TRF2 and protects human telomeres in S phase. *Current biology,* Vol.16, No.13, (May 2006), pp. 1295-1302, ISSN 0960-9822.

Vilenchik, M. M. & A. G. Knudson (2003). Endogenous DNA double-strand breaks: production, fidelity of repair, and induction of cancer. *Proceedings of the National Academy of Sciences of the United States of America,* Vol.100, No.22, (October 2003), pp. 12871-12876, ISSN 0027-8424.

Wang, J., J. M. Pluth, P. K. Cooper, M. J. Cowan, D. J. Chen & S. M. Yannone (2005). Artemis deficiency confers a DNA double-strand break repair defect and Artemis phosphorylation status is altered by DNA damage and cell cycle progression. *DNA repair,* Vol.4, No.5, (April 2005), pp. 556-570, ISSN 1568-7864.

Wang, Z., W. Fast, A. M. Valentine & S. J. Benkovic (1999). Metallo-beta-lactamase: structure and mechanism. *Current opinion in chemical biology,* Vol.3, No.5, (October 1999), pp. 614-622, ISSN 1367-5931.

Williams, G. J., R. S. Williams, J. S. Williams, G. Moncalian, A. S. Arvai, O. Limbo, G. Guenther, S. Sildas, M. Hammel, P. Russell & J. A. Tainer (2011). ABC ATPase signature helices in Rad50 link nucleotide state to Mre11 interface for DNA repair.

Nature structural & molecular biology, Vol.18, No.4, (March 2011), pp. 423-431, ISSN 1545-9985.

Williams, R. S., G. Moncalian, J. S. Williams, Y. Yamada, O. Limbo, D. S. Shin, L. M. Groocock, D. Cahill, C. Hitomi, G. Guenther, D. Moiani, J. P. Carney, P. Russell & J. A. Tainer (2008). Mre11 dimers coordinate DNA end bridging and nuclease processing in double-strand-break repair. *Cell,* Vol.135, No.1, (October 2008), pp. 97-109, ISSN 1097-4172.

Williams, R. S., J. S. Williams & J. A. Tainer (2007). Mre11-Rad50-Nbs1 is a keystone complex connecting DNA repair machinery, double-strand break signaling, and the chromatin template. *Biochem Cell Biol,* Vol.85, No.4, (August 2007), pp. 509-520, ISSN 0829-8211.

Wolter, R., W. Siede & M. Brendel (1996). Regulation of SNM1, an inducible Saccharomyces cerevisiae gene required for repair of DNA cross-links. *Molecular & general genetics : MGG,* Vol.250, No.2, (February 1996), pp. 162-168, ISSN 0026-8925.

Yamaguchi-Iwai, Y., E. Sonoda, M. S. Sasaki, C. Morrison, T. Haraguchi, Y. Hiraoka, Y. M. Yamashita, T. Yagi, M. Takata, C. Price, N. Kakazu & S. Takeda (1999). Mre11 is essential for the maintenance of chromosomal DNA in vertebrate cells. *The EMBO journal,* Vol.18, No.23, (December 1999), pp. 6619-6629, ISSN 0261-4189.

Yang, W. (2010). Nucleases: diversity of structure, function and mechanism. *Q Rev Biophys,* (September 2010), pp. 1-93, ISSN 1469-8994.

Yannone, S. M., I. S. Khan, R. Z. Zhou, T. Zhou, K. Valerie & L. F. Povirk (2008). Coordinate 5' and 3' endonucleolytic trimming of terminally blocked blunt DNA double-strand break ends by Artemis nuclease and DNA-dependent protein kinase. *Nucleic acids research,* Vol.36, No.10, (April 2008), pp. 3354-3365, 1362-4962.

Zhu, Z., W. H. Chung, E. Y. Shim, S. E. Lee & G. Ira (2008). Sgs1 helicase and two nucleases Dna2 and Exo1 resect DNA double-strand break ends. *Cell,* Vol.134, No.6, (2008/09/23), pp. 981-994, ISSN 1097-4172.

12

Mammalian Spermatogenesis, DNA Repair, Poly(ADP-ribose) Turnover: the State of the Art

Maria Rosaria Faraone Mennella
Department of Structural & Functional Biology,
University of Naples "FedericoII"
Italy

1. Introduction

In the last decade an extensive and exhaustive research has focused on morphological and physiological features of mammalian male germ cell development defining a strict correlation between structural and molecular changes occurring from spermatogonia to spermatids and spermatozoa (Hermo et al., 2010 a-e; Inselman et al., 2003). The proliferation and meiotic phases whereby spermatogonia undergo several mitotic divisions to form spermatocytes and then haploid spermatids through two further meioses, have been studied in detail and the morphological, structural and functional features that are common to all generations of germ cells, elucidated (Hermo et al., 2010 a-e). This review will discuss only of those general features strictly related to the molecular events to be treated and will focus on the cell development arising from the dramatic changes in chromatin density and composition taking place during the differentiation process up to the late stages of spermatid maturation (spermiogenesis). The progression from diploid spermatogonia to haploid spermatozoa involves stage- and testis-specific gene expression, mitotic and meiotic division, and the histone-protamine changes (Grimes & Smart, 1985; Inselman et al., 2003; Meistrich et al., 1992, 2003). Alterations in DNA topology that occur in this process require both an exchange of histones to transition proteins and then to protamines, and the formation of DNA strand breaks (Aitken, 2009; Marcon & Boissonneault, 2004; Meistrich et al., 2003; Sakkas et al., 1995, 2010). The high frequency of DNA strand breaks during spermatogenesis needs a finely regulated DNA repair process, involving poly(ADP-ribosyl)ation of proteins among other mechanisms (Aitken & De Juliis, 2010; Meyer-Ficca et al., 2009) as the function of specific histone modifications, chromatin modifiers, DNA repair, DNA methylation, also the knowledge of the meaning of germ cell protein poly-ADP-ribosylation and of its relationship with DNA repair has made a great progress and here will be summarized and discussed.

2. Mammalian spermatogenesis

Mammalian spermatogenesis is an ordered and well-defined process occurring in seminiferous tubules of the testis. It is characterized by mitotical spermatogonia divisions to produce spermatocytes that proceed through meiosis to form a population of haploid cells (spermatids) over a period of several weeks (Hermo et al., 2010 a,b). The three specific

functional phases, proliferation, meiosis, and differentiation of spermatogenesis, involve three different germ cell populations, spermatogonia, spermatocytes, and spermatids. At different steps of development germ cells form various cellular patterns or stages, with 6, 12, and 14 specific stages in various mammals as human, mouse, and rat, respectively. These stages form a cycle of the seminiferous epithelium with a temporarly defined period for a given species (Hermo et al., 2010 a,b).

In the rat, the different classes of spermatogonia are dependent on a specific microenvironment contributed by Sertoli, myoid, and Leydig cells for proper development (Hermo et al., 2010 a, b).

In particular Sertoli cells provide the physical support, nutrients and hormonal signals to get a correct spermatogenesis, thus controlling germ cell proliferation. In the testis, cell adhesion and junctional molecules permit specific interactions and intracellular communication between germ and Sertoli cells. Germ cells are linked to one another by large intercellular bridges which serve to move molecules and even large organelles from the cytoplasm of one cell to another (Hermo et al., 2010 a, b).

With meiosis, spermatocytes go through chromosomal pairing, synapsis, and genetic exchange to be transformed into haploid cells. The synaptonemal complex and sex body are specific structural entities of the meiotic cells (Hermo et al., 2010 a).

Spermiogenesis is the haploid phase of spermatogenesis transforming spermatids into spermatozoa (Hermo et al., 2010 a,b). During this phase of germ cell development, spermatids undergo striking morphological transformations leading to the formation of highly specialized spermatozoa. It is a long process subdivided into distinct steps with 19 being identified in rats, 16 in mouse and 8 in humans. Spermiogenesis extends over 22.7 days in rats and 21.6 days in humans. Several structural and functional key events take place during the development of spermatids. During early spermiogenesis, morphological changes are evident: the Golgi apparatus turns into the acrosome, the endoplasmic reticulum forms the radial body and the annulate lamellae; mitochondria change shape, features and arrangement of location whithin cells; the chromatoid body develops, the shape of the spermatid head is structurally remodelled in a species-specific manner, and the nuclear chromatin becomes compacted to accommodate the fiber-shaped sperm head. Microtubules are described as forming a curtain to maintain sperm head shape and trafficking of proteins in the spermatid cytoplasm (Fouquet et al., 2000; Hermo et al., 2010 c, e).

At molecular level, during spermatogenesis, germ cells express many proteins involved in balance of water, pH, ion transport, etc.. In the nucleus, germ cells contain specialized transcription complexes able to perform the differentiation program of spermatogenesis, with cell-specific differences in the components of this machinery (Hermo et al., 2010, d-e). In mouse spermatocytes expression and localization of proteins critical to events of the meiotic cell division occur, with a temporal order for chromosomal pairing and recombination proteins, kinases and substrates that mediate the cell cycle transition (Inselman et al., 2003). Distinct and protein-specific patterns occur with respect to expression and localization throughout meiotic prophase and division and dramatic relocalization of proteins occurs as spermatocytes enter the meiotic division phase. Such a framework can clarify mechanisms of normal meiosis as well as mutant phenotypes and aberrations of the meiotic process (Inselman et al., 2003; Li et al., 2008; Zhao et al., 2004). Classifying proteins of spermatogenic cells with a view of their functions, and their applications in the regulation of fertility has made it possible to understand the molecular

biology of male gametogenesis in great detail, with the description of specialized proteins, which are dominantly and/or specifically expressed in germ cells and localized in spermatozoa (Gupta, 2005).

At certain periods before and during meiosis, one of the most conspicuous changes involves remodelling of the nucleosomal chromatin into a highly condensed chromatin (Figure 1). The structural reorganization and packaging of the DNA is concomitant with two sequential replacements of spermatid-specific basic nuclear proteins (Oko et al., 1996).

Mammalian, expecially rat spermatogenesis involves a progressive and transient replacement of the classic histones by arginine-rich proteins (Kistler, et al., 1996; Meistrich et al., 1992, 2003). H1t, the testis-specific linker histone, appears in germ cells during the meiotic prophase of mammalian spermatogenesis, when the other variants have already disappeared or are present in traces, except H1a, which is the most abundant somatic subtype in rat testis proteins (Kistler et al., 1996; Meistrich et al., 1992, 2003).

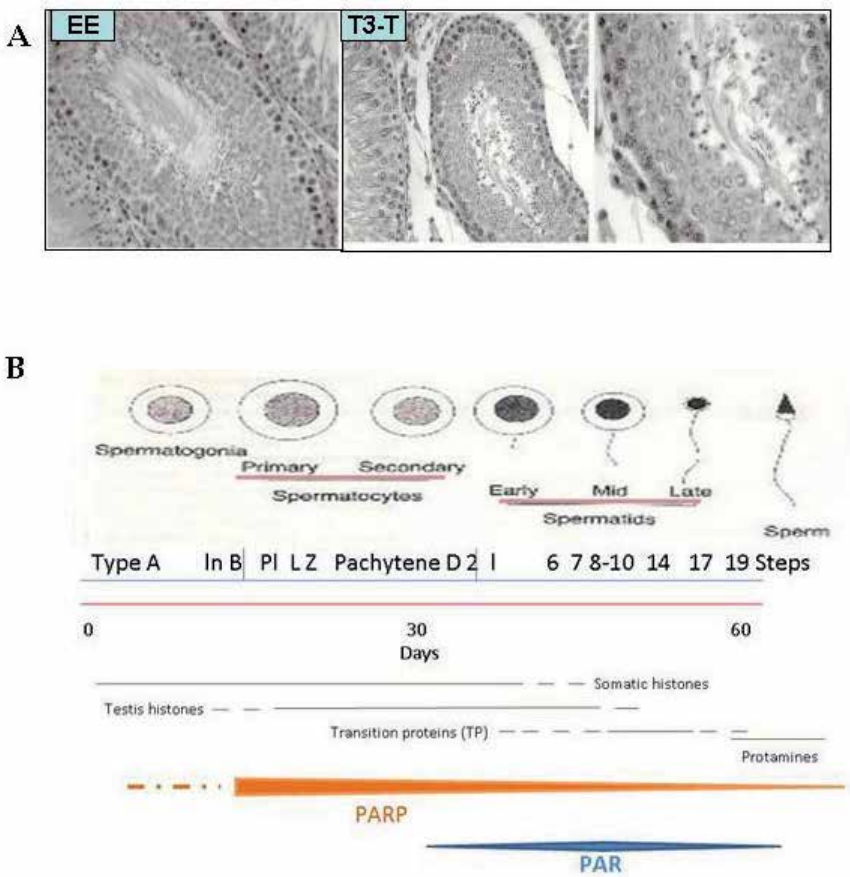

Fig. 1. Rat testis spermatogenesis. A) Optic microscopy of testis sections from euthyroid (EE, control) and hyperthyroid (T3-T) rats. T3-T rats were treated three weeks with dayly administration of thriiodothyronine (Faraone Mennella et al., 2005a). Hormonal stimulus affected normal germ cell differentiation, by reducing spermatozoa in the tubule lumen. B) PARPactivity, PAR, and nuclear proteins during male rat germ cell differentiation.

The transition proteins replace histones during the initial stages of chromatin condensation of spermiogenesis and are later replaced by protamines, which are the only basic nuclear structural proteins in the sperm of most mammals (Dadoune, 2003; Meistrich et al., 1992; Ullas & Rao, 2003). The transition proteins, including the TP family, presumably mediate the replacement of histones by protamines (Kistler et al., 1996; Meistrich et al., 1992; Ullas & Rao, 2003).

The sequential synthesis and replacement of histones and testis specific proteins with protamines must be highly regulated in order to produce large number of spermatozoa with intact and competent DNA. Epigenetic regulation of gene expression and nucleoprotein transition is critical during spermatogenesis. In germ cell nucleus, epigenetic regulation include protein modifications methylation, acetylation, phosphorylation, ubiquitination, poly(ADP-ribosyl)ation, each signaling changes in chromatin structure (Carrel et al., 2007; Godmann et al., 2009; Hermo et al., 2010, d-e; Ullas & Rao, 2003; Yu et al., 2000; Zamudio et al., 2008).

3. Chromatin remodelling and the role(s) of DNA repair during spermatogenesis

Gene expression and other DNA metabolic events involving chromatin are organized specifically within the space of the cell nucleus and are related to nuclear architecture. Local chromatin structures are devoted to maintain genes in an active or silenced configuration, to accommodate DNA replication, chromosome pairing and segregation, and to maintain telomeric integrity. All these processes are highly regulated by chromatin remodelling (Ehrenhofer-Murray, 2004; Falbo, 2006; McNairn, 2003; Morrison, 2004; Phillips & Shaw, 2008; Saha et al., 2006).

Related to chromatin remodelling a large number of modifications are known as signals for the binding of specific proteins and many of them are associated with distinct patterns of gene expression, DNA repair, or replication (Deal et al., 2010; Rajapakse et al., 2010; Talbert & Henikoff, 2010).

Spermatogenesis provides an excellent example of roles for histone variants, post-translational modifications of histone and non-histone proteins, specifically poly (ADP-ribosyl)ation in regulating chromatin structure and function (Faraone Mennella et al., 1999; Govin et al., 2004; Grimes and Smart, 1985; Meyer-Ficca et al., 2005; Nair et al., 2008; Ullas & Rao, 2003).

Histone variants are expecially prevalent during the development of germ cells and some of them play a role to compact DNA less tightly to facilitate rapid nuclear division, DNA replication and access to trans-acting factors (De Lucia et al., 1994; Faraone Mennella et al., 1999; Lewis et al., 2003).

The dual role for H1 in chromatin structure and gene regulation defines different heritable epigenetic states of gene activity which are maintained through mechanisms independent of gene sequence (Zamudio et al., 2008). Linker histone H1 exerts synergistic effects by modulating modifications of core histones either in the presence or absence of its own modification in man and mouse (Yan et al., 2003).

The best example of reversible compaction of DNA by multiple pathways concerns the condensation of DNA into sperm nuclei during spermiogenesis (Govin et al., 2004; Laberge et al., 2005 a,b). Chromatin remodelling is a major event that occurs during mammalian spermiogenesis. Nuclear condensation during germ cell differentiation is accomplished by

replacing somatic histones (linker and core histones) and the testis-specific H1t with transition proteins and, finally, with protamines, Figure 1 (Green et al., 1994; Grimes & Smart, 1985; Meistrich et al., 1992). The transition proteins, the TP family, are mediators in the replacement of histones by protamines (Green et al., 1994; Grimes & Smart, 1985; Meistrich et al., 1992).

Transition proteins and the tail regions of histones are sites of post-translational covalent modifications (Pirhonen et al., 1994; Ullas & Rao, 2003).

Methylation of position-specific lysine residues in the histone H3 and H4 amino-termini has linked with the formation of constitutive and facultative heterochromatin as well as with specifically repressed single gene loci (Cremer et al., 2004). Furthermore ubiquitylation of H2B might be involved in double strand break formation during meiosis (Agarwal et al., 2009).

Core histone acetylation occurs during the late stage of spermatogenesis in several organisms, allowing the removal of histones and their replacement by protamines (Grimes & Smart, 1985). Acetylation of rat testis H3, H4 and of testis histone variants TH2B and TH3 were observed in pachytene spermatocytes and round spermatids, in line with the hypothised roles of acetylation in the deposition of histones onto DNA (early spermatogenesis) and replacement of histones by protamines in spermiogenesis (Grimes & Smart, 1985). In general core histone acetylation has important consequences for the organization of DNA in a nucleosome, loosening interactions at the periphery of he structure. In fact it has been reported that histone acetylation leads to a substantial decrease in nucleosome rigidity. Concurrent with histone acetylation are other post – translational modifications. Simbulan-Rosenthal et al. (1998) reported that acetylated core histones may also be subjected to (ADP-ribosyl)ation. Boulikas et al. (1992) got evidence that acetylated H4 subspecies are predominantly tri- and tetra- (ADP-ribosyl)ated. He proposed that DNA strand breaks induce the formation of poly(ADP-ribosyl)ated species of histones, mainly H1, whereas in the absence of DNA strand breaks histones are mono- and oligo-(ADP-ribosyl)ated. This author hypothesized that newly synthesized core histones may be reversibly oligo(ADP-ribosyl)ated in order to facilitate their assembly into histone complexes and their deposition onto DNA at replication fork, and suggested that the observed simultaneous occurrence of acetylation and oligo (ADP-ribosyl)ation correlates with changes in chromatin structure.

The change in germ cell chromatin architecture requires a global but transient appearance of endogenous stage-specific DNA strand breaks (Laberge and Boissonneault, 2005 b; Leduc et al., 2008, a,b).

Controlling genome integrity is essential to guarantee the fidelity of DNA inheritance. Therefore, maintaining the integrity of sperm DNA is vital to reproduction and male fertility. Sperm contain a number of molecules and pathways for the repair of base excision, base mismatches and DNA strand breaks (Leduc et al., 2008, a,b).

In the mouse, elevated and global increase in DNA strand breaks levels are present in nuclei of round-shaped spermatids when chromatin starts to re-organize.

DNA strand breaks are also detected in the whole population of elongating spermatids (stages IX-XI) of the mouse seminiferous epithelium, coincident with histone H4 hyperacetylation during chromatin remodelling (Marcon & Boissonault, 2004).

In addition to the nuclear protein exchange, the chromatin remodelling process leading to the precise packaging of the paternal genome during spermiogenesis, involves the elimination of the free DNA supercoils created by the nucleosome removal. To reduce the

torsional stress induced by change in DNA topology, DNA strand breaks provide the swivel effect, with the contribution of topoisomerases, to both create and seal DNA nicks, and providing the controlled increase of linking number to relax DNA (Boissonault, 2002). The origin of the transient increase in DNA strand breaks would require an endogenous nuclease activity present up to the late spermiogenesis steps (Boaz et al., 2008). Topoisomerase II may play such a role being able to both create and ligate the DNA nicks during spermiogenesis (Boissonault, 2002; Chen and Longo, 1996; McPherson et al., 1993; Roca &Mezquita, 1989; Shaman et al., 2006; Yamauchi et al., 2007).

Topoisomerase II beta (TOP2B) is the type II topoisomerase present in elongating spermatids between steps 9 and 13, co-exhisting with tyrosyl-DNA phosphodiesterase 1 (TDP1), an enzyme known to resolve topoisomerase-mediated DNA damage, and gamma-H2AX (also known as H2AFX), triggered as a DNA damage response (Boissonault, 2002; Shaman et al., 2006; Yamauchi et al., 2007).

During the normal developmental program of the spermatids, dramatic consequences for the genomic integrity of the developing male gamete may arise from any unresolved double-strand breaks resulting from a failure in the rejoining process of TOP2B (Leduc et al., 2008, a,b).

A correct DNA condensing process is likely to play a key role in the elimination of the strand breaks since DNA breaks appear transiently and are no more present once the nuclear protein transition is completed. A current hypothesis links the DNA condensation process (from the transition proteins to the protamines) with the repair of the DNA strand breaks. An altered sperm chromatin packaging was already correlated with an increase in DNA fragmentation in the mature sperm. In addition, underprotamination seems to be related with DNA nicking, and the transition protein 1 (TP1) stimulates in vitro the repair of a nicked circular plasmid, whereas TP1, TP2 and protamines stimulate oligomerization of short DNA fragments in the presence of T4 DNA ligase (Adham et al., 2001; Carrell et al., 2007; Kierszenbaum, 2001; Leduc et al., 2008 a; Zhao et al., 2001). . The transition proteins or protamines would therefore act as `alignment factors by bridging the free DNA ends created at the break point.

Most of DNA damage in midspermatogenesis is attributed to physiological apoptosis of germ cells (Leduc et al., 2008, a,b; Sinha Hikim et al., 2003).

Apoptosis regulates germ cell over proliferation and eliminates defective germ cells. It is a normal event and occurs to select only high quality germ cells. Uncorrect cells do not achieve maturity; they undergo spontaneous cell death through apoptosis. In somatic cells, the apoptotic cascade involves the formation of apoptotic body; however, in highly differentiated spermatozoa, the sequence of events may differ as a result of the highly condensed sperm nucleus (Leduc et al., 2008, a,b; Sinha Hikim et al., 2003). In adult rat, most apoptotic cells are among spermatogonia (75%) and occur to a lesser extent during maturation divisions of spermatocyte and spermatid development. Increase of apoptotic germ cell death can be triggered by various regulatory stimuli, including deprivation of gonadotropins and intratesticular testosterone by GnRH antagonist, or by hormone treatment (Figure 1A), exposure to local testicular heating, Sertoli cell toxicants, and chemotherapeutic agents (Faraone Mennella et al., 2005a, 2009; Leduc et al., 2008, a,b; Sinha Hikim et al., 2003).

Recent studies have demonstrated that both spontaneous and increased apoptotic programmed cell death in abnormal spermatogenesis play a main role in male fertility (de Boer et al., 2010). Appropriate epigenetic regulation is needed throughout all phases of

spermatogenesis for imprinting, chromatin remodelling, the histone–protamine transition, etc.. Strikingly, aberrant epigenetic profiles, in the form of anomalous DNA and histone modifications, are characteristic of cancerous testis cells. Germ cell development is a critical period during which epigenetic patterns are established and maintained (de Boer et al., 2010).

Some questions about epigenetic modifications regulating these events are still unanswered, as the exact functions, the impact and the order of occurrence of the epigenetic modifications associated with spermatogenesis. Environmental factors may influence the epigenetic state that may be inherited through the male germ line and passed onto more than one generation (Agarwal et al., 2009; de Boer et al., 2010; Godmann et al., 2009; Patrizio et al., 2008).

Origin of DNA damage in human spermatozoa can occur by abortive apoptosis, abnormal chromatin packaging, generation of reactive oxygen species and premature release from Sertoli cells (Leduc et al., 2008 b). For a hypothesis explaining experimental data, de Boer and co-workers (2010) propose that regulation of chromosome structure in the germline, by the occupancy of matrix/scaffold associated regions, contains molecular memory function. The male germline is strikingly dynamic as to chromatin organization. To be installed, such memory requires both S-phase and chromatin reorganization during spermatogenesis. and in the zygote, that likely also involves reorganization of loop domains, where replication occurs.

The authors underline that nuclear structure, chromatin composition and loop domain organization are aspects of human sperm variability that in many cases of assisted reproduction is increased due to inclusion of more incompletely differentiated/maturated sperm nuclei (de Boer et al., 2010).

New work on the function of specific histone modifications, chromatin modifiers, DNA methylation, and the impact of the environment on developing sperm suggests that the correct setting of the epigenome is required for male reproductive health and the prevention of paternal disease transmission (de Boer et al., 2010).

It is clear from the above data that programmed DNA fragmentation and DNA damage response take place during the chromatin remodeling steps in spermatids and are not necessarily synonymous with apoptotic degeneration. Chromatin-remodeling steps in spermatids may be intrinsically mutagenic and is an important source of genetic instability, that can be further enhanced by internal and external factors (De Iuliis et al., 2010; Sakkas & Alvarez, 2010).

4. Poly-ADP-ribosylation in mammalian spermatogenesis

4.1 The scenario of poly(ADP-ribose)polymerases

The modification of proteins by ADP-ribose polymers (PAR) is a reversibile process in which the synthesis of PAR from NAD^+ is catalyzed by poly(ADP-ribose) polymerases (PARPs) and polymer catabolism is due to poly(ADP-ribose) glycohydrolase (PARG) and ADPR-protein lyase (D'Amours et al., 1999; Faraone Mennella, 2005; Hassa & Hottiger, 2008). The PARP family has eighteen members that share the highly conserved PARP catalytic domain, but vary widely in other parts of the proteins (Hottiger et al., 2010). The different PARPs are grouped in subfamilies and are involved in various events mediated by their variable domain structures. Hassa and Hottiger (2008), on the basis of PARPs catalytic domain sequences have identified 3 separate groups, but other classifications can be made

on their different subcellular localization patterns, or on different composition in functional domains (ankyrin repeats, CCCH-, WWE- and macro-domain, etc) and precise functions (regulation of vault proteins, telomere length, DNA protection, etc.) (Citarelli et al., 2010: Hottiger et al., 2010; Otto et al., 2005). More recently it has been found that proteins within the PARP superfamily have altered catalytic sites, and have mono(ADP-ribose) transferase (mART) activity or are enzymatically inactive. These findings suggest that the PARP catalitycally active region has a broader range of functions than initially predicted. Human PARP10 has transferase activity rather than polymerase activity, and enzymes where the catalytically important residues are present, may not act as PARPs. For example, human PARP3 has been reported to act in poly(ADP-ribosyl)ation (Augustin et al., 2003), and mono(ADP-ribosyl)ation (Loseva et al., 2010).

Despite of these recent findings, poly(ADP-ribosyl)ation is the second very important post-translational modification which mostly affects different nuclear acceptor proteins. It is involved in the regulation of several cellular functions related to the maintenance of genomic integrity (DNA repair, gene amplification, apoptosis) and to the expression and propagation of the genetic information (DNA transcription and replication, differentiation, neoplastic transformation) (Hassa & Hottiger, 2008). The synthesis of PAR is an immediate response to DNA damage and is the first step in a cascade of events leading to either DNA repair or apoptosis (Burkle, 2001; Malanga & Althaus, 2005). PARP-1 and PARP-2 are so far the only PARP enzymes whose catalytic activity has been shown to be induced by DNA-strand breaks, providing strong support for sharing key functions in the cellular response to DNA damage. Recent data suggest unique functions for PARP-2 in specific processes, such as genome surveillance, spermatogenesis, adipogenesis and T cell development (Yélamos et al., 2008).

4.2 PAR turnover and spermatogenesis

Since the early discovery in the '80-'90, that mammalian testes are enriched of PARP (Concha, 1989; Corominas & Mezquita, 1985; Farina et al., 1979 a, b), and the identification of in vitro and in vivo poly(ADP-ribosyl)ated testis-specific proteins (Corominas & Mezquita, 1985; Faraone Mennella et al., 1982, 1984, 1988, 1999), it was clear that this reaction is a metabolic event highly involved in mammalian male germ cell differentiation. At that time the presence of more than the 116kDa PARP was unconceivable, being the second enzyme, PARP2, discovered at the end of '90s (Ame' et al., 1999; Babiychuck et al, 1998). In rat testis most PARP activity was found in isolated seminiferous tubules (Quesada et al, 1989) and among linker histone variants, the rat testis specific H1t was preferentially modified with poly(ADP-ribose) (Faraone Mennella et al., 1999; Malanga et al., 1998).

In a study with differently-aged rats, it was found that in isolated intact nuclei of testis from 8-day-old animals (only spermatogonia present in seminiferous tubules), poly(ADP-ribosylation) of nuclear proteins was very low, increased significantly by 16-day (pachytene spermatocytes appear) and reached adult proportions by 32 days (condensing spermatids present), Figure 1B (Quesada et al., 1989). It was concluded that poly(ADP-ribosylation) of nuclear proteins in rat testis is closely correlated with spermatogenesis and was inferred that it is particularly active in the early stages of meiosis, where DNA breaks are frequently produced during DNA replication and transcription. The subcellular distribution of both PARP and Poly(ADPR)glycohydrolase (PARG) was also determined after separation of different germ cell populations, and the results showed that the maximum of both PARP

amount and PARP activity can be detected on tetraploid spermatocytes which undergo meiotic division, whereas PARG activity does not differ in germinal cells (Di Meglio et al., 2003). The authors concluded that regulation of PAR turnover, variations of PARP amount, as well as changes of PARP transcription level, accompany germinal cell differentiation, possibly being implicated in DNA replication, repair and other related events (Quesada et al., 2003; Di Meglio et al., 2003).

The advance in the knowledge of poly(ADP-ribosyl)ation reaction and the discovery of a number of enzymes defined as PARP family led to a great progress of research on PARPs and spermatogenesis (Ame' et al., 2004). Among PARP family members, PARP1 and PARP2 are the two enzymes demonstrated to be directly involved in base excision DNA repair, the former being modulated by PARP3, described as a newcomer in genome integrity and mitotic progression as it is stimulated by DNA double-strand breaks (Rulten et al., 2011; Boehler et al., 2011).

In a study by Tramontano et al. (2007) examining rat primary spermatocytes it was found that both PARP1 and PARP2 are present in these germ cells. However, the vast majority of PAR in these rat primary spermatocytes is produced by PARP1 suggesting possibly different roles of PARP1 and PARP2 in spermatogenesis.

Meyer–Ficca et al. (2005) showed for the first time that poly(ADP-ribose) formation, mediated by poly(ADP-ribose) polymerases (PARP-1 and PARP-2), occurs in spermatids of steps 11-14, steps that immediately precede the most pronounced phase of chromatin condensation in spermiogenesis. High levels of ADP-ribose polymer were observed in spermatid steps 12-13 in which the highest rates of chromatin nucleoprotein exchanges take place. They also detected gamma-H2AX, the histone variant indicating the presence of DNA double-strand breaks during the same step, and hypothesize that transient ADP-ribose polymer formation may facilitate DNA strand break management during the chromatin remodeling steps of sperm cell maturation.

Interestingly, other authors provided *in vivo* evidence for the pleiotropic involvement of Parp-2 in both meiotic and postmeiotic processes (Dantzer et al., 2006). They showed that Parp-2-deficient mice exhibit severely impaired spermatogenesis, with a defect in prophase of meiosis I characterized by massive apoptosis at pachytene and metaphase I stages. Although Parp-2$^{-/-}$ spermatocytes exhibit normal telomere dynamics and normal chromosome synapsis, they display defective meiotic sex chromosome inactivation associated with dis-regulation of histone acetylation and methylation and up-regulated X- and Y-linked gene expression. These findings give evidence that chromatin remodeling steps during spermiogenesis trigger poly(ADP-ribose) formation. Knockout mice deficient in PARP1, PARG (110-kDa isoform), or both display morphological and functional sperm abnormalities that are dependent on the individual genotypes, including residual DNA strand breaks associated with varying degrees of subfertility. The data presented highlighted the importance of PAR metabolism, particularly PARG function, as a prerequisite of proper sperm chromatin quality. PARG is involved in DNA repair by regulating the amount of PAR synthesized in response to DNA damage since excessive accumulation of PAR may result in cell death (Meyer-Ficca, 2009).

*I*n vivo evidence showed that Parp-2$^{-/-}$ spermatids are severely compromised in differentiation and exhibit a marked delay in nuclear elongation (Dantzer et al., 2006).

Altogether, in addition to its well known role in DNA repair, Parp-2 exerts essential functions during meiosis I and haploid gamete differentiation (Dantzer et al., 2006).

The activity of PARP during chromatin remodeling steps of spermatogenesis in terms of repairing double stranded breaks and the poly (ADP-ribosyl)ation of histones, is critical and disregulation of the chromatin remodeling steps of spermiogenesis could have serious consequences for the male gamete. Meyer-Ficca et al (2005) demonstrated the presence of poly (ADP-ribose) in elongated spermatids of rat. They showed that during these steps when a high number of DNA breaks occur directly preceding nuclear condensation, there is correspondingly a higher amount of PAR in rat germ cells. Greater PAR formation through PARP1 and PARP2 action occurs during this phase of spermatogenesis that includes a great deal of chromatin condensation (steps 11-14 of rat spermatogenesis); PAR levels decrease only when protamines appear in the chromatin (Meyer-Ficca et al., 2010). Thus, PAR formation could be important for repairing DNA strand breaks during these crucial chromatin remodeling steps of spermatogenesis. Furthermore, PAR formation could also be important for histone modification because not only is there auto-modification of PARP during spermatogenesis, but much of PARP activity is targeted towards the testes-specific histone, H1t (Agarwal et al., 2009; Malanga et al., 1998).

The presence of poly (ADP-ribose) polymerase and its homologues has been shown specifically during stage VII of human spermatogenesis. High PARP expression has been reported in mature spermatozoa of proven fertile men (Agarwal et al., 2009).

In a recent study, using human testicular samples, the strongest levels of PARP1 were found in spermatogonia. Presence of poly (ADP-ribose) differed slightly with the stage of spermatogenesis. Poly (ADP-ribosyl)ation was strongest in human round and elongating spermatids as well as in a subpopulation of primary spermatocytes. In contrast, mature spermatids had no PARP expression or poly (ADP-ribosyl)ation (Agarwal et al., 2009).

Origin of DNA damage in human spermatozoa can occur by abortive apoptosis, abnormal chromatin packaging, generation of reactive oxygen species, hormone stimuli, all events involving in some way PARP and its reaction (Godman, et al., 2009; Maymon et al., 2006)

In human testis, an increase in DNA strand breaks occurs in 100% of elongating spermatids becoming critical for human fertility (Agarwal et al., 2009). Focus on genomic integrity of the male gametes has increased to relate DNA integrity in mature ejaculated spermatozoa and male infertility with a growing concern about the role of PARP as a DNA damage repair protein (Agarwal et al., 2009).

4.3 PARP and epigenetic state in spermiogenesis

It is widely recognized that environmental factors may influence the epigenetic state and that these epigenetic modifications may be inherited through the male germ line and passed onto more than one generation (Godman et al., 2009).

Since genomic stability of cells is linked to their poly(ADP-ribosyl)ation capacity, the patterns of poly(ADP-ribosyl)ation during human spermatogenesis were studied (Maymon et al., 2006). By testicular biopsy immunohistochemistry evaluation of PARP-1 expression and of poly(ADP-ribose), the detection of PAR expression in germ-line cells and its subcellular localization in meiotic and postmeiotic prophases were demonstrated to link with chromatin modifications occurring during spermatogenesis and confirmed a key role for poly(ADP-ribosyl)ation in germ cell differentiation, to preserve DNA integrity.

Deduction of a mechanism in male transmission is difficult because of the specialized nature of the sperm cell, which requires very compact chromatin to enable transport and protect DNA against oxidative stress (Aitken & De Iuliis, 2009).

In a recent study by Jha et al. (2009) several isoforms of PARP were detected in ejaculated spermatozoa including PARP1, PARP2, and PARP9. Immunolocalization patterns showed that PARP was found near the acrosomal regions in sperm heads. Furthermore, a direct correlation was seen between sperm maturity and the presence of PARP, i.e., an increased presence of PARP1, PARP2, and PARP9 was seen in mature sperm when compared to immature sperm.

Inside seminiferous tubules hyperactivation of PARP and its cleavage accompany the morphological and functional changes induced by apoptotic stimuli (hormonal, oxidative, chemical , etc.) (Atorino et al., 2001; Boissonault et al., 2002; Faraone Mennella et al., 2009; Sinha-Hikkim et al., 2003). In human sperm, in the presence of a PARP inhibitor, 3-aminobenzamide, chemical and oxidative stress-induced apoptosis was reported to increase by nearly two-fold (Argawal et al., 2009). This novel finding suggests that PARP could play an important role in protecting spermatozoa subjected to oxidative and chemical damage (Argawal et al., 2009).

An age-related increase in DNA break repair and apoptosis was also demonstrated in human testicular germ cells. DNA repair markers (PARP-1, PAR, XRCC1, and apoptosis-associated markers (caspase 9, active caspase 3, and cleaved PARP-1) were detected in these cells (El-Domyati et al., 2009).

In summary, DNA damage in spermatozoa can be induced by events involving PARP as regulatory factor and occurring within the testis as apoptosis, and remodelling of sperm chromatin during the process of spermiogenesis, or in the post-testicular phase as induced mainly by radical oxygen species (ROS) and nitric oxide (NO), or by endogenous caspases and endonucleases; or by environmental factors.

To the latter refers epigenetic transmission of information from one generation to the next during chromatin replication in combination with posttranslational histone modification (the histone code) as demonstrated in parental imprinting (de Boer et al., 2010).

An epigenetic memory for male genetic transmission resides in a link between DNA replication and matrix associated DNA repair (Hatch et al., 2007).

Some authors propose that regulation of chromosome structure in the germline, by the occupancy of matrix/scaffold associated regions, contains molecular memory function. Nuclear structure, chromatin composition and loop domain organization are aspects of human sperm variability that in many cases of assisted reproduction is increased due to inclusion of more incompletely differentiated/maturated sperm nuclei.

The association of DNA repair proteins with the nuclear matrix has been demonstrated by a number of authors, as well as topoisomerase II species constitute a significant component of the nuclear matrix (Roca & Mezquita, 1989; Quesada et al., 2000).

For adaptation of loop domain structure during chromatin remodeling at spermatid nuclear elongation, the activity of TopoIIB is essential for removing supercoiling from nucleosomal DNA in transit to protamine toroid chromatin (Leduc et al., 2008 a,b). In rat testes there is evidence that some of PARP activity is associated to nuclear matrix, thus becoming one of those tightly bound components which are not solubilized from chromatin by high salt treatment. By the use of DNA and protein cross-linking reactions, more evidences were provided about the association of PARP-1, PARP-2, and PARPs related proteins with the nuclear matrix. These findings confirmed that nuclear matrix could be seen as a fraction greatly enriched in transcription factors (i.e., C/EBP-beta) and enzymes (DNA Topo II, DNA PK) that co-localize with PARP-1 and -2 at the matrix associated regions (MARs) of chromatin. Moreover, PAR contributes to PARP-1 localization at the nuclear matrix,

showing that PARP(s) activity co-operates to the functions of this nuclear fraction. (Tramontano et al., 2005). Topo II is constantly present as a component of chromatin remodelling. As described in the previous section, alterations in DNA topology that occur in the extreme condensation of the spermatid nucleus have been shown to require the controlled formation of DNA strand breaks to allow the transition from a supercoiled form of DNA to a non-supercoiled form. Supercoiled DNA relaxes by transient formation of physiological strand breaks that spermatids, being haploid, cannot repair by homologous recombination. These DNA strand breaks trigger the activation of poly(ADP-ribose) polymerases PARP1 and PARP2 and any interference with PARP activation causes poor chromatin integrity with abnormal retention of histones in mature sperm and impaired embryonic survival (Meyer-Ficca et al., 2011). In this context, the activity of topoisomerase IIbeta (TOP2B), an enzyme involved in DNA strand break formation in elongating spermatids, is strongly inhibited by the activity of PARP1 and PARP2 in vitro and is restored by the PAR degrading activity of PAR glycohydrolase (PARG). Moreover, genetic and pharmacological PARP inhibition both led to increased TOP2B activity in murine spermatids in vivo, measured as covalent binding of TOP2B to the DNA (Meyer-Ficca et al., 2011). These data suggest a functional relationship between the DNA strand break generating activity of TOP2B and the DNA strand break-dependent activation of PARP enzymes which in turn inhibits TOP2B. Because PARP activity also facilitates histone H1 linker removal and local chromatin decondensation, cycles of PAR formation and degradation may be necessary to coordinate TOP2B dependent DNA relaxation with histone-to-protamine exchange necessary for spermatid chromatin remodelling (Meyer-Ficca et al., 2011). In the light of their own results and those from other groups, Meyer-Ficca et al. (2011) suggest that the activities of the DNA relaxing enzyme TOP2B, and the DNA strand break dependent enzymes PARP1 and PARP2 may be able to directly and dynamically regulate each other via the formation of DNA strand breaks and poly(ADP-ribose) to mediate simultaneous DNA relaxation and histone H1 removal as essential steps of spermatid chromatin remodeling necessary for sperm function (Meyer-Ficca, 2011).

5. Conclusions

In this review the possible biological significance of PARP in mammalian germ cells has been summarized focusing on the role played by PARP during spermatogenesis and sperm maturation, and on recent findings in ejaculated spermatozoa. It is widely demonstrated that molecular events leading to the high condensation of the spermatid nucleus, include an exchange of histones to transition proteins and then to protamines, that replace all other nuclear proteins in sperms, and alterations in DNA topology that require both the controlled formation of DNA strand breaks, and protein modifications, such as poly(ADP-ribosyl)ation. As discussed above, the sequential synthesis and replacement of histones and testis specific proteins with protamines found indeed in the poly(ADP-ribosyl)ation reaction a further regulatory process to control and to produce large number of spermatozoa with intact and competent DNA. Poly(ADP-ribosyl)ation involves the automodified PARP as the main actor in DNA protecting function, and the free enzyme as regulator of most of nuclear proteins demonstrated to be involved in chromatin remodelling, either as modifier enzyme or as a recruiter of partner proteins. PARP is activated whenever there are strand breaks in sperm DNA due to oxidative stress, chromatin remodeling or cell death. The fact that PARP and PAR localize at MARs, recognized as a site of "memory" for transmission of information from one generation to the next, gives further support to the role(s) of PARP in

essential steps of germ cell development. Male germ cells are exposed to a wide variety of endogenous and exogenous genotoxic agents, most of which involve PARP as a common player. Recent findings confirm the occurrence of PARP in ejaculated spermatozoa and the presence of higher levels of caspase 3-cleaved PARP in sperm of infertile men adds a new proof for the correlation between apoptosis and male infertility. In the light of these observations PARP can be regarded as an hallmark of the actual state of germ cells able either to counteract DNA damage or to give a signal of death upon high DNA abnormalities.

6. References

Adham, IM, Nayernia, K, Burkhardt-Gottges, E, Topaloglu, O, Dixkens, C, Holstein, AF, Engel, W. (2001). Teratozoospermia in mice lacking the transition protein 2 (Tnp2). *Mol Hum Reprod,* Vol. *7,* pp. 513–520

Agarwal, A, Mahfouz, RZ, Sharma, RK, Sarkar, O, Mangrola, D, Mathur, PP.(2009). Potential biological role of poly (ADP-ribose) polymerase (PARP) in male gametes. *Reprod Biol Endocrinol*. Vol.7 (Dec 5), pp. 143

Aitken, RJ, De Iuliis, GN, McLachlan, RI. (2009). Biological and clinical significance of DNA damage in the male germ line. *Int J Androl,* Vol. 32, pp.46-56

Aitken RJ, De Iuliis GN. (2010). On the possible origins of DNA damage in human spermatozoa. *Mol Hum Reprod*, Vol. 16, pp. 3-13

Amé, JC, Rolli, V, Schreiber, V, Niedergang, C, Apiou, F, Decker, P, Muller, S, Höger, T, Ménissier-de Murcia, J, de Murcia, G. (1999). PARP-2, a novel mammalian DNA-damage dependent PARP. *J Biol Chem,* Vol. 274, pp. 17860-17868

Ame', JC, Spenlehauer, C, de Murcia, G. (2004). The PARP superfamily. *BioEssays,* Vol. 26, pp.882–893

Atorino, L, Di Meglio, S, Farina, B, Jones, R, Quesada, P. (2001) Rat germinal cells require PARP for repair of DNA damage induced by gamma-irradiation and H2O2 treatment. *Eur J Cell Biol* Vol 80, pp.222–229

Augustin, A, Spenlehauer, C, Dumond, H, Menissier,-De Murcia, J, Piel, M, Schmit, AC, Apiou, F, Vonesch, JL, Kock, M, Bornens, M, De Murcia, G. (2003). PARP-3 localizes preferentially to the daughter centriole and interferes with the G1/S cell cycle progression. *J Cell Sci* Vol.116(Pt 8), pp.1551–1562

Babiychuck, E, Cottrill, PB, Storozhenko, S, Fuangthong, M, Chen, Y, O'Farrell, MK, Van Montagu, M, Inze', D, Kushnir, S.(1998). Higher plants possess two structuralkly different PARPs *Plant J*, Vol. 15, pp. 635-645

Boaz, SM, Dominguez, K, Shaman, JA, Ward, WS. (2008). Mouse spermatozoa contain a nuclease that is activated by pretreatment with EGTA and subsequent calcium incubation. *J Cell Biochem* Vol.103, pp.1636-1645

Boehler, C, Gauthier, LR, Mortusewicz, O, Biard, DS, Saliou, JM, Bresson, A, Sanglier-Cianferani, S, Smith, S, Schreiber, V, Boussin, F, Dantzer, F. (January 26, 2011). Poly(ADP-ribose) polymerase 3 (PARP3), a newcomer in cellular response to DNA damage and mitotic progression. *Proc Natl Acad Sci USA*, doi: 10.1073/pnas.1016574108

Boissonneault, G. (2002). Chromatin remodeling during spermiogenesis:a possible role for the transition proteins in DNA strand breakrepair. *FEBS Lett* Vol. 514, pp. 111–114

Boulikas, T. (1992). Poly(ADPR) synthesis in blocked and damaged cells and its relation to carcinogenesis. *Anticancer Res* Vol.12, pp.885–898

Burkle, A. (2001). PARP-1: a regulator of genomic stability linked with mammalian longevity. *ChemBioChem* Vol.2, pp.725–728

Carrell, DT, Emery, BR, Hammoud, S.(2007). Altered protamine expression and diminished spermatogenesis: what is the link? *Human Reproduction Update*, Vol.13, No.3, pp. 313–327

Chen, JL, Longo, FJ.(1996). Expression and localization of DNA topoisomerase II during rat spermatogenesis. *Mol Reprod Dev*, Vol. 45, pp.61-71

Citarelli, M, Teotia, S, Lamb, RS. (2010). Evolutionary history of the poly(ADP-ribose) polymerase gene family in eukaryotes. *BMC Evol Biol* , 2010 October 13. doi: 10.1186/1471-2148-10-308

Concha, II, Figueroa, J, Concha, MI, Ueda, K, Burzio, LO.(1989). Intracellular distribution of poly(ADP-ribose) synthetase in rat spermatogenic cells. *Exp Cell Res* Vol.180, pp.353–366

Corominas, M, Mezquita, C. (1985). Poly(ADP-ribosylation) at successive stages of rooster spermatogenesis. Levels of polymeric ADP-ribose in vivo and poly(ADP-ribose) polymerase activity and turnover of ADP-ribosyl residues in vitro. *J Biol Chem* Vol.260, pp.16269–16273

Cremer, M, Zinner, R, Stein, S, Albiez, H, Wagler, B, Cremer, C, Cremer, T. (2004). Three dimensional analysis of histone methylation patterns in normal and tumor cell nuclei. *Eur J Histochem* Vol.48(1), pp.15-28

D'Amours, D, Desnoyers, S, D'Silva, I, Poirier, GG. (1999). Poly(ADPribosyl)ation reactions in the regulation of nuclear functions. *Biochem J* Vol. 342(Pt 2), pp.249–268

Dadoune, JP. (2003). Expression of mammalian spermatozoal nucleoproteins. *Microsc Res Tech* Vol.61, pp.56–75

Dantzer, F, Mark, M, Quenet, D, Scherthan, H, Huber, A, Liebe, B, Monaco, L, Chicheportiche, A, Sassone-Corsi, P, de Murcia, G, Ménissier-de Murcia, J. (2006). Poly(ADP-ribose) polymerase-2 contributes to the fidelity of male meiosis I and spermiogenesis. *Proc Natl Acad Sci U S A* Vol. 103(40), pp.14854-14859

Deal, RB, Henikoff, JG, Henikoff, S .(2010). Genome-wide kinetics of nucleosome turnover determined by metabolic labeling of histones., *Science (New York, N.Y.)*, Vol. 328 (5982), pp. 1161-1164

de Boer, P, Ramos, L, de Vries, M, Gochhait, S. (2010) Memoirs of an insult: sperm as a possible source of transgenerational epimutations and genetic instability. *Mol Hum Reprod*., Vol.16(1), pp.48-56

De Iuliis, GN, Thomson, LK, Mitchell, LA. (2009). Sperm abnormalities:DNA damage in human spermatozoa is highly correlated with the efficiency of chromatin remodeling and the formation of 8-hydroxy-2'-deoxyguanosine, a marker of oxidative stress. *Biol Reprod,* Vol.81(3), pp.517-24

De Lucia, F, Faraone-Mennella, MR, D'Erme, M, Quesada, P, Caiafa, P, Farina, B.(1994). HISTONE-INDUCED CONDENSATION OF RAT TESTIS CHROMATIN: TESTIS-SPECIFIC H1t VERSUS SOMATIC H1 VARIANTS. *Biochim Biophys Res Commun* Vol.198 (1), pp. 32-39

Di Meglio, S, Denegri, M, Vallefuoco, S, Tramontano, F, Scovassi, AI, Quesada, P. (2003). Poly(ADPR) polymerase-1 and poly(ADPR)glycohydrolase level and distribution in differentiating rat germinal cells. *Mol Cell Biochem* Vol.248, pp. 85–91

Ehrenhofer-Murray, AE. (2004). Chromatin dynamics at DNA replication, transcription and repair. *Eur J Biochem* Vol.271(12), pp. 2335–2349

El-Domyati, MM, Al-Din, AB, Barakat, MT, El-Fakahany, HM, Xu, J, Sakkas, D. (2009).Deoxyribonucleic acid repair and apoptosis in testicular germ cells of aging fertile men: the role of the poly(adenosine diphosphate-ribosyl)ation pathway. *Fertil Steril,* Vol. 91(5 Suppl), pp. 2221-2229

Falbo, KB, Shen, X.(2006). Chromatin Remodeling in DNA Replication. *J Cell Biochem* Vol.97, pp. 684–689

Faraone Mennella, MR, Gentile, N, De Lucia, F, Quesada, P, Farina, B.(1999) In Vitro Poly (ADPribosyl)ated H1a and H1t variants modulate Rat Testis Chromatin Differently. *J Cell Biochem*, Vol. 76, pp. 20-29

Faraone Mennella, MR, Quesada, P, Farina, B, Leone, E, Jones, R. (1982) ADP-Ribosylation of Nuclear Proteins. *Biochem J*, Vol. 205, pp. 245-248

Faraone Mennella, MR, Quesada, P, Farina, B, Leone, E, Jones, R. (1984). Purification of Non-Histone Acceptor Proteins for ADP-Ribose from Mouse Testis Nuclei. *Biochem J*, Vol.221, pp.223-233

Faraone-Mennella, MR, Ferone, A, Ingino, R, Cardone, A, Venditti, P, Di Meo, S, Farina, B. (2005a). Poly-ADPribosylation of Proteins in Hyperthyroid Rat Testes. In: Curtin N (ed) Poly(ADP-ribose) polymerases: Bench to Bedside. PARP Conference 2005, Gateshead (Newcastle, UK), *Med Sci Monit* 11(S 1), p 35

Faraone-Mennella, MR. (2005b). Chromatin architecture and functions: the role(s) of poly(ADP-RIBOSE) polymerase and poly(ADPribosyl)ation of nuclear proteins. Biochem Cell Biol. 2005 Jun;83(3):396-404

Faraone Mennella, MR, Leone, E, Malanga, M, Farina, B. (1988). ADP-Ribosylation of a specific protein from isolated intact bull testis nuclei. *Biochim Biophys Acta*, Vol. 954, pp. 58-64

Faraone-Mennella, MR, Ferone, A, Marino, L, Cardone, A, Comitato, R, Venditti, P, Di Meo, S, Farina, B. (2009). Poly(ADP-ribosyl)ation of proteins and germ cell development in hyperthyroid rat testes. *Mol Cell Biochem* Vol. 323, pp.119–129

Farina, B, Faraone Mennella, MR, Leone, E. (1979a). Nucleic Acids, Histones and Spermiogenesis: the Pply(Adenosine Diphosphate Ribose)Polymerase System. In *Macromolecules in the Functioning Cell,* Salvatore, F, Marino, G, Volpe, P eds., pp.283-300, Plenum Press Corporation, New York

Farina, B, Faraone Mennella, MR, Mauro, A, Leone, E. (1979b). *IN VIVO* ADP-Ribosylation of Epididymal and Testicular Proteins. *IRCS Med Sci*, Vol. 7, p. 378

Fouquet, J, Kann, M, Soues, S, Melki, R. (2000) ARP1 in Golgi organisation and attachment of manchette microtubules to the nucleus during mammalian spermatogenesis. *J Cell Sci*, Vol. 113 (Pt. 5), pp. 877–886

Godmann, M, Lambrot, R, Kimmins, S. (2009). The dynamic epigenetic program in male germ cells: Its role in spermatogenesis, testis cancer, and its response to the environment. *Microsc Res Tech*, Vol.72(8), pp.603-619

Govin, J, Caron, C, Lestrat, C, Rousseaux, S, Khochbin, S. (2004). The role of histones in chromatin remodelling during mammalian spermiogenesis. *Eur J Biochem* Vol. 271, pp. 3459–3469

Green, GR, Balhorn, R, Poccia, DL, Hecht, NB. (1994). Synthesis and processing of mammalian protamines and transition proteins. *Mol Reprod Dev*, Vol. 37, pp. 255–263

Grimes, SR Jr, Smart, PG. (1985) Changes in the structural organization of chromatin during spermatogenesis in the rat. *Biochim Biophys Acta*, Vol. 824(2), pp. 128-139

Gupta, GS (ed). (2005). *Proteomics of spermatogenesis.* Springer, Berlin

Hassa, PO, Hottiger, MO. (2008). The diverse biological roles of mammalian PARPs, a small but powerful family of poly-ADP-ribose polymerases. *Frontiers in Bioscience* Vol. 13, pp. 3046-3082

Hermo, L, Pelletier, RM, Cyr, DG, Smith, CE.(2010a). Surfing the wave, cycle, life history, and genes/proteins expressed by testicular germ cells. Part 1: background to spermatogenesis, spermatogonia, and spermatocytes. *Microsc Res Tech*, Vol. 73(4), pp.278

Hermo, L, Pelletier, RM, Cyr, DG, Smith, CE.(2010b). Surfing the wave, cycle, life history, and genes/proteins expressed by testicular germ cells. Part 2: changes in spermatid organelles associated with development of spermatozoa. *Microsc Res Tech*, Vol. 73(4), pp. 279-319

Hermo, L, Pelletier, RM, Cyr, DG, Smith, CE.(2010c). Surfing the wave, cycle, life history, and genes/proteins expressed by testicular germ cells. Part 3. *Microsc Res Tech*, Vol. 73(4), pp.320-363

Hermo, L, Pelletier, RM, Cyr, DG, Smith, CE.(2010d). Surfing the wave, cycle, life history, and genes/proteins expressed by testicular germ cells. Part 4: intercellular bridges, mitochondria, nuclear envelope, apoptosis, ubiquitination, membrane/voltage-gated channels, methylation/acetylation, and transcription factors. *Microsc Res Tech*, Vol. 73(4), pp. 364-408

Hermo, L, Pelletier, RM, Cyr, DG, Smith, CE.(2010e). Surfing the wave, cycle, life history, and genes/proteins expressed by testicular germ cells. Part 5: intercellular junctions and contacts between germs cells and Sertoli cells and their regulatory interactions, testicular cholesterol, and genes/proteins associated with more than one germ cell generation. *Microsc Res Tech*, Vol. 73(4), pp. 409-494

Hottiger, MO, Hassa, PO, Luscher, B, Schuler, H, Koch-Nolte, F. (2010). Toward a unified nomenclature for mammalian ADP-ribosyltransferases. *Trends Biochem Sci* Vol. 35(4), pp.208–219

Inselman, A, Eaker, S, Hande, MA. (2003). Temporal expression of cell cycle-related proteins during spermatogenesis: establishing a timeline for onset of the meiotic divisions In: *Molecular Aspects of Mouse Spermatogenesis* Vol. 103 (No. 3-4), pp. 289-296 Benavente, R

Jha, R, Agarwal, A, Mahfouz, R, Paasch, U, Grunewald, S, Sabanegh, E, Yadav, SP, Sharma, R. (2009). Determination of Poly (ADP-ribose) polymerase (PARP) homologues in human ejaculated sperm and its correlation with sperm maturation. *Fertil Steril* Vol. 91(3), pp. 782-790

Kierszenbaum, AL. (2001). Transition nuclear proteins during spermiogenesis: unrepaired DNA breaks not allowed, *Mol Reprod Dev*, Vol. 58, pp. 357–358.

Kistler, WS, Henriksen, K, Mali, P, Parvinen, M. (1996). Sequential expression of nucleoproteins during rat spermiogenesis. *Exp Cell Res*, Vol. 225, pp. 374–381

Laberge, RM, Boissonneault, G. (2005a). Chromatin remodeling in spermatids: a sensitive step for the genetic integrity of the male gamete. *Arch Androl*, Vol. 51, pp. 125–133

Laberge, RM, Boissonneault, G. (2005b) On the nature and origin of DNA strand breaks in elongating spermatids. *Biol Reprod* Vol. 73, pp. 289-296

Leduc, F, Maquennehan, V, Bikond-Nkoma, G, Boissonneault, G. (2008a). DNA damage response during chromatin remodeling in elongating spermatids. *Biology of Reproduction*, Vol. 78, pp. 324-332

Leduc, F, Nkoma, GB, Boissonneault, G. (2008b). Spermiogenesis and DNA repair: A possible etiology of human infertility and genetic disorders. *System Biology in Reproductive Medicine*, Vol. 54, pp. 3-10

Lewis, JD, Abbott, DW, Ausio, J. (2003). A haploid affair: core histone transitions during spermatogenesis. *Biochem Cell Biol* Vol. 81, pp.131–140

Li, Y, Lalancette, C, Miller, D, Krawetz, SA. (2008). Characterization of nucleohistone andnucleoprotamine components in the mature human sperm nucleus. *Asian J Androl,* Vol.10, pp. 535-541

Lodish, HF (ed). (2008). *Molecular cell biology*. St. Martin's Press, 6th ed

Loseva, O, Jemth, AS, Bryant, HE, Schuler, H, Lehtio, L, Karlberg, T, Helleday, T.(2010). Poly(ADP-ribose) polymerase-3 (PARP-3) is a mono-ADP ribosylase that activates PARP-1 in absence of DNA. *J Biol Chem* Vol.285(11), pp. 8054–8064

Malanga, M, Atorino, L, Tramontano, F, Farina, B, Quesada, P. (1998). Poly(ADP-ribose) binding properties of histone H1 variants. *Biochim Biophys Acta*, Vol. 1399, pp.154-160

Malanga, M, Althaus, FR. (2005). The role of poly(ADP-ribose) in the DNA damage signalling network. *Biochem Cell Biol*, Vol. 83, pp. 354-364

Marcon, L, Boissonneault, G. (2004) . Transient DNA strand breaks during mouse and human spermiogenesis new insights in stage specificity and link to chromatin remodeling. *Biol Reprod*, Vol. 70, pp. 910-918

Maymon, BB, Cohen-Armon, M, Yavetz, H, Yogev, L, Lifschitz-Mercer, B, Kleiman, SE, Botchan, A, Hauser, R, Paz G. (2006). Role of poly(ADP-ribosyl)ation during human spermatogenesis. *Fertil Steril,* Vol. 86(5), pp.1402-1407

McNairn, AJ, Gilbert, DM. (2003). Epigenomic replication: Linking epigenetics to DNA replication. *Bioessays*, Vol. 25(7), pp. 647–656

McPherson, SM, Longo, FJ. (1993). Nicking of rat spermatid and spermatozoa DNA: possible involvement of DNA topoisomerase II. *Dev Biol*, Vol.158, pp.122-130

Meistrich, ML, Trostle-Weige, PK, Lin, R, Bhatnagar, YM, Allis, CD. (1992). Highly acetylated H4 is associated with histone displacement in rat spermatids. *Mol Reprod Dev*, Vol. 31, pp. 170-181

Meistrich, ML, Mohapatra, B, Shirley CR, Zhao, M. (2003). Roles of transition nuclear proteins in spermiogenesis. *Chromosoma*, Vol. 111, pp. 483-488

Meyer-Ficca, ML, Scherthan, H, Burkle, A, Meyer, RG. (2005). Poly(ADP-ribosyl)ation during chromatin remodeling steps in rat spermiogenesis. *Chromosoma*, Vol. 114, pp.67-74

Meyer-Ficca, ML, Ihara, M, Lonchar, JD, Meistrich, ML, Austin, CA, Min, W, Wang, ZQ,
Meyer, RG. (2010). Poly(ADP-ribose) Metabolism Is Essential for Proper Nucleoprotein Exchange During Mouse Spermiogenesis. *Biol Reprod* , doi: 10.1095/biolreprod.110.087361

Meyer-Ficca, ML Lonchar, JD, Credidio, C, , Ihara, M, Li, Y, Wang, ZQ, Meyer, RG. (2009). Disruption of poly(ADP-ribose) homeostasis affects spermiogenesis and sperm chromatin integrity in mice. *Biol Reprod*, Vol. 81 (1), pp. 46–55

Meyer-Ficca, ML, Lonchar, JD, Ihara, M, Meistrich, ML, Austin, CA, Meyer, RG. (Jan 2011). Poly(ADP-Ribose) Polymerases PARP1 and PARP2 Modulate Topoisomerase II Beta (TOP2B) Function During Chromatin Condensation in Mouse Spermiogenesis. *Biol Reprod* DOI:10.1095/biolreprod.110.090035

Morrison, AJ, Highland, J, Krogan, NJ, Arbel-Eden, A, Greenblatt, JF. (2004). INO80 and gamma-H2AX interaction links ATP-dependent chromatin remodelling to DNA damage repair. *Cell*, Vol.119(6), pp. 767–775

Nair, M, Nagamori, I, Sun, P, Mishra, DP, Rheaume, C, Li, B, Sassone-Corsi, P, Dai, X. (2008). Nuclear regulator Pygo2 controls spermiogenesis and histone H3 acetylation. *Dev Biol*, Vol. 320, pp. 446–455

Oko, RJ, Jando, V, Wagner, CL, Kistler, WS, Hermo, LS. (1996). Chromatin Reorganization in Rat Spermatids during the Disappearance of Testis-Specific Histone, Hlt, and the Appearance of Transition Proteins TP1 and TP2'. *Biol Repr*, Vol. 54, pp.1141-1157

Otto, H, Reche, PA, Bazan, F, Dittmar, K, Haag, F, Koch-Nolte, F. (2005). In silico characterization of the family of PARP-like poly(ADP-ribosyl)transferases (pARTs) *BMC Genomics* Vol.6, p. 139. doi: 10.1186/1471-2164-6-139.

Patrizio, P, Sanguineti, F, Sakkas, D. (2008). Modern andrology: from semen analysis to postgenomic studies of the male gametes. Annals of NY Acad Sci, Vol. 1127, pp. 59-63

Phillips, T, Shaw, K. (2008). Chromatin remodeling in eukaryotes. Nature Education 1(1)

Pirhonen, A, Linnala-Kankkunen, A, Maenpaa, PH. (1994). Identification of phosphoseryl residues in protamines from mature mammalian spermatozoa, *Biol Reprod*, Vol. 50, pp. 981–986

Quesada, P, Farina, B, Jones, R. (1989). Poly(ADP-ribosylation) of nuclear proteins in rat testis correlates with active spermatogenesis. *Biochim Biophys Acta*, Vol.1007(2), pp.167-175

Quesada, P, Atorino, L, Cardone, A, Ciarcia, G, Farina, B. (1996). Poly(ADPribosyl)ation system in rat germinal cells at different stages of differentiation. *Exp Cell Res*, Vol. 226(1), pp.183-90

Quesada, P, Tramontano, F, Faraone-Mennella, MR, Farina, B. (2000) . The analysis of the poly(ADPR) polymerase mode of action in rat testis nuclear fractions defines a specific poly(ADP-ribosyl)ation system associated with the nuclear matrix. *Mol Cell Biochem* Vol.205(1-2), pp.91-99

Rajapakse, I, Scalzo, D, Tapscott, SJ, Kosak, ST, Groudine, M. (2010). Networking the nucleus. *Molecular systems biology*, 6, 395

Roca, J, Mezquita C. (1989). DNA topoisomerase II activity in nonreplicating, transcriptionally inactive, chicken late spermatids. *EMBO J,* Vol. 8, pp. 1855-1860

Rulten, SL, Fisher, AE, Robert, I, Zuma, MC, Rouleau, M, Ju, L, Poirier, G, Reina-San-Martin, B, Caldecott KW. (2011). PARP-3 and APLF function together to accelerate nonhomologous end-joining. *Mol Cell*, Vol. 41(1), pp.33-45

Saha, A, Wittmeyer, J, Cairns, BR. (2006). Chromatin remodelling: the industrial revolution of DNA around histones. *Nature Rev Mol Cell Biol*, Vol.7, pp. 437-447

Sakkas, D, Manicardi, G, Bianchi, PG, Bizzaro, D, Bianchi, U. (1995). Relationship between the presence of endogenous nicks and sperm chromatin packaging in maturing and fertilizing mouse spermatozoa. *Biol Reprod*, Vol. 52, pp.1149-1155

Sakkas, D, Alvarez, JG. (2010). Sperm DNA fragmentation: mechanisms of origin, impact on reproductive outcome, and analysis. *Fertil Steril*, Vol. 93(4), pp.1027-1036

Shaman, JA, Prisztoka, R, Ward, WS. (2006). Topoisomerase IIB and an extracellular nuclease interact to digest sperm DNA in an apoptotic-like manner. *Biol Reprod*, Vol. 75, pp.741-748

Simbulan-Rosenthal, CM, Rosenthal, DS, Iyer, S, Boulares, AH, Smulson. ME. (1998). Transient poly(ADP-ribosyl)ation of nuclear proteins and role of poly(ADP-R)polymerase in the early stages of apoptosis. *J Biol Chem,* Vol.273, pp.13703–1371

Sinha Hikim, AP, Lue, Y, Diaz-Romero, M, Yen, PH, Wang, C, Swerdloff, RS. (2003). Deciphering the pathways of germ cell apoptosis in the testis. *J Steroid Biochem Mol Biol*, Vol. 85(2-5), pp. 175-82

Talbert, PB, Henikoff, S. (2010). Histone variants-ancient wrap artists of the epigenome. *Nature Rev Mo Cell Biol*, Vol. 11 (4), pp. 264-75

Tramontano, F, Di Meglio, S, Quesada, P. (2005). Co-localization of poly(ADPR)polymerase 1 (PARP-1) poly(ADPR)polymerase 2 (PARP-2) and related proteins in rat testis nuclear matrix defined by chemical cross-linking. *J Cell Biochem*, Vol. 94(1), pp.58-66

Tramontano, F, Malanga, M, Quesada, P. (2007). Differential contribution of poly(ADP-ribose)polymerase-1 and -2 (PARP-1 and -2) to the poly(ADP-ribosyl)ation reaction in rat primary spermatocytes. *Mol Hum Reprod, Vol.* 13 (11), pp. 821-828

Yamauchi, Y, Shaman, JA, Ward, WS. (2007). Topoisomerase II-mediated breaks in spermatozoa cause the specific degradation of paternal DNA in fertilized oocytes. *Biol Reprod*, Vol. 76, pp. 666-672

Yélamos, J, Schreiber, V, Dantzer, F. (2008). Toward specific functions of poly(ADP-ribose) polymerase-2. *Trends Mol Med*, Vol. 14 (N° 4), pp. 169-178

Yu, YE, Zhang, Y, Unni, E, Shirley, CR, Deng, JM, Russell, LD, Weil, MM, Behringer, RR, Meistrich, ML. (2000). Abnormal spermatogenesis and reduced fertility in transition nuclear protein 1-deficient mice. *Proc Natl Acad Sci USA*, Vol. 97 (N° 9), pp. 4683–4688

Ullas, KS, Rao, MR. (2003). Phosphorylation of rat spermatidal protein TP2 by sperm-specific protein kinase A and modulation of its transport into the haploid nucleus. *J Biol Chem*, Vol. 278, pp. 52673–52680

Zamudio, N, Chong, S, O'Bryan, M. (2008). Epigenetic Regulation in Male Germ Cells. *Reproduction*, Vol. 136, p. 131

Zhao, M, Shirley, CR, Yu, YE, Mohapatra, B, Zhang, Y, Unni, E, Deng, JM, Arango, NA, Terry, Weil, MM, Russell, LD, Behringer, RR, Meistrich, ML. (2001) Targeted disruption of the transition protein 2 gene affects sperm chromatin structure and reduces fertility in mice. *Mol Cell Biol*, Vol. 21, pp. 7243–7255

Zhao, M, Shirley, CR, Hayashi, S, Marcon, L, Mohapatra, B, Suganuma, R, Behringer, RR, Boissonneault, G, Yanagimachi, R, Meistrich ML. (2004). Transition nuclear proteins are required for normal chromatin condensation and functional sperm development. *Genesis*, Vol. 38, pp. 200-213

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The Ubiquitin-Proteasome System and DNA Repair

Christine A. Falaschetti, Emily C. Mirkin, Sumita Raha,
Tatjana Paunesku and Gayle E. Woloschak
Department of Radiation Oncology, Feinberg School of Medicine and Robert H. Lurie Cancer Center, Northwestern University USA

1. Introduction

The 2004 Nobel Prize in Chemistry was awarded to Aaron Ciechanover, Avram Hershko, and Irwin Rose for their work in discovering the ubiquitin-proteasome system (UPS), as reviewed by Herrmann and others (Herrmann et al., 2007). The mechanisms by which proteolysis occurs had remained elusive until the late 1970s, when a series of key experiments paved the way for a new area of research (Ciechanover et al., 1978; Ciechanover et al., 1980b; Hershko et al., 1980). These studies revealed that the majority of protein degradation is nonlysosomal and adenosine triphosphate (ATP)-dependent. Most importantly, it was also demonstrated that this proteolysis requires at least two components: one with protease activity and another in the form of an 8.5-kDa heat-stable protein. These elements were later identified as the proteasome and ubiquitin, respectively (Ciechanover et al., 1980a; Wilkinson et al., 1980; Hough et al., 1986; Waxman et al., 1987; Arrigo et al., 1988).
Substrates of the UPS include many short-lived regulatory proteins in addition to misfolded and defective proteins (Dahlmann, 2007; Naiki & Nagai, 2009; Xie, 2010). Conserved from Archaea to humans, the UPS is thought to be responsible for degrading approximately 90% of nuclear and cytoplasmic proteins (Magill et al., 2003). Through regulation of protein expression, the UPS controls processes such as protein homeostasis, cell-cycle, cell division, cellular differentiation, apoptosis, signal transduction, gene expression, immunity, and DNA repair (Magill et al., 2003; Finley, 2009; Liggett et al., 2010; Shabek & Ciechanover, 2010; Xie, 2010). Although much focus on this system revolves around its proteolytic function and regulation, members of the UPS also play non-proteolytic roles in transcription, membrane trafficking, protein kinase activation, chromatin dynamics, and DNA repair (Chen & Sun, 2009; Xie, 2010).
The UPS plays one of the central roles in pathology and disease, and it has become the target of several newer therapeutic modalities. In patients with some forms of cardiac dysfunction, neurodegeneration, autoimmune disease, and viral infections, proteasome activity and/or expression is diminished (Magill et al., 2003; Dahlmann, 2007; Naiki & Nagai, 2009). Conversely, in some cancer patients and patients with cachexia, an increase in proteasome expression has been observed. According to the idea that this increase in proteosome activity is a potential therapeutic target, proteosome inhibitors are developed and isolated from natural products (Orlowski & Kuhn, 2008; Groll et al., 2009; Huang & Chen, 2009).

Most notably, bortezomib (Velcade, PS-341) is the first FDA approved proteasome inhibitor and has been used with some success in the treatment of multiple myeloma and mantle cell lymphoma patients (Palombella et al., 1998; Hideshima et al., 2001; Russo et al., 2007).
This chapter is focused on the UPS and its interaction with DNA repair. DNA can be damaged by a wide variety of environmental stresses (ionizing radiation, Ultraviolet (UV) radiation, chemicals) and endogenous cellular metabolites; these DNA damages include base-damage lesions, single-strand breaks (SSBs), double-strand breaks (DSBs), DNA-DNA and DNA-protein crosslinks, as well as other adducts (Sharova, 2005a; Huang & D'andrea, 2006). Consequently, cells have evolved a number of DNA repair pathways in order to remove or mend these lesions quickly and efficiently to preserve genomic integrity. The connection between DNA repair and the UPS was first revealed when Jentsch and colleagues showed that the DNA repair gene Rad6 encodes a ubiquitin-conjugating enzyme (Jentsch et al., 1987). Following this finding, the study of UPS-regulated DNA repair has expanded immensely. Specifically, this chapter will revolve around the UPS as it relates to the nucleotide excision repair (NER), base excision repair (BER), mismatch repair (MMR), double-strand break (DSB) repair, post-replication repair (PRR), and Fanconi anemia (FA) pathways. We will explore the extent to which representative DNA repair proteins of these pathways directly interact with members of the UPS and depend upon the proteolytic and non-proteolytic roles of the UPS. Finally, despite the fact that the mechanisms of action of proteasome inhibitors are not wholly understood (Drexler, 1997), current evidence suggests that DNA repair pathways are key targets of bortezomib treatment.

2. The ubiquitin-proteasome system

The UPS contains both substrate-recruiting and substrate-degrading machinery (Dahlmann, 2007). The central element of this intricate system, the proteasome, is a highly abundant and stable cellular protein complex (Liggett et al., 2010). A number of proteasome-associated proteins have been identified, which may either enhance or suppress UPS-mediated proteolysis, and yeast genetics has contributed greatly to the understanding of the UPS by facilitating its manipulation (Finley, 2009).

2.1 Structure of the proteasome

The 2.5-MDa proteasome can exist in several forms organized into two main subcomplexes: the 28-subunit core particle (CP or 20S proteasome) and the 19-subunit regulatory particle (RP, 19S proteasome, or PA700) (Finley, 2009; Xie, 2010). The 20S proteasome is a barrel-like structure composed of four stacked heptameric rings. The outer rings consist of one of seven unique alpha-subunits and the inner rings are composed of one of seven unique beta-subunits, a configuration that may be represented as: $\alpha_{1\text{-}7}\beta_{1\text{-}7}\beta_{1\text{-}7}\alpha_{1\text{-}7}$ (Groll et al., 1997; Navon & Ciechanover, 2009). The largest dimensions of the interior of the 20S proteasome are approximately 100 Å axially and 60 Å orthogonally (Finley, 2009). However, crystal structures of the 20S proteasome show that the entry ports of the alpha-subunits are as narrow as 10-13 Å (Groll et al., 1997), which helps to prevent spontaneous protein degradation.
The 20S proteasome supports three main catalytic activities: (1) the β1-subunit has caspase-like activity and cleaves peptide bonds after acidic residues; (2) the β2-subunit possesses trypsin-like activity and cleaves after basic residues; and (3) the β5-subunit carries chymotrypsin-like activity and cleaves after large, hydrophobic residues (Orlowski & Wilk,

2000). The catalytic subunits of the 20S proteasome degrade proteins into a heterogeneous mixture of peptides rather than into single amino acids (Kisselev et al., 1999; Goldberg et al., 2002). Proteasomal inhibitors interfere with these activities to various degrees and with specificities for one or more of the main catalytic activities (Groll et al., 2009).
The 19S proteasome is attached to one or both ends of the 20S proteasome, and the resulting complex is known as the 26S proteasome. Although no crystal structure for the 19S proteasome has yet been solved, it is currently believed that the 19S proteasome can be separated into two additional subcomplexes: the base and the lid. The base is composed of six AAA ATPase (ATPases Associated with diverse cellular Activities) subunits (Rpt1-6 in *Saccharomyces cerevisiae*) as well as three non-ATPase subunits (Rpn1, Rpn2, and Rpn13 in *S. cerevisiae*). The lid includes at least nine non-ATPase subunits (Rpn3, Rpn5-9, Rpn11, Rpn12, and Rpn15 in *S. cerevisiae*). Additionally, the yeast protein Rpn10 appears to stabilize the connection between the base and the lid (Finley, 2009; Xie, 2010).

2.2 Function of the UPS

Protein substrates targeted for proteasomal degradation must first be tagged by ubiquitin, a highly conserved 76 amino acid protein. This process is carried out by the concerted activities of three categories of enzymes: a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2), and a ubiquitin-protein ligase (E3) (Dahlmann, 2007; Chen & Sun, 2009; Navon & Ciechanover, 2009; Shabek & Ciechanover, 2010). According to recent counts, humans have genes for two E1, roughly 40 E2, and approximately 600 E3 enzymes (Chen & Sun, 2009; Hofmann, 2009). In the first step of the reaction, E1 activates the ubiquitin polypeptide in an ATP-dependent process, and the activated ubiquitin molecule is subsequently transferred onto E2 by a thioester bond. E3 can then bind the activated ubiquitin to a substrate protein; the high degree of specificity of this enzyme dictates the specificity by the UPS (Magill et al., 2003). An isopeptide bond is formed between the C-terminal glycine (G76) of ubiquitin and the ε-amino group of a lysine on the targeted protein. Next, a polyubiquitin chain is generated through repetition of this process on the ubiquitin molecules themselves. Although ubiquitin contains seven lysine residues (K6, K11, K27, K29, K33, K48, and K63), new ubiquitin moieties are conjugated most often to K48 of the previously attached ubiquitin molecule (Varshavsky, 1997; Hershko et al., 2000; Schwartz & Ciechanover, 2009). A chain of at least four ubiquitin proteins linked through K48 is recognized by the 26S proteasome for degradation (Figure 1). It appears as though K48 has a rather exclusive role in the formation of ubiquitin-mediated degradation signals; the other six lysines of ubiquitin can play either proteolytic or non-proteolytic roles when engaged to form polyubiquitin chains (Chen & Sun, 2009).
Ubiquitin-like proteins (UBLs) are structurally similar to ubiquitin and utilize a comparable enzyme cascade consisting of UBL-activating (E1), UBL-conjugating (E2), and UBL-ligase (E3) enzymes (Huang & D'andrea, 2006). Among UBLs, SUMO (small ubiquitin-like modifier) and NEDD8 (neuronal-precursor-cell-expressed developmentally downregulated protein-8) are the best characterized. SUMO exists in at least four mammalian isoforms (SUMO-1,2,3,4). The process of sumoylation, which is ATP-dependent and generally has nondegradative functions, is reversibly executed by SUMO isopeptidases (Johnson, 2004). NEDD8 (Rub1 in *S. cerevisiae*) also does not directly signal proteolysis but rather regulates E3 ubiquitin-protein ligases and covalently binds to the Cullin family of E3 complexes (Hori et al., 1999).

Entry of substrates into the multicatalyic 20S proteasome is regulated and stimulated by the 19S proteasome. Rpn11 and Rpn13 of the 19S proteasome, and three proteasome-associated proteins, Rad23, Dsk2, and Ddi1, serve as ubiquitin receptors (Finley, 2009). These latter proteins weakly bind to the proteasome and are termed UBL/UBA shuttle proteins due to their ubiquitin-like (UBL) and ubiquitin-associated (UBA) domains (Finley, 2009). Prior to degradation, the ubiquitin moieties must be removed and the protein substrates unfolded. Rpn11 in yeast (Poh1 in humans) serves as a deubiquitinating enzyme (DUB) in the presence of ATP and cooperates with two other deubiquitinases, Upb6 and Uch37 (Usp14 in mammals) (Finley, 2009). Mammals possess about 90 known DUBs for cleaving polyubiquitin chains or for removing ubiquitin from protein substrates (Chen & Sun, 2009; Hofmann, 2009). Although substrate unfolding and translocation are not completely understood, it is currently believed that the six ATPases of the 19S base unfold target proteins and open the gate of the 20S so that substrate proteins may reach the proteolytic sites (Finley, 2009; Shabek & Ciechanover, 2010).

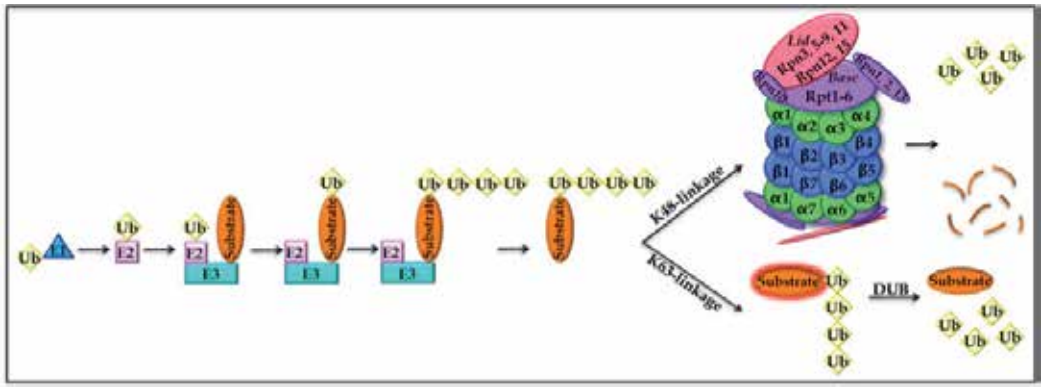

Fig. 1. Schematic representation of UPS-mediated protein regulation. A protein substrate (orange) is tagged with a polyubiquitin chain upon the activities of E1, E2, and E3 enzymes. Substrates modified by a K48-linked polyubiquitin chain are processed and degraded by the 26S proteasome, consisting of a 20S core and 19S regulatory particles. Oppositely, K63-linkages are attributed to non-proteolytic functions and can allow for functional alterations of the tagged substrate.

2.3 The UPS and general repair proteins

Two proteins closely linked with DNA repair in general and regulated by the UPS are p53 and PCNA. In both cases, their UPS regulation can be either proteolytic or non-proteolytic (Coutts et al., 2009; Ulrich, 2009). p53 is regulated by the UPS in a way that prevents unnecessary cell death or cell-cycle arrest (Stehmeier & Muller, 2009). p53 interacts with the 19S proteasome *in vitro* and *in vivo* (Zhu et al., 2007) and can be both mono- or polyubiquitinated (Stehmeier & Muller, 2009). Mdm2, a ubiquitin-protein ligase, promotes the ubiquitination and consequent degradation of p53 as well as the conjugation of NEDD8 (Zhang & Xiong, 2001; Xirodimas et al., 2004). NEDD8 conjugation appears to weaken the inhibition of p53, and this ubiquitination and NEDDylation of p53 are independently regulated upon DNA damage (Xirodimas et al., 2004). Additionally, both p53 and Mdm2 are modified by SUMO-1 (Chen & Chen, 2003). Only a small fraction of p53 is sumoylated, and

the possible role of p53 sumoylation in degradation and DNA repair is still being investigated (Stehmeier & Muller, 2009).
Similarly, proliferating cell nuclear antigen (PCNA), a processivity clamp for the replicative and repair DNA polymerases, is a target of the UPS (Paunesku et al., 2001; Ulrich, 2009). In yeast, PCNA is modified by SUMO at the beginning of S-phase (Papouli et al., 2005; Pfander et al., 2005); however, in response to DNA damage, PCNA is monoubiquitinated in a Rad6/Rad18 dependent manner (Hoege et al., 2002; Lee & Myung, 2008). Further modification with a K63-linked polyubiquitin chain can then occur pending the interaction of Rad5, a ubiquitin-protein ligase, and Ubc13-Mms2, a ubiquitin-conjugating dimer (Hofmann, 2009). Monoubiquitinated and polyubiquitinated PCNA proteins are involved in different DNA repair mechanisms. Proteins PTIP/Swift, an adapter for checkpoint kinases ATM and ATR, and ubiquitin specific protease 1 (USP1) deubiquitinate PCNA (Huang et al., 2006; Ulrich, 2009). Finally, tight regulation of PCNA ubiquitination, keeping ubiquitinated PCNA at low levels in undamaged cells, prevents the recruitment of translesion synthesis (TLS) polymerases, an event that could lead to mutagenesis (Lee & Myung, 2008). For more on PCNA, see section 7.

3. Nucleotide excision repair

Nucleotide excision repair (NER) is an evolutionarily conserved, multistep mechanism responsible for removing bulky chemical adducts and UV-induced photoproducts from DNA (Sharova, 2005a). NER can be divided into repair of overall genomic DNA [global genome repair (GGR)] and repair of actively transcribing genes [transcription-coupled repair (TCR)] (Mueller & Smerdon, 1996). Approximately 30 proteins participate in NER. In this relatively error-free repair process, sites of DNA damage are first recognized and bound by a multi-protein complex. The damaged strand is then cleaved several nucleotides away from the 5′ and 3′ ends of the affected oligonucleotide. Upon removal of this fragment, the resulting ~30 nucleotide gap is filled by DNA polymerases δ and ε in a PCNA-dependent manner, and a DNA ligase covalently attaches the 3′ end of the newly synthesized strand to the flanking DNA (Lommel et al., 2000). Defects in NER are associated with diseases such as Xeroderma Pigmentosum, Cockayne's Syndrome, and Trichothiodystrophy (Bergoglio & Magnaldo, 2006; Leibeling et al., 2006).

3.1 Nucleotide excision repair enzymes and the UPS

In mammalian cells, the first step of NER initiation after damage involves the Damaged-DNA binding protein (DDB) complex that recognizes sites of complex adducts, including those induced by UV radiation (Stoyanova et al., 2009; Iovine et al., 2011). Two protein members of this complex, DDB1 and DDB2, associate with the Cullin 4A (Cul4A) ubiquitin E3 complex, which has been shown to polyubiquitinate Xeroderma pigmentosum C protein (XPC) (Stoyanova et al., 2009; Iovine et al., 2011). DDB1 regulates cyclin-dependent kinase inhibitor 1B (p27) levels after low-dose UV irradiation (Iovine et al., 2011). DDB2 associates with COP9 Signalosome complex (CSN), which has structural homology to the 19S proteasome (Groisman et al., 2003). DDB2 also regulates levels of cyclin-dependent kinase inhibitor 1 (Cip1 or p21) and phospho-p53 through the UPS (Stoyanova et al., 2009). Interestingly, DDB2 is targeted by Cul4A and polyubiquitinated after UV irradiation (Bergink et al., 2007; Stoyanova et al., 2009; Iovine et al., 2011). Therefore, both DDB2 and XPC are downregulated by Cul4A, but protein degradation in this situation promotes a

more efficient DNA repair, as explained further later. No homolog of DDB2 is found in yeast, but a comparable E3 enzyme complex containing Rad16, Rad7, Cul3, and Elc1 is linked to GGR in yeast (Gillette et al., 2006; Dantuma et al., 2009).

Following UV irradiation, ubiquitin and SUMO-1 modify XPC in a manner dependent on DDB2 and XPA (Wang et al., 2005b). XPC in mammalian cells and Rad7 and Rad16 in yeast are required for GGR (Chen et al., 2007). Cockayne syndrome complementation group A and B proteins (CSA and CSB) in mammals and Rpb9 and Rad26 (the homologue of CSB) in yeast are required for TCR (Chen et al., 2007). CSA and CSB are also necessary for ubiquitination of Rpb1, the largest subunit of RNA polymerase II (Pol II) (Chen et al., 2007). When DNA damage causes DNA Polymerase II to become stalled, Rpb1 is polyubiquitinated and degraded by the 26S proteasome (Ribar et al., 2006; Chen et al., 2007). It does not appear as if the whole Pol II complex is degraded (Ribar et al., 2006), and Rpb9, another subunit of Pol II, mediates the ubiquitination of Rpb1 in response to UV radiation (Chen et al., 2007; Daulny & Tansey, 2009). Conversely, the DUB Ubp3 deubiquitinates Pol II and prevents its degradation (Mao & Smerdon, 2010).

Different types of histone modifications are associated with DNA repair, such as monoubiquitination of histone H2A following UV-damage (Bergink et al., 2006). Ubiquitinated H2A (uH2A) is the most common modification of histones in higher eukaryotes, and uH2A foci formation requires either functional GGR or TCR (Zhu et al., 2009). The E3 enzyme Cul4A-DDB has also been shown to ubiquitinate histones H3 and H4 in order to positively regulate NER (Zhu et al., 2009). Histone ubiquitination could play a role in chromatin remodelling and is dependent on RING2, an E3 enzyme, and ATR-kinase (Bergink et al., 2006). The pivotal yeast NER protein Rad4, the yeast homologue of XPC, is involved in this process. Rad4 binds to Snf5 and Snf6 of the Swi/Snf chromatin-remodelling complex (Gong et al., 2006), which correlates with UV-induced chromatin remodelling; this event could induce DNA to be more accessible to repair proteins (Dantuma et al., 2009). Interestingly, proteasome inhibitors such as lactacystin (LC) and N-acetyl-leucyl-leucyl-norleucinal (ALLnL) deplete free cellular ubiquitin, thereby negatively affecting protein dynamics of histones, and promote chromatin condensation (Mimnaugh et al., 2000).

Yeast protein Rad23 is involved in both NER pathways: GGR and TCR (Mueller & Smerdon, 1996). Rad23 itself is not degraded by the 26S proteasome because it lacks an initiation region for the proteasome to engage and unfold it (Fishbain et al., 2011). However, Rad23 has been shown to contact proteins Rpt1 (Cim5), Rpt4 (Sug2), Rpt6 (Cim3/Sug1), Rpn1, and Rpn10 (S5a) of the 19S proteasome (Waters et al., 1993; Motegi et al., 2009), perhaps to recruit it to sites of DNA damage (Russell et al., 1999). The N-terminal domain of Rad23 (the UbL domain) resembles ubiquitin, which enables it to interact with the 26S proteasome directly (Watkins et al., 1993; Schauber et al., 1998). Notably, Rad23 has 22% identical and 43% similar amino acid residues compared to ubiquitin from *S. cerevisiae* (Watkins et al., 1993; Schauber et al., 1998). Deletion of the UbL domain impairs Rad23 function, leading to UV-sensitivity and a 50% decrease of NER activity in yeast (Watkins et al., 1993; Russell et al., 1999). This effect is reversed, however, upon substitution of the UbL domain with the sequence of ubiquitin (Watkins et al., 1993). E1-ubiquitin and E2-ubiquitin thioester intermediate formation does not appear to be affected by Rad23, but the presence of Rad23 remarkably stabilizes proteins *in vivo* and inhibits multi-ubiquitin chain assembly *in vitro* (Ortolan et al., 2000). The two UBA regions of Rad23 participate in noncovalent interactions with ubiquitin, and loss of both UBA regions does not allow Rad23 to block assembly of multi-ubiquitin chains on protein substrates (Chen et al., 2001). Interestingly,

the C-termini of the human homologues to Rad23, hHR23A and hHR23B, contain a stretch of amino acids homologous to E2 enzymes (van der Spek et al., 1996; Masutani et al., 1997).
The C-terminus of Rad23 binds with high affinity to Rad4 (Mimnaugh et al., 2000). Rad23/Rad4 complexes govern damage recognition and bind UV-irradiated DNA (Guzder et al., 1998; Jansen et al., 1998; Guzder et al., 1999); loss of Rad4 leads to severe UV-sensitivity (Ortolan et al., 2000). Rad23 can regulate polyubiquitination of Rad4 *in vivo* and delay its degradation by the 26S proteasome (Lommel et al., 2002). The Rad4 binding domain (R4B) of Rad23 is also sufficient to stabilize Rad4 and enable NER in yeast strains lacking Rad23 (Ortolan et al., 2004). Nevertheless, in a Rad23 null strain, overexpression of Rad4-hemagglutinin does not rescue the impaired repair of cyclobutane pyrimidine dimers (CPDs) (Lommel et al., 2002). Rad23 may protect Rad4 from degradation so that it can participate in NER, but Rad23 may also play proteolytic roles in other pathways (Ortolan et al., 2004). Essentially, optimal NER necessitates two distinct functions of Rad23; both Rad23/proteasome and Rad23/Rad4 interactions are required for maximum UV-resistance (Ortolan et al., 2004).
Other yeast enzymes, Rad7 and Rad16, are also involved in UPS regulation of NER. These proteins are members of the nucleotide excision repair factor 4 (NEF4) complex, which bears E3 activity and regulates Rad4 levels (Ramsey et al., 2004). The DUB Upb3 promotes degradation of Rad4, which leads to a negative regulation NER; conversely, inactivation of *Upb3* stabilizes Rad4 (Mao & Smerdon, 2010).
Ubiquitination is necessary for NER, since E1 enzyme inactivation exerts a negative influence on NER in mammalian cells (Wang et al., 2005a). However, much debate surrounding NER and the UPS still exists, revolving around the question of whether UPS-mediated modifications leading to proteolysis (a proteolytic role of the UPS) are more or less important for NER than the modifications (ubiquitination, sumoylation, etc.) that do not lead to protein degradation (a non-proteolytic role of the UPS). For example, Rad23 is not degraded by the proteasome, and this suggests that it has a non-proteolytic role (Watkins et al., 1993). Antibodies against the ATPases of the 19S have been shown to measurably lower NER activity, but mutations in 20S subunits that severely curb proteolysis and incubation with proteosome inhibitor LC do not appear to change NER activity (Russell et al., 1999; Gillette et al., 2001). Also, efficient NER in yeast relies on two mechanisms related to the necessity of *de novo* protein synthesis, neither of which entails proteolytic behavior (Gillette et al., 2006). Rad23 and the 19S proteasome regulate one such pathway, independently of *de novo* protein synthesis. Another pathway involving Rad4 ubiquitination by a Rad7-containing E3 ligase depends on *de novo* protein synthesis to restore Rad4 to baseline levels such as they were prior to DNA damage (Gillette et al., 2006). All these findings stress a connection between NER and non-proteolytic behavior of the UPS (Gillette et al., 2006).
However, NER has been shown to be increased in yeast strains with mutations in genes encoding the 26S proteasome (Lommel et al., 2002). Proteosome inhibitors LC and ALLnL interfere with removal by NER of cisplatin-damaged DNA and decrease mRNA levels of the excision nuclease Ercc-1 in ovarian cancer cells (Mimnaugh et al., 2000). Treatment of normal human fibroblasts with proteosome inhibitors MG132 or LC decreases repair of CPDs (Wang et al., 2005a). Overexpression of hSug1, one of the six ATPases of the mammalian 19S proteasome, competes with endogenous 19S proteasomes for substrates and negatively affects NER (Wang et al., 2005a). In yeast, conditional proteasome mutants of Rpt1 and Rpt6 exhibit faster NER in a single gene repair study (Lommel et al., 2000). Similarly, overexpression of Rad4 results in increased NER of the transcribed and non-

transcribed strands of the damaged gene. Therefore, proteosome subunit overexpression has an effect similar to overexpression of the NER protein Rad4, which results in increased GGR and TCR (Lommel et al., 2000).
Ultimately, this apparent discrepancy could stem from the use of different model systems because NER proteins and processes differ among different organisms (Wang et al., 2005a). The exact role(s) of the 26S proteasome in NER remain somewhat obscured. There is no consensus on whether the 26S proteasome plays excitatory and/or inhibitory roles in NER, nor is the balance between the roles of Rad23 in DNA repair and proteolysis fully understood (Dantuma et al., 2009). It is unclear whether Rad23 is ubiquitinated itself, and if so, what is the significance of that modification (Dantuma et al., 2009). Nevertheless, evidence of "protein sharing" between the UPS and NER suggests a functional connection that is deeper than our present understanding of this system.

4. Base excision repair

The base excision repair (BER) pathway regulates removal of damaged bases, apurinic/apyrimidinic (AP) sites, and single-strand breaks (SSBs) induced by UV radiation, ionizing radiation, oxidative stressors, and alkylating agents (Dalhus et al., 2009; Wilson et al., 2010). For every double-stranded DNA break (DSB), cells sustain hundreds of SSBs; consequently, BER activity in cells is perpetually engaged. BER can be separated into long-patch repair and short-patch repair, dependent upon the type of lesion incurred as well as the expression levels of BER proteins (Memisoglu & Samson, 2000; Wilson et al., 2010). The most fundamental steps of BER include damaged base recognition and removal by DNA glycosylases, strand cleavage by an AP endonuclease, incised strand processing, DNA synthesis, and ligation (Sharova, 2005a). Processing of gaps in mammalian DNA involves a DNA polymerase (primarily Pol β), DNA ligase IIIα, and X-ray crosscomplementing group-1 protein (Xrcc1) (Parsons et al., 2010). Importantly, the BER, NER, and MMR activities can overlap in protection against DNA damage (Wilson et al., 2010), sharing proteins such as Rad4. Dysfunctional BER can lead to chromosomal rearrangements and cell death, and defects/knock-out animals for BER genes are often associated with embryonic lethality (Wilson & Thompson, 1997).

4.1 Base excision repair and the UPS

While poly (ADP-ribose) polymerase-1 (PARP-1) does not appear to play an enzymatic role in BER, it is the molecule that recognizes DNA damage repaired by BER. Moreover, PARP inhibition delays repair of SSBs in Xrcc1-deficient cells (Strom et al., 2010) and protects SSBs from becoming DSBs, thereby ensuring that the SSBs may be repaired by BER proteins (Woodhouse et al., 2008). PARP-1 has been shown to be polyubiquitinated and modified by SUMO-1 and SUMO-3 *in vitro* and *in vivo* (Wang et al., 2008; Messner et al., 2009). Polyubiquitination of PARP-1 requires proteasomal inhibition, indicating that it is a target of the 26S proteasome (Wang et al., 2008). In a yeast two-hybrid screen, PARP was found to interact with hUbc9, the human protein homologous to Ubc9, a yeast E2 enzyme (Masson et al., 1997). *hUbc9* mRNA has been shown to increase at the beginning of S-phase, suggesting that hUbc9 is involved in degradation of cyclins, as Ubc9 is in yeast (Masson et al., 1997).
The transcription of yeast DNA glycosylase genes *MAG1*, *NTG1*, and *NTG2* is co-regulated with proteasomal genes and modulated by transcription factor Rpn4, a negative regulator of the proteosome (Jelinsky et al., 2000; Hanna & Finley, 2007). Another BER DNA glycosylase,

thymine-DNA glycosylase (TDG), binds SUMO-1 covalently and noncovalently (Takahashi et al., 2005). Covalent modification of TDG with SUMO-1 or SUMO-3 reduces its affinity for DNA (Hardeland et al., 2002b). The enzymatic properties of TDG change upon sumoylation; G•T mismatch processing is repressed, whereas G•U processing augments. TDG sumoylation facilitates its dissociation from AP sites, allowing for AP endonuclease entry (Hardeland et al., 2002b). Mechanistically, TDG changes conformation when it contacts DNA, binding tightly to mismatches; conjugation of SUMO-1 to the C-terminal domain of TDG induces another conformational change in the N-terminus of TDG so that it can dissociate from the AP sites (Steinacher & Schar, 2005b). TDG sumoylation does not seem to affect polyubiquitination and degradation of TDG (Hardeland et al., 2007).
While TDG levels are highest during G2/M- and G1-phases of the cell-cycle but taper during S-phase, UNG2, a uracil-DNA glycosylase, is upregulated during S-phase (Hardeland et al., 2007). Thus, TDG and UNG2 play non-redundant, alternating roles. The UPS regulates these precise fluctuations in TDG and UNG2 protein levels; for example, treatment with the proteasomal inhibitor MG132 measurably increases TDG levels (Hardeland et al., 2007).
Interestingly, a key role of ubiquitination in BER is to modify the proteins that are not members of an active BER complex so that they may be rapidly degraded, which allows clearance of access for BER proteins. CHIP, an E3 enzyme, is responsible for turnover of BER proteins such as Xrcc1, which acts as a scaffold for directing assembly of BER complexes at sites of DNA damage, and Pol β (Parsons et al., 2008b). Although CHIP appears to be the principal E3 in this process, other E3 enzymes may be involved as well. Phosphorylation of Xrcc1 by casein kinase 2 (CK2) appears to be necessary for efficient BER; this phosphorylation improves the stability of Xrcc1 by protecting it from ubiquitination (Parsons et al., 2010). In addition, Xrcc1 recruits JWA to sites of damage, and loss of JWA leads to Xrcc1 degradation by the UPS (Wang et al., 2009). Xrcc1 is also modified by SUMO-1 (Gocke et al., 2005; Moschos & Mo, 2006).

5. Mismatch repair

The mismatch repair (MMR) system corrects noncomplementary base pairs that escape the proofreading activity of DNA polymerases δ and ε in DNA replication (Jiricny, 1998; Sharova, 2005b). The process begins with mispair recognition by the hMutSα complex, a heterodimer of hMSH2 and hMSH6, or by the complex hMutSβ, a heterodimer of hMSH2 and hMSH3 (Modrich & Lahue, 1996; Jiricny, 1998; Kolodner & Marsischky, 1999). Another complex, hMutLα, a heterodimer of hMLH1and hPMS2, regulates the termination of mismatch-stimulated DNA excision (Li, 2008).

5.1 Mismatch repair and the UPS

The UPS is involved in post-transcriptional regulation of hMutSα protein expression (Humbert et al., 2002; Hernandez-Pigeon et al., 2004). Ubiquitination and degradation rates of hMSH2 and hMSH6 appear quite similar, suggesting that UPS-mediated proteolysis may maintain a constant ratio of these two proteins (Hernandez-Pigeon et al., 2004). While no strong correlation between total proteasomal activity and the degradation rate of hMutSα has been observed *in vitro*, low hMutSα expression in cells is a limiting factor for MMR and indicative of proteolytic activity of the UPS in MMR regulation (Ciechanover, 1994; Humbert et al., 2002; Hernandez-Pigeon et al., 2004). This process is regulated also by an

atypical protein kinase C ζ (PKCζ); this kinase increases hMutSα protein levels and the binding of hMutSα to G•T mismatches (Hernandez-Pigeon et al., 2005). Essentially, PKCζ expression and ubiquitination of hMutSα proteins are inversely related, and PKCζ kinase activity interferes in the UPS-mediated degradation of hMutSα (Hernandez-Pigeon et al., 2005). Collectively, these results support a model in which PKCζ serves as a positive regulator for UPS-mediated MMR by directly interacting with the hMutSα complex. There is also evidence of hMutSα protein sumoylation, but the functional importance of this is unclear (Hernandez-Pigeon et al., 2005).
Human exonuclease I (hEXO1) complexes with hMutLα and functions in the excision step of MMR (Schmutte et al., 2001; Genschel et al., 2002). In response to DNA replication arrest, the isoform hEXO1b is polyubiquitinated and degraded, and phosphorylation of hEXO1b correlates with its UPS-mediated degradation (El-Shemerly et al., 2005).

6. Double-strand break repair

DNA double-strand breaks (DSBs) can be lethal to cells predominantly because the most important DSB repair process, non-homologous end joining (NHEJ), is error-prone, leaving mutations in the DNA following repair. Two possible repair processes that correct DSBs are homologous recombination (HR) and NHEJ. HR repair exchanges nucleotide sequences between two homologous chromosomes; this leads to error-free repair of DSB damage, preventing mutagenesis (Li et al., 2000). During the initial stages of mammalian HR, the MRN complex, composed of Mre11-Rad50-Nbs1, recognizes and carries out initial processing of the broken DNA ends. Following DSB resection, single-stranded DNA ends are bound by Rad51, Rad52, and RPA (Zhao et al., 2007). Strand invasion and displacement to initiate repair synthesis from the homologous sequence are mediated by Rad51, Rad54, Brca1, and Brca2, and HR is completed upon DNA annealing and ligation (Li & Heyer, 2008). NHEJ is a rapid, error-prone pathway that does not necessarily restore the sequence around the DSB, leading to local deletions and chromosomal translocations. NHEJ is the predominant form of DSB repair in mammalian cells because it occurs in the G0-, G1-, and early S-phases of the cell-cycle (before duplication of the DNA). The Ku70/Ku80 heterodimer detects the exposed ends of DNA DSBs in NHEJ and then recruits and forms a holoenzyme with DNA-dependent protein kinase (DNA-PK) to sense and to repair damaged DNA (Gottlieb & Jackson, 1993; Downs & Jackson, 2004). The MRN complex also influences NHEJ, and Xrcc4 and ligase IV complete the resealing reaction.

6.1 Double-strand break repair and the UPS

Crosslinking and chromatin immunoprecipitation studies reveal that subunits of the 26S proteasome are recruited to DSB sites, suggesting that proteolysis takes place concurrent with DSB repair (Krogan et al., 2004). Both NHEJ and HR are affected by interaction with the proteasome, such as through DNA polymerase IV (Pol4) and Rad52, respectively (Tseng & Tomkinson, 2002; Krogan et al., 2004). Rad52 forms multimeric ring foci, which are centers of recombination repair capable of processing multiple DNA lesions (West, 2003). Other HR proteins depend on the presence of Rad52 and its interaction with ssDNA regions in HR repair for their function (West, 2003). In yeast, one of the proteins associated with Rad52 is Sem1, a component of the yeast 19S proteasome. In the absence of Rad52, recruitment of Sem1 to damaged DNA is reduced (Krogan et al., 2004). Moreover, knock-out of *Sem1* in yeast strains capable of only HR or NHEJ, but not both, results in impaired cell

growth. Therefore, Sem1 and the proteasome are crucial for HR DSB repair (Krogan et al., 2004). The human homolog of Sem1, Deleted in Split hand/Split foot 1 (DSS1), is a part of the human 19S proteasome and involved in HR through interaction with Brca2 (Marston et al., 1999). Just as knock-out of Brca2 increases dependence of HR on mammalian Rad52 (West, 2003), depletion of DSS1 in human cells significantly reduces HR activity, while treatment with proteasome inhibitors corresponds to a smaller decrease in HR (Kristensen et al., 2010). Thus, it is likely that DSS1 engages in functions in HR other than strictly proteolysis (Kristensen et al., 2010).

DSS1 and Brca2 interact with Rpn3 and Rpn7, though the Brca2/Rpn7 interaction does not depend on DSS1 (Gudmundsdottir et al., 2007). On the contrary, it is Brca2 that secures the presence of the proteasome close to DNA repair machinery. Upon treatment of an ES cell line with the proteasome inhibitor epoxomicin, Gudmundsdottir and colleagues observed a shift in the repair of repetitive elements from the error-free gene conversion pathway to the error-prone single-strand annealing pathway. This suggests that the proteasome plays a functional role in support of HR as a repair mechanism of DSBs (Gudmundsdottir et al., 2007).

As mentioned previously, knock-out of different proteasomal subunits or proteosome inhibition by small molecules interferes with both HR and NHEJ in yeast (Krogan et al., 2004); in higher eukaryotes, association of NHEJ and proteosome appears to be less obligatory. Here, proteasome inhibitors MG132 and LC reduce HR-dependent DSB repair but only marginally affect NHEJ-mediated repair of an artificial substrate (Murakawa et al., 2007). MG132 treatment of Ku70-deficient chicken DT40 cells, which are impaired in NHEJ but not in HR, negatively affects the repair kinetics of ionizing radiation-induced DSBs. However, MG132 treatment of Rad54-deficient chicken DT40 cells, impaired in HR but not in NHEJ, does not delay DSB repair. Ionizing radiation-induced Brca1 and Rad51 foci formation in HeLa cells is reduced upon treatment with MG132. Therefore, it appears as if the proteasome functions at an early step of HR, prior to formation of the Brca2-DSS1 complex (Murakawa et al., 2007).

Brca1 contains a RING domain, which is generally associated with ubiquitin-protein ligase activity (Starita & Parvin, 2003). Human C-terminal binding protein interacting protein (CtIP), which regulates DSB resection and efficient HR, is ubiquitinated when it associates with chromatin following DNA damage. This ubiquitination of CtIP is catalyzed by Brca1 and is not a degradation signal (Yu et al., 2006; Sartori et al., 2007). Brca1 dimerizes with Bard1 to elicit higher ubiquitin-protein ligase activity than either Brca1 or Bard1 alone (Hashizume et al., 2001; Xia et al., 2003). These two proteins appear to stabilize one another *in vivo*, suggesting that each does not participate in the degradation of the other (Hashizume et al., 2001; Xia et al., 2003). Brca1/Bard1 assemble polyubiquitin chain linkages through non-K48 residues, mostly through K63, indicative of non-proteolytic function (Chen et al., 2002). The Brca1/Bard1 complex is capable of autoubiquitination *in vitro* and *in vivo*, and it also mediates monoubiquitination of histone H2A/H2AX *in vitro* (Chen et al., 2002). At a higher level of complexity, Brca1/Bard1, Rap80, which has two domains that bind K63-linked ubiquitin chains, the DUB Brcc36, and Abraxas form a complex that localizes to damaged DNA (Wang & Elledge, 2007). Ubc13, an E2 enzyme, and Rnf8, a Ubc13-associated E3 enzyme, which together catalyze K63-linked ubiquitin chains at DSBs, are both required for Rap80 and Abraxas foci formation induced by ionizing radiation (Huen et al., 2007; Kolas et al., 2007; Wang & Elledge, 2007). The interaction between Rap80 and Abraxas localizes Brca1 and the DUB Brcc36 to ionizing radiation-induced foci (Wang & Elledge, 2007).

In conducting studies revolving around the sensitivity of Ubc13-deficient cells to DNA-damaging agents, Zhao and co-workers discovered a link between Ubc13 and HR in higher eukaryotes. Mammalian cells depleted of *Ubc13* demonstrate defective HR following ionizing radiation (Zhao et al., 2007). This observation corresponds with impaired recruitment of Rad51 to DSBs, reduced Brca1 ubiquitin-protein ligase activity and foci formation, and decreased accumulation of RPA single-strand binding protein at DNA lesions. NHEJ, however, appears to function normally in *Ubc13* null cells. The role of Ubc13 in HR does not appear to involve PCNA ubiquitination, thereby distinguishing the role of Ubc13 in HR from its activities in post-replication repair (PRR). Nevertheless, the mechanism of Ubc13-promoted DSB resection requires further investigation (Zhao et al., 2007).

The E3 ubiquitin-protein ligase, Rnf8, a modulator of the DNA damage response, mediates histone ubiquitination and allows DSB-flanking chromatin to accumulate additional DNA damage regulators (Huen et al., 2007; Kolas et al., 2007; Mailand et al., 2007; Wang & Elledge, 2007; Doil et al., 2009). In particular, Rnf8 is required for accumulation of 53BP1 and Brca1 following ionizing radiation (Huen et al., 2007; Kolas et al., 2007; Mailand et al., 2007; Sakasai & Tibbetts, 2008). Following UV illumination, recruitment of 53BP1 partially depends on Ubc13 and Rnf8, and 53BP1 recruitment is suppressed upon proteasomal inhibition (Sakasai & Tibbetts, 2008). Importantly, 53BP1-deficient cells exhibit defects in NHEJ (Nakamura et al., 2006). Rnf8 is the first E3 to assemble at DSBs and allows for the recruitment of another E3 ligase, Rnf168, which has two ubiquitin-binding domains. Rnf168 appears to increase K63-linked ubiquitination at DSBs necessary to retain 53BP1 and Brca1 (Doil et al., 2009). Stewart and co-workers identified *Rnf168* as the gene mutated in RIDDLE Syndrome (Stewart et al., 2009), a disorder characterized in part by radiosensitivity and immunodeficiency. Cells derived from a RIDDLE patient fail to localize 53BP1 to DSBs (Stewart et al., 2007). Accordingly, these cells display decreased Brca1 foci formation, hypersensitivity to ionizing radiation, and irregular cell-cycle checkpoints (Stewart et al., 2007; Stewart et al., 2009).

With regard to UBLs, SUMO-1 is the most frequently involved in DSB repair; it forms stable complexes with Rad51, Rad52, and Rad51/Rad52 in co-immunoprecipitation experiments (Shen et al., 1996; Li et al., 2000). The role of SUMO-1 in HR appears to be inhibitory, as overexpression of SUMO-1 has been shown to measurably decrease radioresistance and bidirectional gene conversion tracts in mammalian cells (Li et al., 2000). Sumoylation of Rad52 is observed infrequently unless this modification of Rad52 is induced in a cell cycle- and lesion-specific fashion (Ohuchi et al., 2008). In yeast, Rad52 sumoylation appears to be triggered specifically by interactions with Rad50, Mre11 and Xrs2 (but not Rad51), members of the yeast homolog of the MRN complex, the MRX complex (Sacher et al., 2006; Ohuchi et al., 2008). There is conflicting evidence pertaining to the residues required for Rad52 sumoylation, but self-association of Rad52 appears important for its sumoylation. Essentially, sumoylation of Rad52 appears to preserve Rad52 activity and to inhibit rapid degradation of the protein (Sacher et al., 2006).

Two of the key NHEJ enzymes, Ku70 and Ku80, are susceptible to ubiquitination (Gama et al., 2006). Ku70 and Ku 80 are degraded by the proteasome, and MG132 delays proteolysis of these proteins (Postow et al., 2008; Enokido et al., 2010). Ubiquitinated Ku70 in human cells has been detected in the absence of proteasome inhibitors, indicating that this modification may not always serve as a degradation signal for Ku70; yet, apoptotic stress does upregulate degradative Ku70 ubiquitination (Gama et al., 2006). Ku70 and Ku80

stabilize one another, but ubiquitinated Ku70 appears to inhibit Ku70/Ku80 complex formation (Gama et al., 2006). Ku80 is polyubiquitinated when bound to DSBs in *Xenopus laevis* egg extracts, and this K48-linked polyubiquitination is related to removal of Ku80 from DNA (Postow et al., 2008). However, polyubiquitination-induced removal of Ku80 from DSBs is not required for the completion of NHEJ (Postow et al., 2008). In yeast, aberrant expression of key proteosome regulating transcription factor Rpn4 appears to hinder NHEJ but not HR (Ju et al., 2010). When proteosomal degradation of Rpn4 itself is inhibited, the expression levels of *Ku70*, *Ku80*, and *Mre11* are decreased, as well as the accumulation of Ku70 at DSBs (Ju et al., 2010).

Ubiquitination and levels of monoubiquitinated Xrcc4 increase upon etoposide-induced DNA damage (Foster et al., 2006). Xrcc4, however, is a stable protein, and treatment with proteasome inhibitors does not correlate with Xrcc4 accumulation, suggesting that this type of Xrcc4 modification serves a non-proteolytic function. This enzyme also stabilizes ligase IV, whose half-life also increases upon treatment with proteasome inhibitors (Foster et al., 2006). Human Xrcc4 is also sumoylated *in vitro* and *in vivo* (Yurchenko et al., 2006). This modification regulates Xrcc4 localization; a non-sumoylated mutated Xrcc4 protein accumulates in the cytoplasm rather than in the nucleus (Yurchenko et al., 2006). This mutant protein causes radiosensitivity, but fusion of SUMO to its C-terminus leads to nuclear Xrcc4 localization and radiation resistance of cells with this genotype (Yurchenko et al., 2006).

7. Post-replication repair

Post-replication repair (PRR), also known as DNA damage tolerance or damage bypass, is a process that fills gaps formed in newly synthesized single-stranded DNA. This type of repair is essential for avoiding unrepaired DSBs, such as those that result from the prolonged stalling of DNA replication forks (Podlaska et al., 2003; Zhuang et al., 2008). PRR operates by either an error-free or an error-prone mechanism. The error-free/damage avoidance pathway involves HR or template switching to the undamaged sister chromatid (Lee & Myung, 2008; Ulrich, 2009). The error-prone method, referred to as DNA translesion synthesis (TLS), is carried out by a TLS polymerase such as Pol ζ, Pol η, Pol ι, Pol κ, or Rev1 (Prakash et al., 2005). Rad6, an E2 enzyme, and Rad18, a DNA-binding protein, form a complex that governs PRR and is responsible for PCNA modification, a crucial event in this DNA repair pathway (Hoege et al., 2002; McIntyre et al., 2006).

7.1 Post-replication repair and the UPS

Proteasome inhibitors have been shown to disrupt TLS in cancer cells by delaying cisplatin and UV-induced translesion reactions (Takezawa et al., 2008). Studies of defective 20S proteasomal activity in yeast strains have revealed important relationships between the UPS and PRR. The Ump1 proteasomal maturase is required for processing of the β-subunits and proper assembly of the 20S proteasome (Ramos et al., 1998). Deletion of *Ump1* or of the genes encoding the β2- and β5-subunits of the 20S proteasome, *Pup1* and *Pre2*, respectively, correlates with an increase in UV-sensitivity and spontaneous mutagenesis. Importantly, this phenotype is characteristic of yeast strains with defects in PRR, and Podlaska and colleagues have determined that these genes are members of the Rad6/Rad18 epistasis group (Podlaska et al., 2003). This spontaneous mutator phenotype has been verified to be primarily PRR-based and unrelated to the HR and NER pathways (McIntyre et al., 2006).

Knock-out of *Rev3*, which encodes the catalytic subunit of Pol ζ, in an *Ump1*-deficient yeast strain correlates with a dramatic decrease in the frequency of UV-induced and spontaneous mutations, thereby suggesting that *Ump1* may serve as a negative regulator of *Rev3* (Podlaska et al., 2003; McIntyre et al., 2006; Wiltrout & Walker, 2011). In contrast, the presence of *Rad30*, which encodes Pol η, in an *Ump1*-deficient background is associated with increased UV-sensitivity but a decrease in the frequency of spontaneous mutations (Podlaska et al., 2003; McIntyre et al., 2006). Taken together, these results suggest that *Rev3* and *Rad30* are epistatic to *Ump1* and that mutations caused by proteasomal defects depend upon both Pol ζ and Pol η (Podlaska et al., 2003; McIntyre et al., 2006). Rad30 is a short-lived protein post-translationally regulated by the UPS (Skoneczna et al., 2007). Its degradation depends on the Skp1/Cullin/F-box E3 enzyme complex and the Ufo1 F-box protein (Skoneczna et al., 2007). Rev1 is also a relatively short-lived protein but has a longer half-life during G2/M-phases than G1-phase, potentially implicating proteasomal regulation. In support of this idea, Rev1 levels are elevated upon treatment with proteasome inhibitors or in an *Ump1*-deficient background (Wiltrout & Walker, 2011).

Ubiquitin is essential for the regulation, localization, and stability of Rad18 PRR protein (Ulrich, 2009). In response to DNA damage, Rad18 autoubiquitination indirectly regulates PRR by balancing levels of unmodified Rad18 in the nucleus and monoubiquitinated Rad18 in the cytoplasm (Miyase et al., 2005). Polyubiquitinated Rad18 is not present under normal conditions, potentially because it is subject to rapid degradation. However, polyubiquitinated Rad18 may be detected upon treatment with proteasome inhibitors or in an *in vitro* system consisting of Rad18, one E1 enzyme, Rad6, and ubiquitin (Miyase et al., 2005).

Rad18 also influences PCNA monoubiquitination following DNA damage (Hoege et al., 2002; Stelter & Ulrich, 2003; Kannouche et al., 2004). Monoubiquitinated PCNA is thought to promote the error-prone TLS pathway, while polyubiquitinated PCNA may regulate the error-free damage avoidance pathway (Hoege et al., 2002; Stelter & Ulrich, 2003; Kannouche et al., 2004; Watanabe et al., 2004). Monoubiquitinated PCNA is thought to facilitate targeting of Pol η to DNA damage sites and is required for the replacement of stalled Pol δ by Pol η (Kannouche et al., 2004; Watanabe et al., 2004; Zhuang et al., 2008). In response to DNA damage, Pol η and Pol ι also noncovalently interact with ubiquitin and monoubiquitinated PCNA (Bienko et al., 2005; Plosky et al., 2006). Pol η and Pol ι mutants lacking the ability to bind ubiquitin demonstrate downregulated levels of DNA damage-induced replication foci; this suggests that the polymerase-ubiquitin interaction may be an important component in the recruitment of TLS polymerases to stalled replication forks (Plosky et al., 2006). Ubiquitinated PCNA also substantially activates Pol η and Rev1 *in vitro* (Garg & Burgers, 2005). Unmodified PCNA stimulates Pol ζ-mediated TLS, and no significant differences in this type of TLS are observed upon substitution of unmodified PCNA with ubiquitinated PCNA (Garg & Burgers, 2005; Garg et al., 2005). Thus, ubiquitinated PCNA has different functional interactions with the various TLS polymerases.

Importantly, it appears as if ubiquitination of, rather than sumoylation of, PCNA contributes to the observed spontaneous mutator phenotype associated with proteasomal defects (McIntyre et al., 2006). An important role for sumoylation of PCNA seems to be in preventing unscheduled HR during S-phase (Papouli et al., 2005; Pfander et al., 2005). Nevertheless, recent studies suggest that PCNA sumoylation may be required for the stimulation of TLS in addition to the presence of monoubiquitinated PCNA (Halas et al., 2011). In the case of PCNA, SUMO and ubiquitin appear to cooperatively rather than

competitively coordinate the choice in DNA repair pathways of replication lesions (Plosky et al., 2006).

8. Fanconi anemia

Fanconi anemia (FA) is a rare autosomal recessive and X-linked genetic disorder characterized in part by congenital deformities, bone marrow failure, and elevated risk of cancer. FA is associated with hypersensitivity to DNA crosslinking agents, and treatment of FA cells with such agents (mitomycin C, for example) leads to chromosomal instability and cell death (Heinrich et al., 2000). 13 FA complementation groups have been identified (FANCA, B, C, D1, D2, E, F, G, I, J, L, M, and N), and disruption of any one of the genes named after these complementation groups causes FA. Proposed roles for the FA pathway include involvement in circumventing stalled replication forks, HR, TLS, enzymatic DNA processing, and cell-cycle regulation (Kennedy & D'Andrea, 2005; Pang & Andreassen, 2009; Kratz et al., 2010).

8.1 Fanconi anemia and DNA repair

At least 8 of the FA complementation groups (FANCA, B, C, E, F, G, L, and M) and two associated proteins, FAAP24 and FAAP100, form an E3 enzyme complex required for monoubiquitination of FANCD2 (Ciccia et al., 2007; Ling et al., 2007). Loss of any subunit of this FA core complex negatively alters complex formation and prevents FANCD2 monoubiquitination (Huang & D'andrea, 2006). Specifically, monoubiquitination of FANCD2 occurs at K561 during S-phase during normal cell-cycle progression as well as following DNA damage (Kennedy & D'Andrea, 2005). The latter event is linked to FANCD2 localization to chromatin and colocalization with other DNA damage signalling proteins such as FANCD1, Brca2, Rad51, Nbs1, and PCNA (Kennedy & D'Andrea, 2005; Nijman et al., 2005). FANCL is capable of autoubiquitination *in vitro* and is required for monoubiquitination of FANCD2 and its relocation to damage-associated nuclear foci, suggesting that FANCL is the catalytic component of the FA core complex (Meetei et al., 2003; Huang & D'andrea, 2006). Brca1 has the potential to serve as an E3 enzyme for FANCD2 *in vitro*, but genetic evidence hints that Brca1 is not required for FANCD2 monoubiquitination (Vandenberg et al., 2003). Instead, Brca1 may modulate monoubiquitination of FANCD2 or the stability of the modified protein (Meetei et al., 2003). UBE2T, which directly binds FANCL, is the E2 enzyme involved in monoubiquitination of FANCD2 (Machida et al., 2006). UBE2T engages in a negative feedback loop, commencing self-inactivation by automonoubiquitination *in vivo* (Machida et al., 2006). RPA1 and ATR are required for efficient FANCD2 monoubiquitination, and ATM is not necessary in this process (Andreassen et al., 2004). Similarly, silencing of ATR, but not of ATM, suppresses FANCD2 foci formation following exposure of cells to DNA crosslinking agents (Andreassen et al., 2004). As with PCNA, the DUB Usp1 associates with and regulates monoubiquitination of FANCD2, and Usp1 inhibition increases FANCD2 monoubiquitination (Nijman et al., 2005).

FANCD2 and FANCI share sequence homology (Smogorzewska et al., 2007). Small interfering RNAs (siRNAs) directed against FANCD2, FANCI, and FANCA suppress HR, suggesting a role of these proteins in the DSB repair pathway. Like FANCD2, FANCI is ubiquitinated following DNA damage or during normal S-phase. These two enzymes form a complex, known as ID, which dictates FANCD2 localization in damage-associated nuclear

foci. FANCD2 and FANCI are interdependent in that ubiquitin modification of each protein is dependent upon ubiquitin modification of the other (Smogorzewska et al., 2007). FANCI also restricts monoubiquitination of FANCD2 to the correct lysine residue *in vivo* (Alpi et al., 2008). FANCD2-ubiquitin and FANCD2-histone H2B fusion proteins harbouring an arginine substitution to remove the monoubiquitin site of FANCD2 have been shown to colocalize to chromatin and to reverse DNA-crosslink hypersensitivity of FANCD2-deficient cells (Matsushita et al., 2005). However, these fusion proteins do not complement FANCC-, FANCG-, or FANCL-deficient cells, suggesting that the members of the FA core complex play roles outside that of monoubiquitination and chromatin targeting of FANCD2 (Matsushita et al., 2005). The link between monoubiquitination of FANCD2/FANCI and recruitment of the ID complex to chromatin remains unclear (Al-Hakim et al., 2010). Recent findings surrounding the FANCD2-associated nuclease KIAA1018/FAN1 provide valuable information regarding the functional importance of ID complex ubiquitination. FAN1 is a conserved protein with an N-terminal ubiquitin-binding zinc finger (UBZ) and a C-terminal nuclease domain (Kratz et al., 2010; Liu et al., 2010; MacKay et al., 2010; Smogorzewska et al., 2010). FAN1 interacts with FANCD2 and FANCI, and loss of FAN1 is associated with hypersensitivity to and defective repair of intrastrand crosslinks. The UBZ of FAN1 targets the protein to damage-induced nuclear foci since deletion of the UBZ prevents FAN1 from accumulating at sites of damage (Kratz et al., 2010; Liu et al., 2010; MacKay et al., 2010; Smogorzewska et al., 2010). Importantly, this recruitment of FAN1 is dependent upon interaction of its UBZ domain with monoubiquitinated FANCD2. Although the mechanism of FAN1 is still being elucidated, it will be interesting to determine whether other factors which also recognize ID complex monoubiquitination are recruited to sites of damage by crosslinking agents (Al-Hakim et al., 2010).

9. Proteasome inhibitors and DNA repair

Proteasome inhibitors include synthetic molecules and natural products (Groll et al., 2009). Most small molecule proteasome inhibitors are peptide derivatives that bind to the proteolytic active sites of the 20S proteasome (Huang & Chen, 2009). Specifically, bortezomib (PS-341, Velcade) is a dipeptidyl boric acid that selectively and reversibly inhibits the β5-subunit of the 20S with high affinity (Berkers et al., 2005). Clinically, bortezomib administration results in tumor shrinkage through events such as cellular growth arrest, increased apoptosis, and decreased angiogenesis (Russo et al., 2007; Gilardini et al., 2008). The mechanisms by which bortezomib exerts its effects, however, remain controversial. It has been shown that bortezomib stabilizes p21, p27, p53, proapoptotic proteins such as Bid and Bax, and the transcription factor Myc (McConkey & Zhu, 2008; Orlowski & Kuhn, 2008). Inhibition of NF-κB and of anti-apoptotic proteins, generation of reactive oxygen species (ROS), and increases in endoplasmic reticulum (ER) stress have also been implicated by some and repudiated by others as potential bortezomib-stimulated outcomes (Chen et al., 2010). Nevertheless, although the exact mechanisms of bortezomib-induced cell death remain inconclusive, current research hints at a role of DNA repair in this process.

Bortezomib has been shown to enhance the DNA damage response. In a genome-wide siRNA screen to identify genetic components linked to bortezomib-induced cell death, knockdown of proteins responsible for HR was found to be protective against cell death

(Chen et al., 2010). Bortezomib treatment leads to stabilization of Rad51, upregulation of ATM-dependant phosphorylation of H2AFX, CHK2, SMC1A, and TP53 and of ATR-dependent phosphorylation of CHK1, as well as increased monoubiquitination of FANCD2 (Chen et al., 2010). Bortezomib has also been shown to inhibit basal and DNA damage-induced monoubiquitination of FANCD2 but not of PCNA (Jacquemont & Taniguchi, 2007).
A phase 3 clinical trial of bortezomib with melphalan and prednisone, the standard treatment of multiple myeloma patients, is currently ongoing with patients who cannot receive high-dose therapy (Chen et al., 2005; San Miguel et al., 2008). HR and FA are thought to be required for resistance to melphalan, a chemotherapeutic DNA alkylating agent, and bortezomib plus melphalan and prednisone produces outcomes greater than melphalan-prednisone alone (Wang et al., 2001; Chen et al., 2005). Since bortezomib appears to interfere with DNA-damage signaling, sensitization of cancer cells to melphalan and prednisone coud be due to the influence of the proteasome on one or more DNA repair pathway(s). Treatment of cells first with ionizing radiation or DNA crosslinking agents and secondly with bortezomib delays formation of phospho-ATM, 53BP1, Nbs1, and Brca1 foci and inhibits formation of FANCD2 and Rad51 foci. However, γ-H2AX, MDC1, and RPA foci are unaffected by these treatments, suggesting that early DNA damage signaling remains intact in these cells. This and similar results found using the proteasome inhibitors MG132 and epoxomicin (Jacquemont & Taniguchi, 2007; Takeshita et al., 2009) again suggest the requirement for UPS in DNA repair progression.

10. Conclusion

Here, we have summarized some of the current understanding of the relation of UPS to NER, BER, MMR, DSB, PRR, and FA repair. However, the influence of the UPS on DNA repair is not restricted to these repair mechanisms, as evidence suggests that the UPS also affects pathways beyond the scope of this review, such as direct repair by MGMT (O^6-methylguanine-DNA methyltransferase) (Vlachostergios et al., 2009a). Much evidence shows that the UPS is a potent regulator of repair following DNA damage. It will no doubt be interesting and beneficial to expand our knowledge of the UPS and DNA repair by using proteasome inhibitors such as bortezomib in conjunction with different chemotherapeutic and irradiation treatments.
The obligatory cooperation between DNA repair and UPS is clearly preserved throughout eukaryotic evolution (Daulny & Tansey, 2009); upon consideration, this is a very logical example of the interdependence of seemingly disparate biological systems. Sequential enzymatic activity is a hallmark of well-regulated biological processes. In DNA repair, it appears in many cases that replacement of one enzyme with another depends on its tightly regulated proteolysis or other UPS-mediated modification (Hardeland et al., 2002a; Steinacher & Schar, 2005a; Parsons et al., 2008a). The UPS has the necessary degree of control to execute the proteolysis in a manner required, enabling the repair processes to advance through different stages. A well-orchestrated activity of the two processes suggests that DNA repair and the UPS have co-evolved to provide living organisms with a way to cope with environmental DNA injuries. Increasingly complex UPS-mediated regulation of multifunctional repair enzymes such as PCNA, coinciding with evolution of higher eukaryotes, further supports this notion.
It is interesting to note that DNA repair in prokaryotes, where there is no proteasome system, is regulated predominantly at the transcriptional level (Friedberg, 1996). This

regulation often involves the removal of a single protein (a transcriptional repressor), which allows for a particular operon to be expressed. In eukaryotes, while there are a few DNA repair proteins that are transcriptionally regulated (for example, PCNA and DNA polymerase β), most DNA repair is regulated at the level of post-translational modifications such as phosphorylation, acetylation, protein complex formation, as well as monoubiquitination, polyubiquitination, sumoylation, etc. (Vlachostergios et al., 2009b; Vlachostergios et al., 2009a). The removal of specific proteins is needed for the DNA repair process progression in eukaryotic cells, but this removal in eukaryotes is more complex than the repressor system of DNA repair regulation prokaryotes—it requires the complex UPS to regulate protein accumulation and turnover (Motegi et al., 2009). It is possible that the expansion of the eukaryotic genome to contain large numbers genes on several chromosomes made it necessary for proteasome complexes to co-exist and control DNA repair protein quantities at the level of protein degradation.

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12. References

Al-Hakim, A.; Escribano-Diaz, C.; Landry, M.C.; O'Donnell, L.; Panier, S.; Szilard, R.K. & Durocher, D. (2010). The ubiquitous role of ubiquitin in the DNA damage response. *DNA Repair (Amst)*, Vo.9, No.12, pp. 1229-1240

Alpi, A.F.; Pace, P.E.; Babu, M.M. & Patel, K.J. (2008). Mechanistic insight into site-restricted monoubiquitination of FANCD2 by Ube2t, FANCL, and FANCI. *Molecular Cell*, Vo.32, No.6, pp. 767-777

Andreassen, P.R.; D'Andrea, A.D. & Taniguchi, T. (2004). ATR couples FANCD2 monoubiquitination to the DNA-damage response. *Genes and Development*, Vo.18, No.16, pp. 1958-1963

Arrigo, A.P.; Tanaka, K.; Goldberg, A.L. & Welch, W.J. (1988). Identity of the 19S 'prosome' particle with the large multifunctional protease complex of mammalian cells (the proteasome). *Nature*, Vo.331, No.6152, pp. 192-194

Bergink, S.; Jaspers, N.G. & Vermeulen, W. (2007). Regulation of UV-induced DNA damage response by ubiquitylation. *DNA Repair (Amst)*, Vo.6, No.9, pp. 1231-1242

Bergink, S.; Salomons, F.A.; Hoogstraten, D.; Groothuis, T.A.; de Waard, H.; Wu, J.; Yuan, L.; Citterio, E.; Houtsmuller, A.B.; Neefjes, J.; Hoeijmakers, J.H.; Vermeulen, W. & Dantuma, N.P. (2006). DNA damage triggers nucleotide excision repair-dependent monoubiquitylation of histone H2A. *Genes and Development*, Vo.20, No.10, pp. 1343-1352

Bergoglio, V. & Magnaldo, T. (2006). Nucleotide excision repair and related human diseases. *Genome Dyn*, Vo.1, pp. 35-52

Berkers, C.R.; Verdoes, M.; Lichtman, E.; Fiebiger, E.; Kessler, B.M.; Anderson, K.C.; Ploegh, H.L.; Ovaa, H. & Galardy, P.J. (2005). Activity probe for in vivo profiling of the specificity of proteasome inhibitor bortezomib. *Nat Methods*, Vo.2, No.5, pp. 357-362

Bienko, M.; Green, C.M.; Crosetto, N.; Rudolf, F.; Zapart, G.; Coull, B.; Kannouche, P.; Wider, G.; Peter, M.; Lehmann, A.R.; Hofmann, K. & Dikic, I. (2005). Ubiquitin-binding domains in Y-family polymerases regulate translesion synthesis. *Science*, Vo.310, No.5755, pp. 1821-1824

Chen, A.; Kleiman, F.E.; Manley, J.L.; Ouchi, T. & Pan, Z.Q. (2002). Autoubiquitination of the BRCA1*BARD1 RING ubiquitin ligase. *J Biol Chem*, Vo.277, No.24, pp. 22085-22092

Chen, L. & Chen, J. (2003). MDM2-ARF complex regulates p53 sumoylation. *Oncogene*, Vo.22, No.34, pp. 5348-5357

Chen, L.; Shinde, U.; Ortolan, T.G. & Madura, K. (2001). Ubiquitin-associated (UBA) domains in Rad23 bind ubiquitin and promote inhibition of multi-ubiquitin chain assembly. *EMBO Rep*, Vo.2, No.10, pp. 933-938

Chen, Q.; Van der Sluis, P.C.; Boulware, D.; Hazlehurst, L.A. & Dalton, W.S. (2005). The FA/BRCA pathway is involved in melphalan-induced DNA interstrand cross-link repair and accounts for melphalan resistance in multiple myeloma cells. *Blood*, Vo.106, No.2, pp. 698-705

Chen, S.Q.; Blank, J.L.; Peters, T.; Liu, X.Z.J.; Rappoli, D.M.; Pickard, M.D.; Menon, S.; Yu, J.; Driscoll, D.L.; Lingaraj, T.; Burkhardt, A.L.; Chen, W.; Garcia, K.; Sappal, D.S.; Gray, J.; Hales, P.; Leroy, P.J.; Ringeling, J.; Rabino, C.; Spelman, J.J.; Morgenstern, J.P. & Lightcap, E.S. (2010). Genome-Wide siRNA Screen for Modulators of Cell Death Induced by Proteasome Inhibitor Bortezomib. *Cancer Research*, Vo.70, No.11, pp. 4318-4326

Chen, X.; Ruggiero, C. & Li, S. (2007). Yeast Rpb9 plays an important role in ubiquitylation and degradation of Rpb1 in response to UV-induced DNA damage. *Molecular and Cellular Biology*, Vo.27, No.13, pp. 4617-4625

Chen, Z.J. & Sun, L.J. (2009). Nonproteolytic functions of ubiquitin in cell signaling. *Molecular Cell*, Vo.33, No.3, pp. 275-286

Ciccia, A.; Ling, C.; Coulthard, R.; Yan, Z.; Xue, Y.; Meetei, A.R.; Laghmani el, H.; Joenje, H.; McDonald, N.; de Winter, J.P.; Wang, W. & West, S.C. (2007). Identification of FAAP24, a Fanconi anemia core complex protein that interacts with FANCM. *Molecular Cell*, Vo.25, No.3, pp. 331-343

Ciechanover, A. (1994). The ubiquitin-proteasome proteolytic pathway. *Cell*, Vo.79, No.1, pp. 13-21

Ciechanover, A.; Elias, S.; Heller, H.; Ferber, S. & Hershko, A. (1980a). Characterization of the heat-stable polypeptide of the ATP-dependent proteolytic system from reticulocytes. *Journal of Biological Chemistry*, Vo.255, No.16, pp. 7525-7528

Ciechanover, A.; Heller, H.; Elias, S.; Haas, A.L. & Hershko, A. (1980b). Atp-Dependent Conjugation of Reticulocyte Proteins with the Polypeptide Required for Protein-Degradation. *Proceedings of the National Academy of Sciences of the United States of America-Biological Sciences*, Vo.77, No.3, pp. 1365-1368

Ciechanover, A.; Hod, Y. & Hershko, A. (1978). Heat-Stable Polypeptide Component of an Atp-Dependent Proteolytic System from Reticulocytes. *Biochemical and Biophysical Research Communications*, Vo.81, No.4, pp. 1100-1105

Coutts, A.S.; Adams, C.J. & La Thangue, N.B. (2009). p53 ubiquitination by Mdm2: A never ending tail? *DNA Repair*, Vo.8, No.4, pp. 483-490

Dahlmann, B. (2007). Role of proteasomes in disease. *Bmc Biochemistry*, Vo.8, pp. -

Dalhus, B.; Laerdahl, J.K.; Backe, P.H. & Bjoras, M. (2009). DNA base repair--recognition and initiation of catalysis. *FEMS Microbiology Reviews*, Vo.33, No.6, pp. 1044-1078

Dantuma, N.P.; Heinen, C. & Hoogstraten, D. (2009). The ubiquitin receptor Rad23: At the crossroads of nucleotide excision repair and proteasomal degradation. *DNA Repair*, Vo.8, No.4, pp. 449-460

Daulny, A. & Tansey, W.P. (2009). Damage control: DNA repair, transcription, and the ubiquitin-proteasome system. *DNA Repair*, Vo.8, No.4, pp. 444-448

Doil, C.; Mailand, N.; Bekker-Jensen, S.; Menard, P.; Larsen, D.H.; Pepperkok, R.; Ellenberg, J.; Panier, S.; Durocher, D.; Bartek, J.; Lukas, J. & Lukas, C. (2009). RNF168 binds and amplifies ubiquitin conjugates on damaged chromosomes to allow accumulation of repair proteins. *Cell*, Vo.136, No.3, pp. 435-446

Downs, J.A. & Jackson, S.P. (2004). A means to a DNA end: the many roles of Ku. *Nature reviews. Molecular cell biology*, Vo.5, No.5, pp. 367-378

Drexler, H.C. (1997). Activation of the cell death program by inhibition of proteasome function. *Proceedings of the National Academy of Sciences of the United States of America*, Vo.94, No.3, pp. 855-860

El-Shemerly, M.; Janscak, P.; Hess, D.; Jiricny, J. & Ferrari, S. (2005). Degradation of human exonuclease 1b upon DNA synthesis inhibition. *Cancer Research*, Vo.65, No.9, pp. 3604-3609

Enokido, Y.; Tamura, T.; Ito, H.; Arumughan, A.; Komuro, A.; Shiwaku, H.; Sone, M.; Foulle, R.; Sawada, H.; Ishiguro, H.; Ono, T.; Murata, M.; Kanazawa, I.; Tomilin, N.; Tagawa, K.; Wanker, E.E. & Okazawa, H. (2010). Mutant huntingtin impairs Ku70-mediated DNA repair. *J Cell Biol*, Vo.189, No.3, pp. 425-443

Finley, D. (2009). Recognition and Processing of Ubiquitin-Protein Conjugates by the Proteasome. *Annual Review of Biochemistry*, Vo.78, pp. 477-513

Fishbain, S.; Prakash, S.; Herrig, A.; Elsasser, S. & Matouschek, A. (2011). Rad23 escapes degradation because it lacks a proteasome initiation region. *Nat Commun*, Vo.2, pp. 192

Foster, R.E.; Nnakwe, C.; Woo, L. & Frank, K.M. (2006). Monoubiquitination of the nonhomologous end joining protein XRCC4. *Biochemical and Biophysical Research Communications*, Vo.341, No.1, pp. 175-183

Friedberg, E.C. (1996). Relationships between DNA repair and transcription. *Annu Rev Biochem*, Vo.65, pp. 15-42

Gama, V.; Yoshida, T.; Gomez, J.A.; Basile, D.P.; Mayo, L.D.; Haas, A.L. & Matsuyama, S. (2006). Involvement of the ubiquitin pathway in decreasing Ku70 levels in response to drug-induced apoptosis. *Experimental Cell Research*, Vo.312, No.4, pp. 488-499

Garg, P. & Burgers, P.M. (2005). Ubiquitinated proliferating cell nuclear antigen activates translesion DNA polymerases eta and REV1. *Proceedings of the National Academy of Sciences of the United States of America*, Vo.102, No.51, pp. 18361-18366

Garg, P.; Stith, C.M.; Majka, J. & Burgers, P.M. (2005). Proliferating cell nuclear antigen promotes translesion synthesis by DNA polymerase zeta. *Journal of Biological Chemistry*, Vo.280, No.25, pp. 23446-23450

Genschel, J.; Bazemore, L.R. & Modrich, P. (2002). Human exonuclease I is required for 5' and 3' mismatch repair. *Journal of Biological Chemistry*, Vo.277, No.15, pp. 13302-13311

Gilardini, A.; Marmiroli, P. & Cavaletti, G. (2008). Proteasome Inhibition: A Promising Strategy for Treating Cancer, but What About Neurotoxicity? *Current Medicinal Chemistry*, Vo.15, No.29, pp. 3025-3035

Gillette, T.G.; Huang, W.; Russell, S.J.; Reed, S.H.; Johnston, S.A. & Friedberg, E.C. (2001). The 19S complex of the proteasome regulates nucleotide excision repair in yeast. *Genes and Development*, Vo.15, No.12, pp. 1528-1539

Gillette, T.G.; Yu, S.; Zhou, Z.; Waters, R.; Johnston, S.A. & Reed, S.H. (2006). Distinct functions of the ubiquitin-proteasome pathway influence nucleotide excision repair. *EMBO Journal*, Vo.25, No.11, pp. 2529-2538

Gocke, C.B.; Yu, H. & Kang, J. (2005). Systematic identification and analysis of mammalian small ubiquitin-like modifier substrates. *Journal of Biological Chemistry*, Vo.280, No.6, pp. 5004-5012

Goldberg, A.L.; Cascio, P.; Saric, T. & Rock, K.L. (2002). The importance of the proteasome and subsequent proteolytic steps in the generation of antigenic peptides. *Molecular Immunology*, Vo.39, No.3-4, pp. 147-164

Gong, F.; Fahy, D. & Smerdon, M.J. (2006). Rad4-Rad23 interaction with SWI/SNF links ATP-dependent chromatin remodeling with nucleotide excision repair. *Nat Struct Mol Biol*, Vo.13, No.10, pp. 902-907

Gottlieb, T.M. & Jackson, S.P. (1993). The DNA-dependent protein kinase: requirement for DNA ends and association with Ku antigen. *Cell*, Vo.72, No.1, pp. 131-142

Groisman, R.; Polanowska, J.; Kuraoka, I.; Sawada, J.; Saijo, M.; Drapkin, R.; Kisselev, A.F.; Tanaka, K. & Nakatani, Y. (2003). The ubiquitin ligase activity in the DDB2 and CSA complexes is differentially regulated by the COP9 signalosome in response to DNA damage. *Cell*, Vo.113, No.3, pp. 357-367

Groll, M.; Ditzel, L.; Lowe, J.; Stock, D.; Bochtler, M.; Bartunik, H.D. & Huber, R. (1997). Structure of 20S proteasome from yeast at 2.4 A resolution. *Nature*, Vo.386, No.6624, pp. 463-471

Groll, M.; Huber, R. & Moroder, L. (2009). The persisting challenge of selective and specific proteasome inhibition. *Journal of Peptide Science*, Vo.15, No.2, pp. 58-66

Gudmundsdottir, K.; Lord, C.J. & Ashworth, A. (2007). The proteasome is involved in determining differential utilization of double-strand break repair pathways. *Oncogene*, Vo.26, No.54, pp. 7601-7606

Guzder, J.; Paris, J.; Zelkowitz, P. & Feldman, R. (1999). Psychological risk factors for borderline pathology in school-age children. *Journal of the American Academy of Child and Adolescent Psychiatry*, Vo.38, No.2, pp. 206-212

Guzder, S.N.; Sung, P.; Prakash, L. & Prakash, S. (1998). Affinity of yeast nucleotide excision repair factor 2, consisting of the Rad4 and Rad23 proteins, for ultraviolet damaged DNA. *Journal of Biological Chemistry*, Vo.273, No.47, pp. 31541-31546

Halas, A.; Podlaska, A.; Derkacz, J.; McIntyre, J.; Skoneczna, A. & Sledziewska-Gojska, E. (2011). The roles of PCNA SUMOylation, Mms2-Ubc13 and Rad5 in translesion DNA synthesis in Saccharomyces cerevisiae. *Molecular Microbiology*, Vo., pp.

Hanna, J. & Finley, D. (2007). A proteasome for all occasions. *FEBS Letters*, Vo.581, No.15, pp. 2854-2861

Hardeland, U.; Kunz, C.; Focke, F.; Szadkowski, M. & Schar, P. (2007). Cell cycle regulation as a mechanism for functional separation of the apparently redundant uracil DNA glycosylases TDG and UNG2. *Nucleic Acids Res*, Vo.35, No.11, pp. 3859-3867

Hardeland, U.; Steinacher, R.; Jiricny, J. & Schar, P. (2002a). Modification of the human thymine-DNA glycosylase by ubiquitin-like proteins facilitates enzymatic turnover. *EMBO J*, Vo.21, No.6, pp. 1456-1464

Hardeland, U.; Steinacher, R.; Jiricny, J. & Schar, P. (2002b). Modification of the human thymine-DNA glycosylase by ubiquitin-like proteins facilitates enzymatic turnover. *EMBO Journal*, Vo.21, No.6, pp. 1456-1464

Hashizume, R.; Fukuda, M.; Maeda, I.; Nishikawa, H.; Oyake, D.; Yabuki, Y.; Ogata, H. & Ohta, T. (2001). The RING heterodimer BRCA1-BARD1 is a ubiquitin ligase inactivated by a breast cancer-derived mutation. *Journal of Biological Chemistry*, Vo.276, No.18, pp. 14537-14540

Heinrich, M.C.; Silvey, K.V.; Stone, S.; Zigler, A.J.; Griffith, D.J.; Montalto, M.; Chai, L.; Zhi, Y. & Hoatlin, M.E. (2000). Posttranscriptional cell cycle-dependent regulation of human FANCC expression. *Blood*, Vo.95, No.12, pp. 3970-3977

Hernandez-Pigeon, H.; Laurent, G.; Humbert, O.; Salles, B. & Lautier, D. (2004). Degadration of mismatch repair hMutSalpha heterodimer by the ubiquitin-proteasome pathway. *FEBS letters*, Vo.562, No.1-3, pp. 40-44

Hernandez-Pigeon, H.; Quillet-Mary, A.; Louat, T.; Schambourg, A.; Humbert, O.; Selves, J.; Salles, B.; Laurent, G. & Lautier, D. (2005). hMutS alpha is protected from ubiquitin-proteasome-dependent degradation by atypical protein kinase C zeta phosphorylation. *Journal of molecular biology*, Vo.348, No.1, pp. 63-74

Herrmann, J.; Lerman, L.O. & Lerman, A. (2007). Ubiquitin and ubiquitin-like proteins in protein regulation. *Circulation Research*, Vo.100, No.9, pp. 1276-1291

Hershko, A.; Ciechanover, A.; Heller, H.; Haas, A.L. & Rose, I.A. (1980). Proposed Role of Atp in Protein Breakdown - Conjugation of Proteins with Multiple Chains of the Polypeptide of Atp-Dependent Proteolysis. *Proceedings of the National Academy of Sciences of the United States of America-Biological Sciences*, Vo.77, No.4, pp. 1783-1786

Hershko, A.; Ciechanover, A. & Varshavsky, A. (2000). The ubiquitin system. *Nature Medicine*, Vo.6, No.10, pp. 1073-1081

Hideshima, T.; Richardson, P.; Chauhan, D.; Palombella, V.J.; Elliott, P.J.; Adams, J. & Anderson, K.C. (2001). The proteasome inhibitor PS-341 inhibits growth, induces apoptosis, and overcomes drug resistance in human multiple myeloma cells. *Cancer Research*, Vo.61, No.7, pp. 3071-3076

Hoege, C.; Pfander, B.; Moldovan, G.L.; Pyrowolakis, G. & Jentsch, S. (2002). RAD6-dependent DNA repair is linked to modification of PCNA by ubiquitin and SUMO. *Nature*, Vo.419, No.6903, pp. 135-141

Hofmann, K. (2009). Ubiquitin-binding domains and their role in the DNA damage response. *DNA Repair (Amst)*, Vo.8, No.4, pp. 544-556

Hori, T.; Osaka, F.; Chiba, T.; Miyamoto, C.; Okabayashi, K.; Shimbara, N.; Kato, S. & Tanaka, K. (1999). Covalent modification of all members of human cullin family proteins by NEDD8. *Oncogene*, Vo.18, No.48, pp. 6829-6834

Hough, R.; Pratt, G. & Rechsteiner, M. (1986). Ubiquitin-lysozyme conjugates. Identification and characterization of an ATP-dependent protease from rabbit reticulocyte lysates. *Journal of Biological Chemistry*, Vo.261, No.5, pp. 2400-2408

Huang, L. & Chen, C.H. (2009). Proteasome Regulators: Activators and Inhibitors. *Current Medicinal Chemistry*, Vo.16, No.8, pp. 931-939

Huang, T.T. & D'andrea, A.D. (2006). Regulation of DNA repair by ubiquitylation. *Nature Reviews Molecular Cell Biology*, Vo.7, No.5, pp. 323-334

Huang, T.T.; Nijman, S.M.; Mirchandani, K.D.; Galardy, P.J.; Cohn, M.A.; Haas, W.; Gygi, S.P.; Ploegh, H.L.; Bernards, R. & D'Andrea, A.D. (2006). Regulation of monoubiquitinated PCNA by DUB autocleavage. *Nature cell biology*, Vo.8, No.4, pp. 339-347

Huen, M.S.; Grant, R.; Manke, I.; Minn, K.; Yu, X.; Yaffe, M.B. & Chen, J. (2007). RNF8 transduces the DNA-damage signal via histone ubiquitylation and checkpoint protein assembly. *Cell*, Vo.131, No.5, pp. 901-914

Humbert, O.; Hermine, T.; Hernandez, H.; Bouget, T.; Selves, J.; Laurent, G.; Salles, B. & Lautier, D. (2002). Implication of protein kinase C in the regulation of DNA mismatch repair protein expression and function. *Journal of Biological Chemistry*, Vo.277, No.20, pp. 18061-18068

Iovine, B.; Iannella, M.L. & Bevilacqua, M.A. (2011). Damage-specific DNA binding protein 1 (DDB1) is involved in ubiquitin-mediated proteolysis of p27(Kip1) in response to UV irradiation. *Biochimie*, Vo., pp.

Jacquemont, C. & Taniguchi, T. (2007). Proteasome function is required for DNA damage response and fanconi anemia pathway activation. *Cancer Research*, Vo.67, No.15, pp. 7395-7405

Jansen, L.E.; Verhage, R.A. & Brouwer, J. (1998). Preferential binding of yeast Rad4.Rad23 complex to damaged DNA. *Journal of Biological Chemistry*, Vo.273, No.50, pp. 33111-33114

Jelinsky, S.A.; Estep, P.; Church, G.M. & Samson, L.D. (2000). Regulatory networks revealed by transcriptional profiling of damaged Saccharomyces cerevisiae cells: Rpn4 links base excision repair with proteasomes. *Molecular and Cellular Biology*, Vo.20, No.21, pp. 8157-8167

Jentsch, S.; McGrath, J.P. & Varshavsky, A. (1987). The yeast DNA repair gene RAD6 encodes a ubiquitin-conjugating enzyme. *Nature*, Vo.329, No.6135, pp. 131-134

Jiricny, J. (1998). Replication errors: cha(lle)nging the genome. *The EMBO journal*, Vo.17, No.22, pp. 6427-6436

Johnson, E.S. (2004). Protein modification by SUMO. *Annual Review of Biochemistry*, Vo.73, pp. 355-382

Ju, D.; Wang, X.; Ha, S.W.; Fu, J. & Xie, Y. (2010). Inhibition of proteasomal degradation of rpn4 impairs nonhomologous end-joining repair of DNA double-strand breaks. *PloS one*, Vo.5, No.4, pp. e9877

Kannouche, P.L.; Wing, J. & Lehmann, A.R. (2004). Interaction of human DNA polymerase eta with monoubiquitinated PCNA: a possible mechanism for the polymerase switch in response to DNA damage. *Molecular Cell*, Vo.14, No.4, pp. 491-500

Kennedy, R.D. & D'Andrea, A.D. (2005). The Fanconi Anemia/BRCA pathway: new faces in the crowd. *Genes and Development*, Vo.19, No.24, pp. 2925-2940

Kisselev, A.F.; Akopian, T.N.; Woo, K.M. & Goldberg, A.L. (1999). The sizes of peptides generated from protein by mammalian 26 and 20 S proteasomes. Implications for understanding the degradative mechanism and antigen presentation. *Journal of Biological Chemistry*, Vo.274, No.6, pp. 3363-3371

Kolas, N.K.; Chapman, J.R.; Nakada, S.; Ylanko, J.; Chahwan, R.; Sweeney, F.D.; Panier, S.; Mendez, M.; Wildenhain, J.; Thomson, T.M.; Pelletier, L.; Jackson, S.P. & Durocher,

D. (2007). Orchestration of the DNA-damage response by the RNF8 ubiquitin ligase. *Science*, Vo.318, No.5856, pp. 1637-1640

Kolodner, R.D. & Marsischky, G.T. (1999). Eukaryotic DNA mismatch repair. *Current opinion in genetics & development*, Vo.9, No.1, pp. 89-96

Kratz, K.; Schopf, B.; Kaden, S.; Sendoel, A.; Eberhard, R.; Lademann, C.; Cannavo, E.; Sartori, A.A.; Hengartner, M.O. & Jiricny, J. (2010). Deficiency of FANCD2-associated nuclease KIAA1018/FAN1 sensitizes cells to interstrand crosslinking agents. *Cell*, Vo.142, No.1, pp. 77-88

Kristensen, C.N.; Bystol, K.M.; Li, B.; Serrano, L. & Brenneman, M.A. (2010). Depletion of DSS1 protein disables homologous recombinational repair in human cells. *Mutation research*, Vo.694, No.1-2, pp. 60-64

Krogan, N.J.; Lam, M.H.; Fillingham, J.; Keogh, M.C.; Gebbia, M.; Li, J.; Datta, N.; Cagney, G.; Buratowski, S.; Emili, A. & Greenblatt, J.F. (2004). Proteasome involvement in the repair of DNA double-strand breaks. *Molecular cell*, Vo.16, No.6, pp. 1027-1034

Lee, K.Y. & Myung, K. (2008). PCNA modifications for regulation of post-replication repair pathways. *Molecules and cells*, Vo.26, No.1, pp. 5-11

Leibeling, D.; Laspe, P. & Emmert, S. (2006). Nucleotide excision repair and cancer. *J Mol Histol*, Vo.37, No.5-7, pp. 225-238

Li, G.M. (2008). Mechanisms and functions of DNA mismatch repair. *Cell Research*, Vo.18, No.1, pp. 85-98

Li, W.; Hesabi, B.; Babbo, A.; Pacione, C.; Liu, J.; Chen, D.J.; Nickoloff, J.A. & Shen, Z. (2000). Regulation of double-strand break-induced mammalian homologous recombination by UBL1, a RAD51-interacting protein. *Nucleic Acids Res*, Vo.28, No.5, pp. 1145-1153

Li, X. & Heyer, W.D. (2008). Homologous recombination in DNA repair and DNA damage tolerance. *Cell research*, Vo.18, No.1, pp. 99-113

Liggett, A.; Crawford, L.J.; Walker, B.; Morris, T.C.M. & Irvine, A.E. (2010). Methods for measuring proteasome activity: Current limitations and future developments. *Leukemia Research*, Vo.34, No.11, pp. 1403-1409

Ling, C.; Ishiai, M.; Ali, A.M.; Medhurst, A.L.; Neveling, K.; Kalb, R.; Yan, Z.; Xue, Y.; Oostra, A.B.; Auerbach, A.D.; Hoatlin, M.E.; Schindler, D.; Joenje, H.; de Winter, J.P.; Takata, M.; Meetei, A.R. & Wang, W. (2007). FAAP100 is essential for activation of the Fanconi anemia-associated DNA damage response pathway. *EMBO Journal*, Vo.26, No.8, pp. 2104-2114

Liu, T.; Ghosal, G.; Yuan, J.; Chen, J. & Huang, J. (2010). FAN1 acts with FANCI-FANCD2 to promote DNA interstrand cross-link repair. *Science*, Vo.329, No.5992, pp. 693-696

Lommel, L.; Chen, L.; Madura, K. & Sweder, K. (2000). The 26S proteasome negatively regulates the level of overall genomic nucleotide excision repair. *Nucleic Acids Res*, Vo.28, No.24, pp. 4839-4845

Lommel, L.; Ortolan, T.; Chen, L.; Madura, K. & Sweder, K.S. (2002). Proteolysis of a nucleotide excision repair protein by the 26 S proteasome. *Current Genetics*, Vo.42, No.1, pp. 9-20

Machida, Y.J.; Machida, Y.; Chen, Y.; Gurtan, A.M.; Kupfer, G.M.; D'Andrea, A.D. & Dutta, A. (2006). UBE2T is the E2 in the Fanconi anemia pathway and undergoes negative autoregulation. *Molecular Cell*, Vo.23, No.4, pp. 589-596

MacKay, C.; Declais, A.C.; Lundin, C.; Agostinho, A.; Deans, A.J.; MacArtney, T.J.; Hofmann, K.; Gartner, A.; West, S.C.; Helleday, T.; Lilley, D.M. & Rouse, J. (2010). Identification of KIAA1018/FAN1, a DNA repair nuclease recruited to DNA damage by monoubiquitinated FANCD2. *Cell*, Vo.142, No.1, pp. 65-76

Magill, L.; Walker, B. & Irvine, A.E. (2003). The proteasome: a novel therapeutic target in haematopoietic malignancy. *Hematology*, Vo.8, No.5, pp. 275-283

Mailand, N.; Bekker-Jensen, S.; Faustrup, H.; Melander, F.; Bartek, J.; Lukas, C. & Lukas, J. (2007). RNF8 ubiquitylates histones at DNA double-strand breaks and promotes assembly of repair proteins. *Cell*, Vo.131, No.5, pp. 887-900

Mao, P. & Smerdon, M.J. (2010). Yeast deubiquitinase Ubp3 interacts with the 26 S proteasome to facilitate Rad4 degradation. *Journal of Biological Chemistry*, Vo.285, No.48, pp. 37542-37550

Marston, N.J.; Richards, W.J.; Hughes, D.; Bertwistle, D.; Marshall, C.J. & Ashworth, A. (1999). Interaction between the product of the breast cancer susceptibility gene BRCA2 and DSS1, a protein functionally conserved from yeast to mammals. *Molecular and cellular biology*, Vo.19, No.7, pp. 4633-4642

Masson, M.; Menissier-de Murcia, J.; Mattei, M.G.; de Murcia, G. & Niedergang, C.P. (1997). Poly(ADP-ribose) polymerase interacts with a novel human ubiquitin conjugating enzyme: hUbc9. *Gene*, Vo.190, No.2, pp. 287-296

Masutani, C.; Araki, M.; Sugasawa, K.; van der Spek, P.J.; Yamada, A.; Uchida, A.; Maekawa, T.; Bootsma, D.; Hoeijmakers, J.H. & Hanaoka, F. (1997). Identification and characterization of XPC-binding domain of hHR23B. *Molecular and Cellular Biology*, Vo.17, No.12, pp. 6915-6923

Matsushita, N.; Kitao, H.; Ishiai, M.; Nagashima, N.; Hirano, S.; Okawa, K.; Ohta, T.; Yu, D.S.; McHugh, P.J.; Hickson, I.D.; Venkitaraman, A.R.; Kurumizaka, H. & Takata, M. (2005). A FancD2-monoubiquitin fusion reveals hidden functions of Fanconi anemia core complex in DNA repair. *Molecular Cell*, Vo.19, No.6, pp. 841-847

McConkey, D.J. & Zhu, K. (2008). Mechanisms of proteasome inhibitor action and resistance in cancer. *Drug Resist Updat*, Vo.11, No.4-5, pp. 164-179

McIntyre, J.; Podlaska, A.; Skoneczna, A.; Halas, A. & Sledziewska-Gojska, E. (2006). Analysis of the spontaneous mutator phenotype associated with 20S proteasome deficiency in S. cerevisiae. *Mutation research*, Vo.593, No.1-2, pp. 153-163

Meetei, A.R.; de Winter, J.P.; Medhurst, A.L.; Wallisch, M.; Waisfisz, Q.; van de Vrugt, H.J.; Oostra, A.B.; Yan, Z.; Ling, C.; Bishop, C.E.; Hoatlin, M.E.; Joenje, H. & Wang, W. (2003). A novel ubiquitin ligase is deficient in Fanconi anemia. *Nature Genetics*, Vo.35, No.2, pp. 165-170

Memisoglu, A. & Samson, L. (2000). Base excision repair in yeast and mammals. *Mutation Research*, Vo.451, No.1-2, pp. 39-51

Messner, S.; Schuermann, D.; Altmeyer, M.; Kassner, I.; Schmidt, D.; Schar, P.; Muller, S. & Hottiger, M.O. (2009). Sumoylation of poly(ADP-ribose) polymerase 1 inhibits its acetylation and restrains transcriptional coactivator function. *FASEB Journal*, Vo.23, No.11, pp. 3978-3989

Mimnaugh, E.G.; Yunmbam, M.K.; Li, Q.; Bonvini, P.; Hwang, S.G.; Trepel, J.; Reed, E. & Neckers, L. (2000). Prevention of cisplatin-DNA adduct repair and potentiation of cisplatin-induced apoptosis in ovarian carcinoma cells by proteasome inhibitors. *Biochemical Pharmacology*, Vo.60, No.9, pp. 1343-1354

Miyase, S.; Tateishi, S.; Watanabe, K.; Tomita, K.; Suzuki, K.; Inoue, H. & Yamaizumi, M. (2005). Differential regulation of Rad18 through Rad6-dependent mono- and polyubiquitination. *The Journal of biological chemistry*, Vo.280, No.1, pp. 515-524

Modrich, P. & Lahue, R. (1996). Mismatch repair in replication fidelity, genetic recombination, and cancer biology. *Annual Review of Biochemistry*, Vo.65, pp. 101-133

Moschos, S.J. & Mo, Y.Y. (2006). Role of SUMO/Ubc9 in DNA damage repair and tumorigenesis. *J Mol Histol*, Vo.37, No.5-7, pp. 309-319

Motegi, A.; Murakawa, Y. & Takeda, S. (2009). The vital link between the ubiquitin-proteasome pathway and DNA repair: Impact on cancer therapy. *Cancer Letters*, Vo.283, No.1, pp. 1-9

Mueller, J.P. & Smerdon, M.J. (1996). Rad23 is required for transcription-coupled repair and efficient overrall repair in Saccharomyces cerevisiae. *Molecular and Cellular Biology*, Vo.16, No.5, pp. 2361-2368

Murakawa, Y.; Sonoda, E.; Barber, L.J.; Zeng, W.; Yokomori, K.; Kimura, H.; Niimi, A.; Lehmann, A.; Zhao, G.Y.; Hochegger, H.; Boulton, S.J. & Takeda, S. (2007). Inhibitors of the proteasome suppress homologous DNA recombination in mammalian cells. *Cancer Research*, Vo.67, No.18, pp. 8536-8543

Naiki, H. & Nagai, Y. (2009). Molecular Pathogenesis of Protein Misfolding Diseases: Pathological Molecular Environments Versus Quality Control Systems Against Misfolded Proteins. *Journal of Biochemistry*, Vo.146, No.6, pp. 751-756

Nakamura, K.; Sakai, W.; Kawamoto, T.; Bree, R.T.; Lowndes, N.F.; Takeda, S. & Taniguchi, Y. (2006). Genetic dissection of vertebrate 53BP1: a major role in non-homologous end joining of DNA double strand breaks. *DNA Repair (Amst)*, Vo.5, No.6, pp. 741-749

Navon, A. & Ciechanover, A. (2009). The 26 S Proteasome: From Basic Mechanisms to Drug Targeting. *Journal of Biological Chemistry*, Vo.284, No.49, pp. 33713-33718

Nijman, S.M.; Huang, T.T.; Dirac, A.M.; Brummelkamp, T.R.; Kerkhoven, R.M.; D'Andrea, A.D. & Bernards, R. (2005). The deubiquitinating enzyme USP1 regulates the Fanconi anemia pathway. *Molecular Cell*, Vo.17, No.3, pp. 331-339

Ohuchi, T.; Seki, M.; Branzei, D.; Maeda, D.; Ui, A.; Ogiwara, H.; Tada, S. & Enomoto, T. (2008). Rad52 sumoylation and its involvement in the efficient induction of homologous recombination. *DNA Repair (Amst)*, Vo.7, No.6, pp. 879-889

Orlowski, M. & Wilk, S. (2000). Catalytic activities of the 20 S proteasome, a multicatalytic proteinase complex. *Archives of Biochemistry and Biophysics*, Vo.383, No.1, pp. 1-16

Orlowski, R.Z. & Kuhn, D.J. (2008). Proteasome inhibitors in cancer therapy: Lessons from the first decade. *Clinical Cancer Research*, Vo.14, No.6, pp. 1649-1657

Ortolan, T.G.; Chen, L.; Tongaonkar, P. & Madura, K. (2004). Rad23 stabilizes Rad4 from degradation by the Ub/proteasome pathway. *Nucleic Acids Res*, Vo.32, No.22, pp. 6490-6500

Ortolan, T.G.; Tongaonkar, P.; Lambertson, D.; Chen, L.; Schauber, C. & Madura, K. (2000). The DNA repair protein rad23 is a negative regulator of multi-ubiquitin chain assembly. *Nat Cell Biol*, Vo.2, No.9, pp. 601-608

Palombella, V.J.; Conner, E.M.; Fuseler, J.W.; Destree, A.; Davis, J.M.; Laroux, F.S.; Wolf, R.E.; Huang, J.; Brand, S.; Elliott, P.J.; Lazarus, D.; McCormack, T.; Parent, L.; Stein, R.; Adams, J. & Grisham, M.B. (1998). Role of the proteasome and NF-kappaB in

streptococcal cell wall-induced polyarthritis. *Proceedings of the National Academy of Sciences of the United States of America*, Vo.95, No.26, pp. 15671-15676

Pang, Q. & Andreassen, P.R. (2009). Fanconi anemia proteins and endogenous stresses. *Mutation Research*, Vo.668, No.1-2, pp. 42-53

Papouli, E.; Chen, S.; Davies, A.A.; Huttner, D.; Krejci, L.; Sung, P. & Ulrich, H.D. (2005). Crosstalk between SUMO and ubiquitin on PCNA is mediated by recruitment of the helicase Srs2p. *Molecular Cell*, Vo.19, No.1, pp. 123-133

Parsons, J.L.; Dianova, II; Finch, D.; Tait, P.S.; Strom, C.E.; Helleday, T. & Dianov, G.L. (2010). XRCC1 phosphorylation by CK2 is required for its stability and efficient DNA repair. *DNA Repair (Amst)*, Vo.9, No.7, pp. 835-841

Parsons, J.L.; Tait, P.S.; Finch, D.; Dianova, II; Allinson, S.L. & Dianov, G.L. (2008a). CHIP-mediated degradation and DNA damage-dependent stabilization regulate base excision repair proteins. *Mol Cell*, Vo.29, No.4, pp. 477-487

Parsons, J.L.; Tait, P.S.; Finch, D.; Dianova, II; Allinson, S.L. & Dianov, G.L. (2008b). CHIP-mediated degradation and DNA damage-dependent stabilization regulate base excision repair proteins. *Molecular Cell*, Vo.29, No.4, pp. 477-487

Paunesku, T.; Mittal, S.; Protic, M.; Oryhon, J.; Korolev, S.V.; Joachimiak, A. & Woloschak, G.E. (2001). Proliferating cell nuclear antigen (PCNA): ringmaster of the genome. *International journal of radiation biology*, Vo.77, No.10, pp. 1007-1021

Pfander, B.; Moldovan, G.L.; Sacher, M.; Hoege, C. & Jentsch, S. (2005). SUMO-modified PCNA recruits Srs2 to prevent recombination during S phase. *Nature*, Vo.436, No.7049, pp. 428-433

Plosky, B.S.; Vidal, A.E.; Fernandez de Henestrosa, A.R.; McLenigan, M.P.; McDonald, J.P.; Mead, S. & Woodgate, R. (2006). Controlling the subcellular localization of DNA polymerases iota and eta via interactions with ubiquitin. *The EMBO journal*, Vo.25, No.12, pp. 2847-2855

Podlaska, A.; McIntyre, J.; Skoneczna, A. & Sledziewska-Gojska, E. (2003). The link between 20S proteasome activity and post-replication DNA repair in Saccharomyces cerevisiae. *Molecular microbiology*, Vo.49, No.5, pp. 1321-1332

Postow, L.; Ghenoiu, C.; Woo, E.M.; Krutchinsky, A.N.; Chait, B.T. & Funabiki, H. (2008). Ku80 removal from DNA through double strand break-induced ubiquitylation. *The Journal of cell biology*, Vo.182, No.3, pp. 467-479

Prakash, S.; Johnson, R.E. & Prakash, L. (2005). Eukaryotic translesion synthesis DNA polymerases: specificity of structure and function. *Annual Review of Biochemistry*, Vo.74, pp. 317-353

Ramos, P.C.; Hockendorff, J.; Johnson, E.S.; Varshavsky, A. & Dohmen, R.J. (1998). Ump1p is required for proper maturation of the 20S proteasome and becomes its substrate upon completion of the assembly. *Cell*, Vo.92, No.4, pp. 489-499

Ramsey, K.L.; Smith, J.J.; Dasgupta, A.; Maqani, N.; Grant, P. & Auble, D.T. (2004). The NEF4 complex regulates Rad4 levels and utilizes Snf2/Swi2-related ATPase activity for nucleotide excision repair. *Molecular and Cellular Biology*, Vo.24, No.14, pp. 6362-6378

Ribar, B.; Prakash, L. & Prakash, S. (2006). Requirement of ELC1 for RNA polymerase II polyubiquitylation and degradation in response to DNA damage in Saccharomyces cerevisiae. *Molecular and Cellular Biology*, Vo.26, No.11, pp. 3999-4005

Russell, S.J.; Reed, S.H.; Huang, W.; Friedberg, E.C. & Johnston, S.A. (1999). The 19S regulatory complex of the proteasome functions independently of proteolysis in nucleotide excision repair. *Molecular Cell*, Vo.3, No.6, pp. 687-695

Russo, A.; Fratto, M.E.; Bazan, V.; Schiro, V.; Agnese, V.; Cicero, G.; Vincenzi, B.; Tonini, G. & Santini, D. (2007). Targeting apoptosis in solid tumors: the role of bortezomib from preclinical to clinical evidence. *Expert Opinion on Therapeutic Targets*, Vo.11, No.12, pp. 1571-1586

Sacher, M.; Pfander, B.; Hoege, C. & Jentsch, S. (2006). Control of Rad52 recombination activity by double-strand break-induced SUMO modification. *Nat Cell Biol*, Vo.8, No.11, pp. 1284-1290

Sakasai, R. & Tibbetts, R. (2008). RNF8-dependent and RNF8-independent regulation of 53BP1 in response to DNA damage. *The Journal of biological chemistry*, Vo.283, No.20, pp. 13549-13555

San Miguel, J.F.; Schlag, R.; Khuageva, N.K.; Dimopoulos, M.A.; Shpilberg, O.; Kropff, M.; Spicka, I.; Petrucci, M.T.; Palumbo, A.; Samoilova, O.S.; Dmoszynska, A.; Abdulkadyrov, K.M.; Schots, R.; Jiang, B.; Mateos, M.V.; Anderson, K.C.; Esseltine, D.L.; Liu, K.; Cakana, A.; van de Velde, H. & Richardson, P.G. (2008). Bortezomib plus melphalan and prednisone for initial treatment of multiple myeloma. *New England Journal of Medicine*, Vo.359, No.9, pp. 906-917

Sartori, A.A.; Lukas, C.; Coates, J.; Mistrik, M.; Fu, S.; Bartek, J.; Baer, R.; Lukas, J. & Jackson, S.P. (2007). Human CtIP promotes DNA end resection. *Nature*, Vo.450, No.7169, pp. 509-514

Schauber, C.; Chen, L.; Tongaonkar, P.; Vega, I.; Lambertson, D.; Potts, W. & Madura, K. (1998). Rad23 links DNA repair to the ubiquitin/proteasome pathway. *Nature*, Vo.391, No.6668, pp. 715-718

Schmutte, C.; Sadoff, M.M.; Shim, K.S.; Acharya, S. & Fishel, R. (2001). The interaction of DNA mismatch repair proteins with human exonuclease I. *Journal of Biological Chemistry*, Vo.276, No.35, pp. 33011-33018

Schwartz, A.L. & Ciechanover, A. (2009). Targeting Proteins for Destruction by the Ubiquitin System: Implications for Human Pathobiology. *Annual Review of Pharmacology and Toxicology*, Vo.49, pp. 73-96

Shabek, N. & Ciechanover, A. (2010). Degradation of ubiquitin The fate of the cellular reaper. *Cell Cycle*, Vo.9, No.3, pp. 523-530

Sharova, N.P. (2005a). How does a cell repair damaged DNA? *Biochemistry-Moscow*, Vo.70, No.3, pp. 275-291

Sharova, N.P. (2005b). How does a cell repair damaged DNA? *Biochemistry (Mosc)*, Vo.70, No.3, pp. 275-291

Shen, Z.; Pardington-Purtymun, P.E.; Comeaux, J.C.; Moyzis, R.K. & Chen, D.J. (1996). UBL1, a human ubiquitin-like protein associating with human RAD51/RAD52 proteins. *Genomics*, Vo.36, No.2, pp. 271-279

Skoneczna, A.; McIntyre, J.; Skoneczny, M.; Policinska, Z. & Sledziewska-Gojska, E. (2007). Polymerase eta is a short-lived, proteasomally degraded protein that is temporarily stabilized following UV irradiation in Saccharomyces cerevisiae. *Journal of Molecular Biology*, Vo.366, No.4, pp. 1074-1086

Smogorzewska, A.; Desetty, R.; Saito, T.T.; Schlabach, M.; Lach, F.P.; Sowa, M.E.; Clark, A.B.; Kunkel, T.A.; Harper, J.W.; Colaiacovo, M.P. & Elledge, S.J. (2010). A genetic screen

identifies FAN1, a Fanconi anemia-associated nuclease necessary for DNA interstrand crosslink repair. *Molecular Cell*, Vo.39, No.1, pp. 36-47

Smogorzewska, A.; Matsuoka, S.; Vinciguerra, P.; McDonald, E.R., 3rd; Hurov, K.E.; Luo, J.; Ballif, B.A.; Gygi, S.P.; Hofmann, K.; D'Andrea, A.D. & Elledge, S.J. (2007). Identification of the FANCI protein, a monoubiquitinated FANCD2 paralog required for DNA repair. *Cell*, Vo.129, No.2, pp. 289-301

Starita, L.M. & Parvin, J.D. (2003). The multiple nuclear functions of BRCA1: transcription, ubiquitination and DNA repair. *Current Opinion in Cell Biology*, Vo.15, No.3, pp. 345-350

Stehmeier, P. & Muller, S. (2009). Regulation of p53 family members by the ubiquitin-like SUMO system. *DNA Repair (Amst)*, Vo.8, No.4, pp. 491-498

Steinacher, R. & Schar, P. (2005a). Functionality of human thymine DNA glycosylase requires SUMO-regulated changes in protein conformation. *Curr Biol*, Vo.15, No.7, pp. 616-623

Steinacher, R. & Schar, P. (2005b). Functionality of human thymine DNA glycosylase requires SUMO-regulated changes in protein conformation. *Current Biology*, Vo.15, No.7, pp. 616-623

Stelter, P. & Ulrich, H.D. (2003). Control of spontaneous and damage-induced mutagenesis by SUMO and ubiquitin conjugation. *Nature*, Vo.425, No.6954, pp. 188-191

Stewart, G.S.; Panier, S.; Townsend, K.; Al-Hakim, A.K.; Kolas, N.K.; Miller, E.S.; Nakada, S.; Ylanko, J.; Olivarius, S.; Mendez, M.; Oldreive, C.; Wildenhain, J.; Tagliaferro, A.; Pelletier, L.; Taubenheim, N.; Durandy, A.; Byrd, P.J.; Stankovic, T.; Taylor, A.M. & Durocher, D. (2009). The RIDDLE syndrome protein mediates a ubiquitin-dependent signaling cascade at sites of DNA damage. *Cell*, Vo.136, No.3, pp. 420-434

Stewart, G.S.; Stankovic, T.; Byrd, P.J.; Wechsler, T.; Miller, E.S.; Huissoon, A.; Drayson, M.T.; West, S.C.; Elledge, S.J. & Taylor, A.M. (2007). RIDDLE immunodeficiency syndrome is linked to defects in 53BP1-mediated DNA damage signaling. *Proceedings of the National Academy of Sciences of the United States of America*, Vo.104, No.43, pp. 16910-16915

Stoyanova, T.; Roy, N.; Kopanja, D.; Raychaudhuri, P. & Bagchi, S. (2009). DDB2 (damaged-DNA binding protein 2) in nucleotide excision repair and DNA damage response. *Cell Cycle*, Vo.8, No.24, pp. 4067-4071

Strom, C.E.; Johansson, F.; Uhlen, M.; Al-Khalili Szigyarto, C.; Erixon, K. & Helleday, T. (2010). Poly (ADP-ribose) polymerase (PARP) is not involved in base excision repair but PARP inhibition traps a single-strand intermediate. *Nucleic Acids Res*, Vo., pp.

Takahashi, H.; Hatakeyama, S.; Saitoh, H. & Nakayama, K.I. (2005). Noncovalent SUMO-1 binding activity of thymine DNA glycosylase (TDG) is required for its SUMO-1 modification and colocalization with the promyelocytic leukemia protein. *Journal of Biological Chemistry*, Vo.280, No.7, pp. 5611-5621

Takeshita, T.; Wu, W.W.; Koike, A.; Fukuda, M. & Ohta, T. (2009). Perturbation of DNA repair pathways by proteasome inhibitors corresponds to enhanced chemosensitivity of cells to DNA damage-inducing agents. *Cancer Chemotherapy and Pharmacology*, Vo.64, No.5, pp. 1039-1046

Takezawa, J.; Ishimi, Y. & Yamada, K. (2008). Proteasome inhibitors remarkably prevent translesion replication in cancer cells but not normal cells. *Cancer science*, Vo.99, No.5, pp. 863-871

Tseng, H.M. & Tomkinson, A.E. (2002). A physical and functional interaction between yeast Pol4 and Dnl4-Lif1 links DNA synthesis and ligation in nonhomologous end joining. *Journal of Biological Chemistry*, Vo.277, No.47, pp. 45630-45637

Ulrich, H.D. (2009). Regulating post-translational modifications of the eukaryotic replication clamp PCNA. *DNA Repair*, Vo.8, No.4, pp. 461-469

van der Spek, P.J.; Visser, C.E.; Hanaoka, F.; Smit, B.; Hagemeijer, A.; Bootsma, D. & Hoeijmakers, J.H. (1996). Cloning, comparative mapping, and RNA expression of the mouse homologues of the Saccharomyces cerevisiae nucleotide excision repair gene RAD23. *Genomics*, Vo.31, No.1, pp. 20-27

Vandenberg, C.J.; Gergely, F.; Ong, C.Y.; Pace, P.; Mallery, D.L.; Hiom, K. & Patel, K.J. (2003). BRCA1-independent ubiquitination of FANCD2. *Molecular Cell*, Vo.12, No.1, pp. 247-254

Varshavsky, A. (1997). The ubiquitin system. *Trends in Biochemical Sciences*, Vo.22, No.10, pp. 383-387

Vlachostergios, P.J.; Patrikidou, A.; Daliani, D.D. & Papandreou, C.N. (2009a). The ubiquitin-proteasome system in cancer, a major player in DNA repair. Part 1: post-translational regulation. *J Cell Mol Med*, Vo.13, No.9B, pp. 3006-3018

Vlachostergios, P.J.; Patrikidou, A.; Daliani, D.D. & Papandreou, C.N. (2009b). The ubiquitin-proteasome system in cancer, a major player in DNA repair. Part 2: transcriptional regulation. *Journal of Cellular and Molecular Medicine*, Vo.13, No.9B, pp. 3019-3031

Wang, B. & Elledge, S.J. (2007). Ubc13/Rnf8 ubiquitin ligases control foci formation of the Rap80/Abraxas/Brca1/Brcc36 complex in response to DNA damage. *Proceedings of the National Academy of Sciences of the United States of America*, Vo.104, No.52, pp. 20759-20763

Wang, Q.E.; Wani, M.A.; Chen, J.; Zhu, Q.; Wani, G.; El-Mahdy, M.A. & Wani, A.A. (2005a). Cellular ubiquitination and proteasomal functions positively modulate mammalian nucleotide excision repair. *Molecular Carcinogenesis*, Vo.42, No.1, pp. 53-64

Wang, Q.E.; Zhu, Q.; Wani, G.; El-Mahdy, M.A.; Li, J. & Wani, A.A. (2005b). DNA repair factor XPC is modified by SUMO-1 and ubiquitin following UV irradiation. *Nucleic Acids Res*, Vo.33, No.13, pp. 4023-4034

Wang, S.; Gong, Z.; Chen, R.; Liu, Y.; Li, A.; Li, G. & Zhou, J. (2009). JWA regulates XRCC1 and functions as a novel base excision repair protein in oxidative-stress-induced DNA single-strand breaks. *Nucleic Acids Res*, Vo.37, No.6, pp. 1936-1950

Wang, T.; Simbulan-Rosenthal, C.M.; Smulson, M.E.; Chock, P.B. & Yang, D.C. (2008). Polyubiquitylation of PARP-1 through ubiquitin K48 is modulated by activated DNA, NAD+, and dipeptides. *Journal of Cellular Biochemistry*, Vo.104, No.1, pp. 318-328

Wang, Z.M.; Chen, Z.P.; Xu, Z.Y.; Christodoulopoulos, G.; Bello, V.; Mohr, G.; Aloyz, R. & Panasci, L.C. (2001). In vitro evidence for homologous recombinational repair in resistance to melphalan. *Journal of the National Cancer Institute*, Vo.93, No.19, pp. 1473-1478

Watanabe, K.; Tateishi, S.; Kawasuji, M.; Tsurimoto, T.; Inoue, H. & Yamaizumi, M. (2004). Rad18 guides poleta to replication stalling sites through physical interaction and PCNA monoubiquitination. *EMBO Journal*, Vo.23, No.19, pp. 3886-3896

Waters, R.; Zhang, R. & Jones, N.J. (1993). Inducible removal of UV-induced pyrimidine dimers from transcriptionally active and inactive genes of Saccharomyces cerevisiae. *Molecular and General Genetics*, Vo.239, No.1-2, pp. 28-32

Watkins, J.F.; Sung, P.; Prakash, L. & Prakash, S. (1993). The Saccharomyces cerevisiae DNA repair gene RAD23 encodes a nuclear protein containing a ubiquitin-like domain required for biological function. *Molecular and Cellular Biology*, Vo.13, No.12, pp. 7757-7765

Waxman, L.; Fagan, J.M. & Goldberg, A.L. (1987). Demonstration of two distinct high molecular weight proteases in rabbit reticulocytes, one of which degrades ubiquitin conjugates. *Journal of Biological Chemistry*, Vo.262, No.6, pp. 2451-2457

West, S.C. (2003). Molecular views of recombination proteins and their control. *Nat Rev Mol Cell Biol*, Vo.4, No.6, pp. 435-445

Wilkinson, K.D.; Urban, M.K. & Haas, A.L. (1980). Ubiquitin is the ATP-dependent proteolysis factor I of rabbit reticulocytes. *Journal of Biological Chemistry*, Vo.255, No.16, pp. 7529-7532

Wilson, D.M., 3rd & Thompson, L.H. (1997). Life without DNA repair. *Proceedings of the National Academy of Sciences of the United States of America*, Vo.94, No.24, pp. 12754-12757

Wilson, S.H.; Beard, W.A.; Shock, D.D.; Batra, V.K.; Cavanaugh, N.A.; Prasad, R.; Hou, E.W.; Liu, Y.; Asagoshi, K.; Horton, J.K.; Stefanick, D.F.; Kedar, P.S.; Carrozza, M.J.; Masaoka, A. & Heacock, M.L. (2010). Base excision repair and design of small molecule inhibitors of human DNA polymerase beta. *Cellular and Molecular Life Sciences*, Vo.67, No.21, pp. 3633-3647

Wiltrout, M.E. & Walker, G.C. (2011). Proteasomal regulation of the mutagenic translesion DNA polymerase, Saccharomyces cerevisiae Rev1. *DNA Repair (Amst)*, Vo.10, No.2, pp. 169-175

Woodhouse, B.C.; Dianova, II; Parsons, J.L. & Dianov, G.L. (2008). Poly(ADP-ribose) polymerase-1 modulates DNA repair capacity and prevents formation of DNA double strand breaks. *DNA Repair (Amst)*, Vo.7, No.6, pp. 932-940

Xia, Y.; Pao, G.M.; Chen, H.W.; Verma, I.M. & Hunter, T. (2003). Enhancement of BRCA1 E3 ubiquitin ligase activity through direct interaction with the BARD1 protein. *Journal of Biological Chemistry*, Vo.278, No.7, pp. 5255-5263

Xie, Y. (2010). Structure, assembly and homeostatic regulation of the 26S proteasome. *J Mol Cell Biol*, Vo.2, No.6, pp. 308-317

Xirodimas, D.P.; Saville, M.K.; Bourdon, J.C.; Hay, R.T. & Lane, D.P. (2004). Mdm2-mediated NEDD8 conjugation of p53 inhibits its transcriptional activity. *Cell*, Vo.118, No.1, pp. 83-97

Yu, X.; Fu, S.; Lai, M.; Baer, R. & Chen, J. (2006). BRCA1 ubiquitinates its phosphorylation-dependent binding partner CtIP. *Genes and Development*, Vo.20, No.13, pp. 1721-1726

Yurchenko, V.; Xue, Z. & Sadofsky, M.J. (2006). SUMO modification of human XRCC4 regulates its localization and function in DNA double-strand break repair. *Molecular and Cellular Biology*, Vo.26, No.5, pp. 1786-1794

Zhang, Y. & Xiong, Y. (2001). Control of p53 ubiquitination and nuclear export by MDM2 and ARF. *Cell Growth Differ*, Vo.12, No.4, pp. 175-186

Zhao, G.Y.; Sonoda, E.; Barber, L.J.; Oka, H.; Murakawa, Y.; Yamada, K.; Ikura, T.; Wang, X.; Kobayashi, M.; Yamamoto, K.; Boulton, S.J. & Takeda, S. (2007). A critical role for the ubiquitin-conjugating enzyme Ubc13 in initiating homologous recombination. *Molecular cell*, Vo.25, No.5, pp. 663-675

Zhu, Q.; Wani, G.; Arab, H.H.; El-Mahdy, M.A.; Ray, A. & Wani, A.A. (2009). Chromatin restoration following nucleotide excision repair involves the incorporation of ubiquitinated H2A at damaged genomic sites. *DNA Repair (Amst)*, Vo.8, No.2, pp. 262-273

Zhu, Q.; Wani, G.; Yao, J.; Patnaik, S.; Wang, Q.E.; El-Mahdy, M.A.; Praetorius-Ibba, M. & Wani, A.A. (2007). The ubiquitin-proteasome system regulates p53-mediated transcription at p21waf1 promoter. *Oncogene*, Vo.26, No.29, pp. 4199-4208

Zhuang, Z.; Johnson, R.E.; Haracska, L.; Prakash, L.; Prakash, S. & Benkovic, S.J. (2008). Regulation of polymerase exchange between Poleta and Poldelta by monoubiquitination of PCNA and the movement of DNA polymerase holoenzyme. *Proceedings of the National Academy of Sciences of the United States of America*, Vo.105, No.14, pp. 5361-5366

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Virtual Screening for DNA Repair Inhibitors

Barakat, K.[1] and Tuszynski, J.[1,2]
[1]*Department of Physics, University of Alberta,*
[2]*Department of Oncology, University of Alberta,*
Canada

1. Introduction

All living cells have a tendency to maintain their genomic stability with as few mutations as possible. This is of crucial importance to the normal function of cells in complex environments, correctly timed cell cycle progression, and a commitment to apoptosis when appropriate (Wood, et al., 2001). In this context, the balance between constancy and mutability in the context of genomic stability must be precisely regulated and controlled. To achieve this objective, a number of multiple and overlapping DNA repair pathways have been crafted within the cell (Harper & Elledge, 2007). Nevertheless, an elevated activity of these pathways could significantly decrease cancer cells' sensitivity to many known anticancer agents and, consequently, increase their antitumor drug resistance. This unforeseen role stems from the fact that most cancer chemotherapy in clinical use today, directly or indirectly damage DNA by causing single- or double-stranded DNA breaks or by interfering with the functions of crucial DNA interacting proteins. As a natural cellular response, following the detection of damage, DNA repair pathways attempt to restore the genome and restore the normal state of the cell. During this course, the cell's fate is mainly determined by the effectiveness of DNA repair mechanisms which allow the cell to survive or, if the damage is too heavy, induce apoptosis, causing the cell to die (Harper & Elledge, 2007). Consequently, to improve existing cancer therapies, DNA repair pathways have been considered as novel therapeutic targets. Several DNA repair inhibitors have been reported, some of which have been recently proven to be successful (Damia & D'Incalci, 2007)
This review paper focuses on our efforts directed at *in silico* searches for inhibitors of proteins that control the DNA repair circuitry. The targets chosen here play critical roles in tumor cell initiation and progression, hence their regulation offers promise for the improvement of current cancer therapy. Two of these targets are DNA repair proteins that are directly linked to the hallmark "relapse" or "drug resistance" phenomena. These are Excision Repair Cross-Complementation Group 1 (ERCC1) (Kang, et al., 2006), and DNA polymerase beta (pol β) (Parsons, et al., 2004). The former is a key player in Nucleotide Excision Repair (NER), while the latter is the error-prone polymerase of Base Excision Repair (BER). The third target is p53 (Teodoro, et al., 2007), a so-called guardian protein of the genome that is inactivated in more than half of all human cancers investigated. An additional aim of this review is to share with the reader our experience as a computational drug discovery group by describing the virtual screening protocol we have developed in order to successfully address these biological problems. This chapter is divided into two main sections. The first gives a description of the computational workflow that we typically

follow in our virtual screening tasks. The second is a summary of our findings for the individual targets listed above.

2. An improved virtual screening (VS) protocol

Fig. 1 illustrates the essential steps taken in order to execute the virtual screening (VS) protocol used in our lab. In a nutshell, the developed protocol employs molecular docking, molecular dynamics simulations and clustering techniques to filter a given library of compounds for inhibitors of a particular target. The concepts behind VS and other computational tools are described elsewhere (Limin, et al., 2011); (Stahura & Bajorath, 2004). However, a detailed description and rationale behind each step of this workflow are summarized below. Except for a few steps that need carful preparation, the whole process has been automated. It starts with a collection of 3D structures of ligands and a well-prepared target structure. It finally yields a set of top hit structures in their preferred binding modes with the target. Although the following steps were applied to the three specific targets described in the following section, the procedure is general and the same method is applicable to almost any bio-molecular target.

2.1 Target preparation

2.1.1 Primary assessment of target structure

In general, the downloaded "crude" crystal structure of a target contains many details that must be taken into account. This includes non-standard amino acids; co-factors; other small molecules that are present due to the crystallization process; ions and co-crystallized water molecules. For most small molecules like polyethylene glycol, it is advisable to remove them from the structure, since they are not included in the native form of the target but were required for the crystallization process. Moreover, non-standard amino acids must be carefully assessed and modeled. In many protein structures, these unusual amino acids lack several atoms because most structure handling packages do not check automatically inspect for them. Their parameters must be appended to the used Force Field (FF) before starting further simulations. Co-factors, ions and co-crystallized water molecules should be included within the simulated structure.

Water molecules that are located close to or within the binding site can mediate several interactions with the ligands. However, it is important to find out which water molecules are conserved within these regions. Any unpreserved (misplaced) water molecule can obstruct the docking simulation and lead to incorrect results. One way to identify important water molecules is to compare several crystal structures of the same target (if applicable) and choose the water molecules to be kept during the docking procedure. When a limited number of target structures is available, it is important to run different docking exercises by removing/keeping these water molecules and selecting the cases that lead to realistic and favorable binding modes.

An additional decision-making tool for the selection of water molecules is to use prediction software packages (e. g. ConSolv 1.0) that check whether a bound water molecule is likely to be conserved or displaced in other, independently solved crystallographic structures of the same target.

Finally, it is necessary to verify that no parts of the protein structure are missing. These missing residues are usually mentioned at the header of the Protein Data Bank (PDB)-file and must be added and relaxed within the target structure. Regarding the targets that were studied in this work, all missing amino acids were distant away from the binding site.

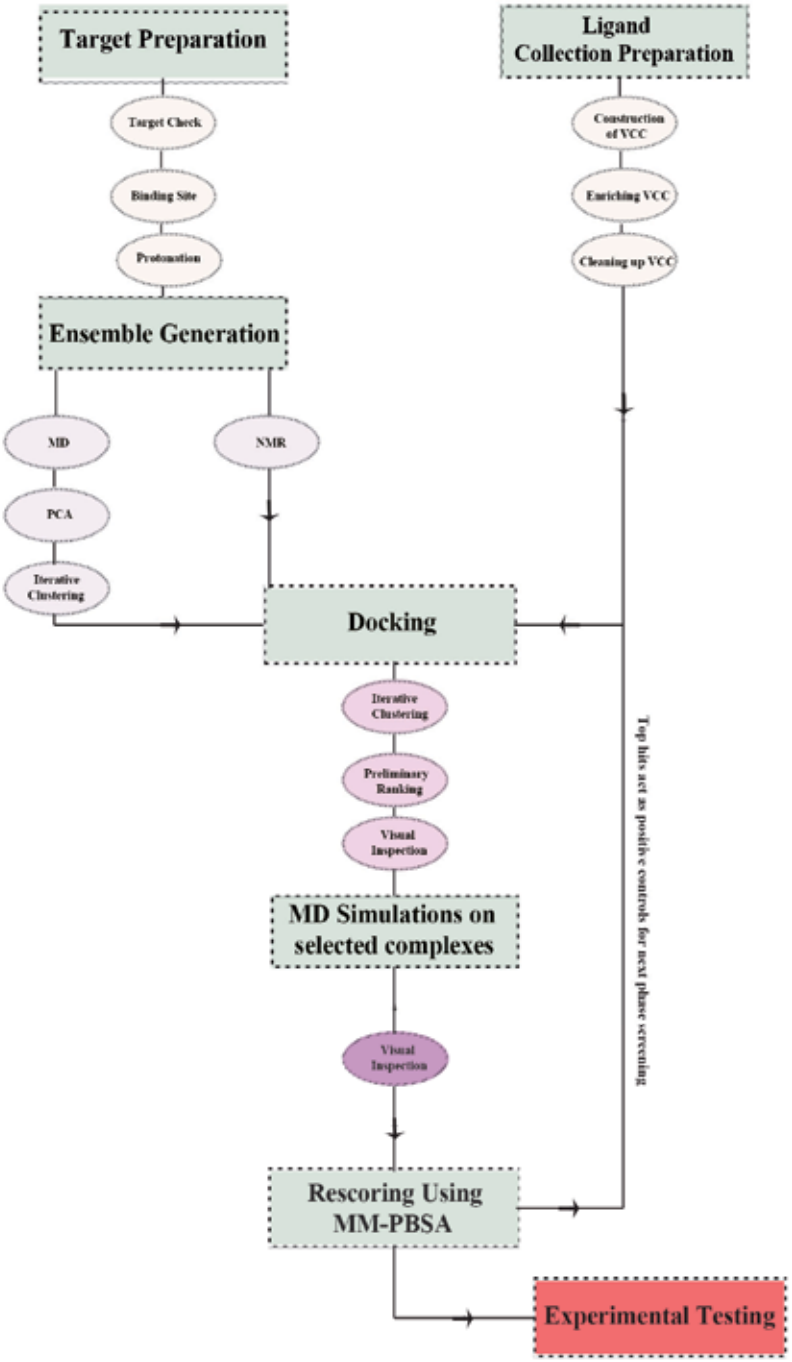


Fig. 1. Description of the implemented computational workflow. See text for more details.

Nonetheless, we added and relaxed them using molecular dynamics (MD) simulations before running the docking experiments (see below).

2.1.2 Identifying the binding site

The starting point of any VS study is the identification of the binding site within the target protein. This portion of the protein is directly related to the biological activity that needs to be regulated. At this stage, it is important to consult previously published work and determine if there are any known active compounds that bind to the target protein (positive controls) and to ascertain their binding location. If the binding site is not exactly known, however, there is a set of active molecular structures that exist, one should run a series of blind docking experiments until a suitable and experimentally verified binding site is found (Bennett, et al., 2010); (Hazan, et al., 2008). Regarding the three targets that we focus on here, the binding sites were accurately known, mainly because they are protein-protein interaction sites (e.g. ERCC1-XPA and p53-MDM2/MDM4), or protein-DNA binding sites (e.g. DNA-pol β) where crystal structures of the interacting subunits are available.

2.1.3 Protonation states of charged residues

Proper adjustment of the protonation states, "the assignment of Hydrogen atoms" of the ionizable groups, contained by the target structure is important for any successful VS simulation. These residues play key roles in inter-protein, protein-solvent and protein-ligand interactions. The protonation states can be determined by predicting the pKa value of charged residues and comparing it to the pH value at which the simulation is performed. In this work, all protonation states of ionizable residues were calculated using the software PROBKA and adjusted at physiological pH of 7.0 (H. Li, et al., 2005). PROBKA is a very fast and accurate method that relates the structure and environment of the charged residues to the change of the pKa values from their intrinsic ones. Once the protonation states have been decided, all hydrogen atoms are then added to the system according to a given force field (FF). For the three targets, the AMBER99SB FF was used (Hornak, et al., 2006). At this stage, the protein structure is ready for the docking or MD simulations.

2.2 Ligand collection preparation

In parallel with target preparation, the organization and cleaning up of the set of compounds is undertaken for *in silico* screening. Currently, there are many suitable, easy to access compound databases that contain millions of molecules spanning various structural families. Prior to any screening exercises, one should decide on a set of compounds to be filtered and build up a virtual compound collection (VCC) of compounds. This collection will be repeatedly used against many targets. A typical VCC should include marketed drugs, lead-like compounds, fragment structures, commercially available chemicals and other high-activity molecules. It is also important to represent these molecules in different protonation, stereo and conformational states. An effective VCC should be constructed from molecules that are suitable for further lead optimizations, after they show biological activity.

2.2.1 Construction of the VCC

Five different databases comprise the core of our VCC. These are the National Cancer Institute diversity set (NCIDS), the DrugBank database (Wishart, et al., 2006), subsets of the ZINC database (Irwin & Shoichet, 2005) and finally, the French national chemical library "la

Chimiothèque Nationale" (CN). Some of them are used in the first iteration of VS and others are retained for higher-order screening exercises.

The NCIDS is a collection of approximately 2,000 compounds that are structurally representative of a wide range of molecules, representing almost 140,000 compounds that are available for testing at the NCI. A number of its ligands contain rare earth elements and cannot be properly parameterized for docking experiments, leaving us with 1,883 compounds that can be actually used. We use a cleaned 3D version of the NCIDS formatted for use in AutoDock (the main docking program used by us) (Goodsell & Olson, 1990) and was prepared by the AutoDock Scripps team. What makes the NCIDS so valuable and extensively screened by many groups (even in HTS) is that its individual molecules have distinctive structures and are the cluster representatives of their parent families. Having screened and ranked the molecules, one can re-screen the subset of the representative structures, instead of screening the entire NCI set of compounds.

The DrugBank database is not only a set of molecules representing FDA-approved (and investigational) drugs, but also it is a unique bioinformatics and cheminformatics resource since it relates each drug to its target(s). It includes details about the different pathways, structural information and chemical characteristics of these targets and the way they are involved in a particular disease. This information is stored on a freely accessible website that is linked to other databases (KEGG, PubChem, ChEBI, PDB, Swiss-Prot and GenBank) and to a range of structure displaying applets. The DrugBank collection includes ~4,800 drug structures including >1,350 FDA-approved small molecule drugs, 123 FDA-approved biotech (protein/peptide) drugs, 71 nutraceuticals and >3,243 experimental drugs. Once a hit is identified from this library, it is simply a drug. This means it overcomes many barriers of preclinical and clinical testing and development and can be directly tested for its novel biological activity. Moreover, a hit from this collection may explain a mysterious side effect that would not be discovered before its identification as a regulator of the examined target.

ZINC is a free database dedicated to VS It includes more than 13 million purchasable compounds most of which are "drug-like" or "lead-like". These compounds are available in several 3D formats and compatible with several docking programs. The ZINC database has many other interesting features. For example, one can easily create a subset of the whole database with any given set of properties such as specific functional groups, molecular weight, and a calculated logP. Most of the compounds also exist in multiple protonation states suitable for different pH values, several tautomeric forms, all possible stereochemistries, and different 3D conformations. The database is also organized so that the origin of each molecule is known. That is, one can determine the vendor and original catalog number for each commercial source of a compound. Similarly to the DrugBank database, a molecule can be annotated for its function or activity. It also has a powerful web server that helps in searching, browsing, creating subsets, and downloading some or all of the molecules in the database.

The CN chemical library (~100,000 compounds) is a repository of all synthetic, natural compounds and natural extracts in the existing French public laboratories. This database is divided into two main categories. The first part includes information about all synthetic products, while the second contains the natural compounds and extracts. In this work, we used the whole CN database in our screening. In contrast to the previously mentioned databases, compounds in this library are represented by 2D SDF structures with no hydrogen atoms attached. This required a number of cleaning and preparation steps before using them in our VS simulations (see below).

2.2.2 Enriching the VCC core

This is where ligand-based methods come to play a significant role in the pre-screening process. Any molecule that is known to bind to the target-binding site can serve as a positive control. Such molecules can be identified through published articles or previous patents. Besides their function in directing and verifying the simulation parameters, they can be used as seeds in the searches for similar chemical structures to enrich the VCC. This is a crucial step, which should be taken even if the identified similar structures have been previously removed from the VCC in its early construction steps.

Following this strategy, we have used known inhibitors for the p53-MDM2 interaction (see section 3.2) and DNA pol β (see section 3.3) to enrich their representative VCCs For the ERCC1-XPA interaction (see section 3.1), initially, there was no active compound confirmed to bind to the ERCC1 pocket. Hence, for the first round of VS, we started from scratch and did not apply this enrichment method. However, it was used in the second round of screening, after the first iteration identified a list of novel binders to the ERCC1 target.

2.2.3 Cleaning up the VCC

Having decided which collection of compounds to use in the screening process, one should spend time and effort to ensure the quality of the used ligand structures. As mentioned before, it is important to adopt proper protonation and conformational states for the ligands. For example, the original CN library of compounds is a collection of 2D structures with no hydrogen atoms. Ligands in this state are not suitable for docking using many of the popular docking programs. These software packages require 3D structures with proper placement of hydrogen atoms. One solution to this problem, which was followed in the ERCC1-XPA case (see section 3.1), is to use conversion software that can translate the 2D information into its 3D representative structure Many of such programs are available (e.g. Open Babel and LigPrep from Schrödinger). We prefer LigPrep for this task because it produces structures with few errors compared to Open Babel, especially in bond connection and hydrogen atoms assignment.

2.3 Generation of an ensemble of target structures

Proteins are inherently dynamical macromolecules. Their dynamical behavior is essential in order to recognize and bind to other molecules inside the cell. Although many attempts have been made to partly include the flexibility of the molecular target within docking algorithms (Schneider & Bohm, 2002), there are still many barriers and challenges that impede progress in this field. One major challenge is the enormous number of conformations that are accessible to the target under equilibrium conditions The range of these conformations is very wide and includes many local and global movements within the structure of the protein. These dynamical transitions can be as small as minor rotations of the side-chains or as large as the complete dislocation of domains within the same target. There are many crystal structures in the PDB that give evidence to this bizarre dynamical behavior. These conformational changes can be illustrated by comparing different crystal structures of the same target, especially, between its bound and unbound forms.

2.3.1 Hybrid MD-docking methods

One way to accommodate receptor flexibility and to offer more accurate scoring techniques is to implement a hybrid method between docking and MD simulations. Originally, the use

of MD simulations in VS studies was intended to create a set of receptor conformations (Broughton, 2000; Carlson, et al., 2000). However, it was always debatable whether to use structures derived from MD simulations or NMR data. In our opinion, if a reasonable ensemble of NMR structures exist, one should consider using them all, instead of running long MD simulations. However, if the VS exercise departs from a single X-ray crystal structure, it is important to generate such an ensemble using MD simulations.
In this context, a successful approach, reported by McCammon and his team, is the relaxed complex scheme (RCS)(Lin, et al., 2002; Amaro, et al., 2008). This method, illustrated in Fig. 2, forms the foundation of the VS protocol presented here. In the RCS approach, all-atom MD simulations (e.g., 2-5 ns simulation) are applied to explore the conformational space of the target, while docking is subsequently used for the fast screening of drug libraries against an ensemble of receptor conformations This ensemble is extracted at predetermined time intervals (e.g., 10 ps) from the simulation, resulting in hundreds of thousands of protein conformations. Each conformation is then used as a target for an independent docking experiment.

2.3.2 Principle component analysis and sampling convergence

A typical MD trajectory displays the time dependence of atomistic Cartesian coordinates. Although the duration of the whole trajectory is typically very short (at best, on the order of hundreds of ns) compared to real life biological dynamics, it involves a huge number of snapshots that contain a mixture of fast and slow modes of motion. It is impossible to segregate or understand this mixed dynamics through simple analysis (e.g. visual inspection). However, covariance, or principle component, analysis (PCA) can break up these two types of motions and extract the essential dynamics (ED) spanned by the protein structure. This essential dynamics represents the collective movements that are directly linked to the function of the protein and are essential for its role. In fact, PCA transforms the original space of correlated variables from a large MD simulation into a reduced space of independent variables (Garcia, 1992); (Amadei, et al., 1993). For a typical protein, the system's dimensionality is thereby reduced from tens of thousands to fewer than fifty degrees of freedom.

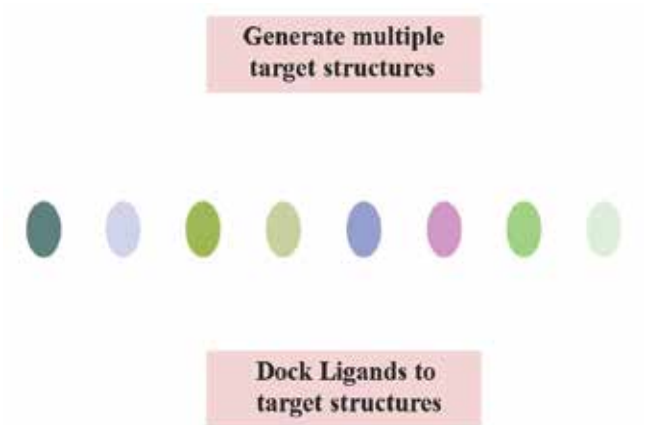


Fig. 2. Schematic illustration of the basic idea behind the relaxed complex scheme developed by McCammon et al.(Lin, et al., 2002).

To perform PCA for a subset of N atoms, the entire MD trajectory is RMSD fitted to a reference structure, in order to remove all rotations and translations. The covariance matrix can then be calculated from their Cartesian atomic co-ordinates as:

$$\sigma_{ij} = \left\langle \left(r_i - \langle r_i \rangle \right) \left(r_j - \langle r_j \rangle \right) \right\rangle \tag{1}$$

where r_i represents the three Cartesian co-ordinates (x_i, y_i or z_i) and the eigenvectors of the covariance matrix constitute the essential vectors of the motion. It is generally accepted that the larger an eigenvalue, the more important its corresponding eigenvector in the collective motion. PCA can also be employed to predict the completeness of sampling during the MD simulation. This step is critical and was used in three cases to answer a very important question: when to stop the simulation and start extracting the dominant conformations of the protein? In this, we follow a method proposed by Hess (Hess, 2002) that divides an MD trajectory into separate parts, and their normalized overlap is calculated using the covariant matrices for each pair of parts:

$$\text{normalized overlap } (C_1, C_2) = 1 - \frac{\sqrt{tr\left(\left(\sqrt{C_1} - \sqrt{C_2} \right)^2 \right)}}{\sqrt{tr(C_1) + tr(C_2)}} \tag{2}$$

where C_1 and C_2 are the covariant matrices, and the symbol *tr* denotes the trace operation. If the overlap is 0, then the two sets are considered to be orthogonal, whereas an overlap of 1 indicates that the matrices are identical. In this context, for the three targets studied described below, the individual whole trajectories were divided into three parts and the normalized overlap between each pair was calculated to determine the completeness of sampling.

2.3.3 Iterative clustering to extract dominant conformations

Once a sufficient sampling is confirmed through the aforementioned PC calculations, clustering analysis is used to extract a set of target structures that represent dominant conformations. Unfortunately, there is no universally accepted clustering algorithm or parameters that can be used to extract all information contained within the MD simulation. However, recent studies suggest that a number of clustering algorithms, such as average-linkage, means and self-organizing maps (SOM) can be used accurately to cluster MD data (Shao, et al., 2007). In this work, the clustering quality was anticipated by calculating a number of clustering metrics. These metrics can reveal the optimal number of clusters to be extracted and their population size. These are the Davies-Bouldin index (DBI)(Davies DL & Bouldin DW, 1979) and the "elbow criterion" (Shao, et al., 2007). A high-quality clustering scheme is correlated with high DBI values. On the other hand, using the elbow criterion, the percentage of variance explained by the data is expected to plateau for cluster counts exceeding the optimal number of clusters. Using these metrics, by varying the number of clusters, one should expect for adequate clustering, a local minimum for DBI and a horizontal line for the percentage of variance explained by the data. Fig. 3 describes an example of such calculations.

Our implementation employs an iterative clustering algorithm using the above-mentioned hypothesis. The procedure is established as an in-house code using the PTRAJ utility of

AMBER10 (Case, et al., 2005). A modified version of the code is also used to cluster the docking results. MD trajectories' clustering runs the average-linkage algorithm for a number of clusters ranging from 5 to 150 clusters. Structures are extracted at 2 ps intervals over the entire simulation time. In order to remove the overall rotation and translation, all $C_{\propto}$atoms are fitted to the minimized initial structure. RMSD-clustering is performed on the residues contained in the investigated binding sites. These residues are clustered into groups of similar conformations using the atom-positional RMSD of the entire amino acid, including side chains and hydrogen atoms, as the similarity criterion. The centroid of each cluster, the structure having the smallest RMSD to all members of the cluster, is chosen as the cluster representative structure and the most dominant structures are used as rigid templates for the ensemble-based docking experiments (see below).

2.4 Docking ligands to the ensemble of target structures

As stated above, the outcome of the iterative clustering step is an ensemble of protein structures that are used as targets for docking. The main docking program that was used for the three cases analyzed was AutoDock version 4 (Garrett MM, et al., 1999). AutoDock is one of the most popular docking packages that utilize different conformational search methods, including Simulated Annealing (SA), traditional Genetic Algorithm (GA), and Lamarckian Genetic Algorithm (LGA). Here, we use the LGA approach. The approach is well-described in the original paper by Morris et al. (Garrett MM, et al., 1999).

2.4.1 Automated clustering of docked poses

AutoDock can cluster output poses into subgroups depending on their RMSD values referred to a reference structure. Although this approach is widely used, the number of clusters and the population size of each cluster strongly depends on the RMSD cut-off used. Consequently, it is impossible to predict the optimal cut-off for the RMSD in order to produce a clustering pattern with the highest confidence. This motivated us to use an alternative approach when clustering the docked ligand structures In fact, we extended and automated the clustering methodology that was used in section 2.3.3 to couple the elbow criterion (Shao J, et al., 2007) with the clustering module of PTRAJ (Case, et al., 2005). This method exploits the fact that the percentage of variance exhibited by the data (λ), is expected to plateau for cluster counts exceeding the optimal number.
The percentage of variance is defined by:

$$\lambda = \frac{SSR}{SST} \tag{3}$$

where (SSR) is the sum-of-squares regression from each cluster summed over all clusters and (SST) is the total sum of squares. Here, we used the SOM algorithm to cluster the docking results. This modified clustering program increases the number of clusters required until the percentage of variance exhibited by the data (λ) plateaus. The convergence of clustering can be determined by calculating the first and second derivatives of the percentage of variance with respect to the clusters number ($d\lambda / dN$ and $d^2\lambda / dN^2$) after each attempt to increase the cluster counts. The clustering process then stops at an acceptable value for these derivatives that is close to zero. In this way, the clustering procedure depends only on the system itself and adjusts itself to arrive at the optimal clustering pattern for that specific system.

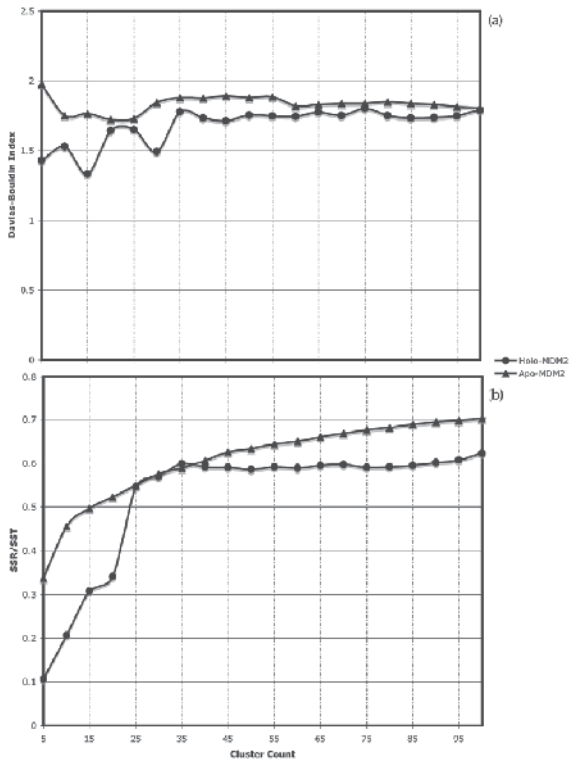


Fig. 3. Clustering Analysis. A high-quality clustering is obtained when a local minimum in DBI correlates with saturation in the SSR/SST ratio (K. Barakat, et al., 2010).

2.4.2 Preliminary ranking of docking results

The VS protocol then sorts the docking results by the lowest binding energy of the most populated cluster. The compounds can also be ranked using their weighted average binding energies according to the following formula:

$$\text{Weighted Average Binding Energy (WABE)} = \sum_{i}^{M} \text{percent ditribution(i) x binding energy} \tag{4}$$

where i is the index number of each ensemble cluster, whose percent distribution sums up to 100% and M is number of different structures included in the ensemble. The VS protocol only considers a compound among the top hits if the most populated cluster from any of the VS experiments includes at least 25% of all docked conformations. The top N hits of the combined docking runs construct an irredundant set of promising compounds that are used for further analysis. In this work, a typical preliminary set (N) includes from 200 to 500 compounds.

2.4.3 Visual inspection and selection of a focused set of hits

Visual inspection of the preliminary set of hits is necessary before proceeding to the later computationally rigorous steps. Although this step involves more human intervention, it

assures the quality of the docking results, which are not precise in terms of ranking or the final selection of binding geometries.

2.5 Molecular dynamics simulations on selected hit-target complexes

There are many factors (e.g. water content, protein flexibility, etc.) that are not well characterized within the docking context (Warren, et al., 2006). During this step, the VS protocol aims at accounting for these factors by performing MD simulations. Each simulation starts from the final docked structure. The important aspect at this stage is the solvation of the docked models. It is generally accepted that water molecules are not only involved in solvation/desolvation of the protein-ligand complexes, but also mediate their interactions and help in generating more suitable binding modes. MD simulations relax the structures, rearrange water and ion molecules and generate trajectories that are used during the next step of binding energy calculations. The output obtained from this step is a set of snapshots representing the trajectory of the MD simulations for each complex. Although this procedure requires extensive computational resources, it tends to improve the protein–ligand interactions and enhance their molecular complementarity.

2.6 Rescoring of hits using the MM-PBSA method

Besides using MD simulations to refine the docked structures, another essential constraint for a successful VS experiment is to accurately predict the binding energies. To correctly perform this task, we need to move away from simple docking scoring methods. However, we are also restricted by the need for a fairly fast method that can be applied to many systems at a reasonable computational cost. In this context, the VS protocol utilizes a fast and efficient scoring method to suggest the final ranked set of top hits. This method is the molecular mechanics Poisson-Boltzmann surface area (MM-PBSA) technique. The method was initially proposed by Kollman et al. (Kollman, et al., 2000) and it combines molecular mechanics with continuum solvation models. The method has been extensively tested on many systems and shown to reproduce, with an acceptable range of accuracy, experimental binding data. It was also validated as a VS refining tool and revealed excellent results in predicting the actual binding affinities and in discriminating true binders from inactive (decoy) compounds (Abagyan & Totrov, 2001; Schneider & Bohm, 2002; Shoichet, et al., 1993). Its main advantages are the lack of adjustable parameters and the option of using a single MD simulation for the complete system to determine all energy values.

In this work we used the MM-PBSA method as implemented in AMBER. The total free energy is the sum of average molecular mechanical gas-phase energies (*EMM*), solvation free energies (*Gsolv*), and entropy contributions (*-TSsolute*) of the binding reaction:

$$G = E_{MM} + G_{solv} - TS_{solute} \tag{5}$$

The total molecular mechanical energies can be further decomposed into contributions from electrostatic (E_{ele}), van der Walls (E_{vdw}) and internal energies (E_{int}):

$$\Delta G^{o} = \Delta G_{gas}^{protein-ligand} + \Delta G_{solv}^{protein-ligand} - \left\{ \Delta G_{solv}^{ligand} + \Delta G_{solv}^{protein} \right\} \tag{6}$$

Furthermore, the solvation free energy can be expressed as a sum of non-electrostatic and electrostatic contributions:

$$\Delta G_{solv} \approx \Delta G_{solv}^{nonele} + \Delta G_{solv}^{ele} \tag{7}$$

The non-electrostatic part was approximated by a linear function of the (*SASA*). That is:

$$\Delta G_{solv}^{nonele} = \gamma \text{ x } SASA, \text{ where } \gamma = 7.2 \text{ cal/mol/A}^2 \quad (8)$$

3. Targeting DNA repair proteins

Below is a description of our target proteins and a summary of the results we found after applying the above-mentioned VS protocol to regulate their activity.

3.1 Case I: inhibitors of the ERCC1-XPA interaction

Platinum-based cancer therapy is one of the most efficacious treatments for many cancer types including testes, ovary, head, neck, and lung cancers (Boulikas & Vougiouka, 2003). While generally efficient at inducing apoptosis, acquired resistance to platinum compounds has limited their efficacy and therefore reduced successful clinical use of these agents (McGuire & Ozols, 1998). One way of acquiring this type of drug resistance is the remarkable high activity of the Nucleotide Excision Repair (NER) pathway (Rabik & Dolan, 2007).

Although many proteins are involved in the NER machinery, only the over-expression ERCC1 (a 33-kDa protein) correlates with the augmented platinum resistance. This spectacular conclusion has been reached from several independent clinical trial investigations on ovarian (Kang, et al., 2006), colorectal (Shirota, et al., 2001), and non–small cell lung cancer (Lord, et al., 2002). ERCC1 forms a tight heterodimer endonuclease complex with XPF. At the final stages of NER, the ERCC1-XPF enzyme cleaves the damaged DNA strand at the phosphodiester bonds on the 5′ side of the damage. Prior to the incision step in NER, the ERCC1-XPF endonuclease is recruited to the damaged DNA site through a secondary interaction between ERCC1 and XPA (L. Li, et al., 1994). This protein-protein interaction is necessary for a functional NER mechanism.

The NMR crystal structure was resolved by Tsodikov's group (Tsodikov, et al., 2007). The critical residue-residue interactions as determined through our binding energy predictions are shown in Fig. 4. A 14-residue peptide from XPA that includes three essential consecutive glycines (residues 72–74) is buried within a hydrophobic cleft within the central domain of ERCC1. This peptide has two critical characteristics (Tsodikov, et al., 2007). First, it is necessary and sufficient for binding to ERCC1. Second, and more importantly, it can compete with the full-length XPA protein in binding to ERCC1 and disrupting NER in vitro. Moreover, there is no other cellular function beyond NER that has been observed for XPA (Rosenberg, et al., 2001). These observations, coupled with the available crystal structure of this interaction make ERCC1 and XPA an extremely attractive target for computationally based development of small molecule inhibitors that are targeted for use in combination therapies involving cisplatin.

3.1.1 Two-stage filtering procedure

Following the VS protocol described in section 2, we carried out a two-stage virtual screening procedure. Top hits from the first iteration were used as positive controls for the second First, we screened relatively small chemical libraries from the constructed VCC (see section 2.2.1). The objective at this stage was to discover novel lead compounds that can be used as positive controls for subsequent screening simulations and experiments.

Furthermore, we wished to construct a pharmacophore model that can guide future research toward discovering potent and specific inhibitors of this interaction. A detailed description of the parameters used and the results obtained in this study can be found in the published manuscript (K. H. Barakat, et al., 2009). Here, we summarize the major steps and report important findings.

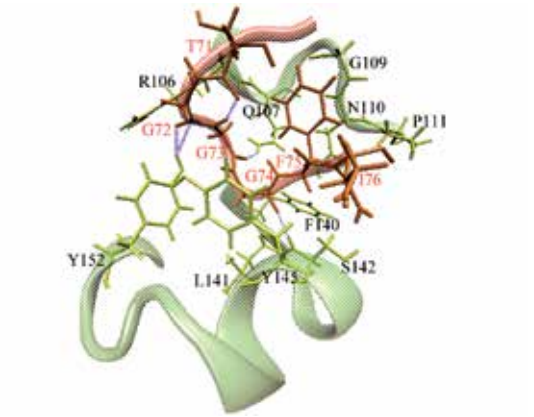


Fig. 4. An illustration of the XPA-ERCC1 protein-protein interaction. The binding between ERCC1 (teal) and XPA (red).

Prior to the screening process, it was important to determine precisely the key residues that mediated the ERCC1-XPA interaction (see Fig. 4). To do so, we used binding energy decomposition analysis using the MM-PBSA method in order to mark the essential residues from both sides. Residues within ERCC1 highlighted the binding site of the target. Following that, we extended the MD simulations up to 50 ns and extracted 6 dominant structures using clustering analysis. The length of the simulation was determined by convergence of the normalized overlap using PCA (see section 2.3.2 for details). Beside the 6 extracted structures we added 2 more protein conformations, one for a minimized initial structure and the other was an equilibrated conformation obtained from PCA. We also tried two docking alternatives. The intention was to compare docking against one target structure with flexible side chains to docking against eight rigid protein conformations. Flexible docking exploited the findings of the earlier analysis of binding energy decomposition, where we found Y145 to contribute more than 25% of the overall binding energy (data not shown). Hence, we decided to allow the full side-chain flexibility for this residue in docking to the minimized initial structure. In the other approach, we docked the two compound libraries to the eight rigid models of the target. We also used 3 different methods to rank the compounds; all of them were based on the AutoDock scoring function. In all ranking methods we used only the binding modes that possessed the largest docking cluster. The first ranking method was based on the minimum energy of the flexible docking run. The second used the average binding energy from the eight different rigid simulations. The final ranking was based on the weighted average binding energy using Equ. 4.

The binding mode for three selected top hits within their most favored binding site conformations is shown in Fig. 5. Electrostatic surface maps are included to provide an additional perspective of the charge distribution in the ERCC1 cavity. The binding cleft is mainly positively charged with small negatively charged spots on boundaries of the binding

site. This electrostatic potential distribution indicates that the binding site may exhibit a weak positive electrostatic potential. Although, the charge distribution changed slightly between the two representative binding sites indicating the perseverance of its overall shape, the positive potential is apparent on closed conformation. It is worth mentioning that thirty compounds out of this study were experimentally tested and two of them (including the one shown in Fig. 5-A) exhibited positive activity. These two identified top hits were then used as positive controls during the second stage of VS (see below).

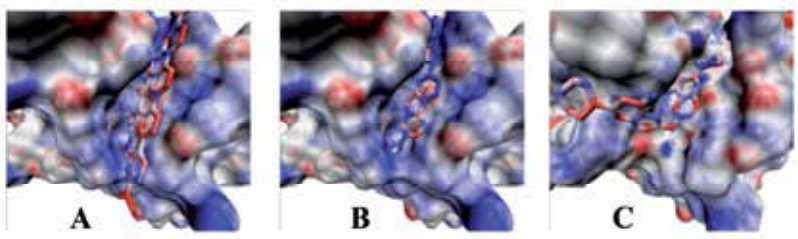


Fig. 5. Three selected hits within their preferred binding site conformations.

Two important lessons were learnt from the first stage and guided us toward more successful outcomes in the second round of screening. The first was to use NMR structures when available, instead of carrying out long MD simulations to extract dominant conformations of the target. The second lesson was to employ more accurate methods in ranking of the compounds, instead of depending solely on the docking scoring function, no matter which combination of scoring methods was used (i.e. average or weighted average binding energies). Hence, in executing the second screening simulation we used 10 NMR target structures, a larger library of compounds (CN chemical library ~100,000 compounds), and the MM-PBSA scoring method to rank the top hits. Eleven compounds from the top hits of this screening were tested experimentally. Five compounds showed positive activity leading to a significantly better-hit rate than the initial round of screening (data not shown). The top hit of the second round of ERCC1 screening is shown in Fig. 6.

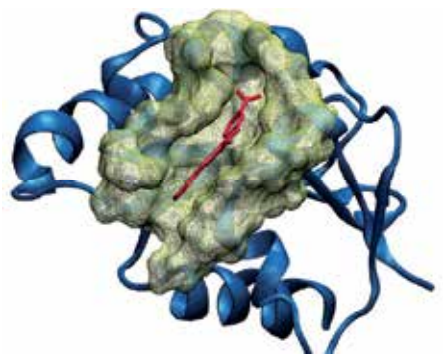


Fig. 6. The top hit from the second screening against the ERCC1 target.

3.2 Case II: dual inhibitors of p53-MDM2/4

Over the last two decades, the tumor suppressor protein p53 has been called the "guardian of the genome". P53 earned this label due to its vital roles in cell cycle, apoptosis, DNA

repair and senescence (Teodoro, et al., 2007). In these processes, p53 responds to cellular stresses, such as hypoxia and DNA damage, by accumulating in the nucleus and activating various pathways to maintain the cell's functional normality (Vogelstein, et al., 2000). As such, tumor cells have developed numerous ways to disable its function. Certainly, the gene *TP53*, which encodes for p53, is mutated or deleted in ~50% of human cancers (Feki & Irminger-Finger, 2004). In the remaining human cancers, while p53 retains its wild type structure, its activity is eradicated by its main cellular inhibitors, murine double minute 2/4 (MDM2/4) proteins (Kubbutat, et al., 1997; Kussie, et al., 1996). Originally, MDM2 was discovered as the main regulator of p53 activity. It acts as an E3 ubiquitin ligase that exports p53 out of the nucleus and promotes its degradation. Moreover, by binding to the transactivation domain of p53 within the nucleus, MDM2 inhibits p53 function as a transcription factor for other proteins. Consequently, over-expression of MDM2 reduces the cellular ability to activate the p53 pathway under stress conditions (Fakharzadeh, et al., 1991).

Structurally related to MDM2, MDM4 (also known as MDMX or HDMX) is a second cellular regulator of p53 (Shvarts, et al., 1996). Although MDM4 lacks the intrinsic E3 ligase activity of MDM2, current models suggest that it acts as a major p53 transcriptional antagonist independent of MDM2 (Toledo, et al., 2006). The binding domains of p53 within MDM2 and MDM4 are very similar (V. Bottger, et al., 1999), offering promise for the discovery of new small molecule compounds that can simultaneously target the two proteins.

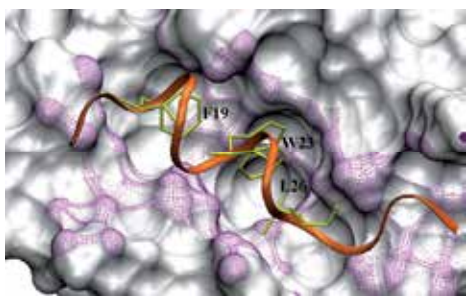


Fig. 7. Illustration of the P53-MDM2 interaction. The p53-binding site within MDM2 (purple) is shown in molecular surface representation with the residues constituting the binding site are highlighted in purple. P53 (orange) is shown in ribbon representation.

The high-resolution crystal structure of the p53-MDM2 complex demonstrated the essentialinteracting regions located in the MDM2-p53 interface (see
Fig. 7) (Kussie, et al., 1996). Essentially, p53 forms an amphipathic-helix peptide (residues 15-29) that is partly buried inside a small but deep, hydrophobic groove on the surface of the MDM2 N-terminal domain (residues 19-102). This interaction involves four key residues from p53, namely F19, L22, W23 and L26 and at least 13 residues from MDM2 (L54, L57, I61, M62, Y67, Q72, V75, F86, F91, V93, I99, Y100 and I103) (A. Bottger, et al., 1997). Interestingly, 10 out of the 13 most important MDM2 residues described above are conserved in MDMX, which indicates that the binding site of p53 within the surface of MDMX is similar to, but not identical with, that of MDM2.

The last decade witnessed the identification of many small-molecule p53-MDM2 inhibitors with promising binding affinities (Patel & Player, 2008). These are analogs of *cis*-imidazoline (Nutlins) (Vassilev, 2004), spiro-oxindole (MI-63 and MI219) (Dastidar, et al., 2008; Ding, et

al., 2006), benzodiazepinedione (TDP665759) (Grasberger, et al., 2005), terphenyl,(L. Chen, et al., 2005), quilinol,(Lu, et al., 2006), chalcone (Stoll, et al., 2001) and sulfonamide (Galatin & Abraham, 2004). Only three compounds, namely, Nutlin-3, MI-219 and TDP665759 showed sufficiently high binding affinity, and desirable pharmacokinetic profiles in cells (Shangary & Wang, 2008) to be seriously considered for clinical development. However, these compounds are more highly selective for MDM2 than for its homolog MDM4. Even nutlin-3 has been shown to be inactive in cancer cells that over-express MDM4 (B. Hu, et al., 2006), opening a new avenue in p53 research and requiring a new generation of MDM2-inhibitors that can target its homolog, MDM4, as well.

3.2.1 Screening against two targets

In our efforts to discover novel compounds that can restore the p53 activity we screened for dual inhibitors of the p53 interactions with both MDM2 and MDM4 (K. Barakat, et al., 2010). Our strategy followed from the evident similarity between the p53 binding sites within the two proteins. We first filtered a subset of our VCC for MDM2 inhibitors Top hits from this search were then screened against the MDM4 target. Compounds that can simultaneously bind to the two targets were considered as potential dual inhibitors. Our VCC-subset included the NCI diversity set, DrugBank compounds. We also enriched the docked compounds with more than 3,168 derivative structures extracted from the known MDM2-inhibitors. This enrichment was obvious, as similar targets are more than likely to bind similar compounds. The initial screening against MDM2 used 28 dominant protein conformations. These conformations represented the apo- and holo-MDM2's collective conformational dynamics and were extracted from MD simulations, PCA and clustering analyses.

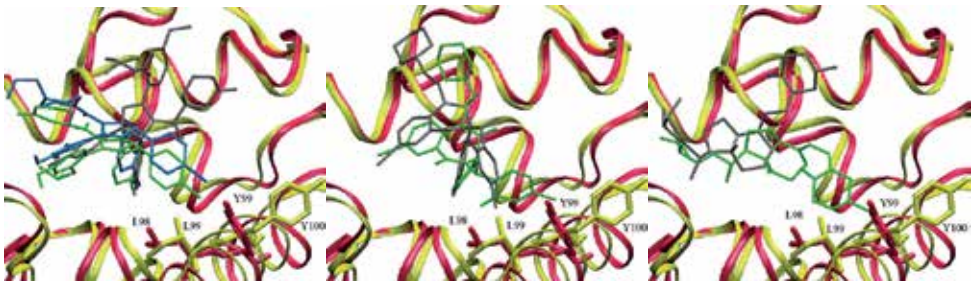
Compound	MDM2 Ranking (kcal/mol)			MDM4 Ranking (kcal/mol)		
	MM-PBSA	AutoDock	Experimental	MM-PBSA	AutoDock	Experimental
MI-219	-10.6 ± 1.5	-9.1 ± 2.2	-11.4	-5.3 ± 1.5	-6.8 ± 2.2	-5.9
Nutlin-3	-9.3 ± 1.3	-8.2 ± 2.2	-9.7	-6.1 ± 1.6	-5.8 ± 2.2	Negligible
TDP665759	-9.5 ± 1.5	-9.1 ± 2.2	-8.4	-5.6 ± 1.4	-8.2 ± 2.2	Negligible
PMI	-10.4 ± 1.6	N/A	-11.6	-12.8 ± 1.5	N/A	-11.5

Table 1. Relative ranking of positive controls using the two scoring methods compared to experimental data.

Scoring of the top MDM2 hits employed two ranking steps. First was a docking-based ranking similar to what was described in the previous ERCC1 study. The objective was to suggest a modest number (300 in this case) of promising hits for the subsequent re-ranking step. This final scoring utilized the MM-PBSA method. Prior to the application of the MM-PBSA method and as described in Fig. 1, all 300 hits were prepared through all-atoms and solvated MD simulations. We also included a recently discovered peptidic MDM2/4 dual inhibitor (PMI) in the rescoring step (Pazgier, et al., 2009). The 300 suggested MDM2 top hits were also docked to the p53-binding site within MDM4 followed by rescoring their binding using MD and MM-PBSA calculations. Table 1 describes the relative ranking of the used positive controls. The apparent IC_{50} values for Nutlin3, MI-219, TDP665759 and PMI in binding to MDM2 are 90 nM, 5 nM, 704 nM (Koblish, et al., 2006; Vassilev, 2007) and 3.4 nM35 at 250C, respectively. We did not find explicit values for the binding affinities of the three non-peptide molecules regarding their binding to MDM4, hoverer, it has been

experimentally confirmed that these compounds are weak binders to MDMX (Vassilev, 2007) (Pazgier, et al., 2009).

Although the discrepancy in the MM-PBSA calculations for the interactions of the four inhibitors with MDM2 was about 1 kcal/mol, the predicted values were in an excellent agreement with the experimental data compared to the values obtained by the AutoDock scoring function (see Table 1). These results also illustrated the limitations of AutoDock scoring function in eliminating false positive ligands, i.e. compounds that cannot practically bind but are predicted to bind, from active compounds. For example, the TDP665759 compound was predicted to bind to MDM4 with a relatively high binding energy compared to the rest of the compounds. On the other hand, the MM-PBSA approach selected the real binders for the two protein targets. For MDM2, the four ligands can bind strongly to the protein, while, for MDM4, only the PMI peptide can bind with a very high binding energy.



(a) (b) (c)

Fig. 8. Structural variations between MDM2 (yellow) and MDMX (red) and their effect on the binding modes of Nutlin-3 (a) and two selected hits form the predicted MDM2/MDM4 inhibitors (b and c). Tyr100 and Leu99 of MDM2 and the same residues in MDM4 are shown in Licorice representations with the same color as that of the two proteins. For each compound, the binding mode within MDM2 is shown in green and within MDM4 is shown in gray. Tyr99 and Leu98 prevent Nutlin-3 from binding to MDM4 with the same binding conformation adopted by Nutlin-2 within the MDM2-pocket (blue). The conformation of nutlin-2 was extracted from the MDM2-nutlin crystal structure 1RV1. On the other hand, compounds Pub#11952782 (b) and ZINC04629876 (c) from the suggested MDM2/MDM4 inhibitor list can tolerate the structural variations in the two binding sites in order to maximize their interactions with the proteins.

Not only did this study reveal a number of promising hits that can simultaneously bind to the two targets, but it also explained why known MDM2-inhibitors such as Nutlin 3 could not bind to MDM4. Although the two binding sites are fairly similar, the MDM4 pocket seemed to be more compact than that of MDM2. This was mainly due to the three residues Pro95, Ser96 and Pro97 in MDM4 that have been replaced by His96, Arg97 and Lys98 in MDM2 (see Fig. 8).

These substitutions are located on one of the alpha helices that comprise the p53 binding site within the two proteins. Consequently, the proline residues (Pro95 and Pro97) in MDM4 shifted this helical domain in MDM4 relative to MDM2 and caused Lys98 and Tyr99 to protrude into the p53-binding cleft within MDM4, making it shallower and less accessible to many of the MDM2 top hits we found. Moreover, we noticed very minor differences in the electrostatic potential distributions around the surfaces of the two proteins (data not

shown), where MDM2 was more positively charged in certain regions deeply located within the binding site. These slight variations in both shape and electrical properties of the two proteins played a considerable role in governing the final conformation adopted by the ligands.

This observation is clear when comparing the binding modes of nutlin within the two pockets (see Fig. 8-a). While Tyr100 and Leu99 of MDM2 extend the binding site allowing nutlin to intimately bind to MDM2, the same residues in MDM4 clash with the drug preventing it from taking the normal conformation that was adopted within MDM2. On the other hand, Fig. 8b-c show how two compounds from the discovered set of proposed MDM2/MDM4 inhibitors were able to tolerate the structural variations between the two binding sites.

3.3 Case III: inhibitors of DNA polymerase beta

DNA polymerase beta (polβ), the smallest naturally occurring DNA polymerase enzyme, belongs to the X-family of DNA polymerases (Uchiyama, et al., 2009). DNA polβ is a vital member of the base excision repair (BER) pathway (Beard & Wilson, 2006). The enzyme plays a significant role in chemotherapeutic agent resistance, as its over-expression reduces the efficacy of anticancer drug therapies including bleomycin (Parsons, et al., 2004), monofunctional alkylating agents (Liu, et al., 2002), cisplatin (Hoffmann, et al., 1996), and other platinum-based compounds.. Furthermore, small-scale studies on different types of cancer showed that polβ is mutated in approximately 30% of tumors, which in turn reduces polβ fidelity in DNA synthesis exposing the genome to serious and often deleterious mutations (Chan, et al., 2007; Starcevic, et al., 2004). Based on these findings, polβ, the error-prone polymerase of BER, has been seriously considered as a promising therapeutic target for cancer treatment.

Many inhibitors of DNA polβ have been identified during the last two decades. To name but a few, this list includes polypeptides (Husain, et al., 1995), fatty acids (Mizushina, et al., 1996), triterpenoids (Tanaka, et al., 1998), sulfolipids (Mizushina, et al., 1998), polar lipids (Ogawa, Murate, Izuta, et al., 1998), secondary bile acids (Ogawa, Murate, Suzuki, et al., 1998), phenalenone-derivatives (Perpelescu, et al., 2002), anacardic acid (J. Z. Chen, Y.; Wang, L.; Sucheck, S.; Snow, A.; Hecht, S., 1998), harbinatic acid (Deng, et al., 1999), flavanoid derivatives (Maloney, et al., 2005), and pamoic acid (H. Y. Hu, et al., 2004). However, most of these inhibitors are either not potent enough or lack sufficient specificity to eventually become approved drugs. Among these compounds pamoic acid (PA) was one of the few compounds that had promising activity against polβ and a well-defined binding mode. The compound was initially discovered by Hu and his co-workers (H. Y. Hu, et al., 2004) Their NMR analysis revealed that PA binds to the 8-kDa domain of polβ and suggested that the binding pocket is located between the two helices: helix-2 and helix-4 of the 8-kDa domain. Interestingly, the same region has been recognized in different studies to be essential in the DNA binding and deoxyribose phosphate lyase activities of the enzyme (Pelletier, et al., 1994). The precise interactions between PA and the lyase domain of polβ were further investigated in a different study (Hazan, et al., 2008) which used a combination protocol of blind docking and NMR analysis to confirm the earlier findings of Hu et al (H. Y. Hu, et al., 2004).

3.3.1 Screening against the lyase active site of polβ

Following the procedure described in Fig. 1, we focused our search space on the binding site of PA, using it as a positive control. Our aim was to discover more potent drug candidates through filtering a library of ~12,500 compounds against 11 protein structures. The molecules tested included the NCI diversity set, the DrugBank set of small-molecules and more than 9,000 fragment structures with drug-like properties extracted from the ZINC database (see section 2.2.1 for a detailed description of these compound libraries). The top 300 hits that showed strong affinity for polβ have been validated and rescored using a more robust scoring function, the MM-PBSA method.

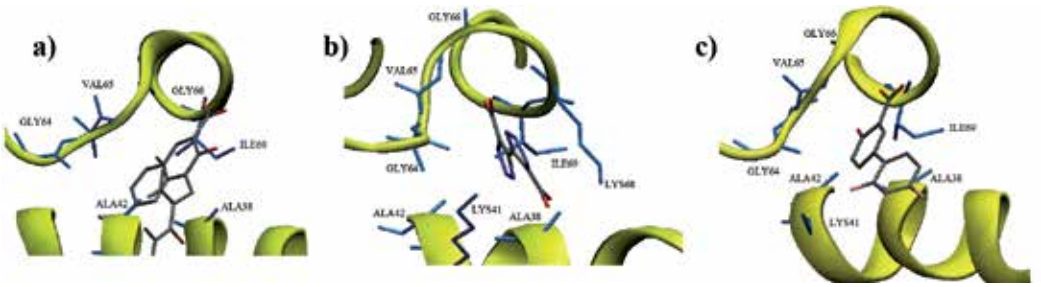


Fig. 9. Binding modes of selected hits. Polβ is shown in yellow, important protein residues are shown in blue, and the different atoms of the bound compounds are shown by their representative colors (carbon in gray, oxygen in red, nitrogen in blue and hydrogen in white).

The reported K_D value for PA binding to polβ is 9 μM (H. Y. Hu, et al., 2004). Using the AutoDock scoring function, we obtained a value of -6.2 kcal/mol as an estimate for this binding energy. Although this value is in excellent agreement with the experimental measurement (-6.9 kcal/mol) as calculated using the K_D value, based on the previously described studies, docking scoring functions were not efficient in discriminating false positives in VS experiments and are biased toward their training set of compounds (Tondi, et al., 1999). Consequently, in this work, the top 300 hits were rescored using the MM-PBSA method in order to validate their docking results and confirm their binding to the protein. Fig. 9 demonstrates the binding modes of the top three hits of the MM-PBSA ranking. Similarly to a substantial number of our suggested top hits, the shown compounds are small in size, however, they occupy a considerable portion of the DNA-binding pocket. These lead compounds can be employed as the basis for a further fragment-based drug design step, in order to construct potent and more specific pol β inhibitors.

4. Conclusion

DNA repair pathways control the balance between genomic stability's constancy and mutability (Harper & Elledge, 2007). The mode of action of modern anticancer treatments is by inducing damage to the DNA. Over-expression of proteins involved in the DNA repair circuitry boosts the repair activity, removes most of the induced damage and hence, reduces the efficacy of DNA-damaging agents. This unexpected mechanism represents one of the

major factors behind the antitumor drug resistance phenomena observed for these agents (Harper & Elledge, 2007). Therefore. DNA repair proteins are currently considered as valuable targets to improve cancer therapy (Damia & D'Incalci, 2007).

Our group has been involved in in silico searches for novel inhibitors of a number of DNA repair proteins. This chapter reviews our efforts in applying computational high throughput screening methods to filter compound libraries for such inhibitors. The chapter contains two main parts. First is a detailed description of all the computational steps that are used in the virtual screening workflow that we follow. Second is a summary of the results we found in applying this protocol to three important DNA repair targets. The three targets are ERCC1-XPA (K. H. Barakat, et al., 2009), an important element of the NER pathway; MDM2 and MDM4 (K. Barakat, et al., 2010), the two cellular inhibitors of p53; and finally DNA polβ (K. Barakat, et al., In press); (K. Barakat & Tuszynski, 2011), the error prone polymerase of BER. The aim of this review is to share with the reader our experience in this field from a computational drug discovery perspective. Furthermore, we have attempted to demonstrate that computational tools can be easily applied to DNA repair proteins and eventually arrive at compounds capable of regulating their activities.

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6. References

Abagyan, R., & Totrov, M. (2001). High-throughput docking for lead generation. *Curr Opin Chem Biol, 5*(4), 375-382.

Amadei, A., Linssen, A. B., & Berendsen, H. J. (1993). Essential dynamics of proteins. *Proteins, 17*(4), 412-425.

Amaro, R. E., Baron, R., & McCammon, J. A. (2008). An improved relaxed complex scheme for receptor flexibility in computer-aided drug design. *J Comput Aided Mol Des, 22*(9), 693-705.

Barakat, K., Gajewski, M., & Tuszynski, J. (In press). DNA polymerase beta (pol ß) inhibitors: a comprehensive overview.

Barakat, K., Mane, J., Friesen, D., & Tuszynski, J. (2010). Ensemble-based virtual screening reveals dual-inhibitors for the p53-MDM2/MDMX interactions. *J Mol Graph Model, 28*(6), 555-568.

Barakat, K., & Tuszynski, J. (2011). Relaxed complex scheme suggests novel inhibitors for the lyase activity of DNA polymerase beta. *J Mol Graph Model, 29*(5), 702-716.

Barakat, K. H., Torin Huzil, J., Luchko, T., Jordheim, L., Dumontet, C., & Tuszynski, J. (2009). Characterization of an inhibitory dynamic pharmacophore for the ERCC1-XPA interaction using a combined molecular dynamics and virtual screening approach. *J Mol Graph Model, 28*(2), 113-130.

Beard, W. A., & Wilson, S. H. (2006). Structure and mechanism of DNA polymerase Beta. *Chem Rev, 106*(2), 361-382.

Bennett, M. J., Barakat, K., Huzil, J. T., Tuszynski, J., & Schriemer, D. C. (2010). Discovery and characterization of the laulimalide-microtubule binding mode by mass shift perturbation mapping. *Chem Biol, 17*(7), 725-734.

Bottger, A., Bottger, V., Garcia-Echeverria, C., Chene, P., Hochkeppel, H. K., Sampson, W., et al. (1997). Molecular characterization of the hdm2-p53 interaction. *J Mol Biol, 269*(5), 744-756.

Bottger, V., Bottger, A., Garcia-Echeverria, C., Ramos, Y. F., van der Eb, A. J., Jochemsen, A. G., et al. (1999). Comparative study of the p53-mdm2 and p53-MDMX interfaces. *Oncogene, 18*(1), 189-199.

Boulikas, T., & Vougiouka, M. (2003). Cisplatin and platinum drugs at the molecular level. (Review). *Oncol Rep, 10*(6), 1663-1682.

Broughton, H. B. (2000). A method for including protein flexibility in protein-ligand docking: improving tools for database mining and virtual screening. *J Mol Graph Model, 18*(3), 247-257, 302-244.

Carlson, H. A., Masukawa, K. M., Rubins, K., Bushman, F. D., Jorgensen, W. L., Lins, R. D., et al. (2000). Developing a dynamic pharmacophore model for HIV-1 integrase. *J Med Chem, 43*(11), 2100-2114.

Case, D. A., Cheatham, T. E., 3rd, Darden, T., Gohlke, H., Luo, R., Merz, K. M., Jr., et al. (2005). The Amber biomolecular simulation programs. *J Comput Chem, 26*(16), 1668-1688.

Chan, K., Houlbrook, S., Zhang, Q. M., Harrison, M., Hickson, I. D., & Dianov, G. L. (2007). Overexpression of DNA polymerase beta results in an increased rate of frameshift mutations during base excision repair. *Mutagenesis, 22*(3), 183-188.

Chen, J. Z., Y.; Wang, L.; Sucheck, S.; Snow, A.; Hecht, S. (1998). Inhibitors of DNA Polymerase b from Schoepfia Californica. *J C S Chem Commun*, 2769–2770.

Chen, L., Yin, H., Farooqi, B., Sebti, S., Hamilton, A. D., & Chen, J. (2005). p53 alpha-Helix mimetics antagonize p53/MDM2 interaction and activate p53. *Mol Cancer Ther, 4*(6), 1019-1025.

Damia, G., & D'Incalci, M. (2007). Targeting DNA repair as a promising approach in cancer therapy. *Eur J Cancer, 43*(12), 1791-1801.

Dastidar, S. G., Lane, D. P., & Verma, C. S. (2008). Multiple peptide conformations give rise to similar binding affinities: molecular simulations of p53-MDM2. *J Am Chem Soc, 130*(41), 13514-13515.

Davies DL, & Bouldin DW. (1979). A cluster separation measure. *IEEE Trans. Pattern Anal. Mach. Intelligence, 1*, 224.

Deng, J. Z., Starck, S. R., Hecht, S. M., Ijames, C. F., & Hemling, M. E. (1999). Harbinatic acid, a novel and potent DNA polymerase beta inhibitor from Hardwickia binata. *J Nat Prod, 62*(7), 1000-1002.

Ding, K., Lu, Y., Nikolovska-Coleska, Z., Wang, G., Qiu, S., Shangary, S., et al. (2006). Structure-based design of spiro-oxindoles as potent, specific small-molecule inhibitors of the MDM2-p53 interaction. *J Med Chem, 49*(12), 3432-3435.

Fakharzadeh, S. S., Trusko, S. P., & George, D. L. (1991). Tumorigenic potential associated with enhanced expression of a gene that is amplified in a mouse tumor cell line. *EMBO J, 10*(6), 1565-1569.

Feki, A., & Irminger-Finger, I. (2004). Mutational spectrum of p53 mutations in primary breast and ovarian tumors. *Crit Rev Oncol Hematol, 52*(2), 103-116.

Galatin, P. S., & Abraham, D. J. (2004). A nonpeptidic sulfonamide inhibits the p53-mdm2 interaction and activates p53-dependent transcription in mdm2-overexpressing cells. *J Med Chem, 47*(17), 4163-4165.

Garcia, A. E. (1992). Large-amplitude nonlinear motions in proteins. *Phys Rev Lett, 68*(17), 2696-2699.

Garrett MM, David SG, Robert SH, Ruth H, William EH, Richard KB, et al. (1999). Automated docking using a Lamarckian genetic algorithm and an empirical binding free energy function. *J Comput Chem., 19*, 1639.

Goodsell, D. S., & Olson, A. J. (1990). Automated docking of substrates to proteins by simulated annealing. *Proteins, 8*(3), 195-202.

Grasberger, B. L., Lu, T., Schubert, C., Parks, D. J., Carver, T. E., Koblish, H. K., et al. (2005). Discovery and cocrystal structure of benzodiazepinedione HDM2 antagonists that activate p53 in cells. *J Med Chem, 48*(4), 909-912.

Harper, J. W., & Elledge, S. J. (2007). The DNA damage response: ten years after. *Mol Cell, 28*(5), 739-745.

Hazan, C., Boudsocq, F., Gervais, V., Saurel, O., Ciais, M., Cazaux, C., et al. (2008). Structural insights on the pamoic acid and the 8 kDa domain of DNA polymerase beta complex: towards the design of higher-affinity inhibitors. *BMC Struct Biol, 8*, 22.

Hess, B. (2002). Convergence of sampling in protein simulations. *Phys Rev E Stat Nonlin Soft Matter Phys, 65*(3 Pt 1), 031910.

Hoffmann, J. S., Pillaire, M. J., Garcia-Estefania, D., Lapalu, S., & Villani, G. (1996). In vitro bypass replication of the cisplatin-d(GpG) lesion by calf thymus DNA polymerase beta and human immunodeficiency virus type I reverse transcriptase is highly mutagenic. *J Biol Chem, 271*(26), 15386-15392.

Hornak, V., Abel, R., Okur, A., Strockbine, B., Roitberg, A., & Simmerling, C. (2006). Comparison of multiple Amber force fields and development of improved protein backbone parameters. *Proteins, 65*(3), 712-725.

Hu, B., Gilkes, D. M., Farooqi, B., Sebti, S. M., & Chen, J. (2006). MDMX overexpression prevents p53 activation by the MDM2 inhibitor Nutlin. *J Biol Chem, 281*(44), 33030-33035.

Hu, H. Y., Horton, J. K., Gryk, M. R., Prasad, R., Naron, J. M., Sun, D. A., et al. (2004). Identification of small molecule synthetic inhibitors of DNA polymerase beta by NMR chemical shift mapping. *J Biol Chem, 279*(38), 39736-39744.

Husain, I., Morton, B. S., Beard, W. A., Singhal, R. K., Prasad, R., Wilson, S. H., et al. (1995). Specific inhibition of DNA polymerase beta by its 14 kDa domain: role of single- and double-stranded DNA binding and 5'-phosphate recognition. *Nucleic Acids Res, 23*(9), 1597-1603.

Irwin, J. J., & Shoichet, B. K. (2005). ZINC--a free database of commercially available compounds for virtual screening. *J Chem Inf Model, 45*(1), 177-182.

Kang, S., Ju, W., Kim, J. W., Park, N. H., Song, Y. S., Kim, S. C., et al. (2006). Association between excision repair cross-complementation group 1 polymorphism and clinical

outcome of platinum-based chemotherapy in patients with epithelial ovarian cancer. *Exp Mol Med, 38*(3), 320-324.

Koblish, H. K., Zhao, S., Franks, C. F., Donatelli, R. R., Tominovich, R. M., LaFrance, L. V., et al. (2006). Benzodiazepinedione inhibitors of the Hdm2:p53 complex suppress human tumor cell proliferation in vitro and sensitize tumors to doxorubicin in vivo. *Mol Cancer Ther, 5*(1), 160-169.

Kollman, P. A., Massova, I., Reyes, C., Kuhn, B., Huo, S., Chong, L., et al. (2000). Calculating structures and free energies of complex molecules: combining molecular mechanics and continuum models. *Acc Chem Res, 33*(12), 889-897.

Kubbutat, M. H., Jones, S. N., & Vousden, K. H. (1997). Regulation of p53 stability by Mdm2. *Nature, 387*(6630), 299-303.

Kussie, P. H., Gorina, S., Marechal, V., Elenbaas, B., Moreau, J., Levine, A. J., et al. (1996). Structure of the MDM2 oncoprotein bound to the p53 tumor suppressor transactivation domain. *Science, 274*(5289), 948-953.

Li, H., Robertson, A. D., & Jensen, J. H. (2005). Very fast empirical prediction and rationalization of protein pKa values. *Proteins, 61*(4), 704-721.

Li, L., Elledge, S. J., Peterson, C. A., Bales, E. S., & Legerski, R. J. (1994). Specific association between the human DNA repair proteins XPA and ERCC1. *Proc Natl Acad Sci U S A, 91*(11), 5012-5016.

Limin, A. L., Dongqing, W., Yixue, L., & Huimin, L. (2011). *Handbook of Research on Computational and Systems Biology: Interdisciplinary Applications (ISBN (ebook))*: IGI Publishing.

Lin, J. H., Perryman, A. L., Schames, J. R., & McCammon, J. A. (2002). Computational drug design accommodating receptor flexibility: the relaxed complex scheme. *J Am Chem Soc, 124*(20), 5632-5633.

Liu, L., Nakatsuru, Y., & Gerson, S. L. (2002). Base excision repair as a therapeutic target in colon cancer. *Clin Cancer Res, 8*(9), 2985-2991.

Lord, R. V., Brabender, J., Gandara, D., Alberola, V., Camps, C., Domine, M., et al. (2002). Low ERCC1 expression correlates with prolonged survival after cisplatin plus gemcitabine chemotherapy in non-small cell lung cancer. *Clin Cancer Res, 8*(7), 2286-2291.

Lu, Y., Nikolovska-Coleska, Z., Fang, X., Gao, W., Shangary, S., Qiu, S., et al. (2006). Discovery of a nanomolar inhibitor of the human murine double minute 2 (MDM2)-p53 interaction through an integrated, virtual database screening strategy. *J Med Chem, 49*(13), 3759-3762.

Maloney, D. J., Deng, J. Z., Starck, S. R., Gao, Z., & Hecht, S. M. (2005). (+)-Myristinin A, a naturally occurring DNA polymerase beta inhibitor and potent DNA-damaging agent. *J Am Chem Soc, 127*(12), 4140-4141.

McGuire, W. P., & Ozols, R. F. (1998). Chemotherapy of advanced ovarian cancer. *Semin Oncol, 25*(3), 340-348.

Mizushina, Y., Tanaka, N., Yagi, H., Kurosawa, T., Onoue, M., Seto, H., et al. (1996). Fatty acids selectively inhibit eukaryotic DNA polymerase activities in vitro. *Biochim Biophys Acta, 1308*(3), 256-262.

Mizushina, Y., Watanabe, I., Ohta, K., Takemura, M., Sahara, H., Takahashi, N., et al. (1998). Studies on inhibitors of mammalian DNA polymerase alpha and beta: sulfolipids from a pteridophyte, Athyrium niponicum. *Biochem Pharmacol, 55*(4), 537-541.

Ogawa, A., Murate, T., Izuta, S., Takemura, M., Furuta, K., Kobayashi, J., et al. (1998). Sulfated glycoglycerolipid from archaebacterium inhibits eukaryotic DNA polymerase alpha, beta and retroviral reverse transcriptase and affects methyl methanesulfonate cytotoxicity. *Int J Cancer, 76*(4), 512-518.

Ogawa, A., Murate, T., Suzuki, M., Nimura, Y., & Yoshida, S. (1998). Lithocholic acid, a putative tumor promoter, inhibits mammalian DNA polymerase beta. *Jpn J Cancer Res, 89*(11), 1154-1159.

Parsons, J. L., Dianova, II, & Dianov, G. L. (2004). APE1 is the major 3'-phosphoglycolate activity in human cell extracts. *Nucleic Acids Res, 32*(12), 3531-3536.

Patel, S., & Player, M. R. (2008). Small-molecule inhibitors of the p53-HDM2 interaction for the treatment of cancer. *Expert Opin Investig Drugs, 17*(12), 1865-1882.

Pazgier, M., Liu, M., Zou, G., Yuan, W., Li, C., Li, J., et al. (2009). Structural basis for high-affinity peptide inhibition of p53 interactions with MDM2 and MDMX. *Proc Natl Acad Sci U S A, 106*(12), 4665-4670.

Pelletier, H., Sawaya, M. R., Kumar, A., Wilson, S. H., & Kraut, J. (1994). Structures of ternary complexes of rat DNA polymerase beta, a DNA template-primer, and ddCTP. *Science, 264*(5167), 1891-1903.

Perpelescu, M., Kobayashi, J., Furuta, M., Ito, Y., Izuta, S., Takemura, M., et al. (2002). Novel phenalenone derivatives from a marine-derived fungus exhibit distinct inhibition spectra against eukaryotic DNA polymerases. *Biochemistry, 41*(24), 7610-7616.

Rabik, C. A., & Dolan, M. E. (2007). Molecular mechanisms of resistance and toxicity associated with platinating agents. *Cancer Treat Rev, 33*(1), 9-23.

Rosenberg, E., Taher, M. M., Kuemmerle, N. B., Farnsworth, J., & Valerie, K. (2001). A truncated human xeroderma pigmentosum complementation group A protein expressed from an adenovirus sensitizes human tumor cells to ultraviolet light and cisplatin. *Cancer Res, 61*(2), 764-770.

Schneider, G., & Bohm, H. J. (2002). Virtual screening and fast automated docking methods. *Drug Discov Today, 7*(1), 64-70.

Shangary, S., & Wang, S. (2008). Targeting the MDM2-p53 interaction for cancer therapy. *Clin Cancer Res, 14*(17), 5318-5324.

Shao J, Tanner SW, Thompson N, & Cheatham TE. (2007). Clustering Molecular Dynamics Trajectories: 1. Characterizing the Performance of Different Clustering Algorithms. Journal of Chemical Theory and Computation. *3*, 2312.

Shao, J., Tanner, S., Thompson, N., & Cheatham, T. (2007). Clustering Molecular Dynamics Trajectories: 1. Characterizing the Performance of Different Clustering Algorithms. *Journal of Chemical Theory and Computation*(3), 2312.

Shirota, Y., Stoehlmacher, J., Brabender, J., Xiong, Y. P., Uetake, H., Danenberg, K. D., et al. (2001). ERCC1 and thymidylate synthase mRNA levels predict survival for colorectal cancer patients receiving combination oxaliplatin and fluorouracil chemotherapy. *J Clin Oncol, 19*(23), 4298-4304.

Shoichet, B. K., Stroud, R. M., Santi, D. V., Kuntz, I. D., & Perry, K. M. (1993). Structure-based discovery of inhibitors of thymidylate synthase. *Science, 259*(5100), 1445-1450.

Shvarts, A., Steegenga, W. T., Riteco, N., van Laar, T., Dekker, P., Bazuine, M., et al. (1996). MDMX: a novel p53-binding protein with some functional properties of MDM2. *EMBO J, 15*(19), 5349-5357.

Stahura, F. L., & Bajorath, J. (2004). Virtual screening methods that complement HTS. *Comb Chem High Throughput Screen, 7*(4), 259-269.

Starcevic, D., Dalal, S., & Sweasy, J. B. (2004). Is there a link between DNA polymerase beta and cancer? *Cell Cycle, 3*(8), 998-1001.

Stoll, R., Renner, C., Hansen, S., Palme, S., Klein, C., Belling, A., et al. (2001). Chalcone derivatives antagonize interactions between the human oncoprotein MDM2 and p53. *Biochemistry, 40*(2), 336-344.

Tanaka, N., Kitamura, A., Mizushina, Y., Sugawara, F., & Sakaguchi, K. (1998). Fomitellic acids, triterpenoid inhibitors of eukaryotic DNA polymerases from a basidiomycete, fomitella fraxinea. *J Nat Prod, 61*(9), 1180.

Teodoro, J. G., Evans, S. K., & Green, M. R. (2007). Inhibition of tumor angiogenesis by p53: a new role for the guardian of the genome. *J Mol Med, 85*(11), 1175-1186.

Toledo, F., Krummel, K. A., Lee, C. J., Liu, C. W., Rodewald, L. W., Tang, M., et al. (2006). A mouse p53 mutant lacking the proline-rich domain rescues Mdm4 deficiency and provides insight into the Mdm2-Mdm4-p53 regulatory network. *Cancer Cell, 9*(4), 273-285.

Tondi, D., Slomczynska, U., Costi, M. P., Watterson, D. M., Ghelli, S., & Shoichet, B. K. (1999). Structure-based discovery and in-parallel optimization of novel competitive inhibitors of thymidylate synthase. *Chem Biol, 6*(5), 319-331.

Tsodikov, O. V., Ivanov, D., Orelli, B., Staresincic, L., Shoshani, I., Oberman, R., et al. (2007). Structural basis for the recruitment of ERCC1-XPF to nucleotide excision repair complexes by XPA. *EMBO J, 26*(22), 4768-4776.

Uchiyama, Y., Takeuchi, R., Kodera, H., & Sakaguchi, K. (2009). Distribution and roles of X-family DNA polymerases in eukaryotes. *Biochimie, 91*(2), 165-170.

Vassilev, L. T. (2004). Small-molecule antagonists of p53-MDM2 binding: research tools and potential therapeutics. *Cell Cycle, 3*(4), 419-421.

Vassilev, L. T. (2007). MDM2 inhibitors for cancer therapy. *Trends Mol Med, 13*(1), 23-31.

Vogelstein, B., Lane, D., & Levine, A. J. (2000). Surfing the p53 network. *Nature, 408*(6810), 307-310.

Warren, G. L., Andrews, C. W., Capelli, A. M., Clarke, B., LaLonde, J., Lambert, M. H., et al. (2006). A critical assessment of docking programs and scoring functions. *J Med Chem, 49*(20), 5912-5931.

Wishart, D. S., Knox, C., Guo, A. C., Shrivastava, S., Hassanali, M., Stothard, P., et al. (2006). DrugBank: a comprehensive resource for in silico drug discovery and exploration. *Nucleic Acids Res, 34*(Database issue), D668-672.

Wood, R. D., Mitchell, M., Sgouros, J., & Lindahl, T. (2001). Human DNA repair genes. *Science, 291*(5507), 1284-1289.

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Mitochondrial DNA Repair

Sarah A. Martin
Barts Cancer Institute, Barts & the London School of Medicine & Dentistry,
Queen Mary University of London
UK

1. Introduction

Each mitochondrion consists of 16,569 base pairs which encodes 37 genes, all of which are essential for normal mitochondrial function (Anderson et al., 1981). Each human cell contains several hundred copies of mitochondrial DNA, encoding 13 genes that are required for oxidative phosphorylation, 22 transfer RNAs and 2 ribosomal RNAs (Anderson et al., 1981). Mitochondria are vital organelles, which generate the majority of the cells energy through oxidative phosphorylation (Wallace, 2005). During this process, reactive oxygen species (ROS) are produced, that can leak out and react with a range of cellular components, including the mitochondrial genome (Richter et al., 1988). Therefore, it has been suggested that levels of oxidative DNA damage are higher in mitochondrial DNA than in nuclear DNA, with mitochondrial DNA accumulating mutations at a 10- to 50- fold higher rate (Hudson et al., 1998; Michikawa et al., 1999; Pakendorf and Stoneking, 2005; Yakes and Van Houten, 1997). If this mitochondrial DNA damage is not repaired, it can lead to disruption of the electron transport chain and increased generation of ROS, possibly resulting in vicious cycle of ROS production and mitochondrial DNA damage, leading to energy depletion and ultimately cell death (Harman, 1972; Miquel et al., 1980). Therefore suggesting that mitochondria must employ some form of repair or defence mechanism against such forms of deleterious damage.

The integrity of mitochondrial DNA repair plays a central role in maintaining homeostasis in the cell and thus the efficient repair of mitochondrial DNA damage serves as an essential function in cellular survival. In comparison to nuclear DNA repair, our knowledge regarding mitochondrial DNA repair is limited. In fact, it was originally believed that mitochondria employed no repair mechanisms and damaged DNA was not repaired, but was merely degraded. This was primarily based on a study published in 1974, which demonstrated the inability of mitochondria to remove cyclobutyl pyrimidine dimers after exposure to ultraviolet light (Clayton et al., 1974). This theory remained for many years, but now it is abundantly clear that multiple DNA repair pathways and the controlled degradation of mitochondrial DNA, work together to maintain the integrity of the mitochondrial genome (Berneburg et al., 2006; Liu and Demple, 2010). Initially the repair of most mitochondrial DNA damage was thought to be limited to short-patch base excision repair (BER) (Stierum et al., 1999). However, the complex range of DNA lesions inflicted on mitochondrial DNA by ROS and potential replication errors indicated that such a restricted repair mechanism would be insufficient. Our knowledge of mitochondrial DNA repair has recently witnessed a rapid expansion and it is now evident that mitochondria also employ

long-patch BER (Akbari et al., 2008; Liu et al., 2008; Szczesny et al., 2008; Zheng et al., 2008), mismatch repair (de Souza-Pinto et al., 2009; Mason et al., 2003), homologous recombination and non-homologous end-joining (Bacman et al., 2009; Fukui and Moraes, 2009; Thyagarajan et al., 1996). In addition, sanitation of the mitochondrial deoxynucleotide triphosphate (dNTP) pool and selective degradation of heavily damaged mitochondrial DNA play important roles in maintaining mitochondrial DNA integrity and preventing cell death (Bacman et al., 2009; Ichikawa et al., 2008; Shokolenko et al., 2009). The majority of the proteins dedicated to DNA repair have to be transcribed and translated from nuclear DNA where they are encoded and imported into the mitochondrion (Bohr, 2002).

Many inherited diseases result from mutations in the mitochondrial genome or due to mutations in nuclear genes that encode mitochondrial components (Chan and Copeland, 2009; Horvath et al., 2009; Tuppen et al., 2010). Somatic mutations in mitochondrial DNA are increasingly linked to common diseases, including age-related degenerative disorders and cancers. Specifically, mitochondrial DNA mutations have been detected in colorectal (Habano et al., 1998; Polyak et al., 1998), breast (Parrella et al., 2001; Radpour et al., 2009) bladder (Copeland et al., 2002; Dasgupta et al., 2008; Wada et al., 2006), lung (Dai et al., 2006; Jin et al., 2007; Suzuki et al., 2003), head and neck cancers (Dasgupta et al., 2010) (Allegra et al., 2006; Mithani et al., 2007), amongst others. Furthermore, some evidence also exists suggesting that mutations in mitochondrial DNA can even accelerate disease progression (Ishikawa and Hayashi, 2010; Lee et al., 2010). Although many associations between mitochondrial DNA mutations and cancer have been shown, a functional link to mitochondrial DNA repair still requires further investigation. Increasing evidence also suggests that mitochondrial DNA damage accumulates with age. However conflicting reports argue whether aging is due to the accumulation of mitochondrial DNA damage or perhaps modifications in mitochondrial DNA repair mechanisms may cause accumulation of DNA damage associated with aging (Boesch et al., 2011; Gruber et al., 2008; Obulesu and Rao, 2010).

2. Mitochondrial DNA repair pathways

Our DNA, both nuclear and mitochondrial, is constantly exposed to endogenous and exogenous agents that induce DNA lesions and genomic instability (De Bont and van Larebeke, 2004; Sander et al., 2005). In the absence of DNA repair, the genome would be unable to survive the multitude of lesions that form throughout the cell cycle. Therefore, a range of molecular mechanisms has evolved that ensures that damaged DNA is effectively repaired. These pathways coordinate the repair of DNA lesions and the stalling of the cell cycle to allow repair to occur (Harper and Elledge, 2007). DNA repair mechanisms have been extensively studied in the nucleus and increasing data demonstrates how distinct DNA lesions are repaired by different DNA repair pathways including homologous recombination, non-homologous end joining, base excision repair, nucleotide excision repair, mismatch repair, and translesion synthesis (Hoeijmakers, 2009). The relevance of the DNA repair pathways in the maintenance of genome integrity and cellular survival is evidenced by the critical consequences in the survival of organisms when deficiencies in key enzymes of the DNA repair pathways occur (Martin et al., 2008).

In contrast to the repertoire of nuclear DNA repair pathways, for many years, the repair of mitochondrial DNA damage was thought to be limited to short-patch BER (Stierum et al., 1999). However more recently with increasing knowledge of the likely array of lesions

inflicted on mitochondrial DNA, it was suggested that such a limited repair repertoire would be insufficient. Studies have identified an expanded range of mitochondrial DNA repair processes including long-patch base excision repair, mismatch repair, homologous recombination and nonhomologous end-joining (Boesch et al., 2011; Liu and Demple, 2010; Yang et al., 2008). It is still generally considered that there is no nucleotide excision repair (NER) in the mitochondria. However, it has been shown that the NER gene, Cockayne syndrome B (CSB) is involved in the removal of oxidative DNA damage from the nucleus, such that CSB-deficient cells demonstrated reduced repair rates of 8-oxoG DNA lesions and extracts from CSB-deficient cells fail to incise oligonucleotides containing 8-oxoG (Balajee et al., 1999) (Dianov et al., 1999; Le Page et al., 2000; Selzer et al., 2002). CSB has also been shown to act in concert with OGG1 in the repair of these lesions (Tuo et al., 2002; Tuo et al., 2001). Due to the generation of ROS in the mitochondria and the increased levels of oxidative damage it was hypothesized that mitochondria-targeted CSB could have a role in repair of mitochondrial DNA. To this end, Stevnsner et al. demonstrated that CSB-deficient cells exhibited a reduced ability to repair 8-oxoG in the mitochondria, suggesting possible NER activity (Stevnsner et al., 2002a). Similarly, the presence of translesion synthesis (TLS) in mitochondria has not been fully elucidated. In the nucleus, TLS is carried out by specialized polymerases, which have the ability to copy defective DNA templates. The possibility of mitochondrial TLS has been suggested due to the fact that the mitochondrial polymerase POLG is capable of mutagenic bypass through DNA lesions introducing dA opposite an AP site or an 8-oxodG (Graziewicz et al., 2007; Pinz et al., 1995) and also opposite benzo[a]pyrene and benzo[c]phenanthrene diol epoxide adducts of deoxyguanosine and deoxyadenosine (Graziewicz et al., 2004). To date, the presence of TLS activity *in vivo* in mitochondria remains to be shown. For both NER and TLS, further research is necessary to define the precise mechanisms of these processes in the mitochondria.

2.1 Base excision repair

The mitochondrial DNA sits on the inner side of the mitochondrial inner membrane, where most reactive oxygen species (ROS) are generated, rendering it highly susceptible to oxidative damage. BER is one of the main pathways for the repair of oxidized modifications both in nuclear and mitochondrial DNA (Slupphaug et al., 2003). As mentioned above, previously the repair of mitochondrial DNA damage and in particular oxidative DNA damage was thought to be limited to short-patch BER (Stierum et al., 1999), which replaces a single nucleotide by the sequential action of DNA glycosylases, an apurinic/apyrimidinic (AP) endonuclease, a DNA polymerase, an abasic lyase activity and DNA ligase (Dianov et al., 2001)(Figure 1). In addition to oxidative DNA damage, BER is the primary pathway required for repair of small DNA modifications induced by alkylaltion and deamination. As in nuclear BER, mitochondrial BER is initiated with recognition of the modified base and its removal is followed by processing of the apurinic/apyrimidinic (AP) site, incorporation of the correct nucleotide and finally strand ligation (Chan et al., 2006; Dianov et al., 2001). A schematic representation of the BER pathway in mitochondria is illustrated in Figure 1.

The 1st step of BER is initiated by DNA glycosylases, which recognize the modified base and cleave the N-glycosidic bond, resulting in an abasic site. It has been shown that a number of glycosylases are bi-functional DNA glycosylases such that they also have AP lyase activity, which enables the cleavage of the DNA backbone (Robertson et al., 2009). Mitochondrial and

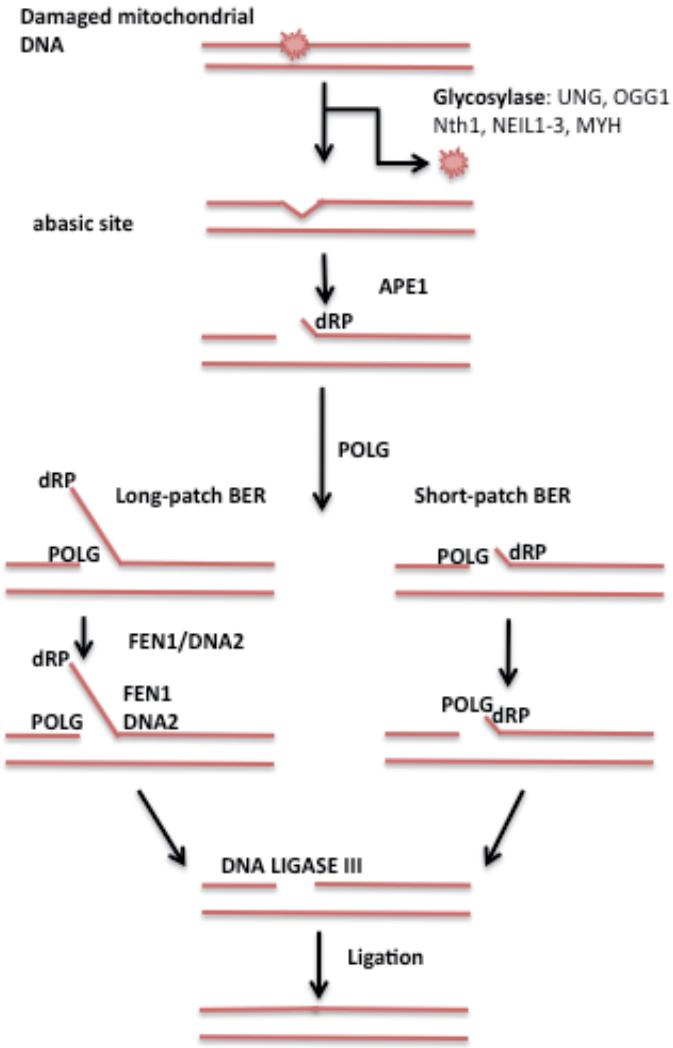

Fig. 1. Schematic representation of the BER pathway in mitochondria.

nuclear glycosylases are encoded by the same nuclear gene, however isoforms are generated by alternative transcription initiation sites and alternative splicing (Bohr, 2002; Nilsen et al., 1997). The mitochondrial DNA glycosylases include the 8-oxoguanine DNA glycosylase-1 (OGG1), the uracil DNA glycosylase (UNG), MYH, endonuclease III homolog (NTH1) and the NEIL glycosylases. OGG1 is a bi-functional glycosylase that is required for the recognition and cleavage of 8-hydroxy-guanine (8-oxoG) oxidative DNA lesions from double-stranded DNA (Kuznetsov et al., 2005). UNG was the 1st glycosylase to be identified and is involved in the removal of uracil from DNA, generated by deamination of cytosine or by misincorporation of dUMP (Lindahl, 1974). The removal of uracil is vital, because of its ability to pair with adenine resulting in GC to AT transition mutations upon replication (Darwanto et al., 2009). MYH is involved in the removal of adenine misinserted opposite 8-oxoG (Takao et al., 1999). NTH1 is also involved in the removal of oxidized DNA lesions

(Takao et al., 2002). The NEIL glycosylases are responsible for excising oxidative DNA lesions such as 2,6-diamino-5-foramidopyrimidine (FapyG) and 4,6-diamino-5-formamidopyrimidine (FapyA) (Doublie et al., 2004). There are three main isoforms, NEIL1, NEIL2 and NEIL3, which are present in both the nucleus and the mitochondria (Gredilla et al., 2010b; Hazra et al., 2002a; Hazra et al., 2002b). Whilst partial redundancy has been described for these glycosylases, NEIL1 knock-out mice accumulate mitochondrial DNA deletions to a greater extent than wild-type mice and also develop symptoms associated with metabolic syndrome (Vartanian et al., 2006).

After recognition and cleavage of the modified base by the specific DNA glycosylase, an abasic site is formed. The AP endonuclease (APE1) is involved in this step of repair. APE1 cleaves on the immediate 5′ side of the AP site, leaving a 3′ hydroxyl and 5′-deoxyribose-5-phosphate (5′-dRP) residue (Masuda et al., 1998). APE1 is the major endonuclease in mammalian cells in both the nucleus and the mitochondria (Tell et al., 2005). The functional importance of APE1 is highlighted by the findings that knockout mice for the APE1 gene are embryonic lethal at very early stages (6–8 days) suggesting that cell survival is critically compromised in the absence of APE1 (Ludwig et al., 1998; Xanthoudakis et al., 1996). Heterologous expression of APE1 restores resistance to DNA-damaging agents in AP endonuclease-deficient cells (Li et al., 2008). APE1 is the only AP endonuclease in mitochondrion, and loss of mitochondrial APE1, not of the nuclear APE1 (Chattopadhyay et al., 2006), is believed to be responsible for triggering apoptosis, therefore highlighting APE1 as a potential therapeutic target. (Li et al., 2008).

Once the AP site has been processed by APE1, the only known mitochondrial DNA polymerase, POLG is required to insert the correct nucleotide in the generated gap (Ropp and Copeland, 1996). Two different BER pathways exist depending on the number of nucleotides that is incorporated by POLG. Short-patch BER involves the incorporation of one single nucleotide into the gap, while long-patch BER involves the incorporation of several nucleotides, usually in the range of 2 to 7 (Robertson et al., 2009). During the long-patch BER process, this incorporation of multiple nucleotides results in the exposure of the original DNA strand as a single-stranded overhang or a flap structure (Xu et al., 2008). Therefore increasing the complexity of long-patch BER, as additional enzymatic activities are required to process this flap. Increasing evidence suggests that in both the nucleus and the mitochondria, this structure is recognized and cleaved by the flap endonuclease, FEN1 (Kalifa et al., 2009; Klungland and Lindahl, 1997). Although FEN1 is clearly involved in mitochondrial BER, studies have suggested the existence of additional activities involving the enzyme Dna2 can also enable the process. Dna2 was originally identified in yeast as a nuclear DNA helicase with an endonuclease activity required for removing part of an RNA or DNA flap structure (Zheng et al., 2008) and yeast Dna2 has been known for some time to function in the nucleus along with FEN1 to process 5′ flaps (Budd and Campbell, 1997). Significantly, the major isoform of Dna2 is localized to the mitochondria. (Copeland and Longley, 2008; Duxin et al., 2009). Current work implies that mammals have evolved to utilize FEN1 as the only nuclear flap endonuclease, whereas both FEN1 and DNA2 appear to function together in mitochondria (Duxin et al., 2009).

The final process in the mitochondrial BER pathway involves sealing of the nick, which requires the mitochondrial DNA ligase, Ligase III. It was shown to be an ATP independent enzyme, similar to the nuclear DNA ligase (Lakshmipathy and Campbell, 1999b). It is involved in both mitochondrial replication and repair. Recently it has been demonstrated that Ligase III is critical for mitochondrial DNA maintenance and viability, but is

dispensable for Xrcc1-mediated nuclear BER (Gao et al., 2011; Simsek et al., 2011). Depletion of DNA ligase III in the mitochondria by antisense DNA ligase III mRNA expression led to a decrease in cellular mitochondrial DNA copy number and increased levels of single-strand DNA breaks within the mitochondrial genome (Lakshmipathy and Campbell, 2001). Ongoing investigations on how the organization of mitochondrial DNA affects BER suggests that mitochondrial DNA association to the inner mitochondrial membrane may be critical for efficient BER (Boesch et al., 2010).

2.2 Mismatch repair

The presence of mismatch repair (MMR) activity in the mitochondria is a controversial area. In 2003, Mason et al. demonstrated that mitochondrial extracts from rat liver exhibited a low but significant MMR activity and that this activity was independent, of one of the main nuclear MMR proteins, MSH2 (Mason et al., 2003). Therefore suggesting that the mitochondrial MMR pathway may be distinct from nuclear MMR. To date, data suggesting the presence of the nuclear MMR proteins in the mitochondria has been conflicting. In 2009, de Souza-Pinto et al. detected the classical MMR proteins MSH3, MSH6 and MLH1 in the nuclei but not in mitochondria (de Souza-Pinto et al., 2009). However we and others, have detected the presence of MLH1, but not MSH2, in the mitochondria of human tumor cells and mouse liver, respectively (Martin et al., 2010; Mootha et al., 2003). Furthermore, our recent data suggests a role for MLH1 in mitochondrial oxidative DNA repair, such that MLH1 deficiency in combination with silencing of the mitochondrial genes, POLG and PINK1, amongst others results in an accumulation in mitochondrial 8-oxoG lesions, incompatible with cell viability (Martin et al., 2011; Martin et al., 2010). Studies have also suggested that mitochondrial DNA mismatch-binding activity is due to the Y-box-binding protein, YB-1 (de Souza-Pinto et al., 2009). Mitochondrial extracts depleted of YB-1 demonstrated a significantly reduced mismatch-binding and repair activity and also a reduced rate of cellular respiration, suggestive of mitochondrial dysfunction. Significantly, silencing of YB-1 by RNA interference (RNAi) also resulted in increased mitochondrial DNA mutagenesis, therefore suggesting that mitochondria do have a MMR pathway, which involves YB-1. The YB-1 mediated mitochondrial mismatch-binding activity was shown to have no bias in favor of the matrix strand and is therefore prone to the introduction of mutations. Recent data has suggested that it can specifically recognize and bind base mismatches and small insertion/deletion loops. In *S. cerevisiae*, Msh1 which is a homologue of the bacterial MutS component, can repair G:A mispairs in mitochondrial DNA, which are generated by replication past 8-oxodG, as well as other mismatches (Chi and Kolodner, 1994). Msh1 is also thought to be involved in mitochondrial DNA recombination, which may help prevent oxidative lesion-induced instability of the mitochondrial genome (Dzierzbicki et al., 2004; Kaniak et al., 2009; Mookerjee et al., 2005). To date the full extent of mismatch repair activity in mammalian mitochondria remains to be elucidated. BER may also be involved in repairing mitochondrial mismatches and therefore it is possible that proteins that participate in mitochondrial BER may have a role in the downstream activities of the mitochondrial MMR pathway.

2.3 Homologous recombination

Double-strand breaks (DSBs) represent one of the most lethal forms of DNA damage. In the nucleus, even one DSB can be lethal whilst in contrast because the mitochondria possess multiple copies of wild type mitochondrial DNA, this can compensate resulting in a less

critical presence of a DSB. Even so, DSB repair has been identified in the mitochondria. In general, homologous recombination (HR) is the primary mechanism for error-free repair of DSBs. HR also plays a critical role in facilitating replication fork progression when the polymerase complex encounters a blocking DNA lesion. In 1995, Ling et al identified the presence of HR in mitochondria in yeast (Ling et al., 1995). It has also been shown that mitochondria are able to repair DSBs in Chinese hamster ovary cells (LeDoux et al., 1992). Rad51, the central mediator of nuclear HR, Rad51C and XRCC3, have all been shown to localize to the mitochondria in human cells (Sage et al., 2010). Rad51 has been shown to bind mitochondrial DNA following exposure to cells upon oxidative stress. Rad51-mediated activity is necessary for regulating mitochondrial DNA copy number under conditions of oxidative stress and this activity requires the functions of Rad51C and XRCC3. In the nucleus, Rad51 and XRCC3 have been shown to cooperate in regulating replication fork progression on damaged chromosomes, therefore it has been suggested that mitochondrial Rad51, Rad51C and XRCC3 ensure faithful completion of mitochondrial DNA replication as the fork encounters blocking lesions. In addition, a study by Thyagarajan et al., have demonstrated that human mitochondrial extracts have the ability to catalyze HR of different DNA substrates (Thyagarajan et al., 1996). Further evidence of mitochondrial HR analyzed segregated mitochondrial DNA mutations in a heteroplasmic mitochondrial DNA population and identified combinations of these two mutations in different mitochondrial DNA molecules indicating HR and crossing over events between mitochondrial DNA molecules with segregated mutations (Zsurka et al., 2004). BRCA1, the breast and ovarian cancer susceptibility gene, which plays a role in the HR pathway, has also been shown to localize to the mitochondria and was found to colocalize with mitochondrial DNA clusters (Coene et al., 2005).

2.4 Non-homologous end joining

Studies have shown that mitochondrial protein extracts possess non-homologous end-joining (NHEJ) activity. NHEJ is highly precise in the case of DNA with cohesive ends while blunt-ended DNA are rejoined with less efficiency and precision (Roth et al., 1985). In mitochondrial extracts, it has been demonstrated that both cohesive and blunt-ended DNA substrates can be rejoined, although the latter with much lower efficiency (Lakshmipathy and Campbell, 1999a). Irrespective of which DNA substrate was used, the majority of recovered products were precisely repaired. Analysis of imprecisely repaired products revealed the presence of deletions that spanned direct repeat sequences. These deletions were similar to those observed in the mitochondrial DNA of certain pathological states as well as in aging cells. Ku80 is required for nuclear NHEJ due to its DNA end-joining activity. Mammalian mitochondrial DNA end-joining activity was reported to be practically indistinguishable from that of the nuclear activity. This observation led to the investigation and subsequent demonstration that Ku80 is also required for mammalian mitochondrial DNA end-joining activity (Feldmann et al., 2000).

3. Mitochondrial DNA degradation

The possibility of mitochondrial degradation was first proposed because of early studies suggesting that UV-induced pyrimidine dimmers were not repaired in mammalian mitochondria (Clayton et al., 1974). Furthermore, in response to treatment with mutagenic agents such as ethylmethane sulfonate, N-methyl-N′-nitrosoguanidine and benzo(a)pyrene, mitochondrial DNA from HeLa cells only accumulated few mutations suggesting that

mitochondrial DNA accumulating excessive amounts of damage or irreparable lesions, is not replicated (Mita et al., 1988). More recently, further investigation into this process has revealed that extensive or persistent DSBs result in mitochondrial DNA degradation (Alexeyev et al., 2008; Bacman et al., 2009; Fukui and Moraes, 2009). Such that the signal that triggers mitochondrial DNA degradation has been attributed to DSBs, generated by stalled DNA or RNA polymerases on the damaged mitochondrial DNA template. Degradation of these molecules prevents mutagenesis and maintains mitochondrial DNA integrity. In the case of UV-induced pyrimidine dimers and benzo(a)pyrene-induced adducts, the stalled RNA or DNA polymerase would trigger the degradation process. More recently, studies have suggested that oxidative stress can lead to the degradation of mitochondrial DNA and that strand breaks and abasic sites prevail over mutagenic base lesions in ROS-damaged mitochondrial DNA (Shokolenko et al., 2009). Furthermore, inhibition of abasic site processing by APE1 and inhibition of BER by methoxyamine treatment enhanced this degradation in response to oxidative damage, suggesting that the inability to repair mitochondrial DNA damage may be the signal for its degradation (Shokolenko et al., 2009). The elimination of damaged mitochondrial DNA was preceded by the accumulation of linear mitochondrial DNA molecules, which potentially represent degradation intermediates. These intermediates, unlike undamaged circular mitochondrial DNA molecules, are susceptible to exonucleolitic degradation thus ensuring the specificity of the process. Therefore supporting the observation by Suter and Richter who demonstrated that 8-oxoG content of circular mitochondrial DNA is low and does not increase in response to oxidative insult in contrast to fragmented mitochondrial DNA which had very high 8-oxoG content, that further increased after oxidative stress (Suter and Richter, 1999).

3.1 Mitochondrial DNA degradation nuclease

The Endonuclease G (EndoG) was initially proposed to be the nuclease responsible for selectively degrading non-replicable mitochondrial DNA. Such that Ikeda and Ozaki showed that mitochondrial EndoG is more active *in vitro* on oxidatively modified DNA compared to undamaged DNA suggesting that it may be involved in the degradation of oxidatively damaged mitochondrial DNA (Ikeda and Ozaki, 1997). However, more recent studies illustrated that EndoG-deficient cells or EndoG null mice showed no accumulation in mitochondria DNA mutation rate or defects in mitochondrial structure, therefore suggesting that EndoG may not be the exclusive nuclease involved (Irvine et al., 2005). Davies et al. reported that upon removal of EndoG activity from the mitochondria, another nuclease activity can be detected internal to the inner mitochondrial membrane (Davies et al., 2003). This exonuclease causes a gradual degradation of amplified DNA and linearized pBR322 plasmid DNA without the site-specific cleavage seen with EndoG. However they also showed that when supercoiled mitochondrial DNA is used as a substrate, both endo- and exonuclease activities could be detected. Whether the endo- and exonucleolytic activities arise from the same nuclease or from separate enzymes remains under investigation.

4. Sanitation of the mitochondrial deoxynucleotide triphosphate pool

So far, we have only discussed repair and damage of mitochondrial DNA, however the free deoxynucleotide triphosphate (dNTP) pool is also exposed to oxidation and other stresses.

dNTPs are the precursors used by DNA polymerases for replication and repair of nuclear and mitochondrial DNA. The cell employs specialized enzymes that remove for example, oxidized dNTPs that otherwise may be incorrectly incorporated during DNA synthesis such as 8-oxo-2′-deoxyguanosine triphosphate (8-oxo-dGTP). 8-oxo-dGTP can be potentially incorporated opposite A by POLG, resulting in 8-oxodG:dA base pairs which are resistant to the proof-reading activity of POLG, ultimately resulting in AT to CG transversions (Hanes et al., 2006; Pursell et al., 2008). As a defense to such activities, MUTYH, present in both the nucleus and mitochondria, has the ability to remove the misincorporated adenine, enabling insertion of dCMP and removal of the 8-oxoguanine by BER (Takao et al., 1999; van Loon and Hubscher, 2009). Oxidation of the mitochondrial dNTP pool represents a significant threat to mitochondrial DNA integrity with the 8-oxo-dGTP concentrations in mitochondrial extracts from rat tissues ranging from 1-10% of the total dGTP (Pursell et al., 2008).
The major defense mechanisms against 8-oxo-dGTP, is its elimination from the dNTP pool by the mitochondrial MTH1 (Kang et al., 1995; Nakabeppu, 2001). MTH1 can hydrolyze 8-oxodGTP to 8-oxodGMP, which is not a substrate for DNA polymerases and therefore would not be incorporated into the DNA. MTH1 can also hydrolyze, 8-oxo-2′-deoxyadenosine triphosphate and 2-hydroxy-2′-deoxtadenosine triphosphate to the monophosphates (Sakai et al., 2002). 8-oxoG accumulation in mitochondrial DNA was observed in MTH1-null mouse embryonic fibroblasts following hydrogen peroxide treatment and in dopaminergic neurons from MTH1-null mice following 1-methyl-4-phenyl-1,2,3,6,-tetreadropyridine treatment (Yamaguchi et al., 2006; Yoshimura et al., 2003). MTH1 was also shown to protect cells from the cytotoxicity of sodium nitoprusside by preventing 8-oxoG accumulation in mitochondrial DNA (Ichikawa et al., 2008). Taken together, this strongly suggests that MTH1 plays a critical role in protecting mitochondrial DNA from oxidized dNTPs.
The *DUT* gene, which encodes a UTPase which can remove dUTP from the nucleotide pool, also encodes an alternative splice variant that is located to mitochondria (Ladner and Caradonna, 1997). dUTP can arise from deamination of dTTP. The mitochondrial protein is 23 kDa and is constitutively expressed, in contrast to the nuclear isoform, which is cell cycle regulated. If modified dNTPs are incorporated into mitochondrial DNA they must be removed via the BER pathway, which can repair modifications of single nucleotides already incorporated in DNA.

5. Mitochondrial DNA repair and disease

Accumulating data increasingly shows the involvement of various mitochondrial DNA mutations in human diseases. Several disorders such as myopathy, optic atrophy and Leigh syndrome arise as a result of mitochondrial alterations (Edmond, 2009). In addition, a number of pathologies are also caused by mutations in nuclear genes that encode for mitochondrial proteins (Chan and Copeland, 2009; Horvath et al., 2009; Tuppen et al., 2010). The most common genetic defect seen in individuals with mitochondrial DNA-associated disease are deletions (Holt et al., 1988; Shoffner et al., 1989) or point mutations (Goto et al., 1990; Wallace et al., 1988). Mitochondrial DNA deletions have been shown to be important in pathogenesis in a number of ways. Single mitochondrial DNA deletions are a common cause of sporadic mitochondrial disease and an identical mitochondrial DNA deletion is present in all cells of the affected tissue (Schaefer et al., 2008). Some individuals with mitochondrial disease have multiple mitochondrial DNA deletions in the affected tissues,

usually the muscle and the central nervous system (Taylor and Turnbull, 2005). These involve nuclear genes encoding proteins involved in either mitochondrial nucleotide metabolism or mitochondrial DNA maintenance. There are also a number of reports of mitochondrial deletions in aged post-mitotic tissues and individuals with neurodegenerative disease (Bender et al., 2006; Kraytsberg et al., 2006; Taylor and Turnbull, 2005). These pathogenic mitochondrial DNA deletions have been suggested to be as a result of mitochondrial DNA repair. It has been postulated that mitochondrial deletions are initiated by single-stranded regions of mitochondrial DNA generated through exonuclease activity at DSBs (Krishnan et al., 2008). Ultimately, these single strands are free to anneal with microhomologous sequences such as repeat sequences on other single-stranded mitochondrial DNA or within the noncoding region (Haber, 2000). Once annealed, subsequent repair, ligation and degradation of the remaining exposed single strands would result in the formation of an intact mitochondrial genome harboring a deleted portion.

5.1 Mitochondrial DNA repair and neurodegenerative disease

Mitochondrial DNA damage is found in affected neurons in the majority of neurodegenerative disorders, and is often associated with oxidative DNA damage and mitochondrial dysfunction (de Moura et al., 2010). Accumulation of nuclear DNA and mitochondrial DNA lesions has been demonstrated to be a critical factor contributing to genomic instability and mitochondrial dysfunction in neurodegenerative diseases (Lin and Beal, 2006; Yang et al., 2008). DNA repair mechanisms are essential for the proper maintenance of the mammalian central nervous system. Therefore, deficiency in DNA repair, particularly in BER, is increasingly recognized as a major contributor to neuronal loss. Neurodegenerative diseases are increasingly associated with mutations in mitochondrial DNA strongly suggesting that neurons are particularly sensitive to mitochondrial dysfunction. Neurons in both the peripheral and central nervous systems are adversely affected by mitochondrial mutations (Wallace, 2001). Examples of neurodegenerative diseases associated with mitochondrial DNA damage and repair (Finsterer, 2006; Servidei, 2004) include but are not limited to: Alzheimers disease, Parkinsons disease and Huntingtons disease. The fact that many of these share similar neuropathological features with multiple neurodegenerative disorders, suggests a significant role for mitochondrial dysfunction in the pathogenesis of neurodegenerative disorders.

Alzheimers Disease, the most common form of age-associated dementia, is a progressive and always fatal disorder characterized clinically by memory loss and behavioral abnormalities, and histopathologically by deposition of amyloid β-peptide (Aβ), cytoskeletal pathology, degeneration of synapses and neuronal death (Mattson, 2004). Several studies have shown that oxidative modification to both nuclear DNA and mitochondrial DNA are increased in brains of Alzheimers disease patients (Gabbita et al., 1998; Mecocci et al., 1994; Wang et al., 2005). An accumulation of 8-hydroxy-2-deoxyguanosine (8-OHdG) was observed in mitochondrial DNA isolated from cortical brain regions of Alzheimers patients (Mecocci et al., 1994). Furthermore significant BER dysfunction was observed in brains of Alzheimers patients, resulting from reduced UDG, OGG1 and POLB activities (Weissman et al., 2007). Parkinson's disease is the second most prevalent neurodegenerative disease, affecting approximately 2% of individuals over the age of 65 years (de Rijk et al., 1997; Mouradian, 2002). It is clinically characterized by resting tremor, postural instability, gait disturbance, bradykinesia and rigidity. Increasing evidence suggests that oxidative damage

to DNA, both nuclear and mitochondrial, contributes to the degeneration of dopaminergic neurons in Parkinsons disease (Alam et al., 1997). Swerdlow et al. demonstrated that mitochondria from Parkinsons patients exhibit increased production of ROS, decreased activity of complex I and increased DNA damage (Swerdlow et al., 1996). Huntington's disease is a dominantly inherited neurodegenerative disorder caused by expanded CAG trinucleotide repeats in the amino-terminal coding region of the huntingtin (Htt) gene (Cepeda et al., 2007). It was suggested that expansion of the CAG trinucleotide repeats in Huntingtons disease requires DNA break repair and involves several DNA repair enzymes including FEN1 (Lee and Park, 2002; Spiro et al., 1999). It was also proposed that faulty processing of strand breaks by FEN-1, initiates CAG repeat instability in mammalian cells (Spiro and McMurray, 2003). It was recently demonstrated that the accumulation of oxidative DNA lesions in brains and livers of Huntingtons mice, including 8-oxoG, 5-hydroxyuracil (5-OHU), 5-hydroxycytosine (5-OHC), and formamidopyrimidine (FAPY), were correlated with the degree of trinucleotide expansion, suggesting that that initiation of CAG repeats may occur during removal of oxidative DNA lesions, and could be specifically associated with OGG1 activity (Kovtun et al., 2007).

5.2 Mitochondrial DNA repair and cancer

The extent to which cancer is caused by or is a consequence of mitochondrial genomic alterations is unknown, but substantial data suggest an involvement of mutations in mitochondrial DNA in the carcinogenic process. Mitochondrial defects have long been suspected to play an important role in the development and progression of cancer (Carew and Huang, 2002; Hockenbery, 2002; Warburg, 1956). However the majority of the existing data currently show an association of increased mitochondrial DNA mutations in different tumours with only little direct evidence for a functional role of these mutations. Tumour cells, in general, have increased levels of mitochondrial DNA transcripts, while both increases and decreases in the levels of tumour cell mitochondrial DNA have been reported.
ROS-triggered mutagenesis of both mitochondrial DNA and nuclear DNA has been suggested to correlate with tumourigenesis. (Klaunig et al., 2010). Decreased nuclear and mitochondrial levels of the OGG1 glycosylase were observed in human lung cancers compared with normal cells (Karahalil et al., 2010). Furthermore, decreased OGG1 expression was also observed in spontaneous hepatocellular carcinomas developed in mutant rats, in association with an accumulation of oxidative DNA damage and ROS generation (Choudhury et al., 2003). Colorectal cancers have been shown to exhibit increased somatic mitochondrial DNA mutations (Habano et al., 1998; Polyak et al., 1998). Significantly, all of these mutations were present in the majority of the tumour cells and 90% of them were detectable in all of the mitochondrial DNA present in cells, strongly suggesting that all mitochondrial DNA molecules in the mitochondrion contain the same mutation. Breast cancer also exhibit somatic mitochondrial DNA mutations (Parrella et al., 2001; Radpour et al., 2009), in addition to kidney (Meierhofer et al., 2006) (Nagy et al., 2003), stomach (Hung et al., 2010; Jeong et al., 2010), prostate (Moro et al., 2009) (Parr et al., 2006) liver (Vivekanandan et al., 2010; Zhang et al., 2010), bladder (Dasgupta et al., 2008), head and neck (Allegra et al., 2006; Dasgupta et al., 2010; Mithani et al., 2007) and lung (Dai et al., 2006; Jin et al., 2007; Suzuki et al., 2003). Furthermore increased mitochondrial DNA mutation frequencies were associated with hereditary paraganglioma (Muller et al., 2005; Taschner et al., 2001) and thyroid cancers (Abu-Amero et al., 2005; Rogounovitch et al., 2004). Clayton and Smith further expanded studies of mitochondrial DNA structural

changes in leukocytes of leukemic patients and also in patients with a variety of solid tumors (Clayton and Smith, 1975).

Data suggesting a role for mitochondrial DNA in cancer regression comes from studies with the chemotherapy drugs, bis-2-chloroethylnitrosourea (BCNU) and temozolomide. These drugs induce cell death by alkylation of DNA bases to form mutagenic O^6 methylguanine and interstrand cross-links (Ludlum, 1997; Newlands et al., 1997). The repair enzyme O^6methyloguanine DNA methyltransferase (MGMT) removes O^6methylguanine DNA damage (Bobola et al., 1995; Bobola et al., 1996). Studies have shown that transfecting haematopoietic cell lines with low repair activity for alkylated DNA damage with mitochondrial-targeted and nuclear-targeted MGMT generated resistance against the cytotoxic effects of BCNU and temozolomide (Cai et al., 2005). Significantly, this effect was more dependent on mitochondrial MGMT in comparison to the nuclear MGMT suggesting the contribution of mitochondrial DNA repair in the generation of drug-resistant tumour cells.

6. Mitochondrial DNA repair and aging

Many theories have been proposed to explain the phenomenon of aging (Kirkwood, 2005). Amongst these is the mitochondrial free radical theory of aging, which states that the accumulation of mitochondrial damage and the progressive accumulation of free radical damage in post-mitotic tissues, is the cause of aging (Harman, 1956). Because mitochondria are the main generators of ROS and consequently the main target of their DNA damaging effects, oxidative damage can result in increasing rates of mitochondrial DNA mutations. A vicious cycle can potentially occur as mitochondria encode for components of the respiratory chain and ATP synthase complexes, therefore mutations in the mitochondrial DNA may cause defects in oxidative phosphorylation resulting in an increased generation of ROS and further mitochondrial DNA damage (Miquel et al., 1980).

The mitochondrial theory of ageing has been controversial, with numerous studies performed to elucidate the precise correlation between oxidative damage, mitochondrial mutations and aging. One prominent study involves the generation of a mouse model that illustrates an increase in mitochondrial DNA mutation and oxidative phosphorylation defects. This mouse model which carries an error-prone form of POLG was generated, and correlated with decreased life expectancy and a premature ageing phenotype (Kujoth et al., 2005; Trifunovic et al., 2004). However there was little evidence of increased ROS or oxidative damage as a result of the mitochondrial DNA replication errors, suggesting the lack of the previously proposed "vicious cycle". Studies of the various tissues of these mice, have suggested that it is the accumulation of mitochondrial DNA deletions and clonal expansion identified in the brain and heart that drive the premature aging phenotype (Vermulst et al., 2007)(Vermulst et al., 2008). More recently, an alternative study has now suggested that it is random point mutations occurring in mitochondrial DNA analyzed in the liver and heart that are the driving force behind the aging phenotype (Edgar et al., 2009). The discrepancies between the studies may be due to the analysis of either mitotic or post-mitotic tissues. Such that, it has been suggested that in post-mitotic tissues, mitochondrial DNA deletions occur initially during repair of damaged DNA whilst in mitotic tissues it is thought that mitochondrial DNA point mutations are likely to be generated during replication (Reeve et al., 2009).

A number of studies suggest that although oxidative damage of mitochondrial DNA does accumulate with age in mammalian cells, this accumulation does not regulate lifespan

(Arnheim and Cortopassi, 1992; Barja and Herrero, 2000). Similarly, in Drosophila, mitochondrial ROS production increases with age but does not influence its lifespan (Sanz et al., 2010). One reason has been postulated such that scavenging free radicals could increase life expectancy whilst increasing ROS may lead to premature cell death. To address this several transgenic models have been generated. Although over-expression of the mitochondrial Mn-superoxide dismutase (MnSOD) extends lifespan in Drosophila (Sun et al., 2002), it had no effect on lifespan in similarly over-expressing mice (Jang et al., 2009; Perez et al., 2009). An increase in ROS levels by inactivation of antioxidants does not display shortened lifespan, such that transgenic mice expressing only one allele of mitochondrial thioredoxin TRX2 do not display any decrease in life expectancy, whilst exhibiting significant defects in oxidative phosphorylation and increased hydrogen peroxide production (Jang et al., 2009). Therefore strongly suggesting that ROS generation during normal metabolism is unlikely to be the main or single cause of aging.

A causative role for mitochondrial DNA damage in the development of aging remains to be proven, however damaged mitochondrial DNA accumulates with age suggesting a potential role for mitochondrial DNA repair. Mitochondrial DNA repair defects may contribute to the accumulation of DNA damage associated with aging (Druzhyna et al., 2008; Gredilla et al., 2010a). Studies suggest that the 8-oxoG DNA lesion is one of the most abundant oxidative lesions which accumulates with age in the mitochondria. However, in apparent contrast the overall OGG1 8-oxoG glycosylase activity has been shown to increase with age in mammalian cells (Stevnsner et al., 2002b). Further studies have postulated that while the overall OGG1 content in the mitochondria increases with age, the amount of OGG1 in the mitochondrial inner compartment decreases resulting in the observed accumulation of 8-oxoG in mitochondrial DNA with a large fraction of the enzyme remaining stuck to the membrane in the precursor form, which could not be translocated to and processed in the mitochondrial matrix. (Szczesny et al., 2003). A similar observation has been reported for the mitochondrial uracil DNA glycosylase, UDG, suggesting a deficiency in import in aged cells (Szczesny et al., 2003).

Caloric restriction has been shown to reduce the accumulation of mitochondrial DNA mutations and increase lifespan (Aspnes et al., 1997; Cassano et al., 2004; Gredilla and Barja, 2005). DNA repair in the nucleus has been shown to be enhanced by caloric restriction and promote genomic stability (Heydari et al., 2007). However, studies in the mitochondria have shown that mitochondrial BER capacity did not change in liver and actually decreased in the brain and kidney of caloric restricted rats (Stuart et al., 2004). This decrease in BER correlates with the observation that mitochondria from caloric restricted rodents generate ROS and accumulate oxidative DNA damage at lower rates than non-restricted animals (Gredilla and Barja, 2005). Therefore it has been suggested that when the levels of ROS and mitochondrial DNA damage are significantly reduced, it may enable the organism to require less energy required for mitochondrial DNA repair.

7. Conclusion

Originally thought to be absent, DNA repair mechanisms in the mitochondria are now well established. Whilst all the core enzymatic components of the BER pathway have been identified in the mitochondria, the precise mechanisms of the remaining pathways have been less well investigated. For example, identification and characterization of the key players in the mitochondrial MMR pathway and a potential role for NER proteins in the

repair of oxidative damage in the mitochondria remain unclear. Inactivation of many nuclear genes encoding key proteins, can impact mitochondrial DNA maintenance and result in an accumulation of DNA damage and ultimately mutations. Controversy surrounds the pathological nature of these mitochondrial DNA mutations, however increasing evidence links mitochondrial DNA integrity with carcinogenesis, neurodegenerative disease and aging. Taken together, future work requires an in dept analysis of the functional role of these mutations in human pathologies and aging.

8. References

Abu-Amero, K.K., Alzahrani, A.S., Zou, M., and Shi, Y. (2005). High frequency of somatic mitochondrial DNA mutations in human thyroid carcinomas and complex I respiratory defect in thyroid cancer cell lines. Oncogene *24*, 1455-1460.

Akbari, M., Visnes, T., Krokan, H.E., and Otterlei, M. (2008). Mitochondrial base excision repair of uracil and AP sites takes place by single-nucleotide insertion and long-patch DNA synthesis. DNA Repair (Amst) *7*, 605-616.

Alam, Z.I., Jenner, A., Daniel, S.E., Lees, A.J., Cairns, N., Marsden, C.D., Jenner, P., and Halliwell, B. (1997). Oxidative DNA damage in the parkinsonian brain: an apparent selective increase in 8-hydroxyguanine levels in substantia nigra. J Neurochem *69*, 1196-1203.

Alexeyev, M.F., Venediktova, N., Pastukh, V., Shokolenko, I., Bonilla, G., and Wilson, G.L. (2008). Selective elimination of mutant mitochondrial genomes as therapeutic strategy for the treatment of NARP and MILS syndromes. Gene Ther *15*, 516-523.

Allegra, E., Garozzo, A., Lombardo, N., De Clemente, M., and Carey, T.E. (2006). Mutations and polymorphisms in mitochondrial DNA in head and neck cancer cell lines. Acta Otorhinolaryngol Ital *26*, 185-190.

Anderson, S., Bankier, A.T., Barrell, B.G., de Bruijn, M.H., Coulson, A.R., Drouin, J., Eperon, I.C., Nierlich, D.P., Roe, B.A., Sanger, F., *et al.* (1981). Sequence and organization of the human mitochondrial genome. Nature *290*, 457-465.

Arnheim, N., and Cortopassi, G. (1992). Deleterious mitochondrial DNA mutations accumulate in aging human tissues. Mutat Res *275*, 157-167.

Aspnes, L.E., Lee, C.M., Weindruch, R., Chung, S.S., Roecker, E.B., and Aiken, J.M. (1997). Caloric restriction reduces fiber loss and mitochondrial abnormalities in aged rat muscle. FASEB J *11*, 573-581.

Bacman, S.R., Williams, S.L., and Moraes, C.T. (2009). Intra- and inter-molecular recombination of mitochondrial DNA after in vivo induction of multiple double-strand breaks. Nucleic Acids Res *37*, 4218-4226.

Balajee, A.S., Dianova, I., and Bohr, V.A. (1999). Oxidative damage-induced PCNA complex formation is efficient in xeroderma pigmentosum group A but reduced in Cockayne syndrome group B cells. Nucleic Acids Res *27*, 4476-4482.

Barja, G., and Herrero, A. (2000). Oxidative damage to mitochondrial DNA is inversely related to maximum life span in the heart and brain of mammals. FASEB J *14*, 312-318.

Bender, A., Krishnan, K.J., Morris, C.M., Taylor, G.A., Reeve, A.K., Perry, R.H., Jaros, E., Hersheson, J.S., Betts, J., Klopstock, T., *et al.* (2006). High levels of mitochondrial DNA deletions in substantia nigra neurons in aging and Parkinson disease. Nat Genet *38*, 515-517.

Berneburg, M., Kamenisch, Y., Krutmann, J., and Rocken, M. (2006). 'To repair or not to repair - no longer a question': repair of mitochondrial DNA shielding against age and cancer. Exp Dermatol *15*, 1005-1015.

Bobola, M.S., Blank, A., Berger, M.S., and Silber, J.R. (1995). Contribution of O6-methylguanine-DNA methyltransferase to monofunctional alkylating-agent resistance in human brain tumor-derived cell lines. Mol Carcinog *13*, 70-80.

Bobola, M.S., Tseng, S.H., Blank, A., Berger, M.S., and Silber, J.R. (1996). Role of O6-methylguanine-DNA methyltransferase in resistance of human brain tumor cell lines to the clinically relevant methylating agents temozolomide and streptozotocin. Clin Cancer Res *2*, 735-741.

Boesch, P., Ibrahim, N., Dietrich, A., and Lightowlers, R.N. (2010). Membrane association of mitochondrial DNA facilitates base excision repair in mammalian mitochondria. Nucleic Acids Res *38*, 1478-1488.

Boesch, P., Weber-Lotfi, F., Ibrahim, N., Tarasenko, V., Cosset, A., Paulus, F., Lightowlers, R.N., and Dietrich, A. (2011). DNA repair in organelles: Pathways, organization, regulation, relevance in disease and aging. Biochim Biophys Acta *1813*, 186-200.

Bohr, V.A. (2002). Repair of oxidative DNA damage in nuclear and mitochondrial DNA, and some changes with aging in mammalian cells. Free Radic Biol Med *32*, 804-812.

Budd, M.E., and Campbell, J.L. (1997). A yeast replicative helicase, Dna2 helicase, interacts with yeast FEN-1 nuclease in carrying out its essential function. Mol Cell Biol *17*, 2136-2142.

Cai, S., Xu, Y., Cooper, R.J., Ferkowicz, M.J., Hartwell, J.R., Pollok, K.E., and Kelley, M.R. (2005). Mitochondrial targeting of human O6-methylguanine DNA methyltransferase protects against cell killing by chemotherapeutic alkylating agents. Cancer Res *65*, 3319-3327.

Carew, J.S., and Huang, P. (2002). Mitochondrial defects in cancer. Mol Cancer *1*, 9.

Cassano, P., Lezza, A.M., Leeuwenburgh, C., Cantatore, P., and Gadaleta, M.N. (2004). Measurement of the 4,834-bp mitochondrial DNA deletion level in aging rat liver and brain subjected or not to caloric restriction diet. Ann N Y Acad Sci *1019*, 269-273.

Cepeda, C., Wu, N., Andre, V.M., Cummings, D.M., and Levine, M.S. (2007). The corticostriatal pathway in Huntington's disease. Prog Neurobiol *81*, 253-271.

Chan, K.K., Zhang, Q.M., and Dianov, G.L. (2006). Base excision repair fidelity in normal and cancer cells. Mutagenesis *21*, 173-178.

Chan, S.S., and Copeland, W.C. (2009). DNA polymerase gamma and mitochondrial disease: understanding the consequence of POLG mutations. Biochim Biophys Acta *1787*, 312-319.

Chattopadhyay, R., Wiederhold, L., Szczesny, B., Boldogh, I., Hazra, T.K., Izumi, T., and Mitra, S. (2006). Identification and characterization of mitochondrial abasic (AP)-endonuclease in mammalian cells. Nucleic Acids Res *34*, 2067-2076.

Chi, N.W., and Kolodner, R.D. (1994). The effect of DNA mismatches on the ATPase activity of MSH1, a protein in yeast mitochondria that recognizes DNA mismatches. J Biol Chem *269*, 29993-29997.

Choudhury, S., Zhang, R., Frenkel, K., Kawamori, T., Chung, F.L., and Roy, R. (2003). Evidence of alterations in base excision repair of oxidative DNA damage during

spontaneous hepatocarcinogenesis in Long Evans Cinnamon rats. Cancer Res *63*, 7704-7707.

Clayton, D.A., Doda, J.N., and Friedberg, E.C. (1974). The absence of a pyrimidine dimer repair mechanism in mammalian mitochondria. Proc Natl Acad Sci U S A *71*, 2777-2781.

Clayton, D.A., and Smith, C.A. (1975). Complex mitochondrial DNA. Int Rev Exp Pathol *14*, 1-67.

Coene, E.D., Hollinshead, M.S., Waeytens, A.A., Schelfhout, V.R., Eechaute, W.P., Shaw, M.K., Van Oostveldt, P.M., and Vaux, D.J. (2005). Phosphorylated BRCA1 is predominantly located in the nucleus and mitochondria. Mol Biol Cell *16*, 997-1010.

Copeland, W.C., and Longley, M.J. (2008). DNA2 resolves expanding flap in mitochondrial base excision repair. Mol Cell *32*, 457-458.

Copeland, W.C., Wachsman, J.T., Johnson, F.M., and Penta, J.S. (2002). Mitochondrial DNA alterations in cancer. Cancer Invest *20*, 557-569.

Dai, J.G., Xiao, Y.B., Min, J.X., Zhang, G.Q., Yao, K., and Zhou, R.J. (2006). Mitochondrial DNA 4977 BP deletion mutations in lung carcinoma. Indian J Cancer *43*, 20-25.

Darwanto, A., Theruvathu, J.A., Sowers, J.L., Rogstad, D.K., Pascal, T., Goddard, W., 3rd, and Sowers, L.C. (2009). Mechanisms of base selection by human single-stranded selective monofunctional uracil-DNA glycosylase. J Biol Chem *284*, 15835-15846.

Dasgupta, S., Hoque, M.O., Upadhyay, S., and Sidransky, D. (2008). Mitochondrial cytochrome B gene mutation promotes tumor growth in bladder cancer. Cancer Res *68*, 700-706.

Dasgupta, S., Koch, R., Westra, W.H., Califano, J.A., Ha, P.K., Sidransky, D., and Koch, W.M. (2010). Mitochondrial DNA mutation in normal margins and tumors of recurrent head and neck squamous cell carcinoma patients. Cancer Prev Res (Phila) *3*, 1205-1211.

Davies, A.M., Hershman, S., Stabley, G.J., Hoek, J.B., Peterson, J., and Cahill, A. (2003). A Ca2+-induced mitochondrial permeability transition causes complete release of rat liver endonuclease G activity from its exclusive location within the mitochondrial intermembrane space. Identification of a novel endo-exonuclease activity residing within the mitochondrial matrix. Nucleic Acids Res *31*, 1364-1373.

De Bont, R., and van Larebeke, N. (2004). Endogenous DNA damage in humans: a review of quantitative data. Mutagenesis *19*, 169-185.

de Moura, M.B., dos Santos, L.S., and Van Houten, B. (2010). Mitochondrial dysfunction in neurodegenerative diseases and cancer. Environ Mol Mutagen *51*, 391-405.

de Rijk, M.C., Rocca, W.A., Anderson, D.W., Melcon, M.O., Breteler, M.M., and Maraganore, D.M. (1997). A population perspective on diagnostic criteria for Parkinson's disease. Neurology *48*, 1277-1281.

de Souza-Pinto, N.C., Mason, P.A., Hashiguchi, K., Weissman, L., Tian, J., Guay, D., Lebel, M., Stevnsner, T.V., Rasmussen, L.J., and Bohr, V.A. (2009). Novel DNA mismatch-repair activity involving YB-1 in human mitochondria. DNA Repair (Amst) *8*, 704-719.

Dianov, G., Bischoff, C., Sunesen, M., and Bohr, V.A. (1999). Repair of 8-oxoguanine in DNA is deficient in Cockayne syndrome group B cells. Nucleic Acids Res *27*, 1365-1368.

Dianov, G.L., Souza-Pinto, N., Nyaga, S.G., Thybo, T., Stevnsner, T., and Bohr, V.A. (2001). Base excision repair in nuclear and mitochondrial DNA. Prog Nucleic Acid Res Mol Biol *68*, 285-297.

Doublie, S., Bandaru, V., Bond, J.P., and Wallace, S.S. (2004). The crystal structure of human endonuclease VIII-like 1 (NEIL1) reveals a zincless finger motif required for glycosylase activity. Proc Natl Acad Sci U S A *101*, 10284-10289.

Druzhyna, N.M., Wilson, G.L., and LeDoux, S.P. (2008). Mitochondrial DNA repair in aging and disease. Mech Ageing Dev *129*, 383-390.

Duxin, J.P., Dao, B., Martinsson, P., Rajala, N., Guittat, L., Campbell, J.L., Spelbrink, J.N., and Stewart, S.A. (2009). Human Dna2 is a nuclear and mitochondrial DNA maintenance protein. Mol Cell Biol *29*, 4274-4282.

Dzierzbicki, P., Koprowski, P., Fikus, M.U., Malc, E., and Ciesla, Z. (2004). Repair of oxidative damage in mitochondrial DNA of Saccharomyces cerevisiae: involvement of the MSH1-dependent pathway. DNA Repair (Amst) *3*, 403-411.

Edgar, D., Shabalina, I., Camara, Y., Wredenberg, A., Calvaruso, M.A., Nijtmans, L., Nedergaard, J., Cannon, B., Larsson, N.G., and Trifunovic, A. (2009). Random point mutations with major effects on protein-coding genes are the driving force behind premature aging in mtDNA mutator mice. Cell Metab *10*, 131-138.

Edmond, J.C. (2009). Mitochondrial disorders. Int Ophthalmol Clin *49*, 27-33.

Feldmann, E., Schmiemann, V., Goedecke, W., Reichenberger, S., and Pfeiffer, P. (2000). DNA double-strand break repair in cell-free extracts from Ku80-deficient cells: implications for Ku serving as an alignment factor in non-homologous DNA end joining. Nucleic Acids Res *28*, 2585-2596.

Finsterer, J. (2006). Central nervous system manifestations of mitochondrial disorders. Acta Neurol Scand *114*, 217-238.

Fukui, H., and Moraes, C.T. (2009). Mechanisms of formation and accumulation of mitochondrial DNA deletions in aging neurons. Hum Mol Genet *18*, 1028-1036.

Gabbita, S.P., Lovell, M.A., and Markesbery, W.R. (1998). Increased nuclear DNA oxidation in the brain in Alzheimer's disease. J Neurochem *71*, 2034-2040.

Gao, Y., Katyal, S., Lee, Y., Zhao, J., Rehg, J.E., Russell, H.R., and McKinnon, P.J. (2011). DNA ligase III is critical for mtDNA integrity but not Xrcc1-mediated nuclear DNA repair. Nature *471*, 240-244.

Goto, Y., Nonaka, I., and Horai, S. (1990). A mutation in the tRNA(Leu)(UUR) gene associated with the MELAS subgroup of mitochondrial encephalomyopathies. Nature *348*, 651-653.

Graziewicz, M.A., Bienstock, R.J., and Copeland, W.C. (2007). The DNA polymerase gamma Y955C disease variant associated with PEO and parkinsonism mediates the incorporation and translesion synthesis opposite 7,8-dihydro-8-oxo-2'-deoxyguanosine. Hum Mol Genet *16*, 2729-2739.

Graziewicz, M.A., Sayer, J.M., Jerina, D.M., and Copeland, W.C. (2004). Nucleotide incorporation by human DNA polymerase gamma opposite benzo[a]pyrene and benzo[c]phenanthrene diol epoxide adducts of deoxyguanosine and deoxyadenosine. Nucleic Acids Res *32*, 397-405.

Gredilla, R., and Barja, G. (2005). Minireview: the role of oxidative stress in relation to caloric restriction and longevity. Endocrinology *146*, 3713-3717.

Gredilla, R., Bohr, V.A., and Stevnsner, T. (2010a). Mitochondrial DNA repair and association with aging--an update. Exp Gerontol *45*, 478-488.

Gredilla, R., Garm, C., Holm, R., Bohr, V.A., and Stevnsner, T. (2010b). Differential age-related changes in mitochondrial DNA repair activities in mouse brain regions. Neurobiol Aging *31*, 993-1002.

Gruber, J., Schaffer, S., and Halliwell, B. (2008). The mitochondrial free radical theory of ageing--where do we stand? Front Biosci *13*, 6554-6579.

Habano, W., Nakamura, S., and Sugai, T. (1998). Microsatellite instability in the mitochondrial DNA of colorectal carcinomas: evidence for mismatch repair systems in mitochondrial genome. Oncogene *17*, 1931-1937.

Haber, J.E. (2000). Partners and pathwaysrepairing a double-strand break. Trends Genet *16*, 259-264.

Hanes, J.W., Thal, D.M., and Johnson, K.A. (2006). Incorporation and replication of 8-oxo-deoxyguanosine by the human mitochondrial DNA polymerase. J Biol Chem *281*, 36241-36248.

Harman, D. (1956). Aging: a theory based on free radical and radiation chemistry. J Gerontol *11*, 298-300.

Harman, D. (1972). The biologic clock: the mitochondria? J Am Geriatr Soc *20*, 145-147.

Harper, J.W., and Elledge, S.J. (2007). The DNA damage response: ten years after. Mol Cell *28*, 739-745.

Hazra, T.K., Izumi, T., Boldogh, I., Imhoff, B., Kow, Y.W., Jaruga, P., Dizdaroglu, M., and Mitra, S. (2002a). Identification and characterization of a human DNA glycosylase for repair of modified bases in oxidatively damaged DNA. Proc Natl Acad Sci U S A *99*, 3523-3528.

Hazra, T.K., Kow, Y.W., Hatahet, Z., Imhoff, B., Boldogh, I., Mokkapati, S.K., Mitra, S., and Izumi, T. (2002b). Identification and characterization of a novel human DNA glycosylase for repair of cytosine-derived lesions. J Biol Chem *277*, 30417-30420.

Heydari, A.R., Unnikrishnan, A., Lucente, L.V., and Richardson, A. (2007). Caloric restriction and genomic stability. Nucleic Acids Res *35*, 7485-7496.

Hockenbery, D.M. (2002). A mitochondrial Achilles' heel in cancer? Cancer Cell *2*, 1-2.

Hoeijmakers, J.H. (2009). DNA damage, aging, and cancer. N Engl J Med *361*, 1475-1485.

Holt, I.J., Harding, A.E., and Morgan-Hughes, J.A. (1988). Deletions of muscle mitochondrial DNA in patients with mitochondrial myopathies. Nature *331*, 717-719.

Horvath, R., Kemp, J.P., Tuppen, H.A., Hudson, G., Oldfors, A., Marie, S.K., Moslemi, A.R., Servidei, S., Holme, E., Shanske, S., *et al.* (2009). Molecular basis of infantile reversible cytochrome c oxidase deficiency myopathy. Brain *132*, 3165-3174.

Hudson, E.K., Hogue, B.A., Souza-Pinto, N.C., Croteau, D.L., Anson, R.M., Bohr, V.A., and Hansford, R.G. (1998). Age-associated change in mitochondrial DNA damage. Free Radic Res *29*, 573-579.

Hung, W.Y., Wu, C.W., Yin, P.H., Chang, C.J., Li, A.F., Chi, C.W., Wei, Y.H., and Lee, H.C. (2010). Somatic mutations in mitochondrial genome and their potential roles in the progression of human gastric cancer. Biochim Biophys Acta *1800*, 264-270.

Ichikawa, J., Tsuchimoto, D., Oka, S., Ohno, M., Furuichi, M., Sakumi, K., and Nakabeppu, Y. (2008). Oxidation of mitochondrial deoxynucleotide pools by exposure to sodium nitroprusside induces cell death. DNA Repair (Amst) *7*, 418-430.

Ikeda, S., and Ozaki, K. (1997). Action of mitochondrial endonuclease G on DNA damaged by L-ascorbic acid, peplomycin, and cis-diamminedichloroplatinum (II). Biochem Biophys Res Commun *235*, 291-294.

Irvine, R.A., Adachi, N., Shibata, D.K., Cassell, G.D., Yu, K., Karanjawala, Z.E., Hsieh, C.L., and Lieber, M.R. (2005). Generation and characterization of endonuclease G null mice. Mol Cell Biol *25*, 294-302.

Ishikawa, K., and Hayashi, J. (2010). A novel function of mtDNA: its involvement in metastasis. Ann N Y Acad Sci *1201*, 40-43.

Jang, Y.C., Perez, V.I., Song, W., Lustgarten, M.S., Salmon, A.B., Mele, J., Qi, W., Liu, Y., Liang, H., Chaudhuri, A., *et al.* (2009). Overexpression of Mn superoxide dismutase does not increase life span in mice. J Gerontol A Biol Sci Med Sci *64*, 1114-1125.

Jeong, C.W., Lee, J.H., Sohn, S.S., Ryu, S.W., and Kim, D.K. (2010). Mitochondrial microsatellite instability in gastric cancer and gastric epithelial dysplasia as a precancerous lesion. Cancer Epidemiol *34*, 323-327.

Jin, X., Zhang, J., Gao, Y., Ding, K., Wang, N., Zhou, D., Jen, J., and Cheng, S. (2007). Relationship between mitochondrial DNA mutations and clinical characteristics in human lung cancer. Mitochondrion *7*, 347-353.

Kalifa, L., Beutner, G., Phadnis, N., Sheu, S.S., and Sia, E.A. (2009). Evidence for a role of FEN1 in maintaining mitochondrial DNA integrity. DNA Repair (Amst) *8*, 1242-1249.

Kang, D., Nishida, J., Iyama, A., Nakabeppu, Y., Furuichi, M., Fujiwara, T., Sekiguchi, M., and Takeshige, K. (1995). Intracellular localization of 8-oxo-dGTPase in human cells, with special reference to the role of the enzyme in mitochondria. J Biol Chem *270*, 14659-14665.

Kaniak, A., Dzierzbicki, P., Rogowska, A.T., Malc, E., Fikus, M., and Ciesla, Z. (2009). Msh1p counteracts oxidative lesion-induced instability of mtDNA and stimulates mitochondrial recombination in Saccharomyces cerevisiae. DNA Repair (Amst) *8*, 318-329.

Karahalil, B., Bohr, V.A., and De Souza-Pinto, N.C. (2010). Base excision repair activities differ in human lung cancer cells and corresponding normal controls. Anticancer Res *30*, 4963-4971.

Kirkwood, T.B. (2005). Understanding the odd science of aging. Cell *120*, 437-447.

Klaunig, J.E., Kamendulis, L.M., and Hocevar, B.A. (2010). Oxidative stress and oxidative damage in carcinogenesis. Toxicol Pathol *38*, 96-109.

Klungland, A., and Lindahl, T. (1997). Second pathway for completion of human DNA base excision-repair: reconstitution with purified proteins and requirement for DNase IV (FEN1). EMBO J *16*, 3341-3348.

Kovtun, I.V., Liu, Y., Bjoras, M., Klungland, A., Wilson, S.H., and McMurray, C.T. (2007). OGG1 initiates age-dependent CAG trinucleotide expansion in somatic cells. Nature *447*, 447-452.

Kraytsberg, Y., Kudryavtseva, E., McKee, A.C., Geula, C., Kowall, N.W., and Khrapko, K. (2006). Mitochondrial DNA deletions are abundant and cause functional impairment in aged human substantia nigra neurons. Nat Genet *38*, 518-520.

Krishnan, K.J., Reeve, A.K., Samuels, D.C., Chinnery, P.F., Blackwood, J.K., Taylor, R.W., Wanrooij, S., Spelbrink, J.N., Lightowlers, R.N., and Turnbull, D.M. (2008). What causes mitochondrial DNA deletions in human cells? Nat Genet *40*, 275-279.

Kujoth, G.C., Hiona, A., Pugh, T.D., Someya, S., Panzer, K., Wohlgemuth, S.E., Hofer, T., Seo, A.Y., Sullivan, R., Jobling, W.A., *et al.* (2005). Mitochondrial DNA mutations, oxidative stress, and apoptosis in mammalian aging. Science *309*, 481-484.

Kuznetsov, N.A., Koval, V.V., Zharkov, D.O., Nevinsky, G.A., Douglas, K.T., and Fedorova, O.S. (2005). Kinetics of substrate recognition and cleavage by human 8-oxoguanine-DNA glycosylase. Nucleic Acids Res *33*, 3919-3931.

Ladner, R.D., and Caradonna, S.J. (1997). The human dUTPase gene encodes both nuclear and mitochondrial isoforms. Differential expression of the isoforms and characterization of a cDNA encoding the mitochondrial species. J Biol Chem *272*, 19072-19080.

Lakshmipathy, U., and Campbell, C. (1999a). Double strand break rejoining by mammalian mitochondrial extracts. Nucleic Acids Res *27*, 1198-1204.

Lakshmipathy, U., and Campbell, C. (1999b). The human DNA ligase III gene encodes nuclear and mitochondrial proteins. Mol Cell Biol *19*, 3869-3876.

Lakshmipathy, U., and Campbell, C. (2001). Antisense-mediated decrease in DNA ligase III expression results in reduced mitochondrial DNA integrity. Nucleic Acids Res *29*, 668-676.

Le Page, F., Kwoh, E.E., Avrutskaya, A., Gentil, A., Leadon, S.A., Sarasin, A., and Cooper, P.K. (2000). Transcription-coupled repair of 8-oxoguanine: requirement for XPG, TFIIH, and CSB and implications for Cockayne syndrome. Cell *101*, 159-171.

LeDoux, S.P., Wilson, G.L., Beecham, E.J., Stevnsner, T., Wassermann, K., and Bohr, V.A. (1992). Repair of mitochondrial DNA after various types of DNA damage in Chinese hamster ovary cells. Carcinogenesis *13*, 1967-1973.

Lee, H.C., Chang, C.M., and Chi, C.W. (2010). Somatic mutations of mitochondrial DNA in aging and cancer progression. Ageing Res Rev *9 Suppl 1*, S47-58.

Lee, S., and Park, M.S. (2002). Human FEN-1 can process the 5'-flap DNA of CTG/CAG triplet repeat derived from human genetic diseases by length and sequence dependent manner. Exp Mol Med *34*, 313-317.

Li, M.X., Wang, D., Zhong, Z.Y., Xiang, D.B., Li, Z.P., Xie, J.Y., Yang, Z.Z., Jin, F., and Qing, Y. (2008). Targeting truncated APE1 in mitochondria enhances cell survival after oxidative stress. Free Radic Biol Med *45*, 592-601.

Lin, M.T., and Beal, M.F. (2006). Mitochondrial dysfunction and oxidative stress in neurodegenerative diseases. Nature *443*, 787-795.

Lindahl, T. (1974). An N-glycosidase from Escherichia coli that releases free uracil from DNA containing deaminated cytosine residues. Proc Natl Acad Sci U S A *71*, 3649-3653.

Ling, F., Makishima, F., Morishima, N., and Shibata, T. (1995). A nuclear mutation defective in mitochondrial recombination in yeast. EMBO J *14*, 4090-4101.

Liu, P., and Demple, B. (2010). DNA repair in mammalian mitochondria: Much more than we thought? Environ Mol Mutagen *51*, 417-426.

Liu, P., Qian, L., Sung, J.S., de Souza-Pinto, N.C., Zheng, L., Bogenhagen, D.F., Bohr, V.A., Wilson, D.M., 3rd, Shen, B., and Demple, B. (2008). Removal of oxidative DNA damage via FEN1-dependent long-patch base excision repair in human cell mitochondria. Mol Cell Biol *28*, 4975-4987.

Ludlum, D.B. (1997). The chloroethylnitrosoureas: sensitivity and resistance to cancer chemotherapy at the molecular level. Cancer Invest *15*, 588-598.

Ludwig, D.L., MacInnes, M.A., Takiguchi, Y., Purtymun, P.E., Henrie, M., Flannery, M., Meneses, J., Pedersen, R.A., and Chen, D.J. (1998). A murine AP-endonuclease gene-targeted deficiency with post-implantation embryonic progression and ionizing radiation sensitivity. Mutat Res *409*, 17-29.

Martin, S.A., Hewish, M., Sims, D., Lord, C.J., and Ashworth, A. (2011). Parallel high-throughput RNA interference screens identify PINK1 as a potential therapeutic target for the treatment of DNA mismatch repair-deficient cancers. Cancer Res *71*, 1836-1848.

Martin, S.A., Lord, C.J., and Ashworth, A. (2008). DNA repair deficiency as a therapeutic target in cancer. Curr Opin Genet Dev *18*, 80-86.

Martin, S.A., McCabe, N., Mullarkey, M., Cummins, R., Burgess, D.J., Nakabeppu, Y., Oka, S., Kay, E., Lord, C.J., and Ashworth, A. (2010). DNA polymerases as potential therapeutic targets for cancers deficient in the DNA mismatch repair proteins MSH2 or MLH1. Cancer Cell *17*, 235-248.

Mason, P.A., Matheson, E.C., Hall, A.G., and Lightowlers, R.N. (2003). Mismatch repair activity in mammalian mitochondria. Nucleic Acids Res *31*, 1052-1058.

Masuda, Y., Bennett, R.A., and Demple, B. (1998). Dynamics of the interaction of human apurinic endonuclease (Ape1) with its substrate and product. J Biol Chem *273*, 30352-30359.

Mattson, M.P. (2004). Pathways towards and away from Alzheimer's disease. Nature *430*, 631-639.

Mecocci, P., MacGarvey, U., and Beal, M.F. (1994). Oxidative damage to mitochondrial DNA is increased in Alzheimer's disease. Ann Neurol *36*, 747-751.

Meierhofer, D., Mayr, J.A., Fink, K., Schmeller, N., Kofler, B., and Sperl, W. (2006). Mitochondrial DNA mutations in renal cell carcinomas revealed no general impact on energy metabolism. Br J Cancer *94*, 268-274.

Michikawa, Y., Mazzucchelli, F., Bresolin, N., Scarlato, G., and Attardi, G. (1999). Aging-dependent large accumulation of point mutations in the human mtDNA control region for replication. Science *286*, 774-779.

Miquel, J., Economos, A.C., Fleming, J., and Johnson, J.E., Jr. (1980). Mitochondrial role in cell aging. Exp Gerontol *15*, 575-591.

Mita, S., Monnat, R.J., Jr., and Loeb, L.A. (1988). Resistance of HeLa cell mitochondrial DNA to mutagenesis by chemical carcinogens. Cancer Res *48*, 4578-4583.

Mithani, S.K., Smith, I.M., Zhou, S., Gray, A., Koch, W.M., Maitra, A., and Califano, J.A. (2007). Mitochondrial resequencing arrays detect tumor-specific mutations in salivary rinses of patients with head and neck cancer. Clin Cancer Res *13*, 7335-7340.

Mookerjee, S.A., Lyon, H.D., and Sia, E.A. (2005). Analysis of the functional domains of the mismatch repair homologue Msh1p and its role in mitochondrial genome maintenance. Curr Genet *47*, 84-99.

Mootha, V.K., Bunkenborg, J., Olsen, J.V., Hjerrild, M., Wisniewski, J.R., Stahl, E., Bolouri, M.S., Ray, H.N., Sihag, S., Kamal, M., *et al.* (2003). Integrated analysis of protein composition, tissue diversity, and gene regulation in mouse mitochondria. Cell *115*, 629-640.

Moro, L., Arbini, A.A., Yao, J.L., di Sant'Agnese, P.A., Marra, E., and Greco, M. (2009). Mitochondrial DNA depletion in prostate epithelial cells promotes anoikis

resistance and invasion through activation of PI3K/Akt2. Cell Death Differ *16*, 571-583.

Mouradian, M.M. (2002). Recent advances in the genetics and pathogenesis of Parkinson disease. Neurology *58*, 179-185.

Muller, U., Troidl, C., and Niemann, S. (2005). SDHC mutations in hereditary paraganglioma /pheochromocytoma. Fam Cancer *4*, 9-12.

Nagy, A., Wilhelm, M., and Kovacs, G. (2003). Mutations of mtDNA in renal cell tumours arising in end-stage renal disease. J Pathol *199*, 237-242.

Nakabeppu, Y. (2001). Molecular genetics and structural biology of human MutT homolog, MTH1. Mutat Res *477*, 59-70.

Newlands, E.S., Stevens, M.F., Wedge, S.R., Wheelhouse, R.T., and Brock, C. (1997). Temozolomide: a review of its discovery, chemical properties, pre-clinical development and clinical trials. Cancer Treat Rev *23*, 35-61.

Nilsen, H., Otterlei, M., Haug, T., Solum, K., Nagelhus, T.A., Skorpen, F., and Krokan, H.E. (1997). Nuclear and mitochondrial uracil-DNA glycosylases are generated by alternative splicing and transcription from different positions in the UNG gene. Nucleic Acids Res *25*, 750-755.

Obulesu, M., and Rao, D.M. (2010). DNA damage and impairment of DNA repair in Alzheimer's disease. Int J Neurosci *120*, 397-403.

Pakendorf, B., and Stoneking, M. (2005). Mitochondrial DNA and human evolution. Annu Rev Genomics Hum Genet *6*, 165-183.

Parr, R.L., Dakubo, G.D., Crandall, K.A., Maki, J., Reguly, B., Aguirre, A., Wittock, R., Robinson, K., Alexander, J.S., Birch-Machin, M.A., *et al.* (2006). Somatic mitochondrial DNA mutations in prostate cancer and normal appearing adjacent glands in comparison to age-matched prostate samples without malignant histology. J Mol Diagn *8*, 312-319.

Parrella, P., Xiao, Y., Fliss, M., Sanchez-Cespedes, M., Mazzarelli, P., Rinaldi, M., Nicol, T., Gabrielson, E., Cuomo, C., Cohen, D., *et al.* (2001). Detection of mitochondrial DNA mutations in primary breast cancer and fine-needle aspirates. Cancer Res *61*, 7623-7626.

Perez, V.I., Van Remmen, H., Bokov, A., Epstein, C.J., Vijg, J., and Richardson, A. (2009). The overexpression of major antioxidant enzymes does not extend the lifespan of mice. Aging Cell *8*, 73-75.

Pinz, K.G., Shibutani, S., and Bogenhagen, D.F. (1995). Action of mitochondrial DNA polymerase gamma at sites of base loss or oxidative damage. J Biol Chem *270*, 9202-9206.

Polyak, K., Li, Y., Zhu, H., Lengauer, C., Willson, J.K., Markowitz, S.D., Trush, M.A., Kinzler, K.W., and ogelstein, B. (1998). Somatic mutations of the mitochondrial genome in human colorectal tumours. Nat Genet *20*, 291-293.

Pursell, Z.F., McDonald, J.T., Mathews, C.K., and Kunkel, T.A. (2008). Trace amounts of 8-oxo-dGTP in mitochondrial dNTP pools reduce DNA polymerase gamma replication fidelity. Nucleic Acids Res *36*, 2174-2181.

Radpour, R., Fan, A.X., Kohler, C., Holzgreve, W., and Zhong, X.Y. (2009). Current understanding of mitochondrial DNA in breast cancer. Breast J *15*, 505-509.

Reeve, A.K., Krishnan, K.J., Taylor, G., Elson, J.L., Bender, A., Taylor, R.W., Morris, C.M., and Turnbull, D.M. (2009). The low abundance of clonally expanded mitochondrial DNA point mutations in aged substantia nigra neurons. Aging Cell *8*, 496-498.

Richter, C., Park, J.W., and Ames, B.N. (1988). Normal oxidative damage to mitochondrial and nuclear DNA is extensive. Proc Natl Acad Sci U S A *85*, 6465-6467.

Robertson, A.B., Klungland, A., Rognes, T., and Leiros, I. (2009). DNA repair in mammalian cells: Base excision repair: the long and short of it. Cell Mol Life Sci *66*, 981-993.

Rogounovitch, T., Saenko, V., and Yamashita, S. (2004). Mitochondrial DNA and human thyroid diseases. Endocr J *51*, 265-277.

Ropp, P.A., and Copeland, W.C. (1996). Cloning and characterization of the human mitochondrial DNA polymerase, DNA polymerase gamma. Genomics *36*, 449-458.

Roth, D.B., Porter, T.N., and Wilson, J.H. (1985). Mechanisms of nonhomologous recombination in mammalian cells. Mol Cell Biol *5*, 2599-2607.

Sage, J.M., Gildemeister, O.S., and Knight, K.L. (2010). Discovery of a novel function for human Rad51: maintenance of the mitochondrial genome. J Biol Chem *285*, 18984-18990.

Sakai, Y., Furuichi, M., Takahashi, M., Mishima, M., Iwai, S., Shirakawa, M., and Nakabeppu, Y. (2002). A molecular basis for the selective recognition of 2-hydroxy-dATP and 8-oxo-dGTP by human MTH1. J Biol Chem *277*, 8579-8587.

Sander, M., Cadet, J., Casciano, D.A., Galloway, S.M., Marnett, L.J., Novak, R.F., Pettit, S.D., Preston, R.J., Skare, J.A., Williams, G.M., *et al.* (2005). Proceedings of a workshop on DNA adducts: biological significance and applications to risk assessment Washington, DC, April 13-14, 2004. Toxicol Appl Pharmacol *208*, 1-20.

Sanz, A., Fernandez-Ayala, D.J., Stefanatos, R.K., and Jacobs, H.T. (2010). Mitochondrial ROS production correlates with, but does not directly regulate lifespan in Drosophila. Aging (Albany NY) *2*, 220-223.

Schaefer, A.M., McFarland, R., Blakely, E.L., He, L., Whittaker, R.G., Taylor, R.W., Chinnery, P.F., and Turnbull, D.M. (2008). Prevalence of mitochondrial DNA disease in adults. Ann Neurol *63*, 35-39.

Selzer, R.R., Nyaga, S., Tuo, J., May, A., Muftuoglu, M., Christiansen, M., Citterio, E., Brosh, R.M., Jr., and Bohr, V.A. (2002). Differential requirement for the ATPase domain of the Cockayne syndrome group B gene in the processing of UV-induced DNA damage and 8-oxoguanine lesions in human cells. Nucleic Acids Res *30*, 782-793.

Servidei, S. (2004). Mitochondrial encephalomyopathies: gene mutation. Neuromuscul Disord *14*, 107-116.

Shoffner, J.M., Lott, M.T., Voljavec, A.S., Soueidan, S.A., Costigan, D.A., and Wallace, D.C. (1989). Spontaneous Kearns-Sayre/chronic external ophthalmoplegia plus syndrome associated with a mitochondrial DNA deletion: a slip-replication model and metabolic therapy. Proc Natl Acad Sci U S A *86*, 7952-7956.

Shokolenko, I., Venediktova, N., Bochkareva, A., Wilson, G.L., and Alexeyev, M.F. (2009). Oxidative stress induces degradation of mitochondrial DNA. Nucleic Acids Res *37*, 2539-2548.

Simsek, D., Furda, A., Gao, Y., Artus, J., Brunet, E., Hadjantonakis, A.K., Van Houten, B., Shuman, S., McKinnon, P.J., and Jasin, M. (2011). Crucial role for DNA ligase III in mitochondria but not in Xrcc1-dependent repair. Nature *471*, 245-248.

Slupphaug, G., Kavli, B., and Krokan, H.E. (2003). The interacting pathways for prevention and repair of oxidative DNA damage. Mutat Res *531*, 231-251.

Spiro, C., and McMurray, C.T. (2003). Nuclease-deficient FEN-1 blocks Rad51/BRCA1-mediated repair and causes trinucleotide repeat instability. Mol Cell Biol *23*, 6063-6074.

Spiro, C., Pelletier, R., Rolfsmeier, M.L., Dixon, M.J., Lahue, R.S., Gupta, G., Park, M.S., Chen, X., Mariappan, S.V., and McMurray, C.T. (1999). Inhibition of FEN-1 processing by DNA secondary structure at trinucleotide repeats. Mol Cell *4*, 1079-1085.

Stevnsner, T., Nyaga, S., de Souza-Pinto, N.C., van der Horst, G.T., Gorgels, T.G., Hogue, B.A., Thorslund, T., and Bohr, V.A. (2002a). Mitochondrial repair of 8-oxoguanine is deficient in Cockayne syndrome group B. Oncogene *21*, 8675-8682.

Stevnsner, T., Thorslund, T., de Souza-Pinto, N.C., and Bohr, V.A. (2002b). Mitochondrial repair of 8-oxoguanine and changes with aging. Exp Gerontol *37*, 1189-1196.

Stierum, R.H., Dianov, G.L., and Bohr, V.A. (1999). Single-nucleotide patch base excision repair of uracil in DNA by mitochondrial protein extracts. Nucleic Acids Res *27*, 3712-3719.

Stuart, J.A., Karahalil, B., Hogue, B.A., Souza-Pinto, N.C., and Bohr, V.A. (2004). Mitochondrial and nuclear DNA base excision repair are affected differently by caloric restriction. FASEB J *18*, 595-597.

Sun, J., Folk, D., Bradley, T.J., and Tower, J. (2002). Induced overexpression of mitochondrial Mn-superoxide dismutase extends the life span of adult Drosophila melanogaster. Genetics *161*, 661-672.

Suter, M., and Richter, C. (1999). Fragmented mitochondrial DNA is the predominant carrier of oxidized DNA bases. Biochemistry *38*, 459-464.

Suzuki, M., Toyooka, S., Miyajima, K., Iizasa, T., Fujisawa, T., Bekele, N.B., and Gazdar, A.F. (2003). Alterations in the mitochondrial displacement loop in lung cancers. Clin Cancer Res *9*, 5636-5641.

Swerdlow, R.H., Parks, J.K., Miller, S.W., Tuttle, J.B., Trimmer, P.A., Sheehan, J.P., Bennett, J.P., Jr., Davis, R.E., and Parker, W.D., Jr. (1996). Origin and functional consequences of the complex I defect in Parkinson's disease. Ann Neurol *40*, 663-671.

Szczesny, B., Hazra, T.K., Papaconstantinou, J., Mitra, S., and Boldogh, I. (2003). Age-dependent deficiency in import of mitochondrial DNA glycosylases required for repair of oxidatively damaged bases. Proc Natl Acad Sci U S A *100*, 10670-10675.

Szczesny, B., Tann, A.W., Longley, M.J., Copeland, W.C., and Mitra, S. (2008). Long patch base excision repair in mammalian mitochondrial genomes. J Biol Chem *283*, 26349-26356.

Takao, M., Kanno, S., Shiromoto, T., Hasegawa, R., Ide, H., Ikeda, S., Sarker, A.H., Seki, S., Xing, J.Z., Le, X.C., *et al.* (2002). Novel nuclear and mitochondrial glycosylases revealed by disruption of the mouse Nth1 gene encoding an endonuclease III homolog for repair of thymine glycols. EMBO J *21*, 3486-3493.

Takao, M., Zhang, Q.M., Yonei, S., and Yasui, A. (1999). Differential subcellular localization of human MutY homolog (hMYH) and the functional activity of adenine:8-oxoguanine DNA glycosylase. Nucleic Acids Res *27*, 3638-3644.

Taschner, P.E., Jansen, J.C., Baysal, B.E., Bosch, A., Rosenberg, E.H., Brocker-Vriends, A.H., van Der Mey, A.G., van Ommen, G.J., Cornelisse, C.J., and Devilee, P. (2001). Nearly all hereditary paragangliomas in the Netherlands are caused by two founder mutations in the SDHD gene. Genes Chromosomes Cancer *31*, 274-281.

Taylor, R.W., and Turnbull, D.M. (2005). Mitochondrial DNA mutations in human disease. Nat Rev Genet *6*, 389-402.

Tell, G., Damante, G., Caldwell, D., and Kelley, M.R. (2005). The intracellular localization of APE1/Ref-1: more than a passive phenomenon? Antioxid Redox Signal *7*, 367-384.

Thyagarajan, B., Padua, R.A., and Campbell, C. (1996). Mammalian mitochondria possess homologous DNA recombination activity. J Biol Chem *271*, 27536-27543.

Trifunovic, A., Wredenberg, A., Falkenberg, M., Spelbrink, J.N., Rovio, A.T., Bruder, C.E., Bohlooly, Y.M., Gidlof, S., Oldfors, A., Wibom, R., *et al.* (2004). Premature ageing in mice expressing defective mitochondrial DNA polymerase. Nature *429*, 417-423.

Tuo, J., Chen, C., Zeng, X., Christiansen, M., and Bohr, V.A. (2002). Functional crosstalk between hOgg1 and the helicase domain of Cockayne syndrome group B protein. DNA Repair (Amst) *1*, 913-927.

Tuo, J., Muftuoglu, M., Chen, C., Jaruga, P., Selzer, R.R., Brosh, R.M., Jr., Rodriguez, H., Dizdaroglu, M., and Bohr, V.A. (2001). The Cockayne Syndrome group B gene product is involved in general genome base excision repair of 8-hydroxyguanine in DNA. J Biol Chem *276*, 45772-45779.

Tuppen, H.A., Blakely, E.L., Turnbull, D.M., and Taylor, R.W. (2010). Mitochondrial DNA mutations and human disease. Biochim Biophys Acta *1797*, 113-128.

van Loon, B., and Hubscher, U. (2009). An 8-oxo-guanine repair pathway coordinated by MUTYH glycosylase and DNA polymerase lambda. Proc Natl Acad Sci U S A *106*, 18201-18206.

Vartanian, V., Lowell, B., Minko, I.G., Wood, T.G., Ceci, J.D., George, S., Ballinger, S.W., Corless, C.L., McCullough, A.K., and Lloyd, R.S. (2006). The metabolic syndrome resulting from a knockout of the NEIL1 DNA glycosylase. Proc Natl Acad Sci U S A *103*, 1864-1869.

Vermulst, M., Bielas, J.H., Kujoth, G.C., Ladiges, W.C., Rabinovitch, P.S., Prolla, T.A., and Loeb, L.A. (2007). Mitochondrial point mutations do not limit the natural lifespan of mice. Nat Genet *39*, 540-543.

Vermulst, M., Wanagat, J., Kujoth, G.C., Bielas, J.H., Rabinovitch, P.S., Prolla, T.A., and Loeb, L.A. (2008). DNA deletions and clonal mutations drive premature aging in mitochondrial mutator mice. Nat Genet *40*, 392-394.

Vivekanandan, P., Daniel, H., Yeh, M.M., and Torbenson, M. (2010). Mitochondrial mutations in hepatocellular carcinomas and fibrolamellar carcinomas. Mod Pathol *23*, 790-798.

Wada, T., Tanji, N., Ozawa, A., Wang, J., Shimamoto, K., Sakayama, K., and Yokoyama, M. (2006). Mitochondrial DNA mutations and 8-hydroxy-2'-deoxyguanosine Content in Japanese patients with urinary bladder and renal cancers. Anticancer Res *26*, 3403-3408.

Wallace, D.C. (2001). Mitochondrial defects in neurodegenerative disease. Ment Retard Dev Disabil Res Rev *7*, 158-166.

Wallace, D.C. (2005). A mitochondrial paradigm of metabolic and degenerative diseases, aging, and cancer: a dawn for evolutionary medicine. Annu Rev Genet *39*, 359-407.

Wallace, D.C., Zheng, X.X., Lott, M.T., Shoffner, J.M., Hodge, J.A., Kelley, R.I., Epstein, C.M., and Hopkins, L.C. (1988). Familial mitochondrial encephalomyopathy (MERRF): genetic, pathophysiological, and biochemical characterization of a mitochondrial DNA disease. Cell *55*, 601-610.

Wang, J., Xiong, S., Xie, C., Markesbery, W.R., and Lovell, M.A. (2005). Increased oxidative damage in nuclear and mitochondrial DNA in Alzheimer's disease. J Neurochem *93*, 953-962.

Warburg, O. (1956). On the origin of cancer cells. Science *123*, 309-314.

Weissman, L., Jo, D.G., Sorensen, M.M., de Souza-Pinto, N.C., Markesbery, W.R., Mattson, M.P., and Bohr, V.A. (2007). Defective DNA base excision repair in brain from individuals with Alzheimer's disease and amnestic mild cognitive impairment. Nucleic Acids Res *35*, 5545-5555.

Xanthoudakis, S., Smeyne, R.J., Wallace, J.D., and Curran, T. (1996). The redox/DNA repair protein, Ref-1, is essential for early embryonic development in mice. Proc Natl Acad Sci U S A *93*, 8919-8923.

Xu, G., Herzig, M., Rotrekl, V., and Walter, C.A. (2008). Base excision repair, aging and health span. Mech Ageing Dev *129*, 366-382.

Yakes, F.M., and Van Houten, B. (1997). Mitochondrial DNA damage is more extensive and persists longer than nuclear DNA damage in human cells following oxidative stress. Proc Natl Acad Sci U S A *94*, 514-519.

Yamaguchi, H., Kajitani, K., Dan, Y., Furuichi, M., Ohno, M., Sakumi, K., Kang, D., and Nakabeppu, Y. (2006). MTH1, an oxidized purine nucleoside triphosphatase, protects the dopamine neurons from oxidative damage in nucleic acids caused by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine. Cell Death Differ *13*, 551-563.

Yang, J.L., Weissman, L., Bohr, V.A., and Mattson, M.P. (2008). Mitochondrial DNA damage and repair in neurodegenerative disorders. DNA Repair (Amst) *7*, 1110-1120.

Yoshimura, D., Sakumi, K., Ohno, M., Sakai, Y., Furuichi, M., Iwai, S., and Nakabeppu, Y. (2003). An oxidized purine nucleoside triphosphatase, MTH1, suppresses cell death caused by oxidative stress. J Biol Chem *278*, 37965-37973.

Zhang, R., Zhang, F., Wang, C., Wang, S., Shiao, Y.H., and Guo, Z. (2010). Identification of sequence polymorphism in the D-Loop region of mitochondrial DNA as a risk factor for hepatocellular carcinoma with distinct etiology. J Exp Clin Cancer Res *29*, 130.

Zheng, L., Zhou, M., Guo, Z., Lu, H., Qian, L., Dai, H., Qiu, J., Yakubovskaya, E., Bogenhagen, D.F., Demple, B., *et al.* (2008). Human DNA2 is a mitochondrial nuclease/helicase for efficient processing of DNA replication and repair intermediates. Mol Cell *32*, 325-336.

Zsurka, G., Schroder, R., Kornblum, C., Rudolph, J., Wiesner, R.J., Elger, C.E., and Kunz, W.S. (2004). Tissue dependent co-segregation of the novel pathogenic G12276A mitochondrial tRNALeu(CUN) mutation with the A185G D-loop polymorphism. J Med Genet *41*, e124.

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Mitochondrial DNA Damage, Repair, Degradation and Experimental Approaches to Studying These Phenomena

Inna Shokolenko, Susan LeDoux, Glenn Wilson and Mikhail Alexeyev
University of South Alabama
USA

1. Introduction

In mammalian cells, genetic information is stored in two locations: in the nucleus and in mitochondria. Nuclear DNA (nDNA) is organized into chromosomes of which two sets are present per cell: one paternal, and one maternal. In contrast, mitochondrial DNA (mtDNA) inheritance is (with few exceptions) exclusively maternal, and is highly redundant, typically a few hundred to a few thousand copies per cell. In many (but not all, (Noll et al., 1990)) cell types the bulk of ATP is produced by oxidative phosphorylation (OXPHOS) in mitochondria. Since mtDNA encodes components of four out of five mitochondrial respiratory complexes, it is not surprising that alterations in mtDNA result in (mitochondrial) disease (Holt et al., 1988; Lestienne & Ponsot, 1988; Wallace et al., 1988). Apart from mitochondrial disease, mutations in mtDNA are linked to a spectrum of diseases including cancer, diabetes, cardiovascular diseases and neurodegenerative disorders, as well as the normal process of aging (Wallace, 2005). Importantly, it has been established that not only mtDNA mutations, but also reduction in the mtDNA copy number can be pathogenic (Clay Montier et al., 2009; Rotig & Poulton, 2009). Understanding cellular mechanisms for the maintenance of mtDNA integrity and copy number is, therefore, of utmost importance since it can provide targets for clinical interventions aimed at prevention and treatment of human disease.

2. Organization of the mitochondrial genome

Human mtDNA (Figue 1) is approximately 16.6 kbp long and encodes two rRNAs, 22 tRNAs and 13 polypeptides of which 7 are subunits of complex I (NADH dehydrogenase), 3 are subunits of complex IV (cytochrome c oxidase), 2 are subunits of complex V (ATP synthase), and cytochrome b (a subunit of complex III). The density of genetic information in mtDNA is relatively high, with very short intergenic regions. To increase this density some genes overlap, and some others lack complete termination codons, which are created by polyadenylation of corresponding mRNAs (Ojala et al., 1981). A short noncoding regulatory region in mtDNA harbours an origin of replication plus two promoters, one on each of the two complementary strands. These promoters generate polycistronic transcripts that are processed to produce mature rRNAs, tRNAs, and mRNAs and also are involved in the generation of the primer for replication of one of the strands.

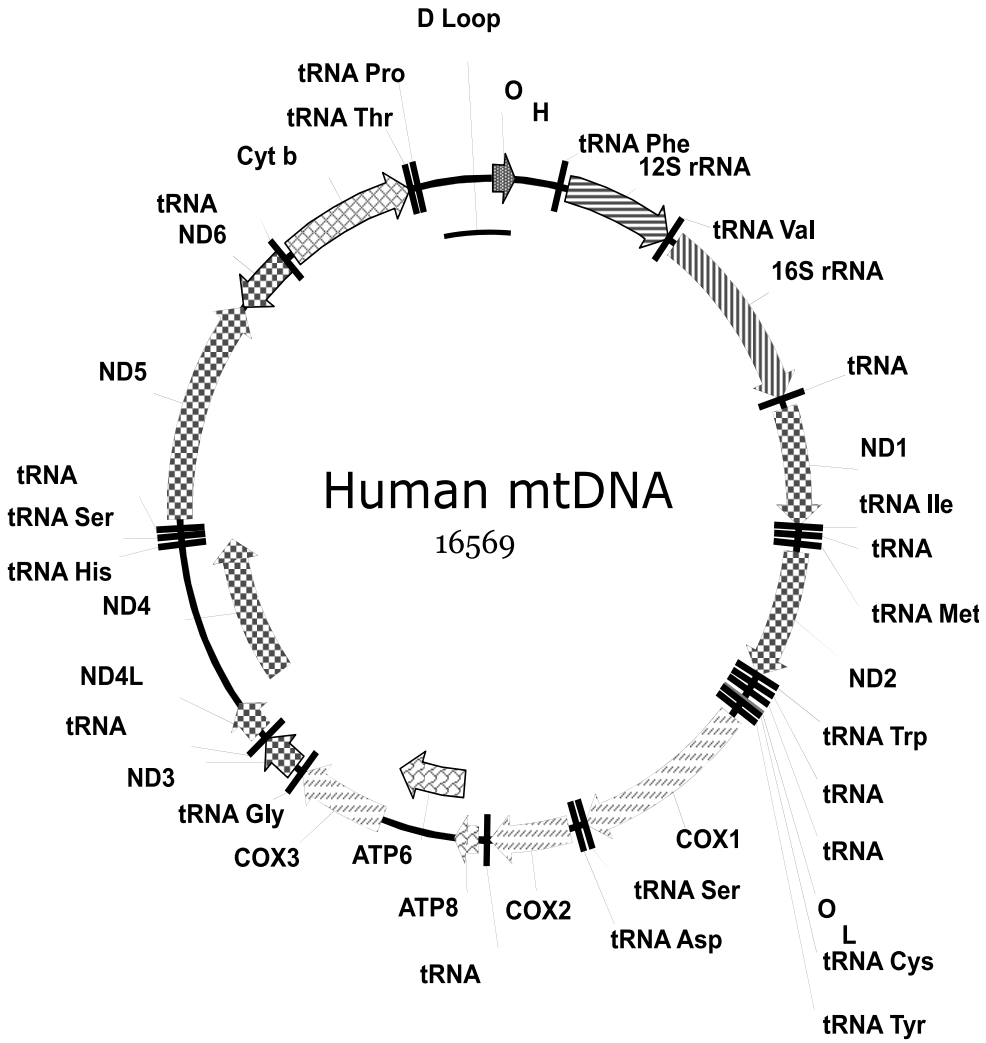

Fig. 1. The map of human Mitochondrial DNA. O_H and O_L, origins of heavy and light strand replication, respectively; ND1-ND6, subunits of NADH dehydrogenase (ETC complex I) subunits 1 through 6; COX1-COX3, subunits of cytochrome oxidase subunits 1 through 3 (ETC complex IV), ATP6 and ATP8, subunits 6 and 8 of mitochondrial ATPase (complex V), Cyt b, cytochrome b (complex III).

It has been determined that mitochondria contain, on average, two molecules of mtDNA (Cavelier et al., 2000). However, mitochondria form a dynamic network which, in different cell types and under different physiological conditions, can assume a variety of conformations, the two extremes being "reticular" (mitochondria in the cell are fused to form a network of extended filaments) and "particular" (network is disintegrated into short fragments). In both conformations, mitochondria perpetually undergo the processes of fission and fusion, thus mixing their contents. Therefore, the above definitions of "reticulate" and "particulate" mitochondrial conformations are relative terms referring to a snapshot of the mitochondrial network in a cell. Nevertheless, these terms are useful as they

describe the prevalence of either mitochondrial fission ("particulate" conformation) or fusion ("reticulate" conformation) in a given cell under given physiological conditions. In this light, the average number of mtDNA copies per mitochondrion determined in some studies (Cavelier et al., 2000) may simply reflect the extent of mitochondrial fragmentation under the assay conditions, which is defined by two factors: a) the mitochondrial conformation inside the cell, and b) the extent of mitochondrial fragmentation during isolation for the analysis of mtDNA content.

Nuclear genetic material is represented by nucleoprotein complexes consisting of DNA wrapped around a core octamer of histones forming "beads on a string". This nucleosomal chromatin is further organized to form chromosomes. In contrast, the mitochondrial genome lacks histones, which has led to the widespread belief that the observed high rate of mtDNA mutagenesis (approximately 10-fold greater than in nDNA (Brown, W.M. et al., 1979; Ballard & Whitlock, 2004; Tatarenkov & Avise, 2007) can be explained by the lack of "protective" histones. This belief lacks direct experimental support and remains controversial as it contradicts some experimental evidence, which suggests that histones may enhance, rather than reduce DNA damage (Liang, R. et al., 1999; Liang, Q. & Dedon, 2001), at least under some conditions, and that mtDNA-associated proteins are at least as protective against mutagenic insults as histones under other conditions (Guliaeva et al., 2006). Moreover, mtDNA may be physically covered with TFAM (Alam et al., 2003), an HMG-like protein involved in mtDNA transcription and replication, a notion which is consistent with the limited accessibility of mtDNA to methytransferases (Rebelo et al., 2009).

Considering the endosymbiotic theory of mitochondrial origin from an ancient prokaryote, it is perhaps not surprising that recent studies revealed similarities in packaging of mtDNA and bacterial chromosomes. Thus, it has been established that in the ECV304 cell line the 3,500 copies of mtDNA are organized into ~475 nucleoids about 70 nm in diameter, each of them carrying 6-10 copies of mtDNA (Iborra et al., 2004). This organization insures similar DNA densities in mitochondrial and E. *coli* nucleoids, about 35 mg/ml (Iborra et al., 2004). Mitochondrial nucleoids are spaced more uniformly than would be expected by random distribution. This uniformity likely results from inability of nucleoids to diffuse freely due to their anchoring in the mitochondrial inner membrane. Nucleoids are found in close association with both microtubules and with KIF5B, a kinesin motor responsible for the movement of mitochondria along microtubules. (Iborra et al., 2004). Subsequent studies refined this model, and now mitochondrial nucleoids are viewed as layered structures consisting of a core, where replication and transcription of mtDNA occur, and peripheral regions, where translation of mitochondrial transcripts and assembly of newly synthesized polypeptides into respiratory complexes occurs (Bogenhagen, D.F. et al., 2008).

3. Maintenance of mtDNA

Normal functioning of the cell and organism critically depends upon proper maintenance of mtDNA integrity and copy number. This is achieved through intricate coordination of the processes of mtDNA replication, repair, and degradation (turnover). Below, we will review each of these processes in some detail.

3.1 mtDNA replication

It is generally accepted that replication of mtDNA is not linked to the cell cycle as strictly as replication of nDNA is. In fact, mtDNA replication occurs in all stages of the cell cycle and

persists even in nondividing cells (Bogenhagen, D. & Clayton, 1977; Clayton, 1982). DNA polymerase γ (Pol γ) is the sole DNA polymerase identified in mitochondria. This enzyme is heterotrimeric and consists of a single 140 kDa catalytic subunit encoded by the POLG gene and two 55 kDa accessory subunits, encoded by POLG2. As the only DNA polymerase found in mitochondria, Pol γ is responsible for both replication and repair of mtDNA. Several other proteins play prominent roles in the mtDNA replication process. These are the DNA helicase Twinkle, a mitochondrial single-strand-binding protein (mtSSB), which mediates unwinding of mtDNA through its physical interaction with Twinkle (St John et al., 2010), and a mitochondrial RNA polymerase, which generates primers for mtDNA replication with the assistance of mitochondrial transcription factors A (TFAM), B1 (TFB1M), and B2 (TFB2M). While the major players in mtDNA replication are fairly well known, the exact mechanism remains controversial (reviewed in (Holt, 2009)).

Electron microscopic observations of purified mtDNA molecules led to the adoption of the strand-displacement model (Robberson et al., 1972). In these experiments, the observation of extensive single-strand regions in mtDNA suggested that synthesis of the leading strand is uncoupled from that of the lagging strand. The leading strand synthesis is initiated at a fixed point and advances about two-thirds of the way around the mtDNA molecule before second strand synthesis is initiated (Holt, 2009). Recently, however, analysis of mtDNA replication intermediates in both mammalian tissues and cultured cells by two-dimensional agarose gel electrophoresis revealed the presence of products consistent with a strand-coupled mechanism of replication (Holt et al., 2000). Subsequently, it was found that RNA is incorporated throughout the lagging strand (RITOLS mechanism, (Yasukawa et al., 2006)). This raised the possibility that the abundant single-strand regions observed in the earlier studies could be an artifact of RNA loss during DNA isolation and processing, and suggested that strand-coupled and RITOLS could be the only two mechanisms involved in mtDNA replication, thus excluding the earlier strand-displacement mechanism (Yasukawa et al., 2006). RITOLS appears to be initiated at several sites in the D-loop and proceeds unidirectionally (Yasukawa et al., 2006), whereas initiation of strand-coupled replication occurs over a broad region and is bidirectional (Yasukawa et al., 2005). However, the observation of stable non-replicative DNA-RNA hybrid loops formed by some mitochondrial transcripts casts a shadow on the authenticity of RITOLS in favor of the original asynchronous strand-displacement mechanism (Brown, T.A. et al., 2008).

3.2 Damage and repair of mtDNA

Mitochondrial genomes accumulate mutations approximately one order of magnitude faster than nDNA (Brown, W.M. et al., 1979; Ballard & Whitlock, 2004; Tatarenkov & Avise, 2007). This could be caused by a variety of factors, including an intrinsically lower fidelity of replication by mitochondria-specific DNA polymerase γ (Pol γ), a lower efficiency of mtDNA repair, or chronic exposure of mtDNA to noxious factors, such as Reactive Oxygen Species (ROS) or environmental genotoxins. However, attempts to experimentally link mtDNA mutagenesis to exposure to carcinogens (Mita et al., 1988) or to reactive oxygen species (Shokolenko et al., 2009) proved unsuccessful, leading to the notion that mtDNA may be resistant to mutagenesis. To confound things even further, several studies have reported that nDNA is at least as sensitive to oxidative damage as mtDNA (Anson et al., 1999; Anson et al., 2000; Lim et al., 2005), which undermines the earlier notion that the higher susceptibility of mtDNA to damage by ROS is the driving force behind its higher rate of mutagenesis (Richter et al., 1988).

The current progress in our understanding of mtDNA repair pathways has been reviewed recently (Liu & Demple, 2010). Historically, the discovery that mitochondria are unable to repair ultraviolet (UV)-induced pyrimidine dimers (Clayton et al., 1974, 1975) and some types of alkylating damage (Miyaki et al., 1977), suggested that they may contain a reduced complement of DNA repair pathways. However, Anderson and Friedberg (Anderson & Friedberg, 1980) found uracil-DNA glycosylase activity in mitochondrial extracts, suggesting the presence of the base excision repair (BER) pathway. This was followed by a report of mitochondrial repair of O^6-ethyl-2'-deoxyguanosine (Myers et al., 1988; Satoh et al., 1988). This can be processed by direct reversal using O^6-methyl guanine methyl transferase or by a nucleotide excision repair pathway. Subsequently, repair of a variety of mtDNA lesions by BER, including those arising from oxidative damage, was demonstrated (Pettepher et al., 1991; LeDoux et al., 1992; Driggers et al., 1993). Recently, long-patch BER of oxidative DNA lesions (Akbari et al., 2008; Liu et al., 2008; Szczesny et al., 2008), and mismatch repair (de Souza-Pinto et al., 2009) have been reported in mammalian mitochondria. The presence in mammalian mitochondria of a DNA end binding activity (Coffey et al., 1999), and a ligase capable of joining both cohesive and blunt ends (Lakshmipathy & Campbell, 1999) suggested the presence of a non-homologous end joining pathway in mitochondria. Similarly, detection of recombination intermediates indicated that mtDNA can be repaired through a homologous recombination pathway (Kajander et al., 2001; Kraytsberg et al., 2004). This notion was further supported by experiments on the induction of mtDNA double-strand breaks (DSBs) in vivo with the help of mitochondrially-targeted restriction endonucleases. In these experiments, DSB repair was accompanied by the formation of mtDNA deletions, some of which had breakpoints flanked by direct repeats, thus implicating homologous recombination in the repair (Srivastava & Moraes, 2005; Fukui & Moraes, 2008). To summarize, current experimental evidence suggests the presence in mitochondria of all major DNA repair pathways, with the exception of the nucleotide excision repair. Moreover, mitochondria appear to possess a unique mechanism for the maintenance of DNA integrity through degradation of damaged molecules (see below). Importantly BER, which is responsible for the repair of oxidative base lesions, is robust in mitochondria, as evidenced by observation that repair of 8-oxodG, the most prominent oxidative base lesion, is more efficient in mitochondria than in the nucleus (Thorslund et al., 2002).

3.3 Degradation and maintenance of mtDNA integrity

Unlike the nuclear genome, the mitochondrial genome is redundant, consisting of hundreds to thousands of copies per cell. Therefore, a "repair or die" constraint is not imposed on mtDNA. Conceivably, a substantial fraction of damaged mtDNA can be lost without detrimental effects, provided that this loss is compensated for by replication of new genomes. In fact, the loss and resynthesis of mtDNA was observed more than 40 years ago by Gross and Rabinowitz, who described mtDNA turnover (Gross & Rabinowitz, 1969). Many cell lines are fairly tolerant to the loss of mtDNA, and can survive both a gradual loss of mtDNA through chronic treatment with ethidium bromide (King & Attardi, 1989), and acute destruction of a fraction (Alexeyev et al., 2008) or even all of their mtDNA (Kukat et al., 2008) by mitochondrially targeted restriction endonucleases. This is in a stark contrast to nDNA, in which persistent DSB can activate apoptosis. However, the hypothesis that turnover (degradation) of damaged mtDNA can be a mechanism used by mitochondria to deal with either excessive damage, or damage that can not be repaired did not take hold in

part due to the lack of direct experimental evidence supporting it and in part due to discovery of mitochondrial BER (Pettepher et al., 1991) , which shifted attention from unrepairable lesions to those that can be repaired. However, recent evidence reignited interest in mtDNA degradation.

Ethanol has been reported to induce mtDNA loss in yeast (Ibeas & Jimenez, 1997). In mice, intragastric administration of ethanol induced oxidative stress and was accompanied by a reversible loss of mtDNA (Mansouri et al., 1999). The loss of mtDNA was approximately 50% in all organs studied. It could be partially prevented by the antioxidants melatonin, vitamin E and coenzymeQ, and was followed by adaptive mtDNA resynthesis (Mansouri et al., 2001). Lipopolysaccharide, a known inducer of in vivo oxidative stress also induced, mtDNA depletion (Suliman et al., 2003). Angiotensin II induced mitochondrial ROS production and decreased skeletal muscle mtDNA content in mice (Mitsuishi et al., 2008). Degradation of mtDNA was observed in the rat model of cerebral ischemia/reperfusion (Chen et al., 2001). Similar to mtDNA depletion induced by intragastric ethanol administration, mtDNA levels returned to normal within 24h of cerebral ischemia/reperfusion (Chen et al., 2001). Finally, H_2O_2-induced oxidative stress in hamster fibroblasts was accompanied by Ca^{2+}-dependent degradation of mtDNA (Crawford et al., 1998). Taken together, these findings strongly suggested a link between oxidative stress (which may result in oxidative mtDNA damage) and mtDNA degradation, yet they stopped short of invoking degradation as protective mechanism. In an unrelated study, it was observed that mtDNA is resistant to mutagenesis induced by alkylating agents, and the authors suggested degradation of damaged mtDNA as one of the potential mechanisms for this resistance (Mita et al., 1988). However, mtDNA degradation under the experimental conditions of that study was not demonstrated (Mita et al., 1988).

Recently, we attempted to study the relationship between experimentally induced oxidative stress and mtDNA mutagenesis. In initial experiments, superoxide radicals were generated on the matrix side of the mitochondrial inner membrane by treating cells with sublethal concentrations of the complex I inhibitor rotenone (St-Pierre et al., 2002; Muller et al., 2004). However, exposing human colon carcinoma cells or mouse embryonic fibroblasts to rotenone for 30 days did not result in a significant increase in the rate of mtDNA mutagenesis (Shokolenko et al., 2009). Similarly, repeated treatment of HCT116 colon cancer cells with H_2O_2 failed to induce significant mtDNA mutagenesis. Instead, DNA lesions that manifest themselves as strands breaks under denaturing conditions (single-strand breaks (SSBs) and DSBs, abasic sites, etc.) prevailed over premutagenic base modifications by a factor of 10. Consistent with the hypothesis that unrepairable mtDNA molecules are degraded, treatment of cells with an inhibitor of BER methoxyamine, enhanced mtDNA degradation in response to both oxidative and alkylating damage (Shokolenko et al., 2009). The elimination of damaged mtDNA was preceded by the accumulation of linear mtDNA molecules, which may represent degradation intermediates, since, unlike undamaged circular molecules, they are susceptible to exonucleolytic degradation.

The high rate of lesions (mostly, SSBs and abasic sites) in mtDNA induced by ROS suggests a mechanism by which mitochondria may maintain the integrity of their genetic information. In this model, oxidative stress induces in mtDNA lesions with a much higher (by the factor of 10, (Shokolenko et al., 2009)) frequency than mutagenic lesions. These lesions represent a block to transcription and replication of mtDNA, and when accumulated above a threshold level, they induce degradation of mtDNA molecule. Therefore,

degradation of mtDNA molecule is triggered before it accumulates mutagenic lesions. This model provides a mechanistic explanation for the observations made by Suter and Richter (Suter & Richter, 1999), who found that the 8-oxodG content of circular mtDNA is low and does not increase in response to oxidative insult. However, fragmented mtDNA had a very high 8-oxodG content, which increased further after oxidative stress. It incorporates the previously suggested notion of a possible contribution of APE1 to mtDNA degradation (Tomkinson et al., 1988; Tomkinson et al., 1990). The model is consistent with the observations of Yakes and van Houten (Yakes & Van Houten, 1997), who found that oxidative stress promoted a higher incidence of polymerase-blocking strand breaks and abasic sites in mtDNA than in nDNA. Recent studies using qPCR for the analysis of mtDNA provide further support for the notion of mtDNA degradation in response to oxidative stress (Rothfuss et al., 2010). Therefore, degradation of severely damaged mtDNA emerges as a unique, mitochondria-specific mechanism for the maintenance of DNA integrity.

Degradation of damaged organellar DNA appears not to be unique to mammalian cells. Known examples of rapid organellar DNA turnover in plants and protists in response to ROS were reviewed recently by Bendich (Bendich, 2010).

3.4 Degradation and maintenance of mtDNA copy number

In most mammalian cells, mtDNA copy number is kept relatively constant at 1,000-10,000 copies per cell, depending on the cell type and physiological conditions (Copeland, 2008). However, antiretroviral therapy (Arnaudo et al., 1991) and genetic defects in the components of the mtDNA replicating machinery (Rotig & Poulton, 2009) were demonstrated to induce a pathologic decrease in mtDNA content of the cell. Also, mtDNA copy number can be decreased in response to increased mtDNA damage, which is not met with a corresponding increase in repair (Shokolenko et al., 2009). For patients with genetic mitochondrial DNA depletion syndromes (MDS), there is no treatment other than supportive therapy (Poulton & Holt, 2009). Liver transplantation proved inefficient in two major forms of MDS associated with liver failure: Alpers-Huttenlocher syndrome and deoxyguanosine kinase (DGUOK) deficiency. In the former instance failure to achieve a therapeutic effect appears to be linked to the inevitable brain involvement, which may not be apparent until after the transplantation. Attempts to correct the hepatocerebral syndrome resulting from DGUOK deficiency through liver transplantation were reviewed recently (Rahman & Poulton, 2009). Infant death was observed in 6 out of the 9 cases reviewed.

Since mtDNA copy number is maintained through an intricate coordination between two opposing processes, mtDNA synthesis and mtDNA degradation, we suggest that MDS should not be viewed merely as diseases of reduced mtDNA synthesis but rather as diseases of imbalance between synthesis and degradation of mtDNA. This view allows for a new, so far unexplored treatment strategy, i.e. inhibition of mtDNA degradation. Indeed, suppressed mtDNA replication due to mutations in Pol γ (patients with Alpers-Huttenlocher syndrome), Twinkle helicase (patients with progressive external ophtalmoplegia), or due to ingestion of nucleotide reverse transcriptase inhibitors (AIDS patients) results in the establishment of a new, lower cellular mtDNA content, which is characterized by reduced rates of both mtDNA synthesis and degradation. Conversely, suppression of mtDNA degradation should lead to a new steady state with increased mtDNA content, and therefore could be therapeutic in patients with MDS.

4. Experimental approaches

4.1 Quantitative southern blotting

Southern Blot analysis can be used for the quantitation of various types of damage to mtDNA. This method is based on the detection of strand breaks within linearized mtDNA. Strand breaks can be generated either directly by noxious agents (e.g., by alkylating compounds or oxidative stress), or indirectly, after the treatment of damaged DNA with lesion-specific glycosylases, which remove damaged bases thus creating abasic sites. Examples of glycosylases widely used for this purpose include E. *coli* DNA-repair enzymes formamido-pyrimidine-DNA-glycosylase (FPG, recognizes oxidized purines) and endonuclease III (EndoIII, recognizes oxidized pyrimidines). Both enzymes are bifunctional glycosylases, i.e. they both remove damaged bases and incise the resulting abasic sites thus creating SSBs. Under alkaline conditions, the mtDNA strands separate and fragment at nicks resulting in a decreased hybridization signal from the treated (damaged) mtDNA (LeDoux et al., 1999). The membrane is exposed to an imaging screen, and the fraction of mtDNA remaining intact is calculated. This fraction can be used to calculate the lesion (break) frequency per length of intact fragment detected by hybridization using the formula:

$$BF = - \ln(\text{Treated}/\text{Control}) \quad (1)$$

In other words, mtDNA break frequency (BF) in treated samples equals the negative natural logarithm of the ratio of mtDNA band intensities in treated and control samples.

Several important caveats have to be noted in relation to this technique:

1. Prior to analysis, circular mtDNA is linearized by digestion with restriction endonuclease.
2. The technique relies on measuring mtDNA band intensities in treated vs. control samples. Therefore, loading equal amounts of total DNA per well of the gel, which depends on accurate DNA quantitation is very important. Since nDNA shows much lower sensitivity to oxidative damage than mtDNA, hybridization of the membrane to nDNA probe in addition to mtDNA probe can be used in addition to visual inspection of ethidium bromide stained gels as loading control when studying oxidative mtDNA damage. However, hybridization to a nDNA probe is not useful as a loading control when studying, certain types of alkylating DNA damage, when the difference in the damage of nuclear and mitochondrial genomes is not as dramatic.
3. Isolation of mtDNA is impractical and is associated with the introduction of artifacts. Therefore, in this technique total cellular DNA is subjected to Southern hybridization. The use of a mtDNA-specific hybridization probe allows one to study only changes in mtDNA integrity. In a typical cell type studied by this technique, mtDNA constitutes only about 1-2% of total DNA.
4. Quantitative Southern Blotting under denaturing (alkaline) conditions, by itself, does not discriminate between SSBs and DSBs. Therefore mtDNA containing DSBs, which repair inefficiently and therefore lead to mtDNA loss (Kukat et al., 2008), will appear the same as SSBs, which repair much better (Fig. 2, Mix 1 vs. Mix 2, left side). To discriminate between SSBs (repairable mtDNA damage) and DSBs (mtDNA degradation) we introduced an approach that involves running the same DNA samples under both alkaline and neutral conditions (Shokolenko et al., 2009). Samples containing DSBs appear the same under both conditions (Fig. 2, Mix 2, left side vs. right side). In contrast, mtDNA containing SSBs appears like mtDNA containing DSBs under

denaturing conditions, but under non-denaturing (neutral) conditions it behaves like undamaged control DNA (Fig. 2, Mix 1, left side vs. right side).

Specific types of DNA damage can be detected as follows:

1. DSBs convert circular mtDNA into a linear molecule. Therefore, qualitative detection of DSBs can be performed by Southern Blotting of total cellular DNA samples under non-denaturing conditions using linearised mtDNA as a standard. The increase in the signal corresponding to linear mtDNA is interpreted as a result of DSB. It is helpful to digest total DNA with a restriction enzyme that does not cut mtDNA (e.g., BglII for human DNA). In our experience, failure to perform this step results in an absence or in a severe reduction of the hybridization signal. However, the method is not quantitative for two reasons: a) DSB repair in mtDNA is inefficient, and most linear mtDNA is degraded fairly quickly (Shokolenko et al., 2009), and b) mtDNA can concatenate, at least in some cell lines (Bedoya et al., 2009), and electrophoretic mobility of linear concatemers is distinct from that of linear mtDNA monomers.
2. SSBs can be quantified as a difference in break frequencies detected using Southern Blotting under alkaline and neutral conditions (Fig. 2). Alternatively, it can be calculated as break frequency in sample ran under the alkaline conditions using the same sample ran under neutral conditions as a control.
3. Abasic sites. This type of lesion can be quantified as a difference in break frequency in two identical aliquots of the sample ran under alkaline conditions if one aliquot has been treated with methoxyamine prior to electrophoresis. Under alkaline conditions, abasic sites are converted into strand breaks through the process of beta-elimination. Modification of abasic sites with methoxyamine renders them alkali-resistant (Liuzzi & Talpaert-Borle, 1985; Scicchitano & Hanawalt, 1989). Alternatively, abasic sites can be quantified by comparing aliquots of methoxyamine-treated DNA run under the alkaline conditions after treatment with APE1 (control) and EndoIII (experimental). Methoxyamine-modified abasic sites are resistant to hydrolysis by APE1, but not by endoIII (Rosa et al., 1991)
4. Base modifications can be quantified using lesion-specific DNA glycosylases. One aliquot of DNA sample is treated with lesion-specific DNA glycosylase, whereas a second aliquot is left untreated. Monofunctional DNA glycosylases (e.g., uracil DNA glycosylase or methylpurine DNA glycosylase) convert a lesion into an abasic site, which can be converted into a strand break under the alkaline conditions thus allowing for the quantitation by comparing hybridization signals obrained from enzyme-treated vs. untreated controls. As indicated above, bifunctional DNA glycosylases, such as FPG or Endo III, will convert a lesion into a strand break allowing for quantitation using the same approach.

The advantages of Quantitative Southern Blotting include its robustness due to reliance on physical interactions rather than on enzymatic reactions and its ability to quantify some lesions (e.g., abasic sites), which can not be quantified by PCR-based techniques (see below). The disadvantages include the fact that the procedure involves multiple steps, is time-consuming, and requires relatively large quantities (1μg or more) of starting DNA.

4.2 Quantitative PCR

An alternative approach for the detection of DNA damage was developed by Govan (Govan et al., 1990) and modified by Yakes and van Houten for studies with mtDNA. This method, QPCR (a.k.a. QXL-PCR), is predicated upon the ability of the lesions present in mtDNA to block the progression of a thermostable DNA polymerase, resulting in a decrease of DNA

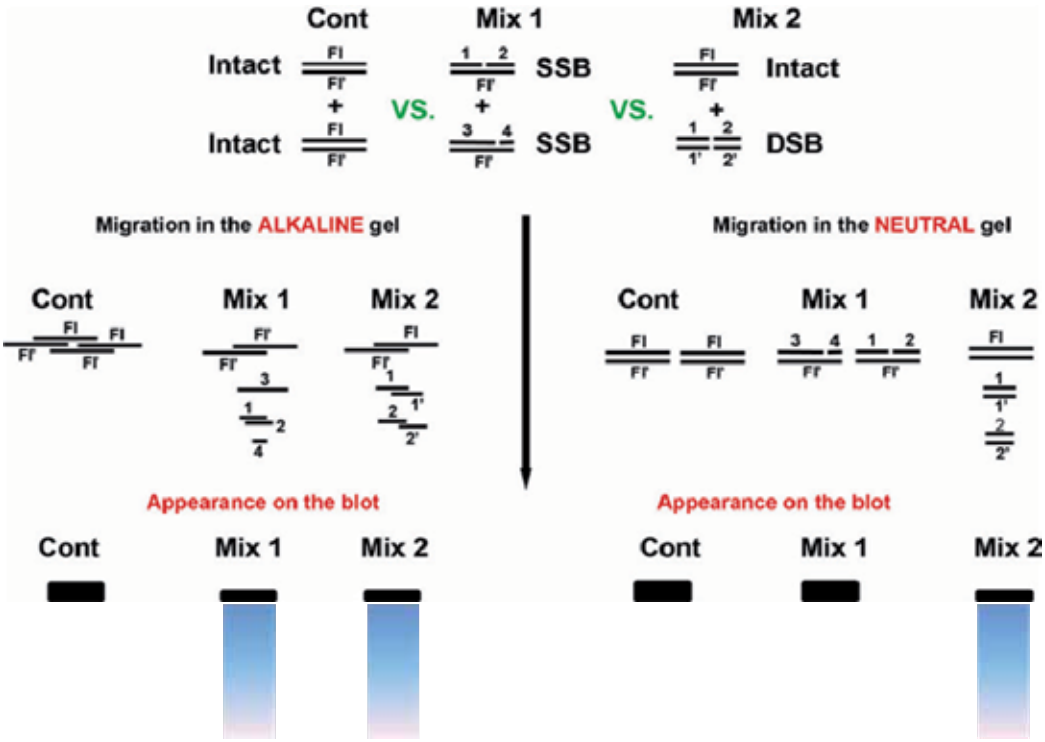

Fig. 2. Analysis of mtDNA damage by quantitative Southern Blotting under denaturing (alkaline) and non-denaturing (neutral) conditions. Behavior of the mtDNA samples that contain either no damage (Cont), SSBs (Mix 1), or a mixture of intact mtDNA and mtDNA containing DSBs (Mix 2) is presented schematically. Under the denaturing conditions (left side of the figure), mtDNA strands separate, and strands containing lesions in the form of SSBs, DSBs, or abasic sites fragment. The resulting fragments migrate faster than intact full-length (Fl) mtDNA strands in the agarose gel thus creating smears (Mix1 and Mix 2, left side). Under conditions depicted in this scheme, the intensity of the Southern Blot signal corresponding to intact mtDNA fragment from Mix 1 equals that of Mix 2, and represents half of the signal strength produced by undamaged control. When the same samples are analyzed under the non-denaturing conditions (right side of the figure), mtDNA fragmentation in Mix 1 containing SSBs does not occur. In contrast, mtDNA in Mix 2 containing DSBs fragments create a smear. As a result, the signal intensity for intact mtDNA in the Mix 1 under non-denaturing conditions is twice as high as that in the Mix 2. The arrow indicates the direction of electrophoresis; Fl', full-length mtDNA strand complementary to Fl strand; 1, 2, 3, and 4 in Mix 1, subfragments into which Fl strand containing a lesion fragments; 1, 1', 2, and 2' in Mix 2, direct and complimentary strands of the subfragments resulting from a DSB in the Fl fragment.

amplification in the damaged template, when compared to undamaged control (Yakes & Van Houten, 1997). Similar to quantitative Southern Blotting, QPCR measures the fraction of undamaged amplifiable template, which decreases with increased number of lesions. Successful outcome of experiments with either quantitative Southern Blot or QPCR is heavily dependent upon the ability to accurately measure the amount of DNA used.

Spectrophotometric methods (A_{260}) appear to be inappropriate for this purpose because of the intrinsic difficulties associated with controlling the quantity and spectrum of contaminants in DNA preparations. Fluorescense based methods (PicoGreen and Hoechst 33258 dyes), unlike spectrophotometric techniques, show little sensitivity to such contaminants as proteins, single-stranded DNA, RNA etc., which are common to genomic DNA preparations and therefore are deemed the methods of choice. Also, when using QPCR, one has to control for changes in the mtDNA copy number. Indeed, a reduction in mtDNA copy number will manifest itself as DNA damage because of the reduction in the number of amplifiable mtDNA genomes in the template. This can be controlled for by amplifying of a short (about 300bp) fragment of mtDNA-encoded gene. The rationale is that encountering DNA damage in such a short fragment is an event with a very low probability and therefore profiles of amplification of such a fragment should be essentially identical between damaged and undamaged DNA. Therefore, variations in the degree of amplification of the small fragment are assumed to be the result of fluctuations in mtDNA copy number and the results of small fragment amplification are used for the normalization of the data obtained for the large (16 kb) mtDNA fragments.

The success of the QPCR approach requires the measurements be made within the linear range of amplification. This requires optimization to find the optimal starting concentration of DNA template (Yakes & Van Houten, 1997). Alternatively, one can identify the range for linear amplification. However, both approaches require a significant amount of optimization. Recently, a real-time PCR approach has been extended to QPCR resulting in the development of the long-range PCR technique (LRPCR, (Edwards, 2008)). Two significant problems had to be addressed in the process: (1) the low processivity and polymerization rates of the DNA polymerases used in comparison to the length of the amplicons, (2) SYBR green inhibition of DNA amplification (Gudnason et al., 2007). In comparison to the earlier semi-quantitative protocols this represents a significant improvement in both the ease of data acquisition and the precision for quantification of mtDNA damage (Edwards, 2008). The most recent variation of the technique, the semi-long run real-time (SLR rt-) PCR method, further simplifies the procedure by amplifying relatively short mtDNA fragments using real-time PCR (qPCR) reagents and instruments (Rothfuss et al., 2010). In this procedure, the reduced length of amplified products enables the use of standard qPCR kits. The flip side of this improvement is the reduced sensitivity of the technique, which is directly related to the length of amplified fragments. Therefore, applicability of this technique for reliable detection of physiological (low) levels of mtDNA damage requires independent validation and is likely to strongly depend upon the instrument used. Indeed, a simple calculation shows that a fairly high level of mtDNA damage of 1 lesion/mtDNA molecule (16.5 kbp) translates into 0.061 lesion per 1 kbp fragment amplified in this method. Using "zero class" Poisson distribution used for the analysis of this type of DNA damage

$$D=-\ln(A_D/A_C) \quad (2)$$

where D= lesion frequency per length of amplified fragment (1kbp), ln is natural logarithm, A_D is amplification of the damaged DNA sample, and A_C is amplification of the control sample) we arrive at the A_D/A_C =0.94. The corresponding shift in the threshold cycle (ΔC_t, derived from the readout of the qPCR instrument) is 0.089. Therefore, a significant mtDNA damage of 1 lesion per mtDNA molecule results in less than a 0.1 threshold cycle shift between amplification curves of treated and untreated samples. This places a very high demand on the

instrument's ability to reproducibly amplify different samples. In our experience, a PCR block that allows for greater than 0.7 C_t spread between identical samples still conforms to the standards of the two major manufacturers of qPCR instruments. In this case, the instrument's well-to-well variability exceeds the measured differences by a factor of 7.

The strength of PCR-based techniques for the analysis of mtDNA damage is in the ability to work with very low starting quantities of DNA. This strength is turned into a weakness when relevant methodological precautions, such as the availability of distinct, dedicated workstations, for different steps of the procedure in physically separate laboratories (Santos et al., 2006) are considered. Another weakness of this approach is that it provides even less information about the nature of DNA damage than Quantitative Southern Blotting. E.g., abasic sites can be quantitated by Quantitative Southern Blotting under alkaline conditions by comparing lesion frequencies in DNA modified with methoxyamine vs. unmodified DNA. Methoxyamine modification protects abasic sites from being converted into strand breaks through beta-elimination under alkaline conditions. In contrast, native abasic sites, methoxyamine-modified abasic sites, and abasic sites converted into strand breaks through beta-elimination all will prevent copying by the DNA-polymerase in PCR-based techniques and therefore will be indistinguishable. Nevertheless, these techniques are the only ones available for analysis of mtDNA damage and repair when amount of the starting material is limited.

5. Conclusion

mtDNA integrity and appropriate copy number appear to be crucial for normal functioning of the cell. Therefore, understanding the processes that govern mtDNA replication, repair and degradation is of critical importance for our ability to prevent and/or clinically intervene in pathological processes associated with mutations in mtDNA and mtDNA depletion. Degradation of mtDNA is now emerging as a promising therapeutic target in the treatment of congenital mtDNA depletion syndromes and mtDNA depletion induced by antiretroviral therapy. However, the molecular identity of the nuclease involved in mtDNA degradation remains enigmatic. Future research will shed light on this and other remaining mysteries of mtDNA biology.

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7. References

Akbari, M., T. Visnes, et al. (2008). Mitochondrial base excision repair of uracil and AP sites takes place by single-nucleotide insertion and long-patch DNA synthesis. *DNA Repair (Amst),* 7, 4, pp. 605-616, 1568-7864 (Print).

Alam, T. I., T. Kanki, et al. (2003). Human mitochondrial DNA is packaged with TFAM. *Nucleic Acids Res,* 31, 6, pp. 1640-1645,

Alexeyev, M. F., N. Venediktova, et al. (2008). Selective elimination of mutant mitochondrial genomes as therapeutic strategy for the treatment of NARP and MILS syndromes. *Gene Ther,* 15, 7, pp. 516-523, 1476-5462 (Electronic).

Anderson, C. T. and E. C. Friedberg (1980). The presence of nuclear and mitochondrial uracil-DNA glycosylase in extracts of human KB cells. *Nucleic Acids Res,* 8, 4, pp. 875-888, 0305-1048 (Print).

Anson, R. M., E. Hudson, et al. (2000). Mitochondrial endogenous oxidative damage has been overestimated. *Faseb J,* 14, 2, pp. 355-360,

Anson, R. M., S. Senturker, et al. (1999). Measurement of oxidatively induced base lesions in liver from Wistar rats of different ages. *Free Radic Biol Med,* 27, 3-4, pp. 456-462, 0891-5849 (Print).

Arnaudo, E., M. Dalakas, et al. (1991). Depletion of muscle mitochondrial DNA in AIDS patients with zidovudine-induced myopathy. *Lancet,* 337, 8740, pp. 508-510, 0140-6736 (Print) 0140-6736 (Linking).

Ballard, J. W. and M. C. Whitlock (2004). The incomplete natural history of mitochondria. *Mol Ecol,* 13, 4, pp. 729-744, 0962-1083 (Print).

Bedoya, F., M. M. Medveczky, et al. (2009). Identification of mitochondrial genome concatemers in AIDS-associated lymphomas and lymphoid cell lines. *Leuk Res*, 1873-5835 (Electronic).

Bendich, A. J. (2010). Mitochondrial DNA, chloroplast DNA and the origins of development in eukaryotic organisms. *Biol Direct,* 5, pp. 42, 1745-6150 (Electronic) 1745-6150 (Linking).

Bogenhagen, D. and D. A. Clayton (1977). Mouse L cell mitochondrial DNA molecules are selected randomly for replication throughout the cell cycle. *Cell,* 11, 4, pp. 719-727, 0092-8674 (Print) 0092-8674 (Linking).

Bogenhagen, D. F., D. Rousseau, et al. (2008). The layered structure of human mitochondrial DNA nucleoids. *J Biol Chem,* 283, 6, pp. 3665-3675, 0021-9258 (Print).

Brown, T. A., A. N. Tkachuk, et al. (2008). Native R-loops persist throughout the mouse mitochondrial DNA genome. *J Biol Chem*, 0021-9258 (Print).

Brown, W. M., M. George, Jr., et al. (1979). Rapid evolution of animal mitochondrial DNA. *Proc Natl Acad Sci U S A,* 76, 4, pp. 1967-1971, 0027-8424 (Print).

Cavelier, L., A. Johannisson, et al. (2000). Analysis of mtDNA copy number and composition of single mitochondrial particles using flow cytometry and PCR. *Exp Cell Res,* 259, 1, pp. 79-85,

Chen, H., C. J. Hu, et al. (2001). Reduction and restoration of mitochondrial dna content after focal cerebral ischemia/reperfusion. *Stroke,* 32, 10, pp. 2382-2387, 1524-4628 (Electronic) 0039-2499 (Linking).

Clay Montier, L. L., J. J. Deng, et al. (2009). Number matters: control of mammalian mitochondrial DNA copy number. *J Genet Genomics,* 36, 3, pp. 125-131, 1673-8527 (Print) 1673-8527 (Linking).

Clayton, D. A. (1982). Replication of animal mitochondrial DNA. *Cell,* 28, 4, pp. 693-705, 0092-8674 (Print) 0092-8674 (Linking).

Clayton, D. A., J. N. Doda, et al. (1974). The absence of a pyrimidine dimer repair mechanism in mammalian mitochondria. *Proc Natl Acad Sci U S A,* 71, 7, pp. 2777-2781,

Clayton, D. A., J. N. Doda, et al. (1975). Absence of a pyrimidine dimer repair mechanism for mitochondrial DNA in mouse and human cells. *Basic Life Sci,* 5B, pp. 589-591,

Coffey, G., U. Lakshmipathy, et al. (1999). Mammalian mitochondrial extracts possess DNA end-binding activity. *Nucleic Acids Res,* 27, 16, pp. 3348-3354, 1362-4962 (Electronic).

Copeland, W. C. (2008). Inherited mitochondrial diseases of DNA replication. *Annu Rev Med,* 59, pp. 131-146, 0066-4219 (Print) 0066-4219 (Linking).

Crawford, D. R., N. E. Abramova, et al. (1998). Oxidative stress causes a general, calcium-dependent degradation of mitochondrial polynucleotides. *Free Radic Biol Med,* 25, 9, pp. 1106-1111, 0891-5849 (Print).

de Souza-Pinto, N. C., P. A. Mason, et al. (2009). Novel DNA mismatch-repair activity involving YB-1 in human mitochondria. *DNA Repair (Amst),* 8, 6, pp. 704-719, 1568-7864 (Print).

Driggers, W. J., S. P. LeDoux, et al. (1993). Repair of oxidative damage within the mitochondrial DNA of RINr 38 cells. *J Biol Chem,* 268, 29, pp. 22042-22045,

Edwards, J. G. (2008). Quantification of mitochondrial DNA (mtDNA) damage and error rates by real-time QPCR. *Mitochondrion*, 1567-7249 (Print).

Fukui, H. and C. T. Moraes (2008). Mechanisms of formation and accumulation of mitochondrial DNA deletions in aging neurons. *Hum Mol Genet*, 1460-2083 (Electronic).

Govan, H. L., 3rd, Y. Valles-Ayoub, et al. (1990). Fine-mapping of DNA damage and repair in specific genomic segments. *Nucleic Acids Res,* 18, 13, pp. 3823-3830, 0305-1048 (Print) 0305-1048 (Linking).

Gross, N. J. and M. Rabinowitz (1969). Synthesis of new strands of mitochondrial and nuclear deoxyribonucleic acid by semiconservative replication. *J Biol Chem,* 244, 6, pp. 1563-1566,

Gudnason, H., M. Dufva, et al. (2007). Comparison of multiple DNA dyes for real-time PCR: effects of dye concentration and sequence composition on DNA amplification and melting temperature. *Nucleic Acids Res,* 35, 19, pp. e127, 1362-4962 (Electronic) 0305-1048 (Linking).

Guliaeva, N. A., E. A. Kuznetsova, et al. (2006). [Proteins associated with mitochondrial DNA protect it against the action of X-rays and hydrogen peroxide]. *Biofizika,* 51, 4, pp. 692-697,

Holt, I. J. (2009). Mitochondrial DNA replication and repair: all a flap. *Trends Biochem Sci,* 34, 7, pp. 358-365, 0968-0004 (Print).

Holt, I. J., A. E. Harding, et al. (1988). Deletions of muscle mitochondrial DNA in patients with mitochondrial myopathies. *Nature,* 331, 6158, pp. 717-719,

Holt, I. J., H. E. Lorimer, et al. (2000). Coupled leading- and lagging-strand synthesis of mammalian mitochondrial DNA. *Cell,* 100, 5, pp. 515-524, 0092-8674 (Print) 0092-8674 (Linking).

Ibeas, J. I. and J. Jimenez (1997). Mitochondrial DNA loss caused by ethanol in Saccharomyces flor yeasts. *Appl Environ Microbiol,* 63, 1, pp. 7-12, 0099-2240 (Print).

Iborra, F. J., H. Kimura, et al. (2004). The functional organization of mitochondrial genomes in human cells. *BMC Biol,* 2, 1, pp. 9,

Kajander, O. A., P. J. Karhunen, et al. (2001). Prominent mitochondrial DNA recombination intermediates in human heart muscle. *EMBO Rep,* 2, 11, pp. 1007-1012, 1469-221X (Print).

King, M. P. and G. Attardi (1989). Human cells lacking mtDNA: repopulation with exogenous mitochondria by complementation. *Science,* 246, 4929, pp. 500-503, 0036-8075 (Print).

Kraytsberg, Y., M. Schwartz, et al. (2004). Recombination of human mitochondrial DNA. *Science,* 304, 5673, pp. 981,

Kukat, A., C. Kukat, et al. (2008). Generation of {rho}0 cells utilizing a mitochondrially targeted restriction endonuclease and comparative analyses. *Nucleic Acids Res*, 1362-4962 (Electronic).

Lakshmipathy, U. and C. Campbell (1999). Double strand break rejoining by mammalian mitochondrial extracts. *Nucleic Acids Res,* 27, 4, pp. 1198-1204, 0305-1048 (Print).

LeDoux, S. P., W. J. Driggers, et al. (1999). Repair of alkylation and oxidative damage in mitochondrial DNA. *Mutat Res,* 434, 3, pp. 149-159,

LeDoux, S. P., G. L. Wilson, et al. (1992). Repair of mitochondrial DNA after various types of DNA damage in Chinese hamster ovary cells. *Carcinogenesis,* 13, 11, pp. 1967-1973, 0143-3334 (Print).

Lestienne, P. and G. Ponsot (1988). Kearns-Sayre syndrome with muscle mitochondrial DNA deletion. *Lancet,* 1, 8590, pp. 885,

Liang, Q. and P. C. Dedon (2001). Cu(II)/H2O2-induced DNA damage is enhanced by packaging of DNA as a nucleosome. *Chem Res Toxicol,* 14, 4, pp. 416-422,

Liang, R., S. Senturker, et al. (1999). Effects of Ni(II) and Cu(II) on DNA interaction with the N-terminal sequence of human protamine P2: enhancement of binding and mediation of oxidative DNA strand scission and base damage. *Carcinogenesis,* 20, 5, pp. 893-898,

Lim, K. S., K. Jeyaseelan, et al. (2005). Oxidative damage in mitochondrial DNA is not extensive. *Ann N Y Acad Sci,* 1042, pp. 210-220,

Liu, P. and B. Demple (2010). DNA repair in mammalian mitochondria: Much more than we thought? *Environ Mol Mutagen,* 51, 5, pp. 417-426, 1098-2280 (Electronic) 0893-6692 (Linking).

Liu, P., L. Qian, et al. (2008). Removal of Oxidative DNA Damage via FEN1-Dependent Long-Patch Base Excision Repair in Human Cell Mitochondria. *Mol Cell Biol*, 1098-5549 (Electronic).

Liuzzi, M. and M. Talpaert-Borle (1985). A new approach to the study of the base-excision repair pathway using methoxyamine. *J Biol Chem,* 260, 9, pp. 5252-5258, 0021-9258 (Print).

Mansouri, A., C. Demeilliers, et al. (2001). Acute ethanol administration oxidatively damages and depletes mitochondrial dna in mouse liver, brain, heart, and skeletal muscles: protective effects of antioxidants. *J Pharmacol Exp Ther,* 298, 2, pp. 737-743, 0022-3565 (Print).

Mansouri, A., I. Gaou, et al. (1999). An alcoholic binge causes massive degradation of hepatic mitochondrial DNA in mice. *Gastroenterology,* 117, 1, pp. 181-190, 0016-5085 (Print).

Mita, S., R. J. Monnat, Jr., et al. (1988). Resistance of HeLa cell mitochondrial DNA to mutagenesis by chemical carcinogens. *Cancer Res,* 48, 16, pp. 4578-4583,

Mitsuishi, M., K. Miyashita, et al. (2008). Angiotensin II Reduces Mitochondrial Content in Skeletal Muscle and Affects Glycemic Control. *Diabetes*, 1939-327X (Electronic).

Miyaki, M., K. Yatagai, et al. (1977). Strand breaks of mammalian mitochondrial DNA induced by carcinogens. *Chem Biol Interact,* 17, 3, pp. 321-329,

Muller, F. L., Y. Liu, et al. (2004). Complex III releases superoxide to both sides of the inner mitochondrial membrane. *J Biol Chem,* 279, 47, pp. 49064-49073,

Myers, K. A., R. Saffhill, et al. (1988). Repair of alkylated purines in the hepatic DNA of mitochondria and nuclei in the rat. *Carcinogenesis,* 9, 2, pp. 285-292,

Noll, T., P. Wissemann, et al. (1990). Hypoxia tolerance of coronary endothelial cells. *Adv Exp Med Biol,* 277, pp. 467-476, 0065-2598 (Print) 0065-2598 (Linking).

Ojala, D., J. Montoya, et al. (1981). tRNA punctuation model of RNA processing in human mitochondria. *Nature,* 290, 5806, pp. 470-474,

Pettepher, C. C., S. P. LeDoux, et al. (1991). Repair of alkali-labile sites within the mitochondrial DNA of RINr 38 cells after exposure to the nitrosourea streptozotocin. *J Biol Chem,* 266, 5, pp. 3113-3117,

Poulton, J. and I. J. Holt (2009). 163rd ENMC International Workshop: nucleoid and nucleotide biology in syndromes of mitochondrial DNA depletion myopathy 12-14 December 2008, Naarden, The Netherlands. *Neuromuscul Disord,* 19, 6, pp. 439-443, 1873-2364 (Electronic) 0960-8966 (Linking).

Rahman, S. and J. Poulton (2009). Diagnosis of mitochondrial DNA depletion syndromes. *Arch Dis Child,* 94, 1, pp. 3-5, 1468-2044 (Electronic) 0003-9888 (Linking).

Rebelo, A. P., S. L. Williams, et al. (2009). In vivo methylation of mtDNA reveals the dynamics of protein-mtDNA interactions. *Nucleic Acids Res*, 1362-4962 (Electronic).

Richter, C., J. W. Park, et al. (1988). Normal oxidative damage to mitochondrial and nuclear DNA is extensive. *Proc Natl Acad Sci U S A,* 85, 17, pp. 6465-6467,

Robberson, D. L., H. Kasamatsu, et al. (1972). Replication of mitochondrial DNA. Circular replicative intermediates in mouse L cells. *Proc Natl Acad Sci U S A,* 69, 3, pp. 737-741, 0027-8424 (Print) 0027-8424 (Linking).

Rosa, S., P. Fortini, et al. (1991). Processing in vitro of an abasic site reacted with methoxyamine: a new assay for the detection of abasic sites formed in vivo. *Nucleic Acids Res,* 19, 20, pp. 5569-5574, 0305-1048 (Print).

Rothfuss, O., T. Gasser, et al. (2010). Analysis of differential DNA damage in the mitochondrial genome employing a semi-long run real-time PCR approach. *Nucleic Acids Res,* 38, 4, pp. e24, 1362-4962 (Electronic) 0305-1048 (Linking).

Rotig, A. and J. Poulton (2009). Genetic causes of mitochondrial DNA depletion in humans. *Biochim Biophys Acta,* 1792, 12, pp. 1103-1108, 0006-3002 (Print) 0006-3002 (Linking).

Santos, J. H., J. N. Meyer, et al. (2006). Quantitative PCR-based measurement of nuclear and mitochondrial DNA damage and repair in mammalian cells. *Methods Mol Biol,* 314, pp. 183-199, 1064-3745 (Print) 1064-3745 (Linking).

Satoh, M. S., N. Huh, et al. (1988). Enzymatic removal of O6-ethylguanine from mitochondrial DNA in rat tissues exposed to N-ethyl-N-nitrosourea in vivo. *J Biol Chem,* 263, 14, pp. 6854-6856,

Scicchitano, D. A. and P. C. Hanawalt (1989). Repair of N-methylpurines in specific DNA sequences in Chinese hamster ovary cells: absence of strand specificity in the

dihydrofolate reductase gene. *Proc Natl Acad Sci U S A,* 86, 9, pp. 3050-3054, 0027-8424 (Print).

Shokolenko, I., N. Venediktova, et al. (2009). Oxidative stress induces degradation of mitochondrial DNA. *Nucleic Acids Res,* 37, 8, pp. 2539-2548, 1362-4962 (Electronic).

Srivastava, S. and C. T. Moraes (2005). Double-strand breaks of mouse muscle mtDNA promote large deletions similar to multiple mtDNA deletions in humans. *Hum Mol Genet,* 14, 7, pp. 893-902, 0964-6906 (Print).

St-Pierre, J., J. A. Buckingham, et al. (2002). Topology of superoxide production from different sites in the mitochondrial electron transport chain. *J Biol Chem,* 277, 47, pp. 44784-44790,

St John, J. C., J. Facucho-Oliveira, et al. (2010). Mitochondrial DNA transmission, replication and inheritance: a journey from the gamete through the embryo and into offspring and embryonic stem cells. *Hum Reprod Update,* 16, 5, pp. 488-509, 1460-2369 (Electronic) 1355-4786 (Linking).

Suliman, H. B., M. S. Carraway, et al. (2003). Postlipopolysaccharide oxidative damage of mitochondrial DNA. *Am J Respir Crit Care Med,* 167, 4, pp. 570-579, 1073-449X (Print).

Suter, M. and C. Richter (1999). Fragmented mitochondrial DNA is the predominant carrier of oxidized DNA bases. *Biochemistry,* 38, 1, pp. 459-464,

Szczesny, B., A. W. Tann, et al. (2008). Long patch base excision repair in mammalian mitochondrial genomes. *J Biol Chem*, 0021-9258 (Print).

Tatarenkov, A. and J. C. Avise (2007). Rapid concerted evolution in animal mitochondrial DNA. *Proc Biol Sci,* 274, 1619, pp. 1795-1798, 0962-8452 (Print).

Thorslund, T., M. Sunesen, et al. (2002). Repair of 8-oxoG is slower in endogenous nuclear genes than in mitochondrial DNA and is without strand bias. *DNA Repair (Amst),* 1, 4, pp. 261-273,

Tomkinson, A. E., R. T. Bonk, et al. (1990). Mammalian mitochondrial endonuclease activities specific for ultraviolet-irradiated DNA. *Nucleic Acids Res,* 18, 4, pp. 929-935, 0305-1048 (Print).

Tomkinson, A. E., R. T. Bonk, et al. (1988). Mitochondrial endonuclease activities specific for apurinic/apyrimidinic sites in DNA from mouse cells. *J Biol Chem,* 263, 25, pp. 12532-12537, 0021-9258 (Print).

Wallace, D. C. (2005). A mitochondrial paradigm of metabolic and degenerative diseases, aging, and cancer: a dawn for evolutionary medicine. *Annu Rev Genet,* 39, pp. 359-407,

Wallace, D. C., G. Singh, et al. (1988). Mitochondrial DNA mutation associated with Leber's hereditary optic neuropathy. *Science,* 242, 4884, pp. 1427-1430,

Yakes, F. M. and B. Van Houten (1997). Mitochondrial DNA damage is more extensive and persists longer than nuclear DNA damage in human cells following oxidative stress. *Proc Natl Acad Sci U S A,* 94, 2, pp. 514-519,

Yasukawa, T., A. Reyes, et al. (2006). Replication of vertebrate mitochondrial DNA entails transient ribonucleotide incorporation throughout the lagging strand. *Embo J,* 25, 22, pp. 5358-5371, 0261-4189 (Print) 0261-4189 (Linking).

Yasukawa, T., M. Y. Yang, et al. (2005). A bidirectional origin of replication maps to the major noncoding region of human mitochondrial DNA. *Mol Cell,* 18, 6, pp. 651-662, 1097-2765 (Print) 1097-2765 (Linking).

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DNA Repair in Embryonic Stem Cells

Volker Middel and Christine Blattner
Karlsruhe Institute of Technology, Institute of Toxicology and Genetics
Germany

1. Introduction

DNA is the largest and most important molecule of a cell. Due to its chemical nature, DNA is particularly prone to numerous lesions. These lesions comprise different sugar and base modifications, deletion of bases as well as single (SSBs) and double DNA strand breaks (DSBs). All alterations in the DNA that a cell experiences sum up to more than 10.000 lesions per day (Lindahl, 1993). DNA damage can be brought about by several endogenous and exogenous factors including reactive oxygen species (ROS), ultraviolet light (UV), ionizing radiation (IR) or by DNA damaging chemicals. The different lesions are removed by several repair mechanisms that help the cell to preserve structure and sequence of the DNA. These repair pathways include mismatch repair (MMR), base excision repair (BER), nucleotide excision repair (NER), non-homologous end joining (NHEJ), microhomology-mediated end joining (MMEJ) and homologous recombination (HR). If DNA damage is too severe, cells can also initiate a cell death program that removes cells with damaged DNA from the population (reviewed in: Roos & Kaina, 2006).

Stem cells comprise a group of self-renewing cells including embryonic and adult stem cells. Embryonic stem cells (ESCs) have the ability of indefinite self-renewal and rapid proliferation. They are pluripotent and can differentiate into cells of all three germ layers (ectoderm, mesoderm and endoderm) as well as of the germ cell lineage. Adult or tissue stem cells, in contrast, are multipotent and differentiate into only one or several specific cell lines (reviewed in: Barilleaux et al, 2006; Thomson & Marshall, 1998; Weissman et al, 2001). By proliferation and differentiation, adult stem cells replenish tissue cells that are lost during normal wear and tear or after injury and thus are key for tissue and organ regeneration as well as for the preservation of homeostasis in a living organism (Reya et al, 2001). ESCs can be isolated from different species, but the most investigated ones are human (hESCs) and murine (mESCs) ESCs. Both, hESCs and mESCs are derived from the inner cell mass of a blastocyst, an early stage of embryonic development (Martin, 1981; Thomson et al, 1998). Human ESCs are of particular interest because of their high potential for medical applications like tissue and organ regeneration (reviewed in: Donovan & Gearhart, 2001; Thomson & Odorico, 2000; Wobus, 2001). However, since hESCs are derived from human embryos, they also raise ethical, social and juristic problems (McLaren, 2000). A potential alternative to the use of hESCs is the employment of induced pluripotent stem (iPS) cells. By transfection of a combination of stem cell markers, iPS cells can be made from basically every differentiated cell (Kim et al, 2009; Okita et al, 2007; Takahashi et al, 2007; Takahashi & Yamanaka, 2006; Wernig et al, 2007; Yu et al, 2007). Thus, differentiated cells can be taken from a patient, modified and induced for pluripotency *in vitro,* and transplanted back into

the same patient. Therefore, iPS cells are not only a great tool for research, they also allow an individualized therapy. Moreover, since donor and acceptor of the cells are the same individual, eventual problems with intolerance or rejection of the cells are prevented. Because of the low rate of reprogramming efficiency and the fact that several of the reprogramming factors are oncogenes, which bears the risk of a cancerous behavior of re-implanted iPS cells, this approach is, though, not as yet used extensively in therapy (Okita et al, 2007; Takahashi et al, 2007; Takahashi & Yamanaka, 2006).

The multitude of lesions that arise per cell and day illustrates that maintaining genomic integrity is an important issue for a cell. This applies even more for stem cells, which comprise the basal set of proliferating cells in an organism. Stem cells thus should have an even higher interest in preserving their genomic integrity than somatic cells. In line with this notion, stem cells have been reported to have to a higher rate of DNA damage-induced apoptosis and a lower frequency of mitotic recombination and mutations (Cervantes et al, 2002; de Waard et al, 2008; Momcilovic et al, 2010; Roos et al, 2007; Saretzki et al, 2004). These differences in the DNA damage response are due to variations in several parameters between stem cells and differentiated cells. One of the most intriguing dissimilarity between ESCs and differentiated cells is the short G1-phase and the absence of a G1 checkpoint in ESCs (Aladjem et al, 1998; Fluckiger et al, 2006; Hong & Stambrook, 2004). In addition, ESCs and differentiated cells vary in the expression of several repair genes (Maynard et al, 2008; Momcilovic et al, 2010; Tichy et al, 2011).

In this chapter we will review the current knowledge about DNA repair of ESCs, highlight differences to DNA repair of differentiated cells and discuss differentiation as an option of ESCs to respond to DNA lesions. Where necessary, we will discriminate between human and murine ESCs.

2. DNA damage recognition

Before damaged DNA can be repaired, the lesion must be detected. Therefore, specific "sensor" proteins are present in a cell, which scan across the DNA. When these sensor proteins find sugar or base modifications, SSBs or DSBs, they become trapped at the lesion and activated. These events mobilize further repair factors and initiate a signaling cascade that transduces the information about the lesion across the cell body. Well known "sensors" of lesions in the DNA are kinases of the PI3K-related kinase (PIKK) family including ataxia-telangiectasia mutated (ATM), ATM- and Rad3-related (ATR) and DNA-dependent protein kinase (DNA-PK; reviewed in: Oberle & Blattner, 2010). ATM, ATR and DNA-PK are specifically activated in response to double strand breaks. Once activated, these kinases initiate DNA repair and checkpoint signaling (Boehme et al., 2008; Bozulic et al., 2008; Dobbs et al., 2010; Motoyama & Naka, 2004; Shiloh, 2003).

One of the first events of differentiated cells in response to a double strand break is autophosphorylation of ATM at serine 1981 and phosphorylation of the histone variant protein H2AX at serine 139, thus generating γ-H2AX (Rogakou et al, 1999). Phosphorylation of H2AX then allows the formation of sub-nuclear structures that can be distinguished as "foci" by immunofluorescence microscopy (Rogaku et al., 1999; Burma et al, 2001; Ward & Chen, 2001). All three PIKKs, ATM, ATR and DNA-PK, phosphorylate H2AX when they are activated (Hammond et al, 2003; Stiff et al, 2004).

ESCs undergo similar events in response to DSBs as differentiated cells. In ESCs, ATM also becomes phosphorylated at serine 1981, relocates to DSBs and phosphorylates the histone

variant protein H2AX within a few minutes post irradiation (Momcilovic et al, 2009). Notably, mESCs display an elevated basal level of γ-H2AX, even in the absence of DSBs (Banath et al, 2009; Chuykin et al, 2008). Nevertheless, when H2AX phosphorylation is followed over time, mESCs and differentiated cells show a similar behavior with respect to foci formation and dissolution (Adams et al, 2010a; Chuykin et al, 2008). The reason for the high basal level of phosphorylated H2AX in mESCs is as yet unclear. A possible explanation is an eventually higher number of double strand breaks in mESCs, even in the absence of exogenous insults, which could result from global chromatin decondensation. Since most ESCs are in S-phase and replicating their DNA, the chromatin is usually kept in a more open structure and may therefore be more accessible for DSBs. Alternatively, the higher number of DSBs could result from an increased number of collapsed replication forks. It is known for several years that DSBs can derive from incomplete disaggregation of replication forks (Strumberg et al, 2000). Since one of the hallmarks of ESCs is their rapid proliferation, ESCs are almost constantly replicating their DNA, which enhances the probability of acquiring DSBs from collapsed replication forks during a given time. Another possibility for the high basal level of γ-H2AX foci is the elevated expression of ATM and ATR in ESCs, which could result in a higher phosphorylation activity even under normal growth conditions (Momcilovic et al, 2010). Besides H2AX, ATM phosphorylates CHK-2 at threonine 68, NBS-1 at serine 343, and TP53 at serine 15 in ESCs (Momcilovic et al, 2010).

In differentiated cells, ATM is mostly involved in the recognition of double strand breaks in response to ionizing irradiation and during the immune response, while ATR becomes activated by double strand breaks that are generated during replication (reviewed in: Cimprich & Cortez, 2008). Despite having similar targets in ESCs and differentiated cells, the division of labor between ATM and ATR appears to be somewhat different in these cell types. While ATM is the first choice for phosphorylation of H2AX after ionizing irradiation in differentiated cells and is absolutely required for the maintenance of genomic stability (Shiloh, 2003), the number of γ-H2AX foci after ionizing radiation was reduced to only thirty percent of wild type ESCs when ATR was genetically deleted albeit abundance of ATM was not altered under these conditions (Adams et al, 2010a). Similarly, inhibition of ATM by KU-55933 reduced NHEJ in hESCs by only twenty-five percent, whereas NHEJ rates were diminished under the same conditions by about fifty percent in neural precursors and by about seventy-five percent in astrocytes (Adams et al, 2010a). Genetic deletion of ATM or inhibition of the kinase yet impaired phosphorylation of ATM at serine 1981, of Tp53 at serine 15, of CHK-2 at threonine 68 and of NBS-1 at serine 343 as well as the implementation of a G2 arrest (Momcilovic et al, 2009).

Other "sensor" proteins recognize different kinds of DNA lesions. The XPA and XPC proteins, for example, recognize photoproducts and bulky adducts in the DNA that are generated upon exposure to UVB- and UVC-light or after exposure to certain chemicals. Glycosylases specifically recognize damaged bases, and heterodimers of MSH2/MSH6 and MSH2/MSH3 identify mismatches and short deletions in the DNA (Beard & Wilson, 2006; Iyer et al, 2006; Jiricny, 2006; Jones & Wood, 1993; Sugasawa et al, 2002). In accordance with the superior role of DNA repair in stem cells, the abundance of the mismatch sensors MLH2, MLH3 and MLH6 is significantly increased in ESCs. The increase in the amount of these repair proteins is at least partly due to enhanced transcription, although other mechanisms e.g. enhanced translation or protein stability may also contribute to the effect (Momcilovic et al, 2010; Osman et al, 2010; Roos et al, 2007; Tichy et al, 2011).

Another enzyme that acts as a DNA damage sensor and that functions in checkpoint signaling and in the preservation of genomic integrity is the poly(ADP-ribose) polymerase 1 (PARP-1), a protein that adds ADP-ribosyl moieties to substrate proteins including histone H1, DNA topoisomerase, DNA-PK and to PARP-1 itself (reviewed in: D'Amours et al, 1999; Shall & de Murcia, 2000). PARP-1 expression is also clearly elevated in mESCs (Tichy et al, 2010; V. Middel, unpublished data).

3. Base excision repair

Base excision repair (BER; Fig. 1) repairs modified bases, e.g. after oxidation or alkylation as well as abasic sites after spontaneous loss of bases (reviewed in: Fleck & Nielson, 2004; Zharkov, 2008). In principal, BER can be divided into two sub-mechanisms: short patch BER and long patch BER. Whereas short patch BER excises and replaces single nucleotides, long patch BER removes a stretch of several nucleotides (reviewed in: Robertson et al, 2009). Both sub-pathways start with the recognition of a damaged base by a DNA glycosylase and its excision, which leaves the DNA with an apurinic or apyrimidinic (AP) site. Proteins like apurinic/apyrimidinic endonuclease 1/redox effector factor 1 (APE-1/REF-1) then hydrolyze the 5'phosphodiesterbond in the sugar-phosphate backbone at the AP-site followed by insertion of a new base into the gap by DNA polymerase β and sealing of the break by DNA ligase III (reviewed in: Fleck & Nielson, 2004; Zharkov, 2008). Cells with lower APE-1/REF-1 activity are hypersensitive to several DNA damaging agents, including methylmethane sulfonate (MMS), hydrogen peroxide (H_2O_2), temozolomide (TMZ), ionizing radiation (IR) and cisplatin (Bapat et al, 2009; Fishel & Kelley, 2007; Ono et al, 1994). Since ESCs proliferate fast, they should produce a higher amount of ROS than differentiated cells, which should result in increased oxidation of bases and an increased amount of 8-oxoG, a damaged base that is normally removed from the DNA by the N-glycosylase/DNA lyase OGG1 (reviewed in: Boiteux & Radicella, 1999). In contrast to this expectation, very little 8-oxoG has been measured in undamaged hESCs (Maynard et al, 2008). The most simple explanation for this low amount of 8-oxoG would be a more effective removal of 8-oxoG in ESCs. However, neither elevated OGG1 protein levels nor enhanced enzyme activity have been observed in ESCs (Maynard et al, 2008). Another principle that could be used by hESCs to efficiently remove 8-oxoG would be recognition of 8-oxoG and initiation of its removal by other glycosylases. In consistency with this notion, other proteins of BER including Flap-endonuclease 1 (FEN-1), APE-1, X-ray repair complementing defective repair in chinese hamster cells 1 (XRCC-1), DNA ligase III, PARP-1 and Uracil-DNA-glycosylase 2 (UNG-2) are expressed in higher amounts in ESCs than in differentiated cells, while DNA-3-methyladenine glycosylase (MPG) is weaker expressed in hESCs than in differentiated cells (Fig.1; Table 1; Maynard et al, 2008; Momcilovic et al, 2010; Tichy et al, 2011, V. Middel, unpublished data). Surprisingly, despite the increase in several BER proteins and a strongly enhanced incision activity, overall BER activity is only moderately elevated in ESCs (Tichy et al, 2011).

4. Nucleotide excision repair

The nucleotide excision repair (NER) pathway preferentially removes bulky adducts and photoproducts from the DNA (Fig. 2). NER can be divided into three sub mechanisms which are transcription-coupled NER (TCR), global-genome NER (GGR) and transcription domain-associated NER (DAR) (reviewed in: Nouspikel, 2009). Defects in genes associated

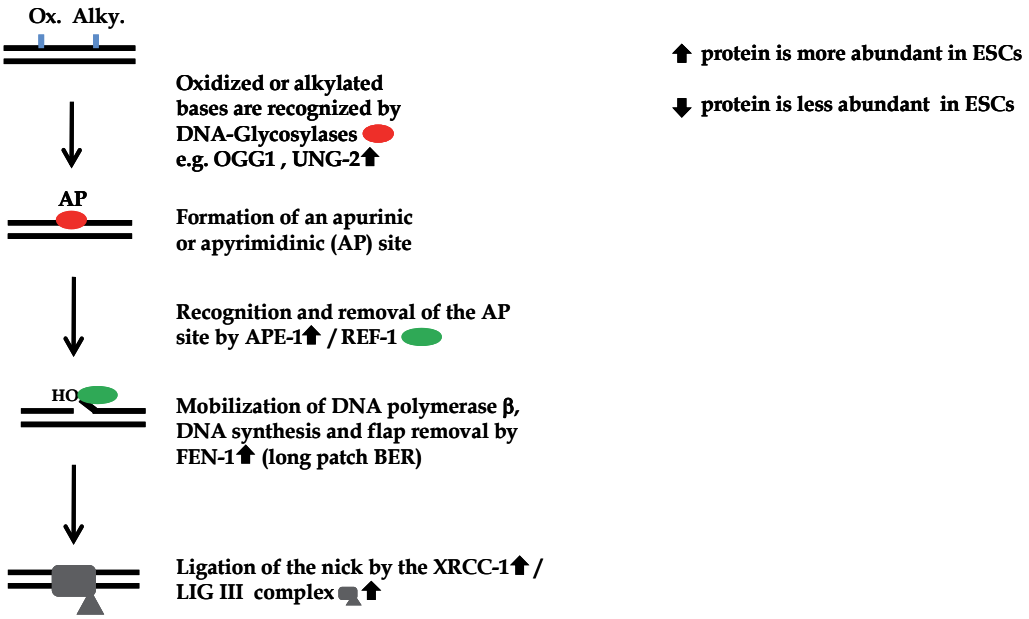

Fig. 1. Base excision repair (BER) in ESCs. Modified bases, e.g. after oxidation or alkylation are recognized by DNA glycosylases and excised, which leaves the DNA with an apurinic or apyrimidinic (AP) site. A complex of apurinic/apyrimidinic endonuclease 1/redox effector factor 1 (APE-1/REF-1) hydrolyses the 5'phosphodiesterbond in the sugar-phosphate backbone at the AP-site followed by insertion of one (short patch BER) or several (long patch BER) new bases into the gap by DNA polymerase β. Eventually occurring base overhangs are removed by flap-endonuclease 1 (FEN-1) prior to sealing of the break by the DNA ligase III (LIG III)/XRCC-1 complex. Arrows next to individual proteins indicate higher (⬆) or lower (⬇) abundance in ESCs.

with one of these pathways lead to several disorders including Xeroderma pigmentosum (XP), Trichothiodystrophy (TTD) and Cockayne syndrome (CS) (reviewed in: Nouspikel, 2009).
The first step in GGR is detection of the lesion by the XPC protein, usually due to an alteration in the helical structure of the DNA. This event mobilizes XPA and replication protein A (RPA) to the lesion, followed by recruitment of the ATPases/helicases XPB and XPD, which unwind the DNA double helix. The endonucleases XPG and XPF cut 3' and 5' of the lesion, leading to the release of a twenty-seven nucleotide (plus/minus two nucleotides) long stretch of DNA. Finally the gap is filled by DNA synthesis performed by DNA polymerase β and the nick is closed by DNA ligase I (Huang et al, 1992; O'Donovan et al, 1994; Riedl et al, 2003). TCR, in contrast, starts with recognition of the lesion by RNA polymerase II, which mobilizes CSA and CSB to the lesion. These proteins attract XPA and RPA, which initiate the same repair process as during GGR (reviewed in: Fousteri & Mullenders, 2008). DAR is a third sub-pathway of NER that has been particularly observed in terminally differentiated cells. In these cells, NER of non-transcribed regions is strongly compromised while transcribed regions are proficiently repaired. In contrast to TCR that only removes lesions in the transcribed strand, DAR also operates on the non-transcribed strand in transcriptionally active regions of the genome. Since DAR has been identified only recently, very little is known as yet about its mechanism (reviewed in: Nouspikel, 2009).

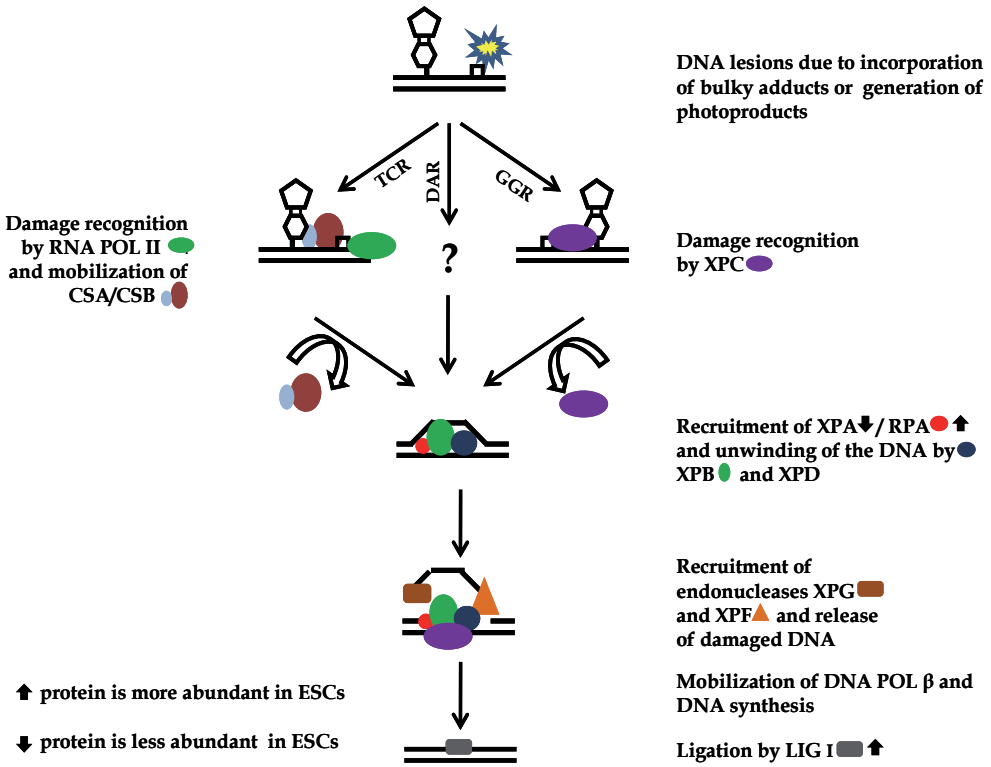

Fig. 2. Nucleotide excision repair (NER) in ESCs. The first step in global genome repair (GGR) is detection of the lesion by the XPC protein, usually due to an alteration in the helical structure of the DNA. This is in contrast to transcription-coupled repair (TCR), which starts with recognition of the lesion by RNA polymerase II (RNA POL II) and attraction of CSA and CSB to the lesion. These events mobilize XPA to the lesion, followed by recruitment of the helicases XPB and XPD which unwind the DNA double helix. The endonucleases XPG and XPF cut 3′ and 5′ of the lesion, leading to the release of a twenty-seven nucleotide (plus/minus two nucleotides) long stretch of DNA. Finally the gap is filled by DNA polymerase β (POL β) and the nick is closed by DNA ligase I (LIG I).The initial recognition step for DAR is as yet unknown. Arrows next to individual proteins indicate higher (⬆) or lower (⬇) abundance in ESCs.

Although most DNA repair pathways are more efficient in ESCs than in differentiated cells, for NER it appears to be the opposite (de Waard et al, 2008). While at low doses of UV-irradiation, MEFs and ESCs have comparable repair efficiencies, exposure to as little as 5 J/m² UVC leads to saturation of the repair capacity in ESCs (Van Sloun et al, 1999). Thus, although repletion is also an issue for differentiated cells, saturation levels are reached three times faster in ESCs than in MEFs (Van Sloun et al, 1999). At doses above 10 J/m² UVC-light, there is nearly no further repair of cyclobutane pyrimidine dimers (CPDs) and only about thirty percent of induced 6-4 photoproducts are repaired in ESCs within twenty-four hours. This is in strong contrast to MEFs or cardiomyocytes, which repair up to seventy percent of 6-4 photoproducts within twelve and more than fifty percent of CPDs in the transcribed region within twenty-four hours after irradiation (Cheo et al, 1997; van der Wees et al, 2003; Van Sloun et al, 1999; reviewed in: van der Wees et al, 2007). Eventually, the

decreased expression of XPA in hESCs contributes to the reduced repair activity, although this has not been seen in all cases (Maynard et al, 2008; Momcilovic et al, 2010). In contrast to XPA, the expression of RPA and DNA ligase I is enhanced in hESCs (Momcilovic et al, 2010), yet this might be due to the involvement of these repair proteins in other repair pathways.

It is known for several years that ESCs are less potent in NER than differentiated cells, but until more recently, it was unclear whether this failure is caused by inefficient GGR, TCR or by disorganization of both. With the establishment of mESCs and MEFs that are deficient in TCR (*csB*$^{-/-}$), GGR (*xpc*$^{-/-}$) or in both NER pathways (TCR/GGR; *xpa*$^{-/-}$), this question could be solved. Genetic deletion of the *xpc* gene (GGR) further enhanced the number of apoptotic mESCs over the already high number of apoptotic ESCs after UV-irradiation, whereas genetic deletion of the *csB* gene (TCR) did not affect the rate of cell death of mESCs but significantly elevated their mutation rate after UV-exposure (de Waard et al, 2008). This is in contrast to differentiated cells where UV sensitivity is rather linked to TCR (Conforti et al, 2000; de Waard et al, 2008; Ljungman & Zhang, 1996; Ljungman et al, 1999). The difference between ESCs and differentiated cells might be due to different responses of the transcriptional machinery to photoproducts in the DNA. While RNA synthesis is rapidly blocked in differentiated cells after exposure to UV light, gene transcription is continued for several hours in mESCs, even after irradiation with up to 10J/m^2 (de Waard et al, 2008).

5. Mismatch repair

The mismatch repair (MMR, Fig. 3) pathway is focused on the repair of mispaired nucleotides, arising after exposure to chemical agents like the methylating agent N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) or after errors during replication (reviewed in: Marra & Jiricny, 2005). Mismatches in the DNA are recognized and processed by a heterodimer of MSH2 and MSH3 or MSH6. The MSH2/MSH6 heterodimer supports repair of bases and mismatches with one or two unpaired nucleotides, while the MSH2/MSH3 heterodimer initiates repair of up to ten unpaired nucleotides and is rather inefficient in the identification of single nucleotide mismatches. Loss of function due to mutations in MMR genes leads frequently to hereditary nonpolyposis colorectal cancer (HNPCC), one of the most common cancers in the Western World (Fishel et al, 1993; Leach et al, 1993). MMR defective mice also showed spontaneous lymphomas upon exposure to DNA damaging agents as well as sterility and microsatellite instability (Friedberg & Meira, 2006).

After recognition of the DNA lesion by MSH2/MSH3 or MSH2/MSH6, a heterodimer of MLH1 and PMS1, MLH1 and PMS2 or of MLH1 and MLH3 is recruited to the damaged site. ATP-binding to MSH2/MSH3 or to MSH2/MSH6 verifies mismatch recognition, recruits MUTL to the lesion and initiates further repair activities. Other proteins that are required for MMR are proliferating cell nuclear antigen (PCNA), exonuclease 1 (EXO-1), RPA, replication factor C (RFC), high mobility group box 1 protein (HMGB-1) and DNA polymerase δ (POLδ). These proteins associate with MSH2, MSH3, MSH6 and/or MLH1 and are required for MMR initiation, excision of the mismatch and for DNA re-synthesis (reviewed in: Iyer et al, 2006; Jiricny, 2006; Li, 2008).

MMR is strongly enhanced in mESCs, which correlates with elevated expression of the MMR genes *msh-2*, *msh-3*, *msh-6*, *mlh-1*; *pms-2*, *mutyH* and *exo-1* (Fig. 3; Table 1), enhanced binding to damaged DNA and increased mismatch repair activity (Momcilovic et al, 2010; Osman et al, 2010; Roos et al, 2007; Tichy et al, 2011). DNA repair, however, seems to be not

the only function of MMR proteins. Overexpression of MSH2 also strongly reduced survival of 3T3 cells in response to treatment with the alkylating agent MNNG, while genetic deletion of the gene increased resistance to cell killing by low level irradiation and alkylating or oxidizing drugs in ESCs (Abuin et al, 2000; DeWeese et al, 1998; Roos et al, 2007). Thus MSH2 and eventually other mismatch repair proteins may also be involved in the induction of apoptosis. The high abundance of some of these repair proteins in ESCs may therefore also contribute to the increased sensitivity of stem cells towards alkylating and oxidizing agents.

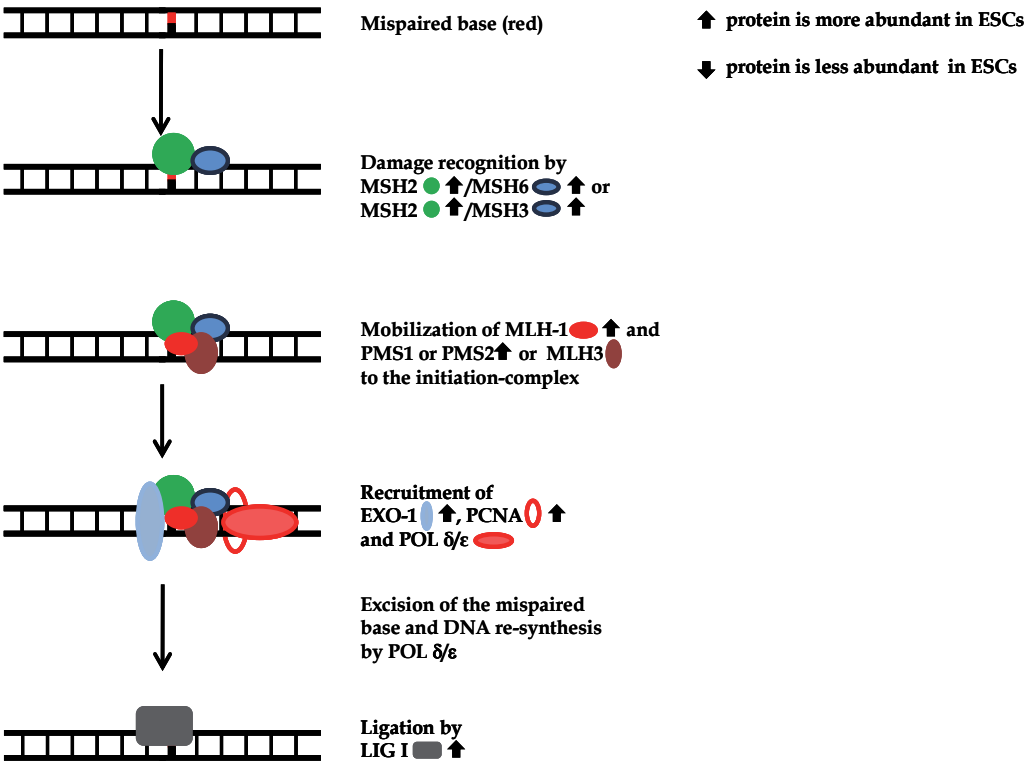

Fig. 3. Mismatch repair (MMR) in ESCs. After recognition of DNA lesions by MSH2/MSH3 or MSH2/MSH6, a heterodimer of MLH1 and PMS1, MLH1 and PMS2 or of MLH1 and MLH3 is recruited to the damaged site. Other proteins that are required for MMR are proliferating cell nuclear antigen (PCNA), exonuclease 1 (EXO-1), and DNA polymerase δ/ε (Pol δ/ε), which synthesizes the missing base(s). The final ligation step is performed by DNA ligase I (LIG I). Arrows next to individual proteins indicate higher (⬆) or lower (⬇) abundance in ESCs.

6. Repair of DNA double strand breaks by homologous recombination and non-homologous end joining

Double strand breaks (DSBs) can occur through normal cell metabolism, as intermediates of programmed genome rearrangements or after exposure to DNA damaging agents such as

ionizing radiation (IR). If not properly repaired, DSBs can result in chromosomal rearrangements and other severe genetic abnormalities as well as in senescence and apoptosis (reviewed in: Cahill et al, 2006). Because of the severe genomic aberrations that can arise, DSBs are considered as being the most harmful DNA lesion. Nonetheless, DSBs are also introduced into DNA on purpose, for example as part of the "mating-type-switch" in *Saccharomyces cerevisiae* or during V(D)J recombination in the course of the maturation of B- and T- cells in mammals (reviewed in: Paques & Haber, 1999; Soulas-Sprauel et al, 2007). DSBs are repaired by two major repair pathways: homologous recombination (HR; reviewed in: Johnson & Jasin, 2001) and non-homologous end joining (NHEJ; reviewed in: Lieber, 2008). HR is usually accurate, while NHEJ is frequently error-prone.

NHEJ starts with the recognition of the DSB by the Ku70/Ku80 heterodimer, a protein complex that binds specifically to broken DNA ends (Fig. 4). Once bound, Ku70/80 recruits the DNA-dependent protein kinase catalytic subunit (DNA-PKcs) to form the DNA-PK holoenzyme. The DNA ends, which often posses damaged bases, are then processed by nucleases such as Artemis or the MRN (Mre11, Rad50, Nbs1) complex. Finally, a complex of XRCC-4 (X-ray repair complementing defective repair in chinese hamster cells 4), DNA Ligase IV and XLF (XRCC4-like factor) seals the break (Ahnesorg et al, 2006; Valerie & Povirk, 2003). HR starts with the recruitment of RAD51 to the DSB and resection of the broken DNA resulting in the generation of single stranded DNA (ssDNA; Fig. 5). RPA then binds to the exposed ssDNA to form a nucleoprotein filament and RAD51 mediates invasion of the nucleoprotein filament into the homologues duplex DNA and formation of a D-loop (Baumann et al, 1996; Sung et al, 2003). The 3'-end of the invading strand is extended by DNA polymerase during branch migration, the Holliday junctions are resolved and the emerging nicks are ligated by DNA ligase I (reviewed in: Heyer et al, 2010).

A major difference in the repair of DSBs between ESCs and differentiated cells lies in the use of the two main pathways for DSB repair, HR and NHEJ (Fig. 6). While somatic cells repair DSBs mostly through NHEJ and less frequently via HR, this is opposite in ESCs, which repair seventy-five to eighty percent of DSBs by HR and only fifteen to twenty percent by NHEJ (Francis & Richardson, 2007; Yang et al, 2004). One reason for the prevalent use of HR by ESCs is the short G1 phase of the cell cycle (Fig. 7). Hence, almost seventy percent of an ESC population is in S-phase (Fluckiger et al, 2006; Savatier et al, 2002). Therefore, a significantly greater number of ESC chromosomes have sister chromatids available that can be used for HR. However, it should be noted that ESCs use HR even in the G1-phase of the cell cycle to repair DSBs (Serrano et al, 2011).

According to the importance of HR in ESCs, the RAD51 protein is about 20 times more abundant in ESCs than in differentiated cells and ES cells exhibit a higher number of cells with Rad51 foci after irradiation (Sioftanos et al, 2010; Tichy et al, 2010; Tichy & Stambrook, 2008). In addition, hESCs express a larger isoform of RAD51 (Adams et al, 2010a). Within several minutes after irradiation, RAD51 foci are observed in hESCs, which reach a maximum at six hours after irradiation. This is different, e.g. to neuronal progenitor cells, which also form RAD51 foci within minutes after irradiation, but in neuronal progenitor cells, it takes about twelve hours for maximal foci formation (Adams et al, 2010a). However, since in MEFs RAD51 foci are already maximally formed at four hours after irradiation (Sioftanos et al, 2010) and thus much earlier than in mESCs, these differences in the kinetics might rather display species- and cell type-specific differences than differences between stem cells and differentiated cells. Beside RAD51, RAD52, EXO-1 and MRE11 are also more abundant in ESCs than in differentiated cells (Table 1; Momcilovic et al, 2010; Tichy et al, 2010).

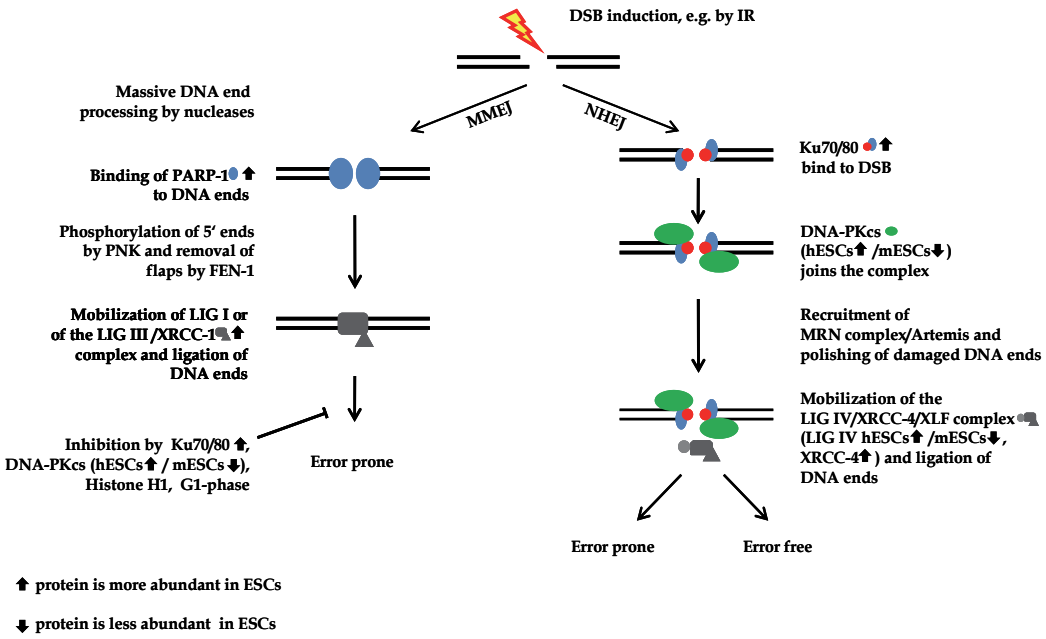

Fig. 4. DSB End joining pathways in ESCs. DSBs can be repaired by two end joining pathways. Microhomology-mediated end joining (MMEJ) starts with massive end processing by cellular nucleases and binding of poly(ADP-ribose) polymerase 1 (PARP-1) to the DNA ends. Binding of PARP-1 mobilizes further DNA end processing enzymes like polynucleotide kinase (PNK) and flap-endonuclease 1 (FEN-1). Finally, Ligase I (LIG I) or a heterodimer of Ligase III and XRCC-1 (LIG III/XRCC1) are attracted for the final ligation step. Proteins like Ku70/Ku80, DNA-PKcs and Histone H1 inhibit MMEJ. Non-homologous end joining (NHEJ) starts with binding of the Ku70/80 heterodimer to broken DNA ends. DNA-PK_{CS} translocates to the DSB and binds to the Ku complex. Afterwards, nucleases such as the MRN complex and Artemis arrive at the lesion and polish the DNA ends. Ligation of the DNA is performed by a complex of DNA ligase IV (LIG IV), XRCC-4 and XLF. Arrows next to individual proteins indicate higher (⬆) or lower (⬇) abundance in ESCs.

Despite the high rate of HR in ESCs for repair of DSBs they also use NHEJ, yet at a low rate. Lesions repaired by NHEJ can feature deletions or insertions of up to several thousand basepairs (Boubakour-Azzouz & Ricchetti, 2008). Genetic deletion of XRCC-4, downregulation of DNA-PK by siRNA or inhibition of DNA-PK activity by small molecules further reduced NHEJ frequency in hESCs, yet to a lesser extent than in differentiated cells (Adams et al, 2010a; Adams et al, 2010b), further supporting the notion that ESCs perform NHEJ, to some extent. In line with the infrequent use of NHEJ is the weak expression of the catalytic subunit of DNA-PK (*dna-pk*$_{CS}$) in mESCs, albeit the kinase is strongly expressed in hESCs (Banuelos et al, 2008; Momcilovic et al, 2010). In contrast to *DNA-PK*$_{CS}$, *ku70* and *ku80* are highly expressed in hESCs and mESCs (Maynard et al, 2008; Momcilovic et al, 2010; Tichy et al, 2010). A potential explanation for the different expression levels of *dna-pk*$_{CS}$ and *ku70* and *ku80* could be that Ku70 and Ku80 might have additional functions in ESCs. The Ku70/Ku80 heterodimer, for example, impedes unwanted recombination processes of

chromosomes and thus reduces mutation rates (Gullo et al, 2006). In addition, the Ku70/Ku80 heterodimer is involved in the regulation of cell growth and G1/S transition, where it keeps p21 levels under control, as well as in DNA replication, where it binds to origins of replication and associates with the replication machinery (Matheos et al, 2003; Matheos et al, 2002; Rampakakis et al, 2008). The repair factor XRCC-4 is expressed in similar amounts in mESCs and differentiated cells, while DNA ligase IV is less abundant in mESCs (Tichy et al, 2010). Only little is known about the principles that regulate the differential abundance of these proteins. However, as RNA levels of DNA ligase IV do not differ between mESCs and differentiated cells (Tichy et al, 2010), abundance of the proteins should be regulated posttranscriptionally.

In addition to cell type-specific differences in the expression of repair factors, there are also species-specific dissimilarities. XRCC-4, for example, is present in similar amounts in mESCs and MEFs, while in hESCs its expression is elevated compared to differentiated cells (Momcilovic et al, 2010; Tichy et al, 2010). Also DNA ligase IV is present in higher amounts in hESCs compared to differentiated human cells (Momcilovic et al, 2010), while its expression is lower in mESC compared to differentiated cells (Tichy et al, 2010). In contrast, DNA-PK_{CS} is present in lower amounts in mESCs compared to MEFs (Banuelos et al, 2008), but its expression is elevated in hESCs in comparison to differentiated human cells (Momcilovic et al, 2010). Also expression of breast cancer 1 (*BRCA-1*), a tumor suppressor protein which is involved in cell cycle regulation, transcription and DNA repair, is much lower in mESCs than in differentiated murine cells (Figure 8), while it is present in higher amounts in hESCs than in differentiated human cells (Table 1; Momcilovic et al, 2010).

7. Microhomology-mediated end joining (MMEJ): an alternative pathway to NHEJ and HR in ESCs

An alternative pathway related to NHEJ is microhomology-mediated end joining (MMEJ; Fig. 4). This repair pathway is, though, very error-prone due to massive DSB end processing and it leads frequently to deletions and occasionally to chromosomal aberrations like insertions, inversions or translocations (reviewed in: McVey & Lee, 2008). While for NHEJ one to five complementary bases are sufficient for the initiation of repair, MMEJ requires ten to twenty-five complementary bases (reviewed in: McVey & Lee, 2008). Preferred substrates for MMEJ are DSBs with blunt and non-cohesive ends, which are poor templates for NHEJ (Zhang & Paull, 2005). Proteins like PARP-1, XRCC-1, the bifunctional polynucleotide phosphatase/kinase (PNK), DNA ligase I and DNA ligase III are major players in this repair pathway (Zhang & Paull, 2005).

After introduction of a DSB, PARP-1 becomes active and is recruited to the DSB where it attracts PNK and the DNA ligase III/XRCC-1-complex. When these proteins are at the lesion, PNK phosphorylates the 5'-end of the DNA at the DSB. After that, the DNA ends are ligated by the DNA ligase III/XRCC-1-complex (Audebert et al, 2004; Audebert et al, 2006; Audebert et al, 2008). Apart from the "core" MMEJ-factors, Ku70, Ku80, Histone H1, CtIP (CtBP-interacting protein), CDK-2, and BRCA-1 are involved in MMEJ, albeit Ku70, Ku80 and Histone H1 are mostly active as regulators of this repair pathway (reviewed in: McVey & Lee, 2008). While Ku70 inhibited MMEJ in fission yeast (*Schizosaccharomyces pombe*), Ku80 promoted MMEJ in CHO (chinese hamster ovary) cells (Decottignies, 2007; Katsura et al, 2007). It remains to be determined whether these two proteins, that usually function as a heterodimer,

regulate MMEJ indeed differently or whether the opposing observations are due to species-specific variations. Also Histone H1 suppresses MMEJ, while CtIP, a component of the CtIP/BRCA-1/MRE-11 complex, supports DSB end resection, an essential process during MMEJ in somatic cells (Liang et al, 2005; Zhong et al, 2002).
There is very little known about the regulation of MMEJ in ESCs, yet, MMEJ is used in ESCs, albeit at a very low frequency (Tichy et al, 2010). Eventually, it only serves as a backup repair system in case of severe damage or when proteins of the classical NHEJ pathway are not available. A possible explanation for the minor contribution of MMEJ to DSB repair in ESCs might lie in the error-proneness of this repair pathway. Since ESCs are masters of preserving their genomic information, MMEJ might not be an appropriate pathway for DNA repair in general in this cell type.

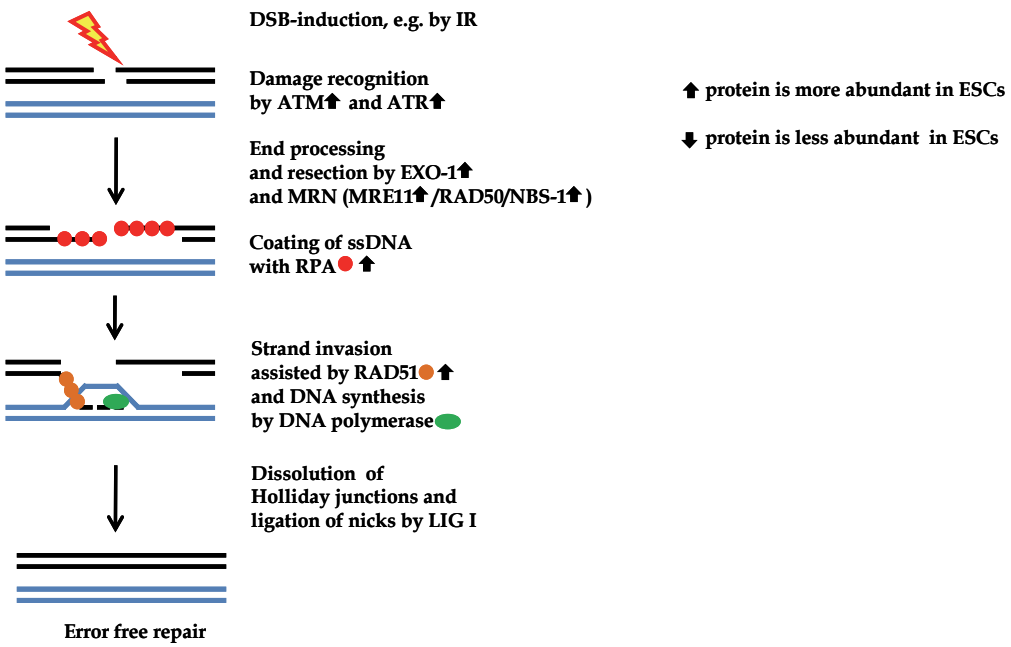

Fig. 5. Homologous recombination in ESCs. DSBs are recognized by ATM and ATR. DSB ends are resected by 5`-3` exonucleases like Exonuclease I (EXO-1) or the MRN complex, resulting in single stranded DNA ends (ssDNA). RPA binds to ssDNA and protects the DNA ends from degradation. Then, RAD51 is mobilized and binds to ssDNA followed by the formation of a presynaptic complex and displacement of RPA. Rad51 finally invades into the sister-chromatid and DNA polymerase synthesizes new DNA using the corresponding sister-chromatid as a template. At the end, Holliday Junctions are dissolved by resolvases and the nicks are ligated by DNA ligase I (LIG I). Arrows next to individual proteins indicate higher (⬆) or lower (⬇) abundance in ESCs.

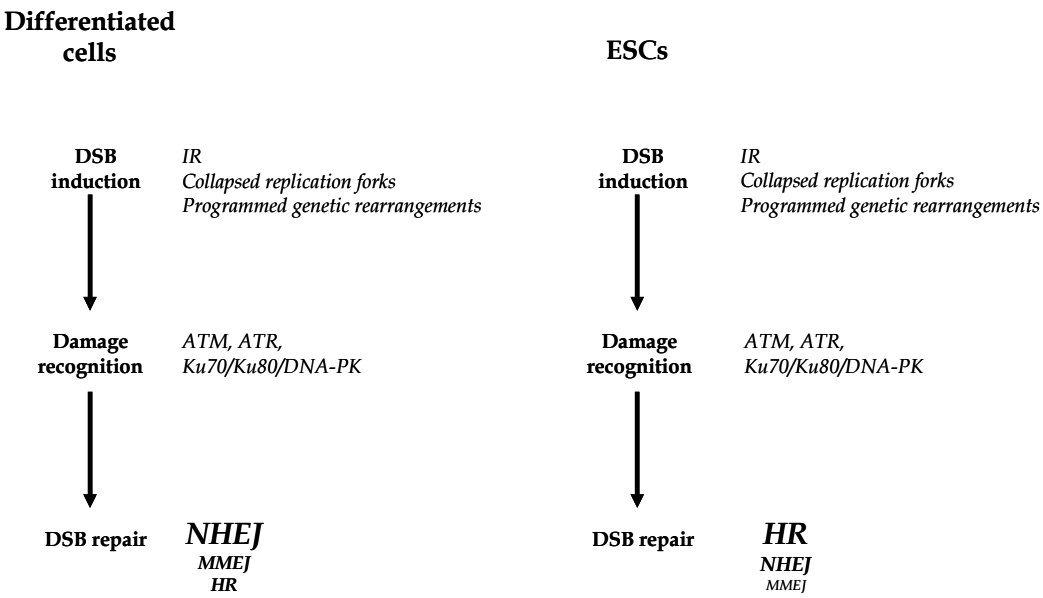

Fig. 6. Double strand break repair in somatic versus embryonic stem cells. After DSB induction, lesions are detected by ATM, ATR or the Ku70/Ku80/DNA-PK complex and repair pathways are switched on. While somatic cells predominantly repair DSBs by non-homologous end joining (NHEJ) and only secondary by microhomology-mediated end joining (MMEJ) and homologous recombination (HR), ESCs favour homologous recombination for DSB repair.

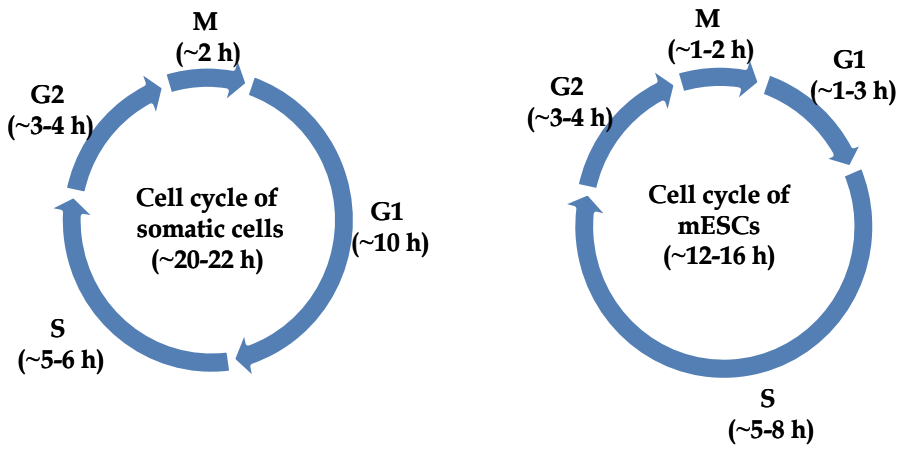

Fig. 7. Duration of the cell cycle of somatic cells and embryonic stem cells. In differentiated cells, progression through the cell cycle takes about twenty-four hours (h) whereas, due to a very short G1-phase it takes only about twelve hours in mESCs. Due to the short G1-phase, S-phase becomes automatically the longest phase of the cell cycle of ESCs resulting in about seventy percent of ESCs of a proliferating population in S-phase (Aladjem et al., 1998; Savatier et al., 2002; Hong and Stambrook, 2004; Fluckiger et al., 2006).

8. Induction of differentiation in response to DNA damage

Differentiation is a way for ESCs to respond to a negatively changing environment or growth condition. During differentiation, stem cells loose their potential to proliferate while they remain vital and functional as a differentiated cell. Differentiation is therefore an attractive option for stem cells to respond to DNA damage, when repair is not possible or has been unsuccessful. Since cell division is discontinued, DNA lesions and mutations cannot be propagated to daughter cells while the cell can still fulfill some functions. Nevertheless, under conditions of severe DNA damage, induction of cell death might certainly be the better choice to maintain a healthy population of ESCs.

There are several routes by which differentiation can be initiated after DNA damage. One option is via activation of the tumor suppressor protein Tp53. Upon DNA damage, the transcription factor Tp53 accumulates in the nucleus of stem cells (Lin et al, 2005; Solozobova et al, 2009). Among the promoters, to which Tp53 binds upon activation is the promoter of *nanog*, an important stem cell marker gene. Binding of Tp53 suppresses transcription of *nanog* and elimination of the Nanog protein from stem cells, leads to their differentiation (Lin et al, 2005). Activation of Tp53 can, however, also have the opposite effect as activated Tp53 also stimulates production of the Wnt ligands Wnt3, Wnt3a, Wnt8a, Wnt8b and Wnt9a (Lee et al, 2010). The Wnt ligands are secreted from cells with damaged DNA and engulfed by neighboring ESCs, where they inhibit differentiation (Lee et al, 2010). In consequence, these cells continue to proliferate, which may help to maintain a healthy pool of ESCs and to replace cells that are lost by apoptosis after genotoxic insults. Another route to differentiation in response to cellular stress is via activation of the c-Jun-N-terminal kinase (JNK). JNK is a negative regulator of the gp130-JAK-STAT3 signaling cascade in somatic cells (Lim & Cao, 1999), a pathway that also controls pluripotency in stem cells (Raz et al, 1999). Since JNK is activated in response to DNA damage (Adler et al, 1995; Nehme et al, 1999; Wilhelm et al, 1997), it is conceivable that this signaling pathway also supports differentiation of stem cells in the presence of DNA lesions. A further signaling cascade that is involved in regulating differentiation in hESCs is the NFκB pathway (Yang et al, 2010). Since DNA damage also activates NFκB (Basu et al, 1998), it may influence stem cell differentiation also via this route.

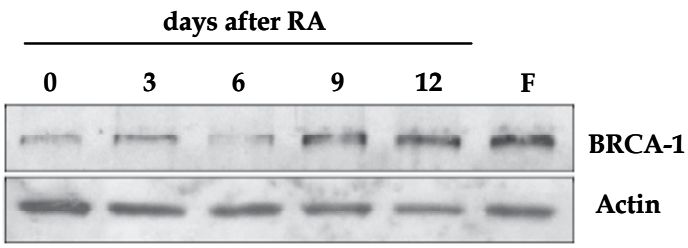

Fig. 8. Expression of BRCA-1 in mESC and during differentiation. D3 cells were treated with retinoic acid for the indicated time. Four hours prior to harvest, MG132 was added to the cultures. Cells were lysed and the amount of BRCA-1 was determined by Western Blotting. Hybridization with an antibody directed against actin served for loading control.

	Proteins			References
	abundance	mESCs	hESCs	
BER	⬆	APE-1, DNA ligase III, PARP-1, PCNA, UNG-2, XRCC-1	DNA ligase III, Endonuclease III-like protein 1 (gene: *NTHL1*), PCNA, UNG-2, FEN-1, XRCC-1	Maynard et al, 2008; Momcilovic et al, 2010; Tichy et al, 2010, 11
	⬇	-	DNA-3-methyladenine glycosylase (gene: *MPG*)	Maynard et al, 2008; Momcilovic et al, 2010
NER	⬆	-	DNA ligase I, RPA	Maynard et al, 2008; Momcilovic et al, 2010; Tichy et al, 2010
	⬇	-	XP-A	Maynard et al., 2008
MMR	⬆	MLH-1, MSH-2, MSH-6, PCNA PMS-2	EXO-1, MSH-2, MSH-3 MSH-6, A/G-specific adenine DNA glycosylase (gene: *MUTYH*), PCNA,	Momcilovic et al, 2010; Roos et al, 2007;
	⬇	-	Three prime repair exonuclease 1 (gene: *TREX1*)	Momcilovic et al, 2010
HRR	⬆	FEN-1, PCNA, γ-H2AX, RAD51, RAD52, RAD54	ATM, ATR, BRCA-1, FEN-1, DNA-ligase I, MRE11, NBS-1 (gene: *NBN*), PCNA, RAD51, RAD52, RPA, XRCC-2	Chuykin et al, 2008; Jirmanova et al, 2005; Maynard et al, 2008; Momcilovic et al, 2010; Tichy & Stambrook, 2008; Tichy et al, 2010
	⬇	BRCA-1	-	Fig. 8
NHEJ	⬆	γ-H2AX, Ku70, Ku80, PARP-1, PCNA, RAD54, XRCC-1	ATM, ATR, BRCA-1, DNA ligase IV, DNA-PK$_{CS}$, Ku70, Ku80, MRE11, PCNA, XRCC-4	Banuelos et al, 2008; Chuykin et al, 2008; Jirmanova et al, 2005; Maynard et al, 2008; Momcilovic et al, 2010; Tichy & Stambrook, 2008; Tichy et al, 2010
	⬇	BRCA-1, DNA-PK$_{CS}$, DNA ligase IV	-	Banuelos et al, 2008; Tichy et al., 2010; Fig. 8
MMEJ	⬆	DNA ligase III, PARP-1, PCNA, XRCC-1	DNA ligase III, PNK, PCNA, XRCC-1	Maynard et al, 2008; Momcilovic et al, 2010; Tichy et al, 2010

BER: Base excision repair, NER: Nucleotide excision repair, MMR: Mismatch repair, HR: Homologous recombination, NHEJ: Non-homologous end-joining, MMEJ: Microhomology-mediated end-joining.

Table 1. Comparison of the expression of genes involved in DNA repair between hESCs and their differentiated counterpart and mESCs and differentiated murine cells. Whether the abundance is increased in ESCs in relation to the differentiated counterpart (⬆) or decreased (⬇) is indicated with either black or blue arrows.

9. Conclusions

It is known for many years that ESCs acquire significantly less mutations and genetic rearrangements in response to DNA damage than differentiated cells. This observation raises the interesting possibility that, by understanding how DNA repair is controlled in stem cells, one could eventually make DNA repair in differentiated cells more efficient and less error-prone. This would eventually help to reduce the accumulation of DNA lesions in the cells and thus combat development of cancer and degenerative diseases and to reduce failures associated with aging. Comparison of the DNA damage responses of ESCs and differentiated cells has shown that ESCs are not only more sensitive to DNA damaging agents, they usually also use the available repair pathways more efficiently and they prioritize error-free repair pathways over error-prone ones. This different behavior is also reflected in differences in the expression of repair proteins. Currently it is unclear, which factors are responsible for the different expression pattern of repair proteins and how they are regulated in stem cells and differentiated cells. One possibility that has been discussed in the past is that, due to the short G1 phase of the cell cycle, a significantly higher number of stem cells are in S-phase compared to differentiated cells. Accordingly, stem cells have higher levels of the transcription factor E2F-1 (Roos at al, 2007). It has, however, only been shown for very few repair proteins, that their expression is indeed controlled by E2F-1. In addition, it needs to be resolved whether the differences in the expression of some repair pathways is indeed the reason for a more effective repair or whether this is only co-incidence. Here, more research is needed to identify the regulatory factors and their interplay.

Another important issue is the sporadically observed species-specific difference in the expression of repair factors. Are these differences indeed due to species-specific gene expression programs or do we eventually look at different types of cells? Although murine and human ESCs are both derived from the inner cell mass of a blastocyst, it is presently unclear how far they are really comparable. A close look at origin and culture conditions of the employed cells is certainly required to allow a direct comparison of the gene expression program and behavior of different stem cell lines.

A matter that we have not addressed in this review is DNA repair of iPS cells, since only a minor amount of data is currently available on DNA repair of these cells. However, since iPS cells are presently intensively investigated for future therapeutic applications, research into DNA repair capabilities of reprogrammed cells is of prime importance.

10. References

Abuin, A., Zhang, H. & Bradley, A. (2000). Genetic analysis of mouse embryonic stem cells bearing Msh3 and Msh2 single and compound mutations. *Mol Cell Biol,* 20, 1, (December 1999), 149-157, ISSN 0270-7306

Adams, B. R., Golding, S. E., Rao, R. R. & Valerie, K. (2010a). Dynamic dependence on ATR and ATM for double-strand break repair in human embryonic stem cells and neural descendants. *PLoS One,* 5, 4, (April 2010), e10001, 1932-6203

Adams, B. R., Hawkins, A. J., Povirk, L. F. & Valerie, K. (2010b). ATM-independent, high-fidelity nonhomologous end joining predominates in human embryonic stem cells. *Aging (Albany NY),* 2, 9, (September 2010), 582-596, ISSN 1945-4589

Adler, V., Fuchs, S. Y., Kim, J., Kraft, A., King, M. P., Pelling, J. & Ronai, Z. (1995). jun-NH2-terminal kinase activation mediated by UV-induced DNA lesions in melanoma and

fibroblast cells. *Cell Growth Differ,* 6, 11, (November 1995), 1437-1446, ISSN 1044-9523

Ahnesorg, P., Smith, P. & Jackson, S. P. (2006). XLF interacts with the XRCC4-DNA ligase IV complex to promote DNA nonhomologous end-joining. *Cell,* 124, 2, (January 2006), 301-313, ISSN 0092-8674

Aladjem, M. I., Spike, B. T., Rodewald, L. W., Hope, T. J., Klemm, M., Jaenisch, R. & Wahl, G. M. (1998). ES cells do not activate p53-dependent stress responses and undergo p53-independent apoptosis in response to DNA damage. *Curr Biol,* 8, 3, (April 1998), 145-155, ISSN 0960-9822

Audebert, M., Salles, B. & Calsou, P. (2004). Involvement of poly(ADP-ribose) polymerase-1 and XRCC1/DNA ligase III in an alternative route for DNA double-strand breaks rejoining. *J Biol Chem,* 279, 53, (October 2004), 55117-55126, ISSN 0021-9258

Audebert, M., Salles, B. & Calsou, P. (2008). Effect of double-strand break DNA sequence on the PARP-1 NHEJ pathway. *Biochem Biophys Res Commun,* 369, 3, (December 2007), 982-988, ISSN 1090-2104

Audebert, M., Salles, B., Weinfeld, M. & Calsou, P. (2006). Involvement of polynucleotide kinase in a poly(ADP-ribose) polymerase-1-dependent DNA double-strand breaks rejoining pathway. *J Mol Biol,* 356, 2, (December 2005), 257-265, ISSN 0022-2836

Banath, J. P., Banuelos, C. A., Klokov, D., MacPhail, S. M., Lansdorp, P. M. & Olive, P. L. (2009). Explanation for excessive DNA single-strand breaks and endogenous repair foci in pluripotent mouse embryonic stem cells. *Exp Cell Res,* 315, 8, (January 2009), 1505-1520, ISSN 1090-2422

Banuelos, C. A., Banath, J. P., MacPhail, S. H., Zhao, J., Eaves, C. A., O'Connor, M. D., Lansdorp, P. M. & Olive, P. L. (2008). Mouse but not human embryonic stem cells are deficient in rejoining of ionizing radiation-induced DNA double-strand breaks. *DNA Repair (Amst),* 7, 9, (July 2008), 1471-1483, ISSN 1568-7864

Bapat, A., Fishel, M. L. & Kelley, M. R. (2009). Going ape as an approach to cancer therapeutics. *Antioxid Redox Signal,* 11, 3, (August 2008), 651-668, ISSN 1557-7716

Barrilleaux, B., Phinney, D. G., Prockop, D. J. & O'Connor, K. C. (2006). Review: ex vivo engineering of living tissues with adult stem cells. *Tissue Eng,* 12, 11, (May 2007), 3007-3019, ISSN 1076-3279

Basu, S., Rosenzweig, K. R., Youmell, M. & Price, B. D. (1998). The DNA-dependent protein kinase participates in the activation of NF kappa B following DNA damage. *Biochem Biophys Res Commun,* 247, 1, (June 1998), 79-83, ISSN 0006-291X

Baumann, P., Benson, F. E. & West, S. C. (1996). Human Rad51 protein promotes ATP-dependent homologous pairing and strand transfer reactions in vitro. *Cell,* 87, 4, (November 1996), 757-766, ISSN 0092-8674

Beard, W. A. & Wilson, S. H. (2006). Structure and mechanism of DNA polymerase Beta. *Chem Rev,* 106, 2, (February 2006), 361-382, ISSN 0009-2665

Boehme, K. A., Kulikov, R. & Blattner, C. (2008). p53 stabilization in response to DNA damage requires Akt/PKB and DNA-PK. *Proc Natl Acad Sci U S A,* 105, 22, (May 2008), 7785-7790, ISSN 1091-6490

Boiteux, S. & Radicella, J. P. (1999). Base excision repair of 8-hydroxyguanine protects DNA from endogenous oxidative stress. *Biochimie,* 81, 1-2, (April 1999), 59-67, ISSN 0300-9084

Boubakour-Azzouz, I. & Ricchetti, M. (2008). Low joining efficiency and non-conservative repair of two distant double-strand breaks in mouse embryonic stem cells. *DNA Repair (Amst),* 7, 2, (October 2007), 149-161, ISSN 1568-7864

Bozulic, L., Surucu, B., Hynx, D. & Hemmings, B. A. (2008). PKBalpha/Akt1 acts downstream of DNA-PK in the DNA double-strand break response and promotes survival. *Mol Cell,* 30, 2, (April 2008), 203-213, ISSN 1097-4164

Burma, S., Chen, B. P., Murphy, M., Kurimasa, A. & Chen, D. J. (2001). ATM phosphorylates histone H2AX in response to DNA double-strand breaks. *J Biol Chem,* 276, 45, (September 2001), 42462-42467, ISSN 0021-9258

Cahill, D., Connor, B. & Carney, J. P. (2006). Mechanisms of eukaryotic DNA double strand break repair. *Front Biosci,* 11, (December 2005), 1958-1976, ISSN 1093-4715

Cervantes, R. B., Stringer, J. R., Shao, C., Tischfield, J. A. & Stambrook, P. J. (2002). Embryonic stem cells and somatic cells differ in mutation frequency and type. *Proc Natl Acad Sci U S A,* 99, 6, (March 2002), 3586-3590, ISSN 0027-8424

Cheo, D. L., Ruven, H. J., Meira, L. B., Hammer, R. E., Burns, D. K., Tappe, N. J., van Zeeland, A. A., Mullenders, L. H. & Friedberg, E. C. (1997). Characterization of defective nucleotide excision repair in XPC mutant mice. *Mutat Res,* 374, 1, (March 1997), 1-9, ISSN 0027-5107

Chuykin, I. A., Lianguzova, M. S., Pospelova, T. V. & Pospelov, V. A. (2008). Activation of DNA damage response signaling in mouse embryonic stem cells. *Cell Cycle,* 7, 18, (September 2008), 2922-2928, ISSN 1551-4005

Cimprich, K. A. & Cortez, D. (2008). ATR: an essential regulator of genome integrity. *Nat Rev Mol Cell Biol,* 9, 8, (July 2008), 616-627, ISSN 1471-0080

Conforti, G., Nardo, T., D'Incalci, M. & Stefanini, M. (2000). Proneness to UV-induced apoptosis in human fibroblasts defective in transcription coupled repair is associated with the lack of Mdm2 transactivation. *Oncogene,* 19, 22, (June 2000), 2714-2720, ISSN 0950-9232

D'Amours, D., Desnoyers, S., D'Silva, I. & Poirier, G. G. (1999). Poly(ADP-ribosyl)ation reactions in the regulation of nuclear functions. *Biochem J,* 342 (Pt 2), (August 1999), 249-268, ISSN 0264-6021

de Waard, H., Sonneveld, E., de Wit, J., Esveldt-van Lange, R., Hoeijmakers, J. H., Vrieling, H. & van der Horst, G. T. (2008). Cell-type-specific consequences of nucleotide excision repair deficiencies: Embryonic stem cells versus fibroblasts. *DNA Repair (Amst),* 7, 10, (July 2008), 1659-1669, ISSN 1568-7864

Decottignies, A. (2007). Microhomology-mediated end joining in fission yeast is repressed by pku70 and relies on genes involved in homologous recombination. *Genetics,* 176, 3, (May 2007), 1403-1415, ISSN 0016-6731

DeWeese, T. L., Shipman, J. M., Larrier, N. A., Buckley, N. M., Kidd, L. R., Groopman, J. D., Cutler, R. G., te Riele, H. & Nelson, W. G. (1998). Mouse embryonic stem cells carrying one or two defective Msh2 alleles respond abnormally to oxidative stress inflicted by low-level radiation. *Proc Natl Acad Sci U S A,* 95, 20, (September 1998), 11915-11920, ISSN 0027-8424

Dobbs, T. A., Tainer, J. A. & Lees-Miller, S. P. (2010). A structural model for regulation of NHEJ by DNA-PKcs autophosphorylation. *DNA Repair (Amst),* 9, 12, (October 2010), 1307-1314, ISSN 1568-7856

Donovan, P. J. & Gearhart, J. (2001). The end of the beginning for pluripotent stem cells. *Nature,* 414, 6859, (November 2001), 92-97, ISSN 0028-0836

Fishel, M. L. & Kelley, M. R. (2007). The DNA base excision repair protein Ape1/Ref-1 as a therapeutic and chemopreventive target. *Mol Aspects Med,* 28, 3-4, (June 2007), 375-395, ISSN 0098-2997

Fishel, R., Lescoe, M. K., Rao, M. R., Copeland, N. G., Jenkins, N. A., Garber, J., Kane, M. & Kolodner, R. (1993). The human mutator gene homolog MSH2 and its association with hereditary nonpolyposis colon cancer. *Cell,* 75, 5, (December 1993), 1027-1038, ISSN 0092-8674

Fleck, O. & Nielsen, O. (2004). DNA repair. *J Cell Sci,* 117, Pt 4, (January 2004), 515-517, ISSN 0021-9533

Fluckiger, A. C., Marcy, G., Marchand, M., Negre, D., Cosset, F. L., Mitalipov, S., Wolf, D., Savatier, P. & Dehay, C. (2006). Cell cycle features of primate embryonic stem cells. *Stem Cells,* 24, 3, (October 2005), 547-556, ISSN 1066-5099

Fousteri, M. & Mullenders, L. H. (2008). Transcription-coupled nucleotide excision repair in mammalian cells: molecular mechanisms and biological effects. *Cell Res,* 18, 1, (January 2008), 73-84, ISSN 1748-7838

Francis, R. & Richardson, C. (2007). Multipotent hematopoietic cells susceptible to alternative double-strand break repair pathways that promote genome rearrangements. *Genes Dev,* 21, 9, (May 2007), 1064-1074, ISSN 0890-9369

Friedberg, E. C. & Meira, L. B. (2006). Database of mouse strains carrying targeted mutations in genes affecting biological responses to DNA damage Version 7. *DNA Repair (Amst),* 5, 2, (November 2005), 189-209, ISSN 1568-7864

Gullo, C., Au, M., Feng, G. & Teoh, G. (2006). The biology of Ku and its potential oncogenic role in cancer. *Biochim Biophys Acta,* 1765, 2, (February 2006), 223-234, ISSN 0006-3002

Hammond, E. M., Dorie, M. J. & Giaccia, A. J. (2003). ATR/ATM targets are phosphorylated by ATR in response to hypoxia and ATM in response to reoxygenation. *J Biol Chem,* 278, 14, (January 2003), 12207-12213, ISSN 0021-9258

Heyer, W. D., Ehmsen, K. T. & Liu, J. (2010). Regulation of homologous recombination in eukaryotes. *Annu Rev Genet,* 44, (August 2010), 113-139, ISSN 1545-2948

Hong, Y. & Stambrook, P. J. (2004). Restoration of an absent G1 arrest and protection from apoptosis in embryonic stem cells after ionizing radiation. *Proc Natl Acad Sci U S A,* 101, 40, (September 2004), 14443-14448, ISSN 0027-8424

Huang, J. C., Svoboda, D. L., Reardon, J. T. & Sancar, A. (1992). Human nucleotide excision nuclease removes thymine dimers from DNA by incising the 22nd phosphodiester bond 5' and the 6th phosphodiester bond 3' to the photodimer. *Proc Natl Acad Sci U S A,* 89, 8, (April 1992), 3664-3668, ISSN 0027-8424

Iyer, R. R., Pluciennik, A., Burdett, V. & Modrich, P. L. (2006). DNA mismatch repair: functions and mechanisms. *Chem Rev,* 106, 2, (February 2006), 302-323, ISSN 0009-2665

Jiricny, J. (2006). The multifaceted mismatch-repair system. *Nat Rev Mol Cell Biol,* 7, 5, (April 2006), 335-346, ISSN 1471-0072

Johnson, R. D. & Jasin, M. (2001). Double-strand-break-induced homologous recombination in mammalian cells. *Biochem Soc Trans,* 29, Pt 2, (May 2001), 196-201, ISSN 0300-5127

Jones, C. J. & Wood, R. D. (1993). Preferential binding of the xeroderma pigmentosum group A complementing protein to damaged DNA. *Biochemistry,* 32, 45, (November 1993), 12096-12104, ISSN 0006-2960

Katsura, Y., Sasaki, S., Sato, M., Yamaoka, K., Suzukawa, K., Nagasawa, T., Yokota, J. & Kohno, T. (2007). Involvement of Ku80 in microhomology-mediated end joining for DNA double-strand breaks in vivo. *DNA Repair (Amst),* 6, 5, (January 2007), 639-648, ISSN 1568-7864

Kim, J. B., Greber, B., Arauzo-Bravo, M. J., Meyer, J., Park, K. I., Zaehres, H. & Scholer, H. R. (2009). Direct reprogramming of human neural stem cells by OCT4. *Nature,* 461, 7264, (September 2009), 649-643, ISSN 1476-4687

Leach, F. S., Nicolaides, N. C., Papadopoulos, N., Liu, B., Jen, J., Parsons, R., Peltomaki, P., Sistonen, P., Aaltonen, L. A., Nystrom-Lahti, M. & et al. (1993). Mutations of a mutS homolog in hereditary nonpolyposis colorectal cancer. *Cell,* 75, 6, (December 1993), 1215-1225, ISSN 0092-8674

Lee, K. H., Li, M., Michalowski, A. M., Zhang, X., Liao, H., Chen, L., Xu, Y., Wu, X. & Huang, J. (2010). A genomewide study identifies the Wnt signaling pathway as a major target of p53 in murine embryonic stem cells. *Proc Natl Acad Sci U S A,* 107, 1, (December 2009), 69-74, ISSN 1091-6490

Li, G. M. (2008). Mechanisms and functions of DNA mismatch repair. *Cell Res,* 18, 1, (December 2007), 85-98, ISSN 1748-7838

Liang, L., Deng, L., Chen, Y., Li, G. C., Shao, C. & Tischfield, J. A. (2005). Modulation of DNA end joining by nuclear proteins. *J Biol Chem,* 280, 36, (July 2005), 31442-31449, ISSN 0021-9258

Lieber, M. R. (2008). The mechanism of human nonhomologous DNA end joining. *J Biol Chem,* 283, 1, (November 2007), 1-5, ISSN 0021-9258

Lim, C. P. & Cao, X. (1999). Serine phosphorylation and negative regulation of Stat3 by JNK. *J Biol Chem,* 274, 43, (October 1999), 31055-31061, ISSN 0021-9258

Lin, T., Chao, C., Saito, S., Mazur, S. J., Murphy, M. E., Appella, E. & Xu, Y. (2005). p53 induces differentiation of mouse embryonic stem cells by suppressing Nanog expression. *Nat Cell Biol,* 7, 2, (December 2004), 165-171, ISSN 1465-7392

Lindahl, T. (1993). Instability and decay of the primary structure of DNA. *Nature,* 362, 6422, (April 1993), 709-715, ISSN 0028-0836

Ljungman, M. & Zhang, F. (1996). Blockage of RNA polymerase as a possible trigger for u.v. light-induced apoptosis. *Oncogene,* 13, 4, (August 1996), 823-831, ISSN 0950-9232

Ljungman, M., Zhang, F., Chen, F., Rainbow, A. J. & McKay, B. C. (1999). Inhibition of RNA polymerase II as a trigger for the p53 response. *Oncogene,* 18, 3, (February 1999), 583-592, ISSN 0950-9232

Marra, G. & Jiricny, J. (2005). DNA mismatch repair and colon cancer. *Adv Exp Med Biol,* 570, (August 2008), 85-123, ISSN 0065-2598

Martin, G. R. (1981). Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. *Proc Natl Acad Sci U S A,* 78, 12, (December 1981), 7634-7638, ISSN 0027-8424

Matheos, D., Novac, O., Price, G. B. & Zannis-Hadjopoulos, M. (2003). Analysis of the DNA replication competence of the xrs-5 mutant cells defective in Ku86. *J Cell Sci,* 116, Pt 1, (November 2002), 111-124, ISSN 0021-9533

Matheos, D., Ruiz, M. T., Price, G. B. & Zannis-Hadjopoulos, M. (2002). Ku antigen, an origin-specific binding protein that associates with replication proteins, is required for mammalian DNA replication. *Biochim Biophys Acta,* 1578, 1-3, (October 2002), 59-72, ISSN 0006-3002

Maynard, S., Swistowska, A. M., Lee, J. W., Liu, Y., Liu, S. T., Da Cruz, A. B., Rao, M., de Souza-Pinto, N. C., Zeng, X. & Bohr, V. A. (2008). Human embryonic stem cells have enhanced repair of multiple forms of DNA damage. *Stem Cells,* 26, 9, (June 2008), 2266-2274, ISSN 1549-4918

McLaren, A. (2000). Stem cells: golden opportunities with ethical baggage. *Science,* 288, 5472, (September 2007), 1778, ISSN 0036-8075

McVey, M. & Lee, S. E. (2008). MMEJ repair of double-strand breaks (director's cut): deleted sequences and alternative endings. *Trends Genet,* 24, 11, (September 2008), 529-538, ISSN 0168-9525

Momcilovic, O., Choi, S., Varum, S., Bakkenist, C., Schatten, G. & Navara, C. (2009). Ionizing radiation induces ataxia telangiectasia mutated-dependent checkpoint signaling and G(2) but not G(1) cell cycle arrest in pluripotent human embryonic stem cells. *Stem Cells,* 27, 8, (June 2009), 1822-1835, ISSN 1549-4918

Momcilovic, O., Knobloch, L., Fornsaglio, J., Varum, S., Easley, C. & Schatten, G. (2010). DNA damage responses in human induced pluripotent stem cells and embryonic stem cells. *PLoS One,* 5, 10, (October 2010), e13410, 1932-6203

Motoyama, N. & Naka, K. (2004). DNA damage tumor suppressor genes and genomic instability. *Curr Opin Genet Dev,* 14, 1, (April 2004), 11-16, ISSN 0959-437X

Nehme, A., Baskaran, R., Nebel, S., Fink, D., Howell, S. B., Wang, J. Y. & Christen, R. D. (1999). Induction of JNK and c-Abl signalling by cisplatin and oxaliplatin in mismatch repair-proficient and -deficient cells. *Br J Cancer,* 79, 7-8, (March 1999), 1104-1110, ISSN 0007-0920

Nouspikel, T. (2009). DNA repair in mammalian cells : Nucleotide excision repair: variations on versatility. *Cell Mol Life Sci,* 66, 6, (January 2009), 994-1009, ISSN 1420-9071

O'Donovan, A., Davies, A. A., Moggs, J. G., West, S. C. & Wood, R. D. (1994). XPG endonuclease makes the 3' incision in human DNA nucleotide excision repair. *Nature,* 371, 6496, (September 1994), 432-435, ISSN 0028-0836

Oberle, C. & Blattner, C. (2010). Regulation of the DNA Damage Response to DSBs by Post-Translational Modifications. *Curr Genomics,* 11, 3, (November 2010), 184-198, ISSN 1875-5488

Okita, K., Ichisaka, T. & Yamanaka, S. (2007). Generation of germline-competent induced pluripotent stem cells. *Nature,* 448, 7151, (June 2007), 313-317, ISSN 1476-4687

Ono, Y., Furuta, T., Ohmoto, T., Akiyama, K. & Seki, S. (1994). Stable expression in rat glioma cells of sense and antisense nucleic acids to a human multifunctional DNA repair enzyme, APEX nuclease. *Mutat Res,* 315, 1, (July 1994), 55-63, ISSN 0027-5107

Osman, A. M., van Dartel, D. A., Zwart, E., Blokland, M., Pennings, J. L. & Piersma, A. H. (2010). Proteome profiling of mouse embryonic stem cells to define markers for cell differentiation and embryotoxicity. *Reprod Toxicol,* 30, 2, (June 2010), 322-332, ISSN 1873-1708

Paques, F. & Haber, J. E. (1999). Multiple pathways of recombination induced by double-strand breaks in Saccharomyces cerevisiae. *Microbiol Mol Biol Rev,* 63, 2, (June 1999), 349-404, ISSN 1092-2172

Rampakakis, E., Di Paola, D. & Zannis-Hadjopoulos, M. (2008). Ku is involved in cell growth, DNA replication and G1-S transition. *J Cell Sci,* 121, Pt 5, (February 2008), 590-600, ISSN 0021-9533

Raz, R., Lee, C. K., Cannizzaro, L. A., d'Eustachio, P. & Levy, D. E. (1999). Essential role of STAT3 for embryonic stem cell pluripotency. *Proc Natl Acad Sci U S A,* 96, 6, (March 1999), 2846-2851, ISSN 0027-8424

Reya, T., Morrison, S. J., Clarke, M. F. & Weissman, I. L. (2001). Stem cells, cancer, and cancer stem cells. *Nature,* 414, 6859, (November 2001), 105-111, ISSN 0028-0836

Riedl, T., Hanaoka, F. & Egly, J. M. (2003). The comings and goings of nucleotide excision repair factors on damaged DNA. *EMBO J,* 22, 19, (October 2003), 5293-5303, ISSN 0261-4189

Robertson, A. B., Klungland, A., Rognes, T. & Leiros, I. (2009). DNA repair in mammalian cells: Base excision repair: the long and short of it. *Cell Mol Life Sci,* 66, 6, (January 2009), 981-993, ISSN 1420-9071

Rogakou, E. P., Boon, C., Redon, C. & Bonner, W. M. (1999). Megabase chromatin domains involved in DNA double-strand breaks in vivo. *J Cell Biol,* 146, 5, (September 1999), 905-916, ISSN 0021-9525

Roos, W. P., Christmann, M., Fraser, S. T. & Kaina, B. (2007). Mouse embryonic stem cells are hypersensitive to apoptosis triggered by the DNA damage O(6)-methylguanine due to high E2F1 regulated mismatch repair. *Cell Death Differ,* 14, 8, (April 2007), 1422-1432, ISSN 1350-9047

Roos, W. P. & Kaina, B. (2006). DNA damage-induced cell death by apoptosis. *Trends Mol Med,* 12, 9, (August 2006), 440-450, ISSN 1471-4914

Saretzki, G., Armstrong, L., Leake, A., Lako, M. & von Zglinicki, T. (2004). Stress defense in murine embryonic stem cells is superior to that of various differentiated murine cells. *Stem Cells,* 22, 6, (November 2004), 962-971, ISSN 1066-5099

Savatier, P., Lapillonne, H., Jirmanova, L., Vitelli, L. & Samarut, J. (2002). Analysis of the cell cycle in mouse embryonic stem cells. *Methods Mol Biol,* 185, (January 2002), 27-33, ISSN 1064-3745

Serrano, L., Liang, L., Chang, Y., Deng, L., Maulion, C., Nguyen, S. & Tischfield, J. A. (2011). Homologous recombination conserves DNA sequence integrity throughout the cell cycle in embryonic stem cells. *Stem Cells Dev,* 20, 2, (May 2010), 363-374, ISSN 1557-8534

Shall, S. & de Murcia, G. (2000). Poly(ADP-ribose) polymerase-1: what have we learned from the deficient mouse model? *Mutat Res,* 460, 1, (June 2000), 1-15, ISSN 0027-5107

Shiloh, Y. (2003). ATM and related protein kinases: safeguarding genome integrity. *Nat Rev Cancer,* 3, 3, (March 2003), 155-168, ISSN 1474-175X

Sioftanos, G., Ismail, A., Fohse, L., Shanley, S., Worku, M. & Short, S. C. (2010). BRCA1 and BRCA2 heterozygosity in embryonic stem cells reduces radiation-induced Rad51 focus formation but is not associated with radiosensitivity. *Int J Radiat Biol,* 86, 12, (October 2010), 1095-1105, ISSN 1362-3095

Solozobova, V., Rolletschek, A. & Blattner, C. (2009). Nuclear accumulation and activation of p53 in embryonic stem cells after DNA damage. *BMC Cell Biol,* 10, (June 2009), 46, ISSN 1471-2121

Soulas-Sprauel, P., Rivera-Munoz, P., Malivert, L., Le Guyader, G., Abramowski, V., Revy, P. & de Villartay, J. P. (2007). V(D)J and immunoglobulin class switch

recombinations: a paradigm to study the regulation of DNA end-joining. *Oncogene,* 26, 56, (December 2007), 7780-7791, ISSN 1476-5594

Stiff, T., O'Driscoll, M., Rief, N., Iwabuchi, K., Lobrich, M. & Jeggo, P. A. (2004). ATM and DNA-PK function redundantly to phosphorylate H2AX after exposure to ionizing radiation. *Cancer Res,* 64, 7, (April 2004), 2390-2396, ISSN 0008-5472

Strumberg, D., Pilon, A. A., Smith, M., Hickey, R., Malkas, L. & Pommier, Y. (2000). Conversion of topoisomerase I cleavage complexes on the leading strand of ribosomal DNA into 5'-phosphorylated DNA double-strand breaks by replication runoff. *Mol Cell Biol,* 20, 11, (May 2000), 3977-3987, ISSN 0270-7306

Sugasawa, K., Shimizu, Y., Iwai, S. & Hanaoka, F. (2002). A molecular mechanism for DNA damage recognition by the xeroderma pigmentosum group C protein complex. *DNA Repair (Amst),* 1, 1, (Januar 2003), 95-107, ISSN 1568-7864

Sung, P., Krejci, L., Van Komen, S. & Sehorn, M. G. (2003). Rad51 recombinase and recombination mediators. *J Biol Chem,* 278, 44, (August 2003), 42729-42732, ISSN 0021-9258

Takahashi, K., Tanabe, K., Ohnuki, M., Narita, M., Ichisaka, T., Tomoda, K. & Yamanaka, S. (2007). Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell,* 131, 5, (November 2007), 861-872, ISSN 0092-8674

Takahashi, K. & Yamanaka, S. (2006). Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell,* 126, 4, (August 2006), 663-676, ISSN 0092-8674

Thomson, J. A., Itskovitz-Eldor, J., Shapiro, S. S., Waknitz, M. A., Swiergiel, J. J., Marshall, V. S. & Jones, J. M. (1998). Embryonic stem cell lines derived from human blastocysts. *Science,* 282, 5391, (November 1998), 1145-1147, ISSN 0036-8075

Thomson, J. A. & Marshall, V. S. (1998). Primate embryonic stem cells. *Curr Top Dev Biol,* 38, (December 1997), 133-165, ISSN 0070-2153

Thomson, J. A. & Odorico, J. S. (2000). Human embryonic stem cell and embryonic germ cell lines. *Trends Biotechnol,* 18, 2, (February 2000), 53-57, ISSN 0167-7799

Tichy, E. D., Liang, L., Deng, L., Tischfield, J., Schwemberger, S., Babcock, G. & Stambrook, P. J. (2011). Mismatch and base excision repair proficiency in murine embryonic stem cells. *DNA Repair (Amst),* (February 2011), ISSN 1568-7856

Tichy, E. D., Pillai, R., Deng, L., Liang, L., Tischfield, J., Schwemberger, S. J., Babcock, G. F. & Stambrook, P. J. (2010). Mouse embryonic stem cells, but not somatic cells, predominantly use homologous recombination to repair double-strand DNA breaks. *Stem Cells Dev,* 19, 11, (May 2010), 1699-1711, ISSN 1557-8534

Tichy, E. D. & Stambrook, P. J. (2008). DNA repair in murine embryonic stem cells and differentiated cells. *Exp Cell Res,* 314, 9, (April 2008), 1929-1936, ISSN 1090-2422

Valerie, K. & Povirk, L. F. (2003). Regulation and mechanisms of mammalian double-strand break repair. *Oncogene,* 22, 37, (August 2003), 5792-5812, ISSN 0950-9232

van der Wees, C., Jansen, J., Vrieling, H., van der Laarse, A., Van Zeeland, A. & Mullenders, L. (2007). Nucleotide excision repair in differentiated cells. *Mutat Res,* 614, 1-2, (August 2006), 16-23, ISSN 0027-5107

van der Wees, C. G., Vreeswijk, M. P., Persoon, M., van der Laarse, A., van Zeeland, A. A. & Mullenders, L. H. (2003). Deficient global genome repair of UV-induced cyclobutane pyrimidine dimers in terminally differentiated myocytes and

proliferating fibroblasts from the rat heart. *DNA Repair (Amst),* 2, 12, (December 2003), 1297-1308, ISSN 1568-7864

Van Sloun, P. P., Jansen, J. G., Weeda, G., Mullenders, L. H., van Zeeland, A. A., Lohman, P. H. & Vrieling, H. (1999). The role of nucleotide excision repair in protecting embryonic stem cells from genotoxic effects of UV-induced DNA damage. *Nucleic Acids Res,* 27, 16, (August 1999), 3276-3282, ISSN 1362-4962

Ward, I. M. & Chen, J. (2001). Histone H2AX is phosphorylated in an ATR-dependent manner in response to replicational stress. *J Biol Chem,* 276, 51, (October 2001), 47759-47762, ISSN 0021-9258

Weissman, I. L., Anderson, D. J. & Gage, F. (2001). Stem and progenitor cells: origins, phenotypes, lineage commitments, and transdifferentiations. *Annu Rev Cell Dev Biol,* 17, (November 2001), 387-403, ISSN 1081-0706

Wernig, M., Meissner, A., Foreman, R., Brambrink, T., Ku, M., Hochedlinger, K., Bernstein, B. E. & Jaenisch, R. (2007). In vitro reprogramming of fibroblasts into a pluripotent ES-cell-like state. *Nature,* 448, 7151, (June 2007), 318-324, ISSN 1476-4687

Wilhelm, D., Bender, K., Knebel, A. & Angel, P. (1997). The level of intracellular glutathione is a key regulator for the induction of stress-activated signal transduction pathways including Jun N-terminal protein kinases and p38 kinase by alkylating agents. *Mol Cell Biol,* 17, 8, (August 1997), 4792-4800, ISSN 0270-7306

Wobus, A. M. (2001). Potential of embryonic stem cells. *Mol Aspects Med,* 22, 3, (July 2001), 149-164, ISSN 0098-2997

Yang, C., Atkinson, S. P., Vilella, F., Lloret, M., Armstrong, L., Mann, D. A. & Lako, M. (2010). Opposing putative roles for canonical and noncanonical NFkappaB signaling on the survival, proliferation, and differentiation potential of human embryonic stem cells. *Stem Cells,* 28, 11, (October 2010), 1970-1980, ISSN 1549-4918

Yang, Y. G., Cortes, U., Patnaik, S., Jasin, M. & Wang, Z. Q. (2004). Ablation of PARP-1 does not interfere with the repair of DNA double-strand breaks, but compromises the reactivation of stalled replication forks. *Oncogene,* 23, 21, (March 2004), 3872-3882, ISSN 0950-9232

Yu, J., Vodyanik, M. A., Smuga-Otto, K., Antosiewicz-Bourget, J., Frane, J. L., Tian, S., Nie, J., Jonsdottir, G. A., Ruotti, V., Stewart, R., Slukvin, II & Thomson, J. A. (2007). Induced pluripotent stem cell lines derived from human somatic cells. *Science,* 318, 5858, (November 2007), 1917-1920, ISSN 1095-9203

Zhang, X. & Paull, T. T. (2005). The Mre11/Rad50/Xrs2 complex and non-homologous end-joining of incompatible ends in S. cerevisiae. *DNA Repair (Amst),* 4, 11, (July 2005), 1281-1294, ISSN 1568-7864

Zharkov, D. O. (2008). Base excision DNA repair. *Cell Mol Life Sci,* 65, 10, (February 2008), 1544-1565, ISSN 1420-682X

Zhong, Q., Chen, C. F., Chen, P. L. & Lee, W. H. (2002). BRCA1 facilitates microhomology-mediated end joining of DNA double strand breaks. *J Biol Chem,* 277, 32, (June 2002), 28641-28647, ISSN 0021-9258

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