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Advances in DNA Repair

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ADVANCES IN DNA REPAIR

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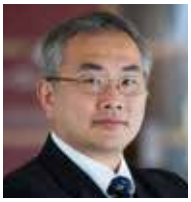
Meet the editor



Dr. Chen is a neurosurgeon with dedicated interest in oncology and a leader in the study of DNA repair and genetic alterations in brain tumors. He received his B.S. in biology from Stanford University, his M.S. in epidemiology from Columbia University, and his M.D., Ph.D. from Harvard Medical School. He subsequently completed his neurosurgery training at the Massachusetts General Hospital, including a clinical fellowship in radiosurgery and a second fellowship on stereotactic neurosurgery. Dr. Chen is the recipient of several highly competitive research awards including: the Damon Runyon Fellowship Award, the Burroughs Wellcome Foundation Career Award in Medical Sciences, the Sontag Foundation Distinguished Scientist Award, the Doris Duke Foundation Clinical Scientist Award, and the Forbeck Scholar award.

Contents

Preface

The 2015 Nobel Prize in Chemistry was awarded to Drs. Tomas Lindah, Paul Modrich, and Aziz Sancar for mechanistic studies of DNA repair. These pioneering investigators have made observations pertaining to base excision repair, mismatch repair, and nucleotide excision repair that fundamentally altered our understanding of the dynamic processes required for maintaining the integrity of DNA. The award recognizes the critical importance of DNA repair in all living organisms and the pertinence of these processes all aspects of basic science and clinical medicine, including aging, evolution, and cancer. Since the pioneering work that elucidated the specific processes mediating DNA repair, significant advances have been toward an understanding of the global regulation of specific repair processes through DNA damage response (DDR) and the roles of these responses in an integrated cellular network. Moreover, many of these DNA repair frameworks have been translated into potential therapy for human disease.

The purpose of this book is not to provide a comprehensive overview of DNA repair and DNA damage response. Such an overview can be found in the excellent text book "DNA Repair and Mutagenesis, 2 nd edition" by Friedberg, Walker, Siede, Wood, Schultz, and Elleberger. Instead, this book aims to provide select work to highlight recent, key insights into the specific DNA repair processes and how these insights are shaping intellectual frameworks underlying cancer, inflammation, virology, and stem cell biology.

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Section 1

DNA Repair Mechanisms

Chapter 1

Recent Advances in Plant DNA Repair

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Additional information is available at the end of the chapter

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1. Introduction

Plants are sessile organisms that are continuously exposed to different environmental factors, which may affect their development and production. In order to support this, plants change their metabolic pathways through different signal transductions in order to survive or set seeds to propagate the next generation [1, 2]. Due to this, it may be said that plants have a sophisticated perception of stress conditions. Moreover, these stress conditions might be biotic (caused by animals, insects, bacteria or virus), abiotic (caused by heat, temperature, drought, flood, salt, sunlight, soil contamination (chemicals mutagenic in soil or air), or endogenous metabolism (Figure 1). The response from plants to these conditions is usually associated with change in gene regulation, gene expression, protein translation, and post-translational modifications. These changes generated a change in plant metabolism, which is correlated to keep plant homeostasis, DNA repair, cell division, cell growth, and expansion [3, 4].

Regarding environmental conditions, plants are exposed continually to sunlight, which has a deleterious UV component. Then, in order to avoid this negative effect these organisms produce phenolic compounds such as flavones to reduce the amount that reaches the plant cells and they have DNA repair pathways to correct lesions and then keep the genome integrity [5-7].

In general, biotic or abiotic stresses are correlated with an increase in the Reactive Oxidative Species (ROS), and this has been associated as a type of oxidative stress [1]. ROS may be produced due to oxygen presence during plant metabolism (photosynthesis), or a consequence of biotic and abiotic stress. The presence of ROS may produce other forms that are more reactive such as superoxide, H_2O_2, OH^-, and singlet oxygen [8-9]. Normally, plants keep a balance between ROS production and degradation with an antioxidant system [9-10]. This system is

formed by superoxide dismutase (SOD), catalase, and ascorbate peroxidase (APX) and others proteins. Furthermore, when plants are exposed to a stress condition (biotic or abiotic), the ROS production is accelerated and then a different physiological response [9-15]. It has been observed in some crops that the oxidative stress unbalances the ROS equilibrium in cells that may promote growth reduction, late development, decrease seed production [11-15].

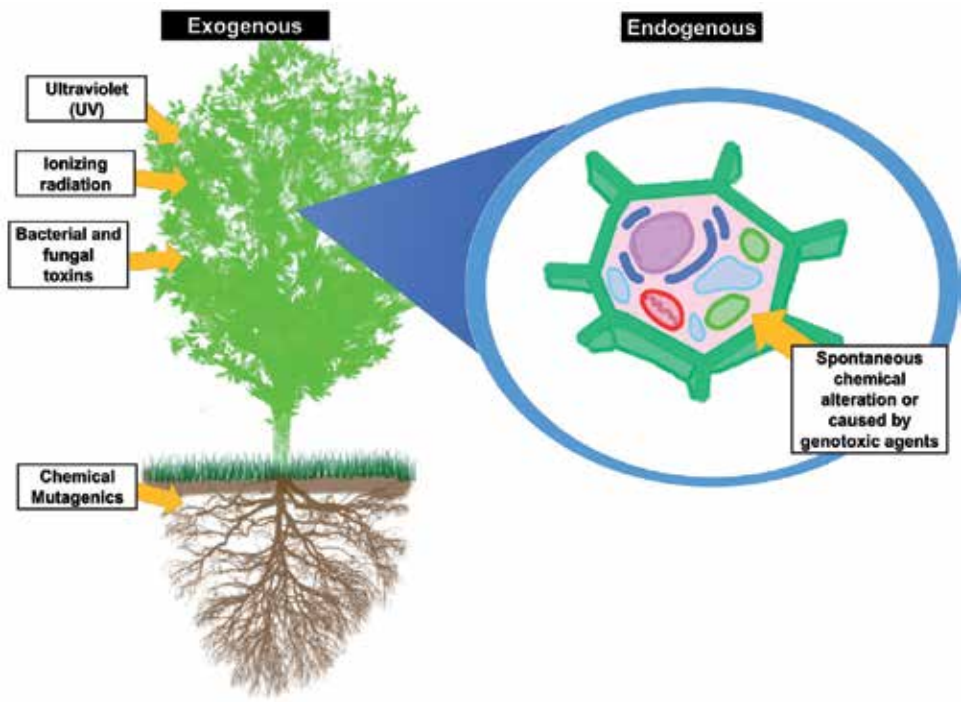

Figure 1. Schematical representation of agents that act in plant organisms. These agents are divided into biotic, abiotic, and endogenous agents. The biotic is formed by bacteria, insects, and virus. The abiotic is formed by sunlight (UV component), chemical mutagenics. And the endogenous components can be spontaneous lesions that happen during DNA replication or during photosynthesis.

When the ROS fine balance is not maintained, then these molecules may interact with DNA and this may produce DNA lesions and lipid peroxidation in membranes [9, 13-15]. Then, in order to avoid the DNA lesions and keep the genome integrity, plants have different DNA repair pathways to detect DNA lesions and correct them [15-16]. The effects of biotic and abiotic stresses on DNA repair have been observed (Figure 2). *Arabidopsis ku80* mutants had an increase in homologous recombination (HR) [16]. But, when these mutants were exposed to abiotic stress, this increase in HR was not observed. It was proposed an independent NHEJ and HR pathways in response to abiotic stress [16]. Another example of the connection between DNA repair and stress was observed by [17]. In this work, they observed an increase in DNA Polymerase λ protein when plants were grown in the presence of H_2O_2 and NaCl. Another protein that has a role in DNA repair and stress is DNA helicase. In rice, it was verified that *OsSUV3* expression (a DNA helicase) increased when rice plants were exposed to 200 mM

NaCl (abiotic stress). Its expression increased fivefold in the first hour and then an interactome analysis indicated that *OsSUV3* plays an important role in other pathways [18]. These are some examples that show the connection among the DNA repair machinery, stress tolerance, and the ROS production (Figure 2) [11-15].

"Omics" are a powerful tool to identify genes/proteins/metabolites that are involved in the plant response to a specific stress and/or to a DNA repair pathway [19]. Besides, transgenic and mutant plants are also helping in the gene characterization function. The data have shown that plant response is more complicated than previously thought. Not only is the presence of transcript (tissue or time presence) important, but also the signals are important for gene regulation, post-translation modifications (ubiquitination or sumolation), protein degradation, and protein targeting. All these may change when plants are exposed to different environmental conditions [19-23]. Furthermore, the *next generation sequence* data have shown that the signal transduction pathways actually form a network and the different networks are interconnected [24-25]. miRNAs have also been connected as a key factor for plant response to the stress and tolerance mechanism [25-28].

Considering the importance of the plants for food production, it is important to identify which genes/proteins/pathways are involved in these different mechanisms. This knowledge is important for plant breeders to produce new cultivars [17, 28]. Moreover, considering all that was explained above, plants are an interesting model to study stresses and DNA repair (Figure 2) due their sessile condition, genome plasticity, and the fact that these organisms do not have a germinative cell lineage. The apical meristem cells (shoot or root) suffer division continually during plant development and then genome integrity is extremely important [2]. Then, this chapter will focus on DNA repair pathways in plants.

This figure illustrates different abiotic factors such as drought, heavy metals, light, heat, ozone, lack of nutrients, cold, freezing, etc. Plants are able to perceive these different conditions or signals (on the right side) and then promote different molecular and physiological responses, which involve changes in gene expression, protein translation, post-translation modifications, degradation, epigenetic changes, and miRNAs. All these together produce a plant response that helps plants to tolerate this stress condition. Represented on the left side are the effects of an imbalance of ROS in DNA repair and the different DNA repair and genes that are involved in these different processes. The DNA repair presented in this figure includes mismatch repair (MMR), excision repair (NER and BER), and double strand breaks (HR and NHEJ).

2. Photoreactivation

Due to the sessile habits of plants, they are exposed continually to sunlight that is composed by UV-A (315 – 400 nm), UV-B (280-315 nm), and UV-C (200 -280 nm). However, due to the ozone layer, the UV-C does not penetrate. Furthermore, it is known that UV-B light affects photosynthesis, reduces productivity, and is also responsible for promoting lesions in DNA, such as (6-4) photoproducts and cyclobutane pyrimidine dimmers (CPDs). Moreover, plants produce compounds such as phenol compounds that act as filters in order to reduce the UV

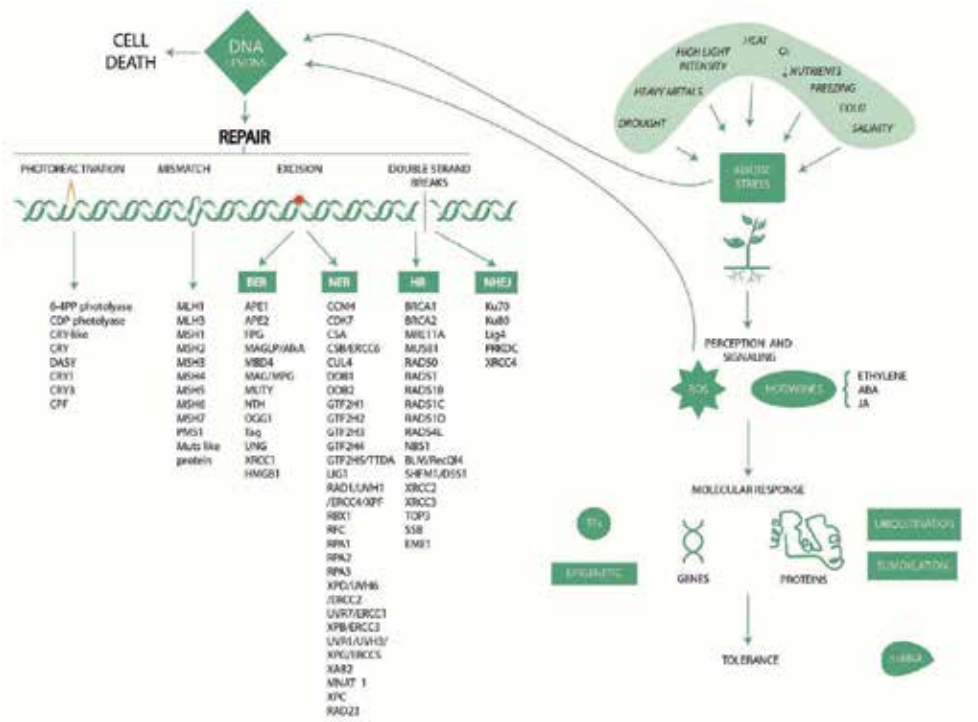

Figure 2. Schematical representation of environmental and endogenous factors and different plant responses.

light in plant cells [15]. Besides this, plant DNA also suffers DNA lesions. In rice cultivars was reported that CPDs may be the principal lesion that affects growth [29]. In *Arabidopsis* seedlings was observed that plants have photorepair mediated by photolyase proteins similar to what had been observed with other organisms [29-32]. The photolyase binds to damaged DNA and it uses the blue light photon to correct these lesions [33]. Furthermore, it was also observed that photolyase enzymes were not affected by the environmental temperature to which plants are exposed [34]. In cucumber leaves it was verified high expression of photolyase between the period of 9 am to 12 pm, and the photoreactivation activity was high between 12 pm to 15 pm [35]. The dark repair was done by Mismatch Repair (MMR), Nucleotide Base Excision (NER), and Base Excision Repair (BER) [1, 35].

The cryptochrome/photolyase superfamily (CPF) is a group of flavoproteins that includes photolyases and cryptochrome (CRY). Photolyases are DNA enzymes that are activated by light and when they recognize the CPD lesion, they are called CPD photolyases; and when they recognize the (6-4) pyrimidine-pyrimidone photoproducts, they are called (6-4) photolyases. CRY proteins do not have DNA repair activity, but they have a photoreceptor or transcription regulation functions [33]. Moreover, by phylogenetic analysis using the genomic data available, the CPF superfamily was divided into the following groups: 1- Class II CPD (involved in CPD DNA repair); 2- Class I and II CPDs and CRY photoreceptors (blue light receptors involved in circadian clock regulation; plant development); 3- Cry

DASHs (proteins that are able to do DNA repair); and 4- 6-4 photolyase (diverse group of proteins that includes DNA repair, photoreceptors, and transcriptional regulators) [33]. Although CPF is a diverse group, these proteins have a conserved photolyase homology region (PHR), with two non-covalent bound chromophoros (FAD and 8-HDF/MTHF). The CPFs may also have an N or C-terminal extension that might be associated with different functions such as signalling, regulation, post-translational modifications, protein targeting, circadian clock regulation [30, 32].

It has been shown that some rice cultivars have different UVB sensitivity, which is related to CPD lesion, photolyase activity, and ability to correct these lesions [29, 36]. It was observed that overexpression of photolyases increases biomass production under UV-B light in *Arabidopsis* [37]. Furthermore, data from the rice photolyase overexpression in different rice cultivars showed that rice UV-B resistance is associated with photolyase activity [38]. It was identified only one nuclear CPD photolyase gene in rice that produces only one mRNA [39]. However, this mRNA is translated in one protein that may be target to nuclei, chloroplast, or mitochondria. This is an example of a protein with triple targeting. Furthermore, their data showed that OsCRY-DASH carried some sequence at the N-terminal region that may be important for the mitochondria and chloroplast target [39].

3. Mismatch repair — MMR

The mismatch repair pathway is important for the genome stability during replication and it has been associated with the correction of the incorrect base incorporation by DNA polymerase during DNA replication or during the process of recombination [1, 49, 50] as well as to correct the photoproducts [42-43]. The other function of this pathway is to reduce recombination events between diverged genomes [42, 44-45].

It has also been observed that MMR is conserved from bacteria, yeast, humans, and plants. This pathway is formed by the following proteins: MutS (recognizes the base-base mismatch and deletion or insertion that promotes mismatch on a DNA strand, ATPase activity); MutL (interacts with MutS, helps in the mismatch detection, recruits and activates MutH, ATPase activity); MutH (an endonuclease - identifies the hemi-methylated sequence and produces a nick on the DNA strand); DNA helicase II (MutU or UvrD); exonucleases (ExoI, ExoVII, ExoX, RecJ); SSB protein (single strand-binding protein); PCNA (important for lagging strand); DNA polymerase III (fill the gap), and DNA ligase (ligates the DNA ends) [41, 47]. Also observed in human cells hemi-methylated sites that may act as important signals for discriminating the strand where nicks is produced. Despite this conservation, some differences have been observed between bacteria, yeast, and humans, and plants, which may be related to the life style where plants are sessile organisms [41, 46].

One model for the MMR pathway is: MutS/MSH (this complex recognizes mismatches or insertions/deletions on the DNA strand and bind to the DNA strand); and MutL/MLH (this complex has been associated with ligation to the DNA strand and the DNA repair complex is assembled). The MutL/MLH protein complex interacts with MutS in helping the detection

of the mismatch and the complex assembling. Then, both proteins activate the MutH protein. This protein now will recognize the hemi-methylation strand and produce a nick in the unmethylated DNA strand in a process that requires ATP-dependent proteins [41- 42]. After these steps, there is the association of PCNA, DNA helicases II, exonucleases, SSB, DNA polymerases, DNA synthesis and DNA ligation; then the mismatch lesion will be corrected. One difference between bacteria and eukaryotes is that MutS and MutL proteins work as homodimers and in the case of eukaryotes these proteins work as heterodimers (Table 1) [41-42].

This MMR pathway is able to identify a mismatch on the DNA strand, remove it, and replace it with the correct base [1, 40, 42]. Mutants in mice have shown that MMR proteins are associated with cancer as well as fertility as some mice mutants were sterile, and then it has been suggested that the MMR may also have a role in meiosis [47]. As plants are sessile organisms and do not have specific germ cells, these organisms have meristematic cells that divide to form gametes. These meristematic cells go through division during the life cycle of the plant and then these cells may accumulate spontaneous mutations that need to be recognized and corrected in order to keep the genome integrity. Due to this aspect, MMR has an important role in the plant genome [1, 40, 42].

The genome projects have allowed researchers to identify gene sequences and it has been observed that in plants some MMR genes were duplicated when compared to a bacteria genome (Table 1). Phylogenetic work on MSH proteins (bacteria MutS homolog) has observed the division into two distinct groups [48]. The idea is that the MutS from eubacteria present in mitochondria suffered duplication, and this new copy was transferred to nuclei where other duplication and specialization processes gave rise to MSH2, MSH3, MSH4, MSH5, and MSH6 in the nuclei. It has been observed that these sequences were related to recognizing and correcting DNA replication errors, and some proteins became involved in meiotic recombination. It has been proposed that these events occurred before the evolution of plants [49]. Furthermore, it was observed that sequence from MSH7 is specific to plants (Table 1).

Considering the role of MMR in recombination, in *Arabidopsis* when the level of genome divergence was 1.6% or 1.9%, a reduction in somatic recombination by to 3.6 or 9.6 fold was observed [50, 51]. Then, it has been proposed that the MMR pathway is also involved in reducing recombination frequency (or plays a role in anti-recombination) between diverged species or in cases of interspecies hybrids [52]. On the other hand, mutation in the MMR pathway has shown an increase in the recombination frequency. One difference in MMR between animals and plants is that it has been observed that many knockouts in animals are lethal; however, in plants it was observed in most cases that knockout may cause some problems in fertility, but a few had embryo lethality. These results shown that plants have plasticity for these lesions, as these organisms may tolerate these mutations [46].

For the MSH genes, it has been proposed that MSH2 is an important gene for MMR pathway and for the recombination repression [52]. This protein has a role in recognition of mismatch lesions (Table 1) and it forms dimmers to MSH3 or MSH6 proteins. The mutation only in the MSH2 sequence increases the homologous recombination by ninefold [52]. It has been observed that mutants in the MSH2 gene had problems in setting seeds and siliques were

abnormal; embryo lethality was also observed. These phenotypes suggested that these mutants probably accumulated many mutations, as it was not able to correct mismatch errors from DNA replication or normal metabolism [53].

In maize and in *Arabidopsis*, it was observed an increase in MSH2 and MSH6 transcriptional levels in leaves after UV-B irradiation. The MSHα (MSH2-MSH6) heterodimer may be involved in DNA repair by UV-B radiation in special to CPD lesions [43]. These authors also investigated the influence of other stress conditions as drought, cold, salt, heat, wounding and osmotic stress (all abiotic stress conditions). It has been observed that all these conditions down-regulated MSH2. On the other hand, the gene MSH6 was only down-regulated by drought, heat, and genotoxic stress. Both sequences were up-regulated by UV-B radiation. The hormone influence was also analyzed, and it was verified that the MSH2 sequence only responds to brassinolides, but MSH6 responds to all hormones tested [43]. Then these results showed that this heterodimer has a regulation to UV-B radiation on leaves, but each gene responds to different abiotic and hormone conditions. The authors also analyzed the cell cycle effect and they verified that MSH6 expression may be regulated by E2F transcription factors during the cell cycle [43].

The MSH7 protein (specific to plants) may interact to MSH2 as well as MSH3 and MSH6 [48]. It has been shown that this protein plays an important role in maintaining the genome integrity in plants and meiotic recombination [49; 54]. In tomato plants was showed a role of MSH7 in anti-recombination activity [55]. It was also shown that the heterodimer MSH2-MHS7 was able to recognize G/T or A/C mismatches, and this heterodimer may have a role in mismatches that arose from cytosine deamination or by UV or oxidative lesions [56].

The heterodimer MUTLα (MLH1-PMS1) has been considered an important heterodimer. The MLH1 has been associated with homologous recombination, as a decrease of 72% was observed [45]. Mutant plants for this sequence had problems in setting seeds, and a problem in pollen formation has also been observed. Then, it was proposed that the MLH1 gene may be important for recombination as well as in limiting the recombination frequency between diverged sequences [45]. For MLH2 it was observed that mutants also had problems in recombination, but the frequency reduction was only 22% [52]. The MLH3 mutants also had a reduction in seed production, but also a verified decrease in frequency of crossovers. MLH3 has a role in meiosis and is expressed in reproductive tissues. The immunolocalization identified the presence of this protein as foci on the chromosome axes during prophase I in meiosis, suggesting a role in Holliday resolution. Furthermore, AtMLH1 protein is dependent on AtMLH3 protein [57]. For the *PMS1* gene, it has been observed that the loss of the *PMS1* sequence creates problems in correcting the loopout (deletion/insertion mispairs), increasing the frequency in mutation in microsatellites [58]. *AtPMS1* mutants also showed problems in setting seeds, suggesting that this may be a characteristic of MUTL mutants. Furthermore, these mutants showed problems in pollen formation; however, the data obtained proposed that the *AtPMS1* gene has an anti-recombination activity as these mutants had an increase in homeologous recombination [58]. This observation is the opposite of what had been observed in yeast.

The comparative genome analysis between *UVRD* sequence from *Arabidopsis* and the rice genome showed that these enzymes may be involved in the following processes: DNA replication, DNA repair, DNA transcription, and DNA recombination. In this work, it was observed that both protein sequences of AtUVRD (129 KDa) and OsUVRD (130 KDa) share identity with E. *coli* REP helicases and both sequences have the ATP binding and helicase C-terminal domains that are important for its activity [18]. Furthermore, the structural modelling from these two sequences showed conservation in their structure to *E. coli* ReP helicases. This protein is an important component from MMR and these data showed the conservation domains, suggesting that the plant proteins may work in a way similar to the *E.coli* protein.

E. coli	Plant	Function	Localization
MutS	MSH4	meiotic recombination	nuclei
	MSH5	meiotic recombination	nuclei
	MSH1	replication errors	mitochondria
	MSH2	replication errors	nuclei
	MSH3	replication errors	
	MSH6	replication errors	
	MSH7 **(sequence found only in plants)**	replication errors	?
	MSHα – MSH2-MSH6	mismatch error and 1-2 bases of deletion/insertion loopout	
	MSHβ – MSH2-MSH3	2-12 bases of deletion/insertion loopout	
	MSHγ – MSH2-MSH7	preference for G/T or A/C base/base mispairs and meiotic recombination	
MutL	MLH1	homologous recombination	
	MLH2		
	MLH3		
	MUTLα - MLH1-PMS1	endonuclease activity, protein/DNA complex	
	MLH6		
Uvr	UVRD	DNA helicase	
PCNA	PCNA	Interacts with MSH2, MLH1, MSH6	

Table 1. MMR sequences identified in plant genomes

These data in plants showed the importance of MMR components and that many of these mechanisms are not clear yet, especially related to meiotic recombination and homeologous recombination.

4. Base Excision Repair (BER)

This pathway is responsible for identifying and correcting lesions associated with alkylation, oxidation, deamination, DNA replication, and base adducts that sometimes block DNA replication and transcription [59-60]. This pathway is a multistep, beginning with the lesion recognition by DNA glycosidase. These enzymes remove the base by an incision on DNA strands at the N-glycosidic bond that connects the base to the deoxyribose-phosphate residue. Then, this incision creates an abasic site or AP site [61, 62]. There are different DNA glycosylases (mono or bifunctional), each one specific to a different type of base lesion. The AP sites may also be produced by spontaneous depurination or by hydrolysis of N-glycosidic bond [63]. After that, the AP site is processed by a bifunctional DNA glycosylase (3′AP lyase function) or by AP endonuclease. This excision creates a gap, which is filled with nucleotides by DNA polymerase and then ligated to the DNA strand by DNA ligase (Figure 3) [59].

The BER may be divided into two pathways: short-patch and long-patch (Figure 3). Choosing the short or long patch will be associated with which type of lesion and which DNA glycosylase will be used in the DNA repair. The short patch is responsible for correcting the lesion of only one nucleotide, and the long-patch will correct a lesion from 2 to 13 nucleotides [64]. The other difference is that in the short-patch the DNA polymerase that acts in this process is Polβ (mammals) or PolI (bacteria) [65]; and the DNA sealing is done by XRCC1 and LigIII (mammals) or LigI (bacteria) [66-67]. It is known that for the long patch Polβ probably introduces the first nucleotide, but the other nucleotides are processed by Polδ or Polε [64]. The short and long-patches were discovered by *in vitro* and *in vivo* experiments [68-72].

Different work using the *Arabidopsis thaliana* has shown that there are homologous sequences for BER pathway in plants [2]. However, one difference found was related to the correction of the 8-oxo-7, 8-dihydroguanine lesion. In bacteria this lesion is corrected by MutM/Fpg (formamidopyrimidine DNA glycosylase) and in eukaryotes by OGG1 (8-Oxoguanine DNA-glycosylase). And it was found that plants have both sequences/proteins in their genome. But, the real function of these proteins is not clear, although an overlap for the lesion subtract was observed [73-74].

The other interesting component of this pathway is the enzyme fosfodiesterase-DNA tirosil (TDP1), which is involved in the repair of lesions *topo I-mediated damage* [75]. The TDP1 enzyme is responsible for hydrolyzing the phosphodiester ligation from DNA at the 3′end ligated to the tirosil radical and this enzyme has been associated with the repair of the topoisomerase complex I (TOP1)-DNA. Some data have shown that TPD is important for oxidative lesions in mitochondrial DNA [76]. This enzyme has been identified in plants. It has been observed in two sequences of the *Medicago truncatul*a genome: MtTDP1α and MtTDP1β. These isophorms are up-regulated in leaves and roots in response to heavy metals and osmotic stress [77].

Another difference observed in plants is related to AP endonuclease. This protein is necessary for survival, as it has been observed that homozygote knockout mice are lethal. [78]. Furthermore, APE1 (also known as HAP1 or APEX) has two types of functions – AP endonuclease and a redox function to *jun* and *fos* transcription factors [79]. In plants at least three sequences

of the *Arabidopsis* genome have been observed: *AtAPE1*, *AtAP2*, and *AtARP*. However, only the *AtARP* has been shown to have the AP endonuclease activity [80]. In sugarcane, two sequences were identified, one directed to nuclei (*ScARP1*) and the second one (*ScARP3*) directed to chloroplast and/or mitochondria [81]. Furthermore, at least nine genes that codified bifunctional DNA glycosylase have been identified, seven of which were characterized by *in vivo* experiments - *AtMMH*, *AtNTH1*, *AtOGG1*, *DME*, *ROS1*, *DML2*, and *DML3* [82-86].

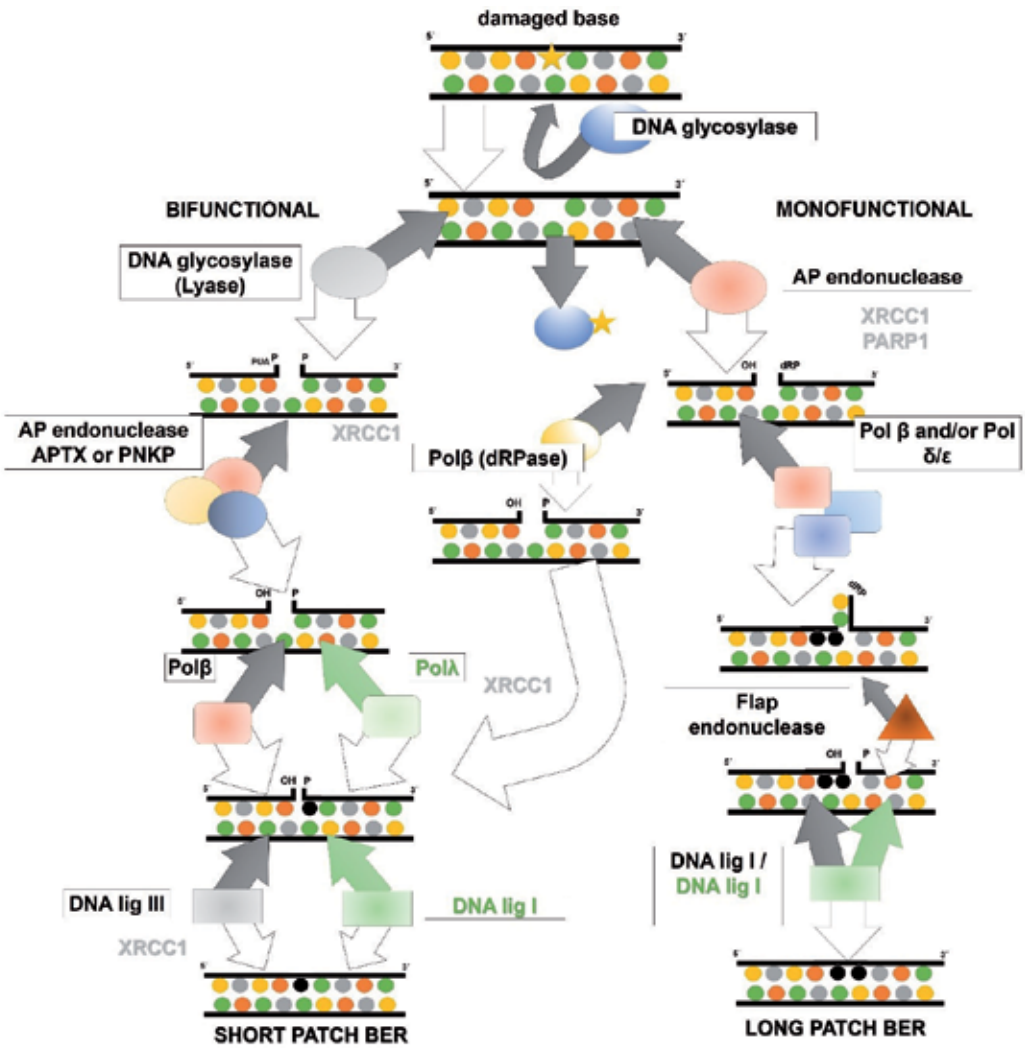

Figure 3. Schematical representation of BER pathway.

The short-patch needs the DNA polymerase β and DNA ligase III, but as these sequences were not identified in *Arabidopsis* and rice genomes, it was thought that plants do not have this pathway, only the long-patch [87] (Figure 3). However, experiments showed by *in vivo* that

plants have both pathways [88]. Another difference verified in plants is related to DNA polymeraseλ, which is a member of the polymeraseX family [89]. Then, DNAPolλ is the only member of this family identified up to now in plants [87]. Furthermore, for DNA ligase only the presence of DNA ligase I was observed in plants. It has been proposed that DNA ligase I may have the function of all DNA ligase in plants for both patches [90]. In XRCC a lack of domains responsible for the interaction to DNAPolβ and LigIII has been observed. One reason for this reinforces the observation that in the plant genome these two sequences were not found. But the presence of the domain PARP1 was observed. Although plants have only DNAPolλ and not DNAPolβ, no protein interaction was observed between XRCC and Polλ, but rather only the interaction from XRCC to PCNA (Figure 3) [91-92].

The first step of the BER pathway is the recognition of the lesion (represented by a star on the DNA strand). This lesion recognition is done by a bifunctional DNA glycosylase that makes an incision on the DNA strand and produces an end with 5′P and the other end 3′P or a 3 ′polinsaturated aldeid (PUA). In the case of the monofunctional DNA glycosylase, the nick is done by AP endonuclease creating 3′OH and 5′dRP. These ends were corrected by Polβ (dRPase function). The short-patch (left side) Polβ adds one nucleotide, and then XRCC1/LigIII or LigI does the ligation. In the long-patch Polβ and/or Polδ/ε adds the nucleotides (2 to 13); then the lesion is removed by the DNA Flap (FEN) and DNA is sealed by LigI. All the proteins and steps present in BER are represented by gray, and the proteins and steps present only in plants are represented by green color.

5. Nucleotide excision repair

Nucleotide excision repair (NER) is the other DNA repair pathway that removes damages caused by UV radiation and bulky covalent adducts that cause helix distortion [5, 93-95]. Furthermore, it has been observed that NER proteins are more conserved considering amino acid identity than the other pathways when compared with bacteria, yeast, humans, and plants [46].

NER is divided into two sub-pathways, each one having distinct damage recognition mechanisms but both using the same machinery to correct the lesion. Transcription-coupled repair (TC-NER) is responsible for the removal of lesions from the transcribed strand of actively expressed genes [96]. This pathway is activated when RNA polymerase is stalled during transcription and it depends on the recruitment of the proteins CSA and CSB (Cockayne syndrome A and B) to the site of the lesion [97]. This process was first discovered in mammals [98] and *E. coli* [99], and then in yeast [100]. CSB interacts with CSA-DDB1(damage-specific DNA binding protein 1)-CUL4 (CULLIN4) E3 ubiquitin ligase [101]. The ubiquitination of CSB in the DNA repair is regulated by the CUL4-DDB1-CSA complex [102].

The regions in the genome that are not actively expressed are repaired by Global Genome repair (GG-NER). In this case, the lesion is detected by the DDB2 (damage-specific DNA binding protein 2)-DDB1-CUL4 complex. The binding of this complex recruits another complex of proteins composed by XPC (*xeroderma pigmentosum* group C), RAD23, and centrin

[94]. After the recognition of the lesion and binding of the protein complex, TCR and GG-NER converge to base reactions common for both pathways. All the components of TC-NER and GG-NER identified in humans are well conserved in the *A. thaliana* and rice genomes, except for the protein XPA (*xeroderma pigmentosum* complementation group A) [46].

In *Arabidopsis* genome, two *DDB1* genes, *DDB1a* and *DDB1b* were found [103], and the protein complex CUL4-DDB1 was also identified in plants [104]. This complex has been associated with different biological processes [104-107]. However, it is not clear yet if plants have a CUL4-DDB1CSA protein complex. It was identified two CSA-like genes from *A. thaliana* (*AtCSA1A* and *AtCS1B*) that form an heterotetramer [107]. A UV sensitivity assay was performed in order to check whether *atcsa-1,1; ddb2-3* and *atcsa-1,1/ddb2-3* double mutants had an increase in UV sensitivity. After the plants had been exposed to UV-B radiation, it was observed that the root growth from single mutants was more severe than in wild-type plants. It was also observed that *atcsa-1, 1* had a stronger reduction in the root growth than *ddb2-3*, and the double mutant did not have an increase in UV treatment sensitivity. These results show the importance of these genes for DNA repair in roots. It has also shown the interaction of ATCSA-1 and DDB1a by two-hybrid assay [108].

For both NER pathways it is important that the TFIIH complex (transcription elongation factor-IIH) unwind the DNA near the lesion site. XPB and XPD proteins are the subunits of this complex that have helicase activity [109]. Orthologs of these two helicases were isolated in *A. thaliana* genome. Furthermore, it is also observed gene duplication for *XPB1* and *XPB2* genes [110-112]. AtXPB1 and AtXPB2 share 95% of amino acid sequence identity and the complementation assay in yeast showed that each protein sequence is able to complement the yeast Rad25 mutant strain in the presence of UV radiation [110-111]. The XPD gene in *Arabidopsis* is essential for plant development, since the *xpd* mutants present yellow-green leaves and their size is smaller than that of the wild-type plants. And after UV plant treatment, these mutants shown brown color and dead leaves in the rosette. These results suggested the involvement of this *XPD* gene to DNA repair [113].

In rice, differential modulation was reported for the genes *OsXPB2*, *OsXPD*, *OsTFIIS*, and *OsTFIIS-like* in response to γ-rays. The expression of the genes *OsXPB2* and *OsXPD* were down-regulated in 5-day-old seedlings and were up-regulated 20-day-old plantlets. The treatment with γ-rays and salinity showed an up-regulation of these two genes in both samples. For the *OsTFIIS* and *OsTFIIS-like* genes a similar pattern was observed [114].

In the study [115] it was observed that OsREX1-S (a TFB5 homolog in yeast and human - a subunit of TFIIH), is involved in NER as it was observed that *Arabidopsis* plants expressing this gene had an UV-B tolerance. Another subunit of TFIIH - MAT1 (ménage à trois 1) from sugarcane gene (ScMAT1) was also characterized. There was sequence conservation between human MAT1 and ScMAT1 for the region related to CAK kinase where XPD and XPB proteins interact to MAT1. This work using yeast two-hybrid assay showed that ScMAT1 interacts with proteins related to stress, but ScMAT1 was not induced by stress conditions such as phosphate deficiency, ABA methyl jasmonate, or cold. Therefore, this data suggest an indirect role of ScMAT1 in NER [116].

Plants also have DNA polymerases that are an important component of the DNA repair machinery. The only family of these proteins known to have a role in DNA repair and recombination is the DNA polymerases X family [117]. It was observed that AtPolλ had the C-terminal and N-terminal motives characteristic of the PolX family [118]. Seedlings from two mutant lines for *AtPolλ* (*AtPoll-1* and *AtPoll-2*) showed growth inhibition when compared to the wild-type lines after UV-B radiation exposure. In the same work, it was observed that the *AtPolλ* overexpression lines had a germination inhibition after UV radiation as well as CPD and DSB lesions. These data reinforce the role of *AtPolλ* in the NER DNA repair pathway [118].

6. Double-Strand Break (DSB) repair in plants

Double-strand breaks (DSBs) can be introduced in DNA mainly by metabolic products, ROS, radiation, replication, or transposon excision [119-120]. This structure is also intermediate in several recombination events in eukaryotic cells [121]. When a DSB is detected in the cell, the cell cycle is stopped or arrested for its damage to be repaired [119] to avoid the serious consequences to the cell that an unrepaired DSB can cause, such as chromosome rearrangements, chromosome loss, or cell death [121]. Prokaryotes and eukaryotes have evolved two main pathways to repair DSBs: non-homologous end joining (NHEJ) and homologous recombination (HR) [121-122].

6.1. Non-Homologous End Joining (NHEJ)

NHEJ promotes the ligation of two DNA ends without homology between them. If the DNA damaged is repaired by this pathway, the integrity of DNA molecule is re-established, but the sequence is altered because of the nucleotides additions or deletions at the junction [123]. In plants, as in all the higher eukaryotes, DSBs in somatic cells are mainly repaired by NHEJ, but depending on the conditions of the cell, like the phase of the cell cycle and availability of homologous repair templates, DSBs can also be corrected by homologous recombination (HR) [120, 124]. Bacterial homologs of all DNA repair pathways were found in the genomes of *Arabidopsis* and rice, but not for all the proteins that form the NHEJ pathway up to now [46]. There are more data about this repair pathway in mammals. It is known that the KU complex (KU70/KU80) recruits DNA-dependent protein kinases (DNAPKcs) that activate nucleases, DNA polymerases (polymerase λ or μ), and the XRCC4-Lig4 complex to the sites of the lesion [125]. This was identified in plant orthologs of KU70, KU80, LIG4, and XRCC4 [144-126].

Another process of error-prone DSB repair was reported by direct ligation of extremities of DNA, without the participation of the KU proteins—microhomology-mediated end joining (MMEJ). This pathway uses a microhomologous sequence with the length of 5-25 base pair (bp) in the alignment of the DNA ends, before the ligation, which causes deletions in the flanking region of the DSB [127].

It was recently reported that PARPs (Poly-ADP-ribose polymerases) are involved in several processes and play a role in MMEJ in *Arabidopsis thaliana* [128]. Homologs of *PARP1* and *PARP2* were identified in plants by [129]. PARP1 is one of the proteins that have a role at base excision

repair [130]. There is evidence that PARP is also involved in abiotic stress response, as it was observed that transgenic plants of *A. thaliana* and *Brassica napus* with *PARP* overexpression were more resistant to abiotic stress [131]. In order to investigate whether these proteins are involved in MMEJ, mutants *parp1, parp2, parp1parp2* (*p1p2*), *ku80* and *parp1parp2ku80* (*p1p2ku80*) were analyzed for the MMS sensitivity (a genotoxic agent) and the *in vitro* ability to join linear DNA [128]. It was observed that the *p1p2* double mutant was more sensitive, and this mutant had the root length about half of what was observed in the wild type. The phenotype for the triple mutant *p1p2k80* was stronger than for the double mutant *p1p2*. To test whether the PARP protein had function in MMEJ, an end-joining experiment using a substrate containing 10 bp microhomology sequences was done with cell extracts from wild-type, *ku70*, *ku80*, *p1p2* and *p1p2k80* mutants. It was observed in this experiment that *p1p2* and *p1p2ku80* mutants had two to 20-fold less MMEJ products while *ku70* and *ku80* mutants had four-fold more of these products when compared with the wild type. This demonstrates that PARP proteins are involved in this DNA repair, and a possible competition from PARP and KU proteins may determine which DNA repair pathway may be used to correct these lesions [128].

The *KU70* gene homolog in rice was identified by genome comparative analysis [153]. Studies using two-hybrid and pull-down assays demonstrated an interaction of OsKU70 and OsKU80 proteins, and the importance of OsKU70 for plant development. It was also observed that the *Osku70* mutant was more sensitive to MMS treatment than wild-type plants, suggesting its participation in the DSB repair. Recently, *KU70* from *Vitis vinifera* was cloned and characterized [133]. The mRNA was found in leaves, stem, and roots and its expression increased in response to gamma irradiation.

Agrobacterium tumefaciens is known to transfer the T-DNA region to the plant genome. The T-DNA is converted in double-DNA strand and it is integrated into the host genome [134]. One mechanism proposed for this integration involves the DNA repair pathway NHEJ using the KU80 protein [135]. The *Agrobacterium* plant transformation using *Arabidopsis ku80* mutants showed a decrease in stable transformation and the plants overexpressing *KU80* had increased in T-DNA integration and MMS resistance. However, up to now this mechanism is not clear.

It was verified in rice that the NHEJ pathway is involved in the *Agrobacterium* stable transformation. The mutants lacking NHEJ-related genes *ku70*, *ku80*, and especially the *lig4* mutant presented a reduction in frequency of stable transformation [137]. In tobacco, the role of XRCC4, another NHEJ factor, was observed in a complex with Lig4 to seal the two ends of the DSB during in the T-DNA integration [138]. In this work, it was also observed by yeast two-hybrid assay that the protein VirE2 (from *Agrobacterium*) interacts with XRCC4, then it is proposed that the VirE2 protein may act in the inactivation of XRCC4, then delaying the final step of NHEJ repair, creating an opportunity for T-DNA integration [138].

Besides, *AtPolλ* has been associated to the NER pathway in the repair of DSBs induced by high salinity and mitomycin C (MMC - a DNA cross-linker agent) [17, 118]. An increase in the levels of *AtPolλ* was observed after treatment with NaCl or MMC. On the other hand, for *AtPolλ* mutants had an increase in sensitivity to these treatments. These data propose a possible role of DNA polymerase in DNA repair for these two genotoxic agents. Furthermore, results obtained from yeast two-hybrid assays showed a protein interaction between AtPolλ and

XRCC4-Lig4 [17]. All these data have demonstrated the role of NHEJ in plant transformation as well as its role in abiotic stress.

6.2. Homologous Recombination (HR)

HR is a repair pathway that uses a homologous donor molecule, being, in principle, a DSB repair pathway which is not mutagenic, since all the genetic information can be recovered in the case that the sequence of the template is identical to the broken site [120]. In the HR process many proteins are recruited. PARP1 and PARP2 proteins are considered a sensor for DSBs and then these proteins are important for the recruitment of the MRN complex, which consists of MRE11–Rad50–NBS1 [139-140].

One of the proteins from the MRN complex, *AtMRE11*, has distinct roles in meiosis [141]. Two *Atmre11* T-DNA mutants (*Atmre11-2* and *Atmre11-4*) were analyzed. These mutants produced truncated forms protein with a small difference in size at the C-terminus region. The *Atmre11-2* had a normal phenotype; on the other hand, the *Atmre11-4* mutant had sterility, yellow leaf margins, and misarranged mesophyll cells. To investigate if these phenotypic differences were associated with abnormalities at the cellular or chromosome level, cytogenetic analysis was performed. After this analysis, it was observed that the mitotic phases for *Atmre11-2* and wild-type plants were fine, but for *Atmre11-4* it was observed chromosomal breaks and fusions suggesting genome instability. Since *Atmre11-4* plants were sterile, it was analyzed the pollen mother cells. It was observed problem in the meiosis process, since the regular prophase was not observed. *In silico* analysis proposes that the *Atmre11-4* T-DNA mutant may not have the RAD50 interaction domain in the sequence [141].

For the homologous recombination process, it is important that a stretch of single strand DNA (ssDNA) be produced before the strand invasion. A 5′-3′ degradation of DSB occurs, generating 3′ssDNA overhangs. This process is called end resection and it requires the action of nucleases and helicases. In yeast and mammals the kinases MEC1/ATR, TEL1/ATM, Rad53/CHK1, Cdc5/PL, and CDKs (cyclin-dependent kinases) are involved in this process [142]. Five RAD51 paralog proteins are involved in the process of recombination repair in mammals: RAD51B [143], RAD51C [144], RAD51D [145], XRCC2 [146], and XRCC3 [147]. RAD51C-XRCC3 form one complex and RAD51B-RAD51C-RAD51D-XRCC2 form another complex [148]. In addition to these five genes, *Arabidopsis* and rice have four more *recA-like* genes [148-150].

In *Arabidopsis*, it was observed that AtRAD51 is necessary for double-strand break repair, as the *Atrad51c* mutant has sensitivity to γ-radiation and cisplatin during development, besides it has lower HR frequency and higher chromosome fragmentation in somatic cells [151]. The *RAD51D Arabidopsis* homolog in rice, *OsRAD51D*, was characterized by [152] and it has an important role for reproductive development. The *Osrad51d* mutant presented normal vegetative growth but it had defects in the reproductive development. The *OsRAD51C* characterization showed that the mutant plants had male and female sterility. The cytological analysis showed that this phenotype was a consequence of fragments produced during early meiosis [153]. The DNA-DAMAGE REPAIR/TOLERANCE 100 (DRT100) is a RECA protein identified in plants [154]. In grapevine, it was observed that this protein plays an important role in DNA damage repair and toleration against UV-B irradiation [155].

During HR, in a few cases, there is a second strand capture after the strand exchange, forming a structure called Holliday junction (HJ) that consists of four DNA strands of two homologous chromosomes or sister chromatids [156]. The removal of this structure is important to correct chromosome segregation. The enzymatic processing of recognition and cleavage or resolving these structures is done by resolvase proteins [157-158]. In *Arabidopsis*, the resolvase AtGEN1 and AtSEND1 are members of the Rad2/XPG family and these proteins work similar as the resolvase in *E. coli* [159].

One important aspect for plant survival and reproduction is the accurate transmission of chromosomes during meiosis. The **s**tructural **m**aintenance of **c**hromosomes (SMC) proteins are essential for the architecture and organization of chromosomes, and these proteins are also responsible for sister chromatid cohesion, chromosome condensation, and HR during meiosis [160]. The complex SMC5/6 is one of the SMC complexes and it is related to HR damage repair [161]. MMS21, a SUMO 3 ligase, is a subunit of this complex. The function of *AtMMS21* in HR repair was verified by [162]. In this work, it was observed that *Atmms21* mutants presented sensitivity to MMS, cisplatin, and γ radiation. It was also observed that in *Atmms21, atm* double mutant has a severe morphology defect and it was verified that the HR frequency is reduced in this mutant line. It was verified that *mms21* mutants had a disrupted meiosis. All this data support the role of *AtMMS21* in the HR repair pathway [162-163].

7. DNA repair in organelles

Plants need to maintain the genome stability of the DNA in three compartments: nuclei, mitochondria, and chloroplast. The endosymbiotic theory postulates that these organelles originated from a prokaryotic organism that developed a symbiotic relationship with a eukaryotic host. In the course of time, chloroplast and mitochondrial genomes have been reduced and studies have shown that the nuclei genome integrated DNA from these organelles' genomes.

Many studies have been done on nuclei, but little is known about plant organelle DNA repair (mitochondria or chloroplast) [15, 31]. Comparative analysis proposed that in the *Arabidopsis* genome 17% and 10% of all genes related to DNA repair and recombination (DRR) have chloroplast and mitochondrial origin, respectively. The same investigation was done in the rice genome and it was seen that 19% and 17% of DRR genes have chloroplast and mitochondrial origin, respectively [46].

7.1. Photoreactivation (DR)

This pathway may be an alternative mechanism to correct NER lesions. It has been shown that DR is functional in mitochondrial and chloroplast plant genomes. It was observed that CPDs and 6-4 photoproducts were corrected after *Arabidopsis* leaves were UV radiated and transferred to blue lights (photoreactivation) [164]. In spinach leaf chloroplast no photolyase activity was detected [165]. These data showed that more work is necessary to understand all the mechanism in organelle.

7.2. BER

In plant organelles the BER pathway is also not completely understood. Some data have shown that this pathway occurs in mitochondria and chloroplast. It has been observed that the 8-oxoG lesion in mitochondria needs the OGG1 protein to correct it. Furthermore, it was verified that the mitochondria and chloroplast have a functional Uracil-DNA-glycosylase (UNG) [166]. Furthermore, there is also the presence of thymine glycol DNA glycosylase proteins [167]. In relation to AP endonuclease, it was verified in *A. thaliana* and *Solanum tuberosum* that mitochondria and chloroplast have AP functional endonuclease [166]

With regard to short or long patch pathways, at least the short patch was detected in mitochondria [166]. And for chloroplast, it was identified the presence of two homologs for endonuclease III and one AP endonuclease [167]. For DNA polymerase γ, duplication was observed: POLγ1 (At3g20540) and POLγ2 (At1g50840). Both sequences had a dual targeting – chloroplast and mitochondria. On the other hand, it was observed for DNA ligase 1 an alternative splicing producing proteins that were targeting nuclei and mitochondria, but it is not clear about chloroplasts [168].

7.3. NER

In yeast it has been proposed that it is possible that the NER pathway does not exist and that some lesions may be corrected by other pathways or alternative mechanisms [37, 169-170].

7.4. MMR

The role of the MSH1 sequence was analyzed in *Arabidopsis* mitochondria and it was verified that this protein may have a role in the recombination process. It is not clear how MMR happens in chloroplast and mitochondrial. It is proposed that BER may have an overlap in these organelles [171].

7.5. NHEJ and HR

Arabidopsis mutants for the polymerase *PolIA* and *PolIB* showed the importance of these genes in organelles as it was observed that these mutants had problems to set seeds [172]. Moreover, single mutant for *polIA or pollB* were viable, showing that each gene partially compensates the function of the other [173]. The observation of higher levels of DNA damage in chloroplasts of *polIB* mutants compared to mitochondria suggests the specialized role of PollB protein in this organelle [172].

Furthermore, it was identified in the *Arabidopsis* genome five putative homologues of the bacterial RecA that were predicted to be targeted to mitochondria or chloroplasts [148-149, 154, 174-175]. In the study done by [176], two RECA chloroplast-targeted proteins were analyzed using T-DNA mutants. Although *drt100-1* mutant had the same phenotype, it was observed 24-fold reduction in mRNA expression in the *drt100-1* genotype when compared to wild-type plants. However, this reduction did not alter the structure or the amount of cpDNA presented in wild-type and mutant plants [176]. The analysis of another *RECA*

mutant - *cprecA* also showed no morphological difference between immature seedlings and the wild type, but only after the fourth generation it was observed change coloration and tissue necrosis in leaves [176].

Despite the existence of the homologous recombination DNA repair pathway in mitochondria and chloroplasts, it was verified in *Arabidopsis* DSBs caused by ciprofloxacin treatment produced DNA rearrangements with microhomology at their junctions. This suggested a DNA repair pathway through a microhomology-mediated break-induced replication (MMBIR) [177]. One of the proteins that act in this pathway is the single-stranded DNA-binding proteins WHIRLY (WHY) [178]. The role of WHIRLY in the stability of the organelle genome was observed by [179]. Later, it was proposed by [209] that these proteins would prevent the error-prone DNA repair binding and protecting resected ends at break sites, independent of the sequence.

The genomic comparative data from coding and non-coding sequences from the mitochondria genome of two *Arabidopsis* ecotypes showed that DNA mitochondrial is repaired by different pathways and these regions (coding and non-coding) had different mutation rates and spectra [180-181]. Recently, it was verified that coding regions that are repaired by inaccurate mechanisms will be eliminated by natural selection while the consequences of inaccurate DNA repair in non-coding regions (e.g. mutations) will be kept in the genome [182]. Then, this data would explain the low mutation rates and rearrangements in genes observed in non-coding regions.

8. Plant-specific factors involved in DNA repair

Triggering the DNA repair machinery in response to lesions in DNA is essential in order to maintain the genome integrity [183]. The cell cycle may be arrested to allow that the DNA damage is repaired before entering the mitosis process. The perception of DNA damage activates proteins that promote the suppression of the cyclin-dependent kinase (CDK) activity and consequently arrest the cell cycle process [184]. B-type CDKs (CDKBs) are plant-specific, under cell-cycle control, and divided into CDKB1 and CDKB2 [185].

Endoreplication or endoreduplication is a common modification of the cell cycle, which consists of multiple rounds of replication from the nuclear genome without cytokinesis [186]. A genotoxic stress may promote endoreduplication in plants and animals, but the function of this process in animals remains unclear, as endoreduplication can block mitosis and may be associated with tumor progression [187]. In plants, the process of endoreplication is important, as it may be observed in *Arabidopsis* trichomes. The precursor cells need the endoreduplication process to complete the development [186]. It has been observed that DSBs enhanced the endoreduplication process in *Arabidopsis* [188]. Also observed was a reduced expression of CDK and cyclin B during endoreduplication in trichomes, cells of the epidermis, or mesophyll cells [189].

One of the largest families of plant-specific transcription factors is NAC, and AtSOG1 is one of the proteins that belong to this family [190]. AtSOG1 protein has been associated with transcription, cell-cycle arrest and programmed cell death, and genome stability [191]. Although there is no similarity in p53 and *AtSOG1* sequences, or in their gene regulation, their functions are similar [192]. The AtSOG1 has also been associated with the endoreduplication process after a genotoxic stress that induces DSBs [188].

ATAXIA TELANGIECTASIA MUTATED (ATM) and RAD3-RELATED (ATR) both are protein kinases that act as DNA damage sensors in mammals [193]. In *Arabidopsis*, mutants were sensitive to γ radiation or other genotoxic agents and these mutants also had problems in DNA replication. However, these mutants were viable [190, 194]. It has been proposed that biogenesis of small interfering RNAs, DNA repair, and tolerance to stress are connected [194, 195].

The work [196] described MAINTANCE OF MERISTEMS (MAIN), one of the 14 members of the DUF1723 protein family in *Arabidopsis*, mainly expressed in meristematic cells. The DUF1723 domains are plant-specific and they were described in a work with WRKY and GCM1 transcription factors [191]. It was proposed that MAIN was essential to the genome stability of meristematic cells because *main* mutants presented a high level of DNA damage when compared to the wild type [196]. Three *MAIN*-related genes are present in *Arabidopsis*, forming a subfamily of plant-specific aminotransferase-like proteins. These genes were named *MAINLIKE 1* (*MAIL1*), *MAIL2* and *MAIL3*, and their proteins are localized in the nuclei [197]. MAIN and MAIL1 proteins had 68% of sequence similarity. The mutant *mail1* presented dead cells in the root apical meristem (RAM), as observed in mutants that display genomic instability. It was not established whether cell death was induced in this mutant or whether DNA damaged led to cell death, because this happened without the participation of the ATM/ATR signaling pathway [197].

Plant mitochondria present a striking homologous recombination activity [181]. Therefore, this organelle has plant-specific ssDNA-binding proteins that function in this process like the Organelle single-strand binding protein (OSB) [198] and the WHIRLY protein (WHY) [199]. OSB1, in *Arabidopsis*, was reported as important to the genome stability of mitochondria [198]. The proteins WHIRLY (WHY) may be found in mitochondria (WHY2) and chloroplasts (WHY1 and WHY3) [199]. The report by [200] described Organellar DNA-binding protein 1 (ODB1), another plant-specific mitochondrial ssDNA-binding protein. The role of OSB1 was similar to that of Rad52, in vitro, in homologous recombination - the stimulation of the pairing of complementary sequences. Moreover, the similarity between the DNA-binding domain of ODB1 and RAD52 suggests that ODB1 is involved in the homologous recombination repair pathway.

9. DNA damage in plant cells

Stem cells in plants are maintained in two regions: shoot apical meristem and root apical meristem, both of which may be referred to simply as meristem. The division of meristem

allows plants to grow continually and produce new organs and tissues. These cells may divide for self-renew and also may produce new tissues and organs throughout their lifetime. For example, in shoot apical meristem these cells may produce leaves during the vegetative stage; however, when the plant reaches the reproductive stage, the meristem now produce flowers [200]. Furthermore, like animal stem cells, plant stem cells are kept in microenvironments known as stem cell niches, where signals act to organize and keep the adjacent stem cells [201]. In animals, it is known that stem cells have a low tolerance for DNA lesions. This process leads to apoptosis in order to avoid cancer and protect germline. In plants this process, as well as the tolerance for DNA damage accumulation at meristem cells, is not well-known [200]. There are some differences between animals and plants with regard to programmed cell death (PDC), as some genes/proteins have not yet been identified [202-204]. It has been observed that PDC is dependent on ATM and ATR [205]. [206] identified the *MERISTEM DISORGANIZATION 1* (*MDO* 1) gene that may interact with ATM kinase and is essential for maintenance of plant stem cells by reducing DNA lesions. In *mdo 1-1* mutants an elevated rate of double strand breaks (DSB) was observed, supporting the hypothesis that *MDO* 1 is *ATM* dependent. Furthermore, also observed was the importance of the *MAINTENANCE OF MERISTEMS* (*MAIN*) gene that produces a nuclear protein that acts as a transcription factor or as chromatin remodeling or DNA replication. [207] proposed that this protein may also be important for genome maintenance of division cells.

Considering that plants are sessile organisms, they are exposed to different environmental conditions (abiotic and biotic stress) as well as exogenous mutagens that may increase ROS, which may induce DNA lesion and increase the accumulation of DNA mutations in cells as described above. [208] worked with Arabidopsis mutants at the MMR pathway in order to test the genome maintenance and integrity. They observed in *Atmsh2-1* mutants a low rate of mutation loading, and this was unable to correct errors due to DNA replication. Only at G5 (5^{th} generation) did they observe problems in seed production and morphological changes such as light green leaf, crinkled leaf, early flower, dwarf, stress-like, sterile plants. These authors calculated the mutation frequency and proposed an error rate of 10^{-7} to 10^{-6}, or one base substitution in 30, 000 to 300, 000 bp. [209] also analyzed the mutation frequency in *Arabidopsis* plants exposed to salinity stress. In their experiments they observed variants in mutation and epigenetic variants only at the 10^{th} generation. These were wild-type plants exposed or not (control plants) to saline condition. They analyzed their data by a new sequencing approach (Illumina) and by bisulfate sequencing (for epigenetic variants). The data analysis allowed them to propose three hypotheses to explain the rate and pattern of mutations observed when Arabidopsis plants were exposed to an abiotic stress. The first hypothesis is related to errors occurring because of DNA replication, the second is based on plant metabolism due to ROS, which increases DNA lesions, consequently increasing the rate of mutation; and the third hypothesis is associated with DNA repair. These authors propose similar SOS and SIM mechanisms that permit an increase in DNA mutation in order for the organism to survive. These data showed that this field is not well known, despite its economic importance, and the

environmental factors have a huge influence on this mutation rate. Further study is needed in order to improve new cultivars and increase seed production.

10. Final considerations

DNA repair has been studied since 1970 in bacteria, but in plants the first gene was cloned only in late 80. Then, the worry about the ozone layer reduction and UV light that may affect plant production, DNA repair in plants came to have a place in this field. Genomics and comparative genomics were powerful tools for searching for homologous sequences in genes or proteins in plant genomes that are well characterized in bacteria, yeast, and humans. This data allowed identifying that many gene or protein sequences were also present in plant models such as *Arabidopsis* and rice. Considering the results from evolution, it has been observed that gene duplication happened in plants. Some data revealed the Whole Genome Duplication (WGD) process in plants that was responsible for the loss or duplication of some gene sequences. The loss may be different mutations on the sequence and the duplication of these sequences may acquire new functions or new organelle targeting by either a sub-functionalization or a neo-functionalization process [210, 211]. In the case of MMR, for example, it was determined that MutS homologs were duplicated in plants and the different proteins target different organelles (Table 1). The work with AP endonuclease in sugarcane identified gene duplication in the genome; and by *in silico* analysis they observed that one sequence may target chloroplasts or mitochondria and the sequence duplicated may target the nuclei [95]. Moreover, this duplication event also changed gene regulation as observed in gene expression. In rice it was observed a triple targeting [39]; the mRNA may be translated into one protein and according to the N-terminal sequence this protein may target nuclei, mitochondria, or chloroplasts. Mutants in different genes in DNA repair pathways generally have problems in germination, setting seeds, plant development, or mitoses or meiosis. Even during the *Agrobacterium* T-DNA transfer process, the DNA repair pathways have an important role. Moreover, the data presented here show a connection between stress (abiotic or biotic) and DNA repair. It has been observed that the overexpression of DNA repair genes in transgenic plant makes these plants more tolerant of drought, salinity, and other stress conditions [18, 212-214]. The other aspect of plant metabolism in which DNA repair pathways have importance is the seed germination process, where there is a burst in ROS. Another difference found in plants is that these organisms have FPG and OGG1 sequences [215, 216].

Then, all these data presented here show how important endogenous and exogenous signals are for the plant response and how these signals are connected to make a interconnected network that helps plant make the fine adjustment in their metabolism in order to tolerate the adverse conditions to which these organisms are exposed continually. Moreover, plants have become an interesting model for research because of their sessile style, photosynthesis, the ROS presence and a possible connection among ROS x stress x DNA repair x food importance. Much progress has been made, but a lack of knowledge still remains; consequently, much work needs to be done.

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Chapter 2

Formation, Repair, and Biological Effects of DNA–Protein Cross-Link Damage

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Additional information is available at the end of the chapter

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1. Introduction

Genomic DNA is constantly associated with the various proteins that are involved in DNA folding and transactions. The association between DNA and proteins is reversible, and when prompted, proteins dissociate from or translocate along the DNA strand, leaving the open nucleotide sequence available for replication, transcription, and repair. This process ensures the faithful expression and propagation of genetic information. However, exposure of cells to DNA-damaging agents can cause proteins to become covalently trapped on DNA, generating DNA–protein cross-links (DPCs) [1, 2]. The formation of DPCs was originally demonstrated in bacterial and mammalian cells that had been heavily irradiated with ultraviolet light [3, 4]. It was subsequently shown that DPCs can be produced by various chemical and physical agents, such as aldehydes [5], metal ions [6], and ionizing radiation [7], and by certain types of anticancer agents [8-10].

DPCs are unique among DNA lesions, since they are extremely bulky and are likely to impose steric hindrances upon proteins involved in DNA transactions, and hamper their function. Despite the potential importance of DPCs as genomic damage, they have received less attention than other DNA lesions. Accordingly, much remains to be learned about how cells alleviate the toxic effects of DPCs and about what happens to cells if DPCs are left unrepaired.

The characteristics of DPCs vary considerably with respect to the size, physicochemical properties, biological function, and cross-linking bonds of the trapped proteins. The currently known DPCs can be subdivided into four groups (types 1–4) according to whether and how they are associated with flanking DNA nicks (Fig. 1) [2, 11]. Type 1 DPCs contain proteins that are covalently attached to an undisrupted DNA strand. They are the most common form of

DPC found under physiological conditions and are produced by chemical and physical agents such as aldehydes, chromate, platinum compounds, ionizing radiation, and ultraviolet light [1]. Type 2 DPCs, which were identified very recently *in vitro* and *in vivo*, contain poly(ADP-ribose) polymerase-1 (PARP-1) attached to the 3′ end of a DNA single-strand break (SSB) [12, 13]. They are formed as a result of abortive DNA repair. Type 3 DPCs contain topoisomerase (TOPO) I attached to the 3′ end of an SSB via a tyrosinyl–phosphodiester bond. Finally, type 4 DPCs are formed via the attachment of TOPO II to the two 5′ ends of a DNA double-strand break (DSB) via tyrosinyl–phosphodiester bonds. Type 3 and type 4 DPCs are produced by inhibition of the covalent reaction intermediate of TOPO I and TOPO II, respectively, by TOPO inhibitors (TOPO poisons) or by flanking DNA damage [14].

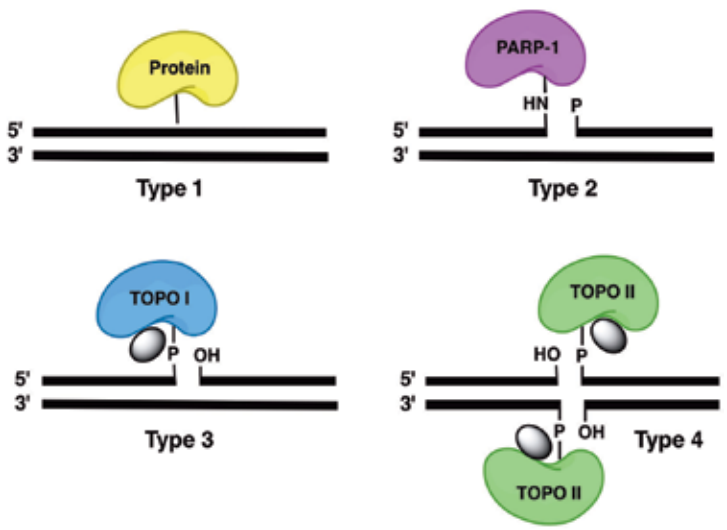

Figure 1. Structures of DPCs of types 1–4. The black ovals shown in DPCs of types 3 and 4 represent TOPO inhibitors, and "P" indicates a 3′- or 5′-terminal phosphate group.

In this article we review the current knowledge regarding the formation, repair, and biological effects of type 1 and 2 DPCs (Fig. 1). There already exist extensive reviews and research papers on similar topics for the TOPO-inhibitor-induced type 3 and type 4 DPCs [15-17], and so these will not be dealt with herein.

2. Detection and characterization of DPCs

2.1. Overview of DPC detection

Analysis of the induction and removal of DPCs in the genome is indispensible when studying the repair and biological effects of DPCs. DPCs can be detected either directly or indirectly; while DNA purification is not required for the indirect detection method (Section 2.2), it is

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required for both direct detection (Section 2.3) and immunodetection (Section 2.4) methods. When required, DNA can be purified by conventional cesium chloride density gradient ultracentrifugation [10, 18, 19] or using the DNAzol-based method [20, 21]. Recently, the rapid and small scale purification methods of DNA were reported and used for immunodetection of DPCs [22, 23]. The methods of DPC detection and their principles are summarized below.

2.2. Indirect detection of DPCs

The indirect methods of detecting DPCs include the alkaline elution, nitrocellulose filter-binding, sodium dodecyl sulfate (SDS)/potassium ion (K^+) precipitation, and single-cell gel-electrophoresis methods. The alkaline elution method is based on the different elutabilities of DNA without and with cross-linked proteins from a filter under alkaline conditions [24, 25]. In brief, cells are filtered onto a polyvinylchloride filter and lysed with sarkosyl; the DNA that is retained on the filter is eluted at pH 12.1. The adsorption of cross-linked proteins to the filter reduces the elutability of unwound single-stranded DNA, thereby changing its elution kinetics. The nitrocellulose filter-binding method depends upon the different abilities of DNA without and with cross-linked proteins to bind to a nitrocellulose filter [26, 27]. In this method, cells are lysed with sarkosyl and passed through the filter, which retains proteins and DNA with cross-linked proteins, but not free DNA. The amount of DNA that is retained on the filter via cross-linked proteins is then assayed for DPCs. The SDS/ K^+ precipitation method is based on SDS binding tightly to proteins to form insoluble precipitates with K^+ [6, 28]. Cells are lysed with SDS, and SDS-bound proteins and DNA with cross-linked proteins, but not free DNA, are selectively precipitated by KCl. The amount of DNA precipitated due to cross-linked proteins is then assayed for DPCs. The single-cell gel-electrophoresis method (the comet assay) detects retarded DNA migration due to a certain type of DPC [29, 30]. Pretreatment of lysed cells with proteinase K enables the distinction between DNA with and without DPCs in these methods.

While a major advantage of these aforementioned indirect methods is that they enable the detection of DPCs without purifying DNA, there is no linear relationship between the amounts of DNA and cross-linked proteins. This makes it difficult to quantitatively interpret the data derived from these indirect measurements of DPCs.

2.3. Direct detection of DPCs

Two techniques have been developed that allow direct and quantitative analysis of DPCs: the ^{125}I-postlabeling and fluorescein isothiocyanate (FITC)-labeling methods. The ^{125}I-postlabeling method is based on the specific incorporation of ^{125}I into tyrosine residues that are associated with purified DNA [31]. A recently reported FITC-labeling method has been shown to provide a more straightforward analysis. Cross-linked proteins in purified DNA are specifically labeled with FITC and directly assayed for the resulting fluorescence [18, 19, 32]. A key advantage of the FITC-labeling method is that the amount of DPCs is proportional to the fluorescence intensity of the labeling.

2.4. Immunodetection of DPCs

Most DPC-inducing agents are fairly nonspecific and covalently trap various proteins. However, DPCs can be detected directly by Western blotting if the identity of the cross-linked protein is known. DNA methyltransferase (DNMT), which is associated with type 1 DPC [10, 22], PARP-1, which is associated with type 2 DPC [12, 13], and TOPOs I and II, which are associated with type 3 and type 4 DPC, respectively [23], can be detected by Western blotting when they are covalently trapped in DNA by inhibitors.

2.5. Proteomic analysis of cross-linked proteins

Considerable insight into the biological effects and repair mechanisms of DPCs can be obtained by identifying the cross-linked proteins in DNA. Comprehensive proteomic analyses of the cross-linked proteins induced by ionizing radiation [20], formaldehyde [33], mechlorethamine (one of the antitumor nitrogen mustards) [34, 35], and butadiene diepoxide (a carcinogenic metabolite of 1,3-butadiene) [36, 37] have been performed. The identified cross-linked proteins include those participating in transcriptional regulation, translation, RNA processing, DNA damage response, DNA repair, cell cycle, homeostasis, cell signaling, and cell architecture. These proteomic approaches may have potential applications in the analysis of DNA-damage interactomes [38].

3. Formation of DPCs

DPCs are produced by various chemical and physical agents or during DNA transactions. Here we summarize the formation of DPCs by selected agents including aldehydes, bifunctional alkylating agents, and ionizing radiation. We also refer to DPC formation by abortive DNA metabolism and repair.

3.1. Formation of DPCs by DNA-damaging agents

3.1.1. Aldehydes

Aldehydes are well-known inducers of type 1 DPCs. Humans are exposed to various aldehydes through anthropogenic and food sources. Aldehydes are also generated by lipid peroxidation and metabolism in cells [39]. Aldehydes that have escaped from the detoxification systems of cells react with DNA, proteins, and other biomolecules and hamper cellular functions. The reactions between aldehydes and DNA result in the formation of DPCs [1, 5] and base adducts [40]. Other DNA lesions such as DNA intrastrand cross-links, DNA interstrand cross-links (ICLs), SSBs, and DSBs may be formed concurrently to varying extents [41].

In light of the reactivity of aldehydes, the side chains of lysine, cysteine, and histidine residues in proteins react with aldehydes to form adducts (Fig. 2). The aromatic amines of DNA bases are weak nucleophiles and are less reactive to aldehydes. The resulting protein adducts react further with the amino group of DNA bases to form various types of cross-linking bond that

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have different chemical stabilities. The cytotoxicities of formaldehyde, chloroacetaldehyde, acrolein, crotonaldehyde, *trans*-2-pentenal, and glutaraldehyde, and their *in vivo* DPC-inducing efficiencies have been analyzed using human MRC5-SV cells and the FITC-labeling method (Table 1) [19]. The results show that chloroacetaldehyde, acrolein, and glutaraldehyde are more potent DPC inducers than are crotonaldehyde, *trans*-2-pentenal, and formaldehyde, and that the DPC-inducing efficiency of aldehydes is correlated with their cytotoxicity. The *in vitro* DPC-inducing efficiencies of these aldehydes (except for chloroacetaldehyde) have also been assessed using plasmid DNA and histone (Table 1) [42]. Comparison of these *in vivo* and *in vitro* data indicates that glutaraldehyde and acrolein are potent DPC-inducers both *in vivo* and *in vitro*, whereas crotonaldehyde and *trans*-2-pentenal are poor DPC-inducers *in vivo* and *in vitro*. Interestingly, formaldehyde is a highly potent DPC-inducer *in vitro*, but a poor DPC-inducer *in vivo*, indicating that formaldehyde is effectively detoxified in cells [43]. The DPCs induced by the aforementioned aldehydes are eliminated from the genome with a half-life 4.8–8.4 h *in vivo*, while they are reversed spontaneously with a half-life 8.0–20.2 h *in vitro* (Table 1) [19]. The shorter half-life *in vivo* may be at least partially attributable to acceleration of DPC reversal by nucleophiles present in cells. There is a positive correlation between the *in vivo* and *in vitro* half-lives of DPCs.

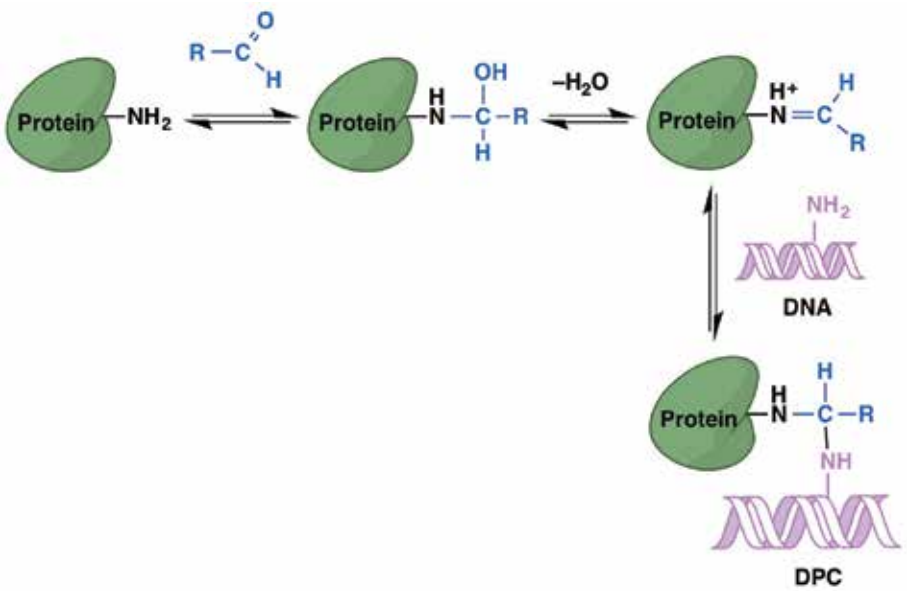

Figure 2. Reaction scheme for DPC formation involving the ε-amino group of lysine (-NH_2 in black) in protein and the amino group of DNA bases (-NH_2 in purple).

Finally, it is also worth noting that the importance of DNA damage induced by endogenous aldehydes has been again acknowledged in recent studies. Studies involving mouse models have revealed that DNA damage induced by endogenous aldehydes is associated with the symptoms of Fanconi anemia (FA), which is a complex heterogenic disorder of genomic

instability, bone marrow failure, and cancer predisposition [44, 45]. In a study involving chicken DT40 cells, it was found that the catabolism of formaldehyde is essential for cells deficient in the FA DNA-repair pathway [46]. The identity of the aldehyde-induced DNA lesion that is responsible for FA remains elusive.

Aldehyde	Cytotoxicity (μM)[a]	DPC-inducing efficiency[b]		Half-life of DPC (h)	
		In vivo	*In vitro*[c]	*In vivo*	*In vitro*[d]
Chloroacetaldehyde	5.6	93	–	7.5	17.0
Acrolein	5.8	64	37	8.2	18.2
Glutaraldehyde	7.1	68	590	7.2	20.2
Crotonaldehyde	110	5.0	0.72	8.4	17.9
trans-2-Pentenal	200	1.0	1.0	5.7	12.4
Formaldehyde	220	4.3	4000	4.8	8.0

[a] Aldehyde concentrations that gave 10% cell survival.

[b] Relative yields of DPCs produced per μM of aldehydes. Data are relative to those for *trans*-2-pentenal.

[c] Data were obtained using plasmid pUC13 and calf thymus histone.

[d] Data were obtained with DNA isolated from aldehyde-treated MRC5-SV cells (pH 7.4 and 37°C).

Table 1. Cytotoxicity and DPC-inducing efficiency of aldehydes. With the exception of that for *in vitro* DPC-inducing efficiency [42], these data were obtained using MRC5-SV cells [19].

3.1.2. Bifunctional alkylating agents

Bifunctional alkylating agents have been generating considerable interest as both health hazards and anticancer drugs. Their biological activity relies on their capacity to cross-link biomolecules, resulting in inactivation of their function. With DNA, bifunctional alkylating agents react with DNA to form monoadducts. The remaining reactive site of these reagents can further react with either DNA to form a DNA–DNA cross-link or a protein to form a DPC [47].

The simple *bis*-electrophiles, such as 1,2-dibromoethane, butadiene diepoxide, and epibromohydrin, are used in industry and are considered to be hazardous to health. In general, many of them are cytotoxic and mutagenic, and induce monoadducts, DNA–DNA cross-links, and DPCs. Interestingly, the cytotoxic and mutagenic effects of 1,2-dibromoethane, butadiene diepoxide, and epibromohydrin in *Escherichia coli* and Chinese hamster cells are significantly increased by the ectopic overexpression of human O^6-alkylguanine-DNA alkyltransferase (hAGT), the primary function of which is to maintain genomic integrity by directly reversing alkylation DNA damage [48, 49]. These agents cross-link hAGT and DNA to form type 1 DPCs together with other DNA damage [50, 51]. An initial reaction occurs between the hAGT and one side of the reagents to produce a reactive intermediate at Cys145 at the active site of hAGT. The resulting intermediate subsequently attacks the *N*7 of guanine in DNA, yielding covalent hAGT–DNA cross-links [50, 51]. It has been proposed that the hAGT–DNA cross-links and/or

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apurinic/apyrimidinic (AP) sites arising from the depurination of hAGT–DNA cross-links are involved in the cytotoxicity and mutagenicity observed in the presence of hAGT.

Glyceraldehyde 3-phosphate dehydrogenase and histones are cross-linked to DNA by butadiene diepoxide *in vitro* [52, 53]. However, in contrast to hAGT, the ectopic expression of these proteins in *E. coli* and concomitant treatment with butadiene diepoxide does not affect cell survival and mutations [52, 53]. As mentioned in Section 2.5, various butadiene diepoxide-induced DPCs were identified in human cells by proteomic analysis [36, 37].

The nitrogen mustards containing *N*-(2-chloroethyl) groups are typical bifunctional alkylating agents and are frequently used for cancer therapy. The *N*-(2-chloroethyl) group cyclizes spontaneously and forms an aziridinium ion, which alkylates DNA and proteins and forms a "half mustard". The resulting half mustard undergoes a similar cycle of reactions to form a DNA–DNA cross-link and a DPC [47]. Accumulated evidence indicates that the chemotherapeutic potential of nitrogen mustards and other cross-linking anticancer drugs such as mitomycin C is mainly attributable to their ability to form ICLs [54, 55]. However, it has not been clarified whether DPCs induced by nitrogen mustards and other cross-linking anticancer drugs potentiate the therapeutic efficacy of the drugs in conjunction with ICLs.

Mechlorethamine belongs to the member of the nitrogen mustards. The formation of DPCs together with ICLs upon the treatment of cells and nuclei with mechlorethamine and other cross-linking agents (nitrosoureas) was initially demonstrated using the alkaline elution method [56]. Recent proteomic analyses have revealed the formation of DPCs in mechlorethamine-treated nuclear extracts and cells, demonstrating the involvement of functionally different proteins in DPCs [34, 35].

Mitomycin C is another class of bifunctional alkylating agent and is also used for cancer therapy. FK973, FK317, and FR900482 are substituted dihydrobenzoxazine derivatives and undergo reductive activation to form the reactive mitosene structures that are similar to that of mitomycin C. Alkaline elution analyses have shown that together with mitomycin C, FK973 forms concentration- and time-dependent ICLs and DPCs in cells, but not SSBs [57]. In addition, chromatin immunoprecipitation analyses have revealed that FR900482 and FK317 cross-link minor-groove binding proteins such as HMGA1, HMGB1, and HMGB2, but not major-groove binding proteins such as NF-κB or Elf-1, to the promoter regions of the IL-2 and IL-2Rα genes to form DPCs *in vivo* [58, 59].

3.1.3. Ionizing radiation

Ionizing radiation causes damage to DNA via both direct and indirect mechanisms. In the direct mechanism, the radiation energy is deposited directly in DNA and produces DNA cation radicals, which are unstable and undergo decomposition. In the indirect mechanism, the radiation energy is deposited to water (i.e., the bulk medium of cells) and produces reactive oxygen species such as hydroxyl radicals, which in turn attack and damage DNA. Various types of radiation-induced DNA lesion have been identified: base damage, SSBs, DSBs, and DPCs. The most critical damage underlying the cell-killing effects of ionizing radiation is attributed to DSBs. The efficiency of DSB formation by ionizing radiation is decreased under

hypoxic conditions relative to normoxic conditions, whereas that of DPC formation is increased under hypoxic conditions [1, 7]. Although the contribution of DPCs to the lethal events in irradiated cells remains to be clarified, the aforementioned opposing effects of oxygen on DSB and DPC formation point to the potential importance of DPCs for hypoxic cells, and in particular those present in tumors.

The induction of DPCs and their removal from the genome following irradiation of normoxic and hypoxic mouse tumors with carbon-ion beams were recently analyzed using the FITC-labeling method [19]. The yield of DPCs was greater by 4-fold in hypoxic tumors than in normoxic tumors. Simultaneously, the yield of DSBs in hypoxic tumors was decreased to 1/2.4 relative to that in normoxic tumors. Interestingly, the carbon-ion beams produced two types of DPC that differed according to their rate of removal from the genome. The half-life of the rapidly removed component of DPCs was less than a few hours *in vivo*, whereas that of the slowly removed component was estimated to be longer than a few days. The rapidly and slowly removed components accounted for 40% and 60% of the total DPCs, respectively, indicating that DPCs remain in the genome much longer than do DSBs, the half-live of which is around several hours *in vivo*. It would be interesting to know whether similar results are observed upon irradiation with X-rays, which are characterized by lower linear energy transfer than that for carbon-ion beams.

It is possible that the rapidly removed DPCs are chemically unstable and reversed spontaneously by hydrolysis as is the case for aldehyde-induced DPCs (see Section 3.1.1 and Fig. 2). Alternatively, they may be peptide-containing DPCs, which are relatively small and are efficiently removed from DNA by nucleotide excision repair (NER) as described in Section 4.4.2. Slowly removed DPCs are virtually irreversible DPCs and are resistant to excision repair as evidenced by their long half-live *in vivo*. These DPCs will contain a stable covalent bond between the DNA and the protein molecules. DPCs containing a stable thymine–tyrosine cross-link bond have been identified in cells irradiated with γ-rays [60]. Furthermore, DPCs containing large proteins are not excised from DNA by NER (see Section 4.4.2). The mechanisms underlying the formation of DPCs through the direct and indirect actions of ionizing radiation and the effect of oxygen on the formation of DPCs have been discussed in a recent review [61].

3.2. Formation of DPCs by abortive DNA metabolism and repair

3.2.1. DPC formation by inhibition of DNA-metabolizing and repair enzymes

Some classes of enzymes form transient covalent complexes with their substrates during catalysis. Those involved in DNA metabolism and repair are no exception to this, and considerable numbers of enzymes have been found that form a transient covalent complex with DNA as a reaction intermediate.

The methylation of cytosine in 5′-CG-3′ sequences is an important carrier of epigenetic information in higher organisms; this methylation is performed by DNMTs including DNMT1 (maintenance methyltransferase), DNMT3a, and DNMT3b (both *de novo* methyltransferases). The methylation reaction proceeds via a covalent intermediate between the DNMTs and the

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target cytosine [62]. The DNMT inhibitor 5-aza-2′-deoxycytidine (azadC; known clinically as decitabine) is metabolized in cells, incorporated into DNA, and partly substituted for cytosine. When DNMTs attempt to methylate DNA, 5-azacytosine (the base moiety of azadC) covalently traps their reaction intermediates and aborts subsequent reactions, leaving type 1 DPCs [62]. AzadC and its analogs are used as anticancer agents; their anticancer activity is at least partly attributable to the toxic effects of the resulting type 1 DPCs [32], although the hypomethylation of DNA due to the passive (covalent trapping) and active (proteasome-mediated degradation) depletion of free DNMT1 may also affect cell viability via the altered gene expression [63-65].

Bifunctional DNA glycosylases and DNA repair proteins such as PARP-1 and Ku have an associated AP lyase activity and react with AP sites in DNA, forming covalent Schiff base intermediates [66-68]. The structure of these covalent Schiff base intermediates is similar to that of type 2 DPCs (Fig. 1). DNA polymerases that have an associated 5′-terminal 2-deoxyribose-5-phosphate (dRP) lyase activity also form covalent Schiff base intermediates [69, 70]. These intermediates mimic type 2 DPCs (Fig. 1), but the protein is tethered to the 5′ end of a SSB via dRP. With the exception of PARP-1, the covalent Schiff base intermediates of the aforementioned glycosylases, repair proteins, and polymerases cannot be isolated, but they can be stabilized by $NaBH_4$-reduction and isolated as DPCs [66]. Interestingly, the formation of stable DPCs containing PARP-1 (type 2) *in vitro* and *in vivo* has recently been demonstrated [12, 13]. Their levels were increased by a PARP-1 inhibitor (4-amino-1,8-naphthalimide) or by the knockout of DNA polymerase β or X-ray repair cross-complementing protein (XRCC)1. The biological and clinical significances of these findings in conjunction with abortive DNA repair remain to be elucidated.

Tyrosyl-DNA phosphodiesterase (Tdp1) is involved in the repair of TOPO I–DNA covalent complexes (see below), and its catalytic cycle involves a covalent reaction intermediate in which a histidine residue is connected to a DNA 3′-phosphate through a phosphoamide linkage [71]. In the strand-breakage reaction catalyzed by TOPO I and TOPO II, a nucleophilic attack of a catalytic tyrosyl residue of the TOPO upon a DNA phosphodiester bond results in transient covalent attachment of the tyrosine to the DNA phosphate either at the 3′-end (TOPO I) or the 5′ end (TOPO II) of the broken DNA (Fig. 1) [15, 16]. TOPO inhibitors (poisons) such as camptothecin (a TOPO I inhibitor) and etoposide (a TOPO II inhibitor) freeze the covalent reaction intermediate and abort the subsequent rejoining of DNA ends, leaving TOPO cleaved complexes that contain strand breaks and protein covalently bound to DNA (type 3 and type 4 DPCs; Fig. 1) [15, 16]. Many chemotherapeutic drugs targeting the covalent TOPO reaction intermediates have been developed since type 3 and type 4 DPCs are complex DNA lesions containing DPC(s) and strand break(s) and would be effective at killing tumor cells.

3.2.2. Suicidal cross-linking DNA damage

Several DNA lesions have been shown to act as suicidal substrates by stably cross-linking base excision repair (BER) enzymes, although the cross-linking reactions have only been demonstrated *in vitro*. The exceptional case with PARP-1 is described in Section 3.2.1.

2-Deoxyribonolactone (dL) is an oxidized form of an AP site and is produced by many DNA-damaging agents. dL in DNA undergoes β-elimination to form α,β-unsaturated lactone at the

3′-terminus. Alternatively, dL can be incised by an AP endonuclease (e.g., APE1) to form a dL phosphate at the 5′-terminus. The lactone in these dL analogs can react with nucleophiles such as lysine to form a stable amide bond. It has been shown that of eight bifunctional DNA glycosylases tested, Nth/Endo III cross-links to dL in DNA, while Fpg and hNEIL1 cross-link to the β-elimination product of dL (Fig. 3A) [72, 73]. The dL phosphate at the 5′-terminus generated by the incision of APE1 also cross-linked to DNA polymerase (Pol) β [74]. Other forms of oxidized 2-deoxyribose (dioxobutane and the C4-oxidized AP site) at the 5′-terminus of DNA produce transient covalent complexes with Pol β and Pol λ, but the complex containing the oxidized sugar and enzyme is subsequently released from DNA, resulting in inactivated free polymerases [75].

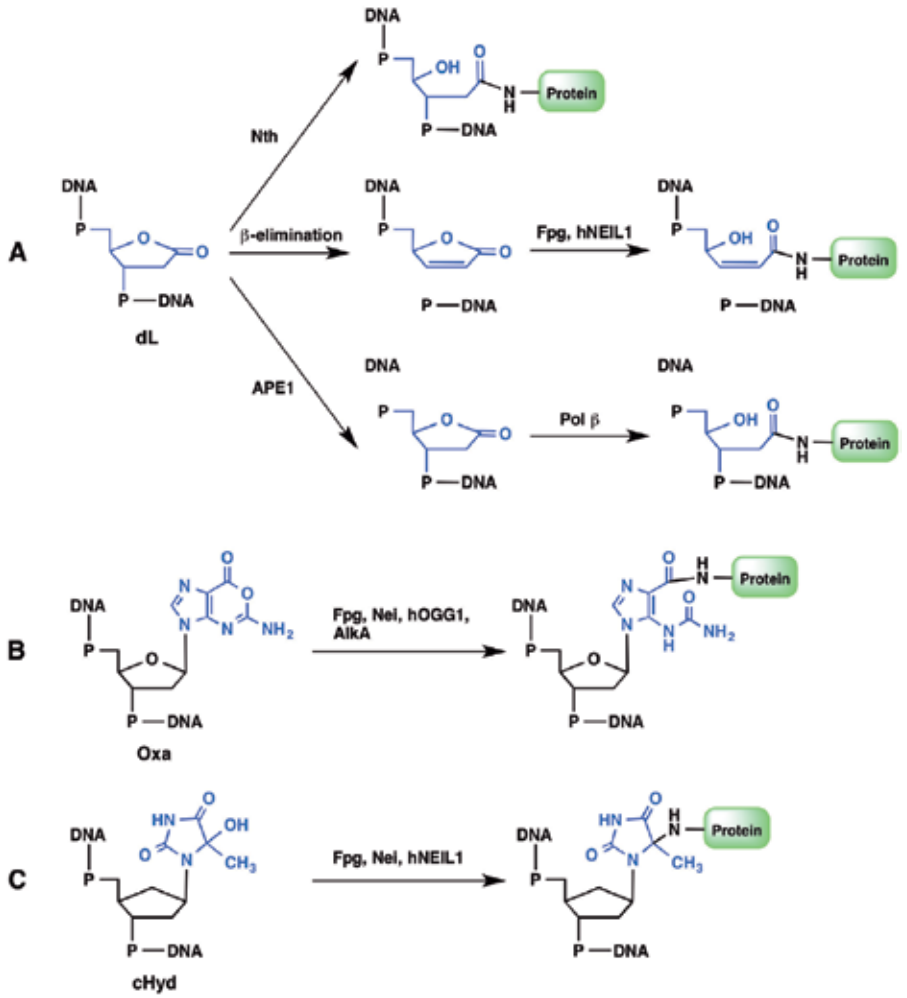

Figure 3. Possible reaction schemes for the suicidal cross-linking reactions of (A) dL, (B) Oxa, and (C) cHyd. The "P" in DNA indicates a phosphate group.

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Oxanine (Oxa) is produced by nitrosative damage of guanine [76] and has a reactive lactone-like structure. It was shown that of seven DNA glycosylase tested, Fpg, Nei/Endo VIII, and hOGG1 (bifunctional glycosylases) and AlkA (monofunctional glycosylase) cross-link to Oxa in DNA to form type 1 DPCs (Fig. 3B) [77]. The glycosylases trapped by Oxa are notably different from those trapped by dL (Nth), suggesting distinct interactions with DNA damage in the active site. Histones also react with Oxa to produce DPCs, but at much lower rates, indicating the importance of specific interactions with proteins in DPC formation. With dL and Oxa, it seems that the catalytic amino acids in the active site (lysine or proline) of the enzymes attack the carbonyl carbon of dL or Oxa, resulting in stable amide bond formation (Fig. 3AB).

5-Hydroxy-5-methylhydantoin is produced by oxidation of thymine, and the carbanucleoside of 5-hydroxy-5-methylhydantoin (cHyd) has been shown to covalently trap Fpg, Nei/Endo VIII, and hNEIL1, serving as a suicidal substrate (Fig. 3C) [78]. The crystal structure of the cHyd-DNA and Fpg covalent complex directly revealed the cross-linking between the N-terminal proline and the C5 of cHyd.

The cross-linking efficiencies of dL, Oxa, and cHyd for glycosylases or polymerases are relatively low. Thus, the biological significance of these lesions as suicidal substrates for repair enzymes remains to be assessed *in vivo*. However, these lesions can be used in mechanistic studies of repair enzymes *in vitro*, and also applied for solving the crystal structure of complexes involving DNA and repair enzymes [78].

4. Repair of DPCs

4.1. DPC repair in bacterial cells

The genes involved in the repair of DPCs have been elucidated by analyzing the sensitivity of a panel of repair-deficient *E. coli* mutants to formaldehyde and 5-azacytidine (azarC; the ribonucleoside form of azadC) that induce type 1 DPCs (these are simply referred to as DPCs in Section 4) [18, 79]. These studies have revealed that two mechanisms underlie the repair of DPCs. The first mechanism involves RecBCD-dependent homologous recombination (HR) and subsequent PriA-dependent replication restart (RR), and the second mechanism involves NER. The sensitivity of mutants (Fig. 4) indicates that the first mechanism is the major mechanism and is effective for DPCs induced by both formaldehyde and azarC, whereas the second mechanism is effective only for those induced by formaldehyde [79]. This finding also suggests the differences in the nature of the DPCs induced by the two reagents. Formaldehyde is a nonspecific DPC inducer and covalently traps various proteins of potentially different sizes. For example, the nucleoid-associated proteins of *E. coli* are the putative candidates of DPCs and contain both small and large proteins (i.e., ranging from 9 to 33 kDa) [80]. Conversely, azarC is incorporated into DNA after metabolic transformation and specifically cross-links a 53 kDa DNMT protein [81]. It was therefore assumed that the DPCs containing large proteins (large DPCs), which are commonly produced by formaldehyde and azarC, are repaired by HR plus RR, while DPCs containing small proteins (small DPCs), which are produced only by

formaldehyde, are repaired by NER. Consistent with this, the removal of formaldehyde-induced small DPCs from the *E. coli* genome was found to be dependent on UvrA protein, which is a component of the UvrABC nuclease involved in bacterial NER [18].

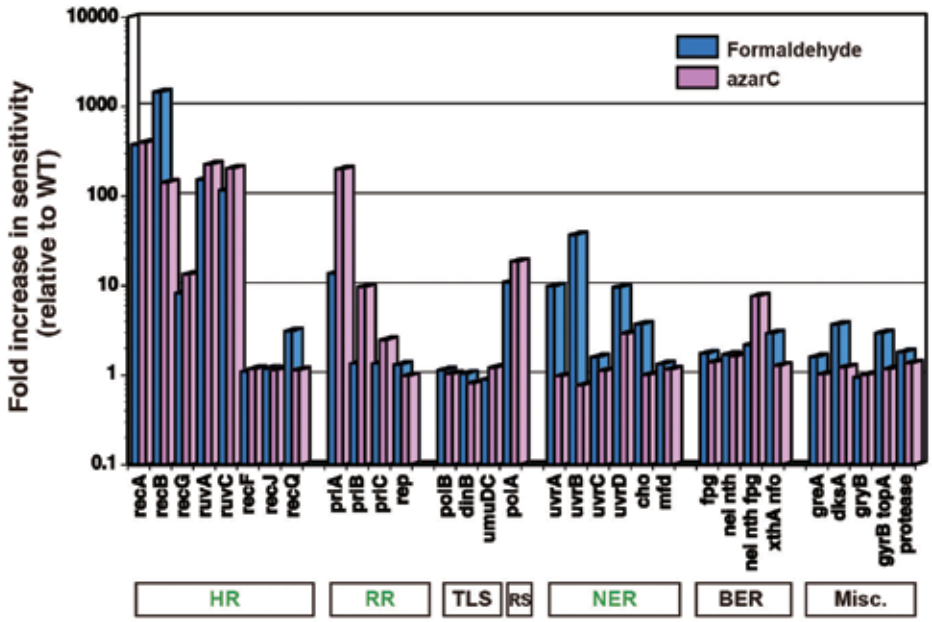

Figure 4. Sensitivity of *E. coli* mutants to formaldehyde (blue) and azarC (red). Mutations were grouped into homologous recombination (HR), replication restart (RR), translesion synthesis (TLS), repair synthesis (RS), nucleotide excision repair (NER), base excision repair (BER), and miscellaneous (Misc.) [79].

In vitro studies involving model substrates have provided further insight into DPC repair by NER. The UvrABC nuclease makes damage-specific incisions for DPCs containing short peptides *in vitro*, but it exhibits poor activity for those containing a T4 endonuclease V protein (16 kDa) [82, 83]. A more systematic study has shown that the dual incision activity of UvrABC is increased for proteins up to 1.6–2.1 kDa, and then decreases for larger proteins, with the activity being negligible for proteins of 25–44 kDa [18]. That study has also revealed the steric inhibition of the loading of UvrB onto the DPC site in the damage-recognition step by large DPCs, abrogating the subsequent recruitment of UvrC that executes the dual DNA incision. Interestingly, the *uvrC* mutant of *E. coli* exhibited a uniquely weak sensitivity to formaldehyde, but the *cho* (a UvrC homolog) mutant exhibited a moderate sensitivity (Fig. 4), suggesting that it has an *in vivo* role as an alternative nuclease in the NER of DPCs [79].

Since large DPCs are not removed from DNA by NER, they stall the replication fork. As mentioned above, the genetic data show the essential role played by HR plus RR in the reactivation of a stalled replication fork by DPCs. The HR of *E. coli* has two subpathways: RecBCD-dependent HR, which is involved in the repair of DSBs, and RecFOR-dependent HR, which is involved in the repair of daughter strand gaps [84]. It has been demonstrated that The

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RecBCD-dependent HR, but not RecFOR-dependent HR, is pivotal to the reactivation of the replication fork stalled by DPCs [79]. Furthermore, other HR components including RuvABC Holliday junction resolvase and the RecG Holliday junction translocase/helicase were required for the HR of DPCs (Fig. 4) [79]. The survival of *E. coli* after treatment with cisplatin and mitomycin C that produce ICLs as well as DPCs requires RecBCD, but it is also moderately dependent upon RecFOR, indicating the distinct requirement of RecFOR for the repair of DNA damage induced by formaldehyde/azarC and cisplatin/mitomycin C [85]. It seems that DNA Pol I (*polA*) are required in HR, but translesion synthesis (TLS) polymerases including Pol II (*polB*), Pol IV (*dinB*), and Pol V (*umuCD*) are dispensable for the damage tolerance pathway associated with DPCs (Fig. 4). It is worth noting that an array of 34 *lacO* repressor sites bound by *lac* repressors impedes fork progression and inhibits cell growth of r*ecA, recB, and recG* mutants but not of *recF* and *ruvABC* mutants [86]. Thus, tolerance to repressors bound to an array of repressor sequences and tolerance to DPCs (Fig. 4) require overlapping recombination genes, with the exception of *ruvABC*.

In *E. coli*, PriA, PriB and PriC proteins play a vital role in RR via two PriA-dependent mechanisms: the PriA–PriB and PriA–PriC pathways [87, 88]. The RR proteins recognize forked DNA structures such as arrested replication forks and D-loops, and load the replicative helicase DnaB for RR [89, 90]. According to the sensitivity to formaldehyde and azarC, the PriA–PriB pathway contributes more to RR than does the PriA–PriC pathway (Fig. 4) [79]. Another Rep–PriC restart pathway [90] appears to be dispensable in RR following the HR of DPCs.

The precise molecular mechanism underlying reactivation of the DPC-induced stalling of the replication fork by HR remains to be established. Inactivation of some replication proteins (DnaB, Rep helicases, DNA Pol III) by mutations results in fork breakage and DSBs in a *recBC* background [91]. However, treatment of the *recB* mutant (and wild type) by formaldehyde did not result in fork breakage, as evidenced by no accumulation of DSBs [18]. This suggests that arrest of the replisome by DPCs does not lead to fork breakage and that the DSB ends processed by the RecBCD helicase/exonuclease are generated by other mechanisms. DSB ends may be formed by the re-replication of incomplete nascent strands [92] or by fork reversal mediated by the RecG helicase [93]. Further study is required to elucidate the underlying molecular mechanism.

4.2. DPC repair in yeast

Yeasts such as *Saccharomyces cerevisiae* are one of the simplest eukaryotic organisms but many essential cellular processes are conserved between yeast and higher organisms. To identify genes that mitigate the cytotoxic effects of DPCs, the *S. cerevisiae* haploid non-essential gene deletion library (ca. 5000 genes) was screened for increased sensitivity to formaldehyde [94]. This screening revealed 44 deletion strains that are sensitive to chronic low-dose exposure to formaldehyde (1.0–1.5 mM for 48 h). The identified genes were those involved in the cell cycle and DNA repair (20 genes), metabolism (6 genes), transcription (7 genes), and others (11 genes). The functions of the identified DNA repair genes were HR [*RAD50*, *RAD51*, *RAD52*, *RAD54*, *RAD55*, *XRS2*(*NBS1*), and *MRE11*], NER [*RAD1*(*XPF*), *RAD4*(*XPC*), and *RAD14*(*XPA*)], post-replication repair (PRR)/TLS [*RAD5*(*SHPRH*) and *MMS2*], and the maintenance of replisome

stability [*SGS1*(*RECQ*) and *TOP3*]. Note that genes within parentheses are mammalian counterparts.

Reexamination of the sensitivity of individual strains to chronic low-dose exposure to formaldehyde has indicated that cell survival is mainly conferred by proteins of the HR pathway and those related to that process [*SGS1*(*RECQ*) and *TOP3*]. The low-to-moderate sensitivity of the NER mutants suggest a less critical contribution of NER to survival following chronic exposure [94]. Although the PRR/TLS-deficient strain *RAD5*(*SHPRH*) was sensitive to chronic formaldehyde exposure, other deletion mutants involved in this pathway did not exhibit sensitivity (*REV1*, *REV3*, *REV7*, *UBC13*, *MMS2*, *RAD6*, *RAD18*, and *RAD30*) [94], suggesting that canonical PRR/TLS is dispensable for cell survival. It is also noteworthy that the deletions of genes involved in ICL repair (*REV3*, *EXO1*, and *PSO2*) did not confer the cells with sensitivity. Thus, it seems that the requirement of repair genes in *S. cerevisiae* [94] reproduces those in *E. coli* [18, 79] with respect to mitigation of the cytotoxic effects of formaldehyde-induced DPCs.

Interestingly, the requirement of repair gene to mitigate the cytotoxic effect of formaldehyde following acute high dose exposure (60 mM, 15 min) differed significantly from that following chronic low-dose exposure to formaldehyde (1.0–1.5 mM for 48 h) [94]. The NER-deletion strains (*RAD1* and *RAD4*) exhibited the highest sensitivity, whereas the HR-deletion strains [*RAD50*, *RAD52*, *MRE11*, *XRS2*(*NBS1*)] and the related strains [*SGS1*(*RECQ*) and *TOP3*] exhibited only moderate sensitivity, suggesting that the relative contributions of DNA repair pathways to protection against formaldehyde-induced DPCs vary with the exposure conditions. Acute high-dose treatment may have changed the cell state analogous to the G_1/G_0 phase of the cell cycle, in which no HR takes place. However, the molecular mechanism underlying the change in the major repair pathway upon acute high-dose exposure remains to be elucidated. Although it is not clear whether relevant to the aforementioned observations with yeast, it has been shown that the concentration and the regimen of formaldehyde treatment affect the formation of DPCs and gene expression in human cells, and that the genes with altered expression are involved in detoxification but not DPC repair [95].

It has been demonstrated very recently that the metalloprotease Wss1 in *S. cerevisiae* is crucial for cell survival after exposure to formaldehyde and camptothecin, which induce type 1 and type 3 DPCs, respectively [96]. The mutants deficient in Wss1 were found to accumulate DPCs. *In vitro* analysis has shown that Wss1 protease cleaves TOPO I-DPCs directly in a DNA dependent manner. In addition, the results with formaldehyde-treated cells suggest that the proteolytic degradation of DPCs by Wss1 enables the TLS of DPC-containing DNA and suppresses gross chromosomal rearrangements that can otherwise occur through the HR of intact DPCs. The authors suggest that proteolysis by Wss1 enables repair of DPCs via downstream canonical DNA repair pathways [96]. Proteins homologous to Wss1 are present in bacteria and several eukaryotes such as fungi, plants, *Plasmodium*, and *Trypanosoma brucei*, but are absent in animals [97]. In higher eukaryotes Dvc1/Spartan, which has a domain organization similar to that of Wss1, may have Wss1-like function [96, 98]. Consistent with the results

with yeast [96], recent analysis of *in vitro* DNA replication using Xenopus egg extracts has shown that the proteolytic degradation of DPCs in leading and lagging strands promotes replication through lesion sites [154].

4.3. DPC repair in chicken DT40 cells

The chicken B lymphocyte cell line DT40 has a high rate of gene targeting and has been used as a model system for reverse genetics studies in higher eukaryotes [99]. The genes involved in the repair of DPCs have been elucidated by assessing the formaldehyde sensitivity of DT40 cells with targeted mutations in various DNA repair genes [100]. In total, 22 mutants involved in different DNA repair pathways were studied: FA (*FANCD2*), HR (*BRCA1*, *BRCA2*, *XRCC2*, *XRCC3*, *RAD51C*, *RAD51D*, *RAD52*, and *RAD54*), PRR/TLS (*REV1*, *REV3*, *RAD18*, and *POLQ*), NER (*XPA*), BER (*PARP1*, *POLB*, and *FEN1*), NHEJ (*DNA-PKcs*, *KU70*, and *LIG4*), and DNA damage response (*ATM* and *CHK1*). With a few exceptions of mutants, the general order of the repair pathways that are critical for cell survival after formaldehyde treatment was FA >> HR > PRR/TLS > NER/BER > NHEJ = DNA damage response = wild type, suggesting that FA, HR, PRR/TLS, and (to a lesser extent) NER are critical for mitigating the cytotoxic effects of formaldehyde. A parallel experiment showed that human *FANCC* and *FANCG* knockout cells were sensitive to formaldehyde [100]. Thus, in addition to HR and NER, which are required in *E. coli* (Section 4.1) and *S. cerevisiae* (Section 4.2), FA and PRR/TLS pathways are also required in DT40 cells. Cells deficient in the FA pathway are sensitive to ICL-inducing agents [101]. Higher eukaryotes use multiple pathways for ICL repair, and the existence of the specialized FA pathway represents a significant difference from yeasts. Accordingly, one possible interpretation of the data with DT40 cells is that formaldehyde simultaneously induces DPCs and ICLs, and that the two lesions are repaired via partially overlapping pathways.

Interestingly, the DT40 *FANCD2* mutant was also sensitive to a simple aldehyde (acetaldehyde), but not to dicarbonyl compounds (glyoxal and methylglyoxal) and α,β-unsaturated aldehydes (acrolein and crotonaldehyde) [100].

4.4. DPC repair in mammalian cells

4.4.1. Sensitivity to DPC-inducing agents

Chinese hamster ovary (CHO) cells deficient in HR (*XRCC3* and *RAD51D*) and NER (*XPD* and *XPF*) were examined for their sensitivity to formaldehyde and azadC [32]. As mentioned in Section 4.1, formaldehyde induces DPCs of various sizes, whereas azadC specifically induces large DPCs containing DNMTs, which are 33–183 kDa in mammalian cells. The HR-deficient CHO mutants were highly sensitive to formaldehyde and azadC. The CHO *XPD* mutant showed slight sensitivity to formaldehyde but not to azadC; similar results were obtained with human NER mutants (*XPA* and *XPD*) [32]. These results indicate that cell survival after treatment with formaldehyde and azadC reagents is mainly conferred by the HR pathway. The low-to-negligible sensitivity of the NER mutants (*XPA* and *XPD*) suggests a less critical contribution of NER to survival after treatment with both reagents. Interestingly, the CHO

XPF mutant was highly sensitive to formaldehyde but not to azadC. The hypersensitivity of the CHO *XPF* mutant but not other CHO NER mutants to formaldehyde was also confirmed by the recent study [102]. The distinct and unique sensitivity of the CHO *XPF* mutant to formaldehyde suggests a role of XPF protein outside canonical NER. As mentioned in Section 4.3, formaldehyde can induce both ICLs and DPCs, and the FA pathway mitigates the cytotoxic effects of ICLs. In this regard, it is noteworthy that *XPF* has been recently added as a member of the family of FA genes, and has been designated *FANCQ* [103].

The sensitivity of FA-pathway-deficient mammalian cells to formaldehyde has also been examined. One study involving mouse and CHO cells found that cell survival was dependent on *FANCD1*/*BRCA2*, *FANCD2*, and *FANCG*, but not on either *FANCA* or *FANCC*, which are present in the core FA complex [104], whereas another study involving human cells found that cell survival was dependent on *FANCC* (and *FANCG*) [100], revealing a different requirement of FA genes. It remains to be seen whether the response of mammalian cells to formaldehyde through the FA pathway is species dependent [105]. With respect to DPCs induced by azadC, human *FANCD1*/*BRCA2* and *FANCD2* cells are only weakly sensitive to azadC [32].

The removal of DPCs induced by formaldehyde and other aldehydes has been analyzed using NER-proficient and NER-deficient (*XPA*) human cells, which revealed that the rate of removal of DPCs from the genome was similar in the two cell types [19]. Another study found that the rate of removal from the genome of hexavalent chromium [Cr(VI)]-induced DPCs was also similar in NER-proficient and NER-deficient (*XPA*) human cells [106]. This study further suggested that the Cr(VI) sensitivity of NER-deficient cells was due to a defect in the repair of Cr-DNA adducts, which are the precursors of Cr(VI)-induced DPCs. With the exception of *XPF* cells that have a defect in ICL repair, the repair of DPC precursors (but not DPCs *per se*) may also account for the slight sensitivity of NER-deficient mammalian cells to formaldehyde. These *in vivo* observations with mammalian cells contrast with those observed with *E. coli*, wherein genomic small DPCs were actively removed by NER *in vivo* [18].

4.4.2. Repair capacity of mammalian NER for DPCs

The capacity of mammalian NER to repair DPCs has been studied *in vitro* using defined DPC substrates. The cell-free extracts (CFEs) from mammalian cells made efficient damage-specific incisions for DPCs containing peptides comprising 4 or 12 amino acids but not for those containing T4 endonuclease V (16 kDa), histone H1 (22 kDa), and HhaI DNMT (37 kDa) [107-109]. A systematic analysis with HeLa CFEs and defined DNA substrates containing DPCs of various sizes demonstrated that the 5′-incision efficiency increased for cross-linked proteins up to 1.6 kDa, and then decreased thereafter (Fig. 5A); the incision activity was negligible for the 11 kDa protein. [32]. This was also the case for 3′-incisions. Together these observations indicate that the upper size limit of cross-linked proteins amenable to mammalian NER is around 8 kDa *in vitro*, which is notably smaller than that for bacterial NER (Fig. 5B). The smaller upper size limit of DPCs for mammalian NER accounts for a less critical contribution of NER to cell survival after treatment with formaldehyde and azadC observed in mammalian cells (see above).

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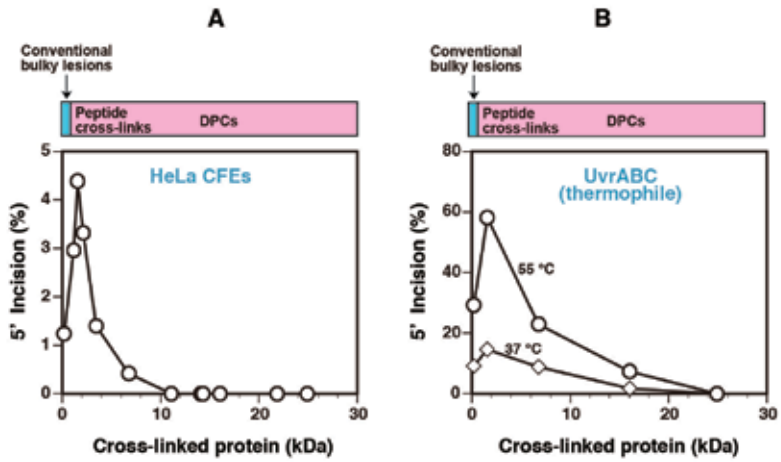

Figure 5. The 5′-incision activity of (A) HeLa CFEs and (B) UvrABC from thermophilic bacteria [32].

As with conventional bulky lesions, the 5′-incision sites were around the 21st phosphodiester bond 5′ to DPCs, and 3′-incision site was at the 6th phosphodiester bond 3′ to DPCs, which indicates that the incision sites are independent of the size of cross-linked proteins [32]. The CFEs from NER-deficient cells exhibited no incision activity for DPCs. Despite significant differences in protein components, bacterial and mammalian NER shares an activity optimum for a cross-linked protein size of around 1.6 kDa, which is several times larger than the sizes of conventional bulky lesions. It would be interesting to know whether this is simply due to a mechanistic reason or if it has some evolutional significance.

An alternative model of DPC repair by mammalian NER has been proposed. In this model cross-linked proteins are initially degraded to short peptides by the proteasome, and due to the robust activity of mammalian NER for DPCs containing short peptides *in vitro*, the resulting DNA-peptide cross-links are removed by NER (Fig. 5A) [11, 83, 108, 109]. Polyubiquitination targets proteins for recognition and degradation by the 26S proteasome [110]. However, it was shown that cross-linked proteins were not polyubiquitinated *in vivo* after treatment with formaldehyde, and hence were not subjected to proteasomal degradation in cells [32]. Very recently the yeast metalloprotease Wss1 and a putative protease in Xenopus egg extracts have been shown to be involved in the repair of DPCs [96, 154]. It would be interesting to elucidate whether the functional homologs are present in mammalian cells, although no clear orthologs of Wss1 seem to exist in mammalian cells (see Section 4.2 for details).

4.4.3. DPC tolerance by HR

Since NER is virtually unable to repair DPCs in mammalian cells, the replication fork will run into the DPC site and become stalled. Given the high sensitivity of HR-deficient CHO mutants to formaldehyde and azadC, HR is pivotal with respect to activation of the DPC-stalled replication fork. Indeed, the formation of RAD51 nuclear foci, which is reminiscent of HR, was observed following treatment with formaldehyde and azadC [32]. Accumulation of DSBs was

observed in HR-deficient CHO cells, but not in HR-proficient CHO cells after treatment with formaldehyde or azadC, suggesting that HR for DPCs is initiated by fork breakage to generate one-sided DSBs [32].

When CHO cells are treated with replication inhibitors such as hydroxyurea and aphidicolin, accumulation of DSBs due to fork breakage is observed even in HR-proficient cells [111, 112], suggesting mechanistic differences in the formation of DSBs by DPCs and replication inhibitors. The MUS81-EME1 and MUS81-EME2 structure specific endonucleases are implicated in fork breakage in mammalian cells [113-115]. However, whether they are also involved in fork breakage at DPCs remains to be elucidated.

The data for DT40 cells suggest that TLS is a crucial component of DPC tolerance [100], but the sensitivity of mammalian TLS mutants to DPC-inducing agents has not been tested. Mouse embryonic stem cells deficient in two major DNA glycosylases (Nth1 and Ogg1) did not exhibit significant sensitivity to formaldehyde and azadC (Ide et al., unpublished data). Regarding the DNA damage response, it was shown that DPCs activate both ATM (ataxia-telangiectasia mutated) and ATR (ATM and Rad3-related) pathways in the late and early stages of damage response, respectively, in human cells [32].

Studies of the tolerance/repair of DPCs have been hampered by the facts that DPC-inducing agents such as aldehydes, bifunctional alkylating agents, and platinum anticancer drugs concurrently produce ICLs, which are also potent lethal lesions, and that tolerance/repair mechanisms for DPCs and ICLs are partly overlapping at least with respect to the requirement of HR and some structure-specific endonucleases. In the replication-dependent ICL repair mechanism, the FA core complex recognizes and binds an ICL that stalled the replication fork. Then, the FA core complex monoubiquitinates FANCD2 and FANCI. The ubiquitinated FANCD2-I heterodimer localizes to the ICL and recruits structure-specific endonucleases (XPF-ERCC1, MUS81-EME1, FAN1, SLX4) that incise the DNA on either side of the lesion to create a DSB. The complementary strand containing the unhooked cross-link is replicated by a TLS polymerase, and downstream FA proteins assist in coordination of HR to repair the DSB [116]. ICLs are also repaired by the replication-independent mechanism involving structure-specific endonucleases and TLS polymerases [117]. Similarly, DPCs can be repaired in a replication-independent manner if NER coupled with DPC-specific proteases (functional Wss1 homologs) operates in mammalian cells (see Section 4.2). The replication-independent repair of DPCs will be important for the survival of nonproliferating cells such as neurons since it ensures faithful gene expression and maintains cellular homeostasis.

5. Biological effects of DPCs

5.1. Overview

Some types of DPC generated by chemical and physical agents are stable and are not spontaneously reversed (Section 3). Furthermore, only small DPCs are actively removed from the genome by NER in bacterial cells (Section 4). Thus, a significant portion of DPCs persist in the

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genome and can affect various aspects of DNA transactions such as replication, transcription, repair, and recombination. This section focuses on the cytotoxic effects of DPCs through DNA replication and transcription.

5.2. Effects on DNA replication

5.2.1. The replisome

DNA is replicated by the replisome, which comprises the replicative DNA helicase, DNA polymerase, and other factors [118, 119]. The replicative helicases unwind the parental double-stranded DNA into two single strands, and DNA polymerases synthesize leading and lagging strands in continuous and discontinuous modes, respectively, using the separated strands as templates. The mechanism is well conserved from phages and bacteria through to higher organisms [118, 119]. The replisome proceeds through the barrier of DNA-associated proteins such as nucleosomes and site-specific DNA-binding proteins. The replicative helicases disrupt nucleosomes in eukaryotes, probably with the aid of histone modifications and chaperones [119]. They can also unwind DNA that is associated with DNA-binding proteins with variable efficiencies [120, 121], and dislodge proteins from double-stranded DNA [122]. Thus, the replisome has an intrinsic capacity to proceed through the protein barrier as long as it is reversible. However, many DNA-damaging agents generate DPCs and immobilize proteins in DNA.

5.2.2. Host-cell reactivation assays

The effects of DPCs on replication were studied *in vitro* using host-cell reactivation assays. Several types of DPC containing DNMT, histone fragments, and T4 endonuclease V were introduced into plasmids and the replication of those plasmids was analyzed in *E. coli* [18, 123, 124]. These studies revealed that DPCs inhibit the replication of plasmids, indicating that the progression of the replisome is impeded by DPCs *in vivo*. Analysis of the replication intermediates of plasmids containing DNMT-DPCs by two-dimensional gel electrophoresis and electron microscopy suggests that replication can switch from the theta to the rolling circles mode after a replication fork is stalled by a DNMT-DPC [123]. A study involving plasmids containing histone fragments as DPCs revealed that the efficiency of replication in *E. coli* varies with the size of the histone fragments and the *uvrA* gene [18]. With plasmids containing T4 endonuclease V as a DPC, the efficiency of replication is dependent on the *uvrD* gene [124].

5.2.3. Effects on DNA polymerases

The effects of DPCs on DNA polymerases have been studied *in vitro* using defined DPC DNA templates. DPCs constitute absolute blocks to DNA polymerase I Klenow fragments with and without 3′-5′ exonuclease and HIV-1 reverse transcriptase [107, 125, 126]. More recently it was shown that DPCs and DNA–peptide cross-links (a 23-mer peptide) completely block DNA replication by TLS DNA polymerases η, κ, ν, and ι *in vitro*, whereas smaller DNA–peptide cross-links (a 10-mer peptide) are bypassed [127]. In addition, TLS polymerase ν can bypass small DNA-peptide cross-links placed in the major but not the minor groove of DNA [128].

The replicative polymerases, such as prokaryotic Pol III holoenzyme and eukaryotic Pol δ and Pol ε, have not been tested for DPCs.

5.2.4. Effects on replicative helicases

The impediment of the replisome by DPCs is more closely associated with replicative helicases that unwind DNA at the front of the replication fork than with replicative polymerases. Replicative helicases such as the phage T7 gene 4 protein (T7gp4), simian virus 40 large T antigen (Tag), and *E. coli* DnaB protein are characterized by their ring-shaped homohexameric structure with a central channel that accommodates DNA [129]. The eukaryotic replicative helicase also assembles into a ring-shaped heterohexamer of minichromosome maintenance (Mcm) proteins 2–7 [130]. In addition, a subcomplex comprised of Mcm4, Mcm6, and Mcm7 (Mcm467) forms a ring-shaped heterohexamer containing two respective subunits and exhibits helicase activity *in vitro* [131]. Coupled with the hydrolysis of NTP (usually ATP), T7gp4 and DnaB helicases translocate along the lagging template strand with 5′–3′ polarity and disrupt the hydrogen bonds between two strands, whereas Tag and Mcm helicases translocate along the leading template strand with 3′–5′ polarity [129, 130].

The effects of DPCs on the DNA-unwinding reaction of replicative helicases have been elucidated *in vitro* using defined DPC substrates [132]. DPCs in the translocating strand, but not those in the nontranslocating strand, were found to impede the progression of the T7gp4, Tag, DnaB, and Mcm467 helicases (a conflicting result with Tag has been reported, [133]). The impediment varied with the size of the cross-linked proteins, with a threshold size for clearance of 5.0–14.1 kDa (Fig. 6), indicating that the central channel of the dynamically translocating hexameric ring helicases can accommodate only small proteins. Although DPCs constitute strong blocks to DNA polymerases, as mentioned above, the results shown in Fig. 6 highlight an alternative mechanism of replisome blockage that involves the inhibition of replicative helicases that unwind DNA at the front of the replication fork.

In addition, the results obtained for helicase suggest the distinct fates of replisomes upon encountering conventional bulky damage and large DPCs. Conventional bulky damage both in the translocating and nontranslocating strands are cleared by helicases and arrest DNA polymerase (Fig. 7A). This can further lead to functional uncoupling of polymerases and helicases as well as that of leading and lagging polymerases. In eukaryotes, the functional uncoupling of polymerases and helicases activates a checkpoint kinase ATR (ATM and Rad3-related), which directs the DNA damage response [134]. DPCs in the translocating strand block the helicase, immediately halting leading- and lagging-strand synthesis (Fig. 7B). This will preclude functional uncoupling of polymerases and helicases and of leading and lagging polymerases. In contrast, DPCs in the nontranslocating strand do not block the helicase, and act like conventional bulky damage. Accordingly, the mechanism underlying stalled fork-processing and the concurrent events of damage signaling may differ significantly for DPCs in the translocating and nontranslocating strands.

Stalled DnaB, T7gp4, and Mcm467 helicases exhibit limited stability and dissociate from DNA with a half-life of 15–36 min *in vitro* [132]. With *E. coli*, replisomes that are blocked by an array of repressor–operator complexes lose the ability to continue replication with a half-life of 4–6

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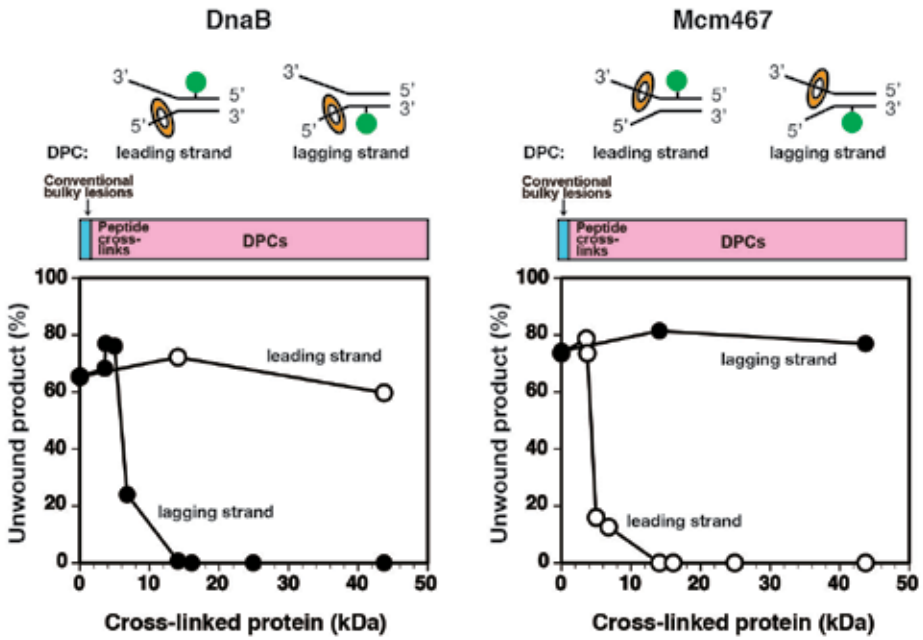

Figure 6. Abilities of the replicative helicases DnaB and Mcm467 to translocate through DPCs [132].

min *in vitro* [135], whereas they retain the ability to resume replication upon removal of the block for several hours *in vivo* [136]. The dissociation of stalled DnaB from DNA accounts at least partially for the inactivation of the replisome *in vitro*. The inactivation of the replisome due to loss of DnaB also seems to be consistent with the finding that reactivation of a stalled replication fork requires reloading of DnaB (or replication machinery) via the PriA helicase in *E. coli* [137], and that the *priA* mutant is hypersensitive to DPC-inducing agents [79].

In yeast, replisomes stalled by tight (but reversible) DNA–protein complexes are stable *in vivo*, and DNA synthesis continues through the barriers after a transient pause (ca. 30 min) [138, 139]. Thus, Mcm is likely to be retained in the stalled replisome in yeast cells. In contrast, a recent study of *in vitro* replication of plasmids with Xenopus egg extracts has shown that Mcm7 (a component of the Mcm complex) dissociates from DNA with an approximate half-life of 10 min when progression of the replisome is blocked by an ICL [140]. This finding with Xenopus egg extracts concurs with the observation that Mcm467 stalled by a DPC dissociates from DNA with a half-life of 33 min. It is possible that the replisome can proceed by gradually disrupting reversible protein roadblocks in cells while retaining the helicase in the replisome. Conversely, this does not occur if the replisome is completely arrested by irreversible roadblocks such as DPCs and ICLs.

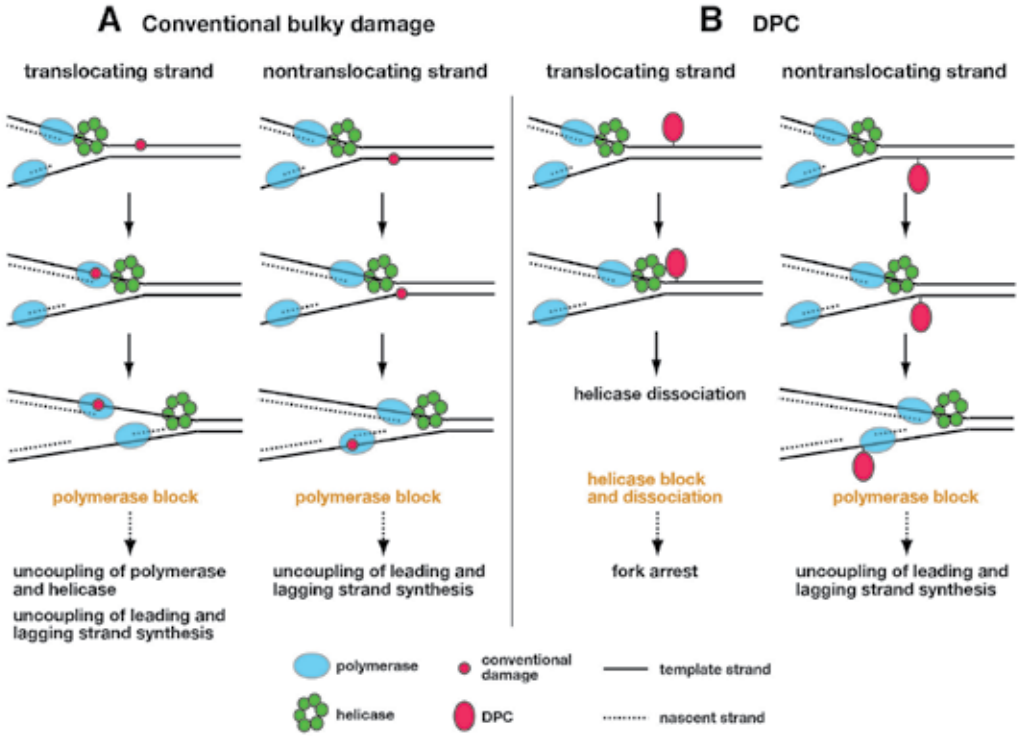

Figure 7. Possible fates of replisomes that encounter (A) conventional bulky damage and (B) DPCs. The scheme is drawn for eukaryotic replication, where the replicative helicase translocates on the leading template strand [132].

5.3. Effects on transcription

5.3.1. RNA polymerase

Viral, prokaryotic, and eukaryotic RNA polymerases (RNAPs) have an ability to transcribe through nucleoproteins and site-specific DNA binding proteins, although the read-through efficiencies vary depending on the roadblocking proteins [141]. ATP-dependent chromatin remodeling complexes, histone chaperones, and covalent histone modifications promote the transcription through nucleosomes [142]. It has also been shown that the trailing RNAP stimulates forward translocation of the stalled leading RNAP through reversibly bound proteins [143, 144], as well as through naturally occurring pausing sites [145, 146].

RNAPs open the downstream DNA duplex at the DNA entry site to generate a transcription bubble, in which the transcribed strand (TS) is delivered deep into the active site and used for nascent RNA synthesis, while the nontranscribed strand (NTS) is relatively exposed to the surface of RNAP [147, 148]. Resolution of the crystal structure of yeast RNAP II revealed that conventional bulky lesions such as a cyclobutane pyrimidine dimer, a cisplatin intrastrand cross-link, and a monofunctional platinum adduct in the TS are delivered to the active site or

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its proximal position and then arrest transcription [149-151]. Conversely, DNA lesions in the NTS impose much less serious problems for transcription than do those in the TS [152].

5.3.2. Reporter assays

Luciferase-based reporter assays are widely used as a tool to study gene expression at the transcriptional level. To assess the effects of DPCs on transcription, histone H1 was cross-linked by formaldehyde to a pGL4.50 plasmid harboring the luciferase gene (Fig. 8A). The pGL4.50 containing histone H1-DPCs was transfected into HeLa cells and the bioluminescence resulting from the expressed luciferase was measured (Ide et al., unpublished data). The luciferase activity was found to decrease with increasing amounts of cross-linked histone H1 protein, indicating that transcription of the luciferase gene by RNAPII was inhibited by DPCs *in vivo* (Fig. 8B).

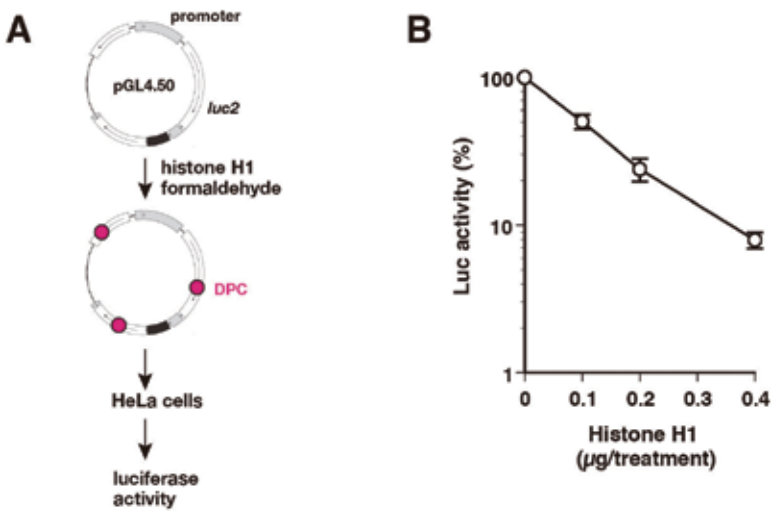

Figure 8. Preparation of a plasmid containing histone H1-DPC (A), and the effect of DPCs on transcription of the luciferase gene (B).

5.3.3. Effects on T7 RNAP

T7 RNAP is a single subunit RNAP and is structurally unrelated to bacterial and eukaryotic multisubunit RNAPs, but all share many functional characteristics in the initiation and elongation phases of transcription [153].

The effects of DPCs on transcription have been analyzed *in vitro* using phage T7 RNAP and defined DNA templates containing DPCs of various sizes (1.6–44 kDa) [126]. When DPCs are present in the TS, both abortive and runoff transcripts were produced, indicating stalling of the T7 RNAP by DPCs. There was trend for the number of copies of runoff transcripts to decrease for larger DPCs. This result indicates that DPCs in the TS pose strong but not absolute

blocks to T7 RNAP, allowing limited but significant lesion bypass even for large DPCs. This property contrasts with that of DNA polymerase I Klenow fragment, which was completely arrested even by the smallest DPC (1.6 kDa). It was also found that when DPCs are present in the NTS, no damage-dependent abortive transcripts are produced, although common weak abortive products form for all templates. The formation of runoff transcripts was retarded only moderately by NTS-DPCs. The number of copies of runoff transcripts was virtually independent of the DPC size, and was 40–60% of that for the control template.

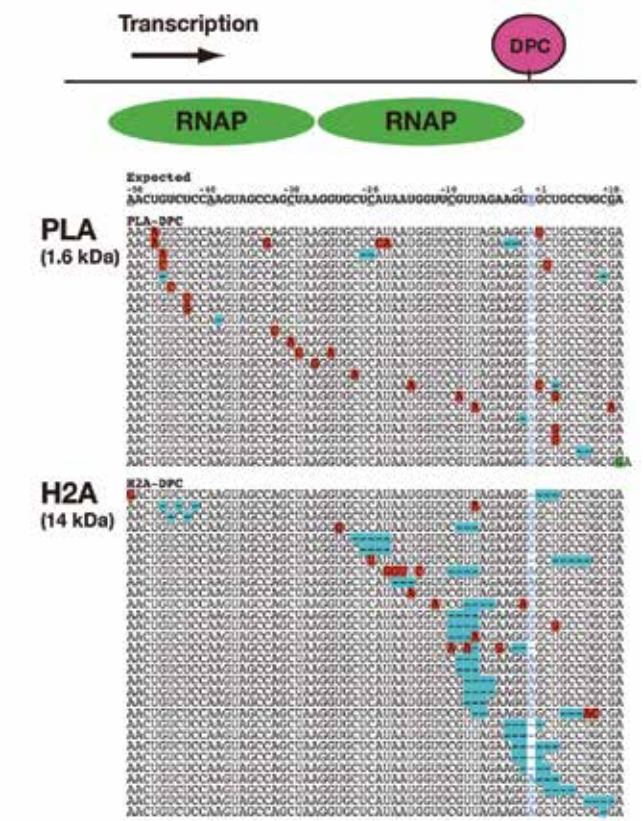

Figure 9. Spectra of untargeted mutations induced by stalled leading and trailing T7 RNAPs. The results obtained with DPC templates containing platelet factor-4 (PLA) and histone H2A (H2A) are shown. Base substitutions, insertions, and deletions [minus signs (–)] in transcripts are highlighted in red, green, and blue, respectively [126].

Stalling of leading T7 RNAP by TS-DPCs caused congestion of the trailing T7 RNAPs. Interestingly, sequence analysis of runoff transcripts has shown that stalled leading and trailing T7 RNAPs become highly error prone and generate untargeted mutations in the upstream intact template regions (Fig. 9); 40–75% of runoff transcripts contained mutations in the region [126]. In contrast, no mutations were induced in runoff transcripts when NTS-DPCs were used. This contrasts with the transcriptional mutations induced by conventional DNA

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lesions, which are delivered to the active site or its proximal position in RNAPs and cause direct misincorporation.

Another interesting observation is that the trailing RNAP stimulates forward translocation of the stalled leading RNAP, promoting the translesion bypass of DPCs [126]. The cooperation of T7 RNAPs enhances transcription through DPCs by a factor of 5.2–17. It has been proposed that bacterial and eukaryotic RNAPs cooperate during elongation so that the trailing RNAP assists in the transcription of the leading RNAP through reversibly bound proteins and pausing sites, by reducing the backtracking of the stalled/paused leading RNAPs [143-146]. Accordingly, similar cooperating mechanism may be working for transcription through DPCs.

How bacterial and eukaryotic multisubunit RNAPs respond to DPCs *in vitro* and *in vivo* remains to be elucidated in future studies.

6. Conclusion

DPCs are superbulky DNA lesions that affect replication, transcription, and repair via mechanisms that differ from those involving conventional bulky lesions.

The findings from *in vitro* studies are summarized below. In DNA replication, DPCs, unlike conventional bulky lesions, block the progression of the replicative helicase and constitute helicase blocks when they are located in the translocating strand. Conversely, DPCs in the nontranslocating strand do not block the helicase. They act like conventional bulky damage and are delivered to polymerases (but not into the active site), constituting polymerase blocks. In transcription, DPCs in the TS block the progression of RNAP, but those in the NTS only moderately affect the transcription through DPCs. T7 RNAPs stalled by DPCs are very error prone. Thus, DPCs exert cytotoxic effects through the impairment of DNA replication and transcription. The impairment of DNA replication and transcription by DPCs has been substantiated *in vivo* by host-cell reactivation and reporter assays using DPC-containing plasmids.

In DNA repair, NER is the major mechanism for the repair of conventional bulky lesions. NER exhibits a robust activity for DNA–peptide cross-links, but a poor to negligible activity for DPCs. The initial recognition of DPCs by NER factors appears to be critical, and is compromised due to the steric hindrance of DPCs. However, the proteolytic degradation of DPCs by proteases may enable NER and TLS to participate in DPC repair. HR plays a principal role in the repair/tolerance of DPCs, but the molecular mechanism by which the DPC-stalled replication fork is reactivated through HR remains to be established.

Studies of the cytotoxic effects and repair of DPCs have been hampered by the facts that DPC-inducing agents concurrently produce other lethal lesions, such as ICLs (aldehydes and bifunctional cross-linking agents) and DSBs (ionizing radiation), and that repair pathways for DPCs, ICLs, and DSBs are partially overlapping. These also pose challenges for studies of the mutagenic and carcinogenic effects of DPCs, which were not addressed in this review. Future

research will overcome these limitations and clarify the importance of DPCs in DNA damage together with the underlying molecular mechanism of the repair/tolerance of DPCs.

Abbreviations

AGT: O^6-alkylguanine-DNA alkyltransferase; AP: apurinic/apyrimidinic; azadC: 5-aza-2'-deoxycytidine; azarC: 5-azacytidine; BER, base excision repair; CHO. Chinese hamster ovary cells; cHyd: carbanucleoside of 5-hydroxy-5-methylhydantoin; dL: 2-deoxyribonolactone; DNMT: DNA methyltransferase; DPC: DNA-protein cross-link; dRP: 2-deoxyribose-5-phosphate; DSB: DNA double-strand break; FA: Fanconi anemia; FITC: fluorescein isothiocyanate; HR: homologous recombination; ICL: interstrand cross-link; Mcm: minichromosome maintenance; NER: nucleotide excision repair; NTS: nontranscribed strand; Oxa: oxanine; PARP-1: poly(ADP-ribose) polymerase-1; PRR: post-replication repair; RNAP: RNA polymerase; RR: replication restart; SDS: sodium dodecyl sulfate; SSB: DNA single-strand break; T7gp4: phage T7 gene 4 protein; Tag: simian virus 40 large T antigen; Tdp1: tyrosyl-DNA phosphodiesterase; TLS: translesion synthesis; TOPO: topoisomerase; TS: transcribed strand; XRCC: X-ray repair cross-complementing protein.

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Chapter 3

Ubiquitination — An Evolving Role in DNA Repair

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Additional information is available at the end of the chapter

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1. Introduction

Upon DNA damage due to endogenous or exogenous causes, chromatin is dynamically modified, especially through posttranslational modifications (PTMs) of histones together with noncoding RNA expression. Both procedures modify accessibility of genes and regulatory genomic loci by protein factors and enzymes involved in gene expression as well as DNA repair processes. In addition, PTMs of proteins involved in genome integrity seem to play important roles in regulating their functions and protein–protein interactions (PPI). The most studied PTMs involved in DNA repair machinery function are histone phosphorylations, methylations/demethylations and to a lesser extent acetylations, allowing/prohibiting accessibility to double-strand break recognition and binding of factors/enzymes. Protein ubiquitination – the covalent link of the small protein ubiquitin (Ub) to lysine residues of a target protein – was classically related to protein degradation, ensuring structural integrity control and/or protein turnover rate. This procedure involves the addition of multiple ubiquitin molecules in a specific manner, which targets the polyubiquitinated protein to the proteasome for degradation. In recent years, accumulating data unveiled a role for nondegrading ubiquitination of proteins involved in DNA repair pathways and cell fate decisions [1,2]. A well-documented overview depicting the exceptional importance of ubiquitination in the restoration of genotoxic insults was carried out by a number of reviews, clearly pointing out ubiquitination and DNA damage response/repair interrelation [3]. The issue comprehensively covers practically all principal aspects of Ub function in the field. Therefore, many issues of the ubiquitin landscape at DNA double-strand breaks (DSBs) have been highlighted. As described, many challenges and puzzles remain to be solved regarding the intimate relationship between the DNA repair machinery and nondegrading ubiquitin signaling at DNA DSBs and the surrounding chromatin (Fig. 1) [3].

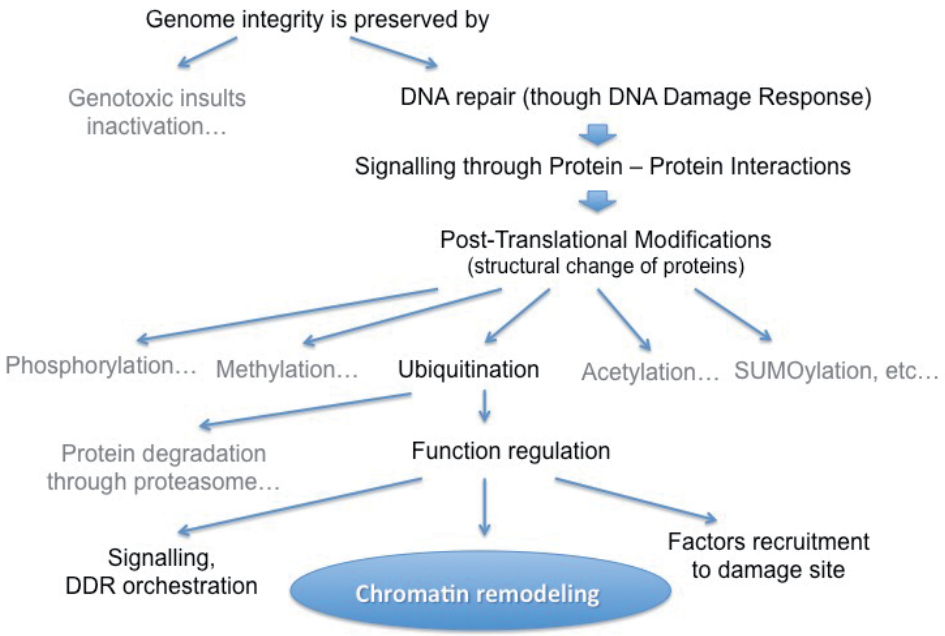

Figure 1. A simplified scheme of ubiquitination involvement in DNA repair through chromatin remodeling. (Details in the text.)

Consequently, the current chapter's objectives are focused on clarifying aspects of ubiquitination involvement in DNA repair regulation as a PTM and not as a signal for degradation and more specifically on chromatin remodeling at the site of DNA damage and its vicinity. Chromatin relaxation and/or histone removal controls, in turn, the accessibility of damaged DNA molecules to the DNA damage response (DDR) machinery and repair processes. In addition, abolishment of ubiquitin add-on, to specific lysine residues of histones H2A and H2B may also play an additional role in malignancy. Clarification of the mechanisms and the pathways involved, apart from contributing to the deeper understanding of basic mechanisms governing genome integrity, may also lead to the development of innovative therapeutic approaches against major cancer types and shed light on aging phenomena related to DNA repair deficiencies.

2. Ubiquitin as a PTM

Ubiquitin (Ub) is a small (76 amino-acid residues, 8.5 kDa molecular mass) regulatory protein expressed in all cell types (ubiquitously) of eukaryotic organisms. It is highly conserved among eukaryotic species: for example, human and yeast ubiquitin share 96% sequence identity.

Ubiquitin is covalently attached to a large range of cellular proteins by specific enzymatic reactions referred to as the ubiquitination system. The ubiquitination reaction is an ATP- and

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Mg2+-dependent process where the carboxyl group of the C-terminal glycine residue of ubiquitin (G76) is the moiety conjugated to substrate lysine residues [4]. Ub performs its myriad functions through conjugation to a large range of target proteins. A variety of different modifications can occur. Key features include its C-terminal tail and the seven lysine residues of the protein. Ubiquitination may exert its function via multiple ways. As depicted in Fig. 2, ubiquitination was primarily attributed to signal proteins for degradation via the proteasome but it may also alter protein cellular location, affect protein activity, and promote or prevent protein–protein interactions [5-7]. More specifically, protein modifications by ubiquitination may de distinct. Either a single ubiquitin molecule (monoubiquitination) or a chain of ubiquitins (polyubiquitination) can be added. The ubiquitin is ligated on the target protein through one of its seven lysine residues. These "linking" lysine residues are defined by a "K" (one-letter lysine symbol) and a number, referring to K position on the ubiquitin molecule, from the amino-terminal to the carboxy-terminal edge of the protein. Initially, a ubiquitin molecule is bonded by its C-terminus (G76) to usually the ε-amino group of a specific lysine residue (e.g., K48, K29, K63) of the target protein. Polyubiquitination occurs when the C-terminus of another ubiquitin, is linked again to a lysine residue (e.g., again K48 or K29) on the previously added ubiquitin molecule, forming a chain. This process is repeated several times, leading to the addition of several ubiquitins on a certain protein molecule. While polyubiquitination mostly on K48 and K29, is related to degradation via the proteasome (referred to as the "molecular kiss of death"), other polyubiquitinations (e.g., on K63, K11, K6) and monoubiquitinations may regulate several cellular processes such as endocytic trafficking, inflammation, gene expression, and DNA repair (Fig. 2) [8]. Recent publications also relate ubiquitination to stem cell differentiation [9].

The most extensively studied ubiquitin chains are the K48-linked ones, which signal proteins to the proteasome for degradation and recycling [10]. This discovery was honored by the Nobel Prize for chemistry in 2004. The ubiquitin-proteasome system is implicated through protein homeostasis in practically all aspects of cellular processes including DNA repair [11-12]. A nice example of DNA repair regulation through ubiquitination by UBR3 E3 ligase and subsequent protein degradation is the control of APE1 (Ref-1), a protein involved in DNA repair (mostly excision repair of abasic sites) and regulation of transcription [13]. On the other hand, proteins ubiquitinized in K63 are primarily involved in protein–protein interactions (PPI) and in turn in cascade signaling and chromatin remodeling. The process may involve both, mono- or polyubiquitination. Overall, protein structural modification through ubiquitination may thus affect its localization, activity, and/or stability together with interactions with partner molecules.

Ubiquitination is carried out in three main steps performed by the concerted action of respective types of enzymes organized in multiple levels of specificity. The activation is performed by a family of ubiquitin-activating enzymes termed as E1 ligases, the conjugation by a group of ubiquitin-conjugating enzymes (E2 ligases), and finally the ligation on the target protein by the ubiquitin ligases of E3 type. This sequential cascade binds ubiquitin to lysine residues on the protein substrate via an isopeptide bond or to the amino group of the protein's N-terminus via a peptide bond [10,14]. Among the ligases, the E2 types act as key mediators

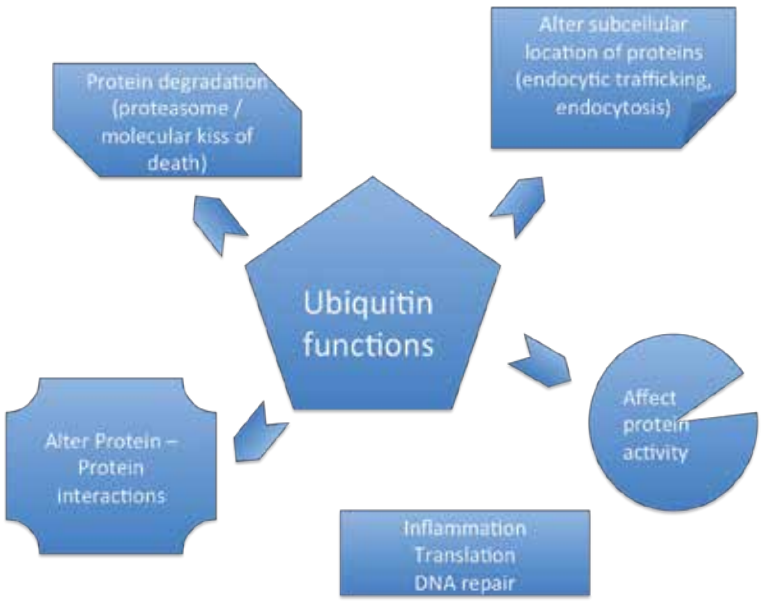

Figure 2. Schematic illustration overviewing ubiquitin functions in the cell.

of chain assembly, thus being able to govern the switch from ubiquitin chain initiation to elongation. By this activity, E2 ligases regulate the processivity of chain formation and establish the topology of assembled chains, thereby determining the consequences of ubiquitination for the modified proteins [15]. Addition of the activated ubiquitin on the target protein substrates is performed by a variety of enzymes collectively termed as E3 ubiiquitin ligases. The human genome codes for only two E1s, about 35 E2s and strikingly, more than 500 E3 type enzymes.

Ubiquitination comprises a dynamic phenomenon; therefore, Ub removal enzymes termed as deubiquitinases have also been detected. A number of specific deubiquitinases (DUBs) recognizing both the Ub position and the target protein have been characterized. DUBs' action reverses the ubiquitination-induced function and serves as a rapid mechanism to adjust cellular responses to stimuli by the fine-tuning of Ub-driven complex formation. A characteristic example of DUB activity in relation to DDR signalling cascade is the fine regulation of the E3 ubiquitin ligases RNF8-RNF168 activity. At the sites of DSBs, lysine63-linked ubiquitin chains are built at the damage sites, through the activity of RNF8-RNF168 complex, driving the effective assembly of DNA repair factors for proper control and repair. RNF168 has a short half-life and upon DNA damage is stabilized by the DUB USP34. Abolishment of USP34 activity results in rapid degradation of RNF168 that in turn, through attenuated DSB-associated ubiquitination, results in defective recruitment of BRCA1 and 53BP1 at the damage sites and compromised cell survival following ionizing radiation [16].

In addition, a number of protein molecules termed Cullin Family proteins, which serve as scaffolds to complex formation of E3 Ub ligases with RING proteins adaptor proteins, and

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substrate recognition receptors, add another degree of complexity in the ubiquitin embroidery of cellular functions [17].

Overall, ubiquitin addition/removal on a specific K residue of a target protein is a highly regulated and finely tuned process involved in regulation of many crucial cellular pathways. During DDR and genome repair, nondegradative protein ubiquitination seems to be implicating in a number of procedures including sensoring/signalling, chromatin remodelling and factor recruitment to damage points.

3. Ubiquitination in DNA damage response

DNA damage is sensed by a number of DNA interacting proteins, mainly histone subunits. The primary signal produced appears to be the phosphorylation of H2Ax (γH2AX), activating in turn ATM/ATR kinases, which serve as sensors and through a series of target molecules phosphorylation the DNA damage alert is transduced to appropriate pathways toward activating DDR mediators and effectors (Fig. 3). One of the primary mediators activated by ATM/ATR kinases is BRCA1, which in turn through its multiple roles orchestrates DDR [18]. BRCA1 dynamically interacts with numerous protein partners and according to these interactions (cell cycle stage dependent) is involved in cell cycle regulation, transcription coupled repair, and repair processes [19]. Non-degradative ubiquitination of histones and a number of DDR factors is also a crucial event upon DNA damage (Fig. 4) and is sensed by a number of DNA interacting proteins, mainly histone subunits and chromatin remodelers [16].

Upon triggering the DNA damage response by introduction of a DNA double stand brake, the initial response step includes histone variant H2AX molecules rapid phosphorylation at the γ position (γH2AX) along chromatin tracks flanking the DSB. Phosphorylation is performed by kinases ATM, ATR, and DNA-PK [20]. H2AX phosphorylation facilitates in turn the accumulation of DNA damage response regulators, Mdc1/NFBD1 [21,22]. RNF8, and RNF168. RNF8 and RNF168 are RING-type E3 ubiquitin ligases, which catalyze the K63-linked polyubiquitin chain formation on histones H2A and H2AX [23-26]. RAP80 is then recognized and recruited to the K63-linked polyubiquitinated histones driven by its ubiquitin interaction motif [27-29]. Recruitment of RAP80 allows docking of BRCA1. Moreover, induction of the intact IR-induced G2/M checkpoint is also dependent on RAP80 and its interaction with K63-linked polyubiquitin chains on H2A and H2AX [27-30]. Abolishment of histone ubiquitination enzymes by knockdown experiments impairs DSB-associated polyubiquitination of H2A and H2AX and inhibits retention of 53BP1 and BRCA1 at the DSB sites, thus resulting in sensitization of the corresponding cells to ionizing radiation [23-26, 31].

Remarkably, the quantity and stoichiometry of ubiquitinated factors at the site of the lesion and the flanking area appear to direct the cell toward selecting the appropriate repair pathway. Recent findings elegantly showed that the stoichiometry of the ubiquitin-binding proteins RAD18 and RNF168 are related to the selection of either error-prone Non Homologous End Joining (NHEJ) or the high-fidelity Homologous Recombination (HR) pathway in IR-treated cells (Fig. 5) [32]. More specifically, the hierarchical assembly of ubiquitin-related factors that

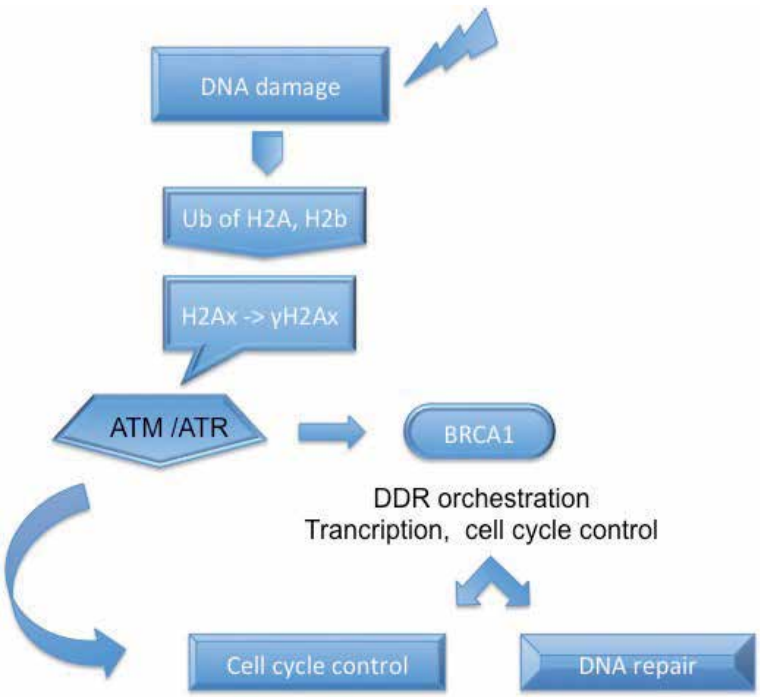

Figure 3. Schematic representation of the initial cascade of reactions following DNA damage. Ubiquitination of DNA packaging proteins (H2A and H2B) result in chromatin remodeling, enabling the binding of signaling and repair factors.

begin with RNF8 assembly is further enhanced by RNF168, facilitating the association of 53BP1 and the Ub ligases BRCA1 and RAD18. As 53BP1 blocks the resection of broken DNA strands, it suppresses HR in favor of the NHEJ pathway. RAD18 overexpression dramatically impairs 53BP1 and in turn favors RAP80–BRCA1 binding to lesion sites, following IR, without affecting damage signaling, repair, or radiosensitivity. In this case, the HR pathway is promoted [32]. In accordance with these data, it seems that the key selection point of the repair pathway, the RAD18 E3 ligase, when monoubiquitinated (RAD18-ub1), does not interact with SNF2 histone linker plant homeodomain RING helicase (SHPRH) nor with helicase-like transcription factor, two downstream E3 ligases required for the promotion of error-free bypass of lesions during genome duplication. Interestingly, the RAD18-ub1 form by its zinc finger domain, binds to nonubiquitinated RAD18, thus inhibiting RAD18's function and resulting in fine-tuning of the ratio of ubiquitinated versus nonubiquitinated forms of RAD18 in the nucleus [33]. It is of interest that ubiquitination not only prevents RAD18 from localizing to the damage site but also, through ubiquitination of the proliferating cell nuclear antigen (PCNA) [34], a factor facilitating DNA replication, suppresses mutagenesis [35]. These data are concomitant with a model where monoubiquitination controls RAD18 function by sequestering active (non-ubiquitinated) RAD18 molecules. The damage-triggered removal of the ubiquitin load by one or more DUBs favors the switch from RAD18-ub1–RAD18 complex to RAD18–SHPRH complex formation required for high-fidelity lesion bypass during DNA replication [33].

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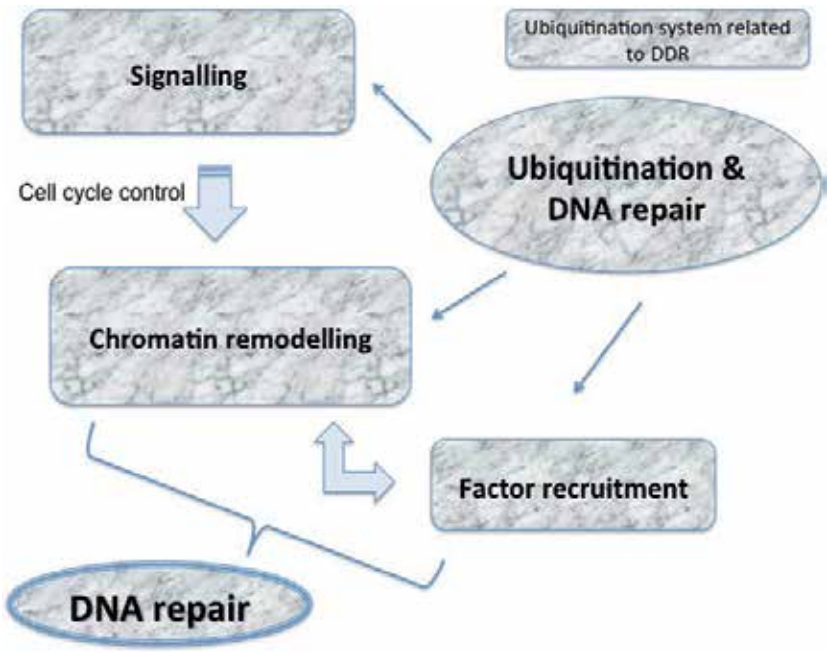

Figure 4. Nondegradative ubiquitination of DNA damage response and repair factors together with chromatin structure modification is a dynamic process essential for genome restoration and cell viability. Ubiquitination has been detected in signaling molecules, mediators, and effectors of DDR as well as histone subunits and histone interacting factors. Deregulation of this cascade is implicated in malignancies.

Therefore, it seems that ubiquitin-family (ubiquitin and ubiquitin-like molecules like SUMO) modifications regulate damage-induced template switching. Moreover, the ratio of ubiquitinated to nonubiquitinated RAD18 appears to be modified during cell cycle progression, thus serving as a posttranslational mechanism controlling RAD18 activity, while it may also be implicated in abnormal cell state conditions and malignancies.

An elegant example of ubiquitination regulation of DNA repair per se comprises the modification of substrate recognition and activities of FBH1 helicase. Single-molecule sorting revealed that ubiquitination affects FBH1 interaction with the RAD51 nucleoprotein filament – the major recombinase of the HR pathway, without perturbing its translocase and helicase activities [36].

Replication Protein A (RPA) complex is another factor involved in the HR pathway (repair based on replicated sister chromatid sequence) by polymerizing onto single-stranded DNA (ssDNA) and coordinating the recruitment and exchange of factors involved in DNA replication, recombination, and repair. The RPA-ssDNA platform also activates the master ATR kinase during replication stress. The RPA complex is regulated by a number of post-translational modifications, one of which is ubiquitination. RPA ubiquitination results in modulation of its interactions with partner proteins, a critical function in the maintenance of genome stability through the error-free HR process [37].

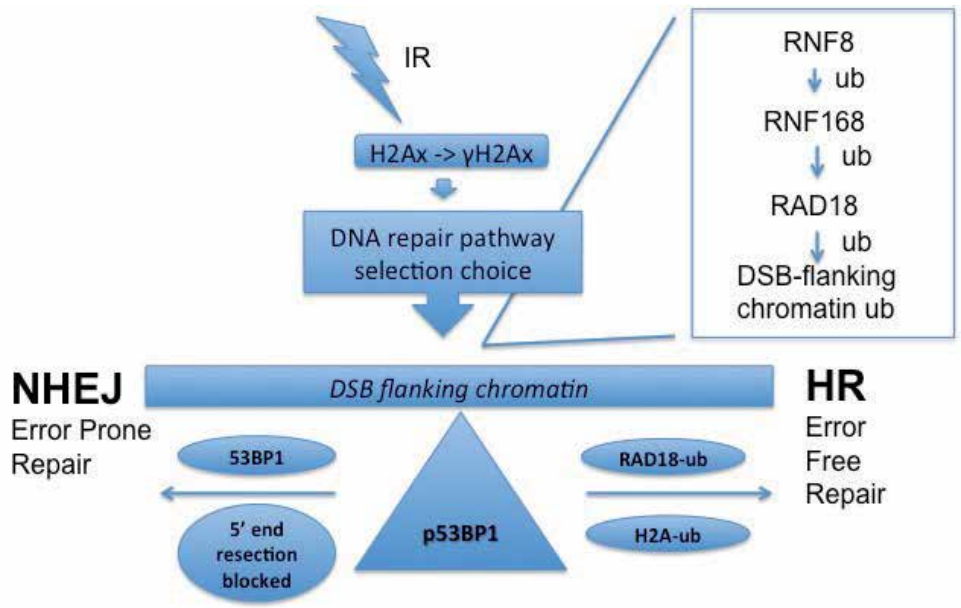

Figure 5. Stoichiometry of Ub-binding proteins RAD18 / RNF168 associated with IR-induced foci influences 53BP1 association and subsequent selection of the repair pathway.

4. Ubiquitination & chromatin remodeling in DNA repair

Chromatin consists of functional units of DNA packed in solid nucleoprotein structures, the nucleosomes. Nucleosomes are composed of the four core histone proteins (H2A, H2B, H3, and H4) wrapped by 147 base-pairs (bp) of DNA. Two nucleosomes are separated by linker DNA, ranging between 20 and 80 bp in length. Nucleosomes, apart from packing the large eukaryotic genomes into the limited volume of the nucleus, are also responsible for the DNA accessibility by interacting with DNA binding factors and modifying enzymes. Monoubiquitination of histone H2B shows a genome-wide distribution in different organisms and is probably related to hetero-/euchromatin determination as well as gene expression profiles.

DNA damage induces structural changes in chromatin, serving as the initial signal for DDR sensors. In order to repair the nuclear DNA, multiple regulated processes facilitate the exposure of DNA at the lesion point and its vicinity. As the DNA repair machinery requires direct access to DNA, nucleosomes should either loosen, move, or be removed from the damaged area. Recruitment of enzymes and factors enabling repair is thus facilitated/allowed by structural changes in histones – the protein components of nucleosomes [38-42]. In general, the local chromatin architecture is mainly driven by nucleosome remodelers and histone and DNA modifiers. Ubiquitination of histones and histone binding factors results in critical chromatin rearrangements either genome-wide or at the local scale, enabling accessibility to factors controlling important biological processes like transcription, genome duplication, chromatin condensation, and DNA repair. Regarding DDR, ubiquitination of several DNA repair machinery components enables interactions with factors recruited from other cellular

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pathways during DNA damage [43-45]. A threatened genome is an extremely stressful and emergency condition for the cell requiring rapid responses. These responses are definitely required for prompt lesion restoration and cell viability [46].

Histones H2A, H2AX and H2B are monoubiquitinated (ub1) at K119 (H2Aub1, H2AXub1) and at K120 (H2Bub1) respectively, at the sites of DNA damage, a reaction catalyzed by RING1B/BMI1 and by a prominent E3 RING finger ubiquitin ligase RNF20/RNF40 [47-54]. H2Bub1 pertains the ability to physically disrupt chromatin strands (due to significant increase in dimension), adopting a more open chromatin structure, accessible to DNA repair proteins, thus facilitating the repair processes, as was shown by *in vitro* studies. Roles of signal recruitment, histone cross-talk and methylation influencing of H3 are also attributed to H2Bub1. Besides, recruitment of DNA repair machine proteins involved in both NHEJ and HR repair pathways to the DSB requires the activity of RNF20 and histone H2B monoubiquitination [52]. It is assumed that the H2B monoubiquitination machinery is temporarily recruited to damage sites. The locally produced H2Bub1 is in turn required for timely recruitment of DSB repair mediators and co-mediators, resulting in DSB repair. This phenomenon represents a crossroad between the DDR pathway and chromatin structure, and represents an example for the intercommunication and tight co-operation of pathways required to ensure genome integrity.

DDR and repair are urgent stress responses of the cell consuming vast amount of energy and recruiting protein components related to various normal functioning pathways in order to address the challenge. Therefore, a dynamic equilibrium of ubiquitination addition/removal is required at the chromatin level and the key player, the RNF20-RNF40 complex, is in dialogue with a number of deubiquitinases (including USP7, USP22, and USP44 enzymes). The active regulation of histone ubiquitination also by DUBs USP3 and K63-Ub DUB BRCC36 plays a critical role in efficient DDR and DNA repair pathways [55]. Apart from the RNF20-RNF40 complex, other key protein factors involved in DNA repair, like BRCA1, may also act as ubiquitin ligases, although this field still remains obscure [19].

Overall, it is hypothesized that the monoubiquitination/deubiquitination interplay of histones H2A and H2B regulates chromatin condensation, thereby facilitating recognition and binding of the repair machinery at the DNA damage site [52] and the restoration of chromatin structure upon damage repair. Despite intensive studies, the underlying mechanisms still remain elusive.

More extensive studies of the role of ubiquitination of histones have attracted particular interest, especially since H2Bub1 presents low to undetectable levels in many cancer types, including breast, colorectal, and lung. Approaches adopted include crosslinking combined with pull-down assays and similar high-throughput/directed strategies and functional assays. Based on these observations, members of the pathway-regulating H2Bub1 may represent promising therapeutic targets for malignancies and aging-related syndromes.

More intensive studies on elucidating mechanisms governing general histone modification induced by DNA damage in health and disease are expected to shed light in DNA repair and chromatin structure intercommunication along with the ability to explore and design pioneer treatment schemes for both cancer and other DNA-impairment-related diseases.

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Chapter 4

Direct Reversal Repair in Mammalian Cells

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Timothy R. O'Connor

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/60037

1. Introduction

Direct reversal repair eliminates some DNA and RNA modifications without using excision, resynthesis, and ligation. Therefore, because direct reversal repair does not require breaking of the phosphodiester backbone, it is error-free and preserves genetic information. Direct reversal is primarily utilized in correcting damage caused by DNA alkylating agents including *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG), *N*-methyl-*N*-nitrosourea (MNU), and methyl methanesulfonate (MMS) that react with DNA to produce various O-alkylated and N-alkylated products. Two major types of proteins conduct direct reversal repair, O6-methylguanine-DNA methyltransferases and ALKBH α-ketoglutarate Fe(II) dioxygenases (FeKGDs) [1]. Although there are numerous methyltransferases in mammalian cells, those enzymes generally catalyze transfer of methyl groups to DNA or transfer of methyl groups to or from proteins [2-4]. In mammalian cells, there is only a single DNA methyltransferase protein, O6-methylguanine-DNA methyltransferase (MGMT or AGT), that removes methyl groups at exocyclic ring oxygens of DNA [5]. The other type of direct reversal repair is performed by ALKBH proteins that are members of a superfamily of FeKGDs [4, 6]. Though the ALKBH family of FeKGDs encompasses nine proteins with conserved active site domains, removal of alkyl damage in DNA has only been established for four family members, ALKBH1 – 3 and FTO [6]. Unlike repair by MGMT, which is inactivated following a single repair reaction, each ALKBH protein can catalyze numerous repair reactions to eliminate N-modifications of cytosine, adenine, thymine and guanine residues [4]. In this review, prior to description of the direct reversal DNA repair enzymes and their functions, we will briefly describe sources of alkylation damage and adducts that are introduced upon exposure of cells to alkylating agents.

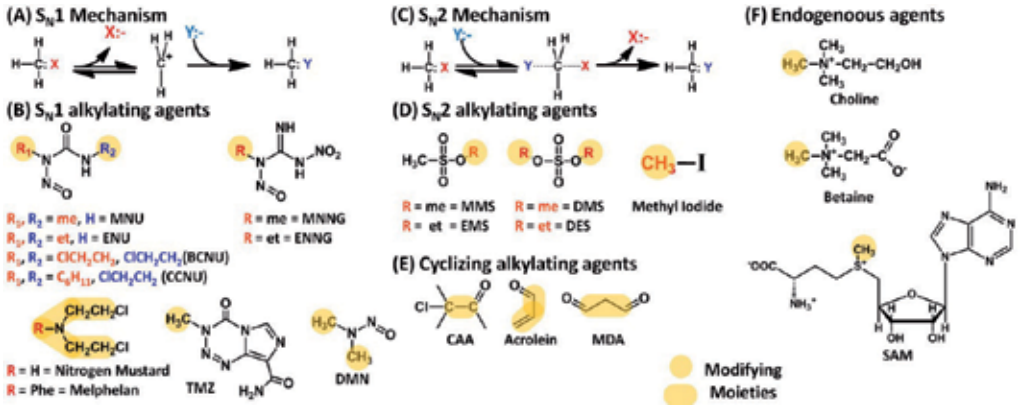

Figure 1. Alkylation mechanisms and selected alkylating agents. **(A)** S_N1 alkylating agent mechanism, **(B)** examples of S_N1 alkylating agents, TMZ-temozolomide, **(C)** S_N2 alkylating agent mechanism, **(D)** examples of S_N2 alkylating agents **(E)** examples of alkylating agents that can form cyclized adducts with DNA bases, CAA-Chloroacetaldehyde, MDA-Malondialdehyde, **(F)** examples of endogenous methylating agents

1.1. Sources of alkylation damage

Alkylating agents are present in the exogenous environment as well as intracellularly via oxidative metabolism. Alkylation damage from exposure to exogenous sources such as *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG), *N*-methyl-*N*-nitrosourea (MNU), and methyl methanesulfonate (MMS) (Figure 1B and 1D) [7] can arise from environmental agents present in various media including air, water, plants, and food [8]. Furthermore, numerous alkylating agents are used in chemotherapy to attack rapidly dividing tumor cells [9]. In addition to exogenous sources of DNA damage, a number of putative endogenous alkylating agents are proposed (Figure 1E). Endogenous agents can also introduce alkylation damage as a consequence of cellular metabolism [10]. Among the possible alkylating agents implicated is the enzyme cofactor *S*-adenosylmethionine (SAM), which is involved in numerous biochemical processes [11]. Methylating agents can also be formed via enzymatically-catalyzed chemical nitrosation reactions [12]. These nitrosation reactions can activate choline and betaine, as well as other lipoperoxidation products (Figure 1F) [10, 12, 13]. Regardless of whether sources are exogenous or endogenous, reaction of alkylating agents with DNA and RNA generates adducts that can disrupt major cellular processes such as replication and transcription, which can trigger cell cycle checkpoints and initiate apoptosis [14]. Importantly, if DNA alkyl adducts are left unrepaired, replication of damaged DNA can result in formation of mutations that, depending on the site within the genome, can lead to long term effects on cellular function.

1.2. Types of alkylating agents

The two major types of alkylating agents are S_N1 (Figure 1A) and S_N2 (Figure 1C). In the S_N1 reaction mechanism, a charged ionic species forms that is generally the rate limiting step (monomolecular) (Figure 1A); whereas S_N2 reactions follow bimolecular kinetics (Figure 1C). Alkylating agents are generally electrophilic compounds that have an affinity for the nucleo-

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philic centers in organic macromolecules and react in either a mono- or bifunctional manner [13, 15, 16]. Monofunctional agents consist of a single reactive group that interacts covalently with a nucleophilic center in DNA and primarily modify ring nitrogens [14, 17]. Common monofunctional nucleophilic reaction centers in DNA include: adenine N1, N3, N6; guanine N7, N1, N2, N3, N7, and O6; cytosine N3, N4, and O2; and thymine N3, O2, and O4 (Figure 2A), as well as phosphate modifications that form phosphotriesters (Figure 2B) [18]. Bifunctional alkylating agents, on the other hand, have two reactive groups that can interact with the DNA and can form cyclized or cross-linked DNA bases in addition to alkylating ring nitrogens (*e.g.*, Melphelan and Nitrogen Mustard (Figure 1B and 1E)) [14, 17].

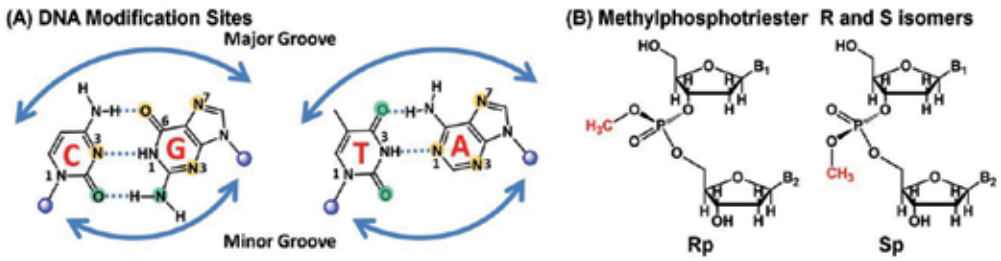

Figure 2. DNA Watson-Crick base pairs with principal damage sites modified by small alkylating agents (methyl and ethyl). **(A)** Sites of base modification by S_N1 and S_N2 alkylating agents. The orange indicates major damage sites and the green minor damage sites. **(B)** Phosphotriester formation indicated by the presence of methyl groups, along with the Rp and Sp isomers.

1.3. Distribution of DNA Damage manifested by simple alkyating agents

Alkylating agents can cause damage at all exocyclic nitrogens and oxygens in DNA and RNA, as well as at ring nitrogens (Figure 2A) [17]. However, the percentage of each base site modified depends on the alkylating agent, the position in DNA or RNA, and whether nucleic acids are single- or double-stranded (Table 1) [14]. Interestingly, O-alkylations are more mutagenic and harmful than N-alkylations, which may be more cytotoxic, but not as mutagenic [14].

Some frequent methylation sites in DNA include 1-methylguanine (1-meG), O6-methylguanine (O6-meG), 7-methylguanine (7-meG), 3-methylguanine (3-meG), 3-methylcytosine (3-meC), and 3-methyladenine (3-meA) (Figure 2A) [7, 10]. Importantly, nitrogens at base pairing positions in double-stranded DNA are less susceptible to alkylating damage than those found in single-stranded regions of DNA; though, methylating agents can react at Watson-Crick hydrogen bonding sites when DNA is singe-stranded. As a result, 1-meA and 3-meC adducts are much more frequent in single-stranded than in double-stranded DNA (Table 1).

Base modifications caused by larger ethylating agents (Table 1) begin to show differences from the corresponding methylating agents. Ethyl methanesulfonate (EMS) reacts similarly with guanine ring nitrogens compared to the methyl methanesulfonate (MMS), but there is a small, yet significant, decrease in the percentage of 1-meA and 3-meC formed by that agent. On the contrary, 1-ethyl-1-nitrosourea (ENU) produces significantly less 7-ethylguanine compared to

the percentage of 7-meG formed by exposure to 1-methyl-1-nitrosourea (MNU) (Table 1). The decrease in modification at the N7 position of guanine (G) is accompanied by a concomitant increase in the formation of phosphotriesters by ENU that represents over 50% of the damage assayed.

Alkylating Agent	**MMS (S_N2)**		**MNU (S_N1)**		**EMS (S_N2)**		**ENU (S_N1)**	
Strand	**ssDNA**	**dsDNA**	**ssDNA**	**dsDNA**	**ssDNA**	**dsDNA**	**ssDNA**	**dsDNA**
Alkylation Site				Adenine				
N1	18.0	3.8	2.8	1.3	8.0	1.7	2.0	0.2
N3	1.4	10.4	2.6	9.0	1.0	4.9	1.2	4.0
N7	3.8	1.8	1.8	1.7	3.0	1.1	0.6	0.3
Alkylation Site				Guanine				
N3	1.0	0.6	0.4	0.8	1.0	0.9	0.5	0.6
O6	nd	0.3	3.0	6.3	1.0	2.0	7.0	7.8
N7	68.0	85.0	69.0	67.0	77.0	65.0	10.0	11.5
Alkylation Site				Thymine				
O2	nd	nd	nd	0.1	nd	nd	6.0	7.4
N3	nd	0.8	nd	0.3	nd	nd	nd	0.8
O4	nd	nd	nd	0.4	nd	nd	4.0	2.5
Alkylation Site				Cytosine				
O2	nd	nd	nd	0.1	nd	nd	5.0	3.5
N3	10.0	<1.0	2.3	0.6	5.0	0.6	1.7	0.2
				Phosphodiester				
	2.0	0.8	~10.0	16.0	10.0	13.0	65.0	57.0

nd-not determined or detected, MMS-methyl methanesulfonate, MNU-1-methyl-1-nitrosourea, EMS-ethyl methanesulfonate, ENU-1-ethyl-1-nitrosourea

Table 1. Alylating agent damage from S_N1 or S_N2 methylating and ethylating agents [17].

Given the number and importance of alkylating agents, much effort has been expended to study the biological effects of alkylated DNA in cells. Interestingly, the biological consequences of unrepaired alkylation damage vary depending on the site in DNA. For instance, exposure of double-stranded DNA to S_N1 or S_N2 alkylating agents results in more frequent generation of 7-meG and 3-meA; however, the consequences of unrepaired 7-meG and 3-meA are different. Specifically, 7-meG does not block DNA replication and therefore is not as cytotoxic as 3-meA, whereas 3-meA adducts arrest DNA synthesis and do not show altered coding specificity [10]. In contrast, 7-meG can spontaneously depurinate and indirectly can lead to

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mutations at the apurinic sites that form. Furthermore, although both 7-meG and 3-meA modifications destabilize the glycosylic linkages, 3-meA repair occurs much more rapidly than 7-meG. Though not formed as frequently as 3-meA and 7-meG adducts, 3-meT and 3-meC lesions block DNA synthesis [19]. Additionally, generation of 1-meA and 3-meC modifications, primarily in single-stranded DNA regions will also halt DNA polymerization [17]. Unique to O6-meG, unrepaired damage is both cytotoxic and mutagenic [17].

2. Repair of DNA alkylation damage

The diversity of the types of DNA alkylation damage necessitates the involvement of a number of DNA repair systems to eliminate the ensemble of alkylation damage. As this chapter is focused on direct reversal repair mechanisms, we will only mention other major systems implicated in repair of alkylation damage in this section (Figure 3). Major repair pathways include, excision repair mechanisms, including base excision repair (BER) and nucleotide excision repair (NER), which require removal of the damage followed by resynthesis and ligation [1]. Additionally, alkylation damage that persists during replication can lead to double strand breaks which are repaired by either non-homologous end-joining (NHEJ) or homologous recombination (HR) mechanisms [20-22].

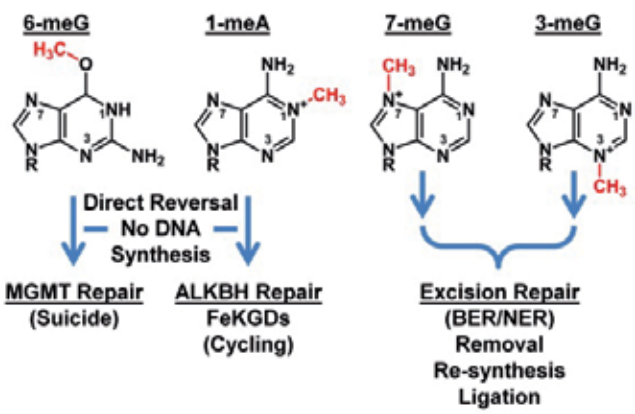

Figure 3. DNA repair systems that regenerate genetic information for the major purine bases damaged by methylating agents. O6-methyl**g**uanine-DNA **m**ethyl**t**ransferase and FeKGDs -α-ketoglutarate Fe(II) dioxygenases represent direct reversal repair. BER (base excision repair) and NER (nucleotide excision repair) make breaks in the DNA backbone and require complete removal of the base or an oligodeoxyribonucleotide.

3. O6-methylguanine-DNA methyltransferases direct reversal repair

Of the aforementioned direct reversal proteins, the first O6-methylguanine methyltransferase was isolated from *E. coli* and named Ada in that it was identified to regulate the adaptive response to alkylation damage [23]. Currently, O6-methylguanine-DNA methyltransferases have been identified in prokaryotes, archea, and many eukaryotes [23]. In initial mechanistic studies of the enzymatic activity of this group, protein extracts from *E. coli* incubated with DNA containing tritiated methyl groups at the O6 position of guanine (G) resulted in an association of the radiolabel with the methyltransferase proteins [24, 25]. Specifically, the active site cysteine of Ada covalently and irreversibly accepted the methyl group from O6-meG converting the cysteine to S-methylcysteine using a mechanism conserved by the homologous human protein MGMT. Although the O6-meG (Figure 4) is the preferred substrate for MGMT to act upon, the protein can also remove longer alkyl chains from DNA, including ethyl- (Figure 4), propyl-, butyl-, benzyl- and 2-chloroethyl groups, as well as O4-meT (Figure 4) [23].

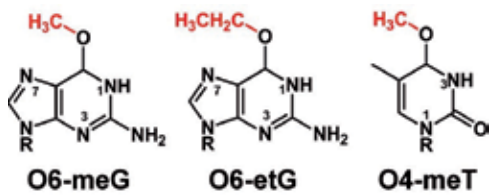

Figure 4. Several O-Methylated bases repaired mainly by MGMT. O6-methylguanine (O6-meG), O6-ethylguanine (O6-etG), and O4-methylthymine (O4-meT).

3.1. MGMT protein structure/ active site recognition

In human cells, O6-methyl**g**uanine-DNA methyl**t**ransferase (MGMT), coded for by *MGMT*, is a monomer with a molecular weight of ~18 kDa [24, 26] that contains numerous conserved structural features found in O6-methylguanine-DNA methyltransferases from different species. For example, a zinc finger structure containing a sole Zn(II) bound within a coordination sphere of four amino acids (Cys5, Cys24, His29, and His85) is preserved near the N-terminus (Figure 5) [27, 28]. The C-terminal domain (residues 86-207) of MGMT consists of an α/β fold that bears a helix-turn-helix (HTH) motif. The second or "recognition" helix of the HTH contains a highly conserved RAV[A/G] motif with an 'arginine finger' that promotes flipping of the target nucleotide from the base stacking arrangement [28]. Additionally, the C-terminal portion is comprised of a short, two-stranded, parallel β sheet, as well as four α helices, and a 3_{10} helix where the active site PCHR cysteine motif is found [28]. Immediately before the active-site, methyl group acceptor cysteine (Cys145) are two tight, overlapping turns stabilized by a conserved asparagine-hinge, Asp137, as well as the helix-turn-helix motif [28]. Finally, the N-terminal portion of MGMT (residues 1-85) encompasses a conserved α/β roll structure with three-stranded, anti-parallel β sheets followed by two helices [28].

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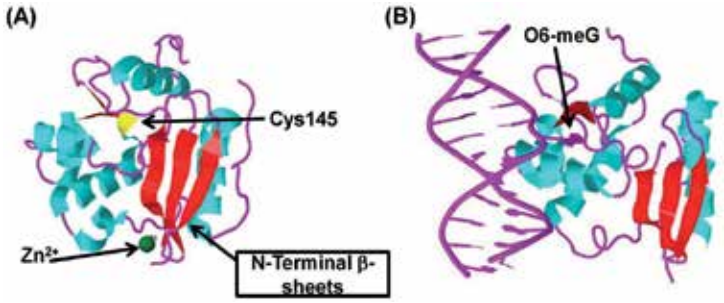

Figure 5. Structure of human **(A)** MGMT (PDBid: 1EH6) [29] and **(B)** MGMT bound to DNA (PDBid: 1T38) [30]. β-sheets and α-helices are indicated in red and blue, respectively. Active site residue Cys145 is highlighted in yellow and the Zn(II) is indicated as a space filled structure in green (Jmol: an open-source Java viewer for chemical structures in 3D). Used for all the structures in this chapter: http://www.jmol.org/.

3.2. MGMT substrate recognition/repair mechanism

The "suicide" mechanism that MGMT employs to directly remove O-alkyl adducts from DNA (Figure 6) is unique in that a single protein molecule is responsible for eliminating each lesion, which is a high energetic cost for cells [24]. The single use of the enzyme means that cellular MGMT activity begins to be depleted as soon as the enzymatic reaction occurs [31]. This suggests that as the enzyme repairs DNA lesions rapidly, saturation of the repair process occurs after which the initial repair rate slows considerably [5]. Unlike most other DNA repair systems, MGMT acts as a single protein and no other enzymes or cofactors are involved in the process [32]. At low MGMT concentrations and in the absence of any cofactors, the transfer of a methyl group occurs in <2 s at 37°C [24].

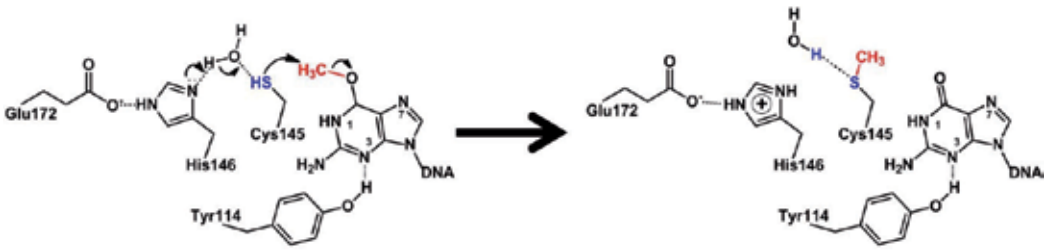

Figure 6. Mechanism of MGMT direct reversal DNA repair of 6-meG from DNA [33]. Repair of 6-meG in MGMT and other 6-meG-DNA methyltransferases is linked to a conserved sequence: Pro-Cys-His-Arg-Val.

The reaction catalyzed by MGMT is similar to the first half of a ping-pong enzyme kinetics mechanism, in which a group, the DNA adduct, is transferred from a substrate to a site in the enzyme, MGMT. In the absence of the second substrate, the group remains covalently attached to the protein, inactivating it [5]. The alkyl group removed from DNA is covalently attached to the Cys145 residue in the active site of the human MGMT protein through an S_N2 mechanism [23]. The MGMT active site is buried inside its structure, therefore it must flip the damaged

DNA base pairs out of the DNA helix in order to access them [34]. In fact, a mispaired base in the helical structure is more likely to be detected by MGMT than the same Watson–Crick base pair, which suggests that DNA structural distortion caused by alkyl base damage is an efficient way for the MGMT protein to locate damage sites on DNA [34]. The proposed reaction mechanism between the alkyl group and the active site of MGMT (Figure 6) requires that His146 acts as a water-mediated general base to deprotonate Cys145, which mediates the attack at the O6-alkyl carbon of guanine and, results in generation of a cysteine thiolate anion and an imidazolium ion stabilized by Glu172. Residue Tyr114 donates a proton to N3 of O6-meG and abstracts a proton from Lys165, facilitating simultaneous transfer of the methyl group on O6-meG to the thiolate anion of the Cys145 residue [33, 35].

3.3. *MGMT* expression/regulation

In human cells, *MGMT* is encoded on chromosome 10q26 [36] and is a housekeeping gene that is expressed in all tissues, though expression levels vary between cell types [37]. The highest *MGMT* expression levels in normal tissue are found in the liver, lung, kidney, and colon [32, 37-41]. Interestingly, the liver has higher levels of endogenous nitrosating agents relative to other organs, which could indicate a need for MGMT in that tissue. In contrast, the lowest levels of MGMT are found in the pancreas, hematopoietic cells, and lymphoid tissues [37, 41, 42]. MGMT levels in different cell types are dependent on various factors such as promoter regulatory elements, microRNAs (miRNAs) and possibly post-translational modifications. However, this correlation is not well-understood as evidenced by the fact that *MGMT* expression is up-regulated in some cancers, but silenced in others [37, 41, 43-45].

MGMT gene transcription is mediated by the 5′ promoter regulatory region of the gene that initiates at a single site within a GC-rich, non-TATA box, non-CAAT box-containing promoter [37]. Expression is additionally mediated by two glucocorticoid response elements (GRE) within MGMT that bind activator protein-1 (AP-1) sequences (Figure 7) [46, 47]. Protection against alkylating agent treatment by MGMT can be induced in response to the glucocorticoid phorbol-12-myristate-13-acetate (TPA), which regulates MGMT expression by Protein Kinase C (PKC) signaling. Thus, control of MGMT expression has implications for the use of chemotherapeutic drugs [48, 49].

In addition to transcript levels, another means of controlling MGMT protein levels is through microRNAs (miRNAs). miRNAs can lead to RNA degradation via the RNA-induced silencing complex (RISC) or by binding to the mRNA and inhibiting translation. *MGMT* mRNA has a number of associated miRNAs [50-55], some of which control expression. A comparison of *MGMT* mRNA and MGMT levels indicated that the production of protein and transcripts are not directly correlated [51], which suggests a means of post-transcriptional control of protein synthesis. One miRNA, miR-181d, was linked to favorable glioblastoma patient responses to temozolomide (TMZ) [52]. Subsequently, analysis of mRNAs from glioma samples showed two alternative poly(A) signals in the 3′-untranslated region (3′UTR) of *MGMT* (Figure 8), producing long and short *MGMT* transcripts with identical full length coding regions [50, 52]. Other *in vitro* analysis in cell lines identified three principal miRNAs that altered protein levels associated with the long *MGMT* transcript: miR-181d, miR-767-3p, and miR-648. All 3 miRNAs

were linked to reduction in transcript levels. Other miRNAs, including miR-661 and miR-370 had lesser effects on MGMT levels [50]. Moreover, there is a direct interaction of the miR-181d with the *MGMT* transcript [52]. Specifically, the longer 3'UTR transcript provides a site for interaction with the miR-181d that leads to degradation of the *MGMT* transcript and thereby regulates cellular MGMT levels. On the contrary, although miR-648 is in the longer transcript, its principal function is to limit translation and not degradation of the mRNA [50]. Of note, cells with lower the MGMT levels caused by association of the miRNAs with the longer transcript are more susceptible to methylating agent treatment than cells that do not have the alternative 3'UTR [50]. Other miRNAs with MGMT target sites that have been associated with response to both chemotherapy and radiotherapy include miR-181b and miR-181c [55]. Additional miRNAs, including miR-661 and miR-370 had lesser effects on MGMT levels; however, this area is only beginning to be explored as a method of regulating direct reversal repair and is associated with patient responses to alkylating agents.

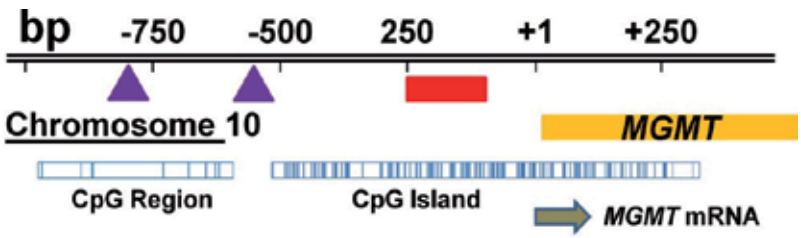

Figure 7. Promoter and first exon CpG islands in the gene coding for *MGMT*. The CpG island is boxed with the individual CpG sequences as vertical blue lines. The numbers on the line at the top refer to the nucleotide distances from the mRNA transcription start site (+1). The gene is indicated by the yellow box and the chromosome on which the gene is located is indicated on the left hand side of the figure. CpG islands were identified using Methprimer (http://www.urogene.org/cgi-bin/methprimer/methprimer.cgi). There is a 'hot spot' indicated in red that is in the CpG island and two AP1 binding sites for which AP1 binding is influenced by CpG methylation in the CpG Region are indicated by the purple triangles.

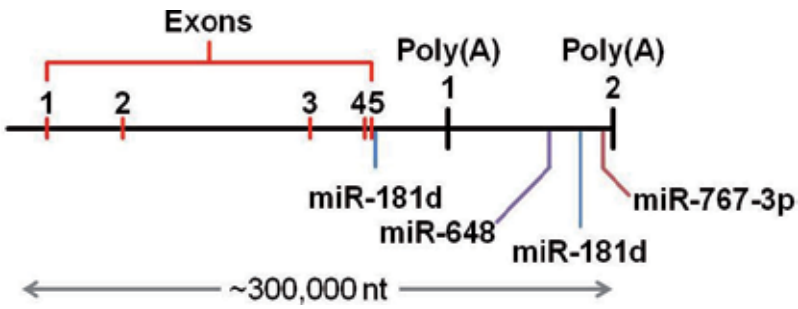

Figure 8. mRNA and miRNAs directly associated with *MGMT* expression and MGMT levels. The complete transcript of *MGMT* is depicted. Exons are represented as red vertical lines and the two poly(A) signal sequences indicated as black vertical lines. miRNA sequences are indicated in different colors: miR-181d in blue, miR-648 in purple, and miR-767-3p in brown. Note that there are two miR-181d sites, but only the longer miR-181d transcript alters MGMT levels. Adapted from References [50] and [52].

Epigenetic regulation is another means by which MGMT expression is controlled. Epigenetic factors are heritable changes not directly related to primary DNA structure and do not involve mutations to the DNA [56]. Two main mechanisms through which epigenetic regulation occurs are DNA methylation and histone modifications [56]. DNA methylation in mammals is observed in CpG sequences by introduction of a methyl group at the 5 position of cytosine [57]. CpG sequences are underrepresented in mammalian genomes based on the random distribution of dimer sequences. Depending on the position of a CpG site, enzymatic methylation can either enhance or reduce gene expression [58-60]. CpG sequences are often organized in promoter regions or early in genes in a concentrated manner defined as CpG islands. The *MGMT* promoter region has such a CpG island with multiple CpG dinucleotides in six different SP1 recognition sites (Figure 7) [46], which can be methylated. Modification of the *MGMT* promoter CpGs at the 5-meC interferes with transcription factor binding, that leads to *MGMT* silencing [61].

The other major type of epigenetic control of gene expression is through post-translational modification of histones. Histone deacetylation has a major role in transcriptional regulation and gene expression by removing acetyl groups from lysine residues in the amino terminal histone tails, which stabilizes DNA-histone interactions and condenses chromatin such that transcription binding sites are blocked and inaccessible [62, 63]. Other factors that can contribute to epigenetic regulation include diet and lifestyle choices [56, 64]. Such histone modifications contribute to the epigenetic regulation of *MGMT* expression. Acetylated H3 and H4 histones in a 'hot spot region' (~-100 to -250 from the transcription start site) and in AP1 binding sites (-605 to -611 and -798 to -804) of the *MGMT* promoter were associated with enhanced *MGMT* expression [65]. *MGMT* expression is also controlled by another group of proteins, methyl-CpG-binding-domain proteins (MBD). When MBD protein levels are high, the *MGMT* promoter is silenced, suggesting that MBD proteins remove lysine acetylation from histones H3 and H4, which result in more condensed chromatin, inhibiting transcription factor access to the MGMT promoter region, consequent inactivating transcription [61, 66]. Thus, multiple epigenetic factors influence *MGMT* expression including, but not limited to CpG methylation at the promoter. Therefore, *MGMT* silencing will additionally be discussed further with respect to biological significance.

3.4. MGMT localization

MGMT is actively transported to the nucleus [67]; however, establishing stable MGMT levels in the nucleus is a two-step process. In the first step, MGMT is transported to the nucleus, and then, once in the nucleus MGMT is localized to regions of active RNA polymerase II transcription [68]. Nevertheless, the localization of MGMT changes dramatically upon treatment with DNA alkylating agents. Following MNU exposure, those MGMT foci co-localized with RNA polymerase II transcription sites diffuse, suggesting dispersal of MGMT to damage sites [68]. After elimination of DNA alkylation damage, and ensuing inactivation of MGMT, ubiquitination of Lys125 and Lys178 targets the inactive protein for degradation via the proteasome [69]. Importantly, retention of MGMT is mediated by the basic PKAAR sequence (codons 124-128) that prevents the loss of the protein from the nucleus [67].

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3.5. Post-translational modifications of MGMT

Transfer of the methyl group to generate the thioester, S-alkylcysteine restores the original guanine base, but in the process irreversibly inactivates the MGMT enzyme [70]. Following the methyl group transfer and inactivation of MGMT, the enzyme is ubiquitinylated and subjected to degradation by the proteasome [34, 71]. There are also predictions for general sites of methylation, acetylation, sumoylation, and phosphorylation in MGMT, which include: methylation of Arg128 and Arg135; acetylation of Lys8, Lys125, Lys178, and Lys193:, sumoylation of Lys75, Lys205, Lys18, and Lys107; and phosphorylation of Ser36, Ser56, Ser130, Ser182, Ser202, Ser206, Ser208, Thr37 Tyr91, and Tyr115 [69, 71-73]. These sites may stimulate or attenuate MGMT activity or assist in translocation to damage sites, but the role of such putative modifications is still not clear and is a possible target for future investigations.

3.6. Biological significance of direct repair by MGMT

Though exposure to alkylating agents introduces numerous DNA and RNA alkyl adducts (Figure 2), specific repair of O6-meG and O4-meT adducts by MGMT reduces cell cytotoxicity and mutagenicity, as exhibited by the increased cell death and mutation frequency displayed in Mgmt-deficient murine models treated with S_N1 or S_N2 alkylating agents [39, 74, 75]. Lack of O6-meG or O4-meT adduct repair can result in cytotoxicity due to ensuing interference with replication and transcription machinery, which leads to apoptosis [76, 77]. Alternatively, if a modified base can form at least two hydrogen bonds, transcription, replication, and translation of templates can continue [17]. In the absence of repair, O6-meG can readily form two hydrogen bonds to base pair with T. That transition mutationcan lead to G→A mutations [78, 79], which has been observed in Ada,Ogt-deficient *E. coli* or Mgmt-deficient murine systems,. Otherwise, in the absence of repair, translesion synthesis (TLS) DNA polymerases can bypass DNA adducts, facilitating progression of replication and transcription past the damaged bases [80, 81]. However, TLS DNA polymerases exhibit reduced fidelity compared to normal replicative polymerases, making TLS DNA polymerases more tolerant to distortions in DNA that may result from alternative hydrogen bonding and non-Watson-Crick base pairing with damaged bases [82]. Consequently, in the absence of MGMT, mispairs are readily incorporated opposite unrepaired bases, reducing cytotoxic effects, but increasing mutagenicity.

Direct reversal repair of O6-meG is a relatively simple repair mechanism, but the biological consequences in the absence of O6-meG repair are of great importance. For instance, *MGMT* promoter silencing is exhibited in numerous types of cancers including breast, lung, colon, head and neck cancers [83-86]. In gliomas, higher *MGMT* promoter methylation is linked to increased overall survival in response to alkylating agents [38, 50, 87, 88], but has also been noted in myeloma, colon, pancreatic, breast, and lung cancers, as well as non-Hodgkin lymphoma [38, 86, 89, 90]. Furthermore, the expression of microRNA, miR-181d [50, 52], which targets MGMT expression, is predictive of patient responses to the chemotherapeutic drug TMZ. Presumably, decreases in MGMT, either by promoter silencing or miRNA inhibition, permit cytotoxic O6-meG adducts to remain in DNA and lead to increased cell death that is more specific to dividing tumor cells.

4. ALKBH Fe(II)/α-ketoglutarate-dependent dioxygenases direct reversal repair

The other family of direct reversal DNA repair proteins found in mammalian cells is the ALKBH family. Similar to MGMT, the AlkB protein was initially discovered in *E. coli*. Though AlkB was originally identified using a screen for methyl methanesulfonate (MMS) sensitive mutants [91], it took almost 20 years to classify AlkB as part of the FeKGD superfamily [6] and to demonstrate its ability to reverse 1-meA and 3-meC damage via oxidative demethylation [92, 93]. Two human homologs of AlkB, ALKBH2 and ALKBH3, were subsequently confirmed to be oxidative DNA demethylases [94, 95]. DNA adducts that are typically repaired by ALKBH proteins are 1-meA, 3-meC, 1-meG, 3-meT, 1-etA, 3-etC, as well as etheno adducts, 1,N^6-ethenoadenine, and 3,N^4-ethenocytosine [6, 91, 93, 96] (Figure 9). Additionally, bacterial AlkB and mammalian ALKBH3 also repair alkyl adducts in RNA [94].

The ALKBH family consists of nine human ALKBH enzymes, ALKBH1-8 and the **F**a**t** Mass and **O**besity associated gene (*FTO*) [1, 97, 98]. Despite primary structure conservation, only ALKBH1-3 and FTO have demonstrated unambiguous DNA repair activity [6, 97, 98]. The prototypical substrates for ALKBH1-3 are 1-meA and 1-meC adducts, but other modifications can also be substrates for those proteins (Figure 9). Cells that are deficient in ALKBH proteins generally show a higher sensitivity to S_N2 type alkylating agents and a higher mutant frequency [75, 99, 100]. Adducts repaired by ALKBH proteins are considered cytotoxic because they prevent hydrogen bonding with a complementary nucleotide and thus arrest DNA and RNA synthesis [101], blocking replication and transcription, which leads to apoptosis [73, 91, 93, 100-102]. Alternatively, increased mutant frequency could result from unrepaired lesions that undergo mutagenic bypass [100]. In murine models, targeted deletion of *Alkbh1* is linked to developmental defects with Alkbh1 enzymatic activity primarily directed at demethylation of histone H2A [103-105]. However, because this review is focused on DNA repair, our discussion of ALKBH1 will be limited. Similarly, although FTO removes 1-meA and 3-meC damage *in vitro*, its role is more closely linked to functions in RNA demethylation or demethylation of 6-meA [69, 106-110], which is not usually linked to DNA repair functions, and will therefore not be discussed further in this review.

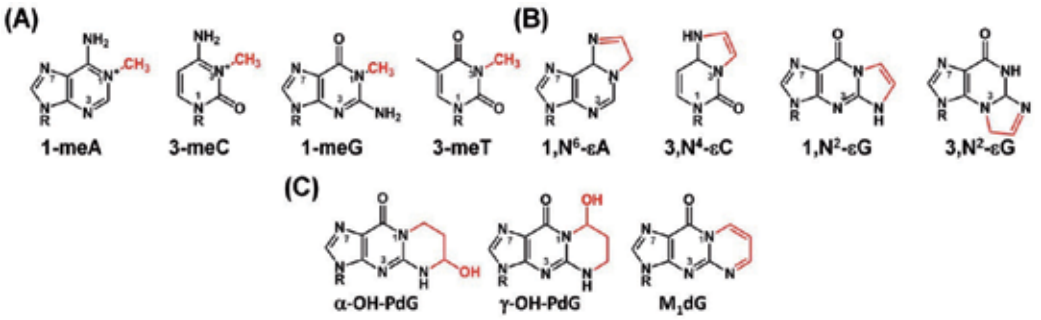

Figure 9. Numerous bases repaired by ALKBH2 and/or ALKBH3 proteins. **(A)** Methylated bases **(B)** Exocyclic 5-member ring bifunctional etheno (ε) adducts **(C)** Exocyclic 6-member ring bifunctional α-hydroxypropano-dG (α-OH-PdG), γ-hydroxypropano-dG (γ-OH-PdG), and M_1dG.

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4.1. ALKBH protein structure/active site organization

Although sequence homology is limited to active site and conserved domains in human ALKBH proteins, the secondary structures are conserved. For instance, all ALKBH family proteins have similar catalytic domains, but varying DNA recognition motifs [111]. Conserved domains in the FeKGD superfamily include a jelly-roll topology with a His_2-X-Asp/Glu-X_n-His_2 motif [112]. Specifically, the jelly-roll is made up of two sheets of antiparallel β-strands that contain His131, Asp133, and Trp69 residues which bind the iron and 2-oxoglutarate co-substrates [102, 112, 113] (Figure 10). Additionally, the His187 residue of the jelly-roll assists in Fe ligation, whereas Arg204 and Arg210 act to form salt bridges with the carboxylates of 2-oxoglutarate [114].

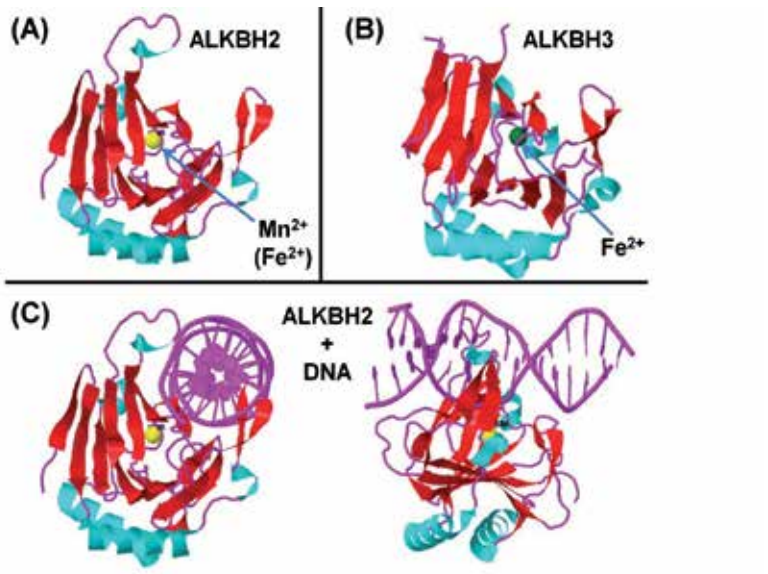

Figure 10. Structures of ALKBH2 (PDBid: 3S57) and ALKBH3 ((PDBid: 2IUW). (**A**) ALKBH2 structure without bound DNA [113]. The space filling yellow Mn(II) was substituted to block catalytic activity, but is normally occupied by an Fe(II). (**B**) ALKBH3 structure without bound DNA. The Fe(II) is shown as space filling in green [112]. (**C**) ALKBH2 with bound DNA in two orientations along the z axis of the DNA (left) and along the y axis of the DNA.

The catalytic core of both ALKBH2 and ALKBH3 is made up of three major components: a double-stranded β-helix, a nucleotide recognition lid, and an N-terminal extension [114, 115]. The Nucleotide Recognition Lid (NRL) is comprised of β-hairpin motifs which create a substrate binding groove that covers the active site until substrate is bound [112]. Despite similar catalytic mechanisms, β-strands and α-helices that create distinct outer walls of the DNA binding groove, which is involved in substrate recognition and specificity, vary between ALKBH proteins [94]. More explicitly, this divergence is present in the two looped structures, or "flips" that lie between the single β-sheet and two α-helices in the N-terminal portion of the catalytic region [112, 113]. In ALKBH2, the first flip is made up of 20 residues that constitute a β-hairpin and a short α-helix that together create a hydrophobic substrate binding groove

[113, 116]. ALKBH3, on the other hand, contains a first flip that is a β-hairpin that is made up of only 17 residues, forming a hydrophilic binding groove that has a preference for single stranded DNA or RNA substrates [111, 112]. The second flip in ALKBH2 consists of 24 residues that is composed of three β-sheets, allowing for interaction with both strands of DNA, whereas flip 2 of ALKBH3 is only 12 residues long and is made up of a single β-sheet [111].

To facilitate repair, ALKBH proteins flip the damaged base into their active site enabling the protein to interact with both strands of the DNA [111, 113]. In the case of ALKBH2, a short loop with a positively charged RKK sequence (Arg241–Lys243) is involved in grasping the complementary strand of the DNA, while a longer, more flexible loop, containing the residues Arg198, Gly204 and Lys205, binds the opposite DNA strand [111, 113, 114]. Additionally, ALKBH2 uses an aromatic finger residue, Phe102, to intercalate within the duplex stack, filling the gap resulting from DNA base flipping [113]. Tyr76 forms hydrogen bonds with the two phosphates 5′ of the methylated base, maintaining the substrate within the active site and residues Asp135 and Glu136 hydrogen bond with the exocyclic amino group via a water molecule [113, 115]. On the contrary, ALKBH3 does not contain the same aromatic finger residue and RKK motif as ALKBH2 and therefore has a greater preference for single-stranded DNA substrates. As a result, "flipping" of the damaged base is accomplished by squeezing the DNA proximal to the damage, causing it to rotate outward [112, 113]. Though the structures of the ALKBH homologs 4 – 8 have not been studied extensively, differences in the organization of the catalytic residues and active sites are predicted to influence the substrate specificities as well as enzymatic activities of these homologs [101].

4.2. Substrate recognition/repair mechanism

The ALKBH family of proteins removes and repairs DNA methyl adducts via a mechanism known as oxidative demethylation which results in the direct restoration of the original base coupled with the release of the hydroxylated methyl group as formaldehyde [92-94]. Other modifications can also be removed using similar mechanisms. Unlike MGMT, the repair mechanism utilized by the ALKBH family requires molecular oxygen, Fe(II), and α-ketoglutarate as co-factors to execute removal of alkyl adducts from DNA [93, 94].The ALKBH repair reaction consists of four steps with various intermediates (Figure 11) [117]. The first step of this mechanism involves a reaction between the active site Fe(II) and O_2 which produces a superoxo anion (O_2^-) bound to Fe(III) [118]. The superoxide attacks the α-keto carbon of the α-ketoglutarate, resulting in a bridged peroxotype intermediate [118]. The α-ketoglutarate intermediate is decarboxylated releasing succinate and CO_2 and undergoes a heterolytic cleavage of the O–O bond to form the high-valence ferryl-oxo intermediate. This intermediate then hydroxylates the alkyl adduct on the DNA producing an unstable intermediate that decomposes in water, with release of formaldehyde for methylated bases (and other aldehydes, depending on the substrate) restoring the original undamaged base (Figure 11) [75, 101, 118, 119].

Though ALKBH proteins primarily repair 1-meA and 3-meC (Figure 9A), those proteins also repair etheno and other exocyclic bases, but to a lesser extent than the methylated bases (Figure 9B and 9C, Figure 11C and 11D) [75, 96, 99, 118, 120, 121].

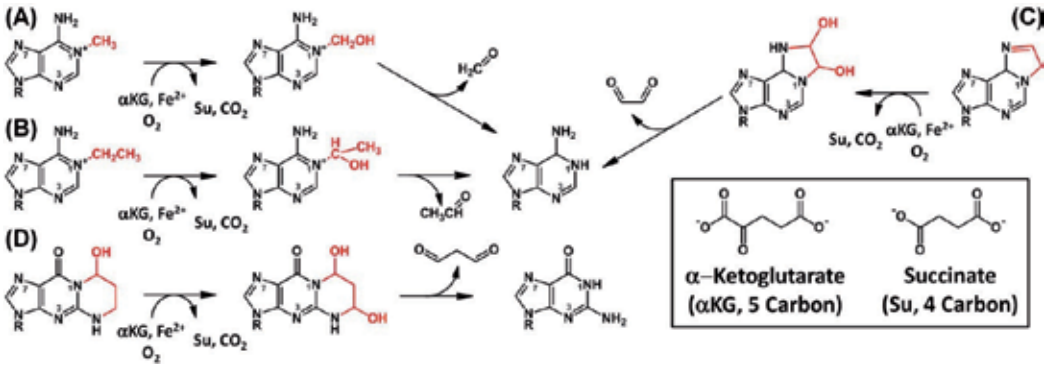

Figure 11. Products and intermediates formed during oxidative dealkylation of methyl, etheno, and exocyclic bases. (**A**) Repair of 1-meA results in release of formaldehyde, (**B**) 1-ethylA adducts release acetaldehyde, (**C**) 5 membered ring cyclized adduct (ethenoA, εA) releases diacetaldehyde, and (**D**) the 6 membered ring cyclized adduct (α-hydroxy-propano-dG) releases malondialdehyde.

4.3. Gene expression/protein regulation

ALKBH2 and *ALKBH3* are located on chromosomes 12q24 and 11p11, respectively and are considered housekeeping genes. The mRNA and protein levels of the ALKBH2 and 3 mRNAs vary with tissue type and the homolog [122-124]. However, both *ALKBH2* and *ALKBH3* are highly expressed in the testis and ovary [123, 124]. *ALKBH2* and *ALKBH3* contain CpG islands in their promoters (Figure 12A and 12C), but the role that those structures play in gene expression remains undefined.

Control of expression for *ALKBH2* and *ALKBH3* has not been as thoroughly studied as for *MGMT*. Interestingly, the arrangements of the ALKBH2 and uracil-DNA glycosylase gene (*UNG*) suggest a possible manner to control gene expression. *ALKBH2* is adjacent to *UNG* on human chromosome 12, but transcribed in the opposite direction [125]. The opposite orientations of these two genes could have an influence on their expression (Figure 12B). For *ALKBH3*, expression could be controlled by a putative ALKBH3 antisense RNA that is also converted into a long non-coding RNA (lncRNA) sequence (Figure 12D). The role that the lncRNA plays in ALKBH3 expression remains to be established. Unlike MGMT, control of *ALKBH2*, and *ALKBH3* expression via micro RNAs has not been examined. However, the mir-505-5p miRNA is reported to target *ALKBH2* (www.Exiqon.com), whereas at least 3 miRNAs (mir-188-3p, mir-4774-3p, and miR5580-5p) that could be involved in regulating ALKBH3 expression have been identified. Thus, the regulation of *ALKBH2* and *ALKBH3* expression has much that is yet unresolved.

4.4. Protein localization

ALKBH2 and ALKBH3 not only have different substrate preferences, but also exhibit different subcellular localization patterns, suggesting distinct biological functions. ALKBH2 is strictly nuclear and is found mainly at replication foci during S-phase [122-124]. Additionally, ALKBH2 co-localizes with PCNA [123, 124], indicating a possible role in DNA repair close to

the replication fork. In contrast, ALKBH3 is found in the nucleus and in the cytoplasm [111, 122]. Association of ALKBH3 with the activating signal cointegrator complex 3 (ASCC3) helicase enzyme is consistent with nuclear localization [126], whereas the role of ALKBH3 in mRNA repair is consistent with its localization in the cytoplasm [123].

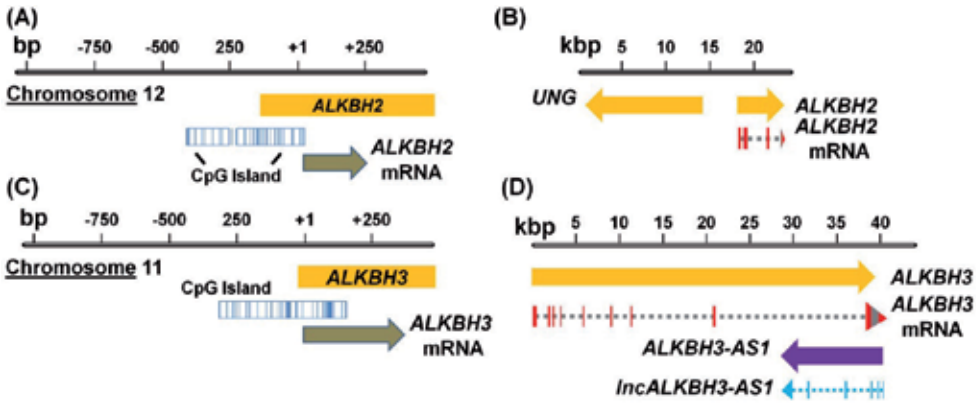

Figure 12. Promoter CpG islands, gene structure and major mRNAs for *ALKBH2* and *ALKBH3*. **(A)** *ALKBH2* promoter region. CpG islands are boxed with individual CpG sequences as vertical lines. Numbers at the top refer to nucleotide distances from mRNA start sites (+1). The gene is the yellow box and the chromosomes are indicated on the left hand side of the figure. CpG islands were identified using Methprimer (http://www.urogene.org/cgi-bin/methprimer/methprimer.cgi). **(B)** *ALKBH2* showing the position and size of the uracil-DNA glycosylase gene (*UNG*). Exons are indicated in red and the gene in brown. **(C)** *ALKBH3* promoter region. The CpG island and gene sequences are as for part A **(D)** *ALKBH3* and the position of the antisense *ALKBH3-AS1* are shown in purple for the unspliced antisense-RNA and in blue for the spliced lncRNA. The exons for the mRNA are indicated in red.

4.5. Post-translational modifications of ALKBH proteins

Post-translational modifications of residues within ALKBH2 and ALKBH3 have been examined using site-specific mutagenesis methods, as well as mass spectrometry [73], but the effects of these modifications are unknown. ALKBH2 residues Lys34 and Lys104 can be acetylated, Tyr91 and Thr93 and Thr252 can be phosphorylated, and Lys104 can be ubiquitinated [73]. Though the effects of these modifications on ALKBH2 activity have not been established, it is important to note that residue Lys104 falls within the variable region of the N-terminus which provides for protein specificity. Similarly, post-translational modification of ALKBH3 includes several phosphorylated residues such as Thr126, Thr212, and Thr214, as well as Tyr127 and Tyr143, some of which have been shown to correlate with acute myelogenous, chronic myelogenous, and T-cell leukemia [73]. Moreover, phosphorylation of active site residues Thr212 and Thr214 have been observed in liver cancer tissue samples, while phosphorylated Tyr residues have been reported in lung and non-small cell lung cancer cell lines [73]. Despite some intriguing results as possible biomarkers for tumors, the functions of these post-translation modifications have not been identified.

4.6. Biological significance of direct repair by ALKBH proteins

Similar to MGMT, the presence of ALKBH2 and/or ALKBH3 reduces cell cytotoxicity and mutagenicity, as shown in Alkbh-deficient murine models treated with S_N2 alkylating agent

methyl methanesulfonate (MMS) [75, 100, 127]. Though the major damage sites repaired by ALKBH proteins are only susceptible to modification when DNA is single-stranded, formation of 1-meA and 3-meC at DNA base-pairing positions prevents proper base insertion which can halt DNA synthesis [99], causing replication fork collapse [20]. As a result, persistence of 1-meA and 3-meC adducts increases cell cytotoxicity by triggering programmed cell death [75, 100, 127]. In contrast to O6-meG, following alkylation of the N1 of a purine or the N3 of pyrimidine, only a single hydrogen bond can be readily formed. Therefore, increased mutant frequency exhibited in Alkbh-deficient murine models is likely due to adduct bypass by TLS DNA polymerases [128]. In *E. coli*, evaluation of 1-meA, 3-meC, 1-meG, or 3-meT mutagenicity revealed that all adducts were highly mutagenic, with the exception of 1-meA, which was only slightly mutagenic [99]. Interestingly, of the adducts repaired by ALKBH proteins, 3-meC is formed at the highest frequency in response to MMS treatment (Table 1) and the mutations identified following MMS treatment in Alkbh2- or Alkbh3-deficient primary MEFs were C:G →A:T and C:G → T:A [129], suggesting that 3-meC is highly mutagenic in absence of repair.
Similar to O6-meG repair by MGMT, direct repair by ALKBH proteins has a biological significance that is not well understood. Varying expression levels of *ALKBH2* and *ALKBH3* also contribute to the progression or suppression of different types of cancers. Down-regulation of *ALKBH2* increases sensitivity of H1299 lung cancer cells to the drug, cisplatin, improving overall survival [130]. However, down-regulation of *ALKBH2* has been observed to promote the growth of gastric cancer cells [130]. Mutations in *ALKBH2* and *ALKBH3* have also been associated with their enhanced expression levels in glioma cells and pediatric brain tumors [131, 132]. ALKBH2 also mediates resistance to the alkylating agent therapeutic temolozomide (TMZ) in glioblastoma cells [133]. *ALKBH3* silencing induced senescence and increased sensitivity to alkylating agent therapies in prostate cancer cells [126, 130]. Therefore, further investigation of the roles of ALKBH2 and ALKBH3 in different types of cancer is important to define the specific roles of individual ALKBH family proteins.

5. Models of direct reversal repair and implications as therapeutic targets

Repair of DNA damage is critical for cell survival and maintenance of genome integrity. Not surprisingly, cells depend on direct repair mechanisms to remove damage that could otherwise be cytotoxic or mutagenic [14]. Understanding the roles that direct reversal repair proteins play in genome stability also enables exploration and exploitation of these proteins in regard to therapeutics. Therefore, use of currently established animal models, as well as generation of additional models is integral in development of diagnostic and therapeutic approaches.

5.1. Current mammalian models defective in DNA direct reversal repair genes

The effects and efficiency of repair pathways is best studied by observing the effects on cell cytotoxicity, replication, transcription, and mutation in the absence of the repair proteins. To evaluate the impact of the absence of direct repair proteins animal models with targeted

deletions have been developed for MGMT, as well as ALKBH proteins. Interestingly, murine knock-out (KO) models for *Mgmt* and *Alkhb2* or *Alkbh3* do not exhibit a detectable phenotype in the absence of alkylating agent treatment [75, 85, 100, 127, 129, 134-137]. As anticipated from *in vitro* cell culture studies, Mgmt-deficient mice treated with a number of alkylating agents including, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG), *N*-methyl-*N*- nitrosourea (MNU), 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU), 1-(-4-amino-2-methyl-5-pyrimidinyl)methyl-3-(2-chloroethyl)3-nitrosourea (ACNU), streptozocin, TMZ, and dacarbazine, exhibited lethality at lower drug concentrations than for the wild type mice [10, 40, 87, 138], consistent with increased toxicity due to the absence of Mgmt. Moreover, *Mgmt* KO mice that were treated with MNU lost hematopoietic stem cells [135, 139] and were prone to the development of thymic lymphomas [139] as well as lung adenomas [103, 120, 139]. However, the sensitivity to methylating agents is lost when the mismatch repair system is not functional [85, 135, 140-143], which has implications for therapeutic treatments. Importantly, mice heterozygous for *Mgmt* do not exhibit decreased survival following alkylating agent treatment [139]. Furthermore, mice exhibiting elevated levels of Mgmt are resistant to alkylating agent-induced tumor formation [135].

Even though Alkbh2- and Albh3-deficient murine models do not show any overt phenotypic changes compared to their wild type counterparts, over time the Alkbh2-deficient mice accumulate high levels of 1-meA in the liver [75]. Similarly, double mutants with targeted deletions in both *Alkbh2* and *Alkbh3* do not demonstrate an obvious phenotype and the mice are fertile and live to normal ages [75, 100, 127]. Of note, a mouse model that targeted both the FeKGDs (Alkbh2 and Alkbh3) and the base excision repair pathway (Mpg or Aag) was generated. All of those proteins have roles in repair of alkylation damage. In response to alkylation damage (chemically-induced colitis), that mouse model deficient in all three proteins manifested a synergistic phenotype that resulted in death with even a single treatment [127]. Due to the lack of phenotypic effect and limitations treating animals, analysis of the effects of Alkbh2 and/or Alkbh3 deficiency have also been conducted using primary and immortalized mouse embryonic fibroblasts (MEFs) [75, 100, 127].

5.2. Generation of new *in vitro* mammalian models defective in DNA direct reversal repair genes

The capacities to directly target human cells to either abrogate protein function or place specific tags on proteins have been limited. Recently, the development of clustered regularly interspaced short palindromic repeats/CRISPR associated endonuclease 9 (CRISPR/Cas9) technologies have permitted the generation of targeted deletions in human and rodent cells rapidly. Implementation of that technology will facilitate the study of DNA repair and mutagenesis in great depth. The basis for targeting genomic DNA to generate deletions is outlined (Figure 13). A guide RNA is designed based on an exon sequence in the genomic DNA. Following transfection of a plasmid expressing the guide RNA and the Cas9 mRNA, the protein-RNA complex will induce a double strand break in the genomic DNA. Repair by non-homologous end-joining leads to a change in the reading frame that inactivates the protein. This powerful technology can also be used to introduce point mutations and to create other cancer prone models in human cells for study *in vitro* and also *in vivo* in animal models. Readers are referred to recent publications that describe the possibilities of using these methods [144-146].

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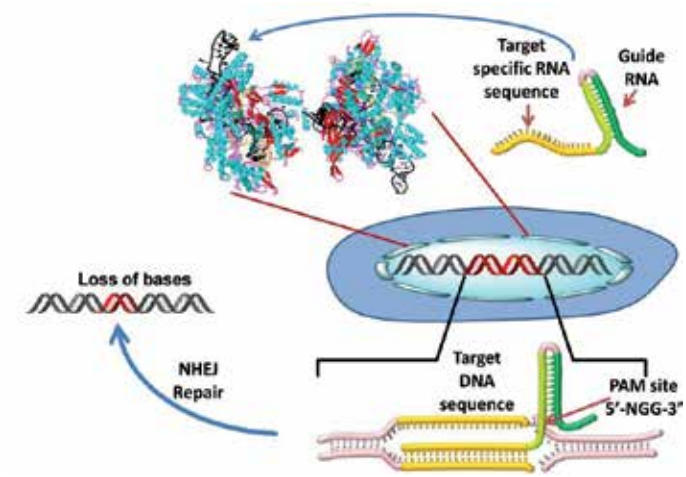

Figure 13. Outline of CRISPR/Cas9 modification of genomic DNA. Structure of the Cas9 complex is from Nishimasu *et al.* [147]. The guide RNA is represented by black in the structure, the target DNA by gold, helical regions by cyan, and β-sheets by red. The cell cytoplasm is represented in blue and the nucleus in light blue. The target region in genomic DNA is in red. The figure was adapted from Pennisi [148].

5.3. Direct repair proteins as therapeutic targets

DNA repair deficiency is associated with increased cancer risk and formation of tumors, but has also been used in therapeutic strategies employing synthetic lethality in an effort to overload the cancer cells with damage that results in apoptosis while normal cells with efficient repair can eliminate the damage invoked by the chemotherapeutic regimen [149-153]. Commonly anti-neoplastic therapies utilize alkylating agents as well as ionizing radiation (IR); however, these treatments not only induce cell death in cancer cells, but can also increase the formation of mutations in normal cells, leading to an increased risk of secondary cancers. Synthetic lethality for DNA repair agents exploits defects in DNA repair found in tumor cells that use alternative repair systems for repair. Inhibiting the alternative repair systems results in increased tumor cell death specifically targeted to the tumors. Currently, both chemotherapy and radiation are used in combination to target specific DNA repair proteins in cancer cells in order to improve therapeutic efficacy and limit drug resistance [154, 155]. One of the advantages of direct reversal repair proteins is that a single protein is ultimately responsible for elimination of the damage and that no breaks are made in the DNA by the repair mechanism. Targeting direct reversal repair proteins to increase sensitivity could supplement the efficacy of the alkylating agents already used in clinical protocols.

MGMT-inhibitors currently exist and include O6-benzylguanine (BG) and O6-(4-bromothenyl) guanine (PaTrin-2 or lomeguartib) [88]. Patient studies indicate that treatment with BG eliminates nearly 99% of MGMT activity in human cells for 24 hours after the removal of the chemotherapeutic drug [156]. Additionally, clinical trials evaluating combination treatment of BG with BCNU have been conducted to evaluate enhancement of alkylating agent chemotherapeutics [38], and treatment with O6-(4-bromothenyl) guanine has been evaluated in glioma patients [88].

The roles of ALKBH2 and ALKBH3 in response to methylating agent chemotherapies (particularly for TMZ) remain unclear. Although MGMT is associated with resistance, there are reports of ALKBH2 also enhancing resistance [133]. Inhibitors of Alkb have already been identified, but inhibitors of the human homologs have not been reported [157-159]. The identification of ALKBH inhibitors and their use with current chemotherapeutics could provide new tailored therapies for patients.

In addition to the development of therapies targeting the proteins, RNA interference-mediated gene silencing (RNAi) [160, 161] is a possible alternative approach for specifically targeting *MGMT* or *ALKBH* family members that allows depletion of proteins for extended periods of time [37]. Lowering protein levels of species that protect against DNA damage would render cells more susceptible to chemotherapeutic agents and should theoretically offer better drug efficacy.

siRNAs can be identified using empirical methods to target direct DNA repair protein mRNAs (*i.e.*, for MGMT, ALKBH2, or ALKBH3). At present, reports indicate that higher MGMT levels are associated with poor response to therapy using TMZ, whereas patients with lower MGMT levels have better responses to TMZ therapy [23, 52, 86-90]. Rather than using siRNA constructs defined empirically, naturally occurring miRNAs could be used to reduce DNA repair protein levels and improve therapeutic responses for tumors (*e.g.*, glioblastomas) that respond to methylating agents (*e.g.*, TMZ). However, a number of miRNAs have already been identified including: miR-181d, miR-195, and miR-196b, that negatively correlate with overall survival in glioma patients [52, 160]. Using miRNAs as targets could attenuate MGMT levels in tumors, rendering the tumors more susceptible to TMZ treatment. That susceptibility of cells to TMZ/miRNA treatment was demonstrated *in vitro* [50, 52]. Using T98G or A1207 cells, miRNA targeting in combination with TMZ reduced cell viability by up to 2.5-fold for the *MGMT* transcript compared to cells in which the MGMT levels were not reduced [50, 52]. To date, the miRNAs in combination with TMZ as treatment are indicated as biomarkers to predict probable patient outcomes [50, 52, 55, 160]. In an evaluation of The Cancer Genome Atlas dataset for glioblastomas, *MGMT* transcript levels and miR-181d correlated with patient survival [52]. In the future, miRNAs against *MGMT* mRNA could be introduced to augment therapeutic response (Figure 7). Other microRNAs associated with ALKBH2 and 3, such as mir-505-5p miRNA for *ALKBH2* (www.Exiqon.com) and mir-188-3p, mir-4774-3p, and miR5580-5p for *ALKBH3*, could also be used as new therapeutic avenues, because there are also reports that TMZ response is linked to the presence of ALKBH2 or ALKBH3 [100, 126, 133]. The use of miRNAs has great potential for high specificity with limited side effects. Targeting transcripts using miRNAs is an exciting area for developing new therapeutic targets and biomarkers for predicting outcomes. Employing miRNAs could have substantial benefits for patients, but much work remains to bring such promising therapies to fruition.

6. Summary

Direct reversal repair is one of the lesser known mechanisms by which cells repair DNA. Unique to direct reversal repair pathways, repair occurs without breakage of the DNA backbone and the processes are error free. As a result, direct reversal repair proteins have

central roles in the preservation of genomic stability. In mammalian cells, direct reversal repair is principally limited to correcting DNA alkylation damage that can arise from exogenous or endogenous sources. Elimination of alkylation damage by direct reversal is achieved by two major types of proteins: O6-methylguanine-DNA methyltransferases and ALKBH α-ketoglutarate Fe(II) dioxygenases. Although much is known biochemically about direct reversal repair enzymes, epigenetic factors, post-translational modifications, as well as genomic and mitochondrial DNA repair mechanisms require further investigation. Recent data establishing the function of direct reversal repair proteins in model system organisms, most prominently in mice has contributed to the comprehension of the biological function of these proteins. Already, the partial understanding of these mechanisms has been translated into clinical use and in the future should lead to an even greater influence on improving therapeutic outcomes.

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Chapter 5

Transcriptional Regulation of the Human Genes that Encode DNA Repair- and Mitochondrial Function-Associated Proteins

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Additional information is available at the end of the chapter

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1. Introduction

Mitochondria are thought to be evolved from primeval prokaryotes after symbiosis in anearobic cells, and they have their own circular DNAs (mtDNAs) and transcription/translation systems [1-3]. However, most of the genes (99%) that encode mitochondrial proteins and components of protein complexes are contained in nuclear genomes [3]. Previous researches revealed that mitochondria play important roles in the regulation of vital biological events, namely production of energy [4]. More importantly, recent studies showed that mitochondria exert signals to affect cell death [5], cellular senescence [6], and DNA repair systems [7]. These observations imply that mitochondria and nuclei are communicating each other to protect nuclear DNAs that encode 99% of mitochondrial proteins [8]. Furthermore, mitochondria also play roles in the responses to various stresses, including immunological reaction [9, 10].

Previously, we surveyed the human genomic DNA data-base and found that promoter regions of several DNA-repair-associated genes, including *ATM*, *BRCA1*, *FANCD2*, *PARG*, and *TP53*, which encode proteins that regulate mitochondrial functions, contain duplicated GGAA-motifs [11]. Moreover, numbers of DNA repair and mitochondrial function-associated genes are linked with partner genes by bidirectional promoter regions containing duplicated GGAA motifs [12]. These observations suggest that expression of the DNA repair and mitochondrial function associated factor-encoding genes are commonly regulated by GGAA-motif binding transcription factors (TFs).

In this chapter, we will focus on and discuss transcriptional mechanisms that regulate both DNA repair and mitochondrial functions. Not only DNA repair systems, but also several metabolic enzyme reactions that depend on an inner cellular NAD^+/NADH ratio, including TCA (Citrate/Krebs) cycle and poly (ADP-ribosyl)ation, are thought to be dys-regulated in cancer or tumor cells. We therefore, propose a novel cancer therapy by introducing GGAA-motif binding TFs or their expression vectors, activating both DNA repair and mitochondrial functions.

2. Relationships between DNA-repair, mitochondrial functions and Immune responses

Telomeres are the specific region of chromosomes that regulate cellular senescence and chromosomal integrity [13]. It has also been indicated that mitochondrial function is regulated by telomeres [6]. Several nuclear DNA-repair factors are suggested to play roles in the maintenance of mitochondrial DNAs (mtDNAs), and damaged mtDNAs in turn exert signals to regulate nuclear transcription [7]. Some of the DNA repair factors have been shown to localize in mitochondria. Furthermore, immune system has been suggested to be under the regulation of DNA repair [14]. Therefore, understanding the co-operation of the telomere-mitochondria-DNA repair-immune response might contribute to reveal molecular mechanism of cellular senescence, cancer and immunological diseases.

2.1. Characterization of the promoter regions of genes encoding enzymes that regulate human poly(ADP-ribose) metabolism

We have found duplicated GGAA-motifs in the bidirectional promoter of the human *PARG* and *TIM23B* genes that encode a poly(ADP-ribose) degrading enzyme and a mitochondrial inner membrane translocase 23B, respectively [11, 15, 16]. Isoforms of the PARG protein localize in the mitochondria [17]. PARP1 enzyme synthesizes poly(ADP-ribose)s on various target proteins, including p53 [18, 19] and PARP1 itself to regulate DNA-repair synthesis [20]. Interestingly, duplicated GGAA motifs are also contained in the promoter region of the human *PARP1* gene [21, 22]. PARP1 and PARP2 enzymes influence mitochondrial function and oxidative metabolism [23]. It has been shown that PARP1 protein localizes in mitochondria to maintain mitochondrial DNA integrity [24]. These observations imply that several DNA-repair factors localize in mitochondria, and that their gene expression may be partly controlled by the GGAA-motif binding protein factors.

2.2. DNA-repair factors and mitochondrial functions

It is widely known that damage on DNAs activates p53, which is transcribed from the *TP53* gene, allowing it to bind to 5′-regulatory elements and activate genes encoding cell cycle regulators, apoptosis- and autophagy-inducers [25, 26]. The p53 protein does not only act as a “guardian of the genome”, but also serves as a metabolism regulator [27, 28]. Moreover, p53

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has been reported to accumulate in mitochondria in response to stress [26]. One recent study revealed that mitochondrial disulfide relay causes translocation of p53 into mitochondria to facilitate its function for repairing oxidative damage to mitochondrial DNA [29]. However, overexpression of p53 in mitochondria would lead to depleted mitochondrial DNA abundance and a reduction in oxidative stress [30]. Oncogenic *RAS*-induced mitochondrial dysfunction, which causes oncogene induced senescence, is dependent on either p53 or RB [31]. As a tumor suppressor protein, RB plays a role in linking cell cycle exit with mitochondrial biogenesis [32]. RB is widely known to control cell cycle progression, maintenance of genome stability and apoptosis by interacting with the E2F family of TFs [33]. Recently, it was reported that mutation of E2F1 leads to mitochondrial defects in human cells [34].

Besides p53 and Rb, various DNA repair factors have been reported to localize in mitochondria or regulate their biological functions. For example, mutations of the *BRCA1* gene have become one of the hallmarks for diagnosis of breast cancer [35, 36]. BRCA1 protein, which plays a part in the repair synthesis of double-strand DNA breaks [37], is also involved in the mitochondrial genome maintenance to be trans-located into mitochondria especially when it is phosphorylated [38]. Deficiency of BRCA1, which interacts with FANCD2 protein, leads to phenotypes that resemble to Fanconi amaemia (FA) [39]. A number of additional DNA repair factors associate with FA proteins [40]. Recent study of transcripts from bone marrow cells revealed that FA patients have deficiencies in mitochondrial, redox and DNA repair pathways [41, 42]. Another DNA-repair deficient disease is Ataxia Telangiectasia (AT) that is caused by mutations on the *ATM* gene [43]. Recently, it was reported that lack of ATM causes reduced mitochondrial DNA integrity and mitochondrial dysfunction [44]. Moreover, it was suggested that mitochondria are required for the oxidative activation of ATM [45]. The duplicated GGAA-motifs are present in the 5′-upstream regions of the *BRCA1*, *FANCD2*, and *ATM*, which have bi-directional partner genes *NBR2*, *CIDECP*, and *NPAT*, respectively [11]. Although *BRCA2*, which encodes a tumor suppressor to repair double-strand DNA breaks [46, 47], has no bi-directional partner gene, the duplication of the GGAA-motif is present near its transcription start site (TSS) [11].

2.3. Apoptosis is executed by signals from mitochondria

Execution of apoptosis or programmed cell death is mediated by mitochondria in response to various stresses including DNA-damage and immunological stress signals [48, 49]. Previously, we reviewed the roles of the ETS family proteins on apoptosis, and found the GGAA-duplications in the 5′-regulatory regions of the human *PDCD1*, *DFFA*, *BCL2*, *FAS*, and *FASL* genes [50]. The findings imply that expression of the apoptosis regulating factor-encoding genes is under the control of the duplicated GGAA-motifs. Previous studies revealed that mitochondrial functions closely associate with apoptosis [5, 48, 51]. For instance, it is one of its characteristics that cytochrome c is released from mitochondria during induction of DNA-damage signals, and that apoptosis regulator proteins BAX and BCL2 localize in mitochondria [48]. Our *in silico* surveillance of the human genomic data base retrieved several interesting examples of duplicated GGAA-containing bidirectional promoters, including *ATG12*/*AP3S1*,

APOPT1/*BAG5*, and *HTRA2*/*AUP1* gene pairs [12]. The *ATG12* and *HTRA2* genes encode an autophagy protein that takes a part in the quality control after mitochondrial damage [52, 53] and a serine protease that is localized in mitochondria [54], respectively. Importantly, with assistance of tumor suppressors, such as p53, RB1 and BRCA1, ATG12 and HTRA2 may contribute to determine cell fate between DNA-repair and cell death after excess cellular stresses.

2.4. Identification of duplicated GGAA (TTCC) motifs in the 5′-upstream of the human genes encoding DNA repair factors and apoptosis regulators

We have reported that duplications of the GGAA-motif are found in the 5′-regulatory regions of the human *TP53* and *RB1* genes [11]. Moreover, we have found the DNA sequence 5′-CAATA**GGAA**CCGCCGCCGTTG**TTCC**CGTC-3′ near the TSS of the human *E2F1* gene. These lines of evidences imply that tumors could be generated from mitochondrial dysfunctions when p53 and RB proteins lose their intrinsic biological functions as tumor suppressors, and that expression of their encoding genes are under the control of GGAA-motif binding TFs.

We have also identified GGAA-motif duplications in the 5′-upstream of the *APEX1* gene, which has a bidirectional partner gene *OSGEP* [11]. The *APEX1* encodes apurinic/apyrimidinic endonuclease 1 (APE1) that regulates both base excision repair and mitochondrial DNA-repair systems [7, 55]. It is noteworthy that APE1 interacts with XRCC1, which is recruited to the poly(ADP-ribosyl)ated site [56]. APE1 does not only function as a regulator of the base excision repair system, but also as a redox regulator [57]. The GGAA-duplication is contained in the regulatory region of the head-head configured *ACO2*/*PHF5A* genes [12]. The *ACO2* encodes aconitase that functions in the TCA cycle to produce citrate and isocitrate and also serves as a mitochondrial redox-sensor [58]. More importantly, a recent study revealed that aconitase and mitochondrial base excision repair enzyme OGG1 (8-oxoguanine DNA glycosylase) cooperatively preserve mitochondrial DNA integrity [59]. Additionally, it has been shown that Cockayne syndrome (CS) proteins CSA and CSB, which play roles in nucleotide excision repair, accumulate in mitochondria upon oxidative stress [60]. A putative ETS1 binding motif is located, though no obvious duplication of the GGAA-motif is present near TSS of the *ERCC8* (*CSA*) gene. Interestingly, it has a bidirectional partner *NDUFAF2* that encodes one of the components of the NADH dehydrogenase (ubiquinone) [12]. The observation implies that not only GGAA-motif-duplication, but also another *cis*-element may take part in supporting transcription from a bi-directional promoter.

Collectively, our *in silico* analysis of the 5′-upstream regions of human genes suggested that transcription of a large numbers of DNA-repair/apoptosis/mitochondrial function associated genes could be regulated by duplicated GGAA-motif-containing promoters.

2.5. DNA-repair and immune responses

It should be noted that duplicated GGAA (TTCC) motifs are frequently contained in numbers of 5′-upstream region of the human interferon (IFN) stimulated genes (ISGs) [61]. BRCA1 has been reported to regulate IFN-gamma signaling by inducing *IRF7* gene expression [62].

MRE11, which is a double-stranded DNA break sensor with Rad50, is required for activation of stimulator of IFN genes, STING [63]. These lines of evidences imply that response to IFN should be co-regulated in accordance with DNA repair system when damage was introduced in chromosomal DNAs. Conversely, IFN signaling affects expression of genes encoding DNA repair factors. Recent studies revealed that immune system is closely associated with DNA-repair system. It has been reported that transcription of the *FANCF* gene is up-regulated by IRF8 during differentiation of myeloid cells [64]. Moreover, IRF1 has been shown to regulate *BRIP1* (*FANCJ*) gene expression [65]. IL-4 decreases DNA damage in murine and human glioblastoma cells when PARP-dependent DNA-repair is required [66]. Over expression of the IFN-related genes are caused by treatment with DNA-damaging agents and following ionizing radiation [67]. Interestingly, this over expression is enhanced in the BRCA2 knockout cells.

Integration of viruses into chromosomes might be damage on DNAs because exogenous DNAs will cause disruption of genes or enhancer insertions. Therefore, DNA repair system should be immediately evoked upon viral infection. Hence, immune sensing is primarily required to anti-viral immunity. It was indicated that oxidized base 8-hydroxyguanosine (8-OHG) potentiates cytosolic immune recognition by decreasing its susceptibility to TREX1-mediated degradation [68]. TREX1, which is also known as DNase III, is a 3′ exonuclease that is thought to play an important role in HIV-1 DNA sensing and viral immune evasion [69]. Interestingly, *TP53* gene expression is induced by type-I IFN signaling in $CD4^+$ T cells upon infection of HIV-1 [70]. Importantly, the concept has been postulated that DNA damage response affects innate immune sensors that drive metabolism, apoptosis, cancer, and aging [14].

Overall, the DNA repair system, including DNA damage sensing, and IFN response are thought to depend on and regulate each other. Previously, we identified duplicated GGAA (TTCC) motifs in a number of DNA repair associated genes, including *TP53*, *RB1*, and *BRCA1* [11]. In order to examine if GGAA (TTCC) duplication is a common feature of the 5′-upstream region of the DNA repair associated genes, we proceeded to re-survey of the data base of the human genomic DNA.

3. Comprehensive analysis of the DNA repair-associated gene promoter regions

First, we retrieved 568 gene IDs from NCBI_GENE data base (http://www.ncbi.nlm.nih.gov/gene/) with a key word "DNA repair" on July 20, 2014. Then, we accessed to their individual sequence data and searched GGAA or TTCC motifs within a region between approximately 540 nucleotides upstream and 90 nucleotides downstream from the putative TSSs. At least one duplicated GGAA (TTCC) motif is contained in the 630 nucleotide region of 358 different genes (Table 1). Our defined GGAA (TTCC) duplications, with no more than ten nucleotide distance between GGAA (TTCC) sequence pairs, are not found in the remaining 210 genes (not shown). These genes, whose putative promoter regions contain duplicated GGAA motifs, could be classified into several groups according to the biological functions of the encoded proteins.

Gene-Partner gene	Sequence
ABL1	CGGCA**GGAA**ATTTGTT**GGAA**GATGA, GTGAC**TTCC**ACA**GGAA**AAGTT
ACTL6A	CTACC**TTCC**CCTACCCGGG**TTCC**CGCCG, GCTTC**TTCC**AGCC**TTCC**TCCTT, TCGCT**TTCC**TCT**TTCC**CGCCC
ADH5-LOC100506113	TGAAA**TTCC**CG**TTCC**CTCACC, CACGG**GGAA**GCCCT**TTCC**CGACA
ADPRHL2(ARH3)-TEKT2	GATGG**GGAA**CACTA**TTCC**TCCGA, CGGAC**GGAA**GTAG**GGAA**ACTGT
AKAP9	GAGTG**GGAA**CCAGTGGAG**GGAA**GAGGG, CCACC**GGAA**CTT**TTCC**GTTGG
ALKBH1-SLIRP	GCCCC**GGAA**AAAAT**TTCC**GGATCC**GGAA**CACGA, CTTTC**GGAA**ACTT**TTCC**GCTTC
ALKBH4-LRWD1	CGACC**GGAAGGAA**GC**GGAA**CCCAG
ALKBH6-LOC101927572	AGACG**GGAA**A**GGAA**GTGC**TTCC**TTCAG
ALPK2	AGTTT**TTCC**T**TTCC**TAAGG, TCTTC**TTCC**AGAAC**TTCC**CCGGGCAT**GGAA**T**TTCC**CCTCTTA**GGAA**GAGAT
ANKRD26	GACAT**GGAA**GG**GGAA**TAAAC, AGATT**GGAA**ACCGCGGAGT**TTCC**TTTGG
APC	AGGAT**TTCC**C**GGAA**GAGGT
APEX1-OSGEP	CAGCT**TTCC**GGAGCGCAGA**GGAA**GCTGG, CACTG**GGAA**AGACACCGC**GGAA**CTCCC, CCGTT**TTCC**TATCTCT**TTCC**CCGTGG
APITD1	CGCAG**TTCC**TG**TTCC**ACTCG
APOBEC3B	CACAC**TTCCTTCC**CCACT, GGAGG**TTCC**TCTGCCAGCG**GGAA**GGGTCCGG**GGAA**AACCA
APOBEC3G	AAGCA**GGAAGGAA**AGAGC
AR	AGGTA**TTCC**TATCGTCCTT**TTCC**TCCCTC, GGGAG**GGAA**AAGGAGGTG**GGAA**GGCAAG
ASCC3	TAATA**GGAA**TTAT**TTCC**TCCAC
ASF1A	AAAGT**TTCC**GAGTCCA**TTCC**GGGAG
ASTE1-NEK11	ATCAC**GGAA**CTGTAC**TTCC**CAGAG, GAGAC**TTCC**GA**TTCC**CGCTC
ATF2	TGCTG**GGAA**GTGAC**GGAA**ACGGA
ATF3	TACTA**GGAAA**G**GAA**TCTGT
ATF4	TCGCC**GGAA**AACGACCT**TTCC**CCGCC
ATM-NPAT	AAAGC**TTCC**CTACCAAG**GGAA**AACCT, CAGCA**GGAA**CCACAATAA**GGAA**CAAGA, CCTTC**GGAA**CTGTCGTCAC**TTCC**GTCCT
ATR	CGGTG**GGAA**CGTGA**GGAA**CTTTT, ACGGC**TTCC**CGGC**TTCC**CCCGG
ATRIP	CATCA**TTCC**TCCTTGGACT**TTCC**TCCTC
ATRX	TTGGT**TTCC**TCATCT**GGAA**AATGT

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Gene-Partner gene	Sequence
AURKB	CTGGG**GGAA**TTTGG**GGAA**ACT**TTCC**TAAACT**GGAA**GCCAA, TCTCA**TTCC**GCCTC**TTCC**ATTGGG**TTCC**CATGA
BACH2	TGCCC**TTCC**G**GGAA**AACGC
BARD1-LOC101928103	GCAGC**TTCC**CTGTGGT**TTCC**CGAGGC**TTCC**TTGC**TTCC**CGCTC
BCCIP-UROS	CTACG**GGAA**GGGGAGG**GGAA**GCTTT, GAGGGT**GGAA**AGC**GGAA**GAAAA, GCCGT**GGAA**AGTGGGG**TTCC**GCAGC, GACGA**GGAA**GA**GGAA**AAAGA
BCL2	TTTTA**GGAA**AAGAG**GGAA**AAAAT
BCR-BCRP8	AAGTG**TTCC**TG**TTCC**AGGAC
BLM	CCGGG**TTCC**AGCTGCCTAC**TTCC**TTTAA, TCGGC**TTCC**CCA**GGAA**GCAGCCAATC**GGAA**TAGGCAAGC**TTCC**GGCG**GGAA**GTG AG
BRCA1-NBR2	ATGCT**GGAA**ATAATTAT**TTCC**CTCCA, AATTC**TTCC**TC**TTCC**GTCTCT**TTCC**TTTTA, TTGGT**TTCC**GTGGCAAC**GGAA**AAGCGCG**GGAA**TTACA
BRCA2(FANCD1)	GACAA**GGAA**T**TTCC**TTTCG
BRE-RBKS	TCTTC**TTCC**T**GGAA**TAGTC, GCTGA**GGAAGGAA**CTGTC
BRIP1 (FANCJ)	GATAC**TTCC**T**TTCC**GCTGG, GAGAC**TTCC**AGT**TTCC**AA**GGAA**TTTGC
BTG2-LINC01136	CCACG**GGAAGGAA**CCGAC
C17orf70	CCCGC**TTCC**CCACCCTGG**GGAA**CCCGT
C19orf60	CTTGG**TTCC**CCTTTC**TTCC**TTCTG
CAGE1-RIOK1	GCGAT**GGAAA**G**GAA**CGGCT
CCNF	GCGGC**GGAAGGAA**GGCCG
CCNO	CTGGC**GGAAGGAA**GGGCA
CDC20	CAAGC**TTCC**CAA**TTCC**GTCCC, TCTCC**TTCC**CCTTCTA**GGAA**CGGCT, AGACT**TTCCCC**G**GAA**GGCCC
CDC25	GCCTC**TTCC**CACTAGG**TTCC**ATCAT, GGAGG**GGAA**AGAG**GGAA**GGAGG
CDK1	TTTTT**GGAA**TCT**GGAA**TATTA**GGAA**TCAAC
CDK2-PMEL	CGAGA**TTCC**CGGC**TTCC**TGGT**TTCC**AAAGG, GCCAG**GGAA**ACGCG**GGAA**GCAGG
CDK5-SLC4A2	CCCAT**TTCC**GCTGCATTCT**GGAA**CGCGT, AAACT**GGAA**AAGATTGG**GGAA**GGTAAT**GGAA**TCTCG
CDK5RAP2	GGTTA**GGAA**CTTTGAGGA**TTCC**TGAGT, CTCGT**TTCC**GTA**GGAA**GAAGCGCCG**GGAA**AGATG
CDK6-LOC101927497	TGTGT**TTCC**TT**GGAA**TCGGC
CDKN1A(p21)	CATTG**TTCC**CAGCAC**TTCC**TCTCCC**TTCC**TAGGC, CCTGC**TTCC**CA**GGAA**CATGC

Gene-Partner gene	Sequence
CDKN2A(p16)-CDKN2AAS1	AGCCA**GGAA**TAAAATAAGG**GGAA**TAGGG
CEP63-ANAPC13	AAGCG**GGAA**AGCCTTG**TTCC**TTGCT, CGATG**GGAA**TAGGG**GGAA**GTCCG, TCGCT**TTCC**TCGGA**TTCC**CGGAT
CFL1	GAGAT**TTCC**TTGTACCT**TTCC**CCTGTGCCTT**TTCC**TCCTA, AGCGG**TTCC**TG**GGAA**ATTGG
CHAF1A	TGGGA**GGAA**T**GGAA**GTCAC
CHAF1B	ATAAA**TTCC**GGCCGGGA**TTCC**GACCC
CHD1L-PRKAB2	GTGGG**TTCC**TTATAG**GGAA**TAAGA
CHEK1	TTTTT**TTCC**TAC**GGAA**TCATG, TCGCC**TTCC**CAAAGTGCT**GGAA**TTACA, CTTAT**TTCC**ATTT**TTCC**TATTT
CINP-TECPR2	ATCGG **TTCC**T**TTCC**CGGGG
CLOCK	CGGCA**GGAA**GCTCTTC**TTCC**TCCTC
CLSPN	CCACG**GGAA**CCTT**GGAATTCC**TCTAA, GCCCA**GGAA**CCGT**TTCC**CAGCTCAC**TTCC**CCCCG
CLU	CTGAT**TTCC**TAACTG**GGAA**GGCTC, GGCTC**TTCC**CTACT**GGAA**GCGCC
COCH	AAGTA**GGAA**CTCT**TTCC**ACGAG
COL6A6	GTCCG**TTCC**ACGG**TTCC**GAGGT, TGCAT**GGAA**GT**TTCC**CCAAG
COPS5	CCTTC**TTCC**GGTGC**GGAA**GACTA
CPT2	GAGTT**GGAAGGAA**TCTTG, TGACA**GGAA**GCCTC**TTCC**AATAG, AACAC**GGAA**GAC**TTCC**TAGAG, GGGGA**TTCC**GCTC**GGAA**GGGGC, CAGCG**GGAA**ACTCCAGGT**TTCC**AACTC
CRY1	AAAAA**TTCC**A**GGAA**GTCCA**GGAA**TGCCT, CTGAA**GGAA**ACCGGACAAT**TTCC**AGGCC
CSNK1D	CGCGA**GGAA**CTCACCTGGC**TTCC**TCGAC
CSNK1E	TCCCA**GGAA**CTGTGC**TTCC**GGGAT
CUL4A	ATCCA**TTCC**CTATAT**TTCC**TATCC
DCK	ACTCC**GGAA**CCTC**TTCC**CGCGC
DCLRE1A-NHLRC2	CAGCG**GGAA**CTTG**TTCC**CGCCA
DCLRE1B	TCCAG**TTCC**AGCCTTAA**TTCC**CCCTC
DCLRE1C-MEIG1	TAAAC**GGAA**GAG**GGAA**TTAATAG**TTCC**TGAAT, AAGCA**GGAA**GC**GGAA**CGAAG, TCGAT**TTCCCTTCC**CGCGA
DDB1-DAK	AGTCC**TTCC**CG**TTCC**CAAAGGA**GGAA**CAGCCC

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Gene-Partner gene	Sequence
DDX1	GAGGA**TTCC**TCATTTACT**TTCC**CCATC
DDX11-DDX11AS1	GAGCG**GGAA**AACA**TTCCGGAA**GTGGA
DEK	ATCTT**TTCC**A**GGAA**GCGACCGT**GGAA**ACAAT, CGTCC**TTCC**G**TTCC**GCGCT, CCGCA**TTCC**CGCTCTCC**TTCC**CGAAC
DHFR-MSH3	GGCTC**TTCC**CACC**TTCC**CCTTC
DNTT	GATCT**GGAA**AACATAG**TTCC**AAGTG, GATGC**TTCC**CTACC**TTCC**TCACG
EGF	GGGAT**TTCC**CTTTGATTT**GGAA**AGAAT, CCTGCT**TTCC**TGTGTGGA**GGAA**TTGCC, TAGCT**GGAA**CT**TTCC**ATCAGTTC**TTCC**TTTCTTT**TTCC**TCTCT
EGFR	AGAAG**GGAA**AGGG**GGAA**GGGGA, TGCTG**GGAA**CGCCCCTCTC**GGAA**ATTAA
EGR1	AGGGC**TTCC**TGC**TTCC**CATAT
EIF2AK3	TTGTA**GGAA**AGGTA**TTCC**G**GGAA**CTGAT, CACCAG**GGAA**AGTCCACC**TTCC**CCAAC
EIF3A	GCTCC**TTCC**T**TTCC**GTCTC
EME2-MRPL34	CGGCC**GGAA**GTCACC**GGAA**GAGGC, CGGCC**GGAA**GCGA**GGAA**GAGGT
ENDOV-LOC100294362	GGGTG**GGAA**GTGCGGCCCG**GGAA**AGCGC, GTCGC**TTCCGGAA**GTGACGTGC**GGAA**GGGGT, CCAGT**TTCC**GGCGC**GGAA**GCGGA
ENG	TCTAA**GGAA**GCGCAT**TTCC**TGCCT
EPC1	TTTTT**TTCC**CAA**GGAA**TTAAA
EPC2	GGAGG**GGAA**GGGAGA**GGAA**GGAGG
ERBB2-PGAP3	GAAGC**TTCC**AC**TTCC**GGAGTAACC**GGAA**G**TTCC**TGTGT
ERCC1	AATTC**GGAA**TT**TTCC**GAGAA
ERCC2(XPD)	GCTCT**TTCCCTTCC**ATGTT
ERCC3(XPB)	GGAGC**TTCC**GGATTGAGCC**GGAA**GTCCC
ERCC4(FANCQ)	CTTAC**TTCC**CC**TTCC**CTTGC
ERCC6L2-LINC00476	CAGAG**GGAA**GAGACATCG**GGAA**GATTG
ERN1	GTTCA**TTCC**AAGC**GGAA**GTGAT, TGAGG**GGAATTCC**TGAGGGCAA**GGAA**AA**GGAA**GAAAG
EWSR1-RHBDD3	CGGAC**GGAA**CCA**TTCC**AAACA
EYA1	CTTTT**GGAA**GAACCGG**TTCC**TCAGC
EYA3	ATGTC**TTCC**AAAAC**TTCC**CACTC, CTTAC**TTCC**GG**TTCC**TAGCG
EYA4	GAGAG**TTCC**AG GCAA**TTCC**GGGGG, GGCCG**TTCC**CGGC**TTCC**GCGCAA AAC**TTCC**ATCCT, GAGGG**GGAA**AGAGCTGCG**GGAA**AAGCC

Gene-Partner gene	Sequence
FANCA	AGTCT**GGAATTCC**TGGGC, AGTCA**TTCC**CGGCA**GGAA**CCACG
FANCB-MOSPD2	GAGTC**TTCC**C**TTCC**CAGGA, GAGAG**GGAAGGAA**GCGGG
FANCC	TCGTC**GGAA**TT**TTCC**CGCGA, CCGCG**GGAA**AA**TTCC**AAAAA
FANCD2-CIDECP	CGGCC**TTCC**AC**TTCC**GGCGC**GGAA**GTTGG
FANCE	CCTCC**TTCC**CT**TTCC**GACAGCGCG**GGAA**CGGCT
FANCF	GATAT**TTCC**AAAGCGAAA**GGAA**GCGCG, CGTGG**TTCCGGAA**ATTCT
FANCG	TCGGT**GGAA**GCG**GGAA**CCCAG
FANCI	CTGCC**TTCC**AGGCTT**TTCCA**GTGC
FANCL	TTCAT**TTCC**GCCCGC**GGAA**TCCTC
FANCM	TCATT**GGAA**AC**GGAA**CTTAA, AATCA**TTCC**CAAC**GGAA**ACTCA
FBXO6	CATAA**GGAAGGAA**CTAGT
FEN1-TMEM258	GGGGC**TTCC**CCC**TTCC**CCACC, CAACC**GGAA**AA**G** **GAA**GTGCC
FGF10	AATTC**GGAA**AGCG**GGAA**GATAC
FNDC4	GAGGT**GGAATTCC**TC**TTCC**CAACT, GGCTC**TTCC**ACGCGGGGA**GGAA**GGGGA
FOXM1	ACGAT**TTCC**CCCAGTGA**GGAA**ATCAA
FOXO3	CTCGT**GGAA**GGGAGGAGGA**GGAA**TGT**GGAA**GGTGG
FUS	CCACA**GGAA**TCTCGG**TTCC**ACCCC
GLB1	TGCAG**TTCC**AAAGGGTCCC**TTCC**CAG**GGAA**GACGC
GSTP1	GCAAT**TTCC**T**TTCC**TCTAA, CTTAG**GGAA**T**TTCC**CCCCG, ACCTG**GGAA**AGAG**GGAA**AGGC**TTCC**CCGGC
GTF2H1-HPS5	TCGCG**TTCC**TCCCC**TTCC**TTGCT, GGGAG**GGAA**CTAGC**GGAA**GGTGT
GTF2H2	GTGAA**TTCC**AGCT**GGAA**CACCGTCCCT**TTCC**GCGCC, GCGGC**GGAA**TGAC**TTCC**GGGGC
GTF2H3-EIF2B1	CCCAC**TTCC**GGCGCAC**TTCC**GTACCCCTC**TTCC**GGCGC
GZMA	AGTGG**GGAAGGAA**AATCC
H2AFX	TGGTC**TTCC**GCTTCTGGT**TTCC**GATTG
HERC2	GGTGT**TTCC**TTCTTCGA**TTCC**CTGCA, GTGGC**GGAA**ATCCCGCC**TTCC**GGCGC
HELQ-MRPS18C	CATGG**TTCC**GCGTTTC**TTCC**AC**TTCC**TTTCG**TTCC**AAATCG**TTCC**GAAAGGCCCC**TT** **CC**GCTGCTC**TTCC**CCTGT
HINFP	CACTG**TTCC**CGCCCC**TTCC**GTGTT
HLTF-HLTFAS1	ATAAA**GGAA**GGTCGT**TTCC**CTCCG

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Gene-Partner gene	Sequence
HMGB2	GGGCT**TTCCTTCC**CGAGC
HNRNPC	CAATA**GGAA**GATTCTCA**GGAA**TGGGG
HSPA1A-HSPA1L	ACCCT**GGAA**TA**TTCC**CGACC
HSD17B6-PRIM1	CCACA**GGAA**TTGGCG**GGAA**CAGCA, CGCCG**GGAA**TTGTAG**TTCC**CACTT, GTCCA**TTCC**A**GGAA**GAGGA
HTATIP2	AGAAA**GGAA**TCAAA**GGAA**TCCTG, GTGAG**GGAA**AACGCG**GGAA**GAGGG, GCAGA**TTCC**AAACTTA**GGAA**GGGTC
HUS1B	GGAGT**GGAA**AC**GGAA**GCATT
HUWE1	TCATG**TTCCC**T**TCC**GCGGC**TTCC**ACCGT
IER3	TCGTC**GGAA**T**TTCC**AGCCC
IGF1	AAATG**TTCC**CCCAGCTGT**TTCC**TGTCT
IGF1R	TGAGC**GGAA**AAAAAAAG**GGAA**AAAAC
IGFBP3	GCCGC**TTCC**TGCCTGGA**TTCC**ACAGC
IGHMBP2-MRPL21	CGGCC**GGAA**AC**GGAA**ACGAC
IL18	TGGGA**GGAA**GG**GGAA**GTCCT, TCGAC**TTCC**ATTGCCCTA**GGAA**AGAGC
INO80B	GGACC**GGAA**CGTTCGTT**GGAA**GGATC, AGTTT**TTCC**GCGGGGC**GGAA**AAGGC
INO80C	ACCTT**TTCC**GCGTG**GGAA**GGCAG
INO80E-HIRIP3	AGTCA**GGAA**CGGCGCT**TTCC**AGCGT, ATACC**GGAA**TCTGAAGC**GGAA**GCTCAAG**TTCC**TCATC
IPO4	GGGCA**TTCCTTCC**CCAGA, GCCCT**TTCC**TCC**GGAA**GTGGG
IPPK	AGGCC**GGAA**GCTTCTC**TTCC**GGCTC, CCGCG**TTCCGGAA**ATGAG
IRS1	TAAAT**TTCC**TGGG**GGAA**ACAGC
JMY	GGGCT**TTCC**TCAGACACC**TTCC**TTTCA, CTCAG**TTCC**TCCGCCTTAG**TTCC**TCTT**TTCC**CCGGGT, TGCGC**GGAAGGAA**GGAGA
KAT5	AGCTA**GGAA**TC**TTCC**CTGAG
KDM4A	GCAGC**TTCCC**T**TCC**CTGTT
KIAA0101-TRIP4	CCATC**TTCC**CCAGCC**GGAA**CCAGC,
KIF2C	GAAGT**TTCC**CAGTTTTCG**GGAA**CCCCG, GCGTA**GGAA**GATGGTTG**GGAA**CTGCG
KIF13B	TGGCA**GGAA**ATGAGCA**GGAA**GAGGT
KIF22	GACTG**GGAA**CC**GGAA**CCGTG
KIN-ATP5C1	CCCGG**TTCC**GT**TTCC**GGCTG
KLF8	CTCCG**TTCC**TTTTAGC**TTCC**TCCCT

Gene-Partner gene	Sequence
KRAS	CCCTC**TTCC**CTC**TTCC**CACAC
LCT	ACATT**TTCC**GGG**TTCC**TCTGC
LMNA-MEX3A	TTTCT**TTCC**ATTA**TTCC**AGATA, GTGGT**GGAA**G**GGAA**AAGAG
LIG1-C19orf68	GGCGC**TTCC**ACCGA**TTCC**TCCTCT**TTCC**CTGCC
LRRCC1	AATGT**TTCC**A**GGAA**CAAGA, TTTTC**TTCC**TCATACAG**GGAA**GTGAC, AGGCG**GGAA**AG**TTCC**CGGCT
MAD2L1	TCTCG**GGAA**AAGCTGCG**TTCC**CACAC
MAD2L2-DRAXIN	GGACT**GGAAGGAA**GGGGG
MCM8-TRMT6	GCCGC**TTCC**GCT**TTCC**GGCCC, ACCGC**TTCCGGAA**GCCTCTCGGC**TTCC**GTCTG, CTTCT**GGAA**GCTGCGGTGG**GGAAA**CTGAGT**TTCC**CGAGC
MCRS1	TCGTG**GGAA**TTT**GGAA**GTCGA, AACTA**GGAA**AGCCTTTACT**TTCC**GCTAT
MDC1	ACGTA**TTCC**CA**GGAA**GAAAG, CAAAA**GGAA**ATGAAA**TTCC**AATGC
MGRN1	CGCTT**GGAA**CGCAGAG**GGAA**GGACC, GTTGG**TTCCTTCC**CTCTG, TCCTG**GGAA**AGATAG**TTCC**CAGACGGGC**TTCC**CGCGCTGC**TTCC**CGGCG
MIR96	ACGTC**GGAA**ACAGGCTGC**TTCC**AAGGG
MMS22L	GTGCT**TTCC**AAGTT**TTCC**ATATC
MNAT1	TCATG**GGAA**TGT**TTCC**AGACA, AAGAT**GGAA**TTTATC**TTCC**TAATT, CCGGG**GGAA**CTGACTGCC**GGAA**CGTTT
MORF4L2	ATTTT**TTCC**TA**GGAA**TGAAC
MPG	GGCTG**TTCC**CACA**GGAA**GGAGA
MPLKIP-SUGCT	GTAGC**GGAA**GCAGC**TTCC**GG**GGAA**CCCCG
MRE11A-ANKRD49	GCAGG**TTCC**CAGGC**GGAA**GCCCA
MSH3-DHFR	GATTC**TTCC**AGTCTACG**GGAA**GCCTG
MSH6	CGCTG**TTCC**CGC**TTCC**GCTCC
MSL1	CCGCT**TTCC**CCTTC**TTCC**CGCGG
MYB	GTGCG**GGAA**T**TTCC**CCCCA
MYO5A	CCCTA**GGAA**TGCTT**GGAA**GGACG
NABP2-RNF41	TCCCG**GGAA**G**GGAA**G**GGAA**AGG**GGAA**GGAGG**GGAA**AGAAG
NBN(NBS1)	GGTTG**GGAA**GCTACT**GGAA**TTAGG, CAGGT**GGAA**GT**GGAA**A**GGAA**GGGTA, CTAGA**TTCC**AAA**GGAA**TACCT, TGCTG**TTCC**TT**TTCC**AACCA
NCOR2	GGCGC**TTCC**CCCCTCC**TTCC**TCCTC
NDNL2	TGCAC**GGAA**AACGCTG**TTCC**TTTTGG
NEIL1	GGCGG**TTCCTTCC**GCCGG

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Gene-Partner gene	Sequence
NEIL2	CCACT**TTCC**AG**GGAA**TGAGC
NES	CCTGG**GGAA**GCA**GGAA**CAGAG
NFRKB(INO80G)	GGACG**GGAA**GGA**GGAA**TGAA**GGAA**CTCGGAAGCACA
NIPBL-LOC646719	GTGGC**GGAA**GTGGAGTGG**GGAA**GAGGG
NLRP11-NLRP4	CGGCT**GGAA**GCG**GGAA**GAAAA, GGAGG**TTCC**TATTGAGAA**TTCC**CAGGG
NONO	TCCCC**TTCC**TCTCCCTCT**TTCC**ACT**TTCC**TCTCC
NPAS2	GCAGA**TTCC**TTG**TTCC**CCCCG
NPM1	CATCT**TTCCTTCC**TAACA
NR2C2	CGCTG**GGAA**GA**GGAA**GAAGA
NSMCE1	CTCAG**TTCC**ACAGATGG**GGAA**ACTGA, CAAGT**GGAA**GCCCC**TTCC**CATTA
NSMCE4A	CGAAC**TTCC**GCCG**TTCC**GAAGT
NUDT1-FTSJ2	CCCGG**GGAAC**TGCGACCC**GGAA**TCCTG
NUPR1	ATCCC**TTCC**CCCTCC**TTCC**TCACG
OFD1	TAAAT**GGAA**TCACTAATGA**GGAA**AGGCA
OTUB2	TACCC**TTCC**TGGA**TTCC**AGAAA
PALB2(FANCN)-DCTN5	AGAGA**TTCC**GGCTAC**TTCC**GGCCG
PARG-TIMM23B	GCCGC**TTCC**CCCGCCTCC**TTCC**ATGGT, TGACA**TTCC**GGGCGCCGG**TTCC**CGTTA, GCCCC**GGAA**GCC**GGAA**GCGCC, CAGCT**TTCC**GGTGGTG**GGAA**AGTGA
PARP1	GCGGG**TTCC**GTGGGCG**TTCC**CGCGG
PARP2-RPPH1	CCCCC**TTCC**CC**TTCC**AGCTC
PARP4	CCTGT**TTCC**ACGAACT**TTCC**CGAAA, CCCGA**TTCC**GGGCGCG**TTCC**GGCTA
PARPBP-NUP37	AAGTG**GGAAGGAA**GAACTCCTG**GGAA**TAGAG
PDE4DIP	TCAAG**GGAA**AATTGAAA**GGAA**AAGATTTTA**GGAA**AGAGA,
PIWIL2-LOC100507071	CACAG**GGAA**CCTGCT**GGAA**AGGAC
PMS2-AIMP2	TGGAG**GGAAC**T**TTCC**CAGTC, CGGCA**TTCC**AACCTCCCT**GGAA**ATGGGG**GGAA**CATGG
PNKP	AGATG**GGAA**AAAAATC**TTCC**TCCCT, GTCAT**TTCC**GTCCGCCGA**GGAA**CCGAC
POLA1	TCGCT**TTCC**CGGCTCTGG**GGAA**AACGA, CTCCT**TTCC**G**GGAA**AATGG, TGGCC**TTCC**GGCC**GGAA**GTCCG
POLB	CCCGT**TTCC**CCTTCTAG**GGAA**AGGA**TTCC**AGATA, AGGTC**TTCC**CATA**GGAA**GGCCC
POLD1	GGCGG**GGAA**CAGC**GGAA**GTGAG

Gene-Partner gene	Sequence
POLD3	CCTCT**GGAA**AAACC**TTCC**CTAAT
POLD4	GCCTA**GGAAGGAA**AACG**GGAA**GTGAG
POLG	CTTCG**TTCC**TGAGGGA**GGAA**TAAAC
POLH-XPO5	AGCCC**TTCC**ATT**TTCCTTCC**AGTAG
POLI	CAGGC**GGAA**GCGGCC**GGAA**GTAGC
POLL	AACCG**TTCC**AGAGGGTCAC**TTCC**GGCTGACTC**GGAA**GCTAT
POLM	GGGGC**TTCCTTCC**GTCTC
POLQ	CCCAA**TTCC**TCATTACATA**TTCC**TCACA
POLR2A-ZBTB4	GGGCG**GGAA**A**GGAA**GGGGC, ACCTT**TTCC**TT**TTCC**CTTCT, AAAAT**TTCC**GGTAAG**GGAA**AGAAG, CTTAT**TTCC**CCGCCTCC**TTCC**CTCCCCCACC**TTCC**CCTCC
POLR2B	TTCTG**GGAA**CGTCGGAGAC**GGAA**GTTAC
POLR2F-C22orf23	CTCCC**GGAA**GTGAT**TTCC**TCTGG, GCCGA**GGAAGGAA**GGGCG
POLR2G	AGTGT**TTCC**GGTGGA**TTCC**CAGGG
POLR2I	CCCCC**TTCC**G**GGAA**CCCCC, GTCCC**TTCC**CCACCGCCA**GGAA**GAGGG
PPM1D	CCTTT**GGAA**GGGAGGT**TTCC**CGCCA
PPP1R15A	CTTAC**TTCC**AC**TTCC**CACCC
PPP5C	AGAGA**GGAA**G**GGA**AGATTT
PRKDC(DNA-PK$_{CS}$)-MCM4	ATCGA**GGAA**CAAACTT**GGAA**CTCTT, CGTTT**TTCC**TTAGGT**TTCC**ATGTT, CCCCG**GGAA**AG**TTCC**TGCCG
PRMT8	GGCAT**GGAA**AACCA**GGAA**GTTTC
PRPF19	TTCTG**GGAA**AGGGCAAT**TTCC**GTTAG
RAD1-BRIX1	TTCAC**TTCC**TCCGCGG**TTCC**TCGGA
RAD9B-VPS29	GTTTA**TTCC**CT**TTCC**CTAGA, TTGCG**GGAA**ACGAGTA**GGAA**CCGTCT**GGAA**ACGGA, CTCCC**TTCCTTCC**CCTAGA, GGGAT**TTCC**CAA**TTCC**TCGCC
RAD17	CCAAC**GGAA**TTAACG**TTCC**GCGTC
RAD23A	AACCC**GGAA**GGC**GGAA**GCTGC
RAD23B	CGACA**TTCC**AGGACCGCC**TTCC**GCCCC
RAD51AP1-C12orf4	TGGGA**GGAA**AACTAAG**GGAA**AAGAC
RAD51B	AAAAT**TTCC**AAACAGGGTG**TTCC**CTTGT, GCGTT**TTCC**GCGG**GGAA**ACTGT
RAD51C(FANCO)-TEX14	TTTGG**GGAA**TCAAAAC**GGAA**TGGTG
RAD51D	ATCCG**TTCC**GTTT**GGAA**C**GGAA**GCTGG, AGCCT**GGAA**CCC**GGAA**GCGGC

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Gene-Partner gene	Sequence
RAD52-ERC1	GGTGA**GGAA**CTG**GGAA**GCGGG, CCGTA**GGAA**GTGGACGCT**GGAA**GCCCG
RAD54L	AAATC**TTCC**C**TTCC**ATAGC, CACTA**TTCC**CGCCTC**TTCC**TTGGG
RAF1	ATGGG**GGAA**AAATGAACTC**GGAA**TTTAC
RASSF	CACTG**GGAA**AAGCAT**GGAA**AGACT, AGGAG**GGAAGGAA**GGGCAA
RB1-LINC00441	CAGGT**TTCC**CAGTTTAA**TTCC**TCATG, CGGGC**GGAA**GTGACGTT**TTCC**CGCGG
RBBP8	TTTGA**TTCC**ATG**TTCC**ACAGA
RBP14	CCCAG**GGAA**TGT**TTCC**AAAGA
RECQL-GOLT1B	ACGTC**TTCCGGAA**ACACG
RECQL5-SAP30BP	CCCGA**TTCC**CCC**TTCC**AGCTT, CCGAC**TTCC**GGGC**GGAA**AGGCA, AACAG**TTCCGGAA**CCAGC
REV3L-TRAF3IP2	GTTCG**GGAA**GGG**GGAA**CGCCA
REXO2	ACTCA**GGAA**ATAACTCCT**GGAA**GCAAA
RFC2	GGGGT**GGAATTCC**CATCT
RFC5	AGGGA**GGAA**GTC**GGAA**ACTGG
RHBDL2	GCCCA**GGAA**GCCT**GGAA**CGCAA
RIF1	GGCAG**GGAA**GGGATG**GGAA**GGGATG**GGAA**GGGAG
RMI2	CCCAT**TTCC**TCCGTTCA**TTCC**TAACT
RNF2	AGAGT**GGAA**GGTCATT**TTCC**CAGGA
RNF4	CGGCT**GGAA**ATCTA**GGAA**TG**GGAA**GGTTC
RNF44	GTAAG**GGAA**GGCCCTCACT**TTCC**CCATC, CTTAG**TTCC**CAGT**TTCC**CTGGC, ACCTG**TTCCC**CGCCTCTC**TTCC**TCCAC
RNF113A	GCTCC**GGAA**GAAGCGAC**GGAA**TCTGC
ROCK1	GCTTC**GGAA**CT**TTCC**CAGTG, GCTGG**TTCC**CCC**TTCC**GAGCG
RPA2	TTTGA**GGAAGGAA**CTGAC
RPA3	TTTTC**TTCC**TCTTT**GGAA**TTAAA
RPS3	CCCCT**TTCC**TG**TTCC**TGCCT, GCCAC**TTCC**T**TTCC**TTTCA
RPM1	TTTTC**TTCC**AG**TTCC**AGAGT
RRM2B	CCAGC**GGAA**GCAGGGAGAT**TTCC**TTAGG
RTEL1	AAGCT**GGAA**CGCAGGAGA**GGAA**GGAGA
RUNX1	ATTCT**TTCC**CT**TTCC**CAGGC, TGGAG**GGAAGGAA**GGGCA, GCTGT**GGAA**AGG**GGAA**CAGTT
RUVBL1-EEFSEC	ACCCG**TTCC**GGCCC**GGAA**GC**TTCC**GCCCT

Gene-Partner gene	Sequence
SAMHD1	CCACT**TTCCTTCC**TCTG**GGAA**TGCAG, AATCA**TTCC**GGGTTC**TTCC**AGTTC, CTGTT**TTCC**TCTTCACTG**GGAA**GGTGC, GCTCT**TTCC**TCCCCCT**TTCC**ACCAG
SAT1	CCTCT**GGAA**AA**TTCC**ATTGT
SERPINE1	CTGGG**GGAA**AAC**TTCC**ACGTT
SETMAR	GTCAA**GGAA**G**GGAA**GCGCC**TTCC**AGGCC
SETX	GGCCT**TTCC**CGCAGTG**TTCC**CCTGG
SF3B3-COG4	CGTGT**GGAA**GCAAGAC**GGAA**GCATT
SFPQ	CTGTA**TTCC**TATGAGG**TTCC**ATAAT
SFR1	CGAGA**GGAA**ACTGGAT**TTCC**AGTTT, CTCTC**TTCC**CCTTTGTAAA**TTCC**TTGGG
SIRT1	CTTCA**GGAA**GACGT**GGAA**A**TTCC**CAGGG
SLC30A9	CATCC**TTCC**CATCT**TTCC**TCCCAT**TTCC**GAAAC, CCCCG**GGAAGGAA**GGCCT
SLX1A-BOLA2B	GCCAC**TTCC**GCT**GGAA**AACTCAC**TTCC**GCCCT, GCGGC**GGAA**CTCAG**GGAA**GGAGC
SLX4 (FANCP)	GCAGA**GGAA**GACC**GGAA**GCGAG
SMAD3	CGTGT**TTCC**CAGGAC**TTCC**TCCCC, GGACT**TTCCTTCC**CGGAG
SMARCB1	AGAGA**GGAA**TGGAGAAGGT**GGAA**GGTGT
SMC1A-RIBC1	GGTCC**TTCC**AA**TTCC**CGACC
SMC2	TCAAA**GGAA**TAAATAG**TTCC**GGCGC
SMC3	CAGCA**TTCC**ATGTG**TTCC**AAGGC, GGCGC**GGAA**CCT**TTCC**CCCTT
SMC4-IFT80	TCAAG**TTCC**A**GGAA**AGCGG, CTCCC**TTCC**TC**TTCC**CGCGA
SMC5-SMC5AS1	CGGTG**GGAACGGAA**GTCGC
SMG1	CTCCC**TTCCCTTCC**ATCGT, GTGCT**TTCC**G**GGAA**GCGTT
SMO	ACGAT**TTCC**ACTCATCTCT**TTCC**CCCGG
SMUG1	AGCAT**TTCC**GGC**GGAA**GTGGC, GTGGG**GGAAA**G**GAA**CC**GGAA**ACGGG
SOX4	TCGGG**TTCC**AAGCCAATG**GGAA**GCCCG
SP1	CTGGT**TTCCTTCC**AAGCC
SPATA22	GATTC**TTCC**A**GGAA**CAACA, GTCGA**GGAATTCC**C**GGAA**ACCTC
SPIDR	CCCGG**GGAAGGAA**GCTCG
SPTAN1	CCTCG**GGAA**AGTGAGCA**GGAA**GAGAC
SSRP1	ACGCG**GGAA**AAGC**TTCC**CCGGT
STAT3	GGACA**TTCC**GGTCATC**TTCC**CTCCCT
STRA13	CCGGC**TTCCGGAA**GGTGA

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Gene-Partner gene	Sequence
STUB1	AACTC**TTCC**CGATACCTGA**GGAA**GGGCG
SUMO1	CCCAT**TTCC**CGCCTTGTCT**TTCC**TCTCT
SUPT16H	TTGTG**GGAAGGAA**CTAAA, GCTCT**TTCC**GCTCCCCC**TTCC**TTTGC
SUZ12	TTTTT**TTCC**TCCCTCC**TTCC**CTCCT
SWI5	TCAGT**TTCC**CAAGACCTG**TTCC**ACAGA, TCCCC**TTCC**AGCT**GGAA**ATTTA, CTACA**TTCC**ACTCCTAGGG**GGAA**CATCA
SYNE2	CAAGG**GGAA**GGA**GGAA**CCCAG, CCTAC**TTCC**AAGACCCA**GGAA**TCTAC
TCEA1	ACGGG**TTCC**ATTT**TTCC**CCGTA, CAAAG**TTCC**ATGCTC**GGAA**TCTGC
TDP1-EFCAB11	GGCAA**GGAA**CGTGGGGCGAG**TTCC**TTTTC (11 nucleotide distance)
TDP2-ACOT13	ACTTC**GGAA**GAGCT**GGAA**AGTCC
TDRD3	TTTTG**GGAA**GACCAAAC**GGAA**TACCC, TCCCC**TTCCTTCC**GTAAC, TACCC**TTCC**GCCTG**TTCC**TCTCT
TERT	TCCCC**TTCC**T**TTCC**GCGGC
TICRR	AAGTT**TTCC**TCGGTCTTG**GGAA**ACGTG, GCTGT**TTCC**CTGAA**GGAA**GGGAC
TIRAP	CCTTT**GGAA**AAG**TTCC**ATCTC
TK1	TAAGC**TTCC**TTCTT**GGAATTCC**AATCT, TCTTC**TTCC**AA**GGAA**CCTTGCTTG**GGAA**ACCCA
TONSL	CGTAC**TTCC**C**GGAA**TGCCC
TOP2A	TCAGT**TTCC**TCA**GGAA**AACGA, ACCCC**TTCC**CGC**TTCC**AAAGC, ATCTC**TTCC**AAGCT**TTCC**GCACG
TOPBP2	ATTGA**GGAA**ATCCTTTCT**TTCC**CTGGC, GTCAC**TTCC**ACC**GGAA**AAGGC
TP53-WRAP53	TCCAT**TTCC**TTTGC**TTCC**TCCGG
TP63	ATCAA**GGAA**T**TTCC**CTGTC
TREX1	GCTTC**TTCC**AGAGG**TTCC**CCAAC, GCCGC**GGAA**AC CGATGT**GGAA**GACCC
TREX2	CTCCC**TTCCTTCC**CCAGC
TRIM56	CTCCA**GGAA**GCCTGTGCTG**TTCCC**TCAG
TRIP12-FBXO36	GAGGG**GGAA**TTAG**TTCC**TGCTA
TTC5	TTTGT**TTCC**AGGATCTG**GGAA**AGAAA
TYMS	CCGCA**GGAA**AACGTG**GGAA**CTGTG, CCAGG**TTCC**CGGGT**TTCC**TAAGA, CCGCG**GGAA**AAGGCGCGC**GGAA**GGGGT
UBA1	CCCAA**GGAA**GAAT**TTCC**AGCAC, ACACG**TTCC**GT**TTCC**TC**TTCC**CACCC
UBA2	CGCCC**TTCC**CCCACCCGC**TTCC**GGCCG

Gene-Partner gene	Sequence
UBB	TGCAA**GGAA**GT**TTCC**AGAGC, ATATT**TTCC**TAAAGAA**GGAA**GAGAA
UBC-MIR5188	GTACA**GGAA**GGT**GGAA**GAACA, GGGGT**TTCC**GCCTCTTTT**TTCC**AAATT
UBE2B-CDKL3	CTTCA**GGAA**GCCCAGGG**GGAA**CCGCG
UBE2T	CTAAC**TTCC**ACTTGAACAT**TTCC**AGTGATGAA**GGAA**TTCAC, ACGAT**TTCC**AGCTCC**TTCC**TTGGT
UBE2V1	GCGCC**GGAAGGAA**TCCTG
UBE2V2	ACAAG**GGAA**TTGC**GGAA**ACAGC
UBE2W	TGGTT**GGAA**ACGAAATA**GGAA**AAAAA
UBR5	ATAAT**TTCC**TTACTT**TTCC**AATAA
UHRF1	AGGCG**GGAA**AACGAGGCG**GGAA**AAGAC
UNG	GGGCA**GGAA**CTTTTC**TTCC**CAGCC
UPF1	GATGG**GGAA**ACTGAG**TTCC**AAGCA
USF1	GAATG**GGAA**TCAAGA**TTCC**TGTCA, CTTCT**TTCC**T**GGAA**TGAAA
USP1	GCGCG**GGAA**CCCTG**GGAA**GCTCC
USP3	CACCC**TTCC**CGGGGCCGG**GGAA**GCGGC, GACTA**GGAA**AGTCACTTC**GGAA**CACAG, GGCCT**GGAA**AGGC**GGAA**GCCTC
USP28	TGATG**GGAA**ATCCTTTA**TTCC**ACGGT, GCAGT**TTCC**CACGGCGGG**GGAA**CAGTT
USP47-LOC102724878	GAGAA**GGAA**G**TTCC**CT**GGAA**GAGGG
UVSSA	AGACC**GGAA**C**TTCC**TTTCG
VCP	GAGAA**TTCC**AATCCGTCGA**GGAA**GCGTA
VWA2	AAAAA**GGAA**AT**GGAA**AACCT
WRN	AGGTG**GGAA**GATG**GGAA**TGAGG
WRNIP1-MYLK4	GGGCC**GGAA**GACGACCCC**TTCC**TTTCG
XIAP	CATCC**TTCC**CTTCTT**GGAA**ACAGA, CTTTC**TTCC**ACTA**TTCC**TCAAC
XPC-LSM3	CATTT**TTCC**TGAGTCT**GGAA**AAAGC, GCTCT**TTCC**TGC**TTCC**CGCAG
XRCC1	GCTAA**GGAA**CGCAGCGCTC**TTCC**CGCTC
XRCC3-ZFYVE21	CGGCG**GGAA**GAGGAGTGC**GGAA**CCCGC
XRCC4-TMEM167A	GTTTT**GGAA**GATACC**GGAA**GTAGA
YBX1	CTCGT**GGAA**GTCACG**TTCC**TTCTG
ZC3HAV1(PARP13)	GCTCT**TTCC**G**GGAA**TGGGT
ZNF143-LOC644656	CCAAT**GGAA**AACC**GGAA**GCGTC, ACGAA**GGAA**TTGTT**GGAA**AATTT

Gene-Partner gene	Sequence
ZNF668-ZNF646	GGGAG**GGAA**G**GGAA**AGAG**GGAA**AGGAG, AGGGG**GGAA**GA**GGAA**GGAGG, TACTA**GGAA**ACA**GGAA**GTGTC, AAAGC**TTCC**CCCGCGAAAC**TTCC**GCTTC, TTAAA**GGAA**ATGTTGT**GGAA**TATAA
ZNRD1-ZNRD1AS1	TGGTG**GGAA**AATTTGCT**GGAA**GCGCAG, CCCCT**GGAA**AGGG**TTCC**AAGTC, GTCTG**GGAATTCC**GGGCG

Table 1. GGAA motifs located in the 5′-upstream regions of human genes encoding DNA-repair associated factors. Duplicated GGAA (TTCC) motifs (bold) that are located between 540-bp upstream and 90-bp downstream of the putative transcription start sites (TSSs) of DNA repair-associated protein encoding genes are shown. Several of them have bidirectional partner genes. In that case, extra sequences containing both of the most upstream were surveyed. Nucleotide sequences contained in the 5′-upstream of the partner cDNAs are also included.

3.1. Classification of DNA repair genes whose upstream regions contain duplicated GGAA motifs

Numbers of genes (Table 1) encode proteins with multiple functions. However, they could be categorized into several groups as follows:

1. Nucleotide excision repair (NER); *ATR, CDKN1A, ERCC1, ERCC2(XPD), ERCC3(XPB), ERCC4 (XPF), GTF2H1, GTF2H2, GTF2H3, H2AFX, LIG1, PARP1, POLD1, POLD3, POLD4, RAD23A, RAD23B, RFC2, RFC5, RPA2, RPA3, UVSSA, XPC, XRCC1...* NER is a DNA repair system that is executed with several functional proteins, which recognize a lesion to form TFIIH complex to excise the lesion introduced DNA chain and the gap is filled by DNA polymerases [71].

2. Transcription coupled repair (TCR); *APEX1, BRCA1, CDK1, CDK2, CDKN1A, CDKN2A, ERCC1, ERCC2, ERCC3, ERCC4, GSTP1, GTF2H1, GTF2H2, GTF2H3, LIG1, MRE11A, NBN, PARP1, POLD1, POLD3, POLD4, POLH, POLR2A, POLR2B, POLR2F, POLR2G, POLR2I, PRKDC, RFC2, RFC5, RPA2, RPA3, SP1, STAT3, TERT, TP53*...TCR is thought to regulate genomic integrity. This process begins with unwinding the double stranded DNA by TFIIH, the next step is that the damaged strand is incised apart by XPF, XPG and endonucleases, then gap is filled and finally the nick is ligated [72].

3. Fanconi anemia proteins*; ATM, ATR, BRCA2 (FANCD1), BRIP (FANCJ), CHEK1, ERCC4(FANCQ), FANCA, FANCB, FANCC, FANCD2, FANCE, FANCF, FANCG, FANCI, FANCL, FANCM, NBN, PALB2 (FANCN), LMNA, RAD51C (FANCO), SLX4(FANCP)*...Encoded proteins are involved in Fanconi anemia pathway that plays a part in the repair of inter strand cross links [73]. Notably, all genes that encode protein components in the Fanconi anemia core complex are listed in Table 1.

4. Double strand break (DSB) repair, Non homologous end joining (NHEJ); *APC, APEX1, ATM, ATR, BLM, BRCA1, BRCA2, CHEK1, DCLRE1A, DCLRE1B, DCLRE1C, DDX1, ERCC1, ERCC4, FEN1, LIG1, LMNA, MCM8, MRE11A, NBN, PARP1, RBBP8, POLA1, POLB, POLH, POLL, POLM, POLR2A, RAD51AP1, RAD51B, RAD51C, RAD51D, RAD52, RECQL, RECQL5, SP1, TERT, TP53, TREX2, XRCC4...* DSBs are caused by stresses on

chromosomal DNAs, such as irradiation of ultra violet or X-ray, and alkylating agents. Unrepaired DSBs will lead to collapse of stalled replication forks and in response to uncapped telomeres [74]. Some of the genes that encode protein components in the MRE11 complex are listed in Table 1. Not only serving as regulator of the mammalian DNA damage response, the MRE11 complex plays an important role in the maintenance of telomeres [75].

5. Base excision repair (BER); *APC, APEX1, BRCA1, BRCA2, CDKN1A, CHEK1, ERCC1, ERCC2, FEN1, LIG1, NBN, NEIL1, NEIL2, PARP1, PARP2, POLA1, POLB, POLD1, POLD3, POLD4, POLG, POLH, POLI, POLL, POLL, POLQ, RAD23B, RECQL5, RPA2, RPA3, RPS3, TP53, TREX1, UNG, WRN, XPC, XRCC*... BER is a fundamental repair system to eliminate nucleotide-bases from mutated nucleotides that is recognized by AP endonucleases. FEN1 and Ligases fill the gap to ligate DNA ends [76, 77].

6. Apoptosis; *ABL1, APC, BARD1, BCL2, BCR, BLM, BRCA1, CNKN1A, CDKN2A, CHEK1, ERCC1, ERCC2, ERCC3, ERCC4, HMGB2, KRAS, LMNA, MRE11A, PARG, PARP1, PARP4, RAD23A, RAD23B, RAD51B, SIRT1, SUMO1, TERT, TOP2A, TP53, TP63, XIAP*... Apoptosis or programmed cell death is executed by a number of proteins, including mitochondrial protein BCL2 and tumor suppressor p53 [48, 51]. It is thought to be an important process to eliminate cells with unrepairable DNA damage.

7. Mitochondrial functions; *ADPRHL2 (ARH3), APEX1, ATM, BARD1, BCL2, BRCA1, BTG2, CDKN1A, CDKN2A, ERBB2, FEN1, FOXO3, LMNA, PARG, POLG, SIRT1, STAT3, SUMO1, TERT, TP53, TP63, XIAP*... Mitochondria and nuclei are communicating to regulate each other [6-9]. Several of the DNA repair factors, including p53 and BRCA1, have been shown to localize in mitochondria [78, 79]. It has been shown that mitochondrial matrix-associated proteins are poly(ADP-ribosyl)ated [80]. Poly(ADP-ribosyl)ation, which is catalyzed by PARP enzymes, is a modification of proteins utilizing NAD^+ molecule as a substrate [81]. The NAD^+/NADH ratio is thought to regulate mitochondrial metabolism. A recent study showed that decrease in NAD^+/NADH ratio, which is thought to occur by an aberrances in mitochondria, does not only enhance cancer progression but also metastasis [82].

8. Response to IFN; *ABL1, BCL2, BCR, FOXO3, IL18, PARP1, PRKDC, RUNX1, RUVBL1, SAMHD1, SP1, STAT3, TERT, TP53, TREX1*... Duplicated GGAA (TTCC) motifs are found in number of the 5′-upstream regions of the human Interferon Stimulated genes (ISGs) [61]. The observation suggests that expression of some of the DNA repair factor-encoding genes might be up-regulated by IFN-induced signals.

3.2. The GGAA (TTCC) motifs are often present in the 5′-flanking regions of the genes that encode protein modification factors

PARP enzymes and p53, which have multiple functions in DNA repair process, are thought to affect mitochondrial metabolism [29, 83]. We previously reported that 5′-upstream regions of many of the mitochondrial function associated genes contain duplicated GGAA (TTCC) motifs [12]. Therefore, mitochondrial function could be up-regulated when cells encounter

with various stresses, including DNA damage, viral infections, or tumorigenesis. It is noteworthy that PARP enzymes consume NAD^+ to synthesize poly(ADP-ribose)s on various target proteins, including p53 [18, 19]. Given that NAD^+ is an essential molecule for energy metabolism, the ratio of inner cellular NAD^+/NADH may keep balance of mitochondria/DNA repair system. SIRT1, which is known as a NAD^+ dependent de-acetylating enzyme, is not only involved in controlling life spans of organisms but also DNA repair system with PARPs [84]. Besides affecting transcription by its de-acetylating activity, SIRT1 may indirectly contribute to regulate inner cellular acetyl-CoA level [85]. SIRT3, which localizes in mitochondria, catalyzes de-acetylation of acyl-CoA dehydrogenase and acetyl CoA synthase 2 [86-88]. It should be noted that *SIRT3* gene is head-head configured with *PSMD13* gene [89], and that the bidirectional promoter contains a sequence, 5′-ACTAG**GGAA**C**TTCC**TCTAC-3′ [12]. It is important to remind that both the NAD^+ and the acetyl-CoA are essential molecules in energy metabolism. Thus, GGAA-mediated transcription might be a biologically constitutional response to nutrition stress.

Not only the ubiquitin encoding genes, *UBB* and *UBC*, ubiquitin metabolism factor encoding genes, including *UBE2B*, *UBE2T*, *UBE2V1*, *UBE2V2*, *UBE2W*, *UBR5*, *UHRF1*, *USP1*, *USP3*, *USP28*, and *USP47*, are included in Table 1. Protein ubiquitination has been suggested to regulate DNA repair [90]. Notably, SUMOylation, which is a modification of proteins with Small Ubiquitin-like Modifier [91], plays an important role in DNA repair [92, 93]. The duplicated GGAA motif is also present in the *SUMO1* promoter [50]. Furthermore, UBA1, whose encoding gene is listed in Table 1, is a SUMO1 activating enzyme [94].

3.3. Possible roles of the duplicated GGAA motif in the 5′-upstream regions of DNA-repair genes

Genome wide analysis of the human promoters revealed that c-ETS binding element is frequently found with another c-ETS binding element [95, 96]. Redundant occupation of the duplicated GGAA motifs by Ets family proteins seems to be a complicated system, but this would enable finely tuned regulation of each promoter through altering composition of Ets family or GGAA-binding proteins, including GABP and STATs, in the nucleus in response to cellular signals [95].

4. Bidirectional promoters that regulate DNA repair factor-encoding genes

From the surveillance of the human DNA data base, not only mitochondrial function associated genes but also DNA repair factor encoding genes have head-head oriented partner genes [12]. Recent studies on RNA sequencing revealed a wide variety of transcripts, and the human DNA data base is continuously updated. Therefore, numbers of known bidirectional promoters are increasing day by day. As the reason why so many genes have bidirectional partners has not yet been elucidated, there is great value in investigating the role of bidirectional promoters driving transcription of DNA-repair factor encoding genes.

4.1. Surveillance of the bidirectional promoters from human genomic database

We have reported that a lot of human mitochondrial function associated genes have a bidirectional partner [12]. Moreover, several DNA repair associated genes are head-head configured with another gene [11]. Re-surveillance of DNA repair factor-encoding genes revealed that a number of the genes have opposite direction transcribed partner genes, utilizing the same regulatory region as their common promoter (Table 1). Although the number of the bidirectional gene pair is increasing according to recent findings of transcripts with next generation sequencing, at least 95 gene pairs were identified from the surveillance of the 358 DNA repair associated genes whose promoter contains GGAA (TTCC) motifs. The observation suggests that duplicated GGAA (TTCC) motifs and the binding factors may play a part in the bidirectional transcription of both mitochondrial function- and DNA repair-associated genes. However, these genes are not always simply controlled by GGAA-motif alone. For example, Sp1 binding element or GC-box is co-localized with ETS binding motifs in human promoters with 28.4% occurrence [95]. Co-operation of the GABP binding motif with Sp1/3 and YY1 binding sites is required for murine *Gabpa-Atp5j* bidirectional promoter [97]. These observations suggest that another *cis*-element, such as GC-box, may play a role in the co-operative transcription.

4.2. Biological relevance of bidirectional transcription

It has been reported that many cancer or DNA repair associated genes have bidirectional partner genes, and that tandem repeated ETS binding sites are frequently found in the 5′-upstream regions of both genes [98-101]. Therefore, expression of many DNA repair factor encoding genes is thought to be regulated by TFs that bind to GGAA motifs.

Surveillance of the human genomic sequence database revealed that several ISGs have bidirectional partner genes [61]. Similar to the bidirectional promoters involved with DNA repair factor encoding genes, bidirectional ISG promoters contain duplicated GGAA motifs. They are *BAG1-CHMP5*, *BLZF1-NME7*, *EIF3L-ANKRP54*, *CCDC75-HEART5B*, *IFI27L1-DDX24*, *PARP10-PLEC*, *PSMA2-MRPL32*, *RPL22-RNF207*, and *TRADD-FBXL8* [61]. It is noteworthy that the bidirectional gene pair *HSPD1-HSPE1*, which encodes the mitochondrial chaperon proteins HSP60 and HSP10, respectively, has been reported to be regulated by IFN gamma [102]. These findings suggest that promoters of the DNA-repair and mitochondrial function associated genes that carry duplicated GGAA-motifs could be simultaneously regulated by IFN-induced signals.

5. Cellular senescence and cancer generation might be simultaneously regulated at transcriptional level

Introduction of several transcription factors (OSKM or Yamanaka factors) into somatic cells could reprogram and generate induced pluripotent stem (iPS) cells [103]. Recently, it was demonstrated that three transcription factors, *Blimp1*, *Prdm14*, and *Tfap2c*, direct epiblast-like

cells into primordial germ cells [104]. Moreover, transcription factor C/EBPα enhances effects of the OSKM factors to reprogram B cells [105]. These lines of evidences imply that the transcriptional profile determines cell fate towards proliferation, cell cycle arrest, differentiation, senescence or programmed cell death. Furthermore, it has been postulated that nutrient or metabolite state may contribute to affecting the balance between quiescence and proliferation of stem cells [106]. In this article, we would propose a hypothesis that DNA-repair and mitochondrial functions are regulated by the same or similar mechanisms that affect transcription of various genes *via* common duplicated GGAA motifs. The scenario is that transcriptional dysregulation should proceed to characteristics of cancer, including mitochondrial dysfunction and genomic instabilities. Conversely, cancer and malignant tumors could be reprogrammed to benign state if transcriptional state in the cells were altered.

5.1. Effect of caloric restriction (CR) mimetic drugs on telomere associated protein-encoding gene promoters

Loss of function mutations on the *WRN* gene, which encodes telomere regulating RecQ helicase, can lead to cancer or premature aging syndrome [107, 108]. On the other hand, caloric restriction (CR) can extend life spans of various organisms [109], and thus CR mimetic drugs may have an anti-aging effect. We therefore hypothesized that CR mimetic drugs activates transcription of telomere-associated genes and demonstrated that promoter activities of the human shelterin encoding genes are up-regulated by 2-deoxy-D-glucose (2DG) and Resveratrol (Rsv) in HeLa S3 cells [110].

2DG and Rsv, which are known as a potent inhibitors of glucose metabolism [109], and an activator of sirtuin-mediated de-acetylation [111], respectively, are often referred as CR mimetic drugs. We observed moderate activation of telomerase activity in HeLa S3 cells with 2DG and Rsv treatment [110, 112], suggesting that CR mimetic drugs have protective effects on telomeres by inducing telomerase activity along with up-regulating expression of the telomere maintenance factor-encoding genes. The human *TERT* (h*TERT*) promoter has been well characterized with c-Ets, GC-box, E-box and other TF-binding elements that are located in its 5′-flanking region [113, 114]. GC-boxes and Sp1-binding sites are frequently found in the human *TERT*, *WRN* and shelterin protein-encoding gene promoters with duplicated GGAA elements that present adjacent to TSSs [110, 115].

Interestingly, both duplicated GGAA-motif and GC-boxes are contained within 500-bp upstream of the TSS of the human *SIRT1* gene [116]. SIRT1, which plays a role in NAD^+ dependent de-acetylation of various proteins including histones, PGC-1α, FOXO1, p53 and HIF1α, is proposed to regulate aging and the healthspan of organisms [117]. Human *SIRT1* gene expression is regulated by PPARβ/γ through Sp1 binding elements [118]. Therefore, signals evoked by CR or CR mimetic drugs might induce Sp1 or GC-box binding TFs, thus simultaneously up-regulating expression of *TERT*, *WRN*, *SIRT1*, and the shelterin-encoding genes. Given that the CR imposes a stress on cells due to the lack of nutrients or energy to survive, cells need to stop growth but need to keep the integrity of chromosomes and telomeres without replication of their genome. Under these circumstances, cells may require full commitment of mitochondria to drive TCA cycle and OXPHOS generating more ATP mole-

cules than glycolysis does. Therefore, CR mimetic compounds with ability to induce telomere maintenance factor encoding genes might be anti-aging drugs simultaneously up-regulating expression of mitochondrial function associated genes.

5.2. Mechanisms that regulate aging or lifespan via mitochondria and metabolic state

Genetic studies of *C. elegans* implied that the insulin/IGF-1 signaling pathway regulates the lifespan of animals [119]. Insulin/IGF-1 signaling and glucose metabolism are thought to be associated with several diabetes/obesity controlling factors, including AKT, FOXO, mTOR and AMPK [120]. The mTOR is a component of mTORC1 and mTORC2 that play key roles in signal transduction in response to changes in energy balance [120]. Recently, it was reported that mTORC1 in the Paneth cell niche plays a role in calorie intake by modulating cADPR release from cells [121]. AMPK is known to be a sensor for energy stress and DNA damage to induce phosphorylation of various TFs, such as FOXO, PGC-1α, CREB and HDAC5 [120, 122]. Moreover, AMPK regulates SIRT1 activity by modulating NAD^+ metabolism [122].

Mitochondrial functions are known to affect lifespan of organisms [123]. Furthermore, a cross talk between telomeres and mitochondria is suggested to regulate aging [124]. This concept was implied from a *Tert* knock down experiment that indicates telomere dysfunction causes suppression of PGC-1α in a p53-mediated manner [6]. The tumor suppressor p53 has been suggested to affect aging of organisms as a pro-aging factor [125]. It does not only affect cell cycle arrest and apoptosis, but also play a role in mitochondrial respiration and glycolysis [126, 127]. These lines of evidences strongly suggest that p53-mediated signaling is transferred to both telomeres and mitochondria to control cellular senescence. Although no canonical GC-boxes are found, duplicated GGAA-motifs are located near the TSS of the human *TP53* promoter (Table 1). Detailed analysis of the *TP53-WRAP53* bidirectional promoter region revealed that both the duplicated GGAA motif and a putative E2F binding sequence are involved in the response to Rsv [128]. Therefore, various stresses from DNA damage, viral infection, or lack of nutrients, will activate expression of genes encoding DNA repair/mitochondrial/telomere maintenance-associated factors *via* duplicated GGAA-motif with help from other *cis*-elements, including GC-box and E2F elements.

5.3. Implication of transcriptional control on genes that encode TCA cycle enzymes

It has long been argued how and why cancers are generated. Recently, diagnosis of cancer and diseases that are thought to occur from genomic alterations could be analyzed by second-generation sequencing [129]. In general, it has been thought that cancer is a genetic disease with several mutations on driver genes, including *PIK3CA*, *IDH1* and *RB1* [130]. Another aspect of cancer is that it is a metabolic disease [79]. It is widely known that cancer consumes more glucose to produce ATP by glycolysis or fermentation. The metabolic state of the cells could be referred to as the "Warburg effect" [131]. Importantly, TCA-cycle enzymes, FH (Fumarate hydratase) and SDH (succinate dehydrogenage) have been suggested as tumor suppressors [132]. We have confirmed that duplicated GGAA motifs are present near TSSs of the *CS*, *ACO2*, *IDH1*, *IDH3A*, *IDH3B*, *SUCLG1*, *SDHAF2*, *SDHB*, *SDHD*, *FH*, and *ACLY* genes that encode enzymes in the TCA-cycle [12]. In this chapter, it was shown that a number of the

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5′-upstream regions of DNA repair factor- and IFN responding factor-encoding genes contain duplicated GGAA (TTCC) motifs near their TSSs. The observation suggests that expression of genes encoding TCA cycle enzymes is mediated by GGAA-motifs in a similar manner to that of DNA repair factor encoding genes.

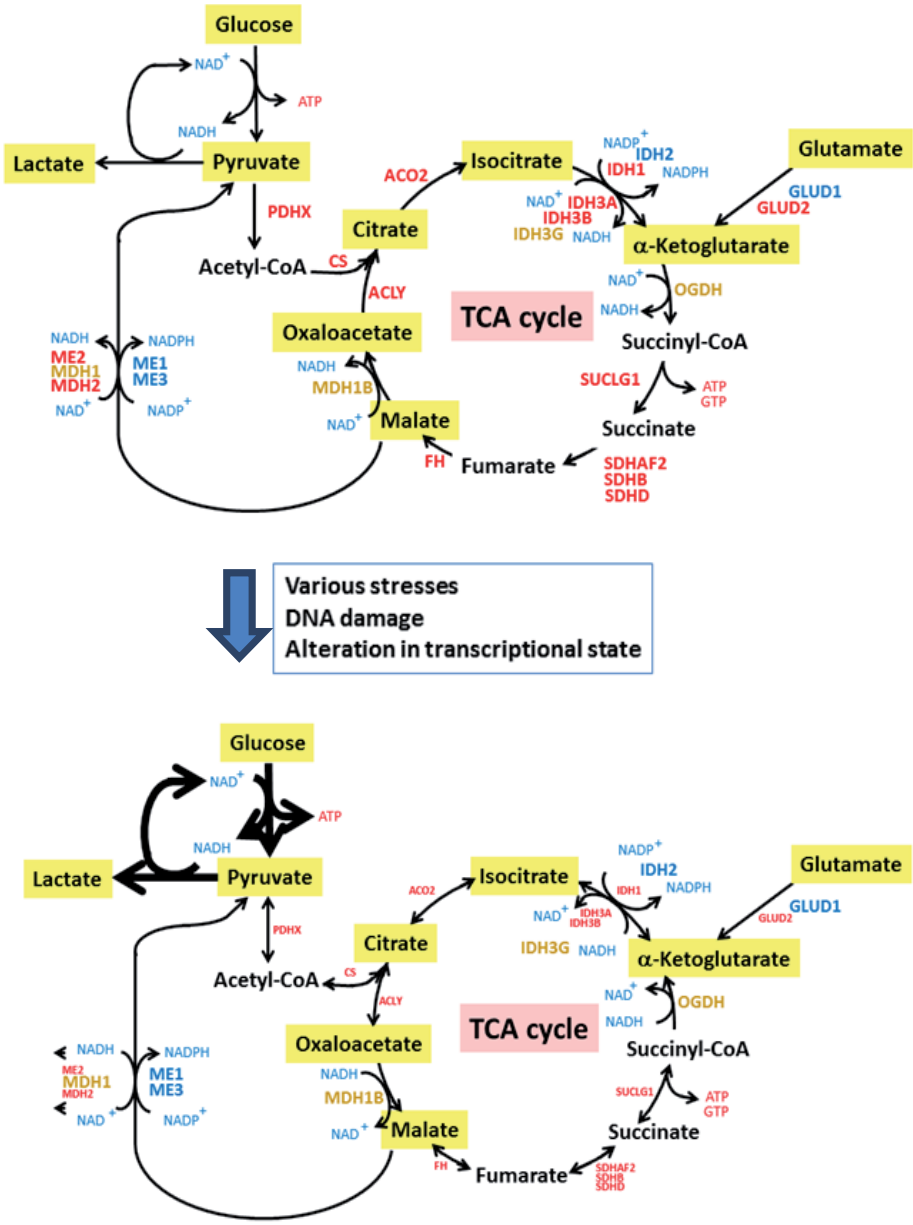

Figure 1. Dysfunction of tricarboxylic acid (TCA) cycle, which could be caused by alteration of GGAA-dependent transcription, may enforce the "Warburg effect". Duplicated GGAA-motifs are contained in the 5′-upstream regions of the

ACO2, *GLUD2*, *IDH1*, *IDH3A*, *IDH3B*, *MDH2*, *ME2*, *SUCLG1*, *SDHAF2*/*SDHB*/*SDHD*, *FH*, *ACLY*, *CS*, and *PDHX* genes (red). The activity of the IDH3 complex, OGDH, and MDH1B will be reduced when NAD^+/NADH ratio was attenuated according to the poly(ADP-ribosyl)ation when cells encountered DNA-damage. Compounds highlighted in yellow indicate metabolites that are produced when dysfunction of mitochondria and PARP activation has occurred, which push the cycle in the counter clockwise direction (reductive carboxylation). When FH and SDHs do not work sufficiently, glutamate will be used as a source to process TCA-cycle. Various cellular stresses, including chemicals, X-ray and UV irradiation, virus infection, and aging, may alter the quality and/or quantity profile of the GGAA-binding factors in a normal cell (upper panel). That will lead to disruption of the mitochondrial- and DNA repair function-associated gene expression. Repeated DNA-damage will enforce PARP activity to consume and deplete NAD^+ molecule from cytoplasm and mitochondria. When NAD^+/NADH ratio decreased to cause dysfunction of the TCA-cycle, cells would synthesize ATP by glycolysis or fermentation (lower panel). The consequence could be referred to as the "Warburg effect".

5.4. Regulation of rate limitting factors in the DNA repair system

As noted in **3.2.**, PARP, which localizes in mitochondria, may play a key role as one of the rate limitting factors in the DNA repair system. Poly(ADP-ribosyl)ation is essential for DNA repair, especially in cells that have deficiencies in BRCA, which is also known to localize in mitochondria [78, 79]. Therefore, highly potent inhibitors for PARP1 have been tested in different clinical trials [47]. Inhibition of the PARP enzyme will not only prevent over consumption of the NAD^+ molecule, but it also blocks DNA repair systems in cancer cells. This balance in metabolism/DNA repair should be taken into account for treatment of cancer patients. The other key factor in mitochondria is the p53, which is frequently referred as a "gurdian of the genome" [27, 28]. Although it has not been elucidated which TFs play essential roles in the regulation of those rate limitting factors in DNA repair, cells have some systems to monitor metabolites. For instance, C terminal-binding protein (CtBP), which is a transcriptional repressor of tumor suppressors [133], regulates *BRCA1* gene expression in a NAD^+/NADH ratio-dependent manner [134]. This implies that a metabolic swich mediated by CtBP plays a role in the regulation of the genes encoding DNA repair factors.

5.5. Alteration in transcriptional profile may cause cancerous state

It has been postulated that epigenetic and/or transcriptional changes play a role to determine chromatin states in tumor cells [135]. Recent genomic studies indicated alterations of gene expression in many human diseases [136, 137]. The transcriptome indicating *cis*- quantitative trait loci (QTLs) has been reported as value to reveal gene expression and transcription state in cells from patients of specific disease [138]. Cancer incidence in humans increase exponentially with age, suggesting that aging is the strongest demographic risk factor for most human malignancies [139, 140]. This has been mainly explained by reactive oxygen species (ROS) generation and accumulation of DNA damage on chromosomes or increased genomic stability, including telomere shortening [140]. Moreover, hypoxia, which will attenuate DNA damage response causing an increased mutation rate and chromosomal instability, has been suggested to modulate senescence [141, 142]. Importantly, aging is accompanied with epigenetic change and alteration of gene expression profile [143, 144]. Numerous GGAA motif-binding TFs acting as positive and negative transcriptional regulators, could drive mitochondrial- and DNA repair factor-encoding genes. The redundancy of the binding factors to the related sequences may help to control expression of a specific gene with accuracy, and subtle changes in the

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profile of the TFs would not cause severe abnormalities in normal cells. However, repeated cell division and extracellular signals will gradually disturb the balance of TFs that bind to GGAA (TTCC) motifs, and finally lead to dysregulation of mitochondrial functions, DNA-repair system, and IFN-response simultaneously. At this stage, abnormalities in metabolism, mutations on DNAs, and aberrant IFN response would be observed in the cells. These features could be referred to as characteristics of cancer and malignant tumors. Moreover, DNA damage-inducing signals will activate poly(ADP-ribosyl)ation to lead to over consumption of NAD^+ molecule for poly (ADP-ribose) synthesis. The reduction of NAD^+/NADH ratio in mitochondria may in turn reverse the direction of the TCA cycle. If the TCA cycle cannot meet demand for ATP levels, cells will abandon dependence on the normal respiratory system in favour of up-regulating glycolysis or fermentation (Fig. 1).

6. Concluding remarks

It remains unclear how the GGAA motif has been duplicated and incorporated into specific regulatory regions of various genes. However, the duplication of TF binding site might have been advantageous for organisms in the course of evolution. The crystal structure of mouse Elf3 with type II TGF-β receptor promoter was reported [145], representing an association model of the *ets* motif binding protein with the duplicated GGAA motifs. Very recently, it was shown that binding sites for MYC and its partner MIZ correlate with Pol II binding and transcription start site [146], implying that two adjacent TF-binding sites play significant roles in the regulation of transcription of multiple genes. At least twenty seven ETS family proteins and other TFs, including GABP, NF-κB/c-Rel, and STAT proteins, recognize the sequence. Therefore, the transcriptional efficiency could be determined only by the distance between GGAA (TTCC) sequences, variation of the flanking sequences and the combination of binding factors, which might have acquired variations as a consequence of evolution.

It has been shown that a lot of head-head configured gene pairs are contained in human genomes [99]. In this chapter, we proposed a mechanism that alterations in the transcriptional state in the cells lead to insufficient mitochondrial function accompanied with impaired DNA repair system. In this regard, cancer could be referred to as a "transcriptional disease". Given that introduction of the four OSKM (Yamanaka) factors enables reprogramming of cell, enforced expression of some TFs could reprogramm metabolic state in cancerous cells [103]. In order to assess the possibility, elucidation of how human genes, especially those that encode mitochondrial function- and DNA repair-associated factors, are regulated by GGAA motif-dependent transcription system, should be done [128]. If the mechanism were revealed, scientists could establish gene therapy to let pre-cancerous cells regain normal TCA cycle/respiration from unhealthy ATP-synthesis, or get rid of "Warburg effect". In addition, this therapy will up-regulate DNA repair system. We believe that the concept is valuable, though not yet fully cultivated, to find a way to next generation cancer treatment with much lower side effects.

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Section 2

DNA Repair and Cancer

Chapter 6

Epigenetic Reduction of DNA Repair in Progression to Cancer

Carol Bernstein and Harris Bernstein

Additional information is available at the end of the chapter

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1. Introduction

DNA damage appears to be a fundamental problem for life [1]. As we show below, the average human cell receives about 60,000 DNA damages per day due to natural endogenous causes. Most DNA damages are repaired by one or more enzyme systems. However, excessive DNA damages are a major primary cause of cancer. Error-prone replication past DNA damages or inaccurate repair of DNA damages give rise to mutations and epimutations that, by a process of natural selection, can cause progression to cancer.

DNA damage is a change in the basic structure of DNA that is not itself replicated when the DNA is replicated. A DNA damage can be a chemical addition or disruption to a base of DNA (creating an abnormal nucleotide or nucleotide fragment) or a break in one or both strands of DNA. When DNA carrying a damaged base is replicated, an incorrect base may be inserted opposite the site of the damaged base in the complementary strand, and this can become a mutation in the next round of replication. Also DNA double-strand breaks may be repaired by an inaccurate end-joining process leading to mutations. In addition, a double strand break can cause rearrangements of the chromosome structure (possibly disrupting a gene, or causing a gene to come under abnormal regulatory control), and, if such a change can be passed to successive cell generations, it is also a form of mutation. Mutations, however, can be avoided if accurate DNA repair systems recognize DNA damages as abnormal structures, and repair the damages prior to replication.

DNA damages occur in both replicating, proliferative cells (e.g. those forming the internal lining of the colon or blood forming "hematopoietic" cells), and in differentiated, non-dividing cells (e.g. neurons in the brain or myocytes in muscle). Cancers occur primarily in proliferative tissues. If DNA damages in proliferating cells are not accurately repaired due to inadequate

expression of a DNA repair gene, the risk of cancer increases. In contrast, when DNA damages occur in non-proliferating cells and are not repaired due to inadequate expression of a DNA repair gene, the damages can accumulate and may cause premature aging [1].

A mutation is a change in the DNA sequence in which normal base pairs are substituted, added, deleted or rearranged. The DNA containing a mutation still consists of a sequence of standard base pairs, and the altered DNA sequence can be copied when the DNA is replicated. A mutation can prevent a gene from carrying out its function, or it can cause a gene to be translated into a protein that functions abnormally. Mutations can activate oncogenes, inactivate tumor suppressor genes or cause genomic instability in replicating cells, and an assemblage of such mutations, together in the same cell, can lead to cancer. Cancers usually arise as a consequence of mutations conferring a selective advantage that leads to clonal expansion. Colon cancers, for example, have an average of 3 or 4 "driver" mutations (mutations occurring repeatedly in different colon cancers) and about 75 "passenger" mutations (mutations occurring infrequently in colon cancers) [2]. Colon cancers also have an average of 17 focal amplifications, 28 recurrent deletions and up to 10 translocations [3]. Since mutations have a normal DNA structure, they cannot be recognized or removed by DNA repair processes in living cells. Removal of a mutation only occurs if it is sufficiently deleterious to cause the death of the cell.

An epigenetic change (epimutation) is a heritable change in gene expression that is not accompanied by a change in DNA sequence. These epigenetic changes can include DNA methylation, constitutive (not facultative or induced) changes in small noncoding RNAs including microRNAs, altered chromatin architecture, histone tail modifications by methylations and acetylations that repress or activate transcription of the DNA wrapped around the histones, and nucleosome re-positioning [4]. These epigenetic changes are very frequent in cancers. For instance, 24 colon cancers were analyzed at more than 3,000 DNA segments within the genome for differentially methylated regions (DMRs). The colon cancers were found to have between 515 and 33,576 DMRs compared to adjacent histologically normal tissues [5]. Most of the differential methylations were increases in methylation, though some were hypomethylations. Increased methylation of the promoter region of a gene generally represses the transcription of that gene. Another epigenetic factor, a microRNA (miRNA), can have several hundred "target genes" [6]. Those target genes are repressed by the miRNA causing the degradation or blocked translation of the messenger RNA produced by those genes. Increased expression of an miRNA can occur due to epigenetic hypomethylation of the promoter region controlling transcription of the miRNA. When 754 miRNAs were evaluated in progression to esophageal adenocarcinoma (EAC), the expression levels of about 130 miRNAs were increased and the expression levels of 16 miRNAs were decreased in tissues with EAC (or in tissues with Barrett's esophagus, a precursor lesion) compared to histologically normal esophageal tissues adjacent to the EACs [7].

As described in detail below, inherited germ-line mutations of DNA repair genes give rise to syndromes characterized by increased risk of cancer. Such inherited mutational defects of

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DNA repair genes can allow excess unrepaired DNA damages to accumulate in somatic cells. Inaccurate translesion synthesis past the unrepaired DNA damages can cause mutations. In addition, error prone DNA repair pathways, such as non-homologous end joining, can also cause mutation. Erroneous or incomplete DNA repair may also cause epigenetic modifications. Thus, deficient DNA repair that leaves behind excess DNA damages can cause increased mutations and epimutations, and these mutations and epimutations can include both the driver mutations and the epigenetic alterations central to progression to cancer.

As will be described below, whole genome sequencing of many different types of cancers show that between thousands to hundreds of thousands of mutations occur in various types of cancers. Mutation frequencies in non-cancerous tissues are substantially lower. Loss-of-function mutations in DNA repair genes are relatively infrequent in sporadic (non-germ-line induced) cancers. However, DNA repair genes frequently express reduced levels of repair proteins in cancers due to epigenetic repression, and this can lead to increased DNA damage, and hence, increased mutation. The epigenetic repression of DNA repair gene expression is also frequent in the field defects that surround and give rise to cancers. Thus, epigenetic reduction of DNA repair appears to be a frequent early step, central to progression to cancer.

2. Inherited mutations in DNA repair genes and cancer syndromes

Hereditary cancer syndromes account for about 5% to 10% of the incidence of cancers [8]. Two reviews list 48 [8] and 55 [9] familial cancer susceptibility syndromes. Mutations in 38 genes related to DNA repair cause hereditary cancer syndromes (Table 1). Since such syndromes are frequently caused by mutations in DNA repair genes, this indicates that sporadic reductions in DNA repair gene expression may also be a frequent and crucial early event in progression to sporadic cancer.

3. Mutations versus epimutations in DNA repair genes during progression to cancer

Upon reviewing the results from sequencing 3,284 tumors and the 294,881 mutations found in these tumors, Vogelstein et al. [10] noted that germ-line mutations that give rise to cancer are infrequent in sporadic tumors. This indicates that if an early step in progression to sporadic cancer (rather than a germ-line syndrome) is reduction in function of a DNA repair gene, the reduction is likely due to an epigenetic alteration in that gene (an epimutation), rather than to a mutation (change in base pair-sequence).

Two examples are given here. In one case, for 113 sequential colorectal cancers, only four had a missense mutation in the DNA repair gene O-6-methylguanine-DNA methyltransferase (MGMT), while the majority had reduced MGMT expression due to methylation of the MGMT promoter region (an epigenetic alteration) [11]. Five reports presented evidence that between

40% and 90% of colorectal cancers have reduced MGMT expression due to methylation of the MGMT promoter region [12-16].

Similarly, out of 119 cases of mismatch repair-deficient colorectal cancers that lacked DNA repair gene PMS2 expression, PMS2 was deficient in 6 due to mutations in the PMS2 gene, while in 103 cases PMS2 expression was deficient because its pairing partner MLH1 was repressed due to promoter methylation (PMS2 protein is unstable in the absence of MLH1) [17]. In the other 10 cases, loss of PMS2 expression was likely due to epigenetic over-expression of the miRNA, miR-155, which down-regulates MLH1 [18].

4. DNA damages are very frequent

As shown in Table 2, an average of more than 60,000 endogenous DNA damages occur per cell per day in humans. These are largely caused by exposure to reactive oxygen molecules, hydrolytic reactions, and interactions with other reactive metabolites (including lipid peroxidation products, endogenous alkylating agents and reactive carbonyl species) [19].

In addition to the damages shown in Table 2, further DNA damages occur due to environmental assaults. Doll and Peto [20], compared cancer rates of specific organs in humans in the United States to cancer rates in these organs in other countries. They concluded that 75 - 80% of the cases of cancer in the United States were likely avoidable, and were due to DNA damaging agents found in occupational, medical and "social" exposures (including diet and tobacco).

Colon cancer is an example of a diet-related cancer that appears to be caused by excessive exposure of the colon to DNA damaging agents, mainly bile acids. Bile acids are released into the intestinal tract in response to consumption of fatty foods to aid in their digestion. As reviewed by Bernstein et al. [21], 14 published reports indicate that the secondary bile acids deoxycholic acid and lithocholic acid cause DNA damage. The concentration of these bile acids in the colon are affected by diet and are doubled in the colonic contents of humans on typical diets in the United States who were experimentally fed a high fat diet [22]. The potential consequences of high fecal bile acid concentrations is illustrated by the following comparison. The concentration of deoxycholic acid (DOC) in the feces of Native Africans in South Africa is 7.30 nmol/g wet weight stool while that of African Americans is 37.51 nmol/g wet weight stool, so that there is 5.14 fold higher concentration of DOC in stools of African Americans than in Native Africans [23]. Native Africans in South Africa have a colon cancer rate of <1:100,000 [24] compared to the incidence rate for male African Americans of 72:100,000 [25], a more than 72-fold difference in rates of colon cancer. In populations migrating from low-incidence to high-incidence countries cancer rates change rapidly, and within one generation may reach the rate in the high-incidence country. This has been observed, for instance, in the colon cancer incidence of migrants from Japan to Hawaii [26]. These changes in colon cancer rates among migrants are thought to be largely due to changes in diet.

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DNA repair gene(s)	Encoded protein	Repair pathway(s) affected*	Cancers with increased risk
breast cancer 1 & 2	BRCA1 BRCA2	HRR of double-strand breaks and daughter strand gaps [27]	Breast, Ovarian [28]
ataxia telangiectasia mutated	ATM	Different mutations in ATM reduce HRR, single-strand annealing (SSA), NHEJ or homology directed double- strand break rejoining (HDR) [29]	Leukemia, Lymphoma, Breast [29,30]
Nijmegen breakage syndrome	NBS	NHEJ [31]	Lymphoid cancers [31]
meiotic recombination 11	MRE11	HRR and NHEJ [32]	Breast [33]
Bloom's Syndrome (helicase)	BLM	HRR [34]	Leukemia, Lymphoma, Colon, Breast, Skin, Lung, Auditory canal, Tongue, Esophagus, Stomach, Tonsil, Larynx, Uterus [35]
Werner Syndrome (helicase)	WRN	HRR, NHEJ, long patch BER [36]	Soft tissue sarcoma, Colorectal, Skin, Thyroid, Pancreatic [37]
Rothman Thomson syndrome Rapadilino syndrome Baller Gerold syndrome	RECQ4	Helicase likely active in HRR [38]	Basal cell carcinoma, Squamous cell carcinoma, Intraepidemial carcinoma [39]
Fanconi's anemia gene FANC A,B,C,D1,D2,E,F,G,I,J,L,M,N	FANCA etc.	HRR and TLS [40]	Leukemia, Liver tumors, Solid tumors many areas [41]
xeroderma pigmentosum C, E [DNA damage binding protein 2 (DDB2)]	XPC XPE	Global genomic NER repairs damage in both transcribed and untranscribed DNA [42,43]	Skin cancer (melanoma and non-melanoma) [42,43]
xeroderma pigmentosum A, B, D, F, G	XPA XPB XPD XPF XPG	Transcription coupled NER repairs the transcribed strands of transcriptionally active genes [44]	Skin cancer (melanoma and non-melanoma) [44]
xeroderma pigmentosum V (also called polymerase H)	XPV (POLH)	Translesion Synthesis (TLS) [45]	Skin cancers (basal cell, squamous cell, melanoma) [45]
mutS (*E. coli*) homolog 2 mutS (*E. coli*) homolog 6 mutL (*E. coli*) homolog 1 postmeiotic segregation increased 2 (*S. cereviciae*)	MSH2 MSH6 MLH1 PMS2	MMR [46]	Colorectal, Endometrial [46]
mutY homolog (*E. coli*)	MUTYH	BER of A mispaired with 8-OHdG [47]	Colon [47]

DNA repair gene(s)	Encoded protein	Repair pathway(s) affected*	Cancers with increased risk
ataxia telaniectasia and *RAD3* related	ATR	DNA damage response likely affects HRR, but not NHEJ [48]	Oropharyngeal cancer [49]
Li Fraumeni syndrome	P53	HRR, BER, NER and DDR for those and NHEJ and MMR [50]	Sarcoma, Breast, Osteo-sarcoma, Brain, Adreno-cortical carcinomas [51]
Severe combined immune deficiency (SCID)	Artemis DCLRE1C	NHEJ [52]	B-cell lymphoma [53]
CHEK2 (a DDR gene)	CHEK2	Double-strand breaks [54]	Breast, Ovarian [55]

*HRR, homologous recombinational repair; NHEJ, non-homologous end joining; BER, base excision repair; TLS, translesion synthesis; MMR, mismatch repair; DDR, DNA damage response

Table 1. Inherited mutations in genes related to DNA repair that increase the risk of cancer

The likely role of bile acids as causative agents in colon cancer is illustrated by experiments with mice. When mice were fed a diet supplemented with the bile acid deoxycholate (DOC) for 10 months, raising their colonic level of DOC to that of humans on a high fat diet, 45% to 56% of these mice developed colon cancers, while mice fed the standard diet alone, with 1/10 the level of colonic DOC, developed no colon cancers [56,57].

DNA damages	Reported rate of occurrence
Oxidative	86,000 per cell per day in rats 10,000 per cell per day in humans [58]
Depurinations	9,000 per cell per day [59]
Depyrimidations	696 per cell per day [60]
Single-strand breaks	55,000 per cell per day [60]
Double-strand breaks	~50 per cell cycle in humans [61]
O^6-methylguanine	3,120 per cell per day [60]
Cytosine deamination	192 per cell per day [60]

Table 2. DNA damages due to natural endogenous causes in mammalian cells

5. DNA repair deficiency allows excess DNA damage accumulation

At least 169 enzymes are either directly employed in DNA repair or influence DNA repair processes [62]. Of these, 139 are directly employed in DNA repair processes including base

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excision repair (BER), nucleotide excision repair (NER), homologous recombinational repair (HRR), non-homologous end joining (NHEJ), mismatch repair (MMR) and direct reversal of lesions (DR). The other 30 enzymes are employed in the DNA damage response (DDR) needed to initiate DNA repair; chromatin structure modification required for repair; reactions needed for the reversible, covalent attachment of ubiquitin and small ubiquitin-like modifier (SUMO) proteins to DDR factors that facilitate DNA repair; or modulation of nucleotide pools.

When the incidence of endogenous and exogenous DNA damages is high, decreases in expression of DNA repair genes or DNA damage response (DDR) genes would be expected to lead to a build-up of DNA damage within a cell. Five examples below indicate that a DNA repair deficiency leads to excess DNA damage accumulation.

BLM deficiency. As reviewed by Manthei and Keck [63] and Croteau et al. [64], Bloom's syndrome helicase (BLM) likely has roles in multiple steps in homologous recombinational repair (HRR) of double-strand breaks (DSBs) in DNA. BLM is able to stimulate nuclease activity in a 5′ end resection at a DSB. This aids in initiation of HRR. This activity may serve to shuttle DSBs away from non-homologous end joining (NHEJ) pathways, which are more error prone. In the second step of HRR, the RAD51 recombinase forms a helical filament on the free 3′ DNA end. A homology search within double-stranded DNA by the RAD51/ssDNA complex produces a D-loop structure as a result of invasion of a ssDNA segment into a homologous sister-chromatid or chromosome. In this step, BLM interacts with RAD51 and is able to migrate and unwind D-loops. BLM is also part of a "dissolvasome" that resolves Holiday junctions during HRR. Humans with a germ-line BLM mutation accumulate chromosomal rearrangements and aneuploidy [35] and have increased susceptibility to several kinds of cancer (Table 1).

MUTYH deficiency. MUTYH protein is a glycosylase that removes an undamaged adenine mispaired with the damaged DNA base 8-OH-deoxyguanine. This removal leaves an apurinic/apyrimidinic (AP) site that initiates a special long-patch base excision repair. This repair depends on accurate translesion synthesis by polymerase lambda (pairing a cytosine opposite the 8-OH-deoxyguanine), creating a cytosine:8-OH-deoxyguanine pair, which then allows other enzymes to recognize and remove the 8-OH-deoxyguanine [47]. If *MUTYH* (or *MYH*) expression is decreased by short hairpin RNA (shRNA) (that makes a tight hairpin turn that can silence target gene expression) in human-origin HeLa cells, external application of H_2O_2 causes increased accumulation of 8-OH-deoxyguanine [65]. This finding shows that deficient expression of DNA repair protein MUTYH allows 8-OH-deoxyguanine accumulation when cells are under oxidative stress. Note that a germ-line *MUTYH* mutation increases the risk of colon cancer (Table 1).

ATM deficiency. In response to double-strand breaks, both ATM and ATR phosphorylate a multitude of protein substrates, including p53, and the checkpoint kinases, CHEK1 and CHEK2. These phosphorylated substrates promote cell cycle arrest and initiate DNA repair. Arresting the cell cycle allows time for enzymes to repair the DNA before DNA synthesis or chromosome segregation initiates [66,67]. As shown by Flockerzi et al. [68] and Rübe et al. [69], when DNA repair is reduced by homozygous loss of function of *ATM*, a low dose of radiation causes more DNA damage, especially double-strand breaks, to accumulate than when *ATM*

is wild-type. Note that germ-line *ATM* mutations increase the risk of leukemia, lymphoma and breast cancer (Table 1).

ERCC1 deficiency. Nucleotide excision repair (NER) removes helix-distorting "blocking" lesions located throughout the genome. Such lesions may block movement of DNA polymerase during DNA replication or a lesion on the transcribed strand may block elongating RNA polymerase movement within an active gene. XPC-RAD23B initiates the repair response by recognizing a damage-induced structural change in DNA and then binds to the strand opposite the lesion and not the chemical adduct itself. After a number of steps, the two endonucleases, XPF-ERCC1 and XPG then carry out incisions 5′ and 3′, respectively, to the DNA damage. The presence of genetic polymorphisms of *ERCC1*, with reduced DNA repair capacity, allow more benzo[a]pyrene-DNA adducts to accumulate in cells exposed to benzo[a]pyrene [70]. Thus, bulky helix-distorting lesions accumulate when ERCC1 protein activity is deficient.

DNA polymerase beta deficiency. As reviewed by Sobol [71], the base excision repair (BER) pathway is used to repair many DNA damages including depurinated and depyrimidinated bases (abasic sites), deaminated cytosine or 5-methylcytosine, and oxidation products such as 8-OH-dG, thymine glycol and lipid peroxidation products. Once the base lesion is removed by one of 11 DNA glycosylases and the abasic site is hydrolysed by APE1 endonuclease, DNA polymerse beta (POLB) is recruited to the lesion and carries out two functions: (1) removal of the sugar-phosphate residue that remains after APE1 cleaves the DNA backbone and (2) addition of the new nucleotide(s) to replace the one(s) removed during repair. A single nucleotide polymorphism (SNP) in POLB (P242R) that acts at half the rate of wild-type POLB occurs in 2.4% of individuals in certain human populations [72,73]. This P242R polymorphism has reduced effectiveness in DNA repair and cells carrying the P242R polymorphism accumulate double-strand breaks at a higher rate than cells carrying the wild type allele [73]. The population of humans examined and carrying the P242R SNP was not large enough to determine whether this germ-line SNP increases the risk of cancer, so POLB is not listed in Table 1. However, while germ-line mutations in *POLB* are not known to increase cancer risk, the human *POLB* gene is mutated in 40% of colorectal tumors, though not in the field defects surrounding the tumors [74]. Absence in the field defect but presence in the tumor suggests that a mutation in *POLB* is a later step in progression to a tumor.

6. DNA damages give rise to mutations and epigenetic alterations

As described below, a substantial proportion of mutations are due to translesion synthesis past otherwise un-repaired single-strand DNA damages, the most frequent endogenous DNA damages in Table 2. However, while only a minority of endogenous DNA damages in the average cell are double-strand breaks, this type of lesion appears to contribute substantially to the mutation rate as well. As indicated by Vilenchik and Knudson [61], the doubling dose for ionizing radiation (IR) induced double-strand breaks is similar to the doubling dose for mutation and induction of carcinomas by IR. Thus, double-strand breaks likely lead frequently to mutations. In addition, as further described below, some portion of the epigenetic alterations

transmissible from one generation to the next (epimutations) appear to have arisen from otherwise temporary alterations needed during steps in DNA repair.

7. Translesion synthesis past a DNA damage

Translesion synthesis (TLS) is a DNA damage tolerance process that allows the DNA replication machinery to replicate past DNA lesions in the template strand. This allows replication to be completed, rather than blocked (which may kill the cell or cause a translocation or other chromosomal aberration) [75].

Humans have four translesion polymerases in the Y family of polymerases [REV1, Pol κ (kappa), Pol η (eta), and Pol ι (iota)] and one in the B family of polymerases [Pol ζ (zeta)]. REV1 inserts cytosine opposite abasic sites in DNA (which may not be the correct base for that site) and has a structural role in regulating Pol ζ. Pol ζ extends replication past distorted DNA pairs, such as mismatched pairs of bases or bases with bulky DNA adducts. Pol η is a DNA polymerase that efficiently replicates DNA templates containing thymine dimers. Pol ι utilizes Hoogsteen base pairing for efficient and correct incorporation of cytosine opposite altered purines, such as 8-oxoguanine, but also tends to incorporate guanine opposite thymine. Pol κ is specialized in performing error-free bypass of bulky minor groove N2-deoxyguanine adducts among other lesions, but is highly error-prone when replicating a normal portion of a template [75].

The temporary tolerance of DNA damage during replication may allow DNA repair processes to remove the damage later [76], and avoid immediate genome instability [77]. However, translesion synthesis is less accurate than the replicative polymerases δ (delta) and ε (epsilon) and tends to introduce mutations [75].

8. Mutation due to translesion synthesis

Deficiency in expression of a DNA repair gene allows excessive DNA damages to accumulate. Some of the excess damages are likely processed by translesion synthesis, causing increased mutation.

As one example, BRCA2 protein is normally active in the accurate homologous recombinational repair (HRR) pathway. Loss of both wild-type DNA repair gene *BRCA2* alleles causes rapid spontaneous acquisition of genome-wide somatic mutations in the replicating tissues of mouse embryos. The mutations were measured in *LacZ*-plasmid transgenic reporter mouse embryos, a system in which large genomic deletions, insertions and translocations can be detected. The mutations found in *BRCA2*$^{(-/-)}$ mouse embryos are predominantly deletion/rearrangement mutations consistent with mis-repair of DNA double-strand breaks arising during DNA replication [78]. The proportion of deletion/rearrangement mutations (76%) in the presence of *BRCA2*$^{(-/-)}$ is close to the proportion of deletion/rearrangement mutations (71%)

among the much less frequent mutations found in the presence of the wild-type alleles *BRCA2*$^{(+/+)}$. This finding suggests that the mode of error-prone translesion synthesis past any un-repaired DNA damages is likely the same in *BRCA2*$^{(-/-)}$ and *BRCA2*$^{(+/+)}$ cells. In *BRCA2*$^{(+/+)}$ cells, the accurate HRR pathway would take care of most of the relevant DNA damages, rather than translesion synthesis.

Kunz et al. [79] summarized a large number of experiments in yeast, in which forward mutations were measured (by sequence analyses of a few selected genes) in cells carrying either wild-type alleles or one of 11 inactivated DNA repair genes. Their results indicated that DNA repair deficient cells accumulate excess DNA damage that could then give rise to mutations after error-prone translesion synthesis. The 11 inactivated DNA repair genes were distributed among mismatch repair, nucleotide excision repair, base excision repair and homologous recombinational repair genes. Deficiencies in DNA repair increased mutation frequencies by factors between 2- and 130-fold, but most often by double digit-fold increases. Overall, the authors concluded that 60% or more of single base pair substitutions and deletions are likely caused by translesion synthesis.

Hegan et al. [80] studied forward mutation in mice to determine the spontaneous mutation frequency in the presence of wild-type alleles or in the presence of knockout mutations in five individual mismatch repair genes and in pairs of double knockout mismatch repair genes. They used two mutation reporter genes within chromosomally integrated, recoverable phage lambda shuttle vectors to measure mutation frequencies and to determine the types of mutations present. The inactivated mismatch repair genes were in *Pms2, Mlh1, Msh2, Msh3* and *Msh6*. All mice with nullizygous mutations in these mismatch repair genes had significantly increased mutation frequencies compared to wild-type mice with both reporter genes tested. The highest two individual mutation frequencies were found in mice defective for *Mlh1* (>72-fold increase) and *Msh2* (65-fold increase). The double knockout mice had still higher frequencies of mutation than the single knockout mice. The greatest increase found was with the Msh3$^{-/-}$/Msh6$^{-/-}$ double knockout mice that had more than a 100-fold increase in mutation frequency with one of the reporter genes compared to wild-type mice. In these mismatch repair deficient mice, the majority of mutations found were generally insertion and deletion mutations.

Stuart et al. [81] examined spontaneous mutation frequencies in a *lacI* transgene (in a Big Blue mutation assay [82]) in either replicating tissues or in largely non-replicating tissues of mice. If most mutations occur during translesion synthesis, then non-replicating brain tissue, which has little or no synthesis once maturity is reached, would have little or no further mutation accumulation. In mouse brain, after 6 months of age, there was no increase in mutation frequency, even at 25 months of age. In bladders of mice, with replicating tissues, mutation frequency increased with age, almost tripling between ages of 1.5 months and 12 months of age. The authors concluded that the age related increases in spontaneous mutation frequencies reflect endogenous DNA damages that were subsequently expressed as mutations following DNA replication. This indicates that translesion synthesis is a major source of mutation in the mouse.

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9. Mutation due to error prone repair of double-strand breaks

As described by Bindra et al. [83], non-homologous end-joining (NHEJ) and homologous recombination repair (HRR) comprise the two major pathways by which double-strand breaks (DSBs) are repaired in cells. NHEJ processes and re-ligates the exposed DNA termini of DSBs without the use of significant homology, whereas HRR uses homologous DNA sequences as a template for repair. HRR predominates in S-phase cells, when a sister chromatid is available as a template for repair, and is a high-fidelity process. NHEJ is thought to be active throughout the cell cycle, and it is more error-prone than HRR. NHEJ repair comprises both canonical NHEJ and non-canonical pathways. The former pathway results in minimal processing of the DSB during repair, whereas the latter pathway, with or without the use of sequence microhomology for re-ligation, typically results in larger insertions or deletions. Mutagenic NHEJ repair is a robust process, yielding percentages of mutated sites at the position of a DSB ranging from 20 to 60%.

As pointed out by Vilenchik and Knudson [61], about 1% of single-strand DNA damages escape repair and are not bypassed, and some of these become converted to double-strand breaks. This may contribute to the impact of double-strand breaks in causing mutations and carcinogenesis.

10. Epigenetic alterations occur due to DNA damage

Experiments have been conducted to determine the molecular steps by which epigenetic alterations arise due to incomplete repair of DNA double-strand breaks. In one experiment O'Hagan et al. [84] used a cell line that was stably transfected with a plasmid containing a consensus I-SceI cut site inserted into a copy of the E-cad promoter. This promoter contained a CpG island (where a cytosine nucleotide frequently occurs next to a guanine nucleotide in the linear sequence of bases). The cytosines in these CpG islands are often hypermethylated, causing epigenetic repression of the associated genes. Such hypermethylations occur in multiple human tumor types. The investigators induced a defined double-strand break in the E-cadherin CpG island, which was not currently hypermethylated. After the onset of repair of the break, they observed the expected recruitment to the site of damage of key proteins involved in establishing and maintaining transcriptional repression, to allow repair of the break. These proteins included SIRT1, EZH2, DNMT1, and DNMT3B. Furthermore, silencing histone modifications appeared including hypoacetyl H4K16, H3K9me2 and me3, and H3K27me3. In most cells selected after the DNA break, DNA repair occurred faithfully with preservation of activity of the promoter, and removal of the silencing factors. However, a small percentage of the plated cells demonstrated induction of heritable silencing. The chromatin around the break site in such a silent clone was enriched for most of the silencing chromatin proteins and histone marks, and the region had increased DNA methylation in the CpG island of the promoter. Their data suggested that repair of a DNA break can occasionally cause heritable silencing of a CpG island-containing promoter by recruitment of proteins involved

in silencing and leading to aberrant CpG island DNA methylation,. Such CpG island methylation is frequently associated with tight gene silencing in cancer.

In a second experiment showing that epigenetic alterations arise as a consequence of DNA damage, Morano et al. [85] studied a system in which recombination between partial duplications of a chromosomal Green Fluorescent Protein (GFP) gene is initiated by a specific double-strand break (DSB) in one copy. The unique DSB is generated by cleavage with the meganuclease I-SceI, which does not otherwise cleave the eukaryotic genome. The DSB is repeatedly formed and repaired, until the I-SceI site is lost by homologous or nonhomologous repair or depletion of the I-SceI enzyme. Recombination products can be detected by direct analysis of the DNA flanking the DSB or by the appearance of functional GFP (green fluorescent cells). Two cell types were generated after recombination: clones expressing high levels of GFP and clones expressing low levels of GFP, referred to as H and L clones, respectively. Relative to the parental gene, the repaired GFP gene was hypomethylated in H clones and hypermethylated in L clones. The altered methylation pattern was largely restricted to a segment just 3′ to the DSB. Hypermethylation of this tract significantly reduced transcription, although it is 2000 base pairs distant from the strong cytomegalovirus (CMV) promoter that drives GFP expression. The ratio between L (hypermethylated) and H (hypomethylated) clones was 1:2 or 1:4, depending on the insertion site of the GFP reporter. These experiments were performed in mouse embryonic (ES) or human cancer (Hela) cells. HRR-induced methylation was dependent on DNA methyltransferase I (DNMT1). These data, taken together, argue for a cause-effect relationship between DNA damage-repair and altered DNA methylation.

The main function of the proteins in the base excision repair (BER) pathway is to repair DNA single-strand breaks and deamination, oxidation, and alkylation-induced DNA base damage. Li et al. [86] reviewed recent studies indicating that one or more BER proteins may also participate in epigenetic alterations involving DNA methylation or reactions coupled to histone modification. Franchini et al. [87] showed that DNA demethylation can be mediated by BER and other DNA repair pathways requiring processive DNA polymerases. Still another form of epigenetic silencing may occur during DNA repair. The enzyme PARP1 [poly(ADP)-ribose polymerase 1] and its product poly(ADP)-ribose (PAR) accumulate at sites of DNA damage as intermediates of a repair process [88]. This, in turn, directs recruitment and activation of the chromatin remodeling protein ALC1 that may cause nucleosome remodeling [89]. Nucleosome remodeling has been found to cause, for instance, epigenetic silencing of DNA repair gene *MLH1* [90]. Thus, DNA damages needing repair can cause epigenetic alterations by a number of different mechanisms.

11. Other causes of epigenetic alterations

Heavy metals and other environmental chemicals cause many epigenetic alterations, including DNA methylation, histone modifications and miRNA alterations [92]. DNA damage itself causes programmed changes in non-coding RNAs, and a large number of miRNAs are

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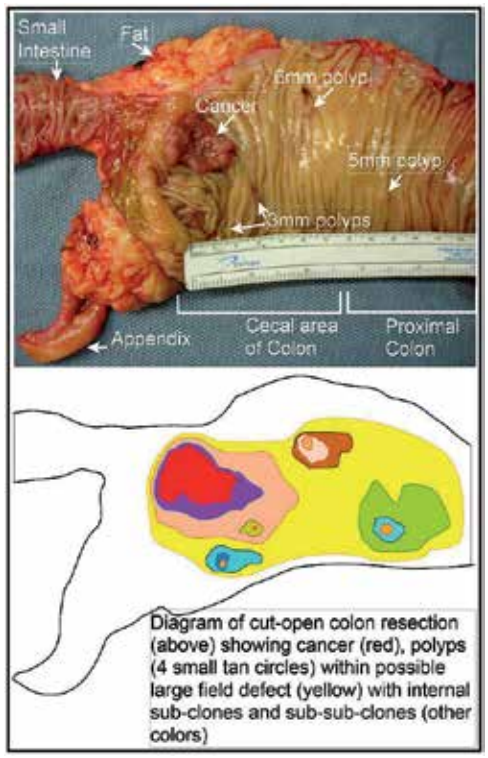

Figure 1. Cut open gross specimen of proximal human colon showing multiple tumors [91]

transcriptionally induced upon DNA damage [93]. However, it is not clear what proportion of these alterations are reversed or are retained as epimutations after the external sources of damage are removed upon repair of the DNA damages [94].

Mutations in *isocitrate dehydrogenase 1* (*IDH1*) and *2* (*IDH2*) are frequent in a number of cancers and they can cause epigenetic alterations. As reviewed by Wang et al. [95], *IDH1* and *IDH2* mutations represent the most frequently mutated metabolic genes in human cancer, mutated in more than 75% of low grade gliomas and secondary glioblastoma multiforme (GBM), 20% of acute myeloid leukemias (AML), 56% of chondrosarcomas, over 80% of Ollier disease and Maffucci syndrome, and 10% of melanomas. The *IDH1* and *IDH2* mutations that give rise to epimutations usually occur in the hotspot codons Arg132 of *IDH1*, or the analogous codon Arg172 of *IDH2*. These mutations allow accumulation of the metabolic intermediate 2-hydroxyglutarate (2-HG), and 2-HG inhibits the activity of alpha ketoglutarate (α-KG) dependent dioxygenases, including α-KG-dependent histone demethylases and the TET family of 5-methylcytosine hydroxylases. Wang et al. [95] found that histone H3K79 dimethylation levels were significantly elevated in cholangiocarcinoma samples that harbored *IDH1* or *IDH2* mutations (80.8%) compared to tumors with wild-type *IDH1* and *IDH2* (45.0%). In addition, they surveyed over 462,000 CpG sites in CpG islands, CpG shores and intragenic regions, and found that 2,309 genes had significantly increased methylation in the presence of

IDH1 or *IDH2* mutations. In particular, Sanson et al. [96] found that methylation of the DNA repair gene *MGMT* was associated with *IDH1* mutation, since 81.3% of *IDH1*-mutated tumors were *MGMT* methylated compared with 58.3% methylated in *IDH1* non-mutated tumors.

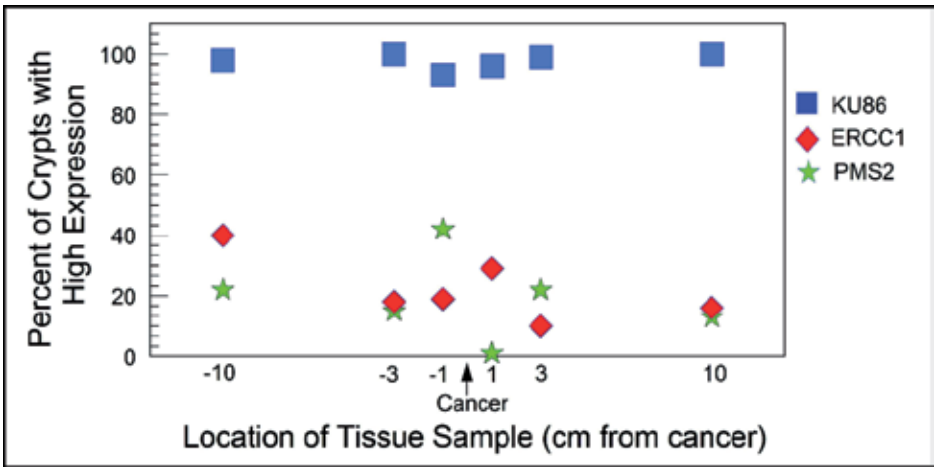

Figure 2. Expression of three DNA repair proteins, KU86, ERCC1 and PMS2, at locations sampled along the 20 cm length of a colon resection that had a cancer at the indicated location. [98]

12. Long-term epigenetic repression of DNA repair genes in progression to cancer

A DNA repair gene that is epigenetically silenced or whose expression is reduced would not likely confer any selective advantage upon a stem cell. However, reduced or absent expression of a DNA repair gene would cause increased rates of mutation, and one or more of the mutated genes could cause the cell to have a selective advantage. The defective DNA repair gene could then be carried along as a selectively neutral or only slightly deleterious passenger (hitch-hiker) gene when there is selective expansion of the mutated stem cell. The continued presence of a DNA repair gene that is epigenetically silenced or has reduced expression would continue to generate further mutations and epigenetic alterations.

The spread of a clone of cells with a selective advantage, but carrying along a gene with epigenetically reduced expression of a DNA repair protein would be expected to generate a field defect, from which smaller clones with still further selective advantage would arise. This is consistent with the finding of field defects in colonic resections, that have both a cancer and multiple small polyps, such as the one shown in Figure 1.

The protein expressions of three DNA repair genes within a 20 cm colon resection were evaluated at six different locations within the resection (Figure 2) [97]. A colon resection, on its inner epithelial surface, has a layer of epithelial crypts (microscopic, test tube like indenta-

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tions about 100 cells deep), with 100 crypts per square millimeter. Each crypt is a clone of about 5,000 cells all generated by the 10 stem cells at the base of the crypt. One of the DNA repair proteins, KU86, was only deficient infrequently, with the deficiencies occurring in small patches (up to three crypts). These KU86 defects are not likely important in progression to colon cancer. However, two evaluated DNA repair proteins, ERCC1 and PMS2, were often deficient in patches of tens to hundreds of adjacent crypts at each of the locations evaluated (see Nguyen et al. [99] at minutes 18 to 24 of a 28 minute video of crypts immunostained for ERCC1 or PMS2).

Overall, ERCC1 was deficient in 100% of 49 colon cancers evaluated, and in 40% of the crypts within 10 cm on either side of the cancer. PMS2 was deficient in 88% of the 49 cancers and in 50% of the crypts within 10 cm of the cancer. As reported by Facista et al. [97], the pattern of expression of ERCC1 in the crypts within 10 cm of a colon cancer indicated that when the ERCC1 protein was deficient, this deficiency was due to an epigenetic reduction in expression of the *ERCC1* gene. When the PMS2 protein is deficient, it is usually due to the epigenetic repression of its pairing partner, MLH1, and the instabilty of PMS2 in the absence of MLH1 [100]. In the study of Facista et al. [97], ERCC1 and PMS2 were also deficient in all 10 tubulovillous adenomas evaluated (precursors to colonic adenocarcinomas). Thus ERCC1 and PMS2 are deficient at early times (in the field defect), at intermediate times (tubulovillus polyps), and late times (within cancer) during progression to colon cancer. Another DNA repair protein, XPF, was deficient in 55% of the cancers, as well [97]. The majority of cancers were simultaneously deficient for ERCC1, PMS2 and XPF.

Deficiencies in multiple DNA repair genes were also observed in gastric cancers. Kitajima et al. [101] evaluated MGMT, MLH1 and MSH2 and found that synchronous losses of MGMT and MLH1 increase during progression and stage of differentiated-type cancers. In un-differentiated-type gastric cancers, the frequency of MGMT deficiency increased from early to late stages of the cancer, while frequencies of MLH1 and MSH2 deficiencies were between 48% and 74% at both early and late stages. Thus, in un-differentiated-type gastric cancers, MLH1 or MSH2 deficiency, if it is present, is an early step, while MGMT deficiency is often a later step in progression of this cancer.

Farkas et al. [102] evaluated 160 genes in 12 paired colorectal tumors and adjacent histologically normal mucosal tissues for differential promoter methylation. They found aberrant methylation in 23 genes, including six DNA repair genes. These DNA repair genes (with DNA repair pathways indicated) were *NEIL1* (base excision repair), *NEIL3* (base excision repair), *DCLRE1C* (non-homologous end joining), *NHEJ1* (non-homologous end joining), *GTF2H5* (nucleotide excision repair), and *CCNH* (nucleotide excision repair).

Jiang et al. [103] evaluated the mRNA expression of 27 DNA repair genes in 40 astrocytomas compared to normal brain tissues from non-astrocytoma individuals. They found that 13 DNA repair genes, *MGMT, NTHL1, OGG1, SMUG1, ERCC1, ERCC2, ERCC3, ERCC4, MLH1, MLH3, RAD50, XRCC4* and *XRCC5* were all significantly down-regulated in all three grades (II, III and IV) of astrocytomas. The deficiencies of these 13 genes in lower grade as well as in higher grade astrocytomas indicated that they may be important in early as well as in later stages of

astrocytoma. For 8 DNA repair genes, *ERCC3, ERCC4, MLH3, MRE11A, NTHL1, RAD50, XRCC4* and *XRCC5*, decreased expression was significantly associated with a poor prognosis.

Based on the examples above, decreased expression of multiple DNA repair genes is likely to occur in many types of neoplasia. This should result in increased mutation frequency in those neoplastic lesions. Most new mutations are expected to be deleterious to the cells in which they arise, and thus would cause negative selection of those cells. This expectation is consistent with the observations of Hofstad et al. [104] who showed that when a colonic polyp was identified during a colonoscopy and followed but not removed, between 11% and 46% of those polyps smaller than 5 mm diameter were not detectable in the succeeding one to three years. For polyps between 5 and 9 mm in diameter, between 4 and 24% became undetectable in the succeeding one to three years. Of the remaining 68 polyps that were followed for three years, 35% decreased in diameter, 25% remained the same size and 40% increased in diameter. The data of Hofstad et al. [104] are also consistent with statistics showing more frequent occurrence of adenomas during colonoscopy and autopsy compared to the frequency of colon cancer, indicating there must be a significant regression rate for adenomas [105].

When infrequent positively selected mutations arise in a cell, this can provide the cell with a competitive advantage that promotes its preferentiail clonal proliferation, leading to cancer. The continued presence of epigenetically repressed DNA repair genes, carried along as passengers in the development of cancers, also predicts that cancers will contain heterogeneous genotypes (multiple subclones). For instance, in one primary renal carcinoma with multiple metastases, 101 non-synonymous point mutations and 32 indels (insertions and deletions) were identified [106]. Five mutations were not validated and excluded from the study. Of the remaining 128 mutations, 40 were "ubiquitous" and present in each region of the tumor sampled. There were 59 "shared" mutations, present in several but not all regions, and 29 "private" mutations, unique to a specific region evaluated. The authors constructed a phylogenetic tree and concluded that the evolution in the tumor and its metastases was branching, and not linear. Similar results were found in a further three tumors evaluated in their study. Every tumor had spatially separated heterogeneous somatic mutations and chromosomal imbalances leading to phenotypic intratumor diversity.

13. Epigenetic repression of DNA repair genes in field defects in progression to cancer

As described in detail by Rubin [107], field defects are of central importance in progression to cancer. While the great majority of studies in cancer research has been done on well-defined tumors formed *in vivo*, evidence indicates that more than 80% of the somatic mutations found in microsatellite instability (MSI) (mutator phenotype) human colorectal tumors occur before the onset of terminal clonal expansion. This evidence included the finding that adenomas were phylogenetically nearly as old as cancers. The origin of field defects was described by Braakhuis et al. [108] as follows. They postulated that a stem cell acquires one (or more) genetic alterations and forms a patch with genetically altered daughter cells. As a result of these and

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subsequent genetic alterations the stem cell escapes normal growth control, gains a growth advantage, and develops into an expanding clone. The lesion, gradually becoming a field, laterally displaces the normal epithelium. The enhanced proliferative capacity of a genetically altered clonal unit is the driving force of the process. As the lesion becomes still larger, additional genetic hits give rise to various subclones within the field. Different clones diverge at a certain time point with respect to genetic alterations but share a common clonal origin. The presence of a relatively large number of genetically altered stem cells in a field is a "ticking time bomb," and as a result of the process of clonal divergence and selection, eventually a subclone evolves into invasive cancer.

Cancer	Gene	Frequency in Cancer	Frequency in Field Defect	Ref.
Colorectal	*MGMT*	46%	34%	[12]
Colorectal	*MGMT*	47%	11%	[109]
Colorectal	*MGMT* with MSI*	70%	60%	[110]
Colorectal	*MSH2*	13%	5%	[109]
Colorectal	*MBD4*	frequent	frequent	[111]
Colorectal	*ERCC1*	100%	40%	[97]
Colorectal	*PMS2*	88%	50%	[97]
Colorectal	*XPF*	55%	40%	[97]
Colorectal	*WRN*	29%	13%	[112]
Head and Neck	*MGMT*	54%	38%	[113]
Head and Neck	*MLH1*	33%	25%	[114]
Head and Neck	*MLH1*	31%	20%	[115]
Lung NSCLC	*ATM*	69%	59%	[116]
Lung NSCLC	*MLH1*	69%	72%	[116]
Stomach	*MGMT*	88%	78%	[117]
Stomach	*MLH1*	73%	20%	[118]
Esophagus	*MLH1*	77%-100%	23%-79%	[119]

*MSI indicates microsatellite instability

Table 3. Frequency of finding epigenetic reductions in protein expression of DNA repair genes in sporadic cancers and in adjacent field defects

Epigenetic reductions in protein expression of DNA repair genes are frequent in cancers (Table 3). For any particular type of cancer, an epigenetic reduction in expression of a specific DNA repair gene, such as an epigenetic reduction of MGMT in colorectal cancer, may be common. In cases where a specific epigenetic reduction of expression of a DNA repair gene occurs in a

cancer, it is also likely to be evident in the field defect surrounding the cancer (Table 3). The lower frequency in the surrounding field defect that is often found (Table 3) likely reflects the process whereby the expanding clone is laterally displacing the normal epithelium. This displacement may be only partial. Thus, areas with the DNA repair deficiency would be present at a lower frequency in the field defect than in the cancer. In the cancer, the cells carrying the DNA repair deficiency are members of a founding clone. Thus, the DNA repair defect, along with other accumulated mutations and epigenetic alterations, would be seen in the cancer at a relatively higher frequency than in the surrounding field defect.

14. Examples of epigenetic repression of DNA repair genes, due to alterations in CpG island methylation, in various cancers

Table 4, below, gives examples of reports of DNA repair genes repressed by CpG island hypermethylation (or with increased expression due to CpG hypomethylation) in 17 different cancers (this is only a partial list). Twenty different DNA repair genes (all listed among the 169 DNA repair and DNA damage response genes previously identified [62]) were often hyper- (or sometimes hypo-) methylated in one or more type of cancer. Such alterations in methylation of promoter regions of DNA repair genes can cause deficient repair of DNA damages. Thus, hyper- (or hypo-) methylations of DNA repair genes are frequently important factors responsible for lack of appropriate repair of DNA damages. Faulty DNA repair leads to increased mutation and epigenetic alteration, central to progression to cancer.

MGMT is one of the DNA repair genes often evaluated for hypermethylation. Of the cancers listed in Table 4, nine were reported to have some frequency of hypermethylation of *MGMT*. Hypermethylation of *MGMT* was particularly frequent in bladder cancer (93%), stomach (88%), thyroid (74%), colorectal (40-90%) and brain (50%).

Other DNA repair genes with high frequencies of hypermethylation (in particular cancers) were *LIG4* (colorectal 82%), *P53* (brain 60-74%), *NEIL1* [head and neck 62% and non-small cell lung cancer (NSCLC) 42%], *ATM* (NSCLC 47%), *MLH1* (NSCLC squamous cell carcinoma 48%) and *FANCB* (head and neck 46%). The DNA repair genes *LIG4, P53, NEIL1* and *FANCB* were frequently not evaluated for hypermethylation in other particular types of cancers, and could be of importance in such cancers as well.

15. DNA repair gene *ERCC1* expression likely can be repressed by multiple processes

A number of the DNA repair genes with reduced expression due to CpG island hypermethylation are also epigenetically repressed by other means. Many protein coding genes are repressed by microRNAs. MicroRNAs (miRNAs) are small noncoding endogenously produced RNAs that play key roles in controlling the expression of many cellular proteins. Once

they are recruited and incorporated into a ribonucleoprotein complex, they can target specific messenger RNAs (mRNAs) in a miRNA sequence-dependent process and interfere with the translation into proteins of the targeted mRNAs *via* several mechanisms (see detailed review by Lages et al. [120]).

Almost one third of miRNAs active in normal mammary cells were found to contain hypermethylated DNA regions in breast cancer cells [121]. This includes, for instance, microRNAs let-7a-3/let-7b.

As indicated by Motoyama et al., [122] the let-7a miRNA normally represses the *HMGA2* gene, and in normal adult tissues, almost no HMGA2 protein is present. In breast cancers, for instance, the promoter region controlling let-7a-3/let-7b microRNA is frequently repressed by hypermethylation [121]. Reduction or absence of let-7a microRNA allows high expression of the HMGA2 protein. HMGA proteins are characterized by three DNA-binding domains, called AT-hooks, and an acidic carboxy-terminal tail. HMGA proteins are chromatin architectural transcription factors that both positively and negatively regulate the transcription of a variety of genes. They do not display direct transcriptional activation capacity, but regulate gene expression by changing local DNA conformation. Regulation is achieved by binding to AT-rich regions in the DNA and/or direct interaction with several transcription factors [123].

HMGA2 targets and modifies the chromatin architecture at the *ERCC1* gene, reducing its expression [124]. The lack of let-7a miRNA repression of HMGA2 could occur through translocation of HMGA2, disrupting the 3'UTR of HMGA2 which is the target of let-7a miRNA (shown in an artificial construct), and this can lead to an oncogenic transformation [125]. However, the promoter controlling let-7a miRNA also can be strongly regulated by hypermethylation in intact cells. When human lung cells are exposed to cigarette smoke condensation, the promoter region controlling let-7a becomes highly hypermethylated [126]. While only 38% of colorectal cancers have CpG island methylation of the *ERCC1* promoter (Table 4), Facista et al. [97] found that 100% of colon cancers have significantly reduced levels of ERRC1 protein expression. In the 49 cancers examined, ERCC1 generally varied from 0% to 45% of the level of ERCC1 expression of neoplasm-free individuals. It is likely that hypermethylated promoter for let-7a microRNA/hyperexpressed HMGA2 or other epigenetic mechanism(s) reduces protein expression of ERCC1 in colorectal cancers in addition to the 38% of colorectal cancers in which the *ERCC1* gene is directly hypermethylated.

16. DNA repair gene *BRCA1* expression likely can be repressed by multiple processes

BRCA1 expression is reduced or undetectable in the majority of high-grade, ductal carcinomas [127]. Among 32 breast cancers examined, none had a sporadic mutation in the *BRCA1* gene [128]. The frequency of *BRCA1* promoter hypermethylation in breast cancer is only 13-16% [129,130] (see Table 4). However, miR-182 targets *BRCA1* [131] and the promoter controlling expression of miR-182 is hypomethylated (would have increased expression) in cancers, as indicated by Shnekenburger and Diederich [132]. Tang et al. [133] showed that transcription

of miR-182 is repressed when the promoter controlling its transcription is methylated so that miR-182 is clearly an epigenetically regulated miRNA. Moskwa et al. [131] showed that basal-like ER-negative breast cancer cell lines had relatively low levels of BRCA1 protein and in five of the six ER negative cell lines there was inverse correlation of BRCA1 protein and miR-182 expression. Thus epigenetically increased expression of miR-182 appears to be implicated in reducing BRCA1 protein expression in breast cancer.

There is a further potential epigenetic mechanism for repressing BRCA1 in breast cancers. miR-34b is repressed by methylation of its promoter [134]. A target of miR-34b is *HMGA1* [135]. When miR-34b is repressed, expression of *HMGA1* is increased [136]. HMGA1 protein appears to target *BRCA1*. Baldaserre et al. [136] found an inverse correlation between HMGA1 and BRCA1 mRNA and protein expression in human mammary carcinoma cell lines and tissues. Thus epigenetically methylated promoter for transcription of miR-34b/increased HMGA1 may be instrumental in reducing BRCA1 protein expression in breast cancer. It is not clear whether increased miR-182 (see paragraph above) or decreased miR-34b is the more important factor in repressing BRCA1 in breast cancers.

17. DNA repair gene *MGMT* expression is repressed by multiple processes

In the most common form of brain cancer, glioblastoma, the DNA repair gene *MGMT* is epigenetically methylated in 29% [137] to 66% [138] of tumors, thereby reducing protein expression of MGMT. However, for 28% of glioblastomas, the MGMT protein is deficient but the *MGMT* promoter is not methylated [138]. Zhang et al. [137] found, in the glioblastomas without methylated *MGMT* promoters, that the level of microRNA miR-181d is inversely correlated with protein expression of MGMT and that the direct target of miR-181d is the MGMT mRNA 3′ UTR (the three prime untranslated region of MGMT mRNA). miR-181d normally occurs at very low levels in the brain [139]. It is not clear whether miR-181d is epigenetically up-regulated, when it occurs at increased levels in the brain. Thus it is not clear if this second process of reducing MGMT expression in progression to glioblastoma is an epigenetic one.

Cancer	Gene	Frequency of hyper- (or hypo-) methylation in cancer	Ref.
Bladder	MGMT	93%	[140]
Bone			
Chondrosarcoma	WRN	33%	[141]
Osteosarcoma	MGMT	24%	[142]
Osteosarcoma	WRN	11%	[141]

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Cancer	Gene	Frequency of hyper- (or hypo-) methylation in cancer	Ref.
Brain (glioma)	P53	60%-74%	[143]
	MGMT	50%	[144]
	ERCC1	38%	[145]
Breast			
	WRN	17%	[141]
	BRCA1	13%-16%	[129, 130]
	P53	12%	[145]
	FEN1	Frequent (hypo-)	[146]
Colorectal			
	LIG4	82%	[147]
	MGMT	40%-90%	[12-16]
	ERCC1	38%	[148]
	WRN	29%-38%	[112, 141]
	MLH1	9%-10%	[100, 149]
	FEN1	Frequent (hypo-)	[146]
	MBD4	Frequent	[150]
Hematological			
Non-Hodgkin lymphoma	WRN	24%	[141]
Acute lymphblasstic leukemia	WRN	10%	[141]
Acute myeloblastic leukemia	WRN	5%	[141]
Head and Neck			
	APEX2	100% hypo-	[151]
	TREX2	79% hypo-	[151]
	NEIL1	62%	[151]
	MSH4	60% hypo-	[151]

Cancer	Gene	Frequency of hyper- (or hypo-) methylation in cancer	Ref.
	MGMT	25%-57%	[151- 153]
	FANCB	46%	[151]
	ATM	25%	[154]
	MLH1	33%	[155]
	MLH1	4%	[152]
Kidney			
	MGMT	9%	[156]
	FEN1	Frequent (hypo-)	[146]
Lung			
NSCLC	NEIL1	42%	[157]
NSCLC	WRN	38%	[141]
NSCLC	MGMT	13%-64%	[157- 159]
NSCLC	ATM	47%	[116]
NSCLC Squamous cell carcinoma	MLH1	48%	[160]
NSCLC Adenocarcinoma	MSH2	42%	[160]
	BRCA2	42%	[161]
	BRCA1	30%	[161]
	XRCC5	20%	[161]
	FEN1	Frequent (hypo-)	[146]
Ovarian	P53	52%	[162]
	MSH2	52%	[163]
	MLH1	30%	[163]
	MBD4	Frequent (hyper-)	[150]
Prostate	WRN	20%	[141]
Stomach	MGMT	88%	[111]
	MLH1	73%	[118]

Cancer	Gene	Frequency of hyper- (or hypo-) methylation in cancer	Ref.
	WRN	24%-25%	[141, 164]
	FEN1	Frequent (hypo-)	[146]
Thyroid	MGMT	74%	[165]
	MLH1	21%	[166]
	PCNA	13%	[166]
	WRN	13%	[141]
	OGG1	5%	[166]
Uterus	MLH1	14%	[167]
	FEN1	Frequent (hypo-)	[146]

Table 4. CpG island hyper- (and hypo-) methylation in DNA repair genes in cancers

18. DNA repair proteins and miRNAs

A number of investigators have tried to relate alteration in DNA repair gene expression to altered level of miRNA expression. For instance, Wouters et al. [168], using "in silico" computer programs (Targetscan and Mirbase), listed 74 DNA repair or DNA Damage Response (DDR) genes and, for each of these genes, listed between 1 and 19 "conserved" miRNAs that were predicted to repress the particular genes. They defined "conserved" miRNAs as miRNAs found in at least five mammalian species. For the purposes of this review, in which we are concerned with epigenetic alterations that control DNA repair, about half of the miRNAs they found "in silico" would not be of interest because they were inducible by UV irradiation, and thus may have been largely controlled by a transient transcriptional regulatory change rather than epigenetically.

More recently, focusing on the DNA repair gene MGMT, Kushiwaha et al. [169] used five different "in silico" computer programs to predict which of 885 miRNAs would repress the DNA repair protein MGMT. Kushiwaha et al. [169] also transfected each of the 885 miRNAs into a glioblastoma cell line where the cell line had a high original expression of MGMT. They found 103 of the tested miRNAs did reduce MGMT expression *in vitro* by more than 50% without causing high cytotoxicity. However, the correspondence of predicted "in silico" interactions of the miRNAs with experimentally found interactions was rather low, 20% at best, indicating that "in silico" predictions often are not biologically relevant. Of the 103 miRNAs that reduced expression of MGMT, 15 had an inverse correlation with MGMT expression *in vivo* in promoter-unmethylated glioblastoma tissue specimens. These 15 miRNAs included miR-181d that Zhang et al. [137] had previously shown to be inversely

correlated with MGMT in glioblastomas. Kushiwaha et al. then focused further on one of the 15 miRNAs, miR-603, which strongly suppressed MGMT. In 23 glioblastoma cell lines, miR-603 was expressed at levels that varied by about 20 fold. It is not known whether the different expression levels were due to epigenetic control. They then determined that miR-603 suppressed MGMT by direct interaction with the 3'UTR region of MGMT mRNA, using mRNA-biotinylated miRNA complex pull down reactions with streptavidin coated magnetic beads. They were able to further show that miR-603 could cooperate with miR-181d to completely silence MGMT expression by jointly binding to nearby locations on the MGMT mRNA 3'UTR. These miRNA controls of expression of a DNA repair enzyme are illustrative of how miRNAs may interact with mRNAs to control their expression. In the experiments discussed in this paragraph, the miRNAs appear to be important in reducing expression of a DNA repair enzyme in progression to glioblastoma, but the extent to which epigenetic mechanisms are employed to control the level of the miRNAs is unclear.

Both Tessitore et al. [170] and Vincent et al. [171] listed about 20 miRNAs that are altered in cancers and that also control expression of DNA repair genes. The lists are not entirely overlapping. However, they do not indicate how these miRNAs are deregulated.

Deregulation of miRNA expression in cancers has been found to occur by a number of non-epigenetic mechanisms [120, 172]. One mechanism includes alterations in genomic miRNA copy numbers and location. Some of these are deletions that include the miRNA clusters *15a*/*16-1* or *let-7g*/*mir-135-1*,or else amplification or translocation of the *mir-17-92* cluster. In some cancers miRNAs were deregulated because of defects in the biogenesis mechanism (the process of creating miRNAs, which has a number of steps). Some cancers have deregulated miRNAs due to single nucleotide polymorphisms (SNPs) in the genes coding for the miRNAs, or SNPs in the target gene area to which the miRNA is targeted. Some miRNAs, that target DNA repair genes, are regulated by oncogenes. For instance ATM is down-regulated by miR-421, but miR-421 is regulated by N-Myc [173]. Thus, not all deregulation of DNA repair genes or DDR genes by miRNAs is due to epigenetic alteration affecting expression of the miRNAs.

19. Examples of epigenetic repression of DNA repair genes, due to alterations in methylation of promoters of miRNAs in various cancers

Table 5 lists nine miRNAs that have three characteristics. (1) Their expression is epigenetically controlled by the methylation level of the promoter region coding for the miRNA, (2) they control expression of DNA repair genes and (3) their level of expression was frequently epigenetically altered in one or more types of cancer. This list is not exhaustive. Many of the 30 miRNAs listed by Tessitore et al. [170] or Vincent et al. [171] might also meet these criteria upon further examination. Four of the miRNAs on this list are not noted by Tessitore et al. [170] or Vincent et al. [171]. Studies of most of these epigenetically controlled miRNAs have not noted the frequencies with which they occur in cancers. This is a very recent area of research, and seems to be less systematic, at this point, than studies of hypermethylation of promoter regions of DNA repair genes.

	DNA repair gene targets	Cancers affected (frequency if measured)	Refs indicating epigenetic control of miRNA	Refs indicating target gene(s) of miRNAs	Refs indicating cancer type affected
MiR-103 MiR-107	*RAD51, RAD51D*	Osteosarcoma, lung, endometrial, stomach	[174]	[175]	[175]
MiR-34c	*UNG* (uracil DNA glycosylase)	Gastric (70%) Field defect gastric (27%) Colon (98%) Field defect colon (60%) Chronic lymphocytic leukemia (18%) Small-cell lung cancer (67%) NSCLC (26%)	[176]	[177]	[49, 176, 178, 179]
MiR-124	*KU70*	Colon	[180]	[181]	[180]
MiR-155	*RAD51* *MLH1* *MSH2* *MSH6*	Breast Colon	[121, 182]	[18, 183]	[18, 121]
Let-7a repression increases HMGA2; HMGA2 alters chromatin architecture of and represses *ERCC1*)	*ERCC1*	(Colon) Anaplastic astrocytoma	[121]	[184, 185]	[185]
Let-7b repression increases HMGA1; HMGA1 targets *P53*	*P53*	Prostate Colon	[121]	[186, 187]	[186, 187]
miR-34b repression increases HMGA1; HMGA1 targets *BRCA1*	*BRCA1*	Breast	[134]	[135, 136]	[136]
MiR-182	*BRCA1*	Breast	[133]	[131]	[131]

Table 5. Epigenetically controlled miRNAs, altered in cancers, that target DNA repair genes

20. Whole genome sequencing indicates a high level of mutagenesis in cancers

Almost 3,000 pairs of tumor/normal tissues were analyzed for mutations by whole exome sequencing (WES) (sequencing the protein coding parts of whole genomes) and more than a hundred pairs of tumor/normal tissues were analyzed for mutations by whole genome sequencing (WGS) by Lawrence et al. [188]. Median mutation frequencies for 27 different types of cancer were found to vary by 1,000-fold. When there was a particular median mutation frequency for a type of cancer, the scatter of values (in individual cancers) for that type of cancer, above and below that median value, also varied by as much as 1,000-fold. Some mutation rates, given as numerical values of median numbers of mutations per megabase in a review of the literature by Tuna and Amos [189], are shown in Table 6, and the values were also converted to mutations per whole diploid genome in the table.

The mutation frequency in the whole genome (not just the protein coding regions) between generations for humans (parent to child) is about 30 - 70 new mutations per generation [190-192]. For protein coding regions of the genome in individuals without cancer, Keightley [193] estimated there would be 0.35 mutations per parent to child generation. Whole genome sequencing was also performed in blood cells for a pair of monozygotic (identical twin) 100 year old centenarians [194]. Only 8 somatic differences were found between the twins, though somatic variation occurring in less than 20% of blood cells would be undetected.

As seen in Table 6, tumors have a substantially higher frequency of mutations than the number of new mutations per generation in individuals without cancer. Also notable in Table 6, tumors with more exposure to DNA damage (lung cancers of smokers, and melanomas in individuals with high UV exposure) had higher mutation frequencies than the comparable tumors for patients with less exposure to DNA damage (lung cancers of non-smokers and melanomas of individuals without high UV exposure).

The information from whole exome sequencing and whole genome sequencing showed that different spectrums of mutations occurred in different tissues [188, 195]. Lung cancers shared a spectrum dominated by C->A mutations, presumably consistent with exposure to polycyclic aromatic hydrocarbons in tobacco smoke. Melanomas had a spectrum with frequent C->T mutations caused by misrepair of UV-induced covalent bonds between adjacent pyrimidines. Jia et al. [195] found 3-5 independent mutational signatures in 9 major types of cancers, indicating a range of 3-5 predominant mutational processes in different cancers. Lawrence et al. [188] also found about a 2.9-fold difference in mutation frequency across the genome depending on expression level of the genes. Genes with higher expression had a lower mutation frequency, possibly due to the availability of extra transcription-coupled repair. Also the mutation frequency of genes replicated early in a cell replication cycle was 2.9 fold lower than that of genes replicated late in the cycle.

While the type of mutation spectrum depended on the most frequent DNA damages in a given tissue, and there were about 5-fold differences in mutation frequency depending on whether genes were frequently transcribed or in a DNA region replicated at early or late times in a

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Parent/child per generation or cancer type	Mutation rate per million bases	Mutation rate per diploid genome
Parent/child per generation	0.00000023	70
Medullablastoma	0.15-0.6	900-3,600
Acute lymphocytic leukemia	0.3	1,800
Chronic lymphocytic leukemia	<1	<6,000
Prostate cancer	0.9	5,400
Multiple myeloma	2.9	17,400
Colorectal carcinoma	~5	~30,000
Microsatellite stable (MSS) colon cancer	2.8	16,800
Microsatellite instable (MSI) colon cancer (mismatch DNA repair deficient)	47	282,000
Hepatocellular carcinoma	4.2	25,200
Breast cancer	1.18-1.66	7,080-9,960
Lung cancer	17.7	106,200
Small cell lung cancer	7.4	44,400
Non-small cell lung cancer (smokers)	10.5	63,000
Non-small cell lung cancer (non-smokers)	0.6	3,600
Lung adenocarcinoma (smokers)	9.8	58,500
Lung adenocarcinoma (non-smokers)	1.7	10,200
Melanoma	~30	~180,000
Chronic UV-irradiation induced melanoma	111	666,000
Non-UV-induced melanoma of hairless skin of extremities	3-14	18,000-84,000
Non-UV-induced melanoma of hair-bearing skin	5-55	30,000-330,000

Table 6. Mutation rates per million bases or per diploid genome

replication cycle, the largest differences in mutation frequency were due to being in a tumor tissue versus a normal tissue (Table 6). These large differences in mutation frequency may frequently be due to whether one or more DNA repair genes are epigenetically reduced in expression in the stem cells giving rise to the development of the cancer.

21. Epigenetically reduced expression of DNA repair genes in DNA repair pathways in cancers

Figure 3 indicates typical DNA damaging agents, some of the lesions they cause and the pathways used to repair these lesions. Many of the genes active in these pathways are indicated by their acronyms. The acronyms listed in red represent genes shown, in Tables 3, 4 and 5, whose expression is frequently reduced due to epigenetic alterations in many types of cancers. The major DNA repair pathways are base excision repair, nucleotide excision repair, homologous recombinational repair, non-homologous end joining, mismatch repair and direct reversal. Each of these repair pathways employs one or more DNA repair enzymes that are frequently epigenetically reduced in expression in one or more types of cancer. This could be a substantial source of the genomic instability that is characteristic of cancers.

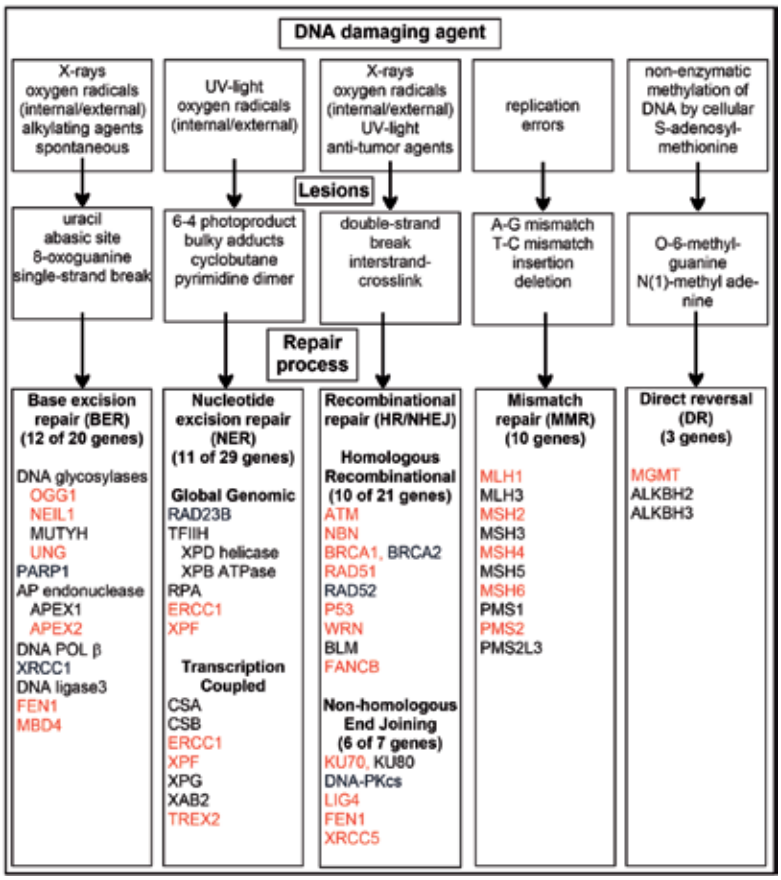

Figure 3. DNA damaging agents, the lesions they produce and the repair pathways that deal with the DNA damages, including acronyms for many of the genes in each of the pathways. Acronyms in red represent genes listed in Tables 3, 4 and 5 and indicate reduction of expression due to epigenetic alteration in one or more types of GI cancer [196].

22. Conclusion

Deficiencies in DNA repair due to inherited germ-line mutations in DNA repair genes cause increased risk of cancer. Such DNA repair gene mutations allow excess unrepaired DNA damages to accumulate in somatic cells. Then either inaccurate translesion synthesis past the un-repaired DNA damages or the error-prone DNA damage response of non-homologous end joining can cause mutations. Erroneous or incomplete DNA repair may also cause epimutations. In sporadic cancers, mutations in DNA repair genes are relatively rare. However, at least 25 DNA repair genes are often epigenetically altered and have reduced expression in sporadic cancers and in the field defects that give rise to the cancers. Such epimutations in DNA repair genes also likely lead to a further increase in mutations and epimutations, and these mutations and epimutations can include both the driver mutations and the other epigenetic alterations central to progression to cancer. Whole genome sequencing of many different types of cancers show that between thousands to hundreds of thousands of mutations occur in various types of cancers. The epimutations in DNA repair genes that occur early in progression to cancer, are a likely source of the high level of genomic instability characteristic of cancers. Epigenetic reduction of DNA repair appears to be a frequent early step, central to progression to cancer.

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Chapter 7

The BRCA1 and BRCA2 Breast and Ovarian Cancer Susceptibility Genes — Implications for DNA Damage Response, DNA Repair and Cancer Therapy

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Additional information is available at the end of the chapter

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1. Introduction

Efficient intracellular DNA repair mechanisms are essential for preventing the accumulation of genetic mutations and protecting against genomic instability, which can lead to cancer development. This is reflected in the increased risk of breast and ovarian cancer conferred by mutations in the breast and ovarian cancer susceptibility genes 1 & 2 (BRCA1 and BRCA2), both of which have important roles in promoting the accurate repair of DNA damage and maintaining genomic integrity. BRCA1 was first identified in 1994 and mapped to chromosome 17q12 through linkage analysis in families with a strong family history of breast and/or ovarian cancer [1, 2]. BRCA2 was discovered a short time later when a second breast cancer susceptibility locus was mapped to chromosome 13q12, again by linkage analysis in similar families [3, 4]. BRCA1/2 mutations may be present in approximately 1/400-1/800 of the general population although a higher incidence of BRCA1 mutations have been observed in certain populations such as in Ashkenazi Jews. Studies estimate that inherited mutations in BRCA1 can increase the cumulative risk of developing breast cancer by age 70 to 80% and ovarian cancer risk to 30-40%, whereas BRCA2 mutation carriers have up to a 50% risk of breast cancer and 10-15% ovarian cancer risk by age 70. Additionally, mutations in BRCA2 also increase susceptibility to male breast cancer, prostate and pancreatic cancer [5, 6]. According to the Breast Cancer Information Core (BIC), over 1,700 distinct mutations have been identified in the BRCA1 gene to date, comprising inactivating truncations and deletions to missense mutations. While approximately 850 of BRCA1 mutations identified have been confirmed to increase cancer risk, the clinical relevance of the remaining mutations is unknown [7].

While BRCA1/2 mutations account for a relatively small proportion of all breast cancers (2.5-5%), mutations in these genes are responsible for approximately 20-25% of inherited breast cancer cases [8-10]. In addition, BRCA1 mutation carriers typically develop cancer before the age of 50 meaning that the number of years affected can be substantially greater than in most subtypes of sporadic breast cancer. This is likely due to the fact that both the BRCA1 and BRCA2 genes adhere to the Knudson "two-hit" hypothesis in which both alleles of a tumour suppressor gene must be mutated for the pathogenic phenotype to become apparent. Hence one inherited copy of mutant BRCA1/2 is the "first hit" and the "second hit" comes from acquiring a somatic mutation.

BRCA1 mutant breast tumours often fall into the basal-like breast cancer subtype, which typically exhibit low or absent expression of the oestrogen receptor α (ERα), progesterone receptor (PR) and the human epidermal growth factor receptor 2 (HER2) and are therefore commonly referred to as triple negative breast tumours. Due to the lack of expression of these receptors, there are no targeted treatments currently available for this type of cancer and as a result these patients tend to have a poor prognosis. Interestingly, in sporadic basal-like breast cancer patients with wildtype BRCA1, BRCA1 expression is often down-regulated possibly as a result of promoter methylation or over-expression of ID4, a negative regulator of BRCA1 expression. A term has been coined, known as "BRCAness", to describe sporadic basal-like tumours with low BRCA1 expression and/or a similar phenotype to BRCA1 mutant tumours. Both BRCA1 mutant and BRCA1-low tumour types are sensitive to DNA damaging agents suggesting a possible common pathogenesis involving dysfunction of BRCA1 or BRCA1-regulated pathways, such as DNA repair [11, 12].

Accordingly, the tumour suppressor function of BRCA1 and BRCA2 is mainly attributed to the role of these proteins in the regulation of conservative DNA repair pathways, thus maintaining genomic integrity. While the main function of BRCA2 identified to date is in promoting the error-free homologous recombination pathway, BRCA1 is a multi-functional protein with roles in many important cellular processes such as transcriptional regulation, ubiquitination, oestrogen metabolism, chromatin remodelling and mRNA splicing [13]. These additional functions of BRCA1 and how they relate to DNA repair will also be discussed, followed by an overview of BRCA2 function in the repair of damaged DNA and how the DNA repair defects in BRCA1/2 mutant related cancers can be exploited for treatment.

2. Structure of BRCA1

The BRCA1 gene encodes 24 exons translating into a 1863 amino acid protein which contains two main functional domains; a **r**eally **i**nteresting **n**ew **g**ene (RING) finger domain and two BRCA1 C-terminal (BRCT) domains (Figure 1). The RING finger domain, located at the N-terminus of BRCA1, is a zinc binding region with a conserved histidine and cysteine motif which is required for binding to the structurally similar **B**RCA1 **A**ssociated **R**ING **D**omain protein 1 (BARD1) which also has a RING finger domain and 2 BRCT domains. The BRCA1-BARD1 interaction is necessary for stability of both the BRCA1 and BARD1 proteins thus

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BRCA1 generally exists in a heterodimeric complex with BARD1 *in vivo* [14]. Furthermore, binding of BARD1 to the RING finger domain of BRCA1 forms an E3 ubiquitin ligase complex, the function of which will be discussed later. A number of tumour associated mutations have been identified within the RING finger of BRCA1 such as C61G and C64G which abolish the ubiquitin ligase activity and confer sensitivity to ionising radiation, suggesting the RING domain of BRCA1 is important for regulating DNA repair [14, 15].

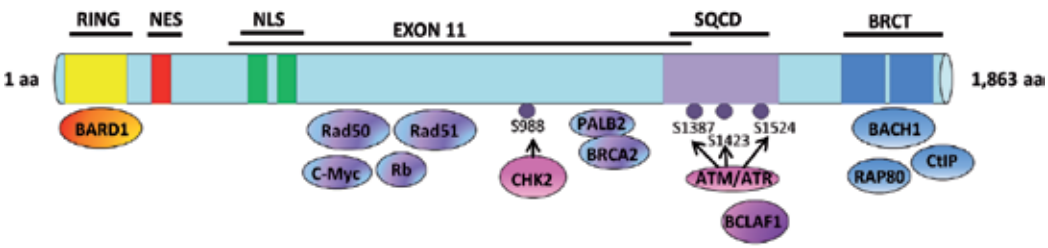

Figure 1. Structure and Binding Partners of BRCA1. Schematic diagram of BRCA1 and it's functional domains illustrating the position of; the RING finger responsible for BARD1 binding and E3 ligase activity, exon 11 which binds important HR proteins including Rad50 and Rad51, and the BRCT domains which mediate binding of RAP80, BACH-1 and CtIP. Phosphorylation sites important for DNA damage signalling are also shown, as well as the kinases responsible for their modification.

At its C-terminus, BRCA1 contains two conserved BRCT domains, each approximately 100 amino acids long. BRCT domains recognise and bind to phospho-peptides containing the pSer-X-X-Phe motif [16]. Phosphorylation is a major mechanism of signalling within the DNA damage response pathway and BRCA1 has been shown to bind to several phosphorylated DNA repair-related proteins through its BRCT domains such as BACH1 and CtIP [17]. As with mutations in the RING finger domain, mutations in the BRCT repeats of BRCA1 have been identified in cases of familial breast cancer. Furthermore, mouse embryonic fibroblast cells harbouring a BRCT mutation that disrupts BACH1 binding exhibit defective homologous recombination, increased sensitivity to genotoxic stress and develop tumours at a similar rate to those lacking BRCA1 [18].

The region of BRCA1 encoded by exons 11 – 13 comprises 65% of the BRCA1 peptide sequence and is also commonly mutated in breast cancer. It contains two nuclear localisation signals (NLS), a less structured central domain and an SQ cluster domain (SQCD) [7]. Mutations in NLS1 in particular, disrupt interactions between BRCA1 and importin-α, resulting in impaired nuclear localisation of BRCA1, which is detrimental to DNA repair [19]. BRCA1 also contains a nuclear export signal (NES) in its N-terminus, which contributes to subcellular shuttling of BRCA1. Numerous proteins with functions in different cellular processes bind the exon 11 – 13 region of BRCA1 including the important DNA repair proteins Rad50 and Rad51 as well as the transcription factor c-Myc and cell cycle regulator, Retinoblastoma (Rb) (reviewed in [7]). The SQCD is also relevant to the function of BRCA1 in DNA repair as it contains numerous serine-glutamine (SQ) or threonine-glutamine (TQ) residues which are targets for phosphorylation via the DNA damage-induced phosphatidylinositol 3-kinase-related kinases (PIKKs);

ataxia-telangiectasia mutated (ATM) and ATM and Rad3-related (ATR) and are responsible for activating numerous functions of BRCA1 depending on the residue(s) phosphorylated [20].

3. BRCA1 in DNA repair

As previously mentioned, BRCA1 mutation or dysfunction has consistently been associated with genomic instability and it is proposed that this is mainly due to defective DNA damage repair pathways [6, 21]. DNA damage occurs frequently within cells due to by-products of normal metabolism such as reactive oxygen species (ROS) but can also occur following exposure to exogenous sources such as ionising radiation (IR), ultraviolet (UV) radiation or chemotherapy. In order to respond to different types of DNA damage, cells have several DNA damage repair pathways including base excision repair (BER) and nucleotide excision repair (NER) for repairing damaged bases and single strand breaks (SSBs) respectively, and non-homologous end joining (NHEJ) or homology-directed repair (HDR) for the repair of double strand breaks (DSBs). NHEJ is the most common form of DSB repair and can occur throughout all phases of the cell cycle. It involves binding of Ku70/Ku80 to the broken DNA ends followed by recruitment of the catalytic subunit of DNA-PK, which phosphorylates numerous substrates at and surrounding the break site thereby promoting the removal of single strand overhangs and subsequent re-ligation of the DNA ends by XRCC4/DNA ligase IV. Although this is the most commonly utilised DSB repair pathway, as it has no regard for sequence homology, it is a relatively error prone repair pathway and utilisation of this pathway is potentially mutagenic.

There are two types of homology-directed repair; single strand annealing (SSA) and homologous recombination (HR). SSA can repair DSBs at short repetitive sequences by annealing the complementary repeats in a Rad52-dependent manner. This always results in loss of genetic material and therefore SSA is the most mutagenic of the DSB repair pathways [22]. In comparison to both NHEJ and SSA, HR is relatively error-free since it uses a sister chromatid as a template to copy and replace damaged DNA. However, HR can only occur in the S and G2 phases of the cell cycle when a homologous sister chromatid is present and in close proximity [23]. Although the most extensively studied role of BRCA1 is its regulation of HR, BRCA1 has also been implicated in NHEJ, SSA and the repair of interstrand crosslinks (ICLs) and how BRCA1 is involved in each of these pathways will be discussed below.

4. Homologous recombination

The process of HR includes several different stages. The MRE11-Rad50-Nbs1 (MRN) complex, in combination with the human single strand binding protein (hSSB1) is responsible for the initial sensing of DNA DSBs within the cell. MRN then binds to the break site leading to the recruitment and activation of ATM which in turn phosphorylates many substrates involved in DNA damage signalling. The histone H2AX is one such substrate of ATM which is phos-

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phorylated at serine 139 (Ser139) forming γH2AX. ATM rapidly phosphorylates H2AX within the chromatin at, and flanking, the DSB site, thereby amplifying and propagating the DNA damage response. Additionally, γH2AX forms a docking site for numerous other proteins involved in DNA damage signalling and repair such as mediator of DNA damage checkpoint protein 1 (MDC1) which binds to γH2AX through its BRCT domain. MDC1 itself is able to recruit and anchor more MRN complexes to γH2AX surrounding the break site through interaction with Nbs1. MRN binding then amplifies ATM activation leading to a positive feedback loop which further amplifies the DNA damage response signalling cascade [24]. Once the necessary proteins have been recruited, DNA end resection must occur for HR to proceed and this involves the generation of 3′ single stranded DNA (ssDNA) overhangs at the DSB ends. MRN has a major role in end resection through the endonuclease and exonuclease activities of Mre11. The 3′ ssDNA overhang is then coated by replication protein A (RPA) which protects the single stranded DNA from degradation and prevents the formation of secondary structures. Next, partner and localiser of BRCA2 (PALB2) recruits BRCA2 which facilitates the displacement of RPA and subsequent loading of the recombinase Rad51 onto ssDNA forming a nucleoprotein filament which is responsible for homology searching and invasion of the homologous sister chromatid. This leads to formation of a transient displacement loop (D-loop) since once strand invasion has taken place, the second strand of the sister chromatid becomes displaced. Elongation by the DNA replication machinery and resolution of the D-loop then completes error-free repair of DNA [24].

BRCA1 was first implicated in DSB repair following the observation that murine embryos harbouring homozygous BRCA1 exon 11 deletions were hypersensitive to ionising radiation and exhibited both structural and numerical chromosomal aberrations in comparison to their heterozygous and wildtype counterparts [21]. Moynahan et al extended these findings by demonstrating that BRCA1-deficient embryonic stem cells were also highly sensitised to the DNA cross-linking agent mitomycin C (MMC) and importantly, correction of the BRCA1 exon 11 deletion restored normal levels of mitomycin C resistance [25] confirming that BRCA1 has a role in mediating resistance to DNA damaging agents. In 1999, the importance of BRCA1 specifically in homologous recombination was demonstrated in $BRCA1^{-/-}$ mouse embryonic stem cells which had 5 to 6 fold lower levels of homologous repair activity compared to $BRCA1^{+/-}$ cells while little effect was observed on non-homologous repair [26]. Over the past couple of decades, multiple roles for BRCA1 in HR have emerged, and BRCA1 appears to have distinct functions depending on its binding partners. Each of these functions will be considered below.

5. BRCA1 in DNA end resection

Processing of DSBs by DNA end resection is necessary to initiate the repair of DSBs by HR. A role for BRCA1 in promoting end resection was first observed when depletion of BRCA1 expression was shown to decrease the generation of ssDNA [27]. Chen et al showed that the interaction between BRCA1-CtIP-MRN (known as the BRCA1-C complex) facilitates end resection in S and G2 phases of the cell cycle and that this interaction is dependent on CDK

phosphorylating CtIP at serine 327. In agreement with this, when the CtIP S327A mutant (which cannot bind to BRCA1) is expressed in U2OS cells it leads to increased radiosensitivity in comparison to U2OS cells transfected with wildtype CtIP. Furthermore, the BRCA1-CtIP interaction was shown to be required for binding of MRN to BRCA1, which is essential for the synthesis of ssDNA overhangs [28, 29].

In direct contrast, a number of recent studies have demonstrated that BRCA1 is in fact dispensable for CtIP-mediated end resection. Reczek et al showed mouse embryonic fibroblasts (MEFs) expressing the CtIP S326A mutation (equivalent to S327A in humans) displayed similar levels of Rad51 and RPA IRIF as CtIP wildtype cells. Accordingly, loss of the CtIP-BRCA1 interaction did not affect HR or tumour development in mice [30]. Polato and colleagues reported similar findings showing that in contrast to the CtIP S327A mutant, mice harbouring the CtIP T847A mutation (which is essential for end resection but does not affect the BRCA1 interaction) had elevated levels of spontaneous chromosomal aberrations as well as decreased levels of IR-induced Rad51 indicating that CtIP functions independently of BRCA1 to promote end resection [31]. Further investigation of the role of the BRCA1-CtIP interaction in end resection was performed using a high resolution technique known as single molecule analysis of resection tracks (SMARTs) which allows visualisation of the length of the resected DNA in a single molecule. This revealed that although BRCA1-CtIP is expendable for the initiation of end resection, disruption of this interaction actually decreases the length and speed of resected DNA generated following IR or etoposide treatment [32]. Therefore, although BRCA1 is not essential for CtIP-mediated end resection it may facilitate the efficiency of the process.

In contrast to the role of BRCA1-CtIP in facilitating end resection, recent evidence suggests that BRCA1 in complex with receptor-associated protein 80 (RAP80) may actually prevent end resection with chromatin immunoprecipitation (ChIP) assays showing an increased abundance of HR proteins RPA and Rad51 on chromatin following RAP80 depletion. The BRCA1-RAP80 complex also contains ABRAXAS, BRCC36, BRCC45, MERIT40 and BARD1 and is known as the BRCA1-A complex, however it appears the BRCA1-RAP80 interaction is most important in the regulation of end resection. Decreased RAP80 has also been shown to increase the BRCA1-CtIP interaction, which may further enhance end resection [33, 34]. Although HR is a relatively error-free method of repair, poorly regulated HR can lead to recombination of inappropriate homologous sequences, which can produce genomic rearrangements and indeed depletion of RAP80 despite increasing HR leads to an increase in multiradial chromosomes, as a result of improper recombination [34]. Thus the BRCA1-RAP80 complex, despite inhibiting end-resection required for HR, may also preserve genomic integrity by preventing excessive end resection, which can lead to chromosomal aberrations [33, 34].

6. BRCA1 in HR/NHEJ pathway choice

End resection is clearly a pivotal step in promoting HR, and the regulation of end resection has recently become an intense area of research in determining the choice between HR and

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NHEJ in S and G2 phases of the cell cycle when both repair pathways are operational. In contrast to the role of BRCA1-CtIP in facilitating end resection in S and G2 phases thus allowing HR to proceed [35], 53BP1 has been shown to prevent end resection therefore inhibiting HR and promoting NHEJ. Several studies have shown that 53BP1 loss at least partially restores HR in BRCA1 deficient cells with Bunting et al observing increased IR-induced RPA phosphorylation in the absence of 53BP1 in BRCA1 mutant cells. Thus it has since been postulated that the antagonistic relationship between BRCA1 and 53BP1 may be responsible for mediating HR/NHEJ pathway choice [36, 37] (Figure 2). Bunting et al have also demonstrated that mice harbouring homozygous deletion of 53BP1 in combination with breast-specific homozygous deletion of BRCA1 exon 11 ($BRCA1^{\Delta 11/\Delta 11}$) display a greatly reduced breast tumour burden in comparison to $BRCA1^{\Delta 11/\Delta 11}$ mice with wildtype 53BP1 [36]. The authors therefore suggest that BRCA1 mutation carriers may benefit from inhibition of 53BP1 to alleviate the repair defect and thus genomic instability. Interestingly, when CtIP was depleted in $BRCA1^{-/-}$ $53BP1^{-/-}$ MEFs, IR-induced ssDNA formation was decreased suggesting CtIP is necessary for the rescue of end resection observed in the absence of both BRCA1 and 53BP1. Furthermore, expression of CtIP in BRCA1 mutant cells decreases levels of genomic instability supporting a model whereby CtIP may partially overcome 53BP1-mediated inhibition of resection following loss of BRCA1 [31]. Despite the inhibitory role of 53BP1 in S phase, Kakarougkas et al propose that 53BP1 actually promotes HR in G2, specifically in DSBs occurring in heterochromatin regions due to 53BP1-dependent formation of phosphorylated KAP1 foci causing relaxation of the heterochromatin and allowing RPA loading in G2, thus 53BP1 can both promote and inhibit HR at different stages of the cell cycle [38].

The mechanism of the antagonism between BRCA1 and 53BP1 was investigated by super-resolution microscopy of IR-induced foci (IRIF), which enabled observations of the precise distribution of 53BP1 and BRCA1 following IR and showed enrichment of 53BP1 within IRIF in G0/G1 cells concomitant with the use of NHEJ mediated DSB repair in these stages of the cell cycle. However, in S phase 53BP1 was redistributed to the periphery of these foci while BRCA1 accumulated in the core of the IRIF. 53BP1 was not repositioned to IRIF margins following siRNA knockdown of BRCA1 showing BRCA1 is necessary for this process. This led the authors of this study to propose a process whereby BRCA1 may inhibit 53BP1 in S phase by preventing its interaction with chromatin at DSB sites allowing end resection and thus HR to proceed [39]. Following on from this, the deubiquitinating enzyme POH1 is also thought to be necessary for formation of the 53BP1 devoid IRIF core in a BRCA1-dependent manner. The suggested model involves BRCA1-mediated redistribution of 53BP1 from the core of the IRIF and this allows access to POH1 which removes RAP80 from the core allowing degradation of ubiquitin chains and complete clearance of 53BP1 from the DNA ends situated within the core of the IRIF thereby facilitating DSB end resection [40].

On the contrary, 53BP1 can also inhibit BRCA1 recruitment to DSBs in G1 phase of the cell cycle and if the 53BP1 effector protein RIF1 is reduced, BRCA1 IRIF form in G1. RIF1 accumulates at DSBs in a 53BP1-dependent manner and has been shown to bind to 53BP1 following activation of ATM. RIF1 IRIF are normally only formed in G1 but down-regulation of BRCA1 leads to a significant increase in RIF1 foci in S/G2. Moreover, this inhibitory effect of BRCA1

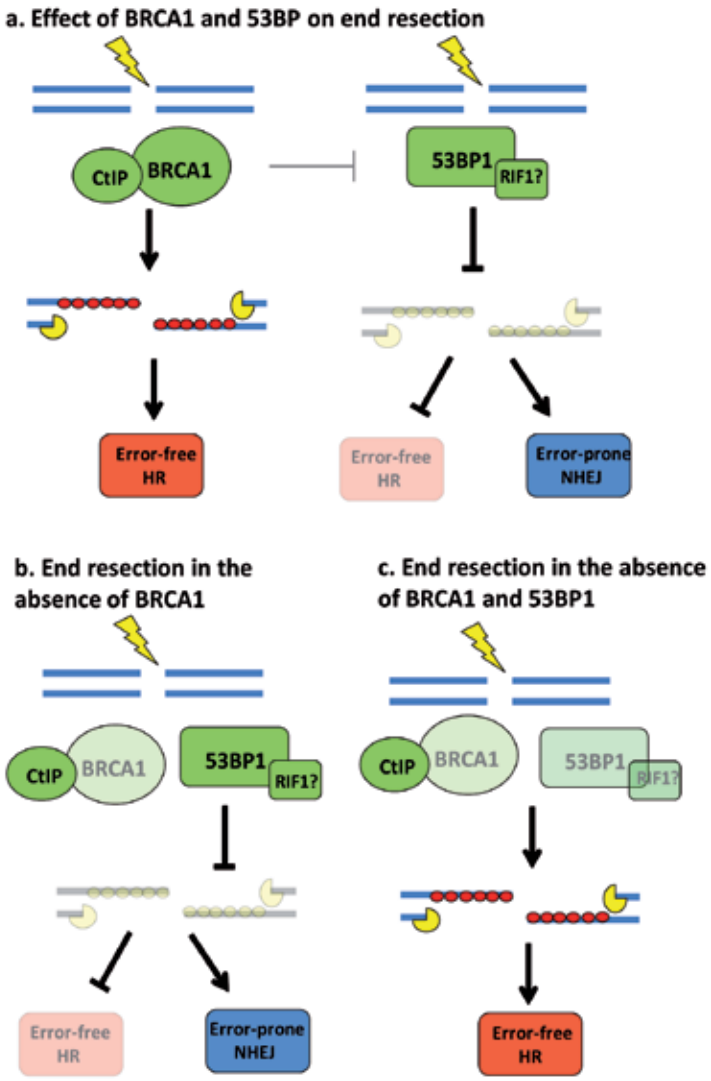

Figure 2. The Antagonistic Relationship of BRCA1 and 53BP1 on End Resection. Both the HR and NHEJ repair pathways can function in S and G2 phases of the cell cycle and end resection has an important role in the choice between HR and NHEJ. In wildtype cells, the BRCA1/CtIP complex promotes HR in S and G2 by stimulating end resection and by inhibiting 53BP1 (and possibly its effector protein RIF1), which acts as a barrier to end resection and thus HR (a). HR defects are observed in the absence of BRCA1, due to loss of its stimulatory effect on end resection and also loss of BRCA1-mediated inhibition of 53BP1 and RIF1. Together this blocks end resection and HR, and DNA is repaired by the potentially mutagenic process of NHEJ, such is the case in BRCA1 mutant breast cancers (b). In the absence of both BRCA1 and 53BP1 the barrier to end resection is removed and error-free HR can once more proceed via a CtIP-dependent mechanism. Therefore the status of 53BP1 in BRCA1 mutant tumours is pivotal for the regulation of HR (c).

on RIF1 IRIF is dependent on the BRCA1-CtIP interaction. Depletion of RIF1 rescues both the end resection and Rad51 loading defect caused by BRCA1 deficiency to a similar degree as loss of 53BP1. Therefore, in G1 RIF1 is recruited to DSBs by 53BP1 following ATM activation and

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inhibits BRCA1 recruitment, while in G2/S RIF1 accumulation is inhibited by BRCA1-CtIP, suggesting that RIF1 and BRCA1 form a cell cycle-regulated circuit to favour NHEJ in G1 and HR in S/G2 [41].

Interestingly, loss of Ring finger Nucleotide Factor 168 (RNF168) which recruits both BRCA1 and 53BP1 to sites of DSBs seems to emulate the effects of 53BP1 loss, with depletion of RNF168 in BRCA1 deficient cells rescuing the homologous recombination defect. This study also demonstrated that expression of a dominant negative form of 53BP1 is able to restore HR in control cells but had no effect in RNF168 depleted cells, suggesting that 53BP1 and RNF168 inhibit HR through a similar mechanism [42].

7. BRCA1 in Rad51 loading

Another key role of BRCA1 in HR is in Rad51 loading which as discussed above is responsible for homologous strand invasion which then allows DNA polymerase to repair DNA using the sister chromatid as a template. BRCA1 has been reported to colocalise with Rad51 at nuclear foci within S phase of the cell cycle. Additionally, BRCA1 and Rad51 physically interact through regions within BRCA1 exon 11 [6, 43, 44]. Furthermore, depletion or mutation of BRCA1 has been shown to result in loss of Rad51 foci formation following DNA damage indicating that BRCA1 is required for Rad51 recruitment to DSB sites [27]. Following this, Sy and colleagues demonstrated that the displacement of RPA and subsequent loading of Rad51 filaments to single stranded DNA was dependent on the interaction between BRCA1, BRCA2 and PALB2 [45]. PALB2 was first identified as a binding partner of BRCA2 and is involved in the recruitment of BRCA2 to DSB sites but PALB2 was later shown to also interact with BRCA1 and this interaction is required for BRCA2-PALB2 localisation to sites of DNA damage. Additionally, depletion of PALB2 results in deficient HR and a PALB2 mutant unable to bind BRCA1 could not restore this repair defect in comparison to wildtype PALB2. This suggests that PALB2 acts as a scaffold between BRCA1 and BRCA2 [45, 46]. Considering the many and varied roles of BRCA1 in HR, it is therefore not surprising that defective HR is a characteristic of BRCA1 deficient cells and as a consequence DNA damage is repaired via error-prone mechanisms such as NHEJ resulting in a higher rate of genetic mutations which increases susceptibility to cancer [47].

8. Single Strand Annealing (SSA)

In comparison to the extensively studied functions of BRCA1 in HR, relatively little is known about the role of BRCA1 in the regulation of the SSA homology-directed repair pathway. Stark et al demonstrated that mouse embryonic stem (ES) cells harbouring homozygous deletion of BRCA1 exon 11 have decreased HR and SSA activity and the same effect was observed following disruption of the BRCA1-binding region of BARD1. Expression of wildtype BRCA1 in the BRCA1 mutant HCC1937 cell line model was also shown to promote SSA, although a

greater increase was observed in HR activity [48]. Although BRCA1 appears to positively regulate both HR and SSA, BRCA2 and Rad51 have opposing effects on these two DSB repair pathways, promoting HR while suppressing SSA. This suggests that BRCA1/BARD1 may function upstream of the branch point between HR and SSA regulating a step common to both pathways while BRCA2 and Rad51 act downstream of BRCA1 to inhibit SSA. Since BRCA1 is known to promote end resection which is required for both HR and SSA this is a possible mechanism by which BRCA1 augments both pathways. Furthermore, loss of the NHEJ factor Ku70, which limits resection of DSB sites, was able to rescue the SSA defect caused by disruption of BARD1, thus overcoming the barrier to resection and allowing SSA to proceed [49]. In contrast to BRCA1 regulating HR and SSA via the same mechanism, a study which analysed the effect of 29 different BRCA1 missense mutations on HR and SSA showed that several mutants with normal HR activity were defective in SSA. Interestingly all of the mutants showing differential regulation of the two pathways exhibited amino acid substitutions between residues 90 and 191 [50]. Therefore, BRCA1 may also have an additional function in the regulation of SSA that has yet to be discovered, possibly regulated by an undefined region within its N-terminus. This is supported by the fact that loss of Ku70 only partially restores SSA in BARD1-mutant cells suggesting BRCA1-BARD1 may also mediate SSA further down the pathway, independent of end resection regulation [49].

9. Non-homologous end joining

As previously mentioned, DNA DSBs can be repaired by homology-directed repair pathways or by NHEJ. However the significance of BRCA1 in the regulation of NHEJ is controversial and early studies produced conflicting results. Snouwaert et al showed a decrease in HR and an increase in NHEJ activity in mouse ES cells with homozygous deletion of BRCA1 between residues 223 and 763 compared to wildtype cells, providing evidence that BRCA1 suppresses NHEJ. Furthermore, expression of a BRCA1 transgene decreased NHEJ to normal levels in these cells [51]. In direct contrast, Zhong et al demonstrated a reduction in NHEJ activity in BRCA1 null mouse embryonic fibroblasts which could also be corrected by reintroduction of BRCA1 [52]. Another study showed no difference in NHEJ in BRCA1 deficient human breast tumour derived cells (HCC1937) compared to cell lines with wildtype BRCA1 [53]. More recent work suggests that the initial discrepancies observed may reflect different functions of BRCA1 depending on cell cycle phase and also on the subtype of NHEJ examined. There are 2 main subtypes of NHEJ – Ku80-dependent canonical NHEJ which is relatively precise and the alternative NHEJ pathway which is Ku80 independent and involves microhomology-mediated end joining (MMEJ) which is similar to SSA except MMEJ can anneal smaller homologous sequences (5-25 bps), but like SSA, MMEJ is extremely mutagenic. In accordance with the role of BRCA1 in tumour suppression, evidence suggests that ATM and Chk2 mediated phosphorylation of BRCA1 promotes precise or canonical end-joining while suppressing the mutagenic MMEJ [54, 55]. Additionally, the BRCA1-BACH1 complex is required for impeding error-prone MMEJ with expression of a BACH1 mutant defective in BRCA1 binding resulting in increased MMEJ activity [56].

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Another study investigated differences in BRCA1 regulation of NHEJ subtypes throughout the cell cycle and interestingly BRCA1 was found to promote canonical NHEJ in G1 but not in G2/S [57]. Furthermore, depletion of BRCA1 increased the number of deletions acquired during NHEJ, confirming that BRCA1 favours precise NHEJ. BRCA1 was shown to interact with the canonical NHEJ factor Ku80, specifically in G1 and this interaction may be critical for stabilising Ku80 to sites of damage as ChIP assays show decreased Ku80 binding at I-Sce-mediated DSB sites following treatment with BRCA1 siRNA. Thus the authors conclude that BRCA1 may maintain genomic stability in G1 via promotion of precise end joining in addition to promotion of error-free HR in S and G2 [57].

10. BRCA1 in interstrand crosslink repair

In addition to the repair of DNA DSBs, BRCA1 has also been implicated in the repair of interstrand crosslinks (ICLs). DNA ICLs are caused endogenously by the by-products of lipid peroxidation and exogenously by DNA crosslinking agents such as the platinum-containing cisplatin and nitrogen mustard compound mitomycin C, commonly used for chemotherapy in the treatment of cancer. ICLs are extremely deleterious lesions due to the covalent bonding of DNA strands which inhibits strand separation and thus DNA replication and transcription. The repair of ICLs can be a complex process and varies depending on the stage of the cell cycle [58]. However, in brief, the presence of an ICL during DNA replication will lead to stalled replication forks and repair of the lesion will then progress by the formation of incisions on either side of the linked nucleotide via the action of NER endonucleases. Translesion synthesis (TLS) polymerases allow bypass of the ICL site, generating a DSB, which can then be repaired by HR. The FANC proteins have a major role in the repair of ICLs. FANC proteins are mutated in Fanconi Anaemia (FA), a syndrome associated with bone marrow failure, developmental defects and susceptibility to cancer. Activation of the FA pathway involves formation of a core complex of eight FANC proteins which together with accessory proteins form a ubiquitin ligase responsible for monoubiquitination of FANCD2 and FANCI which is essential for co-ordinating the incision step of ICL repair [59]. Since ICLs are ultimately repaired by HR, in general the functions of BRCA1 discussed above also apply to the repair of ICLs. The recruitment of BRCA1 in ICL-linked HR however, is dependent on FANC proteins since the BRCA1-RAP80 complex, which modulates HR repair of ICLs, has been shown to bind to K63-linked polyubiquitinated FANCG via the ubiquitin interacting motifs (UIMs) of RAP80 [58].

BRCA1 also regulates ICL repair independently of HR, evidenced by the observation that while loss of 53BP1 restores HR defects in BRCA1-depleted cells, depletion of 53BP1 does not rescue hypersensitivity of BRCA1 null cells to crosslinking agents [60]. Numerous reports suggest loss of BRCA1 impedes the recruitment of the FANCD2 complex to the ICL, but has no effect on the ubiquitination of FANCD2. Depletion of Ku70 rescues FANCD2 foci formation in $BRCA1^{\Delta 11/\Delta 11}$ MEFs following cisplatin or MMC treatment and also decreases the hypersensitivity to these agents suggesting that BRCA1 may recruit or retain FANCD2 at sites of ICLs via inhibition of Ku70/80 [60]. Recently, Long et al have reported another novel role of BRCA1 in ICL repair related to replication fork stalling. It has been shown that replication fork stalling

first occurs approximately 20 bp from the site of the ICL at which point the CMG DNA replicative helicase blocks extension of the leading strand and therefore needs to be removed from the DNA to allow approach towards the ICL, which triggers activation of the FANC pathway and thus ICL repair. Depletion of BRCA1 or defective BRCA1-BARD1 complex formation inhibits CMG 'unloading' in response to ICLs but not during normal DNA replication. As a result of BRCA1 loss, the extension of the leading strand towards the ICL was impeded and a defect in the generation of incisions at the site of the ICL was observed. Therefore localisation of the BRCA1-BARD1 complex is required at an early stage of ICL repair in addition to its later role in HR [61].

11. BRCA1 and the cell cycle

Cell cycle checkpoints are essential for repair of damaged DNA as cell cycle arrest affords time for the DNA to be repaired efficiently ensuring mutations or chromosomal aberrations are not maintained or replicated leading to genomic instability. Cell cycle regulation is mainly orchestrated by the balance of cyclins, cyclin-dependent kinases (CDKs) and CDK inhibitors. However, many other factors are involved in mediating these cell cycle regulators and the timely regulation of cell cycle checkpoints at different phases. Indeed, evidence exists of a role for BRCA1 in the regulation of the G1/S, S and G2/M checkpoints. Initial studies focussed on the role of BRCA1 in the intra-S and G2/M checkpoints, as the BRCA1 deficient cell line HCC1937 also harbours mutations within p53, a master regulator of the G1/S checkpoint, making it difficult to unravel the contribution of BRCA1 to this checkpoint in these cells. However, these cells, which have a defective G1/S checkpoint, also exhibit defective S and G2/M arrest following IR and both of these checkpoints are restored by expression of wildtype BRCA1. While ATM-mediated phosphorylation of BRCA1 at Ser1423 is necessary for IR-induced G2/M arrest, ATM-dependent phosphorylation of BRCA1 Ser1387 is required for S phase arrest [62, 63]. Following this, another study using siRNA mediated depletion of BRCA1 found that the BRCA1/BARD1 heterodimer is required for G1/S checkpoint arrest following IR induced DNA damage [64]. This study found that ATM dependent phosphorylation of BRCA1 on serine 1423 and 1524 is required to allow BRCA1 to function as a scaffold, facilitating the ATM dependent phosphorylation of p53 on Ser-15 thereby stabilising and activating p53 resulting in the transactivation of the cdk inhibitor p21 and activating the G1/S checkpoint. As in HR where BRCA1 function depends on its binding partners, different BRCA1 complexes regulate different cell cycle phases. BACH1 is activated in S phase and forms a complex with BRCA1 and BRCA2 necessary for cells to progress from G1 to S phase [65], and BRCA1 and TOPBP1 colocalise to foci specifically in S phase following treatment with different genotoxic agents, namely hydroxyurea, UV and zeocin [66].

Interaction of BRCA1 with the phosphorylated form of BACH1 is also required for the G2/M checkpoint as BACH1 depletion abolishes G2/M arrest following IR and while the expression of wildtype BACH1 rescues this defect, the BACH1 S990A mutant, which is unable to bind BRCA1, could not restore G2/M arrest [67]. BACH1 exerts cell cycle effects by binding to the BRCT domains of BRCA1 but exon 11 of BRCA1 also appears to play a role in G2/M checkpoint

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activation as MEFs carrying homozygous BRCA1 exon 11 deletions display a defective G2/M checkpoint [68]. A mechanism for BRCA1-regulated G2/M checkpoint arrest was postulated by Yarden and colleagues who showed that in response to IR, ATM activates BRCA1 which inhibits cdk1/cyclinB1 (responsible for G2 to M progression) via activation of Wee1 kinase which antagonises cdk1/cyclinB1. This study also found that, following damage, BRCA1 activates Chk1 leading to inhibition of the cyclinB1 effector Cdc25c, ultimately resulting in G2/M arrest [69]. This work has now been extended to show that BRCA1 E3 ligase activity may regulate this signalling cascade since BRCA1-mediated ubiquitination leads to degradation of Cdc25c and cyclinB1 thus arresting cells before progression into mitosis [70].

Other BRCA1-containing complexes have also been implicated in cell cycle regulation. BRCA1/BARD1/MRN/CtIP is required for activation of the G2/M checkpoint with knockdown of BRCA1 or CtIP leading to increased accumulation of cells in mitosis following IR [29]. Additionally, loss of RAP80 inhibits BRCA1 recruitment to DNA break sites, leading to defective G2/M checkpoint arrest. RAP80 is likely to act upstream of BRCA1 which mediates G2/M arrest partially via Chk1 activation. Therefore, it is not surprising that depletion of RAP80 also results in decreased Chk1 phosphorylation [71], a finding which suggests that RAP80 does indeed regulate the G2/M checkpoint via the same pathway as BRCA1.

12. Post-translational regulation of BRCA1 in the DNA damage response

Many new pathways involved in the regulation of BRCA1 in response to DNA damage have recently emerged, including ubiquitination, SUMOylation and poly-ADP-ribosylation (PARylation) signalling. However, one of the first recognised signalling pathways integral to BRCA1 function in the DNA damage response was phosphorylation. In response to specific types of cellular insults BRCA1 is phosphorylated at different residues by different PIKKs in a cell cycle-dependent manner. Scully et al first demonstrated the phosphorylation of BRCA1 in response to DNA damage following observations of a mobility shift in BRCA1 gel migration. The phosphorylation of BRCA1 was shown to occur specifically in S-phase but not in G1 [72]. The 3 major PIKKs which activate BRCA1 include ATR, which phosphorylates BRCA1 primarily at Ser1423 in response to UV, and ATM and Chk2 which phosphorylate BRCA1 following IR-induced DSBs, with ATM phosphorylating Ser1387, Ser1423 and Ser1524 and Chk2 responsible for Ser988 phosphorylation [73-76]. Chk2-dependent BRCA1 phosphorylation is directly involved in the regulation of HR with expression of the S988A BRCA1 mutant in HCC1937 unable to restore HR activity in comparison to wild-type BRCA1. Similar effects were observed following expression of a dominant negative Chk2 protein which inhibited Chk2 kinase activity [77]. Phosphorylation of BRCA1 also allows formation of complexes with other phosphorylated proteins through interaction with the BRCT domains of BRCA1. Indeed, BRCA1s BRCT domains have been shown to be indispensable for the tumour suppressor functions of BRCA1 and its ability to promote HR [18].

Over the past few years, much progress has been made on the role of ubiquitination in the DNA damage response and DNA repair pathways. In 2007, a number of independent research

groups identified RAP80 as an ubiquitin-binding protein that localises to DSBs following IR and promotes HR mediated DSB repair [78]. Translocation of RAP80 to regions of damage is dependent on 2 ubiquitin-interacting motifs (UIMs) within its N-terminus, which have specific affinity for K63-linked polyubiquitin chains generated at DSBs. RAP80 also binds to the BRCT domains of BRCA1 and loss of RAP80 results in loss of BRCA1 recruitment to sites of damage thus RAP80 targets BRCA1 to ubiquitinated structures at DSBs. BRCA1 is recruited to sites of DNA damage as part of the BRCA1-A complex which contains RAP80, ABRAXAS, BRCC36, BRCC45, MERIT40 and BARD1 [79]. Formation of RAP80 and BRCA1 positive IRIF is dependent on MDC1 therefore interest developed in the upstream signalling controlling this interaction. Phosphorylation of MDC1 by ATM was shown to recruit two ubiquitin E3 ligases, RNF8 and RNF168 and these proteins were shown to be necessary for RAP80-BRCA1 localisation to DSBs via generation of ubiquitin chains on histone 2A, recruiting the ubiquitin-binding protein RAP80 which binds to the K63 linked ubiquitin chains via it's UIM domains and recruits the rest of the BRCA1-A complex to DSBs (Figure 3). Other members of the BRCA1-A complex such as MERIT40 and BRCC45 are thought to not only facilitate accumulation of BRCA1 to sites of damage, but also stabilise and retain the binding of the BRCA1-A complex to DSB sites [80]. Additionally, BRCC36, which is a zinc-dependent metalloprotease and JAMM (JAB1/MPN/Mov34 metalloprotease) domain containing DUB with specific activity to K63-linked polyubiquitin, also forms a cytoplasmic complex known as BRISC, which contains BRCC36 and 45, MERIT40 and KIAA0157. Depletion of BRISC leads to an increase in BRCA1-A complex formation at sites of DNA damage suggesting a balance exists between the 2 complexes [81].

Another post-translational mechanism, SUMOylation, has also been shown to have a role in this signalling cascade and in localising BRCA1 to nuclear foci in response to IR. Small Ubiquitin-like Modifier (SUMO) isoforms 1, 2 and 3, as well as the SUMO-conjugating enzyme Ubc9, have all been shown to interact with BRCA1 in response to genotoxic stress. This interaction is dependent on the PIAS family of SUMO E3 ligases, namely PIAS1 and PIAS4 which are also found in DNA damage induced foci and when depleted inhibit the localisation of BRCA1 to γH2AX foci. The regulation of BRCA1 localisation by PIAS enzymes is thus indirect and it has been shown that PIAS1 and 4 both regulate upstream factors with PIAS1 depletion showing diminished localisation of RAP80, and PIAS4 regulating RNF168, K63-linked ubiquitination and RAP80 further up the damage signalling cascade [82, 83]. RAP80 contains a SUMO-interacting motif (SIM) as well as UIMs which mediate the interaction between the BRCA1-A complex and SUMO [84]. RAP80 can thus bind ubiquitin and SUMO simultaneously and both domains are required for RAP80 recruitment to DNA damage sites [85] (Figure 3). A further ubiquitin E3 ligase RNF4, which ubiquitinates SUMO chains, has been implicated in the recruitment of RAP80 and BRCA1 to DSBs and suggests that SUMOylation and ubiquitination act in concert in the recruitment of DNA repair factors to DSBs [84].

In 2013, a further post-translational modification, PARylation, was identified in the regulation of BRCA1 recruitment to DNA break sites. γH2AX has a major role in BRCA1 recruitment to foci in response to DNA damage but following the observation that γH2AX depletion inhibited maintenance of BRCA1 at foci but did not inhibit the initial accumulation of BRCA1, Li et al

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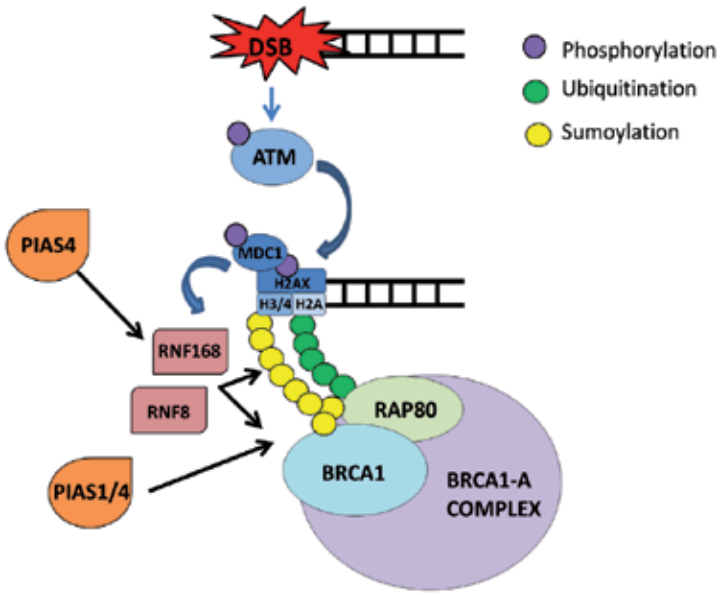

Figure 3. The Role of Post-translational Modifications in the Recruitment of BRCA1 to DNA DSBs. Following formation of DSBs, ATM is activated and phosphorylates H2AX and MDC1 which stimulates RNF8 and RNF168 to generate K63-linked polyubiquitin chains on histone 2A. The UIMs of RAP80 then bind to the polyubiquitin chains and recruit BRCA1 and the rest of the BRCA1-A complex (ABRAXAS, BRCC36, BRCC45, MERIT40 and BARD1). PIAS1 and PIAS4 enzymes directly mediate BRCA1 SUMOylation, which is also required for BRCA1 localisation to DSBs and PIAS4 also indirectly regulates SUMOylation via RNF168. Additionally, RAP80 SIMs bind SUMO and facilitate the SUMOlyation of BRCA1.

postulated that γH2AX was responsible for stabilising BRCA1 at sites of damage but another factor regulated its initial recruitment. This group then showed that the RING finger domain, rather than the BRCT domain of BRCA1, was necessary for recruitment to DNA damage sites, with the BRCT domains of BARD1 also required. Following DNA damage, PARylation is induced and BARD1 was shown to interact with both PAR and the basic unit of PAR, ADP-ribose, through its BRCT domain. BRCA1 and PAR also interact but this interaction is BARD1-dependent. Moreover, BARD1 BRCT germline mutations identified in familial breast cancer patients fail to bind PAR, suggesting this is an important step in the recruitment of BRCA1 and its tumour suppressive functions. In agreement, PARP inhibitors were then shown to suppress early recruitment of BRCA1/BARD1 to DSBs [86].

13. The role of BRCA1 in transcription

The importance of BRCA1 in transcriptional regulation was highlighted by the discovery that the C-terminal domain of BRCA1 forms a complex with RNA polymerase II via interaction with RNA helicase A, which are both members of the core transcriptional machinery [87, 88]. Transcriptional regulation by BRCA1 can occur through either direct or indirect mechanisms. Direct regulation involves BRCA1 binding to the promoter of the gene, however, as BRCA1

does not contain any sequence specific DNA binding domains, specific DNA-binding transcription factors are required to recruit BRCA1 to the promoter regions of target genes [89]. BRCA1 is therefore able to act as either a co-activator or co-repressor of transcription depending on the transcription factor, and other members of the transcriptional complex, to which it is bound. The functional outcome of BRCA1 transcriptional regulation is wide and varied however many BRCA1 regulated genes play a role within the DNA damage response. For example, BRCA1 binds to p53 on the promoter of many p53-regulated genes where it co-activates their transcription. Intriguingly, BRCA1 was shown to selectively induce expression of p53 target genes involved in DNA repair such as p53R2 and Cyclin G2, as opposed to pro-apoptotic genes such as PIDD, PIG and KILLER/DR5. Additionally, BRCA1-mediated transcriptional activation of p53 target genes appears to be particularly important for cell cycle checkpoint control with BRCA1 also transactivating the p53 regulated genes 14-3-3σ and GADD45 both of which are involved in G2/M arrest following DNA damage. BRCA1 also interacts with c-Myc to form a transcriptional repressor complex. This complex binds to the promoters of a large number of genes including basal genes such as psoriasin and p-cadherin down-regulating gene expression [90]. This correlates with the low expression of BRCA1 in basal-like breast cancer.

More recently a comprehensive study by Gorski et al employed microarray analysis to identify almost 1,300 BRCA1-regulated genes in the MCF7 breast cancer cell line and also determined by ChIP-ChIP that BRCA1 was bound directly to promoters of over 600 genes. However, the majority of genes with BRCA1-bound promoters were not transcriptionally regulated by BRCA1 in unperturbed cells although a number of these genes such as MMP3, USP32 and CCL4L2 were commonly altered in response to DNA damaging agents. This implied a model whereby BRCA1 forms an inactive complex on gene promoters in the normal cellular context but in response to DNA damage can regulate the expression of genes involved in DNA repair and/or other DNA damage response processes. This is supported by observations that siRNA mediated knockdown of BRCA1 almost abolished the etoposide-induced activation of CCL4L2 transcription but had little effect on CCL4L2 mRNA expression in untreated cells [89]. Intriguingly, a different study showed that BRCA1 can negatively regulate its own expression by binding to the BRCA1 promoter and inhibiting transcription. However, in response to DNA damage, promoter binding may be inhibited releasing BRCA1 so it can be recruited to sites of DNA damage. The authors suggest that loss of BRCA1 from the promoter then increases BRCA1 transcription in order to replace BRCA1 protein consumed during DNA repair although this hypothesis requires further validation [91].

BRCA1 can also indirectly regulate transcription by binding to chromatin remodelling proteins such as the histone acetyltransferases (HATs) p300 and CBP [92] and the BRG1 and BRD7 subunits of the SWI/SNF chromatin remodelling complex which activates transcription by allowing transcriptional machinery to access DNA. Additionally, BRCA1 can ubiquitinate transcriptional preinitiation proteins which interferes with association of the transcriptional complex and subsequently represses mRNA synthesis [93]. According to Park et al, BRCA1 also binds to the histone deacetylases HDAC1 and HDAC2 and leads to histone deacetylation and transcriptional repression in a SUMO1-dependent manner. Following IR however,

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SUMO1 repression of BRCA1-mediated transcription was alleviated via release of HDAC1 at BRCA1 bound promoters and this enhanced transcriptional activation [94]. BRCA1 mediated transcription is also inhibited by interaction with heterochromatin protein 1γ (HP1γ). Similar to the release of HDAC1 at BRCA1 regulated promoters, HP1γ is removed from the GADD45 promoter in response to etoposide treatment allowing BRCA1 to activate transcription. HP1γ is also attributed a role in recovery from BRCA1-mediated transcription. Following BRCA1 assembly at the promoter, HP1γ is then reassembled at the promoter and once again represses BRCA1 transcription [95]. These studies also propose regulatory mechanisms whereby BRCA1 differentially regulates genes in response to DNA damage.

14. BRCA1 in mRNA splicing

Our group recently characterised a novel function for BRCA1 in the regulation of pre-mRNA splicing of specific DDR genes via an interaction with BCLAF1 following DNA damage [96]. Phospho-peptide pulldown assays carried out with peptides mimicking BRCA1 phosphorylated at serine-1423, revealed that BRCA1 and BCLAF1 interact and further studies went on to show that they only associate following treatment with DNA damaging agents. Further functional assays showed that BCLAF1 depletion, similar to the effects of BRCA1 knockdown, results in increased sensitivity to DNA damage, decreased DNA DSB repair capacity and genomic instability. Furthermore, decreased levels of BCLAF1 failed to sensitise BRCA1 mutant cells to IR suggesting the function of BCLAF1 in DNA repair is dependent on BRCA1.

BCLAF1 was previously identified as a member of a spliceosome complex containing numerous mRNA processing factors. This prompted investigation of the interactions of BRCA1 and BCLAF1 with proteins involved in mRNA splicing. While BCLAF1 is constitutively bound to the core splicing factors PRP8, U2AF64, U2AF35 and SF3B1, BRCA1 binding was only observed following DNA damage and the interaction was abolished when BCLAF1 was depleted. Conversely, BRCA1 was shown to be constitutively bound to the promoters of a large subset of genes (approx. 980) including a large group of DDR genes, such as ATRIP1, BACH1 and EXO1, whereas BCLAF1 and U2AF65 only bound after DNA damage and this was dependent on the presence of BRCA1. This supports a model whereby DNA damage induces BRCA1-mediated recruitment of splicing factors to the promoters of DDR genes via interaction with BCLAF1 and associated mRNA processing factors in order to promote mRNA splicing of these genes. Accordingly, mRNA splicing of BRCA1/BCLAF1 regulated genes is up-regulated following DNA damage, a process which is dependent on both BRCA1 and BCLAF1. Concurrently, protein levels of the target genes tested; ATRIP1, BACH1 and EXO1, were down-regulated following BRCA1 or BCLAF1 depletion after DNA damage. The study then went on to show that proteins encoded by targets of the BRCA1/BCLAF1 complex are turned over more rapidly following DNA damage and that the BRCA1/BCLAF1 mediated up-regulated splicing of these genes following DNA damage, functions to maintain the stability of these proteins, presumably by promoting the processing and subsequent stability of their transcripts.

This suggests that as well as playing a direct role in the repair of DNA DSBs, BRCA1 also regulates the transcription and mRNA processing of a large group of genes, many of which are involved in the DDR, including core DNA repair genes/proteins such as ATRIP, BACH1 and EXO1, in order to maintain the fidelity of the DNA damage response machinery.

15. BRCA1 and ubiquitination

The role of the E3 ubiquitin ligase activity of the BRCA1/BARD1 heterodimer in DNA repair and indeed the biological significance of this function of BRCA1 in general remains elusive. Ubiquitination involves the conjugation of the ubiquitin moiety to its target protein by the formation of a peptide bond between the C-terminal glycine 76 residue of ubiquitin and a lysine residue of the substrate [97]. Monoubiquitination can function as a form of post-translation modification that alters the function of a protein or it can target proteins for lysosomal degradation [98]. However, there is a further degree of complexity to the modification of proteins by ubiquitin owing to the fact that polyubiquitin chains can also be formed and the signal transduced as a consequence depends not only on the number of ubiquitin moieties added, but also on the lysine residue to which the ubiquitin molecule is attached. Since ubiquitin contains 7 lysine residues, there are several conformations a ubiquitin chain can exhibit, each conferring a unique signal to the conjugated substrate [99, 100]. For example, a K48-linked chain consisting of at least 4 ubiquitin adducts typically targets the protein for proteasomal degradation whereas a K63-linked chain may signal a conformational change in protein structure, form a docking site or transduce a signal in another way. K63-linked ubiquitin chains have been reported to be involved in DNA repair, activation of signalling pathways and protein trafficking [97]. The conjugation of ubiquitin to 'tag' proteins involves a cascade of 3 classes of enzymes; E1 (ubiquitin-activating enzyme), E2 (ubiquitin-conjugating enzymes) and E3 (ubiquitin-protein ligase enzymes). It is generally accepted that E3 enzymes such as BRCA1/BARD1 are responsible for specific substrate recognition of the protein [101]. In fundamental terms, the process of ubiquitination begins when E1 activates the C-terminal glycine of ubiquitin in an ATP-dependent reaction. E2 transfers the activated ubiquitin from E1 to E3, which is bound to the substrate protein. E3 then facilitates the formation of an isopeptide bond between ubiquitin and an internal lysine residue of the substrate [102].

The BRCA1/BARD1 E3 ligase was first shown to polyubiquitinate in a K6-linked manner, which does not target proteins for proteasomal degradation however, the biological significance of the K6-linked ubiquitination is unknown. Nevertheless, BRCA1/BARD1 has been shown to autoubiquitinate itself via K6 linkage and this enhances the ubiquitin ligase activity of the complex [103, 104]. Although most E3 ligases only conjugate with one E2 enzyme, BRCA1/BARD1 has been shown to interact with at least 8 different E2 enzymes which determine mono- or polyubiquitination and also the linkage specificity of the ubiquitin chains, therefore BRCA1/BARD1 does not only induce the originally identified K6-linkage mediated via the UbcH5c E2 enzyme [105]. One of the biggest challenges in the BRCA1 field has been the identification of bona-fide BRCA1 ubiquitination targets. This is due to the fact that BRCA1/BARD1 is a relatively promiscuous ubiquitin E3 ligase when studied *in-vitro.* As a result numerous *in-vitro* ubiquitination targets have been identified, including H2AX, RNA Pol II,

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CtIP, ERα, γ-tubulin and NPM1, however few bona fide BRCA1/BARD1 substrates have been confirmed *in-vivo* [106].

Nevertheless, a number of pathogenic mutations in the RING finger of BRCA1/BARD1 such as C61G and C64G have been identified in cases of familial breast cancer suggesting the ubiquitin ligase function of BRCA1/BARD1 may be important for its tumour suppressor functions. Additionally, many of these studies have reported that these mutations result in increased genomic instability, again suggesting that the ubiquitin ligase function of BRCA1 is important for its role in tumour suppression. However, controversy exists over whether this is the case or not. Many studies have employed cells expressing the synthetically engineered RING finger mutation I26A. This mutation inhibits binding of BRCA1 to E2 enzymes but doesn't alter the formation of BRCA1/BARD1 complex therefore allowing separation of the functions of BRCA1 due to ubiquitin ligase activity from those dependent on BARD1 association. Shakya et al have shown that in response to DNA damage, mice harbouring the BRCA1 I26A mutant exhibit similar phenotypes to mice expressing wildtype BRCA1 with comparable levels of chromosomal abnormalities, mitomycin C resistance and ubiquitin foci at sites of DNA damage [18]. Furthermore, Reid et al reported similar findings and extended their study to show the I26A mutation had little effect on Rad51 recruitment at damage-induced foci or on levels of HR activity. However, ubiquitin ligase deficient cells exhibited increased numbers of chromosomal abnormalities following treatment with mitomycin C compared to BRCA1 wildtype ES cells, although the chromosomal aberrations were much more pronounced in cells with deletions of BRCA1 exon 11 [107]. These studies suggest BRCA1 E3 ubiquitin ligase activity is dispensable for its role in DNA damage repair.

On the contrary, several findings support a role for BRCA1-dependent ubiquitination in DNA repair. First, CtIP ubiquitination was shown to be specifically dependent on BRCA1 following DNA damage and this was required for localisation of CtIP to damage-induced foci and association with chromatin as well as G2/M checkpoint regulation [108]. More recently, Shabbeer et al have shown that while re-expression of wildtype BRCA1 rescues cell survival in cell line models with decreased levels of functional BRCA1, introduction of the I26A BRCA1 mutant or the C61G RING domain mutation failed to increase cell survival. This study also demonstrated BRCA1-dependent ubiquitination of cell cycle proteins Cyclin B and Cdc25c in response to IR and HU, in a K48-linked manner via the E2 enzyme UbcH1. This tags these proteins for proteasomal degradation which enables efficient G2/M arrest following DNA damage [70]. Furthermore, mouse models have shown that introduction of the C61G mutation increases genomic instability to a similar level of that observed in BRCA1 null mice and *in-vitro* experiments showed low levels of HR activity in C61G mutant cells. However, in comparison to BRCA1 null mice, mice expressing the BRCA1 C61G mutation displayed a greater number of DNA damage-induced Rad51 foci and γH2AX-positive cells and were less responsive to PARP inhibition indicative of residual HR activity despite loss of ubiquitin ligase activity [109]. Thus, although there are conflicting reports on the role of the BRCA1/BARD1 ubiquitin E3 ligase in DNA repair pathways *per se*, evidence seems to suggest BRCA1/BARD1 E3 ligase activity may be important in HR and cell cycle checkpoint regulation after DNA damage, but the significance of this function remains an active area of research.

16. BRCA1 in tissue-specific tumourigenesis

As mentioned earlier, the majority of BRCA1 mutant tumours do not express the oestrogen receptor. Despite this, the notion that oestrogen may contribute to the development of BRCA1-related tumours is supported by the fact that the risk of breast cancer in BRCA1 mutation carriers is reduced by approx. 50% following oophorectomy, which decreases circulating oestrogen levels. Additionally, pregnancy has been reported to increase the risk of breast cancer in BRCA1 carriers, in contrast to non-carriers for whom pregnancy is protective. This suggests a potential ERα-independent mechanism by which oestrogen may promote tumourigenesis. One such mechanism may be through the conversion of oestrogen to semi-quinone and quinone forms during normal oestrogen metabolism, a process which also results in the release of free radicals. Indeed, our group has recently demonstrated that exposure to the predominant endogenous oestrogen, estradiol (E_2), or its metabolites 2-hydroxyestradiol (2-OHE_2) or 4-hydroxyestradiol (4-OHE_2) induces DNA DSBs in breast cell lines [110]. Additionally, depletion of BRCA1 leads to decreased repair of DSBs generated by treatment with oestrogen metabolites and results in genomic instability marked by increased levels of chromosomal aberrations. Interestingly, cells with decreased BRCA1 expression also exhibited elevated numbers of DSBs at early time-points following 2-OHE_2 and 4-OHE_2 exposure which could not be attributed to a repair defect. Since BRCA1 was previously shown to mediate the transcriptional repression of the CYP1A1 gene, which encodes an enzyme responsible for metabolising androgens to bioactive oestrogens, it appeared plausible that loss of BRCA1 may enhance the production of oestrogen metabolites [111]. Indeed this study then went on to confirm that BRCA1 loss leads to up-regulation of oestrogen metabolising enzymes CYP1A1 and CYP3A4 and down-regulated expression of the detoxification enzyme NQO1 leading to an increase in the production of 2-OHE_2 and 4-OHE_2. Thus BRCA1 has a role in repressing the production of oestrogen metabolite induced DSBs as well as mediating the repair of DSBs in response to 2-OHE_2 and 4-OHE_2 exposure. This finding is particularly significant in explaining why BRCA1 mutation carriers predominantly develop tumours in hormonal tissues such as the breast or ovaries where levels of oestrogen are particularly high [110].

17. Role of BRCA2 in the DNA damage response

Like BRCA1, BRCA2 was identified as a breast/ovarian cancer susceptibility gene by linkage analysis and to date approximately 2000 distinct BRCA2 mutations, polymorphisms or variants have been catalogued in BIC. In comparison to BRCA1, much less is known regarding the functions of BRCA2 owing mainly to the large size of the BRCA2 protein (3418 amino acids), which has been difficult to express and/or purify, hampering functional studies. Additionally, the structure of BRCA2 shares limited homology to other proteins and it's most distinguishing feature is the presence of conserved BRC repeats which are repeated regions of approximately 30 amino acids [112], the number of which varies by species. Human BRCA2 contains eight BRC domains which can mediate interaction with 6-8 Rad51 molecules [113]. BRCA2 also contains a DNA binding domain (DBD) capable of associating with both ssDNA and dsDNA

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and it was the combination of these two properties (Rad51 and DNA binding) that proposed a role for BRCA2 in HR [112, 114, 115]. BRCA2 also contains an N-terminal region, which interacts with PALB2 and is also involved in transcriptional activation and a C-terminal region which can bind multimeric Rad51, in comparison to BRC domains which interact with monomeric Rad51 [116] (Figure 4).

Figure 4. Structural Features and Binding Partners of BRCA2. Schematic diagram of BRCA2 and the functional domains mediating important protein interactions including; PALB2 binding to the RING domain, binding of Rad51 monomers and Rad51 filaments at BRC repeats 1-4 and 5-8 as well as polymeric Rad51 binding at the C-terminus, which is dependent on phosphorylation of Serine3291 by CDK. Also shown are the DNA binding domains and DSS1 binding region which allow BRCA2 to recruit Rad51 to sites of RPA-coated ssDNA thereby promoting HR.

Early phenotypic studies demonstrated that BRCA2 depletion led to increased sensitivity to DNA damaging agents, impaired homologous recombination and decreased formation of Rad51 foci following DNA damage [117, 118]. More recent investigations, following the purification of the full-length BRCA2 protein, have shed light on the mechanism of BRCA2 in HR showing that BRCA2 is responsible for Rad51 nucleation and filament formation by overcoming the inhibitory effects of RPA to allow Rad51 binding to ssDNA at DSB sites [119]. This process is enhanced by Deleted in Split Hand/Split Foot protein 1 (DSS1), which associates with BRCA2 to promote Rad51 nucleofilament formation potentially through stabilisation of the BRCA2 protein [120, 121]. Not only does BRCA2 mediate Rad51-ssDNA interaction but it also inhibits Rad51 binding to dsDNA, which impedes Rad51-mediated DNA strand exchange. Furthermore, BRCA2 can stabilise the Rad51 nucleoprotein filaments by blocking Rad51 ATP hydrolysis, which maintains the active ATP-bound form of Rad51-ssDNA necessary for efficient HR [114].

Carreira et al have demonstrated that the BRC repeats are required for the function of BRCA2 in Rad51-mediated HR and have investigated the functions of individual BRC domains. While all BRC domains bind Rad51 and facilitate the formation of Rad51-ssDNA nucleofilaments, they function via slightly different mechanisms, with BRC domains 5-8 having higher affinity for Rad51-ssDNA filaments and BRC domains 1-4 preferentially binding free Rad51 as well as mediating the inhibition of ATPase activity and preventing binding to dsDNA in order to stimulate DNA strand exchange [122, 123]. In contrast to BRC binding of Rad51 monomers, the C-terminal region of BRCA2 binds only oligomeric Rad51 and the role of this interaction in HR is more controversial than that of BRC repeats. Specifically, serine 3291 of BRCA2 is required for Rad51 association and this residue is phosphorylated by CDK in a cell cycle dependent manner, which abolishes the BRCA2-Rad51 interaction. In response to DNA damage, S3291 phosphorylation is reduced and the affinity of the BRCA2 C-terminal region

for Rad51 is increased which stabilises the Rad51 nucleoprotein filament and may even protect against nucleofilament disassembly. Thus a model is proposed in which IR stimulates ATM-dependent inactivation of CDK, which maintains S3291 in a non-phosphorylated form, promoting HR by allowing the formation and stabilisation of Rad51-ssDNA. On the contrary, it has been suggested that S3291 phosphorylation, which is detected at the highest levels in G2/M, could be involved in the termination of HR, therefore allowing progression into mitosis following efficient repair in S and G2 [124-126].

The CAPAN-1 pancreatic cancer cell line is commonly used for studies of BRCA2 function, as it contains a naturally occurring BRCA2 mutation in which one allele is lost and the other contains the 6174delT frameshift mutation resulting in a truncated BRCA2 protein with loss of 1416 amino acids at the C-terminus. Consequently, CAPAN-1 cells show defective Rad51 foci formation in response to DNA damage and thus decreased HR activity and hypersensitivity to PARP inhibitors [127] which are currently in clinical trials for the treatment of BRCA1/2 deficient tumours (discussed in next section). Intriguingly, Edwards et al were able to produce PARPi resistant clones following treatment of CAPAN-1 cells with the PARPi KU0058948. Edwards et al. then went on to show that many of these cell line clones contained further deletions within the *BRCA2* gene, resulting in restoration of the BRCA2 open reading frame. These restored BRCA2 ORFs always contained the N-terminus of BRCA1 fused to the C-terminus, however, they contained large deletions of other BRCA2 regions thought to be functionally important such as the BRC repeats and the DBD. Despite the loss of these domains, the PARPi resistant clones regained the ability to localise Rad51 to nuclear foci and to repair cells by HR [128]. This adds confusion to the significance of specific BRCA2 regions and suggests possible redundancy of BRCA2 domains. Similarly, Siuad and colleagues demonstrated that deletion of the entire BRCA2 DBD had minimal effects on HR providing PALB2 was present. However, when PALB2 was not bound, mutation of the DBD significantly abrogated HR. Additionally, mutation of the DSS1 binding region within the DBD also decreased HR despite tolerance of the DBD deletion. Additionally "micro-BRCA2" constructs less than 20% of full length BRCA2 were also sufficient for HR providing the C-terminus was intact [116]. Together these studies suggest plasticity of the BRCA2 protein in enhancing HR and also indicate the functional importance of the C-terminal region of the protein. This is in contrast to a number of other studies which claim the BRCA2 C-terminus may be dispensable for HR, therefore the significance of BRCA2 domains in HR requires further clarity [129, 130].

In addition to its role in HR, BRCA2 also maintains genomic integrity by preventing the Mre11 mediated degradation of stalled replication forks. A recent study conducted by Schlacher et al confirmed in a number of mammalian cell lines that the absence of BRCA2 led to shortened nascent DNA strands at stalled replication forks in response to hydroxyurea (HU). Cells with mutations in the C-terminal Rad51 binding region of BRCA2 were defective in protecting nascent DNA strands from Mre11 mediated fork degradation thus stabilisation of Rad51 by the C-terminal region of BRCA2 is essential in the maintenance of stalled replication fork stability. Importantly, degradation of stalled replication forks due to loss of BRCA2 had little effect on cell survival but significantly increased chromosomal aberrations, indicating another mechanism whereby BRCA2 maintains genomic stability. This finding also has clinical

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implications as drugs which elicit replication fork stalling such as HU may actually increase the mutagenic potential of BRCA2 deficient cells and thus may be contraindicated in these patients [129-131].

BRCA2 can also function independently of Rad51 to promote genomic stability through a role in the maintenance of G2/M checkpoint arrest after DNA damage. Depletion of BRCA2 or PALB2 leads to premature recovery of the G2/M checkpoint via Aurora A/PLK1 activation, causing unrepaired cells to enter mitosis. Thus, BRCA2 and PALB2 halt activation of Aurora A/BORA/PLK1 until DNA damage is repaired and it is appropriate for cell cycle progression to occur [132]. A role for BRCA2 in transcriptional regulation and chromatin remodelling has also been reported. In 1997, Milner and colleagues showed that a region of BRCA2 exon 3 fused to the GAL4 DNA binding domain stimulated transcriptional activity in U2OS cells [133]. It was later demonstrated that BRCA2 coactivates androgen receptor (AR) mediated transcription via binding to GRIP1 and P/CAF1 both of which possess histone acetyltransferase activity, also suggesting a role for BRCA2 in chromatin modulation. AR signalling is anti-proliferative and it was therefore postulated that decreased AR-mediated transcription following loss of BRCA2 may contribute to tumourigenesis in BRCA2-linked cancer [134]. On the contrary, the nuclear protein EMSY binds to exon 3 of BRCA2 and silences the transcriptional activation function of BRCA2. EMSY also associates with two additional chromatin remodelling proteins, HP1β and BS69 and localises to sites of DNA repair, potentially implicating BRCA2 in the modulation of access to chromatin during repair. EMSY is overexpressed in a number of sporadic breast and ovarian cancer cases and it has been postulated that increased EMSY expression may emulate BRCA2 mutant familial cancers but this requires further validation [135].

As mentioned earlier another BRCA2 interacting protein, PALB2, is an important mediator of BRCA2 function within HR mediated DSB repair. PALB2 was first identified as a nuclear interactor of BRCA2 following mass spectrometric analysis of protein bands immunoprecipitated in HeLa lysates using a BRCA2 antibody [136]. PALB2 is functionally similar to BRCA2 in several ways. Biallelic mutations in both BRCA2/FANCD1 and PALB2/FANCN have been identified as the cause of Fanconi Anaemia complementation groups D1 and FA-N, respectively. There are now 13 different subtypes of FA attributed to mutations in 12 unique genes but the BRCA2 and PALB2 related FA groups differ from the other identified subgroups, exhibiting a more severe phenotype and increased incidence of solid tumours such as Wilms tumours and medulloblastomas at an early age, suggesting a possible common functionality [137]. Indeed, PALB2 colocalises with BRCA2 in nuclear DNA repair foci and depletion of PALB2 leads to decreased Rad51 localisation to foci, abrogated HR activity and increased sensitivity to the DNA crosslinkers such as MMC [136]. Additionally, 3 of 8 mutations within the PALB2-binding N-terminal region of BRCA2 were shown to disrupt the BRCA2-PALB2 interaction and only these 3 mutations exhibited defective HR when introduced into BRCA2 deficient V-C8 cells [138]. As discussed earlier, PALB2 and BRCA2 recruit BRCA1 to sites of DSBs and promote Rad51 loading and HR. However, the BRCA2/PALB2 complex has also been shown to interact with DNA polymerase η at DNA DBSs induced by collapsed replication

forks and this complex is required for completing efficient HR via initiation of DNA synthesis following Rad51-dependent formation of a D-loop structure [139].

Additionally, mutations within PALB2 itself have now been identified in 0.4 - 4.8% of familial breast cancer patients depending on the population examined [140, 141]. Initial studies estimated that pathogenic PALB2 mutations increase breast cancer risk by ~2.3 fold [142, 143]. However, subsequent analysis of PALB2 mutation carriers estimate that the relative breast cancer risk is approximately 17.6 for 20 to 39 year olds and 8.7 for mutation carriers between 40 and 79 years. The same study found PALB2 mutation carriers with no family history had a 33% risk of breast cancer by 70 years of age whereas two or more cases of early onset breast cancer amongst first degree relatives increased this risk to 58%, comparable to the risk associated with BRCA2 mutation [144]. Therefore, PALB2 is now considered a *bona-fide* breast cancer susceptibility gene.

18. Clinical and therapeutic implications of BRCA1/2 dysfunction

As this chapter reveals, BRCA1 and BRCA2 are involved in the regulation of various DNA damage response and DNA repair pathways at the cellular level, but what are the clinical implications of this? The majority of BRCA1 mutant tumours are of triple negative breast cancer subtype (75%), present at young age (less than 50 years) and typically have a poor prognosis due to lack of targetable receptors. BRCA1 dysfunction has also been noted in over 30% of sporadic breast and ovarian cancers marked by low expression of BRCA1. On the contrary, BRCA2 mutant tumours are normally ERα positive. Although the DNA repair defects in BRCA1/2 mutation or dysfunction increase genomic instability and are associated with breast and ovarian cancer susceptibility, the same repair defect may also be exploited in the treatment of BRCA1/2-related cancers. Breast and ovarian cancer patients harbouring BRCA1/2 mutations are highly sensitive to treatment with platinum compounds, such as cisplatin and carboplatin, as well as alkylating agents as these agents cause ICLs, which are normally repaired by BRCA-dependent HR. On the other hand, BRCA1 mutant tumours are relatively resistant to treatment with taxanes and investigations in mouse models have suggested this may be due to an up-regulation of the drug efflux transporter P-gP, although this is yet to be confirmed in human BRCA1-mutant tumours [145-147]. Based on this it is clear that knowledge of the BRCA status of a tumour may be used to inform treatment regimes.

While platinum agents are relatively effective in treating BRCA-related cancer cells, they also cause a high level of toxicity in normal cells, resulting in severe side effects and intolerance in many patients. Thus the identification of PARP inhibitors as a potential treatment specifically targeting BRCA deficient cells was welcomed [148-150]. Poly(ADP-ribose) polymerase (PARP) is an enzyme responsible for catalysing the transfer of ADP-ribose to target proteins, including itself and many histone proteins, at single strand break sites within DNA. Poly-ADP-ribosylated (PARylated) proteins form docking sites for a number of BER proteins such as XRCC1, DNA ligase III and DNA polymerase β thereby mediating their recruitment to sites of damage [151]. Therefore, when PARP is inhibited,

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SSBs are unrepaired which results in stalled replication forks during S-phase of the cell cycle. As mentioned above the rescue of stalled forks requires the HR machinery including BRCA1 and BRCA2. Because of this, inhibition of PARP in the absence of BRCA1/2 leads to accumulation of DNA DSBs during S-phase (due to collapse of stalled replication forks), resulting in synthetic lethality of these cells. It was therefore initially thought the effectiveness of PARPi's in BRCA-associated cancer was due to the synthetic lethality caused by the lack of HR to repair excessive damage caused by loss of PARP. Accordingly, PARP inhibitors have been well tolerated because normal cells with at least one functional allele of BRCA1/2 can overcome the effects of PARP inhibition [152]. Other mechanisms of action for the effectiveness of PARP inhibitors have since been demonstrated. For example, PARP inhibition has been shown to increase mutagenic NHEJ in HR deficient cells via a DNA-PK-dependent pathway, with inhibition of various NHEJ factors able to abrogate sensitivity to PARPi in cells lacking BRCA1, BRCA2 or ATM. Based on this, it has been suggested that up-regulation of the NHEJ pathway may mediate cytotoxicity of PARPi in an HR-deficient setting [153]. Another model of sensitivity to PARP inhibition involves trapping PARP1 and PARP2 on the damaged DNA causing cytotoxic lesions that cannot be repaired in the absence of an efficient HR pathway. PARP-DNA lesions had a greater effect on cell viability than the accumulation of SSBs due to inactivation of PARP suggesting this may be the primary mechanism of cell death in HR-deficient cells following PARP inhibition [154].

Phase I and II clinical trials treating BRCA mutant patients with the PARPi olaparib have in general been successful with one study showing clinical benefit in 12 out of 19 patients with BRCA-related breast, ovarian or prostate cancer [155, 156]. However, the progression of PARP inhibitors into the clinic suffered a setback in 2011 when Phase III trials investigating the use of iniparib in TNBC failed to prolong the survival of study participants [157]. Although numerous studies have since confirmed that iniparib did not actually inhibit PARP activity and had a different mechanism of action from the other PARP inhibitors currently in trials, the failure of iniparib did hamper the advancement of PARP inhibitors [158, 159]. Nevertheless, Phase III clinical trials are now underway to determine whether PARP inhibitors should be approved in a combination regime with platinum agents or even as single agents for the treatment of BRCA deficient breast and ovarian cancer patients. There are also trials underway in other cancer types with mutations or dysfunction of DNA repair genes. Thus it is hoped that PARP inhibitors will not only be an effective treatment for BRCA-linked breast and ovarian cancers but also for other cancer types with defective HR pathways [160].

19. Conclusion

In conclusion, BRCA1 and BRCA2 both have essential roles in numerous DNA repair pathways and the importance of efficient DNA repair mechanisms is illustrated by the dysfunctional repair observed when BRCA1 or BRCA2 are mutated leading to genomic instability and thus susceptibility to breast and ovarian cancer. While BRCA1 is a multifunctional protein mediating HR, NHEJ, SSA, ICL repair and cell cycle regulation via a variety of mechanisms including

transcriptional regulation, ubiquitination and mRNA splicing, the role of BRCA2 is more straightforward, facilitating Rad51 loading to ssDNA to promote HR as well as protecting stalled replication forks from degradation. Different binding partners of both proteins can modulate their function in repair pathways and therefore the identification of novel interactors of BRCA1 and BRCA2 is likely to shed further light on their mechanism of action. Additionally, the identification of these interactors may identify novel therapeutic targets for the treatment of BRCA-associated breast and ovarian cancers. Furthermore, the regulation of BRCA1 in response to DNA damage is becoming increasingly complex as more signalling pathways such as SUMOylation and PARylation have been shown to mediate recruitment of BRCA1 to DSBs with the intricate control of BRCA1 potentially reflecting it's prominent and varied role in DNA repair. Recent studies have also demonstrated the significance of specific regions of BRCA1 and BRCA2 in mediating different repair functions and therefore it is likely that not all cancer-associated mutations within these genes affect repair in the same way and further investigation of the role of different mutations may be useful, particularly in the case of BRCA2 where PARPi resistant clones have arisen still harbouring BRCA2 mutations albeit different from the original BRCA2 mutation. However, in general PARP inhibitors so far appear promising for the treatment of some BRCA1/2 mutant tumours and provide an example of how DNA repair defects, normally harmful to the cell, may actually be utilised for treatment benefit. Overall, BRCA1/2 mutations lead to highly dysfunctional DNA repair pathways, the catastrophic effects of which are revealed by phenotypic investigations demonstrating accumulation of genetic mutations and chromosomal instability, ultimately predisposing to cancer.

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Chapter 8

The Fanconi Anemia Pathway of DNA Repair and Human Cancer

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Additional information is available at the end of the chapter

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1. Introduction

The accurate repair of DNA damage and the maintenance of genomic integrity is a fundamental property of every cell. Amongst the different classes of DNA damaging agents, DNA interstrand crosslinks (ICLs) represent a class of DNA lesions wherein the two strands of DNA get cross-linked by covalent bonds. Unrepaired, such cross-linking will impede the progress of critical processes like DNA replication and transcription, resulting in a genomic instability-associated disorder called, Fanconi Anemia (FA).

Fanconi anemia is a rare genetic disorder that occurs at the frequency of 1 in 1:100,000 births. The clinical features of Fanconi anemia were first described by the Swiss paediatrician, Guido Fanconi, in the year 1927. The disease is characterized by low birth weight, developmental defects like congenital limb deformities, hearing failure, skin hyperpigmentation, gastrointestinal abnormalities and haematological defects like aplastic anemia, myelodysplastic syndrome (MDS) and bone marrow failure (BMF). During their life time, Fanconi anemia patients have a very high risk for developing leukemias and solid tumors, due to underlying genomic instability.

At the cellular level, cells deficient in the Fanconi anemia pathway show acute sensitivity to DNA interstrand crosslinking agents and the accumulation of chromosomal aberrations. This chapter will focus on the molecular mechanism underlying the Fanconi anemia pathway of ICL repair and the role this pathway plays in preventing human cancer.

2. Molecular control of ICL repair by the FA pathway

The FA pathway of DNA repair is activated when the DNA replication forks are stalled and they encounter an interstrand crosslinked DNA in the S phase of cell cycle. Human FA is caused by mutations in 16 FA gene products, identified so far. A central molecular event in the FA pathway is the monoubiquitination of FANCD2 at lysine 561 and FANCI at lysine 523, a process mediated by the FA core complex. The FA core complex (FANCA, FANCB, FANCC, FANCE, FANCF, FANCG, FANCL and FANCM) is a multi-subunit ubiquitin ligase. Mutations in any of the constituting subunits cause impaired ICL repair and FA. Upon monoubiquitination, FANCD2 recruits the nucleases, FAN1 and SLX4 (FANCP), which together with XPF/ERCC4 (FANCQ) incise on either side of the crosslink. Finally, homologous recombination (HR) repair by the DNA repair proteins FANCD1 (BRCA2), FANCN (PALB2), FANCJ (BRIP1) and FANCO (RAD51C) in conjunction with translesion repair restore the fidelity of the original DNA double helix. Apart from above described bonafide FA genes, there is a growing list of FA-core complex associated proteins, such as FAAP20, FAAP24, FAAP100, MHF1, MHF2, USP1, and S phase checkpoint proteins like ATR and CHK1. As yet, mutations in such accessory proteins have not been uncovered in FA patients, but, they are nevertheless important in controlling the various steps of the repair process. *In the first section of this chapter, the molecular regulation of the Fanconi anemia (FA) pathway of DNA repair will be discussed.*

2.1. Replication-coupled interstrand crosslink repair

DNA interstrand crosslinking occurs when nucleotides on opposing DNA strands undergo a covalent linkage. The resultant crosslinked DNA poses a physical impediment for the movement of the DNA replication and transcription apparatus. Hence, ICL agents are highly toxic and it is estimated that even 1-2 crosslinks can be lethal in repair-deficient yeast strains [1]. Chemicals such as mitomycin C, cisplatin, diepoxybutane, metabolic by-products like acetaldehyde, formaldehyde, malondialdehyde and acrolein containing electrophilic groups are capable of causing DNA interstrand crosslinking.

In seminal work by Raschle *et al*, an elegant cell-free repair assay with Xenopus egg extracts was used to elucidate the exact order of events during ICL repair [2]. A plasmid containing a crosslink was mixed with Xenopus egg extracts and DNA repair was monitored. In this model, two opposing forks collide with crosslinked DNA to activate ICL repair. It was observed that as the leading strand of a replication fork approaches a crosslink, it pauses at the distance of 20-40 nucleotides from the crosslink due to a block posed by the MCM helicase that traverses ahead of the moving replication fork. The 5′ end of the lagging strand also stalls at variable distance from the crosslink. The nascent leading strand that has paused at 20-40 nucleotides then advances to within one nucleotide distance of the ICL after the eviction of the stalled-MCM helicase by BRCA1 [3]. Dual incisions follow that cut on either end of the crosslinked oligonucleotide, to mediate the ‘unhooking’ step. This reaction then generates two sister chromatids with different kinds of DNA lesions: one sister chromatid contains the crosslink whereas the other sister chromatid has a break. The sister chromatid carrying the crosslink is repaired by translesion polymerases that bypass the crosslink. On the other hand, the broken

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sister chromatid is repaired by RAD51-dependent strand invasion into the repaired DNA strand.

This cell-free assay was used to demonstrate that the FA core complex-dependent FANCI/FANCD2 monoubiquitination is a critical step for mediating the nucleolytic incisions and translesion synthesis past the lesion. Also, it was shown that recombination acts downstream of the FANCI/FANCD2 loading step [4, 5].

DNA ICL repair can be subdivided mechanistically into the following key steps: Recognition of lesion, FA core complex-mediated FANCI/FANCD2 monoubiquitination, crosslink unhooking, lesion bypass by translesion synthesis of the sister chromatid carrying the excised nucleotide and HR repair of broken sister chromatid (Figure.1 and Figure.2).

2.2. Recognition of lesion

A stabilized DNA replication intermediate consisting of an ICL-bound stalled-replication fork acts as that activating signal for the FA pathway of repair [6]. Since most of the FA core complex proteins lack recognizable functional domains, the proteins involved in FA pathway activation and lesion recognition remained a mystery until the discovery of FANCM.

One of the first proteins capable of recognising the ICL-bound stalled-replication fork is the FANCM-FAAP24-MHF1 complex. FANCM is a large 230 kDa protein, which complexes with FAAP24 through its C-terminal domain. The FANCM-FAAP24 complex binds to synthetic substrates like ssDNA, splayed arm, and 3′-flap DNA structure, which mimic intermediates during replication or repair [7]. FANCM has a functional DEAH-type helicase domain with DNA-dependent ATPase activity and it translocates on dsDNA in an ATPase-dependent manner to promote the migration of Holliday branches and replication fork branch points [8] [9] [10]. FANCM-dependent translocation stimulates the accumulation of RPA, the ssDNA binding protein [11]. In turn, RPA recruitment is required for ATR loading and activation of the ATR-dependent checkpoint. Thus, the depletion of FANCM or FAAP24 causes defective ATR-mediated checkpoint signalling leading to impaired CHK1, p53 and FANCE phosphorylation after DNA damage [12] [13]. FANCM and FAAP24 also regulate FA core complex relocalization to chromatin during ICL repair. Hence, the depletion of FANCM or FAAP24 with SiRNA cause impaired FANCD2 monoubiquitination and FANCD2 focus formation [14].

Unlike the other FA core complex proteins, FANCM and FAAP24 are constitutively localised on chromatin through their interaction with the histone-fold containing complex MHF1 and MHF2. The MHF complex stimulates DNA binding and replication fork remodelling by FANCM. The depletion of MHF1 or MHF2 caused the destabilization of FANCM, impaired chromatin localization of the FA core complex, reduced FANCD2 monoubiquitination and focus formation and resulted in the accumulation of chromosomal aberrations [15] [16]. Recent structural studies have revealed that the MHF complex senses branched DNA by binding to a pair of crossover DNA duplexes providing mechanistic insights on how the MHF complex stimulates FANCM translocation activity at such a DNA structure [17].

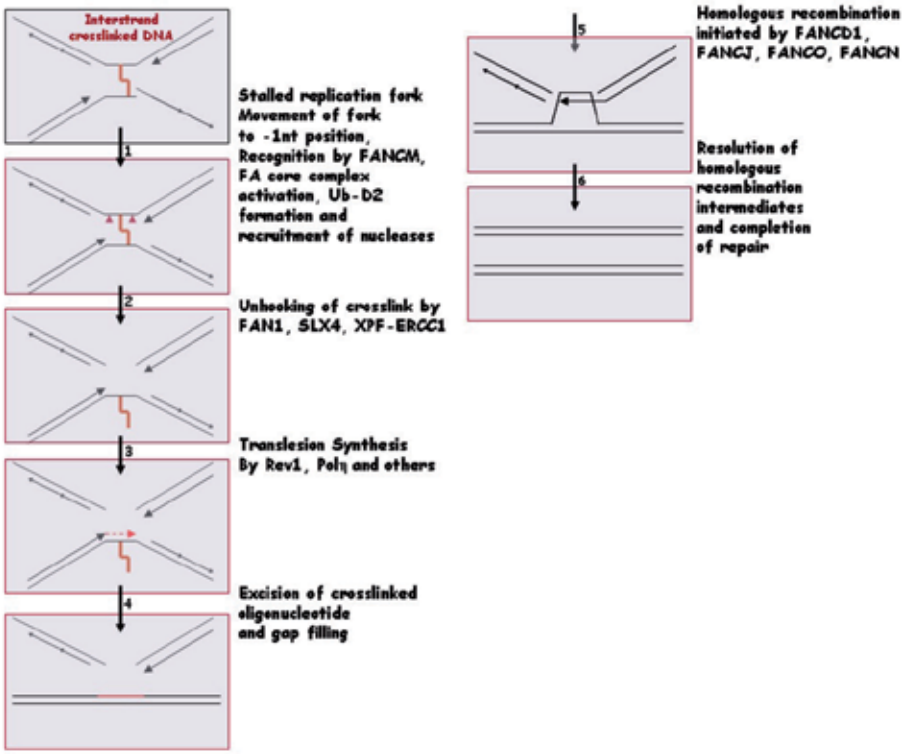

Figure 1. Major steps of the DNA Interstrand crosslink repair pathway

2.3. Role of ATR in ICL repair

ATR or Ataxia telangectasia mutated related is a master regulator of the S-phase checkpoint. In response to different classes of DNA replication stresses, ATR activation is dependent on the presence of RPA-coated single-stranded DNA (ssDNA) containing regions [18]. ATR coordinates checkpoint activation with the completion of DNA repair by phosphorylating CHK1. Defective ATR function results in crosslinker hypersensitivity, impaired FANCD2 monoubiquitination and the accumulation of massive genomic instability in the form of radial chromosomes [19, 20].

Many FA proteins undergo phosphorylation by ATR and these phosphorylation events are necessary for a functional FA pathway. ATR phosphorylates FANCD2 at threonine 691 and serine 717, at clustered SQ/TQ (serine/threonine-glutamine) motifs on FANCI and at ser 1449 on FANCA. In response to replication stress, CHK1 is also activated by ATR and it directly phosphorylates FANCE subunit at threonine 346 and serine 347. All these phosphorylation events are essential for FANCD2 monoubiquitination [21-24]. Interestingly, FANCM which is implicated in ATR activation, itself undergoes ATR-dependent ser 1025 phosphorylation. FANCM phosphorylation at this site controls integrity of the FA pathway, prevents premature mitotic entry and is required for ATR-dependent checkpoint activation [25]. These data

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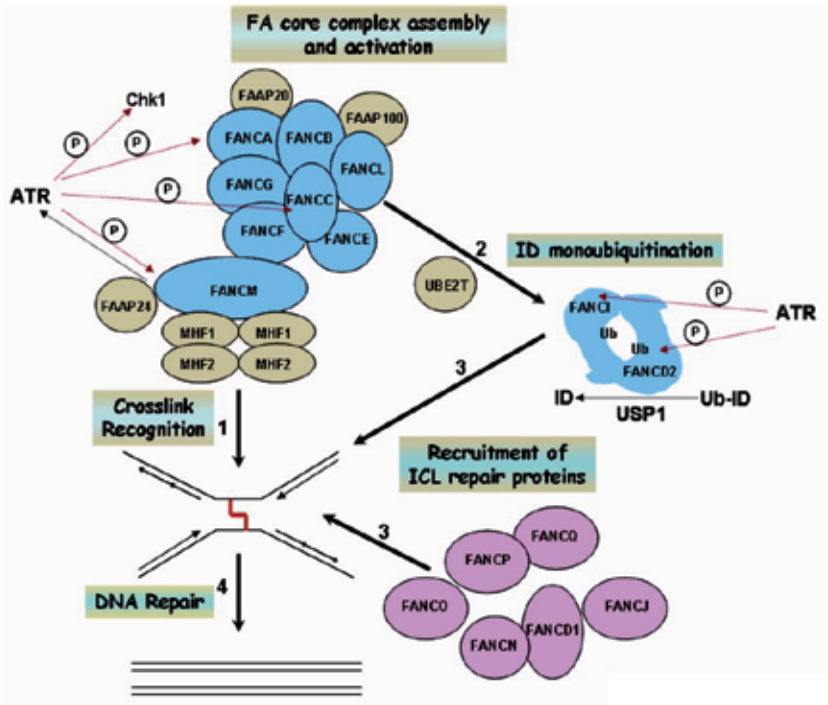

Figure 2. Integrated view of how FA proteins orchestrate the various steps of ICL repair FA core proteins are depicted in blue, FA accessory proteins are indicated in beige and FA downstream proteins involved in homologous recombination are indicated in pink. Step 1: Crosslink Recognition, Step 2: FANCI and FANCD2 monoubiquitination by the FA core complex, Step 3: Recruitment of Ub-FANCD2 and FA downstream proteins, Step 4: Completion of DNA repair by translesion synthesis and homologous recombination.

indicate the existence of a feedback loop wherein the initial signal constituting the ICL-bound stalled-replication fork is recognised by FANCM, which activates ATR. In turn, ATR phosphorylates a number of substrates such as FANCA, FANCI, FANCD2 and lastly FANCM itself, triggering the complete activation of the FA pathway that leads to FANCD2 monoubiquitination.

2.4. The FA core complex

Historically, FA complementation groups were assigned after the pair-wise fusion of patient-derived cell lines followed by assessment of crosslinker sensitivity. Genes mutated in each complementation group were cloned and re-introduced back into mutant cell lines and the ability to rescue crosslinker sensitivity was assessed. Based on this analysis, about 80% of Fanconi anemia patients were assigned to the subtypes FANCA or FANCC [26].

Using a combination of techniques like immunoprecipitation and immunofluorescence, some FA proteins were found to associate to each other, leading to the concept of a FA core complex [27]. It is now known that the FA core complex is constituted by a group proteins mutated in

FA like FANCA, FANCB, FANCC, FANCE, FANCF, FANCG, FANCL, FANCM and the accessory proteins FAAP24, FAAP20 and FAAP100. Out of these subunits, FANCL, which is a RING domain-containing protein, has the ubiquitin ligase E3 subunit. Proper assembly of the core complex is necessary for FANCI and FANCD2 monoubiquitination reaction. Importantly, patient-derived mutations that disrupt the structural integrity of the FA core complex compromise ICL repair [27].

The assembly of the FA core complex depends on protein-protein interactions between the components. One of the first interactions reported between FA core complex proteins was the binding between FANCA and FANCC [28]. FANCA also interacts with FANCL and this interaction is dependent on FANCG, FANCB and FANCM. FANCB interacts with FANCL and a complex of these proteins binds FANCA [29]. FANCE interacts with FANCD2 [30] and FANCG interacts with FANCA [31, 32]. FANCC nuclear localization depends on interaction with FANCE and FANCA requires nuclear localization of FANCC [34].

The accessory protein, FAAP100 is essential for the stability of the core complex and directly interacts with FANCB and FANCL to form a stable sub complex [35]. Another accessory protein, FAAP20 binds to FANCA and ensures the functional integrity of the FA core complex. The depletion of FAAP20 causes hypersensitivity to crosslinking agents, chromosomal aberrations and reduces FANCD2 monoubiquitination [36].

Recently, a modularised organisation has been ascribed for the FA core complex and the catalytic module composed of the FANCL-FANCB-FAAP100 proteins was identified as the minimal subcomplex essential for the ubiquitin ligase function [37]. On the other hand, the other two modules composed of FANCA, FANCG, FAAP20 module and FANCC, FANCE, FANCF modules were proposed to provide non-redundant functions that facilitate the catalytic module to bind sites of DNA damage [38].

2.5. The FANCD2/FANCI complex

A major conundrum in the FA field was solved with the discovery that the FA core complex monoubiquitinates FANCD2 (Ub-FANCD2) at lysine 561 [39]. FANCD2 monoubiquitination is DNA damage-dependent and Ub-FANCD2 localizes to sites of DNA damage to form FANCD2 nuclear foci.

Since most of the FA core proteins described above lacked any enzymatic domain, the next big search was for the FANCD2 ubiquitin ligase that catalyses the monoubiquitination reaction. FANCL was later identified as the key ubiquitin ligase responsible for catalysing the monoubiquitination of FANCD2 [40]. It was the only protein with known enzymatic activity in the form of a ubiquitin ligase activity and it contained a PHD-type ring finger at the C terminal domain and an RWD (RING finger proteins, WD-repeat proteins, and yeast DEAD-like) domain responsible for substrate binding [41-43]. The PHD domain of FANCL interacts with the ubiquitin conjugating enzyme, UBE2T. As expected, UBE2T-depleted cells accumulate abnormal radial chromosomes due to impaired FANCD2 monoubiquitination and defective ICL repair [44]. UBE2T itself undergoes monoubiquitination and inactivation after DNA damage, in a process stimulated by FANCL. Thus, UBE2T was identified as the E2 of the FA

pathway and it was proposed that UBE2T has a self-inactivation mechanism that is important for the negative regulation of the FA pathway.

The identity of the protein responsible for Fanconi complementation group I solved another missing link in the FA field. FANCI was identified as a protein essential for FANCD2 monoubiquitination [45-47]. FANCI is a FANCD2 paralogue and it heterodimerizes with FANCD2 to form the so-called FANCI/FANCD2 complex or the ID complex [45]. Just like FANCD2, FANCI also undergoes monoubiquitination and surprisingly, the monoubiquitination of both proteins are inter-dependent on each other, which suggested a unique regulation for ubiquitin conjugation. Upon treatment with crosslinking agents, both FANCI and FANCD2 form nuclear foci. The solved crystal structure of FANCI and FANCD2 revealed a saxophone-shaped crystal structure, with single-strand and double-strand binding regions, indicating that the complex can bind to DNA structures that arise after replication forks encounter crosslinking lesions [48]. Surprisingly, the FANCI/FANCD2 regulatory and monoubiquitination sites mapped to the interface of FANCI/FANCD2 binding. In order to explain how the ubiquitin ligase can access the buried ubiquitination site, it was proposed that that the monoubiquitination reaction probably acted on monomeric proteins [48].

When the binding activity of the ID complex was tested towards several DNA substrates, it was found that ID complex had better affinity for branched substrates such as splayed arm, Holliday junction, 5'-flap, 3'flap and static fork structures as compared to simple dsDNA, ssDNA or 5'tailed or 3'-tailed structures [49]. Recently, using purified proteins it has been shown that the ID complex is a poor substrate for ubiquitination by UBE2T and FANCL, unless branched or duplex DNA is added to the reaction. Also, mutations in FANCI that inhibit its DNA binding also prevent FANCD2 monoubiquitination. Conversely, FANCI can undergo the monoubiquitination reaction, in a manner independent of FANCD2 [50, 51]. Using Xenopus egg extracts, it was proposed that the ID complex might represent the inactive form and monoubiquitination might break apart the complex into active monomers which then exhibit distinct DNA substrate specificities [52]. According to an evolving model, FANCI and FANCD2 may also function separately at different stages of the repair process. Future research is needed to clarify the function and precise regulation of ID complex formation.

2.6. Negative regulation of FA pathway by USP1

The deubiquitinating enzyme, USP1 is an important regulator of the FA pathway because it deubiquitinates FANCD2 [53]. USP1 depletion increases FANCD2 monoubiquitination both at the steady-state as well as after DNA damage. Unexpectedly, despite there being an increase in Ub-FANCD2 levels, USP1 depletion results in increased crosslinker sensitivity, impaired HR repair, chromosomal aberrations and constitutively chromatin-bound FANCD2. This has led to the model that USP1 is required for recycling and releasing Ub-FANCD2 from chromatin [54, 55]. USP1 exists as a stoichiometric complex with the activator subunit UAF-1 (USP1-associated factor) [56]. The UAF1 contains a tandem repeat of SUMO-like domains at its C-terminus and one of the domains (SLD2) binds directly to the SUMO-like domain interacting motif of FANCI. Thus, UAF1/USP1 proteins get targeted to the FANCI/FANCD2 heterodimer [57]. Both Usp1 and Uaf1 deficiency in mice caused an increase in chromosomal aberrations,

crosslinker sensitization, defective HR and impaired FANCD2 focus assembly. These results indicate that FANCD2 monoubiquitination levels have to be tightly regulated for the proper functioning of the FA pathway [58, 59].

2.7. FANCD2 chromatin localization and nuclear focus formation after DNA damage

The monoubiquitination of FANCD2 is necessary but not sufficient for FANCD2 chromatin retention and nuclear focus formation. This is because monoubiquitination of FANCD2 can be uncoupled from focus formation upon the depletion of certain genes, suggesting the existence of additional layers of regulation in the FA pathway.

For example, the depletion of BRCA1 does not impair FANCD2 monoubiquitination, but only FANCD2 nuclear focus formation [60]. Interestingly, depletion of KU70 overrode the requirement for BRCA1 in FANCD2 recruitment to DNA damage foci, indicating that NHEJ proteins may negatively regulate FANCD2 focus formation. FANCD2 chromatin retention and focus formation are also defective in the absence of XPF-ERCC1 suggesting that a specific DNA structure created by the XPF-ERCC1 mediated-incision might stabilize the chromatin association of Ub-FANCD2 [61]. USP1 and UAF-1 depletion also give rise to impaired FANCD2 focus formation, but increase Ub-FANCD2. Phosphorylated H2AX is also essential for FANCD2 focus formation [62]. Recently, the RUNX family of transcription factors were demonstrated to control FANCD2 chromatin localization and focus formation, but not FANCD2 monoubiquitination. In a non-transcriptional but DNA damage-dependent manner, RUNX proteins interact with the FANCI/FANCD2 heterodimer. The disruption of RUNX proteins in mice gave rise to FA phenotypes such as BMF, MDS and crosslinker sensitivity [63]. Moreover, in at least two FA patients genomic deletions in the region bearing RUNX1 have been identified [64, 65]. In another example, FANCD2 chromatin retention and focus formation are regulated by FANCJ, independent of FANCD2 monoubiquitination [66]. It is possible that multiple proteins co-operatively regulate the FANCD2 focus formation step, because once Ub-FANCD2 lodges itself at the site of DNA damage, it 'licenses' the incision step by orchestrating the recruitment of DNA cleaving nucleases.

2.8. Unhooking of the crosslink

The incision of the parent DNA strand on either end of the crosslink is referred as the 'unhooking step' of ICL repair. Several structure-specific endonucleases have been implicated in the incision process such as the FAN1 nuclease, SLX4-SLX1 heterodimer, XPF-ERCC1 complex, MUS81-EME1 heterodimer and the SNM1 nuclease.

The 'unhooking' step of ICL repair is dependent on FANCD2 monoubiquitination and focus formation [67] because the ubiquitin domain of FANCD2 can recruit both FAN1 and SLX4. In a ShRNA (short hairpin RNA) screen for crosslinker resistance, FANI was identified as a DNA repair nuclease that undergoes recruitment through its UBZ domain (ubiquitin binding domain) by binding to Ub-FANCD2. FANI has both 5′-3′ exonuclease activity and 5′ flap endonuclease activities. Depletion of FAN1 causes crosslinker sensitivity and defective ICL repair [68-70].

In addition to FAN1 nuclease, ICL processing also involves the SLX4 protein, which complexes with several proteins such as the MUS81-EME1, XPF-ERCC1 heterodimers and the SLX1 nuclease. SLX4 is mutated in FA patients belonging to the complementation group FANCP and results in crosslinker sensitivity and chromosomal aberrations [71-73]. Similarly, ERCC4, the gene that encodes for XPF, undergoes biallelic mutations in FA patients, resulting in XPF being designated as FANCQ [74].

The SLX4 complex cleaves 3'flap, 5'flap and replication fork structures and promotes symmetrical cleavage of static and migrating Holliday junctions, identifying it as a Holliday junction (HJ) resolvase [75]. The ubiquitin-binding zinc finger domain (UBZ) of SLX4 mediates its interaction with Ub-FANCD2 leading to its recruitment to DNA damage sites [76]. In more detailed structure-function analysis, it has been found that the N-terminus of SLX4 protein that only binds XPF-ERCC1 is sufficient to confer DNA crosslinker resistance [77, 78]. SLX4 enhances XPF-ERCC1 nuclease activity *in vitro* by 100-fold and stimulates dual incisions around a DNA crosslink embedded within a synthetic replication fork [79].

According to another line of evidence, XPF-ERCC1 might be sufficient to make the dual incision during ICL repair. In elegant *in vitro* biochemical studies, the XPF-ERCC1 complex makes an incision 5 'to a psoralen lesion on a Y-shaped DNA in a damage-dependent manner. Subsequent to the first incision, it creates a second incision specific to the 3'-end side of the ICL. The ICL-specific 5' and 3' incisions result in the separation of the two crosslinked DNA strands resulting in 'unhooking' [80]. Another nuclease that collaborates with XPF-ERCC1 is the mammalian homolog of yeast Pso2 exonuclease, the human SNM1A. hSNM1 exhibits a 5'-3' exonuclease activity and initiates ICL repair by creating a favourable substrate for TLS through its nucleolytic action [81]. In summary, multiple nucleases engender the incisions required for ICL repair to proceed, although the relative contribution of these nucleases and the order of their recruitment are not fully clear.

2.9. Downstream of FANCD2 monoubiquitination- FANCD1 (BRCA2), FANCJ, FANCO and FANCN

The Fanconi proteins FANCD1 (BRCA2), FANCJ (BRIP1), FANCO and FANCN (PALB2) are loosely referred to 'downstream proteins' in the FA pathway because they act downstream to the FANCD2 monoubiquitination step. Mutations in these proteins give rise to more severe phenotypes in FA patients especially with regards to cancer onset.

FANCD1: The biallelic inactivation of FANCD1 or BRCA2 in Fanconi anaemia patient families created tremendous excitement at the time of the discovery, because it linked FA with a gene closely associated with hereditary cancer susceptibility [82]. Also, it strengthened the idea that the FA and BRCA pathway are intimately linked for the successful repair of ICL damage.

BRCA2 is a central protein of HR repair of DNA damage. In response to double strand breaks, BRCA2 mediates the delivery of RAD51 to ssDNA and facilitates the displacement of RPA from ssDNA. RAD51 is a protein that forms a nucleoprotein filament on ssDNA and invades DNA duplex to search for sequence homology. It is suggested that monoubiquitination of

FANCD2 is needed for BRCA2 loading to DNA damage foci, indicating a functional coupling between FANCD2 monoubiquitination and HR [83].

FANCJ is also called as BRCA1-associated helicase or BRIP1. FANCJ-depleted cells show crosslinker sensitivity and chromosomal instability after mitomycin C treatment [84, 85]. Using a chromatin-IP (immunoprecipitation) based method, recruitment of FANCJ and FANCN to DNA crosslinked sites was found to be FA core complex-independent, but DNA replication-dependent [86].

FANCJ has both DNA-dependent ATPase and helicase activities. The helicase activity of FANCJ is a target for mutational inactivation in patients and it depends on the ATPase activity [87]. FANCJ preferentially binds and unwinds forked duplex substrates and 5′ flap substrates which arise during normal replication and repair. Although FANCJ was isolated as a BRCA1-binding protein, the repair function of FANCJ is independent of its BRCA1 binding because a FANCJ mutant deficient for BRCA1 binding can still rescue the crosslinker sensitivity of FANCJ-deficient chicken DT-40 cells [88]. On the other hand, genetic studies have revealed that instead of BRCA1-binding, FANCL–MLH1 binding is essential for FANCJ to be able to correct crosslinker sensitivity of FANCJ-null cells [89].

RAD51C (FANCO) is essential for HR-mediated repair of lesions associated with replication and controls the intra-S checkpoint through CHK2 activation [90]. RAD51C mutation resulted in a Fanconi-anemia like disorder in a family with several congenital abnormalities characteristic of FA [91]. RAD51C is a part of a complex that also contains PALB2 and BRCA2. FA and cancer-associated RAD51C mutants that show reduced complex formation with PALB2 also have a reduced capacity for HR repair [92].

Partner and localizer of BRCA2 or PALB2 mutations were detected in the Fanconi complementation group N patients. FANCN-deficient cells are sensitive to mitomycin C, are defective for BRCA2 loading and RAD51 focus formation and have reduced HR activity [93, 94]. PALB2 was originally identified as a BRCA2-interacting protein and it physically links BRCA1 and BRCA2 to form the so-called BRCA-complex [95, 96]. PALB2 focus formation after DNA damage was itself dependent on BRCA1. PALB2 is important for BRCA2 localization to sites of DNA damage and in supporting BRCA2 function during HR. Clinically relevant point mutations that either disrupt PALB2-BRCA1 or PALB2-BRCA2 binding fail to support HR [97]. Thus, an intact BRCA1-PALB2-BRCA2 pathway is essential for HR and in the suppression of FA phenotypes.

2.10. Translesion synthesis: after unhooking of the crosslink

Translesion synthesis (TLS) is an inherently error-prone pathway that promotes DNA repair by allowing the bypass of a stalled-replication intermediate [98]. Although this process can increase genomic instability in the form of point mutations, the TLS step is required during ICL to generate the intact template for HR. Consistently, genomic analysis of FA patient samples revealed a ‘hypomutability’ phenotype with respect to point mutations [99].

TLS is achieved because the canonical replicative polymerase is replaced by the translesion polymerase that can accommodate DNA lesions into a larger active site. Several classes

of human translesion polymerases like Rev1, Pol zeta, Pol eta, Pol kappa, Pol iota, and Pol nu participate in replication-dependent ICL repair and extent of bypass depends upon the structure of the crosslinking agent as well as the extent of nucleolytic processing of the crosslink [100].

Of the various translesion polymerases, Rev1 and the translesion polymerase eta form foci at sites of damage in a PCNA-dependent manner. After DNA damage, PCNA is monoubiquitinated in a RAD6/RAD18-dependent manner at lysine 164. PCNA is a sliding clamp that carries the replication polymerase delta along DNA during replication. When PCNA undergoes monoubiquitination, it switches the canonical DNA polymerase into the translesion polymerase eta, which allows for lesion bypass due to a larger active site. Monoubiquitinated PCNA also stabilises the recruitment of another translesion polymerase, Rev1. Rev1 is a deoxycytosine monophosphate (dCMP) transferase that can insert a cytosine opposite the unhooked ICL. Rev1 recruitment and focus formation is also promoted in a PCNA-independent manner because the FAAP20 subunit of FA core complex binds to ubiquitinated Rev1 through its Zinc finger 4 domain and stabilizes Rev1 nuclear foci [101]. Intriguingly, PCNA monoubiquitination has TLS-independent role in promoting the monoubiquitination of FANCI and FANCD2 in a FANCL-dependent manner [102]. These findings indicate multiple levels of crosstalk between the FA and TLS pathways.

2.11. ICL repair: HR (HR)

The by-product of the 'unhooking' step is the generation of a broken strand of DNA that is repaired by the FA pathway using HR. HR is initiated by a DNA end-resection reaction that creates the 3' overhangs.

During ICL repair, the MRN complex and CtIP participate in the end resection reaction. ATR phosphorylates the MRN complex which is required for the resection of the double strand created after the excision of the ICL to create 3' overhangs. Recently, CtIP has been found to undergo recruitment to DNA repair sites in a FANCD2 monoubiquitination-dependent manner to start the end resection reaction. The interaction between CtIP and Ub-FANCD2 is required for the formation of mitomyicin C-induced CtIP foci and RPA phosphorylation. It is proposed that Ub-FANCD2 channels ICL repair into the error-free HR pathway by tethering CtIP to damaged chromatin. Thus, CtIP prevents illegitimate recombination during ICL repair [103, 104]. In another study, FANC2 binding to CtIP has been observed to promote replication fork-restart [105].

After end resection, RAD51 plays an important role in HR repair. Although RAD51 functions downstream of FANCD2 monoubiquitination, RAD51 recruitment to stalled -replication forks happens independently of FANCI/FANCD2 and before double strand break (DSB) formation. The depletion of RAD51 from Xenopus egg extracts completely disrupted ICL repair by HR [4].

More recently, the role of FA proteins in HR has been firmly established using the TR-GFP assay. In this assay, ICL formation is achieved by conjugating a triplex-forming oligonucleotide to the crosslinking agent psoralen. Using this system, a profound defect in ICL-induced HR

was observed in FA patient cells, but only upon reporter replication [106], providing evidence that FA proteins are essential for replication-coupled ICL repair by HR.

According to another line of thinking, one of the principal functions of FANCD2 is to channel DSBs away from error-prone NHEJ into error-free HR. Accordingly, the concurrent disruption of FANCC and KU70 suppressed crosslinker sensitivity, reduced chromosomal breaks and reversed defective HR in chicken DT40 cells [107]. Similarly, the DNA repair defects of *C.elegans* FANCD2 mutants can be rescued by simultaneously eliminating the NHEJ pathway, leading to the conclusion that FA-defective phenotype may be consequence of promiscuous end-joining reactions catalysed by NHEJ [108]. However, similar results were not obtained in mice, where the depletion of KU or 53BP1, another NHEJ factor, exacerbated genomic instability in cells lacking FANCD2 [60]. Regardless of whether NHEJ inhibition is the sole function of the FANCD2 monoubiquitination or not, at the least, it can be concluded that channelling DSBs away from toxic NHEJ into error-free HR is one of the important downstream outputs of an activated FA pathway.

2.12. Endogenous sources of DNA interstrand crosslinks

The DNA repair defects associated with the FA pathway have been studied *in vitro* mostly after the exogenous addition of crosslinking agents like cisplatin or mitomycin C. However, FA patients show their disease manifestations without evidence for prior exposure to exogenous crosslinkers. This indicates that human cells are inadvertently exposed to endogenous genotoxic agents and the FA pathway preserves the genomic integrity of cells in the presence of such stresses.

In recent studies, reactive aldehydes have emerged as one of the endogenous agents capable of generating lesions that have to be repaired by the FA pathway. One such example is acetaldehyde, an intermediate by-product of alcohol metabolism. Acetaldehyde is enzymatically catabolised into acetate by the acetaldehyde dehydrogenase, ALDH2. In the absence of ALDH2, acetaldehyde accumulates and binds DNA to form N2-ethylidene-dG adducts. In Aldh2-knockout mice that have been exposed to 8% ethanol for 14 months, increased genotoxic stress in the form of N(2)-ethylidene-dG DNA adduct accumulation is evident in the oesophagus, tongue and submandibular gland [109-111]. Furthermore, epidemiological studies have demonstrated a strong correlation between long-term drinking and a predisposition for oral and esophageal cancers in people bearing the ALDH2*2 polymorphism (a dominant negative isoform of ALDH2).

To understand whether the FA pathway protects cells against the genotoxic effects of aldehydes, mice double-deficient for ALDH2 and FANCD2 were generated. Aldh2 and Fancd2 double knock-out mice exhibited lethality at the embryonic stage. However, when these embryos were transferred into ALDH2-catabolism efficient mothers (*Aldh2*+/-), the mice were born to term, but had developmental defects and leukemic predisposition. Aged-double mutant *Aldh2-/-Fancd2-/-* mice that did not develop leukemia spontaneously developed aplastic anemia, another characteristic feature of human FA patients, together with a drastic 600-fold depletion in haematopoietic stem cell pools [112]. Similar results were obtained in

chicken DT-40 cells, where ADH5 (formaldehyde-catabolising enzyme) and FA pathway factors exhibited a synthetic lethal genetic interaction [113]. The developmental phenotypes and embryonic lethality associated with *Aldh2-/-Fancd2-/-* embryos could be rescued by transferring such embryos into aldehyde catabolism-efficient wild-type mothers. This suggested that maternal aldehydes emanating from ALDH2-deficient mothers can be transferred via the placenta into the growing foetus resulting in embryonic lethality [114]. When such 'rescued' *Aldh2-/- Fancd2-/-* neonates are analysed after birth, they still had severely depleted hematopoietic stem cell pools. This indicated that both fetal and maternal aldehyde detoxification systems are important to counteract the genotoxic effects of aldehydes in the growing embryo. Together, the above studies provided a glimpse on how the repair of aldehyde-mediated damage of the haematopoietic stem cell pool might be one of the underlying functions the FA pathway and can explain the haematopoietic defects of human FA patients.

3. NON-ICL repair function of the FA pathway

It has recently emerged that the FA pathway can also function in an ICL-repair independent manner for the maintenance of genomic integrity. In this section, we will discuss how the FA proteins participate in alternative genome-maintenance pathways.

One of the main ICL repair-independent functions of FANCD2 is that it co-operates with BRCA2 and RAD51 to protect stalled-replication forks from nucleolytic degradation [115]. FANCD2 also co-ordinates the re-start of stalled-replication forks in concert with the BLM helicase and recruits the FAN1 nuclease to promote the re-start of forks [116, 117]. Consistently, FA proteins FANCI and FANCD2 localize to stalled-replication forks in mass spectrometric studies [118]. Apart from FANCD2, other FA proteins FANCM, FAAP24, MHF1 and MHF2 were also found to play an important role in stabilizing stalled replication forks. The ATPase-dependent FANCM translocase activity is needed for replication fork stability. Cells expressing translocase-deficient FANCM showed altered global replication dynamics and stalled replication forks that result in the formation of spontaneous DSBs and 53BP1-marked nuclear bodies called as 53BP1-OPT domains in the G1 phase of cell cycle [119]. Similarly, loss of Fancc exacerbated genomic instability by impairing fork progression during DNA replication in a tumor-prone mouse model that had ~60% loss of dormant origins [120].

FANCI and FANCD2 also bind indirectly to minichromosome maintenance (MCM) proteins that are present in nascent DNA after replication arrest. FANCD2 was found essential for cells to restrain DNA synthesis in the presence of reduced pool of nucleotides. In an ATR-dependent but monoubiquitination-independent way, FANCD2 is required for general replisome surveillance mechanisms [121].

FA proteins may also promote genomic integrity in a transcription-dependent manner. For instance, Fancd2-deficient mice are susceptible to squamous cell carcinomas of the skin in response to Ras oncogene induction. Ub-FANCD2 activates the transcription of a tumor suppressor TAp63 to prevent skin carcinogenesis [122]. FANCD2 also interacts with NFκB in

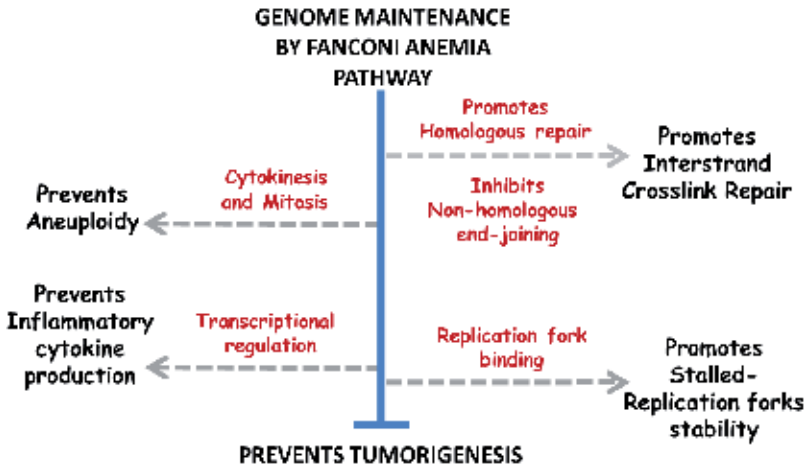

Figure 3. The Fanconi anemia pathway maintains genomic integrity in ICL-repair dependent and independent manner

the TNF-alpha promoter region. Defective FANCD2 causes the activation of TNF-alpha and the production of inflammatory cytokines [123]. Inflammatory cytokines, in turn, are an important source of reactive oxygen species to increase the DNA damage exposure of FA-defective cells.

The FA pathway is also required for maintaining the genomic integrity of B cells during class switch recombination (CSR). FANCA is required for the induction of transition mutations at A/T residues during somatic hypermutation and to prevent short-range recombination downstream of DSB formation during CSR [124].

The FA pathway co-operates with BLM to maintain genomic integrity during mitotic progression. FA pathway-dependent BLM targeting to non-centromeric abnormal structures induced by replication stress has an important role in mitotic progression because it prevents micronucleation and reduces aneuploidy in daughter cells [125]. FA-pathway deficient cells also express a higher number of UBFs (ultra-fine bridges) as compared to wild-type cells. This was attributed to the higher rate of cytokinesis failure in FA-impaired cells resulting in binucleated cells [126] (Figure. 3).

4. The Fanconi anemia pathway as an anti-cancer barrier

In the last section of this chapter, we will examine how the FA proteins suppress tumorigenesis. *There are three lines of evidence linking FA pathway disruption and human cancer. Firstly, FA patients have a heightened risk for developing leukemias and solid tumors in their life time as compared to the general population. Secondly, mouse models deficient for FA genes spontaneously develop tumors. Lastly, FA gene mutations have been uncovered from cases of human sporadic cancers, suggesting that they could be drivers of genomic instability in human cancers.*

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4.1. Cancer Incidence in FA patients

Due to variability in clinical manifestations between FA patients, phenotypes such as BMF, haematological malignancies and solid tumors were used to subdivide patients. In general, anywhere between 20%-80% of FA patients experience BMF. About ~30% of FA patients get cancers, although this number can vary depending on the patient's risk for getting BMF. For example, the high-BMF risk category have a lower chance of getting cancers because they may not live long enough, whereas the patients in the lowest BMF risk group were likely to live long enough and get leukemias or solid tumors [127]. The risk for getting all types of cancers including leukemia and solid tumors is 50-fold higher in the FA population as compared to the non-FA population [128]. In a literature survey published in 2003, cancer incidence was measured in 1300 FA patients for the years in between 1927-2001 [129]. While the median age for cancer development was ~68 years in the normal population, it significantly dropped to a median age of only 16 years for the FA population. Of the patients who developed tumors, about 60% had tumors of haematological origin and 40% were solid tumors. The risk for getting leukemia in the FA population is 800-fold higher than the general population [130, 131]. Haematological cancers, in particular, acute myeloid leukemia and myelodysplastic syndrome accounted for ~50% of cancers in FA patients by age 40. FA-related leukemias were myeloid in 94% of the cases and only 6% of the leukemias were lymphoid in nature. This pattern strongly differed from spontaneous leukemias, where ~84% were lymphoid in nature.

Amongst the solid tumors, ~50% were squamous cell carcinomas of the head and neck, anogenital region, skin, vulvar region, oesophagus and cervix and 10% were liver tumors. When compared to the general population, the risk is for getting all solid tumors is 48-fold higher in the FA population and in the range of 100-1000 fold higher for head and neck cancers and for cancers of the esophagus, liver, vulva, and cervix [130]. Solid tumors had a slower incidence at younger age, but the probability of getting a solid tumor dramatically increased to 76% by age 45, suggesting that older FA patients had a significantly higher risk for developing solid tumors.

It is noteworthy that there is considerable variability between FA patients with respect to the spectrum of cancers manifested and the age of cancer-onset. In general, mutations in FA core proteins result in a milder cancer phenotype as compared to mutations in downstream genes. For example, FA patients belonging to the FANCD1 (BRCA2) subgroup have a much higher risk for an earlier onset of malignancies. The cumulative probability of getting any malignancy such as leukemia or solid tumors in a FANCD1 subtype patient was ~97% by age 5.2 years, indicating that amongst FA patients, FANCD1 subtype was the most severe with respect to their risk for developing malignancies [132, 133]. Similarly, patients of the subtype FANCD1, FANCO and FANCJ show greater predisposition for developing breast cancers, whereas patients of the subtype FANCO are likely to get ovarian cancers. In summary, cancer spectrum and the age of cancer-onset are reflective of the distinct roles played by the FA proteins during ICL repair.

4.2. FA pathway disruption predisposes knockout-mice to cancers

Immediately following the discoveries of mutations responsible for human FA, a number of groups generated mice defective for FA proteins. The first mouse models to be generated were the *Fancc-/-* mice and *Fanca-/-* mice (exons 4-7 deletion). These mice had normal viability and no developmental defects, but impaired fertility. *Fancc-/-* and *Fanca-/-* cells had characteristic crosslinker sensitivity and exhibited several-fold higher accumulation of chromosomal aberrations after treatment with crosslinking agents [134, 135]. Another mouse model for Fanca bore deletions in exons 1-6 and these mice showed more severe phenotypes like prenatal growth retardation, craniofacial abnormalities, and crosslinker sensitivity which were all typical features of human FA patients [136]. *Fancg-/-* mice also showed hypogonadism, impaired fertility and cells isolated from these mice had an accumulation of spontaneous chromosomal aberrations, increased sensitivity to mitomycin C [137, 138]. Together, the characteristics of *Fancc-/-*, *Fancaa-/-* and *Fancg-/-* mice strongly resembled each other, supporting the premise that they function in a common pathway. Yet, phenotypes evident in human FA patients such as typical haematological manifestations and increased risk for spontaneous cancers could not be recapitulated in these mouse models. It was also hypothesised that the benign environment in typical mouse facilities did not provide sufficient exogenous stress to precipitate FA phenotypes. Hence, *Facc-/-* knockout mice were challenged with clastogens such as mitomycin C. Upon crosslinker exposure, *Facc-/-* mice showed progressive pancytopenia and died within a few days [139]. Interestingly, double-deficient mice defective in Fancc and Fancgg, in C57BL/6J background started displaying haematological manifestations seen in FA patients, such as BMF, acute myeloid leukemia (AML), MDS, and complex chromosomal rearrangements that were not seen in the single KO mice. Hence, this mouse model was considered a close mimic of human FA, especially with respect to onset of haematological malignancies [140].

Fancd2-/- mice are viable, but show prenatal and post-natal growth retardation. The severity of the phenotypes in *Fancd2-/-* mice is mouse strain-dependent and mice generated in the C57BL/6J background had more severe phenotypes that those in 129S4 background. *Fancd2-/-* mice shared phenotypes common with *Fanca-/-*, *Fancc-/-* and *Fancg-/-* mice such as impaired fertility, increased chromosomal aberrations and sensitivity to MMC. In addition, *Fancd2-/-* mice also exhibited unique features such as microphthalmia and perinatal lethality. *Fancd2-/-* mice were particularly tumor–prone, and showed epithelial cancers in several tissues that eventually caused their mortality. The tumors found in *Fancd2-/-* mice included ovarian cancers, hepatic adenoma and adenocarcinoma, gastric cancers, mammary B cell lymphoma, lung adenocarcinoma and broncheoalveolar carcinoma [141]. Tumor formation was further accelerated when *Fancd2-/-* mice were crossed with *p53+/-* mice. The tumor spectrum found in the double mutant mice included mammary and lung adenocarcinomas, cancers rarely seen in the *p53+/-* heterozygotes [142]. More recently, mice double-deficient for Fancd2 and Aldh2 were generated. Due to aldehyde-mediated genotoxicity, such mice showed leukemic predisposition and BMF [143]. The above studies reiterate that FA phenotypes are strongly influenced by the type and extent of genotoxic stress exposure. Only when cells accumulate

unrepaired crosslinks beyond a certain threshold, FA phenotypes such as developmental abnormalities, BMF, haematological malignancies and solid tumors are manifested.

Another example, where the absence of FA core proteins results in increased cancer incidence are *Fancf*-/- mice and *Fancm*-/- mice. *Fancf*-/- mice showed increased incidence of ovarian cancers whereas *Fancm*-/- mice showed tumors such as lymphomas, histiocytic sarcoma, hepatoma and lung carcinomas [144, 145].

Apart from core FA proteins, mouse models were also generated for the downstream proteins involved in the unhooking step or in HR. Mice deficient for SLX4 recapitulated several key features of FA. These mice were born at submendelian ratios, had fertility defects and were prone to blood cytopenias. *Slx4*-/- cells show increased chromosomal aberrations and crosslinker sensitivity [72]. Slx4-deficient mice also developed epithelial cancers and died by 100 weeks with haematological cancers and solid tumors such as squamous cell carcinomas, hepatocellular carcinoma and ovarian tumors. Thus, SLX4 deficiency closed mimicked the clinical features of human FA.

According to human genetic data, the heterozygosity of HR proteins cause increased susceptibility to breast, ovarian and other cancers whereas biallelic mutations in HR genes cause FA. A homozygous deletion of HR genes, however, results in an embryonic lethal phenotype. Consistently, mice bearing homozygous deletion of repair proteins involved in HR such as BRCA2, PALB2 and Rad51C show embryonic lethality with gross chromosomal aberrations [146, 147]. But, when hypomorphic strains were derived for these mice, an FA phenotype could be observed. For example, mouse expressing a hypomorphic *Palb2* allele showed reduced fertility, MMC hypersensitivity and chromosomal breakage when treated with mitomycin C [148]. Similarly, thymic lymphomas were observed in mice expressing a truncated form of BRCA2 [149]. Homozygous deletion of exon 27 of *Brca2* prevents its interaction with RAD51. Hence, mice carrying homozygous deletion of exon 27 of *Brca2* were generated and several phenotypes in these mice recapitulated phenotypes observed in human FA patients of the complementation group FANCD1. *Brca2*$^{\Delta}$*27*$^{/\Delta}$*27* mice showed haematological defects such as compromised progenitor cell function, reduced hematopoietic stem cell self renewal, spontaneous accumulation of chromosomal aberrations and sensitivity to crosslinking agents. In addition, *Brca2*$^{\Delta}$*27*$^{/\Delta}$*27* mice were more prone to epithelial tumors such as squamous cell carcinomas of gastric origin and mammary gland, endometrial cancers, sarcomas and lung cancers [150, 151].

4.3. Fanconi anemia pathway in human sporadic cancer

The discovery that bi-allelic mutations in BRCA2 manifests as FA was a major conceptual advance because it linked FA with a gene responsible for familial cancers. FA phenotypes such as cancer predisposition could now be directly linked to HR and DNA repair [82, 152].

However, only about 25% of human hereditary cases of breast cancer are owing to BRCA1 or BRCA2 mutations. Therefore, it was hypothesized that mutations in BRCA1-related FA pathway genes such as FANCJ, FANCN and FANCO could be responsible for BRCA1/BRCA2-negative breast and ovarian human cancers. Amongst these, there is strong evidence that germ-

line mutations in BRIP1 increase the susceptibility to familial ovarian cancer [153]. Similarly, germ-line mutations in FANCO (RAD51C) increase susceptibility to ovarian cancer and breast cancer [154-157]. PALB2 (FANCN) mutations have been uncovered in 1-4% of inherited breast cancer patients [158, 159]. Polymorphic FANCA mutations were also detected in high-risk non-BRCA1/BRCA2 breast cancer patients in a French Canadian population [160]. FANCM has also emerged as a breast cancer susceptibility gene in a Finish population and FANCM mutations confer strong predisposition for triple-negative breast cancers [161]. PALB2, FANCC and FANCG mutations have also been associated with increased risk for pancreatic cancer [162-164]. In summary, a small but significant proportion of familial cancers are driven by mutational inactivation of FA genes.

There is also accumulating evidence that genes of the FA pathway may undergo inactivation by epigenetic silencing in some sporadic human cancers. FANCF promoter methylation and inactivation was observed in lung and oral cancers and promoter methylation correlated with smoking and alcohol use [165]. FANCF promoter methylation and inactivation was also observed in ovarian cancer patients [166]. Epigenetic inactivation of FANCA was observed in non-small cell lung cancer due to microRNA miR-503 dependent de-regulation [167]. An early-stage inactivation of FANCC due to promoter methylation was observed in dysplastic lesions of the head and neck [168]. FANCA and BRCA2 promoters were recurrently methylated in laryngeal squamous cell carcinoma [169]. FANCC and FANCL promoters were hypermethylated in sporadic acute leukemia [170], PALB2 promoter was methylated in sporadic breast and ovarian cancer [171], FANCF promoter was methylated in sporadic breast cancer [172] and ovarian cancer [173, 174] cervical cancer [175] and in AML [176]. In an unknown manner, FANCD2 expression was reduced in human uveal melanoma and influenced spontaneous sister chromatid exchange and RAD51 focus formation [177]. Cancer-associated alterations in the form of deletions were recovered for FANCC in breast cancers [178]. Put together, the inactivation of FA genes might be a common feature in several sporadic cancers.

Lastly, cancer genomics has enabled the analysis of the cancer genome in a high-throughput manner. When cancer genomes are queried for mutational inactivation of the 16 FA genes (FANCA, FANCB, FANCC, FANCD2, FANCE, FANCF, FANCG, FANCI, FANCJ, FANCL, FANCM, FANCD1, FANCN, FANCO, FANCP, FANCQ), nearly 25-30% of solid tumors show a mutational inactivation of one of genes (http://www.cbioportal.org/public-portal, [179, 180] (Figure. 4).

Given that an intact FA pathway is required for the successful repair of ICLs, the inactivation of any of the FA genes can potentially lead to genomic instability during cancer progression. At the same time, genomic instability due to disrupted FA pathway can be exploited for therapy because such cancers by targeted by chemotherapeutics known to cause DNA interstrand crosslinking like Cisplatin. Further functional characterisation of cancer-associated FA mutations is required to provide clearer knowledge on the role of the FA pathway in sporadic human cancer.

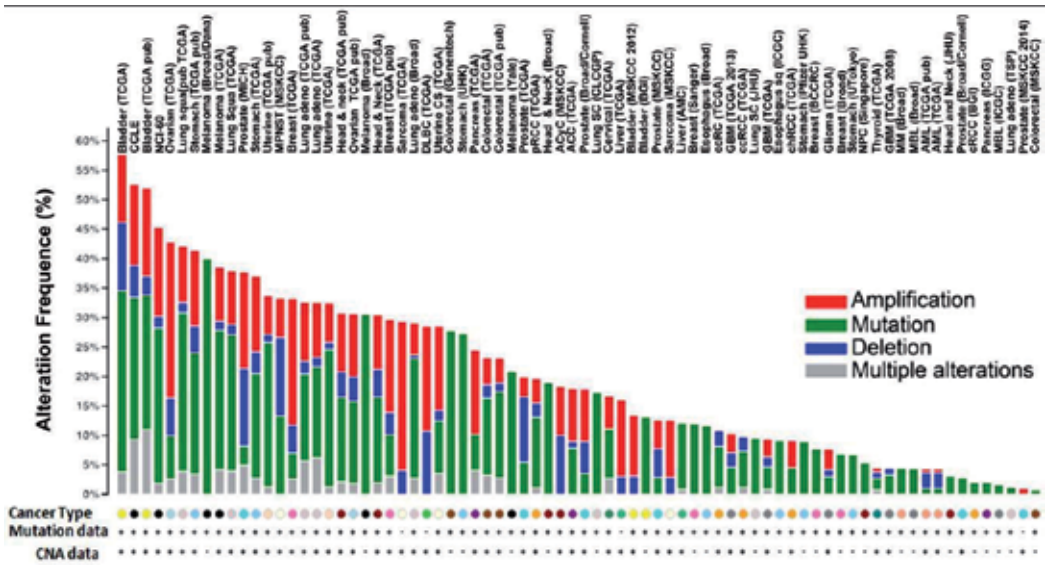

Figure 4. Genomic characterization of FA proteins in human cancer

5. Conclusion

Even though FA was described several decades ago, a thorough knowledge on the functional aspects this pathway has emerged only in the last 15 years. Mechanistic studies have revealed that FA proteins orchestrate the complex process of ICL repair. Unrepaired crosslinks are a major source of genomic instability and responsible for several FA patient phenotypes. But, according to recent studies, FA proteins may also fulfil DNA repair-independent roles to maintain genomic integrity. As even more proteins are being ascertained as accessory factors of this pathway, the study of FA is becoming complex, but exciting.

The most serious complication for FA patients at a young age is aplastic anemia, but, this haematological complication can be mostly treated by stem cell transplantation. An even greater concern for FA patients is their risk for getting leukemias and solid tumors at an older age. The observation that reactive aldehydes are a major source of ICLs has given hope to FA patients that reducing aldehydes levels can perhaps reduce cancer incidence. If environmental or metabolic sources of ICL agents are discovered, then such risk factors can be mitigated to reduce cancer burden in FA patients.

We now know that an intact FA pathway and enzymatic detoxification of aldehydes act together to provide a double-tier protection against crosslinking lesions. Given that ~500 million people world-wide have polymorphisms that make their aldehyde-detoxification pathways inefficient, studying the FA pathway is likely to gain more importance from a human health perspective. Importantly, several known human carcinogens like tobacco smoke, nitrosamines from diet and alcohol can result in potentially genotoxic interstrand crosslinking lesions within cells.

Indeed, the likelihood for getting sporadic human cancer has increased at an alarming rate over the past few decades. Given that the FA pathway lies at the interface of genome maintenance and human cancer, further studies on this pathway can lead to novel strategies for cancer prevention.

Abbreviations

Acute myeloid leukemia (AML)

Ataxia telangectasia-mutated related (ATR)

Bone marrow failure (BMF)

Double strand break (DSB)

Double-stranded DNA (dsDNA)

Fanconi Anemia (FA)

Homologous recombination (HR)

Interstrand crosslink (ICL)

Monoubiquitinated FANCD2 (Ub-FANCD2)

Myelodysplastic syndrome (MDS)

Non-homologous end joining (NHEJ)

Single-stranded DNA (ssDNA)

Translesion synthesis (TLS)

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Chapter 9

Homologous Recombination Repair Polymorphisms, Cancer Susceptibility and Treatment Outcome

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Additional information is available at the end of the chapter

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1. Introduction

DNA repair mechanisms are crucial for the maintenance of genome's integrity. When DNA damage is not repaired promptly, that may pose a serious threat to genomic stability and can contribute to carcinogenesis. On the other hand, the core molecular mechanism of action in several cancer treatments including chemotherapeutic agents and radiation therapy is induction of DNA damage and the efficacy of DNA repair mechanisms may influence the outcome of cancer treatment. Genetic variability of DNA repair proteins can modify the ability to repair DNA damage and may therefore play an important role in both cancer susceptibility and the outcome of cancer treatment.

DNA damage arises from exposure to endogenous or exogenous factors, including chemotherapeutic agents and radiation therapy [1]. There are several forms of DNA damage and therefore several mechanisms involved in their repair. Complex changes such as double strand breaks (DSBs) can lead to chromosome loss, chromosomal rearrangements or apoptosis and as a result can have a significant impact on cellular processes. DSBs represent one of the most detrimental forms of DNA damage because both strands of DNA are damaged and are thus especially challenging for efficient and accurate DNA repair [2]. One of the important pathways involved in DSB repair is HRR, a complex mechanism consisting of several steps that requires coordinated interplay of various enzymes [3]. This chapter focuses on homologous recombination repair (HRR) and summarizes the current knowledge on how genetic variability in this pathway influences cancer susceptibility and treatment outcome.

2. Homologous recombination repair pathway

HRR is crucial for the repair of DSBs, but is also involved in repair of other types of DNA damage, such as interstrand crosslinks. HRR ensures complete repair of DSBs because the undamaged homologous chromosome serves as a template to repair the damage.

In the first step of HRR, MRN complex is essential for recognition of DSBs. MRN complex consists of three proteins: meiotic recombination 11 homologue (MRE11), DNA repair protein RAD50 (RAD50) and nibrin (NBN). MRN recruits different enzymes to the site of DNA damage and activates them [4]. In the beginning, the broken ends of DSBs are processed to single stranded 3′ ends. DNA repair protein RAD51 homolog 1 (RAD51) then binds to DNA and forms a nucleoprotein filament. With the help of mediator proteins such as X-ray repair cross-complementing group 3 (XRCC3) and XRCC2, RAD51 catalyses the central reaction of HRR: the search for a homologous template and strand transfer between the damaged region and the undamaged homologous chromatid. The 3′ end of the damaged strand invades the homologous chromatid and is elongated by DNA polymerase using the complementary strand of the homologous chromatid as a template, resulting in the formation of Holliday junctions. After resynthesis and ligation of the damaged region, resolvase is needed for the resolution of Holliday junctions. Resolution can lead to either crossover or non-crossover products, but it always results in two intact double-stranded DNA molecules [5].

3. Genetic variability in homologous recombination repair genes

DNA repair mechanisms can be less effective in some individuals, leading to increased cancer susceptibility. Rare mutations in DNA repair genes that result in decreased DNA repair capacity have been linked to different hereditary cancers. DNA repair capacity may also be influenced by genetic polymorphisms that were identified in these genes. In particular, common functional single nucleotide polymorphisms (SNPs) leading to amino acid substitutions as well as SNPs in promoter or miRNA binding sites may influence the activity, stability or expression of DNA repair proteins.

The majority of cancer susceptibility and pharmacogenetic studies related to HRR has focused on genetic variability of *NBN*, *RAD51*, *XRCC2*, and *XRCC3*. Most commonly investigated SNPs in these genes, their predicted function and their minor allele frequencies (MAFs) in population of European descent are presented in Table 1.

3.2. *NBN*

MRN complex is involved in DSB recognition in different repair pathways, not only in HRR [14], suggesting that NBN may play a crucial part in DNA repair. NBN consists of three functional regions [6]. The N-terminal region binds to phosphorylated histone H_2AX (γ-H_2AX) and allows the MRN complex to move close to the sites of DSBs [6]. The central region is involved in signal transduction for damage response, while the C-terminal region is involved in MRE11 binding.

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Gene	rs number	Polymorphism	Location	Predicted function	MAF[a]
NBN	rs1805794	p.Glu185Gln	Exon, nonsynonymous	Affects interaction with BRCA1 [6]	0.304
	rs709816	p.Asp399Asp	Exon, synonymous	Affects splicing [7]	0.357
	rs1063054	c.*1209A>C	3′ UTR	Affects miRNA binding [8]	0.317
	rs2735383	c.*541G>C	3′ UTR	Affects miRNA binding [8-10]	0.312
RAD51	rs1801320	c.-98G>C	5′ UTR	Enhances promoter activity [11]	0.067
	rs1801321	c.-61G>T	5′ UTR	Enhances promoter activity [11]	0.467
XRCC3	rs1799794	c.-316A>G	5′ UTR	Affects transcription factor binding [8]	0.184
	rs861539	p.Thr241Met	Exon, nonsynonymous	Might affect protein structure or function [12]	0.433
XRCC2	rs3218536	p.Arg188His	Exon, nonsynonymous	Modified sensitivity to DNA damaging agents [13]	0.094

[a]MAF: minor allele frequency in population of European descent included in HapMap project (HapMap-CEU)

Table 1. Most commonly investigated HRR SNPs and their predicted function.

Mutations in the *NBN* gene may lead to autosomal recessive disorder Nijmegen breakage syndrome, presenting with immunodeficiency, increased cancer risk and radiation sensitivity [6]. Rare *NBN* mutations were associated with chromosomal instability and increased susceptibility to cancer [15] and are presented in Table 2. The most common is a deletion of five nucleotides (675del5), common in Slavic populations [16], that leads to protein truncation [17].

Mutation	rs number	Predicted function
Asp95Asn	rs61753720	May affect protein-protein interactions [18], not highly damaging [19]
Ile171Val	rs61754966	Affects protein structure and protein-protein interactions [20]
Arg215Trp	rs61753718	Impairs histone γ-H_2AX binding [4]
Pro266Leu	rs769420	Probably damaging effect [8]
657del5		Leads to protein truncation [17]

Table 2. Most common mutations in the *NBN* gene.

Besides rare mutations, several common SNPs have been described in both the coding region and the regulatory regions of *NBN* gene (Table 1). By far the most frequently investigated

polymorphism is *NBN* rs1805794 (p.Glu185Gln) that leads to amino acid change in BRCA1 C-terminal domain [6] and could therefore affect protein-protein interactions with other HRR proteins. Polymorphic rs1805794 C allele was previously associated with decreased DNA damage detected with comet assay in healthy individuals [21]. It was also shown to modify the frequencies of chromatid-type aberrations [22]. *NBN* rs709816 (p.Asp399Asp) is a synonymous SNP that does not change the amino acid sequence in the central region of NBN. Two other *NBN* SNPs that may be functionally important, rs2735383 (c.*541G>C) and rs1063054 (c.*1209A>C), are located in the 3′ untranslated region (UTR). Rs1063054 was predicted to affect miRNA binding, but that was not yet validated [4, 8]. On the other hand, studies have already shown that rs2735383 modifies miR-629 and miR-509-5p binding and the polymorphic C allele was associated with lower transcriptional activity [9, 10].

3.2. *RAD51*

RAD51 is a key enzyme of HRR that has both DNA binding and ATPase activities. It interacts with many proteins, for example RAD51 paralogs, BRCA1, BRCA2 and RAD54 [23]. Several SNPs have been described in *RAD51* gene, but only few are located in the coding region. On the other hand, there are SNPs in the 5′ UTR that may affect both gene transcription and protein expression, such as *RAD51* rs1801320 (c.-98G>C) and rs1801321 (c.-61G>T) that were reported to increase promoter activity [11, 24]. *RAD51* rs1801320 polymorphism was also associated with protein over-expression and increased DNA repair [11]. The polymorphic rs1801321 allele facilitates binding of a transcription factor, thus increasing the transcription of the *RAD51* gene [24]. This polymorphism was associated with decreased DNA damage detected with comet assay in healthy individuals [21] and lower amount of gamma radiation-induced chromatid breaks [24], suggesting a protective effect.

3.3. *XRCC3*

XRCC3 is one of XRCC proteins involved in the protection of cell from ionizing radiation and belongs to the RAD51 family [25]. XRCC3 deficiency affects RAD51 foci formation and leads to increased genetic instability and sensitivity to DNA damaging agents [26].

Only a few putatively functional SNPs have been described in the *XRCC3* gene. Among them, non-synonymous polymorphism rs861539 (p.Thr241Met) and rs1799794 (c.-316A>G) polymorphism in 5′ UTR were the most frequently studied. *XRCC3* rs861539 changes the amino acid residue, which could affect protein structure or function [12]. Polymorphic rs861539 allele was previously associated with decreased DNA damage detected with comet assay in healthy individuals [21] and had a protective effect against chromosomal aberrations [27], but not in all studies [28].

3.4. *XRCC2*

XRCC2 is also one of the RAD51 paralogs, necessary for successful HRR. It is essential in the early stages of HRR for the formation of RAD51 foci, but it does not require ATP binding [29].

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Studies have shown that XRCC2 deficiency leads to defects in RAD51 foci formation, markedly decreased HRR and increased DNA damage, as well as hypersensitivity to radiation [29-31].

Among SNPs that have been described in *XRCC2*, the only non-synonymous rs3218536 (p.Arg188His) polymorphism attracted the most attention, despite its relatively low MAF and very few individuals carrying two polymorphic alleles. A deletion or a non-conservative substitution in the position 188 markedly increased sensitivity to mitomycin C induced DNA damage, but the common Arg188His substitution only had a small influence on damage sensitivity [32]. As the variant *XRCC2* 188His allele was associated with increased resistance to cisplatin induced DNA damage, it was suggested that it could be associated with increased DNA repair capacity [13]. The observed differences could be partly due to the use of different DNA damaging agents. It was suggested that lesions caused by different agents could require more precise regulation of protein expression to reach full repair potential [13].

4. Genetic variability in HRR and cancer susceptibility

Due to important role of DSBs in carcinogenesis, several studies have investigated the role of HRR SNPs in cancer susceptibility. To overcome the problem of non-concordant effects observed in some studies, several meta-analyses have been performed. Meta-analyses have the advantage of larger sample sizes and better statistical power. Their results suggested that HRR SNPs may contribute to cancer susceptibility, but their role may not be the same in all cancer types or in all populations, especially as MAFs can differ substantially for some polymorphisms. Another shortcoming of the meta analyses is that gene-gene and gene-environmental interactions could modify the role of SNPs, but the results of meta-analyses are usually not adjusted for confounders. In addition, it is difficult to perform meta-analyses in rare cancers.

4.1. *NBN*

Genetic variability in *NBN* was associated with susceptibility to different hematological and solid tumors. Several meta-analyses have been published to date, showing that *NBN* mutations and polymorphisms may have different effects in different cancer types (Table 3).

Mutation / SNP	Reference	N of studies	N of cases/controls	Cancer type	Major observation*
Ile171Val	Bogdanova, 2008 [33]	4	2954/2531	Breast	No association
	Gao, 2013 [4]	10	4516/9951	Overall	Increased risk
		5	3301/3904	Breast	No association
		2	182/720	Lymphoma	Increased risk
	Zhang, 2012 [34]	5	3273/4004	Breast	No association
Arg215Trp	Gao, 2013 [4]	9	6728/9508	Overall	Increased risk
657del5	Zhang, 2012 [34]	9	7534/14034	Breast	Increased risk

Mutation / SNP	Reference	N of studies	N of cases/controls	Cancer type	Major observation*
rs1805794	Zhang, 2013 [35]	10	25365	Breast	Increased risk
	Gao, 2013 [4]	21	15184/54081	Overall	Increased risk
		10	9091/15154	Breast	Increased risk
		5	1053/9524	Lymphoma	Increased risk
		2	3440/2490	Prostate	Increased risk
	Vineis, 2009 [36]	4	∑4825	Bladder	Increased risk
	Lu, 2009 [37]	17	9734/10325	Overall	Borderline increased risk
		6	4595/3603	Breast	No association
		3	605/639	Lung	No association
		3	1446/1452	Bladder	No association
	Stern, 2009 [38]	13	6348/6752	Bladder	Modestly increased risk
	Wang, 2010 [39]	10	4452/5665	Breast	Decreased risk Not credible, some mistakes [40]
	Wang, 2013 [41]	6	2348/2401	Lung	Increased risk
	Yao, 2013 [42]	14	6642/7138	Breast	No association
	He, 2014 [43]	48	17159/22002	Overall	No association
		7	2837/2973	Urinary system	Increased risk
		5	1682/2213	Digestive system	Decreased risk
	Zhang, 2014 [44]	8	3542/4210	Urinary system cancer	Increased risk, especially in bladder cancer
	Gao, 2013 [4]	42	18901/21430		No association in subgroup analysis by cancer type, heterogeneity too big for overall analysis
rs2735383	Gao, 2013 [4]	13	7561/8432	Overall	Increased risk
		4	2915/3035	Lung	Increased risk
rs1063054	Gao, 2013 [4]	9	2757/5796	Overall	Increased risk

*the direction of association for the mutated or polymorphic allele; ∑ - the total number of cases and controls

Table 3. Observed influence of *NBN* genetic variability on cancer risk in meta-analyses.

Rare mutations in the *NBN* gene have a more deleterious effect on the gene function and therefore have a bigger influence on cancer risk [4]. Even though the results of individual studies differed, several meta-analyses observed similar influence of various *NBN* mutations

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on cancer risk (Table 3). *NBN* 657del5 mutation was associated with increased overall cancer risk, as well as increased risk for breast cancer, prostate cancer, and lymphoma [4, 34, 35]. Interestingly, Ile171Val mutation did not predispose to increased breast cancer risk [4, 33, 34], but it was associated with overall increased risk of cancer and increased lymphoma risk [4]. The results of the meta-analysis showed that Arg215Trp mutation also significantly increased the overall cancer risk, in contrast with Asp95Asn and Pro266Leu mutations that were not associated with increased cancer risk [4].

Most of the meta-analyses investigating the role of *NBN* polymorphisms in cancer susceptibility were limited to the non-synonymous rs1805794 SNP (Table 3). Most studies have confirmed that polymorphic allele modestly increases bladder cancer risk [36, 38, 43]. The results in other cancer types were more ambiguous as some studies observed an association with increased cancer risk, but several did not (Table 3). Interestingly, in one meta-analysis decreased risk was observed for cancers of digestive system [43]. Decreased risk was also reported in some studies in rare cancer types such as acute myeloid leukemia [45] or osteosarcoma [46]. The observed discrepancies could be due to large heterogeneity between studies. Also *NBN* genotype distribution differs among populations, as the variant rs1805794 C allele is more common in some populations [21, 47]. Therefore it is not surprising that meta-analyses observed significant influence of *NBN* SNPs only in specific subgroups: only in Caucasians [37], only in Asians [41], or only among smokers [38]. Further studies should pay special attention to these differences as they could help explain discrepancies among studies. As the effect of a particular SNP may differ among cancer types, analyses should be stratified by cancer type. However, this can present a problem in rare cancer types, as it may be difficult to achieve sufficient power.

Among other *NBN* SNPs, one meta-analysis included two SNPs in the 3′ UTR, rs2735383 and rs1063054. The results suggested that both SNPs contribute to increased overall cancer risk [4]. However, when the analysis was stratified by cancer type, rs2735383 was only associated with increased lung cancer risk, but no significant association with bladder, nasopharyngeal cancer or leukemia was observed. *NBN* rs709816 was not associated with modified cancer risk in any of the studies [17].

4.2. *RAD51*

RAD51 rs1801320 is the most studied polymorphism in this gene despite its relatively low MAF. Several meta-analyses were published on the influence of rs1801320 on breast cancer risk until 2011, but they were mostly inconclusive [48-52]. Several shortcomings in the analyses associated with data and inclusion of these studies were later noted [53], suggesting that many of these studies were unreliable. More recent meta-analyses are presented in Table 4. Some suggested that rs1801320 may increase breast cancer susceptibility [54, 55], but one of the studies suggested a potential role of this polymorphism only in individuals with *BRCA2* mutations [56]. BRCA2 directly interacts with RAD51 and influences intracellular transport as well as function of RAD51 [57], thus playing an important role in HRR, so these observations are biologically plausible.

RAD51 rs1801320 SNP was also associated with increased overall cancer risk in the two largest meta-analyses that included more than 40 individual studies [54, 55], however no association was observed in an earlier study [56]. Increased risk for several cancer types, including hematological malignancies, ovarian, colorectal, and endometrial cancer was observed in a recent study [55], but not all were replicated in other studies (Table 4).

Another *RAD51* polymorphism, rs1801321 was investigated in only one meta-analysis and even though overall cancer risk was not modified [54], the decreased risk in carriers of polymorphic allele for head and neck cancer confirmed the results of previous studies [24]. Decreased breast cancer risk was also observed in carriers of polymorphic allele [58]. The suggested protective role of rs1801321 is in concordance with the described biological effect of this polymorphism.

Reference	N of studies	N of cases/controls	Cancer type	Major observation*
He, 2014 [59]	10	2656/3725	Myelodysplastic syndrome and acute leukemia	No association
	3	726/604	Myelodysplastic syndrome	Increased risk
Wang, 2013 [56]	39	19068/22630	Overall	No association
	7	1605/3121	Acute myeloid leukemia	No association
	14	11709/11291	Breast	No association
	6	2388/4411	Ovarian	No association
Cheng, 2014 [60]	22	6836/8507	Overall	No association
	4	1237/1340	Squamous cell carcinoma of the head and neck	Increased risk
	4	753/720	Colorectal	No association
	5	2001/2420	Ovarian	No association
	9	2845/4027	Acute leukemia	No association
Zhao, 2014 [54]	42	19142/20363	Overall	Increased risk
	17	11716/9839	Breast	Increased risk
Shi, 2014 [61]	10	2648/4369	Ovarian	No association
Li, 2014 [62]	6	1764/3469	Acute myeloid leukemia	No association
Zhang, 2014 [55]	45	28956/28372	Overall	Increased risk
	19	19171/17198	Breast	Increased risk
	7	2169/3629	Hematological malignancies	Increased risk
	4	3598/3002	Ovarian	Increased risk
	4	1202/1216	Head and neck	No association

*the direction of association for the polymorphic allele

Table 4. Observed influence of *RAD51* rs1801320 on cancer risk in meta-analyses.

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4.3. *XRCC3*

XRCC3 is by far the most studied HRR gene in cancer susceptibility studies. More than 50 meta-analyses focusing on *XRCC3* rs861539 SNP have been published, so only recent studies published in 2014 are presented in Table 5. The polymorphic rs861539 allele was associated mostly with increased breast and bladder cancer risk, but decreased lung or skin cancer risk [63-65]. An interesting observation is almost consistently observed increased cancer risk in carriers of polymorphic allele from Asian populations, while usually no association was observed in Caucasian populations or when different populations were combined.

Reference	N of studies	N of cases/controls	Cancer type	Major observation*
Mao, 2014 [66]	36	23812/25349	Breast	Slightly increased risk, especially in Asians
Xing, 2014 [67]	8	3215/3106	Lung	No association
Yuan, 2014 [12]	4	5173/7800	Ovarian	No association
Feng, 2014 [68]	8	3455/4435	Glioma	No association
Li, 2014 [69]	5	1507/3623	Larynx	No association
Adel Fahmideh, 2014 [70]	5	3374/3734	Glioma	No association
Chen, 2014 [26]	15	4329/7291	Overall	No association
	8	2056/3920	Non-melanoma skin cancer	Decreased risk
	5	1324/2209	Basal cell carcinoma	Decreased risk
	3	732/1711	Squamous cell carcinoma	Decreased risk
Qin, 2014 [71]	9	2209/3269	Gastric	No overall, association, increased risk in Asians
Yu, 2014 [72]	6	723/1399	Thyroid	No overall association, increased risk in Caucasians
Yan, 2014 [73]	7	1070/1850	Leukemia	No overall association, increased risk in Asians
Yan, 2014 [74]	7	3635/5473	Ovarian	No association
Qin, 2014 [75]	15	2339/4162	Leukemia	No overall association, increased risk in acute myeloid leukemia

Reference	N of studies	N of cases/controls	Cancer type	Major observation*
Wang, 2014 [76]	12	2209/3269	Gastric	No overall association, decreased risk in Asians
Du, 2014 [77]	23	7777/9868	Overall (Chinese mainland population)	Increased risk, especially cervical and nasopharyngeal cancer
Wang, 2014 [78]	10	4136/5233	Glioma	No overall association, increased risk in Asians
Liu, 2014 [79]	13	4984/7472	Brain tumors	No overall association, increased risk in Asians
Ma, 2014 [80]	18	5667/7609	Bladder	Increased risk
Peng, 2014 [81]	16	5608/6197	Bladder	Increased risk

*the direction of association for the polymorphic allele

Table 5. Observed influence of *XRCC3* rs861539 on cancer risk in recent meta-analyses.

Only a few meta-analyses were performed for *XRCC3* rs1799794. This polymorphism in 5'UTR was associated with increased overall and breast cancer risk in earlier studies [64, 82], but the association with breast cancer was not confirmed [83] and a decreased ovarian cancer risk was observed in a more recent meta-analysis [12].

4.4. *XRCC2*

The majority of cancer susceptibility studies focused solely on the *XRCC2* rs3218536 SNP. Different types of cancer were investigated, but most studies were performed in breast and ovarian cancer. Recent meta-analyses summarized in Table 6 tried to overcome the discrepancies observed between individual studies [61, 84-86]. All meta-analyses observed an association of the polymorphic rs3218536 allele with decreased ovarian cancer risk [61, 84, 86]. On the other hand, no association with breast cancer risk was observed in the most recent meta-analyses [61, 84, 86], confirming the results of a previous meta-analysis [85]. Although overall cancer risk was also not significantly affected by *XRCC2* rs3218536 [86], it was suggested that different cancer types should be evaluated separately [84]. However, a conclusive role of *XRCC2* rs3218536 in other cancer types is still difficult to ascertain, due to the limited number of studies investigating a particular cancer. Nevertheless, polymorphic *XRCC2* rs3218536 could be associated with increased risk for cancer of upper aerodigestive tract [84].

Apart from separate evaluation of different cancer types, further studies should investigate the possible interactions that could modify the role of XRCC2 SNPs. Several studies on breast cancer reported an association only in specific subgroups of patients, suggesting that besides genetic variability, also environmental factors and gene-environment interactions could

contribute to cancer risk. Such interactions could also help to explain the effect of low penetrance variants on cancer risk.

Reference	N of studies	N of cases/controls	Cancer type	Major observation*
Yu, 2010 [85]	16	18341/19028	Breast	No association
He, 2014 [84]	14	17420/17811	Breast	No association
	6	3035/5554	Ovarian	Decreased risk
	3	499/583	Upper aerodigestive tract	Increased risk
Shi, 2014 [61]	9	3279/5934	Ovarian	Decreased risk
Zhang, 2014 [86]	33	26320/28862	Overall	No association
	12	17230/16485	Breast	No association
	6	3035/5554	Ovarian	Decreased risk

*the direction of association for the polymorphic allele

Table 6. Observed influence of *XRCC2* rs3218536 on cancer risk in meta-analyses.

5. Genetic variability in HRR and cancer treatment outcome

Cancer treatment is often associated with severe adverse effects, however there is considerable interindividual variability regarding the occurrence and severity of adverse effects and regarding treatment efficacy. As cancer treatment is usually based on the use of chemotherapeutic agents and radiation therapy, whose cytotoxic effect results from their ability to induce DNA damage, pharmacogenetic factors such as polymorphisms in DNA repair pathways can contribute to observed differences.

Different agents may cause different forms of DNA damage. DSBs can occur due to the formation of strand crosslinks after treatment with alkylating and platinum-based compounds. Mechanisms involved in DSB repair may also lead to increased sensitivity to topoisomerase inhibitors such as camptothecines, anthracycline, and etoposide. DSB repair may be also important for the repair of radiation-induced DNA damage. Genetic variability of HRR may thus play a role in resistance to chemotherapy, in treatment efficacy and in occurrence of treatment related toxicities.

There are a lot less pharmacogenetic studies investigating the role of genetic variability in HRR in cancer treatment outcome compared to studies on cancer susceptibility. In addition, many studies are small and/or inconclusive and the shortcoming of most of the studies is that DNA repair capacity itself was not measured. Most pharmacogenetic studies focused on *XRCC3* polymorphisms and were predominantly investigating their influence on treatment with platinum compounds. *XRCC3* rs861539 was associated with shorter survival in ovarian and colorectal cancer [87, 88]. Most studies were however performed in non-small cell lung cancer

(NSCLC), where *XRCC3* rs861539 was associated with better response rate. Even though this effect was not observed in all the studies, recent meta-analyses confirmed the possible prognostic value of *XRCC3* rs861539 in response to cisplatin-based chemotherapy in NSCLC patients (Table 7). Although individual studies observed the association of this SNP with longer overall survival of NSCLC patients [89, 90], that was not confirmed in meta-analyses [91-93]. Several studies also observed an association of *XRCC3* rs861539 with decreased toxicity of platinum compounds in malignant mesothelioma, colorectal cancer and other malignancies [94, 95]. *XRCC3* rs1799794 was also associated with decreased odds of developing treatment related toxicities in malignant mesothelioma [95]. Some of the discrepancies observed between studies could be explained by different chemotherapy regimens used in different cancer types.

Reference	N of studies	N of cases	Major observation*
Shen, 2013 [92]	7	1186	Better response to chemotherapy, no significant influence on overall survival
Qiu, 2013 [91]	8	1289	Better response to chemotherapy, no significant influence on overall survival
Zhang, 2013 [93]	7	1514	No significant influence on overall survival

*the direction of association for the polymorphic allele

Table 7. Meta-analyses of *XRCC3* rs861539 and treatment outcome in non-small cell lung cancer.

The role of genetic variability in other HRR genes in cancer treatment outcome is currently not well established. Pharmacogenetic studies of other HRR genes were limited to individual studies in particular cancer types. *NBN* polymorphisms have been associated with increased treatment-related toxicity of gemcitabine-platinum combination chemotherapy in patients with malignant mesothelioma [95]. On the other hand, *NBN* rs1805794 was associated with longer progression-free survival in NSCLC patients treated with platinum-based chemotherapy, suggesting it might serve as a favourable prognostic factor [96].

RAD51 rs1801320 and rs1801321 polymorphisms were also associated with altered survival in NSCLC and cervical cancer patients [97-99], but no prognostic role was observed in malignant mesothelioma or sarcoma patients [95, 100].

Similar to other HRR genes, the potential influence of *XRCC2* on cancer treatment outcome was not studied as often as cancer risk. The low MAF of *XRCC2* rs3218536 could be a part of the reason why there is a lack of studies regarding treatment outcome. *XRCC2* rs3218536 was associated with decreased survival in pancreatic cancer and NSCLC patients [99, 101], but the association was significant only in specific subgroups of patients. In pancreatic cancer patients, treated with chemotherapy and radiation, the polymorphic *XRCC2* rs3218536 allele was associated with decreased survival only in patients treated with 5-fluorouracil based chemoradiation, but not in patients treated with gemcitabine based chemoradiation [101]. These

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differences further support observations that the effect of HRR polymorphisms may depend on the type of DNA damage.

Radiation therapy is used for treatment of up to 50% of cancer patients [102]. Adverse events are common and affect patients' quality of life [103]. They occur mainly locally in irradiated sites and therefore vary between cancer types. Acute toxicities affect rapidly proliferating tissues, but are usually transient and reversible [102]. Erythema and dermatitis are common skin acute adverse events, radiation pneumonitis is a typical complication in lung cancer, while urinary and bowel toxicities occur in prostate cancer.

The new field of radiogenomics aims to identify SNPs associated with radiation toxicity that could be used for personalized radiation therapy of cancer patients, for example patients with low risk for adverse events could receive higher doses of radiation [103]. As DSBs represent the most harmful effect of radiation, several studies have been published regarding HRR SNPs and radiation toxicity.

NBN polymorphisms did not influence toxicity in prostate, breast or lung cancer [99, 104, 105], but *NBN* rs1805794 was associated with oral mucositis in head and neck cancer patients treated with radiation or chemoradiation [106]. *RAD51* rs1801320 was also associated with toxicity in head and neck cancer in one study [107], as well as radiation pneumonitis in lung cancer patients [99]. Other studies did not report any association of *RAD51* SNPs and radiation toxicity [104, 108-110]. *XRCC2* rs3218536 was not associated with radiation toxicity in any of the studies [99, 104, 111]. Numerous studies investigated the role of *XRCC3* poymorphisms in radiation toxicity, but the results are not conclusive. Several studies found no association [99, 105-107, 109, 111-113], but carriers of polymorphic *XRCC3* rs1799794 allele had more toxicity after radiation treatment of prostate cancer [104, 114] and *XRCC3* rs861539 was associated with increased radiation toxicity in nasopharyngeal cancer [115, 116].

Comparison of radiogenomics studies is difficult, as they were performed in different cancer types treated with different radiation therapy protocols, sometimes in combination with chemotherapy. Additionally, different toxicities were selected as endpoints. Nevertheless, the published data suggest the impact of some of the HRR polymorphisms on the outcomes of radiation therapy, however meta-analyses are needed to validate these observations.

6. Conclusions

The combined evidence from different studies and meta-analyses suggests that SNPs in HRR genes contribute to carcinogenesis and could serve as markers of cancer susceptibility. As HRR proteins often interact in DNA repair, future studies should evaluate if combinations of SNPs in different HRR genes may serve as a better predictor of susceptibility to various cancers.

Cancer treatments are often characterized by a narrow therapeutic index and a balance between the desired therapeutic effect and the acceptable treatment-related toxicity has to be achieved. In the future, the improved understanding of the role of HRR genetic variability in the response to treatment of a particular cancer with a particular chemotherapeutic regimen

could contribute to identification of predictive or prognostic biomarkers that could help to stratify patients based on their risk for adverse events and guide treatment selection. Thus, treatment from which a particular patient would benefit the most could be selected.

In conclusion, genetic variability in HRR may modify DNA repair capacity and may therefore play an important role in both cancer susceptibility and the outcome of cancer treatment. A better understanding of the role of SNPs in HRR genes in different cancers and cancer treatments is however needed before they could be employed as markers of cancer susceptibility or treatment outcome in personalized medicine.

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Chapter 10

Paulownia tomentosa (Princess Tree) Extract Reduces DNA Damage and Induces DNA Repair Processes in Skin Cells

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Additional information is available at the end of the chapter

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1. Introduction

1.1. Skin DNA damage and repair

Skin is the largest and one of the most complex organs in human body, accounting for almost 15% of total body weight. It serves as an important environmental interface and thus acts as a first line of defense against various environmental insults [1]. Skin is organized into three main layers, epidermis, dermis and subcutaneous layer. The epidermis, an outermost avascular layer, is formed by keratinocytes at the epidermal basal layer that differentiate into corneocytes at the outer layer of the epidermis. The dermis lies below the epidermis separated by a basement membrane and is composed mainly of fibroblasts. The primary function of skin is to constitute an efficient barrier to protect the organism both from water evaporation [2] and from damage, as such skin is exposed to many external and internal aggressors which can induce DNA damage, including ultraviolet radiations (UVR). The ultraviolet radiation component of sunlight is the most important environmental inducer of damage in the skin. Ultraviolet radiations can induce damage on DNA bases by direct absorption of photons resulting in the direct effect of cyclobutane pyrimidine dimers (CPD) or the 6-4 photoproducts formation both created by dimerization of contiguous pyrimidines on the DNA [2,3]. Ultraviolet radiation can also induces significant damage to skin cells through the generation of Reactive Oxygen Species (ROS) which produce secondary damage to DNA nucleobases and the sugar phosphate backbone [2]. Different forms of DNA damage can result from the type of ROS generated (singlet oxygen and hydroxyl radicals through the formation of superoxide radicals), different modifications are generated to DNA such as bulky (8-oxo- guanosine, as

guanine is the most easily oxidized base, Thymidine and Cytosine glycol) and non-bulky (cyclo purine and etheno adducts) base modifications, spontaneous hydrolysis of a normal or damaged nucleobase leading to an abasic site, (see review [4). Finally ROS may also generate other forms of DNA damage such as single strand breaks (SSB) or double strand breaks (DSB) when the free radical attack is located on the poly- deoxy- ribose chain [2]. In addition to ultraviolet radiation, pollution and cigarette smoke can also act as external aggressors and favor DNA damage onset by depleting intracellular anti-oxidant molecules such as glutathione and thus shifting the oxidative balance to favor oxidation by ROS. In addition to external aggression, cells are also subjected to internal aggression from ROS generated by oxidative metabolism or respiration as well as to the attack of genotoxic or photo-sensitizers coming from the diet [2].

The integrity of DNA is one of the key factors affecting the viability of healthy organisms, living cells have developed strategies not only to prevent DNA damage but also to minimize the impact of DNA damage by efficiently repairing any damaged DNA. In human cells, DNA is repaired by different mechanisms: Base Excision Repair (BER), Nucleotide Excision Repair (NER), Single and Double stranded Breaks Repair (SSBR and DSBR), Homologous Recombination (HR) and Mismatched repair. Basically, DNA alterations without strand breaks are repaired mainly by excision repair mechanisms where the damaged bases are removed from the DNA molecule by excision and then replaced the right bases [2]. In the case of the Nucleotide Excision Repair (NER) an oligonucleotide fragment of approximately 25-30 nucleotides is removed around the damaged DNA and the gap generated in the DNA duplex is filled by DNA synthesis using the opposite, normal DNA strand as a template. To complete the process of NER, the last nucleotide incorporated is covalently joined to the extent DNA by ligation [5]. BER consists of four to five steps in which specific enzymes play a role: excision of the damaged base by a glycosylase, incision of the resulting abasic site, processing of the generated termini at the strand break, DNA synthesis and ligation [6,7]. A third mechanism called mismatched repair occurs when only one nucleotide mismatch appears in the DNA double chain. This mechanism is particularly effective for the repair of DNA error arising during replication due to the limited fidelity of the replicative machinery. Finally, DNA double strand breaks can be repaired by a specific process called homologous recombination and non homologous end joining [2,8].

The importance of the DNA repair process and its relevance in the skin physiology is apparent in genetic disorders affecting genes responsible for DNA repair. Xeroderma Pigmentosum (XP), Cockayne syndrome (CS) and Ataxia telangiectasia (AT) are genetic diseases resulting from rare autosomal recessive pathologies involving DNA repair enzymes that are deficient due to inactivating mutation in their genes [9,10,11]. These diseases are characterized at the level of the skin by extreme sensitivity to sunlight, resulting in sunburn, pigmentation changes, an early onset of the appearance of skin aging signs and a greatly elevated incidence of skin cancers in particular for XP disorder [12]. These changes can be explained by long lasting DNA damages that induces prolonged cellular inflammation through the activation of the NF-κB pathway [2,13,14,15,16] and an acquired immune deficiency [17] as well as rapid accumulation of mutation leading to cell apoptosis, senescence and cell tumorigenesis [18,19,20,21].

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Humans share repair pathways with plants, particularly nucleotide excision repair (NER). NER is essential in removing major damage to DNA which interferes with the genetic code. Due to similarities in DNA damage and repair mechanisms in plants and humans, metabolites such as polyphenols produced by plants may provide beneficial effects in humans [22].

2. Photo protective mechanisms in plants

Sun light is a source of energy to sustain all types of life on earth including plants and animals. Sun light exposure specifically excessive sun light exposure can be harmful to plants and animals. Plants do not have the ability of movement therefore plants developed various mechanisms to protect them from harmful effects of sun light. Most harmful effects come from UVB part of sun light. It is known that excess UVB exposure adversely affects plant growth and development in many ways including nutrient uptake and photosynthesis rates [23]. At a cellular and molecular level DNA is the most important target for UV radiation specifically concerning are exposures from UV-B and UV-C regions of the spectrum [24]. The table below documents adverse effects of UV radiation on some additional cellular and molecular targets of plants. The adverse effects on a molecular level also alter the genetic makeup of cells by introducing mutations in DNA.

Target sites of UV radiation in plants	Effects on the targets	Select References
Proteins	Inactivation of proteins & enzymes	[25, 26]
Amino acids	Photooxidation resulting to decomposition and/or generation of reactive species	[27, 28]
Lipids	Lipid peroxidation mostly at a point of unsaturation	[29, 30]
Growth factors	Degradation, Inactivation, Stimulant	[31] [32]
Pigments	Chlorophylls, Carotenoids	[33]
Membranes	Transport Phenomena	[34]
Photosynthesis	70-80% plants are sensitive to UV radiation	[35] [36]

Table 1. Plants are adversely affected by excessive exposure of UV radiation

To adapt to the continuous insult from excessive sun light exposure, plants developed physical, enzymatic and non-enzymatic mechanisms to not only protect but also to repair the damage done from indiscriminate exposure of UV radiation. Some of the secondary metabolites for example polyphenols such as green tea polyphenols have been studied [37]. Green tea polyphenols plays protective role by mediating DNA repair and reduction in skin inflammation. Polyphenols of various classes used topically [38 or consumed via diet as fruit and vegetables [39] helps to scavenge free radicals produced during exposure of light and also to

mediate additional signaling pathways leading to ultimate damage of DNA at a molecular level.

3. Botanical extracts — Princess Tree as an antioxidant

Paulownia tomentosa is commonly known as Princess Tree, Empress Tree, Royal Empress Tree, Royal Paulownia, Fox glove tree, Kiri (in Japan), PaoTong (China), and Odong-Namoo (Korea). Paulownia plants are well respected in Japan, China and most of East Asia for its tradition, uses and quality of wood. According to traditional literature flowers and leaves are cooked and consumed occasionally for the treatment of fever and pain, and skin ailments [40]. Recently the wood of Princess Tree has also been reported to possess anti-oxidant activity. The major polyphenol found in Princess Tree wood is Paulownin which belongs to a class of chemistry called lignan.

4. Phytochemical profile

Paulownia plants are a rich source of phytochemistry documented by many studies and are reviewed comprehensively [41]. Expressed phytochemistry as a function of a part of the Paulownia tomentosa plant is shown in Table-1 below. Fruit, flower, and leave express specific chemistries of prenylated flavonoids, essential oil, and terpenoids resp. There are other examples of non-specific expression of flavonoids and phenolics by aerial parts and woody parts of the plant.

Class of Chemistry	Part of the plant	Select Examples
Flavonoids	Leaves, bark, fruit, Stem, Flowers	Apigenin, kaempferol, Luteolin, Quercetin, Catechin, Naringenin, Taxifolin.
Prenylated Flavonoids	Fruit	Prenylated taxifolin
Geranylated Flavonoids	Flower, Fruit	Mimulone, Diplacone, Diplacol, Schizolaenone C, Prokinawan, Tomentodiplacone, Tomentin
Phenolic glycosides	Bark, Stem, wood	Syringin, coniferin, Acteoside, Campenoside, Ilicifolioside, Isoverbascoside, Cistanoside
Lignan / Phenolics	Wood, Leaves, bark, Flowers	Paulownin, Sesamin, Piperitol, Vanillic acid, gallic acid, cinnamic acid, coumaric acid
Quinones	Stem, Bark	Furanoquionones, plumbagin
Terpenoids	Leaves	Iridoids: Paulownioside, catalpol, aucubin, tomentoside
Glycerides	Leaves exudates	Acyl glycerols
Essential Oil	Flower	Cosanes, benzyl alcohol

Table 2. Phytochemicals reported from parts of Paulownia tomentosa plant (derived from 2014 review cited above)

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Essential oil from Paulownia flower is reported with identification of major components [42, e.g. benzyl alcohol (13.72%), phenol, 3,4-dimethoxy-methyl ester (3.64%), phenol, 2-methoxy-3-(2-popenyl)-methyl ester (6.24%), 1,2,4-Trimethoxybenzene (8.32%), tricosane (3.28%), and pentacosane (3.26%). A number of additional studies are also reported with similar chemical composition of Paulownia flower oil [43 and their anti-microbial activities. The heartwood of Paulownia is known to express Paulownin, Sesamin, lapachones, sterols, and naphoquinones.

5. Princess Tree reduces UV-induced reactive oxygen species and cellular inflammation

5.1. Free radicals & ROS

Antioxidants primarily mitigate the negative effect of free radicals through their radical-scavenging ability. These antioxidants stabilize radicals by donating electrons and thus preventing oxidation of DNA or other cellular components. While the body is equipped with its own defense system against reactive oxygen species (ROS) and other free radicals produced in the body, it also relies on external (exogenous) antioxidants including those contained in food. As environmental conditions lead to premature aging, a search for a suitable antioxidant product is vital [22].

Free radicals cause damage in the body because of their instability and high reactivity. ROS are of particular interest. During aerobic respiration, mitochondrial electron transport results in the formation of a ROS (superoxide) as a by-product. Solar UV radiation also leads to formation of ROS. Oxygen is particularly vulnerable to radical formation due to its electronic configuration with two valence shell unpaired electrons. Thus, there are several types of ROS including superoxide, hydrogen peroxide, nitric oxide, and hydroxyl radical. Free radicals of other atomic species specifically nitrogen are also formed within the body [22].

ROS can potentially react with other cellular entities including DNA which can lead to DNA modification and ultimately bodily harm. The guanine base in DNA is particularly susceptible to attach by ROS formed by solar UV radiation. Oxidation reactions which modify the guanine base may also lead to single-strand breaks in DNA [44]

While the effects of oxidative stress on the body vary according to type and duration, cells often halt division (enter G0 phase) and may even undergo apoptosis under severe stress. The general response to oxidative stress is cell cycle arrest through the expression of various inhibitor proteins (such as p21). Nevertheless, ROS also serve useful roles within the body including intracellular and intercellular communication [44].

5.2. Antioxidants combat oxidative stress

While broad-spectrum sunscreen which absorbs and reflects harmful solar radiation remains the most effective protection against immediate solar UV damage (which result in CPD

formation), antioxidants are crucial in combating oxidative stress caused by ROS. Skin's antioxidant system consists of vitamins (vitamins C and E), enzymes (catalase and superoxide dismutase), glutathione, and coenzyme Q10 (CoQ10). As these antioxidants perform their protective actions and are degraded by ROS, they are reactivated by other antioxidants. Because several types of ROS may be formed through environmental insult, several types of antioxidants are produced in the skin. Thus antioxidants come in various forms (vitamins, enzymes, etc.) and may be either lipophilic or hydrophilic to function in a variety of areas [22].

During tissue damage and the subsequent inflammation, a number of mediators are released which have been shown to modulate DNA repair. The activation of the Melanocortin Receptor 1 (MCR1) by either its natural ligand, the α-Melanocyte stimulating Hormone αMSH or synthetic analogs [20,21] can enhance the DNA repair activity in cells. Also two interleukins (IL), IL12 and IL23 known to display anti-tumor activity [45,46,47,48] have been shown to accelerate the repair of UVB induced CPDs [2]. Activation of detoxifying mechanisms such as the NRf2 pathway may enhance also DNA repair [49]. Finally mono- and poly- ubiquitilation as well as sumoylation play an important role in the regulation of DNA repair (see review [50]). Thus inflammatory mediators can directly affect the DNA repair process and therefore could be regulatory factors either enhancing or repressing DNA repair. Recent studies have identified that the NF-κB pathway, which is a key regulator in the expression of inflammatory proteins, may be an important mediator in DNA damage and the subsequent repair [2].

Paulownin and Paulownin rich extracts from wood of Paulownia tomentosa were studied for their anti-oxidant and for skin protective effects. Preincubation with Princess Tree wood extract at concentrations from 0.1% to 5% significantly attenuated hydrogen peroxide production in a dose-dependent manner (Figure 1A, *P<0.05 compared with UV exposed vehicle treated epidermal equivalents). UV-induced hydrogen peroxide formation was determined using a modification of the method of Martin et al.,. Through free radicals scavenging activity Princess Tree wood extract may protect skin from oxidative stress that could result in DNA damage.

Paulownin and Paulownin rich extracts from wood of Paulownia tomentosa were studied for their anti-inflammatory activity and for skin protective effects. Preincubation with Princess Tree wood extract at concentrations from 0.1% to 5% significantly inhibited pro-inflammatory cytokine release. In the study shown in Figure 1B and 1C, Epidermal equivalents were topically treated (2mg/cm^2) with Princess tree extracts in 70% ethanol/30% propylene glycol vehicle 2 hours before exposure to solar ultraviolet light (1000W-Oriel solar simulator equipped with a 1-mm Schott WG 320 filter; UV dose applied: 70 kJ/m2 as measured at 360nm). Supernatants were analyzed after 24 hours for IL-1A and IL-8 cytokine release using commercially available kits. These results clearly demonstrate that Princess Tree wood extract can reduce the skin inflammation and damage resulting from UV exposure.

5.3. NF-κB Signal transduction

Nuclear factor-κB (NF-κB) consists of a family of transcription factors that play critical roles in inflammation, immunity, cell proliferation, differentiation, and survival [52. The NF-κB family of transcription factors shares a high-conserved sequence of amino acids within their

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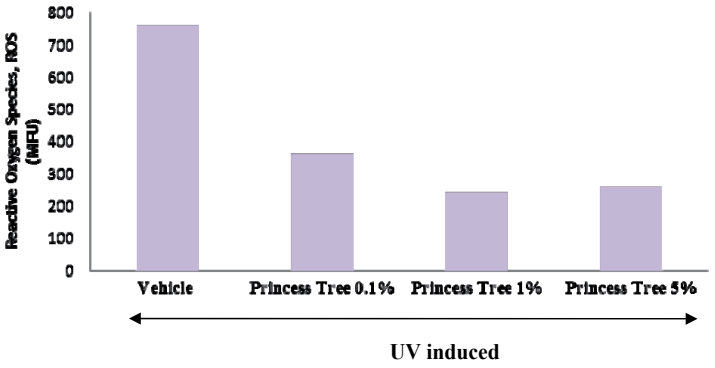

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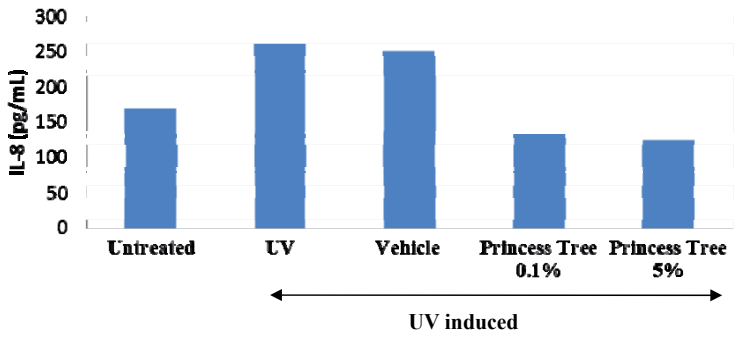

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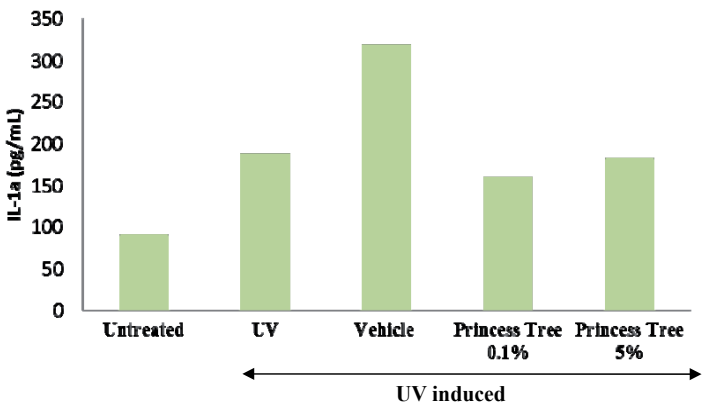

(c)

Figure 1. (a). Princess tree mitigates UV-induced ROS (b). Princess tree inhibits UV-induced pro-inflammatory mediator IL-8 (c). Princess tree inhibits UV-induced pro-inflammatory mediator IL-1α

amino terminus, which contains a nuclear localization sequence that is involved in the dimerization with sequence-specific DNA binding and with the inhibitory IκB proteins [2].

In unstimulated cells, NF-κB family proteins exists as heterodimers or homodimers that are sequestered in the cytoplasm in an inactive form by virtue of their association with a member of the IκB family of inhibitory proteins, most notably IκBα, IκBβ and IκBγ [2,53,54]. About 200 extracellular signals can lead to activation through the dissociation of NF-κB from the IκB proteins. These activating signals include viral and bacterial products, oxidative stress, pro-inflammatory cytokines including IL-1 and TNF-α, and phorbol esters [2,55,56,57,58,59]. Ultraviolet (UV) radiation from sunlight induces IL-1 and TNF-α and creates reactive oxygen species that then leads to NF-κB-mediated inflammation [2,60,61]. The kinase activity of IκK phosphorylates two serine residues (Ser32 and Ser36) on IκB proteins, which results in the ubiquitination and degradation of IκB by the proteasome. The degradation of IκB reveals the nuclear localization sequence of NF-κB [53,54]. Free NF-κB can then translocate to the nucleus and bind to a NF-κB *consensus* sequence present within the promoter region of target genes, thereby upregulating the expression of hundreds of genes, including cytokines (IL-1, -2, -6, etc.) [2], immunoreceptors (immunoglobin kappa light chain, MHC class I, etc.), cellular adhesion molecules (ICAM-1, VCAM-1, ELAM-1), and many others [59].

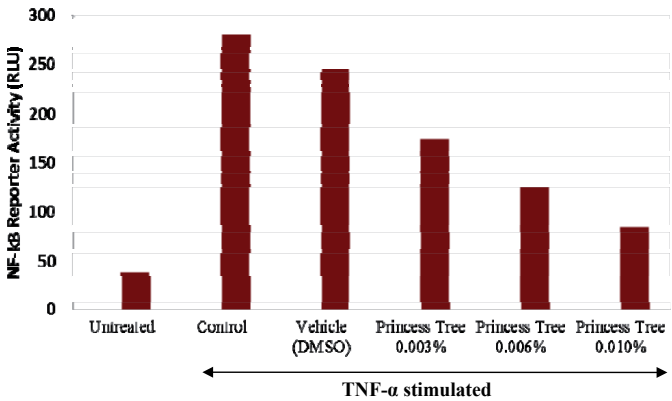

Figure 2. Princess tree inhibited NF-κB promoter activity

Paulownin and Paulownin rich extracts from wood of Paulownia tomentosa were studied for their NF-κB activity. Preincubation with Princess Tree wood extract at concentrations from 0.001% to 0.01% significantly inhibited NF-κB activation. In the study shown in Figure 2, a cell line expressing a NF-κB promoter gene and internal control Renilla luciferase reporter gene were treated with the indicated doses of Princess tree and stimulated with Tumor Necrosis Factor-α (TNFα). Cell lysates were analyzed using Dual-Luciferase Reporter System. These results establish that Princess Tree wood extract can reduce the activation of NF-κB and thus may reduce the cellular damage resulting from UV exposure.

5.4. NF-κB and DNA damage

The NF-κB pathway has been shown to be regulated by ionizing radiation at both the mRNA and protein levels by Brach et al., who demonstrated that NF-κB transcripts were transiently

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increased after irradiation, which was preceded by enhanced DNA binding activity of this transcription factor [62]. Nuclear DNA double strand breaks (DSBs) are one of the most potent DNA damage signals to activate NF-κB [2]. This process can occur within 1–2 h after break induction through activation of the canonical inhibitor of κB (IκB) kinase (IKK) complex and IκBa degradation [15]. NF-κB can be activated by Topoisomerase inhibitors (such as camptothecin) potentially via the generation of double strand breaks as well [16]. Furthermore activation of IKK following treatment with topoisomerase inhibitors was described to be dependent on the zinc finger domain in NF-κB essential modulator (NEMO) [50]. DSBs can trigger two independent signaling cascades that eventually lead to the induction of NF-κB via NEMO [61]. In one case, DSBs can activate ATM, which in turn can bind to and phosphorylate NEMO. In a parallel cascade, the p53-induced protein with a death domain (PIDD) translocates to the nucleus leading to the SUMOylation of NEMO. Consequently, the resulting activation of NF-κB favors cell survival by turning on the transcription of several anti-apoptotic gene [2]s. In response to DSB, PIDD as well as ATM are capable of initiating cascades leading to pro- or antiapoptotic signals, NF-κB presumably being a part of the pro-survival cascade [61]. Miyamoto et al., have summarized this model of NF-κB activation by DNA damage as a 'two signal' model as it requires coincident NEMO SUMOylation and ATM activation by double strand breaks to permit robust NF-κB activation [15]. Taken together these findings suggest that NF-κB may be both have both causal and effector roles in the development of DNA damage [2].

5.5. NF-κB and the DNA repair process

Although the mechanisms by which NF-κB affects DNA damage are not fully established, one possibility is that NF-κB may either directly or indirectly regulate DNA repair processes in cells. Protecting cells from apoptotic cell death following DNA damage is one of the major ways that NF-κB activation regulates the DNA repair process [2]. Wang et al., have demonstrated that NF-κB functions as a positive modulator of cellular senescence, an intrinsic tumor suppression mechanism, by showing that human fibroblasts lacking NF-κB activity prematurely exit from senescence [63]. Others have shown that skin cells devoid of NF-κB activity exhibit deregulated growth correlating with impaired cell-cycle control [64,65]. It has been proposed that the role of NF-κB in cellular senescence could be cell type specific, differentially initiating senescence or acting further downstream in the DNA repair process to maintain the senescent state [2,63]. DNA damage caused by chemical genotoxic agents, such as camptothecin, has been described to activate the Ataxia Telangiectasia-Mutated (ATM) kinase and NEMO (IκB kinase), leading to the inducing of NF-κB p50/p65 heterodimer [66]. In a parallel signaling pathway, ROS can be generated by genotoxic agents in sufficient quantities to activate the NF-κB pathway. ROS can also act as signaling molecules in immune responses, cell death and inflammation, where NF-κB is involved [66]. Depending on the relative degree of DNA damage, multiple mechanisms of NF-κB activation are engaged. Physical genotoxic agents such as UVA or hydrogen peroxide lead to extensive oxidative damage within the cytoplasm which can signal the activation of NF-κB pathway in the absence of DNA damage [2].

Among the various types of DNA damage, repairing double strand breaks can be particularly challenging to cells [67,68], and may contribute to genomic instability associated with most cancers [68,69,70,71]. Wiesmuller et al., have shown that NF-κB is involved in double strand removal and repair via a stimulatory action on homologous repair, involving the targets ATM and the tumor suppressor gene BRCA2 [72]. NF-κB is known to bind to the BRCA2 promoter and activate BRCA2 gene expression [73]. The role of NF-κB in ATM function and DNA repair was demonstrated by Siervi et al., in T-cells where levels of ATM mRNA and protein were significantly reduced by NF-κB blockade [74]. Activation of NF-κB by ATM results in an anti-apoptotic signal in the cells. Wiesmuller et al. have also described that NF-κB utilizes multiple mechanisms to enhance homologous recombination, including stimulation of the activity of CtIP–BRCA1 complexes to trigger DNA end processing, and upregulation of ATM and BRCA2 for strand transfer [72].

The nuclear factor p53 controls several physiological processes including DNA repair and cell cycle arrest. Cross-talk between NF-κB and p53 has been established by multiple groups ([75,76]; see review [77]), including results that suggest NF-κB may have both anti- and pro-apoptotic roles. Only a limited number of studies have investigated the role of NF-κB in DNA damage and repair in skin cells (including: [64,65,78,79,80,81]). Evaluation of the p53-NFκB cross-talk by Puszynski et al. in HaCat keratinocytes cells showed that inactivation of NF-κB improved p53-mediated DNA repair and prevented arsenite-induced malignant transformation of HaCaT cells [80]. Marwaha et al. have shown that in primary skin cells, such as dermal fibroblasts and keratinocytes, treatment with T-oligos led to the up-regulation and activation of p53, coinciding with decreased NF-κB DNA binding activity and inhibition of transcription from NF-κB-driven promoter constructs [79]. Thyss et al. have demonstrated that the sequential activation of NF-κB, Egr-1 and Gadd45 cascade induces UVB-mediated cell death in epidermal cells [81], a process that was crucial in order to eradicate the cells that bear the risk of becoming tumorigenic. In HaCat keratinocytes, hydroxytyrosol (main component of olive oil shown to be an inhibitor of NF-κB), has been shown to significantly reduce the DNA strand breaks caused by UVB, and also attenuate the expression of p53 and NF-κB in a concentration-dependent manner [78].

5.6. Princess Tree reduces DNA damage

The skin is the largest organ protecting the body against external threats such as physical, environmental and biological insults. Among the harmful environmental factors, solar ultraviolet radiation (UVR) is the major one causing cellular and molecular modifications in the skin like photo-aging and eventually leading to genomic instability and cancer ([3, 82]. UV-induced generation of Reactive Oxygen Species (ROS) such as O_2 , H_2O_2, OH° in the skin, develops oxidative stress when their formation exceeds the antioxidant defense ability. UVR penetrates the skin, reaches the cells, and is absorbed by DNA, leading to the formation of photoproducts that inactivate the DNA functions. Oxidation of DNA can product different types of DNA damages: strand breaks, sister chromatid exchange, DNA-protein crosslinks, sugar damage, abasic sites and base modifications [83,84]; [4]. One of the major DNA oxidation products formed as a result of such damage is 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxo-

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dG). DNA damage by UVB irradiation results also from photochemical reactions consequent to direct absorption of photons by DNA bases [51]. The UV-induced DNA lesions that have been studied in most detail are the cyclobutane pyrimidine dimer (CPD) and the 6-4 pyrimidine–pyrimidone photoproduct (6-4PP) at adjacent pyrimidines [51,85,86].

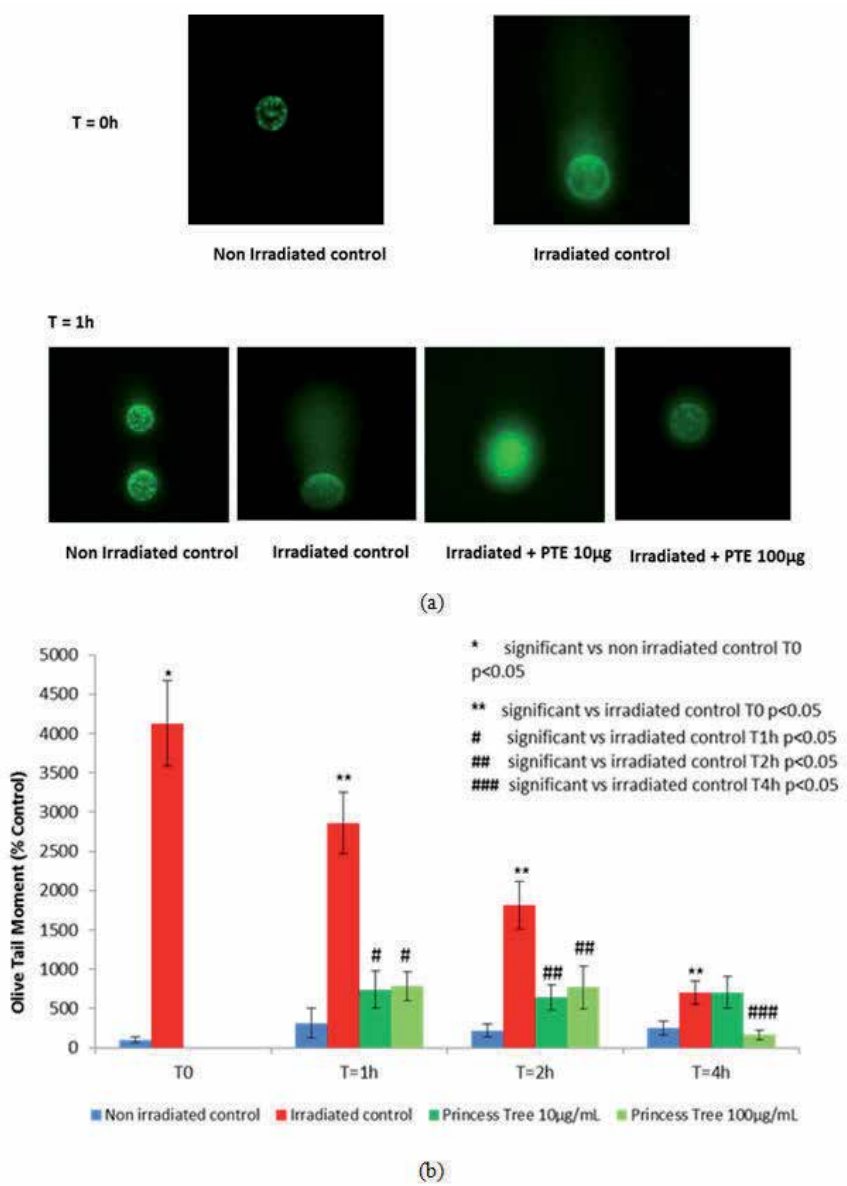

Figure 3. (a). Treatment of primary human keratinocytes with Princess Tree increased repair of UV-induced DNA damage (b). Treatment of primary human keratinocytes with Princess Tree increased repair of UV-induced DNA damage.

Mammalian cells have evolved several DNA-repair pathways to remove all the categories of DNA base lesions, relying in particular on DNA excision mechanisms. One of these, nucleotide excision repair, removes bulky adducts and is thus an essential mechanism for correcting UV-induced DNA damage [87,88]. The base excision repair pathway corrects small base modifications such as oxidized and alkylated bases [88,89]. The importance of repair mechanisms is demonstrated by the hazardous consequences of genetic defects in DNA repair [88,90,91].

Princess Tree wood extract was investigated for the capacity to promote DNA repair after UV insults (6DEM) using Comet assay. After 1 hour treatment, both concentrations of Princess Tree (10 and 100 μg/ml) reduced significantly UV-induced DNA damage in Normal Human Epidermal Keratinocytes (NHEK) when compared to UV-irradiated control as shown by the fluorescent images and the quantification of the comet tail (Fig 3A and 3B, $p<0.05$). The Princess Tree wood extract treatment increased the DNA damage repair rate. Indeed, 4 hours were needed for the UV-irradiated control to reach the same level of DNA damage/repair compared to the Princess Tree wood extract treated conditions.

Princess Tree wood extract direct effects on mitigating DNA damage may be by an indirect mechanism, such as the inhibition of NFkB pathway known to be regulated by ionizing radiation at both the mRNA and protein levels [62]

6. Summary and conclusions

6.1. The use of botanical extracts for protection from DNA damage and DNA repair

Skin is under continual assault from a variety of damaging environmental factors such as ultraviolet irradiation and atmospheric pollutants. As organisms age the cumulative damage exceeds the capacity of endogenous antioxidant defenses resulting in oxidative damage. Furthermore, during oxidative stress the elevation of NF-κB transcriptional activity may contribute to the decrease in DNA repair capacity of skin cells and thereby lead to the accumulation of DNA damage. Since NF-κB is activated by DNA damage, there is a potential for a vicious circle to take place as more NF-κB may decrease the capacity of the cell to repair damages and lead to a longer persistence of the DNA damage. Plants have adapted to chronic exposure to ultraviolet irradiation by producing phytochemicals which can mitigate reactive oxygen species and repair damaged DNA. Botanical extracts such as Princess Tree (Paulownia tomentosa) which can modulate the NF-κB pathway, a primary pathway linking inflammation and DNA damage, can prevent the deleterious effects of DNA damage in cells (Figure 4). Through the ability to scavenge free radicals, inhibit NF-κB activation, reduce DNA damage and induce repair of damaged DNA, Princess Tree may protect skin from numerous external aggressions encountered daily and reduce the damage to oxidatively challenged skin.

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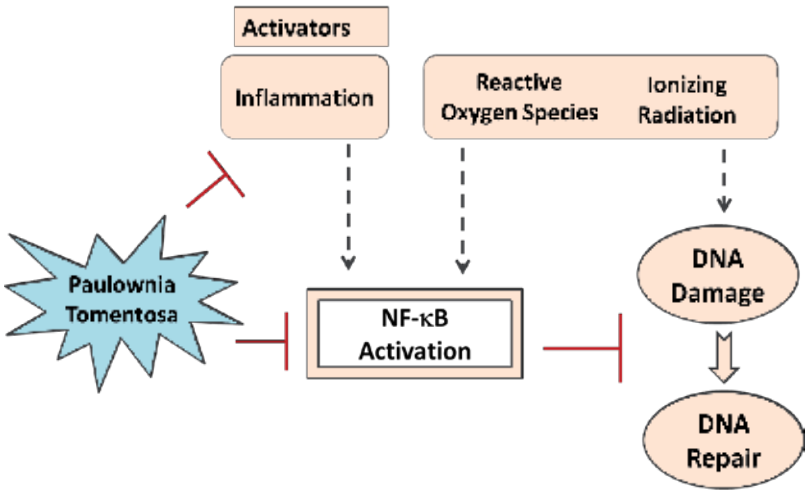

Figure 4. Proposed model showing the effects of Paulownia tomentosa on DNA damage and repair

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Chapter 11

Targeting DNA Repair Mechanisms to Treat Glioblastoma

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Additional information is available at the end of the chapter

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1. Introduction

The current standard of care for the treatment of glioblastoma multiforme (GBM) includes the surgical resection of the tumor in combination with ionizing radiation and the DNA alkylating agent Temozolomide (TMZ). The introduction of TMZ into clinical use has improved patient outcomes [1, 2]. Stupp et al. showed that the addition of TMZ to radiotherapy lengthened the median survival in patients with GBM from 12.1 months to 14.6 months [2]. TMZ exerts an effect upon GBM cells by preferentially damaging the DNA of the rapidly growing tumor cells, ultimately resulting in their death. While the combined use of TMZ and ionizing radiation can increase overall survival, the long-term survival for GBM patients is still poor [3]. What has recently become apparent is that GBM tumors can develop several forms of resistance to the DNA damage-induced cell death caused by radiotherapy and TMZ treatments. In this way, GBM tumors can survive and generate new tumors when they should otherwise not survive. This review will discuss mechanisms of resistance to DNA damage-induced cell death in GBM tumors and will outline some DNA repair functions that can be targeted to potentially improve treatment outcomes.

Maintaining the integrity of the genome is essential for the health and survival of multicellular organisms. The continuous exposure of cellular DNA to potentially harmful environmental and internal insults necessitates redundant and overlapping DNA repair mechanisms. Several excellent reviews have extensively described the wide variety of DNA repair mechanisms used by cells in response to DNA damage [4-6]. Damage to DNA can result in cell cycle arrest to allow for DNA repair mechanisms to occur, or can stall replication forks during DNA repli-

cation causing senescence. Proliferating cells, like those in GBM tumors, are affected to a greater extent than quiescent cells following DNA damage, causing the cells to arrest at particular points within the cell cycle [7]. However, cancers such as GBMs are quite adept at repairing the DNA damage or over-riding the cell cycle checkpoints to allow cell proliferation to continue despite the damage.

GBM tumors respond to DNA damage induced by ionizing radiation and TMZ treatment through increased expression of DNA repair enzymes, including the proteins O-6-methylguanine-DNA methyltransferase (MGMT) and Poly (ADP-ribose) Polymerase 1 (PARP-1) [8]. Furthermore, tumors are able to eliminate chemotherapeutic compounds from cells through the increased expression and activity of ABC transporters, specifically ABC-1 [9]. Compounding this issue is the growing body of evidence indicating that a small population of slow-growing cancer stem cells reside within the GBM tumor (also called glioma-initiating cells) and are responsible for the subsequent recurrence of GBM tumors [10, 11]. Glioma initiating cells are particularly resistant to standard treatments, in part through the elevated expression of enzymes responsible for repair of DNA damage [12, 13]. Therefore, successful destruction of GBM tumors may require a combined approach utilizing standard treatments in combination with inhibition of DNA repair pathways. This approach to cancer treatment, called synthetic lethality, preferentially affects cancer cells by inhibiting several molecular processes necessary for tumor survival without significantly affecting normal tissues [14, 15]. This treatment approach utilizing the DNA repair enzymes MGMT and PARP-1 have been a focus of the research conducted at the Upper Michigan Brain Tumor Center, and this review will be supplemented with findings from our laboratory.

Temozolomide. TMZ is easily absorbed after oral administration, readily crosses the blood-brain barrier and is better tolerated than its parent compound, mitozolomide. TMZ is an imidazotetrazine prodrug that is converted to a compound, 5-(3-methyltriazen-1-yl)imidazole-4-carboximide (MTIC), capable of alkylating DNA and displays antitumor activity in a variety of cancer types [16]. Spontaneous conversion of TMZ to MTIC is pH dependent and is a chemically controlled reaction [1]. MTIC's most common sites of methylation are at the N7 position of guanine followed by the N3 position of adenine. The N7-methylguanine is stable and makes up 80-85% of all alkyl adducts, whereas, N3-methyladenine is readily hydrolyzed and comprises only 8-18% of adducts [8, 17]. The O6 position of guanine only makes up 5% of lesions but is the most stable of the three and persists in the DNA in the absence of MGMT enzyme activity [8, 17]. O6-methylguanine is considered to be the most lethal of the alkyl adducts. For a visual representation of these common adducts, see Figure 1.

O-6-Methylguanine-DNA Methyltransferase. The MGMT enzyme reduces the cytotoxicity of O6-alkylating agents like TMZ by catalyzing the transfer of methyl groups from the O6 position of guanine to a cysteine residue within the active site of the MGMT enzyme [1]. The transfer of ethyl or alkyl groups to MGMT renders the MGMT enzyme inactive, leading to its degradation via the proteosomal pathway [18]. Therefore, continued MGMT function requires the de novo expression of MGMT protein in order to provide resistance to chemotherapeutic agents. Unfortunately, many GBM tumors exhibit increased MGMT expression, which reduces the effectiveness of alkylating agents such as TMZ. GBM tumors with hypermethylated MGMT

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promoter regions do not develop resistance to the drug indicating that reduced MGMT expression in tumors is a clinically relevant and potentially important cellular phenotype to consider during treatment.

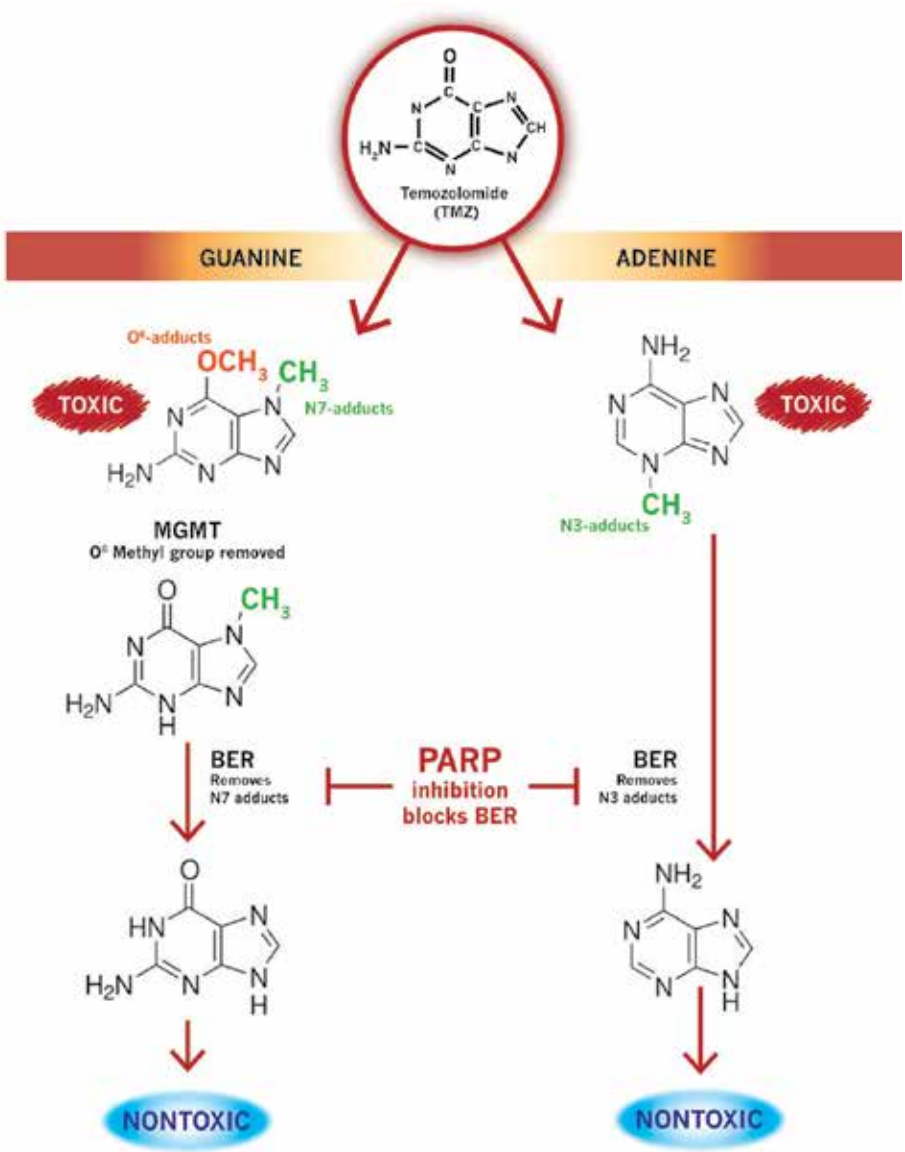

Figure 1. Common temozolomide-induced DNA lesions appear on guanine and adenine. N7-methylguanine, N3-methyladenine and O6-methylguanine DNA adducts account for roughly 70%, 10% and 5% of these lesions respectively. O6-methylguanine adducts are the most cytotoxic, yet are readily repaired by MGMT, resulting in TMZ resistance. The less toxic N7-methylguanine and N3-methyladenine adducts are readily repaired by the base excision repair (BER) system. The inhibition of PARP blocks BER and increases toxicity.

Poly (ADP-ribose) Polymerase 1. PARP-1 is a cell-survival factor that functions in single-stranded break repair (SSBR) to maintain genomic integrity [19]. The zinc-finger domain of PARP-1 binds to DNA nicks and adds the polyanion ADP-ribose (PAR) to histone proteins H1 and H2B [20]. PAR addition to histones relaxes the 30nm chromatin allowing access of DNA repair enzymes to the DNA. Inhibition of PARP-1 in proliferating cells sensitizes cells to DNA damage resulting in cell cycle arrest [21].

2. O-6-methylguanine-DNA methyltransferase

MGMT Mechanism of Action. The MGMT gene is located on chromosome band 10q26.3 spanning 300kb, with 5 exons and 4 introns. It encodes the 207 amino acid MGMT repair enzyme that is highly conserved among species and plays a critical role in maintaining the integrity of genomic DNA. The MGMT 1.2kb promoter region is TATA-and CAAT-box free with numerous CpG islands. Expression levels of MGMT vary significantly between tissues and can be regulated by glucocorticoids, cAMP, protein kinase C, and DNA damage [22].

The transcription factor Sp1 functions in transcriptional regulation of the MGMT gene, and CpG methylation within the promoter sequence affects chromatin structure to affect Sp1 access to the promoter site [23]. Methylation of specific CpG clusters in the promoter is correlated with MGMT gene silencing [24]. However, the overall amount, location, and homogeneity of MGMT promoter methylation is variable in GBM [25]. Dunn et al. investigated 109 newly diagnosed GBMs, and found that 58 tumors had an elevated methylation status compared to non-neoplastic brain tissue (≥ 9% methylated). Furthermore, 19 of the tumors examined with a methylation status greater than 35% correlated with the highest 2-year survival rates [26]. It is not fully understood what determines MGMT promoter methylation levels, but recent evidence indicates that p53 may play a role. Using human lung cancer cells, Lai et al. showed that the knockdown of p53 increases MGMT promoter methylation in wild type p53 lung cancer cells [27] while Srivenugopal et al. reported that inducible p53 expression suppresses MGMT levels in a p53-null lung cancer cell line [28]. In contrast, published work by Wang et al. suggests that hypermethylation of CpG islands within the MGMT gene does not strictly correlate with reduced MGMT protein expression [29]. A number of studies have highlighted the variability in MGMT promoter methylation and MGMT gene expression levels indicating both a variability in MGMT activity within and between tumors [30]. More recently, Kanemoto et al. performed deep sequencing analyses of the entire MGMT promoter to develop a diagnostic assay for progression-free survival of GBM patients based upon hypermethylation of CpG islands. Despite the evidence that variability in MGMT promoter methylation does exist, the data confirm the general hypothesis that hypermethylation of the MGMT promoter does correlate with reduced MGMT enzyme activity [31].

The MGMT enzyme functions as both a transferase and acceptor of alkyl-groups. MGMT activity does not require cofactors or other enzymes, rapidly removes DNA adducts from the O6 position of guanine, and transfers them to an internal cysteine residue (Cys145) within the enzyme active site [32]. This reaction is stoichiometric and once the MGMT protein has been alkylated, it is inactivated and undergoes ubiquitin-mediated degradation [33].

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Most alkylating agents used to therapeutically induce cell death target the O6-methylguanine adduct. While MGMT primarily repairs O6-methylguanine DNA adducts, it has the ability to repair adducts of greater size (i.e. O6-ethylguanine) as well as the minor alkylation product O4-methylthymine. MGMT-mediated repair pathways correct the damage caused by alkylating chemotherapeutic agents utilized in the treatment of gliomas, melanomas, carcinoid tumors, and lymphomas, such as carmustine, temozolomide, streptozotocin, procarbazine, and dacarbazine [32].

Clinical Implications of MGMT. As noted above, MGMT specifically reverses the DNA damaging effects of TMZ, and hypermethylation of the promoter for the MGMT gene correlates with reduced MGMT production. This finding has clinical implications, as highlighted by Stupp et al. and Hegi et al. in companion 2005 papers [2, 34]. Stupp et al. reported results from a multicenter, randomized trial comparing adjuvant radiation therapy to radiation therapy plus temozolomide for the treatment of glioblastoma. The median survival for the temozolomide group was 14.6, versus 12.1 months in the radiation only group. The two year survival rate was 26.5% in the temozolomide group versus 10.4% in the radiation only group. Perhaps the more interesting findings came from subgroup analysis. Hegi et al. stratified the two treatment groups based on MGMT promoter methylation. Though less than half of the patients enrolled in the clinical trial had usable DNA methylation data, the results, summarized in Table 1, are nonetheless compelling. In nearly a decade since the publications of these papers, the conclusion that MGMT promoter methylation sensitizes malignant glioma to temozolomide has been confirmed in multiple clinical and cohort studies [35-38]. MGMT promoter methylation status is an important prognostic biomarker and it appears that MGMT methylation status should be considered when formulating the treatment plan [39].

3. Inhibition of O-6-methylguanine-DNA methyltransferase

The key mechanism of resistance to alkylating agents in GBM is the presence of MGMT enzyme, and most human tumors exhibit high levels of MGMT expression and activity. As mentioned previously, elevated expression of MGMT is inversely correlated with survival [34, 40, 41]. Thus, suppression of MGMT activity could render cells more sensitive to alkylating agents, augmenting cytotoxicity.

O6-benzylguanine. Of the agents to target MGMT suppression, O6-benzylguanine (O6BG) was the first developed and was thought to have the greatest potential. O6BG is an MGMT substrate that inactivates MGMT in a suicide manner by binding to the protein. In the early/mid 1990's, studies using O6BG to suppress MGMT in GBM, both *in vitro* [42, 43] and *in vivo* [44], showed increased sensitivity and cytotoxicity to alkylating agents. These data initiated several clinical trials [45-49]. Unfortunately, patients in these early phase trials also exhibited significant hematological toxicity to O6BG and late phase trials were not pursued. Although O6BG may someday find a very useful place in neuro-oncology, the data from these clinical trials suggested that safer and more effective therapeutic approaches were needed to target MGMT.

Methylation Status	Radiation Alone	Radiation and Temozolomide
MGMT Methylated		
Patients	46	46
Median progression free survival (months)	5.9	10.3
Median overall survival (months)	15.3	21.7
2 year survival (%)	22.7	46
MGMT Unmethylated		
Patients	54	60
Median progression free survival (months)	4.4	5.3
Median overall survival (months)	11.8	12.7
2 year survival (%)	0	13.8

Table 1. Data summarized from Hegi et al. 2005.

Gene Therapy. While O6BG was proving to be too toxic for systemic delivery, a promising method for treating malignant tumors was being developed, gene therapy. Several studies have targeted oncogenes both *in vitro* and *in vivo* using antisense oligonucleotides with varying success, as described in the review by Caffo et al. [50]. Further, multiple studies have applied RNA interference strategies to treatment of gliomas - a Pubmed search in September of 2014 using the key words "RNA interference and glioma" generated 707 citations. The disruption of genes via synthetic nucleotide sequences may provide a specific inhibition of tumor growth with minimal off-target effects.

In 2008, our lab used RNA interference *in vitro* to silence the MGMT gene in GBM cell lines expressing high (U138MG), low (U87MG) and non-detectable levels (LN229) of MGMT [51]. Baseline TMZ dose response curves indicated that GBM cells with high levels of MGMT expression exhibit the greatest resistance. The half maximum effective concentration (EC50) of TMZ was consistently higher in cells with higher MGMT expression, this finding was similar to previous reports [52]. After MGMT mRNA knockdown, U138 cells became more sensitive to TMZ as expected (Figure 2). Interestingly, we were unable to detect a gene expression change in the U87MG cells, even though siRNA treatment rendered these cells more sensitive to TMZ treatment. No gene knockdown occurred in the LN229 cells (MGMT was not detectable at baseline) and the EC50 in these cells did not change after attempting RNA interference. These data are consistent with the work of others [13, 53] suggesting that siRNA could be an effective therapeutic agent.

Although it is possible for siRNA to function efficiently using cells grown in culture, siRNA application within organisms is difficult. These siRNA macromolecules do not cross cell membranes easily and would make crossing the blood brain barrier problematic. Thus, novel delivery systems for siRNA are being investigated. One successful nanoparticle system being developed is the LipoTrust EX Oligo liposome delivery system [13]. Kato et al. reported that liposome delivery of siRNA to downregulate MGMT was effective in sensitizing GBM to TMZ

in both *in vitro* and *in vivo* models [13]. Nanoparticles, whole cells and viruses, alone and in combination, are options being explored for delivery of synthetic nucleotides [54, 55]. It appears that gene therapy is a realistic possibility and may provide patients with "personalized medicine" in the near future. Yet, there are major concerns with the use of the technology, including off-target gene effects. Further, there are concerns about the use of viruses for gene therapy because of their potential to induce mutations. Oncolytic viruses, which preferentially lyse tumor cells, can become more selective and effective when engineered to possess tumor specific transgenes. The conditionally replicating adenovirus is one such oncolytic virus that not only targets the tumor cell but also possesses a gene that blocks the recruitment of the transcriptional coactivator p300 to the MGMT promoter [56].

p53. DNA damage from alkylating agents, ionizing radiation and ultraviolet light induces p53 activity. The role p53 plays in MGMT expression is not fully understood. Knockdown of p53 increases MGMT promoter methylation [27], yet overexpression of wild-type p53 suppresses MGMT and renders human cancer cells more sensitive to TMZ [28]. A previous study also reported MGMT downregulation in p53-null osteosarcoma cells with the introduction of wild-type p53 [57]. Grombacher et al. demonstrated that the overexpression of p53 reduced basal MGMT promoter activity in rodent cells [58]. The mechanism of p53-mediated MGMT knockdown may be through physical binding of the MGMT promoter without altering the methylation status [59]. It has also been shown that p53 prevents Sp1 from binding to the promoter [60]. Because overexpression of p53 may suppress MGMT, targeting p53 may render GBM more sensitive to TMZ. Levetiracetam is often used in GBM patients to manage seizures, but its use may further benefit patients by augmenting p53-mediated MGMT suppression [61]. Interferon beta (IFN-β) was shown to mediate cytotoxicity in human GBM cell lines [62], most likely because IFN-β induces p53 and improves the response of GBM cells to TMZ treatment [63, 64]. More recently, the use of a mitogen-activated protein/extracellular signal-regulated kinase (MEK) inhibitor has shown promise as a TMZ adjuvant by activating p53 [65]. Molecules targeting miRNAs may also be effective at p53-mediated suppression of MGMT. For example, ways to suppress miR-21 may also reduce MGMT levels via p53 activation [66]. The utility of targeting p53 may be limited [64] due to the high incidence of p53 mutation in GBM [63].

Other MGMT Inhibitors. Other targets have been suggested for regulation of MGMT levels in the cell. For example, valproic acid has become an established anti-cancer drug because of it's role in histone deacetylation, but it has also been shown to downregulate MGMT in GBM cells [67]. The proteasome inhibitors Bortezomib and MG132 also suppress MGMT transcription [68, 69]. Altering the expression of tumor suppressor genes may also be an effective treatment strategy. For example, miR-181d has been shown to down regulate MGMT possibly via the K-ras-related Pi3K/AKT and MapK/ERK pathways [70]. Lastly, the cytokine interleukin-24 downregulates MGMT expression in human melanoma cells and the DNA crosslinking agent cisplatin suppresses MGMT in leukemia cells [71, 72].

It is worth noting that individualized therapy based solely on MGMT promoter methylation alone may not always be advantageous. MGMT promoter methylation may not correlate with TMZ sensitivity and survival in some populations [73]. Although it has been reported that the level of MGMT mRNA and protein expression is correlated with promoter methylation status

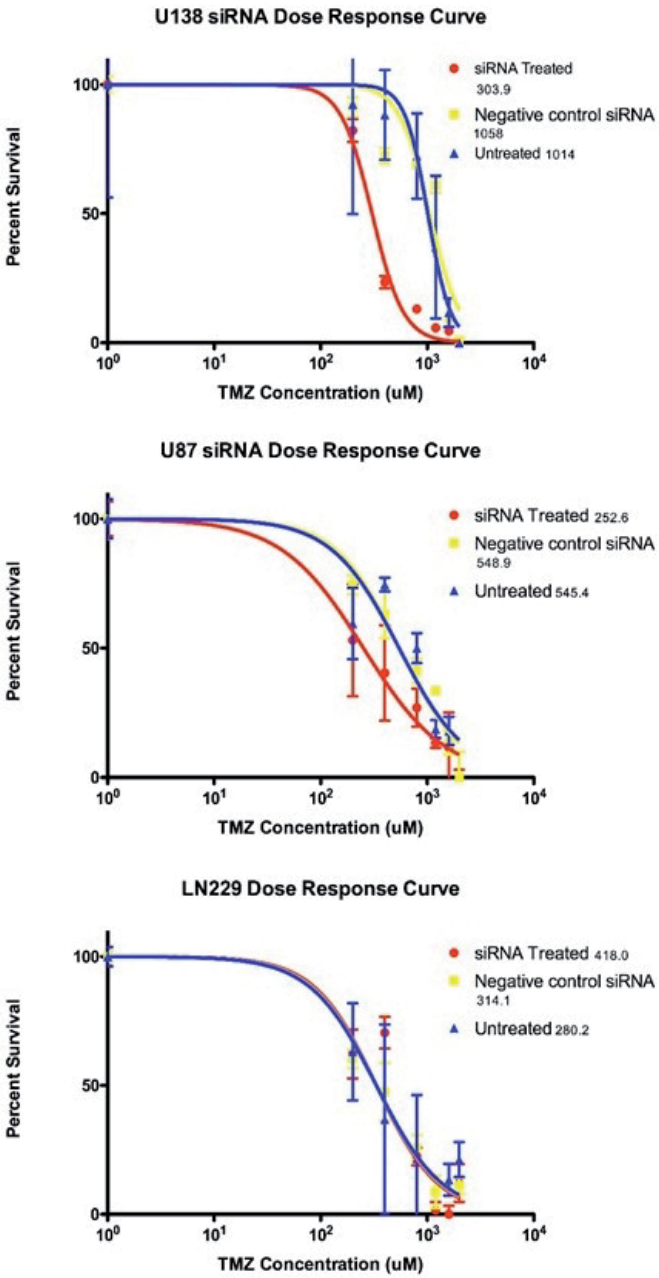

Figure 2. U138MG (high MGMT expression), U87MG (low MGMT expression) and LN229 (no MGMT detected) dose response curves from siRNA experiments (March 2008). The difference in EC50 between the siRNA treated group and the negative and untreated control group was statistically different for U138MG ($P<0.0001$) and U87MG ($P<0.01$), with no difference in the LN229 cell line. The figure legend represents the EC50 values for each treatment group in μM of TMZ.

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[74-76], this correlation does not hold true in all cases [73, 76-80]. Reports of GBMs with unmethylated MGMT promoter regions and low MGMT mRNA expression as well as GBMs with methylated MGMT promoter regions expressing high MGMT mRNA levels suggest that methylation-independent pathways may alter MGMT mRNA levels [74, 76, 77, 81]. Evidence suggests that mechanisms of post-transcriptional regulation alter MGMT protein expression since protein analysis of MGMT does not correlate with mRNA [76]. Recent data suggest that miRNA regulation of MGMT may explain these discrepancies and miRNAs are currently being investigated as therapeutic targets [70, 82, 83]. Because MGMT expression appears be predictive of progression free and overall survival [74], adequate assessment of tumors may need to include MGMT mRNA and/or protein expression.

MGMT Summary. MGMT plays a critical role in maintaining the integrity of genomic DNA. Unfortunately, elevated MGMT expression is correlated with poorer prognosis in cancer patients. Tumors expressing MGMT will not respond well to alkylating chemotherapy because MGMT corrects O6-methylguanine. Screening the tumor tissue not only for MGMT (expression and methylation status), but also for other genes that may suppress or increase MGMT expression will be important for successful management of GBM. Clinicians should be guarded, however, when determining the treatment strategy because not all GBMs display the same magnitude, locations and homogeneity of methylations [25]. Although many therapeutic targets have been found to suppress MGMT activity, research is still needed to determine which of these molecules will effectively suppress MGMT while being safely administered to the patient.

4. Poly (ADP-ribose) polymerase 1

PARP-1 Mechanism of Action. The human Poly (ADP-ribose) Polymerase (PARP) gene family is evolutionarily conserved and codes for 17 different enzymes. The most important member of the PARP family, PARP-1, is a key DNA repair enzyme located on chromosome 1q42 and is responsible for the majority of PARP activity in the cell. As a component of the base excision repair (BER) pathway, PARP-1 binds to single-strand DNA breaks, catalyzes the formation of ADP-ribose polyanions from its substrate nicotinamide adenine dinucleotide, and recruits additional repair enzymes to the damaged strand. In addition to its central role in DNA repair, the PARP-1 enzyme also regulates other vital biological functions as reviewed in Krishnakumar and Kraus, 2010 [84]. While PARP-1 protein expression is typically low in normal brain tissue, it is highly expressed in GBMs [85]. The transcription of PARP-1 appears to be primarily regulated by the transcription factors SP1 and NFI, but, AP2, YY1 and ETS also bind to promoter sites of the PARP-1 gene [86].

Clinical Implications of PARP-1. In GBM, the effectiveness of radiation and chemotherapy is mitigated by normal cellular DNA repair mechanisms. It follows that interfering with DNA repair will enhance DNA damaging treatments. A number of preclinical studies confirm that PARP inhibition strengthens the efficacy of several DNA damaging anticancer therapies including radiation, DNA methylating agents, and topoisomerase I inhibitors [87]. A particu-

larly exciting application of PARP inhibition is in cancer cells with defects in homologous recombination. The loss of both single strand repair and repair of double strand DNA breaks is fatal [88]. This example of synthetic lethality demonstrates that these two loss-of-function mutations prove fatal, whereas either mutation alone is not. Clinical trials have utilized PARP inhibitors in two ways: in combination with DNA damaging therapies and as a single agent for tumors deficient in homologous repair (e.g., BRCA1/2 mutated breast cancers) [87]. Further, epidermal growth factor receptor (EGFR) status may act as a predictive biomarker for PARP inhibition sensitivity [89]. The EGFR gene is overexpressed in ~50% of GBMs [90]. Nearly 20% of grade III and IV tumors possess the constitutively active class III variant (EGFRvIII) and correlates with elevated expression of the gene [91]. Clark Chen's group recently determined that GBM cells over-expressing EGFRvIII were dependent on the BER system and PARP-1 related function for cell survival [89]. Following pharmacological inhibition or PARP-1 silencing with siRNAs, cytotoxicity was increased in GBM cells expressing elevated levels of EGFRvIII. It was suggested the increased cytoxicity was due to the inability to correct the damage caused by reactive oxygen species and the effect was greater when coupled with radiation [89].

5. Inhibition of poly (ADP-ribose) polymerase 1

Cancer treatments utilizing ionizing radiation and DNA alkylating agents damage DNA, which if not repaired causes cell death. Inhibition of PARP-1 contributes to the sensitization of tumor cells to these treatments and is the basis for multiple preclinical and clinical studies with PARP inhibitors in combination with classical therapies [92].

Benzamides. The inhibition of PARP-1 and the role of PARP-1 inhibition has been of interest since the early 1980's when it was demonstrated that PARP could be inhibited using nicotinamide analogues [93]. The initial interest in PARP inhibition was to determine the role of the enzyme in the cell. The ability to inhibit PARP was responsible for elucidation of the function of PARP and its role in DNA repair. Using 3-aminobenzamide, Sidney Shall's group demonstrated that inhibition of PARP resulted in disruption of the repair of DNA breaks created by treatment with the DNA alkylating agent, dimethyl sulfate. Additionally, they reported that PARP inhibition enhanced the efficacy of dimethyl sulfate as a cytotoxic agent [94]. This study was the first to suggest that combined treatments with DNA alkylating agents and PARP inhibition could be effective in the treatment of cancer. The benzamides were essential in determining the role of PARP and providing "proof of principle" that PARP inhibition could play a role in cancer treatment and increase the efficiency of DNA damaging agents. However, the benzamides are relatively weak PARP inhibitors and have been shown to interfere with cellular pathways not associated with PARP [95]. More recently, specific PARP inhibitors have been produced and several are currently in clinical trials. Second and third generation PARP inhibitors are more potent and require markedly lower effective concentrations to reduce 50% of PARP's activity [96].

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New Generation Inhibitors. PARP inhibition results from two different but complementary mechanisms, blocking PARP catalytic activity and the release of the enzyme from the DNA. The enzyme needs to be poly(ADP-ribosyl)ated to dissociate from DNA and if that ability is blocked by the catalytic inhibitor, it remains bound causing a physical obstruction to the repair of the DNA break. This was first proposed by Masahiko Satoh and Tomas Lindahl in 1992, who demonstrated that the repair of nicked plasmid DNA by nuclear extracts was not dependent on PARP, indicating that other repair pathways were still effective [97]. However, if PARP was present then its substrate, NAD+, was necessary for repair to occur. If this PARP-NAD+ repair requirement was blocked by 3-aminobenzamide, which competes for the NAD+ binding site, DNA repair was stopped. This indicated that inactive PARP impeded DNA repair, not only did PARP not recruit repair enzymes to the site of damage but also physically blocked other DNA repair pathways. Recent work expanded our understanding of the role of PARP inhibitors in DNA binding. DNA damage produced by alkylating agents administered concomitantly with a PARP inhibitor resulted in increased PARP–DNA binding compared to PARP–DNA binding with either the alkylating agent or PARP inhibitor alone [98]. In a similar study it was demonstrated that effectiveness of a PARP inhibitor might not only be its ability to inhibit the catalytic ability of the enzyme but also its ability to trap the enzyme on the DNA strand [99]. Using three potent PARP inhibitors (Olaparib (AZD-2281), veliparib (ABT-888), and niraparib (MK-4827)), currently being investigated clinically, it was demonstrated that all are effective in suppression of catalytic activity, having IC50 values that are in the low nanomolar range [100]. If we were to assume that the function of PARP inhibitors is solely explained by catalytic inhibition these three drugs should have similar effect and that effect should not differ from PARP deletion or silencing. However, it was reported that following treatment with the DNA alkylating agent, MMS, that while all three PARP inhibitors were effective in inhibiting catalytic activity, these clinically relevant PARP inhibitors differ markedly in their potency to induce cytotoxic PARP-DNA complexes. The authors indicated that, the potency in trapping PARP differed markedly among inhibitors with niraparib (MK-4827) > olaparib (AZD-2281) >> veliparib (ABT-888), a pattern not correlated with the catalytic inhibitory properties for each drug and suggested that further PARP inhibitor studies should examine both aspects, catalytic inhibition and DNA trapping, in inhibitor evaluation [100].

Inhibitors Versus Deletion or Silencing. It is becoming clear that there are subtle, but important differences between PARP deletion versus PARP inhibition. PARP-1 knockout mice are viable and fertile as are PARP-2 knockout mice. However, the deletion of both enzymes is lethal [101]. This indicates that while either enzyme can fill the DNA repair role, that role is essential. Additionally, it has been shown that PARP-$1^{-/-}$knockout mice are more sensitive to radiation than wildtype mice [102]. In addition to knockout mice, studies examining the effect of PARP silencing using RNA interference have been effective in reduction of PARP and PARP activity and have increased radiosensitivity in a manner equivalent to PARP-$1^{-/-}$knockout mice [103]. In our hands, silencing of PARP-1 in glioblastoma cell lines was generally not as effective as

treatment with a PARP inhibitor [104]. The greater efficacy of PARP inhibitors compared to PARP knockout or PARP silencing likely has to do with the mode of action of the inhibitors. This is an important consideration as it has been shown that inhibitors act on both PARP-1 and PARP-2, usually with similar potency and thus inhibition is more similar to the deletion of both enzymes. Additionally, it is well established that PARP in combination with an inhibitor blocks DNA repair in at least two ways.

PARP and Glioblastoma. Temozolomide acts by specific methylation of the DNA bases guanine and adenine resulting in inappropriate pairing during DNA replication. O6-methylguanine will trigger the DNA mismatch repair (MMR) pathway and become highly cytotoxic unless it is corrected by MGMT. However, nearly 80% of the total methylation events resulting from TMZ treatment are the N7-methylguanine and N3-methyladenine that trigger the BER system. The disruption of the BER pathway through PARP inhibition renders these lesions cytotoxic and helps overcome the MGMT related TMZ resistance. Another important consideration is the use of PARP inhibitors to increase TMZ sensitivity in tumors that exhibit MMR deficiencies, a relatively common occurrence in sporadic cancers. In these tumors, the lack of MMR, causes the cell to overlook the O6-methylguanine lesion rendering TMZ ineffective. Treatment with a PARP inhibitor renders the N3-methyladenine and N7-methylguanine lesions cytotoxic [105]. However, it has been reported that MMR deficiency does not seem to be responsible for mediating TMZ resistance in adult GBM [106].

Targeting PARP-1 and MGMT. In 2011 we examined the role that PARP-1 inhibition plays in altering GBM cell lines' response to TMZ [104]. We examined any changes in TMZ effectiveness in the GBM cells using either the PARP inhibitor, 3-aminobenzamide (3-AB) and compared its effect to silencing of PARP-1 using RNA interference. In our hands, the use of the PARP inhibitor was generally as, or more, effective than PARP silencing. Additionally, we examined the response to TMZ following PARP-1 silencing combined with MGMT silencing by RNA interference. The response to TMZ following silencing of both PARP-1 and MGMT was compared to the response to TMZ following PARP inhibition and MGMT silencing. In three of four GBM cell lines, the PARP inhibitor, 3-AB, combined with MGMT silencing rendered cells more sensitive to TMZ compared to silencing of both PARP-1 and MGMT or TMZ alone. These data suggest that GBM cells may be more sensitive to PARP inhibition versus PARP silencing, but also suggest that targeting both MGMT and the BER system may result in lasting TMZ-induced lesions leading to cell death.

PARP-1 Summary. PARP-1 and PARP-2 play critical roles which have been demonstrated by the lethality seen when both genes are deleted. One well defined role for these enzymes is DNA repair via the base excision repair pathway. Unfortunately, many cancer treatments including radiation and many types of chemotherapeutic agents act by damaging DNA. Much of this damage can be repaired through the actions of PARP. PARP inhibitors have been shown to bind effectively to both PARP-1 and PARP-2, block the enzymatic activity and may trap the inactivated PARP on the DNA lesion, effectively blocking additional DNA repair mechanisms. These actions have been shown to increase

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the sensitivity to radiation and DNA alkylating chemotherapies in preclinical studies. However, while promising, the result of most clinical trials has been somewhat disappointing as the PARP inhibitors as chemopotentiating agents has been limited by an increase in toxicities, necessitating dose reductions of the cytotoxic chemotherapeutic agent and the PARP inhibitor. Ongoing research re-examining the mechanism of action of the PARP inhibitors, including those that bind in sites other than the PARP catalytic site, may eliminate some of the off target binding and reduce inhibition of other members of the PARP family. Also, continued efforts to determine other pathways affected by the PARP inhibitors are necessary to make adequate decisions about clinical usage.

6. Conclusions

Surgical resection, radiation and use of TMZ is currently the standard of care for GBM patients. The alkylating agent, TMZ, induces lesions at the N7 and O6 positions of guanine and N3 position of adenine. However, many of these tumors express MGMT which promptly corrects the most cytotoxic lesion, the O6-methylguanine adduct. Tumors expressing MGMT are, therefore, inherently resistant to TMZ. MGMT inhibition improves the response to TMZ, but MGMT inhibition as standard therapy is still in development. O6BG, although too toxic to give systemically, may find itself useful for future therapy if delivery to the central nervous system can be improved. Gene therapy, with the enhancement of the delivery of synthetic nucleotides like the LipoTrust and oncolytic viruses, may become the standard of care in the future. There are several other molecules targeting pathways that influence MGMT and many more will surely emerge.

The majority (~80%) of these TMZ-induced DNA alkyl adducts, N7-methylguanine and N3-methyladenine, are repaired by the BER system. After the mismatched base has been removed, PARP-1 plays a role in repairing DNA breaks by binding and recruiting other BER proteins. Because of its role in the BER system, PARP inhibitors also improve the response to TMZ. While the results from numerous clinical trials have been disappointing due to systemic toxicities, new inhibitors may improve outcomes.

Modulation of TMZ resistance through the MGMT and BER pathways is clinically viable. New combinations of existing strategies may prove to further compliment TMZ and augment its effectiveness. Although several approaches have been used to modulate PARP and MGMT pathways, molecular screening should be used to identify targets with the greatest therapeutic potential. For example, pre-treatment assessment for MGMT and EGFR expression would provide information regarding the susceptibility to TMZ and PARP inhibitors, respectively. With the growing understanding of the pathways involved with DNA repair, the design of novel strategies or the use of combinations of existing therapies may improve GBM outcome.

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Chapter 12

DNA Repair and Chemotherapy

Seiya Sato and Hiroaki Itamochi

Additional information is available at the end of the chapter

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1. Introduction

Cancer chemotherapy is designed to kill cancer cells, with most agents inducing DNA damage. Highly conserved DNA repair machinery that process DNA damage and maintain genomic integrity developed during the evolution of mammalian cells. Interestingly, in established tumors, DNA repair activity is required to counteract oxidative DNA damage that is prevalent within the tumor microenvironment. If the damaged DNA is successfully repaired, the cell will survive.

In order to specifically and effectively kill cancer cells using chemotherapy that induce DNA damage, it is important to take advantage of specific abnormalities in the DNA damage response machinery that are present in cancer cells but not in normal cells. Such properties of cancer cells may be targets for sensitization and lead to the development of biomarkers. Furthermore, inhibition of a DNA damage response pathway may enhance the therapeutic effects of DNA-damaging agents when employed in combination with these agents.

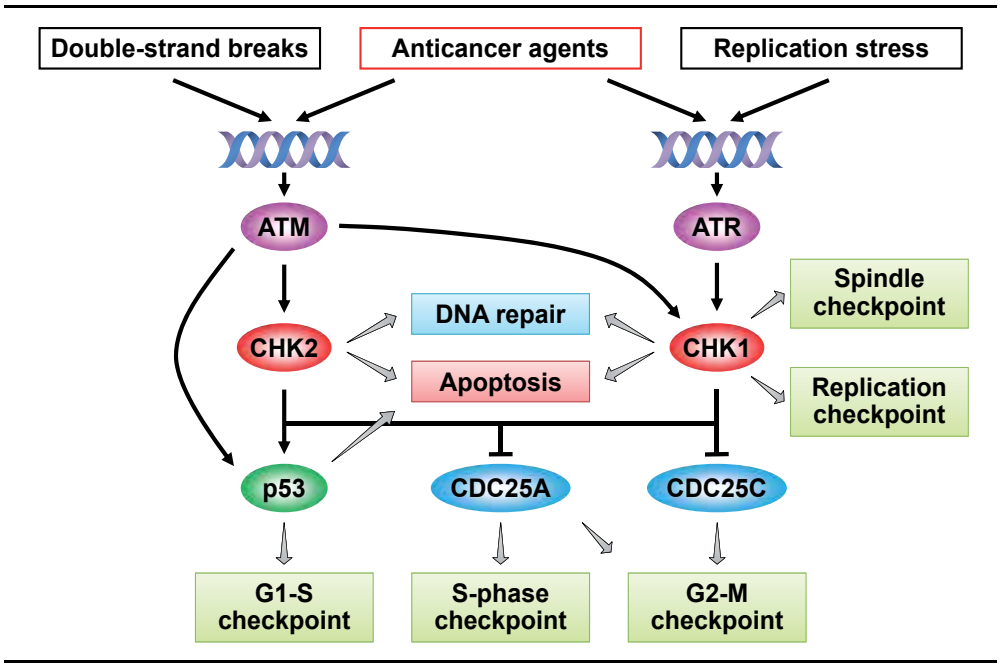
Recently, DNA repair inhibition has emerged as a promising strategy for personalized cancer therapy. Synthetic lethality exploits inter-gene relationships where the loss of function of either one of two related genes is nonlethal, but loss of both causes cell death. Emerging clinical data provide compelling evidence that overexpression of DNA repair factors may have prognostic and predictive significance in patients.

In this chapter, we will provide an overview of major DNA repair pathways and describe recent advances in anticancer therapy with a focus on DNA repair in cancer.

2. DNA damage response

The cellular DNA damage response (DDR) involves activation of cell cycle checkpoints to induce cell cycle arrest while repair mechanisms, transcriptional modulation, and/or apoptotic

pathways are activated. DNA damage induced by anticancer agents triggers recruitment of multiprotein complexes and activates a number of pathways, including ataxia telangiectasia mutated (ATM) and ATM and Rad3-related (ATR) signaling pathways. Cell cycle checkpoint kinases (Chk) of Chk1 and Chk2 are functionally redundant protein kinases that respond to checkpoint signals, initiate ATM and ATR, and play a critical role in determining cellular responses to DNA damage [1, 2]. Chk1 is mainly activated through ATR-mediated phosphorylation. Activated Chk1 phosphorylate Cdc25A, which leads to ubiquitin-and proteasome-dependent protein degradation, and downstream to increased phosphorylation of cyclin-dependent kinase (CDK) 2. In contrast, Chk2 is activated mainly by ATM, and activated Chk2 phosphorylates Cdc25A. Activated Chk1 and Chk2 then phosphorylates diverse downstream effectors, which in turn are involved in cell cycle checkpoints (i.e., G1/S-phase, intra-S-phase, and G2/M-phase checkpoints), the DNA replication checkpoint, and the mitotic spindle checkpoint, as well as DNA repair and apoptosis.



ATM, ataxia telangiectasia–mutated; ATR, ATM and Rad3–related; CHK, checkpoint kinase.

Figure 1. DNA-damage response signaling pathways

Consequently, through regulating the activity of CDKs, the progression from one cell cycle phase to another is delayed. The resulting cell cycle arrest allows time for repair, thereby preventing genome duplication or cell division in the presence of damaged DNA.

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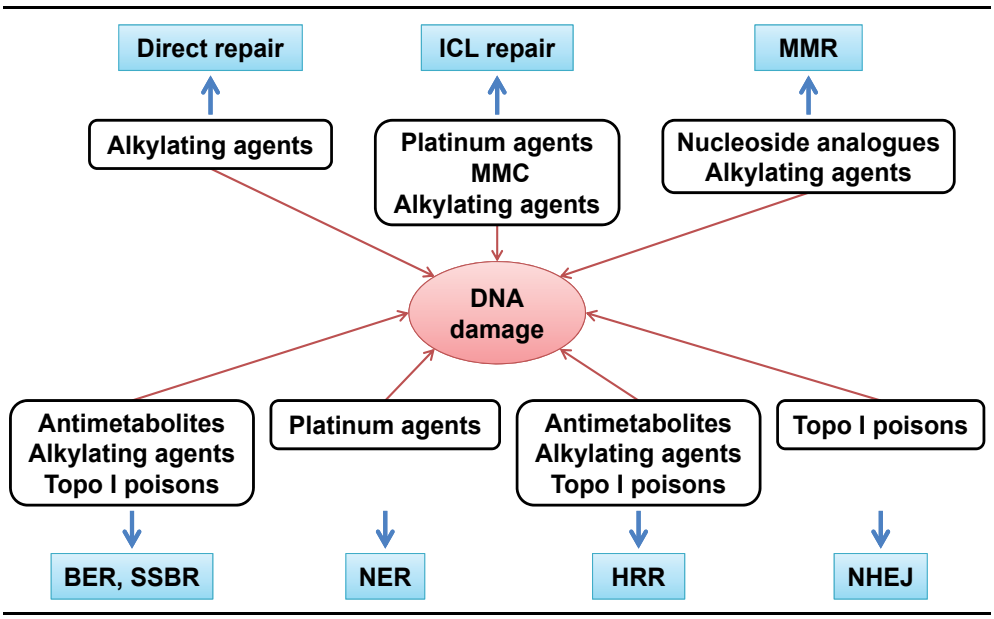
3. DNA repair pathways

DNA repair pathways in mammalian cells maintain genomic integrity. Depending on the type of DNA damage, cells invoke specific DNA repair pathways in order to restore genetic information.

Minor changes to DNA such as oxidized or alkylated bases, small base adducts and single-strand breaks (SSBs) are restored by the base excision repair (BER) pathway [3]. Poly(adenosine diphosphate ribose) (PAR) polymerase (PARP) is important in this process. Upon detection of SSBs, PARP covalently transfers PAR chains to itself and to acceptor proteins in the vicinity of the lesion, thereby facilitating the repair of SSBs. More complex, DNA helix-distorting base lesions, such as those induced by UV light, are repaired by nucleotide excision repair (NER) [4]. Another kind of damage disturbing the helical structure of DNA is represented by base mismatches. Mismatch repair factors recognize and process misincorporated nucleotides as well as insertion or deletion loops that arise during recombination or from errors of DNA polymerases [5].



BER, base excision repair; HRR, homologous recombination repair; ICL, inter-strand crosslink; MMR, mismatch repair; MMC, mytomycin C; NER, nucleotide excision repair; NHEJ, non-homologous end joining; SSBR, single-strand break repair; Topo, topoisomerase.

Figure 2. DNA repair pathways and chemotherapeutic agents

Covalent links between the two strands of the double helix represent a type of DNA damage referred to as interstrand crosslinks (ICLs). ICLs represent the most deleterious lesions produced by chemotherapeutic agents such as mitomycin C (MMC), cisplatin and cyclophos-

phamide. ICL repair is complex and involves the collaboration of several repair pathways, namely Fanconi anaemia, NER, translesion synthesis (TLS) and homologous recombination (HR) [6].

So far, four mechanistically distinct DNA double-strand break (DSB) repair mechanisms in mammalian cells have been described: non-homologous end joining (NHEJ), alternative NHEJ, single-strand annealing and HR [7]. NHEJ and HR represent the two major DSB repair pathways, with NHEJ operating throughout the cell cycle and HR being the most active during S-phase [8].

4. Cancer therapies targeting DNA repair mechanism

Alterations in expression of DNA repair may influence cancer biology and aggressive phenotypes. Clinical evidence supports the hypothesis that overexpression of DNA repair factors may have prognostic and predictive significance in patients [9]. Furthermore, highly proliferative cancer cells are hypersensitive to DNA damage because the S-phase is the most vulnerable period of the cell cycle. Therefore, DDR pathways make an ideal target for therapeutic intervention.

Dysfunction of one DNA repair pathway may be compensated by the function of another compensatory DDR pathway, which may be increased and contribute to resistance to DNA-damaging chemotherapy. So, inhibition of the pathway in combination with DNA damage agents will selectively kill cancer cells. These hypotheses are currently being tested in the laboratory and are being translated into clinical studies.

5. Direct repair

The simplest form of DNA repair is direct reversal of the lesion. Direct reversal of the oxidative lesion O6-methylguanine is carried out by the suicide enzyme methylguanine methyltransferase (MGMT) via an active site Cys145 that acts as a methyl recipient, followed by rapid ubiquitin-induced degradation. MGMT expression is one of several factors governing the response to alkylating chemotherapy agents [10, 11].

MGMT demethylates O6-methylguanine lesions, which are formed as a result of erroneous methylation by S-adenosylmethionine (SAM) and other alkylations at the O6 position of guanine that are induced by dietary nitrosamines or chemotherapy agents such as temozolomide (TMZ), dacarbazine (DTIC) and nitrosoureas [12, 13]. The higher levels of MGMT that are frequently observed in tumor tissue compared with normal tissue suggest that its depletion with pseudo-substrates that resemble O6-methylguanine might be a viable strategy to sensitize tumor cells to O6 alkylating agents. However, these pseudo-substrates have shown only marginal clinical benefit [14, 15].

A more promising approach may be the exploitation of reduced MGMT activity owing to epigenetic silencing in some cancers [16]. MGMT promoter methylation correlated with

sensitivity to BCNU in patients with astrocytomas and also correlated with sensitivity to TMZ plus radiotherapy in patients with gliomas [17]. Therefore, MGMT promoter methylation could be useful for stratifying patients for TMZ treatment.

6. Base excision repair

BER is responsible for detection and repair of damage caused by a number of mechanisms including alkylation, oxidation by reactive oxygen species (ROS), SSBs and base deamination. BER repairs DNA damage that is therapeutically induced by ionizing radiation, DNA-methylating agents, topoisomerase I poisons such as camptothecin, irinotecan and topotecan [18]. Single-strand break repair (SSBR) and BER are often assumed to be synonymous because they involve the same components and are similar after the initial recognition step. The main components of the pathway are glycosylases, endonucleases, DNA polymerases and DNA ligases, with PARP1 and PARP2 facilitating the process. Damaged bases are first removed by BER glycosylases to form apurinic or apyrimidinic (AP) sites. BER endonucleases then generate an SSB, which along with directly induced SSBs and those generated by topoisomerase (topo) I poisons [19, 20], are the substrates for SSBR. On detecting SSBs, PARP1 rapidly becomes bound and poly(ADP-ribosyl)ated, protecting the nick ends from undesirable recombination and allowing the recruitment of the molecular scaffold protein X-ray repair cross-complementing protein (XRCC) 1 for ongoing repair [21].

The BER pathway is an attractive target for the modulation of chemosensitivity. Early inhibitors of DNA polymerase-β (Pol β), flap endonuclease 1 (FEN1), ligase 1 and ligase 3 enhance sensitivity to ionizing radiation and TMZ. However, the most advanced drugs that target this pathway are AP endonuclease 1 (APE1) inhibitors and PARP-inhibitors (PARP-i, described later). Both APE1 and PARP expression and/or activity are generally higher in tumors [9, 22, 23].

There are two classes of APE1 inhibitor: methoxyamine, which binds the AP site in DNA, and inhibitors of APE1 endonuclease activity. Preclinically, methoxyamine potentiates the cytotoxicity of TMZ [24] and pemetrexed. In a phase I trial of methoxyamine, responses were seen in combination with pemetrexed, and there is an ongoing study with TMZ. Lucanthone, a topo II inhibitor, also inhibits APE1 endonuclease activity and potentiates the cytotoxicity of DNA-methylating agents in breast cancer cells [25]. Novel, more specific, APE1 endonuclease inhibitors increased the persistence of AP sites in vitro and increased the cytotoxicity of alkylating agents [26]. The synthetic lethality relationship between HR and APE1 was confirmed by the observed cytotoxicity following ATM inhibitor exposure in $APE1^{-/-}$ cells [27].

7. Nucleotide excision repair

NER recognizes and repairs base lesions associated with distortion of the DNA helical structure, including UV-induced photoproducts not eliminated by direct repair, and an array

of bulky adducts induced by various exogenous chemical agents. NER removes helix-distorting adducts on DNA and contributes to the repair of intrastrand and ICLs; the xeroderma pigmentosum (XP) proteins and excision repair cross-complementation group 1 (ERCC1) also have crucial roles in both the NER and ICL repair pathways [28]. Deficiency in NER confers sensitivity to platinum agent therapy, which reflects a reduced capacity to repair ICLs [29, 30]. There are currently no small molecule inhibitors of NER, although cyclosporine and cetuximab might down-regulate XPG and ERCC1–XPF expression, respectively. Recent evidence suggests that the efficacy of PARP-i–topo I poison combinations may be most effective in tumors that lack ERCC1–XPF, which are involved in the NER pathway [31].

8. Mismatch repair

Mismatch repair (MMR) recognizes and repairs errors introduced during replication. MMR also recognizes and repairs insertion/deletion loops (IDLs), particularly within microsatellite DNA. Hence, "microsatellite instability" (MSI) is recognized as a hallmark of MMR failure [32, 33]. If MSI manifests within tumor suppressor genes, it can produce frameshift mutations that contribute to carcinogenesis in colorectal, endometrial, ovarian, and gastric cancers [34]. Defective MMR increases mutation rates up to 1,000-fold, results in MSI, and is associated with cancer development [35].

Several DDR genes have microsatellites and could be mutated in MSI-high cancer, potentially conferring sensitivity to some DNA-damaging agents [36, 37]. However, defects in MMR cause tolerance to TMZ, platinum agents and some nucleoside analogues, which leads to drug resistance [38, 39]. Some researchers have focused on attempts to reactivate epigenetically silenced MLH1. However, after promising preclinical data that demonstrated chemosensitization [40], clinical trials have shown adverse reactions.

9. Homologous Recombination Repair

HR repair (HRR) is crucial for the maintenance of genomic stability, and is the predominant mechanism for DSB. HRR pathway for DSB repair is a highly complex process that involves multiple proteins, and occurs during the S and G2 phases of the cell cycle [41]. Many tumor suppressors participate in this pathway, including BRCA1, BRCA2 and ATM. As heterozygosity at a BRCA allele is associated with effective HR, DSB accumulation induced by PARP-inhibition specifically occurs only in tumor cells with acquired $BRCA^{-/-}$ homozygosity [42, 43]. Reasons for "BRCAness" are inactivation of BRCA1 or BRCA2 function caused by aberrant epigenetic or posttranslational modifications, and a wider range of mutations in other genes resulting in defective DSB signaling and HRR. Tumors with HRR defects are highly sensitive to crosslinking agents such as cisplatin, carboplatin and nitrosoureas, and DSBs that are induced by ionizing radiation and topo I poisons.

The high frequency of HRR defects in tumors may underlie the efficacy of cytotoxic therapy and provide a rationale for the use of inhibitors of HRR in the sensitization of tumors with

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functional HRR to conventional chemotherapy. Recent evidence suggests that PARP-i induces single agent cytotoxicity in cells with reduced expression of ATM, the checkpoint activator that is activated by DSBs [27, 44]. There are few HRR inhibitors, but mirin is an inhibitor of MRE11 endonuclease activity and thus inhibits HRR function [45]. Germline mutations in the HR protein RAD51D confer susceptibility to ovarian cancer and may be a target for PARP-i in a small subset of women [46]. Other prototype RAD51 inhibitors have been identified but the most common way to target HRR is by inhibition of the ATM–ChK2 or ATR–Chk1 pathways. Hyperactive growth factor signaling and oncogene-induced replicative stress increase DNA breakage that activates the ATR–Chk1 pathway, and some examples of synthetic lethality of checkpoint or DNA repair inhibitors in cells harbouring activated oncogenes have been shown. ATR knockdown was synthetically lethal in cells that were transformed with mutant KRAS [47], and inhibition of Chk1 and Chk2 significantly delayed disease progression of transplanted MYC-overexpressing lymphoma cells in vivo [48].

10. Non-homologous end joining

NHEJ is thought to be the major pathway for DSB repair. Damage recognition in NHEJ is performed by the Ku70/Ku80 heterodimer, which binds to the DSB ends with high affinity, possibly tethering the broken ends together. Ku binding recruits and activates the DNA-dependent protein kinase catalytic subunit (DNA-PKcs), forming the DNA-PK complex that phosphorylates other repair proteins including XRCC4-like factor (XLF), Werner syndrome helicase, DNA ligase IV and XRCC4.

DNA-PKcs is a member of the PI3K-related protein kinase family of enzymes that also includes ATM, ATR and mammalian target of rapamycin (mTOR). PI3K inhibitors, such as wortmannin and LY294002, also inhibit DNA-PKcs, and in proof-of-concept studies, these drugs hindered DSB rejoining and enhanced the cytotoxicity of DSB-inducing agents [49, 50]. More potent and specific DNA-PKcs inhibitors have been developed [51, 52] that substantially slow DSB repair and increase the cytotoxicity and antitumor activity of IR, radiomimetics and topo II poisons in cells and xenografts [53, 54]. However, none of these agents have reached the clinical testing stage.

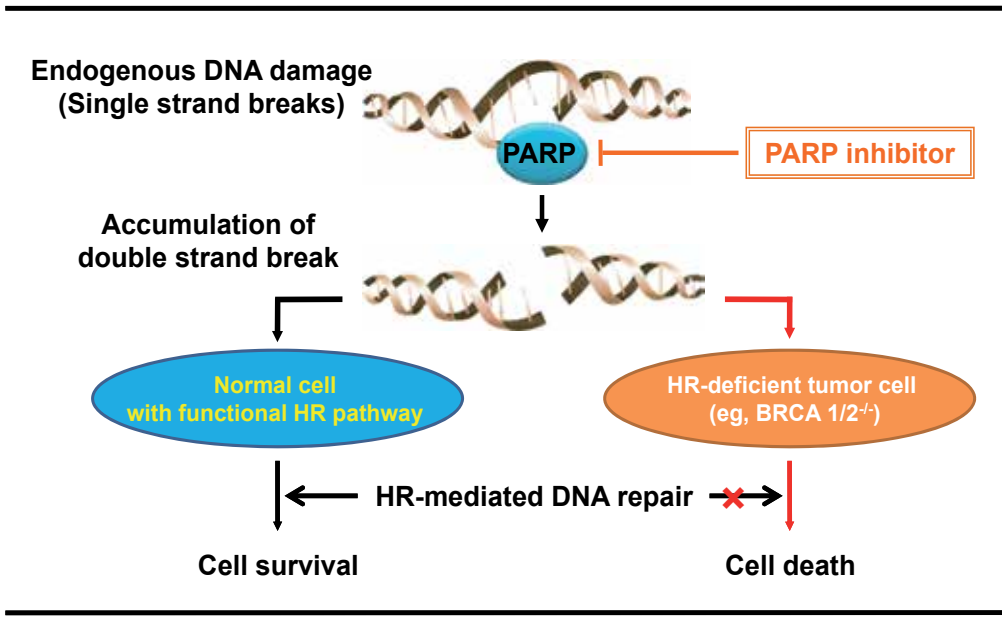
11. Translesion synthesis

If damaged DNA bases or adducts are not repaired, they may stall replication forks, which could contribute to genomic instability [55]. Several DNA polymerases can synthesize DNA past DNA lesions. Such TLS contributes to survival. However, errors can occur because these polymerases have no proofreading function and therefore, TLS should be considered a DNA damage tolerance mechanism rather than a DNA repair mechanism. Defects in TLS polymerases contribute to carcinogenesis but also confer sensitivity to DNA-damaging agents, and inhibitors of these polymerases are starting to emerge [56, 57].

12. Synthetic lethal strategies

Perhaps the most promising prospect for cancer treatment is the exploitation of dysregulated DDR by the synthetic lethality approach. Synthetic lethality exploits inter-gene relationships where the loss of function of either one of two related genes is nonlethal, but loss of both causes cell death. Loss of some elements of one DNA repair pathway may be compensated by the increased activity of other elements or pathways. The discovery of the synthetic lethality relationship between PARP1 and BRCA suggests that other tumor-specific defects in DSB repair factors may be therapeutically targeted by PARP inhibition.

The best characterized synthetic lethality relationship is between BRCA mutation and PARP1 inhibition [58-60]. BRCA1 and-2 have long been known as tumor suppressors, and their inherited mutation increases susceptibility to breast and ovarian tumors [61]. Both BRCA gene products have a role in the HRR pathway [62]. In BRCA-deficient cells, loss of effective HR leads to DSB persistence and cell death. However, resistance to PARP-i can develop owing to secondary mutations in BRCA1 or BRCA2 that restore their function [63, 64]. In addition, even in BRCA-mutant cells, HRR function and PARP-i resistance can be restored if 53BP1 or DNA-PKcs are also inactivated [65, 66].



HR, homologous recombination; PARP, poly-adenosine-diphosphate-ribose (PAR) polymerase.

Figure 3. Tumor selective synthetic lethality

Beyond BRCA1 and BRCA2, their joint interaction partner PALB2 is emerging as a breast cancer susceptibility gene, thus providing another opportunity for PARP-i-based therapies [67]. NVP-BEZ235, a recognized dual PI3K/mTOR inhibitor, was also reported to efficiently

block ATM, ATR and DNA-PK activity. Furthermore, NVP-BEZ235 was found to act as a radio- and chemosensitizer in various cancer cell lines [68, 69] and is currently being tested as a single agent in various phase I/II clinical trials [70, 71].

Synthetic lethality of components of the cell cycle checkpoint machinery could be exploited in cancers harbouring activated oncogenes, since oncogene-induced replication stress activates the ATR-Chk1 signaling pathway. Importantly, more than 50% of human tumors are defective in p53 tumor suppressor function and cell cycle checkpoint inhibitors have been demonstrated to sensitize p53-deficient cancer cells to various anticancer agents in clinical use [72]. The two transducer kinases Chk1 and Chk2 are downstream of ATM and ATR, and several inhibitors of transducer kinases have emerged in recent years. Recently, three novel Chk1 inhibitors, GDC-0425, SCH900776 and LY-2606368, have entered phase I clinical trials either as single agents or in combination with gemcitabine, a nucleoside analogue [73]. Another promising drug that interferes with checkpoint activation is the WEE1 tyrosine kinase inhibitor MK-1775 [74]. MK-1775 is already under investigation in a phase II trial combined with carboplatin in order to assess the benefit for patients with p53-mutated epithelial ovarian cancer. Several agents targeting CDC25 phosphatases that represent key molecules in checkpoint regulation have also been developed [75, 76].

SSBR factors other than PARP1 are potential synthetic lethality partners in DSB repair loss, which is supported by the observed cytotoxicity induced by inhibitors of ATM or DNA-PKcs following knockdown of the BER protein XRCC1 [77]. Recent evidence suggests that relationships between BER and non-HR DNA repair pathways may have potential synthetic lethality. The ATR inhibitor NU6027 was also more profoundly cytotoxic to BER-defective cells and in BER-functional cells treated with a PARP-i, reflecting the complementarity of HRR and BER [78].

Phosphatase and tensin homolog (PTEN) is a negative regulator of the anti-apoptotic PI3K/Akt/mTOR pathway. PTEN has recently been implicated in the maintenance of genomic integrity [79-83]. In the nucleus, PTEN promotes chromosome stability and DNA repair. PTEN loss-of-function could be an effective target for treatment strategies. Since PTEN deficiency causes a defect in HR, cells rely on PARP for the repair of DSBs. PTEN deficiency therefore sensitizes cancer cells to PARP inhibition [84-86]. Mendes-Pereira et al. [84] tested for synthetic lethality in HCT116 colorectal tumor cells transfected with a PTEN-mutant cDNA clone. Homozygosity for PTEN mutation was associated with a 20-fold increase in sensitivity to PARP-i in vitro and in vivo. Ectopic expression of RAD51 in a PTEN-deficient cell line overcame PARP-i sensitivity, supporting the proposed link between PTEN mutation and reduced RAD51 expression. Similar results were demonstrated in uterine endometrial carcinoma [85]. In primary PTEN−/− mouse astrocytes, reduced transcription of the RAD51 paralogs was associated with sensitivity to PARP inhibition [86], while PTEN disruption in colorectal cancer cells resulted in reduced MRE11 accumulation at DSBs that is also associated with PARP-i sensitivity [87]. Prostate cancers exhibiting PTEN loss often harbor a genetic rearrangement leading to TMPRSS22-ERG fusion. The TMPRSS22-ERG protein product promotes the formation of DNA DSBs and interacts with PARP, thus sensitizing cells to PARP inhibition [88, 89]. In lung cancer cells, PTEN deficiency potentiated the synergistic effect of

olaparib and cisplatin combination treatment [90], while rucaparib sensitized PTEN-deficient prostate cancer cells to ionizing radiation [91]. In melanoma cells, PTEN loss may contribute to BRAF and APE1 inhibition [92, 93]. Retrospective analysis of genetic alterations and PTEN status in tumors taken from patients who are participating in an ongoing clinical trial will provide information for the development of synthetic lethal treatment involving PTEN [94].

Mutations of the von Hippel–Lindau (VHL) tumor suppressor gene occur in the majority of sporadic renal cell carcinomas (RCC). The lack of VHL function in cells results in decreased repair capacity [95]. For example, the suppressor of cytokine signaling 1 (SOCS1) promotes nuclear redistribution and K63 ubiquitylation of VHL in response to DSBs. Loss of VHL function or VHL mutation that compromises K63 ubiquitylation attenuates the DDR, resulting in decreased HRR and persistence of DSBs [96]. Furthermore, loss of VHL function is associated with stabilization of hypoxia-inducible factor α (HIFα). The exposure of cells to hypoxia markedly enhances genetic instability caused by exogenous genotoxins, and HIF activation decreased NER [97]. Recently, synthetic lethal (SL) partner of VHL was identified from a screening of large volumes of cancer genomic data using a small interfering RNA screen. The VHL-deficient cells are significantly more sensitive to the knockdown of the predicted VHL-SL partners [98]. DNA repair pathway abnormalities involving VHL dysfunction might be therapeutic targets.

Many strategies based on the concept of synthetic lethality have so far only been investigated in preclinical settings.

13. PARP inhibitor

A number of potential PARP-i have been identified. In xenograft and in vitro models, PARP-i have been demonstrated to potentiate the action of a wide variety of damaging agents including platinums, the alkylating agents TMZ and cyclophosphamide, the nucleoside analogue gemcitabine, the topo inhibitor irinotecan, and ionizing radiation [90]. Furthermore, preclinical studies also suggested the potential use of PARP-i in sporadic cancers that share phenotypical features with cancers arising from hereditary BRCA mutations, a phenomenon that is referred to as "BRCAness" [91]. Many additional phase I and II trials are currently underway, examining the combination with a variety of agents including carboplatin, 5-fluorouracil and oxaliplatin, cisplatin and paclitaxel, topotecan, gemcitabine, and radiotherapy [92]. For example, rucaparib has been evaluated in phase I and II studies in combination with TMZ for malignant melanoma, demonstrating successful PARP inhibition at the tissue level and probable anticancer activity, but significant myelosuppression caused dose-limiting toxicity [93].

An initial phase I study of olaparib in a cohort enriched for BRCA1/2 mutation carriers demonstrated evidence of in vivo anti-PARP activity and evidence of response in 40% of BRCA carriers [60]. Phase II trials of olaparib for breast or ovarian cancer associated with BRCA1/2 mutations were favorable, suggesting antitumor efficacy [94, 95]. Good responses were also seen in patients with BRCA-associated breast and ovarian cancers, and even in unselected

patients with high-grade serous ovarian cancer [96, 97]. However, olaparib did not progress to a phase III trial for hereditary BRCA mutation-associated breast cancer due to economic concerns [98].

Iniparib has been evaluated in a phase II study of metastatic triple-negative breast cancer treatment in combination with gemcitabine and carboplatin. A significantly improved median overall survival was demonstrated compared with gemcitabine and carboplatin, without increased toxicity. However, a phase III trial failed to meet co-primary endpoints of overall and progression-free survival improvement, and after further disappointing results in a phase III non-small cell lung cancer trial, iniparib has been suspended from further development [99].

A good safety profile was also observed with veliparib in combination with TMZ. This was associated with early positive results in metastatic colorectal and BRCA-deficient breast cancers, although the combination was associated with poor response and no progression-free or overall survival improvement in advanced melanoma. Likewise, phase II investigation of rucaparib in BRCA1/2-mutated breast or ovarian cancer demonstrated PARP activity inhibition and evidence of a tumor response. The oral PARP1/2 inhibitor niraparib has also been evaluated at phase I and was shown to possess an acceptable safety profile and probable antitumor activity. Other PARP-i including orally bioavailable agents are currently being tested in clinical trials [100].

Clinical trials of PARP-i have generally been disappointing owing to toxicity, which may be due to use of a dose of PARP-i that was established as safe when used as a single agent. In general, preclinical data indicated that the MTD of single agent PARP-i was much higher than MTD of PARP-i when combined with another cytotoxic agent such as TMZ [101, 102]. This is because almost total inhibition of PARP-i is needed to render endogenous DNA damage cytotoxic, but this level of inhibition is not necessary to render the additional burden of deliberately introduced DNA damage cytotoxic, both in the tumor and in proliferating normal tissues. In addition, secondary BRCA2 mutations have been identified, which restore the full-length protein, thereby re-establishing BRCA2 functions and conferring PARP-i resistance [103]. A major challenge of using PARP-i is the acquired resistance of initially PARP-i-sensitive cancer cells due, for example, to the loss of p53-binding protein-1 (53BP1) or to overexpression of multidrug-resistance efflux transporters [104, 105]. The data described above suggest that the clinical utility of PARP-i in combination with chemotherapy may be limited in tumors in view of its narrow therapeutic index.

14. Predictive biomarkers

Relevant biomarker assays should predict the functionality of DNA repair pathways, rather than just providing information about mutations or expression levels of proteins involved in the DNA repair pathway. Furthermore, such detailed molecular profiling of cancer versus normal tissue from a given patient is critical to maximize the potential of personalized cancer drugs in terms of both therapeutic success and cost-effectiveness.

A general marker of DNA damage is the phosphorylation of histone H2AX by ATM, ATR and DNA-PK. γH2AX foci, formed at sites of DSBs, or increased levels of γH2AX, may be measured by immunofluorescence microscopy, flow cytometry or immunoblotting and used to detect DNA damage [106]. The increase and/or persistence of γH2AX can be used to demonstrate the inhibition of PARP, DNA-PK, ATR and Chk1. To directly measure the effect of a molecularly targeted agent, immunological methods may be used to detect the product. For example, activation of DNA-PK and ATM in response to DNA damage can be determined by measuring their autophosphorylation with phospho-specific antibodies, and PARP activity may be measured by immunodetection of the ADP-ribose polymer product, to guide PARP-i clinical trials [60, 107-109]. In multiple clinical trials, PARP activity in peripheral mononuclear blood cells has been used as a marker of effective inhibition [110, 111].

An alternative approach is to assess HRR function in fresh viable tumor material by measuring the number of RAD51 foci following ex vivo DNA damage induction [112-114]. In the ovarian cancer study, this was further analyzed in BRCA2-mutated pancreatic cancer cell clones to predict RAD51 foci formation as a marker of HR, and to examine for sensitivity to PARP inhibition.

When inactivation of a single gene has been identified as a crucial determinant of sensitivity, it may then be used to select patients for the appropriate therapy. For example, low levels of the NER endonuclease ERCC1 correlate with cisplatin sensitivity in several cancers [30, 115, 116]. Several studies report methods to identify tumors with non-germline HRR defects: gene expression profiling, methylation-specific arrays, immunohistochemistry analysis of tissue microarrays and copy number aberrations by array comparative genomic hybridization. [117-121]

Immunohistochemistry analysis of formalin-fixed, paraffin-embedded samples may be a useful tool for identifying DDR defects in order to stratify patients. To measure the effect of an agent that directly causes DNA DSBs in all phases of the cell cycle, patient-derived lymphocytes can be used [122]. Owing to the invasive procedures that are needed to obtain tumor material, except in the case of hematological malignancies, circulating tumor cells offer the best hope of routinely obtaining suitable material [123].

15. Conclusion

DNA repair mechanisms play an essential role in promoting genomic stability. On the other hand, impaired DNA repair capacity in cancer cells may result in a favorable response to chemotherapy. Many conventional therapeutic regimens that effectively kill cancer cells are based on DNA damage. However, most chemotherapeutic regimens cause severe side effects that limit their therapeutic potential. Inhibition of DNA repair is a new paradigm in cancer therapy, and there is heightened interest in the therapeutic potential of these inhibitors that selectively target tumors with minimal host toxicity.

The synthetic lethal approaches targeting the individual genetic profile of the tumors are under clinical development. The molecular characterization of tumors and reliable biomarkers are

needed for effective personalized therapy. Further research is necessary in order to determine the most appropriate treatment for patients.

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Chapter 13

DNA Replication Restart in Archaea

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Additional information is available at the end of the chapter

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1. Introduction

One fundamental challenge of cells is to accurately copy their genetic material for cell proliferation. This task is performed by core machineries considered conserved in all three domains of life: bacteria, archaea and eukaryotes [1].

For the vast majority of bacteria, the genome consists of one circular DNA molecule. Replication is initiated at a single replication origin from which two replication forks progress in the opposite direction. Replication termination takes place in the terminus region opposite the origin so that each replication fork has copied approximately one half of the genome. Studies of *Escherichia coli* mutants in key proteins for replication restart such as PriA strongly suggested that many replication forks encounter DNA damage or roadblocks leading to replisome inactivation under normal growth conditions. The reactivation of replication forks has been studied for several decades in bacteria. The picture that emerges is that bacterial proteins implicated in homologous recombination also play a key role in stabilizing and/or restoring blocked replication forks.

Unlike bacterial genomes, eukaryotic chromosomes contain numerous replication origins that can be used as backup origins to rescue arrested forks. Consequently, the importance of replication restart pathways in eukaryotes has long been ignored. However, recent studies have demonstrated that fork restart pathways operate also in eukaryotic cells and are important for cell viability under replication stress conditions. Eukaryotic replication restart pathways described also involve recombination proteins, as in bacteria. Thus it appears that general rules regarding replication restart and the key role of recombination proteins in these processes are conserved in bacteria, yeast and higher eukaryotes, but little is known in archaea, the third domain of life. This is of interest as archaea appear to be evolutionary hybrids between bacteria and eukaryotes.

Three main archaeal phyla are currently recognized: Crenarchaeota, Euryarchaeota [2] and Thaumarchaeota [3]. Similarly to most bacteria, archaeal genomes are also formed by a circular DNA molecule. However, unlike bacteria, some archaeal species have a single origin, whereas others have multiple origins per chromosome. Moreover, the ploidy of the genome in archaea varies considerably, with some species having one copy per cell whereas others have up to 25 copies of their genome in proliferating cells. As archaeal DNA replication consists both of evolutionary conserved as well as original features, understanding replication restart in these microorganisms will shed light on these fundamental but very complex pathways crucial to fulfill DNA replication. In this chapter we present recent advances on replication in archaea, followed by focused description of the Hef/XPF protein and its implication in replication restart in archaeal cells.

2. DNA replication origins in archaea

2.1. Multiple replication origins

Bacteria replicate their circular chromosome from a defined site called a replication origin. Two replication forks assemble at the replication origin and move in opposite directions. Each replication fork progresses at the same rate, and termination occurs at specific sites opposite the origin. Archaeal chromosomes are also circular, but whereas some archaea initiate replication from a single origin others replicate their chromosome from multiple replication origins, as observed for eukaryotic linear chromosomes (Table 1).

Phylum	Organism	No. of replication origins	References
Euryarchaeota	*Pyrococcus abyssi*	1	[4]
	Haloferax volcanii	3	[5-7]
	Haloferax mediterranei	2	[7]
	Archaeoglobus fulgibus	1	[8]
	Halobacterium sp. NRC1	4	[9, 10]
	Haloarcula hispanica	2	[11]
	Methanothermobacter thermoautotrophicus	1	[12]
Crenarchaeota	*Sulfulobus acidocaldarius*	3	[13]
	Sulfulobus solfataricus	3	[13]
	Sulfulobus islandicus	3	[14]
	Pyrobaculum calidifontis	4	[15]
	Aeropyrum pernix	At least 2	[16]
Thaumarchaeota	*Nitrosopumilus maritimus*	1	[7]

Table 1. Replication origins experimentally identified in archaeal chromosomes

Replication from a single replication origin was reported experimentally in the euryarchaea *Pyrococcus abyssi* [4] and in *Archaeoglobus fulgibus* [8]. But then several studies showed that various euryarchaea have multiple replication origins like the halophiles *Haloferax volcanii* [5-7] and *Haloarcula hispanica* [11]. Multiple replication origins have also been identified in *Sulfulobus solfataricus*, *Sulfulobus acidocaldarius* and *Sulfulobus islandicus* [13, 14] as well as in *Pyrobaculum calidifondis* [15] and *Aeropyrum pernix* [16] that belong to the crearchaeota phylum. Whether archaea from the recently discovered phylum thaumarchaeota have multiple origins remains unknown but a recent study in *Nitrosopumilus maritimus* identified a single replication origin in this organism that is conserved in the phylum, suggesting they have a single replication origin as also suggested by recent computational analysis [7, 17].

2.2. Archaeal replication initiator Orc1/Cdc6 proteins and origins recognition

How replication is regulated to allow a single circular DNA molecule to be replicated from uneven multiple origins is an ongoing question in archaea (Figure 1).

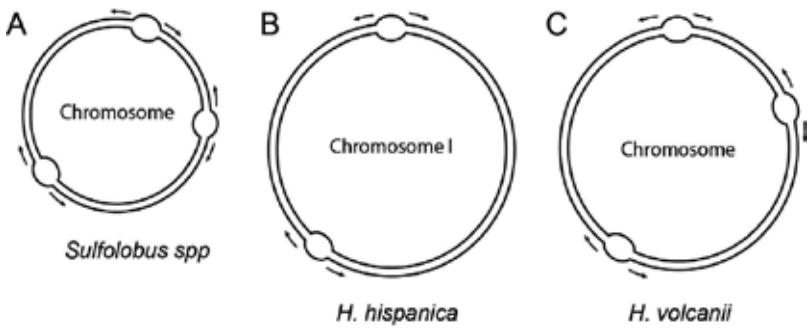

Figure 1. Uneven distribution of multiple replication origins in archaeal chromosomes. (A) in *Sulfolobus* species, (B) in *Haloarcula hispanica*, and (C) in *Haloferax volcanii*. Bubbles on chromosomes indicate replication origins, and arrows indicate bidirectional replication from each origin.

From each replication origin two replication forks are assembled and progress at the same rate so that termination of the replication is asynchronous. The origin region usually has a high content of adenine and thymine residues flanked by several conserved repeated motifs known as Origin Recognition Boxes (ORBs). In manycases archaeal replication origins are linked to replication genes [15, 18] and are located near genes coding initiator proteins. Despite the conservation of the replication origin-initiator structure, archaeal replication origins exhibit considerable diversity in terms of both ORB elements and their initiator genes [7, 11, 12]. Because replication origins can be dramatically diverse, it may facilitate differential usages by these microorganisms to adapt to various harsh environments.

All sequenced archaeal genomes encode proteins homologous to the eukaryotic initiator proteins Orc1 and Cdc6. Because the archaeal proteins are related both to the eukaryotic Orc1 subunit, involved in the replication origin recognition, and Cdc6, involved in the replicative helicase recruitment, they may combine both activities in a single polypeptide. Indeed, several

studies have shown that the archaeal replicative helicase MCM is recruited by Cdc6/Orc1 proteins at replication origins [14, 19, 20]. Archaeal Cdc6/Orc1 proteins also share mechanistic similarities with the bacterial initiator protein DnaA. *In vitro* studies on the binding of Cdc6/Orc1 proteins to ORBs in the *Methanothermobacter thermoautotrophicus* replication origin revealed that they bind cooperatively to the repeated sequences found in the vicinity of the *oriC*, as observed for the association of the bacterial initiator protein DnaA [12, 21].

How multiple replication origins are regulated by Cdc6/Orc1 proteins in archaeal cells is a complex question. The number of Orc1/Cdc6 proteins varies between species, and recent genetic studies attempting to delete *cdc6*/*orc1* genes revealed a complex regulation of replication, highlighting a specificity of initiator proteins at each origin.

For instance, four *cdc6*/*orc1* genes are found on *Halobacterium* NRC-1 chromosome that replicates from four distinct replication origins, but only two *cdc6*/*orc1* genes are located near a replication origin. Genetic studies of replication initiation at one of these origins showed that only the presence of the initiator protein associated was needed, revealing a specific binding of each replication origin by initiator proteins [10]. This is also the case in *S. Solfataricus* cells. Three *cdc6*/*orc1* genes are found in the chromosome. Out of the three replication origins, two were linked to a *cdc6*/*orc1* gene, and different subsets of the three Cdc6/Orc1 proteins recognized these replication origins [22]. The third origin was not recognized by Cdc6/Orc1 initiators. It was specifically recognized by the crenarchaeal-specific WhiP protein (for Winged-Helix initiator protein). WhiP proteins share sequence similarity with the essential eukaryal replication factor Cdt1 and display a domain organization reminiscent of bacterial plasmid initiator proteins. The conservation of WhiP-coding genes located near the replication origin in other crenarchaea suggested that this third replication origin was captured from extrachromosomal elements [16]. A similar situation is found in *Sulfolobus islandicus*. *Sis*OriC-1 was bound by Orc1-1, *Sis*OriC-2 by Orc1-3 while no association of any Cdc6/Orc1 protein was observed at *Ssi*OriC-3 specifically recognized by *Ssi*WhiP protein [14].

Moreover, additional role of initiator proteins independent of replication origins has recently been suggested by serial deletions of *cdc6*/*Orc1* genes in *S. Islandicus* and *H. hispanica*. In *S. Islandicus* none of the three *cdc6*/*Orc1* genes were essential for viability and all three possible double-mutants were viable. However, although one of the Cdc6/Orc1 proteins seemingly did not bind to any replication origin *in vivo*, the triple mutant could not be generated, further suggesting that the observed synthetic lethality may reflect additional role of replication initiator proteins [14]. Similarly, both replication origins in *H. hispanica* chromosome were shown to be controlled independently by specific *cdc6*/*orc1* genes. But while one of the replication origin could be deleted, the deletion of its associated *cdc6*/*orc1* gene lead to a severe growth defect, also suggesting a vital function of the protein outside replication initiation from its associated origin [23].

2.3. Are replication origins essential for viability in archaea?

The specific initiation sites, replication origins, on the chromosome of *H. hispanica* could be deleted separately but it was not possible to generate a mutant deleted for both origins at the same time. Attempt to delete also the replication origins of other replicons found in this

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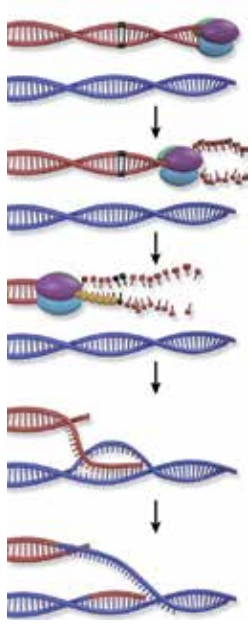

Figure 2. Model for double-strand break repair and replication restart in *E. coli*. RecBCD (purple, green and blue egg-shaped) degrades double-stranded end until it encounters a Chi site (black region). A switch in RecBCD activity produces a 3′-single-stranded DNA on which RecA (yellow ball) proteins are loaded. Homology search and strand exchange forms a Holliday junction (HJ) adjacent to a D-loop. The Holliday junction is resolved by RuvABC. PriA then load the replisome on the D-loop at which replication restarts.

organism suggested that one active *ori-cdc6* pairing on each replicon was essential for genome replication in *H. hispanica* [23]. But recent data obtained in the halophilic archaea *Haloferax volcanii* challenged the notion that replication origins are essential determinants of DNA replication. Indeed, Hawkins *et al*. revealed that not only cells were viable upon deletion of all known replication origins but they even grew faster than the corresponding wild-type cells [5].

How replication initiates in absence of replication origins? Because no evidence for activation of dormant origins has been found, authors favoured the hypothesis that replication initiation occurs randomly on the chromosome at recombination intermediates. Recombination-Dependent Replication (RDR) has first been observed in *E. coli* cells and extensively studied by various laboratories. From those studies it appears that replication fork inactivation occurs very frequently under normal growth conditions. Several replication restart pathways have evolved depending on the cause of arrest. They all share the common feature to involve recombination proteins such as the RecA recombinase and the PriA protein responsible for the loading of a replisome at recombination intermediates (Figure 2).

Indeed Hawkins *et al*. have shown that the archaeal RecA ortholog (RadA) is essential for viability in absence of replication origins.

But the deletion of *radA* alone impaired *H. volcanii* growth, highlighting that viability already relied on recombination [24]. Thus the essentiality of RadA in absence of replication origins may not reflect a direct need for recombination to start replication. Furthermore, in *E. coli* cells

RDR is deleterious for growth and viability. It also seems to be the case in yeast [25] and higher eukaryotes in which replication defects are linked to genome rearrangements and diseases [26, 27]. In that context the better fitness of origins-deleted cells observed in *H. volcanii* is puzzling. Hawkins *et al*. argued that replicative helicases MCM were more efficiently recruited at recombination intermediates as they were not sequestered at replication origin(s). Whether MCM is a limiting factor for replication initiation at replication origins in *H. volcanii* cells is currently not known and would have to be investigated. They also argued that the polyploidy of *H. volcanii* genome (18 copies of the genome in exponential phase [28]) allows viability to rely on stochastic partitioning. This argument implies that all chromosome dimers generated by recombination events (including RDR) do not have to be resolved to provide viable daughter cells, and that proteins involved in resolution of recombination intermediates such as the Holliday junction resolvase Hjc are not essential for viability. This hypothesis is clearly worth of experimental testing.

An alternative explanation for RDR is activation of dormant origins randomly in cells so that no preferential origin emerged at the level of a cell population [29, 30]. In this scenario the essentiality of RadA could imply that randomly-initiated replication forks more often collapse and have to be restarted. This notion would be consistent with an organization of archaeal genes on the genome preventing collision of replication machinery with transcription machinery [18] and physical connections recently suggested between replication and transcription machineries [31].

In conclusion, this study by Hawkins *et al*. raised many interesting questions that need to be experimentally addressed to understand how *H. volcanii* genome is replicated in absence of replication origins. Future work should aim at unravelling the molecular mechanisms that allow archaeal cells lacking origins to be viable and to even show increased fitness. In that context, one interesting protein to focus on might be the helicase/nuclease Hef. Indeed it has recently been shown that Hef (i) is genetically linked to the HJ resolvase Hjc and (ii) is recruited at arrested replication forks in living *H. volcanii* cells [32, 33].

3. Archaeal Hef/XPF proteins from the XPF/MUS81/FANCM family

Proteins belonging to the XPF/MUS81/FANCM endonuclease family act on 3′-flap DNA structures that are formed during DNA repair or replication restart. They are found throughout eukarya and archaea but to date have not been identified in bacteria. Eukaryotes have several XPF/MUS81/FANCM family members that all share a conserved nuclease domain [34] whereas MUS81 proteins possess only an active nuclease domain. In XPF, an active nuclease domain is fused to a SF2-helicase domain that is degenerated and appears to be inactive [35]. By contrast, FANCM consists of a helicase:nuclease fusion in which the nuclease domain is degenerated [36, 37]. Other members can be found that have a degenerated nuclease and/or helicase domain. They assemble into heterodimeric complexes with MUS81, XPF or FANCM proteins to form distinct active complexes involved in DNA repair, meiotic recombination and replication restart [38] (Figure 3).

All archaea encode a protein of the XPF/MUS81/FANCM family of endonucleases. It exists in two forms. The long form, referred as Hef, consists of an N-terminal helicase fused to a C-terminal nuclease and is specific to the euryarchaea. The short form, referred as XPF, lacks the helicase domain and is specific to the crenarchaea and the thaumarchaea (Figure 3).

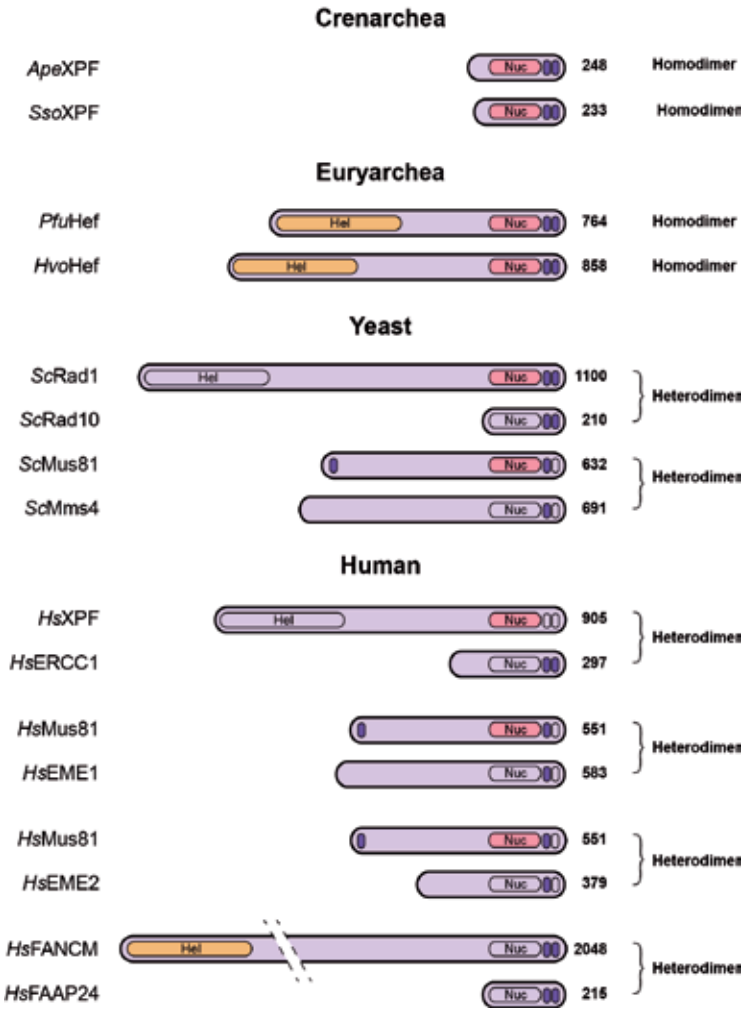

Figure 3. Schematic representation of archaeal and eukaryotic members of XPF/MUS81/FANCM family. Yellow-filled regions represent active helicase domains, pink-filled regions represent active nuclease domains while active HhH domains are represented by dark purple-filled ovals. Numbers of amino acids for each protein are indicated.

The long-formed Hef protein was first identified in *Pyrococcus furiosus* due to its activity on branched DNA structures, Hef meaning **h**elicase-associated **e**ndonuclease **f**ork-structure DNA [39]. Hef has the unique feature among XPF/MUS81/FANCM proteins of having both an active helicase domain and an active nuclease domain, allowing the identification of its human

ortholog FANCM protein also consisting of a helicase:nuclease fusion [37, 40]. What do we know about archaeal Hef/XPF function?

3.1. *In vitro* studies of crenarchaeal XPF proteins

The crystallographic structure of XPF from the crenarchaea *Aeropyrum pernix* was solved in presence and absence of double-stranded DNA [41, 42]. The protein has two domains, a N-terminal nuclease domain and an Helix-hairpin-Helix $(HhH)_2$ domain. *Ape*XPF formed homodimers. The interaction involved the two nuclease domains and the two $(HhH)_2$ domains from each monomer. However, only one monomer seemed catalytically active at a time when the homodimer was bound to DNA. The $(HhH)_2$ domain had a major role in interacting with DNA. This interaction triggered a domain movement coupling the $(HhH)_2$ domain to the nuclease domain to allow subsequent cleavage of the DNA substrate. The DNA was bent by around 90° upon interaction, suggesting that XPF binding causes distortion at double-strand/single-strand DNA junctions.

The nuclease activity of XPF from *Sulfulobus solfataricus* has been studied in more details. The replication factor PCNA (Proliferating Cell Nuclear Antigen) was required *in vitro* for nuclease activity of this "short" XPF [43]. In the cell, the trimeric PCNA ring encircles double-strand DNA (dsDNA) and firmly attaches the replicative polymerase to the template strand, enhancing its processivity. PCNA is a central protein as it also interacts with various proteins involved in replication and/or repair like Fen1. Interaction with PCNA often involves a conserved motif known as PCNA-Interacting Protein (PIP) motif conserved in XPF proteins. Indeed it was shown that *Sso*XPF interacts with PCNA through its conserved PIP motif [43, 44]. Intramolecular FRET experiments showed that the binding of *Sso*XPF to a 3′-flap indeed bent the DNA as observed in *Ape*XPF structure, but that the interaction with PCNA allowed *Sso*XPF to distort the DNA structure in a proper conformation for efficient cleavage [45, 46]. *Sso*XPF preferentially cleaved 3′-flap and processed them into gapped duplex products. It was also observed that *Sso*XPF can act on substrates containing a variety of DNA damages or modifications [47, 48].

3.2. *In vitro* characterization of euryarchaeal Hef proteins

As mentioned previously Hef was identified in *P. furiosus* due to its enzymatic activity on branched DNA structures [39]. *In vitro* experiments on *Pfu*Hef revealed a similar organization of the C-terminal region of archaeal XPF proteins, with a nuclease domain and a helix-hairpin-helix domain. Similarly, homodimers were observed with both the nuclease and the HhH domains forming domain-domain interfaces. Dimer formation appeared crucial for substrate recognition specificity [49]. A variety of branched DNA structures carrying single-strand DNA (ssDNA) portions, such as flapped and fork-structured DNAs, were recognized and cleaved by the C-terminal nuclease domain of *Pfu*Hef [50, 51]. The N-terminal domain of Hef displayed a structured-DNA specific helicase. Two conserved helicase motifs from Super-Family 2 (SF2) helicases were separated by a third domain that shares structure similarity with the "Thumb" domain of polymerases involved in dsDNA binding [51].

In vitro experiments suggested collaboration between the coupled helicase and the nuclease domains of *Pfu*Hef: the helicase domain binds and processes the fork-structured DNA, forming a four-way structure that is then cleaved by the endonuclease domain [52]. Yet replication fork restart can involve the formation of a four-way junction (Holliday junctions) from a three-way junction (fork-like structure). The four-way junction is then resolved by a Holliday junction resolvase. Thus euryarchaeal Hef could be involved in the resolution of stalled replication forks, as suggested for Mus81 complexes in eukaryotes. Indeed in both fission and budding yeast it seems that the primary function of Mus81 complexes is the restart of collapsed-replication forks by homologous recombination [53-57], a role that is functionally redundant with the helicase-nuclease Sgs1-Top3 and the Rqh1-Top3 complexes, respectively [53, 58]. The MUS81 complex is also found in humans [59], and promotes replication fork restart by homologous recombination [60-63].

More recently, *Thermococcus kodakarensis* Hef has been shown to interact with *Tko*PCNA1. The interaction with PCNA did not involve a canonical PIP motif but a disordered region of Hef between the helicase and nuclease domains. Interestingly, these long disordered regions connecting two catalytic domains are a common feature of euryarchael Hef and eukaryotic FANCM proteins [64].

These biochemical studies have indicated that both creanarchaeal XPF and euryarchaeal Hef proteins interact with PCNA and display biochemical activities consistent for being proteins involved in DNA repair and/or replication restart. Is this hypothesis supported by *in vivo* studies of Hef proteins?

3.3. What have we learned deleting *hef* gene in euryarchaea

The *hef* gene has been deleted in two different euryarchaea: in the hyperthermophile *Thermococcus kodakarensis* and in the halophile *Haloferax volcanii*. In both organisms, Hef was non-essential for cell viability under normal laboratory growth conditions.

T. kodakarensis cells deleted for *hef* showed increased sensitivity to a variety of DNA damaging agents [65], consistent with a role of Hef in the maintenance of genomic stability. The sensitivity to UV irradiation suggested that Hef was involved in the repair of UV lesions. Both helicase and nuclease domains of *Tko*Hef were needed as the same phenotype was observed upon deletion of the entire gene and deletion of the helicase-coding region or the nuclease-coding region of the gene [65]. Nucleotide Excision Repair (NER) is the major pathway to repair DNA lesions after UV radiation. But the existence of an archaeal NER pathway has not been established yet. Most archaea have eukaryal-type NER genes, but most of eukaryal NER proteins have multiple cellular functions so the presence of several NER-like protein is not enough to conclude that a functional NER pathway can be found in archaea [66]. Interestingly the sensitivity to UV radiations of *T. kodakarensis* cells deleted for *hef* suggested that Hef was involved in Nucleotide Excision Repair (NER), as its human ortholog XPF-ERCC1 and its counterpart RAD1-RAD10 in the yeast *Saccharomyces cerevisae* [67, 68]. And it also suggested that an active NER pathway exists in archaea. Clearly, additional experiments are now needed to better understand the role of *Tko*Hef in NER and, more generally, to further dissect the pathway responsible for archaeal Nucleotide Excision Repair.

In contrast, the deletion of *hef* in *H. volcanii* neither affected sensitivity to various DNA damaging agents nor recombination frequency. We could only observed a slow-growth phenotype of the Δ*hef* colonies when chronically exposed to mitomycin C (MMC) on plate [32]. Note that the direct comparison between these two studies on phenotypic analyses of Δ*hef* strains is difficult as Table 2 illustrates major differences regarding experimental conditions used including drug concentrations and cell treatment methods.

	H. volcanii			***T. kodakarensis***		
	Concentration	**Exposure**	**Sensitivity**	**Concentration**	**Exposure**	**Sensitivity**
UV irradiation	Up to 150 J/m²	on plate	-	2 or 5 J/m²	on plate	+
MMS	0,04%	1 hour in suspension	-	0,05%	4 hours in suspension	++
γ-rays	Up to 1000 Gy	on plate	-	1700 Gy	in suspension	++
Phleomycin	1 or 2 mg/ml	1 hour in suspension	-			
Mitomycin C	0,02 μg/ml	On plate	slow-growing	100 μg/ml	4 hours in suspension	+++

Table 2. Methods used for exposure of *H. volcanii* and *T. Kodakarensis* Δ*hef* cells to DNA damaging agents as reported in [32, 65, 69].

A possible explanation for these phenotypic differences is that NER proteins in *Thermococcus* and *Haloferax* species are very different. In fact *H. volcanii* also possesses bacterial-like NER proteins most probably acquired by lateral gene transfer [66], and it was shown that they were responsible for the repair of UV lesions [32].

To further investigate the role of Hef in *H. volcanii*, the observed lack of an obvious phenotype for Δ*hef* cells prompted us to combine *hef*-deletion with other endonuclease or helicase deletions that may encode redundant functions with Hef. Among several combinations tested, we demonstrated that Hef was essential for viability in the absence of the Holliday junctions (HJs) resolvase Hjc. Holliday junctions are four-way branched DNA structures formed during homologous recombination strand exchange and recombination-dependent replication restart. HJs resolvases are found in bacteria, archaea and eukarya, although they are not evolutionary related. Hjc is conserved throughout archaea. The single deletion of *hjc* gene in *H. volcanii* cells (as well as in *T. Kodakarensis* cells) did not affect growth rate, DNA repair or recombination [32, 65]. Co-lethality of Hef and Hjc could be explained by redundant roles of Hef and Hjc as HJs resolvase. In this scenario Hef could use its helicase activity on arrested replication forks to process them into four-way DNA structures that can be resolved by its nuclease activity. This scenario was compatible with the *in vitro* studies described above.

Indeed, point mutations inactivating the helicase activity (*Hvo*Hef-K48A) or the nuclease activity (*Hvo*Hef-D679A) of *Hvo*Hef resulted in the same phenotype observed in the absence of the entire protein. This nicely demonstrated that both helicase and nuclease activities of Hef were required for fulfilling its role in the absence of the Hjc resolvase [32]. To test the hypothesis that Hef and Hjc were both acting as HJs resolvase, we deleted *hef* or *hjc* in a strain carrying a *radA* deletion. In absence of RadA recombinase, HJs are no longer formed by homologous recombination so that deleting HJs resolvases should not have any affect. Indeed cells deleted for both *radA* and *hjc* were phenotypically similar to cells only deleted for *radA*. This observation was consistent with a role of Hjc in the resolution of HJs formed by RadA-mediated strand exchange during homologous recombination. However, *radA* gene could not be deleted in *hef*-deleted cells [32], strongly reflecting that functional roles of Hjc and Hef were distinct. This observation also suggested that Hef was required for cell viability in absence of recombination. Which alternative pathways or additional functions could (i) depend on Hef, (ii) be essential for cell viability during normal growth condition, and (iii) implicate recombination proteins and/or Hef? Replication restart is one possible pathway. But how could we obtain more detailed information on functional roles of Hef if *hef*-deleted mutant strains hardly shows any phenotype or cannot be combined with other deletions? We decided to develop tools to allow dynamic localization of fluorescently-labelled Hef proteins in living *Haloferax volcanii* cells.

Genotype	Growth phenotypes	References
WT	+++	
Δ*hef*	+++	[32]
Δ*hjc*	+++	[32]
Δ*radA*	+	[24]
Δ*hef* Δ*hjc*	-	[32]
Δ*radA* Δ*hjc*	+	[32]
Δ*radA* Δ*hef*	-	[32]

Table 3. Growth phenotypes of *H. volcanii* deletion mutants.

4. Dynamic localization of Hef proteins fused to the Green Fluorescent Protein (GFP) in living *H. volcanii* cells

The Green Fluorescent Protein (GFP) was originally isolated from the jellyfish *Aequora Victoria* [70]. It is encoded by a single polypeptide containing the chromophore. After translation of the protein, an autocatalytic process involving oxygen has to take place within the chromophore. Once active, the GFP has a major excitation peak at a wavelength of 395 nm and an emission peak at 509 nm. A deep understanding of the protein has enabled the development

of several GFP variants with modified spectral properties [71, 72]. All those FPs are now used in living cells/organisms to study protein localization, mobility, turnover, interactions, and much more [73]. Such approaches can reveal key features of proteins *in vivo* to complete our understanding of pathways, as illustrated for NER pathway in mammalian cells [74], but their use in archaeal cells has been rather limited until recently.

4.1. Fusion of the green fluorescent protein to the C-terminal end of Hef protein in *H. volcanii*

GFP has been used to investigate proteasome-dependant proteolysis and protein levels in *H. volcanii* [75, 76] as well as biofilm formation by the crenarchaea *Sulfulobus solfataricus* [77], two aerobic archaea. Because GFPs variant can differ not only by their fluorescence properties but also by their maturation rate of the fluorophore, temperature and pH stability or oligomeric state for instance, one has to carefully choose the variants that optimally fit the lifestyle of the organism being studied. We have recently used GFP-fusions to investigate protein localization and behaviour in archaeal cells for the first time. These studies were performed using the halophile *H. volcanii* that has a relatively high intracellular salt concentration (around 2,5M in laboratory growth conditions) and an optimal growth temperature of 45°C. Expression of several GFP variants were previously tested in this species, demonstrating that the smRS-GFP could be used for further studies [75]. This variant has mutations increasing solubility (Phe99Ser, Met153Thr and Val63Ala) as well as a mutation in one of the three amino acids of the chromophore (Ser65Thr) that redshifts the absorption maximum to 488 nm without changing the emission properties of the protein [78, 79]. Based on this observation we fused the smRS-GFP to the C-terminal end of *H. volcanii* Hef. The resulting fusion protein was expressed under physiological expression levels and conditions from the native chromosomal locus of the *hef* gene [33].

Whether GFP-fused Hef proteins remained functional was then tested by comparing cells deleted for *hef* with cells expressing the *hef*::*gfp* allele. No growth delay was observed for *hef*::*gfp* cells on MMC plates, indicating functional complementation by Hef::GFP construct. In agreement with this notion, *hjc* could be deleted, although a growth defect was measured for *hef*::*gfp* Δ*hjc* cells. Because we were interested in the localization of Hef in response to replication arrests, we exposed cells to aphidicolin (APD), an antibiotic that inhibits DNA synthesis in halophilic archaea [80], thus arresting replication forks. Exposing *hef*-deleted cells to increasing concentrations of APD decreased cell viability, showing that indeed Hef is involved in the genomic stability upon replication arrest. Such decrease in cell viability was not observed with cells expressing GFP-fused Hef.

4.2. Localising the fluorescence signal in *H. volcanii* living cells

We then observed the localisation of Hef::GFP proteins by fluorescence microscopy, comparing cells exposed to APD to non-treated control samples. Towards this goal, a drop of cells was spotted on an agarose slice placed on a glass slide. After allowing this drop to dry, the agarose pad was covered with a cover-slip for cell imaging studies using a wield-field microscope to visualize a large number of individual cells. Differential Interference Contrast (DIC) [also

known as Nomarski Interference Contrast (NIC)], was first used to visualized the cells as it enhances the contrast in unstained, transparent samples. Then fluorescence imaging was performed (exciting at 474 nm and collecting at 527-554 nm). Note however that due to the small cell size (around 1 to 2 μm) and the use of the soft agarose, not all cells were in the same focal plane. In order not to lose any information, fluorescence images were acquired at different focal planes on the z-axis. Consecutive slices of cells in focus were then selected and used to perform a maximum intensity z-projection. At each pixel, the highest fluorescence signal was kept when comparing the selected images. This maximum intensity z-projection resulted into a two-dimensional picture where the maximal fluorescence signals from different focal planes were recorded (Figure 4).

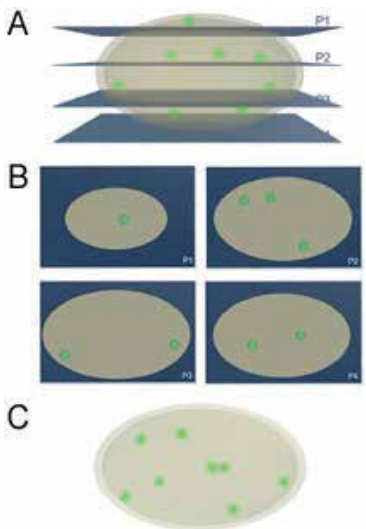

Figure 4. Schematic representation of fluorescence signal analysed in cells. (A) Representation of a cell with fluorescence foci and the different focal planes used for imaging. (B) Representation of fluorescence signal in each focal plane. (C) Resulting image after projection of the maximum fluorescence signal at each pixel for the four focal planes.

Resulting images contained hundreds of cells that were analysed by quantitative image analyses using IMARIS software. Different imaging parameters were optimized to detect cells and fluorescence foci within cells using automatic thresholds to avoid user-bias. This approch allowed thousands of cells to be analysed in each condition tested, providing extremely high statistical power.

4.3. Hef::GFP molecules are recruited at arrested replication forks

Using such approach, we have shown that Hef::GFP proteins formed fluorescence foci even under normal growth condition, in the absence of any DNA damaging agents. The number of these foci was significantly increased from two to four foci *per* cell in response to aphidicolin exposure. We also observed that the number of foci per individual cell changed significantly.

While the majority of cells had one or two foci in normal growth conditions, a higher proportion of cells having more than two foci was observed upon APD exposure (Figure 5).

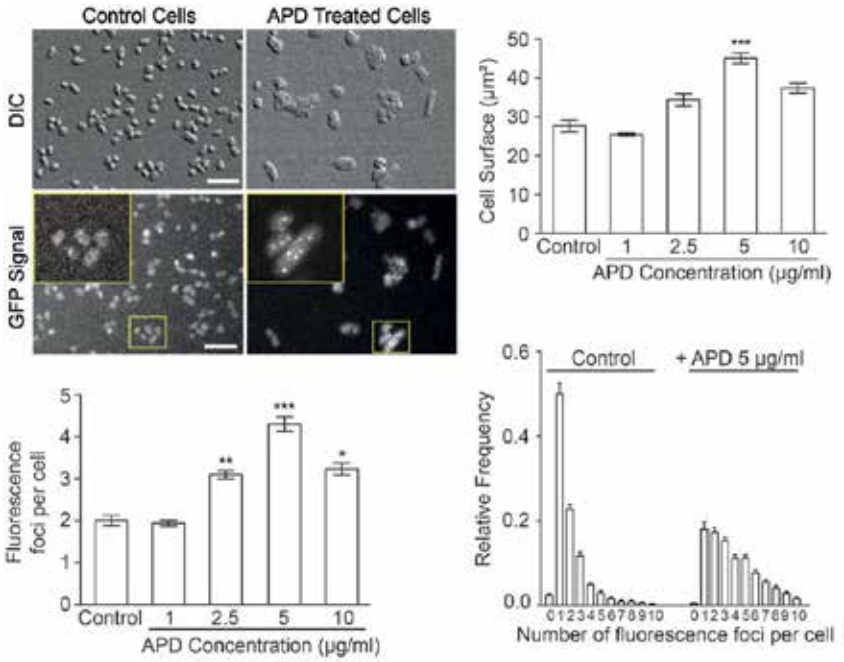

Figure 5. *In vivo* localization of GFP-labeled Hef in response to aphidicolin exposure. A total of 23760 spots within 13666 control cells and 15299 spots within 3721 APD-treated cells were analyzed. (A) Pictures of DIC and GFP signal of *hef::gfp* cells under control conditions and after exposure to 5 µg/ml aphidicolin. Bar equals 10 µm. (B) Average cell surface of *hef::gfp* cells in response to increasing concentrations of aphidicolin. (C) Mean number of GFP-Hef labeled fluorescence foci per cell in response to increasing concentrations of aphidicolin. (D) Relative frequency of number of foci per individual cell. All error bars represent SD. n > 3 experiments, t test are performed in comparison to control without aphidicolin. *** Significantly different, p<0.001. ** Significantly different, p<0.01. * Significantly different, p<0.05. From [33].

We have also observed that cell size was increased from 28 to 45 µm² in response to replication arrest (i.e. APD exposure). We have shown using other DNA damaging agents that increased cell size and number of foci were specific to APD treatment, suggesting that indeed *Hvo*Hef is recruited at arrested replication forks brought about by addition of aphidicolin.

4.4. Diffusing pattern of Hef::GFP molecule upon replication arrests

To investigate the diffusion of Hef::GFP molecules inside and outside fluorescence foci, we performed Fluorescence Recovery After Photobleaching (FRAP) and Number and Brightness (N&B) experiments. These experiments were performed using a confocal microscope on cells immobilized on a poly-D-lysine coated cover-slip.

In FRAP experiments a region of interest was photobleached in a cell. The speed of fluorescence recovery in that region was then measured, reflecting the diffusion of

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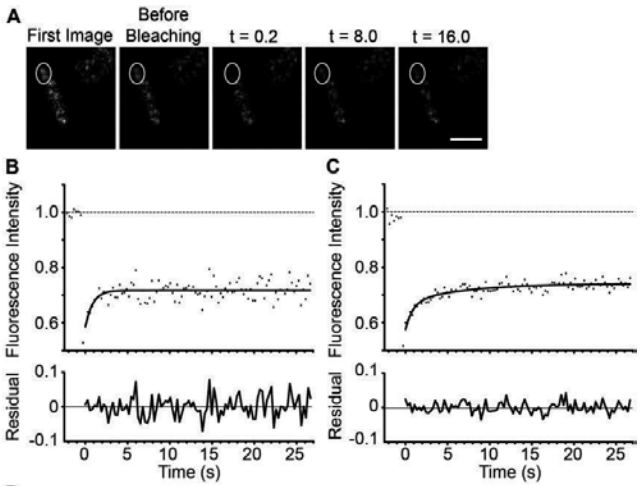

D

Sample	k1 (1/s)	k2 (1/s)	n
Control Cells	1.153 [0.605, 1.702]		9
APD-treated Cells	2.750 [0.512,4.989]	0.240 [0.147,0.334]	8
	1.153 (fixed)	0.144 [0.057,0.232]	8

Figure 6. Fluorescence Recovery After Photobleaching experiments to study the dynamic localization of GFP-labeled Hef molecules at fluorescence foci. (A) Images of a representative cell in response to aphidicolin treatment for FRAP analysis. FRAP regions are shown by white circles. Time after photobleaching in seconds. Bar equals 5 μm. (B) Fluorescence recovery curve averaged for 9 control cells. C) Fluorescence recovery curve averaged for 8 aphidicolin treated-cells. (D) Diffusion constants [Confidence interval at 95%] calculated for GFP-labeled Hef diffusing molecules. From [33].

Hef::GFP fluorescent molecules arriving from the non-photobleached region of the cell. In control cells (no aphidicolin), one major population of Hef::GFP diffusing molecules was observed. From the fit of the recovery curve we obtained the recovery constant, allowing then the apparent two-dimensional diffusion rate of Hef::GFP to be estimated at 0.8 to 2.3 μm^2 per second. This appeared markedly lower than expected for Hef dimer, as revealed by analytical ultracentrifugation experiments on purified *Hvo*Hef further indicating that Hef has a peculiar elongated shape in solution. Several possibilities may explain this limited diffusion. In addition to the non-globular quaternary structure, physical constraints of the cytosol, possibly resulting from high DNA and salt concentration, and/or transient interactions with cellular components (DNA or proteins complexes) may explain this slow diffusion. But FRAP experiments performed on cells exposed to aphidicolin revealed an additional, even more slowly-diffusing population that was clearly induced by APD treatment (Figure 6).

Such changes in the diffusion pattern of Hef::GFP molecules upon replication arrests were also observed using N&B technique that measures fluctuation of fluorescence intensity in each analysed pixel [81]. These analyses were performed on one hundred images taken every 2 seconds, and cell regions including and excluding fluorescence foci were compared. Fluctuation of fluorescence intensity per pixel was then used to

determine the number of diffusing molecules and their brightness. This information can then be used to deduce changes in the oligomeric state of the fluorescent molecules. When N&B technique was applied to diffusing Hef::GFP molecules, changes in the oligomeric state (i.e. higher brightness) were observed upon APD treatment. This observation revealed oligomerization and/or co-localisation of several Hef::GFP molecules induced by APD exposure (Figure 7), and provided a feasible explanation for the slow diffusion in APD treated cells revealed by FRAP experiments.

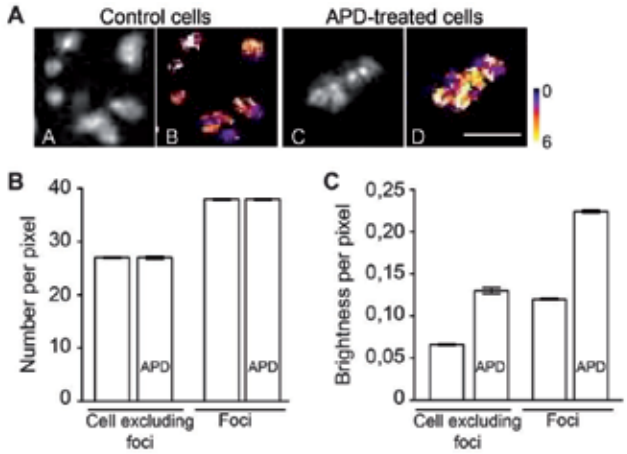

Figure 7. Number & Brightness experiments to study the oligomeric state of GFP-labeled Hef molecules diffusing at fluorescence foci. (A) Images of representative cells for N&B analysis. Average intensity (A and C) and pseudo-coloured normalized brightness values (B and D) for representative control cells (A and B) and cells exposed to 5 μg/ml aphidicolin (C and D). Bar equals 5 μm. (B) Average number of Hef::GFP diffusing molecules per pixel. (C) Average brightness of Hef::GFP diffusing molecules per pixel. From [33].

Overall, the results obtained from FRAP and N&B experiments were consistent with the notion that Hef::GFP molecules are actively recruited at arrested replication forks. Whether the slow-diffusion pattern of Hef::GFP molecules reflects their recruitment directly on DNA and/or as part of protein complexes at arrested replication forks are questions that remain to be addressed. Interestingly, *hjc* deletion had effect neither on cell size nor on the number of foci per cell in normal growth condition as well as in response to APD treatment. These observations showed that recruitment of Hef to arrested replication forks was not increased in the absence of the alternative pathway involving Hjc (and RadA), suggesting that Hef is recruited at arrested replication forks even in the presence of the alternative HR-dependent pathway. We also noted that in eukarya recent studies have indicated that FANCM proteins can prevent homologous recombination [82-85]. This raised the possibility that *Hvo*Hef may prevent access of recombination proteins to arrested replication forks (Figure 8) [33]. Because both the helicase and nuclease activities of Hef are presumably needed for biological function, we also assumed that Hef is directly implicated in processing of arrested replication forks. These hypotheses will be addressed in the future experimental work.

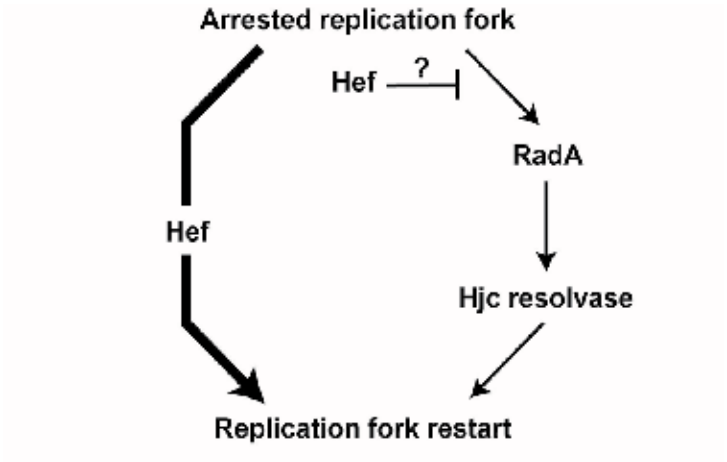

Figure 8. Model for replication restart in *Haloferax volcanii*. Two alternative pathways allow replication restart: one is dependent on the homologous recombination proteins Hjc and RadA (pathway on the right) and one is independent of homologous recombination (pathway on the left). Our data show that Hef has a dominant role during replication restart, even in the presence of Hjc, and are also compatible with Hef preventing the access of recombination proteins at arrested forks. From [33].

Moreover, our work has also shown that replication forks arrest spontaneously occured in *H. volcanii* cells that may contain a high number of replications forks. This is exemplified by the fact that a typical *H. volcanii* cell contains 18 genome copies and that each molecule can be replicated by up to 8 replication forks. Whether all genome copies are replicated simultaneously is not known and this striking question needs to be addressed in future work to better understand replication dynamic in archaea. But as we have observed up to 15 to 20 arrested replication forks in some individual cells, our imaging studies rather suggest that several copies of the genome may be replicated in one cell.

5. Concluding remarks

In vitro characterization of Hef/XPF proteins suggested a key role in genomic stability similarly to what was observed in Eukarya. Genetic experiments coupled to dynamic localization of Hef proteins fused to the Green Fluorescence Protein further revealed that Hef is recruited at arrested replication forks in *Haloferax volcanii* cells. Experimental approaches we have recently developed for halophiles provide a valuable tool for studying functional roles of Hef at arrested replication forks in living archaeal cells. Understanding how Hef is recruited at arrested replication forks and the processing taking place to allow replication restart is our next challenge. Whether XPF protein have similar role in crearchaeal cells remains to be addressed. As genetic tools have been developed for crenarchaeal organisms, future studies might tackle this issue [86, 87].

In conclusion, as archaea possess hallmarks of both bacterial and eukaryotic replication systems we believe that continuation of studies underlined will shed light on the evolutionary

history of replication restart mechanisms and its complex machinery that we are just starting to unravel in eukaryotes and now archaea.

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Chapter 14

DNA Repair Based Therapy in Oncology and Neurodegeneration

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Additional information is available at the end of the chapter

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1. Introduction

There are various types of DNA damages as well as the presence of sophisticated processes utilized by the cells to maintain the integrity of genome. It has been shown that DNA damage is a usual event which is also underlying cause of many disorders such as cancer and other inherited or acquired pathologies. There are many endogenous and exogenous sources which cause DNA damages interfering with genome [1].

In response to genotoxic stress which can be mainly caused by various chemicals, reactive cellular metabolites and ionizing/UV radiation, DNA repair pathways and cell cycle checkpoints can be activated, allowing the cell to repair and prevent the transmission of damaged and/or incompletely replicated chromosomes. The balance between cell cycle arrest, DNA repair and the initiation of cell death can determine whether DNA damage is compatible with cell survival or require elimination of the damaged cell by apoptosis. Defects of DNA repair pathways and cell cycle checkpoints may cause susceptibility to DNA damages, genomic defects, hypersensitivity to cellular stress and resistance to apoptosis, which all characterize cancer cells [2].

Repair of DNA is critical for cell growth, proliferation and for organ development. Genome stability and maintenance require several biochemical pathways involving many different proteins that are having roles in specific DNA repair pathways. Loss of function in these repair proteins may lead to pathologies including growth and developmental defects, like immunodeficiency, cancer and neurodegeneration [3]. As known, cancer is a disease of excessive cell

proliferation, whereas neurodegeneration is a disease of excessive cell dysfunction and death. Those opposite cellular effects can arise from defects of common and/or related processes [4]. DNA repair enable cancer cells to additively accumulate genomic alterations and change into more aggressive phenotypes. As DNA repair pathways are frequently altered in cancer; during anticancer therapies alterations in DNA repair should also be considered [5]. So it may therefore be most effective to search primarily for genetic alterations in those pathways first.

Genomic integrity is important for the survival and is controlled by DNA damage response (DDR) network, this is a complicated signal transduction system sensing DNA damage and recruit repair factors; DDR senses different types of damages and coordinates the responses including cell cycle, apoptosis, senescence and DNA repair processes [6,7]. As it is now known, aging, dementia, and cancer share altered cellular functions in response to DNA damage and/or genotoxic stress. The molecular machinery involved in neural function in neurodegenerative diseases may be shared with oncogenic pathways; so both may be affected by common signaling pathways regulating the balance of cell survival and death [3]. For those regulations to occur, cell cycle check point proteins are extremely important, among those p53 is the most significant because of its role in stopping cell cycle in G_0/G_1 and G_2/M phases, it helps to determine whether the cell will go to apoptosis or DNA repair will occur. Those prevent inappropriate DNA replication, whereas the G_2/M checkpoint prevents cells with DNA damage from entering mitosis. Loss of p53 may increase risk of carcinogenesis, whereas specific gain-of-function in its alleles reduces the incidence of cancer but accelerate aging [3]. The p53 is only one of the targets that may involve in both cancers and aging-related neurological diseases. For instance, in mouse models, experiments showed that the withdrawal of the important myc oncogene was resulting in regression of osteosarcomas, epidermal papillomas and lymphomas [8,9]. As those kinds of gene therapies are now usual for experimental area, in this chapter, we aimed to focus on the current therapeutic approaches focusing on the DNA repair in cancer and neurodegenerative diseases.

DDR is related to a pair of related protein kinases called ATM and ATR and both are activated by DNA damage. ATM works with its regulator MRN complex (MRE11, Rad50-NBS1) by sensing the double-strand breaks (DSBs) [10]. ATR has also its regulator ATRIP (ATR-interacting protein) sensing single-strand DNA (ssDNA). Both of them has many common substrates including Chk1 and Chk2 initiating a cascade that results in cell cycle arrest and DNA repair. Chk1 and Chk2 are serine/threonine kinases; Chk1 is responsible for initiating cell cycle arrest to allow time for DNA repair. After its activation, Chk1 prevents cells from entering S phase [11,12]. Chk2 has a similar effect and is activated by phosphorylation by ATM after DSB [13,14]. In this cascade, phosphorylation of histone H2AX on Ser139 by ATM and ATR leads to the accumulation of repair proteins on DSBs sites [15-17]. Many proteins involved in DDR contain specific H2AX-recognition domains such as BRCT domains (C-terminal domain of BRCA1) [18,19].

Depending on the phase of the cell cycle, there are two major intracellular DNA DSB repair pathways: homologous recombination (HR) or non-homologous-end joining (NHEJ) [1,20]. As chromosomes are duplicated during S-phase of the cell cycle, double strand breaks (DSBs) during S/G_2 can be repaired without any loss of information, by recombination between the

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damaged and its homologous undamaged counterpart. This process is known as homologous recombination (HR), and requires the activity of a number of proteins including BRCA1, BRCA2, XRCC2, XRCC3, and RAD51 [21]. Homozygous HR mutants are rarely survive to birth. Patients carrying such mutations usually have developmental disorders like Fanconi anemia [22]. Homologous recombination uses a sister chromatid in S and G_2 phases as a template; NHEJ is an error-prone method of directly ligating the DSB ends in G_0 and G_1 phases. HR involves BRCA1, BRCA2 and Rad51 proteins and NHEJ involves Ku70/80, the DNA-PK, and DNA ligase IV [1]. During migration and differentiation, there is dependence on NHEJ pathways, so mutations in the NHEJ pathway can result in loss of neuroprogenitor cells, cortical neurons and finally results in microcephaly.

As an example to neurological disorders caused by DSB repair, the severity of the disease in ataxia telangiectasia usually correlates with the nature of the mutation, the amount of active ATM protein within the cells of the patient; as it is a disease with the symptoms of immunodeficiency, sterility, radiosensitivity, cancer predisposition [23,24]. In those cases, loss of ATM results primarily in neuronal dysfunction and ataxia rather than microcephaly. If ATM signal fails, neurons may escape apoptosis and with their unrepaired DSBs they will stay in a dysfunctional state causing juvenile neuropathology. Late-onset progressive neuropathologies, like ataxia telangiectasia, are under debate yet probably depending on the cumulative effects of DNA damages [25].

Single-strand breaks (SSBs) are 3 orders of magnitude more frequent than DSBs. SSBs are usually repaired by the SSBR and NER pathways. Nucleotide-excision repair (NER) is mainly responsible for repairing pyrimidine dimers having important roles during G_1 phase to remove bulky lesions, caused for example by ultraviolet irradiation [1]. But if those pathways are defective, they can trigger apoptosis by blocking the progression of RNA polymerases [26]. The defects in the repair of SSBs are less likely to cause developmental defects, but they are related to neurodegeneration and premature ageing. Deficiencies in single-strand break repair (SSBR) may lead to cellular sensitivity to radiation, oxidative stress and base damaging agents. As poly-ADP-ribose polymerase (PARP1) is the sensor of chromosomal SSBs, it hauls SSBR proteins to the sites of DNA damage [27]. Neuronal cells seem to be particularly sensitive to PARP-induced cell death as shown in cerebral ischaemia experiments [28].

DNA-damaging agents are the corner-stones for the treatment of solid tumors. It is now known that tumors that do not respond to DNA-damaging treatment had proficient DNA repair processes [29,30]. DNA damaging genotoxic therapeutics can be divided into groups due to their mechanism of action and type of damage induced. Alkylating agents and platinum-based agents directly effect DNA to induce bulky DNA damage and those are repaired by the nucleotide excision repair pathway (NER) [1,31,32]. Direct methylating agents cause damages that are repaired mainly via the base excision repair pathway (BER) [31]. DSBs are considered as most toxic forms of DNA damages, induction of DSB via radiation or radio-mimetics is an effective method to induce cellular death. DNA metabolism can also be targetted and DNA intercalating agents, topoisomerase poisons and antimetabolites can be used for this purpose [32].

ATM kinase has been a target for the development of novel anticancer agents. The disease associated with ATM mutation is known as ataxia telangiectasia, an autosomal recessive neurological disorder characterized by cerebellar ataxia and oculocutaneous telangiectasia [33]. This disease has symptoms of growth retardation, premature aging and insulin resistance; patients are known to exhibit hypersensitivity to ionizing radiation. So ATM inhibitors are thought to act as radiosensitizer and/or chemosensitizer [34]. Several ATM and ATM/ATR specific inhibitors have been recently developed: LY294002, KU-55933, KU-60019, CP466722, aminopyrazines [35-37]. As p53 is one of the major substrates for ATM, targetting p53 function also enhances cell sensitivity to ATR disruption.

Inhibitors of poly-ADP-ribosepolymerase (PARP) enzyme, that is normally involved in DNA repair, are also used in DNA repair-based therapies. PARP1 inhibitors are used in the treatment of BRCA1- or BRCA2- defective cancers. ADP-ribosylation is also important in DNA repair and genome stability [5]. As it is known, the BRCA1 and BRCA2 genes play essential roles in HR-mediated DSB repair, PARP1 inhibition induces DNA damages. BRCA1/2- defective cells are sensitive to PARP1 inhibition, but BRCA1/2-proficient cells are resistant [38]. However, although there are main hypotheses, the precise mechanism through which PARP1 inhibition leads to cytotoxicity in HR-defective cells is not exactly known yet. As it is found that PARP1 inhibitors were active against HR-defective tumors, it was thought that their effect could be increased by combination therapies with other genotoxic drugs.

miRNAs are also promising agents to improve efficacy of cancer therapy due to their ability to target DDR components and control cellular responses to DNA damaging agents. For instance, it is known that inhibition of ATM by miR-101, miR-100 and miR-421 or inhibition of DNA-PKcs by miR-101 may cause increased cellular sensitivity to IR [39,40]. It is known that some miRNAs can target multiple genes involved in DDR, so it is thought that modulating endogenous miRNA expression may be a promising way to overcome chemoresistance in cancer treatment [41].

2. Conclusion

Cancer and neurodegeneration are diseases occured because of genomic instability accumulated in large regions of the genome. Many of these abnormalities are eligibilities of inaccurate joining of double-strand break ends, resulting from disruption of DNA repair mechanism [42]. These defects are defined such as single nucleotide polymorphisms, mutations, copy number changes or chromosomal realignments causes inactivation of DNA-repair, tumour-suppressor and proapoptotic genes, leading to defects in the repair of DNA damage. Accordingly, there is a need for diagnostic tests of DNA repair deficiency in clinical trials. Recent studies indicated correlation between a DNA repair profiling methods and prognosis [43]. Clinical development of DDR inhibitors will be expedited in the future by use of next-generation sequencing of key and novel genes included as well as molecular and functional assays for DDR proficiency to identify phenotypes is likely to respond to this approaches and strategies.

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Section 3

DNA Repair, Virus, Inflammation and Stem Cells

Chapter 15

DNA Damage Response/Repair in Cancer Stem Cells — Potential vs. Controversies

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1. Introduction

1.1. Genome integrity and DNA damage response

The genetic material of each cell maintains the information required for all cell processes including cell viability and proliferation. Preservation of genome integrity is essential for living cells, while being a prerequisite for survival of higher-order multicellular organisms. Thus, intact genetic information should pass to the forthcoming generations. On the other hand, genome is constantly subjected to endogenous and exogenous damages by a number of factors, such as reactive radicals, radiation, and genotoxins [1]. Despite the fact that in a few cases mutations may provide selective advantage in species evolution, a DNA damage response system to sense DNA damage, arrest cell cycle, repair DNA lesions, and/or induce programmed cell death is crucial for maintenance of genomic integrity and survival of the organism. Consequently, a coordinated cellular response to DNA damage is required for effective DNA repair, ensuring viability, and preventing disease.

Unfortunately, neither the chemical properties of the DNA molecule nor its interaction with environmental factors guarantee lifelong stability and proper functioning of the genome. Due to its chemical structure, DNA is particularly sensitive to spontaneous hydrolysis reactions that may create both abasic sites and base deamination. Furthermore, ongoing cellular metabolism generates reactive oxygen species and their highly reactive intermediate metabolites, which can create 8-oxoguanine lesions in DNA as well as a variety of base oxidations and DNA strand breaks that are all highly mutagenic. This phenomenon may also lead to genomic instability. DNA is also constantly assaulted by mutagens present in the external

environment. UV light from the sun, as well as various chemical reagents, can react with DNA and induce nucleotide chemical modifications. In addition, ionizing radiation generated by the cosmos, X-rays, and exposure to radioactive substances, as well as treatment with certain chemotherapeutic drugs, can induce base modifications, interstrand cross links, and DNA single - and double-strand breaks (DSBs), which can all lead to genomic instability. It is estimated that each cell is confronted with approximately 10^4–10^5 DNA lesions per day, indicating that clearance of genomic injuries constitutes the maintenance of proper genome function a demanding task [2].

Thus, maintenance of genomic stability through damage repair is essential for cell and organism longevity. Without genomic stability, replication errors and external stress as well as direct forms of DNA damage can induce mutations, which decrease cell survival, cause altered gene expression, and therefore can lead to cellular transformation.

In response to the wide diversity of potential DNA lesions, eukaryotic cells developed a number of highly conserved DNA repair mechanisms that can recognize and repair different types of DNA damage with varying fidelity and mutagenic consequences (Table 1). Irrespective of the type of lesion and the repair mechanism, DNA damage is rapidly sensed and activates evolutionarily conserved signaling pathways, known collectively as the DNA damage response (DDR). DDR components can be separated into four functional groups, extensively described in other chapters of the current edition: damage sensors, signal transducers, repair effectors, and arrest or death effectors. In brief, cells contain multiple DNA repair mechanisms including: base excision repair (BER) that removes damaged bases caused by small chemical alterations (base modifications), mismatch repair (MMR) that recognizes and removes mispaired base incorporation errors and base damage arising from replication errors, nucleotide excision repair (NER) that corrects bulky helix-distorting lesions caused by chemicals and ionizing radiations, and cross-link repair (ICL) that removes interstrand cross links. In addition, the most deleterious DNA lesions, breaks in the DNA backbone, (Double-Strand Breaks, DSBs) are mainly repaired via homologous recombination (HR) and nonhomologous end joining (NHEJ). These most challenging DSBs may be restored by a different degree of repair fidelity, related to the pathway chosen according to the phase of the cell cycle. While the the almost error-free HR repair dominates in dividing cells, the G1 phase acting NHEJ is error-prone, as genome has not yet undergone duplication; hence, a template for recombination used in HR is not yet available. These two pathways seem to repair the majority of chemotherapy- and radiotherapy-induced damage [2-7].

In a rapid overview, regarding the restoration processes of DSBs, HR allows cells to repair DNA damage in an error-free manner and can be performed only during S and G2 phases of the cell cycle due to the requirement for the undamaged sister chromatid as a template. HR starts with 5′-3′ resection of the DNA ends to create 3′ single-stranded DNA tails providing a substrate for assembly of RAD51 filaments which catalyze homology search and DNA strand invasion followed by repair synthesis and annealing with the second end of DSB. Each stage of this multistep pathway requires the sequential involvement of a number of distinct enzymes [8-11]. On the other hand, NHEJ is an error-prone DNA repair mechanism that utilizes the specialized DNA end-binding proteins Ku70/Ku80 and various DNA-specific enzymatic

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proteins such as DNA-dependent protein kinase (DNA-PK), nucleases, polymerases, and ligases. NHEJ does not require DNA sequence homology and can take place throughout the cell cycle [10]. At the chromatin level, an initial event triggering DDR is the phosphorylation of H2A.X, which is called γ-H2A.X, forms nuclear foci on the sites of DNA damage and is necessary for the assembly of repair complexes [12,13].

DNA-DAMAGING CAUSES	- ROS - X-rays - Alkylating agents - Spontaneous reactions	- UV light - Polycyclic aromatic hydrocarbons	- Replication errors	- X-rays - Ionizing radiation - Antitumor agents
DNA LESIONS OBSERVED	- Oxidation (8-oxoG) - Uracil Abasic site - Single strand breaks	- Bulky adducts - Intrastrand cross links	- A->G mismatches - T->C mismatches - Insertions - Deletions	- Double-strand breaks - Interstrand cross links
DNA REPAIR PATHWAYS	- Base Excision Repair, BER	- Nucleotide Excision Repair, NER	- Mismatch Repair, MMR	- DSBs Repair: - Non-Homologous End Joining, NHEJ - Homologous Recombination, HR

Table 1. Common DNA-damaging causes and agents, examples of DNA lesions produced by these sources, and the relevant DNA repair mechanism responsible for their removal.

In normal conditions, under extensive damage or inability of repair, senescence or apoptosis may occur as distinct checkpoints block cell duplication and/or survival. Persistent DNA damage can entail mutagenesis such as base substitutions and small insertions/deletions, as well as gross chromosomal rearrangements. Such genome instability likely contributes to aging and age-related disease and it constitutes an essential step in the development of cancer [14,15].

1.2. Cancer stem cells

Cancer is broadly defined as a group of diseases characterized by uncontrolled growth and spread of abnormal cells bearing genetic or epigenetic alterations resulting in high morbidity and mortality. Since the declaration of "war on cancer" about 50 years ago, significant strides have been made in battling the disease thanks to the worldwide scientific community's concerted effort for a better understanding of cancer biology. As a result, an apparent target of antitumor therapy was proved to be the destruction of tumor genetic material. Tearing up the DNA of carcinoma cells, usually combined with tumor removal by surgery, is suggested as one of the most effective therapeutic schemes resulting in residual cancer cell death. Radiation therapy and common chemotherapeutics are DNA damaging agents targeting the genome of carcinoma cells and nowadays they represent conventional

treatment schemes. A common practice in cancer treatment includes a combination of surgery, chemotherapy, and radiotherapy, depending on the lesion type and the clinical picture of the patient. Nevertheless, cancer still plagues humanity as a largely incurable disease. Poor prognosis and low survival rate are even more prominent in cases where malignancy is detected at a late stage. Another challenge for oncologists arises from frequent metastasis and tumor recurrence, which further frustrates effective treatment protocols currently available. Despite "heavy" therapeutic schemes, a subpopulation of cancer cells seems to evade DNA-damage-induced cell death and retain the ability of tumor regrowth through metastastic spread capacity. This subpopulation was termed cancer stem cells (CSCs) or tumor initiating cells (TICs), and is considered to be a major cause of tumor relapse due to local and/or distant recurrence of carcinoma cells [16-22]. The underlying mechanisms are not clearly understood in detail but a lot of work is accumulating worldwide, aiming to elucidate CSCs resistance etiology to DNA-damaging agents [1,23].

CSCs seem to differentiate into a diverse panel of progeny cells that make up the tumor, and reproduce the original tumor after xenotransplantation. There are several theories regarding the origin of CSCs [24]. According to a widely accepted hypothesis, CSCs are considered to derive from malignant transformation of stem/progenitor cells, when encountering special genetic mutations or environmental alterations, instigating the tumorigenic process. This hypothesis indicates that only CSCs possess tumor-initiating potential whereas non-CSCs do not. CSCs can be distinguished from other cells within the tumor by symmetry of their cell division and alterations in their gene expression. CSCs possess stem-like properties, such as self-renewal, proliferation and differentiation abilities, expression of pluripotency factors (e.g., Sox2, Oct4, Nanog) and functional markers (e.g., ALDH1, CD133+, CD44+, CD24, CD38-), active signaling pathways (e.g., Notch, Hedgehog, Wnt), genetic and epigenetic profiles similar to stem cells, and the capacity to form form 3dimensional spheres *in vitro*. Cell surface markers such as CD44, CD24, CD133, epithelial specific antigen (ESA), and aldehyde dehydrogenase1 (ALDH1) have been used to isolate and enrich CSCs from different tumors. Markedly, CSCs express surface markers, which seem to be tissue-type-specific and in some cases tumor-subtype-specific. For example, breast CSCs are characterized as CD44+/CD24−/low and ALDH +, based on the presence or absence of the respective molecules. In analogy, CD133 expression is characteristic for colon, brain, and lung CSCs, while CD34 presence and CD8 absence (CD34+/CD8-) are characteristic for leukaemia. The list includes CD44+ for head and neck, CD90+ for liver, and CD44+/CD24+/ESA+ for pancreas CSCs [25-28]. Nevertheless, novel combinations of CSC markers are continuously being added to this group or combinations not previously defined are also described (Frangou et al., unpublished data). Therefore, expression of CSC surface markers can only be a manmade criterion to describe tumor stem cells and some CSCs may not fulfil these criteria. Moreover, CSCs have been found to exhibit similarities with normal stem/progenitor cells in cell surface marker expression, properties, phenotype, and function. For example, the mammary gland progenitor cells are characterized as a CD44+/CD24−/low cell population and resemble the CD44+CD24−/low cells identified as CSCs from breast cancer patients. Apart from the theory suggesting that CSCs derive from transformation of normal stem cells, an alternative theory about the origin of CSCs suggests that they may arise from transformation of normal somatic cells. According to this notion, somatic cells

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acquire stem-like characteristics and malignant behavior through genetic and/or heterotypic alterations. The epithelial to mesenchymal transition (EMT) of cancer cells during the metastatic process may provide a mechanism by which cancer cells may gain stem-like characteristics [29,30]. Further research is required to elucidate whether both theories may be correct about CSCs' origin but each explain distinct cases.

In addition, another role attributed to normal stem cells in the various tissues is their implication in the repair process of damaged tissue. In order to fulfill this task, normal stem cells have to overcome genotoxic insults and in turn proliferate to restore eradicated tissue cells. Since much evidence favors cancer originating from stem cells, it is not surprising that many of survival and proliferation pathways of stem cells have aberrant expression in cancer cells. In accordance to this hypothesis, the traditional pathways Notch, Hedgehog (Hh), and Wingless/Int (Wnt) have been proposed to also characterize CSCs [31].

Another aspect to consider is that tumorigenesis is followed by angiogenesis and by cancer cell invasion in other tissues (metastasis) as part of the disease progression. Not surprisingly, CSCs have been associated with the induction of tumor vascularization through the expression of vascular-related factors and by their contribution to metastasis through the induction of the Epithelial to Mesenchymal Transition (EMT) program [30,32].

1.3. Cancer stem cell resistance pathways

For all the aforementioned reasons it is obvious that CSCs' resistance to chemo- and radio therapies is clinically important as most anticancer agents target the tumor bulk but not the CSC population. As previously mentioned, mechanisms helping cells to escape cytostatic and cytotoxic effects after application of DNA-damaging treatment approaches are far from clearly understood despite intensive and extensive studies. Cancer stem cell (CSC) theories attempt to explain how CSCs overcome cell death caused by genotoxic treatment schemes. It is also suggested that CSCs possess specific intracellular molecular properties assisting them to avoid treatment-derived cytotoxicity [23]. Various mechanisms account for CSCs drug resistance. A comprehensive example comes from studies on breast cancer stem cells. Of all the CSCs identified in solid tumors, breast CSCs are one of the most commonly studied. They are defined as $CD44^+/CD24^-$ cells and have been linked to resistance to many forms of treatment, including radiation therapy. $CD44^+/CD24^-$ cells isolated from MCF-7 and MDA-MB-231 breast cancer cells were more radioresistant than non-$CD44^+/CD24^-$ cells [33]. The mechanisms involved possibly include decreased induction of reactive oxygen species and activation of the DNA damage checkpoint response [34]. Moreover, an *in vitro* study has documented that during a fractionated course of radiation, the number of CSCs with activation of Jagged-1 and Notch-1 increased, suggesting the possible induction of radiotherapy resistance via the Notch signaling pathway [33].

Overall, CSCs display features different from the bulk of cancer cells, like high-level expression of ATP-binding cassette membrane transport proteins (ABC transporters), mainly including ABCG2/BCRP and ABCB1/MDR1; high capacity for DNA signaling and repair; reduced immunogenicity; inherent anti-apoptotic properties; and quiescence. Understanding the mechanisms of chemo- and radiation-resistance of CSCs may pave the way towards discov-

ering a set of signaling pathways unique to CSCs. Such mechanisms may be responsible for CSCs' capability to resist and survive the current chemoradiation therapeutic schemes. Targeting such set of pathways would ideally provide more effective and presumably personalized therapeutic potential towards complete tumor eradication. Despite difficulties and though such ideal pathway has not been found yet, elucidation of developmental pathways that control survival, proliferation, and differentiation of stem cells is under extensive investigation.

As summarized in Figure 1, the resistance ability of CSCs appears to be associated with their slow-cycling phenotype, and/or expression of efflux transporters, antiapoptotic proteins, altered profile of cell surface markers, DNA response and repair mechanisms, or presence of free radical scavengers [27,31,35-38].

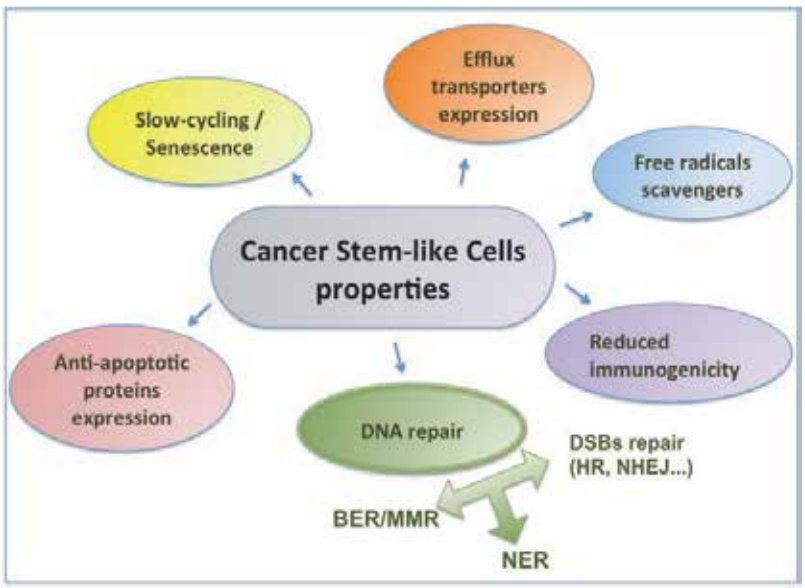

Figure 1. Schematic representation of CSCs properties related to chemo- and/or radioresistance (details in the text).

Among these characteristics, the multifaceted protection of genome integrity by a prompt activation of the DNA damage sensor and repair machinery is one of the key features rendering resistance to applied genotoxic insults. CSCs possess highly effective DNA repair systems mainly consisting of double-strand breaks (DSBs) repair, base excision repair (BER), transcription-coupled nucleotide excision repair (NER), and mismatch repair (MMR) pathways. These pathways seem to be distinctly regulated in CSCs, resulting in significant enhancement of DNA repair capability and finally radio- and chemoresistance [23,39-43].

Therefore, the aim of the current chapter is to highlight issues and discuss controversies of CSC genotoxic resistance through DNA Damage Response (DDR) and DNA repair pathways,

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which seem to play a key role in evading genotoxicity-induced cell death. This is a highly evolving topic as the anticipated information about the basic mechanisms governing DNA repair processes is also expected to contribute in predicting and improving therapy responses and the clinical outcome in cancer patients treated with DNA-damaging agents.

2. DNA damage resistance of CSCs

Accumulating reports tend to indicate that there are CSCs in almost all tumor types (reviewed in [22]). For example, using the CD133 as the brain stem cell marker, Bao et al. at Duke University have described an increased proportion of brain CSCs, from about 2 to about 8% in control versus irradiated tumors, which was associated with tumor radioresistance [34]. A group at UCLA showed that breast-cancer-initiating cells displaying the marker of breast CSCs (CD24−/low/CD44+) are radioresistant and cells expressing these markers increase after short courses of fractional irradiation [33]. All of these findings shed new light on the mechanisms of an accelerated tumor cell proliferation with an increase in the percentage of radioresistant CSCs.

The use of radiation therapy and chemotherapeutic drugs aim to provoke DNA damage in cancer cells. If the damage is quite extensive and cannot be repaired, cell death is inevitable [44]. Research on radio- and chemoresistance of CSCs after exposure to DNA-damaging agents has been extensively conducted and there is great evidence demonstrating that this subpopulation in tumors protects itself from DNA-damaging treatment by multiple mechanisms. First of all, CSCs are considered to have an enhanced DNA repair capability and consequently they are protected more effectively than the rest of tumor cells. Numerous studies suggest that the resistance of cancer stem cells to therapy is mediated by more robust DNA damage response and repair pathways [45-48]. Special regulation and elongation of cell cycle is also regarded to be another protection mechanism incorporated, providing CSCs more available time to repair damaged DNA. Moreover, CSCs demonstrate great efficiency in scavenging of reactive oxygen species (ROS) and therefore eliminate the primary cause of DNA insults [49-51].

In the following paragraphs, information regarding CSCs' resistance to DNA damage through both genotoxicity inactivation and DNA Damage Response/Repair mechanisms will be presented. It is well documented that depending on the type and tumor stage, CSCs adopt distinct main and auxiliary mechanisms to protect their genome and overcome insults. The most prevalent intrinsic molecular determinants of radioresistance appear to be the protection from oxidative DNA damage by enhanced ROS scavenging as well as the enhanced DNA repair capability by post-translational modification of damage signalling factors (ATM and CHK1/CHK2 phosphorylation) and subsequent repair.

2.1. ROS scavenging

The indirect pathway of radiation-induced damage includes the generation of chemically reactive free radicals, including the product of oxygen metabolism called reactive oxygen species (ROS). These products play an important physiological role and participate in many

signaling events regulating cell proliferation, migration, angiogenesis, wound healing, and metabolism [52]. Both normal and cancer cells can control ROS level by balancing their production and elimination by ROS-scavenging molecules such as glutathione, peroxidase, catalase, superoxide dismutase, thioredoxin, etc. [53]. An excessive production of ROS in response to irradiation may lead to their interaction with critical cell macromolecules including DNA, lipids, and proteins, leading to cell death [54]. High resistance of CSC populations in breast and gastrointestinal carcinomas to genotoxic stress is related to a more efficient ROS scavenging system and lower levels of ROS production after irradiation as compared to non-CSC populations [49]. Genes involved in ROS scavenging, including superoxide dismutase, glutathione peroxidase, and catalase are upregulated in CD44+CD24− breast CSCs. The role of ROS scavenging in CSC radioresistance is supported by the observation that pharmacological depletion of ROS scavengers in tumor progenitors by treatment with buthionine sulfoximine (BSO), which inhibits glutamate-cysteine ligase, markedly decreased clonogenic properties and radioresistance of CSCs [49]. The functional link between stem cell markers and ROS metabolism was first demonstrated by Ishimoto and colleagues who showed that CD44 interacts with glutamate-cysteine transporter xCT and controls the intracellular level of ROS scavenger glutathione in gastrointestinal cancer cells [50]. These preclinical results are supported by recent clinical studies, which showed that high expression of CD44 in tumors was correlated with resistance to radiation therapy and associated with early recurrence in HNSCC patients [55-57].

The activity of aldehyde dehydrogenase (ALDH) enzymes is also highly correlated with the existence of cancer stem cells in tumors [58]. Furthermore, there is a relationship between poor clinical prognosis in breast and prostate cancer and increased expression of ALDH1 [59]. It has been proved that ALDH1 and ALDH3A1 play a key role in the cellular response to oxidative stress, since they contribute to the scavenging of radiation-induced free radicals and the production of the antioxidant NAD(P)H [60]. These observations suggest that ALDH activity can be crucial for regulation of cell radio-sensitivity. High ALDH1 activity is another characteristic of human breast and colonic cancer stem/progenitor cells [61]. As few as 500 ALDH1-positive cells (as documented by the ALDEFLOUR assay) can give rise to a new tumor in NOD/SCID mice [59]. Other studies have found overexpression of ALDH1 in cyclophosphamide-resistant leukemic and colonic cancer cells [62]. Thus, overexpression of the detoxification enzyme ALDH1 may also contribute to the resistance of CSCs to various cancer treatments, including chemo- and radiation therapy resistance.

Another characteristic example of ROS management is through the regulation of Ape1/Ref-1, also known as APEX1, a ~37kDa protein containing a redox activity and an endonuclease activity domain. Ape1/Ref-1 is involved in BER. During cell exposure to genotoxic agents its expression is increased in response to reactive oxygen radicals (ROS) production. Activation of Ref-1 redox domain decreases ROS levels resulting in the enhancement of carcinoma cell stemness and self-renewal. On the other hand, inhibition of the Ref-1 domain, which is responsible for ROS scavenging, results in increased intracellular ROS levels, activation of p53, and promotion of cancer cell differentiation and cell death. Permanent ROS production, possibly caused by microenvironmental factors in the CSC niche, results in overexpression of

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Ape1/Ref-1, which may effectively protect CSCs from ROS genotoxic effects produced during treatment with ionizing radiation/DNA-damaging agents. Besides, redox modulation of p53 by this factor seems to contribute significantly to DNA repair in CSCs. Ape1/Ref-1 is also required for redox regulation of HIF-1α, thus regulating downstream controlled DNA repair genes. Likewise, Ape1/Ref-1 is also implicated in the regulation of Rac GTPase activity, a protein closely related with CSC formation, resistance to DNA-damaging agents (BER, G2-M checkpoints activation allowing repair of damaged DNA), and carcinoma cell motility and migration. On the other hand, BER allows mutation accumulation and further genome instability, occurring also due to Raf1, MEK1/2, and ERK1/2 pathway activation, which promotes enhancement of tumor aggressiveness, insensitivity to therapeutic approaches, and metastasis [23,63].

2.2. DNA damage response/signaling

Radiation-induced cell death may occur as a result of direct and indirect energy transfer to critical cellular structures including chromatin, plasma membrane and mitochondria.

Cell-cycle checkpoint components, such as Ataxia Telangiectasia Mutated (ATM) protein, Ataxia Telangiectasia/Rad3-Related kinase (ATR), and checkpoint kinases (Chk1 and Chk2), become engaged under replication stress or in response to DSBs. These cell-cycle arrest mechanisms allow the recruitment of either DNA repair effectors or in case of irreversible damage and repair failure, of proapoptotic molecules [65].

The observed resistance of CSCs to common chemo-/radiotherapy strategies is considered to partly occur through their extensive ability of repairing DNA damage that has been provoked through radiation or chemical drugs. This enhancement of DNA repair capacity can be either direct, through elevated DNA repair mechanisms, or indirect, through delayed cell-cycle progression.

A principal player in DDR, in both normal and malignant cells, is the major sensor of DNA double-strand breaks termed MRN complex (a complex of MRE11, RAD50, and NBS1 proteins). MRN complex binds to and stabilizes broken DNA ends and is required for the activation of ATM. MRN complex functioning through BMl1 is also interconnected with CSC-related molecules like Notch1, ALDH1A1, CD44, and Sonic Hedgehog, together with telomere biology and upon deregulation, with aggressive tumor behavior and unfavorable disease prognosis [66]. Moreover, another mechanism rendering Glioblastoma Multiforme (GBM) stem cells relatively resistant to DNA damage through MRN-ATM-Chk2 network signaling involves L1CAM (CD171) interaction with NBS1. L1CAM intracellular domain (L1-ICD) nuclear translocation mediates NBS1 upregulation via c-Myc. Ectopic expression of NBS1 in GSCs rescues the decreased checkpoint activation and radioresistance caused by L1CAM knockdown. These data demonstrate that L1CAM augments DNA damage checkpoint activation and radioresistance of GSCs through the enhanced MRN-ATM-Chk2 signaling, resulting in GSCs displaying a preferential activation of DNA damage checkpoint and radioresistance [67].

The major sensor and signaling effector of DDR, ATM kinase, seems to also contribute to DNA damage resistance of CSCs. ATM comprises a key DNA damage sensor and downstream effector kinase playing central roles in DNA repair, cell-cycle control regulation, and development of senescence and/or apoptosis (refs). Several studies indicate that ATM activity may play a role in normal stem cell maintenance and proliferation. Two main roles are attributed to ATM: a role in stem cell survival and an implication, as part of DDR, in pathways classically linked to stem cell maintenance [68,69].

Regarding the first role, ATM seems to be implicated in regulation of neuronal stem cell survival (NSCs). More precisely, while ATM is abundantly expressed in NSCs, it is gradually reduced during cell differentiation. This observation suggests that ATM is vital for NSC survival and function [70]. ATM is required to maintain normal self-renewal and proliferation of NSCs due to its role in controlling the redox status. Loss of ATM renders NSCs defective for proliferation through oxidative-stress-dependent p38 MAPK signaling, suggesting that p38 is a central player in the defective proliferation of Atm/NSCs induced by oxidative stress [71, 72]. Moreover, it has been shown that ATM plays a central role in terminal differentiation of a human neural stem cell line model through its function in DDR [73].

In addition, ATM protein is a major player in signaling pathways classically implicated in stem cell maintenance. Lately, ATM was proposed to positively modulate the activity of ITCH E3-ubiquitin ligase. ITCH is a member of the NEDD4-like family of HECT-E3-ubiquitin ligases, a family of proteins that participates in several physiological signaling pathways, including the DNA damage response, tumor necrosis factor (TNFα), Notch, and Sonic-Hedgehog signaling [74]. The ATM-dependent activation of ITCH requires the single amino acid residue S161 of ITCH protein, which is part of an ATM S/T-Q consensus motif. Subsequent *in vitro* and *in vivo* genetic experiments provided evidence showing that ATM kinase enhances ITCH enzymatic activity and triggers ubiquitination/degradation of ITCH (itchy E3 ubiquitin protein ligase) substrates such as FLIP-L and JUN [75]. Possibly, ATM is also implicated in the regulation of other ITCH substrates including the transcription factor GLI-1. GLI-1 mediates Sonic-Hedgehog (SHH) signaling, resulting in regulation of tissue patterning and cell proliferation. These cellular functions are prerequisites ensuring the accurate developmental progress and homeostasis maintenance of adult tissues [76]. Interestingly, several lines of evidence suggest a putative cross talk between ATM and the SHH pathway. The first hint comes from the identification of GLI-1 as a substrate of ITCH [77,78] and from the observation that ATM modulates ITCH [75]. A second hint comes from the observation of the wild-type p53-induced phosphatase 1 (WIP1) function. WIP1 is a Ser/Thr phosphatase, which is aberrantly upregulated in cancer and modulates ATM activity by dephosphorylation [79]. Moreover, WIP1 is involved in the modulation of the SHH signaling [80]. Presumably, during tumorigenesis, overexpression of WIP1 may be implicated to increased GLI-1 activity resulting in both the proliferation and self-renewal of CSCs. This phenomenon may be responsible for CSCs expansion as the derived CSC progenitors retain the ability to sustain tumor growth [80]. In accordance to this hypothesis, it was recently shown that wild-type p53 downregulates GLl-1 function by sequestering the co-activator TATA Binding Protein Associated Factor 9 (TAF9), a process comprising an inhibitory loop controlling stem cell and tumor cell numbers

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[81]. Furthermore, accumulated data suggest a possible bidirectional connection between GLI-1 and the DDR. It has been observed that there is a feedback loop in GLI-1 level regulation as abnormal increase of GLI-1 induces DDR, which in turn may decrease GLI-1 activity. The delicate control of GLI-1 activity may thus be part of the mechanisms controlling the precursors and stem cell numbers and preventing tumorigenesis [82,83]. Whether ATM kinase may directly modulate SHH signaling, therefore contributing to the maintenance of stem cell identity, remains to be elucidated.

Furthermore, DDR and ATM activation are also implicated in the regulation of CSCs' survival. In particular, upon therapy-induced DNA damage, temporal halt of proliferation through cell-cycle elongation may provide cancer stem cells with increased time for repair. Upon genome insults restoration, the replisome is reactivated for genome duplication. Through such pathways genotoxic resistance may be triggered in CSCs. Therefore, by specific inhibition of the DNA damage checkpoint response, a cell-cycle break may occur in CSCs, driving them again towards proliferation and thereby specifically sensitizing them to genotoxic insults caused by radiotherapy. In this context, ATM may serve as a useful candidate target for eliminating cancer stem cells in the tumor. This notion is further supported by recent studies showing that constitutive activation of a DDR started by ATM may promote radioresistance of CSCs. Utilization of this idea was pioneered by Bao and collaborators in glioblastoma multiforme (GBM). In GMB, cancer stem-like cells are characterized by expression of Prominin-1 (CD133). CD133 is a marker for both neural stem cells and brain cancer stem cells. $CD133^+$ CSCs of GMB were shown to preferentially activate the DNA damage checkpoint in response to radiation accompanied by higher expression of activating phosphorylation of ATM, RAD17, CHK1, and CHK2 checkpoint proteins following IR treatment [34]. As a result, CD133+ cells exhibited preferential survival after irradiation, a phenomenon that may be reversed after treatment with CHK2 inhibitor. In consistency to these results, two distinct grade IV glioma cell lines, varying in CSC content (low and high, respectively), were preincubated with a nontoxic concentration of the ATM inhibitors KU-55933 and KU-60019 and then irradiated. The aim of this experiment was to investigate potential improvement of the therapeutic efficacy of radiation on glioma stem cells. Indeed, GSCs were sensitized to IR by ATM inhibitors, as revealed by a significant reduction in their survival. Quite interestingly, IR treatment following cell differentiation showed no sensitization, indicating that ATM inhibitors specifically sensitize GSCs [84]. Recently, a profound radiosensitization of GSCs was obtained by using a combination of PARP and ATR inhibitors which exceed the effect of ATM inhibition alone [85]. However, it is encouraging that similar results following ATM inhibition have been obtained when using CD44+/CD24−/low cells, a subpopulation enriched for CSCs, from two breast cancer cell lines and a primary breast cancer cell culture isolated from breast cancer patient. In these cases, the CSC-enriched subpopulations demonstrated enhanced expression of phosphorylated ATM after radiation, a results concomitant with increased radioresistance. These results were also reverted when the ATM inhibitor KU-55933 was used. In this case also the CD44+/CD24−/low subpopulation isolated both from the cell lines and from the primary culture exhibited significant decrease of radiation resistance [86]. Jointly, a crucial role for ATM signaling in survival capacity of CSCs in response to genotoxicity is supported by these findings, further suggesting that ATM inhibition may be exploited towards the development of novel therapeutic strategies against CSCs.

DNA damage induces checkpoint mechanisms including two distinct kinase signaling pathways, the ATM-Chk2 and ATR-Chk1 pathways, which are activated by DSBs and single-strand DNA breaks, respectively. DNA damage checkpoint signaling inhibits cell-cycle progression to allow DNA repair [87]. Recent findings of Bartucci et al. suggests that chemotherapy-induced activation of the DNA damage checkpoint-Chk1 signaling in a stem-cell-enriched cell subset within Non-Small-Cell Lung Carcinoma (NSCLC) led to cell-cycle arrest, more efficient DNA damage repair and a higher cell survival rate as compared to the differentiated tumor cell population. The use of Chk1 inhibitor AZD7762 in combination with chemotherapy significantly reduced survival of the stem cell population by inducing premature cell-cycle progression and subsequent mitotic catastrophe [88]. As aforementioned, studies of glioblastoma by Bao and co-workers demonstrated that ATR-Chk1 and ATM-Chk2 signaling pathways are preferentially activated in CD133+ progenitor cells, but not in CD133− cells in response to radiation-induced genotoxic stress, and CD133+ cells repair DNA more effectively than CD133− tumor cells [34]. Moreover, relative radioresistance of CD133+ glioblastoma progenitor cells can be reversed by pharmacological inhibition of the Chk1 and Chk2 kinases with debromohymenial-disine (DBH) [34]. Chk1 knockdown in CD133+/CD44+ prostate cancer tumor-initiating cells abrogated the radiation-induced cell-cycle arrest and conferred CSC radiosensitization [89]. In addition, ATR or Chk1 inhibition sensitizes colon cancer stem cells to cisplatin. Remarkably, treatment of human colon cancer cells with caffeine, an unspecific inhibitor of PIKK kinases, led to depletion of the CD133+ chemoresistant and tumor-initiating cell population, which may suggest an overlap of the signaling pathways regulating tumorigenic properties and DNA damage response [90].

In the case of glioma CSCs, high expression levels of checkpoint kinases CHK1 and CHK2 have been observed, indicating expansion of cell cycle. The delay of cell cycle offers more time for DNA repair 1 have been also monitored in non-small-cell lung CSCs. In leukemia, CSCs increased levels of p21, which inhibits cell-cycle progression, providing sufficient time to repair DNA damage.

Chk1 inhibition has been shown to inhibit both the DNA-damage-induced cell-cycle checkpoint response and homologous recombination repair [91,92] and many studies have also shown that cancer stem cells elicit a more robust Chk1-mediated DNA damage response than non-stem cells [34,47,90]. As an example, Chk1 inhibition was used to sensitize pancreatic cancer stem cells to gemcitabine. A combination of gemcitabine and AZD7762 significantly reduced the percentage of marker-positive cells (pChk1 (S345) as a pharmacodynamic biomarker of gemcitabine-AZD7762 activity [93]) and decreased the tumor-initiating capacity of cancer stem cells using a limiting dilution assay. The same study demonstrated that Chk1 inhibition displayed a heightened DNA damage response in stem cells (vs. non-stem cells) overall highlighting the potential efficacy of this approach to target pancreatic cancer stem cells [94]. The possibly underlying greater extent of DNA damage in the stem cells treated by this approach is consistent with their sensitization to gemcitabine by Chk1 inhibition. As inhibition of Chk1 produces more DNA damage that is marked by a more robust DNA damage response in cancer stem cells, an important future challenge remains to better understand the mechanisms contributing to selective cancer stem cell sensitization through the incorporation

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of *in vitro* pancreatic cancer stem cell models. In parallel, other studies have shown that the AKT inhibitor, perifosine, sensitizes breast cancer stem cells to radiation [95], whereas targeting DR5, SHH, or mTOR in combination with gemcitabine reduces pancreatic cancer stem cells [96,97].

3. DNA repair capability

The most lethal form of DNA damage is considered to be the DNA double-strand breaks (DSBs), which can be repaired by either homology-directed recombination (HR) or nonhomologous end joining (NHEJ), depending on the phase of the cycle [98]. During HR, RAD51 filaments assemble to single-stranded DNA tails and catalyze homology search. One of the early and best characterized chromatin modification events in DNA DSB response is histone H2AX phosphorylation on serine 139 of its C-terminal tail by phosphatidylinositol 3-kinase-related kinases (PIKKs) DNA-PK, ATM, or ATR serine/threonine protein kinases, which are activated by DNA damage. A few independent studies demonstrated that CSCs have an activated DNA repair process. Recent finding suggests that human breast CSCs and murine mammary gland CSCs have significantly more RAD51 foci and less γ-H2AX foci after irradiation compared to non-CSC population that is reflective of more efficient DSB repair in these CSC populations [99]. A high DNA repair capability has been attributed to CSCs in a variety of tumor entities including glioma, nasopharyngeal carcinoma, lung, breast, and mouse mammary tumors [34, 100-103]. In a syngeneic p53 null mouse mammary gland tumor model, it was shown that this cell subset has an increased expression of genes involved in DNA damage response including Nek1, Brca1, Chek1, Hus1, Ung, Xrcc5, Sfpq, and Uhrf1 [104].

A paradox seems to emerge in CD133+ glioblastoma stem cells which activate ATM and Chk1 more promptly than the CD133- counterpart [34]. This molecular response enabled CD133+ cells to survive ionizing radiation, as opposed to the CD133- population that underwent cell death. While radiosensitivity is restored by pharmacologic abrogation of Chk1 and Chk2, it seems that the glioblastoma stem cell pool does not possess enhanced DNA repair activity following exposure to ionizing radiation. In this case, radioresistance properties were linked to cell-cycle kinetics, as indicated by the significant increase in the population doubling time and enhanced basal activation of Chk1 and Chk2 [105]. This elongated cell cycle, therefore, theoretically provides more time for repairing DNA damage. To further intricate this picture, a direct comparison of radiosensitivity between glioblastoma stem cells and a panel of established glioma cell lines revealed that CD133+ cells exhibit reduced DSB repair ability [46]. Cell-cycle analysis revealed that although glioblastoma stem cells possessed an intact G2 checkpoint, they displayed deficient activation of the intra-S-phase checkpoint. Because the latter checkpoint is crucial for maintaining genome integrity, chemotherapy could paradoxically lead to the emersion of genetically unstable CSCs, thus explaining the pattern of disease progression during sequential chemotherapeutic regimens.

The genetic heterogeneity of CSCs could mirror a different DNA damage repair proficiency among subtypes, thus providing a possible explanation for the conflicting results discussed above.

High-grade primary brain tumors are also known to aberrantly activate the phosphoinositide 3-kinase (PI3K)/ Akt pathway [106], an oncogenic axis functionally interconnected with the DNA repair machinery, as highlighted by the ability of PI3K or Akt inhibitors to hamper the removal of radiation-induced DNA damage [107]. It is worth considering that the pharmacologic abrogation of Akt impaired glioblastoma stem cell fitness and abrogated neurosphere formation [111], thus allowing postulating that Akt inhibitors could be exploited as chemotherapy-enhancing agents.

Notch and Wnt signaling pathways, utilized in normal stem cell self-renewal, were also found to mediate radioresistance in glioblastoma and breast cancer [33, 108,109].

On the other hand, Facchino et al. have shown that the polycomb group protein BMI1 is enriched in CD133+ glioblastoma stem cells and operates as a recruitment platform for the double-strand break repair (DSB) response and nonhomologous end joining (NHEJ) proteins, resulting in increased cell radioresistance [110].

Based on the above mentioned studies, attempts for stratifying tumor types based on the DNA repair expression index were able to show statistically significant prognostic capability of the clinical outcome, paving the way towards personalized treatment schemes [112]. In addition, inhibitors of DNA damage response are increasingly utilized in order to increase DNA damage sensitivity [113]. Quite recently, an elegant approach developed a NOTCH tumor phenotype in C. elegans delineating the role of NOTCH receptors expression in relation to HR and radiosensitization [114]. Numerous ongoing studies are focusing on deciphering the detailed DDR /repair mechanisms governing CSCs resistance in solid tumors with expected encouraging results [115].

In the field of haematological malignancies, the development of solid and hematological tumors is strongly associated with the presence of small populations of cells known as tumor stem cells. In AML, these cells are referred to as leukemic stem cells (LSCs). LSCs, which are considered to originate from hematopoietic stem or progenitor cells due to defects in their self-renewal and differentiation processes, not only adopt the regulatory machinery operating in normal HSCs but also establish their own mechanisms against apoptosis and senescence. Therefore, hematopoietic stem cell transplantation along with combination of chemotherapy comprises one of the major therapeutic strategies for hematological malignancies, such as Chronic Lymphoid Leukemia (CLL) and Acute Lymphoid Leukemia (ALL) of lymphoid origin, Acute Myeloid Leukemia (AML) and Chronic Myeloid Leukemia (CML) of myeloid and plasma affecting multiple myelomas.

In a similar way to normal hematopoiesis, AML is arranged as a loose hierarchy in which a small population of self-renewing leukemic stem cells (LSCs) give rise to a large population of more mature leukemic blasts, which lack self-renewal capacity. This organization helps to explain the observed clinical scenario in AML whereby current chemotherapeutic regimes frequently induce remissions but often fatal relapses occur. A number of mechanisms have been suggested to explain the exquisite resistance of LSCs to chemotherapy like the expression of various ABC transporters that export drugs out of the cell, the efficient DNA repair mechanism and the protection provided by the bone marrow microenvironment, namely the

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stem cell niche where the LSCs are located in a hypoxic extracellular matrix preventing the exposure of LSCs to chemotherapy [116,117]. Research on AML pathology over the past years has revealed that a variety of polymorphisms in DNA damage repair (DDR) genes are associated with increased risk of developing AML or lead to disease relapse. Moreover, epigenetic silencing of DDR genes affects leukemogenesis, while, on the other hand, elevated levels of DNA repair induces chemotherapy resistance, by allowing cells with severe damage to attempt repair and survive. Therefore, DNA repair mechanisms' status not only influences the genetic predisposition to leukemia but is also very important for refractoriness to treatment [118]. Several major pathways of DNA repair are known to be implicated in AML, including homologous recombination (HR), nonhomologous end joining (NHEJ), base excision repair (BER), nucleotide excision repair (NER), and mismatch repair (MMR). Different DNA repair processes overlap in their function. When the damage is moderate and the repair processes inadequate, the cells acquire mutations and genomic instability occurs, representing an initial step towards malignant transformation, while also, increased processes of DNA repair contribute to resistance to chemotherapy and radiation [118]. Myeloid malignancies, such as AML, are frequently characterized by defects in the DNA repair machinery. Defects of the HR machinery, as well as BER, NER, and MMR, have been mainly associated with therapy-related AML (t-AML) and are rarely seen in *de novo* AML. RAD51 is the central molecule of the HR pathway and its polymorphic variant RAD51-G135C correlates with increased risk of t-AML, a risk that increases when it is combined with XRCC3-Thr241Met variant [119,120], probably because it leads to RAD51 upregulation [121] that might have a dominant negative effect on HR initiation [122]. Although mutations involving NHEJ genes predisposition to leukemia have yet to be revealed, it is known that chromosomal instability in myeloid neoplasms often results from deregulated NHEJ and inadequate DSB repair. The rate of NHEJ in leukemic blasts is two- to sevenfold higher compared to normal cells and results in major mis-repair, accounting for increased genomic instability in AML cells [123] such as chromosomal translocations, mostly depletions [124]. BER mechanism concerns the removal of the bases changed by alkylation, oxidation, or ionizing radiation. BER also takes part in the single-strand breaks repair. Important components of BER pathway are the poly(ADP-ribose) polymerases (PARP) family containing 18 members which allow for access to DNA repair enzymes in the case of single-strand breaks. Cancer cells with defective HR processes switch to PARP-mediated BER mechanisms. Hence, PARP1 inhibitors are extremely active in tumors deficient in HR pathway. Moreover, AML1-ETO fusion gene is implicated in AML pathology [125] and acts by inhibiting differentiation and immortalizing the hematopoietic progenitors [126], through the repression of a variety of genes involved in DDR, in particular in BER (i.e., *OGG1*, *FEN1*, *MPG*, and *ATM*) [127]. The role of NER mechanism has also been investigated in AML. Common polymorphisms in XPD gene belonging to NER pathway are associated with the risk of AML development. XPD Lys 751 Gln variant is an independent prognostic marker for disease-free survival and overall survival in elderly AML patients [128].

Given the great implication of the DNA repair mechanisms in the pathology of AML, over the last years several new therapeutic strategies have been proposed for its treatment, based on DNA repair inhibitors. Therefore, RAD52 aptamers were proposed for BRCA-deficient AML [129], CHK1 inhibitors for AML patients who do not achieve remission with standard

chemotherapy [130], PARP inhibitors in combination with standard chemotherapy to reduce probable secondary leukemias [131] and HDAC inhibitors, which are believed to downregulate several genes of the DDR [132].

4. Ribonucleic acids and chromatin structure modifiers of CSC resistance

4.1. miRNAs

Another intriguing player in regulating CSCs' modified responses to genotoxic insults is gene expression regulation by microRNAs (miRNAs). The relatively recent discovery of miRNAs has added an entirely new dimension to our knowledge about the regulation of gene expression and the control of various cell functions, such as apoptosis, proliferation, differentiation, and therapy resistance [133]. Over the past decade, it has become progressively clearer that these tiny genetic regulators are linked to the development of cancer. The miRNA profiles have been shown to be highly informative, reflecting the developmental history and differentiation state of the tumors, and providing molecular links between cancer and normal stem cells. The fact that miRNAs expression may have adverse consequences for the functional properties of cancer cells has been recently highlighted for tumor radioresistance. Yan and coworkers for the first time demonstrated that miRNAs could be used to target the DNA repair machinery and thus sensitize tumor cells to radiation [134]. Since then, an accumulating body of research demonstrated that miRNAs can modulate tumor radioresistance [135-138]. *In vivo* experiments using xenograft models and clinical studies are needed to ascertain whether manipulation of miRNA expression can be a viable tool to augment current cancer therapies [139].

4.2. Chromatin structure and lncRNAs

Furthermore, CSCs display different epigenetic profiles, in comparison with their nontumorigenic progenies that result in changes of multiple signaling pathways [140]. These pathways may involve cell adaptation to microenvironmental stresses including inflammation, hypoxia, low pH, shortage in nutrients, and anti-cancer therapies as well. As a paradigm, the MGMT promoter methylation status is routinely assessed in patients diagnosed with glioblastoma multiforme. It is known that the MGMT pathway is adopted by glioblastoma cells to overcome temozolomide cytotoxicity and, to a similar extent, this enzyme protects glioblastoma stem cells from alkylating agents [141]. Notwithstanding, a comparative evaluation of the MGMT promoter methylation pattern between surgical samples and paired glioblastoma-derived neurospheres indicated that epigenetic silencing of MGMT is enriched in putative glioblastoma stem cells [142], thus shedding doubts on the biologic relevance of this pathway on survival of temozolomide-treated glioblastoma stem cells.

Another noteworthy example of prevention of mismatch DNA repair in human liver CSCs was revealed by the delineation of the mechanism of long non-coding (lnc) RNA HOTAIR function. HOTAIR, through downregulation of SETD2 gene expression, results indirectly in histone modifications and chromatin structure alterations leading to microsatellite instability and promoting tumorigenesis in liver CSCs [143,144]. In overall, chromatin structure seems to

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be another important player in the maintenance of genome integrity through a plethora of mechanisms and interactions with other nuclear components as the nuclear envelope [145-147]. Therefore, nuclear structure may be also significantly involved in CSC genotoxicity resistance.

5. Conclusion

In general, CSCs can successfully survive and initiate tumor regrowth largely due to mobilization of DNA response and repair mechanisms. Recent advances in high-throughput screening methods, like genomics, transcriptomics, proteomics, or epigenomics provide an enormous amount of data to the scientific community, contributing to the elucidation of the molecular pathways and networks underlying cellular DNA repair mechanisms.

Another interesting aspect is that normal stem cells retain the ability to repair tissue damage. The regenerative capacity of endogenous stem cells decreases with age, is impaired in degenerative diseases, and deregulated in cancer. Recent data reveal that "normal" stem cells detected in the neighborhood of a resected triple-negative breast tumor may be transformed to cancer stem cells and be also responsible for tumor recurrence. DNA repair capacity of these "neighboring" stem cells is under investigation and preliminary data from our group reveal possible deregulation of factors involved in BER and double-strand DNA breaks repair and a likely involvement of RecQ helicase family members in this process.

Despite ongoing progress, the regulation of DNA damage encountered in CSCs and the interrelation of genome surveillance pathways with cell cycle control, regeneration, and apoptosis possess many obscure sides and controversies. It is agreeable that an ideal CSC-specific therapeutic would target the CSC and bulk tumor cells with minimal adverse effects. Nevertheless, the phenotypical and functional properties of CSCs may be dynamically regulated during the course of genotoxic therapy. Understanding the complex mechanisms regulating the CSC population during the course of cancer treatment will turn CSCs into a powerful tool for therapeutic and diagnostics improvement. Integration of such information and further investigation are obviously required towards effectively targeting and eliminating CSCs from the malignant tumors, a feasible goal of the near future, especially in the rising era of genome editing and personalized therapeutic approaches.

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Chapter 16

Screening for Suppression of Inflammatory Responses Against UVB-Induced DNA Damage in Skin Cells Based on Natural Plant Extract Enhances DNA Repair-Related Polymerase Activity

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Additional information is available at the end of the chapter

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1. Introduction

DNA-dependent DNA polymerase (Pol) (E.C. 2.7.7.7) catalyzes the polymerization of deoxyribonucleotides along a DNA strand that is "read" as a template [1]. The newly polymerized molecule is complementary to the template strand. Pol adds free nucleotides only to the 3′ end of a newly formed strand, resulting in elongation of the new strand in the 5′ → 3′ direction. The human genome encodes at least 15 Pols that function in cellular DNA synthesis (Table 1) [2-5]. Eukaryotic cells contain three replicative Pols (α, δ, and ε), one mitochondrial Pol (γ), and at least 11 non-replicative Pols (β, ζ, η, θ, ι, κ, λ, μ, and ν), REV1, and terminal deoxynucleotidyl transferase (TdT) [2-5]. Pols have a highly conserved structure, with their overall catalytic subunits showing little variation among species. Conserved enzyme structures are usually preserved over time because they undertake important cellular functions that confer evolutionary advantages. Based on sequence homology, eukaryotic Pols can be divided into four main families: A, B, X, and Y [2-5]. Family A includes mitochondrial Pol γ as well as Pols θ and ν; family B includes the three replicative Pols α, δ, and ε, and Pol ζ; family X is comprised of Pols β, λ, and μ, as well as TdT; and family Y includes Pols η, ι, and κ, in addition to REV1 [5]. At least two Pols, such as B-family Pol ζ and Y-family REV1, have substantial translesion DNA synthesis (TLS) activity [5]. The most notable TLS Pol that bypasses ultraviolet (UV) radiation-induced DNA damage is Pol η, which bypasses TT-*cis*-syn cyclobutane pyrimidine

dimmers (TT-CPD) with high efficiency and fidelity. Purified human Pol η correctly inserts A deoxynucleotides opposite linked bases of a TT-CPD [6]. Pol β, the base excision repair (BER) Pol, enhances UV-induced genetic instability, and facilitates translesion replication of CPD in a UV lesion bypass [7]. Consequently, activation of DNA repair-related Pols β and η is likely important for maintaining UVB-induced DNA damage.

Name	Catalytic subunit		Family [a)]	Function	KCl (120 mM) inhibition
	Gene	Size of protein (kDa)			
Pol α	*POLA1*	166	B	DNA replication priming	+
Pol β	*POLB*	38	X	BER and meiotic recombination	–
Pol γ	*POLG*	140	A	Mitochondrial DNA replication and repair	–
Pol δ	*POLD1*	124	B	DNA replication, NER, and MMR	+
Pol ε	*POLE*	262	B	DNA replication, NER, and MMR	+
Pol ζ	*REV3L*	353	B	TLS and mutagenesis	–
Pol η	*POLH*	78	Y	Bypass of UV radiation-induced DNA adducts, especially CPDs	–
Pol θ	*POLQ*	290	A	Defense against ionizing radiation-induced DNA damage	–
Pol ι	*POLI*	80	Y	Backup enzyme for UV radiation-induced DNA adducts and BER	–
Pol κ	*POLK*	99	Y	Bypass of bulky adducts, backup enzyme for NER	–
Pol λ	*POLL*	63	X	V(D)J recombination; possibly and joining	–
Pol μ	*POLM*	55	X	V(D)J recombination; possibly and joining	–
Pol ν	*POLN*	100	A	? (ICL repair or testis-specific function)	–
REV1	*REV1*	138	Y	TLS and mutagenesis, anchor for several Pols	–
TdT	*DNTT*	58	X	Immunoglobulin diversity at junctions of coding regions	–

BER, base excision repair; CPD, cyclobutane pyrimidine dimer; ICL, interstrand crosslink; MMR, mismatch repair; NER, nucleotide excision repair; Pol, DNA polymerase; TdT, terminal deoxynucleotidyl transferase; TLS, translesion DNA synthesis; UV, ultraviolet.

[a)] In human cells, these enzymes fall into four distinct families, designated A, B, X, and Y, based on amino acid sequence.

This table was composed based on previous references [1, 5].

Table 1. Human Pol species.

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The skin is the largest organ of the human body and protects against external physical, chemical, and biological insults, including UV radiation and microorganisms. Although many environmental and genetic factors contribute to the development of various skin diseases, one of the most important factors is chronic exposure of the skin to solar UV radiation. Excessive exposure of the skin to UV radiation has many biological consequences, including sunburn, hyperpigmentation, solar keratosis, solar elastosis, skin cancer, immunosuppression, and acute inflammatory responses [8, 9]. UVB (290–320 nm) radiation induces keratinocyte apoptosis, which is evident within the epidermis as sunburn cells. The formation of sunburn cells in UVB-exposed skin reflects the severity of DNA damage. UV absorption produces two main types of DNA damage: CPD and pyrimidone photoproducts. However, the repair of DNA damage in UVB-exposed skin cells prevents accumulation of damaged cells [10]. UV-induced DNA damage is also an important molecular trigger for UV-induced inflammation, as well as various forms of skin cancer [11].

Sunscreens are commonly used for preventing or ameliorating harmful effects of UV radiation on the skin [12]. However, sunscreen alone may not provide sufficient protection against skin photodamage [13]. Non-sunscreen compounds have become more relevant to large parts of the population in preventative skin care [14]. Active compounds that support skin defensive mechanisms or inhibit pathological processes in photodamaged skin are highly desirable. Some plant extracts are reported to protect skin against various UV-induced damage [15], and there has been considerable interest in applying plant polyphenols to the prevention of UV-induced skin photodamage [16].

Therefore, we asked whether components from tropical plants, which absorb strong UV-containing sunlight, might enhance UV-damaged DNA repair-related Pols. In addition, we have been screening mammalian Pol inhibitors from natural products, such as food materials, nutrients, and higher plants, for over 20 years, and have identified more than 100 low-molecular-weight organic compounds as Pol inhibitors [17-19]. Through this process, we have developed a simple *in vitro* assay to screen for mammalian Pol inhibitors [20-22]. Therefore, we initially established an *in vitro* Pol enhancer assay using cell extracts from cultured normal human epidermal keratinocytes (NHEK) using this method. Next, we screened 50 tropical plant extracts for Pol enhancer activity in cultured NHEK, and purified the activity-enhancing compounds from extracts of the most bioactive plants. In this review, we explore the relationship between the DNA repair of UVB-stimulated DNA damage by cellular Pol activity in NHEK and the immunosuppression of UVB-irradiated skin cells treated with bioactive plant extract compounds.

2. Pol assay in UVB-irradiated NHEK

2.1. NHEK cultures

NHEK and serum-free keratinocyte growth medium (KGM, trade names: EpiLife-KG2 and HuMedia-KG2) containing insulin, hydrocortisone, gentamycin/amphotericin B, and growth additives such as bovine pituitary extract and human epidermal growth factor were purchased

from Kurabo Industries Ltd. (Osaka, Japan). NHEK were seeded at 3×10^5 cells/cm^2 into 75-cm^2 cell culture flasks, and cultured in KGM at 37°C under 5% CO_2. Third- or fourth-passage cells were used for all experiments. Test compounds, such as plant extracts and purified compounds, were dissolved in dimethyl sulfoxide (DMSO) and diluted with medium to appropriate concentrations, and the final volume adjusted to 0.05% (v/v) DMSO. NHEK were initially treated with test compound for 24 h (Figure 1). Cultures were then washed with Hank's buffer, irradiated with UVB (0–150 mJ/cm^2), and cultured for 2–24 h in KGM. After treatment, cultured cells were collected by a cell scraper, and sonicated for 10 s in lysis buffer containing 50 mM Tris–HCl (pH 7.5), 1 mM EDTA, 5 mM 2-mercaptoethanol, 15% glycerol, and a protease inhibitor cocktail of Complete Mini (Roche Diagnostics, Mannheim, Germany). Cell extract Pol activity was assayed and quantified *in vitro* as described previously [20-22] with some modification, as described below.

2.2. Measurement of *in vitro* Pol activity

For Pol reactions, poly (dA)/oligo$(dT)_{18}$ and [^{3}H]-labeled 2′-deoxythymidine-5′-triphosphate (dTTP; 43 Ci/mmol) were used as the DNA template-primer substrate and nucleotide (dNTP; 2′-deoxynucleoside-5′-triphosphate) substrate, respectively (Figure 1). A chemically synthesized DNA template, poly (dA), was purchased from Sigma-Aldrich Inc. (St Louis, MO, USA), and a customized oligo $(dT)_{18}$ DNA primer was purchased from Sigma-Aldrich Japan K.K. (Hokkaido, Japan). Radioactive nucleotides [^{3}H]-dTTP were obtained from Moravek Biochemicals Inc. (Brea, CA, USA). The standard reaction mixture for all Pol species (24 μL of final volume) contained 50 mM Tris–HCl, pH 7.5, 1 mM dithiothreitol, 1 mM $MgCl_2$, 5 μM poly (dA)/oligo$(dT)_{18}$ (A/T, 4:1), 10 μM [^{3}H]-dTTP (100 cpm/pmol), and 15% (v/v) glycerol. The standard reaction mixture for DNA repair-related Pol species was the same, except that it also contained 120 mM KCl. Cultured NHEK cell extract (16 μL) was mixed with 8 μL of standard Pol reaction mixture. After incubation at 37°C, for 60 min, the radioactive DNA product was collected on a DEAE–cellulose paper disc (DE81) as described by Lindell *et al.* [23], and the radioactivity measured in a scintillation counter (2300TR TriCarb; PerkinElmer, Downers Grove, IL, USA).

2.3. UVB enhancement of NHEK Pol activity

We first investigated whether cultured NHEK Pol activity was stimulated by UVB radiation. UVB at 12.5 and 25 mJ/cm^2 had no effect on Pol activity, whereas 50 mJ/cm^2 significantly enhanced activity (Figure 2A). Irradiation (150 mJ/cm^2) lead to the largest increase in Pol activity, with 1.59- and 1.78-fold enhanced activity for standard reaction mixtures without or with KCl, respectively. All 15 human Pols are active in the absence of salt (i.e., KCl and NaCl), whereas DNA replicative Pols, such as Pols α, δ, and ε, are inhibited by salt [1] (Table 1). Therefore, the standard reaction mixture for all Pols containing both DNA replication and repair Pols, or DNA repair-related Pols only, was with or without 120 mM KCl, respectively. Because the activity of DNA repair-related Pols (with KCl) is higher than all other Pol species (without KCl), the UV-damaged Pols, especially Pols β and η, might be enhanced in NHEK.

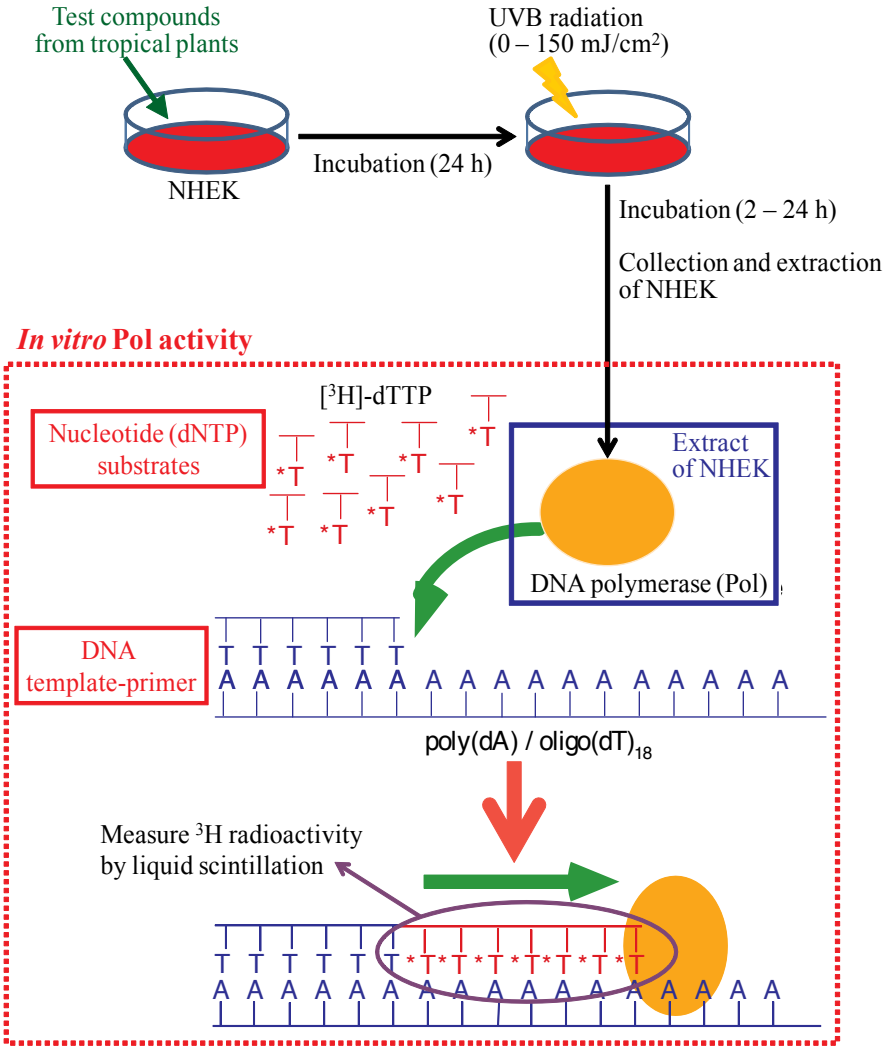

Figure 1. *In vitro* cellular Pol activity assay using UVB-exposed NHEK cell extract.

Interestingly, NHEK Pol activity decreased at 200 mJ/cm^2 because most cells had died (data not shown).

Next, we investigated the effect of incubation time on Pol activity following UVB irradiation. Pol activity was highest among NHEK incubated for 4 h (100 mJ/cm^2), among cells cultured 2–24 h, suggesting that UVB-damaged DNA repair activity peaks 4 h after irradiation (Figure 2B). These results suggest that the culture conditions most suitable for increasing Pol activity is 150 mJ/cm^2 of UVB irradiation, and incubation for 4 h. Next, we screened Pol enhancer activity from tropical plant extracts using these same conditions.

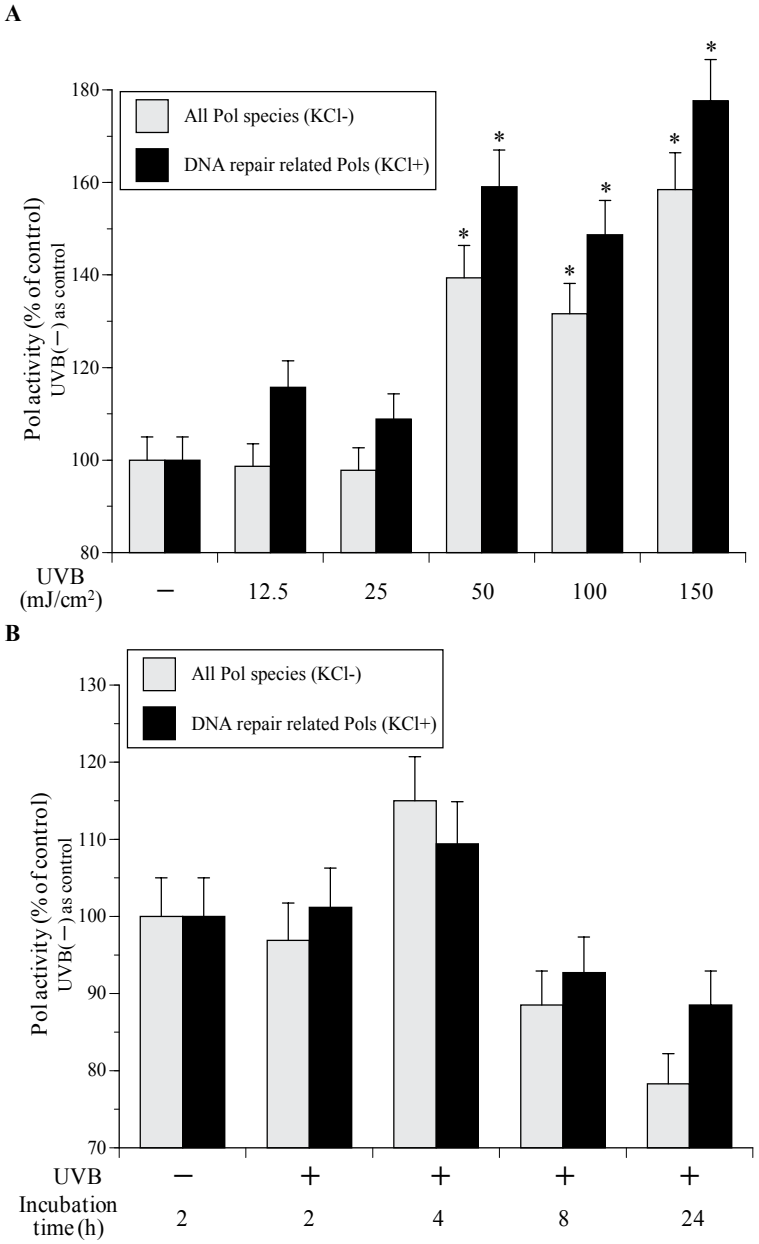

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Figure 2. Examination of cellular Pol activity in UVB-exposed NHEK. (A) Cellular Pol activity was dependent upon the level of UVB irradiation (0–150 mJ/cm^2). NHEK were cultured for 1 h after UVB irradiation. (B) Pol activity is dependent upon incubation time (2–24 h) above 100 mJ/cm^2 UVB-exposed NHEK. Gray bars and black bars are human all Pols and DNA repair-related Pol species, respectively (the standard reaction conditions without or with 120 mM KCl, respectively). The Pol activity of vehicle control without UVB irradiation was taken as 100%. All data are expressed as mean ± SEM (n = 3). *$P < 0.05$ compared with the UVB (−) vehicle control.

3. Screening for plant Pol activity enhancers in UVB-irradiated NHEK

3.1. Screening tropical plant extracts

We screened for UVB-induced Pol active compounds, testing 80% ethanol extracts from 50 tropical plants. The Rose Myrtle extract was the strongest stimulator of Pol activity in UVB-exposed NHEK among the plant extracts tested. Rose Myrtle is a shrub of the Myrtaceae family, originating from Southeast Asia. It grows under various conditions and is an invasive species in some areas where it was introduced as an ornamental plant. Parts of this plant (leaves, roots, buds, and fruits) have long been used in traditional Vietnamese, Chinese, and Malay medicine. In particular, the fruits have been used to treat diarrhea and dysentery, and to boost the immune system [24]. Rose Myrtle fruit has an astringent taste and a deep purple color at maturity. All these properties may be explained, at least partially, by the presence of polyphenols.

3.2. Isolation of the bioactive compound from Rose Myrtle fruit

Rose Myrtle fruit was obtained from Maechu Co. Ltd. (Nara, Japan) and Shinwa Bussan Co., Ltd. (Osaka, Japan) and 100 g extracted with 1 L of 80% ethanol. The evaporated extract (6.6 g) was dissolved in distilled water, and subjected to hydrophobic column chromatography (Diaion HP-20; Sigma Aldrich, St. Louis, MO, USA). Three hydrophobic chromatography fractions were collected: water, methanol, and acetone. The methanol fraction was evaporated (2.6 g) and subjected to silica gel 60 column chromatography and eluted with chloroform: methanol: water (v:v:v, 10:5:1). The active fraction was obtained and purified by reverse-phase silica gel column chromatography (Chromatorex ODS DM1020T; Fuji Silysia Ltd, Durham, NC, USA), and continuous high-performance liquid chromatography. This process resulted in a white powdery compound **1** (1.14 mg).

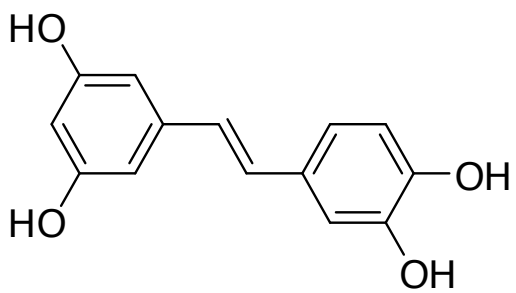

Figure 3. Structure of purified compound **1** (piceatannol) from Rose Myrtle fruit (*Rhodomyrtus tomentosa*).

Compound **1** was identified as the polyphenol piceatannol using high-resolution mass spectrometry and ^{1}H and ^{13}C nuclear magnetic resonance (NMR) data (Figure 3), consistent with previously published spectroscopic data [25]. Therefore, we used purified piceatannol

(98%) as determined by NMR analysis (data not shown). Additionally, we further investigated whether Rose Myrtle extract and piceatannol stimulated cellular Pol activity, blocked UVB-induced cell damage, and suppressed the inflammatory mediator prostaglandin E_2 (PGE_2) in NHEK.

4. Effect of Rose Myrtle fruit extract and piceatannol on Pol activity in UVB-exposed NHEK

The effect of purified piceatannol on Pol activity in cultured NHEK was investigated using the Pol enhancement assay (Figure 1). All Pol species are active in buffer with salts such as NaCl and KCl, whereas Pols α, δ, and ε are inhibited by salt [1] (Table 1). Therefore, the standard reaction mixture with or without 120 mM KCl was used to detect all Pol activity (Figure 4A) or DNA repair-related Pol activity (Figure 4B), respectively. The activity of DNA repair-related Pol species such as X and Y family Pols are enhanced by salt (120 mM KCl) [1]. The activities of purified calf Pol α and rat Pol β, which are B- and X-family Pols, respectively, were one-tenth lower and 1.5-fold higher with 120 mM KCl than those without KCl (data not shown). The ratios of cellular Pol activity in the standard reaction mixture without salt were higher than those with salt (Figure 4).

NHEK Pol activity with or without UVB irradiation and test compound treatment was similar (Figure 4). In non-treated compounds, UVB exposure at 100 mJ/cm^2 resulted in an approximately 1.2-fold increased Pol activity. In non-UVB-irradiated NHEK, extract and piceatannol enhanced NHEK Pol activity slightly. Moreover, Pol activities were raised significantly by the extract and piceatannol treatment in UVB-exposed NHEK. These results indicate a synergistic effect of UVB irradiation and Rose Myrtle extract and/or piceatannol on the induction of Pol enzyme activity, particularly DNA repair-related Pols.

5. Effect of the Rose Myrtle fruit extract and piceatannol on UVB-exposed NHEK cell viability

We next sought to investigate whether piceatannol inhibited NHEK proliferation. NHEK were grown to sub-confluence in KGM in 48-well plates, washed with Hank's buffer, irradiated with UVB (50 mJ/cm^2), and treated with test compound for 24 h in KGM. After treatment, cell viability (percent living cells) was evaluated by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay [26].

Treatment of cultured NHEK with Rose Myrtle fruit extract and isolated piceatannol at concentrations up to 100 and 20 μg/mL, respectively, did not induce cytotoxic effects (cell viability >95% after 24 h treatment, data not shown). Therefore, the following experiments were conducted within the concentration range mentioned above. NHEK were treated after UVB irradiation (50 mJ/cm^2), and cell viability examined 24 h post-irradiation and compared

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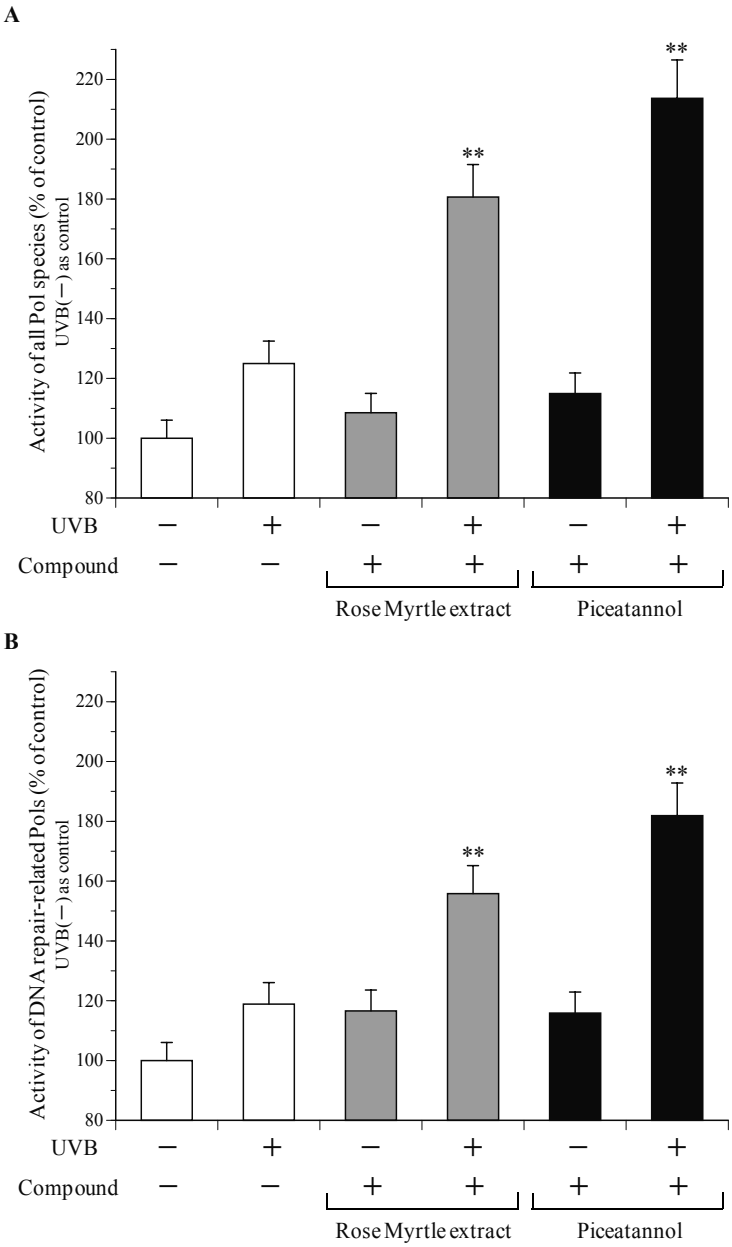

Figure 4. Effect of Rose Myrtle extract and piceatannol on NHEK Pol activity with or without UVB irradiation. (A) Activity of human all Pols species under standard reaction conditions without KCl. (B) Activity of DNA repair-related Pol species under standard reaction conditions with 120 mM KCl. NHEK were incubated for 24 h with or without each compound (10 μg/mL Rose Myrtle extract and 2 μg/mL piceatannol) before UVB (100 mJ/cm^2) irradiation. Pol activity of vehicle control without UVB irradiation was taken as 100%. All data are expressed as mean ± SEM (n = 3). $^{**}P < 0.01$ compared with the UVB (−) vehicle control.

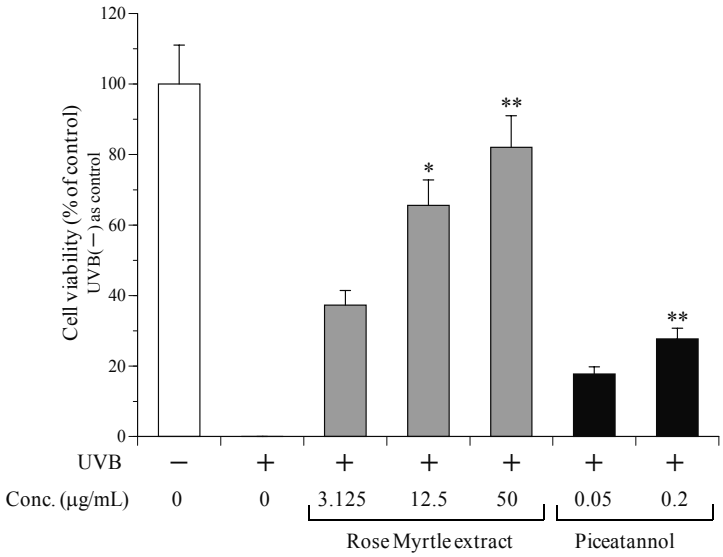

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Figure 5. Effect of Rose Myrtle extract and piceatannol on cell viability of UVB-exposed NHEK. NHEK were irradiated with UVB (50 mJ/cm^2), and treated with each compound at the indicated concentrations. MTT assays were used to evaluate living cells 24 h after treatment. Cell viability of vehicle control with or without UVB irradiation was taken as 0% or 100%, respectively. All data are expressed as mean ± SEM (n = 6). *P < 0.05 and **P < 0.01 compared with the UVB (−) vehicle control.

with non-treated cells. Extract markedly inhibited UVB-induced NHEK cytotoxicity in a dose-dependent manner (Figure 5). Cell viability with 50 μg/mL of extract increased to more than 80% in non-treated cells. Piceatannol also increased UVB-exposed NHEK cell viability in a dose-dependent manner, suggesting it is the protective component of Rose Myrtle extract. At the same time, piceatannol-4'-*O*-β-D-glucopyranoside, a glucoside form of piceatannol, did not exhibit a protective effect (data not shown), suggesting that the aglycone structure is important for protective activity.

6. Effect of Rose Myrtle fruit extract and piceatannol on CPD production in UVB-exposed NHEK

CPD formation is an important product of DNA damage and mutagenesis [27]. We investigated the hypothesis that Rose Myrtle extract and its polyphenolic component, piceatannol, may influence the removal of CPD from DNA in UVB-irradiated NHEK. To measure CPD production, NHEK were grown to sub-confluence using KGM in 60-mm^2 culture dishes, and treated with test compound for 24 h. Cultures were then washed with Hank's buffer, irradiated with UVB (80 mJ/cm^2), and treated with test compound for 6 h in KGM. Cultured cells were collected by a cell scraper after treatment and nuclear DNA purified using a QIAamp Blood Kit (Qiagen, Tokyo, Japan). CPD levels in the quantified DNA were measured by enzyme-

linked immunosorbent assay (ELISA) using an anti-CPD monoclonal antibody (Cosmobio Co. Ltd., Tokyo, Japan), according to the manufacturer's protocol.

Exposure of NHEK (80 mJ/cm^2 UVB) induced CPD formation as measured immediately after irradiation, and served as a reference for DNA damage (Figure 6). To evaluate DNA repair in irradiated cultures, CPD levels were measured after UVB exposure and compared with the non-repaired reference. Both Rose Myrtle extract and piceatannol decreased CPD production in UVB-exposed NHEK in a dose-dependent manner, with 10 μg/mL of extract and 0.5 and 2 μg/mL of piceatannol exhibiting 20% CPD reduction compared with non-treated control cells. These results suggest that Rose Myrtle extract and/or piceatannol might have DNA repair activity against UVB-damaged DNA in NHEK. As shown in Figure 4, both Rose Myrtle extract and piceatannol increased cellular Pol activity in NHEK, suggesting that activation of these enzymes, in particular DNA repair-related Pols β and η, contributes to reduced CPD production.

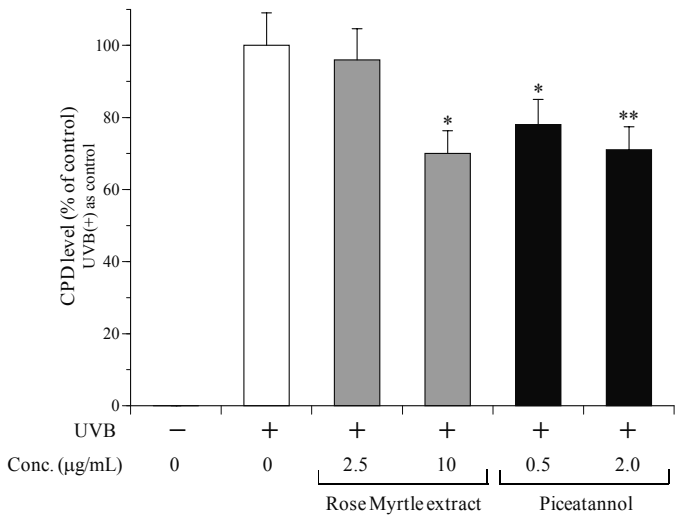

Figure 6. Effect of Rose Myrtle extract and piceatannol on UVB-induced CPD production in NHEK. NHEK were incubated with each compound at the indicated concentrations before and after UVB (80 mJ/cm^2) irradiation. CPD was quantitatively evaluated by DNA-ELISA. CPD production by vehicle control with or without UVB irradiation was taken as 100% or 0%, respectively. All data are expressed as mean ± SEM (n = 6). *P < 0.05 and **P < 0.01 compared with the UVB (+) vehicle control.

7. Effect of Rose Myrtle fruit extract and piceatannol on PGE_2 production in UVB-exposed NHEK

We next examined the possible influence of extract and piceatannol on PGE_2 production to examine whether they are associated with anti-inflammatory properties in NHEK. To measure

PGE_2 production, NHEK were grown to sub-confluence in KGM using 48-well plates. Cells were then cultured in KGM without hydrocortisone for 1 day, irradiated in Hank's buffer with UVB (50 mJ/cm^2), and treated with test compound for 24 h in KGM without hydrocortisone. After treatment, the culture medium was collected and centrifuged. Supernatant PGE_2 was analyzed using PGE_2 EIA Kits (Cayman Chemical Co., Ann Arbor, MI, USA).

UVB irradiation increased PGE_2 secretion by approximately 2.9-fold in non-irradiated NHEK to 238.6 pg/mL, and this amount was set to 100% as a positive control (Figure 7). Both extract and piceatannol lead to decreased PGE_2 production in a dose-dependent manner, implying that Rose Myrtle extract and/or piceatannol suppress UVB-stimulated inflammation.

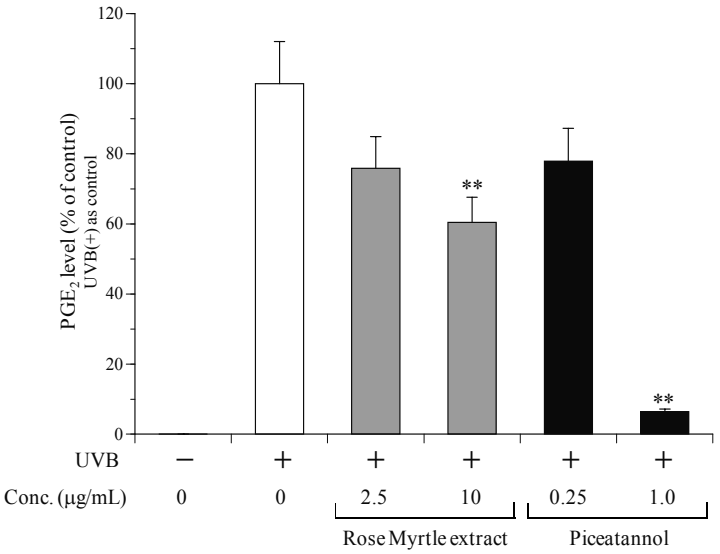

Figure 7. Effect of Rose Myrtle extract and piceatannol on UVB-induced PGE_2 production in NHEK. NHEK were incubated with each compound at the indicated concentrations after UVB (50 mJ/cm^2) irradiation. Supernatant PGE_2 was quantitatively evaluated by ELISA. PGE_2 production by vehicle control with or without UVB irradiation was taken as 100% or 0%, respectively. All data are expressed as mean ± SEM (n = 5). $^{**}P < 0.01$ compared with the UVB (+) vehicle control.

8. Discussion

We established an *in vitro* Pol activator assay using cell extracts from UVB-exposed NHEK (Figures 1 and 2) to demonstrate cellular Pol enhancement in 80% ethanol extracts from Rose Myrtle fruit and its key active ingredient, piceatannol (Figure 3). About 90% of skin inflammation cases are attributed to solar UV radiation, particularly its UVB component, which is absorbed efficiently by cellular DNA [28]. UVB radiation penetrates the skin epidermis, inducing both direct and indirect DNA-damaging effects. Rose Myrtle extract and piceatannol

increased cell viability in UVB-exposed NHEK (Figure 5), and promoted removal of CPD photoproducts (Figure 6), suggesting an improvement in DNA damage repair. The formation of CPD and 6-4 pyrimidine-pyrimidone photoproducts are the most predominant DNA lesions in skin after UVB and UVA exposure [27, 29]. The main repair mechanism of UVB-induced DNA damage is nucleotide excision repair (NER). When skin cells are exposed to excessive UV radiation, NER capacity is reduced and CPD lesions remain, resulting in cell death, senescence, mutagenesis, and carcinogenesis of the skin [29]. Presumably, enhancement of DNA repair is one of the reasons why extract and piceatannol exert a protective effect on UVB-irradiated NHEK viability, and on sun-damaged cell formation in UVB-irradiated human skin explants.

We also analyzed the effect of Rose Myrtle extract and piceatannol on *in vitro* Pol activity in UVB-irradiated cultured NHEK cell extracts, which significantly enhanced enzyme activity (Figure 4). Pols synthesize DNA and have an essential role in genome duplication, but are also crucial for protecting cells against the effects of DNA damage. In both normal and cancerous cells, DNA is subjected to damage from many sources. Water-catalyzed reactions and attack by reactive oxygen species inflict continual damage. Ubiquitous sources of lesions include naturally occurring ionizing radiation, such as UV radiation from the sun. The toxic and mutagenic consequences of such damage are minimized by distinct DNA repair pathways, including BER and NER. These repair mechanisms rely on a Pol to fill gaps in the DNA that are left by the removal of damaged bases. If DNA damage is unrepaired, cells often tolerate it by TLS during DNA replication to insert a base opposite a lesion and bypass the damage. Finally, when breaks and gaps arise in DNA they are repaired by various mechanisms, including homologous recombination and various non-homologous end-joining processes. Pols are also essential components of these pathways.

There are 15 different Pols encoded in mammalian genomes, which are specialized for replication, repair, or tolerance of DNA damage (Table 1). New evidence is emerging for lesion-specific and tissue-specific functions of Pols [5]. The most notable TLS Pol for the bypass of UV radiation-induced DNA damage is Pol η. Currently, Pol η is the only Pol for which a deficiency is known to predispose humans to cancer [30]. The inherited disorder xeroderma pigmentosum (XP) is associated with a greatly increased risk of sunlight-induced carcinomas of the skin, and individuals with the variant type of the condition, XP-V, have disabling mutations in Pol η [30, 31]. $Polh^{-/-}$ mice mirror the XP-V phenotype as they rapidly develop UV radiation-induced tumors. $Polh^{+/-}$ mice are also susceptible to UV radiation-induced skin carcinogenesis, although at a lesser rate [32]. UV irradiation of XP-V cells cause DNA double-strand breaks owing to the absence of Pol η TLS function, which causes DNA replication forks to stall and collapse at sites of DNA damage on the template strand [33]. Prolonged replication delays in the absence of Pol η may also inhibit DNA repair of UV radiation-induced lesions [34]. Unirradiated $POLH^{-/-}$ cells have more chromatid breaks than normal, including breaks at a common fragile site [35]. Patients with XP-V have an increased incidence of squamous cell carcinoma of the skin, and experiments have been conducted to determine whether *POLH* mutations are associated with sporadic skin carcinomas [36] or other human cancers. However, no mutations affecting Pol η function have yet been identified.

We have demonstrated that Rose Myrtle extract and piceatannol decrease UVB-induced secretion of the inflammatory mediator PGE_2 (Figure 7). Notably, NHEK-derived inflammatory mediators play an important role in the development of the inflammatory reaction in UVB-exposed skin [37]. Numerous studies have demonstrated that PGE_2 mediates induction of erythema, angiogenesis, vasodilatation, and vascular permeability [38], and PGE_2 signaling pathways promote photoaging and development of UVB-induced skin carcinogenesis [39]. Taken together, the inhibitory effects of Rose Myrtle extract and piceatannol against UVB-induced PGE_2 expression in NHEK demonstrate the anti-inflammatory properties of these compounds. This observation supports the idea that these compounds have anti-inflammatory capability not only against UVB-induced inflammation, but also against inflammatory reactions caused by other irritants. Thus, there appears to be a relationship between enhanced cellular Pol activity in UVB-irradiated NHEK and reduced PGE_2 anti-inflammation, but further investigation will be required to support this hypothesis. Because both Rose Myrtle extract and piceatannol do not absorb UVB light, we suggest that they act in a non-sunlight dependent manner to protect against UVB-induced inflammatory induction.

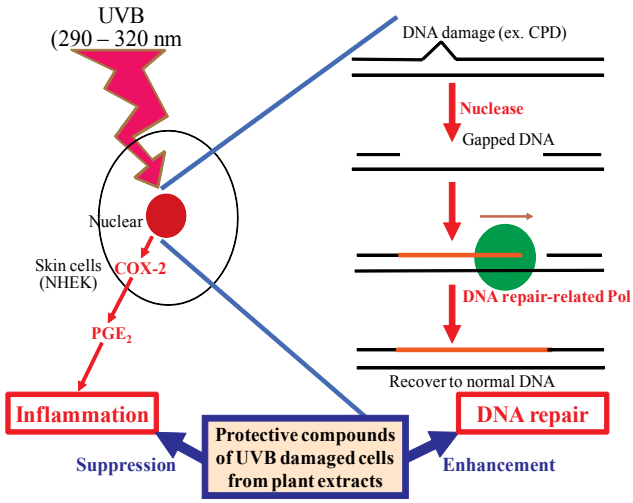

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Figure 8. The relationship between enhanced DNA repair-related Pol activity and suppression of inflammation in UVB-irradiated skin cells treated with plant extract.

9. Conclusion

Rose Myrtle fruit extracts were the most effective among 50 tropical plants at increasing cell viability in UVB-irradiated NHEK. Rose Myrtle fruit extract and its isolated polyphenolic component, piceatannol, were found to decrease production of CPD and PGE_2, a DNA damage photoproduct and an inflammatory mediator, respectively. These results suggest that Rose

Myrtle piceatannol protects skin from UVB-induced damage via enhancement of DNA repair-related Pol enzyme activity and suppresses inflammation. We demonstrate the utility of an *in vitro* Pol activity screening method using UVB-irradiated NHEK (Figure 1), show that it is easy to perform, and provides rapid results. The selected plant extract Pol activity enhancement compounds (Figure 8), may have potential as non-sunscreen cosmetics.

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The authors declare no conflicts of interest.

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Chapter 17

Interaction Between Viral Proteins and Caretakers — Polyomavirus as a Model

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Additional information is available at the end of the chapter

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1. Introduction

DNA repair plays an essential barrier against birth of a precancerous cell [1]. In nature history of cancer, one important characteristic is genomic instability [2]. Among tumor suppressor genes (TSG), the functions of caretakers including DNA repair genes are crucial for cellular genomic integrity. They prevent the mutation of other TSG, e.g. gatekeepers and landscapers [3]. Virueses are always more complicated than human understanding. They not only direct host replication machineries but also interact with a wide variety of cellular proteins. In the past decade, some viruses have been reported to hijack DNA repair proteins and/or collaborate DNA damage response (DDR) which favor their own life cycle or induce carcinogenesis of host cells [4, 5].

Several members of *Polyomaviridae*, a family of circular double-stranded DNA tumor viruses, induces multiple tumors in animal [6]. The family includes some famous animal viruses, i.e. simian virus 40 (SV40) and murine polyomavirus (MPyV), and twelve not very well-characterized human polyomaviruses, e.g. JC virus (JCV), BK virus (BKV), merkle cell polyomavirus (MCV), KIV, WUV, etc. SV40 contributes to numerous pioneer discoveries, including eukaryotic DNA replication, alternative splicing, the interaction/inactivation of tumor suppressor genes etc., and serves as a paradigm in molecular biology [7, 8]. Furthermore, SV40 is suspected as an emergent human pathogen and a co-carcinogen of human mesothelioma which is due to its contamination of poliovirus vaccine [9]. The relationship of SV40 and human cancer has been comprehensively evaluated by the International Agency for Research on Cancer (IARC) in 2012. Human MCV has been identified as a probably human carcinogen and associated with a highly aggressive human skin carcinoma, merkel cell carcinoma (MCC) [10]. Due to the high prevalence of human polyomaviruses and life-long persistent infection in human [11, 12], the interaction between polyomaviruses and host proteins still is the barren areas to be explored.

In this chapter, we will briefly review the recent development regarding the interaction between polyomavirus proteins and cellular caretakers. This chapter will depict the roles of polyomaviruses in deregulating DNA repair, genomic stability and provide a valuable information for the studies of DNA repair affected by viral proteins.

2. Family polyomavirudae

2.1. Genome strutures and gene products of polyomaviruses

The genomes of polyomaviruses, about 5.0~5.4 kb, contain early, late and non-coding regulatory regions (Figure. 1A). The large tumor antigen (LT Ag) and small tumor antigen (ST Ag) are produced by alternative splicing from the early transcript. They have common N terminal but unique C terminal sequences that lead them to interact with different host proteins [13]. The sequence of LT Ag contains the Dna J domain, for heat shock protein 70 binding, and retinoblastoma (pRb) and p53 protein binding domains. The ST Ag sequence contains common N terminal region (Dna J domain) and unique C terminal region which bears binding site for protein phosphatase 2A (PP2A) [14]. However, the number and structure of alternative transcripts are different among polyomaviruses. Besides LT and ST Ags, SV40 also produces 17 kT Ag; MPyV has middle T and TT Ag, JCV has T'135, T'136 and T'165; BKV has trunc T, MCV has 57kT splicing variants. Most studies focus on their LT Ags which play essencial roles for viral replication and transformation. ST Ags are known to be the helper for transformation [15]. The late region codes for structural proteins VP1, VP2 and VP3. Agnoprotein, the smallest one, is also produced from late region. It plays roles in viral relication, transcription and virion synthesis [16].

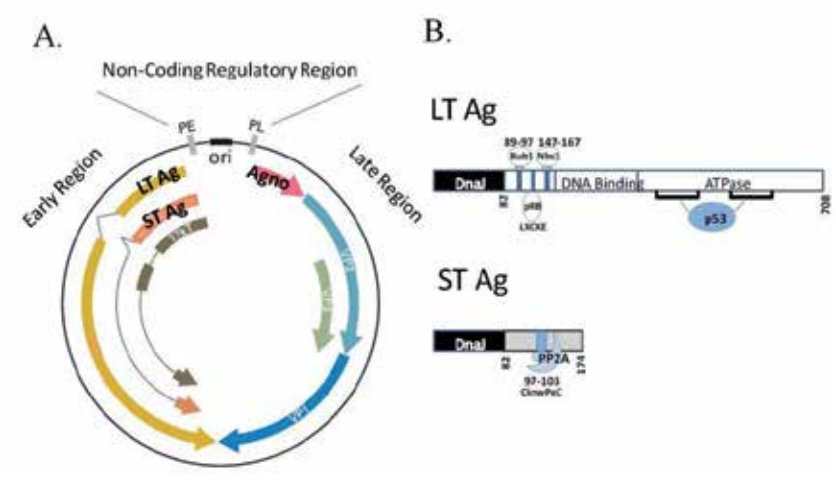

Figure 1. A. The genome structure of SV40 (5243 bp), the representative polyomavirus. B. The LT and ST Ags of SV40. The binding regions of major caretakers are indicated.

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2.2. Polyomaviruses and human cancers

MCV is a new human polyomavirus discovered in 2008 and is found to be clonally integrated into the genome of MCC at a frequence of ~80% [10]. Merkel cells reside in the basal layers of skin and express dual epithelial/neuroendocrine phenotypic markers. MCC is a rare but highly aggressive skin cancer which typically affects elderly and immunocompromised individuals [17]. In 2013, IARC declared that MCV is probably carcinogenic to humans (Group 2A). It is the human polyomavirus which has most tightly association with human cancer so far. Most of the MCV genomes found in MCC carry various C-terminal truncation in LT Ag, while the virus preserves full-length of ST open reading frame. The tumor derived LT Ag is a specific signature for MCV [18].

Another two human polyomaviruses, JCV and BKV, were intially isolated from immunocompromised patients in 1971 [19, 20]. JCV caused progressive multifocal leukoencephalopathy in AIDS patients. BKV led to nephropathy or hemorrhagic cystitis. Importantly, they are oncogenic when inoculated into newborn rodents. These viruses persist in multiple tissues of host for life-long infection. Epidemiological studies found that they are widespread and common in human population [11, 12]. The seropositive rates of JCV and BKV in normal adults can be as high as 72%-98% [21]. Until 2007, only these two human polyomaviruses were known to infect human beings. Currently, several new human polyomaviruses have been detected [10, 22]. Seropoistive rates by using newer method to avoid cross reaction are 9% (SV40), 82% (BKV), 39% (JCV), 42% (MCV), 55% (KIV) and 69% (WUV) [23]. Some studies explored the relationship of BKV and JCV in human cancers [6, 21, 24]. For example, by using polymerase chain reaction (PCR) and immunohistochemistry (IHC) etc. methods, the DNA and LT Ag of neurotropic JCV have been detected with high prevalence in different types of neural cancers [25-27]. Its DNA and LT Ag could be also detected in lung and colon cancers [21, 27-32]. JCV and BKV are highly suspective as human carcinogens. In 2013, IARC evaluated and declared that JCV and BKV are possibly carcinogenic to human as Group 2B carcinogens.

SV40 was discovered in 1960 and long suspected as human emergent virus [33]. The natural host of SV40 is rhesus macaue. However, SV40 contaminated poliovirus vaccines were used to inoculate approximately 100 million peoples in the United States and countless more throughout the world between 1955 and 1963. SV40 DNA had been detected in many types of cancer by PCR-based assays [34, 35]. The PCR-based assays, including false positive by contamination and crossreaction with JCV and BKV ect., raised many debates and controversy about SV40 in human tumors. High seropositve of SV40 were found by crossreaction with JCV and BKV in human serum. Epidemiological studies showed no trend of increased number of cancer cases related to persons who received SV40-contaminated vaccine. The prevalence of SV40 was studied by using high specific assay (virus like particles assay ect.) and found that only 1.0%-1.6% seropositive of SV40 in individuals born before 1963 [36]. However, the longstanding controversies were discussed by IARC group and at present SV40 is classified to Group 3, not classifiable as to its carcinogenicity to humans [37].

3. Anti-cancer barriers

3.1. DNA repair caretakers

Myriad of exogenous and endogenous DNA damaging events threaten cellular genetic information every moment. Cells have to invest abundant proteins for repairing DNA mutation and maintaining their genomic integrity to prevent the birth of cancer [1, 38, 39]. DNA repair systems are crucial and evolutionally conserved [40]. Several DNA repair systems are responsible for dealing with different kinds of DNA damages. Nucleotide excise repair (NER) removes UV-induced cyclopyrimidine dimer (CPD), pyrimidine-6,4-pyrimidone photoproducts (6-4 PP) and polyaromatic hydrocarbons (PAH) bulky adducts; base excision repair (BER) repairs the modified bases (e.g. 8-oxy-guanine) and abasic sites etc. causing by endogenous physical reactions; mismatch repair (MMR) deals with the mismatch nucleotides which raises from error of DNA polymerase. These pathways comprise recognition, excision and polymerization processes to repair the DNA lesions. Non homologous end join (NHEJ) and homologous recombination repair (HRR) are mechanisms to repair double strand breakages (DSBs) induced by exogenous irradiation, e.g. X-rays and anti-tumor agents. These cellular DNA repair pathways are clearly reviewed elsewhere [41]. The main caretaking systems which polyomaviruses interact and interfere are described as follows.

3.1.1. NER caretakers

The repairosome for NER embraces 20-30 distinct proteins to remove CPD, 6-4 PP and bulky adducts. Major effectors which include protein products of XPA-XPG genes, have defect in xeroderma pigmentosum *(XP)* patients who are extremely prone to skin cancer [42]. There are two initial subpathways for NER DNA damage recognition, the transcription coupled NER (TC-NER) and global genomic NER (GG-NER) (Figure 2A). The TC-NER recognizes the DNA damages on transcribed templates. The GG-NER first globally screens the disrupted base pairing by GG-NER specific factor, XPC-hHR23B. These two subpathways differ only in the initial steps of DNA damage recognition. Following XPC-hHR23B GG-NER initiator, the DDB1/DDB2 heterodimer (XPE) recognize and bind the UV lesions to initiate GG-NER cascade. Differently, TC-NER required CSB and CSA, TC-NER specific factor, for blocking elongating PolII on DNA lesions. After lesions recognition, the subsequent stages of two subpathways are identical. The XPB 3′-5′ and XPD 5′-3′ helicases, subunits of TFIIH, unwind the double strand and form DNA bubble. The single-stranded binding protein, replication protein A (RPA), stabilizes the open intermediates. Then, the endonuclease team, XPG and XPF, respectively cleave the 3′ and 5′ of opened damaged strand, excise 24-32-base oligonucleotide to remove the injury. The DNA polymerase δ and/or ε then fill and ligate the gap. The tumor suppressor p53, dual function as gatekeeper and caretaker, plays a pivotal role in NER [43]. Most obviously, the promoters of XPC and DDB2 contain the p53 responsive elements and are regulated transcriptionally by p53 [44]. The functions of NER effector/regulator which are targeted by polyomaviruses are listed on Table 1.

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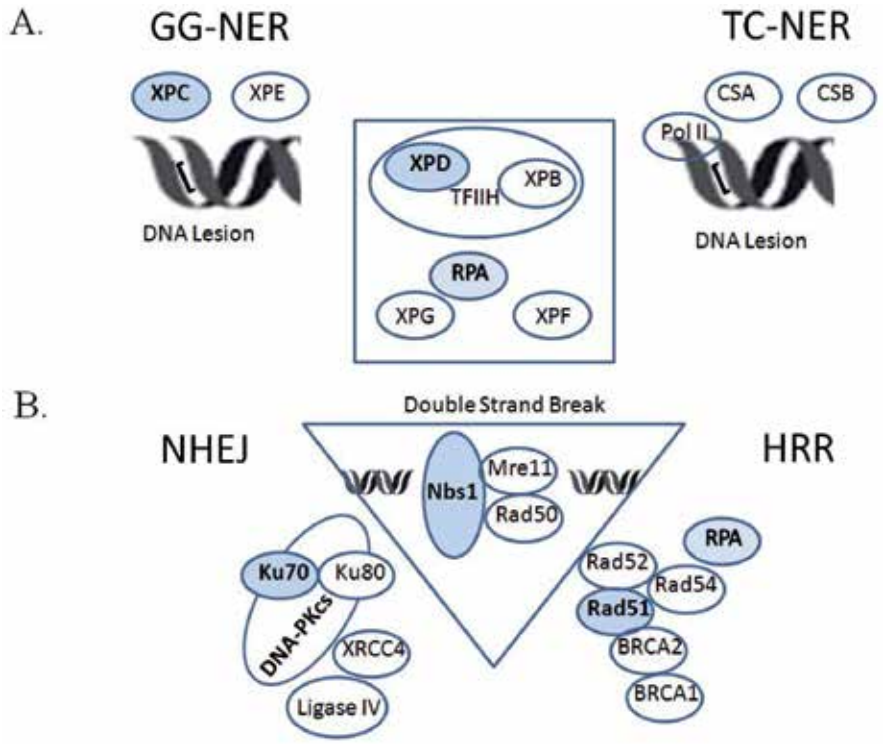

Figure 2. A. Effectors of NER. The common XP components, which participated in both GG-NER and TC-NER, are indicated by square. B. Effectors involved in DSBs repair. MRN complex, which participates in both HRR and NHEJ, is indicated by reverse triangle. The effectors which are targeted by polyomaviruses are colored by light blue.

3.1.2. HRR and NHEJ caretakers

DSBs are serious threats to genomic integrity. The HRR and NHEJ pathways contribute to genetic stability by removing a wide range of DSBs through error-free or error-prone reaction. HRR is error-free homologous recombination-based repair which occurs in S/G2/M phases and uses sister chromatids as the template to repair DSBs. Differently, DSBs in G1/S phase trigger NHEJ for error-prone repair. When DSBs occur, the signal transducing kinase, ataxia telangiectasia mutated (ATM), is autophosphorylated at S1981. It further phosphorylates p53, breast cancer 1 (BRCA1) and Nijmegen breakage syndrome 1 (Nbs1) and so on. Nbs1 and BRCA1 are defected on hereditary disorders which loss genomic stability through problems of DNA repair pathways and can directly contribute to human malignancy.

After ATM activation, it phosphorylates H2AX, also indicated as γ-H2AX (the marker of DNA damage), to form DNA damage foci in the flanking chromatin [45]. The Mre11-Rad50-Nbs1 (MRN) complex is then recruited to DSBs and promotes bridging of the DNA ends. MRN complex, as sensor, participates both in NHEJ and HR pathways (Figure 2B). If cells are in G1/S phase, Ku70/80 hetrodimers, the NHEJ specific caretakers, form complex with DNA-PKcs as a docking site for other NHEJ proteins, XRCC4 and ligase 4, for further end processing and

ligation [46]. For HRR, the single strand binding protein RPA facilitates the assembly. Rad51 recombinase, together with Rad52 and Rad54, catalyzes strand-exchange reaction and interacts with BRCA2/ BRCA1. The functions of HRR/NHEJ effector/regulator which are targeted by polyomaviruses are listed on Table 1.

Caretaking systems	Caretakers	Functions	Refs
Nucleotide Excision Repair (NER)	RPA	Single-strand binding protein	41,42
	XPC	CPD, bulky DNA adduct recognition	41,42
	XPD	5'-3' DNA helicase	41,41
	p53	NER Regulator [Regulate NER and MMR (transcription dependent); NER, BER, MMR, NHEJ and HRR (transcription independent)]	43,44
Homologous Recombination Repair (HRR)	Nbs1	Member of Mre11/Rad50/Nbs1 complex (DSB repair complex)	41,46
	Rad51	Recombinase for HRR (bacterial RecA homolog)	41,46
Non-Homologous End Join (NHEJ)	Ku70/Ku80 hetrodimer	Form complex with DNA protein kinase (DNA-PKcs)	41,46
	PP2A	dephosphorylation of Ku, DNA-PKcs and γ-H2Ax; Centrosome cycle	74,77
Mismatch repair (MMR)	hMSH3, hMSH6	Mismatch, insertion/deletion recognition	41
	hPMS1, hMLH1	form heterodimer	41
Spindle Assembly Checkpoint (SAC)	Bub1	Serine/Threonine-protein kinase For proper chromosome segregation	47
Interstrand Cross-Linked Repair (ICL)	FancD2	Caretaker in ICL	63

Table 1. The functions of caretakers which are targeted by polyomavirual proteins

3.1.3. MMR caretakers

The recognition proteins of DNA mismatch pairing and single base loops are hMSH2/6 heterodimer, whereas insertion/deletion loops detection are performed by hMSH2/3 hetrodimer. The hMLH1/hPMS2 are recruited to mismatch sites by interacting with MSH complex. Additional MMR factors search for a signal that identify the wrong strand and resynthesis the excised one. These include RPA, proliferating cell nuclear antigen (PCNA), RFC, exonuclease 1 and endonuclease FEN1. MMR components also interact with NER and recombination.

3.2. Spindle Assembly Checkpoints (SAC) caretakers

Caretaker genes encode proteins that stabilize the genome including DNA repair factors, cell-cycle checkpoints. Cell-cycle checkpoints stop cell-cycle progression when DNA damages occur. Caretakers do not directly control cell birth or cell death but rather control the rate of mutations of other genes, including gatekeeper genes. Except important G1 and G2 check-

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points, the SAC [47] and centrosome cycle [48] regulate chromosome distribution. To ensure the fidelity of chromosome segregation, the SAC blocks the ubiquitin ligase activity of anaphase-promoting complex (APC)-Cdc20 in response to a sister chromatid which is not properly attached to the mitotic spindle through kinectochore. The components of SAC including Mad1, Mad2, Bub3, Bub1 and Mps1 play crucial roles to guard and initiate sister chromatides segreagation. Among them, Bub1 is a serine/theronine kinase and inhibits Cdc20 by phosphorylation [49]. To ensure equal distribution of sister chromatids, the centrosome has to duplicate before mitosis and serves as the spindle poles during mitosis. Aurora A, a serine/threonine kinases, is associated with centrosomes and localized at the centrosome just prior to the onset of mitosis. The activity of aurora A is regulated by phosphorlyation and proteasomal degradation [48].

Retinoblastoma protein (pRB), a pocket protein, is a famouse cell-cycle moleculer brake. Through phosporylation cascade of cyclin/cyclin dependent kinase, pRB is activated to release E2F for entering into S phase. It directly controls cell birth and is considered as a gatekeeper gene. LT Ags of polyomaviruses also contain the LXCXE sequences and interact with pRB gatekeeper to deregulate cell cycle. This important interaction between polyomaviruses and host is also indicated in Figure 1B. The functions of other effectors in genomic stability which are targeted by polyomaviruses are listed on Table 1.

4. Interaction between SV40 viral antigens and DNA repair proteins

Despite SV40 is not a significant human oncogenic virus, it is a powerful model system for our understanding of the molecular interactions between virus and host. Those are not only important in virology and also in cell and cancer biology. In addition to the well-established effects of SV40 LT Ag in deregulating the cell cycle, this viral protein plays an important role in the development of genomic instability. LT Ag of SV40 is DNA damage regent and is enough to induce DDR in cells [50]. Furthermore, LT Ag binds and inactivates p53 and pRb, which play a significant role in their transformation activity. Although, SV 40 LT Ag simultaneously inactivates the pRb, a gatekeeper TGS, and p53, the gatekeeper/caretaker TGS; however, the studies indicated that complete transformation of human cells requires the additional inactivation of PP2A, the gatekeeper/caretaker TSG, by ST Ag [51, 52]. SV40 cellular targets which involve in genomic instability are described below.

4.1. SV40 LT Ag and p53, Nbs1, Bub1

LT Ag of SV40 interacts with many important cellular proteins. It has served as a useful paradigm for understanding cell transformation. In 1979, scientists reported the discovery of a 53 kDa protein that was present in human and mouse cells [8, 53-57]. The 53 kDa protein was discovered because it bound to the LT Ag in SV40 infected cells. Now, we know that the tumor suppressor TP53 is the most frequently altered gene in human.[58] It plays super star roles on cancer biology in past 30 years [58]. It functions as a transcription factor and regulates hundred of genes through its DNA binding domain. Now, this cellular partner of LT Ag in SV40, p53,

is called "genome guardian". It receives upstream signals (DNA damage, cell stress and oncogene activation) and directs downstream cellular responses (cell cycle arrest, DNA repair and apoptosis) to maintain the genome integrity.

Virus	Viral proteins	Cellular targets	Impaired DNA repair	Refs
SV40	LT Ag	p53	NER, BER, MMR, NHEJ and HRR	53-57
		Bub1	Mitotic Spindle checkpoint	50
		Nbs1	HRR , NHEJ	65
		MRN foci decreased	HRR, NHEJ	65,66
		FancD2 relocalized	HRR, ICL (FancD2/BRCA1 foci)	61
		PML	HRR (Rad51/Nbs1/PML foci)	61
		p53	GG-NER	67
		hMSH3,hMLH1 etc.	MMR	68
	ST Ag	PP2A	NHEJ, centrosome cycle	74-77
MPyV	OBD* in LT Ag	RPA	NER, HRR, NHEJ	92
	ST, Middle T	PP2A	?	94

*: Origin binding domain, residues 264-420 in LT Ag of MPyV

Table 2. Cellular caretakers targeted by animal polyomaviral antigens

DNA damage elicits ATM/ATR activation and p53 phosphorylation. The negative regulator of p53, MdM2, is then displaced. The expression level and transcriptional activity of p53 are increased [59]. Through its transcriptional regulation function, activated p53 regulates NER and MMR. However, p53 can through its transcription-independent process to modulate NER, BER, MMR, NHEJ and HRR [43]. For NER, p53 has essential functions through its transcription-dependent and transcription-independent roles. The NER effectors, XPC and DDB2, are transcriptionally regulated by p53. There are p53 responsive elements in their promoter regions [44]. p53 also modulates the enzymatic activity of XPD and XPB helicases by its transcription-independent function. p53 recruits the histone acetylase p300 to NER sites to acetylate histone H3, thereby through epigenetic regulation relaxing the chromatin and enhancing NER. p53 functions as a 'molecular node' in DDR and plays the pivotal role in NER [43]. There are bipartite p53 binding regions in SV40 LT Ag which are located around the C' terminal ATPase domain (Fig. 1B). SV40 LT Ag binds and inactivates p53. The crystal structure of SV40 LT Ag and p53 complex revealed that LT Ag occupies the whole p53 DNA-binding domain and interferes with formation of p53 tetramer [60].

Gjoerup's groups reported that LT Ag of SV40 deregulated multiple DDR and repair pathways [61]. Individual domains of LT are connected to different subcomponents of the DDR and repair machinery. LT and 17 T bind Bub1 through residues 89-97 [50](Figure 1B). Bub1 is a

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member of mitotic SAC and plays an important role in safeguarding the genome. Bub1 kinase delays anaphase progression if microtubules haven't attached to kinetochores on metaphase. Bub1 mutation results in chromosoml instability (CIN) and aneuploidy in human cancer [62]. SV40 LT Ag attacks the genomic integrity by binding to Bub1 [50]. It doesn't require the viral replication origin of genome. LT Ag alone can induce DDR and Chk1/Chk2 activation. Through Bub1 binding, LT Ag induces significant tetraploidy. It is suggested that p53 inactivation is important for cell survival in tetraploidy. SV40 LT Ag via Bub1 binding induces γ–H2AX and 53BP1 foci, the hallmarks of DDR.

Gjoerup's groups further found that LT Ag induces a distinct set of foci, H2AX/53BP1 in the G1 phase, Fanconi anemia group D2 protein (FancD2)/BRCA1 or Rad51/Nbs1/ promyelocytic leukemia protein (PML) in G2/S phases [61]. LT Ag induces activation of the FancD2 by relocalizing it into foci on chromatin. LT Ag also induces distinct foci of the HRR recombinase Rad51, which are colocalized with Nbs1 and PML. FancD2 protein, a caretaker protein involved in repair of DNA interstrand cross-links (ICLs), is monoubiquitinated in response to DNA damage, resulting in its localization to nuclear foci with BRCA1 and BRCA2 which involved in HRR. Foci of FancD2 and BRCA1 are mainly found in S/G2 and likely connected with a replication stress response [63].

SV40 LT Ag also induces distinct foci of the HRR recombinase Rad51. It colocalizes with PML and Rad51. It targets PML, a transcription factor and tumor suppressor, to Rad51 HRR recombinase and results in inefficient HRR [64].

SV40 LT also interacts with Nbs1, another protein of MRN complex, through its residues 147-167 [65] (Figure 1B). MRN complex forms at DSBs DNA damage foci. Interaction of Nbs1 by SV40 LT Ag impaired both HRR and NHEJ. Nbs1 is a multifunction protein that contributes to proper DNA replication and the maintenance of genomic stability. Nbs1 suppresses rereplication of cellular DNA and SV40 origin-containing replicons. Interaction of SV40 LT Ag and Nbs1 also results in enhancing the yield of new SV40 genomes during viral DNA replication [65].

In irradiated human fibroblast, the presence of SV40 LT Ag disturbs the formation of nuclear trimeric MRN DNA-repair foci. This MRN complex involves in NHEJ and HRR. These strongly elucidate interference of DNA repair by SV40 LT Ag [66].

In addition to the effect of SV40 LT Ag in disrupting HRR and NHEJ, SV40 also impairs GG-NER of CPD, most likely because inactivation of p53 by its LT Ag [67]. SV40 LT Ag also been reported to interfere MMR. In SV40 LT expressing cells, the MMR activity (for G:T, A:C, G:G) was deficient, and MMR genes (hMSH3, hMSH6, and hPMS1) were expressed at a low level and hMLH1 was mutated and/or deleted. This MMR deficiency also contributes to genetic instability [68].

4.2. SV40 ST Ag and PP2A

In 1990, SV40 ST Ag and MPyV ST and middle T Ag have been demonstrated to form stable complexes with PP2A [69]. PP2A, the important cellular target of SV40 ST Ag, is a serine/ threonine phosphatase. Complexly regulated PP2A has been identified as a multiple function

tumor suppressor gene. It plays as a negative regulator for PI3K/AKT, MAPK, Wnt, NF-κB, PKC pathways to control cell growth, division and survival [70]. Inhibition of PP2A activity is essential for cell transformation [71]. SV40 ST Ag, through its residues 97-103 CknwPeC, binds PP2A [72]. This viral protein displaces regulatory subunit B of PP2A to form ST-PP2A/AC complex with structural subunit A and catalytic subunit C heterodimer [14]. Mutiple functions of PP2A were disclosed through interaction and inhibition by SV40 ST Ag. For example, it disrupts cell adhesion and cytoskeletal dynamics which is linked to loss of cell polarity, increased cell motility and invasiveness [73].

Significantly, PP2A, a SV40 ST-targeted tumor suppressor gene, also plays a critical role in DNA repair and genome stability. Inactivation of PP2A via SV40 ST Ag represses cellular NHER repair activity. By using SV40 ST Ag as PP2A inhibitor, PP2A had been demonstrated to promote NHEJ by dephosphorylation of Ku70 and DNA-PKcs, and forms Ku/DNA-PKcs complex to bind to DNA ends [74] (Figure 3). This is a novel mechanism of NHEJ promotion by PP2A through direct dephosphorylation of Ku and DNA-PKcs. The involvement of PP2A to repair DSBs contributes to maintenance of genetic stability. On the other hand, PP2A also facilitates DSBs repair through dephosphorylate γ-H2AX to recruit effectors of NHEJ [75]. Cells overexpressing SV40 ST Ag can't form organized centrosome and alters centrosome cycles [76]. PP2A interacts with Aurora A which regulates centrosome dynamic [77]. The abnormal centrosome cycles in cells overexpressing SV40 ST Ag may be due to inactivation of PP2A. SV40 ST Ag disrupts the caretaker roles of PP2A. Taken these studies together, SV40 ST Ag via PP2A binding, probably impairs chromosomal stability through different mechanisms.

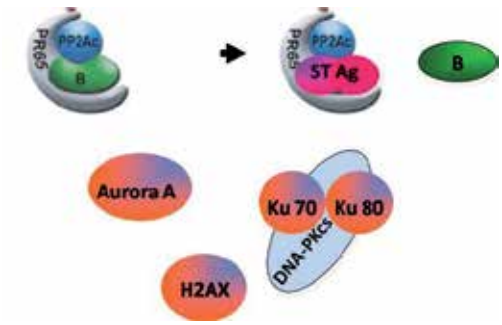

Figure 3. ST Ags of SV40 and JCV disrupt the PP2A holoenzyme. The ST Ag competes with regulatory subunit B of PP2A and inactivates its phosphatase activity. The substrates of PP2A which is involved in genomic stability are indicated.

5. MCV LT Ags, DDR and DNA repair

MCV is the human polyomavirus which is most tightly associated with human cancer. Recently, Li et al. found that the interesting differences between LT Ags of SV40 and MCV. Full length MCV LT Ag, through its C-terminal domain, activates ATR and Chk1 pathway via

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$p53^{s15}$ activation. It induces DDR in host genome (SV 40 LT Ag activates a DDR through ATM and ATR pathway but inhibits p53 function). MCV LT Ag arrests cell cycle. It, just as antitumor effect, inhibites cell proliferation, focus formation and anchorage-independent cell growth [78]. To explain the carcinogenicity of MCV, Feng et al. collected clinical MCC samples and found that the intergrated MCV genomes have mutations which result in prematurely turncated LT Ag or C-terminal truncations of MCV LT Ag [18]. The tumor-derived trunated MCV LT Ag (tLT) contains full open reading frame of ST Ag. They explained that removed of C-terminal region of MCV LT is necessary for MCV carcinogenicity [78].

SV40-transformed cells impair global genomic repair of CPD as mentioned previously [67]. MCV-positive cells also have poor GG-NER activity. In addition, MCV tLT Ag can inhibit GG-NER and XPC expression upon UV irradiation [79].

Protein functions of this new human cancer causing virus have not been well investigated. How the tLT Ag contribute to carcinogenesis? Whether it interacts with some caretakers to disrupt the genomic stability remains to be determined. The ST Ag of MCV also has predicted PP2A binding site [80]. Whether it plays roles on MCV carcinogenesis awaits for further investigation. It will be interesting to explore the common and novel features of the viral tumor Ag of MCV on DNA repair and genomic instability etc. The caretakers targeted by human polyomaviruses are summarized in Table 3.

6. Interaction between JCV/BKV viral Ags and DNA repair proteins

6.1. JCV LT Ag targets IRS-1

LT Ags of JCV and BKV bind and inactivate p53 and pRB as SV40 LT Ag does [21, 81]. In addition to direct interaction with p53 to disrupt DNA repair and genomic stability, JCV indirectly disrupts HRR. Khalili's group had a series of publications about the relationship of JCV and genomic instability. They found a novel mechanism for JCV LT Ag-mediated HRR repression. They examined clinical samples of progressive multifocal leukoencephalopathy and found that there are the Rad51 foci in inclusion bodies bearing oligodendrocytes. They used virus infected system to explore DDR and DNA repair upon viral infection. JCV-infected human astrocytes showed lower NHEJ activity. The γ-H2AX, Rad51 expression and micro-nuclei formation (marker of chromosome instability) increase in these cells. These indicated that induction of DDR and suppression of DNA repair did occur [82]. Additionally, they pinpointed that JCV LT Ag inhibits HRR indirectly. They initially noted that JCV LT Ag translocated insulin receptor substrate 1 (IRS-1) to nucleus [83]. IRS-1 is a cytosolic adaptor protein which involved in insulin receptor (IR) and insulin-like growth factor-1 receptor (IGF-1R) signaling. They found the IRS-1-Rad51 nuclear interaction in JCV LT Ag-positive medulloblastoma cells [84]. They demonstrated that JCV LT Ag translocated IRS-1 to nucleus and forced IRS-1-Rad51 complex formation. JCV LT Ag-positive medulloblastomas, defective in HRR recombinase Rad51 activity, therefore tended to mutation accumulation and sensitized to genotoxic agents. The presence of JCV LT Ag affects faithful HRR and DNA repair fidelity [85]. The DNA damages induced by LT Ag are repaired by error-prone NHEJ and have

threatened to genomic integrity. Given that JCV LT and IGF-1 pathway merge to destroy precise DNA repair and genomic integrity [86], LT Ag of JCV impairs HRR as does LT Ag SV40; however, it uses a novel mechanism to interfere HRR indirectly (Table 3) [86]. By using factor of IGF-1 pathway, JCV LT indirectly suppresses Rad51 activity and forces error-prone NHEJ. Besides, the nuclear IRS-1 was also detected in SV40 LT Ag expressing cells [87].

Virus	Viral proteins	Cellular targets	Impaired DNA repair	Refs
MCV	LT Ag	ATR	Induce DDR	78
	Tumor derived tLT Ag	XPC	GG-NER	79
JCV	LT Ag	p53	NER etc.	21,81
	ST Ag	PP2A	-	14,89,9C
	Agnoprotein#	Ku70	NHEJ	16,88
	LT*	IRS-1 ^	Form nuclear IRS-1Rad51 complex, HRR	83-87
BKV	LT	p53	NER etc.	81

#: JCV agnoprotein bind to Ku70 and ST Ag through its N terminal.

*: JCV LT indirectly inhibit Rad51 via translocating IRS-1 to nuclear.

^: IRS-1 binds Rad51. It, via residue155-302, also binds LT Ags of JCV and SV40.

-: indicates not fully determined

Table 3. Cellular caretakers targeted by human polyomaviral antigens

6.2. JCV agnoprotein, ST Ag and DNA repair

Khalili et al. addressed the issue of low NHEJ activity in JCV-infected human astrocytes. Agnoprotein, a small product of late region (71 a.a.), of JCV was found to impair NHEJ. Agnoprotein reduces the expression of Ku70 and Ku80 NHEJ proteins. Agnoprotein, through its N terminal residues 18-36, directly binds to Ku70 and represses NHEJ activity [16, 88]. As we described on SV40 ST Ag, it interacts and inhibits PP2A. SV 40 ST Ag impairs NHEJ through PP2A binding. JCV ST Ag has been predicted and demonstrated to bind PP2A [14, 89, 90]. Our group found that JCV ST Ag, a PP2A inhibitor, also inhibits NHEJ. We suggest that the NHEJ inhibition activity of JCV-infected cells may be contributed by both agnoprotein and ST Ag. In our laboratory, we found that ST Ag of JCV impairs both NER and NHEJ activity. In JCV ST-expressing cells, the expression of XPD is lower than that in the vector-control cells [91].

The LT and ST Ags among SV40, JCV and BKV have high homology in protein sequence. For example, LT Ags of JCV, BKV and SV40 are above 70% homologous in protein sequence. In brief, LT Ags of JCV (688 a.a.), BKV (695 a.a.) and SV 40 (708 a.a.) bind p53, as well as ST Ag

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of those (below 70% homologous) that bind PP2A. However, they have different downstream effects on host due to host complexity. In permissive cells, they proceed to lytic life cycle, whereas in non-permissive cells, they transformed cells. Interaction of caretakers and SV40 LT Ag plays as a model for mechanism of transformation of other polyomaviruses in non-permissive host.

7. Interaction between MPyV viral Ag and DNA repair proteins

The study of MPyV, another well-studied animal polyomavirus, revealed a novel connection bewteen virus and DNA repair pathways. It represses DNA repair systems through its LT Ag by directly binding to a single-strand DNA binding protein, replication protein A (RPA), an essential DNA replication and repair protein [92]. For DNA repair, RPA plays as a sensor for UV-induced CPD to repair UV induced damage. When DNA encounters double-strand break, it also recuits MRN complex to damage lesions. MPyV LT Ag blocks RPA to DNA damage foci and leads to failure to recruit Rad51 etc. The OBD (origin binding domain, residue 262-420) of LT Ag mediated this inetraction. LT Ag or OBD induces DNA damage which is revealed by comet assay. In UV irradiated-MPyV LT Ag expressing cells, location of RPA is diffusely nuclear, rather than localization to damage foci. Rad51, the critical recombinase for HRR, is not recruited to foci. Rad 9, a component of sliding clamp complex for DNA repair, is also prevented to reach DNA damage foci by MPyV LT Ag.

Another study on MPyV also provides a link between DNA repair and virus replication. MPyV infcetion increases ATM activity and level of ATM^{S1981P}. It activates and utilizes a component(s) of an ATM pathway of DNA repair to prolong S phase and aids in its own replication [93].

Interestingly, SV40 LT antigen targets p53 directly, but MPyV LT does not [94]. However, MPyV ST and middle T antigens, as SV40 ST antigen, also form stable complexes with PP2A [69]. Differentially, these interactions eclict the activation of different cellular signal pathways involved in growth control [95]. There is no related publication about the effect of MPyV ST Ag and PP2A interaction on DNA repair. Nevertheless, the difference of PP2A binding subunits and PP2A substrates between ST Ags of SV40 and MPyV have pointed out the complexity and diversity of these groups of viruses [94].

8. Specific interaction between other viruses and DNA repair

Some other viruses also encode specific proteins to target DNA repair proteins as polyomaviruses do. The most famous DNA repair caretaker which is bound and/or degraded by viral proteins is tumor suppressor p53. In addition to SV40 LT Ag, E1B-55k/E4-ORF6 of adenovirus, E6 of human papillomavirus (HPV), vIRF1 of Kaposi's sarcoma-associated herpesvirus (KSHV) and X protein of hepatitis B viris (HBV) can bind and/or degrade p53 which plays the pivotal role in NER. Other viral proteins such as E1B-55k/E4-ORF6 and E4-ORF3 of adenovirus also interact and/or mislocalize MRN DNA repair complex as SV40 LT Ag. These interactions

repress NHEJ and HRR pathways. Interestingly, recent studies showed that herpes simplex virus-1 manupulates the Fanconi anemia pathway, redistributes FancD2, to inhibits NHEJ and promote viral replication cycle [96]. Several viral proteins target other DNA repair proteins which are not mentioned yet in polyomaviruses studies. X protein of HBV inhibits NER through interrupts DDB1 which recognizes UV damage site. E6 of HPV-8 inhibits XRCC1, a BER and single stranded breakage repair protein. Many viruses also activate or inactivate the ATM/ATR pathways to take the advantages for their life cycles. The interaction of viruses and host DNA repair machineries had been revealed in the past decade and were well reviewed in other articles [4, 5]. These viral proteins serve as useful tools for our understanding the function and important roles of these DNA caretakers.

9. Conclusions

The polyomaviruses are not closely associated with human cancers if compared with the other six Group 1 carcinogenic viruses (HPV, HBV, KSHV, human T-lymphotropic virus 1, hepatitis C virus and Epstein Barr virus). However, they transform cells of non-permissive host efficently and serve as an excellent model to investigate carcinogenesis. Some of polyomaviral antigens not only induce DNA damage but also block DNA repair pathways. They directly induce mutations and simutenously ruin the caretaker defense barriers. The viral proteins cooperate to accummulate mutations/chromosomal instability and initiate the birth of cancer. SV40 targets numerous caretakers to disrupt genomic intergrity and serves as a powerful model to gain insight of the complexity of DNA repair systems. It is worthwhile to note that there are novelties and differences among these viruses. Some LT Ags target p53, some do not. Human MCV is most closely related to human malignancy. The function of MCV LT Ag is dramatically different to that of SV40. The MCC tissues contain tLT and ST Ag. The lessons learned from SV40 will help to reveal the roles of MCV tLT and ST Ag on genomic instability. Especially, the homology among their ST Ags are lower than that of LT Ags. Bollag et al. claim that LT Ags have received much attention. JCV ST Ag binds PP2A and pRB and has only recently become a focus of study [91]. Additionally, SV ST Ag complements LT Ag, hTERT and Ras for the transformation of human mammary epithelial cells, but MPyV ST Ag does not. The differences between their ST Ags may depend on the differential utilization of PP2A. They bind different scaffold subunits of PP2A/A. The two ST Ags can target different proteins for dephosphorylation. As described in the Introduction section, virueses are always more complicated than our understanding. By studying the proteins and RNAs of cancer associated viruses, we can learn more lessons on DNA repair in further investigations.

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This book edition is intended to provide a concise summary for select topics in DNA repair, a field that is ever-expanding in complexity and biologic significance. The topics reviewed ranged from fundamental mechanisms of DNA repair to the interface between DNA repair and a spectrum on cellular process to the clinical relevance of DNA repair in oncologic paradigms. The information in this text should provide a foundation from which one can explore the various topics in depth. The book serve as a supplementary text in seminar courses with focus on DNA repair as well as a general reference for scholars with an interest in DNA repair.

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