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Gene Expression and Regulation in Mammalian Cells

Transcription Toward the Establishment of Novel Therapeutics

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Meet the editor

Fumiaki Uchiumi, professor of Pharmaceutical Sciences, Tokyo University of Science, received his bachelor's degree (Chemistry) from Tokyo University of Science in 1987. In 1993, after obtaining his PhD degree (Molecular Biology) from Tokyo University, he joined Professor S. Tanuma's Laboratory at Tokyo University of Science as an assistant professor. He obtained his second PhD degree (Pharmaceutical Science) from Tokyo University of Science in 1999, and in 2000, he was promoted to the position of lecturer at Tokyo University of Science. Professor Uchiumi then went abroad as a postdoctoral researcher for the United States-Japan Cooperative Cancer Research Program in Professor E. Fanning's Laboratory at Vanderbilt University, 2000–2001. Professor Uchiumi was promoted to associate professor and then full professor at Tokyo University of Science in 2010 and 2016, respectively.

Contents

Preface

Although not all of the relationships between transcription-controlling system and cellular functions or behaviors have been fully explained yet, rapidly accumulating data from transcriptome analyses enabled us to find the differences in gene expression between cells from healthy contributors and cells from patients of certain diseases, including cancer. They are frequently referred to as "driver genes." Presently, a variety of human genomic databases from various experimental systems, including transcriptome, ChIP seq, DNA methylome, and others, are available. The differences in gene expression pattern will surely contribute for diagnosis of specific diseases suggesting target molecules. However, even though the data are reliable, they might only indicate the consequences. Probably, it might be a right time for scientists to start developing prediction system for specific diseases. We know that introducing transcription factors into somatic cells reverses cell fate to revoke pluripotency. In this respect, artificial control of certain transcription factors could be applied for novel therapies for human refractory diseases, including cancer and neurodegenerative diseases.

In Section 1, the importance of the nutrient conditions affecting transcription system is discussed. Beneficial hormetic effects of phytochemicals delaying aging and age-related diseases are explained in Chapter 1. The nutrient conditions to regulate transcription of ribosomal RNAs are reviewed in Chapter 2, and it will provide a novel insight into treatment of cancer.

Section 2 includes one of the most currently popular topics, epigenetic regulation. In Chapter 3, the effect of DNA methylation that could lead to cancer generation and the molecular mechanisms are commented. Histone chaperones, which are also involved in the epigenetic regulation, are thought to be tightly linked with cellular differentiation and development, and that is discussed in depth in Chapter 4. The key epigenetic regulators of genes that are associated with cancer are summarized in Chapter 5. Moreover, the important functions of hydroxymethylation, which is mainly catalyzed by TET enzymes, are discussed in Chapter 6.

In Section 3, alterations in transcription systems that could cause human diseases are discussed. In Chapter 7, functions of the tumor suppressor E2F are reviewed. Cancer-generating mechanisms that could be affected by transcription controlling proteins, such as sirtuins and paired-box transcription factors, are discussed in Chapters 8 and 9, respectively. In Chapter 10, in search of novel cancer therapeutics, the transcription-controlling mechanism by hypoxia-inducible factor is described in detail. Finally, in Chapter 11, the method for analyzing gene expression in Parkinson's disease is presented.

In Section 4, we might have to consider about transcription-based therapies. In Chapter 12, the transcriptional molecular mechanisms in colorectal cancer generation are reviewed. In Chapter 13, the essential functions of the retrotransposons in cancer development are dis-

cussed. Chapter 14 explains the importance in artificial designing of vaccines against infectious and other diseases.

At the end of the "Preface" of Volume 1, I asked if we could have reached the goal successfully. It is my opinion, but the answer is "not yet." Although we have learned a lot about transcription from various studies and variety of experimental data, our ship is still going on the way. However, the light of the lampstand tells us that we are surely approaching to the transcription-based or transcription-inspired therapies on human diseases. The story of our voyage is to be continued, aiming to get to the goal or clinical applications.

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Section 1

Stress Response and Transcription

Chapter 1

Stress Response of Dietary Phytochemicals in a Hormetic Manner for Health and Longevity

Ceren Gezer

Additional information is available at the end of the chapter

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Abstract

The stress responses observed in mammalian cells can be classified as heat shock response, unfolded protein response, autophagic response, deoxyribonucleic acid damage response, antioxidant response, and sirtuin response at the intracellular and molecular levels. Factors that strengthen the hemodynamic structure causing low-level molecular damage and activating one or several stress response pathways are called hormetins. Hormetins can be categorized as physical, physiological, biological, and nutritional hormetins. Nutritional hormetins provide an interesting, comprehensive research topic because of their effects on health and lifespan. Dietary phytochemicals, with their low-level stress-inducing effects, are potential nutritional hormetins. Resveratrol, curcumin, epicatechin, isothiocyanates, ferulic acid, and certain vitamin-minerals can induce a heat shock response, unfolded protein response, autophagic response, deoxyribonucleic acid damage response, antioxidant response, and sirtuin response causing the stimulation of kinases and transcription factors. Studies have shown that these phytochemicals are related to nuclear factor-erythroid 2, sirtuins, nuclear factor-kappa B, and heat shock response pathways. In this chapter, the stress response of dietary phytochemicals will be systematically examined in a hormetic manner for delay of age-related diseases, healthy aging, and longevity based on current data.

Keywords: aging, longevity, health, stress response, hormesis, nutritional hormetin, phytochemical

1. Introduction

The term hormesis, based on toxicology, is described as a biphasic dose response in which environmental factors show a stimulant effect at low doses and a toxic effect at higher doses [1]. A comprehensive current definition of “hormesis” is "chemical and environmental factors

having a beneficial effect to cells in an organism at low doses, whereas they are damaging at high doses" [2]. Hemodynamic is the ability of live systems to provide protection against stress, and to maintain adaptation, survival, and continuity of health. Hemodynamic impairment, increased molecular heterogeneity, altered cellular function, and decreased adaptive stress responses are some factors that determine health status and lifespan [3, 4]. The development of adaptive stress response with mild and periodic stress is hormetically related to the strengthening of the hemodynamic structure, the reduction of disease risks, and healthy aging. Hormesis in aging implies that mild stress produces biologically beneficial effects by inducing protective mechanisms in the cells and the organism [5]. Stress response can be defined as the response of cells, tissues, and organisms to physical, chemical, or biological factor(s) affecting adaptation and lifespan by initiating a series of biological events. In terms of hormetic level, stressors at a mild level activate various signaling pathways, maintaining intrinsic changes leading to a high level of stress-adaptive response. Stress response in mammalian cells can be classified into seven basic pathways at the intracellular and molecular levels: (1) heat shock response; (2) unfolded protein response; (3) autophagic response; (4) deoxyribonucleic acid (DNA) repair response; (5) antioxidant response; (6) sirtuin response; and (7) nuclear factor-kappa B (NF-κB) inflammatory response. The conditions and factors identified as hormetic activate the pathway of one or more stress responses by mild molecular impairment and strengthen the hemodynamic structure. Hormetins can be grouped under three categories: (1) physical hormetins (exercise, thermal shock, and irrigation); (2) physiological hormetins (mental interrogation and focusing); (3) biological and nutritional hormetins (infections, micronutrients, phytochemicals, and energy restriction) [4, 6, 7].

Dietary phytochemicals are potential nutritional hormetins with mild stress-inducing effects. In the Greek language “phyto” means plant, so phytochemical means “plant chemical.” Phytochemicals are non-nutrient biologically active compounds produced to protect plants against microbial infections that occur because of environmental factors damaging the plant. Therefore, phytochemicals, which are secondary plant metabolites found primarily to protect their structures and properties in vegetables, fruits, grains, and various plants, may have positive effects on human health when taken in the diet. Phytochemicals are generally classified according to their chemical structure. The main groups with bioactive properties from these groups are phenolic compounds [8, 9]. Ferulic acid, resveratrol, epigallocatechin gallate (EGCG), luteolin, quercetin, and curcumin as phenolic compounds are dose-dependently responsible for the stimulation of kinases and transcription factors and produce a heat shock response, unfolded protein response, autophagic response, DNA repair response, antioxidant response, and sirtuin response [6, 10–13]. In this chapter, the stress response of dietary phytochemicals will be systematically examined in a hormetic manner for delay of age-related diseases, healthy aging, and longevity based on current data.

2. Dietary Phytochemicals as Nutritional Hormetins

When dietary phytochemicals are invoked in relation to neurodegenerative diseases, cardiovascular diseases, cancer, aging, and longevity, especially in the heat shock response, antioxidant

response, NF-κB inflammatory response, and autophagic response were emphasized regarding their hormetic adaptive stress response pathways. The characteristics and importance of these stress response pathways are summarized in what follows.

The major effectors involved in heat shock response are heat shock proteins (HSPs), which are cytoprotective proteins that facilitate cellular protein folding, prevent protein aggregation, and provide protein degradation activation. They also affect the cell survival by interacting with various molecules in the regulation of apoptosis and mitochondrial activities. HSPs are divided into five main groups: the Hsp100 family, Hsp90 family, Hsp70 family, Hsp60 family, and the small Hsp family. Hsp70 regulates protein homeostasis, thereby, it can provide protection against cancer, neurodegeneration, and infections [14, 15]. Hsp90 regulates the stability and intracellular sorting of client proteins found in many oncogenic processes. Thus, Hsp90 inhibition may prevent cancer progression [16]. Hsp27 can protect against neurodegenerative diseases by controlling apoptosis, cytoskeleton regulation, oxidative stress, and protein folding [17]. In general, HSPs provide the survival of cancer cells by overexpression in cancer cells. Thus, the inhibition of Hsp27, Hsp70, and Hsp90 can be targeted in the treatment of cancers in which HSPs are known to be over-expressed [18]. The nuclear factor-erythroid 2-related factor 2 (Nrf2)/antioxidant response element (ARE) is the main effective pathway in the formation of antioxidant stress responses. Under basal conditions, Nrf-2 is present in the cell cytoplasm bound to Keap1 protein. However, when combined with oxidative stress and chemo-blocking factors, Nrf2 is released from Keap-1 into the nucleus; it activates the ARE and induces the expression of the antioxidant enzymes including glutathione peroxidase (GPx), catalase, hemoxygenase (HO)-1, and the phase II detoxification enzymes, including glutathione S-transferase (GST). Extracellular signaling protein kinases are responsible for the release of Nrf2 from Keap-1 by phosphorylation of extracellular signal-regulated kinases 1 and 2 (ERK1/2), protein kinase C (PKC), and c-Jun N-terminal kinase (JKN). Thus, Nrf2 associated with the cell defense mechanism, may have protective effects against oxidative stress-induced tissue degeneration, premature aging, cancer, neurodegenerative diseases, cardiovascular diseases, acute and chronic lung diseases, and autoimmune and inflammatory diseases [19–22]. Among the factors that induce Nrf2 in the formation of antioxidant stress responses are isothiocyanates and Michael acceptors. Michael acceptors are susceptible to flavonoids, chalkones, terpenoids, curcumin, cinnamic acid derivatives, and thiophenes, and interact with these phytochemicals to modulate the Nrf-2 pathway [23, 24]. The effector NF-κB protein complex action regulates the expression of genes involved in innate and adaptive immunity, inflammation, cellular stress response, cell survival, and proliferation. Therefore, this pathway can be effective in pathogenesis of inflammatory and autoimmune diseases, septic shock, viral infections, tumorigenesis, and neurodegenerative diseases. Various dietary phytochemicals such as curcumin and resveratrol can suppress NF-κB activation and protect against immunological and inflammatory diseases, cancer, and neurodegenerative diseases [12]. In an autophagic response, hypoxia-inducible factor (HIF)-1 and the activated mammalian target of rapamycin (mTOR) are important. mTOR is involved in cell proliferation and protein synthesis via insulin and insulin-like growth factor (IGF)-1 signaling. It can also cause the suppression of autophagy, and reduced autophagy is associated with decreased longevity. Thus, the increase in autophagy is associated with an increase in inflammatory

response, cellular senescence, decreased proteotoxic protein aggregation, and the removal of intracellular pathogens, cumulatively resulting in an increased innate immune response that leads to longevity [25]. HIF-1 regulates genes related to angiogenesis, iron and glucose metabolism, cell proliferation and cell survival. Various dietary phytochemicals, with HIF-1 inhibition, have protective effects against neurodegenerative diseases, cancer, cardiovascular diseases [12, 26]. In this section, hormetic effects of phenolic compounds predominantly expressed as hormetin including ferulic acid, curcumin, resveratrol, EGCG, luteolin, quercetin, and sulforaphane will be discussed in relation to these stress response pathways. The stress pathways, transcription factors, and biological outcomes of these phytochemicals have been summarized in **Table 1**.

2.1. Ferulic acid

Ferulic acid (4-hydroxy-3-methoxycinnamic acid) is a cinnamic acid derivative phenolic compound. It is also the preliminary metabolite for curcumin and lignins. Grain bran, whole grains, artichoke, eggplant, banana, cabbage, and coffee are rich in ferulic acid. Ferulic acid has a positive effect on diseases such as cancer, Alzheimer's disease, Parkinson disease, and diabetes through various pathways. Among the mechanisms of action of ferulic acid are the antioxidant response, heat shock response, and NF-κB inflammatory response, especially in the adaptive stress response pathways [27–29]. Ferulic acid showed a protective effect against heat stress-induced intestinal epithelial barrier dysfunction in IEC-6 intestinal epithelial cells in a dose-dependent manner in male Sprague-Dawley rats *in vitro* and *in vivo* [30]. In a study conducted on the human neuroblastoma cell line SH-SY5Y, ferulic acid increased dose-dependent HO-1 expression through Nrf2 [31]. In a study on PC12 cells, ferulic acid increased HO-1 expression through ERK1/2-Nrf2 signaling pathway and protected against lead acetate-induced neurite outgrowth inhibition [32]. On the other hand, 1-feruloyl glycerol and 1-feruloyl diglycerol predominate in water-soluble forms of ferulic acid in rat primordial astrocytes, suppressing nitric oxide (NO) synthesis and inducible nitric oxide synthase (iNOS) expression by suppressing the NF-κB pathway. Accordingly, these ferulic acid forms may provide a protective effect against neurodegenerative diseases [33]. The tumor necrosis factor (TNF)-α induces endothelial dysfunction by reducing NO bioavailability. Ferulic acid increased tyrosine-dependent NO production and suppressed the NF-κB pathway in TNF-α-stimulated inflammatory human umbilical vein endothelial cells (HUVECs) [34]. Another study showed that ferulic acid demonstrated a cardioprotective effect by increasing Hsp70 through the NO-ERK1/2 pathway in mice cardiomyocytes and suppressing the NF-κB pathway [35]. In another study, HeLa and mouse primary hepatocyte cells activated basal autophagy with an mTOR inhibition almost equivalent to that of rapamycin [36]. As a result, ferulic acid can exert a protective effect against neurodegenerative diseases, cardiovascular diseases, and cancer inflammatory diseases by acting on stress pathways and thus can positively affect longevity.

2.2. Curcumin

Curcumin (1,7-bis (4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione), also known as diferuloylmethane, is a yellow phenolic compound, found in *Curcuma longa* (turmeric) a

Phytochemicals	Stress pathways	Transcription factors	Biological outcomes	References
	Antioxidant response pathway	Nrf-2	HO-1↑	[31, 32]
Ferulic acid	NFκB inflammatory pathway	NFκB	NO↓, iNOS↓	[33]
	Heat shock response pathway	HSF-1	Hsp70↑	[35]
	Autophagic response pathway	—	mTOR inhibition	[36]
	Antioxidant response pathway	Nrf2	Glutathione, GR, GST, HO-1, NQO1	[43, 44]
Curcumin	NFκB inflammatory pathway	NFκB	SOD-2↑, Hsp60↑	[42, 45]
	Heat shock response pathway	HSF-1	Overexpressed Hsp27↓, Hsp70↓, Hsp90↓ Hsp27↑, Hsp70↑	[39, 40] [41, 42]
	Sirtuin response pathway	—	SIRT3↑	[42]
	Antioxidant response pathway	Nrf2	Glutathione↑, HO-1↑	[49, 50]
Resveratrol	NFκB inflammatory pathway	NFκB	iNOS↓, IL-6↓, TNF-α↓	[54, 55]
	Heat shock response pathway	HSF-1	Hsp25↑, Hsp70↑	[47, 48]
	Autophagic response pathway	—	mTOR inhibition	[52, 53]
	Sirtuin response pathway	—	SIRT1↑	[47, 48]
	Antioxidant response pathway	Nrf2	GST↑, NQO1↑, HO-1↑	[59, 60, 62, 64]
EGCG	NFκB inflammatory pathway	NFκB	IL-12p40↓, IL-6↓	[65–67]
	Heat shock response pathway	HSF-1	Overexpressed Hsp90↓	[58]
	Autophagic response pathway	—	HIF-1α, mTOR inhibition	[68, 69]
	Antioxidant response pathway	Nrf2	HO-1↑, CYP1A1↑, NQO1↑, GST-P1↑, GCLC↑, GCLM↑	[76–78, 80, 81, 83]
Luteolin	NFκB inflammatory pathway	NFκB	TNF-α↓, NO↓	[73–75, 81]
	Autophagic response pathway	—	HIF-1α inhibition	[82]
	Sirtuin response pathway	—	SIRT1↑	[81]
	Antioxidant response pathway	Nrf2	GSH↑, GPx↑, GR↑, GST↑, GCLC↑, GCLM↑, HO-1↑	[89–95]
Quercetin	NFκB inflammatory pathway	NFκB	COX-2↓	[90, 94]
	Heat shock response pathway	HSF-1	Overexpressed Hsp27↓, Hsp70↓	[85–87]

Phytochemicals	Stress pathways	Transcription factors	Biological outcomes	References
	Autophagic response pathway	—	HIF-1, mTOR inhibition	[88, 96–101]
	Antioxidant response pathway	Nrf2	HO-1↑, SOD-1↑,NQO1↑	[104–111]
Sulforaphane	NFκB inflammatory pathway	NFκB	TNF- α↓, IL-6↓	[109]
	Autophagic response pathway	—	HIF-1α inhibition	[114]

↑: increased; ↓: decreased.

Table 1. Summary of stress pathways, transcription factors, and biological outcomes of phytochemicals

plant of the ginger family. Curcumin is the compound responsible for the chemical and biological properties of this spice, as well as its color and taste. Numerous studies have shown that curcumin is associated with antioxidant, anti-inflammatory, antimutagenic, antimicrobial, and anticancer effects, mitigating chronic diseases and increasing longevity [37, 38]. HSPs, HSF1, and histone deacetylase (HDAC) 6 are upregulated in cancer. Expression of Hsp 27, Hsp70, Hsp90, HSF1, and HDAC-6, which are overexpressed in K-562 and HL-60 leukemia cells, was reduced when curcumin was administered [39]. Also, curcumin appeared to reverse the inhibition on Hsp70 induced by the gp120 V3 loop peptide and increased the expression of Hsp70 in primary rat cortical neuronal apoptosis [40]. In addition, curcumin can protect against endosulfan toxicity by decreasing endosulfan-induced apoptosis through increased Hsp 27 expression in human peripheral blood mononuclear cells (PBMCs) [41]. In hyperglycemic HepG2 human hepatoma cells, curcumin increased the expression of NF-κB and Hsp70, sirtuin (SIRT)-3, glutathione peroxidase (GPx)-1, and superoxide dismutase (SOD)-2 in a dose-dependent manner [42]. On the other hand, curcumin may act as an antioxidant in the stress-response pathway. Primary cell cultures of cerebellar granule neurons of rats increased the expression of HO-1, glutathione, glutathione reductase (GR), GST, and SOD through Nrf-2 depending on the dose and duration and thereby protected against hemin-induced toxicity [43]. In mice liver cells with T-cell lymphoma, the expression of GST, GR, and NAD(P)H:quinine oxidoreductase (NQO1) enzymes was increased by activation of curcumin Nrf-2 [44]. Lipopolysaccharide (LPS)-stimulated BV2 mouse microglia cells also inhibited microglial activation by inhibiting the curcumin Hsp60/TLR4/MyD88/NF-κB pathways [45]. As a result, curcumin can show protective effects against cancer, neurodegeneration, and inflammation by acting on stress-response pathways.

2.3. Resveratrol

Resveratrol (3,5,4′-trihydroxy-trans-stilbene) is a phenolic compound found in some plants such as grapes, berries, peanuts, and Japanese knotweed, with purported medical uses. Several studies have shown that resveratrol affects chronic diseases and longevity through anti-carcinogenic, anti-inflammatory, and antioxidant properties [46]. Resveratrol dose-dependently increased expression of Hsp70 and SIRT-1 in human neuroblastoma SH-SY5Y

cells induced by neurotoxicity with high-dose homocysteine [47]. It has been reported that resveratrol induced Hsp25 and Hsp70 proteins in G93A-SOD1 mutant mice cells and can prevent motor neuron losses [48]. Resveratrol dose-dependently increased glutathione expression through the Nrf2 pathway in normal human keratocytes [49]. In the human neuroblastoma cell line SH-SY5Y, resveratrol dose-dependently increased HO-1 expression and HO-1-dependent autophagic flux and prevented rotatone-induced apoptosis [50]. It has been determined that resveratrol dose-dependently reduced the vascular endothelial growth factor (VEGF), leptin, interleukin (IL)-6, and IL-8 expression in hypoxia-induced human adipocytes and prevented adipokine-induced inflammation and angiogenesis [51]. In addition, resveratrol induced autophagy by directly inhibiting mTOR in HeLa cells [52]. Prostate cancer cells induced autophagy through inhibition of the Akt/mTOR pathway in PC3 and DU145 cells [53]. In murine RAW 264.7 macrophages and microglial BV-2 cells, resveratrol also inhibited microglial activation by suppressing the NF-κB pathway [54]. In another study, resveratrol showed anti-inflammatory effect by suppressing the NF-κB pathway in RAW 264.7 murine macrophages in a dose-dependent manner [55]. These studies suggest that resveratrol has anti-inflammatory, antioxidant, anti-carcinogenic effects and can strengthen hemodynamic structure, which in turn can positively affect the aging process and longevity.

2.4. Epigallocatechin gallate

The major catechin EGCG, which is found in green tea at a level of 48–55%, has protective effects against chronic diseases such as neurodegenerative diseases, metabolic syndrome, and cancer by its anti-inflammatory and antioxidant effects [56, 57]. EGCG, with Hsp90 inhibition, showed a protective effect against cancer in a novel human prostate cancer progression model [58]. In primary vascular endothelial cells, GST and NQO1 enzymes were increased dose-dependently by Nrf2 [59]. In another study, EGCG increased the level of HO-1 expression by Nrf-2 activation in endothelial cells, resulting in the passage of caveolin-1 from the plasma membrane to the cytosol, accumulating in the caveolae-regulating signaling pathways associated with vascular disease pathology [60]. Accordingly, EGCG may reduce endothelial inflammation and protect against atherosclerosis [61]. EGCG also showed a protective effect against oxidative stress-induced cerebral ischemia through Nrf2/ARE activation [62]. EGCG suppressed the Nrf-2 pathway in a lethal dose with biphasic dose-response effect in mice hepatocytes [63]. EGCG has been shown to inhibit oxidative stress damage induced by HO-1 through Nrf2 in HUVECs with ambient fine particulate matter (≤2.5 μm in aerodynamic diameter PM2.5) [64]. EGCG dose-dependently suppresses endothelial inflammation through NF-κB inhibition in high glucose-induced HUVECs [65]. It can also suppress NF-κB activation in cardiac fibroblasts and can show a protective effect against cardiac fibrosis [66]. EGCG inhibited lipopolysaccharide-induced inflammation with NF-κB suppression in bone marrow-derived macrophages (BMMs) isolated from ICR mice [67]. EGCG also showed a protective effect against human papillomavirus-16 oncoprotein-induced lung cancer and IGF-1 stimulated lung cancer angiogenesis through HIF-1α inhibition [68, 69]. In addition, primary bovine aortic endothelial cells stimulate autophagy in cells, leading to degradation of lipid droplets. In this way, EGCG may be effective in the prevention of cardiovascular diseases [70]. EGCG regulates ultraviolet B (UVB)-mediated autophagy through the mTOR signaling pathway

and significantly alleviates the toxic effects of UVB irradiation in macular retinal pigment epithelial cells. Thus, it may also have a protective effect against macular degeneration [71]. As a result, EGCG can be effective in the prevention of neurodegeneration, cancer, cardiovascular diseases, inflammatory diseases, and macular degeneration through stress pathways.

2.5. Luteolin

Luteolin (3′,4′,5,7-tetrahydroxy flavone) is a phenolic compound found in broccoli, pepper, thyme, celery, lettuce, oregano, artichoke, and carrots; it has antioxidant, anticancer, anti-inflammatory, and neuroprotective effects [72]. Luteolin destabilized the Hsp90 client protein c-Jun and Akt and inhibited LPS-induced production of TNF-α and NO dose-dependently in macrophages [73]. In addition, luteolin prevented TNF-α-induced endolytic monocyte adhesion in mice by suppressing vascular inflammation and the IKBα/NF-κB pathway in HUVECs [74]. In psoriatic skin, luteolin inhibited keratinocyte activation by decreasing NF-κB, which increased dose-dependently [75]. Luteolin and luteolin-7-*O*-glucoside modulated Nrf2/mitogen-activated protein kinase (MAPK) mediated the HO-1 signaling cascade in RAW 264.7 cells [76]. In wild-type mouse traumatic brain injury models, luteolin showed neuroprotective action by Nrf2/ARE pathway activation [77]. Luteolin inhibited tBHP-induced oxidative stress by increasing ERK2/Nrf2/ARE signaling pathway activation and HO-1, glutamate cysteine ligase catalytic (GCLC), and glutamate cysteine ligase modifier (GCLM) subunit transcription in rat primary hepatocytes [78]. In addition, in HepG2, Hepa1c1c7, and RL-34 HepG2 hepatocytes, it dose-dependently inhibited the expression of phase I enzyme cytochrome P450 1A1 (CYP1A1), and phase II enzymes NQO1 and GST-P1 through an aryl hydrocarbon receptor (AhR) and Nrf2 pathways [79]. In HepG2 human hepatocytes, luteolin also dose-dependently activated the PI3K/Nrf2/ARE system, increased HO-1 expression, and reduced the expression of lipopolysaccharide-induced NO, iNOS, and cytosolic phospholipase A2 (cPLA2) in hepatocytes [80]. Luteolin also reduced acute mercuric chloride-induced hepatotoxicity by anti-inflammatory and antioxidant responses by regulating the SIRT1/Nrf2/TNF-α pathways [81]. The induction of VEGF by oxidative stress has an important role in the pathogenesis of premature retinopathy. Luteolin has shown a protective effect against retinal neovascularization by reducing hypoxia-induced VEGF expression through decreasing HIF-1α expression in human retinal microvascular endothelial cells (HRMECs) [82]. Luteolin reduced 4-hydroxy-2-nonenal-induced cell death of neuronal-like catecholaminergic PC12 cells by regulating unfolded protein response and the MAPK, Nrf2/ARE pathways [83]. As a result, luteolin also affects neurodegeneration, endothelial function, and liver function through stress-response pathways as do other hormetic phytochemicals.

2.6. Quercetin

Quercetin (3,3′,4′,5,7-pentahydroxyflavone) is found in many vegetables and fruits. It has anti-inflammatory, anticarcinogenic, and antioxidant effects on cardiovascular diseases, cancer, neurodegenerative diseases, and can reduce aging and positively increase the life span [84]. Quercetin inhibited the growth of A549 and H460 cancer cells with Hsp70 inhibition in lung cancer cells and increased sensitivity to chemotherapy [85]. Quercetin inhibited the t-AUCB-induced autophagy by inhibiting Hsp 27 and Atg 72 in glioblastoma cells [86]. In addition,

quercetin inhibited Hsp70 in U937 human monoblastic leukemia cell line [87]. Quercetin inhibited hypoxia-induced AMPK by dramatically inducing apoptosis in hypoxia and reducing the activity of HIF-1 in HCT116 cancer cells [88]. Quercetin dose-dependently increased glutathione, glutamylcysteine synthetase (GSH), GPx, GR, and GST expression in liver HepG2 cells through p38/MAPK and Nrf-2 activation [89]. Quercetin protected against toxicity and inflammation by increasing Nrf-2 expression and decreasing NF-kB and cyclooxygenase (Cox)-2 expression in a time-dependent manner in mycotoxin ochratoxin A-induced liver HepG2 cells [90]. Furthermore, dose-dependently, through p62 and Nrf2-ARE activation, quercetin increased HO-1, GCLC, and GCLM subunit expression and showed a protective effect against hepatotoxicity [91]. Quercetin, depending on the dose, inhibited the production of LPS-induced NO production in BV2 microglial cells, suppressed the NF-κB pathway, and activated the Nrf2-dependent HO-1 pathway [92, 93]. Quercetin showed a protective effect against indomethacin-induced gastrointestinal oxidative stress and inflammation through Nrf-2 activation and NF-kB inhibition in human intestinal Caco-2 cells [94]. In malignant mesothelioma MSTO-211H and H2452 cells, quercetin also inhibited cell growth and showed cytoprotective effect with Nrf-2 activation [95]. In a study on porcine renal proximal tubule cell line LLC-PK1 cells and C57BL/6j mice, quercetin inhibited renal ischemia/reperfusion injury by increasing AMP phosphorylase, inhibiting mTOR phosphorylation, and activating autophagy [96]. A combination of quercetin, resveratrol, and catechin was administered to human metastatic cancer cell lines MDA-MB-231 and MDA-MB-435; quercetin was shown to be the most effective compound for Akt/mTOR inhibition and can prevent breast cancer growth and metastasis [97]. Quercetin inhibited mTOR by expressing SESTIN 2, p53, and activating AMPK in a dose-dependent manner and induced apoptosis via increased intracellular ROS in HCT116 colon cancer cells [98]. The mTOR complex has an important role in cell growth, protein synthesis, and autophagy, with the inhibition of quercetin mTOR/PI3K/Akt in cancer and other diseases where excessive mTOR complex activity is observed [99]. In addition, quercetin, by affecting autophagy with the inhibition of proteasome and mTOR activity, can be both protective and therapeutic against cancer with the death of human breast cancer cell lines MCF7 and MDA-MB-453, the cervical adenocarcinoma cell line HeLa, the ovarian cancer cell line OVCAR3, and the human B-lymphoblastoid cell line IM-9 [100]. Quercetin inhibited tumor growth and angiogenesis by inhibiting VEGF regulated by AKT/mTOR in HUVECs [101]. As a result, quercetin may exert a protective effect against cancer, especially by acting on stress-response pathways.

2.7. Sulforaphane

Sulforaphane (SulR-1-isothiocyanato-4-methylsulfinyl butane) is an isothiocyanate found extensively in cruciferous vegetables. Studies have shown that sulforaphane has a protective effect against cancer, diabetes, cardiovascular diseases, neurodegenerative diseases, and kidney diseases, and is mostly influenced by an Nrf-2-mediated antioxidant response [102, 103]. Sulforaphane may prevent diabetic auric damage and cardiomyopathy by increasing Nrf2 activation in mice [104, 105]. Sulforaphane showed protective effect against ethanol-induced oxidative stresses and apoptosis in neural crest cells by generating an antioxidant response with Nrf2 activation [106]. Sulforaphane activates the Nrf2/ARE pathway and inhibits 3-nitropropionic acid-induced toxicity in striatal cells by inhibiting MAPKs and NF-κB pathways [107]. In MSTO-211H

cells administered with sulforaphane, Nrf2-mediated HO-1 expression was regulated by the PI3K/Akt pathway [108]. Sulforaphane inhibited muscle inflammation by inhibiting Nrf-2 and NF-kB in dystrophin-deficient mdx mice [109]. Sulforaphane showed a protective effect against acute alcohol-induced liver steatosis by activation of Nrf2 and synthesis of antioxidant proteins in HepG2 E47 liver cells [110]. Sulforaphane increased Nrf2 expression in TRAMP C1 prostate cancer cells and affected epigenetic regulation [111]. Sulforaphane induced autophagy through ERK activation in immortalized mouse CN1.4 cortical and human SHSY5Y neuronal cells [112]. Huntington's disease, a neurodegenerative disease, involves damage to the ubiquitin proteasome system. In a mouse study, sulfate inhibited proteasomal and autophagic activation and cytotoxicity resulting from proteasomal impairment [113]. Sulforaphane inhibited HIF-1α expression in HCT116 human colon cancer cells and AGS human gastric cancer cells, but inhibited hypoxia-induced VEGF expression only in HCT116 cells [114]. Sulforaphane affects the stress-response pathways and can show protective effects, especially against neurodegeneration and cancer.

3. Conclusion

Dietary phytochemicals can exert a protective effect against cancer, neurodegenerative diseases, cardiovascular diseases, inflammatory and immune diseases by acting on multiple stress-response pathways. Therefore, healthy aging and longevity can be achieved by preventing the deterioration of hemodynamics. In addition, it is necessary to emphasize that the hormetic stress pathways of each dietary phytochemical is a very wide ranging subject. Therefore, the mechanisms of action of important phytochemicals and stress response pathways in this chapter have been summarized in the light of data obtained in recent years; this may lead to a broader outlook on this subject and to new studies.

Abbreviations

AhR:	aryl hydrocarbon receptor
ARE:	antioxidant response element
BMMs:	bone marrow-derived macrophages
Cox-2:	cyclooxygenase-2
cPLA2:	cytosolic phospholipase A2
DNA:	deoxyribonucleic acid
EGCG:	epigallocatechin gallate
ERK:	extracellular signal-regulated kinase
GCLC:	glutamate cysteine ligase catalytic
GCLM:	glutamate cysteine ligase modifier

GPx: glutathione peroxidase

GST: glutathione-S-transferase

HDAC: histone deacetylase

HO-1: hemeoxygenase-1

HRMECs: human retinal microvascular endothelial cells

HSP: heat shock protein

HIF-1: hypoxia-inducible factor-1

HUVECs: human umbilical vein endothelial

IGF-1: insulin-like growth factor

iNOS: inducible nitric oxide synthase

JKN: c-Jun N-terminal kinase

LPS: lipopolysaccharide

MAPK: mitogen-activated protein kinase

mTOR: mammalian target of rapamycin

NFκB: nuclear factor kappa B

NO: nitric oxide

Nrf2: nuclear factor-erythroid 2-related factor 2

NQO1: NAD(P)H:quinine oxidoreductase

PBMCs: human peripheral blood mononuclear cells

PKC: protein kinase C

SOD: superoxide dismutase

TNF-α: tumor necrosis factor-α

UVB: ultraviolet B

VEGF: vascular endothelial growth factor

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References

[1] Mattson MP. Hormesis defined. Ageing Research Reviews. 2008;**7**(1):1-7. DOI: 10.1016/j.arr.2007.08.007

[2] Mattson MP, Calabrese EJ. Hormesis: What it is and why it matters? In: Mattson MP, Calabrese EJ, editors. Hormesis a Revolution in Biology, Toxicology and Medicine. 1st ed. New York: Humana Press; 2010. pp. 1-13. DOI: 10.1007/978-1-60761-495-1_1

[3] Rattan SIS. Biogerontology: From here to where? The Lord Cohen medal Lecture-2011. Biogerontology. 2012;**13**(1):83-91. DOI: 10.1007/s10522-011-9354-3

[4] Rattan SIS. Rationale and methods of discovering hormetins as drugs for healthy ageing. Expert Opinion on Drug Discovery. 2012;**7**(5):439-448. DOI: 10.1517/17460441.2012.677430

[5] Rattan SIS. Hormesis in ageing. Ageing Research Reviews. 2008;**7**(1):63-78. DOI: 10.1016/j.arr.2007.03.002

[6] Son TG, Camandola S, Mattson MP. Hormetic dietary phytochemicals. NeuroMolecular Medicine. 2008;**10**(4):236-246. DOI: 10.1007/s12017-008-8037-y

[7] Demirovic D, Rattan SIS. Establishing cellular stress response profiles as biomarkers of homeodynamics, health and hormesis. Experimental Gerontology. 2013;**48**(1):98-98. DOI: 10.1016/j.exger.2012.02.005

[8] Somani SJ, Modi KP, Majumdar AS, Sadarani BN. Phytochemicals and their potential usefulness in inflammatory bowel disease. Phytotherapy Research. 2015;**29**(3):339-350. DOI: 10.1002/ptr.5271

[9] Doughari JH. Phytochemicals: Extraction methods, basic structures and mode of action as potential chemotherapeutic agents, phytochemicals. In: Rao V, editor. A Global Perspective of Their Role in Nutrition and Health. 1st ed. Rijeka: InTech; 2012. pp. 1-32. DOI: 10.5772/26052

[10] Hayes DP. Nutritional hormesis. European Journal of Clinical Nutrition. 2007;**61**:147-159. DOI: 10.1038/sj.ejcn.1602507

[11] Mattson MP. Dietary factors, hormesis and health. Ageing Research Reviews. 2008;**7**(1):43-48. DOI: 10.1016/j.arr.2007.08.004

[12] Lee J, Jo DG, Park D, Chung HY, Mattson MP. Adaptive cellular stress pathways as therapeutic targets of dietary phytochemicals: Focus on the nervous system. Pharmacological Reviews. 2014;**66**(3):815-868. DOI: 10.1124/pr.113.007757

[13] Demirovic D, Rattan SIS. Curcumin induces stress response and hormetically modulates wound healing ability of human skin fibroblasts undergoing ageing in vitro. Biogerontology. 2011;**12**(5):437-444. DOI: 10.1007/s10522-011-9326-7

[14] Evans CG, Chang L, Gestwicki JE. Heat shock protein 70 (Hsp70) as an emerging drug target. Journal of Medicinal Chemistry. 2010;**53**:4585-4602. DOI: 10.1021/jm100054f

[15] Gupta SC, Sharma A, Mishra M, Mishra MK, Chowdhuri DK. Heat shock proteins in toxicology: How close and how far? Life Sciences. 2010;**86**:377-384. DOI: 10.1016/j.lfs.2009.12.015

[16] Hong DS, Banerji U, Tavana B, George GC, Aaron J, Kurzrock R. Targeting the molecular chaperone heat shock protein 90 (HSP90): Lessons learned and future directions. Cancer Treatment Reviews. 2013;**39**:375-387. DOI: 10.1016/j.ctrv.2012.10.001

[17] Mymrikov EV, Seit-Nebi AS, Gusev NB. Large potentials of small heat shock proteins. Physiological Reviews. 2011;**91**:1123-1159. DOI: 10.1152/physrev.00023.2010

[18] Jego G, Hazoume A, Seigneuric R, Garrido C. Targeting heat shock proteins in cancer. Cancer Letters. 2013;**332**(2):275-285. DOI: 10.1016/j.canlet.2010.10.014

[19] Hine CM, Mitchell JR. NRF2 and the phase II response in acute stress resistance induced by dietary restriction. Journal of Clinical and Experimental Pathology. 2012;**Suppl 4**(4):7329-7362. DOI: 10.4172/2161-0681.S4-004

[20] Jung KA, Kwak MK. The Nrf2 system as a potential target for the development of indirect antioxidants. Molecules. 2010;**15**(10):7266-7291. DOI: 10.3390/molecules15107266

[21] Kaspar JW, Niture SK, Jaiswal AK. Nrf2:INrf2(Keap1) signaling in oxidative stress. Free Radical Biology & Medicine. 2009;**47**(9):1304-1309. DOI: 10.1016/j.freeradbiomed.2009.07.035

[22] Zhang DD. Mechanistic studies of the Nrf2-Keap1 signaling pathway. Drug Metabolism Reviews. 2006;**38**(4):769-789. DOI: 10.1080/03602530600971974

[23] Birringer M. Hormetics: Dietary triggers of an adaptive stress response. Pharmaceutical Research. 2011;**28**:2680-2694. DOI: 10.1007/s11095-011-0551-1

[24] Kumar H, Kim IS, More SV, Kim BW, Choi DK. Natural product-derived pharmacological modulators of Nrf2/ARE pathway for chronic diseases. Natural Prodcut Reports. 2014;**31**(1):109-139. DOI: 10.1039/c3np70065h

[25] Madeo F, Tavernarakis N, Kroemer G. Can autophagy promote longevity? Nature Cell Biology. 2010;**12**(9):842-846. DOI: 10.1038/ncb0910-842

[26] Xia Y, Choi HK, Lee K. Recent advances in hypoxia-inducible factor (HIF)-1 inhibitors. European Journal of Medicinal Chemistry. 2012;**49**:24-40. DOI: 10.1016/j.ejmech.2012.01.033

[27] Barone E, Calabrese V, Mancuso C. Ferulic acid and its therapeutic potential as a hormetin for age related diseases. Biogerontology. 2009;**10**:97-108. DOI: 10.1007/s10522-008-9160-8

[28] Mancuso C, Santangelo R. Ferulic acid: Pharmacological and toxicological aspects. Food and Chemical Toxicology. 2014;**65**:185-195. DOI: 10.1016/j.fct.2013.12.024

[29] Ghosh S, Basak P, Dutta S, Chowdhury S, Sil PC. New insights into the ameliorative effects of ferulic acid in pathophysiological conditions. Food and Chemical Toxicology. 2017;**103**:41-55. DOI: 10.1016/j.fct.2017.02.028

[30] He S, Liu F, Xu L, Yin P, Li D, Mei C, Jiang L, Ma Y, Xu J. Protective effects of ferulic acid against heat stress-induced intestinal epithelial barrier dysfunction in vitro and in vivo. PLoS One. 2016;**11**(2):e0145236. DOI: 10.1371/journal.pone.0145236

[31] Catino S, Paciello F, Micell F, Rolesi R, Troiani D, Calabrese V, Santangelo R, Mancuso C. Ferulic acid regulates the Nrf2/Heme oxygenase-1 system and counteracts trimethyltin-induced neuronal damage in the human neuroblastoma cell line SH-SY5Y. Frontiers in Pharmacology. 2016;**6**(305):1-12. DOI: 10.3389/fphar.2015.00305

[32] Yu CL, Zhao XM, Niu YC. Ferulic acid protects against lead acetate-induced inhibition of neurite outgrowth by upregulating HO-1 in PC12 cells: Involvement of ERK1/2-Nrf2 pathway. Molecular Neurobiology. 2016;**53**(9):6589-6500. DOI: 10.1007/s12035-015-9555-x

[33] Kikugawa M, Ida T, Ihara H, Sakamoto T. Ferulic acid and its water-soluble derivatives inhibit nitric oxide production and inducible nitric oxide synthase expression in rat primary astrocytes. Bioscience, Biotechnology, and Biochemistry. 2017;**81**(8):1607-1611. DOI: 10.1080/09168451.2017.1336925

[34] Zhao J, Suyama A, Chung H, Fukuda T, Tanaka M, Matsui T. Ferulic acid enhances nitric oxide production through up-regulation of argininosuccinate synthase in inflammatory human endothelial cells. Life Sciences. 2016;**145**:224-232. DOI: 10.1016/j.lfs.2015.12.044

[35] Liao Z, He H, Zeng G, Liu D, Tang L, Yin D, Chen D, He M. Delayed protection of ferulic acid in isolated hearts and cardiomyocytes: Upregulation of heat-shock protein 70 via NO-ERK1/2 pathway. Journal of Functional Foods. 2017;**34**:18-27. DOI: 10.1016/j.jff.2017.04.012

[36] Bian Z, Furuya N, Zheng DM, Trejo JAO, Tada N, Ezaki J, Ueno T. Ferulic acid induces mammalian target of rapamycin inactivation in cultured mammalian cells. Biological & Pharmaceutical Bulletin. 2013;**36**(1):120-124. DOI: 10.1248/bpb.b12-00695

[37] Pulido-Moran M, Moreno-Fernandez J, Ramirez-Tortosa C, Ramires-Tortosa MC. Curcumin and health. Molecules. 2016;**21**(3):264-286. DOI: 10.3390/molecules21030264

[38] Salem M, Rohani S, Gillies ER. Curcumin, a promising anti-cancer therapeutic: A review of its chemical properties, bioactivity and approaches to cancer cell delivery. RSC Advances. 2014;**4**:10815-10829. DOI: 10.1039/c3ra46396f

[39] Sarkar R, Mukherjee S, Biswas J. Curcumin augments the efficiency of antitumor drugs used in leukemia by modulation of heat shock proteins via HDAC6. Journal of Enviromental Patology, Toxicology and Oncology. 2014;**33**(3):247-263. DOI: 10.1615/JEnvironPatholToxicolOncol.2014010913

[40] Xia C, Cai Y, Li S, Yang J, Xiao G. Curcumin increases HSP70 expression in primary rat cortical neuronal apoptosis induced by gp120 V3 loop peptide. Neurochemical Research. 2015;**40**:1996-2005. DOI: 10.1007/s11064-015-1695-x

[41] Ahmed T, Banerjee BD. HSP27 modulates survival signaling in endosulfan-exposed human peripheral blood mononuclear cells treated with curcumin. Human & Experimental Toxicology. 2016;**35**(7):695-704. DOI: 10.1177/0960327115597986

[42] Gounden S, Chuturgoon A. Curcumin upregulates antioxidant defense, lon protease, and heat-shock protein 70 under hyperglycemic conditions in human hepatoma cells. Journal of Medicinal Food. 2017;**20**(5):465-473. DOI: 10.1089/jmf.2016.0146

[43] González-Reyes S, Guzmán-Beltrán S, Medina-Campos ON, Pedraza-Chaverri J. Curcumin pretreatment induces Nrf2 and an antioxidant response and prevents hemin-induced toxicity in primary cultures of cerebellar granule neurons of rats. Oxidative Medicine and Cellular Longevity. 2013;**2013**:801418. DOI: 10.1155/2013/801418

[44] Das V, Vinayak M. Long term effect of curcumin in restoration of tumour suppressor p53 and phase-II antioxidant enzymes via activation of Nrf2 signalling and modulation of inflammation in prevention of cancer. PLoS One. 2015;**10**(4):e0124000. DOI: 10.1371/journal.pone.0124000

[45] Ding F, Li F, Li Y, Hou X, Ma Y, Zhang N, Ma J, Zhang R, Lang B, Wang H, Wang Y. HSP60 mediates the neuroprotective effects of curcumin by suppressing microglial activation. Experimental and Therapeutic Medicine. 2016;**12**:823-828. DOI: 10.3892/etm.2016.3413

[46] Smoliga JM, Baur JA, Hausenblas HA. Resveratrol and health—A comprehensive review of human clinical trials. Molecular Nutrition & Food Research. 2011;**55**:1129-1141. DOI: 10.1002/mnfr.201100143

[47] Curro M, Trovato-Salinaro A, Gugliandolo A, Koverech G, Lodato F, Caccamo D, Calabrese V, Ientile R. Resveratrol protects against homocysteine-induced cell damage via cell stress response in neuroblastoma cells. Journal of Neuroscience Research. 2015;**93**:149-156. DOI: 10.1002/jnr.23453

[48] Han S, Choi JR, Shin KS, Kang SJ. Resveratrol upregulated heat shock proteins and extended the surviva lof G93A-SOD1 mice. Brain Research. 2012;**1483**:112-117. DOI: 10.1016/j.brainres.2012.09.022

[49] Soueur J, Eilstein J, Léreaux G, Jones C, Marrot L. Skin resistance to oxidative stress induced by resveratrol: From Nrf2 activation to GSH biosynthesis. Free Radical Biology & Medicine. 2015;**78**:213-223. DOI: 10.1016/j.freeradbiomed.2014.10.510

[50] Lin TK, Chen SD, Chuang YC, Lin HY, Huang CR, Chuang JH, Wang PW, Huang ST, Tiao MM, Chen JB, Liou CW. Resveratrol partially prevents rotenone-induced neurotoxicity in dopaminergic SH-SY5Y cells through induction of heme oxygenase-1 dependent autophagy. International Journal of Molecular Sciences. 2014;**15**:1625-1646. DOI: 10.3390/ijms15011625

[51] Cullberg KB, Olholm J, Paulsen SK, Foldager CB, Lind M, Richelsen B, Pedersen SB. Resveratrol has inhibitory effects on the hypoxia-induced inflammation and angiogenesis in human adipose tissue in vitro. European Journal of Pharmaceutical Sciences. 2013;**49**:251-257. DOI: 10.1016/j.ejps.2013.02.014

[52] Park D, Jeong H, Lee NM, Koh A, Kwon O, Yang YR, Noh J, Suh PG, Park H, Ryu SH. Resveratrol induces autophagy by directly inhibiting mTOR through ATP competition. Scientific Reports. 2016;**6**:21772. DOI: 10.1038/srep21772

[53] Selvaraj S, Sun Y, Sukumaran P, Singh BB. Resveratrol activates autophagic cell death in prostate cancer cells via downregulation of STIM1 and the mTOR pathway. Molecular Carcinogenesis. 2016;**55**:818-831. DOI: 10.1002/mc.22324

[54] Capiralla H, Vingtdeux V, Zhao H, Sankowski R, Al-Abed Y, Davies P, Marambaud P. Resveratrol mitigates lipopolysaccharide- and Aβ-mediated microglial inflammation by inhibiting the TLR4/NF-κB/STAT signaling cascade. Journal of Neurochemistry. 2012;**120**:461-472. DOI: 10.1111/j.1471-4159.2011.07594.x

[55] Ma C, Wang Y, Dong L, Li M, Cai W. Anti-inflammatory effect of resveratrol through the suppression of NF-κB and JAK/STAT signaling pathways. Acta Biochimica et Biophysica Sinica. 2015;**47**(3):207-213. DOI: 10.1093/abbs/gmu135

[56] Braicu C, Ladomery MR, Chedea VS, Irimie A, Berindan-Neagoe I. The relationship between the structure and biological actions of green tea catechins. Food Chemistry. 2013;**141**(3):3282-3289. DOI: 10.1016/j.foodchem.2013.05.122

[57] Legeay S, Rodier M, Fillon L, Faure S, Clere N. Epigallocatechin gallate: A review of its beneficial properties to prevent metabolic syndrome. Nutrients. 2015;**7**:5443-5468. DOI: 10.3390/nu7075230

[58] Moses MA, Henry EC, Ricke WA, Gasiewicz TA. The heat shock protein 90 inhibitor, (−)-epigallocatechin gallate, has anticancer activity in a novel human prostate cancer progression model. Cancer Prevention Research. 2015;**8**(3):249-257. DOI: 10.1158/1940-6207.CAPR-14-0224

[59] Han SG, Han SS, Toborek M, Hennig B. EGCG protects endothelial cells against PCB 126-induced inflammation through inhibition of AhR and induction of Nrf2-regulated genes. Toxicology and Applied Pharmacology. 2012;**261**:181-188. DOI: 10.1016/j.taap.2012.03.024

[60] Pullikotil B, Chen H, Muniyappa R, Greenberg CC, Yang S, Reiter CEN, Lee JW, Chung JH, Quon MJ. Epigallocatechin gallate induces expression of heme oxygenase-1 in endothelial cells via p38 MAPK and Nrf-2 that suppresses pro-inflammatory actions of TNF-α. The Journal of Nutritional Biochemistry. 2012;**23**(9):1134-1145. DOI: 10.1016/j.jnutbio.2011.06.007

[61] Zheng Y, Morris A, Sunkara M, Layne J, Toborek M, Hennig B. Epigallocatechin gallate stimulates NF-E2-related factor and heme oxygenase-1 via calveolin-1 displacement. The Journal of Nutritional Biochemistry. 2012;**23**:163-168. DOI: 10.1016/j.jnutbio.2010.12.002

[62] Han J, Wang M, Jing X, Shi H, Ren M, Lou H. (−)-Epigallocatechin gallate protects against cerebral ischemia-induced oxidative stress via Nrf2/ARE signaling. Neurochemical Research. 2014;**39**(7):1292-1299. DOI: 10.1007/s11064-014-1311-5

[63] Wang D, Wang Y, Wan X, Yang CS, Zhang J. Green tea polyphenol (−)-epigallocatechin-3-gallate triggered hepatotoxicity inmice: Responses ofmajor antioxidant enzymes and the Nrf2 rescue pathway. Toxicology and Applied Pharmacology. 2015;**283**:65-74. DOI: 10.1016/j.taap.2014.12.018

[64] Yang GZ, Wang ZJ, Bai F, Qin XJ, Cao J, Lv JY, Zhang MS. Epigallocatechin-3-gallate protects HUVECs from PM2.5-induced oxidative stress injury by activating critical antioxidant pathways. Molecules. 2015;**20**:6626-6639. DOI: 10.3390/molecules20046626

[65] Yang J, Han Y, Chen C, Sun H, He D, Guo J, Jiang B, Zhou L, Zeng C. EGCG attenuates high glucose-induced endothelial cell inflammation by suppression of PKC and NF-κB signaling in human umbilical vein endothelial cells. Life Sciences. 2013;**92**:589-597. DOI: 10.1016/j.lfs.2013.01.025

[66] Cai Y, Yu SS, Chen TT, Gao S, Geng B, Yu Y, Ye JT, Liu PQ. EGCG inhibits CTGF expression via blocking NF-κB activation in cardiac fibroblast. Phytomedicine. 2013;**20**:106-113. DOI: 10.1016/j.phymed.2012.10.002

[67] Joo SY, Song Y, Park YL, Myung E, Chung CY, Park KJ, Cho SB, Lee WS, Kim HS, Rew JS, Kim NS, Joo YE. Epigallocatechin-3-gallate inhibits LPS-induced NF-κB and MAPK signaling pathways in bone marrow-derived macrophages. Gut and Liver. 2012;**6**(2):188-196. DOI: 10.5009/gnl.2012.6.2.188

[68] He L, Zhang E, Shi J, Li X, Zhou K, Zhang Q, Lee AD, Tang X. (2)-Epigallocatechin-3-gallate inhibits human papillomavirus (HPV)-16 oncoprotein-induced angiogenesis in non-small cell lung cancer cells by targeting HIF-1a. Cancer Chemotherapy and Pharmacology. 2013;**71**:713-725. DOI: 10.1007/s00280-012-2063-z

[69] Li X, Feng Y, Liu J, Feng X, Zhou K, Tang X. Epigallocatechin-3-gallate inhibits IGF-1-stimulated lung cancer angiogenesis through downregulation of HIF-1α and VEGF expression. Journal of Nutrigenetica and Nutrigenomics. 2013;**6**:169-178. DOI: 10.1159/000354402

[70] Kim HS, Montana V, Jang HJ, Parpura V, Kim J. Epigallocatechin gallate (EGCG) stimulates autophagy in vascular endothelial cells. The Journal of Biological Chemistry. 2013;**288**(31):22693-22705. DOI: 10.1074/jbc.M113.477505

[71] Li CP, Yao J, Tao ZF, Li XM, Jiang Q, Yan B. Epigallocatechin gallate (EGCG) regulates autophagy in human retinal pigment epithelial cells: A potential role for reducing UVB light-induced retinal damage. Biochemical and Biophysical Research Communications. 2013;**438**:739-745. DOI: 10.1016/j.bbrc.2013.07.097

[72] Nabavi SF, Braidy N, Gortzi O, Sobarzo-Sanchez E, Daglia M, Skalicka-Wozniak K, Nabavi SM. Luteolin as an anti-inflammatory and neuroprotective agent: A brief review. Brain Research Bulletin. 2015;**119**(Pt A):1-11. DOI: 10.1016/j.brainresbull.2015.09.002

[73] Chen D, Bi A, Dong X, Jiang Y, Rui B, Liı J, Yin Z, Luo L. Luteolin exhibits anti-inflammatory effects by blocking the activity of heat shock protein 90 in macrophages. Biochemical and Biophysical Research Communications. 2014;**443**:326-332. DOI: 10.1016/j.bbrc.2013.11.122

[74] Jia Z, Nallasamy P, Liu D, Shah H, Li JZ, Rojin C, Si H, McCormickJ ZH, Zhen W, Li Y. Luteolin protects against vascular inflammation in mice and TNF-alpha-induced monocyte

adhesion to endothelial cells via suppressing IKBα/NF-κB signaling pathway. Journal of Nutritional Biohemistry. 2015;**26**(3):293-302. DOI: 10.1016/j.jnutbio.2014.11.008

[75] Weng Z, Patel AB, Vasiadi M, Therianou A, Theoharides TC. Luteolin inhibits human keratinocyte activation and decreases NF-kB induction that is increased in psoriatic skin. PLoS One. 2014;**9**(2):e90739. DOI: 10.1371/journal.pone.0090739

[76] Song YS, Park CM. Luteolin and luteolin-7-O-glucoside strengthen antioxidative. Food and Chemical Toxicology. 2013;**65**:70-75. DOI: 10.1016/j.fct.2013.12.017

[77] Xu J, Wang H, Ding K, Zhang L, Wang C, Li T, Wei W, Lu X. Luteolin provides neuroprotection in models of traumatic brain injury via the Nrf2–ARE pathway. Free Radical Biology & Medicine. 2014;**71**:186-195. DOI: 10.1016/j.freeradbiomed.2014.03.009

[78] Huang CS, Lii CK, Lin AH, Yeh YW, Yao HT, Li CC, Wang TS, Chen HW. Protection by chrysin, apigenin, and luteolin against oxidative stress is mediated by the Nrf2-dependent up-regulation of heme oxygenase 1 and glutamate cysteine ligase in rat primary hepatocytes. Archives of Toxicology. 2013;**87**:167-178. DOI: 10.1007/s00204-012-0913-4

[79] Zhang T, Kimura Y, Jiang S, Harada K, Yamashita Y, Ashida H. Luteolin modulates expression of drug-metabolizing enzymes through the AhR and Nrf2 pathways in hepatic cells. Archives of Biochemistry and Biophysics. 2014;**557**:36-46. DOI: 10.1016/j.abb.2014.05.023

[80] Paredes-Gonzalez X, Fuentes F, Jeffery S, Saw CLL, Shu L, ZY S, Kong ANT. Induction of NRF2-mediated gene expression by dietary phytochemical flavones apigenin and luteolin. Biopharmaceutics & Drug Disposition. 2015;**36**:440-451. DOI: 10.1002/bdd.1956

[81] Yang D, Tan X, Lv Z, Liu B, Baiyun B, Lu J, Zhang Z. Regulation of SIRT1/Nrf2/TNF-α signaling pathway by luteolin is critical to attenuate acute mercuric chloride exposure induced hepatotoxicity. Scientific Reports. 2016;**6**:37157. DOI: 10.1038/srep37157

[82] Park SW, Cho CS, Jun HO, Ryu NH, Kim JH, YS Y, Kim JS, Kim JH. Anti-angiogenic effect of luteolin on retinal neovascularization via blockade of reactive oxygen species production. Investigative Ophthalmology & Visual Science. 2012;**53**:7718-7726. DOI: 10.1167/iovs.11-8790

[83] Wu PS, Yen JH, Kou MC, Wu MJ. Luteolin and apigenin attenuate 4-hydroxy-2-nonenal-mediated cell death through modulation of UPR, Nrf2-ARE and MAPK pathways in PC12 cells. PLoS One. 2015;**10**(6):e0130599. DOI: 10.1371/journal.pone.0130599

[84] Russo M, Spagnuolo C, Tedesco I, Bilotto S, Russo GL. The flavonoid quercetin in disease prevention and therapy: Facts and fancies. Biochemical Pharmacology. 2012;**83**:6-15. DOI: 10.1016/j.bcp.2011.08.010

[85] Lee SH, Lee EJ, Min KH, Hur GY, Lee SH, Lee SY, Kim JH, Shin C, Shim JJ, In KH, Kang KH, Lee SY. Quercetin enhances chemosensitivity to gemcitabine in lung cancer cells by inhibiting heat shock protein 70 expression. Clinical Lung Cancer. 2015;**16**(6):e235-e243. DOI: 10.1016/j.cllc.2015.05.006

[86] Li J, Tang C, Li L, Li R, Fan Y. Quercetin blocks t-AUCB-induced autophagy by Hsp27 and Atg7 inhibition in glioblastoma cells in vitro. Journal of Neuro-Oncology. 2016;**129**(1):39-45. DOI: 10.1007/s11060-016-2149-2

[87] Storniolo A, Raciti M, Cucina A, Bizzarri M, Di Renzo L. Quercetin affects Hsp70/IRE1α mediated protection from death induced by endoplasmic reticulum stress. Oxidative Medicine and Cellular Longevity. 2015;**2015**:645157. DOI: 10.1155/2015/645157

[88] Kim HS, Wannatung T, Lee S, Yang WK, Chung SH, Lim JS, Choe W, Kang I, Kim SS, Ha J. Quercetin enhances hypoxia-mediated apoptosis via direct inhibition of AMPK activity in HCT116 colon cancer. Apoptosis. 2012;**17**:938-949. DOI: 10.1007/s10495-012-0719-0

[89] Granado-Serrano AB, Martín MA, Bravo L, Goya L, Ramos S. Quercetin modulates Nrf2 and glutathione-related defenses in HepG2 cells: Involvement of p38. Chemico-Biological Interactions. 2012;**195**:154-164. DOI: 10.1016/j.cbi.2011.12.005

[90] Ramyaa P, Krishnaswamy R, Padma PP. Quercetin modulates OTA-induced oxidative stress and redox signalling in HepG2 cells—Up regulation of Nrf2 expression and down regulation of NF-κB and COX-2. Biochimica et Biophysica Acta. 2014;**1840**:681-692. DOI: 10.1016/j.bbagen.2013.10.024

[91] Ji LL, Sheng YC, Zheng ZY, Shi L, Wang ZT. The involvement of p62–Keap1–Nrf2 antioxidative signaling pathway and JNK in the protection of natural flavonoid quercetin against hepatotoxicity. Free Radical Biology & Medicine. 2015;**85**:12-23. DOI: 10.1016/j.freeradbiomed.2015.03.035

[92] Kang CH, Choi YH, Moon SK, Kim WJ, Ki GY. Quercetin inhibits lipopolysaccharide-induced nitric oxide production in BV2 microglial cells by suppressing the NF-κBpathway andactivating the Nrf2-dependent HO-1 pathway. International Immunopharmacology. 2013;**17**:808-813. DOI: 10.1016/j.intimp.2013.09.009

[93] Sun GY, Chen Z, Jasmer KJ, Chuang DY, Gu Z, Hannink M, Simonyi A. Quercetin attenuates inflammatory responses in BV-2 microglial cells: Role of MAPKs on the Nrf2 pathway and induction of heme oxygenase-1. PLoS One. 2015;**10**(10):e0141509. DOI: 10.1371/journal.pone.0141509

[94] Carrasco-Pozo C, Castillo RL, Beltrán C, Miranda A, Fuentes J, Gotteland M. Molecular mechanisms of gastrointestinal protection by quercetin against indomethacin-induced damage: Role of NF-κB and Nrf2. The Journal of Nutritional Biochemistry. 2016;**27**:289-298. DOI: 10.1016/j.jnutbio.2015.09.016

[95] Lee YJ, Lee DM, Lee SH. Nrf2 expression and apoptosis in quercetin-treated malignant mesothelioma cells. Molecules and Cells. 2015;**38**(5):416-425. DOI: 10.14348/molcells.2015.2268

[96] Chen BL, Wang LT, Huang KH, Wang CC, Chiang CK, Liu SH. Quercetin attenuates renal ischemia/reperfusion injury via an activation of AMP-activated protein kinase-regulated autophagy pathway. The Journal of Nutritional Biochemistry. 2014;**25**:1226-1234. DOI: 10.1016/j.jnutbio.2014.05.013

[97] Rivera AR, Castillo-Pichardo L, Gerena Y, Dharmawardhane S. Anti-breast cancer potential of quercetin via the Akt/AMPK/mammalian target of rapamycin (mTOR) signaling cascade. PLoS One. 2016;**11**(6):e0157251. DOI: 10.1371/journal.pone.0157251

[98] Kim GT, Lee SH, Kim YM. Quercetin regulates sestrin 2-AMPK-mTOR signaling pathway and induces apoptosis via increased intracellular ROS in HCT116 colon cancer cells. Journal of Cancer Prevention. 2013;**18**(3):264-270. DOI: 10.15430/JCP.2013.18.3.264

[99] Bruning A. Inhibition of mTOR signaling by quercetin in cancer treatment and prevention. Anti-Cancer Agents in Medical Chemistry. 2013;**13**(7):1025-1031

[100] Klappan AK, Hones S, Mylonas I, Bruning A. Proteasome inhibition by quercetin triggers macroautophagy and blocks mTOR activity. Histochemistry and Cell Biology. 2012;**137**:25-36. DOI: 10.1007/s00418-011-0869-0

[101] Pratheeshkumar P, Budhraja A, Son YO, Wang X, Zhang Z, Ding S, Wang L, Hitron A, Lee JC, Xu M, Chen G, Luo J, Shi X. Quercetin inhibits angiogenesis mediated human prostate tumor growth by targeting VEGFR- 2 regulated AKT/mTOR/P70S6K signaling pathways. PLoS One. 2012;**7**(10):e47516. DOI: 10.1371/journal.pone.0047516

[102] Elbarbry F, Elrody N. Potential health benefits of sulforaphane: A review of the experimental, clinical and epidemiological evidences and underlying mechanisms. Journal of Medicinal Plant Research. 2011;**5**(4):473-484

[103] Dinkova-Kostova AT, Kostov RV. Glucosinolates and isothiocyanates in health and disease. Trends in Molecular Medicine. 2012;**18**(6):337-347. DOI: 10.1016/j.molmed.2012.04.003

[104] Miao X, Bai Y, Sun W, Cui W, Xin Y, Wang Y, Tan Y, Miao L, Fu Y, Su G, Cai L. Sulforaphane prevention of diabetes-induced aortic damage was associated with the up-regulation of Nrf2 and its down-stream antioxidants. Nutrition and Metabolism. 2012;**9**(84):1-9. DOI: 10.1186/1743-7075-9-84

[105] Bai Y, Cui W, Xin Y, Miao X, Barati MT, Zhang C, Chen Q, Tan Y, Cui T, Zheng Y, Cai L. Prevention by sulforaphane of diabetic cardiomyopathy is associated with up-regulation of Nrf2 expression and transcription activation. Journal of Molecular and Cellular Cardiology. 2013;**57**:82-95. DOI: 10.1016/j.yjmcc.2013.01.008

[106] Chen X, Liu J, Chen SY. Sulforaphane protects against ethanol-induced oxidative stress and apoptosis in neural crest cells by the induction of Nrf2-mediated antioxidant response. British Journal of Pharmacology. 2013;**169**:437-448. DOI: 10.1111/bph.12133

[107] Jang M, Cho IH. Sulforaphane ameliorates 3-nitropropionic acid-induced striatal toxicity by activating the Keap1-Nrf2-ARE pathway and inhibiting the MAPKs and NF-κB pathways. Molecular Neurobiology. 2016;**53**:2619-2635. DOI: 10.1007/s12035-015-9230-2

[108] Lee YJ, Jeong HY, Kim YB, Lee YJ, Won SY, Shim JH, Cho MK, Nam HS, Lee SH. Reactive oxygen species and PI3K/Akt signaling play key roles in the induction of Nrf2-driven heme oxygenase-1 expression in sulforaphane-treated human mesothelioma MSTO-211H cells. Food and Chemical Toxicology. 2012;**50**:116-123. DOI: 10.1016/j.fct.2011.10.035

[109] Sun CC, Li SJ, Yang CL, Xue RL, Xi YY, Wang L, Zhao QL, Li DJ. Sulforaphane attenuates muscle inflammation in dystrophin-deficient mdx mice via NF-E2-related factor 2 (Nrf2)-mediated inhibition of NF-κB signaling pathway. The Journal of Biological Chemistry. 2015;**290**(29):17784-17795. DOI: 10.1074/jbc.M115.655019

[110] Zhou R, Lin J, Wu D. Sulforaphane induces Nrf2 and protects against CYP2E1-dependent binge alcohol-induced liver steatosis. Biochimica et Biophysica Acta. 2014;**1840**:209-218. DOI: 10.1016/j.bbagen.2013.09.018

[111] Zhang C, Su ZY, Khor TO, Shu L, Kong ANT. Sulforaphane enhances Nrf2 expression in prostate cancer TRAMP C1 cells through epigenetic regulation. Biochemical Pharmacology. 2013;**85**:1398-1404. DOI: 10.1016/j.bcp.2013.02.010

[112] Jo C, Kim S, Cho SJ, Choi KJ, Yun SM, Koh YH, Johnson YVW, Park SI. Sulforaphane induces autophagy through ERK activation in neuronal cells. FEBS Letters. 2014;**588**: 3081-3088. DOI: 10.1016/j.febslet.2014.06.036

[113] Liu H, Talalay P. Relevance of anti-inflammatory and antioxidant activities of exemestane and synergism with sulforaphane for disease prevention. Proceedings of the National Academy of Sciences of the United States of America. 2013;**110**(47):19065-19070. DOI: 10.1073/pnas.1318247110

[114] Kim DH, Sung B, Kang YJ, Hwang SY, Kim MJ, Yoon JH, Im E, Kim ND. Sulforaphane inhibits hypoxia-induced HIF-1α and VEGF expression and migration of human colon cancer cells. International Journal of Oncology. 2015;**47**:2226-2232. DOI: 10.3892/ijo.2015.3200

Chapter 2

Control of Ribosomal RNA Transcription by Nutrients

Yuji Tanaka and Makoto Tsuneoka

Additional information is available at the end of the chapter

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Abstract

The ribosome is a unique machine for protein synthesis in organisms. The construction of ribosomes is exceedingly complex and consumes the majority of the cell materials and energy. The materials for ribosome production are supplied by nutrients. Therefore, the production of ribosomes is restricted by environmental nutrients, and cells need mechanisms to control ribosome production in order to reconcile demands for cell activities with available resources. Transcription of ribosomal RNA is an essential step in ribosome biogenesis. It strongly affects the total amount of ribosome production, and thus rapidly growing cells have an elevated level of ribosomal RNA transcription. Ribosomal RNA transcription is controlled by many mechanisms, including the efficiency of preinitiation complex formation for RNA polymerase I (Pol I) and epigenetic marks in ribosomal RNA genes. These are affected by cell cycle progression, signal transduction pathways, cell-damaging stresses, nutrients such as glucose, and the metabolites. Recent studies also suggest that the epigenetic marks, acetylation and methylation, may be not only controlled by nutrients but also function as reservoirs for biological resources in chromatin. Further studies would provide information about the mechanisms cells use to adjust production of cellular components to available resources and clues for developing novel anti-cancer treatments.

Keywords: ribosomal RNA (rRNA), transcription, nutrients, glucose, epigenetic

1. Introduction

The ribosome is a unique machine for synthesizing protein in organisms. Protein synthesis is essential for all biological events, and the quantity of ribosomes substantially affects all biological activities. Rapidly growing cancer cells require synthesis of much protein and thus many ribosomes. In vertebrates, a ribosome consists of about 80 proteins and 4 structural ribosomal RNAs (rRNAs): 5S rRNA, 5.8S rRNA, 18S rRNA, and 28S rRNA [1, 2]. The construction processes are exceedingly complex and include rRNA transcription, rRNA processing,

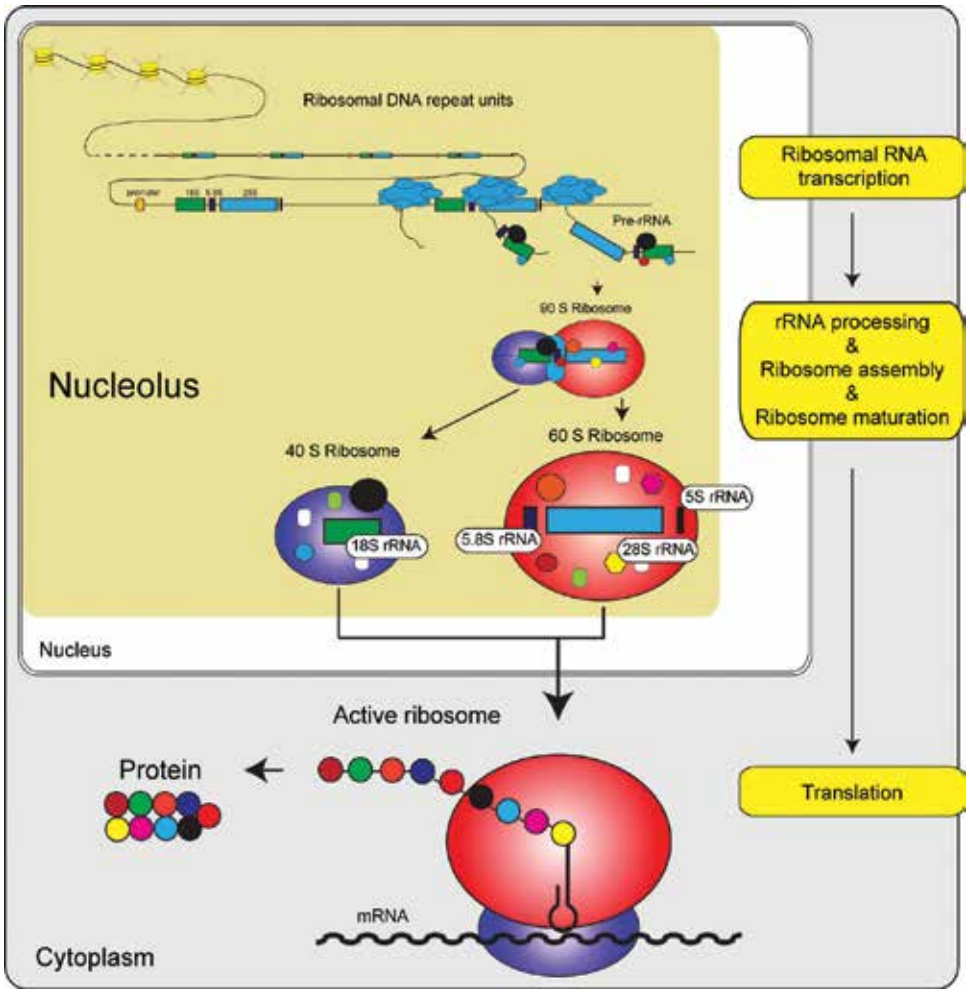

Figure 1. Processes of ribosome construction. Ribosomal RNA transcription, processing, and association of ribosomal proteins occur in the nucleolus. Mature ribosome functions in the cytoplasm. The process is outlined in the yellow box on the right side of this figure. Ribosomes contain four structural ribosomal RNAs (rRNAs): 5S rRNA, 5.8S rRNA, 18S rRNA, and 28S rRNA. The first step of ribosome construction is transcription of ribosomal RNA (rRNA) from the ribosome RNA gene (rDNA) repeating units in the nucleolus. RNA polymerase I (Pol I) transcribes pre-rRNA, which is processed to three structured rRNAs (18S, 28S and 5.8S rRNA). 5S rRNA is synthesized by RNA polymerase III. In the mature ribosome, 18S rRNA is contained in the 40S ribosome (small subunit of ribosome), and 28S, 5.8S, and 5S rRNAs are contained in the 60S ribosome (large subunit of ribosome). Ribosomal protein assembly, rRNA processing, and maturation occur in the nucleolus, and ribosomes are exported to the cytoplasm and perform the translation activity.

synthesis of ribosome proteins and regulatory proteins, assembly of rRNAs and ribosome proteins, and maturation of the ribosome (**Figure 1**). The entire process consumes up to 80% of the cell's materials [3, 4], and 80% of the energy to proliferate cells [4, 5]. The materials for ribosome production are supplied by nutrients that are taken up from the environment. Therefore, the production of ribosomes is restricted by environmental conditions, and cells

should have control of the mechanisms of ribosome production in order to reconcile demands for cell activities with the available biological resources.

Three of the four structured rRNAs (18S, 28S, and 5.8S rRNA) constituting ribosomes are produced by processing of a precursor transcript, pre-ribosomal RNA (pre-rRNA). The pre-rRNA is coded by rRNA genes (rDNA) and specifically transcribed by RNA polymerase I (Pol I) in the nucleolus. Because a single copy of rDNA is not sufficient to supply the number of rRNA molecules required, there are 100–300 copies of tandemly repeated rDNAs per haploid genome in mammals. Paradoxically, only half the copies of rDNA are in transcriptionally active forms and the rest are silent, which may provide a control step for rRNA transcription [6–9]. The transcription of rRNA is an essential step in ribosome biogenesis and affects the total number of ribosomes produced. It was suggested that 75% of total RNAs constitute rRNAs in Hela cells [10], and the rRNA transcription represents about 35% of all transcripts in proliferating cells [6], showing that rRNA synthesis uses a lot of materials. Therefore, the control of rRNA transcription plays a role in maintaining homeostasis in biological resources. In this review, we describe the control of rRNA transcription by various factors such as the cell cycle regulators, signal transduction pathways, growth factors, tumor-related proteins, and cell-damaging stresses. Then, we will discuss the control mechanisms of rRNA transcription in response to nutrients.

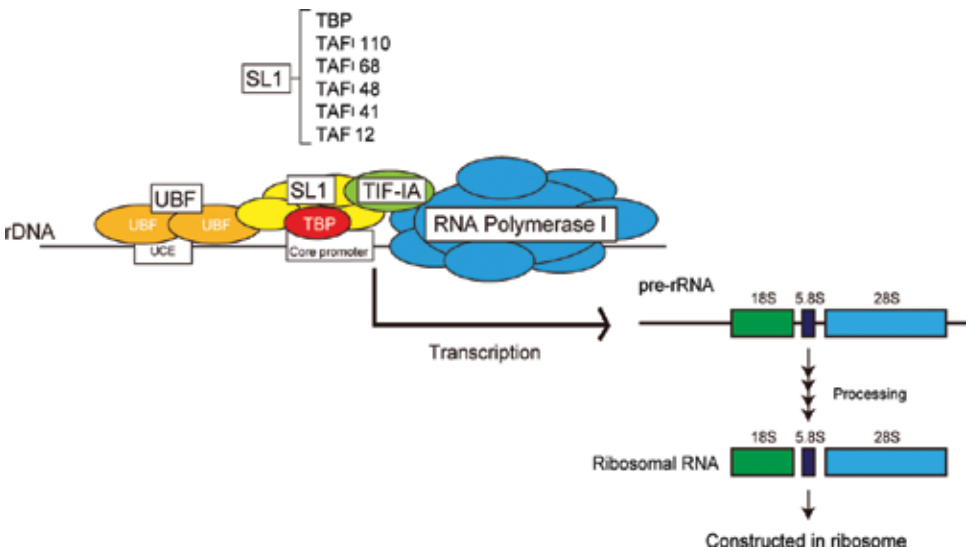

Figure 2. Pre-initiation complex for RNA polymerase I and rRNA processing. The basic composition of the pre-initiation complex (PIC) for RNA polymerase I is illustrated. PIC is assembled on the rDNA promoter by synergistic action of the upstream binding factor (UBF), which is bound at the upstream control element (UCE), selective factor 1 (SL1), which is bound to the core promoter through TATA-box binding protein (TBP), transcription initiation factor IA (TIF-IA), and RNA polymerase I (Pol I). SL1 contains TBP and Pol I-specific TBP-associated factors (TAF_Is: TAF_I110, TAF_I68, TAF_I48, TAF_I41, and TAF12). SL1 on the core promoter recruits RNA polymerase I through TIF-IA, which associates with both components of Pol I and SL1. After the completion of PIC formation, Pol I is released from the promoter by regulation of TIF-IA and starts to transcribe pre-rRNA. This release is the initiation step of rRNA transcription. Pre-rRNA is processed to structured rRNA, 18S, 5.8S, and 28S rRNA to construct ribosomes.

2. Formation of preinitiation complex (PIC) on rDNA promoter

The first step of rRNA transcription is the formation of the preinitiation complex (PIC) on the rDNA promoter. The upstream binding factor (UBF), the promoter selective factor 1 (SL1), transcription initiation factor IA (TIF-IA), and RNA polymerase I (Pol I) synergistically assemble at the rDNA promoter to form PIC (**Figure 2**). While the name SL1 is used for human proteins, that for mice is transcription initiation factor 1B (TIF-IB). UBF and SL1 bind to an upstream control element (UCE) and the core promoter region of rDNA, respectively. SL1 recruits Pol I through TIF-IA, and UBF stabilizes the binding of SL1 to Pol I at the rDNA promoter [6, 11].

SL1 is a protein complex consisting of TATA-box binding protein (TBP), Pol I-specific TBP-associated factors (TAF_Is), including TAF_I110, TAF_I68, and TAF_I48, which were originally identified with TBP as essential transcriptional factors by an *in vitro* transcription assay [12]. TBP is not only a Pol I-specific factor, but also used for transcription by RNA polymerase II and III. Later, TAF_I41 and TAF12 were identified as members of the TAF_Is. TAF12 was originally reported as a transcription factor for RNA polymerase II [13]. One activity of TBP is binding to the TATA box in the core promoter to recruit SL1 complex. TIF-IA binds to the RPA43 subunit of Pol I complex and TAF_I110, TAF_I68, and TAF_I41 of the SL1 complex. These binding activities are essential for TIF-IA to recruit Pol I to the promoter bound by SL1 to facilitate PIC formation on the rDNA promoter. The formation of PIC is controlled by various factors (**Figure 3**). When Pol I starts the rRNA transcription, the interactions forming PIC are disrupted. This disruption is the initiation step of transcription, one of the control mechanisms.

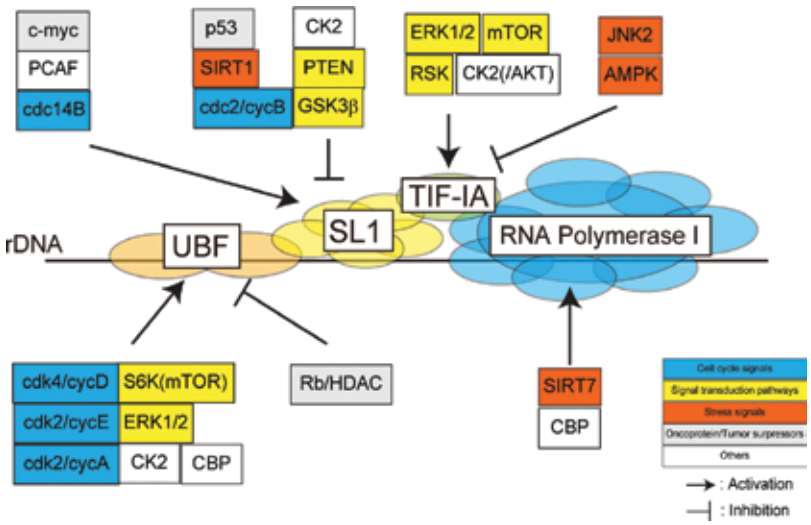

Figure 3. Factors regulating PIC formation. The PIC for Pol I transcription is controlled by various factors, including cell cycle signals, signal transduction pathways, stress signals, oncoprotein/tumor suppressors, and others. The classes of regulatory factors are expressed with specific color boxes as indicated in the box on the right. UBF is activated by cdk family proteins, ribosomal protein S6 kinase (S6K), casein kinase II (CK2), and CBP, and is repressed by the Rb/HDAC complex. SL1 is activated by c-Myc, PCAF, and cdc14B, and repressed by p53, SIRT1,cdc2/cyclin B, CK2, PTEN, and GSK3β. TIF-IA is activated by ERK1/2, RSK, mTOR, and CK2 (activated by Akt), and repressed by JNK2 and AMPK. RNA polymerase I (Pol I) is activated by SIRT7 and CBP.

3. Control of rRNA transcription during cell cycle progression

The activities of all classes of RNA polymerases are controlled during the cell cycle progression [14, 15]. The cell cycle regulator cyclin/cdk complexes control the level of rRNA transcription (**Figure 3**). In the M phase, SL1 is inactivated by cdk1/cyclin B (cdc2/cyclin B) through phosphorylation of TAF_I110 to silence rRNA transcription [16, 17]. On exiting mitosis, the phosphorylation in TAF_I110 is removed by cell division cycle 14B (Cdc14B) [18]. Additionally, mitotic repression of rRNA transcription correlates with the hypo-acetylation of TAF_I68 caused by Sirtuin 1 (SIRT1). The hypo-acetylation makes SL1 instable on binding to the rDNA promoter [18]. It was also reported that the site of deacetylation of TAF_I68 by SIRT1 is acetylated by p300/CBP-associated factor (PCAF), which is correlated with the activation of rRNA transcription [18].

After mitosis, rRNA transcription is re-activated by G1/S-specific cyclins (cdk4/cyclin D, cdk2/cyclin E, cdk2/cyclin A) through phosphorylation of UBF on the specific sites (cdk4/cyclin D (S484), cdk2/cyclin E (S388, S484), cdk2/cyclin A (S388): in mouse) [19, 20].

4. Signal transduction pathways control rRNA transcription

Protein synthesis is required for cell growth, and the signal transduction pathways that affect cell growth, including phosphoinositide 3-kinase (PI3K)-AKT-mammalian target of rapamycin (mTOR) signaling and ERK (MAPK) signaling, are involved in the regulation of rRNA transcription (**Figure 3**).

The PI3K-AKT-mTOR signal pathway is stimulated by binding of insulin/Insulin-like growth factors (IGF) to their cognate receptors on the cell surface. AKT activates rRNA transcription through the phosphorylation of CK2. CK2 regulates rRNA transcription at multiple levels by affecting the formation of PIC, initiation, elongation, and reinitiation, through phosphorylation of several proteins including UBF, TAF_I110 (SL1), and TIF-IA [21–26]. mTOR activates rRNA synthesis by translocating TIF-IA into the nucleolus using kinase activity [27]. The ribosomal protein S6 kinase (S6K), which is a downstream kinase of mTOR, also activates rRNA synthesis through regulation of UBF-SL1 interaction by phosphorylation of UBF. The mTOR activity also enhances the expression of UBF [28]. SNF2 histone linker PHD RING helicase (SHPRH), which was identified as a RAD5 homolog and known as E3 ubiquitin-protein ligase, binds to rDNA promoters using its PHD domain and promotes recruitment of Pol I to rDNA (**Figure 4**). This activation of rRNA transcription by SHPRH is inhibited in an mTOR-dependent manner [29]. K-demethylase 4A (KDM4A)/JMJD2A activates rRNA transcription on serum stimulation (**Figure 4**). This activation is mediated through the PI3K/serum/glucocorticoid regulated kinase 1 (SGK1) signaling cascade independent of the AKT pathway. SGK1 is one of the downstream kinases of PI3K signaling. The serum-stimulated KDM4A decreases a repressive histone H3K9me3 mark modification in rDNA to activate rRNA transcription [30]. In mouse adipocytes, polymerase I transcription and release factor (PTRF)/Cavin-1 promotes rRNA transcription, which is induced by insulin and repressed by fasting (**Figure 4**). The stimulation of rRNA transcription by PTRF is mediated by the formation of the transcription loop that

links the transcriptional start sites and termination sites. The formation may enhance transcriptional reinitiation [31].

The binding of epidermal growth factor (EGF) or its related ligands to their cognate receptors on the cell surface stimulates a signaling cascade including the GTPase Ras, the kinases Raf, MAP kinase-ERK kinase (MEK), and extracellular signal-regulated kinase (ERK). ERK activates rRNA transcription through phosphorylation of UBF in the promoter [32] and gene body regions [33] (**Figure 3**).This phosphorylation decreases the binding capacity of UBF to rDNA. In this case, it was reported that the dissociation of UBF from rDNA enhances Pol I release from the promoter, leading to activation of rRNA transcription. Additionally, ERK/90 kDa ribosomal S6 kinase (RSK) phosphorylates TIF-IA to activate rRNA transcription [34] (**Figure 3**). It is still unclear how the phosphorylation by RSK induces rRNA transcription.

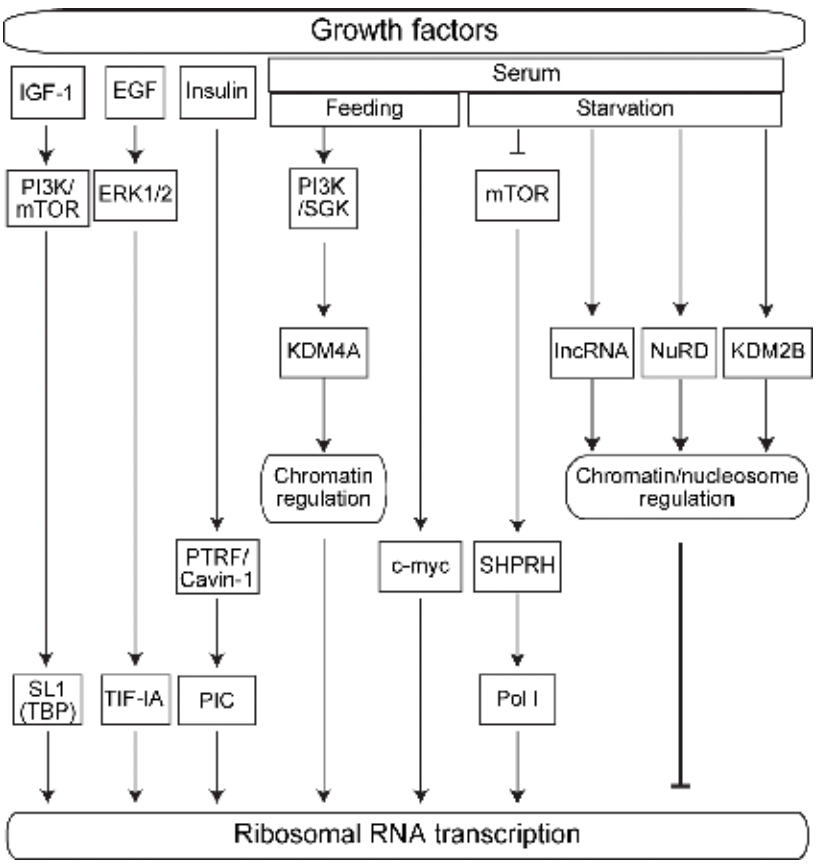

Figure 4. Signal transduction pathways under growth factor controlled rRNA transcription. Growth factors including insulin, insulin-like growth factor (IGF-1), epidermal growth factor (EGF), and unidentified serum factors (Serum) control rRNA transcription through signal transduction pathways, such as PI3K/mTOR, PI3K/SGK and ERK1/2, which control PIC components (SL1, TIF-IA, and Pol I), transcription factors (PTRF/Carvin-1, c-Myc and SHPRH) or chromatin/nucleosome regulators (lncRNA, NuRD, and KDM2B).

Elevation of the concentration of calcium ions (Ca^{2+}) in the cytoplasm stimulates the signaling pathway of calcium/calmodulin-dependent protein kinase II (CaMKII). The stimulated CaMKII activates S6K, which phosphorylates UBF to activate rRNA transcription in colorectal cancer (CRC) (**Figure 3**). In CRC, the function of adenomatous polyposis coli (APC) gene is frequently lost and the level of Ca^{2+} is increased in the cells [35].

5. Control of rRNA transcription by unidentified serum factors

Serum, used to supplement the cell culturing medium, contains many factors that control rRNA transcription. Although all factors and signal cascades are not completely identified, they perform critical functions in the regulation of rRNA transcription (**Figure 4**).

Depletion of serum from a culture medium represses rRNA transcription. c-Myc plays a critical role for cell growth and proliferation in many types of cells, and is deregulated and over-expressed in tumor cells. c-Myc associates with the promoter and transcribed regions of rDNA and activates rRNA transcription in response to serum stimulation [36].

K-demethylase 2B (KDM2B)/JHDM1B is bound to rDNA to repress rRNA transcription. The repression is associated with the demethylation of trimethylated lysine 4 on histone H3 (H3K4me3) by KDM2B. Serum starvation increases the recruitment of KDM2B on rDNA, and resupply of serum decreases it. These data suggest that the activity of KDM2B in controlling rRNA transcription is regulated by serum factors [37].

The specific long non-coding RNAs (lncRNAs) are induced during periods of quiescence, such as serum starvation, and increase the level of histone H4K20me3 on the rDNA promoter in a suppressor of variegation 4-20 homolog (Suv4-20 h)-dependent manner. The elevated level of H4K20me3 leads to chromatin compaction. The lncRNAs are antisense transcripts against rDNA, are termed the promoter and pre-rRNA antisense (PAPAS), and associated with rDNA [38].

A chromatin remodeling complex, nucleosome remodeling deacetylase (NuRD), establishes the poised state of rDNA through regulation of histone modifications and nucleosome positions. The level of the state of rDNA is increased in the growth-arrested conditions induced by serum starvation and differentiation [39].

6. Oncoprotein and tumor suppressors in rDNA transcription

Tumor cells show abnormal growth that is thought to be associated with the elevation of ribosome biogenesis, and regulation of rRNA transcription by oncogenes and tumor-suppressor genes was reported.

The oncoprotein c-Myc is the product of oncogene c-*myc*, and its expression is stimulated by serum and associated with rRNA transcription [36] (**Figure 4** and Section 5). It was reported that c-Myc binds to the sites with the consensus sequences on rDNA and stimulates rRNA

transcription [40]. c-Myc is also reported to control the PIC factors such as UBF and ribosomal proteins [41–43]. Therefore, c-Myc activates ribosome biogenesis at multiple steps [3, 44, 45] .

The *rb* gene is a tumor-suppressor gene. Rb protein binds to UBF, which may be related to restriction of cell proliferation by Rb [46]. The binding of Rb to UBF inhibits the binding of UBF to rDNA [47] or inhibits the binding of UBF to SL1 [48], both of which result in the repression of rRNA transcription (**Figure 3**). Another report suggested that phosphorylated Rb (pRb) creates a complex with histone deacetylase (HDAC) and decreases the acetylation of UBF to repress rRNA transcription [49]. In this study, it was also reported that the acetylation in UBF is modified by CREB-binding protein (CBP) (**Figure 3**).

A tumor-suppressor gene, *p53*, is frequently mutated in tumors, and p53 protein represses rRNA transcription through prevention of the interaction between SL1 and UBF [50] (**Figure 3**). The phosphatase and tensin homolog deleted from chromosome 10 (PTEN) is known as a tumor suppressor. PTEN represses rRNA transcription by disrupting the SL1 complex in its lipid phosphatase activity-dependent manner [51] (**Figure 3**). It was also reported that PTEN is phosphorylated by glycogen synthase kinase (GSK) 3β [52]. GSK3β and PTEN are selectively enriched in the nucleoli of RAS-transformed cells and associate with the promoter and coding region of the rDNA [53]. An activated GSK3β mutant abolishes rRNA transcription and associates with TAF_I110 in the SL1 complex [53]. These results suggest a repressive function for GSK3β on rRNA transcription that supports its role as a tumor suppressor.

7. Controls of rRNA transcription by cell-damaging stresses

A variety of stresses such as UV, ionizing radiation, heat shock, and osmotic shock attack cellular vital components like DNA, proteins, and lipid membranes. These stresses also affect rRNA transcription.

c-Jun N-terminal kinase (JNK) phosphorylates c-Jun at the NH_2-terminal Ser63 and 73 residues in response to UV irradiation and other stress stimuli [54]. JNK2 inactivates rRNA transcription through phosphorylation of TIF-IA to inhibit its function of bridging between Pol I and SL1 [55] (**Figure 3**).

The DNA damage caused by ionizing radiation also induces the repression of rRNA transcription through other pathways, which involves Nijmegen breakage syndrome protein 1 (NBS1)-treacle, Ataxia Telangiectasia Mutated (ATM), and breast cancer susceptibility gene I (BRCA1). In the presence of double strand breaks induced by ionizing radiation, NBS1 translocates and accumulates in nucleoli in a treacle-dependent manner to silence rRNA transcription [56]. The *treacle* gene was found to be mutated in Treacher Collins syndrome, which is characterized by deformation of bones and other tissues in the face. ATM-dependent signaling was shown to shut-down rRNA transcription in response to chromosome breaks [57]. BRCA1, known as a tumor suppressor, was reported to interact with UBF, SL1, and Pol I. In response to DNA damage, BRCA1 bound to rDNA is dissociated, and induces instability of Pol I on rDNA to repress rRNA transcription [58].

Heat shock at 42°C represses rRNA transcription through the inactivation of TIF-IA by inhibition of CK2-dependent phosphorylation of TIF-IA and the lncRNAs PAPAS-dependent nucleosome regulation by NuRD complex [59]. Hypotonic stress represses rRNA transcription through upregulation of PAPAS to trigger nucleosome repositioning by NuRD [60]. In these conditions, Suv420h2 was neddylated and the levels of Suv420h2 and H4K20me3 marks were increased. However, the relationship between PAPAS and Suv420h2 was not clear. Cytoskeletal stress, which is related to cell shape, represses rRNA transcription through Rho-associated protein kinase (ROCK). ROCK is one of the kinases of myosin and induces recruitment of HDAC on rDNA, resulting in deacetylation of histone acetylated lysine 9 and 14 on histone H3 (H3K9/14) [61].

8. Control of rRNA transcription by nutrients

Cells obtain biological resources for cellular activities from their environment. The sensing of environmental nutrients is important for efficient usage of nutrients and maintaining cells. In murine intestinal epithelium, apical transcripts are more efficiently translated, because ribosomes were more abundant on the apical sides. Refeeding of fasted mice induces a basal to apical shift of mRNAs encoding ribosomal proteins, which is associated with an increase in their translation and increased protein production. These mechanisms allow efficient nutrient absorption in response to the rich conditions, although the molecular mechanisms are not clear [62]. It was shown that mTOR senses the levels of amino acids, especially leucine, in cells, and controls the translation activity through regulation of the eukaryotic translation initiation factor 4E binding protein (4E-BP)-eukaryotic translation initiation factor 4E (elF4E) axis and p70 S6K-S6 axis [4, 63, 64]. Recently, increasing evidence shows the presence of specific mechanisms to control rRNA transcription in response to nutrients (**Figure 5**).

8.1. Amino acids

The starvation of amino acids affects the frequency of initiation of nucleolar RNA polymerase, which was later established to be an rRNA transcription by Pol I [65]. The starvation of amino acids decreases the interaction of TIF-IA with SL1 and Pol I [66]. As described above in Section 4, mTOR controls rRNA transcription, and is important for regulation of rRNA transcription in response to amino acid levels. Amino acid starvation inhibits the activity of mTOR and its downstream kinase S6K. mTOR and S6K control TIF-IA and UBF, respectively, to regulate rRNA transcription [27, 67].

c-MYC is also involved in regulation of rDNA transcription in response to starvation of amino acids. Although translation of c-Myc is reported to be controlled by mTOR signaling [68, 69], the stabilization of c-Myc in response to amino acid starvation is controlled by an mTOR-independent pathway [70].

8.2. Guanosine triphosphate (GTP)

It was reported that the sizes of ATP and GTP intracellular pools affect the level of nucleolar RNA synthesis (rRNA transcription) [71]. Recently, the consensus sequences for GTP binding

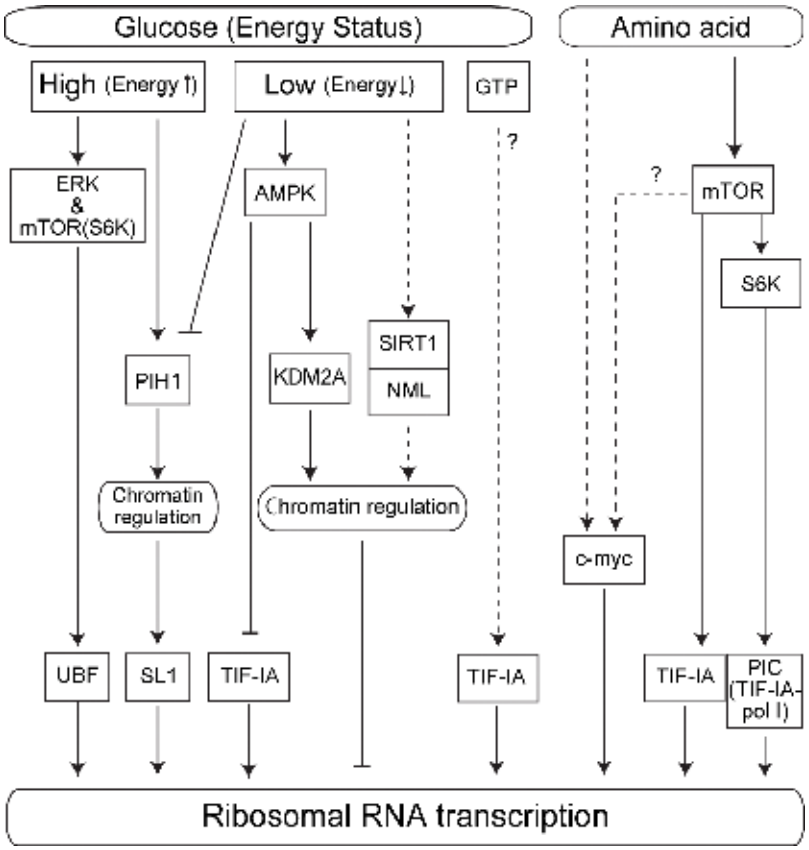

Figure 5. Glucose and amino acids control rRNA transcription. Glucose, amino acids, and GTP control rRNA transcription through several pathways, such as ERK, mTOR/S6K, and AMPK, which control PIC components (UBF, SL1, and TIF-IA), transcription factors (c-Myc), or chromatin regulators (KDM2A, PIH1, and SIRT1/NML). High glucose activates ERK and mTOR/S6K pathways to control UBF. High glucose also activates PIH1 to control chromatin. Glucose depletion or low energy conditions activate SIRT1/NML and the AMPK pathway. Activated AMPK controls the activities of TIF-IA and KDM2A. Activated KDM2A controls rDNA chromatin to inhibit rRNA transcription. Amino acid depletion represses the mTOR pathway, resulting in the repression of TIF-IA, and decreases the expression of c-Myc. Guanosine triphosphate (GTP) is bound to TIF-IA, and the binding is required to control rRNA transcription.

were identified in TIF-IA [72], and the binding of TIF-IA to GTP is required for the interaction of TIF-IA with ErbB3-binding protein (Ebp1). Ebp1 controls ribosomal biogenesis when located in the nucleolus [73]. Therefore, the level of GTP appears to be sensed by TIF-IA to affect rRNA transcription (**Figure 5**).

8.3. Glucose

The major energy source for cells is glucose. Glucose is used to synthesize ATP. ATP is essential for most biological activities, including ribosome biogenesis. Several studies demonstrated that the levels of glucose and ATP production affect rRNA transcription.

Ribosomal biogenesis including rRNA transcription was reported to be induced by high glucose treatment or diabetes. A high level of glucose activates UBF through ERK1/2 and mTOR in kidney glomerular epithelial cells of mice [74].

The PIH1 domain-containing protein 1 (PIH1)/Nop17 is reported to enhance rRNA transcription through the recruitment of SNF5-Brg1 complex on the rRNA promoter [75] (**Figure 5**). The complex increases acetylation of several histones, except histone H4K16Ac, on rDNA in high glucose conditions. Until now, the acetylation marks of histone in rDNA, excluding the acetylation at K16 in histone H4 (H4K16Ac), are linked to activation of transcription. The acetylated histones function as active marks in transcription in many cases because the acetylation of histone weakens the interaction of histone octamers with DNA, and the acetylated histones are recognized by several transcription-activating factors. On the other hand, the H4K16Ac mark is reported to be recognized by nucleolar remodeling complex (NoRC) in rDNA, which induces chromatin-silencing status [76]. Glucose starvation dissociates PIH1 and the SNF5-Brg1 complex from rDNA and increases histone H4K16Ac marks, which repress rRNA transcription [75]. Another report suggested that PIH1 interacts with mTORC1 to stabilize it, resulting in enhancement of rRNA transcription [77].

8.4. AMPK is activated by glucose starvation

Glucose starvation decreases ATP production and activates AMPK (**Figure 5**). The AMP-activated kinase (AMPK) is known as an energy sensor, which recognizes the ratios of AMP, ADP, and ATP and regulates many phenomena in cells to maintain energy homeostasis.

Additionally, a recent study showed the existence of an AMP/ADP-independent mechanism that triggers AMPK activation (**Figure 6**). Glycolysis is a determined sequence of 10 enzyme-catalyzed reactions. In the fourth step, the hexose ring of fructose 1, 6-bisphosphate (FBP) is split by aldolase into two triose sugars: dihydroxyacetone phosphate (a ketose) and glyceraldehyde 3-phosphate (an aldose). When extracellular glucose is decreased, intracellular FBP is decreased, and aldolase unoccupied by FBP promotes the formation of a lysosomal complex containing v-ATPase axin, liver kinase B1 (LKB1), and AMPK, which regulates AMPK activity [78]. These results suggest that the decreased level of the metabolite in glycolysis controls AMPK before the reduction of ATP production just after changing environmental conditions, emphasizing that AMPK is a highly sensitive monitor of energy conditions.

AMPK induces phosphorylation of TIF-IA (**Figure 5**). The phosphorylation of TIF-IA by AMPK reduces interaction of TIF-IA with SL1, decreases the TIF-IA amount on the rDNA promoter, and interrupts PIC assembly, which results in the reduction of rRNA transcription [79].

KDM2A, identified as mono- and di-methylated lysine 36 on histone H3 (H3K36me1/2) demethylase [80], is accumulated in the nucleolus and binds to rDNA [81]. The repression of rRNA transcription by KDM2A is induced in response to serum and glucose starvation (**Figure 5**). The repression requires the demethylase activity of KDM2A on the rDNA promoter [82]. The KDM2A-dependent regulation affects the levels of protein synthesis [81]. The demethylase activity of KDM2A proceeds with a co-reaction in which α-ketoglutarate (α-KG)

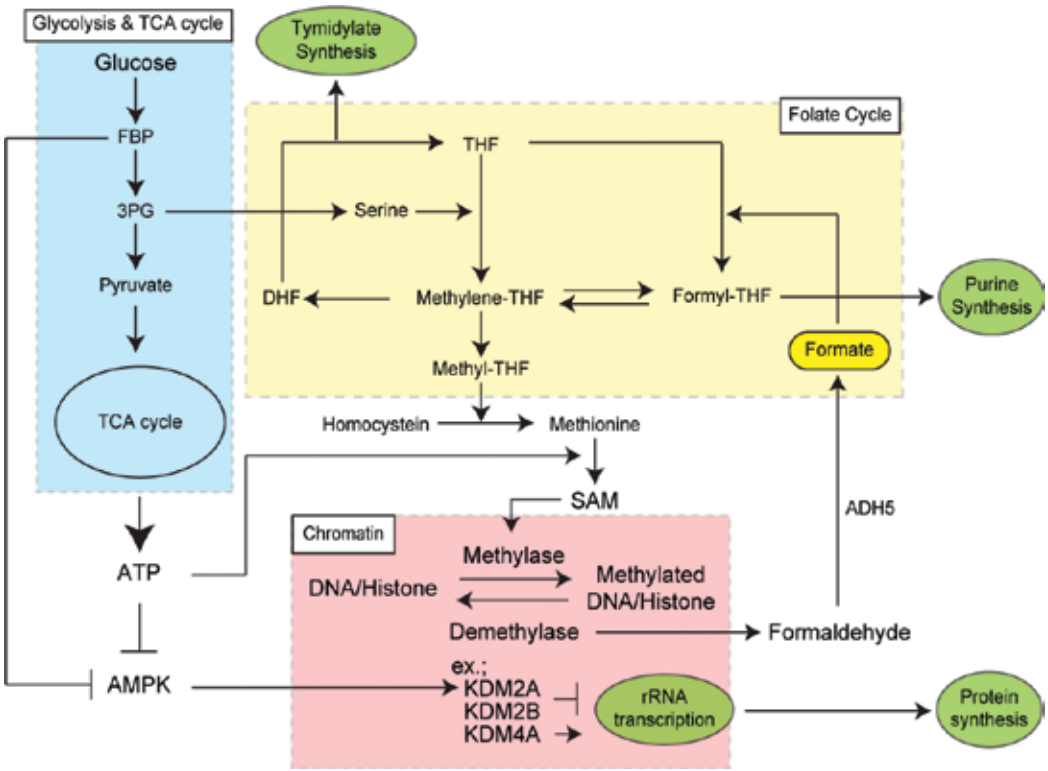

Figure 6. Control of methylation by energy status and methyl marks as a reservoir for biological resources. Chromatin components are methylated and demethylated by specific enzymes influenced by metabolites in energy production. Formaldehyde is produced as a demethylation byproduct, directly generates one carbon unit, fuels the folate cycle through alcohol dehydrogenase 5 (ADH5) activity, and can be used as a source for production of SAM, which is used for methylation and nucleotides. AMPK, AMP-activated protein kinase; DHF, dihydrofolate; FBP, fructose 1, 6-bisphosphate; 3PG, 3-phosphoglycerate; SAM, S-adenosylmethionine; THF, tetrahydrofolate.

is converted to succinate, both of which are organic acids constituting the TCA cycle. Interestingly, the enzyme activity of KDM2A is controlled by cell-permeable succinate (dimethyl succinate: DMS), suggesting that metabolites in the TCA cycle affect KDM2A activity. Recently, it was found that glucose starvation in the presence of serum induces the repression of rRNA transcription by KDM2A, in which activated AMPK induces KDM2A activity [83] (**Figure 5**). Interestingly, treatment with a low concentration of the glycolysis inhibitor 2-deoxy-D-glucose (2DG) induces KDM2A-dependent repression of rRNA transcription associated with histone demethylation on the rDNA promoter, although it does not dissociate TIF-IA from the rDNA promoter. Treatment with a high concentration of 2DG induces both the dissociation of TIF-IA from the rDNA promoter and KDM2A-dependent demethylation of the rDNA promoter. These results suggest that the repression of rRNA transcription in response to glucose starvation is performed by two different mechanisms: epigenetic regulation by KDM2A and TIF-IA regulation, depending on the glucose starvation level.

AMPK phosphorylates dozens of proteins, but until now KDM2A has not been detected as a substrate of AMPK kinase activity. AMPK also controls the activity of mTOR [84], and mTOR

is a candidate kinase for control of the states of histone methylation in the rDNA promoter, but currently there is no evidence connecting mTOR and KDM2A. Further studies are required to determine how the KDM2A activity in the rDNA promoter is induced by AMPK. H3K36me2 on the rDNA promoter which is demethylated by KDM2A on starvation is quickly restored by refeeding glucose and serum [82]. The data suggest that the control of H3K36me2 levels on rDNA promoters is reversible by changes in nutrient status, although which enzyme induces methylation of H3K36me2 on the rDNA promoter in response to refeeding of glucose and serum remains unknown. The control mechanism of rRNA transcription through epigenetic regulation by KDM2A may be a fine tuning device that quickly reflects nutrient states around cells.

8.5. Sirtuins

Sirtuins target a wide range of cellular proteins in the nucleus, cytoplasm, and mitochondria for post-translational modification by acetylation (SirT1, 2, 3, and 5) or ADP-ribosylation (SirT4 and 6). The deacetylase activity of sirtuins is controlled by the cellular NAD^+/NADH ratio, where NAD^+ works as an activator, while nicotinamide and NADH act as inhibitors (**Figure 7**). The acetylation regulates a wide variety of cellular functions. Sirtuins participate in various cellular processes, deacetylating both chromatin and non-histone proteins, and their roles in aging have been extensively studied. Sirtuins may also play a critical role in tumor initiation and progression as well as drug resistance. Reduced compounds such as glucose and fatty acids are oxidized, thereby releasing energy. This energy is transferred to NAD^+ by reduction to NADH, as part of glycolysis, the citric acid cycle, and β-oxidation. The mitochondrial

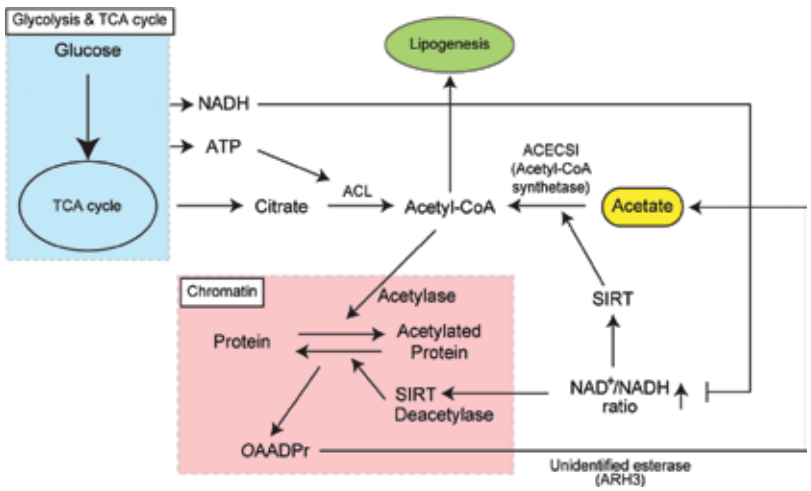

Figure 7. Control of acetylation by energy status and acetyl marks as a reservoir for biological resources. Chromatin components are acetylated and deacetylated by specific enzymes whose activities are influenced by metabolites in energy production. During deacetylation, the acetyl group from the substrate is accepted by the ribose to produce *O*-acetyl-ADP-ribose (*O*AADPr). A cytoplasmic esterase, which was suggested to be ADP-ribosyl hydrolase 3 (ARH3), hydrolyzes *O*AADPr to acetate and ADPr. Acetate that is generated from the deacetylation may be changed to acetyl-CoA, and used as resource for acetyl marks and lipogenesis. ACECSI, acetyl-CoA synthase 1; ACL, ATP-citrate lyase; *O*AADPr, *O*-acetyl ADP-ribose.

NADH is then oxidized in turn by the electron transport chain, which generates ATP through oxidative phosphorylation.

SIRT1 was reported to be required for the recruitment of nucleomethylin (NML) on rDNA. In response to glucose starvation, rRNA transcription is repressed through NML-induced chromatin regulation [85]. Although it is not clear that SIRT1 shows deacetylase activity on starvation, SIRT1 induces the deacetylation of p53, and this deacetylation activity is required for the repression of rRNA transcription. Further, the NAD^+ synthesis enzyme nicotinamide mononucleotide adenylyltransferase (NMNAT1) modulates the repression of rRNA transcription [86]. As described above, SIRT1 is required for mitotic repression of rRNA transcription through deacetylation of TAF_I68 (**Figure 3** and section 3). TAF_I68 is acetylated by PCAF to restart transcription in the mitotic exit phase [18].

On the other hand, SIRT7, another SIRT family member, was reported to activate rRNA transcription depending on the deacetylation activity, through regulation of PAF53, which is an important component of Pol I complex [87–91].

8.6. Epigenetic marks may play a role in conserving biological resources

Acetyl-CoA is used as an acetyl group donor on acetylation of histone and other proteins (**Figure 7**). Acetyl-CoA is produced from pyruvate, acetate, or fatty acid oxidation in multiple metabolisms. The amount of acetyl-CoA affects the activity of histone acetyltransferases (HATs). S-adenosylmethionine (SAM) is known to be used as a methyl donor in DNA/histone (protein) methylation (**Figure 6**). SAM is produced through the condensation of methionine and ATP by methionine adenosyltransferase (MAT). The production of SAM is mediated through the folate and methionine cycles. The amount of SAM is thought to affect the activity of methylase. The production of the two epigenetic marks is clearly affected by energy production processes, suggesting that intracellular energy conditions affect the modifications of epigenetic marks [92–94].

Further, enzymes detaching the marks are also affected by metabolites in energy production. NAD^+ activates sirtuin deacetylase, while nicotinamide and NADH inhibit the activity (**Figure 7**). The demethylase activity of KDM2A is controlled by the amounts of intracellular ATP through AMPK (**Figure 6**), and probably more directly by succinate. In another report, chromatin-associated fumarase generating fumarate inhibits the demethylation activity of KDM2A on the promoter region of the RNA polymerase II gene [95]. Additionally, 2-hydroxy-glutarate (2-HG), which was produced from α-KG by a mutant type isocitrate dehydrogenase (IDH) also modulates several jmjC-type enzymes including the lysine-specific demethylases (KDMs) such as KDM2A and Tet methyl-cytosine dioxygenases (TETs) [96–98]. Therefore, metabolites reflecting intracellular energy conditions can control the enzymes for detaching the epigenetic marks as well as adding them.

Interestingly, during deacetylation, the glycosidic bond of the nicotinamide ribose is cleaved to yield nicotinamide, and the ribose accepts the acetyl group from the substrate to produce *O*-acetyl-ADP-ribose (*O*AADPr). *In vitro*, a cytoplasmic esterase from humans and yeast, which was suggested to be ADP-ribosyl hydrolase 3, hydrolyzes *O*AADPr to acetate and ADPr [99]. Therefore, acetyl marks of proteins that are deacetylated by sirtuin may also be

used as a source of acetate for acetyl marks and lipogenesis (**Figure 7**). KDMs catalyze histone lysine demethylation through an oxidative reaction. The catalytic reaction begins with the coordination of molecular oxygen (O_2) by Fe(II) and the conversion of α-KG to succinate and CO_2 with the concomitant hydroxylation of the methyl group of the peptide substrate. The resulting carbinolamine is unstable and degrades spontaneously to an unmethylated peptide and the cytotoxic molecule formaldehyde (**Figure 6**). Recently, it was shown that formaldehyde reacts spontaneously with glutathione (GSH) to yield S-hydroxymethylglutathione (HMGSH), and subsequently HMGSH is oxidized by alcohol dehydrogenase 5 with NAD(P)$^+$ to create S-formylglutathione. This biochemical route provides a cell with formaldehyde detoxification as well as utilizable one-carbon units, which contribute to nucleotide synthesis. Therefore, cells reserve materials for one-carbon metabolism as methyl marks [100], which are released by KDM2A on starvation. These two examples of acetylation and methylation suggest that modifications of the epigenetic marks are not only controlled by intracellular energy conditions, but also function as reservoirs in chromatin for biological resources.

9. Applications for therapy

Enlarged nucleoli have been recognized as a hallmark of cancer cells [101, 102]. Elevated levels of rRNA transcription and protein synthesis are often observed in cancer cells. These observations suggest the possibility that the control of rRNA transcription could regulate the proliferation of cancer cells. Actually, the anti-cancer effects of some compounds are associated with down-regulation of rRNA transcription. Cisplatin [103], mitomycin C [104], and 5-fluorouracil [105], well-known anti-cancer drugs, are reported to inhibit rRNA transcription [11]. However, it is not clear whether the reduction of rRNA transcription in cancer cells is causal or only as a consequence of inhibition of cell growth.

On the other hand, there are drugs that appear to reduce rRNA transcription and then repress cancer growth. Actinomycin D (Dactinomycin) specifically represses rRNA synthesis at low concentrations through inactivation of transcriptional elongation by Pol I by interaction with GC-rich regions of rDNA, and thus inhibits growth of cancer cells. Actinomycin D is used as a chemotherapy medication to treat a number of types of cancer, including gestational trophoblastic neoplasia [106], Wilms tumor [107], rhabdomyosarcoma [108], Ewing's sarcoma [109], and malignant hydatidiform mole [110].

CX-5461 was identified by screening for selective inhibitors of Pol I but not Pol II transcription. CX-5461 specifically inhibits ribosomal RNA transcription by impairment of SL1 binding to the rDNA promoter [111] and thus exhibits anti-cancer activity [83, 111]. The inhibition of rRNA synthesis by CX-5461 leads to senescence and autophagy in a p53-independent manner in a tumor cell line [111], to activation of p53-dependent apoptotic signaling in Myc-overexpressing B-lymphoma cells (Eμ-Myc lymphoma cells) [112], and to activation of the ATM/Ataxia Telangiectasia and Rad3-related protein (ATR) pathway in acute lymphoblastic leukemia to induce G2 arrest and apoptosis [113]. The potential therapeutic effect of CX-5461 was demonstrated in xenograft models using human pancreatic carcinoma (MIA PaCa-2), melanoma (A375) [111], biphenotypic B myelomonocytic leukemia (MV 4;11) [112] and breast cancer susceptibility gene II (BRCA2) deficient colon cancer (HCT116) [114], and in mice

models transplanted with p53 wild-type Eμ-Myc lymphoma [112]. The treatment of CX-5461 in these experiments hardly affected on the health and body weights of mice [111, 112].

BMH-21, which was identified by cell-based screening, intercalates into GC-rich sequences, which exist at a high frequency in rDNA, and represses Pol I transcription [115]. Treatment with BMH-21 induces proteasome-dependent degradation of the largest catalytic subunit of Pol I, RPA194, resulting in a decrease of the Pol I level on rDNA. These effects were correlated to the anti-cancer activity of BHM-21. The anti-tumor activity of BMH-21 was demonstrated using human melanoma (A375) and colorectal carcinoma (HCT116) xenograft models with little effect on body weight [115]. These studies suggest that the chemicals that repress the rRNA transcription show anti-cancer activities.

Epigenetic controls of rDNA chromatin are also candidates for cancer therapy. For example, specific activation of KDM2A could reduce cancer cell proliferation. Because KDM2A activity is regulated by ATP levels through AMPK and also metabolites in energy production, control of these compound levels may regulate KDM2A activity and cell proliferation. As seen here, there are many elaborate mechanisms for control of rRNA transcription, some of which involve intracellular metabolites, which are produced from environmental nutriments. Further studies of the relationship between rRNA transcription and nutrients will provide information about the mechanisms by which cells reconcile demand and usage of biological resources, and clues for novel methods to treat cancers.

10. Conclusion

The construction of ribosomes consumes the majority of the cell's materials and energy. Because the materials for ribosome production are supplied by nutrients, the production of ribosomes is largely restricted by environmental nutrients and cells need mechanisms to control ribosome production in order to reconcile demands for cell activities with available resources. Transcription of rRNA is an essential step in ribosome biogenesis, and strongly affects the total amount of ribosome production. Ribosomal RNA transcription is controlled by many mechanisms, including the efficiency of PIC formation for Pol I and epigenetic marks in rDNA. These are affected by nutrients. Recent studies suggest that the epigenetic marks, such as acetylation and methylation, may be not only controlled by nutrients but also function as reservoirs for biological resources in chromatin. Elevated levels of rRNA transcription and protein synthesis are often observed in cancer cells, and the control of rRNA transcription can regulate their proliferation. Indeed some chemicals that repress the rRNA transcription show anti-cancer activities. Further studies of the relationship between rRNA transcription and nutrients will provide clues for novel methods to treat cancers.

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Abbreviations

AMPK	AMP-activated kinase
ATM	Ataxia telangiectasia mutated
Cdk	Cyclin dependent kinase
CK2	Casein kinase 2
ERK	Extracellular signal-regulated kinase
FBP	Fructose 1, 6-bisphosphate
GSK3β	Glycogen synthase kinase 3β
H3K36me2	Dimethylated lysine 36 on histone H3
H4K20me3	Trimethylated lysine 20 on histone H4
HDAC	Histone deacetylase
KDM2A	Lysine(K)-specific demethylase 2A
KDMs	Lysine(K)-specific demethylases
lncRNAs	Long non-coding RNAs
mTOR	Mammalian target of rapamycin
NuRD	Nucleosome remodeling deacetylase
PAPAS	Promoter and pre-rRNA antisense
PCAF	p300/CBP-Associated factor
PI3K	Phosphoinositide 3-kinase
PIC	Preinitiation complex
PIH1	PIH1 domain-containing protein 1
Pol I	RNA polymerase I
pre-rRNA	Pre-ribosomal RNA
PTEN	Phosphatase and tensin homolog deleted from chromosome 10
rDNA	Ribosome RNA gene
rRNA	Ribosomal RNA
RSK	ERK/90 kDa ribosomal S6 kinase
S6K	Ribosomal protein S6 kinase
SAM	S-adenosylmethionine

SIRT1	Sirtuin 1
SL1	Promoter selective factor 1
Suv420h2	Suppressor of variegation 4-20 homolog
TAF_Is	TBP-associated factors for RNA polymerase I
TBP	TATA-box binding protein
TIF-IA	Transcription initiation factor IA
UBF	Upstream binding factor

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References

[1] Khatter H, Myasnikov AG, Natchiar SK, Klaholz BP. Structure of the human 80S ribosome. Nature. 2015;**520**(7549):640-645. DOI: 10.1038/nature14427

[2] Melnikov S, Ben-Shem A, Garreau de Loubresse N, Jenner L, Yusupova G, Yusupov M. One core, two shells: Bacterial and eukaryotic ribosomes. Nature Structural & Molecular Biology. 2012;**19**(6):560-567. DOI: 10.1038/nsmb.2313

[3] Schmidt EV. The role of c-myc in regulation of translation initiation. Oncogene. 2004;**23**(18):3217-3221. DOI: 10.1038/sj.onc.1207548

[4] Kusnadi EP, Hannan KM, Hicks RJ, Hannan RD, Pearson RB, Kang J. Regulation of rDNA transcription in response to growth factors, nutrients and energy. Gene. 2015;**556**(1):27-34. DOI: 10.1016/j.gene.2014.11.010

[5] Thomas G. An encore for ribosome biogenesis in the control of cell proliferation. Nature Cell Biology. 2000;**2**(5):E71-E72. DOI: 10.1038/35010581

[6] Olson MOJ. The Nucleolus. Vol. xxv. New York: Springer; 2011. p. 414, DOI: absent

[7] Grummt I. Different epigenetic layers engage in complex crosstalk to define the epigenetic state of mammalian rRNA genes. Human Molecular Genetics. 2007;**16**(Spec No 1): R21-R27. DOI: 10.1093/hmg/ddm020

[8] Grummt I. Life on a planet of its own: Regulation of RNA polymerase I transcription in the nucleolus. Genes & Development. 2003;**17**(14):1691-1702. DOI: 10.1101/gad.1098503R

[9] Grummt I. Wisely chosen paths – regulation of rRNA synthesis: Delivered on 30 June 2010 at the 35th FEBS Congress in Gothenburg, Sweden. The FEBS Journal. 2010;**277**(22): 4626-4639. DOI: 10.1111/j.1742-4658.2010.07892.x

[10] Jackson DA, Pombo A, Iborra F. The balance sheet for transcription: An analysis of nuclear RNA metabolism in mammalian cells. The FASEB Journal 2000;**14**(2):242-254. DOI: absent

[11] Drygin D, Rice WG, Grummt I. The RNA polymerase I transcription machinery: An emerging target for the treatment of cancer. Annual Review of Pharmacology and Toxicology. 2010;**50**:131-156. DOI: 10.1146/annurev.pharmtox.010909.105844

[12] Zomerdijk JC, Beckmann H, Comai L, Tjian R. Assembly of transcriptionally active RNA polymerase I initiation factor SL1 from recombinant subunits. Science 1994;**266**(5193): 2015-2018. DOI: absent

[13] Goodfellow SJ, Zomerdijk JC. Basic mechanisms in RNA polymerase I transcription of the ribosomal RNA genes. Sub-Cellular Biochemistry. 2013;**61**:211-236. DOI: 10.1007/978-94-007-4525-4_10

[14] Loyer P, Trembley JH, Katona R, Kidd VJ, Lahti JM. Role of CDK/cyclin complexes in transcription and RNA splicing. Cellular Signalling. 2005;**17**(9):1033-1051. DOI: 10.1016/j.cellsig.2005.02.005

[15] Lim S, Kaldis P. Cdks, cyclins and CKIs: Roles beyond cell cycle regulation. Development. 2013;**140**(15):3079-3093. DOI: 10.1242/dev.091744

[16] Heix J, Vente A, Voit R, Budde A, Michaelidis TM, Grummt I. Mitotic silencing of human rRNA synthesis: Inactivation of the promoter selectivity factor SL1 by cdc2/cyclin B-mediated phosphorylation. The EMBO Journal. 1998;**17**(24):7373-7381. DOI: 10.1093/emboj/17.24.7373

[17] Kuhn A, Vente A, Doree M, Grummt I. Mitotic phosphorylation of the TBP-containing factor SL1 represses ribosomal gene transcription. Journal of Molecular Biology. 1998; **284**(1):1-5. DOI: 10.1006/jmbi.1998.2164

[18] Voit R, Seiler J, Grummt I. Cooperative action of Cdk1/cyclin B and SIRT1 is required for mitotic repression of rRNA synthesis. PLoS Genetics. 2015;**11**(5):e1005246. DOI: 10.1371/journal.pgen.1005246

[19] Voit R, Hoffmann M, Grummt I. Phosphorylation by G1-specific cdk-cyclin complexes activates the nucleolar transcription factor UBF. The EMBO Journal. 1999;**18**(7):1891-1899. DOI: 10.1093/emboj/18.7.1891

[20] Voit R, Grummt I. Phosphorylation of UBF at serine 388 is required for interaction with RNA polymerase I and activation of rDNA transcription. Proceedings of the National

Academy of Sciences of the United States of America. 2001;**98**(24):13631-13636. DOI: 10.1073/pnas.231071698

[21] Voit R, Schnapp A, Kuhn A, Rosenbauer H, Hirschmann P, Stunnenberg HG, et al. The nucleolar transcription factor mUBF is phosphorylated by casein kinase II in the C-terminal hyperacidic tail which is essential for transactivation. The EMBO Journal 1992;**11**(6):2211-2218. DOI: absent

[22] Kihm AJ, Hershey JC, Haystead TA, Madsen CS, Owens GK. Phosphorylation of the rRNA transcription factor upstream binding factor promotes its association with TATA binding protein. Proceedings of the National Academy of Sciences of the United States of America 1998;**95**(25):14816-14820. DOI: absent

[23] Lin CY, Navarro S, Reddy S, Comai L. CK2-mediated stimulation of Pol I transcription by stabilization of UBF-SL1 interaction. Nucleic Acids Research. 2006;**34**(17):4752-4766. DOI: 10.1093/nar/gkl581

[24] Panova TB, Panov KI, Russell J, Zomerdijk JC. Casein kinase 2 associates with initiation-competent RNA polymerase I and has multiple roles in ribosomal DNA transcription. Molecular and Cellular Biology. 2006;**26**(16):5957-5968. DOI: 10.1128/MCB.00673-06

[25] Bierhoff H, Dundr M, Michels AA, Grummt I. Phosphorylation by casein kinase 2 facilitates rRNA gene transcription by promoting dissociation of TIF-IA from elongating RNA polymerase I. Molecular and Cellular Biology. 2008;**28**(16):4988-4998. DOI: 10.1128/MCB.00492-08

[26] Nguyen le XT, Mitchell BS. Akt activation enhances ribosomal RNA synthesis through casein kinase II and TIF-IA. Proceedings of the National Academy of Sciences of the United States of America. 2013;**110**(51):20681-20686. DOI: 10.1073/pnas.1313097110

[27] Mayer C, Zhao J, Yuan X, Grummt I. mTOR-dependent activation of the transcription factor TIF-IA links rRNA synthesis to nutrient availability. Genes & Development. 2004;**18**(4):423-434. DOI: 10.1101/gad.285504

[28] Hannan KM, Brandenburger Y, Jenkins A, Sharkey K, Cavanaugh A, Rothblum L, et al. mTOR-dependent regulation of ribosomal gene transcription requires S6K1 and is mediated by phosphorylation of the carboxy-terminal activation domain of the nucleolar transcription factor UBF. Molecular and Cellular Biology 2003;**23**(23):8862-8877. DOI: absent

[29] Lee D, An J, Park YU, Liaw H, Woodgate R, Park JH, et al. SHPRH regulates rRNA transcription by recognizing the histone code in an mTOR-dependent manner. Proceedings of the National Academy of Sciences of the United States of America. 2017;**114**(17): E3424-E3433. DOI: 10.1073/pnas.1701978114

[30] Salifou K, Ray S, Verrier L, Aguirrebengoa M, Trouche D, Panov KI, et al. The histone demethylase JMJD2A/KDM4A links ribosomal RNA transcription to nutrients and growth factors availability. Nature Communications. 2016;**7**:10174. DOI: 10.1038/ncomms10174

[31] Liu L, Pilch PF. PTRF/Cavin-1 promotes efficient ribosomal RNA transcription in response to metabolic challenges. eLife. 2016;**5**:e17508. DOI: 10.7554/eLife.17508

[32] Stefanovsky VY, Pelletier G, Hannan R, Gagnon-Kugler T, Rothblum LI, Moss T. An immediate response of ribosomal transcription to growth factor stimulation in mammals is mediated by ERK phosphorylation of UBF. Molecular Cell. 2001;**8**(5):1063-1073. DOI: S1097-2765(01)00384-7

[33] Stefanovsky V, Langlois F, Gagnon-Kugler T, Rothblum LI, Moss T. Growth factor signaling regulates elongation of RNA polymerase I transcription in mammals via UBF phosphorylation and r-chromatin remodeling. Molecular Cell. 2006;**21**(5):629-639. DOI: 10.1016/j.molcel.2006.01.023

[34] Zhao J, Yuan X, Frodin M, Grummt I. ERK-dependent phosphorylation of the transcription initiation factor TIF-IA is required for RNA polymerase I transcription and cell growth. Molecular Cell. 2003;**11**(2):405-413. DOI: S1097-2765(03)00036-4

[35] Tsoi H, Lam KC, Dong Y, Zhang X, Lee CK, Zhang J, et al. Pre-45s rRNA promotes colon cancer and is associated with poor survival of CRC patients. Oncogene. 2017;**36**:6109-6118. DOI: 10.1038/onc.2017.86

[36] Arabi A, Wu S, Ridderstrale K, Bierhoff H, Shiue C, Fatyol K, et al. c-Myc associates with ribosomal DNA and activates RNA polymerase I transcription. Nature Cell Biology. 2005;**7**(3):303-310. DOI: 10.1038/ncb1225

[37] Frescas D, Guardavaccaro D, Bassermann F, Koyama-Nasu R, Pagano M. JHDM1B/FBXL10 is a nucleolar protein that represses transcription of ribosomal RNA genes. Nature. 2007;**450**(7167):309-313. DOI: 10.1038/nature06255

[38] Bierhoff H, Dammert MA, Brocks D, Dambacher S, Schotta G, Grummt I. Quiescence-induced LncRNAs trigger H4K20 trimethylation and transcriptional silencing. Molecular Cell. 2014;**54**(4):675-682. DOI: 10.1016/j.molcel.2014.03.032

[39] Xie W, Ling T, Zhou Y, Feng W, Zhu Q, Stunnenberg HG, et al. The chromatin remodeling complex NuRD establishes the poised state of rRNA genes characterized by bivalent histone modifications and altered nucleosome positions. Proceedings of the National Academy of Sciences of the United States of America. 2012;**109**(21):8161-8166. DOI: 10.1073/pnas.1201262109

[40] Grandori C, Gomez-Roman N, Felton-Edkins ZA, Ngouenet C, Galloway DA, Eisenman RN, et al. c-Myc binds to human ribosomal DNA and stimulates transcription of rRNA genes by RNA polymerase I. Nature Cell Biology. 2005;**7**(3):311-318. DOI: 10.1038/ncb1224

[41] Poortinga G, Hannan KM, Snelling H, Walkley CR, Jenkins A, Sharkey K, et al. MAD1 and c-MYC regulate UBF and rDNA transcription during granulocyte differentiation. The EMBO Journal. 2004;**23**(16):3325-3335. DOI: 10.1038/sj.emboj.7600335

[42] Dai MS, Lu H. Crosstalk between c-Myc and ribosome in ribosomal biogenesis and cancer. Journal of Cellular Biochemistry. 2008;**105**(3):670-677. DOI: 10.1002/jcb.21895

[43] Poortinga G, Wall M, Sanij E, Siwicki K, Ellul J, Brown D, et al. c-MYC coordinately regulates ribosomal gene chromatin remodeling and Pol I availability during granulocyte differentiation. Nucleic Acids Research. 2011;**39**(8):3267-3281. DOI: 10.1093/nar/gkq1205

[44] Schmidt EV. The role of c-myc in cellular growth control. Oncogene. 1999;**18**(19):2988-2996. DOI: 10.1038/sj.onc.1202751

[45] van Riggelen J, Yetil A, Felsher DW. MYC as a regulator of ribosome biogenesis and protein synthesis. Nature Reviews. Cancer 2010;**10**(4):301-309. DOI: 10.1038/nrc2819

[46] Cavanaugh AH, Hempel WM, Taylor LJ, Rogalsky V, Todorov G, Rothblum LI. Activity of RNA polymerase I transcription factor UBF blocked by Rb gene product. Nature. 1995;**374**(6518):177-180. DOI: 10.1038/374177a0

[47] Voit R, Schafer K, Grummt I. Mechanism of repression of RNA polymerase I transcription by the retinoblastoma protein. Molecular and Cellular Biology 1997;**17**(8):4230-4237. DOI: absent

[48] Hannan KM, Hannan RD, Smith SD, Jefferson LS, Lun M, Rothblum LI. Rb and p130 regulate RNA polymerase I transcription: Rb disrupts the interaction between UBF and SL-1. Oncogene. 2000;**19**(43):4988-4999. DOI: 10.1038/sj.onc.1203875

[49] Pelletier G, Stefanovsky VY, Faubladier M, Hirschler-Laszkiewicz I, Savard J, Rothblum LI, et al. Competitive recruitment of CBP and Rb-HDAC regulates UBF acetylation and ribosomal transcription. Molecular Cell. 2000;**6**(5):1059-1066. DOI: S1097-2765(00)00104-0

[50] Zhai W, Comai L. Repression of RNA polymerase I transcription by the tumor suppressor p53. Molecular and Cellular Biology 2000;**20**(16):5930-5938. DOI: absent

[51] Zhang C, Comai L, Johnson DL. PTEN represses RNA polymerase I transcription by disrupting the SL1 complex. Molecular and Cellular Biology. 2005;**25**(16):6899-6911. DOI: 10.1128/MCB.25.16.6899-6911.2005

[52] Al-Khouri AM, Ma Y, Togo SH, Williams S, Mustelin T. Cooperative phosphorylation of the tumor suppressor phosphatase and tensin homologue (PTEN) by casein kinases and glycogen synthase kinase 3beta. The Journal of Biological Chemistry. 2005;**280**(42):35195-35202. DOI: 10.1074/jbc.M503045200

[53] Vincent T, Kukalev A, Andang M, Pettersson R, Percipalle P. The glycogen synthase kinase (GSK) 3beta represses RNA polymerase I transcription. Oncogene. 2008;**27**(39):5254-5259. DOI: 10.1038/onc.2008.152

[54] Johnson GL, Nakamura K. The c-jun kinase/stress-activated pathway: Regulation, function and role in human disease. Biochimica et Biophysica Acta. 2007;**1773**(8):1341-1348. DOI: 10.1016/j.bbamcr.2006.12.009

[55] Mayer C, Bierhoff H, Grummt I. The nucleolus as a stress sensor: JNK2 inactivates the transcription factor TIF-IA and down-regulates rRNA synthesis. Genes & Development. 2005;**19**(8):933-941. DOI: 10.1101/gad.333205

[56] Larsen DH, Hari F, Clapperton JA, Gwerder M, Gutsche K, Altmeyer M, et al. The NBS1-treacle complex controls ribosomal RNA transcription in response to DNA damage. Nature Cell Biology. 2014;**16**(8):792-803. DOI: 10.1038/ncb3007

[57] Kruhlak M, Crouch EE, Orlov M, Montano C, Gorski SA, Nussenzweig A, et al. The ATM repair pathway inhibits RNA polymerase I transcription in response to chromosome breaks. Nature. 2007;**447**(7145):730-734. DOI: 10.1038/nature05842

[58] Johnston R, D'Costa Z, Ray S, Gorski J, Harkin DP, Mullan P, et al. The identification of a novel role for BRCA1 in regulating RNA polymerase I transcription. Oncotarget. 2016;**7**(42):68097-68110. DOI: 10.18632/oncotarget.11770

[59] Zhao Z, Dammert MA, Hoppe S, Bierhoff H, Grummt I. Heat shock represses rRNA synthesis by inactivation of TIF-IA and lncRNA-dependent changes in nucleosome positioning. Nucleic Acids Research. 2016;**44**(17):8144-8152. DOI: 10.1093/nar/gkw496

[60] Zhao Z, Dammert MA, Grummt I, Bierhoff H. lncRNA-induced nucleosome repositioning reinforces transcriptional repression of rRNA genes upon hypotonic stress. Cell Reports. 2016;**14**(8):1876-1882. DOI: 10.1016/j.celrep.2016.01.073

[61] Wu TH, Kuo YY, Lee HH, Kuo JC, Ou MH, Chang ZF. Epigenetic repression of ribosomal RNA transcription by ROCK-dependent aberrant cytoskeletal organization. Scientific Reports. 2016;**6**:28685. DOI: 10.1038/srep28685

[62] Moor AE, Golan M, Massasa EE, Lemze D, Weizman T, Shenhav R, et al. Global mRNA polarization regulates translation efficiency in the intestinal epithelium. Science. 2017;**357**(6357):1299-1303. DOI: 10.1126/science.aan2399

[63] Ma XM, Blenis J. Molecular mechanisms of mTOR-mediated translational control. Nature Reviews. Molecular Cell Biology. 2009;**10**(5):307-318. DOI: 10.1038/nrm2672

[64] Gonzalez A, Hall MN. Nutrient sensing and TOR signaling in yeast and mammals. The EMBO Journal. 2017;**36**(4):397-408. DOI: 10.15252/embj.201696010

[65] Grummt I, Smith VA, Grummt F. Amino acid starvation affects the initiation frequency of nucleolar RNA polymerase. Cell. 1976;**7**(3):439-445. DOI: 0092-8674(76)90174-4

[66] Yuan X, Zhao J, Zentgraf H, Hoffmann-Rohrer U, Grummt I. Multiple interactions between RNA polymerase I, TIF-IA and TAF(I) subunits regulate preinitiation complex assembly at the ribosomal gene promoter. EMBO Reports. 2002;**3**(11):1082-1087. DOI: 10.1093/embo-reports/kvf212

[67] James MJ, Zomerdijk JC. Phosphatidylinositol 3-kinase and mTOR signaling pathways regulate RNA polymerase I transcription in response to IGF-1 and nutrients. The Journal of Biological Chemistry. 2004;**279**(10):8911-8918. DOI: 10.1074/jbc.M307735200

[68] West MJ, Stoneley M, Willis AE. Translational induction of the c-myc oncogene via activation of the FRAP/TOR signalling pathway. Oncogene. 1998;**17**(6):769-780. DOI: 10.1038/sj.onc.1201990

[69] Csibi A, Lee G, Yoon SO, Tong H, Ilter D, Elia I, et al. The mTORC1/S6K1 pathway regulates glutamine metabolism through the eIF4B-dependent control of c-Myc translation. Current Biology. 2014;**24**(19):2274-2280. DOI: 10.1016/j.cub.2014.08.007

[70] Kang J, Kusnadi EP, Ogden AJ, Hicks RJ, Bammert L, Kutay U, et al. Amino acid-dependent signaling via S6K1 and MYC is essential for regulation of rDNA transcription. Oncotarget. 2016;**7**(31):48887-48904. DOI: 10.18632/oncotarget.10346

[71] Grummt I, Grummt F. Control of nucleolar RNA synthesis by the intracellular pool sizes of ATP and GTP. Cell. 1976;**7**(3):447-453. DOI: 0092-8674(76)90175-6

[72] Nguyen le XT, Lee Y, Urbani L, Utz PJ, Hamburger AW, Sunwoo JB, et al. Regulation of ribosomal RNA synthesis in T cells: Requirement for GTP and Ebp1. Blood. 2015;**125**(16):2519-2529. DOI: 10.1182/blood-2014-12-616433

[73] Squatrito M, Mancino M, Donzelli M, Areces LB, Draetta GF. EBP1 is a nucleolar growth-regulating protein that is part of pre-ribosomal ribonucleoprotein complexes. Oncogene. 2004;**23**(25):4454-4465. DOI: 10.1038/sj.onc.1207579

[74] Mariappan MM, D'Silva K, Lee MJ, Sataranatarajan K, Barnes JL, Choudhury GG, et al. Ribosomal biogenesis induction by high glucose requires activation of upstream binding factor in kidney glomerular epithelial cells. American Journal of Physiology. Renal Physiology. 2011;**300**(1):F219-F230. DOI: 10.1152/ajprenal.00207.2010

[75] Zhai N, Zhao ZL, Cheng MB, Di YW, Yan HX, Cao CY, et al. Human PIH1 associates with histone H4 to mediate the glucose-dependent enhancement of pre-rRNA synthesis. Journal of Molecular Cell Biology. 2012;**4**(4):231-241. DOI: 10.1093/jmcb/mjs003

[76] Zhou Y, Grummt I. The PHD finger/bromodomain of NoRC interacts with acetylated histone H4K16 and is sufficient for rDNA silencing. Current Biology. 2005;**15**(15):1434-1438. DOI: 10.1016/j.cub.2005.06.057

[77] Kamano Y, Saeki M, Egusa H, Kakihara Y, Houry WA, Yatani H, et al. PIH1D1 interacts with mTOR complex 1 and enhances ribosome RNA transcription. FEBS Letters. 2013;**587**(20):3303-3308. DOI: 10.1016/j.febslet.2013.09.001

[78] Zhang CS, Hawley SA, Zong Y, Li M, Wang Z, Gray A, et al. Fructose-1,6-bisphosphate and alDOIase mediate glucose sensing by AMPK. Nature. 2017;**548**(7665):112-116. DOI: 10.1038/nature23275

[79] Hoppe S, Bierhoff H, Cado I, Weber A, Tiebe M, Grummt I, et al. AMP-activated protein kinase adapts rRNA synthesis to cellular energy supply. Proceedings of the National Academy of Sciences of the United States of America. 2009;**106**(42):17781-17786. DOI: 10.1073/pnas.0909873106

[80] Tsukada Y, Fang J, Erdjument-Bromage H, Warren ME, Borchers CH, Tempst P, et al. Histone demethylation by a family of JmjC domain-containing proteins. Nature. 2006; **439**(7078):811-816. DOI: 10.1038/nature04433

[81] Tanaka Y, Okamoto K, Teye K, Umata T, Yamagiwa N, Suto Y, et al. JmjC enzyme KDM2A is a regulator of rRNA transcription in response to starvation. The EMBO Journal. 2010;**29**(9): 1510-1522. DOI: 10.1038/emboj.2010.56

[82] Tanaka Y, Umata T, Okamoto K, Obuse C, Tsuneoka M. CxxC-ZF domain is needed for KDM2A to demethylate histone in rDNA promoter in response to starvation. Cell Structure and Function. 2014;**39**(1):79-92. DOI: 10.1247/csf.13022

[83] Tanaka Y, Yano H, Ogasawara S, Yoshioka S, Imamura H, Okamoto K, et al. Mild glucose starvation induces KDM2A-mediated H3K36me2 Demethylation through AMPK to reduce rRNA transcription and cell proliferation. Molecular and Cellular Biology. 2015;**35**(24): 4170-4184. DOI: 10.1128/MCB.00579-15

[84] Inoki K, Zhu T, Guan KL. TSC2 mediates cellular energy response to control cell growth and survival. Cell. 2003;**115**(5):577-590. DOI: 10.1016/S0092-8674(03)00929-2

[85] Yang L, Song T, Chen L, Kabra N, Zheng H, Koomen J, et al. Regulation of SirT1-nucleomethylin binding by rRNA coordinates ribosome biogenesis with nutrient availability. Molecular and Cellular Biology. 2013;**33**(19):3835-3848. DOI: 10.1128/MCB.00476-13

[86] Song T, Yang L, Kabra N, Chen L, Koomen J, Haura EB, et al. The NAD^+ synthesis enzyme nicotinamide mononucleotide adenylyltransferase (NMNAT1) regulates ribosomal RNA transcription. The Journal of Biological Chemistry. 2013;**288**(29):20908-20917. DOI: 10.1074/jbc.M113.470302

[87] Chen S, Seiler J, Santiago-Reichelt M, Felbel K, Grummt I, Voit R. Repression of RNA polymerase I upon stress is caused by inhibition of RNA-dependent deacetylation of PAF53 by SIRT7. Molecular Cell. 2013;**52**(3):303-313. DOI: 10.1016/j.molcel.2013.10.010

[88] Ford E, Voit R, Liszt G, Magin C, Grummt I, Guarente L. Mammalian Sir2 homolog SIRT7 is an activator of RNA polymerase I transcription. Genes & Development. 2006;**20**(9):1075-1080. DOI: 10.1101/gad.1399706

[89] Hanada K, Song CZ, Yamamoto K, Yano K, Maeda Y, Yamaguchi K, et al. RNA polymerase I associated factor 53 binds to the nucleolar transcription factor UBF and functions in specific rDNA transcription. The EMBO Journal 1996;**15**(9):2217-2226. DOI: absent

[90] Meraner J, Lechner M, Loidl A, Goralik-Schramel M, Voit R, Grummt I, et al. Acetylation of UBF changes during the cell cycle and regulates the interaction of UBF with RNA polymerase I. Nucleic Acids Research. 2006;**34**(6):1798-1806. DOI: 10.1093/nar/gkl101

[91] Rothblum LI, Rothblum K, Chang E. PAF53 is essential in mammalian cells: CRISPR/Cas9 fails to eliminate PAF53 expression. Gene. 2017;**612**:55-60. DOI: 10.1016/j.gene.2016.12.023

[92] Wong CC, Qian Y, Yu J. Interplay between epigenetics and metabolism in oncogenesis: Mechanisms and therapeutic approaches. Oncogene. 2017;**36**(24):3359-3374. DOI: 10.1038/onc.2016.485

[93] Kaelin WG, Jr., McKnight SL. Influence of metabolism on epigenetics and disease. Cell 2013;**153**(1):56-69. DOI: 10.1016/j.cell.2013.03.004

[94] Krautkramer KA, Rey FE, Denu JM. Chemical signaling between gut microbiota and host chromatin: What is your gut really saying? The Journal of Biological Chemistry. 2017;**292**(21):8582-8593. DOI: 10.1074/jbc.R116.761577

[95] Wang T, Yu Q, Li J, Hu B, Zhao Q, Ma C, et al. O-GlcNAcylation of fumarase maintains tumour growth under glucose deficiency. Nature Cell Biology. 2017;**19**(7):833-843. DOI: 10.1038/ncb3562

[96] Losman JA, Kaelin WG, Jr. What a difference a hydroxyl makes: Mutant IDH, (R)-2-hydroxyglutarate, and cancer. Genes & Development 2013;**27**(8):836-852. DOI: 10.1101/gad.217406.113

[97] Chowdhury R, Yeoh KK, Tian YM, Hillringhaus L, Bagg EA, Rose NR, et al. The oncometabolite 2-hydroxyglutarate inhibits histone lysine demethylases. EMBO Reports. 2011;**12**(5):463-469. DOI: 10.1038/embor.2011.43

[98] Xu W, Yang H, Liu Y, Yang Y, Wang P, Kim SH, et al. Oncometabolite 2-hydroxygluta rate is a competitive inhibitor of alpha-ketoglutarate-dependent dioxygenases. Cancer Cell. 2011;**19**(1):17-30. DOI: 10.1016/j.ccr.2010.12.014

[99] Tong L, Denu JM. Function and metabolism of sirtuin metabolite O-acetyl-ADP-ribose. Biochimica et Biophysica Acta. 2010;**1804**(8):1617-1625. DOI: 10.1016/j.bbapap.2010.02.007

[100] Burgos-Barragan G, Wit N, Meiser J, Dingler FA, Pietzke M, Mulderrig L, et al. Mammals divert endogenous genotoxic formaldehyde into one-carbon metabolism. Nature. 2017;**548**(7669):549-554. DOI: 10.1038/nature23481

[101] Ruggero D. Revisiting the nucleolus: From marker to dynamic integrator of cancer signaling. Science Signaling. 2012;**5**(241):pe38. DOI: 10.1126/scisignal.2003477

[102] Quin JE, Devlin JR, Cameron D, Hannan KM, Pearson RB, Hannan RD. Targeting the nucleolus for cancer intervention. Biochimica et Biophysica Acta. 2014;**1842**(6):802-816. DOI: 10.1016/j.bbadis.2013.12.009

[103] Jordan P, Carmo-Fonseca M. Cisplatin inhibits synthesis of ribosomal RNA in vivo. Nucleic Acids Research. 1998;**26**(12):2831-2836. DOI: gkb492

[104] Rey JP, Scott R, Muller H. Induction and removal of interstrand crosslinks in the ribosomal RNA genes of lymphoblastoid cell lines from patients with Fanconi anemia. Mutation Research. 1993;**289**(2):171-180. DOI: 0027-5107(93)90067-P

[105] Ghoshal K, Jacob ST. An alternative molecular mechanism of action of 5-fluorouracil, a potent anticancer drug. Biochemical Pharmacology. 1997;**53**(11):1569-1575. DOI: S0006-2952(97)00040-3

[106] Turan T, Karacay O, Tulunay G, Boran N, Koc S, Bozok S, et al. Results with EMA/CO (etoposide, methotrexate, actinomycin D, cyclophosphamide, vincristine) chemotherapy in gestational trophoblastic neoplasia. International Journal of Gynecological Cancer. 2006;**16**(3):1432-1438. DOI: 10.1111/j.1525-1438.2006.00606.x

[107] D'Angio GJ, Evans A, Breslow N, Beckwith B, Bishop H, Farewell V, et al. The treatment of Wilms' tumor: Results of the second National Wilms' tumor study. Cancer 1981;**47**(9):2302-2311. DOI: absent

[108] Khatua S, Nair CN, Ghosh K. Immune-mediated thrombocytopenia following dactinomycin therapy in a child with alveolar rhabdomyosarcoma: The unresolved issues. Journal of Pediatric Hematology/Oncology. 2004;**26**(11):777-779. DOI: 00043426-2004 11000-00020

[109] Jaffe N, Paed D, Traggis D, Salian S, Cassady JR. Improved outlook for Ewing's sarcoma with combination chemotherapy (vincristine, actinomycin D and cyclophosphamide) and radiation therapy. Cancer 1976;**38**(5):1925-1930. DOI: absent

[110] Uberti EM, Fajardo Mdo C, Ferreira SV, Pereira MV, Seger RC, Moreira MA, et al. Reproductive outcome after discharge of patients with high-risk hydatidiform mole with or without use of one bolus dose of actinomycin D, as prophylactic chemotherapy, during the uterine evacuation of molar pregnancy. Gynecologic Oncology. 2009;**115**(3):476 481. DOI: 10.1016/j.ygyno.2009.09.012

[111] Drygin D, Lin A, Bliesath J, Ho CB, O'Brien SE, Proffitt C, et al. Targeting RNA polymerase I with an oral small molecule CX-5461 inhibits ribosomal RNA synthesis and solid tumor growth. Cancer Research. 2011;**71**(4):1418-1430. DOI: 10.1158/0008-5472.CAN-10-1728

[112] Bywater MJ, Poortinga G, Sanij E, Hein N, Peck A, Cullinane C, et al. Inhibition of RNA polymerase I as a therapeutic strategy to promote cancer-specific activation of p53. Cancer Cell. 2012;**22**(1):51-65. DOI: 10.1016/j.ccr.2012.05.019

[113] Negi SS, Brown P. rRNA synthesis inhibitor, CX-5461, activates ATM/ATR pathway in acute lymphoblastic leukemia, arrests cells in G2 phase and induces apoptosis. Oncotarget. 2015;**6**(20):18094-18104. DOI: 10.18632/oncotarget.4093

[114] Xu H, Di Antonio M, McKinney S, Mathew V, Ho B, O'Neil NJ, et al. CX-5461 is a DNA G-quadruplex stabilizer with selective lethality in BRCA1/2 deficient tumours. Nature Communications. 2017;**8**:14432. DOI: 10.1038/ncomms14432

[115] Peltonen K, Colis L, Liu H, Trivedi R, Moubarek MS, Moore HM, et al. A targeting modality for destruction of RNA polymerase I that possesses anticancer activity. Cancer Cell. 2014;**25**(1):77-90. DOI: 10.1016/j.ccr.2013.12.009

Section 2

Epigenetic Regulation and Development of Cells

Chapter 3

DNA Methylation in Mammalian Cells

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Additional information is available at the end of the chapter

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Abstract

Epigenetic regulation was first studied in the 1970s and has quickly gained global interest. It is involved at many different stages of mammalian cell development. The broad nature of epigenetic regulation has led to it being referred to as an 'all stage' mechanism of regulation as it is implicated in many developmental stages including embryonic development, ageing and in cancer progression. The term 'epigenetic' refers to the alteration of gene expression without changing the genomic sequence. Epigenetic regulation involves the main subtypes of DNA methylation and histone modification, and microRNA expression. Epigenetic alteration is used by mammalian cells to 'turn-on and -off' the gene expression. During embryonic development this process is used to induce cell apoptosis of genes that are no longer useful. During cancer development, epigenetics works to repress tumour suppressor gene expression and activate oncogene expression. The stability and sustainability for the detection of epigenetic markers make it an attractive area for biomarker and drug discovery. In this book chapter we will discuss the key epigenetic processes involved in mammalian cell development and disease progression, specifically in cancer.

Keywords: epigenetic, DNA methylation, histone modification, microRNA, tumour suppressors, cell development

1. Introduction

1.1. Types of epigenetic regulation

The term 'epigenetics' describes the stable modifications of gene expression that occur at different stages of cell development and proliferation [1]. Epigenetic modifications are an essential component for human development and cell differentiation, but can also come about

through random changes and external environmental influences. Different genetic makeups produce different individuals, while the differentiation of organs and various diseases is controlled by epigenetic signatures (**Figure 1**). Epigenetic modification can also be a mechanisms used to protect bacterial or viral modification in the host cell that affect cellular function. There are a number of epigenetic alterations that are required for cellular functions including: DNA methylation and histone modification, and microRNA expression. Epigenetic alterations influence cellular function by altering the protein levels without changing the genomic DNA sequences. These post translational modifications are recognised by specific proteins that are closely associated with DNA as a mediator that causes modifications [2]. The DNA methylation process was first discovered in the 1970s and was later recognised as a major contributor to the stabilisation of gene expression [3].

1.1.1. DNA methylation and histone modification

DNA methylation is an essential process for the normal development of biological systems [4] and its dysregulation has been associated with various pathologies including cancer [5]. Although DNA methylation is known to be integral to the development and is strongly associated with disease, there is limited knowledge on the specifics of its changes during cellular differentiation and its relation to histone methylation and chromatin modifications.

DNA methylation can alter the genome to supress gene expression in mammalian cells. It occurs by the methylation of deocycytosine (dC) bases at the 5′prime position of the cytosine to create deoxymethylcytosine (d^mC). The majority of d^mC are located in CG dinucleotides with approximately 80% of CG pairs being methylated. The majority of these CG pairs are localised to CG-rich DNA regions termed CpG islands. An abundance of transcription factor binding sites exist at CpG islands, which exist in the promoter regions for some genes. CpG islands are present in the promoter regions of approximately half of all mammalian genes [6]. DNA methylation occurs at CG dinucleotides termed CpGs that are distributed globally in 80% of genomes in non-embryonic cells. CpGs sites located at short CpG regions generally remain unmethylated at all times [7, 8]. The majority of CpG islands reside in the promoter

Figure 1. Top panel depicts various species, each with a unique genomic signature. Bottom, within the same individual the differentiation of cell function is controlled by a cell specific epigenetic signature.

region of a gene [9] and are also known to be associated with the transcriptional activity of Sp1 [10]. When CpG islands become methylated during mammalian cell development this can lead to long-term silencing of the associated gene [11].

DNA methyltransferases (DNMT) are a family of enzymes consisting of members DNMT1, DNMT3a and DNMT3b that mediate DNA methylation. These members have different and integral roles to execute DNA methylation; DNMT1 is involved in the maintenance of methylation patterns while DNMT3a and DNMT3b are responsible for *de novo* methylation, that is, methylating DNA that is previously unmethylated [12]. DNA methylation signatures are developed during cell differentiation for the purpose of supressing genes with functions that are unnecessary to the mature cell. This *de novo* methylation is carried out by DNMT3a and DNMT3b [13]. After differentiation, DNMT1 replicates these methylation patterns in the process of mitosis [12]. Unstimulated cells express small amounts of DNMT1, but following mitogenic stimulation the enzyme is upregulated by a sequence of pathways including MAPK/ERK and JNK [14]. DNMT1 is then able to maintain methylation patterns in the DNA by producing symmetrically methylated sites in parent and daughter DNA strands. It accomplishes this by recognising hemi-methylated CG dinucleotides in parent DNA strands and transferring methyl groups from S-adenosylmethione (SAM) to cytosine regions of the unmethylated daughter strand [12]. Alternatively, *de novo* methylation rarely occurs during this normal post-gastrulation development stage and is instead prevalent during processes such as establishment of cell lines *in vitro* [15] and in cancer cells.

DNA methylation is an important process during development and researchers have shown the deletion of *de novo* methylation enzyme DNMT1 in mouse embryonic stem cells led to dramatic DNA hypomethylation [16]. DNMT3a and DNMT3b predominantly exert enzyme activity during the oocyte stage and in early pre-implantation embryos [12]. DNMT3b is also transcribed during zygotic gene activation (ZGA) and highly expressed by blastocysts that acquire epiblast lineage. The absence (by deletion) of DNMT3b led to embryonic lethality and the deletion of DNMT3a was partially viable during development [13]. There is a lower requirement for DNMT3L in DNA methylation which is used predominantly during imprinting control region (ICR) methylation in gametes [17]. However, it is a crucial activating cofactor for DNMT3a [18]. DNMT2 differs structurally from other DNMT and does not present with phenotype modification in knockout mice models which are referred to as a misnomer and depicts methylation activity on RNA [19].

DNA methylation is predominantly found in cytosines of the CG dinucleotide in mammalian cells; this modification is post-replicable. The extent of DNA methylation changes in an orchestrated way during mammalian development, starting with a wave of demethylation during cleavage, followed by genome-wide *de novo* methylation after implantation [20]. Demethylation is an active process that strips the male genome off methylation within hours of fertilisation [21]; by contrast, the maternal genome is only passively demethylated during subsequent cleavage divisions [22]. The extent of methylation in the genome of the gastrulating embryo is high owing to *de novo* methylation, but it tends to decrease in specific tissues during differentiation [17]. *De novo* methylation occurs rarely during normal post-gastrulation development but is seen frequently during the establishment of cell lines *in vitro* [15] and in cancer.

A variety of chromatin modifications can halt the initiation of transposable element (TE) transcription in mammalian cells; modifications of histone tails, chromatin packaging alterations, DNA methylation and condensation are all examples of this. Histone amino (N)-terminal tails modification causes changes to protein factor binding and in turn relays information to transcription factors. DNA methylation of histone H3 at lysine 9 (H3K9) occurs in nucleosomes, that are associated with TE's, leading to transcriptional repression and inactivation of chromatin [23]. Sometimes, mutations can occur in genes that are required for the repression of histone tail modifications, subsequently leading to TE reactivation. A specific example occurred in mouse embryonic stem (ES) cells where mutations of the histone H3K9 DNMT gene suppressor of variegation 3–9 (Su(var)3–9) drove the upregulation of TE transcripts [23].

It is widely understood that histones, specifically H3 and H4, are methylated at lysine (Lys) and arginine (Arg) sites. The predominant regions for Lys-specific methylation on histones that have been catalogued in literature are: Lys9, Lys4, Lys36, Lys27, Lys79 on H3 and Ls20 located on H4 [24]. Additionally, Lys site methylation can occur by mono-, di-, or trimethylation. The differential manners of Lys residue methylation dictate the variety of functional consequences of Lys methylation.

The pioneering study that uncovered the functions of H3 Lys-methylation determined that one of the well-understood Su(var) genes encodes a histone methyltransferase (HMT). The *Drosophlia* SU(Var)3–9 gene was discovered to have roles in transcriptional silencing associated with heterochromatin [25]. The human homologue of this gene, Suv39H1, underwent biochemical analysis and its protein was found to methylate histone H3 at Lys9 using its enzymatic functions [26]. Expanding on this, specific antibodies for methylation of H3 at different sites revealed a pathway for heterochromatin formation [27]. An example of this occurs in *S. pombe*, where heterochromatin formation is initiated by the deacetylation of histone H3 at Lys9 by a histone deacetylase (HDAC) complex that allows the methylation of this site by histone-lysine N-methyltransferase (Clr4). The chromodomain of heterochromatin protein (HP1) can then recognise and bind to this methylated Lys9 motif. This *in vivo* study demonstrated that disruption to the Clr4 gene caused delocalisation of the HP1 homologue Swi6, depicting the requirement of H3 methylation for HP1 and heterochromatin assembly [27]. Genetics studies in *S. pombe* and *Tetrahymena* have illustrated that heterochromatin formation is dependent on genes that code for elements of the RNA interference (RNAi) machinery [28]. Additionally, small RNAs are thought to target histone-modifying activities at silenced regions [29].

Although the association of Lys4-methylated H3 with euchromatic regions is well established, its role in transcriptional activation is not completely understood. Lys4-methylated H3 was found to be directly bound to the yeast chromatin remodelling enzyme Isw1p [30]. Further supporting their association with transcriptional sites, Lys4 methylated H3 was shown to inhibit the binding of the HDAC complex NuRD (nucleosome remodelling and HDAC) to chromatin in a mammalian system [31]. Together, these findings suggest that Lys4-methylated H3 regions indirectly regulate transcription by maintaining promoter genomic regions in a state that favours transcriptional activation.

HDACs drive the deacetylation of acetyl-L-lysine side chains in histones to repress transcription by altering the conformation of chromatin [32]. To date, there are 18 known HDACs including: class I HDACs [33]; class IIa HDACs [34]; class IIb HDACs [35]; class III HDAC enzymes sirtuins [35]; and finally the only class IV enzyme HDAC11 [33]. To catalyse the

deacetylation of histone groups, HDACs together with HDAC related deacetylases, must switch a single metal ion at the metal ion binding site $Mn^{2+}B$ in arginase [36].

1.1.2. microRNA expression

Epigenetic mechanisms play a pivotal role in the regulation of gene expression that forms part of the large complex network that regulates the functioning of eukaryotic cells. MicroRNAs (miRNAs) are a class of small RNA molecules that post-transcriptionally repress gene expression through interaction with the three prime untranslated region/s (3′-UTR) of target messenger RNAs (mRNA)s [37]. miRNAs have a wide variety of functional roles in biological systems that have been extensively studied but the mechanisms controlling their expression are not well understood. In most cases, miRNA expression is initiated by transcription of the miRNA gene by RNA polymerase II. Genes transcribed by RNA polymerase II are frequently regulated by epigenetic mechanisms, so it is likely that DNA methylation regulates the expression of miRNAs. The notion of DNA methylation-based regulation of miRNAs is further supported by the tissue-specific or developmental-stage specific pattern of miRNA expression [38].

A specific study carried out to determine if DNA methylation can alter miRNA expression was carried out using HCT116 colon cancer cells with knockout of DNMT. This model illustrated that approximately 10% of miRNAs studied were regulated by DNA methylation. Furthermore, these miRNAs were shown to be tightly regulated by methylation as shown by a high level of CpG site demethylation required to induce their re-expression. Treatment with 5-aza-2′-deoxycytidine (AZA) or induction of partial demethylation was unsuccessful in upregulating these miRNAs. Collectively, these findings could be directly due to the demethylation of CpG islands in the miRNA promoter sites or be explained instead by the indirect epigenetic regulation of transcription factors acting on these miRNAs [39].

Many miRNAs with tumour suppressor functions have been shown to be silenced by hypermethylation in cancer [40]. miR-148a, miR-34b/c, miR-9-1, miR-9-2 and miR-9-3 were observed to have specific CpG island hyper-methylation associated silencing *in vitro* and *in vivo* in metastatic cancer cells. The metastatic carcinoma cell line SIHN-011B was hypermethylated leading to the repression of miR-148a and miR-34b/c. Transfection of SIHN-011B with expression vectors containing the flanking regions of mature miR-148 and miR-34b/c induced a reduction in migration ability compared to controls in wound-healing assays. Tumour and metastasis formation assays in nude mice depicted a reduction in tumour growth over time following miRNA transfection. These findings illustrate the tumour suppressor activity of these miRNAs, which were found to be explicitly downregulated by CpG island hypermethylation in miRNA promoter regions in these cell lines [41].

The epigenetic regulation of miRNAs is not solely limited to DNA methylation. Two separate studies showed no change in miRNA expression following AZA treatment in lung or bladder cancer cell lines. Combination treatments with a histone deacetylase inhibitor induced miRNA upregulation [42]. To study the mechanisms leading to miRNA regulation more closely a separate group compared the miRNA gene expression profile of a DNMT1 and DNMT3b double knockout cell line model to its associated parental cell line HCT116. Their results depicted notable alterations in miRNA expression in the double knockout model, strongly suggesting that DNA methylation significantly regulates gene expression [39].

2. Epigenetic regulations involvement mammalian cell function gene silencing during development

DNA methylation is the most prevalent form of epigenetic alterations, the most studied and therefore the remainder of this chapter will focus on this area. During mammalian cell development, DNA methylation is an essential component to turn genes 'on and off' [13], however this exact mechanism is still undefined. DNMTs cloning techniques [12] has led to the improved understanding of how DNA methylation proteins and methylation signals influence mammalian cells. The DNA binding protein located on cytosines on the 5′ position of the DNA sequence contributes to the major 'on and off' gene mechanism of mammalian cells. DNA methylation is a heritable trait through mammalian cell development, and these inherited changes of methylation status prompted researchers to develop techniques to identify different stages of cell development. DNA methylation has a multitude of roles in development, specifically methylation of CpG-rich promoter regions and is responsible for the inactivation of the X chromosome and to maintain its silencing. The roles of methylation do not stop with cellular development, the normal functioning of DNA can be affected by methylation and is responsible for the development of human diseases including carcinogenesis. Epigenetic alterations can occur in a mammalian system during different stages of development and can also be effected by external stimuli as shown in **Figure 2** that summarises the regulation of mammalian cells by various epigenetic networks.

Different DNMTs are essential during different stages of vertebrate development and will contribute to cell apoptosis in embryos and fibroblasts [43], but not in ES cells or cancer cells [44].

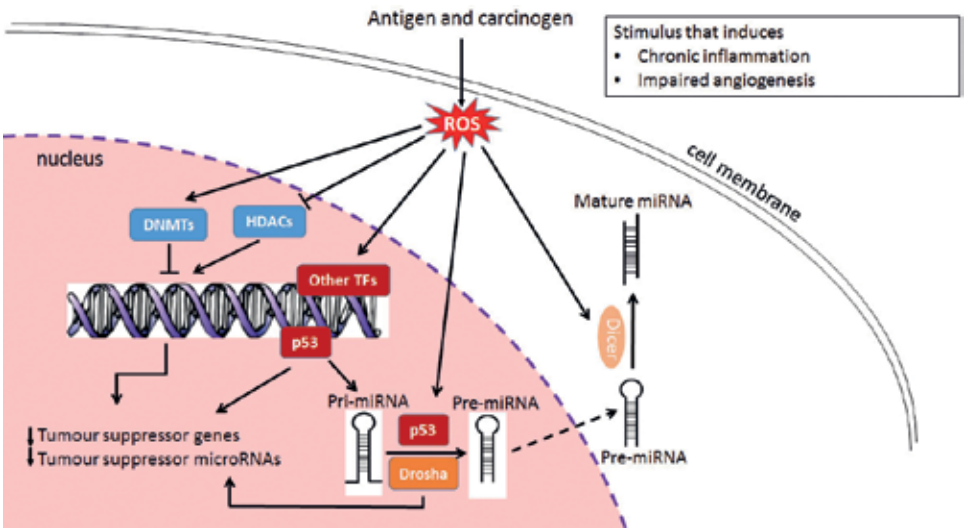

Figure 2. Mammalian cells react to external stimulus such as antigens and carcinogens. The generation of reactive oxygen species (ROS) leading to the alteration of DNA methyltransferase (DNMTs), histone deacetylase (HDACs) and microRNA (miRNA) expression/processing at different stages, will lead to cell apoptosis or epigenetic alterations which allow cells to progress to disease types. TF: transcription factor.

The dysregulated activity of DNMTs results in the repression or activation of gene expression [43] and transcriptional activation elements in diseased mammalian cells [45]. Additionally, aberrant deletion of DNMT1 during brain development has been known to lead to perinatal respiratory distress and malfunction of embryonic development [46]. DNA hypermethylation causes gene silencing via binding of DNMTs to the genome which interferes with the activity of transcription binding proteins that activate gene transcription [47].

2.1. DNA methylation and evaluation of mammalian cells

Genetic variation in individuals can influence phenotypic representation of age and lifespan [48]. However epigenetic and environmental factors also have significant roles in determining physiological changes [49]. This is particularly exemplified by the increasing inherent epigenetic variation present in monozygotic twins with age [50]. DNA methylation status is altered in multiple tissues and specific cell types with age, with the most frequent changes being age-dependent while others remain unchanged with age [51]. Different tissues have specific DNA methylation patterns that results in different cellular functions.

Genome-wide assays have provided a platform for discovering the effect of ageing during the phenotypic alteration in liver cells and a study found that the level of visceral fat is involved in cytosine methylation. This was the first investigation to show that ageing contributes to changes in DNA methylation which is locus-specific in both liver and adipose tissues. These changes appear to cause global hypermethylation in liver specific tissues in genetically identical rat models when exposed to the same environmental conditions throughout their life. This hypermethylated pattern is usually accompanied by hypomethylation at the paired locus. Epigenomic dysregulation has a stable but reversible effect on the genomic sequence. The stability and tissue specific potential of epigenetic marks highlights their potential as biomarkers for the heterogenetic pathophysiology encountered during ageing. In most cases, these epigenetic changes occur in genes that are involved in metabolism and metabolic dysregulation. Therefore, epi-genomic dysregulation is a primary mediator for the pathogenesis of age-related metabolic disease [52]. DNA methylation plays a major role during the ageing process that contributes vastly to loss of tissue homeostasis, the decline of normal cellular functions and the capacity for replication [53]. The accumulation of fat deposits with increasing age in the liver can reduce the capacity of the liver to regenerate and function [54] which may lead to diseases such as diabetes, dyslipidaemia and cardiovascular disease [55]. Ageing is a result of accumulating genomic damage over time that is accompanied by physiological decline [56].

DNMT1 is responsible for the maintenance of DNA methylation levels and also acts as a catalyst for methylation [57]. DNA methylation becomes more frequent when there is existing methylation which ensures the self-perpetuation of methylation status. This process results in *de novo* methylation extending from the already methylated regions to surrounding sequences during ageing and cancer progression, leading to an overall reduction in global methylation as methylation-specific enzymes are concentrated in these areas [58]. The direction and strength of the correlation between age and methylation is dependent on the CpG island status of loci. During ageing CpG island loci gain methylation and non-islanded CpGs become less methylated. Researchers have frequently shown that methylation status is increased within CpG

islands with age [59, 60]. Tra et al. and Bjornsson et al. showed bi-modal age-related methylation in aged tissues vs. normal tissues using microarray. A direct comparison of the top 50 most age-related and normal methylation genomes show an overall decreased methylation status in non-CpG island regions of normal samples [51]. They also indicated an inverse correlation trend in other tissue types, where they found that although methylation occurs at non-CpG island regions, there is a strong correlation between age and methylation at the CpG island regions [61].

Studies have indicated that age-related methylation is a common mechanism of dysregulation regardless of tissue specificity [62]. There is a reduction in the fidelity to maintain DNMTs activity with ageing and this potentially results in the age-related reduction of methylation [63]. On the other hand, age-related hypermethylation is a reflection of accumulating stochastic methylation events over time [64]. However, these methylated CpGs in mammalian cells may not have dramatic functional consequences due to the absence of pathologic phenotype differences [65]. The accumulation of alterations without functional consequences should not be considered biologically insignificant, because age-related alteration of the normal epigenome without changes in gene expression may confer a significantly increased risk to pathologic phenotypes and altered gene expression or genomic instability [64]. For example, the methylation of 'non-functional' CpG islands in promoter regions of an aged individual can continue to accumulate methylation events (methylation spreading) and increase the chance of methylation induced gene silencing in the future [64]. The increasing spread of methylation can also affect gene expression of distant loci via silencing of important genomic regions, such as enhancers resulting in progression to a diseased phenotype [66]. Fortunately, the aberrant CpG methylation causing gene silencing on a single allele can be compensated by the complementary allele that is not methylated. Thus, the clusters of mono-allelic gene expression will increase the risk of pathologic phenotype alteration, for example the loss of the 2nd functional allele. As a result, it is necessary to study the potential of quantification of age and/or environmental exposure that is associated with DNA methylation which can act as an indication of disease risk [67].

Other DNA methylation mechanisms are involved in cellular ageing and provide a large area of discovery, these include: endogenous hypomethylation and exogenous hypermethylation [68]. Age-dependent decreases in DNA methylation, including possible endogenous changes that alter gene expression or the function of DNMT and demethylases, as well as exogenous factors like diet, drugs and UV may also result in gene expression alteration [69]. Endogenous DNMT alteration can arise at different stages of cellular development from new-born, middle aged, to elderly individuals. All of these factors may lead to gene overexpression and the increase of 'transcriptional noise', however this is not fully understood in the mammalian system. Exogenous agents that affect DNA methylation may affect cellular function in the long-term [47]. Cells treated with demethylation agents can become re-methylated due to the action of their DNA methylation maintenance mechanisms which endure multiple insults with accumulating age [70]. Dietary deficiencies in folate, choline, methionine, zinc and/or selenium can result in alteration of DNA methylation status [71]. Folate and choline deficiency may contribute to DNA hypomethylation in the liver that leads to liver cancer development [72]. Folate deficiency can also lead to an increase of homocysteine levels and promote degeneration of neurons in Alzheimer's and Parkinson's disease [73].

The mechanisms involved in hypermethylation of CpG islands with ageing are not well understood. Wu et al., showed increased levels of DNMT1 leading to CpG island methylation and phenotype changes in fibroblasts, suggesting the increased level of the protein contributed to process of phenotypic modification. Under normal cellular development DNMT1 may increase in response to DNA hypomethylation that is caused by drug intake and dietary deficiencies which lead to increased DNA methylation and contributes to the DNA methylation balance of the cell.

2.2. DNA methylation and disease development

Germline and somatic mutations are mainly the result of cytosine methylation during cancer development [74]. Abnormal promoter methylation of the regulatory genes can lead to gene silencing and is an important mechanism of cancer progression [75]. Rare diseases such as immunodeficiency, centromeric region instability, facial anomalies syndrome (ICF) [13] and mental retardation in young girls (Rett Syndrome) are the potential consequence of abnormal methylation alteration [76]. For example, ICF patients are found to be have a mutated DNMT3b gene that leads to the downregulation of satellite DNA methylation and chromosomal de-condensation. Methylation binding domains (MBDs, MeCP2) were found to be aberrantly methylated in Rett Syndrome patients, resulting in the interruption of the methylation signal [76]. Together this suggests methylation is not completed after embryonic development, requires maintenance and is essential in mammalian cells. Alternatively, the increasing methylation of mammalian cells may contribute to the risk of cancer development. Therefore, the balance of methylation is essential in maintaining healthy cellular function.

Many studies have indicated that the imbalance of DNA methylation occurs in the disease mechanism which leads to the discovery of pharmacological agents that reverse epigenetic abnormalities [77]. The interaction of DNA methylation and histone modification machinery were further investigated and proved to be an important contribution that led to disease development. Another group of epigenetic alterations caused by small RNAs also play a major role at different disease stages that could also be exploited to monitor treatment results [78].

2.3. DNA methylation and cancer development

DNA methylation and cancer development was first studied in the 1980s, showing DNA hypomethylation in their normal counterparts of cancer cells [79]. The loss of DNA methylation in the repetitive regions of the genome was referred to as hypomethylation which led to genomic instability and is a hallmark of tumour cells [80]. Hypomethylation can also lead to over-expression of oncogenes that contributes to cancer progression [81]. Transcriptional interference describes the reactivation of transposon promoters via demethylation that contributes to aberrant gene regulation in cancers [82]. Down-regulation of DNA methylation occurs at the early stages of cancer and correlates with disease progression and metastatic potential in many cancer types [81]. The melanoma antigen (MAGE) family of cancer genes are a gene-specific hypomethylation in cancer cells that encodes tumour antigens of unknown function that are frequently demethylated and re-expressed in cancer [83]. Specific genes that are hypomethylated in specific types of cancer include: S100 calcium binding protein A4 (S100A4) upregulated in colon cancer [84], serine protease inhibitor gene SERPINB5 (also known as maspin) in gastric

cancer [85] and the putative oncogene γ-synuclein (SNCG) in breast and ovarian cancers [86]. Global hypomethylation that occurs at early stages of tumorigenesis may be protected by genomic instability and further genetic changes. However, gene-specific hypomethylation could allow tumour cell adaptation to their local environment and promote metastasis. Research on genome-wide demethylation in cancer cells has been largely overshadowed by studies of gene-specific hypermethylation events, which occur concomitantly with the hypomethylation events discussed above. In cancer cells, aberrant hypermethylation usually occurs at CpG islands that are mostly unmethylated in normal somatic cells [87]. Histone deacetylation leads to changes in chromatic structure that effectively silence transcription. In a subset of tumour types that are referred to as CpG island-methylator phenotypes, there was a 3–5 fold increase in aberrant methylation [88]. Most of the involved genes were regulators of cell-cycle, tumour cell invasion, DNA repair, chromatic remodelling, cell signalling, transcription and apoptosis. Additionally these genes are known to be aberrantly hypermethylated and silenced in most cancers, favouring cancer cell growth and increasing their genetic instability causing them to metastasize. In the case of colon cancer, aberrant hypermethylation is detectable in the earliest precursor lesion, indicating DNA hypermethylation is an early, detectable event during colon cancer development and can be used as a biomarker [89].

Cancer can be described as a disease of ageing, Issa et al., reported global (repeat element) hypomethylation and promoter hypermethylation of cancer cells are also found in normal tissues with ageing [58]. Other studies have described the age-related methylation in normal human prostate and colon tissues that contain CpG island bearing genes [90]. The alteration of DNA methylation is age-related but also tissue-dependent. The process of DNA methylation associated with ageing and promoter CpG methylation is complex. CpG islands are known to contribute to gene silencing in cancer cells. Gene silencing of retinoic acid receptor- β (RAR β) 51 is an example of *de novo* methylation of the CpG island that causes leukaemia to develop in humans [91]. During cancer development, interactions between DNMTs and HDACs may facilitate *de novo* methylation to maintain permanent methylation of tumour suppressor genes that are already down-regulated [92].

2.4. DNA methylation, biomarkers, treatment and monitoring of disease

Promoter hypermethylation of DNA can be used as biomarkers for the detection of different cancerous cells when compared with methylation status of 'normal-healthy' cells. There is a growing trend to develop DNA methylation based biomarkers for cancer diagnosis. This area of development is attractive to researchers because of the stable and sustainable nature of detection (even in circulation). Aberrant DNA methylation can also be used as a biomarker for malignant transformation. The development of a methylation-specific PCR technique in 1996 by Herman et al., became popular soon after their first publication [93]. It offers a quick, easy, non-radioactive and sensitive way to detect hypermethylated CpG regions of tumour suppressor genes and can detect unmethylated CpG regions in 'normal-healthy' cells. Recently, the new droplet digital PCR technology and next generation sequencing has moved methylation detection in clinical samples a step forward which is sensitive enough to look at the traced methylation status of tumour suppressor genes in

circulation. The advantage of this detection technology is built on the basis of the following: positive PCR signals are not masked by the contamination of normal cells, promoter hypermethylation can occur at an early stage of cancer which allows early diagnosis and all tumours have one or more loci that contain hypermethylated tumour suppressor genes. The development of detection techniques also allowed promoter hypermethylation to be identified using bronchoalveolar lavages [94], lymph nodes [95], stool and sputum [96] to screen methylation of tumour suppressors. The screening of promoter hypermethylation in serum DNA from non-small cell lung cancer also opens the avenue for researchers to further develop this technology for diagnosis [97]. Septin 9 (Sept9) was the first FDA approved DNA methylation marker that utilised non-invasive serum samples from patients [98]; MLH1 is used for colon cancer diagnosis [99] and O6-methylguanine-DNA-methyltransferase (MGMT1) is used for brain cancer [100] diagnosis in the clinic.

The process of methylating d^mC in cancer is complicated as malignancies have different origins; it can either be direct or indirect influence by oncogenes or as part of cancer cell adaptation to external stress response, environmental factors and exposure to therapeutic genes and others [101, 102]. The distribution of d^mC during cancer development will allow cancer cells to abject their phenotypes, adapt to different tissue microenvironments and also become resistant to therapeutic drugs [103]. Cancer associated upregulation of d^mC at promoters or enhancers of a genome can also cause tumour suppressor genes to become silenced [104]. The commonly silenced tumour suppressor genes in most cancers CDKN2A, RB and MLH1 are associated with aggressive cancer types and a poor prognosis [104]. DNA methylation can also be used to detect silenced genes involved in immune recognition or modulate a response to chemotherapy, resulting in disruption of immune surveillance that induced chemotherapy resistance [105]. However, these epigenetic alterations are reversible and it is possible to reverse aberrant methylation with DNMT inhibitor (DNMTi) or a demethylating agent (i.e. decitabine) which allows the restoration of the genomic functions [67]. The advantages of these drugs are their ability to reprogram cancer cells to undergo terminal differentiation, induce chemosensitisation, loss of self-renewal properties or become visible to immune system. DNMTi and decitabine can also be used to induce anti-tumour response by induction of endogenous stimulation of interferon response pathways [106].

The multifaceted and easy detection of d^mC highlights its great potential to be used as a biomarker with utilisation of the improved detection methods of methylated DNA using modified DNA fragments. d^mC biomarkers have now become a convincing predictor of clinical outcomes and are able to predict response to DNMTi in the clinic. They can also be used to classify cancer into biological and clinically distinct disease subtypes, providing guidance for chemotherapeutic drug selection. The use of tumour suppressor gene CDKN2A methylation status has shown to be prognostically significant in many cancer types [107]. In leukaemia patients, clinicians use the panel 16 methylated gene panel as a biomarker for detection using microarray and showed that differentially methylated regions (DMTs) are useful to predict outcomes of patients and their clinical variability. This d^mC detection technology has been developed further as biomarkers and is validated in acute myeloid leukaemia (AML) patients [108]. The DMRs can also be used to predict response of patients with chronic myelomonocytic leukaemia to DNMTi [109]. Hypermethylation and silencing

of SMAD1 was useful as a predictive biomarker for chemotherapy resistance in patients with high-risk diffuse large B-cell lymphoma (DLBCL) [110]. SMAD1 silencing also showed contribution to chemotherapy resistance that can be reversed by DNMTis in patients [110]. Results were favourable for the use of DNMTi in patients diagnosed with high-risk DLBCL, in combination with decitabine before rituximab in combination of cyclophosphamide, doxorubine, vincristine and prednisone chemo-immunotherapy. MGMT which is a DNA repair enzyme has also proved to be useful as a predictive marker for alkylating agent response [111].

3. Epigenetic regulation and drug discovery

Other epigenetic factors are useful for drug discovery, such as acetylation, methylation and phosphorylation, ubiquitination, sumoylation and ADP-ribosylation [112, 113]. A list of these agents is summarised in **Table 1a** and **Table 1b** [114, 115]. Vincent Alfrey et al., suggested modifications of acetylation have functional roles in modulating transcription and it was later established by others that the process of chromatin and post-translational modification of epigenetic regulation may be interrupting DNA [116]. HDAC are a family of enzymes responsible for chromatin modification. Aberrant regulation of this family of genes has been studied in many cancers and used in pharmacological target discovery. The inhibition of chromatin-modification enzymes is a key process to modulate transcription in eukaryotic cells which led to the development of novel pharmacologic agent discovery. HDAC inhibitor (HDACi), vorinostat, was the FDA approved treatment for patients with advanced refractory cutaneous T-cell lymphoma. This provides evidence that HDACi is a useful therapeutic treatment, however the different subtypes of HDAC should be considered to achieve promising therapeutic interventions. The discovery of both HDACi and DNMTi provide options for clinical treatment alone or possible use with other agents in combination therapies for the treatment of various diseases that are related to epigenetic abnormalities [117].

4. Conclusion

Epigenetic regulation is an attractive area of research; it provides a broad spectrum of discovery as it is involved in almost all developmental processes of the mammalian cell from

Name	DNMT inhibitor	Clinical status	Treatment
Decitabine	5-AZA-CdR	FDA and the European Medicines Agency	Myelodysplastic syndromes (MDS), acute myeloid leukaemia (AML), and chronic myelomonocytic leukaemia (CMML)
5-AZA	Nucleoside analogues azacitidine	FDA and the European Medicines Agency	Myelodysplastic syndromes (MDS), acute myeloid leukaemia (AML), and chronic myelomonocytic leukaemia (CMML)

Table 1a. DNMT inhibitors.

Class	HDAC inhibitor	Target HDAC Class	Clinical status
Hydroxamic acids	SAHA	pan	Approved for cutaneous T-cell lymphoma
	Belinostat	pan	Approved for peripheral T-cell lymphoma
	Panabiostat	pan	Approved for multiple myeloma
	Givinostat	pan	Phase II clinical trials—relapsed leukaemia and multiple myeloma
	Resminostat	pan	Phase I and II clinical trials—hepatocellular carcinoma
	Abexinostat	pan	Phase II clinical trial—B-cell lymphoma
	Quisinostat	pan	Phase I clinical trial—multiple myeloma
	Rocilinostat	II	Phase I clinical trial—multiple myeloma
	Practinostat	I, II and IV	Phase II clinical trial—prostate cancer
	CHR-3996	I	Phase I clinical trial—advanced/metastatic solid tumours refractory to standard therapy
	Valproic acid	I, IIa	Approved for epilepsia, bipolar disorders and migraine, phase II clinical trials—several studies
Short chain fatty acids	Butyric acid	I, II	Phase II clinical trials—several studies
	Phenylbutyric acid	I, II	Phase I clinical trials—several studies
	Entinostat	I	Phase II clinical trials—breast cancer, Hodgkin's lymphoma, non-small cell lung cancer, phase III clinical trial—hormone receptor positive breast cancer
Benzamides	Tacedinaline	I	Phase III clinical trial—non-small cell lung cancer and pancreatic cancer
	4SC202	I	Phase I clinical trial—advanced haematological malignancies
	Mocetinostat	I, IV	Phase II clinical trials—Hodgkin's lymphoma
	Romidepsin	I	Approved for cutaneous T-cell lymphoma
Cyclic tetrapeptides	Nicotinamide	All class III	Phase III clinical trial—laryngeal cancer
Sirtuins inhibitors	EX-527	SIRT 1 and 2	Cancer preclinical, phase I and II clinical trials—Huntington disease, glaucoma

Table 1b. HDAC inhibitors.

early fertilisation, implantation, embryonic development, ageing and carcinogenesis. As evolutionary stages require different types of epigenetic signature, the various epigenetic patterns have been exploited in biomarker discovery to identify distinctive/unique stages of disease (typically cancer) development. Although DNA methylation is the most studied area of epigenetics, there are not many markers that are currently used as standard clinical diagnostic markers. Due to the stability of DNA in cells and in circulation we believe that with the development of new technologies and methods, DNA methylation biomarkers

have the potential to become a favourable clinical diagnostic marker. Further research is required in this field to ensure the widespread application of DNA methylation markers in the clinical setting.

Abbreviations

3′-UTR	three prime untranslated region
AML	acute myeloid leukaemia
Arg	arginine
AZA	5-aza-2′-deoxycytidine
Clr4	histone-lysine N-methyltransferase
dC	deocycytosin
DLBCL	diffuse large B-cell lymphoma
dmC	deoxymethylcytosine
DMTs	differentially methylated regions
DNMT	DNA methyltransferases
ES	embryonic stem
H3K9	histone H3 at lysine 9
HDAC	histone deacetylase
HMT	histone methyltransferase
HP1	heterochromatin protein
ICF	facial anomalies syndrome
ICR	imprinting control region
Lys	lysine
MBD	Methylation binding domains
MGMT1	O6-methylguanine-DNA-methyltransferase
miRNAs	MicroRNAs
mRNA	messenger RNA
NuRD	nucleosome remodelling

RAR β	retinoic acid receptor- β
RNAi	RNA interference
ROS	reactive oxygen species
S100A4	S100 calcium binding protein A4
SAM	S-adenosylmethione
TE	transposable element
TF	transcription factor
ZGA	zygotic gene activation

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References

[1] Issa JP. CpG-island methylation in aging and cancer. Current Topics in Microbiology and Immunology. 2000;**249**:101-118

[2] Urnov FD, Wolffe AP. Above and within the genome: Epigenetics past and present. Journal of Mammary Gland Biology and Neoplasia. 2001;**6**(2):153-167

[3] Holliday R, Pugh JE. DNA modification mechanisms and gene activity during development. Science. 1975;**187**(4173):226-232

[4] Reik W. Stability and flexibility of epigenetic gene regulation in mammalian development. Nature. 2007;**447**(7143):425-432

[5] Jones PA, Baylin SB. The epigenomics of cancer. Cell. 2007;**128**(4):683-692

[6] Antequera F, Bird A. Number of CpG islands and genes in human and mouse. Proceedings of the National Academy of Sciences of the United States of America. 1993;**90**(24):11995-11999

[7] Bird AP. Cpg islands as gene markers in the vertebrate nucleus. Trends in Genetics. 1987;**3**(12):342-347

[8] Larsen F et al. Cpg islands as gene markers in the human genome. Genomics. 1992; **13**(4):1095-1107

[9] Adachi N, Lieber MR. Bidirectional gene organization: A common architectural feature of the human genome. Cell. 2002;**109**(7):807-809

[10] Lee JH, Voo KS, Skalnik DG. Identification and characterization of the DNA binding domain of CpG-binding protein. The Journal of Biological Chemistry. 2001;**276**(48):44669-44676

[11] Rig-gs AD et al. Methylation dynamics, epigenetic fidelity and X chromosome structure. Epigenetics. 2008;**793**:214

[12] Bestor T et al. Cloning and sequencing of a CDNA encoding DNA methyltransferase of mouse cells. The carboxyl-terminal domain of the mammalian enzymes is related to bacterial restriction methyltransferases. Journal of Molecular Biology. 1988;**203**(4):971-983

[13] Okano M et al. DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development. Cell. 1999;**99**(3):247-257

[14] Deng C et al. Decreased ras–mitogen-activated protein kinase signaling may cause DNA hypomethylation in T lymphocytes from lupus patients. Arthritis & Rheumatology. 2001;**44**(2):397-407

[15] Kawai J et al. Comparison of DNA methylation patterns among mouse-cell lines by restriction landmark genomic scanning. Molecular and Cellular Biology. 1994;**14**(11):7421-7427

[16] Chen T, Li E. Structure and function of eukaryotic DNA methyltransferases. Current Topics in Developmental Biology. 2004;**60**:55-89

[17] Bourc'his D, Bestor TH. Meiotic catastrophe and retrotransposon reactivation in male germ cells lacking Dnmt3L. Nature. 2004;**431**(7004):96-99

[18] Jia D et al. Structure of Dnmt3a bound to Dnmt3L suggests a model for de novo DNA methylation. Nature. 2007;**449**(7159):248-251

[19] Goll MG et al. Methylation of tRNAAsp by the DNA methyltransferase homolog Dnmt2. Science. 2006;**311**(5759):395-398

[20] Jaenisch R. DNA methylation and imprinting: Why bother? Trends in Genetics. 1997;**13**(8):323-329

[21] Saitou M, Kagiwada S, Kurimoto K. Epigenetic reprogramming in mouse pre-implantation development and primordial germ cells. Development. 2012;**139**(1):15-31

[22] Li E. Chromatin modification and epigenetic reprogramming in mammalian development. Nature Reviews. Genetics. 2002;**3**(9):662-673

[23] Martens JH et al. The profile of repeat-associated histone lysine methylation states in the mouse epigenome. The EMBO Journal. 2005;**24**(4):800-812

[24] Lee DY et al. Role of protein methylation in regulation of transcription. Endocrine Reviews. 2005;**26**(2):147-170

[25] Jenuwein T et al. SET domain proteins modulate chromatin domains in eu- and heterochromatin. Cellular and Molecular Life Sciences. 1998;**54**(1):80-93

[26] Rea S et al. Regulation of chromatin structure by site-specific histone H3 methyltransferases. Nature. 2000;**406**(6796):593-599

[27] Nakayama J et al. Role of histone H3 lysine 9 methylation in epigenetic control of heterochromatin assembly. Science. 2001;**292**(5514):110-113

[28] Volpe TA et al. Regulation of heterochromatic silencing and histone H3 lysine-9 methylation by RNAi. Science. 2002;**297**(5588):1833-1837

[29] Grewal SI, Moazed D. Heterochromatin and epigenetic control of gene expression. Science. 2003;**301**(5634):798-802

[30] Santos-Rosa H et al. Methylation of histone H3 K4 mediates association of the Isw1p ATPase with chromatin. Molecular Cell. 2003;**12**(5):1325-1332

[31] Zegerman P et al. Histone H3 lysine 4 methylation disrupts binding of nucleosome remodeling and deacetylase (NuRD) repressor complex. The Journal of Biological Chemistry. 2002;**277**(14):11621-11624

[32] Taunton J, Hassig CA, Schreiber SL. A mammalian histone deacetylase related to the yeast transcriptional regulator Rpd3p. Science. 1996;**272**(5260):408-411

[33] Gregoretti IV, Lee YM, Goodson HV. Molecular evolution of the histone deacetylase family: Functional implications of phylogenetic analysis. Journal of Molecular Biology. 2004;**338**(1):17-31

[34] Pang M, Zhuang S. Histone deacetylase: A potential therapeutic target for fibrotic disorders. The Journal of Pharmacology and Experimental Therapeutics. 2010;**335**(2):266-272

[35] Haberland M, Montgomery RL, Olson EN. The many roles of histone deacetylases in development and physiology: Implications for disease and therapy. Nature Reviews. Genetics. 2009;**10**(1):32-42

[36] Dowling DP et al. Evolution of the arginase fold and functional diversity. Cellular and Molecular Life Sciences. 2008;**65**(13):2039-2055

[37] Bartel DP. MicroRNAs: Genomics, biogenesis, mechanism, and function. Cell. 2004;**116**(2):281-297

[38] Deo M et al. Detection of mammalian microRNA expression by in situ hybridization with RNA oligonucleotides. Developmental Dynamics. 2006;**235**(9):2538-2548

[39] Han L et al. DNA methylation regulates MicroRNA expression. Cancer Biology & Therapy. 2007;**6**(8):1284-1288

[40] Kubo T et al. Epigenetic silencing of microRNA-34b/c plays an important role in the pathogenesis of malignant pleural mesothelioma. Clinical Cancer Research. 2011;**17**(15):4965-4974

[41] Lujambio A et al. A microRNA DNA methylation signature for human cancer metastasis. Proceedings of the National Academy of Sciences of the United States of America. 2008;**105**(36):13556-13561

[42] Saito Y et al. Specific activation of microRNA-127 with downregulation of the proto-oncogene BCL6 by chromatin-modifying drugs in human cancer cells. Cancer Cell. 2006;**9**(6):435-443

[43] Jackson-Grusby L et al. Loss of genomic methylation causes p53-dependent apoptosis and epigenetic deregulation. Nature Genetics. 2001;**27**(1):31-39

[44] Rhee I et al. DNMT1 and DNMT3b cooperate to silence genes in human cancer cells. Nature. 2002;**416**(6880):552-556

[45] Walsh CP, Chaillet JR, Bestor TH. Transcription of IAP endogenous retroviruses is constrained by cytosine methylation. Nature Genetics. 1998;**20**(2):116-117

[46] Fan G et al. DNA hypomethylation perturbs the function and survival of CNS neurons in postnatal animals. The Journal of Neuroscience. 2001;**21**(3):788-797

[47] Bird A. DNA methylation patterns and epigenetic memory. Genes & Development. 2002;**16**(1):6-21

[48] Gurland BJ, Page WF, Plassman BL. A twin study of the genetic contribution to age-related functional impairment. The Journals of Gerontology. Series A, Biological Sciences and Medical Sciences. 2004;**59**(8):859-863

[49] Gartner K. A third component causing random variability beside environment and genotype. A reason for the limited success of a 30 year long effort to standardize laboratory animals? International Journal of Epidemiology. 2012;**41**(2):335-341

[50] Kaminsky ZA et al. DNA methylation profiles in monozygotic and dizygotic twins. Nature Genetics. 2009;**41**(2):240-245

[51] Bjornsson HT et al. Intra-individual change over time in DNA methylation with familial clustering. JAMA. 2008;**299**(24):2877-2883

[52] Thompson RF et al. Tissue-specific dysregulation of DNA methylation in aging. Aging Cell. 2010;**9**(4):506-518

[53] Campisi J. Senescent cells, tumor suppression, and organismal aging: Good citizens, bad neighbors. Cell. 2005;**120**(4):513-522

[54] Schmucker DL. Age-related changes in liver structure and function: Implications for disease ? Experimental Gerontology. 2005;**40**(8–9):650-659

[55] Huffman DM, Barzilai N. Role of visceral adipose tissue in aging. Biochimica et Biophysica Acta. 2009;**1790**(10):1117-1123

[56] Maslov AY, Vijg J. Genome instability, cancer and aging. Biochimica et Biophysica Acta. 2009;**1790**(10):963-969

[57] Fatemi M et al. The activity of the murine DNA methyltransferase Dnmt1 is controlled by interaction of the catalytic domain with the N-terminal part of the enzyme leading to an allosteric activation of the enzyme after binding to methylated DNA. Journal of Molecular Biology. 2001;**309**(5):1189-1199

[58] Issa JP et al. Methylation of the oestrogen receptor CpG island links ageing and neoplasia in human colon. Nature Genetics. 1994;**7**(4):536-540

[59] Jahner D, Jaenisch R. Retrovirus-induced de novo methylation of flanking host sequences correlates with gene inactivity. Nature. 1985;**315**(6020):594-597

[60] Magewu AN, Jones PA. Ubiquitous and tenacious methylation of the Cpg site in Codon-248 of the P53 gene may explain its frequent appearance as a mutational hot-spot in human cancer. Molecular and Cellular Biology. 1994;**14**(6):4225-4232

[61] Christensen BC et al. Aging and environmental exposures alter tissue-specific DNA methylation dependent upon CpG island context. PLoS Genetics. 2009;**5**(8):e1000602

[62] Hannum G et al. Genome-wide methylation profiles reveal quantitative views of human aging rates. Molecular Cell. 2013;**49**(2):359-367

[63] Ahuja N et al. Aging and DNA methylation in colorectal mucosa and cancer. Cancer Research. 1998;**58**(23):5489-5494

[64] Johnson AA et al. The role of DNA methylation in aging, rejuvenation, and age-related disease. Rejuvenation Research. 2012;**15**(5):483-494

[65] Bell JT et al. Epigenome-wide scans identify differentially methylated regions for age and age-related phenotypes in a healthy ageing population. PLoS Genetics. 2012;**8**(4): e1002629

[66] Jones PA, Takai D. The role of DNA methylation in mammalian epigenetics. Science. 2001;**293**(5532):1068-1070

[67] Baylin SB. DNA methylation and gene silencing in cancer. Nature Clinical Practice. Oncology. 2005;**2**(Suppl 1):S4-11

[68] Robertson KD, Jones PA. DNA methylation: Past, present and future directions. Carcinogenesis. 2000;**21**(3):461-467

[69] Richardson B. Impact of aging on DNA methylation. Ageing Research Reviews. 2003; **2**(3):245-261

[70] Law JA, Jacobsen SE. Establishing, maintaining and modifying DNA methylation patterns in plants and animals. Nature Reviews. Genetics. 2010;**11**(3):204-220

[71] Niculescu MD, Zeisel SH. Diet, methyl donors and DNA methylation: Interactions between dietary folate, methionine and choline. Journal of Nutrition. 2002;**132**(8):2333s-2335s

[72] Wainfan E, Poirier LA. Methyl groups in carcinogenesis: Effects on DNA methylation and gene expression. Cancer Research. 1992;**52**(7 Suppl):2071s-2077s

[73] Kruman II et al. Folic acid deficiency and homocysteine impair DNA repair in hippocampal neurons and sensitize them to amyloid toxicity in experimental models of Alzheimer's disease. The Journal of Neuroscience. 2002;**22**(5):1752-1762

[74] Cooper DN, Youssoufian H. The CpG dinucleotide and human genetic disease. Human Genetics. 1988;**78**(2):151-155

[75] Jones PA, Laird PW. Cancer epigenetics comes of age. Nature Genetics. 1999;**21**(2):163-167

[76] Amir RE et al. Rett syndrome is caused by mutations in X-linked MECP2, encoding methyl-CpG-binding protein 2. Nature Genetics. 1999;**23**(2):185-188

[77] Brown R, Strathdee G. Epigenomics and epigenetic therapy of cancer. Trends in Molecular Medicine. 2002;**8**(4 Suppl):S43-S48

[78] Novina CD, Sharp PA. The RNAi revolution. Nature. 2004;**430**(6996):161-164

[79] Feinberg AP, Tycko B. The history of cancer epigenetics. Nature Reviews. Cancer. 2004; **4**(2):143-153

[80] Yoder JA, Walsh CP, Bestor TH. Cytosine methylation and the ecology of intragenomic parasites. Trends in Genetics. 1997;**13**(8):335-340

[81] Widschwendter M et al. DNA hypomethylation and ovarian cancer biology. Cancer Research. 2004;**64**(13):4472-4480

[82] Robertson KD, Wolffe AP. DNA methylation in health and disease. Nature Reviews. Genetics. 2000;**1**(1):11-19

[83] De Smet C et al. DNA methylation is the primary silencing mechanism for a set of germ line-and tumor-specific genes with a CpG-rich promoter. Molecular and Cellular Biology. 1999;**19**(11):7327-7335

[84] Nakamura N, Takenaga K. Hypomethylation of the metastasis-associated S100A4 gene correlates with gene activation in human colon adenocarcinoma cell lines. Clinical & Experimental Metastasis. 1998;**16**(5):471-479

[85] Akiyama Y et al. Cell-type-specific repression of the maspin gene is disrupted frequently by demethylation at the promoter region in gastric intestinal metaplasia and cancer cells. American Journal of Pathology. 2003;**163**(5):1911-1919

[86] Gupta A et al. Hypomethylation of the synuclein gamma gene CpG island promotes its aberrant expression in breast carcinoma and ovarian carcinoma. Cancer Research. 2003; **63**(3):664-673

[87] Song F et al. Association of tissue-specific differentially methylated regions (TDMs) with differential gene expression. Proceedings of the National Academy of Sciences of the United States of America. 2005;**102**(9):3336-3341

[88] Issa JP. CpG island methylator phenotype in cancer. Nature Reviews. Cancer. 2004;**4**(12): 988-993

[89] Chan AO et al. CpG island methylation in aberrant crypt foci of the colorectum. The American Journal of Pathology. 2002;**160**(5):1823-1830

[90] Kwabi-Addo B et al. Age-related DNA methylation changes in normal human prostate tissues. Clinical Cancer Research. 2007;**13**(13):3796-3802

[91] Di Croce L et al. Methyltransferase recruitment and DNA hypermethylation of target promoters by an oncogenic transcription factor. Science. 2002;**295**(5557):1079-1082

[92] Fuks F et al. Dnmt3a binds deacetylases and is recruited by a sequence-specific repressor to silence transcription. The EMBO Journal. 2001;**20**(10):2536-2544

[93] Herman JG et al. Methylation-specific PCR: A novel PCR assay for methylation status of CpG islands. Proceedings of the National Academy of Sciences of the United States of America. 1996;**93**(18):9821-9826

[94] Ahrendt SA et al. Molecular detection of tumor cells in bronchoalveolar lavage fluid from patients with early stage lung cancer. Journal of the National Cancer Institute. 1999;**91**(4):332-339

[95] Sanchez-Cespedes M et al. Molecular detection of neoplastic cells in lymph nodes of metastatic colorectal cancer patients predicts recurrence. Clinical Cancer Research. 1999;**5**(9):2450-2454

[96] Palmisano WA et al. Predicting lung cancer by detecting aberrant promoter methylation in sputum. Cancer Research. 2000;**60**(21):5954-5958

[97] Côté S, Sinnett D, Momparler RL. Demethylation by 5-aza-2 [spacing acute]-deoxycytidine of specific 5-methylcytosine sites in the promoter region of the retinoic acid receptor [latin sharp s] gene in human colon carcinoma cells. Anti-Cancer Drugs. 1998;**9**(9):743-750

[98] Heyn H, Esteller M. DNA methylation profiling in the clinic: Applications and challenges. Nature Reviews. Genetics. 2012;**13**(10):679-692

[99] Nakagawa H et al. Age-related hypermethylation of the 5′ region of MLH1 in normal colonic mucosa is associated with microsatellite-unstable colorectal cancer development. Cancer Research. 2001;**61**(19):6991-6995

[100] Kulke MH et al. O6-methylguanine DNA methyltransferase deficiency and response to temozolomide-based therapy in patients with neuroendocrine tumors. Clinical Cancer Research. 2009;**15**(1):338-345

[101] Shen JC, Rideout WM 3rd, Jones PA. The rate of hydrolytic deamination of 5-methylcytosine in double-stranded DNA. Nucleic Acids Research. 1994;**22**(6):972-976

[102] Denissenko MF et al. Cytosine methylation determines hot spots of DNA damage in the human P53 gene. Proceedings of the National Academy of Sciences of the United States of America. 1997;**94**(8):3893-3898

[103] Segura-Pacheco B et al. Global DNA hypermethylation-associated cancer chemotherapy resistance and its reversion with the demethylating agent hydralazine. Journal of Translational Medicine. 2006;**4**:32

[104] David GL et al. MDR1 promoter hypermethylation in MCF-7 human breast cancer cells: Changes in chromatin structure induced by treatment with 5-Aza-cytidine. Cancer Biology & Therapy. 2004;**3**(6):540-548

[105] Herman JG, Baylin SB. Gene silencing in cancer in association with promoter hypermethylation. The New England Journal of Medicine. 2003;**349**(21):2042-2054

[106] Reu FJ et al. Overcoming resistance to interferon-induced apoptosis of renal carcinoma and melanoma cells by DNA demethylation. Journal of Clinical Oncology. 2006;**24**(23):3771-3779

[107] Tang B et al. Clinicopathological significance of CDKN2A promoter Hypermethylation frequency with pancreatic cancer. Scientific Reports. 2015;**5**:13563

[108] Figueroa ME et al. DNA methylation signatures identify biologically distinct subtypes in acute myeloid leukemia. Cancer Cell. 2010;**17**(1):13-27

[109] Meldi K et al. Specific molecular signatures predict decitabine response in chronic myelomonocytic leukemia. The Journal of Clinical Investigation. 2015;**125**(5):1857-1872

[110] Clozel T et al. Mechanism-based epigenetic chemosensitization therapy of diffuse large B-cell lymphoma. Cancer Discovery. 2013;**3**(9):1002-1019

[111] Weller M et al. MGMT promoter methylation is a strong prognostic biomarker for benefit from dose-intensified Temozolomide Rechallenge in progressive glioblastoma: The DIRECTOR trial. Clinical Cancer Research. 2015;**21**(9):2057-2064

[112] Jenuwein T, Allis CD. Translating the histone code. Science. 2001;**293**(5532):1074-1080

[113] Nathan D, Sterner DE, Berger SL. Histone modifications: Now summoning sumoylation. Proceedings of the National Academy of Sciences of the United States of America. 2003;**100**(23):13118-13120

[114] Eckschlager T et al. Histone deacetylase inhibitors as anticancer drugs. International Journal of Molecular Sciences. 2017;**18**(7)

[115] Mazzone R et al. Epi-drugs in combination with immunotherapy: A new avenue to improve anticancer efficacy. Clinical Epigenetics. 2017;**9**:59

[116] Allfrey V, Faulkner R, Mirsky A. Acetylation and methylation of histones and their possible role in the regulation of RNA synthesis. Proceedings of the National Academy of Sciences. 1964;**51**(5):786-794

[117] Gallinari P et al. HDACs, histone deacetylation and gene transcription: From molecular biology to cancer therapeutics. Cell Research. 2007;**17**(3):195-211

Chapter 4

Histone Chaperones Regulate Mammalian Gene Expression

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Additional information is available at the end of the chapter

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Abstract

Histone chaperones are fundamental molecules that aid in the synthesis, translocation, and exchange of histones across the barrier of cytoplasm to nucleus. Regulation in repair, replication, and nucleosome assembly constitute the widely associated functions of histone chaperones. Recently, they have been associated with transcriptional regulation. Different stages of mammalian development have been correlated to the expression of histone chaperones. From oocyte and sperm till the formation and development of zygote, different histone chaperones demonstrated distinct regulatory roles. Efficient models of studying mammalian development include differentiation of embryonic stem cells (ESCs) to different lineages. Both *in vitro* and *in vivo* differentiation of mammalian cells exhibit regulation by different subtypes of histone chaperones. Due to the ethical issues concerning the use of embryos for the derivation of ESCs, induced pluripotent stem cells (iPSCs) were derived from pre-existing differentiated cells by a phenomenon called cellular reprogramming. Cellular reprogramming is characterized by erasure of pre-existing epigenetic signature to a new modulated epigenome. Histone chaperones serve as either facilitator or barrier to reprogramming. Here, we will discuss how histone chaperones could regulate the gene expression pattern by regulating epigenetic modification during the complex process of mammalian development and reprogramming.

Keywords: histone chaperone, reprogramming, epigenetic, development, transcription factor, histone variant

1. Introduction

Nucleosomes comprising of histones and DNA could be considered as the basic unit of regulation of gene expression. Nucleosomes tightly regulate the transcriptional traffic while relaxing the structure of chromatin to bind the chromatin factors across the DNA. The marked

presence of euchromatin and heterochromatin determines the fate of gene expression as a result of presence or absence of the regulatory complex formed at different loci [1]. Thus, the assembly or disassembly of the nucleosome is a major contributory factor in the regulation of gene expression in mammalian cells. Histone chaperone are proteins in nature and regulates the nucleosomal function by the deposition or eviction of corresponding canonical histone subunits or non-canonical histone variants. Their function is not restricted to nucleosomal activity only. They regulate all sorts of histone metabolism throughout the life cycle of a mammalian cell. Histone deposition by histone chaperones may or may not be coupled to DNA replication. The entire dynamics associated with histone chaperones enable us to realize how they could regulate DNA replication, repair, transcription and finally the genomic integrity of the cells (**Figure 1**).

The fundamental unit of chromatin is nucleosome. Nucleosome is composed of histone octamer with each two units of histone H2A, H2B, H3, H4 wrapped around by 147 bp of DNA. A linker DNA, along with other histone subunit H1, connects one nucleosome to the other and forms a beaded chain like structure in the nucleus. The degree of compactness of these nucleosomes determines the fate of gene expression in mammalian cells. Along with the canonical histone units of H2A, H2B, H3.1, H3.2 and H4, replacement histone variants have evolved which are essentially regulatory in nature including H3.3, CENP-A, H2A.Z and deposited in a replication-independent manner unlike the canonical ones which are generally deposited in a replication-coupled fashion and are expressed in the S-phase of the cell cycle. Replacement of canonical subunit with the histone variants contributes to the transcriptional regulation of genes [2]. Histone chaperones are responsible for the recruitment of canonical histones and these histone variants at different loci of the genome and further indulge in the regulation of gene expression.

Till date different subtypes of histone chaperones have been discovered. They are generally classified on the basis of replication-coupled or uncoupled mechanism of action. As mentioned

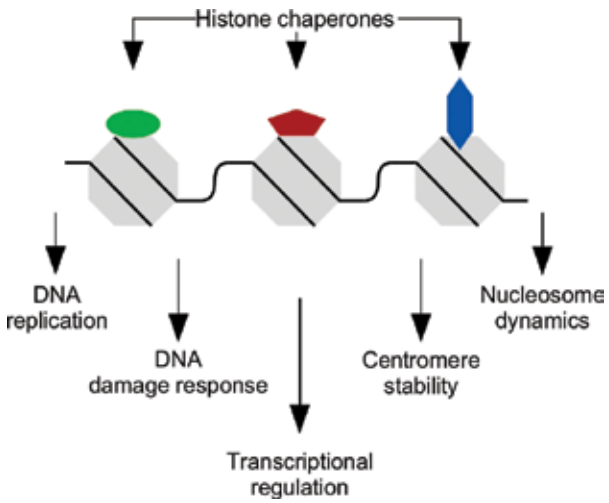


Figure 1. Overview of chromatin functions regulated by histone chaperones.

earlier replication coupled are the ones associated with deposition of newly synthesized canonical histones into the nucleosome, recycling them throughout the cell cycle and that include Chromatin Assembly Factor 1 (CAF1), Facilitates Chromatin Transcription (FACT), Suppressor Of Ty6 (Spt6), among others [2]. Whereas the other phenomenon of DNA repair, centromere deposition, transcriptional regulation and maintenance of heterochromatin dynamics are performed in a replication uncoupled fashion and that include Histone regulation A (HIRA), Anti Silencing Function 1A (ASF1A), Death domain-associated protein 6 (DAXX) among others [2]. Detailed information on the same could be referred from a recent review by Hammond et al. [2].

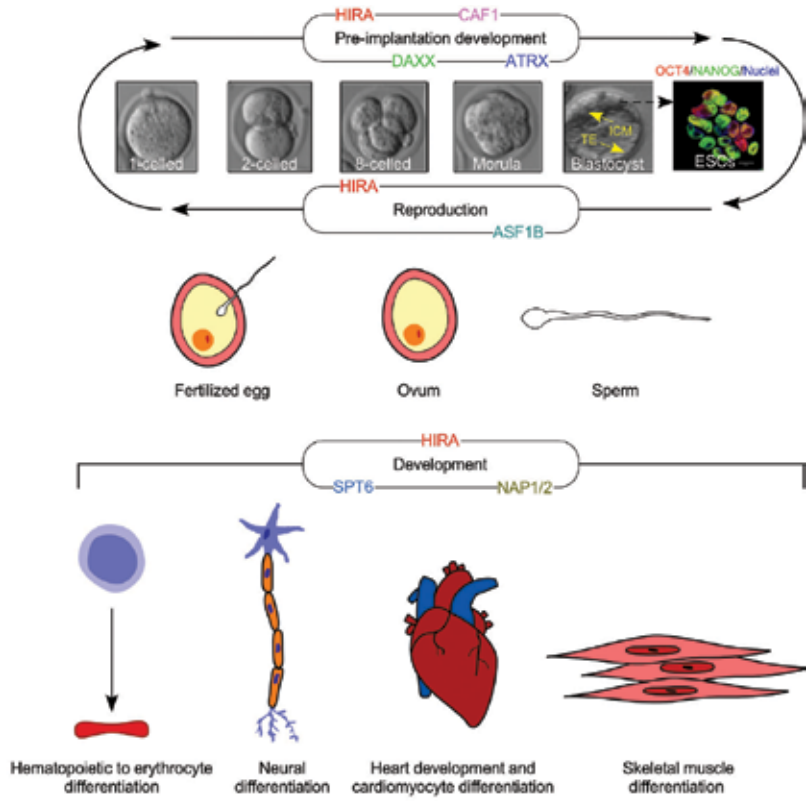
Replication-independent functioning of histone chaperones has a profound impact on the chromatin structure and as well as in different cellular processes. Such is the influence in different cellular context that many knockout phenotypes of histone chaperones demonstrated embryonic lethality. Among these cellular process, in this chapter, we will discuss in detail how histone chaperone influence different mammalian developmental stages and in the context of cellular reprogramming.

2. Histone chaperones in mammalian development

Mammalian development initiates upon the formation of single celled zygote from the sperm and ovum. The zygote undergoes division to form two-celled to four-celled and finally to a stage called morula. Till this stage the embryo is termed as totipotent, that can differentiate into any lineage. But, from the formation of blastocyst, the potency to differentiate becomes restricted from pluripotent to multipotent to finally unipotent (**Figure 2**, top panel). Intricate molecular mechanisms encompassing different transcriptional and epigenetic factors regulate the different stages of mammalian development from the pre-implantation embryo till the attainment of adulthood.

2.1. Pre-implantation and early embryonic development

From one-celled zygote to the formation of totipotent morula, functionally different histone chaperones influence the developmental stages. Even before fertilization, histone chaperone HIRA depletion lead to inhibition of male pronucleus formation due to a lack of nucleosome assembly in the sperm genome [3]. Hira mutant oocytes in *Xenopaus*, lack the potential to divide parthenogenetically [4]. Basically, HIRA-dependent H3.3 incorporation in the paternal genome is a prerequisite for their reprogramming into the development to the mouse zygote [5]. Progression to the two-celled stage is absolutely dependent on the histone chaperone HIRA. Loss in maternal HIRA leads to complete inability to deposit the core histones on to the paternal genome thus resulting in a compromised maternal genome reactivation in mice [5]. Also, ASF1B has been found to be required for retaining the female reproductive capacity in mouse where in loss of ASF1B could introduce change in meiotic entry thereby resulting in a discrepancy in gonad development [6]. The development of morula to blastocyst occurs around E3.5 and E5.5 in mouse and human respectively (**Figure 2**). This is the first cellular differentiation wherein the blastocyst could be partitioned into the inner cell mass (ICM)



Histone chaperones	Stages of development
HIRA	Sperm pronucleus [3], oocyte [4], muscle differentiation [14, 15, 16], skeletal myogenesis [17], cardiomyocyte differentiation and heart development [11, 12, 13], hematopoietic stem cell differentiation [21], hemogenic to hematopoietic differentiation [20], DNA replication and ribosomal RNA transcription in zygote [5], 2-celled stage, maternal genome H3.3 [5], endothelial-specific angiogenesis [19], neural progenitors cell proliferation and neurogenesis [22]
CAF1	Heterochromatin organization in embryonic stem cells [7], protect preimplantation embryos from endogenous retrotransposons [10]
ATRX	Trophoblast development [8]
DAXX	Proliferation of cells in early mouse development [9]
ASF1B	Reproduction [6]
SPT6	Myoblast differentiation [16]
NAP1/2	Neural tube formation [23]

Figure 2. Histone chaperones implicated in mammalian developmental processes. (Top panel) Pre-implantation development—singe-celled mouse embryo undergoes division to form blastocyst at embryonic day 3.5, E3.5. Inner cell mass (ICM) and trophectoderm (TE) constitute the blastocyst surrounding the blastocoel. ICM are source of embryonic stem cells (ESCs). Mouse ESCs when derived from ICM express core pluripotency factor OCT4 and NANOG, as visualized by immunofluorescence microscopy. (Middle panel) The structural organization of the chromatin of sperm and oocyte are also regulated by different set of histone chaperones. (Lower panel) Further development and differentiation to all three lineages, after post-implantation, have been attributed to the proper functioning of histone chaperones. Histone chaperones associated with the regulation of the developmental stages have been summarized in the table.

comprising of epiblast and hypoblast and the outer layer of trophectoderm (TE) covering the blastocoel (**Figure 2**). ICM gives rise to the embryo proper and the TE develops into the extraembryonic tissue forming the link between the embryo and the mother. Embryonic stem

cells (ESCs) are developed *in vitro* from the ICM and with time have evolved into the best tool to understand development as well as serve as the best alternative to be exploited for the generation of cells of different lineages due to the pluripotent nature of these cells. Pluripotent ESCs can differentiate into any lineage except to extraembryonic lineage. Loss in CAF1 leads to the mislocalization, loss of clustering, and decondensation of pericentric heterochromatin domains in ESCs and an altered histone methylation mark at the level of pericentric heterochromatin is formed [7]. Basically, CAF1 targeted mutation led to the developmental arrest at the 16-cell stage due to severe alteration in the nuclear organization of constitutive heterochromatin. The reason was the non-maturation of the heterochromatin and the retention of the 4-celled stage in the 16-cell stage leading to stalled development. So, CAF1p150 is needed for the development of preimplantation embryo [7]. Conditional mutagenesis of the histone chaperone ATRX demonstrated a failure in the development of TE in murine embryo [8]. A defect in methylation pattern and growth pattern lead to the embryonic lethality of mouse embryo. Thus, ATRX serve as one of the deterministic factor for the successful differentiation of embryo into TE and hence in the formation of extraembryonic tissues. During the early embryonic development, histone chaperone DAXX has been associated in the suppression of apoptosis in the embryo wherein its loss resulted in enhanced apoptosis thus resulting to embryonic lethality [9]. In the late pre-implantation stage, the embryo is significantly hypomethylated. Retrotransposons present in the mammalian genome stay in a silenced fashion to avoid their activation that could result in the loss of genomic integrity. In this stage of development, CAF1 mediated deposition of histone variant H3.1/3.2 and repressive histone marks, including H4K20me3 and H3K9me3, at retrotransposon regions repressed the activation of these elements thereby aiding in the proper development of embryo [10]. So, different histone chaperones specifically regulate stages of pre-implantation development or early mammalian development (**Figure 2**).

Development of the embryo to an adult further instils another set of histone chaperones in regulatory mechanism associated with differentiation into different lineages. A classic example is the histone chaperone HIRA. Targeted mutagenesis of HIRA demonstrated that HIRA is indispensable during murine embryogenesis. The defects of mutation were prominently visible during gastrulation, abnormal placentation, cardiac morphogenesis and finally leading to embryonic lethality [11]. There are different lineages, which are preferentially being targeted by few histone chaperones, and in the next section we will discuss the relation between subtypes of histone chaperones in development (**Figure 2**).

2.2. Cardiac differentiation and heart development

Mesodermal differentiation to cardiac progenitors demonstrated a substantial role of histone chaperone HIRA. Cardiomyocyte-specific *Hira* conditional-knockout mice did not disturbed the heart development, but instead resulted in cardiomyocyte hypertrophy and susceptibility to sarcolemmal damage [12]. Cardiomyocyte degeneration led to focal replacement fibrosis and hence resulted in the impaired cardiac function. Gene expression profile in *Hira* conditional-knockout hearts indicated impairment in pathways associated with responses to cellular stress, DNA repair and transcription and could hence implicate HIRA in maintenance of cardiomyocyte homeostasis [12]. HIRA could also individually regulate locus-specific

effects on cardiac-specific genes. Conditional ablation of Hira in the cardiogenic mesoderm of mice demonstrated dysregulation of *Tnni2* and *Tnnt3*, Troponin genes, involved in the cardiac contractility [13]. HIRA bind to the enhancer elements of Troponin genes that are already bound by the cardiac-specific transcription factor NKX2.5. Hence, absence of HIRA during cardiac differentiation results in several defects including edema, which finally aggravate to embryonic lethality [13].

2.3. Muscular differentiation

Transcription factor MyoD is essential in myoblast differentiation. Histone chaperone HIRA and H3.3 play pivotal roles in MyoD regulation [14]. HIRA, phosphorylated by Akt kinase, thereby modulates a switch between its phosphorylated and non-phosphorylated state and thereby dictate the expression of myogenic genes during myogenesis [15]. Another histone chaperone SPT6, through cooperation with RNA Pol II and histone demethylase KDM6A, orchestrates removal of repressive H3K27me3 mark from MyoD, thereby facilitating the expression of MyoD and thus controlling gene expression associated with development and cell differentiation [16]. Simultaneously, HIRA interact with another transcription factor MEF2C and contributes to its activation during muscle differentiation [17]. ASF1A forms a complex with HIRA for MEF2C dependent transcription and is indispensable for myoblast differentiation. Basically, at the chromatin level, HIRA mediated enrichment of active histone modification marks within the myogenin promoter regulated myoblast differentiation [17]. Myofibers lacking HIRA suffer oxidative stress and generate a hypertrophic response in skeletal muscle thereby exposing the myofibers to stress-induced degeneration [18].

2.4. Endothelial/hematopoietic differentiation

Endothelial cells are differentiated from mesodermal lineage. They constitute crucial partners in angiogenesis, the development of new blood vessels. Angiogenic factors like basic fibroblast growth factor and epidermal growth factor could incite angiogenic response in endothelial cells with the significant increase in expression of Vascular endothelial growth factor receptor 1 (VEGFR1) [19]. Interestingly, mechanistic analysis showed that HIRA mediated incorporation of histone H3.3 variants upon acetylated at H3K56 induces the VEGFR1 level in mouse yolk sac endothelial cells. Loss in expression of HIRA eventually reduced *in vitro* angiogenesis and pathological angiogenesis in the choroidal neovascularization model [19].

RUNX1 (Runt-related transcription factor 1) has been attributed to be a pre-requisite for the hemogenic to hematopoietic transition. This cellular transition is transient in nature, but recently it has been shown to be the authentic source for the emergence of hematopoietic stem cells (HSCs). Since, HIRA could influence angiogenesis related endothelial-specific genes, we investigated the role of the histone chaperone HIRA in hemogenic to hematopoietic transition. In mouse hemogenic endothelial cells, HIRA physically interact with RUNX1 and thus could regulate the downstream targets of RUNX1 including *Pu.1*, *Gfi1*, and *Gfi1b* that are implicated in the functioning of HSCs [20]. The *Runx1* + 24 mouse conserved noncoding element, an intronic enhancer, is essential for the expression of *Runx1* during endothelial to hematopoietic transition. The locus is active upon incorporation of histone variant H3.3 in a HIRA-dependent

fashion. Thus, HIRA regulate RUNX1 in driving the hemogenic to hematopoietic transition. Earlier studies in leukemia cells, demonstrated that HIRA interacting with transcription factor EKLF could regulate the β-globin gene expression associated with adult definitive erythropoiesis [21]. It could also control the expression of EKLF and GATA1 by regulating the chromatin modification at the corresponding regulatory genes during differentiation.

2.5. Neuronal differentiation

Neural progenitor cells are enriched in histone chaperone HIRA and have been associated with neural progenitor cell proliferation, terminal mitosis and cell cycle exit. Loss of HIRA leads to premature differentiation in neural progenitors [22]. HIRA is involved in the increased β-catenin expression due to the enrichment of H3K4me3 mark within its promoter by inducing the recruitment of Setd1A methyltransferase at the promoter. Thus, HIRA could regulate neurogenesis. Interestingly, deletion of the neuronal Nap1/2 (nucleosome assembly protein 1-like 2) gene, another histone chaperone, in mice causes neural tube defects [23]. Nap1/2 actually enhance the histone acetylation at H3K9/14 within the *Cdkn1c* locus, responsible for neuronal differentiation.

3. Histone chaperones regulate reprogramming

In 2006, Takahashi and Yamanaka demonstrated how cell fates could be manipulated with the expression of four-transcription factor Oct4, Klf4, Sox2 and c-Myc or OSKM [24, 25]. These Yamanaka factors, when ectopically expressed in terminally differentiated fibroblasts led to the generation of induced pluripotent stem cells (iPSCs) [24, 25]. iPSCs are similar in character to ESCs. The major ethical concern associated with exploitation of embryo to derive ESCs could be successfully avoided by the use of iPSCs. Till now, they have been derived from different mammalian species, including human, utilizing varied protocols. Technically, human iPSCs has been proven to be a very efficient tool in understanding mechanistic basis of several diseases, for drug screening and finally to serve as the huge potential of cells needed for replacement therapy or in regenerative medicine. Although a vivid literature is available on the methods to generate iPSCs, nevertheless it is associated with lot of complexities including its low turnover and the distinct set of cellular machinery that result in reversing the clock. Even after a decade, the molecular mechanisms underlying the reprogramming process remains unclear and hence substantial amount of information still remain elusive.

Functionality of cell depends upon how the chromatin is arranged in each cell type. So, the parts of genome, which are essential for the function of a specialized cell, should be in accessible state to the transcription factors and other chromatin associated proteins. Actively differentiating cell changes its chromatin states progressively toward its mature state. The reversible nature of epigenome to embryonic state is proved with the seminal somatic cell nuclear experiments by the oocyte factors. Further the erasure of epigenetic modifications to pluripotency state is attained with the ectopic expression of transcription factors. iPSCs technology revealed the importance of transcription factors in cell fate change. Different transcription factor cocktail to induce pluripotency have been tried and still OSKM remains the

preferred cocktail mixture to study the cell fate change. These reprogramming factors are transcription factors, which are highly expressed, in embryonic stem cells and they maintain the circuits in the chromatin to change a state to pluripotent, which is likely to differentiate into different lineages including neuron, blood, etc. Thus, transcription factors and chromatin remodeling factors are key elements in cell fate change, which can drive the cell toward other state when maintained in a controlled environment. Thus it is essentially to identify the key factors to reprogram the cells directly into other cell type by bypassing the pluripotent state.

Along with transcriptional regulation, reprogramming entails global epigenetic remodeling. Reprogramming of somatic cells to pluripotent state is marked with change in the global epigenome, with the erasure of donor cell epigenetic modifications. Thus, inducing a somatic cell to reprogram into pluripotent one largely involves structural change at the chromatin in creating a signature expression pattern of genes associated with the generation of iPSCs. The histone code comprising of different histone modification patterns indicates the status of the genomic loci by the presence of active or repressive marks. After the OSKM transduction, initial days of reprogramming results in the loss of somatic cell characteristics, which are due to binding of the OSKM factors. With the OSKM binding the regions of the condensed chromatin leads to the genome wide chromatin changes. A plethora of histone modification marks encompasses different stages of reprogramming including active H3K4me2, H3K3me3 or repressive H3K27me3 [26]. The remodeling of pluripotent gene promoter initiates at an early stage to facilitate the chromatin accessibility for the binding of different remodeling factors.

Histone modifying enzymes responsible for the corresponding histone modifications interact with the core pluripotency factors like OCT4 and promote the activation marks to facilitate attainment of pluripotency.

Interestingly, the barrier for the reprogramming process lies in the histone marks with the repressive function, preventing their removal and directing a shift from change the chromatin structure from heterochromatin to euchromatin. This is an uphill task to be achieved only by the set of transcription factor. This criterion adds up to prolonged time span required to accomplish the process of reprogramming with the low production efficiency.

The non-canonical or replacement histone variants have been implicated in the regulation of cellular reprogramming. Deposition of histone variants by replacing their canonical histones along the genomic sequences changes the expression profiles thereby giving a cell a new identity. MacroH2A histone variants have been shown to resist reprogramming [27]. On the contrary, histone variants TH2A/TH2B, highly enriched in oocytes, typically enhance the reprogramming process [28]. These histone variants are known to be expressed in testes, oocytes and zygotes are associated with open chromatin [28].

But, upon looking into a close proximity, we could infer that basically, it is the tight packaging of nucleosomes that regulates the genetic information driving the morphological change of one phenotype of cell to the other one during reprogramming. And the players regulating the assembly or disassembly of nucleosomes are the histone chaperones. Although, late, but the first report on the role of histone chaperones in cellular reprogramming was established in 2014 [29].

After that, two other reports came out in the simultaneous years proving their role as activator or barrier to reprogramming thereby entrusting another regulatory behavior for the gene expression in mammalian cells. In the following paragraphs, we will understand what molecular mechanism drives the regulation of iPSCs formation as a function of histone chaperone.

3.1. ASF1A: essential for induction of pluripotency

Anti Silencing Function 1 (ASF1) is the most conserved histone H3 and histone H4. It has been implicated in almost all the functions of histone chaperone described in earlier sections. In mammals, ASF1 has two paralogs, ASF1A and ASF1B. The human oocyte in metaphase II is highly enriched in ASF1A and this state of oocyte has been attributed in having a greater reprogramming potential than any cells driven to pluripotency by the addition of exogenous factors [29]. Global transcriptomic profiling demonstrated ASF1A as the most vital oocyte-reprogramming factor across different mammalian species [29]. In mammals, it forms complex with other histone chaperones including HIRA and CAF1 and its role in cellular reprogramming of human dermal fibroblasts was analyzed for the first time. ASF1A is required for the generation of iPSCs from adult human dermal fibroblasts. The promoters of *NANOG*, *OCT4* and *SOX2* are significantly enriched with the active acetylated H3K56 mark during reprogramming. This enrichment was further enhanced upon overexpression of ASF1A while the level was significantly reduced upon its downregulation. So, functionally, ASF1A mediates the maintenance of H3K56ac level in reprogramming cells within the promoters of core pluripotency genes and those pluripotent factors as a consequence bind to their target genes to accomplish the process. OSKM combination is added for the induction of reprogramming, but ASF1A along with only OCT4 and GDF9 (Growth Differentiation Factor 9) could contribute to the generation of authenticated iPSC colonies. GDF9 is again an oocyte specific growth factor. This study proved that ASF1A is required for the reprogramming phenomenon in human dermal fibroblasts and how a histone chaperone could ultimately influence and regulate the transcriptional machinery in cellular reprogramming [29].

3.2. CAF1: restrict induction of pluripotency

Chromatin assembly factor 1 (CAF1) associate with the deposition of newly synthesized histone H3/H4 on DNA. Chaf1a and Chaf1b, two subunits of CAF1, have been recently associated with reprogramming [30]. Downregulation of CAF1 induced the generation of iPSC clones within 5 days of OSKM addition in human dermal fibroblasts. Even the efficiency in formation of iPSCs enhanced from 0.1% in control fibroblasts to 1–5% in *Caf1*-shRNA cells. But, this increase in number does not reflect an accelerated proliferative capacity of cells upon downregulation of CAF1. However, CAF1 presence was needed during the initial period of reprogramming and hence an optimal dosage of CAF1 determines the effect on the reprogramming process. SON-seq and ATAC-seq data revealed that CAF1 downregulation result in the enrichment of accessible ES-cell specific super enhancer elements. This implied that CAF1 regulate the local chromatin structure of the ES-cell specific enhancer elements. Thus, CAF1 regulate the gene expression thereby modulating the chromatin for accessibility of transcription factors. Pluripotency-associated transcription factor, SOX2 binding increased

across the lineage-specific super enhancer elements in CAF1-downregulated cells. Also, CAF1 downregulation resulted in an upregulation of OCT4 independent of OSKM-induced cell-fate changes. CAF1 deprivation resulted in a local depletion of the repressive histone modification mark, H3K9me3 at a subset of somatic heterochromatin areas termed 'reprogramming-resistant regions', linked to those sites that associated with low efficiency in somatic cell nuclear transfer. So, CAF-1 inhibition primed the change in chromatin to a more accessible form being further efficient in transcriptional activation.

3.3. APLF: a barrier of reprogramming

Aprataxin-PNK like Factor was first discovered as a DNA repair factor associated with the Non-homologous End Joining (NHEJ) repair process [31, 32]. Upon DNA damage, APLF aids in the recruitment of Ku, XRCC4 and Lig4 at the damaged site and accelerates the repairing process [31, 32]. On exposure to ionizing radiation, myeloid neoplasms were impeded in APLF-deprived mice, with a minute effect in DNA repair capacity [33]. So, APLF although a part of NHEJ complex, is dispensable and its dosage if modulated could be used in the advantage of diseased state of cancer. In 2011, APLF was demonstrated to possess histone chaperone activity. It could bind to histone H3/H4 and as well as the repressive MacroH2A variants [34]. We observed that the level of APLF was almost undetectable in mouse ESCs whereas, a significant expression was evident in the mouse embryonic fibroblasts (MEFs) [35]. On downregulation of APLF, the efficiency of reprogramming of MEFs to iPSCs was significantly enhanced to ~10 times. The average time for the generation of iPSC clones from APLF-depleted MEFs reduced to half the time required for the control cells to achieve the same. Mechanistically, it influenced mesenchymal-to-epithelial transition (MET) during the process of forming iPSCs from MEFs. Fibroblasts are typically mesenchymal in nature whereas the ESC-like iPSC clones demonstrate epithelial characteristics [36]. So, generation of iPSCs from MEFs involve this cellular transition and is one of the earliest event in the phases of reprogramming. E-Cadherin or CDH1 is the major player for the cells to demonstrate epithelial morphology whereas a group of other transcription factors SNAI1/SNAI2/ZEB along with N-cadherin or CDH2 drives the reverse phenomenon of epithelial-to-mesenchymal transition (EMT). Downregulation of APLF induced the expression of CDH1 while the same resulted in reduced expression of the other transcription factors associated with EMT. The enhanced expression of CDH1 resulted from the loss in recruitment of repressive MacroH2A.1 variant within the *Cdh1* promoter. Histone variant macroH2A.1 drives the compaction of chromatin thereby resulting in a repressed locus. APLF-downregulated cells demonstrated increased level of pluripotency genes, *Nanog* and *Klf4*, in comparison to the control cells. Histone modification H3K4me2 level was significantly enriched within these promoters of core pluripotency genes; one of the earliest histone marks in reprogramming that facilitates the cellular transition of fibroblasts to iPSCs [37]. So, histone chaperone APLF proved to be a barrier in reprogramming and its downregulation did not interfere with the DNA repair capacity whereas induced proliferation, kinetics and efficiency of reprogramming (**Table 1**).

Histone chaperone	Histone/ histone variants	Reprogramming process	Transcription target/signaling target	Role	Chromatin status	Histone modification
ASF1A [29]	—	Human fibroblasts	GDF9/K56ac increased at Nanog/Sox2/ Oct4 regulatory regions	Enhancing	Open	H3K56ac
CAF1A [30]	—	Mouse fibroblasts, direct conversion of B cells into macrophages and fibroblasts into neurons	Increased binding of Sox2 to pluripotency targets	Inhibitory	Heterochromatin inducer	H3K9me3
APLF [35]	MacroH2A.1	Mouse fibroblasts	MET targets, core pluripotency factors	Inhibitory	Heterochromatin inducer	H3K4me2

Table 1. Histone chaperones in reprogramming.

4. Conclusion

Basic fundamental molecule histone chaperones constitutes one of the epigenetic modulator responsible for different extent of epigenetic modifications [1, 2, 38]. They work in conjunction with histone modifying enzymes, histone modification and in few instances with transcription factors to induce the change occurring within the chromatin. Regulatory roles of histone chaperones are recently being highlighted and if exploited to their full potential, could serve as target molecules to be modulated in diseases as well. Again on that front also, a limited number of studies were performed. Being a component that take care of the entire histone metabolism and which in turn drives the major chunk of epigenetic status of a cell demands a better viewing and hence we initiated this approach for this chapter on gene expression in mammalian cells. We could only cover the development part as this field represents the most dynamicity in present day and the days to come. But, their role in replication, repair and heterochromatin is far from fully exploited. More studies are required to understand the importance of histone chaperones in mammalian system. Histone chaperones have the tremendous potential to modulate epigenetic changes and understanding their functions would give insights into how a cell converts its epigenome into another and can further unravel the secrets of developmental processes.

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Abbreviations

ESCs	embryonic stem cells
iPSCs	induced pluripotent stem cells
CENP-A	centromere protein A
CAF1	chromatin assembly factor
Spt6	Suppressor of Ty's
FACT	facilitates chromatin transcription
HIRA	histone cell cycle regulator A
DAXX	death associated protein 6
ASF1	antisilencing factor
ICM	inner cell mass
TE	trophectoderm
ATRX	alpha thalassemia/mental retardation syndrome X-linked
NKX2.5	NK2 transcription factor related, locus 5
KDM	lysine (K)-specific demethylase
MEF2C	myocyte-specific enhancer factor 2C
VEGFR1	vascular endothelial growth factor receptor
RUNX1	Runt-related transcription factor
Gfi1	growth factor independent protein 1
EKLF	erythroid Krüppel-like factor
GATA1	GATA-binding factor 1
NAP1/2	nucleosome assembly protein 1;2
CDKN1C	cyclin-dependent kinase inhibitor 1C
OSKM	Oct4-Sox2-Klf4-cMyc
OCT4	octamer 4
TH2A/TH2B	testis-specific counterparts for canonical H2A and H2B
SOX2	sex determining region Y-box 2
GDF9	growth differentiation factor
ATAC-seq	assay for transposase accessible chromatin with high-throughput sequencing

XRCC4	X-ray repair cross-complementing protein 4
LIG4	DNA ligase 4
NHEJ	non homologous end joining
MEF	mouse embryonic fibroblast
CDH1	Cadherin-1
CDH2	N-Cadherin
SNAI1/2	snail family transcriptional repressor 1/2
ZEB	zinc finger E-box-binding homeobox
EMT	epithelial-mesenchymal transition
APLF	aprataxin-PNK-like factor
PNK	polynucleotide kinase

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References

[1] Gurard-Levin ZA, Quivy JP, Almouzni G. Histone chaperones: Assisting histone traffic and nucleosome dynamics. Annual Review of Biochemistry. 2014;**83**:487-517. DOI: 10.1146/annurev-biochem-060713-035536

[2] Hammond CM, Strømme CB, Huang H, Patel DJ, Groth A. Histone chaperone networks shaping chromatin function. Nature Reviews. Molecular Cell Biology. 2017;**18**:141-158. DOI: 10.1038/nrm.2016.159

[3] Loppin B, Bonnefoy E, Anselme C, Laurençon A, Karr TL, Couble P. The histone H3.3 chaperone HIRA is essential for chromatin assembly in the male pronucleus. Nature. 2005;**437**:1386-1390

[4] Jullien J, Astrand C, Szenker E, Garrett N, Almouzni G, Gurdon JB. HIRA dependent H3.3 deposition is required for transcriptional reprogramming following nuclear transfer to Xenopus oocytes. Epigenetics & Chromatin. 2012;**5**:17. DOI: 10.1186/1756-8935-5-17

[5] Lin CJ, Koh FM, Wong P, Conti M, Ramalho-Santos M. Hira-mediated H3.3 incorporation is required for DNA replication and ribosomal DNA transcription in the mouse zygote. Developmental Cell. 2014;**30**:268-279. DOI: 10.1016/j.devcel.2014.06.022

[6] Messiaen S, Guiard J, Aigueperse C, Fliniaux I, Tourpin S, Barroca V, Allemand I, Fouchet P, Livera G, Vernet M. Loss of the histone chaperone ASF1B reduces female reproductive capacity in mice. Reproduction. 2016;**151**:477-489. DOI: 10.1530/REP-15-0327

[7] Houlard M, Berlivet S, Probst AV, Quivy JP, Héry P, Almouzni G, Gérard M. CAF-1 is essential for heterochromatin organization in pluripotent embryonic cells. PLoS Genetics. 2006;**2**:e181

[8] Garrick D, Sharpe JA, Arkell R, Dobbie L, Smith AJ, Wood WG, Higgs DR, Gibbons RJ. Loss of Atrx affects trophoblast development and the pattern of X-inactivation in extraembryonic tissues. PLoS Genetics. 2006;**2**(4):e58

[9] Michaelson JS, Bader D, Kuo F, Kozak C, Leder P. Loss of Daxx, a promiscuously interacting protein, results in extensive apoptosis in early mouse development. Genes & Development. 1999;**13**:1918-1923

[10] Hatanaka Y, Inoue K, Oikawa M, Kamimura S, Ogonuki N, Kodama EN, Ohkawa Y, Tsukada Y, Ogura A. Histone chaperone CAF-1 mediates repressive histone modifications to protect preimplantation mouse embryos from endogenous retrotransposons. Proceedings of the National Academy of Sciences of the United States of America. 2015;**112**:14641-14646. DOI: 10.1073/pnas.1512775112

[11] Roberts C, Sutherland HF, Farmer H, Kimber W, Halford S, Carey A, Brickman JM, Wynshaw-Boris A, Scambler PJ. Targeted mutagenesis of the Hira gene results in gastrulation defects and patterning abnormalities of mesoendodermal derivatives prior to early embryonic lethality. Molecular and Cellular Biology. 2002;**22**:2318-2328

[12] Valenzuela N, Fan Q, Fa'ak F, Soibam B, Nagandla H, Liu Y, Schwartz RJ, McConnell BK, Stewart MD. Cardiomyocyte-specific conditional knockout of the histone chaperone HIRA in mice results in hypertrophy, sarcolemmal damage and focal replacement fibrosis. Disease Models & Mechanisms. 2016;**9**:335-345. DOI: 10.1242/dmm.022889

[13] Dilg D, Saleh RN, Phelps SE, Rose Y, Dupays L, Murphy C, Mohun T, Anderson RH, Scambler PJ, Chapgier AL. HIRA is required for heart development and directly regulates Tnni2 and Tnnt3. PLoS One. 2016;**11**:e0161096. DOI: 10.1371/journal.pone.0161096

[14] Yang JH, Song Y, Seol JH, Park JY, Yang YJ, Han JW, Youn HD, Cho EJ. Myogenic transcriptional activation of MyoD mediated by replication-independent histone deposition. Proceedings of the National Academy of Sciences of the United States of America. 2011;**108**:85-90. DOI: 10.1073/pnas.1009830108

[15] Yang JH, Song TY, Jo C, Park J, Lee HY, Song I, Hong S, Jung KY, Kim J, Han JW, Youn HD, Cho EJ. Differential regulation of the histone chaperone HIRA during muscle cell differentiation by a phosphorylation switch. Experimental & Molecular Medicine. 2016;**48**:e252. DOI: 10.1038/emm.2016.68

[16] Wang AH, Zare H, Mousavi K, Wang C, Moravec CE, Sirotkin HI, Ge K, Gutierrez-Cruz G, Sartorelli V. The histone chaperone Spt6 coordinates histone H3K27 demethylation and myogenesis. The EMBO Journal. 2013;**32**:1075-1086. DOI: 10.1038/emboj.2013.54

[17] Yang JH, Choi JH, Jang H, Park JY, Han JW, Youn HD, Cho EJ. Histone chaperones cooperate to mediate Mef2-targeted transcriptional regulation during skeletal myogenesis. Biochemical and Biophysical Research Communications. 2011;**407**:541-547. DOI: 10.1016/j.bbrc.2011.03.055

[18] Valenzuela N, Soibam B, Li L, Wang J, Byers LA, Liu Y, Schwartz RJ, Stewart MD. HIRA deficiency in muscle fibers causes hypertrophy and susceptibility to oxidative stress. Journal of Cell Science. 2017;**130**:2551-2563. DOI: 10.1242/jcs.200642

[19] Dutta D, Ray S, Home P, Saha B, Wang S, Sheibani N, Tawfik O, Cheng N, Paul S. Regulation of angiogenesis by histone chaperone HIRA-mediated incorporation of lysine 56-acetylated histone H3.3 at chromatin domains of endothelial genes. The Journal of Biological Chemistry. 2010;**285**:41567-41577. DOI: 10.1074/jbc.M110.190025

[20] Majumder A, Syed KM, Joseph S, Scambler PJ, Dutta D. Histone chaperone HIRA in regulation of transcription factor RUNX1. The Journal of Biological Chemistry. 2015;**290**:13053-13063. DOI: 10.1074/jbc.M114.615492

[21] Soni S, Pchelintsev N, Adams PD, Bieker JJ. Transcription factor EKLF (KLF1) recruitment of the histone chaperone HIRA is essential for β-globin gene expression. Proceedings of the National Academy of Sciences of the United States of America. 2014;**111**:13337-13342. DOI: 10.1073/pnas.1405422111

[22] Li Y, Jiao J. Histone chaperone HIRA regulates neural progenitor cell proliferation and neurogenesis via β-catenin. The Journal of Cell Biology. 2017;**216**:1975-1992. DOI: 10.1083/jcb.201610014

[23] Attia M, Rachez C, De Pauw A, Avner P, Rogner UC. Nap1l2 promotes histone acetylation activity during neuronal differentiation. Molecular and Cellular Biology. 2007;**27**:6093-6102

[24] Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell. 2006;**126**:663-676. DOI: 10.1016/j.cell.2006.07.024

[25] Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, Yamanaka S. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell. 2007;**131**:861-872

[26] Papp B, Plath K. Epigenetics of reprogramming to induced Pluripotency. Cell. 2016;**152**:1324-1343

[27] Gaspar-Maia A, Qadeer ZA, Hasson D, Ratnakumar K, Leu NA, Leroy G, Liu S, Costanzi C, Valle-Garcia D, Schaniel C, Lemischka I, Garcia B, Pehrson JR, Bernstein E. MacroH2A histone variants act as a barrier upon reprogramming towards pluripotency. Nature Communications. 2013;**4**:1565. DOI: 10.1038/ncomms2582

[28] Shinagawa T, Takagi T, Tsukamoto D, Tomaru C, Huynh LM, Sivaraman P, Kumarevel T, Inoue K, Nakato R, Katou Y, Sado T, Takahashi S, Ogura A, Shirahige K, Ishii S. Histone variants enriched in oocytes enhance reprogramming to induced pluripotent stem cells. Cell Stem Cell. 2014;**14**:217-227. DOI: 10.1016/j.stem.2013.12.015

[29] Gonzalez-Muñoz E, Arboleda-Estudillo Y, Otu HH, Cibelli JB. Cell reprogramming. Histone chaperone ASF1A is required for maintenance of pluripotency and cellular reprogramming. Science. 2014;**345**:822-825. DOI: 10.1126/science.1254745

[30] Cheloufi S, Elling U, Hopfgartner B, Jung YL, Murn J, Ninova M, Hubmann M, Badeaux AI, Euong Ang C, Tenen D, Wesche DJ, Abazova N, Hogue M, Tasdemir N, Brumbaugh J, Rathert P, Jude J, Ferrari F, Blanco A, Fellner M, Wenzel D, Zinner M, Vidal SE, Bell O, Stadtfeld M, Chang HY, Almouzni G, Lowe SW, Rinn J, Wernig M, Aravin A, Shi Y, Park PJ, Penninger JM, Zuber J, Hochedlinger K. The histone chaperone CAF-1 safeguards somatic cell identity. Nature. 2015;**528**:218-224. DOI: 10.1038/nature15749

[31] Grundy GJ, Rulten SL, Zeng Z, Arribas-Bosacoma R, Iles N, Manley K, Oliver A, Caldecott KWAPLF. Promotes the assembly and activity of non-homologous end joining protein complexes. The EMBO Journal. 2013;**32**:112-125. DOI: 10.1038/emboj.2012.304

[32] Macrae CJ, McCulloch RD, Ylanko J, Durocher D, Koch CA. APLF (C2orf13) facilitates nonhomologous end-joining and undergoes ATM-dependent hyperphosphorylation following ionizing radiation. DNA Repair (Amst). 2008;**7**:292-302

[33] Tong KI, Ota K, Komuro A, Ueda T, Ito A, Anne Koch C, Okada H. Attenuated DNA damage repair delays therapy-related myeloid neoplasms in a mouse model. Cell Death & Disease. 2016;**7**:e2401. DOI: 10.1038/cddis.2016.298

[34] Mehrotra PV, Ahel D, Ryan DP, Weston R, Wiechens N, Kraehenbuehl R, Owen-Hughes T, Ahel I. DNA repair factor APLF is a histone chaperone. Molecular Cell. 2011;**41**:46-55. DOI: 10.1016/j.molcel.2010.12.008

[35] Syed KM, Joseph S, Mukherjee A, Majumder A, Teixeira JM, Dutta D, Pillai MR. Histone chaperone APLF regulates induction of pluripotency in murine fibroblasts. Journal of Cell Science. 2016;**129**:4576-4591

[36] Li R, Liang J, Ni S, Zhou T, Qing X, Li H, He W, Chen J, Li F, Zhuang Q, Qin B, Xu J, Li W, Yang J, Gan Y, Qin D, Feng S, Song H, Yang D, Zhang B, Zeng L, Lai L, Esteban MA, Pei D. A mesenchymal-to-epithelial transition initiates and is required for the nuclear reprogramming of mouse fibroblasts. Cell Stem Cell. 2010;**7**:51-63. DOI: 10.1016/j.stem.2010.04.014

[37] Barrero MJ, Sese B, Kuebler B, Bilic J, Boue S, Martí M, Izpisua Belmonte JC. Macrohistone variants preserve cell identity by preventing the gain of H3K4me2 during reprogramming to pluripotency. Cell Reports. 2013;**3**:1005-1011. DOI: 10.1016/j.celrep.2013.02.029

[38] Avvakumov N, Nourani A, Côté J. Histone chaperones: Modulators of chromatin marks. Molecular Cell. 2011;**41**:502-514. DOI: 10.1016/j.molcel.2011.02.013

Chapter 5

Epigenetic Factors: Key Regulators Targeted in Cancers

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Additional information is available at the end of the chapter

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Abstract

Gene expression is tightly regulated via a myriad of mechanisms in the cell to allow canonical processes to occur. However, in the context of cancer, some of these mechanisms are dysregulated, and aberrant gene expression ensues. Some of the dysregulated mechanisms include changes to transcription factor activity, epigenetic marks (such as DNA methylation, histone modifications and chromatin state), or the stability of mRNA and protein. Disruption of these regulators would result changes in transcriptional landscape, affecting multiple pathways and eventually lead to continual cell proliferation and the formation of the tumor. Here, we discuss epigenetic factors that affect gene expression which are dysregulated in cancer, and summarize the therapeutic options available to target these factors.

Keywords: cancer, gene regulation, epigenetics, chromatin remodelers, histone modifications, DNA methylation, transcriptional regulation

1. Introduction

Genetic information in cells is stored as DNA, and are the same in all cells of a single organism. The method in which the same code can lead to the translation of multiple different proteins in a tissue-specific manner lies in the regulation of expression of specific genes encoded by DNA. Gene expression involves the transcription of DNA to RNA, and in some cases, translation into proteins. The gene products which consist of translated and non-coding RNAs (RNAs of which the RNA is its final product and does not get translated to protein) have different but very important functions in the cell. Collectively, the combination of the genes which are expressed and those which are silenced are crucial in maintaining normal processes in the cell, determining when it should proliferate or divide etc.

To ensure that gene expression is kept in check, its regulation is multi-tiered and can be altered/halted at every step of the gene expression process. This ensures that even if one of

these regulatory events goes awry, there are other mechanisms in the cell in place to curb aberrant gene expression. Numerous alterations in the multi-tiered process often lead to aberrant gene expression and abnormal function in the cell, which occurs in the case of cancer.

Cancer is the result of a cell escaping from its natural cell cycle, evading apoptosis which leads to uncontrolled and abnormal proliferation. The transformation of a normal cell into a malignant one results from the increase in expression of *oncogenes*, with a concomitant decrease in *tumor suppressor* expression. Oncogenes are involved in functions which lead to uncontrolled proliferation and growth, evading the canonical apoptotic mechanisms, while tumor suppressors curb these mechanisms. Although cancer cells of different tissue types have the same outcome of uncontrolled growth, the mechanisms involved are varied. Even within the same tissue, malignancies are very heterogeneous, contributing to the challenge in treating this disease.

In this chapter, we will summarize the multiple layers of gene regulation, focusing on the dysregulated epigenetic changes in cancer involved in gene expression regulation. We then summarize the therapeutic options available which seek to curb these gene regulation changes.

2. Transcriptional regulation

Gene expression is regulated by many varying factors governing different stages of this complex process. The regulatory process begins from the chromatin conformation defining its state, either euchromatin/open chromatin, allowing active transcription, or a repressive heterochromatin/closed chromatin, illustrated in **Figure 1A**. The open chromatin is actioned by several factors, including acetylated histone tails and the inclusion of specific histone variants which act to destabilize the nucleosome. This is often tested by DNase hypersensitivity assays, which measure the sensitivity of DNA to enzymatic digestion. Portions of the DNA with nucleosomes loaded would be protected from DNase digestion, while nucleosome depleted regions (NDR) are sensitive.

The idea of topological domains was suggested recently by Dixon et al. [1], which describe a section of the genome of which its enclosed genes are generally co-regulated. The boundaries of these topological domains interact in 3D space and are marked by the presence of CCCTC-binding factor (CTCF) and cohesin. There have been mutations observed in these topologically associated domains (TADs), which will be described later in this chapter.

On the DNA level, the region of the genome around the transcription start site (TSS) is particularly important in the regulation of gene expression as that is where transcription machinery and co-regulators bind. Proteins known as transcription factors are able to recognize motifs/transcription factor binding sites on the promoters of genes, and recruit RNA Pol II and/or phosphorylate Pol II to initiate transcription. Alternatively, transcription regulators can also inhibit the binding or recruitment of the transcription complex. In addition to the TSS, recent studies have also identified that distal regulatory elements, such as enhancers, are able to regulate expression as well. Both enhancers and promoters can be marked by different histone modifications which can be read and have an impact on the expression of its corresponding gene.

Regulatory processes that affect the stability of mRNA and proteins are equally as important as gene expression factors but will not be addressed in this chapter. These include microRNA

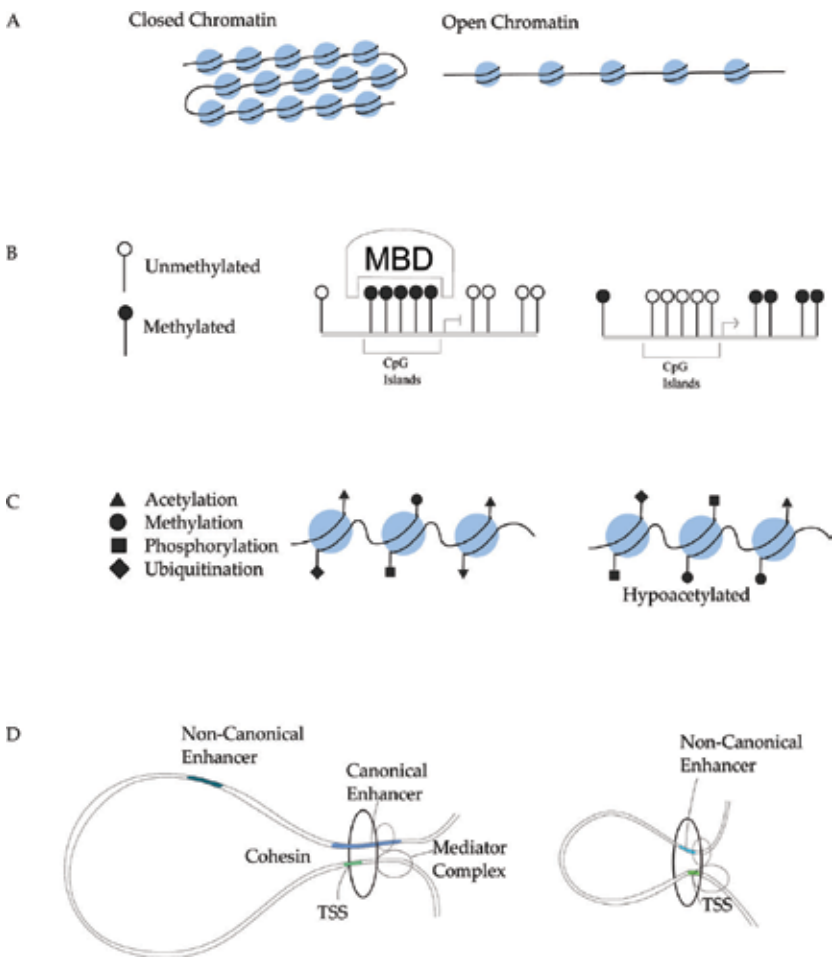

Figure 1. Illustration of selected methods of gene regulation. (A) Repressive heterochromatin state wherein transcription machinery is not able to bind to DNA, compared to activating, euchromatic state permissive of transcription activation. (B) CpG islands upstream of TSS are often hypermethylated in cancer, repressing transcription, while CpG islands in the rest of the genome are hypomethylated. These methylated CpG marks are read by protiens containing methyl CpG-binding domains (MBD). These methylation states are reversed in cancer, allowing transcriptional activation. (C) Histone tails are able to undergo multiple covalent modifications such as acetylation, methylation, phosphorylation and ubiquitination. In cancer, histone tails are often observed to be hypoacetylated and therefore repressing transcription. (D) Mediator and cohesin enabling long-range chromatin interactions to occur, therefore bringing together the proximal (promoter/TSS) and distal (enhancer) regulatory regions. During the process of tumor growth, non-canonical enhancer usage often occurs, resulting in aberrant gene expression.

(miRNA) which have the ability to degrade mRNA and thus prevent it from being translated to proteins, and also endogenous systems which degrade proteins.

Studies have shown that every step of the gene expression regulation can be exploited by cancer cells to prolong survival and contribute to tumorigenesis. Considering the vast nature of this topic, we will be focusing on the multiple epigenetic factors regulating gene expression which are dysregulated in cancer. However, dysregulated transcription factors form a huge

topic of interest in cancer research. This includes work on tumor suppressor p53 (reviewed in [2–4]) and the oncogene MYC (reviewed in [5, 6]), amongst others.

3. Epigenetic regulators of gene expression

Epigenetics is an additional layer of complexity to the genetic code, and comprise of additional information (in the form of compounds added or secondary structures) on top of the four basic nucleotides: adenine, thymine, cytosine and guanine. This allows a gene with the same genetic sequence to be differentially regulated according to cell type or context. This occurs through mechanisms such as binding of transcription factors and machinery. These epigenetic changes and modifications allow for greater control and regulation for gene expression by transcription factors. Here, we will discuss the myriad of epigenetic features that can contribute to gene expression.

3.1. Chromatin modifications

It is only through the compaction of DNA in a nucleosome that the long length of DNA is packaged into the nucleus of each cell. A length of 146 bp of DNA is coiled around a histone octamer, consisting of two residues each of H2A, H2B, H3 and H4, secured in place by H1. It is linked to its neighboring nucleosome via linker DNA, varying between 20 and 80 nucleotides in length. The placement and composition of nucleosomes are not at all random. Rather, its content and position is strategically coordinated to regulate gene expression on several different layers.

Although the terms are used interchangeably in literature, in this chapter, we will address chromatin modifiers and remodelers as two separate groups of enzymes, the former covalently modifying histone, and the latter regulating the position and composition of the nucleosomes.

3.1.1. Chromatin modifiers

Chromatin modifiers consist of a group of enzymes that post-translationally modify histones, resulting in histone modifications that make up the histone code. Covalent modifications on these histones can consist of acetyl, methyl, ubiquitin, phosphoryl groups, amongst others. The specific modifications which are added to different histones determines its function and its role in the cell, as represented in **Figure 1C**. A summary of these modifications and its related downstream effect was investigated by the ENCODE team, and summarized in their paper in 2012 [7].

Histone modifications can occur on different regulatory regions of a gene, such as at its promoter, enhancer or even along the gene body. The presence of an active mark on the promoter, for an example, recruits other transcriptional machinery factors, and allows transcription to occur. These histone modifications are not permanent, and can differ between tissue types or depending on its cellular state. The regulation of histone modifications is a balance between its *epigenetic writers* and *epigenetic erasers*, and the dysregulation of either would result in aberrant histone modifications and thus a change in the transcription. The group of proteins that are

involved in interpreting these histone marks, *epigenetic readers*, are also crucial, whose dysregulation could result in the misinterpretation of the epigenetic marks and therefore a change in transcriptional landscape.

A point to note is only a subset of these residues can undergo multiple modifications. For example, lysine 27 on histone 3, can be either acetylated (in active enhancers) or tri-methylated (a mark of repressed promoters), each of which contributes to a different transcriptional outcome. It is also hypothesized that the addition of a particular covalent modification sterically inhibits the alternate modification. Additionally, histone marks on the enhancers such as H3K4me and H3K27ac are capable of regulating the 3D structure of chromatin. Since this method of regulation is indeed another layer that the cell regulates gene expression in a normal setting, it comes as no surprise that chromatin modifiers are known to be dysregulated in cancer, resulting in the aberrant expression of its downstream genes (lysine acetyltransferases (KATs) reviewed in [8], histone methyltransferases (HMTs) reviewed in [9], histone deacetylases (HDACs) reviewed in [10–12], histone demethylases reviewed in [13, 14]). Here, we will focus on epigenetic factors which are dysregulated in cancer, resulting in a transcriptional change.

3.1.1.1. Epigenetic writers

Epigenetic writers are enzymes that have the ability to deposit the moiety onto histone tails, and have to work in balance with epigenetic erasers to ensure the presence of the correct histone modification to govern the required transcriptional program. All epigenetic writers require a catalytic domain which allows the enzymatic reaction of the moiety transfer to occur, and another domain which allows the recognition of the chromatin.

Another class of chromatin modifiers are KATs which are involved in acetylating lysine residues on histones. This is perhaps the most crucial modification on histone tails as it not only marks histones to be read by epigenetic readers, but the acetylated histones also allow the relaxation of chromatin conformation. Acetyl groups neutralize the positive charge of histones, therefore loosening the conformation of nucleosomes in turn allowing the binding of transcription initiation complex to chromatin, resulting in gene activation. The decrease in acetylated histones is a phenomenon observed in multiple cancers as depicted in **Figure 1C**, along with its permissive state, as seen with global levels of H4K16ac decreased in lymphomas when compared to normal [15].

TIP60 (HIV-Tat1 interactive protein 60 kDa) is an acetyltransferase, a member of the MYST (Moz, Ybf2p/Sas3p, Sas2p and TIP60) family, known to acetylate both histones and non-histone proteins. Although it has been shown to have a bivalent role in the process of carcinogenesis (dependent on cancer type), strong evidence has supported its role as a tumor suppressor [16, 17]. TIP60 exerts its tumor suppressive phenotype through acetylating several substrates in the cell, one of which is ataxia-telangiectasia mutated (ATM) at DNA damage sites [18, 19]. Additionally, TIP60 is also known to acetylate p53 at lysine 120, crucial in mediating the switch between cell-cycle arrest or apoptosis [20]. It was also recently shown that TIP60 is able to repress telomerase transcription by acetylating Sp1, therefore inhibiting its binding on *TERT* promoter [21].

In support of its role as a tumor suppressor, the levels of TIP60 was found to be lower in tumor compared to its matched normal in multiple cancers including breast [16] and colon [22].

The downregulation of TIP60 occurs through several mechanisms, including regulation at mRNA level by miR-22 [23] or via proteosomal degradation by the human papillomavirus (HPV) oncogene E6 through EDD1, an E3 ubiquitin ligase [17]. TIP60 has been shown to regulate transcription at the integrated HPV promoter via the acetylation of H4, and therefore repress the expression of E6 [24, 25].

GCN5 (general control of amino acid synthesis protein 5-like 2) is another acetyltransferase which acetylates H3K9, H3K14, marks of active transcription [26], and when part of the SAGA (Spt-Ada-GCN5-Aceyl transferase) complex, acetylates H3 and H2B. Its link with cancer is primarily through the oncogene MYC, which recruits the SAGA complex to chromatin, where GCN5 functions to activate its gene targets [27]. Since MYC is a substrate of GCN5 and when acetylated at K323 increases its stability, both proteins are maintained in a positive feedback loop [28]. GCN5 is also crucial in ALL (acute lymphoblastic leukemia) via the acetylation and stabilization of the oncogenic fusion protein E2A-PBX1 [29], leading to aberrant expression of HOX genes, therefore leukemogenesis [30].

The dysregulation of methyltransferases have also been implicated in the severity and progression of cancer. In particular, G9a is responsible for the mono and di-methylation of H3K9, which are characteristic of a transcriptionally repressed gene. G9a has been found to be involved in epigenetically silencing numerous tumor suppressor genes, such as *DSC3* (desmocollin 3) and *CDH1* (cadherin 1), with the repression of G9a resulting in rescue of tumor suppressive gene expression [31]. Recent studies have shown the upregulation of G9a in tumors, leading to the aberrant methylation of H3K9 and thus silencing of tumor suppressor and growth inhibitory factors [32, 33]. In both lung and breast cancers, G9a exerts these effects through regulating epithelial-mesenchymal transition (EMT) factors such as EpCAM (epithelial cell adhesion molecule) and Snai1 (snail family transcriptional repressor 1) [34, 35]. In AML (acute myeloid leukemia), the depletion of G9a results in late disease onset and a reduction of leukemia stem cell frequency, although there was no observable function in hematopoietic stem cells [36]. This was identified to occur through the regulation of transcription in a HOXA9-dependent manner. In addition, G9a also has alternate roles in the cell, acting as both a transcriptional co-repressor and co-activator. As a transcriptional co-repressor, G9a has been found to be present in the same protein complex as JARID1A, the H3K4 demethylase, while it acts as transcriptional co-activator through stabilization of the mediator complex [37].

EZH2 is the enzymatic subunit of the polycomb repressive complex 2 (PRC2) which methylates lysine 27 of histone H3, resulting in chromatin compaction and transcriptional silencing [38, 39]. EZH2 overexpression has been observed in a myriad of different cancers including prostate, breast, bladder and endometrial (reviewed in [40]). Several independent studies have shown that this gain-of-function mutation on EZH2 is able to contribute to cell proliferation [41] and neoplastic transformation in breast epithelial cells [42], which is dependent on EZH2's methyltransferase domain. In addition, mutations have been found in the H3K27me3 demethylase, UTX [43], further contributing to dysregulated tri-methylation of H3K27.

3.1.1.2. Epigenetic readers

The faithful expression and activity of *epigenetic readers* are also crucial in regulating histone modifications, the dysregulation of which would lead to histones being modified for an

extended amount of time, resulting in a cascade of downstream effects. A group of epigenetic readers are the ING (inhibitor of growth) family which contains PHD (plant homeodomain) finger at its C terminus, with the ability to read methylated lysine 4 of histone 3 [44, 45]. ING readers are present in numerous protein complexes, which allow the interpretation of the histone tails to be actioned. ING1 and ING2 are able to recruit mSin3-HDAC transcriptional repressors while ING3, ING4 and ING5 interact with HATs to activate a downstream gene expression [46, 47]. ING family members have been implicated in many cellular processes with tumorigenic features such as cell cycle progression, apoptosis, DNA repair and senescence [48]. Because of its prominent role in the development of tumors, cancer cells have exploited this mechanism, with loss-of-function mutations in INGs observed in many solid tumors [48].

Although acetylated histones can exert transcriptional change by itself through the regulation of chromosomal conformation, the acetylated marks can be read by epigenetic readers and result in further gene expression changes. Extensive studies have been carried investigating the readers of acetylation marks—bromodomain-containing proteins. Bromodomain and extra-terminal (BET) proteins are a subset of this family, consisting of BRD2, BRD3, BRD4 and BRDT. BRD4 has shown to recruit the elongation factor P-TEFβ [49, 50], thus facilitating the transcription by RNA Pol II, resulting in gene activation. In other cases, BRD4 is also known to recruit repressive machinery [24].

3.1.1.3. Epigenetic erasers

Epigenetic erasers are capable of removing the histone modifications applied by the epigenetic writers, and are crucial in ensuring that histone modifications are removed in a timely manner to prevent aberrant transcription from occurring.

The larger of the two classes of histone demethylases are the family of proteins that contain the Jumonji C (JmJC) domain, of which JARID1B (also known as KDM5A) is a member. It has been identified to remove the methylation marks from lysine 3 of histone 4. Its downstream targets comprise of tumor suppressor genes, including BRCA1 and Caveolin 1, whose promoters JARID1B demethylates and therefore suppresses its expression [51, 52]. Not surprisingly, JARID1B was found to be overexpressed in late stage breast and prostate cancer [51, 53]. Similarly, KDM4A and KDM4B have been identified as proto-oncogenes, interacting with ERα to regulate pro-tumorigenic factors such as MYC [54]. KDM4A, in particular, blocks cellular senescence through transcriptionally repressing the tumor suppressor CHD5 [55]. Interestingly, KMD4C was discovered to increase the amount of euchromatin in the cell through delocalizing HP1 (a repressive protein), therefore allowing transcription [56].

HDACs are able to remove the acetyl groups from histone tails, and are divided into four classes based on their similarity with their yeast homologs. The most well-studied class of HDACs is the class I subfamily (consisting of HDAC1, HDAC2, HDAC3 and HDAC8), where all the members have been linked to cancer. The upregulation of HDAC1 has been associated with poor prognosis in several solid tumors such as lung, prostate and liver [57, 58], and even as an independent prognostic marker in breast tissues [59]. Along similar lines, the transient depletion of HDAC1 and HDAC3 the cervical cancer cell line, HeLa, resulted in decreased cell proliferation [60]. Links between the class II HDAC genes and lung cancer has also been drawn, when HDAC genes from 72 NSCLC (non-small cell lung cancer) patients

were measured via real-time PCR [61]. It was found that lower expression of class II HDAC genes was correlated with poorer prognosis, of which HDAC10 was the strongest predictor of patient outcome.

3.1.2. Chromatin remodelers

Chromatin remodelers are enzymes that are able to make structural changes to the nucleosome, either by adding or ejecting a nucleosome, or by moving the nucleosome along the string of DNA (reviewed in [62]). This acts as one of the first steps of gene expression regulation, allowing the DNA to be exposed to other biological factors to be read and therefore expressed.

There are four chromatin remodeler families that utilize ATP hydrolysis to facilitate the catalysis of these movements along the string of DNA, NuRD/Mi-2/CHD, switch/sucrose nonfermenting (SWI-SNF), inositol requiring 80 (INO80) and imitation switch (ISWI). There is at least one epigenetic reader protein in each of the complexes, which allow the recognition of the nucleosome prior to its ejection or relocation.

Similar to other dysregulated factors in cancer, chromatin remodelers have an important responsibility in regular gene expression, and therefore have been exploited in cancer cells as a mechanism which leads to uncontrolled proliferation. Although there has been extensive research into mutations of members of chromatin remodeling families, limited evidence has linked these mutations to epigenetic alterations and changes in the chromatin architecture.

In the SWI/SNF complex, BRG1 and SNF5 are required for maintaining nucleosome positioning at the −1 and +1 positions around the TSS of repressed genes. Up to 20% of human tumors are known to contain at least one mutation in SWI/SNF [63], although BRG1 was found to have dual effects in both the promotion and suppression of tumorigenesis [64–67]. In spite of many studies carried out to characterize the mutations of SWI/SNF components, there are far fewer studies that identified the epigenetic implications of these mutations (reviewed in [68]). It was found that in the absence of either BRG1 or SNF5, there was a decrease in the distance between nucleosomes on both sides of the TSS, indicating that chromatin condensation is augmented upon SWI/SNF dysregulation [69]. SWI/SNF is also known to interact with other chromatin modifiers, whose interaction is altered when there is a change observed in SWI/SNF. As an example, SWI/SNF complex antagonizes PRC2's repressive activity by removing it from gene promoters, resulting in open chromatin conformation and therefore increase in gene expression [70, 71].

3.1.3. Histone variants

Histone variants are non-canonical versions of three histone subunits (all but H4), some with as few as one amino acid difference between its wildtype counterpart. Histone variants being highly conserved across different species eludes to its important cellular function separate from that of the canonical histones [72, 73].

The placement of histone variants in nucleosomes in specific portions of the genome have different abilities to regulate nucleosomal stability, dynamics and structure, and therefore transcriptional machinery. For example, the presence of H2A.Bbd results in loosened chromatin

and therefore encouraging transcription [74]. As with canonical histones, histone variants are also subject to covalent modifications (acetylation, methylation etc.) and mutations, and therefore add an additional layer of complexity to understanding its function and role.

Many of these histone variants have been found to have a role in cancer (reviewed in [75]), some with oncogenic and others with tumor suppressive abilities, differing based on its role in the cell. Some of the strongest correlations between histone variants and regulating transcription occurs at a macro level, in which histone variants regulates the stability of its nucleosome. Nucleosomes that contain H2A.Z or H3.3 were shown to be less stable, although they are known to occupy the normally nucleosome-depleted regulatory regions [76]. Their presence on these regulatory sites inhibit the formation of stable repressive nucleosomes, and due to its labile nature, can be displaced easily by transcription regulators, therefore facilitating gene expression [77].

In addition to histone variants affecting the overall nucleosome structure, in some instances, the readers of histone variants are different from that of canonical histones. The reader of H3.3K36me3 was identified to be the tumor suppressor protein ZMYND11 (zinc finger MYND-type containing 11), which regulates RNA Pol II, hence linking histone variants and transcriptional elongation [78]. Further, an increase in acetylation of H2A.Z was observed in prostate cancer, particularly around the promoters of actively transcribed genes, thus resulting in the aberrant activation of genes [79].

3.1.4. Chromatin conformation

Regions of the DNA which are known to interact frequently are classified as TADs, which can range up to several million nucleotides in length, and several factors are thought to be associated with the boundaries of these domains, including CTCF and cohesin [1]. Characteristic features of TADs include the lower frequency of interaction of gene domains between TADs while genes within the same TADs are often co-regulated, sharing the same genetic profile (reviewed in [80]).

Given the role of TADs in regulating gene expression, it should come as no surprise that this cellular process is also exploited in cancer cells. Disrupted TAD boundaries have been found present in cancer cells, allowing 'enhancer hijacking' to occur, where enhancers do not act on their canonical targets alone, resulting in aberrant expression of non-canonical genes, illustrated in **Figure 1D**. It was found that GFI1 and GFI1B, members of the growth factor independent 1 family of proto-oncogenes, were upregulated not by amplification in medulloblastoma, but instead activated by enhancer hijacking, coming under the control of an aberrant active enhancer [81].

Similarly, viral oncogenes encoded by Epstein–Barr virus (EBV) was found to hijack DNA looping, leading to the association of two key genes, *MYC* and *BCL2L11* (a pro-apoptotic factor) to non-canonical enhancers [82]. Through the transactivator EBNA2, the MYC locus was reconfigured to be regulated by a non-canonical enhancer, resulting in the activation of the oncogene, promoting tumor formation. Concurrently, EBV repressors EBNA3A and EBNA3C were shown to be capable of recruiting EZH2, thus silencing the upstream regulatory enhancer hub.

3.1.5. Mediator complex

The mediator complex is a large, 26 subunit complex which coordinates the many different elements required for the activation of gene transcription. This includes the cross-talk between RNA Pol II and transcription factors that possess sequence-specific recognition sites, and also distal regulatory regions such as enhancers. The CDK8 module, consisting of MED12, CDK8, Cyclin C and MED13 [83], has been identified as a key component of the complex, functioning as a molecular switch [84] and therefore regulating the activity of the mediator complex. Due to its crucial role in regulating transcription, cancer cells have exploited this mechanism to lead to aberrant gene expression.

MED12 is a member of the complex, of which frequent mutations at the N-terminus have been found in prostate cancer, uterine leiomyosarcomas [85], breast adenomas [86] and phyllodes [87]. Specifically, the mutations in MED12 disrupts the interaction between MED12 and CDK8, therefore rendering the CDK8 module inactive, therefore decreasing the activity of the mediator complex [88–90].

3.2. DNA methylation

One form of epigenetic regulation is CpG (5′ cytosine phosphate guanine 3′) methylation, which refer to the addition of methyl groups to the carbon residue at the fifth position on cytosine, exclusively where cytosine directly precedes guanine. These methyl moieties are modified and interpreted by three distinct groups of proteins, DNA methylation *writers*, *readers* and *editors*.

DNA methylation writers consist of proteins from the DNA methyltransferase (DNMT) family, namely DNMT1, DNMT3A, DNMT3B [91, 92]. *De novo* methylation patterns are added by DNMT3A and DNMT3B in response to stimuli in different contexts, while DNMT1's primary role is in the maintenance of the methyl groups, allowing it to be inherited across cell divisions. The effects of CpG methylation is mediated by the *reader* proteins from three separate families of proteins- methyl-CpG-binding domain (MBD) proteins, the SET- and Ring finger-associated (SRA) domain family and the Kaiso family of proteins [93–96]. These proteins are endowed with the ability to bind to CpG methylation and recruit other factors to exert regulatory roles in the cell [97, 98]. Finally, DNA methylation *editors* are able to oxidize the existing methyl group on carbon-5, and convert it to form a 5-hydroxylmethylcytosine (5-hmC), which undergo further chemical modifications before resuming its unmethylated state [99].

Not surprisingly, there have been reports linking all three groups of the above-mentioned proteins with cancer, leading to a global hypomethylation of repetitive elements and CpG-poor regions but a hypermethylation at CpG islands [100]. Approximately 15% of CpG sites are situated directly upstream of genes within CpG islands, with the remainder of the genome having relatively sparse CpG sites (reviewed in [101]). CpG islands are regions in the genome spanning between 300 to 3000 nucleotides which contain a high density of CpG dinucleotides, and are present at about 60% of human promoters [102], while CpG island shores are regions 2 kb flanking the CpG islands [103]. CpG islands have been shown to be sites of transcription initiation, evidenced by several features; TSS have been found within CpG islands, RNA Pol II found to co-localize to the islands, and the active histone mark H3K4me3 was found to be within the islands [104, 105]. CpG islands are thought to regulate gene expression in two distinct manners.

First, it has been shown that the methylated CpG dinucleotide is capable of sterically hindering the binding of transcription factors and co-activators [106]. Cancer cells have exploited this mechanism to silence tumor suppressor genes, with a global hypermethylation of CpG islands observed across multiple cancer types, depicted in **Figure 1B** [101, 107–109]. Secondly, the MeCP1 proteins, a class of DNA methylation readers, have been shown to recruit HDACs, responsible for deacetylating histones, therefore condensing the chromatin, ultimately leading to a decrease in transcription [107].

MBD proteins have been implicated in multiple cancers (reviewed in [110]), with its mutation and overexpression resulting in uncontrolled cell proliferation. In prostate cancer, it was discovered that MBD2 overexpression is associated in the aberrant hypermethylation and therefore suppression of *GSTP1* tumor suppressor, as is with *TERT* in HPV-positive cells [111–113]. Recently, there was an unexpected finding that MBD2 was associated with DNMT1 and DNMT3A, and the loss of MBD2 resulting in global hypomethylation, eventuating in both downstream gene activation and repression [114]. In particular, the hypomethylation observed at CpG islands and shores were the same regions that were hypermethylated in prostate cancer patients, eluding to the critical role of MBD2 in rewriting the cancer methylome.

The TET (ten-eleven translocation) family of proteins has also been implicated in several different types of cancer, with most studies carried out in hematological malignancies. It was in blood that TET1 was first implicated in cancers, identified as a fusion partner in mixed lineage leukemia (MLL)-rearranged AML [115, 116]. Subsequent studies in blood cancers focused on TET2's role, discovering numerous mutations [117], resulting in a truncated enzyme, or one with compromised enzymatic activity. This was reflected in patients where a global decrease in 5hmC was observed in patients with homozygous or heterozygous *TET2*, suggesting that mutations in *TET2* were haplo-insufficient loss-of-function mutations [118]. Aside from hematological malignancies, overall decreased levels of *TET2*, and its concomitant decrease in 5hmC levels have been observed in cancer of other origins such as breast, lung, liver [119], prostate, gastric, and melanoma [120] and glioblastomas [121].

In addition, *IDH1* (isocitrate dehydrogenase 1) and *IDH2*, genes involved in the tricarboxylic acid (TCA) cycle, were found to be mutated in gliomas and AML, leading to the hypermethylation of the genome. This is attributed to the production of a metabolite which inhibits histone and DNA demethylation [121, 122].

3.3. Chromosomal translocations

Cancer genomes are notorious for being unstable- that is, prone to mutations in the nucleic acid sequences, chromosomal rearrangements, inversions, translocations and deletions. The consequence of this is widespread and severe, resulting in aberrant expression of genes which are crucial in evading apoptosis, eventuating in tumor growth.

Chromosomal translocations are an important aspect of genomic instability, where a section of the genome is inserted into an alternate location. This can be large sections of the genome spanning millions of base pairs, as in the formation of the Philadelphia chromosome through the swap of sections of chromosome 9 and 22 (first described in 1960 [123]), or through a small translocation (<1 kb), as is with MLL fusion genes. Interestingly, the largest proportion of chromosomal

translocation targets are transcription factors, wherein the fusion gene produced is still active, but in an aberrant manner [124]. These chromosome abnormalities are most often observed in hematopoietic and lymphoid tumors [125], with fusion genes involving MLL gene accounting for up to 5–10% of ALL/AML cases, resulting in unfavorable prognoses [126].

There are two variations of MLL's resultant fusion genes, with the chromosomal insertion resulting in the retention of the N or C terminus of the MLL located at 11q23, both of which have been identified to have oncogenic potential. The function of normal MLL is that of a histone methyltransferase, with its N terminus containing a CxxC domain, allowing it to recognize unmethylated CpG dinucleotides and its corresponding target genes [127]. The C terminus of MLL, on the other hand, contains features responsible for its histone methyltransferase activity such as a SET domain, responsible for methylation of lysine 4 of histone H3 [127]. The more prevalent class of fusion genes are the chimeras with the N terminus of MLL fused with the C terminus of the fusion partner, MLL-r (MLL-rearranged), that are also known to have more oncogenic potential. It has been observed that most MLL-r function to augment its canonical downstream targets such as the HOX cluster of genes rather than gain a new profile of target genes. However, the exact function of the fusion genes is entirely dependent on the fusion partner. The two most common fusion gene partners of MLL are AF9 and AF4, which are present in the super elongation complex (SEC), and confer the fusion product's function of a transcription activator [128, 129]. In the chimeric gene, the fusion partner of MLL acts as an adaptor to the MLL portion of the gene (with DNA binding abilities) to the rest of the SEC, therefore resulting in aberrant expression of the downstream genes.

In a recent study, it was shown that MLL-AF9 and MLL-AF4 also bound to distal regulatory elements such as enhancers, and are able to deregulate its target gene expression, through interplay with RUNX1 [130]. Further, enhancer regions enriched for MLL-AF9 were found to be CTCF-rich, suggesting a novel role of MLL-AF9 in mediating 3D chromatin conformation [130].

Although most studies on fusion genes have been published in blood malignancies, recent studies have turned their attention to solid tumors. Fusion genes have also been found to be prevalent in non-blood cancers, with a similar trend of fusion partners being transcription factors, resulting in rampant aberrant gene expression changes (reviewed in [131]).

Another example of a fusion protein is BRD4-NUT, prominent in NUT midline carcinoma (NMC). The N terminus of BRD4 is conjugated with the C terminus of NUT, with retention of both bromodomains (from BRD4) and the KAT catalytic domain (from NUT) in the resultant fusion protein. This fusion protein has oncogenic potential through the formation of large active chromatin (1 Mb) where BRD4-NUT and histone hyperacetylation are co-localized [132]. In spite of the large size of chromatin which is activated, there is surprisingly only a small subset of genes which are upregulated, including *MYC* and *TP63* [132].

4. Therapies targeting epigenetic factors

When considering the different ways in which biological processes are dysregulated in cancer, aberrant activity of epigenetic regulators is considered one of the easiest to treat. This is mainly due to the fact that epigenetic dysregulation typically only occurs in specific cell types, and the

aberrancies are not present in all somatic cells. As such, therapies can be targeted to affected cancer cells, instead of requiring gene therapy to correct all somatic cells. Furthermore, epigenetic factors are often enzymes whose activity can be targeted, and inhibited. Therefore, diseases linked to epigenetic dysregulation often have a more positive prognosis with better treatment possibilities. Most of the epigenetic therapies currently being used are inhibitors, preventing the enzyme from performing its canonical function, as summarized in **Table 1** and **Figure 2**.

The development of 5-azacytidine has been one of the most promising epigenetic therapies thus far, the treatment of which was seen to increase survival rate when compared to conventional care in MDS and AML patients [133]. 5-azacytidine is a cytosine analogue and incorporates into DNA and RNA, binding irreversibly to all three DNMTs, sequestering the enzymes and preventing it from performing its canonical functions. At low doses, treatment with DNMTi results in global hypomethylation (observed in LINE and Alu repetitive elements as surrogate markers of global hypomethylation) [134] while it is cytotoxic at higher doses [135]. 5-azacytidine also cannot be methylated by DNMTs, therefore curbing the phenomenon of CpG hypermethylation seen in cancer cells. However, different tumor types have yielded varied response rates to DNMTi, with solid tumors demonstrating limited sensitivity in comparison to myeloid malignancies [136]. This could be explained in part because DNMTi function during the S-phase of cell cycle, and are therefore less efficacious in solid tumors [137]. In tumors where DNMTi was found to be effective, aberrantly silenced tumor suppressor genes were reactivated upon treatment [138], contributing to the mechanism in which DNMTi can lessen tumor burden. Additionally, treatment with DNMTi was found to increase the presentation of tumor antigens (such as cancer testis antigens (CTA)) and interferon signaling, increasing the visibility and therefore recognition and destruction of the tumor cells by the host [139, 140]. Endogenous retroviral elements (ERVs) were also observed to be increased upon treatment with DNMTi, which lead to the increase in cytoplasmic double-stranded RNA, inducing viral mimicry, and eventually leading to apoptosis [141, 142]. In contrast, treatment of IDH inhibitors have been met with limited success, with only a small subset of IDH-mutant cell lines demonstrating sensitivity to treatment [143]. Currently, there are no known TET inhibitors which prevents the demethylation of CpG islands.

Similarly, aberrant histone modifications are observed in cancer cells, and therefore drugs have been developed to block the activity of the enzymes that are responsible for the maintenance of these modifications. The majority of HDAC inhibitors that have been developed can be termed *broad reprogrammers*, which target entire classes of deacetylases instead of specific enzymes. Class I, II and IV of HDAC enzymes all share a similarity- that they require zinc ion to perform its enzymatic function, whilst class III of HDACs require NAD+ as its cofactor. As a result, it is easier to target these HDACs as two separate entities. There are now four inhibitors which have been approved by the FDA- vorinostat/SAHA (suberoylanilide hydroxamic acid), romidepsin, belinostat, panobinostat. However, research focus has now turned to targeting the readers of these acetylated marks- proteins which contain bromodomains. After reading the acetylated histone marks, bromodomain-containing proteins can act as a scaffold to recruit other activating or repressive machinery to act on the acetylated histone tails, regulating downstream gene expression. Inhibitors of the bromodomains of bromdomain and extra-terminal motif proteins (iBETs) have gained exceptional interest as of late. One of the most prominent drugs targeting bromodomain-containing proteins that have been developed is JQ1, named after its founding chemist, Jun Qi [144, 145], initially found in NUT midline carcinoma. JQ1 acts as a competitive inhibitor

Targeted mechanism	Canonical function	Tumor type	Therapeutic compound
DNA methylation			
DNMT1, 3A, 3B	DNA methylation, methylating and therefore silencing tumor suppressor genes	Myelodysplastic syndrome (MDS), AML	Inhibitors: 5- Azacytidine/Vidaza (FDA and EMA approved), decitabine (EMA and FDA approved) (reviewed in [169])
Epigenetic erasers			
LSD1	Mono and di-methylated H3K4 demethylase	Promyelocytic leukemia, AML, small cell lung cancer	TCP, GSK2879552 [155]
JARID1	Di and tri-methylated H3K4 demethylase	Lung cancer	Compound 6j, prodrug 7j
Classes I, II and IV histone deacetylases	Removes acetyl groups from histone tails	Cutaneous or peripheral T cell lymphoma, glioblastoma	Inhibitor: Vorinostat/suberoylanilide hydroxamic acid (SAHA) (FDA approved) [170], panobinostat (FDA approved), belinostat (FDA approved) Reviewed in [171, 172]
Class I histone deacetylases	Removes acetyl groups from histone tails	Drug-resistant multiple myeloma, T-cell lymphoma	Inhibitor: Romidepsin (FDA approved) Reviewed in [173]
Epigenetic writers			
Histone acetyltransferases-GCN5, p300, PCAF	Acetylates histone tails	Neuroblastoma	Inhibitor: PU139, PU141 [174]
EZH2	Methylation of H3K27, and repression of tumor suppressor genes	Acute myeloid leukemia (AML), lymphomaNon-small cell lung cancer	Inhibitor: EPZ-005687 [158], GSK-126 [160], EPZ-6438/Tazemetostat [159], UNC1999, GSK2816126, CPI-1205, Reviewed in [40]
DOT1L	Methylation of H3K79, and activation of genes involved in DNA damage response and cell cycle progression	Advanced hematological malignancies (Reviewed in [153]) Acute myeloid leukemia (AML), lymphoma	Inhibitor: EPZ-5676 [165], EPZ004777 [163, 164], SYC-522GSK2816126, CPI-1205, [166]
G9a	Methylation of H3K9	Non-small cell lung cancer	Inhibitor: UNC0642 [168], A-366 [175]
Epigenetic readers			
Bromodomain-containing proteins	Reads acetylated histone tails	Solid tumors, AML, MDS	iBET compounds: I-BET762, I-BET151, RVX-208, RVX-2135 Reviewed in [138, 176]

Table 1. Summary of selected therapeutics targeting dysregulated gene expression regulators in cancer.

of BRD2, BRD3, BRD4 and BRDT by reversibly binding to the hydrophobic bromodomain pockets, therefore not allowing it to bind to and recognize acetylated histone tails. Since MYC is a known target of BRD4, the bulk of the tumorigenic effect can be attributed to the decrease in

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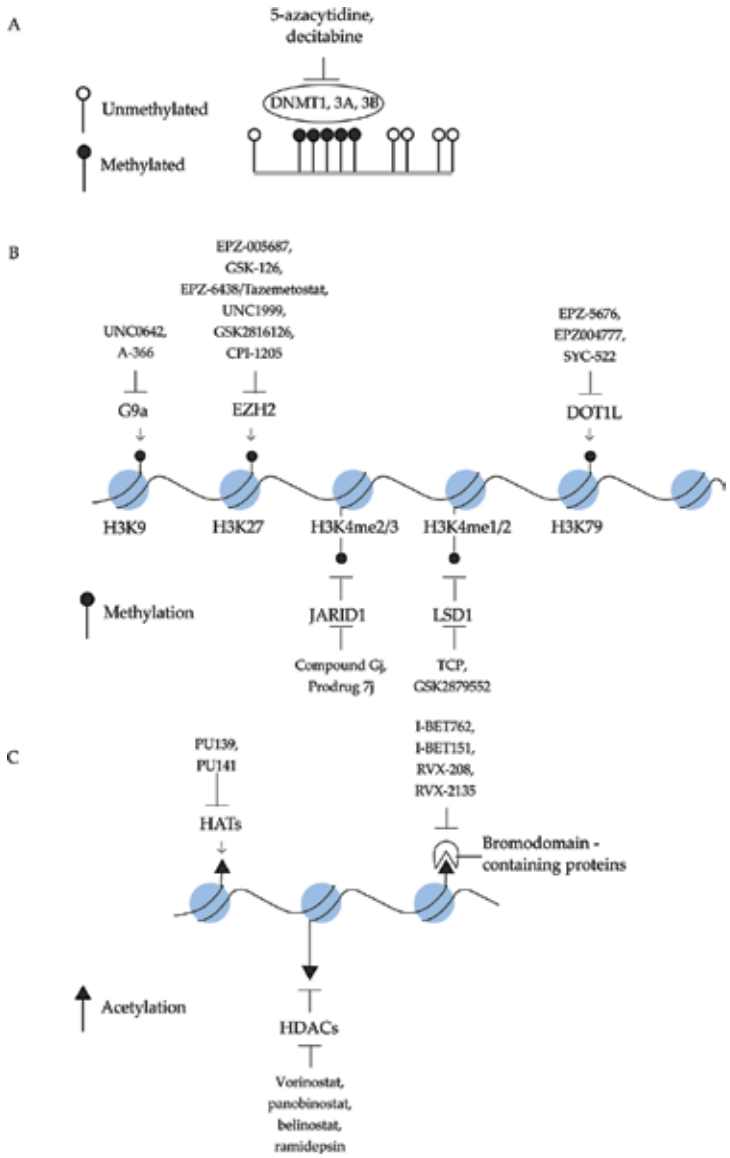

Figure 2. Graphical representation of the therapeutics against epigenetic modifiers. (A) Inhibitors of DNA methyltransferases, (B) histone methylases and demethylases, (C) histone acetyltransferases and deacetylases.

the oncogene expression. However, the effects of iBET compounds have been shown to be not entirely dependent on MYC [146]. Across different tumor types, JQ1 has been shown to suppress tumor growth in a myriad of different ways. In glioblastoma, JQ1 has been shown to induce G1 cell-cycle arrest and apoptosis through regulating expression of key genes such as *MYC*, *hTERT* and *p21* [147]. Similarly in medulloblastoma, JQ1 was shown to affect cell cycle genes via activating cyclin-dependent kinase inhibitors (CDKi), reducing E2F activity and affecting p53 signaling [148]. However, JQ1 is not able to selectively target either of the two bromodomains on the BET proteins, nor between the four BRD proteins, limiting the function of JQ1 [145]. Although BRD4

is known to regulate the transcription of many cellular genes, the treatment of JQ1 only represses a subset of these genes. This raises the question of whether BRD4 regulates transcription in a manner independent from reading acetylated histone tails. This mechanism of action was later elucidated, where BRD4 was found to be located at super-enhancers, therefore regulating transcription in a distinct manner [149]. The abovementioned iBET compounds function to only competitively inhibit the function of the bromodomain-containing enzymes. Thus, recent research has attempted to degrade the iBET substrates by conjugating iBET to E3 ubiquitin ligases in a method known as proteolysis targeting chimera (PROTAC) [150, 151]. iBET compounds have also shown promise in NMC (where BRD4-NUT fusion protein is formed), wherein the treatment with JQ1 significantly reduced tumor formation *in vivo* with limited cytotoxic effects [145].

Histone demethylases are another class of epigenetic erasers which can be targeted in clinic, of which inhibitors against LSD1 has seen the most progress. LSD1 is a member of the lysine demethylase (KDM) 1 family, with the ability to remove mono and di-methylated H3K4, therefore leading to transcriptional repression [152]. Its overexpression is linked to more aggressive breast and esophageal cancers, while its downregulation limits cell proliferation [153]. Combinatorial therapies involving the LSD1 inhibitor tranylcypromine (TCP) and all-trans-retinoic acid have been found efficacious in AML mouse models, and function by accumulating H3K4 methylation and therefore the activation of previously silenced tumor suppressor genes [154]. Other drugs such as GSK2879552, a derivative of TCP, has been developed and are currently in clinical trials for acute small cell lung cancer and AML [155]. Similarly, JARID1 is the demethylase of tri and di-methylated H3K4, and is observed to be aberrantly expressed in several cancers. Compound 6j and prodrug 7j are inhibitors which have been developed to inhibit JARID1 activity, with suppression of growth seen in a lung cancer cell line [156].

Targeted therapies are another group of drugs which have higher specificity and target specific epigenetic modifiers. EZH2 is one such target, where it has been found to be overexpressed in multiple cancers. The first drug to target EZH2 was 3-deazaneplanocin-A (DZNep), which initiates the degradation of the PRC2 complex to restore expression of silenced genes [157]. However, therapeutics targeting EZH2 has since evolved to target its enzymatic activity instead. EPZ-005687 is a competitive inhibitor which has high specificity for EZH2, and induces apoptosis via the reduction of H3K27 methylation levels in lymphoma cells [158]. Similar effects were observed with EPZ-6438/Tazemostat treatment, with decreased H3K27 methylation and decreased tumor size in non-Hodgkin lymphoma mouse models [159]. Several small molecule inhibitors such as GSK126 [160] and UNC1999 [161] have been identified to function as EZH2 inhibitors. In mice xenografts with gain-of-function EZH2 mutations, GSK126 has been shown to be effective in decreasing global levels of H3K27me3 and reactivating genes silenced by the PRC2 complex [160].

DOT1L is the only known histone methyltransferase of H3K79, which is often misregulated in AML as a result of gene translocations, leading to aberrant expression of hematopoietic stem-cell renewal genes [162]. EPZ004777 has been shown to reduce H3K79 methylation and its subsequent downregulation of downstream genes, prolonging the survival of MLL mice model [163, 164]. EPZ-5676 [165] and SYC-522 [166] are two drugs which are currently in clinical trials,

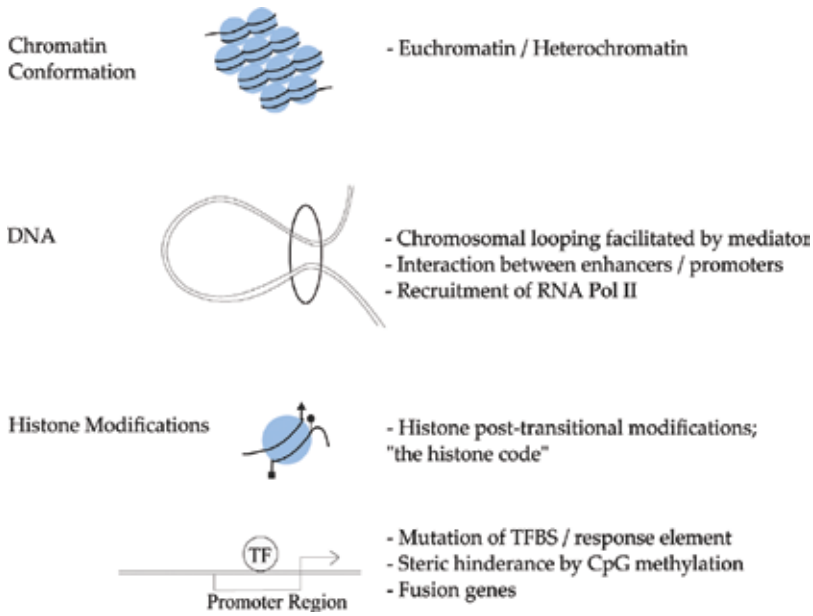

Figure 3. Graphical summary of gene expression regulators. Regulation of gene expression occurs at every step of the process, broadly divided into chromatin conformation, DNA, histone modifications, RNA and protein. Condensation of chromatin prevents the access of transcriptional machinery, thus repressing transcription. The mediator complex facilitates chromosomal looping, bringing together distal regulatory regions (and RNA Pol II) in 3D space. Histones undergo post-translational modification which comprise the histone code, recruiting different readers, resulting in different expression signatures. The proximal promoter region which contains transcription factor binding sites can undergo mutations and therefore inhibit canonical transcription factors from binding, or have its CpG island hypermethylated, therefore sterically inhibiting transcription factor binding. RNA is subject to post-transcriptional regulation by methylation (m^6A) or via degradation by miRNA. Finally, the activity of proteins can be regulated by covalent modifications such as activation by acetylation/phosphorylation or by a shortened half-life by degradation.

both of which have been demonstrated to efficiently decrease H3K79 methylation. G9a is the able to methylate H3K9, and has had inhibitors developed against it. UNC0638 is one such small molecule inhibitor, which results in genetic changes which phenocopy a transient depletion of G9a. Expectedly, there was a concomitant global decrease in H3K9 which was observed [167]. However, soon after, UNC0642 was developed with improved pharmacokinetic properties [168].

As an alternative to directly targeting epigenetic modifiers, research is now expanding into targeting the upstream regulators of these factors such that the activity or expression of the histone modifiers are regulated, affecting the downstream histone modifications.

5. Conclusion

As discussed in this chapter, the regulation of gene expression is a highly complex and multi-tiered process, regulated by a multitude of factors, summarized in **Figure 3**. Cancer cells have

evolved over time to exploit these mechanisms to dysregulate many cellular processes to evade cell cycle checkpoints and apoptosis, to allow continued proliferation. In particular, oncogenic viruses have also been shown to target some of these processes to dysregulate normal cell function, as in the case of BRD4 and TIP60, both targeted by HPV oncogenes. It can be assumed that oncogenic viruses would have evolved to maximize its carcinogenic potential, and therefore have minimal redundant functions. Therefore, the mere fact that these cellular components are targeted by oncogenic viruses eludes to its high canonical importance in the normal cell.

We have presented epigenetic regulating gene expression, one of the main methods in which either the profile of genes expression is changed, or the existing profile of genes are dysregulated, leading to aberrant upregulation or downregulation. In cancer cells, the dysregulated pathways have to overpower the canonical functions, to tip the balance so that processes occur in their favor, for sustained growth. It is therefore crucial to understand the mechanisms which are dysregulated in cancer cells so that further therapies can be developed to target these aberrancies.

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Abbreviation

5-hmC	5-hydroxylmethylcytosine
ALL	Acute Lymphoblastic Leukemia
AML	Acute Myeloid Leukemia
ATM	Ataxia-Telangiectasia Mutated
BET	Bromodomain and Extra-Terminal
CDH1	Cadherin 1
CDKi	Cyclin-Dependent Kinase Inhibitor
CTA	Cancer Testis Antigens
CTCF	CCCTC-binding Factor

CpG	Cytosine Phosphate Guanine
DSC3	Desmocollin 3
DNMT	DNA Methyltransferase
EBV	Epstein–Barr Virus
EMT	Epithelial-Mesenchymal Transition
EpCAM	Epithelial Cell Adhesion Molecule
ERV	Endogenous Retroviral Element
GCN5	General Control of Amino Acid Synthesis Protein 5-like 2
HDAC	Histone Deacetylase
HMT	Histone Methyltransferase
HPV	Human Papillomavirus
iBET	Inhibitors targeting Bromodomain and Extra-Terminal Motif Protein
ING	Inhibitor of Growth
INO80	Inositol Requiring 80
ISWI	Imitation Switch
JmJC	Jumonji C
KAT	Lysine Acetyltransferase
KDM	Lysine Demethylase
MBD	Methyl-CpG Binding Domain
MDS	Myelodysplastic Syndrome
miRNA	MicroRNA
MLL	Mixed-lineage leukemia
MLL-r	MLL-rearranged
MYST	Moz, Ybf2p/Sas3p, Sas2p and TIP60
NDR	Nucleosome depleted region
NMC	NUT midline carcinoma
NSCLC	Non-small cell lung cancer
PHD	Plant homeodomain
PRC2	Polycomb repressive complex 2
PROTAC	Proteolysis targeting chimera
SAGA	Spt-Ada-GCN5-Aceyl transferase
SAHA	Suberoylanilide hydroxamic acid
SEC	Super elongation complex
Snai1	Snail family transcriptional repressor 1

SRA	SET- and Ring family-associated
SWI-SNF	Switch/sucrose non-fermenting
TAD	Topologically associated domain
TCP	Tranylcypromine
TET	Ten-eleven translocation
TIP60	HIV-Tat1 interactive protein 60 kDa
TSA	Trichostatin A
TSS	Transcription start site
ZMYND11	Zinc finger MYND-type containing 11

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References

[1] Dixon JR, Selvaraj S, Yue F, Kim A, Li Y, Shen Y, et al. Topological domains in mammalian genomes identified by analysis of chromatin interactions. Nature. 2012;**485**(7398): 376-380

[2] Bieging KT, Mello SS, Attardi LD. Unravelling mechanisms of p53-mediated tumour suppression. Nature Reviews. Cancer. 2014;**14**(5):359-370

[3] Lane D, Levine A. p53 research: The past thirty years and the next thirty years. Cold Spring Harbor Perspectives in Biology. 2010;**2**(12):a000893

[4] Muller PA, Vousden KH. p53 mutations in cancer. Nature Cell Biology. 2013;**15**(1):2-8

[5] Dang CV. MYC on the path to cancer. Cell. 2012;**149**(1):22-35

[6] Meyer N, Penn LZ. Reflecting on 25 years with MYC. Nature Reviews. Cancer. 2008;**8**(12):976-990

[7] Consortium EP. An integrated encyclopedia of DNA elements in the human genome. Nature. 2012;**489**(7414):57-74

[8] Farria A, Li W, Dent SY. KATs in cancer: Functions and therapies. Oncogene. 2015;**34**(38): 4901-4913

[9] Albert M, Helin K. Histone methyltransferases in cancer. Seminars in Cell & Developmental Biology. 2010;**21**(2):209-220

[10] Glozak MA, Seto E. Histone deacetylases and cancer. Oncogene. 2007;**26**(37):5420-5432

[11] Ropero S, Esteller M. The role of histone deacetylases (HDACs) in human cancer. Molecular Oncology. 2007;**1**(1):19-25

[12] Barneda-Zahonero B, Parra M. Histone deacetylases and cancer. Molecular Oncology. 2012;**6**(6):579-589

[13] D'Oto A, Tian QW, Davidoff AM, Yang J. Histone demethylases and their roles in cancer epigenetics. Journal of Medical Oncology and Therapeutics. 2016;**1**(2):34-40

[14] Kampranis SC, Tsichlis PN. Histone demethylases and cancer. Advances in Cancer Research. 2009;**102**:103-169

[15] Fraga MF, Ballestar E, Villar-Garea A, Boix-Chornet M, Espada J, Schotta G, et al. Loss of acetylation at Lys16 and trimethylation at Lys20 of histone H4 is a common hallmark of human cancer. Nature Genetics. 2005;**37**(4):391-400

[16] Gorrini C, Squatrito M, Luise C, Syed N, Perna D, Wark L, et al. Tip60 is a haplo-insufficient tumour suppressor required for an oncogene-induced DNA damage response. Nature. 2007;**448**(7157):1063-1067

[17] Subbaiah VK, Zhang Y, Rajagopalan D, Abdullah LN, Yeo-Teh NS, Tomaic V, et al. E3 ligase EDD1/UBR5 is utilized by the HPV E6 oncogene to destabilize tumor suppressor TIP60. Oncogene. 2015

[18] Sun Y, Jiang X, Chen S, Fernandes N, Price BD. A role for the Tip60 histone acetyltransferase in the acetylation and activation of ATM. Proceedings of the National Academy of Sciences of the United States of America. 2005;**102**(37):13182-13187

[19] Sun Y, Jiang X, Xu Y, Ayrapetov MK, Moreau LA, Whetstine JR, et al. Histone H3 methylation links DNA damage detection to activation of the tumour suppressor Tip60. Nature Cell Biology. 2009;**11**(11):1376-1382

[20] Tang Y, Luo J, Zhang W, Gu W. Tip60-dependent acetylation of p53 modulates the decision between cell-cycle arrest and apoptosis. Molecular Cell. 2006;**24**(6): 827-839

[21] Rajagopalan D, Pandey AK, Xiuzhen MC, Lee KK, Hora S, Zhang Y, et al. TIP60 represses telomerase expression by inhibiting Sp1 binding to the TERT promoter. PLoS Pathogens. 2017;**13**(10):e1006681

[22] Mattera L, Escaffit F, Pillaire MJ, Selves J, Tyteca S, Hoffmann JS, et al. The p400/Tip60 ratio is critical for colorectal cancer cell proliferation through DNA damage response pathways. Oncogene. 2009;**28**(12):1506-1517

[23] Pandey AK, Zhang Y, Zhang S, Li Y, Tucker-Kellogg G, Yang H, et al. TIP60-miR-22 axis as a prognostic marker of breast cancer progression. Oncotarget. 2015;**6**(38):41290-41306

[24] Jha S, Vande Pol S, Banerjee NS, Dutta AB, Chow LT, Dutta A. Destabilization of TIP60 by human papillomavirus E6 results in attenuation of TIP60-dependent transcriptional regulation and apoptotic pathway. Molecular Cell. 2010;**38**(5):700-711

[25] Jha S, Shibata E, Dutta A. Human Rvb1/Tip49 is required for the histone acetyltransferase activity of Tip60/NuA4 and for the downregulation of phosphorylation on H2AX after DNA damage. Molecular and Cellular Biology. 2008;**28**(8):2690-2700

[26] Kuo MH, Brownell JE, Sobel RE, Ranalli TA, Cook RG, Edmondson DG, et al. Transcription-linked acetylation by Gcn5p of histones H3 and H4 at specific lysines. Nature. 1996;**383**(6597):269-272

[27] Flinn EM, Wallberg AE, Hermann S, Grant PA, Workman JL, Wright AP. Recruitment of Gcn5-containing complexes during c-Myc-dependent gene activation. Structure and function aspects. The Journal of Biological Chemistry. 2002;**277**(26):23399-23406

[28] Patel JH, Du Y, Ard PG, Phillips C, Carella B, Chen CJ, et al. The c-MYC oncoprotein is a substrate of the acetyltransferases hGCN5/PCAF and TIP60. Molecular and Cellular Biology. 2004;**24**(24):10826-10834

[29] Holmlund T, Lindberg MJ, Grander D, Wallberg AE. GCN5 acetylates and regulates the stability of the oncoprotein E2A-PBX1 in acute lymphoblastic leukemia. Leukemia. 2013;**27**(3):578-585

[30] Lu Q, Kamps MP. Heterodimerization of Hox proteins with Pbx1 and oncoprotein E2a-Pbx1 generates unique DNA-binding specifities at nucleotides predicted to contact the N-terminal arm of the Hox homeodomain--demonstration of Hox-dependent targeting of E2a-Pbx1 *in vivo*. Oncogene. 1997;**14**(1):75-83

[31] Wozniak RJ, Klimecki WT, Lau SS, Feinstein Y, Futscher BW. 5-Aza-2′-deoxycytidine-mediated reductions in G9A histone methyltransferase and histone H3 K9 di-methylation levels are linked to tumor suppressor gene reactivation. Oncogene. 2007;**26**(1):77-90

[32] Kondo Y, Shen L, Suzuki S, Kurokawa T, Masuko K, Tanaka Y, et al. Alterations of DNA methylation and histone modifications contribute to gene silencing in hepatocellular carcinomas. Hepatology Research. 2007;**37**(11):974-983

[33] Li KC, Hua KT, Lin YS, CY S, Ko JY, Hsiao M, et al. Inhibition of G9a induces DUSP4-dependent autophagic cell death in head and neck squamous cell carcinoma. Molecular Cancer. 2014;**13**:172

[34] Chen MW, Hua KT, Kao HJ, Chi CC, Wei LH, Johansson G, et al. H3K9 histone methyltransferase G9a promotes lung cancer invasion and metastasis by silencing the cell adhesion molecule Ep-CAM. Cancer Research. 2010;**70**(20):7830-7840

[35] Dong C, Wu Y, Yao J, Wang Y, Yu Y, Rychahou PG, et al. G9a interacts with snail and is critical for snail-mediated E-cadherin repression in human breast cancer. The Journal of Clinical Investigation. 2012;**122**(4):1469-1486

[36] Lehnertz B, Pabst C, Su L, Miller M, Liu F, Yi L, et al. The methyltransferase G9a regulates HoxA9-dependent transcription in AML. Genes & Development. 2014;**28**(4):317-327

[37] Chaturvedi CP, Somasundaram B, Singh K, Carpenedo RL, Stanford WL, Dilworth FJ, et al. Maintenance of gene silencing by the coordinate action of the H3K9 methyltransferase G9a/KMT1C and the H3K4 demethylase Jarid1a/KDM5A. Proceedings of the National Academy of Sciences of the United States of America. 2012;**109**(46): 18845-18850

[38] Margueron R, Reinberg D. The Polycomb complex PRC2 and its mark in life. Nature. 2011;**469**(7330):343-349

[39] Di Croce L, Helin K. Transcriptional regulation by Polycomb group proteins. Nature Structural & Molecular Biology. 2013;**20**(10):1147-1155

[40] Kim KH, Roberts CW. Targeting EZH2 in cancer. Nature Medicine. 2016;**22**(2):128-134

[41] Bracken AP, Pasini D, Capra M, Prosperini E, Colli E, Helin K. EZH2 is downstream of the pRB-E2F pathway, essential for proliferation and amplified in cancer. The EMBO Journal. 2003;**22**(20):5323-5335

[42] Kleer CG, Cao Q, Varambally S, Shen R, Ota I, Tomlins SA, et al. EZH2 is a marker of aggressive breast cancer and promotes neoplastic transformation of breast epithelial cells. Proceedings of the National Academy of Sciences of the United States of America. 2003;**100**(20):11606-11611

[43] van Haaften G, Dalgliesh GL, Davies H, Chen L, Bignell G, Greenman C, et al. Somatic mutations of the histone H3K27 demethylase gene UTX in human cancer. Nature Genetics. 2009;**41**(5):521-523

[44] Shi X, Hong T, Walter KL, Ewalt M, Michishita E, Hung T, et al. ING2 PHD domain links histone H3 lysine 4 methylation to active gene repression. Nature. 2006;**442**(7098): 96-99

[45] Hung T, Binda O, Champagne KS, Kuo AJ, Johnson K, Chang HY, et al. ING4 mediates crosstalk between histone H3 K4 trimethylation and H3 acetylation to attenuate cellular transformation. Molecular Cell. 2009;**33**(2):248-256

[46] Saksouk N, Avvakumov N, Champagne KS, Hung T, Doyon Y, Cayrou C, et al. HBO1 HAT complexes target chromatin throughout gene coding regions via multiple PHD finger interactions with histone H3 tail. Molecular Cell. 2009;**33**(2):257-265

[47] Doyon Y, Cayrou C, Ullah M, Landry AJ, Cote V, Selleck W, et al. ING tumor suppressor proteins are critical regulators of chromatin acetylation required for genome expression and perpetuation. Molecular Cell. 2006;**21**(1):51-64

[48] Ythier D, Larrieu D, Brambilla C, Brambilla E, Pedeux R. The new tumor suppressor genes ING: Genomic structure and status in cancer. International Journal of Cancer. 2008;**123**(7):1483-1490

[49] Jang MK, Mochizuki K, Zhou M, Jeong HS, Brady JN, Ozato K. The bromodomain protein Brd4 is a positive regulatory component of P-TEFb and stimulates RNA polymerase II-dependent transcription. Molecular Cell. 2005;**19**(4):523-534

[50] Yang Z, Yik JH, Chen R, He N, Jang MK, Ozato K, et al. Recruitment of P-TEFb for stimulation of transcriptional elongation by the bromodomain protein Brd4. Molecular Cell. 2005;**19**(4):535-545

[51] Yamane K, Tateishi K, Klose RJ, Fang J, Fabrizio LA, Erdjument-Bromage H, et al. PLU-1 is an H3K4 demethylase involved in transcriptional repression and breast cancer cell proliferation. Molecular Cell. 2007;**25**(6):801-812

[52] Scibetta AG, Santangelo S, Coleman J, Hall D, Chaplin T, Copier J, et al. Functional analysis of the transcription repressor PLU-1/JARID1B. Molecular and Cellular Biology. 2007;**27**(20):7220-7235

[53] Xiang Y, Zhu Z, Han G, Ye X, Xu B, Peng Z, et al. JARID1B is a histone H3 lysine 4 demethylase up-regulated in prostate cancer. Proceedings of the National Academy of Sciences of the United States of America. 2007;**104**(49):19226-19231

[54] Kawazu M, Saso K, Tong KI, McQuire T, Goto K, Son DO, et al. Histone demethylase JMJD2B functions as a co-factor of estrogen receptor in breast cancer proliferation and mammary gland development. PLoS One. 2011;**6**(3):e17830

[55] Mallette FA, Richard S. JMJD2A promotes cellular transformation by blocking cellular senescence through transcriptional repression of the tumor suppressor CHD5. Cell Reports. 2012;**2**(5):1233-1243

[56] Cloos PA, Christensen J, Agger K, Maiolica A, Rappsilber J, Antal T, et al. The putative oncogene GASC1 demethylates tri- and dimethylated lysine 9 on histone H3. Nature. 2006;**442**(7100):307-311

[57] Choi YW, Bae SM, Kim YW, Lee HN, Kim YW, Park TC, et al. Gene expression profiles in squamous cell cervical carcinoma using array-based comparative genomic hybridization analysis. International Journal of Gynecological Cancer. 2007;**17**(3):687-696

[58] Minamiya Y, Ono T, Saito H, Takahashi N, Ito M, Mitsui M, et al. Expression of histone deacetylase 1 correlates with a poor prognosis in patients with adenocarcinoma of the lung. Lung Cancer. 2011;**74**(2):300-304

[59] Krusche CA, Wulfing P, Kersting C, Vloet A, Bocker W, Kiesel L, et al. Histone deacetylase-1 and -3 protein expression in human breast cancer: A tissue microarray analysis. Breast Cancer Research and Treatment. 2005;**90**(1):15-23

[60] Glaser KB, Li J, Staver MJ, Wei RQ, Albert DH, Davidsen SK. Role of class I and class II histone deacetylases in carcinoma cells using siRNA. Biochemical and Biophysical Research Communications. 2003;**310**(2):529-536

[61] Osada H, Tatematsu Y, Saito H, Yatabe Y, Mitsudomi T, Takahashi T. Reduced expression of class II histone deacetylase genes is associated with poor prognosis in lung cancer patients. International Journal of Cancer. 2004;**112**(1):26-32

[62] Lai WKM, Pugh BF. Understanding nucleosome dynamics and their links to gene expression and DNA replication. Nature Reviews. Molecular Cell Biology. 2017;**18**(9): 548-562

[63] Langst G, Manelyte L. Chromatin remodelers: From function to dysfunction. Genes (Basel). 2015;**6**(2):299-324

[64] Buscarlet M, Krasteva V, Ho L, Simon C, Hebert J, Wilhelm B, et al. Essential role of BRG, the ATPase subunit of BAF chromatin remodeling complexes, in leukemia maintenance. Blood. 2014;**123**(11):1720-1728

[65] Shi J, Whyte WA, Zepeda-Mendoza CJ, Milazzo JP, Shen C, Roe JS, et al. Role of SWI/SNF in acute leukemia maintenance and enhancer-mediated Myc regulation. Genes & Development. 2013;**27**(24):2648-2662

[66] Shain AH, Pollack JR. The spectrum of SWI/SNF mutations, ubiquitous in human cancers. PLoS One. 2013;**8**(1):e55119

[67] Bai J, Mei P, Zhang C, Chen F, Li C, Pan Z, et al. BRG1 is a prognostic marker and potential therapeutic target in human breast cancer. PLoS One. 2013;**8**(3):e59772

[68] Skulte KA, Phan L, Clark SJ, Taberlay PC. Chromatin remodeler mutations in human cancers: Epigenetic implications. Epigenomics. 2014;**6**(4):397-414

[69] Tolstorukov MY, Sansam CG, Lu P, Koellhoffer EC, Helming KC, Alver BH, et al. Swi/Snf chromatin remodeling/tumor suppressor complex establishes nucleosome occupancy at target promoters. Proceedings of the National Academy of Sciences of the United States of America. 2013;**110**(25):10165-10170

[70] Wilson BG, Wang X, Shen X, McKenna ES, Lemieux ME, Cho YJ, et al. Epigenetic antagonism between polycomb and SWI/SNF complexes during oncogenic transformation. Cancer Cell. 2010;**18**(4):316-328

[71] Kia SK, Gorski MM, Giannakopoulos S, Verrijzer CP. SWI/SNF mediates polycomb eviction and epigenetic reprogramming of the INK4b-ARF-INK4a locus. Molecular and Cellular Biology. 2008;**28**(10):3457-3464

[72] Pehrson JR, Fuji RN. Evolutionary conservation of histone macroH2A subtypes and domains. Nucleic Acids Research. 1998;**26**(12):2837-2842

[73] Waterborg JH. Evolution of histone H3: Emergence of variants and conservation of post-translational modification sites. Biochemistry and Cell Biology. 2012;**90**(1):79-95

[74] Zhou J, Fan JY, Rangasamy D, Tremethick DJ. The nucleosome surface regulates chromatin compaction and couples it with transcriptional repression. Nature Structural & Molecular Biology. 2007;**14**(11):1070-1076

[75] Buschbeck M, Hake SB. Variants of core histones and their roles in cell fate decisions, development and cancer. Nature Reviews. Molecular Cell Biology. 2017;**18**(5): 299-314

[76] Jin C, Felsenfeld G. Nucleosome stability mediated by histone variants H3.3 and H2A.Z. Genes & Development. 2007;**21**(12):1519-1529

[77] Jin C, Zang C, Wei G, Cui K, Peng W, Zhao K, et al. H3.3/H2A.Z double variant-containing nucleosomes mark 'nucleosome-free regions' of active promoters and other regulatory regions. Nature Genetics. 2009;**41**(8):941-945

[78] Wen H, Li Y, Xi Y, Jiang S, Stratton S, Peng D, et al. ZMYND11 links histone H3.3K36me3 to transcription elongation and tumour suppression. Nature. 2014;**508**(7495):263-268

[79] Valdes-Mora F, Song JZ, Statham AL, Strbenac D, Robinson MD, Nair SS, et al. Acetylation of H2A.Z is a key epigenetic modification associated with gene deregulation and epigenetic remodeling in cancer. Genome Research. 2012;**22**(2):307-321

[80] Dixon JR, Gorkin DU, Ren B. Chromatin domains: The unit of chromosome organization. Molecular Cell. 2016;**62**(5):668-680

[81] Northcott PA, Lee C, Zichner T, Stutz AM, Erkek S, Kawauchi D, et al. Enhancer hijacking activates GFI1 family oncogenes in medulloblastoma. Nature. 2014;**511**(7510): 428-434

[82] Wood CD, Veenstra H, Khasnis S, Gunnell A, Webb HM, Shannon-Lowe C, et al. MYC activation and BCL2L11 silencing by a tumour virus through the large-scale reconfiguration of enhancer-promoter hubs. eLife. 2016;**5**

[83] Allen BL, Taatjes DJ. The mediator complex: A central integrator of transcription. Nature Reviews. Molecular Cell Biology. 2015;**16**(3):155-166

[84] Tsai KL, Sato S, Tomomori-Sato C, Conaway RC, Conaway JW, Asturias FJ. A conserved mediator-CDK8 kinase module association regulates mediator-RNA polymerase II interaction. Nature Structural & Molecular Biology. 2013;**20**(5):611-619

[85] Makinen N, Mehine M, Tolvanen J, Kaasinen E, Li Y, Lehtonen HJ, et al. MED12, the mediator complex subunit 12 gene, is mutated at high frequency in uterine leiomyomas. Science. 2011;**334**(6053):252-255

[86] Lim WK, Ong CK, Tan J, Thike AA, Ng CC, Rajasegaran V, et al. Exome sequencing identifies highly recurrent MED12 somatic mutations in breast fibroadenoma. Nature Genetics. 2014;**46**(8):877-880

[87] Ng CC, Tan J, Ong CK, Lim WK, Rajasegaran V, Nasir ND, et al. MED12 is frequently mutated in breast phyllodes tumours: A study of 112 cases. Journal of Clinical Pathology. 2015;**68**(9):685-691

[88] Kampjarvi K, Park MJ, Mehine M, Kim NH, Clark AD, Butzow R, et al. Mutations in exon 1 highlight the role of MED12 in uterine leiomyomas. Human Mutation. 2014;**35**(9):1136-1141

[89] Turunen M, Spaeth JM, Keskitalo S, Park MJ, Kivioja T, Clark AD, et al. Uterine leiomyoma-linked MED12 mutations disrupt mediator-associated CDK activity. Cell Reports. 2014;**7**(3):654-660

[90] Banaganapalli B, Mohammed K, Khan IA, Al-Aama JY, Elango R, Shaik NA. A computational protein phenotype prediction approach to analyze the deleterious mutations of human MED12 gene. Journal of Cellular Biochemistry. 2016;**117**(9):2023-2035

[91] Bestor T, Laudano A, Mattaliano R, Ingram V. Cloning and sequencing of a cDNA encoding DNA methyltransferase of mouse cells. The carboxyl-terminal domain of the mammalian enzymes is related to bacterial restriction methyltransferases. Journal of Molecular Biology. 1988;**203**(4):971-983

[92] Okano M, Xie S, Li E. Cloning and characterization of a family of novel mammalian DNA (cytosine-5) methyltransferases. Nature Genetics. 1998;**19**(3):219-220

[93] Filion GJ, Zhenilo S, Salozhin S, Yamada D, Prokhortchouk E, Defossez PA. A family of human zinc finger proteins that bind methylated DNA and repress transcription. Molecular and Cellular Biology. 2006;**26**(1):169-181

[94] Unoki M, Nishidate T, Nakamura Y. ICBP90, an E2F-1 target, recruits HDAC1 and binds to methyl-CpG through its SRA domain. Oncogene. 2004;**23**(46):7601-7610

[95] Prokhortchouk A, Hendrich B, Jorgensen H, Ruzov A, Wilm M, Georgiev G, et al. The p120 catenin partner Kaiso is a DNA methylation-dependent transcriptional repressor. Genes & Development. 2001;**15**(13):1613-1618

[96] Hendrich B, Bird A. Identification and characterization of a family of mammalian methyl-CpG binding proteins. Molecular and Cellular Biology. 1998;**18**(11): 6538-6547

[97] Bogdanovic O, Veenstra GJ. DNA methylation and methyl-CpG binding proteins: Developmental requirements and function. Chromosoma. 2009;**118**(5):549-565

[98] Hashimoto H, Vertino PM, Cheng X. Molecular coupling of DNA methylation and histone methylation. Epigenomics. 2010;**2**(5):657-669

[99] Tahiliani M, Koh KP, Shen Y, Pastor WA, Bandukwala H, Brudno Y, et al. Conversion of 5-methylcytosine to 5-hydroxymethylcytosine in mammalian DNA by MLL partner TET1. Science. 2009;**324**(5929):930-935

[100] Ehrlich M, Wang RY. 5-Methylcytosine in eukaryotic DNA. Science. 1981;**212**(4501): 1350-1357

[101] Esteller M. CpG island hypermethylation and tumor suppressor genes: A booming present, a brighter future. Oncogene. 2002;**21**(35):5427-5440

[102] Saxonov S, Berg P, Brutlag DL. A genome-wide analysis of CpG dinucleotides in the human genome distinguishes two distinct classes of promoters. Proceedings of the National Academy of Sciences of the United States of America. 2006;**103**(5):1412-1417

[103] Irizarry RA, Ladd-Acosta C, Wen B, Wu Z, Montano C, Onyango P, et al. The human colon cancer methylome shows similar hypo- and hypermethylation at conserved tissue-specific CpG island shores. Nature Genetics. 2009;**41**(2):178-186

[104] Guenther MG, Levine SS, Boyer LA, Jaenisch R, Young RA. A chromatin landmark and transcription initiation at most promoters in human cells. Cell. 2007;**130**(1):77-88

[105] Mikkelsen TS, Ku M, Jaffe DB, Issac B, Lieberman E, Giannoukos G, et al. Genome-wide maps of chromatin state in pluripotent and lineage-committed cells. Nature. 2007;**448**(7153):553-560

[106] Watt F, Molloy PL. Cytosine methylation prevents binding to DNA of a HeLa cell transcription factor required for optimal expression of the adenovirus major late promoter. Genes & Development. 1988;**2**(9):1136-1143

[107] Jones PA, Laird PW. Cancer epigenetics comes of age. Nature Genetics. 1999;**21**(2):163-167

[108] Esteller M, Corn PG, Baylin SB, Herman JGA. Gene hypermethylation profile of human cancer. Cancer Research. 2001;**61**(8):3225-3229

[109] Herman JG, Baylin SB. Gene silencing in cancer in association with promoter hypermethylation. The New England Journal of Medicine. 2003;**349**(21):2042-2054

[110] Du Q, Luu PL, Stirzaker C, Clark SJ. Methyl-CpG-binding domain proteins: Readers of the epigenome. Epigenomics. 2015;**7**(6):1051-1073

[111] Song JZ, Stirzaker C, Harrison J, Melki JR, Clark SJ. Hypermethylation trigger of the glutathione-S-transferase gene (GSTP1) in prostate cancer cells. Oncogene. 2002;**21**(7):1048-1061

[112] Stirzaker C, Song JZ, Davidson B, Clark SJ. Transcriptional gene silencing promotes DNA hypermethylation through a sequential change in chromatin modifications in cancer cells. Cancer Research. 2004;**64**(11):3871-3877

[113] Chatagnon A, Bougel S, Perriaud L, Lachuer J, Benhattar J, Dante R. Specific association between the methyl-CpG-binding domain protein 2 and the hypermethylated region of

the human telomerase reverse transcriptase promoter in cancer cells. Carcinogenesis. 2009;**30**(1):28-34

[114] Stirzaker C, Song JZ, Ng W, Du Q, Armstrong NJ, Locke WJ, et al. Methyl-CpG-binding protein MBD2 plays a key role in maintenance and spread of DNA methylation at CpG islands and shores in cancer. Oncogene. 2017;**36**(10):1328-1338

[115] Ono R, Taki T, Taketani T, Taniwaki M, Kobayashi H, Hayashi Y. LCX, leukemia-associated protein with a CXXC domain, is fused to MLL in acute myeloid leukemia with trilineage dysplasia having t(10;11)(q22;q23). Cancer Research. 2002;**62**(14): 4075-4080

[116] Lorsbach RB, Moore J, Mathew S, Raimondi SC, Mukatira ST, Downing JR. TET1, a member of a novel protein family, is fused to MLL in acute myeloid leukemia containing the t(10;11)(q22;q23). Leukemia. 2003;**17**(3):637-641

[117] Weissmann S, Alpermann T, Grossmann V, Kowarsch A, Nadarajah N, Eder C, et al. Landscape of TET2 mutations in acute myeloid leukemia. Leukemia. 2012;**26**(5): 934-942

[118] Ko M, Huang Y, Jankowska AM, Pape UJ, Tahiliani M, Bandukwala HS, et al. Impaired hydroxylation of 5-methylcytosine in myeloid cancers with mutant TET2. Nature. 2010;**468**(7325):839-843

[119] Liu C, Liu L, Chen X, Shen J, Shan J, Xu Y, et al. Decrease of 5-hydroxymethylcytosine is associated with progression of hepatocellular carcinoma through downregulation of TET1. PLoS One. 2013;**8**(5):e62828

[120] Lian CG, Xu Y, Ceol C, Wu F, Larson A, Dresser K, et al. Loss of 5-hydroxymethylcytosine is an epigenetic hallmark of melanoma. Cell. 2012;**150**(6):1135-1146

[121] Turcan S, Rohle D, Goenka A, Walsh LA, Fang F, Yilmaz E, et al. IDH1 mutation is sufficient to establish the glioma hypermethylator phenotype. Nature. 2012;**483**(7390): 479-483

[122] Lu C, Ward PS, Kapoor GS, Rohle D, Turcan S, Abdel-Wahab O, et al. IDH mutation impairs histone demethylation and results in a block to cell differentiation. Nature. 2012;**483**(7390):474-478

[123] Nowell PC, Hungerford DA. Chromosome studies on normal and leukemic human leukocytes. Journal of the National Cancer Institute. 1960;**25**:85-109

[124] Look AT. Oncogenic transcription factors in the human acute leukemias. Science. 1997;**278**(5340):1059-1064

[125] Mitelman F, Johansson B, Mertens F. The impact of translocations and gene fusions on cancer causation. Nature Reviews. Cancer. 2007;**7**(4):233-245

[126] Yokoyama A. Transcriptional activation by MLL fusion proteins in leukemogenesis. Experimental Hematology. 2017;**46**:21-30

[127] Slany RK. The molecular biology of mixed lineage leukemia. Haematologica. 2009; **94**(7):984-993

[128] Yokoyama A, Lin M, Naresh A, Kitabayashi I, Cleary ML. A higher-order complex containing AF4 and ENL family proteins with P-TEFb facilitates oncogenic and physiologic MLL-dependent transcription. Cancer Cell. 2010;**17**(2):198-212

[129] Lin C, Smith ER, Takahashi H, Lai KC, Martin-Brown S, Florens L, et al. AFF4, a component of the ELL/P-TEFb elongation complex and a shared subunit of MLL chimeras, can link transcription elongation to leukemia. Molecular Cell. 2010;**37**(3):429-437

[130] Prange KHM, Mandoli A, Kuznetsova T, Wang SY, Sotoca AM, Marneth AE, et al. MLL-AF9 and MLL-AF4 oncofusion proteins bind a distinct enhancer repertoire and target the RUNX1 program in 11q23 acute myeloid leukemia. Oncogene. 2017;**36**(23):3346-3356

[131] Parker BC, Zhang W. Fusion genes in solid tumors: An emerging target for cancer diagnosis and treatment. Chinese Journal of Cancer. 2013;**32**(11):594-603

[132] Alekseyenko AA, Walsh EM, Wang X, Grayson AR, Hsi PT, Kharchenko PV, et al. The oncogenic BRD4-NUT chromatin regulator drives aberrant transcription within large topological domains. Genes & Development. 2015;**29**(14):1507-1523

[133] Fenaux P, Mufti GJ, Hellstrom-Lindberg E, Santini V, Finelli C, Giagounidis A, et al. Efficacy of azacitidine compared with that of conventional care regimens in the treatment of higher-risk myelodysplastic syndromes: A randomised, open-label, phase III study. The Lancet Oncology. 2009;**10**(3):223-232

[134] Qin T, Youssef EM, Jelinek J, Chen R, Yang AS, Garcia-Manero G, et al. Effect of cytarabine and decitabine in combination in human leukemic cell lines. Clinical Cancer Research. 2007;**13**(14):4225-4232

[135] Jones PA, Taylor SM. Cellular differentiation, cytidine analogs and DNA methylation. Cell. 1980;**20**(1):85-93

[136] Issa JP, Kantarjian HM. Targeting DNA methylation. Clinical Cancer Research. 2009; **15**(12):3938-3946

[137] Stewart DJ, Issa JP, Kurzrock R, Nunez MI, Jelinek J, Hong D, et al. Decitabine effect on tumor global DNA methylation and other parameters in a phase I trial in refractory solid tumors and lymphomas. Clinical Cancer Research. 2009;**15**(11):3881-3888

[138] Jones PA, Issa JP, Baylin S. Targeting the cancer epigenome for therapy. Nature Reviews. Genetics. 2016;**17**(10):630-641

[139] Karpf AR, Jones DA. Reactivating the expression of methylation silenced genes in human cancer. Oncogene. 2002;**21**(35):5496-5503

[140] Karpf AR, Peterson PW, Rawlins JT, Dalley BK, Yang Q, Albertsen H, et al. Inhibition of DNA methyltransferase stimulates the expression of signal transducer and activator of transcription 1, 2, and 3 genes in colon tumor cells. Proceedings of the National Academy of Sciences of the United States of America. 1999;**96**(24):14007-14012

[141] Chiappinelli KB, Strissel PL, Desrichard A, Li H, Henke C, Akman B, et al. Inhibiting DNA methylation causes an interferon response in cancer via dsRNA including endogenous retroviruses. Cell. 2015;**162**(5):974-986

[142] Roulois D, Loo Yau H, Singhania R, Wang Y, Danesh A, Shen SY, et al. DNA-Demethylating agents target colorectal cancer cells by inducing viral mimicry by endogenous transcripts. Cell. 2015;**162**(5):961-973

[143] Tateishi K, Wakimoto H, Iafrate AJ, Tanaka S, Loebel F, Lelic N, et al. Extreme vulnerability of IDH1 mutant cancers to NAD+ depletion. Cancer Cell. 2015;**28**(6):773-784

[144] Qi J. Bromodomain and extraterminal domain inhibitors (BETi) for cancer therapy: Chemical modulation of chromatin structure. Cold Spring Harbor Perspectives in Biology. 2014;**6**(12):a018663

[145] Filippakopoulos P, Qi J, Picaud S, Shen Y, Smith WB, Fedorov O, et al. Selective inhibition of BET bromodomains. Nature. 2010;**468**(7327):1067-1073

[146] Rodriguez RM, Suarez-Alvarez B, Salvanes R, Huidobro C, Torano EG, Garcia-Perez JL, et al. Role of BRD4 in hematopoietic differentiation of embryonic stem cells. Epigenetics. 2014;**9**(4):566-578

[147] Cheng Z, Gong Y, Ma Y, Lu K, Lu X, Pierce LA, et al. Inhibition of BET bromodomain targets genetically diverse glioblastoma. Clinical Cancer Research. 2013;**19**(7):1748-1759

[148] Henssen A, Thor T, Odersky A, Heukamp L, El-Hindy N, Beckers A, et al. BET bromodomain protein inhibition is a therapeutic option for medulloblastoma. Oncotarget. 2013;**4**(11):2080-2095

[149] Loven J, Hoke HA, Lin CY, Lau A, Orlando DA, Vakoc CR, et al. Selective inhibition of tumor oncogenes by disruption of super-enhancers. Cell. 2013;**153**(2):320-334

[150] Lu J, Qian Y, Altieri M, Dong H, Wang J, Raina K, et al. Hijacking the E3 ubiquitin ligase Cereblon to efficiently target BRD4. Chemistry & Biology. 2015;**22**(6):755-763

[151] Winter GE, Buckley DL, Paulk J, Roberts JM, Souza A, Dhe-Paganon S, et al. DRUG DEVELOPMENT. Phthalimide conjugation as a strategy for *in vivo* target protein degradation. Science. 2015;**348**(6241):1376-1381

[152] Shi Y, Lan F, Matson C, Mulligan P, Whetstine JR, Cole PA, et al. Histone demethylation mediated by the nuclear amine oxidase homolog LSD1. Cell. 2004;**119**(7): 941-953

[153] Gelato KA, Shaikhibrahim Z, Ocker M, Haendler B. Targeting epigenetic regulators for cancer therapy: Modulation of bromodomain proteins, methyltransferases, demethylases, and microRNAs. Expert Opinion on Therapeutic Targets. 2016;**20**(7): 783-799

[154] Schenk T, Chen WC, Gollner S, Howell L, Jin L, Hebestreit K, et al. Inhibition of the LSD1 (KDM1A) demethylase reactivates the all-trans-retinoic acid differentiation pathway in acute myeloid leukemia. Nature Medicine. 2012;**18**(4):605-611

[155] Mohammad HP, Smitheman KN, Kamat CD, Soong D, Federowicz KE, Van Aller GS, et al. A DNA Hypomethylation signature predicts antitumor activity of LSD1 inhibitors in SCLC. Cancer Cell. 2015;**28**(1):57-69

[156] Itoh Y, Sawada H, Suzuki M, Tojo T, Sasaki R, Hasegawa M, et al. Identification of Jumonji AT-rich interactive domain 1A inhibitors and their effect on cancer cells. ACS Medicinal Chemistry Letters. 2015;**6**(6):665-670

[157] Tan J, Yang X, Zhuang L, Jiang X, Chen W, Lee PL, et al. Pharmacologic disruption of Polycomb-repressive complex 2-mediated gene repression selectively induces apoptosis in cancer cells. Genes & Development. 2007;**21**(9):1050-1063

[158] Knutson SK, Wigle TJ, Warholic NM, Sneeringer CJ, Allain CJ, Klaus CR, et al. A selective inhibitor of EZH2 blocks H3K27 methylation and kills mutant lymphoma cells. Nature Chemical Biology. 2012;**8**(11):890-896

[159] Knutson SK, Kawano S, Minoshima Y, Warholic NM, Huang KC, Xiao Y, et al. Selective inhibition of EZH2 by EPZ-6438 leads to potent antitumor activity in EZH2-mutant non-Hodgkin lymphoma. Molecular Cancer Therapeutics. 2014;**13**(4):842-854

[160] McCabe MT, Ott HM, Ganji G, Korenchuk S, Thompson C, Van Aller GS, et al. EZH2 inhibition as a therapeutic strategy for lymphoma with EZH2-activating mutations. Nature. 2012;**492**(7427):108-112

[161] Konze KD, Ma A, Li F, Barsyte-Lovejoy D, Parton T, Macnevin CJ, et al. An orally bioavailable chemical probe of the lysine Methyltransferases EZH2 and EZH1. ACS Chemical Biology. 2013;**8**(6):1324-1334

[162] Bernt KM, Zhu N, Sinha AU, Vempati S, Faber J, Krivtsov AV, et al. MLL-rearranged leukemia is dependent on aberrant H3K79 methylation by DOT1L. Cancer Cell. 2011;**20**(1):66-78

[163] Deshpande AJ, Chen L, Fazio M, Sinha AU, Bernt KM, Banka D, et al. Leukemic transformation by the MLL-AF6 fusion oncogene requires the H3K79 methyltransferase Dot1l. Blood. 2013;**121**(13):2533-2541

[164] Daigle SR, Olhava EJ, Therkelsen CA, Majer CR, Sneeringer CJ, Song J, et al. Selective killing of mixed lineage leukemia cells by a potent small-molecule DOT1L inhibitor. Cancer Cell. 2011;**20**(1):53-65

[165] Daigle SR, Olhava EJ, Therkelsen CA, Basavapathruni A, Jin L, Boriack-Sjodin PA, et al. Potent inhibition of DOT1L as treatment of MLL-fusion leukemia. Blood. 2013;**122**(6):1017-1025

[166] Liu W, Deng L, Song Y, Redell M. DOT1L inhibition sensitizes MLL-rearranged AML to chemotherapy. PLoS One. 2014;**9**(5):e98270

[167] Vedadi M, Barsyte-Lovejoy D, Liu F, Rival-Gervier S, Allali-Hassani A, Labrie V, et al. A chemical probe selectively inhibits G9a and GLP methyltransferase activity in cells. Nature Chemical Biology. 2011;**7**(8):566-574

[168] Liu F, Barsyte-Lovejoy D, Li F, Xiong Y, Korboukh V, Huang XP, et al. Discovery of an *in vivo* chemical probe of the lysine methyltransferases G9a and GLP. Journal of Medicinal Chemistry. 2013;**56**(21):8931-8942

[169] Kelly TK, De Carvalho DD, Jones PA. Epigenetic modifications as therapeutic targets. Nature Biotechnology. 2010;**28**(10):1069-1078

[170] Marks PA. Discovery and development of SAHA as an anticancer agent. Oncogene. 2007;**26**(9):1351-1356

[171] Halsall JA, Turner BM. Histone deacetylase inhibitors for cancer therapy: An evolutionarily ancient resistance response may explain their limited success. BioEssays. 2016;**38**(11):1102-1110

[172] Bezecny P. Histone deacetylase inhibitors in glioblastoma: Pre-clinical and clinical experience. Medical Oncology. 2014;**31**(6):985

[173] Smolewski P, Robak T. The discovery and development of romidepsin for the treatment of T-cell lymphoma. Expert Opinion on Drug Discovery. 2017;**12**(8):859-873

[174] Gajer JM, Furdas SD, Grunder A, Gothwal M, Heinicke U, Keller K, et al. Histone acetyltransferase inhibitors block neuroblastoma cell growth *in vivo*. Oncogene. 2015; **4**:e137

[175] Agarwal P, Jackson SP. G9a inhibition potentiates the anti-tumour activity of DNA double-strand break inducing agents by impairing DNA repair independent of p53 status. Cancer Letters. 2016;**380**(2):467-475

[176] Chaidos A, Caputo V, Karadimitris A. Inhibition of bromodomain and extra-terminal proteins (BET) as a potential therapeutic approach in haematological malignancies: Emerging preclinical and clinical evidence. Therapeutic Advances in Hematology. 2015;**6**(3):128-141

Chapter 6

Hydroxymethylation Influences on Intestinal Epithelial Cells in Health and Disease

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Additional information is available at the end of the chapter

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Abstract

Epigenetics describes modifications that affect gene expression that are not encoded within the DNA sequence. DNA methylation is the longest appreciated epigenetic modification and has been accepted to play a critical role in maintaining euchromatin and silencing genes. Recently, a separate and distinct covalent modification has been recognized; hydroxymethylation, which has been associated with increased gene expression as opposed to gene silencing. However, traditional methods to study DNA methylation also recognized hydroxymethylation and did not distinguish between these two distinct DNA covalent modifications. Furthermore, TET enzymes have been identified to play a critical role in active hydroxymethylation of previously methylated cytosine residues and may further result in conversion to cytosine. TET1 plays a critical role in intestinal epithelial differentiation and development, and this is also correlated with increased hydroxymethylation in terminally differentiated epithelial cells. Colon cancer, which arises from the colonic epithelium, exhibits decreased hydroxymethylation and altered gene expression.

Keywords: hydroxymethylation, intestinal epithelium, TET1

1. Introduction

Every cell in the human (and all mammalian) body contains the exact same genetic makeup with the exception of the gametes, which are haploid. However, despite containing the same DNA and genetic information there are a vast number of different cell types that perform functions essential to life. The difference in cell types is due to the genes that are selectively expressed, and those that are silenced combined with genes that are primed for activation in response to a stimulus. In this regard, the field of epigenetics is the study of mitotically

heritable changes/modifications to the genome that alters gene expression without changing the DNA sequence. There are three main types of epigenetic changes; specifically, noncoding RNAs, covalent modifications or methylation to DNA, and histone modifications. In this chapter, we will focus primarily on covalent modifications to DNA: DNA methylation and hydroxymethylation and the role this process plays in colonic epithelial cells. The intestinal epithelium has a rapid cellular turnover with the lifespan of a colonocyte being about 4–5 days [1]. Within the colonic epithelium, there are several cell types, including colonocytes, goblet cells, and endocrine cells, all of which arise and differentiate from a common intestinal stem cell located at the base of the crypts [1–3]. In this regard, the colonic epithelium is a unique system in which cellular differentiation and epigenetic alterations can be studied. Furthermore, two disease processes, colon cancer and ulcerative colitis integrally involve colonic epithelial cells. Alterations in DNA methylation and hydroxymethylation profiles are likely to play an important role in these processes. At the end of the chapter, we will discuss altered DNA hydroxymethylation in these disease states.

2. DNA methylation

DNA methylation was first discovered in 1948 in the calf thymus [4]. In the mid-1970s, two papers hypothesized that cytosine methylation could be a *de novo* process, could be inherited through somatic mutation through an enzymatic process, and result in the silencing of genes [5–7]. Methylation occurs at the 5C position of cytosine in regions of DNA that are rich in cytosine-guanine (CpG) dinucleotide. About 98% of the genome is deficient in CpG, with enrichment of CpGs clustered into "CpG islands," which are typically located near the centromere and within/near promoters. Furthermore, we are now recognizing that CpGs exist within introns and exons of genes and these residues may also be methylated [8, 9]. DNA methylation plays a critical role in imprinting, X-chromosome inactivation in females and silencing of transposons, and maintaining chromosomal stability [9–11]. Given the critical role that methylation plays in imprinting and X-chromosome inactivation and the pathology that results from alterations in these processes, DNA methylation was thought to be a static process and only reprogrammed during gametogenesis and embryogenesis.

DNA methylation has been thought to play an important role in gene silencing. When CpG islands within a gene promoter are methylated, the gene is silenced. This occurs through binding of methyl binding proteins to methylated cytosine which results in histone recruitment and heterochromatin, which is tightly condensed, thereby making the promoter inaccessible to transcription factors. However, recently, with improved technology and genetic sequencing, we have recognized that 5-mc may occur within a gene in introns and/or exons. Furthermore, 5-mc in intragenic regions may result in alternate splicing or transcripts [9, 12].

Importantly, cytosine methylation (5-mc) is preserved during mitosis through DNA methyltransferase 1 (DNMT), which recognizes hemi-methylated DNA during the S-phase of mitosis and copies this pattern to the daughter strand [13–15]. This mechanism is essential to maintain tissue homogeneity and cell lineage in terminally differentiated cells, and there are many publications that have established DNA methylation profiles specific to certain tissues

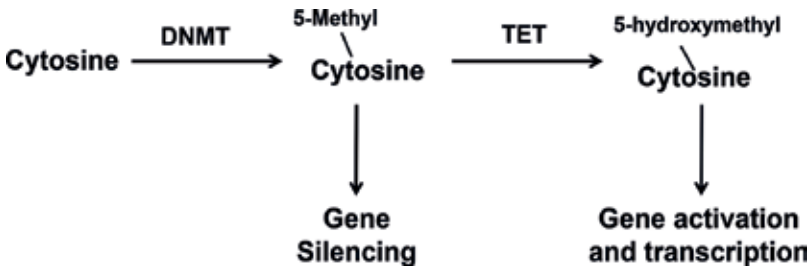

Figure 1. Cytosine may be methylated by DNA methyltransferases (DNMT) resulting in 5-methylcytosine (5-mc) which is associated with gene silencing when located in the promoter. 5-mc may be reduced by ten eleven ten (TET) enzymes resulting in 5-hydroxymethylcytosine (5-hmc) which is associated with euchromatin and gene transcription/activation.

and additionally tissues or cells with similar functions have similar methylation profiles [16, 17]. Maintenance of DNA methylation is an essential function and a recent study on human embryonic stem cells demonstrated lethality when DNMT1 was deleted. Specifically, when DNMT1 was conditionally deleted after initial development, the cells rapidly lost methylation and underwent cell death [18]. In addition to DNTM1, which copies DNA methylation marks during cell division, DNMT 3a and 3b are able to methylate DNA *de novo* (**Figure 1**). In mouse models, deletion of DNMT1 or DNMT3b is embryonically lethal and deletion of DNMT3a results in postnatal lethality [19, 20].

3. Hydroxymethylation

Hydroxymethylation of cytosine was described shortly after DNA methylation in 1950. At that time hydroxymethylated cytosine was described in a bacteriophage and was suggested to be a mechanism by which the virus evaded DNA degradation by the host [21, 22]. Several studies in the 1970s described hydroxymethylated cytosine in mammals but it was not until 2009 when high levels of hydroxymethylation were found in Purkinje cells [23]. Further studies revealed that there were detectable levels of 5-hmc across all tissue types with cells of the central nervous system containing the highest percent of hydroxymethylated cytosine residues [23].

Importantly, bisulfite sequencing that has traditionally been used to identify methylated cytosine bases only distinguishes covalently modified cytosine from unmodified cytosine, therefore methylated and hydroxymethylated cytosine were recognized as the same. With advances in technology and new interest in hydroxymethylation as having a potentially separate function from methylated cytosine, we are now able to distinguish one from the other at the single nucleotide level [7, 24]. Importantly, while 5-mc rich regions are associated with heterochromatin and gene silencing, 5-hmc is associated with euchromatin and genes with rich in 5-hmc are accessible to transcription factors. Methylated cytosines may undergo oxidation by the ten eleven ten (TET) enzymes resulting in 5-hydroxymethylcytosine (**Figure 1**). Further oxidation of 5-hmc by TET enzymes leads to 5-formylcytosine and 5-carboxylcytosine which are then excised and replaced by a new cytosine residue. In this regard, TET enzymes

also function in active demethylation [25, 26]. Additionally as indicated previously in this chapter, 5-methylcytosine associates with methyl binding proteins which promote heterochromatin formation, these methyl binding proteins are not able to recognize hydroxymethylated cytosine and this is another mechanism by which hydroxymethylation may serve to make genes more accessible. Finally, hydroxymethylation may facilitate passive demethylation as DNTM1 has low affinity for 5-hmc and during DNA replication lack of recognition of a previously methylated cytosine, now 5-hydroxymethylcytosine would not have the methyl mark copied to the daughter strand. Hydroxymethylation not only serves as an intermediate step in active demethylation, but is also fairly stable and present in relative abundance compared to 5-flucytosine and 5-carboxylcytosine; therefore, hydroxymethylation may serve an additional and unique function [21, 26–29].

Studies that evaluate global DNA methylation or hydroxymethylation and gene expression have inconsistent results. As our sequencing technology and ability to distinguish 5-hmc from 5-mc has improved, we are coming to understand that regions that are rich in 5-hmc may not be "activated" but rather be "poised for activation" through a delicate balance between 5-mc, 5-hmc and activating (H3K4me3) and repressive (H3K27me3) histone marks [21, 30]. Furthermore, studies in the central nervous system have indicated that changes in specific 5-hmc residues without change in overall methylation can have profound effects on gene expression [31]. Importantly the dynamics and abundance of 5-hmc is cell type specific and changes during development. In this regard, here we will examine the role of 5-hmc in intestinal epithelial cell development and differentiation.

4. Intestinal epithelial cells

The colonic epithelium is one of the largest cellular compartments with a very rapid turnover. The intestinal epithelium is a single cell thick barrier that serves not only as a barrier to protect the underlying lamina propria immune cells from the luminal antigens and bacteria, but these cells also play a role in metabolism, water and nutrient absorption, sensing the luminal environment for potential pathogens, and maintaining a mucus barrier. The colonic epithelium forms crypts and villi which increases the surface area and absorptive surface area. The intestinal epithelium is composed of four major cell types that arise from a common precursor cell. Enterocytes/colonocytes arise from intestinal stem cells which divide to become rapidly dividing transition zone cells, these cells divide up to six more times and migrate up to the tips of the villi during their differentiation. The enterocytes/colonocytes at the tips of the villi play a role in maintaining tight junctions as well as metabolic and absorptive functions, and have a short lifespan of 4–5 days after which they are sloughed off of the surface and mew cells must replace them. The intestinal epithelium also consists of mucin-producing goblet cells, endocrine cells and Paneth cells, which migrate down to the base of the crypts and play a role in bacterial sensing and are relatively long-lived. Over the past decade, we have come to understand that all of these cell types in the intestinal epithelium arise for a common progenitor cell that resides in the transitional zone between the crypts and villi at the +5 position and are referred to as $LGR5^{+}$ cells (intestinal stem cells) [1, 2].

Culture of LGR^{+}5 cells in a 3D matrigel (substituting for the extracellular membrane) supplemented with growth factors: WNT and Noggin to allow for stem cell expansion, R-spondin which maintains stem cell populations, and EGF to promote cell proliferation results in the development of a 3D structure with distinct crypt-like and villus-like structure with a central lumen referred to as organoids. Organoids have successfully been generated from both human and mouse LGR5 cells. These organoids contain all of the cell types present in the intestinal epithelium with crypt-like domains containing Paneth cells and LGR5+ stem cells, villi with villin-positive cells and also enteroendocrine and goblet cells scattered throughout the organoid [3]. Organoids have successfully been generated from isolated LGR5+ cells supporting this notion of LGR5 cells as a pluripotent progenitor for the intestinal/colonic epithelium [3, 32].

Recently, studies have evaluated global methylation and hydroxymethylation in the colonic epithelium. These studies showed relatively similar levels of methylation in the crypts and in association with rapidly dividing (Ki67+) cells in the transition zone; whereas, there was an enrichment of hydroxymethylation at the tips of the villi and a decreased prevalence of hydroxymethylation in the crypts in Ki67-positive cells. These data suggest that hydroxymethylation is gained during differentiation and preferentially expressed in non-dividing, terminally differentiated cells [33].

Alterations in hydroxymethylation profiles in colonic epithelial cells can also be recapitulated *in vitro*. When T84 colonic epithelial cell line is cultured at low density *in vitro*, the cells lack polarity, rapidly divide, and express low levels of global hydroxymethylation. However, as these cells divide and come into contact with other cells they differentiate and polarize to form a monolayer. This process is associated with an increase in hydroxymethylation specifically in promoters of genes involved in maintaining tight junctions, regulation of actin and endocytosis. Furthermore, enrichment in hydroxymethylation co-localizes with binding sites for colonic epithelial-specific transcription factors including HNF4A, RXRA and CDX2 with relatively little change in the hydroxymethylation status of GATA6 (**Figure 2A**). Importantly, this increase in hydroxymethylation also positively correlated with gene expression in a dose-dependent manner with the genes with the highest concentration of hydroxymethylation being the most highly expressed [34].

In mouse models, it is possible to separate the colonocytes at the villous from LGR5^{+} cells using a combination of cell scraping and LGR5 isolation by FACS when LGR5 expression is linked to a fluorescent indicator. This study also confirms that not only is there a difference in the abundance of hydroxymethylation in the LGR5^{+} cells compared to the terminally differentiated enterocytes, but also that hydroxymethylation correlates with highly expressed or inducible cells and changes during differentiation. This study demonstrated that there were over 10,000 differentially hydroxymethylated regions between LGR5^{+} cells and colonocytes. Hydroxymethylation in LGR5^{+} cells localized to promoters of intestinal stem cell markers, such as LGR5, and gene ontology analysis revealed that the functions of the preferentially hydroxymethylated genes were involved in developmental processes, cell differentiation and other stem cell functions. In contrast, in the LGR5^{-} colonocytes that were terminally differentiated, hydroxymethylation was enhanced in the promoters and within genes that control metabolic processes, nutrient transport and other enterocyte functions, and genes with enriched hydroxymethylation also exhibited higher expression at the RNA level [33].

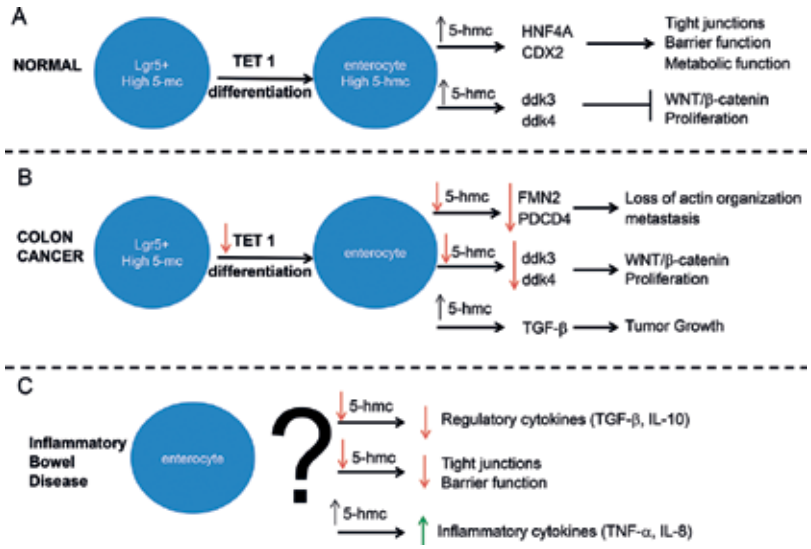

Figure 2. Overview of the role of hydroxymethylation in (A) normal intestinal epithelium, (B) colon cancer and (C) proposed role in inflammatory bowel disease. (A) TET1 activity results in hydroxymethylation of specific cytosine residues as cells rapidly divide and migrate. This results in downregulation of the WNT signaling pathway (reduced proliferation) and upregulation of genes associated with the function of these terminally differentiated cells. (B) In colon cancer, there is decreased TET1 activity and therefore decreased overall hydroxymethylation which leads to a loss of organization, and increased cellular proliferation and inappropriate growth. (C) In IBD, we propose that aberrant hydroxymethylation may result in a decrease in hydroxymethylation of genes associated with barrier function, and downregulatory cytokines and/or increased hydroxymethylation (and therefore increased expression) of pro-inflammatory cytokines.

TET1, which actively can convert methylated cytosine to hydroxymethylated cytosine in epithelial cells, is critical for intestinal epithelial cell maintenance and differentiation. In this regard, TET1-deficient mice exhibit colonic shortening, with shorter villi, with fewer $LGR5^+$ cells. These mice are smaller than their littermate controls and ultimately deletion of Tet1 is lethal. Furthermore, $LGR5^+$ cells derived from these TET1-deficient mice are also unable to form organoids, which confirms the role of TET1 and hydroxymethylation as a critical step in epithelial cell differentiation and maintenance [33, 35].

5. Hydroxymethylation in colon cancer

As we have discussed above, hydroxymethylation is critical in the differentiation of colonic epithelial cells, and there is a change specifically in the distribution of hydroxymethylation as cells divide and differentiate from the LGR5 cells in the crypts (hydroxymethylation in genes involved in proliferation and stem cell functions) and differentiated colonocytes at the tips of the villi (metabolic functions and tight junction). Colonic adenocarcinoma arises from the colonic epithelial cells and is the number two cause of cancer-related deaths in the United States. Traditionally, cancer progresses through four stages from adenomatous-type polyps to adenomas, to invasive disease and then metastasis. It has long been recognized that there

are aberrant methylation profiles in cancers including colon cancer, and the thought was that increased methylation of tumor suppressor genes would result in silencing of these genes and lead to malignant transformation and growth. However, until recently, bisulfite sequencing could not differentiate between methylation (associated with gene silencing when in the promoter) and hydroxymethylation (associated with gene activation).

Since hydroxymethylation has now been appreciated in mammalian cells and it is now recognized that hydroxymethylation has a distinct role in gene regulation and expression, recent attention has been turned to the role of hydroxymethylation in cancer. A recent study evaluated global hydroxymethylation in colon cancer tissue compared to matched, adjacent normal tissue and demonstrated that hydroxymethylation was profoundly less in colon cancer tissue compared to normal [36]. Furthermore, Tet1, which as discussed is essential for active hydroxymethylation in intestinal epithelium cells, is decreased in colon cancer and occurs as an early event [37, 38]. Additionally, in other cancers such as breast cancer, decreased Tet1 expression is associated with more aggressive malignancy and metastatic disease [11]. Taken together, we may hypothesize that decreased Tet1 expression may result in altered/decreased hydroxymethylation. In support of this hypothesis, the role of Tet1 in colon cancer was evaluated in the colonic epithelial cell line derived from colon adenocarcinoma $CaCo_2$. $CaCo_2$ cells were transfected with inducible Tet1 under control of doxycycline. When these cells were treated with doxycycline for 96 hours, these cells had slower growth and there were 300 genes with altered expression, and 60% of the genes with increased expression in the Tet1-induced state had enriched hydroxymethylation and correlated with genes that had high levels of methylation in the wild type ($TET1^{low}$) cells. Gene ontology analysis showed significant enrichment in the WNT/β-catenin signaling pathway of the TET1-deregulated genes. To support this notion, the nuclear level of b-catenin was decreased when TET1 expression was induced, supporting the notion that TET1 expression leads to decreased WNT/β-catenin signaling. Further studies showed that DDK3 and DDK4 which are upstream regulator of the WNT pathway were upregulated in the TET1-induced cells and these genes expressed a higher level of hydroxymethylation when compared to the wild type ($Tet1^{low}$) cells (**Figure 2B**), this in turn provides a mechanistic explanation as to Tet1-induced downregulation of WNT through increased expression of WNT pathway inhibitors. Further studies were done using a xenograft system and TET1-induced CaCo2 cells implanted into a mouse resulted in smaller tumors than the wild-type CaCo2 xenografts [39].

Our group also evaluated hydroxymethylation in colon cancer compared to adjacent normal tissue in patients with colon cancer. While we did not find any specific pathways that exhibited decreased hydroxymethylation in colon cancer, we did identify numerous genes that lost hydroxymethylation and expression. Specifically, two of these genes include: FMN2 (formin 2) and PCDC4 (programmed cell death 4). FMN2 is involved in the organization of actin cytoskeleton and cell polarity; therefore, decreased expression of this gene may result in decreased contact inhibition and the disorganization, which is seen in colonic adenocarcinoma. Furthermore, PDCD4 is also downregulated and exhibits loss of hydroxymethylation in colon cancer. Loss of PDCD4 has been associated with colon adenocarcinoma and these tumors tend to be more aggressive [34, 40]. Additionally, there were also genes that gained hydroxymethylation and exhibited increased expression including BMP7 and TGFB1. Bone

morphogenetic protein 7 (BMP7) is a secreted protein of the TGF-β superfamily, and TGFB1 (transforming growth factor beta 1) is known to be increased in tumor cells and may induce the canonical WNT signaling (**Figure 2B**) [34]. Taken together there is strong evidence that alterations to gene-specific hydroxymethylation profiles may be an early step that occurs in colon cancer leading to progression of disease. Altered hydroxymethylation may serve as a biomarker or predictor of disease severity and/or targets for future therapy.

6. Hydroxymethylation in IBD

Inflammatory bowel disease (IBD) affects more than 3 million adults (estimated 1.3% of adults) in the United States [41]. Importantly, the incidence of IBD has been increasing over the past 10 years especially in developed and developing countries. Furthermore, environmental influences appear to influence IBD with the two major phenotypes, ulcerative colitis and Crohn's disease, demonstrating opposite correlation with smoking. Specifically, smoking is protective in ulcerative colitis and patients may experience flares with cessation of smoking; whereas in Crohn's disease, smoking worsens disease [42–44]. Recent studies including the GWAS have identified an important role for genetics as well as gene regulation and epigenetics in IBD [45, 46]. To date the GWAS have identified 163 loci that are associated with IBD; however, only approximately 10% of these loci are located in DNA coding regions suggesting an important role for gene regulation [47, 48]. Given that genetics account for only 10% of cases of IBD, the increased incidence in developing countries and the identified environmental influences, IBD is likely due to a gene by environment interaction, which also implicates epigenetics as a key driver in disease pathogenesis. Previous studies have implicated a role for alterations in DNA methylation in IBD; however, these studies showed discordant results in genes with "methylated" promoters and RNA expression. This may be due to the fact that these studies were performed prior to the development of the technology to distinguish methylated cytosine (silenced genes) from hydroxymethylated cytosine (activated or primed for activation). Furthermore, these studies were performed in intact tissues or mixed cell populations [49]. Since methylation plays a critical role in maintaining tissue homogeneity, and as described previously, hydroxymethylation is increased in terminally differentiated cells, methylation and hydroxymethylation are best evaluated in a single cell population. Since colonic epithelial cells are the most widely affected cell type in IBD, hydroxymethylation plays a critical role in colonic epithelial cell differentiation and function, and prior studies have suggested altered methylation profiles in IBD tissues, we may infer that there is a role for aberrant hydroxymethylation of genes, potentially involved in barrier function or cytokine production, that contributes to the pathogenesis of IBD (**Figure 2C**). Future studies to evaluate differential hydroxymethylation in epithelial cells from affected/unaffected mucosa from IBD patients may provide further insights into novel pathways or genes implicated in IBD pathophysiology. Additionally further understanding into the mechanisms by which hydroxymethylation profiles are altered either through changes in TET expression or environmental exposures that promote or inhibit hydroxymethylation we may identify novel targets for therapy.

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7. Conclusion

Epigenetics, mitotically heritable changes/modifications to the genome that alter gene expression without changing the DNA sequence, plays a critical role in embryogenesis, normal development and disease. Specifically, DNA methylation has long been recognized as a critical epigenetic modification that is critical to normal function and tissue homogeneity. Alterations in methylation profiles have also been identified in and are thought to play a role in autoimmune diseases as well as malignancy. However, until recently technology did not allow for the differentiation between methylated cytosine (5-mc) and hydroxymethylated cytosine (5-hmc). Given that recent studies have indicated that hydroxymethylated promoters is associated with gene activation, it is important to further evaluate the role of hydroxymethylation in development and disease.

In this chapter, we have discussed the critical role of DNA hydroxymethylation in the differentiation of intestinal epithelial cells. Differential hydroxymethylation of specific gene promoters results in appropriate function of the LGR5 stem cells and the differentiated enterocytes. We have also discussed that higher levels of hydroxymethylation are associated with terminally differentiated, non-dividing cells in the normal tissue. Furthermore, we implicated a role for hydroxymethylation in the pathogenesis of colonic adenocarcinoma through increased WNT signaling, and also differential hydroxymethylation in specific genes that may act as tumor suppressors or growth factors in paired tissue samples. Finally, we have presented evidence that supports a potential role for altered hydroxymethylation in IBD.

Future studies evaluating hydroxymethylation at the single nucleotide level in a single cell population may be extremely valuable in identifying potential novel pathways or drug targets in disease. Importantly, as discussed, hydroxymethylation is a stable epigenetic modification, but is also dynamic in that hydroxymethylation may be gained or lost at a specific location. This is important because altered hydroxymethylation in a cell population may result from a gene x environment interaction and then be propagated as cells divide, thereby leading to disease pathogenesis. In regards to the intestinal epithelium, use of organoids may allow researchers to identify environmental exposures to the epithelium that may result in altered hydroxymethylation. Furthermore, since the intestinal epithelium can easily be isolated from tissue specimens, further studies may be performed on paired specimens to delineate normal and diseased tissue in patients with IBD and colonic adenocarcinoma.

Abbreviations

5-hmC	5-hydroxymethylcytosine
5-mC	5-methylcytosine
CDX2	Caudal type homeobox 2
CpG	Cytosine-guanine dinucleotides

ddk	Dickkopf WNT signaling pathway inhibitor
DNMT	DNA methyltransferase
FMN2	Formin-2
GATA6	GATA-binding protein 6
HNF4A	Hepatocyte nuclear factor 4 alpha
iSC	Intestinal stem cell
LGR5	Leucine-rich repeat-containing G-protein-coupled receptor 5
PDCD4	Programmed cell death 4
RXRA	Retinoid X receptor alpha
TET	Ten eleven ten enzyme

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References

[1] van der Flier LG, Clevers H. Stem cells, self-renewal, and differentiation in the intestinal epithelium. Annual Review of Physiology. Jan 2009;**71**:241-260

[2] Clevers H. The intestinal crypt, a prototype stem cell compartment. Cell. Jul 2013;**154**(2): 274-284

[3] Fatehullah A, Tan SH, Barker N. Organoids as an in vitro model of human development and disease. Nature Cell Biology. Mar 2016;**18**(3):246-254

[4] Hotchkiss RD. The quantitative separation of purines, pyrimidines, and nucleosides by paper chromatography. The Journal of Biological Chemistry. Aug 1948;**175**(1):315-332

[5] Jones PA. Two key papers in 1975 independently suggested that methylation of cytosine residues in the context of CpG dinucleotides could serve as an epigenetic mark in vertebrates. Nature Reviews Genetics. 2012;**13**:484-492

[6] Holliday R, Pugh JE. DNA modification mechanisms and gene activity during development. Science. Jan 1975;**187**(4173):226-232

[7] Yu M, Hon GC, Szulwach KE, Song C-X, Zhang L, Kim A, Li X, Dai Q, Shen Y, Park B, Min J-H, Jin P, Ren B, He C, et al. Base-resolution analysis of 5-hydroxymethylcytosine in the mammalian genome. Cell. 2012;**149**(6):1368-1380

[8] Guibert S, Weber M. Functions of DNA methylation and hydroxymethylation in mammalian development. Current Topics in Developmental Biology. Jan 2013;**104**:47-83

[9] Leenen FAD, Muller CP, Turner JD, et al. DNA methylation: conducting the orchestra from exposure to phenotype? Clinical Epigenetics. Dec 2016;**28**(1):92

[10] Guibert S, Weber M. Chapter Two – Functions of DNA methylation and hydroxymethylation in mammalian development. Current Topics in Developmental Biology. 2013;**104**:47-83

[11] Sun X, He Y, Huang C, Ma T-T, Li J. The epigenetic feedback loop between DNA methylation and microRNAs in fibrotic disease with an emphasis on DNA methyltransferases. Cellular Signalling. 2013;**25**(9):1870-1876

[12] Jones PA. Functions of DNA methylation: Islands, start sites, gene bodies and beyond. Nature Reviews Genetics. May 2012;**13**(7):484-492

[13] Suetake I, Watanebe M, Takeshita K, Takahashi S, Carlton P. In: Kaneda A, Tsukada Y, editors. The Molecular Basis of DNA Methylation BT – DNA and Histone Methylation as Cancer Targets. Cham: Springer International Publishing; 2017. pp. 19-51

[14] Elliott EN, Sheaffer KL, Schug J, Stappenbeck TS, Kaestner KH. Dnmt1 is essential to maintain progenitors in the perinatal intestinal epithelium. Development. 2015; **142**(12):2163-2172

[15] Michael KEB, Rountree R, Herman JG, Baylin SB. DNA methylation, chromatin inheritance, and cancer. Oncogene. 2001;**20**:3156-3165

[16] Ponnaluri VKC, Ehrlich KC, Zhang G, et al. Association of 5-hydroxymethylation and 5-methylation of DNA cytosine with tissue-specific gene expression. Epigenetics. 2017;**12**(2):123-138. DOI: 10.1080/15592294.2016.1265713.

[17] Lokk K, Modhukur V, Rajashekar B, Märtens K, Mägi R, Kolde R, Koltšina M, Nilsson TK, Vilo J, Salumets A, Tõnisson N. DNA methylome profiling of human tissues identifies global and tissue-specific methylation patterns. Genome Biology. Apr 2014;**15**(4):r54-r54

[18] Liao J, Karnik R, Gu H, Ziller MJ, Clement K, Tsankov AM, Akopian V, Gifford CA, Donaghey J, Galonska C, Pop R, Reyon D, Tsai SQ, Mallard W, Joung JK, Rinn JL, Gnirke A, Meissner A. Targeted disruption of DNMT1, DNMT3A and DNMT3B in human embryonic stem cells. Nature Genetics. May 2015;**47**(5):469-478

[19] Okano M, Bell DW, Haber DA, Li E. DNA Methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development. Cell. Sep 1999; **99**(3):247-257

[20] Li E, Bestor TH, Jaenisch R. Targeted mutation of the DNA methyltransferase gene results in embryonic lethality. Cell. Sep 1992;**69**(6):915-926

[21] Shi D-Q, Ali I, Tang J, Yang W-C. New insights into 5hmC DNA modification: Generation, distribution and function. Frontiers in Genetics. Jul 2017;**8**:100

[22] Wyatt GR, Cohen SS. A new pyrimidine base from bacteriophage nucleic acids. Nature. Dec 1952;**170**(4338):1072-1073

[23] Kriaucionis S, Heintz N. The nuclear DNA base 5-hydroxymethylcytosine is present in Purkinje neurons and the brain. Science. May 2009;**324**(5929):929-930

[24] Chun-Xiao Song C, Szulwach KE, Fu Y, Dai Q, Yi C, Li X, Li Y, Wen Zhang L, Jian X, Wang J, Zhang L, Looney TJ, Zhang B, Godley LA, Hicks LM, Lahn BT, Jin P, He C. Selective chemical labeling reveals the genome-wide distribution of 5-hydroxymethylcytosine. Nature Biotechnology. 2011;**29**(1):68-75

[25] Tahiliani M, Koh KP, Shen Y, Pastor WA, Bandukwala H, Brudno Y, Agarwal S, Iyer LM, Liu DR, Aravind L, Rao A. Conversion of 5-methylcytosine to 5-hydroxymethylcytosine in mammalian DNA by MLL partner TET1. Science. May 2009;**324**(5929):930-935

[26] Moen EL, Mariani CJ, Zullow H, Jeff-Eke M, Litwin E, Nikitas JN, Godley LA. New themes in the biological functions of 5-methylcytosine and 5-hydroxymethylcytosine. Immunological Reviews. 2015;**263**(1):36-49

[27] Hahn MA, Szabó PE, Pfeifer GP. 5-Hydroxymethylcytosine: A stable or transient DNA modification? Genomics. 2014;**104**:314-323

[28] Branco MR, Ficz G, Reik W. Uncovering the role of 5-hydroxymethylcytosine in the epigenome. Nature Reviews Genetics. Nov 2011;**13**(1):7

[29] Bachman M, Uribe-Lewis S, Yang X, Williams M, Murrell A, Balasubramanian S. 5-Hydroxymethylcytosine is a predominantly stable DNA modification. 2014. DOI: 10.1038/NCHEM.2064

[30] Hammoud SS, Cairns BR, Jones DA. Epigenetic regulation of colon cancer and intestinal stem cells. Current Opinion in Cell Biology. Apr 2013;**25**(2):177-183

[31] Szulwach KE, Li X, Li Y, Song C-X, Wu H, Dai Q, Irier H, Upadhyay AK, Gearing M, Levey AI, Vasanthakumar A, Godley LA, Chang Q, Cheng X, He C, Jin P. 5-hmC-mediated epigenetic dynamics during postnatal neurodevelopment and aging. Nature Neuroscience. 2011;**14**(12):1607-1618

[32] Sato T, van Es JH, Snippert HJ, Stange DE, Vries RG, Van den Born M, Barker N, Shroyer NF, Van de Wetering M, Clevers H. Paneth cells constitute the niche for Lgr5 stem cells in intestinal crypts. Nature. Jan 2011;**469**(7330):415-418

[33] Kim R, Sheaffer KL, Choi I, Won K, Kaestner KH. Epigenetic regulation of intestinal stem cells by Tet1-mediated DNA hydroxymethylation. Genes & Development. 2016;**30**(21):2433-2442

[34] Chapman CG, Mariani CJ, Wu F, Meckel K, Butun F, Chuang A, Madzo J, Bissonette MB, Kwon JH, Godley LA. TET-catalyzed 5-hydroxymethylcytosine regulates gene expression in differentiating colonocytes and colon cancer. Scientific Reports. 2015;**5**:17568

[35] Dawlaty MM, Breiling A, Le T, Raddatz G, Barrasa MI, Cheng AW, Gao Q, Powell BE, Li Z, Xu M, Faull KF, Lyko F, Jaenisch R. Combined deficiency of Tet1 and Tet2 causes epigenetic abnormalities but is compatible with postnatal development. Developmental Cell. Feb 2013;**24**(3):310-323

[36] Haffner MC, Chaux A, Meeker AK, Esopi D, Gerber J, Pellakuru LG, Toubaji A, Argani P, Iacobuzio-Donahue C, Nelson WG, Netto GJ, DeMarzo A, Yegnasubramanian S. Global 5-hydroxymethylcytosine content is significantly reduced in tissue stem/progenitor cell compartments and in human cancers. Oncotarget. 2011;**2**(8):627-637

[37] Tian Y, Pan F, Sun X, Gan M, Lin A, Zhang D, Zhu Y, Lai M. Association of TET1 expression with colorectal cancer progression. Scandinavian Journal of Gastroenterology. Mar 2017;**52**(3):312-320

[38] Neri F, Dettori D, Incarnato D, Krepelova A, Rapelli S, Maldotti M, Parlato C, Paliogiannis P, Oliviero S. TET1 is a tumour suppressor that inhibits colon cancer growth by derepressing inhibitors of the WNT pathway. Oncogene. 2015;**34**(32):4168-4176

[39] Maurano MT, Humbert R, Rynes E, Thurman RE, Haugen E, Wang H, Reynolds AP, Sandstrom R, Qu H, Brody J, Shafer A, Neri F, Lee K, Kutyavin T, Stehling-Sun S, Johnson AK, Canfield TK, Giste E, Diegel M, Bates D, Hansen RS, Neph S, Sabo PJ, Heimfeld S, Raubitschek A, Ziegler S, Cotsapas C, Sotoodehnia N, Glass I, Sunyaev SR, Kaul R, Stamatoyannopoulos JA. Systematic localization of common disease-associated variation in regulatory DNA. Science (80-.). Sep 2012;**337**(6099):1190-1195

[40] Mudduluru G, Medved F, Grobholz R, Jost C, Gruber A, Leupold JH, Post S, Jansen A, Colburn NH, Allgayer H. Loss of programmed cell death 4 expression marks adenoma-carcinoma transition, correlates inversely with phosphorylated protein kinase B, and is an independent prognostic factor in resected colorectal cancer. Cancer. 2007; **110**(8):1697-1707

[41] Dahlhamer JM, Zammitti EP, Ward BW, Wheaton AG, Croft JB. Prevalence of inflammatory bowel disease among adults aged ≥18 years — United States, 2015. MMWR. 2016;**65**(42):1166-1169. Available at https://www.cdc.gov/mmwr/volumes/65/wr/mm6542a3.htm

[42] Legaki E, Gazouli M. Influence of environmental factors in the development of inflammatory bowel diseases. World Journal of Gastrointestinal Pharmacology and Therapeutics. Feb 2016;**7**(1):112-125

[43] Kuenzig ME, Yim J, Coward S, Eksteen B, Seow CH, Barnabe C, Barkema HW, Silverberg MS, Lakatos PL, Beck PL, Fedorak R, Dieleman LA, Madsen K, Panaccione R, Ghosh S, Kaplan GG. The NOD2-smoking interaction in Crohn's disease is likely specific to the 1007 fs mutation and may be explained by age at diagnosis: A meta-analysis and case-only study. eBioMedicine. Jul 2017;**21**:188-196

[44] Guslandi M. Nicotine treatment for ulcerative colitis. British Journal of Clinical Pharmacology. Oct. 1999;**48**(4):481-484

[45] Loddo I, Romano C. Inflammatory bowel disease: Genetics, epigenetics, and pathogenesis. Frontiers in Immunology. 2015;**6**:551

[46] Ventham NT, Kennedy NA, Nimmo ER, Satsangi J. Beyond gene discovery in inflammatory bowel disease: The emerging role of epigenetics. Gastroenterology. Aug 2013; **145**(2):293-308

[47] Ricaño-Ponce I, Wijmenga C. Mapping of immune-mediated disease genes. Annual Review of Genomics and Human Genetics. Aug 2013;**14**(1):325-353

[48] Hrdlickova B, de Almeida RC, Borek Z, Withoff S. Genetic variation in the non-coding genome: Involvement of micro-RNAs and long non-coding RNAs in disease. Biochimica et Biophysica Acta (BBA) – Molecular Basis of Disease. 2014;**1842**(10):1910-1922

[49] Nimmo ER, Prendergast JG, Aldhous MC, Kennedy NA, Henderson P, Drummond HE, Ramsahoye BH, Wilson DC, Semple CA, Satsangi J. Genome-wide methylation profiling in Crohn's disease identifies altered epigenetic regulation of key host defense mechanisms including the Th17 pathway. Inflammatory Bowel Diseases. May 2012; **18**(5):889-899

Section 3

Transcription and Human Diseases

Chapter 7

The Key Role of E2F in Tumor Suppression through Specific Regulation of Tumor Suppressor Genes in Response to Oncogenic Changes

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Additional information is available at the end of the chapter

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Abstract

E2F, the principal target of the tumor suppressor pRB, plays crucial roles in tumor suppression. Upon dysfunction of pRB, E2F activates tumor suppressor genes such as *ARF*, an upstream activator of the tumor suppressor p53, resulting in the induction of apoptosis and tumor suppression. The E2F activity that activates the tumor suppressor genes is detected only in cancer cells and not in normal growing cells. The E2F activity can drive selective suicide gene expression and induce apoptosis specifically in cancer cells. Thus, the E2F activity provides a beneficial tool to specifically target cancer cells in cancer treatment.

Keywords: E2F, RB, ARF, apoptosis, cancer specific gene expression

1. Introduction

A human body consists of 37 trillion cells and the cell number is maintained by a balance of cell death and cell proliferation. As aged cells are eliminated by cell death, new cells are supplied by cell proliferation to retain appropriate cell numbers. To maintain homeostasis, cell proliferation is strictly regulated by growth signals. Cell proliferation is also induced by abnormal growth stimulation such as overexpression or constitutive activation of oncogenes, which leads to tumorigenesis [1]. To protect cells from tumorigenesis, mammalian cells harbor tumor suppressor pathways, principally mediated by pRB and p53 [2, 3]. The RB pathway consists of pRB and upstream regulators such as cyclin-dependent kinases (CDKs) and CDK inhibitors.

The p53 pathway consists of p53 and upstream regulators such as HDM2 and ARF. The RB pathway and the p53 pathway suppress tumor formation by the induction of cell cycle arrest or apoptosis. The forced inactivation of both pathways in normal cells renders cells tumorigenic and both pathways are disabled in most cancers, indicating that these two pathways play pivotal roles in tumor suppression in normal cells.

The transcription factor E2F, the principal target of the RB pathway, plays central roles in cell proliferation by activating a repertoire of growth-related genes. Consistent with this, overexpression of E2F1, an activator type of E2F family members, in quiescent cells induces progression into S phase [4]. Since E2F plays central roles in cell proliferation, it has generally been thought that defects in the RB pathway upregulate E2F and promote hyperplasia, contributing to tumorigenesis. However, it has also been reported that E2F plays a pivotal role in tumor suppression. E2F1 knockout mice showed increased incidence of tumor formation [5], suggesting a role of E2F1 in tumor suppression. Overexpression of E2F1 also activates p53, the main effector of the p53 pathway, and promotes apoptosis [6], rather than cell proliferation. Knocking out p53 attenuates E2F1-induced apoptosis [7], supporting that the induction of apoptosis is mediated through activation of p53. Of note, the overexpression of E2F1 activates the tumor suppressor gene *ARF*, an upstream activator of p53 [3]. These observations suggest that E2F plays a pivotal role in tumor suppression by activating ARF, and consequently p53. Interestingly, E2F selectively induces the *ARF* gene upon forced inactivation of pRB, which mimics dysfunction of the RB pathway, but not in response to physiological inactivation of pRB by growth stimulation [8, 9]. This observation implies that E2F activates the *ARF* gene specifically in response to oncogenic changes, contributing to tumor suppression. Consistent with this notion, the E2F activation of the *ARF* gene is detected only in cancer cells and is not observed in normal growing cells [8, 9]. Thus, E2F stimulation of *ARF* gene expression can serve as a tool to discriminate cancer cells and normal growing cells. In this chapter, we describe the roles of E2F in cell proliferation and tumor suppression, focusing on the mechanism of E2F dependent, selective regulation of tumor suppressor genes, specifically in response to oncogenic changes.

2. E2F plays central roles in cell proliferation

The proliferation of mammalian cells is dependent on growth stimulation, which promotes cell cycle progression. Once a cell passes through the restriction (R) point, located in late G1 phase, it is programmed to automatically proceed to the end of M phase. Thus, the regulation of the R point is a critical determinant of cell cycle progression and cell proliferation. Key regulators of the R point are the transcription factor E2F, which activates a repertoire of growth-related genes, and the tumor suppressor pRB, which inhibits E2F.

E2F consists of eight family members (E2F1-8), which, based on their function, are divided into transcriptional activators (E2F1–E2F3a) and transcriptional repressors (E2F3b–E2F8). E2F regulates thousands of genes important for cell cycle progression, DNA replication, DNA damage checkpoint, and DNA repair, and plays central roles in cell proliferation [10]. E2F-modulated cell cycle regulatory genes include *Cyclin E* [11], *Cyclin A* [12], and *CDC2* [13, 14].

Cyclin E/CDK2 promotes G1 to S phase transition by inactivating pRB through phosphorylation. Cyclin A/CDK2 promotes progression through S phase. Cyclin A/CDC2 and Cyclin B/CDC2 promote progression through G2 and progression into and through M phase, respectively. E2F-modulated DNA replication genes include *Cdc6* [15], *Cdt1* [16], *Cyclin E* [11], *ASK* [17], and *Cdc45* [18]. Origin recognition complex (ORC) binds to replication origins and marks where DNA replication takes place. Cdc6 and Cdt1 bind to ORC and promote initiation of DNA replication by recruiting the DNA helicase MCM complex to replication origins (**Figure 1**). Cyclin E/CDK2 phosphorylates MCM complex and promotes loading of it onto chromatin. ASK/Cdc7 activates MCM complex by phosphorylation and Cdc45 recruits DNA polymerase α onto chromatin. These E2F targets are essential for DNA replication and G1-S phase transition [19–23]. Accordingly, knocking out all members of activator-type *E2Fs* (*E2F1~E2F3*) abolishes cell proliferation [24]. Precise replication of genomic DNA is important to avoid mutation. E2F also activates genes involved in DNA damage checkpoint, such as *ATM* [25] and *Chk1* [26], and DNA repair, including *Claspin* [27], *BRCA1* [28], and *Rad51* [27]. Thus, E2F plays a pivotal role in cell proliferation by activating a number of genes critical for cell cycle progression and precise DNA replication.

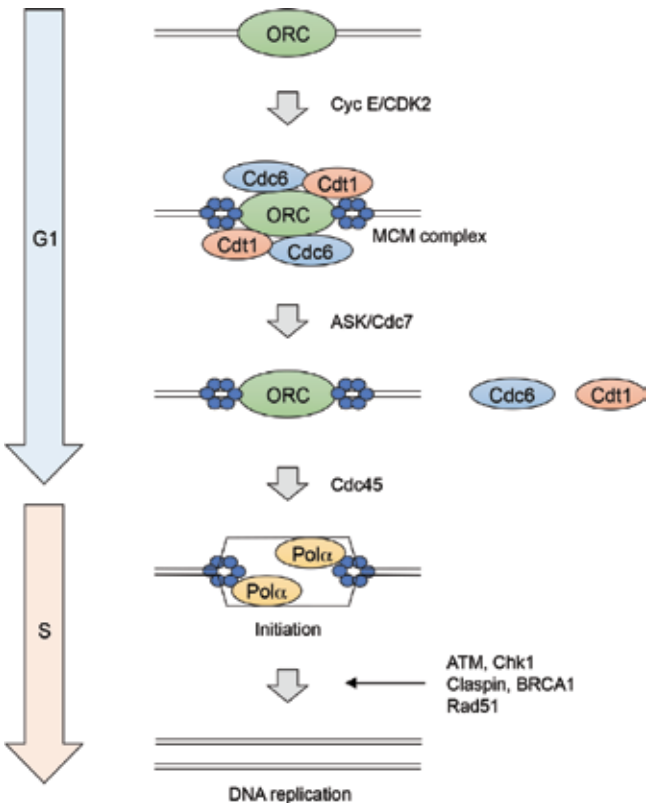

Figure 1. Role of E2F targets in DNA replication. E2F plays central roles in DNA replication by activating genes coding for factors involved in initiation of DNA replication, DNA synthesis, DNA damage checkpoint, and DNA repair.

3. The RB pathway in the control of cell proliferation

pRB is the product of the first identified tumor suppressor gene *retinoblastoma* (*RB1*) [29]. pRB is the principal regulator of G1 to S phase transition by restraining E2F and plays a crucial role in tumor suppression. Based on considerable structural homology, p107 and p130, together with pRB, comprise the RB family. During transition from G1 to S phase upon growth stimulation, RB is inactivated through phosphorylation by CDKs, thereby unleashing E2F and allowing cell cycle progression.

In quiescence, RB family members (pRB and p130) bind to E2F3b-E2F5 on its target promoters and repress their expression (**Figure 2**). The interaction of RB with the transactivation domain of E2F inhibits E2F's transcriptional activity. Furthermore, RB actively represses the expression of E2F target genes by changing chromatin structure through recruitment of histone deacetylase (HDAC) [30], histone methyltransferase (Suv39H1) [31], components of the chromatin remodeling complex (hBrm and BRG1) [32], and DNA methyltransferase (DNMT1) [33] onto their promoters. Upon growth stimulation, D-type cyclin-dependent kinases (CDK4 and 6) are activated, and inactivate p130 and pRB through phosphorylation inhibit binding of RB to E2F3b-5

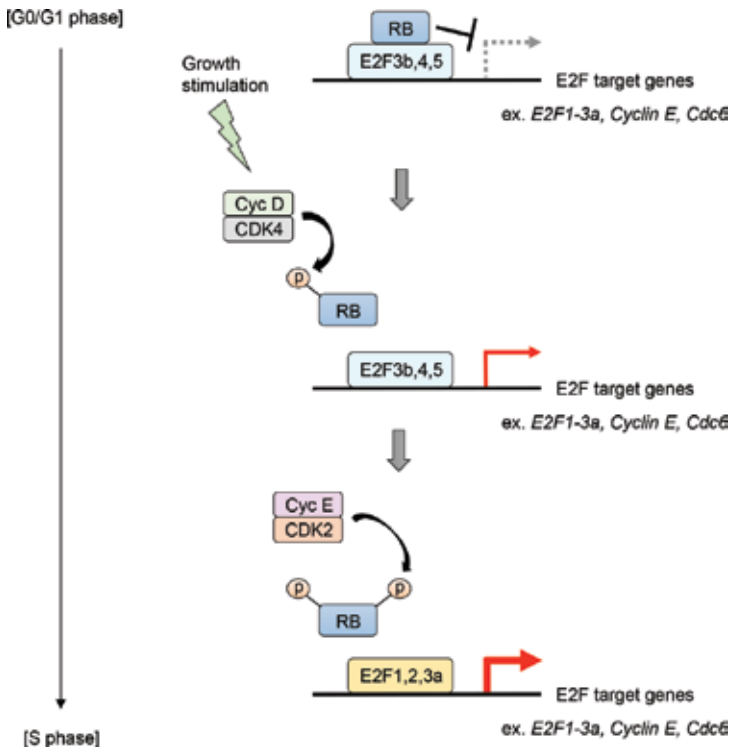

Figure 2. Regulatory mechanism of E2F target genes by E2F and RB. In quiescence, RB family members bind to E2Fs on its target promoters and repress their expression. In response to growth stimulation, Cyclin/CDK inactivates RB family through phosphorylation, activating E2F and its target gene expression.

and its target promoters. This leads to the release of E2F from suppression by RB and induces its target genes including *E2F1-3a* and *Cyclin E* [11, 34]. Cyclin E activates CDK2, which further inactivates RB through phosphorylation. This constitutes a positive feedback loop inactivating RB and activating E2F, resulting in the further induction of E2F targets and initiation of S phase [35]. Thus, regulated functional interactions of E2F and RB play pivotal roles in promoting and restraining cell proliferation, respectively. Given its importance in restraining cell proliferation, RB is often referred to as a "gatekeeper" in the control of cell proliferation.

Consistent with the critical role of RB in restraining cell proliferation, mutation or deletion of the *RB1* gene is responsible for retinoblastoma and various types of cancers including breast cancer [36], osteosarcoma [37], and small cell lung cancer (SCLC) [38]. Since mutation of p107 and p130 are uncommon and considering their function and frequency of inactivation, pRB is thought to be the pivotal tumor suppressor regulating G1-S phase transition [2, 39]. However, although increased tumorigenesis is not detected in $p107^{-/-}$ or $p130^{-/-}$ mice, $RB1^{-/+}p107^{-/-}$ or $RB1^{-/+}p130^{-/-}$ compound mice are more prone to tumor formation than $RB1^{-/+}$ mice [40]. This suggests that, upon loss of pRB function, p107 and p130 can, to some extent, compensate for the tumor suppressor function of pRB [41].

In cancer cells, regulation of G1-S phase transition is lost by the disruption of the RB pathway, which is regarded as a hallmark of cancer [2, 42] (**Figure 3**). Defects in the RB pathway such as deletion or mutation of *RB1* or silencing of its promoter by hypermethylation have been found in breast cancer [36], osteosarcoma [37], and SCLC [38]. Mutation or deletion of the CDK inhibitor

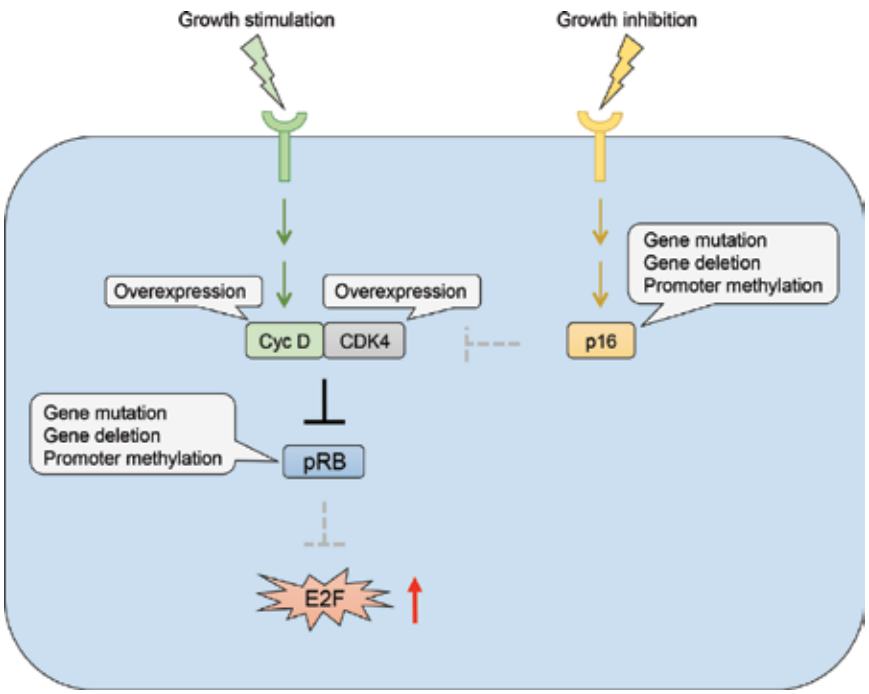

Figure 3. Defects in the RB pathway. pRB, CDKs, or CDK inhibitors are mutated in cancers, resulting in upregulation of E2F activity and its target gene expression.

p16^{INK4a} or silencing of its promoter by hypermethylation was detected at high frequency in a variety of cancers including prostate, renal, and colon cancer [43, 44]. Gene amplification and consequent overexpression of cyclin D1 or CDK4 are also detected in various cancers [45, 46]. Upstream activators of the *Cyclin D1* gene such as c-Myc and Ras are overexpressed or constitutively activated in cancers [47, 48], suggesting that these mutations also contribute to the overexpression of Cyclin D1. Taken together, the RB pathway is, at least at some point, disabled or compromised in almost all cancers. Consequently, pRB is functionally inactivated and E2F activity and its target gene expression are upregulated, leading to the aberrant cell proliferation. This underscores the importance of the RB pathway in tumor suppression.

4. The p53 pathway in the control of cell cycle arrest and apoptosis

p53 plays crucial roles in tumor suppression through the induction of cell cycle arrest or apoptosis (programmed cell death). *TP53*, which codes for p53, is the most frequently mutated gene in a variety of cancers including skin cancer [49], nonsmall cell lung cancer (NSCLC) [50], and breast cancer [51]. *TP53* knockout mice are prone to tumor formation [52, 53] and enhanced expression of p53 induces cell cycle arrest or apoptosis [54]. The target genes involved in cell cycle arrest include CDK inhibitor *p21*^{Cip1}, *14–3-3σ*, and *GADD45* [55] (**Figure 4**). CDK inhibitor p21^{Cip1}, which binds to and inhibits Cyclin D/CDK4, 6, Cyclin E/CDK2, Cyclin A/CDK2, and Cyclin B/CDK1, induces G1 and G2/M arrest [56]. 14–3-3σ binds to the phosphatase Cdc25C, which activates Cyclin B/CDK1, and inhibits its activity by the translocation of the complex from the nucleus into the cytoplasm [57]. GADD45 binds to and inactivates Cdc25C, consequently inhibiting CDK1 to induce G2/M arrest [58]. Activation of these genes by p53 is thought to contribute to tumor suppression through the induction of cell cycle arrest. The target genes involved in apoptosis include *Bax* [59], *Bak* [60], *Noxa* [61], and *Puma* [62] (**Figure 4**). Bax and Bak are Bax family members, whose insertion into mitochondrial membrane induces release of cytochrome *c* and apoptosis. Apoptosis induced by various stimulations is disabled in Bax/Bak-knocked out cells, indicating that Bax and Bak are central players in the induction of programmed cell death [63]. Noxa and Puma directly and indirectly activate Bax and Bak [64]. These observations suggest that p53 contributes to tumor suppression by the induction of apoptosis through activation of Bax and Bak.

The transcriptional activity of p53 is strictly regulated by its binding factors. The oncogene product HDM2, an E3 ubiquitin ligase, induces proteolysis of p53 through ubiquitination and inhibits its activity (**Figure 5**). Under nonstressed conditions, expression of p53 is kept at low levels by binding of HDM2. In response to DNA damage, Chk2 and ATM phosphorylate and activate p53 by inhibiting binding of HDM2 [65]. The tumor suppressor ARF stabilizes p53 by inhibiting HDM2 activity though its sequestration into the nucleolus [66]. Importantly, the expression of ARF is induced by oncogenic changes such as defects in the RB pathway including overexpression of c-myc and Ras [67], and expression of ARF is upregulated in various cancer cells [68]. Based on these observations, ARF is described as a "sensor of oncogenic stresses" and is thought to play crucial roles in tumor suppression, through up-regulation of p53, in response to oncogenic changes. Supporting the importance of its function, mutation, and deletion of *ARF* is detected in various cancers [69] and *ARF*$^{-/-}$ mice are prone to tumor formation [70]. The

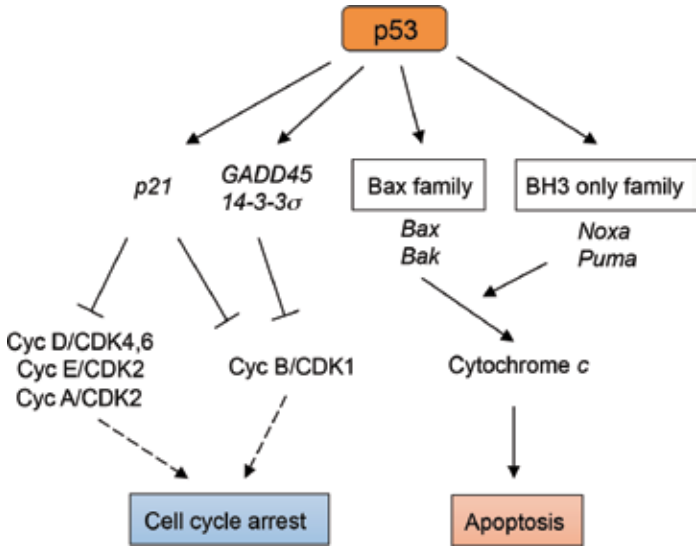

Figure 4. Induction of cell cycle arrest or apoptosis by p53. p53 contributes to cell cycle arrest through the induction of $p21^{Cip1}$, *14–3-3σ*, and *GADD45*, and apoptosis through *Bax*, *Bak*, *Noxa*, and *Puma*.

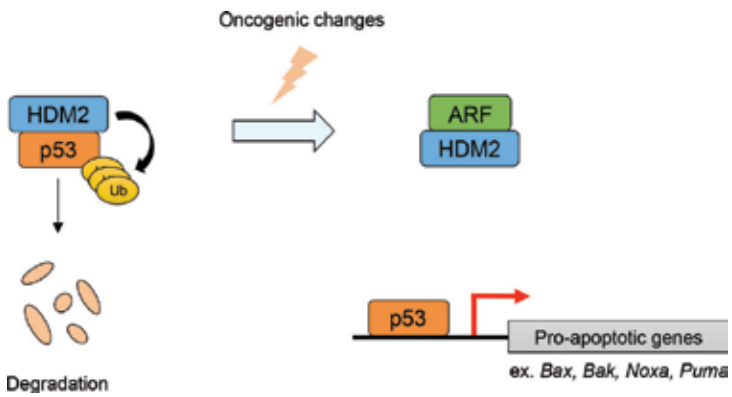

Figure 5. The mechanism of ARF activation of p53. In response to oncogenic changes, ARF stabilizes p53 by inhibiting HDM2 activity.

signal transduction network, from ARF to p53, is referred to as the p53 pathway. Studies using animal models revealed that forced inactivation of the RB and p53 pathways efficiently induce tumorigenesis, suggesting that both play pivotal roles in tumor suppression [71, 72].

5. Pivotal roles of E2F in tumor suppression

E2F plays crucial roles not only in cell proliferation but also in tumor suppression. $E2F1^{-/-}$ mice are prone to tumor formation [5] and overexpression of E2F1 induces apoptosis, suggesting that E2F contributes to tumor suppression through the induction of apoptosis.

The target genes involved in apoptosis include *ARF* and *TAp73* [7, 67] (**Figure 6**). ARF is an upstream activator of p53 and plays an important role in transmitting oncogenic signals to p53. The transcription factor TAp73 is a homolog of p53 and induces apoptosis through upregulation of p53 target genes in a p53-independent manner [73]. Apoptosis induced by the overexpression of E2F1 is attenuated in *TP53*$^{-/-}$ cells and *TAp73*$^{-/-}$ cells, and is disabled in *TP53*$^{-/-}$/*TAp73*$^{-/-}$ cells [7]. Moreover, *PPP1R13B* and *JMY*, whose products function as coactivators of p53 and TAp73, are also E2F targets (**Figure 6**) [74], indicating that E2F1 induces apoptosis primarily via p53 and TAp73. Other tumor suppressor genes that are E2F targets include *MOAP1*, *RASSF1*, and *BIM* (**Figure 6**). MOAP1 forms a complex with RASSF1 and activates the proapoptotic protein Bax. BIM is a member of the BH3-only family, which induces apoptosis through direct or indirect activation of Bax [75]. In addition, *Bax* is also a target of p53 and TAp73 [60]. These observations indicate that E2F suppresses tumor formation by the induction of apoptosis through upregulation of p53, TAp73, and their downstream effectors. Importantly, we demonstrated that E2F activates *ARF* and *TAp73* genes upon forced inactivation of pRB, which mimics dysfunction of the RB pathway, but not in response to the physiological inactivation of pRB through growth stimulation [8, 9]. Moreover, a search for genes regulated by E2F in a similar manner to *ARF* and *TAp73* identified *PPP1R13B*, *JMY*, *MOAP1*, *RASSF1*, and *BIM* [76]. These results suggest that E2F contributes to tumor suppression by inducing these genes specifically upon dysfunction of the RB pathway. Consistent with this

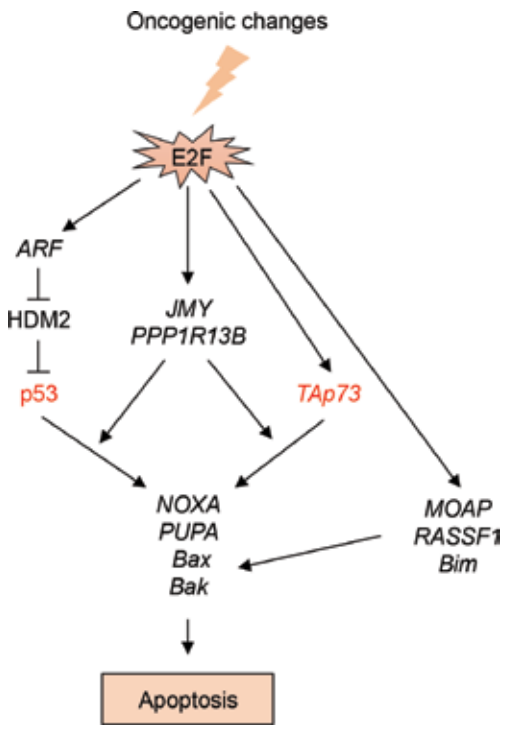

Figure 6. The pathway of E2F-induced apoptosis. In response to oncogenic changes, E2F induces apoptosis through upregulation of p53, TAp73, and their downstream effectors.

notion, E2F activity that activates the *ARF* and *TAp73* genes is detected only in cancer cells and is not present in normal cells [8, 9], underscoring the importance of E2F in tumor suppression. Since E2F selectively activates these tumor suppressor genes in the context of dysfunctional pRB, such E2F activity is referred to as "deregulated E2F activity." This E2F-dependent tumor suppression mechanism implies that disruption of both the p53 and RB pathways is necessary for tumor formation.

6. Regulation of E2F activity to induce apoptosis

Among E2F family members, activator-type E2Fs (E2F1-3) induce tumor suppressor genes such as *ARF* and *TAp73*, with E2F1 exhibiting the highest such activity [9, 76]. Therefore, to understand the regulation of tumor suppression by E2F, elucidation of the mechanism, by which E2F1 activates tumor suppressor genes, is important. Several factors that bind E2F1 and affect its activity are summarized in **Table 1**. TopBP1 is phosphorylated by Akt/PKB upon growth stimulation. The phosphorylated TopBP1 associates with E2F1 [77] and recruits Brg1, a component of chromatin remodeling complex, to E2F1, resulting in the inhibition of E2F1 induction of the *ARF* gene [78]. Jab1, a coactivator of c-Jun [79], binds to E2F1 through the marked box domain and promotes the induction of apoptosis by E2F1 [80, 81]. RIP140 and VHL repress the activation of the ARF promoter by E2F1 [82, 83]. ARF also functions as a transcription cofactor that binds to the transactivation domain of E2F1 to repress E2F1 activation of the ARF promoter [84]. PRMT5 methylates E2F1 on arginine residues 111 and 113, and destabilizes E2F1 [85]. SENP8 deNEDDylates (removes NEDD8) and stabilizes E2F1, resulting in enhancing activation of TAp73 promoter [86]. Sirt1, a histone deacetylase, represses E2F stimulation of the TAp73 promoter [87]. These studies revealed that E2F's ability to activate tumor suppressor genes is regulated by various factors such as transcription cofactors, post-translational modifiers, and histone modifiers. The mechanism of the regulation of E2F activity by these factors is not known in detail and its elucidation is imperative.

Gene name	Function	Monitoring promoter	Effect on E2F activity
Jab1	Transcription cofactor of c-jun	ARF	Upregulation
SENP8	Sentrin-specific protease	TAp73	Upregulation
ARF	Inhibitor of HDM2, transcription cofactor of c-myc	ARF	Repression
PRMT5	Methylase	TAp73	Repression
RIP140	Transcription cofactor of estrogen receptor	ARF	Repression
Sirt1	Histone deacetylase	TAp73	Repression
TopBP1	Transcription cofactor of Miz	ARF	Repression
VHL	E3 ubiquitin ligase, transcription cofactor of p53	ARF	Repression

Table 1. E2F-binding factors and their effects on its activity to activate tumor suppressor genes.

7. Utility of deregulated E2F activity in cancer cell-specific gene expression

In cancer treatment, specifically targeting cancer cells is important for optimal therapeutic efficacy. One strategy is to utilize a cancer-specific promoter to express a cytotoxic gene or a viral gene required for the replication. By regulating a suicide gene such as *HSV-TK* or a proapoptotic gene under the control of cancer-specific promoters, the gene is expressed specifically in cancer cells and causes cell death [88–90]. Alternatively, by regulating a viral gene required for viral replication under the control of these promoters, the gene is expressed specifically in cancer cells, allowing viral replication and cell lysis in a cancer cell-specific manner [91–93]. In this approach, therapeutic effects and side effects are dependent on the promoter activity in cancer cells and normal cells, respectively. Therefore, a promoter with optimal cancer cell-specificity should be used.

For a promoter to be cancer specific, it should have two important characteristics. First, the promoter should have low activity in normal cells to avoid side effects. Second, it should exhibit high activity in a wide variety of cancer cells for maximum therapeutic effects. As promoters thought to exemplify these parameters, hTERT and E2F1 promoters have been utilized. hTERT is a catalytic component of telomerase, which is not expressed in most somatic cells but is present in many types of cancers [94]. Thus, the hTERT promoter exhibits strong promoter activity in many types of cancer cells. However, given that normal stem cells also express hTERT, the hTERT promoter may exhibit strong promoter activity in these cells [95]. The E2F1 promoter is activated by E2F, whose activity is upregulated in cancer cells due to defects in the RB pathway. Thus, E2F1 promoter also exhibits strong promoter activity in many types of cancer cells. However, the E2F1 promoter is also stimulated by physiological E2F activity induced by growth stimulation and thus has a strong promoter activity in normal growing cells [34].

In contrast to the hTERT and E2F1 promoters, which may exhibit strong promoter activity in normal cells, the tumor suppressor ARF promoter, which specifically responds to deregulated E2F activity, is thought to be a better candidate. E2F activity stimulating the ARF promoter, is detected only in cancer cells and not in normal cells [8]. ARF is expressed at high levels in various cancer cells, but not in normally growing cells [68]. Furthermore, the activity of the ARF promoter is detected specifically in tumor tissues and not in normal tissues *in vivo* as revealed using $ARF^{GFP/GFP}$ mice [96]. These observations indicate that the ARF promoter shows optimal cancer cell specificity in a wide variety of cell types and has excellent therapeutic potential.

We showed that the ARF promoter exhibited greater cancer cell specificity than the E2F1 promoter [97]. Adenovirus expressing *HSV-TK*, a suicide gene, under the control of the ARF promoter (Ad-ARF-TK) had more selective cytotoxicity in cancer cells than the analogous E2F1 promoter construct [97]. Moreover, overexpression of the CDK inhibitor $p21^{Cip}$ upregulated deregulated E2F activity specifically in cancer cells and augmented cancer cell-specific cytotoxicity of Ad-ARF-TK [98]. These observations underscore the utility of the ARF promoter and deregulated E2F activity in mediating cancer-specific gene expression (**Figure 7**, upper panel). Furthermore, overexpression $p21^{Cip}$ alone could induce E2F dependent apoptosis specifically in cancer cells [98], suggesting that induction or enhancement of deregulated E2F activity could be a drug target to induce cancer cell-specific apoptosis (**Figure 7**, lower panel). It must be worth testing whether drug-based CDK inhibitors also exhibit similar effects to

p21^{Cip}. Since p21^{Cip} inhibits most of CDKs, identification of responsible CDK, which inhibits deregulated E2F activity, is also important. By using specific inhibitor to the responsible CDK, deregulated E2F activity could be more efficiently enhanced. The combination of deregulated E2F-mediated suicide gene therapy and enhancement of deregulated E2F activity using appropriate CDK inhibitor should also improve deregulated E2F-mediated cancer therapy.

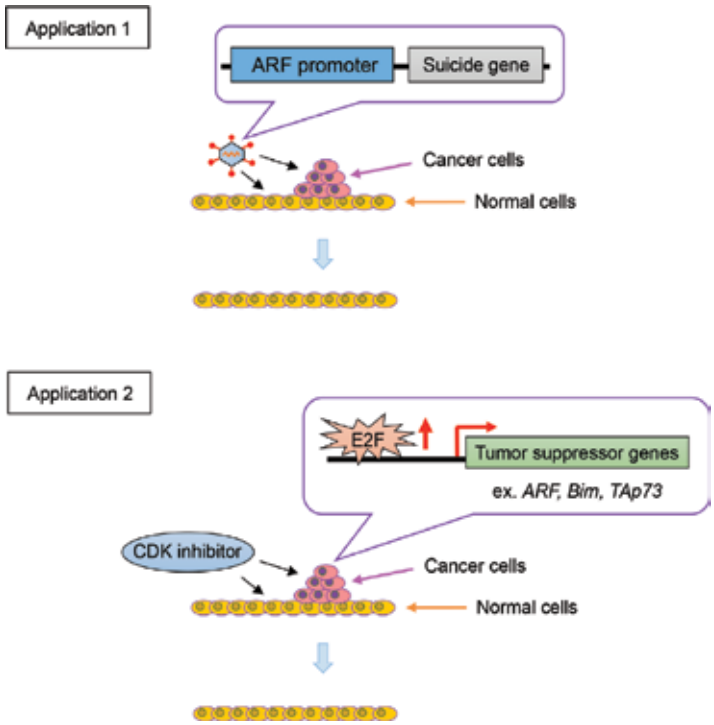

Figure 7. Application of deregulated E2F activity for cancer-specific treatment. Application 1: ARF promoter, which responds to deregulated E2F activity in cancer cells but not to physiological E2F activity in normal cells, drives suicide gene expression, and induces apoptosis specifically in cancer cells. Application 2: Upregulation of deregulated E2F activity by CDK inhibitors activates endogenous tumor suppressor genes and induces apoptosis specifically in cancer cells.

8. Conclusion

E2F is the principal target of the tumor suppressor pRB and defects in the RB pathway are observed in almost all cancers. Upon oncogenic changes, E2F activates *ARF*, an upstream activator of p53 and *TAp73*, resulting in the induction of apoptosis. Importantly, the E2F activity to stimulate *ARF* and *TAp73* expression is not induced by the physiological activation of E2F, such as growth stimulation. Therefore, E2F suppresses tumor formation by inducing apoptosis specifically in response to oncogenic changes through the activation of *ARF* and *TAp73*. Moreover, deregulated E2F-dependent activation of the *ARF* gene is observed only in cancer cells, and not in normal cells, suggesting that deregulated E2F activity represents a beneficial tool to specifically target cancer cells in cancer treatment.

Evidence supporting the potential availability of deregulated E2F activity in cancer therapy is accumulating. The regulation of suicide genes by the ARF promoter has more selective cytotoxicity in cancer cells than the analogous E2F1 promoter construct. Moreover, overexpression of $p21^{Cip}$ upregulates deregulated E2F activity and augments cancer-specific cytotoxicity of the ARF promoter construct. Furthermore, overexpression $p21^{Cip}$ alone can induce E2F-dependent apoptosis specifically in cancer cells. Therefore, deregulated E2F activity can drive selective gene expression and induce apoptosis specifically in cancer cells, supporting its therapeutic potential in a variety of cancers. The development of cancer therapies based upon deregulated E2F activity will require detailed characterization of the components and molecular mechanisms underlying its functional role in oncogenesis and tumor suppression and merits further investigation.

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Abbreviations

ARF	Alternative reading frame
ASK	Activator of S-phase kinase
ATM	Ataxia telangiectasia mutated
Bax	Bcl-2-associated X protein
Bak	Bcl-2 homologs antagonist/killer
BIM	BCL-2 interacting mediator of cell death
BRCA1	Breast cancer susceptibility genes 1
BRG1	Brahma-related gene-1
Cdc	Cell division cycle
CDK	Cyclin-dependent kinase
Cdt1	Chromatin licensing and DNA replication factor 1
Chk1	Checkpoint kinase 1
Chk2	Checkpoint kinase 2
DNMT1	DNA methyltransferase 1
E2F	E2 transcription factor
GADD45	Growth arrest and DNA-damage-inducible gene 45
GFP	Green fluorescent protein
hBrm	human Brahma
HDAC	histone deacetylase
HDM2	Human double minute 2

HSV-TK	Herpes simplex virus-1 thymidine kinase
hTERT	Human telomerase reverse transcriptase
Jab1	Jun activation domain-binding protein 1
JMY	Junction-mediating and regulatory protein
MCM	Minichromosome maintenance
MOAP1	Modulator of apoptosis 1
NSCLC	nonsmall cell lung cancer
ORC	Origin recognition complex
PKB	protein kinase B
PPP1R13B	Protein phosphatase 1 regulatory subunit 13B
PRMT5	Protein arginine methyltransferase 5
Puma	p53 upregulated modulator of apoptosis
RASSF1	Ras association domain family member 1
RB	Retinoblastoma
RIP140	Receptor-interacting protein 140
SCLC	Small cell lung cancer
SENP8	Sentrin specific protease family member
Suv39H1	suppressor of variegation 3–9 homolog 1
TopBP1	DNA topoisomerase II-binding protein 1
VHL	Von Hippel–Lindau

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References

[1] Hanahan D, Weinberg RA. The hallmarks of cancer. Cell. 2000;**100**:57-70

[2] Nevins JR. The Rb/E2F pathway and cancer. Human Molecular Genetics. 2001;**10**:699-703

[3] Bates S, Phillips AC, Clark PA, Stott F, Peters G, Ludwig RL, et al. $p14^{ARF}$ links the tumour suppressors RB and p53. Nature. 1998;**395**:124-125

[4] Johnson DG, Schwarz JK, Cress WD, Nevins JR. Expression of transcription factor E2F1 induces quiescent cells to enter S-phase. Nature. 1993;**365**:349-352

[5] Yamasaki L, Jacks T, Bronson R, Goillot E, Harlow E, Dyson NJ. Tumor induction and tissue atrophy in mice lacking E2F-1. Cell. 1996;**85**:537-548

[6] Qin XQ, Livingston DM, Kaelin WG, Adams PD. Deregulated transcription factor E2F-1 expression leads to S-phase entry and p53-mediated apoptosis. Proceedings of the National Academy of Sciences of the United States of America. 1994;**91**:10918-10922

[7] Irwin M, Marin MC, Phillips AC, Seelan RS, Smith DI, Liu WG, et al. Role for the p53 homologue p73 in E2F-1-induced apoptosis. Nature. 2000;**407**:645-648

[8] Komori H, Enomoto M, Nakamura M, Iwanaga R, Ohtani K. Distinct E2F-mediated transcriptional program regulates *$p14^{ARF}$* gene expression. The EMBO Journal. 2005;**24**: 3724-3736

[9] Ozono E, Komori H, Iwanaga R, Tanaka T, Sakae T, Kitamura H, et al. Tumor suppressor *TAp73* gene specifically responds to deregulated E2F activity in human normal fibroblasts. Genes to Cells. 2012;**17**:660-672

[10] DeGregori J. The genetics of the E2F family of transcription factors: Shared functions and unique roles. Biochimica et Biophysica Acta. 2002;**1602**:131-150

[11] Ohtani K, DeGregori J, Nevins JR. Regulation of the *cyclin E* gene by transcription factor E2F1. Proceedings of the National Academy of Sciences of the United States of America. 1995;**92**:12146-12150

[12] Schulze A, Zerfass K, Spitkovsky D, Middendorp S, Berges J, Helin K, et al. Cell-cycle regulation of the Cyclin-a gene promoter is mediated by a variant E2F site. Proceedings of the National Academy of Sciences of the United States of America. 1995;**92**:11264-11268

[13] Ren B, Cam H, Takahashi Y, Volkert T, Terragni J, Young RA, et al. E2F integrates cell cycle progression with DNA repair, replication, and G2/M checkpoints. Genes & Development. 2002;**16**:245-256

[14] Ishida S, Huang E, Zuzan H, Spang R, Leone G, West M, et al. Role for E2F in control of both DNA replication and mitotic functions as revealed from DNA microarray analysis. Molecular and Cellular Biology. 2001;**21**:4684-4699

[15] Ohtani K, Tsujimoto A, Ikeda M, Nakamura M. Regulation of cell growth-dependent expression of mammalian *CDC6* gene by the cell cycle transcription factor E2F. Oncogene. 1998;**17**:1777-1785

[16] Yoshida K, Inoue I. Regulation of Geminin and Cdt1 expression by E2F transcription factors. Oncogene. 2004;**23**:3802-3812

[17] Yamada M, Sato N, Taniyama C, Ohtani K, Arai K, Masai H. A 63-base pair DNA segment containing an Sp1 site but not a canonical E2F site can confer growth-dependent and E2F-mediated transcriptional stimulation of the human ASK gene encoding the regulatory subunit for human Cdc7-related kinase. The Journal of Biological Chemistry. 2002;**277**:27668-27681

[18] Arata Y, Fujita M, Ohtani K, Kijima S, Kato JY. Cdk2-dependent and -independent pathways in E2F-mediated S phase induction. The Journal of Biological Chemistry. 2000;**275**:6337-6345

[19] Yan Z, DeGregori J, Shohet R, Leone G, Stillman B, Nevins JR, et al. Cdc6 is regulated by E2F and is essential for DNA replication in mammalian cells. Proceedings of the National Academy of Sciences of the United States of America. 1998;**95**:3603-3608

[20] Rialland M, Sola F, Santocanale C. Essential role of human CDT1 in DNA replication and chromatin licensing. Journal of Cell Science. 2002;**115**:1435-1440

[21] Jackson PK, Chevalier S, Philippe M, Kirschner MW. Early events in DNA replication require cyclin E and are blocked by $p21^{CIP1}$. The Journal of Cell Biology. 1995;**130**:755-769

[22] Jiang W, McDonald D, Hope TJ, Hunter T. Mammalian Cdc7-Dbf4 protein kinase complex is essential for initiation of DNA replication. The EMBO Journal. 1999;**18**:5703-5713

[23] Hardy CFJ. Identification of Cdc45p, an essential factor required for DNA replication. Gene. 1997;**187**:239-246

[24] LZ W, Timmers C, Maiti B, Saavedra HI, Sang L, Chong GT, et al. The E2F1-3 transcription factors are essential for cellular proliferation. Nature. 2001;**414**:457-462

[25] Berkovich E, Ginsberg D. ATM is a target for positive regulation by E2F-1. Oncogene. 2003;**22**:161-167

[26] Verlinden L, Bempt IV, Eelen G, Drijkoningen M, Verlinden I, Marchal K, et al. The E2F-regulated gene Chk1 is highly expressed in triple-negative estrogen receptor−/progesterone receptor−/HER-2- breast carcinomas. Cancer Research. 2007;**67**:6574-6581

[27] Iwanaga R, Komori H, Ishida S, Okamura N, Nakayama K, Nakayama KI, et al. Identification of novel E2F1 target genes regulated in cell cycle-dependent and independent manners. Oncogene. 2006;**25**:1786-1798

[28] Wang A, Schneider-Broussard R, Kumar AP, MacLeod MC, Johnson DG. Regulation of BRCA1 expression by the Rb-E2F pathway. The Journal of Biological Chemistry. 2000;**275**:4532-4536

[29] Lee WH, Bookstein R, Hong F, Young LJ, Shew JY, Lee E. Human retinoblastoma susceptibility gene: Cloning, identification, and sequence. Science. 1987;**235**:1394-1399

[30] Luo RX, Postigo AA, Dean DC. Rb interacts with histone deacetylase to repress transcription. Cell. 1998;**92**:463-473

[31] Nielsen SJ, Schneider R, Bauer UM, Bannister AJ, Morrison A, O'Carroll D, et al. Rb targets histone H3 methylation and HP1 to promoters. Nature. 2001;**412**:561-565

[32] Trouche D, LeChalony C, Muchardt C, Yaniv M, Kouzarides T. RB and hbrm cooperate to repress the activation functions of E2F1. Proceedings of the National Academy of Sciences of the United States of America. 1997;**94**:11268-11273

[33] Robertson KD, Ait-Si-Ali S, Yokochi T, Wade PA, Jones PL, Wolffe AP. DNMT1 forms a complex with Rb, E2F1 and HDAC1 and represses transcription from E2F-responsive promoters. Nature Genetics. 2000;**25**:338-342

[34] Johnson DG, Ohtani K, Nevins JR. Autoregulatory control of E2F1 expression in response to positive and negative regulators of cell cycle progression. Genes & Development. 1994;**8**:1514-1525

[35] Henley SA, Dick FA. The retinoblastoma family of proteins and their regulatory functions in the mammalian cell division cycle. Cell Division. 2012;**7**:10

[36] Ertel A, Dean JL, Rui H, Liu C, Witkiewicz AK, Knudsen KE, et al. RB-pathway disruption in breast cancer: Differential association with disease subtypes, disease-specific prognosis and therapeutic response. Cell Cycle. 2010;**9**:4153-4163

[37] Miller CW, Aslo A, Won A, Tan M, Lampkin B, Koeffler HP. Alterations of the p53, Rb and MDM2 genes in osteosarcoma. Journal of Cancer Research and Clinical Oncology. 1996;**122**:559-565

[38] Mori N, Yokota J, Akiyama T, Sameshima Y, Okamoto A, Mizoguchi H, et al. Variable mutations of the RB gene in small-cell lung carcinoma. Oncogene. 1990;**5**:1713-1717

[39] Sherr CJ, McCormick F. The RB and p53 pathways in cancer. Cancer Cell. 2002;**2**:103-112

[40] Donovan SL, Schweers B, Martins R, Johnson D, Dyer MA. Compensation by tumor suppressor genes during retinal development in mice and humans. BMC Biology. 2006;**4**:14

[41] Burkhart DL, Sage J. Cellular mechanisms of tumour suppression by the retinoblastoma gene. Nature Reviews. Cancer. 2008;**8**:671-682

[42] Hanahan D, Weinberg RA. Hallmarks of cancer: The next generation. Cell. 2011;**144**: 646-674

[43] Herman JG, Merlo A, Mao L, Lapidus RG, Issa JPJ, Davidson NE, et al. Inactivation of the CDKN2/p16/MTS1 gene is frequently associated with aberrant DNA methylation in all common human cancers. Cancer Research. 1995;**55**:4525-4530

[44] Rocco JW, Sidransky D. p16(MTS-1/CDKN2/INK4a) in cancer progression. Experimental Cell Research. 2001;**264**:42-55

[45] Musgrove EA, Caldon CE, Barraclough J, Stone A, Sutherland RL. Cyclin D as a therapeutic target in cancer. Nature Reviews. Cancer. 2011;**11**:558-572

[46] An HX, Beckmann MW, Reifenberger G, Bender HG, Niederacher D. Gene amplification and overexpression of CDK4 in sporadic breast carcinomas is associated with high tumor cell proliferation. The American Journal of Pathology. 1999;**154**:113-118

[47] Prior IA, Lewis PD, Mattos C. A comprehensive survey of Ras mutations in cancer. Cancer Research. 2012;**72**:2457-2467

[48] Dang CV. C-myc target genes involved in cell growth, apoptosis, and metabolism. Molecular and Cellular Biology. 1999;**19**:1-11

[49] Benjamin CL, Ananthaswamy HN. p53 and the pathogenesis of skin cancer. Toxicology and Applied Pharmacology. 2007;**224**:241-248

[50] Ahrendt SA, YC H, Buta M, McDermott MP, Benoit N, Yang SC, et al. p53 mutations and survival in stage I non-small-cell lung cancer: Results of a prospective study. Journal of the National Cancer Institute. 2003;**95**:961-970

[51] Gasco M, Shami S, Crook T. The p53 pathway in breast cancer. Breast Cancer Research. 2002;**4**:70-76

[52] Donehower LA, Harvey M, Slagle BL, McArthur MJ, Montgomery CA, Butel JS, et al. Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. Nature. 1992;**356**:215-221

[53] Harvey M, McArthur MJ, Montgomery CA, Butel JS, Bradley A, Donehower LA. Spontaneous and carcinogen-induced tumorigenesis in p53-deficient mice. Nature Genetics. 1993;**5**:225-229

[54] Xu-Monette ZY, Medeiros LJ, Li Y, Orlowski RZ, Andreeff M, Bueso-Ramos CE, et al. Dysfunction of the TP53 tumor suppressor gene in lymphoid malignancies. Blood. 2012;**119**:3668-3683

[55] Taylor WR, Stark GR. Regulation of the G2/M transition by p53. Oncogene. 2001;**20**: 1803-1815

[56] Cayrol C, Knibiehler M, Ducommun B. p21 binding to PCNA causes G1 and G2 cell cycle arrest in p53-deficient cells. Oncogene. 1998;**16**:311-320

[57] Kumagai A, Dunphy WG. Binding of 14-3-3 proteins and nuclear export control the intracellular localization of the mitotic inducer Cdc25. Genes & Development. 1999;**13**:1067-1072

[58] Wang XW, Zhan QM, Coursen JD, Khan MA, Kontny HU, LJ Y, et al. GADD45 induction of a G2/M cell cycle checkpoint. Proceedings of the National Academy of Sciences of the United States of America. 1999;**96**:3706-3711

[59] Miyashita T, Reed JC. Tumor suppressor p53 is a direct transcriptional activator of the human bax gene. Cell. 1995;**80**:293-299

[60] Graupner V, Alexander E, Overkamp T, Rothfuss O, De Laurenzi V, Gillissen BF, et al. Differential regulation of the proapoptotic multidomain protein Bak by p53 and p73 at the promoter level. Cell Death and Differentiation. 2011;**18**:1130-1139

[61] Oda E, Ohki R, Murasawa H, Nemoto J, Shibue T, Yamashita T, et al. Nora, a BH3-only member of the Bcl-2 family and candidate mediator of p53-induced apoptosis. Science. 2000;**288**:1053-1058

[62] Nakano K, Vousden KH. PUMA, a novel proapoptotic gene, is induced by p53. Molecular Cell. 2001;**7**:683-694

[63] Wei MC, Zong WX, Cheng EHY, Lindsten T, Panoutsakopoulou V, Ross AJ, et al. Proapoptotic BAX and BAK: A requisite gateway to mitochondrial dysfunction and death. Science. 2001;**292**:727-730

[64] Shibue T, Suzuki S, Okamoto H, Yoshida H, Ohba Y, Takaoka A, et al. Differential contribution of Puma and Noxa in dual regulation of p53-mediated apoptotic pathways. The EMBO Journal. 2006;**25**:4952-4962

[65] YH O, Chung PH, Sun TP, Shieh SY. p53 C-terminal phosphorylation by CHM and CHK2 participates in the regulation of DNA-damage-induced C-terminal acetylation. Molecular Biology of the Cell. 2005;**16**:1684-1695

[66] Zhang YP, Xiong Y, Yarbrough WG. ARF promotes MDM2 degradation and stabilizes p53: ARF-INK4a locus deletion impairs both the Rb and p53 tumor suppression pathways. Cell. 1998;**92**:725-734

[67] Lowe SW, Sherr CJ. Tumor suppression by Ink4a-Arf: Progress and puzzles. Current Opinion in Genetics & Development. 2003;**13**:77-83

[68] Stott FJ, Bates S, James MC, McConnell BB, Starborg M, Brookes S, et al. The alternative product from the human *CDKN2A* locus, $p14^{ARF}$, participates in a regulatory feedback loop with p53 and MDM2. The EMBO Journal. 1998;**17**:5001-5014

[69] Sherr CJ. Cancer cell cycles. Science. 1996;**274**:1672-1677

[70] Sharpless NE, Ramsey MR, Balasubramanian P, Castrillon DH, DePinho RA. The differential impact of $p16^{INK4a}$ or $p19^{ARF}$ deficiency on cell growth and tumorigenesis. Oncogene. 2004;**23**:379-385

[71] Polager S, Ginsberg D. p53 and E2f: Partners in life and death. Nature Reviews. Cancer. 2009;**9**:738-748

[72] Levine AJ. The common mechanisms of transformation by the small DNA tumor viruses: The inactivation of tumor suppressor gene products: p53. Virology. 2009;**384**:285-293

[73] Candi E, Agostini M, Melino G, Bernassola F. How the TP53 family proteins TP63 and TP73 contribute to tumorigenesis: Regulators and effectors. Human Mutation. 2014;**35**:702-714

[74] Fogal V, Kartasheva NN, Trigiante G, Llanos S, Yap D, Vousden KH, et al. ASPP1 and ASPP2 are new transcriptional targets of E2F. Cell Death and Differentiation. 2005;**12**:369-376

[75] Youle RJ, Strasser A. The BCL-2 protein family: Opposing activities that mediate cell death. Nature Reviews. Molecular Cell Biology. 2008;**9**:47-59

[76] Kitamura H, Ozono E, Iwanaga R, Bradford AP, Okuno J, Shimizu E, et al. Identification of novel target genes specifically activated by deregulated E2F in human normal fibroblasts. Genes to Cells. 2015;**20**:739-757

[77] Liu K, Paik JC, Wang B, Lin FT, Lin WC. Regulation of TopBP1 oligomerization by Akt/PKB for cell survival. The EMBO Journal. 2006;**25**:4795-4807

[78] Liu K, Luo YH, Lin FT, Lin WC. TopBP1 recruits Brg1/Brm to repress E2F1-induced apoptosis, a novel pRb-independent and E2F1-specific control for cell survival. Genes & Development. 2004;**18**:673-686

[79] Claret FX, Hibi M, Dhut S, Toda T, Karin M. A new group of conserved coactivators that increase the specificity of AP-1 transcription factors. Nature. 1996;**383**:453-457

[80] HR L, Liang XD, Issaenko OA, Hallstrom TC. Jab1/CSN5 mediates E2F dependent expression of mitotic and apoptotic but not DNA replication targets. Cell Cycle. 2011;**10**:3317-3326

[81] Hallstrom TC, Nevins JR. Jab1 is a specificity factor for E2F1-induced apoptosis. Genes & Development. 2006;**20**:613-623

[82] Docquier A, Harmand PO, Fritsch S, Chanrion M, Darbon JM, Cavailles V. The transcriptional coregulator RIP140 represses E2F1 activity and discriminates breast cancer subtypes. Clinical Cancer Research. 2010;**16**:2959-2970

[83] Mans DA, Vermaat JS, Weijts BG, van Rooijen E, van Reeuwijk J, Boldt K, et al. Regulation of E2F1 by the von Hippel-Lindau tumour suppressor protein predicts survival in renal cell cancer patients. The Journal of Pathology 2013;**231**:117-129

[84] Eymin B, Karayan L, Seite P, Brambilla C, Brambilla E, Larsen CJ, et al. Human ARF binds E2F1 and inhibits its transcriptional activity. Oncogene. 2001;**20**:1033-1041

[85] Cho EC, Zheng SS, Munro S, Liu G, Carr SM, Moehlenbrink J, et al. Arginine methylation controls growth regulation by E2F-1. The EMBO Journal. 2012;**31**:1785-1797

[86] Aoki I, Higuchi M, Gotoh Y. NEDDylation controls the target specificity of E2F1 and apoptosis induction. Oncogene. 2013;**32**:3954-3964

[87] Wang CG, Chen LH, Hou XH, Li ZY, Kabra N, Ma YH, et al. Interactions between E2F1 and SirT1 regulate apoptotic response to DNA damage. Nature Cell Biology. 2006;**8**:1025-1031

[88] Duarte S, Carle G, Faneca H, de Lima MCP, Pierrefite-Carle V. Suicide gene therapy in cancer: Where do we stand now? Cancer Letters 2012;**324**:160-170

[89] Gu J, Kagawa S, Takakura M, Kyo S, Inoue M, Roth JA, et al. Tumor-specific transgene expression from the human telomerase reverse transcriptase promoter enables targeting of the therapeutic effects of the Bax gene to cancers. Cancer Research. 2000;**60**:5359-5364

[90] Komata T, Kondo Y, Kanzawa T, Ito H, Hirohata S, Koga S, et al. Caspase-8 gene therapy using the human telomerase reverse transcriptase promoter for malignant glioma cells. Human Gene Therapy. 2002;**13**:1015-1025

[91] Russell SJ, Peng KW, Bell JC. Oncolytic virotherapy. Nature Biotechnology. 2012;**30**: 658-670

[92] Kawashima T, Kagawa S, Kobayashi N, Shirakiya Y, Umeoka T, Teraishi F, et al. Telomerase-specific replication-selective virotherapy for human cancer. Clinical Cancer Research. 2004;**10**:285-292

[93] Tsukuda K, Wiewrodt R, Molnar-Kimber K, Jovanovic VP, Amin KM. An E2F-responsive replication-selective adenovirus targeted to the defective cell cycle in cancer cells: Potent antitumoral efficacy but no toxicity to normal cell. Cancer Research. 2002;**62**:3438-3447

[94] Ducrest AL, Szutorisz H, Lingner J, Nabholz M. Regulation of the human telomerase reverse transcriptase gene. Oncogene. 2002;**21**:541-552

[95] Hara T, Matsumura-Arioka Y, Ohtani K, Nakamura M. Role of human T-cell leukemia virus type I tax in expression of the human telomerase reverse transcriptase (hTERT) gene in human T-cells. Cancer Science. 2008;**99**:1155-1163

[96] Zindy F, Williams RT, Baudino TA, Rehg JE, Skapek SX, Cleveland JL, et al. *Arf* tumor suppressor promoter monitors latent oncogenic signals *in vivo*. Proceedings of the National Academy of Sciences of the United States of America. 2003;**100**:15930-15935

[97] Kurayoshi K, Ozono E, Iwanaga R, Bradford AP, Komori H, Ohtani K. Cancer cell specific cytotoxic gene expression mediated by ARF tumor suppressor promoter constructs. Biochemical and Biophysical Research Communications. 2014;**450**:240-246

[98] Kurayoshi K, Shiromoto A, Ozono E, Iwanaga R, Bradford AP, Araki K, et al. Ectopic expression of the CDK inhibitor $p21^{Cip1}$ enhances deregulated E2F activity and increases cancer cell-specific cytotoxic gene expression mediated by the ARF tumor suppressor promoter. Biochemical and Biophysical Research Communications. 2017;**483**:107-114

Chapter 8

Changes in the Expression and the Role of Sirtuin 3 in Cancer Cells and in Cardiovascular Health and Disease

Ozkan Ozden and Kevser Tural

Additional information is available at the end of the chapter

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Abstract

Sirtuin 3, an NAD^+-dependent deacetylase, whose expression is considered a marker in life extension, is downregulated with age and in various diseases. Sirtuin 3 is predominantly localized to the mitochondria and considered a fidelity protein for the integrity and function of this organelle. Some studies report its localization in the nucleus to regulate the expression of stress response–related genes and that reduced expression of SIRT3 produces a cellular milieu permissive for human pathologies. Since the expression and activity of Sirtuin 3 are important for the regulation of antioxidant defense, metabolism, and apoptosis initiation, the expression of SIRT3 is also important in the context of age-associated illnesses. A variety of small molecules are being developed to modulate the expression or activity of Sirtuin 3 and are potentially a valuable strategy to change mitochondrial acetylome to treat several diseases. The AMPK-PGC1α-SIRT3 axis plays a critical role in preserving mitochondrial biogenesis and function. Here, we summarize how changes in Sirtuin 3 expression are regulated in cancer and dysfunctions in cardiovascular diseases. The potential therapeutic strategies by targeting Sirtuin 3 expression to improve mitochondrial function in cancer and cardiovascular diseases are summarized.

Keywords: sirtuin, SIRT3 expression, cancer, cardiovascular diseases, stress

1. Introduction

With the identification of sirtuins (SIRTs), acetylation/deacetylation of proteins has become evident as an essential and highly regulated posttranslational modification, especially for the majority of mitochondrial proteins [1]. Acetylation can regulate the activity of an enzyme, stability or subcellular localization of a protein, transcriptional activity, and DNA-protein interactions. Silent information regulator 2 (Sir2) is the founding member of sirtuins, which was characterized in yeast, *Saccharomyces cerevisiae*. Sir2 functions in silencing gene expression by histone deacetylation [2]. It has been proposed that overexpression of Sir2 leads to an

increase in life expectancy in yeast and other model organisms, such as *Caenorhabditis elegans* and Drosophila [3]. In these model organisms, the activity of Sir2 is stimulated by multiple physiological events and stress signals, including starvation, calorie restriction, osmotic stress, and heat shock [4, 5].

SIRT histone deacetylases differ from traditional class I and II histone deacetylases (HDACs) in two ways: first, the substrates of SIRTs are not limited to histones and they can target key enzymes or proteins in the cytoplasm and mitochondria in addition to histones in the nucleus [6]. Second, SIRTs require nicotinamide adenine dinucleotide (NAD^+) for their enzymatic activity. Their dependence on NAD^+ is important for the regulation of metabolism and links the activity of SIRTs to the energy status of the cells. This is important for a cell to respond to different stress factors with a suitable stress response to sustain homeostasis. For example, in starvation, calorie restriction, exercise, or a cellular genotoxicity, increased cellular NAD^+ levels can activate SIRTs. Like Sir2, activated and/or upregulated SIRTs deacetylate their numerous targets to create a proper cellular stress response.

In mammals, seven SIRT isoforms have been identified, which can be found in different subcellular compartments. SIRT1, SIRT6, and SIRT7 are localized to the nucleus; SIRT3, SIRT4, and SIRT5 are localized mainly in the mitochondria; and SIRT2 is mostly present in the cytoplasm, but it might translocate into the nucleus [7]. Mammalian SIRTs have been proposed to have numerous beneficial effects, such as increasing insulin secretion, ATP synthesis, and lipid mobilization; however, the mechanism of how these beneficial effects translate into life span extension is poorly understood. SIRT3 gene expression has been observed to be upregulated with high frequency in long-lived individuals [8, 9]. In these individuals, mutations in an enhancer region of the SIRT3 gene are believed to upregulate its expression, and high SIRT3 expression can be considered a marker for longevity.

Calorie restriction is defined as lowering dietary calorie intake without malnutrition and has been described to extend the life span of many organisms from yeast to mammals and decrease the occurrence of age-related diseases, such as cancer, cardiovascular diseases, neurodegenerative diseases, and diabetes [10, 11]. In both yeast and *C. elegans*, caloric restriction–induced longevity is reliant on the existence of Sir2 [4]. Parallel to studies on Sir2, subsequent studies suggested that beneficial effects of calorie restriction might be associated with the upregulation of SIRT expression. SIRT3 transcription was stimulated in hepatocytes and skeletal muscle of mice on calorie restricted–diet, while a long-term high-fat diet resulted in lowering SIRT3 expression and increasing mitochondrial protein acetylation [12, 13]. SIRT3 expression declines in individuals over 59 years of age [14, 15], which may contribute to the increased incidence of cardiovascular diseases and cancer in aging population. Studying SIRT3 in these diseases may provide important mechanistic connection between the mitochondrial function and age-associated disorders [16–20]. SIRT3 expression is important for mitochondrial biogenesis, regulation of metabolism, ATP synthesis, suppression of reactive oxygen species (ROS), stress responses, and cell signaling [15, 19, 21–23].

Investigators report that SIRT3 regulates the activity of the transcription factors, Forkhead box O 3a (FOXO3a), and nuclear factor κB (NF-κB) [24, 25]; however, regulation of SIRT3 transcription itself is not completely understood up-to-date. *SIRT3* gene is located in a bidirectional

arrangement with another gene, called *PSMD13*, in a phylogenetically conserved manner. The promoter of the two genes is separated by a 788-bp intergenic region. SIRT3 holds a rich GC content but lacks a TATA box. Human SIRT3 promoter has binding sites for activator protein (AP-1), NF-κB, ZF5 transcription factor, GATAs, and specificity protein 1 (SP1) [26]. There is evidence that peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α) upregulates SIRT3 transcription, conveyed by an estrogen-related receptor (ERR)-binding element in the promoter of SIRT3 in mouse muscle cells and hepatocytes [27]. PGC-1α and ERRα display a synergic action on *SIRT3* promoter activity [27]. Furthermore, SIRT3 increases PGC1-α gene expression by activating AMPK signaling pathway and provides a positive feedback loop. Activation of AMPK signaling pathway leads to phosphorylation of cAMP response element-binding protein (CREB), which directly activates the PGC1-α promoter [28]. The positive feedback loop between SIRT3 and PGC1-α is important for mitochondrial biogenesis and activation of enzymes associated with the antioxidant system and regulation of metabolism [6, 27, 28]. In another study, bioinformatics analysis has been revealed that transcription factor binding motifs might be present in SIRT3 promoter. Nuclear respiratory factor 2 (NRF-2) transcription factor regulates mitochondrial genes, including antioxidant enzymes. NRF-2 has been reported to directly bind to the SIRT3 promoter and increase the expression of SIRT3 mRNA during nutrient stress [29].

2. Changes in Sirtuin 3 expression in carcinogenesis

Acetylation/deacetylation of specific lysine amino acids of proteins is a prevalent regulatory mechanism responsible for modulating signaling pathways, survival, apoptosis, and energy metabolism that takes part in important roles in cellular transformation [30]. SIRTs regulate various cellular activities, such as gene silencing, cellular proliferation, survival, apoptosis, stress response, and energy generation by protein deacetylation [31, 32].

SIRT3 is mostly localized in the mitochondria, and it deacetylates many critical metabolism-related proteins; decreases the levels of mitochondrial ROS; and ultimately regulates proliferation, differentiation, and survival in response to a stress stimulus [20, 33]. SIRT3 is considered a fidelity protein because it plays important roles in integrity and maintenance of mitochondrial function [6, 34, 35]. SIRT3 expression has been determined to be the highest in the heart, liver, brain, and brown adipose tissue where metabolic activity is relatively high [36]. Genetic deletion of SIRT3 in mouse has been reported not to produce any significant phenotypic abnormalities when the mouse is younger than 1 year old. SIRT3 knockout mice are viable, fertile, and metabolically active when these animals are still young. The difference between SIRT3-deficient mice and wild-type animals is that knockout mice express increased numbers of hyperacetylated proteins in their mitochondria, suggesting SIRT3 is the primary deacetylase in this organelle [37]. In normal conditions, SIRT3 gene deficiency does not produce any abnormalities; however, a different picture emerges when SIRT3 knockout mice get older than 12 months or encounter a stress stimulus. These mice might be prone to tumor formation, especially in the breast [38].

SIRT3-deficient mice older than 1 year old develop well-differentiated estrogen- and progesterone-positive mammary tumors [38]. This subtype of mammary tumors is more commonly observed in

women over 60 years old. In human estrogen- and progesterone-positive mammary tumor samples, SIRT3 expression is found to be reduced compared to noncancerous breast tissues [38]. The SIRT3 knockout mouse is suggested to be a convenient model to study this subtype of breast tumors. Additional studies on SIRT3-deficient mice revealed that ionizing radiation causes vacuolization in SIRT3 knockout mouse hepatocytes, suggesting SIRT3 protects hepatocytes against ionizing radiation–induced damage [38]. Moreover, SIRT3-deficient mice may develop age-related hearing loss [19]. SIRT3 expression is reduced in various tumors, and at least one allele of SIRT3 gene is deleted in about 40% of breast and ovarian tumors and 20% of all human cancer samples [38, 39]. SIRT3 expression is associated closely with cancer because it has essential regulatory roles in mitochondrial ROS scavenging, ATP synthesis, metabolism, and mitochondrial function [38].

Electron transport chain in mitochondria is the main source of the generation of ROS, such as superoxide. ROS homeostasis is strictly regulated in the cell, and while ROS play a part as secondary messengers at normal conditions, excessive ROS can damage cellular biomolecules and contribute to mitochondrial dysfunction and carcinogenesis [40]. SIRT3 has been shown to directly deacetylate and stimulate manganese superoxide dismutase (MnSOD) activity, which is the principle ROS scavenger in the mitochondria [20]. In addition, SIRT3 could induce expression of MnSOD, catalase, and isocitrate dehydrogenase (IDH2) by deacetylating FOXO3a transcription factor, triggering its translocation into the nucleus and transcription of these antioxidant enzymes [20, 25, 41, 42].

In the majority of tumors, pyruvate is preferentially transformed into lactate even in the existence of adequate oxygen. In this process, termed the Warburg effect, metabolism shifts in favor of glycolysis to increase the raw materials necessary for making new cancer cells [30, 43, 44]. In other words, glycolytic rate is increased in cancer cells. SIRT3 has been revealed to bring about degradation of HIF-1α, which results in suppression of the expression genes involved in glycolysis and angiogenesis [45]. SIRT3 displays this action indirectly through reductions in ROS level, which activates oxygen-dependent prolyl hydroxylases (PHD). In this regard, profilin1 (Pfn1) has been reported to have an anticancer feature in pancreatic cancer by upregulating SIRT3, which in turn results in degradation of HIF-1α and reduction in the expression of glycolytic genes [46]. Furthermore, SIRT3 has a second action to stimulate mitochondrial respiration by directly targeting electron transport chain and some metabolic enzymes, such as pyruvate dehydrogenase complex, and induce higher ATP production [22, 47–49].

Since SIRT3 takes part in mitochondrial ROS scavenging, regulation of metabolism, and mitochondrial function, it is not surprising that reduced expression of SIRT3 is highly associated with carcinogenesis [6, 30]. In addition, reduced SIRT3 expression is suggested to be a biomarker for breast cancer associated with poor prognosis [50, 51]. In addition to breast cancer, SIRT3 might play tumor suppressive roles in pancreatic cancer [52], hepatocellular carcinoma [53, 54], B cell lymphoma cells [55], and metastatic ovarian cancer [56]. In addition, kaempferol, a flavonoid, increases SIRT3 expression and its mitochondrial import; hence, it stimulates apoptosis in leukemia cell lines [57].

SIRT3 has also been reported to take part in an oncogenic function by promoting cancer initiation or progression depending on the tissue of origin and intracellular signal pathways

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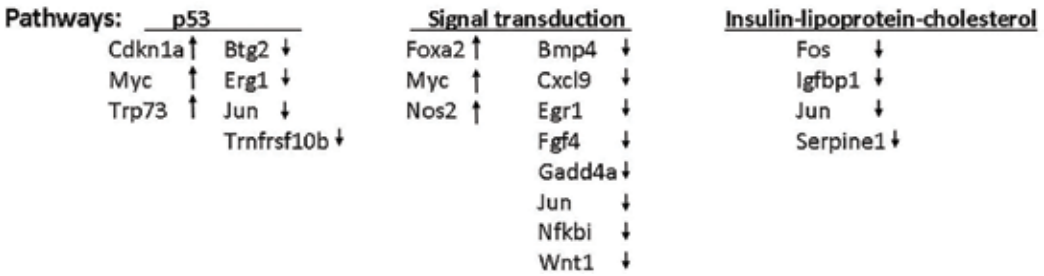

Figure 1. Changes in the expression of p53, signal transduction pathway, and insulin-lipoprotein-cholesterol genes in SIRT3-deficient livers compared to SIRT3 wild-type mouse based on [66].

[30]. Oncogenic properties of SIRT3 are attributed to its actions in stimulation of proliferation, resistance to oxidative stress, and suppression of apoptosis. SIRT3 could play an oncogenic role in a spectrum of cancers including oral squamous cell carcinoma [58], breast cancer [59], esophageal cancer [60], gastric cancer [61], colorectal cancer [62], and melanoma cell lines [63].

SIRT3 is predominantly located in the mitochondria; however, there are studies reporting its localization in the nucleus and having a function in regulating gene expression response to stress factors [64, 65]. We have previously shown that the loss of expression of SIRT3 in mouse liver and cultured mouse embryonic fibroblasts results in a cellular environment susceptible to carcinogenesis and cellular transformation [38, 66]. In the subsequent study, we investigated how SIRT3 alters the gene expression of cancer-related pathways in SIRT3 wild-type and SIRT3 knockout mouse hepatocytes. We studied how deficiency of SIRT3 expression might change the gene expression profile of various transcription factors and proteins linked to tumor formation in addition to genes associated with metabolism using a commercially available real-time polymerase chain reaction kit that screens gene expression profiling of diverse pathways [66]. We found upregulated expression of several genes having oncogenic properties including cyclin-dependent kinase inhibitor 1A P21 (Cdkn1a), myelocytomatosis oncogene (Myc), and nitric oxide synthase (NOS2) in SIRT3-deficient mouse liver. These genes are often overexpressed in human cancers, primarily in breast tumors. In contrast, several genes that were previously reported to be downregulated in human breast cancer containing B-cell translocation gene 2 (BTG2), early growth response 1 (EGR1), and Gadd4 had decreased expression with larger than 2-folds in the SIRT3-deficient hepatocytes [66]. The list of genes with larger than 2-folds in the cancer-associated pathways and genes associated with insulin-lipoprotein-cholesterol metabolism is presented in **Figure 1**.

3. Protective effects of SIRT3 in stress responses in the heart and changes in SIRT3 expression in cardiovascular diseases

The heart produces and uses more than 90% of its ATP from mitochondrial aerobic respiration in the cardiomyocytes, which have one of the highest mitochondrial density among all mammalian cells [67]. Mitochondria are critical in regulating oxidative stress signaling during cardiovascular

physiology and pathology by regulating cell death, ROS homeostasis, and ATP levels in cardiomyocytes. Heart failure might be caused by the imbalance in cardiac metabolism, oxidative stress, and opening of the mitochondrial permeability transition pore, which are important cellular contributors to myocardial ischemia/reperfusion (IR) injury and the development of cardiac hypertrophy [68–70]. Recent evidence demonstrated that the mitochondrial NAD^+-dependent enzyme, SIRT3, may regulate critical intracellular processes, such as oxidative stress, cell survival, and cellular metabolism for a healthy cardiac function [71].

In mammals, SIRT3 is one of seven NAD^+-dependent protein deacetylases or ADP-ribosyltransferases that regulates mitochondrial enzyme activity important in maintaining the integrity of the mitochondria and having a cardioprotective role [17, 72, 73]. Lanza et al. found that SIRT3 expression is downregulated with age, especially pronounced after 60 years old, and chronic endurance training causes elevation of SIRT3 expression along with beneficial health effects and potential lifespan-extending properties [74]. SIRT3, whose expression is rich in the heart, is the main deacetylase in the mitochondria and its absence produces hyperacetylation of numerous proteins in this organelle [75]. Increased acetylation of mitochondrial proteins, such as cyclophilin D, an important regulator of the permeability transition pore (mPTP), in the heart in response to IR injury has been reported [69, 76].

The changes in expression of SIRT3 are appealing to the study of cardiovascular diseases because of its presence mainly in the mitochondria, where a large part of the reactive oxygen species (ROS) is generated in the cardiomyocytes [77]. Overproduction of ROS in mitochondria has been linked to the development of cardiac hypertrophy [71]. High levels of cellular ROS damage biomolecules and accelerate the death of cardiomyocytes via apoptosis and necrosis [78–81]. Increased ROS was measured in cardiomyocytes isolated from hearts with hypertrophy induced by α-adrenergic agonists, namely angiotensin II, endothelin 1, norepinephrine, tumor necrosis factor, or cyclic mechanical stretch. Furthermore, induced hypertrophy could be repressed by the application of antioxidants [82–84]. ROS generation has been shown to activate diverse hypertrophic signal transduction pathways, including NF-κB, mitogen-activated protein kinase (MAPK), and protein kinase C (PKC) to stimulate hypertrophy [85–87]. SIRT3 mediates the mitochondrial ROS levels by inducing transcriptional expression of mitochondrial antioxidant enzymes by deacetylating FOXO3a transcription factor and its translocation into the nucleus [42]. In other words, SIRT3 has been well shown to participate in preventing hypertrophy by restricting ROS through activating MnSOD and catalase.

SIRT3 knockout mice are born without any significant abnormalities in their hearts; however, as they become 2 months of age, they display some indications of cardiac hypertrophic response and interstitial fibrosis, suggesting a protective role of SIRT3 against cardiac hypertrophy [42]. SIRT3 gene–removed mice have been reported to develop an accelerated age-related weakening in cardiac contractile function, which is characterized as an increase in end-diastolic volume, and are more prone to transaortic constriction-induced left ventricular hypertrophy [68, 88]. SIRT3 gene–removed mice can also display spontaneous pulmonary hypertension and have reduced oxygen consumption rate in their pulmonary artery smooth muscle cells [89]. In those knockout cells, the investigators pointed out that the expression of HIF1α, STAT3, and NFATc2 transcription factors increased, which might be responsible for the

development of this disease [89]. Knockdown of SIRT3 expression increases the vulnerability of both H9c2 cardiomyocytes and Langendorff preparations to simulate IR injury [18]. Moreover, IR injury is more pronounced in the aged hearts where SIRT3 expression is reduced, suggesting SIRT3 deficiency contributes to age-related loss of resistance to IR injury [18]. Consistently, exposure of SIRT3-deficient mouse hearts to global IR using a Langendorff-mode perfusion leads to significantly reduced postischemic recovery of cardiac function relative to wild-type mouse hearts due to both elevated mitochondrial ROS production and protein oxidation [90]. Expression of *SIRT3* is upregulated in response to stress and its overexpression has a protective role for cardiomyocytes from stress-mediated cell death by deacetylating Ku70 and preventing translocation of BAX to mitochondria [91]. Additionally, SIRT3 overexpression protects cardiomyocytes from oxidative stress by downregulating apoptosis regulator BAX and BCL-2 by inducing NF-κB transcription factor [24].

SIRT3 also has crucial roles in regulating metabolism of cardiovascular cells. SIRT3 improves mitochondrial oxidative phosphorylation for the production of ATP [47]. SIRT3 also regulates lipid metabolism by directly activating long-chain acyl CoA dehydrogenase (LCAD) enzyme activity, which diminishes lipid accumulation–induced cardiac hypertrophy [92]. In this regard, SIRT3 expression is downregulated in mice fed with high-fat diet; correspondingly, SIRT3 gene–removed mouse heart displays more noticeable hypertrophy [93]. Heart isolated from SIRT3-deficient mice shows impaired mitochondrial and cardiac contractile function accompanied by increased glycolysis and decreased palmitate oxidation and oxygen consumption [88]. Additionally, in SIRT3-deficient heart cells, 84 hyperacetylated mitochondrial proteins including enzymes for fatty acid metabolism, several subunits of electron transport chain, and enzymes involved in the Krebs cycle have been identified, proposing the importance of SIRT3 in maintaining a stable myocardial energy status [88].

Exogenous SIRT3 expression decreases mitochondrial ROS production and improves respiratory capacity in vitro [94, 95]. In recent years, increasing numbers of pharmacological agents to stimulate the expression or activity of SIRT3 to support a healthy cardiac function have been reported. Resveratrol, which is a general SIRT activator, is proposed to have a cardioprotective effect by decreasing the levels of mitochondrial ROS through upregulation of SIRT3 expression [96]. Resveratrol activates AMPK-PGC-1α, which activates the binding of ERRα to the SIRT3 promoter and increases SIRT3 mRNA transcription. Increased SIRT3 expression in the mitochondria in turn increases the deacetylation and activation of antioxidant enzymes, primarily MnSOD, and stimulates ATP synthesis to contribute to reduction in oxidative injury in endothelial cells [96]. In a recent study, it has been reported that adjudin, which is a lonidamine analog, upregulated the expression of SIRT3 and consequently protected cells against oxidative damage by eliminating ROS [97]. This agent might also have a cardioprotective potential.

In addition to resveratrol, other agents use the same signaling pathway to support cardioprotection. Melatonin has been reported to have an important cardioprotective action, which also uses AMPK-PGC-1α-SIRT3 signaling pathway. The investigators of the study reported that the protective effect of melatonin on diabetic myocardial IR injury is prevented by silencing SIRT3 expression [98].

Honokiol, which is a polyphenol derived from magnolia tree, lessens cardiac hypertrophy and fibrosis by activating SIRT3 and protects cardiomyocytes from doxorubicin-induced cell destruction and death by promoting mitochondrial fusion and limiting mitochondrial ROS levels and mtDNA damage [99, 100]. Adenovirus-mediated overexpression of SIRT3 might decrease pathogenesis of cardiovascular diseases by inhibition of Dox-induced cardiac hypertrophy and mitochondrial defects. Overexpression of the transcriptional cofactor receptor-interacting protein 140 (RIP140) increased cardiomyocyte hypertrophy and decreased ATP production along with mitochondrial dysfunction by decreasing the expression of SIRT3 in neonatal rat cardiomyocytes [101]. Repression of SIRT3 expression by RIP140 is dependent on ERRα [101]. A hexokinase inhibitor, 2-deoxy-d-glucose, administration significantly improves cardiac function and reduces myocardial apoptosis [102]. Zhen et al. pointed out that upregulation of SIRT3 expression along with SIRT1 by this agent might be important contributors to its protective action against septic cardiomyopathy [102]. Stimulation of SIRT3 expression improves the mitochondrial respiratory function and improves the cardiac function of mice. Metformin, which is a drug used for the treatment of diabetes, upregulates the expression of SIRT3 in about 8 weeks old mice with heart failure after myocardial infarction [103]. It was suggested that metformin-mediated SIRT3 upregulation and deacetylation of PGC-1α increase mitochondrial ATP production and mitochondrial oxygen consumption rates in the

SIRT3 stimulating potential cardioprotective agents	Upstream signaling of SIRT3	Direct or indirect SIRT3 substrates and targets	Potential effects of agents in cardiovascular system and cardiomyocytes	Associated references
Resveratrol	AMPK, PGC-1α, and ERRα	MnSOD, IDH2, GSH-Px, FOXO3a	Scavenges mtROS	[96]
Melatonin	AMPK and PGC1α	MnSOD, NRF1, TFAM, cytochrome c	Scavenges mtROS, stimulates mitochondrial biogenesis, and reduces apoptosis	[98]
Honokiol	N/A	MnSOD, OGG1, MFN1, OPA1, Ku70, BCL-2, BAX, NF-κB	Scavenges mtROS, reduces apoptosis, and enhances mitochondrial fusion	[24, 91, 100]
2-deoxy-d-glucose	N/A	BAX, BAK	Reduces apoptosis	[24, 102]
Metformin	N/A	PGC-1α	Stimulates mitochondrial ATP production and oxygen consumption rates	[103]
Adjudin	N/A	IDH2	Scavenges mtROS	[96, 97]
RIP140 (SIRT3 repressing TF)	ERRα	LCAD	Increases mtROS and lipid accumulation	[92, 101]

MnSOD: manganese superoxide dismutase; IDH2: isocitrate dehydrogenase; Forkhead box O 3a (FOXO3a); LCAD: long-chain acyl-CoA dehydrogenase; AMPK: AMP-activated protein kinase; GSH-Px: glutathione peroxidase; PGC-1α: peroxisome proliferator-activated receptor gamma coactivator 1-alpha; ERRα: estrogen-related receptor alpha; NRF1: nuclear respiratory factor 1; TFAM: transcription factor A, mitochondrial; OGG1: 8-oxoguanine glycosylase; MFN-1: mitofusin-1; OPA1: dynamin-like 120 kDa protein; Ku70; ATP-dependent DNA helicase II, 70 kDa subunit; BCL-2: B-cell lymphoma 2; NF-κB: nuclear factor kappa-light-chain enhancer of activated B cells; TF: transcription factor.

Table 1. Various agents to stimulate the expression of SIRT3 and the cardioprotective actions of SIRT3.

mouse hearts of myocardial infarction and decrease the associated damage [103]. A summary of agents to stimulate the expression of SIRT3 to support a healthy cardiac function and the possible targets of SIRT3 is presented in **Table 1**.

4. Conclusions

Cardiovascular diseases and cancer are most common causes of age-associated death around the world. SIRT3 has been shown to have essential roles in aging, longevity, and stress response since reduced expression or loss of function of SIRT3 brings about an intracellular milieu permissive for age-related illnesses. The mechanisms of how SIRT3 protects cells against cancer formation or cardiovascular diseases are not well understood because of the fact that SIRT3 has several targets or interacting partners in diverse pathways. Beneficial possessions of SIRT3 on cancer and particularly on various cardiovascular diseases have been reported; however, translating the modulation of SIRT3 expression using small molecules for clinical benefit is in its initial stages. Identification of agents to target SIRT3 expression to improve mitochondrial function will harvest new therapeutic strategies in the treatment of cancer and cardiovascular diseases.

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References

[1] Choudhary C, Kumar C, Gnad F, Nielsen ML, Rehman M, Walther TC, et al. Lysine acetylation targets protein complexes and co-regulates major cellular functions. Science. 2009;**325**:834-840. DOI: 10.1126/science.1175371

[2] Guarente L, Kenyon C. Genetic pathways that regulate ageing in model organisms. Nature. 2000;**408**:255-262. DOI: 10.1038/35041700

[3] Kaeberlein M, McVey M, Guarente L. The SIR2/3/4 complex and SIR2 alone promote longevity in *Saccharomyces cerevisiae* by two different mechanisms. Genes & Development. 1999;**13**:2570-2580

[4] Lin SJ, Defossez PA, Guarente L. Requirement of NAD and SIR2 for life-span extension by calorie restriction in *Saccharomyces cerevisiae*. Science. 2000;**289**:2126-2128

[5] Wood JG, Rogina B, Lavu S, Howitz K, Helfand SL, Tatar M, et al. Sirtuin activators mimic caloric restriction and delay ageing in metazoans. Nature. 2004;**430**:686-689. DOI: 10.1038/nature02789

[6] Torrens-Mas M, Oliver J, Roca P, Sastre-Serra J. SIRT3: Oncogene and tumor suppressor in cancer. Cancers (Basel). 2017;**9**. DOI: 10.3390/cancers9070090

[7] Frye RA. Phylogenetic classification of prokaryotic and eukaryotic Sir2-like proteins. Biochemical and Biophysical Research Communications. 2000;**273**:793-798. DOI: 10.1006/bbrc.2000.3000

[8] Bellizzi D, Rose G, Cavalcante P, Covello G, Dato S, De Rango F, et al. A novel VNTR enhancer within the SIRT3 gene, a human homologue of SIR2, is associated with survival at oldest ages. Genomics. 2005;**85**:258-263. DOI: 10.1016/j.ygeno.2004.11.003

[9] Rose G, Dato S, Altomare K, Bellizzi D, Garasto S, Greco V, et al. Variability of the SIRT3 gene, human silent information regulator Sir2 homologue, and survivorship in the elderly. Experimental Gerontology. 2003;**38**:1065-1070

[10] McCay CM, Crowell MF, Maynard LA. The effect of retarded growth upon the length of life span and upon the ultimate body size. Nutrition. 1989;**5**:155-171 discussion 72. 1935

[11] Weindruch R, Walford RL, Fligiel S, Guthrie D. The retardation of aging in mice by dietary restriction: Longevity, cancer, immunity and lifetime energy intake. The Journal of Nutrition. 1986;**116**:641-654

[12] Hirschey MD, Shimazu T, Jing E, Grueter CA, Collins AM, Aouizerat B, et al. SIRT3 deficiency and mitochondrial protein hyperacetylation accelerate the development of the metabolic syndrome. Molecular Cell. 2011;**44**:177-190. DOI: 10.1016/j.molcel.2011.07.019

[13] Tauriainen E, Luostarinen M, Martonen E, Finckenberg P, Kovalainen M, Huotari A, et al. Distinct effects of calorie restriction and resveratrol on diet-induced obesity and fatty liver formation. Journal of Nutrition and Metabolism. 2011;**2011**:525094. DOI: 10.1155/2011/525094

[14] Joseph AM, Adhihetty PJ, Buford TW, Wohlgemuth SE, Lees HA, Nguyen LM, et al. The impact of aging on mitochondrial function and biogenesis pathways in skeletal muscle of sedentary high- and low-functioning elderly individuals. Aging Cell. 2012;**11**:801-809. DOI: 10.1111/j.1474-9726.2012.00844.x

[15] Zeng L, Yang Y, Hu Y, Sun Y, Du Z, Xie Z, et al. Age-related decrease in the mitochondrial sirtuin deacetylase Sirt3 expression associated with ROS accumulation in the

auditory cortex of the mimetic aging rat model. PLoS One. 2014;**9**:e88019. DOI: 10.1371/journal.pone.0088019

[16] Houtkooper RH, Pirinen E, Auwerx J. Sirtuins as regulators of metabolism and healthspan. Nature Reviews. Molecular Cell Biology. 2012;**13**:225-238. DOI: 10.1038/nrm3293

[17] Ozden O, Park SH, Kim HS, Jiang H, Coleman MC, Spitz DR, et al. Acetylation of MnSOD directs enzymatic activity responding to cellular nutrient status or oxidative stress. Aging (Albany NY). 2011;**3**:102-107. DOI: 10.1016/j.freeradbiomed.2014.08.001

[18] Porter GA, Urciuoli WR, Brookes PS, Nadtochiy SM. SIRT3 deficiency exacerbates ischemia-reperfusion injury: Implication for aged hearts. American Journal of Physiology. Heart and Circulatory Physiology. 2014;**306**:H1602-H1609. DOI: 10.1152/ajpheart.00027.2014

[19] Someya S, Yu W, Hallows WC, JZ X, Vann JM, Leeuwenburgh C, et al. Sirt3 mediates reduction of oxidative damage and prevention of age-related hearing loss under caloric restriction. Cell. 2010;**143**:802-812. DOI: 10.1016/j.cell.2010.10.002

[20] Tao RD, Coleman MC, Pennington JD, Ozden O, Park SH, Jiang HY, et al. Sirt3-mediated deacetylation of evolutionarily conserved lysine 122 regulates MnSOD activity in response to stress. Molecular Cell. 2010;**40**:893-904. DOI: 10.1016/j.molcel.2010.12.013

[21] D'Aquila P, Rose G, Panno ML, Passarino G, Bellizzi D. SIRT3 gene expression: A link between inherited mitochondrial DNA variants and oxidative stress. Gene. 2012;**497**:323-329. DOI: 10.1016/j.gene.2012.01.04

[22] Ozden O, Park SH, Wagner BA, Song HY, Zhu Y, Vassilopoulos A, et al. SIRT3 deacetylates and increases pyruvate dehydrogenase activity in cancer cells. Free Radical Biology & Medicine. 2014;**76**:163-172. DOI: 10.1016/j.freeradbiomed.2014.08.001

[23] Sack MN, Finkel T. Mitochondrial metabolism, sirtuins, and aging. Cold Spring Harbor Perspectives in Biology. 2012;**4**. DOI: 10.1101/cshperspect.a013102

[24] Chen CJ, YC F, Yu W, Wang W. SIRT3 protects cardiomyocytes from oxidative stress-mediated cell death by activating NF-kappaB. Biochemical and Biophysical Research Communications. 2013;**430**:798-803. DOI: 10.1016/j.bbrc.2012.11.066

[25] Jacobs KM, Pennington JD, Bisht KS, Aykin-Burns N, Kim HS, Mishra M, et al. SIRT3 interacts with the daf-16 homolog FOXO3a in the mitochondria, as well as increases FOXO3a dependent gene expression. International Journal of Biological Sciences. 2008;**4**:291-299

[26] Bellizzi D, Dato S, Cavalcante P, Covello G, Di Cianni F, Passarino G, et al. Characterization of a bidirectional promoter shared between two human genes related to aging: SIRT3 and PSMD13. Genomics. 2007;**89**:143-150. DOI: 10.1016/j.ygeno.2006.09.004

[27] Kong X, Wang R, Xue Y, Liu X, Zhang H, Chen Y, et al. Sirtuin 3, a new target of PGC-1alpha, plays an important role in the suppression of ROS and mitochondrial biogenesis. PLoS One. 2010;**5**:e11707. DOI: 10.1371/journal.pone.0011707

[28] Shi T, Wang F, Stieren E, Tong Q. SIRT3, a mitochondrial sirtuin deacetylase, regulates mitochondrial function and thermogenesis in brown adipocytes. The Journal of Biological Chemistry. 2005;**280**:13560-13567. DOI: 10.1074/jbc.M414670200

[29] Satterstrom FK, Swindell WR, Laurent G, Vyas S, Bulyk ML, Haigis MC. Nuclear respiratory factor 2 induces SIRT3 expression. Aging Cell. 2015;**14**:818-825. DOI: 10.1111/acel.12360

[30] Xiong Y, Wang M, Zhao J, Han Y, Jia L. Sirtuin 3: A Janus face in cancer (review). International Journal of Oncology. 2016;**49**:2227-2235. DOI: 10.3892/ijo.2016.3767

[31] Haigis MC, Guarente LP. Mammalian sirtuins—Emerging roles in physiology, aging, and calorie restriction. Genes & Development. 2006;**20**:2913-2921. DOI: 10.1101/gad.467506

[32] Weir HJ, Lane JD, Balthasar N. SIRT3: A central regulator of mitochondrial adaptation in health and disease. Genes & Cancer. 2013;**4**:118-124

[33] Haigis MC, Deng CX, Finley LW, Kim HS, Gius D. SIRT3 is a mitochondrial tumor suppressor: A scientific tale that connects aberrant cellular ROS, the Warburg effect, and carcinogenesis. Cancer Research. 2012;**72**:2468-2472. DOI: 10.1158/0008-5472.CAN-11-3633

[34] Park SH, Ozden O, Jiang H, Cha YI, Pennington JD, Aykin-Burns N, et al. Sirt3, mitochondrial ROS, ageing, and carcinogenesis. International Journal of Molecular Sciences. 2011;**12**:6226-6239. DOI: 10.3390/ijms12096226

[35] Zhu Y, Yan Y, Principe DR, Zou X, Vassilopoulos A, Gius D. SIRT3 and SIRT4 are mitochondrial tumor suppressor proteins that connect mitochondrial metabolism and carcinogenesis. Cancer Metab. 2014;**2**:15. DOI: 10.1186/2049-3002-2-15

[36] Lombard DB, Alt FW, Cheng HL, Bunkenborg J, Streeper RS, Mostoslavsky R, et al. Mammalian Sir2 homolog SIRT3 regulates global mitochondrial lysine acetylation. Molecular and Cellular Biology. 2007;**27**:8807-8814. DOI: 10.1128/MCB.01636-07

[37] Hebert AS, Dittenhafer-Reed KE, Yu W, Bailey DJ, Selen ES, Boersma MD, et al. Calorie restriction and SIRT3 trigger global reprogramming of the mitochondrial protein acetylome. Molecular Cell. 2013;**49**:186-199. DOI: 10.1016/j.molcel.2012.10.024

[38] Kim HS, Patel K, Muldoon-Jacobs K, Bisht KS, Aykin-Burns N, Pennington JD, et al. SIRT3 is a mitochondria-localized tumor suppressor required for maintenance of mitochondrial integrity and metabolism during stress. Cancer Cell. 2010;**17**:41-52. DOI: 10.1016/j.ccr.2009.11.023

[39] Finley LW, Carracedo A, Lee J, Souza A, Egia A, Zhang J, et al. SIRT3 opposes reprogramming of cancer cell metabolism through HIF1alpha destabilization. Cancer Cell. 2011;**19**:416-428. DOI: 10.1016/j.ccr.2011.02.014

[40] Zhu Y, Park SH, Ozden O, Kim HS, Jiang H, Vassilopoulos A, et al. Exploring the electrostatic repulsion model in the role of Sirt3 in directing MnSOD acetylation status and enzymatic activity. Free Radical Biology & Medicine. 2012;**53**:828-833. DOI: 10.1016/j.freeradbiomed.2012.06.020

[41] Rangarajan P, Karthikeyan A, Lu J, Ling EA, Dheen ST. Sirtuin 3 regulates Foxo3a-mediated antioxidant pathway in microglia. Neuroscience. 2015;**311**:398-414. DOI: 10.1016/j.neuroscience.2015.10.048

[42] Sundaresan NR, Gupta M, Kim G, Rajamohan SB, Isbatan A, Gupta MP. Sirt3 blocks the cardiac hypertrophic response by augmenting Foxo3a-dependent antioxidant defense mechanisms in mice. The Journal of Clinical Investigation. 2009;**119**:2758-2771. DOI: 10.1172/JCI39162

[43] Heiden MGV, Cantley LC, Thompson CB. Understanding the Warburg effect: The metabolic requirements of cell proliferation. Science. 2009;**324**:1029-1033. DOI: 10.1126/science.1160809

[44] DeBerardinis RJ, Lum JJ, Hatzivassiliou G, Thompson CB. The biology of cancer: Metabolic reprogramming fuels cell growth and proliferation. Cell Metabolism. 2008;**7**:11-20. DOI: 10.1016/j.cmet.2007.10.002

[45] Bell EL, Emerling BM, Ricoult SJH, Guarente L. SirT3 suppresses hypoxia inducible factor 1 alpha and tumor growth by inhibiting mitochondrial ROS production. Oncogene. 2011;**30**:2986-2996. DOI: 10.1038/onc.2011.37

[46] Yao WT, Ji SR, Qin Y, Yang JX, Xu J, Zhang B, et al. Profilin-1 suppresses tumorigenicity in pancreatic cancer through regulation of the SIRT3-HIF1 alpha axis. Molecular Cancer. 2014;**13**:1-12. DOI: 10.1186/1476-4598-13-187

[47] Ahn BH, Kim HS, Song S, Lee IH, Liu J, Vassilopoulos A, et al. A role for the mitochondrial deacetylase Sirt3 in regulating energy homeostasis. Proceedings of the National Academy of Sciences of the United States of America. 2008;**105**:14447-14452. DOI: 10.1073/pnas.0803790105

[48] Fan J, Shan CL, Kang HB, Elf S, Xie JX, Tucker M, et al. Tyr phosphorylation of PDP1 toggles recruitment between ACAT1 and SIRT3 to regulate the pyruvate dehydrogenase complex. Molecular Cell. 2014;**53**:534-548. DOI: 10.1016/j.molcel.2013.12.026

[49] Shan CL, Kang HB, Elf S, Xie JX, TL G, Aguiar M, et al. Tyr-94 phosphorylation inhibits pyruvate dehydrogenase phosphatase 1 and promotes tumor growth. The Journal of Biological Chemistry. 2014;**289**:21413-21422. DOI: 10.1074/jbc.M114.581124

[50] Desouki MM, Doubinskaia I, Gius D, Abdulkadir SA. Decreased mitochondrial SIRT3 expression is a potential molecular biomarker associated with poor outcome in breast cancer. Human Pathology. 2014;**45**:1071-1077. DOI: 10.1016/j.humpath.2014.01.004

[51] Wei L, Zhou Y, Dai Q, Qiao C, Zhao L, Hui H, et al. Oroxylin A induces dissociation of hexokinase II from the mitochondria and inhibits glycolysis by SIRT3-mediated deacetylation of cyclophilin D in breast carcinoma. Cell Death & Disease. 2013;**4**:1-12. DOI: 10.1038/cddis.2013.131

[52] McGlynn LM, McCluney S, Jamieson NB, Thomson J, MacDonald AI, Oien K, et al. SIRT3 & SIRT7: Potential novel biomarkers for determining outcome in pancreatic cancer patients. PLoS One. 2015;**10**:e0131344. DOI: 10.1371/journal.pone.0131344

[53] Wang JX, Yi Y, Li YW, Cai XY, He HW, Ni XC, et al. Down-regulation of sirtuin 3 is associated with poor prognosis in hepatocellular carcinoma after resection. BMC Cancer. 2014;**14**:1-9. DOI: 10.1186/1471-2407-14-297

[54] Zhang B, Qin L, Zhou CJ, Liu YL, Qian HX, He SB. SIRT3 expression in hepatocellular carcinoma and its impact on proliferation and invasion of hepatoma cells. Asian Pacific Journal of Tropical Medicine. 2013;**6**:649-652. DOI: 10.1016/S1995-7645(13)60112-1

[55] Yu W, Denu RA, Krautkramer KA, Grindle KM, Yang DT, Asimakopoulos F, et al. Loss of SIRT3 provides growth advantage for B cell malignancies. The Journal of Biological Chemistry. 2016;**291**:3268-3279. DOI: 10.1074/jbc.M115.702076

[56] Dong XC, Jing LM, Wang WX, Gao YX. Down-regulation of SIRT3 promotes ovarian carcinoma metastasis. Biochemical and Biophysical Research Communications. 2016;**475**:245-250. DOI: 10.1016/j.bbrc.2016.05.098

[57] Marfe G, Tafani M, Indelicato M, Sinibaldi-Salimei P, Reali V, Pucci B, et al. Kaempferol induces apoptosis in two different cell lines via Akt inactivation, Bax and SIRT3 activation, and mitochondrial dysfunction. Journal of Cellular Biochemistry. 2009;**106**:643-650. DOI: 10.1002/jcb.22044

[58] Alhazzazi TY, Kamarajan P, Joo N, Huang JY, Verdin E, D'Silva NJ, et al. Sirtuin-3 (SIRT3), a novel potential therapeutic target for oral cancer. Cancer. 2011;**117**(8):1670. DOI: 10.1002/cncr.25676

[59] Torrens-Mas M, Pons DG, Sastre-Serra J, Oliver J, Roca P. SIRT3 silencing sensitizes breast cancer cells to cytotoxic treatments through an increment in ROS production. Journal of Cellular Biochemistry. 2016;**118**:397-406. DOI: 10.1002/jcb.25653

[60] Zhao Y, Yang H, Wang X, Zhang R, Wang C, Guo Z. Sirtuin-3 (SIRT3) expression is associated with overall survival in esophageal cancer. Annals of Diagnostic Pathology. 2013;**17**:483-485. DOI: 10.1016/j.anndiagpath.2013.06.001

[61] Cui Y, Qin L, Wu J, Qu X, Hou C, Sun W, et al. SIRT3 enhances glycolysis and proliferation in SIRT3-expressing gastric cancer cells. PLoS One. 2015;**10**:e0129834. DOI: 10.1371/journal.pone.0129834

[62] Liu C, Huang Z, Jiang H, Shi F. The sirtuin 3 expression profile is associated with pathological and clinical outcomes in colon cancer patients. BioMed Research International. 2014;**2014**:871263. DOI: 10.1155/2014/871263

[63] George J, Nihal M, Singh CK, Zhong W, Liu X, Ahmad N. Pro-proliferative function of mitochondrial sirtuin deacetylase SIRT3 in human melanoma. The Journal of Investigative Dermatology. 2016;**136**:809-818. DOI: 10.1016/j.jid.2015.12.026

[64] Iwahara T, Bonasio R, Narendra V, Reinberg D. SIRT3 functions in the nucleus in the control of stress-related gene expression. Molecular and Cellular Biology. 2012;**32**:5022-5034. DOI: 10.1128/MCB.00822-12

[65] Scher MB, Vaquero A, Reinberg D. SirT3 is a nuclear NAD+−dependent histone deacetylase that translocates to the mitochondria upon cellular stress. Genes & Development. 2007;**21**:920-928. DOI: 10.1101/gad.1527307

[66] Tao RD, Leclerc J, Yildiz K, Park SH, Jung B, Gius D, et al. Changes in gene expression in SIRT3 knockout liver cells. Turkish Journal of Biology. 2015;**39**:380-387. DOI: 10.1038/srep24156

[67] Ventura-Clapier R, Garnier A, Veksler V. Energy metabolism in heart failure. The Journal of Physiology. 2004;**555**:1-13. DOI: 10.1113/jphysiol.2003.055095

[68] Yellon DM, Hausenloy DJ. Myocardial reperfusion injury. The New England Journal of Medicine. 2007;**357**:1121-1135. DOI: 10.1056/NEJMra071667

[69] Di Lisa F, Menabo R, Canton M, Barile M, Bernardi P. Opening of the mitochondrial permeability transition pore causes depletion of mitochondrial and cytosolic NAD+ and is a causative event in the death of myocytes in postischemic reperfusion of the heart. The Journal of Biological Chemistry. 2001;**276**:2571-2575. DOI: 10.1074/jbc.M006825200

[70] Hafner AV, Dai J, Gomes AP, Xiao CY, Palmeira CM, Rosenzweig A, et al. Regulation of the mPTP by SIRT3-mediated deacetylation of CypD at lysine 166 suppresses age-related cardiac hypertrophy. Aging (Albany NY). 2010;**2**:914-923. DOI: 10.18632/aging.100252

[71] DX H, Liu XB, Song WC, Wang JA. Roles of SIRT3 in heart failure: From bench to bedside. Journal of Zhejiang University. Science. B. 2016;**17**:821-830. DOI: 10.1631/jzus.B1600253

[72] Michishita E, Park JY, Burneskis JM, Barrett JC, Horikawa I. Evolutionarily conserved and nonconserved cellular localizations and functions of human SIRT proteins. Molecular Biology of the Cell. 2005;**16**:4623-4635. DOI: 10.1091/mbc.E05-01-0033

[73] Winnik S, Auwerx J, Sinclair DA, Matter CM. Protective effects of sirtuins in cardiovascular diseases: From bench to bedside. European Heart Journal. 2015;**36**:3404-3412. DOI: 10.1093/eurheartj/ehv290

[74] Lanza IR, Short DK, Short KR, Raghavakaimal S, Basu R, Joyner MJ, et al. Endurance exercise as a countermeasure for aging. Diabetes. 2008;**57**:2933-2942. DOI: 10.2337/db08-0349

[75] Dittenhafer-Reed KE, Richards AL, Fan J, Smallegan MJ, Fotuhi Siahpirani A, Kemmerer ZA, et al. SIRT3 mediates multi-tissue coupling for metabolic fuel switching. Cell Metabolism. 2015;**21**:637-646. DOI: 10.1016/j.cmet.2015.03.007

[76] Parodi-Rullan R, Barreto-Torres G, Ruiz L, Casasnovas J, Javadov S. Direct renin inhibition exerts an anti-hypertrophic effect associated with improved mitochondrial function in post-infarction heart failure in diabetic rats. Cellular Physiology and Biochemistry. 2012;**29**:841-850. DOI: 10.1159/000178526

[77] Koentges C, Bode C, Bugger H. SIRT3 in cardiac physiology and disease. Frontiers in Cardiovascular Medicine. 2016;**3**:38. DOI: 10.3389/fcvm.2016.00038

[78] Bao W, Behm DJ, Nerurkar SS, Ao Z, Bentley R, Mirabile RC, et al. Effects of p38 MAPK inhibitor on angiotensin II-dependent hypertension, organ damage, and superoxide anion production. Journal of Cardiovascular Pharmacology. 2007;**49**:362-368. DOI: 10.1097/FJC.0b013e318046f34a

[79] Seddon M, Looi YH, Shah AM. Oxidative stress and redox signalling in cardiac hypertrophy and heart failure. Heart. 2007;**93**:903-907. DOI: 10.1136/hrt.2005.068270

[80] Ide T, Tsutsui H, Kinugawa S, Suematsu N, Hayashidani S, Ichikawa K, et al. Direct evidence for increased hydroxyl radicals originating from superoxide in the failing myocardium. Circulation Research. 2000;**86**:152-157

[81] Cave A, Grieve D, Johar S, Zhang M, Shah AM. NADPH oxidase-derived reactive oxygen species in cardiac pathophysiology. Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences. 2005;**360**:2327-2334. DOI: 10.1098/rstb.2005.1772

[82] Nakamura K, Fushimi K, Kouchi H, Mihara K, Miyazaki M, Ohe T, et al. Inhibitory effects of antioxidants on neonatal rat cardiac myocyte hypertrophy induced by tumor necrosis factor-alpha and angiotensin II. Circulation. 1998;**98**:794-799

[83] Pimentel DR, Amin JK, Xiao L, Miller T, Viereck J, Oliver-Krasinski J, et al. Reactive oxygen species mediate amplitude-dependent hypertrophic and apoptotic responses to mechanical stretch in cardiac myocytes. Circulation Research. 2001;**89**:453-460

[84] Hirotani S, Otsu K, Nishida K, Higuchi Y, Morita T, Nakayama H, et al. Involvement of nuclear factor-kappaB and apoptosis signal-regulating kinase 1 in G-protein-coupled receptor agonist-induced cardiomyocyte hypertrophy. Circulation. 2002;**105**:509-515

[85] Li HL, Huang Y, Zhang CN, Liu G, Wei YS, Wang AB, et al. Epigallocathechin-3 gallate inhibits cardiac hypertrophy through blocking reactive oxidative species-dependent and -independent signal pathways. Free Radical Biology & Medicine. 2006;**40**:1756-1775

[86] Higuchi Y, Otsu K, Nishida K, Hirotani S, Nakayama H, Yamaguchi O, et al. Involvement of reactive oxygen species-mediated NF-kappa B activation in TNF-alpha-induced cardiomyocyte hypertrophy. Journal of Molecular and Cellular Cardiology. 2002;**34**:233-240. DOI: 10.1006/jmcc.2001.1505

[87] Adiga IK, Nair RR. Multiple signaling pathways coordinately mediate reactive oxygen species dependent cardiomyocyte hypertrophy. Cell Biochemistry and Function. 2008;**26**:346-351. DOI: 10.1002/cbf.1449

[88] Koentges C, Pfeil K, Schnick T, Wiese S, Dahlbock R, Cimolai MC, et al. SIRT3 deficiency impairs mitochondrial and contractile function in the heart. Basic Research in Cardiology. 2015;**110**:36. DOI: 10.1007/s00395-015-0493-6

[89] Paulin R, Dromparis P, Sutendra G, Gurtu V, Zervopoulos S, Bowers L, et al. Sirtuin 3 deficiency is associated with inhibited mitochondrial function and pulmonary arterial

hypertension in rodents and humans. Cell Metabolism. 2014;**20**:827-839. DOI: 10.1016/j.cmet.2014.08.011

[90] Parodi-Rullan RM, Chapa-Dubocq X, Rullan PJ, Jang S, Javadov S. High sensitivity of SIRT3 deficient hearts to ischemia-reperfusion is associated with mitochondrial abnormalities. Frontiers in Pharmacology. 2017;**8**. DOI: 10.3389/fphar.2017.00275

[91] Sundaresan NR, Samant SA, Pillai VB, Rajamohan SB, Gupta MP. SIRT3 is a stress-responsive deacetylase in cardiomyocytes that protects cells from stress-mediated cell death by deacetylation of Ku70. Molecular and Cellular Biology. 2008;**28**:6384-6401. DOI: 10.1128/MCB.00426-08

[92] Hirschey MD, Shimazu T, Goetzman E, Jing E, Schwer B, Lombard DB, et al. SIRT3 regulates mitochondrial fatty-acid oxidation by reversible enzyme deacetylation. Nature. 2010;**464**:121-125. DOI: 10.1038/nature08778

[93] Zeng H, Vaka VR, He X, Booz GW, Chen JX. High-fat diet induces cardiac remodelling and dysfunction: Assessment of the role played by SIRT3 loss. Journal of Cellular and Molecular Medicine. 2015;**19**:1847-1856. DOI: 10.1111/jcmm.12556

[94] Du Q, Zhu B, Zhai Q, Yu B. Sirt3 attenuates doxorubicin-induced cardiac hypertrophy and mitochondrial dysfunction via suppression of Bnip3. American Journal of Translational Research. 2017;**9**:3360-3373

[95] Cheung KG, Cole LK, Xiang B, Chen K, Ma X, Myal Y, et al. Sirtuin-3 (SIRT3) protein attenuates doxorubicin-induced oxidative stress and improves mitochondrial respiration in H9c2 cardiomyocytes. The Journal of Biological Chemistry. 2015;**290**:10981-10993. DOI: 10.1074/jbc.M114.607960

[96] Zhou X, Chen M, Zeng X, Yang J, Deng H, Yi L, et al. Resveratrol regulates mitochondrial reactive oxygen species homeostasis through Sirt3 signaling pathway in human vascular endothelial cells. Cell Death & Disease. 2014;**5**:e1576. DOI: 10.1038/cddis.2014.530

[97] Quan Y, Xia L, Shao J, Yin S, Cheng CY, Xia W, et al. Adjudin protects rodent cochlear hair cells against gentamicin ototoxicity via the SIRT3-ROS pathway. Scientific Reports. 2015;**5**:8181. DOI: 10.1038/srep08181

[98] Yu L, Gong B, Duan W, Fan C, Zhang J, Li Z, et al. Melatonin ameliorates myocardial ischemia/reperfusion injury in type 1 diabetic rats by preserving mitochondrial function: Role of AMPK-PGC-1alpha-SIRT3 signaling. Scientific Reports. 2017;**7**:41337. DOI: 10.1038/srep41337

[99] Pillai VB, Kanwal A, Fang YH, Sharp WW, Samant S, Arbiser J, et al. Honokiol, an activator of Sirtuin-3 (SIRT3) preserves mitochondria and protects the heart from doxorubicin-induced cardiomyopathy in mice. Oncotarget. 2017;**8**:34082-34098. DOI: 10.18632/oncotarget.16133

[100] Pillai VB, Bindu S, Sharp W, Fang YH, Kim G, Gupta M, et al. Sirt3 protects mitochondrial DNA damage and blocks the development of doxorubicin-induced cardiomyopathy in

mice. American Journal of Physiology. Heart and Circulatory Physiology. 2016;**310**:H962-H972. DOI: 10.1152/ajpheart.00832.2015

[101] You J, Yue Z, Chen S, Chen Y, Lu X, Zhang X, et al. Receptor-interacting protein 140 represses sirtuin 3 to facilitate hypertrophy, mitochondrial dysfunction and energy metabolic dysfunction in cardiomyocytes. Acta Physiologica (Oxford, England). 2017;**220**:58-71. DOI: 10.1111/apha.12800

[102] Zheng Z, Ma H, Zhang X, Tu F, Wang X, Ha T, et al. Enhanced glycolytic metabolism contributes to cardiac dysfunction in polymicrobial sepsis. The Journal of Infectious Diseases. 2017;**215**:1396-1406. DOI: 10.1093/infdis/jix138

[103] Sun D, Yang F. Metformin improves cardiac function in mice with heart failure after myocardial infarction by regulating mitochondrial energy metabolism. Biochemical and Biophysical Research Communications. 2017;**486**:329-335. DOI: 10.1016/j.bbrc.2017.03.036

Chapter 9

Regulatory Functions of *Pax1* and *Pax9* in Mammalian Cells

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Additional information is available at the end of the chapter

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Abstract

Pax1 and *Pax9* are paired-box transcription factors, which play vital roles in axial skeletogenesis, thymus organogenesis, palatogenesis and odontogenesis among others. The importance of these closely related transcription factors can be perceived from the various human anomalies associated with their disruption. Vertebral column abnormalities such as kyphoscoliosis, seen in Jarcho-Levine and Klippel-Feil syndromes, secondary cleft palate, oligodontia/ hypodontia (missing teeth) and thymus developmental defects have all been associated with mutations in *PAX1* and/or *PAX9*. In this chapter, we describe the molecular functions of *Pax1* and *Pax9* in various tissues during mouse development.

Keywords: *Pax1*/*Pax9*, intervertebral disc, palatogenesis, odontogenesis, thymus

1. Introduction

A cell is the functional unit of any living organism and the genome is its underlying blueprint. Transcription factors (TFs) are proteins that bind to the DNA in a sequence-specific manner, where they modulate (activate, repress or insulate) the expression of a particular set of genes. Spatio-temporal regulation of a combination of genes, the "gene battery", is the basis of individual cell type determination in a multicellular organism [1].

Gene regulation is a tremendous feat. A single gene can be regulated by multiple TFs, acting on multiple cis-regulatory elements (CREs), in different cells and at different times (i.e. spatio-temporal regulation). Non-coding RNAs (e.g. microRNAs, small nucleolar RNAs etc.) also play a role at a post-transcriptional level [2]. This complex interplay of the various transfactors acting on the CREs to determine a gene battery can be mapped into a transcriptional

network. Such networks execute downstream processes like specification, commitment and differentiation of stem cells or progenitors into a particular lineage during development. Dysregulation of transcriptional networks manifests as aberrations in the cells which in turn results in developmental defects or diseases [1, 3].

In this chapter, we will describe the roles of two developmental TFs – *Pax1* and *Pax9*, in mammalian development. The importance of studying the closely related *Pax1* and *Pax9* can be appreciated from the various human anomalies associated with them. Vertebral column abnormalities such as kyphoscoliosis, seen in Jarcho-Levine and Klippel-Feil syndromes, secondary cleft palate, oligodontia/hypodontia (missing teeth) and thymus developmental defects have all been associated with mutations or SNPs in *PAX1* and/or *PAX9* [4–7].

The role of *Pax1* was discovered serendipitously, involving a spontaneous mouse mutant with a kinked tail – named "*undulated*". This mouse mutant carried a point mutation in *Pax1*, which resulted in vertebral anomalies, whereby certain segments of the lumbar vertebrae were missing. This led to a misalignment of the vertebral column hence the kinked tail phenotype. More spontaneous variants of the *undulated* (*un*) mutant were discovered, all of which mapped to some defect in the *Pax1* gene or deletion of its entire locus [8]. *Pax1* paralog, *Pax9*, was also mapped and shown to have a role in the development of various organs. What is more intriguing is how well-conserved the functions of these genes are, such that the defects observed in the loss-of- function *Pax1* or *Pax9* mouse models are phenocopied in humans as well. Thus, analyses of such mouse models help us to glean into the functions of these genes and decipher what organs they are important in.

Pax1 and *Pax9* have a variety of roles in multiple tissues (e.g. scapula, pelvic girdle, limb and salivary gland epithelium) yet their functions have been most extensively studied in axial skeletogenesis, palatogenesis, odontogenesis, and thymus development [9–12]. Hence, in this chapter we will focus on their regulatory functions in the context of these tissues.

2. The evolutionary history of *Pax1* and *Pax9*

Pax genes are a family of developmental TFs with crucial functions in early patterning and organogenesis. The paired box, encoding a highly conserved segment of 128 amino acids with DNA-binding activity, was initially identified in the *Drosophila melanogaster* genes: *paired* (*prd*), and *gooseberry* (*gsb*) by Markus Noll and team in 1986 [13].

Similarity to the paired box led to the identification of the *Pax* gene family in other vertebrates and invertebrates. The ancestral *proto-pax* existed prior to the Cambrian explosion, and the two-rounds of whole genome duplication during or prior to this period, and subsequent divergence with uneven deletion events are believed to have given rise to the various paralogs and orthologues in the vertebrates and invertebrates [14]. The paired box is believed to have originated through domestication of the *Tc1/mariner* transposon, which is prevalent in all orders of living organisms. Currently, *Pax* genes have been identified in all orders of the metazoan species, with nine in mammals (human and mouse), and up to fifteen in *Danio rerio* [15].

The *Pax* genes are divided into two supergroups (PAXB-like and PAXD-like) and four subgroups/subfamilies (I to IV) based on their sequence similarity, the combination of functional domains they possess and overlapping regions of tissue expression. PAXB group contain the paired-domain (with two Helix-Turn-Helix, HTH motifs) (PD), octapeptide motif (HSVSNILG) (OP), and paired type homeodomain (PTHD) (full or truncated). The PAXD-like group contains an additional paired type homeodomain tail (PTH). It is as yet unclear whether *proto-pax* originated from PAXB or PAXD supergroups. These supergroups are further categorized as four subfamilies in vertebrates: Group I (*Pax1* and *Pax9*), Group II (*Pax2, Pax5, Pax8*), Group III (*Pax3, Pax7*) and Group IV (*Pax4, Pax6*) (**Figure 1**) [15].

Pax1 and *Pax9* belong to the same subfamily (Group 1/PAXD-like), containing only the PD and OP. Mouse *Pax1* and *Pax9* share a high amino acid sequence similarity of 79%, diverging mainly at their C-terminal ends. Their paired-domains share 98% identity and differ only at five sites - at the first two amino acids of the PD and at positions 82, 89 and 93 of the proteins, which belong to the C-terminal half of the PD [16]. The amino acid substitution from Tyr to Phe at position 2 of the PD is described to be class-specific [17]. Between species, *Pax* orthologs are highly conserved whereby the coding sequences of human PAX1 and mouse Pax1 share 88.1% identity while the PD share 100% identity. Similarly the PD of human PAX9 and mouse Pax9 share 100% identity, while overall identity is 98% [16, 18]. This high conservation in mouse has allowed it to serve as a suitable model to study the functions of *Pax* genes.

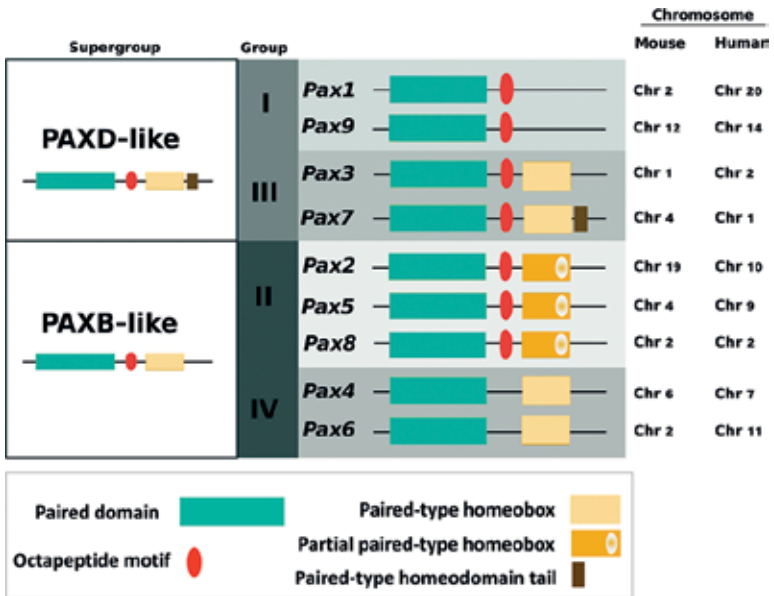

Figure 1. *Pax* genes, structure and grouping in mouse and human. *Pax* genes are divided into supergroups and subgroups. The PAXD-like supergroup is defined by the additional presence of a paired type homeodomain tail. The PD and PTHD have DNA-binding ability and so are drivers of the transcriptional program. The OP is believed to assist in protein-protein interactions, mostly mediating repressive effects of the TFs.

3. Paired domain and DNA recognition

Pax TFs execute their function through their DNA-binding ability aided by the PD and/or homeodomain. DNA-binding ability of the PD was initially demonstrated through *in vitro* biochemical assays on *Drosophila* prd protein binding to the e5 sequence from the *even-skipped* promoter [19]. Since Pax1 and Pax9 do not possess a homeodomain, they are fully reliant upon the PD for binding specificity and affinity. The PD of Pax1 recognizes a 24 bp sequence [20].

Biochemical and crystallographic studies revealed that the PD is a bipartite structure with the N-terminal (PAI) and C-terminal (RED) sub domains, each with a helix-turn-helix (HTH) motif [15, 19, 21, 22]. These subdomains recognize a non-palindromic consensus sequence with two half sites (5′ and 3′) positioned on adjacent major grooves on the same side of the DNA. The PAI subdomain recognizes the 3′ half site of the consensus sequence while RED recognizes the 5′ half site [22]. Our own analysis of *in vivo* Pax9 binding sites in the intervertebral disc (IVD) anlagen revealed a motif "5′-C/A G/A CGTGAACCG-3′" that highly resembles the 3′ half site of the consensus PD motif "5′-GCG G/T A/G AC G/C G/A-3′" (**Figure 2**) [19, 23].

While the PAI domain is most critical for DNA binding, in some scenarios, the Pax protein can bind solely through the RED domain. For instance, in the *undulated* mutants, point mutation

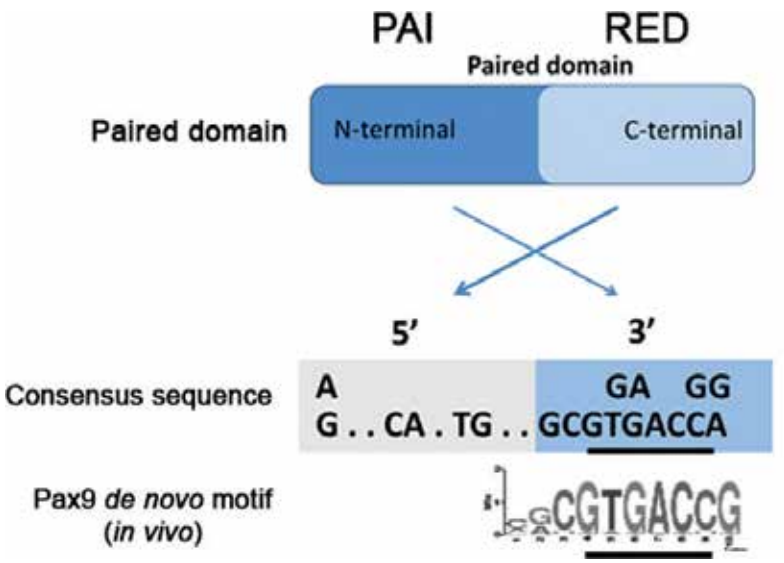

Figure 2. Paired domain and consensus recognition sequence. The paired domain consists of the N-terminal (PAI) and C-terminal (RED) domains. RED recognizes the 5′ half site sequence while PAI recognizes the 3′ half site sequence. The pentanucleotide motif "GGAAC" described by Chalepakis et al. [20] as the core DNA-binding motif of paired domain is underlined. In E12.5 mouse IVD anlagen, Pax9 recognizes an *in vivo* motif resembling the 3′ half site. Abbreviation(s): IVD, intervertebral disc.

in the N-terminal half of the PD in Pax1 drastically reduced its binding affinity and altered the specificity, and so resulted in its loss of function [20]. In contrast, particular isoforms of Pax6 and Pax8 bind DNA exclusively through their REI subdomains [24, 25]. Moreover, binding to both half sites by both subdomains confers greater affinity and specificity *in vitro*. The truncated form of Pax5 PD (missing the last 36 amino acid residues of the PD) retained the capacity to bind to a subset of the sequences bound by the complete form, albeit with lower affinity [19]. Thus, these subdomains are modular. Their ability to bind independently or in combination is postulated to confer greater diversity in the repertoire of sequences that can be bound by the PD.

4. Expression patterns of *Pax1* and *Pax9* in mouse development

Like numerous other developmental TFs, Pax TFs are characterized by spatio-temporally restricted expression during embryogenesis, playing essential roles in early patterning and organogenesis. They can be generalized to have a role in proliferation, migration, condensation and differentiation functions in different cell types. Their expression is often down-regulated or turned off in terminally differentiated tissues. Dysregulation of *Pax* gene expression often results in various developmental abnormalities and has also been observed in various cancers such as esophageal squamous cell carcinoma, non-small cell lung cancer and cervical intraepithelial neoplasia [26–28].

During development, *Pax1*/*Pax9* are the only *Pax* genes not expressed in neural tissues but instead are expressed in the endoderm- and mesoderm-derived tissues [29]. They share similar tissue sites of expression namely the foregut epithelium, sclerotome, pharyngeal pouch endoderm and limb bud mesenchyme [30]. However, unlike *Pax1*, *Pax9* is expressed in neural crest-derived tissues. Both *Pax* genes begin to be expressed in the somites and foregut as early as E8.5, the pharyngeal pouches at E9.0, limb buds at E10.0 to E11.5 and thymus anlagen at E12.5 [9, 16, 31]. In tissues where they are co-expressed, especially the sclerotome-derived axial skeleton, they are known to have redundant, compensatory roles. On the other hand, they are unable to rescue each other's functions in tissues where they are not co-expressed.

5. Pleiotropic roles of *Pax1* and *Pax9* in mouse development

Developmental TFs are pleiotropic. While the very definition of pleiotropy has several meanings in development, evolution and genetics, here we employ the definition of one gene affecting multiple phenotypes [32]. *Pax1* and *Pax9* are no exception. They have multiple roles and act on different tissues which are derived from different germ layers. Therefore, when disrupted, they exhibit complex phenotypes depending on which tissues are disrupted during development.

5.1. *Pax1* and *Pax9* in sclerotome-derived IVD of the axial skeleton

The axial skeleton is a critical load-bearing structure of the vertebral body plan and also functions to protect essential spinal nerves. It is composed of the metameric arrangement of vertebral bodies (VBs) connected by fibrocartilaginous intervertebral discs (IVDs) [33].

Axial skeletogenesis in mouse is a precisely coordinated series of processes; an interplay between the notochord and paraxial mesoderm-derived somites. It begins with the specification of the ventral somites into sclerotome by Sonic hedgehog (Shh) signals emanating from the notochord and floor plate of the neural tube [34–37]. Shh acts partly by antagonizing Wnt signals from the dorsal neural tube and surface ectoderm and BMP signals from the dorsal neural tube or lateral plate mesoderm.

Throughout IVD development, *Pax1* and *Pax9* share largely overlapping expression domains. *Pax1* expression can be detected in the de-epithelializing ventral somites as early as E8.5, while *Pax9* expression is detected slightly later at E9.0. These sclerotomal cells proliferate and then migrate to surround the notochord and form the mesenchymal prevertebrae. By E11.5, these give rise to metameric condensations along the anteroposterior (A/P) axis. Within these condensed segments, *Pax1* is uniformly expressed in rostral and caudal regions, while *Pax9* remains restricted to the caudal portion, but by E12.5, *Pax1* also becomes restricted to the caudal half which will give rise to the IVD anlagen [16, 38, 39]. Sclerotomal cells in close proximity to the notochord give rise to VBs and IVDs while the lateral regions develop into the proximal parts of the ribs, vertebral pedicles and laminae of the neural arch. Subsequently, the condensed portions of the prevertebrae give rise to the IVD and the less condensed regions give rise to the VB. Formation of these condensations is mandatory for the subsequent chondrogenesis into IVD segments of the axial skeleton [16, 40–42].

By E12.5, *Pax1* and *Pax9* expression are restricted to the IVD and are not expressed in the VB. Within the IVD anlagen *Pax1* and *Pax9* expression domains differ slightly. While *Pax1* is strongly expressed in the medial segment, *Pax9* is stronger in the lateral regions. Then the distinction between IVD and VB becomes more apparent at E13.5. The IVD mesenchyme further differentiates into the inner cartilaginous annulus fibrous (IAF) and outer annulus fibrous (OAF) at around E14.5. *Pax1* remains expressed in the IVD and perichondrium of the VB, while *Pax9* is weakly expressed in the IVD. At E15.5, their expression declines within the IAF and become restricted to the OAF. *Pax9* is no longer detected in the vertebral column at E16.5 but mild *Pax1* expression has been detected in the OAF [16, 23, 39, 43].

5.1.1. Regulation of Pax1 and Pax9 and their role in sclerotome maintenance

Pax1 and *Pax9* can be regulated by multiple mechanisms in the somites and sclerotome. Shh induces the expression of *Pax1*, *Pax9* and *Mesenchyme forkhead-1* (*Mfh1*) in the ventral somites which communicate its proliferative function [35, 37]. *Pax1*, *Pax9* and *Mfh1* are vital for maintaining the sclerotome cell numbers. In fact, *Pax1* and *Mfh1* genetically interact as $Pax1^{-/-}Mfh1^{-/-}$ mutants show reduced cell proliferation [35]. Noggin (Nog) also induces *Pax1* expression in

the absence of Hh signaling (in *Shh*$^{-/-}$ mutants) [44, 45]. Other factors which do not independently induce *Pax1*/*Pax9* expression but can regulate their expression in the somites are *Pbx1*/*Pbx2* and *Meox1*/*Meox2*. In both *Pbx1*$^{-/-}$*Pbx2*$^{-/-}$ mutants and *Meox1*$^{-/-}$*Meox2*$^{-/-}$ mutants, *Pax1* and *Pax9* expression is diminished in the somites/sclerotome, although *Pax9* to a lesser extent [46, 47]. Furthermore, Pax1 potentially auto-regulates itself as *Pax1*$^{-/-}$ mutants show reduced *Pax1* mRNA expression. *Pax9* however is independent of *Pax1* in the sclerotome, as *Pax1*$^{-/-}$ mutants do not show any reduction in *Pax9* mRNA [23]. Thus, *Pax1* and *Pax9* can be regulated by different upstream regulators most of which remain to be identified.

5.1.2. Molecular functions of Pax1 and Pax9 in axial skeletogenesis

The roles of *Pax1* and *Pax9* in vertebral column development were first identified through spontaneous mouse mutants – *undulated* (*un*) [48], *Undulated short-tail* (*Un*^{s}) [49], *undulated-extensive* (*un*^{ex}) [50] and *undulated intermediate* (*un-i*) [51] – which encompass a mutation in *Pax1* or deletion of the loci containing *Pax1* [8]. Subsequent gene-targeted knock-out models of *Pax1* [9] and *Pax9* [30] and generation of compound mutants revealed their synergistic, gene-dosage dependent, redundant roles in axial skeletogenesis [23, 52].

Pax1$^{-/-}$ mice exhibit a characteristic short, kinked tail phenotype with defects in the vertebral column (cervical and lumbar), scapula (loss of acromion process) and sternum (inappropriate ossification of some of the inter sternebrae). Within the vertebrae, the lumbar regions show a more pronounced phenotype of split vertebrae with loss of IVDs and formation of a ventral rod-like cartilaginous structure. They also lack the pharyngeal pouch derivatives thymus and parathyroid glands. However, these mice were viable and fertile. Even though *Pax1*$^{+/-}$ show an overall normal phenotype externally, they possess slight abnormalities in the vertebral column and sternum with varying penetrance, indicating haploinsufficiency of *Pax1* in these structures [9].

Contrary to *Pax1*$^{-/-}$ mice, *Pax9*$^{-/-}$ mutants surprisingly do not possess any vertebral column defects. Instead they show defects in all the pharyngeal pouch-derived structures. They exhibit cleft secondary palate, and lack all teeth, both of which are derived from 1st pharyngeal pouch. Further, they lack thymus, parathyroid glands and ultimobranchial bodies, which are derived from the 3rd and 4th pharyngeal pouches. They also display preaxial polydactyly of fore- and hind-limbs. These mice display post-natal lethality, and inability to feed owing to a cleft palate. While *Pax9*$^{+/-}$ mutants did not exhibit any overt defects, a hypomorphic allele, *Pax9*^{neo} showed that *Pax9* is haploinsufficient for tooth development, but not for other structures [30, 53].

Considering the overlapping expression domains in the vertebral structures, compound mutants of *Pax1* and *Pax9* were generated [52]. Increasing severity in vertebral column defects was observed with successive loss of *Pax1* and *Pax9* alleles. The most severe phenotype was displayed by *Pax1*$^{-/-}$*Pax9*$^{-/-}$ mutants that exhibited a complete loss of VB and IVDs, no caudal vertebrae and malformed proximal parts of the ribs. These vertebral column abnormalities, however, were more severe than those seen in individual null mutants of *Pax1* and *Pax9*, indicating their synergistic roles in the vertebral column. The lack of vertebral elements did

not result from lack of sclerotome specification, since sclerotomal cells were present in compound mutants, albeit in reduced numbers. Therefore it was hypothesized that *Pax1*/*Pax9* are required to maintain the proliferative capacity of the sclerotomal cells. Intriguingly, it was discovered that *Pax9* was unable to fully compensate for the loss of *Pax1* but *Pax1* could fully rescue *Pax9* deficiency in the axial skeleton. Notably, *Pax1* was unable to rescue orofacial defects seen in *Pax9*-null mutants since *Pax1* is not expressed in the dental primordia [52].

From these studies and others from our lab, it became evident that *Pax1*/*Pax9* have dual roles in axial skeletogenesis: (1) they maintain sclerotome cells in sufficient numbers and in appropriate locations for IVD anlagen formation through the regulation of proliferation and cell migration; (2) they contribute to the IVD mesenchymal condensation process through the activation of early chondrogenic genes (*Sox5, Bmp4, Co2a1, Acan, Wwp2*), likely in conjunction with *Sox* trio, TGF-b and BMP pathways. In fact, we will observe in the later parts of this chapter that proliferation, migration and mesenchymal condensation are fundamental functions of *Pax1* and *Pax9*, themes which will be replayed in the development of dental mesenchyme and thymus.

A certain number of sclerotomal cells are necessary for a critical size of condensation to form, upon which endochondral ossification can occur. As mentioned earlier, *Pax1* is known to genetically interact with *Mfh1*, another TF expressed in the sclerotome, to synergistically control sclerotome proliferation [35]. Indeed, regulation of proliferation could be a general conserved function among *Pax* genes; *Pax5* is known to regulate B cell proliferation and *Pax6* diencephalic precursor cells proliferation [54, 55]. We further confirmed a role for *Pax1*/*Pax9* in cell proliferation through a combinatorial approach of performing transcriptomic profiling on *Pax1*- and *Pax9*-specific cells and identifying the direct binding targets using Chromatin immunoprecipitation sequencing (ChIP-seq) [23]. Befitting their dosage effect on axial skeletogenesis, increasing numbers of targets were dysregulated with increasing loss of *Pax1* and *Pax9* alleles. Especially, a substantial number of genes associated with proliferation were affected only upon the loss of three ($Pax1^{+/-}Pax9^{-/-}$ and $Pax1^{-/-}Pax9^{+/-}$) or four ($Pax1^{-/-}Pax9^{-/-}$) alleles of *Pax1*/*Pax9* compared to the loss of two alleles ($Pax1^{-/-}$). Corroborating this, phenotypical decrease in the number of sclerotomal cells was more apparent in mutants with the loss of three or four alleles [23].

Besides proliferation, *Pax1* and *Pax9* also have roles in cell motion, adhesion and mesenchymal condensation through extracellular matrix (ECM) organization. Sclerotomal cells become mislocalized to the lateral sides in E14.5 $Pax1^{-/-}Pax9^{-/-}$ embryos; a defect not observed in $Pax1^{-/-}$ mutants. Cellular motion associated genes were also dramatically affected in the double null mutants, thus affirming the role of *Pax1* and *Pax9* in regulating cell motion [23].

The cell-type-specific molecular approach also revealed novel functions of *Pax1*/*Pax9* in regulating genes associated with collagen fibrillogenesis and cartilage development independent of *Sox9*, like *Col2a1, Bmp4, Acan, Sox5* and *Wwp2*. *Col2a1, Wwp2* and *Sox5* are also directly regulated by Pax9 in the vertebral column, and a single copy of *Pax1* or *Pax9* can independently maintain transcription of these critical IVD genes [23]. Additionally, Pax1 has been shown to induce *Acan* in chick presomitic mesoderm explants, independent of *Shh* [56]. A further confirmation of genetic linkage of these genes with *Pax1*/*Pax9* is that knock-out mouse mutants of

	Gene	Expression sites in developing embryo	Function	References
1	*Col2a1*, Collagen Type II, apha 1	(1) Sclerotome (2) Vertebral, intervertebral disc, tail, limb and craniofacial cartilage condensations (3) Limb, head and shoulder mesenchyme	(1) Major ECM component of cartilage (2) Collagen fibrillogenesis (3) Cartilage development (4) TGF-beta tethering in extracellular matrix (ECM) to modulate its signaling.	[23, 52, 57, 61–63]
2	*Acan*, Aggrecan	(1) Vertebral, intervertebral disc, tail, limb and craniofacial cartilage condensations (2) Limb, head, nasal mesenchyme	(1) Major ECM component of cartilage (2) Cartilage development (3) Water retention and maintain osmotic pressure in cartilage	[61–63]
3	*Sox5*, SRY-box-containing gene 5	(1) Vertebral, intervertebral disc, tail, limb and craniofacial cartilage condensations (2) Forebrain	(1) ECM synthesis (2) Cartilage development (3) Chondrocyte differentiation	[23, 60–65]
4	*Wwp2*, WW domain containing E3 ubiquitin	(1) Maxilla and mandible (2) Vertebral and intervertebral disc condensations	(1) Ubiquitylation of proteins (2) Mono-ubiquitylates Sox9 and enhances its transcriptional activity (3) Forms a complex with Sox9: Sox9-Wwp2-Med25 complex which drives *Col2a1* expression. (4) Palatogenesis	[23, 62, 67]
5	*Bmp4*, Bone morphogenetic protein 4	(1) Limb and head mesenchyme (2) Nasal pit epithelium (3) Vertebrae and intervertebral disc cartilage condensations (4) Dental and palatal mesenchyme	(1) Growth factor to activate BMP signaling (2) BMP signaling promotes ECM production and chondrocyte proliferation (3) Cartilage development and chondrocyte differentiation (4) Bmp4 up-regulates cartilage marker genes likes *Acan, Sox5, Sox6* and *Sox9*.	[23, 62, 68, 71]

Abbreviation(s): ECM, extracellular matrix.

Table 1. Expression sites and functions of selected *Pax1*/*Pax9* downstream targets essential in axial skeletogenesis.

Col2a1 [57], *Acan* [58], *Wwp2* [59] and *Sox5* [60] exhibit axial skeletal and craniofacial defects that phenocopy $Pax1^{-/-}Pax9^{-/-}$ mutants (**Table 1**) [52, 57, 61–68].

Importantly, *Pax1*/*Pax9* and *Sox5*/*Sox6* were linked by a negative feedback loop in the vertebral column. This *Pax-Sox* network might be essential in the segregation of IAF and OAF. *Sox5*

and *Sox6* play redundant but vital roles in IVD morphogenesis by regulating the timely maturation of chondroblasts and promoting inner annulus differentiation [60]. They are known to regulate ECM genes *Col2a1* and *Acan* in conjunction with Sox9 as *Sox* trio (*Sox5*/*Sox6*/*Sox9*) [60, 63, 66, 69]. On the other hand, *Pax1* and *Pax9* are down-regulated during the maturation of pre-chondrogenic cells into chondrocytes in the IAF and become restricted to the fibrotic OAF. Cell-type-specific analysis of EGFP-targeted $Sox5^{-/-}Sox6^{-/-}$ mutants (generated in our lab by a similar strategy as the *Pax1*/*Pax9* alleles) revealed that *Sox5*/*Sox6* repressed *Pax1*, while *Pax1*/*Pax9* positively regulated *Sox5* in the IVD anlagen cells [70]. This negative feedback circuit between *Pax* and *Sox* could therefore explain the initial co-expression of *Sox* and *Pax* in the IVD mesenchyme at E12.5-E13.5, and the subsequent restriction of *Pax1*/*Pax9* to the OAF by E15.5 [23].

Pax1 and *Pax9* also have a subsequent role in IVD differentiation through their connection with *Sox5*/*Sox6*, BMP and TGF-b pathways. First, TGF-b and BMP components - *Smad3*, *Tgfbr2*, *Tgfb3* and *Bmp4* are all expressed in the IVD anlagen at E12.5 and become restricted to the OAF by E14.5 [23, 71]. Second, TGF-b signaling is essential to maintain the boundary between VB and IVD, by preventing the inappropriate chondrogenic differentiation in the future IVD segment of the sclerotome and promoting annulus fibrosus development of the IVD [71–73]. Conversely, BMP signaling promotes chondrogenic differentiation of sclerotome cells by regulating the *Sox* trio and cartilage genes (*Acan* and *Wwp2*) [71]. Third, *Pax1*/*Pax9* regulate *Bmp4* and BMP- and TGF-b- regulated targets in the IVD anlagen (**Figure 3**). The continued expression of *Pax1*/*Pax9, Bmp4* and TGF-b pathway components in the OAF at E14.5 suggests their involvement in further differentiation of the OAF [23].

In terms of compensatory roles, compared to *Pax9*, *Pax1* is the more dominant player in axial skeleton development. The primary reason is that *Pax1* has the ability to fully compensate for *Pax9* deficiency in the vertebral column, by up-regulating its own expression through auto-regulation. $Pax9^{-/-}$ mutants show upregulated *Pax1* expression. The inverse, however, is not true as *Pax9* is incapable of upregulating itself in $Pax1^{-/-}$, thus being unable to match the dosage required to rescue *Pax1* function [23, 30, 52]. While dosage may partly explain the defect, the high homology shared between the PD of Pax1 and Pax9 makes one wonder if Pax9 can truly regulate all of the Pax1 targets if knocked into the *Pax1* locus. In fact, *Pax1* and *Pax9* can independently regulate some of the same set of critical IVD genes (e.g. *Sox5*, *Col2a1* and *Wwp2*). Thus, a *Pax9*-knock-in to *Pax1* locus would abrogate any temporal and spatial differences between *Pax1* and *Pax9*, and allow us to investigate if *Pax9* is truly capable of performing the functions of *Pax1* or if both inherently regulate different set of targets.

In humans, *PAX1* and *PAX9* have been linked to Jarcho-Levine and Klippel-Feil syndromes, characterized by vertebral anomalies such as kyphoscoliosis or vertebral segmentation defects that phenocopy $Pax1^{-/-}Pax9^{-/-}$ mouse mutants [4, 74, 75]. Indeed, several of the *Pax1*/*Pax9* regulated genes have been associated with similar axial skeleton defects [23]. Of these, mutations in *ACAN* have been linked to spondyloepiphyseal dysplasia (SEMD) and mutations in *COL2A1* is responsible for certain forms of SEMD [76, 77]. Identification of *Pax1*/*Pax9* as upstream regulators of these genes suggests that dysregulation of *PAX1*/*PAX9* function can reduce the levels of downstream targets like *Acan* and *Col2a1* which in turn lead to vertebral anomalies.

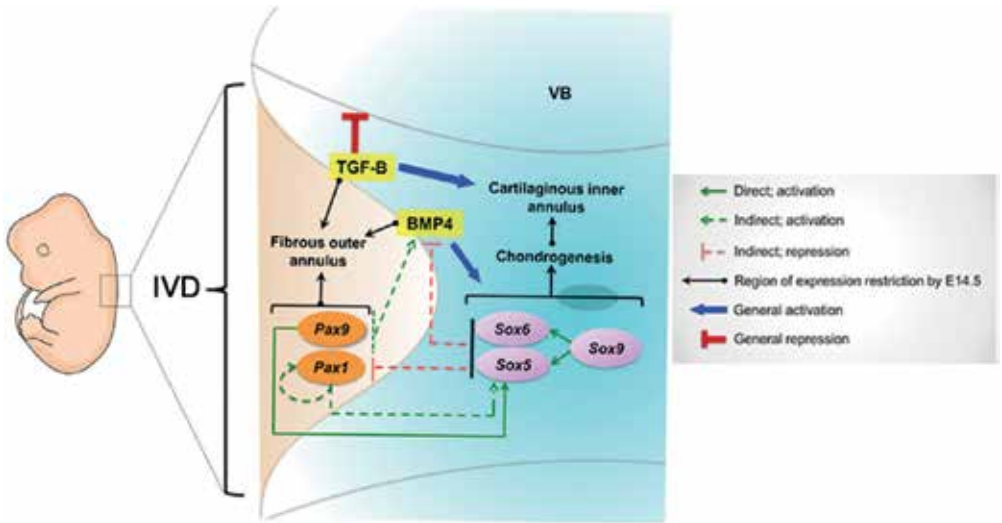

Figure 3. Schematic of *Pax-Sox*-TGFb-BMP4 network in the development of embryonic IVD. TGF-b signaling maintains the boundary between vertebral body (VB) and intervertebral disc (IVD), by preventing the inappropriate chondrogenic differentiation in the future IVD segment. *Bmp4* is regulated by *Pax1*/*Pax9* and the *Sox* trio. *Bmp4* itself regulates the *Sox* trio. The negative feedback loop mechanism between *Pax1*/*Pax9* and *Sox5*/*Sox6*, and their connection to *Bmp4* is postulated to be essential in the segregation of IAF and OAF during IVD development. At E14.5, expression of *Bmp4*, *Pax1*, *Pax9* and *Tgfb3* are restricted to the OAF while the *Sox* trio is retained in the IAF. Abbreviations: VB, vertebral body; IVD, intervertebral disc; TGF-b, transforming growth factor, beta; BMP4, bone morphogenetic protein 4.

5.2. *Pax1* and *Pax9* in pharyngeal-derived tissues

The pharyngeal endodermal pouches (Pp) are pockets that develop successively from the foregut endoderm in a rostro-caudal fashion. They are depressions found in between the branchial/pharyngeal arches which form in the cranial lateral parts of the embryo. These Pp and arches encompass cells derived from the three different germ layers – ectoderm, endoderm and mesoderm- as well as neural crest-derived mesenchyme [78, 79].

Each Pp gives rise to different craniofacial and glandular structures. The 1st Pp (Pp1) gives rise to the maxillary and mandibular structures, 3rd Pp (Pp3) gives rise to thymus and parathyroid glands, and the 4th Pp (Pp4) gives rise to the ultimobranchial bodies which subsequently give rise to thyroid C cells. The 2nd Pp (Pp2) is known to give rise to the palatine tonsil epithelium in all mammals except rodents, and in non-mammals (e.g. avian) it is suspected to give rise to salivary glands, although the latter remains to be investigated in more species [78, 79].

5.2.1. Pax9 in palatogenesis

Pp1-derived maxillary and mandibular prominence are the foundation structures for proper palatogenesis and odontogenesis. The shared developmental ontology of palate and teeth thus result in the co-occurrence of orofacial clefts and tooth agenesis when genes underlying Pp development are disrupted [80]. In fact, the molecular networks that regulate palatogenesis

and odontogenesis share mostly the same set of genes, although the hierarchy and connections between them is tissue-dependent.

Palate, the roof of the mouth, is the structure that helps separate the nasal from the oral cavity. It consists of the anterior hard palate and posterior soft palate. The primary palate forms the anterior portion, and is derived from the medial nasal process. The pair of medial outgrowth of the maxillary processes form the palatal shelves which elevate horizontally above the dorsum of the tongue and fuse to form the secondary palate [81]. *Pax9* is expressed in the neural crest-derived medial nasal process at E10.5, which subsequently develops into the maxillary prominence (upper jaw). *Pax9* then begins to be expressed in the palatal shelf mesenchyme at E12.5 onwards in a posterior-to-anterior gradient.

Pax9$^{-/-}$ mutants exhibit deficiency in primary palate outgrowth. Also, their palatal shelves are abnormally shaped and fail to elevate, resulting in failure of palatal fusion [30, 82]. Conditional knock-out of *Pax9* specifically in neural crest cells (*Pax9flox/Wnt1-Cre*) showed definitive proof that defects in the neural crest-derived mesenchymal components are the underlying basis for the palatal defects seen in *Pax9*-null mutants [83]. Disrupted anterior-posterior (A/P) patterning of the palatal shelves and decreased posterior palate mesenchymal proliferation are believed to be underlying cause of the palate defects in *Pax9*-null mutants [82].

Current studies begin to reveal a molecular network involving *Pax9, Msx1, Bmp4, Osr2, Fgf10* and *Shh* in palatogenesis. In *Pax9*-deficient mutants, *Shh* in the palatal epithelium and rugae, and *Msx1*, *Bmp4*, *Osr2* and *Fgf10* in the palate mesenchyme were all reduced, indicating *Pax9* is located upstream of these factors in the network hierarchy. Studies suggest that *Pax9* modulates A/P patterning through the *Bmp4*/*Shh* axis, and palate growth and elevation through *Osr2*/*Fgf10*/*Shh* cascade, whereby both *Shh* and *Pax9* independently regulate *Osr2* (**Figure 4**) [81, 82, 84, 85]. A more recent study has shown the involvement of Wnt signaling downstream of *Pax9* to play a role in palate elevation as well. How these multiple factors are integrated in this complex morphogenetic process remains to be fully understood. Especially, we still lack information on which targets are directly regulating each other and how these networks are integrated at a single cell level.

Contrary to *Pax9, Pax1* is not expressed in the dental and palatal mesenchyme. This explains the differential phenotypic abnormalities seen in *Pax1*$^{-/-}$ vs. *Pax9*$^{-/-}$ mutants. *Pax1*$^{-/-}$ mutants never exhibit the striking craniofacial defects - cleft secondary palate, defective primary palate and tooth agenesis seen in *Pax9*$^{-/-}$ mutants [9, 30]. *Pax1*, however, is expressed in a different domain of the facial mesenchyme, but its function in this tissue remains to be investigated [16, 43].

5.2.2. Pax9 in odontogenesis

Even though anatomical differences exist between mouse and human odontogenesis, the genetic basis of tooth development is conserved between vertebrates [53]. In humans, among the orofacial developmental defects, two most common anomalies are tooth agenesis and orofacial clefts. Worldwide, about 1 in 1000 individuals suffers from oligodontia [86]. Dominant heterozygous mutations in PAX9 have been identified to be the underlying genetic cause

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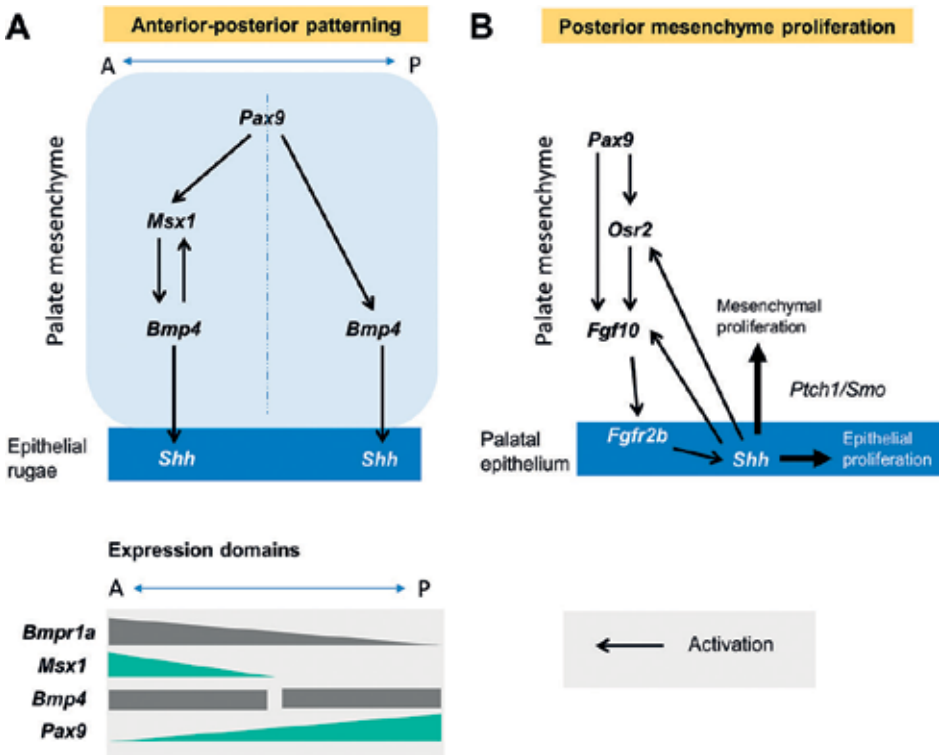

Figure 4. Pax9 molecular network in palatogenesis. (A) *Shh* expressing epithelial rugae serve as signaling centers for coordinating the A/P patterning and anterior outgrowth of the palate. *Shh* expression is maintained in the anterior epithelium by *Msx1*/*Bmp4* and in posterior epithelium by *Pax9*-mediated *Bmp4,* independent of *Msx1*. The expression level of the various factors in the anteroposterior axis is illustrated below. *Bmp4* expression was not detected in the mid-region of the palate at E12.5-E13.5 [82]. (B) *Pax9* regulates mesenchymal proliferation in the posterior palate through the *Osr2*/*Fgf10*/*Shh* axis. Shh also regulates *Osr2* independently. This mesenchymal-epithelial signaling in the palate is essential to maintain proliferation of both the mesenchyme and epithelium [85]. Abbreviations: A/P, anterior-posterior; A, anterior; P, posterior.

of non-syndromic forms of tooth agenesis in some cases [80]. Identification of the genetic cascade involved in odontogenesis in mouse will therefore greatly assist in rectifying tooth agenesis in humans. Elucidation of these pathways is also important for stem cell directed therapies for tooth agenesis.

Similar to the palate, dental mesenchyme is also derived from cranial neural crest cells, and so show defects in *Pax9*-null and *Pax9*-cKO mutants. After patterning during early embryonic stages, which determine the sites, size of tooth field and type of teeth that should develop, tooth development at the specified regions begins. Tooth development happens through a succession of morphogenetic changes and differentiation involving the proverbial epithelial-mesenchymal interactions for signal exchange - between the dental mesenchyme (of the Pp1-derived mandible and maxilla), and the overlying dental epithelium [87]. It involves a back-and-forth, dynamic "developmental power" shift between the epithelium and mesenchyme throughout development.

The epithelium and mesenchyme together go through a series of stages from the epithelial thickening (at E11.0) to bud (E13.5), cap (E14.5), bell (E16.5-E18.5) and tooth eruption stages. Tissue recombination experiments early on showed that oral epithelium from E9.0 to E11.5 possessed odontogenic potential to induce tooth development in the underlying non-dental, neural crest-derived mesenchyme, but not in the non-neural crest-derived limb mesenchyme [88, 89]. This tooth inductive potential then shifts to the dental mesenchyme. Indeed the dental mesenchyme was able to induce tooth development when combined with a non-oral epithelium, but the dental epithelium had lost this ability at E13.0. In a similar manner, at E14.5, the odontogenic potential shifts to the epithelial enamel knot, a transient signaling core that drives the progression from cap to bell stages [90].

In early tooth morphogenesis, *Pax9* is known to play dual roles in patterning the dental mesenchyme: (1) maintenance of *Bmp4* mesenchymal expression to drive tooth progression from bud to cap stage; (2) restricting *Msx1*/*Bmp4* signal mediated dental mesenchyme proliferation to the buccal side by maintaining *Osr2* expression on the lingual side.

Pax9 is not needed for tooth bud initiation, but is required for its subsequent progression to the cap stage. *Pax9* is initially induced in the dental mesenchyme of prospective molar and then incisor regions at E10.0 by diffusible FGF8 signals derived from the oral epithelium. In turn, *Pax9* expression is restricted to specific domains by the counter inhibition of Bmp4 from the epithelium and Bmp2 in the lateral mandibular mesenchyme [91]. Once initiated *Pax9* expression is maintained and is no longer dependent on inductive signals from the oral epithelium. *Pax9* remains expressed in tooth mesenchyme up to E16.5 performing its role in patterning, proliferation and condensation. Hypomorphic *Pax9* mutants revealed a gene-dosage dependency on *Pax9* for tooth formation. In these mice, decreased *Pax9* levels led to reduction in number of dental mesenchymal cells, hence defective mesenchymal condensation and subsequent developmental delay in molar development. However, *Pax9*-null mutants exhibit a dramatic phenotype where they lack all teeth [30, 53].

In vivo and *in vitro* studies revealed more complexity in the tooth morphogenetic process, involving a *Pax9*/*Msx1*/*Bmp4*/*Osr2* signaling axis [92, 93]. *Pax9*-null mutants showed reduced *Msx1*, *Bmp4* and *Osr2* expression in the dental mesenchyme suggesting that it is on top of the network hierarchy. In addition, *Pax9* and *Msx1* are co-expressed in the dental mesenchyme and synergistically regulate tooth development through *Bmp4*. Single homozygous mutants of $Pax9^{-/-}$ and $Msx1^{-/-}$ show cleft palate with arrested tooth development [30, 94]. *Msx1*-null mutants however showed reduction only in *Bmp4* but not in *Pax9* or *Osr2* [95]. Although *Pax9* is upstream of *Msx1*, it is not necessary for *Msx1* expression during tooth initiation at E12.5, but is required for its activation at later stages (E13.5-E14.5). In turn, both Pax9 and Msx1 interact at the protein level to synergistically drive *Bmp4* expression [92, 96], which appears to be primarily driven by the paired domain of Pax9. The epistatic relationship between *Pax9*, *Msx1* and *Bmp4* was further evident through the partial rescue of dentition defects in $Pax9^{+/-}Msx1^{+/-}$ mutants by re-expression of *Bmp4* [92].

BMP4 signaling is required downstream of *Pax9* and *Msx1* for tooth morphogenesis to progress from the bud to the cap stage, failure of which will result in tooth agenesis. Mice with

neural crest-specific inactivation of *Bmp4* (*Bmp4f/f;Wnt1Cre*) exhibit arrested development at the bud-stage in mandibular molar teeth [95]. While in early tooth initiation Bmp4 from the oral epithelium has a repressive role on *Pax9*, once *Pax9* expression becomes independent of epithelial signals, the *Bmp4*/*Pax9* hierarchy becomes inverted and *Bmp4* is no longer able to inhibit *Pax9*. Rather *Bmp4* expression becomes dependent on Pax9 and Msx1 [92].

Besides *Pax9*, another layer of patterning of the dental field is driven by *Osr2*, a negative regulator of odontogenic potential, mediated by its inhibition of *Bmp4* in the lingual region. Both *Osr2* and *Bmp4* are expressed in opposing gradients in the dental mesenchyme: *Osr2* is expressed in a lingual-buccal gradient while *Bmp4* is expressed in a buccal-lingual gradient. Moreover, $Osr2^{-/-}$ mutants exhibit supernumerary teeth lingual to molars. Genetic inactivation of *Osr2* in $Msx1^{-/-}$;$Bmp4^{cKO}$ mice rescued the dental defects. Additionally, *Bmp4* expression in the dental mesenchyme was rescued in the $Msx1^{-/-}Osr2^{-/-}$ mutants. Osr2 could stably interact with Msx1 at the protein level and weakly with Pax9, suggesting a potential competition between Osr2 and Pax9 in partnering with Msx1 to drive *Bmp4* expression [93]. These observations thus put forth a more defined but complex regulatory mechanism at play in the dental domain (**Figure 5**).

In humans, mutations in paired domain of *PAX9*, which in turn lead to defective PAX9 function, or mutations in the conserved regulatory elements of *PAX9*, which lead to reduced PAX9 levels, have been associated with autosomal dominant hypodontia [97–101]. In certain severe cases of non-syndromic oligodontia, the heterozygous deletion of *PAX9* locus, or mutations in

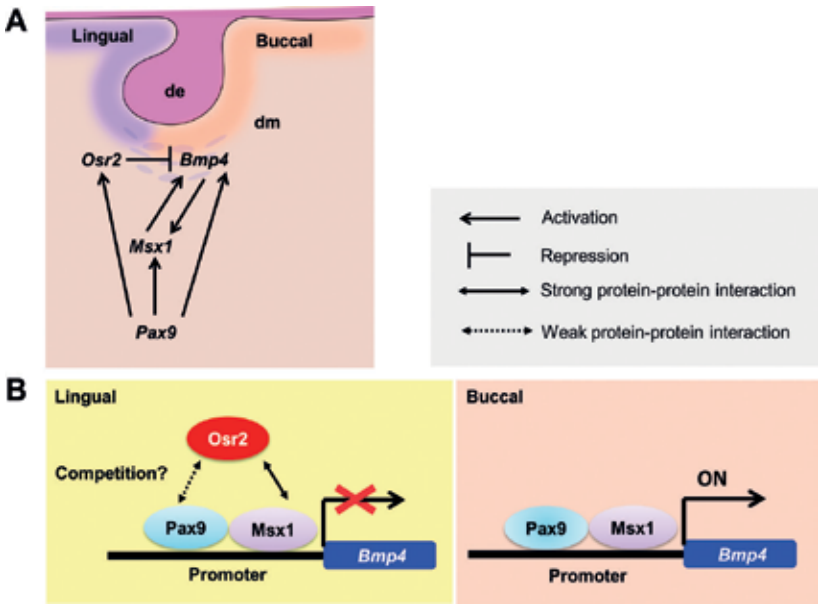

Figure 5. Pax9 molecular network in odontogenesis. (A) *Pax9* regulates a *Msx1*/*Bmp4* axis in the dental mesenchyme. *Osr2* expression in the lingual side restricts *Bmp4* to the buccal mesenchyme. *Pax9* is postulated to indirectly regulate *Osr2* expression. (B) Pax9 and Msx1 interaction at the protein level to regulate *Bmp4* expression. *In vitro*, Osr2 also has the ability to strongly bind to Msx1, and weakly to Pax9. Osr2 may compete with Pax9 for Msx1 to inhibit Bmp4 expression. Abbreviation(s): de, dental epithelium; dm, dental mesenchyme.

the initiation codon of *PAX9* have been noted [102, 103]. Considering the crucial role of *Pax9* in regulating BMP signaling for tooth morphogenesis to progress from bud to cap stage, it can be discerned that in humans with defective PAX9 function, tooth morphogenesis would be incomplete, resulting in missing teeth.

5.2.3. Pax1 and Pax9 in thymus development

The thymus is a bi-lobular epithelial organ surrounded by a mesenchymal capsule, located in the thoracic cavity. It is the niche site for T cell selection and maturation. The parathyroid glands, on the other hand, are endocrine glands located adjacent to the thyroid gland and produce parathyroid hormone for calcium homeostasis [104].

During development, the entire thymic epithelial component (cortical and medullary) is derived from the Pp3 endoderm. But proper formation of a functional thymus requires interaction with the surrounding neural crest-derived mesenchymal capsule [105, 106]. The mesenchymal capsule is essential for the proper thymic epithelial cell (TEC) proliferation and differentiation (by secreting FGF signals) and the collective migration of the thymic rudiment into their appropriate final location - the thoracic cavity, above the heart [107].

Although *Pax9* is known to be expressed in neural crest-derived mesenchyme, it has clear endodermal contributions for thymus development as it is expressed only in the endoderm-derived epithelium of the Pp [83, 108]. Unlike the sclerotome, *Pax9* is first expressed in the Pp3 endoderm at E9.5, while *Pax1* is only weakly detected at this stage [31]. *Pax1* expression becomes stronger a day later at E10.5 along with *Pax9* [16, 31]. Both *Pax1* and *Pax9* are expressed in the E12.5 thymic anlagen and become restricted to the thymic cortical epithelial cells by E14.5 [31]. Their expression remains in a subset of cortical epithelial cells in adults [109]. Since both thymus and parathyroid glands are derived from the Pp3, their formation is closely interconnected during development and show defects in the absence of *Pax1* or *Pax9* [104].

Hoxa3 is the earliest known regulator of Pp patterning toward parathyroid and thymic fates [110]. Even though the thymus and parathyroid glands develop from the same primordium, parathyroid patterning is initiated by E9.5, marked by *Gcm2* expression, whereas thymus epithelium marker *Foxn1* is detected only around E11.0. While *Shh*/*Tbx1*/*Gcm2* pathways are essential for parathyroid patterning, the *Hoxa3*/*Pax1*/*9*/*Eya1*/*Six1*/*4* axis drives thymus anlage formation and patterning [109, 111]. The hierarchy of genes within the latter cascade however remains to be clarified. Both *Pax1* and *Pax9* are down-regulated in E10.5 thymic primordia in *Hoxa3*$^{-/-}$ mutants [112]. While *Hoxa3* is not essential for initiation of *Pax1* and *Pax9* in the primordium, it is essential for their maintenance later. *Pax1* and *Pax9* expression is normal in the *Eya1*-null and *Six1*/*Six4*-null mutants indicating they are upstream of *Eya1* and *Six1*/*Six4* in this cascade [113]. But this is complicated by the observation that *Eya1*$^{-/-}$*Six1*$^{-/-}$ mutants show reduced *Pax1* but unaltered *Pax9* expression [114]. Regardless of the hierarchy, it is clear that *Pax1* and *Pax9* have important roles in thymus/parathyroid development.

Pax1 loss of function mutants exhibit a hypoplastic thymus with defects in thymocyte maturation [8, 31]. Furthermore, *Hoxa3*$^{+/-}$*Pax1*$^{-/-}$ compound mutants show a more drastic thymus phenotype than single null mutants. They possess hypoplastic thymi that are ectopically located due to delay in separation from the pharynx, indicating that *Hoxa3* and *Pax1* genetically interact and synergize to regulate proliferation of the thymus primordium [115].

A more drastic phenotype has been described for *Pax9* targeted-null mutants, whereby all the Pp3 and Pp4 derivatives - the entire thymus, parathyroid gland and ultimobranchial bodies – are absent [30]. However a subsequent study showed that *Pax9*$^{-/-}$ mutants indeed possess a hypoplastic, rudimentary thymic structure, colonized by T cell precursors, albeit ectopically localized in the larynx owing to failure of separation from the pharynx [116]. Furthermore, *Pax9* mutants exhibit defects in certain lymphocyte (T cell) subtypes. These data indicated that *Pax9* is not necessary for thymic primordium formation, but essential for its correct localization and normal thymopoiesis [116].

6. Conclusion

Accumulating evidences suggest the emergence of a central role of *Pax1* and *Pax9* in cell proliferation, cell motility and ECM regulation for condensation. Despite increasing knowledge of how these two TFs are interconnected with other factors, a myriad of questions still remain unanswered. For example, what tissue-restricted co-factors do Pax1 and Pax9 interact with to regulate the formation of axial skeleton and pharyngeal-derived tissues? If the PD of Pax1 and Pax9 are highly conserved, can Pax1 compensate for Pax9 and *vice versa* in the above-mentioned tissues if knocked-into the locus of its paralog? Furthermore, substantial progress in understanding the thymic and parathyroid development remains to be made. The exact molecular mechanisms of *Pax1* and *Pax9* initiation and their downstream targets are yet unknown in these tissues. Future studies on enriched specific cell-types and emerging state-of-the-art technologies will allow us to interrogate these questions at a single-cell resolution. High throughput technologies such as single cell transcriptomics, spatial transcriptomics (FISSEQ, MERFISH), multi-parameter profiling of proteins at single cell (CyTOF) and spatial levels (Imaging mass cytometry) will help to elucidate the pathways and the regulatory networks governing the development of these tissues [117–120]. These technologies in combination with ChIP-seq and utilization of the various gene-targeted mouse models will help to accelerate our understanding of these factors and their gene regulatory networks in the years to come.

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References

[1] Arnone MI, Davidson EH. The hardwiring of development: Organization and function of genomic regulatory systems. Development. 1997;**124**:1851-1864

[2] Walhout AJ. Unraveling transcription regulatory networks by protein-DNA and protein-protein interaction mapping. Genome Research. 2006;**16**:1445-1454. DOI: 10.1101/gr.5321506

[3] del Sol A, Balling R, Hood L, Galas D. Diseases as network perturbations. Current Opinion in Biotechnology. 2010;**21**:566-571. DOI: 10.1016/j.copbio.2010.07.010

[4] Bannykh SI, Emery SC, Gerber JK, Jones KL, Benirschke K, Masliah E. Aberrant Pax1 and Pax9 expression in Jarcho-Levin syndrome: Report of two Caucasian siblings and literature review. American Journal of Medical Genetics. Part A. 2003;**120A**:241-246. DOI: 10.1002/ajmg.a.20192

[5] Giampietro PF, Raggio CL, Reynolds CE, Shukla SK, McPherson E, Ghebranious N, et al. An analysis of PAX1 in the development of vertebral malformations. Clinical Genetics. 2005;**68**:448-453. DOI: 10.1111/j.1399-0004.2005.00520.x

[6] Bonczek O, Balcar VJ, Sery O. PAX9 gene mutations and tooth agenesis: A review. Clinical Genetics. 2017;467-476. DOI: 10.1111/cge.12986

[7] Lee JK, Park JW, Kim YH, Baek SH. Association between PAX9 single-nucleotide polymorphisms and nonsyndromic cleft lip with or without cleft palate. The Journal of Craniofacial Surgery. 2012;**23**:1262-1266. DOI: 10.1097/SCS.0b013e31824e27c7

[8] Dietrich S, Gruss P. undulated phenotypes suggest a role of Pax-1 for the development of vertebral and extravertebral structures. Developmental Biology. 1995;**167**:529-548. DOI: 10.1006/dbio.1995.1047

[9] Wilm B, Dahl E, Peters H, Balling R, Imai K. Targeted disruption of Pax1 defines its null phenotype and proves haploinsufficiency. Proceedings of the National Academy of Sciences of the United States of America. 1998;**95**:8692-8697

[10] Aubin J, Lemieux M, Moreau J, Lapointe J, Jeannotte L. Cooperation of Hoxa5 and Pax1 genes during formation of the pectoral girdle. Developmental Biology. 2002;**244**:96-113. DOI: 10.1006/dbio.2002.0596

[11] LeClair EE, Bonfiglio L, Tuan RS. Expression of the paired-box genes Pax-1 and Pax-9 in limb skeleton development. Developmental Dynamics. 1999;**214**:101-115. DOI: 10.1002/(SICI)1097-0177(199902)214:2<101:AID-AJA1>3.0.CO;2-4

[12] Hofmann C, Drossopoulou G, McMahon A, Balling R, Tickle C. Inhibitory action of BMPs on Pax1 expression and on shoulder girdle formation during limb development. Developmental Dynamics. 1998;**213**:199-206. DOI: 10.1002/(SICI)1097-0177(199810)213:2<199:AID-AJA5>3.0.CO;2-B

[13] Bopp D, Burri M, Baumgartner S, Frigerio G, Noll M. Conservation of a large protein domain in the segmentation gene paired and in functionally related genes of Drosophila. Cell. 1986;**47**:1033-1040

[14] Short S, Holland LZ. The evolution of alternative splicing in the Pax family: The view from the Basal chordate amphioxus. Journal of Molecular Evolution. 2008;**66**:605-620. DOI: 10.1007/s00239-008-9113-5

[15] Paixao-Cortes VR, Salzano FM, Bortolini MC. Origins and evolvability of the PAX family. Seminars in Cell & Developmental Biology. 2015;**44**:64-74. DOI: 10.1016/j.semcdb.2015.08.014

[16] Neubuser A, Koseki H, Balling R. Characterization and developmental expression of Pax9, a paired-box-containing gene related to Pax1. Developmental Biology. 1995;**170**:701-716. DOI: 10.1006/dbio.1995.1248

[17] Walther C, Guenet JL, Simon D, Deutsch U, Jostes B, Goulding MD, et al. Pax: A murine multigene family of paired box-containing genes. Genomics. 1991;**11**:424-434

[18] Stapleton P, Weith A, Urbanek P, Kozmik Z, Busslinger M. Chromosomal localization of seven PAX genes and cloning of a novel family member, PAX-9. Nature Genetics. 1993;**3**:292-298. DOI: 10.1038/ng0493-292

[19] Czerny T, Schaffner G, Busslinger M. DNA sequence recognition by Pax proteins: Bipartite structure of the paired domain and its binding site. Genes & Development. 1993;**7**:2048-2061

[20] Chalepakis G, Fritsch R, Fickenscher H, Deutsch U, Goulding M, Gruss P. The molecular basis of the undulated/Pax-1 mutation. Cell. 1991;**66**:873-884

[21] Jun S, Desplan C. Cooperative interactions between paired domain and homeodomain. Development. 1996;**122**:2639-2650

[22] Xu W, Rould MA, Jun S, Desplan C, Pabo CO. Crystal structure of a paired domain-DNA complex at 2.5 A resolution reveals structural basis for Pax developmental mutations. Cell. 1995;**80**:639-650

[23] Sivakamasundari V, Kraus P, Sun W, Hu X, Lim SL, Prabhakar S, et al. A developmental transcriptomic analysis of Pax1 and Pax9 in embryonic intervertebral disc development. Biology Open. 2017;**6**:187-199. DOI: 10.1242/bio.023218

[24] Epstein JA, Glaser T, Cai J, Jepeal L, Walton DS, Maas RL. Two independent and interactive DNA-binding subdomains of the Pax6 paired domain are regulated by alternative splicing. Genes & Development. 1994;**8**:2022-2034

[25] Kozmik Z, Czerny T, Busslinger M. Alternatively spliced insertions in the paired domain restrict the DNA sequence specificity of Pax6 and Pax8. The EMBO Journal. 1997;**16**:6793-6803. DOI: 10.1093/emboj/16.22.6793

[26] Tan B, Wang J, Song Q, Wang N, Jia Y, Wang C, et al. Prognostic value of PAX9 in patients with esophageal squamous cell carcinoma and its prediction value to radiation sensitivity. Molecular Medicine Reports. 2017;**16**:806-816. DOI: 10.3892/mmr.2017.6626

[27] Hsu DS, Acharya CR, Balakumaran BS, Riedel RF, Kim MK, Stevenson M, et al. Characterizing the developmental pathways TTF-1, NKX2-8, and PAX9 in lung cancer. Proceedings of the National Academy of Sciences of the United States of America. 2009;**106**:5312-5317. DOI: 10.1073/pnas.0900827106

[28] Luan T, Hua Q, Liu X, Xu P, Gu Y, Qian H, et al. PAX1 methylation as a potential biomarker to predict the progression of cervical intraepithelial neoplasia: A meta-analysis of related studies. International Journal of Gynecological Cancer. 2017;**27**:1480-1488. DOI: 10.1097/IGC.0000000000001011

[29] Strachan T, Read AP. PAX genes. Current Opinion in Genetics & Development. 1994; **4**:427-438

[30] Peters H, Neubuser A, Kratochwil K, Balling R. Pax9-deficient mice lack pharyngeal pouch derivatives and teeth and exhibit craniofacial and limb abnormalities. Genes & Development. 1998;**12**:2735-2747

[31] Wallin J, Eibel H, Neubuser A, Wilting J, Koseki H, Balling R. Pax1 is expressed during development of the thymus epithelium and is required for normal T-cell maturation. Development. 1996;**122**:23-30

[32] Paaby AB, Rockman MV. The many faces of pleiotropy. Trends in Genetics. 2013;**29**: 66-73. DOI: 10.1016/j.tig.2012.10.010

[33] Raj PP. Intervertebral disc: Anatomy-physiology-pathophysiology-treatment. Pain Practice. 2008;**8**:18-44. DOI: 10.1111/j.1533-2500.2007.00171.x

[34] Fan CM, Tessier-Lavigne M. Patterning of mammalian somites by surface ectoderm and notochord: Evidence for sclerotome induction by a hedgehog homolog. Cell. 1994;**79**:1175-1186

[35] Furumoto TA, Miura N, Akasaka T, Mizutani-Koseki Y, Sudo H, Fukuda K, et al. Notochord-dependent expression of MFH1 and PAX1 cooperates to maintain the proliferation of sclerotome cells during the vertebral column development. Developmental Biology. 1999;**210**:15-29. DOI: 10.1006/dbio.1999.9261

[36] Johnson RL, Laufer E, Riddle RD, Tabin C. Ectopic expression of Sonic hedgehog alters dorsal-ventral patterning of somites. Cell. 1994;**79**:1165-1173

[37] Koseki H, Wallin J, Wilting J, Mizutani Y, Kispert A, Ebensperger C, et al. A role for Pax-1 as a mediator of notochordal signals during the dorsoventral specification of vertebrae. Development. 1993;**119**:649-660

[38] DiPaola CP, Farmer JC, Manova K, Niswander LA. Molecular signaling in intervertebral disk development. Journal of Orthopaedic Research. 2005;**23**:1112-1119. DOI: 10.1016/j.orthres.2005.03.008

[39] Wallin J, Wilting J, Koseki H, Fritsch R, Christ B, Balling R. The role of Pax-1 in axial skeleton development. Development. 1994;**120**:1109-1121

[40] Christ B, Wilting J. From somites to vertebral column. Annals of Anatomy. 1992;**174**:23-32

[41] Brand-Saberi B, Christ B. Evolution and development of distinct cell lineages derived from somites. Current Topics in Developmental Biology. 2000;**48**:1-42

[42] Sivakamasundari V, Lufkin T. Bridging the gap: Understanding embryonic intervertebral disc development. Cell & Developmental Biology. 2012;**1**(2). pii: 103. PMID: 23106046

[43] Sivakamasundari V, Kraus P, Jie S, Lufkin T. Pax1(EGFP): New wildtype and mutant EGFP mouse lines for molecular and fate mapping studies. Genesis. 2013;**51**:420-429. DOI: 10.1002/dvg.22379

[44] McMahon JA, Takada S, Zimmerman LB, Fan CM, Harland RM, McMahon AP. Noggin-mediated antagonism of BMP signaling is required for growth and patterning of the neural tube and somite. Genes & Development. 1998;**12**:1438-1452

[45] Chiang C, Litingtung Y, Lee E, Young KE, Corden JL, Westphal H, et al. Cyclopia and defective axial patterning in mice lacking Sonic hedgehog gene function. Nature. 1996;**383**:407-413. DOI: 10.1038/383407a0

[46] Capellini TD, Zewdu R, Di Giacomo G, Asciutti S, Kugler JE, Di Gregorio A, et al. Pbx1/Pbx2 govern axial skeletal development by controlling Polycomb and Hox in mesoderm and Pax1/Pax9 in sclerotome. Developmental Biology. 2008;**321**:500-514. DOI: 10.1016/j.ydbio.2008.04.005

[47] Mankoo BS, Skuntz S, Harrigan I, Grigorieva E, Candia A, Wright CV, et al. The concerted action of Meox homeobox genes is required upstream of genetic pathways essential for the formation, patterning and differentiation of somites. Development. 2003;**130**:4655-4664. DOI: 10.1242/dev.00687

[48] Balling R, Deutsch U, Gruss P. undulated, a mutation affecting the development of the mouse skeleton, has a point mutation in the paired box of Pax 1. Cell. 1988;**55**:531-535

[49] Blandova, Y.R., and Egorov, I.U. Sut allelic with un. Mouse News Letters. 1975;**52**:43

[50] Wallace ME. An inherited agent of mutation with chromosome damage in wild mice. The Journal of Heredity. 1985;**76**:271-278

[51] Adham IM, Gille M, Gamel AJ, Reis A, Dressel R, Steding G, et al. The scoliosis (sco) mouse: A new allele of Pax1. Cytogenetic and Genome Research. 2005;**111**:16-26. DOI: 10.1159/000085665

[52] Peters H, Wilm B, Sakai N, Imai K, Maas R, Balling R. Pax1 and Pax9 synergistically regulate vertebral column development. Development. 1999;**126**:5399-5408

[53] Kist R, Watson M, Wang X, Cairns P, Miles C, Reid DJ, et al. Reduction of Pax9 gene dosage in an allelic series of mouse mutants causes hypodontia and oligodontia. Human Molecular Genetics. 2005;**14**:3605-3617. DOI: 10.1093/hmg/ddi388

[54] Wakatsuki Y, Neurath MF, Max EE, Strober W. The B cell-specific transcription factor BSAP regulates B cell proliferation. The Journal of Experimental Medicine. 1994; **179**:1099-1108

[55] Warren N, Price DJ. Roles of Pax-6 in murine diencephalic development. Development. 1997;**124**:1573-1582

[56] Rodrigo I, Hill RE, Balling R, Munsterberg A, Imai K. Pax1 and Pax9 activate Bapx1 to induce chondrogenic differentiation in the sclerotome. Development. 2003;**130**:473-482

[57] Aszodi A, Chan D, Hunziker E, Bateman JF, Fassler R. Collagen II is essential for the removal of the notochord and the formation of intervertebral discs. The Journal of Cell Biology. 1998;**143**:1399-1412

[58] Watanabe H, Nakata K, Kimata K, Nakanishi I, Yamada Y. Dwarfism and age-associated spinal degeneration of heterozygote cmd mice defective in aggrecan. Proceedings of the National Academy of Sciences of the United States of America. 1997;**94**:6943-6947

[59] Zou W, Chen X, Shim JH, Huang Z, Brady N, Hu D, et al. The E3 ubiquitin ligase Wwp2 regulates craniofacial development through mono-ubiquitylation of Goosecoid. Nature Cell Biology. 2011;**13**:59-65. DOI: 10.1038/ncb2134

[60] Smits P, Lefebvre V. Sox5 and Sox6 are required for notochord extracellular matrix sheath formation, notochord cell survival and development of the nucleus pulposus of intervertebral discs. Development. 2003;**130**:1135-1148

[61] Visel A, Thaller C, Eichele G. GenePaint.org: An atlas of gene expression patterns in the mouse embryo. Nucleic Acids Research. 2004;**32**:D552-D556. DOI: 10.1093/nar/gkh029

[62] Diez-Roux G, Banfi S, Sultan M, Geffers L, Anand S, Rozado D, et al. A high-resolution anatomical atlas of the transcriptome in the mouse embryo. PLoS Biology. 2011;**9**:e1000582. DOI: 10.1371/journal.pbio.1000582

[63] Han Y, Lefebvre V. L-Sox5 and Sox6 drive expression of the aggrecan gene in cartilage by securing binding of Sox9 to a far-upstream enhancer. Molecular and Cellular Biology. 2008;**28**:4999-5013. DOI: 10.1128/MCB.00695-08

[64] Smits P, Li P, Mandel J, Zhang Z, Deng JM, Behringer RR, et al. The transcription factors L-Sox5 and Sox6 are essential for cartilage formation. Developmental Cell. 2001;**1**: 277-290

[65] Lefebvre V, Behringer RR, de Crombrugghe B. L-Sox5, Sox6 and Sox9 control essential steps of the chondrocyte differentiation pathway. Osteoarthritis and Cartilage. 2001;**9**(Suppl A):S69-S75. PMID: 11680692. 89

[66] Lefebvre V, Li P, de Crombrugghe B. A new long form of Sox5 (L-Sox5), Sox6 and Sox9 are coexpressed in chondrogenesis and cooperatively activate the type II collagen gene. The EMBO Journal. 1998;**17**:5718-5733. DOI: 10.1093/emboj/17.19.5718

[67] Nakamura Y, Yamamoto K, He X, Otsuki B, Kim Y, Murao H, et al. Wwp2 is essential for palatogenesis mediated by the interaction between Sox9 and mediator subunit 25. Nature Communications. 2011;**2**:251. DOI: 10.1038/ncomms1242

[68] Takae R, Matsunaga S, Origuchi N, Yamamoto T, Morimoto N, Suzuki S, et al. Immunolocalization of bone morphogenetic protein and its receptors in degeneration of intervertebral disc. Spine (Phila Pa 1976). 1999;**24**:1397-1401

[69] Lefebvre V, Huang W, Harley VR, Goodfellow PN, de Crombrugghe B. SOX9 is a potent activator of the chondrocyte-specific enhancer of the pro alpha1(II) collagen gene. Molecular and Cellular Biology. 1997;**17**:2336-2346

[70] Lee WJ, Chatterjee S, Yap SP, Lim SL, Xing X, Kraus P, et al. An integrative developmental genomics and systems biology approach to identify an in vivo Sox trio-mediated gene regulatory network in murine embryos. BioMed Research International. 2017;**2017**:8932583. DOI: 10.1155/2017/8932583

[71] Sohn P, Cox M, Chen D, Serra R. Molecular profiling of the developing mouse axial skeleton: A role for Tgfbr2 in the development of the intervertebral disc. BMC Developmental Biology. 2010;**10**:29. DOI: 10.1186/1471-213X-10-29

[72] Baffi MO, Moran MA, Serra R. Tgfbr2 regulates the maintenance of boundaries in the axial skeleton. Developmental Biology. 2006;**296**:363-374. DOI: 10.1016/j.ydbio.2006.06.002

[73] Baffi MO, Slattery E, Sohn P, Moses HL, Chytil A, Serra R. Conditional deletion of the TGF-beta type II receptor in Col2a expressing cells results in defects in the axial skeleton without alterations in chondrocyte differentiation or embryonic development of long bones. Developmental Biology. 2004;**276**:124-142. DOI: 10.1016/j.ydbio.2004.08.027

[74] McGaughran JM, Oates A, Donnai D, Read AP, Tassabehji M. Mutations in PAX1 may be associated with Klippel-Feil syndrome. European Journal of Human Genetics. 2003;**11**:468-474. DOI: 10.1038/sj.ejhg.5200987

[75] Tracy MR, Dormans JP, Kusumi K. Klippel-Feil syndrome: Clinical features and current understanding of etiology. Clinical Orthopaedics and Related Research. 2004 Jul;**424**:183-90. PMID: 15241163

[76] Gleghorn L, Ramesar R, Beighton P, Wallis G. A mutation in the variable repeat region of the aggrecan gene (AGC1) causes a form of spondyloepiphyseal dysplasia associated with severe, premature osteoarthritis. American Journal of Human Genetics. 2005;**77**:484-490. DOI: 10.1086/444401

[77] Nakane T, Tando T, Aoyagi K, Hatakeyama K, Nishimura G, Coucke IP, et al. Dysspondyloenchondromatosis: Another COL2A1-related skeletal dysplasia? Molecular Syndromology. 2011;**2**:21-26. DOI: 10.1159/000333098

[78] Graham A, Smith A. Patterning the pharyngeal arches. BioEssays. 2001;**23**:54-61. DOI: 10.1002/1521-1878(200101)23:1<54:AID-BIES1007>3.0.CO;2-5

[79] Grevellec A, Tucker AS. The pharyngeal pouches and clefts: Development, evolution, structure and derivatives. Seminars in Cell & Developmental Biology. 2010;**21**:325-332. DOI: 10.1016/j.semcdb.2010.01.022

[80] Phan M, Conte F, Khandelwal KD, Ockeloen CW, Bartzela T, Kleefstra T, et al. Tooth agenesis and orofacial clefting: Genetic brothers in arms? Human Genetics. 2016;**135**:1299-1327. DOI: 10.1007/s00439-016-1733-z

[81] Lan Y, Xu J, Jiang R. Cellular and molecular mechanisms of palatogenesis. Current Topics in Developmental Biology. 2015;**115**:59-84. DOI: 10.1016/bs.ctdb.2015.07.002

[82] Zhou J, Gao Y, Lan Y, Jia S, Jiang R. Pax9 regulates a molecular network involving Bmp4, Fgf10, Shh signaling and the Osr2 transcription factor to control palate morphogenesis. Development. 2013;**140**:4709-4718. DOI: 10.1242/dev.099028

[83] Kist R, Greally E, Peters H. Derivation of a mouse model for conditional inactivation of Pax9. Genesis. 2007;**45**:460-464. DOI: 10.1002/dvg.20295

[84] Lan Y, Ovitt CE, Cho ES, Maltby KM, Wang Q, Jiang R. Odd-skipped related 2 (Osr2) encodes a key intrinsic regulator of secondary palate growth and morphogenesis. Development. 2004;**131**:3207-3216. DOI: 10.1242/dev.01175

[85] Rice R, Spencer-Dene B, Connor EC, Gritli-Linde A, McMahon AP, Dickson C, et al. Disruption of Fgf10/Fgfr2b-coordinated epithelial-mesenchymal interactions causes cleft palate. The Journal of Clinical Investigation. 2004;**113**:1692-1700. DOI: 10.1172/JCI20384

[86] Rakhshan V, Rakhshan H. Meta-analysis of congenitally missing teeth in the permanent dentition: Prevalence, variations across ethnicities, regions and time. International Orthodontics. 2015;**13**:261-273. DOI: 10.1016/j.ortho.2015.06.008

[87] Cobourne MT, Mitsiadis T. Neural crest cells and patterning of the mammalian dentition. Journal of Experimental Zoology. Part B, Molecular and Developmental Evolution. 2006;**306**:251-260. DOI: 10.1002/jez.b.21084

[88] Mina M, Kollar EJ. The induction of odontogenesis in non-dental mesenchyme combined with early murine mandibular arch epithelium. Archives of Oral Biology. 1987;**32**:123-127

[89] Lumsden AG. Spatial organization of the epithelium and the role of neural crest cells in the initiation of the mammalian tooth germ. Development. 1988;**103**(Suppl):155-169. PMID: 3250849

[90] Tucker A, Sharpe P. The cutting-edge of mammalian development; how the embryo makes teeth. Nature Reviews. Genetics. 2004;**5**:499-508. DOI: 10.1038/nrg1380

[91] Neubuser A, Peters H, Balling R, Martin GR. Antagonistic interactions between FGF and BMP signaling pathways: A mechanism for positioning the sites of tooth formation. Cell. 1997;**90**:247-255

[92] Ogawa T, Kapadia H, Feng JQ, Raghow R, Peters H, D'Souza RN. Functional consequences of interactions between Pax9 and Msx1 genes in normal and abnormal tooth development. The Journal of Biological Chemistry. 2006;**281**:18363-18369. DOI: 10.1074/jbc.M601543200

[93] Zhou J, Gao Y, Zhang Z, Zhang Y, Maltby KM, Liu Z, et al. Osr2 acts downstream of Pax9 and interacts with both Msx1 and Pax9 to pattern the tooth developmental field. Developmental Biology. 2011;**353**:344-353. DOI: 10.1016/j.ydbio.2011.03.012

[94] Satokata I, Maas R. Msx1 deficient mice exhibit cleft palate and abnormalities of craniofacial and tooth development. Nature Genetics. 1994;**6**:348-356. DOI: 10.1038/ng0494-348

[95] Jia S, Zhou J, Gao Y, Baek JA, Martin JF, Lan Y, et al. Roles of Bmp4 during tooth morphogenesis and sequential tooth formation. Development. 2013;**140**:423-432. DOI: 10.1242/dev.081927

[96] Ogawa T, Kapadia H, Wang B, D'Souza RN. Studies on Pax9-Msx1 protein interactions. Archives of Oral Biology. 2005;**50**:141-145. DOI: 10.1016/j.archoralbio.2004.09.011

[97] Nieminen P, Arte S, Tanner D, Paulin L, Alaluusua S, Thesleff I, et al. Identification of a nonsense mutation in the PAX9 gene in molar oligodontia. European Journal of Human Genetics. 2001;**9**:743-746. DOI: 10.1038/sj.ejhg.5200715

[98] Frazier-Bowers SA, Guo DC, Cavender A, Xue L, Evans B, King T, et al. A novel mutation in human PAX9 causes molar oligodontia. Journal of Dental Research. 2002;**81**:129-133

[99] Das P, Hai M, Elcock C, Leal SM, Brown DT, Brook AH, et al. Novel missense mutations and a 288-bp exonic insertion in PAX9 in families with autosomal dominant hypodontia. American Journal of Medical Genetics. Part A. 2003;**118A**:35-42. DOI: 10.1002/ajmg.a.10011

[100] Mendoza-Fandino GA, Gee JM, Ben-Dor S, Gonzalez-Quevedo C, Lee K, Kobayashi Y, et al. A novel g.-1258G>A mutation in a conserved putative regulatory element of PAX9 is associated with autosomal dominant molar hypodontia. Clinical Genetics. 2011;**80**:265-272. DOI: 10.1111/j.1399-0004.2010.01529.x

[101] Mues G, Kapadia H, Wang Y, D'Souza RN. Genetics and human malformations. The Journal of Craniofacial Surgery. 2009;**20**(Suppl 2):1652-1654. DOI: 10.1097/SCS.0b013e3181b2d3f3

[102] Das P, Stockton DW, Bauer C, Shaffer LG, D'Souza RN, Wright T, et al. Haploinsufficiency of PAX9 is associated with autosomal dominant hypodontia. Human Genetics. 2002;**110**:371-376. DOI: 10.1007/s00439-002-0699-1

[103] Klein ML, Nieminen P, Lammi L, Niebuhr E, Kreiborg S. Novel mutation of the initiation codon of PAX9 causes oligodontia. Journal of Dental Research. 2005;**84**:43-47. DOI: 10.1177/154405910508400107

[104] Gordon J, Manley NR. Mechanisms of thymus organogenesis and morphogenesis. Development. 2011;**138**:3865-3878. DOI: 10.1242/dev.059998

[105] Gordon J, Wilson VA, Blair NF, Sheridan J, Farley A, Wilson L, et al. Functional evidence for a single endodermal origin for the thymic epithelium. Nature Immunology. 2004;**5**:546-553. DOI: 10.1038/ni1064

[106] Blackburn CC, Manley NR. Developing a new paradigm for thymus organogenesis. Nature Reviews. Immunology. 2004;**4**:278-289. DOI: 10.1038/nri1331

[107] Foster KE, Gordon J, Cardenas K, Veiga-Fernandes H, Makinen T, Grigorieva E, et al. EphB-ephrin-B2 interactions are required for thymus migration during organogenesis. Proceedings of the National Academy of Sciences of the United States of America. 2010;**107**:13414-13419. DOI: 10.1073/pnas.1003747107

[108] Feng J, Jing J, Sanchez-Lara PA, Bootwalla MS, Buckley J, Wu N, et al. Generation and characterization of tamoxifen-inducible Pax9-CreER knock-in mice using CrispR/Cas9. Genesis. 2016;**54**:490-496. DOI: 10.1002/dvg.22956

[109] Manley NR, Condie BG. Transcriptional regulation of thymus organogenesis and thymic epithelial cell differentiation. Progress in Molecular Biology and Translational Science. 2010;**92**:103-120. DOI: 10.1016/S1877-1173(10)92005-X

[110] Manley NR, Capecchi MR. The role of Hoxa-3 in mouse thymus and thyroid development. Development. 1995;**121**:1989-2003

[111] Bain VE, Gordon J, O'Neil JD, Ramos I, Richie ER, Manley NR. Tissue-specific roles for sonic hedgehog signaling in establishing thymus and parathyroid organ fate. Development. 2016;**143**:4027-4037. DOI: 10.1242/dev.141903

[112] Chisaka O, Capecchi MR. Regionally restricted developmental defects resulting from targeted disruption of the mouse homeobox gene hox-1.5. Nature. 1991;**350**:473-479. DOI: 10.1038/350473a0

[113] Xu PX, Zheng W, Laclef C, Maire P, Maas RL, Peters H, et al. Eya1 is required for the morphogenesis of mammalian thymus, parathyroid and thyroid. Development. 2002;**129**:3033-3044

[114] Zou D, Silvius D, Davenport J, Grifone R, Maire P, Xu PX. Patterning of the third pharyngeal pouch into thymus/parathyroid by Six and Eya1. Developmental Biology. 2006;**293**:499-512. DOI: 10.1016/j.ydbio.2005.12.015

[115] Su D, Ellis S, Napier A, Lee K, Manley NR. Hoxa3 and pax1 regulate epithelial cell death and proliferation during thymus and parathyroid organogenesis. Developmental Biology. 2001;**236**:316-329. DOI: 10.1006/dbio.2001.0342

[116] Hetzer-Egger C, Schorpp M, Haas-Assenbaum A, Balling R, Peters H, Boehm T. Thymopoiesis requires Pax9 function in thymic epithelial cells. European Journal of Immunology. 2002;**32**:1175-1181. DOI: 10.1002/1521-4141(200204)32:4<1175:AID-IMMU1175>3.0.CO;2-U

[117] Crosetto N, Bienko M, van Oudenaarden A. Spatially resolved transcriptomics and beyond. Nature Reviews. Genetics. 2015;**16**:57-66. DOI: 10.1038/nrg3832

[118] Chen KH, Boettiger AN, Moffitt JR, Wang S, Zhuang X. RNA imaging. Spatially resolved, highly multiplexed RNA profiling in single cells. Science. 2015;**348**:aaa6090. DOI: 10.1126/science.aaa6090

[119] Giesen C, Wang HA, Schapiro D, Zivanovic N, Jacobs A, Hattendorf B, et al. Highly multiplexed imaging of tumor tissues with subcellular resolution by mass cytometry. Nature Methods. 2014;**11**:417-422. DOI: 10.1038/nmeth.2869

[120] Bendall SC, Nolan GP, Roederer M, Chattopadhyay PK. A deep profiler's guide to cytometry. Trends in Immunology. 2012;**33**:323-332. DOI: 10.1016/j.it.2012.02.010

Chapter 10

The Hypoxia-Inducible Factor-1α in Angiogenesis and Cancer: Insights from the *Drosophila* Model

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Additional information is available at the end of the chapter

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Abstract

The hypoxia-inducible factor-1α (HIF-1α) is an evolutionarily conserved transcription factor with prominent roles in the hypoxic response, cell survival, angiogenesis and cancer. HIF-1α functions as a sensor of molecular oxygen: in the presence of oxygen, it is degraded by the proteasome, whereas in reduced oxygen tensions, it heterodimerizes with the constitutively expressed HIF-1b subunit forming the functional HIF1 transcription factor, which enters the nucleus to control expression of hypoxia-inducible genes. Since HIF-1α has been found upregulated in several cancers, it has attracted a lot of clinical interest, because it represents an interesting candidate for pharmacological chemotherapy interventions. In this chapter, we discuss our current knowledge on the HIF1 transcription factors and their major roles in development, physiology, angiogenesis and cancer using examples of recent studies in the model organism *Drosophila melanogaster*. Given the striking functional conservation between the mammalian and fruit fly HIF-1α, we expect that future studies in the *Drosophila* model will not only expand our knowledge on the basic HIF1 biology, but they will also pinpoint conserved molecular regulators of HIF1 that might lead to the discovery of novel cancer therapeutics.

Keywords: hypoxia, tumorigenesis, Warburg effect, metabolism, tracheogenesis, inflammation, *Drosophila*

1. HIF-1α in mammalian angiogenesis, inflammation and cancer

1.1. Oxygen is required for survival of all animals

Oxygen (O_2) is the main ingredient of the atmospheric air and is required for the survival of all living organisms. It is also present in the seas and oceans, and it is necessary for survival of all aquatic living organisms. Oxygen accumulated on Earth's atmosphere about 2.5 billion

years ago [1]. However, it was discovered only 245 years ago, by the chemist Carl Wilhelm Scheele [2]. Its main role in the survival of animals derives from its utilization during cellular respiration. Specifically, oxygen is involved in oxidative phosphorylation, the process that transfers the chemical energy stored in carbon bonds to the phosphate bonds of Adenosine Tri-Phosphate (ATP), which is the main energy carrier in all cell types of living organisms [3]. In addition, oxygen is the main component of ATP production, because it is the final electron acceptor of the respiratory chain. Oxygen-electron reaction leads to the release of reactive oxygen species (ROS), which, when accumulated, results in oxidative stress and eventually cell death [4, 5]. Oxygen is necessary as an energy substrate, and the danger of oxidative damage needs to be kept at equilibrium. Therefore, oxygen homeostasis is critical for all cellular processes, and its intermittent supply results in many pathophysiological conditions, such as myocardial ischemia, sepsis, pulmonary hypertension, chronic obstructive pulmonary disease and cancer, which correspond to frequent causes of mortality in the Western world [6].

The ambient oxygen concentration is 21%, and the cells of the majority of healthy tissues are exposed to less oxygen, which varies between 2 and 16% [6, 7]. Normoxia is defined as normal oxygen levels, whereas hypoxia is a situation where the organism is underprivileged of sufficient oxygen supply. Hypoxia can be continuous or intermittent [4]. In anoxia, oxygen levels are strictly or totally insufficient, and in hyperoxia, oxygen is superfluous in the tissues and organs of the body. Low oxygen levels are mostly observed in acute inflammatory conditions and also within solid tumors [7]. In contrast, hyperoxia might be the result of immoderate oxygen delivery to the organism due to unlimited angiogenesis [8].

Transport of oxygen throughout the bodies of animals is achieved via different mechanisms that depend on the living environment and the size of each organism. For example, in small animals, such as the nematode *Caenorhabditis elegans*, atmospheric oxygen enters the body via diffusion. Insects, such as *Drosophila melanogaster*, use an elaborate network of tubules, and the tracheal system transports oxygen from the outside environment via the spiracular openings to all the cells of the body. Specialized cells of the insect tracheal system, the terminal cells, are involved in actual gas exchange and come in close contact with different cells in the body that need oxygen. The respiration process and the allocation of oxygen to the trillions of cells in the bodies of organisms, such as vertebrates, seem to be more intricate because of their large body size. The delivery of oxygen to each part of the human/vertebrate body is achieved through the lungs, the diaphragm, the erythrocytes, the heart and the vasculature [3, 9].

1.2. The transcription factor HIF1 is key in oxygen sensing

The hypoxia-inducible factor-1α, HIF-1α, was characterized in 1990 as a transcription factor with a key role in oxygen sensing [10]. The discovery of HIF-1α opened new research avenues focusing on oxygen sensing and oxygen poverty [10]. Despite the discovery of two additional HIFs (the HIF-2α & the HIF-3α) and the variable response of all HIFs to hypoxia, HIF-1α remains the molecule with the major role in oxygen sensing [11, 12].

The importance of HIF-1α and oxygen sensing for living organisms is underscored by the evolutionary conservation of this transcription factor in animals. The genomes of different species ranging from corals to insects to mammals (i.e., *Acropora millepora, Nematostella vectensis, Caenorhabditis elegans, Palaemonetes pugio, Drosophila melanogaster, Anopheles gambiae, Apis mellifera,*

Nasonia vitripennis, *Eurosta solidaginis*, *Tribolium castaneum*, *Mus musculus*) encode homologs of the HIF-1α [13]. HIF-1α is present in all metazoans [14] and is characterized as a master regulator of hypoxia-inducible genes in mammals [15]. HIF1 is a heterodimer of two subunits: one labile oxygen-sensitive subunit, the HIF-1α and one stable constitutively expressed subunit, the HIF-1β [3, 16–18]. The HIF subunits form a subfamily of the basic-Helix-Loop-Helix-Per/ARNT/Sim (bHLH-PAS) superfamily of transcription factors. The bHLH proteins comprise a superfamily of eukaryotic transcription factors that can dimerize via their HLH domain, and the bHLH-PAS proteins are only a small group of this superfamily [18, 19]. The HIF1 heterodimer forms a bHLH transcription factor that recognizes and binds the hypoxia-response elements (HREs) on DNA. HREs are present in the promoters of HIF target genes, which are involved in intracellular homeostatic processes, such as energy metabolism, angiogenesis, erythropoiesis and apoptosis [15, 17]. HIF-1α is the main sensor of low oxygen concentrations [20] and is stabilized upon hypoxia and also in response to divalent cations and iron chelators [21].

The human HIF-1α protein is composed of eight regulatory domains: the bHLH DNA binding and dimerization domain, the PAS dimerization domain, the amino-terminal and carboxy-terminal nuclear localization signals (NLS-N and NLS-C), the proline-serine-threonine-rich protein stabilization domain (PSTD), the amino-terminal and the carboxy-terminal transactivation domains (TAD-N and TAD-C), and the transcriptional inhibitory domain (ID). The HIF-1α peptide consists of 826 amino acids, whereas the HIF-1β peptide is smaller with 774 amino acids, because of alternative splicing in a region that encodes 15 residues [9]. Thus, the two HIF1 subunits have highly conserved amino acid sequences for the majority of the regulatory domains described above [22, 23]. Apart from HIF1 (HIF-1α and HIF-1β), there are also another two HIFs, the HIF-2α and the HIF-3α, also known as ARNT2 and ARNT3. Their expression is more restricted in human and mouse tissues compared with the HIF-1α and the HIF-1β subunits [24–26].

The expression of functional HIF-1α is controlled at multiple levels, such as transcription, nuclear transport, protein stability, and transactivation. Most of the studies have focused on the stabilization of HIF-1α protein at *in vivo* and *in vitro* changes of oxygen levels [9]. Under sufficient oxygen concentrations (normoxia, 21% O_2), the von Hippel-Lindau (VHL) E3 ubiquitin ligase recognizes and binds to the hydroxylated HIF-1α subunit. VHL binds to HIF-1α only when HIF-1α is hydroxylated by the prolyl-4-hydroxylase (PHD). PHD operates as a direct sensor of oxygen, because it uses O_2 as a substrate and attaches –OH groups to particular proline residues (Pro402 and Pro564 of human HIF-1α) [27]. Binding of VHL recruits a ubiquitin ligase complex composed of Elongin C, Elongin B, Cullin 2 (Cul2), and a Ring box protein (Rbx1) and attracts a ubiquitin-conjugating enzyme that attaches a polyubiquitin chain to HIF-1α to target it for proteasomal degradation [17]. The Pro564 residue of HIF-1α has a higher affinity for PHDs compared to the Pro402 residue [28]. However, the hydroxylation of the one can affect the hydroxylation of the other [28]. Other factors that play critical roles in the regulation of the pathway is the availability of O_2 and Fe(II), and additionally, whether VHL protein can function properly or whether it has a mutation that makes it dysfunctional [28]. Furthermore, the control of the process is related to the cell type and the developmental phase of the tissue or organ. This regulation can be affected by post-translational events [28].

In hypoxia (1–2% O_2), the PHD enzyme cannot hydroxylate HIF-1α because oxygen is lacking, VHL cannot bind to the HIF-1α subunit, and therefore, HIF-1α is not degraded by the proteasome [20]. Stabilized HIF-1α is quickly transported to the nucleus to induce the transcription

of target genes [20, 29]. In the nucleus, the HIF-1α/HIF-1β heterodimer binds the p300 coactivator [30]. HIF1 and p300 form a complex that binds the double-stranded DNA and promotes transcription. HIF-1α has a plethora of target genes, which encode proteins involved in critical biological processes, such as erythropoiesis, vascular remodeling, metabolism, cell proliferation, cell viability and angiogenesis [9, 10] (**Table 1**).

1.3. HIF-1α controls tumor angiogenesis

Angiogenesis is the process of forming new blood vessels from pre-existing ones, which differentiates into a vascular network [53, 54]. Blood vessels supply the body with oxygen, nutrients, and immune surveillance. The extensive growth of veins and their non-physiological remodeling result in multiple illnesses, such as cancer and ischemic and inflammatory diseases (e.g., arthritis, atherosclerosis, and diabetes) [54–58]. The veins are used as pathways for the migration of cancer cells [54]. Angiogenesis may be adversely affected by infection with pathogenic bacteria. Additionally, angiogenesis is a feature of cancer, as tumor cells induce the process in order to grow and become metastatic [59].

Previous studies have shown a correlation between tumor growth and angiogenesis and have established molecular links between the signaling pathways induced upon infection, gene regulation, and cancer [60–63]. According to the angiogenesis dogma, a tumor cannot grow more than a few millimeters in diameter, if it does not come in contact with the blood vessels by which it receives enough oxygen [54, 61]. Furthermore, due to the irregular shape and organization of the tumor vasculature, some cells are more than 100 mm away from the blood vessels and they also become hypoxic. The oxygen within the tumor is not static but fluctuates spatially and temporally [64]. Angiogenesis is regulated by molecules that act as "activators" (pro-angiogenic factors) or "inhibitors" (anti-angiogenic factors) [65]. Several studies have shown that the angiogenic activators play an important role in the growth and spread of tumors [66]. Key activators of angiogenesis belong to the family of VEGFs, and their receptors were found expressed in about half of human cancers investigated so far [66].

Importantly, HIF-1α has been shown to control the expression of proangiogenesis regulators, such as VEGF and other growth factors and often activation of their respective pathways feedback to enhance HIF-1α activity [9]. For example, the epidermal growth factors (EGFs) act as angiogenesis activators. The binding of EGF to the epidermal growth factor receptor (EGFR) activates the MAP kinase cascade and also induces the PI3K (phosphatidylinositol 3-kinase)—AKT/PKB (Protein Kinase B) pathway. The PI3K enzyme catalyzes the transfer of a phosphate group, which converts PI phospholipid (phosphatidylinositol) into PI-3P phospholipid (Phosphatidylinositol 3-phosphate). This conversion results in the full activation of the serine/threonine kinase PDK-1 (Phosphoinositide-dependent kinase), which phosphorylates and activates another serine/threonine kinase, known as AKT. PTEN (phosphatase and TENsing homolog), which functions as a kinase with tumor suppressor activity, is a negative regulator of PI3K, which mediates cell proliferation [67]. The protein kinase p70S6 is a target of mTOR. Through phosphorylation, it induces the translation of mRNAs, which encompass a 5′ end rich in pyrimidines. Such sites are found in the HIF-1α mRNA [68].

1.4. Tumor hypoxia, HIF-1α, and the Warburg effect

The hypoxic regions of a tumor are resistant to chemotherapy, exhibit modified metabolism, and often acquire metastatic and invasive properties [69–71]. Chronic cell proliferation, which

appears to correlate with tumor incidence, does not only involve cellular dysfunction but also energy metabolism adjustments through which the organism acquires enough energy by producing ATP, which is used by cancer cells for cell division and growth. In 1924, the Nobelist Otto Warburg first described the preference of cancer cells to convert glucose into lactic acid

Gene name	Gene symbol	Gene function	NCBI Gene ID	Refs
Angiopoietin 1	*ANGPT1 ANGPT2*	Angiogenesis	284	[31]
Angiopoietin 2			285	
BCL2 interacting protein 3	*BNIP3*	Apoptosis	664	[32, 33]
BCL2 interacting protein 3-like	*BNIP3L/NIX*	Apoptosis	665	[33]
Endothelin-1	*ET1*	Angiogenesis	1906	[34]
Enolase 1	*ENO1*	Energy metabolism	2023	[35]
Erythropoietin	*EPO*	Proliferation, survival	2056	[36]
Glyceraldehyde-3-phosphate dehydrogenase	*GAPDH*	Energy metabolism	2597	[37]
Glucose transporter-1	*GLUT1*	Energy metabolism	6513	[38]
Hexokinase 1	*HEX1*	Energy metabolism	3098	[39]
Hexokinase 2	*HEX2*		3099	
Prolyl 4-hydroxylase subunit alpha 1	*P4HA*	Energy metabolism	5033	[40]
Insulin growth factor 2	*IGF2*	Growth and survival	3481	[41]
Insulin growth factor binding protein 1, 2 and 3	*IGFBP-1*	Growth and survival	3484	[41, 42]
Lactate dehydrogenase A	*LDHA*	Energy metabolism	3939	[35]
MAX interactor 1	*MXI1*	Apoptosis, c-Myc activity	4601	[43, 44]
Nitric oxide synthase 2	*NOS2*	Angiogenesis	4843	[45]
Pyruvate kinase M	*PKM*	Energy metabolism	5315	[35]
Pyruvate dehydrogenase kinase 1	*PDK1*	Energy metabolism	5163	[46, 47]
Transforming growth factor beta 3	*TGF-b3*	Invasion, metastasis, growth, survival	7043	[48]
Tumor protein p53	*p53*	Apoptosis	7157	[49]
Twist	*TWIST*	Metastasis	7291	[50]
Vascular endothelial growth factor A	*VEGFA*	Angiogenesis, growth, survival	7422	[51]
Vascular endothelial growth factor receptor 1	*VEGFR-1/ FLT-1*	Angiogenesis, growth, survival	2321	[52]

Although several genomic studies have identified a plethora of potential HIF1 targets, here we focus only on direct targets of HIF-1α with characterized HREs

Table 1. A list of HIF-1α targets with key functions in a variety of physiological cellular processes, such as angiogenesis, survival, and energy metabolism.

even in the presence of oxygen [72]. By measuring lactic acid production and oxygen consumption in thin sections from healthy and tumorous rat livers, he concluded that normal liver cells inhibit the production of lactic acid in the presence of oxygen, whereas cancer cells produced lactic acid irrespective of the availability of oxygen [73, 74]. In aerobic conditions, normal cells convert glucose to pyruvic acid via glycolysis in the cytoplasm, and then, pyruvic acid is used in the mitochondria to produce acetyl Coenzyme A (CoA) and carbon dioxide (CO_2) during oxidative phosphorylation. In anaerobic conditions, normal cells favor glycolysis, and pyruvic acid is used in the cytoplasm to produce lactic acid. Instead, according to Warburg, cancer cells change their metabolism, and even in the presence of oxygen, glucose enters glycolysis and produces lactic acid. Cancer cells use 10 times more glucose than the amount of the cellular breathing process can use, while the amount of lactic acid produced is two times greater than that produced by healthy cells [73]. This phenomenon is known as the "Warburg effect" or "aerobic glycolysis" [70, 75–77]. At first sight, this phenomenon seems paradoxical, since aerobic glycolysis produces significantly less energy (4 mol ATP/mol glucose) compared to oxidative phosphorylation (36 mol ATP/mol glucose). Nevertheless, cancer cells exhibit an increased expression of glucose transporters, such as GLUT1, which correlates with enhanced glucose uptake [78–80]. The feeding of cancer cells with glucose is often associated with oncogene activation and loss-of-function of tumor suppressor genes [78, 79, 81]. The *myc* oncogene is an important regulator of cancer metabolism, since among its many targets, are those of GLUTs as well as genes encoding pyruvate dehydrogenase kinase 1 (PDK1) and lactate dehydrogenase A (LDHA) that promote the Warburg effect by increasing the flow of glucose through glycolysis, while inhibiting the entry of pyruvic acid into the Krebs cycle [82–84]. Furthermore, both the oncoprotein Ras and hypoxia can independently increase the levels of the HIF-1α and HIF-2α transcription factors, which in turn positively regulate glycolysis [85–87]. In addition, loss-of-function mutations in tumor suppressor genes, such as *vhl* [88] and *p53* [89, 90], lead to elevated levels of HIF-1α and VEGF. Gain-of-function mutations in oncogenes, such as the *v-src* [91], activation of EGF, and insulin growth factor I (IGF-I) receptors, also induce HIF-1α [41, 92].

A series of major discoveries remained as milestones in the field of cancer biology followed Warburg's observations. These include the purification and cloning of the HIF-1 in 1995 [36], the effects of HIF-1 in cancer progression in mice [93], the description of VHL [94], the identification of the PHD enzymes, and the establishment of the HIF-α subunit prolyl hydroxylation [69]. The area of hypoxia remains an attractive subject for intensive research, although over a century has passed, since it was first taken into account. With the discovery of HIFs, an extremely attractive field of research emerged and novel proteins came into play, such as the glucose regulated proteins (GRPs), oxygen regulated proteins (ORPs), PDGF, interleukin-1α (IL-1α), endothelin-1, VEGF and erythropoietin (EPO) [95–100]. The characterization of HIF-1 led to the discovery of upstream activators and downstream signals as potential new therapeutic targets. Such targets include the VEGF, fibroblast growth factor (FGF), TGFα, the PI3K/AKT/mTOR and RAS signaling pathways [101]. In addition, reduced oxygen tensions can repress mTOR in the cells similar to the effects of rapamycin. mTOR in hypoxic environments acts as an oxygen sensor and leads to reduced protein translation [102].

The PI3K/AKT pathway inhibits programmed cell death and alters cell proliferation [103]. Loss of PTEN, which is a negative regulator of the pathway, can lead to increased angiogenesis

in the case of prostate cancer. This has been associated with the induction of HIF-1α that guides elevated VEGF expression [103, 104]. In colon tumors, transfection of cells with a HIF-1α expression vector resulted in elevated VEGF mRNA levels and increased angiogenesis [90]. The EGF/PI3K/AKT/TOR pathway promotes VEGF and the transcriptional activity of HIF-1α protein in prostate cancer [89]. Chemical inhibitors of PI3K and TOR, the LY294002 and rapamycin, respectively, inhibited growth factor-induced and mitogen-induced secretion of VEGF. This connected the PI3K/PTEN/AKT/TOR pathway with HIF1 and the process of angiogenesis [105]. In the absence of HIF-1α, the development of a tumor is dramatically reduced although not completely stalled [106]. Moreover, HIF-1α is overexpressed in different cancer types, such as colon, breast and lung carcinomas. HIF-1α is also overexpressed in preneoplastic, premalignant adenomas and other intraepithelial neoplasia and also in malignant and metastatic tumors [89]. Therefore, discovery of chemicals that could potentially control the HIF-1α pathway is of major clinical importance.

1.5. HIF-1α and inflammation

Another important aspect of HIF in tissue maintenance is its role in regulating inflammation and innate immunity. HIFs appear to have different functions in different immune cell types. For example, HIF-1α mediates bacterial killing via regulation of pro-inflammatory gene expression in macrophages [107]. On the other hand, in the case of neutrophils, HIF-1α promotes cell survival upon hypoxia and promotes extensive angiogenesis which is regulated by β2-integrin expression. Furthermore, there is a link between the effect of HIFs in immune cells, inflammation, and tumorigenesis [107]. It is known that HIF-1α is regulated by the availability of oxygen. Interestingly, not only hypoxia but also bacterial products, such as cytokines and growth factors, induce HIF-1α. Inflammatory cytokines, such as tumor necrosis factor a (TNF-α) and interleukin-1β (IL-1β), induce HIF-1α transcription. TNF-α induces HIF transcription and the Nuclear Factor-kappa B (NF-κB) pathway is needed for stabilization of the protein [108, 109]. In the case of IL-1β, the stability of HIF is promoted by the activation of NF-kB activity and the inhibition of VHL function [110]. There is an important crosstalk between NF-kB and HIF upon inflammation and cancer [111]. NF-kB is activated in inflammatory conditions, including cancer, and the activation of the pathway is a characteristic of inflammatory disease [59]. Its role in malignant situations is controversial such that it can act both as a tumor promoter and as a tumor suppressor [112]. Its activated form is implicated in excessive cell proliferation, metastasis, inhibition of apoptosis and angiogenesis [112]. Importantly, cells expressing wild-type p53 undergo apoptosis in hypoxic conditions, in contrast to the mutant p53 cells that are resistant to apoptosis. These results reveal that HIF-1α can promote cell proliferation and thus tumorigenesis by inhibiting apoptosis [113]. The chemokine interleukin-8 (IL-8) [114] and the VEGF [115] NF-kB target genes promote angiogenesis. Importantly, these are also targets of HIF-1α [116, 117], revealing that there is a crosstalk between NF-kB and HIF-1α. This crosstalk is bi-directional, because although NF-kB promotes the activation of HIF, HIF restricts the transcriptional activity of NF-kB [117]. Inflammation promotes NF-kB activity, which leads to tumorigenesis [118]. Not only NF-kB, but also other transcription factors (e.g. STAT3) can induce HIF [119]. PHDs antagonize NF-kB in different tumor cells [120–122]. In colorectal cancer, NF-kB promotes tumorigenesis. Different signaling pathways drive its oncogenic role.

These pathways regulate the production of ROS, the activation of pro-inflammatory cytokines, the uncontrolled cell proliferation, migration, metastasis and angiogenesis [123]. The absence of NF-kB has a negative effect on tumor progression in mouse models of colorectal cancer [124]. ROS affect the hydroxylation of HIF-1α and, thus, modify its activity [125, 126]. Specific defects, not necessarily mitochondrial defects, that restrict the consumption of oxygen, result in enhanced prolyl-hydroxylation accompanied with reduced HIF levels [125, 127].

The induction of HIF by such inflammatory cytokines indicates that HIF has a crucial role in inflammatory responses. Apart from inflammatory cytokines, various signaling pathways seem to also have important roles in the stimulation of HIF. Such pathways include PHDs [128], NF-κB [129–131], MAPKs [130], and ROS [129]. In addition, ROS are released by the mitochondria as a cause of low oxygen tensions, and they can control transcriptional and posttranslational events [132]. Another important crosstalk in colorectal cancer is that between HIF-1α, β-catenin and APC: when repressed under insufficient oxygen levels, APC can lead to activation of the Wnt/β catening signaling and increased proliferation that drives tumorigenesis [133]. Interestingly, NF-kB is regulated by Wnt/β-catenin [134].

2. The HIF-1α pathway in *Drosophila melanogaster*

2.1. The HIF-1α pathway is conserved in *Drosophila*

The *Drosophila melanogaster* genome encodes homologs of the core proteins involved in the HIF-1α pathway. For example, there are two HIF-1α homologs in fruit flies, one is encoded by the gene *similar* (*sima)* and its paralogue is known as *trachealess (trh)* [20, 27, 135]. Sima responds to changing oxygen levels, whereas Trh acts as a patterning gene during *Drosophila* development. The HIF-1β homolog in flies is the product of the gene *tango* (*tgo*) [136]. Both *sima* and *tgo* are transcribed during larval stages [136]. Although Tgo is expressed uniformly throughout development [137], Sima accumulates in the majority of tissues only in hypoxic conditions [138]. Tgo and Sima heterodimerize to control transcription in environments with decreased oxygen levels [139, 140].

Sima has a molecular weight of 180 kDa, is larger compared to the mammalian HIF-1α, and bears 45% similarity in the PAS domain and 63% in the bHLH domain with its human homolog (**Figure 1**) [141, 142]. The single prolyl-4-hydroxylase (PHD) enzyme homolog in *Drosophila* is encoded by the gene *fatiga* (*fga*) and acts as an oxygen sensor. Fga uses O_2 as a substrate and hydroxylates a single Pro residue in Sima (Pro850) [143]. Another factor that plays crucial role in the process is the availability of Fe (II) [28]. In normoxia, where O_2 is abundant, Sima is hydroxylated and targeted to the proteasome for degradation via association with the von Hippel-Lindau ubiquitin ligase, which in *Drosophila* is encoded by the dVhl gene [20]. In low oxygen tensions (environmental hypoxia and tumors), the HIF-1α/Sima transcription factor is not degraded, because it cannot be hydroxylated by Fga due to the lack of oxygen. Consequently, HIF-1α/Sima binds to the constitutively expressed HIF-1β/Tgo forming the HIF1 heterodimer, translocates to the nucleus and binds to hypoxia-response elements (HREs) to control transcription of target genes [20, 29] (**Table 2**).

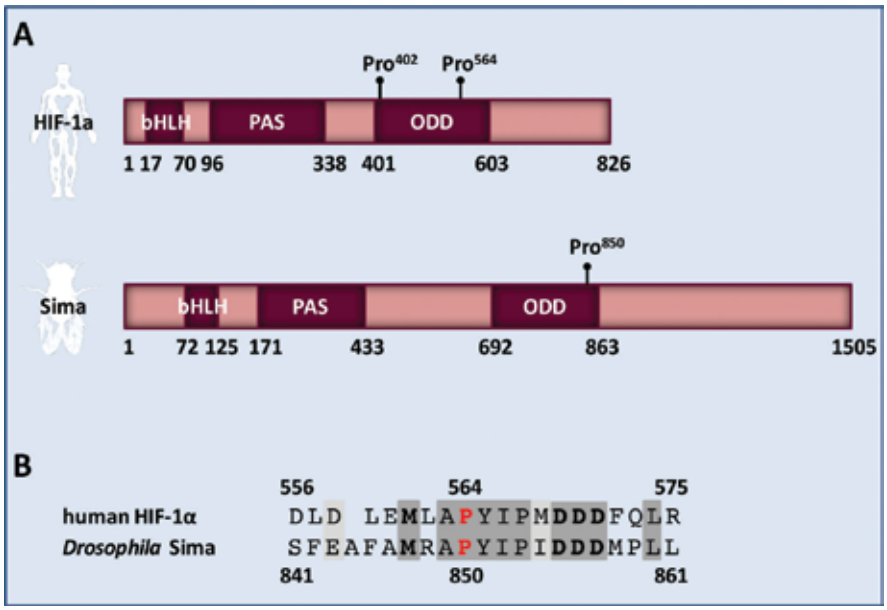

Figure 1. The human HIF-1α and *Drosophila* Sima are homologous proteins. (A) The human HIF-1α protein has a length of 826 amino acids, whereas its *Drosophila* homolog Sima is much larger and consists of 1505 amino acids. The shared functional domains of the two proteins are indicated with darker color: the bHLH DNA binding and dimerization domain, the PAS dimerization domain and the oxygen-dependent degradation domain (ODD). Two Pro residues (Pro^{402} and Pro^{564}) are substrates of the propyl-4-hydroxylase PHD in the human HIF-1α, whereas in *Drosophila*, the propyl-hydroxylase Fga targets a single Sima Pro residue (Pro^{850}). (B) Amino acid alignment of the human HIF-1α (amino acids 556–575) and the *Drosophila* Sima (amino acids 841–861) highlighting in red the hydroxylation targets Pro^{564} and Pro^{850}, respectively. Dark gray and light gray boxes indicate identical residues and conservative amino acid substitutions in the two proteins, respectively.

Gene name	Gene symbol	Human homolog	Gene function	NCBI Gene ID	Refs
branchless	*bnl*	*FGF10*	Tracheal development, cell migration	42356	[144]
breathless	*btl*	*FGFR*	Tracheal development, cell migration	39564	[144]
Ecdysone-inducible gene L3	*ImpL3*	*LDHA*	Energy metabolism	45880	[145]
fatiga B	*fgaB*	*HPH3*	Energy metabolism	40633	[146]
heat shock factor	*hsf*	*HSF1*	Heat response, defense response	37068	[147]

Table 2. A list of Sima targets identified by genetics and direct binding assays.

2.2. HIF-1α/Sima controls remodeling of the tracheal gas-transporting tubes

In *Drosophila melanogaster*, an extensive network of interconnected tubes, the tracheal system, transfers oxygen throughout the body. The *Drosophila* trachea is therefore functionally analogous to the mammalian respiratory system [148]. It is responsible for the oxygenation of the

flight muscles, the brain and all internal organs, such as the intestine. The tracheal system of the fly is a good model for studying tracheal cell migration and the mechanisms that direct the movement of cells in different directions [149]. Systematic studies of the molecular markers labeling the trachea, mutational studies on specific genes and cellular imaging have identified the basic steps in the development of the tracheal system. Embryonic tracheal morphogenesis is initiated at stage 10 of embryogenesis and proceeds through four sequential steps: the formation of the tracheal placode and the sprouting of the primary, secondary and tertiary branches [148]. Tracheal cell specification and primary and secondary branch formation are genetically controlled stereotypical processes, whereas terminal branching is environmentally controlled and adjusted according to the needs of the tissue for oxygen [148]. After embryogenesis and during larval life, the fly trachea grows in size to accommodate the increased oxygen needs of the larva. At the same time, specialized airway progenitors, the tracheoblasts, get activated to proliferate and differentiate to remodel the pupal and adult tracheal system during metamorphosis [150–154]. The FGF/FGFR pathway controls all aspects of tracheal morphogenesis. In flies, the FGF homolog encoded by the gene *branchless* (*bnl*) and the FGFR homolog encoded by the gene *breathless* (*btl*) are repeatedly utilized for tracheal development and remodeling. Bnl/FGF acts as a chemoattractant that can direct tracheal sprouting in cells expressing the Btl/FGFR [155–157].

Remarkably, *Drosophila* adults and larvae present a different mode/pattern of response during hypoxia. In adult *Drosophila*, this response includes the opening of the spiracles, the aeration of the body and the transposition of the fluids from the tracheoles [158, 159]. It has been shown that the majority of insects can survive in complete anoxia for quite a long period in contrast to mammals. The survival of adult *Drosophila melanogaster* under anoxia without any tissue damage and reaches a period of about 4 hours [160]. This time interval depends on the developmental stage of the animal. For example, larvae illustrate escape mobility for 20 minutes in these conditions [161], whereas adult *Drosophila* remains stationary within a period of only 60 seconds, and this is because of the effacement of the electrical responses of the insect muscles [160]. The retention of flies in anoxia for 12 hours leads to death [162]. Furthermore, the survival of the flies in anoxia depends on the suppression of ATP synthesis, with simultaneous reduction of the harmful reverberations of low energy availability [161]. Interestingly, although flies challenged for 6 hours in 0.5% O_2 stop responding and remain motionless, following their reoxygenation, they behave physiologically without any defects [163]. Moreover, the behavior of *Drosophila* under low oxygen conditions depends on the degree of hypoxia and whether it is constant or intermittent [164]. Different gene families have been found regulated in different types of hypoxia. For example, in flies that experienced intermittent hypoxia, a smaller proportion of altered genes has been observed, in comparison with the flies that experienced constant hypoxia. The same flies revealed decreased metabolic rates and loss of spiracular control [165, 166]. In *Drosophila,* the adaptation to hypoxia involves mechanisms that increase oxygen delivery, such as the expansion of the spiracular openings, as noted above, that are able to propel oxygen to the whole organism [158]. The expression of HIF1 increases the diameter of tracheal tubules and induces the expansion of cells that directly contact target tissues, the tracheoles [167].

Interestingly, the embryonic and larval fly trachea encompasses specialized cells, which extend cytoplasmic processes to carry oxygen to the tissue. These cells, known as tracheal terminal cells, are very similar to the tip cells of the mammalian blood vessels; they are plastics and they

respond to hypoxia by extending cytoplasmic tubular processes, the terminal branches, toward the hypoxic tissue [148]. The sprouting and growth of the terminal branches are carefully adjusted according to the needs of tissue in oxygen, just as in the case of sprouting angiogenesis in mammals. Hypoxia induces terminal branching, whereas hyperoxia (increased oxygen supply) suppresses the formation of terminal branches [148, 168]. Hypoxia induces the expression of *bnl*/*FGF*, which acts as a chemoattractant that can direct the newly formed branches of every cell that expresses the FGFR/Btl [148]. The formation of new branches depends on the HIF-1α homolog Sima and the HIF-propyl hydroxylase Fga, which acts as an oxygen sensor [144]. In hypoxia, HIF-1α/Sima accumulation in tracheal cells induces the expression of *btl*/*FGFR* and thus causes further sprouting of new branches, whereas in nontracheal cells, Sima contributes to the induction of *bnl*/*FGF* [144]. Therefore, the hypoxia-induced trachea-specific *btl*/*FGFR* expression, probably, enhances their sensitivity in the presence of high levels of Bnl [144]. In nontracheal target tissues, such as the larval muscle, the Archipelago (Ago) F-box/WD-repeat protein substrate specificity factor for a Skp/Cullin/F-box (SCF)-type polyubiquitin ligase has been shown to antagonize HIF-1α/Sima-dependent *bnl*/*FGF* expression. Ago physically associates with HIF-1α/Sima, reduces its levels, and inhibits the hypoxic response [29, 169]. Thus, terminal cell remodeling in *Drosophila* is controlled by the evolutionarily conserved HIF-1α/Sima pathway, similar to tip cell remodeling of mammalian blood vessels [136, 170].

2.3. HIF-1α/Sima and growth control in *Drosophila*

Insects have a mechanism of body size plasticity. Oxygen sensing has a major role in this mechanism. Hypoxia causes a reduction of body size in the fruit fly *Drosophila* and the moth *Manduca* [171]. The primary regulators of this phenotype are the HIF1 and nitric oxide synthase (NOS) signaling pathways, which are activated in hypoxia. In normoxia, NO inhibits HIF-1α at the level of protein hydroxylation that targets it to the proteasome for degradation [171].

Over the last decade, many scientists tried to address the role of HIF in cell growth and cell size control. Overexpression of *HIF-1α*/*sima* in the fat body resulted in smaller cells compared to the control wild type cells, indicating that Sima operates as a cell-autonomous negative growth regulator [140]. In addition, *fga* mutant pupae revealed reduced rate of growth and smaller size compared to wild type [140]. Cells with *fga* loss-of-function in the larval fat body were found smaller compared to the wild type cells in the same tissue [172]. In contrast, overexpression of the same gene in the wing imaginal discs led to the growth of the cells [140]. HIF uses at least two mechanisms to control the cellular growth in *Drosophila*. It can block protein synthesis, by targeting the insulin-like peptide (ILP)-TOR-S6 K pathway, and as a consequence, there is a reduction of cellular growth of the whole animal. This has been so far seen in the fat tissue, the eye and the gut of *Drosophila* [173]. In addition, *Drosophila* mutants overexpressing or lacking the gene *fga* revealed that HIF controls the function of the cyclin-dependent protein kinase 4 (Cdk4), which is responsible for the activation of cellular growth [172]. Moreover, HIF is also implicated in the expression of *scylla* and *charybdis* genes that downregulate the S6 K-dependent activation of protein synthesis, and this results in the reduction of cellular and body growth [173].

It is firmly established that insulin growth factors and components of the insulin pathway upregulate the HIF-1α protein, thus promoting the expression of hypoxia-sensitive genes

[174]. The PI3K/Akt/TOR signaling pathway is directed by insulin to induce the transcription of *HIF-1α/Sima* both in S2 cell lines and in *Drosophila* embryos [175]. Thus, the transcriptional activation of HIF-1α/Sima is guided by the insulin receptor (InR)-regulated PI3K-AKT and TOR signaling pathways [175]. RNAi silencing experiments showed that the mRNA levels of *HIF-1α/sima*, upon induction with insulin, depended on those two pathways. Experiments in fly embryos, where components of these pathways were overactivated, also revealed the upregulation of *sima* mRNA levels [175]. As noted above, the overexpression of *sima* in different tissues of the fly resulted in the reduction of cell size in these specific tissues [140]. But this is not consistent with our knowledge about the role of PI3K-AKT and TOR pathways in correlation with Sima and cell growth. Particularly, it is known that the activation of these pathways induces growth in different levels, and in parallel with this, it also induces the transcription of genes that are targeted by Sima. However, Sima is a negative regulator of growth. It seems like there is a negative feedback loop, in which the two pathways upregulate growth and Sima simultaneously, and then, Sima downregulates the two pathways, and this consequently results in growth limitation [176]. Interestingly, Sima induces *scylla* in hypoxic conditions, which in turn feeds back on the TSC1 complex to inhibit the TOR pathway and growth. Thus, s*cylla* seems to be part of the negative feedback loop that coordinates InR-mediated growth with HIF-1α/Sima induction during hypoxia [173].

2.4. Other functions of HIF-1α/Sima in *Drosophila*

2.4.1. Epithelial cell migration

A 2010 study dealt with the role of Sima in the rate of cell migration and invasion of the ovarian border cells in *Drosophila*. In more detail, the researchers studied the role of hypoxic response and HIF-1α/Sima during invasion and metastasis. It was shown that the HIF pathway controlled the rate or invasion in the ovary cells in a dose-dependent manner [177]. It seems that precise amounts of Sima are needed for the actual border cell migration. It became also clear that Sima was important for the specificity of the leading cells to reside at the edge of their cluster. Changes in the expression of the *DE*-cadherin adhesion protein also implicated Sima [177]. Notably, Sima regulates the activity of the transcription factor slow border cells (Slbo) that is necessary in border cells for their migration [177, 178]. Changes in HIF expression and activity in just a single migrating border cell of the cluster can lead to metastasis in this model [177]. Overexpression of *vhl* led to delay, blockage and acceleration of the border cell migration. Overexpression of *fga* exhibited only acceleration of border cell migration. Moreover, *sima* and *tgo* mutants demonstrate a delayed or accelerative behavior for border cell migration. Therefore, HIF1 activity is required for the conservation of invasive dynamics of the cells that migrate [177].

2.4.2. Blood cell differentiation

A recent study dealing with the role of HIF-1α in *Drosophila* blood cells focused on the interactions between Notch and HIF-1α. A specific lineage of *Drosophila* blood cells, the crystal cells, expressed elevated levels of Sima, even in normoxia [179]. Overexpression of *sima* results in expansion of the population of crystal cells, phenocopying the effect of Notch overexpression.

Thus, both molecules act in the same pathway in the lymph gland. Elevated activation of Notch in crystal cells is further increased in a *sima* overexpression background [179] and the full-length Notch (N^{fl}) receptor can be activated by *sima* in a ligand-independent manner. This happens also in hemocytes that also express *sima*. The N^{fl} is sufficient to increase the number of crystal cells. Even though Tgo acts together with Sima, *tgo* mutants did not reduce the numbers of crystal cells, but they led to an increase in their number [179]. In *Drosophila*, nitric oxide (NO) inhibits PHD and thus promotes the stabilization of Sima, whereas upon hypoxic conditions, it leads to reduced induction of HIF [180–182]. Nitric oxide synthase 1 (NOS1) is highly expressed in mature crystal cells, and its knockdown in the lymph gland by RNA interference (RNAi) leads to reduced numbers of crystal cells. $NOS1^{RNAi}$ clones revealed low levels of the Sima protein and were unable to form crystal cells. In conclusion, Sima is necessary for differentiation of crystal cells in the fly lymph gland [179].

2.5. Upstream regulators of HIF-1α/Sima in hypoxia

A genome-wide RNAi screen was deployed in *Drosophila* cells in culture to reveal genes required for the activation of HIF-1α/Sima [183]. More specifically, 30 genes appeared for the first time as candidate regulators of HIF in low oxygen concentration conditions, and these specific genes mediated the alteration to oxygen starvation. These genes included transcription elongation factors, translation regulators and components of chromatin remodeling complexes [183]. The *ago1* (*argonaute 1*) gene, which has a critical role in microRNA silencing processes, was also identified in this screen as a regulator of Sima in hypoxia. Given the involvement of Ago1 in Sima regulation, the authors went further to show that the microRNA pathway has a central role in HIF-dependent transcription, and also that Sima mRNA stabilization has a critical role in the *Drosophila* response to hypoxia [183].

Further work on Sima regulators has uncovered several modifiers of Sima function in hypoxia in *Drosophila*, such as the microRNA miR-190, the TIP60 chromatin remodeling complex and the RNA-binding protein Musashi [184–186] (**Figure 2**). miR-190 acts as a positive regulator of the hypoxic response by targeting directly the propyl-4-hydroxylase Fga, which is the principal negative regulator of Sima. Specifically, miR-190 is upregulated in hypoxia, reduces Fga activity and, thus, allows the Sima-mediated response to hypoxia [184]. In addition, the TIP60 complex is required for HIF1-dependent gene expression in fly cells and embryos, as well as colorectal cancer cells. TIP60 is recruited by HIF1 to chromatin during hypoxia and functions as a coactivator of HIF1 action by recruiting RNA-Polymerase II onto chromatin [186]. Finally, Musashi (Msi, dMsi in *Drosophila*) represses *sima* mRNA translation by binding an Msi-binding element within the 3′ UTR of the *sima* transcript. dMsi protein levels are reduced in hypoxia, allowing Sima transcription. Thus, dMsi mediates translational repression of the *Drosophila* HIF-1α, Sima. Moreover, association of murine Msi with the HIF-1α transcript suggests that a similar mechanism might be conserved in mammals [185].

2.6. HIF-1α/Sima in *Drosophila* tumorigenesis

The connection between metabolism deregulation and tumorigenesis is already established [187] and various signaling pathways with crucial roles in cancer progression regulate the

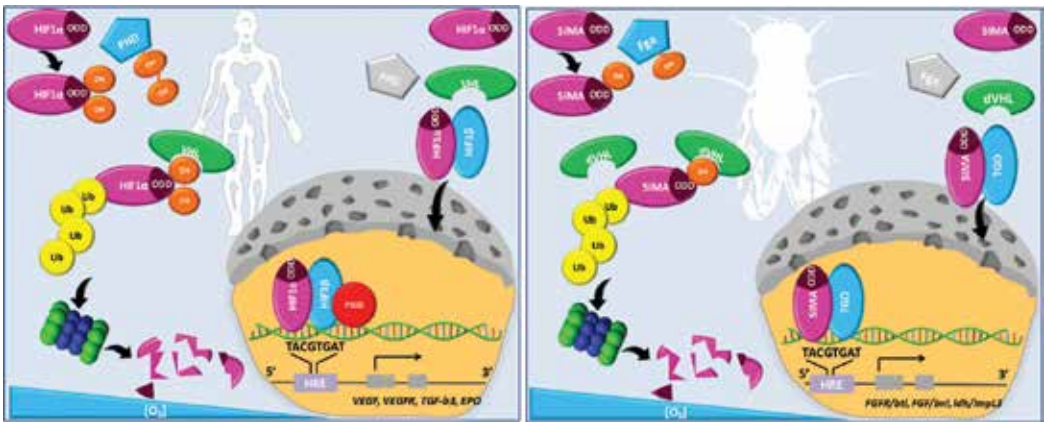

Figure 2. The HIF-1α signaling pathway is conserved between *Drosophila* and mammals. In normoxia, the PHD (Fga in flies) catalyzes the hydroxylation of HIF-1α (Sima in flies). Binding of the hydroxylated HIF-1α by the tumor suppressor protein VHL (dVHL in flies) leads to HIF-1α/Sima polyubiquitination and degradation by the proteasome. In hypoxia, HIF-1α/Sima is stabilized and dimerizes with HIF-1β (Tgo in flies) forming the HIF1 heterodimer. HIF1 is translocated to the nucleus, binds HREs, and induces the transcription of hypoxia-responsive genes.

expression of metabolic genes encoding key glycolytic enzymes [78, 79, 188]. In addition, the transcription factor HIF-1α controls expression of a number of genes involved in different hallmarks of cancer including modifiers of cellular metabolism that facilitate neoplasia [189]. A recent study in *Drosophila* investigated the mechanisms impinging on metabolism deregulation under sufficient oxygen levels and the role of HIF-1α in this process [145] (**Figure 3**). The authors induced a glycolytic tumor in the fly wing imaginal disk by expressing an activated form of the PDGF/VEGF-receptor (Pvr) oncogene, and they observed whether various oncogenic signaling pathways can interact with HIF-1α/*sima* and successively upregulate key metabolic enzymes [145]. Pvr was found to induce strong lactate dehydrogenase (LDH) enzymatic activity, which is a hallmark of aerobic glycolysis and the Warburg effect. Pvr^{act} expression along the anterior-posterior boundary of the wing disk caused extensive growth and dysplasia, and the cells within the mass of this tumor expressed LDH. Thus, the activation of Pvr resulted in *ldh* upregulation, which led to increased LDH enzymatic activity in the tumor. Since Pvr is an RTK, the authors asked if other activated RTKs that lead to overproliferation may also cause increased LDH activity. Interestingly, they found that, unlike Pvr^{act}, InR^{act} and Egfr^{act} did not induce any LDH expression [145]. Sima, one of the key inducers of LDH in tumors [189], was assessed next, and it was found that Sima protein was stabilized in a Pvr^{act} background and its overexpression led to LDH-induced activity [145]. When silencing *sima* in the same background, LDH expression was suppressed. Thus, Sima is necessary for the activation of LDH. Ras^{act}, an effector of all RTKs, was also sufficient to increase LDH activity. Blocking PI3K signaling by targeting different components of the signaling cascade (PI3K, Akt and TOR) led to the suppression of the *LDH-GFP* reporter proving that the PI3K/Akt/TOR axis is necessary for LDH regulation. Co-expression of a gain-of-function allele of human Raf (hRaf^{act}) and activated PI3K (PI3K^{act}) led to extensive LDH activation, whereas

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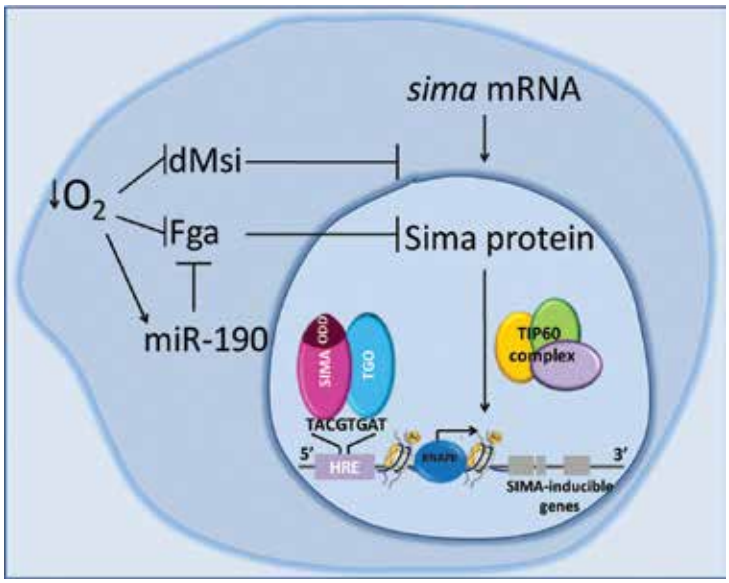

Figure 3. Sima activity is regulated at different levels. In hypoxia, the propyl-4-hydroxylase Fga regulates Sima protein levels post-translationally by promoting its degradation by the proteasome, whereas the RNA-binding protein dMsi represses *sima* mRNA translation. miR-190 is upregulated in hypoxia and inhibits Fga directly, which, in turn, downregulates Sima. Binding of the Sima/Tgo HIF1 heterodimer on the HREs of promoters of hypoxia-responsive genes is necessary for recruitment of the TIP60 chromatin remodeling complex, which, in turn, promotes recruitment of RNA-PolII and efficient transcription.

PI3K/ERK activation led to extensive Sima expression. In the absence of Sima, the PI3K/ERK LDH-GFP expression was reduced. Thus, PI3K and ERK together are necessary and sufficient to induce LDH expression; each signal alone is necessary, but not sufficient, to induce LDH expression. Another known factor that stabilizes HIF-1α is the secretion of ROS even in the presence of sufficient oxygen levels [190]. Peroxidasin (Pxn), a general antioxidant, reduced ROS activity, and when it was expressed together with Pvr^{act} led to the deactivation of the JNK pathway (previously active in a Pvr^{act} background), the suppression of Sima protein accumulation and the reduction of LDH activity. Thus, ROS have a crucial role in metabolism regulation [145]. Moreover, ROS are the central players in the metabolic profile of the cell [191]. They can activate the JNK signaling pathway and use the same mechanisms as hypoxia to stabilize HIF-1α [190, 192]. ROS use a positive feedback loop to strengthen the upstream members of the glycolytic pathway, which is the key point in the metabolic reprogramming of cancer tissues, not only in the absence of sufficient oxygen tensions. Even though hypoxia stabilizes HIF-1α, in the metabolic reprogramming of the cell, hypoxia, unlike HIF-1α, does not play an important role [145]. Interestingly in this study, although Pvr activation was linked to many tumor phenotypes, such as cell shape changes, overproliferation, aerobic glycolysis and local migration, it did not possess any metastatic capability, which correlated with no loss of the cell epithelial polarity that leads to invasive and metastatic abilities [193].

An independent study also assessed Sima expression, as well as induction of tracheogenesis in *Drosophila* epithelial tumors [194] (**Figure 3**). In *Drosophila*, genes involved in the maintenance of apicobasal polarity, such as *lethal giant larvae* (*lgl*), *discs large* (*dlg*) and *scribble* (*scrib*), have been shown to also regulate growth and act as tumor suppressors [195]. Tumors caused by expression of an *lgl* knockdown (lgl^{KD}) in a Minute background in the wing disc of *Drosophila melanogaster* revealed a conserved response to hypoxic stress and also migratory and tracheogenic behaviors [194]. In addition, tumors generated when a loss-of-function mutation of the gene $l(2)gl^{4}$ was combined with the oncogenic form of *Ras*, Ras^{V12}, expressed *bnl*/*FGF* and formed new trachea-like branched structures. Strikingly, cells within the tumor expressed the gene *trh*, a paralogue of *sima*, which is necessary for *btl*/*FGFR* expression and serves as a classic tracheal cell marker in *Drosophila* [135, 196]. These tumor cells induced extra branching of the trachea that was associated with the tumor (similar to sprouting angiogenesis), they synthesized de novo new patterns of trachea-like branches (similar to vascular mimicry), and they migrated toward and incorporated into neighboring pre-existing tracheal tubes (similar to vascular cooption). Importantly, expression of *trh* in the tumor cells transformed some of them to tracheal cells, giving them a different cellular identity and contributing to tumor heterogeneity [194]. Sima exhibited nuclear localization in some of the tumor cells, and this led to activation of the *bnl*/*FGF* promoter. Furthermore, the Polycomb group (PcG) of proteins, which are known epigenetic regulators leading to transcriptional repression in *Drosophila*, was downregulated in the undifferentiated cancer cells, whereas when these cells turned into tracheal cells, they started expressing PcG. This is in agreement with the known function of PcG, which is lowly expressed in stem cells/undifferentiated cells and is upregulated in differentiated cells to lock a particular cell fate [197]. During this epithelial-to-tracheal switch, the JAK/STAT signaling pathway activity was also observed. A correlation between PcG repression and JNK activity was found, whereas the inhibition of JNK led to the opposite result [194]. This publication characterized tracheogenesis of *Drosophila melanogaster* as a novel hallmark of cancer reminiscent of tumor angiogenesis, establishing this model organism as a powerful and promising model for the study of the molecular alterations in the hypoxic microenvironment of the tumor.

3. Conclusions and future perspectives

This book chapter discusses our current knowledge on HIFs and their major roles in development, physiology and disease pathology, using examples of studies in the model organism *Drosophila melanogaster*. The mammalian HIF-1α transcription factor regulates a plethora of genes that promote various aspects of cancer, such as metabolism, invasive motility, growth, angiogenesis and drug resistance (**Table 1**) [189, 198, 199].

The HIF-1α transcription factor has been extensively studied in mammals and in a variety of model organisms due to its highly conserved sequence and function (**Figure 1**). The extensive literature on the *Drosophila* HIF1 pathway suggests that the fruit fly is a potentially good model to study the basic mechanisms of HIF-1α/Sima regulation (**Figure 2**) and identify novel members of the pathway in normoxia and hypoxia (see above). Indeed, a series of studies in *Drosophila* have identified novel

regulators of HIF-1α, which are conserved in mammals and potentially could function in a similar way on mammalian HIF1 [169, 184, 185, 200] (**Figure 3**). Strikingly, recent studies in *Drosophila* [145, 194] underscore the key role of HIF-1α/Sima in tumorigenesis and tumor angiogenesis and suggest that the fruit fly can serve as a great model for studies of the Warburg effect and pathological angiogenesis (**Figure 4**). Impressively, expression of a single oncogene, the activated Pvr, in *Drosophila* can cause glycolytic epithelial tumors that turn on HIF-1α/Sima expression and the glycolysis pathway at the expense of oxidative phosphorylation [145]. Moreover, epithelial neoplasias caused by inactivation of tumor suppressor genes, such as *lgl*, in combination with oncogenic Ras are heterogeneous, and some cells in the tumor induce expression of *HIF-1α*/*Sima*, *trh*, and *bnl*/*FGF*, and a subset of them differentiate into tracheal cells. Therefore, these tumors can promote processes reminiscent of sprouting angiogenesis, vascular cooption and vascular mimicry and indicate that, although *Drosophila* does not possess typical blood vessels, its tracheal system may function similar to mammalian blood vessels in pathological situations.

The contribution of HIF-1α/Sima in epithelial tumorigenesis and tracheogenesis in *Drosophila* is intriguing especially in the light of various studies that show a close proximity of tracheal cells with healthy and tumorous epithelia. For example, metastatic tumor cells have been shown to adhere and move alongside the tracheal network [193, 201] and expansion of the trachea has been observed in a PI3K/Ras glioma model in larval and adult brains [202]. In addition, the adult

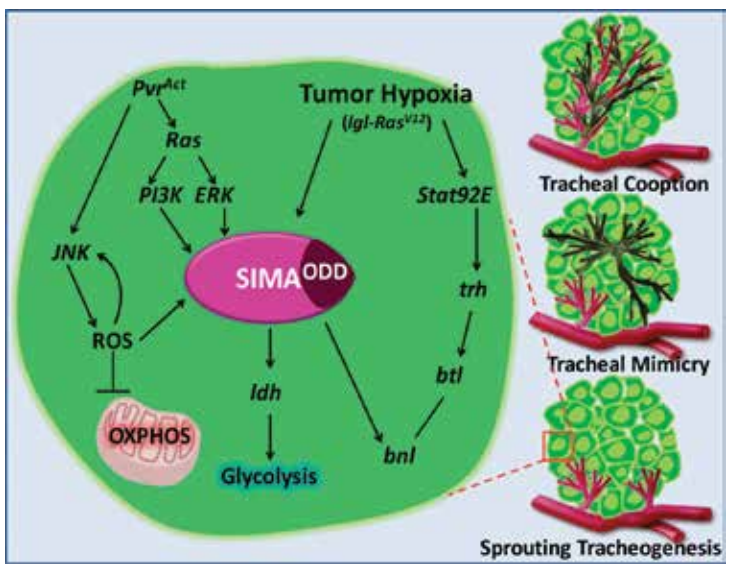

Figure 4. *Drosophila* epithelial tumors induce Sima, the Warburg effect and angiogenesis-like phenotypes. Pvr^{act} triggers the activation of the PI3K/Akt and Raf/ERK signaling pathways in a Ras-dependent manner controlling Sima activity. Pvr^{act} requires JNK activation, which blocks OXPHOS through the production of ROS. ROS produced by the metabolic reprogramming of the tumor responds to the upstream components in order to maintain the Warburg effect. Thus, there is a positive feedback loop that sustains the Warburg effect over time through the activation of LDH, which is induced by Sima. Overall, this mechanism is HIF-dependent but hypoxia-independent. In *lgl-Ras* epithelial neoplasias, tumor hypoxia leads to stabilization of Sima and *bnl*/*FGF* transcription, as well as STAT92E-dependent *trh* expression, which strengthens activation of the *btl*/*FGFR* promoter. All these events promote tumor tracheogenesis via three different strategies reminiscent of tumor angiogenesis: sprouting tracheogenesis, tracheal cooption and tracheal mimicry.

fly intestine is oxygenated by an extensive network of visceral trachea that has been shown to contribute growth and regeneration signals to the intestinal stem cells [203–205]. Recently, it has been shown that mutations of the Sox21α transcription factor cause heterogeneous intestinal neoplasias, inside which some tumor cells express *btl*/*FGFR*, suggesting maybe a phenomenon of tracheal mimicry or cooption [206]. Since many oncogenes and tumor suppressors as well as combinations of mutations with cellular stress caused by pathogens or chemicals can lead to intestinal dysplasia in flies [207, 208], it remains to be seen if and how the HIF-1α/Sima-promoted metabolic changes and tracheogenesis interact with the tumorous environment.

Undoubtedly, *Drosophila melanogaster* has still a lot to contribute toward our understanding of HIF-1α regulation in physiology, hypoxia, tumorigenesis, and angiogenesis. Understanding the function of the HIF pathway in genetically tractable invertebrates will allow the discovery of novel-conserved pathway components that may be used as therapeutic targets in humans.

Abbreviation

ago1	*argonaute 1*
Ago	Archipelago
ARNT2	aryl hydrocarbon receptor nuclear translocator 2
ARNT3	aryl hydrocarbon receptor nuclear translocator 3
ATP	adenosine tri-phosphate
bHLH-PAS	basic-Helix-Loop-Helix-Per/ARNT/Sim
bnl	*branchless*
btl	*breathless*
Cdk4	cyclin-dependent protein kinase 4
CoA	acetyl coenzyme A
CO_2	carbon di oxide
Cul2	cullin 2
dlg	*discs large*
dMsi	*Drosophila* Mushashi
EGFR	epidermal growth factor receptor
EGFs	epidermal growth factors
EPO	erythropoietin
fga	*fatiga*
FGF	fibroblast growth factor
GLUTs	glucose transporters
GLUT1	glucose transporter 1
GRPs	glucose regulated proteins
HIFs	hypoxia-inducible factors
HIF1	hypoxia-inducible factor 1

HIF-1α	hypoxia-inducible factor-1α
HIF-1β	hypoxia-inducible factor-1β
HIF-2α	hypoxia-inducible factor-2α
HIF-3α	hypoxia-inducible factor-3α
$hRaf^{act}$	human Raf gain-of-function
HREs	hypoxia-response elements
ID	inhibitory domain
IGF-I	insulin growth factor I
ILP	insulin-like peptide
IL-1α	interleukin-1α
IL-1β	interleukin-1β
IL-8	interleukin-8
InR	insulin receptor
LDH	lactate dehydrogenase
LDHA	lactate dehydrogenase A
lgl	*lethal giant larvae*
lgl^{KD}	*lgl* knockdown
miR	microRNA
Msi	Musashi
NF-κB	nuclear factor-kappa B
N^{fl}	Notch full-length
NLS-C	carboxy-terminal Nuclear Localization Signals
NLS-N	amino-terminal Nuclear Localization Signals
NO	nitric oxide
NOS	nitric oxide synthase
NOS1	nitric oxide synthase1
ORPs	oxygen regulated Proteins
OXPHOS	oxidative phosphorylation
O_2	oxygen
PcG	polycomb group
PDGF-β	platelet-derived growth factor beta
PDK1	pyruvate dehydrogenase kinase 1
PDK-1	phosphoinositide-dependent kinase 1
PHD	prolyl-4-hydroxylase
PI	phosphatidylinositol
PI3K	phosphatidylinositol 3-kinase

$PI3K^{act}$	PI3K activated
PI-3P	phosphatidylinositol 3-phosphate
PKB	protein Kinase B
Pro	proline
PSTD	Proline-Serine–Threonine-rich protein stabilization Domain
PTEN	phosphatase and TENsing homolog
Pvr	PDGF/VEGF-receptor
Pxn	peroxidasin
Rbx1	ring box protein 1
RNAi	RNA interference
ROSs	reactive oxygen species
scrib	*scribble*
sima	*similar*
Slbo	slow border cells
TAD-C	carboxy-terminal transactivation domains
TAD-N	amino-terminal transactivation domains
TGF-α	transforming growth factor alpha
tgo	*tango*
TNF-α	tumor necrosis factor α
trh	*trachealess*
VEGF	vascular endothelial growth factor
VHL	von Hippel-Lindau

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References

[1] Lane N, Martin W. The energetics of genome complexity. Nature. 2010;**467**(7318):929-934

[2] West JB. Carl Wilhelm Scheele, the discoverer of oxygen, and a very productive chemist. American Journal of Physiology. Lung Cellular and Molecular Physiology. 2014: p. ajplung 00223 2014

[3] Semenza GL. Hypoxia-inducible factors in physiology and medicine. Cell. 2012;**148**(3): 399-408

[4] Semenza GL. Regulation of oxygen homeostasis by hypoxia-inducible factor 1. Physiology (Bethesda). 2009;**24**:97-106

[5] Semenza GL. Hydroxylation of HIF-1: Oxygen sensing at the molecular level. Physiology (Bethesda). 2004;**19**:176-182

[6] Semenza GL. Hypoxia-inducible factor 1: Oxygen homeostasis and disease pathophysiology. Trends in Molecular Medicine. 2001;**7**(8):345-350

[7] Shay JE, Celeste Simon M. Hypoxia-inducible factors: Crosstalk between inflammation and metabolism. Seminars in Cell & Developmental Biology. 2012;**23**(4):389-394

[8] Stone J et al. Development of retinal vasculature is mediated by hypoxia-induced vascular endothelial growth factor (VEGF) expression by neuroglia. The Journal of Neuroscience. 1995;**15**(7 Pt 1):4738-4747

[9] Semenza GL. HIF-1: Mediator of physiological and pathophysiological responses to hypoxia. Journal of Applied Physiology (1985). 2000;**88**(4):1474-1480

[10] Semenza GL, Wang GL. A nuclear factor induced by hypoxia via de novo protein synthesis binds to the human erythropoietin gene enhancer at a site required for transcriptional activation. Molecular and Cellular Biology. 1992;**12**(12):5447-5454

[11] Tian H, McKnight SL, Russell DW. Endothelial PAS domain protein 1 (EPAS1), a transcription factor selectively expressed in endothelial cells. Genes & Development. 1997;**11**(1):72-82

[12] Gu YZ et al. Molecular characterization and chromosomal localization of a third alpha-class hypoxia inducible factor subunit, HIF3alpha. Gene Expression. 1998;**7**(3):205-213

[13] Hampton-Smith RJ, Peet DJ. From polyps to people: A highly familiar response to hypoxia. Annals of the New York Academy of Sciences. 2009;**1177**:19-29

[14] Loenarz C et al. The hypoxia-inducible transcription factor pathway regulates oxygen sensing in the simplest animal, *Trichoplax adhaerens*. EMBO Reports. 2011;**12**(1):63-70

[15] Manalo DJ et al. Transcriptional regulation of vascular endothelial cell responses to hypoxia by HIF-1. Blood. 2005;**105**(2):659-669

[16] Ivan M et al. HIFalpha targeted for VHL-mediated destruction by proline hydroxylation: Implications for O_2 sensing. Science. 2001;**292**(5516):464-468

[17] Kaelin Jr WG, Ratcliffe PJ. Oxygen sensing by metazoans: The central role of the HIF hydroxylase pathway. Molecular Cell. 2008;**30**(4):393-402

[18] Wang GL, Semenza GL. Purification and characterization of hypoxia-inducible factor 1. The Journal of Biological Chemistry. 1995;**270**(3):1230-1237

[19] Wang GL et al. Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O_2 tension. Proceedings of the National Academy of Sciences of the United States of America. 1995;**92**(12):5510-5514

[20] Arquier N et al. Analysis of the hypoxia-sensing pathway in *Drosophila melanogaster*. The Biochemical Journal. 2006;**393**(Pt 2):471-480

[21] Schofield CJ, Ratcliffe PJ. Oxygen sensing by HIF hydroxylases. Nature Reviews. Molecular Cell Biology. 2004;**5**(5):343-354

[22] O'Rourke JF et al. Oxygen-regulated and transactivating domains in endothelial PAS protein 1: Comparison with hypoxia-inducible factor-1alpha. The Journal of Biological Chemistry. 1999;**274**(4):2060-2071

[23] Wiesener MS et al. Induction of endothelial PAS domain protein-1 by hypoxia: Characterization and comparison with hypoxia-inducible factor-1alpha. Blood. 1998; **92**(7):2260-2268

[24] Wenger RH et al. Nucleotide sequence, chromosomal assignment and mRNA expression of mouse hypoxia-inducible factor-1 alpha. Biochemical and Biophysical Research Communications. 1996;**223**(1):54-59

[25] Wiener CM, Booth G, Semenza GL. In vivo expression of mRNAs encoding hypoxia-inducible factor 1. Biochemical and Biophysical Research Communications. 1996; **225**(2):485-488

[26] Jain S et al. Expression of ARNT, ARNT2, HIF1 alpha, HIF2 alpha and ah receptor mRNAs in the developing mouse. Mechanisms of Development. 1998;**73**(1):117-123

[27] Epstein AC et al. *C. elegans* EGL-9 and mammalian homologs define a family of dioxygenases that regulate HIF by prolyl hydroxylation. Cell. 2001;**107**(1):43-54

[28] Giaccia AJ, Simon MC, Johnson R. The biology of hypoxia: The role of oxygen sensing in development, normal function, and disease. Genes & Development. 2004;**18**(18):2183-2194

[29] Mortimer NT, Moberg KH. Regulation of *Drosophila* embryonic tracheogenesis by dVHL and hypoxia. Developmental Biology. 2009;**329**(2):294-305

[30] Lando D et al. FIH-1 is an asparaginyl hydroxylase enzyme that regulates the transcriptional activity of hypoxia-inducible factor. Genes & Development. 2002;**16**(12):1466-1471

[31] Kelly BD et al. Cell type-specific regulation of angiogenic growth factor gene expression and induction of angiogenesis in nonischemic tissue by a constitutively active form of hypoxia-inducible factor 1. Circulation Research. 2003;**93**(11):1074-1081

[32] Bruick RK. Expression of the gene encoding the proapoptotic Nip3 protein is induced by hypoxia. Proceedings of the National Academy of Sciences of the United States of America. 2000;**97**(16):9082-9087

[33] Sowter HM et al. HIF-1-dependent regulation of hypoxic induction of the cell death factors BNIP3 and NIX in human tumors. Cancer Research. 2001;**61**(18):6669-6673

[34] Hu J et al. Hypoxia regulates expression of the endothelin-1 gene through a proximal hypoxia-inducible factor-1 binding site on the antisense strand. Biochemical and Biophysical Research Communications. 1998;**245**(3):894-899

[35] Semenza GL et al. Transcriptional regulation of genes encoding glycolytic enzymes by hypoxia-inducible factor 1. The Journal of Biological Chemistry. 1994;**269**(38):23757-23763

[36] Wang GL, Semenza GL. Characterization of hypoxia-inducible factor 1 and regulation of DNA binding activity by hypoxia. The Journal of Biological Chemistry. 1993; **268**(29):21513-21518

[37] Graven KK et al. Identification of an oxygen responsive enhancer element in the glyceraldehyde-3-phosphate dehydrogenase gene. Biochimica et Biophysica Acta. 1999; **1447**(2-3):208-218

[38] Ebert BL, Firth JD, Ratcliffe PJ. Hypoxia and mitochondrial inhibitors regulate expression of glucose transporter-1 via distinct cis-acting sequences. The Journal of Biological Chemistry. 1995;**270**(49):29083-29089

[39] Iyer NV et al. Cellular and developmental control of O_2 homeostasis by hypoxia-inducible factor 1 alpha. Genes & Development. 1998;**12**(2):149-162

[40] Takahashi Y et al. Hypoxic induction of prolyl 4-hydroxylase alpha (I) in cultured cells. The Journal of Biological Chemistry. 2000;**275**(19):14139-14146

[41] Feldser D et al. Reciprocal positive regulation of hypoxia-inducible factor 1alpha and insulin-like growth factor 2. Cancer Research. 1999;**59**(16):3915-3918

[42] Tazuke SI et al. Hypoxia stimulates insulin-like growth factor binding protein 1 (IGFBP-1) gene expression in HepG2 cells: A possible model for IGFBP-1 expression in fetal hypoxia. Proceedings of the National Academy of Sciences of the United States of America. 1998;**95**(17):10188-10193

[43] Zhang H et al. HIF-1 inhibits mitochondrial biogenesis and cellular respiration in VHL-deficient renal cell carcinoma by repression of C-MYC activity. Cancer Cell. 2007; **11**(5):407-420

[44] Corn PG et al. Mxi1 is induced by hypoxia in a HIF-1-dependent manner and protects cells from c-Myc-induced apoptosis. Cancer Biology & Therapy. 2005;**4**(11):1285-1294

[45] Palmer LA et al. Hypoxia induces type II NOS gene expression in pulmonary artery endothelial cells via HIF-1. The American Journal of Physiology. 1998;**274**(2 Pt 1):L212-L219

[46] Kim JW et al. HIF-1-mediated expression of pyruvate dehydrogenase kinase: A metabolic switch required for cellular adaptation to hypoxia. Cell Metabolism. 2006;**3**(3):177-185

[47] Papandreou I et al. HIF-1 mediates adaptation to hypoxia by actively downregulating mitochondrial oxygen consumption. Cell Metabolism. 2006;**3**(3):187-197

[48] Schaffer L et al. Oxygen-regulated expression of TGF-beta 3, a growth factor involved in trophoblast differentiation. Placenta. 2003;**24**(10):941-950

[49] Hansson LO et al. Two sequence motifs from HIF-1alpha bind to the DNA-binding site of p53. Proceedings of the National Academy of Sciences of the United States of America. 2002;**99**(16):10305-10309

[50] Yang MH et al. Direct regulation of TWIST by HIF-1alpha promotes metastasis. Nature Cell Biology. 2008;**10**(3):295-305

[51] Forsythe JA et al. Activation of vascular endothelial growth factor gene transcription by hypoxia-inducible factor 1. Molecular and Cellular Biology. 1996;**16**(9):4604-4613

[52] Gerber HP et al. Differential transcriptional regulation of the two vascular endothelial growth factor receptor genes. Flt-1, but not Flk-1/KDR, is up-regulated by hypoxia. The Journal of Biological Chemistry. 1997;**272**(38):23659-23667

[53] Swift MR, Weinstein BM. Arterial-venous specification during development. Circulation Research. 2009;**104**(5):576-588

[54] Potente M, Gerhardt H, Carmeliet P. Basic and therapeutic aspects of angiogenesis. Cell. 2011;**146**(6):873-887

[55] Cerimele F et al. Infectious angiogenesis: *Bartonella bacilliformis* infection results in endothelial production of angiopoetin-2 and epidermal production of vascular endothelial growth factor. The American Journal of Pathology. 2003;**163**(4):1321-1327

[56] Carmeliet P. Angiogenesis in health and disease. Nature Medicine. 2003;**9**(6):653-660

[57] Folkman J. Angiogenesis: An organizing principle for drug discovery? Nature Reviews. Drug Discovery. 2007;**6**(4):273-286

[58] Arroyo AG, Iruela-Arispe ML. Extracellular matrix, inflammation, and the angiogenic response. Cardiovascular Research. 2010;**86**(2):226-235

[59] Hanahan D, Weinberg RA. Hallmarks of cancer: The next generation. Cell. 2011;**144**(5): 646-674

[60] Iliopoulos D, Hirsch HA, Struhl K. An epigenetic switch involving NF-kappaB, Lin28, Let-7 MicroRNA, and IL6 links inflammation to cell transformation. Cell. 2009; **139**(4):693-706

[61] Folkman J. Fundamental concepts of the angiogenic process. Current Molecular Medicine. 2003;**3**(7):643-651

[62] Trinchieri G. Cancer and inflammation: An old intuition with rapidly evolving new concepts. Annual Review of Immunology. 2012;**30**:677-706

[63] Weis SM, Cheresh DA. Tumor angiogenesis: Molecular pathways and therapeutic targets. Nature Medicine. 2011;**17**(11):1359-1370

[64] Hardee ME et al. Novel imaging provides new insights into mechanisms of oxygen transport in tumors. Current Molecular Medicine. 2009;**9**(4):435-441

[65] Carmeliet P, Jain RK. Angiogenesis in cancer and other diseases. Nature. 2000;**407**(6801): 249-257

[66] Nishida N et al. Angiogenesis in cancer. Vascular Health and Risk Management. 2006;**2**(3):213-219

[67] Winder T, Lenz HJ. Vascular endothelial growth factor and epidermal growth factor signaling pathways as therapeutic targets for colorectal cancer. Gastroenterology. 2010;**138**(6):2163-2176

[68] Baba Y et al. HIF1A overexpression is associated with poor prognosis in a cohort of 731 colorectal cancers. The American Journal of Pathology. 2010;**176**(5):2292-2301

[69] Bertout JA, Patel SA, Simon MC. The impact of O_2 availability on human cancer. Nature Reviews. Cancer. 2008;**8**(12):967-975

[70] Warburg O. On the origin of cancer cells. Science. 1956;**123**(3191):309-314

[71] Dewhirst MW, Cao Y, Moeller B. Cycling hypoxia and free radicals regulate angiogenesis and radiotherapy response. Nature Reviews. Cancer. 2008;**8**(6):425-437

[72] Cairns RA. Drivers of the Warburg phenotype. Cancer Journal. 2015;**21**(2):56-61

[73] Warburg O. Über den Stoffwechsel der Carcinomzelle. Naturwissenschaften. 1924;**12**(50): 1131-1137

[74] Bensinger SJ, Christofk HR. New aspects of the Warburg effect in cancer cell biology. Seminars in Cell & Developmental Biology. 2012;**23**(4):352-361

[75] The metabolism of tumours. Investigations from the Kaiser-Wilhelm Institute for Biology, Berlin-Dahlem. Edited by Otto Warburg, Kaiser-Wilhelm Institute for Biology, Berlin-Dahlem. Translated from the German edition, with accounts of additional recent researches, by Frank Dickens, M.A., Ph.D., whole-time worker for the Medical Research Council, Courtauld Institute of Biochemistry, Middlesex Hospital, London. Demy 8vo. Pp. 327 + xxix. Illustrated. 1930. London: Constable & Co. Ltd. 40s. net. British Journal of Surgery, 1931;**19**(73):168-168

[76] Warburg O. On respiratory impairment in cancer cells. Science. 1956;**124**(3215):269-270

[77] Warburg O. Origin of cancer cells. Oncologia. 1956;**9**(2):75-83

[78] DeBerardinis RJ et al. The biology of cancer: Metabolic reprogramming fuels cell growth and proliferation. Cell Metabolism. 2008;**7**(1):11-20

[79] Jones RG, Thompson CB. Tumor suppressors and cell metabolism: A recipe for cancer growth. Genes & Development. 2009;**23**(5):537-548

[80] Hsu PP, Sabatini DM. Cancer cell metabolism: Warburg and beyond. Cell. 2008;**134**(5): 703-707

[81] Cairns RA, Harris IS, Mak TW. Regulation of cancer cell metabolism. Nature Reviews. Cancer. 2011;**11**(2):85-95

[82] Osthus RC et al. Deregulation of glucose transporter 1 and glycolytic gene expression by c-Myc. The Journal of Biological Chemistry. 2000;**275**(29):21797-21800

[83] Shim H et al. C-Myc transactivation of LDH-A: Implications for tumor metabolism and growth. Proceedings of the National Academy of Sciences of the United States of America. 1997;**94**(13):6658-6663

[84] Dang CV et al. The interplay between MYC and HIF in cancer. Nature Reviews. Cancer. 2008;**8**(1):51-56

[85] Semenza GL. Defining the role of hypoxia-inducible factor 1 in cancer biology and therapeutics. Oncogene. 2010;**29**(5):625-634

[86] Semenza GL. HIF-1: Upstream and downstream of cancer metabolism. Current Opinion in Genetics & Development. 2010;**20**(1):51-56

[87] Kroemer G, Pouyssegur J. Tumor cell metabolism: cancer's Achilles' heel. Cancer Cell. 2008;**13**(6):472-482

[88] Maxwell PH et al. The tumour suppressor protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis. Nature. 1999;**399**(6733):271-275

[89] Zhong H et al. Overexpression of hypoxia-inducible factor 1alpha in common human cancers and their metastases. Cancer Research. 1999;**59**(22):5830-5835

[90] Ravi R et al. Regulation of tumor angiogenesis by p53-induced degradation of hypoxia-inducible factor 1alpha. Genes & Development. 2000;**14**(1):34-44

[91] Jiang BH et al. V-SRC induces expression of hypoxia-inducible factor 1 (HIF-1) and transcription of genes encoding vascular endothelial growth factor and enolase 1: Involvement of HIF-1 in tumor progression. Cancer Research. 1997;**57**(23):5328-5335

[92] Agani F, Semenza GL. Mersalyl is a novel inducer of vascular endothelial growth factor gene expression and hypoxia-inducible factor 1 activity. Molecular Pharmacology. 1998;**54**(5):749-754

[93] Maxwell PH et al. Hypoxia-inducible factor-1 modulates gene expression in solid tumors and influences both angiogenesis and tumor growth. Proceedings of the National Academy of Sciences of the United States of America. 1997;**94**(15):8104-8109

[94] Maxwell PH et al. The tumour suppressor protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis. Nature. 1999;**399**(6733):271-275

[95] Heacock CS, Sutherland RM. Enhanced synthesis of stress proteins caused by hypoxia and relation to altered cell growth and metabolism. British Journal of Cancer. 1990;**62**(2):217-225

[96] Sciandra JJ, Subjeck JR, Hughes CS. Induction of glucose-regulated proteins during anaerobic exposure and of heat-shock proteins after reoxygenation. Proceedings of the National Academy of Sciences of the United States of America. 1984;**81**(15):4843-4847

[97] Shweiki D et al. Vascular endothelial growth factor induced by hypoxia may mediate hypoxia-initiated angiogenesis. Nature. 1992;**359**(6398):843-845

[98] Kourembanas S et al. Hypoxia induces endothelin gene expression and secretion in cultured human endothelium. The Journal of Clinical Investigation. 1991;**88**(3):1054-1057

[99] Kourembanas S, Hannan RL, Faller DV. Oxygen tension regulates the expression of the platelet-derived growth factor-B chain gene in human endothelial cells. The Journal of Clinical Investigation. 1990;**86**(2):670-674

[100] Goldberg MA, Dunning SP, Bunn HF. Regulation of the erythropoietin gene: Evidence that the oxygen sensor is a heme protein. Science. 1988;**242**(4884):1412-1415

[101] Majumder PK et al. mTOR inhibition reverses Akt-dependent prostate intraepithelial neoplasia through regulation of apoptotic and HIF-1-dependent pathways. Nature Medicine. 2004;**10**(6):594-601

[102] Arsham AM, Howell JJ, Simon MC. A novel hypoxia-inducible factor-independent hypoxic response regulating mammalian target of rapamycin and its targets. The Journal of Biological Chemistry. 2003;**278**(32):29655-29660

[103] Cantley LC, Neel BG. New insights into tumor suppression: PTEN suppresses tumor formation by restraining the phosphoinositide 3-kinase/AKT pathway. Proceedings of the National Academy of Sciences of the United States of America. 1999;**96**(8):4240-4245

[104] Giri D, Ittmann M. Inactivation of the PTEN tumor suppressor gene is associated with increased angiogenesis in clinically localized prostate carcinoma. Human Pathology. 1999;**30**(4):419-424

[105] Zhong H et al. Modulation of hypoxia-inducible factor 1alpha expression by the epidermal growth factor/phosphatidylinositol 3-kinase/PTEN/AKT/FRAP pathway in human prostate cancer cells: Implications for tumor angiogenesis and therapeutics. Cancer Research. 2000;**60**(6):1541-1545

[106] Sutphin PD, Chan DA, Giaccia AJ. Dead cells don't form tumors: HIF-dependent cytotoxins. Cell Cycle. 2004;**3**(2):160-163

[107] Imtiyaz HZ, Simon MC. Hypoxia-inducible factors as essential regulators of inflammation. Current Topics in Microbiology and Immunology. 2010;**345**:105-120

[108] Jung Y et al. Hypoxia-inducible factor induction by tumour necrosis factor in normoxic cells requires receptor-interacting protein-dependent nuclear factor kappa B activation. The Biochemical Journal. 2003;**370**(Pt 3):1011-1017

[109] Zhou J, Schmid T, Brune B. Tumor necrosis factor-alpha causes accumulation of a ubiquitinated form of hypoxia inducible factor-1alpha through a nuclear factor-kappaB-dependent pathway. Molecular Biology of the Cell. 2003;**14**(6):2216-2225

[110] Jung YJ et al. IL-1beta-mediated up-regulation of HIF-1alpha via an NFkappaB/COX-2 pathway identifies HIF-1 as a critical link between inflammation and oncogenesis. The FASEB Journal. 2003;**17**(14):2115-2117

[111] D'Ignazio L, Batie M, Rocha S. Hypoxia and inflammation in cancer, focus on HIF and NF-kappaB. Biomedicine. 2017;**5**(2)

[112] Xia Y, Shen S, Verma IM. NF-kappaB, an active player in human cancers. Cancer Immunology Research. 2014;**2**(9):823-830

[113] Graeber TG et al. Hypoxia-mediated selection of cells with diminished apoptotic potential in solid tumours. Nature. 1996;**379**(6560):88-91

[114] Koch AE et al. Interleukin-8 as a macrophage-derived mediator of angiogenesis. Science. 1992;**258**(5089):1798-1801

[115] Huang S et al. Blockade of nuclear factor-kappaB signaling inhibits angiogenesis and tumorigenicity of human ovarian cancer cells by suppressing expression of vascular endothelial growth factor and interleukin 8. Cancer Research. 2000;**60**(19):5334-5339

[116] Liu W et al. Targeted genes and interacting proteins of hypoxia inducible factor-1. International Journal of Biochemistry and Molecular Biology. 2012;**3**(2):165-178

[117] Bandarra D et al. HIF-1alpha restricts NF-kappaB-dependent gene expression to control innate immunity signals. Disease Models & Mechanisms. 2015;**8**(2):169-181

[118] Kim HJ, Hawke N, Baldwin AS. NF-kappaB and IKK as therapeutic targets in cancer. Cell Death and Differentiation. 2006;**13**(5):738-747

[119] Pawlus MR, Wang L, Hu CJ. STAT3 and HIF1alpha cooperatively activate HIF1 target genes in MDA-MB-231 and RCC4 cells. Oncogene. 2014;**33**(13):1670-1679

[120] Cummins EP et al. Prolyl hydroxylase-1 negatively regulates IkappaB kinase-beta, giving insight into hypoxia-induced NFkappaB activity. Proceedings of the National Academy of Sciences of the United States of America. 2006;**103**(48):18154-18159

[121] Fu J, Taubman MB. Prolyl hydroxylase EGLN3 regulates skeletal myoblast differentiation through an NF-kappaB-dependent pathway. The Journal of Biological Chemistry. 2010;**285**(12):8927-8935

[122] Takeda Y et al. Macrophage skewing by Phd2 haplodeficiency prevents ischaemia by inducing arteriogenesis. Nature. 2011;**479**(7371):122-126

[123] Biddlestone J, Bandarra D, Rocha S. The role of hypoxia in inflammatory disease (review). International Journal of Molecular Medicine. 2015;**35**(4):859-869

[124] Fernandez-Majada V et al. Nuclear IKK activity leads to dysregulated notch-dependent gene expression in colorectal cancer. Proceedings of the National Academy of Sciences of the United States of America. 2007;**104**(1):276-281

[125] Brunelle JK et al. Oxygen sensing requires mitochondrial ROS but not oxidative phosphorylation. Cell Metabolism. 2005;**1**(6):409-414

[126] Guzy RD et al. Mitochondrial complex III is required for hypoxia-induced ROS production and cellular oxygen sensing. Cell Metabolism. 2005;**1**(6):401-408

[127] Bell EL et al. Mitochondrial reactive oxygen species trigger hypoxia-inducible factor-dependent extension of the replicative life span during hypoxia. Molecular and Cellular Biology. 2007;**27**(16):5737-5745

[128] Peyssonnaux C et al. Cutting edge: Essential role of hypoxia inducible factor-1alpha in development of lipopolysaccharide-induced sepsis. Journal of Immunology. 2007; **178**(12):7516-7519

[129] Nishi K et al. LPS induces hypoxia-inducible factor 1 activation in macrophage-differentiated cells in a reactive oxygen species-dependent manner. Antioxidants & Redox Signaling. 2008;**10**(5):983-995

[130] Frede S et al. Bacterial lipopolysaccharide induces HIF-1 activation in human monocytes via p44/42 MAPK and NF-kappaB. The Biochemical Journal. 2006;**396**(3):517-527

[131] Fang HY et al. Hypoxia-inducible factors 1 and 2 are important transcriptional effectors in primary macrophages experiencing hypoxia. Blood. 2009;**114**(4):844-859

[132] Schumacker PT. Current paradigms in cellular oxygen sensing. Advances in Experimental Medicine and Biology. 2003;**543**:57-71

[133] Newton IP et al. Adenomatous polyposis coli and hypoxia-inducible factor-1{alpha} have an antagonistic connection. Molecular Biology of the Cell. 2010;**21**(21):3630-3638

[134] Nathke I, Rocha S. Antagonistic crosstalk between APC and HIF-1alpha. Cell Cycle. 2011;**10**(10):1545-1547

[135] Wilk R, Weizman I, Shilo BZ. *trachealess* encodes a bHLH-PAS protein that is an inducer of tracheal cell fates in *Drosophila*. Genes & Development. 1996;**10**(1):93-102

[136] Gorr TA, Gassmann M, Wappner P. Sensing and responding to hypoxia via HIF in model invertebrates. Journal of Insect Physiology. 2006;**52**(4):349-364

[137] Nambu JR et al. The *Drosophila single-minded* gene encodes a helix-loop-helix protein that acts as a master regulator of CNS midline development. Cell. 1991;**67**(6):1157-1167

[138] Lavista-Llanos S et al. Control of the hypoxic response in *Drosophila melanogaster* by the basic helix-loop-helix PAS protein Similar. Molecular and Cellular Biology. 2002;**22**(19):6842-6853

[139] Bruick RK, McKnight SL. A conserved family of prolyl-4-hydroxylases that modify HIF. Science. 2001;**294**(5545):1337-1340

[140] Centanin L, Ratcliffe PJ, Wappner P. Reversion of lethality and growth defects in Fatiga oxygen-sensor mutant flies by loss of hypoxia-inducible factor-alpha/Sima. EMBO Reports. 2005;**6**(11):1070-1075

[141] Huang LE et al. Regulation of hypoxia-inducible factor 1alpha is mediated by an O_2-dependent degradation domain via the ubiquitin-proteasome pathway. Proceedings of the National Academy of Sciences of the United States of America. 1998;**95**(14):7987-7992

[142] Jiang BH et al. Transactivation and inhibitory domains of hypoxia-inducible factor 1alpha. Modulation of transcriptional activity by oxygen tension. The Journal of Biological Chemistry. 1997;**272**(31):19253-19260

[143] Gorr TA et al. Regulation of *Drosophila* hypoxia-inducible factor (HIF) activity in SL2 cells: Identification of a hypoxia-induced variant isoform of the HIFalpha homolog gene *similar*. The Journal of Biological Chemistry. 2004;**279**(34):36048-36058

[144] Centanin L et al. Cell autonomy of HIF effects in *Drosophila*: Tracheal cells sense hypoxia and induce terminal branch sprouting. Developmental Cell. 2008;**14**(4):547-558

[145] Wang CW et al. In vivo genetic dissection of tumor growth and the Warburg effect. eLife. 2016;**5**

[146] Acevedo JM et al. Oxygen sensing in *Drosophila*: Multiple isoforms of the prolyl hydroxylase Fatiga have different capacity to regulate HIFalpha/Sima. PLoS One. 2010;**5**(8): e12390

[147] Baird NA, Turnbull DW, Johnson EA. Induction of the heat shock pathway during hypoxia requires regulation of heat shock factor by hypoxia-inducible factor-1. The Journal of Biological Chemistry. 2006;**281**(50):38675-38681

[148] Ghabrial A et al. Branching morphogenesis of the *Drosophila* tracheal system. Annual Review of Cell and Developmental Biology. 2003;**19**:623-647

[149] Lebreton G, Casanova J. Specification of leading and trailing cell features during collective migration in the *Drosophila* Trachea. Journal of Cell Science. 2014;**127**(Pt 2):465-474

[150] Sato M, Kornberg TB. FGF is an essential mitogen and chemoattractant for the air sacs of the *Drosophila* tracheal system. Developmental Cell. 2002;**3**(2):195-207

[151] Weaver M, Krasnow MA. Dual origin of tissue-specific progenitor cells in *Drosophila* tracheal remodeling. Science. 2008;**321**(5895):1496-1499

[152] Guha A, Lin L, Kornberg TB. Organ renewal and cell divisions by differentiated cells in *Drosophila*. Proceedings of the National Academy of Sciences of the United States of America. 2008;**105**(31):10832-10836

[153] Sato M, Kitada Y, Tabata T. Larval cells become imaginal cells under the control of homothorax prior to metamorphosis in the *Drosophila* tracheal system. Developmental Biology. 2008;**318**(2):247-257

[154] Pitsouli C, Perrimon N. Embryonic multipotent progenitors remodel the *Drosophila* airways during metamorphosis. Development. 2010;**137**(21):3615-3624

[155] Sutherland D, Samakovlis C, Krasnow MA. Branchless encodes a *Drosophila* FGF homolog that controls tracheal cell migration and the pattern of branching. Cell. 1996;**87**(6):1091-1101

[156] Klambt C, Glazer L, Shilo BZ. Breathless, a *Drosophila* FGF receptor homolog, is essential for migration of tracheal and specific midline glial cells. Genes & Development. 1992;**6**(9):1668-1678

[157] Krasnow MA. Genes that control organ form: Lessons from bone and branching morphogenesis. Cold Spring Harbor Symposia on Quantitative Biology. 1997;**62**:235-240

[158] Harrison J et al. Responses of terrestrial insects to hypoxia or hyperoxia. Respiratory Physiology & Neurobiology. 2006;**154**(1-2):4-17

[159] Lehmann FO, Heymann N. Unconventional mechanisms control cyclic respiratory gas release in flying *Drosophila*. The Journal of Experimental Biology. 2005;**208**(Pt 19): 3645-3654

[160] Haddad GG et al. Behavioral and Electrophysiologic responses of *Drosophila melanogaster* to prolonged periods of anoxia. Journal of Insect Physiology. 1997;**43**(3): 203-210

[161] Klok CJ et al. Critical oxygen partial pressures and maximal tracheal conductances for *Drosophila Melanogaster* reared for multiple generations in hypoxia or hyperoxia. Journal of Insect Physiology. 2010;**56**(5):461-469

[162] Van Voorhies WA. Metabolic function in *Drosophila melanogaster* in response to hypoxia and pure oxygen. The Journal of Experimental Biology. 2009;**212**(19):3132-3141

[163] Liu G, Roy J, Johnson EA. Identification and function of hypoxia-response genes in *Drosophila melanogaster*. Physiological Genomics. 2006;**25**(1):134-141

[164] Harrison JF, Haddad GG. Effects of oxygen on growth and size: Synthesis of molecular, organismal, and evolutionary studies with *Drosophila melanogaster*. Annual Review of Physiology. 2011;**73**:95-113

[165] Lighton JR, Schilman PE. Oxygen reperfusion damage in an insect. PLoS One. 2007; **2**(12):e1267

[166] Azad P et al. Distinct mechanisms underlying tolerance to intermittent and constant hypoxia in *Drosophila melanogaster*. PLoS One. 2009;**4**(4):e5371

[167] Henry JR, Harrison JF. Plastic and evolved responses of larval tracheae and mass to varying atmospheric oxygen content in *Drosophila melanogaster*. The Journal of Experimental Biology. 2004;**207**(Pt 20):3559-3567

[168] Jarecki J, Johnson E, Krasnow MA. Oxygen regulation of airway branching in *Drosophila* is mediated by *branchless* FGF. Cell. 1999;**99**(2):211-220

[169] Mortimer NT, Moberg KH. The archipelago ubiquitin ligase subunit acts in target tissue to restrict tracheal terminal cell branching and hypoxic-induced gene expression. PLoS Genetics. 2013;**9**(2):e1003314

[170] Irisarri M et al. Central role of the oxygen-dependent degradation domain of *Drosophila* HIFalpha/Sima in oxygen-dependent nuclear export. Molecular Biology of the Cell. 2009;**20**(17):3878-3887

[171] Callier V, Nijhout HF. Plasticity of insect body size in response to oxygen: Integrating molecular and physiological mechanisms. Current Opinion in Insect Science. 2014;**1**: 59-65

[172] Frei C, Edgar BA. *Drosophila* cyclin D/Cdk4 requires Hif-1 prolyl hydroxylase to drive cell growth. Developmental Cell. 2004;**6**(2):241-251

[173] Reiling JH, Hafen E. The hypoxia-induced paralogs Scylla and Charybdis inhibit growth by down-regulating S6K activity upstream of TSC in *Drosophila*. Genes & Development. 2004;**18**(23):2879-2892

[174] Zelzer E et al. Insulin induces transcription of target genes through the hypoxia-inducible factor HIF-1alpha/ARNT. The EMBO Journal. 1998;**17**(17):5085-5094

[175] Dekanty A et al. The insulin-PI3K/TOR pathway induces a HIF-dependent transcriptional response in *Drosophila* by promoting nuclear localization of HIF-alpha/Sima. Journal of Cell Science. 2005;**118**(Pt 23):5431-5441

[176] Romero NM, Dekanty A, Wappner P. Cellular and developmental adaptations to hypoxia: A *Drosophila* perspective. Methods in Enzymology. 2007;**435**:123-144

[177] Doronkin S et al. Dose-dependent modulation of HIF-1alpha/Sima controls the rate of cell migration and invasion in *Drosophila* ovary border cells. Oncogene. 2010;**29**(8): 1123-1134

[178] Montell DJ, Rorth P, Spradling AC. *Slow border cells*, a locus required for a developmentally regulated cell migration during oogenesis, encodes *Drosophila* C/EBP. Cell. 1992;**71**(1):51-62

[179] Mukherjee T et al. Interaction between Notch and Hif-alpha in development and survival of *Drosophila* blood cells. Science. 2011;**332**(6034):1210-1213

[180] Kaelin WG. Proline hydroxylation and gene expression. Annual Review of Biochemistry. 2005;**74**:115-128

[181] Hagen T et al. Redistribution of intracellular oxygen in hypoxia by nitric oxide: Effect on HIF1alpha. Science. 2003;**302**(5652):1975-1978

[182] Wingrove JA, O'Farrell PH. Nitric oxide contributes to behavioral, cellular, and developmental responses to low oxygen in *Drosophila*. Cell. 1999;**98**(1):105-114

[183] Dekanty A et al. *Drosophila* genome-wide RNAi screen identifies multiple regulators of HIF-dependent transcription in hypoxia. PLoS Genetics. 2010;**6**(6):e1000994

[184] De Lella Ezcurra AL et al. miR-190 enhances HIF-dependent responses to hypoxia in *Drosophila* by inhibiting the Prolyl-4-hydroxylase Fatiga. PLoS Genetics. 2016;**12**(5): e1006073

[185] Bertolin AP et al. Musashi mediates translational repression of the *Drosophila* hypoxia inducible factor. Nucleic Acids Research. 2016;**44**(16):7555-7567

[186] Perez-Perri JI et al. The TIP60 complex is a conserved coactivator of HIF1A. Cell Reports. 2016;**16**(1):37-47

[187] Pavlova NN, Thompson CB. The emerging hallmarks of cancer metabolism. Cell Metabolism. 2016;**23**(1):27-47

[188] Chambers JW, LoGrasso PV. Mitochondrial c-Jun N-terminal kinase (JNK) signaling initiates physiological changes resulting in amplification of reactive oxygen species generation. The Journal of Biological Chemistry. 2011;**286**(18):16052-16062

[189] Dang CV, Semenza GL. Oncogenic alterations of metabolism. Trends in Biochemical Sciences. 1999;**24**(2):68-72

[190] Chandel NS et al. Reactive oxygen species generated at mitochondrial complex III stabilize hypoxia-inducible factor-1alpha during hypoxia: A mechanism of O_2 sensing. The Journal of Biological Chemistry. 2000;**275**(33):25130-25138

[191] Sun W et al. Mitochondrial mutations contribute to HIF1alpha accumulation via increased reactive oxygen species and up-regulated pyruvate dehydrogenease kinase 2 in head and neck squamous cell carcinoma. Clinical Cancer Research. 2009;**15**(2): 476-484

[192] Tobiume K et al. ASK1 is required for sustained activations of JNK/p38 MAP kinases and apoptosis. EMBO Reports. 2001;**2**(3):222-228

[193] Pagliarini RA, Xu T. A genetic screen in *Drosophila* for metastatic behavior. Science. 2003;**302**(5648):1227-1231

[194] Grifoni D et al. Multiple strategies of oxygen supply in *Drosophila* malignancies identify tracheogenesis as a novel cancer hallmark. Scientific Reports. 2015;**5**:9061

[195] Bilder D. Epithelial polarity and proliferation control: Links from the *Drosophila* neoplastic tumor suppressors. Genes & Development. 2004;**18**(16):1909-1925

[196] Isaac DD, Andrew DJ. Tubulogenesis in *Drosophila*: A requirement for the *trachealess* gene product. Genes & Development. 1996;**10**(1):103-117

[197] Grossniklaus U, Paro R. Transcriptional silencing by polycomb-group proteins. Cold Spring Harbor Perspectives in Biology. 2014;**6**(11):a019331

[198] Carmeliet P et al. Role of HIF-1alpha in hypoxia-mediated apoptosis, cell proliferation and tumour angiogenesis. Nature. 1998;**394**(6692):485-490

[199] Semenza GL. Targeting HIF-1 for cancer therapy. Nature Reviews. Cancer. 2003;**3**(10): 721-732

[200] Mortimer NT, Moberg KH. The *Drosophila* F-box protein Archipelago controls levels of the Trachealess transcription factor in the embryonic tracheal system. Developmental Biology. 2007;**312**(2):560-571

[201] Hirabayashi S, Baranski TJ, Cagan RL. Transformed *Drosophila* cells evade diet-mediated insulin resistance through *Wingless* signaling. Cell. 2013;**154**(3):664-675

[202] Read RD et al. A *Drosophila* model for EGFR-Ras and PI3K-dependent human glioma. PLoS Genetics. 2009;**5**(2):e1000374

[203] Kux K, Pitsouli C. Tissue communication in regenerative inflammatory signaling: Lessons from the fly gut. Frontiers in Cellular and Infection Microbiology. 2014;**4**:49

[204] Linneweber GA et al. Neuronal control of metabolism through nutrient-dependent modulation of tracheal branching. Cell. 2014;**156**(1-2):69-83

[205] Li Z et al. Trachea-derived Dpp controls adult midgut homeostasis in *Drosophila*. Developmental Cell. 2013;**24**(2):133-143

[206] Zhai Z et al. Accumulation of differentiating intestinal stem cell progenies drives tumorigenesis. Nature Communications. 2015;**6**:10219

[207] Apidianakis Y et al. Synergy between bacterial infection and genetic predisposition in intestinal dysplasia. Proceedings of the National Academy of Sciences of the United States of America. 2009;**106**(49):20883-20888

[208] Patel PH, Dutta D, Edgar BA. Niche appropriation by *Drosophila* intestinal stem cell tumours. Nature Cell Biology. 2015;**17**(9):1182-1192

Chapter 11

Manipulation and Study of Gene Expression in Neurotoxin-Treated Neuronal PC12 and SH-SY5Y Cells for *In Vitro* Studies of Parkinson's Disease

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Additional information is available at the end of the chapter

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Abstract

Neuronal PC12 and SH-SY5Y cells are highly suitable *in vitro* models for study of the neurodegenerative mechanisms occurring in Parkinson's disease (PD). Differentiated PC12 and SH-SY5Y cells bear many similarities to the neuronal populations affected in PD, and they provide a convenient source of large amounts of homogeneous material for biochemical and molecular downstream applications. In the present review, we describe how to differentiate PC12 and SH-SY5Y cells into neuron-like cells and provide protocols for their transfection with plasmids and infection with viral particles to manipulate gene expression. We also describe how to treat neuronal PC12 and SH-SY5Y cells with the classical PD neurotoxins 6-hydroxydopamine (6-OHDA) and 1-methyl-4-phenyl-pyridinium ion (MPP+). Finally, we give detailed methods for several downstream applications useful for the analysis of cell death pathways in PD.

Keywords: Parkinson's disease, PC12, SH-SY5Y, differentiation, 6-hydroxydopamine (6-OHDA), 1-methyl-4-phenylpyridinium (MPP+), transfection, lentiviral infection, survival assay, immunofluorescence, quantitative polymerase chain reaction, Western immunoblotting

1. Introduction

The etiology of Parkinson's disease (PD) is still unknown and likely due to combinations of environmental and genetic factors, ultimately leading to the degeneration of various neuronal populations [1–4]. Neuron death in models of PD requires the transcription-dependent induction of specific pro-death genes. Identifying and manipulating transcriptionally regulated genes that mediate neuron degeneration in PD is a promising strategy for treating PD without knowing

the proximal initiating causes of the disease [5]. Tumor-derived cell lines expressing neuronal properties such as PC12 and SH-SY5Y cells are highly suitable *in vitro* models to apply this strategy. PC12 is a cell line initially isolated from a pheochromocytoma of the rat adrenal medulla [6]. Undifferentiated PC12 cells exit the cell cycle and differentiate into neurons after 1 week of exposure to nerve growth factor (NGF). SH-SY5Y is a human neuroblastoma cell line originally derived from a metastatic bone tumor biopsy. Neuroblast-like SH-SY5Y cells can be withdrawn from the cell cycle and differentiated into a more mature neuron-like phenotype by sequential exposure to retinoic acid and brain-derived neurotrophic factor (BDNF) [7]. Differentiated PC12 and SH-SY5Y cells extend long neurite-like processes, express neuron-specific markers and synthesize the catecholamine neurotransmitters dopamine and norepinephrine. PC12 and SH-SY5Y neurons highly resemble some of the neuronal populations affected in PD, such as the dopaminergic neurons of the substantia nigra *pars compacta* and the noradrenergic neurons of the locus coeruleus and peripheral sympathetic ganglia [1–4]. Under appropriate conditions, neuronal PC12 and SH-SY5Y cells are dependent on the trophic actions of NGF and BDNF, respectively [6, 7]. They are also sensitive to the classical PD neurotoxins 6-hydroxydopamine (6-OHDA) and 1-methyl-4-phenyl-pyridinium ion (MPP+). Therefore, differentiated PC12 and SH-SY5Y cells have been widely used as models to study PD neurodegenerative mechanisms [8–13]. They are easily transfected or infected with viral particles to manipulate gene expression. They additionally provide a convenient source of large amount of homogeneous material for biochemical and molecular downstream applications and the analysis of cell death pathways. These properties also make these lines very useful for drug screening studies.

In this chapter, we will introduce how to apply 6-OHDA and MPP+ to differentiated PC12 and SH-SY5Y cells *in vitro*. Then, we will describe two ways to manipulate gene expression: plasmid transfection and lentiviral-mediated plasmid delivery in cellular PD models. In addition, we will discuss different methods to assess cell survival.

2. Materials

2.1. Coating plasticware

1. For PC12 cells: Reconstitute 10 mg of lyophilized rat tail collagen (Roche) with 5 mL of 0.2% acetic acid. Working dilution is 1:20 in sterile double distilled or deionized water (ddH_2O).
2. For SH-SY5Y cells: Matrigel (BD) should be thawed on ice overnight. Swirl vial gently and make 200 μL aliquots in microcentrifuge tubes using a pre-cooled pipet. Aliquots can be stored at −20°C.

2.2. Cell medium

2.2.1. PC12 cell medium

1. Complete growth medium: RPMI 1640 cell culture medium supplemented with 10% heat inactivated horse serum (Sigma), 5% fetal bovine serum (FBS) and the antibiotics penicillin/streptomycin (pen/strep) (50 units/50 μg/mL final concentration).

2. Differentiation medium: RPMI 1640 cell culture medium supplemented with 1% heat-inactivated horse serum and pen/strep (50 units/50 μg/mL final concentration). NGF should be added to the differentiation medium for PC12 cell neuronal differentiation. Recombinant human or murine NGF stock concentration is 50 μg/mL and should be diluted 1000-fold directly into the culture medium. Medium with diluted NGF should not be stored and should be freshly prepared.
3. Freezing medium: Complete medium containing 10% dimethyl sulfoxide (DMSO).

2.2.2. SH-SY5Y cell medium

1. Growth medium: DMEM supplemented with 10% heat inactivated FBS (Gemini Bioproducts), 2-mM L-glutamine and antibiotics pen/strep (50 units/50 μg/mL final concentration).
2. Differentiation medium I: DMEM supplemented with 5% heat inactivated FBS (Gemini Bioproducts), 2 mM L-glutamine and antibiotics pen/strep (50 units/50 μg/mL final concentration). Retinoic acid is added freshly before use from a 0.5 mM stock (50 ×) in sterile ddH_2O.
3. Differentiation medium II:
   a. Neurobasal medium
   b. B27 supplement (50×)
   c. Antibiotics pen/strep (50 units/50 μg/mL final concentration).
   d. GlutaMAX (Life Technologies) supplied as a 200 mM (100×) liquid stock.
   e. 0.25 M (125×) dibutyryl cyclic adenosine monophosphate (dibutyryl cAMP) in sterile phosphate-buffered saline (PBS).
   f. 100 mM (2000×) stock solution of recombinant human brain-derived neurotrophic factor (rhBDNF) in sterile ddH_2O. Store at −20°C. Avoid multiple freeze and thaw cycles.
   g. 2 M (100×) stock solution of KCl in sterile ddH_2O.

Reagents a–c can be added to Neurobasal medium in advance and kept at 4°C. Reagents d-g should be added to Neurobasal medium with serum just before use.

4. Trypsin: 0.05% trypsin solution (Gibco).
5. Freezing medium: Growth medium containing 10% DMSO.

2.2.3. HEK293T cell medium

1. DMEM cell culture medium supplemented with 10% FBS (Gemini Bioproducts).
2. 0.05% Trypsin-EDTA (Gibco).

2.3. Parkinson's disease toxins

Just before the treatment, prepare a 10 mM 6-OHDA (Tocris) or a 100 mM MPP+ (Sigma) stock solution in sterile ddH_2O.

2.4. Transfection and immunostaining reagents

1. Lipofectamine 2000 (Invitrogen).
2. 1 mg/mL Hoechst 33342 dye (Molecular Probes).
3. 4% formaldehyde: Dilute 16% (4×) formaldehyde aqueous solution (EMS) in PBS before use.

2.5. Lentiviral vector production

1. Calcium phosphate transfection buffer: Prepare and sterile-filter a 250 mM $CaCl_2$ solution in ddH_2O. Sterile-filter a 2 × HBSS (Hank's balanced salt solution) containing 50 mM HEPES, 280 mM NaCl and 15 mM Na_2HPO_4 in ddH_2O. The pH of the 2 × HBSS solution should be precisely adjusted to 7.03–7.04 with 1 M NaOH.

2.6. Western blot

1. 1 × cell lysis buffer: In a 15-mL conical tube, add 1 mL of 10 × cell lysis buffer (Cell Signaling), one tablet of complete mini EDTA-free protease inhibitor cocktail (Roche) and 9 mL of ddH_2O. Mix for 30 min by rotation, prepare 1 mL aliquots and freeze at −20°C.
2. Protein sample buffer: 4 × NupageLDS sample buffer (Life Technologies), 10 × (500 mM) dithiothreitol (Nupage Reducing Agent, Life Technologies) and ddH_2O to achieve a 1–2 μg/μL final protein concentration.
3. 1 × running buffer (1 L): 50 mL of 20 × MOPS running buffer (Life Technologies) and 950 mL of ddH_2O.
4. 1 × transfer buffer (1 L): 50 mL of 20 × transfer buffer (Life Technologies), 150 mL of 95% ethanol and 800 mL of ddH_2O.
5. Ponceau S: 0.1% w/v Ponceau and 5% acetic acid in ddH_2O.
6. Washing buffer: 1 × TBS (Tris-buffered saline) + 0.1% Tween-20 (TBST).
7. Blocking solution: TBST + 5% dry milk. Store at 4°C.

2.7. Survival assay

1. 10 × cell lysis counting buffer (100 mL): cetyldimethyl-ethanolammonium bromide (5 g), NaCl (0.165 g), glacial acetic acid (2.8 mL), 10% Triton X-100 (50 mL), 1 M $MgCl_2$ (2 mL), 10 × PBS (10 mL) and ddH_2O (35.2 mL). The working dilution is 1 × in distilled water.

3. Methods

3.1. PC12 cells

PC12 is a cell line derived from a pheochromocytoma of the rat adrenal medulla. It has been found that PC12 cells stop dividing and differentiate to a neuronal phenotype after treatment with NGF [6]. PC12 cells are a good model for studying neuron differentiation and degenerative disease.

3.1.1. Coating plasticware

Dilute 2 mg/ml stock collagen solution in sterile water into a 0.1 mg/ml working collagen solution as described in section 2.1 and ensure the solution covers the entire surface of the dish. Use 1 mL of the diluted collagen solution per 10-cm cell culture dish. The volume should be adjusted accordingly for different dish sizes. Incubate the coated dishes for 4–6 h at room temperature and remove the excess and air-dry in a cell culture hood. Alternatively, do not remove the excess and allow the culture dishes to dry in the cell-culture hood at room temperature overnight.[1]

3.1.2. Maintaining PC12 cells

1. Seed undifferentiated PC12 cells in 5–8 mL of complete growth medium in a collagen-coated 10-cm dish and split them, when they reach 80–90% confluence.
2. When cells reach confluence, aspirate ¾ of the medium from the plate.
3. Add 2 mL of fresh complete medium and pipet up and down over the cell monolayer to detach the cells and break up cell clumps.
4. Triturate the cells several times with the pipette to further break up cell clumps.
5. Split the cells into 2–3 10-cm dishes and add complete medium to a final volume of 5–8 mL.
6. Change the complete medium every 2 days by removing ¾ of the old medium and gently adding fresh complete medium up to 5–8 mL, leaving the cell monolayer undisturbed.

3.1.3. Differentiating PC12 cells

1. Detach PC12 cells from the plate as described above with complete medium.
2. Dilute the cell suspension with the differentiation medium and add NGF to a final concentration of 50 ng/mL. One 10-cm confluent plate of PC12 cells can be used to generate 5–20 cultures of the same size for differentiation. The dilution of the cell suspension should be adjusted according to specific experimental requirements.[2]
3. Plate the cells on the appropriate collagen-coated dish.
4. Change the differentiation medium every 2–3 days (using fresh NGF) for up to 7–10 days.

After 24 h, cells begin to extend neurites. 7–10 days of differentiation is necessary before proceeding to toxin treatment.

[1]Once coated, these dishes can be kept for 2–3 weeks. Because PC12 cells tend to detach from the plasticware easily, the coated plasticware should not be used beyond 3 weeks.

[2]Usually, naïve PC12 cells are grown in 10-cm dishes. When using differentiated PC12 cells, different type of plates can be used to satisfy experimental requirements. For example, 24 or 48 well plates can be used for viability assays. 6-well or 12-well plates can be used for mRNA purification and protein extracts.

3.1.4. Freezing PC12 cells for storage

1. Detach the cells from the plates using complete medium and place them in 15 mL conical tubes.
2. Centrifuge the cell suspension at 1000 rpm for 5 min and discard the supernatant.
3. Add 2 mL of complete medium containing 10% DMSO and mix them gently.
4. Place the cell suspension in cryotubes.
5. Place the cryotubes in an isopropanol-freezing container.
6. Keep the container at −80°C overnight and transfer the tubes into liquid nitrogen for long-term storage. The cryotubes can be stored at −80°C for up to several months. For longer periods of storage, the cells must be kept in liquid nitrogen.

3.2. SH-SY5Y cells

For SH-SY5Y cell differentiation, several protocols are commonly used. Here, we will introduce an easy and reproducible procedure to generate a fully differentiated homogeneous population of neuron-like cells.

3.2.1. Coating plasticware

1. Thaw BD Matrigel in a vial covered by foil on ice overnight, and swirl it to ensure that it is evenly mixed.
2. Dilute Matrigel using pre-cooled pipets in cold DMEM (1:10).
3. Add enough Matrigel solution to cover the entire cell-culture dish surface.
4. Incubate at room temperature for at least 1 h.
5. Rinse with serum-free DMEM two times before use.

3.2.2. Plating cells

1. If SH-SY5Y cells are received or stored in a frozen state (−80°C or in liquid nitrogen), thaw the vial at 37°C in a water bath and plate them as soon as possible to limit exposure to the toxicity of DMSO.
2. Prepare 10 mL of warm (37°C) growth medium in a 15-mL Falcon tube.
3. Remove the thawed cell suspension from the cryotube and place it into the pre-warmed complete medium.
4. Carefully mix the cell suspension and centrifuge for 3 min at 1000 rpm at room temperature.

5. Discard the supernatant and add 2 mL of fresh growth medium and pipet up and down to resuspend the cell pellet.
6. Place the cells into the Matrigel-coated plate and add another 8 mL of growth medium (for a 10-cm plate). Gently swirl the plate back and forth and left to right to prevent the cells from concentrating at the center of the plate.

3.2.3. Maintaining SH-SY5Y cells

Once the cells are plated, passage them at every 2–3 days at 1:3 to 1:4 ratio (it is recommended to subculture the cells when they reach 70–90% confluence), depending on your experimental requirement.

1. Aspirate the entire medium from the 15-cm plate.
2. Wash the cells with 3–8 mL of warm PBS (37°C).
3. Add 1–2 mL of trypsin. Swirl the plate to cover the entire cell surface with trypsin solution and for 4–5 min.
4. Use hands to tap the bottom of the dish. At this point, the cells will detach from the plate.
5. After applying the medium onto the entire surface, triturate the cells several times with the pipette to break down cell clumps.
6. Split cells at different ratios as experimentally required into Matrigel-coated plates.
7. Bring the total volume per plate up to 6–9 mL.

3.2.4. Differentiation of SH-SY5Y cells

1. Remove growth medium and wash the cells with warm PBS in a 15-cm plate.[3]
2. Add 2 mL of trypsin and incubate for 2–3 min at room temperature.
3. Dilute the cell suspension in growth medium and triturate it to get a homogenous cell suspension prior to transfer to a new flask.[4]
4. Seed 2–3 × 10^3 cells/cm^2 cells into Matrigel-coated plates and let them sit overnight in the cell culture incubator so that the cells attach to the plates.
5. The next day, remove the growth medium and add the differentiation medium I (DMEM, 5% FBS, 2 mM L-glutamine and pen/strep) supplemented with retinoic acid at a final concentration of 10 μM.

[3]The passage number of SH-SY5Y cells required for differentiation should be as low as possible. If cells switch to a fibroblast-like phenotype after several passages, they should not be used for differentiation.
[4]Excessive dilution (more than 1:4) and splitting of SH-SY5Y cells before they reach confluence will block the cells from achieving differentiation.

6. Change the culture medium every 2 days and culture the cells under these conditions for 5 days.
7. Subsequently, remove the medium and replace it with differentiation medium II (Neurobasal medium, 5% B-27, 2 mM GlutaMAX, 2 mM dibutyryl-cAMP, 20 mM KCl, 50 ng/mL rhBDNF and pen/strep).
8. Keep the cells in differentiation medium II for an additional 5 days before using them.

3.2.5. Conserving SH-SY5Y cells

1. Wash SH-SY5Y cells with PBS.
2. Add 2–4 mL of 0.05% trypsin for 2–3 min at room temperature.
3. Detach the cells from the plate with 2 mL of growth medium.
4. Centrifuge the cell suspension at 1000 rpm for 5 min and remove the supernatant.
5. Add 2 mL of growth medium containing 10% DMSO and mix them gently.
6. Place the cell suspension in cryotubes.
7. Place the cryotubes in an isopropanol-freezing container and place it at −80°C overnight. Transfer the cryotubes into liquid nitrogen for long-term storage.

3.3. Parkinson's disease cellular models

Addition of 6-OHDA or MPP+ to NGF-differentiated PC12 and SH-SY5Y cells is used as PD cellular models.

1. Replace the medium with fresh medium before any treatment.
2. Prepare stock solutions of the toxin before use.
3. Since 6-OHDA and MPP+ are light sensitive and unstable, their solutions should be wrapped in aluminum foil to avoid light and prepared just before each use. Minimize exposure to light and air.
4. To treat neuronal PC12 cells[5]:
   a. 6-OHDA:

Measure 6-OHDA into a small tube covered with foil. Prepare a 10 mM stock in sterile ddH_2O. Filter-sterilize using a 10 mL syringe and a 0.2 μm filter. Use 6-OHDA at a 50–100 μM

[5]Both 6-OHDA and MPP+ are usually used as toxins to mimic PD. Dopamine transporters will specifically take up these toxins. Therefore, the density of the dopamine transporter on cells and the total number of cells determine the toxicity range. To get a certain amount of cell death, the cell density and the toxin concentrations should be determined empirically. The concentrations listed above are intended to cause 40–60% cell death. For initial toxin treatment experiments, viability should be monitored as recommended at 24 and 48 h.

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final concentration. Take appropriate caution at all times to keep from coming into contact with the 6-OHDA powder or solution.

b. MPP+:

Prepare a 100-mM MPP+ stock solution in a tube covered with foil with ddH_2O and sterile-filter using a 0.2-μm filter. Use MPP+ at a 500 μM to 1 mM working concentration. Take appropriate caution at all times to keep from coming into contact with the MPP+ powder or solution.

5. To treat differentiated SH-SY5Y cells (see footnote 5):

Prepare toxin stocks as above, but the final concentration of 6-OHDA should be much lower: 10–20 μM for 6-OHDA. The final concentration of MPP+, however, should be between 1 and 3 mM. These concentrations of toxins will result in about 50% cell death.

6. For small wells (24- or 48-well plates), because small volumes are more prone to pipetting errors, use a diluted stock of 6-OHDA. Dilute the 10 mM stock at 1:10 in medium to make a 1 mM stock before use.

7. After treatments, assess the experiment at the desired time.

3.3.1. Manipulation of gene expression in cultured cells to study PD

To study gene function in PD cellular models, gene overexpression and silencing are powerful tools. Here, we take PC12 cells as an example to introduce two ways to manipulate gene expression: plasmid transfection and lentiviral-mediated plasmid delivery. We will also introduce several methods to assay PC12 cell survival in these two systems.

3.4. Manipulation of gene expression by transfection

3.4.1. Transfection

Neuronal PC12 cells are very difficult to transfect with plasmids (less than 5% transfection rate), but transfection is still a good way to study some gene functions following PD toxin treatments. The low transfection rate makes it easy to observe individual cell phenotype changes, and transfection is also easier to handle than virus infection. Usually the plasmids used for transfection contain fluorescent markers such as GFP, which makes it possible to observe the transfected population. Transfection is also useful for gene regulation studies using luminescent reporters such as luciferase.

1. Seed PC12 cells as mentioned above.

2. Add NGF to differentiate PC12 cells for at least 3–4 days.

3. Transfect neuronal PC12 cells with plasmids (usually with some fluorescent marker, such as green fluorescent protein (GFP)) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

4. After 48 h, observe transfected cells (as judged by expression of the fluorophore) with a fluorescence microscope. If sufficient cells are transfected, the cultures can be treated with 6-OHDA or MPP+ as experiments require.
5. At various times after treatment with the toxins, viable transfected neuronal PC12 cells can be identified and target genes in the transfected cells can be studied by co-immunostaining.

3.4.2. Viability assay for transfected cells

Cell number counting or apoptotic nuclei assessment can be used to assess cell survival.[6]

a. Strip counting or whole-well counting: the number of healthy, GFP positive cells is counted in a field consisting of a strip across the diameter of each well or the whole well. The survival rate is calculated and normalized to the number of cells counted in the control wells. The number of transfected cells counted should be at least 400.[7]

b. Apoptotic nuclei assessment: Hoechst dye 33342 is applied to stain nuclei either of living cells or after fixation with formaldehyde.

For living cells:

1. 1 mg/mL Hoechst 33342 is added the medium to a final concentration of 1 μg/mL for 5 min.
2. Remove the culture medium and replace with fresh medium that does not contain Hoechst 33342.

For fixed cells:

1. Remove the medium.
2. Add 4% formaldehyde for 10 min, wash 3 times with PBS.
3. Stain with Hoechst 33342 (at 1 μg/mL final concentration) for 5 min.
4. Replace with PBS without dye.

The cells that possess both GFP and condensed nuclei/fragmented chromatin are scored as apoptotic. The number of apoptotic cells is calculated relative to the number of GFP+ cells in the same well (see footnote 7).

3.4.3. Immunostaining after transfection

1. 24 or 48 h after transfection, fix the cells with 4% formaldehyde for 10 min.
2. Permeabilize cells with 0.5% Triton X-100 for 10 min at room temperature.

[6]All the results should be repeated as least three times, and analysis by Student's test or ANOVA with Tukey's *post hoc* test.

[7]If the fluorescence signal is weak, immunostaining will enhance the signal. GFP antibody can be used enhance the signals. After that, assess cell viability through cell survival assessments as described.

3. Block cells with 5% bovine serum albumin (BSA) in PBS for 40 min.
4. Stain PC12 cells with specific primary antibody at appropriate dilution rate in 5% BSA overnight at 4°C.
5. Wash cells 3 times with PBS, 10 min each time.
6. Incubate cells with secondary antibody for 1 h at room temperature.
7. Dilute Hoechst 33342 dye to 1 μg/mL in PBS.
8. Stain cells for another 5 min.
9. Wash cells with PBS.

Cells are observed and scored under a fluorescence microscope. An example of transfected and immunostained neuronal PC12 cell culture is shown in **Figure 1**.

3.5. Manipulation of gene expression by lentiviral vector infection

Lentiviral vectors derived from Human immunodeficiency virus type 1 are able to infect differentiated neurons and stably integrate into the host genome, resulting in long-term expression of the transgene. A major advantage of lentiviral-mediated gene delivery, compared to the transfection procedure described above, is that it will typically achieve a 80–90% transduction rate in a PC12 or SH-SY5Y neuronal cell population. This high transduction rate is crucial for the use of downstream applications requiring highly efficient manipulation of gene expression, such as qPCR or Western immunoblotting.

The strategy to produce replication-defective lentiviral particles has been to remove all dispensable genes from the HIV-1 genome and separate the cis-acting sequences from the trans-acting elements required for viral particle production, infection and integration [14–16]. The

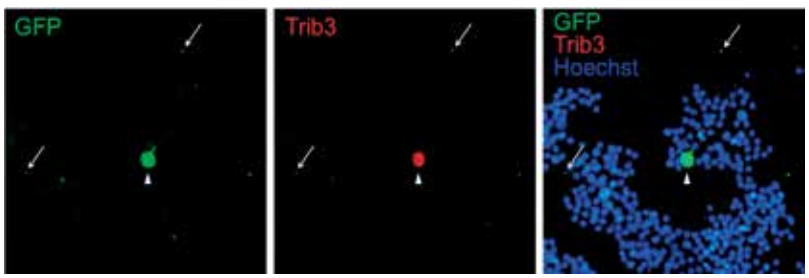

Figure 1. Transfection of the pro-apoptotic Tribbles pseudokinase 3 (Trib3) in neuronal PC12 cells. PC12 cells were differentiated into neurons with NGF for 3 days and transfected with a construct co-expressing full length Trib3 and GFP using Lipofectamine 2000. After 48 h, cells were fixed with 4% PFA and immunostained for GFP (chicken anti-GFP primary antibody and goat anti-chicken Alexa fluor 488 secondary antibody, Life Technologies), Trib3 (rabbit anti-Trib3 primary antibody, Calbiochem, and goat anti-rabbit Alexa fluor 568 secondary antibody, Life Technologies) and nuclei were stained with Hoechst 33342. Under basal conditions, neuronal PC12 cells express undetectable levels of endogenous Trib3. A successfully transfected neuronal PC12 cell is shown here co-expressing GFP (left) and Trib3 (center) in the cell body (arrowhead) and in discrete puncta in the processes (arrows). A merged image with additional Hoechst staining of the nuclei is shown on the right.

third-generation lentiviral vector system contains four plasmids: an expression vector containing the cis-acting sequences and three additional packaging plasmids: pMDL, pRev and pVSVG, providing the trans-acting factors. pMDL encodes a gag-pol precursor that is processed into an integrase and a reverse-transcriptase, as well as structural capsid proteins. Rev interacts with RRE, a cis-acting element enhancing the nuclear export of viral mRNAs. pVSVG encodes an envelope glycoprotein that confers the viral particle the ability to transduce a broad range of cell types. The second-generation lentiviral vector system contains only three plasmids: an expression vector containing the cis-acting sequences including Rev and two additional packaging plasmids: psPAX2 and pVSVG, providing the other trans-acting factors.

Manipulation of gene expression by lentiviral vector infection is a multistep process requiring, first, design and cloning of lentiviral constructs; then transfection of these constructs into packaging cells (HEK 293 cells); followed by collection, purification and concentration of the lentiviral particles; and finally, infection of neuronal cells. Design and cloning of lentiviral vectors will not be discussed in this methodological review, please refer to Tiscornia et al. [17] for a detailed protocol.

3.5.1. HEK 293T cells growth and maintenance

Grow and propagate HEK293T cells in DMEM +10% FBS in 15-cm dishes. Split the cells when they reach 80–90% confluence.[8]

1. Aspirate the medium of a confluent plate of HEK293T cells.
2. Add 5 mL of 1 × trypsin-EDTA. Swirl the plate to cover the entire cell surface with trypsin solution and place the plate in a 37°C incubator for 1 min.
3. Add 10 mL of DMEM +10% FBS to dilute and inactivate the trypsin solution and pipet up and down over the cell monolayer to detach the cells and break down cell clumps.
4. Transfer 5 mL of the cell suspension in a new 15-cm dish with 13 mL of fresh DMEM +10% FBS. Swirl the plate to evenly spread the cells.

3.5.2. Seeding HEK293T cells for lentivirus production (Day 1)

This protocol describes the production of two batches of virus (i.e., four 15-cm dishes total: two 15-cm dishes are needed for each batch of lentivirus), as it is usually needed for a given experiment: one batch (two 15-cm dishes) of lentivirus containing an expression vector designed to manipulate gene expression and one batch (two 15-cm dishes) of lentivirus containing a control expression vector. The lentiviral vectors usually contain fluorescent markers such as GFP.

[8]HEK293T cells should not be allowed to become more than 80–90% confluent during maintenance. To ensure high transfection efficiency, HEK293T cells should be of low passage number and should demonstrate rapid growth when seeded for lentivirus production.

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1. Resuspend HEK293T cells from 80 to 90% confluent 15-cm dishes with trypsin-EDTA and DMEM +10% FBS, as described above.
2. Count the cells concentrated in the trypsinized cell suspension with a hemacytometer.
3. Seed about 2×10^7 HEK293T cells per 15-cm plate. Adjust the total volume to 18 mL/plate with DMEM +10% FBS and swirl the plate to evenly spread the cells.

3.5.3. Calcium phosphate transfection of the lentiviral plasmids (Day 2)

1. Make sure that the cells seeded the day before are healthy and close to 80–90% confluent: they should still have room to undergo 1–2 cell divisions.
2. For each batch of virus, prepare the corresponding plasmid mix in a 15-mL tube containing 4 mL of 250 mM $CaCl_2$. For second-generation lentiviral system, use 30 μg of psPAX2, 20 μg of VSVG and 40 μg of expression vector. For third-generation lentiviral system, use 20 μg of CMV-VSVG, 20 μg of pMDLg/pRRE, 20 μg of RSV/REV and 40 μg of expression vector.
3. Prepare two separate 15-mL tubes, each containing 4 mL of 2× HBSS solution. To ensure high transfection efficiency, the pH of the HBSS solution should be precisely adjusted to 7.03–7.04 with NaOH.
4. Drop by drop, and by continuously vortexing, add the 4 mL of the $CaCl_2$ + plasmid mix solution to the 4 mL of HBSS solution, for each virus batch. Avoid the formation of precipitates. You now have 8 mL of transfection mix ready for each virus batch.
5. Add 4 mL of the transfection mix to each 15-cm dish, swirl the plates gently and place them back into the incubator.
6. After 4–5 h, replace the medium of the transfected cells with 18 mL of fresh DMEM +10% FBS.

3.5.4. Collection of the lentiviral particles (Day 4 and Day 5)

From this point on, viral particles accumulate in the supernatant. Proceed with appropriate precautions when manipulating the supernatants.[9]

1. On Day 4, observe the cells and check the transfection efficiency. Cells should be reaching confluency and, if a marker (such as GFP) is present in the expression vectors, transfection may be assessed visually. The transfection efficiency should be >90%.
2. Harvest the first supernatant by pooling 18 mL from each plate of the same virus batch in a 50-mL conical tube. Seal the tubes with Parafilm and place them on a designated rack at 4°C.

[9]Perform all manipulations in a biosafety cell culture cabinet. When working in the cabinet, wear two pair of gloves at all times and remove the second pair when leaving the area. All containers should be sealed with Parafilm (or placed in a second vessel) when transported outside of the biosafety cabinet. All the material and reagents should be decontaminated in 10% bleach. The biosafety cabinet (containing all the contaminated material and reagents) should be placed under UV light for at least 30 min at the end of each procedure involving contaminated material and reagents.

3. Replace the supernatant with 18 mL of fresh DMEM +10% FBS.
4. On Day 5, harvest the second supernatant, following the same procedure.

3.5.5. Purification of the lentiviral particles

1. Spin the 50-mL conical tubes containing the supernatants for 5 min at 1000 g to pellet the cellular debris.
2. Pool and filter the first and the second supernatants from the same virus batch in 0.45 μM 115 mL filter units. Then proceed to concentrate the virus particles as below.

3.5.6. Concentration of the lentiviral particles

Several methods can be used to concentrate the virus: Ultracentrifugation, the most well-described method [18], filtration with a centrifugal filter unit equipped with a 100 kDa molecular weight cut-off membrane (Millipore cat. No. UFC910024) and regular centrifugation with Lenti-X concentrator. The Lenti-X method gives the same yield as the other methods and is, in our hands, the quickest and the most convenient. Therefore, we will describe this method here.

1. Re-distribute the filtered viral supernatant in a 50-mL conical tube (36 mL/tube) and add 1:3 of the supernatant volume of Lenti-X concentrator (12 mL/tube).
2. Seal the tubes with Parafilm and mix by gentle inversion.
3. Incubate the supernatant + Lenti-X mix for 2 h up to 72 h at 4°C.
4. Centrifuge at 1500 g for 45 min at 4°C. An off-white pellet will become visible.
5. Discard the supernatant and resuspend the pellet with 200 μL of PBS (no $CaCl_2$, no $MgCl_2$).
6. Store the concentrated viral particles in single-use aliquots at −80°C. Avoid freeze–thaw cycles.

3.5.7. Titration of the lentiviral particles

Depending on your experimental needs, a precise determination of the biological titer of the purified lentiviral vectors might be necessary and conducted as follows.

1. The day before titration, seed 10^5 HEK293T cells in each well of a 24-well plate.
2. On the day of titration, prepare 6 tubes, each containing 45 μL of DMEM +10% FBS.
3. Add 5 μL of viral preparation in the first tube and mix.
4. Make a 10-fold serial dilution, by pipetting 5 μL of the diluted virus into the next tube, and so forth.
5. Add 450 μL of DMEM +10% FBS in each tube.

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6. Aspirate the entire medium of each HEK293T cell-culture well and add 500 μL of the lentivirus dilutions.
7. 48 h after infection, count the total number of GFP+ cells in a well that shows a number of GFP+ cells between 50 and 500. If the signal is too weak, perform an immunofluorescence procedure with an anti-GFP primary antibody.
8. The biological titer (BT) of the virus preparation corresponds to the number of GFP+ cells counted in a given well (N) divided by the dilution factor (DF) of the same well multiplied by 5. BT = (N/5*DF).

3.5.8. Lentiviral infection of neuronal PC12 cells

1. Seed PC12 cells as described above.
2. Add NGF to differentiate PC12 cells for at least 3–4 days.
3. On the day of lentiviral infection, replace the medium with fresh differentiation medium.
4. Add concentrated viral particles (1×10^7 viral particles/cm^2 of culture area[10]) and do not change the medium for 24 h.
5. Maintain the infected cells by changing the differentiation medium every 2–3 days, as usual.
6. After 3–7 days, observe the infected cells. Check the transduction efficiency by evaluating the expression of the fluorescent marker (such as GFP) under a fluorescence microscope. The transduction efficiency should be 80–90%. If enough cells are infected, the cultures are ready to be treated with 6-OHDA or MPP+ and used for downstream applications.

3.6. Downstream application: qPCR

The amount of an expressed gene in a cell can be measured by the number of copies of the corresponding mRNA transcript present in a given sample. In order to robustly detect and quantify gene expression from small amounts of RNA, amplification of the gene transcript is necessary. The polymerase chain reaction (PCR) is the most common method for amplifying DNA. For mRNA-based PCR, the extracted mRNA is converted to cDNA by reverse transcription.

3.6.1. Harvesting cells for qPCR

1. Take the cells out of the incubator and place them on ice.
2. Remove the medium gently and add TRI reagent (Molecular Research Center) in each well (100 μL of TRI reagent/cm^2 of culture dish area). Refer to the manufacturer's protocol and handling notes for additional information on TRI reagent.[11]

[10]The amount of concentrated virus vector to use is mostly dependent on cell density in your culture dish and should be determined empirically. Typically, when infecting moderately sparse neuronal PC12 cultures (avoid clumps at all cost), we use 0.1 up to 5× 10 viral particles/cm of culture area, and usually get a 80% infection rate. If the viral preparation is good but your infection rate is low, consider decreasing the density of your cell culture.
[11]TRI reagent contains harmful compounds such as phenol and guanidine thiocyanate. Manipulate with caution under a chemical hood and wear gloves at all times.

3. Use a cell lifter to detach the cells from the bottom of the well and homogenize the cell lysate.
4. Pipette the cell lysate into labeled microcentrifuge tubes and pipette up and down 2–3 more times to further homogenize the cell lysate. At this point store the homogenates at −80°C before proceeding to RNA extraction.

3.6.2. RNA extraction

1. Take the samples out of the −80°C freezer and allow them to thaw at room temperature for 10 min.
2. Add 10 μL of bromochloropropane/100 μL of TRI reagent.
3. Cap the tubes tightly and vortex vigorously for 15 s.
4. Incubate the mixture at room temperature for 10 min.
5. Centrifuge at 12000 g for 10–15 min at 4°C. Meanwhile, label a new set of microcentrifuge tubes.
6. Transfer the aqueous phase (top layer) to a new tube. RNA remains in the aqueous phase whereas DNA is in the interphase and proteins remain in the organic phase.
7. Add 50 μL of isopropanol/100 μL of TRI reagent solution.
8. Vortex for 10 s.
9. Incubate at room temperature for 10 min.
10. Centrifuge at 12000 g for 8 min at 4–25°C. At this point, a translucent (gel-like) to white pellet should become visible at the bottom of the tube.
11. Carefully remove the supernatant without disturbing the pellet.
12. Add 100 μL of 75% ethanol/100 μL of TRI reagent to wash the pellet.
13. Centrifuge at 7500 g for 5 min at 4–25°C.
14. Remove all the ethanol carefully without disturbing the pellet.
15. Air dry the pellet for 5 min.
16. Dissolve the RNA in 20 μL of nuclease-free water and mix vigorously.
17. Store on ice for immediate analysis, or place at −80°C for long-term storage.

3.6.3. Assessment of RNA concentration and quality

Assess the RNA yield and quality by spectrophotometry using a nanodrop. RNA concentration can be assessed by measuring its absorbance at 260 nM.

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1. Use 1–2 μL of non-diluted sample and read the absorbance at 260 nm(A260) to measure RNA concentration (C). C = A260*40 μg/mL.
2. To assess purity of the extracted RNA, note the A260/A280 ratio given by the nanodrop: it should be between 1.8 and 2.2.

3.6.4. Reverse transcription

To create cDNAs form the extracted mRNAs, use the Origene First Strand cDNA synthesis system for qPCR, following the manufacturer's instructions. Keep your mRNAs and all the components of the kit (except the enzyme) on ice.

1. In thin-walled PCR tubes, prepare a 20 μL reaction mix including 1 μg of extracted mRNA, 4 μL of 5× cDNA synthesis mix, and 1 μl of reverse transcriptase (take it out of the −20°C freezer at the very last minute). Adjust the total volume to 20 μL with the provided nuclease-free distilled water.
2. Mix gently and spin down to collect contents.
3. Place the tubes in a thermocycler programmed as follows: 1 cycle at 22°C for 5 min, 1 cycle at 42°C for 30 min, 1 cycle at 85°C for 5 min, hold at 4°C. At this point, you can store the cDNAs at −80°C.

3.6.5. Real-time quantitative PCR

To perform real-time quantitative PCR, you will need to design a set of primers specific to the transcript of the gene(s) of interest. Primer design is beyond the scope of this review and will not be described here.[12]

1. Prepare a 10 μM primer mix containing an equimolar concentration of forward and reverse primers in ddH_20
2. Dilute the template cDNAs 11-fold by adding 200 μL of PCR-grade water to the 20 μL product of the reverse-transcription.
3. Prepare the qPCR reaction mix in a 96-well plate as follow: Total reaction volume: 25 μl = 11 μL of diluted template cDNA +1.5 μL of 10 μM forward/reverse primers mix +12.5 μL of 2× SYBR green mix. The 2× SYBR green mix is a buffer containing all the components necessary for DNA amplification and detection: Taq DNA polymerase, dNTPs, Mg^{2+} and the DNA intercalating dye SYBR Green. Run each sample in duplicate or triplicate. To detect DNA contamination, always include a negative control in each run. To prepare this control, replace template cDNA with PCR-grade water.
4. Place the 96-well plate in the real-time PCR cycler.

[12]Note that several programs for primer designing are freely available on the web such as primer blast: http://www.ncbi.nlm.nih.gov/tools/primer-blast.

5. Set the detection channel to SYBR Green and the reaction volume at 25 μL.
6. Run the reaction as follow: 1 cycle at 95°C for 10 min to activate the Taq DNA polymerase, followed by 40 cycles of amplification: 95°C for 15 s, 58–60°C (the optimum temperature must be determined for each primer set) for 30–60 s, 72°C for 30–60 s.
7. Repeat the same procedure with a set of primers designed to amplify a control housekeeping gene or other species that should not be affected by your experimental conditions. In studies involving cell death induced by PD toxins (6-OHDA, MPP+), we found that 18S rRNA is a good control and can be used for normalization. At the end of the reaction, proceed to quantification and analysis.

3.6.6. Quantification and analysis

Real-time monitoring of the PCR reaction displays the amount of fluorescence signal emitted from the SYBR green dye. During the amplification reaction, the SYBR green dye is progressively inserted in the newly synthesized double-stranded DNA fragments. The amplified fragments correspond to the region of the cDNA from the gene of interest flanked by the forward/reverse primers binding sites. The initial copy number can be quantitated during real-time PCR analysis based on the threshold cycle (Ct). Ct is defined as the cycle at which fluorescence is determined to be statistically significant above background. The more template is initially present, the fewer number of cycles is needed to reach a threshold at which the fluorescence is statistically significant above background. To calculate the fold induction of your gene of interest (GI) normalized to a control housekeeping gene (HKG) in an experimental condition (Exp) compared to a control condition (Ctl), use the following formula:

$$\text{fold induction} = 2^{-\left[\left(Ct_{GIExp} - Ct_{HGKExp}\right) - \left(Ct_{GICtl} - Ct_{HKGCtl}\right)\right]} \quad (1)$$

3.7. Downstream application: Western blot

3.7.1. Harvesting cells and extracting proteins

1. Take the cells out of the incubator and place them on ice.
2. Remove the medium gently and add 1× cell lysis buffer supplemented with protease inhibitor (30 μL of buffer/cm^2 of culture dish area).
3. Use a cell lifter to detach the cells from the bottom of the well.
4. Pipette the cell lysate into labeled microcentrifuge tubes and place them on ice.
5. Pipette up and down 2–3 more times and briefly vortex to further homogenize the cell lysate. Check the homogenate: if cellular debris is still visible, proceed to sonicate the samples.
6. Sonicate for 20 s (10 pulses over 10 s followed by one continuous 10-s pulse) on ice. At this point, the homogenate should be clear of debris.
7. Store the samples at −80°C before measuring the protein concentration in each sample.

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3.7.2. Assessment of protein concentration and sample preparation

1. Use a technique of your choice to measure protein concentration. We recommend using Thermo Scientific's BCA assay following the manufacturer's instructions (Thermo Scientific #23225).
2. Label a new set of tubes in order to prepare the samples in loading buffer. The following steps (sample preparation, gel electrophoresis and protein transfer) are made using the NuPAGE Novex system from Life Technologies. Please refer to the manufacturer's protocol for further information.
3. In each tube add: Protein sample (to achieve a final concentration of 1–2 μg/μL of protein), 10× dithiothreitol (NuPAGE reducing agent, 500 mM dithiothreitol), 4× loading dye and ddH_2O up to the desired volume.
4. Samples can be stored at −20°C at this point.

3.7.3. Gel electrophoresis

Gel electrophoresis is achieved with the NuPAGE Novex Mini system with Bis-Tris precast polyacrylamide gels.[13]

1. Prepare 1 L of 1× MOPS running buffer (50 mL of 20× MOPS running buffer in 950 mL of ddH_2O).
2. Insert the precast gel in the gel box and fill the inside chamber with 1× running buffer.
3. Boil protein samples for 5 min.
4. Remove bubbles and residues from the gel wells by pipetting.
5. Load 4 μL of molecular weight markers and the protein samples in the desired order.
6. Run the gel under constant voltage at 100–140 V (400 mAmps) for 30–45 min.
7. Monitor frequently to assess the degree of separation and to make sure that the protein samples are not running out of the gel.

3.7.4. Protein transfer

1. Prepare 2 L of 1× transfer buffer (100 mL of 20× transfer buffer with 15% ethanol (300 mL) and 1600 mL of ddH20).
2. Cut a polyvinylidene fluoride (PVDF) membrane (pore size 0.2 μm) and 2 pieces of filter paper to the appropriate dimensions of a transfer cassette, or use a premade sandwich (Biorad).

[13]This system is optimized for separation and resolution of small- to medium-sized proteins (1–200 kDa) under denaturing gel electrophoresis conditions. These precast gels are available in different polyacrylamide percentages, well formats and thicknesses and must be chosen according to specific experimental needs. Acrylamide is a toxic compound: manipulate with caution and wear gloves at all time.

3. Soak the PVDF membrane for few seconds in 95% ethanol then soak in transfer buffer for 5 min.
4. Soak filter paper and sponges in transfer buffer for 5 min.
5. Prepare a "transfer sandwich" with 2–3 sponges, 1–2 pieces of filter paper, polyacrylamide gel containing the separated proteins, PVDF membrane, 1–2 pieces of filter paper, 2–3 sponges. Keep the sandwich wet at all times and avoid the formation of bubbles in between layers.
6. Place the sandwich in a transfer cassette and fill up the gel box with 1× transfer buffer. Apply a constant voltage at 40 V (400 mAmps) for 94 min to transfer the proteins form the gel to the membrane.

3.7.5. Protein detection

1. Check the uniformity and overall efficiency of the transfer by staining the membrane with Ponceau S dye (0.1% w/v Ponceau, 5% acetic acid in ddH_2O).
2. Wash the membrane with TBST (1× TBS + 0.1% Tween) for 5 min. Wash again quickly with TBST to discard the excess of Ponceau.
3. Block non-specific protein binding by incubating the membrane with TBST +5% powdered milk for 45 min.
4. Rinse 3 times for 5 min with TBST.
5. Incubate the membrane with a solution of primary antibody in blocking solution under gentle agitation at 4–25°C from 30 min to overnight.[14]
6. Wash the membrane with TBST 3 times for 5–15 min.
7. Incubate the membrane with a secondary antibody targeted to the primary antibody and bound with horseradish peroxidase.
8. Incubate at room temperature for 1–2 h on a shaker.
9. Wash the membrane with TBST three times for 5–15 min.
10. Incubate the membrane with a chemiluminescent agent.
11. Expose a light-sensitive autoradiographic film against the membrane and develop to reveal the protein signal.
12. Repeat the same procedure with a primary antibody against a control housekeeping protein that should not be affected by your experimental conditions. In studies involving cell

[14]The final concentration of primary antibody as well as the duration and temperature of incubation should be determined empirically and according to the manufacturer's guidelines.

death induced by PD toxins (6-OHDA, MPP+), we found that extracellular signal–regulated kinases (ERK) or glyceraldehyde 3-phosphate dehydrogenase (GAPDH) are good loading controls and can be used for normalization.

3.8. Downstream application: survival assay

The quantification of viable cells following treatment with PD-toxins and/or gene manipulation can be achieved by several methods. A number of methods (such as MTT or LDH assays) rely on measuring the metabolic activity of the cultured cells and give an indirect and potentially biased measurement of cell viability. We consider that the most accurate and robust measure of cell death is an absolute count of the numbers of surviving cells remaining in the cell culture dish following toxin treatment and/or gene manipulations. We routinely use a method in which the nuclei of the remaining cells are counted by incubating the cell cultures with a detergent solution that lyses the plasma membrane and leaves the nuclei intact.

1. Prepare 100 mL of 10× counting lysis buffer by combining: 5 g of cetyldimethyl-ethanolammonium bromide, 0.165 g of NaCl, 2.8 mL of glacial acetic acid, 50 mL of 10% Triton-X, 2 mL of 1 M $MgCl_2$, 10 mL of 10× PBS, 35.2 mL of H_2O.
2. Take the cells out of the incubator and aspirate all the medium.
3. Add 1× of counting lysis buffer to the wells (250 μL/cm^2 of culture dish area) and leave sit for 5 min.
4. Pipette 10 μL of the suspended nuclei into a hemacytometer and count at least 100 nuclei.
5. Cell survival in a given condition is expressed as the percentage of remaining nuclei in experimental cultures compared to control cultures.

4. Outcomes: examples of findings using the above techniques

4.1. Published findings

Application of the above techniques by our and other groups has identified a variety of transcriptionally regulated genes with potential relevance to the pathophysiology and treatment of PD. Here, we provide a few examples arising from our own studies (**Figure 2**).

An early serial analysis of gene expression (SAGE) study of transcriptional responses of PC12 cells to 6-OHDA treatment revealed a strong endoplasmic reticulum (ER) stress response signature [19, 20]. Such work contributed to the current focus on the role of ER stress in PD [21, 22]. Among the upregulated stress, genes were the transcription factors *ATF4* (Activating transcription factor 4) and *Ddit3* (DNA damage-inducible transcript 3, which encode the ATF4 and CHOP (C/EBP homologous protein) proteins, respectively). *In vitro* work, including with PC12 cells as well as *in vivo* studies have indicated that CHOP is death promoting in the

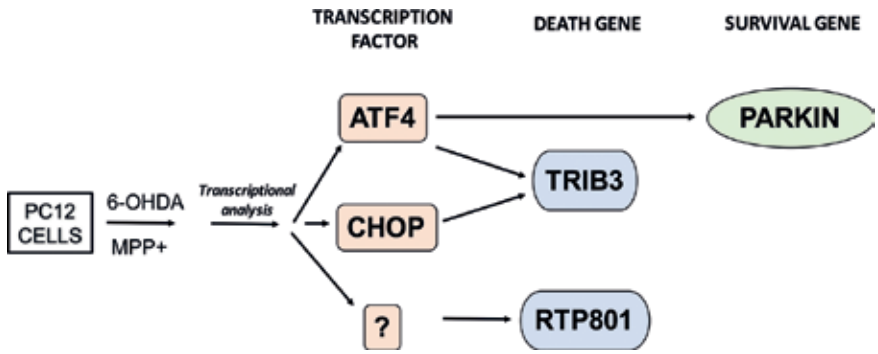

Figure 2. Example of transcriptionally regulated genes in PD cellular models identified by our group using the methods described in this chapter.

context of PD [23–25]. The potential role of ATF4 in contrast has proved to be more complex, and here again, studies with PC12 cells have proved useful for enlightenment. On one hand, ATF4 appears to have protective actions in PD models and does so by causing stabilization of the anti-death protein Parkin [13]. On the other hand, ATF4 appears to cooperate with CHOP in transcriptional induction of Trib3, a protein with pro-apoptotic actions in neurons [25, 26]. *Trib3* was among the genes found to be induced by 6-OHDA [19, 20, 25] in PC12 cells, and subsequently, Trib3 protein was found to be elevated in dopaminergic neurons of PD patients [25]. Down-regulation of Trib3 or inhibition of its transcription is highly protective in multiple cellular PD models including PC12 cells, suggesting it as a potential therapeutic target for PD treatment [25]. An additional transcriptionally regulated gene of interest to arise from the initial SAGE study was *Ddit4* (DNA-damage-inducible transcript 4), which encodes the pro-death RTP801 protein. A series of studies has indicated that like Trib3, RTP801 is elevated in dopaminergic neurons of PD patients and that eliminating its expression is protective in cellular models of PD such as PC12 cells [27–29]. Interference with RTP801 induction in an animal model of PD proved to be protective, thus identifying this as an additional potential target for PD therapy [29].

4.2. Nupr1 is induced but does not regulate Trib3 upregulation in a PD cellular model

As noted above, we recently described Trib3 as a gene-mediating cell death and degeneration in PD and identified two transcription factors, ATF4 and CHOP, responsible for Trib3 upregulation in PD cellular models, including neuronal PC12 cells treated with 6-OHDA [25]. However, ATF4 and CHOP downregulation only partially abrogated Trib3 induction in response to PD toxin mimetics, indicating that other transcription factors might be responsible for Trib3 induction in these models. Several reports indicate that Nupr1 (also known as p8) is upregulated and leads to apoptosis of cancer cells through activation of the ATF4/CHOP-Trib3 pathway [30, 31]. Therefore, we tested whether Nupr1 was also upregulated in PD cellular models and, if so, whether it could regulate Trib3 expression (**Figure 3**).

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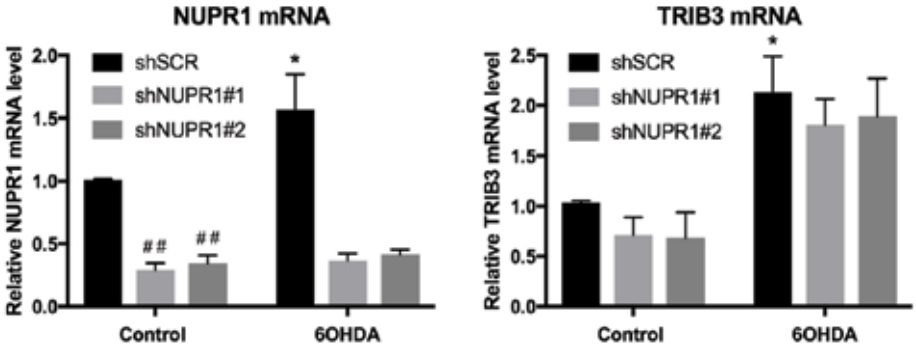

Figure 3. Nupr1 is upregulated but does not regulate Trib3 expression in PD cellular models. PC12 cells were differentiated with NGF for 3 days and infected with three different lentiviruses: two lentiviruses carrying constructs expressing shRNAs directed against two distinct regions of the Nupr1 mRNA (shNUPR1#1 and shNUPR1#2) or a control lentivirus carrying a scrambled shRNA sequence (shSCR). 4 days later, the cells were treated with sterile water (control) or 150 μM 6-OHDA for 8 h (6OHDA). At the end of the treatment, total mRNAs were extracted and RT-qPCR was performed to measure Nupr1 and Trib3 mRNA levels. Nupr1 and Trib3 mRNA levels were normalized to GAPDH. For Nupr1 RT-qPCR primers: (forward) GGGCAAGTTAGGAGCGAGAA and (reverse) GGGCATCCAGTTTTTCCCAC. For Trib3 RT-qPCR primers: (forward) GTTGCGTCGATTTGTCTTCA and (reverse) CGGGAGCTGAGTATCTCTGG. Values are expressed as mean ± SEM of three independent experiments. Statistical analysis was performed using two-way ANOVA with Sidak's multiple comparisons test. $^{*}p < 0.05$ comparing shSCR/6OHDA relative to shSCR/control; $^{\#\#}p < 0.005$ comparing shNUPR1#1/control or shNUPR1#2/control relative to shSCR/control.

Consistent with the upregulation of Nupr1 seen in apoptotic cancer cells [30, 31], we found that 6-OHDA induced a significant 1.6 fold increase in Nupr1. Under these conditions, we measured a 2.1-fold increase in Trib3 mRNA levels, as reported previously [25]. To assess whether Nupr1 is required for this Trib3 induction, we employed lentivirally delivered Nupr1 shRNAs as described in this chapter. Although both shRNA constructs achieved a 70% reduction of Nupr1 mRNA levels, Nupr1 knockdown had no effect on Trib3 mRNA levels neither at baseline nor under 6-OHDA treatment. These results suggest that although Nupr1 is upregulated in PD cellular models, it is not one of the transcription factors responsible for orchestrating Trib3 upregulation in these models.

4.3. xCT/SLC7A11 is induced in PD cellular models

Our PC12 cell experiments indicate upregulation of ATF4 in multiple PD models, and that while ATF4 can play a protective role in PD by reducing loss of the anti-death protein Parkin, it also contributes to induction of the pro-apoptotic protein Trib3 (**Figure 2**). An additional way that ATF4 could affect cell survival or death is by transcriptional regulation of xCT protein (product of the *SLC7A11* gene) [32]. xCT protein levels are reported to be increased in animal PD models such as 6-OHDA and chronic MPTP treatment [33, 34]. These findings are capitulated in our PC12 cell studies, in which we found massive upregulation of xCT mRNA in both the MPTP and 6-OHDA models (**Figure 4**).

xCT is a subunit of the cystine/glutamate antiporter system that transports cystine into cells in exchange for exported glutamate. This property has suggested the possibility of both pro-survival

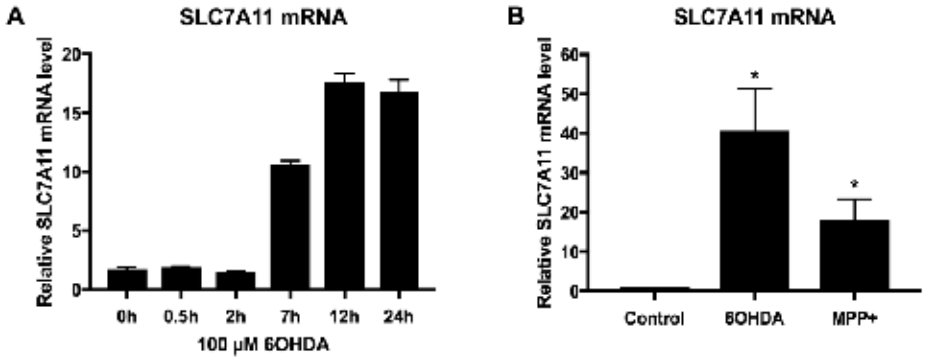

Figure 4. xCT/SLC7A11 mRNA is upregulated in differentiated PC12 cells by PD-mimetic toxin treatment. Neuronally differentiated PC12 cells were treated with 100-µM 6-OHDA for different times as indicated (A) or with 150 µM 6-OHDA for 10 h or 1 mM MPP+ for 16 h (B). Total mRNA was extracted, and RT-qPCR was performed to measure SLC7A11 mRNA levels (normalized to tubulin). For xCT RT-qPCR primers: (forward) GACAGTGTGTGCATCCCCTT and GCATGCATTTCTTGCACAGTTC (reverse). Values are expressed as mean ± SEM. In B, statistical analysis was performed using one-way ANOVA with Tukey's multiple comparisons test. $^{*}p < 0.05$ comparing 6-OHDA or MPP+ relative to control.

and pro-apoptotic activities in the context of PD. By promoting cystine uptake, xCT increases synthesis of glutathione (GSH), an intracellular antioxidant that has been suggested to play a protective role in PD [35, 36]. One possibility is that the upregulation of xCT might be a compensatory protective reaction to increased oxidative stress. Consistent with this idea, treatments that elevate neuronal levels of GSH show protection in a variety of PD models [36, 37]. On the other hand, as an exchanger, xCT elevates extracellular levels of glutamate that in turn may have toxic effects on dopaminergic neurons via glutamate receptors. In agreement with this possibility, 6-OHDA-treated $xCT^{-/-}$ mice were reported to have less striatal extracellular glutamate than wt mice, and their dopaminergic neurons showed substantial protection from 6-OHDA [38]. Such findings raise the challenge of sorting out and appropriately manipulating the pro- and anti-apoptotic actions of xCT for therapeutic advantage in PD. Cellular models of PD with induction of xCT such as described here, have the potential to serve as convenient, first-line screening systems to this end.

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Abbreviations

6-OHDA: 6-hydroxydopamine

ATF4: activating transcription factor 4

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BDNF: brain-derived neurotrophic factor

BSA: bovine serum albumin

CHOP: C/EBP homologous protein

ddH_2O: double distilled or deionized water

DDIT3: DNA damage-inducible transcript 3

DDIT4: DNA-damage-inducible transcript 4

dibutyryl cAMP: dibutyryl cyclic adenosine monophosphate

DMSO: dimethyl sulfoxide

ER: endoplasmic reticulum

ERK: extracellular signal–regulated kinases

FBS: fetal bovine serum

GAPDH: glyceraldehyde 3-phosphate dehydrogenase

GFP: green fluorescent protein

GSH: glutathione

HBSS: Hank's balanced salt solution

MPTP: 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

MPP+: 1-methyl-4-phenylpyridinium

NGF: nerve growth factor

PBS: phosphate-buffered saline

PCR: polymerase chain reaction

PD: Parkinson's disease

pen/strep: penicillin/streptomycin

PVDF: polyvinylidene fluoride

qPCR: quantitative polymerase chain reaction

rhBDNF: recombinant human brain-derived neurotrophic factor

RT-PCR: reverse transcription polymerase chain reaction

SAGE: serial analysis of gene expression

TBS: Tris-buffered saline

TBST: Tris-buffered saline with Tween

Trib3: Tribbles pseudokinase 3

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References

[1] Fahn S. Description of Parkinson's disease as a clinical syndrome. Annals of the New York Academy of Sciences. 2003;**991**:1-14

[2] Fahn S, Sulzer D. Neurodegeneration and neuroprotection in Parkinson disease. NeuroRx. 2004;**1**(1):139-154

[3] Davie CA. A review of Parkinson's disease. British Medical Bulletin. 2008;**86**:109-127

[4] Schapira AH. Neurobiology and treatment of Parkinson's disease. Trends in Pharmacological Sciences. 2009;**30**(1):41-47

[5] Levy OA, Malagelada C, Greene LA. Cell death pathways in Parkinson's disease: Proximal triggers, distal effectors, and final steps. Apoptosis. 2009;**14**(4):478-500

[6] Greene LA, Tischler AS. Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. Proceedings of the National Academy of Sciences of the United States of America. 1976;**73**(7):2424-2428

[7] Encinas M, Iglesias M, Liu Y, et al. Sequential treatment of SH-SY5Y cells with retinoic acid and brain-derived neurotrophic factor gives rise to fully differentiated, neurotrophic factor-dependent, human neuron-like cells. Journal of Neurochemistry. 2000;**75**(3):991-1003

[8] Walkinshaw G, Waters CM. Neurotoxin-induced cell death in neuronal PC12 cells is mediated by induction of apoptosis. Neuroscience. 1994;**63**(4):975-987

[9] Sheehan JP, Palmer PE, Helm GA, et al. MPP+ induced apoptotic cell death in SH-SY5Y neuroblastoma cells: An electron microscope study. Journal of Neuroscience Research. 1997;**48**(3):226-237

[10] Nakamura K, Bindokas VP, Marks JD, et al. The selective toxicity of 1-methyl-4-phenyl-pyridinium to dopaminergic neurons: The role of mitochondrial complex I and reactive oxygen species revisited. Molecular Pharmacology. 2000;**58**(2):271-278

[11] Gimenez-Cassina A, Lim F, Diaz-Nido J. Differentiation of a human neuroblastoma into neuron-like cells increases their susceptibility to transduction by herpesviral vectors. Journal of Neuroscience Research. 2006;**84**(4):755-767

[12] Cheung YT, Lau WK, Yu MS, et al. Effects of all-trans-retinoic acid on human SH-SY5Y neuroblastoma as in vitro model in neurotoxicity research. Neurotoxicology. 2009;**30**(1): 127-135

[13] Sun X, Liu J, Crary JF, et al. ATF4 protects against neuronal death in cellular Parkinson's disease models by maintaining levels of parkin. The Journal of Neuroscience. 2013;**33**(6):2398-2407

[14] Naldini L, Blömer U, Gallay P, et al. In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector. Science. 1996;**272**(5259):263-267

[15] Zufferey R, Nagy D, Mandel RJ, et al. Multiply attenuated lentiviral vector achieves efficient gene delivery in vivo. Nature Biotechnology. 1997;**15**(9):871-875

[16] Dull T, Zufferey R, Kelly M, et al. A third-generation lentivirus vector with a conditional packaging system. Journal of Virology. 1998;**72**(11):8463-8471

[17] Tiscornia G, Singer O, Verma IM. Design and cloning of lentiviral vectors expressing small interfering RNAs. Nature Protocols. 2006;**1**(1):234-240

[18] Tiscornia G, Singer O, Verma IM. Production and purification of lentiviral vectors. Nature Protocols. 2006;**1**(1):241-245

[19] Ryu EJ, Harding HP, Angelastro JM, et al. Endoplasmic reticulum stress and the unfolded protein response in cellular models of Parkinson's disease. The Journal of Neuroscience. 2002;**22**(24):10690-10698

[20] Ryu EJ, Angelastro JM, Greene LA. Analysis of gene expression changes in a cellular model of Parkinson disease. Neurobiology of Disease. 2005;**18**(1):54-74

[21] Michel PP, Hirsch EC, Hunot S. Understanding dopaminergic cell death pathways in Parkinson disease. Neuron. 2016;**90**(4):675-691

[22] Xiang C, Wang Y, Zhang H, et al. The role of endoplasmic reticulum stress in neurodegenerative disease. Apoptosis. 2017;**22**(1):1-26

[23] Silva RM, Ries V, Oo TF, et al. CHOP/GADD153 is a mediator of apoptotic death in substantia nigra dopamine neurons in an in vivo neurotoxin model of parkinsonism. Journal of Neurochemistry. 2005;**95**(4):974-986

[24] Zhao Q, Yang X, Cai D, et al. Echinacoside protects against MPP(+)-induced neuronal apoptosis via ROS/ATF3/CHOP pathway regulation. Neuroscience Bulletin. 2016;**32**(4):349-362

[25] Aimé P, Sun X, Zareen N, et al. Trib3 is elevated in Parkinson's disease and mediates death in Parkinson's disease models. The Journal of Neuroscience. 2015;**35**(30):10731-10749

[26] Zareen N, Biswas SC, Greene LA. A feed-forward loop involving Trib3, Akt and FoxO mediates death of NGF-deprived neurons. Cell Death and Differentiation. 2013;**20**(12): 1719-1730

[27] Malagelada C, Ryu EJ, Biswas SC, et al. RTP801 is elevated in Parkinson brain substantia nigral neurons and mediates death in cellular models of Parkinson's disease by a mechanism involving mammalian target of rapamycin inactivation. The Journal of Neuroscience. 2006;**26**(39):9996-10005

[28] Malagelada C, Jin ZH, Greene LA. RTP801 is induced in Parkinson's disease and mediates neuron death by inhibiting Akt phosphorylation/activation. The Journal of Neuroscience. 2008;**28**(53):14363-14371

[29] Malagelada C, Jin ZH, Jackson-Lewis V, et al. Rapamycin protects against neuron death in in vitro and in vivo models of Parkinson's disease. The Journal of Neuroscience. 2010;**30**(3):1166-1175

[30] Carracedo A, Gironella M, Lorente M, et al. Cannabinoids induce apoptosis of pancreatic tumor cells via endoplasmic reticulum stress-related genes. Cancer Research. 2006;**66**(13):6748-6755

[31] Salazar M, Carracedo A, Salanueva IJ, et al. Cannabinoid action induces autophagy-mediated cell death through stimulation of ER stress in human glioma cells. The Journal of Clinical Investigation. 2009;**119**(5):1359-1372

[32] Lewerenz J, Maher P. Basal levels of eIF2alpha phosphorylation determine cellular antioxidant status by regulating ATF4 and xCT expression. The Journal of Biological Chemistry. 2009;**284**(2):1106-1115

[33] Hashida K, Kitao Y, Sudo H, et al. ATF6alpha promotes astroglial activation and neuronal survival in a chronic mouse model of Parkinson's disease. PLoS One. 2012;**7**(10):e47950

[34] Massie A, Schallier A, Mertens B, et al. Time-dependent changes in striatal xCT protein expression in hemi-Parkinson rats. Neuroreport. 2008;**19**(16):1589-1592

[35] Sian J, Dexter DT, Lees AJ, et al. Alterations in glutathione levels in Parkinson's disease and other neurodegenerative disorders affecting basal ganglia. Annals of Neurology. 1994;**36**(3):348-355

[36] Smeyne M, Smeyne RJ. Glutathione metabolism and Parkinson's disease. Free Radical Biology & Medicine. 2013;**62**:13-25

[37] Miyazaki I, Murakami S, Torigoe N, et al. Neuroprotective effects of levetiracetam target xCT in astrocytes in parkinsonian mice. Journal of Neurochemistry. 2016;**136**(1):194-204

[38] Massie A, Schallier A, Kim SW, et al. Dopaminergic neurons of system x(c)(−)-deficient mice are highly protected against 6-hydroxydopamine-induced toxicity. The FASEB Journal. 2011;**25**(4):1359-1369

Section 4

Application of Transcription-Based Therapies on Human

Chapter 12

Transcriptional Regulation of the Intestinal Cancer Stem Cell Phenotype

Antoine Gleizes, Vincent Cavaillès and Marion Lapierre

Additional information is available at the end of the chapter

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Abstract

Colorectal cancer (CRC) is one of the most frequent cancers worldwide. Current treatments include surgery and chemotherapy, but disease recurrence occurs frequently. The continuous renewal of intestinal epithelium relies on the presence of intestinal stem cells that are also at the origin of CRC and contribute to therapy resistance and metastatic dissemination. Several nuclear signaling pathways and transcription factors regulate both intestinal cell homeostasis and tumorigenesis. However, the transcriptional events that govern the emergence of aggressive therapy-resistant cancer stem cells are still poorly defined. This review summarizes the relevance of transcription factors in intestinal stem cell biology and their involvement in colon cancer development and drug resistance.

Keywords: transcription factors, intestinal cancer stem cells, colon cancer, chemotherapy

1. Introduction

Colorectal cancer (CRC) is one of the most frequent cancers worldwide. The current standard-of-care management includes surgery, radiotherapy and chemotherapy, sometimes in association with targeted agents to block tyrosine kinase receptors or their ligands. However, cancer recurs in 30–50% of patients [1].

The intestinal epithelium is continuously renewing, thanks to the presence of multipotent stem cells (SCs) within the intestinal crypts that give rise to all the differentiated cell types [2]. Different signaling pathways, including Wnt and Notch, and transcription factors are involved in intestinal development, homeostasis and maintenance of the intestinal SC properties [3]. These signaling cascades must be finely controlled because their deregulation is involved in gut

tumorigenesis. Importantly, recent studies suggest that tumor-initiating cells or cancer stem cells (CSCs) can regenerate a tumor and might be at the origin of CRC [4, 5]. Thus, a better understanding of CSC function in tumor initiation, progression and resistance to treatment is necessary to improve the screening, prevention and clinical management of patients with CRC.

In this review, we propose an overview of key transcriptional regulations that are involved in intestinal SC/CSC biology. We present the major signaling pathways and the main transcription factors involved in intestinal homeostasis as well as their roles in the transcriptional regulation of intestinal CSCs.

2. The intestinal epithelium and the stem cell compartment

The main functions of the small intestine are food digestion and absorption and production of gastrointestinal hormones. It is subdivided in duodenum, jejunum and ileum, and is one of the most rapidly self-renewing tissues [6]. It is characterized by the presence of villi and Lieberkühn crypts. The large intestine (cecum, colon and rectum) is specialized in compacting stool for rapid excretion, and is arranged in multiple crypts associated with a flat luminal surface. It shows slower renewal capacities than the small intestine [7].

The intestinal epithelium develops from the embryonic endoderm [8] and its cellular composition is quite similar along the entire intestinal tract. The intestine incredible self-renewal capacity is supported by the SC compartment located at the bottom of the crypts. Specifically, transit-amplifying (TA) cells undergo four to five rounds of rapid cell division and then move out of the crypt and terminally differentiate into enterocytes, goblet cells, Tuft cells and enteroendocrine cells (**Figure 1**). These differentiated cells continue to move up along the villus and die by anoikis 2 or 3 days after having reached the villus tip. Paneth cells also derive from intestinal SC, but migrate downwards and settle at the crypt base where they live for 6–8 weeks [9]. Two other cell types have been detected in the intestinal epithelium: M cells that are associated with Peyer's patches and Cup cells that are located in the ileum.

To date, two SC populations have been identified in the crypts, highlighting the high plasticity of the intestinal epithelial SC compartment. The first one corresponds to crypt-based columnar (CBC) cells that express the leucine-rich receptor, LGR5 and are interspersed between Paneth cells (**Figure 1**). CBC cells are required for the long-term maintenance of the self-renewing epithelium. Indeed, they cycle steadily to produce the rapidly proliferating TA cells that can differentiate into all lineages [6]. In the colon, LGR5+ cells are considered to be SCs because they are pluripotent and can maintain epithelial cell self-renewal over long periods of time. However, LGR5+ cells in the small intestine seem to divide more actively than in the colon, possibly due to differences in the epithelial turnover rates [6].

The second crucial SC population corresponds to 'reserve' SCs that can be rapidly recruited to maintain epithelial homeostasis following injury [7]. They are located at position four from the crypt base (hence, the name of +4 SCs) and are generally considered to be relatively quiescent and resistant to acute injury (**Figure 1**). This population was discovered by Potten et al.

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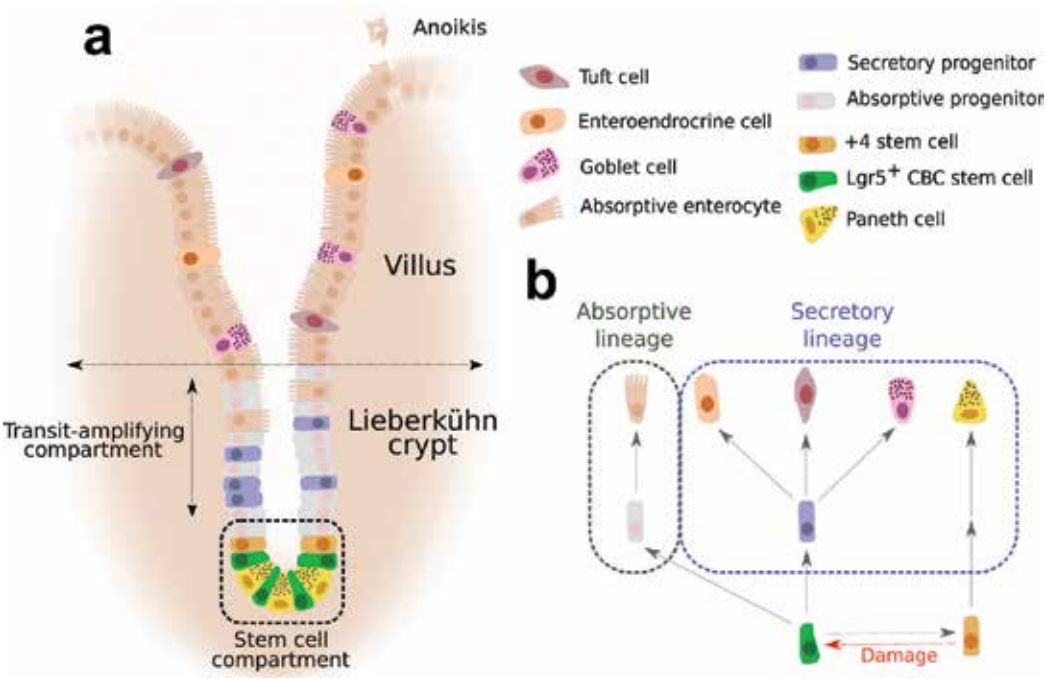

Figure 1. Schematic representation of the intestinal epithelium and the hierarchy of intestinal lineages. Self-renewal of the intestinal epithelium is fueled by small intestinal stem cells (at the bottom of the crypt) that give rise to progenitor cells. These can subsequently differentiate into the mature cell types required for normal gut function.

and was described as the only one responsible for the maintenance of intestinal homeostasis, but without counterpart in the colon [10]. They can retain DNA labels (a surrogate SC marker), possibly due to their infrequent replication or selective retention of labeled DNA during division. Their relative quiescence also explains their resistance to radiation. This SC population was identified thanks to its strong and localized expression of the *BMI1* gene that encodes a component of the Polycomb repressor complex [7]. Lineage tracing of these cells revealed strict terminal differentiation toward the Paneth cell lineage. However, following injury, this population can start cycling and show typical intestinal SC activity and multipotency [11]. These features are typical of SCs, despite the fact that, differently from CBC cells, they do not generate all epithelial lineages.

3. Colorectal cancer and intestinal cancer stem cells

3.1. Colorectal cancer

Genetic or epigenetic changes can lead to deregulated cell proliferation, resulting in tumor growth [12]. In the intestine, tumors start with the formation of small lesions called aberrant crypt foci (ACF). ACF expansion gives rise to an adenoma that can progress to *in situ* carcinoma

and finally to invasive adenocarcinoma [12]. Studies in humans and in animal models suggest that intestinal tumor development is a process where each successive genetic change confers growth advantage to tumor cells. Collectively, these genetic changes in cancer cells allow tumor progression through different stages [12]. Indeed, CRC development is considered as a paradigm of stepwise tumorigenesis with subsequent histopathological stages that precede invasive neoplastic growth and are associated with a progressively increasing number of specific genetic aberrations [11].

3.2. Intestinal cancer stem cells

Intriguingly, the biology of intestinal SCs and CRCs is highly interconnected. In many intestinal malignancies, it is assumed that the 'cell of origin' is a SC that acquired the initial mutation(s) necessary for malignant conversion [11]. These genetic alterations promote self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, programmed cell death evasion, limitless replicative potential, sustained angiogenesis, tissue invasion and ultimately metastasis formation [13]. Additionally, heterogeneously differentiated cell types are found in individual CRC specimens, contributing to the idea that such tumors are 'caricatures' of the normal intestinal tissue. This notion is further strengthened by the discovery of SC-like cancer cells that express intestinal SC markers and display multipotency and self-renewal capacities.

It is thought that intestinal CSCs are the cells that drive tumor growth and progression [11]. Indeed, intestinal CSCs, but not intestinal SCs, can regenerate tumors upon transplantation in animals [14]. CSCs are defined by four main characteristics: (i) they can be serially transplanted for multiple generations because of their self-renewal capacity; (ii) CSCs can generate bulk populations of non-tumorigenic cells by asymmetrical division, which is consistent with the hierarchical model of tumor development. Conversely, symmetrical division allows CSC maintenance within the tumor; (iii) CSCs retain their tumorigenic potential when transplanted into animals and (iv) CSCs can be separated from non-SCs using specific surface markers [14, 15]. In the last decades, the concept of CSC hierarchical arrangement has changed our understanding of tumor cell heterogeneity. The current CSC model postulates that CSCs reside at the top of the tumor hierarchy and differentiate unidirectionally into highly proliferative non-CSCs [12].

4. Nuclear signaling pathways that control intestinal CSCs

In this part, we will focus on the major signaling pathways and transcription factors that are involved in the transcriptional regulation of intestinal SC/CSCs (**Figure 2**) and that could consequently be associated with tumor development/progression and/or cancer cell resistance to therapy.

4.1. The Wnt pathway and its effectors

4.1.1. The Wnt pathway

The Wnt pathway is involved in many biological processes and is essential for epithelial intestinal homeostasis (**Figure 2**) [16]. Accumulation and translocation of β-catenin into the

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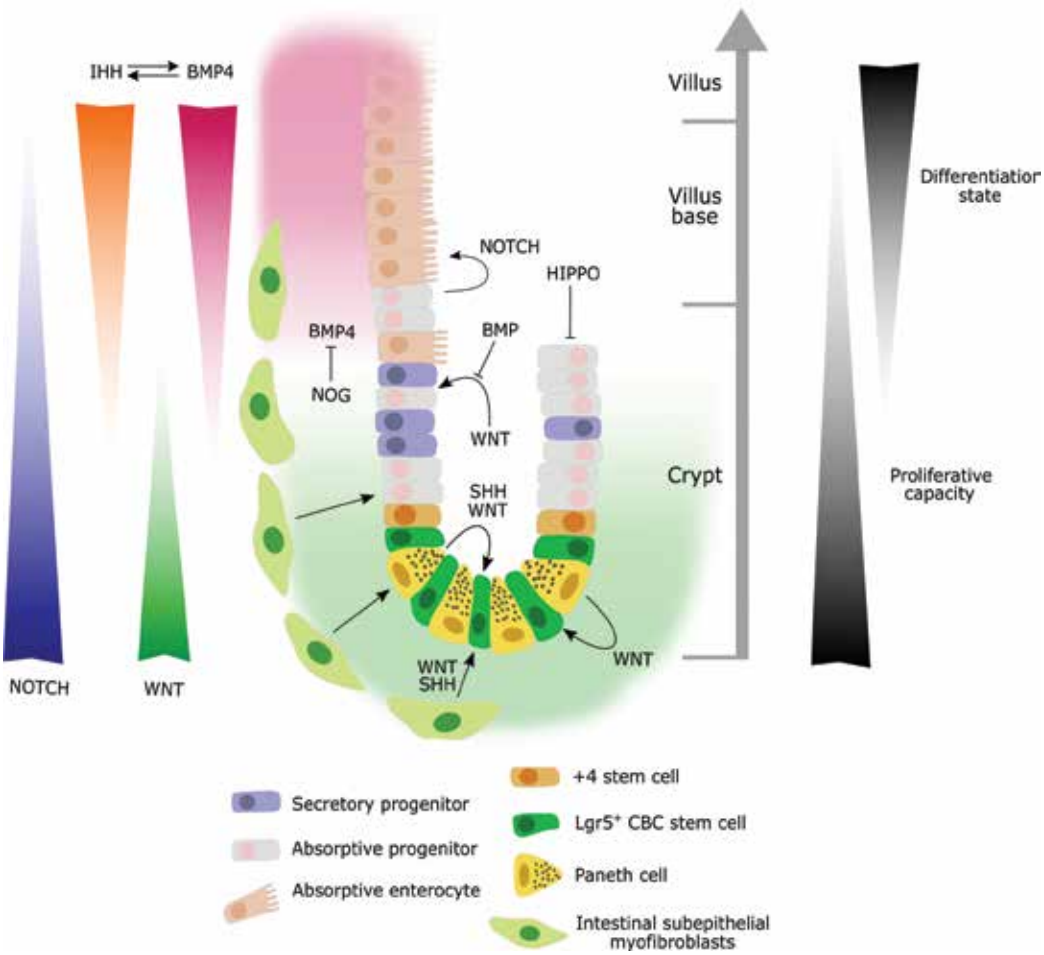

Figure 2. Schematic representation of the major signaling pathways involved in cancer stem cell biology. A gradient of BMP and Hh signaling, with relatively high activity in the villus and less activity within the crypt, regulates cell renewal and lineage specification. Wnt and Notch signaling gradients in the opposite direction (highest expression at the crypt base) play an important role in maintaining the stem cell compartment.

nucleus are the hallmark of the canonical Wnt pathway activity. In the absence of Wnt ligands, β-catenin is phosphorylated, ubiquitinylated and degraded by the proteasomal machinery. Binding of Wnt ligands to their receptors results in the cytoplasmic accumulation of β-catenin that then translocates into the nucleus where it functions as a transcriptional co-activator of Wnt-target genes. The best characterized binding partners of β-catenin in the nucleus are the members of the lymphoid enhancer factor (LEF)/T cell factor (TCF) DNA-binding transcription factors [17]. Some of the downstream targets of the Wnt signaling pathway, such as SOX9 and KLF4/5, are involved in the control of the intestinal CSC phenotype and in CRC development and will be described below (see Sections 4.1.2 and 4.1.3).

In up to 80% of colorectal carcinomas, mutations in molecules that are part of the Wnt/β-catenin pathway (notably truncating mutations in the *Apc* gene) lead to the formation of constitutive nuclear TCF/β-catenin complexes and to uncontrolled transcription of TCF-4 target genes [18]. In

the mouse, specific deletion of the *Apc* gene in LGR5+ SCs triggers the formation of many LGR5+ adenomas in the small and large intestine [5]. Similarly, lack of β-catenin repression in intestinal +4 SCs promotes the formation of BMI1+ adenomas [19]. Moreover, loss of APC negative control induces constitutive nuclear β-catenin/TCF complex activation and hyper-proliferation of the SC compartment [19].

The Wnt signaling pathway has a role also in human intestinal CSCs. In spheroid cultures of CSCs isolated from biopsies of patients with CRC, Wnt expression is heterogeneous. Injection of Wnt^{high} cells in mice results in more effective tumor formation compared with Wnt^{low} cells. The heterogeneous Wnt expression pattern is maintained in the tumors and is related to the expression of several intestinal SC markers, such as LGR5 and ASCL2 [20]. Additionally, colonospheres developed from human CSCs show increased β-catenin expression, associated with transcriptional activation of TCF/LEF [21]. Hence, activation of Wnt/β-catenin signaling can convert intestinal SCs into CSCs, which corresponds to the first step of malignant transformation [19].

Several studies tried to correlate β-catenin activation/expression level with the outcome of patients with CRC. For instance, in 2007, Lugli et al. analyzed tissue microarray data on more than 1400 CRC biopsies and found that high level of β-catenin nuclear expression is an independent adverse prognostic factor [22].

4.1.2. SOX9

The SRY-related high-mobility group box 9 (*SOX9*) gene is a physiological target of the TCF/β-catenin complex that promotes cell proliferation. This key terminal effector of the Wnt pathway is required for +4 SC differentiation into Paneth cells [23]. In the intestinal epithelium, SOX9 expression pattern in the SC compartment almost perfectly overlaps with that of the proliferative marker Ki-67. Interestingly, SOX9 positively regulates its own expression in many cell types and exerts a negative feedback-loop on TCF/β-catenin activity, leading to restriction of intestinal SC proliferation [23, 24].

SOX9-deficient mice exhibit higher cell proliferation, extensive colon hyperplasia with numerous enlarged crypts. However, SOX9 deletion is not sufficient to induce malignancy [25]. Moreover, SOX9 overexpression in human CRC cells results in cell cycle progression and apoptosis bypass, due to increased *BMI1* gene expression [26]. Additionally, in colon epithelial cells, high SOX9 expression is associated with undifferentiated states, SC-like properties and high LGR5 mRNA level *in vitro* [27]. SOX9 has several pro-oncogenic properties, including the ability to promote cell proliferation, to inhibit senescence and to collaborate with other oncogenes in neoplastic transformation [26]. However, recent *in vitro* and *in vivo* studies have described SOX9 tumor suppressor activities in CRC cells. Specifically, SOX9 inhibits β-catenin activity by interacting physically with this protein and removing it from chromatin. It also decreases expression of the c-Myc oncogene, a target of the Wnt/β-catenin pathway [28].

The strong expression of SOX9 in CRC cells due to the constitutive activity of the Wnt pathway can contribute to cancer progression and/or influence tumor differentiation. *SOX9* displays missense or frameshift mutations in almost 10% of CRC [29]. SOX9 mutation rate is higher in more advanced tumors and is correlated with activated KRAS, an oncogene frequently mutated during

CRC development, thus facilitating transformation and tumor progression [29]. Furthermore, a SOX9 splice variant (MiniSOX9) that contains the HMG domain responsible for binding to DNA but devoid of the trans-activating domain has been discovered [30]. MiniSOX9 inhibits SOX9 activity by a dominant-negative effect *in vitro* and can promote the Wnt/β-catenin pathway, resulting in β-catenin over-activation. In addition, strong MiniSOX9 expression is observed in CRC tumor tissue, while it is undetectable in the adjacent normal tissue [30]. Wild type and many SOX9 mutants regulate tumor proliferation capacity, notably through regulation of the CSC pool. Nevertheless, SOX9 protein level could not be clearly associated with patient prognosis [31].

4.1.3. Krüppel-like factors (KLF)

4.1.3.1. KLF4

KLF4 was originally identified as a gut-enriched transcription factor in the intestine and is expressed in terminally differentiated columnar intestinal epithelial cells [32]. KLF4 regulates intestinal epithelial homeostasis and has a critical role in the development and terminal differentiation of goblet cells [32]. In human HT-29 CRC cells, KLF4 inhibits cell proliferation by blocking progression from the G1 to S phase of the cell cycle through inhibition of cyclin D1 expression [33].

Moreover, mutations in the Wnt/β-catenin pathway are associated with KLF4 downregulation in human CRC cell lines. Indeed, KLF4 is an indirect APC target and is considered to be a repressor of BMI1 transcriptional activity [34, 35]. Furthermore, using a KLF4 inducible system in CRC cell lines, it was demonstrated that KLF4 reduces colony formation, cell migration and invasion [34]. Additionally, KLF4 overexpression in human adenocarcinoma cells leads to reduced [3H]-thymidine uptake, whereas inhibition of KFL4 expression increases DNA synthesis, confirming that KLF4 plays an essential role in colon cell growth arrest [36]. Surprisingly, despite its tumor suppressor activity, KLF4 is overexpressed in colon CSC-enriched spheroids compared with the parental CRC cells from which the spheroids were derived [37]. Moreover, KLF4 knockdown affects the stemness phenotype and decreases the malignant profile of these CSC-enriched spheroid cells, in line with its role in reprogramming murine fibroblasts into stem cells [37, 38].

In agreement with its tumor suppressor activity, KLF4 expression is frequently lost in CRC and its downregulation is strongly associated with tumor development. Moreover, loss of heterozygosity on chromosome 9q31, where the *KLF4* gene is localized, is frequently found in human CRC, and could lead to uncontrolled cell proliferation and to a SC-like phenotype of differentiated cells [33]. Low KLF4 expression levels are also found in colon adenomas and metastases [33]. Lee et al. confirmed that *KLF4* mRNA expression levels are lower in CRC tumor tissue compared with normal tissue [39]. However and surprisingly, they observed that high KLF4 level in normal tissue is correlated with high KLF4 expression in tumors and is associated with poor patient survival [39].

The conflicting results between clinical studies concerning KLF4 prognostic value could be explained by the differential regulation of KLF4 mRNA and protein expression in CRC or by the presence also of KLF4+ stromal cells in the tumor samples. Additional investigations are needed to elucidate these data; nevertheless, KLF4 expression levels in normal and tumor tissues are prognostic markers for CRC.

4.1.3.2. KLF5

The transcription factor KLF5 can interact with several components of different signaling pathways (e.g., the Wnt, Hippo, TGF-β and Notch signaling cascades) and mediate their activity [40]. In physiological conditions, KLF5 is strongly expressed by intestinal progenitor and stem cells, suggesting a role in cell proliferation control [41].

Stable KLF5 overexpression in HT-29 CRC cells promotes spheroid formation [40]. Conversely, deletion of the *KLF5* gene in mouse LGR5+ SCs promotes β-catenin nuclear localization and the appearance of abnormal apoptotic cells in the intestinal crypts, due to inhibition of their proliferation and survival capacities [41]. In agreement, KLF5 is required for the tumor-initiating activity of β-catenin during intestinal tumorigenesis in Apc^{Min} mice [41]. Inhibition of *KLF5* gene expression in CRC cell lines reduces cell proliferation and transformation as well as anchorage-independent growth [42].

In patients with CRC, intestinal tumor progression is associated with *KLF5* gene upregulation in the primary tumor and also in metastases, compared with healthy tissues [41]. Moreover, comparative genomic hybridization (CGH) array analysis of human CRC samples highlighted the frequent chromosomal amplification of the *KLF5* locus [41]. CRC samples with mutated KRAS also display KLF5 upregulation, associated with increased cell proliferation [42]. As activating KRAS mutations are found in more than 50% of CRC, KLF5 appears to be an important downstream mediator of activated KRAS during CRC development. These findings indicate that KLF5 is a major regulator of intestinal SC proliferation in normal and pathological conditions.

4.2. The Notch pathway and BMI1

4.2.1. The Notch pathway

The Notch signaling cascade is one of the major pathway involved in intestinal homeostasis and in the direct regulation of cell fate [43]. The initiating step of the Notch signaling cascade is the interaction between one of its five ligands (Delta-like1/3/4, Jagged1/2) and a Notch receptor (Notch1–4). Upon ligand binding, the receptor conformational change through proteolytic cleavage leads to nuclear translocation of cleaved Notch intracellular domain (NICD) and its association with the DNA-binding transcription factor CSL (also called RBP-Jκ). This turns the CSL complex from a transcriptional repressor into a transcriptional activator. The best known targets of the CSL/NICD complex are members of the *HES* gene family and their homologs, the *Hey* (also called HERP) gene family of basic helix-loop-helix transcription factors. This is known as the canonical Notch pathway [17, 43, 44].

In the colon, Notch signaling is an essential gatekeeper of intestinal progenitors and clearly plays an important role in the maintenance of the colon crypt compartment [45] (**Figure 2**). Using small-molecule inhibitors and short hairpin RNA-mediated knock-down, it has been demonstrated that Notch prevents apoptosis of colon cancer-initiating cells (CCICs) and is critical for self-renewal [46]. Moreover, the Notch pathway supports slow-cycling BMI1+ CCICs, by promoting their self-renewal, tumorigenicity and chemoresistance in tumor xenografts [47].

In CRC, the Notch pathway is strongly activated compared with normal tissue. Moreover, expression analysis of resection biopsies from patients with CRC showed that Notch1 expression level is correlated with poor prognosis and is a good predictive marker of cancer progression [48]. Intriguingly, the expression level of Notch2 is negatively correlated with that of Notch1 in CRC and Notch2 has anti-tumoral properties [48]. These opposite features could be used to develop a fine prognostic marker of CRC progression and recurrence.

4.2.2. BMI1

BMI1 is a downstream target of Notch signaling and a key component of the Polycomb group [49]. BMI1 is expressed in almost all tissue types and regulates a myriad of cellular processes that are critical for cell growth, cell fate decision, development, senescence, aging, DNA damage repair, apoptosis and SC self-renewal [49, 50]. BMI1 is highly expressed in intestinal SCs and isolated BMI1+ cells can generate epithelial organoids in culture [7]. Additionally, BMI1 loss decreases murine intestinal SC proliferation and promotes their differentiation into goblet cells [49]. BMI1 also contributes to the tumor-initiating and self-renewal abilities of human CRC cells because its downregulation inhibits tumor cell growth and is associated with reduction of tumor-initiating cells [51]. Moreover, BMI1 is involved in intestinal CSC invasion and migration. Indeed, a recent study demonstrated that BMI1 represses E-cadherin expression in colon CSCs, thus promoting metastasis formation via epithelial to mesenchymal transition [50].

Altogether, these data strongly support BMI1 role in the maintenance of the intestinal CSC phenotype. In agreement, clinical studies showed that BMI1 expression is a negative prognostic marker in CRC [52]. BMI1 mRNA and protein are overexpressed in colorectal adenomas and carcinomas compared with normal tissues [53]. A gradient of BMI1 expression has been reported in human colon precancerous and cancerous tissues and is correlated with the cancer stage, suggesting that BMI1 contributes to CRC progression [53].

Indeed, BMI1 is considered to be a negative CRC prognostic biomarker, and patients with BMI1-positive tumors are at higher risk of disease recurrence and/or metastases compared with those with BMI1-negative tumors. As BMI1 has a role in maintaining the intestinal CSC phenotype, high BMI1 expression could indicate the presence of a large CSC population in the tumor. Consequently, high proportion of CSCs in a tumor could be an indicator of poor prognosis [31, 54, 55].

4.3. Other signaling pathways

4.3.1. The Hedgehog pathway

The Hedgehog (Hh) signaling pathway is a key regulator of intestinal homeostasis. Hh proteins are part of a family of secreted proteins that are involved in the development and maintenance of the gastrointestinal tract [17]. Aberrant activation of the Hh signaling pathway is associated with tumorigenesis in various tissues. The roles of Hh signaling differ at each CRC stage, from adenoma to adenocarcinoma [56]. Moreover, Sonic Hedgehog (SHH), one of the Hh effectors, promotes CRC development, while Indian Hedgehog (IHH) inhibits CRC formation [56].

IHH regulates intestinal SC fates by interfering with the maturation and localization of the underlying stromal cells that in turn generate signaling molecules needed for the maintenance of the intestinal SC niche [56] (**Figure 2**). IHH, expressed by differentiated enterocytes, indirectly inhibits Wnt signaling at the crypt base and reduces the number of proliferating precursor cells [17, 57]. A decrease in Hh signaling is correlated with the expansion of the intestinal SC pool, with blunted enterocyte differentiation and activation of the Wnt pathway. Moreover, *IHH* gene knock-out leads to intestinal SC accumulation [57]. In addition, specific Hh activation in murine stromal cells induces complex transcriptional changes, leading to loss of colon SC-specific gene expression and upregulation of epithelial differentiation markers [58]. Most of the components of the Hh signaling pathway are upregulated (mRNA and protein) in CRC, with the exception of IHH that appears to be downregulated. Overexpression of members of the Hh signaling pathway is associated with poor survival and adverse clinical features [59]. However, in metastatic CRC, treatment with vismodegib, an Hh pathway inhibitor, in combination with standard chemotherapy, does not significantly improve patient survival [60].

4.3.2. The BMP pathway

The BMP pathway regulates many cellular mechanisms, including apoptosis and cell growth, depending on the specific cellular context. BMP ligands are secreted in their active form and homodimerize before binding to their cognate BMP receptors (BMPR). SMAD transcription factors are the main downstream effectors of BMP signaling that plays key roles in adult gut homeostasis, inflammation and cancer.

Specific inhibition of BMP signaling in intestinal epithelial cell does not lead to initiation of colon tumors *in vivo*, while suppression in mesenchymal myofibroblasts is associated with spontaneous tumor formation. This suggests that inhibition of BMP signaling in the mesenchymal cells surrounding the intestinal epithelium acts as a trigger of gastrointestinal tumorigenesis [61]. Moreover, BMP4 expression is lost in intestinal CSCs, leading to deregulation of the proliferative compartment [62].

Nevertheless, it is still unclear whether BMP limits expansion of intestinal epithelial cells by repressing LGR5+ intestinal SC self-renewal or by inhibiting epithelial cell proliferation. In addition, BMP type Ia receptor (*Bmpr1a*) conditional knock-out in the intestinal epithelium leads to intestine hyperplasia with multiple intestinal polyps due to hyperactive SCs [63]. Moreover, in these mice, the LGR5+ SC pool is enlarged due to increased survival, allowing better intestinal regeneration [63]. Among the BMP family members, BMP2 and BMP4 are specifically involved in intestinal CSC regulation by promoting their differentiation and antagonizing Wnt/β-catenin signaling [64]. Furthermore, a recent study showed that the transcription factor GATA6 is a key regulator of CSC expansion and self-renewal through downregulation of BMP genes [65].

Finally, mutations that affect BMP signaling are frequently observed in patients with juvenile polyposis syndrome that is characterized by non-cancerous polyps, as well as in patients with progressing CRC. Analysis of SMAD4 expression levels in patients with CRC showed that it is downregulated in CRC and associated with poor prognosis [66].

4.3.3. The Hippo pathway

The Hippo pathway regulates various cellular processes, including cell survival, proliferation and differentiation, but has been involved only recently in SC biology [67]. Yes-associated protein (YAP) and transcriptional co-activator with PDZ binding motif (TAZ) are the central effector molecules of this signaling cascade and are abundantly expressed in the cytoplasm of both proliferating and post-mitotic cells [17, 68].

In cancer, the Hippo pathway inhibits cell proliferation, promotes apoptosis and regulates stem/progenitor cell expansion. In cancer cells, YAP and TAZ are localized mainly in the nucleus and promotes cell and tumor growth. There is considerable evidence that abnormal Hippo signaling is associated with tumor progression and YAP/TAZ overexpression is frequently observed in CRC [67]. This overexpression could be linked to Wnt/β-catenin over-activation because YAP is a specific target of this pathway. Furthermore, the major components of the Hippo pathway (i.e., MST1/2 and MOBKL1A/B) that control YAP/TAZ activity display low expression levels in colon carcinomas [69]. YAP deletion in Apc^{min} mice prevents polyp formation and blocks the differentiation of $Apc^{-/-}$ organoids. Moreover, using a mosaic model of *Yap* and *Apc* gene deletion in intestinal SCs, YAP appears to be dispensable for tumor initiation, but crucial for progression of tumor-initiating cells to adenoma [70].

Hippo pathway dysregulation, leading to loss of YAP repression, has been observed in different cancer types [71]. In patients with CRC, YAP over-activation is closely related to β-catenin over-activation. Moreover, the tyrosine kinase c-Yes is hyper-phosphorylated in 5-fluorouracil-resistant cells with CSC features, thus preventing YAP nuclear translocation [72]. Finally, *YES1* and *YAP* levels are correlated with worse prognosis in chemotherapy-treated patients with CRC, suggesting that chemotherapy favors the selection of intestinal CSCs with deregulated c-Yes and YAP [72].

4.4. Other intestinal CSC-related transcription factors

4.4.1. PXR

Pregnane X Receptor (PXR, NR1I2), a member of the nuclear receptor superfamily, is highly expressed in the colon. PXR targets are genes that encode phase I and II metabolic enzymes and phase III drug transporters. Members of the nuclear receptor superfamily function as ligand-activated transcription factors and play critical roles in nearly every aspect of development and adult physiology [73]. Interestingly, it has been reported that the Wnt/β-catenin signaling pathway is crucial for PXR activity and notably that β-catenin is required for PXR-mediated induction of target gene expression [74].

Planque et al. have recently demonstrated that PXR is a potent intestinal CSC phenotype driver by regulating a network of downstream genes involved in self-renewal and chemoresistance [75]. PXR expression is associated with CSC enrichment, after cell sorting of cancer cells using ALDH activity to identify CSCs and after spheroid passaging. In addition, expression of CSC

markers and self-renewal are increased in CRC cells with enhanced PXR transcriptional activity [75]. PXR expression in intestinal CSCs is also associated with tumor aggressiveness and chemoresistance [76]. Specifically, PXR increases the oxaliplatin efflux capacity of cancer cells, thus reducing the cell drug concentration and preventing its effects on cell proliferation and apoptosis [76]. Another study demonstrated that PXR is a master regulator of chemoresistance by regulating genes involved in drug resistance, such as cytochrome P450, multidrug resistance 1 and multidrug resistance-associated protein 2 [77]. Furthermore, PXR is associated with poor survival, particularly after drug treatment. Indeed, in patients with CRC, it allows clonal selection after treatment, leading to the emergence of resistant and more aggressive clones with molecular signatures of poor prognosis [75, 77].

4.4.2. HOPX

The homeodomain-only protein homeobox (HOPX) is strongly expressed in normal colorectal mucosa, and is considered a marker of the +4 SC population in the intestine [78]. Conversely, HOPX-β (an isoform of HOPX) represses conversion to the CBC phenotype in +4 SCs in physiological contexts in mice [78].

HOPX shows tumor suppressor functions in CRC by regulating cell proliferation and inhibiting angiogenesis [79]. Microarray data analysis revealed that, in CRC samples, HOPX downregulates oncoproteins, such as c-FOS and EGR-1. Moreover, EphA2 (which increases tumor invasion and survival) is overexpressed in patients with *HOPX* gene hypermethylation. In addition, HOPX-β promoter is frequently hypermethylated in CRC cell lines and tissues. This methylation results in the downregulation of HOPX mRNA and protein levels. Importantly, in patients with stage III CRC, HOPX-β promoter hypermethylation is associated with worse prognosis [79]. Moreover, in patients with CRC, *HOPX* gene hypermethylation is accompanied by increased expression of Cyr61/CCN1, a critical downstream member of the Hh signaling pathway that affects the pro-angiogenic tumor microenvironment [80].

4.4.3. Sp1

Specificity protein 1 (Sp1) is a transcription factor ubiquitously expressed in mammalian cells that recruits the basal transcription machinery. Sp1 is active in all cell types, but it is also tightly regulated because Sp1 activity can alter the expression of genes involved in cell cycle and growth (including many tumor suppressor genes and oncogenes) in response to signaling pathways and specific cellular conditions [81].

Interestingly, Sp1 levels are higher in colon CSCs than in the parental tumor cells [82]. Moreover, siRNA-mediated *SP1* silencing suppresses the specific features of CSCs derived from CRC cells and promotes apoptosis of colon CSCs *in vitro* [82]. *SP1* silencing also decreases the expression of several CSC markers. Hence, colon CSC self-renewal ability, drug resistance and metastasis potential could be partially related to high Sp1 expression. In agreement, Sp1 overexpression correlates with tumor stage and poor prognosis [81].

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5. Conclusion

Cancer management is one of the major issues in our society and therefore, much research is focused on improving our understanding of cancer development and progression. Here, we presented an overview of the transcriptional dysregulation that affect intestinal epithelium homeostasis and that can lead to tumor initiation and development. In the last decade, considerable progress has been made in understanding the molecular and cellular mechanisms linked to CRC development/progression and a major breakthrough was the identification of cells with CSC properties. Studies in mouse models have shown that CRC development is mainly supported by intestinal CSCs that can self-renew and generate tumor cell heterogeneity even after *in vitro* or *in vivo* passaging. However, CSCs do not cycle as fast as cancer cells. This means that the current therapies that target cycling cancer cells are not efficient against the relatively quiescent CSCs.

CSC fate and properties are regulated through a wide transcriptional network controlled by signaling cascades that often crosstalk and regulate each other (**Figure 3**).

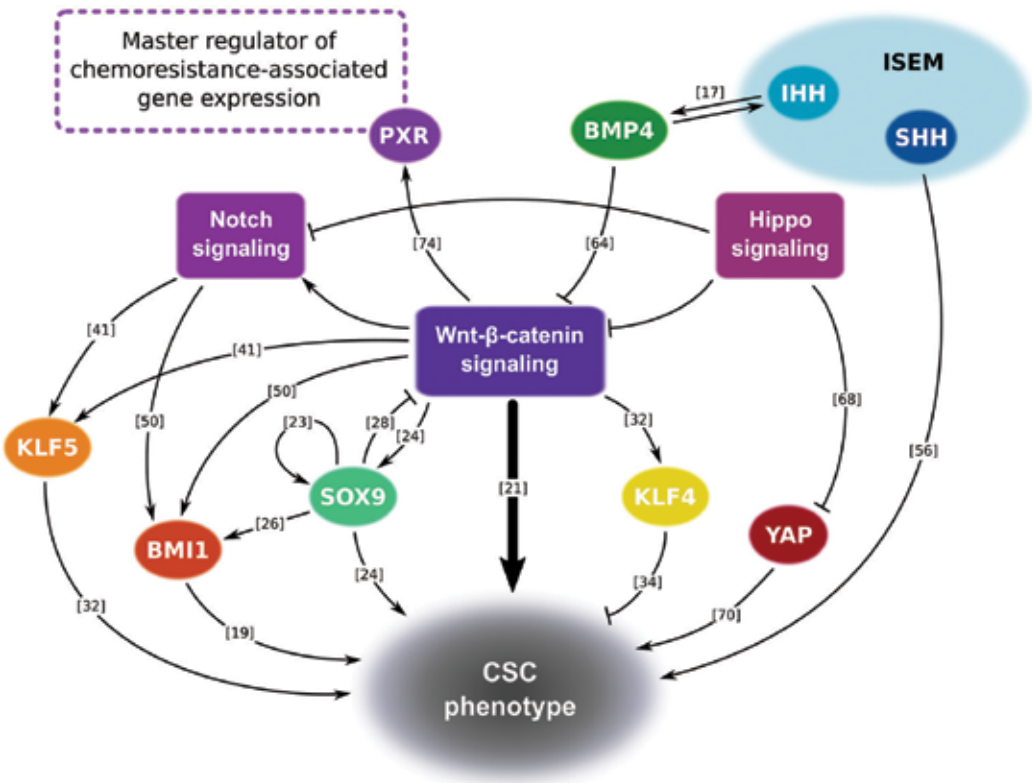

Figure 3. Transcriptional landscape associated with the CSC phenotype in CRC. Schematic representation of the positive (arrows) and negative (bar-ended arrows) regulations between transcription factors and signaling pathways and associated with the CSC phenotype within the tumor. ISEM = intestinal subepithelial myofibroblasts.

Transcription factors	Physiological roles	Status in CRC	CRC-associated phenotype	Prognosis	References
SOX9	Differentiation of Paneth cells Promotion of SC proliferation	Overexpression	Cell cycle progression Apoptosis bypassing undifferentiated state	No correlation	[23–27, 31]
KLF4	Differentiation of Goblet cells	Low expression	Increased DNA synthesis Uncontrolled cell proliferation CSC-like phenotype	Poor	[32, 33, 36, 39]
KLF5	Promotion of cell proliferation	Overexpression	Promotion of cell proliferation Increase of cell survival capacities	No correlation	[41]
BMI1	Promotion of SC proliferation and renewal Prevention of senescence DNA damage repair	Overexpression	Tumor initiation Self-renewal of CRC cells Promotion of cell invasion and migration	Poor	[31, 49–51, 53–55]
IHH	Differentiation of enterocyte cells Inhibition of cell proliferation	Low expression	Expansion of the CSC pool Promotion of cell proliferation		[17, 56, 57]
SHH	Promotion of cell proliferation	Overexpression	Promotion of CRC development	Poor	[56, 59]
SMAD	Differentiation of enterocyte cells Inhibition of $Lgr5^{+}$ SC expansion	Low expression		Poor	[61, 66]
YAP/TAZ	Promotion of cell proliferation	Overexpression and over-activation	Tumor progression	Poor	[67, 70, 72]
PXR	Increase of cholesterol uptake Promotion of intestinal epithelial wound healing and repair		CSC self-renewal Drug resistance	Poor	[73, 75, 77]
HOPX	Maintenance of +4 SC identity	Low expression	Promotion of cell proliferation Promotion of angiogenesis	Poor	[78, 79]
SP1	Cell cycle and growth control	Overexpression	CSC renewal ability Drug resistance Metastasis potential	Poor	[81, 82]

Table 1. Phenotypic outcomes associated with the different transcription factors in normal and tumoral intestinal epithelium.

In this review, we focused on some of these transcription factors and major signaling pathways involved in the regulation of the intestinal CSC phenotype and in CRC development. The basis of CRC development is the over-activation of the Wnt/β-catenin signaling cascade. Then, the disruption of other signaling pathways potentiates the oncogenic process by maintaining or even amplifying these alterations. Similarly, mutations or altered expression of different transcription factors also contribute to the oncogenic network. All these mechanisms concur to promote tumor growth and aggressiveness due to CSC enrichment. Moreover, some of these pathways and transcription factors might confer chemoresistance to the CSC population and are involved in CRC relapse (**Table 1**). Therefore, they are considered poor prognostic markers. Consequently, effective CRC therapies should target not only the highly proliferative cancer cells but also colon CSCs, or sensitize them to therapies. These different signaling pathways and their downstream effectors could represent biomarkers of CRC progression and therapeutic targets.

To conclude, these data do not give the solution on how to cure CRC, but help understanding why its management is not simple. Several topics presented in this review are field of active research. Indeed, there are multiple and complex interactions between key signaling pathways known to control SC behavior. The knowledge on the transcriptional networks that control intestinal CSCs is not complete yet, and some findings are controversial. A better characterization and comprehension of these regulatory mechanisms, notably through network analysis, are needed to identify new therapeutic targets in order to improve patient care.

Abbreviations

ACF	Aberrant crypt foci
ALDH	Aldehyde dehydrogenase
APC	Adenomatous polyposis coli
ASCL2	Achaete-scute family bHLH transcription factor 2
BMI1	B lymphoma Mo-MLV insertion region 1 homolog
BMP4	Bone morphogenetic protein 4
BMPR	Bone morphogenetic protein receptor
CBC	Crypt-based columnar
CCICs	Colon cancer-initiating cells
CGH	Comparative growth hybridization
CRC	Colorectal cancer
CSCs	Cancer stem cells
Cyr61	Cysteine-rich angiogenic inducer 61
EGR1	Early growth factor response 1

EphA2	Ephrin receptor A2
GATA6	GATA binding protein 6
HES	Hairy and enhancer of split
HH	Hedgehog
HMG	High-mobility group
HOPX	Homeodomain-only protein homeobox
IHH	Indian hedgehog
ISEM	Intestinal subepithelial myofibroblasts
KLF	Kruppel-like factor
LEF	Lymphoid enhancer factor
LGR5	Leucine-rich repeat containing G protein-coupled receptor 5
MOBKL1A/B	Mps one binder kinase activator-like 1A and B
MST1/2	Mammalian Ste2-like kinases 1 and 2
MYC	Myelocytomatosis oncogene
NICD	Notch intracellular domain
NOG	Noggin
PXR	Pregnane X receptor
RBP-Jκ	Recombination signal binding protein for immunoglobulin kappa J region
SC	Stem cells
SHH	Sonic hedgehog
SMAD	Mother against Dpp
SOX9	SRY (sex-determining region Y)-related HMG box 9
SP1	Specificity protein 1
TAZ	Transcriptional co-activator with PDZ binding motif
TCF	T cell factor
TGF-β	Tumor growth factor-β
Wnt	Wingless-type MMTV (mouse mammary tumor virus) integration site family
YAP	Yes-associated protein

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References

[1] Cunningham D, Atkin W, Lenz HJ, Lynch HT, Minsky B, Nordlinger B, et al. Colorectal cancer. Lancet (London, England). 2010;**375**(9719):1030-1047

[2] Stappenbeck TS, Wong MH, Saam JR, Mysorekar IU, Gordon JI. Notes from some crypt watchers: Regulation of renewal in the mouse intestinal epithelium. Current Opinion in Cell Biology. 1998;**10**(6):702-709

[3] Clevers H. The intestinal crypt, a prototype stem cell compartment. Cell. 2013;**154**(2): 274-284

[4] Merlos-Suarez A, Barriga FM, Jung P, Iglesias M, Cespedes MV, Rossell D, et al. The intestinal stem cell signature identifies colorectal cancer stem cells and predicts disease relapse. Cell Stem Cell. 2011;**8**(5):511-524

[5] Barker N, Ridgway RA, van Es JH, van de Wetering M, Begthel H, van den Born M, et al. Crypt stem cells as the cells-of-origin of intestinal cancer. Nature. 2009;**457**(7229):608-611

[6] Barker N, van Es JH, Kuipers J, Kujala P, van den Born M, Cozijnsen M, et al. Identification of stem cells in small intestine and colon by marker gene Lgr5. Nature. 2007;**449**(7165):1003-1007

[7] Barker N. Adult intestinal stem cells: Critical drivers of epithelial homeostasis and regeneration. Nature Reviews Molecular Cell Biology. 2014;**15**(1):19-33

[8] Noah TK, Donahue B, Shroyer NF. Intestinal development and differentiation. Experimental Cell Research. 2011;**317**(19):2702-2710

[9] Snippert HJ, van der Flier LG, Sato T, van Es JH, van den Born M, Kroon-Veenboer C, et al. Intestinal crypt homeostasis results from neutral competition between symmetrically dividing Lgr5 stem cells. Cell. 2010;**143**(1):134-144

[10] Potten CS. Extreme sensitivity of some intestinal crypt cells to X and gamma irradiation. Nature. 1977;**269**(5628):518-521

[11] Vermeulen L, Snippert HJ. Stem cell dynamics in homeostasis and cancer of the intestine. Nature Reviews Cancer. 2014;**14**(7):468-480

[12] Vries RG, Huch M, Clevers H. Stem cells and cancer of the stomach and intestine. Molecular Oncology. 2010;**4**(5):373-384

[13] Hanahan D, Weinberg RA. The hallmarks of cancer. Cell. 2000;**100**(1):57-70

[14] Clarke MF, Fuller M. Stem cells and cancer: Two faces of eve. Cell. 2006;**124**(6):1111-1115

[15] Wicha MS, Liu S, Dontu G. Cancer stem cells: An old idea—A paradigm shift. Cancer Research. 2006;**66**(4):1883-1890 discussion 95-96

[16] Fevr T, Robine S, Louvard D, Huelsken J. Wnt/beta-catenin is essential for intestinal homeostasis and maintenance of intestinal stem cells. Molecular and Cellular Biology. 2007; **27**(21):7551-7559

[17] Vanuytsel T, Senger S, Fasano A, Shea-Donohue T. Major signaling pathways in intestinal stem cells. Biochimica et Biophysica Acta. 2013;**1830**(2):2410-2426

[18] Schneikert J, Behrens J. The canonical Wnt signalling pathway and its APC partner in colon cancer development. Gut. 2007;**56**(3):417-425

[19] Sangiorgi E, Capecchi MR. Bmi1 is expressed in vivo in intestinal stem cells. Nature Genetics. 2008;**40**(7):915-920

[20] Vermeulen L, De Sousa EMF, van der Heijden M, Cameron K, de Jong JH, Borovski T, et al. Wnt activity defines colon cancer stem cells and is regulated by the microenvironment. Nature Cell Biology. 2010;**12**(5):468-476

[21] Kanwar SS, Yu Y, Nautiyal J, Patel BB, Majumdar AP. The Wnt/beta-catenin pathway regulates growth and maintenance of colonospheres. Molecular Cancer. 2010;**9**:212

[22] Lugli A, Zlobec I, Minoo P, Baker K, Tornillo L, Terracciano L, et al. Prognostic significance of the wnt signalling pathway molecules APC, beta-catenin and E-cadherin in colorectal cancer: A tissue microarray-based analysis. Histopathology. 2007;**50**(4):453-464

[23] Symon A, Harley V. SOX9: A genomic view of tissue specific expression and action. The International Journal of Biochemistry & Cell Biology. 2017;**87**:18-22

[24] Blache P, van de Wetering M, Duluc I, Domon C, Berta P, Freund JN, et al. SOX9 is an intestine crypt transcription factor, is regulated by the Wnt pathway, and represses the CDX2 and MUC2 genes. The Journal of Cell Biology. 2004;**166**(1):37-47

[25] Bastide P, Darido C, Pannequin J, Kist R, Robine S, Marty-Double C, et al. Sox9 regulates cell proliferation and is required for Paneth cell differentiation in the intestinal epithelium. The Journal of Cell Biology. 2007;**178**(4):635-648

[26] Matheu A, Collado M, Wise C, Manterola L, Cekaite L, Tye AJ, et al. Oncogenicity of the developmental transcription factor Sox9. Cancer Research. 2012;**72**(5):1301-1315

[27] Ramalingam S, Daughtridge GW, Johnston MJ, Gracz AD, Magness ST. Distinct levels of Sox9 expression mark colon epithelial stem cells that form colonoids in culture. American Journal of Physiology Gastrointestinal and Liver Physiology. 2012;**302**(1):G10-G20

[28] Prevostel C, Rammah-Bouazza C, Trauchessec H, Canterel-Thouennon L, Busson M, Ychou M, et al. SOX9 is an atypical intestinal tumor suppressor controlling the oncogenic Wnt/ss-catenin signaling. Oncotarget. 2016;**7**(50):82228-82243

[29] Javier BM, Yaeger R, Wang L, Sanchez-Vega F, Zehir A, Middha S, et al. Recurrent, truncating SOX9 mutations are associated with SOX9 overexpression, KRAS mutation, and TP53 wild type status in colorectal carcinoma. Oncotarget. 2016;**7**(32):50875-50882

[30] Abdel-Samad R, Zalzali H, Rammah C, Giraud J, Naudin C, Dupasquier S, et al. MiniSOX9, a dominant-negative variant in colon cancer cells. Oncogene. 2011;**30**(22):2493-2503

[31] Espersen ML, Olsen J, Linnemann D, Hogdall E, Troelsen JT. Clinical implications of intestinal stem cell markers in colorectal cancer. Clinical Colorectal Cancer. 2015;**14**(2):63-71

[32] Ghaleb AM, Yang VW. The pathobiology of Kruppel-like factors in colorectal cancer. Current Colorectal Cancer Reports. 2008;**4**(2):59-64

[33] Wei D, Kanai M, Huang S, Xie K. Emerging role of KLF4 in human gastrointestinal cancer. Carcinogenesis. 2006;**27**(1):23-31

[34] Dang DT, Chen X, Feng J, Torbenson M, Dang LH, Yang VW. Overexpression of Kruppel-like factor 4 in the human colon cancer cell line RKO leads to reduced tumorigenecity. Oncogene. 2003;**22**(22):3424-3430

[35] Yu T, Chen X, Zhang W, Colon D, Shi J, Napier D, et al. Regulation of the potential marker for intestinal cells, Bmi1, by beta-catenin and the zinc finger protein KLF4: Implications for colon cancer. The Journal of Biological Chemistry. 2012;**287**(6):3760-3768

[36] Shie JL, Chen ZY, O'Brien MJ, Pestell RG, Lee ME, Tseng CC. Role of gut-enriched Kruppel-like factor in colonic cell growth and differentiation. American Journal of Physiology Gastrointestinal and Liver Physiology. 2000;**279**(4):G806-G814

[37] Leng Z, Tao K, Xia Q, Tan J, Yue Z, Chen J, et al. Kruppel-like factor 4 acts as an oncogene in colon cancer stem cell-enriched spheroid cells. PLoS One. 2013;**8**(2):e56082

[38] Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell. 2006;**126**(4):663-676

[39] Lee HY, Ahn JB, Rha SY, Chung HC, Park KH, Kim TS, et al. High KLF4 level in normal tissue predicts poor survival in colorectal cancer patients. World Journal of Surgical Oncology. 2014;**12**:232

[40] Gao Y, Ding Y, Chen H, Chen H, Zhou J. Targeting Kruppel-like factor 5 (KLF5) for cancer therapy. Current Topics in Medicinal Chemistry. 2015;**15**(8):699-713

[41] Nakaya T, Ogawa S, Manabe I, Tanaka M, Sanada M, Sato T, et al. KLF5 regulates the integrity and oncogenicity of intestinal stem cells. Cancer Research. 2014;**74**(10):2882-2891

[42] Nandan MO, McConnell BB, Ghaleb AM, Bialkowska AB, Sheng H, Shao J, et al. Kruppel-like factor 5 mediates cellular transformation during oncogenic KRAS-induced intestinal tumorigenesis. Gastroenterology. 2008;**134**(1):120-130

[43] Chiba S. Notch signaling in stem cell systems. Stem Cells. 2006;**24**(11):2437-2447

[44] Vooijs M, Liu Z, Kopan R. Notch: Architect, landscaper, and guardian of the intestine. Gastroenterology. 2011;**141**(2):448-459

[45] Miyamoto S, Rosenberg DW. Role of Notch signaling in colon homeostasis and carcinogenesis. Cancer Science. 2011;**102**(11):1938-1942

[46] Sikandar SS, Pate KT, Anderson S, Dizon D, Edwards RA, Waterman ML, et al. NOTCH signaling is required for formation and self-renewal of tumor-initiating cells and for repression of secretory cell differentiation in colon cancer. Cancer Research. 2010;**70**(4):1469-1478

[47] Srinivasan T, Walters J, Bu P, Than EB, Tung KL, Chen KY, et al. NOTCH signaling regulates asymmetric cell fate of fast- and slow-cycling colon cancer-initiating cells. Cancer Research. 2016;**76**(11):3411-3421

[48] Chu D, Li Y, Wang W, Zhao Q, Li J, Lu Y, et al. High level of Notch1 protein is associated with poor overall survival in colorectal cancer. Annals of Surgical Oncology. 2010;**17**(5): 1337-1342

[49] Lopez-Arribillaga E, Rodilla V, Pellegrinet L, Guiu J, Iglesias M, Roman AC, et al. Bmi1 regulates murine intestinal stem cell proliferation and self-renewal downstream of Notch. Development. 2015;**142**(1):41-50

[50] Zhang Z, Bu X, Chen H, Wang Q, Sha W. Bmi-1 promotes the invasion and migration of colon cancer stem cells through the downregulation of E-cadherin. International Journal of Molecular Medicine. 2016;**38**(4):1199-1207

[51] Kreso A, van Galen P, Pedley NM, Lima-Fernandes E, Frelin C, Davis T, et al. Self-renewal as a therapeutic target in human colorectal cancer. Nature Medicine. 2014;**20**(1):29-36

[52] Siddique HR, Saleem M. Role of BMI1, a stem cell factor, in cancer recurrence and chemoresistance: Preclinical and clinical evidences. Stem Cells. 2012;**30**(3):372-378

[53] Sanchez-Beato M, Sanchez E, Gonzalez-Carrero J, Morente M, Diez A, Sanchez-Verde L, et al. Variability in the expression of polycomb proteins in different normal and tumoral tissues. A pilot study using tissue microarrays. Modern Pathology: An Official Journal of the United States and Canadian Academy of Pathology, Inc. 2006;**19**(5):684-694

[54] Li DW, Tang HM, Fan JW, Yan DW, Zhou CZ, Li SX, et al. Expression level of Bmi-1 oncoprotein is associated with progression and prognosis in colon cancer. Journal of Cancer Research and Clinical Oncology. 2010;**136**(7):997-1006

[55] Du J, Li Y, Li J, Zheng J. Polycomb group protein Bmi1 expression in colon cancers predicts the survival. Medical Oncology (Northwood, London, England). 2010;**27**(4):1273-1276

[56] Wu C, Zhu X, Liu W, Ruan T, Tao K. Hedgehog signaling pathway in colorectal cancer: Function, mechanism, and therapy. OncoTargets and Therapy. 2017;**10**:3249-3259

[57] Kosinski C, Stange DE, Xu C, Chan AS, Ho C, Yuen ST, et al. Indian hedgehog regulates intestinal stem cell fate through epithelial-mesenchymal interactions during development. Gastroenterology. 2010;**139**(3):893-903

[58] Gerling M, Buller NV, Kirn LM, Joost S, Frings O, Englert B, et al. Stromal Hedgehog signalling is downregulated in colon cancer and its restoration restrains tumour growth. Nature Communications. 2016;**7**:12321

[59] Papadopoulos V, Tsapakidis K, Riobo Del Galdo NA, Papandreou CN, Del Galdo F, Anthoney A, et al. The prognostic significance of the hedgehog Signaling pathway in colorectal cancer. Clinical Colorectal Cancer. 2016;**15**(2):116-127

[60] Berlin J, Bendell JC, Hart LL, Firdaus I, Gore I, Hermann RC, et al. A randomized phase II trial of vismodegib versus placebo with FOLFOX or FOLFIRI and bevacizumab in patients with previously untreated metastatic colorectal cancer. Clinical Cancer Research: An Official Journal of the American Association for Cancer Research. 2013;**19**(1):258-267

[61] Allaire JM, Roy SA, Ouellet C, Lemieux E, Jones C, Paquet M, et al. Bmp signaling in colonic mesenchyme regulates stromal microenvironment and protects from polyposis initiation. International Journal of Cancer. 2016;**138**(11):2700-2712

[62] Ashley N. Regulation of intestinal cancer stem cells. Cancer Letters. 2013;**338**(1):120-126

[63] Qi Z, Li Y, Zhao B, Xu C, Liu Y, Li H, et al. BMP restricts stemness of intestinal Lgr5+ stem cells by directly suppressing their signature genes. Nature Communications. 2017;**8**:13824

[64] Lombardo Y, Scopelliti A, Cammareri P, Todaro M, Iovino F, Ricci-Vitiani L, et al. Bone morphogenetic protein 4 induces differentiation of colorectal cancer stem cells and increases their response to chemotherapy in mice. Gastroenterology. 2011;**140**(1):297-309

[65] Whissell G, Montagni E, Martinelli P, Hernando-Momblona X, Sevillano M, Jung P, et al. The transcription factor GATA6 enables self-renewal of colon adenoma stem cells by repressing BMP gene expression. Nature Cell Biology. 2014;**16**(7):695-707

[66] Voorneveld PW, Jacobs RJ, Kodach LL, Hardwick JC. A meta-analysis of SMAD4 immunohistochemistry as a prognostic marker in colorectal cancer. Translational Oncology. 2015; **8**(1):18-24

[67] Pan D. The hippo signaling pathway in development and cancer. Developmental Cell. 2010;**19**(4):491-505

[68] Mo JS, Park HW, Guan KL. The hippo signaling pathway in stem cell biology and cancer. EMBO Reports. 2014;**15**(6):642-656

[69] Konsavage WM Jr, Yochum GS. Intersection of Hippo/YAP and Wnt/beta-catenin signaling pathways. Acta Biochimica et Biophysica Sinica (Shanghai). 2013;**45**(2):71-79

[70] Gregorieff A, Wrana JL. Multiple roles for the hippo effector yap in gut regeneration and cancer initiation. Molecular & Cellular Oncology. 2016;**3**(3):e1143992

[71] Wang Y, Xie C, Li Q, Xu K, Wang E. Clinical and prognostic significance of Yes-associated protein in colorectal cancer. Tumour Biology: The Journal of the International Society for Oncodevelopmental Biology and Medicine. 2013;**34**(4):2169-2174

[72] Touil Y, Igoudjil W, Corvaisier M, Dessein AF, Vandomme J, Monte D, et al. Colon cancer cells escape 5FU chemotherapy-induced cell death by entering stemness and quiescence associated with the c-Yes/YAP axis. Clinical Cancer Research: An Official Journal of the American Association for Cancer Research. 2014;**20**(4):837-846

[73] Oladimeji P, Cui H, Zhang C, Chen T. Regulation of PXR and CAR by protein-protein interaction and signaling crosstalk. Expert Opinion on Drug Metabolism & Toxicology. 2016;**12**(9):997-1010

[74] Thomas M, Bayha C, Vetter S, Hofmann U, Schwarz M, Zanger UM, et al. Activating and inhibitory functions of WNT/beta-catenin in the induction of cytochromes P450 by nuclear receptors in HepaRG cells. Molecular Pharmacology. 2015;**87**(6):1013-1020

[75] Planque C, Rajabi F, Grillet F, Finetti P, Bertucci F, Gironella M, et al. Pregnane X-receptor promotes stem cell-mediated colon cancer relapse. Oncotarget. 2016;**7**(35):56558-56573

[76] Wang H, Venkatesh M, Li H, Goetz R, Mukherjee S, Biswas A, et al. Pregnane X receptor activation induces FGF19-dependent tumor aggressiveness in humans and mice. The Journal of Clinical Investigation. 2011;**121**(8):3220-3232

[77] Dong Y, Wang Z, Xie GF, Li C, Zuo WW, Meng G, et al. Pregnane X receptor is associated with unfavorable survival and induces chemotherapeutic resistance by transcriptional activating multidrug resistance-related protein 3 in colorectal cancer. Molecular Cancer. 2017;**16**(1):71

[78] Takeda N, Jain R, LeBoeuf MR, Wang Q, MM L, Epstein JA. Interconversion between intestinal stem cell populations in distinct niches. Science. 2011;**334**(6061):1420-1424

[79] Katoh H, Yamashita K, Waraya M, Margalit O, Ooki A, Tamaki H, et al. Epigenetic silencing of HOPX promotes cancer progression in colorectal cancer. Neoplasia. 2012;**14**(7):559-571

[80] Yamashita K, Katoh H, Watanabe M. The homeobox only protein homeobox (HOPX) and colorectal cancer. International Journal of Molecular Sciences. 2013;**14**(12):23231-23243

[81] O'Connor L, Gilmour J, Bonifer C. The role of the ubiquitously expressed transcription factor Sp1 in tissue-specific transcriptional regulation and in disease. The Yale Journal of Biology and Medicine. 2016;**89**(4):513-525

[82] Zhao Y, Zhang W, Guo Z, Ma F, Wu Y, Bai Y, et al. Inhibition of the transcription factor Sp1 suppresses colon cancer stem cell growth and induces apoptosis in vitro and in nude mouse xenografts. Oncology Reports. 2013;**30**(4):1782-1792

Chapter 13

Passenger or Driver: Can Gene Expression Profiling Tell Us Anything about LINE-1 in Cancer?

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Additional information is available at the end of the chapter

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Abstract

LINE-1 retrotransposons are expressed in epithelial cancers but not normal adult tissues. Previously, we demonstrated repression of cell proliferation, migration, and invasion genes in L1-reverse transcriptase-inhibited T47D cells, while genes involved in cell projection, formation of vacuolar membranes, and intercellular junctions were upregulated. Extending this, we examined microarray data from L1-silenced and Efavirenz-treated T47D cells by Weighted Gene Correlation Network Analysis and literature mining. Hub genes in the most significant module comparing L1-silenced and untreated controls included HSP90AB2p, DDX39A, PANK2, MT1M, and LIMK2. HSP90AB2p is related to HSP90, a master regulator of cancer, cancer evolvability and chemo-resistance. DDX39A is a known cancer driver gene while PANK2 and MT1M affect multiple pathways. LIMK2 and SYBL1 impact actin cytoskeletal dynamics and the cofilin pathway, cancer cell motility, and the epithelial-to-mesenchymal transition. Also affected were signal transduction, HIF1 pathways, iron/redox metabolism, stress granules and cancer stem cell-related metabolic reprogramming and the eIF4F translation initiation complex. Hub genes in other modules, including BTRC, MDM2, and FBXW7, stabilize oncoproteins like MYC, p53, and NOTCH1 or reflect CXCL12–CXCR4 signalling. Our findings support mounting evidence that L1 activity is a cause, rather than a consequence of oncogenesis, with L1 affecting the formation of cancer stem cells.

Keywords: LINE-1, breast cancer, cancer stem cells, CSC, WGCNA, module eigengene, stress granule, protein kinase R, proteomics, cancer driver genes, cancer evolvability, epithelial-mesenchymal transition, mesenchymal-epithelial transition, EMT, MET, LINE-1 ORF1 protein interactome, MYC Coding Region Instability Determinant (CRD), HSP90, ROS, iron

1. Introduction

Retrotransposons are mobile genetic elements that replicate through an RNA intermediate, which is copied into genomic DNA by a retrotransposon-encoded reverse transcriptase. Retrotransposons are classified into two subclasses, the long terminal repeat (LTR) elements (human endogenous retroviruses or HERVs) and non-LTR elements (long interspersed elements [LINEs], including LINE-1 (L1) elements, and short interspersed elements [SINEs], including SVA and Alu elements). L1 elements are the most prolific type of retrotransposon and can mediate insertional mutations and other forms of genome reorganization leading to several human disorders and genomic plasticity [1, 2]. There are approximately 7000 full-length L1 copies in the human genome, at least 100 of which are classified as highly active or retrotransposition-competent [3, 4]. An active L1 element is composed of a 5′-untranslated region containing an internal promoter, two open reading frames (ORF1 and ORF2), and a 3′ poly-A tail. ORF1 encodes an RNA-binding protein with nucleic acid chaperone activity, while ORF2 encodes reverse transcriptase (RT) and endonuclease enzymes, required for reverse transcription and integration of the L1 RNA intermediate into new genomic sites [2].

It has long been speculated that somatic L1 insertions might drive tumorigenesis by activating oncogenes or inactivating tumor suppressor genes. This seems to be rare in practice, although the failure to detect frequent L1 retrotransposition in tumors may reflect the fact that sequencing traditionally focuses on exons, whereas L1 insertions may be capable of exerting effects when inserted into introns by creating new promoters, altering transcription, or creating new polyadenylation sites [5–7].

Although adult tissues do not normally express L1 ORF1 protein (ORF1p) [8, 9], many human neoplasms do express L1 RNA and proteins, including epithelial neoplasms [9–11], multiple myeloma, and leukemias [12, 13]. This topic has been the subject of numerous reviews, many of which are recent (listed in **Table 1**), indicating that the role of L1 in cancer is gaining ever-increasing attention.

In summary, while a clear correlation has been established between L1 and cancer, whether L1 expression and activity is a cause rather than a consequence of oncogenesis has been unclear. Probably, the strongest evidence that L1 drives cancer is the finding that L1 induces hTERT and ensures telomere maintenance in tumor cell lines [33]. L1 knockdown also leads to decreased cMyc and KLF4 mRNA and protein expression, two of the main transcription factors of telomerase, and changes in mRNA levels of other stem cell-associated proteins like CD44 and hMyb, with correspondingly reduced growth in spheroids. In addition, knockdown of KLF4 or cMyc decreases L1-ORF1 mRNA levels, suggesting specific reciprocal regulation with L1 [33].

Furthermore, L1 activity is dependent on phosphorylation of L1 ORF1p by the peptidyl prolyl isomerase 1 (Pin1) and is thus integrated with regulatory phosphorylation cascades [34]. This suggests that, like many pathogens, L1 can appropriate a major regulatory cascade of the host, and that competition for kinases by ORF1p could perturb signaling cascades.

Title	Reference
Transposable elements in cancer	[14]
The role of somatic L1 retrotransposition in human cancers	[15]
LINE-1 methylation level and prognosis in pancreas cancer: Pyrosequencing technology and literature review	[16]
Methylation levels of LINE-1 as a useful marker for venous invasion in both FFPE and frozen tumor tissues of gastric cancer	[17]
The function of LINE-1-encoded reverse transcriptase in tumorigenesis	[18]
The human long interspersed element-1 retrotransposon: An emerging biomarker of neoplasia	[19]
Links between human LINE-1 retrotransposons and hepatitis virus-related hepatocellular carcinoma	[20]
The connection between LINE-1 retrotransposition and human tumorigenesis	[21]
The reverse transcriptase encoded by LINE-1 retrotransposons in the genesis, progression, and therapy of cancer	[22]
Crossing the LINE toward genomic instability: LINE-1 retrotransposition in cancer	[23]
LINE-1 in cancer: Multifaceted functions and potential clinical implications	[24]
Regulatory roles of LINE-1-encoded reverse transcriptase in cancer onset and progression	[25]
LINE-1 hypomethylation in blood and tissue samples as an epigenetic marker for cancer risk: A systematic review and meta-analysis	[26]
L1 retrotransposons, cancer stem cells and oncogenesis	[27]
Clinical implications of the LINE-1 methylation levels in patients with gastrointestinal cancer	[28]
Long interspersed element-1 (LINE-1): Passenger or driver in human neoplasms?	[29]
The human L1 element: A potential biomarker in cancer prognosis, current status and future directions	[30]
L1 retrotransposon and retinoblastoma: Molecular linkages between epigenetics and cancer.	[31]
A role for endogenous reverse transcriptase in tumorigenesis and as a target in differentiating cancer therapy	[32]

The list above is the subset of the results returned by a search in PUBMED using the search term: ((LINE-1) AND cancer) AND review.

Table 1. Reviews of LINE-1 involvement in cancer.

Further evidence for an active role for L1 in cancer comes from studies with anti-retroviral drugs that target the reverse transcriptase of L1. Efavirenz is a first-line antiretroviral drug used in the treatment of HIV-1 but also reported to suppress the activity of L1-RT and, remarkably, to promote morphological differentiation in a range of cancer cell lines [35, 36]. In addition to these reports, in another study, we showed that RT expression is widespread in MCF7 and T47D breast cancer cells and decreased markedly after treatment with Efavirenz [11]. Both cell types showed significantly reduced proliferation, accompanied by cell-specific differences in morphology. MCF7 cells displayed elongated microtubule extensions that adhered tightly to their substrate, while T47D cells formed long filopodial projections. These morphological changes were reversible upon stopping RT inhibition, confirming their dependence on RT activity. Microarray gene expression profiling showed that genes involved in proliferation, cell migration, and invasive activity were repressed in RT-inhibited cells.

Concomitantly, genes involved in cell projection, formation of vacuolar membranes, and cell-to-cell junctions were upregulated.

Standard microarray or RNA-seq analyses seek to identify differentially expressed genes in which each gene is analyzed independently. This approach fails to use much of the information that is captured in the transcriptome profiling experiment, namely that the expression of many genes is correlated. Thus, WGCNA quantifies the correlations between individual pairs of gene expression profiles and also the extent to which any two genes are highly correlated with the same neighbors (called topological overlap). The underlying assumption is that the correlated gene profiles and genes that overlap topologically must reflect common regulatory mechanisms or biological function.

In gene networks, a gene that has many interactions with other genes is called a hub gene and usually plays an essential role in gene regulation and biological processes [37, 38]. Compared to standard gene-wise methods of analysis, WGCNA has the advantage of enabling the identification of these hub genes and, in addition, overcomes the problem of multiplicity of hypothesis testing. This is because the number of modules of co-expressed genes is far less than the number of genes on the microarray and a single consensus gene profile from each module is subjected to statistical testing in preference to individual genes. Another advantage of WGCNA is that hub genes and other interesting genes in a module that are relevant to the phenotype under investigation may not be differentially expressed and would escape notice in a conventional gene-wise analysis.

Motivated by our initial findings described above, we decided to reanalyze the transcriptome data in greater detail using the more powerful WGCNA method [39], combining the data from Efavirenz (Efa)-treated cells [11] with our unpublished microarray data from T47D cells subjected to L1-silencing mediated by siRNA. An additional reason for combining the data was that the reproducibility of the co-expressed gene modules found by WGCNA increases as the number of samples increases with 12–15 samples currently being regarded as the practical minimum.

2. Methods

The details of the gene expression profiling in Efa-treated T47D cells have been published previously [11]. The siRNA-treated T47D cells were treated and harvested at the same time to minimize batch effects. Briefly, total RNA was isolated from cells and labeled cDNA hybridized to Roche NimbleGen Human Whole Genome 12-plex arrays. Gene expression levels were calculated with NimbleScan Version 2.4. Relative signal intensities for each gene were generated using the Robust Multi-Array Average algorithm with quantile normalization and summarized by the median polish method with NimbleScan Version 2.4. The biological samples included four experimental groups (L1 silenced by siRNA (pUTR), controls with scrambled vector (pSM2), Efavirenz-treated (Efa), and dimethyl sulfoxide-treated controls (DMSO)). There were three replicate samples in each group. To calculate individual gene-wise p-values and fold changes for the contrasts between L1-silenced or Efavirenz-treated cells and untreated controls, the (Robust Multi-array Average) RMA-normalized

calls files were imported into Partek Genomics Suite v6.2 (St. Louis, Missouri, USA), and the log2 gene expression values were analyzed with a one-factor ANOVA design: (Treatment—with four levels—"DMSO","Efa", "pSM2", "pUTR"). Contrasts were calculated for pUTR versus pSM2 and Efa versus DMSO. 4951 probes passed a false discovery rate threshold of 0.001 for the pUTR versus pSM2 contrast and 9946 for the Efa versus DMSO contrast.

For the WGCNA analysis, the RMA-normalized calls files were imported into R (version 3.1.0) [40] as log2 values, and a subset of the 10,000 most variable probesets was selected to remove noise genes (measured by variance of the expression values of each gene across the 12 samples). A weighted gene coexpression network was constructed using the WGCNA package. Plots of scale-free fit using the pickSoftThreshold and softConnectivity functions indicated that a reasonable scale-free fit could be achieved by setting the soft-thresholding power (beta, β) for network construction to 20. The other parameters used for the blockwiseModules function in WGCNA included a minimum module size of 40, and the dendrogram cut height for module detection set to 0.10 to define modules of co-expressed probesets. networkType was set to "signed," maxBlockSize was set to 10,000, and other parameters were left at their default values.

The statistical enrichment of the overlap between the genes in some modules and relevant gene lists identified in literature was calculated using an online program at http://nemates.org/MA/progs/overlap_stats.html which uses the hypergeometric distribution. This program calculates a representation factor, which is the number of overlapping genes between any two gene lists divided by the expected number of overlapping genes drawn from two randomly chosen gene lists of similar size and is a measure of the enrichment of a gene list with genes from another list. A representation factor > 1 indicates more overlap than expected between two independent groups. A genome size of 19,000 genes was used in all overlap calculations.

3. Results and discussion

3.1. Module discovery

Based on a correlation threshold, WGCNA assigns genes to modules (clusters) in which the expression of genes in a module varies in a similar manner across the different experimental conditions. The modules are labeled automatically by WGCNA with a color code according to the number of genes in the module: turquoise denotes the largest module, blue the next, then brown, green, yellow, etc. WGCNA identified 34 modules (excluding a gray module containing unassigned probesets) ranging in size from a darkmagenta module (58 probes) to a turquoise module (1359 probesets). For statistical analysis, each module is represented by a consensus profile of all the genes in the module, by default, the first principal component, to calculate a module eigengene. A one-factor ANOVA analysis was carried out on the module eigengenes in R using the same ANOVA design (Treatment—with four levels—"DMSO","Efa", "pSM2", "pUTR") used for the gene-wise analysis in Partek. After correcting for multiplicity by multiplying all p-values by 34, the most significant module eigengene for the contrast between

L1-silenced and scrambled vector controls was for the darkmagenta module (**Figure 1**) with a Bonferroni-corrected p-value of 6.55E − 11 (uncorrected p-value 1.93E − 12) (**Table 2**). All genes in the darkmagenta module were downregulated in a range from −1.53 to −2.62 in the contrast between L1-silenced and scrambled vector controls (**Figure 2**). The most significant module eigengene for the contrast between Efavirenz-treated and DMSO controls was for the black module (Bonferroni-corrected p-value 1.15E − 09) (**Figure 3**). Due to space limitations, the following results and discussion focus mainly on the darkmagenta module with references to genes in other modules (**Figures 4**–**8**) that can be linked in common pathways or processes to those in the darkmagenta module.

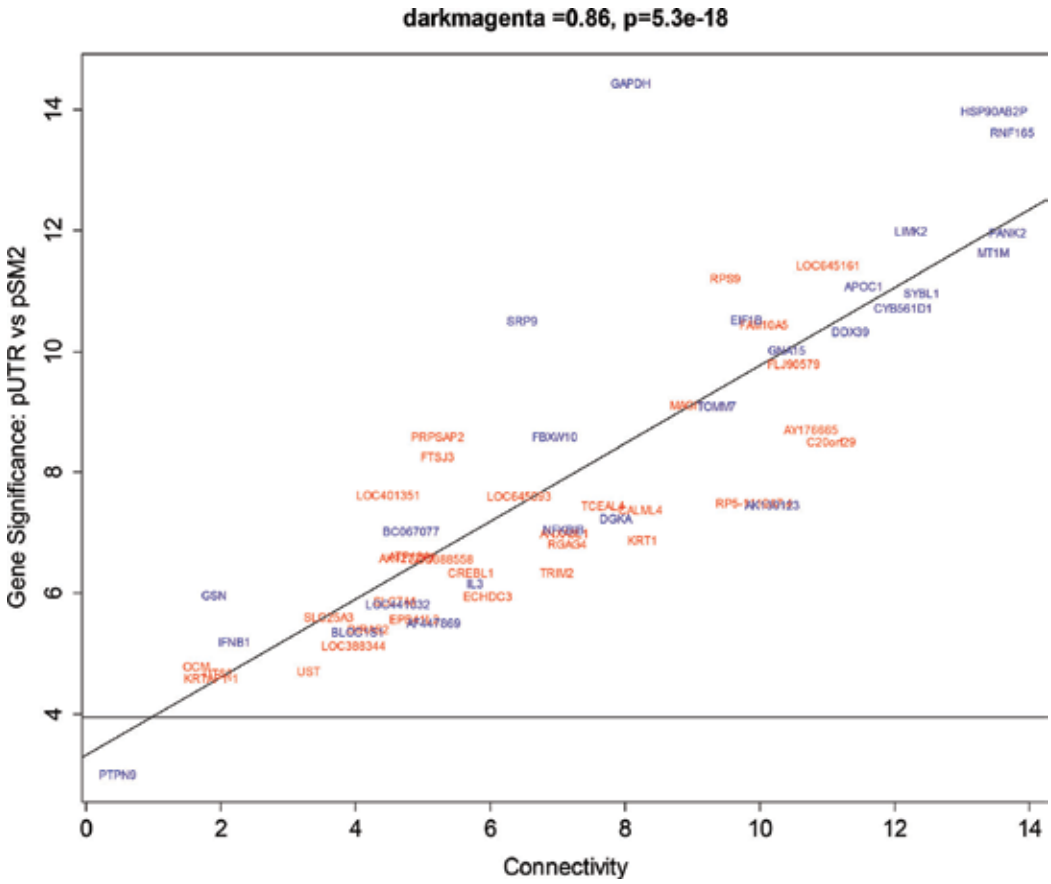

Figure 1. Scatterplot for the darkmagenta module. In Figures 1–8, genes specifically mentioned in the text are labeled blue, otherwise they are labeled red. The vertical axis (Gene Significance) is the -log10(p-value) for the contrast between pUTR versus pSM2 (**Figures 1**, **2**, **4**–**8**) or for Efavirenz versus DMSO (**Figure 3**). The intramodular connectivity for each gene is plotted on the horizontal axis. Genes with higher values of gene significance have smaller p-values in the gene-wise analysis in Partek Genomics Suite. Genes towards the right of the plots have higher intramodular connectivities and are hub genes. Intramodular connectivities were calculated with the WGCNA/intramodularConnectivity function from an adjacency matrix calculated by the WGCNA/adjacency function on the 10,000 most variable probes and with a soft thresholding power = 20. The horizontal line is the false discovery rate (FDR) 0.001 threshold calculated in Partek GS for the gene-wise ANOVA contrast. All genes above this line pass the FDR threshold at the 0.001 level. The plot was created with the WGCNA/verboseScatterplot function.

Module eigengene	pUTR vs. pSM2	Efa vs. DMSO	pUTR vs. pSM2 (Bonferroni)	Efa vs. DMSO (Bonferroni)
MEdarkmagenta	1.93E − 12	6.60E − 11	6.55E − 11	2.25E − 09
MEpurple	2.42E − 11	2.79E − 07	8.24E − 10	9.47E − 06
MEviolet	6.64E − 11	0.05308796	2.26E − 09	1.80E + 00
MEorange	8.03E − 11	0.01438891	2.73E − 09	4.89E − 01
MEwhite	1.38E − 10	1.16E − 09	4.68E − 09	3.95E − 08
MEroyalblue	1.85E − 10	5.02E − 11	6.31E − 09	1.71E − 09
MElightyellow	5.11E − 10	0.01025057	1.74E − 08	3.49E − 01
MEmagenta	1.09E − 09	1.21E − 08	3.69E − 08	4.10E − 07
MEdarkgreen	1.57E − 09	6.28E − 10	5.35E − 08	2.13E − 08
MEdarkgrey	2.73E − 09	5.28E − 08	9.29E − 08	1.80E − 06
MEsteelblue	7.19E − 09	1.05E − 07	2.45E − 07	3.57E − 06
MEdarkolivegreen	7.37E − 09	1.00E − 07	2.51E − 07	3.41E − 06
MEred	7.67E − 09	2.17E − 07	2.61E − 07	7.37E − 06
MElightgreen	7.98E − 09	0.000116041	2.71E − 07	3.95E − 03
MEcyan	1.02E − 08	0.001002342	3.47E − 07	3.41E − 02
MEgreenyellow	2.16E − 08	0.001305863	7.36E − 07	4.44E − 02
MEtan	4.37E − 08	1.36E − 08	1.49E − 06	4.64E − 07
MEyellow	6.60E − 08	2.77E − 10	2.24E − 06	9.40E − 09
MEgrey60	6.76E − 08	6.75E − 10	2.30E − 06	2.29E − 08
MEmidnightblue	9.07E − 08	0.1888604	3.08E − 06	6.42E + 00
MEdarkorange	6.92E − 07	9.11E − 09	2.35E − 05	3.10E − 07
MEblack	1.18E − 06	3.37E − 11	4.00E − 05	1.15E − 09
MElightcyan	2.67E − 06	2.93E − 10	9.08E − 05	9.95E − 09
MEgreen	5.77E − 06	5.31E − 10	1.96E − 04	1.80E − 08
MEsaddlebrown	1.15E − 05	1.44E − 09	3.89E − 04	4.88E − 08
MEblue	1.73E − 05	3.75E − 09	5.89E − 04	1.27E − 07
MEskyblue	4.73E − 05	9.32E − 10	1.61E − 03	3.17E − 08
MEturquoise	0.000172871	8.22E − 08	5.88E − 03	2.79E − 06
MEdarkred	0.00019623	4.79E − 11	6.67E − 03	1.63E − 09
MEpink	0.000201452	0.182879	6.85E − 03	6.22E + 00
MEsalmon	0.000286858	3.75E − 11	9.75E − 03	1.27E − 09
MEpaleturquoise	0.000292228	8.01E − 08	9.94E − 03	2.72E − 06
MEdarkturquoise	0.002355459	1.15E − 06	8.01E − 02	3.91E − 05
MEbrown	0.03581247	1.16E − 09	1.22E + 00	3.94E − 08

pUTR vs. pSM2 is the ANOVA contrast p-value for L1 silenced by siRNA versus scrambled vector controls. Efa vs. DMSO is the ANOVA contrast for Efavirenz-treated versus DMSO controls.

Table 2. Uncorrected and Bonferroni-corrected p-values for ANOVA contrasts for module eigengenes.

3.2. LINE-1 silencing affects HSP90, a master regulator of cancer

The most extreme outlier in the darkmagenta module is HSP90AB2P (**Figure 1**). Despite its classification as a pseudogene, the existence of this protein is supported by mass spectrometry evidence [41]. The parent gene, heat shock protein 90 (HSP90) is a ubiquitously expressed molecular chaperone representing 1–2% of all cellular protein that controls the folding, assembly, intracellular disposition, and proteolytic turnover of approximately 100 proteins, most of which are involved in signal transduction [42]. HSP90 proteins also stabilize and refold denatured proteins under stress, with two major cytosolic forms, an inducible form (HSP90AA1, a hub in the cyan module, **Figure 4**) and HSP90AB1, a constitutive form. Significantly, both HSP90AA1 and HSP90AB1 have been identified as members of L1 ORF2p complexes in isotopic differentiation of interactions as random or targeted (I-DIRT) affinity proteomics experiments and quantitative MS [43], thus supporting the presence of HSP90AB2P and

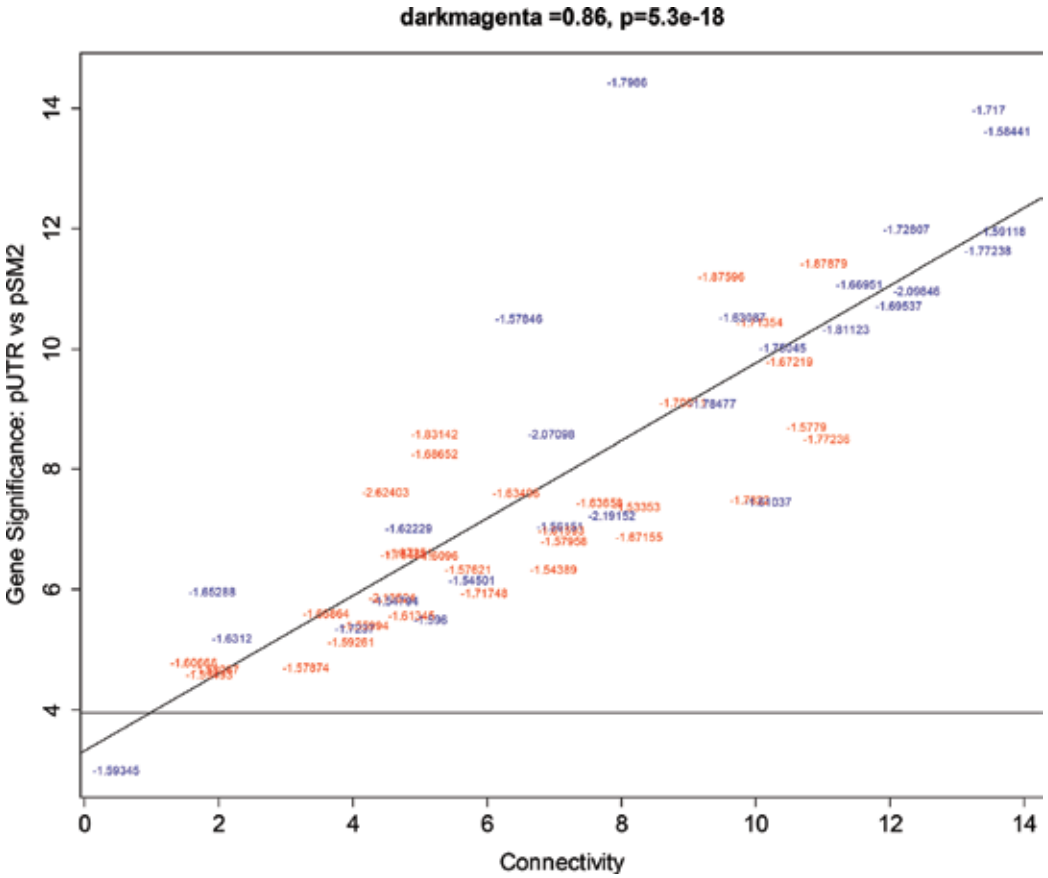

Figure 2. Similar plot to **Figure 1** but points are labelled with the fold-change for the gene in the comparison between pUTR and pSM2 (L1-silenced versus controls). All genes in the darkmagenta module are downregulated in a range from −1.53 to −2.62 for this comparison.

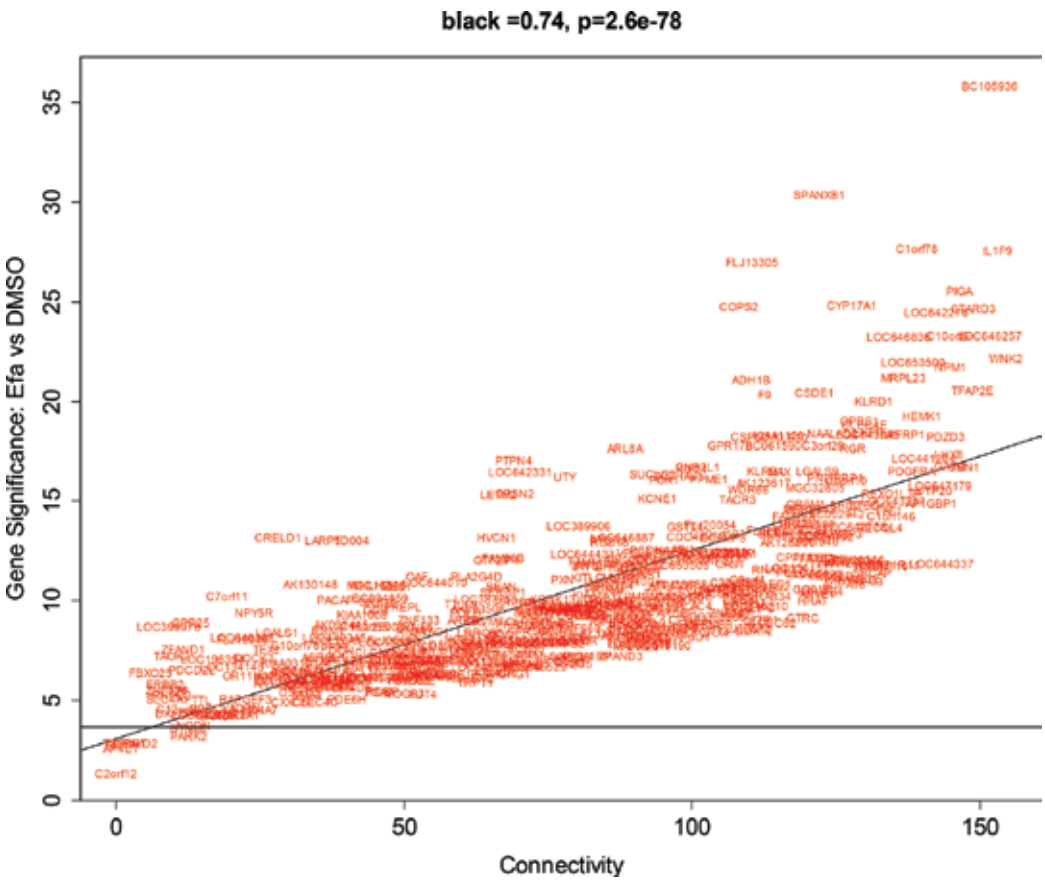

Figure 3. Scatterplot for the black module.

HSP90AA1 in the darkmagenta and cyan modules, respectively. I-DIRT has the advantage of allowing the discrimination of protein-protein interactions formed in-cell from those occurring post-extraction.

HSP90 is a master regulator of cancer [44]. HSP90 family members are overexpressed in many human cancers, and many HSP90 clients are nodes of oncogenic pathways. Cytosolic HSP90 interacts with HER-2, a member of the ErbB family of receptor tyrosine kinases that play central roles in cellular proliferation, differentiation, cell migration, and cancer progression. The interaction may involve stabilization of the cytoplasmic kinase domain of HER-2, and disruption of this association with HSP90 inhibitors leads to proteosomal degradation of the receptor [45].

Cell surface and secreted forms of HSP90 also exist. An HSP90AA α isoform is secreted and associated with matrix metalloproteinase 2 (MMP-2), incriminating extracellular HSP90 (eHSP90) in cancer metastasis [46]. eHSP90 can also initiate the EMT in prostate cancer cells by modulating EZH2 expression and activity [47]. Surface HSP90 appears to interact with the

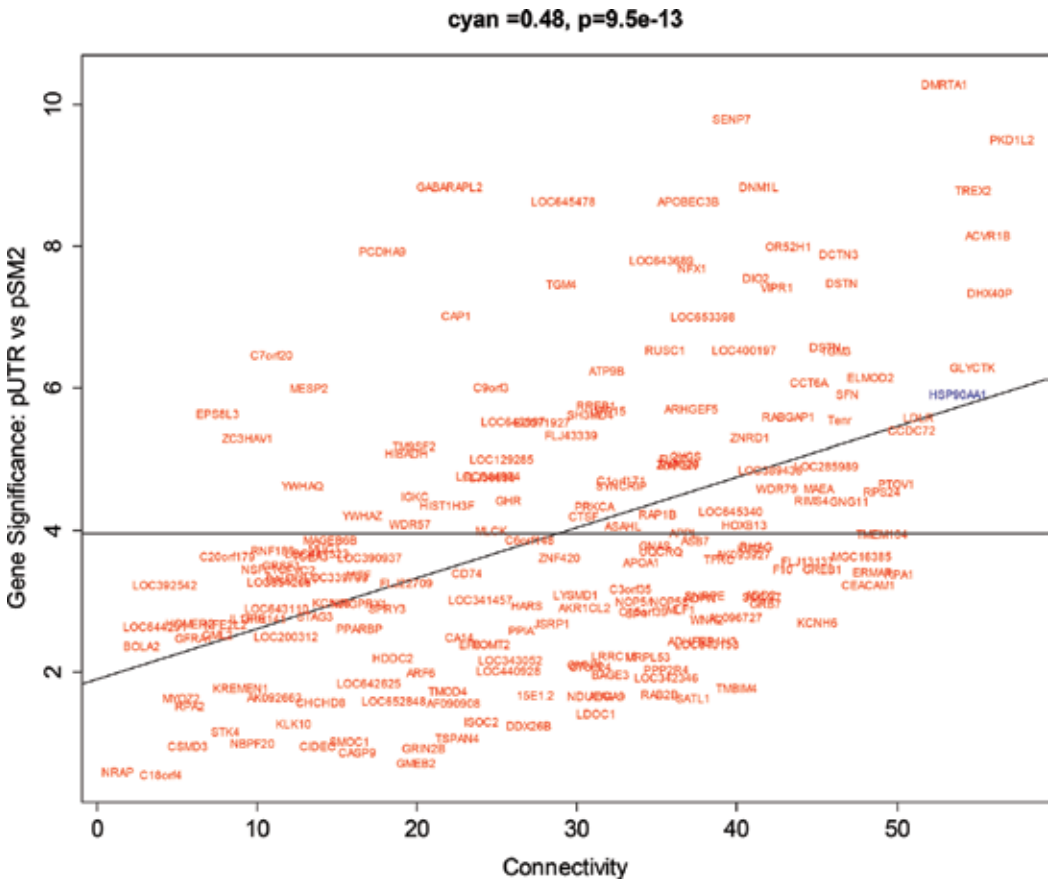

Figure 4. Scatterplot for the cyan module.

extracellular domain of HER-2. Disruption of the interaction inhibits cell invasion and is accompanied by altered actin dynamics in human breast cancer cells. In addition, the protein-tyrosine phosphatase PTPN9 negatively regulates ErbB2/HER-2 signaling in breast cancer cells and its presence in the darkmagenta module also supports involvement of HER-2.

Hsp90 also plays an essential role regulating pluripotency factors, including Oct4, Nanog, and Stat3 in mouse embryonic stem cells (ESCs) [48]. Inhibition of Hsp90 with 17-N-Allylamino-17-demethoxygeldanamycin or miRNA leads to ESC differentiation while overexpression of Hsp90β partially rescues the phenotype restoring Oct4 and Nanog levels.

The normal cellular proteome is only marginally thermodynamically stable, and this problem is exacerbated in cancer since most mutations destabilize proteins [49]. As a protein chaperone, HSP90 has a critical role in the protein homeostasis that supports cancer cell evolvability and that facilitates the rapid evolution of drug resistance in cancer [49]. HSP90 is also involved in the maturation of Piwi [50, 51], which enables piRNA-mediated silencing of transposons,

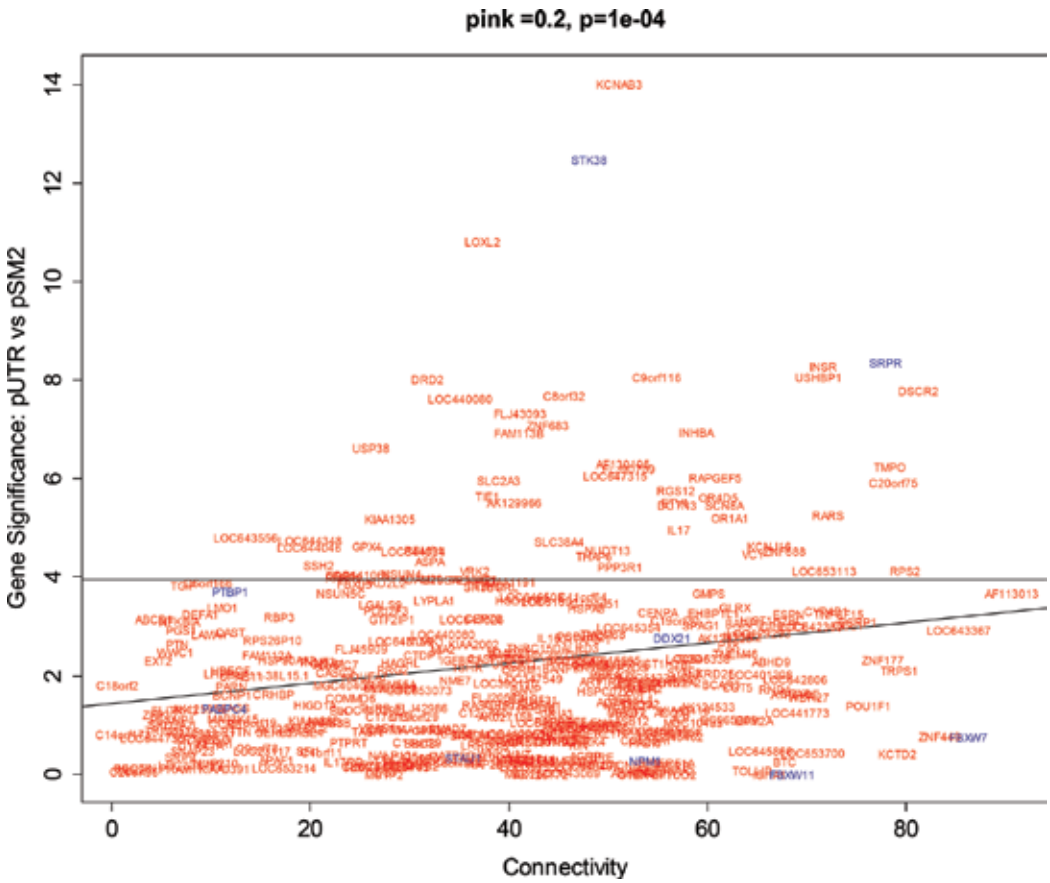

Figure 5. Scatterplot for the pink module. The pink module is enriched in genes from the LINE-1 ORF1 protein interactome (DDX21, NPM1, PABPC4, PTBP1, STAU1, STK38) with a representation factor of 3.2 and p-value <0.011.

including LINE-1, with the co-chaperone Fkbp6 having a critical role in delivering piRNAs to Miwi2 in the mouse [52].

3.3. LINE-1 silencing potentially affects the THOC/TREX nuclear export complex through DDX39A

DDX39A and SRP9 in the central region of the darkmagenta module plot (**Figure 1**) were among 96 proteins associated with the L1 ORF1p and its ribonucleoprotein identified by co-immunoprecipitation of tagged L1 constructs and mass spectrometry [53]. DDX39A (also known as DDX39 or URH49) is a member of the DEAD box RNA helicase family implicated in processes involving alteration of RNA secondary structure, including translation initiation, nuclear and mitochondrial splicing, and ribosome and spliceosome assembly. There are two closely related paralogs, DDX39A and DDX39B (also known as UAP56 or BAT1), both of

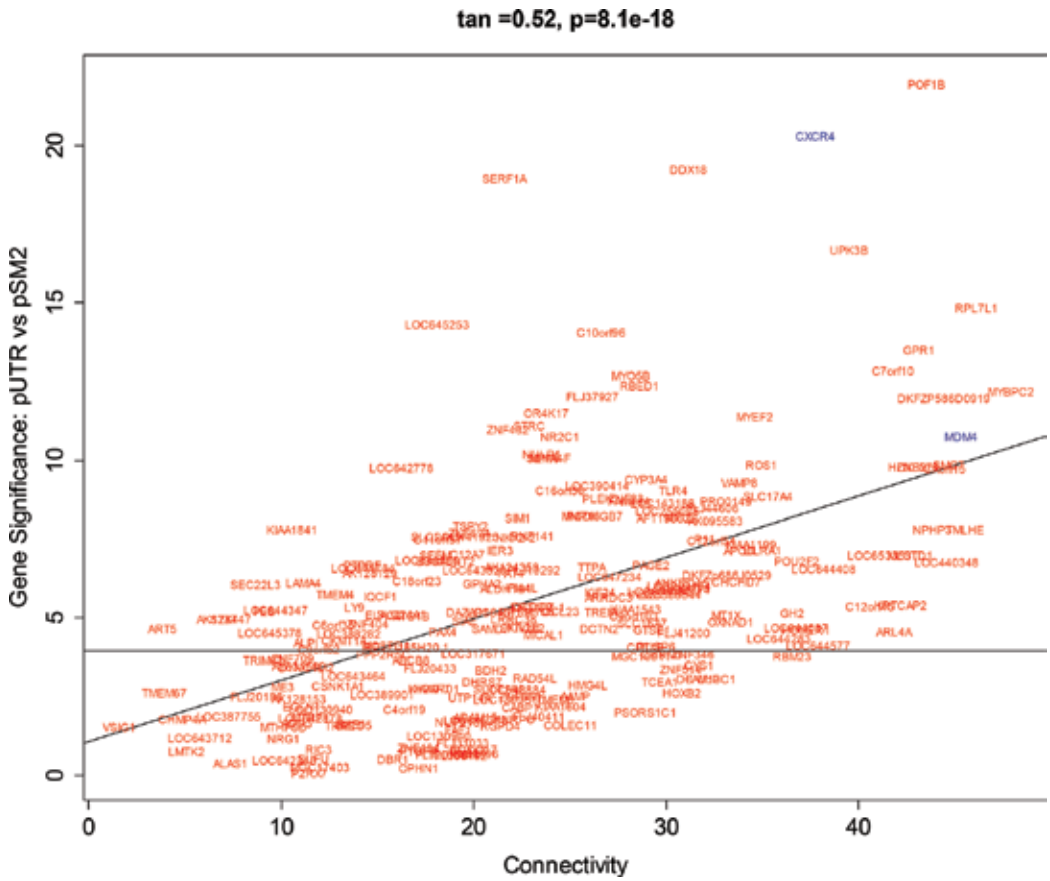

Figure 6. Scatterplot for the tan module.

which have roles in the THO nuclear export complex. The nuclear THO/TREX complex regulates the export of pluripotency-related transcripts and controls ESC self-renewal and somatic cell reprogramming, including controlling the nuclear export of ESRRB, Nanog, Sox2, and Klf4 transcripts. DDX39A interacts physically and functionally with other export factors in the THO/TREX complex [54, 55] and mediates interactions between the THO complex and the general export receptor Nxf1 that binds mRNAs and transports them through the nuclear pore complex (NPC) [56].

An impact of L1-silencing on the THOC/TREX complex is supported by findings from other studies. First, SR (Serine And Arginine Rich Splicing Factor) proteins, three members of which are members of the L1 ORF1p interactome (SRSF1, 6, and 10), interact with NXF1 [57]. Secondly, CDC5L, another member of the L1 ORF1p interactome, is present in the DDX39B/UAP56 immunoprecipitate [58]. Thirdly, the L1 3′ UTR contains a novel sequence element that binds NXF1 suggesting a role in L1 RNP transport from the nucleus, and possibly its reimport into the nucleus for retro-integration in the genome [59].

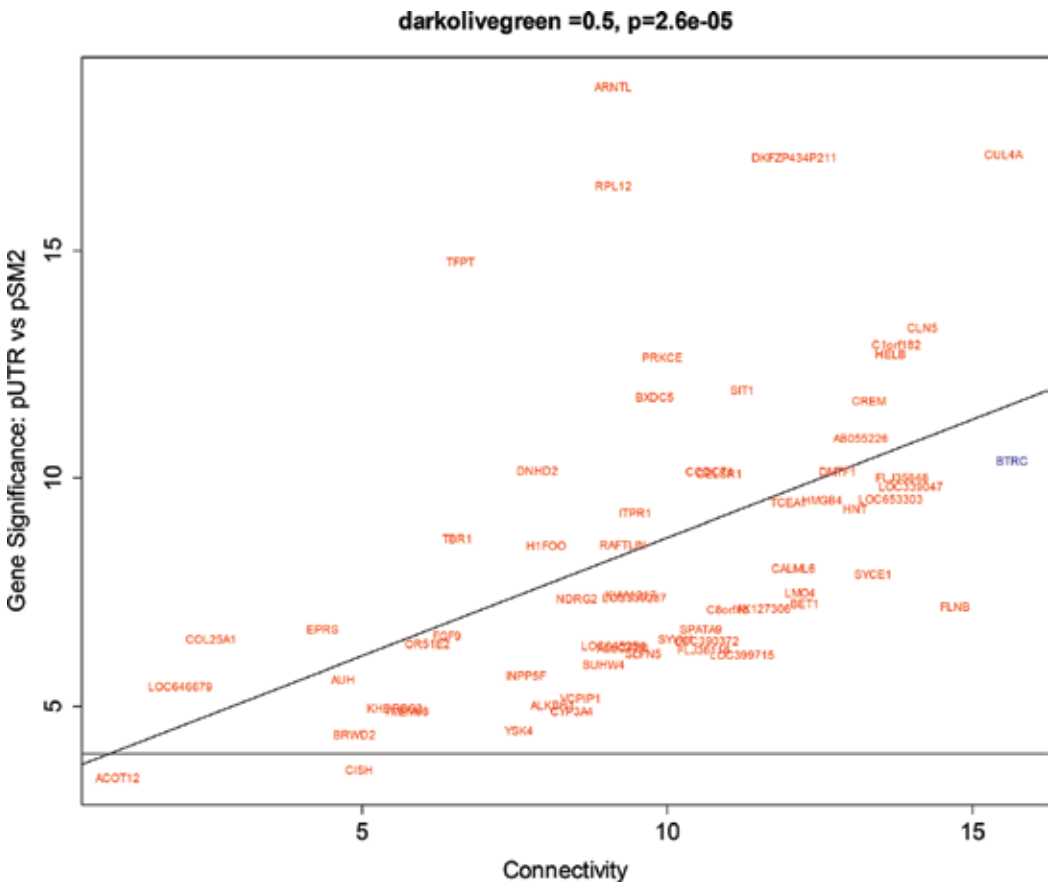

Figure 7. Scatterplot for the darkolivegreen module.

DDX39 has also been identified as a cancer driver gene in two studies. Firstly, DDX39 was identified as a marker predicting urinary bladder cancer progression by proteome analysis [60]. Secondly, DDX39 was identified as a key driver gene and anti-cancer drug target by data mining in the "Sanger Genomics of Drug Sensitivity in Cancer dataset from the Cancer Genome Project" [61]. This dataset contains gene expression levels, copy number, and mutation status for 654 cell lines and IC50 values of 138 anti-cancer drugs. The string-db network [62] of the potential driver genes with the highest 10 largest importance measures among the selected genes for each anti-cancer drug is shown in **Figure 9**.

Four of the markers identified by Kato et al. [60] (CCT4, IDH1, NPM1, YBX1) overlap the L1 ORF1p interactome resulting in a statistically significant overlap with a representation factor of 56.5 and $p < 5.893E-07$. There are also five overlaps between the L1 ORF1p interactome and the cancer driver gene set identified by Park et al. [61] (DDX39A, NPM1, PABPC4, TCP1, YBX1) resulting in a representation factor of 9.9 and $p < 1.528E-04$. This is, in itself, strong evidence for LINE-1 having an active rather than a passive role in cancer.

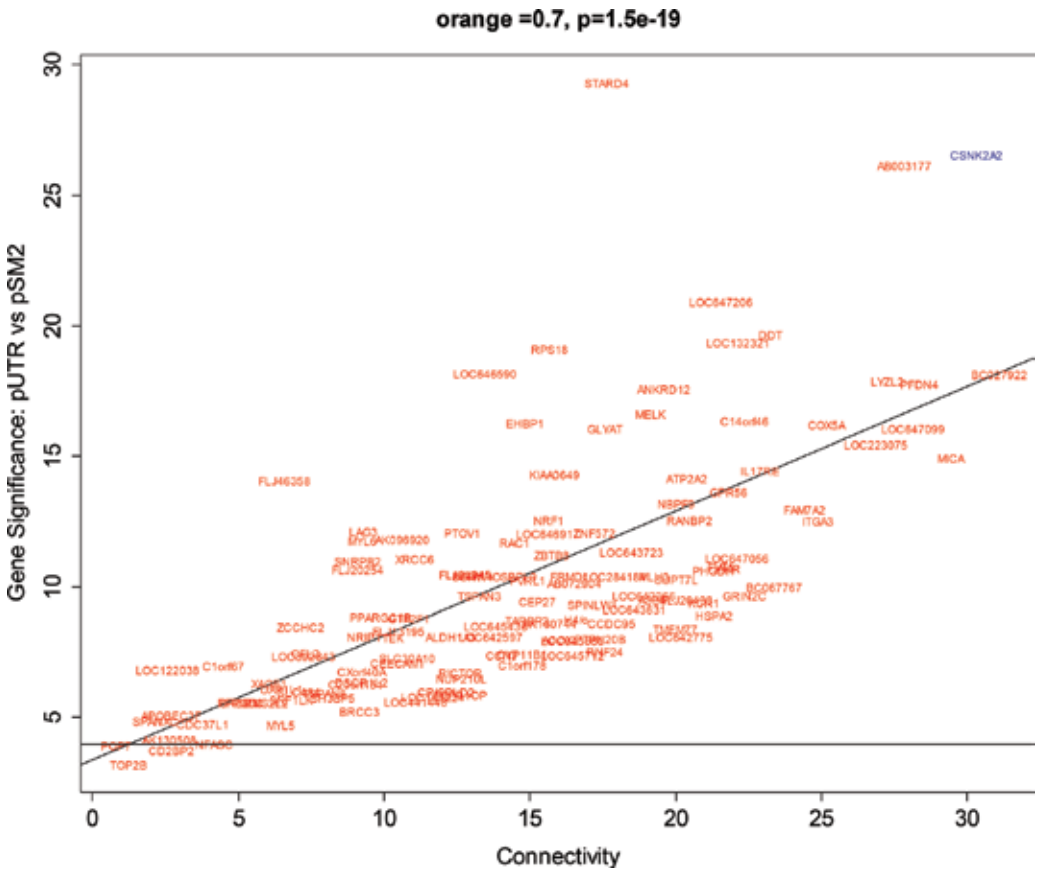

Figure 8. Scatterplot for the orange module.

Furthermore, three genes in the darkmagenta module either match, or are closely related to genes in the Park et al., cancer driver gene signature (DDX39A, EEF1A1, HSP90AA1). In the case of three perfect matches, this would result in a representation factor of 9.8 and $p < 0.004$, further supporting the biological plausibility of this module.

3.4. LINE-1 silencing affects genes with fundamental roles in cancer including PANK2, MT1M, and GAPDH

Pantothenate kinase 2 (PANK2), a master regulator of coenzyme A synthesis, and metallothionein 1M (MT1M), a protein mostly associated with cellular metabolism of metal ions, are among the most highly connected hub genes in the darkmagenta module (**Figure 1**).

PANK2 is the mitochondrial enzyme essential for converting dietary pantothenate into 4′ phosphopantethenic acid, the first regulatory step in the synthesis of coenzyme A (CoA). CoA is an essential cofactor in nearly 100 enzymatic reactions including those involved in the citric acid cycle, amino acid synthesis, and the beta-oxidation of fatty acids.

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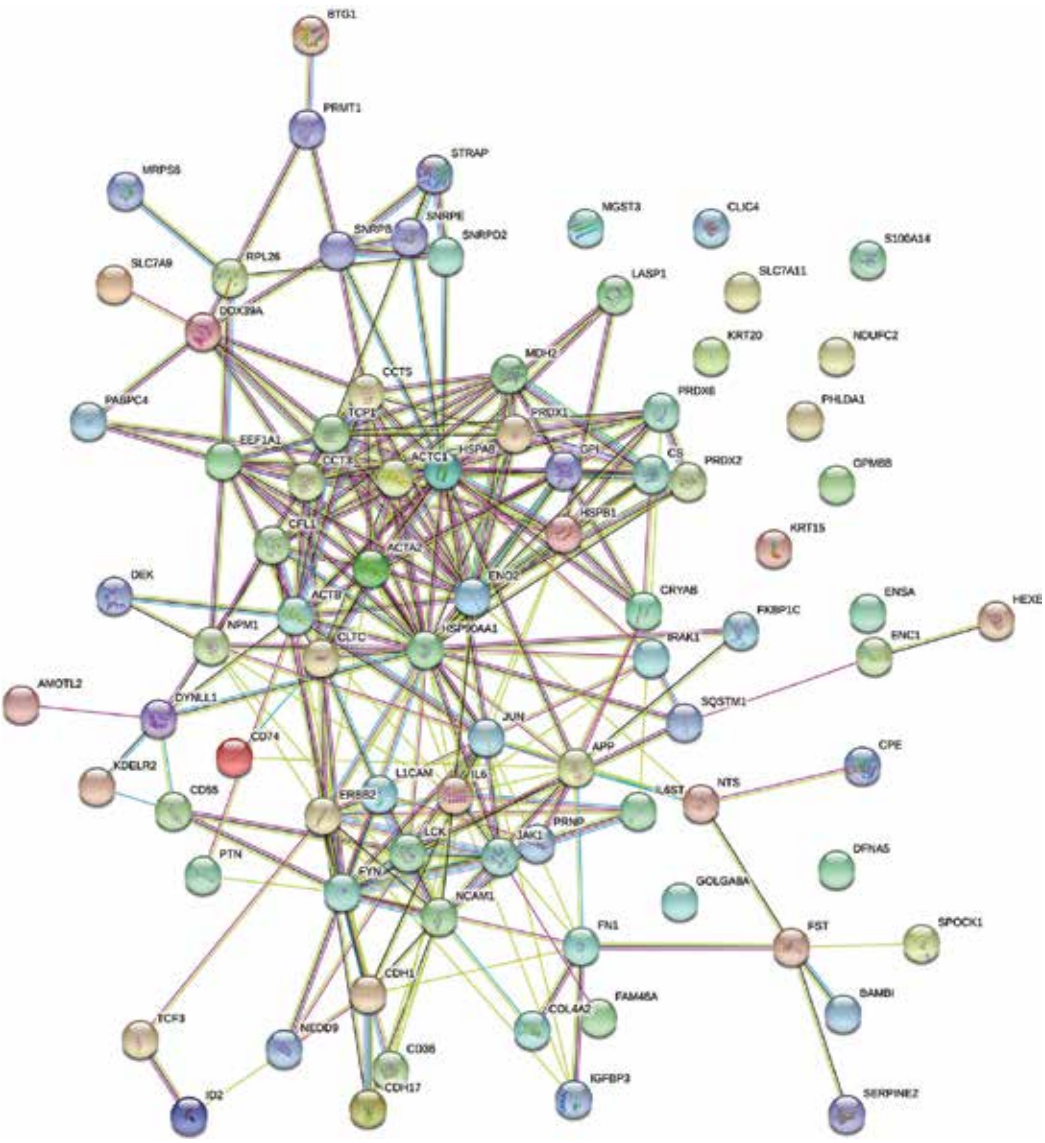

Figure 9. String-db gene network for cancer driver genes identified using data mining by Park et al. [61]. Network connections are based on known and predicted protein-protein interactions. Medium confidence interactions are shown. The network shows the central location of HSP90AA1 and ERBB2. Genes from the darkmagenta module and the LINE-1 ORF1p interactome are also present.

Mutations in the Drosophila PANK homolog (dPANK) lead to reduced CoA levels, impaired acetylation of histones leading to downstream epigenetic effects, and impaired acetylation and stability of tubulin [63].

PANK deficiency in Drosophila and human neuronal cell cultures leads to abnormalities in F-actin organization and abnormally high levels of phosphorylated cofilin (CFL1) (**Figure 9**),

a conserved actin filament severing protein. The increased levels of phosphorylated cofilin coincide with morphological changes in PANK-deficient Drosophila S2 cells and human neuronal SHSY-5Y cells with the latter also forming markedly fewer neurites in culture—a process that is strongly dependent on actin remodeling [63]. Cofilin also plays a critical role in breast cancer invasion and metastasis [64] with the cofilin pathway comprising a group of kinases and phosphatases that regulate cofilin and coordinately initiate actin polymerization and cell motility in response to stimuli in the microenvironment of mammary tumors.

Mutations in the human PANK2 gene lead to neurodegeneration with brain iron accumulation and are linked to changes in ferroportin expression, the only known protein to mediate the export of intracellular iron [65]. Downregulation of PANK2 by siRNA in HeLa cells leads to a 12-fold induction of ferroportin mRNA [66]. Ferroportin is strongly downregulated in breast cancer, possibly being required for phenotypic transitions occurring during metastasis [67]. High ferroportin gene expression identifies an extremely favorable cohort of breast cancer patients with a 10-year survival of >90% [68].

Iron-dependent oxidative demethylation mediated by the Jumonji family of enzymes is linked to the epigenetic regulation of cancer [69, 70]. H3K4 methylation is a key determinant of epithelial-mesenchymal plasticity, and loss of H3K4me3 correlates with poor survival in breast cancer [71]. In addition, the ten–eleven translocation (TET) enzymes promote the iron-dependent oxidative demethylation of 5-methylcytosine and regulate the epithelial-mesenchymal transition (EMT) and the reverse mesenchymal-epithelial transition (MET) [72–74]. Iron may also be directly involved in promoting selective oxidative demethylation of key DNA or histone residues in chromatin to control the epithelial-mesenchymal status in a dynamic manner.

Iron and iron-mediated processes appear to have a central role in the formation of breast cancer stem cells (CSCs) and to be potential therapeutic targets in breast CSCs [67]. Salinomycin and a derivative, Ironomycin, exhibit potent selective activity against breast CSCs in vitro and in vivo, by accumulating and sequestering iron in lysosomes [67]. Preferential iron trafficking also characterizes glioblastoma (GBM) stem-like cells [75]. GBM CSCs have been shown to potently extract iron from the microenvironment more effectively than other tumor cells and preferentially require the transferrin receptor and ferritin, two core iron regulators, to propagate and form tumors in vivo. Transferrin was the top upregulated gene compared with tissue-specific progenitors [75].

The presence of CYB561D1, a putative mitochondrial ferrireductase in the darkmagenta module close to PANK2 (**Figure 1**), further supports perturbation of iron-related metabolism by L1-silencing. A paralog, CYB561D2 (101F6), is highly expressed in lung tumor cell lines [76]. Its forced expression in NSCLC tumor cell lines or tumor xenografts significantly reduces cell viability by inducing apoptosis while lung metastases in nu/nu mice are also greatly reduced following systemic delivery of 101F6-encoding adenoviral vectors [77].

PANK2 also affects NADH levels [78, 79]. Hepatocytes from dKO PANK2 mouse pups cannot maintain NADH levels compared to wild-type hepatocytes [80]. In addition, induced pluripotent stem cell (iPSC)-derived neuronal models of PANK2-associated neurodegeneration reveal

mitochondrial dysfunction with activated NADH-related and inhibited FADH-related respiration, leading to increased reactive oxygen species generation and lipid peroxidation [78].

The link between CoA and NADH also supports an important role for PANK2 in the metabolism of breast CSCs. Reactive oxygen species (ROS) and ROS-dependent signaling pathways and transcriptional activities appear to be critical to both normal stem cell self-renewal and differentiation and to CSCs [81]. CSCs possess low levels of ROS but how they control ROS production and scavenging and how ROS-dependent signaling pathways contribute to CSC function remain poorly understood.

In close proximity to PANK2 in **Figure 1**, MT1M is a member of the metallothionein (MT) family; metallothioneins are small cysteine-rich proteins involved in metal metabolism and detoxification and redox metabolism. Metallothioneins may form a critical surveillance system protecting cells from damage caused by electrophilic carcinogens [82]. However, several studies suggest that metallothioneins have wider roles, contributing to numerous fundamental carcinogenic processes, including proliferation, survival, metabolism, invasion, and metastasis [83, 84].

Metallothionein expression is also strongly associated with tumor grade in breast, ovarian, uterine, and prostate cancers [85].

Hypoxia-inducible factor-1 (HIF-1α) can co-activate MT gene transcription by interacting with the metal-responsive transcription factor (MTF1) in hypoxic conditions increasing the biological aggressiveness of cancer cells [86, 87]. Conversely, metallothioneins can increase HIF-1α transcriptional activity by suppressing ROS accumulation or activating the ERK/mTOR pathway [88, 89]. Also, even though MTF1 is not inducible by iron, expression of ferroportin is induced directly via MTF1 [90]. HIF-1α also transcriptionally activates SLUG expression in hypoxic conditions [91, 92], and because upregulation of HIF-1α and metallothionein expression is self-reinforcing, MT1M may also affect SLUG expression. SLUG is a member of the SNAIL superfamily of zinc finger transcriptional factors involved in the EMT. SLUG expression correlates with reduced cell adhesion, increased cell migration and invasion, and biological aggressiveness in several tumor types including breast cancer [93, 94].

While not a hub gene, GAPDH is the most significantly differentially expressed gene in the darkmagenta module (**Figure 1**). Overexpression of GAPDH occurs in diverse human cancers. Several cancer-related factors, such as insulin, HIF-1, p53, nitric oxide (NO), and acetylated histones, modulate GAPDH gene expression and affect GAPDH protein function [95]. In addition to its role in glycolysis, in which it catalyzes the oxidation and phosphorylation of glyceraldehyde-3-phosphate to 1,3-bisphosphoglycerate in conjunction with NAD+, GAPDH is a key mediator of oxidative stress responses, involving GAPDH nuclear translocation and induction of cell death [96]. GAPDH also inhibits telomerase activity and induces breast cancer cell senescence [96].

3.5. LINE1-silencing affects genes involved in MET-related metabolic reprogramming

The reprogramming of somatic cells to iPSCs by transgene expression of the transcription factors Oct4, Sox2, Klf4, and Myc triggers a mesenchymal-epithelial transition (MET) [97]. This

transformation is promoted by the TET enzymes and blocked by kinase-dependent cytoskeletal reorganization [98]. Two closely associated hub genes in the darkmagenta module (**Figure 1**), LIM Domain Kinase 2 (LIMK2) and Apolipoprotein C1 (APOC1), have roles in the MET, with the presence of APOC1 also suggesting the involvement of TET1. The TET proteins are DNA hydroxylases that mediate oxidation of methylcytosines and thus regulate hypoxia-sensitive gene expression. Among its many actions, TET1 regulates the hypoxia-induced EMT by acting as a co-activator of genes involved in cholesterol metabolism including APOC1 [73]. Significant changes in APOC1 expression are seen in leukemia cell lines in the NCI60 cancer cell line collection [99, 100], while APOC1 is highly expressed at the protein level and protects pancreatic cancer cells from apoptosis [101]. In addition, APOC1 is highly expressed in late-stage lung cancer [102] and is also one of a small number of genes undergoing late-stage upregulation downstream of KLF4 during the metabolic shift that facilitates reprogramming during the generation of iPSCs in an SeVdp(KOSM)-based system [103].

3.6. LINE-1 silencing targets DICER by acting though miR-103/107 embedded in the PANK2 gene

In addition to their central role in metabolism, the PANK1–3 genes contain the microRNAs, miR-103 and miR-107, in their intronic regions, with PANK1, 2, and 3 corresponding to pri-miR-107, pri-miR-103-2, and pri-miR-103-1, respectively. Expression of miR-103/107 has been shown to parallel that of the PANK genes in a series of cell lines and in normal human tissues [104]. Furthermore, miR-103/107 are predicted bioinformatically to affect multiple mRNA targets in pathways that involve cellular acetyl-CoA and lipid levels and thus to act synergistically with their host genes [105].

Although specific microRNAs can be upregulated in cancer, global miRNA downregulation is a common trait of human malignancies. This can be attributed, at least in part, to miR-103/107, which have been shown to target the 3′-UTR of Dicer leading to its downregulation and, in turn, to global downregulation of microRNA expression [106]. In human breast cancer, high levels of miR-103/107 are associated with metastasis and poor outcomes and this has been attributed to the miR-103/107-Dicer axis controlling epithelial plasticity and induction of the EMT, in part via regulation of miR-200 [106].

3.7. LINE-1 silencing is linked to the mitophagy-driven regulation of stem cell fate through TOMM7

The presence of Translocase Of Outer Mitochondrial Membrane 7 (TOMM7) in the darkmagenta module (**Figure 1**) is further evidence of L1 having an impact on cancer cell metabolism acting through HIF1α. TOMM7 encodes a member of the TOM pre-protein translocase complex of the outer mitochondrial membrane, the main entry portal for protein precursors from the cytosol into mitochondria.

TOMM7 has a crucial role in mitophagy, the autophagic elimination of damaged mitochondria that has a role regulating stem cell fate [107]. Mitophagy is regulated by the PTEN-induced putative kinase 1 (PINK1). TOMM7 stabilizes PINK1 on the outer mitochondrial

membrane, and accumulation of PINK1 bound to the TOM complex is completely blocked by the loss of TOMM7 from the TOM complex [108]. PINK1 loss-of-function compromises both mitochondrial autophagy and oxidative phosphorylation and reprograms glucose metabolism through HIF1 [109]. Pink1 deficiency also stabilizes HIF1α in cultured mouse embryonic fibroblasts and primary cortical neurons as well as in vivo [109]. This effect, mediated by mitochondrial ROS, leads to upregulation of the HIF1 target, PDK1 (pyruvate dehydrogenase kinase-1), which inhibits pyruvate dehydrogenase (PDH) activity. HIF1α stimulates glycolysis in the absence of Pink1, and the promotion of glucose metabolism by HIF1α stabilization is required for cell proliferation in Pink1$^{-/-}$ mice. Thus, it is possible that loss of Pink1 reprograms glucose metabolism through HIF1α, sustaining increased cell proliferation.

Independent support for the presence of TOMM7 in the darkmagenta module comes from an I-DIRT affinity proteomics study of L1 interactors [110]. TOMM40, another member of the TOM complex, was one of 37 high-confidence L1 ORF2-interactors in addition to Translocase Of Inner Mitochondrial Membrane 13 (TIMM13), a member of the TIMM family of proteins, that import proteins from the cytoplasm into the mitochondrial inner membrane in conjunction with the TOM complex.

3.8. LINE-1 silencing affects cytoskeletal dynamics and the MET through LIMK2, GSN, SYBL1, BLOC1S1, and RNF165

LIMK2 (darkmagenta module, **Figure 1**) has a key role in the MET, controlling the depolymerization of filamentous actin, by phosphorylating the actin stabilizer, cofilin. LIMK2 is one of the two kinases that have been shown to phosphorylate cofilin and stabilize actin stress fibers in fibroblasts, thus blocking the MET and preventing iPSC generation from mouse embryonic fibroblasts or human fibroblasts [98]. In the MET, the actin cytoskeleton is reorganized from actin stress fibres to cortical actin, the expression of mesenchymal transcription factors such as Zeb1 and Snai1 is lost, and the cells establish tight and adherens junctions stabilized by Par3/ZO-1 or E-cadherin [111].

Gelsolin (GSN) (lower left in darkmagenta module, **Figure 1**) is another key regulator of actin filament assembly and disassembly. Gelsolin is highly expressed at tumor borders infiltrating into adjacent liver tissues, contributes to lamellipodia formation in migrating cells, and induces tumor invasion by modulating the urokinase-type plasminogen activator cascade [112].

LIMK2 also acts with SYBL1 (darkmagenta module) in the assembly and maturation of invadopodia. Invadopodia are actin-rich protrusions that degrade extracellular matrix and are required for penetration through the basement membrane, stromal invasion, and intravasation. SYBL1 encodes VAMP-7, a transmembrane protein from the soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) family. VAMP-7 localizes to late endosomes and lysosomes and is involved in the fusion of transport vesicles to their target membranes. MT1-MMP is delivered by the IQGAP1-WASH-exocyst complex and fuses to the membrane via VAMP-7, resulting in matrix degradation [113].

Biogenesis of Lysosomal Organelles Complex 1 Subunit 1 (BLOC1S1) (darkmagenta module, **Figure 1**) is a component of the ubiquitous BLOC1 multisubunit protein complex required for the biogenesis of specialized organelles of the endosomal-lysosomal system, including melanosomes and platelet dense granules. Loss of BLOC1 function results in downregulation of the actin-related protein-2/3 complex (Arp2/3), a seven-subunit protein complex that plays a major role in the regulation of the actin cytoskeleton. This complex is present in cellular regions characterized by dynamic actin filament activity, including the leading edges of motile cells in lamellipodia, and also has a role in invadopodia [114]. The Arp2/3 complex is also potently activated by WASH [115].

The presence of RNF165/ARKL2 as a hub gene in the darkmagenta module (**Figure 1**), in the context of changes in expression of actin-related genes, is consistent with a bone morphogenetic protein (BMP)-driven MET. BMP has a key role in the induction of the MET [116] and RNF165/ARKL2 is an E3 ubiquitin-protein ligase that regulates motor axon elongation downstream of BMP [117]. A close homolog, RNF111/Arkadia is a key component of TGFβ signaling [118] and amplifies TGFβ and BMP signaling through degradation of the inhibitory Smad7. Aberrant RNF111/Arkadia activity occurs in clear-cell renal-cell carcinoma, colorectal cancer, and non-small cell lung cancer [119–122]. In contrast, not a great deal is known about RNF165 outside the nervous system, although it appears to have a significant role in metastatic prostate carcinoma [123].

3.9. LINE-1 affects stress granule formation through SRP9

The signal recognition particle (SRP) is a cytoplasmic ribonucleoprotein consisting of six polypeptides and a 300-nucleotide (7SL) RNA molecule. SRP9, a key member of the SRP is a member of darkmagenta module (**Figure 1**), while another member, SRP14, is a member of the L1 ORF1p interactome. The SRP9 and SRP14 polypeptides form a heterodimer and bind to the 3′ and 5′ ends of the SRP 7SL RNA. The SRP functions in the co-translational targeting of secretory and membrane proteins to the rough endoplasmic reticulum by complexing with ribosomes associated with the membrane of the RER via its receptor, SRPR, a hub gene in the pink module (**Figure 5**).

Remarkably, the Alu family of SINEs is thought to have originated from a 7SL RNA gene early in primate evolution [124] and subsequently amplified by retrotransposition so that over 1 million copies are now present in the human genome [125]. Binding of the SRP 9/14 proteins to the RNA of Alu elements precedes and is likely to be necessary for efficient L1-mediated Alu retrotransposition [126, 127].

In addition, the SRP9/14 heterodimer can bind to cytoplasmic Alu RNA and 40S ribosomal subunits in a pathway involving the formation of stress granules (SGs) [128]. Cellular stress triggers the formation of dense cytosolic aggregations that sequester mRNA, 40S ribosomal subunits, initiation factors, and RNA-binding and signaling proteins to promote cell survival. SRP9/14 localizes to SGs following arsenite or hippuristanol treatment. The localization and function of SRP9/14 in SGs is mediated by direct binding to 40S ribosomal subunits. Binding of SRP9/14 to 40S or Alu RNA is mutually exclusive indicating that the heterodimer alone is

bound to 40S in SGs and that Alu RNA may competitively regulate 40S binding. Following resolution of stress, cells actively increase cytoplasmic Alu RNA levels to promote disassembly of SGs by disengaging SRP9/14 from 40S [128].

The involvement of stress granules in tumor initiation in breast cancer cells was discovered by screening for intracellular proteins enhancing the effect of chemotherapeutic agents on TIC-enriched breast cancer cells [129]. This screen identified 44 proteins that interacted with the lead compound, C108, including the stress granule-associated protein and GTPase-activating protein (SH3 domain)-binding protein 2 (G3BP2). G3BP2 was shown to regulate breast tumor initiation through the stabilization of squamous cell carcinoma antigen recognized by T cells 3 (SART3) mRNA, leading to increased expression of the pluripotency transcription factors Oct4 and Nanog. THOC6, an interaction partner of DDX39B in the THO complex and involved in the nuclear export of pluripotency-related transcripts, was also among the 44 interacting partners of C108.

At least two genes in the darkmagenta module (**Figure 1**) are linked to the C108 protein interactome, thus supporting the involvement of this module in SG formation. PTPN9 is present in C108 protein interactome, while AK130123 is highly similar to PPP2R2A, whose gene product interacts with those of PPP2R1A and PPP2R1B (present in the C108 protein interactome).

Another three interaction partners of C108 (IGF2BP1, IGF2BP2, and PABPC1) and SART1, but not SART3, are also present in the L1 ORF1p interactome. The degree of overlap between these two interactomes is statistically significant with a representation factor of 12.6 and $p < 0.002$. L1 ORF1 protein has, in fact, been shown by yeast two-hybrid screening to localize in stress granules with other RNA-binding proteins, including components of the RISC complex [130].

3.10. LINE-1 is likely to promote the cancer stem cell phenotype through SART1 and SART3

Although not members of the darkmagenta or any other module, SART1/TIP110, a member of the L1 ORF1p interactome and the functionally related SART3 implicate the L1 ORF1 protein in promotion of the cancer stem cell phenotype. SART1 (also known as U4/U6.U5 Tri-SnRNP-Associated Protein 1) encodes two proteins, the SART1(800) protein expressed in the nucleus of the majority of proliferating cells and the SART1(259) protein expressed in the cytosol of epithelial cancers. The SART1(259) protein is translated by −1 frameshifting during post-transcriptional regulation. SART1(259) plays an essential role in mRNA splicing by recruiting the tri-snRNP to the pre-spliceosome during spliceosome assembly. In contrast, SART3 associates transiently with U6 and U4/U6 snRNPs during the recycling phase of the spliceosome cycle. As mentioned before, stabilization of SART3 mRNA leads to increased expression of the pluripotency transcription factors, Oct-4 and Nanog [129]. SART3 also regulates OCT4 splicing in hESCs [131].

A recent proteomics study identified 13 SART3/TIP110-interacting cellular proteins, 5 of which are also present in the L1 ORF1p interactome [132]. This degree of overlap is highly significant with a representation factor of 76.1 and a p-value < 3.694E-09. These observations suggest that L1 affects SART3 in some way, thus implicating L1 in SART3-mediated breast cancer initiation.

Like SART3, SART1(800) also has fundamental roles in the formation of cancer stem cells. SART1(800), also known as hypoxia-associated factor (HAF), is overexpressed in a variety of tumor types. HAF is an E3 ubiquitin ligase that binds to and ubiquitinates HIF-1α by an oxygen- and pVHL-independent mechanism, targeting HIF-1α for proteasomal degradation [133]. HAF expression lowers HIF-1α levels and decreases HIF-1 transactivating activity. HAF also binds to HIF-2α but does not lead to its degradation and instead *increases* HIF-2 transactivating activity. Thus, HAF expression switches the hypoxia response of the cancer cell from HIF-1α- to HIF-2α-dependent transcription of genes such as MMP9 and OCT-3/4. This switch by HAF promotes the cancer stem cell phenotype and invasion, resulting in highly aggressive tumors in vivo [134].

3.11. LINE-1 silencing affects cancer-related signal transduction pathways by downregulating DGKA and GNA15

The presence of diacylglycerol kinase alpha (DGKA) and G protein subunit alpha 15 (GNA15) in the darkmagenta module (**Figure 1**) implicates LINE-1 silencing in affecting signal transduction pathways. Increasing evidence points to DGKA (DGKα) being a major node in oncogenic signaling [135]. DGKA converts diacylglycerol (DAG) to phosphatidic acid (PA), with both being critical lipid second messengers found in the plasma membrane. DGKA activity terminates DAG signaling and has been linked to activation of NF-κB, HIF-1α, c-Met, ALK, and VEGF [136]. DAG, in turn, binds directly to protein kinase C and D family members, to Ras family members, and to diacylglycerol kinase family members, while PA controls the activity of mTOR, Akt, and Erk.

DGKA plays an important role in the spread and invasion of breast cancer cells [137]. Among the microenvironment signals sustaining cancer cell invasiveness, stromal cell-derived factor-1α (SDF-1α, or CXCL12) plays a major role in several cancers, including breast cancer [138]. SDF-1α is a chemokine secreted by tumor-associated fibroblasts and bone marrow stromal cells, which by activating its CXCR4 receptor (tan module, **Figure 6**), promotes migration and invasion of malignant cells and their homing to target organs [139, 140]. Following SDF-1α stimulation, DGKA is activated and localized at cell protrusions, promoting their elongation and mediating SDF-1α-induced MMP-9 metalloproteinase secretion and matrix invasion. PA generated by DGKA promotes recruitment of atypical PKCs (protein kinase C's) to cell protrusions or ruffling sites, which play an essential role by promoting Rac-mediated protrusion elongation and localized recruitment of β1 integrin and MMP-9. Moreover, DGKA activity sustains the pro-invasive activity of metastatic p53 mutations, by promoting the recycling of α5β1 integrin to invasive protrusions in tridimensional matrix [141].

GNA15 (also known as G15, Gα15 or GNA16) is a heterotrimeric G protein selectively expressed in immature hematopoietic and epithelial cells with high renewal potential. GNA15 is notable for its ability to bypass the usual selectivity of receptor G-protein interactions and to non-selectively couple structurally and functionally diverse receptors to phospholipase C [142]. Following activation of GPCRs, rapid desensitization of receptor responsiveness normally prevents uncontrolled signaling and is initiated by phosphorylation of the receptor by GPCR kinases [143, 144] followed by uncoupling of GPCR-G protein interactions mediated

by β-arrestin protein family members [145, 146]. Intriguingly however, GNA15 is not affected by GPCR desensitization. In certain cell lineages, GNA15 amplifies incoming stimuli regardless of β-arrestin-induced desensitization, thus promoting sustained activation of its downstream effectors, including key players in cancer signal transduction such as PKD1, Ras, Raf, PI3K, MEK, PKCs, and STATs [147–150]. Based on its resistance to desensitization and extraordinarily poor coupling selectivity [147], GNA15 may promote unconventional stimulation based on prolonged auto/paracrine activation of GPCRs. These may include GPCRs known for supporting the immature stages of pancreatic cancer, such as CXCR4 [151, 152], S1PRs [153–155], Frizzled [156, 157], and Smoothened (SMOH) [158–160].

GNA15 was recently identified in a three gene signature highly expressed in a leukemic stem cell-enriched CD34 + cell fraction in normal karyotype acute myeloid leukemia [161]. Ectopic expression of GNA15 is also found in pancreatic carcinoma [160]. In contrast, GNA15 mRNA and protein expression were found to be severely downregulated in a panel of non-small cell lung cancer cell lines and in human lung adenocarcinoma and squamous carcinoma patients [162]. Additionally, GNA15 has been identified as a regulator of non-small cell lung cancer cell proliferation and anchorage-independent cell growth [162].

3.12. Genes involved in protein kinase R stress signaling are enriched in the darkmagenta module

We uploaded the gene list from the darkmagenta module to the MetaCore web server (Clarivate Analytics; https://clarivate.com/) to search for enriched cellular pathways. The most significant pathway identified was that of "Apoptosis and survival_Role of PKR in stress-induced apoptosis" with a raw p-value = 4.925E-6 and a FDR-corrected p-value = 8.126E-4. The darkmagenta module contains 3 of the 53 genes in this pathway. These are NFKBIB, IFNB1, and AK130123 (a probable transcript variant of PPP2R2A). Although not identified as a member of this pathway, IL3 (also present in the darkmagenta module) appears to positively regulate protein synthesis by inducing the inactivation of PKR via a growth factor signaling pathway.

Protein kinase R (PKR) (also known as eukaryotic translation initiation factor 2 alpha kinase 2/EIF2AK2) is a serine/threonine protein kinase that is activated by autophosphorylation after binding to dsRNA. By this mechanism, PKR inhibits the replication of a wide range of DNA and RNA viruses by phosphorylating the alpha subunit of eukaryotic initiation factor 2 (EIF2S1/eIF2α), a central node of the cellular response to stress signals. This impairs the recycling of EIF2S1 between successive rounds of initiation leading to inhibition of translation, which eventually results in shutdown of cellular and viral protein synthesis.

Stress-induced phosphorylation of EIF2S1 also induces stress granule assembly by preventing or delaying translational initiation and, additionally, is involved in the restriction of LINE-1 retrotransposition by SAMHD1. The HIV-1 restriction factor SAMHD1 can negatively modulate retrotransposition of LINE-1 by a mechanism that involves sequestration of L1 RNP in stress granules [163]. SAMHD1 promotes the formation of these stress granules by inducing phosphorylation of EIF2S1 and disrupting the interaction between eIF4A and eIF4G [163].

In addition to its role in stress granule formation, PKR phosphorylates p53/TP53, PPP2R5A, DHX9, ILF3, and IRS1 with DHX9 and ILF3 being members of the LINE-1 ORF1p interactome. Either as an adapter protein and/or via its kinase activity, PKR can also regulate the p38 MAP kinase, NFKB, and insulin signaling pathways and transcription factors (JUN, STAT1, STAT3, IRF1, ATF3) involved in the expression of genes encoding pro-inflammatory cytokines and interferons. PKR also has a role in the regulation of the cytoskeleton by binding to Gelsolin (GSN; darkmagenta module, **Figure 1**), sequestering the protein in an inactive conformation away from actin [164].

The downregulation of NFKBIB in the darkmagenta module suggests activation of NFkB signaling. Hyperactivation of NFkB induces the expression of stemness-associated genes and inflammatory genes in CSCs but this is likely to be context-dependent involving Toll-like receptor signaling and saturated fatty acids [165, 166].

3.13. LINE-1 silencing affects the initiation, elongation, and termination steps of protein translation

Dysregulation of three of the four major steps of mRNA translation: initiation, elongation, and termination, has been implicated in the development and progression of cancer. In addition to the role of PKR signaling in initiation mentioned above, several genes in the darkmagenta module can be directly linked to these steps as can several members of the L1 ORF1p interactome.

Elevated protein synthesis arises as a consequence of increased signaling flux channeled to eIF4F, the key regulator of the mRNA-ribosome recruitment phase of translation initiation and a critical nexus for cancer development. The eIF4F complex is a trimeric complex consisting of the eIF4E cap-binding protein, the eIF4G scaffold protein, and the eIF4A helicase and is subject to regulation by major oncogenic pathways, including the PI3K/AKT/mTOR and MAPK cascades [167]. At least three members of the L1 ORF1p interactome (eIF4B, PABPC1, and PABPC4) interact with eIF4A [167]. In addition, based on a string-db [62] analysis by us of the LINE-1 ORF1p interactome members including eIF4E, there is suggestive literature evidence for interactions between other components of the L1 ORF1p (PCBP2, LARP1, SSB, DDX39A, RNMT, HNRNPA1, and PCBP2) and eIF4E (data not shown). In addition, eIF1B, a highly connected gene in the darkmagenta module (**Figure 1**), is a key player in start codon selection, a critical step in translation initiation that sets the reading frame for decoding [168].

EEF1A1P9 or EEF1AL7 (LOC441032 in the darkmagenta module, **Figure 1**) is a pseudogene related to eukaryotic translation elongation factor 1A1 (eEF1A1/EEF1-α 1), an isoform of eEF1A. eEF1A is a protein subunit of the eukaryotic translation elongation 1 (eEF1) complex, which is composed of eEF1A, valyl-tRNA, and the eEF1B complex, comprising eEF1G, eEF1B, and eEF1D. Overexpression of EEF1D/eEF-1δ in cadmium-transformed Balb/c-3T3 cells in conjunction with eIF3 is a major mechanism responsible for cell transformation and tumorigenesis induced by cadmium [169]. In addition to eEF1A's canonical role in translational elongation, eEF1A has a growing list of functions beyond protein synthesis, including protein degradation [170, 171], apoptosis [172, 173], nucleocytoplasmic trafficking [174], heat shock [175], and

multiple aspects of cytoskeletal regulation [176]. eEF1A1 may also mediate turnover of the LINE-1 restriction factor, SAMHD1, by targeting it to the proteosome for degradation [177].

While translation termination is generally not considered a major target of tumorigenesis, eukaryotic release factors such as AF447869/GSPT1/eRF3 (darkmagenta module, **Figure 1**) are implicated in gastric cancer [178]. GSPT1/eRF3 is also involved in the regulation of cytoplasmic mRNA decay in association with Poly(A)-binding protein (PABP), two isoforms of which, PABPC1 and PABPC4, are present in the L1 ORF1p interactome. GSPT1 also has a role in nonsense-mediated decay [179].

There are five known GSPT1/eRF3a human alleles, one of which has been correlated with increased cancer risk in several studies and which may act by decreasing the binding affinity of GSPT1 for PABP [180]. Alternatively, GSPT1/eRF3 may be involved in tumorigenesis as a result of its non-translational roles, which affect cell cycle dysregulation, apoptosis, and transcription [178].

3.14. E3 ubiquitin protein ligases that affect oncoprotein stability are hub genes in several modules

Proteins that promote cell proliferation must be expressed in a controlled manner but also efficiently degraded. A major pathway for such targeted protein degradation is the ubiquitin-proteasome system (UPS), and oncoproteins that drive tumor development are often deregulated and stabilized in malignant cells. Several E3 ubiquitin protein ligases targeting oncoproteins are hub genes in other modules, including BTRC (a hub gene in the darkolivegreen module, **Figure 7**, fold change -1.68x downregulated in L1-silenced versus controls) and FBXW11 and FBXW7 (hub genes in the pink module (**Figure 5**), although neither are differentially expressed in L1-silenced versus controls). FBXW10 and BC067077 /MDM2, although not hub genes, are present in the darkmagenta module (**Figure 1**).

A number of proteins driving the development and progression of cancer are direct or indirect targets of the UPS. For example, FBXW7 (FBW7 or F-box and WD repeat domain containing 7 E3 Ub protein ligase) promotes ubiquitination and proteasomal degradation of mTOR [181]. This leads to breast cancer suppression in cooperation with PTEN. BTRC also regulates mTOR activity through the targeted degradation of DEP domain-containing mTOR-interacting protein (DEPTOR), an inhibitor of both mTORC1 and mTORC2 [182]. NOTCH signaling is involved in the short-range communication between neighboring cells, and its activation plays a key role in cancer progression. NOTCH receptors are regulated by multiple E3s, and turnover of the unstable NOTCH intracellular domains is also mediated by FBXW7 [183, 184]. In addition, the RING finger E3 Ubiquitin ligase BC067077/MDM2 (E3 Ub ligase mouse double minute 2), present in the darkmagenta module (BC067077, **Figure 1**), is an oncoprotein in its own right and a negative regulator of p53 protein expression [185].

MYC proteins are regulated by at least five different E3 ubiquitin ligases, including FBXW7 and BTRC [186]. FBXW7 acts as a negative regulator of MYC [187], while BTRC positively regulates MYC protein stability [188]. In addition to control of MYC protein by the UPS, a number of other modulators of MYC activity have prominent positions in key modules. The

STK38 kinase (pink module, **Figure 5**) (upregulated 1.69x in L1-silenced versus controls) regulates MYC protein stability and turnover in a kinase activity-dependent manner. In human B-cell lymphomas, STK38 kinase inactivation prevents apoptosis following B-cell receptor activation, whereas silencing of STK38 decreases MYC levels and promotes apoptosis [189]. STK38 knockdown also suppresses growth of MYC-addicted tumors in vivo [189]. CSNK2A2 (a hub gene in the orange module; **Figure 8**) (fold change -1.76x downregulated in L1-silenced versus controls) also phosphorylates and regulates MYC in addition to multiple transcription factors and Hsp90 and its co-chaperones and regulates Wnt signaling by phosphorylating CTNNB1 [190, 191].

Other oncoproteins targeted by E3 ubiquitin ligases in the modules described here include p53 and NFKBIB/IκKB (with NFKBIB/IκKB being present in the darkmagenta module, **Figure 1**). The p53 transcription factor is a tightly regulated sensor of cellular stress and its activation can lead to cell cycle arrest, apoptosis, senescence, DNA repair, altered metabolism, or autophagy [192]. Under normal conditions, protein levels of p53 are kept low by proteasomal degradation, promoted in part through continuous targeting by MDM2 [185]. The transcription of MDM2 is also upregulated by p53, creating a feedback loop in which MDM2 targets both p53 and itself for proteasomal degradation [193]. MDM2 also blocks the transactivating activity of p53, preventing transcriptional activation of p53 target genes [194]. In addition, MDM2 can heterodimerize with the homologous RING finger protein MDM4/MDMX (a hub in the tan module, **Figure 6**). MDM4 binds p53 although it has no intrinsic ubiquitin ligase activity [195]. MDM2 can either mono-ubiquitinate p53, facilitating its transport to the cytoplasm and terminating p53's nuclear activity, or cooperate with MDM4 and other Ub ligases to poly-ubiquitinate and thereby target p53 for degradation by proteasomes [196].

In unstimulated cells, NFκB proteins are generally kept inactive by binding to proteins known as inhibitors of NFκB (IκBs) [197]. In addition to its actions described above, BTRC triggers ubiquitination of the NFκB inhibitor, IκBA [198], with the closely related NFKBIB/IκBβ being a member of the darkmagenta module (**Figure 1**). NFκB signaling controls many cellular functions, including cell growth and survival, differentiation, development, immunity, and inflammation [199], and is subject to tight post-translational regulation by protein kinases, deubiquitinating enzymes [200], and ubiquitin ligases.

Phosphorylation of IκBA by IKK targets it for ubiquitination and proteasomal degradation by BTRC, allowing the NFκB protein, RelA, to translocate to the nucleus and activate gene expression. BTRC also contributes to NFκB pathway activation by promoting the formation of specific NFκB protein complexes in the nucleus through ubiquitination and partial proteolysis of IκBs, such as p105 and p100. Furthermore, FBXW7 also targets p100 for degradation in a GSK3β-dependent manner [201–203].

3.15. LINE-1 may affect MYC mRNA stability via MYC's coding region instability determinant

MYC is also subject to regulation at the transcript level. In the mouse, the IGF2BP1 RNA-binding protein stabilizes c-myc RNA by associating with a coding region instability

determinant (CRD) located in the last 249 nucleotides of the coding region of c-myc [204]. Four RNA-binding proteins present in the LINE-1 ORF1p interactome (HNRNPU, SYNCRIP, YBX1, and DHX9) associate with IGF2BP1 in an RNA-dependent fashion and are essential to ensure stabilization of MYC mRNA via its CRD [205]. Complex formation at the CRD may limit transfer of MYC mRNA to polysomes and subsequent translation-coupled decay. Furthermore, IGF2BP2-3, two members of the LINE-1 ORF1p interactome appear to operate redundantly with IGF2BP1 in regulating MYC mRNA in addition to having important roles in modulating tumor cell fate [206].

In further evidence of links between the L1 ORF1p and the IGF2BP1 protein interactomes, Weidensdorfer et al. [205] identified 24 proteins associating with IGF2BP1 by immune-purification and mass spectrometry, 14 of which are also present in the L1 ORF1p interactome. This degree of overlap is highly significant with a representation factor of 115.5 and p-value < 4.927E-27.

4. Conclusions

The findings from our WGCNA analysis of the L1-silenced transcriptome in T47D breast cancer cells add weight to the growing body of evidence that L1 expression and activity is a cause rather than a consequence of oncogenesis. In our WGCNA analysis, the observed changes in expression of numerous genes with fundamental roles in cancer and the formation of cancer stem cells or the phenotypic transitions of the EMT/MET seem too concerted and related by function for L1 to be dismissed as a passenger gene or epiphenomenon. Furthermore, a number of these changes in gene expression are consistent with the changes in cancer cell morphology observed upon pharmacological blockade of L1-RT. Our results also support a central hypothesis of the WGCNA method; that the similar expression profiles of genes in a module reflect common regulatory mechanisms or biological functions.

In addition to our gene expression profiling of L1-silencing, we present evidence from independent studies showing statistically significant overlaps between the L1 ORF1p and ORF2p interactomes and cancer driver genes identified by proteomics and data mining. This alone is strongly suggestive of a driver role for L1 in cancer. We also present evidence from independent proteomics studies consistent with L1 having a role in the stabilization of MYC, an oncoprotein with a key role in the global metabolic reprogramming that occurs in cancer.

In summary, we find evidence of L1 activity mounting a concerted attack on cancer cell gene expression consistent with EMT/MET-related phenotypic transitions. L1 activity is also important in the formation of breast cancer stem cells, the support of cancer cell evolvability and, probably, the development of chemoresistance.

Future directions include a more intensive transcriptomic investigation of the effects of L1 on the formation of cancer stem cells with a wider range of cancer cell types and larger sample sizes. Another high priority will be further investigation of the effects of L1 on non-coding RNA and integrating this with the effects seen here on gene expression. In this context, we

have already shown global upregulation of microRNA expression mainly due to a marked increase in let-7 expression following L1-silencing by siRNA [207]. This is consistent with the effects of PANK2 downregulation on Dicer described earlier. It is also likely that the effects of L1-silencing by siRNA differ from those induced by pharmacological blockade of L1-RT and these will need to be investigated to establish whether the concept of pharmacological blockade of L1-RT is therapeutically viable. Chemotherapy is implicated in the formation of drug-resistant cancer stem cells, and NNRTI drugs like Efavirenz are probably no exception to this issue. Finally, thought should be given to targeting the L1 ORF1 protein pharmacologically as it is likely that this has a more important role than L1-RT.

Abbreviations

APOC1	Apolipoprotein C1
BLOC1S1	Biogenesis of lysosomal organelles complex 1 subunit 1
BMP	Bone morphogenetic protein
CML	Chronic myeloid leukemia
CoA	Coenzyme A
CSCs	Cancer stem cells
DAG	Diacylglycerol
DEPTOR	DEP domain containing mTOR-interacting protein
DMSO	Dimethyl sulfoxide
dPANK	Drosophila PANK homolog
eEF1	Eukaryotic translation elongation 1
EIF2S1/eIF2α	Eukaryotic initiation factor 2
EMT	Epithelial-mesenchymal transition
ESCs	Embryonic stem cells
EZH2	Enhancer of zeste 2 polycomb repressive complex 2 subunit
FKBP6	FK506 binding protein 6
G3BP2	GTPase-activating protein (SH3 domain)-binding protein 2
GBM	Glioblastoma
GSN	Gelsolin
HAF	Hypoxia-associated factor
HERV	Human endogenous retrovirus

HIF-1	Hypoxia inducible factor-1
HSP90	Heat shock protein 90
hTERT	Human telomerase reverse transcriptase
I-DIRT	Isotopic Differentiation of Interactions as Random or Targeted
IkBs	Inhibitors of NFkB
iPSC	Induced pluripotent stem cell
IQGAP1-WASH-exocyst complex:	
IQGAP1	IQ Motif Containing GTPase Activating Protein 1
WASH	Arp2/3 activating protein localized at surface of endosomes where it induces formation of branched actin networks
Exocyst	Octameric protein complex involved in vesicle trafficking and cell migration
L1-KD	L1 knockdown
L1 ORF1p	L1 ORF1 protein
LIMK2	LIM Domain Kinase 2
L1	LINE-1
L1-RT	LINE-1 reverse transcriptase
MET	Mesenchymal-epithelial transition
Miwi2	Mouse homolog of PIWIL4 (Piwi Like RNA-Mediated Gene Silencing 4)
MMP-2	Matrix metalloproteinase 2
MT	Metallothionein
MT1M	Metallothionein 1 M
MT1-MMP	Membrane type 1 metalloprotease
MTF1	Metal-responsive transcription factor
NO	Nitric oxide
NPC	Nuclear pore complex
PA	Phosphatidic acid
PANK2	Pantothenate kinase 2
PDH	Pyruvate dehydrogenase

Pin1	Peptidyl prolyl isomerase 1
PINK1	PTEN-induced putative kinase 1
piRNA	Piwi-interacting RNA
Piwi	P-element Induced WImpy testis (a subfamily of Argonaute proteins)
PKR	Protein Kinase R
RMA	Robust multi-array average
ROS	Reactive oxygen species
RT	Reverse transcriptase
SART3	Squamous cell carcinoma antigen recognized by T cells 3
SDF-1α, or CXCL12	Stromal cell-derived factor-1α
SINEs	Short interspersed elements
SMOH	Smoothened
SNARE	Soluble N-ethylmaleimide-sensitive factor attachment protein receptor
SRP	Signal recognition particle
TET	Ten–eleven translocation
TIC	Tumor-initiating cell
TOMM7	Translocase of Outer Mitochondrial Membrane 7
UPS	Ubiquitin–proteasome system
WGCNA	Weighted gene correlation network analysis

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References

[1] Belancio VP, Hedges DJ, Deininger P. Mammalian non-LTR retrotransposons: For better or worse, in sickness and in health. Genome Research. 2008;**18**(3):343-358

[2] Cordaux R, Batzer MA. The impact of retrotransposons on human genome evolution. Nature Reviews Genetics. 2009;**10**(10):691-703

[3] Beck CR, Collier P, Macfarlane C, Malig M, Kidd JM, Eichler EE, et al. LINE-1 retrotransposition activity in human genomes. Cell. 2010;**141**(7):1159-1170

[4] Brouha B, Schustak J, Badge RM, Lutz-Prigge S, Farley AH, Moran JV, et al. Hot L1s account for the bulk of retrotransposition in the human population. Proceedings of the National Academy of Sciences. 2003;**100**(9):5280-5285

[5] Han JS, Szak ST, Boeke JD. Transcriptional disruption by the L1 retrotransposon and implications for mammalian transcriptomes. Nature. 2004;**429**(6989):268-274

[6] Han JS, Boeke JD. LINE-1 retrotransposons: Modulators of quantity and quality of mammalian gene expression? BioEssays. 2005;**27**(8):775-784

[7] Speek M. Antisense promoter of human L1 retrotransposon drives transcription of adjacent cellular genes. Molecular and Cellular Biology. 2001;**21**(6):1973-1985

[8] Asch H, Eliacin E, Fanning T, Connolly J, Bratthauer G, Asch B. Comparative expression of the LINE-1 p40 protein in human breast carcinomas and normal breast tissues. Oncology Research. 1996;**8**(6):239-247

[9] Harris CR, Normart R, Yang Q, Stevenson E, Haffty BG, Ganesan S, et al. Association of nuclear localization of a long interspersed nuclear element-1 protein in breast tumors with poor prognostic outcomes. Genes & Cancer. 2010;**1**(2):115-124

[10] Ting DT, Lipson D, Paul S, Brannigan BW, Akhavanfard S, Coffman EJ, et al. Aberrant overexpression of satellite repeats in pancreatic and other epithelial cancers. Science. 2011;**331**(6017):593-596

[11] Patnala R, Lee S-H, Dahlstrom JE, Ohms S, Chen L, Dheen ST, et al. Inhibition of LINE-1 retrotransposon-encoded reverse transcriptase modulates the expression of cell differentiation genes in breast cancer cells. Breast Cancer Research and Treatment. 2014;**143**(2):239-253

[12] Bollati V, Fabris S, Pegoraro V, Ronchetti D, Mosca L, Deliliers GL, et al. Differential repetitive DNA methylation in multiple myeloma molecular subgroups. Carcinogenesis. 2009;**30**(8):1330-1335

[13] Fabris S, Bollati V, Agnelli L, Morabito F, Motta V, Cutrona G, et al. Biological and clinical relevance of quantitative global methylation of repetitive DNA sequences in chronic lymphocytic leukemia. Epigenetics. 2011;**6**(2):188-194

[14] Burns KH. Transposable elements in cancer. Nature Reviews Cancer. Jul 2017;**17**(7):415-424

[15] Scott EC, Devine SE. The role of somatic L1 retrotransposition in human cancers. Viruses. May 31, 2017;**9**(6). pii: E131

[16] Yamamura K, Kosumi K, Baba Y, Harada K, Gao F, Zhang X, et al. LINE-1 methylation level and prognosis in pancreas cancer: pyrosequencing technology and literature review. Surgery Today. Dec 2017;**47**(12):1450-1459

[17] Min J, Choi B, Han T-S, Lee H-J, Kong S-H, Suh Y-S, et al. Methylation Levels of LINE-1 as a useful marker for venous invasion in both FFPE and frozen tumor tissues of gastric cancer. Molecules and Cells. May 31, 2017;**40**(5):346-354

[18] Ye ZJ, Liu QP, Cen S, Li XY. The function of LINE-1-encoded reverse transcriptase in tumorigenesis. Yi Chuan. May 20, 2017;**39**(5):368-376

[19] Ardeljan D, Taylor MS, Ting DT, Burns KH. The human long interspersed element-1 retrotransposon: An emerging biomarker of neoplasia. Clinical Chemistry. Apr 2017;**63** (4):816-822

[20] Honda T. Links between human LINE-1 retrotransposons and hepatitis virus-related hepatocellular carcinoma. Frontiers in Chemistry. May 11, 2016;**4**:21

[21] Liu Q, Wang JH, Li XY, Cen S. The connection between LINE-1 retrotransposition and human tumorigenesis. Yi Chuan. Feb 2016;**38**(2):93-102

[22] Sciamanna I, De Luca C, Spadafora C. The reverse transcriptase encoded by LINE-1 retrotransposons in the genesis, progression, and therapy of cancer. Frontiers in Chemistry. Feb 11, 2016;**4**:6

[23] Kemp JR, Longworth MS. Crossing the LINE toward genomic instability: LINE-1 retrotransposition in cancer. Frontiers in Chemistry. Dec 16, 2015;**3**:68

[24] Xiao-Jie L, Hui-Ying X, Qi X, Jiang X, Shi-Jie M. LINE-1 in cancer: Multifaceted functions and potential clinical implications. Genetics in Medicine. May 2016;**18**(5):431-439

[25] Sciamanna I, Gualtieri A, Piazza PF, Spadafora C. Regulatory roles of LINE-1-encoded reverse transcriptase in cancer onset and progression. Oncotarget. Sep 30, 2014;**5**(18): 8039-8051

[26] Barchitta M, Quattrocchi A, Maugeri A, Vinciguerra M, Agodi A. LINE-1 hypomethylation in blood and tissue samples as an epigenetic marker for cancer risk: A systematic review and meta-analysis. PLoS One. Oct 2, 2014;**9**(10):e109478

[27] Carreira PE, Richardson SR, Faulkner GJ. L1 retrotransposons, cancer stem cells and oncogenesis. FEBS Journal. Jan 2014;**281**(1):63-73

[28] Baba Y, Murata A, Watanabe M, Baba H. Clinical implications of the LINE-1 methylation levels in patients with gastrointestinal cancer. Surgery Today. Oct 2014;**44**(10):1807-1816

[29] Rodic N, Burns KH. Long interspersed element-1 (LINE-1): Passenger or driver in human neoplasms? PLoS Genetics. Mar 2013;**9**(3):e1003402

[30] Piskareva O, Lackington W, Lemass D, Hendrick C, Doolan P, Barron N. The human L1 element: A potential biomarker in cancer prognosis, current status and future directions. Current Molecular Medicine. Jun 2011;**11**(4):286-303

[31] Montoya-Durango DE, Ramos KS. L1 retrotransposon and retinoblastoma: Molecular linkages between epigenetics and cancer. Current Molecular Medicine. Jul 2010;**10**(5): 511-521

[32] Sinibaldi-Vallebona P, Lavia P, Garaci E, Spadafora C. A role for endogenous reverse transcriptase in tumorigenesis and as a target in differentiating cancer therapy. Genes, Chromosomes and Cancer. Jan 2006;**45**(1):1-10

[33] Aschacher T, Wolf B, Enzmann F, Kienzl P, Messner B, Sampl S, et al. LINE-1 induces hTERT and ensures telomere maintenance in tumour cell lines. Oncogene. 2016;**35**(1):94-104

[34] Cook PR, Jones CE, Furano AV. Phosphorylation of ORF1p is required for L1 retrotransposition. Proceedings of the National Academy of Sciences of the United States of America. 2015;**112**(14):4298-4303

[35] Sciamanna I, Landriscina M, Pittoggi C, Quirino M, Mearelli C, Beraldi R, et al. Inhibition of endogenous reverse transcriptase antagonizes human tumor growth. Oncogene. 2005;**24**(24):3923-3931

[36] Oricchio E, Sciamanna I, Beraldi R, Tolstonog GV, Schumann GG, Spadafora C. Distinct roles for LINE-1 and HERV-K retroelements in cell proliferation, differentiation and tumor progression. Oncogene. 2007;**26**(29):4226-4233

[37] Horvath S, Zhang B, Carlson M, Lu KV, Zhu S, Felciano RM, et al. Analysis of oncogenic signaling networks in glioblastoma identifies ASPM as a molecular target. Proceedings of the National Academy of Sciences. 2006;**103**(46):17402-17407

[38] Lehner B, Crombie C, Tischler J, Fortunato A, Fraser AG. Systematic mapping of genetic interactions in *Caenorhabditis elegans* identifies common modifiers of diverse signaling pathways. Nature Genetics. 2006;**38**(8):896-903

[39] Langfelder P, Horvath S. WGCNA: An R package for weighted correlation network analysis. BMC Bioinformatics. 2008;**9**(1):559

[40] R Core Team. R: A Language and Environment for Statistical Computing. Vienna, Austria: R Foundation for Statistical Computing; 2014. Available from: http://www.R-project.org/

[41] Zhu X, Wang F, Zhao Y, Yang P, Chen J, Sun H, et al. A gain-of-function mutation in Tnni2 impeded bone development through increasing Hif3a expression in DA2B mice. PLoS Genetics. 2014;**10**(10):e1004589

[42] Sidera K, Gaitanou M, Stellas D, Matsas R, Patsavoudi E. A critical role for HSP90 in cancer cell invasion involves interaction with the extracellular domain of HER-2. Journal of Biological Chemistry. 2007;**283**(4):2031-2041

[43] Taylor M, LaCava J, Mita P, Molloy K, Huang C, Li D, et al. Affinity proteomics reveals human host factors implicated in discrete stages of LINE-1 retrotransposition. Cell. 2013;**155**(5):1034-1048

[44] McDonald E, Workman P, Jones K. Inhibitors of the HSP90 molecular chaperone: Attacking the master regulator in cancer. Current Topics in Medicinal Chemistry. 2006;**6**(11):1091-1107

[45] Citri A, Kochupurakkal BS, Yarden Y. The Achilles heel of ErbB-2/HER2: Regulation by the Hsp90 chaperone machine and potential for pharmacological intervention. Cell Cycle. 2004;**3**(1):50-59

[46] Eustace BK, Jay DG. Extracellular roles for the molecular chaperone, HSP90. Cell Cycle. 2004;**3**(9):1096-1098

[47] Nolan KD, Franco OE, Hance MW, Hayward SW, Isaacs JS. Tumor-secreted Hsp90 subverts polycomb function to drive prostate tumor growth and invasion. The Journal of Biological Chemistry. 2015;**290**:8271-8282

[48] Bradley E, Bieberich E, Mivechi N, Tangpisuthipongsa D, Wang G. Regulation of embryonic stem cell pluripotency by heat shock protein 90. Stem Cells. 2012;**30**(8):1624-1633

[49] Jarosz D. Hsp90: A global regulator of the genotype-to-phenotype map in cancers. Advances in Cancer Research. 2016;**129**:225-247

[50] Piacentini L, Fanti, L, SpecchiaV, BozzettiMP, BerlocoM, PalumboG, Pimpinelli S. Transposons, environmental changes, and heritable induced phenotypic variability. Chromosoma. 2014;**123**(4):345-354

[51] Specchia V, Piacentini, L, Tritto P, Fanti L, D'Alessandro R, Palumbo G, Pimpinelli S, Bozzetti MP. Hsp90 prevents phenotypic variation by suppressing the mutagenic activity of transposons. Nature. 2010;**463**:662-665

[52] Xiol J, Cora E, Koglgruber R, Chuma S, Subramanian S, Hosokawa M, et al. A role for Fkbp6 and the chaperone machinery in piRNA amplification and transposon silencing. Molecular Cell. 2012;**47**(6):970-979

[53] Goodier JL, Cheung LE, Kazazian HH. Mapping the LINE1 ORF1 protein interactome reveals associated inhibitors of human retrotransposition. Nucleic Acids Research. 2013;**41**(15):7401-7419

[54] Chang C, Hautbergue G, Walsh M, Viphakone N, van Dijk T, Philipsen S, et al. Chtop is a component of the dynamic TREX mRNA export complex. The EMBO Journal. 2013;**32**(3):473-486

[55] Luna R, Rondón AG, Aguilera A. New clues to understand the role of THO and other functionally related factors in mRNP biogenesis. Biochimica et Biophysica Acta (BBA) – Gene Regulatory Mechanisms. 2012;**1819**(6):514-520

[56] Viphakone N, Hautbergue GM, Walsh M, Chang C, Holland A, Folco EG, et al. TREX exposes the RNA-binding domain of Nxf1 to enable mRNA export. Nature Communications. 2012;**3**:1006

[57] Müller-McNicoll M, Botti V, de Jesus Domingues AM, Brandl H, Schwich OD, Steiner MC, et al. SR proteins are NXF1 adaptors that link alternative RNA processing to mRNA export. Genes & Development. 2016;**30**(5):553-566

[58] Dufu K, Livingstone MJ, Seebacher J, Gygi SP, Wilson SA, Reed R. ATP is required for interactions between UAP56 and two conserved mRNA export proteins, Aly and CIP29, to assemble the TREX complex. Genes & Development. 2010;**24**(18): 2043-2053

[59] Lindtner S, Felber B, Kjems J. An element in the 3′ untranslated region of human LINE-1 retrotransposon mRNA binds NXF1(TAP) and can function as a nuclear export element. RNA. 2002;**8**(3):345-356

[60] Kato M, Wei M, Yamano S, Kakehashi A, Tamada S, Nakatani T, et al. DDX39 acts as a suppressor of invasion for bladder cancer. Cancer Science. 2012;**103**(7):1363-1369

[61] Park H, Imoto S, Miyano S. Recursive random lasso (RRLasso) for identifying anti-cancer drug targets. PLoS One. 2015;**10**(11):e0141869

[62] Szklarczyk D, Morris JH, Cook H, Kuhn M, Wyder S, Simonovic M, et al. The STRING database in 2017: Quality-controlled protein–protein association networks, made broadly accessible. Nucleic Acids Research. 2017;**45**(Database issue):D362-D3D8

[63] Siudeja K, Srinivasan B, Xu L, Rana A, de Jong J, Nollen E, et al. Impaired Coenzyme A metabolism affects histone and tubulin acetylation in Drosophila and human cell models of pantothenate kinase associated neurodegeneration. EMBO Molecular Medicine. 2011;**3**(12):755-766

[64] Wang W, Eddy R, Condeelis J. The cofilin pathway in breast cancer invasion and metastasis. Nature Reviews. Cancer. 2007;**7**(6):429-440

[65] Ward DM, Kaplan J. Ferroportin-mediated iron transport: Expression and regulation. Biochimica et Biophysica Acta (BBA) – Molecular Cell Research. 2012;**1823**(9):1426-1433

[66] Poli M, Derosas M, Luscieti S, Cavadini P, Campanella A, Verardi R, et al. Pantothenate kinase-2 (Pank2) silencing causes cell growth reduction, cell-specific ferroportin up-regulation and iron deregulation. Neurobiology of Disease. 2010;**39**(2):204-210

[67] Mai TT, Hamaï A, Hienzsch A, Cañeque T, Müller S, Wicinski J, et al. Salinomycin kills cancer stem cells by sequestering iron in lysosomes. Nature Chemistry. 2017;**9**: 1025-1033

[68] Pinnix ZK, Miller LD, Wang W, D'Agostino R, Kute T, Willingham MC, et al. Ferroportin and iron regulation in breast cancer progression and prognosis. Science Translational Medicine. 2010;**2**(43):43ra56-43ra56

[69] Yamane K, Tateishi K, Klose RJ, Fang J, Fabrizio LA, Erdjument-Bromage H, et al. PLU-1 is an H3K4 demethylase involved in transcriptional repression and breast cancer cell proliferation. Molecular Cell. 2007;**25**(6):801-812

[70] Yamamoto S, Wu Z, Russnes HG, Takagi S, Peluffo G, Vaske C, et al. JARID1B is a luminal lineage-driving oncogene in breast cancer. Cancer Cell. 2014;**25**(6):762-777

[71] Greer EL, Shi Y. Histone methylation: A dynamic mark in health, disease and inheritance. Nature Reviews Genetics. 2012;**13**(5):343-357

[72] Shen L, Wu H, Diep D, Yamaguchi S, D'Alessio AC, Fung H-L, et al. Genome-wide analysis reveals TET- and TDG-dependent 5-methylcytosine oxidation dynamics. Cell. 2013;**153**(3):692-706

[73] Tsai Y-P, Chen H-F, Chen S-Y, Cheng W-C, Wang H-W, Shen Z-J, et al. TET1 regulates hypoxia-induced epithelial-mesenchymal transition by acting as a co-activator. Genome Biology. 2014 Dec 3;**15**(12):513. DOI: 10.1186/s13059-014-0513-0

[74] Hu X, Zhang L, Mao S-Q, Li Z, Chen J, Zhang R-R, et al. Tet and TDG mediate DNA demethylation essential for mesenchymal-to-epithelial transition in somatic cell reprogramming. Cell Stem Cell. 2014;**14**(4):512-522

[75] Schonberg DL, Miller TE, Wu Q, Flavahan WA, Das NK, Hale JS, et al. Preferential iron trafficking characterizes glioblastoma stem-like cells. Cancer Cell. 2015;**28**(4):441-455

[76] Lerman MI, Minna JD. The 630-kb lung cancer homozygous deletion region on human chromosome 3p21.3: Identification and evaluation of the resident candidate tumor suppressor genes. Cancer Research. 2000;**60**(21):6116

[77] Ji L, Nishizaki M, Gao B, Burbee D, Kondo M, Kamibayashi C, et al. Expression of several genes in the human chromosome 3p21.3 homozygous deletion region by an adenovirus vector results in tumor suppressor activities in vitro and in vivo. Cancer Research. 2002;**62**(9):2715-2720

[78] Arber C, Angelova PR, Wiethoff S, Tsuchiya Y, Mazzacuva F, Preza E, et al. iPSC-derived neuronal models of PANK2-associated neurodegeneration reveal mitochondrial dysfunction contributing to early disease. PLoS One. 2017;**12**(9):e0184104

[79] Dansie LE, Reeves S, Miller K, Zano SP, Frank M, Pate C, et al. Physiological roles of the pantothenate kinases. Biochemical Society transactions. 2014;**42**(4):1033-1036

[80] Garcia M, Leonardi R, Zhang Y-M, Rehg JE, Jackowski S. Germline deletion of pantothenate kinases 1 and 2 reveals the key roles for CoA in postnatal metabolism. PLoS One. 2012;**7**(7):e40871

[81] Ding S, Li C, Cheng N, Cui X, Xu X, Zhou G. Redox regulation in cancer stem cells. Oxidative Medicine and Cellular Longevity. 2015;**2015**:750798

[82] Babula P, Masarik M, Adam V, Eckschlager T, Stiborova M, Trnkova L, et al. Mammalian metallothioneins: Properties and functions. Metallomics. 2012;**4**(8):739

[83] Coyle P, Mathew G, Game PA, Myers JC, Philcox JC, Rofe AM, et al. Metallothionein in human oesophagus, Barrett's epithelium and adenocarcinoma. British Journal of Cancer. 2002;**87**(5):533-536

[84] Palmiter RD. The elusive function of metallothioneins. Proceedings of the National Academy of Sciences. 1998;**95**(15):8428-8430

[85] Gumulec J, Raudenska M, Adam V, Kizek R, Masarik M. Metallothionein – Immunohistochemical cancer biomarker: A meta-analysis. PLoS One. 2014;**9**(1)

[86] Murphy BJ, Kimura T, Sato BG, Shi Y, Andrews GK. Metallothionein induction by hypoxia involves cooperative interactions between metal-responsive transcription factor-1 and hypoxia-inducible transcription factor-1. Molecular Cancer Research. 2008;**6**(3): 483-490

[87] Dubé A, Harrisson J, Saint-Gelais G, Séguin C. Hypoxia acts through multiple signaling pathways to induce metallothionein transactivation by the metal-responsive transcription factor-1 (MTF-1). Biochemistry and Cell Biology. 2011;**89**(6):562-577

[88] Feng W, Wang Y, Cai L, Kang YJ. Metallothionein rescues hypoxia-inducible factor-1 transcriptional activity in cardiomyocytes under diabetic conditions. Biochemical and Biophysical Research Communications. 2007;**360**(1):286-289

[89] Kojima I, Tanaka T, Inagi R, Nishi H, Aburatani H, Kato H, et al. Metallothionein is upregulated by hypoxia and stabilizes hypoxia-inducible factor in the kidney. Kidney International. 2009;**75**(3):268-277

[90] Troadec M, Ward D, Lo E, Kaplan J, De Domenico I. Induction of FPN1 transcription by MTF-1 reveals a role for ferroportin in transition metal efflux. Blood. 2010;**116**(22): 4657-4664

[91] Huang C, Yang W, Chang S, Tai S, Tzeng C, Kao J, et al. Regulation of membrane-type 4 matrix metalloproteinase by SLUG contributes to hypoxia-mediated metastasis. Neoplasia. 2009;**11**(12):1371-IN14

[92] Storci G, Sansone P, Mari S, D'Uva G, Tavolari S, Guarnieri T, et al. TNFalpha upregulates SLUG via the NF-kappaB/HIF1alpha axis, which imparts breast cancer cells with a stem cell-like phenotype. Journal of Cellular Physiology. 2010;**225**(3):682-691

[93] Alves CC, Carneiro, F, Hoefler H, Becker KF. Role of the epithelial-mesenchymal transition regulator Slug in primary human cancers. Frontiers in Bioscience. 2009;(14):3035-3050

[94] Storci G, Sansone P, Trere D, Tavolari S, Taffurelli M, Ceccarelli C, et al. The basal-like breast carcinoma phenotype is regulated by SLUG gene expression. The Journal of Pathology. 2008;**214**(1):25-37

[95] Zhang J, Zhang F, Hong C, Giuliano A, Cui X, Zhou G, et al. Critical protein GAPDH and its regulatory mechanisms in cancer cells. Cancer Biology & Medicine. 2015;**12**(1): 10-22

[96] Nicholls C, Pinto AR, Li H, Li L, Wang L, Simpson R, et al. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) induces cancer cell senescence by interacting with telomerase RNA component. Proceedings of the National Academy of Sciences. 2012;**109**(33): 13308-13313

[97] David L, Polo JM. Phases of reprogramming. Stem Cell Research. 2014;**12**(3):754-761

[98] Sakurai K, Talukdar I, Patil VS, Dang J, Li Z, Chang K, et al. Kinome-wide functional analysis highlights the role of cytoskeletal remodeling in somatic cell reprogramming. Cell Stem Cell. 2014;**14**(4):523-534

[99] Ross DT, Scherf U, Eisen MB, Perou CM, Rees C, Spellman P, et al. Systematic variation in gene expression patterns in human cancer cell lines. Nature Genetics. 2000;**24**(3):227-235

[100] Lightfoot J, Hitzler JK, Zipursky A, Albert M, Macgregor PF. Distinct gene signatures of transient and acute megakaryoblastic leukemia in down syndrome. Leukemia. 2004; **18**(10):1617-1623

[101] Takano S, Yoshitomi H, Togawa A, Sogawa K, Shida T, Kimura F, et al. Apolipoprotein C-1 maintains cell survival by preventing from apoptosis in pancreatic cancer cells. Oncogene. 2007;**27**(20):2810-2822

[102] Ko H, Wang Y, Fong W, Chi M, Chi K, Kao S. Apolipoprotein C1 (APOC1) as a novel diagnostic and prognostic biomarker for lung cancer: A marker phase I trial. Thoracic Cancer. 2014;**5**(6):500-508

[103] Nishimura K, Aizawa S, Nugroho FL, Shiomitsu E, Tran YTH, Bui PL, et al. A role for KLF4 in promoting the metabolic shift via TCL1 during induced pluripotent stem cell generation. Stem Cell Reports. 2017;**8**(3):787-801

[104] Baskerville S, Bartel DP. Microarray profiling of microRNAs reveals frequent coexpression with neighboring miRNAs and host genes. RNA. 2005;**11**(3):241-247

[105] Wilfred BR, Wang W-X, Nelson PT. Energizing miRNA research: A review of the role of miRNAs in lipid metabolism, with a prediction that miR-103/107 regulates human metabolic pathways. Molecular Genetics and Metabolism. 2007;**91**(3):209-217

[106] Martello G, Rosato A, Ferrari F, Manfrin A, Cordenonsi M, Dupont S, et al. A MicroRNA targeting dicer for metastasis control. Cell. 2010;**141**(7):1195-1207

[107] Vazquez-Martin A, den Haute C, Cufí S, Faja B, Cuyàs E, Lopez-Bonet E, et al. Mitophagy-driven mitochondrial rejuvenation regulates stem cell fate. Aging. 2016;**8**(7): 1330-1352

[108] Hasson SA, Kane LA, Yamano K, Huang C-H, Sliter DA, Buehler E, et al. High-content genome-wide RNAi screens identify regulators of parkin upstream of mitophagy. Nature. 2013;**504**(7479):291-295

[109] Requejo-Aguilar R, Lopez-Fabuel I, Fernandez E, Martins LM, Almeida A, Bolaños JP. PINK1 deficiency sustains cell proliferation by reprogramming glucose metabolism through HIF1. Nature Communications. 2014;**5**:4514

[110] Molloy KR, Taylor MS, Altukhov I, Mita P, Jiang H, Adney EM, et al. Dissection of purified LINE-1 reveals distinct nuclear and cytoplasmic intermediates. bioRxiv 157818, cold Spring Harbor. The Laboratory. 2017

[111] Lamouille S, Xu J, Derynck R. Molecular mechanisms of epithelial–mesenchymal transition. Nature reviews Molecular cell biology. 2014;**15**(3):178-196

[112] Zhuo J, Tan EH, Yan B, Tochhawng L, Jayapal M, Koh S, et al. Gelsolin induces colorectal tumor cell invasion via modulation of the Urokinase-type plasminogen activator Cascade. PLoS One. 2012;**7**(8):e43594

[113] Monteiro P, Rossé C, Castro-Castro A, Irondelle M, Lagoutte E, Paul-Gilloteaux P, et al. Endosomal WASH and exocyst complexes control exocytosis of MT1-MMP at invadopodia. The Journal of Cell Biology. 2013;**203**(6):1063-1079

[114] Morris HT, Machesky LM. Actin cytoskeletal control during epithelial to mesenchymal transition: Focus on the pancreas and intestinal tract. British Journal of Cancer. 2015; **112**(4):613-620

[115] Duleh SN, Welch MD. WASH and the Arp2/3 complex regulate endosome shape and trafficking. Cytoskeleton (Hoboken). 2010;**67**(3):193-206

[116] Samavarchi-Tehrani P, Golipour, A, David L, Sung HK, Beyer TA, Datti A, et al. Functional genomics reveals a BMP-driven mesenchymal-to-epithelial transition in the initiation of somatic cell reprogramming. Cell Stem Cell. 2010;**7**(1):64-77

[117] Kelly CE, Thymiakou E, Dixon JE, Tanaka S, Godwin J, Episkopou V. Rnf165/Ark2C enhances BMP-Smad signaling to mediate motor axon extension. PLoS Biology. 2013; **11**(4):e1001538

[118] van Cuijk L, van Belle GJ, Turkyilmaz Y, Poulsen SL, Janssens RC, Theil AF, et al. SUMO and ubiquitin-dependent XPC exchange drives nucleotide excision repair. Nature Communications. 2015;**6**:7499

[119] Sharma V, Antonacopoulou AG, Tanaka S, Panoutsopoulos AA, Bravou V, Kalofonos HP, et al. Enhancement of TGF-signaling responses by the E3 ubiquitin ligase Arkadia provides tumor suppression in colorectal cancer. Cancer Research. 2011;**71**(20): 6438-6449

[120] Briones-Orta MA, Levy L, Madsen CD, Das D, Erker Y, Sahai E, et al. Arkadia regulates tumor metastasis by modulation of the TGF- pathway. Cancer Research. 2013;**73**(6): 1800-1810

[121] Chen H, Yang T, Lei Z, Wang L, Yang H, Tong X, et al. RNF111/Arkadia is regulated by DNA methylation and affects TGF-β/Smad signaling associated invasion in NSCLC cells. Lung Cancer. 2015;**90**(1):32-40

[122] Mizutani A, Koinuma D, Seimiya H, Miyazono K. The Arkadia-ESRP2 axis suppresses tumor progression: Analyses in clear-cell renal cell carcinoma. Oncogene. 2016;**35**(27): 3514-3523

[123] Xu A, Sun S. Genomic profiling screens small molecules of metastatic prostate carcinoma. Oncology Letters. 2015 Sep;**10**(3):1402-1408

[124] Ullu E, Tschudi C. Alu sequences are processed 7SL RNA genes. Nature. 1984;**312**(5990): 171-172

[125] Lander ES, Linton LM, Birren B, Nusbaum C, Zody MC, Baldwin J. Initial sequencing and analysis of the human genome. Nature. 2001;**409**(6822):860-921

[126] Hsu K, Chang D, Maraia R. Human signal recognition particle (SRP) Alu-associated protein also binds Alu interspersed repeat sequence RNAs. Journal of Biological Chemistry. 1995;**270**(17):10179-10186

[127] Bennett EA, Keller H, Mills RE, Schmidt S, Moran JV, Weichenrieder O, et al. Active Alu retrotransposons in the human genome. Genome Research. 2008;**18**:1875-1883

[128] Berger A, Ivanova E, Gareau C, Scherrer A, Mazroui R, Strub K. Direct binding of the Alu binding protein dimer SRP9/14 to 40S ribosomal subunits promotes stress granule formation and is regulated by Alu RNA. Nucleic Acids Research. 2014;**42**(17):11203-11217

[129] Gupta N, Badeaux M, Liu Y, Naxerova K, Sgroi D, Munn LL, et al. Stress granule-associated protein G3BP2 regulates breast tumor initiation. Proceedings of the National Academy of Sciences. 2017;**114**(5):1033-1038

[130] Goodier JL, Zhang L, Vetter MR, Kazazian HH. LINE-1 ORF1 protein localizes in stress granules with other RNA-binding proteins, including components of RNA interference RNA-induced silencing complex. Molecular and Cellular Biology. 2007;**27**(18):6469-6483

[131] Liu Y, Timani K, Ou X, Broxmeyer HE, He JJ. C-MYC controlled TIP110 protein expression regulates OCT4 mRNA splicing in human embryonic stem cells. Stem Cells and Development. 2013;**22**(5):689-694

[132] Timani KA, Liu Y, He JJ. Tip110 interacts with YB-1 and regulates each other's function. BMC Molecular Biology. 2013;**14**:14

[133] Koh MY, Darnay BG, Powis G. Hypoxia-associated factor, a novel E3-ubiquitin ligase, binds and ubiquitinates hypoxia-inducible factor 1α, leading to its oxygen-independent degradation. Molecular and Cellular Biology. 2008;**28**(23):7081-7095

[134] Koh MY, Lemos R, Liu X, Powis G. The hypoxia-associated factor switches cells from HIF-1α– To HIF-2α–dependent signaling promoting stem cell characteristics, aggressive tumor growth and invasion. Cancer Research. 2011;**71**(11):4015-4027

[135] Dominguez CL, Floyd DH, Xiao A, Mullins GR, Kefas BA, Xin W, et al. Diacylglycerol kinase alpha is a critical signaling node and novel therapeutic target in glioblastoma and other cancers. Cancer Discovery. 2013;**3**(7):782-797

[136] Purow B. Molecular pathways: Targeting diacylglycerol kinase alpha in cancer. Clinical Cancer Research. 2015;**21**(22):5008-5012

[137] Rainero E, Cianflone C, Porporato PE, Chianale F, Malacarne V, Bettio V, et al. The diacylglycerol kinase α/atypical PKC/β1 integrin pathway in SDF-1α mammary carcinoma invasiveness. PLoS One. 2014;**9**(6):e97144

[138] Müller A, Homey B, Soto H, Ge N, Catron D, Buchanan ME, et al. Involvement of chemokine receptors in breast cancer metastasis. Nature. 2001;**410**(6824):50-56

[139] Korkaya H, Liu S, Wicha MS. Breast cancer stem cells, cytokine networks, and the tumor microenvironment. The Journal of Clinical Investigation. 2011;**121**(10):3804-3809

[140] Teicher BA, Fricker SP. CXCL12 (SDF-1)/CXCR4 pathway in cancer. Clinical Cancer Research. 2010;**16**(11):2927-2931

[141] Rainero E, Caswell PT, Muller PA, Grindlay J, McCaffrey MW, Zhang Q, et al. Diacylglycerol kinase α controls RCP-dependent integrin trafficking to promote invasive migration. The Journal of Cell Biology. 2012;**196**(2):277-295

[142] Offermanns S, Simon MI. Gα15 and Gα16 couple a wide variety of receptors to phospholipase C. Journal of Biological Chemistry. 1995;**270**(25):15175-15180

[143] Strasser RH, Benovic JL, Caron MG, Lefkowitz RJ. Beta-agonist- and prostaglandin E1-induced translocation of the beta-adrenergic receptor kinase: Evidence that the kinase may act on multiple adenylate cyclase-coupled receptors. Proceedings of the National Academy of Sciences. 1986;**83**(17):6362-6366

[144] Benovic JL, Strasser RH, Caron MG, Lefkowitz RJ. Beta-adrenergic receptor kinase: Identification of a novel protein kinase that phosphorylates the agonist-occupied form of the receptor. Proceedings of the National Academy of Sciences. 1986;**83**(9):2797-2801

[145] Attramadal H, Arriza JL, Aoki C, Dawson TM, Codina J, Kwatra MM, et al. Beta-arrestin2, a novel member of the arrestin/beta-arrestin gene family. Journal of Biological Chemistry. 1992;**267**(25):17882-17890

[146] Lohse M, Benovic J, Codina J, Caron M, Lefkowitz R. Beta-arrestin: A protein that regulates beta-adrenergic receptor function. Science. 1990;**248**(4962):1547-1550

[147] Giannone F, Malpeli G, Lisi V, Grasso S, Shukla P, Ramarli D, et al. The puzzling uniqueness of the heterotrimeric G15 protein and its potential beyond hematopoiesis. Journal of Molecular Endocrinology. 2010;**44**(5):259-269

[148] Philip PA, Mooney M, Jaffe D, Eckhardt G, Moore M, Meropol N, et al. Consensus report of the National Cancer Institute clinical trials planning meeting on pancreas cancer treatment. Journal of Clinical Oncology. 2009;**27**(33):5660-5569

[149] Waldron RT, Innamorati G, Torres-Marquez ME, Sinnett-Smith J, Rozengurt E. Differential PKC-dependent and -independent PKD activation by G protein α subunits of the Gq family: Selective stimulation of PKD Ser748 autophosphorylation by Gαq. Cellular Signalling. 2012;**24**(4):914-921

[150] Harikumar KB, Kunnumakkara AB, Ochi N, Tong Z, Deorukhkar A, Sung B, et al. A novel small molecule inhibitor of protein kinase D blocks pancreatic cancer growth in vitro and in vivo. Molecular Cancer Therapeutics. 2010;**9**(5):1136-1146

[151] Singh S, Srivastava SK, Bhardwaj A, Owen LB, Singh AP. CXCL12–CXCR4 signalling axis confers gemcitabine resistance to pancreatic cancer cells: A novel target for therapy. British Journal of Cancer. 2010;**103**(11):1671-1679

[152] Kayali AG, Van Gunst K, Campbell IL, Stotland A, Kritzik M, Liu G, et al. The stromal cell–derived factor-1α/CXCR4 ligand–receptor axis is critical for progenitor survival and migration in the pancreas. The Journal of Cell Biology. 2003;**163**(4):859-869

[153] Contos JJA, Ye X, Sah VP, Chun J. Tandem genomic arrangement of a G protein (Gna15) and G protein-coupled receptor (s1p4/lpC1/Edg6) gene. FEBS Letters. 2002;**531**(1):99-102

[154] Inniss K, Moore H. Mediation of apoptosis and proliferation of human embryonic stem cells by Sphingosine-1-phosphate. Stem Cells and Development. 2006;**15**(6):789-796

[155] Serafimidis I, Heximer S, Beis D, Gavalas A. G protein-coupled receptor signaling and Sphingosine-1-phosphate play a Phylogenetically conserved role in endocrine pancreas morphogenesis. Molecular and Cellular Biology. 2011;**31**(22):4442-4453

[156] Morris JP, Wang SC, Hebrok M. KRAS, hedgehog, Wnt and the twisted developmental biology of pancreatic ductal adenocarcinoma. Nature Reviews Cancer. 2010;**10**(10):683-695

[157] Wray J, Hartmann C. WNTing embryonic stem cells. Trends in Cell Biology. 2012; **22**(3):159-168

[158] Masdeu C, Faure H, Coulombe J, Schoenfelder A, Mann A, Brabet I, et al. Identification and characterization of hedgehog modulator properties after functional coupling of smoothened to G15. Biochemical and Biophysical Research Communications. 2006; **349**(2):471-479

[159] Morton JP, Mongeau ME, Klimstra DS, Morris JP, Lee YC, Kawaguchi Y, et al. Sonic hedgehog acts at multiple stages during pancreatic tumorigenesis. Proceedings of the National Academy of Sciences. 2007;**104**(12):5103-5108

[160] Giovinazzo F, Malpeli G, Zanini S, Parenti M, Piemonti L, Colombatti M, et al. Ectopic expression of the heterotrimeric G15 protein in pancreatic carcinoma and its potential in cancer signal transduction. Cellular Signalling. 2013;**25**(3):651-659

[161] de Jonge HJM, Woolthuis CM, Vos AZ, Mulder A, van den Berg E, Kluin PM, et al. Gene expression profiling in the leukemic stem cell-enriched CD34+ fraction identifies target genes that predict prognosis in normal karyotype AML. Leukemia. 2011;**25**(12):1825-1833

[162] Avasarala S, Bikkavilli RK, Van Scoyk M, Zhang W, Lapite A, Hostetter L, et al. Heterotrimeric G-protein, Gα16, is a critical downstream effector of non-canonical Wnt signaling and a potent inhibitor of transformed cell growth in non-small cell lung cancer. PLoS One. 2013;**8**(10):e76895

[163] Hu S, Li J, Xu F, Mei S, Le Duff Y, Yin L, et al. SAMHD1 inhibits LINE-1 retrotransposition by promoting stress granule formation. PLOS Genetics. 2015;**11**(7):e1005367

[164] Irving A, Wang D, Vasilevski O, Latchoumanin O, Kozer N, Clayton A, et al. Regulation of actin dynamics by protein kinase R control of gelsolin enforces basal innate immune defense. Immunity. 2012;**36**(5):795-806

[165] DiDonato JA, Mercurio F, Karin M. NF-κB and the link between inflammation and cancer. Immunological Reviews. 2012;**246**(1):379-400

[166] Tirinato L, Pagliari F, Limongi T, Marini M, Falqui A, Seco J, et al. An overview of lipid droplets in cancer and cancer stem cells. Stem Cells International. 2017;**2017**(2017): 1656053

[167] Pelletier J, Graff J, Ruggero D, Sonenberg N. Targeting the eIF4F translation initiation complex: A critical nexus for cancer development. Cancer Research. 2015;**75**(2):250-263

[168] Mitchell SF, Lorsch JR. Should I stay or should I go? Eukaryotic translation initiation factors 1 and 1A control start codon recognition. Journal of Biological Chemistry. 2008;**283**(41):27345-27349

[169] Joseph P, Lei Y-X, Ong T-m. Up-regulation of expression of translation factors – A novel molecular mechanism for cadmium carcinogenesis. Molecular and Cellular Biochemistry. 2004;**255**(1):93-101

[170] Gonen H, Smith CE, Siegel NR, Kahana C, Merrick WC, Chakraburtty K, et al. Protein synthesis elongation factor EF-1 alpha is essential for ubiquitin-dependent degradation of certain N alpha-acetylated proteins and may be substituted for by the bacterial elongation factor EF-Tu. Proceedings of the National Academy of Sciences of the United States of America. 1994;**91**(16):7648-7652

[171] Hotokezaka Y, Többen U, Hotokezaka H, van Leyen K, Beatrix B, Smith DH, et al. Interaction of the eukaryotic elongation factor 1A with newly synthesized polypeptides. Journal of Biological Chemistry. 2002;**277**(21):18545-18551

[172] Ruest L-B, Marcotte R, Wang E. Peptide elongation factor eEF1A-2/S1 expression in cultured differentiated myotubes and its protective effect against Caspase-3-mediated apoptosis. Journal of Biological Chemistry. 2002;**277**(7):5418-5425

[173] Chang R, Wang E. Mouse translation elongation factor eEF1A-2 interacts with Prdx-I to protect cells against apoptotic death induced by oxidative stress. Journal of Cellular Biochemistry. 2007;**100**(2):267-278

[174] Murthi A, Shaheen HH, Huang H-Y, Preston MA, Lai T-P, Phizicky EM, et al. Regulation of tRNA bidirectional nuclear-cytoplasmic trafficking in *Saccharomyces cerevisiae*. Molecular Biology of the Cell. 2010;**21**(4):639-649

[175] Shamovsky I, Ivannikov M, Kandel ES, Gershon D, Nudler E. RNA-mediated response to heat shock in mammalian cells. Nature. 2006;**440**(7083):556-560

[176] Kim S, Coulombe PA. Emerging role for the cytoskeleton as an organizer and regulator of translation. Nature Reviews. Molecular Cell Biology. 2010;**11**(1):75-81

[177] Morrissey C, Schwefel D, Ennis-Adeniran V, Taylor IA, Crow YJ, Webb M. The eukaryotic elongation factor eEF1A1 interacts with SAMHD1. Biochemical Journal. 2015;**466**(1): 69-76

[178] Malta-Vacas J, Aires C, Costa P, Conde AR, Ramos S, Martins AP, et al. Differential expression of the eukaryotic release factor 3 (eRF3/GSPT1) according to gastric cancer histological types. Journal of Clinical Pathology. 2005;**58**(6):621-625

[179] Schoenberg DR, Maquat LE. Regulation of cytoplasmic mRNA decay. Nature Reviews. Genetics 2012;**13**(4):246-259

[180] Jerbi S, Jolles B, Bouceba T, Jean-Jean O. Studies on human eRF3-PABP interaction reveal the influence of eRF3a N-terminal glycin repeat on eRF3-PABP binding affinity and the lower affinity of eRF3a 12-GGC allele involved in cancer susceptibility. RNA Biology. 2016;**13**(3):306-315

[181] Mao JH, Kim IJ, Wu D, Climent J, Kang HC, DelRosario R, et al. FBXW7 targets mTOR for degradation and cooperates with PTEN in tumor suppression. Science. 2008;**321**(5895): 1499-1502

[182] Zhao Y, Sun Y. Targeting the mTOR-DEPTOR pathway by CRL E3 ubiquitin ligases: Therapeutic application. Neoplasia. 2012;**14**(5):360-367

[183] Öberg C, Li J, Pauley A, Wolf E, Gurney M, Lendahl U. The Notch intracellular domain is ubiquitinated and negatively regulated by the mammalian Sel-10 homolog. Journal of Biological Chemistry. 2001;**276**(38):35847-35853

[184] Gupta-Rossi N, Le Bail O, Gonen H, Brou C, Logeat F, Six E, et al. Functional interaction between SEL-10, an F-box protein, and the nuclear form of activated Notch1 receptor. Journal of Biological Chemistry. 2001;**276**(37):34371-34378

[185] Haupt Y, Maya R, Kazaz A, Oren M. Mdm2 promotes the rapid degradation of p53. Nature. 1997;**387**(6630):296-299

[186] Thomas L, Tansey W. Proteolytic Control of the Oncoprotein Transcription Factor Myc. Advances in Cancer Research. 2011;**110**:77-106

[187] Choi SH, Wright JB, Gerber SA, Cole MD. Myc protein is stabilized by suppression of a novel E3 ligase complex in cancer cells. Genes & Development. 2010;**24**(12):1236-1241

[188] Popov N, Schülein C, Jaenicke LA, Eilers M. Ubiquitylation of the amino terminus of Myc by SCFβ-TrCP antagonizes SCFFbw7-mediated turnover. Nature Cell Biology. 2010;**12**(10):973-981

[189] Bisikirska BC, Adam SJ, Alvarez MJ, Rajbhandari P, Cox R, Lefebvre C, et al. STK38 is a critical upstream regulator of MYC's oncogenic activity in human B-cell lymphoma. Oncogene. 2013;**32**(45):5283-5291

[190] Song DH, Sussman DJ, Seldin DC. Endogenous protein kinase CK2 participates in Wnt signaling in mammary epithelial cells. Journal of Biological Chemistry. 2000;**275**(31): 23790-23797

[191] Dominguez I, Sonenshein GE, Seldin DC. CK2 and its role in Wnt and NF-κB signaling: Linking development and cancer. Cellular and Molecular Life Sciences. 2009;**66**(11-12): 1850-1857

[192] Vousden KH, Prives C. Blinded by the light: The growing complexity of p53. Cell. 2009;**137**(3):413-431

[193] Fang S, Jensen JP, Ludwig RL, Vousden KH, Weissman AM. Mdm2 is a RING finger-dependent ubiquitin protein ligase for itself and p53. Journal of Biological Chemistry. 2000;**275**(12):8945-8951

[194] Momand J, Zambetti GP, Olson DC, George D, Levine AJ. The mdm-2 oncogene product forms a complex with the p53 protein and inhibits p53-mediated transactivation. Cell. 1992;**69**(7):1237-1245

[195] Jackson MW, Berberich SJ. MdmX protects p53 from Mdm2-mediated degradation. Molecular and Cellular Biology. 2000;**20**(3):1001-1007

[196] Wang X, Jiang X. Mdm2 and MdmX partner to regulate p53. FEBS Letters. 2012;**586**(10): 1390-1396

[197] Gilmore TD. Introduction to NF-κB: Players, pathways, perspectives. Oncogene. 2006; **25**(51):6680-6684

[198] Karin M. Nuclear factor-κB in cancer development and progression. Nature. 2006; **441**(7092):431-436

[199] Ben-Neriah Y, Karin M. Inflammation meets cancer, with NF-κB as the matchmaker. Nature Immunology. 2011;**12**(8):715-723

[200] Harhaj EW, Dixit VM. Deubiquitinases in the regulation of NF-κB signaling. Cell Research. 2011;**21**(1):22-39

[201] Arabi A, Ullah K, Branca RM, Johansson J, Bandarra D, Haneklaus M, et al. Proteomic screen reveals Fbw7 as a modulator of the NF-κB pathway. Nature Communications. 2012;**3**:976

[202] Busino L, Millman SE, Scotto L, Kyratsous CA, Basrur V, O'Connor O, et al. Fbxw7α- and GSK3-mediated degradation of p100 is a pro-survival mechanism in multiple myeloma. Nature Cell Biology. 2012;**14**(4):375-385

[203] Fukushima H, Matsumoto A, Inuzuka H, Zhai B, Lau AW, Wan L, et al. SCF(Fbw7) modulates the NFκB signaling pathway by targeting NFκB2 for ubiquitination and destruction. Cell Reports. 2012;**1**(5):434-443

[204] Wisdom R, Lee W. The protein-coding region of c-myc mRNA contains a sequence that specifies rapid mRNA turnover and induction by protein synthesis inhibitors. Genes & Development. 1991;**5**(2):232-243

[205] Weidensdorfer D, Stohr N, Baude A, Lederer M, Kohn M, Schierhorn A, et al. Control of c-myc mRNA stability by IGF2BP1-associated cytoplasmic RNPs. Ribonucleic Acid. 2009;**15**(1):104-115

[206] Lederer M, Bley N, Schleifer C, Hüttelmaier S. The role of the oncofetal IGF2 mRNA-binding protein 3 (IGF2BP3) in cancer. Seminars in Cancer Biology. 2014;**29**:3-12

[207] Ohms S, Rangasamy D. Silencing of LINE-1 retrotransposons contributes to variation in small noncoding RNA expression in human cancer cells. Oncotarget. 2014;**5**(12):4103-4117

Chapter 14

Viral Modulation of Host Translation and Implications for Vaccine Development

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Additional information is available at the end of the chapter

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Abstract

Translation of mRNAs into protein is an essential mechanism of regulating gene expression—and a step exploited by viruses for their own propagation. In this article, we review mechanisms that govern translation and provide an overview of the translation machinery, discuss some of the components involved in this process, and discuss how viruses modulate host translational controls and implications in vaccine design.

Keywords: translation, transfer RNAs, transfer RNA fragments, tRNAs, viruses

1. Introduction

The central dogma of molecular biology is that data are organized by DNA, mRNA, and protein and that this information is translated during transcription leading to the execution of cellular programs via proteins, which are fundamental to the functioning of a cell. A vast body of literature has added to our understanding of the molecular interplay during translation; however, it is far from comprehensive as (1) biological systems are complex where there is little correlation between the sizes of an organism, its genome size, and the number of protein coding/noncoding genes; (2) biological systems respond acutely to changes in the environment or upon infection with a pathogen; (3) all biological systems are in a state of continuous evolution as they learn from new stimuli and adapt accordingly; (4) posttranslational modifications are normally required for assembly into molecular complexes/proteins to elicit a function; and (5) many proteins are multifunctional across different pathways. We begin this article with a succinct overview of the main components involved in protein translation and the translation process itself and then consider the multiple roles transfer RNAs (tRNAs) have during translation in virus-infected cells and how viruses modify tRNA expression and

function. We conclude with a discussion of how understanding the mechanisms by which viruses modulate host translation pathway can aid in an effective vaccine design.

Protein synthesis is a multistep process involving various error-checking mechanisms. For example, genes are transcribed in the nucleus, and mature messenger RNAs (mRNAs) are exported into the cytoplasm as ribonucleoprotein particles, and immediately they are associated with ribosomes (either free in the cytoplasm or bound to endoplasmic reticulum) for initiation of translation. In eukaryotes, ribosomes consist of two subunits, a small 40S (Svedberg) and a large 60S, which together form 80S macromolecular ribonucleoprotein complexes of ribosomal RNA and ribosomal proteins [1]. The 40S subunit scans the mRNA until it recognizes the first codon (triplet AUG) at which point the first amino acid (a.a) methionine (Met) which is bound to its cognate transfer RNA (tRNA) with the UAC anticodon enters and binds to the AUG codon via sequence complementarity. The 60S subunit binds to this complex forming two distinct pockets, the peptidyl (P) site containing the Met-tRNA and an amino-acyl (A) site where the next aa-tRNA comes in. The chain initiator Met from the P site is transferred to the a.a. at the A site with the formation of a peptide bond, and the empty tRNA at A site is released. The 80S ribosome scans the next codon and the dipeptide-tRNA complex moves to the P site, the next aa-tRNA is brought in and peptide chain elongation continues until the ribosome reads the special codon (stop codon) that signals chain ending. When stop codons are read, the peptide chain from the tRNA and the ribosome is released [2]. Typically, each mRNA is processed by multiple ribosomes simultaneously as polysome complexes [3]. Native peptides so formed may need substantial posttranslational modifications before they are transported to their cellular niche and become functional. Mistranslated peptides are degraded by a variety of proteolytic mechanisms and components are recycled. Some mRNAs are long-lived in the host cytoplasm, while others are rapidly degraded following protein synthesis [4].

2. Principal components of translation

2.1. Ribosomes

Both prokaryotic and eukaryotic ribosomes are macromolecular complexes consisting of ribosomal RNAs (rRNAs) and ribosomal proteins. Ribosomes are separated for structural and related studies using isopycnic ultracentrifugation [5] where eukaryotic ribosomes typically pellet at 80 Svedberg units of sedimentation and are referred to as 80S ribosomes though they consist of the smaller 40S and the larger 60S subunit [6–11]. The complete ribosome is 4.3 MDa where the larger 60S subunit contains 28S rRNA, 5S rRNA, 5.8S rRNA, and 47 distinct ribosomal proteins, while the smaller 40S contains a single 18S rRNA and 33 distinct ribosomal proteins [12]. Mammalian ribosomes contain all the sites necessary for interaction with the components of the translation machinery such as eukaryotic initiation factor 1 [13]. Structural studies have identified conserved cores in mammalian ribosomes as well as proteins that are unique to the human ribosome [14]. The main features of the ribosome involved in translation include the amino-acyl (A) site where aa-tRNAs bind, the P site where peptide bond formation occurs, and the E site where uncharged tRNAs exit the ribosome (**Figure 1**). Ribosomal RNAs are also posttranscriptionally modified at multiple positions and these modifications

are essential for proper folding and function [15, 16]. Typical rRNA modifications are catalyzed by small nucleolar RNAs (snoRNAs) and include 2′-O ribose methylation and pseudouridylation, which is a very abundant posttranscriptionally modified nucleotide in various stable RNAs of all organisms. These specific bases in the rRNA stabilize rRNA structure and function. Ribose modifications are guided by C/D box snoRNAs, while pseudouridylation modifications are regulated by H/ACA box snoRNAs [17–24].

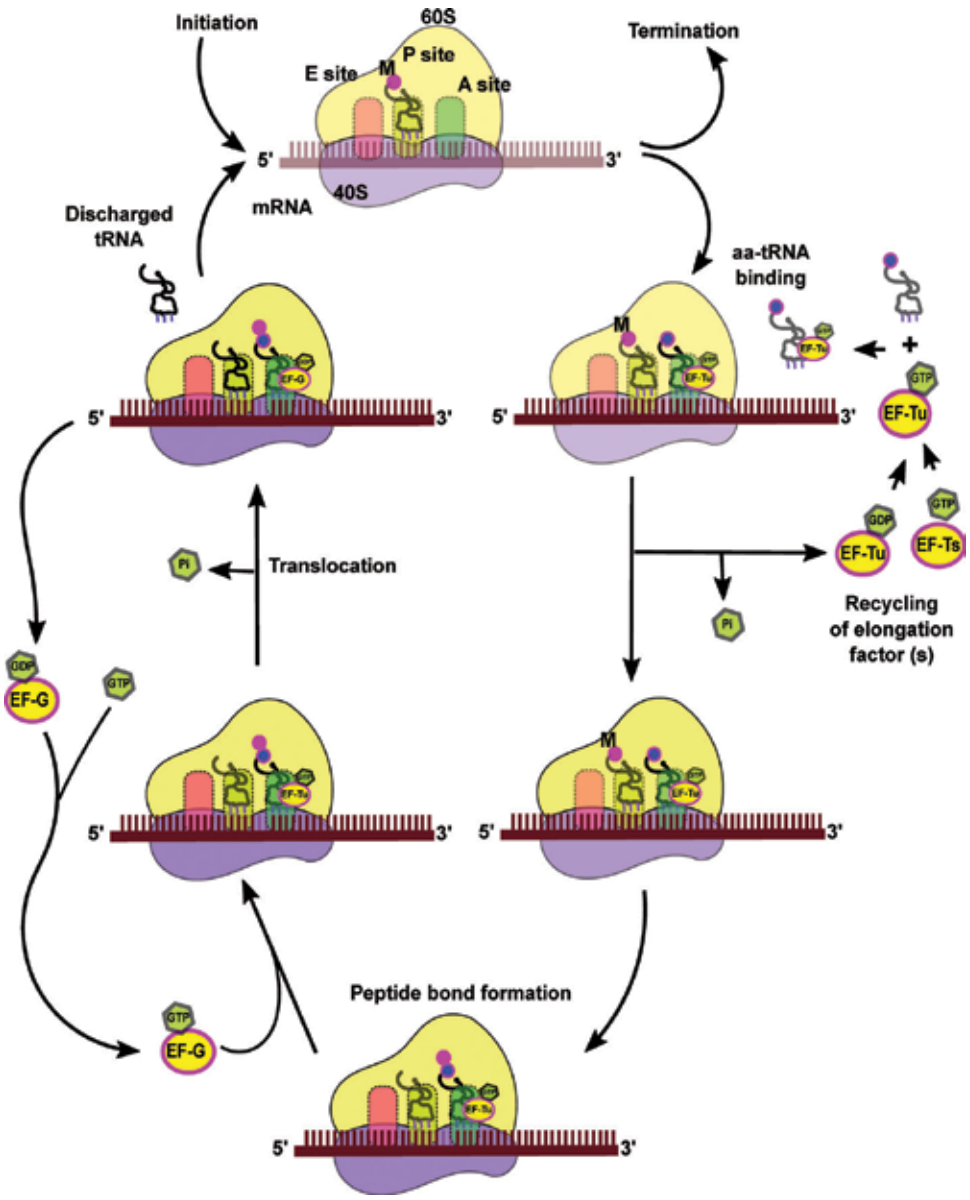

Figure 1. Overview of host translation.

2.2. Messenger RNA (mRNA)

The human genome is 3.4 billion base pair long and encodes ~32,000 protein-coding genes with a median gene size of ~1 kb containing 7 exons [25]. Protein-coding genes are transcribed by RNA polymerase II, and primary transcripts are spliced to remove introns to generate mature mRNAs, which are polyadenylated by a poly A polymerase at the 3′ end while the 5′ end carries a specific 7-methyl guanosine (m7G) modification that stimulates canonical translation initiation [26]. Mature mRNAs associate with several RNA-binding proteins and exit the nucleus as ribonucleoprotein complexes, which then associate with ribosomes to initiate translation. Multiple factors such as number of transcripts, half-life of the mRNA, etc. determine the level to which a particular mRNA is translated. Housekeeping mRNAs have long half-lives, while transcription factors and inducible genes constitute the bulk of mRNAs with short half-lives in concordance with their transient roles.

2.3. Transfer RNAs

The human genome encodes 610 tRNA genes [25] that are interspersed throughout the nuclear genome and can be classified into 51 anticodon families targeting the 64 codons. Significant intraspecies [26] and interspecies [25] copy number variation has been previously demonstrated and may extend to the tissue or cellular level. Approximately 50% of the nuclear tRNA genes are transcribed. The standard 20 a.a are decoded by 597 different tRNAs, and 3 tRNAs encode selenocysteine, where incorporation of selenocysteine into the growing peptide chain occurs by a unique suppressor tRNA and a stop codon. Moreover, 2 tRNAs have potential suppressor function, and 6 tRNAs have unknown a.a. that they carry. Additionally, the mitochondrial genome encodes 22 mitochondrial tRNAs (mtRNAs) [27]. Nuclear tRNAs are encoded by intronic or intergenic tRNA genes that are transcribed by RNA polymerase III in conjunction with transcription factors TBP, BDP1, BRF1, TFIIIB, and TFIIIC in a 3D spatially distinct region in the nucleus termed the nucleolus.

The prototypical tRNA genes consist of a 5′-UTR and signature A and B box motif [28, 29], followed downstream by a stretch of U residues that signal transcript termination. tRNA genes can be located within introns of protein coding genes where they are cotranscribed with their encoding genes. For all intergenic tRNAs, transcription is a concerted process initiating with binding of transcription factor TFIIIC to the A and B box region, recruiting TFIIIB upstream, and culminating in recruitment of RNA Pol III. Primary transcript is next processed by RNAse P- and RNAse Z-mediated removal of the 5′ leader and the 3′ trailer sequence, where tRNA nucleotidyl transferase mediates addition of the 3′-CCA trinucleotides [30–32]. Several posttranscriptional modifications on the tRNA are followed by coupling of the tRNA with the cognate a.a., a process mediated by aminoacyl tRNA synthetases. The process of tRNA charging involves recognition of several modifications on the tRNA body especially N73 near the CCA motif at the 3′ end [33]. Aberrant primary tRNA transcripts are recycled through a nonsense-mediated decay pathway involving degradation of their 3′ ends. Additionally, mature tRNAs lacking modifications are degraded via a 5′ exonucleolytic cleavage. Eukaryotic cells encode for 20 distinct tRNA synthetases for each of the 20 standard a.a. It remains unclear if amino acylation is restricted to the nucleus or also occurs in the cytoplasm. Mitochondrial tRNAs (mtRNAs) that are encoded on the circular mitochondrial genome between the rRNA and mRNA genes [27] are transcribed by

the mitochondrial RNA polymerase in conjunction with transcription factors Tfam and mtTFB from the bidirectional promoters on the circular mitochondrial genome.

Both cytosolic and mtRNAs are posttranscriptionally modified [34], though nuclear tRNAs [35] can have additional modifications presumably due to the mechanisms of action for nuclear tRNAs and the bacterial origin of mitochondrial tRNAs [27, 36, 37]. These modifications have at least three important functions: (1) modifications affecting the anticodon loop, which alter translation efficiency; (2) modifications to the tRNA body affecting tRNA secondary structure; and (3) modifications at other positions that determine aminoacyl transferase recognition and amino acid loading on the CCA motif [38]. More than 100 diverse modifications have been reported for nuclear tRNAs, while mtRNAs exhibit about 16 conserved posttranscriptional modified nucleosides [39]. The nature and role of tRNA modifications are beyond the scope of this review, but they have an essential role in tRNA function both canonical and noncanonical functions. Specifically, modifications in the anticodon loop affect tRNA translational function and increase translational accuracy by preventing translational frameshifting. Posttranscriptional modifications to tRNAs considerably increase tRNA complexity since the presence/absence of certain modifications can affect tRNA function, and it is estimated that the major tRNA modifications can lead to 8192 possible different species of tRNAs for each tRNA. Most mature nuclear tRNA molecules are ~76–93 nts, while mtRNAs are 57–73 nts. Nuclear tRNAs exhibit an evolutionarily conserved cloverleaf secondary structure across pro- and eukaryotic kingdoms consisting of four arms designated the acceptor arm, dihydrouridine arm, anticodon stem loop, and the TψC arm (ψ representing pseudouridine). The 3′ end of all tRNA molecules terminates in a CCA sequence, the 2′ or 3′-OH of the terminal adenosine being the site of aminoacyl-tRNA addition. In 3-D, tRNA molecules assume an L-shaped structure where the TψC arm stacks on the acceptor stem to form a 12 bp acceptor-TψC minihelix flanking the anticoding stem loop. mtRNAs can be structurally classified into three classes [40]: (1) class I mtRNAs (e.g., $tRNA^{Ser(UCN)}$), which contain a short and an extended anticodon stem [40], (2) class II mtRNAs lack the canonical D- and T-loop interaction and have variable lengths and are stabilized via an interaction between the D-stem and the extra loop [41, 42], and (3) class III mtRNAs (e.g., $mtRNA^{Ser(AGY)}$) lack the D-loop and do not exhibit the classical cloverleaf structure [43, 44].

2.4. Wobble-hypothesis and associated implications on translation

The specificity of the codon: anticodon interaction is crucial for incorporation of the correct amino acid into the growing peptide chain and determines the composition of the proteome [45–47], rate of a.a misincorporation [48–52], and ultimately protein folding [53, 54]. However, the standard genetic code is degenerate (i.e., more than one codon can specify the same amino acid). For example, six different codons can specify the a.a. lysine (K); $tRNA^{Lys}$ is thus able to bind to six different codons for K in any given mRNA. This is because the ribosome can determine if the interactions between the first two bases of the anticodon on the tRNA and the corresponding complements on the mRNA are of Watson-Crick-type, but cannot distinguish if the third base interaction is perfectly complementary. Nuclear magnetic resonance (NMR) studies with anticodon stem loops of the smaller 40S unit of *E. coli* $tRNA^{Lys}$ have clearly shown three modifications in this region, a N6-threonylcarbamoyladenosine (t^6A) modification at position 37, a 5-methylaminomethyl-2-thiouridine (S,mnm^5s^2U) modification at position 34, and a pseudouridine at position 39, which

force the dynamic loop structure to assume an open U-turn structure that perfectly fits the ribosomal decoding center [55]. Ribosomal profiling studies have shown that wobble positions slow the rates of protein translation [56]. Controlling the rate of translation via wobble base pairing has important implications: (1) utilizing infrequent tRNAs that are expressed only under particular stimuli, (2) allowing for stable and correct folding of the protein, and (3) allowing information for regulation of translation rate to be hard-coded in the mRNA [57, 58].

Recent studies have shown that in cellular organelles that do not encode all the tRNAs necessary to read the genetic code, a single tRNA species containing a U in the wobble position in the anticodon can read fourfold degenerate codon, a phenomenon described as superwobbling [58]. The superwobbling allows codons to be decoded not only by tRNAs containing a perfectly complementary or wobble 3rd base but also by tRNAs that employ superwobbling allowing for smaller genomes [58, 59].

2.5. Alternative functions of tRNAs

In addition to their normal function in protein synthesis, tRNAs acutely respond to cellular and environmental stresses. Cells with different proteomic profiles also exhibit diversity of tRNA iso-acceptor types, i.e., tRNAs with different anticodons but same a.a. tRNA expression, post-transcriptional modifications, and abundance (both copy numbers and expression) typically reflect the cellular state of tRNAs that code for the most abundant codons and are found in high copy numbers. tRNA expression levels in a particular cell type reflect the codon bias of that cell and indicate the proliferation status of a cell type, a feature that supports the proposition that tRNA gene expression is modulated in response to the host cell needs. The ribosomal tempo is thus regulated by abundance and diversity of the tRNA pool available during translation.

tRNAs are cleaved during cellular stress [60] and in immune response to infection generating specific tRNA fragments (tRFs) that contain the 5′ (5′tRFs) or the 3′ (3′tRFs) ends of the parent tRNA molecule (**Figure 2**). The most known tRFs are nuclear in origin though a few tRFs have been shown to originate from plastid genomes [61] or mitochondria [62]. tRFs have also been reported to originate from the pre-tRNA moiety instead of the mature tRNA molecule, and these are labeled as 3′-U tRFs since they match the 3′-trailer region of the precursor tRNA [63–65]. Many tRFs that result from cellular stress conditions consist of two 30–40 nt long fragments split across the anticodon loop and are referred to as tRNA-derived stress-induced RNAs (tiRNAs) [66–68]. tiRNAs reflect universal hallmarks of cellular stress across all kingdoms of life [69–75].

The level of parent tRNA molecules is maintained during tRF generation suggesting that tRF formation may be a mechanism to regulate translation via inhibition of initiation [76, 77]. Among tRFs, 5′-tRFs primarily function as signaling intermediates [78] and reduce translation [79] via induction of stress granule formation [80]. The complete biosynthesis of tRFs involves either degradation of pre-tRNA molecules via the TRAMP pathway in the nucleus [81–85] or via cytosolic degradation of mature tRNAs via the rapid tRNA decay (RTD) pathway. The TRAMP pathway consists of a polyadenylase Trf4 (topoisomerase 1-related 4), a RNA helicase Mtr4p (mRNA transport regulator 4 protein), and Air2 (arginine methyltransferase-interacting RING finger protein 2), which interacts with Rrp6, a 3′ exoribonuclease of the nuclear exosome. The RTD pathway involves methionine-requiring protein 22 (Met22) [86] and cytosolic 5′-3′ exonucleases such as ribonucleic acid trafficking protein 1 (Rat 1) [86], exoribonuclease 1 (Xrn1) [86, 87], endonucleases ELAC2 [65], Dicer [64, 88, 89], and angiogenin (ANG) [71]. Though the exact

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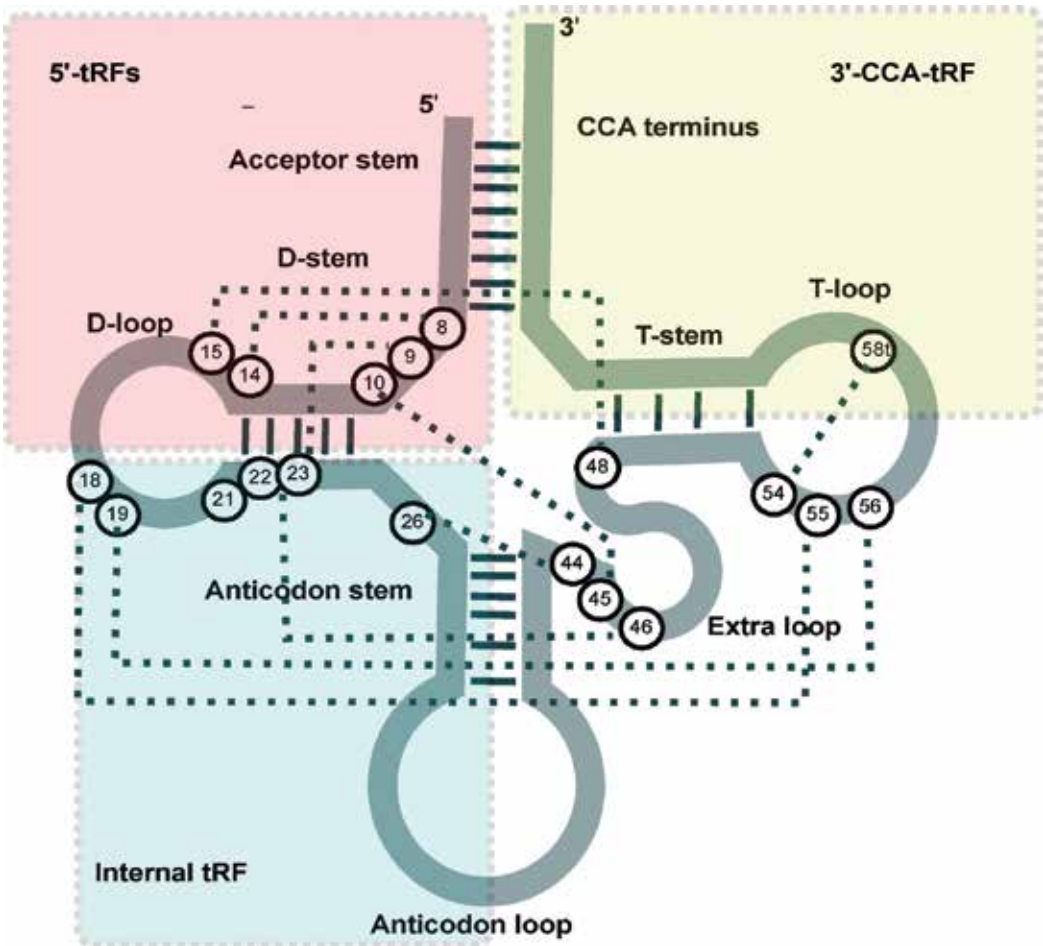

Figure 2. Transfer RNA structure and biogenesis of transfer RNA fragments (tRFs). The cloverleaf model of a canonical nuclear tRNA is shown. Bold lines indicate Watson-Crick base pairing in the tRNA stems while dotted lines indicate base pairing in the tertiary structure of the tRNA. Shaded areas indicate regions from where the 5′/3′ or internal tRFs are produced.

function of tRFs is not known, evidence indicates that tRFs can behave as siRNAs by degrading transcripts [90] and can regulate ribosomal loading and protein chain elongation [91]. Mechanisms of how tRFs are produced are most likely stimulus and species specific. Similarly, the functional roles of tRFs are yet to be elucidated (reviewed previously [92]). In yeast, tRFs are associated with starvation-induced vacuoles where they are degraded to provide phosphate and nitrogen [93]. tRFs also accumulate in plants during conditions of phosphate paucity [70]. Cleavage of the 3′ end CCA by angiogenin has been shown to reduce rate of protein translation [94], as well as initiation by competing with the eukaryotic initiation factor eIF4F.

2.6. How do tRNAs affect vaccine production and potentially efficacy?

Among the variety of stimuli host cells respond to, intracellular pathogens are a special case as many pathogens regulate host cell translation themselves. Viruses in particular regulate multiple facets of the host translation process since inhibition of host protein synthesis (1) makes

available crucial resources for translation of viral proteins, (2) reduces intracellular antiviral responses, and (3) reduces intercellular signaling helping viral spread in neighboring tissue. Immunization of a host with viral vaccine antigen can prevent viral modulation of the host translation machinery. Most viral antigens are considered "foreign" by the host cell—a feature tied to their codon usage that differs from the host.

The standard vertebrate genetic code contains 64 codons (61 coding for an amino acid and 3 stop codons); however, most eukaryotic proteins contain 20 standard amino acids, and thus, more than one codon can encode the same amino acid. Codons that specify the same amino acid are referred to as synonymous codons. Those that do not specify the same amino acid are termed nonsynonymous codons. However, most biological systems have evolved to preferentially utilize one or few codons for each amino acid during translation, a feature referred to as codon usage bias [95–98]. Thus, in an infected cell, viral and host proteins may be translated by very different collections of codons. Accumulating data show that many viruses evolve to adapt their codon usage to the host [99], and this can be specific for each virus or viral gene to regulate the tempo and pattern of expression. This raises a challenge in commercial vaccine production because rare codon usage can lead to low yield of the immunogen and increase production costs [100]. Secondly, while most host protein synthesis begins with an initiator codon (AUG) coding for methionine, viral genomes utilize multiple mechanisms of noncanonical translation such as internal ribosomal entry sites (IRES), ribosome shunting, leaky scanning of the viral open reading frame, non-AUG initiation, and reinitiation from AUG with frame shifts; read through translation and alternative stop; and carry on translation [101]. A detailed description of this is out of the scope of this examination, and it is important to understand how these mechanisms can be used to improve vaccine yield and/or efficacy.

A commonly employed strategy to improve vaccine yield is to optimize the codon usage pattern to overcome bias for the antigen in question [57, 102]. Codon usage bias is calculated by counting the number of time a particular codon is observed in a gene or set of genes. This can be extended to calculate the relative synonymous codon usage, which reflects the abundance of a particular codon relative to all other codons in the absence of a codon usage bias. By tabulating the most frequently used codons in the host genome and comparing to those used in the viral genome, it is possible to discern codon usage bias (CUB) for the virus. Immunogens in vaccines can then be expressed either in cells that overexpress the rare tRNA used by the viral protein to increase protein yield or engineered through molecular tools (site-directed mutagenesis, cloning, etc.) to utilize the most common host codons. This codon optimization strategy has been employed for developing a variety of vaccines [57, 103–140]. Codon optimization has been reported to reduce vaccine efficacy by increasing antigenicity and changing conformation of the native immunogen [141–145]. Codon optimization as a way to increase immunogen (vaccine) production suffers from the assumptions that: (1) rare codons limit rate of translation, (2) synonymous codons have redundant function, (3) replacing rare codons with high-frequency codons improves protein yield, and (4) sites of posttranslational modifications are preserved upon codon optimization. However, multiple studies have shown that these are not necessarily true and multiple other factors such as mRNA secondary structure [146] and posttranscriptional modifications on mRNAs [147] can alter rates of translation.

Conversely, incorporation of rare (nonpreferred) codons in viral genes used for antigen production can lead to decreased production of viral antigens and lead to attenuation. This codon deoptimization strategy has also been employed for a variety of viral vaccine candidates [148–163]. These studies have clearly shown attenuation of viral replication and improved immune responses. Further, it was recently shown that deoptimized live attenuated viral vaccines in case of respiratory syncytial virus (RSV) remain genetically stable if these changes in the genome are distributed throughout and not restricted to one viral gene or antigen [149]. Codon deoptimization strategies are still being explored for viral vaccine design; however, like codon optimization strategies, the rules for design of a safe and effective candidate are only partly recognized. Both optimization and deoptimization require extensive computational analysis, which needs to be followed up with measures of attenuation, antigenicity, and structural analysis of the antigen coupled with analysis of alternative peptides and proteins. An overview of codon optimization strategies currently used for viral antigens is shown in **Figure 3**.

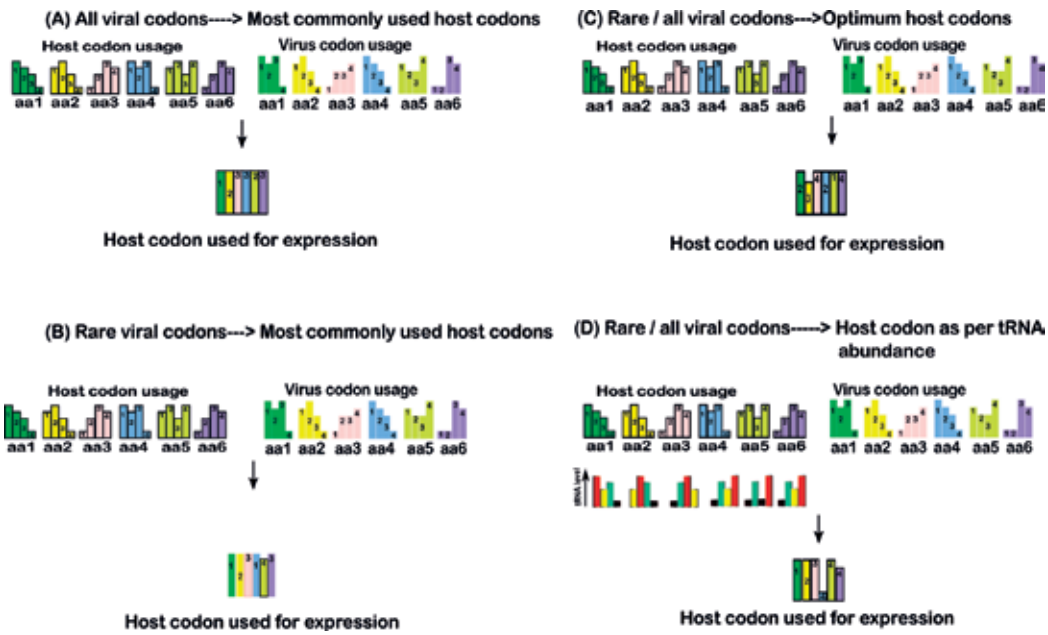

Figure 3. Strategies for codon optimization for viral gene expression. Schematic showing how poor translation of viral antigens owing to differential codon usage between viral and host genes can be overcome. In all panels, host/viral codon usage for six hypothetical amino acids (aa1–6) is shown using a color-coded histogram. Each bar represents a separate codon (1–4) used for that amino acid. Height of the bar is proportional to the frequency of that codon used. Host histograms are distinguished by bold outlines. (A) In this approach, the entire viral coding sequence is modified to reflect the most abundant codons used by the host. (B) In this strategy, only those viral codons that are rare in the host are mutated to the host codon. (C) In this approach, viral coding sequences are mutated to reflect optimum (not necessarily maximal) codon usage (D) This approach utilizes information on host transfer RNA (tRNA) expression to determine which codons in the viral coding sequence need to be mutated to the host. This strategy can include/exclude host codon usage bias. Host tRNA expression is depicted on a color scale with low (black) to red (high) expression.

3. Future directions

tRNAs and other molecules involved in host translation are an important target for disease intervention especially for intracellular viral pathogens, which are completely reliant on the host translation machinery for their successful replication and propagation in the host. However, mechanisms by which viruses and their hosts regulate translation are still being elucidated and this information is critical for development of novel interventions for both infectious and noninfectious diseases. Several vaccine production platforms use codon optimization strategies so that vaccine candidates mimic host codon usage and can be produced more efficiently with lower production costs. This results in selective usage of certain tRNAs to carry particular amino acids and to be recognized by the host cells. It is important that viral proteins can be synthesized preferentially over host proteins stimulating an immune response using these viral antigens and can be used to educate the host immunity to reduce or block damage due to subsequent infections. Inherently, every vaccine is foreign in nature for its host, which triggers an immune response. Prevalent vaccines used against infectious disease broadly fall into three categories: (1) those involving attenuated/killed pathogen, (2) subunit vaccines that contain one or more pathogen antigens (pathogen-derived or recombinant), and (3) recombinant plasmids that express one or more antigens as above. Additionally, vaccines are formulated considering delivery routes, speed of antigen release, need for adjuvants, and desired immune response. Irrespective of these criteria, the primary criterion that defines a vaccine is its antigenicity and it is important to understand mechanisms that regulate antigenicity of vaccine candidates to retain efficacy *in vivo*.

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References

[1] Ramakrishnan V. Ribosome structure and the mechanism of translation. Cell. 2002;**108**(4): 557-572

[2] Frolova LY, Merkulova TI, Kisselev LL. Translation termination in eukaryotes: Polypeptide release factor eRF1 is composed of functionally and structurally distinct domains. RNA. 2000;**6**(3):381-390

[3] Wells JN, Bergendahl LT, Marsh JA. Co-translational assembly of protein complexes. Biochemical Society Transactions. 2015;**43**(6):1221-1226

[4] Schwanhausser B et al. Global quantification of mammalian gene expression control. Nature. 2011;**473**(7347):337-342

[5] Nieuwenhuysen P, Slegers H. Purification of eukaryotic ribosomes by isopycnic centrifugation in sucrose. Analytical Biochemistry. 1978;**89**(2):472-480

[6] Yusupova G, Yusupov M. Crystal structure of eukaryotic ribosome and its complexes with inhibitors. Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences. 2017;**372**(1716)

[7] Yusupova G, Yusupov M. High-resolution structure of the eukaryotic 80S ribosome. Annual Review of Biochemistry. 2014;**83**:467-486

[8] Wilson DN, Doudna Cate JH. The structure and function of the eukaryotic ribosome. Cold Spring Harbor Perspectives in Biology. 2012;**4**(5)

[9] Ben-Shem A et al. The structure of the eukaryotic ribosome at 3.0 Å resolution. Science. 2011;**334**(6062):1524-1529

[10] Ben-Shem A et al. Crystal structure of the eukaryotic ribosome. Science. 2010;**330**(6008): 1203-1209

[11] Taylor DJ et al. Comprehensive molecular structure of the eukaryotic ribosome. Structure. 2009;**17**(12):1591-1604

[12] Khatter H et al. Structure of the human 80S ribosome. Nature. 2015;**520**(7549):640-645

[13] Klinge S et al. Crystal structure of the eukaryotic 60S ribosomal subunit in complex with initiation factor 6. Science. 2011;**334**(6058):941-948

[14] Anger AM et al. Structures of the human and drosophila 80S ribosome. Nature. 2013; **497**(7447):80-85

[15] Roundtree IA et al. Dynamic RNA modifications in gene expression regulation. Cell. 2017;**169**(7):1187-1200

[16] Chow CS, Lamichhane TN, Mahto SK. Expanding the nucleotide repertoire of the ribosome with post-transcriptional modifications. ACS Chemical Biology. 2007;**2**(9):610-619

[17] Bachellerie JP, Cavaille J, Huttenhofer A. The expanding snoRNA world. Biochimie. 2002;**84**(8):775-790

[18] Dieci G, Preti M, Montanini B. Eukaryotic snoRNAs: A paradigm for gene expression flexibility. Genomics. 2009;**94**(2):83-88

[19] Dupuis-Sandoval F, Poirier M, Scott MS. The emerging landscape of small nucleolar RNAs in cell biology. Wiley Interdisciplinary Reviews. RNA. 2015;**6**(4):381-397

[20] Falaleeva M et al. C/D-box snoRNAs form methylating and non-methylating ribonucleoprotein complexes: Old dogs show new tricks. BioEssays. 2017;**39**(6)

[21] Kiss T et al. Biogenesis and intranuclear trafficking of human box C/D and H/ACA RNPs. Cold Spring Harbor Symposia on Quantitative Biology. 2006;**71**:407-417

[22] Maxwell ES, Fournier MJ. The small nucleolar RNAs. Annual Review of Biochemistry. 1995;**64**:897-934

[23] McMahon M, Contreras A, Ruggero D. Small RNAs with big implications: New insights into H/ACA snoRNA function and their role in human disease. Wiley Interdisciplinary Reviews. RNA. 2015;**6**(2):173-189

[24] Mleczko AM, Bakowska-Zywicka K. When small RNAs become smaller: Emerging functions of snoRNAs and their derivatives. Acta Biochimica Polonica. 2016;**63**(4):601-607

[25] Chan PP, Lowe TM. GtRNAdb 2.0: An expanded database of transfer RNA genes identified in complete and draft genomes. Nucleic Acids Research. 2016;**44**(D1):D184-D189

[26] Iben JR, Maraia RJ. tRNA gene copy number variation in humans. Gene. 2014;**536**(2): 376-384

[27] Suzuki T, Nagao A, Suzuki T. Human mitochondrial tRNAs: Biogenesis, function, structural aspects, and diseases. Annual Review of Genetics. 2011;**45**:299-329

[28] Green CJ. Transfer RNA gene organization and RNase P. Molecular Biology Reports. 1995;**22**(2-3):181-185

[29] Lassar AB, Martin PL, Roeder RG. Transcription of class III genes: Formation of preinitiation complexes. Science. 1983;**222**(4625):740-748

[30] Xiong Y, Steitz TA. Mechanism of transfer RNA maturation by CCA-adding enzyme without using an oligonucleotide template. Nature. 2004;**430**(7000):640-645

[31] Xiong Y, Steitz TA. A story with a good ending: tRNA 3′-end maturation by CCA-adding enzymes. Current Opinion in Structural Biology. 2006;**16**(1):12-17

[32] Lizano E et al. A comparative analysis of CCA-adding enzymes from human and E. coli: Differences in CCA addition and tRNA 3′-end repair. Biochimie. 2008;**90**(5):762-772

[33] Ibba M, Soll D. Aminoacyl-tRNA synthesis. Annual Review of Biochemistry. 2000;**69**: 617-650

[34] Cantara WA et al. The RNA modification database, RNAMDB: 2011 update. Nucleic Acids Research. 2011;**39**(Database issue):D195-D201

[35] Czerwoniec A et al. MODOMICS: A database of RNA modification pathways. 2008 update. Nucleic Acids Research. 2009;**37**(Database issue):D118-D121

[36] Lang BF, Gray MW, Burger G. Mitochondrial genome evolution and the origin of eukaryotes. Annual Review of Genetics. 1999;**33**:351-397

[37] Suzuki T, Suzuki T. A complete landscape of post-transcriptional modifications in mammalian mitochondrial tRNAs. Nucleic Acids Research. 2014;**42**(11):7346-7357

[38] Phizicky EM, Hopper AK. tRNA biology charges to the front. Genes & Development. 2010;**24**(17):1832-1860

[39] Machnicka MA et al. MODOMICS: A database of RNA modification pathways—2013 update. Nucleic Acids Research. 2013;**41**(Database issue):D262-D267

[40] Watanabe K. Unique features of animal mitochondrial translation systems. The non-universal genetic code, unusual features of the translational apparatus and their relevance to human mitochondrial diseases. Proceedings of the Japan Academy. Series B, Physical and Biological Sciences. 2010;**86**(1):11-39

[41] Wakita K et al. Higher-order structure of bovine mitochondrial tRNA(Phe) lacking the 'conserved' GG and T psi CG sequences as inferred by enzymatic and chemical probing. Nucleic Acids Research. 1994;**22**(3):347-353

[42] Messmer M et al. Tertiary network in mammalian mitochondrial tRNAAsp revealed by solution probing and phylogeny. Nucleic Acids Research. 2009;**37**(20):6881-6895

[43] Anderson S et al. Complete sequence of bovine mitochondrial DNA. Conserved features of the mammalian mitochondrial genome. Journal of Molecular Biology. 1982;**156**(4): 683-717

[44] Anderson S et al. Sequence and organization of the human mitochondrial genome. Nature. 1981;**290**(5806):457-465

[45] de Godoy LM et al. Comprehensive mass-spectrometry-based proteome quantification of haploid versus diploid yeast. Nature. 2008;**455**(7217):1251-1254

[46] Arava Y et al. Genome-wide analysis of mRNA translation profiles in Saccharomyces cerevisiae. Proceedings of the National Academy of Sciences of the United States of America. 2003;**100**(7):3889-3894

[47] Ingolia NT et al. Genome-wide analysis in vivo of translation with nucleotide resolution using ribosome profiling. Science. 2009;**324**(5924):218-223

[48] Drummond DA, Wilke CO. Mistranslation-induced protein misfolding as a dominant constraint on coding-sequence evolution. Cell. 2008;**134**(2):341-352

[49] Kramer EB, Farabaugh PJ. The frequency of translational misreading errors in E. coli is largely determined by tRNA competition. RNA. 2007;**13**(1):87-96

[50] Kramer EB et al. A comprehensive analysis of translational missense errors in the yeast Saccharomyces cerevisiae. RNA. 2010;**16**(9):1797-1808

[51] Kurland CG. Translational accuracy and the fitness of bacteria. Annual Review of Genetics. 1992;**26**:29-50

[52] Salas-Marco J, Bedwell DM. Discrimination between defects in elongation fidelity and termination efficiency provides mechanistic insights into translational readthrough. Journal of Molecular Biology. 2005;**348**(4):801-815

[53] Kaiser CM et al. The ribosome modulates nascent protein folding. Science. 2011;**334**(6063): 1723-1727

[54] Jacobson GN, Clark PL. Quality over quantity: Optimizing co-translational protein folding with non-'optimal' synonymous codons. Current Opinion in Structural Biology. 2016;**38**:102-110

[55] Kumar RK, Davis DR. Synthesis and studies on the effect of 2-thiouridine and 4-thiouridine on sugar conformation and RNA duplex stability. Nucleic Acids Research. 1997;**25**(6):1272-1280

[56] Stadler M, Fire A. Wobble base-pairing slows in vivo translation elongation in metazoans. RNA. 2011;**17**(12):2063-2073

[57] Mauro VP, Chappell SA. A critical analysis of codon optimization in human therapeutics. Trends in Molecular Medicine. 2014;**20**(11):604-613

[58] Rogalski M, Karcher D, Bock R. Superwobbling facilitates translation with reduced tRNA sets. Nature Structural & Molecular Biology. 2008;**15**(2):192-198

[59] Alkatib S et al. The contributions of wobbling and superwobbling to the reading of the genetic code. PLoS Genetics. 2012;**8**(11):e1003076

[60] Gebetsberger J, Polacek N. Slicing tRNAs to boost functional ncRNA diversity. RNA Biology. 2013;**10**(12):1798-1806

[61] Wang L et al. A novel class of heat-responsive small RNAs derived from the chloroplast genome of Chinese cabbage (Brassica rapa). BMC Genomics. 2011;**12**:289

[62] Burroughs AM et al. Deep-sequencing of human Argonaute-associated small RNAs provides insight into miRNA sorting and reveals Argonaute association with RNA fragments of diverse origin. RNA Biology. 2011;**8**(1):158-177

[63] Liao JY et al. Deep sequencing of human nuclear and cytoplasmic small RNAs reveals an unexpectedly complex subcellular distribution of miRNAs and tRNA 3′ trailers. PLoS One. 2010;**5**(5):e10563

[64] Haussecker D et al. Human tRNA-derived small RNAs in the global regulation of RNA silencing. RNA. 2010;**16**(4):673-695

[65] Lee YS et al. A novel class of small RNAs: tRNA-derived RNA fragments (tRFs). Genes & Development. 2009;**23**(22):2639-2649

[66] Raina M, Ibba M. tRNAs as regulators of biological processes. Frontiers in Genetics. 2014;**5**:171

[67] Ivanov P et al. G-quadruplex structures contribute to the neuroprotective effects of angiogenin-induced tRNA fragments. Proceedings of the National Academy of Sciences of the United States of America. 2014;**111**(51):18201-18206

[68] Anderson P, Ivanov P. tRNA fragments in human health and disease. FEBS Letters. 2014;**588**(23):4297-4304

[69] Haiser HJ et al. Developmentally regulated cleavage of tRNAs in the bacterium Streptomyces coelicolor. Nucleic Acids Research. 2008;**36**(3):732-741

[70] Hsieh LC et al. Abundance of tRNA-derived small RNAs in phosphate-starved Arabidopsis roots. Plant Signaling & Behavior. 2010;**5**(5):537-539

[71] Yamasaki S et al. Angiogenin cleaves tRNA and promotes stress-induced translational repression. The Journal of Cell Biology. 2009;**185**(1):35-42

[72] Dhahbi JM et al. 5′ tRNA halves are present as abundant complexes in serum, concentrated in blood cells, and modulated by aging and calorie restriction. BMC Genomics. 2013;**14**:298

[73] Ogawa T et al. A cytotoxic ribonuclease targeting specific transfer RNA anticodons. Science. 1999;**283**(5410):2097-2100

[74] Tomita K et al. A cytotoxic ribonuclease which specifically cleaves four isoaccepting arginine tRNAs at their anticodon loops. Proceedings of the National Academy of Sciences of the United States of America. 2000;**97**(15):8278-8283

[75] Lee SR, Collins K. Starvation-induced cleavage of the tRNA anticodon loop in Tetrahymena thermophila. The Journal of Biological Chemistry. 2005;**280**(52):42744-42749

[76] Thompson DM, Parker R. Stressing out over tRNA cleavage. Cell. 2009;**138**(2):215-219

[77] Saikia M et al. Genome-wide identification and quantitative analysis of cleaved tRNA fragments induced by cellular stress. The Journal of Biological Chemistry. 2012;**287**(51):42708-42725

[78] Zhang Y et al. Identification and characterization of an ancient class of small RNAs enriched in serum associating with active infection. Journal of Molecular Cell Biology. 2014;**6**(2):172-174

[79] Ivanov P et al. Angiogenin-induced tRNA fragments inhibit translation initiation. Molecular Cell. 2011;**43**(4):613-623

[80] Emara MM et al. Angiogenin-induced tRNA-derived stress-induced RNAs promote stress-induced stress granule assembly. The Journal of Biological Chemistry. 2010; **285**(14):10959-10968

[81] LaCava J et al. RNA degradation by the exosome is promoted by a nuclear polyadenylation complex. Cell. 2005;**121**(5):713-724

[82] Kadaba S et al. Nuclear surveillance and degradation of hypomodified initiator tRNAMet in S. cerevisiae. Genes & Development. 2004;**18**(11):1227-1240

[83] Anderson JT. RNA turnover: Unexpected consequences of being tailed. Current Biology. 2005;**15**(16):R635-R638

[84] Wang X et al. Degradation of hypomodified tRNA(iMet) in vivo involves RNA-dependent ATPase activity of the DExH helicase Mtr4p. RNA. 2008;**14**(1):107-116

[85] Maraia RJ, Lamichhane TN. 3′ processing of eukaryotic precursor tRNAs. Wiley Interdisciplinary Reviews. RNA. 2011;**2**(3):362-375

[86] Chernyakov I et al. Degradation of several hypomodified mature tRNA species in Saccharomyces cerevisiae is mediated by Met22 and the 5′-3′ exonucleases Rat1 and Xrn1. Genes & Development. 2008;**22**(10):1369-1380

[87] Watanabe K et al. Degradation of initiator tRNAMet by Xrn1/2 via its accumulation in the nucleus of heat-treated HeLa cells. Nucleic Acids Research. 2013;**41**(8):4671-4685

[88] Cole C et al. Filtering of deep sequencing data reveals the existence of abundant dicer-dependent small RNAs derived from tRNAs. RNA. 2009;**15**(12):2147-2160

[89] Babiarz JE et al. Mouse ES cells express endogenous shRNAs, siRNAs, and other microprocessor-independent, dicer-dependent small RNAs. Genes & Development. 2008;**22**(20):2773-2785

[90] Maute RL et al. tRNA-derived microRNA modulates proliferation and the DNA damage response and is down-regulated in B cell lymphoma. Proceedings of the National Academy of Sciences of the United States of America. 2013;**110**(4):1404-1409

[91] Sobala A, Hutvagner G. Small RNAs derived from the 5′ end of tRNA can inhibit protein translation in human cells. RNA Biology. 2013;**10**(4):553-563

[92] Megel C et al. Surveillance and cleavage of eukaryotic tRNAs. International Journal of Molecular Sciences. 2015;**16**(1):1873-1893

[93] Huang H et al. Bulk RNA degradation by nitrogen starvation-induced autophagy in yeast. The EMBO Journal. 2015;**34**(2):154-168

[94] Czech A et al. Reversible and rapid transfer-RNA deactivation as a mechanism of translational repression in stress. PLoS Genetics. 2013;**9**(8):e1003767

[95] Supek F. The code of silence: Widespread associations between synonymous codon biases and gene function. Journal of Molecular Evolution. 2016;**82**(1):65-73

[96] Behura SK, Severson DW. Codon usage bias: Causative factors, quantification methods and genome-wide patterns: With emphasis on insect genomes. Biological Reviews of the Cambridge Philosophical Society. 2013;**88**(1):49-61

[97] Novoa EM, Ribas de Pouplana L. Speeding with control: Codon usage, tRNAs, and ribosomes. Trends in Genetics. 2012;**28**(11):574-581

[98] Angov E. Codon usage: Nature's roadmap to expression and folding of proteins. Biotechnology Journal. 2011;**6**(6):650-659

[99] Barrai I et al. Oligonucleotide correlations between infector and host genomes hint at evolutionary relationships. Nucleic Acids Research. 1990;**18**(10):3021-3025

[100] Quax TE et al. Codon bias as a means to fine-tune gene expression. Molecular Cell. 2015;**59**(2):149-161

[101] Firth AE, Brierley I. Non-canonical translation in RNA viruses. The Journal of General Virology. 2012;**93**(Pt 7):1385-1409

[102] Kim CH, Oh Y, Lee TH. Codon optimization for high-level expression of human erythropoietin (EPO) in mammalian cells. Gene. 1997;**199**(1-2):293-301

[103] Lopes A et al. Codon-optimized P1A-encoding DNA vaccine: Toward a therapeutic vaccination against P815 mastocytoma. Molecular Therapy: Nucleic Acids. 2017;**8**:404-415

[104] Chen Q et al. Induction of humoral and cellular immune responses in mice by multiepitope vaccines composing of both T and B lymphocyte epitopes of MAGE-A3 which are recombined into HBcAg. Protein and Peptide Letters. 2017

[105] Wang D et al. Stop codon mutagenesis for homogenous expression of human papillomavirus L1 protein in Escherichia coli. Protein Expression and Purification. 2017;**133**: 110-120

[106] Stachyra A et al. Codon optimization of antigen coding sequences improves the immune potential of DNA vaccines against avian influenza virus H5N1 in mice and chickens. Virology Journal. 2016;**13**(1):143

[107] Yu TW et al. Novel GM-CSF-based vaccines: One small step in GM-CSF gene optimization, one giant leap for human vaccines. Human Vaccines & Immunotherapeutics. 2016;**12**(12):3020-3028

[108] Velazquez-Salinas L et al. Recoding structural glycoprotein E2 in classical swine fever virus (CSFV) produces complete virus attenuation in swine and protects infected animals against disease. Virology. 2016;**494**:178-189

[109] Bang HB et al. High-level production of human papillomavirus (HPV) type 16 L1 in Escherichia coli. Journal of Microbiology and Biotechnology. 2016;**26**(2):356-363

[110] Liang B et al. Enhanced neutralizing antibody response induced by respiratory syncytial virus prefusion F protein expressed by a vaccine candidate. Journal of Virology. 2015;**89**(18):9499-9510

[111] Datta D et al. Evaluation of the impact of codon optimization and N-linked glycosylation on functional immunogenicity of Pfs25 DNA vaccines delivered by in vivo electroporation in preclinical studies in mice. Clinical and Vaccine Immunology. 2015;**22**(9):1013-1019

[112] Zhang Y et al. Effects of the fusion design and immunization route on the immunogenicity of Ag85A-Mtb32 in adenoviral vectored tuberculosis vaccine. Human Vaccines & Immunotherapeutics. 2015;**11**(7):1803-1813

[113] Willet M et al. Preclinical development of inactivated rabies virus-based polyvalent vaccine against rabies and filoviruses. The Journal of Infectious Diseases. 2015;**212**(Suppl 2): S414-S424

[114] Liu Y et al. Expression, purification and identification of Pla a1 in a codon-optimized Platanus pollen allergen. Molecular Medicine Reports. 2015;**12**(2):2197-2202

[115] Lorenz FK et al. Codon optimization of the human papillomavirus E7 oncogene induces a CD8+ T cell response to a cryptic epitope not harbored by wild-type E7. PLoS One. 2015;**10**(3):e0121633

[116] Wang YY et al. Optimized codon usage enhances the expression and immunogenicity of DNA vaccine encoding Taenia solium oncosphere TSOL18 gene. Molecular Medicine Reports. 2015;**12**(1):281-288

[117] Williams JA. Improving DNA vaccine performance through vector design. Current Gene Therapy. 2014;**14**(3):170-189

[118] Fan X et al. Targeting the HA2 subunit of influenza A virus hemagglutinin via CD40L provides universal protection against diverse subtypes. Mucosal Immunology. 2015;**8**(1):211-220

[119] Cheng CY et al. Enhancing expression of the classical swine fever virus glycoprotein E2 in yeast and its application to a blocking ELISA. Journal of Biotechnology. 2014;**174**:1-6

[120] Hung YF et al. Recombinant production of the amino terminal cytoplasmic region of dengue virus non-structural protein 4A for structural studies. PLoS One. 2014;**9**(1):e86482

[121] Dutton JL et al. A novel DNA vaccine technology conveying protection against a lethal herpes simplex viral challenge in mice. PLoS One. 2013;**8**(10):e76407

[122] Spatz SJ et al. Expression of chicken parvovirus VP2 in chicken embryo fibroblasts requires codon optimization for production of naked DNA and vectored meleagrid herpesvirus type 1 vaccines. Virus Genes. 2013;**47**(2):259-267

[123] Gao J et al. Codon optimization of the rabbit hemorrhagic disease virus (RHDV) capsid gene leads to increased gene expression in Spodoptera frugiperda 9 (Sf9) cells. Journal of Veterinary Science. 2013;**14**(4):441-447

[124] Li K et al. Codon optimization and woodchuck hepatitis virus posttranscriptional regulatory element enhance the immune responses of DNA vaccines against infectious bursal disease virus in chickens. Virus Research. 2013;**175**(2):120-127

[125] Seo JY, Chung HJ, Kim TJ. Codon-optimized expression of fish iridovirus capsid protein in yeast and its application as an oral vaccine candidate. Journal of Fish Diseases. 2013;**36**(9):763-768

[126] Arora U et al. Virus-like particles displaying envelope domain III of dengue virus type 2 induce virus-specific antibody response in mice. Vaccine. 2013;**31**(6):873-878

[127] Kim HJ, Kwag HL, Kim HJ. Codon optimization of the human papillomavirus type 58 L1 gene enhances the expression of soluble L1 protein in Saccharomyces cerevisiae. Biotechnology Letters. 2013;**35**(3):413-421

[128] Tu Y et al. High-level expression and immunogenicity of a porcine circovirus type 2 capsid protein through codon optimization in Pichia pastoris. Applied Microbiology and Biotechnology. 2013;**97**(7):2867-2875

[129] Shin TH et al. Induction of robust cellular immunity against HPV6 and HPV11 in mice by DNA vaccine encoding for E6/E7 antigen. Human Vaccines & Immunotherapeutics. 2012;**8**(4):470-478

[130] Zhao KN, Chen J. Codon usage roles in human papillomavirus. Reviews in Medical Virology. 2011;**21**(6):397-411

[131] Mani I et al. Codon optimization of the major antigen encoding genes of diverse strains of influenza A virus. Interdisciplinary Sciences. 2011;**3**(1):36-42

[132] Laguia-Becher M et al. Effect of codon optimization and subcellular targeting on Toxoplasma gondii antigen SAG1 expression in tobacco leaves to use in subcutaneous and oral immunization in mice. BMC Biotechnology. 2010;**10**:52

[133] Zhu Y et al. Synergistic enhancement of immunogenicity and protection in mice against Schistosoma japonicum with codon optimization and electroporation delivery of SjTPI DNA vaccines. Vaccine. 2010;**28**(32):5347-5355

[134] Tenbusch M et al. Codon-optimization of the hemagglutinin gene from the novel swine origin H1N1 influenza virus has differential effects on CD4(+) T-cell responses and immune effector mechanisms following DNA electroporation in mice. Vaccine. 2010;**28**(19):3273-3277

[135] Siegismund CS et al. Enhanced T- and B-cell responses to simian immunodeficiency virus (SIV)agm, SIVmac and human immunodeficiency virus type 1 Gag DNA immunization and identification of novel T-cell epitopes in mice via codon optimization. The Journal of General Virology. 2009;**90**(Pt 10):2513-2518

[136] Ahn I, Jeong BJ, Son HS. Comparative study of synonymous codon usage variations between the nucleocapsid and spike genes of coronavirus, and C-type lectin domain genes of human and mouse. Experimental & Molecular Medicine. 2009;**41**(10):746-756

[137] Richardson JS et al. Enhanced protection against Ebola virus mediated by an improved adenovirus-based vaccine. PLoS One. 2009;**4**(4):e5308

[138] Muthumani K et al. Immunogenicity of novel consensus-based DNA vaccines against Chikungunya virus. Vaccine. 2008;**26**(40):5128-5134

[139] Ternette N et al. Immunogenicity and efficacy of codon optimized DNA vaccines encoding the F-protein of respiratory syncytial virus. Vaccine. 2007;**25**(41):7271-7279

[140] Zakhartchouk AN et al. Optimization of a DNA vaccine against SARS. DNA and Cell Biology. 2007;**26**(10):721-726

[141] Tsai CJ et al. Synonymous mutations and ribosome stalling can lead to altered folding pathways and distinct minima. Journal of Molecular Biology. 2008;**383**(2):281-291

[142] Spencer PS et al. Silent substitutions predictably alter translation elongation rates and protein folding efficiencies. Journal of Molecular Biology. 2012;**422**(3):328-335

[143] Zhou JH et al. The effects of the synonymous codon usage and tRNA abundance on protein folding of the 3C protease of foot-and-mouth disease virus. Infection, Genetics and Evolution. 2013;**16**:270-274

[144] Zhang F et al. Differential arginylation of actin isoforms is regulated by coding sequence-dependent degradation. Science. 2010;**329**(5998):1534-1537

[145] Katsnelson A. Breaking the silence. Nature Medicine. 2011;**17**(12):1536-1538

[146] Chen C et al. Dynamics of translation by single ribosomes through mRNA secondary structures. Nature Structural & Molecular Biology. 2013;**20**(5):582-588

[147] Irimia M et al. Evolutionarily conserved A-to-I editing increases protein stability of the alternative splicing factor Nova1. RNA Biology. 2012;**9**(1):12-21

[148] Jack BR et al. Reduced protein expression in a virus attenuated by codon deoptimization. G3 (Bethesda). 2017;**7**(9):2957-2968

[149] Le Nouen C et al. Genetic stability of genome-scale deoptimized RNA virus vaccine candidates under selective pressure. Proceedings of the National Academy of Sciences of the United States of America. 2017;**114**(3):E386-E395

[150] Stobart CC et al. A live RSV vaccine with engineered thermostability is immunogenic in cotton rats despite high attenuation. Nature Communications. 2016;**7**:13916

[151] Cheng BY et al. Development of live-attenuated arenavirus vaccines based on codon deoptimization of the viral glycoprotein. Virology. 2017;**501**:35-46

[152] Butt AM et al. Evolution of codon usage in Zika virus genomes is host and vector specific. Emerging Microbes & Infections. 2016;**5**(10):e107

[153] Rostad CA et al. A recombinant respiratory syncytial virus vaccine candidate attenuated by a low-fusion F protein is immunogenic and protective against challenge in cotton rats. Journal of Virology. 2016;**90**(16):7508-7518

[154] Diaz-San Segundo F et al. Synonymous deoptimization of foot-and-mouth disease virus causes attenuation in vivo while inducing a strong neutralizing antibody response. Journal of Virology. 2015;**90**(3):1298-1310

[155] Baker SF, Nogales A, Martinez-Sobrido L. Downregulating viral gene expression: Codon usage bias manipulation for the generation of novel influenza A virus vaccines. Future Virology. 2015;**10**(6):715-730

[156] Cheng BY et al. Development of live-attenuated arenavirus vaccines based on codon deoptimization. Journal of Virology. 2015;**89**(7):3523-3533

[157] Meng J et al. Refining the balance of attenuation and immunogenicity of respiratory syncytial virus by targeted codon deoptimization of virulence genes. MBio. 2014;**5**(5):e01704-14

[158] Le Nouen C et al. Attenuation of human respiratory syncytial virus by genome-scale codon-pair deoptimization. Proceedings of the National Academy of Sciences of the United States of America. 2014;**111**(36):13169-13174

[159] Nogales A et al. Influenza A virus attenuation by codon deoptimization of the NS gene for vaccine development. Journal of Virology. 2014;**88**(18):10525-10540

[160] Ni YY et al. Computer-aided codon-pairs deoptimization of the major envelope GP5 gene attenuates porcine reproductive and respiratory syndrome virus. Virology. 2014; **450-451**:132-139

[161] Aragones L et al. Fine-tuning translation kinetics selection as the driving force of codon usage bias in the hepatitis A virus capsid. PLoS Pathogens. 2010;**6**(3):e1000797

[162] Mueller S et al. Reduction of the rate of poliovirus protein synthesis through large-scale codon deoptimization causes attenuation of viral virulence by lowering specific infectivity. Journal of Virology. 2006;**80**(19):9687-9696

[163] Burns CC et al. Modulation of poliovirus replicative fitness in HeLa cells by deoptimization of synonymous codon usage in the capsid region. Journal of Virology. 2006;**80**(7):3259-3272

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