

Epigenetic Regulation of Cellular Senescence

by WNT-MYC-Polycomb Signaling

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4. Tegla CA, Cudrici C, Rozycka M, Soloviova K, Ito T, Singh AK, Khan A, Azimzadeh P, Andrian-Albescu M, Niculescu F, Rus V, Judge SI, Rus H. C5b-9-activated, K(v)1.3 channels mediate oligodendrocyte cell cycle activation and dedifferentiation. *Exp Mol Pathol*. 2011; 91(1):335-45.
5. Yu J, Proddutur A, Elgammal F, Ito T, Santhakumar V. Status epilepticus enhances tonic GABA currents and depolarizes GABA reversal potential in dentate fast-spiking basket cells. *Journal of Neurophysiology*. 2013; 109: 1746-63.

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2. Oh S, Cudrici C, Ito T, Rus H. B-cells and humoral immunity in multiple sclerosis. Implications for therapy. *Immunologic research.* 2008; 40: 224-.
3. Tegla A, Cudrici C, Rus V, Ito T, Vlaicu S, Singh A, et al. Neuroprotective effects of the complement terminal pathway during demyelination: implications for oligodendrocyte survival. *Journal of Neuroimmunol.* 2009; 213(1-2), 3-11.
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To my parents and my wife

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Chapter 1. Introduction

1.1 Cellular Senescence

Hayflick and Moorhead originally observed that normal human diploid fibroblasts (HDF) have a finite lifespan and strains of embryonic fibroblasts grew for longer time than adult fibroblasts referring to the phenomenon as cellular senescence (Hayflick, 1965; Hayflick and Moorhead, 1961). Since the initial discovery, tremendous progress identifying the triggers and the regulators driving senescence has been elucidated. Recent advances have also identified multiple roles of cellular senescence in physiological processes.

Triggers of Cellular Senescence

Cellular senescence is a state of irreversible growth arrest that is triggered in response to variety of detrimental stimuli to limit the propagation of stressed and damaged cells (Figure 1.1A). Originally, senescence was believed to be induced primarily by telomere attrition. Telomeres are repetitive DNA sequences at the linear ends of the chromosomes for protection from degradation and recombination. Telomeres erode with each cell division due to the use of RNA-based priming of the lagging strand and the unidirectionality of DNA polymerases. Eventually the telomeres become critically short and are recognized as double stranded breaks (DSBs) by the DNA damage response (DDR) pathway. Activation of DDR pathway as a result of shortened telomeres leads to inhibition of cell cycle progression, which is referred to as replicative senescence (d'Adda di Fagagna et al., 2003; Harley et al., 1990). However, not all human cells undergo cellular senescence through telomere attrition. For example, expression of human

telomerase reverse transcriptase (*hTERT*) in human keratinocytes or mammary epithelial cells is not sufficient to immortalize the cells (Kiyono et al., 1998). Similarly many cells from rodents do not undergo senescence due to telomere attrition as they have very long telomeres compared to humans and in some cases express telomerase (Itahana et al., 2004). Telomerase is also expressed in many human stem and cancer cells (Martinez and Blasco, 2011).

Telomere-independent senescence can occur in response to other triggers such as activation of oncogenes. Expression of the G12V oncogenic variant of *hRAS* (*HRAS*^{V12}) or over-activation of downstream effectors such as *RAF* or *MEK* in normal cells leads to what is now referred to as oncogene-induced senescence (OIS) (Lin et al., 1998; Peeper, 2011; Serrano et al., 1997; Zhu et al., 1998). In addition, induction of many other oncogenes such as *AKT*, *E2F1*, *CCNE1*, and *CDC6* as well as inactivation of many tumor-suppressor genes such as *PTEN* and *NF1* have been shown to induce OIS (Courtois-Cox et al., 2008).

It is also well established that exposure to DNA damaging agents or oxidative stress like reactive oxygen species (ROS) are strong inducers of senescence and are often called stress-induces premature senescence (SIPS). Sublethal dose of ultraviolet (UV) light or irradiation has been shown to induce premature senescence (Chainiaux et al., 2002; Kastan et al., 1992; Toussaint et al., 2000). Similarly, sublethal dose of stressors produced intrinsically or extrinsically like hydrogen peroxide, hypoxic atmosphere, or *tert*-Butyl hydroperoxide can also induce senescence (Saretzki et al., 1998; Toussaint et al., 1992; von Zglinicki et al., 2000). Treatment of cells with antioxidants or free radical scavengers has been shown to delay the onset of replicative senescence (Chen et al.,

1995). Reduction in mitochondrial content *in vivo* prevents senescence in aging mouse liver (Correia-Melo et al., 2016).

Regulators of Cellular Senescence

DNA-Damage Response (DDR) Pathway

A variety of triggers can induce multiple effector pathways to initiate many of the phenotypes of senescent cells (Figure 1.1B). One common feature of senescence is the generation of DNA damage, primarily DSBs that are considered most deleterious. DDR is a signaling pathway in which ataxia telangiectasia mutated (*ATM*) or ataxia telangiectasia and Rad3 related (*ATR*) kinases block cell cycle progression through stabilization of tumor suppressor protein p53 (*TP53*) and its transcriptional regulation of cyclin-dependent kinase inhibitor 1A (*CDKN1A* also referred to as p21). Many senescent cells show accumulation of DNA damage markers including γ -H2AX, CHK1, CHK2, 53BP1, and phosphorylated ATM and inactivation of DDR delays entry into senescence (d'Adda di Fagagna et al., 2003; Takai et al., 2003). However, different senescence stimuli generate DNA damage through varying mechanisms. For example, during replicative senescence telomeres become critically short and are sensed as DSBs triggering DDR-induced senescence (d'Adda di Fagagna et al., 2003; Herbig et al., 2004).

Senescence and DDR can be triggered independently of telomere shortening as exemplified by senescence of post-mitotic cells (Jurk et al., 2012). Overexpression of oncogenic RAS triggers DNA damage and senescence by driving hyper-replication resulting in terminated replication forks (Bartkova et al., 2006; Di Micco et al., 2006). DNA damage can also be triggered by a variety of stimuli such as irradiation, genotoxic

drugs, or hydrogen peroxide leading to DDR activation. Long-term DDR results in mitochondrial damage and widespread ROS production (Passos et al., 2010). The production of ROS can also result in DNA damage providing a mechanism by which ROS and DNA damage stimulate each other to induce senescence.

DSB formation is not uniform throughout the chromosome. Even though DNA damage by these triggers can occur randomly throughout the genome, they frequently persist at the telomeres (Fumagalli et al., 2012). It is postulated that proteins of the shelterin complex, which normally inhibit DNA end joining and chromosomal fusion, block the recruitment of many of the DNA damage repair proteins within the telomeric repeats resulting in persistent damages at the telomeres. Certain chromosomal loci called common fragile sites (CFS) are also hotspots for DNA damage and are frequently in the repetitive regions of the genome that can form secondary structures to alter the replication fork progression (Glover, 2006). It is not surprising that OIS increases the frequency of DNA replication stress at these repetitive regions and at the telomeres (Suram et al., 2012).

Although many triggers eventually activate DDR pathway, several groups have identified DDR-independent senescence. For examples, *BRAF(V600E)* induces senescence through metabolic mechanism involving increases in mitochondrial pyruvates dehydrogenase (Kaplun et al., 2013). Senescence induced by *E2F3* activation has also been shown to be independent of DDR and are primarily due to upregulation of p16 (Lazzerini Denchi et al., 2005).

p53-p21 and p16 Pathways

It has been proposed that entry into senescence is mediated by activation of the p53-p21 and p16 tumor suppressor pathways. DNA damage resulting in DDR pathway inhibits cell cycle by promoting the accumulation of tumor suppressor p53 that regulates transcription of p21 (Herbig et al., 2004). p16 is regulated in a more complex manner that includes the Polycomb group (PcG) of epigenetic regulators (Gil and Peters, 2006). The *INK4a/ARF* locus encoding *CDKN2A* (p16) and p14 (p19 in mice), is repressed by the PcG mediated trimethylation of histone H3 lysine 27 (H3K27me3) and ubiquitination of histone H2A lysine 119 (H2AK119ub1) (LaPak and Burd, 2014). p21 and p16 inhibit the activities of cyclin dependent kinases (CDKs) 2, 4 and 6, hence antagonizing the phosphorylation and inactivation of the tumor suppressor and master cell cycle regulator pRb (retinoblastoma, *RBI*) (Palmero et al., 2002; Pei and Xiong, 2005). Euchromatization of the genome using histone deacetylase inhibitors triggers senescence in p21-dependent mechanism (Romanov et al., 2010). Both the p53-p21 and p16 tumor suppressor pathways can become activated to regulate senescence. For example, cell cycle arrest induced by DNA damage is initially mediated by stabilization of p53 and activation of p21, but if the lesion persists, this results in induction of p16 through p38 mitogen-activated protein kinase (p38MAPK)-mediated mitochondrial dysfunction and ROS production (Passos et al., 2010).

Epigenetic Changes in Senescence

Another hallmark of human cells undergoing senescence is the formation of regions of punctate heterochromatin called Senescence-Associated Heterochromatin Foci (SAHF).

Formation of SAHF can be visualized by immunofluorescence (IF) microscopy as domains of high intensity DAPI staining. SAHF are formed by the compaction of individual chromosomes into densely packed heterochromatic domains. Recruitment of the histone chaperone, HIRA, to Promyelocytic Leukemia (PML) bodies is required for formation of SAHF (Ye et al., 2007; Zhang et al., 2007). SAHF contain several markers of transcriptionally silent heterochromatin, such as heterochromatin-associated protein 1 (HP1), the histone variant macro H2A (mH2A) and methylation of histone 3 at lysine 9 (H3K9me3) (Narita et al., 2003). This is also accompanied by the high mobility group proteins HMGA replacing the linker histone H1 (Zhang et al., 2007). The formation of SAHF is thought to induce permanent growth arrest by transcriptionally silencing proliferative genes such as cyclin A2 (Narita et al., 2003).

However, the formation of SAHF varies with the cell type as well as the cause of senescence (Narita et al., 2003). For example, OIS induces formation of SAHF, while replicative senescence elicits varying levels of SAHF (Narita et al., 2003). There also seems to be differences between mouse and human cells in regards to SAHF formation. Mouse embryonic fibroblasts (MEF) and adult mouse tail fibroblasts (MTF) do not form robust SAHF upon OIS. However, normal diploid human fibroblasts (HDF) have been shown to form SAHF (Narita et al., 2003). Despite the differences in formation of SAHF between species, protein levels of components of SAHF, such as mH2A and HP1, increase in senescent cells in baboon and mouse cells (Kreiling et al., 2011).

Lamin B1 (LMNB1) is downregulated in senescence and triggers global and local modifications in chromatin structure (Sadaie et al., 2013; Shah et al., 2013). As cells approach senescence, there is decondensation of pericentromeric satellite

heterochromatin that precedes SAHF formation (Swanson et al., 2013). Similarly, there is “smoothening” of chromatin between euchromatic and heterochromatic regions in senescent cells, where euchromatic regions become more compact and heterochromatic regions become more unfolded leading to expression and activation of transposable elements (De Cecco et al., 2013). The gene-poor lamin-associated domains exhibit DNA hypomethylation due to mislocalization of DNA methyltransferase (DNMT1) and hypermethylation in CpG islands as cells approach senescence (Cruickshanks et al., 2013). Senescent cells also degrade and eliminate damaged DNA. Senescent cells exhibit autophagy and lysosome-mediated proteolysis referred to as cytoplasmic chromatin fragments (CCFs) that buds off the nuclei and are positive for H3K27me3, γ H2AX, and DNA (Ivanov et al., 2013). In senescent cells, microtubule-associated protein 1 light chain 3 (LC3: key protein in autophagy) mobilized LMNB1 into the cytoplasm for degradation (Dou et al., 2015).

Phenotypes of Cellular Senescence

Senescence-Associated β -Galactosidase and Morphological Changes

There has been great effort in identifying molecular markers of cellular senescence besides the loss of replicative capacity (Figure 1.1C). One of the most widely used markers of senescence is the increase in senescence-associated β -galactosidase (SA- β -GAL) that is detectable at pH 6.0 (Dimri et al., 1995). Nonsenescent cells display β -galactosidase activity in the lysosome at the optimal pH of 4.0. The increase in SA- β -GAL activity is primarily believed to be due to the increase in galactosidase beta 1 (*GLB1*), gene encoding lysosome enzyme, and expansion of the lysosomal compartment

to compensate for the accumulation of damage macromolecules (Kurz et al., 2000; Lee et al., 2006a).

Senescence is accompanied by morphological changes. Senescence induced by telomere erosion, expression of *HRAS*^{V12} or DNA damage becomes large, flat and multinucleated (Chen et al., 1995; Serrano et al., 1997). Cells undergoing senescence by expression of *BRAF*(V600E) or silencing of *p400* acquire a spindle-like morphology (Chan et al., 2005; Michaloglou et al., 2005).

Senescence-Associated Secretory Phenotype (SASP)

Another hallmark of cellular senescence is the senescence-associated secretory phenotype (SASP). The senescent cells and fibroblasts derived from subjects with premature aging syndrome express and secrete broad range of proinflammatory factors such as cytokines, chemokines, growth factors, and proteases that adds a non-autonomous functionality of senescent cells (Coppe et al., 2010). The biological effect of SASP on neighboring cells is diverse depending on the cell type.

Regulation of SASP has been shown to be dependent on persistent DDR observed in senescence induced by genotoxic stress, replicative exhaustion, and oncogenic stress (Coppe et al., 2011; Rodier et al., 2011). Loss of ATM or other DDR factors reduces secretion of SASP factors but seems to be independent of p16 or p21 expression (Rodier et al., 2009).

Many of the SASP components are transcriptionally regulated by two transcription factors, nuclear factor- κ B (*NF- κ B*) and CCAAT-enhancer-binding protein beta (*C/EBP β*) (Freund et al., 2011; Kuilman et al., 2008). The expression of SASP

factors mediated by *NF-κB* is regulated by multiple factors. One effector that has been identified is *p38MAPK*. This is not surprising as ATM and p38MAPK converge on NF-κB that work cooperatively with C/EBPβ to drive SASP expression (Freund et al., 2011). The mammalian target of rapamycin (mTOR) promotes SASP by enhancing the translation of cytokine IL1A, which in turn increases the activity of NF-κB for transcription of many SASP genes (Laberge et al., 2015). Similarly mTOR also control the translation of mitogen-activated protein kinase-activated protein kinase 2 (MAPKAPK2 or MK2) that is downstream of p38MAPK. MK2 phosphorylates RNA-binding protein ZFP36L1 that is normally responsible for the degradation of many SASP factors (Herranz et al., 2015). It has also been shown that DNA damage stabilizes transcription factor GATA binding protein 4 (GATA4), which is normally degraded by p62-mediated autophagy, to activate NF-κB expression of SASP factors (Kang et al., 2015).

Physiological Roles of Senescence *in vivo*

Tumor Suppression and Promotion

Although research on cellular senescence has been ongoing for over 50 years, the role of senescence *in vivo* has only emerged in the last decade and is now accepted to harbor important functions in many physiological processes (Figure 1.1D). As cells enter irreversible cell cycle arrest upon oncogene activation, cellular senescence is an ideal mechanism for tumor suppression. Senescent cells were found in many premalignant neoplasm such as lung adenoma, pancreatic intraductal neoplasma, prostatic intraepithelial neoplasm and melanocytic naevi (Braig et al., 2005; Chen et al., 2005;

Collado et al., 2005; Michaloglou et al., 2005). Similarly, oncogenic BRAF(V600E) are expressed in senescent melanocytes and benign human naevi (Dhomen et al., 2009). On the other hand, markers of senescent cells are absent in malignant lesions such as lung carcinoma, pancreatic ductal adenocarcinoma, prostate carcinoma and melanoma (Chen et al., 2005; Collado et al., 2005; Gray-Schopfer et al., 2006). Mouse model with inactivation of tumor suppressor PTEN or expression of oncogenic NRAS induce senescence in malignant lesions similar to that of *in vitro* experiments and loss of p53 in those mouse led to development of malignant tumors (Braig et al., 2005; Chen et al., 2005). Member of the bromodomain and extra terminal domain (BET) family, BRD4, recognized H3K27ac at super enhancers of SASP-associated genes to regulates the expression of immune surveillance genes during OIS suggesting another mechanism by which senescence has a tumor suppression function (Tasdemir et al., 2016). Together, these data suggest that premalignant cells must bypass or escape senescence response to become malignant and cellular senescence is a possible evolutionary selected mechanism for tumor suppression.

Several evidences suggest that secretion of SASP factors by senescent cells can have a protumorigenic effect to promote cell proliferation in certain cell types such as epithelial cells. For example, senescent fibroblast can stimulate proliferation of mammary or prostate epithelial cells by secretion of C-X-C motif chemokine ligand 1 (CXCL1) or amphiregulin (AREG) known to promote growth in tumors cells (Bavik et al., 2006; Krtolica et al., 2001). Several SASP factors like IL-6 and IL-8 have been implicated in invasiveness of cancer cells (Coppe et al., 2008). Xenografts of senescent cells in mice increase the tumorigenicity of breast epithelial cells due to the secretion of matrix

metalloproteinase (MMP) that allow for increased access of mitogenic and chemotactic signals in these cells (Liu and Hornsby, 2007). Many of the SASP factors have varying physiological effects involving paracrine signaling that affects angiogenesis (Coppe et al., 2006), inflammation (Freund et al., 2010), and epithelial-to-mesenchymal transitions (EMT) (Laberge et al., 2012).

Tissue Repair

Cellular senescence has also been shown to play a role in tissue repair. Tissue damage in the liver stimulates hyperproliferation of the hepatic stellate cells (HSCs) that produces extracellular matrix to form fibrotic scars (Krizhanovsky et al., 2008). The hyperproliferation of HSCs results in entry into cellular senescence and reduced secretion of extracellular matrix and increased secretion of extracellular matrix degrading enzyme to limit fibrosis. The senescent HSCs also secrete or express factors that stimulate its own clearance by immune cells like natural killer (NK) cells although this is not universal as benign naevi can persist of decades without clearance (Michaloglou 2005). Similar function of senescent cells in limiting fibrosis was observed in pancreatic stellate cells (Fitzner et al., 2012). Cellular senescence also aid in tissue repair at sites of cutaneous wound healing. During this process, myofibroblast undergoes senescence induced by the extracellular matrix protein CCN1, which acts through integrin and heparin sulfate proteoglycan (HSPG)-mediated activation of the RAC1-dependent NADPH oxidase 1 to produce accumulation of ROS (Jun and Lau, 2010). Similarly to what was observed in hepatic and pancreatic stellate cells, CCN1-mediated senescence induced expression of anti-fibrotic genes that limits fibrosis.

Development

Recent studies suggest the role of cellular senescence in embryonic development. Senescent cells are present throughout mouse and human embryos, including embryonic kidney, endolymphatic sac of the inner ear, apical ectodermal ridge, and the neural roof plate (Munoz-Espin et al., 2013; Storer et al., 2013). Senescence during development is independent of DDR, p53 and p16 but express high levels of p21 and subsets of SASP proteins. The p21 expression in the embryo was induced by TGF- β /SMAD and PI3K/FOXO signaling pathways. Although speculative, it is believed that senescence during development fine-tunes and optimizes embryogenesis.

Organismal Aging

Cellular senescence seems to have many beneficial functions *in vivo* by regulating tumor suppression, tissue repair, and development. However, recent evidences suggest a detrimental role of cellular senescence as a contributor of organismal aging. As we are exposed to variety of senescence triggers throughout our life, senescent cells accumulate with age that in turn may promote or contribute to the aging process. The role of replicative senescence *in vivo* has been seen in livers with chronic hepatitis, cirrhosis and hepatocellular carcinoma (Paradis et al., 2001; Wiemann et al., 2002). Due to the fact that mice have long telomeres, mutant mice with decreased telomere length were more prone to liver cirrhosis suggesting the role of replicative senescence in liver damage (Rudolph et al., 2000). Frequency of senescent cells increase in skin of aging primates detected by increase in p16 expression and DDR markers co-localizing to the telomeres although others have implicated increase of senescence with age to be independent of telomere

length (Bhat et al., 2012; Herbig et al., 2006; Jurk et al., 2012). Increase in key tumor suppressors genes that regulates senescence like p19, p53, p21, and p16 have been observed in the fat, skeletal muscle, and eye of BubR1 progeroid mice that correlates with decline in physiological functions (Baker et al., 2008; Baker et al., 2013). Deletion of p21 improves intestinal crypt and bone marrow stem cell functions and increase longevity in mutant mice with short telomeres (Choudhury et al., 2007).

Examination of senescent knockout mice has been difficult as p16 knockout mice are more prone to tumorigenesis (Sharpless et al., 2001). However, recent methods to clear senescent cells by promoting them to undergo apoptosis in mice show improvement in healthspan and lifespan (Baker et al., 2016; Baker et al., 2011). Similarly, senolytics drugs that selectively kill senescent cells delayed age-associated disorders (Chang et al., 2016). Together, these finding suggest the importance of senescent cells in contributing to organismal aging and age-associated pathologies.

1.2 Polycomb Repressive Complex

DNA is compacted into nucleosomes consisting of octamers with two copies of histone proteins H2A, H2B, H3, and H4 to form chromatin in the cells. The chromatin structure contributes to the transcription of specific loci. The chromatin can either be open (euchromatin) to permit transcription or compacted (heterochromatin) to limit transcription (Chen and Dent, 2014). The chromatin structure is regulated by modifications to the histone tails, N-terminal region of histones, and DNA. There are varieties of post-translational modifications that occur at the histone tails including

acetylation, sumoylation, phosphorylation, methylation, and ubiquitination. PcG proteins were initially discovered by Edward Lewis as regulator of gene expression of developmental genes in *Drosophila melanogaster*. PcG proteins have gained significant attention as it plays critical role in development, cancer and stem cell biology by modulating both the methylation and ubiquitination of the histone tails.

PRC1 and PRC2 Components and Functions

Polycomb group proteins play a role in transcriptional repression and functions in complex referred to as Polycomb repressive complex (PRC). PcG proteins are highly conserved across species. It is composed of two major complexes, PRC1 and PRC2, although many studies have identified extreme diversity in these complexes. *Drosophila melanogaster* contains 15 Polycomb genes whereas mammals contain 37 Polycomb genes.

In mammalian systems, PRC2 is comprised of three major core proteins: enhancer of zeste homolog 2 (EZH2), embryonic ectoderm development (EED) and suppressor of zeste 12 (SUZ12) (Figure 1.2). EZH2 harbors the SET domain that is responsible of catalyzing the di- and trimethylation of H3K27, heterochromatic mark that function to compact chromatin and repress transcription. EED contains five WD40 repeats served to function in binding to both EZH2 and accessory PRC2 proteins for catalytic activity of PRC2. The carboxyl-terminal of EED has been shown to bind to pre-existing H3K27me3 mark leading to allosteric activation of EZH2 implicating its role in transmission of histone modification from mother to daughter cells (Margueron et al., 2009). SUZ12 is

also critical for the stability of the complex and is required for the catalytic activity of PRC2.

Major components of the PRC1 consist of the CBX (Chromobox; CBX2, 4, 6, 7, 8), RING1 (E3 ligase; RING1-2), PCGF (Polycomb group factor; PCGF1-6) and the PHC (Human polyhomeotic homology; PHC1-3) family (Figure 1.2). PRC1 complex functions to compact chromatin and to catalyze the monoubiquitination of histone H2A on lysine 119 (H2AK119ub1) by the E3 ubiquitin ligase RING1. CBX functions for proper targeting of the PRC1 complex to the genomic loci by binding to the H3K27me3 mark placed by the PRC2 complex. PCGF family enhances the activity of RING1 protein.

Due to the high number of paralogues for each of the PRC1 components, diverse PRC1 complexes exist with different functionality. PRC1 complexes can also form non-canonical PRC1 to repress transcription that lacks the CBX protein. For example, non-canonical RING1/YY1-binding protein (RYBP)-PRC1 that lacks CBX mediates ubiquitination of H2AK119 independently of H3K27me3 and is critical for the global maintenance of ESCs (Gao et al., 2012).

Gene Regulation by Polycomb Group

Polycomb group proteins regulate gene expression by several mechanisms. Genome-wide binding studies have shown the presence of PcG proteins on repressed genes and enriched at CpG islands close to the transcriptional start site (TSS) of many developmental genes. These CpG islands harbored both repressing

marks H3K27me3 and activating marks H3K4me3 referred to as bivalent domains and is believed to be present in genes loci for activation during cell-fate decision. The activities of both PRC1 and PRC2 result in chromatin compaction at these loci (Figure 1.3A). The compaction of the chromatin reduces the accessibility of many transcription factors and chromatin remodeling machineries like ATP-dependent chromatin remodeling complex SWI/SNF to inhibit transcription (Gebuhr et al., 2000).

The RING1 is the major component responsible for catalyzing the deposition of H2AK119ub1. Several studies have shown that RING1 and presence of H2AK119ub1 at bivalent domains interferes with transcriptional elongation by pausing or impeding the RNA polymerase II (RNAPII) at the TSS to limit the expression of differentiation genes (Figure 1.3A) (Stock et al., 2007; Zhou et al., 2008). H3K27me3 enriched at the proximal promoters, enhancers and gene bodies could also limit the RNAPII recruitment and activity (Jonkers and Lis, 2015).

Recent studies have also brought into question whether modulation of chromatin by PcG are responsible for initiating gene repression. Kinetics analysis of cells expressing oncogenic RAS induced transcriptional repression prior to H3K27me3 acquisition (Hosogane et al., 2013). Similarly, treatment of mouse embryonic stem cells with transcription inhibitors induced recruitment of PRC2 components and H3K27me3 to previously nucleosome-free CpG islands of untranscribed genes (Riising et al., 2014). These studies together suggest that establishment of PcG domain occurs only at sites where transcriptional silencing has already occurred and possibly are critical in the maintenance and not the initiation of transcriptional repression.

Although PcG complexes normally act as epigenetic repressors, some evidences suggest possible function as contributor to gene expression. Many components of PRC1 are enriched in transcriptionally expressed genes (Barradas et al., 2009; Pemberton et al., 2014). Switch in PRC1 subunits from CBX7 to CBX8 during ESC differentiation alters the PRC1 role from repressor to activator of developmental genes (Creppe et al., 2014). Non-canonical KDM2B-PRC1 complex are recruited to unmethylated CpG islands. KDM2B functions as a demethylase for H3K36me3 (euchromatin mark) and are enriched at promoters of actively transcribed genes (Farcas et al., 2012; He et al., 2013; Wu et al., 2013).

How does PRC1 activate gene expression? The Aurora kinase B (AURKB) occupies target genes with PRC1 and phosphorylates and activates ubiquitin specific peptidase 16 (USP16) that deubiquitinate H2AK119ub1 allowing recruitment of RNAPII for gene transcription (Figure 1.3B) (Creppe et al., 2014). AURKB also phosphorylates UBE2D3 that inhibits the ubiquitin ligase activity of RING1 (Frangini et al., 2013). Another mechanism involves non-canonical PRC1 complex where casein kinase 2 (CSNK2A1 also referred to as CK2) phosphorylates and inactivates RING1B. The non-canonical PRC1 can also complex with autism susceptibility candidate 2 (AUTS2) recruiting p300 for acetylation of histone tail and successive transcription activation (Figure 1.3B) (Gao et al., 2014).

Recruitment of Polycomb Group

There has been large effort to understand the mechanism of how PcG complexes are recruited to target genes. In *Drosophila melanogaster*, PcG proteins are recruited to

specific DNA elements that are a few hundreds base pairs long referred to as Polycomb repressive element (PRE) (Chan et al., 1994; Simon and Kingston, 2009). Currently, out of the thousands of PcG binding regions, only two functional PRE-like DNA elements have been identified in mammals and may have adapted different mechanisms in recruiting PcG complexes.

PRC1 complexes are recruited to specific loci by two major mechanisms. The canonical PRC1 complexes are dependent on PRC2 activity in part by the binding of CBX chromodomain to H3K27me3 (Figure 1.4A). However, PRC1 can also be recruited independently of PRC2 activity by the non-canonical PRC1 complexes that do not contain the chromodomain-containing CBX protein. This is evident as genome-wide distribution of PRC1 do not completely overlap with PRC2 or H3K27me3 (Gao et al., 2012). These PRC1 complexes bind to sequence specific DNA-binding proteins such as KDM2B, RYBP, FBXL10 or E2F6, which drive the localization of the PRC1 to specific genomic loci (Figure 1.4B) (Gao et al., 2012; Ogawa et al., 2002; Wu et al., 2013).

How are the PRC2 complex recruited? Genome-wide studies have shown enrichment of PRC2 proteins at the CpG islands (Tanay et al., 2007). Introduction of artificial GC-rich elements from bacterial genome into the mammalian genome was able to recruit PRC2 but not PRC1 complex (Mendenhall et al., 2010). Currently, there has been three main mechanisms by which PRC2 complexes are recruited although these mechanisms are not mutually exclusive.

First mechanism is the DNA-based recruitment where PRC2 components interact with DNA-binding proteins (Figure 1.4C). Many of the PcG have been shown to interact with sequence specific DNA-binding factors such as Ying and Yang 1 (YY1), RE1-

silencing transcription factor (REST), zinc-finger protein SNAI1, RUNT-related transcription factor 1 (RUNX1), or zinc-finger domains (ZNF518) (Dietrich et al., 2012; Herranz et al., 2008; Maier et al., 2015; Yu et al., 2012). This is an attractive model as many of these transcriptional regulators have high specificity for specific loci and can interact with PRC2 components. On the other hand, since the interaction between PRC2 components and the transcription factors are transient, this mechanism may only function for the initial recruitment of PRC2 and may not account for the propagation and maintenance of the histone modification. Recruitment of PRC2 to the CpG islands is initiated by interaction with PRC2-associated members like JARID2 or AEBP2 that also have DNA-binding capacity. AEBP2 contain the zinc-finger domain for binding to GC-rich region. Amongst the PRC2-associated factors that may be responsible for recruitment of PcG to the specific loci, JARID2 is the most studied factor. JARID2 is part of the Jumonji family group that contains the JmjC domain known for demethylase activity; however, JARID2 lacks the crucial residues for cofactor binding and is catalytically inactive (Shen et al., 2009b). JARID2 is highly enriched at PRC2 targets and loss of JARID2 results in reduction in PRC2 binding to target genes, although the recruitment was not completely abolished suggesting that this cannot be the only recruitment mechanism (Landeira et al., 2010; Shen et al., 2009b).

The second mechanism of how PcG complexes are recruited is mediated by pre-existing histone modifications (Figure 1.4D). For example, PRC2 was found to occupy subset of genes together with H3K9me3 methyltransferase EHMT2 in ESCs and EED can bind to histone methylation catalyzed by EHMT2 (Mozzetta et al., 2014). Acetylation of H3K27 (H3K27Ac) by acetyltransferases CBP and p300 results in the specific loci to

become nonpermissive for PRC2 binding and deacetylation by Nucleosome Remodeling Deacetylase (NuRD) facilitate recruitment of PRC2 (Reynolds et al., 2012). PRC2 can also be recruited to actively transcribed genes mediated by Polycomb-like (PCL) protein that binds to H3K36me3 possibly to recruit PRC2 to set up repression of the specific loci (Ballare et al., 2012; Cai et al., 2013).

Third mechanism is the role of noncoding RNAs (ncRNA) in recruitment of PcG complexes (Figure 1.4E). Noncoding RNAs can act as a scaffold to assemble complexes. During inactivation of X chromosome during dosage compensation, ncRNA X inactive specific transcript (*XIST*) recruits the localization of PRC2 components. ncRNA HOX transcript antisense RNA (*HOTAIR*) from the HOXC cluster can bind to PRC2 and recruit the complex in *cis* to HOXC genes and in *trans* to HOXD genes (Rinn et al., 2007). *HOTAIR* can simultaneously bind to KDM1A, H3K4 demethylase, to coordinate the demethylation at these bivalent domains (Tsai et al., 2010). The antisense long non-coding RNA (lncRNA) *ANRIL* binds to CBX7 at the *INK4a/ARF* locus to regulate the expression of p16 for regulation in cell cycle progression (Yap et al., 2010).

Regulation of PcG Complexes

The activities of PcG complexes are modulated at different levels. First, the surrounding chromatin environment regulates PRC2 activity. The presence of H3K4me3, H3K36me2 and H3K36me3 decreases the activity of PRC2 complex (Figure 1.5A) (Schmitges et al., 2011; Yuan et al., 2011). On the other hand, presence of H3K27me2 and H3K27me3 induces PRC2 activity to maintain the heterochromatic environment.

Second, there is variety of factors that binds to the *EZH2* promoter to drive its expression (Figure 1.5B). The MEK-ERK-ELK1 pathway was responsible for EZH2 overexpression in ERBB2-overexpressing breast cancer where ELK1 binds to *EZH2* promoter for expression of EZH2 (Fujii et al., 2011). The pRB-E2F also has been implicated in EZH2 transcription upon discovery of E2F binding motif at the EZH2 promoter (Bracken et al., 2003). Other transcription factors like MYC and ETS directly regulate EZH2 transcription in prostate cancer (Koh et al., 2011b; Kunderfranco et al., 2010). Similarly NF- κ B and STAT3 regulate EZH2 expression in variety of cancer cells (Duan et al., 2013; Xu et al., 2016). The hypoxia-inducible factor 1 α (HIF1 α) also binds to the consensus sequence of hypoxia response element (HRE) within the EZH2 promoter in breast cancer (Mahara et al., 2016).

There have been emerging evidences that multiple micro-RNAs (miRs) interact with the EZH2 3'UTR to repress the abundance of EZH2 protein. The miR-25, -26a, -30d, -98, -101, -124, -137, -138, -144, -214 and let-7 are downregulated or mutated in different aggressive cancers (Sun et al., 2016a) (Figure 1.5C). EZH2 can also be phosphorylated at serine 21 by AKT to suppress EZH2 methyltransferase activity by binding and methylating the androgen receptor to regulate androgen-receptor target genes instead of the PcG-mediated genes (Cha et al., 2005; Xu et al., 2012) (Figure 1.5D).

Transmission of Polycomb Domain

One of the major challenges in maintaining chromatin integrity is during DNA replication. The segregation of histones is cooperative and dispersive where histones are transferred behind the replication fork by histone chaperones equally and randomly

between the daughter strands. These events occur close to the replication machinery as nucleosomes are present 200-300 bases behind the replication fork on both the leading and lagging strands (Xu et al., 2010). Most of the H3-H4 is transferred onto the DNA as intact (H3-H4)₂ tetramer whereas the newly synthesized cytoplasmic histones are imported into the nucleus together with Anti-Silencing Factor 1 (ASF1) histone chaperone and transferred to Chromatin Assembly Factor 1 (CAF-1) chaperone onto the replicating DNA as H3-H4 dimers. CAF-1 binds with the PCNA scaffold ring and assembles the (H3-H4)₂ tetrasome intermediate (Liu et al., 2012; Shibahara and Stillman, 1999). Currently, it is not exactly known how the two juxtaposed H2A-H2B dimers are transferred to the new chromatin.

How does post-translational modification (PTM), specifically involving PcG propagate during DNA replication? Several studies have shown that PRCs are able to bind to the histone modifications that were placed by the PRCs themselves. For example, the carboxyl-terminal of EED can bind to H3K27me₃ to stimulate further trimethylation of H3K27 within neighboring histones by allosteric activation of EZH2 (Margueron et al., 2009). The PTMs from the inherited parental histones are thought to regulate the establishment of histone marks over maturing chromatin. In *Drosophila melanogaster*, there is an increase in recruitment of PcG proteins and H3K27me₃ mark prior to replication to possibly compensate for the dilution of these factors after replication (Lanzuolo et al., 2011). PcG proteins were found to be associated within 200bp of the replication fork on the nascent DNA (Petruk et al., 2012). This observation was consistent with mammalian cells where PRC2 proteins were purified at the replication fork.

Polycomb Group and Senescence

Several reports have implicated PcG proteins in cellular senescence. The key observation was that the PcG proteins suppress p16 expression in normal cells by laying down repressive marks along the *INK4A/ARF* loci like H3K27me3 and H2AK119ub1 by PRC1 and PRC2 complexes, respectively (Jacobs et al., 1999). During cellular senescence however, PcG proteins are downregulated leading to loss of the H3K27me3 mark along the *INK4A* locus resulting in the upregulation of p16 (Agger et al., 2009; Barradas et al., 2009; Bracken et al., 2007; Jacobs et al., 1999).

Importance of PcG in regulation of p16 has been exemplified *in vivo*. Since PcG proteins regulate the expression of many developmental genes, knockout (KO) mice of PRC2 members are embryonically lethal. PRC1 KO mice that survive gestation present defects that are regulated by PcG regulation of p16. *Bmi1* KO mice show decrease in number of hematopoietic cells that are unresponsive to mitogens and show neurological abnormalities from proliferative defects in neural progenitor and neural stem cells (van der Lugt et al., 1994). Removal of the p16 in *Bmi1* KO mice show improvement in many of the defects suggesting the importance of p16 regulation by PcG components (Bruggeman et al., 2005; Jacobs et al., 1999). Overexpression of CBX proteins represses the *INK4A/ARF* locus and bypasses senescence and mutation in the chromodomain region of CBX protein results in premature senescence (Gil et al., 2004). Additionally, CBX7 binds to *ANRIL* to be recruited to the *INK4a/ARF* locus to regulate proliferation in prostate cancer (Yap et al., 2010). CBX7 also binds to RNA helicase MOV10 and was involved inhibiting transcription of p16 (El Messaoudi-Aubert et al., 2010).

Since PRC2 KO mice are embryonically lethal, many studies using conditional KO mice have been utilized. For example, loss of EZH2 in pancreatic islet induced expression of p16 in β -cell that resulted in diabetic phenotype (Chen et al., 2009). However, in some tissues, homolog EZH1 plays a compensatory role upon loss of EZH2. Deletion of EZH2 in the brain and skin resulted in only mild defects whereas loss of both EZH1 and EZH2 in skin led to severe defects (Ezhkova et al., 2011; Pereira et al., 2010).

Genome-wide enrichment analysis of H3K27me3 between proliferating and senescent cells shows increase enrichment of H3K27me3 together with LMNB1 in small subset of gene-rich regions where gene expression becomes repressed (Sadaie et al., 2013). Another group observed loss of H3K27me3 at gene-rich regions and enhancers and increase in H3K27me3 and H3K4me3 at lamin B1-associated domains (LADs) (Shah et al., 2013).

Polycomb Group in Cancer

Overexpression and aberrant function of EZH2 have been implicated in cancer. Many studies have focused on gain-of-function mutations of EZH2 in B-cell lymphoma. About 7% of large follicular lymphomas and 22% of diffuse B-cell lymphoma harbor mutation in tyrosine 641 (Y641) of the EZH2 SET domain resulting in high levels of H3K27me3 mark (Sneeringer et al., 2010). Transgenic mice harboring the EZH2 (Y641F) mutation in lymphocytes develop lymphoma when combined with MYC expression (Yap et al., 2011). Other gain-of-function mutations have been observed to occur at the EZH2 SET domain such as alanine 677 (A677G) and alanine 687 (A687V) in lymphomas (Majer et al., 2012; McCabe et al., 2012a). Increase in recruitment of PRC2 to the chromatin has

also been implicated in cancer progression. Overexpression of lncRNA like *HOTAIR* increases invasiveness and metastatic potential of epithelial cancer cells (Rinn et al., 2007).

It is also important to note that not all cancers are associated with gain-of-function EZH2 mutations. Myelodysplastic syndrome frequently harbor missense and frameshift mutations that suggests that EZH2 can also function as a tumor suppressor (Nikoloski et al., 2010). Loss-of-function EZH2 mutations are also detected in pediatric cancers such as acute myeloid leukemia and T-lineage acute lymphoblastic leukemia (Muto et al., 2013). Glioblastoma multiforms (GBM) and diffuse intrinsic pontine gliomas (DIPG), frequently harbor lysine to methionine substitution at position 27 (K27M) in histone H3.3 resulting in loss of H3K27me3 by acting as a dominant negative inhibitor of EZH2 to interfere with its methyltransferase activity (Lewis et al., 2013).

Polycomb Group in Aging

It is well established that senescent cells increase with age. Loss of H3K27me3 was seen in cells from Hutchinson-Gilford Progeria Syndrome (HGPS) patients, disease resembling aspects of premature aging (Shumaker et al., 2006). Analyses of H3K27me3 methyltransferase and demethylase expressions in aging human brains show significant decrease in EZH2 expression while expression of one of the H3K27me3 demethylase UTX increases with age (Jin et al., 2011; Lu et al., 2004). Some of these features seem to be evolutionarily conserved. In *C. elegans*, *utx-1* activity increases with age while H3K27me3 levels decrease in somatic cells and knockdown or heterozygous mutation of *utx-1* extends lifespan that is dependent on the insulin-FoxO signaling pathway (Jin et al.,

2011; Maures et al., 2011; Ni et al., 2012). However in *Drosophila melanogaster*, mutation in PRC2 components increased longevity and stress resistance due to reduced H3K27me3 levels possibly suggesting beneficial roles of reduced PcG activity at the system-level (Siebold et al., 2010).

1.3 WNT Pathway

WNT is family of signaling proteins that are evolutionarily conserved implicated in proliferation, cell fate determination, development and tissue renewal in adults. There are 19 different genes encoding the WNT ligands in humans that are expressed in tissue-specific manner. Binding of the WNT ligand to the receptor activates downstream signaling cascade and activation through β -catenin is the defining factor between canonical and non-canonical WNT pathways.

Canonical WNT Signaling

In the canonical WNT pathway, extracellular WNT ligands bind to the Frizzled family of receptors (FZD) and the LRP5/6 co-receptor leading to the activation of Dishevelled (DVL) that inhibits the β -catenin destruction complex composed of glycogen synthase kinase-3 β (GSK-3 β), adenomatosis polyposis coli (APC) and AXIN. β -catenin then accumulates in its unphosphorylated form, relocalizes into the nucleus in complex with DNA-binding LEF/TCF to promote the transcription of a variety of target genes, including *MYC* and *CCND1*. Extracellular inhibitors like DKK1 or sFRP can antagonize

WNT signaling. In absence of WNT ligand, the destruction complex proteins degrade β -catenin through ubiquitin-mediated proteolysis.

WNT Signaling and Senescence

The role of WNT signaling has been implicated in cellular senescence and has been shown to contribute to the change in chromatin structure observed during senescence (Ye et al., 2007). In human diploid fibroblasts, *WNT2* is the only WNT ligand to be downregulated in both replicative senescence and OIS. *WNT2* normally regulates the canonical WNT pathway and this was supported by increase in GSK-3 β kinase activity and reduction in active β -catenin levels in senescent cells. Ectopic repression of *WNT2* is sufficient in inducing premature senescence. It was proposed that increased GSK-3 β activity phosphorylates HIRA, triggering its recruitment to PML body for further activation of the SAHF signaling cascade (Figure 1.6). Conversely, activation of WNT signaling by addition of WNT3a delays onset of RAS-induced senescence. Similarly, inhibition of GSK-3 β with lithium treatment reduces accumulation of p53 and induces the cells to become quiescent instead senescent (Zmijewski and Jope, 2004). Overexpression of *WNT3A* in pancreatic stem cells induces expression of proliferating gene like *MYC* and increases proliferation and toleration to mitochondrial ROS (He et al., 2015).

However, there have been several reports of WNT signaling in promoting senescence. Expression of constitutively active *β -catenin* activates p53 to induce senescence-like phenotype in MEF (Damalas et al., 2001). In the same study, *Klotho* knockout mice show accelerated aging phenotypes, which were proposed to be as a result

of the increased WNT activity due to the loss of interaction between secreted Klotho and WNT ligands. Additionally, activation of WNT signaling increases ROS in mesenchymal stem cells (MSC) and expresses markers of senescence such as p21, p53, p16 and increase in DNA damage (Zhang et al., 2013). MSCs treated with old rat serum induces senescence, which is rescued by treatment with WNT inhibitor like DKK 1 or knockdown of *β-catenin* suggesting that MSCs from old age induces senescence by excessive activation of WNT signaling (Zhang et al., 2011). Large-scale RNAi screen for mitochondrial abundance revealed WNT as potent activator of mitochondrial biogenesis and ROS production, which is dependent on the insulin receptor substrate-1 (IRS-1) in mouse muscle cells (Yoon et al., 2010).

WNT and Aging

Canonical WNT signaling has been implicated in positively regulating the self-renewal of stem cells and progenitor cells to delay the onset of age-related alterations. For example, activation of WNT signaling improves adult stem cell maintenance, neurogenesis and regenerations in adult mice (Ring et al., 2014). Analysis of 84 WNT-related genes in aging lung and brain indicated a general decrease in WNT signaling with age (Hofmann et al., 2014). Reduced WNT signaling has been also associated with many age-related diseases. For example, common genetic variation within the *LRP6* gene and decreased *β-catenin* signaling are associated with late-onset of Alzheimer's disease (AD) (De Ferrari et al., 2007). The connection between WNT signaling and Alzheimer's disease has received attention as GSK-3 β is the major kinase that phosphorylates tau (MAPT) protein and increase in phosphorylated GSK-3 β precedes many of the pathophysiological

changes that occurs in AD (Inestrosa et al., 2007). Similarly, aged rats have increased levels of GSK-3 β phosphorylation in pyramidal cells in the cortical region of the central nervous system possibly contributing to the learning and memory deficits with age (Lee et al., 2006b). Reduction in WNT-mediated signaling by mutation in the *LRP5* co-receptor is associated with osteoporosis-pseudoglioma syndrome (Gong et al., 2001). Mutation in the *LRP6* was observed in families with early coronary diseases (Mani et al., 2007). Decrease in WNT signaling in aging mouse heart showed correlation with many of the phenotypes seen in with cardiac aging such as decreased regenerative capacity, cell death, angiogenesis, and cardiac remodeling (Ashton et al., 2006). There is also increase in the secretion of WNT antagonist, sFRP2, in aged dermal fibroblasts that decreases β -catenin and ultimately results in loss of key redox effector, APE1 (Kaur et al., 2016). The loss of APE1 attenuates the response of these cells to DNA damage and thus proposes increased resistance to targeted therapy in older cancer patients.

However, increased WNT signaling has also been implicated in pro-aging phenotypes. Aging in muscle stems cells (satellite cells) show conversion from myogenic to fibrogenic lineage that is induced by canonical WNT signaling and can be suppressed by WNT inhibitors (Brack et al., 2007). This conversion is associated with reduced levels of injury-induced regeneration, increase in fibroblasts and decrease in differentiation of muscle cells observed in old mice. Along the same lines, *klotho* knockout mice featuring premature aging phenotypes have elevated WNT signaling and accelerated cellular senescence in stem cell compartments of hair follicles, bone marrow and intestinal crypts (Liu et al., 2007). Analysis of WNT ligands in *Caenorhabditis elegans* shows that all five WNT proteins are expressed past the development stages. In general WNT signaling

activity increases as the worm ages but is dependent on the specific WNT ligands. Subsets of the WNT ligands were found to have pro-aging effects and others possess anti-aging effects on lifespan (Lezzerini and Budovskaya, 2014). In *Drosophila melanogaster*, overexpression of *wingless* expressed conditionally in adult flies decreases lifespan both in males and females (Shen et al., 2009a). Overexpression of constitutive active β -*catenin* leads to rapid exhaustion of hematopoietic stem cells in mice (Kirstetter et al., 2006; Scheller et al., 2006).

1.4 MYC

The proto-oncogene *MYC* belongs to family that includes *MYCL* and *MYCN*. *MYC* was originally discovered from chicken tumor by oncogenic retrovirus identified as *v-myc* that caused spontaneous myelocytomatosis (Duesberg et al., 1977; Hu et al., 1979). The mammalian *MYC* was later discovered in Burkitt's lymphoma as a human oncogene (Dalla-Favera et al., 1982).

Regulation of MYC

MYC is under tight transcriptional control by whole host of transcription factors (Levens, 2010). One prominent transcription factor known to regulate *MYC* is *TCF4* downstream of the WNT pathway. Single-nucleotide polymorphisms (SNPs) at the *TCF4* binding site of *MYC* enhancer are commonly observed in multiple cancers (Tuupanen et al., 2009). Defects in the canonical WNT signaling in colon carcinoma results in enhanced transcriptional activation of *MYC* (He et al., 1998). BRD4, containing the bromodomain

and extra terminal domain (BET), can also bind to *MYC* promoter (Loven et al., 2013). *MYC* is downstream of other growth mediated signaling such as *ERK/MAPK* signaling and T cell receptor signaling. In T cell leukemia, *MYC* acts downstream of the NOTCH signaling pathway (Palomero et al., 2006)

The half-life of *MYC* mRNA is extremely short, regulated by miRNAs like let-7, miR-34, and miR-145 (Christoffersen et al., 2010; Sachdeva et al., 2009; Sampson et al., 2007). *MYC* protein also has a short half-life (15-20 minutes) as they are ubiquitinated and degraded (Gregory and Hann, 2000). Phosphorylation of *MYC* at Ser62 and Thr58 stabilizes *MYC* protein and increases *MYC* transcriptional activity (Asano et al., 2004; Seth et al., 1991). These residues are common sites for mutation in many cancer cells.

Transcriptional Regulation by *MYC*

MYC protein binds to *MAX* through the carboxyl-terminal helix-loop-helix-leucine zipper (HLH-LZ) region to form *MYC-MAX* heterodimer. Reports of *MYC* activity in *MAX*-independent mechanism have also been observed in pheochromocytoma and *Drosophila* cells (Hopewell and Ziff, 1995; Steiger et al., 2008). *MYC-MAX* heterodimer binds to CACGTG binding motifs referred to as E-boxes with high affinity (Blackwood and Eisenman, 1991; Park et al., 2004). There are also non-canonical E-boxes with varying sequences. Recent studies show that *MYC* occupies promoters and enhancers to amplify expression of all active transcripts without any specificity referred to as the amplifier model (Lin et al., 2012; Nie et al., 2012). This theory has been questioned by two studies showing that *MYC* regulates specific subset of target genes and the amplification of active transcripts observed in previous studies were possibly due

to MYC regulation of genes involved in nucleotide synthesis that can exert global effects on RNA and DNA synthesis (Sabo et al., 2014; Walz et al., 2014). However, all these studies agree that if MYC is abnormally abundant, it can bind to active enhancers and promote gene expression through chromosome looping to bring distant sections of DNA close together.

MYC can regulate gene expression through different mechanisms. Genome-wide studies on MYC binding sites suggest that *MYC* is a general transcriptional factor and can possibly bind to 15% of all promoters (Cawley et al., 2004). However, MYC requires additional factors for expression of target genes and MYC at the promoter is not sufficient to drive expression of corresponding gene (Li et al., 2003; Zeller et al., 2006). Along the same lines, binding profiles of key iPSC factors MYC, SOX2, OCT4, and KLF4 in ESCs suggests that gene whose expression changes the most are bound by multiple factors (Kim et al., 2010). The MYC-MAX heterodimer can also recruit complexes that modify chromatin like transformation/transcription domain-associated protein (TRRAP) that further recruits histone acetyltransferase GCN5 permitting transcription of target genes (Liu et al., 2003; McMahon et al., 2000).

Although MYC is known mostly for gene expression, MYC can also mediate gene repression. For example, MYC can form complex with MIZ1 sequestering MIZ1 from the initiator element to mediate the repression of MIZ1-target genes (Walz et al., 2014). Another mechanism MYC mediates gene repression is through activation of miRNAs. MYC activates miR-17-92 cluster that mediates number of biological activities of MYC like attenuation of E2F1 and PTEN activity (Chang et al., 2008). MYC can also repress networks of miRNAs including let-7 to mediate insulin signaling, miR-23 to

mediate glutaminase expression, and miR-34a to mediate lactate dehydrogenase (LDHA) expression (Gao et al., 2009; Xiao et al., 2016).

***MYC* and Cancer**

Since *MYC* is deregulated in many cancer cells, the role of *MYC* in initiating transformation of normal cells into cancer cells have been proposed for many years. Initial *in vitro* studies showed that *MYC* is required to transform normal embryonic fibroblasts into tumor cells upon expression of *RAS* oncogene (Land et al., 1983). A transgenic mice with deregulated *MYC* form tumor in many tissues (Adams et al., 1985; Leder et al., 1986). Many other studies have shown *MYC* as important regulator in cancer phenotypes like proliferation, differentiation, and self-renewal. However, overexpression of *MYC* alone is not sufficient to induce tumorigenesis. *MYC*-induced tumorigenesis is restrained through different mechanisms such as apoptosis, proliferation arrest, or cellular senescence. Therefore, tumorigenic potential upon overexpression of *MYC* is dependent on deregulation of additional factors. *MYC* overexpression in normal cells triggers activation of tumor suppressor checkpoint by *ARF-p53* pathway and mutations in this pathway are required for transformation to occur (Eischen et al., 1999). Regardless, *MYC* seems to play vital role in initiation of tumor formation.

MYC may also contribute to the maintenance of tumors. Downregulation of *MYC* in cancer cell lines reduced proliferation (Cappellen et al., 2007; Koh et al., 2011a). Partial reduction of *MYC* using conditional *MYC* mice regresses tumors referred to as *MYC* addiction (Arvanitis and Felsher, 2006; Sodir and Evan, 2011). Even brief

suppression of *MYC* is sufficient to reverse tumorigenesis in some cells (Jain et al., 2002).

Many of the signaling pathways that regulate *MYC* are also deregulated in many cancers. For example, constitutive activation of *MYC* can occur as a result of deregulation in the upstream activators like in WNT. Elevated and constitutive *MYC* expression could potentially drive promiscuous activation of E-box-dependent genes to alter normal non-proliferative cells to become tumorigenic.

MYC not only regulates proliferation but many features of cancer cells such as increased genomic instability, epithelial-mesenchymal transition (EMT), and increased ability to metastasize (Karlsson et al., 2003). For example, overexpression of *MYC* can repress genes involved in cell-cell contact inhibition (Dang et al., 2006). *MYC* regulates EMT through regulation of miR-9 that targets E-cadherin and activation of *BMII* (Ma et al., 2010; Song et al., 2009). *MYC* also increases ROS production by increasing mitochondrial biogenesis and metabolism, which can also trigger genomic instability (Egler et al., 2005).

***MYC* in Senescence**

Tumor regression due to inactivation of *MYC* can be associated with cellular senescence (Wu et al., 2007). *MYC* antagonizes senescence-associated gene expressions. *MYC* directly regulates genes involved in the cell cycle, like *CDK4* or *CCND1/2* (Hermeking et al., 2000; Orian et al., 2003). *MYC* also represses cell cycle inhibitors like p15, p53, and p21 by binding to MIZ1 and inhibiting *MIZ1*-mediated transactivation at the respective promoters (Seoane et al., 2002; Staller et al., 2001; Wu et al., 2003). Transgenic mice

expressing transcriptionally active mutant of *MYC* (*MYCV394D*), deficient in binding to MIZ, show delayed lymphoma development due to increase in p16 expression and onset of senescence that is dependent on *TGF β* signaling (van Riggelen et al., 2010). Human diploid fibroblasts expressing *hTERT* harboring one copy of *MYC* results in onset of premature senescence by expression of p16, which was dependent on reduced expression of PRC1 member *BMII* (Guney et al., 2006).

MYC has also been shown to antagonize senescence in non-cell-autonomous mechanisms. Reduction in *MYC* in mouse model lacking both mature B- and T-cells failed to senesce suggesting that immune response are critical mediator of cellular senescence *in vivo* (Rakhra et al., 2010). Since *MYC* has been shown to inhibit T-cell immune recognition by suppressing *NF- κ B* and interferon-related antigen presentation, *MYC* inactivation provides pro-senescent T-cell activation (Schlee et al., 2007).

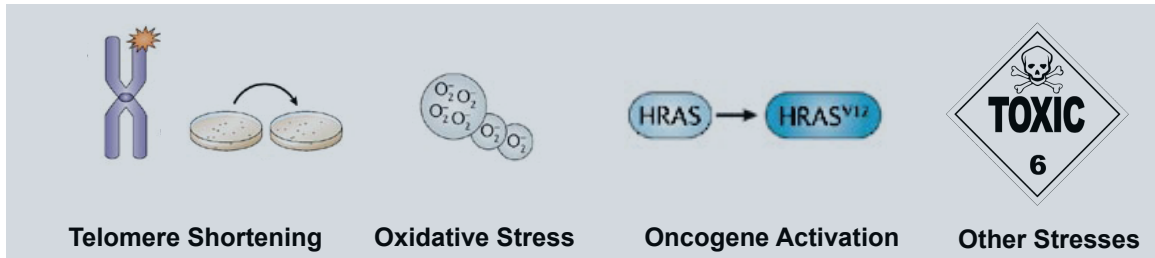
In contrast to what is known about other oncogenes like *RAS* in initiating cellular senescence, *MYC* overexpression does not induce senescence by itself in most cases. *MYC* overexpression can induce senescence in cells in concert with other alterations like absence of the Werner gene (*WRN*) (Grandori et al., 2003). *MYC* overexpression in non-transformed cells deficient of *CDK2* are susceptible to cellular senescence (Campaner et al., 2010). Aberrant *MYC* activity or overexpression has also been implicated in many senescence-associated features like DNA damage from increased ROS, inappropriate DNA synthesis and abrogation of DNA repair (Karlsson et al., 2003; Tanaka et al., 2002; Vafa et al., 2002).

***MYC* in Aging**

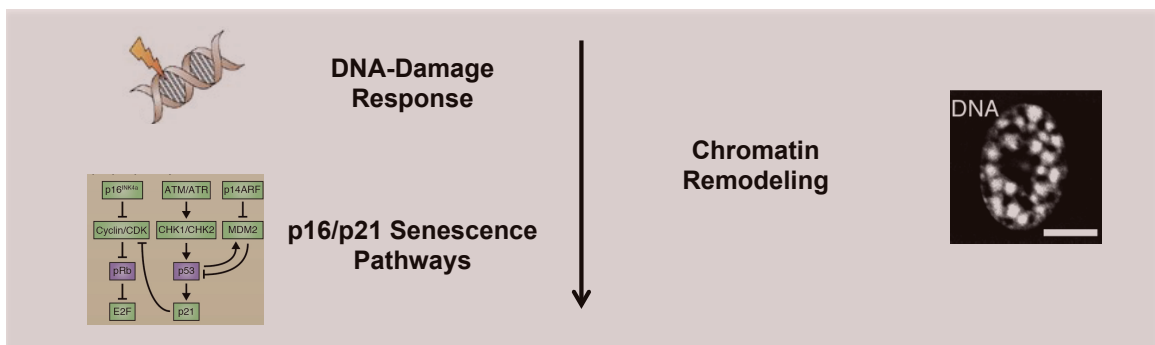
Myc^{+/-} mice extended median and maximum lifespan compared to control in both sexes and ameliorated many age-associated phenotypes such as cardiac fibrosis, osteoporosis, lipid metabolism and motor performance (Hofmann et al., 2015). The protection against aging in many tissues suggests that *Myc*^{+/-} mice were aging slower and was not due to preservation of function in specific tissues or prevention of specific diseases. Similarly, reduced *Myc* activity in *Drosophila melanogaster* extended lifespan suggesting the evolutionary conservation of *Myc* in the aging process (Greer et al., 2013).

Figure 1.1

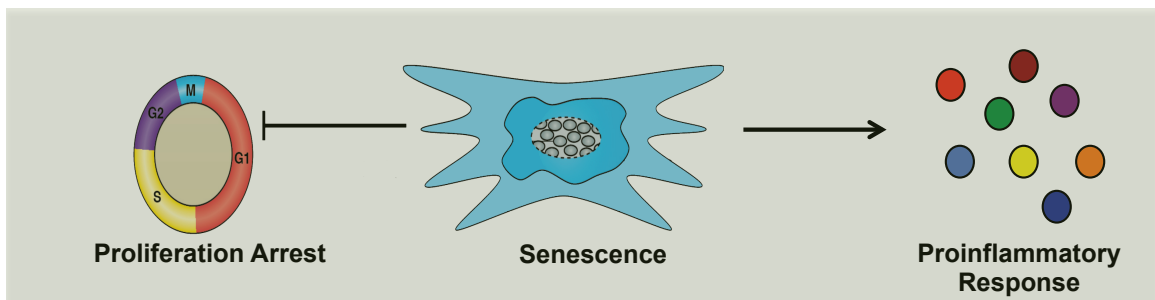
A Triggers



B Effector Pathways



C Phenotype



D Physiological Function

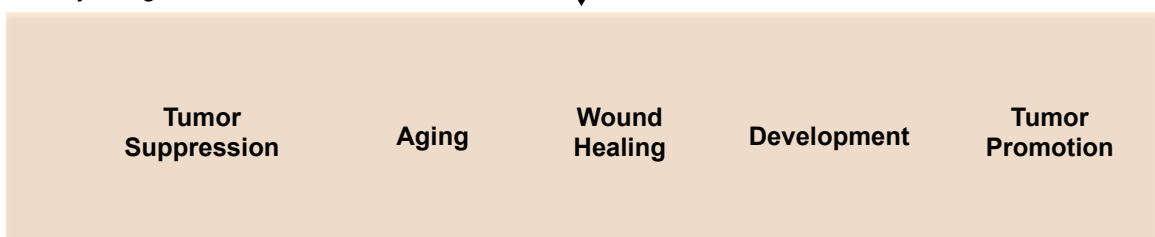


Figure 1.1 Senescence Model

(A) Senescence is triggered by different stimuli such as telomere shortening resulting in telomere dysfunction, oxidative stress from production of reactive oxygen species, expression of oncogenes or suppression of tumor suppressors, and intrinsic and extrinsic stresses to inhibit the proliferation of damaged or potentially dangerous cells.

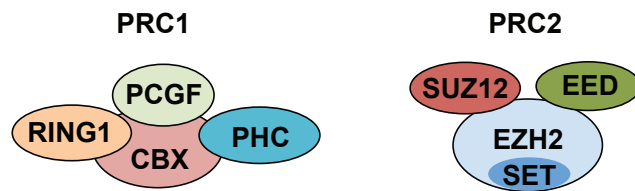
(B) Many triggers induce DNA damage initiating the DNA-damage response (DDR) pathway, which stabilizes p53 to activate its downstream transcriptional target p21. Senescence is associated with epigenetic derepression of p16 locus. There are also global epigenetic changes exemplified by formation of senescence-associated heterochromatin foci (SAHF).

(C) Main feature of cellular senescence is the onset of irreversible proliferation arrest accompanied by morphological changes. Senescence cells initiate a complex pro-inflammatory response known as senescence-associated secretory phenotype (SASP). SASP is the secretion of cytokines, chemokines, growth factors, and proteases.

(D) Senescence plays a role in tumor suppression to inhibit the proliferation of cells expressing oncogenes or suppressing tumor suppressors. Senescent cells are prevalent in lesion of premalignant tissues. The frequency of senescent cells increases with age in many tissues and contributes to age-associated phenotypes. Senescence occurs during development in mesonephros, endolymphatic sac and apical ectodermal ridge through variety of signaling pathways that converge on p21. Subsets of SASP factors have pro-tumorigenic effects on specific cell types like endothelial cells.

Figure adapted from (Pawlikowski et al., 2013a)

Figure 1.2



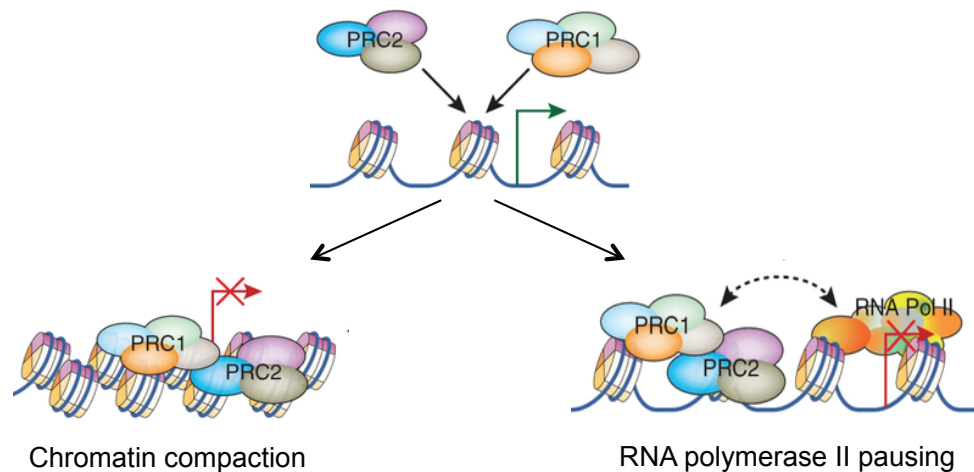
PRC1		Function
RING	RING1A	- Ubiquitinates H2AK119
	RING1B	
CBX	CBX2	- Chromodomain that binds H3K27me3 and RNA
	CBX4	
	CBX6	
	CBX7	
	CXB8	
PCGF	PGCF1 (NSPC1)	- Enhancer of RING1A and RING1B activity
	PGCF2 (MEL18)	
	PGCF3	
	PGCF4 (BMI1)	
	PGCF5	
PHC	PGCF6 (MBLR)	
	PHC1	
	PHC2	
	PHC3	
PRC2		Function
EZH	EZH1	- Di- and trimethylation of H3K27
	EZH2	
EED		- Binds to H3K27me3 - Required for catalytic activity of PRC2
SUZ12		- Stability of complex - Required for catalytic activity of PRC2

Figure 1.2 Compositions and Functions of Polycomb Complexes

The core Polycomb repressive complex 1 (PRC1) is composed of CBX, RING1, PCGF and PHC family of proteins. PRC1 plays a role in ubiquitination of H2AK119, binding to the H3K27me3 mark, compacting the chromatin, and blocking RNA polymerase II elongation. The core PRC2 components include EZH2, EED and SUZ12 and are important for methylation of H3K27 and chromatin compaction. The diversity of Polycomb complexes is achieved by incorporation of homologous proteins as well as binding to other factors to form non-canonical PRC complexes.

Figure 1.3

A Polycomb-mediated transcriptional repression



B Polycomb-mediated transcriptional activation

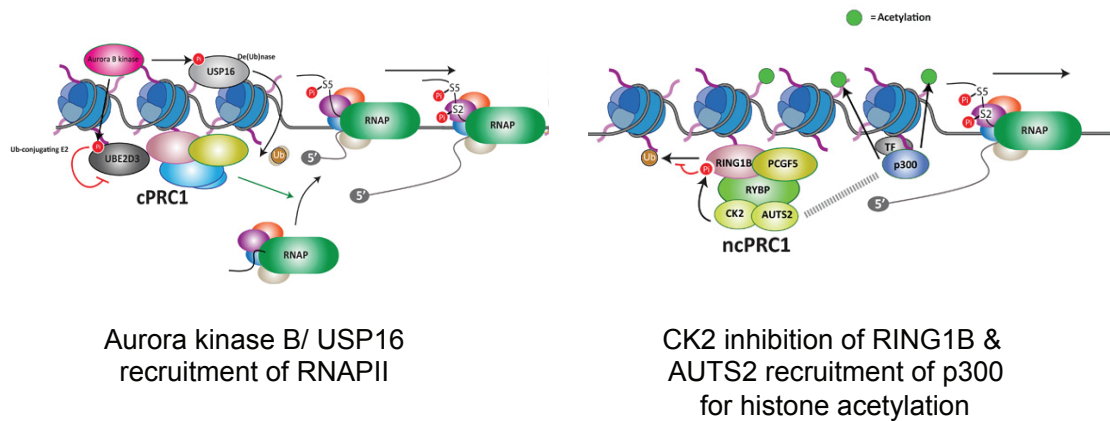


Figure 1.3 Polycomb-mediated Gene Repression and Activation

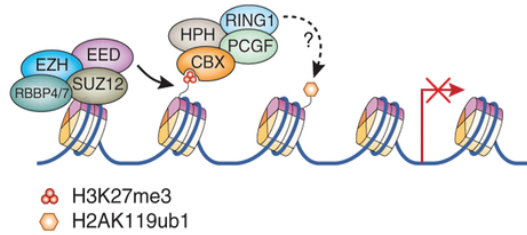
(A) PcG proteins mediate repression of transcription through two major mechanisms. First, the PcG complexes compact chromatin to block the accessibility of chromatin remodeling complexes required for transcription as well as many other transcriptional proteins (*left*). Second, PcG complexes hold the poised RNAPII at the transcriptional start site to inhibit its release for transcription as well as impeding the transcriptional elongation by RNAPII at gene bodies (*right*).

(B) PRC1 complex can also mediate gene activation by actions with protein kinases. First, Aurora kinase B phosphorylates and activates USP16 and inactivates E2-conjugating enzyme UBE2D3 to block PRC1 activity. The activated USP16 removes the ubiquitin from H2AK119. The inactive UBE2D3 impairs the E3 ubiquitin ligase activity of PRC1. The impaired PRC1 results in recruitment of RNAPII to activate gene transcription (*left*). Second mechanism of gene activation involves the non-canonical PRC1 complex composed of CK2 that phosphorylates RING1 to inhibit its catalytic activity. Complex with ATRX also recruits acetyltransferase P300 to enhance transcription (*right*).

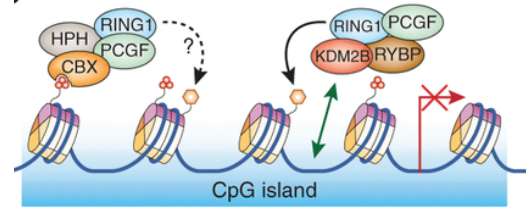
Figure adapted from (Aranda et al., 2015; Di Croce and Helin, 2013)

Figure 1.4

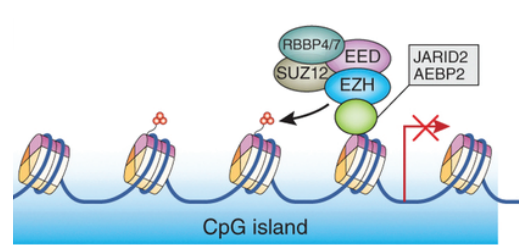
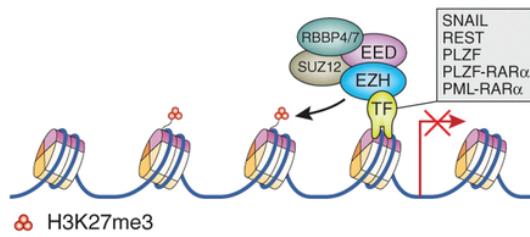
A Canonical PRC1 targeting



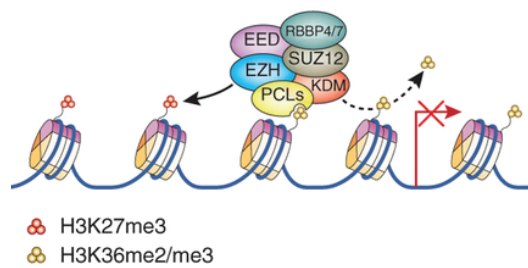
B Non-canonical PRC1 targeting



C DNA-based recruitments



D Histone PTM-based recruitment



E PRC2 recruitment by ncRNA

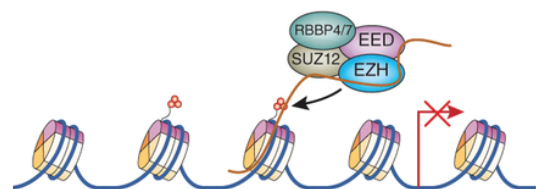


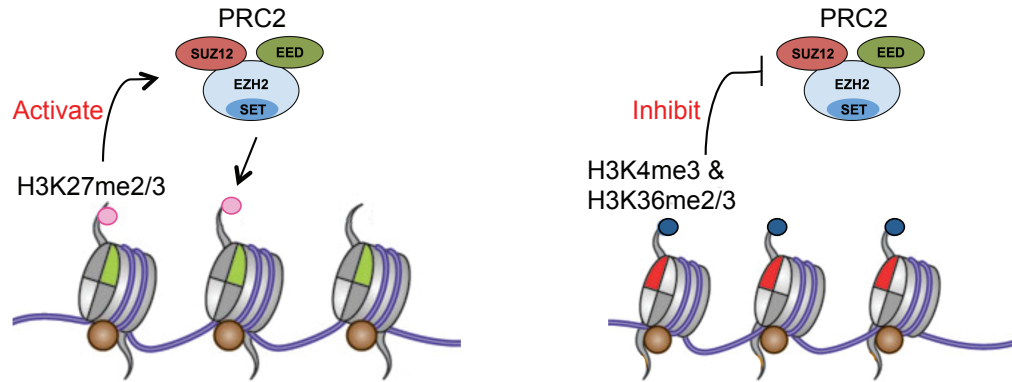
Figure 1.4 Recruitment of PcG to Chromatin

- (A) The canonical PRC1 recognizes and binds to the H3K27me3 mark deposited by PRC2 complex.
- (B) The non-canonical PRC1 containing KDM2B or RYBP are recruited to the CpG islands based on the high affinity of KDM2B for CG-rich regions.
- (C) PRC2 is able to be recruited based on DNA-based mechanisms in which PcG complexes are targeted to defined DNA sequences. PRC2 complex can interact with transcription factors (TF) to mediate recruitment of PRC2 to DNA-specific sequences targeted by the TF (*left*). Similar to the mechanism by TF, PRC2 binds to PRC2 accessory proteins like JARID2 or AEBP2 containing DNA binding domains to be recruited to the CpG islands (*right*).
- (D) PRC2 can be recruited by preexisting histone modifications by interaction with Polycomb-like (PCL) proteins that binds to H3K36me3.
- (E) ncRNAs interact with PcG proteins and are recruited to the chromatin.

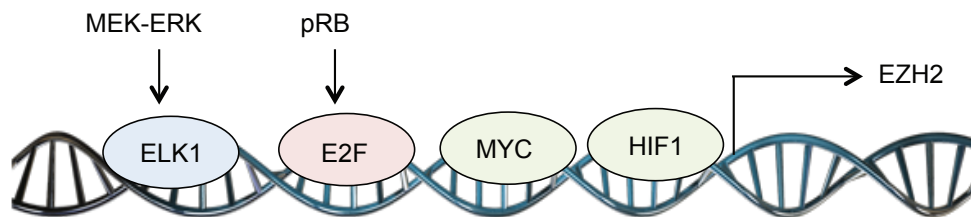
Figure adapted from (Di Croce and Helin, 2013)

Figure 1.5

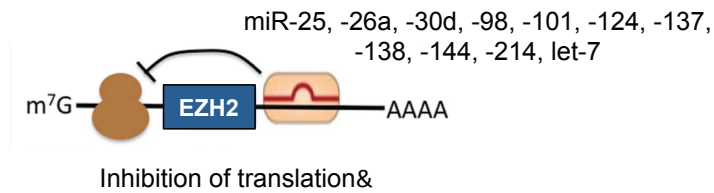
A Chromatin environment



B Upstream regulation of EZH2



C Post-transcriptional regulation of EZH2 by miRNAs



D Phosphorylation and association with non-PRC2 complex

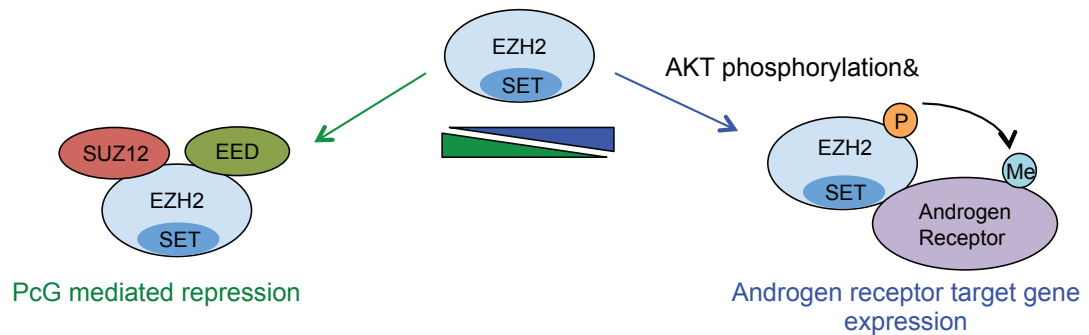


Figure 1.5 Regulation of Polycomb Group

(A) Histone methylation by PRC2 is activated by its own heterochromatin marks, H3K27me2 and H3K27me3, and inhibited by euchromatin marks H3K4me3, H3K36me2, H3K36me3, and H3K27ac.

(B) EZH2 expression is achieved at transcriptional level through binding of transcription factors like HIF1, MYC, E2F, or ELK1 to its promoter.

(C) EZH2 abundance is controlled at the post-translational levels by multiple microRNAs.

(D) Unphosphorylated EZH2 is incorporated into PRC2 and participates in repression of PcG-target genes. Phosphorylation of EZH2 at serine 21 by AKT sequesters EZH2 to interact and methylate the androgen receptor leading to transcription of androgen receptor-target genes.

Figure 1.6

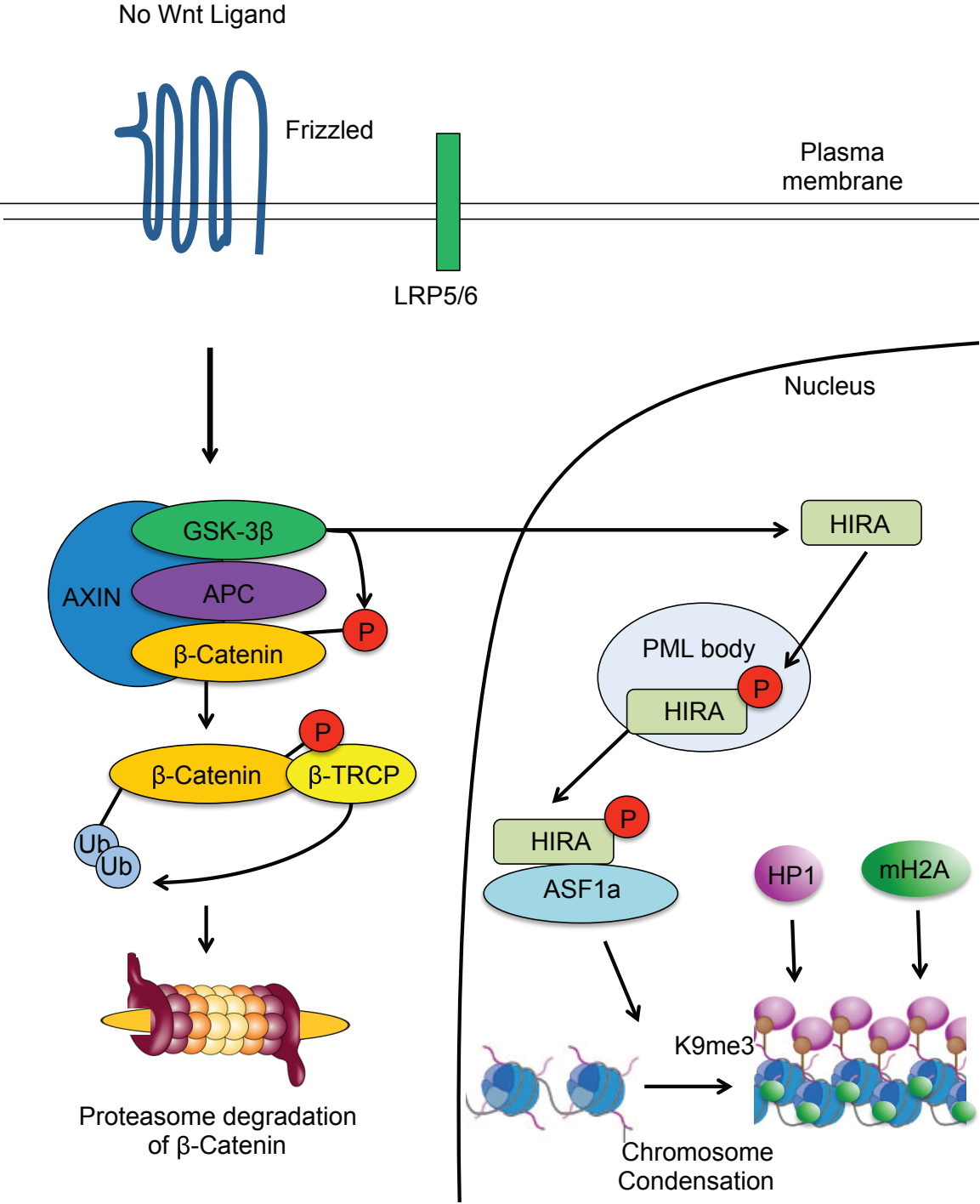


Figure 1.6 Induction of SAHF by Reduction of WNT Signaling

WNT2 ligand is downregulated during replicative and oncogene-induced senescence.

Absence of WNT ligand leads to the formation of the APC/AXIN/GSK-3 β destruction complex. GSK-3 β can phosphorylate β -catenin for targeted degradation. GSK-3 β can also phosphorylate HIRA. Phosphorylated HIRA is translocated into a specific organelle, the PML nuclear body. HIRA forms complex with ASF1a that leads to chromosome condensation and subsequent accumulation of mH2A and HP1.

Chapter 2. Materials and Methods

2.1 Culture Conditions

All HDF cultures were passaged using a 1:4 subculture regimen and incubated at 37°C in an atmosphere of 2.5% O₂, 92.5% N₂ and 5% CO₂. 293T cells were cultured in air supplemented with CO₂ to 5% (20% O₂, 75% N₂, 5% CO₂). LF1 cells were cultured in Ham's F10 nutrient mixture (Invitrogen) supplemented with 15% fetal bovine serum (FBS; Hyclone). Quiescent cells were supplemented with 0.25% FBS. IMR90, WI-38 and 293T cells were cultured in Dulbeco's modified Eagle's medium (DMEM) supplemented with 10% FBS. All media were supplemented with 2 mM L-glutamine and penicillin/streptomycin (Sigma-Aldrich).

2.2 Viral Vectors

293T cells were co-transfected with a lentiviral vector and the helper vectors pVSV-G and pCMV Δ8.9 using FuGENE HD (Promega). Medium was collected 24, 36, and 48 hrs after transfection, filtered through a 0.45 μm filter and used to infect HDF cells in the presence of 8 μg/ml polybrene (Stewart et al., 2003). Infected cells were selected with 2 μg/ml puromycin for 2 days, or 5 μg/ml blasticidin for 5 days. pLKO.1 vectors were used for shRNA-mediated gene knockdowns (Moffat et al., 2006). The control used throughout this study was an shRNA against an irrelevant target (GFP, 5'-TACAACAGCCACAACGTCTAT). The following shRNA vectors were obtained from Open Biosystems: EZH2, TRCN0000018365 (#1 in Figure 3.3B), TRCN0000040075 (#2 in Figure 3.3B), TRCN0000040076 (#3 in Figure 3.3B); WNT2, TRCN0000033373; MYC, TRCN0000010390; and CDKN1A (p21, TRCN0000287021). A pSUPER retrovirus vector was used for the knockdown of ATM (5'-

GATACCAGATCCTTGGAGA) (Ortega-Atienza et al., 2015). TRCN0000040076 (EZH2 #3) was used throughout this study. TRCN0000033373 (WNT2) corresponds to shWnt2-5 described and validated by (Ye et al., 2007). TRCN0000010390 (MYC) was described and validated by (Guney et al., 2006). For double knockdowns of p21 and EZH2, and ATM and EZH2, early passage LF1 cells were infected with p21 or ATM shRNA vectors, drug selected, grown up as pools, and superinfected with EZH2 shRNA vectors. Full-length *EZH2* cDNA was obtained from the ATCC; *MYC* cDNA was a kind gift of Dr. Michael Cole (Dartmouth Medical School). Both were PCR cloned into pLX303 (plasmid 25897, Addgene) modified by removal of the Gateway Destination sequences and incorporation of multiple cloning sites and sequence verified. Empty pLX vectors were used as controls. For the expression of *HRAS*(G12V) we used lentivirus vector pLenti CMV RasV12 Neo (plasmid 22259, Addgene). The empty vector (plasmid 17485, Addgene) was used as control. In combined cDNA expression and knockdown experiments, cells were first infected with *EZH2* or *MYC* cDNAs, stable pools were selected as indicated above, and subsequently infected with shRNA vectors.

2.3 Immunoblotting

Cells were harvested in Laemmli sample buffer (60 mM Tris pH 6.8, 2% SDS, 10% glycerol, 100 mM DTT). Whole cell extracts were separated by SDS-PAGE and transferred onto immobilon-FL membranes (Millipore). Signals were detected using the LI-COR Odyssey infrared imaging system (LI-COR Biosciences). The antibodies used were as follows: Millipore, active β -catenin (05-665, 1:1000), H3K27me3 (07-449, 1:1000), EED (09-774, 1:2000), MYC (06-340, 1:2000); Cell Signaling, GAPDH (5174,

1:1000), histone H3 (4499, 1:1000), EZH2 (5246, 1:1000), SUZ12 (3737, 1:1000), IκBα (4814, 1:1000); Santa Cruz, p16 (sc-756, 1:500), p21 (sc-397, 1:200), lamin A/C (sc-6215, 1:200). Immunoblots shown are representative images of three independent experiments.

2.4 Real-Time Quantitative PCR

RT-qPCR was performed using the SYBR Green system (Applied Biosystems) on the ABI 7900 Fast Sequence Detection instrument as specified by the manufacturer. Total RNA was harvested from cells using Trizol reagent (Invitrogen) and further purified using RNeasy columns (Qiagen). 1 µg of total RNA was transcribed into cDNA in 50 µl reactions using the Taqman kit (Applied Biosystems). 1 µl of this reaction was used in subsequent RT-qPCR reactions. GAPDH was used as the normalization control. All reactions were performed in triplicates. The primers used for RT-qPCR are provided in Table 2.1.

2.5 Chromatin Immunoprecipitation

The procedure was based on the Magna ChIP protocol (Millipore, 17-10085). Cells (2×10^6) were cross-linked in 1% formaldehyde for 10 min. at room temperature, quenched by adding glycine to 125 mM, and washed three times in ice-cold phosphate-buffered saline (PBS) containing 1x protease inhibitor (Roche). Cells were harvested by scraping, centrifuged and resuspended in SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris pH 8.1, 1x protease inhibitor cocktail), and incubated for 10 min. on ice. Lysed cells were diluted two-fold in ChIP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2

mM EDTA, 16.7 mM Tris-HCl pH 8.1, 167 mM NaCl), and disrupted with a Bioruptor UCD-200 sonicator (Diagenode) at a power setting of high for 5 min. (30 sec. sonification followed by 30 sec. rest), yielding genomic DNA fragments of 300-600 bp. The samples were centrifuged and the supernatants were diluted five-fold with ChIP dilution buffer. An aliquot (1%) of each chromatin preparation was saved as input. Samples were immunoprecipitated overnight at 4°C with 4 mg of antibody (MYC, 06-340, or H3K27me3, 07-449; both from Millipore). 20 ml of magnetic protein A beads were added and the immunocomplexes were washed as described in the Magna ChIP protocol (once with each of low salt, high salt and LiCl wash buffers, followed by two washes with TE buffer). The DNA was recovered by a 2 hrs. digestion with proteinase K at 65°C, followed by a 10 min. incubation at 95°C. The DNA was cleaned up by phenol/chloroform extraction and ethanol precipitation, and finally resuspended in 30 ml of TE. Yields were quantified with the Qubit 2.0 dsDNA HS assay kit (Invitrogen, Q32851). The ChIP and input were then used for qPCR with primers described in Table 2.2.

2.6 Immunofluorescence and ImmunoFISH

Cells were fixed for 20 min. with freshly prepared 4% paraformaldehyde in PBS. Permeablization was with 0.2% Triton X-100 in PBS for 20 min. at room temperature. Nonspecific binding was blocked by incubation in 4% bovine serum albumin (BSA; Thermo Fisher Scientific), 2% donkey serum, and 0.1% Triton X-100 in PBS for 1 hr. at room temperature. Antibodies were diluted in the blocking solution and incubated overnight at 4°C in a humidified chamber. Secondary antibodies were diluted in blocking

solution and incubated for 1 hr. at room temperature. Nuclei were counterstained with 2 µg/ml DAPI in PBS containing 0.2% Triton X-100 for 15 min. The antibodies used were as follows: Santa Cruz, p16 (sc-756, 1:100), p21 (sc-397; 1:100); Millipore, gH2AX (05-636, 1:100); Becton Dickinson, 53BP1 (NB100-304, 1:100); Cell Signaling, phospho-ATM (Ser1981, 4526, 1:100). The rabbit polyclonal antibody against human mH2A was a kind gift from Dr. Peter Adams (University of Glasgow) and was used as indicated before (Kreiling et al., 2011). The telomere dysfunction induced foci (TIF) assay, an immuno-FISH procedure, was performed as described previously (Herbig et al., 2004). Briefly, cells were fixed, permeabilized and immuno-stained for 53BP1. The samples were then dehydrated in a 70%, 90%, and 100% ethanol (3 min. each) and air dried. Nuclear DNA was denatured for 5 min. at 80°C in hybridization buffer containing 0.5 mg/ml Cy5-conjugated peptide nucleic acid (PNA) telomere C probe (PNA bio, #F1003), 70% formamide, 12 mM Tris HCl (pH 8), 5 mM KCl, 1 mM MgCl₂, 0.001% Triton X-100, and 2.5 mg/ml acetylated BSA. After denaturation, incubation was continued for 14 hrs. at room temperature in a humidified chamber. Cells were washed two times for 15 min. with 70% formamide in 2x SSC (0.3 M NaCl, 30 mM Na-citrate), followed by a 10 min. wash with 2x SSC, and a 10 min. wash with PBS. For EdU labeling cells were incubated with 10 µM of EdU stock solution (Click-iT 647, Invitrogen) for 1 hr (pulse-labeling) or 12 hrs (to assay proliferation) prior to fixation.

2.7 Microscopy

Imaging was done on either a Zeiss Axiovert 200M fluorescence microscope equipped with a Roper CoolSnap HQ monochrome camera controlled by MetaMorph software 6.1

(Molecular Devices), or a Zeiss LSM 710 Confocal Laser Scanning Microscope. Image analysis was performed using ImageJ open source software from the NIH (<http://rsbweb.nih.gov/ij/>).

2.8 Detection of SA- β -Gal Activity

SA- β -Gal was detected as described (Debacq-Chainiaux et al., 2009). Briefly, cells grown on coverslips were fixed with 0.2% glutaraldehyde and 2% formaldehyde for 5 min. Following two washes with PBS, cells were incubated overnight at 37°C in a solution containing 5-bromo-4-chloro-3-indolyl- β -galactopyranoside.

2.9 Mouse Husbandry

Mice with one floxed allele of *Myc* (*Myc*^{+/*lox*}) on a mixed 129 and C57BL/6 background (de Alboran et al., 2001) were obtained from Dr. Ignacio de Alboran, Department of Immunology and Oncology, University of Madrid, Spain. Upon receipt the mice were rederived and subsequently housed in a Helicobacter-free barrier facility at Brown University. The floxed allele was converted to a germline deletion allele (*Myc*^{+/*D*}, referred to here as *Myc*^{+/-}) by breeding heterozygous floxed animals with the Cre deleter line TgN(CMVCre)1AN (Nagy et al., 1998), which was backcrossed by Dr. Tomas Prolla (Departments of Genetics and Medical Genetics, University of Wisconsin, Madison) to C57BL/6 for over 6 generations. After deleting the *Myc*^{*lox*} allele *Cre* was segregated away, and the *Myc* heterozygous line was backcrossed more than 10 times to C57BL/6NCrl mice purchased from Charles River (strain code 027). The *Myc* deletion line has been propagated by breeding *Myc* heterozygous males (*Myc*^{+/-}) with

C57BL/6NCrl females purchased from Charles River. These crosses produce the two genotypes ($Myc^{+/+}$ and $Myc^{+/-}$) and the two sexes at the expected Mendelian ratio (1:1). The lung, liver and brain tissues from $Apc^{fl/fl}$ mice were obtained from Dr. Peter Adams, Institute of Cancer Sciences, Beatson Institute and Glasgow University, United Kingdom. $Apc^{fl/fl}$ mice in which exon 14 of the *Apc* gene is flanked by two loxP sites (Shibata et al., 1997) were maintained on a C57BL/6 background. Mice were kept in conventional animal facilities, monitored frequently, and experiments were carried out in compliance with UK Home Office guidelines at the Beatson Institute for Cancer Research mouse facility. The mouse tissues of strain C57BL/6 were collected in house from mice obtained from NIA Aged Rodent Colonies.

2.10 Flow Cytometry and ROS Detection

Reactive oxygen species and mitochondrial superoxide were measured by incubation with 5 μ M CellROX Green Reagent (ThermoFisher) for 30 min. at 37°C or 5 μ M of MitoSOX (ThermoFisher) for 10 min. at 37°C, respectively. Flow cytometry data were acquired on Becton Dickinson Biosciences FACSCalibur Flow Cytometer.

2.11 Statistical Analysis

Data are shown as means with standard deviations (SD). Statistical significance was determined using the two-tailed Student's test (t-test). $P < 0.05$ was considered significant.

Table 2.1 List of Primer Sequences for RT-qPCR

Gene	Orientation	Sequences (5' to 3')
<i>EZH2</i>	Sense	GCAACACCCAACACTTATAAGC
	Antisense	CTCCCTCCAAATGCTGGTA
<i>WNT2</i>	Sense	TGGCAGGAAGGCTGTAAAGC
	Antisense	ATGGCCAGCCAGCATGTC
<i>MMP1</i>	Sense	AGCCTTCCAACCTCTGGAGTAATGT
	Antisense	CCGATGATCTCCCCTGACAA
<i>MMP3</i>	Sense	CCCACCTTACATACAGGATTGTGA
	Antisense	CCCAGACTTTCAGAGCTTTCTCA
<i>IL6</i>	Sense	CACTGGCAGAAAACAACCTGAA
	Antisense	ACCAGGCAAGTCTCCTCATTGA
<i>CXCL8 (IL8)</i>	Sense	GTTTTTGAAGAGGGCTGAGAATTC
	Antisense	CCCTACAACAGACCCACACAATAC
<i>CCL2</i>	Sense	AAGACCATTGTGGCCAAGGA
	Antisense	TTCGGAGTTTGGGTTTGCT
<i>CXCL1</i>	Sense	CAATCCTGCATCCCCCATAG
	Antisense	CAGCCACCAGTGAGCTTCCT
<i>CXCL2</i>	Sense	AGAATGGGCAGAAAGCTTGTCT
	Antisense	CCTTCTGGTCAGTTGGATTTC
<i>CDKN2A (p16-INK4A)</i>	Sense	CGGAAGGTCCCTCAGACATC
	Antisense	CCCTGTAGGACCTTCGGTGA
<i>CDKN1A (p21-CIP1)</i>	Sense	GAGACTCTCAGGGTCGAAAACG
	Antisense	TTCCTGTGGGCGGATTAGG
<i>GAPDH</i>	Sense	GGAGTCAACGGATTTGGTCGT
	Antisense	GTTGAGGTCAATGAAGGGGTCA
<i>MYC</i>	Sense	GGATTCTCTGCTCTCCTCGAC
	Antisense	TTGTTCCCTCCTCAGAGTCGC
<i>EED</i>	Sense	AAAGATGCTTGCATTGGGCA
	Antisense	TGAGTCAGTGTTGTACATTGGC
<i>SUZ12</i>	Sense	ACTCCAGAACAAACATCAAAAGG
	Antisense	GCAAATGAGCTGACAAGCTATGA
<i>RBBP7</i>	Sense	ATGCCGCAGAATCCTCACAT
	Antisense	TCCACTTGGGTCTGGTTTAGC
<i>DKK1</i>	Sense	TGTATCACACCAAAGGACAAGA
	Antisense	TGGACCAGAAGTGTCTAGCA

Table 2.2 List of Primer Sequences for ChIP-qPCR

Gene/location	Orientation	Sequences (5' to 3')	Notes
<i>ARF</i> Exon 1 β	Sense	GTGGGTCCCAGTCTGCAGTTA	Fig. 3.5A, #1
	Antisense	CCTTTGGCACCAGAGGTGAG	
15 kb downstream of <i>ARF</i> promoter	Sense	GCACTTGCCCTTCCAGGTATA	Fig. 3.5A, #2
	Antisense	TGATAGTTCAAGGCCCTATGCC	
200 bp upstream <i>p16-INK4A</i> TSS	Sense	ACCCCGATTCAATTTGGCAG	Fig. 3.5A, #3
	Antisense	AAAAAGAAATCCGCCCCCG	
<i>p16-INK4A</i> exon 1 α	Sense	AGAGGGTCTGCAGCGG	Fig. 3.5A, #4
	Antisense	TCGAAGCGCTACCTGATTCC	
200 bp downstream of <i>p16-INK4A</i> exon	Sense	GCCAAGGAAGAGGAATGAGGAG	Fig. 3.5A, #5
	Antisense	CCTTCAGATCTTCTCAGCATTCG	
<i>EZH2</i> , 1034 bp upstream of TSS	Sense	ACATTGCTGCCATTTTCAGAC	Fig. 4.2E, #1
	Antisense	AGTGAACAGTGCTCATCTTGA	
<i>EZH2</i> , 156 bp upstream of TSS	Sense	TTCGCTGTAAGGGACGC	Fig. 4.2E, #2
	Antisense	TGTGTTTCAGCGAAAGAACAA	
<i>EZH2</i> TSS	Sense	CCAATCGCCATCGCTTTTAT	Fig. 4.2E, #3
	Antisense	GGGCCCTGTGATTGGAC	

Chapter 3. Repression of EZH2 Regulates Senescence Through H3K27me3-independent and H3K27me3-dependent Pathways

3.1 Summary

Polycomb Group epigenetic regulators are important but not well-understood components of cellular senescence. EZH2, a PcG histone H3 lysine-27 methyltransferase, is repressed in senescent cells and activated in many tumours. We show that repression of EZH2 promotes senescence through two independent mechanisms. First, downregulation of EZH2 in proliferating cells initiates a rapid DNA damage response prior to depletion of H3K27me3 marks. Second, reduction of EZH2 activity causes a gradual loss of H3K27me3 that induces p16 (CDKN2A) expression and contributes to the activation of the senescence-associated secretory phenotype. These mechanisms elucidate how PcG-mediated epigenetic changes are involved in the regulation of cellular senescence.

RESULTS

3.2 Downregulation of EZH2 Leads to Cellular Senescence with Features of SASP

We and others have performed gene expression profiling of proliferating and replicatively senescent human diploid fibroblasts (HDFs) and *EZH2* is among the genes that are reproducibly downregulated in senescent cells (Lackner et al., 2014; Nelson et al., 2014). Quantitative RT-PCR (RT-qPCR) confirmed that *EZH2* transcripts were decreased in senescent cells of three commonly used strains of normal HDFs (LF1, WI-38, IMR90) (Figure 3.1A). In addition, cells made senescent by expression of *HRAS*(G12V) or exposure to DNA damaging agents also showed reduced expression of *EZH2* (Figure 3.1B and 3.1C). Immunoblotting showed that the levels of *EZH2* protein decreased gradually as LF1 cells approached replicative senescence (Figure 3.1D). *EZH2* protein became undetectable after two weeks of senescence, however the depletion of the H3K27me3 mark was not apparent until much later. Although we observed some loss of core histones H3 and H4 as well as histone H1 during senescence due to histones budding out into the cytoplasm (Figure 3.2A-3.2C), this did not account for the major reduction of H3K27me3 marks (Ivanov et al., 2013). Similarly to LF1 cells, WI-38 and IMR90 cells showed loss of *EZH2* at (or shortly after) the onset of senescence, while the disappearance of H3K37me3 lagged (Figure 3.1E).

To confirm that downregulation of *EZH2* is sufficient to induce senescence we introduced short hairpin RNAs (shRNAs) targeting its transcript into early passage LF1 cells (Figure 3.3A). Three shRNA target sequences were tested using lentivirus vectors and resulted in a 63-97% decrease of *EZH2* mRNA (Figure 3.3B). Knockdown of *EZH2*

resulted in a rapid depletion of EZH2 protein seen at 2 days after infection with no changes in H3K27me3 levels at that time (Figure 3.3C). Significant depletion of H3K27me3 was not evident until 8 days. We observed that downregulation of EZH2 induced premature senescence by several criteria. First, cell proliferation was arrested within 2 days of infection (Figure 3.4A). Second, the cells expressed the senescence-associated beta galactosidase (SA- β -Gal) and SAHF markers (Figure 3.4B and 3.4C). In accord with the increase in SAHF, repression of EZH2 increased levels of the histone variant macroH2A (Figure 3.4D and 3.4E) (Ye et al., 2007). Third, knockdown of EZH2 upregulated components of the SASP, such as matrix-degrading enzymes *MMP1* and *MMP3*, and inflammatory cytokines *IL6*, *IL8*, *CCL2*, *CXCL1* and *CXCL2* (Figure 3.4F). Interestingly, this phenotype was observed only at late times after infection (6-8 days), while growth arrest, SA- β -Gal and SAHF were induced much more rapidly (2-4 days). Collectively, we found that downregulation EZH2 is sufficient to rapidly induce cellular senescence, apparently independently of the depletion of the H3K27me3 mark.

3.3 Downregulation of EZH2 Results in DNA Damage and Induces p21 in a H3K27me3-Independent Manner

Downregulation of EZH2 has been reported to result in the loss of H3K27me3 along the *CDKN2A* locus (Bracken et al., 2007). Since there were no significant changes in the global levels of H3K27me3 at the onset of replicative senescence, or senescence induced by knockdown of EZH2, we examined the presence of H3K27me3 at the *CDKN2A* locus by performing chromatin immunoprecipitation coupled with qPCR (ChIP-qPCR). No significant changes in H3K27me3 enrichment were observed 4 days after infection with

shEZH2, with a decrease in H3K27me3 becoming apparent at 8 days (Figure 3.5A and 3.5B). These data reflect closely the global levels of H3K27me3, and suggest that loss of EZH2 can induce senescence independently of epigenetic changes at the *CDKN2A* locus.

To examine the activation of senescence pathways elicited by the downregulation of EZH2 in more detail, we monitored the time course of p21 and p16 expression changes. p21 mRNA was induced at 4 days after EZH2 knockdown (Figure 3.6A), while p16 mRNA was unchanged at this time, becoming upregulated only later (8 days; Figure 3.6B). The earliest time after infection we could assess was 2 days, at which point SA- β -Gal staining showed that the majority of cells (93%) were already senescent (Figure 3.4B). Likewise, using a sensitive single-cell immunofluorescence (IF) assay, we found that at the 2-day time point 83% of cells were positive for p21 protein (Figure 3.6C). The frequency of p21-positive cells declined over time, with only 10% of cells being positive at 8 days. A similar single-cell IF assay for p16 protein showed no expression at early time points, with the frequency of p16-positive cells becoming significant at 6 and 8 days (Figure 3.6D). To examine whether the upregulation of p21 is required for the induction of cellular senescence, we performed double knockdowns of p21 and EZH2. Indeed, senescence induced by EZH2 depletion was reduced by p21 knockdown (Figure 3.6E and 3.6F). Collectively, these results indicate that p21 is an important and early effector engaged by EZH2 downregulation, and that p16 upregulation occurs much later and coincides with the loss of H3K27me3 at the *CDKN2A* locus.

Since the primary regulator of p21 in senescence is p53, which in turn is activated by DNA damage, we examined components of the DNA damage response (DDR) after knockdown of EZH2. An early event in the DDR is the recruitment of the ataxia

telangiectasia mutated (ATM) kinase to sites of DNA double-strand breaks (DSB), where it becomes activated by autophosphorylation and locally phosphorylates histone H2A.X (γ H2AX). This results in the recruitment of other DDR components, including 53BP1, and these events can be visualized in intact nuclei by IF. Knockdown of EZH2 led to a strong and rapid induction of DNA damage foci, detectable with γ H2AX, 53BP1, or phospho-ATM(S1981) staining (Figure 3.7A). We also observed the recruitment of activated ATM to sites of DSB, as evidenced by the colocalization of ATM(S1981) foci with 53BP1 foci (data not shown). Quantification of 53BP1 foci showed a significant increase in DNA damage based both on the frequency of foci-containing cells as well as the number of foci per nucleus (Figure 3.7B).

To determine whether activation of ATM is required for the induction of senescence by EZH2 downregulation, we performed double knockdowns of ATM and EZH2. We found that ATM knockdown significantly alleviated the early onset of senescence, monitored by SA- β -Gal staining (Figure 3.7C). To determine whether the DDR elicited by EZH2 downregulation is localized to telomeres we performed an assay that colocalizes DNA damage foci with telomeres (Herbig et al., 2004). We found no changes in DNA damage occurring specifically at telomeres upon knockdown of EZH2 (Figure 3.8A). Similarly, knockdown of EZH2 in LF1 cells immortalized by expression of telomerase robustly induced senescence as indicated by SA- β -Gal staining (Figure 3.8B), suggesting that telomere maintenance is not sufficient to prevent senescence due to EZH2 depletion. Altogether, these data indicate that senescence elicited by EZH2 downregulation is triggered initially by non-telomeric DSBs and mediated by the ATM-p53-p21 DDR pathway.

3.4 Induction of DNA Damage by EZH2 Depletion is Dependent on DNA Replication

The expression of EZH2 is cell cycle dependent (Bracken et al., 2003). Quiescence elicited by serum deprivation reduced the levels of EZH2 protein by some 50% (Figure 3.9A and 3.9B) which is relatively mild compared to the >90% reduction in senescent cells (Figure 3.1D). Importantly, quiescence is not associated with a DDR (Figure 3.9C). Since EZH2 and other PRC2 components localize to sites of DNA replication (Hansen et al., 2008; Leung et al., 2013) we examined the effect of cell cycle progression on the ability of EZH2 knockdown to elicit a DDR. We found that arresting the cell cycle, either in G0 by serum withdrawal or in late G1 by a thymidine block, strongly protected against the induction of a DDR (Figure 3.9D) or upregulation of p21 (Figure 3.9E). To determine whether DNA damage localizes to sites of DNA replication, we knocked down EZH2 under proliferative conditions and pulse-labeled with EdU before the cultures became fully senescent. We found that in many of the cells still capable of EdU incorporation, 53BP1 foci frequently colocalized with sites of EdU labeling (Figure 3.9F). Altogether these data suggest that the DDR and ensuing senescence triggered by EZH2 depletion are dependent on DNA replication.

3.5 DNA Damage by EZH2 Depletion Induces Increase in ROS Production

Senescent cells produce ROS upon DNA damages as a positive feedback loop to increase ROS-mediated damage (Passos et al., 2010). Therefore, we investigated whether DNA damage induced at the replication fork upon EZH2 depletion increases the production of ROS. We detected higher levels of ROS in cells with knockdown of EZH2 indicated by

CellROX flow cytometry (Figure 3.10A). Cellular ROS can arise from varying sources (Torres and Forman, 2003) and ROS in EZH2 knockdown cell are produced from the mitochondria (Figure 3.10B).

3.6 Loss of H3K27me3 Marks Induces Senescence by Upregulating p16 and SASP

The temporal correlations between declining H3K27me3 levels, p16 expression and upregulation of SASP genes suggest a possible contribution of H3K27me3 loss to the phenotypes of senescent cells. We therefore examined the effects of GSK126, a highly selective *S*-adenosyl-methionine (SAM) competitive inhibitor of EZH2, on early passage HDFs (McCabe et al., 2012b). GSK126 inhibited EZH2 enzymatic activity within 4 days of treatment, as indicated by the loss of H3K27me3, without altering EZH2 protein levels (Figure 3.11A). Treatment with GSK126 also elicited, in a dose-dependent manner, cellular senescence as evidenced by the inhibition of proliferation and increase in SA- β -Gal staining. These effects were first evident at 8-10 days and continued to increase over a period of 30 days (Figure 3.11B and 3.11C).

To gain further insights into the mechanisms by which loss of the H3K27me3 modification induces cellular senescence, we investigated the expression of p21 and p16 proteins using single-cell IF assays. Treatment of LF1 cells with GSK126 did not affect the levels of p21, while p16 levels were significantly increased (Figure 3.12A and 3.12B). To explore another method of inhibiting EZH2 activity, we used a catalytically inactive EZH2 mutant that lacks the conserved SET domain (EZH2 Δ SET). Similar to what was observed after treatment with GSK126, infection with an EZH2 Δ SET-expressing lentivirus vector induced senescence accompanied by the upregulation of p16

but not p21 (Figure 3.12C and 3.12D). In contrast to the knockdown of EZH2, inhibition of its activity with GSK126 did not elicit a DDR, as indicated by quantification of DNA damage (53BP1) foci (Figure 3.12E). These data agree with the absence of p21 induction.

H3K27me3 marks have been reported to decrease at enhancer regions of many SASP genes during cellular senescence (Shah et al., 2013). We observed an increase in the expression of SASP genes at late times after knockdown of EZH2, which coincided with a decrease in global H3K27me3 levels (Figure 3.3C and 3.4F). We therefore examined the expression of SASP genes after treatment with GSK126, and found that they were also upregulated (Figure 3.13A).

SASP gene expression in senescent cells is also promoted by the mammalian target of rapamycin (mTOR) by enhancing the translation of the cytokine IL1A which in turn increases the activity of nuclear factor- κ B (NF- κ B), a transcription factor that regulates most of the SASP genes (Laberge et al., 2015). We examined the expression of SASP genes after treatment with GSK126 and the mTOR inhibitor rapamycin. Rapamycin reduced the transcript levels of six out of the seven SASP genes upregulated by GSK126 (Figure 3.13B). We next examined the expression of I κ B α , which inhibits NF- κ B by sequestering it in the cytoplasm, and found that treatment with GSK126 decreased the levels of I κ B α (Figure 3.13C). This was rescued by treatment with rapamycin, indicating that the loss of H3K27me3 induces transcription of SASP genes in a manner that is mediated by NF- κ B and regulated by mTOR. Taken together, these data indicate that inhibition of EZH2 methyltransferase activity is sufficient to induce cellular senescence in the absence of DNA damage, and that this response is characterized by the upregulation of p16 and accompanied by SASP.

3.7 Loss of H3K27me3 Marks Induces Expression of NKG2D Ligands

Senescent cells activate form of immune surveillance as part of the SASP to clear senescent cells from tissues. Senescent hepatic stellate cells express ligands for natural killer cell receptors (NKG2D) to enhance the immune clearance of senescent cells by NK cells (Krizhanovsky et al., 2008). Expression of several NKG2D ligands in tumor cell lines is dependent on the DDR pathway (Gasser et al., 2005). Replicative senescent cells increase the expression of NKG2D ligands UL16 binding protein (ULBP) 1, 2, 5 and 6 compared to proliferating cells (Figure 3.14A). Treatment with GSK126 induced expression of the same subset of NKG2D ligands (Figure 3.14B). Similar to the kinetic expressions of other SASP factors upon ectopic repression of EZH2, expression of NKG2D ligands were observed only at late times after infection (8 days) although ULBP1 was not upregulated (Figure 3.14C). Together, these data suggest that decrease in H3K27me3 activates expression of NKG2D ligands, independently of DDR, to possibly enhance immune surveillance of these senescent cells.

Figure 3.1

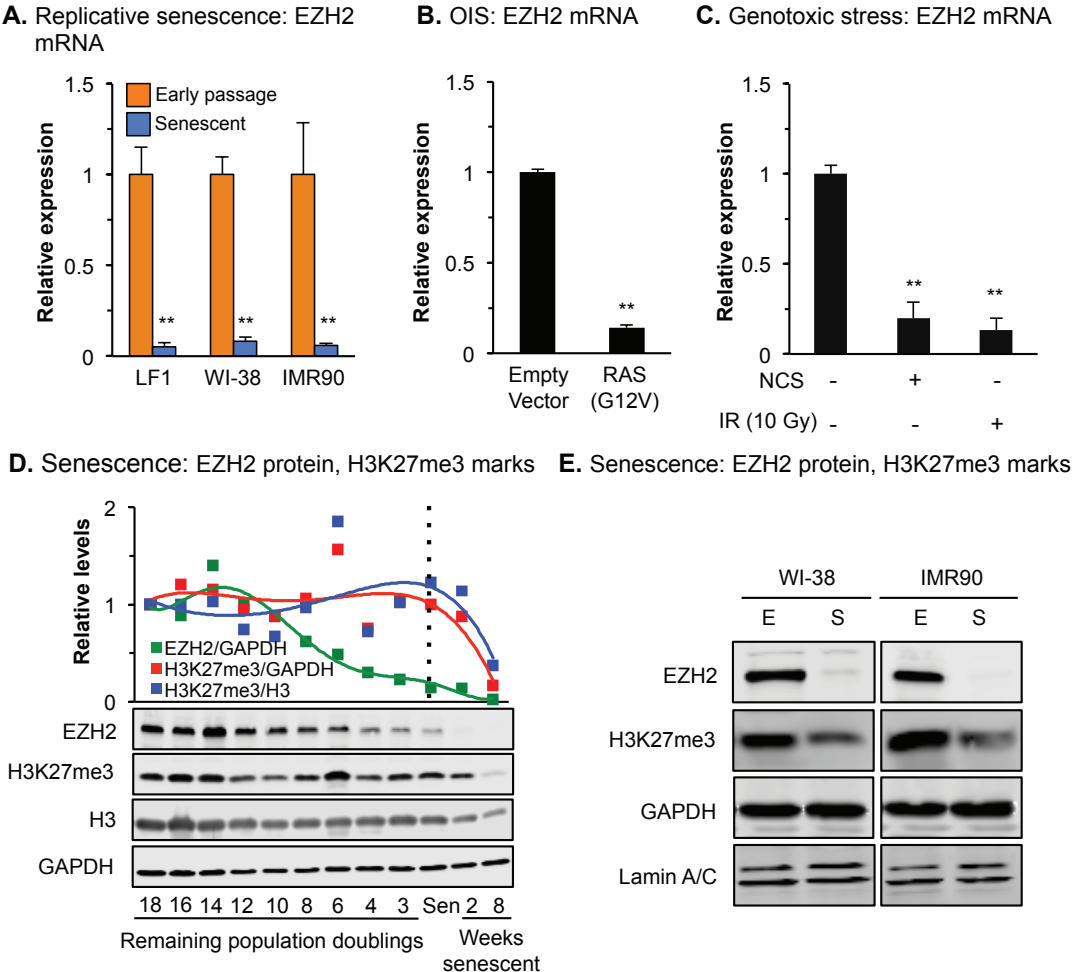


Figure 3.1 EZH2 Expression Decreases During Senescence

(A) EZH2 mRNA levels were determined by RT-qPCR in early passage proliferating and senescent cells of strains LF1, WI-38 and IMR90 (** $p < 0.01$, $n=3$).

(B) Expression of EZH2 mRNA was compared between LF1 cells expressing empty vector or *HRAS*(G12V) 6 days after infection. (** $p < 0.01$, $n=3$).

(C) Early passage LF1 cells were irradiated with ionizing radiation (10 Gy) or treated with 200 ng/ml neocarzinostatin and EZH2 mRNA levels were determined after 6 days (** $p < 0.01$, $n=3$).

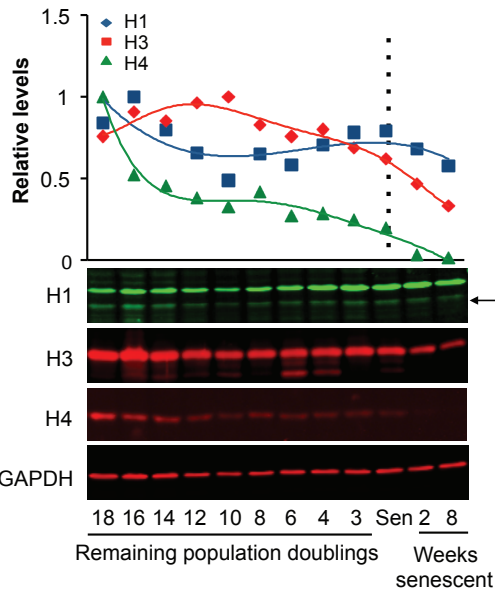
(D) EZH2 protein levels and the presence of K27me3 marks on H3 were examined in LF1 cells by immunoblotting, starting at 18 population doublings before senescence and continuing until 8 weeks after the onset of senescence. EZH2 and H3K27me3 levels were normalized to GAPDH (green, red); H3K27me3 is also shown normalized to total H3 (blue).

(E) Levels of EZH2 protein and the H3K27me3 mark were examined by immunoblotting in the HDF strains WI-38 and IMR90 at early passage (E) and 5 weeks after the onset of senescence (S).

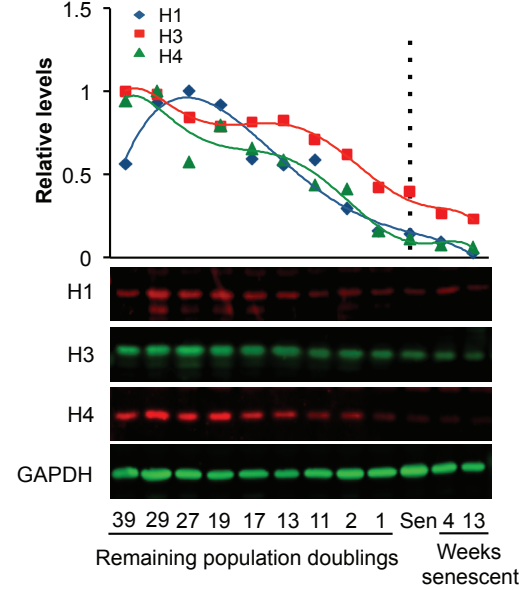
Error bars represent SD, * $p < 0.05$, ** $p < 0.01$

Figure 3.2

A. Senescence (LF1): H1, H3, H4



B. Senescence (IMR90): H1, H3, H4



C. Senescence (LF1): H3 IF

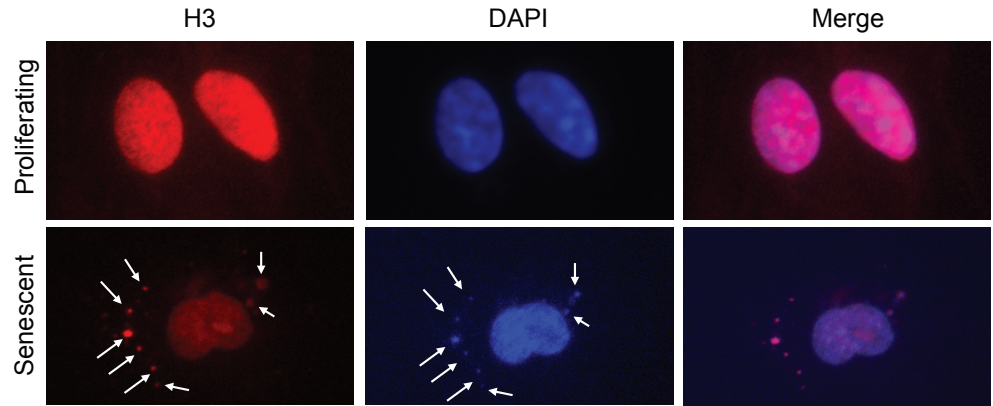


Figure 3.2 Decrease in Histones During Senescence

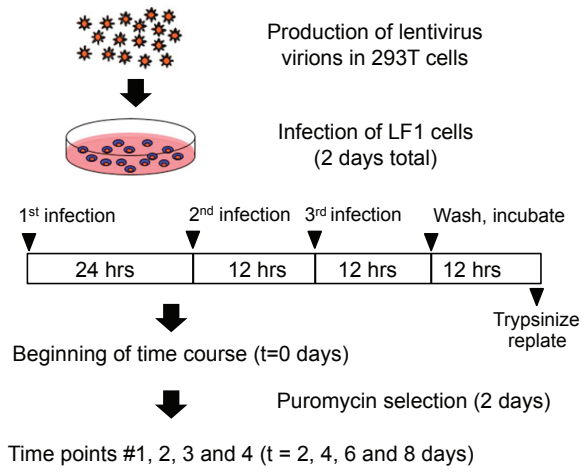
(A) Histones H1, H3, and H4 protein levels were examined in LF1 cells by immunoblotting, starting at 18 population doublings before senescence and continuing until 8 weeks after the onset of senescence. Quantifications of the histone levels were normalized to GAPDH.

(B) Histones H1, H3, and H4 protein levels were examined as in (A) in IMR90 cells, starting 39 population doubling before senescence and continuing until 13 weeks after onset of senescence.

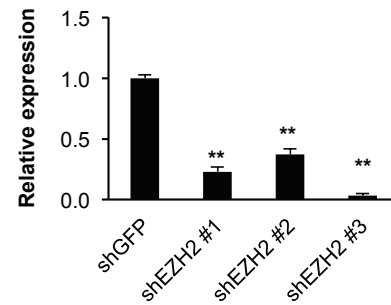
(C) Representative images of LF1 cells immunostained with antibodies to H3 (red) and counterstained with DAPI (blue) in proliferating and 8 weeks senescent LF1 cells. Arrows points to the cytoplasmic DNA and histone fragments in senescent cells. Note the lower intensity of H3 immunostaining in senescent cells compared to proliferating cells.

Figure 3.3

A. shRNA lentivirus infection timeline



B. shEZH2: EZH2 mRNA



C. shEZH2: EZH2 protein, H3K27me3 marks

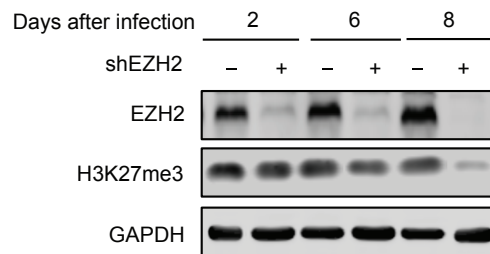


Figure 3.3 Knockdown of EZH2

(A) Schematic overview and timeline of the lentivirus shRNA infection experiments. LF1 cells were infected with lentiviral particles for a total of 2 days. After a wash and 12 hrs further incubation the cultures were replated, and this time point was designated as t=0 for all the time courses presented in this study. Cells were treated with puromycin for 2 days, at which time all sensitive cells had been effectively removed. Cells were harvested at successive time points as indicated in individual experiments.

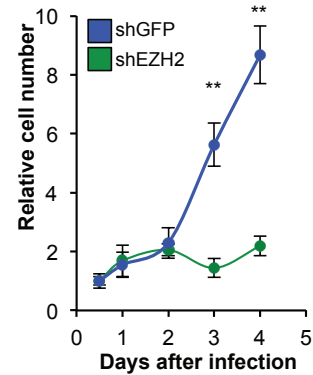
(B) LF1 cells were infected with lentivirus vectors expressing three different shRNAs against the EZH2 transcript. The effectiveness of knockdowns was examined by RT-qPCR for the EZH2 mRNA 2 days after infection (**p<0.01, n=3). shRNA against GFP was used as the control.

(C) LF1 cells were infected with a lentivirus vector expressing shRNA #3 against EZH2 as in (B). shRNA to GFP was used as the control. Protein levels of EZH2 and H3K27me3 were examined by immunoblotting at the indicated times after infection.

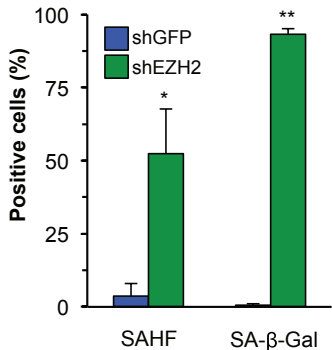
Error bars represent SD, * p<0.05, ** p<0.01

Figure 3.4

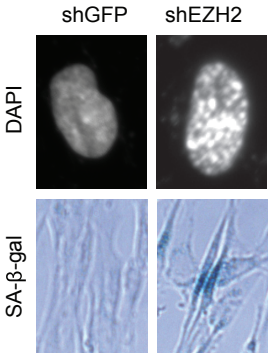
A. shEZH2: proliferation



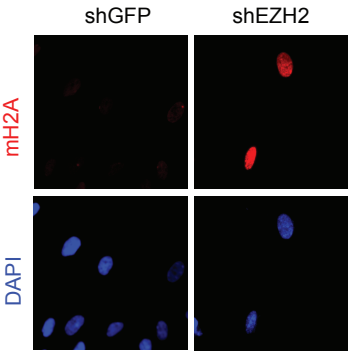
B. shEZH2: SAHF, SA-β-Gal



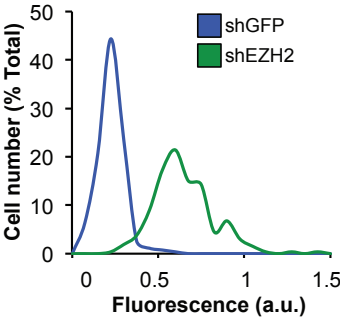
C. shEZH2: SAHF, SA-β-Gal



D. shEZH2: macroH2A IF



E. shEZH2: macroH2A IF quantification



F. shEZH2: SASP genes

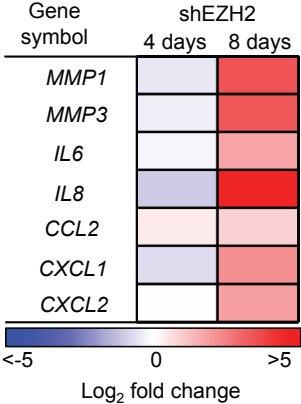


Figure 3.4 Ectopic Repression of EZH2 Elicits Premature Senescence

(A) EZH2 was knocked down as in Figure 3.3C and proliferation was assessed by counting cell numbers (** $p < 0.01$, $n=3$).

(B) EZH2 was knocked down as in (A) and SAHF formation (by DAPI staining) and SA- β -Gal activity were determined 2 days after infection. SA- β -Gal or SAHF-positive cells were scored as % of total cells in randomly selected fields (* $p < 0.05$, ** $p < 0.01$, $n=3$).

(C) Representative images of cells displaying SAHF and SA- β -Gal activity are shown. SAHF formation was visualized by DAPI staining. Images were acquired 2 days after infection with EZH2 shRNA.

(D) EZH2 was knocked down as in (A) and mH2A expression was examined using IF microscopy.

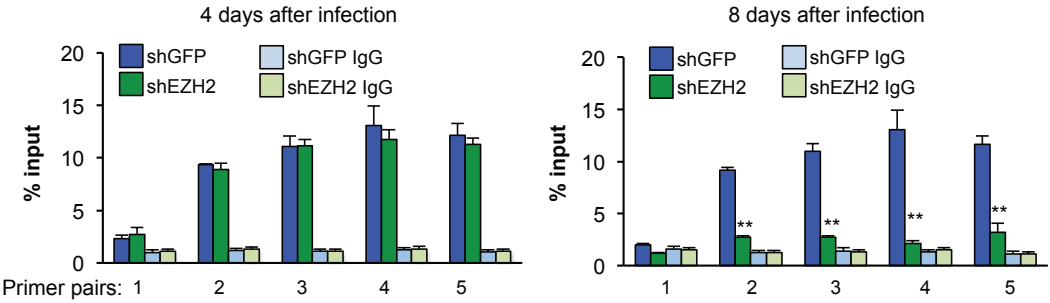
(E) mH2A nuclear signals were quantified in the IF images using ImageJ software and plotted as histograms of cell number (% of total) against fluorescent intensity in arbitrary units (a.u.). At least 260 nuclei were observed for each condition.

(F) EZH2 was knocked down as in (A) and expression of the indicated genes was determined by RT-qPCR at 4 and 8 days after infection ($n=3$). The data are expressed as heat maps relative to control shRNA infected cells.

Error bars represent SD, * $p < 0.05$, ** $p < 0.01$.

Figure 3.5

A. H3K27me3 ChIP of *CDKN2A*



B. Schematic

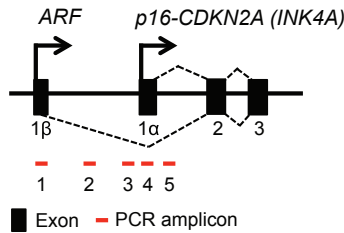


Figure 3.5 Changes in H3K27me3 Enrichment Along the *CDKN2A* Locus

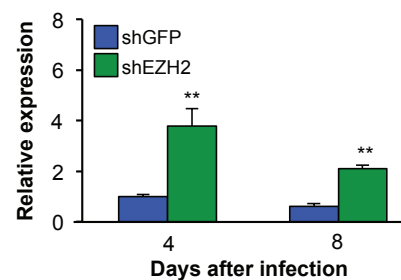
(A) EZH2 was knocked down as in Figure 3.3C and H3K27me3 enrichment at the *CDKN2A* locus was determined by ChIP 4 and 8 days after infection. For locations of primer pairs see panel B. Normal rabbit IgG was used as the IP control (** $p < 0.01$, $n=3$).

(B) Schematic of *CDKN2A* locus.

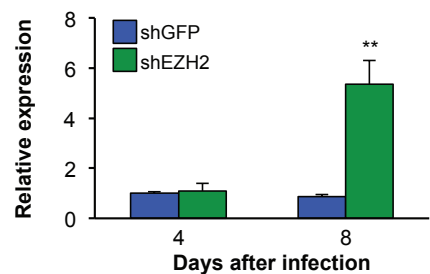
Error bars represent SD, * $p < 0.05$, ** $p < 0.01$

Figure 3.6

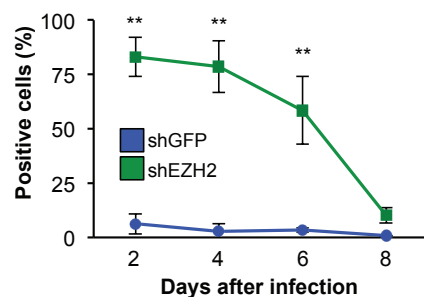
A. shEZH2: p21 mRNA



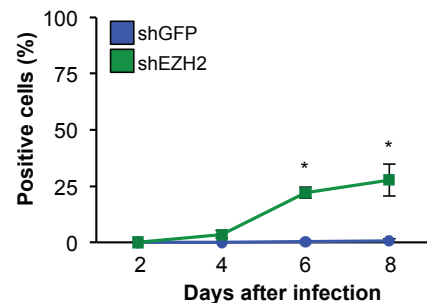
B. shEZH2: p16 mRNA



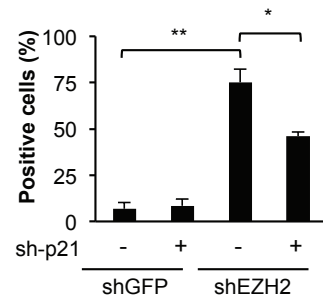
C. shEZH2: p21 IF



D. shEZH2: p16 IF



E. shEZH2, sh-p21: SA- β -Gal



F. shEZH2, sh-p21: p21 protein

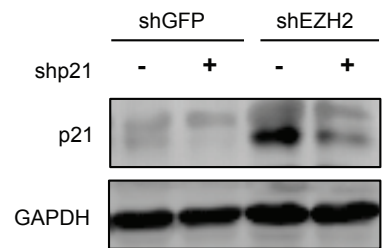


Figure 3.6 Downregulation of EZH2 Rapidly Triggers Activation of p21 in H3K27me3-independent Manner with Delayed Activation of p16

(A) EZH2 was knocked down as in Figure 3.3C and expression of p21 mRNA was measured by RT-qPCR (** $p < 0.01$, $n = 3$).

(B) Expression of p16 was measured as in (A).

(C) EZH2 was knocked down as in (A) and the frequency of p21-expressing cells was scored using IF microscopy (% of total cells, random fields, >400 cells per condition, ** $p < 0.01$).

(D) Frequency of p16-expressing cells was determined as in (C, * $p < 0.05$).

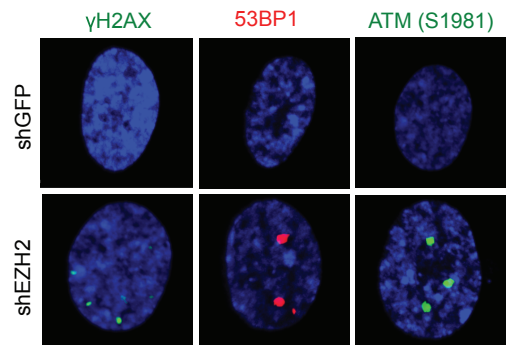
(E) Double knockdowns: a p21 shRNA (+) or the empty vector (-) were combined with an EZH2 shRNA (shEZH2) or a shGFP control, and SA- β -Gal-positive cells were scored 4 days after infection (* $p < 0.05$, ** $p < 0.01$, $n = 3$).

(F) A double knockdown of EZH2 and p21 was performed as in (E), and the levels of p21 protein were determined by immunoblotting. The control for EZH2 knockdown was shGFP, and for p21, the empty vector.

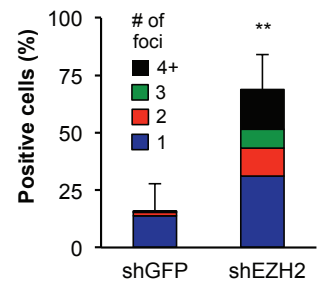
Error bars represent SD, * $p < 0.05$, ** $p < 0.01$.

Figure 3.7

A. shEZH2: γ H2AX, 53BP1 and ATM (S1981) IF



B. shEZH2: 53BP1



C. shEZH2, shATM: SA- β -Gal

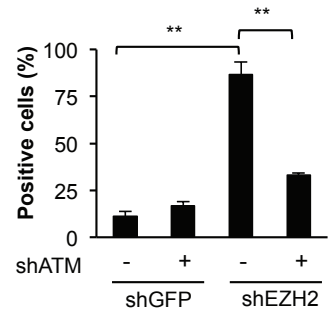


Figure 3.7 Knockdown of EZH2 Induces DNA Damage in a H3K27me3-independent Manner

(A) EZH2 was knocked down as in Figure 3.3C and cells were immunostained with antibodies to γ H2AX, 53BP1 or ATM phosphorylated at serine 1981. Representative images are shown. Cells were harvested 4 days after infection; DAPI was used to visualize nuclei. Scale bar represents 3 μ m.

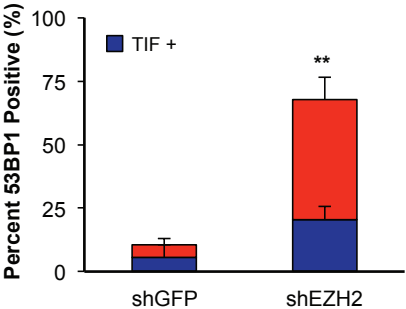
(B) 53BP1 foci were visualized 4 days after EZH2 knockdown by IF. The stacked bars depict the fraction of cells with 1 (blue), 2 (red), 3 (green), and >4 (black) foci per nucleus (**P<0.01, n=4).

(C) Double knockdowns combining shATM and shEZH2 interventions were assessed for SA- β -Gal-positive cells 4 days after infection (**p<0.01, n=3).

Error bars represent SD, * p<0.05, ** p<0.01

Figure 3.8

A. shEZH2: TIF assay



B. shEZH2, hTERT: SA- β -Gal

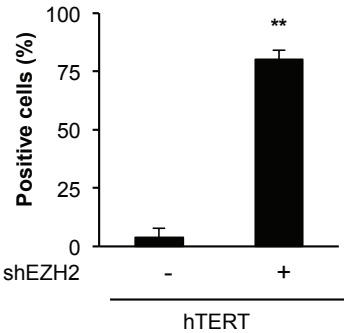


Figure 3.8 Senescence Induced by EZH2 Downregulation is Triggered by Non-telomeric DSBs

(A) EZH2 was knocked down as in Figure 3.3C and cells were processed, at 4 days after infection by an immuno-FISH procedure to examine the presence telomere dysfunction induced foci (TIFs). The TIF assay allows the simultaneous visualization of 53BP1 foci (by IF) and telomeres (by FISH). A nucleus with one (or more) 53BP1 foci is scored as a 53BP1-positive cell, and a cell in which $\geq 50\%$ of 53BP1 foci colocalize with a telomere is scored as a TIF-positive cell. At least 150 nuclei were observed for each condition (** $p < 0.01$, $n=3$).

(B) EZH2 was knocked down as in (A) in LF1 cells immortalized with human telomerase (hTERT) and SA- β -Gal-positive cells were scored 4 days after infection (** $p < 0.01$, $n=3$).

Error bars represent SD, * $p < 0.05$, ** $p < 0.01$.

Figure 3.9

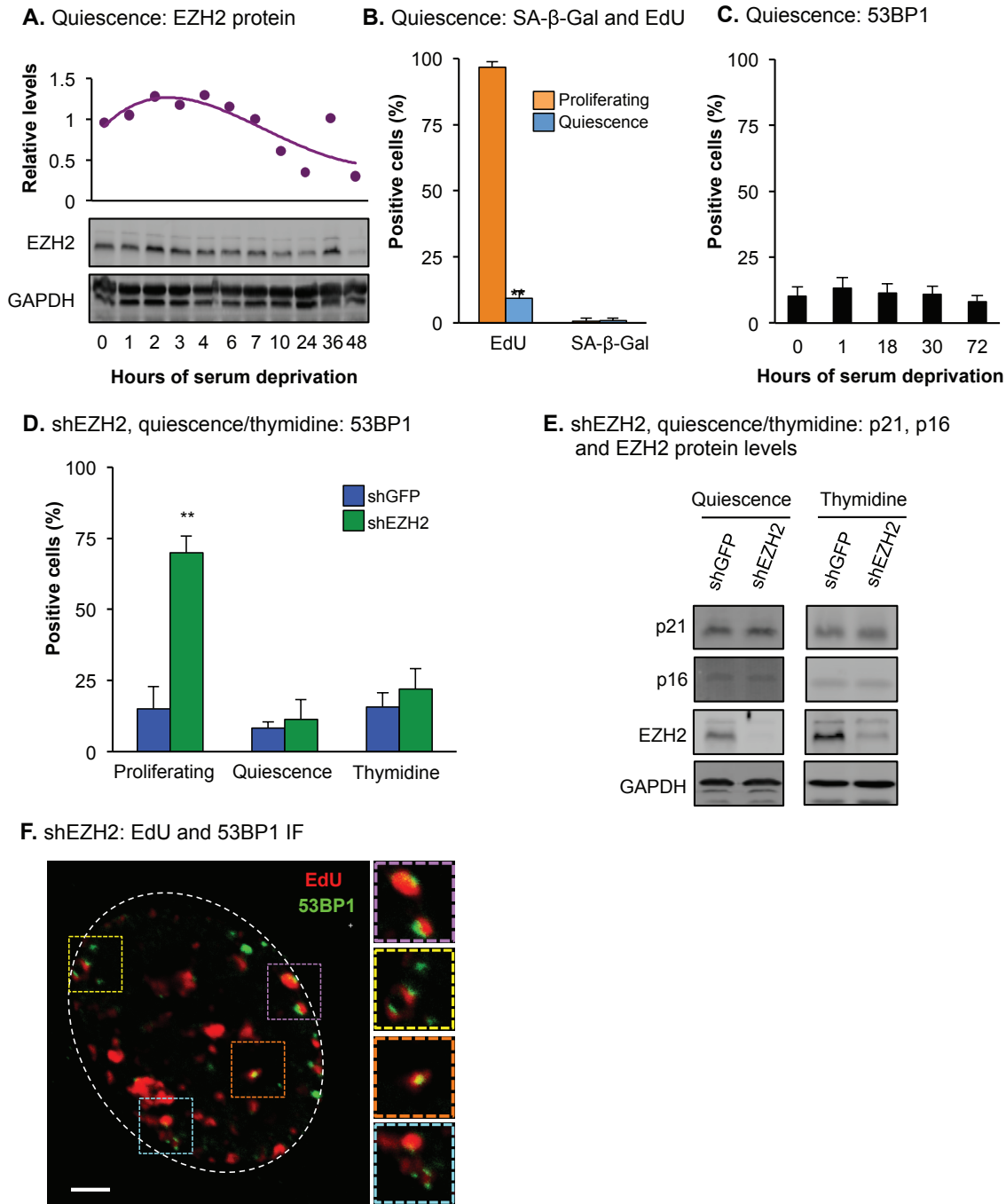


Figure 3.9 DNA Replication is Required for Induction of DNA Damage by Downregulation of EZH2

(A) LF1 cells were made quiescent by incubation in medium supplemented with 0.25% FBS for the indicated times and EZH2 protein levels were examined by immunoblotting.

(B) Proliferating and quiescent cells were scored for EdU incorporation (after 12 hrs of labeling) and the presence of SA- β -Gal positive cells. At least 220 nuclei were observed for each condition (**p<0.01, n=3).

(C) Cells were examined for 53BP1 foci by IF at the indicated times (% of total cells, random fields, >200 cells per condition).

(D) At t=0 of shEZH2 infection (Figure 3.3A) the cells were reseeded into either normal medium (15% FBS), quiescence-inducing medium (0.25% FBS), or medium supplemented with thymidine. 53BP1 foci were scored after t=4 days (**p<0.01, n=3).

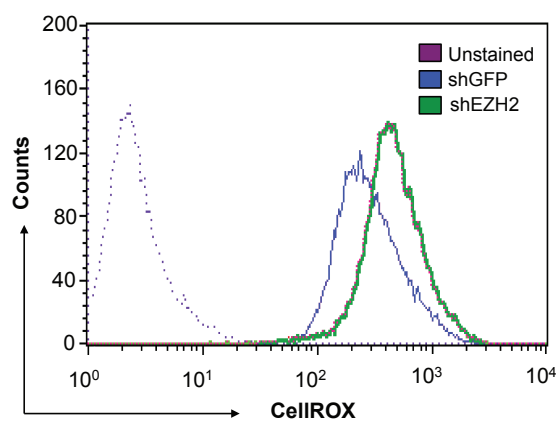
(E) EZH2 was knocked down as in (D), and p21, p16 and EZH2 protein levels were determined by immunoblotting 4 days after infection.

(F) EZH2 was knocked down as in (D) and pulse-labeled with EdU for 1 hour at 2 days after infection. A representative image showing colocalization of EdU (red) and 53BP1 (green) signals is shown. Right panels: magnified views of the indicated areas. Scale bar represents 3 μ m.

Error bars represent SD, * p<0.05, ** p<0.01

Figure 3.10

A. shEZH2: Reactive oxygen species



B. shEZH2: Mitochondrial superoxide

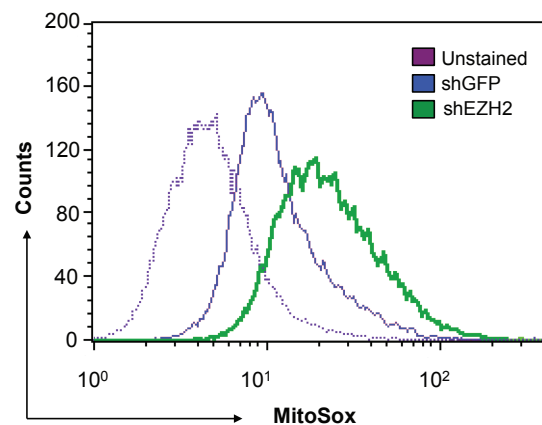


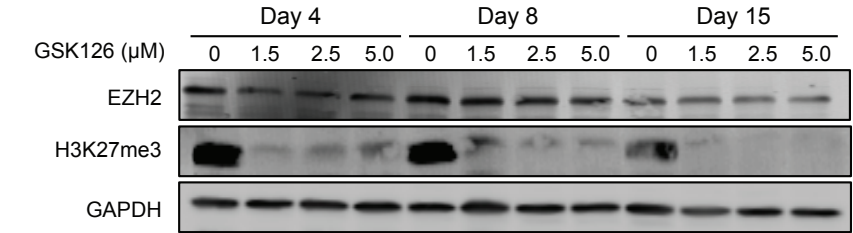
Figure 3.10 Repression of EZH2 Induces Increased Production of ROS

(A) ROS in cells with knocked down EZH2 as in Figure 3.3C were measured by CellROX flow cytometry 4 days after infection.

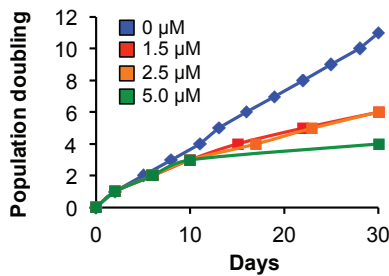
(B) Mitochondrial superoxide in cells with knocked down EZH2 as (A) were measured by MitoSOX flow cytometry 4 days after infection.

Figure 3.11

A. GSK126: EZH2 protein, H3K27me3 marks



B. GSK126: proliferation



C. GSK126: SA- β -Gal

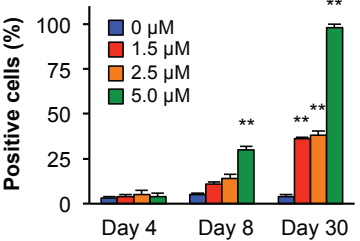


Figure 3.11 Inhibition of EZH2 Activity Induces Senescence

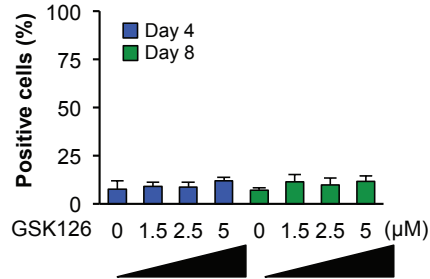
(A) Early passage LF1 cells were grown continuously in the presence of GSK126 and levels of EZH2 protein and H3K27me3 marks were examined by immunoblotting.

(B,C) Cells were treated with GSK126 as in (A) and proliferation (B) or SA- β -Gal staining (C) were determined (** $p < 0.01$, $n=3$).

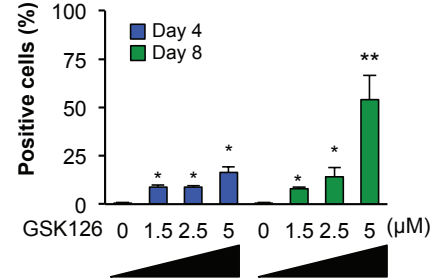
Error bars represent SD, * $p < 0.05$, ** $p < 0.01$.

Figure 3.12

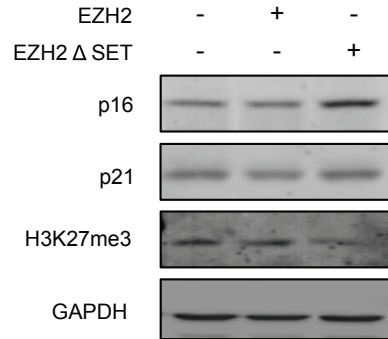
A. GSK126: p21 IF



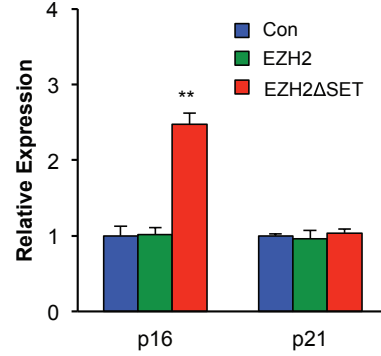
B. GSK: p16 IF



C. EZH2 SET domain deletion: p21, p16 proteins, H3K27me3 marks



D. EZH2 SET domain deletion: p16, p21 mRNA



E. GSK126: 53BP1

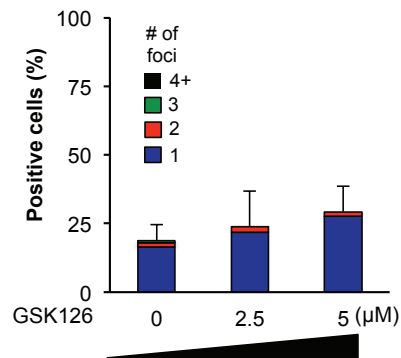


Figure 3.12 Inhibition of EZH2 Activity Induces Senescence in the Absence of DNA Damage by Activating p16

(A,B) Cells were treated as in Figure 3.11A with GSK126 and the frequency of p21 (A) and p16 (B) positive cells was scored by IF microscopy (% of total cells, random fields, >400 cells per condition, * $p<0.05$, ** $p<0.01$).

(C) Cells were infected with lentivirus vectors expressing *EZH2* cDNA, *EZH2* with an in-frame deletion of the SET domain (*EZH2 Δ SET*), or empty vector as control. The expression levels of the indicated proteins were determined by immunoblotting 9 days after infection.

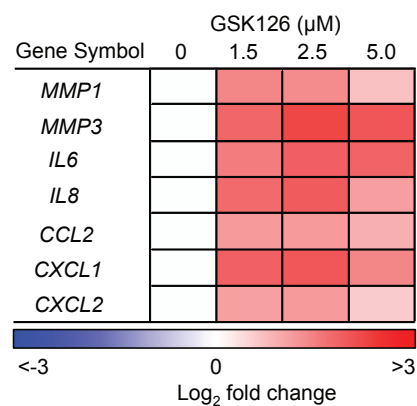
(D) For the experiment shown in (C), expression of p16 and p21 mRNAs were measured by RT-qPCR (** $p<0.01$, $n=3$).

(E) Cells were treated with GSK126 for 8 days and examined for 53BP1 foci ($n=3$).

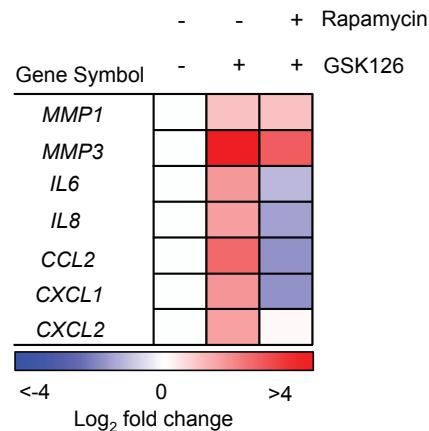
Error bars represent SD, * $p<0.05$, ** $p<0.01$.

Figure 3.13

A. GSK126: SASP genes



B. GSK126, rapamycin: SASP genes



C. GSK126, rapamycin: I κ B α protein

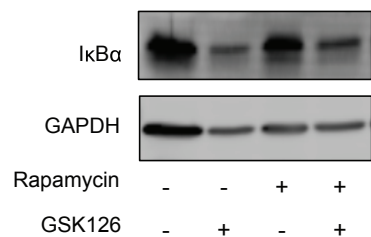


Figure 3.13 Induction of SASP with GSK126 is Rescued by Rapamycin

(A) Cells were treated with GSK126 and expression of the indicated SASP genes was determined by RT-qPCR after 4 days (n=3). The data are expressed as heat maps relative to control (vehicle-treated) cells.

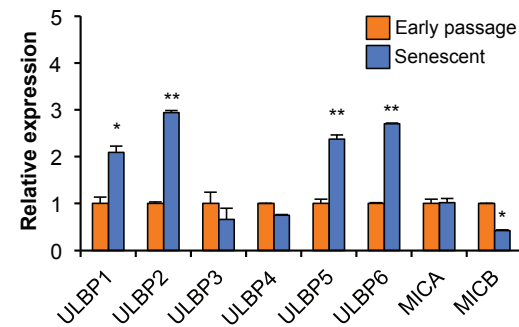
(B) Cells were treated with GSK126 for 10 days, followed by 4 days of combined treatment with GSK126 and rapamycin (12.5 nM). Expression of the indicated SASP genes was determined by RT-qPCR (n=3). The data are expressed as heat maps relative to control (vehicle-treated) cells.

(C) Cells were treated as in (B) and I κ B α protein levels were determined by immunoblotting.

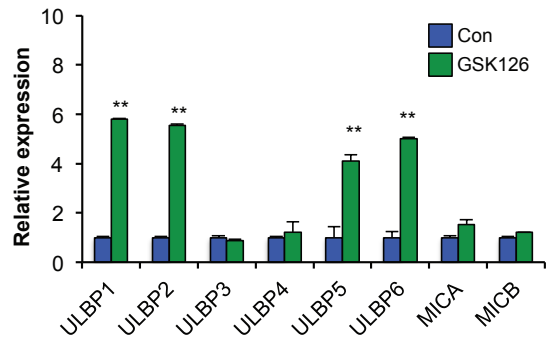
Error bars represent SD, * p<0.05, ** p<0.01.

Figure 3.14

A. Replicative senescence: NKG2D ligands



B. GSK126: NKG2D ligands



C. shEZH2: NKG2D ligands

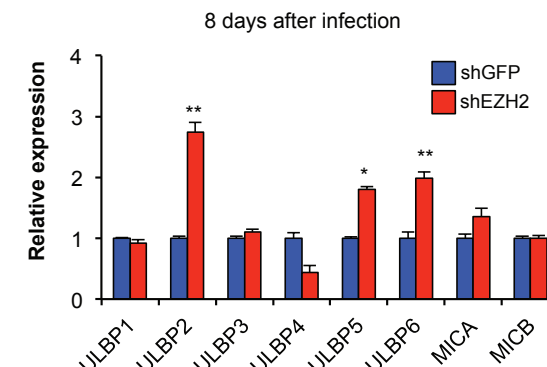
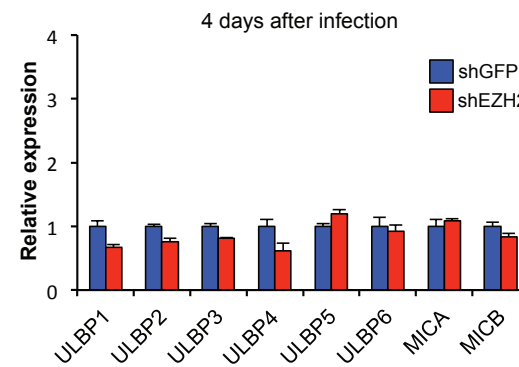


Figure 3.14 Loss of H3K27me3 Upregulates Expression of NKG2D Ligands

(A) ULBP1-6, MICA, and MICB mRNA levels were determined by RT-qPCR in early passage and senescent LF1 cells (* $p < 0.05$, ** $p < 0.01$, $n = 3$).

(B) Cells were treated with GSK126 for 24 days and expression of the indicated NKG2D ligand genes was determined by RT-qPCR as in (A) (** $p < 0.01$, $n = 3$).

(C) EZH2 was knocked down as in Figure 3.3C and expression of the indicated NKG2D ligand genes was determined by RT-qPCR as in (A) 4 and 8 days after infection (* $p < 0.05$, ** $p < 0.01$, $n = 3$)

Error bars represent SD, * $p < 0.05$, ** $p < 0.01$.

Chapter 4. Elevated EZH2 Rescues Senescence Induced by Depletion of WNT

Signaling or MYC

4.1 Summary

Downregulation of WNT signaling and MYC is sufficient to induce premature senescence. The decrease in expression of EZH2 is mediated by repression of WNT and MYC signaling. MYC binds to the canonical and non-canonical E-boxes at the EZH2 promoter to regulate the expression of EZH2. Repression of *Myc in vivo* using the *Myc* heterozygous mice also shows decrease in EZH2 levels in some tissues. We also show that EZH2 is required for senescence induced by WNT2 or MYC depletion. These mechanisms suggest potential therapeutic targets downstream of WNT and MYC in the context of cancer, and provide insights on the generation of senescent cells during aging.

RESULTS

4.2 Regulation of *EZH2* Expression in Cellular Senescence by WNT and MYC Signaling

WNT2 is a WNT ligand gene whose expression decreases in both replicative senescence and OIS (Ye et al., 2007), and *MYC* is a well-known downstream effector of WNT signaling (Herbst et al., 2014). We confirmed that *WNT2* and *MYC* are both downregulated and activity of β -catenin declines in HDFs undergoing replicative senescence (Figure 4.1A and 4.1B), and knockdown of either *WNT2* or *MYC* is sufficient to induce senescence (Figure 4.1C and 4.1D). *MYC* is a direct regulator of *BMII*, a PRC1 component (Guney et al., 2006). We thus examined whether WNT and MYC signaling also regulate *EZH2* during senescence.

Knockdown of *WNT2* in proliferating HDFs reduced the levels of active β -catenin (ABC) protein and strongly downregulated *EZH2*, with weaker effects observed on other PRC2 proteins (Figure 4.2A). Similar results were found after knockdown of *MYC* (Figure 4.2B). Conversely, activation of WNT signaling with the GSK-3 inhibitor CHIR99021 increased the levels of both ABC and *EZH2* proteins in a concentration-dependent manner (Figure 4.2C). Finally, ectopic expression of *MYC* cDNA using a lentiviral vector increased *EZH2* protein levels (Figure 4.2D). These data indicate that WNT signaling and MYC act upstream of *EZH2* to regulate its expression.

We next asked whether MYC is a direct regulator of *EZH2*. Examination of ENCODE datasets revealed a broad region of MYC binding centered at 150 bp upstream of the TSS of the *EZH2* gene. We therefore performed quantitative ChIP analysis with

several primer pairs, and found that MYC is indeed recruited to this region in proliferating HDFs (Figure 4.2E). Binding was reduced in cells after knockdown of WNT2, and eliminated after knockdown of MYC. These results show that EZH2 is a direct transcriptional target of MYC.

4.3 Repression of *EZH2* is Required for Senescence Induced by Loss of WNT and *MYC* Signaling

To test whether EZH2 is a necessary component of this pathway we knocked down WNT2 or MYC in HDFs that were previously engineered to ectopically express *EZH2* cDNA. Ectopic expression of EZH2 suppressed the activation p21 and partially rescued the onset of senescence after WNT2 knockdown (Figure 4.3A-4.3C). Similarly, expression of EZH2 alleviated the activation of p21 and onset of senescence after knockdown of MYC (Figure 4.4A-4.4C). This analysis thus places EZH2 downstream of both WNT2 and MYC. To confirm that MYC is acting downstream of WNT2, we knocked down WNT2 in HDFs ectopically expressing *MYC* cDNA and found that activation of p21 and entry into senescence were alleviated (Figure 4.5A-4.5C). These data further confirm the existence of a WNT–MYC–EZH2 pathway for the regulation of senescence.

4.4 *Myc* Heterozygous Mice Show Decrease in *Ezh2* Levels

To examine whether the WNT–MYC–EZH2 axis occurs *in vivo*, we utilized two transgenic mice. We examined the muscle and liver tissues from the long-lived *Myc* heterozygous (*Myc*^{+/-}) mice (Hofmann et al., 2015). There was significant increase in

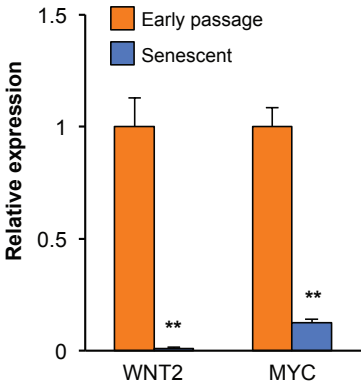
mH2A levels in both liver and muscle from young *Myc*^{-/+} mice compared to WT (*Myc*^{+/+}) mice (Figure 4.6A and 4.6B). Although we did not observe significant changes in H3K27me3 mark in the *Myc*^{-/+} mice (Figure 4.6C and 4.6D), there was a dramatic decrease in EZH2 level in the liver of old *Myc*^{-/+} mice compared to WT possibly suggesting the recapitulation MYC-EZH2 axis observed in LF1 cells (Figure 4.6E and 4.6F).

We also examined liver, lung, and muscle tissues from old (20-21 months) mice with activated Wnt signaling using homozygous inactivation of *Apc* in *Apc*^{fl/fl} mice (Pawlikowski et al., 2013b). Although these mice are devoid of Cre, they appear to be hypomorphic for APC, possibly due to the two flox sites decreasing the expression of the gene. These mice show activation of Wnt signaling, enough to get liver cancer and increase the expression of Wnt-target genes such as *Myc* (data not shown). The modest increase in Wnt signaling in these mice was not sufficient to change the levels of mH2A or H3K27me3 in all the tissues that were examined (Figure 4.7A-4.7F).

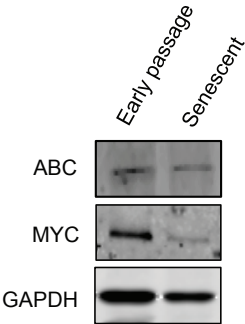
Analysis of human brain shows decrease in expression of EZH2 and increase in expression of demethylase UTX with age (Jin et al., 2011; Lu et al., 2004). We examined the EZH2 levels in the aging mice liver and did not observe significant changes (Figure 4.8).

Figure 4.1

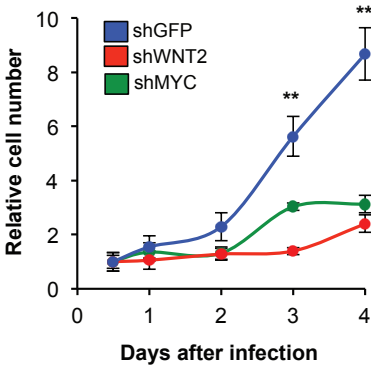
A. WNT2 and MYC mRNA



B. ABC and MYC proteins



C. shWNT2 or shMYC: proliferation



D. shWNT2 or shMYC: SA- β -Gal

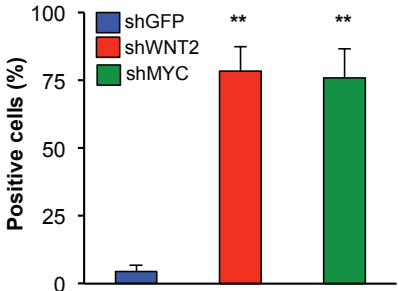


Figure 4.1 WNT2 and MYC Signaling in Senescent Cells

(A) WNT2 and MYC mRNA levels were determined by RT-qPCR in early passage proliferating and senescent LF1 fibroblasts (** $p < 0.01$, $n=3$). .

(B) Active β -catenin (ABC) and MYC protein levels were determined by immunoblotting in proliferating and senescent LF1 fibroblasts as in (A).

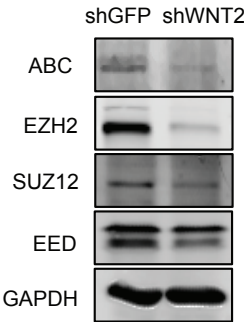
(C) LF1 cells were infected with lentivirus vectors expressing shRNAs against WNT2 or MYC. shGFP was used as the control. Proliferation was assessed by counting cell numbers at successive times after infection (** $p < 0.01$, $n=3$).

(D) Cells were infected with shRNAs targeting WNT2, MYC, or GFP as in (C) and the presence of SA- β -Gal positive cells was scored (** $p < 0.01$, $n=3$).

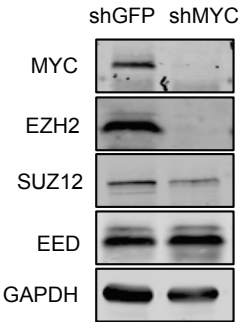
Error bars represent SD, * $p < 0.05$, ** $p < 0.01$.

Figure 4.2

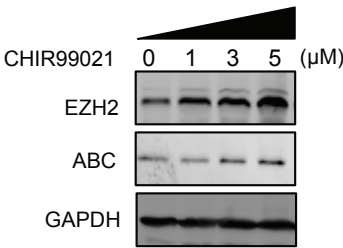
A. shWNT2: PRC2 proteins



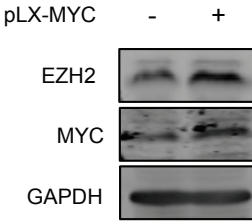
B. shMYC: PRC2 proteins



C. CHIR: EZH2, ABC proteins



D. MYC cDNA: EZH2, MYC proteins



E. MYC ChIP of *EZH2* Promoter

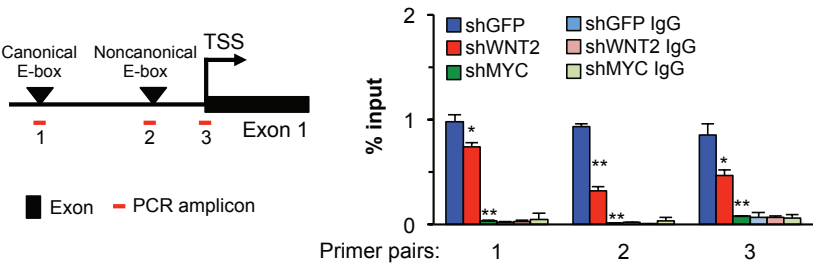


Figure 4.2 WNT Signaling and MYC Regulate *EZH2* Expression

(A) LF1 cells were infected with a lentivirus vector expressing a shRNA against WNT2. shRNA against GFP was used as the control. Levels of active β -catenin (ABC), EZH2, SUZ12 and EED proteins were examined 4 days after infection by immunoblotting.

(B) Cells were infected with a shRNA targeting MYC, treated as in (A), and MYC, EZH2, SUZ12 and EED proteins were visualized by immunoblotting.

(C) Cells were treated continuously for 8 days with the indicated concentrations of the GSK3 inhibitor CHIR99021 and levels of EZH2 and ABC proteins were determined by immunoblotting.

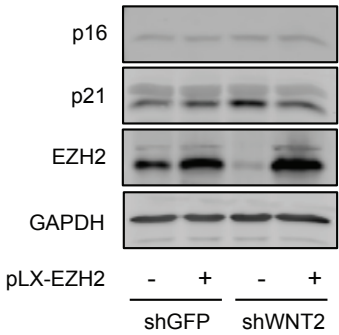
(D) Cells were infected with a lentivirus vector expressing *MYC* cDNA, and levels of EZH2 and MYC proteins were determined by immunoblotting. Empty pLX vector (–) was used as the control.

(E) MYC enrichment at the promoter region of the *EZH2* gene was determined by ChIP after knockdown of WNT2 or MYC. Locations of primer pairs are shown on left. Normal rabbit IgG was used as the IP control (* $p < 0.05$, ** $p < 0.01$, $n = 3$).

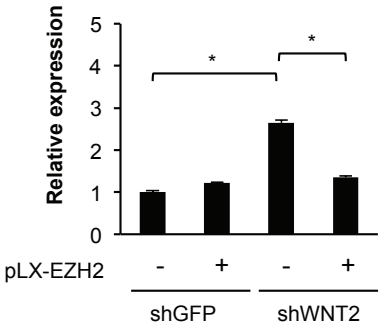
Error bars represent SD, * $p < 0.05$, ** $p < 0.01$.

Figure 4.3

A. *EZH2* cDNA, shWNT2: p16, p21, EZH2 proteins



B. *EZH2* cDNA, shWNT2: p21 mRNA



C. *EZH2* cDNA, shWNT2: SA- β -Gal

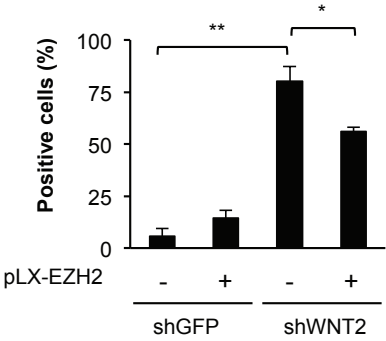


Figure 4.3 EZH2 is Required for Senescence Induced by Downregulation of WNT

(A) Ectopic expression of *EZH2* cDNA was combined with a shRNA knockdown of WNT2. Controls were empty pLX lentivirus vector for *EZH2* (–) and shGFP for WNT2. Levels of p16, p21, and EZH2 proteins were determined by immunoblotting. Note that while endogenous EZH2 expression is effectively downregulated by shWNT2 (lane 3, also see Figure 4.2A), ectopic expression is maintained by the pLX-EZH2 vector (lane 4).

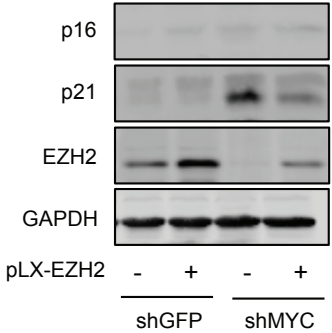
(B) p21 mRNA expression was determined by RT-qPCR in the experiment shown in (A) (* $p < 0.05$, $n = 3$).

(C) The presence of SA- β -Gal positive cells was scored in the experiment shown in (A) (* $p < 0.05$, ** $p < 0.01$, $n = 3$).

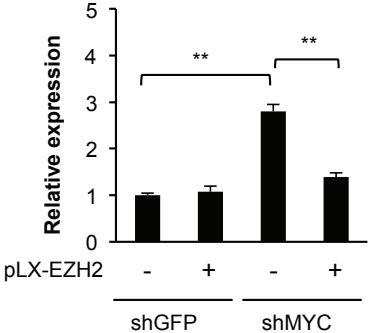
Error bars represent SD, * $p < 0.05$, ** $p < 0.01$.

Figure 4.4

A. *EZH2* cDNA, shMYC: p16, p21, EZH2 proteins



B. *EZH2* cDNA, shMYC: p21 mRNA



C. *EZH2* cDNA, shMYC: SA- β -Gal

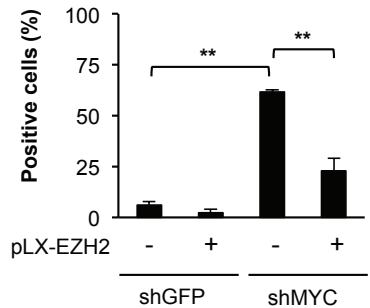


Figure 4.4 EZH2 is Required for Senescence Induced by Downregulation of MYC

(A) The double intervention shown in Figure 4.3A was performed with MYC shRNA (instead of WNT2 shRNA) and the levels of p16, p21, and EZH2 proteins were examined. Note again that while endogenous EZH2 expression is effectively downregulated by shMYC (lane 3, also see Figure 4.2B), ectopic expression is maintained by the pLX-EZH2 vector (lane 4).

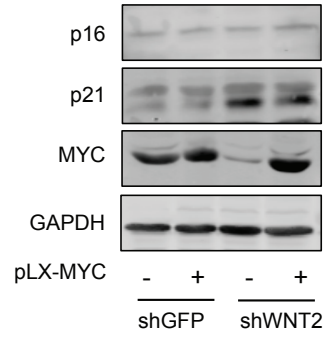
(B) p21 mRNA expression was determined in the experiment shown in (A) (**p<0.01, n=3).

(C) SA- β -Gal positive cells were quantified in the experiment shown in (A) (**p<0.01, n=3).

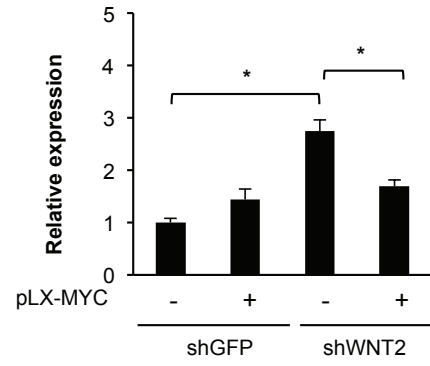
Error bars represent SD, * p<0.05, ** p<0.01.

Figure 4.5

A. MYC cDNA, shWNT2: p16, 21, EZH2 proteins



B. MYC cDNA, shWNT2: p21 mRNA



C. MYC cDNA, shWNT2: SA- β -Gal

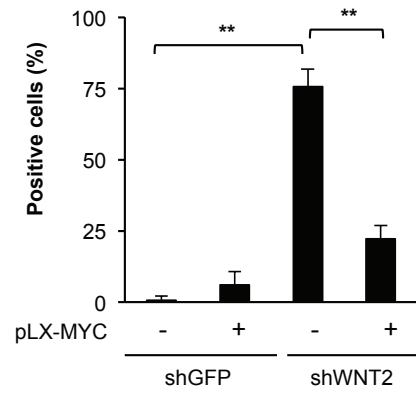


Figure 4.5. Ectopic Expression of *MYC* Rescues a Knockdown of WNT2

(A) Ectopic expression of *MYC* cDNA was combined with a shRNA knockdown of WNT2. Controls were empty pLX lentivirus vector for *MYC* (–) and shGFP for WNT2. Levels of p16, p21, and MYC proteins were determined by immunoblotting. Note that while endogenous MYC expression is effectively downregulated by shWNT2 (lane 3), ectopic expression is maintained by the pLX-MYC vector (lane 4).

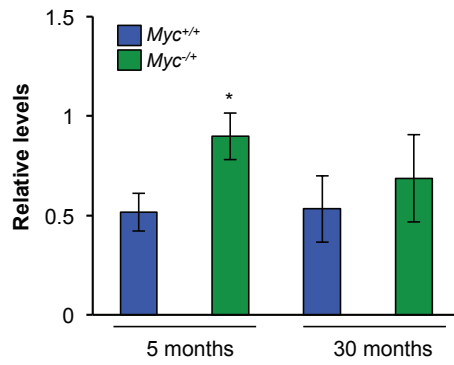
(B) p21 mRNA expression was determined by RT-qPCR in the experiment shown in (A) (* $p < 0.05$, $n = 3$).

(C) The presence of SA- β -Gal positive cells was scored in the experiment shown in (A) (** $p < 0.01$, $n = 3$).

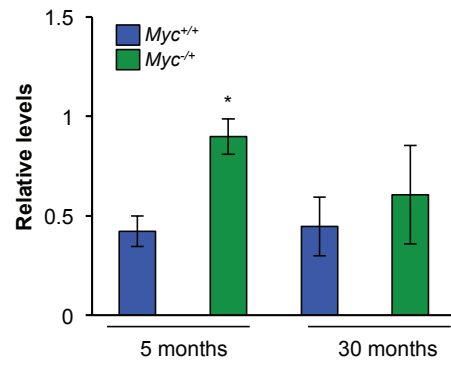
Error bars represent SD, * $p < 0.05$, ** $p < 0.01$.

Figure 4.6

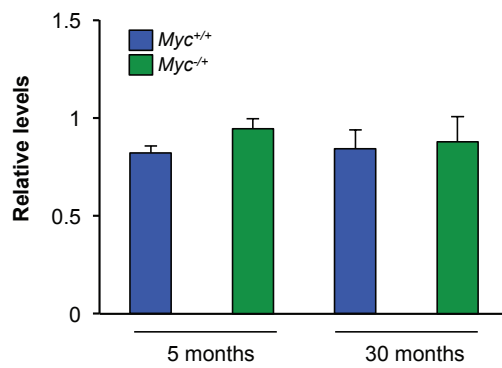
A. *Myc*^{-/-} mice muscle: mH2A



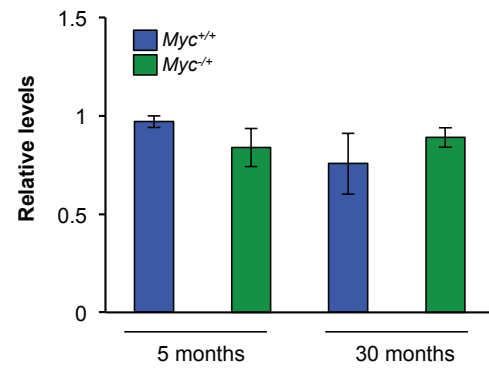
B. *Myc*^{-/-} mice liver: mH2A



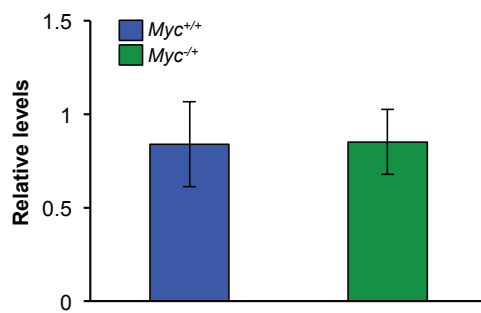
C. *Myc*^{-/-} mice muscle: H3K27me3 mark



D. *Myc*^{-/-} mice liver: H3K27me3 mark



E. Old *Myc*^{-/-} mice muscle: Ezh2 protein



F. Old *Myc*^{-/-} mice liver: Ezh2 protein

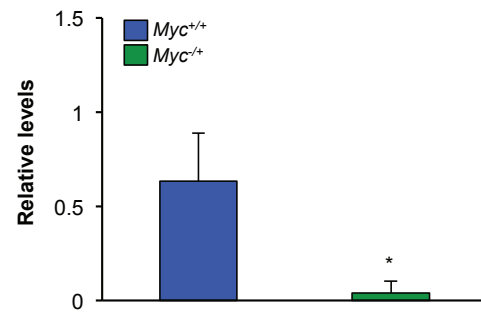


Figure 4.6 *Myc* Heterozygous Mice Have Increased Heterochromatin Mark and Decreased EZH2

(A) MacroH2A (mH2A) and H3 protein levels were examined by immunoblotting from skeletal muscle extracts of young (5 months; n=3) and old (30 months, n=3) *Myc*^{-/+} (blue) and *Myc*^{+/+} (green) mice. The ratio of mH2a to H3 was determined for each mice and the average ratio for each cohort was determined (*p<0.05).

(B) mH2A to H3 ratio was determined as in (A) from liver extracts (*p<0.05).

(C) H3K27me3 to H3 ratio was determined as in (A) from muscle extracts.

(D) H3K27me3 to H3 ratio was determined as in (A) from liver extracts.

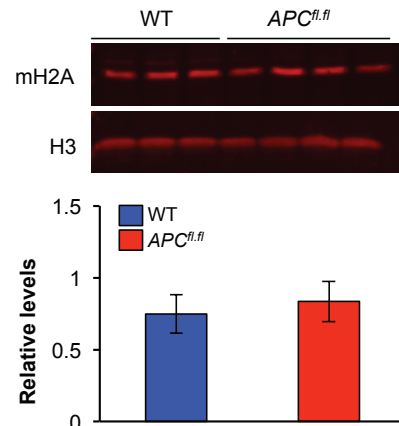
(E) EZH2 and GAPDH protein levels were examined by immunoblotting from skeletal muscle extracts of old (30 months, n=3) *Myc*^{-/+} (blue) and *Myc*^{+/+} (green) mice. The ratio of EZH2 to GAPDH was determined for each mice and the average ratio for each cohort was determined.

(F) EZH2 to GAPDH ratio was determined as in (E) from liver extracts (*p<0.05).

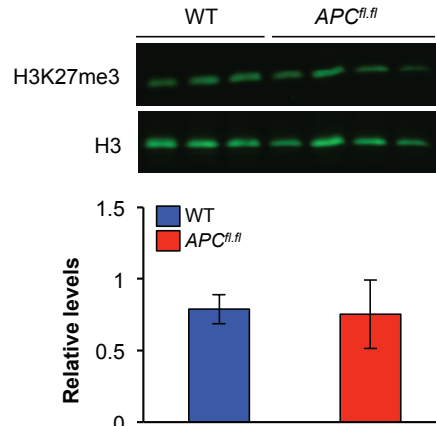
Error bars represent SD, * p<0.05, ** p<0.01.

Figure 4.7

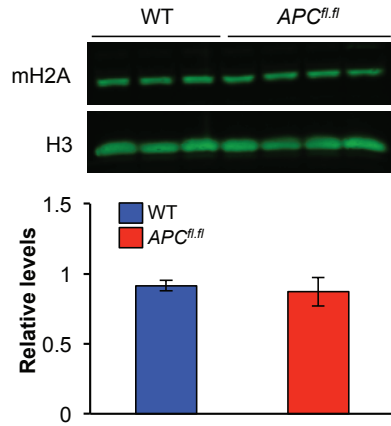
A. *APC^{fl/fl}* mice liver: mH2A protein



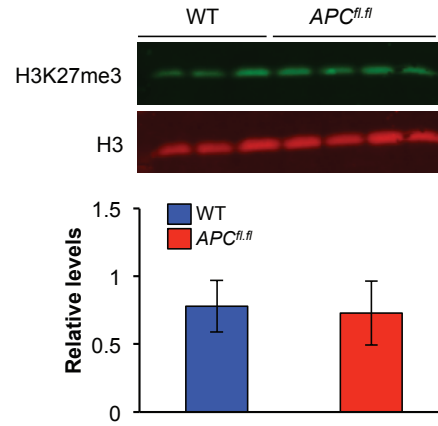
B. *APC^{fl/fl}* mice liver: H3K27me3 mark



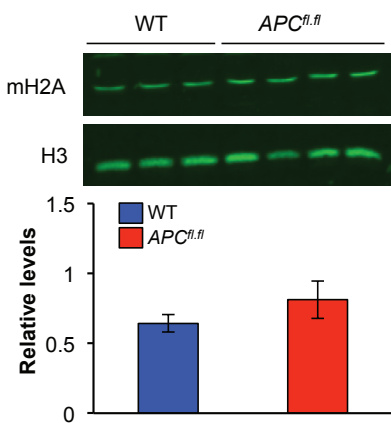
C. *APC^{fl/fl}* mice lung: mH2A protein



D. *APC^{fl/fl}* mice lung: H3K27me3 mark



E. *APC^{fl/fl}* mice muscle: mH2A protein



F. *APC^{fl/fl}* mice muscle: H3K27me3 mark

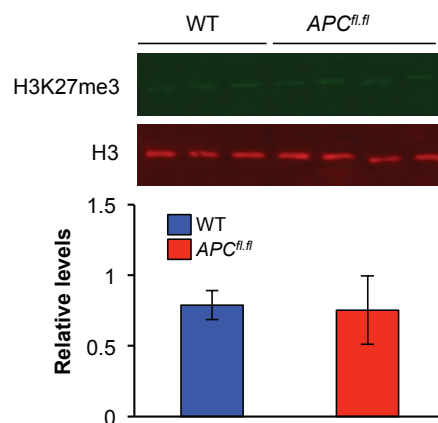


Figure 4.7 Heterochromatin Marks in Mice with Elevated WNT Signaling

(A) MacroH2A (mH2A) and H3 protein levels were examined by immunoblotting from liver extracts for old (20-21 months) WT (blue; n=3) and *APC^{fl/fl}* (red; n=4) mice. The ratio of mH2a to H3 was determined for each mice and the average ratio for each cohort was determined (*p<0.05).

(B) H3K27me3 to H3 ratio was determined as in (A) from liver extracts.

(C) mH2A to H3 ratio was determined as in (A) from lung extracts.

(D) H3K27me3 to H3 ratio was determined as in (A) from lung extracts.

(E) mH2A to H3 ratio was determined as in (A) from skeletal muscle extracts.

(F) H3K27me3 to H3 ratio was determined as in (A) from skeletal muscle extracts.

Error bars represent SD, * p<0.05, ** p<0.01.

Figure 4.8

A. Aging mice liver: Ezh2 protein

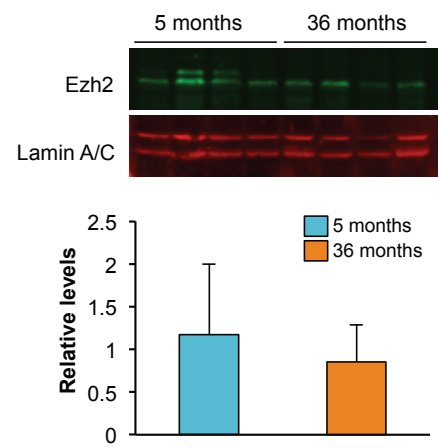


Figure 4.8 Ezh2 Levels with Organismal Aging

(A) Ezh2 and Lamin A/C protein levels were examined by immunoblotting from liver extracts for young (5 months) and old (36 months) mice (n=4 per age cohort). The ratio of Ezh2 to Lamin A/C (average of two bands) was determined for each mice and the average ratio for each cohort was determined.

Error bars represent SD, * $p < 0.05$, ** $p < 0.01$.

Chapter 5. Discussion

5.1 Senescence by Downregulation of EZH2

In this study we have explored how the Polycomb group (PcG) regulates cellular senescence. The EZH2 protein, with its histone lysine methyltransferase activity, is the catalytic core of PcG complexes, depositing the H3K27me3 mark that is the characteristic feature of facultative heterochromatin. We found that downregulation of EZH2 in normal, proliferating cells rapidly induces cellular senescence that is initiated by widespread DNA damage leading to a DDR mediated by the ATM–p53–p21 signaling pathway. The ensuing cell cycle arrest clearly precedes the depletion of H3K27me3 marks, and is hence likely to be independent of expression changes at PcG-regulated loci. PcG complexes are localized at replication forks during S phase, and their depletion can cause DNA damage (Hansen et al., 2008; Piunti et al., 2014). The DDR we observed after depletion of EZH2 likely proceeds through this mechanism, since it is dependent on cell cycle progression, and DNA damage (53BP1) foci frequently colocalize with sites of DNA replication.

The CDK inhibitor p16 (CDKN2A) is an important regulator of senescence, and arguably the best current marker of senescent cells (van Deursen, 2014). Its regulation by PcG is well established (Bracken et al., 2007) and our data support those studies. However, the upregulation of p16 is a late event after EZH2 downregulation, clearly separated from the appearance of DNA damage, induction of SA- β -Gal activity, and cell cycle arrest. While irreversible cell cycle arrest is the classical feature of senescence, senescent cells display many gene expression changes that are involved in unrelated processes (Lackner et al., 2014; Nelson et al., 2014). Among these the SASP is of considerable interest, as it is likely to mediate many of the deleterious effects of

senescent cells. Chromatin changes during senescence, in particular those involving H3K27me3 marks, have been associated with the upregulation of SASP genes (Shah et al., 2013). We found that downregulation of EZH2 resulted in the activation of SASP genes at late times that coincided with the depletion of H3K27me3 marks and induction of p16.

We thus propose a model for the induction of senescence by PcG downregulation that consists of two distinct phases: an early phase characterized by DNA damage and a late phase of epigenetic changes and chromatin remodeling (Figure 5.1). That these processes can be independent is evidenced by the strong activation of senescence by the simple inhibition of EZH2 activity, either with the drug GSK126 or expression of a catalytically inactive EZH2 protein. This senescence response appears to be completely devoid of DNA damage and activation of p21, is late in onset, and characterized by the upregulation of p16 and SASP. While the early phase appears to proceed through a physical loss of PcG complexes resulting in DNA replication fork defects, the late phase is mediated by the absence of EZH2 activity leading to the depletion of H3K27me3 marks, facultative heterochromatin, and ensuing gene expression changes.

p16 is an important regulator of senescence, yet its upregulation alone, while sufficient to cause cell cycle arrest, does not induce SASP (Coppe et al., 2011). This underscores the importance of epigenetic changes, which regulate p16 and contribute to the regulation of SASP in eliciting the full phenotype of senescent cells. NF- κ B is a key regulator of SASP (Laberge et al., 2015) and PcG is known to interact with the NF- κ B pathway (Lee et al., 2011). Recently, BRD4 has been shown to drive enrichment of H3K27ac at super-enhancers of key SASP genes in senescence cells and inhibition of

BRD4 using BET inhibitors prevents proper immune targeting of senescent cells *in vitro* and *in vivo* (Tasdemir et al., 2016). Our data indicate that loss of H3K27me3 mark regulates the expression of NKG2D ligands that play critical role in the immune clearance of senescence cells. We also show that loss of H3K27me3 marks facilitates the expression of SASP genes in senescence.

5.2 WNT-MYC-PcG Axis

How might EZH2 be regulated during senescence? We previously found that downregulating MYC by as little as 50% makes cells increasingly prone to senescence, and that this response involves the PRC1 component BMI1 (Guney et al., 2006). Here we document that downregulation of MYC closely phenocopies the senescence response induced by depletion of EZH2, including DNA damage, biphasic kinetics and activation of SASP, and that EZH2 is a direct transcriptional target of MYC. It appears that MYC can regulate multiple PcG components (Krepelova et al., 2014), although in our experiments the effect on EZH2 was the strongest. MYC has been reported to downregulate miR-26a/b, a negative effector of EZH2 (Sander et al., 2008), and through its effects on the AKT pathway to indirectly activate and stabilize the EZH2 protein (Kaur and Cole, 2013). These posttranscriptional and posttranslational mechanisms likely reinforce the direct regulation of EZH2 by MYC we document here, and underscore the importance of MYC as a regulator of EZH2, and by extension, the PcG pathway.

Ye et al. (2007) first reported that downregulation of WNT2 triggered senescence and was required for subsequent epigenetic changes, in particular the formation SAHF that were the focus of that study. Since canonical WNT signaling is known to activate

MYC expression, it was of interest to examine the regulatory relationship between WNT2, MYC and EZH2 in the biological system under investigation here. We did this by an epistasis analysis, performing a series of double interventions. In particular, ectopic expression of MYC antagonized the senescence-inducing effects of WNT2 depletion, and likewise expression of EZH2 partially reversed the effects of both MYC and WNT2 downregulation. These compensatory effects were highly significant but not complete, most likely because PcG genes in addition to EZH2 are involved, and overexpression of MYC in normal cells is deleterious. Nevertheless, the genetic relationships are clearly indicative of a WNT2–MYC–EZH2 hierarchy.

WNT, MYC or EZH2 overexpression or increased activities have been strongly implicated in tumorigenesis and correlate with poor prognosis in many tumor types. Downregulation of MYC in cancer cells has been found to have considerable beneficial effects, sometimes referred to as "oncogene addiction" (Gabay et al., 2014), and is likely to be an important therapeutic strategy (Sodir and Evan, 2011). The development of drugs that inhibit EZH2 activity presents new opportunities for intervention given the difficulty of pharmacologically targeting MYC. These compounds (such as GSK126) are SAM competitive inhibitors and are effective against normal and hyperactivated mutant EZH2, providing promising clinical potential for use against different cancers. In B cell lymphoma and melanoma, the lack of additive effects between MYC overexpression and EZH2 gain-of-function mutation as well as overlap in gene expression profiles suggests that the transforming effects of MYC are mediated by EZH2 (Souroullas et al., 2016).

However, long-term inhibition of EZH2 activity is likely to eventually compromise the maintenance of facultative heterochromatin in postmitotic cells, which

may have deleterious consequences. In contrast, given that PcG complexes are localized at the replication fork for the transmission of H3K27me3 marks to daughter cells, targeting this process, for example by inhibiting the binding of the PcG PRC2 complex to the H3K27me3 marks on parental chromatin (Margueron et al., 2009), might be an effective way of provoking replication fork defects and DNA damage that are largely confined to cycling cells.

5.3 Outstanding Questions/Future Directions

In this study, we show EZH2 is downregulated by various stimuli that induce DNA damage such as telomere shortening, oncogenic stress, irradiation and treatment with DNA damaging agent. The loss of EZH2 during the S-phase further induces additional DNA damage through EZH2-mediated replication fork defects. Why would cells develop a mechanism to induce more DNA damage upon sensing DNA damage? This may be explained by two possible mechanisms that are not mutually exclusive.

First, this may be a mechanism to amplify the DNA damage signals. Senescent cells have shown to amplify DNA damage in other ways such as production of ROS upon long-term DDR as a positive feedback loop to increase ROS-mediated DNA damage (Passos et al., 2010). The DDR-ROS-DDR loop was also observed upon knockdown of EZH2. The additional DNA damage from the replication fork defect may provide an additional positive feedback loop to ensure that the cells stop dividing, at least temporarily.

The roles of p21 and p16 in senescent cells have been well established and have been thought to be distinct senescence programs. Senescence triggered by telomere shortening activates the ATM-p53-p21 pathway without initial induction of p16 (Herbig

et al., 2004). Fluorescence-activated cell sorting (FACS) for p16-positive cells in hTERT-immortalized HDF do not show activation of p21 (Jeyapalan and Sedivy, 2013). However, we show that activation of p21 induced by replication fork defects and activation of p16 by epigenetic alterations are both mediated by downregulation of EZH2. We propose a model where downregulation of EZH2 acts as senescence “clock” to mediate the transition for pre-senescent cells to fully senescent cells. In this model, loss of EZH2 accentuates the senescence triggers by inducing additional DNA damage and by activating p21. This is the point at which the cells stop dividing at S-phase and the “clock” begins for cells to repair the DNA damage. In one scenario, the DNA damage is repaired and cells can go back into the cell cycle by re-expressing EZH2. However, if the DNA damage cannot be repaired and the “clock” runs out, the loss of H3K27me3 leads to expression of p16 and SASP factors. If this model holds true, conditional expression of EZH2 upon stimulation by DNA damaging stimuli should rescue the cells back into the cell cycle and prohibit the activation of p16 and SASP factors.

Under what circumstances might these processes operate *in vivo*? The loss of PcG complexes during S phase triggers DNA damage at replication forks (Piunti et al., 2014), and the induction of senescence by the downregulation of the WNT–MYC–PcG "axis" we document here likewise requires cell cycle progression. Recent work has revealed the induction of single-strand DNA breaks in enhancers (Puc et al., 2015) and DSB in promoters (Madabhushi et al., 2015) in some contexts during physiological regulation of gene expression. We show that the induction of quiescence does not elicit DNA damage and DDR, likely because the downregulation is more gradual and allows cell cycle withdrawal. The literature also indicates that widespread DDR induction is unlikely to be

associated with normal developmental or cell differentiation events regulated by WNT or MYC.

In contrast, DNA damage, ROS and DDR signaling are almost universally associated with cellular senescence. Since DNA damage and ROS can promote each other, their roles are difficult to disentangle. DSBs at telomeres or replication forks are the triggering events in replicative senescence and OIS, respectively, the two most studied senescence states. MYC is downregulated during DDR as well as in response to ROS (Cannell et al., 2010; Guney et al., 2006; Popov et al., 2007), which we show here leads to the downregulation of PcG and the epigenetic changes characteristic of the late phase of senescence. In some cases WNT signaling has also been found to decrease in response to DNA damage or ROS (Shin et al., 2004; Sun et al., 2016b).

While DNA damage is closely associated with senescence, and in the case of telomere-induced senescence is the initiating event, this is not always the case. Senescence induced during wound healing is initiated by a cell extrinsic signaling event that triggers an oxidative burst followed by a DDR (Jun and Lau, 2010). Expression of activated *Ras* creates a signaling imbalance that leads to over-replication and ultimately a DDR. Imbalances in the opposite direction, such as downregulation of WNT or MYC can likewise lead to DDR and senescence. What appears to unite these signaling imbalances as initiators of senescence is the requirement for cell cycle progression. In this context it is interesting to note that the age-associated increase in senescent cells has been observed mostly in proliferative cell compartments, such as stellate cells in the liver (Krizhanovsky et al., 2008), adipocyte stem cells and preadipocytes in fat (Baker et al., 2013) and type II pneumocytes in the lung (Alder et al., 2015). Such signaling imbalances may also explain

the strong upregulation of p16 in stromal cells surrounding a tumor (Burd et al., 2013). Although we did not observe downregulation of EZH2 in the aging liver tissue, we predict the expression of EZH2 to decrease in normally proliferating cells in aged organisms. Although knockdown of EZH2 in quiescent cells did not elicit significant DNA damage or activation of p21, inhibition of EZH2 activity in quiescent or post-mitotic cells may induce senescence through epigenetic activation of p16. The question remains whether non-proliferating cells are more resistant in depleting EZH2 upon DNA damage. The quiescence to senescence conversion, referred to as geroconversion, is of increasing interest in stem cell and cancer biology and EZH2 may potentially be significant contributor in this process.

Figure 5.1

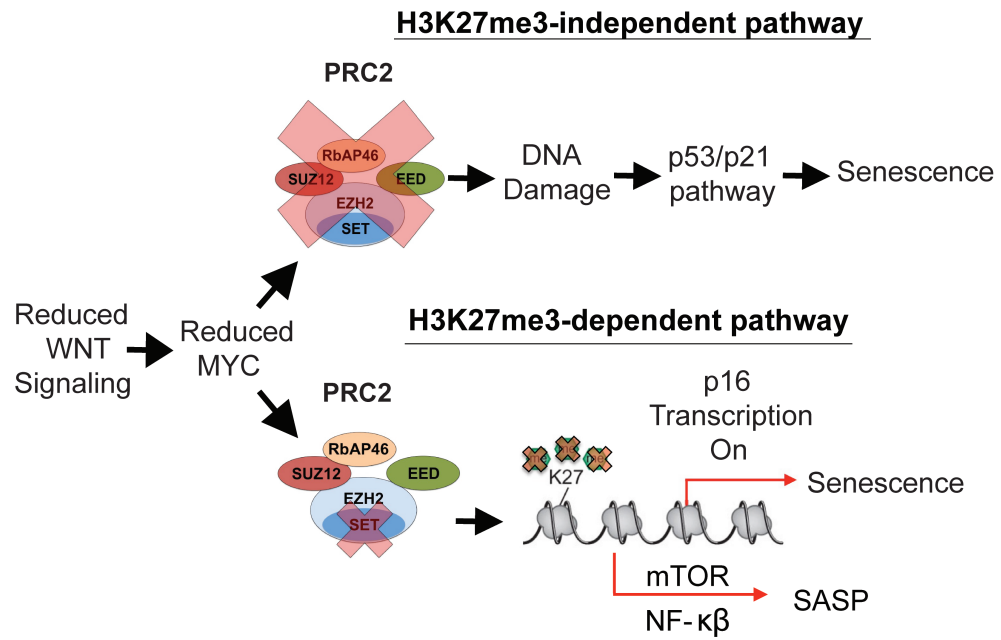


Figure 5.1 Induction of Senescence by the WNT–MYC–EZH2 Pathway

WNT signaling is downregulated leading to inhibition of *MYC* transcription. Subsequent loss of MYC at the promoter of *EZH2* downregulates the transcription of *EZH2*. In the H3K27me3-independent pathway (top) loss of *EZH2* protein disrupts the PRC2 complex, which during S phase progression results in replication errors and activation of DDR, leading to a senescence response. In the H3K27me3-dependent pathway (bottom) loss of *EZH2* activity results in the depletion of H3K27me3 at the *CDKN2A* locus, leading to transcriptional activation of p16. Global loss of H3K27me3 also contributes to the expression of SASP genes.

References

Adams, J.M., Harris, A.W., Pinkert, C.A., Corcoran, L.M., Alexander, W.S., Cory, S., Palmiter, R.D., and Brinster, R.L. (1985). The c-myc oncogene driven by immunoglobulin enhancers induces lymphoid malignancy in transgenic mice. *Nature* 318, 533-538.

Agger, K., Cloos, P.A., Rudkjaer, L., Williams, K., Andersen, G., Christensen, J., and Helin, K. (2009). The H3K27me3 demethylase JMJD3 contributes to the activation of the INK4A-ARF locus in response to oncogene- and stress-induced senescence. *Genes Dev* 23, 1171-1176.

Alder, J.K., Barkauskas, C.E., Limjunyawong, N., Stanley, S.E., Kembou, F., Tuder, R.M., Hogan, B.L., Mitzner, W., and Armanios, M. (2015). Telomere dysfunction causes alveolar stem cell failure. *Proc Natl Acad Sci U S A* 112, 5099-5104.

Aranda, S., Mas, G., and Di Croce, L. (2015). Regulation of gene transcription by Polycomb proteins. *Sci Adv* 1, e1500737.

Arvanitis, C., and Felsher, D.W. (2006). Conditional transgenic models define how MYC initiates and maintains tumorigenesis. *Semin Cancer Biol* 16, 313-317.

Asano, T., Yao, Y., Zhu, J., Li, D., Abbruzzese, J.L., and Reddy, S.A. (2004). The PI 3-kinase/Akt signaling pathway is activated due to aberrant Pten expression and targets transcription factors NF-kappaB and c-Myc in pancreatic cancer cells. *Oncogene* 23, 8571-8580.

Ashton, K.J., Willems, L., Holmgren, K., Ferreira, L., and Headrick, J.P. (2006). Age-associated shifts in cardiac gene transcription and transcriptional responses to ischemic stress. *Exp Gerontol* 41, 189-204.

Baker, D.J., Childs, B.G., Durik, M., Wijers, M.E., Sieben, C.J., Zhong, J., Saltness, R.A., Jeganathan, K.B., Verzos, G.C., Pezeshki, A., *et al.* (2016). Naturally occurring p16(Ink4a)-positive cells shorten healthy lifespan. *Nature* 530, 184-189.

Baker, D.J., Perez-Terzic, C., Jin, F., Pitel, K.S., Niederlander, N.J., Jeganathan, K., Yamada, S., Reyes, S., Rowe, L., Hiddinga, H.J., *et al.* (2008). Opposing roles for p16Ink4a and p19Arf in senescence and ageing caused by BubR1 insufficiency. *Nat Cell Biol* 10, 825-836.

Baker, D.J., Weaver, R.L., and van Deursen, J.M. (2013). p21 both attenuates and drives senescence and aging in BubR1 progeroid mice. *Cell Rep* 3, 1164-1174.

Baker, D.J., Wijshake, T., Tchkonia, T., LeBrasseur, N.K., Childs, B.G., van de Sluis, B., Kirkland, J.L., and van Deursen, J.M. (2011). Clearance of p16Ink4a-positive senescent cells delays ageing-associated disorders. *Nature* 479, 232-236.

Ballare, C., Lange, M., Lapinaite, A., Martin, G.M., Morey, L., Pascual, G., Liefke, R., Simon, B., Shi, Y., Gozani, O., *et al.* (2012). Phf19 links methylated Lys36 of histone H3 to regulation of Polycomb activity. *Nat Struct Mol Biol* 19, 1257-1265.

Barradas, M., Anderton, E., Acosta, J.C., Li, S., Banito, A., Rodriguez-Niedenfuhr, M., Maertens, G., Banck, M., Zhou, M.M., Walsh, M.J., *et al.* (2009). Histone demethylase JMJD3 contributes to epigenetic control of INK4a/ARF by oncogenic RAS. *Genes Dev* 23, 1177-1182.

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

Bavik, C., Coleman, I., Dean, J.P., Knudsen, B., Plymate, S., and Nelson, P.S. (2006). The gene expression program of prostate fibroblast senescence modulates neoplastic epithelial cell proliferation through paracrine mechanisms. *Cancer Res* 66, 794-802.

Bhat, R., Crowe, E.P., Bitto, A., Moh, M., Katsetos, C.D., Garcia, F.U., Johnson, F.B., Trojanowski, J.Q., Sell, C., and Torres, C. (2012). Astrocyte senescence as a component of Alzheimer's disease. *PLoS One* 7, e45069.

Blackwood, E.M., and Eisenman, R.N. (1991). Max: a helix-loop-helix zipper protein that forms a sequence-specific DNA-binding complex with Myc. *Science* 251, 1211-1217.

Brack, A.S., Conboy, M.J., Roy, S., Lee, M., Kuo, C.J., Keller, C., and Rando, T.A. (2007). Increased Wnt signaling during aging alters muscle stem cell fate and increases fibrosis. *Science* 317, 807-810.

Bracken, A.P., Kleine-Kohlbrecher, D., Dietrich, N., Pasini, D., Gargiulo, G., Beekman, C., Theilgaard-Monch, K., Minucci, S., Porse, B.T., Marine, J.C., *et al.* (2007). The Polycomb group proteins bind throughout the INK4A-ARF locus and are disassociated in senescent cells. *Genes Dev* 21, 525-530.

Bracken, A.P., Pasini, D., Capra, M., Prosperini, E., Colli, E., and Helin, K. (2003). EZH2 is downstream of the pRB-E2F pathway, essential for proliferation and amplified in cancer. *EMBO J* 22, 5323-5335.

Braig, M., Lee, S., Loddenkemper, C., Rudolph, C., Peters, A.H., Schlegelberger, B., Stein, H., Dorken, B., Jenuwein, T., and Schmitt, C.A. (2005). Oncogene-induced senescence as an initial barrier in lymphoma development. *Nature* *436*, 660-665.

Bruggeman, S.W., Valk-Lingbeek, M.E., van der Stoop, P.P., Jacobs, J.J., Kieboom, K., Tanger, E., Hulsman, D., Leung, C., Arsenijevic, Y., Marino, S., *et al.* (2005). Ink4a and Arf differentially affect cell proliferation and neural stem cell self-renewal in Bmi1-deficient mice. *Genes Dev* *19*, 1438-1443.

Burd, C.E., Sorrentino, J.A., Clark, K.S., Darr, D.B., Krishnamurthy, J., Deal, A.M., Bardeesy, N., Castrillon, D.H., Beach, D.H., and Sharpless, N.E. (2013). Monitoring tumorigenesis and senescence in vivo with a p16(INK4a)-luciferase model. *Cell* *152*, 340-351.

Cai, L., Rothbart, S.B., Lu, R., Xu, B., Chen, W.Y., Tripathy, A., Rockowitz, S., Zheng, D., Patel, D.J., Allis, C.D., *et al.* (2013). An H3K36 methylation-engaging Tudor motif of polycomb-like proteins mediates PRC2 complex targeting. *Mol Cell* *49*, 571-582.

Campaner, S., Doni, M., Hydbring, P., Verrecchia, A., Bianchi, L., Sardella, D., Schleker, T., Perna, D., Tronnersjo, S., Murga, M., *et al.* (2010). Cdk2 suppresses cellular senescence induced by the c-myc oncogene. *Nat Cell Biol* *12*, 54-59; sup pp 51-14.

Cannell, I.G., Kong, Y.W., Johnston, S.J., Chen, M.L., Collins, H.M., Dobbyn, H.C., Elia, A., Kress, T.R., Dickens, M., Clemens, M.J., *et al.* (2010). p38 MAPK/MK2-mediated induction of miR-34c following DNA damage prevents Myc-dependent DNA replication. *Proc Natl Acad Sci U S A* *107*, 5375-5380.

Cappellen, D., Schlange, T., Bauer, M., Maurer, F., and Hynes, N.E. (2007). Novel c-MYC target genes mediate differential effects on cell proliferation and migration. *EMBO Rep* *8*, 70-76.

Cawley, S., Bekiranov, S., Ng, H.H., Kapranov, P., Sekinger, E.A., Kampa, D., Piccolboni, A., Sementchenko, V., Cheng, J., Williams, A.J., *et al.* (2004). Unbiased mapping of transcription factor binding sites along human chromosomes 21 and 22 points to widespread regulation of noncoding RNAs. *Cell* *116*, 499-509.

Cha, T.L., Zhou, B.P., Xia, W., Wu, Y., Yang, C.C., Chen, C.T., Ping, B., Otte, A.P., and Hung, M.C. (2005). Akt-mediated phosphorylation of EZH2 suppresses methylation of lysine 27 in histone H3. *Science* *310*, 306-310.

Chainiaux, F., Magalhaes, J.P., Eliaers, F., Remacle, J., and Toussaint, O. (2002). UVB-induced premature senescence of human diploid skin fibroblasts. *Int J Biochem Cell Biol* *34*, 1331-1339.

Chan, C.S., Rastelli, L., and Pirrotta, V. (1994). A Polycomb response element in the Ubx gene that determines an epigenetically inherited state of repression. *EMBO J* 13, 2553-2564.

Chan, H.M., Narita, M., Lowe, S.W., and Livingston, D.M. (2005). The p400 E1A-associated protein is a novel component of the p53 --> p21 senescence pathway. *Genes Dev* 19, 196-201.

Chang, J., Wang, Y., Shao, L., Laberge, R.M., Demaria, M., Campisi, J., Janakiraman, K., Sharpless, N.E., Ding, S., Feng, W., *et al.* (2016). Clearance of senescent cells by ABT263 rejuvenates aged hematopoietic stem cells in mice. *Nat Med* 22, 78-83.

Chang, T.C., Yu, D., Lee, Y.S., Wentzel, E.A., Arking, D.E., West, K.M., Dang, C.V., Thomas-Tikhonenko, A., and Mendell, J.T. (2008). Widespread microRNA repression by Myc contributes to tumorigenesis. *Nat Genet* 40, 43-50.

Chen, H., Gu, X., Su, I.H., Bottino, R., Contreras, J.L., Tarakhovsky, A., and Kim, S.K. (2009). Polycomb protein Ezh2 regulates pancreatic beta-cell Ink4a/Arf expression and regeneration in diabetes mellitus. *Genes Dev* 23, 975-985.

Chen, Q., Fischer, A., Reagan, J.D., Yan, L.J., and Ames, B.N. (1995). Oxidative DNA damage and senescence of human diploid fibroblast cells. *Proc Natl Acad Sci U S A* 92, 4337-4341.

Chen, T., and Dent, S.Y. (2014). Chromatin modifiers and remodellers: regulators of cellular differentiation. *Nat Rev Genet* 15, 93-106.

Chen, Z., Trotman, L.C., Shaffer, D., Lin, H.K., Dotan, Z.A., Niki, M., Koutcher, J.A., Scher, H.I., Ludwig, T., Gerald, W., *et al.* (2005). Crucial role of p53-dependent cellular senescence in suppression of Pten-deficient tumorigenesis. *Nature* 436, 725-730.

Choudhury, A.R., Ju, Z., Djojosebroto, M.W., Schienke, A., Lechel, A., Schaetzlein, S., Jiang, H., Stepczynska, A., Wang, C., Buer, J., *et al.* (2007). Cdkn1a deletion improves stem cell function and lifespan of mice with dysfunctional telomeres without accelerating cancer formation. *Nat Genet* 39, 99-105.

Christoffersen, N.R., Shalgi, R., Frankel, L.B., Leucci, E., Lees, M., Klausen, M., Pilpel, Y., Nielsen, F.C., Oren, M., and Lund, A.H. (2010). p53-independent upregulation of miR-34a during oncogene-induced senescence represses MYC. *Cell Death Differ* 17, 236-245.

Collado, M., Gil, J., Efeyan, A., Guerra, C., Schuhmacher, A.J., Barradas, M., Benguria, A., Zaballos, A., Flores, J.M., Barbacid, M., *et al.* (2005). Tumour biology: senescence in premalignant tumours. *Nature* *436*, 642.

Coppe, J.P., Desprez, P.Y., Krtolica, A., and Campisi, J. (2010). The senescence-associated secretory phenotype: the dark side of tumor suppression. *Annu Rev Pathol* *5*, 99-118.

Coppe, J.P., Kauser, K., Campisi, J., and Beausejour, C.M. (2006). Secretion of vascular endothelial growth factor by primary human fibroblasts at senescence. *J Biol Chem* *281*, 29568-29574.

Coppe, J.P., Patil, C.K., Rodier, F., Sun, Y., Munoz, D.P., Goldstein, J., Nelson, P.S., Desprez, P.Y., and Campisi, J. (2008). Senescence-associated secretory phenotypes reveal cell-nonautonomous functions of oncogenic RAS and the p53 tumor suppressor. *PLoS Biol* *6*, 2853-2868.

Coppe, J.P., Rodier, F., Patil, C.K., Freund, A., Desprez, P.Y., and Campisi, J. (2011). Tumor suppressor and aging biomarker p16(INK4a) induces cellular senescence without the associated inflammatory secretory phenotype. *J Biol Chem* *286*, 36396-36403.

Correia-Melo, C., Marques, F.D., Anderson, R., Hewitt, G., Hewitt, R., Cole, J., Carroll, B.M., Miwa, S., Birch, J., Merz, A., *et al.* (2016). Mitochondria are required for pro-ageing features of the senescent phenotype. *EMBO J* *35*, 724-742.

Courtois-Cox, S., Jones, S.L., and Cichowski, K. (2008). Many roads lead to oncogene-induced senescence. *Oncogene* *27*, 2801-2809.

Creppe, C., Palau, A., Malinverni, R., Valero, V., and Buschbeck, M. (2014). A Cbx8-containing polycomb complex facilitates the transition to gene activation during ES cell differentiation. *PLoS Genet* *10*, e1004851.

Cruickshanks, H.A., McBryan, T., Nelson, D.M., Vanderkraats, N.D., Shah, P.P., van Tuyn, J., Singh Rai, T., Brock, C., Donahue, G., Dunican, D.S., *et al.* (2013). Senescent cells harbour features of the cancer epigenome. *Nat Cell Biol* *15*, 1495-1506.

d'Adda di Fagagna, F., Reaper, P.M., Clay-Farrace, L., Fiegler, H., Carr, P., Von Zglinicki, T., Saretzki, G., Carter, N.P., and Jackson, S.P. (2003). A DNA damage checkpoint response in telomere-initiated senescence. *Nature* *426*, 194-198.

Dalla-Favera, R., Bregni, M., Erikson, J., Patterson, D., Gallo, R.C., and Croce, C.M. (1982). Human c-myc onc gene is located on the region of chromosome 8 that is translocated in Burkitt lymphoma cells. *Proc Natl Acad Sci U S A* *79*, 7824-7827.

Damalas, A., Kahan, S., Shtutman, M., Ben-Ze'ev, A., and Oren, M. (2001). Deregulated beta-catenin induces a p53- and ARF-dependent growth arrest and cooperates with Ras in transformation. *EMBO J* 20, 4912-4922.

Dang, C.V., O'Donnell, K.A., Zeller, K.I., Nguyen, T., Osthus, R.C., and Li, F. (2006). The c-Myc target gene network. *Semin Cancer Biol* 16, 253-264.

de Alboran, I.M., O'Hagan, R.C., Gartner, F., Malynn, B., Davidson, L., Rickert, R., Rajewsky, K., DePinho, R.A., and Alt, F.W. (2001). Analysis of C-MYC function in normal cells via conditional gene-targeted mutation. *Immunity* 14, 45-55.

De Cecco, M., Criscione, S.W., Peckham, E.J., Hillenmeyer, S., Hamm, E.A., Manivannan, J., Peterson, A.L., Kreiling, J.A., Neretti, N., and Sedivy, J.M. (2013). Genomes of replicatively senescent cells undergo global epigenetic changes leading to gene silencing and activation of transposable elements. *Aging Cell* 12, 247-256.

De Ferrari, G.V., Papassotiropoulos, A., Biechele, T., Wavrant De-Vrieze, F., Avila, M.E., Major, M.B., Myers, A., Saez, K., Henriquez, J.P., Zhao, A., *et al.* (2007). Common genetic variation within the low-density lipoprotein receptor-related protein 6 and late-onset Alzheimer's disease. *Proc Natl Acad Sci U S A* 104, 9434-9439.

Debacq-Chainiaux, F., Erusalimsky, J.D., Campisi, J., and Toussaint, O. (2009). Protocols to detect senescence-associated beta-galactosidase (SA-beta-gal) activity, a biomarker of senescent cells in culture and in vivo. *Nat Protoc* 4, 1798-1806.

Dhomen, N., Reis-Filho, J.S., da Rocha Dias, S., Hayward, R., Savage, K., Delmas, V., Larue, L., Pritchard, C., and Marais, R. (2009). Oncogenic Braf induces melanocyte senescence and melanoma in mice. *Cancer Cell* 15, 294-303.

Di Croce, L., and Helin, K. (2013). Transcriptional regulation by Polycomb group proteins. *Nat Struct Mol Biol* 20, 1147-1155.

Di Micco, R., Fumagalli, M., Cicalese, A., Piccinin, S., Gasparini, P., Luise, C., Schurra, C., Garre, M., Nuciforo, P.G., Bensimon, A., *et al.* (2006). Oncogene-induced senescence is a DNA damage response triggered by DNA hyper-replication. *Nature* 444, 638-642.

Dietrich, N., Lerdrup, M., Landt, E., Agrawal-Singh, S., Bak, M., Tommerup, N., Rappsilber, J., Sodersten, E., and Hansen, K. (2012). REST-mediated recruitment of polycomb repressor complexes in mammalian cells. *PLoS Genet* 8, e1002494.

Dimri, G.P., Lee, X., Basile, G., Acosta, M., Scott, G., Roskelley, C., Medrano, E.E., Linskens, M., Rubelj, I., Pereira-Smith, O., *et al.* (1995). A biomarker that identifies

senescent human cells in culture and in aging skin in vivo. *Proc Natl Acad Sci U S A* 92, 9363-9367.

Dou, Z., Xu, C., Donahue, G., Shimi, T., Pan, J.A., Zhu, J., Ivanov, A., Capell, B.C., Drake, A.M., Shah, P.P., *et al.* (2015). Autophagy mediates degradation of nuclear lamina. *Nature* 527, 105-109.

Duan, Z., Zou, J.X., Yang, P., Wang, Y., Borowsky, A.D., Gao, A.C., and Chen, H.W. (2013). Developmental and androgenic regulation of chromatin regulators EZH2 and ANCCA/ATAD2 in the prostate Via MLL histone methylase complex. *Prostate* 73, 455-466.

Duesberg, P.H., Bister, K., and Vogt, P.K. (1977). The RNA of avian acute leukemia virus MC29. *Proc Natl Acad Sci U S A* 74, 4320-4324.

Egler, R.A., Fernandes, E., Rothermund, K., Sereika, S., de Souza-Pinto, N., Jaruga, P., Dizdaroglu, M., and Prochownik, E.V. (2005). Regulation of reactive oxygen species, DNA damage, and c-Myc function by peroxiredoxin 1. *Oncogene* 24, 8038-8050.

Eischen, C.M., Weber, J.D., Roussel, M.F., Sherr, C.J., and Cleveland, J.L. (1999). Disruption of the ARF-Mdm2-p53 tumor suppressor pathway in Myc-induced lymphomagenesis. *Genes Dev* 13, 2658-2669.

El Messaoudi-Aubert, S., Nicholls, J., Maertens, G.N., Brookes, S., Bernstein, E., and Peters, G. (2010). Role for the MOV10 RNA helicase in polycomb-mediated repression of the INK4a tumor suppressor. *Nat Struct Mol Biol* 17, 862-868.

Ezhkova, E., Lien, W.H., Stokes, N., Pasolli, H.A., Silva, J.M., and Fuchs, E. (2011). EZH1 and EZH2 cogovern histone H3K27 trimethylation and are essential for hair follicle homeostasis and wound repair. *Genes Dev* 25, 485-498.

Farcas, A.M., Blackledge, N.P., Sudbery, I., Long, H.K., McGouran, J.F., Rose, N.R., Lee, S., Sims, D., Cerase, A., Sheahan, T.W., *et al.* (2012). KDM2B links the Polycomb Repressive Complex 1 (PRC1) to recognition of CpG islands. *Elife* 1, e00205.

Fitzner, B., Muller, S., Walther, M., Fischer, M., Engelmann, R., Muller-Hilke, B., Putzer, B.M., Kreutzer, M., Nizze, H., and Jaster, R. (2012). Senescence determines the fate of activated rat pancreatic stellate cells. *J Cell Mol Med* 16, 2620-2630.

Frangini, A., Sjoberg, M., Roman-Trufero, M., Dharmalingam, G., Haberle, V., Bartke, T., Lenhard, B., Malumbres, M., Vidal, M., and Dillon, N. (2013). The aurora B kinase and the polycomb protein ring1B combine to regulate active promoters in quiescent lymphocytes. *Mol Cell* 51, 647-661.

Freund, A., Orjalo, A.V., Desprez, P.Y., and Campisi, J. (2010). Inflammatory networks during cellular senescence: causes and consequences. *Trends Mol Med* 16, 238-246.

Freund, A., Patil, C.K., and Campisi, J. (2011). p38MAPK is a novel DNA damage response-independent regulator of the senescence-associated secretory phenotype. *EMBO J* 30, 1536-1548.

Fujii, S., Tokita, K., Wada, N., Ito, K., Yamauchi, C., Ito, Y., and Ochiai, A. (2011). MEK-ERK pathway regulates EZH2 overexpression in association with aggressive breast cancer subtypes. *Oncogene* 30, 4118-4128.

Fumagalli, M., Rossiello, F., Clerici, M., Barozzi, S., Cittaro, D., Kaplunov, J.M., Bucci, G., Dobrev, M., Matti, V., Beausejour, C.M., *et al.* (2012). Telomeric DNA damage is irreparable and causes persistent DNA-damage-response activation. *Nat Cell Biol* 14, 355-365.

Gabay, M., Li, Y., and Felsher, D.W. (2014). MYC activation is a hallmark of cancer initiation and maintenance. *Cold Spring Harb Perspect Med* 4.

Gao, P., Tchernyshyov, I., Chang, T.C., Lee, Y.S., Kita, K., Ochi, T., Zeller, K.I., De Marzo, A.M., Van Eyk, J.E., Mendell, J.T., *et al.* (2009). c-Myc suppression of miR-23a/b enhances mitochondrial glutaminase expression and glutamine metabolism. *Nature* 458, 762-765.

Gao, Z., Lee, P., Stafford, J.M., von Schimmelmann, M., Schaefer, A., and Reinberg, D. (2014). An AUTS2-Polycomb complex activates gene expression in the CNS. *Nature* 516, 349-354.

Gao, Z., Zhang, J., Bonasio, R., Strino, F., Sawai, A., Parisi, F., Kluger, Y., and Reinberg, D. (2012). PCGF homologs, CBX proteins, and RYBP define functionally distinct PRC1 family complexes. *Mol Cell* 45, 344-356.

Gasser, S., Orsulic, S., Brown, E.J., and Raulet, D.H. (2005). The DNA damage pathway regulates innate immune system ligands of the NKG2D receptor. *Nature* 436, 1186-1190.

Gebuhr, T.C., Bultman, S.J., and Magnuson, T. (2000). Pc-G/trx-G and the SWI/SNF connection: developmental gene regulation through chromatin remodeling. *Genesis* 26, 189-197.

Gil, J., Bernard, D., Martinez, D., and Beach, D. (2004). Polycomb CBX7 has a unifying role in cellular lifespan. *Nat Cell Biol* 6, 67-72.

- Gil, J., and Peters, G. (2006). Regulation of the INK4b-ARF-INK4a tumour suppressor locus: all for one or one for all. *Nat Rev Mol Cell Biol* 7, 667-677.
- Glover, T.W. (2006). Common fragile sites. *Cancer Lett* 232, 4-12.
- Gong, Y., Slee, R.B., Fukai, N., Rawadi, G., Roman-Roman, S., Reginato, A.M., Wang, H., Cundy, T., Glorieux, F.H., Lev, D., *et al.* (2001). LDL receptor-related protein 5 (LRP5) affects bone accrual and eye development. *Cell* 107, 513-523.
- Grandori, C., Wu, K.J., Fernandez, P., Ngouenet, C., Grim, J., Clurman, B.E., Moser, M.J., Oshima, J., Russell, D.W., Swisshelm, K., *et al.* (2003). Werner syndrome protein limits MYC-induced cellular senescence. *Genes Dev* 17, 1569-1574.
- Gray-Schopfer, V.C., Cheong, S.C., Chong, H., Chow, J., Moss, T., Abdel-Malek, Z.A., Marais, R., Wynford-Thomas, D., and Bennett, D.C. (2006). Cellular senescence in naevi and immortalisation in melanoma: a role for p16? *Br J Cancer* 95, 496-505.
- Greer, C., Lee, M., Westerhof, M., Milholland, B., Spokony, R., Vijg, J., and Secombe, J. (2013). Myc-dependent genome instability and lifespan in *Drosophila*. *PLoS One* 8, e74641.
- Gregory, M.A., and Hann, S.R. (2000). c-Myc proteolysis by the ubiquitin-proteasome pathway: stabilization of c-Myc in Burkitt's lymphoma cells. *Mol Cell Biol* 20, 2423-2435.
- Guney, I., Wu, S., and Sedivy, J.M. (2006). Reduced c-Myc signaling triggers telomere-independent senescence by regulating Bmi-1 and p16(INK4a). *Proc Natl Acad Sci U S A* 103, 3645-3650.
- Hansen, K.H., Bracken, A.P., Pasini, D., Dietrich, N., Gehani, S.S., Monrad, A., Rappsilber, J., Lerdrup, M., and Helin, K. (2008). A model for transmission of the H3K27me3 epigenetic mark. *Nat Cell Biol* 10, 1291-1300.
- Harley, C.B., Futcher, A.B., and Greider, C.W. (1990). Telomeres shorten during ageing of human fibroblasts. *Nature* 345, 458-460.
- Hayflick, L. (1965). The Limited in Vitro Lifetime of Human Diploid Cell Strains. *Exp Cell Res* 37, 614-636.
- Hayflick, L., and Moorhead, P.S. (1961). The serial cultivation of human diploid cell strains. *Exp Cell Res* 25, 585-621.

He, J., Shen, L., Wan, M., Taranova, O., Wu, H., and Zhang, Y. (2013). Kdm2b maintains murine embryonic stem cell status by recruiting PRC1 complex to CpG islands of developmental genes. *Nat Cell Biol* 15, 373-384.

He, T.C., Sparks, A.B., Rago, C., Hermeking, H., Zawel, L., da Costa, L.T., Morin, P.J., Vogelstein, B., and Kinzler, K.W. (1998). Identification of c-MYC as a target of the APC pathway. *Science* 281, 1509-1512.

He, X., Han, W., Hu, S.X., Zhang, M.Z., Hua, J.L., and Peng, S. (2015). Canonical Wnt signaling pathway contributes to the proliferation and survival in porcine pancreatic stem cells (PSCs). *Cell Tissue Res*.

Herbig, U., Ferreira, M., Condel, L., Carey, D., and Sedivy, J.M. (2006). Cellular senescence in aging primates. *Science* 311, 1257.

Herbig, U., Jobling, W.A., Chen, B.P., Chen, D.J., and Sedivy, J.M. (2004). Telomere shortening triggers senescence of human cells through a pathway involving ATM, p53, and p21(CIP1), but not p16(INK4a). *Mol Cell* 14, 501-513.

Herbst, A., Jurinovic, V., Krebs, S., Thieme, S.E., Blum, H., Goke, B., and Kolligs, F.T. (2014). Comprehensive analysis of beta-catenin target genes in colorectal carcinoma cell lines with deregulated Wnt/beta-catenin signaling. *BMC Genomics* 15, 74.

Hermeking, H., Rago, C., Schuhmacher, M., Li, Q., Barrett, J.F., Obaya, A.J., O'Connell, B.C., Mateyak, M.K., Tam, W., Kohlhuber, F., *et al.* (2000). Identification of CDK4 as a target of c-MYC. *Proc Natl Acad Sci U S A* 97, 2229-2234.

Herranz, N., Gallage, S., Mellone, M., Wuestefeld, T., Klotz, S., Hanley, C.J., Raguz, S., Acosta, J.C., Innes, A.J., Banito, A., *et al.* (2015). mTOR regulates MAPKAPK2 translation to control the senescence-associated secretory phenotype. *Nat Cell Biol* 17, 1205-1217.

Herranz, N., Pasini, D., Diaz, V.M., Franci, C., Gutierrez, A., Dave, N., Escriva, M., Hernandez-Munoz, I., Di Croce, L., Helin, K., *et al.* (2008). Polycomb complex 2 is required for E-cadherin repression by the Snail1 transcription factor. *Mol Cell Biol* 28, 4772-4781.

Hofmann, J.W., McBryan, T., Adams, P.D., and Sedivy, J.M. (2014). The effects of aging on the expression of Wnt pathway genes in mouse tissues. *Age* 36, 9618.

Hofmann, J.W., Zhao, X., De Cecco, M., Peterson, A.L., Pagliaroli, L., Manivannan, J., Hubbard, G.B., Ikeno, Y., Zhang, Y., Feng, B., *et al.* (2015). Reduced expression of MYC increases longevity and enhances healthspan. *Cell* 160, 477-488.

Hopewell, R., and Ziff, E.B. (1995). The nerve growth factor-responsive PC12 cell line does not express the Myc dimerization partner Max. *Mol Cell Biol* 15, 3470-3478.

Hosogane, M., Funayama, R., Nishida, Y., Nagashima, T., and Nakayama, K. (2013). Ras-induced changes in H3K27me3 occur after those in transcriptional activity. *PLoS Genet* 9, e1003698.

Hu, S.S., Lai, M.M., and Vogt, P.K. (1979). Genome of avian myelocytomatosis virus MC29: analysis by heteroduplex mapping. *Proc Natl Acad Sci U S A* 76, 1265-1268.

Inestrosa, N.C., Varela-Nallar, L., Grabowski, C.P., and Colombres, M. (2007). Synaptotoxicity in Alzheimer's disease: the Wnt signaling pathway as a molecular target. *IUBMB Life* 59, 316-321.

Itahana, K., Campisi, J., and Dimri, G.P. (2004). Mechanisms of cellular senescence in human and mouse cells. *Biogerontology* 5, 1-10.

Ivanov, A., Pawlikowski, J., Manoharan, I., van Tuyn, J., Nelson, D.M., Rai, T.S., Shah, P.P., Hewitt, G., Korolchuk, V.I., Passos, J.F., *et al.* (2013). Lysosome-mediated processing of chromatin in senescence. *J Cell Biol* 202, 129-143.

Jacobs, J.J., Kieboom, K., Marino, S., DePinho, R.A., and van Lohuizen, M. (1999). The oncogene and Polycomb-group gene *bmi-1* regulates cell proliferation and senescence through the *ink4a* locus. *Nature* 397, 164-168.

Jain, M., Arvanitis, C., Chu, K., Dewey, W., Leonhardt, E., Trinh, M., Sundberg, C.D., Bishop, J.M., and Felsher, D.W. (2002). Sustained loss of a neoplastic phenotype by brief inactivation of MYC. *Science* 297, 102-104.

Jeyapalan, J.C., and Sedivy, J.M. (2013). How to measure RNA expression in rare senescent cells expressing any specific protein such as p16Ink4a. *Aging (Albany NY)* 5, 120-129.

Jin, C., Li, J., Green, C.D., Yu, X., Tang, X., Han, D., Xian, B., Wang, D., Huang, X., Cao, X., *et al.* (2011). Histone demethylase UTX-1 regulates *C. elegans* life span by targeting the insulin/IGF-1 signaling pathway. *Cell Metab* 14, 161-172.

Jonkers, I., and Lis, J.T. (2015). Getting up to speed with transcription elongation by RNA polymerase II. *Nat Rev Mol Cell Biol* 16, 167-177.

Jun, J.I., and Lau, L.F. (2010). The matricellular protein CCN1 induces fibroblast senescence and restricts fibrosis in cutaneous wound healing. *Nat Cell Biol* 12, 676-685.

Jurk, D., Wang, C., Miwa, S., Maddick, M., Korolchuk, V., Tzolou, A., Gonos, E.S., Thrasyvoulou, C., Saffrey, M.J., Cameron, K., *et al.* (2012). Postmitotic neurons develop a p21-dependent senescence-like phenotype driven by a DNA damage response. *Aging Cell* *11*, 996-1004.

Kang, C., Xu, Q., Martin, T.D., Li, M.Z., Demaria, M., Aron, L., Lu, T., Yankner, B.A., Campisi, J., and Elledge, S.J. (2015). The DNA damage response induces inflammation and senescence by inhibiting autophagy of GATA4. *Science* *349*, aaa5612.

Kaplon, J., Zheng, L., Meissl, K., Chaneton, B., Selivanov, V.A., Mackay, G., van der Burg, S.H., Verdegaal, E.M., Cascante, M., Shlomi, T., *et al.* (2013). A key role for mitochondrial gatekeeper pyruvate dehydrogenase in oncogene-induced senescence. *Nature* *498*, 109-112.

Karlsson, A., Deb-Basu, D., Cherry, A., Turner, S., Ford, J., and Felsher, D.W. (2003). Defective double-strand DNA break repair and chromosomal translocations by MYC overexpression. *Proc Natl Acad Sci U S A* *100*, 9974-9979.

Kastan, M.B., Zhan, Q., el-Deiry, W.S., Carrier, F., Jacks, T., Walsh, W.V., Plunkett, B.S., Vogelstein, B., and Fornace, A.J., Jr. (1992). A mammalian cell cycle checkpoint pathway utilizing p53 and GADD45 is defective in ataxia-telangiectasia. *Cell* *71*, 587-597.

Kaur, A., Webster, M.R., Marchbank, K., Behera, R., Ndoeye, A., Kugel, C.H., 3rd, Dang, V.M., Appleton, J., O'Connell, M.P., Cheng, P., *et al.* (2016). sFRP2 in the aged microenvironment drives melanoma metastasis and therapy resistance. *Nature* *532*, 250-254.

Kaur, M., and Cole, M.D. (2013). MYC acts via the PTEN tumor suppressor to elicit autoregulation and genome-wide gene repression by activation of the Ezh2 methyltransferase. *Cancer Res* *73*, 695-705.

Kim, J., Woo, A.J., Chu, J., Snow, J.W., Fujiwara, Y., Kim, C.G., Cantor, A.B., and Orkin, S.H. (2010). A Myc network accounts for similarities between embryonic stem and cancer cell transcription programs. *Cell* *143*, 313-324.

Kirstetter, P., Anderson, K., Porse, B.T., Jacobsen, S.E., and Nerlov, C. (2006). Activation of the canonical Wnt pathway leads to loss of hematopoietic stem cell repopulation and multilineage differentiation block. *Nat Immunol* *7*, 1048-1056.

Kiyono, T., Foster, S.A., Koop, J.I., McDougall, J.K., Galloway, D.A., and Klingelhutz, A.J. (1998). Both Rb/p16INK4a inactivation and telomerase activity are required to immortalize human epithelial cells. *Nature* *396*, 84-88.

Koh, C.M., Gurel, B., Sutcliffe, S., Aryee, M.J., Schultz, D., Iwata, T., Uemura, M., Zeller, K.I., Anele, U., Zheng, Q., *et al.* (2011a). Alterations in nucleolar structure and gene expression programs in prostatic neoplasia are driven by the MYC oncogene. *Am J Pathol* 178, 1824-1834.

Koh, C.M., Iwata, T., Zheng, Q., Bethel, C., Yegnasubramanian, S., and De Marzo, A.M. (2011b). Myc enforces overexpression of EZH2 in early prostatic neoplasia via transcriptional and post-transcriptional mechanisms. *Oncotarget* 2, 669-683.

Kreiling, J.A., Tamamori-Adachi, M., Sexton, A.N., Jeyapalan, J.C., Munoz-Najar, U., Peterson, A.L., Manivannan, J., Rogers, E.S., Pchelintsev, N.A., Adams, P.D., *et al.* (2011). Age-associated increase in heterochromatic marks in murine and primate tissues. *Aging Cell* 10, 292-304.

Krepelova, A., Neri, F., Maldotti, M., Rapelli, S., and Oliviero, S. (2014). Myc and max genome-wide binding sites analysis links the Myc regulatory network with the polycomb and the core pluripotency networks in mouse embryonic stem cells. *PLoS One* 9, e88933.

Krizhanovsky, V., Yon, M., Dickins, R.A., Hearn, S., Simon, J., Miething, C., Yee, H., Zender, L., and Lowe, S.W. (2008). Senescence of activated stellate cells limits liver fibrosis. *Cell* 134, 657-667.

Krtolica, A., Parrinello, S., Lockett, S., Desprez, P.Y., and Campisi, J. (2001). Senescent fibroblasts promote epithelial cell growth and tumorigenesis: a link between cancer and aging. *Proc Natl Acad Sci U S A* 98, 12072-12077.

Kuilman, T., Michaloglou, C., Vredeveld, L.C., Douma, S., van Doorn, R., Desmet, C.J., Aarden, L.A., Mooi, W.J., and Peeper, D.S. (2008). Oncogene-induced senescence relayed by an interleukin-dependent inflammatory network. *Cell* 133, 1019-1031.

Kunderfranco, P., Mello-Grand, M., Cangemi, R., Pellini, S., Mensah, A., Albertini, V., Malek, A., Chiorino, G., Catapano, C.V., and Carbone, G.M. (2010). ETS transcription factors control transcription of EZH2 and epigenetic silencing of the tumor suppressor gene Nkx3.1 in prostate cancer. *PLoS One* 5, e10547.

Kurz, D.J., Decary, S., Hong, Y., and Erusalimsky, J.D. (2000). Senescence-associated (beta)-galactosidase reflects an increase in lysosomal mass during replicative ageing of human endothelial cells. *J Cell Sci* 113 (Pt 20), 3613-3622.

Laberge, R.M., Awad, P., Campisi, J., and Desprez, P.Y. (2012). Epithelial-mesenchymal transition induced by senescent fibroblasts. *Cancer Microenviron* 5, 39-44.

Laberge, R.M., Sun, Y., Orjalo, A.V., Patil, C.K., Freund, A., Zhou, L., Curran, S.C., Davalos, A.R., Wilson-Edell, K.A., Liu, S., *et al.* (2015). MTOR regulates the pro-tumorigenic senescence-associated secretory phenotype by promoting IL1A translation. *Nat Cell Biol* 17, 1049-1061.

Lackner, D.H., Hayashi, M.T., Cesare, A.J., and Karlseder, J. (2014). A genomics approach identifies senescence-specific gene expression regulation. *Aging Cell* 13, 946-950.

Land, H., Parada, L.F., and Weinberg, R.A. (1983). Tumorigenic conversion of primary embryo fibroblasts requires at least two cooperating oncogenes. *Nature* 304, 596-602.

Landeira, D., Sauer, S., Poot, R., Dvorkina, M., Mazzarella, L., Jorgensen, H.F., Pereira, C.F., Leleu, M., Piccolo, F.M., Spivakov, M., *et al.* (2010). Jarid2 is a PRC2 component in embryonic stem cells required for multi-lineage differentiation and recruitment of PRC1 and RNA Polymerase II to developmental regulators. *Nat Cell Biol* 12, 618-624.

Lanzuolo, C., Lo Sardo, F., Diamantini, A., and Orlando, V. (2011). PcG complexes set the stage for epigenetic inheritance of gene silencing in early S phase before replication. *PLoS Genet* 7, e1002370.

LaPak, K.M., and Burd, C.E. (2014). The molecular balancing act of p16(INK4a) in cancer and aging. *Mol Cancer Res* 12, 167-183.

Lazzerini Denchi, E., Attwooll, C., Pasini, D., and Helin, K. (2005). Deregulated E2F activity induces hyperplasia and senescence-like features in the mouse pituitary gland. *Mol Cell Biol* 25, 2660-2672.

Leder, A., Pattengale, P.K., Kuo, A., Stewart, T.A., and Leder, P. (1986). Consequences of widespread deregulation of the c-myc gene in transgenic mice: multiple neoplasms and normal development. *Cell* 45, 485-495.

Lee, B.Y., Han, J.A., Im, J.S., Morrone, A., Johung, K., Goodwin, E.C., Kleijer, W.J., DiMaio, D., and Hwang, E.S. (2006a). Senescence-associated beta-galactosidase is lysosomal beta-galactosidase. *Aging Cell* 5, 187-195.

Lee, S.J., Chung, Y.H., Joo, K.M., Lim, H.C., Jeon, G.S., Kim, D., Lee, W.B., Kim, Y.S., and Cha, C.I. (2006b). Age-related changes in glycogen synthase kinase 3beta (GSK3beta) immunoreactivity in the central nervous system of rats. *Neurosci Lett* 409, 134-139.

Lee, S.T., Li, Z., Wu, Z., Aau, M., Guan, P., Karuturi, R.K., Liou, Y.C., and Yu, Q. (2011). Context-specific regulation of NF-kappaB target gene expression by EZH2 in breast cancers. *Mol Cell* 43, 798-810.

Leung, K.H., Abou El Hassan, M., and Bremner, R. (2013). A rapid and efficient method to purify proteins at replication forks under native conditions. *Biotechniques* 55, 204-206.

Levens, D. (2010). You Don't Muck with MYC. *Genes Cancer* 1, 547-554.

Lewis, P.W., Muller, M.M., Koletsky, M.S., Cordero, F., Lin, S., Banaszynski, L.A., Garcia, B.A., Muir, T.W., Becher, O.J., and Allis, C.D. (2013). Inhibition of PRC2 activity by a gain-of-function H3 mutation found in pediatric glioblastoma. *Science* 340, 857-861.

Lezzerini, M., and Budovskaya, Y. (2014). A dual role of the Wnt signaling pathway during aging in *Caenorhabditis elegans*. *Aging Cell* 13, 8-18.

Li, Z., Van Calcar, S., Qu, C., Cavenee, W.K., Zhang, M.Q., and Ren, B. (2003). A global transcriptional regulatory role for c-Myc in Burkitt's lymphoma cells. *Proc Natl Acad Sci U S A* 100, 8164-8169.

Lin, A.W., Barradas, M., Stone, J.C., van Aelst, L., Serrano, M., and Lowe, S.W. (1998). Premature senescence involving p53 and p16 is activated in response to constitutive MEK/MAPK mitogenic signaling. *Genes Dev* 12, 3008-3019.

Lin, C.Y., Loven, J., Rahl, P.B., Paranal, R.M., Burge, C.B., Bradner, J.E., Lee, T.I., and Young, R.A. (2012). Transcriptional amplification in tumor cells with elevated c-Myc. *Cell* 151, 56-67.

Liu, D., and Hornsby, P.J. (2007). Senescent human fibroblasts increase the early growth of xenograft tumors via matrix metalloproteinase secretion. *Cancer Res* 67, 3117-3126.

Liu, H., Fergusson, M.M., Castilho, R.M., Liu, J., Cao, L., Chen, J., Malide, D., Rovira, II, Schimel, D., Kuo, C.J., *et al.* (2007). Augmented Wnt signaling in a mammalian model of accelerated aging. *Science* 317, 803-806.

Liu, W.H., Roemer, S.C., Port, A.M., and Churchill, M.E. (2012). CAF-1-induced oligomerization of histones H3/H4 and mutually exclusive interactions with Asf1 guide H3/H4 transitions among histone chaperones and DNA. *Nucleic Acids Res* 40, 11229-11239.

- Liu, X., Tesfai, J., Evrard, Y.A., Dent, S.Y., and Martinez, E. (2003). c-Myc transformation domain recruits the human STAGA complex and requires TRRAP and GCN5 acetylase activity for transcription activation. *J Biol Chem* 278, 20405-20412.
- Loven, J., Hoke, H.A., Lin, C.Y., Lau, A., Orlando, D.A., Vakoc, C.R., Bradner, J.E., Lee, T.I., and Young, R.A. (2013). Selective inhibition of tumor oncogenes by disruption of super-enhancers. *Cell* 153, 320-334.
- Lu, T., Pan, Y., Kao, S.Y., Li, C., Kohane, I., Chan, J., and Yankner, B.A. (2004). Gene regulation and DNA damage in the ageing human brain. *Nature* 429, 883-891.
- Ma, L., Young, J., Prabhala, H., Pan, E., Mestdagh, P., Muth, D., Teruya-Feldstein, J., Reinhardt, F., Onder, T.T., Valastyan, S., *et al.* (2010). miR-9, a MYC/MYCN-activated microRNA, regulates E-cadherin and cancer metastasis. *Nat Cell Biol* 12, 247-256.
- Madabhushi, R., Gao, F., Pfenning, A.R., Pan, L., Yamakawa, S., Seo, J., Rueda, R., Phan, T.X., Yamakawa, H., Pao, P.C., *et al.* (2015). Activity-Induced DNA Breaks Govern the Expression of Neuronal Early-Response Genes. *Cell* 161, 1592-1605.
- Mahara, S., Lee, P.L., Feng, M., Tergaonkar, V., Chng, W.J., and Yu, Q. (2016). HIF1- α activation underlies a functional switch in the paradoxical role of Ezh2/PRC2 in breast cancer. *Proc Natl Acad Sci U S A*.
- Maier, V.K., Feeney, C.M., Taylor, J.E., Creech, A.L., Qiao, J.W., Szanto, A., Das, P.P., Chevrier, N., Cifuentes-Rojas, C., Orkin, S.H., *et al.* (2015). Functional Proteomic Analysis of Repressive Histone Methyltransferase Complexes Reveals ZNF518B as a G9A Regulator. *Mol Cell Proteomics* 14, 1435-1446.
- Majer, C.R., Jin, L., Scott, M.P., Knutson, S.K., Kuntz, K.W., Keilhack, H., Smith, J.J., Moyer, M.P., Richon, V.M., Copeland, R.A., *et al.* (2012). A687V EZH2 is a gain-of-function mutation found in lymphoma patients. *FEBS Lett* 586, 3448-3451.
- Mani, A., Radhakrishnan, J., Wang, H., Mani, A., Mani, M.A., Nelson-Williams, C., Carew, K.S., Mane, S., Najmabadi, H., Wu, D., *et al.* (2007). LRP6 mutation in a family with early coronary disease and metabolic risk factors. *Science* 315, 1278-1282.
- Margueron, R., Justin, N., Ohno, K., Sharpe, M.L., Son, J., Drury, W.J., 3rd, Voigt, P., Martin, S.R., Taylor, W.R., De Marco, V., *et al.* (2009). Role of the polycomb protein EED in the propagation of repressive histone marks. *Nature* 461, 762-767.
- Martinez, P., and Blasco, M.A. (2011). Telomeric and extra-telomeric roles for telomerase and the telomere-binding proteins. *Nat Rev Cancer* 11, 161-176.

Petruk, S., Sedkov, Y., Johnston, D.M., Hodgson, J.W., Black, K.L., Kovermann, S.K., Beck, S., Canaani, E., Brock, H.W., and Mazo, A. (2012). TrxG and PcG proteins but not methylated histones remain associated with DNA through replication. *Cell* *150*, 922-933.

Piunti, A., Rossi, A., Cerutti, A., Albert, M., Jammula, S., Scelfo, A., Cedrone, L., Fragola, G., Olsson, L., Koseki, H., *et al.* (2014). Polycomb proteins control proliferation and transformation independently of cell cycle checkpoints by regulating DNA replication. *Nat Commun* *5*, 3649.

Popov, N., Herold, S., Llamazares, M., Schulein, C., and Eilers, M. (2007). Fbw7 and Usp28 regulate myc protein stability in response to DNA damage. *Cell Cycle* *6*, 2327-2331.

Puc, J., Kozbial, P., Li, W., Tan, Y., Liu, Z., Suter, T., Ohgi, K.A., Zhang, J., Aggarwal, A.K., and Rosenfeld, M.G. (2015). Ligand-dependent enhancer activation regulated by topoisomerase-I activity. *Cell* *160*, 367-380.

Rakhra, K., Bachireddy, P., Zabuawala, T., Zeiser, R., Xu, L., Kopelman, A., Fan, A.C., Yang, Q., Braunstein, L., Crosby, E., *et al.* (2010). CD4(+) T cells contribute to the remodeling of the microenvironment required for sustained tumor regression upon oncogene inactivation. *Cancer Cell* *18*, 485-498.

Reynolds, N., Salmon-Divon, M., Dvinge, H., Hynes-Allen, A., Balasooriya, G., Leaford, D., Behrens, A., Bertone, P., and Hendrich, B. (2012). NuRD-mediated deacetylation of H3K27 facilitates recruitment of Polycomb Repressive Complex 2 to direct gene repression. *EMBO J* *31*, 593-605.

Riising, E.M., Comet, I., Leblanc, B., Wu, X., Johansen, J.V., and Helin, K. (2014). Gene silencing triggers polycomb repressive complex 2 recruitment to CpG islands genome wide. *Mol Cell* *55*, 347-360.

Ring, A., Kim, Y.M., and Kahn, M. (2014). Wnt/catenin signaling in adult stem cell physiology and disease. *Stem Cell Rev* *10*, 512-525.

Rinn, J.L., Kertesz, M., Wang, J.K., Squazzo, S.L., Xu, X., Brugmann, S.A., Goodnough, L.H., Helms, J.A., Farnham, P.J., Segal, E., *et al.* (2007). Functional demarcation of active and silent chromatin domains in human HOX loci by noncoding RNAs. *Cell* *129*, 1311-1323.

Rodier, F., Coppe, J.P., Patil, C.K., Hoeijmakers, W.A., Munoz, D.P., Raza, S.R., Freund, A., Campeau, E., Davalos, A.R., and Campisi, J. (2009). Persistent DNA damage signalling triggers senescence-associated inflammatory cytokine secretion. *Nat Cell Biol* *11*, 973-979.

- Maures, T.J., Greer, E.L., Hauswirth, A.G., and Brunet, A. (2011). The H3K27 demethylase UTX-1 regulates *C. elegans* lifespan in a germline-independent, insulin-dependent manner. *Aging Cell* *10*, 980-990.
- McCabe, M.T., Graves, A.P., Ganji, G., Diaz, E., Halsey, W.S., Jiang, Y., Smitheman, K.N., Ott, H.M., Pappalardi, M.B., Allen, K.E., *et al.* (2012a). Mutation of A677 in histone methyltransferase EZH2 in human B-cell lymphoma promotes hypertrimethylation of histone H3 on lysine 27 (H3K27). *Proc Natl Acad Sci U S A* *109*, 2989-2994.
- McCabe, M.T., Ott, H.M., Ganji, G., Korenchuk, S., Thompson, C., Van Aller, G.S., Liu, Y., Graves, A.P., Della Pietra, A., 3rd, Diaz, E., *et al.* (2012b). EZH2 inhibition as a therapeutic strategy for lymphoma with EZH2-activating mutations. *Nature* *492*, 108-112.
- McMahon, S.B., Wood, M.A., and Cole, M.D. (2000). The essential cofactor TRRAP recruits the histone acetyltransferase hGCN5 to c-Myc. *Mol Cell Biol* *20*, 556-562.
- Mendenhall, E.M., Koche, R.P., Truong, T., Zhou, V.W., Issac, B., Chi, A.S., Ku, M., and Bernstein, B.E. (2010). GC-rich sequence elements recruit PRC2 in mammalian ES cells. *PLoS Genet* *6*, e1001244.
- Michaloglou, C., Vredeveld, L.C., Soengas, M.S., Denoyelle, C., Kuilman, T., van der Horst, C.M., Majoor, D.M., Shay, J.W., Mooi, W.J., and Peeper, D.S. (2005). BRAFE600-associated senescence-like cell cycle arrest of human naevi. *Nature* *436*, 720-724.
- Moffat, J., Grueneberg, D.A., Yang, X., Kim, S.Y., Kloepper, A.M., Hinkle, G., Piqani, B., Eisenhaure, T.M., Luo, B., Grenier, J.K., *et al.* (2006). A lentiviral RNAi library for human and mouse genes applied to an arrayed viral high-content screen. *Cell* *124*, 1283-1298.
- Mozzetta, C., Pontis, J., Fritsch, L., Robin, P., Portoso, M., Proux, C., Margueron, R., and Ait-Si-Ali, S. (2014). The histone H3 lysine 9 methyltransferases G9a and GLP regulate polycomb repressive complex 2-mediated gene silencing. *Mol Cell* *53*, 277-289.
- Munoz-Espin, D., Canamero, M., Maraver, A., Gomez-Lopez, G., Contreras, J., Murillo-Cuesta, S., Rodriguez-Baeza, A., Varela-Nieto, I., Ruberte, J., Collado, M., *et al.* (2013). Programmed cell senescence during mammalian embryonic development. *Cell* *155*, 1104-1118.
- Muto, T., Sashida, G., Oshima, M., Wendt, G.R., Mochizuki-Kashio, M., Nagata, Y., Sanada, M., Miyagi, S., Saraya, A., Kamio, A., *et al.* (2013). Concurrent loss of Ezh2 and

Tet2 cooperates in the pathogenesis of myelodysplastic disorders. *J Exp Med* 210, 2627-2639.

Nagy, A., Moens, C., Ivanyi, E., Pawling, J., Gertsenstein, M., Hadjantonakis, A.K., Pirity, M., and Rossant, J. (1998). Dissecting the role of N-myc in development using a single targeting vector to generate a series of alleles. *Curr Biol* 8, 661-664.

Narita, M., Nunez, S., Heard, E., Narita, M., Lin, A.W., Hearn, S.A., Spector, D.L., Hannon, G.J., and Lowe, S.W. (2003). Rb-mediated heterochromatin formation and silencing of E2F target genes during cellular senescence. *Cell* 113, 703-716.

Nelson, D.M., McBryan, T., Jeyapalan, J.C., Sedivy, J.M., and Adams, P.D. (2014). A comparison of oncogene-induced senescence and replicative senescence: implications for tumor suppression and aging. *Age* 36, 9637.

Ni, Z., Ebata, A., Alipanahramandi, E., and Lee, S.S. (2012). Two SET domain containing genes link epigenetic changes and aging in *Caenorhabditis elegans*. *Aging Cell* 11, 315-325.

Nie, Z., Hu, G., Wei, G., Cui, K., Yamane, A., Resch, W., Wang, R., Green, D.R., Tessarollo, L., Casellas, R., *et al.* (2012). c-Myc is a universal amplifier of expressed genes in lymphocytes and embryonic stem cells. *Cell* 151, 68-79.

Nikoloski, G., Langemeijer, S.M., Kuiper, R.P., Knops, R., Massop, M., Tonnissen, E.R., van der Heijden, A., Scheele, T.N., Vandenberghe, P., de Witte, T., *et al.* (2010). Somatic mutations of the histone methyltransferase gene EZH2 in myelodysplastic syndromes. *Nat Genet* 42, 665-667.

Ogawa, H., Ishiguro, K., Gaubatz, S., Livingston, D.M., and Nakatani, Y. (2002). A complex with chromatin modifiers that occupies E2F- and Myc-responsive genes in G0 cells. *Science* 296, 1132-1136.

Orian, A., van Steensel, B., Delrow, J., Bussemaker, H.J., Li, L., Sawado, T., Williams, E., Loo, L.W., Cowley, S.M., Yost, C., *et al.* (2003). Genomic binding by the *Drosophila* Myc, Max, Mad/Mnt transcription factor network. *Genes Dev* 17, 1101-1114.

Ortega-Atienza, S., Wong, V.C., DeLoughery, Z., Luczak, M.W., and Zhitkovich, A. (2015). ATM and KAT5 safeguard replicating chromatin against formaldehyde damage. *Nucleic Acids Res.*

Palmero, I., Murga, M., Zubiaga, A., and Serrano, M. (2002). Activation of ARF by oncogenic stress in mouse fibroblasts is independent of E2F1 and E2F2. *Oncogene* 21, 2939-2947.

- Palomero, T., Lim, W.K., Odom, D.T., Sulis, M.L., Real, P.J., Margolin, A., Barnes, K.C., O'Neil, J., Neuberg, D., Weng, A.P., *et al.* (2006). NOTCH1 directly regulates c-MYC and activates a feed-forward-loop transcriptional network promoting leukemic cell growth. *Proc Natl Acad Sci U S A* *103*, 18261-18266.
- Paradis, V., Youssef, N., Dargere, D., Ba, N., Bonvoust, F., Deschatrette, J., and Bedossa, P. (2001). Replicative senescence in normal liver, chronic hepatitis C, and hepatocellular carcinomas. *Hum Pathol* *32*, 327-332.
- Park, S., Chung, S., Kim, K.M., Jung, K.C., Park, C., Hahm, E.R., and Yang, C.H. (2004). Determination of binding constant of transcription factor myc-max/max-max and E-box DNA: the effect of inhibitors on the binding. *Biochim Biophys Acta* *1670*, 217-228.
- Passos, J.F., Nelson, G., Wang, C., Richter, T., Simillion, C., Proctor, C.J., Miwa, S., Olijslagers, S., Hallinan, J., Wipat, A., *et al.* (2010). Feedback between p21 and reactive oxygen production is necessary for cell senescence. *Mol Syst Biol* *6*, 347.
- Pawlikowski, J.S., Adams, P.D., and Nelson, D.M. (2013a). Senescence at a glance. *J Cell Sci* *126*, 4061-4067.
- Pawlikowski, J.S., McBryan, T., van Tuyn, J., Drotar, M.E., Hewitt, R.N., Maier, A.B., King, A., Blyth, K., Wu, H., and Adams, P.D. (2013b). Wnt signaling potentiates neovogenesis. *Proc Natl Acad Sci U S A* *110*, 16009-16014.
- Peeper, D.S. (2011). Oncogene-induced senescence and melanoma: where do we stand? *Pigment Cell Melanoma Res* *24*, 1107-1111.
- Pei, X.H., and Xiong, Y. (2005). Biochemical and cellular mechanisms of mammalian CDK inhibitors: a few unresolved issues. *Oncogene* *24*, 2787-2795.
- Pemberton, H., Anderton, E., Patel, H., Brookes, S., Chandler, H., Palermo, R., Stock, J., Rodriguez-Niedenfuhr, M., Racek, T., de Breed, L., *et al.* (2014). Genome-wide co-localization of Polycomb orthologs and their effects on gene expression in human fibroblasts. *Genome Biol* *15*, R23.
- Pereira, J.D., Sansom, S.N., Smith, J., Dobenecker, M.W., Tarakhovsky, A., and Livesey, F.J. (2010). Ezh2, the histone methyltransferase of PRC2, regulates the balance between self-renewal and differentiation in the cerebral cortex. *Proc Natl Acad Sci U S A* *107*, 15957-15962.

Rodier, F., Munoz, D.P., Teachenor, R., Chu, V., Le, O., Bhaumik, D., Coppe, J.P., Campeau, E., Beausejour, C.M., Kim, S.H., *et al.* (2011). DNA-SCARS: distinct nuclear structures that sustain damage-induced senescence growth arrest and inflammatory cytokine secretion. *J Cell Sci* *124*, 68-81.

Romanov, V.S., Abramova, M.V., Svetlikova, S.B., Bykova, T.V., Zubova, S.G., Aksenov, N.D., Fornace, A.J., Jr., Pospelova, T.V., and Pospelov, V.A. (2010). p21(Waf1) is required for cellular senescence but not for cell cycle arrest induced by the HDAC inhibitor sodium butyrate. *Cell Cycle* *9*, 3945-3955.

Rudolph, K.L., Chang, S., Millard, M., Schreiber-Agus, N., and DePinho, R.A. (2000). Inhibition of experimental liver cirrhosis in mice by telomerase gene delivery. *Science* *287*, 1253-1258.

Sabo, A., Kress, T.R., Pelizzola, M., de Pretis, S., Gorski, M.M., Tesi, A., Morelli, M.J., Bora, P., Doni, M., Verrecchia, A., *et al.* (2014). Selective transcriptional regulation by Myc in cellular growth control and lymphomagenesis. *Nature* *511*, 488-492.

Sachdeva, M., Zhu, S., Wu, F., Wu, H., Walia, V., Kumar, S., Elble, R., Watabe, K., and Mo, Y.Y. (2009). p53 represses c-Myc through induction of the tumor suppressor miR-145. *Proc Natl Acad Sci U S A* *106*, 3207-3212.

Sadaie, M., Salama, R., Carroll, T., Tomimatsu, K., Chandra, T., Young, A.R., Narita, M., Perez-Mancera, P.A., Bennett, D.C., Chong, H., *et al.* (2013). Redistribution of the Lamin B1 genomic binding profile affects rearrangement of heterochromatic domains and SAHF formation during senescence. *Genes Dev* *27*, 1800-1808.

Sampson, V.B., Rong, N.H., Han, J., Yang, Q., Aris, V., Soteropoulos, P., Petrelli, N.J., Dunn, S.P., and Krueger, L.J. (2007). MicroRNA let-7a down-regulates MYC and reverts MYC-induced growth in Burkitt lymphoma cells. *Cancer Res* *67*, 9762-9770.

Sander, S., Bullinger, L., Klapproth, K., Fiedler, K., Kestler, H.A., Barth, T.F., Moller, P., Stilgenbauer, S., Pollack, J.R., and Wirth, T. (2008). MYC stimulates EZH2 expression by repression of its negative regulator miR-26a. *Blood* *112*, 4202-4212.

Saretzki, G., Feng, J., von Zglinicki, T., and Villeponteau, B. (1998). Similar gene expression pattern in senescent and hyperoxic-treated fibroblasts. *J Gerontol A Biol Sci Med Sci* *53*, B438-442.

Scheller, M., Huelsken, J., Rosenbauer, F., Taketo, M.M., Birchmeier, W., Tenen, D.G., and Leutz, A. (2006). Hematopoietic stem cell and multilineage defects generated by constitutive beta-catenin activation. *Nat Immunol* *7*, 1037-1047.

Schlee, M., Holzel, M., Bernard, S., Mailhammer, R., Schuhmacher, M., Reschke, J., Eick, D., Marinkovic, D., Wirth, T., Rosenwald, A., *et al.* (2007). C-myc activation impairs the NF-kappaB and the interferon response: implications for the pathogenesis of Burkitt's lymphoma. *Int J Cancer* 120, 1387-1395.

Schmitges, F.W., Prusty, A.B., Faty, M., Stutzer, A., Lingaraju, G.M., Aiwazian, J., Sack, R., Hess, D., Li, L., Zhou, S., *et al.* (2011). Histone methylation by PRC2 is inhibited by active chromatin marks. *Mol Cell* 42, 330-341.

Seoane, J., Le, H.V., and Massague, J. (2002). Myc suppression of the p21(Cip1) Cdk inhibitor influences the outcome of the p53 response to DNA damage. *Nature* 419, 729-734.

Serrano, M., Lin, A.W., McCurrach, M.E., Beach, D., and Lowe, S.W. (1997). Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a. *Cell* 88, 593-602.

Seth, A., Alvarez, E., Gupta, S., and Davis, R.J. (1991). A phosphorylation site located in the NH2-terminal domain of c-Myc increases transactivation of gene expression. *J Biol Chem* 266, 23521-23524.

Shah, P.P., Donahue, G., Otte, G.L., Capell, B.C., Nelson, D.M., Cao, K., Aggarwala, V., Cruickshanks, H.A., Rai, T.S., McBryan, T., *et al.* (2013). Lamin B1 depletion in senescent cells triggers large-scale changes in gene expression and the chromatin landscape. *Genes Dev* 27, 1787-1799.

Sharpless, N.E., Bardeesy, N., Lee, K.H., Carrasco, D., Castrillon, D.H., Aguirre, A.J., Wu, E.A., Horner, J.W., and DePinho, R.A. (2001). Loss of p16Ink4a with retention of p19Arf predisposes mice to tumorigenesis. *Nature* 413, 86-91.

Shen, J., Curtis, C., Tavaré, S., and Tower, J. (2009a). A screen of apoptosis and senescence regulatory genes for life span effects when over-expressed in *Drosophila*. *Aging (Albany NY)* 1, 191-211.

Shen, X., Kim, W., Fujiwara, Y., Simon, M.D., Liu, Y., Mysliwiec, M.R., Yuan, G.C., Lee, Y., and Orkin, S.H. (2009b). Jumonji modulates polycomb activity and self-renewal versus differentiation of stem cells. *Cell* 139, 1303-1314.

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

- Shibata, H., Toyama, K., Shioya, H., Ito, M., Hirota, M., Hasegawa, S., Matsumoto, H., Takano, H., Akiyama, T., Toyoshima, K., *et al.* (1997). Rapid colorectal adenoma formation initiated by conditional targeting of the Apc gene. *Science* 278, 120-123.
- Shin, S.Y., Kim, C.G., Jho, E.H., Rho, M.S., Kim, Y.S., Kim, Y.H., and Lee, Y.H. (2004). Hydrogen peroxide negatively modulates Wnt signaling through downregulation of beta-catenin. *Cancer Lett* 212, 225-231.
- Shumaker, D.K., Dechat, T., Kohlmaier, A., Adam, S.A., Bozovsky, M.R., Erdos, M.R., Eriksson, M., Goldman, A.E., Khuon, S., Collins, F.S., *et al.* (2006). Mutant nuclear lamin A leads to progressive alterations of epigenetic control in premature aging. *Proc Natl Acad Sci U S A* 103, 8703-8708.
- Siebold, A.P., Banerjee, R., Tie, F., Kiss, D.L., Moskowitz, J., and Harte, P.J. (2010). Polycomb Repressive Complex 2 and Trithorax modulate *Drosophila* longevity and stress resistance. *Proc Natl Acad Sci U S A* 107, 169-174.
- Simon, J.A., and Kingston, R.E. (2009). Mechanisms of polycomb gene silencing: knowns and unknowns. *Nat Rev Mol Cell Biol* 10, 697-708.
- Sneeringer, C.J., Scott, M.P., Kuntz, K.W., Knutson, S.K., Pollock, R.M., Richon, V.M., and Copeland, R.A. (2010). Coordinated activities of wild-type plus mutant EZH2 drive tumor-associated hypertrimethylation of lysine 27 on histone H3 (H3K27) in human B-cell lymphomas. *Proc Natl Acad Sci U S A* 107, 20980-20985.
- Sodir, N.M., and Evan, G.I. (2011). Finding cancer's weakest link. *Oncotarget* 2, 1307-1313.
- Song, L.B., Li, J., Liao, W.T., Feng, Y., Yu, C.P., Hu, L.J., Kong, Q.L., Xu, L.H., Zhang, X., Liu, W.L., *et al.* (2009). The polycomb group protein Bmi-1 represses the tumor suppressor PTEN and induces epithelial-mesenchymal transition in human nasopharyngeal epithelial cells. *J Clin Invest* 119, 3626-3636.
- Souroullas, G.P., Jeck, W.R., Parker, J.S., Simon, J.M., Liu, J.Y., Paulk, J., Xiong, J., Clark, K.S., Fedoriw, Y., Qi, J., *et al.* (2016). An oncogenic Ezh2 mutation induces tumors through global redistribution of histone 3 lysine 27 trimethylation. *Nat Med*.
- Staller, P., Peukert, K., Kiermaier, A., Seoane, J., Lukas, J., Karsunky, H., Moroy, T., Bartek, J., Massague, J., Hanel, F., *et al.* (2001). Repression of p15INK4b expression by Myc through association with Miz-1. *Nat Cell Biol* 3, 392-399.
- Steiger, D., Furrer, M., Schwinkendorf, D., and Gallant, P. (2008). Max-independent functions of Myc in *Drosophila melanogaster*. *Nat Genet* 40, 1084-1091.

Stewart, S.A., Dykxhoorn, D.M., Palliser, D., Mizuno, H., Yu, E.Y., An, D.S., Sabatini, D.M., Chen, I.S., Hahn, W.C., Sharp, P.A., *et al.* (2003). Lentivirus-delivered stable gene silencing by RNAi in primary cells. *RNA* 9, 493-501.

Stock, J.K., Giadrossi, S., Casanova, M., Brookes, E., Vidal, M., Koseki, H., Brockdorff, N., Fisher, A.G., and Pombo, A. (2007). Ring1-mediated ubiquitination of H2A restrains poised RNA polymerase II at bivalent genes in mouse ES cells. *Nat Cell Biol* 9, 1428-1435.

Storer, M., Mas, A., Robert-Moreno, A., Pecoraro, M., Ortells, M.C., Di Giacomo, V., Yosef, R., Pilpel, N., Krizhanovsky, V., Sharpe, J., *et al.* (2013). Senescence is a developmental mechanism that contributes to embryonic growth and patterning. *Cell* 155, 1119-1130.

Sun, S., Yu, F., Zhang, L., and Zhou, X. (2016a). EZH2, an on-off valve in signal network of tumor cells. *Cell Signal* 28, 481-487.

Sun, Y., Zhu, D., Chen, F., Qian, M., Wei, H., Chen, W., and Xu, J. (2016b). SFRP2 augments WNT16B signaling to promote therapeutic resistance in the damaged tumor microenvironment. *Oncogene*.

Suram, A., Kaplunov, J., Patel, P.L., Ruan, H., Cerutti, A., Boccardi, V., Fumagalli, M., Di Micco, R., Mirani, N., Gurung, R.L., *et al.* (2012). Oncogene-induced telomere dysfunction enforces cellular senescence in human cancer precursor lesions. *EMBO J* 31, 2839-2851.

Swanson, E.C., Manning, B., Zhang, H., and Lawrence, J.B. (2013). Higher-order unfolding of satellite heterochromatin is a consistent and early event in cell senescence. *J Cell Biol* 203, 929-942.

Takai, H., Smogorzewska, A., and de Lange, T. (2003). DNA damage foci at dysfunctional telomeres. *Curr Biol* 13, 1549-1556.

Tanaka, H., Matsumura, I., Ezoe, S., Satoh, Y., Sakamaki, T., Albanese, C., Machii, T., Pestell, R.G., and Kanakura, Y. (2002). E2F1 and c-Myc potentiate apoptosis through inhibition of NF-kappaB activity that facilitates MnSOD-mediated ROS elimination. *Mol Cell* 9, 1017-1029.

Tanay, A., O'Donnell, A.H., Damelin, M., and Bestor, T.H. (2007). Hyperconserved CpG domains underlie Polycomb-binding sites. *Proc Natl Acad Sci U S A* 104, 5521-5526.

Tasdemir, N., Banito, A., Roe, J.S., Alonso-Curbelo, D., Camiolo, M., Tschaharganeh, D.F., Huang, C.H., Aksoy, O., Bolden, J.E., Chen, C.C., *et al.* (2016). BRD4 Connects Enhancer Remodeling to Senescence Immune Surveillance. *Cancer Discov* 6, 612-629.

Torres, M., and Forman, H.J. (2003). Redox signaling and the MAP kinase pathways. *Biofactors* 17, 287-296.

Toussaint, O., Houbion, A., and Remacle, J. (1992). Aging as a multi-step process characterized by a lowering of entropy production leading the cell to a sequence of defined stages. II. Testing some predictions on aging human fibroblasts in culture. *Mech Ageing Dev* 65, 65-83.

Toussaint, O., Medrano, E.E., and von Zglinicki, T. (2000). Cellular and molecular mechanisms of stress-induced premature senescence (SIPS) of human diploid fibroblasts and melanocytes. *Exp Gerontol* 35, 927-945.

Tsai, M.C., Manor, O., Wan, Y., Mosammaparast, N., Wang, J.K., Lan, F., Shi, Y., Segal, E., and Chang, H.Y. (2010). Long noncoding RNA as modular scaffold of histone modification complexes. *Science* 329, 689-693.

Tuupanen, S., Turunen, M., Lehtonen, R., Hallikas, O., Vanharanta, S., Kivioja, T., Bjorklund, M., Wei, G., Yan, J., Niittymaki, I., *et al.* (2009). The common colorectal cancer predisposition SNP rs6983267 at chromosome 8q24 confers potential to enhanced Wnt signaling. *Nat Genet* 41, 885-890.

Vafa, O., Wade, M., Kern, S., Beeche, M., Pandita, T.K., Hampton, G.M., and Wahl, G.M. (2002). c-Myc can induce DNA damage, increase reactive oxygen species, and mitigate p53 function: a mechanism for oncogene-induced genetic instability. *Mol Cell* 9, 1031-1044.

van der Lugt, N.M., Domen, J., Linders, K., van Roon, M., Robanus-Maandag, E., te Riele, H., van der Valk, M., Deschamps, J., Sofroniew, M., van Lohuizen, M., *et al.* (1994). Posterior transformation, neurological abnormalities, and severe hematopoietic defects in mice with a targeted deletion of the bmi-1 proto-oncogene. *Genes Dev* 8, 757-769.

van Deursen, J.M. (2014). The role of senescent cells in ageing. *Nature* 509, 439-446.

van Riggelen, J., Muller, J., Otto, T., Beuger, V., Yetil, A., Choi, P.S., Kosan, C., Moroy, T., Felsher, D.W., and Eilers, M. (2010). The interaction between Myc and Miz1 is required to antagonize TGFbeta-dependent autocrine signaling during lymphoma formation and maintenance. *Genes Dev* 24, 1281-1294.

von Zglinicki, T., Pilger, R., and Sitte, N. (2000). Accumulation of single-strand breaks is the major cause of telomere shortening in human fibroblasts. *Free Radic Biol Med* 28, 64-74.

Walz, S., Lorenzin, F., Morton, J., Wiese, K.E., von Eyss, B., Herold, S., Rycak, L., Dumay-Odelot, H., Karim, S., Bartkuhn, M., *et al.* (2014). Activation and repression by oncogenic MYC shape tumour-specific gene expression profiles. *Nature* 511, 483-487.

Wiemann, S.U., Satyanarayana, A., Tsahuridu, M., Tillmann, H.L., Zender, L., Klempnauer, J., Flemming, P., Franco, S., Blasco, M.A., Manns, M.P., *et al.* (2002). Hepatocyte telomere shortening and senescence are general markers of human liver cirrhosis. *FASEB J* 16, 935-942.

Wu, C.H., van Riggelen, J., Yetil, A., Fan, A.C., Bachiredy, P., and Felsher, D.W. (2007). Cellular senescence is an important mechanism of tumor regression upon c-Myc inactivation. *Proc Natl Acad Sci U S A* 104, 13028-13033.

Wu, S., Cetinkaya, C., Munoz-Alonso, M.J., von der Lehr, N., Bahram, F., Beuger, V., Eilers, M., Leon, J., and Larsson, L.G. (2003). Myc represses differentiation-induced p21CIP1 expression via Miz-1-dependent interaction with the p21 core promoter. *Oncogene* 22, 351-360.

Wu, X., Johansen, J.V., and Helin, K. (2013). Fbxl10/Kdm2b recruits polycomb repressive complex 1 to CpG islands and regulates H2A ubiquitylation. *Mol Cell* 49, 1134-1146.

Xiao, X., Huang, X., Ye, F., Chen, B., Song, C., Wen, J., Zhang, Z., Zheng, G., Tang, H., and Xie, X. (2016). The miR-34a-LDHA axis regulates glucose metabolism and tumor growth in breast cancer. *Sci Rep* 6, 21735.

Xu, K., Wu, Z.J., Groner, A.C., He, H.H., Cai, C., Lis, R.T., Wu, X., Stack, E.C., Loda, M., Liu, T., *et al.* (2012). EZH2 oncogenic activity in castration-resistant prostate cancer cells is Polycomb-independent. *Science* 338, 1465-1469.

Xu, M., Long, C., Chen, X., Huang, C., Chen, S., and Zhu, B. (2010). Partitioning of histone H3-H4 tetramers during DNA replication-dependent chromatin assembly. *Science* 328, 94-98.

Xu, Z., Sun, Y., Guo, Y., Qin, G., Mu, S., Fan, R., Wang, B., Gao, W., Wu, H., Wang, G., *et al.* (2016). NF-YA promotes invasion and angiogenesis by upregulating EZH2-STAT3 signaling in human melanoma cells. *Oncol Rep* 35, 3630-3638.

- Yap, D.B., Chu, J., Berg, T., Schapira, M., Cheng, S.W., Moradian, A., Morin, R.D., Mungall, A.J., Meissner, B., Boyle, M., *et al.* (2011). Somatic mutations at EZH2 Y641 act dominantly through a mechanism of selectively altered PRC2 catalytic activity, to increase H3K27 trimethylation. *Blood* 117, 2451-2459.
- Yap, K.L., Li, S., Munoz-Cabello, A.M., Raguz, S., Zeng, L., Mujtaba, S., Gil, J., Walsh, M.J., and Zhou, M.M. (2010). Molecular interplay of the noncoding RNA ANRIL and methylated histone H3 lysine 27 by polycomb CBX7 in transcriptional silencing of INK4a. *Mol Cell* 38, 662-674.
- Ye, X., Zerlanko, B., Kennedy, A., Banumathy, G., Zhang, R., and Adams, P.D. (2007). Downregulation of Wnt signaling is a trigger for formation of facultative heterochromatin and onset of cell senescence in primary human cells. *Mol Cell* 27, 183-196.
- Yoon, J.C., Ng, A., Kim, B.H., Bianco, A., Xavier, R.J., and Elledge, S.J. (2010). Wnt signaling regulates mitochondrial physiology and insulin sensitivity. *Genes Dev* 24, 1507-1518.
- Yu, M., Mazor, T., Huang, H., Huang, H.T., Kathrein, K.L., Woo, A.J., Chouinard, C.R., Labadorf, A., Akie, T.E., Moran, T.B., *et al.* (2012). Direct recruitment of polycomb repressive complex 1 to chromatin by core binding transcription factors. *Mol Cell* 45, 330-343.
- Yuan, W., Xu, M., Huang, C., Liu, N., Chen, S., and Zhu, B. (2011). H3K36 methylation antagonizes PRC2-mediated H3K27 methylation. *J Biol Chem* 286, 7983-7989.
- Zeller, K.I., Zhao, X., Lee, C.W., Chiu, K.P., Yao, F., Yustein, J.T., Ooi, H.S., Orlov, Y.L., Shahab, A., Yong, H.C., *et al.* (2006). Global mapping of c-Myc binding sites and target gene networks in human B cells. *Proc Natl Acad Sci U S A* 103, 17834-17839.
- Zhang, D.Y., Pan, Y., Zhang, C., Yan, B.X., Yu, S.S., Wu, D.L., Shi, M.M., Shi, K., Cai, X.X., Zhou, S.S., *et al.* (2013). Wnt/beta-catenin signaling induces the aging of mesenchymal stem cells through promoting the ROS production. *Mol Cell Biochem* 374, 13-20.
- Zhang, D.Y., Wang, H.J., and Tan, Y.Z. (2011). Wnt/beta-catenin signaling induces the aging of mesenchymal stem cells through the DNA damage response and the p53/p21 pathway. *PLoS One* 6, e21397.
- Zhang, R., Chen, W., and Adams, P.D. (2007). Molecular dissection of formation of senescence-associated heterochromatin foci. *Mol Cell Biol* 27, 2343-2358.

Zhou, W., Zhu, P., Wang, J., Pascual, G., Ohgi, K.A., Lozach, J., Glass, C.K., and Rosenfeld, M.G. (2008). Histone H2A monoubiquitination represses transcription by inhibiting RNA polymerase II transcriptional elongation. *Mol Cell* 29, 69-80.

Zhu, J., Woods, D., McMahon, M., and Bishop, J.M. (1998). Senescence of human fibroblasts induced by oncogenic Raf. *Genes Dev* 12, 2997-3007.

Zmijewski, J.W., and Jope, R.S. (2004). Nuclear accumulation of glycogen synthase kinase-3 during replicative senescence of human fibroblasts. *Aging Cell* 3, 309-317.