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Molecular Mechanisms of Cellular Senescence

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1. Introduction

Normal mammalian cells in culture have a limited life span and will eventually maintain a growth arrested state, referred to as replicative senescence. Usually induced by telomere shortening this form of arrest is irreversible in the sense that cells cannot be triggered to re-enter proliferation by physiological mitotic stimuli like growth factors. Senescence may also occur prematurely in response to various stress stimuli such as oxidative stress, DNA damage or active oncogenes. Thereby premature senescence acts as an important tumor suppressive mechanism and not surprisingly there is emerging evidence that senescence is indeed not only a result of tissue culture but markers of senescence have been identified in vivo in human and animal tissue.

The function of the retinoblastoma protein (pRb) is central to the onset of senescence. pRb, in its active hypophosphorylated form, is a potent repressor of genes that function during DNA replication and thereby pRb causes cell cycle arrest. The cell cycle inhibitors p16^{INK4a} and p21^{Waf1} and their homologues work in concert with pRb by inhibiting cyclin dependent kinases (CDKs) from phosphorylating pRb and thus maintaining in its active growth inhibitory state.

Additionally to the transient role in growth inhibition active, hypophosphorylated pRb coordinates major changes in direct and epigenetic gene regulation leading to changes in chromatin structure, which are crucial to the onset and maintenance of senescence.

This chapter provides an insight in these molecular mechanisms of cellular senescence.

2. Senescence features and biomarkers

Senescent cells display several characteristic morphological and biochemical features. The detection of these markers has been used to identify senescent cells in vitro and in vivo. The

typical senescence phenotype consist of enlarged cell with multiple or enlarged nuclei, prominent Golgi apparatus and sometimes a vacuolated cytoplasm (Figure1). Recently a novel method to measure protein levels with fluorescence microscopy confirmed that indeed senescent cells accumulate increased levels of protein in the cytoplasm and nucleus [1]. In addition to the detection of characteristic morphological changes the most common method used to identify senescent cells is measurement of the lysosomal beta-galactosidase activity with a simple biochemical assay [2]. Due to an expansion of the lysosomes senescent cells show an increased activity of this enzyme, which is therefore often referred to as senescence-associated beta-galactosidase (SA-beta-gal), [3, 4]. However, it should be noted, that an increased beta-gal activity is an unreliable marker of senescence since it is also detectable in vitro after prolonged cell culture, serum withdrawal, TGF-beta, heparin or TPA treatment [3, 5-8]. The tumour suppressors p16^{INK4a} and p21^{Waf1} are mediators of cell cycle arrest and senescence and therefore often used as biomarkers. Since neither p16^{INK4a} nor p21^{Waf1} is strictly required for the induction or maintenance of the senescence program their predictive value is limited if used individually. A specific feature of senescent cells are condensed heterochromatic regions, known as senescence-associated heterochromatic foci (SAHF). These heterochromatin spots are enriched with i) histone H3-methylated at lysine 9 (H3K9meth), its binding partner ii) heterochromatin protein-1γ (HP- 1γ) and iii) the non-histone chromatin protein, HMGA2, which all have been used as markers of SAHF [9, 10].

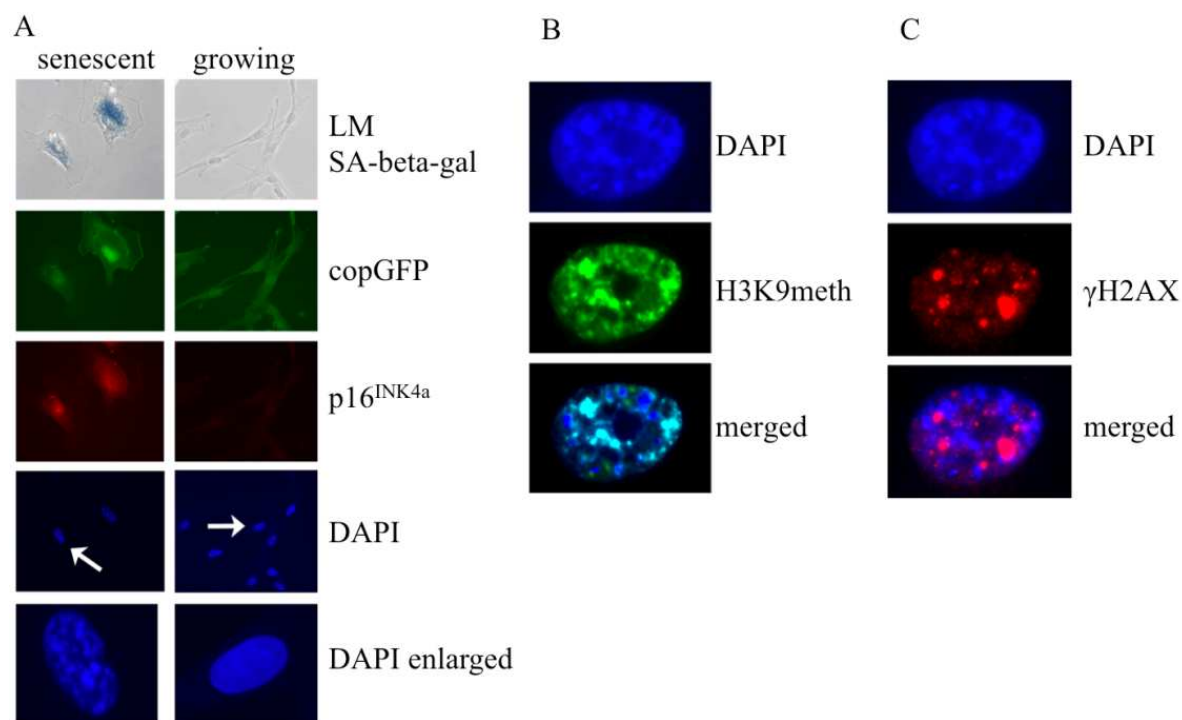


Figure 1. Senescence characteristics

A) The typical senescence phenotype consist of enlarged cell with multiple or enlarged nuclei, and an increased SA-beta-gal activity is visible after N-RAS^{Q61K} induced senescence. Hu-

man diploid fibroblasts (HDF) were transduced with lentiviruses expressing N-RAS^{Q61K} or copGFP control. The efficiency of transduction was controlled with the co-expression of copGFP and was consistently above 90%. p16^{INK4a} expression, chromatin condensation (DAPI), and the appearance of increased SA- β -Gal activity were analyzed and quantified 15 days after infection. Cells enlarged to show DAPI-stained chromatin foci are indicated with arrows. B, C) HDF induced to senesce with oncogenic N-RAS^{Q61K} were stained with DAPI and an antibody to H3K9meth or γ H2AX to highlight senescence-associated heterochromatin foci or DNA damage foci respectively. H2AX is a member of the histone H2A family that gets instantly phosphorylated after DNA damage and forms foci at DNA break sites.

3. pRb in cell cycle regulation

The retinoblastoma protein (pRb) is often referred to as the “master brake” of the cell cycle because its main function is to inhibit E2F transcription factors from inducing a range of genes essential for DNA replication and thus proliferation [11]. Consequently active pRb causes cell cycle arrest. In contrast during proliferation when cells are promoted towards cell division, pRb is sequentially phosphorylated by a series of cyclin dependent kinases (CDKs) and this results in pRb inactivation and consequently derepression of proliferation genes.

Initiation of cell proliferation is normally triggered by growth factors. These external molecules function as ligands to a number of growth factor receptors expressed on the cell surface and thus activate signalling cascades, most prominently the mitogen activated protein kinase (MAPK) pathway, and ultimately lead to the expression of a number of genes including cyclin D [12]. CDK4 and 6 initiate phosphorylation of pRb in the presence of cyclin D and this leads to de-repression of early cell cycle genes including cyclin E and thus the entry into the cell cycle. Subsequently, CDK2 and CDK1, in co-operation with cyclins E, A and B, continue to stepwise further phosphorylate and inactivate pRb, which leads to cell cycle progression and finally cell division. As the “master brake” of the cell cycle pRb is an important tumor suppressor and alterations of its pathway have been associated with the childhood cancer retinoblastoma and are known to occur in over 90% of cancers [13]. There are two important types of cell cycle inhibitors represented most prominently by p16^{INK4a} and p21^{Waf1}. p16^{INK4a} is at the forefront of cell cycle inhibition as it binds specifically to the cyclin D dependent kinases CDK4 and CDK6 and displaces cyclin D and thereby it prevents the entry into the cell cycle and arrests cells in G1 phase (Figure 2). p21^{Waf1} is more promiscuous and is able to inhibit all CDK molecules at any stage during the cell cycle. p21^{Waf1} molecules do not necessarily displace cyclin partners from their CDK target and importantly it may require several p21^{Waf1} molecules to effectively inhibit CDKs [14]. In normal cells p16^{INK4a} and p21^{Waf1} are able to work hand in hand, the accumulation of p16^{INK4a} and binding to CDK4 and 6 frees p21^{Waf1} molecules from these kinases to bind and inhibit CDK2 and 1 more efficiently [15]. These basic cell cycle regulatory functions of pRb, p16^{INK4a} and p21^{Waf1} are essential to initiate and maintain senescence and it is not surprising that all three molecules are considered important tumor suppressors.

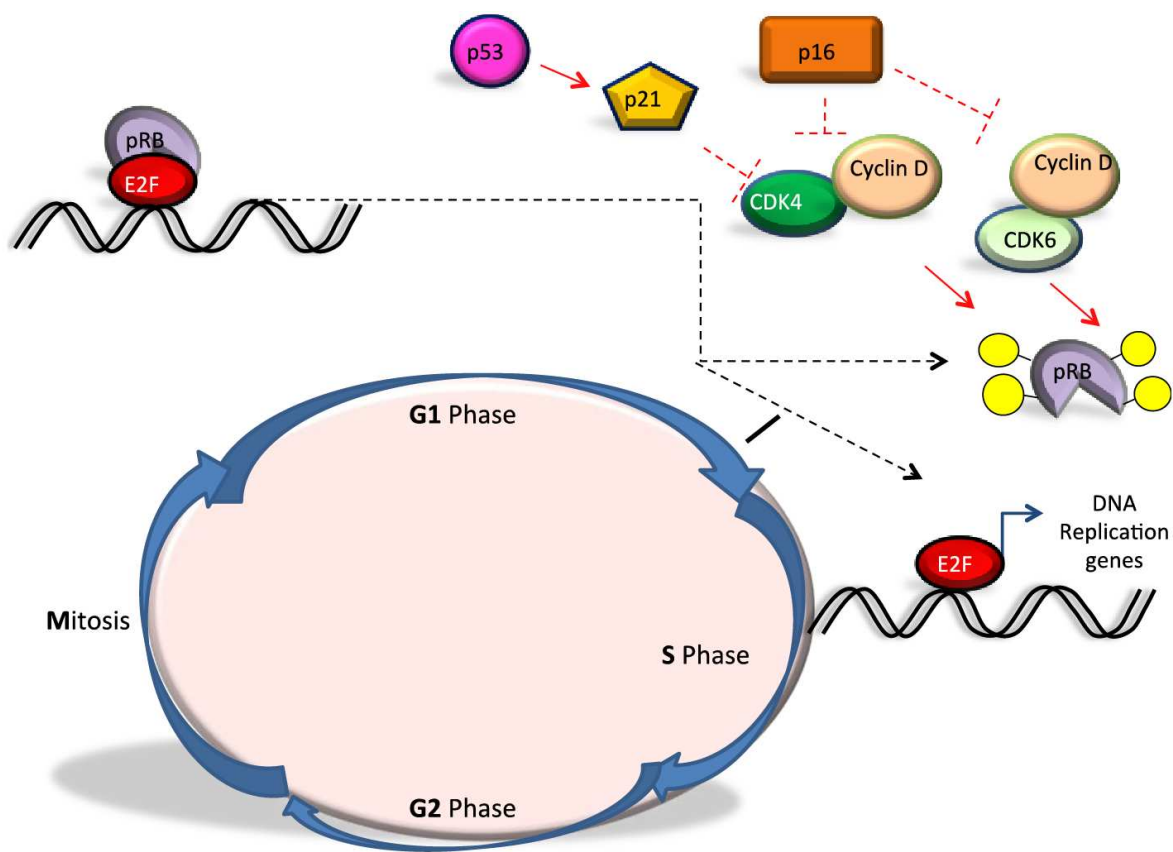


Figure 2. The Cell Cycle

This simplified model, focusing on early cell cycle entry, illustrates that hypophosphorylated, active pRb represses E2F-mediated transcription. The action of CDKs, exemplified by the cyclin D dependent CDK4 and 6, phosphorylate pRb and thus release E2F to activate transcription of early DNA replication genes. p16^{INK4a} and p21^{Waf1}, the latter usually activated by p53, inhibit CDKs and retain pRb in its active cell cycle inhibitory state.

4. The role of the tumor suppressor p16INK4a in senescence

With regard to senescence, it is long known that p16^{INK4a} levels accumulate and cause growth arrest and senescence when cells approach their replicative life span [16-23]. Moreover, in long term tissue culture studies cells that were able to overcome senescence commonly had lost p16^{INK4a} and p53 expression [24]. Increased p16^{INK4a} expression is also linked to oncogene induced and other forms of premature senescence [25-33].

Interestingly, despite this clear correlation of p16^{INK4a} up-regulation with senescence there is some evidence from p16^{INK4a} is not strictly required for senescence to occur. Evidence from mouse models show that mouse embryonic fibroblasts (MEFs) of p16-null mice undergo a comparable number of cell divisions as wild type MEFs before entering senescence [34] [35], while in primary melanocytes, which were lentivirally transduced to express oncogenic

HRAS or NRAS, silencing of p16^{INK4a} did not abolish most senescent features. Interestingly however, the formation of SAHF did only occur in the presence of p16^{INK4a} [29, 36, 37]. These findings show two important points: first there are other redundant mechanisms able to compensate for p16^{INK4a} loss and rescue senescence and second the p16^{INK4a}-pRb pathway has a specific role in SAHF formation and, importantly, these heterochromatin foci have been suggested to abolish expression of proliferation associated genes and secure senescent features so senescence becomes irreversible [9] (see section 7 for more detail). This idea is supported by a report that senescence was only reversible, via p53 inactivation, in fibroblasts and mammary epithelial cells with low but not with high p16^{INK4a} expression [38]. It is noteworthy that the importance of SAHF in securing senescence has been challenged recently and SAHF are thought dispensable for senescence by some investigators and/or only associated with oncogene-induced senescence [39, 40]. The fact that SAHF formation only occurs in the presence of increased p16^{INK4a} levels remains undebated and it is therefore tempting to speculate a direct p16^{INK4a} role in the formation of these structures.

Even though cells may be able to compensate for p16^{INK4a} loss and still undergo a growth arrest characterised by most if not all senescent features, the importance of the tumor suppressor p16^{INK4a} in senescence is clear as p16-null tumor cells can be driven into senescence by the sole re-expression of p16^{INK4a}. Induced p16^{INK4a} expression in glioma cells caused a typical senescent phenotype [41], reversing promoter hypermethylation allowed for the re-expression of endogenous p16^{INK4a} in oral squamous cell carcinoma cells leading to senescence [42], inducible p16^{INK4a} expression in osteosarcoma cells induced senescence after 3-6 days, potentially irreversible after 6 days [43] and inducible p16^{INK4a} in human melanoma cells caused a senescent phenotype after 3-5 days in the absence of p53 [44, 45]. Moreover, even in normal early passage human fibroblasts the ectopic introduction of p16^{INK4a} or functional peptides thereof initiated cell cycle arrest and senescent features [46, 47]. In line with this, melanoma associated germline mutations of p16^{INK4a} are impaired in inducing a cellular senescence program in melanoma cells and this disability to promote senescence may contribute to the melanoma-risk of p16^{INK4a} linked melanoma-prone families [45].

5. Timing of Senescence by repression and activation of p16^{INK4a}

5.1. p16^{INK4a} repression

In fact, the ability to induce senescence in response to accumulated or sudden genomic stress is probably the most important tumor suppressive function of p16^{INK4a}. In line with this consideration it is not surprising that p16^{INK4a} expression is tightly repressed at the chromatin and transcriptional level in “young” proliferating cells and in cells with extensive renewal capacities, such as stem cells. The polycomb protein Bmi1, which is also known as “stem cell factor” is facilitating repression of the INK4a locus at the chromatin level [48, 49]. Intriguingly, in a functional feedback loop, in human fibroblasts the Bmi1-mediated repression of p16^{INK4a} requires active pRb and also H3K27 (histone 3/lysine 27) trimethylation facilitated by the histone methyltransferase EZH2 in concert with a second polycomb protein,

SUZ12 [50]. Crucially, Bmi1 chromatin binding can be inhibited by its phosphorylation through the MAPK and p38 signalling pathways [51]. Hence these pathways are able to directly oppose p16^{INK4a} repression and lead to its transcriptional activation via Ets and Sp-1 during oncogene-induced senescence.

In concert with Bmi-linked chromatin-remodelling events a number of transcription factors facilitate p16^{INK4a} repression during the proliferative life-time of cells. The perhaps most important transcriptional repressors of p16^{INK4a} are Id proteins, with the main representative Id1. Id proteins function by binding to E-box DNA sequences to repress the INK4a promoter and importantly by interfering with Ets transcriptional complex formation and thereby inhibiting the main INK4a transcriptional activator, Ets, in two ways [52, 53]. In line with this, high Id1 levels were associated with early stage melanoma whereas premalignant and interestingly also more advanced melanoma showed limited Id1 expression [54]. This suggests a role of the Id1/p16^{INK4a} regulative connection during melanomagenesis and Id1 may be dispensable in later melanoma stages once p16^{INK4a} is either more tightly repressed by engaging repressive histone modifications or inactivated by other mechanisms. Id1 down-regulation on the other hand is usually associated with cell differentiation and senescence [55]. Consequently, ectopic expression of Id1 delayed senescence in melanocytes [56] and keratinocytes [57], while MEFs lacking Id1 prematurely senesced due to increased p16^{INK4a} levels [58, 59]. Another way to oppose Ets driven p16^{INK4a} transcription was identified, when Cdh1, an adaptor protein of the anaphase promoting complex, was shown to bind to and promote degradation of Ets2 and thereby increased the replicative life span of MEFs [60], while the Epstein-Barr virus protein LMP1 represses p16^{INK4a} by promoting the nuclear export of Ets2 [61, 62]

Interestingly, p16^{INK4a} may also be repressed by the oncogene β -catenin, which has been linked to melanoma. β -catenin binds the INK4a promoter at a conservative β -catenin/Lef/Tcf binding site and thereby directly represses its transcription. Consequently β -catenin silencing increased p16^{INK4a} levels in A375P human melanoma cells, while stabilization of β -catenin together with oncogenic N-RAS led to prevention of senescence and thus, immortalization [63]. Importantly, a role of β -catenin in melanocyte senescence is controversial as nuclear β -catenin was commonly found in benign melanocytic nevi [64-66] and these lesions were proposed to be senescent by some investigators [29, 67], this again is controversial, as benign nevi are not distinguished from normal melanocytes or primary melanomas using a range of common senescence markers [68]. It would clearly be interesting to test whether nuclear β -catenin does overlap with the expression of p16^{INK4a} in benign nevi as the latter is mosaic and not found in all cells [29] and co-localisation or lack of it could help clarify this debate. Another repressor of p16^{INK4a} is the "T-box transcription factor" Tbx2, this transcription factor binds to corresponding T-box DNA sequence elements [69]. Tbx2 overexpression was identified in melanomas and associated with melanoma progression [70].

5.2. p16^{INK4a} expression

When cells reach their finite life span the pendulum at the INK4a promoter swings from repression to activation and the SWI/SNF chromatin remodeling complex, replaces Bmi1 repressors and relaxes chromatin structures around the INK4 promoter region, which is strictly depend-

ent on the SWI/SNF subunits BRG1 and hSnf5, and the relaxed chromatin structure allows transcription factor access [71] (Figure 3a). Alterations of hSnf5 are associated with early childhood rhabdoid cancer and re-expression of hSnf5 in rhabdoid cancer cells leads to p16^{INK4a} accumulation, growth arrest and senescence [71, 72] and this requires functional p16^{INK4a} [73].

The best understood transcription factors driving p16^{INK4a} expression and thereby growth arrest and senescence are Ets1 and Ets2. They are effectors of MAPK signalling, often in response to oncogenic stress, such as activating N-RAS or B-RAF mutations, which induce an increase in p16^{INK4a} levels [25, 29, 37, 52]. In line with this, human fibroblasts with biallelic mutations in p16^{INK4a} did increase mutant p16^{INK4a} expression in response to RAS signaling or expression of ectopic Ets, but failed to arrest or undergo senescence [74]. Interestingly increased p16^{INK4a} expression has also been linked to loss of p53 and this appears to be correlated with increased Ets protein half-life [75]. Another member of the Ets transcription factor family, ESE-3, was independently identified as a down stream target of p38 signalling and caused senescence via p16^{INK4a} up-regulation [76]. p38 signalling has been linked to enhanced p16^{INK4a} expression before and this involved the downstream transcription factor Sp-1, which was proposed to be required for p16^{INK4a} up-regulation during senescence in human fibroblasts [77]. Sp-1 was reported to engage the p300 enhancer leading to further p16^{INK4a} upregulation [78] (Figure 3b).

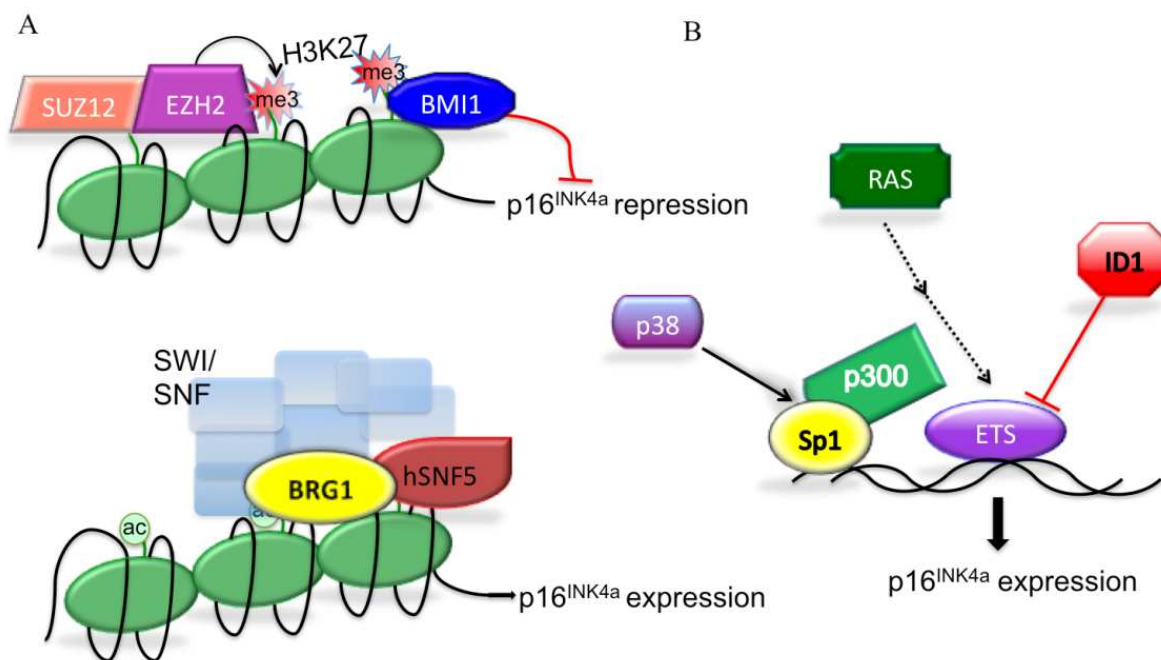


Figure 3. Schematic presentation of p16^{INK4a} regulation

(A) During the proliferative cellular life-time, EZH2 in cooperation with SUZ12 trimethylates H3K27 at the genomic INK4a locus and these histone modifications attract the polycomb repressor BMI1, which maintains the p16^{INK4a} promoter region inaccessible for

transcriptional activation. Once cells reach their finite life span or during premature senescence the histone modifications at the INK4a locus change from repressive methylations to activating acetylations, which attract the SWI/SNF complex. The SWI/SNF subunits hSnf5 and BRG1 are instrumental in opening the chromatin structure and allowing access of the transcriptional machinery to the p16^{INK4a} promoter. (B) Ets transcription factors are downstream targets of MAPK signalling, exemplified here by RAS, and bind to E-box p16^{INK4a} promoter motifs to activate gene expression. Id transcription factors can compete with Ets for DNA binding and oppose transcriptional expression. The levels of Id proteins decline with onset of senescence and Ets are able to promote p16^{INK4a} expression.

6. The role of the p53/p21 pathway in senescence

The transcription factor and tumor suppressor p53 is often referred to as “guardian of the genome” and inactivating mutations in p53 are observed in about half of all human cancer cases. The p53 protein is a critical regulator of cell survival in response to cellular stress signals including DNA damage, oncogene activation, hypoxia and viral infection (reviewed in [79]). In the absence of stress stimuli p53 gets rapidly ubiquitinated by one of several E3 ligases including MDM2, MDM4, TOPORS, COP1, and ARF-BP1 and subsequently degraded in the proteasome [80, 81]. Stress signals, on the other hand, induce covalent modification usually by disrupting the interaction between p53 and the E3 ubiquitin ligases which prevents its degradation. Oncogenic stress, for example, activates the alternative reading frame product of the INK4a locus (p14ARF) that stabilizes p53 by binding and thereby inhibiting its negative regulator MDM2.

Several lines of evidence show convincingly that the p53 and its downstream effector p21^{Waf1} play a crucial role in the regulation of cell cycle arrest and senescence. Overexpression of p53 [82] and p21^{Waf1} [83-86] autonomously induced senescence in human cells and activation of p53 by either overexpression of p14ARF [87] or nutlin-3 treatment [88] induced senescence in a p21^{Waf1} dependent manner in human diploid fibroblasts (HDF) and human glioblastoma cells respectively. Furthermore, inactivation of p53 or p19ARF (mouse homologue of human p14ARF) prevents senescence in mouse embryonic fibroblasts (MEF) [89-91] and human fibroblast lacking p21Waf1 can bypass the senescence growth arrest [83]. Further supporting evidence comes from studies that show that, inactivation of p53 using viral oncoproteins, anti-p53 antibodies or anti-sense oligonucleotides can extend the replicative lifespan or even reverse the senescence growth arrest in human cells [38, 92-94]. However, it should be noted that although inactivation of the p53 pathway can weaken or even reverse the senescence arrest in some cells, there is emerging evidence that it fails to do so in cells with an activated p16^{INK4a}/pRb pathway [38, 95-97]. Despite the clear evidence of p53's role in promoting senescence a study conducted by Demidenko and co-workers suggested that p53 can also act as an inhibitor of senescence [98]. Surprisingly, p53 was able to reverse a p16^{INK4a} and p21^{Waf1} driven senescence response in human fibroblasts. Although the underlying mechanisms are not fully understood, inhibition of the mTor (mammalian target of rapamycin) pathway seems to be involved in the repression of cellular senescence [98-101].

6.1. DNA damage and senescence

The human genome is constantly exposed to genotoxic stress such as ultraviolet light, reactive oxygen species (ROS) as well as chemical and biological mutagens. To ensure the integrity of the genome cells have evolved a sophisticated safety system that can implement a cell cycle arrest to allow the repair of the incurred damage. The initial step in the complicated DNA damage repair process is the detection of DNA damage. The Mre11/Rad50/NBS1 (MRN) complex function as a DNA damage sensor is localized in nuclear foci at the sites of double strand breaks (DSBs) [102-104]. Here, the complex tethers DNA ends, processes and repairs free strands via endonuclease and exonuclease activities [105-107]. The MRN complex is also involved in the recruitment and activation of ATM (ataxia-telangiectasia mutated) and ATR (ATM and Rad3-related) protein kinases, which in turn activate checkpoint-1/2 (CHK1/2) kinases leading to phosphorylation and thereby stabilization of a variety of target genes including p53 [108]. In the case of irreparable damage, the cell will be permanently retracted from the pool of dividing cells by the induction of apoptosis or senescence (Figure 4) [109, 110].

6.2. Drug induced senescence

Several sources of genotoxic stress including cisplatin, cyclophosphamide, doxorubicine, taxol, vincristine, cytarabine, etoposide, hydroxyurea, bromodeoxyuridine, adriamycin, bleomycin mitomycin D, interferon beta, radiation, ROS, H₂O₂ have been used to trigger senescence-like growth arrest in vitro and in vivo allowing a detailed analysis of the underlying signaling events [28, 111-122]. The crosslinking agent cisplatin, for example, induced senescence in primary human fibroblasts and HCT116 colon carcinoma cells in a p53 and dose dependent manner [123, 124]). Interestingly, treatment of HCT116 cells with low cisplatin concentrations lead to DNA damage and senescence whereas high drug concentrations induced apoptosis via superoxide production. Furthermore, in human lung cancer cells induced p53 expression enhanced the cytotoxic effect of cisplatin whereas p21^{waf1} overexpression surprisingly lead to increased drug resistance [82]. Similarly, cisplatin-resistant human non-small cell lung cancer (NSCLC) cells could be sensitized to drug-induced senescence by re-expressing p16^{Ink4a} [118]. Duale and co-workers compared changes in the gene expression profiles of a cisplatin treated human colon carcinoma cells (HCT116) and cell lines derived from testicular germ cell tumors (TGCTs). The systematic approach combining the acquired gene expression data and other publicly available microarray data identified 1794 genes that were differentially expressed including 29 senescence-related genes such as IGFBP 7, interleukin 1 and MAPK8 [125]. These findings highlight the notion that cellular response to chemotherapeutic agents is generally depending on the cellular context as well as the type and level of the stress signal.

6.3. Oncogene-induced senescence and DNA damage

In contrast to chemotherapeutic agents that directly damage the DNA, activated oncogenes do the harm by forcing the cell in uncontrolled, constitute replication cycles which leads to DNA replication stress and subsequent DNA damage [126, 127]. During this process DNA replication forks stall making the DNA more susceptible to single or double strand breaks.

The subsequent activation of the DNA damage response (DDR) machinery delays cell-cycle progression by initiation of the ATM/ATR-CHK1/2 pathway and stable knockdown of any one of these DDR genes was sufficient to bypass RAS induced senescence in human fibroblasts. Specifically, in the absence of ATM, ATR, Chk1 or Chk2, human fibroblasts continued to proliferate despite Ras expression, and BrdU incorporation increased from approximately 5% in the control sample to 15% in cells deficient for a DNA damage protein. Interestingly, the inhibition of both the p16INK4a and DNA damage pathway enhanced the effect and enabled over 60% of the cells to replicate DNA [126]. In a similar study, Bartkova and co-workers showed that the suppression of ATM or p53 allowed the MRC5 and BJ human fibroblasts to bypass oncogene-induced senescence using overexpressed Mos (an activator of the MAPK pathway) or Cdc6 (a DNA replication licensing factor). However, in this report p16INK4a depletion alone did not weaken the senescence response [127].

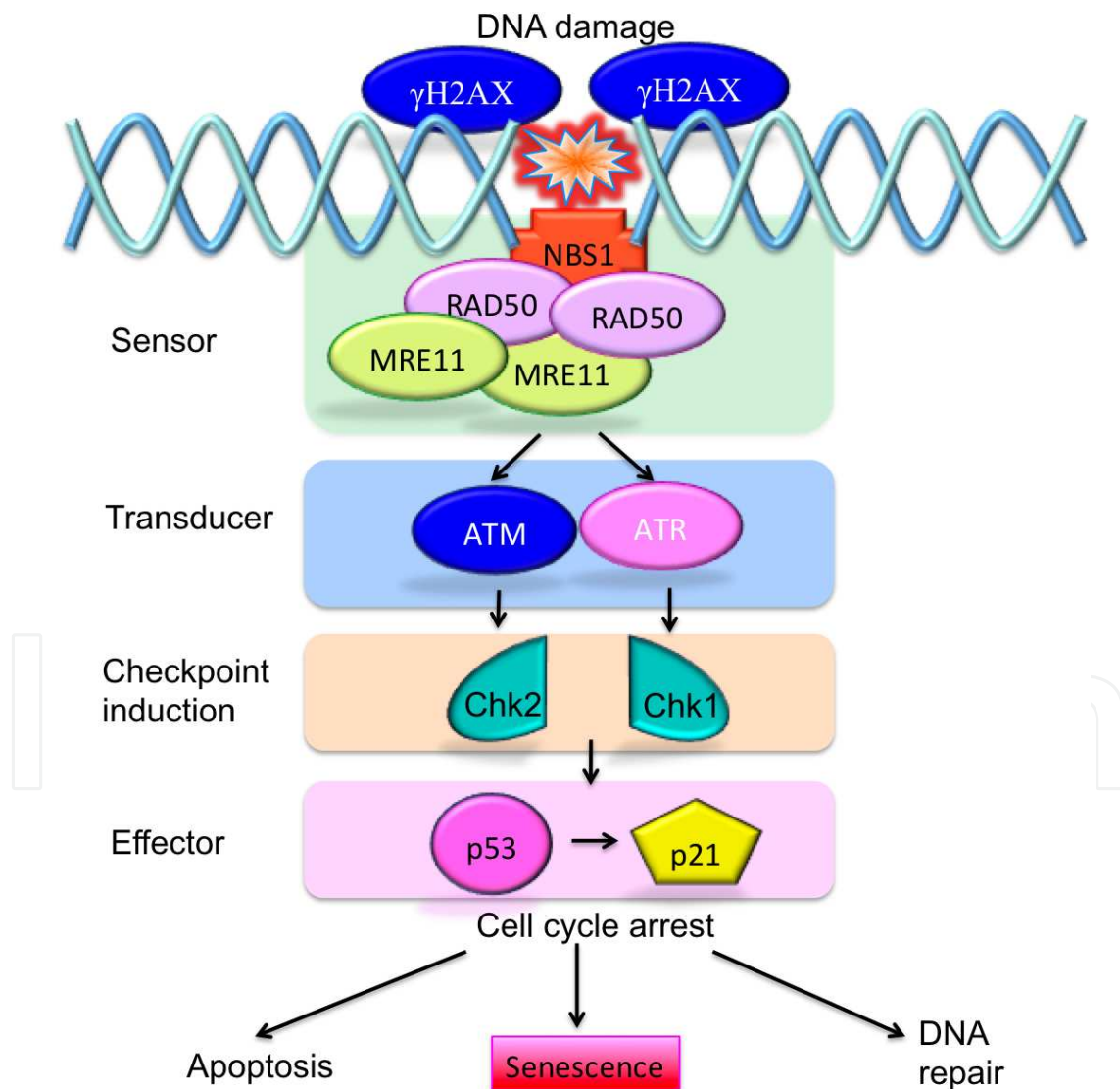


Figure 4. DNA damage pathway in senescence

To protect the integrity of their DNA, cells need to be able to sense DNA damage and activate response pathways that coordinate cell cycle progression and DNA repair. Ataxia telangiectasia mutated (ATM) and ATM-Rad3-related (ATR) kinases are important DNA damage checkpoints proteins that transduce signals from the DNA damage sensors to the effector proteins that control cell cycle progression, chromatin restructuring, and DNA repair. ATM and ATR activate other kinases such as Chk1 (activated by ATR) and Chk2 (activated by ATM) that phosphorylate and activate the tumour suppressor protein p53. ATM and ATR can additionally enhance p53 activity by directing p53 phosphorylation on Ser15. Activated p53 can halt progression of the cell cycle in the G1 phase, allowing DNA repair to occur and preventing the transmission of damaged DNA to the daughter cells.

7. pRb in senescence: maintaining a secure proliferative arrest

In the previous sections we have reviewed the pivotal role of the cell cycle inhibitors p16^{INK4a} and p21^{Waf1} in keeping pRb in a hypophosphorylated, active state and explored the mechanisms of timing the expression of these tumor suppressors with the onset and maintenance of senescence. Here we explore the roles of active pRb in the senescence program, which go beyond the antiproliferative functions described in the cell cycle section 3.

There is longstanding evidence that pRb is required for an intact senescence program, for instance re-introduction of pRb into SAOS osteosarcoma cells that have lost its expression can cause senescence [128]. On the other hand inactivation of pRb with the viral oncoprotein E1A prevents senescence, while a mutant form of E1A that is impaired in pRb binding is unable to prevent senescence [9]. Interestingly, another mutant E1A, that is able to bind pRb but is unable to interact with the histone deacetylases p300 and p400 leads to less efficient development of senescence [9].

7.1. pRb and SAHFs

These data highlight that chromatin remodeling plays an important role in the cellular senescence program. Indeed, during senescence E2F responsive genomic promoter regions are stably repressed from transcription by the establishment of heterochromatin regions. As mentioned above (section 3) these regions are visible as microscopical “senescence associated heterochromatin foci” (SAHF) when cells are stained with certain DNA intercalating dyes such as DAPI. The term heterochromatin refers to a highly condensed protein-DNA structure, which is facilitated by modifications of the histone core molecules of chromatin and suppresses DNA access by the transcriptional machinery and thus gene expression of these regions is tightly suppressed (reviewed in [129]). The investigation of SAHF remains a progressing research field and is developing forward with improved microscopic imaging technology and the ability to isolate these complex structures and analyse them more precisely and we can expect to understand even more about their role and configuration in the future. To date we know that SAHF formation coincides with the recruitment of heterochromatin proteins by pRb when interacting with the histone deacetylase 1 (HDAC1) at E2F-re-

sponsive promoters [130]. A number of heterochromatin-associated histone modifications were characteristically found associated with SAHF. These include the reversion of histone 3/lysine 9 acetylation (H3K9Ac) and histone 3/lysine 4 trimethylation (H3K4me3) and significantly, since it is often used as a marker of senescence, the promotion of histone 3/lysine 9 trimethylation (H3K9me3) and H3K27me3. H3K9 trimethylation is a fundamental step during heterochromatin formation as it attracts HP1 proteins, which are pivotal for heterochromatin assembly [9]. It is important to highlight that senescence does not always lead to a net increase in overall H3K9me3, suggesting that SAHF formation is local and indeed targeted via pRb to E2F promoters [131]. H3K9me3 is thought to be very stable and is able to prevent histone acetylases (HATs) to catalyze histone acetylations. Histone acetylations are commonly associated with “euchromatin”, which describes actively transcribed chromatin regions. Accordingly, SAHF formation is thought to “lock” chromatin regions that encode proliferation genes and thus contributes to the very secure silencing of E2F responsive proliferation genes, which is not reversible by physiological stimuli [9]. H3K9 trimethylation associated with SAHF formation is thought to be catalyzed by either the SUV39H1 methyltransferase, which together with HP1 interacts with pRb during E2F promoter silencing in senescence [132] or the RIZ1/PRDM2 H3K9 methyltransferase, which was also shown to co-operate in pRb gene repression and is inactivated in colon, breast and gastric carcinoma [133]. Furthermore, a search for H3K9me3 interacting proteins to identify proteins involved in the senescence program identified JMJD2C, which is a H3K9 specific demethylase. Thereby it is the direct antagonist of the SUV39H1 H3K9 methyltransferase and its over-expression is not surprisingly associated with several forms of cancer [134].

Lately there is a different view about the importance of SAHF emerging and SAHF formation, may according to a recent study only be of major relevance in the oncogene induced senescence process that crucially involves DDR and the corresponding ATR signalling, as knock-down of ATR prevents SAHF formation during RAS induced senescence. The same study also found that SAHF resembling structures containing H3K9me3 do independently occur in proliferating oncogene expressing cells, however the investigators data show that these structures are not associated with E2F target promoters, which remain active. The authors concluded that SAHF structures might not be an essential feature of senescence [40].

More recently a groundbreaking study by the Narita group has combined microscopic, electron microscopic and genomic data analysis to shed more light on SAHF formation and structure. Their significant findings are that H3K9me3 and H3K27me3 may in fact not directly be involved in SAHF. Although these markers are usually associated with SAHF, the overall methylation status of histone tail lysines does not change within the genomic DNA during senescence. Moreover, the investigators found that SAHF formation was linked to pre-senescent replication timing and specific histone modifications were characteristic for early-, mid- and late replications genes. Significantly though, when they prevented SAHF formation by silencing of either pRb or the high mobility group AT-hook 1 (HMGA-1), which they and others had previously linked to SAHF formation, there was no change to H3K9me3 in proliferating cells [135] and this may explain the above-mentioned data by Micco et al [40]. Moreover, Chandra et al. reversed H3K9 and H3K27 trimethylation by either

overexpressing the H3K9me3 favoring histone demethylase JMJD2D or by silencing SUZ12, a polycomb protein involved in H3K9 and K27 methylation. Importantly, even without these methylated histone markers RAS induction still led to senescence with the occurrence of DAPI dense SAHF structures. The investigators concluded that SAHF formation is mediated through spatial rearrangement of pre-existing H3K9me3 and H3K27me3 regions but that these histone marks are not a prerequisite for the SAHF formation process [135]. Clearly, further studies are needed to fully define the combined roles of histone modifications, heterochromatin and SAHF in senescence.

Consistent with gene silencing and decreased acetylation during senescence is the down-regulation of the histone acetylases p300/CBP, which was observed in melanocytes reaching in their finite lifespan [136].

With regard to the role of chromatin remodelling in senescence, there is also evidence that ATP dependent chromatin remodeling complexes, such as SWI/SNF are required for senescence onset in roles other than the timely expression of p16^{INK4a} as described in section 5.1.2: The introduction of the ATPase active component of the SWI/SNF complex, BRG1, into various cell lines induced senescent features. This is attributed to the BRG1 ability to interact with pRb and participate in E2F target promoter repression [137]. However development of senescence in response to BRG1 introduction was only convincing in cells that also lack the BRG1 homologue ATPase BRM [138]. Interestingly we found that BRG1 is able to interact with p16^{INK4a}, and since p16^{INK4a} is required for SAHF formation it might directly be involved in this process in co-operation with BRG1. However, the absence of BRG1, by specific silencing, does neither prevent p16^{INK4a} induced growth arrest nor senescence associated SAHF formation. It should be noted however, that the WMM1175 melanoma cells used in these experiments also express the BRG1 homologue BRM and therefore they still have functional SWI/SNF complexes even in the absence of BRG1. [139]. It still needs to be tested whether BRM also interacts with p16^{INK4a} and whether the BRM-p16^{INK4a} complex is involved in SAHF formation. This idea is in line with a suggested role for BRM in melanocyte senescence as it was demonstrated that BRM was recruited and required, albeit transiently, by the pRb-HDAC1 complex during the initiation of SAHF [140].

Importantly, although the p53-p21 pathway was demonstrated to be capable of inducing a number of senescent features and does so in response to DNA damage, telomere dysfunction and oncogene induced senescence [38, 97], E2F promoter silencing via SAHF formation requires an intact p16-pRb pathway [9, 141]. As expected, SAHF formation is prevented by cancer associated p16^{INK4a} mutations [45]. Since p21Waf1 is capable of inhibiting all CDKs it remains unclear why it appears less effective in promoting a secure senescence program in involving heterochromatin formation (Figure5).

The current view is that pRb binds HDAC1 and attracts H3K9 and H3K27 methylating complexes (exemplified by SUV39H1 and EZH2/SUZ12) to promoters of E2F responsive proliferation genes leading to histone trimethylation and heterochromatin and thereby DAPI dense microscopic structures. Recent work indicates that H3K9 and H3K27, although usually associated with SAHF, may not be a strict requirement. Instead the formation of DAPI dense structures is linked to the timing of presenescent replication events [135]. This view

would explain the fact that SWI/SNF including BRM has been associated in a transient manner with the build up of SAHF structures; BRM can also interact with pRb but strictly is associated with acetylated histones and replicating chromatin is acetylated [140]. The complete understanding of SAHF formation is still an evolving field. Regardless, it is widely accepted that functional p16^{INK4a} and pRb as well as the HMGA, which accumulate at E2F target promoters during senescence, are critically required for SAHF arrangement. In regards to p16^{INK4a} it is tempting to speculate that its specific role in SAHF formation goes beyond CDK inhibition and thus maintaining pRb in an active state.

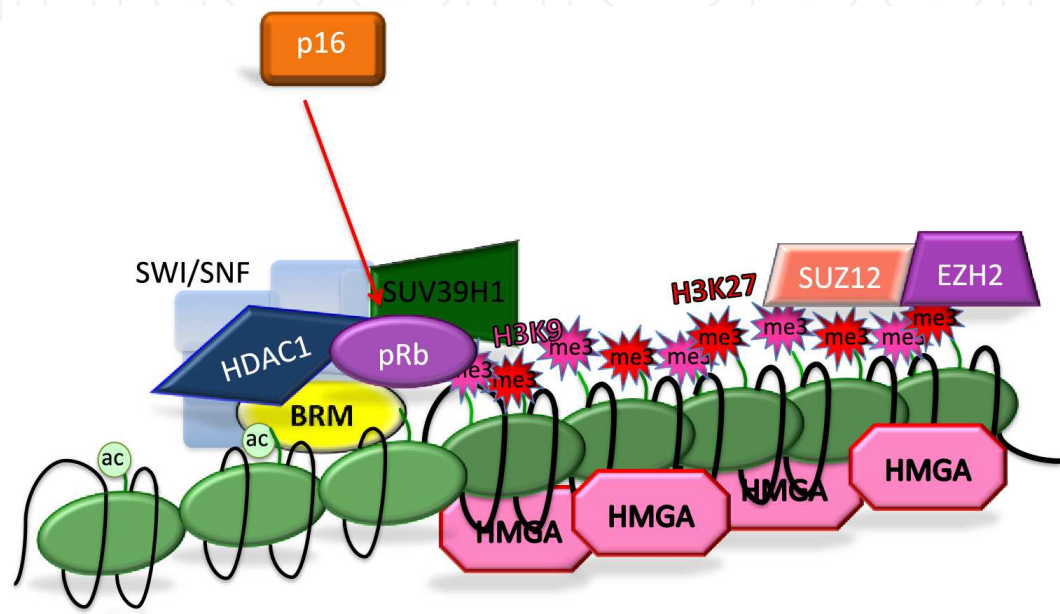


Figure 5. Chromatin remodeling and SAHF formation

8. Conclusion

Senescence is a potent means of tumor suppression. The mechanisms of senescence involve cell cycle regulatory protein functions in concert with the chromatin remodeling machinery to maintain a complex and secure withdrawal from proliferation. The understanding of these mechanisms is still evolving and is predicted to identify novel targets for cancer therapy.

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