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Review

Epigenetics and senescence: Learning from the INK4-ARF locus

Elisabeth Simboeck [a,1], Joana D. Ribeiro [a,1], Sophia Teichmann [a,1], Luciano Di Croce [a,b,*]

[a] Centre de Regulació Genómica, Universitat Pompeu Fabra, Barcelona, Spain
[b] Institució Catalana de Recerca i Estudis Avançats (ICREA), Barcelona, Spain

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ABSTRACT

Cellular senescence is the biological consequence of aging. However, the same mechanisms that provoke senescence during aging have been proven to act in tumor suppression and thus to occur in premalignant cells. All the diverse aspects of the senescent phenotype, as are observed for many other cell fates, arise from alterations of the chromatin architecture. Relatively little is known overall about the changes in chromatin structure, and which regulatory networks are implicated in these. Major insight into the epigenetic contributions to senescence has been gained by studying the regulation of the INK4-ARF locus. Activation of the tumor suppressors encoded by this locus leads to an irreversible cell cycle exit. Importantly, epigenetic alterations at this locus have been associated with the onset of cancer. Here we discuss the recent findings that link epigenetics to the senescence pathway.

Contents

1. Introduction

More than 40 years ago, Hayflick reported that primary human fibroblasts could not be propagated infinitely in vitro. Rather, they displayed a limited proliferative lifespan under culture conditions, followed by an irreversible growth arrest. The halt of mitotic cell divisions, however, did not abrogate viability or metabolic activity, and the mere loss of proliferative potential was designated senescence [1]. Exiting the cell cycle and entering in a stage of non-division also goes in hand with changes in cell morphology. Senescent cells share several common features, such as large sizes, flattened, enlarged shapes [2], and nuclei that can display the appearance of senescence-associated heterochromatic foci (SAHF) [3].

The senescent stage is also reflected by changes in protein expression levels and activity. One of the key players involved is the tumor suppressor p53, whose activity is enhanced by progressive passaging of human fibroblasts [4]. Additionally, transgenic mice carrying an active mutant allele of p53 displayed a precipitated onset of aging and, interestingly, a decreased incidence of tumor development [5]. Microarray analyses of different cell lines have identified additional relevant genes that are differentially expressed upon entering senescence. Understanding their implications in the molecular regulation of this cell stage is of particular interest, as it will help to decipher aging and tumor suppressing mechanisms [6,7].

* Corresponding author at: Department of Differentiation and Cancer, CRG, 08003 Barcelona, Spain. Tel.: +34 93 3160132.
E-mail address: luciano.dicroce@crg.es (L. Di Croce).
[1] These authors contributed equally to this work.

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In order to distinguish quiescent from senescent cells in culture, as well as to monitor senescence in vivo, several biomarkers can be used. Whether or not senescence networks have been activated can be established by the detection of several key proteins, such as p53 or the retinoblastoma protein (pRB) [8]. The most common test, however, for detecting senescent cells in tissue preparations is to measure the senescence-associated (SA) β-galactosidase activity. In senescent cells, β-galactosidase is active at pH 6 rather than at its optimum of pH 4, due to an enlarged lysosomal compartment and a consequentially higher amount of the enzyme present [9].

Many molecular pathways are involved in triggering the senescent cell response. In general, senescence is considered to be a cell-protective mechanism and to be as important as apoptosis because it irreversibly stops proliferation of stressed and damaged cells and, in this way, impedes potential further aberrations. In fact, bypassing of senescence occurs frequently in tumorigenesis.

Irreversible growth arrest can be induced by numerous factors in addition to proliferative exhaustion of cells, such as by several forms of physiologic stress (Fig. 1). Another well-characterized trigger of the senescence phenotype is telomere shortening following serial passaging of human fibroblasts [10]. The reason for the continuous shortening is that during S phase the lagging strand is synthesized only incompletely at its 5′ telomeres. Therefore, this kind of senescence is designated replicative senescence. The shortened telomeres are recognized as damaged DNA by the cellular repair machinery, which leads to activation of the CHK1 and CHK2 kinases, as well as other downstream components of the DNA repair signaling pathways [11]. In addition to telomere attrition after serial passages, there are also other stimuli that provoke the onset of the so-called premature senescence program. One important stimulus is the high expression of oncogenes, which leads to oncogene-induced senescence (OIS) in vivo. The best studied example for OIS is that induced by activated H-ras. When H-ras is expressed in primary mouse embryonic fibroblasts (MEFs), senescence is induced and the levels of the tumor suppressors p53 and p16INK4a are correspondingly elevated [12]. However, upon mutation of either p53 or p16INK4a in MEFs, senescence is circumvented by provoking cell transformation. This effect underscores the function of senescence in tumor suppression [12]. In addition, inactivation of tumor suppressors can also cause cells to go into irreversible growth arrest. The tumor suppressor PTEN is very often mutated in prostate cancer; it acts by preventing proteosomal degradation of p53 by its E3 ubiquitin-ligase Mdm2. MEFs that are deficient for PTEN show morphologic characteristics of senescence as well as elevated p16INK4a expression, and are also positive for SA β-galactosidase staining. However, when p53 was deleted in a PTEN-negative background, tumor growth was enhanced and tumors were more invasive [13].

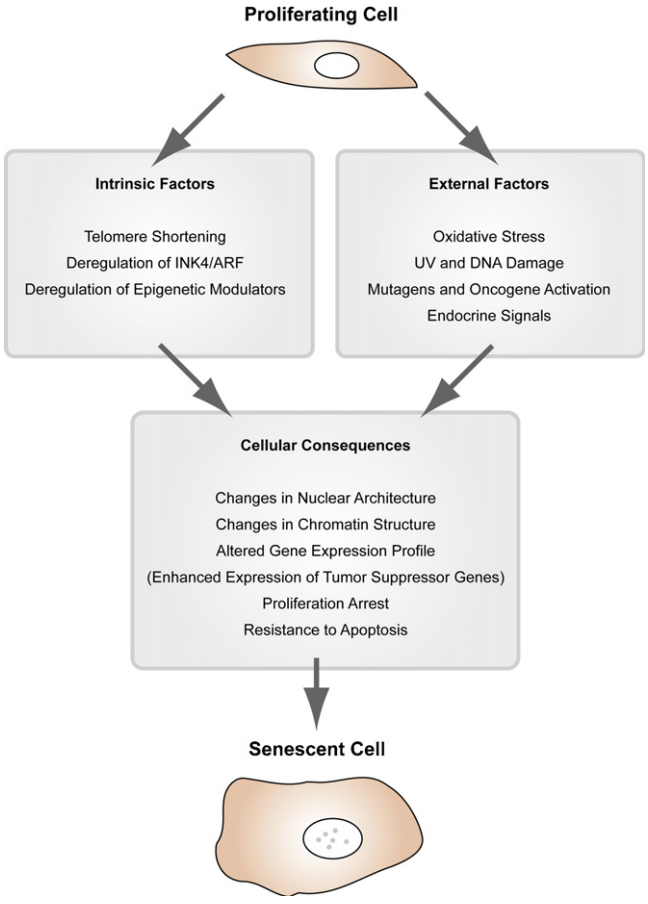

Fig. 1. Scheme of intrinsic and external factors leading to cellular senescence, including the cellular characteristics of a senescent cell.

Most of the signaling pathways in senescence converge on the activation of the tumor suppressors p53 and pRB. Transcriptional regulation of the INK4A-ARF locus plays the pivotal role here at the regulatory level, as it encodes the two unrelated tumor suppressors p16INK4a and p19ARF, both of which can induce cell cycle arrest [14]. p19ARF for instance interacts with Mdm2, the p53-associated E3 ubiquitin-ligase. Ubiquitination of p53 ends in the proteosomal degradation of p53. However, when Mdm2 is bound by p19ARF, it itself is degraded, which in turn leads to an accumulation of p53, followed by growth arrest of the cells [15].

At the transcriptional level, all of the above mentioned senescence pathways are modulated by the chromatin state. This applies equally for the DNA damage pathway as for the transcriptional control of tumor suppressors, such as that of the INK4-ARF locus. Chromatin is the functional entity of DNA made up of nucleosomes, which consist of 146 bp of DNA wrapped around histone octamers. Histone octamers are composed of an (H3/H4)2 heterotetramer and two H2A/H2B heterodimers. Chromatin is subjected to a series of highly dynamic posttranslational modifications (PTMs), such as DNA methylation at cytosine, and methylation, phosphorylation or acetylation of the histone proteins, that regulate its packaging density as well as the recruitment of further chromatin modifying enzymes. This allows the execution of a large variety of chromatin-associated processes, like regulation of gene transcription, establishment of heterochromatic transcriptional silent regions, and DNA repair. Therefore, alterations in the modification state of chromatin can lead to cancer development by affecting chromosome stability and gene expression [16]. Several key events have been reported in senescence that rely on chromatin regulation, such as telomere shortening [17], senescence-associated heterochromatic foci (SAHF) [3], and a general decline in DNA methylation [18]. Better understanding of senescence pathways will therefore be crucial to unravel their underlying mechanisms as well as the role that alterations in chromatin structure play in aging and cancer.

1.1. The INK4-ARF locus

The INK4-ARF locus is a critical regulator of senescence. Proteins encoded by the INK4-ARF locus accumulate during senescence induction and drive the cells to growth arrest. Both replicative and oncogenic stresses activate the INK4-ARF locus and lead to cellular senescence [12].

Three gene products are encoded within the INK4-ARF locus. The p16INK4a and p15INK4b proteins are cyclin-dependent kinase inhibitors (CDKi) of cyclin/cdk4 (or cdk6) complexes that prevent

pRB phosphorylation and therefore regulate cell cycle progression. In contrast, p19ARF prevents p53 degradation and ensures its stabilization. Consequently, p53 activation can lead to apoptosis or cell cycle arrest. p21CIP1/WAF1 expression is induced by p53, and this pathway is important to establish and maintain growth arrest during senescence. p16INK4a and p15INK4b are well-established senescence markers both in vitro and in vivo, since their expression has been detected in several pre-malignant lesions [19].

The INK4-ARF locus is often mutated or deleted in primary tumors, indicating its important role in counteracting tumorigenesis [20]. The biological contribution of the locus to growth control has been assessed through several mouse models. For instance, mice that had lost ARF expression escaped senescence and were prone to transformation by oncogenic RAS [21]. Interestingly, mice deficient for either of the three tumor suppressors developed skin tumors and soft tissue sarcomas, and the range of tumors increased when p15INK4b was lost in mice already lacking p16INK4a or p16INK4a–p19ARF [21]. These results suggest that the three genes work synergistically to counteract tumor development.

In mice, cells respond to neoplastic signals by activating p16INK4a and p19ARF. These genes also appear to be coregulated during aging. In rodents, p19ARF seems to play a predominant role in senescence entry, since in MEFs, the loss of the p19ARF–p53, but not of the p16INK4a–pRB axis, leads to spontaneous escape from senescence [22,23]. However, mouse models deficient for or overexpressing p16INK4a revealed the impact of the p16INK4a–pRB pathway on the self-renewal of hematopoietic stem cells, neural stem cells, and pancreatic islets [23]. In humans, the p16INK4a–pRB axis is prevalently activated, both upon oncogene-induced senescence and during aging [23]. Thus, the INK4-ARF locus plays a pivotal role in promoting aging by limiting proliferation and in self-renewal in several tissues.

1.2. Epigenetic repression of INK4-ARF by Polycomb group proteins

Epigenetic transcriptional regulators of the INK4-ARF locus play a crucial role in senescence, placing chromatin regulation as a critical pathway in senescence. The polycomb group (PcG) proteins are direct regulators of the INK4-ARF locus (Fig. 2). PcG proteins catalyze histone modifications that promote changes in chromatin structure, leading to transcriptional repression. PcG are organized into at least two different complexes, the Polycomb repressive complexes 1 and 2 (PRC1 and PRC2). One of the subunits of PRC2 is EZH2, a histone methyltransferase (HMTase) specific for lysine 27 of histone H3 (H3K27). Consequently, PRC2 specifically trimethylates H3K27 (H3K27me3), a histone modification believed to recruit PRC1 to chromatin. The PRC1 core complex contains a RING finger protein, RING1B, as well as the BMI1, HPH, and CBX proteins. PRC1 orchestrates the mono-ubiquitination of histone H2A at lysine 119 (H2AK119ub). Both H3K27me3 and H2AK119ub are repressive marks and trigger gene silencing through DNA compactation [24].

Overexpression of the PcG proteins BMI1, CBX7, and CBX8 delays the onset of replicative senescence in both human and mouse embryonic fibroblasts, which can be explained through regulation of the INK4-ARF locus by PcGs [25,26]. In proliferating cells, PRC2 restores the levels of H3K27me3, leading to the recruitment of PRC1 to the INK4-ARF locus that is consequently kept repressed. Replicative senescence is characterized by down-regulation of EZH2, removal of the H3K27me3 mark at the INK4-ARF locus, and loss of PRC1 binding. In this condition, the INK4-ARF locus is prone to activation (Fig. 2) [27].

Recent studies show that epigenetic control also occurs through the active de-methylation of H3K27 within the INK4-ARF locus. In oncogene-induced senescence (OIS), the expression of JMJD3, a histone lysine demethylase that catalyzes the de-methylation of di- and tri-methylated HK27, is induced. JMJD3 is recruited to the INK4-ARF locus and, once there, removes the H3K27me3 repressive mark; the locus can then be activated upon cellular stress [28]. Down regulation of JMJD3 is associated with decreased expression of the INK4-ARF locus and immortalization in MEFs. Interestingly, JMJD3 is down regulated in several types of cancer, reinforcing the idea that it is important in the regulation of the INK4-ARF locus [28].

1.3. Trithorax proteins activate the INK4-ARF locus

The SWI/SNF complex provides another layer of epigenetic regulation at the INK4-ARF locus, which is required for p16INK4a activation in malignant rhabdoid tumors (MRT). SWI/SNF is a chromatin remodeling complex that plays an important role in gene expression control. Chromatin remodeling complexes use ATP to reposition nucleosomes and remodel chromatin. In MRT, it has been reported that the SWI/SNF complex lacks the SNF5

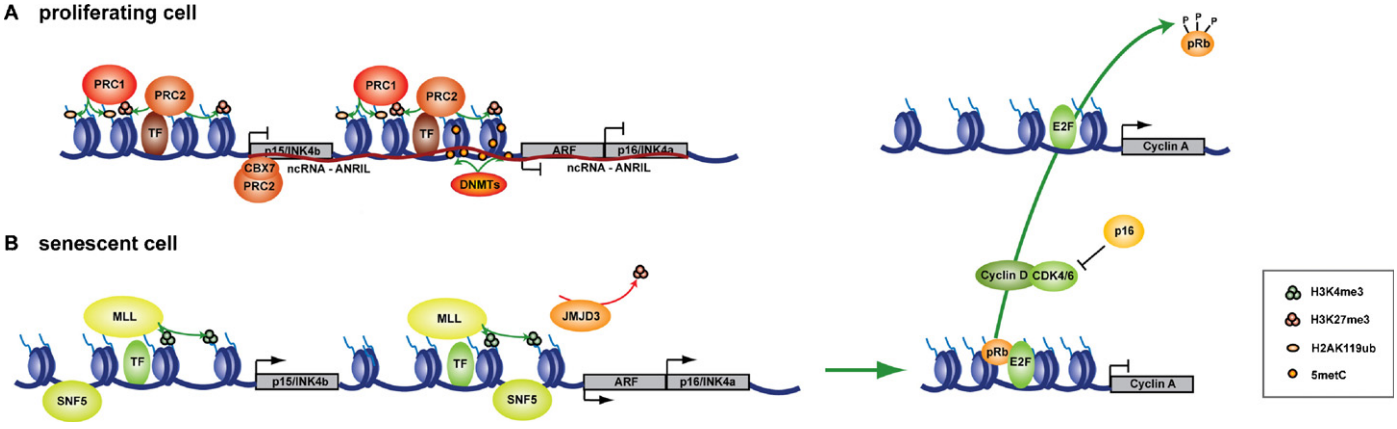

Fig. 2. Schematic representation of the INK4-ARF locus, as well as of the E2F target gene Cyclin A. (A) In a proliferating cell the repression of the INK4-ARF locus is controlled by a crosstalk of several chromatin modulators. Generally, PRC2 methylates H3K27 within the regulatory regions of the INK4-ARF locus. The H3K27me3 mark is recognized by PRC1, finally leading to H2AK119ub and the establishment of repressed heterochromatin. In addition, CpG islands within the p16INK4a promoter are hyper-methylated and most likely the whole locus is under the control of a long non-coding RNA (ncRNA-ANRIL), which contributes to enhanced recruitment of PRC2 by directly interacting with CBX7. E2F target genes, like Cyclin A, are required to ensure cell cycle progression and are therefore activated. Typically, they are under the regulation of E2F transcription factors that in this situation do not interact with pRB as it is hyper-phosphorylated by the action of Cyclin D/CDK4/6 complexes. (B) In a senescent cell the INK4-ARF locus is activated. Repressive heterochromatic marks are removed, e.g. JMJD3 demethylates H3K27me3, while active marks are placed. MLL complexes methylate H3K4 and SWI/SNF complexes (including the SNF5 subunit) contribute in remodeling and opening the chromatin within the INK4-ARF locus. The gene products, CDK inhibitors, inhibit the action of Cyclin/CDK complexes, as is depicted for p16INK4a. As a consequence pRB stays hypo-methylated and bound to E2F. Target genes, like Cyclin A, are therefore repressed.

subunit and that the tumor suppressor p16INK4a is therefore silenced [29]. It was shown that reintroduction of SNF5 in MRT, in addition to displacing PcG proteins, leads to the displacement of DNA methyltransferases (DNMTs) and histone deacetylases (HDACs) from the p16INK4a locus; this allows further acetylation of H4K16 (H4K16ac), a histone mark associated with transcriptional activation [29].

A further epigenetic regulation is presented by the mixed-lineage leukemia (MLL1) HMTase, which methylates histone H3 at lysine 4 (H3K4me3) and thus guides the epigenetic activation of the INK4-ARF locus (Fig. 2) [29]. MLL and SWI/SNF belong to the trithorax group proteins (TrxG), which have been characterized to have an opposing force to PcG-mediated gene silencing. Mechanistically, both complexes function by driving histone methylation at specific lysine residues, which then recruit additional proteins responsible in turn for other types of histone modifications [29,30].

1.4. Other epigenetic regulators of the INK4-ARF

Modulation of histone acetylation marks contributes to the senescence phenotype, as exemplified by the observation that HDAC interference induced senescence in human fibroblasts [31]. It was additionally shown that HDAC levels decreased upon senescence in human fibroblasts, which could trigger p21CIP1/WAF1 expression that would consequently lead to growth arrest [31]. Another study in human diploid fibroblasts demonstrated that HDAC1 and HDAC2 are targeted to the p16INK4a promoter, and that this leads to a delay in the onset of senescence [32]. A further histone mark that is implicated in replicative senescence is trimethylation of lysine 20 on histone H4 (H4K20me3): this mark has been associated with aging in liver and kidney of rats, and has been shown to be downregulated in cancer [33]. Recently, the trimethylation of lysine 36 on histone H3 (H3K36me3) has also appeared in the senescence map, through the work characterizing the H3K36 demethylase Jhdm1b and its importance in regulating the INK4-ARF locus: p15INK4b is specifically repressed by Jhdm1b, and this repression promotes cellular proliferation and inhibits cellular senescence [34].

Noncoding RNAs (ncRNAs), which are transcripts that are not translated into proteins, have also been implicated in the regulation of the INK4-ARF locus [35]. Functionally, ncRNAs have been shown to be recruiters of histone modifiers and therefore actively participate in transcription regulation. An example of a ncRNA that is directly involved in epigenetic transcriptional repression is ANRIL, a long, noncoding RNA transcript that is antisense to and overlaps with the INK4-ARF locus that has been found to recruit CBX7 to the p16aINK4-ARF locus (Fig. 2). Compromising the binding of ANRIL to CBX7 affects its ability to represses the INK4-ARF locus. By mediating CBX7 repression of the p16INK4a-ARF locus, ANRIL also regulates senescence. Moreover ANRIL has also been shown to recruit PRC2 to the p15INK4b gene mediating its repression [36]. Further, in human fibroblasts, expression of ANRIL decreases upon replicative senescence [35].

1.5. Regulation of the INK4-ARF locus by DNA methylation

Another epigenetic modification that changes characteristically during the aging process is the DNA methylation. DNA methylation of chromatin involves the addition of a methyl group on a cytosine on position 5 within the pyrimidine ring and can be synthesized by 2 different isoforms of DNMTs. DNMT1, the so-called maintenance DNMT, is responsible for copying DNA methylation from the parental strand to the daughter strand after DNA replication, while DNMT3a and DNMT3b catalyze de novo DNA methylation.

During aging of mice an overall general loss of the level of methylated cytosine is observed, and this is hypothesized to lead to gene re-expression [18]. The decline in the methylation level takes place specifically at interspersed repetitive sequences (IRS) [37]. Nevertheless, there are also specific DNA sequences for which, rather than hypomethylation, a gain of methylated cytosine is observed. This is the case for CpG islands, which are regions of high C and G content mainly found within or in close proximity to gene promoters. Those sequences are unmethylated in active genes, and their methylation at cytosine provokes gene silencing [16].

Interestingly, in various cancer cells DNMTs are overexpressed, which leads to aberrant hypermethylation. As a consequence especially the expression of tumor suppressor genes has been shown to be frequently impaired by methylation of the CpG islands within promoter regions [38–41]. For instance, the CpG islands of pRB are methylated in sporadic unilateral retinoblastoma tumors, an event that is suggested to regulate tumor onset [42]. Epigenetic silencing of p16INK4a in several types of human cancer is a consequence of promoter hypermethylation. There are also reports mentioning silencing of p15INK4b by promoter hypermethylation in glial tumor and leukemia [43]. In various cancer cell lines the promoters of the CDK inhibitors p16INK4a and p21CIP1/WAF1 are hypermethylated and therefore repressed, suggesting that DNA methylation may also be involved in cellular senescence [38,44,45]. Only recently it was shown that DNMTs indeed play a role in controlling stem cell aging [46]. Inhibition of DNMTs by 5-azacytidine (5-AzaC) or siRNA in human umbilical cord blood-derived multipotent stem cells induced p16INK4a and p21CIP1/WAF1 expression and concomitant cellular senescence [46]. Interfering for DNA methylation goes along with changes on histone modification patterns, suggesting a crosstalk between DNA methylation and histone modifications. For instance it was shown that SUZ12, a member of the PRC2 complex, specifically binds to methylated CpGs [47]. In addition, in the study of the role of DNMTs within stem cell aging it was shown that PcG proteins are specifically targeted and repressed by microRNAs (miRNAs) that are upregulated upon inhibition of DNMTs, thereby enabling the activation of p16INK4a and p21CIP1/WAF1 genes [46].

1.6. Senescence-associated heterochromatic foci (SAHFs)

In contrast to quiescent cells, senescent cells undergo an irreversible cell cycle arrest. One major reason is that senescent cells permanently shut down the expression of E2F target genes required for proliferation (such as cyclin A and PCNA), even in a promitogenic environment [48,49]. It has been suggested that changes in chromatin architecture can contribute to the cell fate and can help to determine quiescent and senescent cells [3,50].

One characteristic of senescent cells is the formation of SAHFs. Human primary fibroblasts that were forced into senescence by serial passaging or oncogenic stress developed heterochromatic foci that are absent in quiescent cells [3]. These foci are intensively stained by DAPI and are resistant to digestion by nucleases (Fig. 3). In addition, they are enriched in constitutive heterochromatin markers, as chromatin hypoacetylation, H3K9me3, and associated Heterochromatin protein 1γ (HP1γ). By RNA fluorescence in situ hybridization (RNA FISH), it was shown that active transcription sites are absent within SAHFs [51]. Importantly, SAHF formation is dependent on the pRB tumor suppressor pathway, and p16INK4a has been shown to have a causative role in SAHF formation. While oncogene-induced senescence always goes along with induced p16INK4a levels, only some replicative senescent human fibroblast strains show increased p16INK4a expression. Strikingly, in some strains that enter into senescence triggered by telomere shorting, p16INK4a levels are low, and SAHF formation is impaired [3,52]. In fact, it was recently shown that SAHF formation is not a common feature of cellular senescence. Kosar et al. observed SAHF formation in diverse cell types under oncogenic stress, yet found

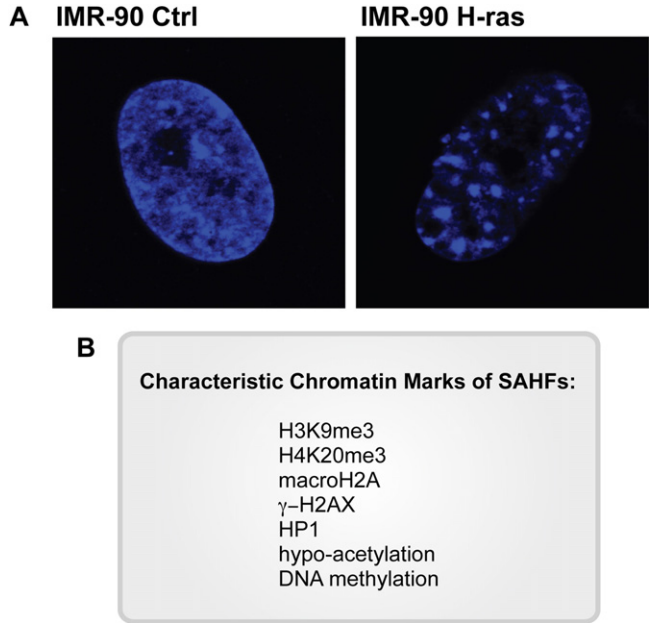

Fig. 3. Senescence associated heterochromatin foci (SAHFs). (A) Pictures of a non-senescent and a senescent human fibroblast (IMR-90). While the non-senescent fibroblast (infected with a control plasmid) shows disperse DAPI-stained DNA, which points to a more loose and open chromatin structure, the oncogene induced senescent cell (infected with a plasmid to overexpress the oncogene H-ras) shows classical heavily DAPI-stained SAHF structures. (B) List of characteristic chromatin marks of SAHFs: SAHFs are enriched in methylated H3K9 and H4K20, macroH2A, phosphorylated H2AX, HP1 and DNA methylation, while they lack histone acetylation.

that SAHF is cell-type restricted with genotoxin-induced and replicative senescence [53]. Senescence signaling pathways and their consequences can be very different between mice and humans, which is also illustrated by the aspect of SAHF formation: MEFs and mouse skin fibroblasts do not form the heterochromatic foci observed in human cells upon senescence stimuli [54].

In senescent cells, E2F target genes are permanently silenced by marking them with H3K9me3 and recruiting HP1γ to them. In contrast, in cells that exit the cell cycle into a reversible quiescent state, E2F target gene expression is under control of the competing action of histone acetyltransferases (HATs) and HDACs. Interestingly, it was also shown that pRB family protein members assume distinct roles when cells exit the cell cycle. p107 and p130 are the predominant pRB members bound to E2F responsive promoters in quiescent cells [55,56], while pRB protein is recruited in senescent cells [3]. pRB can also interact with HP1 and HMTases, such as SUV39H1, leading to the hypothesis that pRB could be actively involved in stably and irreversibly silencing E2F target genes in senescent cells [3]. It was also suggested that pRB might control the nucleation of heterochromatin at specific sites throughout the genome, and that this then spreads by the action of HMTases and HP1, leading to SAHF formation [57].

Importantly, each SAHF corresponds to one chromosome territory (CT), and it was shown that not only E2F target genes but the whole chromosome becomes condensed, similar to the inactive X chromosomes in female mammals [58,59]. By chromosome painting and in situ RNA FISH, it was determined that telomeric and centromeric chromatin is located predominantly at the periphery of SAHFs. In addition, when probed for the E2F target cyclin A2, which gives a diffused RNA staining on chromosome 4 in growing cells, no staining was detected in senescent cells, suggesting that the cyclin A2 gene lies within the repressive and non-hybridizing interior of SAHFs.

SAHFs are enriched for the histone H2A variant macroH2A [51]. Time course experiments revealed that macroH2A was incorporated after the appearance of DAPI staining, in both replicative-senescent and oncogene-induced-senescent human fibroblasts. The knockdown of macroH2A resulted in a reduction of the number of SAHFs, suggesting that macroH2A plays an important role in maintaining and stabilizing SAHFs and is not just a consequence of induced heterochromatin formation [51].

Interestingly, SAHF-positive senescent cells lose the linker histone H1 and exhibit increased levels of high-mobility group protein A proteins (HMGAs) [59,60]. HMGA1 and HMGA2 are abundant, non-histone chromatin proteins that compete with histone H1 for binding to the minor groove of AT-rich DNA sequences (linker DNA) [61,62]. HMGAs are associated with transcriptionally active chromatin, can promote tumorigenesis, and are overexpressed in some human cancers. On the other hand, histone H1 is required for higher-order chromatin structures; it is therefore believed to facilitate chromatin condensation and to act as a transcriptional repressor [63]. Therefore, it was unexpected to find a specific accumulation of HMGA1 and HMGA2, and loss of histone H1, within SAHFs in senescent human fibroblasts [52,59].

SAHF formation is driven by the histone chaperones ASF1a (but not ASF1b) and HIRA. Overexpression of either chaperones in human fibroblasts leads to senescence, with increased numbers of SAHFs; this is even more pronounced when they are simultaneously overexpressed, while knockdown of either showed a dramatic decrease of SAHFs [51]. This finding was also rather surprising since HIRA specifically directs the incorporation of the histone variant H3.3 into chromatin, independently of replication [64]. H3.3 differs from the canonical histone variant histone H3.1 only in 5 amino acids, yet they are believed to have very distinct biological roles. While H3.1 is expressed periodically in the S phase of the cell cycle and is incorporated into chromatin by a replication-coupled assembly, H3.3 is expressed throughout the cell cycle and is incorporated into chromatin by the HIRA/ASF1a complex independently of replication or DNA repair [65]. In contrast, the yeast orthologs of Asf1a and HIRA (Asf1p, Hir1p, and Hir2p) are involved in heterochromatin formation and silencing of telomers, pericentromers, and mating loci [66–69]. In the context of senescence, however, ASF1a/HIRA activity promotes deposition of macroH2A, even though neither ASF1a nor HIRA interact directly with this histone variant. As ASF1a was shown to have nucleosome disassembly activity [70], it was suggested that ASF1a could contribute to macroH2A incorporation by disassembling chromatin, prior to the insertion of macroH2A by other factors [51]. However, the responsible macroH2A chaperone has not yet been identified.

Results based on kinetic studies and monitoring the formation of SAHFs, and the involvement of chromatin modifiers (described above), suggest a multistep process for SAHF formation. Zhang et al., present a model for the SAHF formation, and the spatial and temporal requirements of several regulators in senescent human cells [58]. The first step at the onset of senescence is the transient recruitment of HP1 proteins to PML bodies prior to the incorporation of HP1 into SAHFs. A similar localization tendency was observed for HIRA [51,71]. At this time point, chromatin is still decondensed. Why HP1 and HIRA are localization within PML bodies is not known. However, PML bodies were also described to be implicated in the induction of senescence. PML bodies are built by PML and other proteins and, upon onset of senescence, become larger and more numerous [72–74]. PML bodies possess tumor suppressor functions, and disruption of these structures promotes cell transformation [75]. PML bodies have been suggested to be sites of macromolecular complex assembly and protein modifications [74,76,77]. Importantly, HP1γ is phosphorylated prior to SAHF incorporation, and it was suggested that this might be occur within the PML body [58].

In the next step, individual chromosomes condense to form single SAHFs by the actions of the HIRA/ASF1a chaperone complex. Zhang et al. suggest that chromosome condensation, as well as gene silencing, might be the consequence of increased nucleosome density. Although HIRA specifically deposits H3.3, a histone variant associated with transcriptionally active chromatin, they suggest that H3.3 might be associated with any major remodeling of chromatin, perhaps as a way to "reset" pre-existing histone modifications. Whether senescent heterochromatic structures are indeed enriched in H3.3 has still to be shown. However, at this stage of SAHF formation, HMGAs are found associated with chromatin [52].

Finally, upon chromosome condensation by HIRA/ASF1a, an accumulation of H3K9me3, HP1, and macroH2A occurs. HP1 and macroH2A are most likely recruited in parallel but independently of each other. It was suggested that these characteristic histone modifications and associated proteins are not required to drive chromosome condensation but rather to maintain and stabilize this structure.

Although the model initially suggested by Zhang et al. provides an initial understanding of SAHF formation, plenty of questions still need to be addressed. For instance, which stimuli trigger HIRA recruitment to the PML bodies prior to SAHF formation are not known. Also, the responsible enzymes/factors for HP1 phosphorylation and H3K9me3 and macroH2A deposition have not yet been identified so far.

1.7. Chromatin structure of telomeres

Telomeres are heterochromatic domains composed of TTAGGG repeats that are generated by reverse transcription of the telomerase [78]. They are responsible for protecting the ends of eukaryotic chromosomes and for preventing them from being recognized as DNA breaks. The length of telomeres, as well as the integrity of their binding proteins, is fundamental for their protective function. In addition, telomere- and subtelomere-structures are regulated by a number of epigenetic modifications and by the association of chromatin modifiers and readers [79]. Multicellular eukaryotes have a limited amount of telomerase and, with each cell division, telomeres become shorter. When telomeres reach a critical short length, the DNA damage pathway is activated, leading to replicative senescence. Telomere shortening and concomitant senescence is observed with increased aging in various human tissues [17], as well as in normal cells in culture [80].

It is evident that epigenetic modifications play a fundamental role in the regulation of telomere and subtelomere-structures. Similar to pericentromeres, subtelomeres are gene-poor, and the few genes present are silenced through an effect known as "telomere position effect" (TPE) [81,82].

In general, mammalian telomeres are enriched in classic heterochromatin marks, such as hypoacetylation, H3K9me3, and H4K20me3, and are highly bound by HP1 proteins. As telomeres become shorter after several cell divisions, telomeres are devoid of

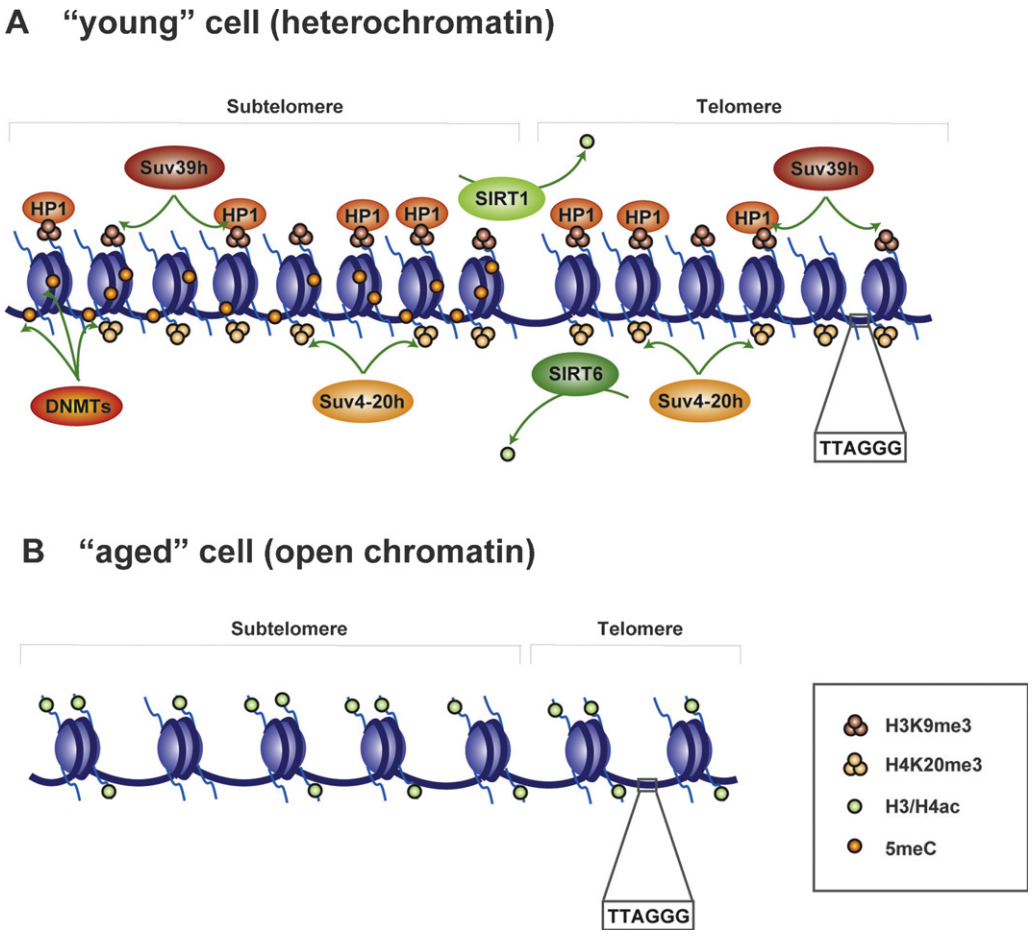

Fig. 4. Illustration of telomeres and subtelomeres. (A) Within a "young" non-senescent cell telomeres and subtelomeres are highly heterochromatized. As a consequence, chromatin is enriched in methylated H3K9 and H4K20, HP1 binding as well as DNA methylation (only within subtelomere structures). Sirtuins, especially SIRT1 and SIRT6, play an important role in maintaining the chromatin hypo-acetylated. (B) In contrast, telomeres and subtelomeres become shorter in "aging" senescent cells. In addition, repressive heterochromatic marks are lost and chromatin becomes looser due to hyperacetylation.

repressive heterochromatin marks but are enriched in active marks, predominantly histone acetylation (Fig. 4). Mice with a double knockout for the HMTases Suv39h1 and Suv39h2, which are specific for H3K9 methylation, displayed abnormally long telomeres that were devoid of H3K9me3 and H3K9me2 marks and HP1 binding [83]. Similar effects were observed in cells following interference for Suv4-20h1 and/or Suv4-20h2, the HMTases that target H4K20: deficient cells showed a dramatic loss of H4K20me3 that was accompanied by telomere elongation [84]. Suv4-20 HMTases are recruited to telomeres by a direct interaction of pRB family proteins. Cells deficient for all three pRB proteins show a general genomic instability that is accompanied with decreased DNA methylation, hyperacetylation, and decreased H4K20me3. Chromatin immunoprecipitation (ChIP) experiments have shown that the loss in H4K20me3 happens particularly at pericentromeric and telomeric regions [85].

In addition to the classic heterochromatin marks H3K9me3 and H4K20me3, DNA methylation is also responsible for correct telomere structures. Even though the telomere repeats (TTAGGG) lack the canonical CpG methylation site, sub-telomeric DNA is heavily methylated [86]. Blasco and coworkers used mouse embryonic stem (ES) cells that are deficient for either DNMT1 or DNMT3a and DNMT3b [87]. In comparison to wild-type ES cells, DNA methylation was strongly reduced in the DNMT-deficient cells, while no changes in H3K9 and H4K20 methylation levels were observed. Concomitant with the loss in DNA-methylation, telomeres were elongated and more prone to telomeric recombination [87]. Finally, telomeres and sub-telomeres are also characterized by low levels of acetylated histone H3 and histone H4, which can be disrupted by the histone deacetylase inhibitor (HDACI) Trichostatin A (TSA), indicating that the equilibrium of HDACs and HATs at telomeres generally is shifted in favor of HDACs [81,82].

The histone H3 lysine 9 (H3K9) deacetylase SIRT6 in particular plays an important role in replicative senescence [88]. Importantly, SIRT6 is a member of the evolutionary conserved sirtuin family of NAD+-dependent deacetylases and is not sensitive to HDACIs such as TSA. Mice deficient for SIRT6 have a shortened lifespan and show a pre-mature aging phenotype [89]. It has been shown that SIRT6 in human fibroblasts specifically associates with telomeres and is required to modulate telomeric chromatin that leads to abrogation of pre-mature senescence [90]. Cells depleted for SIRT6 exhibit abnormal telomere structures that resemble defects observed in Werner syndrome, an autosomal recessive accelerated aging syndrom. It was shown that SIRT6 deacetylates H3K9 at telomeres, which enables its association with WRN, a RecQ class of helicase known as the factor mutated in Werner syndrome [90].

Another member of the sirtuin family, SIRT1, was also recently reported to contribute to telomere maintenance. Using SIRT1 deficient and overexpressing mice, Palacios et al. showed that SIRT1 associated with telomeric repeats and had a positive effect on telomere length [91].

1.8. Nuclear architecture – the role of lamins in aging

It is becoming more and more evident that nuclear architecture, and therefore the arrangement of chromatin within the nucleus, can influence the epigenetic status and thus the cell fate. For instance, during cell cycle or terminal differentiation, the nuclear architecture undergoes dramatic changes [92,93]. Chromosomes are organized into chromosome territories (CTs), which are arranged in a non-random fashion within the nucleus. It has been shown that gene-rich chromosomes reside preferentially deep inside the nucleus, while gene-poor chromosomes are located close the nuclear membrane or lamina [94]. Areas of active gene transcription within the CTs are found in a decondensed chromatin state located at the periphery of CTs. In contrast, transcriptionally silent and condensed chromatin is located in the interior of CTs.

Recent models suggest that lamins and lamin-associated proteins are responsible for chromatin positioning within the nucleus [95]. This is achieved by direct interactions between the C-terminal domain of lamins and the N- and C-terminal domains of core histones [96]. The lamin A gene, LMNA, is prone to numerous mutations, leading to severe diseases known as laminopathies [97]. The Hutchinson–Gilford progeria syndrome (HGPS) and Werner syndrome are premature aging diseases that are used as models to study human aging. HGPS is caused by a single nucleotide substitution within the LMNA gene, which creates a cryptic splice site and therefore a truncated Lamin A protein lacking its C-terminal domain [98,99]. This leads to a number of abnormalities, including loss of peripheral heterochromatin and severe changes in the epigenetic organization of chromatin. For instance, a global decrease was observed for H3K9me3 and its associated HP1 proteins, and of H3K27me3 was observed, while an increase of H4K20me3 was observed [100–102]. It was suggested that these changes in chromatin architecture are due to the expression of the truncated lamin A protein, which contributes to the phenotypes of laminopathies. Importantly, similar changes in chromatin organization, and in the expression of truncated lamin A protein, are also observed in healthy but old humans [103].

The molecular mechanisms linking lamin A to heterochromatin organization are still not fully understood. However, two possible scenarios have been discussed. First, mutated lamin A can lead to changes in the organization of CTs and therefore also to changes in actively transcribed areas [104]. Second, pRB proteins directly bind to lamin A, which is fundamental for its regulation [105,106]. pRB influences methylation of histones, including H3K9, H3K27, and H4K20, by interacting and recruiting the responsible HMTases, suggesting that the impairment of the pRB pathway by truncated lamin A could have an effect on heterochromatic histone marks [105,107].

1.9. Future prospects

Several studies emphasize the role of chromatin in the activation of senescence. The possibility to induce senescence in tumors with chemotherapeutic drugs would be important in cases were the apoptotic response is no longer inducible. Understanding the mechanistics of the epigenetic regulation pathways offers the possibility to design novel drugs that could also contribute to inducing a senescence response. Indeed, HDACIs such as suberoylanilide hydroxamic acid (SAHA) are currently being tested in clinical trials as promising anti-cancer drugs. While HDACIs induce a reversible cell cycle arrest, it is tempting to speculate that, by specifically targeting additional chromatin modifiers in parallel, an irreversible exit of cell cycle could be achieved. Nevertheless problems could arise in selecting only a senescence induction based therapy since accumulation of senescent cells could potentially hazard the normal tissue function. This occurs, as senescent cells are known to secrete factors like immune and proliferation regulators as well as remodelers of the extracellular matrix. The so-called secretome could severely affect neighboring cells and even stimulate their malignant progression limiting in this manner the success of the therapy [108,109].

On the other hand premature aging syndromes therapy is still in the beginning. Regarding the Werner syndrome a kinase inhibitor of the p38 MAP kinase (SB203580) has been shown to reduce cellular senescence. One mechanism proposed for p38 MAP kinase senescence induction is the phosphorylation of PcG proteins by one of the targets of p38 MAP kinases: the kinase MAPKAPK3. Phosphorylation of PcG proteins has been previously shown to lead to their displacement from chromatin and derepression of

p16INK4a [110,111]. Further studies should be performed in order to know if the application of the inhibitor SB203580 could indirectly favor the INK4-ARF locus epigenetic silencing and therefore alleviating the impact of senescence in this premature aging syndrome.

Drug design to circumvent deficiencies in the components of the telomerase complex in aging syndromes is a nodal point that will benefit from disease modeling in induced pluripotent stem cells (iPS) derived from patients. A recent study with iPS derived from dyskeratosis congenital patients allowed identifying failure in stem cell self-renewal associated with this disease. iPS-based systems will allow future drug screening and cell replacement therapy for several diseases [112].

In addition to cancer and pre-mature aging syndroms, special attention is being paid to senescence with respect to anti-aging cosmetics. Pharmaceutical research is investing a lot of resources in understanding how low-caloric diets impact senescence, and in developing special anti-aging creams that contain epidermal growth factors, anti-oxidants, and other components that are included to prevent the onset of senescence in skin. However, what we should keep in mind is that aging is a biological consequence. Even though mankind is getting older, in particular due to recent breakthroughs in medicine, we will only be able to postpone aging but not completely bypass it.

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