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Lessons from senescence: chromatin maintenance in non-proliferating cells

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Abstract

Cellular senescence is an irreversible proliferation arrest, thought to contribute to tumour suppression, proper wound healing and, perhaps, tissue and organismal aging. Two classical tumor suppressors, p53 and pRB, control cell cycle arrest associated with senescence. Profound molecular changes occur in cells undergoing senescence. At the level of chromatin, for example, senescence associated heterochromatic foci (SAHF) form in some cell types. Chromatin is inherently dynamic and likely needs to be actively maintained to achieve a stable cell phenotype. In proliferating cells chromatin is maintained in conjunction with DNA replication, but how non-proliferating cells maintain chromatin structure is poorly understood. Some histone variants, such as H3.3 and macroH2A increase as cells undergo senescence, suggesting histone variants and their associated chaperones could be important in chromatin structure maintenance in senescent cells. Here, we discuss options available for senescent cells to maintain chromatin structure and the relative contribution of histone variants and chaperones in this process.

Introduction

Cellular senescence is an irreversible proliferation arrest, thought to mediate tumour suppression, wound healing and, perhaps, contribute to aging [1]. This proliferative arrest is accompanied by profound changes in chromatin structure. Activated oncogenes, shortened telomeres that result from repeated rounds of cell division, oxidative stress, inadequate *in vitro* growth conditions and other cellular stresses trigger cell senescence [1-5].

Maintenance of chromatin structure and its proper regulation are a fundamental cellular requirement. Chromatin consists of DNA, histones and many accessory proteins and RNAs that contribute to its structure and regulation [6]. Proliferating cells maintain chromatin in conjunction with DNA replication during S phase of the cell cycle [7]. However, non-proliferating senescent cells can persist in the body for decades; for example, senescent melanocytes in benign human nevi [8]. In the absence of DNA replication, these cells maintain their senescent phenotype and, presumably, their chromatin structure. This raises the question as to how chromatin dynamics are regulated and chromatin structure is maintained in senescent cells. In this review we discuss how histone chaperones and their substrates might contribute to chromatin structure maintenance in senescent cells. While we focus on non-proliferating senescent cells, the ideas discussed are also relevant to other non-proliferating cells, such as terminally differentiated cells.

Overview of senescence

Triggers of senescence

Since its original description by Leonard Hayflick in early 1960s [9, 10], much has been learnt about senescence. As outlined above, senescence is an irreversible proliferation arrest triggered by a multitude of factors. These triggers can be subdivided into four broad categories: 1, DNA damage and short telomeres which are also recognized as DNA damage and caused by excess rounds of replication of linear chromosomes (the so-called “end replication” problem). Senescence caused by short telomeres is called replicative senescence; 2, oxidative stress; 3, activated oncogenes; 4, other stresses such as inadequate *in vitro* growth conditions. Irrespective of the specific trigger, these triggers share a key physiological output, which is cell proliferation arrest.

Effectors of senescence

pRB and p5—Senescence associated proliferation arrest can be enforced and reinforced by a network of effector pathways. At the heart of this network are the pRB and p53 pathways, which are activated by most triggers of senescence. One of the effectors of the p53 response is p21CIP1 (p21). p21 is a cyclin dependent kinase inhibitor (CDKI), and therefore, inhibitor of cell proliferation. p21 is a direct transcriptional target of p53 and mediates p53-dependent cell cycle arrest [11]. Another CDKI p16INK4a (p16) governs pRB and the senescence-associated growth arrest. Ultimately, through inhibition of cyclin/cdk kinases, p21 and p16 both maintain pRB in a hypophosphorylated [11]. This, in turn, stops cellular proliferation, in part, by inhibiting expression of E2F target genes that are required for progression through the cell cycle. To activate senescence, numerous upstream effector pathways converge on the pRB and p53 tumor suppressors. For example, DNA damage signaling activated by telomere shortening and/or oncogene activation results in activation of various effector proteins, e.g. kinases such as ataxia telangiectasia mutated (ATM) and CHK2, and subsequent stabilization of p53 and hypophosphorylation of pRB [12-16]. Hence two classical tumor suppressors, p53 and pRB, are the major components of effector pathways required for establishment of senescence.

The senescence secretome—Senescent cells secrete a cocktail of growth factors, proteases, and inflammatory cytokines, termed the senescence-associated secretory phenotype (SASP) [2, 17], or senescence-messaging secretome (SMS) [18], or more simply the senescence secretome. The senescence secretome is complex and includes increased IGFBP, PAI-1, TGFβ1 [19-21], increased expression of immune regulators such as IL6, IL6R, IL8, CXCL1, 5 and 7 [22-24] and increased expression of extracellular remodeling proteins such as matrix metalloproteinase (MMP) 1 and 3 [25]. This altered secretory program contributes to and reinforces the senescence-associated proliferation arrest. For example, cytokines such as IL6 and its receptor IL6R are required in a cell-autonomous manner to implement cell-cycle arrest, and depletion of IL6 results in bypass of oncogene induced senescence (OIS) [22]. Similarly, increased expression of the chemokine receptor, CXCR2, and some of its ligands, such as, IL8, help to reinforce the senescence program [23]. Thus senescence-associated secretion helps senescent cells to enforce the senescent phenotype.

Autophagy—Recent studies have implicated autophagy as important effector of the senescence program [26-28]. Autophagy is a conserved lysosomal degradation process, which recycles cytoplasmic proteins and small organelles. Autophagy genes such as ATG1, ATG6/Beclin1 and ATG8/LC3 drive autophagy through sequestration of cytosolic contents and organelles in double membrane vesicles called autophagosomes, which fuse with lysosomes to form autolysosomes. Autophagy has been found to increase in senescence, by

various autophagic markers such as lipidation and cleavage of LC3, upregulation of ATG1-related genes such as ULK1 and 3, and detection of autolysosomes [26, 27]. Senescent cells also express a senescence-associated p-galactosidase (SA β-gal) [29], which partly reflects the increase in lysosomal mass [26, 30]. Other lysosomal proteins such as Cathepsin D are also upregulated in senescent cells [28, 31], suggesting active senescence-associated autophagic processing. Autophagy helps senescent cells to generate raw materials for protein synthesis. Recently it was shown that the mammalian target of rapamycin (mTOR) protein and autolysosomes colocalize in senescent cells, suggesting coupling of protein degradation and biosynthetic pathways. This was shown to be important for generation of components of the secretome, such as IL6 and IL8 [26]. In line with this, previous reports have shown that mTOR inhibition by rapamycin treatment suppresses senescence [32, 33]. The demonstration that autophagy potentiates oncogene-induced senescence *in vitro* [27], and yet rapamycin, a potent activator of autophagy, can suppress replicative senescence [32, 33] underscores the fine control of the level of autophagy required to enact senescence. Together, these results indicate that senescence-associated autophagy coupled to senescence-associated protein synthesis is involved in production of essential metabolites and secreted components, indicative of collaboration of various effector pathways in imposing senescent associated proliferative arrest.

Functions of senescence

Tumor suppression—Senescence *in vivo* is thought to be an important tumor suppression process (Figure 1). Senescence triggered by activated oncogenes, such as RAS and BRAF, is thought to block progression to a transformed cell phenotype. For example, in primary human fibroblasts, primary lymphocytes and melanocytes, an oncogene mutation, such as RASG12V or BRAFV600E, results in senescence associated growth arrest [8, 34, 35]. *In vivo*, senescent cells are known to exist in premalignant neoplasms, including nevi, neurofibromas, prostatic intraepithelial neoplasia (PIN), pancreatic intraepithelial neoplasia (PanIN) and mouse lung adenomas [8, 36-39]. Inactivation of senescence pathways allows progression to cancer in multiple tumors, notably in melanoma, lymphoma, prostate and pancreas [35, 36, 40, 41]. Further highlighting the role of senescence as a tumor suppression mechanism and its therapeutic potential, reactivation of p53 in murine tumors causes cell senescence and associated tumor regression [24, 42].

Programmed cell differentiation—Recently senescence was also shown to have another normal physiological function in maturation of megakaryocytes [43]. As part of the normal differentiation process, mature megakaryocytes enter a senescent state in which cell division stops. Megakaryocytes express increased SA β-gal, p21 and Cathepsin D, indicative of cell senescence. By studying megakaryocytes in culture, the authors were able to determine that thrombopoetin induces a differentiation program that leads to gene activation associated with senescence. Thus, it appears that senescence can be a normal programmed physiological endpoint in differentiated cell lineages (Figure 1).

Wound healin—Another proposed normal physiological function of the senescence program is wound healing, a process which is accompanied by inflammation, new tissue formation and tissue remodelling (Figure 1). The role of senescence in wound healing was first described by Lowe and coworkers [25] in a mouse model of chemical-induced liver damage. Upon liver damage, stellate cells differentiate into myofibroblasts and secrete a similar cocktail of inflammatory mediators as cells undergoing replicative senescence and oncogene induced senescence, including MMPs. MMPs are thought to help in the wound healing response through restraining and reversing the fibrotic component of the wound, and the senescence secretome also triggers clearance of senescent stellate cells by NK cells. In mice deficient in master senescence regulators, senescence of liver stellate cells is impaired,

resulting in excessive liver fibrosis. This implicates senescence in maintenance and repair of normal tissue integrity.

Pathological impact of senescence

On the one hand the senescence secretome is beneficial for the cell and organism. But, senescence can also be harmful to neighboring cells through its effects on the microenvironment (Figure 1). For example, senescent stromal cells can promote malignant transformation of epithelial cells, in part by altering epithelial cell differentiation [44]. Similarly, senescent human fibroblasts can stimulate premalignant and malignant epithelial cells to proliferate in culture and form tumors in mice [45]. Further underscoring the harmful effects of senescence, two recent papers provide direct genetic evidence that inactivation of proteins included in the senescence secretome, such as IL6 and MMP7, results in decreased tumorigenesis [46, 47]. Both groups utilized the KRASG12D mouse model, which develop benign pancreatic intraepithelial neoplasia (PanINs), due to oncogene-induced senescence [37, 39, 48]. Some of these PanIN lesions acquire additional mutations that bypass senescence program and progress to pancreatic ductal adenocarcinoma (PDAC). Specifically, Lesina et al. (2011) found that genetic deletion of IL6 in these mice significantly decreased development of PDACs, highlighting the importance of interleukin signaling in promoting cancerous transformation of PanIN lesions into PDACs. Similarly, Fukuda et al. (2011) found that deletion of MMP7 in the same KRASG12D mice limits tumor size, progression and metastasis, without affecting PanIN formation. This suggests that onset of senescence is not affected by loss of MMP7, but MMP7 can drive tumor progression and metastasis. This argues that senescence signaling can also promote tumorigenesis under certain contexts.

Overview of chromatin

A chromatin language—Chromatin consists of DNA and a large number of proteins that contribute toits proper folding and regulation in the cell nucleus [6]. The basic repeatingunit of chromatin is the nucleosome, approximately 147 base pairs of DNAfolded around a histone octamer comprised of two copies each of histones H2A, H2B, H3 and H4. The DNA between each nucleosome is called linkerDNA and can be bound by H1 histone. The N and C terminal tails of thehistones in nucleosomes are unstructured and protrude from the globularnucleosome itself [49]. These tails are decorated with various posttranslational modifications (PTMs), such as acetylation, phosphorylation, ubiquitination and methylation. These modifications impact function of the underlying DNA. For example, histone acetylation is generally associated with active transcription [6]. In contrast, histone lysine methylation can be associated with either active or repressed chromatin [6]. For example H3K4 methylation is generally associated with active transcription [50, 51]. H3K9 momomethylation has been reported in association with promoters of active genes [52], and di and trimethylation of H3K9 are typically associated with gene repression, constitutive and facultative heterochromatin [52-57]. Similarly, H3K27 trimethylation is associated with gene repression and silenced chromatin [58-61]. Therefore, the sites of lysine methylation and mono-, di- or tri- status govern the activity status of genes.

Histone PTMs frequently mediate interactions between nucleosomes and other regulatory proteins. For example, acetylated lysines serve as a recognition platform for proteins with bromodomains such as, P/CAF (p300/CBP-associated factor) and SWI/SNF chromatin remodeling complexes [62-66]. Such chromatin remodelers remodel chromatin structure by utilizing energy generated from ATP hydrolysis to restructure the nucleosomes, and consequently are called ATP-dependent chromatin remodelers. Similar to recognition of acetylated lysines by bromodomains, methylated lysines can serve as a recognition platform for proteins with chromodomains such as HP1 and CHD chromatin remodeler complexes

[67, 68]. These molecular switches behave in a context dependent way but generally, the acetylation-bromo-remodeler interactions result in gene activation [62, 66, 69, 70], and the methylation-chromo-remodeler interactions result in gene repression [67, 68, 71].

Histone post-translational modifications also dictate access and release of various chromatin-binding proteins, which establish a repressive or permissive state of chromatin. These modifications interact in complex ways to regulate protein binding and the functional output of chromatin. For example, Heterochromatin Protein 1 (HP1) is a transcriptional repressor that directly binds to di or tri methylated lysine 9 residue of histone H3 (H3K9me2/3), a modification that is often associated with transcriptionally silenced heterochromatin [71-73]. However, phosphorylation of neighboring serine 10 results in displacement of HP1 from chromatin, and this helps facilitate chromatin condensation and proper chromosome segregation in mitosis [74, 75]. Similarly H3K27 methylation recruits the repressive polycomb PRC1 complex of proteins [76-78]. Phosphorylation of neighboring S28 results in the displacement of polycomb proteins, leading to gene activation [79]. Such phospho-switches help to change the chromatin status without the requirement for active histone demethylation.

An additional layer of complexity is overlaid by the presence of histone variants and their associated marks in chromatin [80]. In mammals, except for histone H4, all histones have variants. Variant histones differ in amino acid sequence from the canonical histones, and, unlike the canonical histones, are typically encoded as single copy genes in the genome, are expressed throughout the cell cycle and in non-proliferating cells, and give rise to polyadenylated mRNAs [80]. Variant histones are implicated in diverse chromatin regulation events such as gene activation, differentiation, DNA repair and gene silencing. Histones H2A and H3 have the most documented variants [81]. H2A variants include macroH2A, H2AX, H2AZ and H2ABbd. MacroH2A has roles in gene silencing, for example X-chromosome inactivation [82]. H2AX has roles in DNA repair [83]. H2AZ has roles linked to active transcription [84] Variants of canonical H3.1 include H3.2, H3.3, CENP-A and a testis specific variant H3.1t. Histone H3.3 often marks sites of active transcription and is enriched for histone modifications associated with active transcription in flies, plants and mammals [85-88]. More specifically, histone H3.3 is enriched at transcription start sites (TSS) and gene bodies of actively transcribed genes, TSS of genes that are likely poised for activation and some other transcription regulatory sites, likely including promoters and enhancers [89, 90]. At the TSS, nucleosomes containing both H3.3 and histone H2A variant H2A.Z are more labile, hence facilitating access to transcription factors [91].

Regulation of chromatin by histone modifications and variants is invariably context dependent, and, illustrating this, histone H3.3 is also deposited at telomeres and pericentromeres, regions considered to be transcriptionally silenced [89, 92-94]. Histone H3.3 is also incorporated into the × and Y-chromosomes during formation of the transcriptionally silent sex body by meiotic sex chromosome inactivation [95]. Similarly H3.3 is also required for the establishment of heterochromatin in the mouse embryo [96]. This context-dependent combinatorial effect of histone modifications, histone variants and binding proteins culminates in a so-called “chromatin language” [97], which orchestrates a vast array of physiological processes from gene transcription to epigenetic inheritance.

Histone chaperones—One class of molecules important in setting out and maintaining this language are histone chaperones [98]. Histone chaperones facilitate the assembly of nucleosomes from DNA and histones. During S-phase of proliferating cells, histone chaperones, such as Chromatin Assembly Factor 1 (CAF1), contribute to assembly of nucleosomes on newly replicated DNA. While non-proliferating senescent cells do not

undergo S-phase and S-phase coupled chromatin assembly, other processes such as transcription and DNA repair are active in senescent cells and are associated with nucleosome disassembly and reassembly. Presumably, therefore, senescent cells have active DNA replication-independent chromatin assembly pathways.

Histone H3.3 is one histone variant that is incorporated into chromatin predominantly via the replication-independent pathway [99]. Several histone chaperone complexes facilitate deposition of histone H3.3 into chromatin, including the mammalian HIRA/UBN1/CABIN1/ASF1a (HUCA) complex, DAXX/ATRX complex and the DEK complex. Like H3.3 itself (see above), these chaperones are implicated in diverse processes from gene activation to gene repression. For example, HUCA (and orthologous complexes in other species) is implicated in both gene silencing and gene activation [19, 95, 100-105]. DAXX/ATRX deposits H3.3 at pericentromeres and telomeres, regions of chromatin that are generally considered to be heterochromatic [89, 93, 94]. Drosophila DEK (dDEK) facilitates H3.3 assembly during transcription puff formation [106]. Subsequently, it was also shown that DEK is also crucial to global heterochromatin integrity [107]. *In vivo*, loss of DEK in Drosophila leads to a Suppressor of Variegation Su(var) phenotype and global reduction in heterochromatin formation. In sum, several different histone chaperones are known to be involved in deposition of H3.3, and the full extent to which these deposition pathways are functionally distinct or overlapping likely remains to be determined. H3.3 and its chaperones have functions linked to both gene activation and repression. Understanding regulation and function of histone variant H3.3 is likely to be instructive regarding chromatin regulation in non-proliferating senescent cells, because H3.3 is deposited into chromatin in a DNA replication independent manner.

Chromatin and Senescence

Structure and formation of SAHF—Chromatin reorganization plays an important role in the senescence program. In many senescent human cells, this reorganization is visible at the global level when senescent cells are stained with 4′-6-Diamidino-2-phenylindole (DAPI). Proliferating cells exhibit a diffuse distribution of DNA throughout the cell nucleus. However in DAPI-stained senescent cells, punctate DNA foci become visible (Figure 2). These foci have been described as heterochromatic (so-called senescent associated heterochromatin foci (SAHF)), based on the presence of heterochromatic proteins, such as HP1, repressive histone modifications such as H3K9 methylation and generally hypoacetylated histones [108]. Each SAHF focus in a senescent cell is thought to represent an individual chromosome [109, 110]. Significantly, SAHF do not contain pericentromeres and telomeres, pointing to massive heterochromatization of euchromatin in senescent cells [109-112]. Formation of SAHF has been reported by some to be wholly or partly dependent on major effectors of senescence, such as pRB and p53 [111-113] (but see also below). pRB colocalizes with SAHF and its inactivation blocks formation of SAHF [111, 112]. Formation of SAHF is also dependent on the histone chaperone activity of the HUCA histone chaperone complex and so is likely to involve histone variant H3.3 [114]. Further, SAHF-positive senescent cells lose linker histone H1 and exhibit increased levels of chromatin-bound high mobility group A proteins (HMGA) [108, 109], which cooperate with p16 to promote SAHF formation. Therefore, there is large-scale reorganization and heterochromatization during formation of SAHF in senescent cells

Molecular function of chromatin changes in senescence—Inclusion of proliferation-promoting genes, such as cyclin A, into SAHF correlates with silencing of expression of these genes and senescence-associated cell cycle arrest [110, 111]. So, it has been suggested that the heterochromatinization of proliferation genes through SAHF could

directly contribute to senescence-associated silencing of these genes and proliferation arrest (Figure 2).

Recently, an alternate viewpoint emerged for the function of SAHF from Fabrizio d'Adda di Fagagna's lab [115]. These authors concluded that SAHF is formed as a result of persistent DNA damage and helps to dampen the cellular responses to DNA damage. In fact, d'Adda di Fagagna's recent work showed the existence of two types of heterochromatin in senescent cells; one which represses proliferation-promoting genes and another, SAHF, which suppresses the DNA damage response [115]. Inactivation of ATM or p53 was sufficient to release proliferation-promoting genes from heterochromatin-mediated repression and reactivate cell proliferation. Remarkably, these proliferating cells retained SAHF, showing that SAHF are not sufficient to repress proliferation genes and drive proliferation arrest. Previous reports have similarly shown that inactivation of p53 does not completely bypass SAHF formation [111, 112]. Also some studies have shown an shown increase in abundance of heterochromatin proteins and marks in senescence, without formation of SAHF [116]. Hence, local heterochromatinization of proliferation-promoting genes, but not large scale SAHF, appears to mediate silencing of proliferation genes and senescence-associated proliferation arrest. Instead, d'Adda di Fagagna and coworkers went on to show that SAHF serve to restrict senescence-associated DNA damage signaling to sublethal levels, thereby contributing to the prolonged viability of senescent cells (Figure 2).

Another recent report suggests that, rather than suppressing DNA damage signaling, chromatin changes in senescence amplify DNA damage signaling. Karlseder and coworkers showed that levels of all core histones and the linker histone H1 decrease during senescence [117]. This coincides with reduced expression of the stem loop–binding protein SLBP, which is important for stability and expression of histone mRNAs [117, 118]. Karlseder and coworkers proposed that DNA damage signals at short telomeres destabilize SLBP, leading, in turn, to reduced production of H3 and H4. This leads to genome-wide chromatin changes, which amplify the damage signal that triggers senescence. Moreover, chromatin changes at telomeres allow DNA damage and repair proteins to associate with chromosome ends, further elevating the DNA damage signal. Eventually, the DNA damage signal exceeds a threshold and triggers cell cycle arrest. Taken together with the work of d'Adda di Fagagna this work suggest that chromatin finely balances the level of DNA damage signaling in a senescent cell. On the one hand, chromatin changes, particularly at telomere ends, upregulate DNA damage signaling to promote cell cycle arrest. On the other hand, chromatin changes in the form of SAHF suppress DNA damage signaling to prevent cell death. This dual role is consistent with our previous observation that telomeres in senescent cells are excluded from SAHF [110].

Physiological function of chromatin changes in senescence—Since cell senescence is a tumor suppression process, it may be that SAHF and other chromatin changes contribute to tumor suppression (Figure 2). Defining the molecular function of SAHF will shed light on whether or not these structures are likely to contribute to tumor suppression. If SAHF suppress cell proliferation, they might be tumor suppressive. On the other hand, if they suppress DNA damage-induced cell killing in mutated cells, they might actually be oncogenic (Figure 2). Significantly, inactivation of effectors involved in SAHF formation, such as HMGA2, contributes to bypass of senescence [108]. This implies that SAHF are tumor suppressive and inactivation of gene products that are required for SAHF formation, such as HUCA and HMGA2, might contribute to development of human tumors. Interestingly, a member of the HUCA complex, CABIN1, is hypermethylated in ovarian carcinoma [119], and missense mutations have been reported in schwannomatosis, a rare tumor of the tissue covering nerves [120]. Similarly, HIRA was reported to be deleted in 22.7% of meningiomas, which are brain tumors that develop in the meninges, the tissue that

surrounds and protects the brain and spinal cord [121]. Further, other chaperones that deposit H3.3, such as DAXX and ATRX, while not yet implicated in SAHF formation, have been reported to be mutated in 43% of pancreatic neuroendocrine tumors (PanNETs) [122]. Other chromatin remodelling factors, such as BRG1 and SNF5, also mediate tumor suppression, in part by upregulation of p16 and activation of senescence [123-133]. Taken together, it is clear that some chromatin regulators act as tumor suppressors, but whether any do so through formation of SAHF remains to be established.

Chromatin maintenance in non-proliferating senescent cells—Chromatin is a highly dynamic entity in which nucleosomes and associated proteins actively turnover. By controlling accessibility of DNA to chromatin binding proteins, regulated nucleosome turnover controls gene expression states and facilitates processes, such as replication and DNA repair [134]. The dynamic nature of chromatin is highlighted by the fact that nucleosomes at active genes and at epigenetic regulatory elements are replaced multiple times during a cell cycle time of approximately 20 hours [135]. Similarly, regulatory proteins associated with chromatin also show dynamic binding and dissociation. For example even apparently stable heterochromatin domains show transient binding and dynamic exchange of HP1 on chromatin [136]. Moreover, heterochromatin seems to be a dynamic structure which can spread to neighboring sites [137]. Together, these results imply that there is active maintenance of euchromatin and heterochromatin in eukaryotic cells.

In proliferating cells, chromatin is maintained in conjunction with DNA replication. Nucleosomes are transiently disrupted ahead of the replication fork and are rapidly deposited and positioned behind the replication fork. One factor, which actively participates in DNA replication-coupled nucleosomal deposition is CAF1 that is recruited to the DNA by interacting with proliferating cell nuclear antigen (PCNA) [138-141]. Consistent with this, human CAF-1 is found in a complex containing two of the major histones expressed in S phase, H3.1 and H4 [99, 142]. Loss of CAF-1 leads to defects in DNA replication and S-phase progression, suggesting that in human cells, CAF-1 couples chromatin maintenance and assembly to DNA replication [143, 144]. PCNA, in addition to recruiting CAF1, also serves as platform for recruitment of DNMT1 (a maintenance DNA methyltransferase) [145], various chromatin remodelling complexes and histone modifying enzymes such as HDACs [146, 147]. DNMT1 preferentially methylates hemimethylated DNA, suggesting the parental strand serves as template for copying methylation patterns to the daughter strand [145]. Likewise DNMT1 also interacts with EZH2 and helps in establishment of H3K27Me3 repressive chromatin coupled to DNA replication [148]. In sum, the activities of DNMT1, CAF1, PCNA, HDACs and other chromatin remodelers recruited to the replication fork can achieve faithful propagation of DNA methylation and histone PTMs and “replication coupled” maintenance of chromatin.

However, since we have already noted that chromatin is inherently dynamic, even outside of S phase, this raises the question of how chromatin structure is maintained in a non-proliferating cell. For example, in a senescent nevus melanocyte chromatin structure should be maintained for decades, and a failure to do so could result in malignant transformation of such a cell, which already harbors an activated oncogene.

Chromatin maintenance linked to transcription—In non-proliferating cells, processes other than DNA replication disrupt chromatin and provide an opportunity for maintenance and/or remodelling. For example, during transcription, passage of RNA polymerase II results in displacement of histones followed by reassembly. An important factor in facilitating transcription through chromatin is FACT, which removes one copy of the H2A–H2B dimer in a transcription-coupled manner [149, 150]. FACT acts by destabilizing nucleosomes as RNA polymerase II passes along chromatin and restores

nucleosomal structure after the DNA has been transcribed [150]. Although senescent cells are replication deficient, they are transcription competent, metabolically active and secrete lots of inflammatory proteins. Thus, senescent cells have active ongoing transcription, which could provide a window of opportunity for chromatin maintenance (Figure 3).

Chromatin maintenance linked to DNA repair—Another example of chromatin metabolism independent of replication is DNA repair, which involves remodeling of the chromatin via nucleosome exchange and eviction. DNA damage results in rapid recruitment of histone acetyltransferases at the damage loci, which, via acetylation of histones, open up the chromatin for repair factors [151]. Damage loci also show rapid phosphorylation of the histone H2AX variant [83]. After completion of the repair, the chromatin must be restored to its original format. In flies, the histone acetyltransferase dTip60 and the ATPase Domino/p400 catalyze the replacement of phospho-H2Av by unmodified H2Av (the fly ortholog of H2Ax) [152]. Another PTM that needs to be removed is acetylation. The acetylated histones present at the DNA damage site could result in inappropriate gene activation and so should be either removed or deactylated. Indeed, there is decrease in histone acetylation post DNA repair, via recruitment of HDACs at the repaired loci [153]. Thus DNA damage repair provides an opportunity for chromatin restructuring and maintenance independent of DNA replication. Indeed, the DNA damage-signaling pathway is a major effector of the cell senescence program [12-14, 115]. Therefore, replication-independent chromatin assembly coupled to DNA damage repair may be important for chromatin maintenance in senescent cells (Figure 3).

Chromatin maintenance and histone variants—Transcription is known to be coupled to deposition of the histone variant, H3.3 which marks actively transcribed genes [85]. Given H3.3′s diverse role in gene activation and silencing it could be involved in both active transcription and heterochromatin maintenance in senescent and/or non-proliferating cells. Although differentiation is different from senescence in many aspects, both processes share absence of cell division and, perhaps, utilization of histone variants. Many lines of evidence points to H3.3's role in senescent and/or non proliferating differentiated cells. Firstly, mouse erythroleukemia (MEL) cells undergoing differentiation induce expression of both H3.3 genes, H3.3B and H3.3A [154]. This was shown at the mRNA level, but presumably precedes H3.3 deposition. Secondly, histone H3 variant, H3.3 accumulates in fibroblasts approaching senescence and in non-dividing differentiated cells [154-158]. Thirdly, H3.3 also accumulates in quiescent T lymphocytes and the proportion of H3.3 in the chromatin of a cell is related to how long that cell has been quiescent [159]. Fourthly, differentiating and mature rat brain cortical neurons accumulate H3.3 [160]. Consistent with an increase of H3.3 in senescent and differentiated cells, the H3.3 chaperone HUCA pathway is apparently activated in primary senescent cells and *in vivo* in the skin of aging primates [114, 161, 162]. In addition to H3.3, other histone variants are also associated with chromatin of senescent cells. Specifically, histone H2A variant, macroH2A, is enriched in SAHF of senescent human fibroblasts [114].

Consistent with this, levels of macroH2A increase in human diploid fibroblasts (TIG3) undergoing replicative and oncogene induced senescence [163, 164]. Further, differentiating and mature rat brain cortical neurons accumulate histone variants such as H2AX in chromatin, whereas, H3.1, and H3.2 decrease [160]. Taken together this suggests that histone variants and their chaperones, such as HUCA, could provide a molecular framework for chromatin maintenance in non-proliferating and senescent cells (Figure 3). Therefore, incorporation of histone variants, such as H3.3, into chromatin of senescent cells might be important for maintenance of chromatin structure and function and cell phenotype.

A role for PML bodies? Interestingly, HIRA, a member of the HUCA H3.3 chaperone complex, is recruited to PML nuclear bodies in senescent cells. PML bodies are 0.2–1.0-micrometer diameter structures that are implicated in diverse biological processes, such as senescence, the antiviral response, apoptosis and tumor suppression [165]. Why HIRA goes to PML bodies in senescence is not known, but disruption of HIRA's translocation to PML bodies impairs the ability of senescent cells to form SAHF [112]. Specifically, dominant negative HIRA mutants that block HIRA's localization to PML bodies prevent formation of SAHF. Similarly, a PML-RARα fusion protein which disrupts PML bodies also prevents formation of SAHF [112]. Interestingly, UBN1 and CABIN1 also localize to PML bodies in senescent cells [166] (TSR and PDA, unpublished). This implies that the HUCA histone chaperone's localization to PML bodies might be important for remodeling or maintenance of chromatin structure in senescent cells. Further, PML bodies contribute to senescence by recruiting pRB/E2F complexes and suppressing E2F target gene expression [167]. In fact, in senescent cells there may be multiple replication independent nucleosome assembly pathways operating via PML bodies. For example, DAXX/ATRX also co-localize to PML bodies [168, 169], perhaps for replication independent chromatin assembly [106, 169]. Since both HUCA and DAXX/ATRX complex interact with PML nuclear bodies, it's tempting to speculate that several replication-independent histone assembly pathways converge at PML nuclear bodies for chromatin maintenance. Therefore, PML bodies might be important regulatory sites of replication-independent chromatin assembly in senescence (Figure 3).

Concluding remarks

Although much as been learnt about histone variants, chaperones and chromatin remodelers, and their respective roles in chromatin structure and function in proliferating cells, little is known in the context of senescence and/or differentiation and quiescent stem cells. Recent identification of multiple replication-independent histone deposition pathways has opened doors for investigation of chromatin structure maintenance and function in the context of senescence, differentiation and quiescence. Future studies will address the relative contributions of these chaperones and their associated histone variants in senescence-associated replication-independent chromatin maintenance, and whether or not alterations in these factors are drivers of cancers.

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***Highlights**

- Overview of cell senescence
- Overview of chromatin structure
- Implications of senescence for chromatin maintenance
- Model for maintenance of chromatin structure in senescence.

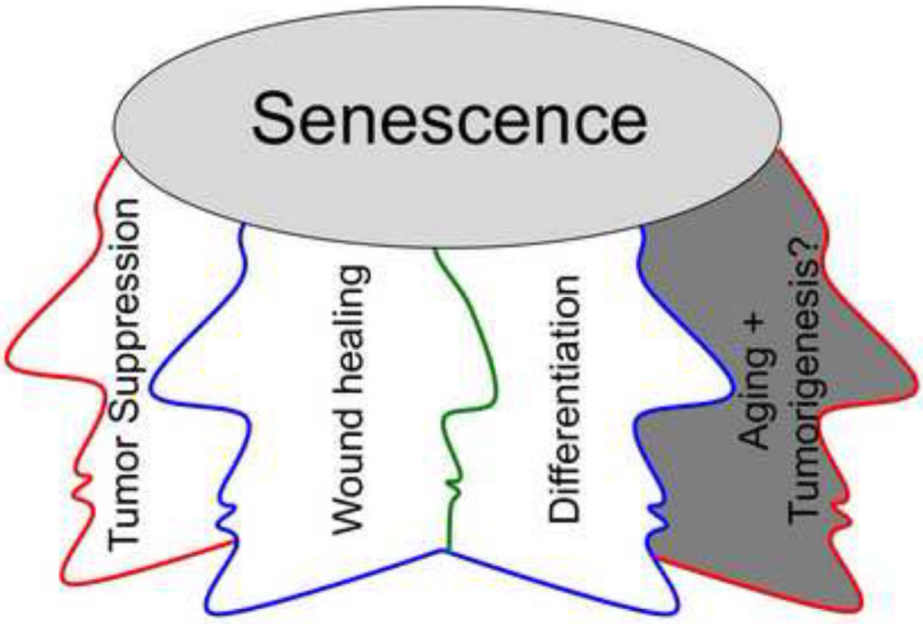

Figure 1. The multifaceted effects of cell senescence

Senescence is a well- established tumor suppression process. Senescence is likely also important for wound healing, and perhaps for generation of terminally differentiated cells in the body, highlighting the importance of senescence in normal physiology. On the darker side, senescence may also contribute to tissue aging. The secretome and senescence-associated inflammation have been proposed to contribute to tumorigenesis.

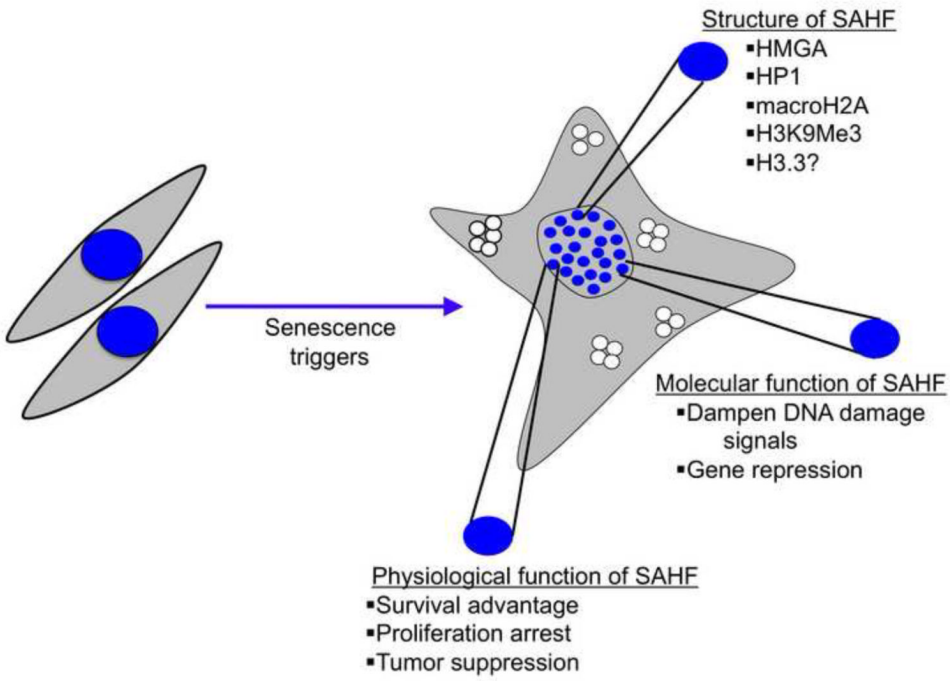

Figure 2. Structure function of SAHF

Normal proliferating cells have diffuse DAPI staining associated with euchromatin. Senescent cells form punctate foci termed SAHF, which are heterochromatic based on markers such as HP1 proteins, H3K9Me3 and hypoacetylated histones. On the one hand, SAHF might contribute to tumor suppression via repression of proliferating promoting genes such as cyclin A. On the other hand, SAHF may dampen the DNA damage response, suppress apoptosis and promote viability of senescent cells.

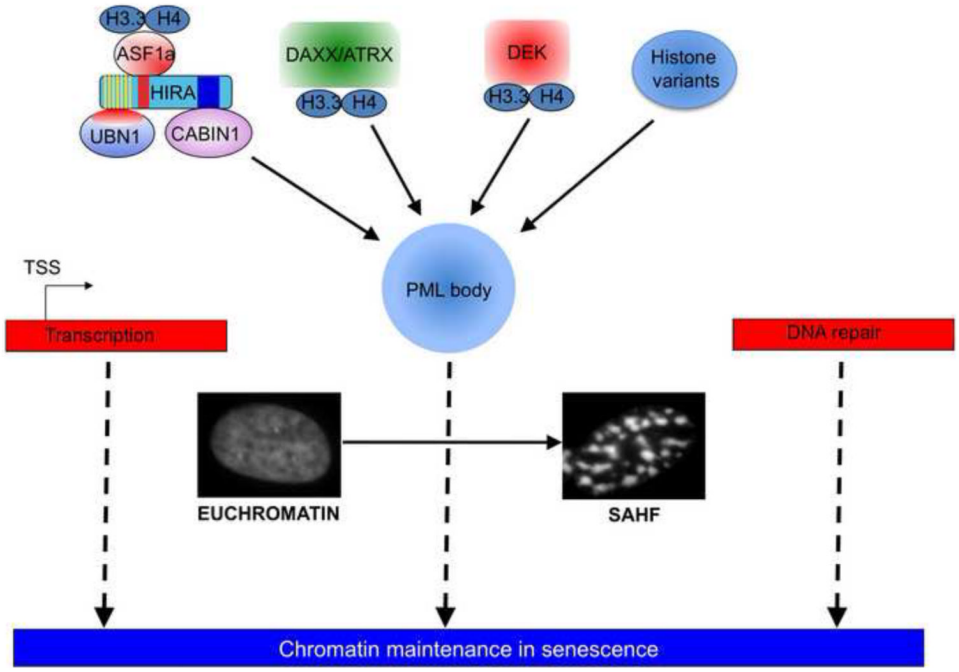

Figure 3. Some options available for chromatin regulation and maintenance in senescence

Multiple histone chaperones and some histone variants, e.g. H3.3, converge on PML bodies and help in formation of SAHF and chromatin maintenance. Other histone variants such as macroH2A also get incorporated into SAHF and may contribute to maintenance of chromatin structure in senescenc Finally e chromatin can also be maintained in senescent cells in conjunction with ongoing transcription and DNA repair, processes that depend on turnover of chromatin.