Title: Beyond SAHF: an integrative view of chromatin compartmentalization during senescence

Authors

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Abstract

Cellular senescence, a persistent form of cell cycle arrest, has been linked to the formation of heterochromatic foci, accompanied by additional concentric epigenetic layers. However, senescence is a highly heterogeneous phenotype, and the formation of these structures is context dependent. Recent developments in the understanding of the high-order chromatin organization have opened new avenues for contextualizing the nuclear and chromatin phenotypes of senescence. Oncogene-induced senescence displays prominent foci and typically exhibits increased chromatin compartmentalization, based on the chromosome conformation assays, marked by increased transcompaction and segregation of the heterochromatin and euchromatin. However, other types of senescence (e.g., replicative senescence) exhibit comparatively lower levels of compartmentalization. Thus, a more integrative view of the global rearrangement of the chromatin architecture that occurs during senescence is emerging, with potential functional implications for the heterogeneity of the senescence phenotype.

Introduction

Cellular senescence is a distinct phenotype universally marked by highly stable cell cycle arrest in response to diverse stimuli (Figure 1). While it was originally described as a stress response, the triggers also include physiological cues, as exemplified by developmental senescence, which, akin to apoptosis, contributes to biological patterning [1–4].

Aside from this common feature of stable cell cycle inhibition, senescence is a heterogeneous phenotype, involving diverse effectors, including the senescence-associated secretory program (SASP), chromatin modulation, altered sensitivity to cell death, and persistent DNA damage signalling (Figure 1). The mosaic nature of senescence markers and effectors is context-dependent, varying depending on the cell type and stimulus' strength and duration [5]. Thus, senescence is a collective phenotype and, depending on its associated sub-phenotypes, the physiological and

pathological relevance of senescence varies in different tissues and organisms. Accounting for this heterogeneity in defining senescence is therefore critical, but remains challenging [6,7]. While the stable exit from the cell cycle is the essential feature which distinguishes senescence from quiescence (a reversible state of cell cycle arrest), senescence is typically characterised through a combination of features, each of which is not necessarily specific to senescence on its own.

The heterogeneity of the senescent phenotype is also evident within the same cell population, with transcriptomic studies at both the bulk and the single-cell level delineating multiple co-existing senescent profiles in human fibroblast models [8,9]. Oncogene-induced senescence (OIS) is marked by two distinct states: primary (oncogenic RAS-driven) and secondary (NOTCH-driven) senescence which is marked by blunted SASP expression [8,10]. Replicative senescence (RS) has also been shown to induce divergent programmes, with some variability arising as a function of time between the early and late phases of senescence induction [11,12]. Interestingly, a more recent study of DNA-damage senescence (DDIS) detected a distinct senescent cluster that is characterised by increased expression of long non-coding RNA and aberrant RNA splicing, suggesting that the proliferative state upon the damage exposure affects the diversity of the senescence phenotype [12].

Senescence plays a key role within the tissue microenvironment through its non-cell-autonomous (i.e., autocrine, paracrine and juxtacrine) aspects. We and others have shown that one of the hallmarks of senescence, the SASP, changes dynamically from senescence induction through to established senescence [8]. Notably, the expression of genes encoding secreted factors and cell surface proteins tend to be tissue-/cell-type-specific and this specificity can be achieved largely through the epigenetic machinery [13]. Thus, senescence may be viewed as a shift in cell identity. As we have recently discussed this concept extensively elsewhere [14], here we will focus on delineating an integrative view of the architectural features of senescence chromatin, stemming from SAHF formation.

Senescence-associated heterochromatic foci

Senescence-associated heterochromatic foci (SAHFs) can be detected as a DNA/chromatin-dense foci, which are enriched for typical heterochromatin marks in senescent human diploid fibroblasts (HDFs), and it can be induced by various triggers [15–17]. The degree to which SAHF form varies considerably depending on the context. Typically, OIS is accompanied by prominent SAHF in HDFs, and senescence induced by telomere shortening (i.e., replicative senescence) or other forms of DNA damage response show less SAHF [18]. At the other end of the spectrum is NOTCH-induced senescence (NIS), exhibiting a 'smoothened' chromatin appearance and suppressed SAHF formation [19]. The propensity of cells to form SAHF also varies depending on the cell type, with most cells exhibiting SAHF in response to oncogene activation, but not to DNA-damage agents or telomere shortening, with BJ fibroblasts failing to form SAHF under these conditions [18]. Other cell types such as microglia, astrocytes and melanocytes may also form SAHF, but additional studies are required to establish this [20,21]. MEFs do not form SAHF upon *Ras* overexpression but they do show increased levels of SAHF components [22]. The wide spectrum of the SAHF propensity reflects the heterogeneous nature of senescence.

SAHF regulators are classified into i) indirect effectors which promote the process, and ii) direct components that are physically associated with SAHF, such as structural proteins and histone marks (Table 1, Figure 2a). Not all these factors are essential for SAHF integrity, at least on their own. p16 and its downstream mediator RB (their activation is a hallmark of senescence) play a critical role in SAHF formation and endogenous levels of p16 expression appear to correlate with SAHF propensity [7,15,23]. It is still unclear how p16-RB signalling modulates SAHF formation, but various factors, such as BRG1/SMARCA4 (a component of SWI/SNF chromatin remodelling complex) [24] and JMJD3 (histone demethylase) [25] appear to modulate senescence and SAHF through interacting with RB. The endogenous level of HMGA1, an essential structural component of SAHF (Table 1), is also critical: in the NIS context, NOTCH signalling represses HMGA1, leading to reduced SAHF formation. Interestingly, inhibition of the basal activity of NOTCH signalling in OIS IMR90 HDFs further promotes SAHF formation [19]. NOTCH can spread the signal across neighbouring cells ('senescence lateral induction') [8], indicating an underlying mechanism of non-cell-autonomous regulation of SAHF formation.

Therefore, the degree of the availability and activity of the SAHF effectors, including both positive and negative regulators, in individual scenarios can contribute to the SAHF heterogeneity.

Table 1: List of SAHF effectors

Name	Study	Effect on SAHF
p16/Rb signalling	Narita et al. 2003 [15]	Indirect; promoting SAHF
H1	Funayama et al. 2006 [26]	Indirect; disruption promoting SAHF
BRG1/SMARCA4	Tu et al. 2013 [24]	Indirect; promoting SAHF
Lamin B1	Sadaie et al. 2013 [27]	Direct: disruption promoting SAHF
HMGA (1 and 2)	Narita et al. 2006 [16], Funayama et al. 2006 [26]	Direct; promoting SAHF
Nuclear pore density and TPR	Boumendil et al. 2019 [28]	Indirect; disruption suppresses SAHF
DNMT1	Sati et al. 2020 [29]	Indirect; disruption suppresses SAHF
NOTCH signalling	Parry et al. 2018 [19]	Indirect; suppresses SAHF
HP1	Narita et al. 2003 [15]	Colocalization
MacroH2A	Zhang et al. 2005 [17]	Colocalization

HIRA/UBN1/CABIN1/ASF1a (HUCA) histone chaperone complex	Zhang et al. 2005 [17] , Banumathy et al. 2009 [30], Rai et al. 2011 [31]	Indirect; promoting SAHF

Architecture of SAHFs

SAHFs are multi-layered structures centred on a condensed heterochromatic core (Figure 2a), enriched in histone H3K9me3 (a constitutive heterochromatin marker) and its reader, HP1 proteins [15,17], surrounded by a facultative heterochromatin mark, a H3K27me3 'shell', and excluding euchromatic histone marks [32]. SAHF also colocalize with other heterochromatin markers, such as macroH2A and the HMGA proteins [16,17]. Notably, while H3K9me3-marked heterochromatin is typically enriched at the perinuclear region, this pattern is diminished during senescence [27], concomitant with reduced nuclear lamina components (Lamin B1 [33]) releasing perinuclear heterochromatin. The increased nuclear pore density during senescence may work in concert with Lamin depletion and impact the radial positioning of heterochromatin [28]. A reduction of Lamin B1 is not sufficient for SAHF, which further requires chromatin-binding components that promote condensation, such as the HMGA proteins [27]. Therefore, classically, SAHF formation has been viewed as a two-step process, heterochromatin mobilisation and condensation.

Classic model: repositioning

In the OIS HDF model, it has been suggested that SAHF formation involves a 'topological' redistribution of predefined chromatin states, rather than newly formed heterochromatin or heterochromatin spreading. This is best reflected by the correlation between SAHF layers and the DNA replication 'history': a pulse-chase labelling of newly synthesised DNA before senescence induction revealed the concentric organisation of genomic DNA, largely according to the pre-senescence replication timing: later timing was linked to more central positioning within SAHF [32].

Principles of chromatin organization

SAHF and its effectors have been largely studied using microscopy techniques. Over the last decade, large scale chromosome capture assays (Hi-C) have mapped chromatin interactions at increasingly high resolution [34], opening new avenues for characterising the chromatin changes behind SAHF formation.

Hi-C experiments have enabled efforts to derive the principles of chromatin organisation, such as the presence of insulating domains formed by hierarchical loop extrusion or the compartmentalization of the genome by preferential interactions within the A and B compartments, representing environments with similar epigenetic features, euchromatin and heterochromatin, respectively [35]. These represent two emerging models of chromatin organisation, with a potential dynamic interplay [36,37], delimiting the large field of potential chromatin interactions. Loop extrusion occurs by entrapment of DNA within a cohesin ring,

constrained by CTCF binding, whereas compartmentalization has been proposed to occur due to phase separation [38], driven by weak interactions between the intrinsically disordered regions (IDR) of certain promoting molecules and has been studied by chromatin conformation assays, polymer modelling, as well as experimental assays targeting nuclear condensates.

The chromatin structure is highly dynamic, and the trends detected at the cell population level by Hi-C studies exhibit substantial variability at the single-cell level. Both loops and the segregation between chromatin compartments are stochastic, with the current view pointing to a probabilistic (rather than a binary) model of gene regulation by the chromatin organisation [39].

Senescence has been associated with *de novo* loop formation which may support the SASP phenotype [40], but limited compartment switching [29,40,41]. Instead, the strength of the chromatin compartments has been shown to be altered during senescence [29]. Increased contacts between distant (on the linear genome) heterochromatin domains, which may correspond to SAHF, were reported in all [29,40–42], but one study [43]. A recent study by Sati et al. [29] comprehensively compares the high-order epigenomic reconfiguration of OIS and RS. They show that OIS exhibits increased compartmentalization (Figure 2b), represented by an increase in B-B interactions (reflecting SAHF formation) and a decrease in A-B interactions. Although RS also shows a contact frequency shift from local to distal contacts, compartmentalization is reduced, marked by a decrease in A-A interactions and an increase in A-B interactions [29]. This study uncovered genomic domains which form SAHF during OIS in a DNMT1-dependent manner (potentially acting upstream of the HMGA proteins) and that, interestingly, the Hi-C profile of DNMT1-depleted OIS is more similar to RS, where SAHFs are not prominent, highlighting that SAHF might confer additional complexity and diversity to the senescence phenotype.

Polymer modelling

3D structure polymer models, validated by a comparison with Hi-C and microscopy assays, were used to study chromatin organisation and yielded additional insights into heterochromatin organisation, as the driving force behind genome compartmentalization. One particularly interesting study focused on deriving a model for compartmentalization, based on observations made from Hi-C and microscopy experiments, that accounted for both the conventional organisation of heterochromatin at the nuclear periphery, as well as for the architecture of the inverted nuclei observed in rod photoreceptor cells of nocturnal mammals with heterochromatin at their nuclear centre [44].

Inverted nuclei require the loss of both the Lamin B receptor (LBR) and Lamin A/C [45] and have been frequently discussed as a parallel to SAHF formation which also results in the translocation of heterochromatin from the nuclear periphery towards the centre in a Lamin B1 loss-dependent manner. A hierarchy of the interaction strengths between different compartments was derived, with inter-heterochromatin (HC-HC) contacts dominating the interactions field, with A-A and A-B interactions being much less represented. Comparing the nuclei of cells with and without the Lamin B receptor and considering the heterochromatin-Lamin interface (HC-NL) in the model

revealed that these interactions are not required for the separation of the euchromatin and heterochromatin compartments, but instead are required for the conventional radial positioning of heterochromatin at the nuclear periphery.

Similarly, polymer modelling has been used to understand chromatin rearrangement during senescence [46], establishing the critical role of HC-HC and HC-NL contacts towards the chromatin re-organisation. A senescence (OIS) and a progeroid (no SAHF) model, both with weak HC-NL interactions due to Lamin B1 disruption and a mutation in Lamin A/C, respectively, are compared to normal, growing cells. Four possible states were derived, with different degrees of mixture between euchromatin and heterochromatin. The model captures the dichotomy between the increased separation between HC and EC during senescence, and the increased mixing of the two states in progeria, despite a similar decrease in HC-NL contacts. This is consistent with the differences observed between OIS and replicative senescence in terms of the increased and decreased compartmentalization, respectively [29].

These studies mainly focused on modelling compartmentalization driven by attraction forces between regions with similar epigenetic environments (B-B, A-A). However, considering instead repulsion forces in a polymer model revealed that the attraction to the nuclear lamina is not necessary for recapitulating the chromatin organisation of interphase nuclei and that tethering factors may only delay the dissociation of Lamin-associated domains from the nuclear periphery [47]. Similarly, the presence of nucleoli represents a perturbation to the structure and is not a driving force of phase-separation.

SAHF may therefore represent the formation of a strongly interacting heterochromatin compartment, accompanied by further re-arrangement and separation of other epigenetic layers, reflecting a global trend of increased compartmentalization during senescence. This is supported by the polymer modelling studies, as well as by the Hi-C-based observations in OIS. Consistent with the necessary but insufficient role of Lamin B1 loss for SAHF [27], the nuclear-lamina interactions may contribute but not drive the formation of this compartment. It remains to be determined whether the global chromatin re-arrangement is also partially altered by chromatin associations with nuclear condensates which change their behaviour during (oncogene-induced) senescence, reflected by nucleolar fusion [48], an increased number and size of PML bodies [49], increased nuclear pore density [28], and *de novo* super-enhancers [50].

Phase separation facilitators

The emerging model of chromatin re-organisation during senescence goes beyond SAHF and offers a more integrative view of global chromatin compartmentalization, potentially facilitated by phase-separation events (model representation in Figure 3). Indeed, several factors which were shown to contribute to SAHF serve as potential candidates for the increased compartmentalization in OIS.

SAHF components HP1a and HMGA1 have been shown to phase separate *in vitro* and in cells [51,52]. The propensities for phase separation of both proteins are largely facilitated by post-

translational modifications (phosphorylation) and the presence of DNA. H3K9me3-positive nucleosomes also engage in phase separation through the cooperative recruitment of reader complexes, including HP1 [53]. Notably, HP1b, which, unlike HP1a, fails to phase separate *in vitro*, participates in this process by docking with H3K9me3. While these experiments are mostly conducted either *in vitro* or in supraphysiological conditions, and hence any physiological relevance needs careful interpretation, they highlight the multivalent nature of the process with cooperation of multiple factors being required. This notion was reinforced by a 'liquid chromatin' Hi-C study which analysed the stability of chromatin compartmentalization through modifying the Hi-C technique to introduce chromatin fragmentation before fixation and Hi-C [54]. This study shows that the chromatin fragment length is crucial, with fragments larger than 10-25 kb yielding stable compartmentalization possibly due to a higher valency.

Consistent with the interplay of attraction and repulsion forces driving compartmentalization in a multivalent fashion, proteomics assays show the accumulation and depletion of 'promoter' and 'repressor' proteins, respectively, in the presence of optogenetically-induced condensates. Interestingly, this study suggests that 'condensation' at specific genomic loci may be sufficient for the re-organisation of the macromolecular composition, and for the epigenetic changes at the associated regions [55].

In the case of SAHF, HP1 depletion (by expression of a dominant-negative form of HP1b, which depletes a large part of all three endogenous HP1 proteins from chromatin) does not impair SAHF [56]. Downregulation of either H3K9me3 (by ectopic expression of the demethylase JMJD2D) or H3K27me3 (by knock-down of SUZ12) also has little impact on SAHF [32]. While HMGA1 is an essential SAHF component, depletion of its close relative HMGA2 (also enriched in SAHF), has a modest effect on SAHF formation [32]. These observations imply a degree of redundancy and potential cooperativity between various SAHF components.

Some indirect SAHF effectors are also known to phase separate, such as linker histone H1 [57] and components of the nuclear pore complex (NPCs) [58]. Linker histone H1 controls nucleosome spacing and folding [59], and depletion of H1 (by ~ 50%) leads to local chromatin decompaction [60]. While the mechanism is still unclear, it was suggested that canonical H1 is diminished during senescence and disruption of endogenous H1 promotes senescence and SAHF [26]. NPC regions in nuclei are known to be devoid of heterochromatin and NPCs indeed interact with active enhancers [61]. As mentioned earlier, NPC density during senescence promotes SAHF [28]. Although intrinsically disordered phenylalanine-glycine repeat-containing nucleoporins (FG Nups) undergo phase separation mostly in the NPC lumen, contributing to selective and specific cargo transportation through the channel [58], they might also play a role in the association between active enhancers and NPCs (Tyagi et al., [preprint]). This suggests that the compartmentalization process behind SAHF may also be multivalent, with multiple contributors, but also exhibiting redundancy.

Chromatin layers

The concentric distribution of the chromatin layers accompanying SAHF reflect more general principles of chromatin organisation, as revealed by a super-resolution and scanning electron microscopy study describing the 3D zonation [62] of nanoscale chromatin domains with H3K9me3 and H4K20me3 marking the interior, followed by H3K27me3, and with active marks such as H3K4me3 and H3K36me3 occupying the perichromatin space, excluded from the domains' interior. This zonation was also reflected in the differences in replication timing, similar to SAHF.

The universality of the layering may also be reflected in the identification of additional sub-compartments in high-resolution Hi-C interaction maps, with specific epigenetic identities ranging from highly euchromatic and transcriptionally active classes to highly heterochromatic ones, such as the A1-A2, B1-B3 sub-compartments, the A (active), B (H3K27me3), C (H3K9me3) and D (H3K9me2) classification [63], or an 'intermediate' compartment separating heterochromatin and euchromatin, which is disrupted in cancer [64]. Recently, a new classification into eight 'interaction profile groups' was reported [37], including several heterochromatic types and highlighting a more continuous nature of the compartments, rather than well-defined separation. The strength of the sub-compartments varies in a cell type-specific manner, across development and cancer, and likely corresponds to a set of constraints to genome organisation. The functional contribution of these variations to gene regulation and their potential association to changes in nuclear condensates, remain to be resolved.

Functional impact

SAHF formation contributes to both gene activation and repression. RB mediates the deposition of heterochromatic marks at cell cycle genes [65], and earlier chromatin-immunoprecipitation studies suggested that such deposition increases at some cell cycles genes during OIS [15], particularly when the DNA damage response signalling is intact [66]. Consistently, a 3D DNA FISH study suggests a physical involvement of *CCNA2*, a cell cycle gene, in SAHF [56]. However, when using the more recent next generation sequencing techniques and highly specific monoclonal antibodies against individual heterochromatic marks [32], the heterochromatin deposition at these genes is less pronounced. Thus, the precise physical proximity between SAHF layers and specific genes requires further study in the context of chromatin compartments. Although Lamin loss during OIS is preferentially linked to H3K9me3 domains, a number of H3K27me3 marked lamina-associated domains (LAD) show increased lamina-association during OIS, linked to repression of a small set of genes, including some cell cycle genes [27].

Consistent with the idea that SAHFs and peri-SAHF structures can affect each other, SAHFs indirectly facilitate new contacts which may contribute to gene regulation. A recent study highlighted the activation during OIS of a set of genes, including *RHOB* and *CXCR4* [29], adjacent to the heterochromatic regions involved in SAHFs, which gain interactions in a SAHF-dependent manner. A set of highly tissue specific genes were also shown to be activated during OIS in a SAHF-dependent manner by being specifically excluded from the highly heterochromatin environment of SAHF [67]. Genes in the epidermal differentiation complex (EDC) locus are typically expressed in terminally differentiated keratinocytes and hence, in normal fibroblasts, the EDC is tightly

repressed and positioned at the nuclear periphery in a H3K9me3-rich LAD. Genes, such as the *LCE2,* become active in senescent fibroblasts, as a result of the EDC being decondensed at peri-SAHF areas by a mechanism which remains to be elucidated.

Such diverse modes of SAHF-dependent expression may contribute to the heterogeneity of the senescent phenotype and these studies highlight the specificity of such expression and its absence in scenarios where SAHF are absent or limited, such as replicative senescence.

Outlook

The dynamics and conservation of the SAHF folding pattern across different cell types and stimuli, and its potential contribution to the heterogeneity of senescence, remains to be determined. A robust query of the presence and activity of genes incorporated or excluded from SAHF and accompanying epigenetic layers, or within sub-compartments of different strengths in growing and senescence cells, may lead to insights into the impact of chromatin architecture on transcriptional heterogeneity.

Single-cell transcriptomic senescence studies showed high heterogeneity even within the same cell population, with divergent transcriptional programmes co-existing (e.g., primary, and secondary senescence). This may be reflected at the chromatin organization level in part, e.g., microscopy studies showing different proportions of cells forming SAHF depending on the trigger and cell type. Intrinsic constraints may be present in some cell types, reducing their capacity to form SAHF or to increase compartmentalization. Whether or not the same cellular 'archetypes' (in terms of chromatin organization and associated transcriptional profile) exist across different types of senescence, but in different proportions, remains to be answered. Technological advancements targeting the 3D epigenome at the single cell level will help elucidate these questions.

Heterochromatin is an important barrier for cell fate transitions as highlighted by cell differentiation studies and reprogramming assays. Loss or reduction of heterochromatin is often linked to pro-tumorigenic phenotypes. SAHF, or more generally, increased compartmentalization, may act either as a barrier, reinforcing cell fate determination, or as a bypass of the heterochromatin related constraints on gene expression by further isolating heterochromatin from euchromatic active regions. The increased trans-compaction accompanied by a decrease in local chromatin interactions is reminiscent of a similar shift during differentiation [68], suggesting that senescence may exhibit a less plastic, cell fate-determined nuclear conformation.

Acknowledgements

We thank all members of the Narita laboratory for useful discussion and suggestions and Dr. Andrew Young for editing. Figure 1 was created using BioRender.com (2023).

Funding

This work is supported by a Cancer Research UK Cambridge Institute core grant (no. C9545/A29580) to MN laboratory. MN is also supported by BBSRC (BB/S013466/1 and

BB/T013486/1) and Diabetes UK via BIRAX and the British Council (65BX18MNIB). IO is also supported by a Cancer Research UK Pioneer Award (C63389/A30462).

Declaration of competing interest

None.

Data availability

No data was used for the research described in the article.

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A comprehensive analysis of the 3D genome organisation in OIS and replicative senescence revealing distinct compartmentalization trends. OIS exhibits increased B-

B interactions reflecting SAHF, whereas replicative senescence exhibits decreased A-A and increased A-B interactions. They also showed that DNMT1 is required for SAHF formation in OIS.

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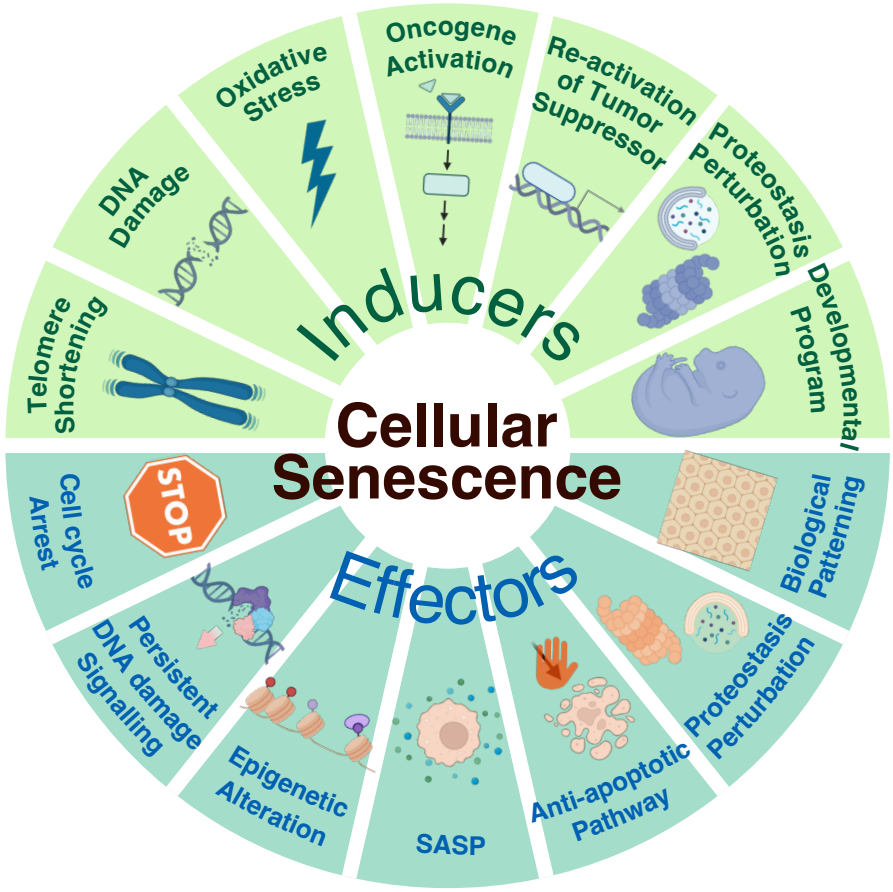
Legends

Figure 1. Inducers and effector programmes of cellular senescence. Created with BioRender.com (2023).

Figure 2. Model of the changes in nuclear architecture occurring during cellular senescence **a)** Heterochromatin repositioning from the nuclear periphery in normal, growing cells (left) to OIS cells (right) and SAHF effectors; SAHF heterochromatic layers and exclusion of euchromatin. **b)**

592 Conceptual representation of the Hi-C maps of growing (left) and OIS cells (right) and
593 compartmentalization patterns, with increased B-B and decreased A-B interactions.
594
595 **Figure 3.** Model representation of chromatin segregation. Two representative examples involving
596 phase separation: heterochromatic and euchromatic condensates with dynamic inclusion
597 (attraction) and exclusion (repulsion) of chromatin and associated proteins in a multivalent manner.
598
599 **Table 1. List of direct and indirect SAHF effectors.**



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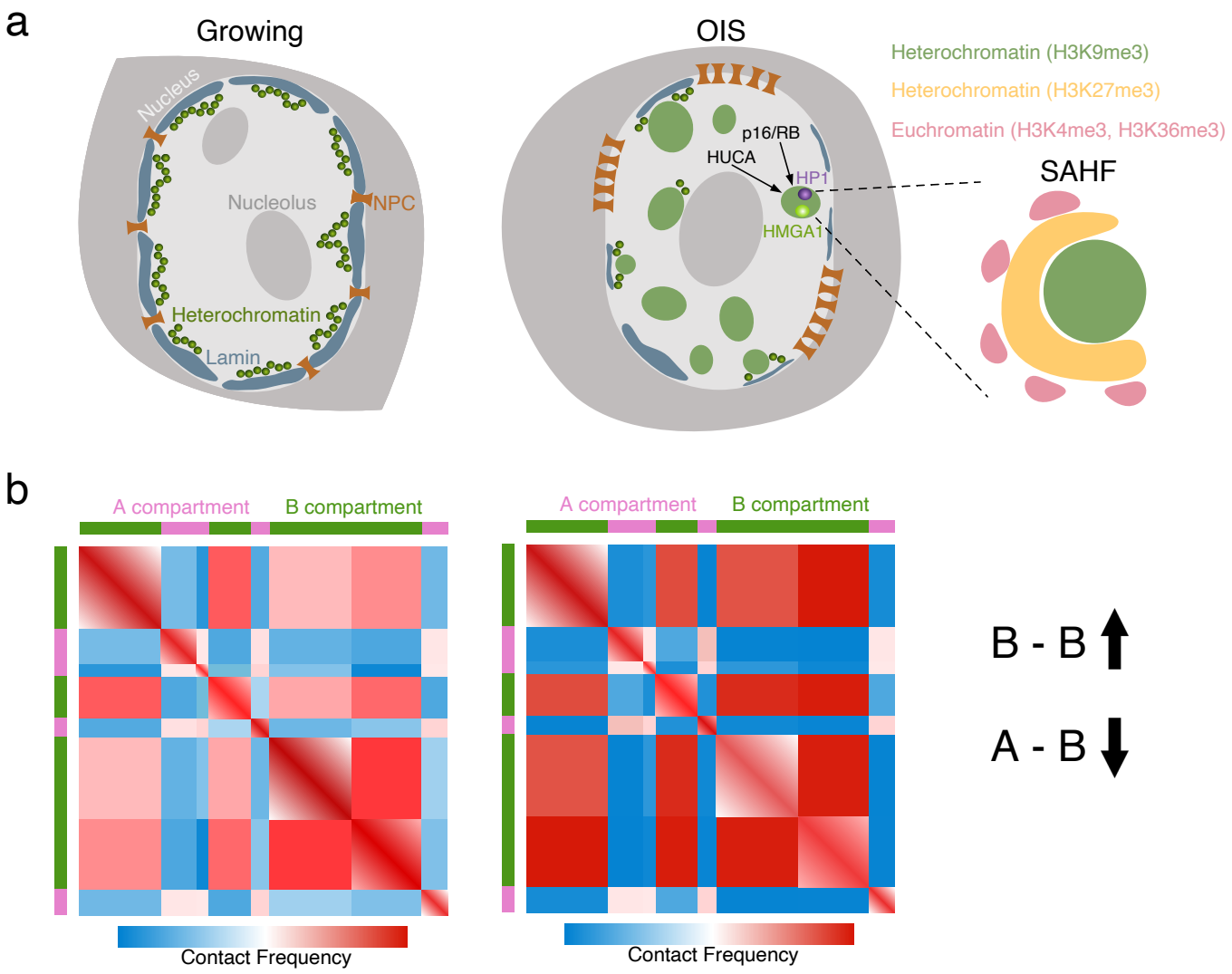

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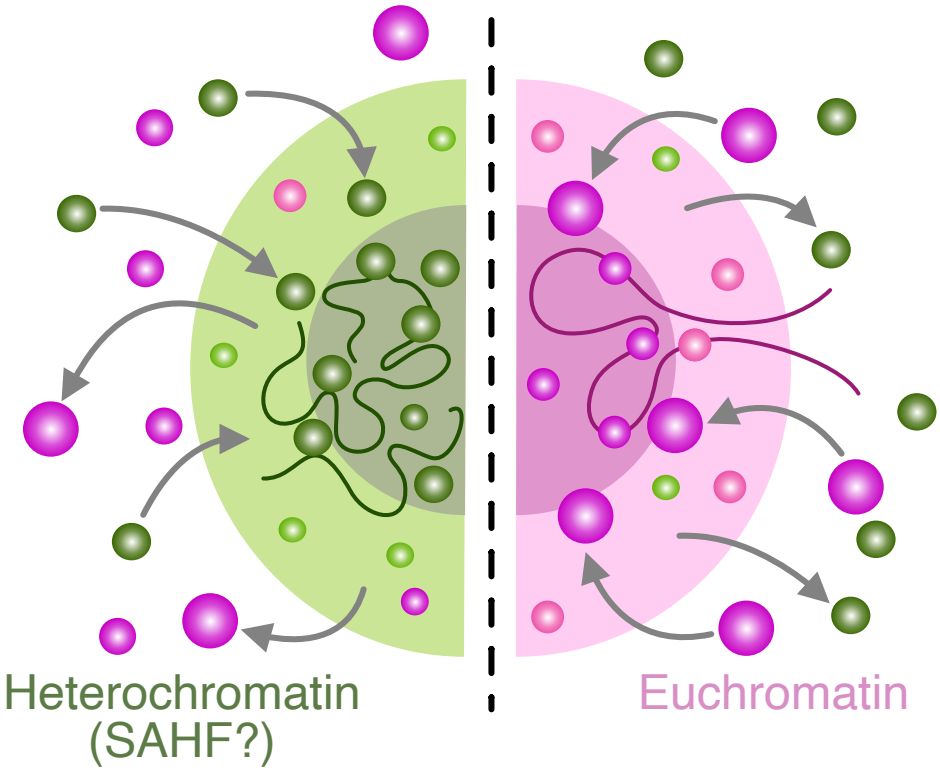

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