High-order chromatin structure and the epigenome in SAHFs

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Introduction

Cellular senescence is a stable state of cell cycle arrest that may be induced by a wide range of cellular stresses, including DNA damage, oxidative stress and oncogene activation.1,2 Senescence has been widely studied in human diploid fibroblasts (HDFs), in which a number of markers and effector mechanisms of senescence have been identified. One marker is a typical chromatin structural change, senescence-associated heterochromatic foci (SAHFs).3 Upon the induction of senescence in HDFs, SAHFs become readily visible as distinct DAPI-dense foci. These foci are enriched for heterochromatic markers, such as H3K9me3, heterochromatin protein 1 (HP1) proteins and core histone macroH2A; while excluding euchromatic markers, such as H3K9-acetylation and H3K4me3.4 SAHFs are also characterized by their accumulation of HMGA proteins (non-histone chromatin architectural proteins) and depletion of linker Histone H1.5 Initial studies suggested a correlation between SAHF formation and specific gene expression during oncogenic Ras-induced senescence. One example is the derepression of selected cell cycle genes upon disruption of SAHFs through depletion of the retinoblastoma (Rb) tumor suppressor in Ras-induced senescence, even though Rb depletion is not sufficient to rescue senescence arrest in HDFs.6 In addition, SAHFs have been suggested to contribute to the irreversibility of senescence arrest.7,8 Aside from the potential relevance of SAHFs in senescence and thus tumor suppression, our recent study proposes that SAHF can be a useful tool for chromatin biology. In that
Integrative phosphorus and nitrogen density analyses using electron spectroscopy imaging (ESI) identified differences in chromatin compaction between the H3K9me3 core and the surrounding H3K27me3 ring. Interestingly, the H3K9me3 core was even more compacted than H3K9me3 enriched perinuclear constitutive heterochromatin, while the H3K27me3 ring was found to be less compacted than the perinuclear heterochromatin. During SAHF formation global H3K9me3 levels change only moderately, whereas the level of chromatin-bound HP1 proteins, which bind H3K9me3, significantly increases. Despite the non-stoichiometric increase of HP1 proteins compared with its binding target, HP1 localization was still restricted to the H3K9me3 core—as judged by confocal microscopy. Thus, it is possible that the affinity of HP1 binding to the histone mark is altered during senescence. However, it is also possible that HP1 proteins associate with different histone marks, as recently shown.11 HP1 proteins can form dimers and exist in complexes with other proteins and thereby introduce another layer of complexity to the H3K9me3 regions. However, the Adam lab has shown that overexpression of a dominant negative mutant of HP1β, which removes 50–80% of endogenous HP1 proteins, fails to inhibit SAHF formation.10 This result suggests that accumulation of HP1 proteins may not be required for SAHF formation per se, but whether or not HP1 proteins contribute to the organization of the chromatin within SAHFs and/or the compactness of the SAHF-core remains to be tested.

In contrast to H3K9me3, which provides a docking site to HP1 proteins, H3K27me3 can be recognized by polycomb (Pc) proteins. So far studies have not been able to find any endogenous Pc protein associated with the H3K27me3 layer. Consistent with our ESI result, indicating that the H3K27me3 ring is less compacted than the SAHF core or perinuclear constitutive heterochromatin, Pc bodies have also been found in rationally DAPI-poor regions in Drosophila.12

In addition, a recent report showed that,
in mammals, Pc group (PcG) proteins are highly concentrated in perichromatin compartments, the surface rather than the interior of condensed chromatin domains.13 These perichromatin compartments seem to be the site for pre-mRNA synthesis.14 Of note, our ESI data suggest that the H3K27me3 layer is more protein- and/or RNA-rich than the core or perinuclear heterochromatin. An attractive hypothesis is that these additional molecules might be specifically involved in the high-order chromatin structure (HOCS) of the H3K27me3 layer of SAHFs.

**How Does the Chromatin Landscape Change During SAHF Formation?**

We profiled repressive histone marks using ChIP-seq to find changes in the linear epigenomic landscape of senescent HDFs. We analyzed the linear profiles with kbp to Mb window resolution. Although we found changes in the distribution of repressive marks on a genic level, we found that the overall pattern of the repressive histone marks remain unchanged (Fig. 2). This suggests that SAHFs are formed through the spatial rearrangement of pre-existing heterochromatin (Fig. 1B). In agreement with this model, late replicating chromatin, showing a perinuclear pattern in growing cells, relocated to the SAHF core upon senescence induction (as discussed in the next section). We were able to reduce the number of SAHF-positive cells in an established senescent population by depletion of the chromosomal architectural protein HMGA1.5,8 However, the histone mark profiles remained static upon HMGA1 depletion. In addition to the mechanistic insight into SAHF formation, the data also suggest that dynamic HOCS and deposition of repressive histone marks can be two distinct processes, at least in somatic cells. The highly static distribution of repressive histone marks during a dramatic HOCS change was surprising. Especially considering, the prevailing assumption that regional spreading of repressive histone marks is structurally associated with heterochromatin formation. However, the spreading model is largely based on data using unicellular organisms, or development systems of higher eukaryotes. For example, in position effect variegation (PEV) in Drosophila, where an euchromatic gene can be silenced when transposed adjacent to a broken segment of heterochromatin, decisions as to which genes are silenced are made during early development, leading to a variegated pattern.15,16 Xi formation is also developmentally regulated and also associated with the expansion of H3K27me3 and PcG proteins.17-20 Indeed, the spreading of H3K9me3 and H3K27me3 repressive marks has been confirmed in embryonic stem cell (ESC) differentiation,21,22 where ESCs exhibit lower levels of repressive histone modifications, a more diffuse heterochromatin structure as well as globally active transcription.23,24 It may therefore be that HOCS formation in development and somatic cells is mechanistically distinct, although a causative role for the spreading of repressive marks in HOCS during ESC differentiation remains to be demonstrated.

**Repressive Marks and HOCS**

In proliferating HDFs late replication timing is associated with heterochromatic regions.11-12 Since senescent cells do not

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**Figure 2. Chromatin pattern dynamics at different resolutions.** (A) Chromosome-wide landscape of H3K27me3 in Growing and Ras-induced senescent (RasInd) fibroblasts (reanalysed from ref. 8). ChIP-seq data intensities have been windowed in 1kb bins. A rolling mean of 1,000 units has been applied. The plot shows a globally static landscape of H3K27me3 between Growing and Senescent cells. The black rectangle approximately indicates the position of CDKN2A (also called p16INK4A) on chromosome 9. (B) A uniformly scaled UCSC genome browser shot of H3K27me3 ChIP-seq in Growing and Ras-induced senescent cells for the CDKN2A locus. Note, CDKN2A is a marker of senescence and its transcription is activated during Ras-induced senescence.
proliferate, we pulse-labeled late replicating regions while the cells were proliferating and chased them through until after senescence induction. Later replicating regions became the SAHF-core. The core was surrounded by earlier replicating regions in concentric layers visually similar to the arrangement of the heterochromatic layers. Having described correlations between histone marks/repetitive elements and the spatial pattern of chromatin regions in SAHFs, we wondered what determines the spatial destination of the genomic regions. We went on to directly test for the causal involvement of the repressive histone marks in SAHF formation, by perturbing the establishment of repressive histone marks before SAHF induction. Depletion of either H3K9me3 (using ectopic expression of a demethylase) or H3K27me3 (using RNAi against a PGR component) in HDFs did not cause an apparent defect in SAHF formation, although a detailed structural analysis remains to be performed. Moreover, the layer structure within SAHFs seems to be preserved in these conditions. Therefore, despite the close spatial correlation, the repressive marks do not appear to be obligatory for SAHF formation. This is consistent with the aforementioned result from the Adams lab, suggesting that HP1 may not be required for SAHF formation.

Another observation, which implies a non-causal correlation between chromatin marks and HOCS has been described for topological domains or topologically associated domains (TADs). TADs were recently discovered through the study of the three-dimensional organization of the genome using high-throughput chromosome conformation capture (3C) approaches in mammalian cells. TADs are Mbp-sized genomic blocks in which chromatin is highly self-interacting and they are correlated with repressive marks, such as H3K9me3, H3K27me2 and H3K27me3. Interestingly, TADs are conserved in both ESCs and fibroblasts, suggesting that TAD formation is not a consequence of spreading of the repressive marks. Furthermore, Nora et al. confirmed that the absence of H3K9me2 or H3K27me3 in G9a or Eed knockout mouse ESCs, respectively, does not affect TADs in their size or position. Thus these studies suggest that repressive marks are not required for TAD formation and that repressive function, the facultative heterochromatin spreading occurs within the framework of TADs during early embryonic development. While it is not yet known how TADs relate to SAHFs, the results of these studies reinforce the significance of the spatial repositioning of pre-existing heterochromatin in HOCS change in somatic conditions.

The molecular basis, beyond the repressive histone marks, required for the clustering and segregation of the different chromatin types remains to be elucidated. A number of factors have been associated with the process of SAHF formation, either directly or indirectly. These factors include HMGA proteins and linker histone H1, which share a common binding site (the linker DNA). Other factors include Rb, p400 (a core subunit of a chromatin-remodeling complex), macroH2A and histone chaperones, HIRA and ASF1a. However, the mechanisms of how these factors affect HOCS and whether or not they are involved in chromatin type segregation upon SAHF formation remains unknown. It may be useful to search for factors that are specifically enriched in (but not dependent on) either H3K9me3 or H3K27me3-positive genomic regions. The resulting candidates might include SAHF-modulating factors.

The Function of SAHF

Although SAHFs have been correlated with gene regulation and the irreversibility of cell cycle arrest, neither can we define a causal role for SAHF in senescence, nor can we explain the complex chromatin layer arrangement. We have shown that the chromatin segregates into layers enriched for H3K9me3, H3K27me3 and H3K36me3, which may roughly correspond to constitutive heterochromatin, facultative heterochromatin and euchromatin, respectively. Notably, core components of constitutive heterochromatin, such as SUV39H1 (histone H3 lysine 9 methyltransferase) and HP1 homologs, were identified as dominant suppressors of PEV in Drosophila, while most Pcg mutations do not appear to suppress PEV. One might speculate that clustering of constitutive heterochromatin leads to a large heterochromatic entity (the SAHF core), which would put adjacent euchromatic regions at risk of epigenetic silencing through the position effect in trans. Therefore, in addition to its own gene repression function, the facultative heterochromatin layer might act as a buffer between the transcribed and constitutive heterochromatin layers.

Beyond the potential relevance of SAHFs in gene regulation, a recent study suggested that SAHFs suppress DNA damage signaling. It was further suggested that some tumor cells, as well as cells having escaped oncogene-induced senescence, can exhibit SAHF-like structures and that in these cells the SAHF-like structure restrains DNA damage signaling and thereby contributing to cell survival in tumor cells. It remains to be seen to what extent these heterochromatin structures in proliferating cells originated from or resemble SAHFs, for example by live microscopy. However, it is possible that these heterochromatic structures contribute to maintaining the gene expression profile of these cells. Whether or not the layer structure of chromatin shown in SAHFs is preserved in the tumor-associated heterochromatin structures remains to be determined, but one possibility is that such a spatial arrangement of heterochromatin and euchromatin enables highly efficient transcription, similar to what has been proposed for transcription factories. Transcription factories are spatially defined and yet are shared by a number of genes to be transcribed from the same or different chromosomes. By the time SAHFs are formed during Ras-induced senescence, gross transcriptional changes might have been established leading to a static gene expression profile in senescence. SAHFs could contribute by locking in the phenotype; by stably silencing as well as expressing genes through spatial clustering, maybe at the cost of plasticity. If this is a fundamental function of SAHFs, the relevance of SAHFs or SAHF-like structures might go beyond senescence. The concept may apply to other “fate determined” conditions, where many genes are either stably expressed or repressed, including differentiated cells or even some tumor cells.
SAHF as a Model System

The only cell cycle states where it is possible to directly observe individual chromosomes under the light microscope are the different stages of mitosis. However, nuclei spend most of their time in interphase and our knowledge about HOCS in interphase chromatin is limited. Studying SAHFs may provide a unique model system to examine chromatin behavior in several ways. For example, SAHFs could extend the Xi model system, the best example of facultative heterochromatin. It has been proposed that SAHFs consist of individual chromosomes but can be a sub-chromosomal phenotype. This implies not only that SAHFs may enrich our knowledge of interphase chromosome arrangement makes SAHFs a unique model system to study structural and functional properties of distinct chromatin types.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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