HHS Public Access

Author manuscript

Transl Med Aging. Author manuscript; available in PMC 2020 October 05.

Published in final edited form as:

Transl Med Aging. 2020 ; 4: 121–131. doi:10.1016/j.tma.2020.08.002.

The eroding chromatin landscape of aging stem cells

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Abstract

Adult stem cells undergo both replicative and chronological aging in their niches, with catastrophic declines in regenerative potential with age. Due to repeated environmental insults during aging, the chromatin landscape of stem cells erodes, with changes in both DNA and histone modifications, accumulation of damage, and altered transcriptional response. A body of work has shown that altered chromatin is a driver of cell fate changes and a regulator of self-renewal in stem cells and therefore a prime target for juvenescence therapeutics. This review focuses on chromatin changes in stem cell aging and provides a composite view of both common and unique epigenetic themes apparent from the studies of multiple stem cell types.

Keywords

Stem cell; Aging; Self-renewal; Differentiation; Epigenetics/chromatin

1. Why do we need stem cells?

In most tissues, adult stem cells occupy a rare but powerful functional compartment, capable of differentiating into multiple tissue-specific lineages. Some stem cell types can remain quiescent until environmental signals prompt them to divide whereas other types continuously divide to repopulate lost or injured tissue. This process is critical and is harnessed during injury and disease to enhance tissue repair [1].

That a multipotent stem cell population capable of regenerating lost tissue exists was first speculated in the 1960s when irradiated mice receiving bone marrow transplants were able to rescue a deficit of white blood cells [2]. This observation gave birth to an explosive field of hematopoietic stem cell (HSC) biology which to this date remains the most well-studied adult stem cell population. HSCs crown the top of a hierarchical ladder consisting primarily of two branches: the myeloid branch and the lymphoid branch (Fig. 1A). In a series of orchestrated steps, HSCs progress down the ladder via progenitor cells to ultimately form terminally differentiated B cells or T cells (lymphoid branch) or basophils, eosinophils,

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Declaration of competing interest

None.

neutrophils, monocyte/macrophages, and megakaryocytes/platelets (myeloid branch). At the same time, through asymmetric division, HSCs self-renew to maintain a small reserve of itself at the top of the ladder [3].

In addition to HSCs, the bone marrow stroma consists of mesenchymal stem cells (MSCs) that generate bone, cartilage, and fat cells to support the formation of connective tissue. Over the years, numerous other solid tissue stem cells were discovered in the liver, pancreas, intestine, epidermis, lung, brain, muscle, taste papillae, hair follicles, mammary gland etc. In contrast to HSCs, most of these solid tissue stem cells show more limited potential, are slow-cycling and proximal to basement membrane structures [1]. Some organs such as the intestine, liver, pancreas, taste buds, hair follicles etc. contain in addition to slow-cycling progenitors, a population of highly proliferative Lgr5+ cells expressing leucine-rich repeat-containing G protein-coupled receptor 5 (Lgr5) and capable of dividing numerous times [4]. To add further complexity, some tissues contain substantial numbers of uni- or bi-potent cells that can respond to injury and divide to replace lost parenchyma. Thus, it appears that regenerative function of individual tissues is distributed among multiple cell types.

2. When stem cells age

All professional and potential stem cells in adult tissues, show dramatic reductions in regenerative capacity with age [5]. Stem cells undergo replicative aging (due to repeat proliferative cycles), chronological aging (due to chronic changes during prolonged quiescent state) and even show senescence/exhaustion phenotypes. Prolonged quiescence can accumulate DNA damage and cause chronological aging due to additive insults and error-prone damage repair mechanisms. In response to replication signals, stem cells are activated to divide. Two major aspects of stem cell division are self-renewal and differentiation. Studies across multiple organisms and stem cell types have revealed distinct effects on self-renewal capacity and differentiation potential depending on stem cell type [6,7]. This is manifested in either loss or gain of stem cell numbers, delay in activation kinetics, altered fate, lineage bias and/or compromised function of differentiated cells with age. Ultimately, these changes in aged stem cells eventually lead to physiological disorders and age-dependent pathologies in the organism.

The number of HSCs increase with age, pointing to a favor towards symmetric division [8]. Furthermore, aged HSCs differentiate along a myeloid-biased lineage tree resulting in an increased incidence of acute myeloid leukemia with age [9–12]. Concomitantly, there is a decline in adaptive immunity with age mediated by the loss in lymphoid cell numbers. The high rate of unexplained anemia in the elderly over 65 years is likely linked to this decline in hematopoietic function mediated in part by the unbalanced lineage potential of aged HSCs [13]. Much like HSCs, intestinal stem cells (ISCs) show an age-related expansion of stem cell and enteroblast numbers and decreased differentiated enterocyte numbers in adult flies [14,15]. Regenerative capacity of ISCs is also attenuated in old mice. Nalapareddy and colleagues reported that the age-related decline in regenerative capacity of ISCs is due to a decline in canonical Wnt signaling [16]. They also found a decrease in *Notch1* expression and an increase in Atonal homolog 1 (*Atoh1*) gene expression in ISCs and their niche. Reduced Notch and Wnt signaling along with elevated *Atoh1* expression favor ISC

differentiation towards the secretory lineage [17,18]. Interestingly, aggressive colorectal cancers which show increased incidence with age, have an ISC-like gene signature and this signature but not their proliferative capacity is predictive of disease relapse in patients. These data indicate that age-related defects in the aged ISC populations might be involved in the occurrence of colorectal cancer in humans [19]. Unlike HSCs, the number of muscle stem cells (MuSCs or satellite cells) decreases during aging, although like HSCs, aged satellite cells exhibit a skewed differentiation potential towards a fibrogenic rather than a myogenic lineage [20,21]. The decline in MuSC function with age leads to the decrease of muscle recovery from injury, eventually reducing muscle mass and inducing muscle fibrosis in the elderly [22]. Old age is also accompanied by fewer activated neural stem cells (NSCs), neural progenitor cells (NPCs) and neuroblasts [7,23]. Interestingly, a morphologically distinct subpopulation of NSCs called horizontal NSCs undergo selective attrition of numbers with age [23]. This age-related decrease in NSC numbers together with decreased neurogenesis might underlie the impaired learning and memory in the elderly [24]. Aged MuSCs and NSCs also show delayed activation kinetics in single-cell transcriptomic studies [25,26]. The lineage trees of NSCs, MuSCs and ISCs are shown in Fig. 1B–D.

3. Focus on cell-intrinsic determinants of stem cell aging

While extrinsic factors such as niche alterations and metabolic changes may contribute to stem cell loss-of-function with age, in this review, we will focus on cell-intrinsic epigenetic or chromatin alterations that profoundly alter gene expression programs [27,28]. In fact, heterochronic transplants have shown that old HSCs transplanted to young niches behave like old cells, lending support to the importance of cell-intrinsic detriments [29].

The understanding of epigenetic changes during stem cell aging has been greatly accelerated by multi-omic technologies such as whole genome bisulfite sequencing (WGBS), chromatin immuno-precipitation sequencing (ChIP-seq), RNA-sequencing (RNA-seq), chromatin accessibility profiling (ATAC-seq) and proteomics. Furthermore, very recent advances in chromatin conformational studies such as Hi-C and more recently, single-cell transcriptomics have greatly advanced our understanding of stem cell aging. These futuristic studies in different stem cell types have revealed key underlying themes of age-related epigenetic erosion. Focusing primarily on HSCs, MuSCs, NSCs and ISCs, where much of the chromatin profiling has been done, we describe below some key epigenetic features of aging.

3.1. The state of global and local DNA methylation in aged stem cells

Cytosine 5-methylation (mC or 5mC) is the major DNA modification found throughout the genome at high frequency, but predominantly located at promoter regions of housekeeping and developmentally regulated genes. Contrary to aged post-mitotic somatic cells which show global hypomethylation, old HSCs are characterized by an increase in global DNA methylation levels [30]. Locus-specific alterations in DNA methylation show hypermethylation at promoters of polycomb group (PcG) target genes and hypomethylation at repeat regions [31]. Correlative analysis between the DNA methylome and transcriptome revealed an increase of DNA methylation at promoters of genes associated with

differentiation and a reduction at genes associated with HSC maintenance, consistent with impaired differentiation potential and increased HSC numbers during aging [30]. Additionally, regions of the genome in myeloid cells that have open chromatin show decreased DNA methylation in aged HSCs [31]. Along the same lines, promoter DNA hypermethylation, which is generally associated with gene repression, does not show any correlation with transcription of genes in stem cells, but instead affects the transcriptional profiles of downstream lineage cells that inherit the altered DNA methylation from the aged stem cell parent [30–33]. DNA methylome studies in human and murine MuSCs, much like aged HSCs, suggest a global DNA hypermethylation across the genome [34,35].

DNA methyltransferase 1 (DNMT1) maintains parental cell methylation patterns by adding a methyl group to cytosines on newly synthesized daughter strands [36]. DNMT1 has been shown to be essential for HSC self-renewal [37] and loss of DNMT1 leads to a skewed lineage output biased toward myelopoiesis [38,39]. DNMT1 inhibition and reduction of DNA methylation in aged MuSCs by 5-Aza-2'–deoxycytidine treatment *in vitro* improves the ability to self-renew [40]. Conditional ablation of *Dnmt1* in the adult ISCs causes crypt expansion and inhibits differentiation potential [41]. In NSCs, DNMT1 is highly expressed in the central nervous system (CNS) during embryogenesis and after birth. DNMT1 deficiency in mitotic CNS precursor cells results in DNA hypomethylation in daughter cells. The mutant CNS neurons are impaired functionally and selected against at postnatal stages [42]. In NPCs, which include all classes of immature and proliferating cells, conditional gene deletion of *Dnmt1* prematurely drives differentiation towards astrocytes, a pattern seen with aging [43,44]. These results indicate that decreased DNA methylation in NSCs and NPCs is linked to the observed functional decline associated with aging.

DNMT3A and DNMT3B are *de novo* methyltransferases that establish new DNA methylation patterns during development and stem cell differentiation. Conditional knockout of *Dnmt3a* alone in HSCs increases self-renewal at the expense of differentiation potential after serial transplantation [45]. DNMT3B is essential for HSC differentiation and loss of both *Dnmt3a* and *Dnmt3b* in HSCs leads to an even more severe arrest of HSC differentiation [45]. DNMT3A is also expressed in postnatal NSCs and is required for neurogenesis. Genome-wide analysis of DNMT3A in NSCs shows that it occupies and methylates intergenic regions and promoters of genes that encode regulators of neurogenesis. DNMT3A-dependent non-promoter DNA methylation promotes expression of neurogenic genes while knockout of DNMT3A in postnatal NSCs show impaired differentiation [46].

Ten-Eleven-Translocation (TET) proteins perform iterative oxidation of 5mC to produce 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC) followed by 5-carboxylcytosine (5caC) and ultimately unmethylated cytosine (C) [47]. In concordance with the global DNA hypermethylation, mass spectrometry revealed reduced 5hmC levels in mouse HSCs [30] as well as human peripheral blood cells with age [48]. Additionally, the levels of all three TET enzymes (TET1–3) decrease in aged HSCs [49]. *Tet2* deletion has been reported to increase HSC self-renewal but also increase myeloid output upon differentiation [50,51]. *Tet1* deletion also leads to enhanced self-renewal of HSCs, but interestingly *Tet1* null HSCs have an increased lymphoid bias toward B cell production and propensity to develop B cell

malignancies [52]. In contrast to HSCs, 5hmc is quite prevalent in mice and human brains at neuronal genes and increases during differentiation [53]. TET1 knockout mice show impaired learning memory with increased methylation and decreased expression of genes involved in the proliferation of NPCs [54]. *In vitro* experiments show that loss of TET3 leads to dysregulation of the maintenance of the NPC population associated with increased apoptosis [55]. 5hmC is also highly dynamic during differentiation of ISCs into functional villus epithelial cells [56]. Studies have found that 5hmC is enriched at highly expressed genes such as the WNT target genes in ISCs, and at genes encoding metabolic and transport function in differentiated intestinal cells [57]. TET1 deficient mice have significantly reduced numbers of proliferative cells in the postnatal intestine. TET1-mediated DNA hydroxymethylation plays a critical role in the epigenetic regulation of the WNT pathway in intestinal stem and progenitor cells, and consequently in the self-renewal of the intestinal epithelium [56].

Clonal hematopoiesis of indeterminate potential (CHIP) is a phenomenon observed in healthy elderly people, where there is a predominant presence of blood lineages with somatic mutations derived from only a few early stem or progenitor cells [58]. Genetic analyses of blood indicate that more than 2% of blood cells carry somatic mutations in cancer-associated genes, this frequency increased to 5–6% for individuals older than 70 years. Remarkably, 43% of these mutations were found in genes encoding for epigenetic modifiers involved in DNA methylation (DNMT3A and TET2) and chromatin compaction (ASXL1). These aberrant epigenetic changes are likely to drive the expansion of a genetically identical clone of hematopoietic cells and ultimately promote the development of leukemias [59].

DNA methylation-based epigenetic clocks have provided new ways to measure quantitative and qualitative parameters of biological aging [60]. Allogeneic transplants of human HSCs completely reset the epigenetic age of the recipient’s blood to that of the donor. A further benefit was a remarkable short-term rejuvenation of an additional few years in the recipient blood that gradually disappears with time to completely match the age of the donor [61,62]. The rise of pioneering induced pluripotent stem cell (iPSC) technology makes it possible to reprogram adult differentiated cells into a pluripotent state by erasing marks of cellular aging and resetting the epigenetic clock [63,64]. In iPSC and iPSC-derived cells, epigenetic age is totally rejuvenated and then slowly accelerated upon differentiation [65]. In contrast, direct conversion of fibroblasts into induced neurons retained some of the age-associated epigenetic and transcriptional signatures [66,67]. Direct conversion of peripheral blood cells to NSCs however, showed a loss of age-related DNA methylation patterns initially but increased epigenetic age upon differentiation and serial passaging [68].

Taken together, aged HSCs and MuSCs show increased DNA methylation with age perhaps as a protective measure to maintain stem cell function. The global methylation status of aged ISCs and NSCs has not been investigated. However, focal loss of DNA methylation across these stem cell types results in increased self-renewal but reduced stem cell function with phenotypes frequently projecting those observed with aging. Age-associated phenomena such as CHIP can be attributed to mutations in DNA modifier enzymes while DNA methylation-based epigenetic clocks can be modulated by reprogramming factors to convert

differentiated cells to stem cell. These data indicate that alterations in DNA methylation may partly underlie age-related decline in tissue regeneration. The changes in DNA methylation in aged stem cells are summarized in Table 1.

3.2. Changes in breadth and intensity of H3K4 methylation during aging

An additional layer of epigenetic information is mediated by histone modifications. Post-translational modifications at various residues on histones and particularly on the N-terminal tails, impact gene expression by altering chromatin structure and making it more/less accessible to transcription factors (TFs) and the general transcriptional machinery [69]. Proper regulation of lysine 4 methylation on histone H3 (H3K4me) has been implicated in HSC self-renewal [70] and differentiation [71]. Mono- and dimethylation of H3K4 (H3K4me1/me2) at regulatory enhancer elements in HSCs do not directly regulate active transcription in the stem cell state, but rather prime genes for expression in the differentiated progeny [72,73]. H3K4me3 is an activating histone modification present at the transcription start sites (TSS) of protein-coding genes. H3K4me3 protects promoters from DNA methylation and helps keep the chromatin in an open state for access of the transcriptional machinery [69]. H3K4me3 in ChIP-seq studies on HSCs isolated from old mice, revealed only a modest increase in the total number H3K4me3 peaks compared to their young counterparts. Although there was a strong correlation between increased H3K4me3 levels and the most up-regulated genes [30], in general H3K4me3 cannot always predict the steady state RNA levels. Intriguingly, the breadth of existing H3K4me3 peaks expanded considerably in old HSCs particularly at genes linked to HSC identity. Previously, a meta-analysis of H3K4me3 ChIP-seq datasets established that broad H3K4me3 domains may provide transcriptional consistency supporting its role at HSC identity genes [74]. However, the spreading of H3K4me3 may also be linked to the dysfunction of aging HSCs as this pattern is enriched at genes associated with self-renewal and loss of differentiation capacity [30]. This is consistent with studies that show a decrease in H3K4 demethylases such as KDM5B can expand the stem cell compartment and decrease differentiation [31]. ChIP-seq studies in HSC-enriched populations from young and old healthy human bone marrow showed a ~31% reduction in H3K4me3 peaks in old donors particularly at genes involved in cancer-related pathways [75].

In contrast to HSCs, examination of H3K4me3 peaks in MuSCs showed few differences between cells isolated from young and aged mice. The intensity, but not the distribution, of the H3K4me3 mark was modestly decreased upon aging with a slight (~5%) increase in breadth [76]. However, in injury-activated old MuSCs, H3K4me3 is upregulated at several cell cycle inhibitor genes such as p21 and p16, which partly explains the decline in the proliferative capacity of these cells [77]. In genome-wide studies using NSC populations from postnatal day 5 mice, H3K4me3 was also a stable mark at promoters of genes that are differentially expressed with NSC activation, consistent with its enrichment at constitutively accessible chromatin rather than correlating with the change in transcription [78]. Furthermore, as in HSCs, broad H3K4me3 domains promote the self-renewal and differentiation of mouse NSCs [79]. Deletion of MLL1 (the methyltransferase for H3K4me3) in adult NSCs does not affect self-renewal, but rather alters multi-lineage

potential of NSCs and loss of neurogenesis [80]. Measurements of H3K4me3 in aged NPCs are yet to be performed.

Thus, the H3K4me3 mark seems to be a relatively invariant (or even decreased) modification at accessible sites in stem cells with some local changes in breadth and intensity driving age-related phenotypes.

3.3. Loss of facultative heterochromatin induces a pre-senescence phenotype in aged stem cells

In contrast to H3K4 methylation, which is mostly associated with active transcription, different forms of H3K27me have distinct functions. H3K27me1 is associated with permissive gene expression, while H3K27me2/me3 mark facultative heterochromatin and are correlated to gene repression [81]. H3K27me3 is exclusively catalyzed by the polycomb repressive complex 2 (PRC2) via its catalytic subunit EZH1/2. The PRC1 complex stabilizes and maintains PRC2-initiated heterochromatin domains. Depletion of the core PRC2 components (*Ezh2*, *Suz12*, *Eed*) in HSCs severely compromises hematopoiesis. Loss of Eed and Suz12 leads to a significant reduction in the number of HSCs, compromised function in competitive repopulation assays, and reductions in global H3K27me3 [82–84]. As HSCs age, the number and genomic location of H3K27me3 peaks remains largely unchanged, but there is a broadening of coverage and a significant increase in intensity of the H3K27me3 signal [30]. H3K27me3 and PcG proteins safeguard HSCs from exhaustion by repressing the *p16* locus and delaying or bypassing senescence [85]. This finding contradicts with the loss of *Suz12*, *Eed* and especially *Ezh2* expression during HSC aging although Ezh1 may play a compensatory role [86].

The ablation of H3K27 demethylase JMJD3/UTX/KDM6A in MuSCs blocks myofiber regeneration and results in defective muscle repair in mice. Furthermore, *Jmjd3* knockdown causes a significant reduction in HOXA9 (Homeobox Protein A9) protein levels in old MuSCs and deletion of HOXA9 *in vivo* improves regeneration following injury [87] supporting the importance of proper maintenance of H3K27me3 levels for MuSC function. MuSCs deficient in SUV420H1 lose H4K20me2, another histone modification linked to facultative heterochromatin which disrupts quiescence, and depletes stem cell numbers in repeatedly injured muscles [88]. These observations suggest that a general loss of facultative heterochromatin is detrimental to MuSC quiescence and productive regeneration.

In young NSCs, the p16 locus is marked by H3K27me3, allowing for proficient stem cell self-renewal. By contrast, in old NSCs, the activity and/or levels of BMI1 (a PRC1 component) and EZH2 decrease and the levels of JMJD3 increase, leading to the loss of this repressive mark. Consequently, the *p16* locus is derepressed, leading to cell cycle arrest and senescence of aged stem cells [89]. Deletion of BMI1 in adult NSCs, triggers increased glial cell production and decreases neurogenic capacity after serial passaging [90]. As the increase of astrocyte number is known to be accompanied with brain aging, the decline of BMI1 during aging may contribute to the loss of NSC multipotency. The deletion of *Ezh2* in NSCs/NPCs results in the upregulation of differentiation genes normally decorated by H3K27me3 and a reduction in progenitor cell proliferation which leads to impaired neurogenesis [91]. Failure of aged stem cells to induce JMJD3 may render NSCs less

responsive to differentiation signals and thereby contribute to decreased differentiation programs during aging [92,93]. Loss of PRC2 components in adult ISCs also causes cell cycle arrest and spontaneous differentiation towards the secretory lineage without affecting the enterocyte-specific differentiation program [94–96].

These studies suggest that an increase in facultative heterochromatin in aged stem cells protects from age-related derepression of senescence inducing genes such as *p16*. Aberrant loss of repressive facultative heterochromatin marks such as H4K20me2 (by loss of *Suv420h1*) or H3K27me3 (either by loss of *Ezh2*, *Bmi1* or overexpression of *Jmjd3*) impairs proliferation and promotes a skewed differentiation program.

3.4. Amplification of bivalent domains during stem cell aging

The presence of bivalent domains (with both the repressive H3K27me3 and the active H3K4me3 mark) are thought to represent a poised status ready for rapid activation or repression of lineage commitment genes [97]. Indeed, bivalent domains are present at lineage-specification genes in HSCs [30] and are resolved to single modification states during differentiation. Consistently, the frequency of bivalent promoters in the young hematopoietic system is highest in HSCs and progressively declines during differentiation into lineage-restricted progeny [98]. In young HSCs, bivalent domains are present at the promoters of many master TFs involved in HSC fate determination and glycoproteins involved in key HSC signaling pathways. As HSCs age, there is a net ~4-fold increase in the number of bivalent domains, which occurs due to gains in both H3K4me3 and H3K27me3 (new bivalent domains), or a gain in only H3K27me3 at promoters with existing H3K4me3 [30]. In contrast, human HSCs from young donors show many bivalent domains over developmentally regulated gene promoters. With aging, there is a bivalency-to-repression switch mediated by loss of H3K4me3 with no change in H3K27me3 [75]. Such a switch while not altering steady state gene expression, is thought to reduce the chances of improper gene activation. In quiescent MuSCs from old mice, bivalency is observed specifically at histone genes within histone gene clusters resulting in the downregulation of expression. Interestingly, these domains are rapidly resolved upon activation with restoration of histone gene expression [76].

The mechanisms contributing to increase in bivalent domain numbers during aging is unclear and could simply be a consequence of invariant H3K4me3 levels and global increase in H3K27me3. The functional relevance for bivalent domain gains during aging remains to be determined.

3.5. Enhancer remodeling predisposes aged stem cells to cancer

Enhancers are regulatory elements in the genome that are decorated with H3K4me1. Active enhancers show high levels of H3K27ac and H3K4me1, loop over to core promoters and activate gene transcription [99]. ChIP-seq studies in HSC-enriched samples from healthy human donors showed a sharp decrease in enhancer modifications with aging [75]. Specifically, there were many more active enhancers marked by H3K4me1 and H3K27ac that were lost during aging than poised enhancers marked by H3K4me1 and H3K27me3. Importantly, several genes regulated by these delicensed enhancers were transcription factors

(such as *RUNX1/2/3*, *HIF1A*, *MEIS1*, *IKZF1*, *KLF6*, *ETV6*, *GFI1* etc.) encoding tumor suppressors partly explaining the propensity for developing leukemias with age. Notably, knockdown of *KLF6* in normal HSC contributes to impaired differentiation and generates similar expression profiles with those in acute myeloid leukemia.

3.6. Disruption of chromosome territories in aging stem cells

Young HSCs express high levels of the activating mark H4K16ac with a polarized distribution (also known as epipolarity), being opposite to the cytoplasmic tubulin pole. A subpopulation of aged HSCs showed low H4K16ac and displayed a more diffuse nuclear signal. The altered H4K16ac in these aged HSCs was reversed by pharmacological inhibition of the small RhoGTPase CDC42, concomitant with partial restoration of HSC function [100]. Interestingly, H4K5ac, H4K8ac, and H3K27ac also displayed epipolarity in young HSCs, although only H3K27ac showed an age-related loss of polar distribution. ChIP-seq of H4K16ac on young and old HSCs, revealed enrichments at intronic and intergenic enhancers and some correlation to expression of specific genes. However, when comparing normalized H4K16ac peak distribution, mostly enrichments were found to be on chromosome 11. DNA FISH experiments showed that chromosome 11 probes were indeed colocalized to the H4K16ac pole but the distance between chromosome 11 homologs to each other significantly increased in aged HSCs. It was thus concluded that H4K16ac in HSCs, maintains chromosome 11 nuclear territory and that disruption of this higher order structure may compromise HSC function in aging. In support of these chromosome scale changes, it was noted that aged HSCs show changes in nuclear shape and size including a higher nuclei volume with fewer invaginations [101].

3.7. Loss of lamina-bound heterochromatin during aging

Constitutive heterochromatin is found in pericentromeric regions, interacts with the nuclear lamina and is broken down in senescence and aging [27]. The nuclear lamina is a complex meshwork of intermediate filament proteins lamin A (LMNA), lamin B1 (LMNB1), lamin B2 (LMNB2) and lamin C (LMNC). They not only provide structural support maintaining the size and shape of nuclei, but also serve non-structural functions such as heterochromatin organization, nuclear pore positioning and transcriptional regulation [102]. Heterochromatin loss is also a characteristic in many laminopathies including premature aging diseases such as Progeria and Werner Syndrome [103].

The heterochromatin tethered to the nuclear lamina (lamin-associated domains or LADs) is decorated with a unique epigenetic signature of DNA methylation, H3K9me2, H3K9me3, H4K20me3 and HP1. SUV39H1, the enzyme responsible for H3K9me3, decreases with age in both human and mouse HSCs, resulting in a global reduction in H3K9me3 and perturbed heterochromatin function [104]. Lamin A/C, peripheral H3K9me2 and H4K16ac epipolarity are also reduced in old HSCs with a concomitant increase in frequency of larger and misshapen nuclei [101]. Disruption of G9a and GLP (responsible for H3K9me2) in postnatal neurons alters the transcription of neuronal genes. These dimethyltransferases also participate in adult NSC self-renewal and regulates the balance between neurogenesis and gliogenesis [105].

Taken together, age-related loss of lamina-bound heterochromatin is highly disruptive to stem cell function. Table 2 highlights the key histone modification changes occurring during stem cell aging.

4. Regulation of stem cell function by metabolites and implications in aging

Epigenetic enzymes use intermediary metabolites as cofactors to modify DNA and histone components. For example, acetyltransferases use acetyl-CoA, methyltransferases use S-adenosyl methionine (SAM), deacylases use NAD+, demethylases use alpha-ketoglutarate and flavin adenine dinucleotide (FAD), kinases and chromatin remodelers use ATP [79,106]. Additionally, mitochondrial reactive oxygen species (ROS) produced via oxidative phosphorylation can have DNA damaging effects [107]. Thus, the cellular metabolic state and cofactor abundance can have profound effects on the epigenome. Importantly, how change in metabolic state during aging affects chromatin state is an exciting area of research.

Intracellular pools of acetyl-CoA can have direct effects on histone acetylation and gene expression. Acetyl-CoA is produced by two enzymes: acetate-dependent acetyl-CoA synthetase 2 (ACSS2) and citrate-dependent ATP-citrate lyase (ACLY). In neuronal cultures, acetyl-CoA derived from chromatin bound ACSS2 can directly fuel histone acetylation and upregulate neuronal gene expression. *In vivo*, hippocampal depletion of ACSS2 impairs spatial memory [108]. In NSC cultures, knockdown of TP53 inducible glycolysis and apoptosis regulator (TIGAR), an important regulator of NSC differentiation, decreased ACLY, acetyl-CoA levels and H3K9ac at neuronal genes [109]. To our knowledge, the role of acetyl-CoA or acetate supplementation has not been investigated in HSCs, MuSCs or ISCs.

SAM is the primary methyl donor in cells and enzymatic donation of the methyl group to chromatin or phosphoethanolamine (the two major sinks of SAM) produces S-adenosylhomocysteine (SAH). Intracellular SAM/SAH ratio dictates the overall methylation potential. Restriction of SAM precursor methionine or SAM depletion itself impairs the proliferation of ISCs in the *Drosophila* gut by a direct effect on protein translation that does not involve transfer of a methyl group [110]. The effect of SAM on other stem cell types have not been investigated and thus remains an intriguing area of future research. For example, it has recently been shown that SAM depletion leads to elevation of H3K9me1 with loss of the di- and trimethyl forms. The H3K9me1 allows for heterochromatin stability under low SAM conditions and epigenetic persistence upon metabolic recovery [111]. Whether similar resilience mechanisms occur in stem cells and how they are changed during aging remain to be investigated.

Nicotinamide adenine dinucleotide (NAD+) is an essential cofactor implicated in various biological processes such as DNA repair, gene expression, mitochondrial function etc. and its levels decline with aging. Numerous studies have shown benefits of NAD + supplementation (in the form of precursors) in mitigating the degenerative effects of aging [112]. Importantly, NAD +acts as a substrate for a class of deacylases called sirtuins, for example, the nuclear SIRT1 and SIRT7. NAD + booster, nicotinamide riboside (NR)

increased mitochondrial clearance in HSCs and improved blood reconstitution in both mice and humans [113]. Dietary NR given to aged mice significantly increased MuSC numbers, stem marker expression and regeneration post-injury. This effect was primarily due to improved mitochondrial function and reduction of DNA damage. NR treatment also increased NSC numbers and neurogenesis in aged mice [114]. Similarly, administration of resveratrol, a SIRT1 activating plant polyphenol, improved neurogenesis in aged rats [115]. While much of the SIRT1 activation/NAD + supplementation work seems to be linked to mitochondrial function, given the histone deacetylase activity and role in DNA repair of some sirtuins [116], a direct impact on chromatin cannot be disregarded.

Many quiescent adult stem cells (HSCs and NSCs) maintain a glycolytic metabolism adapting to their hypoxic microenvironment and are sensitive to ROS. In presence of excess ROS, they undergo apoptosis or differentiation [117,118]. FOXO TFs, a subset of Fork-head Box (FOX) family TFs with longstanding roles in longevity [119], confer protection against oxidative stress and enhance stem cell survival [117]. ROS-induced hyperproliferation of ISCs in *Drosophila* can lead to stem cell exhaustion and degeneration [120]. In the muscle, a small reserve population of Pax3+ MuSCs shows exceptional radiotolerance (i.e. protection from irradiation induced ROS burst) and muscle regeneration capacity [121]. ROS production can negatively affect the epigenome. γ-H2A.X (phosphorylated histone variant H2A.X), an early sensor of ROS-induced DNA damage can be incorporated into chromatin and upregulate senescence-associated inflammatory gene expression as has been previously reported [122].

Thus, metabolic status can have important consequences on the epigenome and transcriptome, but their regulation in tissue-specific stem cells especially during aging, has been explored only marginally.

5. Stem cell rejuvenation by longevity interventions

Longevity extending interventions such as dietary restriction (DR), rapamycin treatment, reprogramming and senolytics have the potential to improve multiple health parameters, delay age-related disease and extend lifespan. Can these interventions rejuvenate stem cell function, and if so, how? A potential mechanism is through reprogramming of the epigenome to favor a balance of self-renewal and differentiation that is characteristic of young stem cells.

While periodic or short-term DR improves HSC, MuSC and NSC function, long-term or life-long restriction did not protect organisms from age-related functional decline [123]. For example, a 9-month 30% DR subdued age-related increase in HSC numbers, showed less myeloid skewing and improved transplantation efficiencies. However, DR also lead to decreased B lymphopoiesis and enhanced erythropoiesis/myelopoiesis suggesting DR can have pleiotropic effects on hematopoiesis [124]. Short-term calorie restriction in young and old mice increased the frequency and function of MuSCs accompanied by elevated expression of epigenetic regulators SIRT1 and FOXO3a [125]. Both SIRT1 and FOXO3a induce autophagy for rapid activation of MuSCs in case of injury [126]. In fact, FOXO3 TFs were targeted to many autophagy genes [127]. DR in middle-aged mice elicits a dominant

Paneth cell response in the gut by downregulating the mammalian target of rapamycin complex 1 (mTORC1) and augmenting ISC numbers and function [128]. This effect is mimicked by rapamycin treatment. mTOR signaling is generally upregulated in aged HSCs, and rapamycin treatment protects from age-related increase in HSC number while also improving blood reconstitution in irradiated recipients [129]. A 24-h short-term DR in aged mice induced a fatty acid oxidation (FAO) program as assessed by RNA-seq and increased ISC number and function. These FAO genes were targets of peroxisome proliferator-activated receptor (PPAR), a family of nuclear receptor TFs. Importantly, activation of this PPAR-FAO axis by an agonist improved aged ISC function [130]. In NSCs, a stepwise DR regimen initiated at 14 weeks showed transient increase in numbers, improved olfactory memory and protection against loss of neurogenesis with age [131]. To our knowledge, how dietary interventions affect the NSC epigenome has not been explored.

Reprogramming by overexpression of OCT4, SOX2, KLF4, and MYC (OSKM) is another strategy to rejuvenate aged cells. Complete reprogramming of aged HSCs into induced pluripotent stem cells followed by blastocyst complementation, re-differentiation into HSCs and serial transplantation showed remarkable repopulation capacity invariant with young cells. Since the genetic material of the stem cells was unchanged, this type of rejuvenation was attributed to an epigenetic resetting although the exact mechanisms remain to be identified [132]. Partial reprogramming by short-term cyclic expression of OSKM also had positive outcomes in aged mice [133]. OSKM induction partially restored the MuSC numbers in old LAKI-4F mice and significantly improved regenerative capacity post cardiotoxin injury. Fibroblasts isolated from tails of old LAKI-4F, upon partial reprogramming, showed restoration of high H3K9me3 to youthful levels and a reduction of H4K20me3 that is normally upregulated with age. There is also evidence from other studies that partial reprogramming can turn back the DNA methylation clock further supporting the notion that reprogramming directly affects the epigenome [134]. However, whether similar changes occur in stem cells remains to be investigated.

Clearance of senescent cells that accumulate in aged tissues has recently been shown to improve healthspan and lifespan in mice and ameliorate age-related disease symptoms [135–137]. Oral administration of ABT263, a potent senolytic drug, effectively depleted senescent HSCs and MuSCs in irradiated or naturally aged mice and rejuvenated aged tissue. Treatment of ABT263 improved clonogenicity, engraftment ability, quiescence and reduced myeloid skewing [138]. Treatment of obese mice with another senolytic cocktail (dasanitib and quercetin), improved neurogenesis and ameliorate anxiety behavior [139]. The reversibility of age-related changes upon senescent cell clearance strongly implies that senescent cells have a negative effect on the epigenome of the surrounding non-senescent cells.

Thus, the diverse strategies that rejuvenate aged tissues, mediate changes in the tissue-specific stem cell population. It is likely that the longevity interventions have direct impact on the epigenome although the exact mechanisms remain to be investigated.

6. The advent of single-cell technologies to study stem cell aging in high resolution

The development of single-cell (sc) technologies have accelerated discoveries in stem cell biology allowing the investigation of DNA methylation, histone modifications, chromatin accessibility, and chromosome conformation in high resolution [140].

Combined scRNA-seq, scATAC-seq and 3D confocal microscopy showed that young HSCs divided asymmetrically, while aged HSCs divided more symmetrically. Moreover, the potential of daughter cells was linked to the amount of H4K16ac and open chromatin distributed to it during cell division but not on the transcriptome [141]. scRNA-seq of young and old human HSCs show a preponderance of non-cycling cells (nc-HSCs) with minor populations of multi-lineage progenitors. Closer analysis of young and old nc-HSCs was able to segregate them into two minimally overlapping groups suggesting that the aged cells were transcriptionally distinct from the young cells. Another study used a multiplexed mass cytometry time-of-flight (EpiTOF) analysis to profile the global levels of chromatin modifications in primary human immune cells at the single-cell level. Age increased variability of modification levels between individuals as well as between cells. PRC1/2-mediated repressive modifications showed the highest variability with age [142]. In support, a very elegant single-cell multi-omics study simultaneously profiling transcriptome and DNA methylome in young and aged MuSCs highlighted a similar increase in cell-to-cell variability with aging primarily affecting cell-niche interactions [34]. In further support, an independent study using scRNA-seq showed increased gene variance in aging despite the overall similarity of the young and old transcriptomes [25]. RNA-velocity analysis of this scRNA-seq dataset showed a delayed and dampened rate of activation during aging, although aged MuSCs followed the same trajectory as young. Interestingly, aged MuSCs stochastically progress along the trajectory, often reverting to the previous state.

Single-cell transcriptomics of aging neurogenic niches showed an infiltration and clonal expansion of T cells [26]. These T cells were hypothesized to recognize neoantigens in the aging brain such as protein aggregates in old NSCs or respond to a chemokine released by glial cells. Furthermore, these T cells release interferon gamma and a subset of NSCs that show high interferon response, also show reduced proliferation that could explain the decline in NSC number in the aging brain. This dataset additionally revealed that the aging neurogenic niche had lesser number of activated NSCs compared to younger mice. Similar single-cell studies in regenerating intestines of irradiated mice have been used in the discovery of novel stem cells although age-related studies remain to be done [143].

Single-cell genomic/epigenomic analyses have contributed and will continue to enrich our understanding of the changes in cell type, cell state, behavior, and activation trajectory in stem cells during aging. Additionally, a wealth of information is available through the Tabula Muris Senis, a multi-tissue bulk and single-cell RNA-seq database profiled across mouse lifespan [144]. Together, they promise to provide critical insights into stem cell aging biology.

Fig. 2 summarizes all the epigenetic themes of stem cell aging discussed in this review.

7. Concluding remarks

Our review attempts to provide a comprehensive summary of the “epigenetic drift” patterns observed during stem cell aging. The evidences suggest a reconfiguration of the chromatin state to a global increase in DNA hypermethylation, an imbalanced constitutive and facultative heterochromatin, a loss of active enhancers, increased bivalency and even a disruption of chromosome territories. The consequences of these epigenomic changes are reflected in functional outcomes such as altered self-renewal patterns and/or senescence phenotypes that impact stem cell number. Additionally, there is dramatic change in stem cell potential, lineage bias, delayed activation kinetics and ultimately higher frequencies of disease phenotypes such as cancer. While “drift” patterns are not necessarily programmed, it may be possible to delay their accumulation or even reverse the changes by late-life epigenetic drug interventions or cellular epigenome reprogramming strategies that “wipe out and start over”.

Multi-omic approaches and single-cell genomics have greatly accelerated stem cell research, but it is important to note that due to limited numbers of stem cells in tissues, these methods have been difficult to implement. Advances in organoid cultures that afford stable expansion of adult stem cells may greatly add and improve upon these findings in the future. Currently, there are large gaps in the field including the lack of information on 3D genome organization, genome-wide TF maps and enhancer landscapes. Additionally, the underlying epigenetic mechanisms employed by longevity altering treatments to rejuvenate stem cells and improve tissue regeneration remains to be explored.

We predict that the next decade of aging research will make leaps and bounds in addressing these unknowns in stem cell biology and greatly improve the possibility of stem cell-based rejuvenation therapies.

Acknowledgements

We wish to acknowledge the National Institute on Aging Intramural Research Program, National Institutes of Health, for financial and resource support. We also thank Myriam Gorospe and Maire Doyle for critical reading of the review. All figures were created with BioRender.com.

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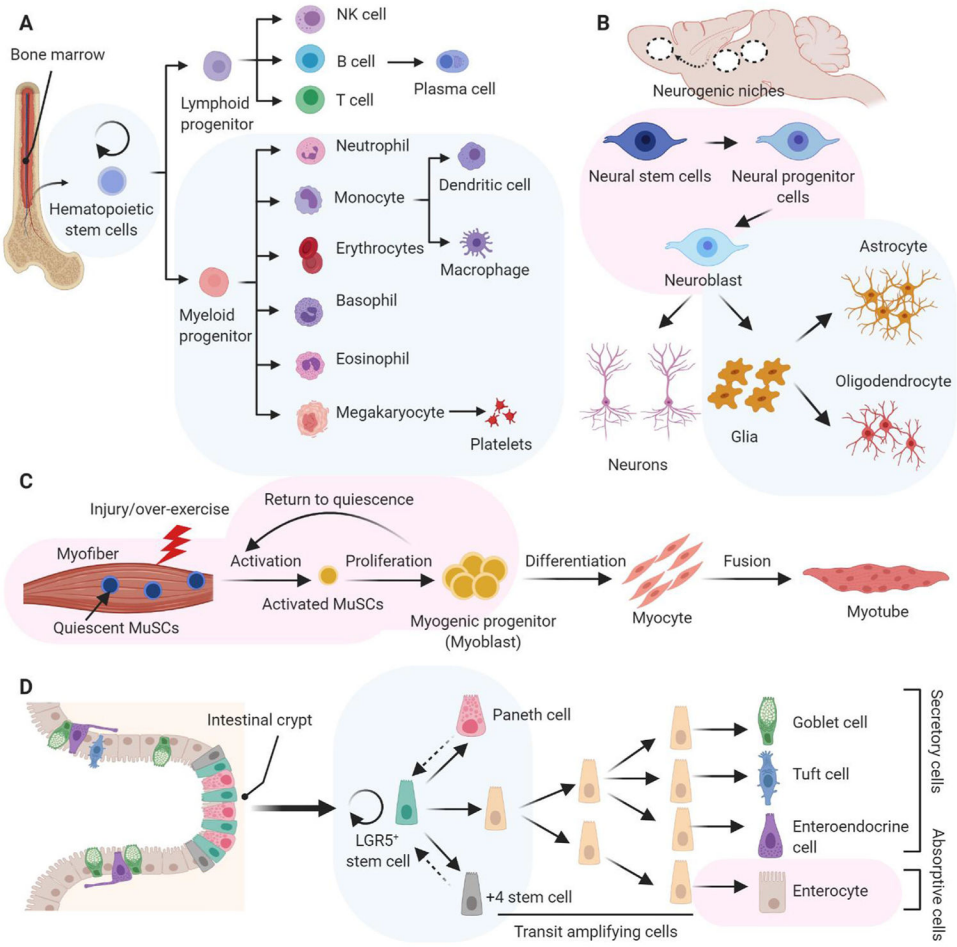

Fig. 1. Lineage tree of the main stem cell types described in this review.

(A) Hematopoietic stem cells undergo asymmetric division to self-renew as well as differentiate into lineage restricted myeloid and lymphoid progenitors. Myeloid progenitors produce monocytes, neutrophils, basophils, eosinophils, erythrocytes, and megakaryocytes. Monocytes further differentiate into macrophages and dendritic cells while megakaryocytes produce platelets. Lymphoid cells include T cells, B cells and natural killer (NK) cells. **(B)** Neural stem cells reside in special neurogenic niches in the brain and produce all neuronal cell types in the central nervous system including neurons and glia. Glial cells include astrocytes and oligodendrocytes. **(C)** In the skeletal muscle, quiescent stem cells called satellite cells or simply muscle stem cells (MuSCs) are activated upon injury or over-exercise and proliferate to produce myoblasts which differentiate into myocytes. Myocytes can fuse to form multinucleated muscle fibers. Meanwhile, a fraction of myoblasts returns to quiescence to avoid exhaustion of the stem cell pool. **(D)** In the intestinal crypts, two types of stem cells exist. The rapidly dividing Lgr5+ cells and the plastic 4+ cells. These differentiate to produce either secretory cells (paneth, goblet, tuft and enteroendocrine) or absorptive enterocytes via intermediate transit-amplifying cells. +4 stem cells and paneth cells under conditions of damage, can revert to Lgr5+ cell type (dotted arrows). Steps in the stem cell self-renewal, activation and differentiation process that are affected with age are shown in colored rectangles. Steps upregulated during aging are shown in blue and those

downregulated are shown in red. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

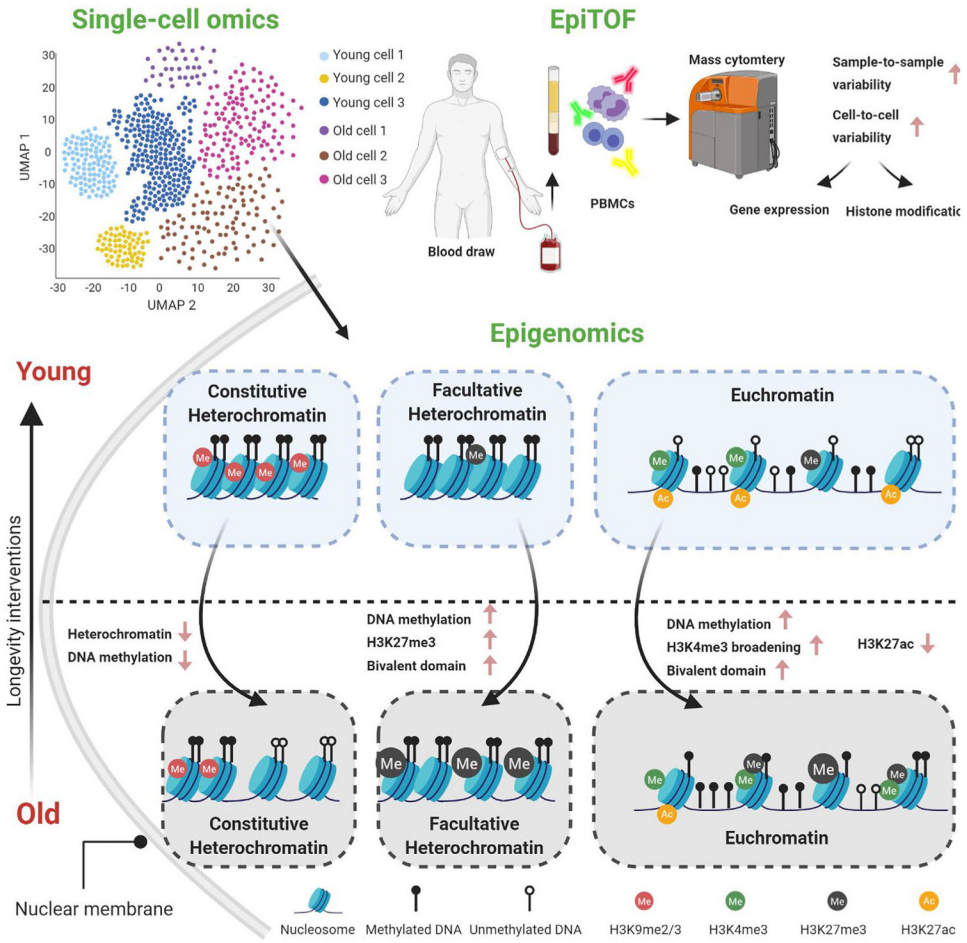

Fig. 2. Overarching epigenetic themes of stem cell aging.

Ensemble and single-cell omics studies have identified some key concepts in stem cell aging. Single-cell transcriptomics (RNA), epigenomics (ATAC) and EpiTOF (histone modification) analyses have shown remarkable increases in variability with aging, both sample-to-sample and cell-to-cell. Genome-wide assessments of DNA methylation and histone modifications have identified loss of constitutive heterochromatin (reduced H3K9me2/3, focal loss of DNA methylation), gain of facultative heterochromatin (increase in H3K27me3), increases in bivalent domains (dually modified H3K4me3/H3K27me3), decrease in active enhancers (reduced H3K27ac), global DNA hypermethylation and broadening of H3K4me3. Collectively, these changes alter self-renewal and reduce stem cell potential but are partially reversible through longevity interventions.

Table 1

Alterations in the DNA methylome during stem cell aging.

	Function	Change during aging	Effect on stem cells during aging
DNMT1	DNA methyltransferase: maintenance parental cell DNA methylation	Decrease	Lineage bias and self-renewal defects [37–41]
DNMT3A	DNA methyltransferase: *de novo* DNA methylation	Decrease	Increase in self-renewal with age at the expense of differentiation [45]
DNMT3A/3B	DNA methyltransferase: *de novo* DNA methylation	Decrease	Severe arrest of HSC differentiation [45,46]
TET1	DNA demethylation; converts 5mC into 5hmC	Decrease	Enhance HSC self-renewal, lymphoid bias [52] Enhance ISC proliferation [56]
TET2	DNA demethylation; convert 5mC into 5hmC	Decrease	Attenuate HSC differentiation, myeloid bias [50,51]
5mC	Transcriptionally repressive	Global increase, site specific gains and losses	Hypermethylation at promoters associated with lineage potential, selectively targeting PRC2 binding sites, HSC fingerprint genes and rRNA genes [30,31]
5hmC	Transcriptionally activating	Decrease	Important for ISC self-renewal, enriched at WNT target genes [56]

Table 2

Alterations in histone modification levels during stem cell aging.

	Function	Change during aging	Effect on stem cells during aging
H3K4me3	Protects promoters from DNA methylation, keeps chromatin in an open state	Either remains constant or slightly decreases, local broadening of peaks	Correlates to most upregulated genes, broadening may occur at genes related to self-renewal and loss of differentiation [30,79]
H3K27me3	Inhibits transcription by preventing access to RNA polymerase II and other trans-factors	Breadth and intensity of peak increases	Protects from age-related derepression of senescence genes [30,89]
H3K4me3/ H3K27me3	Bivalent marks: maintain pluripotency	Amplification	Histone gene downregulation in HSCs, restricts the potential of the aged stem cells [30,76]
H3K27ac	Active enhancer and promoter mark	Decrease	Alters expression of tumor suppressor genes [75]
H4K16ac	Maintenance of chromosome territories	Loss of epipolarity, reduced levels and more diffuse pattern	Loss of regenerative capacity and myeloid lineage skewing, larger and misshapen nuclei [100,101]
H3K9me2/3	Constitutive heterochromatin	Decrease	Loss of pericentric heterochromatin, larger and misshapen nuclei [101]
H4K20me2	Facultative heterochromatin	Unknown	Knockdown of SUV420H1 reduces facultative heterochromatin [88]