Epigenomics and the regulation of aging

It is tempting to assume that a gradual accumulation of damage 'causes' an organism to age, but other biological processes present during the lifespan, whether 'programmed' or 'hijacked', could control the type and speed of aging. Theories of aging have classically focused on changes at the genomic level; however, individuals with similar genetic backgrounds can age very differently. Epigenetic modifications include DNA methylation, histone modifications and ncRNA. Environmental cues may be 'remembered' during lifespan through changes to the epigenome that affect the rate of aging. Changes to the epigenomic landscape are now known to associate with aging, but so far causal links to longevity are only beginning to be revealed. Nevertheless, it is becoming apparent that there is significant reciprocal regulation occurring between the epigenomic levels. Future work utilizing new technologies and techniques should build a clearer picture of the link between epigenomic changes and aging.

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In humans and many other complex organisms, aging is characterized by an increase in damage to the system, and by a decrease in the adaptability and energy efficiency of the system. However, although it is tempting to draw the conclusion that it is the accumulation of damage within the system that 'causes' an organism to age, there may also be certain biological processes, whether 'programmed' or 'hijacked', that control what, and how fast, changes occur. For example, the regulatory mechanisms necessary at specific points in the lifespan for the correct differentiation, sexual development or adaptation to an environment may lead to aging phenotypes later in life [1,2]. In addition, agents that cause damage, such as reactive oxygen species (ROS), may be required by the system for important signaling or feedback mechanisms [3].

In this review we will begin by introducing some theories on how we age, and the key regulators that have been identified. Then we will focus in depth on the epigenetic mechanisms of genome regulation during aging – specifically DNA methylation and histone modifications. Although the inclusion of ncRNA as a true epigenetic mechanism is controversial, some ncRNA are now known to be heritable ([4], and reviewed in [1]), and we introduce ncRNA briefly here. We will also discuss the interactions between the levels of epigenetic regulation, and look forward into the future to the questions still to be investigated.

There are many theories that have attempted to name the sources of altering agents that have a role in aging, the critical locations where changes are related to system-wide aging, the pathways that are affected by aging and influence the rate of aging, and the key regulators of these processes. DNA has generally been the focus of such research as it was hypothesized that aging was derived from the perturbation of the normal expression of key genes – for example, those effecting authophagic pathways – which have recently been linked to aging (reviewed in [5]). Perturbation during aging could occur through the accumulation of mutations, especially via oxidative damage of DNA by ROS, or shortening of the telomeric ends of the chromosomes due to the inactivity of telomerase [6]. Indeed, the most significant risk factor for cancer is age, which would seem to support this notion. We can also take clues from diseases that affect the rate of aging. The accelerated aging seen in Werner syndrome and Cockayne syndrome is caused by deficiencies in DNA repair, allowing mutations to become fixed and accumulate rapidly (reviewed in [7]). It is clear, therefore, that nuclear DNA can acquire changes during the lifespan that may influence the aging process. However, in healthy individuals, DNA is protected to some degree by packaging into chromatin, and repair processes are also sufficient in most cases to keep damage to a minimum (at least away from the gene-sparse telomeric ends of the chromosomes). So can the accumulation of nuclear mutations explain the normal aging phenotype? Outside of the nucleus, mitochondria are now considered

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to be important to the aging process as they are the major site of ROS production, and possess a vulnerable genome (mtDNA) that lacks chromatin (reviewed in [8]). DNA repair deficiencies in the mitochondria also caused accelerated aging when modeled in the mouse [9], but it is still not clear how important mtDNA mutations are to the 'normal' aging phenotype. In addition, the role of ROS as a mutator is being superseded by an understanding of ROS as a small signaling molecule, especially as stimulation by low levels of ROS (known as hormesis) may actually be beneficial to aging, through adaptation to stress and increased stress resistance ([10], and reviewed in [11]). This would suggest that the aging process may be more complicated than simply the accumulation of genetic mutations. When changes to the DNA code occur, there should be an immediate, localized change in gene expression. However, aging is characterized by gradual changes to the whole system, implying a coordinated global process and possibly a 'program' for aging. Studies investigating twins, populations or animal models have also shown that organisms with largely similar genetic backgrounds can age very differently (reviewed in [12]). Therefore, it would appear that, in addition to any genetic changes, environmental or other stimuli experienced during the lifespan may modify the system directly, or stimulate adaptations in the system, perturbing gene expression. The possibilities that this raises are attractive; a better understanding of genome–environment interactions could propose lifestyle interventions or drug targets that may improve the aging process (reviewed in [13]).

DNA methylation was the first true epigenetic (from the greek *epi* or 'above' genetics) mechanism of variation identified in the genome [14]. Subsequently, a plethora of histone modifications has been identified (reviewed in [15]). These two epigenomic levels are known to be linked to distinct gene expression and replication phenotypes, and to be modified by environmental factors. It has been hypothesized that epigenetic marks at key genomic regions, such as transcription start sites, splice sites, and especially in the coding or control regions of known aging regulators, could be an important way that environmental cues are 'remembered' by a system, effecting the wider aging process. The question is, where are the effects most important; to what genes, proteins, processes, and in which cells and tissues? In the epigenomics field we choose to focus not on individual modifications to specific gene loci, but instead on an understanding of epigenetics in a whole genome context.

The most widely applicable way to increase lifespan is through dietary restriction (DR) (reviewed in [16]). DR has been shown to increase the lifespan of yeast by 25% [17], *Caenorhabditis elegans* by 17–25% [18], *Drosophila* by 100% [19] and rodents by 30–40% [20]. However, it should be noted that two studies on primates that are presently underway disagree on whether or not there is reduction in age-related mortality with DR [21,22]. Researchers have sought to tease apart the system-wide changes brought on by DR to identify the key changes that are occurring at the cellular and tissue scale, and then attempt to mimic these effects specifically. DR has been postulated to increase lifespan by decreasing ROS production (or by stimulating hormesis), increasing autophagy and mitophagy, and stimulating expression of some key aging regulators. The known lifespan regulators all reside in nutrient-sensing or stress-response pathways (reviewed in [23]):

- Foxo/Daf-16 and IGF1R/Daf-2, in the insulin/IGF signaling pathway;

- AMPK/AAK-2, in the ROS signaling pathway;

- mTOR/LET-363, in the autophagy and translation pathway;

- SIRT1/2, in the genome stability pathway (histone deacetylation).

One important route for the effects of DR could be via changes to the epigenome, which in turn effect lifespan. In support of this hypothesis, SIRT1/2 are epigenetic modifiers, and there is much data that suggests they have some involvement in healthy aging ([24], and reviewed in [25]). However, it should be noted that recent evidence suggests that their involvement in lifespan regulation may not be as dramatic as was once thought [26]. On the other hand, a recent systems biology study of the DR interactome gene network has shown that genes involved in chromatin organization and modification are significantly enriched in DR-stimulated network interactions, emphasizing chromatin regulation as an important mediator for DR effects on aging [27].

Approaches to study the epigenomics of aging

Epigenomics, like other 'omics', is benefiting from the accessibility of affordable high-throughput techniques such as the rapidly developing second-generation sequencing platforms of 454 Pyrosequencing® [28], Illumina®

and SOLiD™ [29]. The analysis of raw data from these technologies requires the availability of significant but affordable computer power, and complex computational methodologies. Epigenomics utilizes these approaches to evaluate the genome as a whole from specific cells or tissues and in specific situations (e.g., samples of different ages). This is also the basis of so-called epigenome-wide association studies which, in a similar way to genome-wide association studies, link phenotypes to the epigenome (reviewed in [30]). As biases exist for different platforms, library construction and experimental protocols, consideration must be taken into account when designing and analyzing next-generation sequencing data. This is particularly important for avoiding false-positive sequence changes between samples, especially for epigenomic analyses of DNA methylation [31]. Epigenomic studies must also be cautious when assuming the universality of epigenetic changes observed in a heterogenous tissue. It is possible that apparent changes to the epigenome may only represent changes in cell-type ratios within a tissue, and should be confirmed across different tissues and ideally on the level of individual cell types. In addition to human tissues and cell lines, there are several model organisms that have been used extensively in epigenomics. Plant methylation is well studied, but it would now appear that there are significant differences between the mechanisms in plants compared with animals, which prevent us from drawing parallels. *C. elegans* has been widely used for histone epigenomics but is a poor model for DNA methylation, which appears to be absent in the *C. elegans* genome [32]. On the other hand, insect models such as honeybees for DNA methylation, and *Drosophila* for a variety of epigenetic modifications, are widely used, and increasingly mice and rats. There have been suggestions that epigenetic modifications may be more speciated than the DNA sequence itself, possibly even more so than histone modifications, and comparative studies between humans and primates could be an interesting new approach to epigenomics [33]. The major hurdle of current epigenomic studies has been the requirement of large quantities of starting material. Many of the techniques used in the preparation of samples before sequencing (see **Boxes 1 & 2**), such as bisulfite treatment and chromatin immunoprecipitation (ChIP), have poor yields. Third-generation sequencing technologies have great potential to streamline analyses, including single-molecule sequencers, such as the HeliScope™ system, that remove the

need for library construction and PCR amplification [34]. Research continues towards miniaturization and/or automization of techniques, allowing for the processing of smaller quantities of precious samples with the eventual goal of single-cell epigenomics.

There are now databases and projects designed to curate different layers of epigenetic data for future examination of epigenetic associations with phenotypes, such as the rate of aging for an organism. For example, MethylomeDB and NGSmethDB curate DNA methylation profiles for human and mouse tissues and cell lines [35], the Human Epigenome Project analyzes DNA methylation in each tissue, the ENCODE and modENCODE projects curate epigenomic data for human cell types and model organisms (*C. elegans* and *D. melanogaster*), respectively, and expression databases can allow for functional comparisons to epigenetic patterns. To interpret this data and predict which changes, or artificial perturbations, are the most important, requires us to consider the system as a whole. Therefore, many epigenomics studies choose a systems biology approach, which computationally integrates the data from various levels of regulation, for example, ChIP-seq and RNA-seq data, to predict the key regulators of aging.

Regulation of epigenomic marks
■ DNA methylation

DNA methylation occurs at the 5′ position of a cytosine to form 5-methylcytosine (5mC), through the donation of a methionine group from *S*-adenosylmethionine (SAM), predominantly at cytosines in a CpG context [36]. It can also occur in rarer cases at non-CpG cytosines, especially, but not exclusively, in embryonic stem cells, the function of which remains unclear [36,37]. 60–70% of all CpGs are methylated; however, CpG-rich regions of up to 1 kb (known as CpG islands or CGI) are much more likely to be present in an unmethylated state [36]. Outside of CGIs the genome is depleted of CpGs, and studies have therefore mainly focused on the functional implications of the methylation state of CGIs, and in particular, CGIs at transcriptional start sites (TSSs) and promoters. The role of methylation in the gene body, and other regulatory regions such as enhancers and insulators, is only now beginning to be appreciated.

Although methylation can exist as a relatively stable and inheritable modification, it is widely accepted that methylation and demethylation is a dynamic process that is ongoing throughout an organism's lifespan. DNA methylation

Box 1. Global DNA methylation analysis.

Nonsequencing approaches

- Immunochemistry: a crude estimation of genome-wide 5-methylcytosine (5mC) and hydroxymethylated cytosine (5hmC) levels can be made using a simple ELISA or other immunochemical approach [106]
- Liquid chromatography–mass spectrometry (LC-MS): a method whereby enzymatic hydrolysis fragments genomic DNA, which is then analyzed on a liquid chromatography electrospray ionization tandem mass spectrometer to detect 5mC bases [83,233]. Whether this method can accurately differentiate between 5mC and 5hmC is unclear
- Methylated DNA immunoprecipitation (MeDIP): the precursor to methylation-dependent immunoprecipitation sequencing (IP-seq), it uses antibodies targeted to 5mC (and potentially 5hmC) to precipitate methylated regions of the genome. The precipitated DNA can be hybridized to DNA microarrays [234]
- Infinium arrays: Illumina®'s Infinium HumanMethylation27 BeadChip (27,578 CpG sites), and the new Infinium HumanMethylation450 BeadChip (485,000 CpG sites) use hybridization of bisulfite-treated DNA to beads sensitive to either methylated or unmethylated DNA that are fixed on a chip [235]

Sequencing approaches

- Whole-genome bisulfite sequencing: sodium bisulfite treatment of DNA deaminates and subsequently desulfonates unmethylated cytosines to uracil. Optional amplification and subsequent sequencing and comparison of treated and untreated DNA identifies methylated cytosines in the genome at the single nucleotide level [236]. However, bisulfite treatment cannot distinguish between 5mC and 5hmC [237], and the harsh alkaline conditions lead to poor recovery
- Reduced representation bisulfite sequencing (RRBS): a modified bisulfite sequencing approach that enriches for CpG-containing DNA using a CpG-dependent restriction enzyme (e.g., *Msp*I) and adaptor ligation. The technique allows for more efficient sequencing as only the genomic regions containing CpGs are sequenced [238]. However, this technique under-represents non-CpG methylation
- Methyl-mapping analysis by paired end sequencing (methyl-MAPS) or methylation-sensitive restriction enzyme (MRE-seq): an approach that uses methylation-sensitive, or insensitive, enzymatic digestion of genome DNA coupled with next-generation sequencing [239]
- Methylation-dependent IP-seq or methylation capture (MethylCap)-seq: this technique uses antibodies targeted at methylated 5mC and 5hmC to precipitate or affinity capture methylated regions of the genome for downstream next-generation sequencing [240]. This approach has several advantages – using specific antibodies it is possible to specifically target mC or hmC [44], and the technique identifies mC (or hmC) outside of CpG
- Tet-assisted bisulfite-sequencing (TAB-seq): a specific whole-genome approach to map 5hmC at a single-base resolution. By first chemically adding a glucose moiety to 5hmC, using the β-glucosyltransferase enzyme, and treatment with the recombinant Tet1 enzyme, which demethylates all 5mC to 5-carboxylcytosine, only 5hmC is protected during bisulfite conversion. Comparing the data from TAB-seq and whole-genome bisulfite sequencing allows an accurate representation of the relative 5mC and 5hmC landscapes [45]

is catalyzed by the DNA methyltransferase enzymes (DNMTs). In humans there are three DNMTs: DNMT1, DNMT3a and DNMT3b. DNMT1 is generally thought of as the 'maintenance' methylase, due to its preference for hemi-methylated CpG sequences, and preserves methylation during DNA replication [38]. *In vitro* studies suggested DNMT3a and 3b had no such preference, and thus they were considered *de novo* methylases [39]. However, it is likely that this is an oversimplification. Accumulating evidence demonstrates that in specialized circumstances, such as single copy sequences and certain repetitive elements [40], DNMT1 can also *de novo* methylate and DNMT3a and DNMT3b have a role in maintenance methylation [41,42].

Demethylation of 5mC is not fully understood. The most important finding in recent years was the presence of hydroxymethylated cytosines (5hmC) in the genome, albeit present at much lower levels than 5mC [43–45]. The Tet (ten eleven translocases) family of enzymes catalyze the conversion of 5mC to 5hmC [43]. They may also convert 5hmC further to 5-formyl-cytosine and 5-carboxylcytosine, which can then be further reconstituted to unmethylated cytosine through the base excision repair actions of TDG [46,47]. It should be noted that the distribution of 5hmC does not necessarily relate to the distribution of 5mC, which suggests that 5hmC may have other unknown functional roles, a hypothesis now being explored [44,48].

■ **Epigenomic patterns of DNA methylation**

Distinct patterns of DNA methylation exist within the human genome (FIGURE 1). Changes to these patterns have been proposed as one of the major mechanisms for differential gene expression between tissues [49], and are critical for correct differentiation of stem cells [50]. Aging-associated pattern changes may therefore have a significant role in the aging process.

Methylation of CGIs in promoter regions (CGI-rich promoters) is inversely associated with transcription (FIGURE 1). Methylation can inhibit transcription factor binding or recruit methyl-CpG-binding proteins that help block transcription [51,52]. Conversely, hydroxymethylation at promoter regions is associated with a release of methyl-binding proteins and an increase in transcription [48,53]. Methylation in the gene body is

Box 2. Global histone modification analysis.

Nonsequencing approaches

- Immunochemistry: a crude estimation of genome-wide histone modification levels can be made using a simple immunochemical approach
- Liquid chromatography–mass spectrometry (LC-MS): using acid-extracted histones, a reliable approach to this method involves chemical derivatization, trypsin digestion and stable isotope labeling for simultaneous quantitative analysis of total levels of multiple histone modifications [241]
- Chromatin immunoprecipitation (ChIP)-on-chip: this technique involves ChIP and fluorophore labeling of a DNA library followed by hybridization of DNA fragments onto microarrays, where fluorescent signal intensity of the immunoprecipitation (IP) versus input samples represents ChIP enrichment [242]

Sequencing approaches

- ChIP-seq: ChIP, highly dependent on antibody quality, is followed by library construction of DNA fragments and single-end deep sequencing, where computational methods define ChIP enrichment based on peaks identified over background (input) from sequencing reads [243]
- Micrococcal nuclease digestion followed by sequencing (MNase-seq): entails native chromatin preparation and treatment with micrococcal nuclease followed by ChIP (NChIP), library construction and sequencing. This method allows for single-nucleosome resolution [244]
- ChIP combined with paired-end tag sequencing (ChIP-PET): after ChIP, segments of DNA from the ends of ChIP'd DNA fragments, either cloned into a vector or directly used, are ligated together and sequenced, producing two sequences used to identify the boundaries of target binding/modification site [245]
- Chromatin interaction analysis by paired-end tag sequencing (ChIA-PET): ChIA-PET combines ChIP with chromatin proximity ligation and paired-end tag sequencing to generate a high-resolution map of chromatin interactions, used for both transcription factors and histone modifications to increase the signal-to-noise ratio of traditional chromosome conformation capture (3C) [246,247]

not associated with transcriptional repression, but is a feature of actively transcribed genes [54,55] (FIGURE 1). Methylation may act to prevent initiation outside of the TSS, and regulate the use of alternative promoters [56]. Whole-genome studies have also shown that 5mC and 5hmC levels in gene bodies are not uniform; exons are more highly methylated (and hydroxymethylated) than introns, and there is a clear transition at exon–intron boundaries suggesting 5mC and 5hmC may help define splicing boundaries (FIGURE 1) [44,57,58]. Specifically, methylation spikes at 5′ splice sites, and dips at 3′ splice sites [58,59]. This has recently been supported by observations in the honeybee, where highly methylated exons were more likely to be included in transcripts than poorly methylated exons [60].

Methylation of repeat elements such as Alu and L1, endogenous retrotransposons and proto-genes is necessary to prevent aberrant transcription, and methylation at centromeres is important for chromosomal stability (FIGURE 1) [61]. Enhancers are characterized by the fact that they are CpG sparse [36,55]. Although the function of methylation at enhancers is unclear, most enhancers are neither 100% methylated nor unmethylated, which suggests extensive dynamic regulation [62]. Methylation could also decrease enhancer activity, or prevent promoter–enhancer interactions by modifying insulator regions (e.g., *IGF2* enhancer, an aging regulator) [63,64].

DNMTs themselves have very little specificity for target sequences. Although *trans*-acting cofactors of the DNMTs do exist, such as DNMT3L, a noncatalytic cofactor of DNMT3a/3b [65], and Uhrf1 (or Np95), which interacts with DNMTs [66,67], the distinct patterns of DNA methylation across the genomic landscape must be established and maintained by additional mechanisms. Several groups have reported that they can recreate or 'seed' methylation patterns in the genome using artificially inserted sequences, such as promoter-derived sequences [68] or repeat elements proximal to the region of interest [69,70]. In addition, there is a significant overlap between transcription factor binding sites in loci with similar methylation levels [71]. This implies that methylation patterns can be regulated in *cis*.

■ Histone modification

Chromatin is composed of nucleosome units containing an octamer of histone proteins, with two copies of each histone H2A, H2B, H3 and H4, wrapped by DNA. As a whole, chromatin can be divided into regions of euchromatin; more open, accessible and transcriptionally active, and heterochromatin, which is stably closed and repressed. The ability to maintain chromatin stability and regulate gene expression requires post-translational modifications of histone tails, including acetylation, methylation, phosphorylation and ubiquitylation, as well as novel modifications such as crotonylation [72]. Studies using ChIP for single genes and more in-depth analyses, combining ChIP with microarray (ChIP-on-chip) or deep sequencing
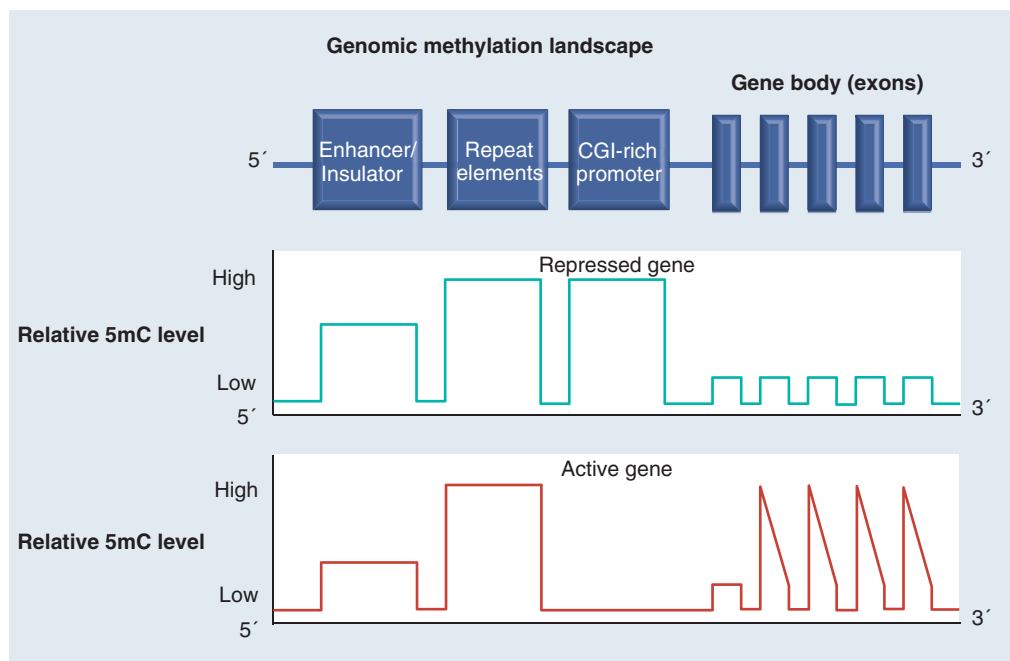
Figure 1. Model of the genomic methylation landscape at enhancers/insulators, repeat elements, CGI-rich promoters and gene exons. Enhancers/insulators are neither completely unmethylated nor methylated, but active enhancers may be less methylated. Repeat elements are generally methylated. CGI-rich promoters are methylated in repressed genes and unmethylated in active genes. Apart from the 5′ exon, actively transcribed exons will be methylated, and methylation will spike near 5′ splice sites and dip near 3′ splice sites.
5mC: 5-methylcytosine; CGI: CpG islands.

(ChIP-seq) and other modified approaches (**Box 2**), has revealed that different modifications mark different transcriptional states. Histone acetylation is generally associated with an open chromatin structure, marking sites of active transcription at the gene locus and at distal enhancer regions bound by active transcription factors [73,74]. Methylation marks many different states, with active gene expression exhibiting trimethylated histone 3 on lysine 4 (H3K4me3) at TSSs, active enhancers and TSSs displaying H3K4me2, and repressed genes associated with H3K27me3 [75,76]. A recent analysis comparing ENCODE ChIP-seq data from seven cell lines illustrates the generality of some histone marks, such as H3K9ac and H3K4me3, for detecting active genes, while H3K79me2 and H3K36me3 are more useful for determining the level of gene expression [77,78]. Different regions of chromatin are also characterized by different histone marks. H3K79me is enriched in euchromatin, whereas H3K9me3 and H4K20me3 largely occur in heterochromatin [79]. In addition, other marks associate with different events, such as phosphorylation of H2AX (γ-H2AX) at sites of DNA damage. Mechanistically, histone acetylation often functions by creating access to DNA by neutralizing positive lysine charges, while histone methylation and sometimes acetylation and other marks function by providing docking sites for other proteins. For example, bromodomain-containing proteins, such as the SWI/SNF chromatin remodelers, bind acetylated lysines, and HP1 binds to methylated H3K9 in regions of heterochromatin (reviewed in [80]).

Importantly, histone modifications are dynamic in that they are actively added and removed by histone-modifying enzymes in a site-specific manner, which is essential for coordinated transcriptional control. Illustrating specificity of histone acetylation, the histone acetyltransferases GCN5/PCAF and CBP/P300 mediate, respectively, H3K9ac and H3K18/K27ac [81]. Histone lysine deacetylases (HDACs) are grouped into four classes based on homology to yeast genes, such as the class III NAD+-dependent sirtuin family that have important roles in metabolism and aging. Thus far, methylation is known to occur through arginine- and lysine-specific methyltransferases. For example, histone H3K4 trimethylation at transcriptionally active genes is mediated by enzymes containing the general lysine methyltransferase SET domain, which reside in one of three complexes: complex proteins associated with SET1 (COMPASS); the trithorax complex; or the trithorax-related

complex. Histone demethylases are divided into three classes: methyl-arginine histone deiminases; flavin-dependent lysine demethylases; and the third and largest class that contains a JmjC domain requiring iron Fe(II) and α-ketoglutarate. In the same manner, other modifications depend on their own subset of various enzymes. Together, understanding the dynamic and coordinated regulation of epigenetic modifications has become an area of significant interest in aging research.

Epigenomic changes associated with aging

■ DNA methylation & aging

Global hypomethylation, but specific hypermethylation, is associated with both aging and cancer (TABLE 1) [82,83]. Whole-genome bisulfite sequencing (WGBS; BOX 1) of newborns (cord blood) and centenarians (peripheral whole blood) showed that age is associated with a decrease in global genomic methylation, including CpG poor promoters, exonic, intronic and intergenic sequences [84]. Although the blood sources (cord and peripheral) in that study were not identical, allowing the possibility that epigenomic differences were due to the blood source, the findings have been supported by others using consistent blood sources [85,86]. This pattern has also consistently been observed in a variety of other tissues, including the heart and bladder [87–89]. The issue of intraindividual (using different tissues) and interindividual variation in DNA methylation is important, as naïvely averaging between samples could abrogate observable trends, and may have

been the cause of initial reports that suggested that DNA methylation did not change with age [90]. Therefore, although we will now look in more detail at the genes and genomic elements that show age-related changes in DNA methylation, we must be cautious in assuming that any will show a consistent change in all tissues (or individuals). With this in mind, although we provide examples for genes that are differently methylated with age, gene ontology may provide a better picture of the types of cellular and tissue functions that are affected.

Intriguingly, age-related hypomethylated regions are mostly transposable repetitive elements, including Alu and L1, which are normally methylated and repressed, implying that there is increasing genomic instability with age (TABLE 1 & FIGURE 2) [91]. Additional significantly hypomethylated loci include normally methylated CpG-rich promoters, such as the imprinted genes *IGF2* [92], *PWCR1* and *TRPM5* [85]. Other groups have also identified many hypomethylated CpG loci involved in the inflammatory response, such as *TNF* [93], *iNOS* and *TLR2* [85], the modulation of which is associated with aging (TABLE 1 & FIGURE 2) [86,94,95].

In the other direction, there is a tendency for CGIs in promoter regions to become hypermethylated with age, although there are many exceptions. Illumina bead arrays (BOX 1) have been used extensively to investigate methylation in human aging sample sets, and hypermethylated genes have been shown to be enriched in tissue-specific functions, such as sequence-specific DNA binding, TF activity,

Table 1. DNA methylation signatures associated with aging.

| DNA methylation status	Example genes	Example reference	Ref.
Hypomethylation			
Repeat elements	*Alu*, *L1*	Wilson *et al.* (2007)	[91]
Imprinted genes	*IGF2*, *PWCR1* and *TRPM5*	Bjornsson *et al.* (2008)	[85]
Inflammatory response genes	*TNF*, *iNOS* and *TLR2*	Gowers *et al.* (2011)	[95]
Hypermethylation			
Duplicate genes, pseudogenes	*ECEL1P2*	Salpea *et al.* (2012)	[105]
DNA-binding factor genes	*GAS2L3*	Hernandez *et al.* (2011)	[98]
TF activity genes	*MYOD1*	Christensen *et al.* (2009)	[97]
Transcription genes	*ZNF169*	Gentilini *et al.* (2012)	[86]
Development and differentiation genes	*FGF17*, *FZD1* and *FZD7*	Salpea *et al.* (2012)	[105]
Apoptosis genes	*FAS*, *XAF1*	Murphy *et al.* (2008)	[99]
Senescence genes, senescence-associated heterochromatin foci	*ECRG4*	Funayama *et al.* (2007)	[100]
Cancer-associated genes	*P16INK4α*, *TET2* and *LOX*	Grönniger *et al.* (2010)	[103]
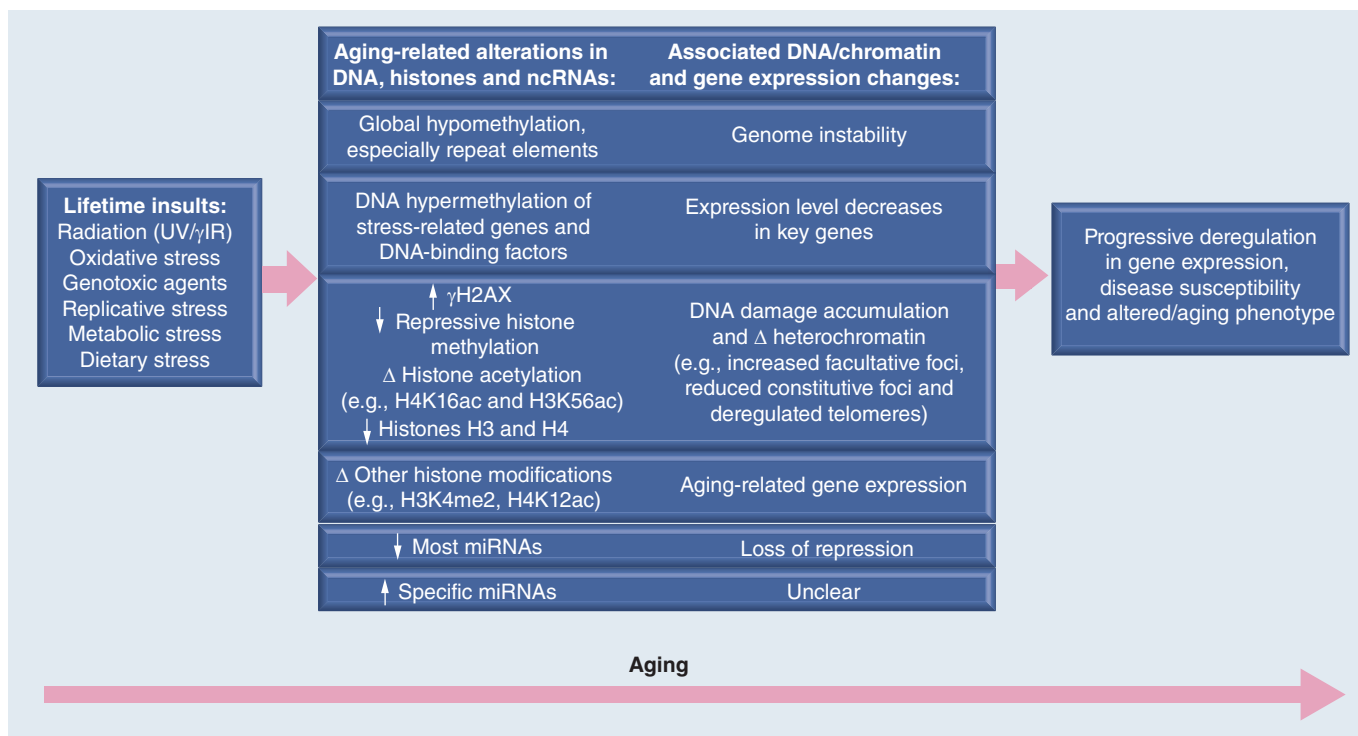
Figure 2. Epigenetic changes associated with aging. The process of aging and exposure to a lifetime of insults are reflected in various alterations in DNA methylation, histone modifications and ncRNA expression. These events are associated with global and gene-specific changes to the DNA/chromatin modification landscape as well as differential patterns of gene expression (e.g., the insulin signaling pathway, stress response genes and cancer-associated genes), which contribute to disease susceptibility and progressive changes related to an altered/aging phenotype.
γIR: γ irradiation; UV: Ultraviolet.

transcription regulation and genes involved in development and differentiation (**Table 1**) [86,96–98]. Some groups have also found enrichment in DNA hypermethylation in genes involved in apoptosis, and domains associated with senescence (**Table 1**) [99–101]. Bocklandt *et al.* used this approach in a twins study that sought to derive a regression model that could accurately predict age. From the 88 age-related loci that they identified in saliva, including ten identified previously in whole blood, they found that the methylation state of the promoters of just three genes (*EDARDD*, *TOM1L1* and *NPTX2*) could predict age with an accuracy of 5.2 years [102]. Overall, it is becoming increasingly apparent that there are similarities between those genes that are hypermethylated in aging and hypermethylated in cancer, including *P16INK4α*, *TET2*, *LOX* and *NPTX2* [102–104].

There have been very few studies on the association of methylation outside of CGIs with age. Salpea *et al.* used methylation-dependent-immunoprecipitation-sequencing (**Box 1**) to identify regions of the genome that show differential methylation between newborns and adults [105]. They observed that the proto-cadherin gene cluster shows extensive age-related increases in

DNA methylation. These changes were in the gene body and not the regulatory regions, and could therefore be affecting splicing.

Finally, as a recently identified component of the epigenomic landscape, studies on 5hmC pattern change with age are also lacking. However, an age-related increase in 5hmC content has been observed in the mouse hippocampus, independent of changes in the expression of TET enzymes, and without a corresponding decrease in 5mC [106]. Enrichment is at discrete locations, primarily intragenic, with the highest levels on the exons [107]. This underlines the hypothesis that 5hmC may be regulated as an epigenetic mark in its own right, not only as an intermediate of 5mC demethylation.

■ Causes of age-related DNA methylation
Initially, reports suggested DNMT1 and DNMT3a decreased with age in some somatic cells, but DNMT3b increased [108,109]. Oliveira *et al.* noted a decreased expression of DNMT3a2 in mice during aging, which was associated with decreased cognitive function, and rescue of DNMT3a2 levels restored these functions [110]. Interestingly, neuronal activity itself has been

linked to modification of the DNA methylation landscape, both dynamic demethylation and *de novo* methylation, in some cases on the scale of hours [111]. However, others suggest that there is commonly no significant differences in DNMTs in mice that could explain the global decrease in DNA methylation, implying other influences are at work [112].

Genome-wide DNA methylation studies have revealed that the greatest differences occur between populations, and there is little overlap in genetic associations of methylation patterns. This implies that there are complex gene–environment interactions (**Figure 2**) [113]. Data from twin studies appears to support this; DNA methylation patterns in twins are concordant in early life, but gradually diverge during lifespan, implying a large environmental influence on genome methylation [114–116]. There is a clear link between metabolism and DNA methylation. SAM, which provides the methyl group for DNA methylation by DNMTs, produces *S*-adenosyl homocysteine during the reaction. Diet is known to modulate SAM levels, although the rate of *S*-adenosyl homocysteine clearance, which inhibits DNMTs, is also important [117,118]. Regulating dietary folate and other methyl donors can also alter the level of DNA methylation [119]. Although the molecular mechanisms responsible are opaque, it has also been shown that moderate exercise is associated with a delay in the aging-related hypomethylation of genes involved in the inflammatory response, such as *ASC* [120]. However, are these changes to methylation important to aging, or simply a phenotype? If we return to dietary restriction, DR attenuates the DNMT3a changes seen in the mouse brain and increases DNMT1 expression [121,122]. DR is also associated with an increase in methylation of proto-oncogenes, such as *Ras* in rats, and *P16INK4α* in mammalian cell culture [122,123]. DR has also been observed to cause DNA methylation changes in genes linked to aging including *TNF-α*, and TFs such as *WT1* (**Figure 2**) [124,125].

■ Age-related histone modification dynamics: DNA damage response

Aging is coupled with a gradual deregulation of chromatin stability and gene expression. Proposed theories for why this deregulation occurs with age include DNA damage (e.g., via replication errors, radiation and genotoxic agents), telomere maintenance and redistribution of heterochromatin foci (**Figure 2**) [126]. Interestingly, histone modifications have an integral role in responding to these events, maintaining chromatin stability, and as a consequence of prolonged chronic stress, contributing to functional decline in later life. Although genome-wide histone modification analyses during aging are scarce, a recent study identified Rhesus macaque brain to exhibit an age-related global increase in dimethylated H3K4me2, associated with transcriptionally active sites, at both promoter and enhancer regions [127]. Elevated H3K4me2-marked genes enriched for response to DNA damage stimuli and genes highly associated with stress, reflecting the close connection between histone modification and stress response during aging. Here, we describe histone modification changes associated with aging in the context of mechanisms proposed for deregulation of chromatin and gene expression (**Table 2**).

Unrepaired DNA damage can cause various adverse effects including accelerated aging [128]. The DNA damage response (DDR) utilizes multiple pathways to remove damaged nucleotides and repair single-stranded breaks and double-stranded breaks. After DNA damage is sensed by various DDR complexes, transducer kinases ATM, DNA-dependent protein kinase catalytic subunit (DNA-PKcs) and ATR are subsequently brought in to initiate the DDR [129–131]. This is followed by recruitment of mediator proteins, such as MDC1, 53BP1, BRCA1 (early onset), TopBP1 and Claspin, activation of effector kinases, and propagation of the DDR to the rest of the cell.

Coordinated recruitment of DDR factors requires interactions with a number of histone modifications. Phosphorylation of H2AX (γ-H2AX) by transducer kinases ATM and DNA-PKcs [132,133] is bound by MDC1, which aids in recruiting other factors to enhance the DDR [134]. On a megabase scale, γ-H2AX spreads outward from the damage site [135], and longer-lasting γ-H2AX foci typically exist outside of heterochromatin [136]. Interestingly, γ-H2AX foci accumulate with age and in patients with accelerated aging [137,138]. Another DNA damage-induced mark is ubiquitinylation (Ub), accumulating at foci particularly on H2A and H2B. As Ub-H2B is associated with transcription elongation, it is proposed that Ub-H2B promotes chromatin relaxation and mediator binding at the damaged site, whereas Ub-H2A creates a repressed environment that, along with γ-H2AX, spreads outward from the site [139–141]. The importance of histone modifications to the DDR is emphasized in studies of DDR mediator recruitment. Mediator protein 53BP1 contains a

Table 2. Histone modification signatures associated with aging.

Modification	Change	Location/function	Example reference(s)	Ref.
γH2AX	Up	DNA damage foci/DNA damage response signaling	Sedelnikova et al. (2004)	[137]
H3K4me2	Up	Enhancer and promoter/transcription activation	Han et al. (2012)	[127]
H3K9me3	Up/down	Heterochromatin maintenance	Scaffidi et al. (2006)	[163]
H3K27me3	Down	Heterochromatin and gene regulation (e.g., IGF1R)	Jin et al. (2011); Maures et al. (2011)	[179,180]
H3K56ac	Down	Telomere and DNA damage	O'Sullivan et al. (2010); Dang et al. (2009)	[147,162]
H4K12ac	Loss of induction	Memory impairment	Peleg et al. (2010)	[177]
H4K16ac	Up	Heterochromatin, telomere and DNA damage	O'Sullivan et al. (2010); Dang et al. (2009)	[147,162]
H4K20me2	Up	Heterochromatin and DNA damage	O'Sullivan et al. (2010)	[147]
H4K20me3	Up/down	Heterochromatin maintenance	O'Sullivan et al. (2010)	[147]

tudor domain with which the yeast orthologs bind H4K20me2 (Crb2) and H3K79me3 (Rad9), and human 53BP1 binds both H4K20me1/2 and H3K79me3 [142–146]. In fibroblasts recovered from individuals, old cells displayed higher levels of H4K20me2 and H3K79me2 compared with young cells [147]. Studies have shown that the H4 methyltransferases PR-Set7/Set8 and MMSET are involved in DNA damage-induced H4K20me1/2, which is then bound by 53BP1 [146,148]. While total H3K79 methylation did not change with DNA damage, the placement of this modification by the only identified H3K79 methyltransferase DOT1L, conserved across species, allows proper 53BP1 binding in human cells and activation of the DDR in yeast [142]. Besides methylation, recruitment of MDC1, 53BP1 and BRCA1 was demonstrated to require MOF-mediated H4K16ac, as well as general H4 acetylation via TIP60, albeit including K16ac, around sites of DNA damage [149–151].

■ Age-related histone modification dynamics: heterochromatin alterations

For over 20 years, aging has been known to be associated with altered heterochromatin abundance [152]. Accumulating evidence links the DDR with maintenance of constitutive heterochromatin and the aging process (TABLE 2 & FIGURE 2). Constitutive heterochromatin regions reside in repetitive DNA elements such as centromeres, telomeres, rDNA and transposable elements. A prime example of the DDR/heterochromatin link is the activity of the deacetylase SIRT1, which acts on acetylated H1K26, H3K9 and H4K16 [153]. Upon oxidative stress, repression of pericentromere heterochromatic repetitive DNA

transcripts by SIRT1 and the yeast homolog SIR2 is lost [154]. Genome-wide analysis further showed oxidative stress caused a dramatic SIRT1 redistribution away from repetitive DNA elements, and other diverse genes, to a more random localization and increased H1K26ac at sites previously repressed by SIRT1 [154]. Similar to yeast SIR protein relocation from telomeres [155], SIRT1 is recruited to sites of DNA damage and required for the DDR [154]. Interestingly, genes repressed by SIRT1 prior to redistribution are also deregulated in aged mouse brain, demonstrating a link between the age-related DDR and altered gene expression [154]. In addition, SIRT1 affects H3K9me3 by activating the H3K9 methyltransferase SUV39H1 by direct deacetylation [156]. Another sirtuin member, SIRT6, was also shown to be required for the normal DDR, and its deficiency reduces lifespan and causes premature aging [157]. SIRT6, which deacetylates H3K9 and H3K56, locates to telomeres and regulates their stability, in part, through modulating telomere association with WRN, which is mutated in the premature aging disorder Werner syndrome [158,159]. Genome-wide SIRT6 promoter binding analysis revealed a close association with NFκB-regulated genes, particularly those related to cell senescence and aging [160]. In aging yeast and late-passage cells, reduced telomere silencing is associated with decreased H3K56ac and increased H4K16ac, a SIRT1 substrate that opposes higher order chromatin structure by preventing interactions between neighboring nucleosomes [147,161,162]. Acetylated H3K56 resides in the globular core at the point where DNA interacts with the histone, and modulating H3K56ac effects nucleosome

assembly and disassembly. These findings provide evidence for reduced heterochromatic silencing, partially via SIRT1/6 redistribution, as a major contributor to altered gene expression during aging.

Chromatin dynamics identified in premature aging syndromes also occur in normal aging. For example, the Hutchinson–Gilford progeria syndrome, linked to a single base change in the lamin A gene, results in alterations to the nuclear membrane architecture, increased expression at pericentromeric repeats, HP1 loss, and altered histone modifications at heterochromatin regions, including decreased H3K9me3, H3K27me3 and increased histone acetylation [163,164]. Intriguingly, cells from older individuals are also associated with reduced H3K9me3, H3K27me3 and HP1. Further analysis of healthy individuals found naturally occurring aberrant splicing present in lamin A gene [163]. In the same distribution as in Hutchinson–Gilford progeria syndrome individuals, older individuals showed altered lamin A/C localization, with lamin A accumulating at the nuclear membrane. Thus, although the spliced form of lamin A did not accumulate with age, the study suggests that the long-term presence of this form becomes progressively intolerable during aging.

The age-related decline in constitutive heterochromatin at the nuclear membrane and repetitive elements coincides with a gain in heterochromatin in other areas, termed facultative heterochromatin (FIGURE 2). A proposed model involves DNA damage leading to redistribution of perinuclear heterochromatin towards nonperinuclear sites, resulting in age-related changes in gene expression [126]. Notably, senescent cells accumulate nonpericentromeric facultative heterochromatin, or senescence-associated heterochromatin foci (SAHF) [165]. SAHF, marked by H3K9me3 and macroH2A, contribute to maintaining senescence and reducing the DDR, in part, through inducing heterochromatin protein and retinoblastoma (RB) tumor suppressor recruitment to cell-cycle genes regulated by the E2F transcription factor [165,166]. Genome-wide RB and H3K4me3 localization further showed a significant senescence-related correlation between binding of RB and loss of H3K4me3, particularly at E2F target genes [167]. In aging primates, senescent skin cells increase with age, and this coincides with elevated ATM activity, telomere γ-H2AX and 53BP1 recruitment, as well as SAHF marked by H3K9me2/3 and HP1 [168].

Epigenomic changes that increase longevity

■ DNA methylation & longevity

Presently, there is only limited data that links DNA methylation to longevity. In flies, overexpression of dDNMT2, the only DNMT present in the *Drosophila* genome, increased mean lifespan by up to 58% [169]. However, the mammalian homologue DNMT2 is not a DNA methyltransferase, instead methylating RNA [170]. Nevertheless, this does suggest that DNA methylation could be important to lifespan in other organisms. In one study in mice, DNMT1 haploinsufficiency effected learning and memory functions in an age-dependent manner, but this was not associated with an effect on survival or mortality [83]. To investigate human longevity, Bell *et al.* used an epigenome-wide association approach to study 172 individuals of various age. They noted that the majority of age-related changes in DNA methylation were not associated with phenotypic measures of healthy aging. However, for a small subset of genes, DNA methylation may mediate environmental and genetic effects on age-related phenotypes [171]. Most significant were the transcription factors *TBX20*, *STAT5A* and *WT1*. It should be noted that the study of Bell *et al.* was promoter-specific; therefore, they did not explore the effects of intragenic DNA methylation. Offspring of centenarians also show a slower decrease in global methylation, which could represent a link to longevity [86]. The specific DNA methylation pattern differences include genes involved in DNA/RNA synthesis, metabolism and cell-signaling. Metabolism-linked signaling processes, especially stress response pathways, are related to longevity through the known lifespan regulators, such as IGF1R, and it is therefore possible that differentially methylated genes showing association with longevity could be causing lifespan effects (FIGURE 2). Indeed, overexpression of dDNMT2 in the flies was associated with increased expression of heat shock proteins, and increased stress resistance [169]. There is accumulating evidence that alternative splicing is also associated with aging; for example, genome-wide association studies have noted novel variants in genes involved in splicing regulation [172]. Other studies focusing on alternative splicing itself observe age-related splicing changes, especially in metabolism genes and genes involved in the repair of oxidative damage [173]. Given the proposed roles for DNA methylation in marking splicing boundaries, and the observed global hypomethylation with age, this may be an important role for epigenetics in

the aging process. Although it is possible that the majority of the data mentioned here may simply reflect the phenotype of aging (with the exception of dDNMT2 in flies), and cannot be used to conclude a causal effect, it does hint that DNA methylation, especially intragenic methylation, may have significant underappreciated influences on longevity that warrant further study.

■ Histone modifiers regulating longevity

Intriguingly, modulating the expression of some enzymes and cofactors capable of coordinating histone modification dynamics significantly affects longevity. Early work discovered that yeast replicative lifespan is modulated by the histone deacetylase SIR2, and this was later shown to involve SIR2-dependent changes in H4K16 acetylation [162,174]. As SIR2 is evolutionarily conserved, *C. elegans* SIR2.1 overexpression was also observed to extend lifespan [175]. However, manipulation of the mouse mammalian homolog SIRT1 regulated age-dependent gene expression and greatly improves health span under metabolically challenging conditions, but had little effect on lifespan [154,176]. In contrast to SIR2/SIRT1, blocking class I and II HDAC activities reversed age-dependent learning impairment associated with altered H4K12ac, at both TSSs and coding regions, in mice [177]. These findings illustrate the divergent roles of histone acetylation on aging and, particularly, aging-related pathologies in a site- and deacetylase-specific manner.

Using a targeted RNAi screen for *C. elegans* histone methyltransferases and core subunits, knockdown of the *WDR-5* and *ASH-2* subunits, and the *SET-2* methyltransferase, were each found to significantly extend lifespan [178]. Characterization of reduced expression of all three genes showed decreased total levels of H3K4me3 [178]. Although altered methylation of nonhistone proteins could be involved, support for histone methylation was demonstrated by reduced lifespan after knockdown of the *RBR-2* H3K4me3 demethylase [178]. Lifespan extension by reduced *WDR-5*, *ASH-2* and *SET-2* requires an intact germline, and microarray analysis of *ASH-2* RNAi worms found deferentially expressed genes related to determination of adult lifespan [178]. Nevertheless, exactly how modulation of H3K4 methylation results in altered lifespan, and whether histone methyltransferases similarly affect mammalian lifespan, remains to be established.

Aged human brain and *C. elegans* exhibit elevated expression of *UTX/UTX-1*, an enzyme that demethylates the repressive mark H3K27me3 [179]. In *C. elegans*, this increased *UTX-1* expression correlated with reduced H3K27me3 [179,180]. Interestingly, *C. elegans* treated with RNAi or heterozygous null for *UTX-1* showed a significant increase in lifespan and stress tolerance, which was dependent on a functional insulin/IGF-1 signaling pathway [179,180]. Analysis of the *IGF1R* locus identified H3K27me3 to decrease with age in both worms and rhesus macaque, and RNAi of *UTX/UTX-1* in *C. elegans* and a mouse cell line led to increased H3K27me3 at the *IGF1R* locus and repressed *IGF1R* expression [179]. As repression of the conserved insulin/IGF1 pathway is known to extend lifespan, these results reveal that altered expression and activity of UTX/UTX-1 may represent a mechanism of lifespan regulation across species. Although the mechanism is unclear, a modifier regulating H3K36me3 also affects lifespan in *C. elegans* [180]. Since histone modifications represent a reversible means to regulate gene expression, it will be of interest to know whether altering the activity of these modifiers in mammals also affects aging in a similar manner.

ncRNAs

■ Definition of ncRNAs

ncRNAs regulate the expression of many genes, and accumulating evidence has shown that at least some ncRNAs are heritable. Studies have also been aided by advances in next-generation sequencing of RNA (RNA-seq), which has now superseded microarrays for identifying and measuring ncRNA expression. Short ncRNAs less than 200 nucleotides long include miRNAs, siRNAs, piRNAs and snoRNAs. The remaining ncRNAs are broadly termed lncRNAs, which are further characterized by distinct properties and genome localization. Unlike DNA methylation and histone modifications, the ncRNA field is younger, and much less data exists on its role in aging. Here, we describe studies characterizing ncRNAs, miRNAs, piRNAs and lncRNAs, with known or potential roles in aging and longevity.

■ miRNAs

miRNAs are the best studied of the ncRNAs, and have recently been implicated in aging, cellular senescence, and age-related diseases [181]. The pool of miRNAs regulates gene expression by complementarity to consensus sequences within the 3′UTR of mRNAs, which leads to translational repression and mRNA degradation.

miRNAs (18–25 nucleotides) are transcribed by RNA polymerase II or III and regulate several cell processes, including proliferation, development, stress responses and apoptosis.

Although miRNAs are essential for normal development, they are also important to health and lifespan in adults [182]. Global miRNA sequencing analysis of *C. elegans* identified expression changes associated with age, and modulation of some of these miRNAs could regulate longevity [183,184]. In addition, three miRNA biomarkers have been found that can predict up to 47% of lifespan differences, which further suggests that miRNAs could control longevity [185]. In support of this, the first miRNA to be shown to regulate longevity in *C. elegans*, lin-4, also targets the insulin signaling pathway, which is well known to modify lifespan [186]. In higher organisms, miRNAs have been associated with tissue specificity and differentiation and, therefore, their effects on aging may also be tissue-specific. To date, studies on miRNA's role in mammalian aging have predominantly focused on the brain or blood. Genome-wide studies of miRNAs in mammalian aging have shown that, in general, miRNA levels gradually decline. Interestingly, declining miRNAs in human blood could be associated with the loss of repression of cancer-associated genes as they target p53 signaling, the citrate cycle and other cancer processes, while target genes of miRNAs declining in mouse brain have known roles in aging, such as miR-145 and miR-375 that target the insulin signaling pathway [187–189]. Some miRNAs that were observed to increase with age include miR-144 in chimpanzees and humans, and let-7 and miR-34a in mouse [189–191]. In mice, miR34-a has now been proposed as a biomarker of aging [191]. Multiple miRNAs are differentially expressed in *C. elegans* aging including miR-71, miR-238, miR-239 and miR-246 [184]. These miRNAs both positively and negatively influence lifespan in *C. elegans* by interacting with genes involved in the DDR and insulin signaling pathways. Boulias *et al.* screened 81 miRNA mutants in *C. elegans* and found that miR-58, miR-61, miR-71 and miR-238 knockdown significantly shorten lifespan [192].

There appears to be clustering of miRNAs that are differentially expressed with age, possibly due to shared promoter sequences, or genomic location [187]. Lanceta *et al.* propose that miRNAs act in concert and are therefore regulated as 'packs' [193]. Overall, miRNAs implicated in aging show many target pathways that have been previously implicated in regulating longevity. However, despite growing consensus of their importance to aging, until new data arises, an understanding of the roles and regulation of miRNAs and their targets remains elusive.

■ Piwi-interacting RNAs

It was recently shown that RNAi can be heritable in *C. elegans*, implying that small ncRNAs may also have transgenerational effects [194,195]. A conserved role is now emerging from work in *C. elegans* and *Drosophila*, and the ncRNA responsible for this appears to be maternal inheritance of piRNAs [4,196,197]. piRNAs are slightly longer than miRNAs and are known to associate into RNA–protein complexes, mediated by their binding to Piwi proteins. Although less is understood about piRNAs, they represent a much bigger class of ncRNA than miRNAs. piRNAs are also associated with gene silencing, most especially at repeat elements such as transposons [196–198]. Thus far, piRNAs seem to be most important in the germline, where they can aid in establishing other epigenetic patterns, such as DNA methylation and histone modifications. The expression of piRNA was also found to be downregulated during aging in *C. elegans* [183]. Nevertheless, specific roles of piRNAs in longevity are not known.

■ lncRNAs

lncRNAs are essential for multiple aspects of gene regulation, such as recruitment of histone and chromatin-modifying complexes, transcription factor recruitment and RNA processing [199]. To date, the roles of lncRNAs in aging are only speculative. RNA-seq identified 14 novel putative lncRNAs that are differentially expressed in rat brain during aging, although their functional roles are unknown [200]. lncRNAs are required for genomic imprinting. However, one study showed that altered imprinting of IGF2 did not affect longevity [201]. Healthy aging and protection against age-related diseases requires an adequate response to a variety of stresses. Interestingly, activation of the heat shock response through the HSF1 requires a lncRNA termed HSR1 [202]. In a study of glioblastoma brain tissue, lncRNA profiling found changes in expression between glioblastoma and healthy control samples, suggesting a role of lncRNAs in disease [203]. More genome-wide and RNA-specific lncRNA studies are imperative for elucidating the functional roles of these ncRNAs.

Cross interactions of epigenetic marks

■ Histone modification & DNA methylation

DNA methylation in CGIs has typically been associated with a nucleosome-occupied repressive environment, displaying polycomb-mediated repression and the repressive histone mark H3K27me3 (FIGURE 3) [105,204]. *De novo* methylation by DNMT3 requires it to form a complex with a nucleosome, but, in contrast, DNMT1 prefers linker DNA [205]. The amino-terminal tail of H3 appears to be especially important in interactions that control methylation; for example, DNMT3a/b can be anchored to nucleosomes via H3K36me3 marks, and DNMT3L can also bind H3K4 tails [206]. Histone modifiers are also associated with methylation. Uhrf1 can bind to histone modification factors such as histone methyltransferase G9a and HDAC1 [207], and SIRT1 can deacetylate various lysines on the DNMT1 protein to regulate its activity [208]. Unmethylated CGIs at TSSs are associated with nucleosome-depleted regions, flanked by active histone marks such as the histone variant H2.AZ and H3K4me3, which may in fact block methylation [209]. Conversely, 5hmC and Tet1 are associated with poised chromatin signatures of H3K27me3 and K3K4me3 [44]. Interestingly, CTCF, a chromatin-binding factor involved in transcriptional activation/repression, enhancer blocking and barrier insulation, among others, has recently been implicated in linking methylation with splicing [210]. CTCF binding is inhibited by the presence of 5mC, but is enriched at 3′ splice sites where methylation dips [210]. CTCF can cause RNAPII to pause, and pausing is associated with the kinetic regulation of splicing [211]. Analyzing genome-wide CTCF binding in 19 human cell types revealed that alterations in DNA methylation could account for 41% of differential CTCF binding [212]. 5hmC marks, on the other hand, overlap with CTCF binding sites hinting at a possible functional association [48].

Mechanistically, methylation-sensitive binding proteins can also link DNA methylation to chromatin. Methyl-CpG-binding proteins (MBPs) often form complexes with HDACs, such as MeCP2 with HDAC1/2-associated complexes, and the zinc finger-containing kaiso proteins ZBTB4 and ZBTB38 with the HDAC-associated NCoR repressive complex [213,214]. MBD1 also interacts directly with the H3K9 histone methyltransferase SetDB1. Finally, the KRAB-ZFP57 zinc-finger protein has been shown in mice to bind to methylated hexanucleotide sequences in so called *cis*-regulating imprinting control regions through interaction with KAP1, a known recruiter of DNMTs and histone modifiers, and SetDB1 [215].

Although DNA methylation can be a long-term stable and repressive mark, lasting an entire lifespan, there has been much discussion about whether methylation triggers silencing [216], or whether a gene under repression triggers the
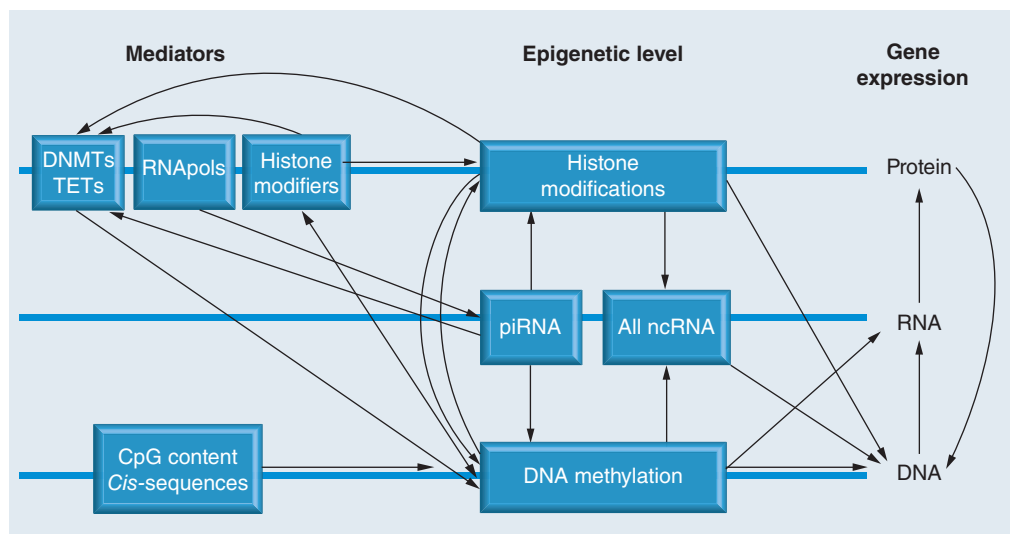

Figure 3. Interactions of the epigenome. There are three levels of epigenetic regulation in the genome that modulate gene expression: histone methylation, ncRNA and DNA methylation. These levels can exert control on each other, for example histone H3, piRNAs and various histone modifiers interact with the mediators of DNA methylation, DNMTs, to control establishment of DNA methylation patterns. For more details see text.
DNMT: DNA methyltransferase; RNApol: RNA polymerase.

promoter to become methylated (FIGURE 3) [217]. It is now believed that methylation itself does not cause repression, but rather it recruits complexes that apply repressive histone marks and package chromatin [218,219]. Acetylated histones, associated with active genes, can stimulate expression even if the DNA is methylated [220]. This hypothesis was recently supported by a study using a GFP reporter controlled by a hypermethylated CMV promoter [221]. Using a histone deacetylase inhibitor, both the GFP reporter and other endogenously 'silenced' genes could be reactivated, and importantly, the reactivated genes were marked by active histone modifications without any change to the underlying methylation state. After 2 weeks the loci had reverted back to a silenced state, demonstrating that DNA methylation can recruit repressive histones. In addition, DNA methylation inhibition can stably activate genes for much longer periods [221].

■ ncRNA

In yeast and flies, ncRNAs are known to direct *de novo* DNMTs to homologous target sequences for methylation (FIGURE 3) [222]. Using DICER knockout mouse embryonic stem cells, defects in the RNAi machinery showed defective methylation patterns [223]. More recently, ncRNAs were found to target mammalian DNMT3b to rRNA genes, and RNA was also found to bind in the catalytic domain of human DNMT3a to modulate DNMT3a activity *in vitro* [224,225]. Interestingly, a methylation-determining region identified by Park *et al.* produced a specialized form of RNA called pit-RNA (a form of piRNA) that was essential for methylation in *cis* [70]. This adds further support to the involvement of ncRNA in *cis*-regulation of methylation. Transgenerational inheritance of ncRNA, including piRNAs and other small RNAs, has been identified in *C. elegans* [4,194] and *Drosophila* [197], where it is associated with heritable gene and transposon silencing [195,196]. Heritable piRNA also appears to direct methylation occurring in mammalian germ cells, and mice lacking certain piRNAs cells fail to remethylate properly after the loss of methylation during fertilization (FIGURE 3) [198,223]. Small RNA-seq profiles show that piRNAs cluster at L1 promoter elements, supporting an involvement of the establishment of DNA methylation at least at this feature [196]. siRNA was responsible for inheritance of histone marks in *C. elegans*, which lack DNA methylation, implying further important interactions with histone modifiers [195]. Therefore,

inheritance of histone modifications in humans could be at least partially mediated by inheritance of ncRNA and the underlying DNA methylation state, representing a coordinated effect across the epigenetic levels ncRNA–DNA methylation–histone modification. However, ncRNA involvement with methylation patterns in somatic cells is still unsupported at this time.

Conclusion & future perspective

Theories of aging that focus on the genome cannot explain the gradual decline in the system that is the phenotype of aging. Changes at all epigenomic levels – DNA methylation, histone modifications and circulating ncRNA levels – are associated with aging, and potentially represent an important way that environmental effects may be linked to the rate of aging (FIGURE 2). It is also important to recognize the close interplay of the various epigenetic marks when considering system-wide implications (FIGURE 3). For example, ncRNA expression influences patterns of DNA methylation, which in turn affect histone modification. Nevertheless, to date, evidence for a direct link between epigenetic modifications and longevity in humans remains lacking and should be a focus of future research.

A systematic approach for combining multiple high-throughput data to produce a meaningful and testable hypothesis is now becoming a priority as more data sets are produced. Work has begun on collating the available data for the interactions of various epigenetic marks and deciphering how this affects epigenome patterns and system-wide phenotypes. Beyond combining DNA methylation and histone modifications for general characterization of enhancer, promoter and coding regions [36,226,227], Wrzodek *et al.* presented a computational synthesis of epigenomic data and used it to predict patterns in cell types with incomplete data sets [228]. This has added support to several of the hypotheses outlined here, such as the interaction between DNA methylation and H3K4me3. Xiao *et al.* have proposed a similar approach, comparing the epigenomes of humans and other model species and revealing regulatory regions of the genome that cannot be identified from sequence alone [229]. These attempts at synthesizing all the data will be bolstered by further data on intragenic methylation, CpG and non-CpG methylation in other regulatory regions outside of promoters, and 5hmC patterns during aging. The first high-resolution genomic maps of 5hmC patterns are now becoming available [230], and we expect a

clearer picture of 5hmC's role in epigenetics to be forthcoming.

The links between ncRNA, DNA methylation and histone modification is, in our opinion, one of the most interesting future directions for epigenomics research, especially aging research. Changes in alternative splicing could have an important role in aging that is regulated by intragenic epigenetic patterns. In addition, a better understanding of the regulation of DNA methylation and histone modifications, directly and indirectly, through ncRNA both in the inheritance of patterns, and establishment and maintenance of patterns, will be enlightening. Finally, however the epigenome changes during aging, approaches for targeted manipulation of the epigenome, including targeted application of specific histone marks [231] and modification of DNA methylation patterns (reviewed in [232]), will be essential for further establishing causative roles of epigenomic changes in aging and lifespan regulation.

Executive summary

Approaches to study the epigenomics of aging
- Advances in sequencing and sample preparation techniques have allowed for genome-wide analyses of the epigenome.
- Databases developed to curate high-throughput data from humans and model organisms can link the epigenome to diverse phenotypes.

Regulation of epigenomic marks
- DNA methylation is established and maintained by DNA methyltransferases, and demethylated by TET proteins.
- A plethora of histone modifications are controlled by the actions of a variety of modifiers including methylases, acetylases, demethylases and deacteylases.
- Patterns of DNA methylation and histone modifications are associated with defined genomic elements.

Epigenomic changes associated with aging
- Aging is characterized by global DNA hypomethylation, and specific DNA hypermethylation on key genes.
- Aging is characterized by a gradual deregulation of chromatin stability.
- Aging-related histone modification patterns are associated with the DNA damage response and loss of heterochromatin.

Epigenomic changes that increase longevity
- Modulating several histone modifiers has been shown to affect lifespan in model organisms, but data for a link between DNA methylation and longevity are limited.

ncRNAs
- ncRNAs, and their effect on aging, is a young field that is quickly gaining interest. Thus far, miRNAs are the most well studied, with evidence for lifespan effects in model organisms.

Cross-interactions of epigenetic marks
- Histone modification and DNA methylation complexes interact to regulate the epigenome.
- Emerging data show that ncRNAs are involved in the establishment of DNA methylation and histone modification patterns.

Conclusion & future perspective
- The development of new methods for integrating high-throughput epigenomic data will aid in-depth study of the association of epigenomic patterns with the regulation of aging and longevity.

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