Making sense of the ageing methylome

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Abstract | Over time, the human DNA methylation landscape accrues substantial damage, which has been associated with a broad range of age-related diseases, including cardiovascular disease and cancer. Various age-related DNA methylation changes have been described, including at the level of individual CpGs, such as differential and variable methylation, and at the level of the whole methylome, including entropy and correlation networks. Here, we review these changes in the ageing methylome as well as the statistical tools that can be used to quantify them. We detail the evidence linking DNA methylation to ageing phenotypes and the longevity strategies aimed at altering both DNA methylation patterns and machinery to extend healthspan and lifespan. Lastly, we discuss theories on the mechanistic causes of epigenetic ageing.

CpGs
(Cytosine–guanine dinucleotides). Regions of DNA whereby a cytosine resides alongside a guanine to form a CpG.

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The world's rapidly ageing population has become one of society's greatest challenges[1]. By 2050, it is projected that in many parts of the world 25% of the population will be aged >65 years[2], yet steady increases in life expectancy (lifespan) are not concomitant with an equivalent increase in healthspan (disease-free, healthy lifespan). Instead, the ageing population exhibits rising morbidity rates and a decline in quality of life[3,4], which comes at social and economic costs[1,5].

Ageing is the time-dependent decline in functional capacity across the lifespan, characterized by the accumulation of molecular damage resulting from a diminished damage-repair capacity[6–8]. Such damage includes changes that impair the structure and function of all tissues over chronological time (that is, primary ageing), as well as deleterious changes that are aggravated by environmental perturbation and disease (that is, secondary ageing)[9–11]. Theories of ageing can be grouped into two main schools of thought[12]. One theory is that ageing is a tightly regulated, programmed process, the pathological consequences of which are an extension of normal biological processes, such as growth and development. The second theory is that ageing is a consequence of accumulated, lifelong damage and stochastic errors that eventually impair the capacity for tissue maintenance. In either case, pan-tissue deterioration associated with ageing is hypothesized to be underpinned by a common set of cellular and molecular defects, considered the 'hallmarks of ageing', which can be grouped into three categories, namely the primary hallmarks that cause the damage; the antagonistic hallmarks that compensate for the primary hallmark-induced damage; and the integrative hallmarks responsible for ageing phenotypes, which emerge when the damage accumulation caused by the primary and antagonistic hallmarks can no longer be compensated for[7]. Alterations to the epigenome are considered primary hallmarks of ageing[7].

The epigenome is a dynamic maintenance system operating via a range of chemical modifications that control chromatin organization and regulate gene activity without altering the DNA sequence[13,14]. The best characterized epigenetic mark is DNA methylation (DNAm), which is the covalent attachment of a methyl group to the fifth carbon of a cytosine residue (5-methylcytosine (5mC)). In mammals, DNAm usually occurs at cytosine–guanine dinucleotides (CpGs)[6] and carries out distinct functions in different genomic regions[15]. Patterns of DNAm (the 'methylome') are laid down early during embryonic development and are maintained through cell divisions to preserve cell identity. As such, the methylome strongly differs between cell types within the same tissue and between tissues[16,17]. In addition, throughout the lifespan, DNAm can be added by DNA methyltransferases (DNMTs) or removed by ten–eleven translocation (TET) enzymes[14] and, because the methylome operates at the interface between the genome and the environment[6], it can 'shift' in response to environmental stimuli[18], such as exercise[19–21], diet[22,23], smoking[24–26] or pollutants[27,28]. Moreover, the integrity of the methylome is closely associated with healthy ageing, with altered DNAm patterns being associated with a broad range of age-related diseases, including Alzheimer disease[29–33], cardiovascular disease (CVD)[34–36] and cancer[37–40].

Despite our current understanding of the ageing methylome, several critical questions remain unresolved. Indeed, it is unclear which differences in DNAm are associated with primary and secondary ageing, whether the methylome ages differently in different tissues and which biological mechanisms underpin changes in DNAm throughout the lifespan. This Review aims to

answer these questions by painting a comprehensive picture of the ageing human methylome. First, we review the different types of changes in DNAm that have been observed with age, including those occurring at the level of individual CpGs (such as differential and variable DNAm) and those that occur at the level of the whole methylome, including epigenetic clocks, entropy and correlation networks. We also describe the strength of evidence linking these changes to age-related diseases. Then, we propose molecular mechanisms that explain these changes and show how environmental factors that accelerate or decelerate age-related changes in DNAm can provide evidence that helps to demonstrate these mechanisms. Finally, we propose future directions for the field.

The ageing human methylome

Our understanding of DNAm changes that accrue over time is bound by the statistical and computational methods used to quantify these changes (TABLE 1). Two common approaches to quantify age-related DNAm changes are to look for CpG sites displaying differences in average DNAm levels or in DNAm variance between younger and older individuals[17]. Although these approaches are widely used, they are one-dimensional measurements that focus only on individual, rather than multiple, CpGs. Changes at the whole methylome level can also be quantified using single measurements, such as entropy, or changes in coordinated DNAm levels at multiple CpGs, such as correlation networks[41–43].

Changes in average DNAm

Early studies investigating global changes in DNAm, using various chromatography techniques, reported that a global loss of DNAm occurs with age in certain human and rodent tissues[44–47]. However, later studies using advanced sequencing technologies, such as whole-genome bisulfite sequencing, reported conflicting evidence. Whereas a global decrease in DNAm with ageing has been observed in human T cells[48], studies in other human cell types (for example, in cells of the brain, epidermis, muscle, heart and liver) and rodent tissues (for example, in the liver and hippocampus) have observed no notable shifts in global DNAm levels during ageing[49–54]. These discrepancies suggest that the overall effect of global DNAm might be dependent on the investigative methods used, which involve vastly different detection techniques, or the specific tissue of interest.

Technological advancements, such as microarrays, reduced representation bisulfite sequencing and whole-genome bisulfite sequencing, have since prompted the development of epigenome-wide association studies (EWAS), which have revealed predictable and consistent shifts in DNAm at specific CpG sites (termed differentially methylated positions (DMPs)) across the lifespan[27,40,55–58]. DMPs exhibit a 'shift' in their average methylation level as humans age[17] (FIG. 1), with DNAm either increasing (hypermethylation) or decreasing (hypomethylation) at the CpG site[59,60]. Age-related DMPs can be identified with linear models, implemented in packages such as limma[61] (TABLE 1). Differential methylation can also occur over a whole genomic region, as CpG sites within ~500 bp are typically highly correlated; these regions form age-related changes in mean DNAm levels over multiple, contiguous CpGs, and are referred to as differentially methylated regions (DMRs)[17]. Various statistical algorithms can detect DMRs, such as DMRcate[62], bumphunter[63], comb-p[64], blockFinder[65] and Probe Lasso[66]. Given that DMPs and DMRs are closely correlated with chronological age, these sites capture age-associated changes in DNAm over the lifespan[67].

Epigenetic clocks: predictors of age

Subsets of DMPs have been used to build both multi-tissue and tissue-specific 'epigenetic clocks' capable of predicting the chronological age of a sample with high accuracy[60,68–71]. The first epigenetic clocks, including the saliva clock by Bocklandt et al.[71], Hannum et al.'s whole blood clock[41] and Horvath's pan-tissue clock[60], were developed using a machine learning algorithm trained to predict chronological age. By computationally distilling the widespread DMPs shared by individuals across the lifespan, these algorithms can accurately select several CpG sites that predict chronological age, a parameter known as 'DNAm age' or 'epigenetic age'[41,60,67,72]. The majority of epigenetic clocks are built using the elastic net regression algorithm[67,73,74] (TABLE 1). Typically, chronological age (or a transformed version of age or mortality risk) is regressed on a set of CpG sites and the algorithm selects the most informative CpGs from a pool of tens of thousands of potential sites to make an age prediction[72,75].

A multitude of specialized epigenetic clocks have been developed, including tissue-specific clocks[70,76–78], clocks for different animal species[79–90] and even multi-tissue, multispecies clocks[56]. For example, a multi-tissue epigenetic clock captures the DNAm changes that are intrinsic to the ageing methylome, reflected by age-related DNAm patterns that are consistently observed across many tissues and cell types[55,60,76,91–93]. Conversely, tissue-specific clocks capture intrinsic changes as well as extrinsic or within-tissue DNAm changes, including those that reflect age-related changes in tissue or cell composition[72,92,93]. In the last year, a novel statistical framework for profiling epigenetic age at the single-cell resolution, 'scAge', was introduced, providing novel insights into the heterogeneity in epigenetic ageing of individual cell types[94].

Notwithstanding the ability of epigenetic clocks to predict chronological age, it is important to remember that each clock is simply a multivariate age predictor generated from a subset of CpGs and does not capture the entire ageing methylome[95]. Indeed, the specific CpG sites selected by different clocks often do not overlap and different epigenetic clocks capture different biological signals[67]. As such, the clock concept should not be confused with global methylation signatures of ageing.

Increase in DNAm variance

Some CpG sites exhibit increased variability in DNAm with age and are known as age-associated variably methylated positions (VMPs)[41,59,96–101]. This phenomenon of 'epigenetic drift' in ageing was first identified in twin studies, which found that the methylomes of monozygotic

Whole-genome bisulfite sequencing
A sequencing technology to survey and quantify DNA methylation (DNAm) at the single-base resolution on a genome-wide scale.

Reduced representation bisulfite sequencing
A sequencing technology to quantify DNA methylation (DNAm) at the single-base resolution at specific regions of interest, such as promoters and repeat regions.

Epigenome-wide association studies
(EWAS). The genome-wide investigations of the association of epigenetic marks, such as DNA methylation (DNAm), and a disease or trait of interest.

Table 1 | **Statistical tests and software for analysing age-associated changes in DNAm**

Feature (description)	Statistical test	Software package (function)	Advantages	Disadvantages	Refs
DMP (CpG site that changes in average DNAm with age)	Linear model	limma (lmFit)	Provides a genome-wide view of methylome shifts shared by individuals over time Useful for age-prediction algorithms Building blocks of epigenetic clocks Detected in relatively small sample sizes	Analysis excludes informative CpGs that change in variance as a function of age but not in average methylation	59,61,282
DMR (region of multiple, contiguous DMPs, such as of CpGs)	Different software use different statistical approaches	minfi (DMRcate, blockFinder), bumphunter, comb-p, ChAMP (Probe Lasso)	DNAm within ~500 bp is typically highly correlated DMR analysis reduces spatial redundancy DNAm altered over a region may offer better functional relevance (that is, directly linking to gene expression changes)	Isolated CpG sites that might be informative are discarded Methylation arrays offer unequal coverage, potentially limiting the number of important regions discovered	17,63–66, 283–285
VMP (CpG site with increased variability in DNAm with age)	Breusch–Pagan test and White test to identify VMPs with age as continuous variables; Bartlett's test, Levene's test and Brown–Forsythe test to identify VMPs between discrete groups	lmtest (bptest), DiffVar (modelled off Levene's test)	Individual CpGs that change in variability with age inform of DNAm changes that differ between individuals over time	Large sample sizes across a broad age range are required for sufficient statistical power Sparsity of large data sets in tissues other than blood could hamper detection of VMPs across different tissues and cells	17,59,99
VMR (genomic region of CpGs displaying variable DNAm changes with age)	Different software packages use different statistical approaches	minfi (DMRcate)	Genomic regions that are rich in VMPs may offer better functional relevance	Isolated CpGs that might be informative are discarded Methylation arrays have limited coverage, which could exclude regions of importance	59,62,65
Entropy (a single quantifiable measure of the methylome-wide DNAm changes for a sample at a point in time)	A Shannon entropy probability formula adapted for DNAm data	None currently available	A single entropy value provides a snapshot of the amount of epigenetic 'noise' or information loss for a single sample at a particular age Can be calculated for a specific set of CpGs to identify regions of high versus low methylation disorder associated with ageing	Two samples of the same age from two different batches are difficult to compare	41,59, 109,111
Correlation networks (clusters of co-methylated CpGs form 'modules' that change with age)	Pairwise correlations are used to construct co-methylation networks; modules are identified using hierarchical clustering	WGCNA	Adopts a systems biology approach to explore the interconnectedness of the entire methylome, alleviating the burden of testing individual CpGs Multiple data sets can be simultaneously analysed as a network-based meta-analysis technique	Technique does not assess modules that become disconnected with age	42,43,126
Epigenetic age (output of an epigenetic clock, which estimates age from a subset of CpGs correlated with chronological age and age-related phenotypes)	Machine learning algorithms, such as elastic net regression	glmnet	Easy method of obtaining the epigenetic state of a single sample at a point in time Clocks that capture biological ageing parameters can predict healthspan and mortality risk Clocks are used to assess the success of longevity interventions and rejuvenation experiments	A narrow measure of the methylome Age estimation depends on the data sets used to build the clock, as well as parameters that the clock was trained against	72,286

CpG, cytosine–guanine dinucleotide; DMP, differentially methylated position; DMR, differentially methylated region; DNAm, DNA methylation; VMP, variably methylated position; VMR, variably methylated region; WGCNA, weighted gene correlation network analysis.

twins diverge as they get older[97,102–104]. VMPs therefore capture the stochastic changes in DNAm that accumulate with age[59] and are largely driven by differences in exposure to environmental factors that accumulate throughout the lifespan[27,93,97,105]. Unlike DMPs, VMPs do not necessarily display shifts in their mean DNAm over time but, instead, show increases in DNAm variance, as a result of aberrant and unpredictable changes (that is, divergence from the mean)[17,41,59,97,98] (FIG. 1). Although some overlap between DMPs and VMPs does exist,

Methylation fraction
For a given DNA methylation (DNAm) locus in the human genome, the proportion of DNA strands that are methylated relative to the total number of DNA strands (0–100%), in a given population of cells.

Heteroscedastic
The variance of the residuals of a variable is non-constant or unequal across a range of values.

characterized by CpG sites that display both a change in mean DNAm and variance with age, a notable proportion of VMPs are independent of differential DNAm shifts, and represent their own class of age-related DNAm changes[59]. Intriguingly, although the overwhelming majority of VMPs increase in variance across the lifespan, a small proportion of VMPs decrease in variance with age[41,97–99,101], with a tendency to approach fully methylated or unmethylated states (a methylation fraction of 0 or 100%)[101]. Nonetheless, we cannot rule out the possibility that variability relates to differences in technologies, which can show more error at intermediate DNAm levels (DNAm levels near 50%).

Various statistical tests have been used to identify VMPs, all of which detect heteroscedastic VMPs (that is, change in variance) (TABLE 1). For example, to test for heteroscedasticity in DNAm with age as a continuous variable, either the Breusch–Pagan test[41,59,100,106,107] or the White test[102] can be used. To test for heteroscedasticity in DNAm between discrete groups (for example, between newborns and centenarians), Bartlett's test, Levene's test or the Brown–Forsythe test can be employed[17,97]. The R package DiffVar has been developed to detect VMPs in microarray data modelled off Levene's test[99]. Variably methylated regions (VMRs), which are genomic regions of CpGs displaying variable DNAm, can be detected using packages such as DMRcate[17,62,65]. Thus, unlike DMPs, VMPs increasingly diverge from chronological age, and may therefore capture the inter-individual variation in DNAm that occurs with age.

Increase in DNAm entropy

Although DNAm is binary in nature, the DNAm fraction of a particular CpG site is often measured over a population of cells and is represented as a gradient (from 0 to 100%). In the mammalian genome, CpG sites are typically highly methylated or unmethylated, with few sites showing intermediate levels of methylation. However, at many CpG sites, methylation levels shift over time from states of high or low methylation to an

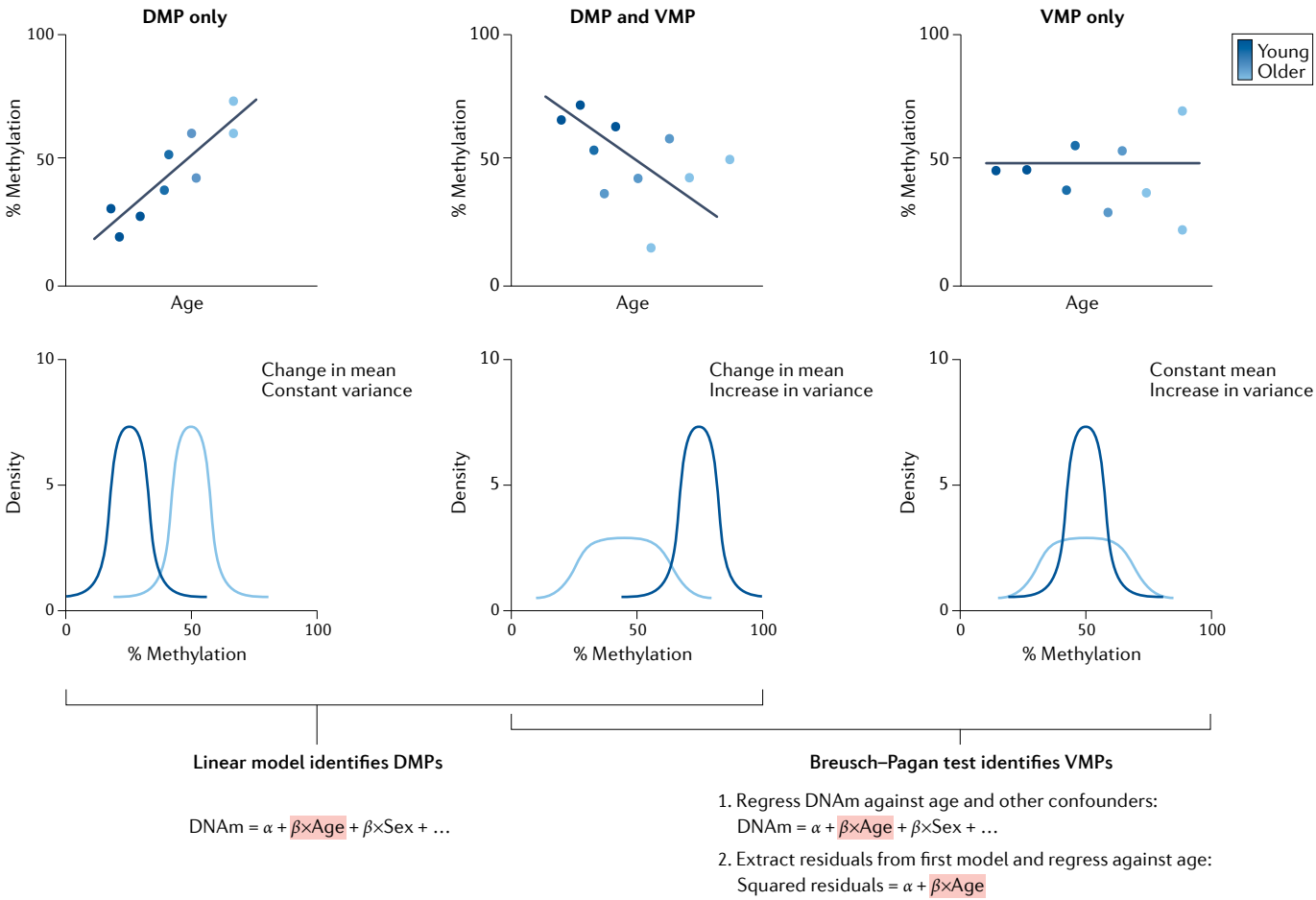


Fig. 1 | **Linear models classify age-associated changes in DNAm.** Linear plots (top) and corresponding density plots (bottom) represent chronological changes at individual cytosine–guanine dinucleotides (CpGs) during ageing. CpGs that change in mean methylation are classified as differentially methylated positions (DMPs). A young individual with high methylation fraction at a particular DMP can be distinguished from an older person with low methylation at the same DMP. DMPs are identified using a linear model and can be homoscedastic (no variance in residuals) or heteroscedastic (variance of residuals increases with age). Variably methylated positions (VMPs) are classified according to the relative changes in variability with age. All VMPs are heteroscedastic. For example, older individuals will show striking variability in methylation status at a particular VMP, compared with young individuals. VMPs can be identified using the Breusch–Pagan statistical test, which is a two-way linear regression formula. First linear model regresses DNA methylation (DNAm) against age and other confounders, second linear model regresses the squared residuals of the first model against age. α, y intercept; β, regression coefficient for each predictor variable. Figure adapted with permission from REF.[41], Elsevier.

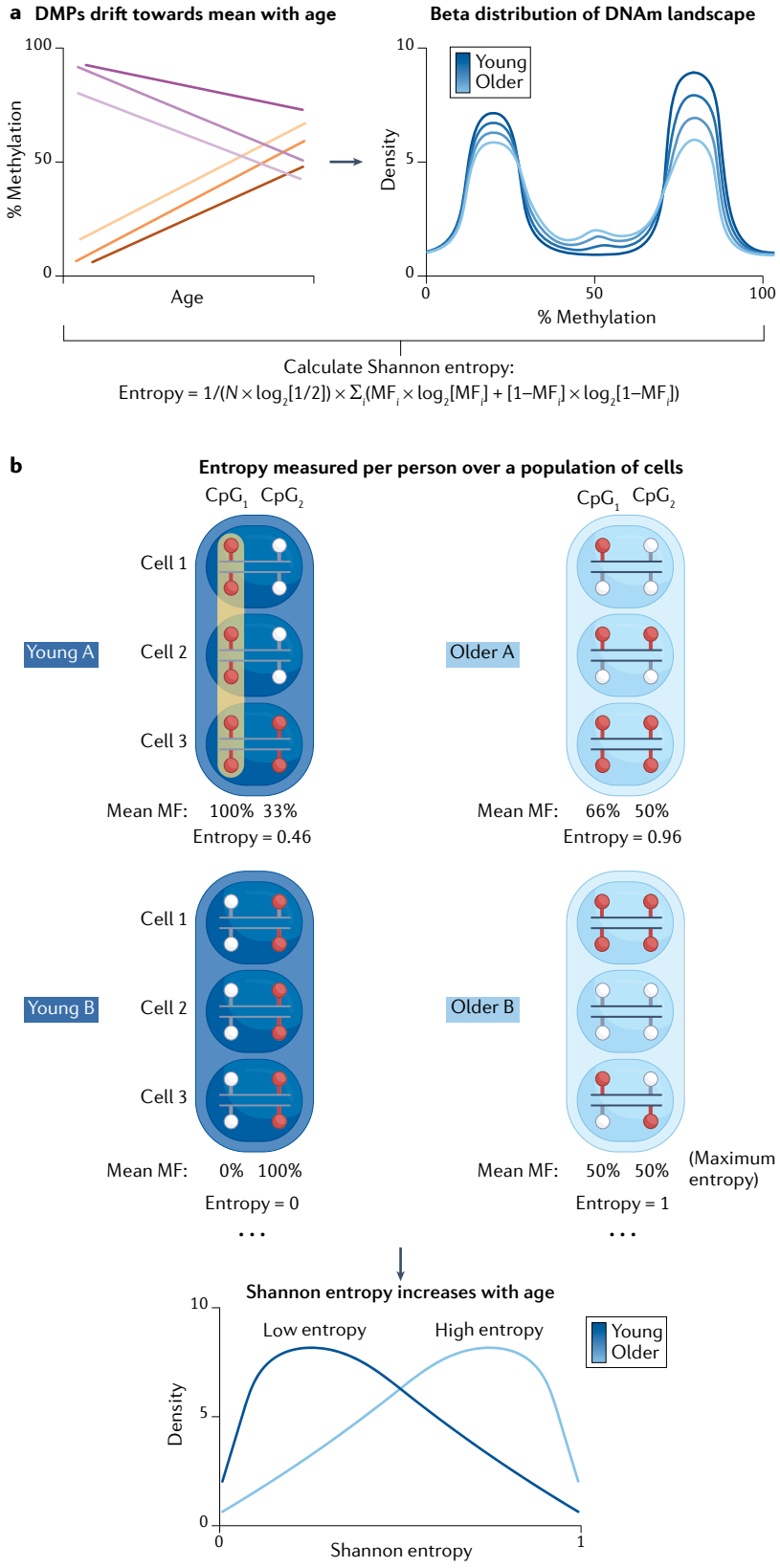
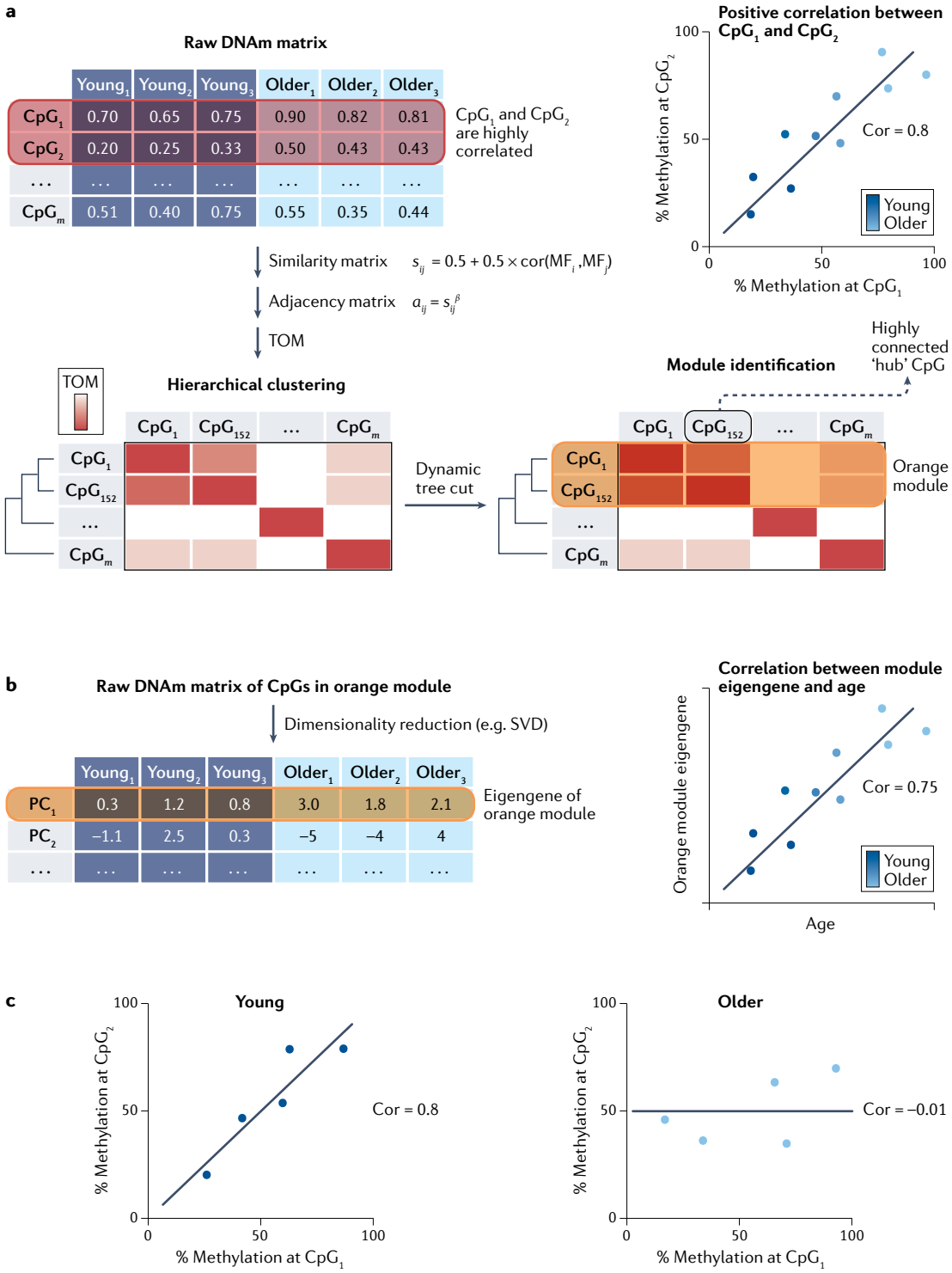


Fig. 2 | **Entropy measures chaos in the ageing methylome. a** | Pattern of differentially methylated positions (DMPs) that drift towards a methylation fraction (MF) of 50% with increasing age (left). These data can also be shown as a beta distribution, whereby all cytosine–guanine dinucleotides (CpGs) in the methylome in young individuals show a bimodal distribution with two distinct peaks, which tend to shift to the middle with age (right). To quantify total accumulation of these age-related changes in DNA methylation (DNAm) across all CpGs, a Shannon entropy probability formula can be used. **b** | To calculate entropy for each individual, mean MF of each CpG is first measured over a population of cells and then input into the entropy formula. If a CpG takes on one or only a few values, it is easy to predict its value and entropy is considered low ('young B'). By contrast, if a CpG takes on numerous values, it is more difficult to predict and entropy is high. Entropy is at a maximum if mean methylation of a CpG is 50% ('older B'). As the methylome accumulates changes at multiple CpGs with age, Shannon entropy increases. *i*, single CpG; *N*, total number of CpGs. Panel **a** adapted with permission from REF.[41], Elsevier.

intermediate fraction of ~50%, representing a 'smoothening' of the epigenetic landscape[41,67] (FIG. 2). CpG sites that are hypermethylated or hypomethylated in youth become less ordered and predictable at older ages[41,67]. Ageing is therefore associated with a reduction in the stringency of epigenetic maintenance systems[6,108], where small perturbations at individual CpG sites (that is, at DMPs and VMPs) cumulatively result in the inability of youthful DNAm patterns to be maintained throughout the lifespan. This observation has been described as 'epigenetic chaos', or loss of information, which increases with age[109,110].

Studies in blood have quantified these methylome-wide DNAm changes as a single measure of 'entropy', or methylation disorder[41,59,100,109,111,112] (TABLE 1). Entropy is a scientific concept, as well as a quantifiable measure of randomness, uncertainty or disorder. Information can be subject to entropy, as first introduced by Claude Shannon in 1948 as 'Shannon entropy' or 'information entropy', which measures the amount of information in a given variable, such as a set of CpG sites[113]. Entropy is low if it is easy to predict the information stored in a given variable, because there is less surprise or uncertainty. Entropy increases if a parameter is difficult to predict, as it takes on many possible values, and thus there is more uncertainty with an increasing number of possible outcomes[113]. For clarity, the uncertainty of a single CpG associated with entropy is distinct from the stochasticity of a VMP. Whereas VMPs capture a variable change with age, entropy is linked to the ability to predict the methylation status of a CpG for any given cell (FIG. 2). For instance, estimating the CpG status by sampling a pool of cells, where half of the cells are methylated and the other half are unmethylated, would yield a high entropy value because there is only a 50% chance one could accurately estimate the methylation fraction.

In a single measure, Shannon entropy can therefore estimate the amount of information within a set of CpG sites and quantify the total accumulation of differential age-related changes in DNAm at all CpG sites[41,109] (FIG. 2). An increase in Shannon entropy with age implies that the ageing methylome shifts to an epigenetic state of high disorder (that is, it tends towards a methylation fraction of 50%), displaying increased 'chaos' or information loss over time[41,59,84,109,112,114]. Shannon entropy is



Breusch–Pagan test
A statistical test for heteroscedasticity of the errors in a linear regression model. It works by determining whether the errors of the response variable (such as DNA methylation (DNAm)) are dependent on the independent variable with continuous values (such as age).

measured using a probability formula, which has been adapted to handle DNAm data[115].

Our current understanding of epigenetic entropy and age is largely derived from studies in blood. Considering that tissues age at different rates[116], future investigations should explore whether these tissue-specific differences are underpinned by differing rates of entropic decay. Moreover, our understanding of the mechanisms underlying entropy are confined to studies on bulk tissue, rather than single cells. As cell to cell heterogeneities increase with age[117], it is plausible that increases in entropy measured at the tissue level might simply reflect DNAm variability between individual cells. Indeed, novel approaches to estimate the epigenetic age of single cells suggests that individual cell types do not age at the same rate[94]. Thus, measuring entropy at the single-cell resolution might reveal interesting entropy dynamics compared with whole tissue analyses, such as whether cells exhibiting a reduced epigenetic ageing rate have a slower rate of entropic decay.

◀ Fig. 3 | **Correlation networks reveal connectivity in the ageing methylome. a** | Highly correlated cytosine–guanine dinucleotides (CpGs) that exhibit coordinated methylation changes with age (top panel; raw DNA methylation (DNAm) matrix and graph) can be summarized into highly informative modules (bottom right) using weighted gene correlation network analysis (WGCNA). The WGCNA package measures strength of correlation between CpGs, taking into account the methylation fraction (MF) for each sample at each CpG. First step involves constructing a similarity matrix from the raw DNAm matrix, a matrix of absolute values of correlation coefficients between CpGs. Note that *s* is similarity measure of MFs for *i*th and *j*th CpGs. This is followed by constructing an adjacency matrix, that uses a soft-thresholding parameter to measure strength of connection, whilst preserving underlying correlation relationship. Adjacency measure (*a*) for *i*th and *j*th CpGs calculated by raising the similarity measure to the power of β, the soft-thresholding parameter. For module detection, a topological overlap measure (TOM) is used to measure interconnectedness (proximity) of CpGs and is combined with unsupervised hierarchical clustering to organize CpGs with similar co-methylation dynamics. Gradient of TOM represents degree of interconnectedness, whereby white denotes low TOM (or low interconnectedness) and red denotes higher TOM (or higher interconnectedness). Modules then defined by 'cutting' branches of identified clusters, using methods such as Dynamic Tree Cut, which cuts branches of a module based on their shape. **b** | Module eigengenes are a mathematical construct used to summarize module connections into a single value using a dimensionality reduction technique such as singular value decomposition (SVD) (left). This is useful because modules often contain hundreds or thousands of CpGs. Module eigengenes can be correlated to specific traits of interest, such as age (right). Module eigengene that is positively correlated with age implies that all CpGs within that module become similarly hypermethylated with age. PC refers to the principal component; the output of the dimensionality reduction technique. The first PC is the module eigengene. **c** | Future work could investigate co-methylation relationships that become disconnected with age.

White test
A statistical test for heteroscedasticity of the errors in a regression model. Unlike the Breusch–Pagan test, the White test can be used to identify both linear and non-linear forms of heteroscedasticity.

Bartlett's test
A statistical test for heteroscedasticity between two discrete groups. This test assumes normality for each group and is thus sensitive to departures from normality.

Levene's test
A statistical test for heteroscedasticity that compares deviations from the mean between groups.

Brown–Forsythe test
A statistical test for heteroscedasticity that compares deviations from the median between groups.

R package
A collection of functions, code, documentation and data bundled into a standardized format that can be downloaded and installed by R users.

Singular value decomposition
(SVD). A technique used to reduce the dimensionality of a data matrix. This is useful for identifying sources of variation.

Correlation networks

The identification of DMPs and VMPs provides valuable insights into the shift of DNAm patterns with age at individual CpGs. However, the methylome forms a complex network of coordinated CpG sites that show similar methylation status (that is, they are co-methylated)[54,58,118–120]. CpG co-methylation can have a physical explanation, as CpGs tend to influence the methylation status of other nearby CpGs, owing to the presence of DNMT and TET enzymes that maintain co-methylation dynamics and epigenetic status during cell divisions[119,121]. Long-range co-methylation relationships also exist[118,122,123], as distal CpGs can be brought into spatial proximity upon chromatin folding[120]. Both local and long-range CpG correlations reflect the three-dimensional architecture of DNA, and such coordinated DNAm patterns play an important role in regulating cellular activities[118,120,123]. As such, co-methylation between individual CpGs can reflect which biological pathways are active, and thus regulate cellular functions, as the activity of these pathways depends on the coordinated activation and silencing of multiple genes[17,124].

DNAm analysis may therefore benefit from adopting a 'systems biology' approach that encompasses the interconnectedness of the entire methylome[42,125]. Focusing on interconnected CpGs narrows the focus on a reduced set of entities, which alleviates the issues associated with making multiple statistical comparisons (consider that typical DNAm data contain hundreds of thousands of CpGs) and enhances biological signals. Weighted correlation network analysis, also known as weighted gene correlation network analysis (WGCNA), has been used to identify clusters of co-methylated CpG sites (modules) that are associated with ageing in humans, both in single tissues, such as saliva[71], and across tissues such as brain and blood[43]. WGCNA constructs co-methylation networks by measuring the pairwise correlations between CpG sites (FIG. 3) and then transforming this correlation into a measure of proximity (that is, network interconnectedness)[42,126] (TABLE 1). Highly interconnected CpGs are then clustered into modules that typically contain hundreds or thousands of CpGs. To represent a sample's profile at each of these modules, DNAm levels at the CpGs contained within each module are 'summarized' using a data reduction technique such as singular value decomposition (SVD). For a given module, each sample's profile is then represented by the module eigengene, which represents a 'summary' of the DNAm levels at the CpGs within that module. Module membership, which is measured by the correlation between a CpG and the module eigengene, determines the connectivity of the CpGs in the module. CpGs with high module membership are considered highly connected 'hub' genes[42,126,127]. Modules that are present in multiple data sets represent common and robust CpG relationships that reflect true underlying biology and not technical noise. These data can be used to determine whether older individuals display distinct DNAm patterns at those modules (that is, at highly correlated CpGs)[72] and what biological pathways these modules reflect.

Although WGCNA reveals whether interconnected CpGs become simultaneously hypomethylated or hypermethylated with age, it does not show whether there are CpGs that become increasingly disconnected with age. This disconnect would translate to CpGs that are highly correlated in young people but poorly correlated in older people (FIG. 3). Such loss in connectivity could impact cellular function, owing to a loss of coordinated gene expression and the reduced activation of biological pathways. Few studies have assessed methylome connectivity using methodologies other than WGCNA[48,124]. However, in one such study comparing the correlation patterns of neighbouring CpGs by calculating Spearman's rank correlation coefficient, reduced correlations in the methylomes of centenarians compared with neonates were observed[48]. As the interdependence between neighbouring CpGs ensures epigenetic fidelity during mitosis[119,121], neighbouring CpGs that lose their correlation with age could gradually introduce noise that is propagated during subsequent cell divisions. Coordinated changes in DNAm can also give rise to differential transcription factor–DNA binding during ageing[91], due to altered DNAm at transcription factor binding sites compromising binding of transcription factors[91,128].

DNAm changes as hallmarks of ageing

Despite identical rates of chronological ageing, there are marked disparities in individual rates of biological ageing[3,129,130]. As such, biological age represents the functional status of body tissues and the organism as a whole, as well as the age-associated risk of disease and disability[3,8,67,68], all of which are influenced by intrinsic factors (for example, sex and genetics) and the cumulative, lifelong exposure to environmental stimuli[105].

In this section, we review the evidence that DNAm is a hallmark of ageing against the previously published criteria[7] that changes arise during normal ageing, the

Homoscedastic
The variance of the residuals of a variable is constant across a range of values.

experimental acceleration of epigenetic ageing accelerates ageing or the experimental deceleration of epigenetic ageing slows ageing down.

Age-related changes in DNAm and disease

Altered DNAm patterns, compared with healthy individuals, have been observed for many age-related diseases. Changes in DNAm are seen in left ventricles and blood of patients with CVD[34–36]. Individuals with atherosclerosis show aberrant DNAm patterns in blood (for example, monocytes) and in endothelial and vascular smooth muscle cells (for example, aorta and arteries)[131–134]. Changes in blood DNAm patterns are associated with hypertension[135] and are suggested to be linked to blood pressure alterations[136]. DNAm changes are also observed in adipose, liver and pancreatic islets in individuals with type 2 diabetes (T2D)[137–140]. There are DNAm changes in cartilage in individuals with osteoarthritis[141,142] and in bone in individuals with osteoporosis and osteoarthritis[143]. Changes in DNAm in multiple brain regions have been reported in individuals with Alzheimer disease[29–33] and are likely to play a functional role in Alzheimer disease pathogenesis[31]. DNAm changes in the lens epithelium can cause alterations in gene expression that are associated with the development of cataracts[144]. Moreover, aberrant DNAm is a feature across multiple types of cancer[145–149].

Many age-associated DMPs overlap with the changes in DNAm that are associated with disease, such as in Alzheimer disease[32], T2D[137] and cancer[40,148–150]. A potential causal role of age-associated DNAm in endometrial cancer pathogenesis has also been suggested[151]. Epigenetic age acceleration (that is, the difference between chronological age and epigenetic age estimated by epigenetic clocks) is associated with Alzheimer disease[152,153], dementia[152], blood pressure[154], cancer[155,156], CVD[68], frailty[68,157], insulin[68,154], osteoarthritis[158,159] and Parkinson disease[160]. Similarly, age-associated VMPs have been reported in cancer[59,161]. Higher entropy has been associated with chronic lymphocytic leukaemia, a cancer affecting older people[162]. Moreover, an age-related co-methylation module present in blood and brain tissue contains CpGs associated with early Alzheimer disease[43]. Considering the importance of methylome integrity to health, it is plausible that ageing phenotypes are the downstream consequences of disrupted DNAm patterns[163,164].

The precise mechanisms by which DMPs and VMPs each contribute to ageing are not well defined. Two individuals with identical chronological ages (and therefore similar patterns at DMPs) may display divergent patterns across VMPs[59] (FIG. 4); these divergent patterns might reflect the introduction of additional noise at key genomic regions that become naturally dysregulated with age. As such, identifying DMPs might pinpoint sites, genes and pathways related to primary ageing, whereas VMPs might pinpoint those related to secondary ageing[6]. If this holds true, the stochasticity introduced by VMPs could reflect mosaicism in ageing cells and tissues, reflecting the inter-individual variation in risk of disease, in addition to the age-related changes that more or less track chronological age[67]. In support of this hypothesis, age-associated epigenetic heterogeneity (or epigenetic 'chaos') is associated with an increase in age-related gene expression noise (for example, transcriptional variation during ageing)[13]. Moreover, a discernible relationship between VMPs and gene expression exists, that is, VMPs associate with the expression of genes in *trans*[59].

Heterogeneity in biological ageing, and therefore age-associated risk of disease, is mirrored at the epigenetic level. An individual whose biological age deviates from their chronological age, due to either positive (for example, exercise or healthy diet) or negative (for example, pollution) environmental influences, could display patterns of VMPs that more closely resemble the methylome of a younger or older individual, respectively (FIG. 4). We propose that homoscedastic DMPs represent DNAm changes that precisely track chronological ageing (that is, primary ageing), whereas VMPs represent DNAm changes that reflect intrinsic and environmental factors (that is, biological and secondary ageing)[8,41,59,105,165].

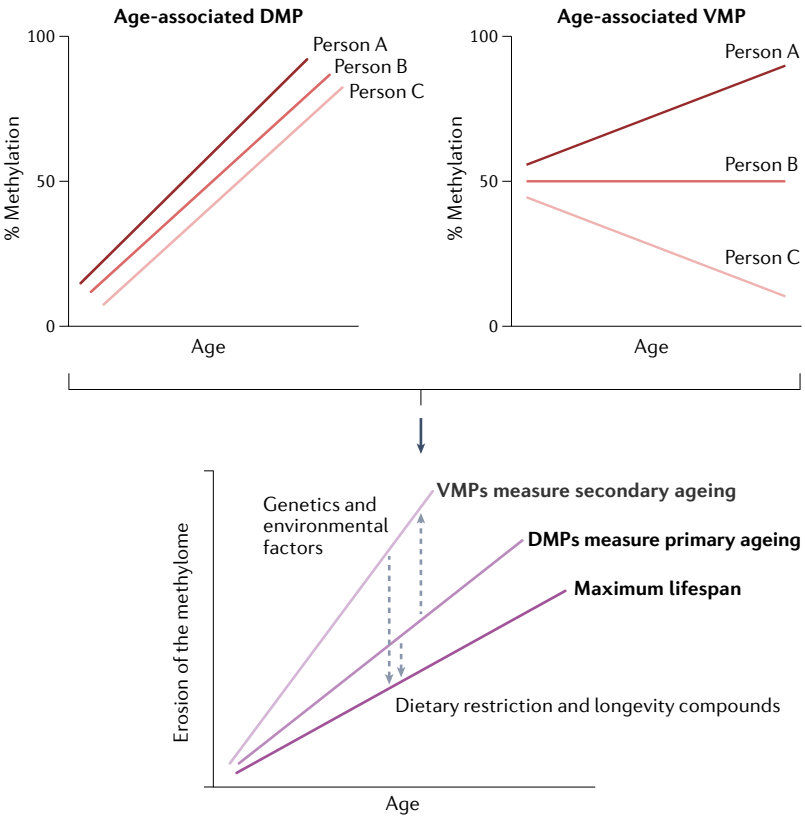


Fig. 4 | **DMPs and VMPs reflect primary and secondary ageing processes.** Differentially methylated position (DMPs) represent changes in DNA methylation (DNAm) that are shared across the lifespan (top left). Variably methylated positions (VMPs) represent DNAm changes that differ across the lifespan (top right). Two individuals of same chronological age may have similar patterns of DMPs but display divergent patterns across VMPs. DMPs likely track a primary ageing process (intrinsic age-related deterioration occurring with chronological time), whereas VMPs likely track a secondary ageing process (additional and heterogeneous age-related changes accelerated or decelerated by environmental influence, genetics or disease). VMPs may therefore represent biological ageing at the epigenetic level. 'Maximum lifespan' indicates that longevity interventions delay DNAm changes associated with primary and secondary ageing. Dashed arrows indicate direction of effect on the methylome. For example, dietary restriction and longevity compounds attenuate age-associated erosion of the methylome. Conversely, unfavourable environmental influences and disease can accelerate erosion of the methylome.

This, however, remains a hypothesis that is yet to be tested. Whereas primary and secondary ageing are considered two distinct processes at the level of DNAm, the overlap that exists between DMPs and VMPs could reflect biological processes that are part of normal primary ageing and that are susceptible to erroneous, secondary changes. As such, these processes may not be completely independent of one another.

Evidence to support this hypothesis stems from the link between age-associated DNAm variability and cancer, whereby VMPs that undergo age-associated changes overlap with VMPs in healthy tissue that will go on to develop cancer[161]. Ageing and cancer might share a common origin[166], which hints at a causal role for DNAm in cancer initiation that might extend to other diseases[93], as suggested by the correlation between DNAm variability and predisposition to type 1 diabetes[167].

Despite numerous studies linking ageing with widespread changes to the methylome, some studies investigating the associations of DNAm with age-related disease have not yielded similar results, identifying only a handful of disease-related CpGs[36,135–137]. One possibility is that disease-affected tissues (for example, pancreatic islets and heart) are often difficult to obtain in large sample numbers, and blood might not serve as a useful surrogate. Cell-type heterogeneity that is unaccounted for could also obscure findings[168,169]. Moreover, EWAS have so far been largely limited to identifying DMPs. A shift in focus to VMPs, entropy and co-methylation networks in the context of human disease will help to answer important questions related to the biology of ageing, such as whether age-related diseases have different rates of entropic decay, which co-methylation networks and underlying mechanisms become disrupted with diseases of ageing and whether VMPs display different patterns across cell types and tissues.

The lack of causality is another major challenge in the interpretation of DNAm studies. The relationship between DNAm and gene expression is complex and improving our knowledge of how DNAm functions in different genomic contexts is necessary to accurately interpret how these DNAm changes affect ageing and disease[170]. Confounding variables, including cell-type heterogeneity, genetic variation and reverse causation (that is, where DNAm is a consequence, rather than cause, of a given phenotype), can challenge the understanding of DNAm alterations[117,169]. Whereas causal inference can be improved using multi-omics[17], few DNAm data sets with matched gene expression and other omics data are available. Moreover, functional studies are not straightforward, as DNAm changes that arise during ageing are spread throughout the methylome. Whether DNAm changes at a single locus or multiple loci cause a particular phenotype is therefore difficult to determine.

Longevity-promoting interventions

If accumulated alterations to the methylome have a causal role in age-related decline, then such changes should, in theory, be alleviated or reversed by longevity-promoting interventions. This concept underlies certain dietary, drug and epigenetic rejuvenation strategies.

Dietary restriction. Dietary restriction entails limiting the quantity or timing of food intake. Calorie restriction is the long-term reduction of calories below standard requirements without incurring malnutrition, and is a well-known longevity-enhancing strategy[171–173]. In humans, calorie restriction reduces the risk of age-associated diseases, including T2D, cancer and CVD, and extends both healthspan and lifespan in mammals[171,172,174]. Intermittent fasting, or time-restricted feeding, also seems to promote healthy ageing[171]. Because calorie restriction and fasting are often combined during dietary restriction strategies[171], future interventions, particularly in humans, should explore which is necessary to reap benefits.

Several animal studies in rodents and monkeys have investigated the effect of calorie restriction on the DNAm signatures of ageing, reporting that calorie restriction shifts the methylome to a younger profile by attenuating the age-related DNAm alterations in several tissues, such as the blood[114,174], liver[53,175], kidney[176] and hippocampus[177]. For instance, a study in liver tissue of female mice reported that calorie restriction increased methylation at DMPs that usually become hypomethylated with age and decreased methylation at DMPs that usually become hypermethylated with age[53]. Another study in rhesus monkeys and mice reported that calorie restriction counteracts epigenetic drift in the blood, shifting the methylation patterns of calorie-restricted older mice and monkeys to resemble their younger counterparts[174]. Moreover, calorie restriction decelerates epigenetic ageing by directly affecting the DNAm changes that underpin the epigenetic clock[84,174]. Co-methylation networks exploring the effects of calorie restriction on conserved ageing modules in multiple species and tissues have also reported that calorie restriction leads to DNAm changes in the opposite direction to age (for example, CpGs within a module that become hypermethylated with age exhibit a decrease in DNAm following calorie restriction)[178].

Although the evidence outlined above is limited, it suggests that calorie restriction promotes longevity, at least in part, by slowing the epigenetic changes associated with primary ageing. However, data on calorie restriction and longevity in humans are needed to confirm this hypothesis. More research is required to determine whether the influence of calorie restriction on DNAm is the causative factor underpinning the healthspan and lifespan-extending properties of dietary restriction. To our knowledge, no studies have examined the influence of dietary restriction on age-related DNAm signatures in humans.

Longevity drugs. Longevity drugs with promise to slow ageing, and their influence on the hallmarks of ageing, have been previously reviewed[179]. Here, we focus on drugs that have been shown to act directly upon the methylome and/or its machinery.

Rapamycin is a lifespan-extending compound that inhibits the mammalian target of rapamycin complex 1 (mTORC1), a master regulator of metabolism and cell growth[179]. In mice, repression of mTORC1 using rapamycin slows ageing by extending the lifespan[180,181] and

healthspan by protecting against age-related diseases, such as Alzheimer disease[182], cancer[183] and T2D[184]. However, one study in mice reported that, although rapamycin protects against many ageing traits, some ageing phenotypes remained unaltered or worsened following treatment with rapamycin[185]. Moreover, not all tissues respond equally to rapamycin treatment[181], and species-specific differences exist. In dogs, rapamycin improved several healthspan parameters, including cardiac function[186], but in non-human primates, long-term rapamycin treatment had few metabolic consequences in adipose or liver tissue[187]. Notably, in humans, rapamycin seems to have geroprotective properties, such as the ability to improve immune function in older people[188]. At the epigenetic level, rapamycin slows the epigenetic ageing of human keratinocytes in culture[189] and, in vivo, age-associated DNAm changes are supressed in mouse livers after rapamycin treatment[84]. However, rapamycin has no apparent effect on the epigenetic age of marmosets according to a marmoset-specific epigenetic clock[89], possibly due to tissue-related differences. Interestingly, rapamycin seems to be less effective at protecting against ageing than calorie restriction[53,84], suggesting that calorie restriction and rapamycin operate through independent mechanisms to slow epigenetic ageing.

Metformin is a drug widely used to treat T2D that is known to target several key nutrient-sensing pathways such as AMP-activated protein kinase (AMPK) and mTORC1 (REFS[179,190]). Metformin also seems to protect against ageing phenotypes, such as cancer and inflammation[190]. Animal studies support metformin as a promising drug for extending healthspan and lifespan[190]. The Targeting Ageing with Metformin trial (TAME trial) has been initiated to investigate the effect of metformin in delaying age-related diseases in humans[179]. Showing some promise, one study in humans reported that a cocktail of drugs, including growth hormone, metformin and dehydroepiandrosterone (DHEA), reversed epigenetic ageing in thymus tissue and increased the predicted human lifespan by approximately 2 years, as measured by the GrimAge clock[191]. However, these results should be interpreted with caution owing to the small sample size and lack of an appropriate control group. It is also difficult to tease apart the effect of each drug on their own. Preliminary evidence from another small study reported that metformin slows epigenetic ageing of peripheral blood in patients with T2D[192].

Nicotinamide adenine dinucleotide (NAD^+) plays a central role in metabolism, acting as an essential co-enzyme for redox reactions[193]. In humans, NAD^+ levels have been shown to decline with age in the skin[194], brain[195], liver[196] and blood[197]. Indicating a causal role of NAD^+ decline in ageing, dietary supplementation with nicotinamide riboside (NR) and nicotinamide mononucleotide (NMN), two NAD^+ precursor molecules, can ameliorate age-associated diseases and extend the healthy lifespan[179,198]. Although the mechanisms of these effects are not fully understood, it is likely relevant that NAD^+ serves as a cofactor for various NAD^+-dependent enzymes that coordinate epigenetic modifications, such as sirtuins, CD38 and poly(ADP-ribose) polymerases (PARP)[193]. For example, NAD^+ is required for the activity of epigenetic regulators, such as sirtuin 1 (SIRT1), and a decline in NAD^+ causes changes to histone modifications, altering chromatin structure and gene expression[193,199]. Moreover, SIRT1 affects DNAm at regions that become specifically altered with age[200]. To our knowledge, no studies to date have explored the influence of NAD^+ enhancers on the global DNAm signatures of ageing.

α-Ketoglutarate (α-KG) is a key metabolite in the Krebs cycle, but also assists in demethylation as a cofactor for TET enzymes[201]. In mice, supplementation with α-KG decreases the severity of ageing phenotypes, such as osteoporosis[202], delays the age-related decline in fertility[203] and extends both healthspan and lifespan[204]. Altered levels of α-KG during ageing may alter the activity of TET enzymes required for regulating patterns of DNAm, and supplementation with α-KG may increase its availability to act as a cofactor for TET enzymes[67,205]. A recent study in humans reported that a cocktail containing α-KG and vitamins, known as Rejuvant, taken for 4–10 months decreased biological age, measured by the TruAge clock, by an average of 8 years[206]. More robust evidence from placebo-controlled experiments is needed to corroborate these results.

Spermidine is a naturally occurring polyamine that has an essential role in metabolism[179,207]. Spermidine synthesis declines during ageing in both humans and mice[208]. In mice, spermidine supplementation extends the lifespan and healthspan, offering cardioprotective benefits[209] and preventing liver fibrosis and hepatocellular carcinoma[210]. In humans, spermidine intake is correlated with lower blood pressure and reduced incidence of heart disease[209], and lowers the mortality risk by up to 5.7 years[211]. Spermidine might promote these effects by altering DNAm[208]. Indeed, studies in mice have shown that lifelong consumption of a polyamine-rich diet inhibits aberrant age-associated DNAm[212]. Mechanistically, elevated polyamine metabolism increases the availability of essential substrate *S*-adenosylmethionine (SAM), which favourably alters the activity of the DNMT enzymes that maintain patterns of DNAm[212–214]. Although these effects seem to be driven by increases in the levels of spermine (a derivative of spermidine), more research is required to determine the influence of spermidine supplementation, particularly in humans, on the DNAm signatures of ageing.

Epigenetic rejuvenation. DNAm is at the core of epigenetic reprogramming experiments, which aim to 'reset' epigenetic patterns to youthful states by reversing cellular age. Overexpression of four transcription factors (OCT4, SOX2, KLF4 and MYC, collectively termed Yamanaka factors or OSKM factors) is the most common strategy for cellular reprogramming and epigenetic rejuvenation[215]. By inducing these four factors in vitro, somatic cells can regain pluripotency and reset their DNAm age to zero, as measured by the pan-tissue epigenetic clock[13].

One issue with reprogramming a somatic cell to a pluripotent state is a loss of original cell identity, and therefore function. Continuous expression of OSKM in vivo also induces teratomas (a type of rare tumour)[215].

GrimAge clock
An epigenetic clock that is a predictor of both lifespan and healthspan. DNA methylation (DNAm) GrimAge is the output of an epigenetic clock, which utilizes a machine learning algorithm trained against a linear combination of chronological age, sex, DNAm-based surrogate biomarkers for seven plasma proteins and smoking pack-years.

TruAge clock
A direct-to-consumer epigenetic age test built using a machine learning algorithm trained to predict chronological age of a saliva sample from a limited number of cytosine–guanine dinucleotides (CpGs) in CpG islands (CGIs) and promoter regions.

Polycomb repressive complexes
(PRCs). Multiprotein complexes of Polycomb group proteins. PRCs modify epigenetic marks to control gene repression and are involved in regulating developmental genes in a multitude of cell types, including embryonic and adult stem cells.

CpG islands
(CGIs). Regions of the genome containing a high frequency of cytosine–guanine dinucleotide (CpG) repeats.

To overcome this issue, transient reprogramming experiments (that is, partial reprogramming) have been introduced to achieve rejuvenation while maintaining somatic identity[215]. In mouse retinas, in vivo overexpression of just three of the four Yamanaka factors (OSK) showed that global DNAm signatures arising from both normal ageing and following injury can be reversed such that cells do not lose their identity, yet recover youthful DNAm signatures[216]. Moreover, this strategy yields a younger transcriptome and restores youthful vision in old, vision-impaired mice[216]. Interestingly, TET demethylating enzymes were necessary for this reprogramming to occur, as evidenced by knockdown experiments, suggesting that DNAm changes are intrinsic to the ageing process and its reversal[216]. Similarly in human dermal fibroblasts, early evidence suggests that in vitro transient reprogramming using OSKM induces a marked reduction in DNAm age by approximately 30 years, measured by the pan-tissue epigenetic clock; transient reprogramming also rescued the transcriptional and morphological features of youthful fibroblasts[217]. These experiments highlight a very important aspect of epigenetic ageing, that is, youthful epigenetic information can be recovered, a feat that requires maintenance methylation enzymes. How the 'lost' information is recovered to reprogramme the methylome is not completely understood, but could involve persistent epigenetic memory at enhancer regions that allows cells to restore their initial identity[217].

Exercise. Another promising healthspan-promoting strategy is exercise. The effect of exercise on the global DNAm signatures of ageing in humans is largely understudied, despite the plethora of healthy ageing benefits offered by exercise[218]. Future work should determine whether exercise (and if so, which type of exercise) retards the ageing process by directly targeting the methylome. Tightly controlled human exercise studies, such as Gene SMART (Skeletal Muscle Adaptive Response to Training)[219] and the Wellderly Project[220], which include large biobanks of skeletal muscle and blood epigenetic data across the lifespan for healthy males and females, are well designed to answer this question.

In summary, DNAm alterations satisfy, at least to some degree, the criteria to be considered a hallmark of ageing[7]: they arise during normal ageing in, arguably, every cell, tissue and species; DNAm ageing can be accelerated experimentally in model organisms and is associated with many age-related phenotypes in humans and animal models; longevity interventions rescue age-associated DNAm changes in model organisms; and resetting the methylome is necessary to reverse the age of a cell in mouse models and human cells. More evidence that longevity interventions (such as exercise training or dietary restriction) or unfavourable lifestyle changes modify DNAm would further solidify DNAm as a true hallmark of ageing in humans. More data are also required to determine whether DNAm alterations are a primary hallmark (and cause) of ageing or whether they are a consequence of another feature of ageing, such as molecular damage.

Origins of age-related DNAm changes

Location of age-related methylome changes

EWAS have identified hypermethylated or hypomethylated DMPs associated with ageing in multiple human tissues and cell types, for example whole blood, monocytes, mesenchymal stem cells and buccal, brain and skeletal muscle, to name a few[27,40,55,57,76,78,137,148,221,222]. Although tissues and cells have unique DNAm ageing signatures, there are conserved DNAm changes across cell types during ageing[60,76,93].

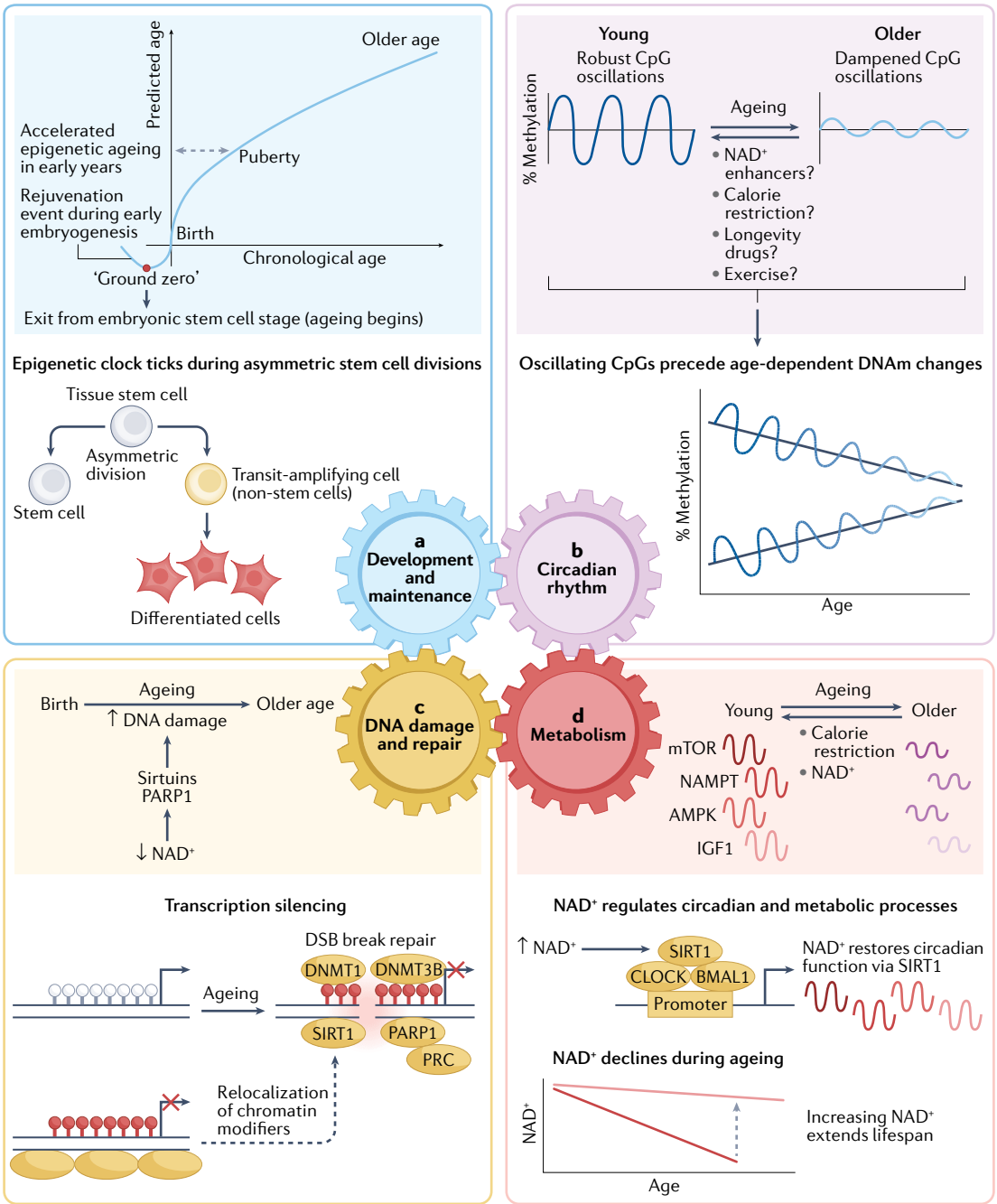
Gains in methylation with age accrue more frequently in CpG-rich regions, as these regions tend to be naturally unmethylated. Specifically, hypermethylated DMPs occur at the promoters of key developmental genes harbouring both active and inactive histone marks (known as bivalent chromatin domains), as well as in regions actively repressed by Polycomb complexes[40,55,57,78].

Polycomb group proteins form Polycomb repressive complexes (PRCs) that associate with DNA and chromatin to control developmental regulators[223,224]. In embryonic stem cells, PRCs maintain pluripotency by repressing developmental genes that are preferentially activated upon cellular differentiation[223]. Hypermethylation might therefore be associated with decreased plasticity due to the permanent silencing of genes required for differentiation[57].

By contrast, hypomethylation occurs preferentially in regions of low CpG density, often at intronic and intergenic regions[148]. Hypomethylated DMPs generally harbour active histone marks that are associated with enhancers[97,148]. Compared with gains in methylation, losses of methylation are less conserved across tissues and are functionally enriched for disparate pathways[55,148]. For example, in a study comparing CpG methylation across blood, brain, kidney and skeletal muscle tissues, only hypomethylated DMPs in the kidney and blood were enriched for a similar pathway related to immune response, and hypomethylated DMPs in skeletal muscle were strongly enriched for muscle-specific pathways[55]. These differences are possibly owing to the role of enhancers in tissue-specific gene expression[15,55].

Convincing evidence recently came from a large meta-analysis of age across multiple species and tissues corroborating results from individual EWAS[56]. This study confirmed that hypermethylation in CpG islands (CGIs) is conserved across tissues, and the majority of universal hyper-DMPs are proximal to genes encoding transcription factors that bind to Homeobox domains. These transcription factors are involved in central developmental processes (that is, morphogenesis and cell differentiation), and many of the hyper-DMPs are located in regions targeted by the PRCs and in bivalent chromatin domains[56]. Moreover, hyper-DMPs were enriched across tissues compared with the number of hypo-DMPs. This observation echoes previous findings suggesting that hypo-DMPs reflect tissue-specific operations. For example, hypo-DMPs in brain and cerebral cortex tissues were enriched for circadian rhythm pathways, but not in other tissues such as skin or blood[56].

Although VMPs are less well characterized when compared with DMPs, a study in blood reported increases in DNAm variability with age at bivalent regions and sites



residing in Polycomb-repressed regions[59]. Similar to hyper-DMPs, VMPs that increase in both mean methylation and variance were strongly enriched for CGIs, and VMPs that decrease in mean methylation but increase in variability were enriched for non-CGIs[59]. In another smaller study of mesenchymal stem cells, increases in DNAm variability with age were preferentially located at non-CGIs and intergenic regions[97]. VMPs do not appear to be driven by changes in cell composition during ageing[59]. Interestingly, a significant proportion of VMPs are associated with gene expression changes in *cis* in pathways involved in neuron differentiation and neuron development, whereas VMPs associated with gene expression in *trans* (that is, the CpG and gene are located on different chromosomes or on the same chromosome >5 Mb apart) correspond to pathways such as metabolism, apoptosis and the DNA damage response[59]. Although there is evidence from cancer studies that 'epigenetic drift' occurs in other healthy tissues, such as the colon, these studies were not focused on identifying and characterizing age-related VMPs[225,226]. More studies using large sample sizes across a broad age range are needed to characterize VMPs in other tissues and in the context of ageing.

Co-methylation network analysis corroborates the EWAS results for hyper-DMPs in ageing mammals[56,178]. WGCNA was recently used to cluster co-methylated CpGs across 63 tissues and in 176 mammalian species, including humans. Pooling DNAm data from ~11,000 samples, 55 co-methylation modules were identified,

◀ Fig. 5 | **Proposed mechanisms of epigenetic ageing.** Epigenome maintains development and maintenance programmes, circadian rhythms, DNA repair and metabolic fitness to maintain health of an organism, and we propose that, during ageing, it contributes to decline of these coordinated processes. **a** | Epigenetic clock, stem cells and developmental process are thought to be linked, with the clock tracking human growth and development from embryogenesis (during which the methylome is at the lowest biological age) into older age[60,94,228]. Ageing thus begins soon after embryonic stem cell state and continues throughout lifespan until death. Epigenetic clock is proposed to 'tick' when tissue stem cells undergo asymmetric division and differentiate into non-stem cells (trans-amplifying cells), and rate of differentiation reflects rate of tissue turnover, which is measured as rate of epigenetic ageing by the clock[231]. **b** | Chrono-epigenetic theory proposes that cytosine–guanine dinucleotides (CpGs) that exhibit circadian oscillations are robust in young people but dampen with age[241], possibly due to altered activity of ten–eleven translocation (TET) and DNA methyltransferase (DNMT) maintenance enzymes. Age-related changes in amplitudes of the oscillations precede linear DNA methylation (DNAm) changes and might predict age-dependent linear outcomes. Whether nicotinamide adenine dinucleotide (NAD^+) enhancers, dietary restriction, longevity compounds or exercise restores CpG rhythmicity that declines with age is unknown (?). **c** | DNA damage, which is present from birth until death, might also be an upstream cause of ageing. Cellular pool of NAD^+ is utilized by DNA repair processes, suggesting that cell metabolism and DNA repair activities are interdependent. Upon DNA damage, NAD^+-dependent enzymes, including sirtuin 1 (SIRT1) and poly(ADP-ribose) polymerase 1 (PARP1), are recruited to double-stranded breaks (DSBs) to assist in repair[193]. Chromatin modifiers, including DNMT1, DNMT3B and Polycomb repressive complex (PRC), are also recruited to damage sites to silence transcription. CpG-rich promoter regions preferentially targeted by the repair machinery are susceptible to stable silencing events, possibly explaining why hypermethylation at Polycomb target genes is conserved in ageing[264]. As DNA damage increases with age, this targeting puts strain on DNA repair machinery and reduces pool of NAD^+ available for cellular metabolism. **d** | Circadian clock controls rhythmic expression of metabolic genes, which lose robust oscillations with age. NAD^+ regulates circadian and metabolic processes[193], and increasing NAD^+ levels (possibly via calorie restriction) restores metabolic rhythms by increasing SIRT1 activity; SIRT1 interacts directly with core clock components to reprogramme healthy circadian function[252]. NAD^+ levels decline with age, and repleting NAD^+ extends healthy lifespan in model organisms. AMPK, AMP-activated protein kinase; IGF1, insulin-like growth factor 1 ; NAMPT, nicotinamide phosphoribosyltransferase. Panel **b** adapted from REF.[241], Springer Nature Limited.

several of which were associated with biological traits, such as chronological age, sex and maximum lifespan[178]. To harmonize chronological ageing between species, relative age was used, defined as the ratio between age of the organism and maximum lifespan of the species (for example, the relative age of a 40-year-old human is 0.33 because the maximum human lifespan is 122.5 years). Strikingly, the most conserved module in mammalian tissues was positively correlated with the relative age of all mammalian species, meaning that CpG sites within this module are correlated with each other and are collectively hypermethylated with age across all tissues and species. This module was enriched for pathways such as those that regulate embryonic stem cells, axonal fasciculation, angiogenesis and diabetes-related processes. An earlier study of co-methylation networks in blood and brain tissue of humans had reported similar findings[43]. In both studies, the top 'hub' CpGs (that is, the most highly connected CpGs in the module occupying central network positions) reside in genomic regions adjacent to Polycomb targets and repressive histone marks. This observation is indicative of a conserved ageing phenomenon across tissues and species, whereby a subset of highly correlated CpGs that are unmethylated in young people become methylated with age at distinct regions that control development[56,178].

Mechanisms of the ageing methylome

Pinpointing the precise mechanisms that underpin the ageing methylome, as well as those that control how fast the methylome ages, is challenging. Nonetheless, several tenable hypotheses have been put forward and are discussed below.

The epigenetic clock theory of ageing. It has been hypothesized that the ageing methylome reflects an innate process that is intricately linked with development and differentiation[75]. In support of this hypothesis, epigenetic clocks can estimate chronological age with remarkable accuracy and across a broad spectrum of the lifespan, from prenatal mammals to the oldest living mammals on earth[56,60]. Furthermore, the epigenetic clock can provide an accurate estimation of gestational age, which involves a highly coordinated developmental process with little noise perturbing the system[227], and can even measure rejuvenation events that occur during embryogenesis, marking the beginning of organismal ageing[94,228] (FIG. 5). As such, the epigenetic clock reflects an ageing process that is not monotonous but, instead, aligns with the non-linear periods of growth and development[75,229,230]. For example, the epigenetic clock is accelerated during the first few years of life and slows after puberty, paralleling the human developmental process[231]. Consistent with these observations, the fundamental processes established in early life ultimately cause an organism to age[75,231].

A tenable hypothesis to explain this phenomenon was proposed in a recent review[231]. Albeit limited in mechanistic evidence, a link between stemness and epigenetic ageing has been proposed, whereby the 'ticking' of the epigenetic clock represents a measure of asymmetric stem cell or progenitor cell divisions (that is, when a stem cell gives rise to two daughter cells, one which retains stemness of the mother cell and one which becomes a non-stem cell) (FIG. 5), or the change in stem cell numbers in different tissues[231,232]. Epigenome-wide investigations substantiate this hypothesis, finding that the dysregulation of developmental genes that govern cell identity is a conserved feature of mammalian ageing[40,55–57,78]. Simply, the methylome is precisely altered in the genomic locations that function to preserve stem cell identity and function. Further support for this hypothesis comes from recent evidence from profiling epigenetic age in individual murine cells, showing that epigenetic ageing is precisely tracked in hepatocytes (that is, epigenetic age increases in old versus young hepatocytes), whereas muscle stem cells display minimal changes in epigenetic age[94], suggesting that the epigenetic clock ticks when stem cells are stimulated to divide[117].

A study in humans analysing the methylome and transcriptome of CpGs from four epigenetic clocks reported that DNAm of some of the clock CpGs associated with gene expression in *trans*, and that the genes involved have a role in T cell processes[233]. It was proposed that the differences in DNAm between immune cells, namely naïve T cells, activated T cells and natural killer cells, may drive the progression of epigenetic clocks. However, these conclusions were drawn from only a subset of clock CpGs and it remains unknown

what percentage of the clock's predictive capability is attributable to T cells and natural killer cells.

Although the maintenance of DNAm patterns established during development is key to maintaining youthful epigenetic states and robust cell identity, this maintenance is challenging due to the plasticity of DNAm[234]. DNAm dynamically responds to environmental cues, DNA repair, transcription and replication. Consequently, failures in DNAm maintenance and tissue homeostasis can have detrimental effects on the organism, leading to ageing and age-associated disease[232,234]. Molecular damage, metabolism or activated developmental programmes, for example, can alter the methylome of adult stem cells, leading to stem cell dysfunction and a decline in tissue and organ function[232]. The clock theory of ageing therefore proposes that the widespread decay of the methylome reflects an 'epigenetic maintenance system' that is operating to support development, cell differentiation and maintenance of cell identity[60,75].

The importance of TET and DNMT enzymes in maintaining DNAm patterns is highlighted by genetic disorders underpinned by mutations in their genes. For example, mutations in *TET2* and *DNMT3A* are implicated in the early onset of haematological malignancies in older humans[235]. Unlike other Mendelian disorders, conditions associated with *DNMT1* mutations uniquely display a gradual and progressive onset of symptoms, such as hearing loss and dementia, that are absent in youth but manifest in adulthood[236,237]. It is plausible that supplementation with longevity drugs, such as α-KG and polyamines, could promote methylation maintenance by altering the activity DNMT and TET enzymes and the methylation of their substrates[205,213]. Evidence from epigenetic rejuvenation experiments, albeit in mice, also suggests that TET and DNMT enzymes are necessary for reprogramming an aged cell to a youthful epigenetic state[216].

In summary, the epigenetic maintenance system responsible for primary ageing is also susceptible to the gradual accumulation of errors or biological 'noise', leading to changes associated with secondary ageing. The biological ageing process is therefore proposed to reflect the unintended consequence of both developmental and maintenance programmes (that is, the ability of the stem cell niche to maintain tissue homeostasis)[75,231]. However, it is not yet clear how epigenetic enzymes lose their ability to perform their function with advancing age, what mechanistic insight can be gained from single-cell epigenetic age analyses or whether a single or multifaceted upstream mechanism drives these changes.

Metabolic signalling and chrono-epigenetic ageing. The circadian system is an autonomous internal oscillator that provides rhythmic coordination to physiological, behavioural and metabolic processes, synchronizing the external environment with internal processes to maintain organismal health[238]. Ageing is accompanied by the loss of robust circadian oscillations and the desynchronization of these processes, which has been linked with metabolic disorders and multiple ageing pathologies in humans and in mice[239,240]. On the contrary, interventions that restore circadian rhythms in rodents are associated with longevity[239]. 'Chrono-epigenetics' is the umbrella term used to describe the circadian dynamics of the epigenome, which affect histone modifications, chromatin architecture and DNAm[241]. Specifically, CpGs exhibit circadian behaviour, which is facilitated by the rhythmic action of DNMT and TET enzymes[241]. Studies in mice suggest that the light entrainment of the circadian clock is dependent on DNAm, supporting the role of DNAm as a mediator between the external environment and internal rhythms[242]. Moreover, experiments in liver and lung tissue of mice, as well as in human neutrophils, have shown that CpGs exhibit rhythmic oscillations that markedly overlap with CpGs that are differentially methylated with age[243,244] (FIG. 5). One caveat of these findings is that, even if DNAm oscillations are detected in a 'purified' cell type, the overlap with age-associated DNAm could reflect subtle shifts in cell subtypes if adjustments for cell-type heterogeneity are imperfect. Enrichment of oscillating CpGs was observed at distal regions and enhancers of both highly expressed and circadian genes[243,244]. Notably, oscillating CpGs preceded age-dependent changes in CpGs, and the amplitudes of the oscillating CpG correlated with the magnitude of the linear age-dependent change[243,244] (FIG. 5). The authors characterized CpGs that oscillate consistently between individuals as DMPs and those under more lenient control (and that are more susceptible to environmental perturbation) as VMPs[241]. In support of this, there are linear age-dependent DNAm changes in the *CLOCK* gene, which is one of the 353 CpGs in Horvath's pan-tissue clock[60].

The role of enhancers in coordinating rhythmic expression might shed light on shared mechanisms between circadian disruption and epigenetic ageing. In mammals, a 'core clock' involving four key factors (the transcription factors CLOCK and BMAL1, and the genes cryptochrome (*CRY*) and period (*PER*)) regulates the 24-h cycles via a transcription–translation feedback loop[245,246]. In addition, cell-autonomous peripheral clocks drive tissue-specific rhythmic gene expression by coordinating the activity of cell-specific enhancers[247–249]. Age-associated hypomethylation at enhancers of highly expressed genes might therefore involve the reduced precision of DNMT and TET following circadian disruption, altering gene–enhancer interactions (that potentially disturb co-methylation networks) and gene regulation[67].

If disruption of the chrono-epigenome is a proximal cause of ageing, it remains unclear what drives this disruption in such a precise manner that it parallels the 'ticking' of the epigenetic clock. We hypothesize that metabolic processes are part of this equation. Indeed, the circadian clock and metabolism have a reciprocal relationship, in that the rhythmicity of metabolic processes is an output of the clock, and metabolic signals and states feed back to the clock[245]. Many metabolic genes that oscillate in tissue-specific rhythms encode proteins that participate in the same metabolic pathways implicated in both ageing and longevity[239,245] (FIG. 5). For example, dietary restriction extends the lifespan through beneficial effects on nutrient sensors that are under circadian control, including the inhibition of pro-ageing factors

such as insulin-like growth factor 1 (IGF1), and the activation of the longevity factors AMPK, sirtuins, nicotinamide phosphoribosyltransferase (NAMPT) and the forkhead transcription factors (FOXOs)[171,250]. Similarly, 'longevity drugs' mimic the longevity effects of dietary restriction by targeting the same metabolic pathways[239]. Animal studies have demonstrated that calorie restriction can restore the tissue-specific circadian rhythmicity of key metabolic genes, suggesting that longevity benefits involve the restoration of healthy circadian cycles[239]. Polyamines also show circadian rhythmicity and, in turn, regulate the circadian period. In mice, supplementation with spermidine counteracts the age-associated decline in circadian cycles by regulating the interaction between the core clock factors[251].

A vital link between ageing, metabolism and circadian rhythms is NAD^+, the central catalyst of metabolism. A reciprocal relationship between NAD^+ and circadian rhythm exists, meditated by the rate-limiting enzyme in NAD^+ biosynthesis, NAMPT[193]. In mice livers, NAD^+ reprogrammes metabolic and stress-response pathways by restoring circadian function that declines with age[252]. Mechanistically, NAD^+ acts through SIRT1, an NAD^+-dependent enzyme, which interacts with the core clock components to restore robust circadian oscillations[252] (FIG. 5). PARP1 is another NAD^+-dependent enzyme that has been linked to entrainment of the circadian clock[253,254], although whether PARP1 also participates in the reprogramming of circadian rhythms is unknown. As well as supplementation of its precursors, NAD^+ levels are increased by dietary restriction and interventions, exercise and healthy circadian cycles[193]. As discussed above, NAD^+ levels decline with age, although the mechanism is poorly understood, and likely involves multiple pathways, such as altered metabolic activity and inflammatory processes. PARP enzymes also assist with DNA repair processes, which likely contributes to the depletion of the NAD^+ pool (and decline in SIRT1 activity) during ageing[193,255].

Considering the above evidence, daily metabolic stress might contribute to the gradual deterioration of circadian function that explains, in part, the age-dependent DNAm changes associated with primary ageing. If so, a natural consequence might be that such changes would be exaggerated in situations of more severe metabolic and circadian disruption, driving variability in the rates of epigenetic ageing. Future work should determine how different stimuli (for example, light, nutrients or DNA damage) regulate DNMT and TET activity and the associated temporal changes in methylation and demethylation, and thus how maintenance enzymes modulate these epigenetic, circadian and metabolic processes. Moreover, elucidating whether longevity interventions (for example, calorie restriction, exercise and supplementing with NAD^+ enhancers) can restore CpG oscillations to youthful states would contribute to our understanding of ageing from a chrono-epigenetic–metabolic perspective.

DNA damage and the re-localization of chromatin modifiers. There is also emerging evidence that DNA damage, particularly in the form of double-stranded breaks (DSBs), might drive the ageing process[110,256,257]. DNA damage causes various DNA lesions, arising from both exogenous (for example, ultraviolet, chemicals and X-rays) and endogenous (for example, oxidative stress, metabolic stress, replication errors and spontaneous hydrolytic reactions) sources[7,257]. The accumulation of DNA damage leads to a collection of molecular consequences, such as genomic instability and epigenetic alterations, that underpin a spectrum of ageing phenotypes[257].

DSBs activate the DNA damage and repair machinery[257]. DSB signals recruit epigenetic modifiers, such as sirtuins and PARP enzymes, from their native loci to repair damaged loci and remodel the epigenetic landscape[258,259] (FIG. 5). Epigenome integrity is restored after DNA repair, which preserves cell identity and function[234]. However, according to the 're-localization of chromatin modifiers hypothesis', during ageing the incomplete return of these epigenetic modifiers to their original positions introduces noise into the epigenome at predictable locations, such as at key developmental regions that govern cell identity, and further increases genome susceptibility to more DSBs[110,256]. Taken together, in addition to their role in NAD^+ metabolism, sirtuins and PARP1 also form part of the DNA damage and repair machinery, suggesting that the regulation of DNA repair and cellular metabolism are coordinated[260] (FIG. 5). In worms and mice, PARP1 is chronically activated during ageing, potentially due to overactivation of DNA repair enzymes. This point is important because the increased requirements for DNA repair that activate PARP1 deplete NAD^+ pools and inhibit sirtuin activity[255], both of which are required for maintaining healthy metabolic and circadian processes.

The role of sirtuins in the re-localization of chromatin modifiers has been described in yeast and mice[261]. In a recent study, it has been experimentally shown in mice that non-mutagenic DSBs cause the loss of cell identity and accelerate the epigenetic clock, and it is hypothesized that the re-localization of chromatin modifiers, such as sirtuins, may underly this process[110,256]. This is an attractive hypothesis to explain how 'random' DNA damage can induce a precise and predictable pattern of DNAm changes and contribute to mammalian ageing.

The specific mechanism that causes DSBs to accelerate the epigenetic clock is unclear, but might involve the re-localization of methylation enzymes to sites of DNA repair[110,256] (FIG. 5). DNMTs, including DNMT1 and DNMT3B, along with other chromatin modifiers, namely SIRT1, PARP1 and Polycomb group proteins, are recruited to DSBs and sites of oxidative damage[262,263]. Localization of these repressive proteins might inhibit transcription at damage sites to prevent interference with repair. Most DNAm alterations that occur during repair are likely transient and can be restored through demethylation; however, chronic DNA damage (that is, DNA damage that occurs during ageing) might lead to DNAm modifications that accumulate with age[264]. Specifically, promoter regions are susceptible to persistent repressive DNAm[262] and it has been postulated that transcription protects promoter regions from silencing; even transient inhibition of transcription would lend

promoters more vulnerable to an increase in stable silencing events[264]. Interestingly, CpG-rich regions are preferentially targeted by the proteins recruited to sites of damage, which are translocated away from CpG-poor regions; this targeting could explain the age-associated hypermethylation at CGI promoters and hypomethylation at CpG-poor regions[263,265]. It has also been hypothesized that Polycomb group target genes are susceptible to hypermethylation due to age-related degradation of the Polycomb machinery. This degradation might lead to PRCs being unable to recognize and target unmethylated CpG-rich regions. Unmethylated CpG-rich regions ordinarily protected by PRCs become increasingly accessible to de novo DNMT3A and DNMT3B, facilitating increased methylation at these sites[266]. The pattern of Polycomb histone marks also alters during ageing; for example, trimethylation of histone H3 on lysine 27 (H3K27me3) changes in a context-dependent manner[267]. How Polycomb histone marks play a role in the DNA damage and re-localization of chromatin modifiers process may depend on specific loci and cells, and remains an interesting avenue for exploration.

DNA damage-induced re-localization of chromatin modifiers as a driver of ageing neatly positions itself in the ageing puzzle, alongside the developmental, metabolic and chrono-epigenetic hypotheses. Sources of DNA damage are ubiquitous in daily life, even arising as early as embryonic development[268], with an estimated rate of ~50 DSBs per cell per cell cycle[269]. The DNA repair process might therefore begin during early development, with insidious DNA damage ensuing throughout life. An overly simplistic explanation is therefore that certain enzymes and proteins orchestrate DNA repair with other crucial processes, including epigenetic maintenance, metabolic regulation and circadian control. The hyperactivity of the repair machinery during ageing is, unfortunately, at the cost of these processes. From this perspective, the DNAm changes that arise during ageing are the response to damage, signalling to the cell to suppress instability, yet result in the unintended consequence of introducing epigenetic noise, compromising cell identity, impairing transcription and, ultimately, causing biological ageing.

Pioneering epigenetic research

Much remains poorly understood about the upstream causes of the mammalian ageing process, despite ageing itself driving the progression of most chronic diseases[1]. Although we know that the methylome is extensively remodelled over the lifespan, the full extent to which chronological and biological ageing is reflected at the DNAm level is far from complete. In recent years, much attention in this field has focused on building epigenetic clocks; however, we propose that instead of building new clocks, the emphasis should be placed on breaking down the entire ageing methylome into its individual parts to first understand the mechanistic processes underling the extraordinary phenomenon of epigenetic ageing. Despite attempts to separate the clocks into their various components[155,156,270], we propose that teasing apart the global changes in DNAm, by measuring DMPs, VMPs, co-methylation networks and entropy, in multiple tissues is needed to obtain a better understanding of the ageing methylome in its entirety. The separation of these factors, down to specific sets of CpGs, could bring new mechanistic insights into both the chronological and biological ageing process, such as which mechanisms are responsible for age-related hypomethylation at enhancer regions.

At the epigenome-wide level, focusing only on patterns of DMPs is limiting when trying to understand aspects of biological ageing, particularly when making sense of why individuals of the same age may display vastly different biological ageing rates. However, there is a lack of research focusing specifically on VMPs in different tissues. Importantly, the classification of CpGs as DMPs and VMPs largely depends on the specifics of the cohort under investigation. For example, smoking exposure changes the mean methylation status at certain CpGs (that is, at DMPs) that can be used to predict smoke exposure[145,271]; however, in the context of ageing, it is plausible that these CpGs might be VMPs. Important questions to consider are to what extent variable methylation underpins biological ageing and whether there are tissue-specific differences. As very large sample sizes distributed across a broad age range are required to detect VMPs, this gap in knowledge is likely owing to the difficulty in sampling tissues other than blood, which can easily be sampled. Nonetheless, uncovering VMPs presents a promising avenue for identifying markers of biological age[41,59]. Future experiments could then investigate whether slowing down ageing at DMPs will lengthen the lifespan or, conversely, whether slowing down ageing at VMPs will lengthen healthspan.

Many conclusions related to the ageing methylome are drawn from mechanistic studies and longevity interventions on laboratory animals, which may or may not extrapolate to humans[272]. One potential remedy to this limitation is the use of third-generation dual-species epigenetic clocks, which are based on CpGs that are highly conserved across mammals[273] and can be used in both humans and model organisms such as rats, mice, pigs, sheep and primates[56,81,82,87,89,90,274]. Experiments in humans that can measure the effect of age-supressing or anti-ageing interventions at the DNAm level would be an invaluable contribution to the field. Furthermore, exploration of whether age and sex need to be considered when adopting different longevity protocols (for example, intermittent fasting) is largely unknown. This point brings to the forefront another challenge in epigenetic research, that is, we know surprisingly little about sex-specific differences in ageing. There is consistent evidence that females tend to outlive males[275], suggesting the existence of a robust feature of biology at play. Multi-tissue epigenetic clocks have also shown that males exhibit accelerated epigenetic ageing when compared with females[41,69]. Less understood, however, are the global patterns of DNAm that diverge between sexes across the lifetime.

Cell-type heterogeneity, which we have not addressed extensively in this Review, is also a notable confounder in experiments involving solid tissues and blood, and is therefore a major challenge in epigenetic research[17,276]. Considering that cell-type proportions change with age, tackling the issue of cellular heterogeneity would be of

great benefit to the ageing field. Recent advancements in single-cell DNAm analysis stem from the development of a novel computation tool capable of delineating the differences in cell type-specific epigenetic ageing[94,117]. This technology has the potential to accelerate our understanding of the functional and mechanistic consequences of DNAm changes during ageing[117], an exciting prospect for future explorations into biological ageing.

The potential confounding effect of 5-hydroxymethylcytosine (5hmC) in DNAm studies is also not covered in this Review. Some platforms, such as the widely used Illumina HumanMethylation arrays, rely on bisulfite sequencing, which cannot distinguish between 5mC and 5hmC[277,278]. This issue has been shown in brain tissue[279], highlighting the possibility that age-related changes in DNAm attributed to 5mC could be due to changes in 5hmC.

Pioneering epigenetic research cannot be achieved without access to large amounts of data from multiple human tissues. Epigenetic data sets of sufficient size in healthy, non-diseased human tissues across a broad age range are in short supply, and even more so in tissues other than blood. Nonetheless, existing data sets present a valuable resource for omics research, which relies on large sample sizes to detect small effect sizes. Large-scale meta-analyses overcome many limitations from small study designs, and are a valuable tool in epigenetic research[280]. Lastly, DNAm does not act in isolation but is simply a cog in a very large epigenetic machine. Future work on the ageing methylome should consider the entire epigenetic network, such as histone marks and chromatin changes, which may become disrupted with age.

Conclusions

The goal of ageing research is to target biological processes that make us live longer and help us to do so in a more youthful state. An important step towards achieving this goal is to identify the epigenetic processes that 'unravel' across the lifespan.

Although the manifestations of ageing are a feature of later life, age-associated alterations to the methylome show that the underlying cellular and molecular changes begin much earlier, even during development[116,281]. Arguably the most astounding feature of the ageing methylome is the consistency with which DNAm changes universally track chronological ageing[60], hinting at the existence of a molecular clock ticking inside our cells. However, the picture of biological ageing is far from complete.

Making sense of the ageing methylome is not an easy feat. It requires the application of computational tools that accurately analyse and interpret the versatile DNAm marks that change across the lifespan. Notwithstanding the challenges, we are at the precipice of major geroscience discoveries, but extensive collaborate efforts from researchers across multiple fields, sharing ideas and data, are needed to collectively move the ageing field forward.

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K.S. and S.V. researched the literature and wrote the manuscript. All authors provided substantial contributions to discussions of the content and reviewed and/or edited the manuscript before submission.

Competing interests
S.H. is a founder of the non-profit Epigenetic Clock Development Foundation, which plans to license several patents from his employer UC Regents. These patents list S.H. as inventor. All other authors declare no competing interests.

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