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Opinion

The ticking of aging clocks

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Computational models that measure biological age and aging rate regardless of chronological age are called aging clocks. The underlying counting mechanisms of the intrinsic timers of these clocks are still unclear. Molecular mediators and determinants of aging rate point to the key roles of DNA damage, epigenetic drift, and inflammation. Persistent DNA damage leads to cellular senescence and the senescence-associated secretory phenotype (SASP), which induces cytotoxic immune cell infiltration; this further induces DNA damage through reactive oxygen and nitrogen species (RONS). I discuss the possibility that DNA damage (or the response to it, including epigenetic changes) is the fundamental counting unit of cell cycles and cellular senescence, that ultimately accounts for cell composition changes and functional decline in tissues, as well as the key intervention points.

Highlights

Aging clocks determine aging rate by the difference between predicted and chronological age.

Clock-determined aging rate is associated with health, morbidity, and mortality.

The DNA damage response and its associated epigenetic changes may be the fundamental counting unit of aging clocks.

Cellular senescence could be the next upper-level unit of aging clocks.

Oxidative stress and inflammation contribute to DNA damage and accelerate aging clocks.

Why are aging clocks needed?

Lifespan is not equal to healthspan. One can have a long lifespan with decades in bad health without enjoying an equally long healthspan. Similarly, chronological age is not necessarily the same as biological age. Supercentenarians have very old chronological ages but relatively young biological ages [1]. Therefore, an important goal of aging research is to compress the time spent in bad health (the morbidity span), thus allowing humans to live long lives at relatively young biological ages. One way to achieve this is to accurately assess and intervene in aging itself instead of in lifespan. Using large omic datasets, **aging clock models** (see Glossary) trained on either chronological or perceived biological age can now accurately measure aging by calculating the **aging rate (ΔAge) –** the difference between model-predicted and chronological age – that is independent of age (Figure 1 and Table 1) [2,3]. Machine-learning methods for clock generation typically select multiple biomarkers, sometimes hundreds. Discussing their selection and combination is beyond the scope of this article because these features are specific to each clock and are well documented in the original publications (Table 1). Despite the growing number of aging clocks that have been developed, a consensus on their underlying counting mechanisms is still missing. I propose here that DNA damage and the response to it are the fundamental units of the aging clocks.

Association of aging rate with health, disease, and mortality

Many metabolic and inflammatory biomarkers, such as blood pressure and levels of low-density lipoprotein (LDL), total cholesterol (TC), and triglycerides (TGs) in the blood, as well as bone density, correlate positively or negatively with epigenetic or aging ratings based on **3D facial image clocks** [4–6]. This indicates that health and aging rate go hand in hand, and that ΔAge itself should be considered as a health parameter.

Acceleration of the **DNA methylation clock** has been found to be associated with a higher incidence of complex diseases or disease severity (Table 1). A >20% higher all-cause mortality risk with every 5 year increase in DNA methylation ΔAge was observed in cross-sectional [7] and longitudinal studies [8–10]. Progeroid diseases such as Hutchinson–Gilford progeria syndrome are associated with a high DNA methylation ΔAge [11]. Between identical twins, the twin with

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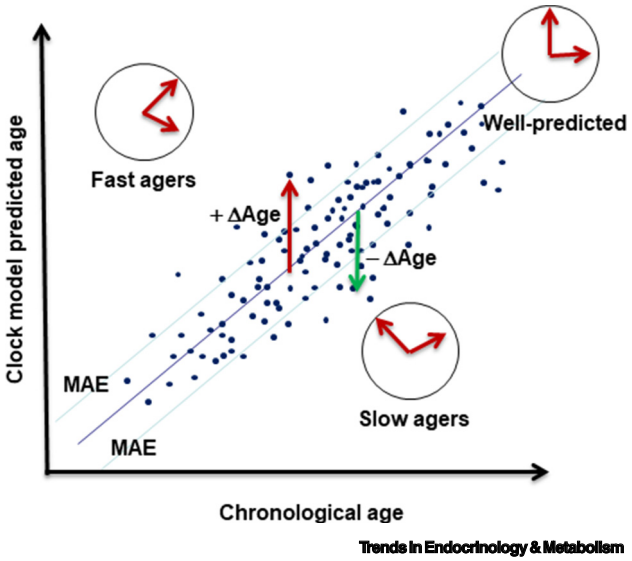

Figure 1. Measuring aging by aging clocks. Linear or non-linear aging clock models can be trained on various omic datasets from large cohorts. The age-independent difference between the model-predicted and chronological age is called ΔAge, where positive and negative ΔAge scores indicate accelerated and deaccelerated aging, respectively. When the ΔAge is outside the mean absolute error (MAE) of the model (light-blue lines), the individuals can be reliably labeled as fast or slow agers beyond the error of the model, whereas those with ΔAge within the MAE are called well-predicted.

the higher DNA methylation age shows a >twofold higher risk of mortality [10], highlighting the important impact of individual epigenomes shaped by different lifestyles on aging rate and mortality. Strong associations with health, morbidity, and mortality have also been observed for many other clocks, including transcriptomic, proteomic, metabolomic, and phenotypic clocks, as well as brain-imaging and psychological clocks [2,3,12] (Table 1). Thus, more than age, ΔAge is a general health indicator and disease risk factor that is an important measure of vitality, and might be a convenient summation of many individual health parameters. Moreover, when measured at various levels or together with specific disease conditions or risks, it could also be used to pinpoint the most problematic tissues or organs that are declining to an unhealthy state.

Pitfalls of clock models

Part of ΔAge estimates comes from errors intrinsic to the model instead of from a true difference between biological and chronological age. Building more accurate models can minimize the model error; however, models built to fit 'perfectly' to chronological age become increasingly useless in predicting biological age [13]. Models trained on some estimates of biological age avoid such a dilemma. Compared to models trained on chronological age, GrimAge clocks, that are trained on health-related surrogate indicators, better predict morbidity and mortality [13], but it is important to note that these remain 'surrogate indicators', and as such do not allow us to understand the full complexities of biological age. Convolutional neural network (CNN) clock models trained on human-perceived ages of facial images, one measure of the 'biological age' of the human face, are more strongly associated with health status than are clock models trained on chronological age [6]. However, the latter can still capture the deviation of an individual from the population average, in particular when the models are not over-fitted to the training data. Linear models, compared to non-linear models such as deep neural network models, although not as accurate, often have better interpretability – in other words, the exact features (biomarkers) and the importance of their weights in contributing to the models can be readily extracted from the models.

Do individual clocks tick at the same rate across adult lifespan?

Analysis of **frailty index** (a measure of vulnerability to external stressors) in a Canadian cohort of 13 000 individuals aged >16 years showed that the rate of functional deficit accumulation is

Glossary

Aging clock models: linear or nonlinear models can be trained on large sets of omic data to predict either chronological or biological age. The difference between the model-calculated age and the true input age shows the error of the model, often shown as the mean absolute error (MAE), median absolute error (MedAE), or root mean square error (RMSE). The model can also be measured by the goodness of fit to the input age, for example, by using the Pearson correlation coefficient (PCC).
Aging rate (ΔAge): the age-independent departure from the predictions of clock models, often called ΔAge or AgeDiff. When associated with health status, ΔAge reflects the difference between the biological age of and individual and their chronological age, and thus can be used as an estimate of aging rate.
Cell cycle: when a cell divides into two daughter cells, the cell must go through interphase (G1, S, and G2 phases) followed by the mitotic (M) phase, which together make up a cell cycle. In G1 and G2 phases the cell grows, in S phase it replicates its chromosomes, and in M phase it undergoes mitosis.
Cell-cycle checkpoints: surveillance gatekeepers that monitor the order, integrity, and fidelity of cell-cycle events, among them DNA damage, where genome and chromosome integrity are key factors. They consist of at least G1/S, S, G2/M, and M phase exit checkpoints.
3D facial image clock: a clock model that is trained on large datasets of human 3D facial images to estimate the age of a person.
DNA damage response (DDR): the ability of cells to sense and transduce DNA damage signals and respond by epigenetic changes, chromatin remodeling, damage repair, cell-cycle arrest, or senescence induction depending on the type and extent of damage. In proliferating cells, DNA damage elicits cell-cycle arrest that allows time for DNA repair before committing to subsequent phases of the cell cycle. Although the DDR often intervenes at the cell checkpoints in proliferating cells, it also occurs in non-proliferating cells.
DNA methylation clock: a clock model that is trained on selected genomic DNA methylation sites using

~4.5% ± 0.75 per year from age 20 to 105 years; however, two spikes of 6% and 7% were found at ~40 and ~80 years of age [14]. A blood aging proteome of two American cohorts also revealed three major peaks of changes at 34, 60, and 78 years of age. The first peak was associated with body mass index (BMI), whereas the middle and last peaks were strongly associated with cardiovascular and neurodegenerative diseases [15]. In Chinese cohorts aged between 20 and 77 years, the facial and transcriptomic aging rates varied the most at ~40–50 years of age [6]. These observations suggest that, although the aging clocks are constantly ticking, the rate can spike in particular periods, and mid-life and ~80 years of age are crucial times during the course of human aging.

Do different clocks tick at the same rate?

Clocks measuring aging rate at different levels or in different tissues are not always synchronized. Among the many tissues examined morphologically in worms, muscles deteriorate the first, displaying mid-life onset, whereas neurons are largely intact throughout the lifespan. Plasma membrane disruption of the cells of the intestine and hypodermis falls in between, and largely coincides with death of the animal. These changes are variable both among animals of the same age and between cells of the same type within individuals [16]. Recent single-cell RNA sequencing (scRNA-seq) analyses also invariably reveal large variations of cellular aging within the same tissue [17–20]. The first and last aging tissues are very important, but equally or perhaps more important is whether aging of a particular tissue confers a turning point that accelerates whole-body aging and makes aging a point of no return, or even elicits death. Because aging acceleration appears to peak at ~40 and ~80 years in humans [14], are these peaks attributable to the initiation of aging of different tissues, such as aging onset in the first tissue (e.g., muscle in worms) or a turning point or system-failure point (e.g., intestine and hypodermis in worms)? This seems possible given that muscle mass and function in humans start to deteriorate in midlife, whereas ~80 years is the average human lifespan in most developed countries.

A multiomic study that followed 44 individuals over 4 years found four patterns of aging (kidney, liver, immunity, and metabolic) which were called 'ageotypes'. Different individuals appear to display one or multiple ageotypes [21]. DNA methylation-based clock-calculated ages showed that breast tissue has substantial aging acceleration compared to other tissues [11]. Low correlation was found between blood telomere, epigenetic clock, and biomarker-composite aging-rate estimators [22]. However, comparisons between the mean error ranges of the models are unreliable. For example, despite a lack of significant correlation within the well-predicted populations whose Δages were smaller than the mean error of the models, there was significant overlap between the fast- or slow-aging outliers determined by the face and blood transcriptomic clocks, especially between the slow agers of the two clocks [6]. Overall, it seems clear that there is substantial heterogeneity in aging rates across individuals, tissues, and cells. However, the coherency (significant overlap) at the very extremity of aging acceleration and deceleration (fast and slow outliers) suggests that there is crosstalk between the aging clocks of different tissues.

Is there a master clock?

Clocks that measure different parameters, in different tissues, or even in different cells, do not tick at the same rate, but they do seem to influence each other across multiple tissues. Therefore, is there a master clock that drives or coordinates the aging clocks at different levels and in different tissues? One such clock could come from the immune system. As the surveillance system of the whole body, the immune system is present in all tissues, and engineered immune system aging in mice did drive all tissues to age [23,24]. Similarly, heterochronic parabiosis studies show that young and old blood rejuvenates and ages, respectively, many tissues and organs of the reciprocal recipients down to the single-cell level [25]. Other than the immune system and blood, the hypothalamus–pituitary–adrenal (HPA) axis and other neural endocrine pathways may also serve

data from cell(s) or tissue(s) to estimate the age of an individual.

Frailty index: a score between 0 and 1 to assess frailty, which is a state of increased vulnerability to external stressors. It is the ratio of present deficits to all the deficits measured (usually 30–40).

Metabolic cycle: in yeast, cells cycle between an oxidative metabolism phase and a reductive metabolism phase, and the reductive phase is temporally coupled to the cell-cycle S phase by checkpoint genes. Although a clear metabolic cycle has not been elucidated for mammalian cells, there are obvious cell cycle-coupled metabolic oscillations.

Senescence: a cellular state where the cell permanently withdraws from the cell cycle but remains metabolically active. Major causes of this state are persistent DNA damage or reaching a replication limit.

Senescence-associated secretory phenotype (SASP): the phenomenon that senescent cells express and secrete a large number of inflammatory cytokines and extracellular matrix modulators that attract immune cells for clearance. However, in old tissues the senescent cells are too many for the immune cells to clear; they therefore remain in the tissues and elicit a local inflammatory microenvironment.

Table 1. Aging clocks developed at various levels from molecular to phenotypic[a,b]

Level	Refs	Prediction	Tissues	Associations	Error (in years)
DNA methylome	[86]	Age	Saliva		MAE = 5.2
	[87]	Age	Blood	Acceleration in tumor	RMSE = 3.88–4.9
	[11]	Age	51 tissues and cell types	Acceleration in 20 cancer types	MedAE = 2.9–3.6
	[88]	Age	Blood	Association with alcohol assumption and number of children; age acceleration in aplastic anemia and dyskeratosis congenita	MAE = 3.4–5.4
	[89]	Age	Blood	Association with life expectancy	MedAE = 3.45
	[90]	Age	Blood	Acceleration in cancer and immune disease	MedAE = 2.77
	[91]		Blood	Association with all-cause mortality	
	[92]	Phenotypic age	Blood	Association with mortality and morbidity	
	[93]	All-cause mortality	Blood	Association with mortality, positive in cancer	
	[13]	Lifespan	Blood	Association with mortality and morbidity	
	[94]	Age	Five cell types, blood, skin, and saliva	Acceleration in HGPS	MedAE = 1–6.3
	[95]	Age	Muscle		MedAE = 4.6
	[96]	Age	Blood	Association with mortality	
Transcriptome	[97]	Age	Blood	Association with six health parameters	MAE = 7.8
	[98]	Age	Dermal fibroblasts	Acceleration in HGPS	MedAE = 4.0
	[99]	Age	Muscle		MAE = 6.24
	[100]	Age	Blood	Associated with all-cause mortality, CHD, hypertension, blood pressure, and glucose levels	PCC = 0.65–0.70
	[6]	Age	PBMC	Association with health parameters and lifestyles and facial clocks	MAE = 5.68
Proteome	[101]	Age	Plasma	Association with 12 health parameters	MAE = 9.7
	[102]	Age	Plasma	Association with aerobic exercise training	MAE = 1.84–2.44
	[103]	Age	Plasma	Association with all-cause mortality	PCC = 0.8
Metabolome	[104]	Age	Urine	Association with all-cause mortality, and clinical phenotypes	RMSE = 11.19 for men RMSE = 10.37 for women
	[105]	Age	Blood	Association with all-cause mortality and clinical phenotypes	PCC = 0.654, MAE = 7.3
	[106]	Age	Cerebrospinal fluid		R^2 = 0.41–0.83 MAE = 6.91–12.85
	[107]	Age	Urine and serum	Association with overweight/obesity, diabetes, heavy alcohol use, and depression	MAE = 3.71–6.49
Metagenome	[108]	Age	Stool	Acceleration in type I diabetes	MAE = 5.91
Imaging	[5]	Age	3D facial images	Association with blood test indicators	MAE = 6.2
	[109]	Age	Eye corner images		MAE = 2.30
	[110]		Brain MRI	Association with mortality	MAE = 5–7
	[111]		Brain MRI	Association with cognitive impairment	MAE = 4.29
	[6]	Age	3D facial images	Association with health parameters/lifestyles and PBMC clocks	MAE = 2.8
	[6]	Perceived age	3D facial images	Association with health parameters/lifestyles and PBMC clocks	MAE = 2.9
Clinical blood tests	[112]	Age	Blood		MAE = 5.55
	[113]	Age	Blood	Associated with hazard ratio	MAE = 5.94
	[114]	Age	Blood	Acceleration in smokers	MAE = 5.72

Table 1. (continued)

Level	Refs	Prediction	Tissues	Associations	Error (in years)
Psychological	[12]	Age and perceived age	Psychosocial questionnaires	Predictive of all-cause mortality risk	MAE = 6.70 for age; MAE = 7.32 for perceived age

[a]This table is adapted, modified, and updated based on [2].
[b]Abbreviations: CHD, coronary heart disease; HGPS, Hutchinson–Gilford progeria syndrome; MAE, mean absolute error; MedAE, median absolute error; MRI, magnetic resonance imaging; PCC, Pearson correlation coefficient; PBMC, peripheral blood mononuclear cell; RMSE, root mean square error.

as coordinators of aging across different tissues [26]. The local **SASP** of **senescent** cells can also be carried to distal tissues, making aging 'contagious' [27]. Indeed, even skin aging has an impact on whole-body aging through all the aforementioned channels [28]. However, it remains unclear whether any of the clocks may serve as a master clock.

What are the counting units of the clocks?

Most timepieces use an oscillator as the unit to track time, including mechanical, quartz, and circadian clocks, but what about aging clocks? GWAS studies on premature aging syndromes revealed that **DNA damage response (DDR)** deficits underlie the acceleration in aging rate [29]. GWAS analysis of DNA methylation ΔAge also identified variants of DDR genes as top hits [30]. Positive facial ΔAge was found to be associated with infections, inflammation, and lysosomes, and expression of the DDR gene p53 (*TP53*) was inferred to decrease blood transcriptome ΔAge [6]. Based on scRNA-seq data, increased inflammatory cytokine expression, ligand–receptor interactions, and cellular senescence markers were hallmarks of aging at the single-cell level, and these were exacerbated by disease conditions such as interstitial lung disease [31,32] and severe coronavirus disease 2019 (COVID-19) [33,34]. Senescent cells were found to increase dramatically in the tissues of severe COVID-19 patients, and senolytics ameliorated COVID-19 outcomes in mouse models [35,36]. Caloric restriction (CR) and intermittent fasting (IF) are effective means to decelerate aging. In *Caenorhabditis elegans*, CR/IF first induced lysosome and metabolic changes downstream of the mTOR pathway, followed by phosphorylation changes downstream of AMPK and TAX-6, and finally a long-term effect on **cell cycle** and DDR functions downstream of FOXO/DAF-16, where the three pathways form a synergistic feedback loop [37]. These findings suggest that nutrition signals impinge on cell-cycle control and in turn affect the DDR. Do enhanced **cell-cycle checkpoints** and DDR actually slow down the cell cycle and thus alter the internal cellular clock? This has not yet been tested. However, scRNA-seq analysis of *in vitro* neural differentiation shows that the length of the differentiation process for a single stem cell is controlled by a cell-intrinsic clock at multiple cell-cycle checkpoints, and the M-phase exit checkpoint this has a particularly large impact [38]. It would thus be interesting to ask whether aging clocks are similarly measured by the cell-cycle interval and are controlled by cell-cycle checkpoints (Figure 2, Key figure).

A plethora of studies demonstrate that cell-cycle and cellular senescence are largely controlled by the DDR. As an alternative state to the cell cycle, does senescence mark another, larger type of time unit? In other words, an overwhelmed DDR might mark the basic counting unit of the clock in both proliferating and non-proliferating cells; cell cycles would be the next level time-units counted in proliferating cells, whereas senescence would count the endings of cell cycles. These would be similar to counting in hours (DDR threshold), days (cell cycle), and months (senescence) for human. In short-lived species, the capacity of the DDR is lower, and the cell cycle and senescence are much faster, and all count toward a final readout of cellular composition and tissue integrity with age (Figure 2). Intriguingly, consistent with this hypothesis, a study of 16 mammalian species showed that the mutation rate per year in intestinal crypts is precisely

Key figure

Ticking components of aging clocks

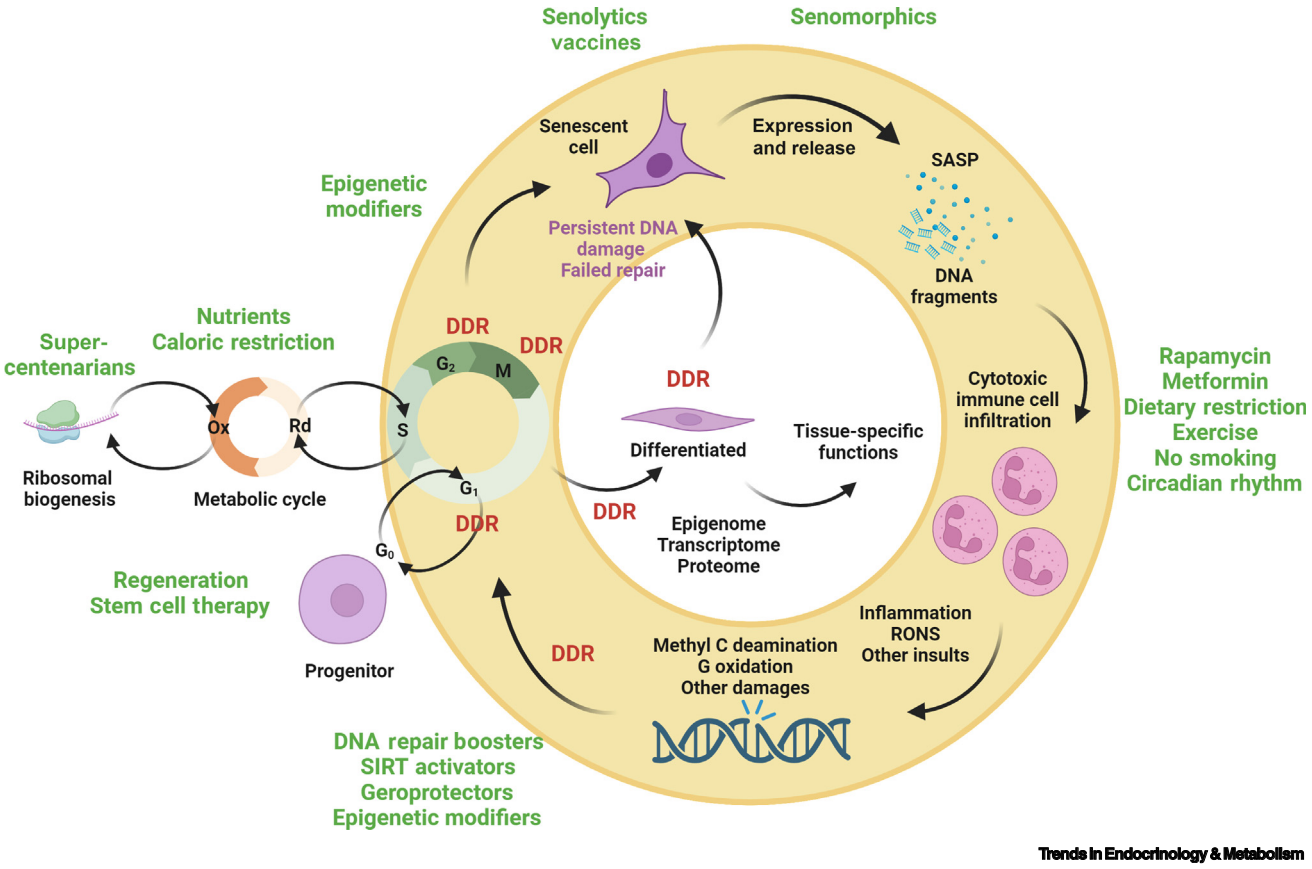

Figure 2. DNA damage and the DNA damage response (DDR) may play a fundamental role in the cascade of aging clock units, often mediated through cell-cycle checkpoints. S-phase cell-cycle checkpoints ensure faithful temporal coupling of DNA replication with the reductive (Rd) phase of the metabolic cycle, whereas the opposite oxidative (Ox) phase is temporally coupled with ribosomal biogenesis. The decline in ribosomal protein expression is a key determinant in single-cell aging clocks in many species including human. Supercentenarians have a unique tendency for high ribosomal protein gene expression. Cell cycles might be a small ticking unit of the aging clock by accumulating unrepaired damage in each cycle. Persistent DNA damage leads to cellular senescence, a middle-level ticking unit of the aging clock, which by triggering the senescence-associated secretory phenotype (SASP), elicits immune cell infiltration. Cytotoxic immune cell infiltration causes the release of reactive oxygen and nitrogen species (RONS). These lead to further DNA damage, in particular deamination of methylcytosine and oxidation of guanine. This in turn leads to an increased DDR, thus contributing to a large higher level ticking unit of the aging clock. The DNA damage–inflammation cycle further leads to compromised cell differentiation through interplay between the genome, epigenome, transcriptome, and proteome. On the other hand, epigenome modifiers and geroproctectors can protect DNA from damage and enhance DNA repair. Other, especially larger, ticking cycles may also exist and feed into or influence these cumulative oscillating units. Potential intervention points to delay or reverse aging clocks are marked in green fonts (text for details). Figure created using BioRender (www.biorender.com).

inversely correlated with the lifespan of the species [39], indicating that mutation rate and total mutations accumulated act as a precise timer that determines the mean lifetime across species. It would be interesting to see whether mutation rate also correlates inversely with lifespan and positively with aging rate across individuals.

Coupling of cell metabolism to the DDR and cell-cycle checkpoints

Recent single-cell aging clock or lifespan analyses show that ribosomal and mitochondrial activities decline with age [1,40,41]. Ribosomal protein gene expression is a key determinant

of single-cell biological age in human blood [1], as well as in fly and mouse tissues [42], and correlates with lifespan across 41 mammalian species with mean lifespans ranging from 3 to >200 years [43]. Highly elevated ribosomal protein levels are observed in supercentenarians, and could be causal for the low SASP gene expression in their blood [1]. Simultaneously elevating or stabilizing ribosomal and mitochondrial gene expression by overexpression or by circuitry rewiring from a toggle switch to negative oscillation can extend the replicative lifespan of budding yeast cells [40,41]. How do these feed into the cell-cycle units of aging clocks? The answer might lie in the coupling of cell cycles to **metabolic cycles**. In yeast cells synchronized by glucose starvation, cells cycle between an oxidative metabolism phase and a reductive metabolism phase every 4–5 h. The oxidative metabolism phase resembles a short and sharp burst that mainly involves mitochondrial energy production and ribosomal biogenesis – in other words, temporal coupling of ribosomal protein gene expression with oxidative metabolism [44]. Hence, it would be interesting to test whether the highly energy-demanding ribosomal biogenesis relies on efficient production of oxidative energy, and is compromised without it, as is seen during aging. Although further evidence will be necessary to support the existence of metabolic cycles in higher organisms, our study has shown that yeast genes expressed in the oxidative and reductive phases, when mapped to *Drosophila melanogaster*, decrease or increase their expression linearly with age, respectively, throughout the entire adult lifespan [45]. Moreover, the expression of oxidative phosphorylation genes correlates strongly and positively with the expression of mitochondrial ribosomal proteins, which in turn correlates strongly with cytosolic ribosomal protein expression and lifespan across 41 mammalian species [43]. By contrast, DNA replication (S phase) is tightly coupled to the beginning of the reductive metabolism phase. Cell-cycle checkpoint mutants that uncouple the S phase from the reductive phase (permitting the S phase to occur in the oxidative metabolic phase) result in more frequent and truncated metabolic cycles and more spontaneous mutations [46]. This suggests that cell metabolism, in particular oxidative metabolism, is an important source for the accumulation of cellular DNA mutations induced by oxidative damage if the cell-cycle checkpoints are not properly implemented to coordinate the cell cycle and the metabolic cycle [46]. Interestingly, across murine species of different lifespans, the expression of genes related to mitochondrial oxidative phosphorylation correlates negatively with lifespan, consistent with the notion that oxidative metabolism undermines lifespan [47]. The opposite is observed across mammalian species with a larger lifespan diversity, ranging from shrews to whales [43], indicating that higher mammals might have circumvented the mutation burden of oxidative metabolism by evolutionary innovations. Consistently, scRNA-seq-based oligodendrocyte, astrocyte, and microglia aging clocks in mouse brain are highly associated with oxidative stress and interferon response [48].

Free radical-induced DNA damage accumulation has been proposed to be a cause of aging since the 1950s [49]. However, this hypothesis has not been substantiated because of the rarity of the DNA mutations at the cell population level in human tissues, which can only now finally be studied with the development of single-cell genomic sequencing technologies. Indeed, age-related mutations often cluster in rare hypermutated cells that harbor many mutations and tend to give rise to clones and clonal expansion if they gain a growth and/or survival advantage [50]. Using single-cell technologies, nearly all aging single-cell DNA mutation studies invariably identified a progressive accumulation of DNA mutations, including functional mutations and hypermutations, with age; the most common age-related mutation signatures include a DNA methylation-associated deamination signature, followed by signatures of DNA damage and repair, including oxidative and other sources of lifestyle incurred (such as smoking) DNA damage [39,51–55]. In particular, smokers have faster mutation accumulation than non-smokers in bronchial epithelial cells [52], and patients with Alzheimer's disease have more and faster DNA mutations in the prefrontal cortex than age-matched controls, and oxidative damage was the most dominant signature in

these individuals [55]. These findings imply that life history, epigenome, and microenvironment might be important factors that drive DNA mutation rate and thus the ticking of aging clocks. In fact, local inflammation and inflammatory cell infiltration to tissues are a major source of RONS that can induce both deamination and oxidative damage to DNA. It is well known that oxidative damage can convert G to 8-oxo-G, thus inducing DNA mutations. However, common deamination-induced DNA mutations can be also induced by an inflammatory environment, in particular by nitrosative RONS such as NO, that are generated by immune cells [56]. Further, the damaged DNA can further trigger more cellular senescence, SASP [57], local inflammation, and inflammatory cell infiltration [58], thus forming a vicious positive feedback cycle (Figure 2). Unrepaired DNA damage, when persistent, will not only result in cellular senescence – even when no permanent cell-cycle arrest occurs, it may compromise the differentiation potential of the cells or bias the lineage of progeny cells (Figure 2), for example, via clonal expansion induced by somatic mutations [59].

It would be interesting to determine whether the various clocks can be decomposed to small cumulative and cycling units such as DNA mutation burden, DDR length, and cell-cycle length and number at the cellular level, or senescent cell count, cell state, and cell composition at the tissue level, that all add up to the minutes, hours, days, and years in biological age of the tissue and system (Figure 2). Furthermore, if DNA damage accumulation is the fundamental counting unit of tissue aging clocks, a clock based on DNA mutation burden would be more precise and fundamental than other omic data-based clocks which act as proxies to the real clock or measure in larger units.

Erosion of epigenetic modifications linked to the DDR

Transposable element (TE) derepression has long been recognized as a hallmark of aging and cellular senescence. In particular, derepression of evolutionarily young 'hot L1' elements capable of transposition induces pervasive DNA damage and genome instability and interferon response during aging [29,60–62]. The reduced levels of the long non-coding RNA (lncRNA) *KCNQ1OT1* in early senescence result in loss of DNA methylation of evolutionarily young L1 and *Alu* TEs and induce retrotransposition, DNA damage, and SASP production. *KCNQ1OT1* sequence-specifically silences these young TE elements through RNA–double-stranded DNA (dsDNA) triplexes and the recruitment of epigenetic silencing machineries [63]. Similarly, endogenous retroviruses have also been observed to be derepressed, and even packaged, inducing inflammatory responses [64]. In addition, clonal expansions in cancers and aging often involve mutations in DNA methylation and demethylation enzyme genes such as *DNMT3A* and *TET2* [65]. In general, DNA methylation and repressive histone modifications such as trimethylation of histone H3 lysine 27 (H3K27me3) and lysine 9 (H3K9me3) decrease with age, whereas active H3K4 methylation increases with age. The repressive marks that decrease with aging not only repress repetitive elements and protect the DNA from damage, but they also play important roles in the DDR. By contrast, actively expressed coding genes often lose active chromatin marks during aging. Together, these lead to aging-related epigenetic drift [61]. Interestingly, the dsDNA break-induced epigenetic changes and epigenetic clock advancement can be reversed by OCT4–SOX2–KLF4 (OSK) overexpression in mice [66], suggesting that epigenetic changes induced by DNA damage, rather than DNA damage *per se*, might be the direct counting mechanism for DNA damage.

Clocks to assess the effectiveness of delayed aging and rejuvenation therapies

If DDR and senescence are at the center of the clocks, the reversal of aging clocks would require (i) reversing the (epi)genetic states marked by the DDR, (ii) removing the senescent cells, or (iii) replenishing the tissues with cells differentiated from progenitor cells that have less mutated

genomes. So far, although biochemical and physiological parameters suggest that genome integrity protectors and DDR boosters such as sirtuin/SIRT activators [67,68] and senolytic treatments [69–71] can confer more youthful appearance and functionalities in mice, direct aging clock measurements of these treatments are still lacking. Senomorphics inhibit the SASP of senescent cells, such as by suppressing retrotransposition [60,62] and reducing NF-κB activity via suppression of NF-κB/*MARL-1* [72], but have also not been quantified by aging clocks. On the other hand, rejuvenation of retinal ganglion cells in aged mice by the OSK combination was shown to restore ribosomal DNA methylation age of the retinal ganglion cells in addition to functional regeneration [73]. Partial reprogramming by OSK plus MYC (OSKM) has also been shown to confer a younger DNA methylation age in mice [74,75]. A small 12 month clinical trial on six individuals of a combination of human growth factor, dehydroepiandrosterone (DHEA), and metformin revealed an average 2.5 years decrease in saliva DNA methylation age measured by four clocks [76]. Interestingly, a proinflammatory response is associated with reprogramming and needs to be quenched for successful chemical reprogramming of human fibroblast to pluripotent cells [77]. In accord, a recent study also shows that an effective aging delay and lifespan extension can by induced by inhibiting the central inflammatory regulatory cGAS [57].

In addition to rejuvenation treatments, aging clocks have been used to quantify the effect of metabolic interventions. DNA methylation clocks show that Rhesus monkeys under 30% calorie restriction are 7 years younger in epigenetic age compared to *ad libitum* controls [78]. Male mice under CR show an average 20% reduction in epigenetic age versus their chronological age [79]. A randomized controlled clinical trial conducted in 43 healthy men aged 50–72 years of an 8 week intervention program including diet, sleep, exercise, and relaxation guidance, as well as supplemental probiotics and phytonutrients, showed an average 1.96 years decrease in blood DNA methylation age at the end versus the beginning of the program in the treatment group [80]. Many endogenous metabolites, such as the NAD^+ precursors nicotinamide riboside (NR) and nicotinamide mononucleotide (NMN) [81], as well as spermidine [82], α-ketoglutarate [83], myoinositol [84], and uridine [85], have been shown to delay aging, but remain to be quantified by aging clocks. Different treatments can only be quantitatively and panoramically compared, and personalized according to the particular aging rate acceleration of an individual, by evaluation of multiple clocks at many different levels. A catalog of aging clocks and matching interventions will be useful to guide both clinicians and the general public on the journey toward a 'young' old age.

Concluding remarks and future perspectives

Because aging clocks range from molecular to phenotypic (Table 1), many of the age scores may not be directly attributable to DNA damage. Error-prone DNA replication itself can lead to DNA damage, and global changes such as systemic inflammation or dysregulated hormonal circuitry, or local events such as uncoupling of the cell cycle from metabolic cycles and oxidative metabolic activity, may also result in DNA damage. Consequentially, these global and local events feed into and feedback to DDR checkpoints.

Many outstanding questions remain regarding the underlying mechanisms of aging clocks and their ticking units. At this stage, the hypothesis that DNA damage serves as the fundamental counting unit for aging clocks remains preliminary. Further evidence, particularly through single-cell analysis, will be necessary to establish a causal link between DNA damage and ΔAge across individuals (see Outstanding questions). Mapping of the molecular underpinnings of clock ΔAge scores at different levels by GWAS or epigenome-wide association studies (EWAS) will reveal whether a composite clock can capture the weakest link and provide the way to reset the clock(s). Importantly, single-cell whole-genome sequencing in large human populations and causal quantitative associations between DNA damage and ΔAge will be necessary to establish

Outstanding questions

What is the master aging clock, if there is one? What are the molecular interactions between clocks at different levels?

What is the fundamental counting unit of aging clocks? What are the different counting units at different levels? Do they form precise and cumulative hierarchies?

Is DNA damage or the damage-induced epigenetic changes the most direct cause of an increase in ΔAge?

What is the most crucial point of intervention in any clock or compilation of clocks?

How can cellular metabolism be leveraged to slow down the clocks? Can this be done in a personalized manner?

beyond doubt that DNA damage and the DDR are a fundamental precise counting unit of aging clocks.

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Declaration of interests

The authors have no interests to declare.

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