Aging leads to epigenetic alterations, including changes in DNA methylation, through both multiple distinct and intersecting age-related mechanisms [6, 41]. Many DNA methylation aging clocks have now been derived, and due to their individual strengths and weaknesses, explicit reference must be made to the specific clock employed (see further in "Challenge 2"). Captured age-related epigenetic variation can be firstly split into intrinsic, or intra-cellular, and extrinsic, or broadly within-tissue and external, aspects of the aging process [27]. The former is a surrogate readout of multiple cellular and genomic processes, including possible deterioration of mechanisms involved in maintaining the epigenome, while the latter includes age-related cell proportion changes within a tissue. While these first clocks are markers capturing these effects to a greater or lesser extent [42], both can predict all-cause mortality at a population, but not individual level, even after correcting for known risk factors [27]. To investigate biological age more directly, clocks have also been trained on age-related and disease phenotypes in combination with chronological age, such as the "PhenoAge" DNA methylation clock that incorporates nine age-related biochemical measures [43]. Cigarette smoking, a significant disease-related factor, is observed to strongly drive mortality-associated predictive DNA methylation changes [44]. However, these tobacco-related methylation changes do not influence the Horvath or Hannum et al. clocks, but are captured in "PhenoAge" [9]. Of note, a very recently constructed mortality predictive DNA methylation clock, termed "GrimAge," directly incorporates smoking-related changes through an estimate of "pack-years" smoking. This clock also includes certain plasma protein levels estimated by DNA methylation, and this leads to an even stronger prediction of both lifespan and healthspan [45].

Current uncertainty

The first DNA methylation clocks devised were found to be useful for estimating actual age, as well as capturing associations with biological aspects of aging. Data gathered from these early clocks can still be exploited for both these chronological and biological measures. However, now this duality has been recognized, we can attempt to improve our assessment of these two characteristics. Specialized clocks are likely to be more powerful for accurate age prediction or to capture specific biological aging-related functional deterioration or disease-related predictions [45]. How far these two distinct uses can be separated into discrete clocks and improved for their specific role is presently unknown. However, clearly if the DNA methylation clock measurement of actual age was perfect, the loss of any variability removes the window where biological aging associations can be made [46]. Empirical calculations estimate that near-perfect forensic age determination may be possible with large enough sample size, even with current DNA methylation array platforms (see Fig. 1a) [46], although this statistically derived view that chronological clocks can approach extreme precision is not held by all in the field.

Each DNA methylation clock that is constructed is unique to its method of calibration [47], indicating the importance of tissue/s employed, number of samples, and statistical methodology. Clearly, small sample sizes are more susceptible to multiple aging-related confounders, measurement errors, and imperfect statistical predictions. Even when clocks are directly trained on actual chronological age, the strong influence of age-related biological processes may skew the CpGs selected for the clock, underscoring the importance of an appropriate population of sample donors. Furthermore, as discussed in "Challenge 3," Zhang et al. recently highlighted the impact of not only sample size but also cell type correction, in heterogeneous cell type-derived DNA, on improving chronological age prediction [46].

For "Biological" clocks, another obvious area of uncertainty is that there is not one measure or "gold standard" of biological aging [6, 7, 41]. This phenomenon encompasses a wide range of age-associated changes from the merely visible to disease-risk related. To understand how aging may be characterized by chronological and biological age-related epigenetic changes, we need more detailed understanding of what mechanisms may be underlying these observations. There is no evidence that the Horvath or Hannum et al. clock CpGs are enriched for functionality over and above the promoter-focused arrays from which they were constructed. Furthermore, the clocks have shown variability in their ability to capture measures of mitotic age, such as telomere length [9

[54–57]. Testing across the range of routinely collected DNA samples will be needed, such as those gathered from peripheral blood or buccal swabs, but also other sources of DNA, such as hair root, skin, and other tissues. However, this is currently only likely to be tractable in data derived from peripheral blood, as these are available at large scale. For the other tissues, the approach is likely to be insufficiently powered in the intermediate future. Specific CpGs will be selected to construct clocks for high-precision forensic age estimation, when chronological age is not known or disputed. They will employ those CpGs that are the most robust and accurate for particular tissues and their constituent cell types [58]. We will need to define the influence of genetic variation and environmental factors on these measures. Accumulating this knowledge of the various DNA methylation clocks will guide their future legal or forensic application [59].

The biological aging component captured by epigenetic age acceleration consists of a large range of drivers, including tissue-specific, cellular aging pathology, stochastic deterioration, and disease-related factors. As mentioned, there is no single measure of biological age; therefore, spe-

Tissue-specific clocks have the potential to be highly clinically useful as prognostic and diagnostic markers of disease, as discussed in the following "Challenge 2." However, we should not forget about the potentially intriguing insights into aging biology that could be identified by modifications that occur across all tissue types in the body, or pan-tissue changes [62]. Strong outlier candidates for pan-tissue changes identified to date should be further evaluated, such as DNA hypermethylation in *2*, as well as looking for novel aging-related chromatin marks. To confirm any consistency of changes across tissue types will ultimately require large-scale and detailed

Table 1 Major biological and analytic issues with epigenetic DNA methylation clocks (Continued)

Significant issue	Current problem	Potential solutions/advances
or active enzymatic-driven loss	prevalent at dynamic enhancer regions. Neuronal cells have high post-mitotic expression of DNMTs and TETs plus high 5-hydroxymethylcytosine (5hmC)	Assaying the specific products of TET activity, such as 5hmC

activity of many genes, although currently without strong evidence of expression disruption [82]. Changes with aging have been observed in both the binding sites of the transcriptional repressor REST [83] and insulator CTCF [84]. However, instead of targeting housekeeping or essential genes, epigenetic drift changes tend to occur in the periphery of the protein-protein interactive network [85].

Current uncertainty

There is uncertainty around how the DNA methylation changes observed in clocks can accrue without replication, i.e., due to processes not related to cell replication (see Table 1). Most tissues are comprised of non- or slowly dividing cells, and different division rates occur in different tissues. Aging-related aberration of the epigenetic machinery is implicated in DNA methylation change over time. However, understanding this will require more detailed characterization of the levels of instability aside from DNA replication, and the extent to which this process is cell-, genetic-sequence-, or regulatory element-specific. Cumulative changes, as well as potentially stochastic factors, most likely influence mitotic rate and fidelity, repair, chromatin remodeling, and transcription. These aggregating mechanisms are not exclusive to each other and could be important in differing degrees at different loci or in different cell types. The Horvath clock is derived from a wide variety of tissue types and works

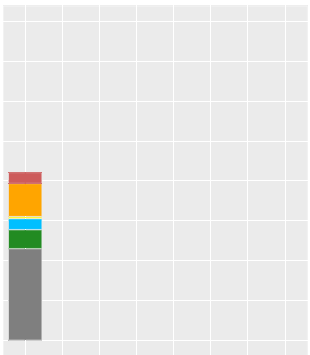
understanding patterns of DNA methylation heterogeneity in aging stem cell populations [99] and for understanding the relationship between age-associated patterns of DNA

the association with mortality lessened, even without cell type correction, with increased training set size. The bio-

cardiovascular risk could combine genetic PRS for this trait with GrimAge clock measures, which estimate cardiovascular disease-related risk, such as smoking pack-years, plasma beta-2 microglobulin, and other plasma proteins, and predicts time to coronary heart disease [45].

Regarding the issue of cell type deconvolution for clock association, this will be specific to the disease or trait being examined. Single-cell analysis, as detailed in "Challenge 5," will also help pinpoint which cell type(s) is the most important and guide the use of cell type corrections in heterogeneous DNA samples for larger longitudinal and epidemiological studies.

Another very important issue is that all these genetic



designing a custom array that could be used at very low cost on very large numbers.

While the focus so far has been on DNA methylation, other DNA modifications, as well as known and currently unrecognized chromatin modifications, should be explored and may reveal exciting clock-like properties. Suggestively, the premature autosomal recessive aging disorder, Werner syndrome, while showing DNA methylation clock age acceleration [163], also has identified significant heterochromatin changes [164]. The optimum analysis of chromatin modifications requires fresh samples, but epigenome-wide association studies have been recently performed successfully with histone acetylation derived from post-mortem specimens [165]. These data can also be further integrated with DNA modification changes. Larger scale mass spectrometry quantitation of histone modifications could also be evaluated. Additional DNA modification analysis by oxidative BS-seq via array for 5hmC [166] should be further evaluated in aging, although this is still currently expensive to perform in large numbers. However, new methodologies, such as a non-destructive DNA deaminase [167], may help to propel these on.

Repetitive elements, where currently technically possible, may be sites for identifying aging-associated DNA modification in order to construct novel clocks, and these loci are clearly under-represented by arrays presently (see Fig. 2h). In this exploration, smaller scale whole-genome sequencing DNA methylome analyses should not be deterred. Analyzing repetitive elements by these methods is the only realistic option, and for the longer repeats, third-generation direct long-read sequen-

Bulk analysis of isolated cell populations can still give

coverage of scBS-seq it will be possible to generate a single-cell clock; this may also be aided by further methodological or technical breakthroughs, including potentially single-cell multi-omics measurements [188]. Furthermore, modulation by experimental models may give further insight into the influence of particular sub-

acceptable to all stakeholders will require transparent governance based on scientific accuracy, which will require significantly more rigorous scientific evaluation.

Conclusion

With this perspective, we have detailed seven challenges alongside the experiments and recommendations to explore these (summarized in Table 2), which we hope will help to further the fascinating biological discoveries that have accompanied DNA methylation clocks. These detailed strengths, weaknesses, and areas of inquiry should stimulate new discussion and experimentation.

The power of epigenomic analysis is clearly displayed by these precise aging-related changes. Detailed evaluation of DNA methylation clocks may reveal unique insights into the aging process itself, as well as act as a

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