Trends in Genetics

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Review

On correlative and causal links of replicative epimutations

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The mitotic inheritability of DNA methylation as an epigenetic marker in higher-order eukaryotes has been established for >40 years. The DNA methylome and mitotic division interplay is now considered bidirectional and highly intertwined. Various epigenetic writers, erasers, and modulators shape the perceived replicative methylation dynamics. This Review surveys the principles and complexity of mitotic transmission of DNA methylation, emphasizing the awareness of mitotic aging in analyzing DNA methylation dynamics in development and disease. We reviewed how DNA methylation changes alter mitotic proliferation capacity, implicating age-related diseases like cancer. We link replicative epimutation to stem cell dysfunction, inflammatory response, cancer risks, and epigenetic clocks, discussing the causative role of DNA methylation in health and disease.

Highlights

The causality between DNA methylation changes and mitosis can be bidirectional, conditioned on biochemical, genomic, cellular, and developmental factors.

Replicative epimutations contribute to aging and age-related diseases like cancer through various mechanisms, acting as passengers, drivers, or both.

Replicative epimutation serves as a mechanism underlying epigenetic clocks.

Epigenetic editing enables a systematic identification of epimutations affecting replicative systems.

Biochemical foundation of DNA methylation inheritance

DNA methylation is defined as the methylation of the 5′-carbon of the DNA cytosine base, or 5mC in short, primarily in the context of cytosine–phosphate–guanine (CpG) dinucleotides [1]. The chemically stable methyl group is enzymatically deposited by DNA methyltransferases (DNMTs) [2]. It can be iteratively oxidized by the TET enzymes [3] and AID/APOBEC-mediated deamination [4] or entirely removed if followed by base-excision repair [5].

The key property that puts DNA 5mC in the spotlight of genomics is that it is mitotically heritable. The progeny cells from mitotic division copy the methylation pattern of the parental cells. This mitotic transmission of information is achieved by the maintenance DNA methyltransferases, mostly through DNMT1 in humans in the cell cycle S phase [6]. A multidomain RING-type E3 ubiquitin ligase UHRF1 first recognizes hemimethylated cytosines via its SRA domain [7–9]. It then flips the methylated cytosine on the parent strand and binds DNMT1 to its replication focus targeting sequence (RFTS) via the SRA domain [10]. The recruited DNMT1 then methylates the newly synthesized DNA at the replication foci. DNMT1 often remains tethered to UHRF1 and PCNA during the methylation reaction and processively methylates neighboring cytosines [11]. This recruitment is critically timed as DNMT1 is diffusely distributed in the non-S phase [6], largely associating heterochromatin [12]. DNMT1 cannot copy the oxidative intermediate, 5-hdyroxymethylcytosine (5hmC), generated by the TET enzymes [13,14]. Thus, although 5hmC is chemically stable [15], it cannot be copied during the cell cycle and will be passively diluted in the cell cycle [16].

Mitotic epimutation rates

Genome-wide DNA methylation patterns are modified in a programmed manner during embryonic development. After fertilization, the embryo undergoes genome-wide demethylation, partly dependent on the TET proteins. TET-dependent demethylation at the early embryonic stage requires cellular replication [17]. Later, an extensive genome-wide wave of *de novo* methylation is executed at implantation, except for CpG islands, which remain hypomethylated. Then, during tissue specification,

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thousands of cell-type-specific enhancers undergo demethylation [18,19]. Low DNA methylation levels in enhancer and promoter sequences allow the binding of transcription factors that often prefer unmethylated cytosine [20–22] and exclude DNMTs from methylating these genome regions in coordination with histone modifications [23]. Thus, tissue-specific demethylation orchestrates the expression of cell-type-specific gene repertoire [19].

Besides these programmed methylation changes, mitotic divisions, akin to Muller's ratchet [24], accumulate random methylation changes over cell replication due to imperfect methylation maintenance [1]. The measured rates of mitotic epimutation (Table 1) represent methylation alterations between two temporal moments of parent and daughter cells. Most measurements have found dramatically higher error rates in the transmission of DNA methylation compared to the genetic code, indicating a less stringent mechanism for preserving the methyl mark. The inconsistency between error rates measured in different studies is related to cell identity, which includes *in vitro* versus *in vivo* environment, stem cells versus differentiated cells, and cancer versus normal cells. In addition, differences in the accuracy of copying DNA methylation could be attributed to the specific genomic region. For example, actively transcribed genes [25] and transcription factor binding sites tend to have fewer errors. Promoter CGIs have more stable methylation inheritance than nonpromoter CGIs [26]. Finally, the epimutation rate is highly dependent on sequence context. For example, it was found that isolated CpGs and CpGs flanked by A or Ts, solo-WCGWs, lose more methylation over cell divisions [25]. Previous enzymology study suggests variations in the NNCGNN flanking sequences can lead to a ~100-fold difference in base-flipping efficiency of DNMTs [27]. Further studies are needed to characterize epimutation rates and their dependence on cell, genomic, and sequence context (see Outstanding questions).

Enzymatic governors of replicative epimutation rates

Errors in the DNA sequence are typically introduced by imperfect DNA polymerase action and are subject to correction through DNA repair mechanisms. Similarly, epimutations, which mostly occur during DNMT1-driven methylation maintenance, are subject to correction and remodeling through active methylation and demethylation by DNMT3s and TETs [28]. The dominance of passive (DNMT1) versus active (DNMT3A/B and TETs) mechanisms can vary significantly between genomic regions [29] (Figure 1). Transcribed gene bodies exhibit high *de novo* DNA methylation as their histone H3K36me3 marks recruit DNMT3B via the PWWP domain of the enzymes [29,30].

Table 1. Replicative epimutation rate measurements from prior studies

Study	Epimutation rate	Method
Wigler *et al.* [1]	91–97%, <85%, and ~95% for three different sites	Three sites in DNA cloned to cultured mouse cells
Pfeifer *et al.*, [175]	99.9% per site per generation	PCR-aided sequencing of PGK1 promoter of X8/6T2 human cells
Issa *et al.*, [65]	1% every 3 years	ERA promoter methylation gain in colon mucosa
Ushijima *et al.*, [26]	99.85–99.92% per site per generation (error = 0.018–0.032 errors/site/21.6 generations)	Bisulfite sequencing of specific CpGs in HMEC cells
Siegmund *et al.*, [176]	0.0003 or 0.0005 per CpG site per division	Bisulfite sequencing of primary colorectal cancers
Ming *et al.*, [38]	Methylation maintenance ratio reach 50% in 4 min, 80% in 30 min, and 10 h to close to complete.	Single-molecule measurement of parent and daughter strand methylation using Hammer-seq in HeLa cells

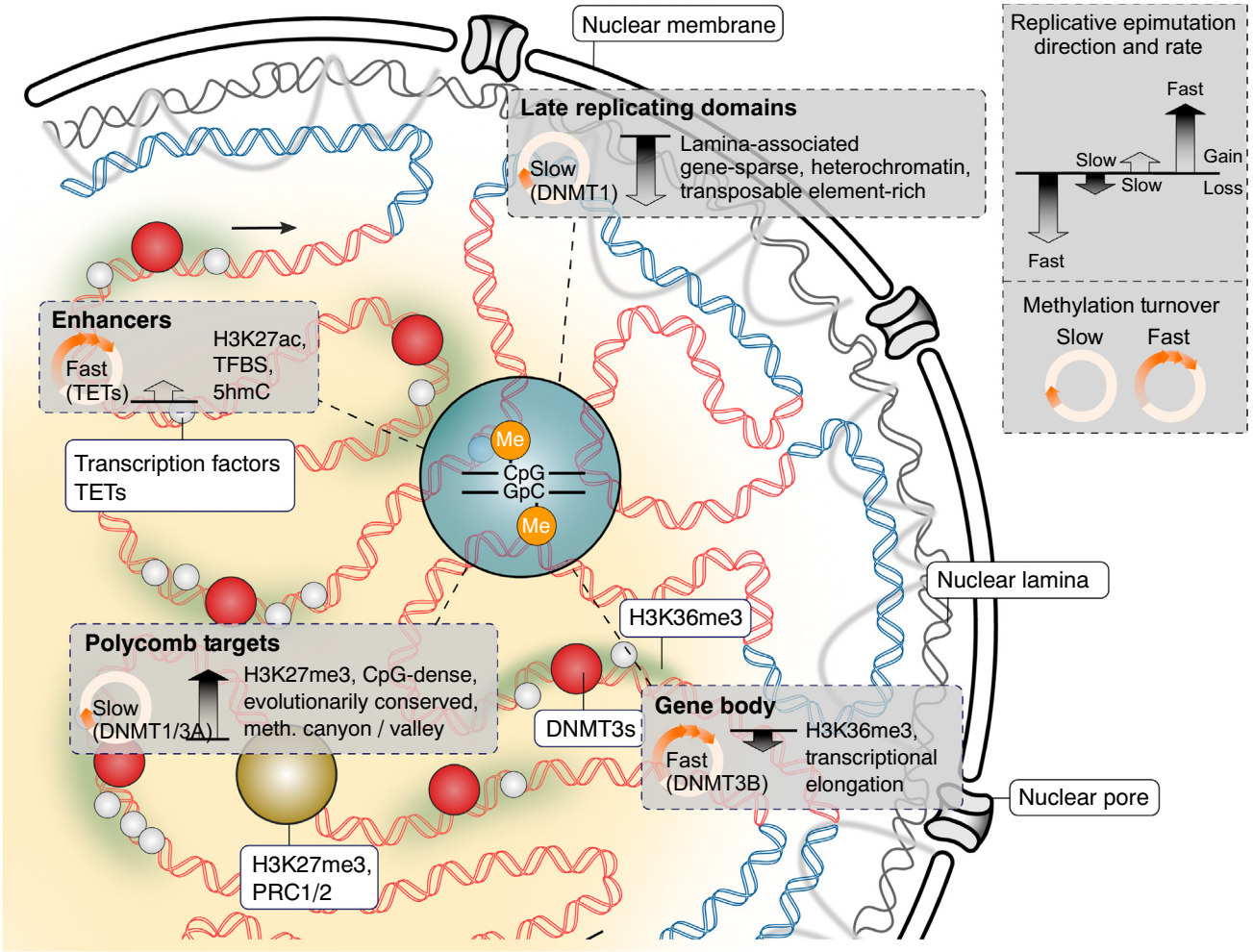

Figure 1. Cellular governors of the replicative epimutation rates. The direction and magnitude of methylation change per cell division is illustrated at four distinct genomic regions: enhancers, Polycomb targets, gene bodies, and late replicating domains, dominated by TETs and DNA methyltransferases (DNMTs), respectively. The methylation turnover rates are labeled in orange circles.

Similar enhanced maintenance might also be true for the H3K36me2-marked intergenic genome, which recruits DNMT3A [31–33]. Conversely, TET-mediated active demethylation predominantly influences transcription factor binding at *cis*-regulatory enhancers [34–36]. Consequently, gene bodies and enhancers are associated with lower replicative epimutation rates [25]. Their alterations in DNA methylation during differentiation are typically not classified as epimutations, although their presence can often complicate the measurement of epimutation rates.

When active correction mechanisms are reduced, observed methylation levels predominantly reflect the dynamics of DNMT1-mediated maintenance (Figure 1). In such cases, the replicative epimutation rate is significantly influenced by the writer enzyme activities and cell proliferation dynamics [199]. DNMT1 and UHRF1 mutations lead to impaired interaction with the replication fork and reduced DNA methylation maintenance [38]. Rapidly proliferating cells exhibited higher epimutation rates in the late-replicating DNA [25], while cells in cell cycle arrest do not exhibit methylation changes at these sites [199].

Important caveats to the described paradigm are that active methylation turnover, switching DNA cytosines to 5-methylcytosines and back by DNMT3s/TETs, also introduce programmed changes for cell differentiation purposes and stochastic age-related changes. Moreover, the maintenance methyltransferase (DNMT1) itself, can also actively correct epimutation through nonreplicative *de novo* methylation capacity [37,38]. The former is exemplified by TETs being master regulators of stem cell differentiation [39] and DNMT3A contributing to Polycomb target methylation gains [40]. The latter has been observed mainly in certain repetitive elements [41] and the neighbor-guided activity of DNMT1 [42], where the enzyme actively *de novo* methylates or avoids *de novo* methylating certain CpGs, depending on the methylation state of neighboring CpGs. DNMTs [11] and potentially TET enzymes [43] already exhibit processivity in their action, leaving neighboring sites consistently methylated. Therefore, the neighbor correction mechanisms may modulate the epimutation rate in both passive and active maintenance models [42].

Genomic contexts of replicative epimutations

In early embryonic development, reprogramming events uniformly establish the baseline methylation state across the cell population (Figure 2). As aging progresses, cells independently and randomly alter their methylation states, leading to a population-wide average methylation percentage nearing 50%. This convergence in aging results from replicative epimutations, indicating a state of high disorder, as quantified by information entropy [44].

The epimutation directionality (gain or loss) depends on the baseline methylation levels. Several decades of research across cancer tissues [45–47], immortalized cell cultures [48,49], primary non-malignant tissues with varying turnover rates [49], and rodents of different donor ages [48] have quantitatively characterized mitotic hypo- and hypermethylation in mammalian genomes (Figure 3). Mitotic hypomethylation commonly occurs at the late replicating genome (Figure 1). This is due to the delayed remethylation of the nascent strand synthesized in the S phase of the cell cycle [50]. The late replicating genome is heterochromatic, sparse in CpG density, rich in transposable elements, attached to the nuclear lamina, and marked by histone H3K9me2/3 [53,182–184,186] (Figure 1). Related genomic features; for example, large organized chromatin K9 modifications

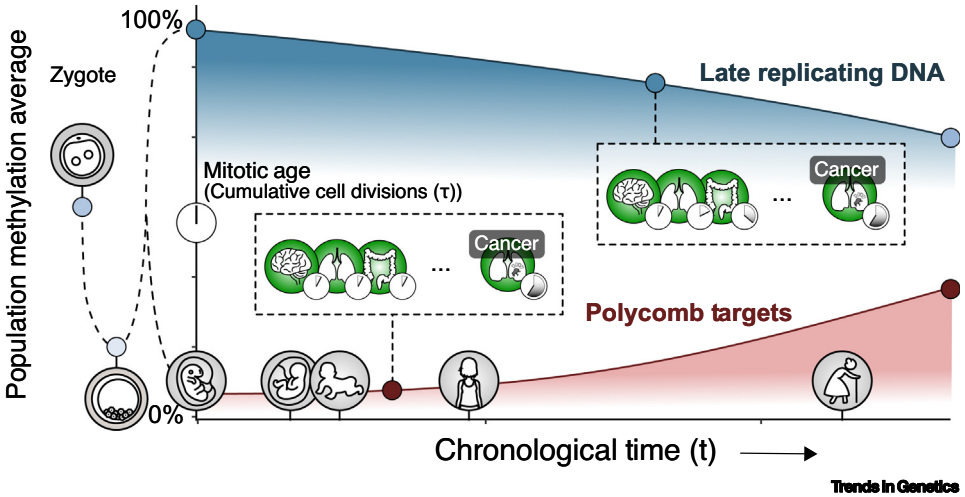

Figure 2. Replicative epimutation rates over organismal development. Hyper- and hypomethylation processes are dichotomized and converge to 50% methylation levels starting from organogenesis and progressing over chronological time and cancer. Different cell types have different mitotic histories depending on intrinsic turnover rates.

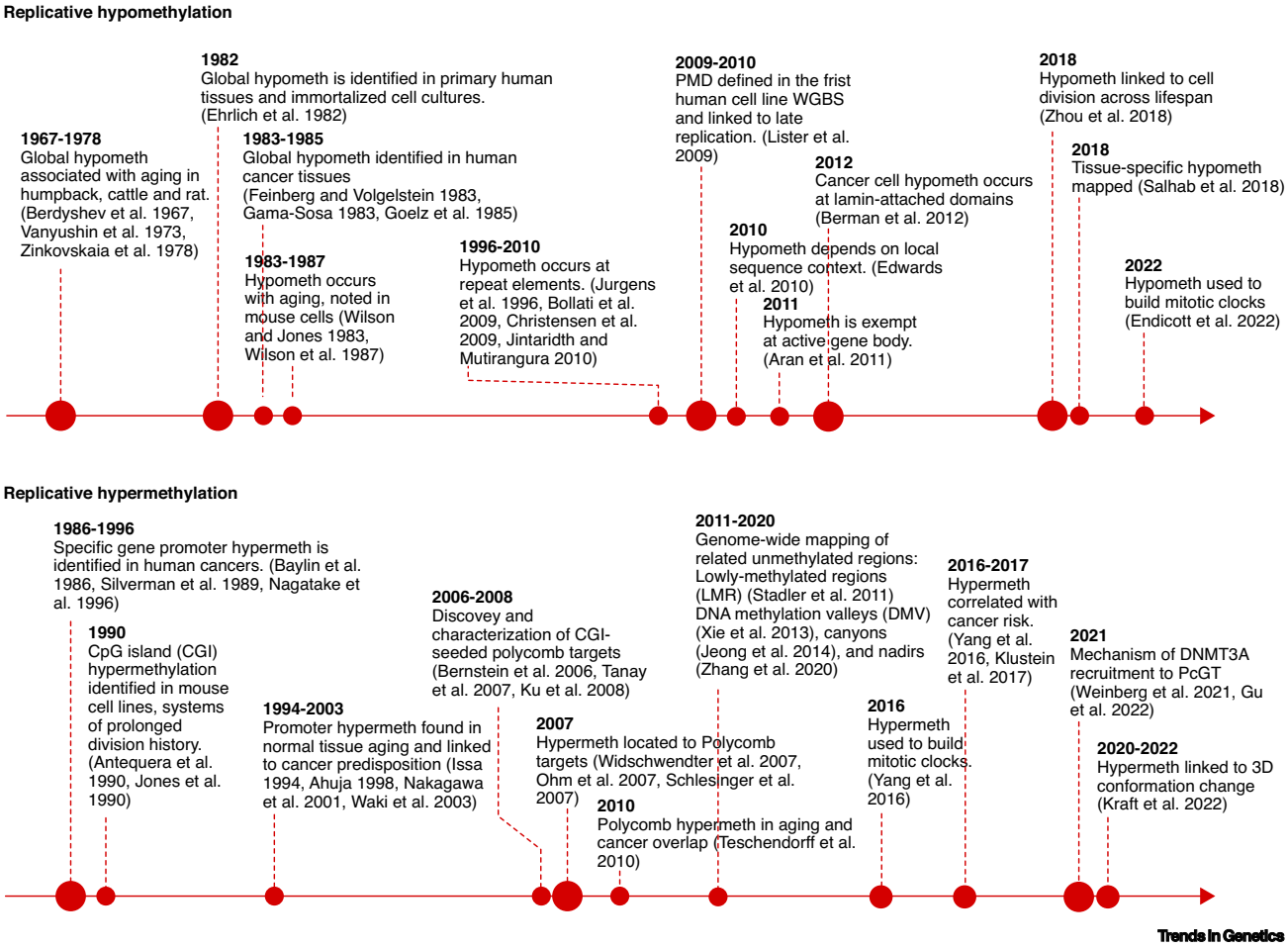

Figure 3. Historical timeline of the discovery of replicative hypo- and hypermethylations. Full reference links follow. Replicative hypomethylation: Berdyshev *et al*. [177], Vanyushin *et al*. [178], Zinkovskaia *et al.* [179], Ehrlich *et al.* [49], Feinberg and Volgelstein [47], Gama-Sosa [46], Goelz *et al*. [45], Wilson and Jones [180], Wilson *et al*. [48], Jurgens *et al.* [181], Bollati *et al*. [182], Christensen *et al*. [183], Jintaridth and Mutirangura [184], Lister *et al*. [54], Edwards *et al*. [185], Aran *et al*. [186], Berman *et al*. [53], Zhou *et al*. [25], Salhab *et al*. [85], Endicott *et al*. [145]. Replicative hypermethylation: Baylin *et al*. [66], Silverman *et al*. [187], Nagatake *et al*. [188], Antequera *et al*. [189], Jones *et al*. [190], Issa [65], Ahuja [64], Nakagawa *et al*. [63], Waki *et al*. [62], Bernstein *et al*. [59], Tanay *et al*. [60], Ku *et al*. [61], Widschwendter *et al*. [56], Ohm *et al*. [57], Schlesinger *et al*. [58], Teschendorff *et al*. [82], Stadler *et al*. [191], Xie *et al.* [192], Jeong *et al*. [193], Zhang *et al*. [194], Yang *et al*. [195], Klustein *et al*. [102], Weinberg *et al*. [40], Gu *et al*. [196], Kraft *et al*. [197].

(LOCKS) [51], lamina-associated domains (LADs) [52,53], partially methylated domains (PMDs) [54], and DNA methylation prairies [55], display similar behavior of replicative hypomethylation.

In contrast, mitotic hypermethylation preferentially occurs at Polycomb targets [56–58], broad CpG-dense genomic regions [59–61] implicated in normal tissue aging, cancer, and cancer predisposition [60,62–66]. These genomic regions hierarchically associate Polycomb repressive complexes (PRCs), depositing the histone H3K27me3 modification [67]. PRCs, particularly PRC2.1 and ncPRC1.1, nucleate at unmethylated CpG islands [60,68,69], as clustered CpGs facilitate the Polycomb protein dimerization and stabilization [68]. Once nucleated, PRCs further spread H3K27me3 to neighboring unmethylated DNA to form broader repressive chromatin [69,70]. Strong evidence suggests that DNA methylation and its void are sufficient in excluding and triggering H3K27me3 [71–75]. The recruiting histone marks of DNA methylation H3K36me2/

3 also inhibit PRC activity [76–79]. Therefore, the Polycomb target status stems from an earlier, unmethylated state. Overlapping genomic features, such as low methylated regions (LMRs) [191], DNA methylation valleys (DMVs) [192], canyons [193], nonmethylated islands [200], and DNA methylation nadirs [194,197], display similar replicative hypermethylation.

Mechanistically, the replicative methylation gain at Polycomb targets can directly be attributed to Polycomb products. Indeed, PRC1 was shown to produce monoubiquitylated histone H2A lysine 119 (H2AK119ub) that interacts with DNMT3A [40]. Histone monoubiquitylation might also involve disrupted functions of other factors maintaining the hypomethylation state, such as TET enzymes [80] and QSER1 [81]. Since Polycomb targets play a key role in rapid stem cell differentiation [60], their methylations irreversibly block gene activation [82]. This change represents an epigenetic switch that perpetuates gene repression [83] and affects the capacity of stem cells for rapid differentiation [60]. Moreover, this switch affecting tumor suppressors may promote tumorigenesis [58].

Cell-type specificity of replicative epimutations

The extent to which replicative epimutation is consistent across cell types or possesses unique cell-type-specific characteristics is not fully characterized. The expression variation of methylation writers and erasers across cell types suggests a diverse landscape of replicative epimutation in scale and genomic distribution. Neurons and embryonic stem cells, with higher expression of DNMT3s and TETs, exhibit broader active methylation maintenance, not limited to gene bodies and enhancers. High TET expression in embryonic stem cells prevents methylation of bivalent chromatin [59,61,198]. While over 80% PMDs are shared across cancer types [25], 26% of the genome is reported to comprise cell-type-invariant PMDs, with the remainder serving as cell-type discriminators [85]. Waki *et al.* observed that cancer-related genes became hypermethylated in nonmalignant tissue aging, and such age-associated hypermethylations are tissue specific [62]. Consistent with the cell-type specificity in replicative hypermethylation, Polycomb targets vary among specific tumors due to their occupation by transcription factors that activate these sites and prevent hypermethylation [84]. Despite this evidence, fully delineating replicative epimutation by cell and cancer type requires future study (see Outstanding questions).

Replicative epimutation as a contributing mechanism of epigenetic aging clocks

Epigenetic clocks, epitomized by the Horvath clock [86] and other clocks [44,87–89], have successfully tracked aging and related phenotypes like multimorbidity and disability [90]. Contrary to viewing epimutations merely as aging markers, there is evidence that loss of epigenetic information might be a reversible cause of aging [91]. Given the precision of epigenetic clocks in predicting chronological age and age-related phenotypes, nonstochastic deviations from predicted age indicate biological abnormalities, reflecting an older or younger epigenomic state [92]. Such deviations are linked to age-related pathologies, including drinking and smoking [93], obesity [94], cancer [95], cardiovascular disease [96], Down syndrome [97], and caloric restriction [98].

While epigenetic clocks are effective predictive models, their mechanistic basis remains unclear, aside from reflecting epigenetic changes during aging processes [99]. Several hypotheses have been proposed to explain their accuracy in predicting chronological age [100]. These mechanisms may involve age-related gene expression changes, shifts in cell composition, and more. One intriguing hypothesis suggests that methylation clocks capture the cumulative trend of cell division, a process linked to organismal aging and stem cell renewal [101,102] (Figure 4). The feasibility is evident in actively renewing cells and tissues containing such cells.

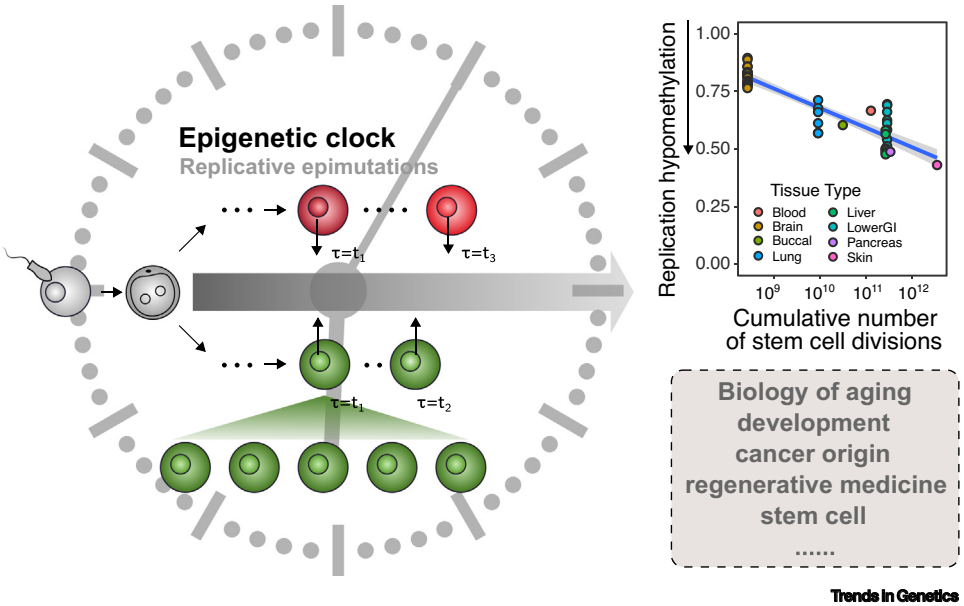

Figure 4. Replicative methylation drift as a contributing mechanism of epigenetic aging clocks. Epigenetic aging clocks capture chronological age by tracking the overall proliferation history in stem cells and their expansions in tissues that contain proliferative cell types. Tissues with longer cumulative stem cell renewal are associated with more pronounced replicative epimutation (showing hypomethylation, data source: [25])

Multiple lines of evidence support this hypothesis. First, the methylation features selected by several clock models colocalize to genomic regions occupied by replicative epimutations, such as the Polycomb targets [100,103,104]. Second, epigenetic clocks track aging across all life stages, including fetuses [105], pediatric populations [106,107], and the elderly [108]. This resembles the lifelong renewal of some proliferative cell types, such as hematopoietic stem cells [109]. Third, excessive cell replication is linked to epigenetic clock acceleration, evident in cancer and immortalized cell cultures [96]. These systems show faster epigenetic clock ticking, uncoupling from chronological age [86]. Caloric restriction reduces cell replication rates [110] and delays age-related epimutation [98,104,111]. Both replicative epimutation and clock methylation changes reset in reprogrammed iPSCs [112]. Certain DNA methylation changes can detect adult stem cell dynamics [113].

Other evidence suggests that replicative epimutation is not the sole mechanism of epigenetic aging clocks. Many age-related cellular processes are replication independent. In liver tissues of obese subjects, accelerated aging is observed at CpGs linked to genes involved in oxidative stress and energy metabolism [94], indicating that the clock might track disease-specific gene expression changes. The interplay between gene-centric and replicative mechanisms in the epigenetic clock might be hard to dissect cleanly. For example, while the Polycomb target genes are intrinsically susceptible to replicative hypermethylation, they are also important genes for development and thus may be epigenetically regulated. Thus, while epigenetic clocks capture replicative epimutations, they also reflect other age-related processes.

Replicative epimutations confound the identification of epigenetic drivers

Establishing the causal role of DNA methylation in cancers is complex. Global epigenetic changes have been linked to causality in cell line and mouse experiments, which inhibited DNMT1 activity [114], introduced hypomorphic mutations [201], and manipulated the expression levels of DNA

methylation writers [202]. However, in diseases, particularly proliferative disorders like cancers, discerning the causal effect of DNA methylation from passive events remains a significant challenge (Figure 5). To conclude the causal role of DNA methylation in replicative diseases (Figure 5, right), one must discriminate against correlative events (Figure 5, model M1, M2) that are either (i) replicative epimutations, which are a direct outcome of cell divisions; (ii) epigenetic hitchhiking events – methylation changes that predate cell division but got clonally amplified through linkage with other driver events rather than conferring selective advantages by themselves [115]; or (iii) direct passengers of the disease. The genomic distribution of the replicative epimutations may be modeled to rule out the replicative origin of these methylation changes [116,117].

Functional consequences of replicative epimutations

Passenger methylation variation can create epigenetic mosaics as cells replicate, resulting in heterogeneity among otherwise identical cells (Figure 5, model M3). This epigenetic mosaicism, demonstrated in various cancers via single-cell methylome mapping [118,119], increases with age and impacts cellular fitness. It contributes to stem cell function deficits, proinflammatory responses, and elevated risks of malignant transformation.

Replicative epimutation at crucial developmental genes can impair stem cell differentiation [120] and renewal capacities [121]. Replicative hypermethylation causes a lymphopoietic-to-myelopoietic shift

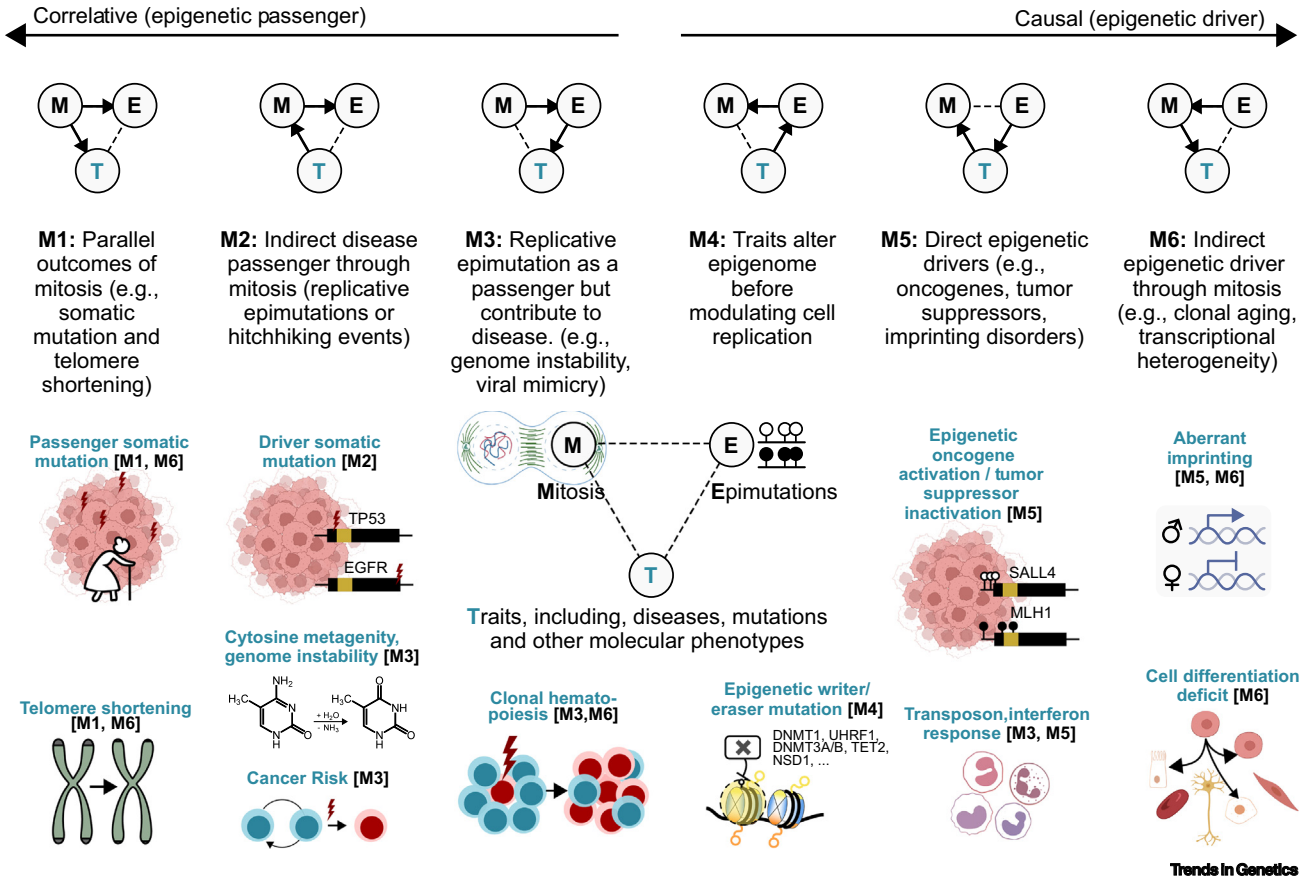

Figure 5. Dissecting causal relationships of epimutations, mitosis, and diseases. The logic flows of causality among mitosis, epimutation, and diseases, stratified by epimutation being an outcome (passenger), an upstream cause (driver), or both, where epimutation arises from mitosis and contributes to disease onset and risk.

in aging hematopoietic stem cells (HSCs) [120,122], mimicking the effects seen with DNMT1 depletion [123]. Such hypermethylation also diminishes the repopulating ability of HSCs [121], leading to transcriptional changes in older HSCs associated with inflammation and stress responses. Early life DNA methylation differences influence epigenome and transcriptome aging [203–205]. Future studies are needed to determine if replicative epimutation affects disease development through this mechanism (see Outstanding questions).

Replicative epimutation can trigger inflammatory responses by activating endogenous retroviruses (ERVs) and immune genes in late-replicating regions, leading to interferon responses and viral mimicry [127–129]. In contrast, a recent prostate cancer study showed that this effect might also be mediated by immunosurveillance gene activation due to DNA hypomethylation, which enhances repression by histone modifications [130]. Increased methylation at certain gene promoters has also been linked to inflammatory diseases in tissues such as the esophagus [131] and colon [132].

Replicative epimutation can disrupt gene regulation, thereby promoting cancer [133,134]. This correlation is evident from the association between lifetime cancer risks and the number of stem cell renewals [102,135,136]. Age-related clonal expansion of epigenetically mosaic stem cells is observed in hematopoiesis [137], intestinal [138], and muscle tissues [119]. These mosaicisms contribute to increased transcriptional heterogeneity [122]. Replicative epimutation at Polycomb targets may lock cells in a proliferative state conducive to oncogenesis [56–58], for example, to enhance self-renewal and inhibit differentiation of HSCs [139].

Lastly, replicative epimutation contributes to genome instability. Studies show that aging mobilizes autonomous retroviruses, increasing mutagenic retrotransposition [129,206]. Additionally, DNA methylation alters the mutagenic potential of cytosine, with varying CpG-to-TpG somatic mutations at different replicative epimutation rates [25]. The interaction of these mechanisms with DNA repair and their impact on genome integrity remain unresolved (see Outstanding questions).

Replication-independent epigenetic changes

It is worth noting that many epimutations are not coupled with cell/DNA replication. Like somatic mutations [140], replication-independent processes contribute to DNA methylation alterations observed in non-proliferating and proliferating cells. For example, the pancreatic beta cells, a long-living endocrine cell type [141], were observed to have experienced age-dependent demethylation of regulatory elements [124], leading to continuous maturation of the beta cell functional phenotypes [125]. DNA methylation [126,142] and demethylation [143] can be triggered by age-related DNA repair, some of which are nonreplicative [144]. For example, DNA methylation loss may occur due to nonreplicative DNA synthesis, such as those due to oxidative DNA damage [145]. Barreto *et al*. showed that *Gadd45a*, a key gene in DNA damage-induced growth arrest, promoted DNA demethylation at DNA damage sites, leading to gene activation [143]. Endicott *et al.* showed that ambient oxidative stress expedited DNA hypomethylation [145]. When cells enter replicative senescence, they acquire additional methylation changes at certain biosynthetic and metabolic genes besides the replicative methylation changes [146,147].

The causal roles of epimutations on cancer-associated mitotic aging

The role of DNA methylation as a driver of mitosis has become increasingly evident in oncology, where it serves as a proxy for studying cell proliferation (Figure 5, M4–6). Aberrant methylation patterns associated with cancer, often rooted in early development, manifest across enhancers, imprinted genes, CpG island promoters, and retrotransposons. Yet, the relevance of these patterns to mitosis in non-cancerous contexts, including normal tissue development and overall organismal aging, warrants comprehensive investigation.

Impaired imprinting as a driver of cancer-associated mitotic aging

Imbalanced genomic imprinting is a well-established mechanism by which aberrant DNA methylation patterns lead to uncontrolled proliferation and cancer. Genomic imprinting is an epigenetic phenomenon by which only one allele is expressed depending on whether it is inherited from the mother or the father. In many cases, DNA methylation is responsible for silencing the relevant allele. In certain pathologies such as Beckwith–Wiedemann syndrome, abnormal DNA methylation in a cluster of imprinted genes results in activation of oncogenes (*IGF2*) or repression of tumor suppressors (*CDKN1C*). This results in various childhood tumors. Targeting demethylases to the *CDKN1C/p57* locus induced proliferation in primary human pancreatic beta cells [148]. This epigenetic approach indicates a direct link between the DNA methylation state of imprinting loci and cellular proliferation.

Tumor suppressor and oncogene dysregulation as drivers of cancer-associated mitotic aging

The disruption of tumor suppressor genes and oncogenes by methylation alterations, which can result in oncogenesis and contribute to mitotic aging, is well-documented in oncology. The effects of such methylation changes have been substantiated by demethylation experiments and applications of epigenetic editing technologies (Figure 6).

Demethylating agents such as 5′-aza-2′-deoxycytidine are cytosine analogs that generate a covalent bond with DNMT1, thus inhibiting its activity and reducing global DNA methylation levels [149–151]. Demethylating agents effectively prevent microadenomas and reduce tumor size. The FDA approved them for treating different leukemias and myelodysplastic syndrome [152,153]. For many years, the main mechanism by which demethylating agents affect tumorigenesis was

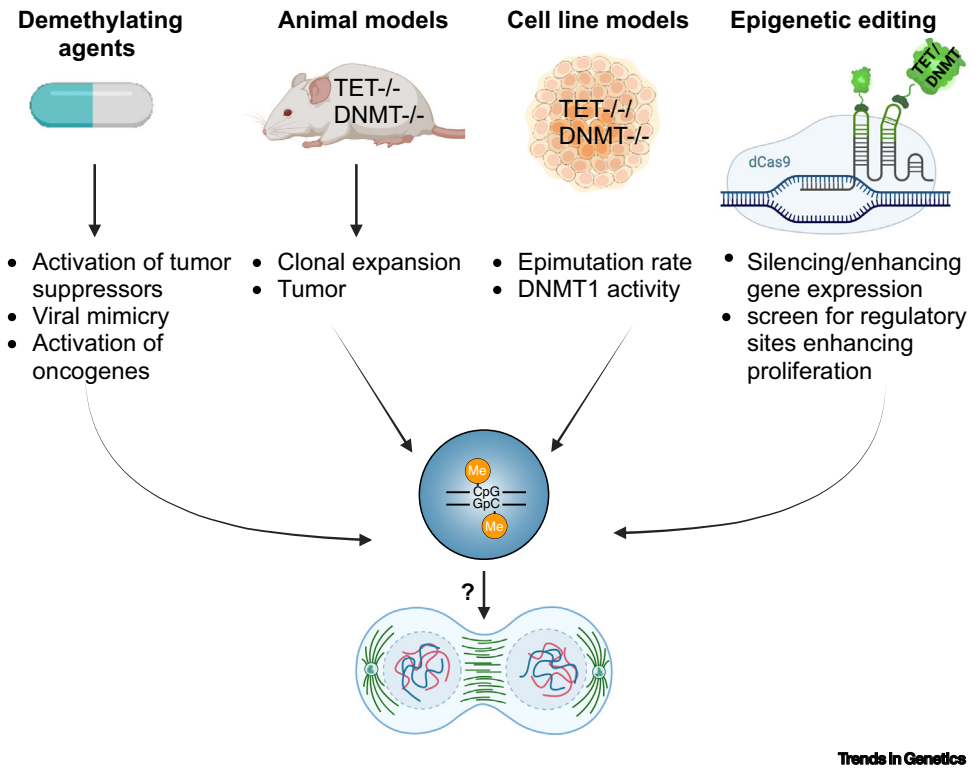

Figure 6. Tools for testing causal epimutations on cell replication, showing demethylating agent, transgenic animal models, cell line models, and CRIPSR dCas9-based epigenetic editing. Abbreviation; DNMT, DNA methyltransferase.

thought to be via reducing CpG island promoter methylation and activating tumor suppressor genes. Reduced DNA methylation levels following demethylating agents were observed in CpG islands residing in the promoters of tumor suppressors such as hMlh1 involved in DNA repair [154–156], p14ARF participating in the cell cycle [157], and DAPK mediating apoptosis [158]. These genes undergo *de novo* methylation early during cancer progression, and they have a low mutation burden, indicating that their silencing is epigenetic [159]. When cancerous cells were treated with demethylating agents, the expression of various tumor suppressor genes was restored, and tumor characteristics associated with the loss of expression of these genes were diminished [156,158,160]. For example, when *hMlh1* expression was restored in colorectal and endometrial cells using demethylating agents, DNA mismatch repair capacity was regained in these cells [156]. Since demethylating agents have genome-wide methylation effects, it is reasonable that other genomic entities are activated in addition to tumor suppressor activation (see viral mimicry effect below). Indeed, demethylating agents were shown to activate the *SALL4* oncogene in MDS patients. *SALL4* expression is associated with worse outcomes. The impact of DNA methylation on *SALL4* expression was further exemplified by epigenetic editing reducing methylation levels at the promoter [161]. Hypermethylation of tumor suppressor genes could stem from *Dnmt3b* overexpression in the aforementioned tumors [115] (Figure 5, models M4 and M5).

Epigenetic editing further supports these observations by targeting the TET catalytic domain to demethylate a specific promoter and restore its activity. Indeed, when the TET1 catalytic domain was recruited to the *hMlh1* promoter, a 4000-fold elevation in *Mlh1* expression was observed [162]. This set of experiments provides solid proof for the role of CpG islands hypermethylation in silencing tumor suppressor genes.

Hypomethylation-induced viral mimicry as a driver of cancer-associated mitotic aging

Cancer proliferation is not solely the result of conventional gene dysregulation but can also be driven by the activity of nontraditional genes or transcripts, such as those from transposable elements, best exemplified by endogenous retroviruses. For example, the use of demethylating agents has been shown to inhibit the growth and self-renewal capabilities of colon-cancer-initiating cells by inducing a viral mimicry pathway that specifically acts through the activation of MDA5/MAVS/IRF7 pathway [127]. This pathway is activated as ERV elements are expressed following hypomethylation, which generates dsRNA that induces an immunological response. The significance of this pathway is not directly related to the CpG island methylator phenotype in cancers. Additionally, the activation of retrotransposons has been linked to reduced DNA methylation levels in promoter regions [127]. Notably, the likelihood of activation by demethylating agents is influenced by the CpG density within the promoters of endogenous retroviruses [163], a principle that likely extends beyond these elements to all promoters. Moreover, the hypomethylation of transposable elements can also precipitate the activation of oncogenes in cancers [164].

Role of DNMTs and TETs in regulating replication in normal development and in cancer

Numerous studies have demonstrated the crucial role of TET and DNMT proteins in replicating normal and cancer cells, with a particular focus on the neurons and hematopoietic lineages. For instance, depletion of DNMT1 in HSCs leads to increased proliferation of myeloid progenitors [123]. Studies have also shown that TET2 depletion alone can slowly activate malignancy, whereas deletion of both TET2 and TET3 leads to rapid tumorigenesis in many models [165,166]. For example, double knockout for TET2 and TET3 during T cell differentiation resulted in uncontrolled proliferation of iNKT cells [167]. In addition, TET2 and TET3 deletion abrogated B cell differentiation and induced myeloid tumorigenesis [166]. Importantly, the TET proteins have been shown to induce tumorigenesis even without catalytic activity, which disconnects the tumorigenic behavior

of TET mutants from DNA methylation dynamics [168]. Nevertheless, TET catalytic activity is essential in certain tumor models to induce tumorigenesis [168]. In cases where the lack of TET proteins leads to cancer, a major factor leading to tumorigenesis is the lack of tissue-specific demethylation, which results in attenuated chromatin accessibility at enhancer sequences [166,169].

Epigenetic editing for studying the causal role of DNA methylation on replication

Epigenetic editing utilizes genetic editing techniques such as CRISPR-Cas9 or TALEN to recruit epigenetic modifiers to specific sites in the genome [170,171]. By disabling the nickase activity in these tools, known as dead Cas9 or dCas9, the technique aims to recruit TET or DNMT catalytic domains to target DNA sequences [170,171]. This approach has been successful in activating or silencing genes [172]. Initially, a single catalytic domain was attached to each dCas9/TALEN. For example, targeted imprinted genes in primary pancreatic beta cells led to the proliferation of beta cells when transplanted *in vivo* [148]. Recently, scientists have fused multiple epigenetic writers into each dCas9/TALEN for enhanced editing. Furthermore, integrating transcriptional activators or repressors on top of using DNA methylation writers has significantly improved the modulation of gene expression and the persistence of modified expression [173]. However, targeting transcriptional activators/repressors without recruiting DNA methylation writers has reduced the capacity to modulate and sustain the modified expression of target genes [173]. Epigenetic editing approaches have also been used to silence most promoters in the genome using a gRNA library [173]. This has helped measure the impact on the replication of tumor cell lines and identified several genes that can successfully attenuate or accelerate cell proliferation [173]. Future studies may identify if activating epigenetic editing can also screen for genes affecting cell proliferation. Furthermore, using epigenetic editing to silence or activate large pools of enhancer regions using large gRNA libraries could advance our understanding of the importance of these regulatory sequences in regulating cell replication. Lastly, recent studies have demonstrated efficient epigenetic silencing *in vivo* [174]. Future studies will utilize this approach to suppress or enhance replication in health and disease (see Outstanding questions).

Outstanding questions

How do we distinguish driver and passenger replicative epimutation from cancer omics profiles?

Are replicative epimutation programmed or stochastic in disease?

How does the replicative epimutation rate vary by cell type, developmental stage, and cancer type?

Could the accumulation of epimutations in early life alter later development, potentially leading to the early onset of age-related diseases?

How does nonreplicative epimutation track aging?

How does replicative epimutation interact with DNA repair and impact genome stability?

To what extent do replicative epimutation and alternative mechanisms contribute to aging clocks?

What is the contribution of animal models to understanding the causal role of DNA methylation on proliferation?

How do demethylating agents affect proliferation and suppress and enhance proliferation simultaneously?

How can epigenetic editing be used to screen genes affecting cell proliferation?

Can epigenetic editing suppress and enhance cell replication in healthy and disease systems?

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Declaration of interests

The authors declare no competing interests.

Declaration of generative AI in scientific writing

During the preparation of this work the authors used Grammarly in order to check grammatical mistakes. After using this tool/service, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

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