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Review

Dynamics of replication timing during mammalian development

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Recent developments in low-input genomics techniques have greatly advanced the analysis of the order in which DNA is replicated in the genome – that is, replication timing (RT) – and its interrelationships with other processes. RT correlates or anticorrelates with genomic-specific parameters such as gene expression, chromatin accessibility, histone modifications, and the 3D structure of the genome, but the significance of how they influence each other and how they relate to biological processes remains unclear. In this review I discuss the results of recent analyses of RT, the time at which it is remodeled and consolidated during embryogenesis, how it influences development and differentiation, and the regulatory mechanisms and factors involved.

Highlights

Replication timing (RT) before the four-cell embryo in the mouse does not show a clear pattern, as is observed in embryonic stem cells for example. As development and differentiation progresses thereafter, a clear RT pattern emerges in conjunction with the formation of 3D genome organization, changes in gene expression, the establishment of nucleosome organization and chromatin marks, and the accelerated rate of replication fork progression.

In the two-cell stage mouse embryo, specific regions of earlier RT in the maternal allele are enriched in H3K27me3.

The balance between RT and fork speed is not maintained in the four-cell stage mouse embryo, which may be the cause of the loss of genomic integrity as it divides toward the eight-cell stage.

Rif1 depletion alters the distribution of both RT and lamina-associated domains (LADs), but there is little correspondence in the changes, suggesting that they are controlled by different mechanisms during mouse embryogenesis.

Emergence and consolidation of replication timing during embryonic development

When mammalian cells replicate their DNA, they initiate replication from a number of different regions of the genome (origins of replication). This leads to the formation of replication domains, and each of them completes replication during a specific time period of S phase. This temporal control of DNA replication – that is, the RT – is subject to regulation, suggesting that the cell is programmed to know which regions replicate when [1,2]. RT correlates with features of genome organization: for example, late-replicating regions correspond to B-compartments while early-replicating regions correspond to A-compartments. In addition, replication domains mostly correspond to topologically associating domains. However, no relationship has been established between RT and other structural domains such as Polycomb-associated domains (PADs) [3,4]. RT is fundamental to the maintenance of the chromatin state and epigenome which enable the emergence and subsequent stabilization of cell fates throughout development. Therefore, understanding the molecular regulation of RT is critical to our understanding of the faithful transmission and re-establishment of chromatin states [5,6]. How and when the RT program is set up during development and differentiation has been an important question for many years, but only recently have data on this theme been reported [7–10]. Interestingly, the pattern of RT in mouse embryos immediately after fertilization is not well defined [7–9], and the results of genome-wide clustering also show that the early RT program differs from later-stage embryos [8]. Clear replication domains are observed in embryos at the four-cell stage, suggesting that this is the stage at which the DNA replication program of the embryo starts to be established [11–13]. Moreover, the variability score, which measures the variance of the replication program across cells, is highest from the zygote to the four-cell stage during embryogenesis and decreases thereafter [8]. Analysis of the genome-wide distribution of RT values also showed that the RT values of eight-cell and morula embryos segregate bimodally, toward earlier and later RT values, but the four-cell embryos do not (Nakatani *et al.*, unpublished). Remarkably, these features of the RT in early embryos are also accompanied by a distinctive slow replication fork speed, which increases progressively as development proceeds [7,14,15]. The speed of replication fork progression (Box 1) in the four-cell stage is also slower than in the blastocyst stage [7,14], consistent with the notion that the

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Box 1. Fork speed inversely correlates with cellular plasticity

DNA replication begins at the replication origin, from which DNA synthesis proceeds in both directions, and the replication fork is the site where DNA synthesis is carried out. The rate at which this replication fork proceeds can be measured at the single-molecule level using the DNA fiber method [97,98] and has been described in several reports during embryogenesis [7,8,15]. In the zygote and two-cell-stage embryo immediately after fertilization in both mice and humans, the rate of fork progression is very slow and tends to increase with developmental progression [7,8,15]. Consistent with these results, the identification of potential replication origins from DNA fiber data and the measurement of their spacing on the DNA showed that replication origins are densely activated in the zygote and two-cell embryo compared with later-stage embryos. Similarly, two-cell-like cells, which are derived from ESCs and have expanded totipotent-like properties, have a slower fork rate than ESCs, and mouse embryonic fibroblasts (which are differentiated somatic cells) have a faster fork rate than ESCs [14,41]. These results suggest an inverse relationship between fork rate during DNA replication and cell plasticity, at least in a number of developmental processes.

four-cell stage appears to be both a watershed in cell fate determination and a stage in which different DNA replication parameters are remodeled (Figure 1).

RT in the zygote and two-cell stage embryo is not well defined, but when compared with ESCs, clear differences in RT were found in specific regions, and these differences are consistent with the pattern of gene expression [9]. Interestingly, regions with differences in RTs between alleles of parental origin were also found at early stages, specifically in the pericentromeric region of maternal origin, where relatively later replication was observed compared with the paternal pericentromeric regions [9,16]. However, allele-specific early-replicating regions of maternal origin were found to be enriched in the histone marks H2AK119ub and H3K27me3 in the two-cell stage. These histone modifications are deposited by Polycomb repressive complexes (PRCs) [4,17,18] and are typically enriched in the heterochromatin region normally associated with facultative LADs in differentiated cells [19,20]. As these histone marks are acquired during oogenesis, it would be interesting to analyze the functional role of PRCs in setting up embryonic RT and to ascertain whether they have direct effects on RT in the early embryo [21–23]. Currently, there are no

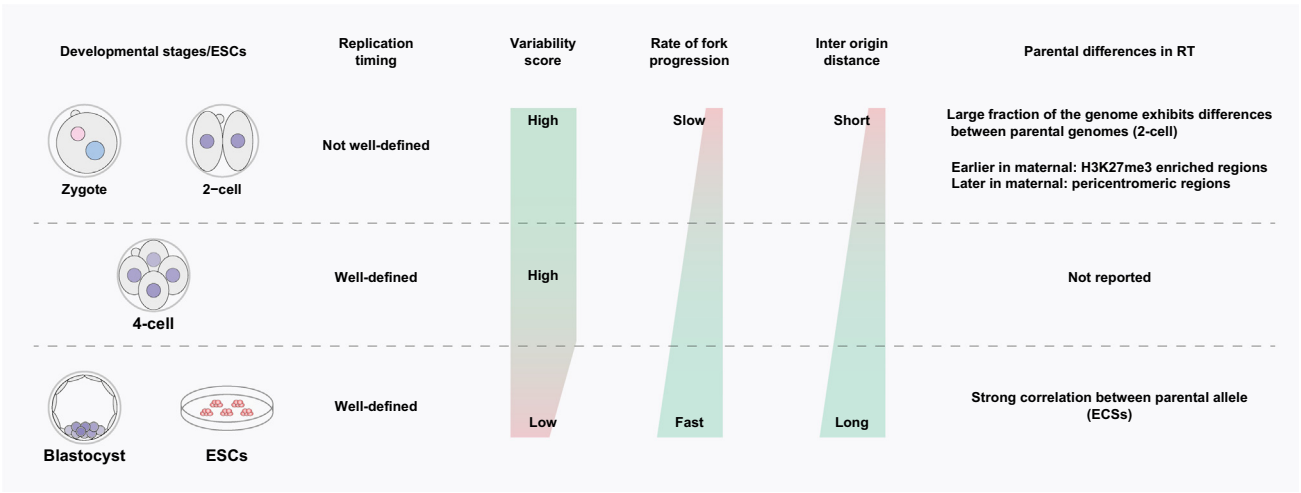

Figure 1. Cell-type-specific features of DNA replication during embryonic development. Summary of changes to features relating to replication timing during embryonic development and in embryonic stem cells (ESCs). Variability scores, which measure the variation in replication programs between cells, are shown in green for high periods and red for low periods. The rate of replication fork progression and the distance between replication start sites (inter-origin distance) are shown in green for fast and long periods, and red for slow and short periods, respectively. I have added a textual explanation of the uncoordinated replication timing and replication fork speed of four-cell stage embryos to the figure. Four-cell stage embryos have a well-defined replication timing compared with zygote and two-cell-stage embryos, but the rate of progression of the replication forks is slower than in late-stage embryos and ESCs, suggesting that the DNA replication program is in the process of being remodeled.

data directly addressing the causal link between RT and these histone marks, but interestingly, H3K27me3 has been shown to mark regions of the maternal genome that dissociate from the nuclear lamina in the two-cell stage, which correlates with the acquisition of an earlier RT profile on these regions [21]. Moreover, depletion of H3K27me3 prevented this large-scale rearrangement of nuclear architecture that occurs at the two-cell stage [3,4,21] (Pal *et al.*, unpublished). The pattern of allele-specific RT is not only explained by genomic canonical imprinting, which is mediated by parent-specific DNA methylation [8,9,24], with the exception of the inactive X-chromosome [25,26]. Therefore, the presence of Polycomb-mediated histone marks may be expected to play an important role in allele-specific RT and 3D genome structure. These results also confirm that RT is not well defined in early mouse embryogenesis. Because human embryos have a larger proportion of the genome with parent-specific epigenetic states than mouse embryos [27], there may also be more differences in RT between alleles. Detailed mechanistic insight in both mouse and human regulation of the RT program is expected in the future. The mechanism by which the egg and sperm – cells with very different shapes and properties – fuse, initiate DNA replication with different patterns, and synchronize during development, is complex but fascinating, and its understanding will further stimulate future work in this field.

Risk management during DNA replication via RT

Disruptions that occur during the process of replication of DNA in cells are collectively referred to as replication stress, and they are a source of genome instability [28–30]. Replication stress is caused by a variety of factors, including slow or fast fork progression [31,32], deficiency or misregulation of essential replication factors or accessory replisome factors [33–37], and conflicts between replication and transcription [38,39], among others. It was suggested that four-cell mouse embryos have already established a well-defined RT program but maintain an early embryonic-type slow fork speed (Box 1), and that temporal uncoupling of these replication features causes replication stress [7]. The factors that determine the rate of replication fork progression in the embryo are still unknown, but nucleoside supplementation at the four-cell stage led to the acceleration of the rate of fork progression, a decrease in the phosphorylated forms of the DNA damage response (DDR) markers, Chk1 and H2A.X, and a predominant reduction in the number of segregation error events [7]. Accelerated fork speed was also observed in human zygotes, but only when challenged with drugs that disturb the fork, for example poly(ADP-ribose) polymerase (PARP) inhibitor; PARP plays an important role in DNA damage repair [10,40]. These results indicate that insufficient levels of substrates for DNA replication and DDR could be the limiting steps for fork progression in early embryos. However, the increased fork speed resulting from these treatments is still slow compared with that observed in blastocyst stage embryos and ESCs, and in embryonic fibroblasts [7,14,15,41], suggesting the existence of multiple mechanisms that regulate the rate of fork progression at the earliest stages of embryogenesis [42].

A correlation between late-replicating regions and mutation rate has been reported in mice [43,44], humans [45–47], and flies [48], and indeed >90% of DNA breakpoints at the four-cell stage in mice occur in the mid-late or late-replicating regions in cultured situations *in vitro* and *in vivo*, respectively [7]. γH2A.X, which is widely used as a marker for DDR, generally shows a signal in response to replication stress during the whole DNA replication process, as exemplified in mouse zygotes and human cancer cells [49,50]. No breakpoints are detected in the regions that are replicated during early S phase at the four-cell stage [7], probably because these regions are more likely to be repaired, as euchromatin is more accessible to repair factors [51–58], although differences in genome fragility cannot be excluded [59]. Thus, the early-replicating regions are less likely to lead to the induction of abnormal karyotypes (Figure 2A). These observations suggest that cells prioritize each region of the genome by regulating the timing of replication, replicating gene-rich regions early to maintain genomic integrity, and leaving gene-poor regions that are

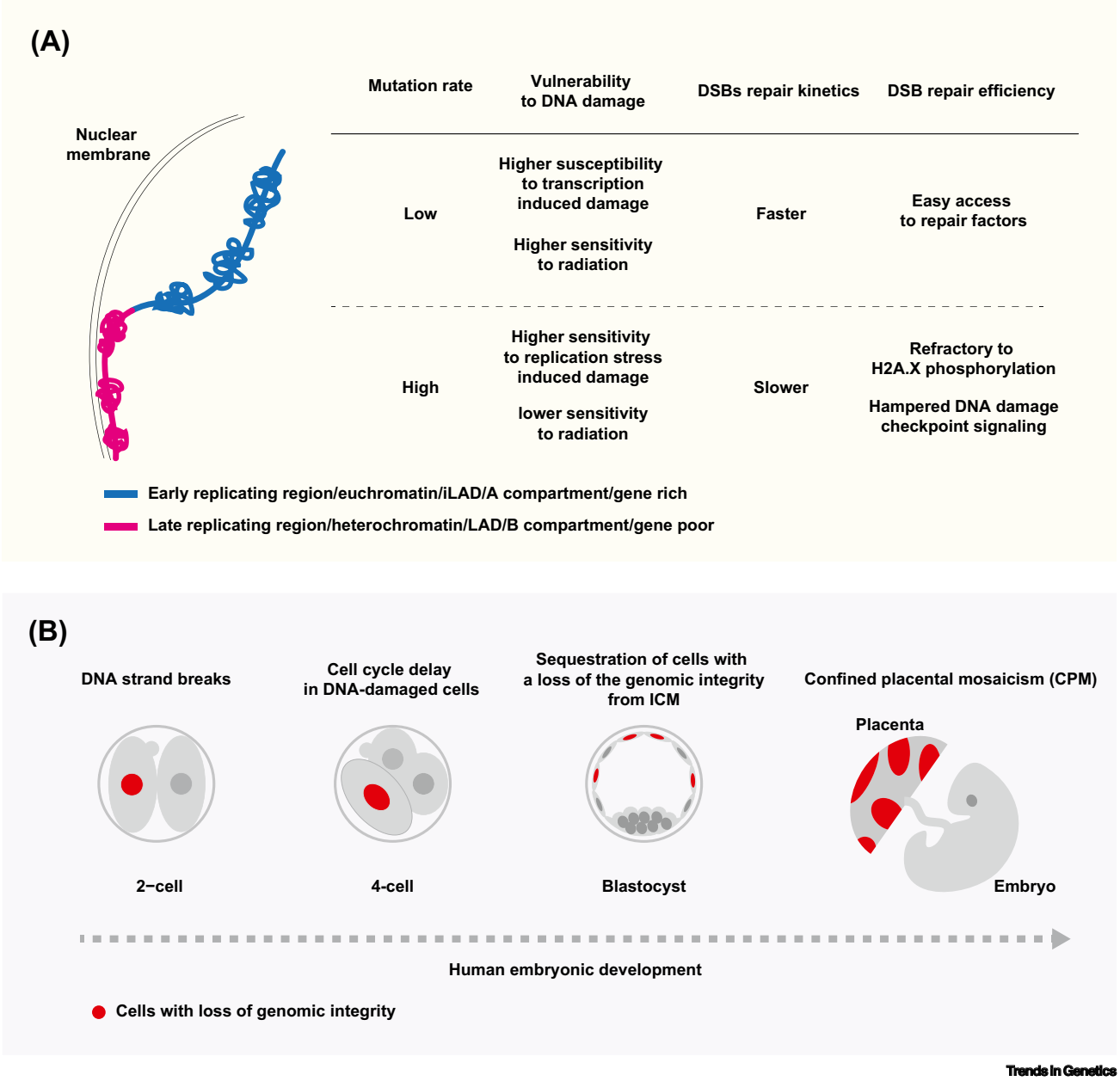

Figure 2. The relationship between 3D genome structure, replication timing, and genome instability. (A) Mechanisms to prevent replication timing (RT)-mediated mutations from remaining in gene-rich regions. (B) Potential safeguards to prevent the loss of genomic integrity in human embryos from being passed on to the next generation. Abbreviations: DSB, double-strand break; ICM, inner cell mass; LAD, lamina-associated domain.

likely to be less affected by the introduction of mutations until later. It is important to remember, however, that heterochromatin in early mouse embryos is not fully formed [60], and therefore the significance of the relationship between heterochromatin and the more inefficient DDR in those regions documented in other cell types remains to be determined. RT in two-cell embryos for major satellites differs between the maternal and the paternal genome [16]. Whether this is relevant for genome stability in embryos remains to be determined.

There is a possibility that induction of replication stress is programmed and regulated in the early preimplantation embryo through regulation of DNA replication, and may contribute to the process of development and differentiation. For example, the findings that induction of replication stress by the depletion of dNTPs induces differentiation of hematopoietic cells [61], that ATR regulates the speed of DNA replication by regulating the amount of dNTPs in the early S phase in naïve B cells [62], and that γH2A.X is involved in the maintenance of stemness in ESCs [63], are interesting examples in support of this notion. Additionally, manipulations that lead to slow fork progression can reprogram ESCs into two-cell-like cells [14]. However, information on replication stress and whether DDR is associated with DNA strand breaks in early embryos is still controversial, and further research is needed [64].

It is possible that cells that activate DDR are targeted for removal from a particular cell fate lineage by negative selection. Extraembryonic tissue, such as the placenta, has higher rates of mosaicism than the embryonic tissue [65,66]. This phenomenon is called confined placental mosaicism (CPM) and suggests the existence of a safeguard mechanism by which the organism prevents cells with abnormal karyotypes from being transferred to the embryo [67]. The mechanism by which the early embryo allocates cells with abnormal karyotypes to extraembryonic tissues is still unclear, but given that the blastomere that divides faster in human two-cell embryos contributes more frequently to the inner cell mass (ICM) at the blastocyst stage [68], the length of the cell cycle may underlie this mechanism (Figure 2B). This can be orchestrated by the embryo through sensing small delays in the cell cycle, particularly in cells whose genomic integrity is threatened by DNA damage, which is easily distinguished by a DDR-mediated delay during or after the DNA replication phase [69]. Thus, during the critical event of DNA replication, where genetic information is transmitted to the next generation, one would expect a multilayered system of safeguards to prevent mutations from being introduced into the embryo.

Underlying mechanisms regulating RT

RT is correlated with various aspects of cellular identities such as gene expression, chromatin accessibility, and the 3D genome structure [70,71]. These features are also thought to be mechanistically linked [72], although the causal relationship is only now beginning to be understood [73]. The 3D genome structure has been reported to be one of the strongest predictors of RT among many features, and changes in topologically associating domains (TADs) with ESC differentiation change concomitantly with RT [74]. By contrast, when RNA polymerase II (RNA Pol II) elongation was inhibited with 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) during the initial wave of transcription after fertilization – zygotic genome activation (ZGA) (Box 2) – a dramatic remodeling of LAD distribution was observed. This remodeling occurred concomitantly with the redistribution of H3K4me3 regions toward the nuclear lamina, but only a mild effect on RT was observed [8,75]. The results were similar when α-amanitin, which degrades the RNA Pol II protein itself, was used to inhibit ZGA. No dramatic global changes in RT were observed, by contrast with the dramatic changes in LAD distribution [8,75], although significant changes in RT were detected on ZGA genes. In human cancer cells, ablation of a key regulator of loop extrusion, cohesin, does

Box 2. Initial transcription: ZGA is essential for preimplantation development

Embryonic transcripts are dependent on RNA stored during oogenesis, and gene expression remains suspended for several hours after fertilization. After a period of time, depending on the species, transcription is initiated through a process called ZGA. This ZGA event consists of two waves: the minor ZGA, which, in mice, begins in the zygote stage, and the major ZGA, which begins in the two-cell-stage embryo [99,100]. Inhibition of either or both ZGAs causes embryonic arrest, demonstrating that ZGAs are essential for embryonic development [101,102]. RNA Pol II binding and gene expression levels at the two-cell stage correlate with RT, and changes in ZGA gene expression levels correlate with changes in LAD distribution [8,9,75].

not globally impact RT patterns, but insertion of a TAD boundary revealed a striking shift in RT from late to early, suggesting that structural boundaries can deterministically influence the initiation of DNA replication [72,76]. These results show that although LAD distribution correlates with RT in the steady state, the changes do not always show the same dynamics as RT. However, the different experimental results obtained from DRB and α-amanitin treatments in mouse embryos suggest that RNA Pol II loading rather than elongation plays an important role in the regulation of RT on ZGA genes [8]. It has been confirmed in cells (including the embryo) that nucleosome positioning is well aligned at active transcription sites, that is, regions where RNA Pol II binds to chromatin to initiate transcription [77–79], and this may play an important role in creating a stable scaffold for loading the pre-replication complex or origin licensing [80–82], which are essential for initiating DNA replication. A similar example of such a mechanism is the G-quadruplex motif, which affects the origin positioning and efficiency of replication [83,84], but it is not entirely clear how, mechanistically, G-quadruplexes affect RT.

Typically, when transcription factors (TFs) bind to their target DNA, the nucleosome must be removed from the promoter region because the chromatin acts as a strong barrier to the binding of TFs, with the exception of pioneer TFs [85–88]. However, the chromatin structure – including nucleosome position – is not well defined globally in early-stage embryos prior to ZGA, and the acquisition of an ordered nucleosome position landscape is disrupted when the zinc finger-containing TF YY1 is depleted, suggesting that chromatin-binding factors play a proactive role in determining the nucleosome position [78]. Nucleosome positions are defined throughout the genome, and this includes regions that are bound by RNA Pol II and transcribed [78,89] but also regions that are transcriptionally silent. Therefore, it will be interesting to determine how these regions are shaped during development and differentiation, and how they influence the binding of DNA replication factors, subsequent origin firing, and regulation of RT. As there is currently no technique for determining replication origins that is applicable to low-input samples such as mammalian embryos, further developments of techniques are expected to provide a more detailed understanding of DNA replication mechanisms during mammalian development and differentiation. Once the locations of the replication origins are known, the factors that bind origins and their chromatin structure could be analyzed in more detail, leading to further elucidation of the molecular mechanism directing origin firing and replication in mammalian embryos.

Rap1-interacting factor 1 (Rif1) is currently one of the most analyzed regulators of RT, and is the only protein known to affect both chromatin organization and RT [90]. Depletion of RIF1 in human cells leads to an almost complete loss of RT [73], and importantly this also results in a robust redistribution of both active (H3K27ac) and repressive (H3K9me3) histone modifications and in alteration of the 3D genome structure, phenotypes that were exacerbated through each subsequent DNA replication cycle. This suggests an important role of RT in regulating the epigenome. During mouse embryo development, the expression level of full-length RIF1 protein is significantly reduced by truncation immediately after fertilization, and is upregulated at the time when the RT is well defined from the eight-cell stage [91]. Depletion of RIF1 at the four-cell stage did not have a major effect on global RT at this stage, but a marked inhibition of RT consolidation was observed from the eight-cell stage onwards, indicating that in early embryos RIF1 plays an important role in RT consolidation during embryonic development (Nakatani *et al.*, unpublished). Interestingly, although RIF1 depletion also led to changes in LAD distribution in both four- and eight-cell embryos, there is no correlation with changes in RT (Nakatani *et al.*, unpublished). Zebrafish, frogs, and flies are also similar to mice and humans in that Rif1 is involved in RT, but the phenotypes upon depletion are diverse, including sex determination [92], increased speed of embryonic development and reduced eye size [93], and survival of only a few days at most with infertility [94], respectively. These reports show that RT is regulated by Rif1 across widely different species, but

that the role Rif1 plays in RT and the effect on cells when Rif1 is depleted varies between species and cell types, which is of great interest from a molecular biological and evolutionary point of view.

Concluding remarks

While advances in analytical techniques have made it possible to study the RT of different species and developmental processes [95,96], and datasets have been enriched, much of the biological roles and molecular mechanisms controlling RT remain unknown. Herein I have reviewed the process of how RT emerges during embryonic development, the potential role of DNA replication through regulation of RT, and interplay between 3D genome organization and RT by focusing on their regulator, Rif1. It is anticipated that the next decade will address the questions raised in this review, while further enriching the database of RT in more cell types and diseases and establishing systems that directly manipulate RT to determine how it affects cell fate decisions (see Outstanding questions). Thanks to the efforts of predecessors, the field of RT has received a great deal of attention and has made significant progress in the last decade, but many unsolved problems remain and new questions continue to arise. It is hoped that this review will stimulate experimental and theoretical research into such issues.

Outstanding questions

What is the relationship between RT and fork speed, and what are the molecular mechanisms of their regulation?

Is there a relationship between maternally-biased regions enriched in H3K27me3 inherited from the oocyte that dislodge from the nuclear lamina after fertilization and are also replicated earlier than the equivalent regions on the paternally-derived chromosome?

What is the molecular mechanism by which RNA Pol II fine-tunes the RT of ZGA genes?

What factors are involved in the truncation of Rif1 in early-stage embryos? Can Rif1 truncation in somatic cells recapitulate the RT seen in early-stage embryos, and are there factors other than Rif1 that shape the specific RT of early-stage embryos?

Can changes in RT be a driver for development, differentiation, and cell fate decisions that can alter histone marks, gene expression, and 3D genome organization, or vice versa?

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Declaration of interests

The author declares no conflicts of interest.

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