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Mammalian DNA Replication Timing

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Immediately following the discovery of the structure of DNA and the semi-conservative replication of the parental DNA sequence into two new DNA strands, it became apparent that DNA replication is organized in a temporal and spatial fashion during the S phase of the cell cycle, correlated with the large-scale organization of chromatin in the nucleus. After many decades of limited progress, technological advances in genomics, genome engineering, and imaging have finally positioned the field to tackle mechanisms underpinning the temporal and spatial regulation of DNA replication and the causal relationships between DNA replication and other features of large-scale chromosome structure and function. In this review, we discuss these major recent discoveries as well as expectations for the coming decade.

DNA replication is the process by which the genome replicates faithfully, in its entirety, and exactly once per cell cycle prior to each cell division. Importantly, and particularly in eukaryotes with large genomes, it is not just DNA that duplicates. The entire epigenome must be stripped down and reassembled at the replication fork. It is thus reasonable to presume that the events occurring at the time of replication are important to maintain epigenomic integrity and to facilitate changes in the epigenome during cell fate transitions. Indeed, eukaryotes employ a defined and highly conserved spatiotemporal program known as the replication timing (RT) program. RT is the temporal order in which parts of the genome replicate during S phase of the cell cycle. This program is closely correlated with many aspects of large-scale chromatin structure and function, including 3D chromatin folding, proximity to subnuclear bodies such as nucleoli, the lamina, and speckles, transcriptional activity and its associated chromatin features, as well as mutation and recombination rates (Fu et al. 2018; Marchal et al. 2019; Nathanailidou et al. 2020). Understanding the biological significance of these structure–function correlations and the causal mechanisms that may link them together has been a major challenge in the genome architecture field. In this review, we summarize results from genomics and imaging methods that established these correlations. Because of space limitations, we focus on mammalian cells, occasionally referring readers to literature showing similarities or differences in other model systems. We then highlight recent breakthroughs that provide long-awaited evidence as to how the RT program is regulated, how it may be mechanistically coordinated with other structural and functional features of the genome, and the bio-

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logical significance of an RT program. We expect the coming decade to be one in which we make mechanistic headway into these long-standing questions and obtain much needed insight into large-scale chromosome structure–function relationships in the cell nucleus.

SPATIAL AND TEMPORAL ORGANIZATION OF THE GENOME FOR REPLICATION

Genome-Wide Methods Define Large-Scale Reorganization of Replication Domains during Cell Fate Transitions

Genome-wide RT profiles are typically generated in one of two ways (Gilbert 2010; Hulke et al. 2020). One measures the copy number of any given DNA segment in an asynchronously growing cell population; sequences that replicate earlier will be slightly more abundant than sequences that replicate later. A second approach, which yields higher signal-to-noise, involves labeling newly synthesized DNA with chemically tagged nucleotides, synchronizing cells in early and late S phase, and then purifying and sequencing the labeled DNA synthesized at each of these times (Fig. 1A, top). The resultant RT profiles consist of long regions with similar RT, termed constant timing regions (CTRs). Because bidirectional replication fork rates are typically ~2 kb/min (Conti et al. 2007), CTRs larger than ~500 kb must arise from multiple, nearly synchronous initiation events. CTRs are punctuated by timing transition regions (TTRs) whose slopes (~2 kb/min) are consistent with unidirectional replication forks traveling long distances, interspersed with regions of occasional initiation (Petryk et al. 2016; Zhao et al. 2020).

The development of genome-wide methods gave the ability to measure RT in multiple cell types and stages during the differentiation process (Hiratani et al. 2008). Studying RT across cell types revealed that most CTRs can be subdivided into regions that coordinately change their RT in at least one cell type. In $\log_2$(E/L) ratio data (e.g., Fig. 1A, top), these changes occur in 400–800 kb units, termed replication domains (Fig. 1B, middle; Hiratani et al. 2008). Thus, CTRs consist of multiple replication domains, many of which alter their RT during differentiation to create cell-type-specific CTRs and TTRs (Pope et al. 2014). Further, it was found that during the course of human and mouse embryonic stem cell (mESC) differentiation, the RT of adjacent replication domains align to form fewer, larger CTRs, a process termed domain consolidation (Hiratani et al. 2008; Rivera-Mulia et al. 2015). Tracking these changes during stem cell differentiation showed that consolidation involved changes in the volume and subnuclear location of replication domains (Hiratani et al. 2008, 2010; Takebayashi et al. 2012). Genome-wide RT profiles also demonstrated that different cancer types, patient-specific cancer clones, and other human diseases are characterized by unique RT profiles resulting from alterations in the RT of specific replication domains (Rivera-Mulia et al. 2017, 2019b). Overall, ~50% of replication domains replicate at similar times in all cell types (constitutive domains), while the other 50% switch RT at some point during development and/or in disease (developmental domains) (Dileep et al. 2015; Rivera-Mulia et al. 2015). Dynamic changes in RT during stem cell lineage commitment are coordinated with changes in transcription, chromatin features, and 3D organization (Marchal et al. 2019; Rivera-Mulia et al. 2019a; Nathanailidou et al. 2020) and the genes that are most difficult to reactivate transcriptionally when generating induced pluripotent stem cells reside within a set of domains that are replicated early only in pluripotent cells (Hiratani et al. 2010), suggesting that late replication is associated with a barrier to reprogramming.

High-Resolution and Single-Cell Measurements of Replication Timing

Methods that simply plot the average RT of genomic bins in a population of cells (Fig. 1A, top) can rapidly compare many cell types, experimental conditions, or individuals (Hiratani et al. 2010; Koren et al. 2014; Rivera-Mulia et al. 2015, 2017, 2019b; Hulke et al. 2019). However, these methods suffer from poor temporal and spatial resolution and are not designed to identify cell-to-cell variation in RT. An alterna-

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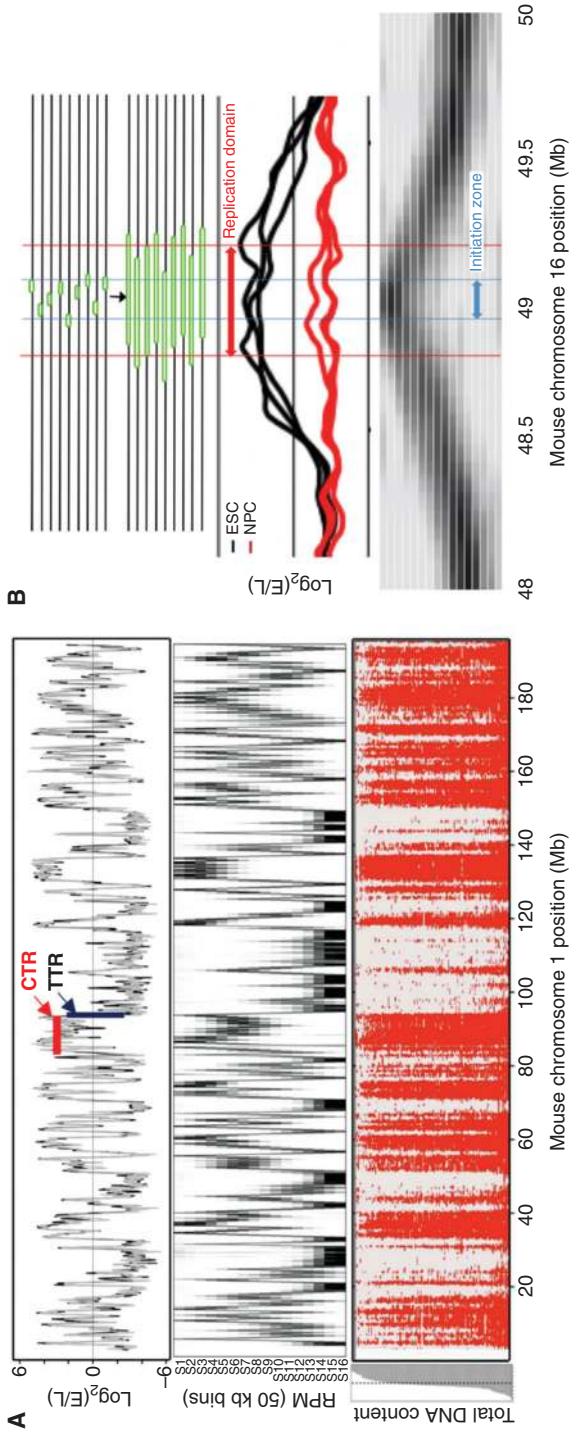

Figure 1. Measuring and interpreting replication timing (RT) profiles. (*A*) DNA RT profiles of chromosome 1 in mouse embryonic stem cells (mESCs). Profiles were generated using three different methods: (*Top*) E/L Repli-seq displays the population-averaged $\log_2$ ratio of DNA synthesized in early S phase to DNA synthesized in late S phase. One example each of a constant timing region ([CTR], red) and a timing transition region ([TTR], blue) are shown. (*Middle*) High-resolution Repli-seq displays a heat map (reads per million per 50 kb bin for each of 16 temporal intervals) of the sequences replicated in each of many S phase temporal fractions, in this case 16 fractions. (Data in *A* is adapted from Zhao et al. 2020 and available at data.4dnucleome.org). (*Bottom*) Single-cell Repli-seq displays the copy number (red = 2; gray = 1) of sequences per chromosome in each of many (in this case 71) individual cells, ranked from top to bottom in order of their total cellular DNA content. (*Bottom* panel adapted from Dileep and Gilbert 2018 with permission from the authors who are also the copyright holders.) (*B*) Replication domains and initiation zones. (*Top*) Schematic of how initiation events appear as they can be observed on single DNA fibers. There is usually one, occasionally more, initiation site(s) per locally earliest replicating peak. Different cells initiate at different sites throughout initiation zones that elongate(s) with time to give a population-averaged RT profile. (*Middle*) An ~0.5-Mb replication domain (red lines) on mouse chromosome 16 defined by E/L Repli-seq as a region of coordinated RT change from early to late during differentiation of mESCs (ESC, black) to neural precursor cells ([NPCs], red). Boundaries of the domain are determined by computing the point of significant slope change. (*Bottom*) High-resolution Repli-seq of this same region in mESCs reveals a single initiation zone (blue lines) within which this domain initiates. Because E/L Repli-seq sums the reds of the first and second half of S phase, the $\log_2$ ratio peak is relatively flat and extends until the point at which cells in the second half of S phase begin to replicate the region.

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tive is to collect multiple temporal intervals of S phase and quantify the amount of replication occurring in each interval independently (Chen et al. 2010; Hansen et al. 2010). High-resolution Repli-seq (Zhao et al. 2020), a genome-wide RT mapping method with high spatial and temporal resolution (<50 kb), uses shortened DNA synthesis labeling times and numerous temporal windows of S phase. This approach resolved the boundaries of replication domains into smaller (~200 kb) initiation zones (IZs) (Fig. 1B, bottom) and resolved CTRs into multiple IZs (Fig. 1A, middle). It also resolved TTRs into regions of uniform replication rates consistent with unidirectional forks moving at ~2 kb/min, and punctuated at specific sites by inefficient IZs (Zhao et al. 2020). High-resolution Repli-seq can also indirectly infer RT heterogeneity by quantifying the breadth of the temporal window over which a genomic bin replicates (*Y* axis spread in Fig. 1A, middle), revealing variability in the degree of cell-to-cell heterogeneity at different genomic locations (Zhao et al. 2020), Altogether, high-resolution Repli-seq has provided the most detailed view to date of where and when replication initiates, elongates, and terminates in several mammalian cell lines.

Direct measurements of cell-to-cell heterogeneity require single-cell approaches. Recently, techniques such as live-cell imaging of specific targeted loci (Duriez et al. 2019) and single-cell Repli-seq (Dileep and Gilbert 2018; Takahashi et al. 2019) have provided such measurements. Live-cell imaging studies assay one locus at a time and are thus laborious and low throughput, but they are the only way to obtain direct real-time single-chromosome measurements of locus duplication (Duriez et al. 2019). Single-cell Repli-seq is low resolution (<200 kb), but it can reveal cell-to-cell heterogeneity of RT at a genome-wide scale (Dileep and Gilbert 2018; Takahashi et al. 2019). Both single-locus live-cell tracking and single-cell Repli-seq have concluded that a majority of cells replicate any given segment of the genome within a relatively defined time period.

One drawback of "seq" or "omics" single-cell approaches is that they compare the average of two diploid genomes; single-chromosome data can only be obtained when maternal and paternal chromosomes can be resolved or haploid cells are available (Dileep and Gilbert 2018; Klein et al. 2019). However, because most domains on homologous chromosomes replicate at similar times (Dileep and Gilbert 2018), diploid single-cell Repli-seq can provide a reasonable view of replication domains and is a useful tool for assaying RT in cell types that are difficult to obtain in large numbers such as the cells of early embryos or to track single cells through cell fate transitions (Miura et al. 2019).

Cytological Studies of Replication: Replication Foci and Spatiotemporal Compartments

Long before the development of high-throughput genomic techniques, cytological and imaging techniques served as the major avenue for studying replication. Labeling cells with nucleotide analogs, initially tritiated thymidine and later halogenated nucleotides that could be detected with fluorescent antibodies, identified both a temporal and a spatial regulation of replication within the nucleus (Taylor 1960; Stubblefield 1975; Nakamura et al. 1986). DNA synthesis could be seen to take place in punctate "replication foci" that were localized to the interior of the nucleus in early S phase, moving to the nuclear and nucleolar periphery during late S phase, defining "early and late" spatiotemporal chromatin compartments (Fig. 2A). Pulse-chase-pulse experiments using two different labels (Ma et al. 1998; Dimitrova and Gilbert 1999), as well as tracking replication fork proteins in living cells (Sporbert et al. 2002; Löb et al. 2016), revealed that replication foci took 45–60 min to complete replication. When chased for multiple consecutive cell cycles, the labeled chromatin remained together as a stable unit of chromosome substructure (Sparvoli et al. 1994; Ferreira et al. 1997; Jackson and Pombo 1998). While the spatial arrangement of these units is rearranged during mitosis to form bands resembling chromomeric banding patterns (Stubblefield 1975), they return to their general subnuclear locations (Dimitrova and Gilbert 1999) and exhibit very little motion during in-

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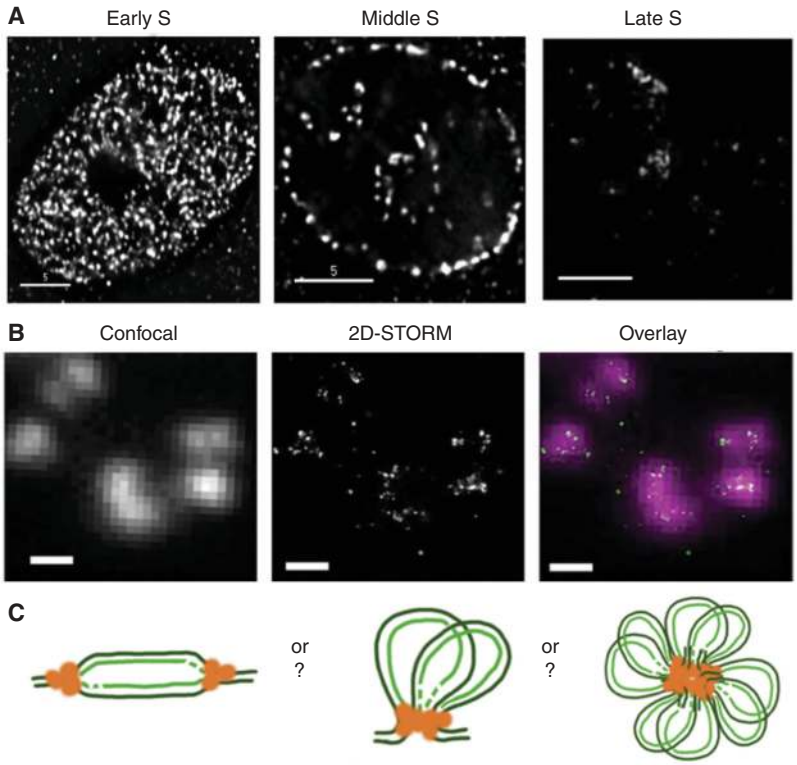

Figure 2. Organization of replicons. (*A*) Chinese hamster ovary cells were labeled for 10 min with BrdU, fixed and stained with anti-BrdU antibodies to reveal the spatial patterns of DNA synthesis in early, middle, and late S phase. (Images in *A* courtesy of J. Lu.) Scale bar, 5 µM. (*B*) Normal rat kidney cells were labeled live with ATTO 633-dUTP and then chased for several generations. Shown is a comparison of a high magnification (scale bar, 500 nm) confocal image of a cluster of replication foci to the super-resolution (2D-STORM) image of the same replication foci, demonstrating that each replication focus consists of a cluster of labeled sites. (Panel *B* reprinted from Xiang et al. 2018 courtesy W. Xiang © 2018 in conjunction with a Creative Commons License [Attribution 4.0 International] www.ncbi.nlm.nih.gov/pmc/articles/PMC5987722.) (*C*) The organization of replicons in these foci remains unknown. Sister forks could be replicated independently (*left*), by a common replisome or clustered replisomes (*middle*) or multiple replicons, and their sister forks could be replicated by a common replisome or cluster of replisomes (*right*). (Panel *C* courtesy of C. Marchal.)

terphase (Abney et al. 1997). Sites of replication can also be tracked in living cells using fluorescently labeled nucleotides (Panning and Gilbert 2005; Wilson et al. 2016), and recent live-cell super-resolution studies have shown that replication foci labeled this way are stable units of coordinated Brownian motion in living cells (Fig. 2B; Nozaki et al. 2017; Xiang et al. 2018).

It is tempting to think of replication foci as the cytological manifestation of replication domains or IZs, but many questions remain as to the molecular architecture and organization of replicons within replication foci. Attempts to count the total number of foci with both conventional and super-resolution microscopy resolution and relate those numbers to genome size suggest that foci are close to the size of replication domains (400–800 kb) but this analysis still relies on indirect estimates of fork rates and interorigin distances (Berezney et al. 2000; Chagin et al. 2016). A single replicon moving bidirectionally at an estimated average fork rate of 1.8 kb/min (Conti et al. 2007) would replicate ~200 kb in the 45–60 min that replication endures at any given focus. Interestingly, that is approximately the size of IZs identified by

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high-resolution Repli-seq (Fig. 1A, middle). However, high-throughput single DNA fiber analyses suggest that typically only one initiation event occurs per IZ (Fig. 1B, top; Wang et al. 2020a), and that those events can occur at any of many potential sites within the IZ so the 200 kb would be staggered in different chromosomes within the cell population (Fig. 1B, top). Also, identifying whether the two emerging sister forks, or in some cases multiple replicons, are located together in space or travel independently will require further technical innovations to discern (Fig. 2C). In yeast, where replication initiates from well-defined sites, it has been shown that equidistant flanking sites come together in space at the time that they replicate (Kitamura et al. 2006; Meister et al. 2007), suggesting that bidirectionally emanating sister forks are synthesized in a single location. Advances in mammalian live-cell and super-resolution imaging should soon shed light on some of these questions (Deng et al. 2016; Bintu et al. 2018; Tasan et al. 2018; Boettiger and Murphy 2020).

The intriguing relationship between spatial and temporal aspects of replication was addressed by chasing early- and late-labeled foci into the following G1 phase (Dimitrova and Gilbert 1999). This demonstrated that the spatial positions of the foci were re-established 1–2 h after mitosis and remained static for the rest of interphase. By introducing these nuclei into a cell-free replication initiation system, it was shown that the temporal program for replication was established coincident with stable repositioning of the labeled foci, an event termed the timing decision point (TDP) (Dimitrova and Gilbert 1999). It was proposed that anchorage of chromatin domains could seed the assembly of subnuclear microenvironments that could set thresholds for initiation of replication (Dimitrova and Gilbert 1999; Gilbert 2001). Although the mechanisms and molecules establishing RT at the TDP have still not been elucidated, the concept of seeding microenvironments in the nucleus is now quite popular and is thought to occur through liquid–liquid phase separation (Strom and Brangwynne 2019). We will return to this concept below when we discuss recent advances in understanding mechanisms regulating RT (see Fig. 4). Understanding the molecular events occurring at the TDP will be a major challenge for the coming decade.

REPLICATION AND GENOME ARCHITECTURE

Hi-C Compartments Strongly Correlate with Replication Timing

3D chromatin organization can also be inferred by studying the interactions of loci at a global scale using high-resolution chromatin conformation capture (Hi-C), a technique that maps chromatin interactions genome wide (Lieberman-Aiden et al. 2009; Mota-Gómez and Lupiáñez 2019). A first principal component analysis of Hi-C data identifies two spatially and functionally distinct compartments of chromatin folding, named compartments A and B (Lieberman-Aiden et al. 2009), which correspond remarkably well to the early and late replicating compartments of the genome, both spatially (Fig. 2A) and temporally (Ryba et al. 2010; Yaffe et al. 2010). The A compartment is enriched in transcriptionally active chromatin marks (e.g., H3K27ac, H3K4me3), located in the interior of the nucleus while the B compartment consists of more transcriptionally silenced chromatin marks (e.g., H3K9me3 and H3K27me3) located at the periphery and perinucleolar regions (Lieberman-Aiden et al. 2009; Rao et al. 2014). Given what was already known about the developmental plasticity of RT and the positions of replication domains in the nucleus (Fig. 1B and see the section Genome-Wide Methods Define Large-Scale Reorganization of Replication Domains during Cell Fate Transitions), it was predicted that chromatin compartments would also be developmentally regulated and spatially consolidate during differentiation, coordinated with RT changes and temporal consolidation. This prediction was later borne out with Hi-C analyses in multiple cell types (Dixon et al. 2012; Xie et al. 2013; Dileep and Gilbert 2018; Miura et al. 2019). Interestingly, one study found that A/B compartments and RT become uncoupled during the early stages of human embryonic stem cell (hESC) lineage specification, after which

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strong alignment between the two is re-established (Dileep et al. 2019). Together these results show that while there is not a one-to-one correspondence of Hi-C compartments and RT—and they can be uncoupled—the two tend to highly correlate with each other, and more so in differentiated cells than in stem cells.

Recently, higher resolution Hi-C methods substratified the binary A/B compartments into five distinct subcompartments, A1-2 and B1-3, with distinct histone modification patterns (Rao et al. 2014). These five subcompartments also correlate strongly with replication in distinct temporal intervals of S phase (Fig. 3B). While A1 replicates very early, A2 does not finish replicating until mid–S phase, has lower guanine–cytosine content, longer genes, and higher levels of H3K9me3 than A1. Large differences are observed in the three B subcompartments. B1 is enriched in H3K27me3, depleted in H3K36me3, and replicates in mid–S phase, whereas B2 and B3 are depleted in H3K27me3 and replicate in very late S phase. B2 can be found in both the nuclear lamina and the nucleolus, while B3 is exclusively found at the nuclear lamina (Rao et al. 2014). This demonstrates a clear subcompartmentalization of the genome that goes beyond the division between A and B compartments yet still correlates strongly with RT.

Topologically Associated Domains (TADs) and Their Relationship to Replication Domains

In addition to large-scale compartments, Hi-C demonstrated that chromosomes are organized into smaller self-interacting domains, hundreds of kilobases in length, called TADs (Dixon et al. 2012; Nora et al. 2012; De Laat and Duboule 2013). Interactions between adjacent domains are depleted, allowing TADs to be mapped by features such as directionality index (interactions of chromosomal sites significantly more frequent in one direction) or insulation score (interactions across a region significantly depleted). TADs, as originally described, were the same size range as replication domains, raising the question as to whether TADs are the structural equivalents of replication domains (Fig. 1B) and, possibly, replication foci (Fig. 2). In fact, it was shown that the boundaries of replication domains (Fig. 1B), defined as domains whose RT changes during cell fate transitions or differs between cell types, align strongly with the boundaries of TADs (Pope et al. 2014). This gave rise to the "replication domain model" (Pope et al. 2013), in which RT is regulated in units corresponding to TADs, which fold in such a manner that TADs with similar RT come into close proximity (at the TDP) to form larger-scale compartments.

Mechanisms linking replication domains to TADs remain uncertain. Because adjacent domains with similar RT reside within the same interaction compartment, TAD boundaries within those interaction compartments were originally difficult to detect with low-resolution data (Dixon et al. 2012; Pope et al. 2014). More recent high-resolution Hi-C resolves these boundaries and reveals many smaller domains nested within larger ones that were previously averaged together in the low-resolution data (Rao et al. 2014; Beagan and Phillips-Cremins 2020). We now recognize two basic forces that shape the 3D organization of chromatin (Sanborn et al. 2015; Rao et al. 2017; Schwarzer et al. 2017). One is driven by the tendency of similar chromatin types to aggregate nonspecifically into compartments, with the borders between different chromatin types manifesting as boundaries. The second is driven by the extrusion of chromatin through the central pore of cohesin rings until encountering roadblocks (e.g., CTCF) that constitute fixed boundary elements (Fudenberg et al. 2017; Rao et al. 2017). Consistently, depletion of either the cohesin subunit RAD21 (Rao et al. 2017), the cohesin loading factor Nipbl (Schwarzer et al. 2017), or CTCF (Nora et al. 2017) eliminates loop domains but has no detectable effect on A/B chromatin compartments (Nora et al. 2017; Rao et al. 2017). Different methods and scales of analysis highlight different features of these domains to different extents (Hsieh et al. 2015, 2020). Super-resolution imaging can also visualize these domains, revealing chromosome-to-chromosome heterogeneity in the specific CTCF sites where cohesin pauses that cannot be

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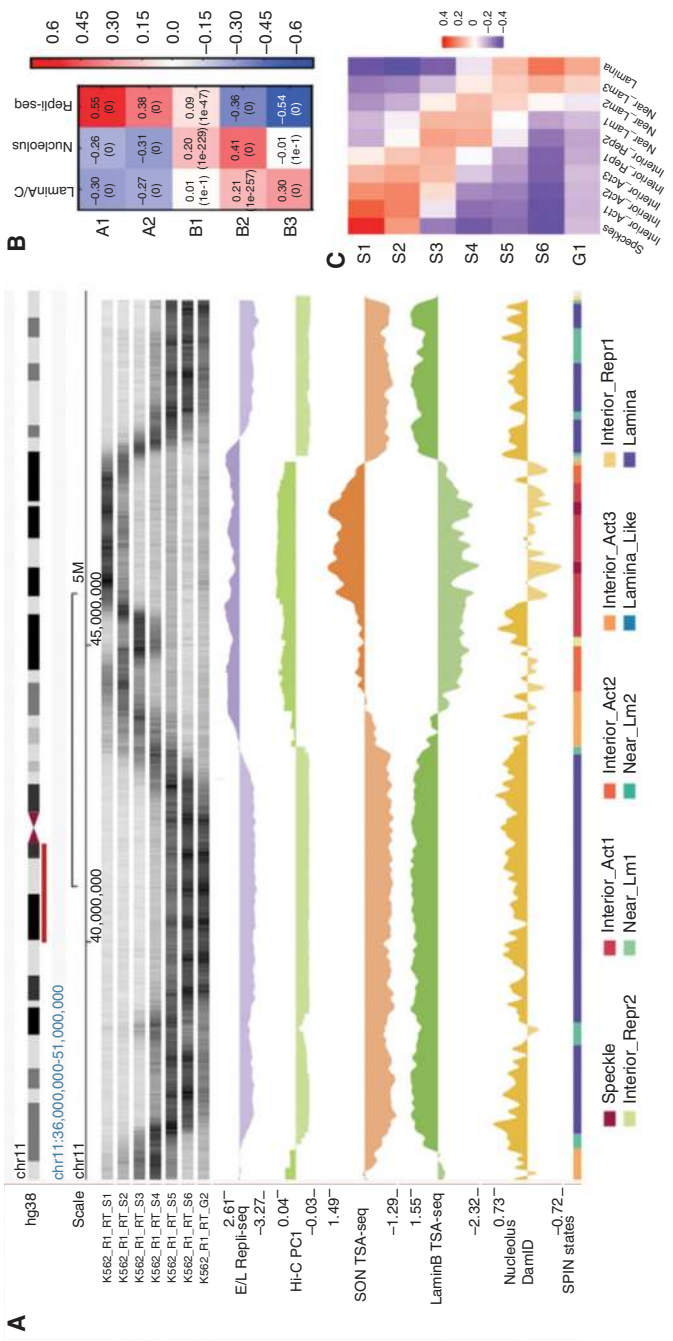

Figure 3. Nuclear organization and chromatin architecture. (*A*) Replication timing (RT), displayed both in high-resolution Repli-seq and E/L Repli-seq, highly correlates with Hi-C-derived first principal component (PC1), the distance of the locus from the nuclear speckle protein SON and the nuclear lamina protein LaminB measured via TSA-seq, and with the contact frequency of chromatin with the nucleolus measured via DamID, an alternative to ChIP, which uses *Escherichia coli* adenine methyltransferase (Dam) fused to a protein of interest to methylate adenines in DNA sequences that come to close proximity to the protein of interest. Information on the distance and contact frequency of chromatin loci from subnuclear landmarks is combined with Hi-C and histone modification data to stratify the genome into 10 SPIN states. A SPIN state is composed of genomic loci sharing unique combinations of histone modifications, compartmentalization, and association with nuclear landmarks. The data displayed originate from a 15 Mb window in chromosome 11 of human bone marrow lymphoblast K562 cells, mapped using the Nucleome Browser (vis.nucleome.org). (*B*) Spearman correlation of five subcompartments in Rao et al. (2014) to the nuclear lamina, the nucleolus, and early RT. (Data in *B* adapted from Rao et al. 2014.) (*C*) Spearman correlation of the 10 SPIN states in Wang et al. (2020b) to six fractions of the S phase and G2. (Data in *C* plotted using data adapted from Wang et al. 2020b.)

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detected with population-averaged methods (Bintu et al. 2018).

Although these mechanisms are not in dispute, the nomenclature has become complicated as some investigators use TAD to mean any self-interacting domain, others reserve the term TAD exclusively to mean cohesin-mediated loop domains, while still others avoid TAD altogether. Also, the term "contact domain," can be used to mean either all domains or only "loop domains." Another source of confusion is that mammals (the focus of this review) have extensive loop extrusion, while many other species mainly use chromatin compartmentalization (Rowley and Corces 2018; Szabo et al. 2019). It is important to be aware of this nomenclature variation when reading the literature.

Importantly for our discussion, depletion of either RAD21 RT (Oldach and Nieduszynski 2019; Cremer et al. 2020) or CTCF RT (Sima et al. 2019) was reported to have no detectable effect on the global RT program. Moreover, deletion of CTCF sites at replication/TAD/loop domain boundaries can cause fusion of neighboring domains (Despang et al. 2019) while having no effect on RT of the locus (Sima et al. 2019). By corollary, inversion of part of an early replicating domain, transplanting it inside of a late replicating domain, can create a new boundary (Sima et al. 2019). It remains to be seen whether in these cases the boundaries are between compartment domains or have created new loop domain boundaries; however, in at least some cases the creation of a new early-to-late RT transition (TTR) leads to the presence of a new RAD21-binding site (Klein et al. 2019). These results demonstrate the independence of global RT on fixed boundaries and suggest that, in at least some cases, RT differences can drive the formation of boundaries. One hint at how this could occur comes from recent evidence suggesting that the replicative helicase MCM2-7 complex, which designates sites of initiation (discussed below), can impede cohesin extrusion (Dequeker et al. 2020) and could thus influence the positions of boundaries. Other evidence suggests that cohesin extrusion confines the replicative helicase to domain boundaries (Emerson et al. 2021). Clearly there is much work to be done to understand the mechanisms linking replication and genome architecture but new tools to manipulate the replication program (Klein et al. 2019; Sima et al. 2019) should reveal new insights in the near future.

REPLICATION AND SUBNUCLEAR LANDMARKS

Lamina-Associated Domains (LADs) and Nucleolus-Associated Domains (NADs): Overlapping Compartments

The nucleus is a highly heterogeneous landscape with myriad neighborhoods of markedly different molecular composition and functional activities all residing in close proximity with no apparent physical boundaries to separate them (Hildebrand and Dekker 2020). These neighborhoods or subnuclear microenvironments can have profound effects on chromatin organization, accessibility, and activities. Those whose composition is well defined form landmarks of subnuclear position. Two of the most prominent landmarks are the nuclear lamina and the nucleolus. Chromatin domains coming into contact with either of these landmarks are called lamina-associated domains (LADs) and nucleolus-associated domains (NADs), respectively. They contain chromatin marks associated with repressed chromatin, and harbor lowly expressed or more frequently silenced genes, broadly referred to as heterochromatin (van Steensel and Belmont 2017). Techniques to map the proximity of chromatin loci to such subnuclear landmarks fall into two basic categories (Fig. 3A): those that map contact frequency, such as chromatin immunoprecipitation (ChIP) and DNA adenine methyltransferase identification (DamID) (Guelen et al. 2008; Kind et al. 2013, 2015), and those that measure the chromosomal distance of loci to nuclear structures, such as TSA-seq, a cytological ruler that measures the distance of a sequence from a specific subnuclear feature (Chen et al. 2018). A subset of LADs associate with the lamina independent of cell type (constitutive LADs), while others associate with the lamina in a cell-type-specific manner (developmental LADs) (Peric-Hupkes et al. 2010; Meuleman et al. 2013). As expected,

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lamina association is dynamic very early in G1, as the nucleus is reassembled, with distinctions between LADs and inter-LADs becoming more pronounced over time (Abney et al. 1997; Marshall et al. 1997; Thomson et al. 2004; Schaik et al. 2020), during the same period of G1 phase in which RT is established (TDP).

Recent studies have indicated that inducing the expression of a gene in contact with the nuclear lamina can dissociate the gene from the lamina and move it to the interior of the nucleus, confined to the transcription unit and 50–100 kb of flanking DNA. This can be accompanied by an RT shift across a significantly larger region containing the expressed gene (Therizols et al. 2014; Brueckner et al. 2020). Interestingly, longer transcripts can advance RT under conditions and in positions where short transcripts do not (Blin et al. 2019). These findings indicate that some aspect associated with the complex process of transcription can directly or indirectly reposition a locus and advance its RT. An important future goal will be to determine whether or not repositioning or RT changes require mitotic disassembly and reassembly of nuclear architecture or whether they occur in direct response to transcriptional activation.

While NADs are traditionally thought to be exclusively heterochromatic and late replicating, it was recently discovered that there are two types of NADs, type I and type II, each having its own distinct epigenetic marks and RT (Vertii et al. 2019). A big distinction between the two is that type I NADs can also be found near the lamina, while type II NADs are exclusively found near the nucleolus. This is consistent with the earlier finding that some LADs can be positioned either at the lamina or the nucleolar periphery between cell cycles (Kind et al. 2013; Politz et al. 2014) and that single-cell Lamin DamID finds some LADs to be associated with the nuclear lamina in every cell while others are variably associated from cell to cell (Kind et al. 2015). Type I NADs tend to replicate very late during S phase, have very low levels of gene expression, and are enriched in H3K9me3, similar to LADs. On the other hand, type II NADs replicate in the mid-late S phase, have higher levels of gene expression, and are enriched in H3K27me3. Overall, close examination of LADs and NADs reveals that domains belonging to these two classes are not homogeneous, with variations in chromatin structure, strength of association with subnuclear landmarks, and correlation to RT.

Speckle-Associated Domains (SPADs)

First observed by Ramon y Cajal in the early twentieth century through histochemical stains and later confirmed, in the 1990s, via electron microscopy (EM), nuclear speckles are subnuclear bodies enriched in pre-mRNA splicing factors that facilitate the maturation of mRNA, although their precise functional role is still under debate (Hall et al. 2006; Spector and Lamond 2011; Chen and Belmont 2019). In contrast to LADs and NADs, loci residing near the nuclear speckles, termed speckle-associated domains (SPADs), are often associated with open chromatin, have high levels of transcription, and are decorated with active chromatin histone marks, making these regions euchromatic (Chen and Belmont 2019).

TSA-seq has been used to map SPADs, by calculating the distance of genomic loci from SON, a protein essential for speckle organization (Chen et al. 2018; Chen and Belmont 2019). Interestingly, plots showing the distance of genomic loci from nuclear speckles show a correlation of SON TSA-seq peaks with early RT peaks (Fig. 3A). Genes close to nuclear speckles tend to have higher levels of expression, and tethering of a locus to the nuclear speckles has been shown to be sufficient to amplify gene expression levels (Kim et al. 2020). Early replication of chromatin in proximity to SPADs could be related to the high levels of transcription close to speckles, either directly through the process of transcription itself or through epigenetic changes occurring during transcription that correlate with early replication. It is important to appreciate that although transcription and early RT are correlated, there are many genes that can be expressed while late replicating so the act of stimulating transcription itself is not sufficient for early RT (Rivera-Mulia et al. 2015), and the correlation may be more related to chro-

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matin changes elicited by transcriptional factor binding (Goren et al. 2008; Ostrow et al. 2017; Rivera-Mulia et al. 2019a; Sima et al. 2019). Alternatively, speckle-associated factors themselves or activities associated with splicing could promote early replication, or early replication could promote association with speckles. As with most correlations between genome organization and RT, the underpinning causal mechanisms remain a major future challenge.

Replication Timing and Models of Nuclear Organization

Thus far, we have described the relationship of RT with chromatin architecture and subnuclear localization as separate correlations. Recently, a computational method has been developed to integrate Hi-C with genome-wide information about contact frequencies with and proximity to specific subnuclear landmarks, termed spatial position inference of the nuclear genome (SPIN) (Wang et al. 2020b). SPIN stratifies chromatin by its subnuclear addresses, dubbed as "SPIN states." SPIN separates the genome into 10 SPIN states, which feature distinct localization patterns within the nucleus (Fig. 3A; Wang et al. 2020b). These SPIN states strongly associate with the five primary subcompartments defined by Rao et al. (2014). However, the incorporation of distance relationships between subnuclear landmarks and chromatin marks gives finer structure and functional significance to the SPIN reference map over Hi-C contact maps alone. Interestingly, each SPIN state correlates strongly with chromatin replicated in a specific time interval of S phase (Fig. 3C). Additionally, certain states highly correlate with constitutive and developmental replication domains, as defined by Dileep et al. (2015); 85% of the domains found in the speckle state were constitutively early replicating, 55% of the domains in the lamina state were constitutively late, while the rest of the states contain higher percentages of developmentally regulated replication domains (Wang et al. 2020b). Overall, SPIN is a newly developed computational tool with the ability to integrate multiple data types and provide a unified correlation between RT and subnuclear chromatin organization. The development of SPIN exemplifies the collaborative efforts that are being undertaken to use newly acquired computational power to gain better insight into the association of RT with other features of the nucleus.

NEW INSIGHTS INTO MECHANISMS REGULATING REPLICATION TIMING PROVIDE CLUES TO CAUSALITY

Initiating Replication: A Precision Mechanism that Requires Flexibility

To understand how RT is regulated, it would seem logical to start with the sites where replication initiates, often called "origins" of replication. In mammalian cells, however, sites of initiation are highly variable, such that any given site in the genome is used in only a small fraction of cell cycles (Demczuk et al. 2012). To restrict initiation to once and only once per cell cycle, cells employ a sequence-agnostic "two-cycle engine" strategy (Gilbert 2001; Deegan and Diffley 2016). The ring-shaped Mcm2-7 helicase is loaded around double-stranded DNA in an inactive form early in G1 strictly under conditions that prevent initiation and with no apparent DNA sequence requirement. Upon entry into S phase, Mcm is converted into an active helicase by Db4-dependent kinase (DDK) and cyclin-dependent kinase (CDK) under conditions that prevent new Mcm complexes from being loaded (Deegan and Diffley 2016). Moreover, unique among chromatin proteins, inactive Mcm is irreversibly locked down on DNA until replication begins (Kuipers et al. 2011). However, it can slide when chromatin is disassembled and DNA unwound during transcription (Foss et al. 2019), shifting potential sites of initiation long after Mcm loading (Sasaki et al. 2006). Finally, the number of Mcm complexes loaded exceeds the number of origins used to replicate the genome by several fold (Ibarra et al. 2008; Limas and Cook 2019). These excess Mcms can either remain dormant until removed by passing replication forks, or they can be recruited to initiate replication when segments of DNA remain unreplicated such as under conditions of replication stress (Ge et al. 2007; Courtot et al.

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2018; Moiseeva and Bakkenist 2019). Thus, mechanisms regulating initiation of replication ensure fail-safe, once per cell cycle initiation without the need for specific sequences or site preference (Gilbert 2001; Deegan and Diffley 2016). Possibly, reliance on specific origin sequences would render large chromosomes dangerously vulnerable to mutation and structural variation (Rivera-Mulia and Gilbert 2016).

While highly flexible and stochastic, origin selection is not random; rather, initiation is confined to zones of several 10s of kilobases and sites within these IZs initiate at different frequencies via mechanisms that are not understood (Petryk et al. 2016; Wang et al. 2020a; Zhao et al. 2020). Addressing these mechanisms will require the ability to make large numbers of single-molecule measurements to accurately quantify the number and frequencies of usage of all initiation sites. It has not yet been possible to map the sites of Mcm loading on single molecules. However, single-molecule measurements of initiation efficiency on long purified DNA fibers have now achieved at >2000× genome coverage (Wang et al. 2020a). While the resolution is still too low (~15 kb) to determine variation in specific site usage, it is clear that replication will initiate at some location within any given ~40 kb IZ with frequencies ranging from <0.5% to ~40% of S phases (Wang et al. 2020a) but at any given site within an IZ at a much lower frequency. This large degree of flexibility in replication initiation sites explains why the results of population-based methods for mapping origins are so methodology dependent; high-resolution methods pick up specific sites within IZs that initiate frequently enough to be detected above noise, while low-resolution methods detect the sum frequency of initiation within larger IZs (Gilbert 2010; Hyrien 2015). Taken together, what emerges is a stochastic view of origin firing in which each segment of the genome has a characteristic probability of firing. Because segments with higher probability are more likely to fire early in the S phase, the mechanisms regulating the probability of initiation within zones, rather than specific origin sequences, are what determines the RT program. We will now turn our attention to how zones of initiation might acquire different probabilities of firing.

Early Replication Control Elements (ERCEs)

The fact that initiation does not require specific DNA sequences does not rule out the possibility that sequence elements may govern the probability that a replication domain will initiate somewhere within its IZ(s). However, demonstrating the existence of sequence elements has remained controversial. Shortly after the discovery of an RT program (Taylor 1960), it was found that the active and inactive X chromosomes in female mammals replicate early and late during S phase, respectively, demonstrating that at least in some cases, sequence-independent (epigenetic) mechanisms can influence RT. On the other hand, there are several examples of synthetically combined DNA segments that can alter RT when inserted into a locus (Simon et al. 2001; Hassan-Zadeh et al. 2012; Blin et al. 2019; Brueckner et al. 2020). The ability to identify bona fide *cis*-elements in their native context required the ability to make genetic lesions in mammalian genomes, which remained extremely low throughput until the advent of CRISPR-Cas9 genome-editing techniques. Recently, a large series of CRISPR-mediated deletions and inversions revealed the existence of discrete *cis*-regulatory elements of RT, termed early replication control elements (ERCEs), in several distinct replication domains in mESCs with 1835 more predicted ERCEs identified computationally across the mouse genome (Sima et al. 2019). Deletions of combinations of ERCEs in Dppa2/4 revealed that the presence of one ERCE is sufficient for a region to replicate in mid-S, while two or more ERCEs gave rise to early-S replication. Intriguingly, ERCEs were also found to be necessary to maintain a subnuclear A/B compartment, proximity to the nuclear lamina, TAD architecture, and transcription, with the effects confined to within the domain in which they reside (Sima et al. 2019; Brueckner et al. 2020). By contrast, the boundaries of TADs as well as CTCF/cohesin loop domains were dispensable for global RT and subnuclear compartments (Rao et al. 2017; Oldach and Niedus-

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zynski, 2019; Sima et al. 2019; Cremer et al. 2020). Thus, ERCEs are not only the long sought *cis*-elements of RT control, but they coordinate replication with transcription and chromosome architecture, providing an experimental handle into mechanisms behind these long-standing correlations.

ERCEs, so far identified only in mESCs, are decorated with large patches of H3K27ac and have binding sites for mESC pluripotency factors, Oct4, Sox2, and Nanog; as such, they resemble super-enhancers, clusters of enhancers occupied by master regulators (Whyte et al. 2013). ERCEs also interact strongly with each other, independent of CTCF and cohesin (Sima et al. 2019). The interaction of multiple ERCEs therefore creates a 3D hub rich in histone acetylation. It is thus likely that ERCEs attract the Brd2/4 protein, which binds acetylated histones and has been shown to form phase-separated droplets (Gibson et al. 2019; Borck et al. 2020; Han et al. 2020). Because Brd2/4 has been shown to interact strongly with the replication-initiation protein Treslin (Sansam et al. 2018), Brd2/4 microenvironments would be replete with Treslin and poised for replication initiation as soon as DDK and CDK are activated at the onset of S phase. The assembly of a microenvironment, potentially through phase separation, could explain domain level regulation of replication (Gilbert 2001), as it could then initiate at any site where Mcm would reside throughout the domain. In this way, ERCEs could promote highly deterministic early replication, via stochastic origin specification (Fig. 4).

This model for ERCE function is reminiscent of that proposed for how Fkh1,2 TFs promote early replication in budding yeast (Knott et al. 2012). Fkh1,2 dimerize to mediate 3D clustering of a set of early origins and recruit the essential replication initiation kinase subunit, Dbf4. Interestingly, mutations that impair their ability to dimerize without affecting their transcription activation function result in a delay of early origin firing, suggesting that formation of a 3D hub rather than transcription is the critical replication role of Fkh1,2 (Ostrow et al. 2017). It is possible that Oct4, Sox2, Nanog mediate the 3D interactions and/or high density of histone acetylation of ERCEs (de Wit et al. 2015; Wu et al. 2015, 2018). Because Oct4, Sox2, and Nanog are cell-type-specific TFs, and since the expression of core transcriptional regulatory network factors correlates with cell-type-specific RT changes (Rivera-Mulia et al. 2019a), this predicts that ERCEs will be found to be cell-type-specific regulatory elements of large-scale genome organization and function.

Trans-Acting Regulators of Replication Timing

The search for *trans*-acting regulators of mammalian RT has been nearly as arduous as the search for *cis*-regulators. Chromatin readers, writers, and remodelers, as well as architectural proteins, have been hypothesized to regulate RT, and mutations in some chromatin regulators have strong effects on RT in yeast (Aparicio et al. 2004; Knott et al. 2009; Yoshida et al. 2014; Zhang et al. 2019). However, depletion of homologs to these factors has little to no effect on RT in mammalian cells. Depletion or loss of some gene products, such as ESBAF subunits, PREP1 and DNA polymerase θ (Takebayashi et al. 2013; Fernandez-Vidal et al. 2014; Palmigiano et al. 2018), have been shown to cause partial or localized RT changes. However, there is only one gene to date, Rif1, whose deletion has profound effects on the global RT program in yeast, mouse, humans, *Drosophila*, and Zebrafish (Hayano et al. 2012; Hiraga et al. 2014, 2018; Foti et al. 2016; Seller and O'Farrell 2018). In budding yeast, Rif1 has been shown to recruit protein phosphatase 1 (PP1), which can antagonize phosphorylation of the MCM complex by Dbf4-dependent kinase (DDK, Cdc7/Dbf4 complex), a critical step in origin firing. This PP1-binding domain is conserved and evidence points to a similar mechanism in *Drosophila* and mammalian cells (Sukackaite et al. 2017; Seller and O'Farrell 2018). In mammalian cells, the loss of the PP1 interacting domain only partially accounts for the RT regulatory activity of Rif1, suggesting that other mechanisms are also in play (Gnan et al. 2019). Although Rif1 is believed to act by delaying RT, and Rif1 is predominantly found in late replicating regions, its elimination leads to both delays and advances in RT (Foti et al. 2016; Klein et al. 2019). By analogy to

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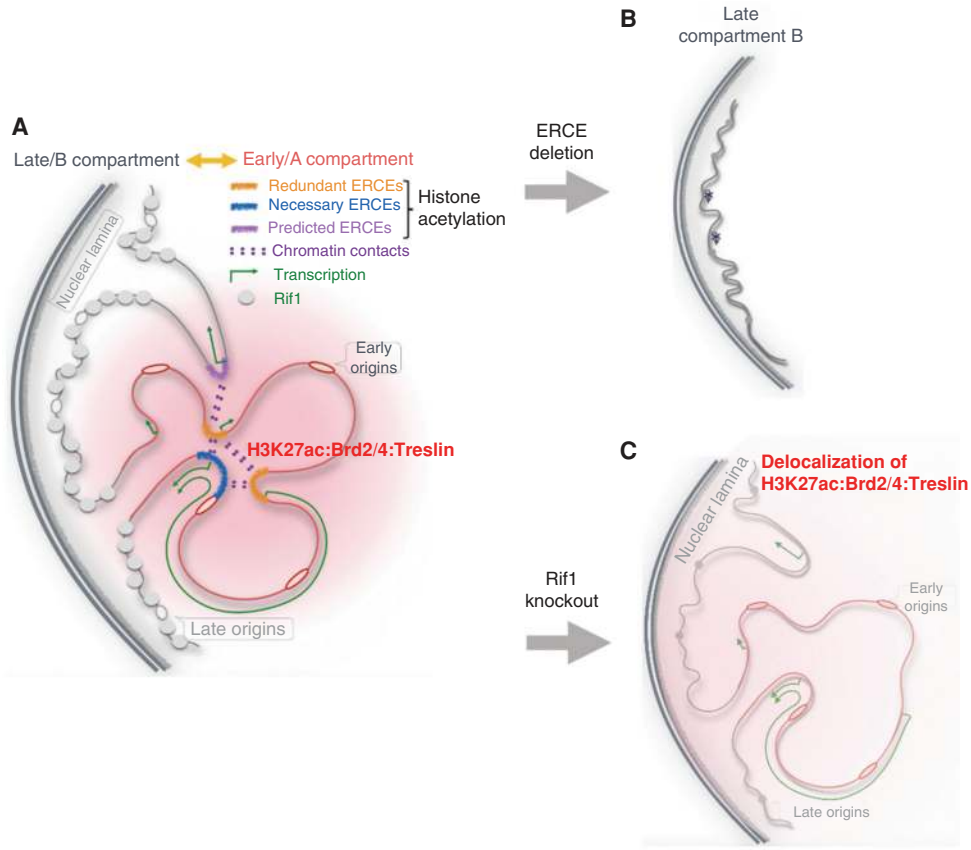

Figure 4. Model of early replication control element (ERCE) interactions in the Dppa2/4 domain. (*A*) ERCEs interact to influence the 3D architecture of topologically associated domains (TADs), the interaction of TADs with other domains (compartmentalization), transcription, and early replication timing (RT) (Sima et al. 2019). ERCEs resemble enhancers, being occupied by H3K27ac, the p300 acetyltransferase, and the major pluripotency transcription factors (TFs) Oct4, Sox2, and Nanog (OSN). In this working model, lineage-specific TFs such as OSN promote histone acetylation, recruiting acetylation readers Brd2/4 and promoting chromatin interactions (Kim 2009; Wu et al. 2015, 2018) to form a 3D hub highly enriched for Brd2/4. The replication initiation protein Treslin interacts with Brd2/4, linking OSN and histone acetylation to initiation of replication (Sansam et al. 2018). Meanwhile, Rif1 coats late replicating regions to prevent them from replicating early during S phase. (*B*) Deletion of all three ERCEs in this domain causes the domain to switch from early to late replicating, eliminates all intradomain transcription, changes compartments (*A* to *B*), and shifts the domain toward the nuclear lamina (NL). (*C*) Rif1 knockout allows for late replicating regions near the NL to become accessible to replication factors, thus diverting replication resources toward these late replicating regions, resulting in highly stochastic RT, redistribution of chromatin marks, and alterations in chromatin compartments (Panels *A* and *B* adapted from Sima et al. 2019 courtesy of Creative Commons Public License.)

studies of the effects of Sir2 on RT in budding yeast (Yoshida et al. 2014), this may be due to competition of the normally late replicating regions, now advanced in their timing, for binding limiting concentrations of replication initiation factors (Mantiero et al. 2011; Tanaka et al. 2011; Collart et al. 2013), thus sequestering them from normally early replicating chromatin, which in turn delays their replication.

The biological significance of RT has remained a puzzle. There is no a priori reason that the genome should be replicated in a par-

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ticular order simply to duplicate the genome. Early replicating genes are at a higher gene dosage per cell than late replicating genes, and there has been some evidence in budding yeast that there is selective pressure for this gene dosage effect mediated by RT (Müller and Nieduszynski 2017). However, the notion that RT could regulate the assembly of different types of chromatin at different times, possibly maintaining or changing the entire epigenomic landscape, has remained a speculative hypothesis (Gilbert 2002; Lande-Diner et al. 2009) because there has been no way to eliminate the temporal order of replication. In the case of Rif1 disruption, until recently, only the $\log_2$(E/L) RT method (Fig. 1A, top) had been applied to study RT, leading to the conclusion that Rif1 disruption led to widespread discrete RT shifts, rather than a loss of timing control. However, high-resolution and single-cell Repli-seq (Fig. 1A, middle) has now revealed that the primary effect of Rif1 loss is to vastly increase cell-to-cell heterogeneity of the affected loci, which become averaged in $\log_2$(E/L) data to appear as discrete shifts. Highly stochastic replication in Rif1 depleted cells provided an opportunity to determine the role of RT in maintaining epigenetic states. A time course using a conditional Rif1-AID degron fusion showed that, following depletion of RIF1, a complete disruption ocurred in the first S phase, followed by the gradual delocalization of multiple histone marks, alteration of genome architecture, and transcription changes that continued to increase for many cell generations, consistent with the gradual alteration of histone marks via the dilution of old histones with each cell cycle (Klein et al. 2019; Stewart-Morgan et al. 2020). Changes in histone marks did not occur after RIF1 depletion until replication initiated (Klein et al. 2019). Intriguingly, despite widespread disruption of the epigenome, three different human cell types null for RIF1 were viable with near normal growth rates. RIF1-null hESCs also retained their pluripotency transcriptional network. This is consistent with the finding that Rif1-null mice do not die until after gastrulation and suggests the intriguing possibility that the epigenome is critical for cell fate transitions but not for self-renewal. Also emerging from this study was the finding that different cell types use different mechanisms to regulate RT to different extents. In hESCs, RIF1 loss led to an almost random replication program genome wide, while in colon cancer cell line HCT116 RIF1, null cells retained RT at large blocks of enriched H3K9me3, and knockdown of the Su(var)39h1/2 writer for this mark further advanced RT of those regions. Thus, the ability to eliminate the RT program genome wide through deletion of RIF1 has provided long-awaited evidence that RT is necessary for maintaining the epigenome and has revealed novel mechanisms of RT regulation (Fig. 4C).

Whole Chromosome Territory Regulation of Replication Timing

Another remarkable finding of the last few years is the discovery of asynchronous replication and autosomal RNAs (ASARs), which are required for the timely replication and mitotic condensation of entire chromosomes. Discovery of ASARs stems from the observation that 80% of cancers harbor one chromosome that enters mitosis with an incompletely replicated and uncondensed chromosome (Fig. 5A; Smith et al. 2001), which is highly unstable resulting in breakages resembling chromothripsis, a mutational phenomenon characterized by a large number of concentrated genomic rearrangements (Donley and Thayer 2013). A systematic series of deletions in human chromosomes 6 and 15 identified ASAR6 and ASAR15, very long noncoding RNAs (vlncRNAs; >200 kb) whose disruption eliminates the coordinated replication of homologous chromosome pairs giving rise to the phenotype of delayed RT (DRT), which in turn delays mitotic chromosome condensation (DMC) (Stoffregen et al. 2011; Donley et al. 2015). ASAR genes are mono-allelically expressed, producing non-spliced Poly A-, very long noncoding RNAs, display asynchronous replication and have a high L1 content. ASAR RNAs coat the chromosome from which they are expressed in *cis*, and their functional activity is mediated by L1 elements that are transcribed in the antisense orientation (Platt et al. 2018). Deletion or inversion

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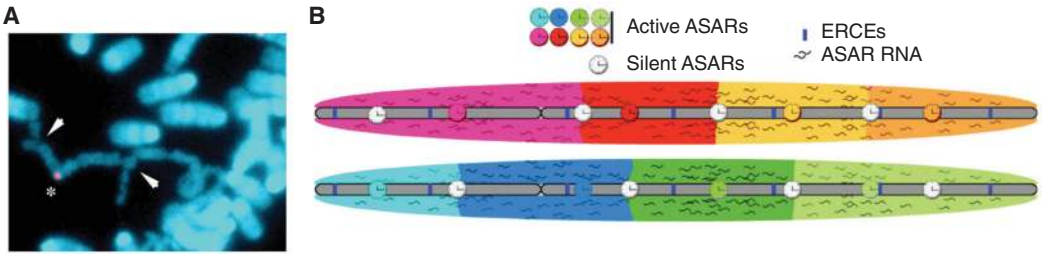

Figure 5. Asynchronous replication and autosomal RNAs (ASARs) are necessary to ensure that whole chromosomes are replicated in a timely fashion. (*A*) Delayed mitotic condensation and spontaneous damage. Mitotic cells containing an uncondensed i(3q), from human rhabdomyosarcoma cells RH30. The i(3q) was identified by FISH with a centromeric probe (red; *). DNA was stained with DAPI (blue), and arrows mark sites of spontaneous damage. (*B*) Monoallelic expression of multiple ASARs from a hypothetical chromosome pair. The expressed (Active; colored clocks) alleles of different ASARs result in "clouds" of RNA that are retained within the chromosome territories from which they are transcribed. ASAR RNA clouds are speculated to regulate early replication control element (ERCE) activity. The nonexpressed ASARs (Silent; white clocks) are inactive. (Panels *A* and *B* courtesy of M. Thayer.)

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of an antisense L1 element within ASAR6 causes a delay in RT of the entire chromosome. Intriguingly, despite L1 elements not being well conserved across species, ectopic insertion of a single L1 element from within ASAR6 into mouse chromosomes was sufficient to affect replication and mitotic condensation of entire chromosomes. Recently, a second mono-allelically expressed and asynchronously replicating vlncRNA, ASAR6-141, was discovered to be expressed from the alternate chromosome 6 homolog, to the homolog expressing ASAR6 (Heskett et al. 2020), suggesting that multiple ASARs in each autosome work together to maintain proper replication control across each chromosome pair (Fig. 5B). There are >2000 vlncRNAs with unknown function (St Laurent et al. 2013, 2016; Caron et al. 2018). The coming years are expected to provide us with critical information on the genome-wide network of ASARs, and how these elements interact with other *cis*-regulatory elements such as ERCEs to control chromosome-wide RT.

CONCLUSIONS AND FUTURE PERSPECTIVES

Replication is a function regulated at the level of large-scale chromatin domains and also is the time at which chromatin is assembled; thus, it provides a unique window and functional readout of large-scale structure–function. In the decade since the last publication of *The Nucleus* (Misteli and Spector 2011), the development of several new molecular, imaging, and computational methods as well as the ability to sequence at much greater depth, have given us a deep molecular description of relationships between RT, chromatin architecture, and subnuclear localization. While their strong correlation suggests a tight interdependence between them, causal mechanistic links remain elusive. It is possible that the architectural state of chromatin and the physical localization of loci within the nucleus dictate a predetermined time range in S phase during which particular loci can replicate, by recruiting or antagonizing key replication initiation factors. Alternatively, replication at a particular time and place could result in the assembly of chromatin that interacts with similar chromatin types and targets to specific subnuclear addresses. These are not mutually exclusive; a familiar theme in epigenetics is the chicken or the egg problem: self-reinforcing feedforward loops of causality.

Overall, it is clear that there is still a need for a comprehensive set of studies that will illuminate the mechanistic link between the organizational state of a locus and the time it replicates during the S phase. Fortunately, robust new experimen-

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tal and computational tools have recently emerged that have already paved the way for mechanistic studies of RT control and its causal relationships to chromosome structure and other functions. Recent work has shown that specific *cis*-acting elements, ERCEs, regulate early replication of loci in mESCs and their characterization, sequence characteristics, and interactors are forthcoming. This regulation appears to be cell-type-specific and soon we expect ERCEs in other cell types to be identified. Also, for the first time researchers were able to show that the global disruption of RT by depletion of a single protein, Rif1, results in widespread disruption of the epigenome, providing long-awaited evidence that the proper timing of chromatin assembly is necessary for epigenome maintenance. We are also on the verge of understanding the enigmatic control of whole chromosome replication that coordinates its completion with proper condensation and segregation during the cell cycle, something that is awry in most cancers. Indeed, six decades after the initial description of replication control in mammalian cells, we are poised for the seventh decade to bring significant progress in understanding the mechanism and biological significance of RT control and its relationship to the overall organization and function of the genome.

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