Review

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Replication timing regulation of eukaryotic replicons: Rif1 as a global regulator of replication timing

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Origins of DNA replication on eukaryotic genomes have been observed to fire during S phase in a coordinated manner. Studies in yeast indicate that origin firing is affected by several factors, including checkpoint regulators and chromatin modifiers. However, it is unclear what the mechanisms orchestrating this coordinated process are. Recent studies have identified factors that regulate the timing of origin activation, including Rif1 which plays crucial roles in the regulation of the replication timing program in yeast as well as in higher eukaryotes. In mammalian cells, Rif1 appears to regulate the structures of replication timing domains through its ability to organize chromatin loop structures. Regulation of chromatin architecture by Rif1 may be linked to other chromosome transactions including recombination, repair, or transcription. This review summarizes recent progress in the effort to elucidate the regulatory mechanisms of replication timing of eukaryotic replicons.

Origins of DNA replication

Marking and activation

DNA replication is initiated at defined loci known as replication origins. In prokaryotic replicons, replication is initiated from a single locus in most cases, and the sequence specificity of origin activation is very high; generally one base substitution within an origin leads to loss of initiation [1]. By contrast, replication initiates at multiple loci on eukaryotic genomes [2]. Although initiation occurs within specific loci on each genome at specific times during S phase, the sequence specificity can be significantly relaxed compared to bacteria. It appears that cells prepare many potential origins for possible uses during S phase, but only a subset of these are utilized during the normal course of S phase. Other origins may be used at later stages of S phase or may not be used at all (dormant origins [3]).

Preparation for DNA replication starts as early as late M or early G1 with assembly of pre-RCs (pre-replicative complexes) at selected locations on chromosomes. This step, also called origin licensing, proceeds through the stepwise assembly of Orc, Cdc6, and Cdt1–Mcm, resulting in the loading of Mcm onto the chromatin (helicase loading). The selected pre-RCs are activated by the actions of Cdc7 kinase and Cdk when cells enter S phase [2,4]. Once in S phase, origin licensing is strictly inhibited by layers of mechanisms that prevent rereplication [5]. These mechanisms are largely conserved from yeasts to human.

Regulation of origin firing during S phase

Once S phase is initiated, origins are fired (i.e., pre-RCs are activated by a series of phosphorylation events to generate active replication forks) in a coordinated and regulated manner, until the entire genome is replicated. There are origins every 50–150 kb; about 300 in budding yeast, about 1100 in fission yeast, and more than 20000 in human (Figure 1). Yeasts (budding and fission yeasts) have served as excellent model organisms for the study of regulation of origin firing due to their small genome sizes and ease of genetic manipulation. Thus, the precise locations of all the origins and the order in which they are fired have been established (Figure 1). In metazoans, firing of origins appears to be regulated on a domain basis [6,7] – that is, clusters of nearby origins present in the same domain may be spatially and temporally coregulated [8,9]. Recent genomics studies demonstrated the presence of cell type-specific 'replication domains' that define the segments of the chromosomes containing the coregulated origins (ranging in size from several hundred kb to 1 Mb) [10]. How these replication domains are generated and regulated in different cell types remains an open question [10,11]. Rif1, originally identified in budding yeast as a Rap1-interacting factor1 involved in telomere length regulation [12], has recently come into the spotlight because of the unexpected discovery of its participation in origin regulation.

Here, we summarize various factors and conditions that regulate the origin firing/replication timing program in various eukaryotes (Table 1; for factor nomenclature in different species see Table S1 in the supplementary material online; also Box 1). We then discuss Rif1, which may be a global regulator of replication timing domains in metazoans.

Regulation of the origin firing/replication timing program

Possible mechanisms

For simplicity, let us assume that there are two classes of replication origins; early- and late-firing. There are two

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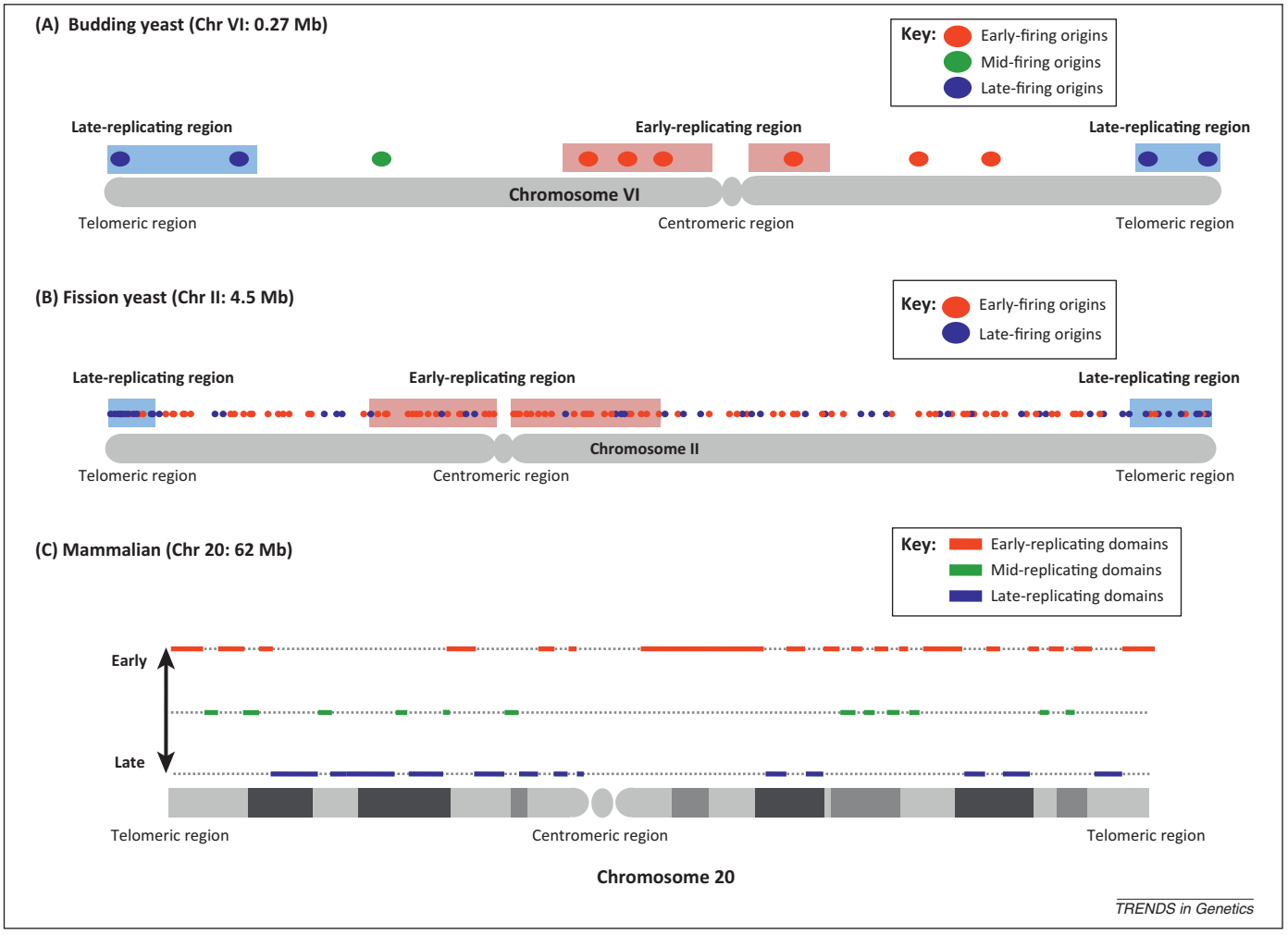

Figure 1. Replication programs of budding yeast, fission yeast, and human chromosomes. **(A)** The locations of replication origins on chromosome (Chr) VI (0.27 Mb) of budding yeast. Red, early-firing origins; green, mid-firing origins; blue, late-firing origins [80]. **(B)** The locations of replication origins on the chromosome II (4.5 Mb) of fission yeast. Red early-firing origins (origins firing in the presence of HU); blue late-firing or dormant origins [53]. **(C)** Replication timing profile of the human chromosome 20 (62 Mb) in K562 cells. Early-, mid-, and late-replicating domains are deduced from data in [11] and are shown by red, green, and blue horizontal bars, respectively. For simplicity, subtle timing differences within the early or late domains are not reflected in this drawing.

possible mechanisms by which the cell could distinguish between early- and late-firing origins. The first is to mark the early-firing origins. The chromatin structures in which the origins are embedded could dictate early-firing. Another way to mark early-firing origins could be through covalent modification of the pre-RC components or another factor(s) that selectively associates with specific origins before initiation (Figure 2A). The late origins might be differentially marked or marked later in S phase for initiation. The second possibility is to assume that all the origins are ready to fire at the onset of the S phase (the default state), but late origins are somehow actively prevented from firing by being sequestered from interacting with replication initiation factors (Figure 2B). At later S phase, this constraint is released and late origins are fired. Combinations of these two mechanisms are also certainly possible, and data supporting both mechanisms have recently been reported.

Temporal and spatial consideration

The replication timing program is established at a discrete point during the early G1 phase [13]. At this point, termed the TDP (timing decision point), major chromatin repositioning takes place, relocating chromatin to its respective subnuclear positions [14].

In mammals, it is well known that chromatin in the nuclear interior is replicated in early S phase whereas chromatin at the nuclear periphery is preferentially replicated during late S phase. For example, the late-replicating inactive X chromosome allele is associated with the nuclear periphery whereas the early-replicating active chromosome is located in the interior of the nuclei [15]. Thus, the spatial arrangement of chromosomes may play an important role in the origin firing program. In budding yeast, forced cell cycle-specific dissociation of telomeres from the origins demonstrated that the decision for late activation is made between mitosis and START (corresponding to the restriction point in mammalian cells) in the subsequent G1 phase. It is also interesting to note that, once established, late origin activation can be enforced even if telomeres are released from the target origin [16,17]. Furthermore, late origins associate with the nuclear envelope during G1 phase whereas early origins are randomly localized within the nucleus throughout the cell

Table 1. Factors affecting replication timing

Organism	Protein(s)	Experimental method	Function	Replication timing phenotype	Refs
Budding yeast	**Rad53**	Release from α-factor into HU (early S-phase)	Checkpoint	Strong firing of late/dormant origins in the presence of HU	[21–26,28]
	Mec1	Release from α-factor into HU (early S-phase)	Checkpoint	Strong firing of late/dormant origins in the presence of HU	[22,23,25,28]
	Rad9	Release from α-factor into HU (early S-phase)	DNA damage checkpoint	Very weak firing of late/dormant origins in the presence of HU	[28]
	Rad24	Release from α-factor into HU (early S-phase)	RFC^{Rad24} Clamp loader	Weak firing of late/dormant origins in the presence of HU	[28]
	Elg1	Release from α-factor into HU (early S-phase)	RFC^{Elg1} Clamp loader	Weak firing of late/dormant origins in the presence of HU	[28]
	Tof1	Release from α-factor into HU (early S-phase)	Replication fork protection	Weak firing of late/dormant origins in the presence of HU	[28]
	Mrc1	Release from α-factor into HU (early S-phase): FACS and microarray analyses	Replication fork protection, checkpoint	Strong firing of late/dormant origins in the presence of HU	[28,81]
	Ku70/80	Release from α-factor or release from a cdc7ts block	Non-homologous end joining	Repression of telomeric or subtelomeric origins firing	[54,55]
	Orc2	Release from α-factor into MMS (early S-phase)	DNA replication	Firing of late/dormant origins in the presence of HU	[21]
	Ctf18	Release from α-factor into HU (early S-phase)	RFC^{Ctf18} Clamp loader	Strong firing of late/dormant origins in the presence of HU	[28]
	Ctf8	Release from α-factor into HU (early S-phase)	RFC^{Ctf18} Clamp loader	Strong firing of late/dormant origins in the presence of HU	[28]
	Dcc1	Release from α-factor into HU (early S-phase)	RFC^{Ctf18} Clamp loader	Strong firing of late/dormant origins in the presence of HU	[28]
	Ctf4	Release from α-factor into HU (early S-phase)	Cohesion of sister chromatids	Weak firing of late/dormant origins in the presence of HU	[28]
	Eco1	Release from α-factor into HU (early S-phase)	Cohesion of sister chromatids	Weak firing of late/dormant origins in the presence of HU	[28]
	Rpd3 complexes	Release from α-factor into +/– HU (early S-phase)	Histone deacetylase	Strong firing of late/dormant origins in the presence of HU	[49–51,61]
	Sir proteins	Release from α-factor	Histone deacetylase	Regulation of telomeric or subtelomeric origin firing	[48,54,82,83]
	Rif1	Release from α-factor or release from a cdc7ts block	Telomere length regulation	Firing of telomeric or subtelomeric origins in the presence of HU	[55]
	Fkh1/2	Release from α-factor into HU (early S-phase)	Transcription factor	Global change of origin activation profiles	[38]
	Clb5	Release from α-factor	Cell cycle regulation	Absence of late origin firing leading to elongated S phase	[84,85]
Fission yeast	**Rad3**	HU synchronization (early S-phase)	DNA repair	Firing of late origins or subtelomeric origins in presence of HU	[86]
	Cds1	HU synchronization (early S-phase)	DNA repair	Firing of late origins or subtelomeric origins in presence of HU	[24,70]
	Mrc1	HU synchronization (early S-phase)	DNA repair	Firing of late origins or subtelomeric origins in presence of HU	[58,70]
	Swi6	HU synchronization (early S-phase)	Maintenance of heterochromatin structure	Regulation of peri-centromeric or silent mating type heterochromatin origin firing	[52]
	Taz1	HU synchronization (early S-phase)	Telomere length regulation	Firing of subtelomeric origins and some origins in the arm regions	[56]
	Rif1	HU synchronization (early S-phase)	Telomere length regulation	Global change of origin activation profiles	[64]
Drosophila	**HP1**	FACS and microarray analyses: siRNA Knockdown	Maintenance of heterochromatin structure	Change of replication timing at centromeric repeats regions and euchromatic regions containing repeat sequences	[87]

Table 1 (*Continued*)

Organism	Protein(s)	Experimental method	Function	Replication timing phenotype	Refs
Mouse	Chk1	FACS and qPCR analyses: conditional knockout MEF cells	Checkpoint	Firing of late origins	[88]
	MII	FACS and qPCR analyses: knockout ES cells	Histone methyl transferase	Modest change of replication timing at specific regions	[89]
	G9a	FACS and qPCR/ microarray analyses: knockout ES cells	Histone methyl transferase	Modest change of replication timing at specific regions	[89,90]
	Suv39h1/h2	FACS and qPCR analyses: knockout ES cells	Histone methyl transferase	Modest change of replication timing at specific regions	[89]
	Dnmt1	FACS and qPCR analyses: knockout ES cells	DNA methyl transferase	Modest change of replication timing at specific regions	[89]
	Dicer	FACS and qPCR analyses: knockout ES cells	RNA degrading enzyme	Modest change of replication timing at specific regions; delayed replication at rDNA	[89]
	Eed	FACS and qPCR analyses: knockout ES cells	Subunit of PRC2	Modest change of replication timing at specific regions; delayed replication at rDNA	[89]
	mRif1	FACS and microarray analyses: knockout MEF cells	Regulation of repair, chromatin architecture	Global change of replication timing	[69]
Human	hRif1	FACS and microarray analyses: siRNA knockdown HeLa cells	Regulation of repair, chromatin architecture	Global change of replication timing	[68]
	SATB1	FISH: shRNA knockdown Jurkat cells	Transcription factor	*phenotype of overexpression: slow down of replication fork at TTR by SATB1	[91]
	Bold	Genome-wide analyses			

cycle [17]. However, the artificial tethering of an early replicating origin to the nuclear membrane in budding yeast did not cause late replication of this origin [18], suggesting that the association with the nuclear periphery alone is not sufficient for enforcing late replication at an early-firing origin.

Factors involved in regulating the origin firing program

Checkpoint signaling

In yeasts, treatment of cells with hydroxyurea (HU) or methyl methanesulfonate (MMS) delays or blocks initiation of late-firing origins [19,20]. However, they are precociously activated in the checkpoint mutants *rad53* and *mec1* [21–26], indicating that the origin firing program is regulated by the checkpoint pathway. Tof1–Csm3–Mrc1 are conserved factors that play important roles in mediating the stalled fork signal to downstream checkpoint machinery [27]. In *mrc1*Δ, late or dormant origins are activated in the presence of HU or in early S phase both in budding and fission yeasts. Testing other checkpoint mutants in budding yeast for their ability to suppress late origin firing showed that the RFC^{ctf18} clamp loader [a complex consisting of replication factor C (RFC), Ctf18, Ctf8, and Dcc1] plays an important role in replication stress-induced repression of late origin firing [28]. This complex is essential for mediating replication stress-induced checkpoint signaling, leading to Rad53 kinase activation. Suppression of dormant or late-firing origins by the checkpoint pathway was also reported in human cells [29].

Transcription and transcription factors

Although not obvious in yeast [30], a correlation between active transcription and early replication has been shown in other organisms including *Drosophila* and mammals [31–34]. DNA combing analyses of replicated molecules at the mammalian IgH locus showed that replication origin activation correlated with changes in the chromatin structure and transcriptional activity at different stages of B cell differentiation [35]. Activation of Pax5, a crucial B cell-specific transcription factor, increased the rate of origin firing on the segment containing Pax5 binding sites, showing that transcription factors can affect the temporal order of replication [36]. It appears that open chromatin or a potential for transcription activity, rather than active transcription *per se*, correlates with replication in early S phase. However, more recently, high-resolution mapping of replication initiation sites in human cells revealed that replication initiation events are associated with moderately transcribed regions and are depleted in highly transcribed regions. Moreover, they do not colocalize with transcription start sites but are enriched in the adjacent downstream sequences and showed strong correlation with methylated CpG [37].

Recently, the Forkhead transcription factors, Fkh1 and Fkh2, were shown to regulate the origin firing-time of many replication origins in budding yeast by binding near a subset of early-firing origins and facilitating the association of Cdc45 with these origins in G1 phase, possibly by promoting origin clustering. Fkh1/2 binding sites are enriched near Fkh-activated origins and depleted near Fkh-repressed origins. Fkh1/2 interacts with Orc and potentially tether together Fkh-activated origins in *trans*, which may facilitate the early firing of these origins by increasing the effective concentration of replication factors [38]. These functions of Fkh1/2 are independent of their

Box 1. Origin firing timing and replication timing

Origin firing timing and replication timing are not identical. One can determine the firing timing of a given origin in a given cell, which can be at a specific time during S phase. Replication timing at a particular genome position could be similar to the firing time of the nearest origin, but could be very different if the nearest origins are far away. Moreover, firing efficiency of an origin can be very low, and a given origin may be fired in only a small subset of the cells during a given S phase. Thus, the average timing of DNA replication (usually replicated passively from origins further away) can be very different from the time of origin firing. However, firing occurs more or less simultaneously in the clusters of the neighboring origins. Thus, even though firing efficiency of each origin is low, the segments harboring early-firing origins are most likely to be replicated early and those harboring late-firing origins late. Therefore, in this article, origin firing program and replication timing program are generally used interchangeably unless specified otherwise. However, special note should be made on the timing transition region (TTR) that separates two distinct timing zones. The TTR, identified more notably on the chromosomes of higher eukaryotes, is generally composed of a long stretch of chromosome with suppressed origins that is replicated unidirectionally [92,93]. The replication timing of the TTR can obviously be different from the firing timing of the even nearest origin. It should be noted that a recent study has suggested that some TTRs may be composed of multiple active origins that are sequentially activated [94].

transcriptional activity. The roles of Fkh1/2 in replication timing regulation is discussed in detail in a recent review [39]. Although it is not known whether similar mechanisms operate in other species, transcription factors have been implicated in origin activation and selection in mammals [40,41]. The EBNA1 transcription factor was shown to facilitate the recruitment of Orc during replication initiation at *oriP*, the origin of replication of Epstein–Barr Virus [42].

Chromatin structure

Histone modifiers play crucial roles in the regulation of the origin firing program. For example, Sir3, which interacts with the histone deacetylase Sir2, was shown to delay or suppress firing of subtelomeric origins in budding yeast [43]. Sir2 suppresses pre-RC formation through a specific sequence present near a subset of origins by generating chromatin structures unfavorable for pre-RC assembly [44]. In human cells, HBO1, a histone acetylase that binds to Cdt1 [45], was shown to promote pre-RC assembly through acetylation of histone H4, which is inhibited by Geminin, a repressor of rereplication [46]. It was also reported that the artificial tethering of HAT1, the *Drosophila* homolog of HBO1, to an origin presumably leads to efficient pre-RC formation, which could in turn cause early firing at this origin in S phase [47]. The bromo-adjacent homology domain of yeast Orc1 (Orc1BAH) was shown to be required for efficient initiation at origins associated with a distinct nucleosome structure, suggesting that interaction of Orc with nucleosomes can play significant roles in origin selection [48].

The histone H3 deacetylase Rpd3 is arguably the most important histone modifier with regard to origin firing. It exerts a profound effect on genome-wide origin firing profiles in budding yeast in a manner independent of checkpoint signaling [49–51]. Correlations between regional transcriptional activation and origin deregulation suggested that hyper-acetylation near origins caused by the lack of Rpd3 facilitates both transcription and the origin activation process potentially through induction of open chromatin structures. It should be noted that chromatin structures can affect the efficiency of both the formation and activation of pre-RC. Although efficient pre-RC formation can lead to early-firing events, mechanistically how

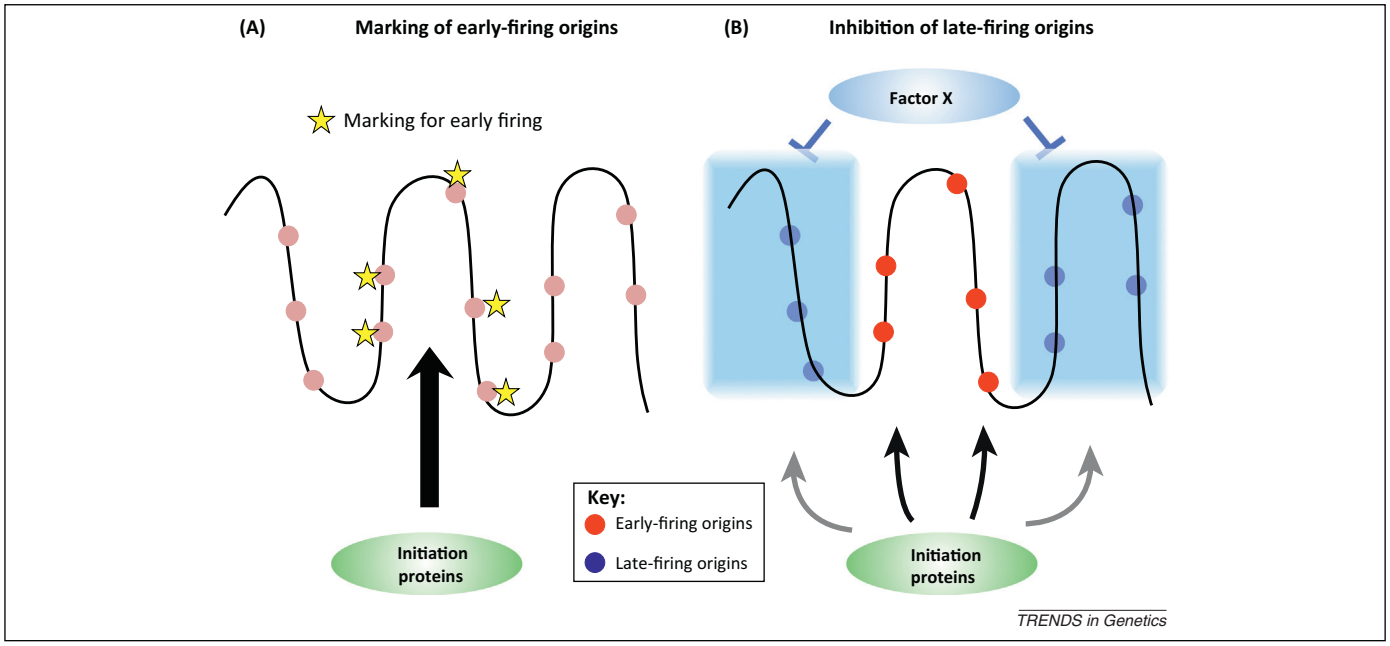

Figure 2. Possible mechanisms for determination of origin firing timing. **(A)** Early-firing origins are molecularly marked. Marking can be chromatin structures favorable for firing, covalent modification of pre-RC components, or via the association of other factor(s) that facilitate recruitment of initiation proteins or via clustering through a pre-RC component and a transcription factor [38]. **(B)** A hypothetical Factor X inhibits firing of late origins by preventing the recruitment of initiation factors (shown by a half-transparent blue cloud). As a result, only the early-firing origins can get access to initiation factors during early S phase (black arrows).

this is achieved is unknown. Therefore, we focus on replication timing regulation that acts at the stage of the pre-RC activation (firing step).

Replication timing of chromosomes with special chromatin structures may be regulated by distinct mechanisms. The fission yeast centromere segments are replicated early despite their heterochromatic character (Figure 1). This is made possible by Swi6/HP1 which recruits Hsk1 (the Cdc7 homolog) [52]. Telomere/subtelomere segments are replicated very late in S phase even though they contain clusters of pre-RCs [53] (Figure 1). Transfer of an early-firing origin on the arm region to the subtelomere region renders it late-firing. Special chromatin structures in subtelomere regions may be responsible for the suppression of initiation on these regions because disruption of telomere binding factors, known to be required for generation of telomere-specific chromatin structures, deregulates replication and causes theses regions to replicate early [54–56]. It was also reported that telomere shortening by the yKu mutation stimulates the firing in the subtelomere segment up to ~40 kb from the end [57]. These results indicate that chromatin structure is a major regulatory element for origin firing.

Other factors

It was reported that weak early-firing origins are stimulated in the fission yeast *mrc1*Δ, but not in a *mrc1* mutant specifically deficient in checkpoint responses, suggesting that Mrc1 also regulates early origin firing in a checkpoint-independent manner, although its precise mechanism is unknown. Mrc1 selectively binds to early-firing origins at the pre-firing stage at the onset of S phase, leading to a proposal that Mrc1 may mark the early-firing origins [58].

A simpler model was also proposed. In fission yeast, early recruitment of Orc to chromatin during the preceding M phase facilitates pre-RC formation in G1, as well as early firing of these origins in S phase, presumably through increased recruitment of initiation factors [59]. Extension of M phase resulted in more uniform levels of initiation at 'early' and 'late' origins, leading the authors to conclude that differential recruitment of Orc during M phase determines the timing and efficiency of origin firing. However, it is possible that the altered chromatin structures caused during the extended M phase are responsible for the change in origin activation.

In both budding and fission yeasts, late-firing or dormant origins can be precociously activated by overexpression of some replication factors, including Cdc7–Dbf4, Sld3, Sld7, and Cdc45, which are limiting in numbers and appear to be preferentially utilized by 'early' origins [60,61]. It is not known whether this is the case in higher eukaryotes.

In summary, although several factors regulate the origin firing program, local chromatin structures are likely to be one of the most crucial elements. In yeasts, preferential recruitment to the early-firing origins of the limiting factors could determine the origin firing pattern. This could be facilitated not only by preferred chromatin structures but also by origin tethering mediated by a factor such as Fkh, or by origin marking with some early origin-specific binding factor such as Mrc1. Late origins may be available for firing at early S phase but are fired only after early origins are fired and limiting factors become available. By contrast another model, which is not mutually exclusive, hypothesizes that the late origins are actively suppressed by some unknown mechanism. In the following sections we discuss a novel factor that might participate in this latter mechanism.

Rif1 as a novel regulator of genome-wide replication timing

Fission yeast cells

Understanding the molecular basis for the replication origin firing program requires the identification of the factors that determine the genome-wide pattern of early- and late-firing of origins. In budding yeast, the Cdc7 kinase is a crucial regulator of origin firing and may determine which potential origins are activated. In the absence of Cdc7, firing efficiency is too low to support the completion of S phase under normal growth conditions. Similarly, *hsk1*Δ fission yeast exhibit a delayed S phase and eventually die [62,63]. Systematic screening for mutations that permit growth of *hsk1*Δ cells led to the identification of Rif1. *rif1*Δ efficiently restores growth of *hsk1*Δ, and this bypass appears to be distinct from that of checkpoint mutants (Box 2) [64].

Box 2. Cdc7 kinase and its bypass mutations

Cdc7, originally identified in budding yeast in the Hartwell collection, encodes a serine-threonine kinase crucial for initiation of S phase. Cdc7 was later shown to form a complex with Dbf4, which was identified in an independent screen for mutants defective in S phase initiation. Formation of this complex activates Cdc7. Cdc7 then phosphorylates Mcm in the pre-RC on the chromatin, and this phosphorylation stimulates interaction of Cdc45 with the pre-RC [95,96], facilitating the generation of an active helicase complex [CMG (Cdc45–Mcm–GINS) helicase] [97]. The haploid budding yeast *cdc7*(ts) mutant cells arrest with 1C DNA content (representing the cell cycle stage before DNA replication) at the non-permissive temperature and can be released into the cell cycle upon return to the permissive temperature. In fission yeast, *hsk1*(ts) at the non-permissive temperature temporarily arrests with 1C DNA, later starts to synthesize DNA, arrests in S phase, and eventually undergoes cell death [63]. In embryonic stem cells, Cdc7 knockout results in S phase arrest, G2–M checkpoint induction, and eventually in p53-dependent cell death [98]. Thus, Cdc7 is generally essential for DNA replication and for growth under normal conditions.

The first bypass mutation of *cdc7*Δ, *bob1*, was isolated in budding yeast and was identified as *mcm5* P83L. This mutation compensates for loss of Cdc7 by modifying the structure of the Mcm complex such that it can initiate replication in the absence of Cdc7-mediated phosphorylation [99]. More recently, deletions within the N-terminal segments of Mcm4 were found to bypass *cdc7* [100]. In fission yeast, the checkpoint mutants *mrc1*Δ and *cds1*Δ were found to bypass *hsk1*Δ. In *mrc1*Δ and *cds1*Δ, late or dormant origins were activated early, presumably due to loss of inhibitory checkpoint signals preventing replication [58,70]. Thus, increased initiation potential in checkpoint mutants appears to reduce the requirement for Cdc7 in initiation. Notably, growth at a higher temperature (37 °C) also restores the growth of *hsk1*Δ cells, which do not grow at 30 °C or below. Firing of some dormant origins was observed in the wild type cells at 37 °C, although the precise mechanisms of this deregulation of origin firing at a higher temperature are not known [70]. Random screening of bypass mutants of *hsk1*Δ yielded *rif1*Δ, which restores the growth of *hsk1*Δ most efficiently [64]. The efficiency of bypass appears to correlate with the extent of late/dormant origin firing in the presence of HU. Indeed, about twofold more origins are activated in *rif1*Δ than in checkpoint mutants.

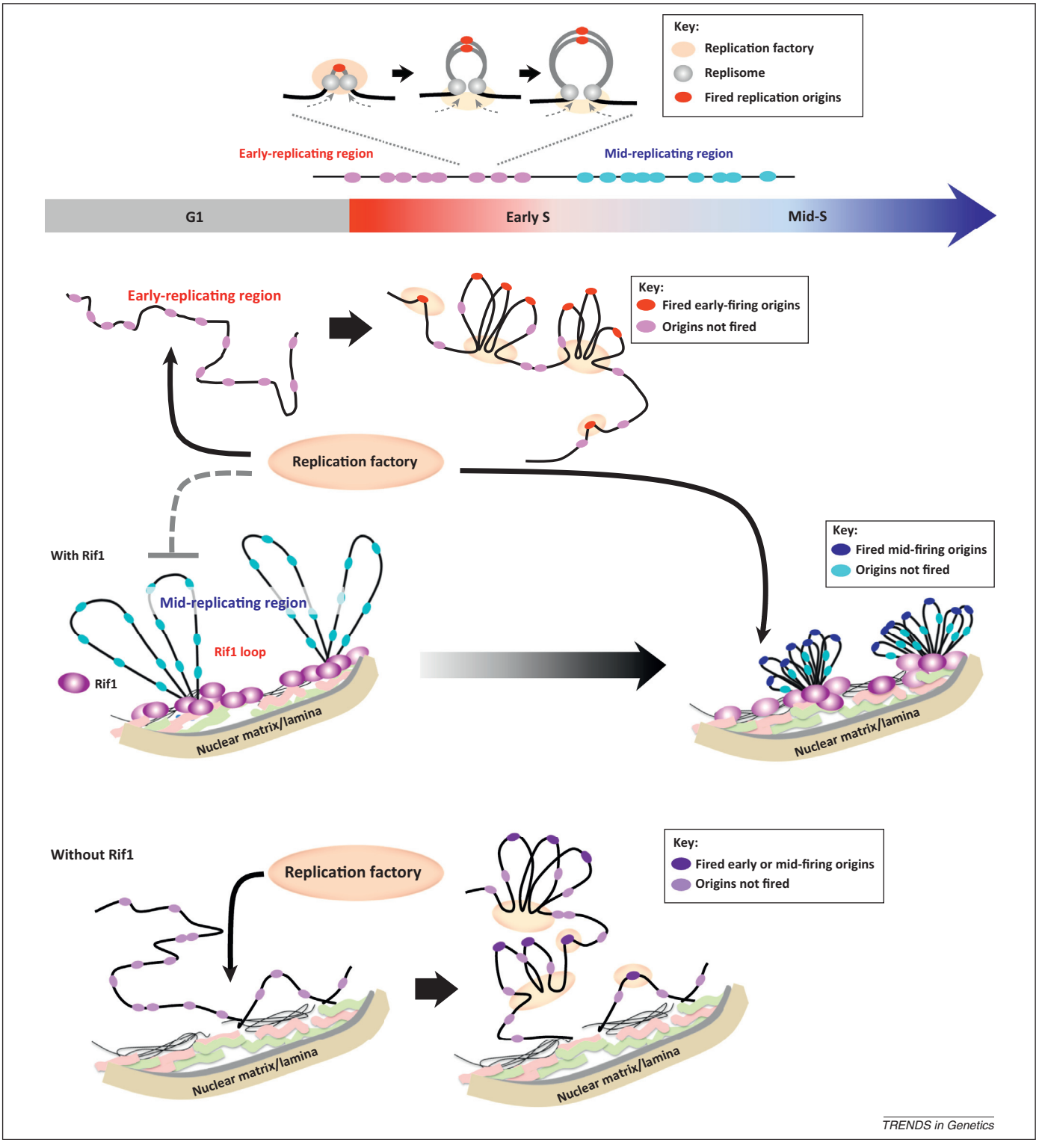

Figure 3. A model for regulation of replication timing domains in higher eukaryotes. Replication occurs at factories where two replisomes are held together and replicating DNA strands are passed through as bidirectional DNA synthesis proceeds, generating a loop consisting of the replicated daughter molecules (replication loops; shown in gray above the cell cycle bar) [101]. In the early-replicating domains (upper), chromosomes, whose conformations are not constrained during G1, can associate with replication factories where the clusters of early origins are simultaneously replicated. In the mid-replicating domains (middle), Rif1 generates specific chromatin loop structures (which we call 'Rif1-loops' to distinguish them from replication loops) in G1, and origins present in the Rif1-loops are sequestered and kept inactive until mid-S phase. Rif1 associates with insoluble nuclear structures which could hold together multiple Rif1-loops. At mid-S phase, the origins in the Rif1-loop are activated through association with the axis of the Rif1-loop, generating an active replication factory. Multiple origins could be simultaneously activated within the Rif1-loop, generating multiple smaller replicating loops. Again, the selection of origins to be activated could be dynamic and stochastic, and thus the sizes and numbers of replication loops generated from one Rif1-loop may vary from one cell to another and from one cell cycle to the next. How the origins in the Rif1-loops are kept from activation in early S phase and how they become activated after mid-S phase are unknown. We also do not know if any factors are responsible for replication loop formation during early S phase and if any factors sequester the late-replicating domains, which are not described here but show a distinct spatial distribution. In the absence of Rif1 (lower), the early S phase domains are intact but the Rif1-loops are disrupted, releasing mid-S phase origins from sequestration. Thus, the majority of the chromosomes (except for the late-replicating heterochromatin segments) are replicated in the early S phase pattern throughout the S phase except for very late

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Rif1 was originally discovered as a telomere-binding factor in budding yeast and is involved in telomere length regulation in both budding and fission yeasts. It binds to subtelomeric regions and suppresses their replication. In *rif1*Δ fission yeast, not only the subtelomeric regions but also many late or dormant origins on the arms of the chromosome are vigorously fired in the presence of HU (Figure 1). Twice as many origins are activated in *rif1*Δ than in checkpoint mutants, including 90% of those activated in the latter. More interestingly, more than 100 early-firing origins are downregulated or their firing timing is delayed in *rif1*Δ cells, suggesting that Rif1 negatively regulates late-firing origins and positively regulates early-firing origins. The combination of these opposing effects results in replication timing that occurs most frequently in mid-S phase in *rif1*Δ. It is possible, however, that extensive deregulation of late-firing/dormant origins in *rif1*Δ causes titration of limiting initiation factors, resulting in reduced firing of origins normally fired early. Regardless, these results indicate that Rif1 plays a global role in regulating the origin firing program in fission yeast [64].

Rif1 binding sites identified by ChIP-chip analyses do not generally overlap with the known origins in yeast. However, Rif1 tends to bind at sites closer to the upregulated origins than to downregulated origins. Analyses of sequences of the binding sites near the upregulated origins revealed the presence of a conserved sequence, although its functional significance is not clear. Rif1 deletion does not affect the pre-RC formation but the loading of Cdc45 onto the pre-RC is either stimulated or decreased at the upregulated or downregulated origins, respectively [64].

The telomere-binding protein Taz1 (a homolog of human TRF1/2) is also involved in the origin activation program in fission yeast [56]. Deregulation of origin firing was observed at telomeres as well as some of the non-telomeric late origins in the arm of the chromosomes in *taz1*Δ. Sequences similar to Taz1 binding sites were identified near the suppressed origins, and these sequences were shown to be required for suppression. Taz1 may enforce late origin firing through binding to these telomere-like repeats present near the affected origins [56]. Unlike *rif1*Δ, suppression of early-firing origins was not observed in *taz1*Δ. All the Taz1-regulated origins are also regulated by Rif1, suggesting that suppression of late origins by Taz1 involves the function of Rif1.

Mammalian cells

In mammalian cells, clusters of origins [65], constituting 'replication (timing) domains', are replicated at specific timings within S phase. Replication domains were initially observed as adjacent chromosome segments that incorporated [^{3}H]-thymidine asynchronously during the S phase. Recent genome-wide studies revealed the presence of distinct replication timing domains that presumably contain sets of temporally coregulated origins, and their distribution pattern on the entire chromosomes has been clarified in many cell types [66] (Figure 1).

It has long been known that replication occurs at specific locations within nuclei [67]. These foci, detectable by incorporated nucleotide analogs, adopt characteristic patterns at distinct stages of S phase. Most notably, foci are detected at nuclear and nucleoli periphery at the mid-S phase stage, whereas small foci are scattered uniformly over nuclei during early S phase [65]. These foci may correspond to each replication domain replicating at a distinct time within S phase. Recent studies demonstrated that replication timing profiles change during development [10]. During the differentiation of ES cells into neural stem cells, changes in replication timing occur in 20% of the genome, often resulting in a shift of the boundary or consolidation of replication domains into a large coordinatedly regulated regions [10]. Similarly, the genome-wide patterns of replication domains change significantly in different cell types [11].

Mammalian Rif1 does not play a role in telomere maintenance but it is required for establishment of replication timing domains in mammalian cells [68,69]. The most striking effect of Rif1 depletion in mammalian cells is the loss of the mid-S phase-specific pattern of replication foci. In Rif1-depleted cells, an early S phase-like pattern of foci prevails throughout S phase, and the late S phase pattern, characterized by replication foci at the heterochromatin segments, appears at the end of S phase. Replication timing profiles also undergo dramatic changes. Genome-wide analyses in Rif1 knockout mouse MEF cells indicated that both early-to-late and late-to-early changes in replication timing occurred in over 40% of the replication segments, resulting in fragmentation of replication timing domains [69]. Knockdown of Rif1 in human cancer cells led to marked alteration of replication domain profiles in the 42 Mb segment of chromosome 5. These changes are complex, including late to early and early-to-mid conversion, and consolidation of domains (fusion of smaller domains) [68]. Generally, replication timing, on average, is distributed toward mid-S phase in the absence of Rif1, suggesting that the replication timing regulation is generally lost, consistent with the analyses of *rif1*Δ fission yeast cells. During early S phase, Cdc7-mediated phosphorylation and chromatin loading of replication factors are enhanced in Rif1-depleted cells, most likely pointing to the fact that more pre-RCs gain access to replication initiation factors and are fired, albeit during a limited time-window. Rif1 is localized in nuclease-insoluble structures within nuclei and maintains chromatin loops and facilitates nucleosome formation. Rif1 localization overlaps with mid-S replication foci, suggesting that Rif1 generates nuclear structures that are specifically required for establishing mid-S replication domains (Figure 3). Chromatin loop sizes increase in Rif1-depleted cells, indicating that Rif1 is required for correct chromatin loop formation. Chromatin loop sizes may be related to the sizes of the replication timing domains because chromatin loops can generate chromatin domains that contain synchronously firing origins and are insulated from the neighboring segment. Rif1

S phase. The replication loops in the main part of the figure are shown by single lines, even though they comprise two daughter molecules; both replicated and unreplicated DNA strands are shown in black. Below the cell cycle bar, darker-colored circles show fired origins whereas lighter-colored circles show origins not fired. Origins in early-replicating domains, in mid-replicating domains, and in disrupted replication domains (due to loss of Rif1) are shown in red, blue, and purple, respectively.

is highly expressed in undifferentiated ES cells, and this may be related to the smaller replication domain sizes observed in these cells. In differentiated cells, Rif1 levels are much lower, whereas replication domain sizes are larger [10].

During the mitosis, Rif1 dissociates from chromatin and re-associates with it at late M/early G1 in a manner that is resistant to nuclease treatment. Thus, Rif1 must generate mid-S replication domain structures by early G1. The TDP was reported to occur during early G1 concomitant with chromatin repositioning [13,14]. It is an intriguing possibility that Rif1 may play a role at the TDP in establishing replication timing domains.

Based on the current evidence, we propose a model for Rif1-dependent regulation of replication timing domains (Figure 3). Rif1 sequesters some replication domains during G1 phase in nuclear insoluble structures, forming 'Rif1-loops' that will be prevented from firing until early S phase replicating domains, which are recruited to replication factories, have fired and replicated. The choice of which origins are brought to the factories may be made stochastically, although origins in 'open chromatin' structures may have a higher probability of being recruited and firing. In the absence of Rif1, the Rif1-loops are not generated and the mid-S phase replication domains are released from constraint and fired prematurely at early S phase. Under these conditions, more origins would compete for limiting replication factors, and some of the origins normally firing early may be outcompeted by more 'active' origins, resulting in delayed or reduced firing, although more positive roles of Rif1 in generating early S replication domains cannot be ruled out.

Concluding remarks

Precise and complete replication of the entire genome is crucial for cell growth and survival. Many different measures are taken to ensure that replication occurs once and only once. After entering S phase, long chromosomes need to be replicated in an ordered manner. Regulation of the timing of replication and the nuclear localization of chromosome are both important for efficient and coordinated replication of the entire chromosome.

In contrast to prokaryotic genomes, which typically carry only a single, very efficient origin, eukaryotic chromosomes carry many potential origins that are typically less efficient. In addition, any given origin is utilized only in a fraction of the cells and cell cycles [8,9]. These features may provide eukaryotic cells with plasticity and adaptability to respond to various environmental or epigenetic changes [10,70,71]. Although the firing at each origin may be stochastic, the order of origin firing and the timing of replication in a population of cells are predetermined and this feature is evolutionally conserved. Chromatin structures may play a major role in determination of the activity of each origin. Generally, 'open chromatin' configurations characterized by 'transcription-permissive' histone marks promote early replication.

We propose the presence of two layers of regulation for origin firing timing. The first is on the level of large replication domains, that coregulate the timing of firing of the origins present in each domain. Additional regulation exists at the level of individual replicons or smaller clusters of replicons, the firing of which may be affected by local chromatin structures. The mid-S replication 'factories' appear to be generated as early as late M/early G1. The pre-RC, which is generated at a similar time during the cell cycle, is not affected by the absence of Rif1 in either fission yeast or in human cells, and thus origin licensing and so-called 'mid-S replication domain licensing' occur independently at a similar timing during the cell cycle, and may constitute the two major steps for regulated and coordinated replication of the genome during S phase.

Rif1 has been shown to interact with transcription factors and was also implicated in DNA damage checkpoint pathways [72–79]. The chromatin architecture generated by Rif1 may regulate not only DNA replication but also

Box 3. Biological significance of replication timing

The regulation of the replication timing program is likely to be evolutionarily conserved because (i) factors regulating origin firing/replication timing are conserved, and (ii) in animal cells, replication domain structures are conserved between different species despite significant divergence of primary sequences and rearrangement of genomic segments [66,102]. However, the apparent normal growth of *rif1*Δ fission yeast despite severely misregulated origin firing patterns suggests that cells can tolerate aberrant origin activation. Similarly, Rif1-depleted cancer cells do not show apparent defects in S phase progression despite genome-wide alteration of the replication timing program. These observations are in line with the speculation that the order and spatial location of origin firing in nuclei are quite flexible and adapt to changes in the environment.

However, the biological significance of replication timing regulation in eukaryotes should not be dismissed altogether. Regulated timing may function to avoid too many replication forks at one time that would cause a shortage of nucleotide pools, resulting in stalled replication forks and eventually in DNA damage. This was indeed shown to occur when early firing of late origins was artificially induced by high-level ectopic expression of multiple replication factors that may be limiting [61]. Precise replication timing may facilitate coordination with transcription timing, which may be required for proper progression of the cell cycle. Early replication may facilitate more protein synthesis of the genes present on the early-replicating segments owing to doubled copy numbers, and this increased expression may be needed for progression through the cell cycle.

Replication timing could affect chromatin structures [34]. The process of DNA replication can induce changes in chromatin status by altering the histone modification patterns or the spatial arrangement of chromatin within the nucleus. Detailed genome-wide studies on replication timing and chromatin features in human cells showed a correlation between chromatin modification features and replication timing in the TTR [103], a long stretch of chromosome that is unidirectionally replicated. This correlation is similar to what is found in other constant replication timing segments, suggesting that replication timing broadly impacts upon chromatin structures. Thus, DNA replication with the proper timing may in turn affect various chromatin activities including transcription, recombination, and repair.

Special mid-S phase replication timing domains may play roles in cell type-specific chromatin packaging or efficient condensation of chromosomes. Indeed, Rif1 knockout MEF cells grow poorly with an extended S phase [69]. Finally, it was reported that the mutation frequency varies during S phase (Box 4). It is higher in late S phase than in early S phase, although the significance of differential mutation rates regulated by replication timing is not clear.

Box 4. DNA replication timing and diseases

Altered replication timing was reported in various diseases such as the ICF (immunodeficiency, centromeric region instability, and facial anomalies) syndrome, Gilles de la Tourette syndrome, and DiGeorge and velocardiofacial syndromes [104–106]. In some cases, transcriptional silencing and late replication were strongly correlated.

Genome-wide analyses of DNA replication timing in cancer cells from patients indicated an altered DNA replication timing pattern that was specific to cancer cells [107]. The changes occur along the developmentally regulated boundary of replication timing domains, supporting the concept that replication domains represent units of chromosome structure and function. In cancer cells, misregulation occurs at the level of these units [107].

Replication timing profile data are useful for detecting karyotypic abnormalities and copy-number variation. Aneuploidies or translocation breakpoints can be detected as lower signals or unnaturally sharp transitions in replication. Cancer SCNAs (somatic copy-number alterations) arise preferentially in genomic regions that have both the same replication timing and share long-range interactions in the nucleus [108].

All these data indicate that the genome rearrangements responsible for genome duplications, deletions, and translocations tend to occur at the boundaries of replication domains or chromosome interaction units. These results suggest the potential of replication timing profiles as a diagnostic biomarker.

It has been suggested that there is an association between mutation rate and DNA replication timing. Initial observation suggested that cancer-related genes are frequently present at the TTR (boundary of domains with different replication timing) [109]. Similarly, single-nucleotide polymorphisms (SNPs) are more enriched in the TTR and in late-replicating regions [109]. Analyses using ENCODE data representing 1% of the human genome revealed that mutation rate, as reflected in evolutionary divergence [110] and human nucleotide diversity (SNPs), is markedly increased in later-replicating segments of the human genome [111]. Analyses of somatic mutations in >400 cancer genomes also showed that the frequency of somatic SNPs increases with replication time during S phase. The late-replicating regions are enriched for recurrently mutated genes but are depleted of cancer driver genes. A higher mutation rate in the late-replicating chromosome segments was also reported in yeast [80].

The mechanisms for increased mutation rate in the late-replicating regions are not clear. The depletion of nucleotide pools in late S phase may cause increased incidence of fork arrest. Alternatively, replication through heterochromatic regions may pause at obstacles to fork progression. The stalled replication fork could generate unstable single-stranded DNA segments that could be the cause for mutagenesis.

Box 5. Outstanding questions

Unsolved issues include

- How does the putative Rif1-loop prevent the origins in the mid-S replication domain from firing in early S phase?
- How is this inhibition released to permit the firing of origins in the mid-S replication domain?
- How does Rif1 (close to 2500 amino acids in humans and lacking any notable motifs except for Heat repeats at the N-terminus) promote the formation of chromatin structures that permit mid-S specific replication?
- Does localization of Rif1 at nuclear periphery play any roles in preferred localization of late-replicating chromatin close to nuclear membrane?
- Are there similar mechanisms by which the firing of late-firing origins (potentially associated with heterochromatin) is inhibited until late S phase?
- Is the regulation of DNA double-stranded break repair and non-homologous end joining by Rif1 related to its role in DNA replication timing regulation?
- What are the roles of Rif1 protein in regulation of differentiation or development?

other chromosome transactions. The importance of Rif1 in early development has been underscored by its high expression level in undifferentiated embryonic stem cells and by the early embryonic lethality of the knockout mice [72,78]. Thus, the chromatin architecture defined by Rif1 may be established early in development and continues to influence various biological events throughout the life of the organism. Further elucidation of its roles will help to clarify the biological significance of replication timing regulation (Box 3) and how it might be linked to various human diseases (Box 4). There are a number of issues to be resolved on DNA replication timing regulation. Several questions that need to addressed about Rif1 in the near future are listed in Box 5.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tig.2013.05.001.

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