REVIEW

Location, location, location: it's all in the timing for replication origins

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The differential replication timing of eukaryotic replication origins has long been linked with epigenetic regulation of gene expression and more recently with genome stability and mutation rates; however, the mechanism has remained obscure. Recent studies have shed new light by identifying novel factors that determine origin timing in yeasts and mammalian cells and implicate the spatial organization of origins within nuclear territories in the mechanism. These new insights, along with recent findings that several initiation factors are limiting relative to licensed origins, support and shape an emerging model for replication timing control. The mechanisms that control the spatial organization of replication origins have potential impacts for genome regulation beyond replication.

Scope

Several excellent reviews have recently examined the significance of replication timing in epigenetic gene regulation and reprogramming, genetic instability, and mutation rates and evolution (for review, see Aladjem 2007; Gondor and Ohlsson 2009; Mendez 2009; Gilbert et al. 2010; Natsume and Tanaka 2010; Burgess 2011; Herrick 2011). Despite substantial progress in characterizing replication timing in various model systems, our understanding of the underlying mechanisms that establish the timing of origin activation has remained rather vague. This review highlights recently published studies that provide new mechanistic insights by identifying novel factors that control replication timing throughout the budding yeast and fission yeast genomes. These factors are conserved in higher eukaryotes, and one has already been shown to control replication timing in mammalian genomes, where replication timing is linked with gene regulation and the developmental program.

Replication origins and initiation control

The discovery of autonomously replicating sequences (ARSs) in the budding yeast *Saccharomyces cerevisiae*

enabled the first detailed studies of chromosomal DNA replication origins in eukaryotic cells (Stinchcomb et al. 1979). ARSs were identified as relatively short (100- to 200-base-pair [bp]) chromosomal DNA sequences that confer replication to plasmids, allowing their mitotic propagation (for review, see Newlon and Theis 1993). Further analysis of these sequences within their native chromosomal loci confirmed their function as replication origins within chromosomes. As prototypes for replication origins in higher cells, ARS studies in yeast propelled much of the genetic and biochemical elucidation of the macromolecular events involved in replication initiation in all eukaryotes. Although yeast origins differ in sequence specificity from mammalian origins, both share highly conserved regulation at the molecular and cellular levels (for review, see Cvetič and Walter 2005).

Eukaryotic replication origins are governed by a series of protein recruitments, beginning with binding of the origin recognition complex (ORC), which marks potential sites of origin assembly (for review, see Bell and Dutta 2002). In G1 phase, ORC, Cdc6, and Cdt1 together load inactive minichromosome maintenance (MCM) helicase complexes at origins. This MCM loading step, which is also referred to as "licensing" or "pre-RC assembly," potentiates origins for initiation. Initiation is controlled by Dbf4-dependent kinase (DDK) and cyclin-dependent kinase (CDK), which are sequentially activated during the G1-to-S-phase transition (for review, see Labib 2010). DDK acts first, phosphorylating MCM subunits to load Cdc45 and Sld3 (Heller et al. 2011). Next, CDK phosphorylates Sld3 and Sld2, leading to the recruitment of GINS (which, together with Cdc45, stimulates MCM helicase) and additional factors, including DNA polymerases, to assemble a complete replisome and initiate DNA synthesis. Whereas this requisite sequence of events is shared by all active origins, they differ in timing and efficiency of initiation during S phase.

Timing and efficiency are distinct measures that are frequently used to characterize origin activity. These measures are population averages and are typically determined through distinct methods (Kalejta and Hamlin 1996; Huberman 1997; van Brabant et al. 1998). Because origins differ in their characteristic initiation timing and exhibit substantial variance in this timing across a population, replication forks from adjacent origins sometimes replicate origins "passively," precluding initiation.

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Thus, most replication timing measurements represent a composite of initiation time and passive replication time. Efficiency is the resulting frequency of initiation (Friedman and Brewer 1995; Kalejta and Hamlin 1996). The replication timing and efficiency of neighboring origins are interrelated in that earlier or more efficient origins will often passively replicate their later or less efficient neighbors. Consequently, timing and efficiency are correlated with earlier origins generally being more efficient and later origins being less efficient (Yamashita et al. 1997; Yang et al. 2010). Both timing and efficiency may be thought of as reflecting the probability of origin firing, with early timing reflecting a high probability per unit time (at least in early S phase) and high efficiency reflecting a high, cumulative probability over the entire period of S phase. It has been suggested, therefore, that timing and efficiency derive from a common underlying mechanism that determines these probabilities (Rhind 2006). The central challenge has been to define this mechanism.

Chromosomal position and chromatin structure

Chromosomal context and local chromatin structure are major determinants of replication origin timing and efficiency, as demonstrated by seminal ARS relocation experiments (Ferguson and Fangman 1992). This study found that *ARS501* residing near a subtelomeric region initiated replication later than *ARS1* residing near a centromere (CEN). Both origins initiated efficiently, indicating that both were efficiently licensed and competent to fire. Relocating *ARS1* to a subtelomeric location near *ARS501* delayed its replication, whereas *ARS501* initiated early when relocated outside of the subtelomeric region (onto a plasmid). These findings suggested that early firing is the normal state and that late timing is imposed by repressive chromatin flanking the origin, such as subtelomeric heterochromatin. Supporting and extending this idea, late-firing origins were identified in nontelomeric regions that fired early when located on a plasmid but fired later when 6–15 kb of native, flanking DNA was included on the plasmid, with the length of delay roughly proportional to the amount of flanking DNA (Friedman et al. 1996). Thus, chromosomal environment influences origin timing.

In some chromosomal contexts, origin firing is suppressed to the point of origin dormancy (Dubey et al. 1991). This phenomenon suggested the possibility that dormancy might result from interference with origin licensing. However, analysis of *ARS301*—a dormant origin associated with the heterochromatic, silent mating type locus *HML*—indicated that *ARS301* was licensed and competent to initiate replication (Santocanale et al. 1999; Vujcic et al. 1999). Indeed, *ARS301* initiated replication when given additional time, which was achieved by blocking its passive replication with hydroxyurea or deletion of flanking origins. Under these circumstances, *ARS301* fired very late, suggesting that a robust delay of firing is sufficient to cause dormancy by favoring passive replication.

Due to differences in origin efficiencies and timings, different subsets of origins are used in different cells of a population (Fangman and Brewer 1991; Kalejta and Hamlin 1996; Patel et al. 2006; Czajkowsky et al. 2008). Nevertheless, at the population level, replication timing profiles emerge reflecting individual origins' timings and efficiencies. Complete analysis of origin timings and efficiencies of all known origins on *S. cerevisiae* chromosomes III and VI, confirmed by whole-genome studies, has revealed general features of chromosomal replication, including early replication of at least one origin near each CEN, late firing and/or dormancy of origins in subtelomeric regions as well as those associated with the heterochromatic silencers, and a wide range of origin timings and efficiencies throughout other chromosomal regions, with some origins notably earlier than the CEN-proximal origins (Newlon et al. 1993; Friedman et al. 1997; Yamashita et al. 1997; Poloumienko et al. 2001; Raghuraman et al. 2001; Yabuki et al. 2002). Together with the ARS relocation studies, these findings implicated some aspect of these different chromosomal domains, such as chromatin structure, transcription, or subnuclear localization, in origin control.

The idea that chromatin structure, subnuclear localization, and/or gene expression regulate initiation gained direct support from studies that analyzed replication in strains with deletions of chromatin modifiers and related factors, such as *SIR3*, which is required for assembly of subtelomeric heterochromatin; *YKU70*, which is required for telomere localization to the nuclear periphery; and *RPD3*, which deacetylates histones to regulate gene expression. Deletion of *SIR3* resulted in earlier replication of subtelomeric origins, and when early origin *ARS1* was transplanted into a subtelomeric region, its timing was delayed in a *SIR3*-dependent manner (Stevenson and Gottschling 1999). Deletion of *YKU70* also advanced replication timing of subtelomeric origins, suggesting a role for localization of subtelomeric chromatin to the nuclear periphery (Cosgrove et al. 2002). Moreover, deletion of *RPD3* advanced the timing of most nontelomeric, late-firing origins, and tethering *GCN5* histone acetylase to a late-firing origin advanced its initiation timing, indicating that histone acetylation levels surrounding origins influence origin activity (Vogelauer et al. 2002; Aparicio et al. 2004; Knott et al. 2009). However, increased histone acetylation by *GCN5* targeting or *RPD3* deletion did not cause late origins to fire as early as the earliest-firing origins in the genome, suggesting the existence of unidentified, early-firing determinants. Recent studies in yeast have revealed such determinants.

CENs impart early timing to proximal origins

The finding that yeast CENs replicate early suggested that CENs might themselves influence replication timing; however, it was also possible that CENs reside in early-replicating regions but do not determine the timing of the regional origins. Studies of CEN replication timing in the related budding yeast *Candida albicans* provided

further evidence that the presence of a functional CEN establishes early replication timing (Koren et al. 2010). In *C. albicans*, ablation of the CEN sequence on a chromosome resulted in the epigenetic formation of a functional, new CEN (neo-CEN) at a different site on the chromosome. Remarkably, a new origin was formed near the neo-CEN through novel recruitment of ORC to the site, and the origin fired early. This occurred at several different neo-CEN loci in different strains, consistent with the neo-CEN establishing early timing; however, the possibility remained that these sites preferentially formed neo-CENs due to the presence of early determinants and/or cryptic origins.

Recent experiments analyzing the effect of relocating a CEN within a chromosome recently provided direct evidence that CENs promote the earlier activation of proximal origins in *S. cerevisiae* (Pohl et al. 2012). This study analyzed a strain with a chromosomal rearrangement that relocated the CEN from its normal, early-firing locus next to a late-firing origin. This rearrangement advanced the late origin's timing while delaying activation of an origin near the original CEN locus. The advanced timing was dependent on the insertion of a functional CEN, demonstrating that the effect was not caused by extraneous sequences associated with the transplanted CEN. The effect of the CEN on replication timing was limited in *cis* to ~19 kb, and hence CENs do not account for the timing of most early origins genome-wide.

CENs also replicate early in the fission yeast *Schizosaccharomyces pombe*, although the mechanism appears to be quite distinct from that of budding yeast, which remains undefined. *S. pombe* CENs are substantially different in chromatin structure from *S. cerevisiae* CENs, undergoing H3K9 methylation, to which Swi6 (equivalent to metazoan HP1, which is apparently absent in *S. cerevisiae*) is recruited (Bannister et al. 2001; Nakayama et al. 2001). Similar heterochromatin exists in subtelomeric regions, which is at least partly responsible for late replication of subtelomeric domains. However, at pericentric origins and at the early-replicating, heterochromatic *matK* mating type locus, Swi6 directly binds and recruits Dfp1, the *S. pombe* equivalent of the DDK-activating subunit Dbf4 (Hayashi et al. 2009). Tethered recruitment of Dfp1 to the pericentric region can bypass the requirement of Swi6 for early firing of the pericentric origins. Thus, Swi6-mediated recruitment of DDK specifically ensures the early replication of CENs and *matK* in fission yeast, despite their heterochromatic structure, which otherwise would delay their replication. Swi6 was not responsible for early firing of a euchromatic origin.

Global determinants

Forkhead box (Fox) transcription factors Fkh1 and Fkh2 (Fkh1/2) have now been shown to account for the timing of most early (and late) origins in the budding yeast genome (Knott et al. 2012). Fkh1/2-binding sites were discovered as an anti-correlated genomic feature with the global set of Rpd3-regulated (late) origins (Knott et al. 2009), suggesting that Fkh1/2 might regulate replication

timing. Indeed, cells lacking *FKH1* and *FKH2* exhibited profound changes in chromosomal replication profiles with significantly delayed firing of 106 normally early origins (termed Fkh-activated) and significantly advanced firing of 82 normally late origins (Fkh-repressed) out of 352 total origins detected. Strikingly, CEN-proximal origins (within 25 kb) did not depend on Fkh1/2 for their early replication, consistent with the conclusion that CENs intrinsically determine origin timing locally. Fkh1/2 consensus binding sites were found to be enriched near Fkh-activated origins, and deletion of a pair of Fkh1/2-binding sites proximal to one of these (*ARS305*) specifically delayed its firing in otherwise wild-type cells, demonstrating that Fkh1/2 act directly in *cis* to advance origin timing (Fig. 1A). In contrast, Fkh1/2 consensus binding sites were found to be depleted near Fkh-repressed origins, suggesting that the earlier firing of these origins derives indirectly from the delayed firing of Fkh-activated origins in *fkh1Δ fkh2Δ* cells (discussed further below).

Two recent studies have identified telomere-binding proteins Rif1 and Taz1 as regulators of replication timing in the fission yeast *S. pombe* (Hayano et al. 2012; Tazumi et al. 2012). Taz1 binds telomeric repeat sequences directly and binds Rif1, thus recruiting Rif1 to telomeres (Kanoh and Ishikawa 2001). Although telomeric chromatin has been implicated previously in replication timing control of subtelomeric regions, Rif1 and Taz1 are now found to regulate replication timing of numerous internal chromosomal loci as well. Tazumi et al. (2012) discovered a replication timing control (RTC) sequence adjacent to an internal, late-firing origin (*AT2088/ars745*) and showed that deletion of this site advanced origin timing (Fig. 1B). Furthermore, introduction of two copies of the 25-bp RTC sequence next to an early origin (*ars2004*) delayed its firing. The RTC contains a Taz1-binding sequence (a tandem repeat of telomere sequence), and deletion of *taz1⁺* advanced the firing time of 26 (of 110) internal and 46 (of 46) subtelomeric late or dormant origins. RTCs were identified near 13 of the 26 internal origins, and chromatin immunoprecipitation (ChIP) analysis showed Taz1 binding to all 13 of the internal origins with an RTC but not to 10 of the 13 deregulated origins lacking an apparent RTC. Similarly, Taz1 binding was most enriched near the telomeres and did not appear to bind some of the more internal, subtelomeric origins that were deregulated by its deletion. Taz1 was suggested to act from a distance to regulate these origins.

Rif1 plays an even broader role in replication timing control. Hayano et al. (2012) discovered that deletion of *rif1⁺* suppressed the lethality of a deletion of *hsk1⁺*, the catalytic subunit of DDK, suggesting that Rif1 acts in opposition to DDK in origin firing. Deletion of *rif1⁺* resulted in delayed activation of 134 normally early-firing origins (designated "EL" for early to late) and advanced activation timing of 189 normally late-firing origins ("LE" for late to early), out of 1083 origins detected; the LE group included all of the Taz1-regulated origins plus an additional 47 internal late or dormant origins. ChIP–chip analysis showed Rif1 binding near telomeres, as expected,

Figure 1. Yeast replication origins and factors regulating initiation timing. Structure models of origins regulated by Fkh1/2 (A), Taz1-Rif1 (B), or Rif1 (C). The *left* panels show regulation of Cdc45 recruitment in wild-type cells in G1/early S phase and resulting initiation timing, and the *right* panels show the effects of deleting indicated genes or protein-binding sites on Cdc45 recruitment and initiation timing. Arrows and blocking arrows regulating Cdc45 recruitment are not implied to act directly. DNA sequence elements and DNA-binding proteins are defined in the key. Rif1 is hypothesized to bind LCS directly or indirectly.

but also at many internal loci, where Rif1 binding was independent of Taz1. Rif1-binding sites mapped most frequently and closely to LE origins and least frequently and closely to EL origins, suggesting that Rif1 acts directly at LE origins to delay their activation and that the effect on EL origins occurs over a distance or is indirect (discussed further below). A significant exception, however, occurred at pericentric origins, which are in the EL group and bind Rif1 robustly, suggesting a direct effect on the timing of these origins. Whether Rif1 interacts with Swi6 to promote early firing of pericentric origins is unclear. Although it is not yet understood how Rif1 binds the Taz1-independent internal sites, Hayano et al. (2012) found a sequence motif associated with about half of the Rif1-binding sites near LE origins. This sequence is very similar to the previously identified late consensus sequence (LCS) responsible for late firing of *ars727* (Yompakdee and Huberman 2004), a Rif1-bound LE origin (Fig. 1C); however, it remains to be tested whether the LCS is required for Rif1 binding, directly or indirectly. Despite this uncertainty, the data suggest that Rif1 acts directly to delay the firing of many internal and subtelomeric origins while also promoting the early firing of pericentric origins.

The role of Rif1 in replication timing control appears to be broadly conserved. Although Taz1 is not present in *S. cerevisiae*, its function in recruiting Rif1 to telomeres is carried out by Rap1, which binds telomeres directly (Marcand et al. 1997). Budding yeast cells lacking Rif1 exhibit advanced replication timing of subtelomeric

origins (Lian et al. 2011). This effect of *RIF1* deletion was attributed to loss of the Rif1-dependent telomere length-sensing mechanism, in which critically short telomeres are replicated early in contrast to the late replication of normal-length telomeres (for review, see Shore and Bianchi 2009). Similarly, the effect of *YKU70* deletion on replication timing was attributed to telomere shortening in *yku70Δ* cells causing activation of the telomere length-sensing mechanism. However, the finding that Rif1 also regulates timing of internal origins in fission yeast suggests a simpler mechanism in which shorter telomeres result in reduced Taz1 and Rif1 binding at telomeres, which is directly required for late replication timing of subtelomeric origins. The budding yeast Rif1 study analyzed only a handful of origins, so the broader effects are unclear, but intriguingly, the one CEN-proximal origin that was analyzed (*ARS1*) replicated later in *rif1Δ* cells, as occurred at all three CENs in *S. pombe rif1Δ* cells. It will be interesting to determine whether budding yeast CENs bind Rif1 and require Rif1 for early replication.

Rif1 has now been shown to control replication timing in mammalian cells as well. Yamazaki et al. (2012) showed that depletion of Rif1 altered the replication program in human (HeLa) cells, and Cornacchia et al. (2012) showed that deletion of Rif1 altered replication timing patterns in mouse (MEF [mouse embryonic fibroblast]) cells. Both studies showed loss of the normal cytological pattern of mid-S-phase replication foci with an apparent accumulation of early S-phase patterns, suggesting an increase of early replication events. These

patterns suggest that Rif1 normally delays the activation of many origins, which fire earlier in Rif1's absence. In addition, analysis of replication timing with BrdU incorporation revealed many early-replicating domains being delayed and late-replicating domains advancing; overall, the replication timing programs were compressed, consistent with a global loss of replication timing control.

Timing mechanism is independent of transcription

The identification of Fkh1/2 transcription factors as regulators of origin timing recalls early studies of ARS elements that identified *trans*-acting factor as stimulators of origin function (for review, see DePamphilis 1993). For example, binding sites for ARS-binding factor 1 (Abf1) were identified at a subset of ARSs, including the well-studied *ARS1*. Deletion of the Abf1-binding site at chromosomal *ARS1* reduced firing efficiency, which was restored by introduction of a binding site for a heterologous protein containing a transcriptional activation domain (Marahrens and Stillman 1992). Subsequent studies indicated that the role of Abf1 at *ARS1* is to maintain a nucleosome-free region for MCM loading, which is required for assembly of competent origins (Lipford and Bell 2001). It has not been reported whether Abf1 affects initiation timing; however, nucleosomal profiles are similar between early and late origins, suggesting that whereas a nucleosome-free region is essential to establish origin competence, it is not a determinant of origin timing (Berbenetz et al. 2010; Eaton et al. 2010).

Origin timing regulation by Fkh1/2 and by Rif1 and Taz1 appears to function by a mechanism distinct from Abf1. Deletion of *FKH1* and *FKH2* did not affect levels of ORC or MCM loading at origins based on genome-wide ChIP-chip analysis (Knott et al. 2012). Similarly, the timing and level of MCM loading at origins were unaffected by *rif1*⁺ or *taz1*⁺ deletion (Hayano et al. 2012; Tazumi et al. 2012). Thus, Fkh1/2, Rif1, and Taz1 do not appear to affect origin licensing.

The involvement of transcription regulators and telomeric chromatin proteins in origin regulation also raised the possibility that transcriptional changes in the mutant cells were responsible for the effects on origin activity. Transcription through an origin sequence can directly disrupt origin activity by interfering with ORC binding and MCM loading (Snyder et al. 1988; Mori and Shirahige 2007; Blitzblau et al. 2012). Thus, changes in transcription might alter origin competence positively or negatively depending on the directionality of transcriptional change. Both *fkh1Δ fkh2Δ* and *rif1Δ* yeast and Rif1-depleted HeLa cells exhibit substantial changes in transcription (Zhu et al. 2000; Hayano et al. 2012; Knott et al. 2012; Yamazaki et al. 2012). However, no changes in ORC and/or MCM origin-binding levels or -binding time were observed at origins showing deregulated firing in *fkh1Δ fkh2Δ*, *rif1Δ*, or *taz1Δ* yeast or Rif1-depleted HeLa cells, inconsistent with transcriptional effects on origin competence (Hayano et al. 2012; Knott et al. 2012; Tazumi et al. 2012; Yamazaki et al. 2012). Furthermore, overexpression of C-terminally truncated Fkh2 in *fkh1Δ*

fkh2Δ cells rescued most of the transcriptional deregulation but did not rescue origin deregulation, strongly suggesting that transcriptional changes are not responsible for the effects on origins (Knott et al. 2012). In addition, transcriptome analysis in these cells showed no correlation between any residual changes in transcription and origin deregulation. Transcriptome analysis of Rif1^{-/-} MEFs showed no changes in transcription (Cornacchia et al. 2012). This result appears to contrast with the analysis in yeast and HeLa cells, which, however, were not further scrutinized. Overall, these results suggest that replication timing control, at least by the Fkh1/2, Rif1, and Taz1 mechanisms, is mechanistically independent of transcription.

Regulation of Cdc45-Sld3 loading

The timing of Cdc45 loading, along with its loading factor, Sld3, corresponds with the differential timing of origin initiation, suggesting that the DDK-dependent recruitment of Cdc45-Sld3 is the temporally regulated step (Aparicio et al. 1999; Kamimura et al. 2001). Furthermore, these studies detected binding of Cdc45 and Sld3 at early origins in G1-arrested budding yeast cells (Fig. 2), leading to the suggestion that Cdc45 might be present in a limiting amount, which is somehow preferentially accessible to early origins (Aparicio et al. 1999). The G1-phase association of Cdc45 with early origins is particularly significant because origin timing is established in G1 phase in budding yeast and mammalian cells (Raghuraman et al. 1997; Dimitrova and Gilbert 1999). The Fkh1/2 study found that Fkh-activated origins were significantly enriched for Cdc45 binding in G1 phase, and, remarkably, this binding was Fkh1/2-dependent (Fig. 1A). Furthermore, Cdc45 binding at CEN-proximal origins was not Fkh1/2-dependent, but instead increased in the absence of Fkh1/2, likely explaining the even earlier activation of CEN-proximal origins in *fkh1Δ fkh2Δ* cells. Thus, Fkh1/2 enhance the ability of Fkh-activated origins to recruit Cdc45 in G1 phase, in anticipation of S phase. Given the dependence of Cdc45 loading on DDK, it is possible that Fkh1/2 act to recruit DDK instead.

Taz1 and Rif1 also regulate the timing of the Cdc45-Sld3-loading step (Hayano et al. 2012; Tazumi et al. 2012). In *taz1Δ* and *rif1Δ* fission yeast cells, Cdc45 and Sld3 binding was advanced at origins that fired earlier in the mutant strains, consistent with inhibitory roles for Taz1 and Rif1 in Cdc45-Sld3 loading (Fig. 1B,C). Similarly, Rif1-depleted HeLa cells showed increased chromatin association of Cdc45 in G1-synchronized cells, suggesting that Rif1 acts prior to replication initiation to inhibit Cdc45 access to origins (Yamazaki et al. 2012). However, Rif1 was required for early S-phase binding of Cdc45 to pericentric origins in fission yeast, which require Rif1 for early firing, suggesting that Rif1 is not simply an inhibitor of Cdc45 loading (Hayano et al. 2012). Further consistent with the idea that Rif1 acts in G1 phase, Rif1 chromatin binding occurs in G1 phase in both fission yeast and mammalian cells (Cornacchia et al. 2012; Hayano et al. 2012; Yamazaki et al. 2012). Interestingly, cytological

Figure 2. Preferential recruitment and recycling of limiting initiation factors as a timing mechanism. Recruitment of Cdc45 requires Sld3 and DDK activities, which are not shown. Additional initiation factors and replisome components are also excluded for simplicity.

analysis indicated that Rif1 binding preceded replication in MEFs but did not localize with ongoing replication (Cornacchia et al. 2012).

Supporting the idea that Cdc45-Sld3 abundance is limiting and may contribute to replication timing control, studies in several replication systems have provided direct evidence that Cdc45 and a handful of other replication initiation factors are indeed limiting for origin firing in vivo. In *Xenopus* (frog) egg extracts, only a subset of licensed origins bound Cdc45, and decreasing the abundance of Cdc45 reduced the level of replication (Edwards et al. 2002). In *S. pombe*, overexpression of Cdc45 or a subunit of DDK (Hsk1 or Dfp1), which is required to load Cdc45-Sld3, increased the level of replication that occurred in early S phase (Patel et al. 2008; Wu and Nurse 2009). In mammalian cells, Cdc45 was present at low levels relative to licensed origins, and increasing the dosage of Cdc45 resulted in an increased number of replication foci, consistent with an increased number of replisomes (Wong et al. 2011). In *S. cerevisiae*, overexpression of Cdc45 alone did not advance late origin timing; however, overexpression of Cdc45 plus its binding partners, Sld3 and Sld7, or overexpression of DDK subunits Cdc7 and Dbf4 did (Tanaka et al. 2011). In a different study in budding yeast, overexpression of Sld3, Dbf4, Sld2, and Dpb11 advanced late origin firing (Mantiero et al. 2011). Furthermore, in the context of overexpressed Sld3, Dbf4, Sld2, and Dpb11, increasing the dosages of Cdc45 and Sld7 increased the overall levels of origin firing, consistent with Cdc45 and Sld7 limiting the total number of active replisomes. Together, these studies indicate that levels of initiation factors, particularly those

involved with DDK-dependent Cdc45-Sld3 loading, are rate-limiting for replication initiation.

Limited abundance of Cdc45-Sld3 would allow only a subset of licensed origins to fire upon S-phase entry. Because Cdc45 is a replisome component (Aparicio et al. 1997; Tercero et al. 2000), it would be unavailable to fire additional origins until early replicons terminated and recycled Cdc45, thus creating a mechanism to distribute origin firing temporally (Fig. 2). Replication factor recycling has been implicated as a component of the timing program (Sporbert et al. 2002; Ma et al. 2012). It is also possible that Cdc45-Sld3 loading and recycling are temporally regulated through checkpoint modulation of Sld3 and DDK activities (Lopez-Mosqueda et al. 2010; Zegerman and Diffley 2010), which may establish an upper limit on the number of simultaneously active replisomes. Nevertheless, the timing program is established independently of the checkpoint signaling pathway, at least in yeast (Santocanale and Diffley 1998; Shirahige et al. 1998).

Spatial organization of origins in timing control

Whereas limiting levels and recycling of initiation factors provide a mechanism to stagger origin firings temporally (Fig. 2), this mechanism does not explain the preferential recruitment of initiation factors to some origins over others, resulting in characteristically distinct firing times at a population level. Indeed, even in the context of overexpressed factors, deletion of *RPD3* was required to activate dormant origins, albeit inefficiently, suggesting that Rpd3-dependent chromatin still impedes accessibility

of replication factors even at increased dosages (Mantiero et al. 2011). Similarly, a subtelomeric region with tethered Dfp1 replicated later than a pericentric region with tethered Dfp1, suggesting that telomeric chromatin hinders replication factors (Hayashi et al. 2009).

The involvement of telomere-binding proteins as global regulators of the replication timing of subtelomeric and many internal origins implies a role for these proteins in the organization or localization of origins within the nucleus. Taz1 and Yku70 are required for localization of telomeres to the nuclear periphery in yeast (Cooper et al. 1997; Laroche et al. 1998). Interestingly, palmitoylation of Rif1 regulates the association of telomeres with the nuclear periphery, suggesting that palmitoylated Rif1 anchors chromatin to the nuclear membrane (Park et al. 2011). Experiments tethering early origins *ARS305* and *ARS607* to the inner nuclear envelope suggest that positioning to the nuclear periphery is not sufficient to delay origin timing (Zappulla et al. 2002; Ebrahimi et al. 2010). However, both *ARS305* and *ARS607* are Fkh-activated origins, suggesting that the Fkh1/2 mechanism responsible for the early firing of these origins might be dominant over peripheral tethering, at least in these contexts. In contrast, targeted heterochromatin assembly and peripheral positioning of *ARS305* through Sir4 recruitment resulted in delayed firing of *ARS305* (Zappulla et al. 2002). Experiments disrupting either SIR chromatin or tethering of telomeres to the nuclear periphery suggest that neither mechanism by itself is essential for late replication (Santocanale et al. 1999; Zappulla et al. 2002; Hiraga et al. 2006). Thus, the combination of peripheral positioning and heterochromatic structure may be required to delay origin firing reliably, particularly in the context of a competing early-firing mechanism.

In mouse and human cells, Rif1 was found in the insoluble nuclear fraction containing nuclear lamins, suggesting association with the nuclear matrix (Cornacchia et al. 2012; Yamazaki et al. 2012). Rif1 depletion in HeLa cells resulted in larger chromatin loops, consistent with a role for Rif1 in anchoring chromatin to nuclear substructures such as the matrix. The N terminus of Rif1 from yeast to humans is predicted to contain 14–21 HEAT domain repeats that have been implicated in protein–protein interactions (Xu et al. 2010), suggesting a potential mechanism to anchor chromatin loops.

The spatial organization of chromosomes and origins has also been implicated in replication timing control by Fkh1/2. Fkh-regulated origins are nonrandomly distributed, with linear groups of Fkh-activated (early) origins frequently separated from linear groups of Fkh-repressed (late) origins (Knott et al. 2012), which is consistent with a previous study that found that origins of similar timings tend to colocalize along budding yeast chromosomes (Raghuraman et al. 2001). These timing domains (up to a few hundred kilobases in length) were lost in *fkh1Δ fkh2Δ* cells, suggesting that Fkh1/2 establish replication timing domains between linear groups of origins. Similarly, studies of mammalian genome replication also show that chromosomes are subdivided into replication timing domains (of several hundred kilobases to a few

megabases in length) comprising groups of origins with similar timing (Hand 1975; Hiratani et al. 2008). In fact, the replication timing of these domains correlates more strongly with their spatial positioning in the nucleus than with other correlated features such as gene expression or chromatin modifications, suggesting that spatial organization may play a mechanistic role in defining replication timing (Ryba et al. 2010; Yaffe et al. 2010). Loss of Rif1 function in MEFs and HeLa cells led to disruption of replication timing domains, suggesting a role for Rif1 in defining these spatial domains (Cornacchia et al. 2012; Yamazaki et al. 2012).

Consistent with the mammalian studies of replication domain organization, whole-genome chromosome conformation capture analysis of the yeast genome found enriched interactions between early origins, suggesting that these origins colocalize or associate with each other in the nuclear space (Duan et al. 2010). Further analysis of this data set indicated that Fkh-activated origins formed a distinct interaction cluster from Fkh-repressed origins, suggesting that these groups occupy distinct spaces in the nucleus (Knott et al. 2012). Chromosome conformation capture analysis comparing wild-type and *fkh1Δ fkh2Δ* mutant cells showed that interaction between early origin *ARS305* and other Fkh-activated origins (e.g., *ARS607*) was Fkh1/2-dependent. Together, these results suggest that Fkh1/2 regulate the spatial organization of origins, which controls replication timing. A role for Fkh1/2 in mediating long-range interaction between chromosomal loci is supported by the role of Fkh1 in regulating donor preference during mating type switching in budding yeast, which involves recombination between distal chromosomal loci (Sun et al. 2002).

Fkh1/2 were shown to interact physically with ORC by coimmunoprecipitation (Knott et al. 2012). Hence, Fkh1/2 bound at one origin might interact in *trans* with ORC at another origin, with multiple such interactions forming a cluster of origins. This mechanism does not necessarily exclude origins lacking Fkh1/2-binding sites. As a group, Fkh-activated origins are enriched for Fkh1/2 consensus binding sites; however, existing ChIP–chip analyses did not detect Fkh1/2 binding at many consensus sites, so it is not clear whether Fkh1/2 uniformly bind Fkh-activated origins (Harbison et al. 2004; MacIsaac et al. 2006). Renewed analysis of Fkh1/2 binding, particularly during G1 phase, should provide important insight.

A compelling alternative mechanism for Fkh1/2 to mediate interactions in *trans* specifically between Fkh1/2-bound origins involves dimerization of Fkh1/2. Crystal structures of the human FoxP2 and FoxP3 forkhead DNA-binding domains revealed their ability to form domain-swapped dimers, suggesting that these proteins might bridge two DNA molecules (Stroud et al. 2006; Bandukwala et al. 2011). Furthermore, mutations that disrupt potential dimerization of FoxP3 have phenotypic consequences, supporting a functional role for dimerization. Structural modeling of yeast Fkh1/2 based on the FoxP2 and FoxP3 structures predicts that Fkh1/2 also dimerize, and mutations of Fkh1/2 predicted to disrupt dimerization deregulate origin timing, supporting a functional

requirement for dimerization (A Ostrow, R Kalhor, L Chen, and O Aparicio, unpubl.). Dimerization of Fkh1/2 might enable clustering specifically between Fkh1/2-bound origins, and the presence of multiple Fkh1/2-binding sites at an origin, as demonstrated at *ARS305* (Knott et al. 2012), would further permit the clustering of multiple origins together (Fig. 3).

The elucidation of mechanisms promoting early firing helps explain the robustness of the replication timing program, at least in budding yeast. These findings support a model based on competition between origins for limiting replication factors where Fkh1/2 promote early firing by binding specific origins and drawing these origins into clusters or preferred locations in G1 phase (Fig. 3). Clustering of licensed origins having an affinity for Cdc45 and other factors may create a cooperative effect that locally aggregates these factors in anticipation of initiation. Alternatively, Fkh1/2 might position origins with replication factors localized by an unknown mechanism. CENs (at least in budding yeast) might act similarly to promote early firing of proximal origins due to the characteristic clustering and subnuclear positioning of CENs (Jin et al. 1998). Present support for the idea that limiting factors are localized within the nucleus is indirect, based on Cdc45's association with origins that are themselves distinctly localized according to the chromosome conformation capture studies; nevertheless, this feature is not essential for the model. Meanwhile, Yku70, Taz1, and Rif1 generally promote later firing through origin localization to the nuclear periphery and/or through heterochromatic chromatin assembly, effectively

sequestering these origins from initiation factors (Fig. 3). The presence of opposing mechanisms should magnify the effect of competition between individual origins for limiting replication initiation factors. In addition, the limited availability of multiple initiation factors would likely enhance the differences in origins' probabilities of recruiting all of the necessary factors for initiation and provide a strong kinetic advantage to some origins over others.

Initiation transforms early origin clusters into the replication foci that are first observed during S phase. As replication ensues, significant mobilization of chromosomes occurs as DNA is spooled through replisomes within the replication foci (Ma et al. 1998; Leonhardt et al. 2000). This may relocate other, unfired origins to the proximity of the replication foci, where they may capture limiting initiation factors as these are released from terminating, early replicons (Sporbert et al. 2002; Ma et al. 2012). Eventually, telomeres and other sequences associated with the nuclear periphery are released to gain access to replication factors (Heun et al. 2001; Ebrahimi and Donaldson 2008). In mammalian cells, however, replication foci assemble near the nuclear periphery later in S phase, suggesting a relocation of replication factors rather than the chromosomal loci themselves (for review, see Berezney et al. 2000).

Interestingly, subtelomeric origins became even later firing in *fkh1Δ fkh2Δ* yeast cells (Knott et al. 2012), suggesting that their relocation from the nuclear periphery depends on prior replication events, such as the firing of internal earlier origins, perhaps proximal to the sub-

Figure 3. Replication timing model based on spatial organization of origins and limiting factor accumulation. Licensed origins are represented by a single MCM hexamer for simplicity. In G1 phase, interactions between Fkh1/2-bound origins create clusters that localize with Cdc45 and likely other limiting factors (not shown), creating a high probability of Cdc45 recruitment (although not all clustered origins are expected to recruit Cdc45 early in every cell). A Cdc45 gradient may form due to cooperative recruitment by clustered origins or another mechanism but remains speculative. CEN localizes proximal origin (1) with Cdc45, perhaps with Rif1 involvement, and/or clustering with other CENs (not shown). At other loci, Rif1 anchors Taz1- and Rif1-bound sites to the nuclear membrane, thereby localizing proximal origins to the nuclear periphery, sequestered from Cdc45 accumulation. Local heterochromatin may also contribute to Cdc45 exclusion from these domains. The cluster of Cdc45-bound origins, likely including origins from other chromosomes (not shown), establishes a replication focus in early S phase comprising these multiple replisomes. Replication draws flanking origins (2) toward the focus, where they access Cdc45, possibly recycled from earlier replisomes, and fire or replicate passively. Eventually, peripheral origins (3) relocate and fire or replicate passively, although in mammalian cells, the replication factors instead relocate to the periphery.

telomeric regions. This repositioning may increase the probability that these origins will fire during this later period. In addition, the diminishing numbers of unfired origins competing for limiting factors over time should also increase the chance that these origins will eventually fire (Lucas et al. 2000; Goldar et al. 2008; de Moura et al. 2010; Rhind et al. 2010). The probability of passive replication also increases over time until firing or passive replication of all origins has occurred. This model can explain how some late origins fire efficiently in late S phase despite firing inefficiently in early S phase.

In the context of the above model, deletion of *FKH1* and *FKH2*, *taz1*⁺, or *rif1*⁺ would alter the favorable or unfavorable positioning of origins bound directly by these proteins and delay or advance their firing time, respectively. However, deletion of these factors also impacted many origins where binding of the corresponding protein was not detected. This might reflect the repositioning of these loci due to their chromosomal *cis*-linkage with directly bound sites. This “*cis*-effect” might be expected to increase the likelihood that certain origins will access the pool of limiting factors and decrease this likelihood at other origins. In addition, reduced firing of early origins (e.g., Fkh-activated origins in *fkh1Δ fkh2Δ* cells) may contribute to earlier firing of other origins (e.g., CEN-proximal and Fkh-repressed origins in *fkh1Δ fkh2Δ* cells) by reducing competition for limiting factors. Conversely, the earlier activation of LE origins in *rif1Δ* cells may increase competition for limiting factors and account for the delayed activation of EL origins.

This generic model combines the early-determining CEN and Fkh1/2 mechanisms shown in budding yeast with the late-determining Yku70, Taz1, and Rif1 mechanisms, of which at least the Rif1 mechanism seems to be broadly conserved. It remains to be determined whether the Fkh1/2 mechanism is conserved in *S. pombe* and other eukaryotes. Despite conservation of Fkh2 in *S. pombe*, its sequence suggests an inability to dimerize, which appears to be required for origin regulation (O Aparicio, unpubl.). The absence of this mechanism could explain why origins in *S. pombe* generally fire more stochastically and less efficiently than in *S. cerevisiae* (Dai et al. 2005; Heinricher et al. 2006; Patel et al. 2006). Indeed, most internal origin firing appears to be more stochastic in *S. cerevisiae* cells lacking *FKH1* and *FKH2* (Knott et al. 2012). On the other hand, the role of Fox proteins as pioneer transcription factors during early mammalian development suggests a potential link between the changes in transcription and replication timing that occur during early differentiation (for review, see Gilbert et al. 2010; Lalmansingh et al. 2012). The ability of FoxP2 and FoxP3 to dimerize and potentially mediate long-range chromosomal interactions supports the possibility that these proteins regulate replication timing in human cells.

Future prospects and implications

These recent studies begin to reveal a more defined picture of the replication process, particularly in the identity of those factors that determine when, and

perhaps where, distinct chromosomal domains replicate. The identification of new factors in yeasts provides important candidates to study the mechanisms and significance of replication timing in metazoan cells. Moreover, Fkh1/2 and Rif1 are excellent candidates for organizers of replication foci where clusters of replication origins may act coordinately, but which remain imprecisely defined. Indeed, the foci are dynamic structures that likely must be disassembled to permit replication to progress without undue spatial constraints. Also exciting is the potential identification of proteins that regulate the three-dimensional organization of chromosomes, which is emerging as an important mechanism of genome regulation, having been implicated in regulation of transcription, recombination, genome stability, and epigenetic inheritance (for review, see Misteli 2007). Once again, studies in yeasts are elucidating a fundamental mechanism of genome regulation in all eukaryotes. This is worth celebrating with your favorite yeast product.

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