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Article

Origin Association of Sld3, Sld7, and Cdc45 Proteins Is a Key Step for Determination of Origin-Firing Timing

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Summary

Background: Chromosomal DNA replication in eukaryotes initiates from multiple origins of replication, and because of this multiplicity, activation of replication origins is likely to be highly coordinated; origins fire at characteristic times, with some origins firing on average earlier (early-firing origins) and others later (late-firing origins) in the S phase of the budding yeast cell cycle. However, the molecular basis for such temporal regulation is poorly understood.
Results: We show that origin association of the low-abundance replication proteins Sld3, Sld7, and Cdc45 is the key to determining the temporal order of origin firing. These proteins form a complex and associate with the early-firing origins in G1 phase in a manner that depends on Dbf4-dependent kinase (DDK), which is essential for the initiation of DNA replication. An increased dosage of Sld3, Sld7, and Cdc45 allows the late-firing origins to fire earlier in S phase. Additionally, an increased dosage of DDK also allows the late-firing origins to fire earlier.
Conclusions: The DDK-dependent limited association between origins and Sld3-Sld7-Cdc45 is a key step for determining the timing of origin firing.

Introduction

Chromosomal DNA replication in eukaryotes initiates from multiple specific sites for replication, replication origins. Replication origins are activated in the S phase of the cell cycle. The activation mechanisms for individual replication origins are thought to be common. However, timings of activation of individual origins in S phase are different between origins. For example, some origins fire early in S phase (early-firing origins), and some fire late (late-firing origins). Therefore, eukaryotic cells are likely to have regulatory mechanisms to coordinate the activation of multiple replication origins.

The budding yeast *Saccharomyces cerevisiae* is a good model to study eukaryotic DNA replication, because the average firing time of each origin has been mapped [1] and the molecular steps in the initiation of DNA replication are best described in this organism (see reviews [2–4]). Briefly, an origin-recognition complex (ORC) associates with replication origins throughout the cell cycle, and the Mcm2-7 complex is loaded in all of the potential replication origins to form the prereplicative complex (pre-RC) from late M phase to G1 phase. Once S phase CDK (S-CDK) is activated, Sld2 and Sld3 are phosphorylated to promote DNA replication [5, 6]. Phosphorylated Sld2 interacts with the C-terminal BRCT pair of Dpb11 and promotes further the formation of a complex, called a pre-loading complex (pre-LC), which contains phosphorylated Sld2, Dpb11, DNA polymerase epsilon, and a GINS complex [7, 8]. Dpb11 harbors four BRCT motifs, and the N-terminal BRCT pair interacts with CDK-phosphorylated Sld3 [5, 6]. It has been shown that Sld3 associates with replication origins together with another essential replication protein, Cdc45, in a mutually dependent manner [9, 10]. These associations depend on the presence of the pre-RC. Therefore, when S-CDK is activated and phosphorylates Sld3, Dpb11 in the pre-LC can bind to replication origin through the origin-bound Sld3 [8]. Such an interaction promotes new complex formation at replication origin and, as a result, may promote the establishment of a bidirectional replication fork.

Interestingly, the association of Sld3-Cdc45 with replication origin is observed even in G1 phase only at the early-firing replication origins [9, 10]. Therefore, this association might be a key in the early-firing property of the early-firing origins. In this study, we show that the protein level of Sld3, Cdc45, and Sld7, a recently identified replication protein [11], is the rate-limiting factor for specifying the early-firing origins, and that their association with the early-firing origins is regulated by the conserved protein kinase DDK (*D*bf4-*d*ependent *k*inase; Dbf4-Cdc7 in the budding yeast), whose activity is required for the activation of replication origins.

Results

Sld3, Sld7, and Cdc45 Are the Rate-Limiting Factors for Specifying Origin Activation

To obtain insight into the association of Sld3 and Cdc45 with origins in G1 phase, we first measured the copy numbers of cellular Sld3 and Cdc45, using antibodies against these proteins, and using the purified proteins as standards (see the Experimental Procedures and Supplemental Experimental Procedures available online for details). The copy numbers of Sld3 and Cdc45 were estimated to be 300 and 700 per cell, respectively, in an asynchronous culture (Table 1; Figure S1). A previous measurement of their copy numbers employed a method using tagged proteins [12], and these values differ from ours. The copy numbers of Sld3 and Cdc45 were below the number of replication origins, which was estimated to be about 700–1,000 per haploid cell (see Supplemental Experimental Procedures). The higher copy number of Cdc45 than that of Sld3 is consistent with their role in DNA replication: Cdc45 stays at the replication fork after initiation as a part of the putative active replicative helicase, the CMG complex [13], whereas the role of Sld3 is initiation specific [5, 6, 10, 11]. In contrast, the copy number of Orc5, a subunit of ORC, was 2,000 (Table 1), and this value approximately matched the number of origins, because the number of replication origins doubles (1,400–2,000) after DNA replication. Because Sld3 and Cdc45 proteins are constitutively

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Table 1. Copy Numbers of Initiation-Related Replication Proteins

Protein	Copy Number
Cdc45	700
Sld3	300
Sld7	300
Orc5	2,000
Dpb11	200
Sld2	500
Mcm10	3,000
Psf3 (GINS)	15,000
Dpb2 (Pol ε)	3,000

All proteins shown here, except Sld2, are expressed constitutively in the cell cycle (data not shown). Therefore, their copy numbers were measured using asynchronous cells (see Figure S1). The copy number of Sld2 was measured in G1-arrested cells with alpha factor, because Sld2 expression fluctuates in the cell cycle and accumulates in S phase [5, 8, 20].

expressed throughout the cell cycle, their low copy number suggests that they are limiting factors that contribute to determination of the timing of origin firing.

To test the possibility that the association of Sld3 and Cdc45 with origins in G1 phase induces early origin firing, we overexpressed Sld3, Sld7, and Cdc45 and examined the profile of origin firing. Sld7 binds to Sld3, stabilizes it, and is localized at the early-firing origins in G1 phase in an Sld3-dependent manner [11]. Interestingly, the copy number of Sld7 was as low as that of Sld3 (Table 1; Figure S1). To observe the effects of high-level Sld3, Sld7, and Cdc45, cells were arrested in G1 phase with alpha factor, and Sld3, Sld7, and Cdc45 were induced from the galactose-inducible promoter (*GALp*) by galactose addition. Finally, the cells were synchronously released into hydroxyurea (HU)-containing medium (Figures 1A–1C). HU treatment arrests the cell cycle in early S phase because of the depletion of dNTPs (Figure 1A). Under this condition, nascent DNA strands (replication intermediates, RIs) are synthesized only from the early-firing origin-containing regions, and not from the late-firing origin-containing regions. RIs can be separated from unreplicated DNA, which has a high molecular weight, using an alkaline denaturing gel, and they were detected with specific probes (Figures 1A and 1B; see also [14]). As shown previously, RIs were only detected using the early-firing origin-specific probes in the cells harboring the *GALp* vector (Figure 1C; Figure S2A). Overexpression of any one of Sld3, Sld7, and Cdc45, or simultaneous expression of Sld3 and Cdc45 or Sld7 and Cdc45, did not change the profile (Figure 1C; Figures S2A and S2C). In contrast, the late-firing origins *ARS501* and *ARS1412* fired when Sld3 and Sld7 or all three proteins were simultaneously expressed (Figure 1C; Figure S2A).

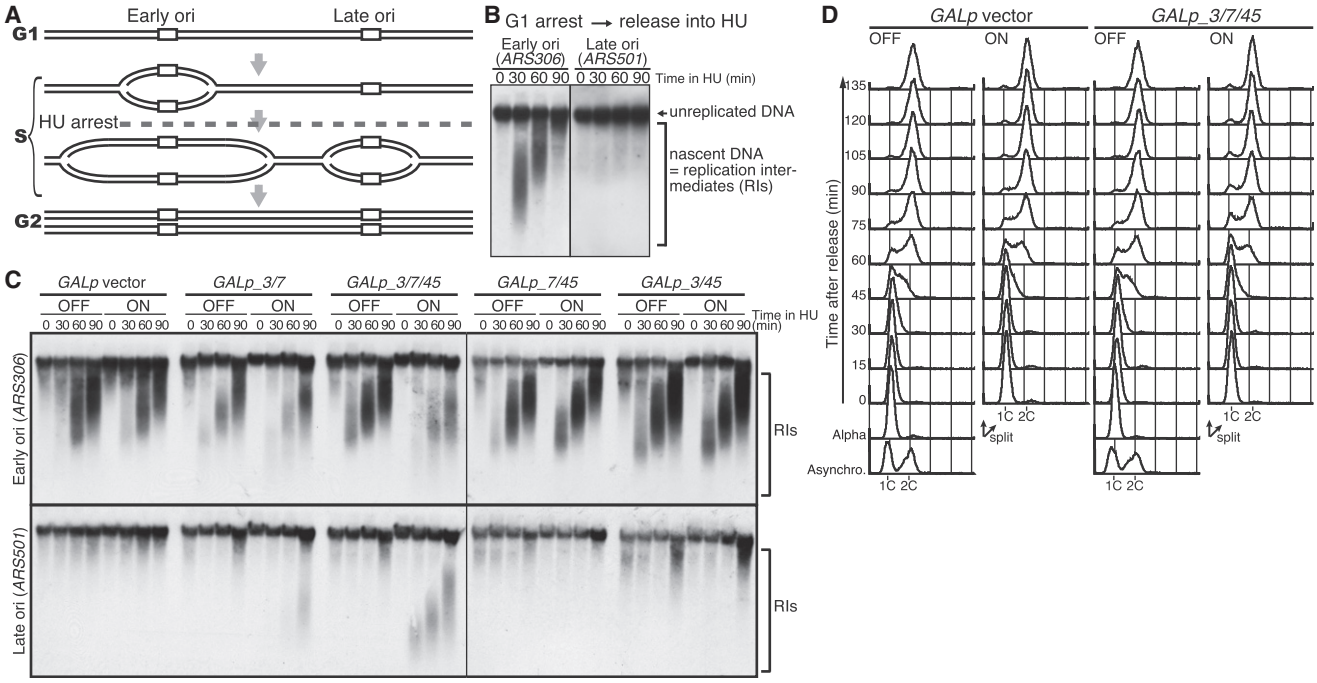

Figure 1. Simultaneous Expression of Sld3, Sld7, and Cdc45 Allows Late Origins to Fire Early

(A) Schematic drawing of DNA replication. Only the early-firing origins fire before the hydroxyurea (HU) arrest point (dashed line).

(B) Alkaline gels. Cells were arrested in G1 phase with alpha factor, synchronously released into HU-containing medium at 30°C, and collected at the indicated times. Chromosome DNA samples were isolated, separated using an alkaline gel, and detected with the early-firing origin (*ARS306*)- and the late-firing origin (*ARS501*)-specific probes, respectively.

(C) Cells harboring *GALp* vector (YST1019), *GALp-SLD3/SLD7* (YST1050, indicated by *GALp_3/7*), *GALp-SLD3/SLD7/CDC45* (YST1053: *GALp_3/7/45*), *GALp-SLD7/CDC45* (YST1190: *GALp_7/45*), or *GALp-SLD3/CDC45* (YST1042: *GALp_3/45*) were synchronously released into HU-containing media after induction of the genes by galactose addition (ON). Chromosome DNA were isolated, separated using an alkaline gel, and detected with *ARS306*- and *ARS501*-specific probes, respectively. Protein induction after galactose addition was also confirmed (see Figure S2B).

(D) Flow cytometry of cells harboring *GALp* vector (YST1019) and *GALp-SLD3/SLD7/CDC45* (YST1053, *GALp_3/7/45*). These were grown at 20°C, arrested in G1 phase with alpha factor, and separated into two halves. Galactose was added to one half (ON), and cells were synchronously released and collected at the indicated time.

See also Figure S2.

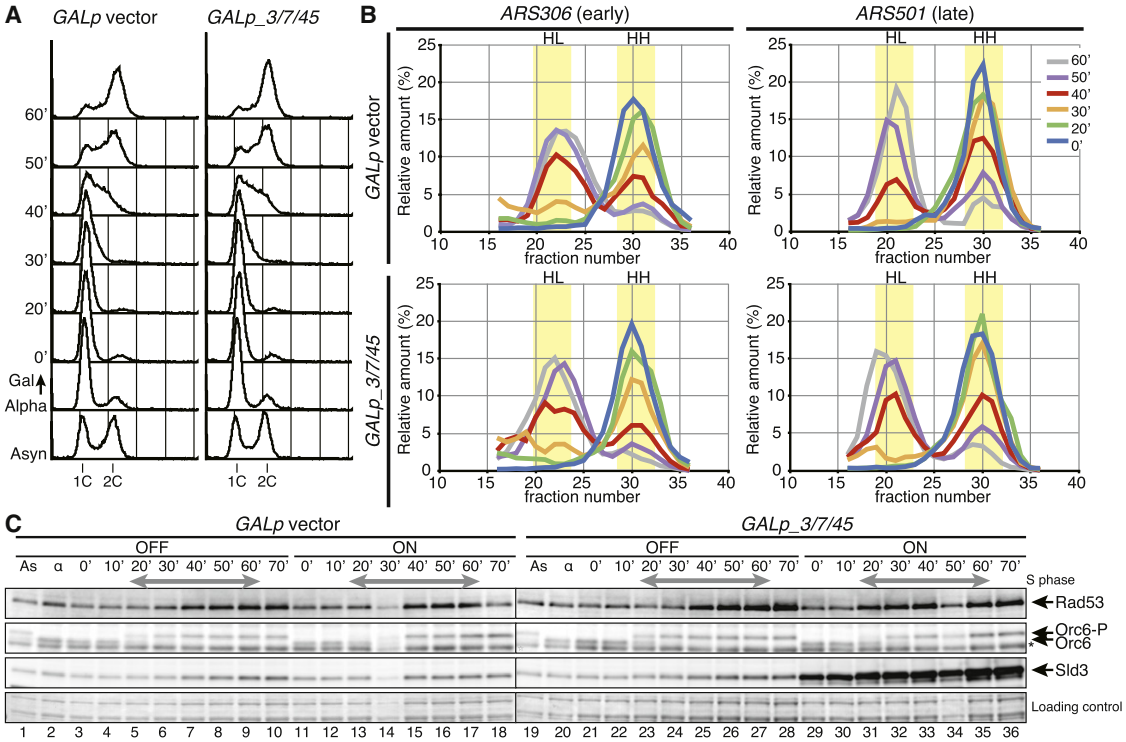

Figure 2. Simultaneous Expression of Sld3, Sld7, and Cdc45 Affects Origin Firing without Abrogating Checkpoint Response

(A) Cells harboring *GALp* vector (YST1019) or *GALp-SLD3/SLD7/CDC45* (YST1053, *GALp_3/7/45*) were grown in [^{13}C]glucose- and [^{15}N]ammonium sulfate-containing medium and arrested in G1 phase with alpha factor. Medium was switched to YPAGal, and after 2 hr incubation, cells were synchronously released and collected. Progression of the cell cycle was monitored by flow cytometry.

(B) Genomic DNA was extracted from cells collected in (A) and separated in a CsCl gradient. The amount of DNA contained in each fraction was analyzed with quantitative PCR and plotted.

(C) Cells harboring *GALp* vector (YST1019) or *GALp-SLD3/SLD7/CDC45* (YST1053, *GALp_3/7/45*) were grown at 25°C, arrested in G1 phase with alpha factor, and separated into two halves. Galactose was added to one half (ON), and cells were synchronously released and collected at the indicated time. Rad53, Orc6, and Sld3 were detected with specific antibodies. The asterisk indicates nonspecific bands. Positions of S phase judged from flow cytometry (Figure S3) are also indicated.

Simultaneous Increase of Sld3, Sld7, and Cdc45 Affects Origin Activation without Abrogating Intra-S Phase Checkpoint

HU treatment induces the intra-S phase checkpoint response and blocks the activation of the late-firing origins [14–17]. Abrogation of checkpoints allows the late-firing origins to fire but does not alter the timing in the presence of HU [14–16]. However, in cells inducing *SLD3*, *SLD7*, and *CDC45* from the galactose promoter (*GALp-SLD3/SLD7/CDC45* cells; *GALp-3/7/45* in Figure 1C and Figure S2A), the size of the RIs from the late-firing origins, *ARS501* and *ARS1412*, increased during the time course, as observed in the early-firing origins, *ARS305* and *ARS306* (Figure 1C; Figure S2A). Thus, simultaneous expression of Sld3, Sld7, and Cdc45 converts the timing of the firing of the late-firing origins to that of the early-firing origins.

To examine whether or not the simultaneous expression of Sld3, Sld7, and Cdc45 affects the late origin firing under the unperturbed condition, we performed the density-transfer experiment without HU. *GALp* vector-harboring or *GALp-SLD3/SLD7/CDC45*-harboring cells were grown in [^{13}C]glucose- and [^{15}N]ammonium sulfate-containing heavy medium, arrested in G1, transferred into galactose-containing light medium, and then finally released from a G1 block. Overall progression of DNA replication was not affected by Sld3, Sld7, and Cdc45 expression (Figure 2A). Replication initiated at 30 min after release, and the heavy-light (HL) DNA appeared at the early-firing origin *ARS306* in both cells (Figures 2A and 2B, orange lines). At the early-firing origin *ARS306*, the progression of DNA replication in *GALp-SLD3/SLD7/CDC45* cells (transition of DNA peak from HH to HL in Figure 2B) was similar to that in *GALp* vector cells. In contrast, the progression of DNA replication in *GALp-SLD3/SLD7/CDC45* cells was enhanced at the late-firing origin *ARS501*: at 40 min after release, unreplicated (HH) peak was higher than replicated (HL) peak in the vector control cells, whereas they were almost equal in *GALp-SLD3/SLD7/CDC45* cells (Figure 2B, red lines in right panels). Thus, the late-firing origin *ARS501* replicated earlier when Sld3, Sld7, and Cdc45 were expressed under the unperturbed condition.

Global Effect of the Simultaneous Expression of Sld3, Sld7, and Cdc45

If all origins fire at once early in S phase, overall progression of DNA replication would accelerate, and S phase would finish earlier than usual. However, the overall profile of replication in the *GALp-SLD3/SLD7/CDC45* cells was apparently the same as in the wild-type cells in flow cytometry (Figure 1D). Because phosphorylation of Rad53, an indicator of checkpoint activation, was not observed in *GALp-SLD3/SLD7/CDC45* cells as in wild-type (vector) cells (Figure 2C; Figure S3), this

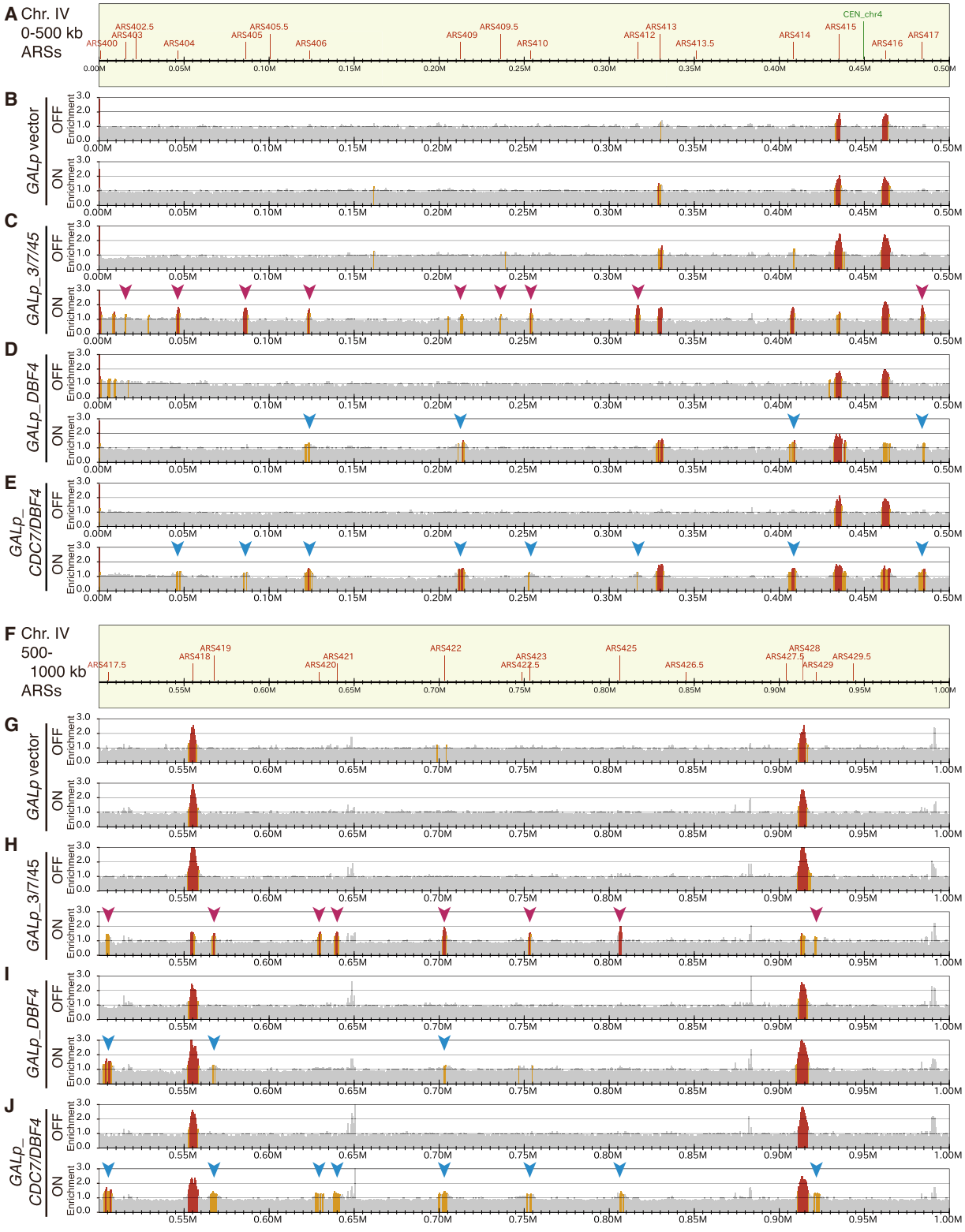

Figure 3. Expression of Sld3, Sld7, and Cdc45 or DDK Affects Genome-Wide Origin Firing Timing

(A) Schematic representation of the 0–0.5 Mb region of chromosome IV. The positions of the confirmed origins (see OriDB; http://www.oridb.org/; [18]) are shown in red.

(B) Replication profile of cells harboring the *GALp* vector (YST1019). The sequence read from the sample 60 min after G1 release in HU-containing YP-raffinose (top, indicated by OFF) or YP-galactose (bottom, indicated by ON) was compared with the sequence read at 0 min, and the ratio of the numbers at 60 and 0 min (enrichment) was plotted over the entire chromosome region. Ratios $\geq$ 1.25 and $\geq$ 1.5 are shown in orange and red, respectively.

(C) Replication profile of cells harboring *GALp-SLD3/SLD7/CDC45* (YST1053, *GALp_3/7/45*). The origins specifically fired in the galactose-containing medium (ON) are indicated by the arrowheads.

strongly suggests that stall or collapse of replication forks is not occurring in *GALp-SLD3/SLD7/CDC45* cells. Moreover, we noticed that the signals of the RIs from the early-firing origins in *GALp-SLD3/SLD7/CDC45* cells were weaker than those of wild-type cells (Figure 1C; Figure S2A). This suggests that simultaneous expression of Sld3, Sld7, and Cdc45 affects not only the timing but also the efficiency of the origin firing.

We further analyzed the replication profile of all chromosomes to elucidate the firing of the mapped replication origins, by monitoring the change in the copy number of the chromosomal DNA. The same DNA samples used for the alkaline gel assays were subjected to sequencing by oligonucleotide ligation and detection (SOLiD) (Figure 3; Figure S4). The sequence reads from the sample 60 min after release were compared with those at 0 min, and the relative enrichment, which is the ratio of the numbers of sequence reads at 60 and at 0 min, was plotted over the entire genome. As a result, the origins that fired in the 60 min samples appeared as peaks in the plot (Figure 3; Figure S4). For example, when wild-type (*GALp* vector) cells were collected from HU-containing raffinose (OFF) or galactose (ON) medium 60 min after release from G1 arrest, we found four origins (*ARS415*, *ARS416*, *ARS418*, and *ARS428*) that fired efficiently and one origin (*ARS413*) that fired modestly (Figures 3B and 3G) in the region from 0 to 1 Mb of chromosome IV, where 31 confirmed origins were mapped (Figures 3A and 3F) [18]. Signals from the efficient origins were weaker when Sld3, Sld7, and Cdc45 were simultaneously expressed from the galactose-inducible promoter (Figures 3C and 3H), and importantly, 17 origins were newly fired in this region (Figures 3C and 3H, indicated by the arrowheads). As observed in the alkaline gel analysis, the signals from the early-firing origins, *ARS305* and *ARS306*, were also weakened, and the late-firing origins, *ARS501* (denoted by *ARS522* in the genome-wide map) and *ARS1412*, fired (Figure S4). Of the confirmed origins (total number = 351) in oriDB [18], 88 and 114 origins were fired in the wild-type (*GALp* vector) and *GALp-SLD3/SLD7/CDC45* cells, respectively, when the galactose-inducible promoter was turned off (Figure 4, *GALp*: OFF). Most of these fired in both cells (67 + 20 origins), although some additional origins fired in the *GALp-SLD3/SLD7/CDC45* cells. When the galactose-inducible promoter was turned on, the number of origins that fired in the *GALp-SLD3/SLD7/CDC45* cells was almost three times higher (increased from 114 to 284; Figure 4, *GALp*: ON). Therefore, these results indicate that simultaneous expression of Sld3, Sld7, and Cdc45 affects not only the timing but also the efficiency of the origin firing. Therefore, it may be possible that there are other rate-limiting factors influencing efficient origin firing. Among other initiation proteins, the copy number of the Dpb11 protein is lower than the number of origins (Table 1; Figure S1). However, simultaneous expression of Dpb11 and its partner, Sld2, in addition to Sld3, Sld7, and Cdc45 did not enhance the overall progression of S phase (Figures S2D and S2E). These results indicate that a simple increase of replication proteins does not alter the timing of origin firing and further suggest that a very specific process determines the timing of origin firing.

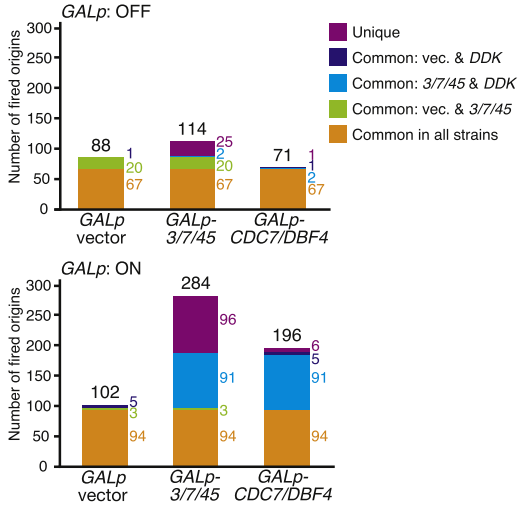

Figure 4. The Numbers of Fired Origins Increase in Cells Expressing Sld3, Sld7, and Cdc45 or DDK

The number of origins that fired in YP-raffinose (indicated by *GALp*: OFF) or YP-galactose (*GALp*: ON). Of the 351 confirmed origins [18], if the corresponding regions had enrichment $\geq$ 1.25 (Figure 3; Figure S4), they were counted as fired origins. Origins that fired in all three strains (*GALp* vector [YST1019], *GALp-3/7/45* [YST1053], and *GALp-CDC7/DBF4* [YST1287]), in only the *GALp* vector and *GALp-3/7/45* strains, in only the *GALp-3/7/45* and *GALp-CDC7/DBF4* strains, in only the *GALp* vector and *GALp-CDC7/DBF4* strains, or in only one of the strains are shown in orange, green, pale blue, blue, or purple, respectively.

Association of Sld3, Sld7, and Cdc45 with the Early-Firing Origins Is Dependent on DDK

The above-mentioned data indicate that an unequal association of Sld3, Sld7, and Cdc45 with origins allows replication origins to fire with different timings in S phase. In budding yeast, the associations of Sld3 and Cdc45 with the early-firing origins are mutually dependent, and the association of Sld7 is dependent on Sld3 [9, 11]. In the fission yeast *Schizosaccharomyces pombe*, Sld3 associates with replication origins in a DDK-dependent manner [19]. Our previous data indicated that budding yeast has a residual DDK activity, even in G1 phase, which promotes the initiation of DNA replication when the CDK requirement is bypassed [5, 20]. In accordance with this, a genome-wide analysis of Dbf4 chromatin immunoprecipitation (ChIP) showed that Dbf4 associates with the early-firing origins in G1-arrested cells [21]. Therefore, we examined whether the association of Sld3, Sld7, and Cdc45 with the early-firing origins in G1 phase requires DDK. As shown in Figures 5A–5H, only the early-firing origins, and not the late-firing origins or nonorigin loci, were recovered from G1-arrested cells using ChIP with 5×FLAG-tagged Sld3, Cdc45, and Sld7. In wild-type cells, a high temperature (36°C) did not alter the ChIP pattern, whereas in cells bearing the temperature-sensitive mutations *cdc7-4* or *dbf4-1*, the ChIP signal was lost at the nonpermissive temperature (36°C), although significant amounts of 5×FLAG-tagged Sld3, Cdc45, and

(D) Replication profile of cells harboring *GALp-DBF4* (YST1076). The origins specifically fired in the galactose-containing medium (ON) are indicated by the arrowheads.
(E) Replication profile of cells harboring *GALp-CDC7/DBF4* (YST1287). The origins specifically fired in the galactose-containing medium (ON) are indicated by the arrowheads.
(F–J) Replication profiles of the 0.5–1 Mb region of chromosome IV. (F)–(J) correspond to (A)–(E), except in the region of the chromosome.
See also Figure S4.

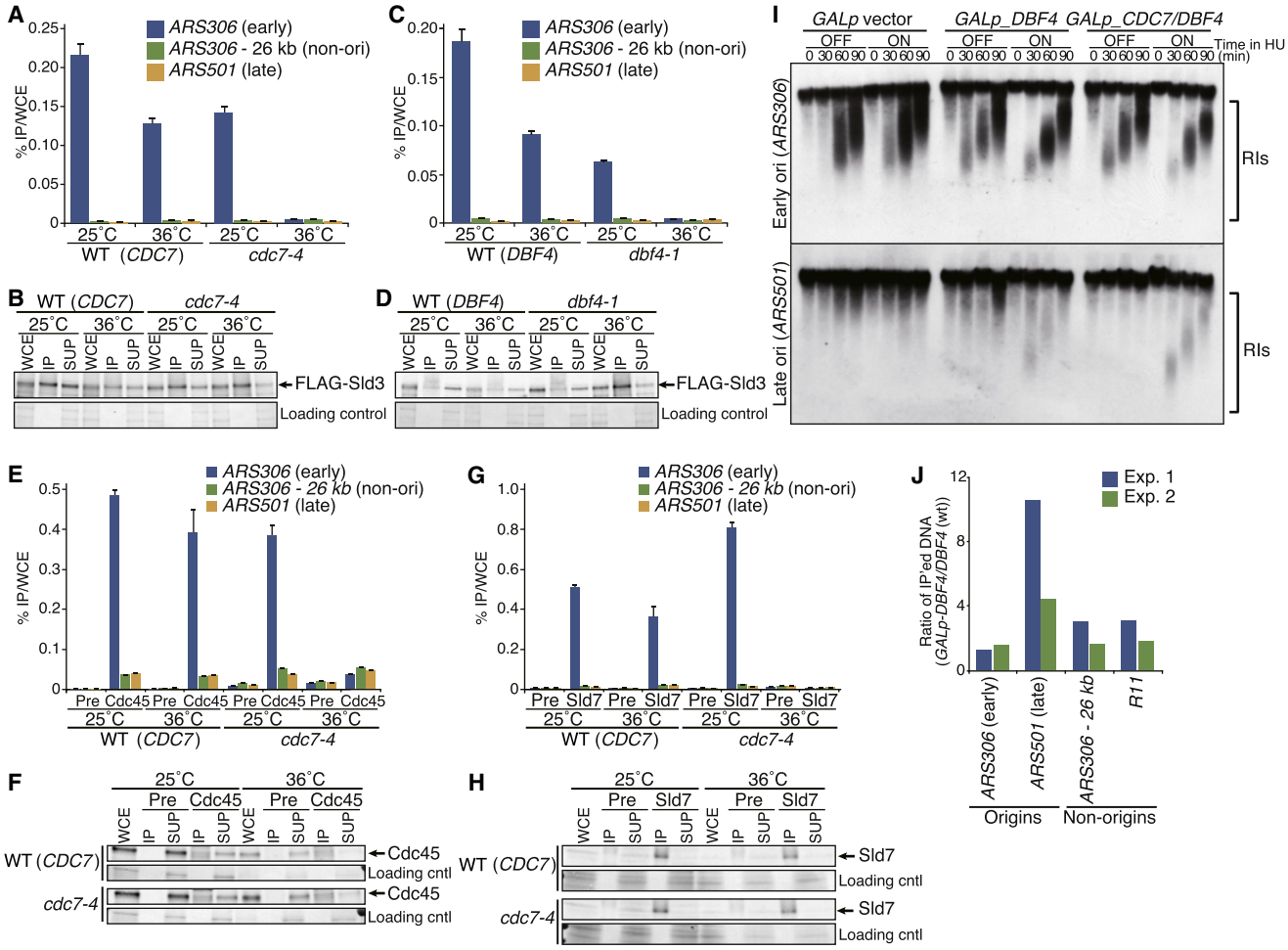

Figure 5. DDK Modulates the Timing of Late Origin Firing via Controlling the Association of Sld3, Sld7, and Cdc45 with Origins

(A) Sld3 association with the early-firing origin in G1 is DDK dependent. YST1204 [*5×FLAG-SLD3*, indicated as WT (*CDC7*)] and YST1340 (*5×FLAG-SLD3 cdc7-4*) cells were grown at 25°C and arrested in G2/M phase with nocodazole. The culture was then split into two halves, and one half was shifted to 36°C, while the other half was kept at 25°C. Finally, the cells were released into alpha factor-containing medium at the indicated temperature and collected. The 5×FLAG-tagged Sld3 was precipitated using an anti-FLAG antibody, and the coprecipitated chromatin DNA was analyzed with quantitative PCR. An outline of experiments and profiles of the flow cytometry data are shown in Figure S5.

(B) FLAG-tagged Sld3 in the samples collected in (A) was detected with anti-FLAG antibodies. The following abbreviations are used: WCE, whole-cell extracts; IP, immunoprecipitates (8.5 times concentrated); SUP, supernatant after immunoprecipitation.

(C) YST1365 [*5×FLAG-SLD3*, indicated as WT (*DBF4*)] and YST1355 (*5×FLAG-SLD3 dbf4-1*, indicated as *dbf4-1*) cells were grown and analyzed as in (A).

(D) FLAG-tagged Sld3 in the samples collected in (C) was detected with anti-FLAG antibodies as in (B). IP samples were 10 times concentrated as compared to WCE and SUP.

(E) W303-1a *Δbar1* [indicated as WT (*CDC7*)] and YST616 (*cdc7-4*) cells were grown and analyzed as in (A), except that the immunoprecipitation was performed using anti-Cdc45 antibodies (Cdc45) and the preimmune IgG of anti-Cdc45 (Pre).

(F) Cdc45 in the samples collected in (E) was detected with anti-Cdc45. IP samples were 3.75 times concentrated as compared to WCE and SUP.

(G) W303-1a *Δbar1* [indicated as WT (*CDC7*)] and YST616 (*cdc7-4*) cells were grown and analyzed as in (A), except that the immunoprecipitation was performed using anti-Sld7 antibodies (Sld7) and the preimmune IgG of anti-Sld7 (Pre).

(H) Sld7 in the samples collected in (E) was detected with anti-Sld7. IP samples were 5 times concentrated as compared to WCE and SUP.

(I) Alkaline gel analysis of YST513 (*GALp* vector), YST1076 (*GALp_DBF4*), and YST1287 (*GALp_CDC7/DBF4*). Cells were grown and analyzed as in Figure 1C.

(J) Association of Sld3 with the late-firing origins increases specifically in *GALp-DBF4* cells. Cells harboring *5×FLAG-SLD3* (WT) and *5×FLAG-SLD3 dbf4::GALp-DBF4* (*GALp-DBF4*) were arrested in G1 with alpha factor, collected, and analyzed as in (A). The ratio of the amounts of coimmunoprecipitated DNA from these two strains obtained from two independent experiments (Exp. 1 and Exp. 2) is shown.

Error bars represent SD.

Sld7 proteins were recovered (Figures 5B, 5D, 5F, and 5H). These results indicate that the association of Sld3, Cdc45, and Sld7 with the early-firing origins in G1 phase requires DDK.

High-Level DDK Facilitates Late Origin Firing

We also expected that an increase of DDK would enhance the redistribution of Sld3, Sld7, and Cdc45 over replication origins including the late-firing origins in G1 phase by facilitating the association of Sld3, Sld7, and Cdc45 with origins. When *GALp-DBF4* or *GALp-CDC7/DBF4* cells were arrested in G1 and released into HU-containing medium after the induction of *DBF4* or *CDC7-DBF4*, the late-firing origins, *ARS501* and *ARS1412*, fired (Figure 5I; Figure S5D) without changing the amount of Sld3, Sld7, and Cdc45 (Figure S2B). Consistent

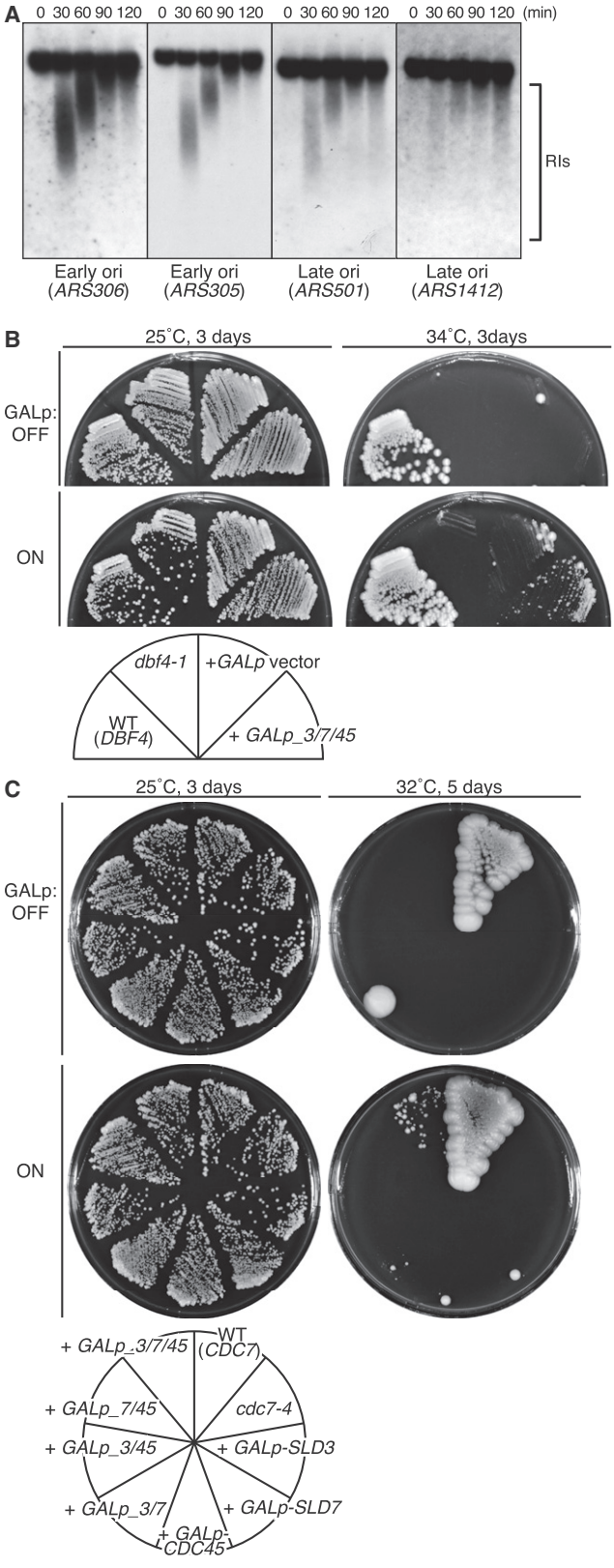

Figure 6. The DDK-Dependent Association of Sld3, Sld7, and Cdc45 with Origins Is a Novel Regulatory Step

(A) *bob1-1* cells (YK504) were synchronously released at 30˚C from G1 (alpha factor) arrest, collected at the indicated time, and analyzed using an alkaline gel, as shown in Figure 1B.

(B) Wild-type (WT, W303-1a *Δbar1*), *dbf4-1* (YST1439) cells, and *dbf4-1* cells harboring the *GALp* vector (YST1650) or *GALp_3/7/45* (YST1651) were grown on YPA-raffinose (GALp: OFF) and YPA-galactose (GALp: ON) plates at the indicated temperature.

(C) Wild-type (WT, W303-1a *Δbar1*), *cdc7-4* (YST1568) cells, and *cdc7-4* cells harboring *GALp-SLD3* (YST1607), *GALp-SLD7* (YST1582), *GALp-CDC45* (YST1569), *GALp_3/7* (YST1577), *GALp_3/45* (YST1573), *GALp_7/45* (YST1580), or *GALp_3/7/45* (YST1579) were grown on YPAD (GALp: OFF) and YPA-galactose (GALp: ON) plates at the indicated temperature.

with this, the recovery of the late-firing origin, *ARS501*, using ChIP employing 5×FLAG-Sld3 specifically increased in *GALp-DBF4* cells (Figure 5J). Moreover, genome-wide sequencing revealed that additional origins fired in the HU-arrested cells expressing Dbf4 or Cdc7 and Dbf4 (arrowheads in Figures 3D, 3E, 3I, and 3J; Figure S4). Most of these origins overlapped with those fired by the simultaneous expression of Sld3, Sld7, and Cdc45, although the number of newly fired origins was lower in *GALp-CDC7/DBF4* cells than in *GALp-SLD3/SLD7/CDC45* cells (Figure 3; Figure 4; Figure S4).

DDK-Dependent Associations of Sld3, Sld7, and Cdc45 with Origins Are Important for the Initiation of Replication

The essential role played by DDK in the initiation of DNA replication is the phosphorylation of the Mcm2-7 complex to activate a replicative helicase [22, 23]. Therefore, we further analyzed the regulation of Sld3-Sld7-Cdc45 association with origins by DDK, using the *bob1-1* mutation that occurs in *MCM5* and bypasses the requirement for DDK [24]. Consistent with the observations above, the late-firing origins, *ARS501* and *ARS1412*, were fired in the presence of HU in the *bob1-1* cells (Figure 6A). Moreover, simultaneous expression of *SLD3*, *SLD7*, and *CDC45* genes from the galactose promoter partially restored the growth of *dbf4-1* and *cdc7-4* cells at the non-permissive temperature (Figures 6B and 6C). These results suggest that the association of Sld3, Sld7, and Cdc45 with replication origins is one of the essential processes regulated by DDK and contributes to the firing timing of replication origins in budding yeast.

Discussion

How the timing of origin firing is determined is a long-standing question. Patel et al. [25] showed that Cdc7 is rate limiting in fission yeast for origin firing, and Wu and Nurse [26] proposed that differential recruitment of ORC, a component of the pre-RC, to origins, followed by competition among origins for the limiting replication factors, establishes the timing and the efficiency of origin firing in fission yeast. Wu and Nurse also showed that an increased dosage of Cdc45 and DDK accelerated firing of both efficient and inefficient origins, and thus they concluded that Cdc45 and DDK are limiting factors. Moreover, at the centromere of fission yeast, the interaction between Dbf4 and Swi6, which binds to the heterochromatin-like regions that include centromeres, facilitates the initiation of replication [27]. We have revealed the rate-limiting process that defines the timing of origin firing in budding yeast. Namely, the association between origins and Sld3-Sld7-Cdc45, which requires DDK, is a key step for determining the timing.

Although the simultaneous expression of Sld3, Sld7, and Cdc45 clearly affected the activation of origin firing, any combination of two of them, except Sld3 and Sld7, could not activate the late-firing origins (Figure 1C; Figure S2A). However, an increase of Sld3 and Sld7 proteins weakly activated the

late-firing origins at later time points (Figure 1C; Figure S2A), unlike the simultaneous expression of Sld3, Sld7, and Cdc45. At the moment, we do not know whether cells expressing both proteins activate the late origins specifically or escape the checkpoint. Further analysis is required for understanding this phenomenon.

One of the merits of limiting the number of origins that fire at once might be having unfired origins until late in S phase to rescue stalled or collapsed replication forks. Cells expressing high levels of Sld3, Sld7, and Cdc45 grew a little more slowly than wild-type but did not show remarkable sensitivity to DNA-damaging agents, such as HU and methyl methanesulfonate, which cause stalled or collapsed forks. Therefore, many origins may be kept inactive even in such cells by unknown mechanisms, although the activation pattern of origins is randomized and thus more origins are apparently activated in the cellular population. We do not know which factors or processes limit the replication fork establishment. The intra-S phase checkpoint might not be involved in this process, because simultaneous expression of Sld3, Sld7, and Cdc45 did not induce the phosphorylation of Rad53 (Figure 2C).

Overall progression of DNA replication did not accelerate in cells overproducing Sld3, Sld7, and Cdc45 (Figure 1D), whereas the genome-wide sequencing detected an almost three times higher number of origins in the same cells in comparison with wild-type cells (Figure 4). There are two ways to explain these observations. First, although the number of origins activated in a single cell might be higher than in wild-type cells, the elongation rate of replication forks would be slower. Second, although a higher number of origins activated in these cells, the number of activated origins in a single cell might be the same as in wild-type cells; the origins activated differ between cells because of randomized activation. So far, we cannot distinguish these possibilities.

The association between origins and Sld3-Sld7-Cdc45 requires DDK activity. This observation suggests a novel level of control of replication origins. The association between origins and Sld3-Sld7-Cdc45 is dynamic, so that these proteins can dissociate from the early-firing origins and reassociate with other origins, including the late-firing origins, in the presence of DDK activity. Thus, in the very early stages of initiation, a reversible association between origins and Sld3-Sld7-Cdc45 seems to take place. This reversible association mainly occurs at the early-firing origins during a period with low DDK activity (G1 phase in a normal cell cycle) in budding yeast, and this process is important for the temporal control of origin firing.

Overexpression of DDK allowed the late-firing origins to fire earlier without an increase of the protein levels of Sld3, Sld7, and Cdc45 (Figure 5; Figure S2B; Figure S5). Thus, DDK phosphorylates some of the replication proteins and/or proteins affecting the environment of replication origins to facilitate the interactions between replication proteins and origin DNAs. The Mcm2-7 complex is one candidate. DDK phosphorylates Mcm2-7 and functions in the association between the Mcm2-7-bound origins and Sld3-Sld7-Cdc45 to initiate DNA replication (Figures 5A–5H) [22, 23, 28]. Moreover, the *bob1-1* mutation in the Mcm5 subunit of the Mcm2-7 complex, which bypasses the requirement of DDK for the initiation of DNA replication [24], allows the late-firing origins to fire earlier (Figure 6A).

The reversible association between origins and Sld3-Sld7-Cdc45 may confer plasticity and probability on origin firing. During the period of the reversible association, some origins are preferably selected for the association of Sld3-Sld7-Cdc45, and this must be the key step of the temporal regulation. Although we still do not know what determines such preferentiality, chromatin structure might be one of the candidates. For example, silenced loci like *HMR* and telomeres replicate late [1]; a histone deacetylase, Rpd3, acts to regulate the timing of origin [29]; and artificial targeting of a HAT, Gcn5, to a late-firing origin causes hyperacetylation of the surrounding chromatin and earlier origin firing [29]. Therefore, the relationship between such chromatin modification and the origin association of Sld3, Sld7, and Cdc45 should be further elucidated.

The simultaneous expression of Sld3, Sld7, and Cdc45 allowed the activation of most of the confirmed origins in HU (Figure 3; Figure 4; Figure S4). Some of them are dormant origins (e.g., *ARS300*, *ARS600.2*, and *ARS608*), and some are origins located in the silenced *HML* locus (*ARS301*, *ARS302*, and *ARS303*) (Figure S4). However, about 20% of confirmed origins were still not activated. Therefore, the threshold of the protein levels of Sld3, Sld7, and Cdc45 for firing origins might vary between origins. This is also true for DDK expression, although the effect was much weaker than that of Sld3, Sld7, and Cdc45 expression (Figure S4). Very recently, a similar observation was reported [28]: the Cdc45 associations with the early-firing origins are reduced in a *cdc7-4* ts mutant even at the permissive temperature, and the reduced levels differ between origins. Thus, as we observed, origins may be classified by their sensitivity to the increased level of Sld3, Sld7, and Cdc45.

Experimental Procedures

Strains and Cell Growth

The yeast strains used had a W303 background and listed in Table S1. Conditions for cell growth and synchronization were performed as described previously [5, 30].

Antibodies

Rabbit polyclonal antibodies specific to Cdc45, Dbf4, Dpb11, Sld3, Sld7, and Mcm10 were raised against Cdc45-FLAG, Dbf4-His, His-Dpb11, and Mcm10-FLAG proteins expressed in *E. coli* and FLAG-Sld3 and Sld7 proteins expressed in yeast. The anti-Cdc45 and anti-Mcm10 antibodies did not recognize FLAG tags. The anti-Cdc45, Dpb11, Sld3, and Sld7 antibodies were purified using GST-Cdc45, His-Dpb11, GST-Sld3, and His-Sld7 protein-conjugated agarose beads, respectively. The rabbit polyclonal antibodies specific to Sld2, Dpb2, and Psf3 and the mouse monoclonal antibodies specific to Orc5 and Orc6 (SB5 and SB49, a generous gift from B. Stillman) have been described previously [5, 8, 31]. Goat polyclonal antibodies (sc-6749, Santa Cruz Biotechnology) were used to detect Rad53.

Alkaline Gel Analysis

Cells were lysed in a chromosome extraction solution (10 mM Tris [pH 8.0], 1 mM EDTA, 100 mM NaCl, 1% SDS, 1% Triton X-100, and 0.1 mg/ml zymolyase 100T [Seikagaku Biobusiness Corporation]), and chromosomal DNA was extracted using a mixture of phenol, chloroform, and 3-methyl-1-butanol. The isolated DNA was analyzed using alkaline gel as described previously [14].

Density-Transfer Experiment

The Messelson-Stahl density-transfer experiment was performed as described previously [5].

SOLiD and Data Analysis

The same DNA prepared for alkaline gel analysis was further purified using RNase A, Proteinase K treatment, and phenol/chloroform/3-methyl-1-butanol extraction. SOLiD deep sequencing was performed according to the manufacturer's standard protocol (Applied Biosystems).

Chromatin Immunoprecipitation

ChIP was performed as described previously [9, 32] using 0.5 μg of anti-FLAG M2 antibody, 1.0 μg of purified anti-Cdc45 or anti-Sld7 antibodies,

or 1.0 μg of the corresponding preimmune IgGs. The amount of DNA in the whole-cell extracts and coimmunoprecipitated fraction was measured using quantitative PCR employing specific primer sets (Table S2).

Flow Cytometry

Flow cytometry was performed as described previously [30].

Accession Numbers

Sequence data have been deposited to the Sequence Read Archive (http://www.ncbi.nlm.nih.gov/sra) with the accession number SRA048138.

Supplemental Information

Supplemental Information includes five figures, Supplemental Experimental Procedures, and two tables and can be found with this article online at doi:10.1016/j.cub.2011.11.038.

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