

Eukaryotic Origin-Dependent DNA Replication In Vitro Reveals Sequential Action of DDK and S-CDK Kinases

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SUMMARY

Proper eukaryotic DNA replication requires temporal separation of helicase loading from helicase activation and replisome assembly. Using an in vitro assay for eukaryotic origin-dependent replication initiation, we investigated the control of these events. After helicase loading, we found that the Dbf4-dependent Cdc7 kinase (DDK) but not S phase cyclin-dependent kinase (S-CDK) is required for the initial origin recruitment of Sld3 and the Cdc45 helicase-activating protein. Likewise, in vivo, DDK drives early-firing-origin recruitment of Cdc45 before activation of S-CDK. After S-CDK activation, a second helicase-activating protein (GINS) and the remainder of the replisome are recruited to the origin. Finally, recruitment of lagging but not leading strand DNA polymerases depends on Mcm10 and DNA unwinding. Our studies identify distinct roles for DDK and S-CDK during helicase activation and support a model in which the leading strand DNA polymerase is recruited prior to origin DNA unwinding and RNA primer synthesis.

INTRODUCTION

Since the identification of the first defined eukaryotic origins of replication in *S. cerevisiae* cells (Stinchcomb et al., 1979), a major goal has been to define the molecular mechanisms by which these sequences direct replication initiation. These short (80–120 bp) origins of replication contain an essential, conserved element called the ARS consensus sequence (ACS) that is bound by the eukaryotic initiator, the origin recognition complex (ORC, Bell and Dutta, 2002). With the exception of some embryonic tissues, the initiation of replication in metazoan organisms also occurs at reproducible positions; however, no consensus sequence is associated with these sites (Cadoret and Prioleau, 2010). Although in vitro assays for the initial helicase loading event at a defined origin exist (Remus and Diffley, 2009), the loaded helicases are inactive and assays for their activation

and for origin-dependent replication initiation have not been described.

The events of replication initiation are conserved throughout eukaryotes and occur in two temporally separated steps. Helicase loading occurs during G1, when Cdc6 and Cdt1 are recruited by ORC to origin DNA. These factors coordinately load a head-to-head double-hexamer of the Mcm2–7 replicative helicase around the origin DNA (Evrin et al., 2009; Gambus et al., 2011; Remus et al., 2009). The resulting pre-replicative complex (pre-RC) licenses the associated origin, but Mcm2–7 helicases remain inactive until S phase.

Initiation of replication is triggered by the activation of the S phase cyclin-dependent kinase (S-CDK) and Dbf4-dependent Cdc7 kinase (DDK) (Labib, 2010). These kinases stimulate binding of Cdc45 and GINS to Mcm2–7, resulting in the formation of the Cdc45-Mcm2–7-GINS (CMG) complex and helicase activation (Ilves et al., 2010). This event is also referred to as preinitiation complex formation (Sclafani and Holzen, 2007). In *S. cerevisiae* cells, S-CDK must phosphorylate two proteins, Sld2 and Sld3, to promote DNA replication (Tanaka et al., 2007; Zegerman and Diffley, 2007). Upon phosphorylation, Sld2 and Sld3 bind the BRCT-repeat protein Dpb11, and the formation of this complex facilitates GINS recruitment (Labib, 2010). S-CDK also stimulates formation of the preloading complex (pre-LC, Muramatsu et al., 2010), which is an unstable complex between Sld2, Dpb11, Pol ϵ , and GINS that forms independently of DNA. Mcm4 and Mcm6 are the only essential targets for DDK (Randell et al., 2010; Sheu and Stillman, 2010), although how this phosphorylation facilitates subsequent recruitment of Cdc45 and GINS is unclear. Recent data suggest that DDK phosphorylation of Mcm2–7 removes an inhibitory function of the Mcm4 N terminus (Sheu and Stillman, 2010) and that this event is regulated by at least two additional kinases (Randell et al., 2010). Although their targets are clear, the order of action of S-CDK and DDK has been controversial (Sclafani and Holzen, 2007).

Origin DNA must be unwound to generate the single-stranded DNA (ssDNA) template needed for polymerase function. The ssDNA-binding protein RPA associates with origin DNA prior to replication initiation (Tanaka and Nasmyth, 1998; Walter and Newport, 2000). After origin unwinding, Pol α /primase primes

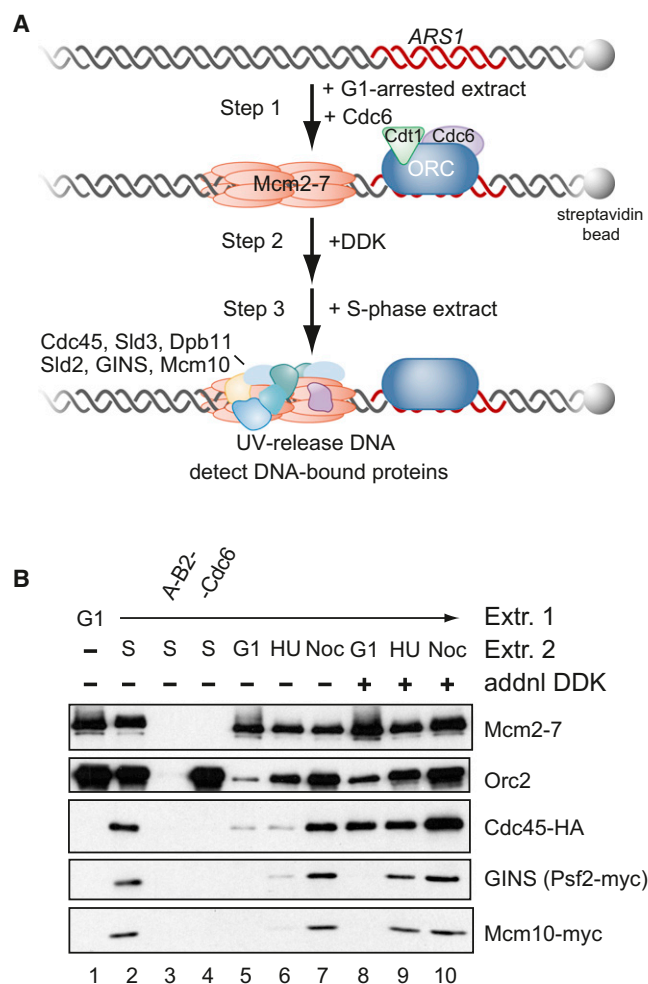


Figure 1. An Assay for Replisome Assembly In Vitro

(A) Schematic of replisome assembly assay. *ARS1* origin DNA was treated with three sequential incubations: step 1, Mcm2-7 loading in G1 extract supplemented with Cdc6; step 2, DDK phosphorylation of Mcm2-7; step 3, replisome assembly in S phase extract.

(B) Protein, substrate, and extract requirements for the replisome assembly assay. Replisome assembly assays were performed with or without DDK-inactivated yRH182 S phase extract (yRH182-S, lanes 1–4) or with α -factor (G1), hydroxyurea (HU)-, or nocodazol (Noc)-arrested extracts made from yRH182 expressing active DDK and overexpressing Cdc45, Dpb11, Sld2, and Sld3 (lanes 5–10). Unless indicated, wild-type (WT) *ARS1* DNA and Cdc6 were used in all reactions. *ARS1-A-B2-* is an *ARS1* mutant lacking ORC-binding sites. Additional DDK (125 ng) was added to the 2nd extract in lanes 8–10. Changes in ORC DNA association were likely due to release of ORC after pre-RC formation (lanes 5 and 8; Tsakraklides and Bell, 2010) and ORC rebinding after Cdc45, GINS, and Mcm10 recruitment. See also Table S2 and Table S3.

DNA synthesis on both strands, whereas Pol ϵ and Pol δ elongate the leading and lagging strands, respectively (Burgers, 2009). Although the Mcm10 protein moves with the replication fork and is required to stabilize the large subunit of DNA polymerase alpha (Pol α , Ricke and Bielinsky, 2004; Zhu et al., 2007), whether Mcm10 is involved in the initial recruitment of Pol α or other DNA polymerases to the replisome is unclear. The order of DNA poly-

merase origin recruitment and how their assembly depends on DNA unwinding also is uncertain.

Using a combination of purified initiation proteins and *S. cerevisiae* extracts, we describe assays that recapitulate events at replication origins as the cell cycle proceeds from G1 into S phase. In an S-CDK- and DDK-dependent manner, previously loaded Mcm2-7 helicases recruit multiple proteins required for origin activation. These interactions lead to helicase activation, recruitment of replicative DNA polymerases, and DNA replication initiation and elongation. Analysis of these assays reveals a preferred order of DDK and S-CDK function, and in vivo studies show that DDK is required during G1 for Cdc45 binding at early firing origins. In addition, we find that the recruitment of the leading and lagging strand DNA polymerases show different requirements for Mcm10 and DNA unwinding.

RESULTS

Recapitulating the G1 to S Phase Events of Replication In Vitro

A major obstacle to the recapitulation of eukaryotic DNA replication initiation in vitro is the incompatibility of the cell-cycle conditions required for helicase loading (G1) and activation (S). To overcome this hurdle, we simulated the G1 to S phase transition using a combination of *S. cerevisiae* extracts, similar to the approach used for nucleus- and origin-independent replication using *Xenopus* egg extracts (Walter et al., 1998). First, we used G1-arrested extract supplemented with purified Cdc6 to load the replicative helicase onto immobilized *ARS1* origin DNA (Bowers et al., 2004; Seki and Diffley, 2000). The loaded Mcm2-7 complexes were isolated from the G1 extract and activated by incubation with an S phase extract (Figure 1A).

S phase extracts were prepared from cells modified in two ways to enhance their replication capacity. First, these cells contained a temperature-sensitive allele in the DDK catalytic subunit Cdc7 and were arrested at the nonpermissive temperature before extract preparation. Thus, the arrested cells are poised for replication initiation with unreplicated DNA but elevated S-CDK levels. To compensate for a lack of DDK activity, we treated loaded Mcm2-7 with purified DDK prior to addition of S phase extract. Second, these cells overproduced Sld2, Sld3, Dpb11, and Cdc45, which are normally expressed at low levels (Ghaemmaghami et al., 2003). Thus, after origin loading in the G1 extract, the Mcm2-7 helicase is exposed to both essential replication-activating kinases and an extract containing a robust source of the proteins required for origin activation.

After sequential treatment of the loaded Mcm2-7 with DDK and S phase extracts, we observed origin association of the helicase activators Cdc45 and Psf2 (a GINS subunit) as well as Mcm10 (Figure 1B). These associations were dependent on the addition of S phase extract (Figure 1B, lanes 1 and 2), an intact origin sequence (A-B2-, lane 3), and prior Mcm2-7 loading (-Cdc6, lane 4). If the temperature-arrested S phase extract was replaced with extracts prepared from hydroxyurea (HU) or G1-arrested cells overexpressing Cdc45, Dpb11, Sld2, and Sld3, then Cdc45, GINS, and Mcm10 failed to associate (Figure 1B, lanes 5 and 6). Interestingly, providing additional DDK to these extracts restored recruitment of all three proteins to the HU

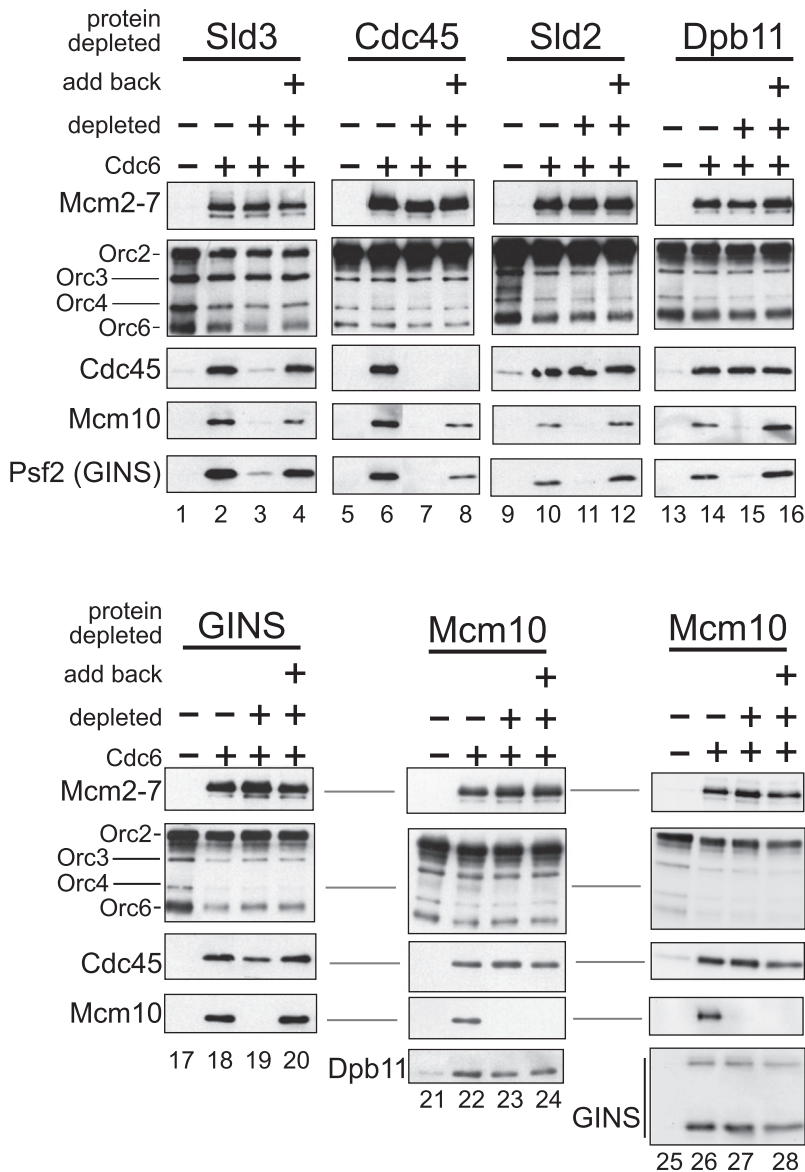


Figure 2. Interdependent Recruitment of Replication Proteins

Depletion of replication proteins reveals interdependent origin DNA association. S phase extracts were depleted for the indicated protein prior to replisome assembly assays. Associated proteins were analyzed by immunoblot. Depleted protein and extracts were as follows: Sld3, yRH208-S; Cdc45, yRH182-S; Sld2, yRH207-S; Dpb11, yRH209-S; GINS, yRH223-S; Mcm10, yRH183-S. For each panel, Cdc6 was omitted from reaction 1, S phase extracts were depleted for the indicated protein in reactions 3–4, and the corresponding purified protein (see Figure S1) was added in reaction 4. Note: purified Cdc45-FLAG and MBP-Mcm10 lack HA and myc tags, respectively. GINS was detected with a polyclonal antibody in lanes 25–28. See also Figure S1 and Figure S2 and Table S2 and Table S3.

indicating that the depleted extracts remained active and that other essential proteins had not been codepleted.

Analysis of the depleted extracts uncovered distinct requirements for the recruitment of the helicase-activating proteins Cdc45 and GINS (Figure 2). Only Sld3 depletion resulted in a loss of Cdc45 association, although depletion of GINS showed reduced Cdc45 recruitment. In contrast, Cdc45, Sld3, Sld2, and Dpb11 were each required for stable GINS recruitment. Finally, unlike studies of *Xenopus* Mcm10 (Wohlschlegel et al., 2002), Cdc45, Dpb11, and GINS associated with the origin DNA in the absence of Mcm10 (Figure 2). In addition, Mcm10 recruitment was eliminated by depletion of any of the other proteins tested.

A Biochemical Assay for Origin-Dependent Replication Initiation

Given that helicase-activating proteins were recruited to the origin-containing DNA template, we probed the reaction for the completion of later steps in the replication initiation process.

extract and Cdc45 recruitment to the G1 extract. In contrast, a nocodazole-arrested extract overexpressing the same four proteins (Figure 1B, lane 7) showed a similar pattern of protein recruitment with or without added DDK, suggesting that when all factors are present, there is no M phase barrier to replisome assembly. Together, these properties mirrored the hallmarks of origin activation in vivo.

Distinct Requirements for Cdc45 and GINS Origin Recruitment

We investigated the interdependencies of replication protein recruitment to origin DNA (Figure 2) by immunodepleting individual factors from the S phase extract and assessing the ability of other replication proteins to associate with the origin. In each case, addition of purified forms of the depleted protein (Figure S1 available online) restored replication protein recruitment, indi-

An ~1 kb linear template poorly supported Pol α recruitment and nucleotide incorporation (Figure 3A and data not shown). In contrast, a larger, 5.9 kb *ARS1* plasmid robustly supported both activities (Figures 3A and 3B). Reactions containing the plasmid template included 6-fold fewer copies of *ARS1* (due to less efficient bead attachment) than reactions with the 1 kb linear template (Figure 3A, compare ORC levels). Nevertheless, the templates showed similar levels of Mcm2–7 loading, and Pol α and replication levels were much higher for the plasmid template. Thus, plasmid DNA was more efficient for helicase loading, polymerase loading, and replication initiation.

To exclude the possibility that the observed nucleotide incorporation is the result of nonspecific repair events, we tested for properties expected for genuine replication products. Nucleotide incorporation was dependent on prior pre-RC formation and ATP hydrolysis (Figure 3C, lanes 1–3). Examination of the

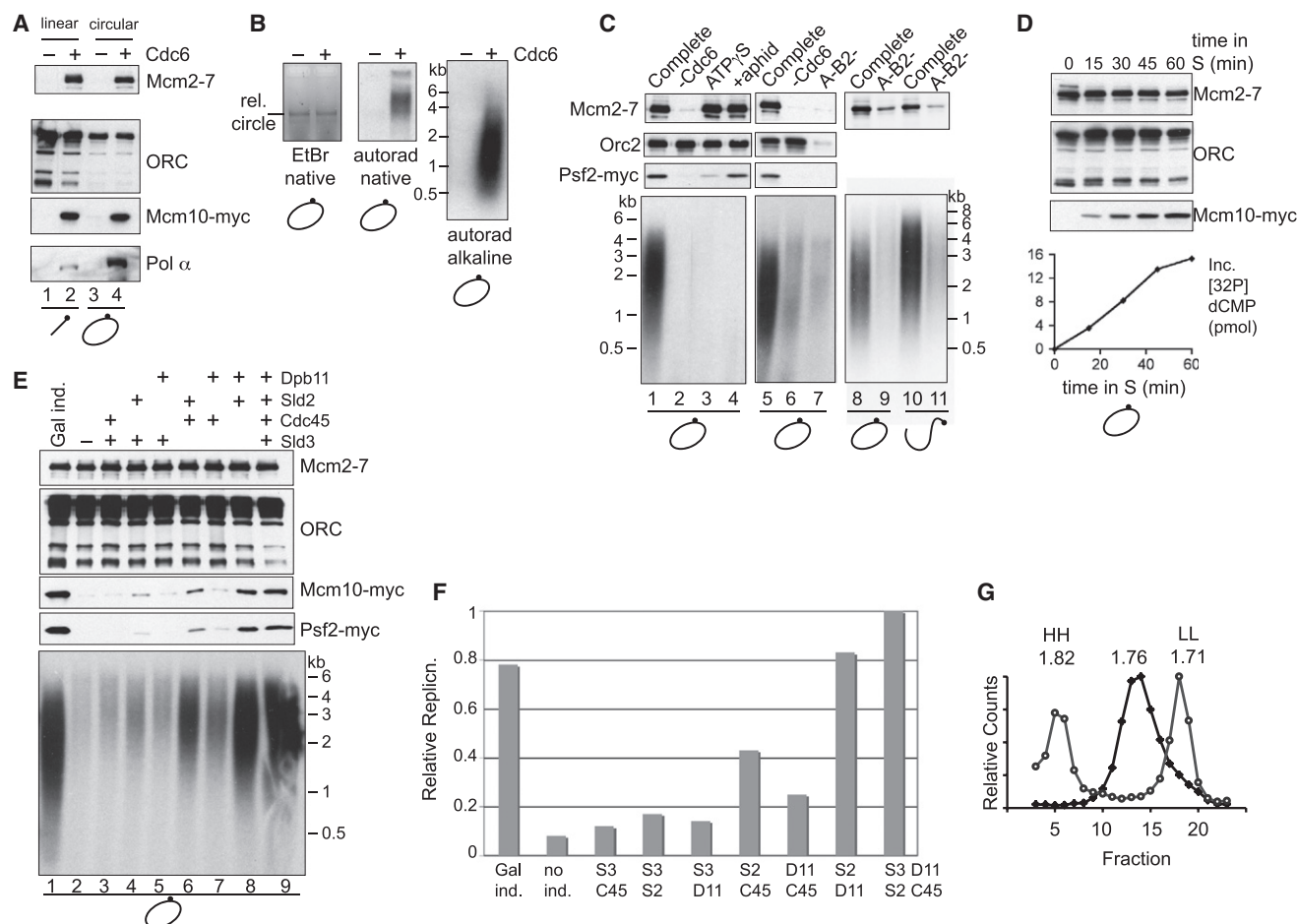


Figure 3. Long DNA Templates Support Polymerase Loading and Replication Initiation

(A) Circular templates show increased DNA Pol α association. Replisome assembly assays were performed with 1 kb linear *ARS1* DNA or pARS1/WT plasmid. Throughout this figure, lines and ovals below images indicate biotinylated linear and circular templates, respectively.

(B) Analysis of replication products. Replication assays were performed using yRH182-S extract on pARS1/WT template. Left, native gel of DNA products, ethidium bromide stain. The location of relaxed plasmid is indicated. Center, autoradiogram of the native gel. Right, autoradiogram of replication products analyzed by alkaline gel electrophoresis. The presence of Cdc6 during Mcm2-7 loading is indicated.

(C) Protein, template, and nucleotide requirements of the replication assay. Replication assays were performed with yRH182-S extract. Reactions lacking Cdc6 during helicase loading are indicated. Immunoblot (upper panels) and alkaline gel analysis (lower panels) of proteins and replication products are shown. Templates used: lanes 1-4, circular pARS1/WT (5.6 kb); lanes 5-7, circular pUC19-ARS1 (3.7 kb); lanes 8 and 9, circular pARS1/Nco-Nco (7.6 kb); lanes 10 and 11, linear pARS1/Nco-Nco (7.6 kb). Lanes 7, 9, and 11 use A-B2- derivatives of the indicated DNA. ATP γ S reactions replaced ATP and the ATP-regenerating system with 1 mM ATP γ S in step 3 of the assay. + aphid, 100 μ g/ml aphidicolin in step 3.

(D) Timecourse of Mcm10 recruitment and replication product accumulation. Replication assays using yRH182-S extract and pARS1/WT were analyzed by immunoblot of origin-associated proteins (upper panels) and nucleotide incorporation (lower panel).

(E and F) Relative contributions of overexpressed Cdc45 (C45), Dpb11 (D11), Sld2 (S2), and Sld3 (S3). Replication assays using pARS1/WT plasmid template and yRH182-S (lane 1) or yRH191-S (no overexpressed proteins, lanes 2-9) extracts were supplemented with the indicated purified replication proteins. Relative level of replication products (lower panel) was quantified and plotted in (F).

(G) Heavy-light analysis of replication products. Replication reactions were performed in the presence of 500 μ M BrdUTP in place of dTTP. Replication products were fractionated by CsCl gradient and detected by scintillation counter (black line). Heavy-heavy and light-light controls are shown (gray line). The CsCl density (g/ml) of the highest point in each peak is indicated.

See also Figure S1 and Table S2 and Table S3.

replication products by native agarose electrophoresis revealed that nucleotide incorporation was associated with a shift in the mobility of the plasmid (Figure 3B), consistent with formation of theta replication intermediates. Replication products synthesized in the presence of BrdUTP migrated at the position expected for heavy:light DNA in a CsCl gradient, indicating that

replication was semiconservative (Figure 3G). In addition, no reaction products were detected in the presence of aphidicolin, a potent inhibitor of eukaryotic replicative DNA polymerases (Figure 3C, lane 4), and nucleotide incorporation accumulated for at least 60 min of incubation (Figure 3D). Most importantly, the replication reaction depended on defined origin sequences.

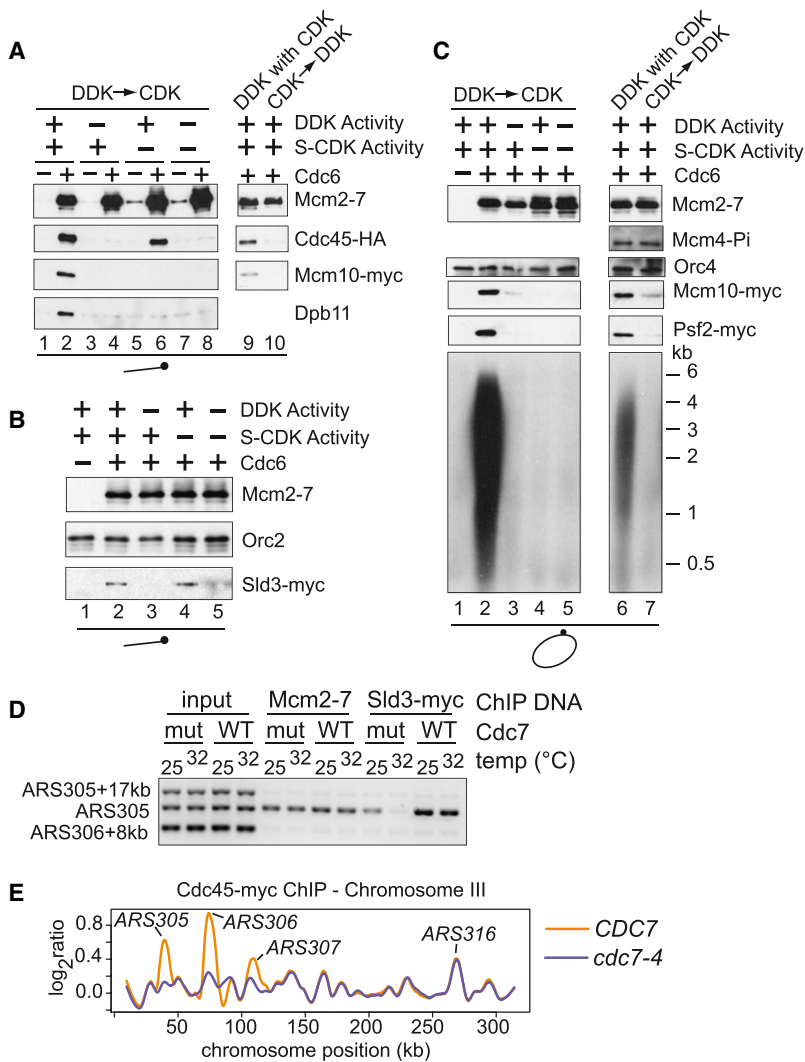


Figure 4. DDK and S-CDK Are Required for Distinct Stages of Origin Activation

(A and B) S-CDK and DDK are required for replisome assembly. Replisome assembly assays using extracts yRH166-S (A) or yRH229-S (B) were analyzed as described in Figure 1. DDK kinase activity was eliminated by omitting DDK from reaction step 2. S-CDK activity was blocked by the addition of GST-Sic1 to reaction step 3. *DDK with CDK*, DDK was omitted from reaction step 2 and added to reaction step 3 in which S-CDK is also active. *CDK → DDK*, reaction step 2 was eliminated. After step 3, purified GST-Sic1 and DDK were added sequentially and incubation was continued for 20 min.

(C) S-CDK and DDK are required for DNA replication. Replication assays used yRH182-S and pARS1/WT plasmid template. *Mcm4-Pi* immunoblot was probed with the Mcm4-phospho-S82-D83 phosphospecific antibody that recognizes a DDK target site in Mcm4 (Randell et al., 2010).

(D) Sld3 binding to ARS305 in G1 requires DDK. Either wild-type *CDC7* or congeneric *cdc7-4* strains including myc-tagged Sld3 were arrested in nocodazole and released into 25°C or 32°C media containing α -factor (Figure S3) and analyzed by ChIP using anti-Mcm2-7 or anti-myc antibodies. Samples were analyzed by PCR using primers recognizing ARS305 and two non-origin sequences (ARS305+17kb and ARS306+8kb) (Table S4).

(E) Cdc45 binding to early origins in G1 requires DDK. Either wild-type *CDC7* or congeneric *cdc7-4* strains including myc-tagged Cdc45 were arrested in media containing α -factor at 25°C (Figure S3) and analyzed by ChIP-Chip using anti-myc antibodies. The average log₂ ratios of immunoprecipitate (IP) to input signal from two experiments are plotted for chromosome III (wild-type, orange; *cdc7-4*, blue). Three early origins (ARS305, ARS306, and ARS307) and one late origin (ARS316) are indicated.

See also Figure S2 and Figure S3 and Table S1, Table S2, and Table S3.

DNA templates with an *ARS1* origin lacking an ORC binding site (A-B2) showed dramatically reduced replication (Figure 3C, lanes 5–11).

DNA length rather than supercoiling or the circular nature of the plasmid DNA was required for replication. Direct comparison of replication using a 7.6 kb *ARS1* plasmid (randomly biotinylated) or a linearized version of the same plasmid (biotinylated at one end) showed that the linear template replicated 2-fold more efficiently than its circular counterpart (Figure 3C, lanes 8–11), due to longer replication products. This finding suggests that the random attachment of the circular DNA to the magnetic bead inhibited replication by impeding replication forks. We determined total nucleotide incorporation and found that ~3% of the total plasmid DNA is replicated in the assay. Incomplete Mcm2-7 loading and replication elongation appear to be the primary reasons for the low levels of incorporation (see Discussion).

To determine whether the overexpression of Cdc45, Dpb11, Sld2, and Sld3 was important for DNA replication, we tested S phase extracts from cells with endogenous protein levels.

These extracts failed to either initiate replication or recruit GINS or Mcm10 (Figure 3E, lane 2). By adding purified and active forms of the limiting proteins (see Figure 2) to the S phase extract, we observed that Cdc45, Sld2, and Dpb11 were each limiting for both events (Figures 3E and 3F). Together, these data indicate that this assay accurately recapitulates replication initiation, displaying a dependence on a defined origin, the replicative DNA polymerases and multiple essential replication initiation proteins.

DDK and CDK Are Required For Distinct Steps in Origin Activation

We next asked how the DDK and CDK kinases affected replication factor recruitment and replication initiation. We eliminated DDK activity by omitting purified DDK and S-CDK activity by addition of the S-CDK inhibitor Sic1. Both kinases were essential for replication initiation, but their loss had distinct effects on replication protein recruitment (Figure 4). In the absence of DDK, none of the factors examined associated with the helicase-loaded origin DNA (Figures 4A–4C). In contrast, S-CDK

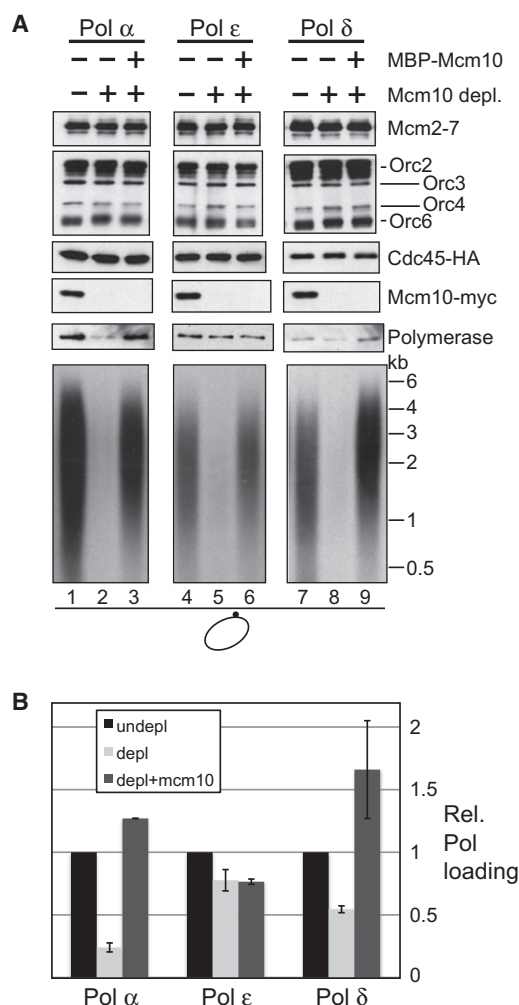


Figure 5. Mcm10 Is Required for the Recruitment of Pol α and Pol δ to Origin DNA

(A) Effect of Mcm10 depletion on DNA polymerase origin association. Replication assays were performed with pARS1/WT plasmid template and extract yRH183-S (lanes 1–3), yRH185-S (lanes 4–6), or yRH187-S (lanes 7–9). As indicated, extracts were depleted of Mcm10 and supplemented with MBP-Mcm10.

(B) Relative levels of DNA polymerase association. Two (Pol α and Pol ϵ) or three (Pol δ) iterations of the experiment in (A) were quantified and plotted. Polymerase recruitment in the undepleted extract was set to 1. Error bars = standard deviation from the mean.

See also Figure S1 and Table S2 and Table S3.

activity was required for Mcm10, Dpb11, and GINS association but not for Sld3 and Cdc45. The association of Cdc45 and Sld3 in the absence of S-CDK suggested that DDK drives the formation of an initial complex (DDK-dependent complex) that is then acted upon in an S-CDK-catalyzed event to recruit Dpb11, GINS, and Mcm10. Consistent with a more robust association of Cdc45 with origins upon entry into S phase (Aparicio et al., 1999), salt extraction experiments showed that Cdc45 association is stabilized by the recruitment of the S-CDK-dependent factors (Figure S2).

Our findings predict that Sld3 and Cdc45 origin recruitment depends on DDK; however, Sld3 and Cdc45 associate with early origins in G1 (Aparicio et al., 1999; Kamimura et al., 2001; Kanemaki and Labib, 2006), a time when Dbf4 is a target of APC-dependent degradation (Sclafani and Holzen, 2007). To address the role of DDK during the G1 recruitment of Cdc45 and Sld3, we compared their origin association in *CDC7* wild-type and temperature-sensitive (*cdc7-4*) cells. Due to reduced Cdc45 and Sld3 origin binding at 37°C in wild-type cells (data not shown), we performed this analysis at 25°C (Cdc45) or 32°C (Sld3). Using either ChIP-Chip (Cdc45) or ChIP-PCR (Sld3, Table S4), we found that association of Cdc45 and Sld3 with early firing origins during G1 (*ARS305*, *ARS306*, and *ARS307*) was reduced in *cdc7-4* cells (Figures 4D and 4E). Weak Cdc45 association with some late firing origins was not reduced by the *cdc7* mutation (Figure 4E, *ARS316*), potentially due to residual Cdc7 activity at 25°C. Analysis of sites of Cdc45 binding reduced in the *cdc7-4* background identified 49 origins, most of which initiate in the first 20% of S phase (Table S1). Thus, DDK is active in late G1 cells and drives the association of Cdc45 and Sld3 with early origins prior to the S-CDK-dependent recruitment of Sld2, Dpb11, and GINS.

Additional experiments support a model in which DDK acts prior to CDK at the origin. We observed the highest levels of replication protein origin association and replication initiation when the loaded helicases were exposed to DDK first, then exposed to CDK in the S phase extract (Figures 4A and 4C, DDK \rightarrow CDK). Addition of DDK to the S phase extract exposed the loaded helicases to both kinases simultaneously (DDK with CDK) and resulted in lower protein association and initiation. Finally, if loaded helicases were exposed to CDK and S phase extracts followed by CDK inactivation and addition of DDK (CDK \rightarrow DDK), we observed no replication initiation (Figure 4C). Intriguingly, under these conditions, association of Cdc45, GINS, and Mcm10 was dramatically reduced (Figures 4A and 4C), even though equivalent DDK phosphorylation of Mcm4 was observed (Figure 4C, lanes 6 and 7). This suggests that prior exposure to CDK prevents subsequent DDK phosphorylation of Mcm2–7 from driving origin recruitment of Cdc45 or GINS.

Distinct Requirements for Leading and Lagging DNA Polymerase Recruitment

Three DNA polymerases act at the eukaryotic replication fork, but the assembly of these enzymes at the replisome is poorly understood. Because of their affinity for ssDNA templates, DNA polymerase recruitment could require origin unwinding, but this has only been addressed for DNA Pol α (Walter and Newport, 2000). Alternatively, DNA polymerases could directly or indirectly interact with loaded helicase even in the absence of DNA unwinding. Mcm10 has been shown to interact with and stabilize Pol α /primase; however, its role in the initial recruitment of DNA polymerases is unknown.

We first asked whether Mcm10 was involved in DNA polymerase recruitment. Depletion of Mcm10 dramatically reduced Pol α loading and DNA replication (Figures 5A and 5B). These effects were not due to the destabilization of Pol α in the absence of Mcm10 (Ricke and Bielinsky, 2004) because addition of purified Mcm10 restored both events. Mcm10 depletion had little effect

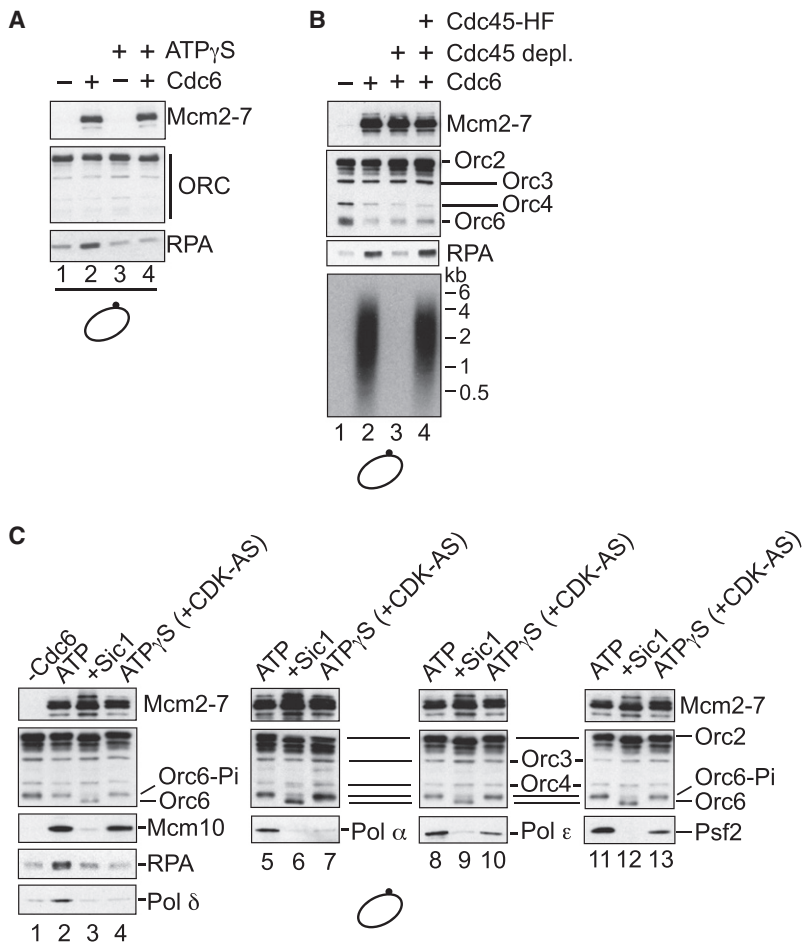


Figure 6. ATP Hydrolysis Is Required for RPA Loading and for the Loading of a Subset of Polymerases

(A) ATP hydrolysis requirement for RPA loading. Replisome assembly assays were performed using pARS1/WT plasmid templates and extract yRH184-S. Where indicated, ATP γ S was added after DDK phosphorylation of Mcm2-7 in place of ATP and the ATP-regenerating system in reaction step 3.

(B) Cdc45 was required for RPA loading and DNA replication. Replication assays were performed using pARS1/WT templates and extract yRH188-S. As indicated, extract was depleted for Cdc45 and supplemented with purified Cdc45-3HA/3Flag.

(C) ATP hydrolysis requirement for DNA polymerase recruitment. Replisome assembly reactions were performed using pARS1/WT plasmid templates and extracts yRH188-S (lanes 1–4), yRH184-S (lanes 5–7), yRH186-S (lanes 8–10), or yRH182-S (lanes 11–13). ATP: Reactions were performed under standard conditions. +Sic1: GST-Sic1 added to reaction step 3. ATP γ S+AS-CDK: During reaction step 3, ATP and the ATP-regenerating system were replaced with 1 mM ATP γ S, analog-specific CDK (CDK-AS), and 0.5 mM 6-benzyl-ATP.

See also Figure S1 and Figure S4 and Table S2 and Table S3.

ATP (Ubersax et al., 2003). We added purified Clb5-Cdk1-AS and a hydrolyzable form of the modified ATP (6-benzyl-ATP) along with ATP γ S to the S phase extract (Figure 6C). Importantly, in these conditions we observed CDK-dependent phosphorylation of Orc6 (Figure 6C) but only background levels of RPA association with origin DNA. Thus, in these conditions,

on Pol ϵ recruitment but reduced Pol δ association by half. Notably, addition of purified MBP-Mcm10 stimulated Pol δ recruitment and DNA synthesis compared with the unperturbed extract (Figure 5A, lanes 7–9, Figure 5B), suggesting that Mcm10 facilitates Pol δ origin recruitment and that Mcm10 was limiting for this event.

To investigate the connection between origin unwinding and replisome assembly, we monitored association of the ssDNA-binding protein RPA with the template DNA. RPA association with the circular template was dependent on ATP hydrolysis (Figure 6A, +ATP γ S), pre-RC formation (Figure 6A, -Cdc6), and Cdc45 (Figure 6B). ATP γ S was added after DDK phosphorylation of the Mcm2-7 complex. Consistent with DDK functioning in the ATP γ S reaction, Sld3 and Cdc45 are recruited to the origin under these conditions (Figure S4). Thus, Cdc45 and Sld3 do not require DNA unwinding for their recruitment, consistent with studies showing that inactivation of Mcm2-7 ATP-binding motifs does not interfere with Cdc45 recruitment (Ying and Gautier, 2005).

Because it was added to the reaction after DDK phosphorylation of loaded Mcm2-7, ATP γ S could prevent origin unwinding in two ways: inhibition of CDK activity and/or inhibition of Mcm2-7 ATPase activity. Because we knew the effects of CDK inhibition (Figure 4), we sought conditions in which ATP γ S specifically inhibited Mcm2-7. To this end, we exploited a mutant in Cdk1 (Cdk1-AS) that preferentially binds and hydrolyzes modified

ATP γ S inhibits an event downstream of CDK function required for DNA unwinding, most likely ATP hydrolysis by Mcm2-7.

To determine which replication proteins and DNA polymerases required DNA unwinding for origin recruitment, we assessed replication protein recruitment in the presence of ATP γ S, Clb5-Cdk1-AS, and 6-benzyl ATP (Figure 6C). Consistent with the restoration of CDK activity, the CDK-dependent recruitment of Mcm10 and GINS (Psf2) was not blocked under these conditions (Figure 6C, lanes 4 and 13). Thus, DNA unwinding is not required for Mcm10 or GINS origin association. Even though Mcm10 is present at the origin and is required for the loading of Pol α and Pol δ (Figure 5), these polymerases were not recruited in the absence of DNA unwinding. In contrast, in the same conditions, Pol ϵ was present at the origin DNA at similar levels as GINS. Both proteins show reduced recruitment in the presence of ATP γ S relative to ATP, most likely due to incomplete restoration of S-CDK activity. Thus, our findings support a model in which DNA unwinding is required for Pol α and δ recruitment, but Mcm10 and Pol ϵ are recruited independently of this event.

DISCUSSION

Although the temporal separation of helicase loading and activation in eukaryotes is critical for preventing genomic stability, the

multiple layers of control that prevent the re-replication of chromosomal DNA have made the examination of replication initiation *in vitro* difficult. To recapitulate origin-dependent replication *in vitro*, we used two extracts derived from different cell-cycle stages to independently drive the G1 and S phase events of DNA replication initiation. Importantly, the helicase activation and replisome initiation observed here show the hallmarks of these events *in vivo*: they are dependent on origin DNA, previously loaded Mcm2–7, as well as DDK and S-CDK and helicase-activating proteins. Furthermore, the reaction supports loading of all three replicative polymerases onto DNA and substantial, semiconservative duplication of the DNA template. Analysis of these assays revealed different roles for DDK and S-CDK during helicase activation and distinct requirements for leading and lagging strand polymerase recruitment.

Requirements for Origin-Dependent Replication Initiation

The *in vitro* origin-dependent replication assay provides insights into the fundamental requirements for this event. The difficulty of developing such an assay has led to many proposals to explain a lack of success. Because we see replication using a soluble extract and non-nucleosomal DNA templates (data not shown), we can conclude that an intact nucleus (Pasero and Gasser, 2002), chromatin loops (Cayrou et al., 2010), and a defined chromatin state are not required for origin-dependent replication initiation. Unlike *E. coli* replication (Bramhill and Kornberg, 1988), these studies indicate that eukaryotic origin DNA unwinding is not driven by negative DNA supercoiling, as long linear templates function well (Figure 3C). Finally, the absence of nucleosomal DNA argues that negative superhelicity stored in nucleosomes is not required for origin-dependent initiation. Although not essential, it is likely that one or more of these factors enhances replication *in vivo*. In contrast to these nonessential factors, we found that overexpression of Cdc45, Dpb11, and Sld2 (Figure 3) and the sequential addition of G1 and S phase extracts (Figure 1) were critical for replication initiation. Consistent with the intra-S phase checkpoint inhibiting DDK and Sld3 in the HU-arrested extracts (Lopez-Mosqueda et al., 2010; Zegerman and Diffley, 2010), we found that addition of excess DDK in the context of overexpressed Sld3 restored origin association of Cdc45, GINS, and Mcm10 (Figure 1B). Finally, these assays demonstrate that *in vitro* loaded Mcm2–7 complexes (Randell et al., 2006; Seki and Diffley, 2000) are competent for replication initiation.

The length of the DNA template also contributed to origin-dependent replication initiation (Figure 3). Studies in *Xenopus* egg extracts also reported a lack of initiation on short, linear DNA templates, although the reason for this defect was unclear (Edwards et al., 2002). Interestingly, we found that the 1 kb template showed dramatically reduced association of DNA Pol α . The ORC-binding site is only 180 bp from the unattached end of the 1 kb template, suggesting that a larger region of ORC-adjacent DNA is required either to unwind origin DNA (required for DNA Pol α recruitment, Figure 6) or to assemble a pair of complete replisomes.

Although the proportion of DNA that was replicated in the assay was modest (~3% of total circular plasmid DNA repli-

cated), considering the length of the replication products as well as the extent of Mcm2–7 loading, the efficiency of Mcm2–7 activation was much higher. Because on average only 1/4 to 1/3 of the length of the circular plasmid DNA was replicated, the percentage of plasmids that undergo replication initiation is 3–4 times the amount of total DNA replicated (9%–12%). Assuming that two Mcm2–7 hexamers are assembled at each origin (Remus et al., 2009), we find that 12%–20% of plasmids have loaded Mcm2–7 complexes. Comparing the percentage of plasmids that underwent replication (9%–12%) with those that loaded Mcm2–7 (12%–20%) suggests that 45% or more of the loaded Mcm2–7 complexes initiate replication in the assay. Modified assays that do not require bead-coupling of the DNA or that improve the extent of Mcm2–7 loading are likely to enhance the extent of replication. Importantly, despite the incomplete replication observed, the strong dependence of the assay on origin DNA and all of the replication initiation proteins tested makes it a powerful tool to investigate their function.

DDK Acts before CDK during the Initiation Reaction

Our findings support a model in which DDK drives the association of Cdc45 and Sld3 with Mcm2–7 prior to CDK action and GINS recruitment. First, we found that DDK but not S-CDK was required for the initial origin recruitment of Cdc45 and Sld3 (Figure 4) and that addition of DDK to G1 extracts overexpressing limiting replication proteins also drove Cdc45 association (Figure 1B). Second, our depletion studies are consistent with Cdc45 and Sld3 interacting prior to GINS and Mcm10 (Figure 2). Third, *in vivo* studies showed that DDK was required for the previously described (Aparicio et al., 1999; Kamimura et al., 2001; Kanemaki and Labib, 2006) association of Cdc45 and Sld3 with early firing origins during G1, a time when S-CDK is inactive. Finally, we found that the order of kinase action influenced both replication factor recruitment and replication initiation, with the most robust replication being observed when Mcm2–7 was treated with DDK prior to CDK (Figure 4). In contrast to some previous studies (Kubota et al., 2003; Takayama et al., 2003), we did not see a requirement for GINS to observe Cdc45 association, although we did see more stable Cdc45 origin association when GINS was present (Figure 2 and Figure S2). This difference is consistent with increased Cdc45 origin association in S phase relative to G1 (Aparicio et al., 1999) and the GINS independence of Cdc45 origin binding versus the requirement of GINS for Cdc45 association with origin-adjacent DNA (Kanemaki and Labib, 2006). These phenomena almost certainly reflect interactions before and after the completion of CMG complex assembly (Ilves et al., 2010). Our findings also are consistent with studies indicating that Cdc45 and Sld3 require each other for their origin recruitment (Kamimura et al., 2001; Takayama et al., 2003) and *S. pombe* studies indicating that Sld3 recruitment is dependent on DDK (Yabuuchi et al., 2006). In addition, the lack of Sld3 in the soluble, S-CDK-dependent complex composed of Sld2, Dpb11, GINS, and Pol ϵ (pre-LC, Muramatsu et al., 2010) is consistent with the recruitment of these proteins to the origin through interaction with the already origin-associated Sld3.

This order of events has important implications for the control of helicase activation. Loading Cdc45 and Sld3 before S-CDK action would ensure that the S-CDK-dependent interaction between Sld3, Dpb11, and Sld2 (Tanaka et al., 2007; Zegerman and Diffley, 2007) and the associated recruitment of GINS always occur at origins and not in solution, preventing formation of soluble CMG complexes. The DDK-dependent loading of Cdc45 during G1 is most robust at the earliest firing origins (Figure 4 and Figure S3), suggesting that Mcm2–7 complexes loaded at these origins are particularly sensitive to levels of DDK phosphorylation. Interestingly, we observed low levels of Cdc45 at a subset of later-firing origins in G1 cells, suggesting that G1 recruitment of Cdc45 is not exclusive to early firing origins. Finally our studies provide clear evidence that DDK acts during G1 phase. Thus, the primary mechanism preventing helicase activation prior to S phase is the inhibition of S-CDK activity.

The order of DDK and S-CDK function we observe is consistent with findings in cell-free *Xenopus* egg extracts (Jares and Blow, 2000; Walter, 2000) where it was observed that DDK acts before CDK to drive replication initiation. These studies also observed an inability to initiate if DDK acted after CDK was inhibited. Under these conditions we observed an inability to recruit Cdc45 and GINS, despite similar levels of Mcm4 phosphorylation by DDK (Figures 4A and 4C). This suggests that exposure of loaded Mcm2–7 to S-CDK prior to DDK inhibits Cdc45 association but not DDK phosphorylation. Because we see reduced but detectable initiation when loaded Mcm2–7 is exposed to DDK and S-CDK simultaneously (Figure 4C), we propose that mechanisms exist to coordinate the DDK- and S-CDK-dependent events when both kinases are present. In contrast to these findings, studies of budding yeast DDK and S-CDK function in vivo suggested that DDK could only function for DNA replication after S-CDK has been activated (Nougarède et al., 2000). Although it has previously been suggested that species-specific differences accounted for this discrepancy (Sclafani and Holzen, 2007), our studies suggest that the difference is more likely to be due to different experimental approaches. In particular, the more complex requirements for kinase activity as hundreds of origins initiate during S phase passage in vivo may not reflect the kinase function at individual origins.

Leading and Lagging Strand DNA Polymerase Recruitment

Our studies reveal distinct requirements for origin recruitment of the leading (Pol ϵ) and lagging strand (Pol α and δ) DNA polymerases and suggest that Pol ϵ is recruited prior to Pol α and δ at the origin. Two prior in vivo findings support this order of polymerase assembly. First, the observation that Pol α is not required for Pol ϵ association at stalled replication forks (Masumoto et al., 2000) is consistent with independent association of these factors with the replisome. Second, the presence of Pol ϵ in the pre-LC (Muramatsu et al., 2010) suggests that Pol ϵ associates with the origin at the same time as Sld2, Dpb11, and the GINS. Because only Pol α /primase can initiate DNA synthesis, the prior recruitment of Pol ϵ would ensure that leading strand DNA polymerases are present prior to synthesis of any RNA primers. Whether

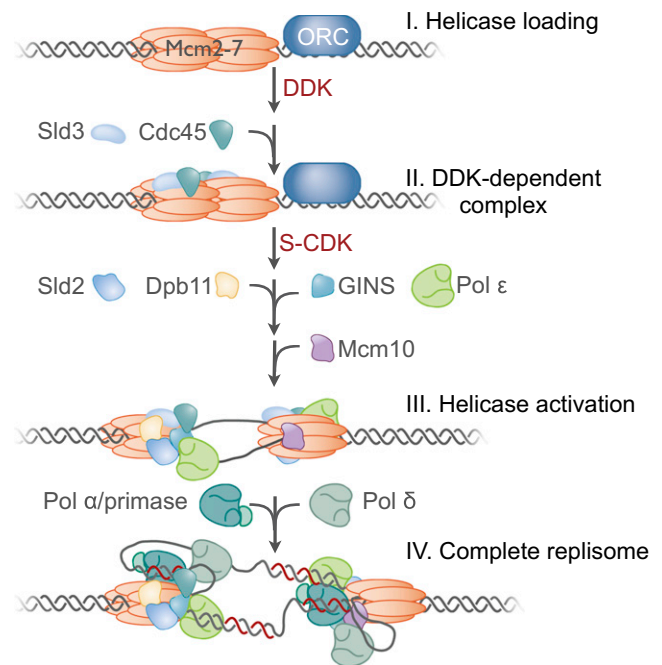


Figure 7. A Model for the Events Leading to Replication Initiation

(I) Helicase loading. The Mcm2–7 helicase is loaded to origin DNA in an inactive form during late M/G1 phase of the cell cycle to form the pre-RC. (II) DDK-dependent complex formation. In late G1 or S phase, DDK targets Mcm2–7 for phosphorylation, allowing recruitment of Sld3 and Cdc45. (III) Helicase activation and phosphorylation of Sld2 and Sld3 trigger the recruitment of Sld2, Dpb11, GINS, and Pol ϵ , and the subsequent recruitment of Mcm10. The formation of the Cdc45-Mcm2–7-GINS complex activates the helicase, triggering melting of origin DNA. (IV) Complete replisome assembly. Pol α and Pol δ are loaded on the unwound DNA in an Mcm10-dependent process to complete replisome assembly.

there is a mechanism to ensure that Pol δ is present prior to Pol α /primase remains to be determined.

Our analysis of Mcm10 function contrasts with earlier studies in yeast and *Xenopus*. We found that Mcm10 origin recruitment required Sld3, Cdc45, Sld2, Dpb11, and GINS; however, *S. cerevisiae* studies found that Mcm10 is recruited to origins in G1 (Homesley et al., 2000; Ricke and Bielinsky, 2004). Similarly, studies in *Xenopus* extracts found that Mcm10 associates with chromatin before S-CDK and DDK are activated (Wohlschlegel et al., 2002). Although we found that Mcm10 is required for replication and DNA polymerase recruitment, both Cdc45 and GINS were recruited in the absence of Mcm10. In contrast, the *Xenopus* studies found that Mcm10 is required for Cdc45 chromatin association. The importance of Mcm10 for Cdc45 DNA association is unclear in yeast cells (Gregan et al., 2003; Ricke and Bielinsky, 2004; Sawyer et al., 2004). The simplest explanation for these discrepancies is that the absence of nucleosomal DNA alters the requirements for Mcm10 and Cdc45 recruitment in our assay. Although we may be assessing a subset of Mcm10 functions, the extensive protein requirements for Mcm10 origin recruitment and the requirement of Mcm10 for Pol α and δ recruitment and replication support the functional relevance of our observations. Indeed, Mcm10

is known to move with the replication fork and interact with and stabilize the large subunit of DNA Pol α (Ricke and Bielinsky, 2004; Zhu et al., 2007), all of which are consistent with our observations.

Based on previous work and our investigation of helicase activation factor and polymerase recruitment in this study, we propose a framework for the assembly of the replisome (Figure 7). Briefly, during late G1 or early S phase, DDK phosphorylates the Mcm2–7 helicase, promoting the stable recruitment of Sld3 and Cdc45. Next, S-CDK-dependent phosphorylation of Sld2 and Sld3 leads to their Dpb11 binding and recruitment of GINS and Pol ϵ (most likely as a complex). These proteins then serve to both recruit Mcm10 and activate the Mcm2–7 helicase, which uses ATP hydrolysis to unwind the origin DNA. Pol α and Pol δ can then be loaded on ssDNA, leading to the formation of a complete replisome with accessory proteins such as PCNA, Mrc1, and Ctf4.

The development of an *S. cerevisiae* in vitro origin-dependent replication assay provides powerful tools to analyze replication in the future. One important goal will be to attribute more specific molecular function to the different initiation proteins and to elucidate the mechanism and regulation of DNA transactions such as origin melting and initial primer synthesis. The ability to substitute seven different purified proteins into corresponding depleted extracts will allow rapid analysis of mutant protein function in vitro and analysis of the corresponding mutants in vivo. In addition, the ease of epitope tagging in budding yeast cells will facilitate the identification of additional proteins that contribute to replication. The development of related assays that use nucleosomal DNA templates will allow direct assessment of the effects of nucleosomes on replication initiation. Finally, the origin dependence of the assay provides approaches to assess the interactions between the origin DNA and replication proteins during the initiation process.

EXPERIMENTAL PROCEDURES

Experimental procedures are described in detail in the [Extended Experimental Procedures](#).

Yeast Strains and Plasmid Construction

The *S. cerevisiae* strains and plasmids used in this study are listed in [Table S2](#) and [Table S3](#), respectively.

Protein Purification

Cdc6 and MBP-Mcm10 were purified from *E. coli* cells. DDK, Clb5-Cdk1-AS, Sld2, Sld3, Dpb11, and GINS were purified from *S. cerevisiae* cells.

Preparation of ARS1 DNA-Coupled Beads

The 1 kb linear ARS1 DNA template was generated by PCR as described (Tsakraklides and Bell, 2010). Plasmid DNAs were biotinylated and purified using photoprobe (long arm) biotinylation reagent (Vector Laboratories). Linear pARS1/Nco-Nco templates were prepared by restriction digest followed by biotinylation at one end. Biotinylated DNAs were coupled to streptavidin-coated magnetic beads.

Preparation of Whole-Cell Extracts

Yeast cultures were grown to mid-log phase in YP-glycerol followed by cell-cycle arrest and induction of GAL1, 10 expression by galactose. Cells were arrested in G1 by addition of α -factor, in S phase by incubation at 38°C or addi-

tion of HU, or at G2/M using nocodazole. Whole-cell extracts were prepared using a SPEX 6870 Freezer/Mill. Extracts were immunodepleted for 1 hr at 4°C by incubating with 1/10 volume of antibody-linked agarose beads with 3–4 repetitions.

Replisome Assembly and Replication Assays

Replisome assembly and replication assays were performed in three steps: helicase loading, DDK phosphorylation, and replisome assembly/replication. Helicase loading was performed in reactions including an ATP regenerating system, purified Cdc6, and G1-arrested whole-cell extract. Replisome assembly assays contained 1 pmol of the 1 kb ARS1 linear DNA template, and replication assays contained 175 fmol of ARS1-containing plasmid or linear DNA as indicated. Reactions were incubated at 25°C for 20 min while shaking. After helicase loading, beads were magnetically isolated and soluble material removed. The beads were resuspended in a DDK reaction mixture including ATP and purified DDK. Reactions were incubated at 25°C for 15 min with shaking followed by removal of the soluble material. For replisome assembly assays, DDK-treated bead-associated protein-DNA complexes were transferred to reaction mixtures containing an ATP-regenerating system and S phase extract and incubated for 20 min at 25°C with shaking. After incubation, beads were washed and the DNA was released from the beads by exposure to UV light and analyzed by SDS-PAGE and immunoblotting. Unless noted, replisome assembly assays were performed with the 1 kb ARS1 linear DNA template. Replication assays were performed as for the replisome assembly assays except for the following: (1) step 3 included 200 μ M ribonucleoside triphosphates (rNTPs) and 40 μ M deoxyribonucleoside triphosphates (dNTPs) (including [α -³²P]dCTP) and were incubated for 45–60 min with shaking; (2) after washing beads, DNA and associated proteins were released by boiling in SDS-containing buffer; and (3) DNA replication products were analyzed by alkaline or native agarose gel electrophoresis. Reactions supplemented with purified proteins contained 300 nM Sld2-Flag, 100 nM Sld3-Flag, 175 nM Dpb11-Flag, 300 nM Cdc45-3HA/3Flag, 225 nM GINS, 35 nM MBP-Mcm10, 800 nM GST-Sic1, or 240 nM Clb5-Cdk1-AS as indicated. Antibodies used for immunoblotting were as follows: Mcm2–7, UM185; ORC, 1108 (Bowers et al., 2004); anti-HA (12CA5); anti-myc (9E10); Dpb11, sc-12004 (Santa Cruz Biotech); Mcm4-Pi, 213/Mcm4-phospho-S82-D83 (Randell et al., 2010); Rfa1, gift from S. Brill (Rutgers University, Piscataway, NJ, USA).

ACCESSION NUMBERS

The GEO accession number for Cdc45 ChIP-Chip is GSE29646.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, four figures, and four tables and can be found with this article online at doi:10.1016/j.cell.2011.06.012.

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REFERENCES

- Aparicio, O.M., Stout, A.M., and Bell, S.P. (1999). Differential assembly of Cdc45p and DNA polymerases at early and late origins of DNA replication. *Proc. Natl. Acad. Sci. USA* *96*, 9130–9135.
- Bell, S.P., and Dutta, A. (2002). DNA replication in eukaryotic cells. *Annu. Rev. Biochem.* *71*, 333–374.
- Bowers, J.L., Randell, J.C., Chen, S., and Bell, S.P. (2004). ATP hydrolysis by ORC catalyzes reiterative Mcm2-7 assembly at a defined origin of replication. *Mol. Cell* *16*, 967–978.
- Bramhill, D., and Kornberg, A. (1988). Duplex opening by dnaA protein at novel sequences in initiation of replication at the origin of the *E. coli* chromosome. *Cell* *52*, 743–755.
- Burgers, P.M. (2009). Polymerase dynamics at the eukaryotic DNA replication fork. *J. Biol. Chem.* *284*, 4041–4045.
- Cadoret, J.C., and Prioleau, M.N. (2010). Genome-wide approaches to determining origin distribution. *Chromosome Res.* *18*, 79–89.
- Cayrou, C., Coulombe, P., and Méchali, M. (2010). Programming DNA replication origins and chromosome organization. *Chromosome Res.* *18*, 137–145.
- Edwards, M.C., Tutter, A.V., Cvetic, C., Gilbert, C.H., Prokhorova, T.A., and Walter, J.C. (2002). MCM2-7 complexes bind chromatin in a distributed pattern surrounding the origin recognition complex in *Xenopus* egg extracts. *J. Biol. Chem.* *277*, 33049–33057.
- Evrin, C., Clarke, P., Zech, J., Lurz, R., Sun, J., Uhle, S., Li, H., Stillman, B., and Speck, C. (2009). A double-hexameric MCM2-7 complex is loaded onto origin DNA during licensing of eukaryotic DNA replication. *Proc. Natl. Acad. Sci. USA* *106*, 20240–20245.
- Gambus, A., Khoudoli, G.A., Jones, R.C., and Blow, J.J. (2011). MCM2-7 form double hexamers at licensed origins in *Xenopus* egg extract. *J. Biol. Chem.* *286*, 11855–11864.
- Ghaemmaghami, S., Huh, W.K., Bower, K., Howson, R.W., Belle, A., Dephoure, N., O'Shea, E.K., and Weissman, J.S. (2003). Global analysis of protein expression in yeast. *Nature* *425*, 737–741.
- Gregan, J., Lindner, K., Brimage, L., Franklin, R., Namdar, M., Hart, E.A., Aves, S.J., and Kearsley, S.E. (2003). Fission yeast Cdc23/Mcm10 functions after pre-replicative complex formation to promote Cdc45 chromatin binding. *Mol. Biol. Cell* *14*, 3876–3887.
- Homesley, L., Lei, M., Kawasaki, Y., Sawyer, S., Christensen, T., and Tye, B.K. (2000). Mcm10 and the MCM2-7 complex interact to initiate DNA synthesis and to release replication factors from origins. *Genes Dev.* *14*, 913–926.
- Ilves, I., Petojevic, T., Pesavento, J.J., and Botchan, M.R. (2010). Activation of the MCM2-7 helicase by association with Cdc45 and GINS proteins. *Mol. Cell* *37*, 247–258.
- Jares, P., and Blow, J.J. (2000). *Xenopus* cdc7 function is dependent on licensing but not on XORC, XCdc6, or CDK activity and is required for XCdc45 loading. *Genes Dev.* *14*, 1528–1540.
- Kamimura, Y., Tak, Y.S., Sugino, A., and Araki, H. (2001). Sld3, which interacts with Cdc45 (Sld4), functions for chromosomal DNA replication in *Saccharomyces cerevisiae*. *EMBO J.* *20*, 2097–2107.
- Kanemaki, M., and Labib, K. (2006). Distinct roles for Sld3 and GINS during establishment and progression of eukaryotic DNA replication forks. *EMBO J.* *25*, 1753–1763.
- Kubota, Y., Takase, Y., Komori, Y., Hashimoto, Y., Arata, T., Kamimura, Y., Araki, H., and Takisawa, H. (2003). A novel ring-like complex of *Xenopus* proteins essential for the initiation of DNA replication. *Genes Dev.* *17*, 1141–1152.
- Labib, K. (2010). How do Cdc7 and cyclin-dependent kinases trigger the initiation of chromosome replication in eukaryotic cells? *Genes Dev.* *24*, 1208–1219.
- Lopez-Mosqueda, J., Maas, N.L., Jonsson, Z.O., Defazio-Eli, L.G., Wohlschlegel, J., and Toczyski, D.P. (2010). Damage-induced phosphorylation of Sld3 is important to block late origin firing. *Nature* *467*, 479–483.
- Masumoto, H., Sugino, A., and Araki, H. (2000). Dpb11 controls the association between DNA polymerases alpha and epsilon and the autonomously replicating sequence region of budding yeast. *Mol. Cell. Biol.* *20*, 2809–2817.
- Muramatsu, S., Hirai, K., Tak, Y.S., Kamimura, Y., and Araki, H. (2010). CDK-dependent complex formation between replication proteins Dpb11, Sld2, Pol (epsilon), and GINS in budding yeast. *Genes Dev.* *24*, 602–612.
- Nougarède, R., Della Seta, F., Zarzov, P., and Schwob, E. (2000). Hierarchy of S-phase-promoting factors: yeast Dbf4-Cdc7 kinase requires prior S-phase cyclin-dependent kinase activation. *Mol. Cell. Biol.* *20*, 3795–3806.
- Pasero, P., and Gasser, S.M. (2002). In vitro DNA replication assays in yeast extracts. *Methods Enzymol.* *351*, 184–199.
- Randell, J.C., Bowers, J.L., Rodríguez, H.K., and Bell, S.P. (2006). Sequential ATP hydrolysis by Cdc6 and ORC directs loading of the Mcm2-7 helicase. *Mol. Cell* *21*, 29–39.
- Randell, J.C., Fan, A., Chan, C., Francis, L.I., Heller, R.C., Galani, K., and Bell, S.P. (2010). Mec1 is one of multiple kinases that prime the Mcm2-7 helicase for phosphorylation by Cdc7. *Mol. Cell* *40*, 353–363.
- Remus, D., and Diffley, J.F. (2009). Eukaryotic DNA replication control: lock and load, then fire. *Curr. Opin. Cell Biol.* *21*, 771–777.
- Remus, D., Beuron, F., Tolun, G., Griffith, J.D., Morris, E.P., and Diffley, J.F. (2009). Concerted loading of Mcm2-7 double hexamers around DNA during DNA replication origin licensing. *Cell* *139*, 719–730.
- Ricke, R.M., and Bielinsky, A.K. (2004). Mcm10 regulates the stability and chromatin association of DNA polymerase-alpha. *Mol. Cell* *16*, 173–185.
- Sawyer, S.L., Cheng, I.H., Chai, W., and Tye, B.K. (2004). Mcm10 and Cdc45 cooperate in origin activation in *Saccharomyces cerevisiae*. *J. Mol. Biol.* *340*, 195–202.
- Sclafani, R.A., and Holzen, T.M. (2007). Cell cycle regulation of DNA replication. *Annu. Rev. Genet.* *41*, 237–280.
- Seki, T., and Diffley, J.F. (2000). Stepwise assembly of initiation proteins at budding yeast replication origins in vitro. *Proc. Natl. Acad. Sci. USA* *97*, 14115–14120.
- Sheu, Y.J., and Stillman, B. (2010). The Dbf4-Cdc7 kinase promotes S phase by alleviating an inhibitory activity in Mcm4. *Nature* *463*, 113–117.
- Stinchcomb, D.T., Struhl, K., and Davis, R.W. (1979). Isolation and characterization of a yeast chromosomal replicator. *Nature* *282*, 39–43.
- Takayama, Y., Kamimura, Y., Okawa, M., Muramatsu, S., Sugino, A., and Araki, H. (2003). GINS, a novel multiprotein complex required for chromosomal DNA replication in budding yeast. *Genes Dev.* *17*, 1153–1165.
- Tanaka, S., Umemori, T., Hirai, K., Muramatsu, S., Kamimura, Y., and Araki, H. (2007). CDK-dependent phosphorylation of Sld2 and Sld3 initiates DNA replication in budding yeast. *Nature* *445*, 328–332.
- Tanaka, T., and Nasmyth, K. (1998). Association of RPA with chromosomal replication origins requires an Mcm protein, and is regulated by Rad53, and cyclin- and Dbf4-dependent kinases. *EMBO J.* *17*, 5182–5191.
- Tsakraklides, V., and Bell, S.P. (2010). Dynamics of pre-replicative complex assembly. *J. Biol. Chem.* *285*, 9437–9443.
- Ubersax, J.A., Woodbury, E.L., Quang, P.N., Paraz, M., Blethrow, J.D., Shah, K., Shokat, K.M., and Morgan, D.O. (2003). Targets of the cyclin-dependent kinase Cdk1. *Nature* *425*, 859–864.
- Walter, J., and Newport, J. (2000). Initiation of eukaryotic DNA replication: origin unwinding and sequential chromatin association of Cdc45, RPA, and DNA polymerase alpha. *Mol. Cell* *5*, 617–627.
- Walter, J., Sun, L., and Newport, J. (1998). Regulated chromosomal DNA replication in the absence of a nucleus. *Mol. Cell* *1*, 519–529.
- Walter, J.C. (2000). Evidence for sequential action of cdc7 and cdk2 protein kinases during initiation of DNA replication in *Xenopus* egg extracts. *J. Biol. Chem.* *275*, 39773–39778.
- Wohlschlegel, J.A., Dhar, S.K., Prokhorova, T.A., Dutta, A., and Walter, J.C. (2002). *Xenopus* Mcm10 binds to origins of DNA replication after Mcm2-7 and stimulates origin binding of Cdc45. *Mol. Cell* *9*, 233–240.
- Yabuuchi, H., Yamada, Y., Uchida, T., Sunathvanichkul, T., Nakagawa, T., and Masukata, H. (2006). Ordered assembly of Sld3, GINS and Cdc45 is distinctly regulated by DDK and CDK for activation of replication origins. *EMBO J.* *25*, 4663–4674.

Ying, C.Y., and Gautier, J. (2005). The ATPase activity of MCM2-7 is dispensable for pre-RC assembly but is required for DNA unwinding. *EMBO J.* 24, 4334–4344.

Zegerman, P., and Diffley, J.F. (2007). Phosphorylation of Sld2 and Sld3 by cyclin-dependent kinases promotes DNA replication in budding yeast. *Nature* 445, 281–285.

Zegerman, P., and Diffley, J.F. (2010). Checkpoint-dependent inhibition of DNA replication initiation by Sld3 and Dbf4 phosphorylation. *Nature* 467, 474–478.

Zhu, W., Ukomadu, C., Jha, S., Senga, T., Dhar, S.K., Wohlschlegel, J.A., Nutt, L.K., Kornbluth, S., and Dutta, A. (2007). Mcm10 and And-1/CTF4 recruit DNA polymerase alpha to chromatin for initiation of DNA replication. *Genes Dev.* 21, 2288–2299.

EXTENDED EXPERIMENTAL PROCEDURES

Yeast Strains and Plasmid Construction

DNAs encoding Dpb11 and Cdc45-3HA were amplified and cloned on either side of the *GAL1,10* promoter in the vector pAS584 to create pRH113. DNAs encoding Sld3-Flag (pRH105), Dpb11-Flag (pRH105), or Sld2-Flag (pRH120) were amplified and cloned adjacent to the *GAL1,10* promoter in pAS584. DNAs encoding Psf2-Flag and Sld5 were amplified and cloned on either side of the *GAL1,10* promoter in the vector pAS584 to create pRH108. The open reading frames of Psf1 and Psf3 were amplified and cloned on either side of the *GAL1-10* promoter and cloned into pRS403 to create pRH109. The open reading frames of Sld2 and Sld3 were amplified and cloned either individually adjacent to (Sld2, pRH110 and Sld3, pRH111) or on either side of (pRH114) the *GAL1-10* promoter and cloned into pRS307. To create the ATP analog-specific Cdk1-AS expression vector pCib5FLAG-Cdc28_as1_His6, DNA encoding Cib5-Flag and Cdk1-as1-His6 [F88G, I291T] were amplified and ligated into pAS584 digested with XhoI/BssHII and NotI, respectively. The Mcm10 coding region was ligated into EcoRI- and XbaI-digested pMAL-c2PP to create plasmid pRH121. The Sic1 coding region was ligated into BamHI- and XhoI-digested pGEX-4T-2 (GE Healthcare) to create pGEX-Sic1. The Cdc45-3HA coding region was cloned into pAS584 downstream of the *GAL1* promoter to generate pAS584-CDC45-3HA. A DNA fragment encoding 3xFlag was cloned in frame with and at the 3' end of CDC45-3HA to generate pAS584-CDC45-CHA3FLAG. pARS1/Nco-Nco was constructed by cloning the genomic NcoI fragment containing *ARS1* into pBS/SK+. pUC19-ARS1/wild-type (WT) was constructed by PCR amplification of a *ARS1*-containing genomic fragment using the following primers:

ARS1 500 upstream: TCATTCTTTCAAGAATTCCAC

ARS1 Down 500: GAGACGCATGCGGATGTTTTGGCTCTGGTCA

ARS1 is approximately in the center of the resulting 1.1 kb DNA fragment which was cloned into the EcoRI and SphI sites of pUC19. Site directed mutagenesis was used to generate A-B2-mutations of pUC19/*ARS1* and pARS1/Nco-Nco. A complete list of plasmids used in this study is shown in Table S3.

The plasmids pRH113 and pRH114 were integrated into the genome of yRH116 to construct strain yRH166. Additional epitope tagging of initiation proteins was done as previously described (Longtine et al., 1998). ySC331 and yWL17 were made by epitope tagging the Sld3 and Cdc45 proteins in the *cdc7-4* strain OAy711. ySC332 and yWL18 were made by transforming ySC331 and yWL17 with the wild-type *CDC7* gene and selecting for growth at 37°C. ySC303 was constructed by integrating pCib5FLAG-Cdc28_as1_His6 into yRH122. A complete list of the yeast strains used in this study is shown in Table S2.

Purification of FLAG-Tagged Proteins from Yeast

C-terminally Flag-tagged Sld2, Sld3, and Dpb11 were overexpressed and purified from *S. cerevisiae* strains yRH152, yRH153, and yRH154, respectively. Cdc45-3HA/3Flag was purified from strain ySK-Cdc45. GINS was purified from strain yRH156, which overexpressed the GINS subunits Sld5, Psf1, Psf2-Flag, and Psf3. Yeast cells were grown to saturation at 30°C in YP-galactose, harvested, washed, and resuspended in 1/4 volume of 100 mM HEPES-KOH (pH 7.6), 0.8 M sorbitol, 5 mM EDTA, 300 mM KGlut (buffer L + 300 mM KGlut). The resulting cell suspension was frozen dropwise in liquid N₂, lysed using a SPEX SamplePrep 6870 Freezer/Mill for 10 cycles of 2 min crushing at a rate of 10 impacts per second. After the resulting cell powder was thawed, 5 M NaCl was added to the extract while mixing to obtain a final concentration of 0.7 M NaCl. The lysate was then incubated for 15 min at 4°C before centrifugation. After centrifugation at 75,000 g for 1 hr, the soluble extract was diluted to a conductivity equivalent to 150 mM NaCl, and purified using anti-Flag M2 affinity gel (Sigma) according to manufacturer instructions using 50 mM HEPES-KOH (pH 7.6), 2 mM DTT, 1 mM EDTA, 10% glycerol, 0.02% NP-40, 300 mM KGlut (buffer H + 300 mM KGlut) as the equilibration and wash buffer. Proteins were eluted in buffer H + 100 mM KGlut containing 0.15 mg/ml 3xFLAG peptide.

Purification of Cib5-Cdk1-AS

ySC303 cells were grown to mid-log phase at 25°C in YP-glycerol overnight before shifting to 37°C. After one hour at 37°C, expression of Cib5-Flag and Cdk1(as1)-His6 was induced by the addition of 2% galactose for 5 hr at 37°C. Cells were harvested, washed, and resuspended in 1/4 volume of buffer L + 1.5 M KGlut and frozen dropwise in liquid N₂. Extracts were made by grinding frozen cell pellets with SPEX SamplePrep 6870 Freezer/Mill as described above followed by centrifugation in a Beckman 70Ti rotor at 45,000 rpm for 45 min. Extract was incubated with anti-FLAG M2 affinity gel (Sigma) according to the manufacturer instructions for 4 hr at 4°C, and the resin was washed with buffer H + 300 mM KGlut lacking DTT and EDTA. Proteins were eluted with 0.15 mg/ml 3xFLAG peptide in the same buffer. Cib5-FLAG/Cdk1(as1)-His6 was further purified by incubating peak fractions eluted from the FLAG column with Talon resin (Clontech) using buffer H + 300 mM KGlut lacking DTT and EDTA as the column equilibration and wash buffer. Cib5-Cdk1-AS was eluted with 200 mM imidazole in the same buffer.

Purification of MBP-Mcm10

pRH121 was transformed into Rosetta 2(DE3, pLysS) *E. coli* cells and grown to log phase. Cells were induced with 0.5 mM IPTG at 30°C for 4 hr and resuspended in 20 mM Tris-HCl (pH 7.4), 200 mM NaCl, 1 mM EDTA, 1 mM DTT, 10% glycerol (buffer T). After lysis, cells were spun at 37,000 rpm in a Beckman 70Ti rotor for 1 hr, then soluble extract was subjected to amylose resin affinity chromatography (New England Biolabs) according to manufacturer's instructions using buffer T as the equilibration and wash buffer, and buffer T + 10 mM maltose as the elution buffer. MBP-Mcm10 containing fractions were pooled, diluted to a conductivity of

150 mM NaCl, and then further purified by chromatography on SP-sepharose. This column was equilibrated in buffer T + 150 mM NaCl, washed with the same buffer, and proteins were eluted using a linear gradient of buffer T containing 150 mM–500 mM NaCl. Peak fractions containing MBP-Mcm10 eluted at approximately 400 mM NaCl.

Purification of GST-Sic1

pGEX-Sic1 was transformed into BL21(DE3, pLysS) *E. coli* cells and grown to log phase. Cells were induced with 0.5 mM IPTG, and resuspended in buffer H + 400 mM KCl. After lysis, soluble extract was subjected to affinity chromatography on glutathione sepharose (GE Healthcare) according to manufacturer instructions using buffer H + 400 mM KCl as the column equilibration and wash buffers. Protein was eluted using buffer H + 400 mM KCl containing 33 mM glutathione.

Preparation of ARS1-DNA-Coupled Beads

The 1 kb linear *ARS1* DNA template was generated and coupled to beads as described (Tsakraklides and Bell, 2010). Plasmids were biotinylated and purified using photoprobe (long arm) biotinylation reagent (Vector Laboratories) according to manufacturer instructions except UV exposure was reduced to 20 s. Linear pARS1/Nco-Nco templates were prepared by digesting with BamHI and SacII and filled in with Klenow 3' → 5' Exo-minus (New England Biolabs) using biotin-14-dATP (Invitrogen), dGTP, dCTP and dTTP. Only the BamHI site incorporates nucleotides. Biotinylated DNAs were coupled to streptavidin-coated magnetic beads (5 pmol DNA/mg beads) in kilobase binding buffer (Invitrogen) overnight at room temperature.

Preparation of Whole-Cell Extracts

Yeast pre-cultures grown to saturation in YPD were used to inoculate larger (typically 2–4 l) cultures using YP +2% glycerol for the growth media. Cells were grown overnight at 25°C until mid-log phase (OD_{600} of 0.5–1.0). Then, galactose was added to 2% to activate the *GAL1-10* promoter and incubation was continued for 2 hr. Cells were arrested in G1 by addition of α -factor to a final concentration of 100 ng/ml and incubated for an additional 3.5 hr. Growth of cells for S phase extract was identical except that cells were arrested at 38°C for 5 hr in place of α -factor arrest. For other extracts, 200mM HU or 10 μ g/ml nocodazole were added 2 hr after galactose induction and the cells grown for an additional 3.5 hr.

After harvesting cells by centrifugation at 5000 rpm for 5 min at 4°C, cells were washed twice with buffer containing 50 mM HEPES-KOH (pH 7.6), 2 mM EDTA, 0.8 M sorbitol, 300 mM KGlut, and 3 mM DTT. Cells were then resuspended in 1/4 to 1/3 packed cell volume of a lysis buffer containing 100 mM HEPES-KOH (pH 7.6), 0.8 M sorbitol, 10 mM Mg(OAc)₂, 1.5 M KGlut, 5 mM DTT, and 2X Complete protease inhibitors (Roche), and frozen dropwise in liquid N₂. Frozen cell “beads” were stored at –80°C until needed.

Yeast cell beads were loaded into a vial pre-chilled in liquid N₂ and crushed using a SPEX 6870 Freezer/Mill for ten cycles of 2 min at a rate of 10 impacts per second. The resulting powder was transferred to a centrifuge tube and allowed to thaw completely on ice. Lysate was then centrifuged in a Beckman 70Ti rotor at 45,000 rpm for 1 hr, and the resulting supernatant was dialyzed against 50 mM HEPES-KOH (pH 7.6), 1 mM EDTA, 1 mM EGTA, 5 mM Mg(OAc)₂, 10% glycerol, 300 mM KGlut, and 3 mM DTT at 4°C for 3–4 hr to reduce the salt concentration. Extracts were flash frozen in liquid N₂ and stored at –80°C.

To immunodeplete extracts, 1/10 volume of antibody-linked agarose beads pre-equilibrated with dialysis buffer was added to extracts and incubated for 1 hr at 4°C with gentle rotation. Tubes were centrifuged at 3000 rpm for 30 s at 4°C and the depleted extracts were removed to new tubes. Immunodepletion was repeated for 2–3 more times until the target protein was undetectable. Replication proteins were immunodepleted using the following resins: Cdc45, anti-HA agarose (Sigma); Sld2, Sld3, Dpb11, and GINS, anti-Flag M2-agarose (Sigma); Mcm10, anti-c-myc-agarose (Sigma).

Replisome Assembly and Replication Reactions

Helicases were loaded onto bead-bound DNA in 40 μ l reactions containing 25 mM HEPES-KOH (pH 7.6), 20 mM creatine phosphate, 40 μ g/ml creatine kinase, 2 mM DTT, 225 mM potassium glutamate (KGlut), 12 mM Mg(OAc)₂, 3 mM ATP, 300 nM purified Cdc6, and 750 μ g of G1 arrested whole-cell extract (typically from strain ySC15). Replisome assembly assays contained 1 pmol of the 1 kb *ARS1* linear DNA template and replication assays contained 175 fmol of *ARS1*-containing plasmid or linear DNA as indicated. Reactions were incubated at 25°C for 20 min while shaking in an Eppendorf Thermomixer at 1250 rpm.

After helicase loading, the beads were isolated by placing the tubes in a magnetic stand and the pre-RC reaction mixtures were removed and discarded. The beads were then resuspended in a 30 μ l DDK reaction mixture containing 50 mM HEPES-KOH (pH 7.6), 3.5 mM Mg(OAc)₂, 225 mM KGlut, 3 mM DTT, 1 mM ATP, 5% glycerol, 1 mM spermine, and purified DDK. The DDK concentration used in the assay varied depending on the activity of a particular preparation and was determined by prior titration. Reactions were incubated at 25°C for an additional 15 min with continuous shaking.

After DDK-phosphorylation, the DNA-beads were isolated by placing tubes in a magnetic stand and the DDK reaction mixture discarded and replaced with 40 μ l replisome-assembly mixtures containing 25 mM HEPES-KOH (pH 7.6), 20 mM creatine phosphate, 2 mM DTT, 40 μ g/ml creatine kinase, 225 mM KGlut, 12 mM Mg(OAc)₂, 3 mM ATP, and 750 μ g S phase extract. Beads were resuspended and incubated for 20 min at 25°C with shaking. Upon completion of the reaction, the beads were washed three times with 50 mM HEPES-KOH (pH 7.6), 1 mM EDTA, 1 mM EGTA, 5 mM Mg(OAc)₂, 10% glycerol, 225 mM KGlut, and 0.02% NP-40. The protein-DNA complexes were released from the beads by suspending them in 10 mM Tris-HCl (pH 7.6), 1 mM EDTA, 50 mM KGlut (15 μ l) and exposed to 254 nm UV light for 30 min at 4°C. Proteins were analyzed by SDS-PAGE and immunoblotted with the

antibodies listed in the [Experimental Procedures](#). Replication assays were performed as for the replisome assembly assays except that the final reaction step also contained 200 μ M NTPs, 40 μ M [α - 32 P]dCTP (4000–5000 cpm/pmol), 40 μ M dATP, dGTP, and dTTP and were incubated at 25°C for 45 min with shaking. After washing beads, DNA and associated proteins were released for immunoblot analysis and replication product analysis by boiling in Laemmli buffer for 5 min. Replication products were analyzed by alkaline gel electrophoresis through a 0.8% agarose gel at 6 V/cm for 3 hr with 30 mM NaOH and 2 mM EDTA as the buffer. Native gel analysis was performed by incubation of bead-associated DNA in 20 mM EDTA, 0.5% SDS, and 0.2 mg/ml proteinase K at 37°C for 30 min. Released DNA was analyzed by electrophoresis at 6 V/cm for 1 hr through a 0.8% TAE agarose gel. Both types of gels were dried, exposed to a phosphorimager screen, and then autoradiographed.

CsCl Analysis of Replication Products

Replication reactions were performed as described above with the linear pARS1/Nco-Nco template except 500 μ M BrdUTP replaced dTTP in the reaction. After completion of the replication reaction, the products were released from the beads by cutting with HaeIII and separated in a CsCl gradient as described ([Walter et al., 1998](#)). Gradients were fractionated by dripping from the bottom. The location of newly replicated DNA was determined by scintillation counting. Control DNA was generated by PCR in the presence of either dTTP (light-light) or BrdUTP (heavy-heavy) as well as [α - 32 P] dCTP. The resulting DNA products were mixed and separated by CsCl gradient and the location of the DNAs was determined by scintillation counting.

Chromatin Immunoprecipitation

Cdc45 ChIP-Chip was performed using yWL17 and yWL18. Sld3 ChIP-PCR was performed using ySC331 and ySC332. For Sld3 ChIP, prior to performing ChIP, cells were initially arrested using nocodazole at G2/M for 3 hr at 25°C. After nocodazole arrest, cells were washed with water and resuspended in YPD containing α -factor either at 25°C (Cdc45) or 32°C (Sld3) and grown for an additional 3 hr. For the Cdc45 ChIP cells were grown at the permissive temperature and arrested in α -factor for three hours prior to ChIP analysis. FACS analysis confirmed the nocodazole (Sld3) and α -factor arrest ([Figure S3](#)). ChIP analysis ([Aparicio et al., 1997](#)) and ChIP-chip ([Chen et al., 2007](#)) was performed as previously described.

Primers used for ChIP-PCR analysis are shown in [Table S4](#).

Analysis of Genome-wide Cdc45 ChIP

We used the R package *sma* (described in [Dudoit et al., 2000](#)) to normalize the ratio of immunoprecipitate (IP) to input signal from four Cdc45 ChIP-chip microarray experiments (two using yWL17 (*cdc45-13myc cdc7-4*) and two using yWL18 (*cdc45-13myc CDC7*). We then averaged these numbers for the two yWL17 replicates, as well as for the two yWL18 replicates. Using R's loess function (R development team, 2008) we smoothed these averaged datasets and plotted them against each other to visualize the extent to which Cdc45 binding is dependent on Cdc7 (shown for Chromosome 3 in [Figure 4E](#)).

To quantify the dependence of Cdc45 binding on Cdc7, we computed the ratio of the unsmoothed WT average to the unsmoothed *cdc7-4* average at each probed position across the genome. We identified peak regions in this set of ratios by computing p-values of the ratios and calling peak regions where ever at least three consecutive probes have p values less than 0.05. The resulting peak regions all overlapped with OriDB ([Nieduszynski et al., 2007](#)) identified origins: 46 “confirmed” (named) origins and 3 “likely” (unnamed) origins ([Table S1](#)).

We determined Trep numbers (replication times, in minutes) at each of these origins from a heavy:light timing study ([Raghuraman et al., 2001](#)) and a copy number timing study ([Yabuki et al., 2002](#)) and computed the corresponding Trep indices as follows. The index of an origin is the ratio of the difference between the Trep at that origin and the minimum Trep across the genome, to the difference between the maximum and minimum Treps across the genome. These numbers are shown in the last two columns of [Table S1](#).

We then compared the Trep numbers at our 49 Cdc7-dependent origins to the Trep numbers at 562 OriDB identified origins (only origins identified by OriDB as “confirmed” or “likely” were considered). For the heavy:light timing study ([Raghuraman et al., 2001](#)) we found that the mean of the Trep indices at the sites of Cdc7-dependent Cdc45 binding is 0.15 with standard deviation 0.08, compared to mean 0.34 and standard deviation 0.16 for all OriDB origins. In the copy number timing study ([Yabuki et al., 2002](#)) we found that the mean of the Trep indices at the sites of Cdc7-dependent Cdc45 binding is 0.15 with standard deviation 0.09, compared to mean 0.40 and standard deviation 0.19 for the OriDB origins.

To compare these Trep numbers we superimposed the histogram of the Trep numbers for our Cdc7-dependent origins over the histogram of the Trep numbers for the 562 OriDB origins, binning the numbers in bins of 2 min for both the heavy:light ([Figure S3A](#)) and the copy number study ([Figure S3B](#)).

SUPPLEMENTAL REFERENCES

Aparicio, O.M., Weinstein, D.M., and Bell, S.P. (1997). Components and dynamics of DNA replication complexes in *S. cerevisiae*: Redistribution of MCM proteins and Cdc45p during S phase. *Cell* 91, 59–69.

Bowers, J.L., Randell, J.C., Chen, S., and Bell, S.P. (2004). ATP hydrolysis by ORC catalyzes reiterative Mcm2-7 assembly at a defined origin of replication. *Mol. Cell* 16, 967–978.

Chen, S., de Vries, M.A., and Bell, S.P. (2007). Orc6 is required for dynamic recruitment of Cdt1 during repeated Mcm2-7 loading. *Genes Dev.* 21, 2897–2907.

- Dudoit, S., Yang, Y.H., Callow, M., and Speed, T. (2000). Statistical methods for identifying differentially expressed genes in replicated experiments, Technical report #578, August 2000, <http://www.stat.berkeley.edu/users/terry/zarray/Html/matt.html>.
- Francis, L.I., Randell, J.C., Takara, T.J., Uchima, L., and Bell, S.P. (2009). Incorporation into the prereplicative complex activates the Mcm2-7 helicase for Cdc7-Dbf4 phosphorylation. *Genes Dev.* 23, 643–654.
- Longtine, M.S., McKenzie, A., 3rd, Demarini, D.J., Shah, N.G., Wach, A., Brachat, A., Philippsen, P., and Pringle, J.R. (1998). Additional modules for versatile and economical PCR-based gene deletion and modification in *Saccharomyces cerevisiae*. *Yeast* 14, 953–961.
- Marahrens, Y., and Stillman, B. (1992). A yeast chromosomal origin of DNA replication defined by multiple functional elements. *Science* 255, 817–823.
- Nieduszynski, C.A., Hiraga, S., Ak, P., Benham, C.J., and Donaldson, A.D. (2007). OriDB: a DNA replication origin database. *Nucleic Acids Res.* 35 (*Database issue*), D40–D46.
- R Development Core Team (2008). R: A Language and Environment for Statistical Computing (Vienna, Austria: R Foundation for Statistical Computing).
- Raghuraman, M.K., Winzeler, E.A., Collingwood, D., Hunt, S., Wodicka, L., Conway, A., Lockhart, D.J., Davis, R.W., Brewer, B.J., and Fangman, W.L. (2001). Replication dynamics of the yeast genome. *Science* 294, 115–121.
- Tsakraklides, V., and Bell, S.P. (2010). Dynamics of pre-replicative complex assembly. *J. Biol. Chem.* 285, 9437–9443.
- Walter, J., Sun, L., and Newport, J. (1998). Regulated chromosomal DNA replication in the absence of a nucleus. *Mol. Cell* 1, 519–529.
- Yabuki, N., Terashima, H., and Kitada, K. (2002). Mapping of early firing origins on a replication profile of budding yeast. *Genes Cells* 7, 781–789.

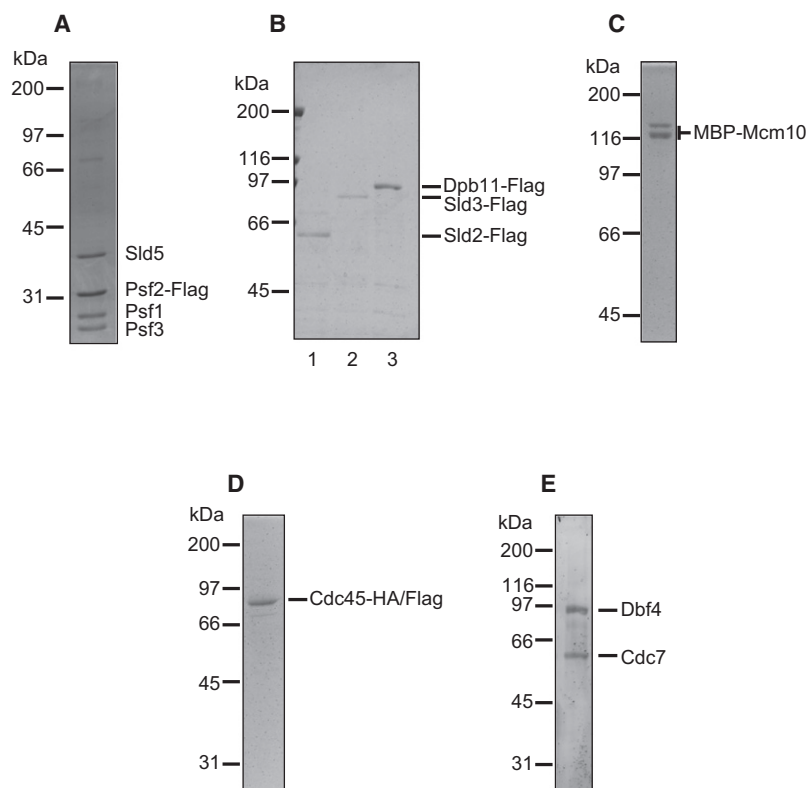


Figure S1. Purified Proteins Used in This Study, Related to Figure 1, Figure 2, Figure 3, and Figure 5

(A–E) Purified proteins were analyzed by SDS-PAGE.

(A) Coomassie blue stain of GINS complex purified from yeast strain yRH156, which overexpresses Sld5, Psf1, Psf2-Flag, and Psf3.

(B) Coomassie blue stain of proteins purified from the following yeast strains: Sld2-Flag, yRH152 (lane 1); Sld3-Flag, yRH153 (lane 2); Dpb11-Flag, yRH154 (lane 3).

(C) Coomassie blue stain of MBP-Mcm10 purified from *E. coli* Rosetta 2(DE3)(pLysS)(pRH121).

(D) Coomassie blue stain of Cdc45-3HA/3Flag purified from yeast strain ySK-Cdc45.

(E) Sypro Orange stain of DDK complex purified from yeast strain yLF52, which overexpresses Cdc7-proA and Dbf4-CBP.

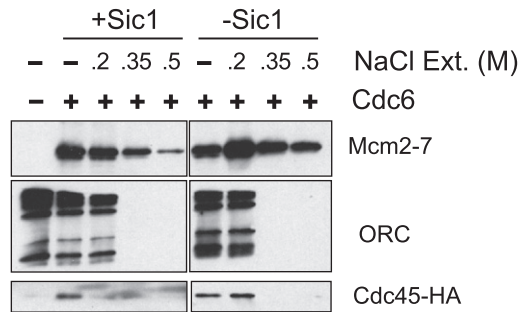


Figure S2. Cdc45 Origin Association Is Stabilized by S-CDK-Dependent Events, Related to Figure 2 and Figures 4A–4C

Cdc45 is more sensitive to salt extraction in the absence of S-CDK activity. Replisome assembly assays were performed with 1 kb linear *ARS1* DNA template and yRH182 S phase extract under conditions where S-CDK activity is blocked by GST-Sic1 (+ Sic1) or unaltered (- Sic1) and then analyzed by immunoblot. Before UV-release of bead-bound proteins, the beads were washed an additional time with H buffer containing the indicated concentration of NaCl. Unless indicated, Cdc6 was added to the G1 extract used in step one of all reactions.

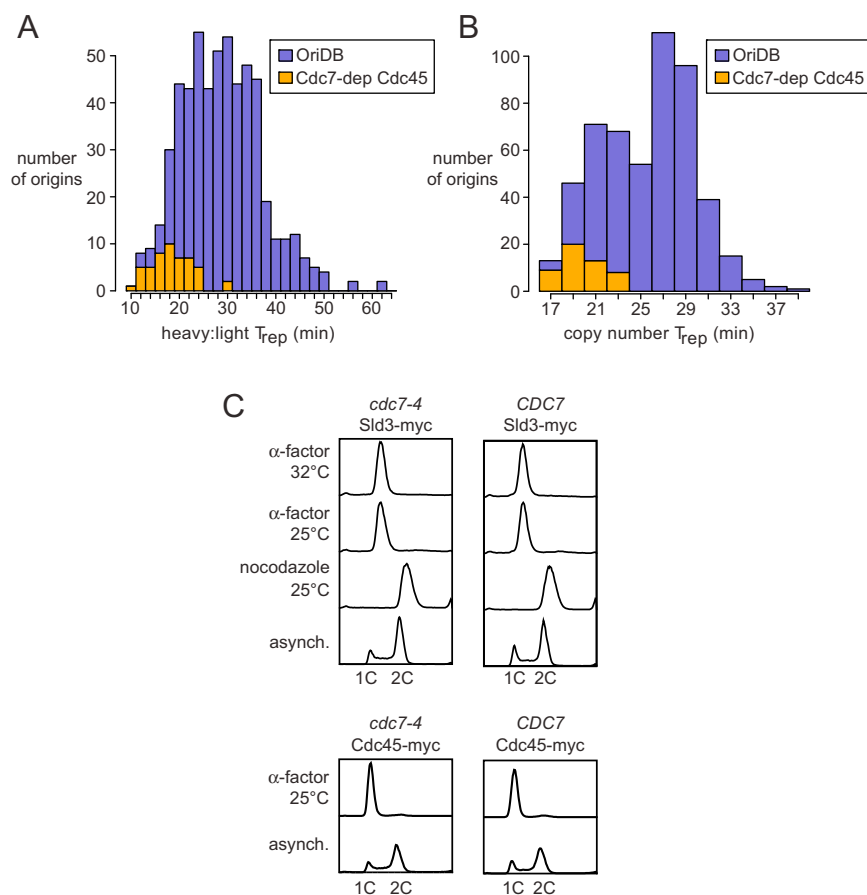


Figure S3. Origins Bound by Cdc45 in G1 in a Cdc7-Dependent Manner Initiate Early in S phase, Related to Figures 4E and 4F

(A and B) Replication timing of origins that bound Cdc45 in a Cdc7-dependent manner during G1. We identified 49 Cdc7-dependent sites of Cdc45-binding in G1-arrested cells (Table S1), all of which correspond to OriDB “confirmed” or “likely” origins (Nieduszynski et al., 2007). For heavy:light (A, Raghuraman et al., 2001) or copy-number (B, Yabuki et al., 2002) replication timing studies, we plotted a histogram of the T_{rep} numbers for these sites (in orange), and superimposed this over the histogram of the T_{rep} numbers at all 562 OriDB “confirmed” or “likely” origins (in blue). Histogram bin sizes are 2 min each.

(C) FACS analysis of cells prior to Cdc45 and Sld3 ChIP. Top: FACS profiles of ySC331 (*sld3-13myc cdc7-4*) and ySC332 (*sld3-13myc CDC7*) prior to and after the nocodazole and α -factor arrest (at 25°C and 32°C). Bottom: FACS profiles of yWL17 (*cdc45-13myc cdc7-4*) and yWL18 (*cdc45-13myc CDC7*) before and after α -factor arrest.

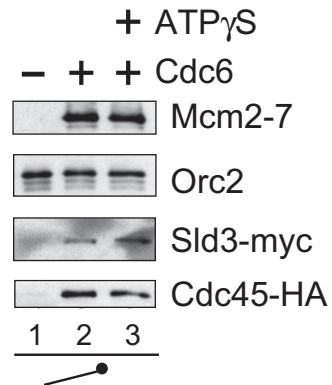


Figure S4. Cdc45 and Sld3 Do Not Require DNA Unwinding for Recruitment, Related to Figure 6

Sld3 and Cdc45 are recruited to origin DNA when ATP γ S is present in step 3 of the replisome assembly assay. Immunoblot analysis of replisome assembly reaction utilizing 1 kb linear *ARS1* DNA template and extract yRH229-S. Cdc6 was present or omitted as indicated. In the ATP γ S reaction (lane 3), step three incubation contained 1 mM ATP γ S instead of ATP and the ATP-regenerating system.