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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-hexamers 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

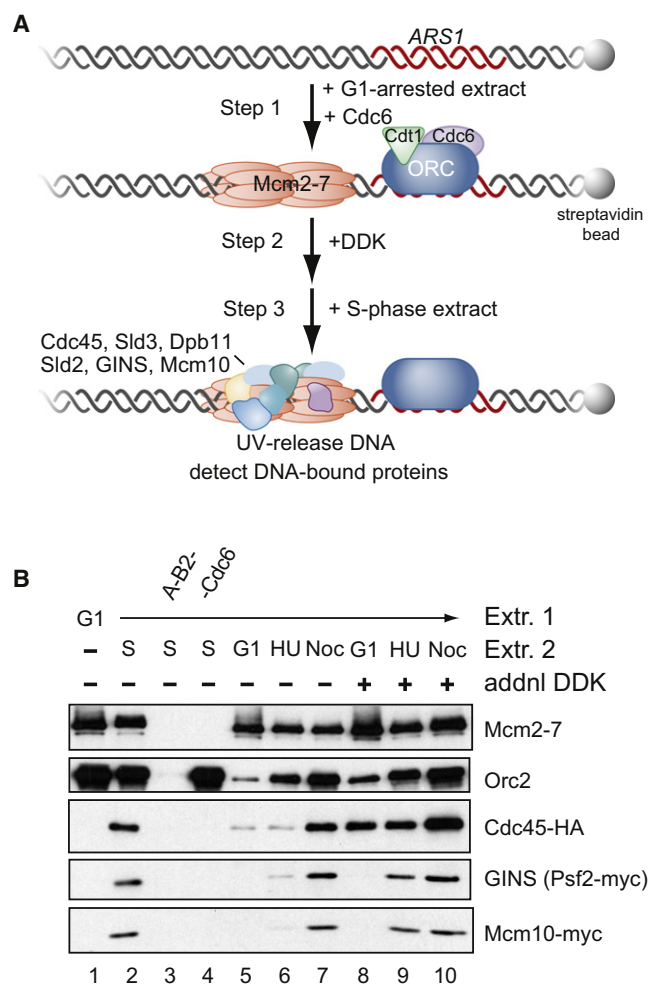


Figure 1. An Assay for Replisome Assembly In Vitro

(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

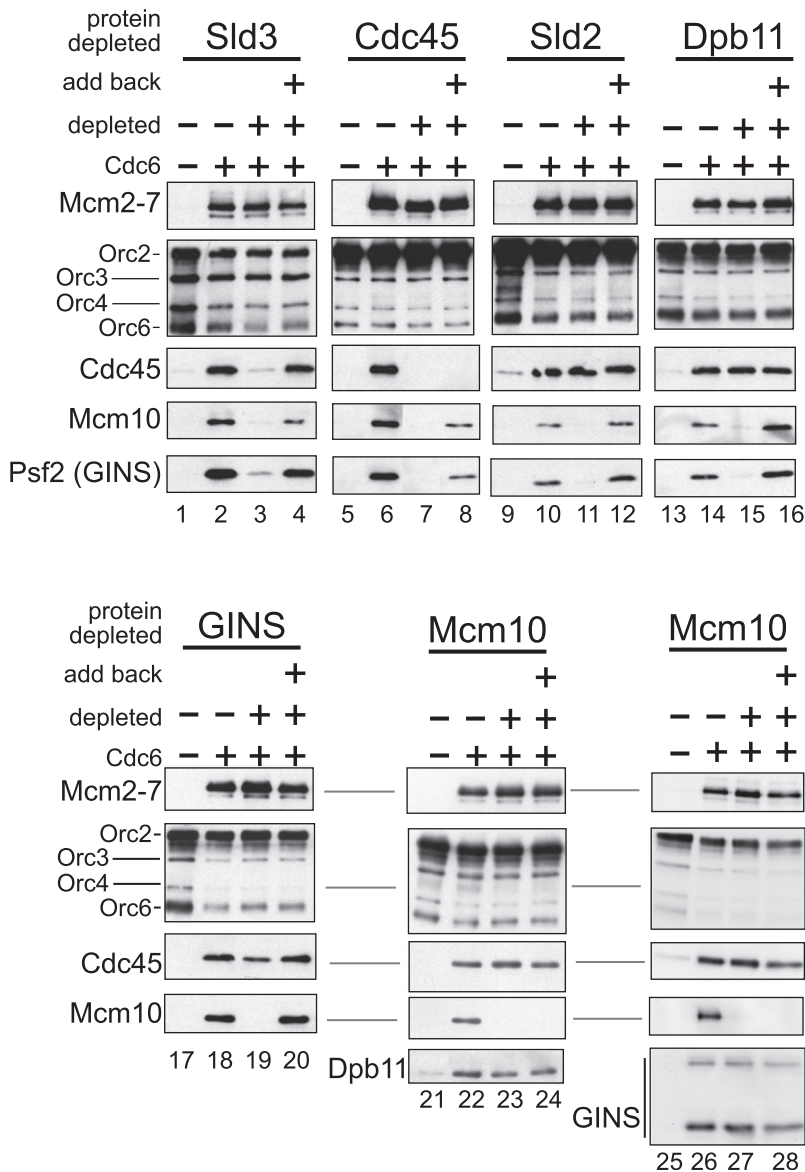


Figure 2. Interdependent Recruitment of Replication Proteins

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

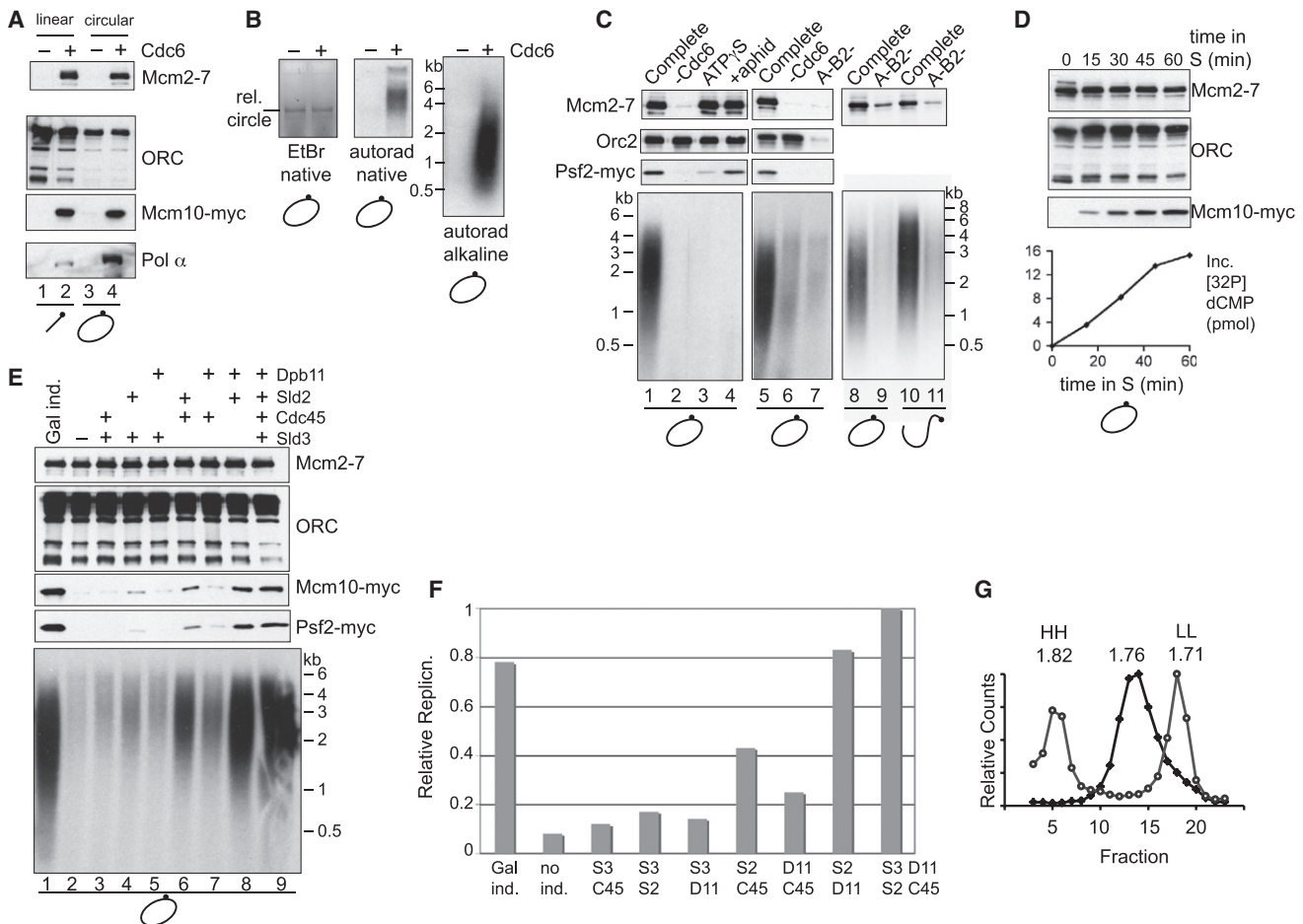


Figure 3. Long DNA Templates Support Polymerase Loading and Replication Initiation

(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.

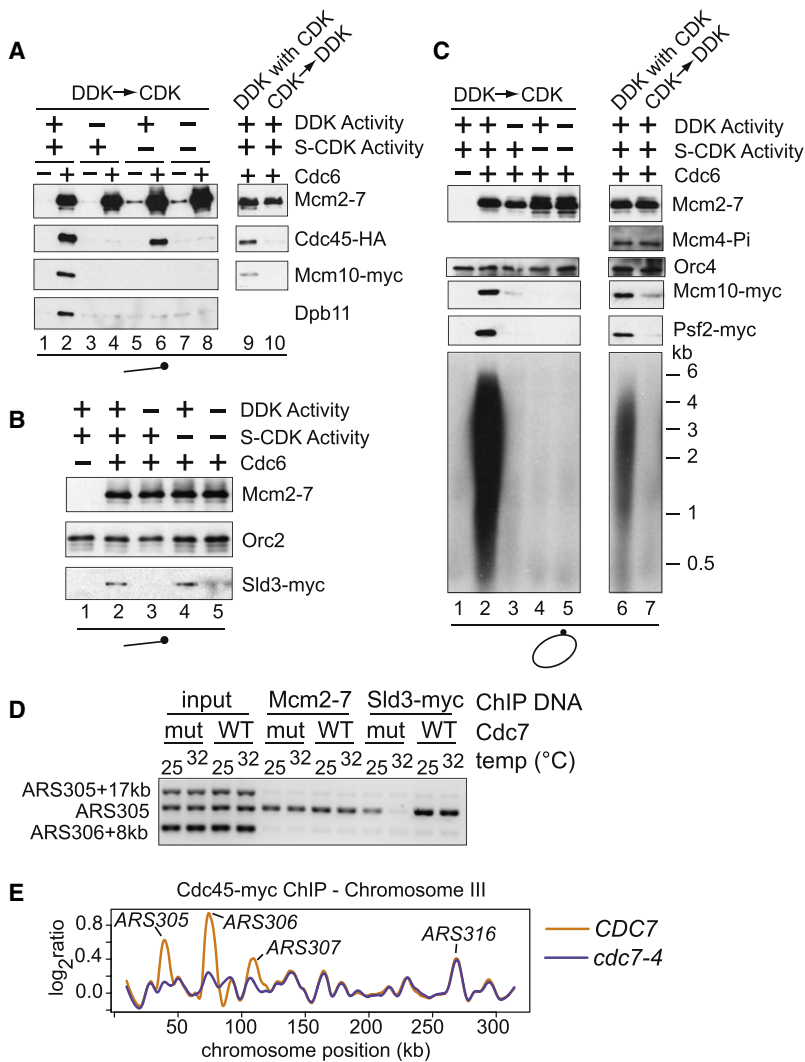


Figure 4. DDK and S-CDK Are Required for Distinct Stages of Origin Activation

(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

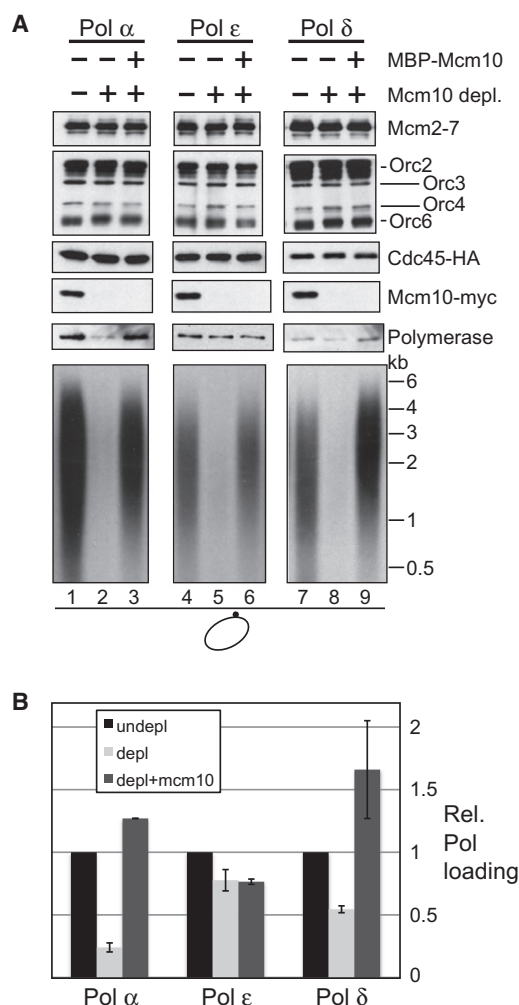


Figure 5. Mcm10 Is Required for the Recruitment of Pol α and Pol δ to Origin DNA

(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

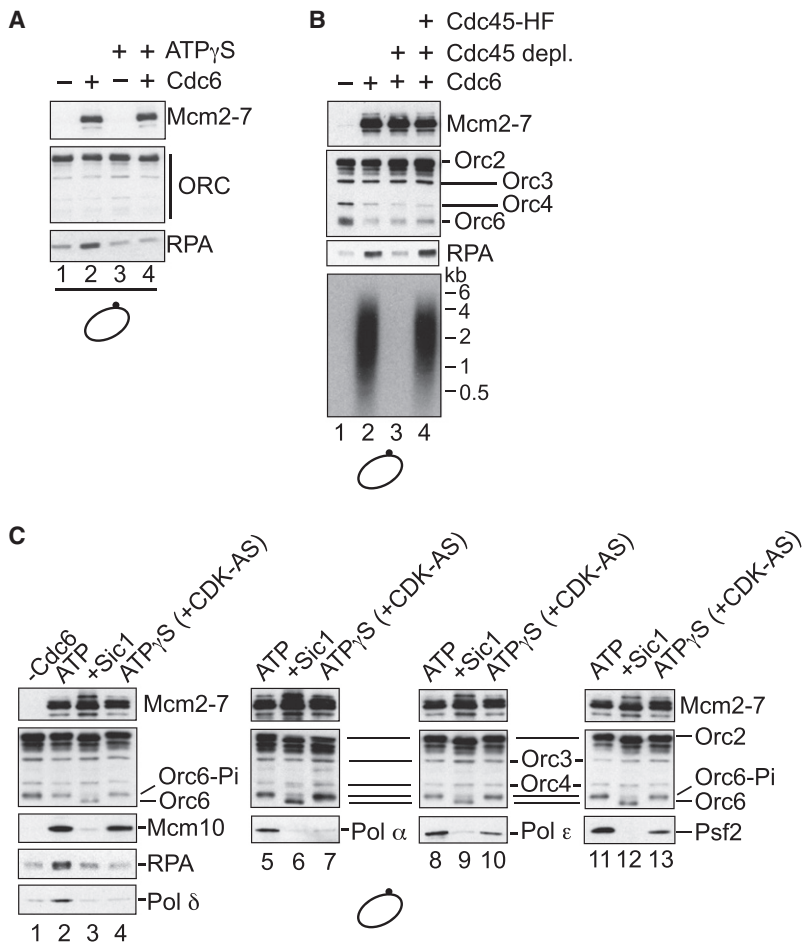


Figure 6. ATP Hydrolysis Is Required for RPA Loading and for the Loading of a Subset of Polymerases

(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

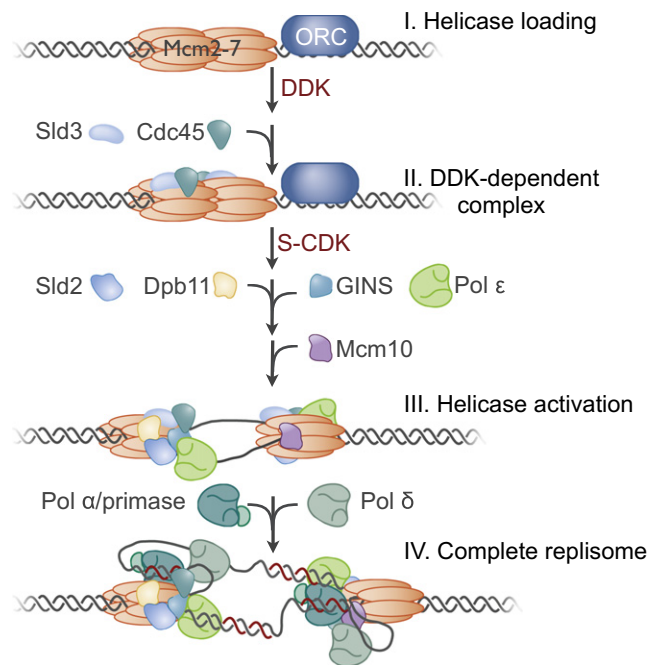


Figure 7. A Model for the Events Leading to Replication Initiation

(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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