A Positive Amplification Mechanism Involving a Kinase and Replication Initiation Factor Helps Assemble the Replication Fork Helicase*

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The assembly of the replication fork helicase during S phase is key to the initiation of DNA replication in eukaryotic cells. One step in this assembly in budding yeast is the association of Cdc45 with the Mcm2–7 heterohexameric ATPase, and a second step is the assembly of the tetrameric GINS (GG-Ichi_Nii-San) complex with Mcm2–7. Dbf4-dependent kinase (DDK) and S-phase cyclin-dependent kinase (S-CDK) are two S phase-specific kinases that phosphorylate replication proteins during S phase, and Dpb11, Sld2, Sld3, Pol e, and Mcm10 are factors that are also required for replication initiation. However, the exact roles of these initiation factors in assembly of the replication fork helicase remain unclear. We show here that Dpb11 stimulates DDK phosphorylation of the minichromosome maintenance complex protein Mcm4 alone and also of the Mcm2–7 complex and the dsDNA-loaded Mcm2–7 complex. We further demonstrate that Dpb11 can directly recruit DDK to Mcm4. A DDK phosphomimetic mutant of Mcm4 bound Dpb11 with substantially higher affinity than wild-type Mcm4, suggesting a mechanism to recruit Dpb11 to DDK-phosphorylated Mcm2–7. Furthermore, dsDNA-loaded Mcm2–7 harboring the DDK phosphomimetic Mcm4 mutant bound GINS in the presence of Dpb11, suggesting a mechanism for how GINS is recruited to Mcm2–7. We isolated a mutant of Dpb11 that is specifically defective for binding to Mcm4. This mutant, when expressed in budding yeast, diminished cell growth and DNA replication, substantially decreased Mcm4 phosphorylation, and decreased association of GINS with replication origins. We conclude that Dpb11 functions with DDK and Mcm4 in a positive amplification mechanism to trigger the assembly of the replication fork helicase.

Key to the initiation of DNA replication is the assembly and activation of the replication fork helicase, the 11-subunit assembly that provides single-stranded DNA templates for the replicative polymerases (1). The replication fork helicase is called Cdc45, Mcm2–7, GINS (CMG)3 and is comprised of the Mcm2–7 heterohexameric ATPase and the essential helicase stimulatory factors Cdc45 and GINS (2–5). Cdc45 is a single protein, whereas GINS is a four-subunit complex comprised of Sld5, Paf1, Paf2, and Psf3 (5, 6).

The Mcm2–7 complex is a single hexamer when it is free in solution, but when loaded onto DNA in late M phase and G1 by Orc, Cdc6, and Cdt1 in the presence of ATP, Mcm2–7 forms a double hexamer, with the N termini of the Mcm2–7 proteins forming interhexameric contacts (7, 8). In S phase, several changes occur that lead to assembly and activation of the replication fork helicase. Mcm2–7 assembles with Cdc45 and GINS to form the CMG helicase, single-stranded DNA is extruded from the central channel of Mcm2–7 (i.e. origin melting), and the Mcm2–7 double hexamers separate to initiate bidirectional unwinding (8–11).

Five essential DNA replication initiation proteins are also required for replication initiation, and these proteins are Sld2, Sld3, Dpb11, Pol e, and Mcm10 (12–17). In addition, two S phase-specific kinases, S-CDK and DDK, are required for replication initiation (18–20). S-CDK phosphorylates Sld2 and Sld3, and phosphorylation of Sld2 and Sld3 results in the formation of a ternary complex with Dpb11 (21, 22). In addition, DDK phosphorylates Mcm2, Mcm4, and Mcm6, and DDK phosphorylation of Mcm2–7 is required for cell growth (23–25).

It was shown previously, using an in vitro reconstitution assay, that DDK phosphorylation of Mcm4 and Mcm6 stimulates Sld3 binding to Mcm2–7 with subsequent recruitment of Cdc45 to Mcm2–7 (26). GINS recruitment to Mcm2–7 requires all of the initiation factors aside from Mcm10 (27), and GINS binds directly to Dpb11 (28).

We report here that Dpb11 stimulates DDK phosphorylation of Mcm4 alone, soluble Mcm2–7, or Mcm2–7 that is loaded onto origin dsDNA. The human homolog of Dpb11, TopBP1, stimulates human DDK phosphorylation of human Mcm4, suggesting that the reaction is conserved from yeast to human. We also find that the DDK phosphomimetic mutant of Mcm4 binds substantially more tightly to Dpb11 compared with Mcm4 with no mutation, suggesting a mechanism for positive amplifica-

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tion. Moreover, Dpb11 recruits DDK to Mcm4 by binding to both Mcm4 and Dbf4. In addition, we found that dsDNA-loaded Mcm2–7 harboring the DDK phosphorymimetic mutant of Mcm4 binds to GINS in the presence of Dpb11, suggesting a mechanism for the recruitment of GINS to Mcm2–7. We found that the BRCT4 motif of Dpb11 is responsible for Mcm4 binding activity, and we isolated a mutant within this BRCT4 motif that is specifically defective for binding to Mcm4. When this mutant is expressed in budding yeast cells, we observe decreased cell growth and DNA replication, decreased phosphorylation of Mcm4, and diminished recruitment of GINS to Mcm2–7. We conclude that Dpb11 functions with DDK and Mcm4 by a positive amplification mechanism to assemble the replication fork helicase.

Results

Dpb11 Substantially Stimulates DDK Phosphorylation of Mcm4—It has been shown previously that Sld3 and Mcm10 stimulate DDK phosphorylation of Mcm2 (25, 29, 30). We reasoned that one of the replication initiation proteins might also stimulate DDK phosphorylation of Mcm4. We therefore screened each of the five essential replication initiation factors, Sld3, Sld2, Dpb11, Pol ε, and Mcm10, for stimulation of DDK phosphorylation of Mcm4. We overexpressed proteins in *Escherichia coli* and purified them to homogeneity as described previously (31), and then assessed stimulation of DDK phosphorylation of the Mcm4 subunit. We found that Dpb11, but not Sld3, Sld2, Pol ε, or Mcm10, substantially stimulates DDK phosphorylation of Mcm4 (Fig. 1A). In contrast, Dpb11 does not stimulate DDK phosphorylation of Mcm2 or Mcm6 (Fig. 1A).

The N-terminal region of Mcm4 bears the binding site for Dbf4 and the kinase sites for Cdc7 (24). We next deleted the N terminus of Mcm4 (mcm4–Δ1–275) and found that Dpb11 does not stimulate DDK phosphorylation of mcm4–Δ1–275, as expected (Fig. 1A). We next examined whether DDK phosphorylates Mcm4 when Mcm4 is incorporated into the Mcm2–7 complex. We found that Dpb11 substantially stimulated DDK phosphorylation of Mcm2–7 when Mcm2–7 is overexpressed and purified from *E. coli* (Fig. 1B). However, when we assembled Mcm2–7 complexes with the mcm4–Δ1–275 mutant instead of Mcm4 wild-type, we found that Dpb11 does not stimulate DDK phosphorylation of Mcm2–7 (Fig. 1B). These data suggest that Dpb11 stimulates DDK phosphorylation of Mcm4 when Mcm4 is incorporated into the Mcm2–7 complex.

We then determined whether Dpb11 stimulates DDK phosphorylation of Mcm4 when Mcm2–7 is loaded onto dsDNA as a double hexamer. Using purified ORC, Cdc6, and Cdt1, we first loaded Mcm2–7 onto linear dsDNA tagged with biotin, followed by a high-salt wash (Fig. 2). This procedure results in Mcm2–7 loaded onto dsDNA (Fig. 2). We found that Dpb11 substantially stimulates DDK phosphorylation of Mcm2–7 that is preloaded onto double-stranded DNA (Fig. 1C). In contrast, Dpb11 does not stimulate DDK phosphorylation of loaded Mcm2–7 harboring the mcm4–Δ1–275 mutant (Fig. 1C), suggesting that Dpb11 stimulates DDK phosphorylation of Mcm4. Furthermore, the level of phosphate incorporation was higher for loaded Mcm2–7 compared with free Mcm2–7 or free Mcm4 alone (Fig. 1, A–C).

There have been suggestions in the literature that the N-terminal region of Mcm2, which also binds directly to DDK, is important for DDK phosphorylation of Mcm4 (33). Thus, we deleted the DDK binding site from Mcm2 (amino acids 1–277), loaded the mutant Mcm2–7 complex onto double-stranded DNA, and tested for Dpb11-induced stimulation of DDK phosphorylation of Mcm4. We found equivalent levels of Mcm4 phosphorylation when the N-terminal (1–277 amino acids) residues of Mcm2 were deleted (Fig. 1C), suggesting that DDK binding to Mcm2 is not important for the Dpb11-induced stimulation of DDK phosphorylation of Mcm4.

To determine whether the human homolog of Dpb11, TopBP1, stimulates human DDK phosphorylation of human Mcm4, we overexpressed the human proteins in *E. coli* and purified them to homogeneity. We found that human TopBP1 substantially stimulated DDK phosphorylation of Mcm4, suggesting that this biochemical reaction is conserved from yeast to human (Fig. 1D).

For budding yeast Mcm4 (Fig. 1, A–C), two prominent bands are visible. The fast-migrating band is close to the molecular weight of Mcm4, suggesting that it is phosphorylated at one or a few positions. In contrast, the slow-migrating band is considerably above the molecular weight of Mcm4, suggesting that it represents a hyperphosphorylated form of Mcm4, as observed *in vivo* (24). Fig. 4D, which shows human Mcm4, shows a large number of bands of roughly equal intensity. These data suggest that, for human Mcm4, TopBP1 stimulates the phosphorylation of different Mcm4 residues with varying efficiency. In Fig. 1, A–D, all radioactive bands are included for quantitation purposes.

Identification of a Mutant of Dpb11 That Is Specifically Defective for Binding to Mcm4—We next examined the mechanism for Dpb11-induced stimulation of DDK phosphorylation of Mcm4 (Fig. 3). We used purified proteins in GST pulldown assays to show that Dpb11 binds directly to Mcm4 (Fig. 3A) and Dbf4 (Fig. 3B) but not Cdc7 (data not shown). We also found, using a GST pulldown assay, that Dpb11 recruits DDK directly to Mcm4 (Fig. 3C).

We next sought to identify a separation-of-function mutation of Dpb11 that was specifically defective for binding to Mcm4. The BRCT 1 and BRCT2 motifs of Dpb11 bind CDK-phosphorylated Sld3 (21, 22), the BRCT3 and BRCT 4 motifs of Dpb11 bind CDK-phosphorylated Sld2 (34), and the region between BRCT2 and BRCT3 binds GINS (28). The C-terminal region of Dpb11 is dispensable for DNA replication, and this region is involved in the DNA damage response (35). We separated Dpb11 into a region encompassing the BRCT1 and BRCT2 motifs (amino acids 1–252), a region encompassing the BRCT3 motif (amino acids 253–430), a region encompassing the BRCT4 motif (amino acids 430–615), and a C-terminal region (amino acids 616–764). Note that region 430–615 of Dpb11 was determined to be a BRCT motif by Koonin and co-workers (36). We found that, of these regions, the BRCT4 motif was responsible for binding to Mcm4 (Fig. 3D). This BRCT4 motif is also functional for stimulating DDK phosphorylation of dsDNA-loaded Mcm2–7 (Fig. 1C).

There are 15 charge residues in the BRCT4 motif of Dpb11 that are conserved among yeast species (37). We systematically...
engineered charge reversal mutations of these residues and screened for binding to Mcm4. We found that four of these mutations, m4 (E477K), m7 (D509K), m9 (K542E,R543E,K544E), and m11 (K549E), are each slightly defective for binding to Mcm4 (data not shown). When these mutations are combined, the resulting mutant, dpb11-m4,7,9,11, is completely defective for binding to Mcm4 (Fig. 3A). Furthermore, this mutant is completely defective for stimulating DDK phosphorylation of dsDNA-loaded Mcm2–7 (Fig. 1C). We then studied whether this mutant is defective for other known functions of Dpb11, including binding to CDK-phosphorylated Sld2 and Sld3, GINS binding, and single-stranded DNA binding. We found that dpb11-m4,7,9,11 is like the wild type for binding CDK-phosphorylated Sld2 (Fig. 3E) and Sld3 (Fig. 3F), GINS (Fig. 3G), and single-stranded DNA (Fig. 3H). These data suggest that we have isolated a separation-of-function mutant for Dpb11 that is specifically defective for binding to Mcm4.

Dpb11 Recruits GINS to Loaded Mcm2–7 in a Manner That Is Substantially Stimulated by DDK Phosphorylation of Mcm4—We next determined the consequence for Dpb11-stimulated DDK phosphorylation of Mcm4 on assembly of the CMG replication fork helicase. First, we wanted to determine whether phosphorylation of Mcm4 at the DDK consensus sites stimulates the interaction between Mcm4 and the initiation factors. To do this, we employed a GST pulldown assay using

![Diagram](https://example.com/diagram.png)
Dpb11 Functions with DDK and Mcm4

GST-Mcm4-wild-type, GST-Mcm4–7D, or GST alone. Mcm4–7D bears phosphomimetic mutations at the seven DDK consensus sites at the N-terminal region of Mcm4 (between amino acids 146 and 178). The seven DDK consensus sites on Mcm4 have been established previously by a published in vitro and in vivo study (24). We found that GST-Mcm4–7D pulled down more Sld3 compared with Mcm4-wild-type, suggesting that phosphorylation of these seven DDK sites is sufficient to recruit Sld3 to Mcm4 alone (Fig. 4A). However, this stimulation was suppressed when Mcm4–7D was incorporated into the dsDNA-loaded Mcm2–7 complex (data not shown), confirming the result of a previous study demonstrating that additional DDK sites on Mcm4 and Mcm6 are required for Sld3 recruitment to dsDNA-loaded Mcm2–7 (26). We also found that GST-Mcm4–7D pulled down substantially more Dpb11 compared with Mcm4-wild-type (Fig. 4B). Furthermore, this substantial increase in pulldown efficiency of Dpb11 was observed when Mcm4–7D was incorporated into dsDNA-loaded Mcm2–7 (Fig. 4C). These data suggest that DDK phosphorylation of Mcm4 stimulates Dpb11 recruitment to dsDNA-loaded Mcm2–7 complexes. These data are consistent with the observation that the BRCT motifs of Dpb11 bind specifically to phosphorylated proteins, suggesting a mechanism for the recruitment of Dpb11 to DNA-loaded Mcm2–7 in a manner that depends on DDK (Fig. 4C). The binding of Dpb11 to Mcm4–7D is substantially inhibited by the BRCT4 mutations of Dpb11 (dpb11-m4,7,9,11) (Fig. 4, B and C), suggesting that binding of Dpb11 to DDK-phosphorylated Mcm4 is dependent on the BRCT4 motif of Dpb11.

Next we sought to determine the consequence for Dpb11 recruitment to loaded Mcm2–7 complexes harboring DDK-phosphorylated-Mcm4. To test this, we incubated dsDNA-loaded Mcm2–7 complexes harboring wild-type Mcm4 or Mcm4–7D with or without Dpb11 and with increasing concentrations of radiolabeled GINS. We found that Dpb11 substantially stimulated GINS recruitment to dsDNA-loaded Mcm2–7, and this loading was only observed for Mcm4–7D and not Mcm4-wild-type (Fig. 4D). Furthermore, this stimulatory effect was dependent on dsDNA-loading because free Mcm2–7 single hexamers did not exhibit Dpb11/DDK-dependent stimulation of GINS loading (data not shown).

Expression of the Mutant of Dpb11 That Is Specifically Defective for Binding to Mcm4 Results in Defective DNA Replication and Helicase Assembly—We next investigated the consequences for expressing the mutant of dpb11 that is specifically defective for binding to Mcm4. To accomplish this, we used a previously published dpb11-td degron strain (37, 38). This strain results in complete degradation of the native Dpb11 protein under restrictive conditions (+ galactose, + doxycycline, 37 °C). We transformed these cells with a plasmid expressing wild-type Dpb11 or mutant dpb11 (dpb11-m4,7,9,11) under inducible regulation by the low-copy GAL-S promoter. We found that, under permissive conditions (− galactose, − doxycycline, 30 °C), there is equal growth of cells transformed with wild-type Dpb11 or dpb11-m4,7,9,11 (Fig. 5A). However, under restrictive conditions, we found that cells with dpb11-m4,7,9,11 exhibit a severe growth defect compared with cells expressing wild-type Dpb11 (Fig. 5B).

Western blotting analysis of whole cell extracts reveals equal levels of Dpb11 expression for cells harboring dpb11-m4,7,9,11 compared with Dpb11-wild-type under restrictive conditions (Fig. 5C). Taken together, these data suggest that Dpb11-binding to Mcm4 is required for cell growth. We also examined DNA replication in these cells under restrictive conditions by FACS analysis and found that cells with dpb11-m4,7,9,11 exhibit a severe DNA replication defect compared with cells expressing wild-type Dpb11 (Fig. 5D). These data suggest that Dpb11-binding to Mcm4 is required for DNA replication.

We then determined whether expression of dpb11-m4,7,9,11 results in decreased phosphorylation of Mcm4 in vivo. It was shown previously that isolating the chromatin-bound fraction from budding yeast cells (SN2 fraction), followed by Western blotting analysis of the chromatin-bound proteins, reveals a mobility shift specific for DDK phosphorylation of Mcm4 (24). We isolated the SN2, chromatin-bound fraction of dpb11-td cells and found that cells complemented with the plasmid expressing wild-type Dpb11 demonstrated a substantial mobility shift of Mcm4 as cells progressed from G1 to S phase (Fig. 1, E and F). In contrast, cells complemented with dpb11-m4,7,9,11 demonstrated little mobility shift as cells progressed from G1 to S phase (Fig. 1, E and F), suggesting that cells
expressing dpb11-td cells harboring dpb11-m4,7,9,11 under restrictive conditions to ChIP. We arrested the cells in G1 with α-factor and then released the cells into medium lacking α-factor for 15 or 30 min to allow the cells to enter S phase. We found that Cdc45 accumulation at the early origins ARS305 and ARS306 was like the wild type for cells expressing dpb11-td compared with cells harboring wild-type DPB11 (Fig. 5G).

These data suggest that Dpb11-binding to Mcm4 is not required for Cdc45 recruitment to an origin of replication. However, GINS accumulation at the early origins ARS305 and ARS306 was defective for cells harboring dpb11-td (Fig. 5H). The severe defect in GINS recruitment is consistent with our in vitro data and with previous studies demonstrating a DDK requirement for GINS recruitment to replication origins.
suggesting that Dpb11/DDK binding to Mcm4 is required for GINS recruitment to dsDNA-loaded Mcm2–7.

We next performed co-immunoprecipitation experiments with antibodies directed against loaded Mcm2 using a protocol published previously (40). We found that cells harboring dbp11-m4,7,9,11 exhibited wild-type levels of Cdc45-Mcm2–7 interaction (Fig. 6) and substantially reduced GINS-Mcm2 interaction (Figs. 6). These data are consistent with the ChIP data and with our in vitro data demonstrating that Dpb11 binding to Mcm4 is required for GINS recruitment to Mcm2–7. The data also suggest that Sld3-dependent recruitment of Cdc45 to Mcm2–7 in vivo does not depend on Dpb11 binding to Mcm4. There may be sufficient DDK phosphorylation of Mcm4 and or Mcm6 in vivo to recruit Sld3-Cdc45 to Mcm2–7 when the Dpb11-Mcm4 interaction is disrupted; alternatively, the recruitment of Cdc45 to Mcm2–7 may occur by some backup mechanism in these mutant cells.

Discussion

Summary of New Findings in This Report—We found, using purified proteins, that Dpb11 substantially stimulates DDK phosphorylation of Mcm4, regardless of Mcm4 is alone, part of the
free single-hexameric Mcm2–7 complex, or part of the dsDNA loaded, double-hexameric Mcm2–7 complex. Furthermore, replacement of the seven consensus DDK sites on Mcm4 with the phosphomimetic mutant (aspartate) results in substantially enhanced interaction between Mcm4 and Dpb11, and, again, this reaction is observed for Mcm4 alone or Mcm4

FIGURE 5. Expression of the mutant of Dpb11 that is specifically defective for binding to Mcm4 results in defective DNA replication and helicase assembly. A and B, 10-fold serial dilutions of dpb11-td cells expressing DPB11 wild type, vector only control, and dpb11-m4,m7,m9,m11 under permissive conditions (CSM-Ura, 30 °C, A) or restrictive conditions (CSM-Ura + galactose (Gal) + doxycycline (Dox), 37 °C, B). C, Western blotting analysis of DPB11-WT, dpb11-m4,m7,m9,m11, and vector-only control whole cell extracts showing equivalent levels of Dpb11 expression under restrictive conditions for the wild type compared with the mutant. MWM, molecular weight marker. D, FACS analysis was performed as described under “Experimental Procedures” on dpb11-td cells expressing DPB11 wild type, vector-only control, and dpb11-m4,m7,m9,m11. Cells were synchronized in G1 with α-factor and then released into medium lacking α-factor for the time points indicated. E and F, analysis of SN2 fractions (chromatin-bound fractions) reveal a slower-migrating Mcm4 band by Western blotting analysis, shown previously to be DDK-hyperphosphorylated Mcm4 (24). F, the percentage of Mcm4 that is hyperphosphorylated was quantified from experiments like shown in E and plotted as mean ± S.E. G and H, dpb11-td cells expressing DPB11-WT or dpb11-m4,m7,m9,m11 under restrictive conditions were arrested with α-factor and then released into medium lacking α-factor for 15 or 30 min. Chromatin immunoprecipitation was performed as described under “Experimental Procedures” using antibodies directed against Cdc45 (G) or Pef1 (H). PCR primers were used that targeted the early yeast origin ARS305, ARS306, or a region positioned midway between ARS305 and ARS306. [α-32P]dCTP was included in the PCR reaction for quantification. Radioactive PCR bands were quantified and plotted.
as part of the free or loaded Mcm2–7 complex. We also studied the mechanism for Dpb11 stimulation of DDK phosphorylation of Mcm4 and found that Dpb11 binds to Dbf4 and Mcm4 and that Dpb11 can thus recruit DDK to Mcm4. Interestingly, the human homolog of Dpb11, TopBP1, substantially stimulates human DDK phosphorylation of human Mcm4, suggesting that the reaction is conserved from yeast to human.

We then isolated the region of Dpb11 that binds to Mcm4 and found that the BRCT4 motif of Dpb11 binds directly to Mcm4 (Fig. 7). We then systematically mutated conserved charged residues in the BRCT4 motif of Dpb11 and identified key residues responsible for Mcm4 binding. We combined the mutations into a single construct for dpb11 (dpb11-m4,m7,m9,m11) and found that this mutant is specifically defective for binding to Mcm4, whereas binding to CDK-phosphorylated Sld2, CDK-phosphorylated Sld3, GINS, and single-stranded DNA is as for the wild type. Expression of dpb11-m4,m7,m9,11 in vivo results in decreased cell growth, defective DNA replication, and diminished DDK phosphorylation of Mcm4. Furthermore, the recruitment of GINS to replication origins is defective in this mutant, suggesting that Dpb11 binding to Mcm4 is required for helicase assembly.

A Positive Amplification Mechanism for Dpb11 and DDK—
We found that Dpb11 substantially stimulates DDK phosphorylation of Mcm4 and that DDK phosphorylation of Mcm4 substantially stimulates Dpb11 binding to Mcm4. These data suggest a positive amplification mechanism, with Dpb11 stimulating DDK recruitment to Mcm4 and DDK phosphorylation of Mcm4 stimulating Dpb11 recruitment to Mcm4. This would result in maximal phosphorylation of Mcm4 in the presence of Dpb11. DDK and Dpb11 are limiting for replication in the cell (41), and this mechanism may ensure that Dpb11 binding to Mcm4 is coordinated with DDK phosphorylation of Mcm4, thus ensuring that the Mcm4 is activated by DDK and loaded with Dpb11 at the same time. A positive amplification cascade, as proposed here, is a paradigm for biology in a number of other different signal cascades (42).

A Recruitment Model for GINS onto Double-hexameric Mcm2–7—
Dpb11 does not stimulate GINS binding to free Mcm2–7 whether free Mcm2–7 harbors Mcm4-wild-type Mcm4 or Mcm4–7D. However, Dpb11 can directly load GINS to Mcm2–7 when Mcm2–7 is loaded onto double-stranded DNA and harbors phosphomimetic mutants for Mcm4. Thus, the Dpb11-directed recruitment of GINS to Mcm2–7 is DDK-
Dpb11 Functions with DDK and Mcm4

Figure 7. Schematics of the domain structures of Dpb11 and Mcm4. A and B, schematics of the structures of yeast Dpb11 (A) and Mcm4 (B). Binding sites for critical macromolecules and domain architectures are indicated. New observations in this manuscript report that the BRCT4 motif of Dpb11 binds to DDK-phosphorylated Mcm4 (A), and the N terminus of Mcm4 binds to DDK (B).

dependent and DNA loading-dependent. DNA-loaded Mcm2–7 is a double hexamer, whereas free Mcm2–7 is a single hexamer, and thus the DNA loading dependence for Dpb11 recruitment of GINS may be related to the double hexamer Mcm2–7 structure. Consistent with these data, the electron microscopy structure of DNA-loaded Mcm2–7 positions the N terminus of Mcm4 of one Mcm2–7 hexamer next to the N terminus of Mcm5 of the second Mcm2–7 hexamer (8, 43). Furthermore, the GINS binding sites on loaded Mcm2–7 are Mcm5 and Mcm3 (44). In contrast, the free Mcm2–7 structure shows that Mcm4 is positioned considerably away from Mcm5 and Mcm3 (7), suggesting a mechanism for how Dpb11 accomplishes GINS recruitment only to DNA-loaded-Mcm2–7. In this model, Dpb11 binds to DDK-phosphorylated Mcm4, and Dpb11 recruits GINS directly to Mcm5/Mcm3 of the other Mcm2–7 hexamer of the double hexamer. This interhexamer loading model would ensure that GINS is only recruited to loaded, double-hexamer Mcm2–7 complexes. The in vivo mechanism may be more complex because multiple cooperative and competitive interactions are potentially functional on the cellular genome. Indeed, in a DNA replication initiation reconstitution assay, the loading of GINS to Mcm2–7 depends on Dpb11 and DDK and also Sld2, Sld3, Pol ε, and S-CDK (39).

A Two-step Model for the Assembly of GINS with Mcm2–7—We propose a two-step model for the cellular recruitment of GINS to Mcm2–7. In this model, Dpb11 stimulates DDK phosphorylation of Mcm4, and CDK phosphorylates Sld2 and Sld3 (21, 22). Dpb11 binds to GINS (28), to S-CDK-phosphorylated Sld2 and Sld3 (21, 22), and to DDK-phosphorylated Mcm2–7, explaining how Dpb11 recruits GINS to Mcm2–7. The recruitment of GINS to Mcm2–7 also requires Pol ε because Pol ε binds directly to Dpb11 and GINS (27, 39). However, we have found previously that Sld2 binds to the GINS binding site on Mcm2–7 and that Sld2 competes with GINS for Mcm2–7 interaction (45). We also have found previously that the melting of origin DNA, with the extrusion of single-stranded DNA from the central channel of Mcm2–7, sequesters Sld2 from Mcm2–7, allowing GINS to bind by a passive sequestration mechanism (46). Origin melting also requires S-CDK-phosphorylation of Sld2 because S-CDK-phosphorylation of Sld2 is required for Sld2 binding to single-stranded DNA (47). Thus, GINS binding to Mcm2–7 requires DDK attachment to Mcm2–7, but GINS binding to Mcm2–7 also requires origin melting to sequester Sld2 away from Mcm2–7. For these reasons, the attachment of GINS with Mcm2–7 requires both DDK and S-CDK, consistent with the requirement of S-CDK and DDK for GINS recruitment to Mcm2–7 suggested by other studies (27, 39).

Experimental Procedures

Antibodies—Antibodies against Mcm4 and Arp3 were purchased from Santa Cruz Biotechnology. Antibodies directed against Mcm2, Cdc45, Psf1, and Dpb11 were validated as described previously (25, 37, 48).

Yeast Strains—Degron strain dbp11-td (YJ770, MAT a ade2–1 ura3–1 his 3–11, 15 trp 1–1 leu23, 112 can 1–100 dbp11-td (DBP11 5’ upstream −100 to −1 is replaced with kanMX-t(A tetR-VP16-tetO2-Ub-DHFRts-HA-linker) UBR1:: GAL-Ubiquitin-M-lacl fragment-Myc-UBR1 (HIS3) leu2–3,112:: pCM244 (tetR’-SSN6, LEU2)) was a generous gift from John F. X. Diffley (London Research Institute, Cancer Research UK, London, UK (22)). The degron strain dbp11-td was transformed with a PRS416 vector containing an empty vector, DBP11 wild-type, or dbp11-m4,m7,m9,m11 under the control of the native DBP11 promoter. Positive transformants were selected on CSM-Ura plates.

Plasmids and Protein Purification—The vectors for overexpression of Cdc6, Cdt1, and Orc1–6 were generously provided by John Diffley as described previously (32). Cdc6, Cdt1, and Orc1–6 were overexpressed and purified as described previously (32). The vectors for overexpression of human TopBP1 (full-length and fragments) were generously provided by Aziz Sancar through Addgene as described previously (49). Human TopBP1 was purified as described previously (49). Plasmids for E. coli recombinant expression of Dpb11 (wild-type and mutant) for GINS and for yeast in vivo expression of DBP11 (wild-type and mutants) were prepared as described previously (37, 50). Yeast Dpb11 (wild-type and mutant), Mcm2–7, Cdc45, Sld3, Sld2, CDK, DDK, GINS, and GST were purified as described previously (37, 46, 48, 51). Protein kinase A was a generous gift from Susan Taylor (University of California, San Diego, CA).

Kinase Labeling of Proteins—PKA labeling of proteins was performed as described previously (46, 51, 52). Proteins containing a PKA tag at the N terminus were radiolabeled in a reaction volume of 100 μl that contained 20 μM PKA-tagged protein in kinase reaction buffer (5 mM Tris-HCl (pH 8.5), 10 mM MgCl2, 1 mM DTT, 500 μM ATP, 500 μCi [γ-32P]ATP containing 5 μg PKA, DDK, or CDK. Reactions were incubated for 1 h at 30 °C. The kinase was then removed from the mixture by affinity chromatography.
GSTM Pulldown—The GST pulldown assays were performed as described previously (48). GST pulldown reactions were performed in a volume of 100 μl and contained GST-tagged protein in GST-binding buffer (40 mM Tris-HCl (pH 7.5), 100 mM NaCl, 0.1 mM EDTA, 10% glycerol, 0.1% Triton X-100, 1 mM DTT, 0.7 mg/ml pepstatin, 0.1 mM PMSF, and 0.1 mg/ml BSA) and varying amounts of radiolabeled protein as described in each figure. Each reaction was incubated at 25 °C for 1 h. Following incubation, reactions were added to 40 μl of glutathione-Sepharose and gently mixed. Binding of GST-tagged protein to the protein was performed for 20 min with gentle mixing every 2 min. When the binding was complete, the beads were allowed to settle, the supernatant was removed, and the glutathione beads were washed twice with 0.5 ml GST-binding buffer. After the last wash, 30 μl of 5× SDS sample buffer was added to each reaction, and the samples were heated to 95 °C for 10 min. Samples (20 μl) were then analyzed by SDS-PAGE followed by phosphorimaging and quantitation. The mean ± S.E. is shown.

Mcm2–7 Loading—The Mcm2–7 heterohexameric complex was loaded onto dsDNA according to an established protocol (32) (Fig. 2). Briefly, Orc1–6, Cdc6, Mcm2–7, and Cdt1 were added to linear dsDNA bearing an early origin sequence (ARS305) in the presence of ATP (Fig. 2). After a high-salt wash, only Mcm2–7 remains bound to the dsDNA (Fig. 2). DNA templates, 1 kb in size, were generated by PCR using a 5’ primer containing a photocleavable biotin (Integrated DNA Technologies) and a plasmid containing ARS305 as a template. PCR products were purified, and, for each reaction, 300 ng (0.45 pmol) was coupled to 5 μl of slurry Dynabeads M-280 streptavidin magnetic beads (Life Technologies). Sufficient Mcm2–7 was loaded onto dsDNA for 10 subsequent assays. For the 10-assay dsDNA-loading step, there was a final volume of 400 μl and contained GST-tagged protein in GST-binding buffer (40 mM Tris-HCl (pH 7.5), 100 mM NaCl, 0.1 mM EDTA, 10% glycerol, 0.1% Triton X-100, 1 mM DTT, 0.7 mg/ml pepstatin, 0.1 mM PMSF, and 0.1 mg/ml BSA) and varying amounts of radiolabeled protein as described in each figure. Each reaction was incubated at 25 °C for 1 h. Following incubation, reactions were added to 40 μl of glutathione-Sepharose and gently mixed. Binding of GST-tagged protein to the protein was performed for 20 min with gentle mixing every 2 min. When the binding was complete, the beads were allowed to settle, the supernatant was removed, and the glutathione beads were washed twice with 0.5 ml GST-binding buffer. After the last wash, 30 μl of 5× SDS sample buffer was added to each reaction, and the samples were heated to 95 °C for 10 min. Samples (20 μl) were then analyzed by SDS-PAGE followed by phosphorimaging and quantitation. The mean ± S.E. is shown.

Biotin Pulldown Assay—Biotin pulldown assays were performed as described previously (39). Large preloaded complex mixtures were resuspended in a biotin pulldown buffer (40 mM Tris-HCl (pH 7.5), 500 mM NaCl, 0.1 mM EDTA, 10% glycerol, 0.1% Triton X-100, 1 mM DTT, 0.7 mg/ml pepstatin, 0.1 mM PMSF, and 0.1 mg/ml BSA). dsDNA-loaded Mcm2–7 complexes were aliquoted into 10 individual tubes, and radiolabeled Dpb11 or GINS was added to the reaction in the absence or presence of Dpb11, as shown in Fig. 4, in biotin pulldown buffer, incubated for 15 min at 30 °C, and washed twice with 200 μl of the biotin pulldown buffer using the magnet. The samples were then resuspended in sample buffer, boiled, and analyzed by SDS-PAGE.

Yeast Dilutions—Serial dilution was performed as described previously (45). Yeast strains in overnight culture (CSM-Ura containing raffinose, 30 °C) were transferred into YPGal medium containing 50 μg/ml doxycycline and incubated for 2 h at 37 °C. The 10-fold serial dilution was performed and spotted onto a plate containing CSM-Ura, which was incubated at 30 °C (permissive conditions) and a plate containing CSM-Ura + Gal + 50 μg of doxycycline, which was incubated at 37 °C (restrictive conditions) for 2 days.

FACS Analysis—FACS was performed as described previously (45). The strains were grown overnight in CSM-Ura medium containing raffinose at 30 °C. For Gt arrest, 6 × 10⁶ cells/ml were treated with α-factor (Zymo Research) for 3 h at 37 °C in YPGal medium containing 50 μg/ml doxycycline. Following extensive washes and addition of 50 μg/ml Pronase (Calbiochem) to fresh YPGal + doxycycline, cells were further incubated at 37 °C. Cells were collected at the indicated time intervals and stained with propidium iodide. Cell cycle progression data were obtained using the BD FACSCanto RUO Special Order System and analyzed using FACS Diva Software.

ChIP—For Gt arrest and release, 6 × 10⁶ cells/ml were treated with α-factor (Zymo Research) for 3 h at 37 °C in YPGal medium containing 50 μg/ml doxycycline. Following extensive washes and addition of 50 μg/ml Pronase (Calbiochem) to fresh YPGal + doxycycline, cells were further incubated at 37 °C for the indicated time. Chromatin immunoprecipitation was performed as described previously (53). We performed PCR with [α-32P]dCTP as a component of the PCR reaction to quantify the amplified product. Formaldehyde-cross-linked cells were lysed with glass beads in a Bead Beater. DNA was fragmented by sonication (Branson 450). Cdc45 or Psf1 antibody and magnetic protein A beads (Dynabeads protein A, Invitrogen, 100.02D) were added to the cleared lysate to immunoprecipitate the DNA. Immunoprecipitates were washed extensively to remove nonspecific DNA. Eluted DNA was then subjected to PCR analysis using primers directed against ARS305, ARS306, or a site midway between ARS305 and ARS306 as described previously (54). The radioactive band in the native gel, representing specific PCR-amplified DNA product, was quantified by phosphorimaging and normalized by a reference standard run in the same gel. The reference standard was a PCR reaction with a known quantity of template DNA replacing the immunoprecipitate. The mean ± S.E. is shown from biological replicates from independent cultures.

Co-immunoprecipitation (Co-IP)—For Gt arrest and release, 6 × 10⁶ cells/ml were treated with α-factor (Zymo Research) for 3 h at 37 °C in YPGal medium containing 50 μg/ml doxycycline. Following extensive washes and addition of 50 μg/ml Pronase (Calbiochem) to fresh YPGal + doxycycline, cells were further incubated at 37 °C for the indicated time. Co-immunoprecipitation was performed as described previously (46). Cells were collected and lysed at 4 °C with glass beads in IP buffer (100 mM HEPES-KOH (pH 7.9), 100 mM potassium acetate, 10 mM magnesium acetate, 2 mM sodium fluoride, 1 mM PMSF, 0.1 mM leupeptin, pepstatin, and 1× complete protease inhibitor mixture without EDTA (Roche)). Lysed material was treated with 200 units of Benzonase nuclease (Novagen, 70746-3) at 4 °C for
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The clarified extract was then mixed with 2 μl of specified antibody and rotated for 2 h at 4°C. Following this, 7 μl of Dynabeads protein A (Invitrogen, 100.01D) beads equilibrated with IP buffer were added to the extract and further rotated for 1 h at 4°C. Beads were washed twice with 500 μl of IP buffer and finally resuspended in SDS sample buffer. Western blotting analysis was performed, and the blots were scanned using the LI-COR Odyssey infrared imager and analyzed in Image Studio. FACS analysis was performed, and the blots were scanned using the LI-COR Odyssey infrared imager and analyzed in Image Studio.

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