

# Conserved mechanism for coordinating replication fork helicase assembly with phosphorylation of the helicase

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Edited by Mike E. O'Donnell, The Rockefeller University, Howard Hughes Medical Institute, New York, NY, and approved July 28, 2015 (received for review May 15, 2015)

**Dbf4-dependent kinase (DDK) phosphorylates minichromosome maintenance 2 (Mcm2) during S phase in yeast, and Sld3 recruits cell division cycle 45 (Cdc45) to minichromosome maintenance 2-7 (Mcm2-7). We show here DDK-phosphorylated Mcm2 preferentially interacts with Cdc45 in vivo, and that Sld3 stimulates DDK phosphorylation of Mcm2 by 11-fold. We identified a mutation of the replication initiation factor Sld3, Sld3-m16, that is specifically defective in stimulating DDK phosphorylation of Mcm2. Wild-type expression levels of *sld3-m16* result in severe growth and DNA replication defects. Cells expressing *sld3-m16* exhibit no detectable Mcm2 phosphorylation in vivo, reduced replication protein A-ChIP signal at an origin, and diminished Go, Ichi, Ni, and San association with Mcm2-7. Treslin, the human homolog of Sld3, stimulates human DDK phosphorylation of human Mcm2 by 15-fold. DDK phosphorylation of human Mcm2 decreases the affinity of Mcm5 for Mcm2, suggesting a potential mechanism for helicase ring opening. These data suggest a conserved mechanism for replication initiation: Sld3/Treslin coordinates Cdc45 recruitment to Mcm2-7 with DDK phosphorylation of Mcm2 during S phase.**

DNA replication | kinase | phosphorylation | helicase | initiation

The replication fork helicase in eukaryotes is composed of Cdc45, the Mcm2-7 heterohexameric ATPase, and the tetrameric GINS (Go, Ichi, Ni, and San) complex (CMG assembly) (1). The replication fork helicase (CMG) assembles in S phase in a manner that is dependent upon the replication initiation factors Sld2, Sld3, and Dpb11 (2). Sld3 (Treslin/TICRR in humans), Sld2 (RecQL4/RecQ4 in humans), and Dpb11 (TopBP1 in humans) are required for the initiation of DNA replication, but these proteins do not travel with the replication fork (3). The S-phase-specific kinases, cyclin-dependent kinase (CDK) and the Dbf4-dependent kinase (DDK), are also required for CMG assembly and origin activation (4, 5). In late M and G<sub>1</sub> phases, the Mcm2-7 complex loads to encircle dsDNA as a double hexamer (6, 7). During S phase, a single strand of DNA is extruded from the central channel of Mcm2-7, and this event is required because the CMG complex unwinds DNA by a steric exclusion mechanism (8).

Central to the initiation of DNA replication is the coordination of entry into S phase with origin firing (4, 5). Levels of the S phase-specific kinases, S-CDK and DDK, rise during the onset of S phase, and these two kinases are central to coordinating S-phase entry with origin firing (4, 5). S-CDK phosphorylates Sld2 and Sld3, and these phosphorylation events are the essential functions of S-CDK (9, 10). S-CDK phosphorylation of Sld3 is conserved in human Treslin (11). S-CDK phosphorylation of Sld2 promotes the association of Sld2 with yeast Dpb11 (12), and also the association of Sld2 with T-rich ssDNA (13). S-CDK phosphorylation of Sld3 stimulates the association of Sld3 with Dpb11 (9, 10). The associations of Sld2 with Dpb11 and Sld3 with Dpb11 have been proposed to be important for the recruitment of GINS to origins, through the generation of a preloading complex (Pre-LC), composed of Sld2, GINS, Pole, and Dpb11 (14). S-CDK-catalyzed formation of an Sld3-Dpb11-Sld2 complex has also been proposed to be important to generate a ternary ssDNA-binding complex of high affinity, because Sld2, Sld3, and Dpb11 bind to T-rich ssDNA (13, 15, 16).

The essential role of DDK in yeast cells is the phosphorylation of subunits of the Mcm2-7 complex (17). DDK phosphorylation of Mcm4 is important for cell growth, and this phosphorylation event alleviates an inhibitory function of the N terminus of Mcm4 (18). DDK phosphorylation of Mcm4 may also promote the interaction between Cdc45 and Mcm2-7 (18). DDK phosphorylation of Mcm6 may also be important for cell growth (19). Mcm2 is also a target for DDK (20), and DDK phosphorylation of Mcm2 is also required for DNA replication under normal growth conditions (21). Furthermore, expression of a mutant of *mcm2* (*mcm2-S164A,S170A*) that is not phosphorylated by DDK exerts a dominant-negative severe growth defect in budding yeast that is bypassed by the *mcm5-bob1* (*mcm5-P83L*) mutation (21). The biochemical mechanism of this genetic suppression has also been examined. DDK phosphorylation of Mcm2 reduces the affinity of budding yeast Mcm2 for Mcm5, and the *mcm5-bob1* mutation also reduces this affinity (21). This reduced affinity may help open the “Mcm2-Mcm5 gate,” which may be important for the extrusion of ssDNA from the central channel of Mcm2-7 during S phase, a requirement for origin activation (22).

Cdc45 binds weakly to Mcm2-7 in the absence of accessory factors (23). Sld3 binds tightly to Mcm2-7 and Cdc45, and thus Sld3 recruits Cdc45 to Mcm2-7 complexes (2, 23). This step may further require DDK and involve the nonessential initiation factor Sld7 (24). During origin activation, Sld3 is removed from Mcm2-7, presumably through the exposure of sequestering T-rich ssDNA (16). GINS can substitute for Sld3 as a factor that promotes the association of Cdc45 with Mcm2-7, thereby forming the stable Cdc45-Mcm2-7-GINS (CMG) replicative helicase complex (23, 25).

The mechanism of GINS recruitment may involve the formation of the S-CDK-dependent preloading complex, wherein the pre-LC recruits GINS to Mcm2-7, analogous to how Sld3 recruits Cdc45 to Mcm2-7 (14). A second proposal posits that Sld3, Sld2, and Dpb11 compete with GINS for binding to Mcm2-7 before origin activation, blocking the premature interaction between GINS and Mcm2-7

## Significance

The replication fork helicase unwinds double-stranded DNA at a replication fork, and assembly and activation of this helicase are tightly controlled. This paper describes a biological mechanism to link two essential functions related to helicase assembly and activation. Sld3, a protein required for initiation of DNA replication, coordinates the assembly of the replication fork helicase with the chemical modification of a helicase protein subunit. This chemical modification is important for subsequent activation of the helicase.

Author contributions: I.B. and D.L.K. designed research; I.B. performed research; I.B. contributed new reagents/analytic tools; I.B. and D.L.K. analyzed data; and I.B. and D.L.K. wrote the paper.

Conflict of interest statement: Patent pending for assay of human Treslin-induced stimulation of human DDK phosphorylation of human Mcm2.

This article is a PNAS Direct Submission.

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This article contains supporting information online at [www.pnas.org/lookup/suppl/doi:10.1073/pnas.1509608112/-DCSupplemental](http://www.pnas.org/lookup/suppl/doi:10.1073/pnas.1509608112/-DCSupplemental).

before origin activation (15, 16, 26). However, when T-rich ssDNA is extruded from the central channel of Mcm2-7, an ssDNA binding surface for Sld3-Sld2-Dpb11 is generated (15, 16, 26). Sld3-Sld2-Dpb11 dissociates from Mcm2-7 once the origin is melted, because Sld3-Sld2-Dpb11 has a higher affinity for ssDNA than Mcm2-7 (15, 16, 26). The dissociation of Sld3-Sld2-Dpb11 from Mcm2-7 allows GINS to bind Mcm2-7 by a passive, sequestration mechanism (15, 16, 26). The two models are not incompatible with one another, and they may both be correct.

There is an excess of Mcm2-7 double hexamer complexes loaded onto dsDNA in M phase and G<sub>1</sub> relative to the number of Mcm2-7 double hexamer complexes that actually fire. Remarkably, the activated Mcm2-7 complexes share several features in common: DDK phosphorylation of Mcm2-7, initiation factor (Sld3, Sld2, Dpb11, Mcm10, and Sld7) binding to Mcm2-7, and Cdc45/GINS attachment to Mcm2-7. What coordinates these different activities at a particular Mcm2-7 double hexamer? In other words, what prevents Cdc45 from binding to one Mcm2-7 double hexamer while DDK phosphorylates a different Mcm2-7 double hexamer? We sought to address this fundamental question in this paper.

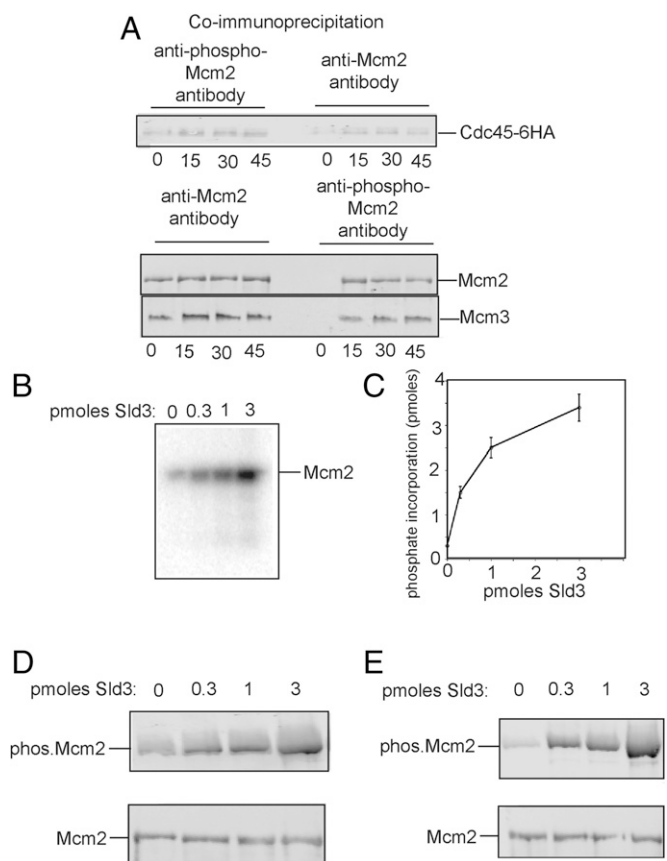
We show that DDK-phosphorylated Mcm2 preferentially interacts with Cdc45 *in vivo* compared with Mcm2 (all Mcm2 in cell, phosphorylated and unphosphorylated), suggesting that Cdc45 recruitment to Mcm2-7 is correlated with DDK phosphorylation of Mcm2. We also show that Sld3 substantially stimulates DDK phosphorylation of Mcm2 *in vitro*. We identified a mutant of Sld3, *sld3-m16*, that is defective in the stimulation of DDK phosphorylation of Mcm2. When *sld3-m16* is expressed in budding yeast cells, a dominant negative severe growth defect is observed that is bypassed by *mcm5-bob1*. Wild-type expression levels of *sld3-m16* confer a growth and DNA replication defect, with decreased DDK phosphorylation of Mcm2. Furthermore, expression of *sld3-m16* results in diminished replication protein A (RPA)-ChIP signal at an origin, and decreased GINS interaction with Mcm2-7. Furthermore, human Treslin substantially stimulates DDK phosphorylation of Mcm2 at serines 53 and 108. Finally, a mutant of human Mcm2 that mimics DDK-phosphorylated Mcm2 exhibits diminished interaction with human Mcm5, suggesting a mechanism for "gate" opening. We conclude that Sld3 coordinates helicase phosphorylation with helicase assembly.

## Results

**Strain Harboring Constitutively Expressed DDK-Dead *mcm2* Mutant (*mcm2-S164A,S170A* Expressed from Native Promoter) Exhibits Suppression.** We previously found that induced expression of *mcm2-S164A,S170A*, which is a DDK-dead mutant of *mcm2*, confers a dominant-negative severe growth defect in budding yeast. Researchers from a different laboratory found that expression of *mcm2-S164A,S170A*, when incorporated into the genome under constitutive expression by its native promoter, exhibited a defect only when exposed to DNA-damaging agents such as methyl methanesulfonate (27, 28). Given the dominant-negative phenotype that we observed, we predicted that this native promoter strain harbored a suppressor mutation. Thus, we obtained the native promoter strain from a coauthor on the paper and expressed the *mcm2-S164A,S170A* gene from a galactose-inducible promoter. Whereas wild-type yeast cells exhibit a severe growth defect upon expression of *mcm2-S164A,S170A* (Fig. S1, *Top* and ref. 21), the native promoter strain exhibited no growth defect (Fig. S1, *Bottom*). These results suggest that the native promoter strain harbors a suppressor mutation, explaining the differences in results between our two laboratories. These data also lend further support that expression of *mcm2-S164A,S170A* confers a dominant negative growth defect under normal growth conditions, and DDK phosphorylation of Mcm2 is required for growth under normal conditions.

**Mcm2 Association with Cdc45 Is Correlated with DDK Phosphorylation of Mcm2 During S Phase.** Mcm2-7 complexes are loaded in excess relative to those Mcm2-7 complexes in late M phase and G<sub>1</sub>

relative to those Mcm2-7 complexes that unwind DNA in S phase (29). Although there are many steps involved in activation of the helicase, one key to Mcm2-7 activation is the association of Mcm2 with Cdc45 (25), and a second key is the DDK phosphorylation of Mcm2 (20). We wondered whether these two events are correlated. Thus, we synchronized cells in G<sub>1</sub> with  $\alpha$ -factor, and then released into medium lacking  $\alpha$ -factor for 0, 15, 30, and 45 min. We next immunoprecipitated wild-type yeast cell extracts with antibody directed against Mcm2 [anti-Mcm2-1-160, insensitive to DDK phosphorylation of Mcm2 (21)] or antibody directed against phospho-Mcm2 [anti-mcm2-phospho-S164-phospho-S170, sensitive to DDK phosphorylation of Mcm2 (21)]. We then examined the immunoprecipitate by Western analysis using antibodies directed against Cdc45-6HA (Fig. 1A). Anti-phospho-Mcm2 antibody yielded a substantially stronger signal at 15-, 30-, and 45-min time points compared with anti-Mcm2. These data suggest that DDK phosphorylation of Mcm2 is correlated with Cdc45 attachment to Mcm2 during S phase. Although a simple explanation is that DDK



**Fig. 1.** Sld3 substantially stimulates DDK phosphorylation of Mcm2. (A) Coimmunoprecipitation analysis of wild-type budding yeast cells using antibody against the N terminus of Mcm2 (residues 1–160) or an antibody directed against the DDK-phosphorylated Mcm2 (anti-phosphoserines 164 and 170). Cells were arrested in G<sub>1</sub> with  $\alpha$ -factor and release into medium lacking  $\alpha$ -factor for the times incubated. (B) Three picomoles budding yeast DDK (Dbf4-Cdc7) was incubated with 3 pmol Mcm2 and [ $\gamma$ -<sup>32</sup>P]ATP as described in *SI Materials and Methods*. The amount of Sld3 added is indicated on top of the gel. The products were analyzed by SDS/PAGE followed by phosphorimaging. Molecular weight markers were used to identify the position of Mcm2 in the gel. A known amount of [ $\gamma$ -<sup>32</sup>P]ATP was also spotted on the gel to quantify the amount of phosphate incorporation. (C) Experiments similar to those in B were quantified and plotted. (D) Similar experiment to B, except the products were analyzed by Western analysis using antibody specific for DDK phosphorylation of Mcm2 at S164 and S170, as described previously (21). (E) Similar to D, except the Mcm2-7 complex was used as a substrate.

phosphorylation of Mcm2 increases the affinity of Mcm2 for Cdc45, our previously published *in vitro* experimental data argue against this idea (21). Thus, DDK phosphorylation of Mcm2 does not directly cause Cdc45 to associate with Mcm2-7. We therefore wondered whether Sld3, which recruits Cdc45 to Mcm2-7 (2), plays a role in coordinating Cdc45 recruitment to Mcm2-7 with DDK phosphorylation of Mcm2.

**Sld3 Substantially Stimulates DDK Phosphorylation of Mcm2 *In Vitro*.** DDK phosphorylates Mcm2 weakly *in vitro* (30), and we next investigated whether Sld3 stimulates this phosphorylation event. DDK was incubated with Mcm2, [ $\gamma$ - $^{32}$ P]ATP, and increasing amounts of Sld3. The results were analyzed by SDS/PAGE followed by phosphorimaging and quantitation (Fig. 1*B* and *C*). We found a substantial, 11-fold increase in DDK phosphorylation of Mcm2 with the addition of Sld3. These data suggest that Sld3 substantially stimulates DDK phosphorylation of Mcm2 *in vitro*. We next examined whether the anti-phospho-Mcm2 antibody, which recognizes Mcm2-phosphoserine-164-phosphoserine-170, detects higher levels of DDK phosphorylation of Mcm2 by Western analysis, using Mcm2 as a substrate (Fig. 1*D*). Indeed, there is a substantial increase in phospho-antibody signal, suggesting that Sld3 stimulates DDK phosphorylation of Mcm2 by stimulating phosphorylation at S164/S170. We then repeated the experiment reported in Fig. 1*D*, but used Mcm2-7 complex as a substrate (Fig. 1*E*). Western analysis demonstrates substantial stimulation of the DDK phosphorylation of Mcm2 at S164/170. These data suggest that Sld3 substantially stimulates DDK phosphorylation of Mcm2 when Mcm2 is incorporated into the Mcm2-7 complex.

**Sld3-m16 Is a Separation-of-Function Mutant That Is Specifically Defective in Stimulating DDK Phosphorylation of Mcm2.** We next worked to identify a separation-of-function mutant of Sld3 that is specifically defective in stimulating DDK phosphorylation of Mcm2, to pursue *in vivo* studies. We therefore divided Sld3 into two fragments, an N-terminal fragment (Sld3-1-510), and a C-terminal fragment (Sld3-511-C). We next repeated the phosphorylation assay described in Fig. 1*C* and *D*. We found that the C-terminal region of Sld3 (Sld3-511-C) functions like wild-type Sld3 in stimulating DDK phosphorylation of Mcm2, whereas the N-terminal region of Sld3 (Sld3-1-510) had no effect (Fig. S24). These results suggest that the C-terminal region of Sld3 is responsible for DDK phosphorylation of Mcm2. We next performed site-directed mutagenesis in the 511-C region of Sld3. We screened a large number of mutants, and the 16th mutant we tried, Sld3-m16 (Sld3-S556A,H557A,S558A,T559A), exhibited a substantial defect in stimulating DDK phosphorylation of Mcm2 (Fig. S24).

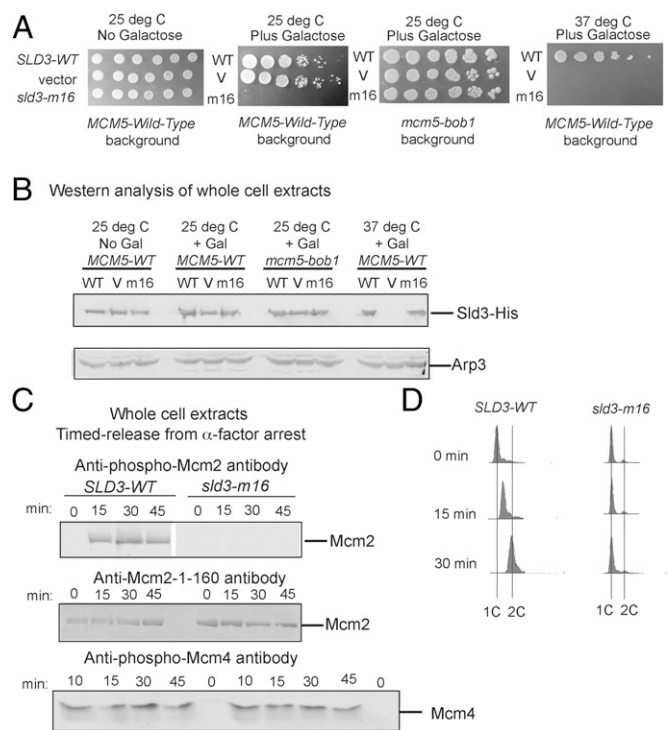
We next determined whether Sld3-m16 was specifically defective for stimulating DDK phosphorylation of Mcm2, or whether it is defective in some other known function of Sld3. CDK-phosphorylated Sld3 binds Dpb11 (9, 10). We thus determined the interaction between CDK-phosphorylated Sld3-m16 and Dpb11 and found that it bound like wild-type Sld3 (Fig. S2B). Sld3 is also known to bind T-rich origin ssDNA, Mcm2-7, and Cdc45 (16, 23). Therefore, we determined the interaction between Sld3-m16 and origin ssDNA (Fig. S2C), Mcm2-7 (Fig. S2D), or Cdc45 (Fig. S2E). In each instance, Sld3-m16 bound to ssDNA, Mcm2-7, or Cdc45 like wild-type Sld3. These data suggest that Sld3-m16 is a separation-of-function mutant that is specifically defective in stimulating DDK phosphorylation of Mcm2.

**Expression of *sld3-m16* Results in a Dominant-Negative Severe Growth Defect with Substantially Decreased Phosphorylation of Mcm2.** An *sld3-7 td* strain was generously provided by Karim Labib, University of Dundee, Dundee, Scotland, for our *in vivo* studies (3). In this strain, the genomic copy of *SLD3* is degraded at the restrictive temperature (37 °C). Given that expression of *mcm2-S164A,S170A* confers a dominant-negative severe growth defect

in budding yeast cells (21), we anticipated that expression of *sld3-m16* may also confer a dominant negative phenotype. We thus expressed *sld3-m16* from the GAL S inducible expression system, a low-copy inducible expression system, and varied the concentration of galactose until we achieved wild-type levels of Sld3-m16 at the restrictive temperature (Fig. 2*B*). In the absence of galactose, no *sld3-m16* is induced, and the cells grow like wild-type *SLD3* (Fig. 2*A*, first panel). However, once galactose is added at the permissive temperature (25 °C), the cells exhibit a severe growth defect (Fig. 2*A*, second panel). These results suggest that expression of *sld3-m16* results in a dominant-negative severe growth defect.

We next performed the same experiment, but in a *mcm5-bob1* genetic background, because the *mcm5-bob1* mutation suppresses the growth defect conferred by expression of *mcm2-S164A,S170A* (21). Indeed, the *mcm5-bob1* mutation suppressed the growth defect conferred by expression of *sld3-m16* (Fig. 2*A*, third panel), suggesting that the mechanism for growth inhibition conferred by expression of *mcm2-S164A,S170A* or *sld3-m16* is similar. We next expressed *sld3-m16* at the restrictive temperature at wild-type expression levels (Fig. 2*B*) and found a severe growth defect (Fig. 2*A*, fourth panel). These data suggest that under normal growth conditions and wild-type expression of *sld3-m16* cell growth is severely impaired.

We next examined whether for the cells examined at the restrictive temperature there is a defect in Mcm2 phosphorylation by DDK (Fig. 2*C*). We thus performed whole-cell-extract analysis followed by blotting with antibody directed against DDK-phosphorylated Mcm2 (Mcm2-phospho-serine-164-phosphoserine-170).



**Fig. 2.** Sld3 stimulation of DDK phosphorylation of Mcm2 is required for cell growth and DNA replication. (A) Tenfold serial dilution analysis of budding yeast *sld3-7 td* (*sld3*-temperature-sensitive degon) cells expressing SLD3-WT, vector, or *sld3-m16* from the GAL-S plasmid inducible promoter system (pRS415). The growth conditions are described at the top of the image, and the strain background is described at the bottom of the image. (B) Western analysis of whole cell extracts from cells used in A, probing with antibody directed against Sld3. (C) Similar to B, except probing with antibody directed against DDK-phosphorylated Mcm2. A white line in the image indicates where the image was spliced from different gels. (D) FACS analysis of cells described in A, using propidium iodide as a stain for DNA content.

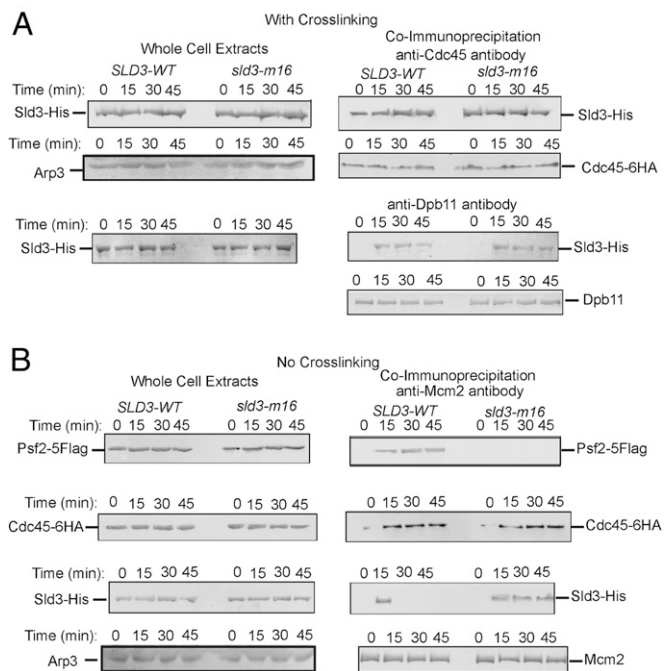
We synchronized cells in G<sub>1</sub> with  $\alpha$ -factor and then released in the absence of  $\alpha$ -factor with no hydroxyurea for 0, 15, 30, and 45 min. Whereas wild-type cells exhibited an increase in phospho-Mcm2 as cells entered S phase, cells expressing *sld3-m16* exhibited no detectable levels of phospho-Mcm2 during S phase. These results suggest that expression of *sld3-m16* confers a substantial decrease in phospho-Mcm2 expression (Fig. 2C). This result is specific for phospho-Mcm2 expression, and not phospho-Mcm4 expression (Fig. 2C). Next, we examined whether there was a defect in DNA replication for cells examined at the restrictive temperature, and indeed we found slow progression through S phase for cells expressing *sld3-m16* (Fig. 2D).

**Expression of *sld3-m16* Results in Decreased RPA-ChIP Signal at an Origin of Replication.** We next determined whether cells expressing *sld3-m16* at the restrictive temperature exhibit a defect in RPA-ChIP signal at an origin of replication. For this experiment, we synchronized cells in G<sub>1</sub> with  $\alpha$ -factor and then released into medium lacking  $\alpha$ -factor for 20 min (S phase cells). RPA-ChIP signal at an origin increases in wild-type cells in S phase at the early origins *ARS305* and *ARS306*, coincident with the formation of ssDNA at an origin of replication during S phase (origin melting and replication initiation, Fig. S3). In contrast, cells expressing *sld3-m16* do not exhibit an increase in RPA-ChIP signal at an origin of replication as cells are released from  $\alpha$ -factor arrest, suggesting that these cells have no origin melting or replication initiation. These results are similar to those observed for expression of *mcm2-S164A,S170A* (21), suggesting that Sld3-stimulated DDK phosphorylation of Mcm2 is required for origin melting and replication initiation. One possible mechanism for the lack of origin melting for cells expressing *sld3-m16* may be that Sld3 stimulation of DDK phosphorylation of Mcm2 is required to disengage Mcm2 from Mcm5 during S phase, allowing for the extrusion of ssDNA from the central channel of Mcm2-7 (21).

**Expression of *sld3-m16* Results in Decreased GINS-Mcm2-7 Signal at an Origin of Replication.** We next examined the result of expressing *sld3-m16* at the restrictive temperature on in vivo protein-protein interactions by coimmunoprecipitation analysis (Fig. 3). We first examined the effect of Sld3-Cdc45 interaction and Sld3-Dpb11 interaction using cross-linking, because cross-linking is required to detect these interactions (Fig. 3A). Cells were arrested in G<sub>1</sub> with  $\alpha$ -factor and then released into medium lacking  $\alpha$ -factor for 0, 15, 30, and 45 min. We found no difference in Sld3-Cdc45 interaction (Fig. 3A, Top) or Sld3-Dpb11 interaction (Fig. 3A, Bottom) with this analysis. These data suggest that expression of *sld3-m16* does not affect Sld3-Cdc45 or Sld3-Dpb11 interaction in vivo.

Next we performed experiments in the absence of cross-linking (Fig. 3B) to observe the interaction between Mcm2-7 and GINS (Psf2, Fig. 3B, Top), Mcm2-7 and Cdc45 (Fig. 3B, Middle), or Mcm2-7 and Sld3 (Fig. 3B, Bottom). No cross-linking is required to observe these interactions. Although there is no effect of *sld3-m16* on the loading of Cdc45 with Mcm2-7 during S phase (Fig. 3B, Middle), there is a substantial decrease in GINS interaction with Mcm2-7 in cells expressing *sld3-m16* (Fig. 3B, Top). These data suggest that Sld3 stimulation of DDK phosphorylation of Mcm2 is required for GINS assembly with Mcm2-7. Because there is no origin melting in cells expressing *sld3-m16* (Fig. S3), the lack of GINS assembly with Mcm2-7 in mutant cells may reflect that origin melting is required for GINS assembly with Mcm2-7 (15, 16, 26). We also observed that Sld3-Mcm2-7 interaction is increased at 30- and 45-min time points in mutant cells (Fig. 3B, Bottom), suggesting that expression of *sld3-m16* results in prolonged interaction between Sld3 and Mcm2-7. This observation is consistent with the idea that origin melting is required for Sld3 sequestration from Mcm2-7 (16).

**Expression of *sld3-m16* Yields a Phenotype Similar to Expression of *mcm2-S164A,S170A*.** Our data from this paper, compared with that of previous work from our laboratory (21), indicate that expression



**Fig. 3.** Sld3 stimulation of DDK phosphorylation of Mcm2 is required for GINS association with Mcm2-7 during S phase. (A) Cells were fixed and analyzed for interaction between Sld3 and Dpb11 or Sld3 and Cdc45. Cells were synchronized in G<sub>1</sub> with  $\alpha$ -factor and released into medium lacking hydroxyurea for the indicated times. (B) Cells were not fixed and analyzed for interaction between Mcm2-7 and Cdc45, GINS, or Sld3.

of *sld3-m16* yields a phenotype similar to expression of *mcm2-S164A,S170A*. Expression of either mutation yields no growth on agar plates and is suppressed by *mcm5-bob1*. Furthermore, FACS analysis of S phase progression, RPA-ChIP analysis, and Co-IP analysis yield very similar results. To further compare the phenotypes of these two mutants, we analyzed the growth rate in solution of *sld3-m16;mcm5-bob1* cells compared with *mcm2-S164A,S170A;mcm5-bob1* cells (Fig. S4). The growth rates of these two strains are very similar, suggesting that the phenotypes of these two mutations are indeed very similar. These data lend further support to the idea that the primary defect in *sld3-m16* cells is a lack of DDK phosphorylation of Mcm2. We thus sought to test whether expression of *mcm2-S164D,S170D* can suppress the growth defect conferred by expression of *sld3-m16*, but unfortunately cells expressing *mcm2-S164D,S170D* cells are dead on agar plates (Fig. S5). These data suggest that the unphosphorylated state of Mcm2 may be required for cell growth.

**Human Treslin Stimulates Human DDK Phosphorylation of Human Mcm2.** DDK phosphorylation of Mcm2 occurs in humans as well as yeast (31), and the human homolog of Sld3 is Treslin (TICRR) (11). We therefore wondered whether Sld3 stimulation of DDK phosphorylation of Mcm2 was conserved from yeast to humans. We purified human Mcm2, human DDK, and human Treslin. We then incubated human Mcm2 with human DDK, [ $\gamma$ -<sup>32</sup>P]ATP, and increasing concentrations of Treslin and analyzed the results by phosphorimaging and quantitation. We found that human Treslin stimulates human DDK phosphorylation of human Mcm2 by 15-fold (Fig. S6A and B). Human DDK phosphorylates human Mcm2 at serines 40, 53, and 108 (31). We therefore wondered whether human Treslin stimulates DDK phosphorylation of these residues. We thus obtained antibodies specific for phospho-40/41, phospho-53, or phospho-108 from Bethyl laboratories. We found that human Treslin substantially stimulates human DDK phosphorylation of human Mcm2 at serines 53 (Fig. S6C) and 108 (Fig. S6D), but not

at 40/41. These data suggest that Sld3/Treslin stimulation of DDK phosphorylation of Mcm2 is conserved from yeast to human.

**Treslin Stimulation of DDK Phosphorylation of Human Mcm2 May Weaken Mcm2/Mcm5 Interaction.** We also found previously that a mutant of yeast Mcm2 that mimics DDK phosphorylation (Mcm2-S164D,S170D) exhibits substantially diminished interaction with Mcm5 compared with wild-type Mcm2 (21). The Mcm2–Mcm5 interaction functions as a “gate” to allow the Mcm2-7 complex to encircle single- or double-stranded DNA (22). To determine whether DDK phosphorylation of Mcm2 inhibits the interaction with Mcm5 in humans, we incubated human GST-Mcm2 with radiolabeled Mcm5 in the presence of ATP (Fig. S6E). Whereas wild-type Mcm2 bound tightly to Mcm5, the DDK-phospho-mimic form of human Mcm2 (Mcm2-2D or Mcm2-S53D,S108D) exhibited substantially diminished interaction with Mcm5. Furthermore, Mcm2-2D bound to human Mcm6 (Fig. S6F) and human Cdc45 (Fig. S6G) like wild-type Mcm2, suggesting that the loss of interaction of Mcm2 with Mcm5 is specific. These data also suggest that Sld3/Treslin stimulation of DDK phosphorylation of Mcm2 reduces the interaction between Mcm2 and Mcm5 in a manner that is conserved from yeast to humans, and thus a potential regulatory mechanism may be conserved to open the Mcm2-7 “gate” during S phase. This mechanism may function along with other factors to allow for the extrusion of ssDNA from the central channel of Mcm2-7 during S phase (origin melting).

## Discussion

**DDK Phosphorylation of Mcm2 Is Coordinated with Cdc45 Recruitment to Mcm2-7.** We found that the yeast strain harboring genomic *mcm2-S164A,S170A* under control by native promoter (27, 28) exhibits normal growth upon galactose-induced overexpression of *mcm2-S164A,S170A* (Fig. S1). These data suggest that this native promoter strain harbors a suppressor mutation, and the data further support that DDK phosphorylation of Mcm2 is required for growth under normal growth conditions. We also found with coimmunoprecipitation analysis that DDK-phosphorylated Mcm2 is enriched for Cdc45 interaction in vivo compared with Mcm2 (Fig. 1A), suggesting that Mcm2-7 complexes that bind to Cdc45 are also phosphorylated by DDK. These data suggest that some mechanism exists to coordinate DDK phosphorylation of Mcm2 with Cdc45 recruitment to Mcm2-7.

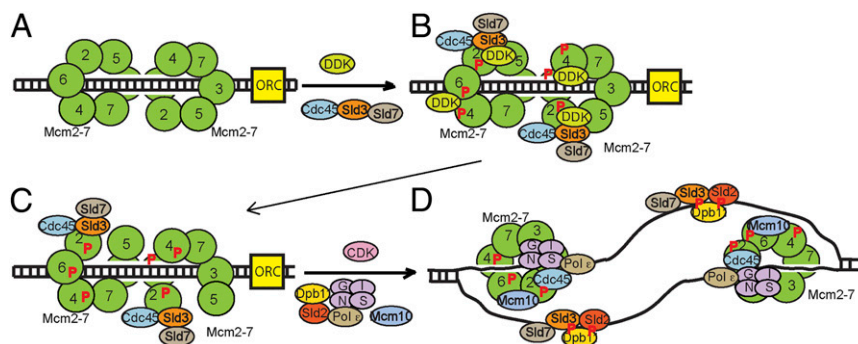
**Sld3 Stimulates DDK Phosphorylation of Mcm2.** We found that yeast Sld3 stimulates yeast DDK phosphorylation of yeast Mcm2 by 11-fold using purified proteins (Fig. 1B and C). We also found that phosphorylation of serine 164 and serine 170 of yeast Mcm2

is substantially enhanced by the addition of Sld3 to DDK (Fig. 1D). This stimulation also occurs when Mcm2-7 is present as a hexameric complex (Fig. 1E). We found that the C-terminal region of Sld3 is responsible for the stimulation of DDK phosphorylation of Mcm2, and after extensive screening of the C-terminal region we identified a mutant of Sld3, Sld3-m16 (Sld3-S556A, H557A,S558A,T559A) that is specifically defective in stimulating DDK phosphorylation of Mcm2 (Fig. S2).

**Sld3 Stimulation of DDK Phosphorylation of Mcm2 May Be Important for Origin Melting and GINS Assembly with Mcm2-7.** Expression of *sld3-m16* confers a dominant negative severe growth defect in budding yeast cells that is suppressed by *mcm5-bob1* (Fig. 2A). Expression of wild-type levels of *sld3-m16* also results in a severe growth and DNA replication defect (Fig. 2A, B, and D). DDK phosphorylation of Mcm2 is undetectable in cells expressing *sld3-m16* compared with wild-type cells (Fig. 2C), suggesting that Sld3 substantially stimulates DDK phosphorylation of Mcm2 in vivo. Cells expressing *sld3-m16* exhibit a substantially reduced RPA-ChIP signal in S phase (Fig. S3), suggesting that Sld3 stimulation of DDK phosphorylation of Mcm2 is important for origin melting and subsequent replication initiation. Coimmunoprecipitation analysis supports that Sld3 stimulation of DDK phosphorylation of Mcm2 is important for GINS association with Mcm2-7 and also timely disengagement of Sld3 from Mcm2-7 (Fig. 3). These results are consistent with previous data suggesting that DDK phosphorylation of Mcm2 is required for origin melting and subsequent sequestration of Sld3 from Mcm2-7, allowing the assembly of GINS with Mcm2-7 (21). Furthermore, the phenotypes of cells expressing *sld3-m16* and *mcm2-S164A,S170A* are very similar, suggesting that the primary defect in cells expressing *sld3-m16* is the lack of DDK phosphorylation of Mcm2.

**Human Treslin Stimulates DDK Phosphorylation of Mcm2.** We also found that human Treslin stimulates human DDK phosphorylation of human Mcm2 by 15-fold (Fig. S6A and B), and we found that serines 53 and 108 are phosphorylated in response to Treslin stimulation of DDK phosphorylation of Mcm2 (Fig. S6C and D). Furthermore, the phosphomimic form of human Mcm2 (Mcm2-2D or Mcm2-S53D,S108D) binds substantially more weakly to Mcm5 compared with unmodified Mcm2, suggesting a potential mechanism for Mcm2-7 “gate” opening with subsequent origin melting.

**A Conserved Mechanism to Couple Helicase Assembly with Helicase Phosphorylation.** A large excess of Mcm2-7 complexes are loaded onto dsDNA relative to the number of Mcm2-7 complexes that actually fire during replication initiation (29). Furthermore, Sld3



**Fig. 4.** Model for the initiation of DNA replication. (A) Mcm2-7 loads as double hexamer to encircle dsDNA during late M and G<sub>1</sub> phases. (B) In S phase, Sld3, along with Sld7, recruits Cdc45 to Mcm2-7. Sld3, while bound to Mcm2-7, substantially stimulates DDK phosphorylation of Mcm2. DDK also phosphorylates Mcm4 and Mcm6. Sld3 also blocks the premature interaction between GINS and Mcm2-7. (C) Once Mcm2 is phosphorylated by DDK, the interaction between Mcm2 and Mcm5 weakens, allowing single-strand extrusion from the central channel of Mcm2-7 (origin melting). (D) Sld3-Sld2-Dpb11 form a CDK-dependent ternary complex in S phase. Once the origin is melted, Sld3-Sld2-Dpb11 is released from Mcm2-7, because Sld3-Sld2-Dpb11 binds preferentially to ssDNA. The sequestration of Sld3-Sld2-Dpb11 onto ssDNA allows GINS to engage with Cdc45-Mcm2-7, and the CMG helicase is assembled and activated for unwinding.

and DDK are two limiting factors for DNA replication, along with Sld2 and Dpb11 (32). Thus, a question arises: What orchestrates the set of events that are required for origin firing at an activated Mcm2-7 double hexamer? We show in the paper that Sld3 may be critical for this orchestration, because Sld3 may couple recruiting Cdc45 to Mcm2-7 with stimulation of DDK phosphorylation of Mcm2. It is important to emphasize that DDK phosphorylation of Mcm2 does not cause the Cdc45 recruitment to Mcm2-7. Instead, DDK phosphorylation of Mcm2 is correlated with Cdc45 recruitment to Mcm2-7. This correlation is the result of Sld3 binding to Mcm2-7. When Sld3 binds to Mcm2-7, Sld3 simultaneously recruits Cdc45 to Mcm2-7, and Sld3 stimulates DDK phosphorylation of Mcm2.

**A Model for Replication Initiation.** A model for the initiation of DNA replication is shown in Fig. 4. In late M and G<sub>1</sub>, the origin recognition complex (Orc), along with Cdc6 and Cdt1, promotes the loading of Mcm2-7 as a double hexamer to surround dsDNA (Fig. S3A) (6, 7, 33, 34). In S phase, Sld3-Sld7 recruits Cdc45 to Mcm2-7 (2, 17, 24, 35) (Fig. 4B). Sld3, once bound to Mcm2-7, substantially stimulates DDK phosphorylation of Mcm2 (this paper). Sld3 also blocks the premature interaction between GINS and Mcm2-7 (16, 23). DDK also phosphorylates Mcm4 and Mcm6 (18, 19, 36–38). The Mcm2-7 double hexamer then dissociates to single hexamers by an unknown mechanism (39). Once DDK phosphorylates Mcm2, and possibly with the aid of accessory proteins, Mcm2 loses its affinity for Mcm5 (Fig. 4C) (21). This allows for single-strand extrusion from the central channel of Mcm2-7 (i.e., origin melting). Origin melting generates ssDNA that sequesters the Sld3-Sld2-

Dpb11 complex onto ssDNA, removing Sld3-Sld2-Dpb11 from Mcm2-7 (15, 16, 26). This sequestration allows GINS to bind Cdc45-Mcm2-7 (Fig. 4D). The CMG replication fork helicase is now fully assembled and activated.

## Materials and Methods

Antibodies were obtained from a commercial supplier (Pierce or Bethyl laboratories, see *SI Materials and Methods* for details). Plasmids and proteins were generated using established methods (see *SI Materials and Methods* for details). Yeast strains were generated from material supplied by Karim Labib; Robert Sclafani, University of Colorado, Denver, CO; and the Yeast Genetic Resource (see *SI Materials and Methods* for details). Kinase labeling of proteins were performed as described. DDK reactions were performed at 30 °C for 60 min (see *SI Materials and Methods* for details). Yeast dilutions were performed in 1:10 dilutions on agar plates (see *SI Materials and Methods* for details). FACS analysis was performed with propidium iodide staining (see *SI Materials and Methods* for details). Chromatin Immunoprecipitation was performed with cross-linking reagent as previously described (see *SI Materials and Methods* for details). Coimmunoprecipitation analyses were performed with no cross-linking agent and no hydroxyurea as described (see *SI Materials and Methods* for details).

**ACKNOWLEDGMENTS.** We thank Christopher Brandl for the yeast strain harboring *mcm2-5164A,5170A* regulated by native promoter; Jerard Hurwitz for cDNA for human Mcm2, Mcm5, Mcm6 and Cdc45; William Dunphy for cDNA for human Treslin; Robert Sclafani for the *mcm5-bob1* strain; Karim Labib for the *sld3-7 td* strain; and Dr. Susan Taylor for supplying purified PKA. We thank the FACS facility at Florida State University College of Medicine for the use of the FACS equipment. Funding was provided by Florida State University and National Science Foundation Grant 1265431 (to D.L.K.).

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