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Mec1 Is One of Multiple Kinases that Prime the Mcm2-7 Helicase for Phosphorylation by Cdc7

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SUMMARY

Activation of the eukaryotic replicative DNA helicase, the Mcm2-7 complex, requires phosphorylation by Cdc7/Dbf4 (*Dbf4-dependent kinase* or DDK), which, in turn, depends on prior phosphorylation of Mcm2-7 by an unknown kinase (or kinases). We identified DDK phosphorylation sites on Mcm4 and Mcm6 and found that phosphorylation of either subunit suffices for cell proliferation. Importantly, prior phosphorylation of either S/T-P or S/T-Q motifs on these subunits is required for DDK phosphorylation of Mcm2-7 and for normal S phase passage. Phosphomimetic mutations of DDK target sites bypass both DDK function and mutation of the priming phosphorylation sites. Mrc1 facilitates Mec1 phosphorylation of the S/T-Q motifs of chromatin-bound Mcm2-7 during S phase to activate replication. Genetic interactions between priming site mutations and *MRC1* or *TOF1* deletion support a role for these modifications in replication fork stability. These findings identify regulatory mechanisms that modulate origin firing and replication fork assembly during cell cycle progression.

INTRODUCTION

The replication process in eukaryotic cells temporally separates helicase loading and helicase activation to ensure that no origin of replication can initiate more than once per cell cycle. Helicase loading (also referred to as prereplicative complex [pre-RC] formation, or origin licensing) is restricted to the G1 phase of the cell cycle. This process requires the origin recognition complex (ORC), Cdc6, and Cdt1 to assemble a dimer of the eukaryotic helicase, the Mcm2-7 complex, around dsDNA (Evrin et al., 2009; Remus et al., 2009). As cells enter S phase, the resulting head-to-head Mcm2-7 dimer is activated by initiation factor binding to the Mcm2-7 complex. Most notably, Cdc45 and the GINS complex activate Mcm2-7 ATPase and helicase activities (Ilves et al., 2010).

Two kinases trigger eukaryotic helicase activation: the Cdc7 kinase and S phase cyclin-dependent kinases (S-CDKs). Cdc7 directly phosphorylates the Mcm2-7 complex and is activated by the cell-cycle-regulated accessory subunit Dbf4 or the related Drf1 (Sclafani and Holzen, 2007). Thus, activated Cdc7 is known as *Dbf4/Drf1-dependent kinase*, or DDK. DDK phosphorylation of Mcm2-7 is required for the association of the helicase activators Cdc45 and GINS (Labib, 2010). The role of S-CDKs is best understood in *Saccharomyces cerevisiae* cells, where they phosphorylate Sld2 and Sld3, leading to the recruitment of Sld2 and Dpb11 to the origin (Tanaka et al., 2007; Zegerman and Diffley, 2007). These three proteins also stimulate Cdc45 and GINS origin association (Labib, 2010), suggesting that S-CDKs and DDK function in parallel to trigger helicase activation.

Two additional kinases, Mec1 and Rad53, also coordinate the events of DNA replication in *S. cerevisiae* cells. The essential function of both kinases is to trigger the activation of ribonucleotide reductase (RNR) and the induction of deoxyribonucleotide synthesis as cells enter S phase (Zhao et al., 2001). This function can be bypassed by artificially increasing dNTP levels by a variety of means, including deletion of the RNR inhibitor *SML1* (Zegerman and Diffley, 2009). The activation of Mec1 and Rad53 upon S phase entry is poorly understood but may involve the detection of ongoing DNA synthesis. Mec1 and Rad53 also mediate the checkpoint response to DNA damage and stalled DNA replication forks (Zegerman and Diffley, 2009). In each of these functions, Mec1 acts at the top of a signal transduction pathway that requires the Mrc1 or Rad9 proteins to activate Rad53 kinase.

Consistent with its role in activating loaded Mcm2-7 complexes, DDK associates with replication origins (Dowell et al., 1994) and preferentially binds and phosphorylates loaded Mcm2-7 complexes (Masai et al., 2006; Sheu and Stillman, 2006). Early in vitro studies using purified Mcm2-7 identified Mcm2 as a primary DDK target, whereas in vivo phosphorylation studies suggested that Mcm4 was the primary DDK target (Francis et al., 2009; Masai et al., 2000, 2006; Sheu and Stillman, 2006). DDK phosphorylation of Mcm4 and Mcm6 is stimulated in the context of the pre-RC (Francis et al., 2009), explaining the distinct in vivo and in vitro phosphorylation profiles. Intriguingly, DDK cannot bind Mcm2-7 or phosphorylate Mcm4 or Mcm6 without prior Mcm2-7 phosphorylation by an unidentified kinase (Francis et al., 2009).

Two classes of DDK target sequences have been identified previously. "Intrinsic" sites (S/T-D/E) are characterized by an acidic residue at the +1 position (Charych et al., 2008; Cho

et al., 2006; Montagnoli et al., 2006). In a second class of sites, a negative charge at the +1 position is provided by a phosphoserine or phosphothreonine. Thus, the recognition of these “phosphorylation-generated” (PG) sites by DDK requires prior phosphorylation by a second kinase. For example, DDK can phosphorylate the initial serine in an S-S-P motif when the second serine is previously phosphorylated by CDK, as occurs with DDK phosphorylation of *S. cerevisiae* Mer2 (Sasanuma et al., 2008; Wan et al., 2008). Phosphorylation of similar sites by DDK on human Mcm2 and Mcm4 has been observed (Masai et al., 2006; Montagnoli et al., 2006); however, the biological relevance of these modifications remains unknown. S-CDK is unlikely to act as an essential priming kinase for DDK targeting of Mcm2-7 because Sld2 and Sld3 are the only essential S-CDK targets for DNA replication in budding yeast (Tanaka et al., 2007; Zegerman and Diffley, 2007). Despite our knowledge of potential DDK target sites, the essential Mcm2-7 sites targeted by DDK and how these modifications are stimulated by prior phosphorylation remain unknown.

In this study, we identify DDK phosphorylation sites on Mcm4 and Mcm6 as well as “priming” phosphorylation sites required for DDK phosphorylation of the same subunits. Phosphomimetic mutations of the identified DDK target sites bypass both DDK function and mutation of the priming sites. The majority of the priming sites are either S/T-P or S/T-Q motifs preceded by one or more S/T residues. Mutational analysis demonstrated overlapping function between the S/T-P and S/T-Q sites as well as between Mcm4 and Mcm6 phosphorylation. Mec1 phosphorylates the S/T-Q sites on chromatin-bound Mcm2-7 during S phase, whereas the S/T-P sites appear to be constitutively phosphorylated. Synthetic lethality between priming site mutations and *MRC1* deletion suggests that incomplete priming site phosphorylation results in defective replication fork assembly. Our findings demonstrate that phosphorylation of Mcm4 and Mcm6 by Mec1 and a proline-directed kinase activates these proteins for phosphorylation by DDK and proper replication fork assembly.

RESULTS

Identification of Phosphorylation Sites on Mcm2-7

We used mass spectrometry to map phosphorylation sites on the Mcm2-7 complex. Our previous studies indicated that DDK phosphorylation of Mcm2-7 is strongly influenced by pre-RC formation and by prior phosphorylation of Mcm2-7 by one or more kinases (Francis et al., 2009). For this reason, we used in vitro assembled pre-RCs as a DDK substrate to identify phosphorylated residues. Because Mcm2-7 is phosphorylated during an in vitro helicase loading reaction lacking DDK, we identified residues that were phosphorylated during pre-RC assembly with and without DDK treatment.

The majority of phosphorylation sites localized to four clusters outside the conserved OB-fold-like and AAA+ motifs of the Mcm subunits: the amino termini of Mcm2, Mcm4, and Mcm6 and the carboxyl terminus of Mcm3 (Figure 1A; only unambiguously assigned phosphates are shown). The cluster of phosphosites in the Mcm3 C terminus was mostly DDK independent (ten of 12 sites) and corresponds to a CDK-regulated nuclear transport

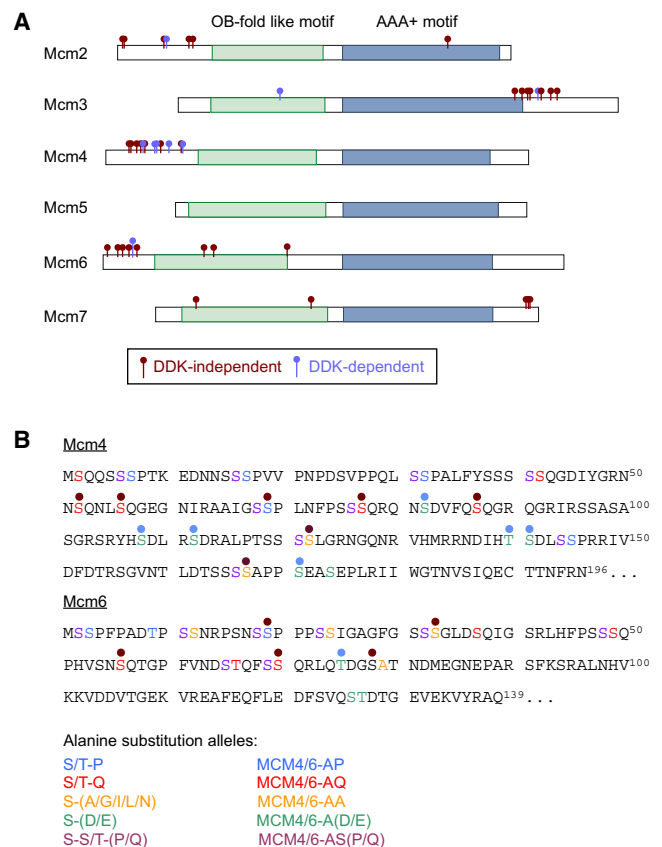


Figure 1. Identification of Priming and Target Sites for DDK on Mcm2-7

(A) Cartoon showing locations of phosphorylation sites on Mcm2-7 either before (red) or after (blue) phosphorylation by recombinant DDK. Pre-RCs were assembled on origin-DNA-bound magnetic beads by using whole-cell extract. Origin-bound pre-RC proteins were separated by SDS-PAGE and the bands representing Mcm2-7 were analyzed for phosphorylation using LC/MS/MS.

(B) Phosphorylation sites on the Mcm4 and Mcm6 N-terminal tails. The colored residues correspond to sites mutated in this study and are coded according to the residue at the +1 position. Blue, Pro; red, Gln; yellow, A/G/I/L/N; green, Asp/Glu; purple, Ser/Thr. Each illustration shows only unambiguously assigned sites of phosphorylation. See also Figure S1.

signal (Liku et al., 2005). The four DDK-independent sites found in the N terminus of Mcm2 are casein kinase 2 (CK2) consensus sites (S/T-D/E-X-D/E; Pinna, 2002), and depleting CK2 from extracts prior to pre-RC assembly eliminated Mcm2 phosphorylation (data not shown). In contrast to a recent study (Bruck and Kaplan, 2009), we found that these sites are not critical for the essential functions of Mcm2 (Figure S1, available online). Because Mcm4 is a critical DDK target in vivo (Sheu and Stillman, 2006, 2010), and because Mcm4 and Mcm6 are the two phosphorylation- and pre-RC-dependent targets of budding yeast DDK (Francis et al., 2009), we focused our analysis on the phosphosites in the Mcm4 and Mcm6 amino termini.

We found 13 phosphorylation sites in the Mcm4 N terminus and six sites in the Mcm6 N terminus (Figure 1B and Table S1). Seven of the Mcm4 and five of the Mcm6 phosphorylation sites

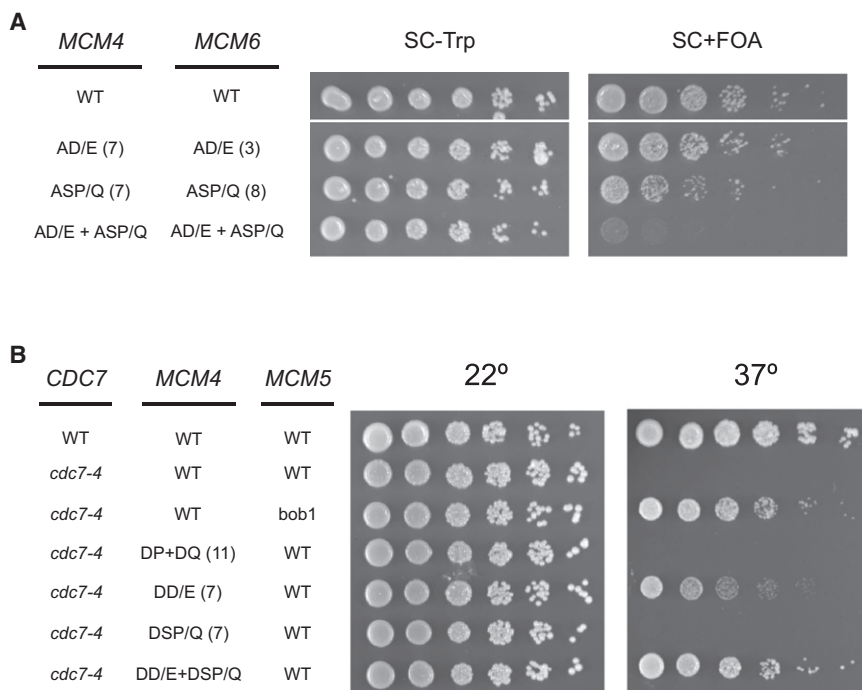


Figure 2. Phenotype of Putative DDK Target Site Mutants

(A) Effect of alanine substitutions at putative DDK target sites. Strains yJCWR185, yJCWR176, yJCWR180, and yJCWR184 (see Table S2) carrying the indicated alleles of *MCM4* and *MCM6* in addition to a URA⁺ *ARS/CEN MCM4+* plasmid were grown on ura⁺ media for 3–4 generations to allow loss of the URA⁺ *MCM4+* plasmid. A 5-fold serial dilution of cells was then spotted on synthetic complete (SC) plates without tryptophan (left) or with FOA (right) to select against the URA⁺ *MCM4+* plasmid. The numbers in parentheses adjacent to the allele name indicate the number of sites mutated in each allele (see also Figure S1). (B) *CDC7* bypass by phosphomimetic *MCM4* alleles. The indicated *MCM4* alleles were introduced into strain OAY711 (*cdc7-4*) by direct replacement at the chromosomal *MCM4* locus. Cells were spotted on YPD plates at 22°C and 37°C.

were DDK independent (Figure 1B, red dots, and Table S1). We categorized the DDK-independent sites by the identity of the +1 residue: two S/T-P sites, six S/T-Q sites, and four sites characterized by an aliphatic residue (A, G, or L) at the +1 position. Intriguingly, many of these phosphoserines (seven of 12) were preceded by one or more serines or threonines, suggesting that these sites could be PG DDK target sites.

Six Mcm4 sites and one Mcm6 site were only observed in the DDK-treated reaction (Figure 1B, blue dots, and Table S1). Each of these phosphosites is followed by an acidic residue at the +1 position as expected for intrinsic DDK sites. We also found evidence that DDK targeted PG sites in Mcm4 and Mcm6. In these cases, DDK targeted serines preceding a phospho-SP or phospho-SQ motif, although in all cases the abundance of serines in the analyzed peptides precluded unambiguous assignment of the phosphorylated residues. For example, mass spectrometry identified the triphosphorylated Mcm4 peptide AAGSSPLNFPSSSR (residues 64–79; Table S1). Because the SP and SQ sites in this peptide are both phosphorylated in a DDK-independent manner (Figure 1B, Table S1, and see below), DDK must phosphorylate at least one of the serines at the –1 or –2 position relative to the DDK-independent sites. We conclude that DDK phosphorylates both intrinsic and PG sites in Mcm4 and Mcm6.

DDK Phosphorylation of Mcm4 and Mcm6 Has Overlapping Functions

To test their in vivo relevance, we eliminated the potential DDK phosphorylation sites in Mcm4 and Mcm6. We hypothesized that there are two classes of DDK target sites: S/T-D/E sites (intrinsic sites) and the first serine in S-S-P/Q sites (PG sites). We created *MCM4* and *MCM6* mutations that eliminated each

class of sites either individually or together. Mutation of intrinsic or PG sites alone or together in Mcm4 or Mcm6 resulted in no significant proliferation defect (Figure S1). Similarly, no proliferation defect was observed in a strain when the intrinsic sites were eliminated (A-D/E) in Mcm4 and Mcm6 simultaneously (Figure 2A). Elimination of the PG sites (A-S-P/Q) from both Mcm4 and Mcm6 resulted in a small proliferation defect. We observed a severe proliferation defect, however, when the intrinsic and PG sites in both *MCM4* and *MCM6* were mutated. This observation suggests that DDK phosphorylation of *MCM4* and *MCM6* can compensate for one another. Furthermore, phosphorylation of either the intrinsic sites or the PG sites is sufficient for cell proliferation.

To confirm that the intrinsic and PG sites are targeted by DDK in vivo, we mutated these residues in *MCM4* to the phosphomimetic amino acid aspartate and asked whether these mutations could bypass the temperature-sensitive proliferation defect of a *cdc7-4* allele. We compared the proliferation of this strain to that of a previously isolated *CDC7* bypass allele, *MCM5-bob1* (Hardy et al., 1997). Mutating the seven intrinsic (S/T-D/E) sites in *MCM4* to D-D/E allowed proliferation of a *cdc7-4* strain at the nonpermissive temperature (Figure 2B). Phosphomimetic mutations in the 11 SP and SQ sites in *MCM4* did not allow growth at elevated temperature, demonstrating that the DDK bypass conferred by the D-D/E mutations was not due to a general increase in the negative charge of the Mcm4 N terminus. Phosphomimetic mutations in the PG class of *MCM4* DDK target sites (S-S-P/Q to D-S-P/Q) did not bypass *cdc7-4* function. Instead, combining the phosphomimetic mutations in the intrinsic and PG sites enhanced *CDC7* bypass relative to the D-D/E mutations alone (Figure 2B). Similar mutations in *MCM6* did not bypass the *cdc7-4* proliferation defect independently but did enhance proliferation in the *MCM4* phosphomimetic background (data not shown). We conclude that both the S/T-D/E and the S-S-P/Q sites in Mcm4 and Mcm6 are targeted by DDK in vivo.

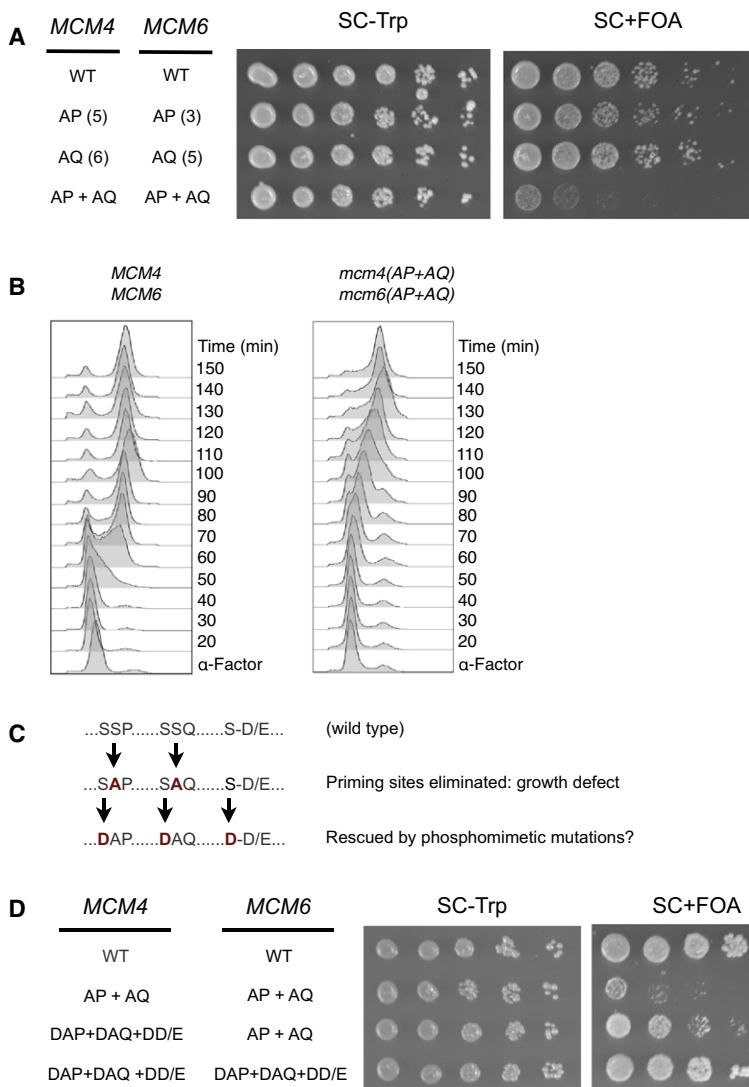


Figure 3. Phenotype of Putative Priming Site Mutants

(A) Effect of alanine substitutions at putative priming sites. Analysis as in Figure 2A of the wild-type control yJCWR185 and *mcm4/mcm6* double-mutant strains yJCWR167, yJCWR171, and yJCWR175. The numbers in parentheses adjacent to the allele name indicate the number of sites mutated in each allele (see also Figure S1). (B) S phase progression defect of *mcm4(AP+AQ) mcm6(AP+AQ)* strain. DNA content was measured by fluorescence-activated cell sorting analysis after release from α factor at 25°C.

(C) Schematic of bypass strategy used in (D).

(D) Rescue of priming site mutations by *MCM4* and/or *MCM6* phosphomimetic mutations at DDK target sites. Analysis of strains yJCWR185, yJCWR175, yJCWR236, and yJCWR237 was performed as in Figure 2A.

populations, we found that relative to wild-type cells, the priming-site-deficient strain showed a 10 to 20 min delay in the onset of DNA replication and a doubling of S phase length (60 versus 30 min). Thus, eliminating the S/T-P and S/T-Q priming phosphorylation sites altered both the onset of bulk DNA replication and the time required to complete DNA synthesis.

If phosphorylation of the S/T-Q and S/T-P sites facilitates DDK targeting, then mutations that bypass DDK activity should also bypass the defects of the AP and AQ mutations. Indeed, the proliferation defect of the priming-site-deficient strain was partially rescued by incorporating phosphomimetic mutations in the predicted DDK sites of *MCM4* (D-A-P/Q and D-D/E mutations, Figures 3C and 3D). This rescue was strongly enhanced when DDK phosphomimetic mutations were also incorporated into *MCM6*. These studies strongly suggest that the primary role of the S/T-P and S/T-Q sites

S/T-P and S/T-Q Sites Prime DDK Phosphorylation

Having established the importance of the predicted DDK sites in vivo, we examined the importance of the proposed S/T-P and S/T-Q priming sites. We mutated each class of site either alone or in combination and assayed the effects of these mutations on cell viability. As with the DDK sites, mutating one or both classes of site in *MCM4* or *MCM6* alone had no effect on cell proliferation (Figure S1). Similarly, mutating all of the *MCM4* or *MCM6* S/T-P sites to AP sites, or all of the *MCM4* and *MCM6* S/T-Q sites to AQ sites, revealed no phenotype (Figure 3A). Consistent with the compensatory functions of Mcm4 and Mcm6 DDK phosphorylation, a severe proliferation defect was observed only when the AP and AQ mutations were simultaneously introduced into both Mcm4 and Mcm6 [*mcm4(AP+AQ) mcm6(AP+AQ)*]; Figure 3A].

The priming-site-deficient strain [*mcm4(AP+AQ) mcm6(AP+AQ)*] was defective for passage through S phase (Figure 3B). By monitoring DNA content of synchronous cell

is to activate Mcm2-7 for targeting by DDK during replication initiation.

To test the hypothesis that phosphorylation of the S/T-P or S/T-Q sites influences DDK targeting, we assembled pre-RCs in extracts derived from strains in which the Mcm6 priming sites were mutated. The resulting pre-RCs were treated with DDK in the presence of radiolabeled ATP. We also performed this experiment using an *mcm6* mutant lacking the putative DDK targets to test whether these mutations eliminated DDK phosphorylation. None of the mutations affected Mcm2-7 loading (Figure 4A).

DDK treatment of the loaded, mutant Mcm2-7 complexes demonstrated that the S/T-P and S/T-Q sites control DDK phosphorylation of both classes of DDK target sites. As expected, simultaneous mutation of the DDK target residues in the intrinsic and PG (S/T-S-P/Q) sites resulted in an ~20-fold reduction in Mcm6 phosphorylation (Figure 4A, compare lanes 2 and 10). Importantly, elimination of both S/T-P and S/T-Q sites on Mcm6 resulted in a similar reduction in DDK phosphorylation

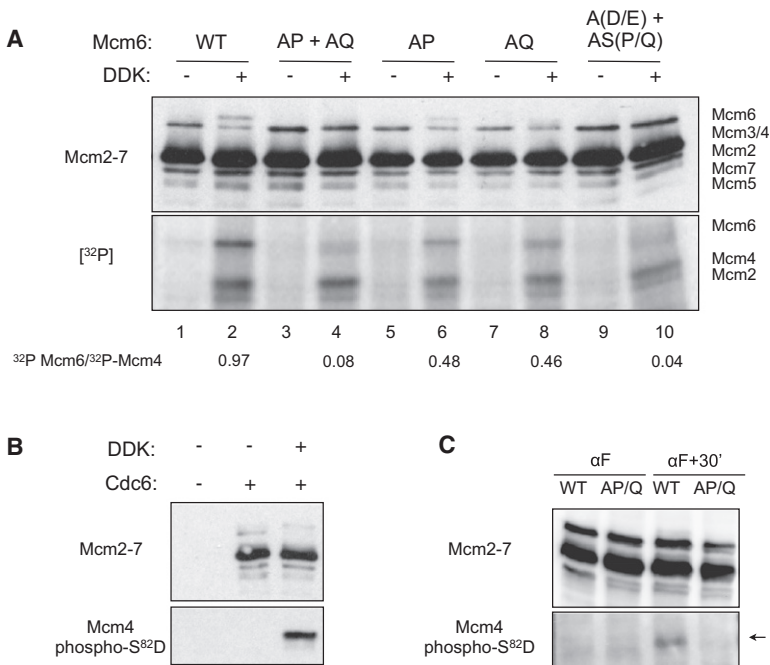


Figure 4. S/T-P and S/T-Q Sites Reduce DDK Targeting of Mcm2-7

(A) Elimination of S/T-P and S/T-Q sites in Mcm6 inhibits DDK phosphorylation. Pre-RCs were assembled using G1-arrested extracts from strains yJCWR128, yRH163, yJCWR129, yJCWR130, and yJCWR135 carrying the indicated sets of alanine substitutions in *MCM6*. All strains are wild-type for *MCM4*. The resulting pre-RCs were then phosphorylated by DDK using radiolabeled ATP, and the labeled proteins were analyzed by immunoblotting (top panel) or autoradiography (bottom panel).

(B) Mcm4-S82-D83 is targeted by DDK. Pre-RCs assembly reactions were performed using G1-arrested extracts with or without Cdc6 and DDK. The resulting DNA-associated proteins were analyzed by immunoblotting with anti-Mcm2-7 (UM-174, top) or Mcm4-phospho-S82-D83 antibodies. See also Figure S2.

(C) S/T-P and S/T-Q sites are required for DDK targeting of Mcm4 in vivo. *MCM4 MCM6* or *mcm4(AP+AQ) mcm6(AP+AQ)* cultures (yJCWR205 or yJCWR208) were arrested in G1 phase with α factor. Cells were washed and released into medium lacking α factor for 30 min at 30°C. Total cell protein was collected by TCA precipitation and analyzed by immunoblotting using anti-Mcm2-7 (top) and a phosphospecific antibody targeting Mcm4-S82-D83 (bottom).

of Mcm6 (Figure 4A, compare lanes 2, 4, and 10). In both cases, the Mcm6 mutations did not affect Mcm4 phosphorylation, indicating that these mutations acted in *cis*. Interestingly, pre-RCs containing either Mcm6-AP or Mcm6-AQ mutations reduced DDK phosphorylation equivalently (~50%, lanes 6 and 8). Thus, in contrast to their apparently redundant function in the proliferation studies, direct phosphorylation assays showed that S/T-P and S/T-Q sites both contribute to full DDK phosphorylation of Mcm6.

We addressed the role of priming phosphorylation in DDK targeting in vivo using a phosphospecific antibody that recognized the phosphorylation of Mcm4-S82-D83, an intrinsic DDK target site. Studies using in vitro assembled pre-RCs showed that recognition of this site by the phosphospecific antibody was dependent on treatment of the pre-RCs with DDK (Figure 4B). In vivo, phosphorylation of this intrinsic DDK site was detected in wild-type S phase cells, but was eliminated in the priming-deficient-strain (Figure 4C). Thus, as observed in the in vitro studies, phosphorylation of the S/T-P and S/T-Q sites was critical for DDK targeting in vivo, even for an intrinsic DDK target site.

Cell Cycle Regulation of Mcm2-7 Phosphorylation

We next addressed the cell cycle regulation of Mcm2-7 phosphorylation. To this end, we used both the Mcm4-phospho-S82-D83 antibody and additional phosphospecific antibodies that recognized specific SP and SQ sites in Mcm4 and Mcm6. Analysis of the phosphoSP and phosphoSQ antibodies showed that their recognition of Mcm2-7 was inhibited by phosphatase treatment (Figure S2) and by mutations that eliminated their target sites (Figure 5A). As expected for a DDK target, phosphorylation of Mcm4-S82-D83 was only seen in the S phase sample (Figure 5A, lane 3). The S/T-P and S/T-Q sites had

distinct patterns of phosphorylation during the cell cycle. Two SP sites (Mcm4-S17-P18 and Mcm6-S19-P20) were phosphorylated in G1, S, and M phase cells (Figure 5A, lanes 1 and 3, and Figure 5C). In contrast, both SQ sites analyzed (Mcm4-S87-Q88 and Mcm6-S70-Q71) were phosphorylated in S phase cells but not in G1 or M phase cells. To further define the time of SQ phosphorylation, we arrested cells at the G1/S transition using *cdc7-4* or *cdc4-1* temperature-sensitive alleles. For both mutants, Mcm4-S87-Q88 was not phosphorylated in cells arrested at the nonpermissive temperature (Figure 5B). Thus, unlike the phosphorylation of SP sites, SQ phosphorylation on Mcm4 and Mcm6 occurs after entry into S phase.

To ask whether phosphorylation of S/T-P or S/T-Q sites on Mcm2-7 is dependent on helicase loading, we fractionated chromatin-bound and -unbound proteins from G1, S, and G2/M phase cells and detected Mcm4 and Mcm6 phosphorylation using the phosphospecific antisera (Figure 5C). Mcm6-S19-P20 was phosphorylated both on and off chromatin, and the amount of phosphorylation was correlated with total Mcm2-7 levels. In contrast, phosphorylation of Mcm4-S87-Q88 in S phase was exclusive to chromatin-associated Mcm2-7 (Figure 5C, lanes 4–6).

Mrc1 Facilitates Mec1 Phosphorylation of Mcm4

S/T-Q sites are frequently targets of PI3 kinases, which in *S. cerevisiae* include the Mec1 and Tel1 kinases. To assess whether Mec1 or Tel1 is responsible for the phosphorylation of S/T-Q sites on Mcm4 in vivo, we deleted these kinases and assessed phosphorylation of Mcm4-S87-Q88 at various cell cycle stages. *SML1* was deleted in all strains to rescue the lethality of *MEC1* deletion (Zhao et al., 2001). S phase phosphorylation of Mcm4-S87-Q88 was eliminated in the *mec1Δ* and *mec1Δ tel1Δ*, but not the

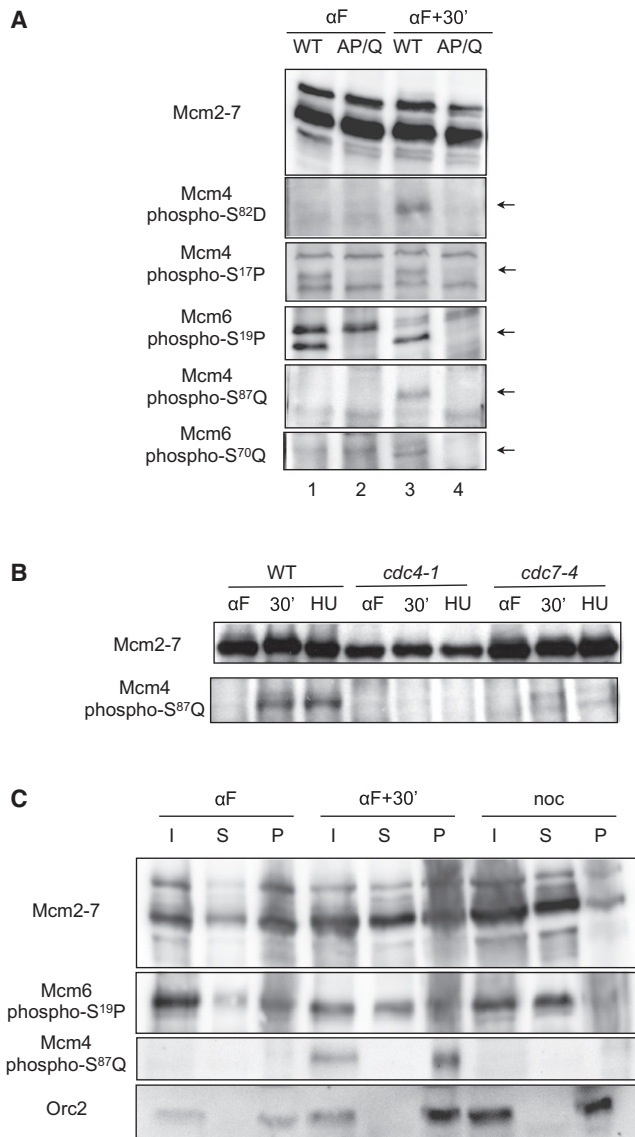


Figure 5. Mcm2-7 SQ Sites Are Phosphorylated on Chromatin in S Phase

(A) S/T-P and S/T-Q phosphorylation in G1 and S phase. Extracts of *MCM4 MCM6* or *mcm4(AP+AQ) mcm6(AP+AQ)* cells were prepared as described in Figure 4C and analyzed by immunoblotting using anti-Mcm2-7 and the indicated phosphospecific antisera. Arrows indicate the phosphospecific bands. (B) SQ phosphorylation in cells arrested at the G1/S transition. Wild-type, *cdc4-1*, or *cdc7-4* cells were grown to mid-log phase at 25°C, arrested in G1 using α factor, then released at 37°C. TCA-precipitated protein was analyzed by immunoblotting using anti-Mcm2-7 or anti-Mcm4-phospho-S87-Q sera. (C) S/T-Q phosphorylation is specific for chromatin-associated Mcm2-7. W303BLa cells were arrested in nocodazole (noc) or α factor (α F) with or without 30 min release at 30°C. Total cell protein was separated by a chromatin fractionation procedure into input (I), supernatant (S), and pelleted chromatin (P) fractions and analyzed by immunoblotting with sera detecting Mcm2-7, Mcm6-phospho-S19-P20, Mcm4-phospho-S87-Q88, or Orc2.

tel1Δ strain (Figure 6A), strongly suggesting that Mec1 is the principal kinase responsible for phosphorylating this site in vivo. Given their association with the Mcm2-7 complex and their

known roles in Mec1 function (Mordes et al., 2008; Navadgi-Patil and Burgers, 2008; Naylor et al., 2009), we asked whether Mrc1 or Dpb11 was required for Mcm4-SQ phosphorylation. Elimination of Mrc1 reduced but did not eliminate phosphorylation of Mcm4-S87-Q88 during normal S phase passage and upon hydroxyurea (HU) treatment (Figure 6A). In contrast, a temperature-sensitive mutation in Dpb11 had no effect on the same phosphorylation event (Figure S3).

If Mec1 is the SQ-directed Mcm4/6 priming kinase, deleting *MEC1* should phenocopy the effect of mutating the S/T-Q sites in these subunits. Because the proliferation role of S/T-Q sites in *MCM4* and *MCM6* is overlapping with that of the S/T-P sites (Figure 3A), we deleted *MEC1* in an *mcm4-AP mcm6-AP* strain. Deletion of *MEC1* in the context of the AP mutations caused the same severe proliferation defect as combining the AP and AQ mutations in *MCM4* and *MCM6* (compare Figure 6B to Figure 3A). Deletion of *TEL1* in the same strain background caused no defect either in the presence or the absence of *MEC1* (Figure 6B). Similarly, no proliferation defect was observed when *MEC1* was deleted in a strain carrying the wild-type or AQ alleles of *MCM4* and *MCM6* (Figure 6B). Together, these data strongly support the conclusion that Mec1 directly targets the S/T-Q sites in Mcm4 and Mcm6, although it is possible that Mec1 and Mrc1 activate a different S/T-Q-directed kinase to target Mcm4 and Mcm6.

Checkpoint Proteins Facilitate Growth in the Absence of SP Phosphorylation

It is possible that eliminating the S/T-P motifs in Mcm4 and Mcm6 causes replication stress that increases the cell's reliance on a *MEC1*-dependent S phase checkpoint. We first tested whether any of the replication defects caused by priming site mutations (Figure 3) conferred sensitivity to HU treatment. Cells lacking the Mcm4 and Mcm6 S/T-P, S-T/Q, or both types of priming sites showed no additional growth defects relative to wild-type cells in the presence of 50 mM HU (Figure 6C). Thus, neither S/T-Q nor S/T-P sites are required for the checkpoint response to stalled replication forks. Next, we tested whether deletion of *RAD53*, *RAD9*, *MRC1*, or *TOF1* also inhibited proliferation of the *mcm4-AP mcm6-AP* cells. These proteins mediate either the DNA-damage (Rad9, Rad53) or the stalled-replication-fork (Mrc1, Tof1, Rad53) checkpoints (Zegerman and Diffley, 2009). Mrc1 and Tof1 also regulate replication fork progression (Hodgson et al., 2007; Szyjka et al., 2005; Tourrière et al., 2005). As with *MEC1*, deletion of each of these genes reduced proliferation of a *mcm4-AP mcm6-AP* strain, but did not affect strains with wild-type or AQ alleles of *MCM4* and *MCM6* (Figure S3). The extent of these defects varied significantly (Figure 6D and Figure S3). Deletion of *RAD53* and *RAD9* showed the smallest proliferation defect. *MEC1* deletion showed intermediate defects equivalent to a strain lacking both the SP and SQ phosphosites. The *RAD53*, *RAD9*, and *MEC1* deletion defects suggest a role of the intra-S phase checkpoint in suppressing the proliferation defects of the *mcm4-AP mcm6-AP* strain. The more severe defect of the *MEC1* deletion suggests that Mec1 plays a role in Mcm4/6 SQ phosphorylation that is distinct from the intra-S phase checkpoint.

Unlike the deletions of other checkpoint proteins, deletion of *MRC1* in the *mcm4-AP mcm6-AP* background resulted in

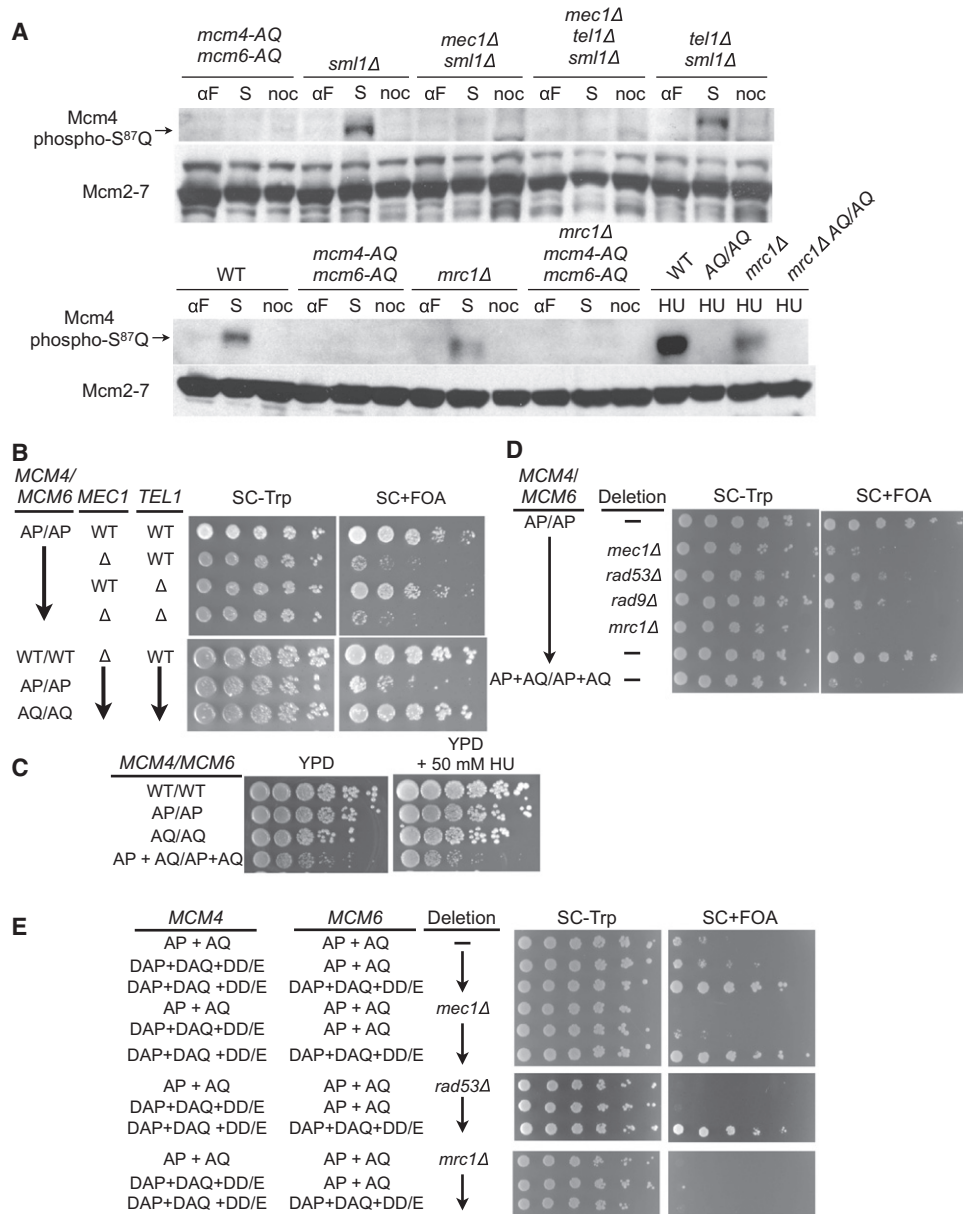


Figure 6. Mec1 Targets Mcm4 In Vivo Independent of Checkpoint Activation

(A) Effect of deletion of *MEC1*, *TEL1*, and *MRC1* on Mcm4 SQ phosphorylation. Extracts were prepared from cells after arrest in α factor (α F), hydroxyurea (HU), nocodazole (noc), or 30 min after α factor release (S phase, S) as indicated and analyzed by immunoblotting using anti-Mcm2-7 and the indicated phosphospecific antisera. Upper panel: yCC29, yRH109, yRH110, yJCWR79, yJCWR78; lower panel: yJCWR200, yJCWR220, yCC98, yCC100.

(B) Deletion of *MEC1* reduces viability of *mcm4-AP mcm6-AP* cells. Strains yJCWR214, yJCWR216, yJCWR217, yJCWR218, yJCWR214, yJCWR216, and yJCWR221 were analyzed as in Figure 2A. The relevant genotype is indicated to the left of each strain.

(C) Eliminating Mec1 target sites on Mcm4 and Mcm6 does not render cells sensitive to HU. Strains yJCWR205, yJCWR206, yJCWR207, and yJCWR208 carrying the indicated wild-type or mutant alleles of *MCM4* and *MCM6* as the only copy of those genes were plated on YPD in the absence or presence of 50 mM HU, as indicated.

(D) Deletion of checkpoint genes reduces viability of *mcm4-AP mcm6-AP* cells. Strains yJCWR214, yJCWR216, yJCWR219, yCC48, yCC55, yJCWR167, and yJCWR175 were analyzed as in Figure 2A. All of these strains include a deletion of *SML1*. See also Figure S3.

(E) Phosphomimetic mutations at DDK target sites rescue the deletion of *MEC1* and *RAD53* but not *MRC1* in the *mcm4(AP+AQ) mcm6(AP+AQ)* strain background. Strains yJCWR175, yJCWR236, yJCWR237, yCC90, yCC94, yCC95, yCC114, yCC116, yCC117, yCC73, yCC75, and yCC77 were analyzed as in Figure 2A.

a loss of viability (in Figure 6D, the few small colonies do not support further growth). Because loss of *MEC1* has a more dramatic effect on SQ phosphorylation than loss of *MRC1* (Fig-

ure 6A), the more severe proliferation defect of *MRC1* relative to *MEC1* deletion suggests that Mrc1 has an additional function unrelated to priming phosphorylation and DDK targeting. To

address this possibility, we took advantage of strains that eliminated SP and SQ priming phosphorylation and used phosphomimetic mutations at DDK target sites to rescue the resulting proliferation defect (Figure 3D). In these strains, eliminating proteins that mediate priming or DDK phosphorylation or that mediate a checkpoint response to incomplete DDK phosphorylation should have no effect on proliferation. Consistent with this logic, deleting either *MEC1* or *RAD53* in the DDK phosphomimetic background has no effect on proliferation (in Figure 6E, compare rows 3 and 6). In contrast, deletion of *MRC1* in the same background resulted in a loss of viability. This result strongly suggests that the synthetic lethality of the *MRC1* deletion with the *mcm4-AP mcm6-AP* allele is due to a role other than facilitating DDK phosphorylation of Mcm4 and Mcm6.

DISCUSSION

Studies of Mcm2-7 activation via phosphorylation have been complicated by the existence of multiple kinases that phosphorylate multiple Mcm2-7 subunits (Devault et al., 2008; Lei et al., 1997; Masai et al., 2000, 2006; Montagnoli et al., 2006). Our results provide a comprehensive picture of Mcm2-7 phosphorylation and reveal that a series of kinases collaborate in Mcm2-7 activation. We identified two classes of DDK target sites on Mcm4 and Mcm6, intrinsic and PG, and showed that both classes of target sites are important for Mcm2-7 function. Importantly, DDK phosphorylation required prior phosphorylation of Mcm4 or Mcm6 at either S/T-P or S/T-Q motifs. We demonstrated that Mec1 targets the S/T-Q priming sites and that these modifications positively regulate DNA replication. Finally, our studies suggest that phosphorylation of S/T-P sites in the N termini of Mcm4 and Mcm6 is important for proper replication fork function. These findings unravel a key step in the initiation of DNA replication and make a positive connection between the critical S phase regulator Mec1 and origin firing. Because DDK acts at each origin as it initiates replication (Labib, 2010), our findings identify mechanisms by which Mec1 and other kinases could modulate origin firing throughout S phase by controlling the extent of DDK phosphorylation of Mcm4 and Mcm6.

Priming Phosphorylation Stimulates DDK Targeting by Multiple Mechanisms

Our findings indicate that priming phosphorylation stimulates DDK phosphorylation of Mcm4 and Mcm6 in two ways. First, at S-S/T-P and S-S/T-Q sites, phosphorylation creates an acidic phosphoserine/threonine residue that targets DDK to an adjacent upstream serine residue. By this mechanism, priming phosphorylation approximately doubles the number of potential DDK target sites in Mcm4 and Mcm6. Many of these PG DDK target sites are preceded by additional serines or threonines, and it is likely that sequential phosphorylation in the C- to N-terminal direction of these S/T strings is responsible for the reported processive phosphorylation of the Mcm4 N terminus by DDK (Sheu and Stillman, 2006).

Mutation of the priming kinase target sequences also interferes with DDK modification of intrinsic (S/T-D/E) sites such as Mcm4-Ser82 (Figures 4A and 5A). Because prior phosphorylation is required for DDK association with loaded Mcm2-7

complexes (Francis et al., 2009), we propose that phosphorylation of S-S-P/Q sites by priming kinases (and possibly DDK) creates DDK binding sites that facilitate subsequent DDK phosphorylation of Mcm2-7. The stronger proliferation defect of the priming-deficient-strain [*mcm4* (AP+AQ) *mcm6* (AP+AQ) strain, Figure 3A] relative to a strain lacking only the PG DDK sites (A-S-P/Q mutations, Figure 2A) also supports a role for the priming sites in S/T-D/E site phosphorylation. Importantly, the severe proliferation defect caused by elimination of the priming sites is bypassed by phosphomimetic mutations of the DDK target sites (Figure 3D), strongly suggesting that the primary role for the S/T-P and S/T-Q priming sites is to promote DDK targeting to Mcm2-7 during origin firing.

Differential Regulation of Mcm2-7 Priming Phosphorylation

Our analysis of Mcm2-7 phosphorylation indicates that the two types of priming sites are regulated differently. The Mec1-dependent phosphorylation of S/T-Q sites on Mcm4 and Mcm6 is spatially restricted to chromatin-bound Mcm2-7 and temporally restricted to S phase (Figure 5). The spatial and temporal regulation of S/T-Q phosphorylation of Mcm4 and Mcm6 is consistent with this modification mediating the preference of DDK for chromatin-associated Mcm2-7 (Sheu and Stillman, 2006). In contrast, Mcm4 and Mcm6 S/T-P sites are phosphorylated in G1, S, and G2/M phases, and this phosphorylation is independent of chromatin loading (Figure 5).

It is likely that phosphorylation of S/T-P priming sites is mediated by S-CDKs. A role for S-CDKs in modifying Mcm subunits has been proposed previously (Devault et al., 2008; Masai et al., 2000; Montagnoli et al., 2006; Sheu and Stillman, 2010). If so, our studies provide a clear mechanism for S-CDKs to stimulate DDK targeting of Mcm2-7 (Nougarède et al., 2000). The apparently overlapping function between the S/T-P and S/T-Q sites would explain why Mcm4/6 S/T-P modification is not an essential CDK target (Tanaka et al., 2007; Zegerman and Diffley, 2007). Both our studies and a previous study of S/T-P phosphorylation of human Mcm2 found that these modifications were not cell-cycle regulated (Montagnoli et al., 2006). Although it is possible that S-CDKs are solely responsible for the S/T-P phosphorylation and these phosphates are not readily removed outside of S phase, the constitutive nature of these modifications suggests that additional kinases phosphorylate these sites. The S/T-P sites on Mcm4 and Mcm6 also could be targeted by G1- and M-phase CDKs, although G1-CDK activity should be inhibited in α -factor-arrested cells. Alternatively, another proline-directed kinase (e.g., Pho85, Huang et al., 2007) could also act as an Mcm2-7 priming kinase.

S-S/T-Q sites are found in the N termini of Mcm subunits in other yeasts, including *Schizosaccharomyces pombe*, but this motif is not present in the N termini of metazoan Mcm2-7. This suggests that the Mec1/Tel1 homologs ATR/ATM are not involved in Mcm2-7 priming in metazoan cells. Nonetheless, there are an abundance of Ser-Ser-X motifs (including, but not limited to, S-S/T-P motifs) in metazoan Mcm2-7 amino termini. Such sites would allow CDKs and other kinases to interface with replication by modulating DDK activation of Mcm2-7 in these organisms. Such alternative priming sites

may also function in *S. cerevisiae*, because elimination of all S/T-P and S/T-Q sites does not completely eliminate replication (Figure 3).

Roles for Rad53, Rad9, Mec1, and Mrc1 in Suppressing Defects in Priming Phosphorylation

The genetic interactions between mutations that eliminate Mcm4 and Mcm6 S/T-P priming phosphorylation and the deletion of the checkpoint genes *RAD53*, *RAD9*, *MEC1*, and *MRC1* suggest that these genes function at three levels to suppress defects caused by the loss of Mcm4/6 S/T-P phosphorylation. First, all of these genes can function in the intra-S phase checkpoint to suppress these replication defects. Second, Mrc1-facilitated Mec1 phosphorylation of Mcm4/6 S/T-Q sites provides a compensatory mechanism to target DDK in the absence of Mcm4/6 S/T-P phosphorylation. Third, in the presence of incomplete priming and DDK phosphorylation, cell proliferation is dependent on Mrc1 (and, to a lesser extent, on Tof1). This dependence is probably related to the role of Mrc1 and Tof1 in replication fork stability and progression (Hodgson et al., 2007). Mrc1 interacts with Mcm6, Cdc45, and Pol ϵ (Komata et al., 2009; Lou et al., 2008; Naylor et al., 2009), and the synthetic lethality between *MRC1* deletion and impaired Mcm2-7 priming is consistent with the requirement of DDK phosphorylation of Mcm2-7 for Cdc45 recruitment to the origins (Labib, 2010). Notably, the severity of the proliferation defects caused by elimination of each protein in the *mcm4-AP mcm6-AP* strain corresponds to the participation of each protein in these three functions: Rad53 and Rad9 are expected to be exclusive to the intra-S phase checkpoint response, Mec1 is implicated both in the checkpoint and in Mcm4/6 S/T-Q phosphorylation, and Mrc1 is important for these functions and fork progression.

These findings have implications for the function of Mcm2-7 phosphorylation. The finding that elimination of checkpoint proteins is deleterious only in the S/T-P background suggests that loss of these sites is more deleterious than loss of S/T-Q sites. It is noteworthy, however, that the specific interaction of *MEC1* and *MRC1* deletion with the *mcm4/6-AP* background is very likely influenced by the role of these proteins in S/T-Q phosphorylation (Figure 6A) and the overlapping functions of the S/T-P and S/T-Q modifications (Figure 3A). The lethality observed when *MRC1* is deleted in a strain lacking Mcm4/6 priming phosphosites, but including DDK phosphomimetic mutations, suggests that priming phosphorylation also contributes to the recruitment of Cdc45 or another downstream factor stabilized at the origin by Mrc1 (Figure 6E). This result is consistent with a previous finding that S/T-P mutations in the Mcm4 N terminus inhibit growth of a DDK bypass mutant (Sheu and Stillman, 2010). The finding that a deletion of the Mcm4 N terminus bypasses DDK function (Sheu and Stillman, 2010) suggests that phosphorylation of these regions alleviates an inhibitory function of the Mcm4 N terminus. Our findings indicate that this mechanism must be dependent on phosphorylation of specific residues, rather than the total level of phosphorylation, because only certain subsets of phosphomimetic mutations bypass DDK activity (Figure 2B). Finally, our findings suggest that strains engineered to replicate their DNA in the

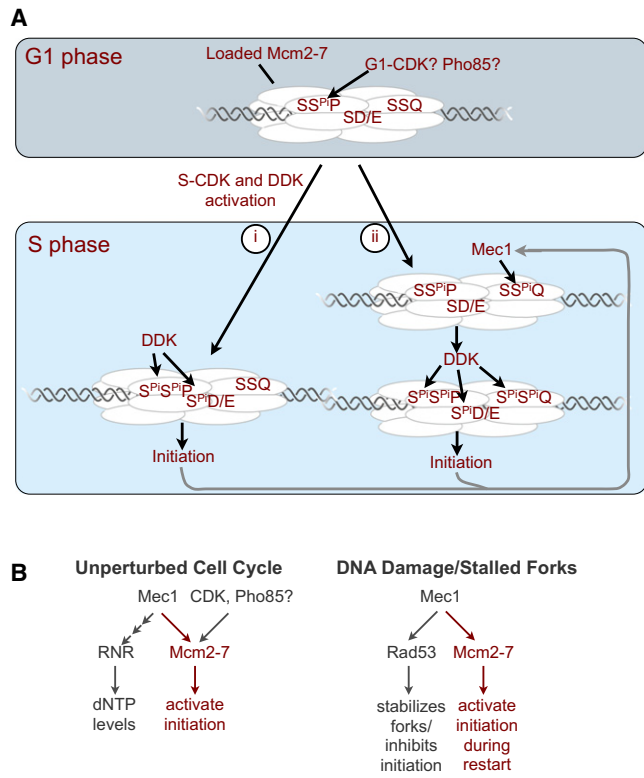


Figure 7. A Model for Phosphoregulation of Mcm2-7

(A) We propose that prior to replication SP sites in Mcm4 and Mcm6 are phosphorylated either by G1-CDKs or Pho85. It is also possible that this phosphorylation occurs earlier (e.g., by S-CDKs) and is not readily removed during G1. Upon entry into S phase we envision two possible mechanisms of DDK targeting: (1) DDK targets S-S^{PI}-P and S-D/E sites to trigger helicase activation; or (2) Mec1 targets S-S-Q sites followed by DDK targeting of S-S^{PI}-P, S-S^{PI}-Q, and S-D/E sites to trigger initiation. Based on our cell cycle studies, we propose that Mec1 is activated initially as a consequence of initiation events mediated by type (i) DDK targeting. Additional activation of Mec1 could be mediated by replication intermediates derived from type (ii) DDK-driven initiation or checkpoint signaling.

(B) Mec1 coordinates multiple events in both unperturbed and perturbed cell cycles. In a normal S phase, Mec1 activates both dNTP synthesis and DDK targeting to Mcm2-7. Under conditions of DNA damage or with stalled replication forks, Mec1 activates a checkpoint pathway that stabilizes stalled forks and inhibits further origin firing. We propose that Mec1 activation could also facilitate firing of additional origins upon checkpoint recovery.

absence of CDK (Tanaka et al., 2007; Zegerman and Diffley, 2007) are likely to be dependent on *MRC1* for replication.

A Model for Mcm2-7 Priming by Mec1 and Other Kinases

Based on our findings, we propose the following model for the cell cycle regulation of Mcm2-7 phosphorylation (Figure 7A). As cells exit G1, DDK is activated by the accumulation of Dbf4 (Sclafani and Holzen, 2007). We propose that phosphorylation of Mcm4/6 S/T-P sites, which are already phosphorylated in G1, allows initial Mcm2-7 phosphorylation by DDK and initiation from the first origins of replication (Figure 7A). Because we observe S phase-dependent S/T-Q phosphorylation in an unperturbed cell cycle (Figure 5), we propose that the same mechanism that activates Mec1 to regulate dNTP synthesis after cells

enter S phase also results in Mec1 phosphorylation of chromatin-associated Mcm4 and Mcm6. Based on our finding that S/T-P and S/T-Q sites both contribute to full DDK phosphorylation of Mcm2-7 (Figure 4), we propose that the resulting Mec1 modification of Mcm4 and Mcm6 further activates DDK phosphorylation of Mcm2-7 (Figure 7A). Our findings suggest that priming and DDK phosphorylation of Mcm4 and Mcm6 both facilitate the subsequent recruitment of Cdc45 or another critical replication factor stabilized at the replication fork by Mrc1. Thus, in this model Mec1 functions as a coordinator of two key S phase events: origin initiation and dNTP synthesis (Figure 7B, “unperturbed”). Such an activity could facilitate the completion of DNA replication by increasing the probability of origin firing as S phase progresses and is consistent with recent data that link DDK activity to the efficient completion of DNA synthesis (Patel et al., 2008).

The HU insensitivity of the *mcm4(AP+AQ) mcm6(AP+AQ)* strain (Figure 6C) and the weaker effects of deleting *RAD53* or *RAD9* relative to *MEC1* in the absence of Mcm4/6 S/T-P sites suggest that Mec1 phosphorylation of Mcm2-7 is not essential for the intra-S phase checkpoint. It remains possible that Mrc1 and Mec1 promote the activation of additional replication origins during checkpoint recovery (Figure 7B, “DNA damage/stalled forks”). Elevated Mec1 activity during replication stress could increase Mcm2-7 S/T-Q phosphorylation (as we observe after HU treatment, Figure 6A) and thus subsequent Mcm2-7 phosphorylation by DDK. Such a mechanism would be distinct from Mec1-dependent inhibition of initiation after fork stalling or DNA damage (Zegerman and Diffley, 2009). In this context, we propose that S/T-Q phosphorylation acts to rescue Mcm2-7 complexes that have not been activated, either because of mutation (e.g., *mcm4-AP mcm6-AP*) or because they are normally dormant and need activation under conditions of replication stress. Although these potential stress-related functions are intriguing, we emphasize that S/T-Q phosphorylation of Mcm4 and Mcm6 is observed in the absence of replication stress (Figure 5), supporting a function for Mec1-dependent Mcm2-7 modification in normal S phase.

EXPERIMENTAL PROCEDURES

Identification of Phosphorylation Sites by Mass Spectrometry

Pre-RC samples for mass spectrometry were generated in a 1 ml *in vitro* reaction using recombinant Cdc6 and extracts from α -factor-arrested cells overexpressing ORC. This reaction corresponds to a 25-fold scale-up of the standard pre-RC assembly reaction (described in Randell et al., 2006). Where indicated, pre-RCs were phosphorylated by recombinant DDK as described previously (Francis et al., 2009). Pre-RCs were concentrated by trichloroacetic acid (TCA) precipitation, and the entire sample was loaded on an 8% SDS-PAGE gel. Bands corresponding to Mcm2-7 proteins were excised from the gel and digested with trypsin or Asp-N. The resulting peptides were analyzed by liquid chromatography coupled with tandem mass spectrometry (LC/MS/MS) to identify phosphorylated residues (Taplin Mass Spectrometry Facility, Harvard Medical School). Peptides covering more than 80% of each Mcm2-7 protein were identified. Table S1 lists all of the Mcm2-7 phosphopeptides identified.

Generation of Phosphospecific Antisera

Phosphospecific antisera were generated by Abgent (San Diego, CA) and were used in immunoblotting assays at a dilution of 1:400.

Generation and Analysis of Mcm2/4/6 Mutations

Mutant alleles of the *MCM2*, *MCM4*, and *MCM6* N termini were synthesized as 712, 602, or 430 bp DNAs by gene synthesis (BioBasic). These synthetic constructs were cloned into TRP+ *ARS/CEN* vectors containing *MCM2*, *MCM4*, or *MCM6* under the control of the *MCM5* promoter (Schwacha and Bell, 2001). The resultant constructs were then tested for *MCM2*, *MCM4*, or *MCM6* function by a plasmid shuffle assay (Schwacha and Bell, 2001). The precise sites mutated in the different alleles are indicated in Figure S1 and further details of strain construction are in the Supplemental Experimental Procedures. The genotypes of all the strains used are presented in Table S2.

Preparation of Phosphorylated Pre-RCs

Pre-RC assembly assays, pre-RC dephosphorylation, purification of DDK, and DDK kinase assays were performed as described (Francis et al., 2009).

SUPPLEMENTAL INFORMATION

Supplemental information includes Supplemental Experimental Procedures, three figures, and two tables and can be found with this article online at doi:10.1016/j.molcel.2010.10.017.

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