Phosphorylation of MCM3 on Ser-112 regulates its incorporation into the MCM2–7 complex

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During late M and early G1, MCM2–7 assemblies and is loaded onto chromatin in the final step of prereplicative complex (pre-RC) formation. However, the regulation of MCM assembly remains poorly understood. Cyclin-dependent kinase (CDK)-dependent phosphorylation contributes to DNA replication by initially activating pre-RCs and subsequently inhibiting refiring of origins during S and M phases, thus limiting DNA replication to a single round. Although the precise roles of specific MCM phosphorylation events are poorly characterized, we now demonstrate that CDK1 phosphorylates MCM3 at Ser-112, Ser-611, and Thr-719. In vivo, CDK1-dependent phosphorylation of Ser-112 triggers the assembly of MCM3 with the remaining MCM subunits and subsequent chromatin loading of MCMs. Strikingly, loss of MCM3 triggers the destabilization of other MCM proteins, suggesting that phosphorylation-dependent assembly is essential for stable accumulation of MCM proteins. These data reveal that CDK-dependent MCM3 phosphorylation contributes to the regulated formation of the MCM2–7 complex.

The DNA replication licensing system ensures that replication of the genome occurs only once per cell division. Central to this system is the formation of prereplicative complexes (pre-RCs) in late M and early G1 phases and their subsequent activation at the G1–S boundary. Assembly of pre-RCs requires loading of the putative DNA helicase, the MCM2–7 complex, onto chromatin in an ORC1–6, CDC6, and CDT1-dependent manner (licensing) (1–4). At the G1–S transition, the activity of two kinases, CDC7 and cyclins E/A-CDK2, recruit additional factors to pre-RCs, resulting in the formation of preinitiation complexes (pre-ICs) (5–7). In addition, CDC7 and CDK2 activate the putative MCM2–7 helicase, which together with pre-IC formation results in recruitment of DNA polymerases and initiation of DNA replication. Paradoxically, during late S and M phases, high cyclin-dependent kinase (CDK) activity results in dissolution of the pre-RCs and destruction of select pre-RC components, thereby preventing DNA rereplication (8).

The importance of the replication licensing system is highlighted by the prevalence of genomic instability resulting from hyperactivation of pre-RCs during the neoplastic process and impaired cell growth associated with failure to assemble pre-RCs (9–11). The MCM2–7 helicase is thought to exist as a hexamer containing individual MCM polypeptides (MCM2/3/4/5/6/7). MCM2–7 subunits are evolutionarily conserved and essential genes; each member possesses conserved Walker A, Walker B, and arginine finger motifs required for ATP binding and hydrolysis. The MCM complex is implicated in both unwinding of origins of DNA replication during initiation and in replication fork progression (12, 13). In addition to the hexamer, MCM subcomplexes (MCM7/4/6, MCM7/4/6/2, and MCM3/5) have also been reported (11, 14, 15). Within these subcomplexes, MCM7/4/6 is thought to possess core ATPase and DNA helicase activity, whereas MCM5/3/2 provides regulatory roles (16, 17). Furthermore, pairwise ATPase studies in vitro have revealed that MCM3/7, MCM4/7, and MCM2/6 pairs are able to catalyze ATP hydrolysis (18). As a hexamer, ATPase activity of MCM2–7 is not required for loading of the complex onto chromatin, whereas ATP hydrolysis by the hexameric complex is essential for initiation of DNA replication (19).

Although the mechanisms that regulate MCM2–7 complex formation are poorly understood, individual MCM polypeptides are subject to phosphorylation, providing a point of potential regulation. MCM2/3/4 are phosphoproteins; MCM4 is targeted by CDC7/DBF4, and MCM2 can be phosphorylated by CDC7, CDK2, and CDK1. However, the CDK-dependent MCM3 phosphorylation sites and the significance of MCM3 phosphorylation have not been elucidated. We undertook this study to determine whether mammalian cyclin–CDK complexes phosphorylate MCM3 at specific phosphoacceptor residues and to unravel the functional significance of these phosphorylation events. This work reveals that cyclin B–CDK1 catalyzes phosphorylation of MCM3 at Ser-112, thereby regulating MCM3 association with other MCM2–7 subunits and loading of MCM3 onto chromatin. Our data suggest that MCM3 phosphorylation at Ser-112 is a critical posttranslational modification that regulates assembly and activity of the MCM2–7 complex.

Results

Components of pre-RCs, including MCM2/3/4, are putative substrates of CDKs (20, 21). Because neither the specific CDK nor the functional consequence of MCM3 phosphorylation is known, we functionally characterized CDK-dependent phosphorylation of MCM3. MCM3 contains four putative CDK phosphorylation sites, Ser-112, Ser-611, and Thr-719 [supporting information (SI) Fig. 1 A and C]; Ser-112 is conserved in human, Xenopus, and Saccharomyces cerevisiae MCM3 orthologs (Fig. 1 A and B). In vitro kinase reactions with MCM3 as a substrate for various cyclin–CDK complexes revealed that cyclin E–CDK2, cyclin A–CDK2, cyclin A–CDK1, and cyclin B–CDK1 phosphorylate MCM3 (Fig. 1 A and Fig. S2 C) in contrast to cyclin D1–CDK4 complexes, which did not (Fig. S2 A and B). Mutation of all putative CDK sites within MCM3 significantly diminished MCM3 phosphorylation (Fig. 1 A). Similarly, mutation of both serine consensus sites diminished MCM3 phosphorylation by CDK2 and CDK1, suggesting that serine/proline (SP) sites are the major phosphoacceptor residues (Fig. 1 B). Mutation of a single consensus site did not significantly abrogate MCM3 phosphorylation in vitro (data not shown). Incubation of CDK2 with p21 and CDK1 complexes

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with roscovitine inhibited MCM3 phosphorylation, confirming the specificity of in vitro reactions (Fig. 1C).

To identify the CDK-dependent phosphoacceptor sites of MCM3, we performed phosphotryptic peptide mapping of MCM3 phosphorylated by CDK1/2 kinases. This analysis revealed one major phosphopeptide when MCM3 was phosphorylated by cyclin A–CDK2 (Fig. S3, peptide A). This phosphopeptide was absent in a MCM3-S611A mutant, suggesting that cyclin A–CDK2 phosphorylates Ser-611. Likewise, tryptic maps indicate that cyclin E–CDK2 also targets Ser-611 (Fig. S4). Phosphorylation of MCM3 by cyclin B–CDK1 revealed additional phosphopeptides, suggesting that this kinase targets additional SP/threonine/proline (TP) residues (Fig. 2A, peptides B–E). Site-directed mutagenesis revealed that peptides B and D contain phosphorylated Ser-112 (Fig. 2B), suggesting that peptides B and D result from partial protease cleavage. We note that phosphate incorporation into peptide C is low in the S112A single mutant but is present in a S112A/S611A double mutant (data not shown), which suggests that it represents a distinct phosphopeptide. Strikingly, alanine substitution of Ser-611 inhibited phosphorylation of peptide A (Ser-611) and peptides B and D but not C (Fig. 2D), implying that the integrity of Ser-611 is critical for CDK1-dependent phosphorylation of Ser-112. Finally, substitution of Thr-719 to alanine abolished phosphorylation of peptide E (Fig. 2E). Collectively, these results indicate that MCM3 is a CDK1/2 substrate and CDK2 preferentially phosphorylates MCM3 at Ser-611, whereas CDK1 targets MCM3 at Ser-112, Ser-611, and Thr-719 in vitro.

To determine whether endogenous MCM3 is phosphorylated by CDK1/2 in vivo, cells were released from a G0 block; 2 h before harvest, 32P was added. Accumulation of phospho-MCM3 is apparent by mid-G1, peaked at the G1–S boundary, and remained elevated throughout S and M phases (Fig. 3A). Importantly, treatment of proliferating cells with roscovitine, a pharmacological inhibitor of CDK1/2, attenuated 32P labeling of MCM3 (Fig. 3B, Top), consistent with CDK1 or 2 being relevant in vivo kinases.

Next, we determined whether MCM3 was phosphorylated in vivo on SP/TP residues. MCM3 immunoprecipitates were probed with a phospho-SP/TP antibody, which detects phosphoserine/threonine only when either residue is followed by a proline. Endogenous MCM3 was readily recognized by the p-SP/TP antibody (Fig. 3B, Middle), and treatment with roscovitine inhibited this phosphorylation, confirming that CDK1/2 phosphorylates at least one CDK consensus site of MCM3 in vivo (Fig. 3B, Middle). Similar to 32P labeling, MCM3 SP/TP phosphorylation and specifically Ser-112, was low in G0 and peaked during S and M phases (Fig. 3E and Fig. S5C). Mutation of either Ser-112 or Thr-719, but of not Thr-464 or Ser-611, inhibited recognition of MCM3 by the p-TP/SP antibody (Fig. 3C; data not shown). Mutation of both Ser-112 and Thr-719 further abolished MCM3 TP/SP phosphorylation (data not shown), revealing that both Ser-112 and Thr-719 are phosphorylated in vivo. In vivo phosphorylation of Ser-112 was further confirmed with a phospho-specific antibody that specifically recognized p-Ser-112 (Fig. 3D and E). These data demonstrate that CDK1 phosphorylates MCM3 Ser-112 and Thr-719 both in vitro and in vivo.

Because Ser-112 was specifically targeted by cyclin B–CDK1 in vitro, we ascertained the association of MCM3 to other MCMs in cells arrested during mitosis, a period of high cyclin B–CDK1 activity. NIH 3T3 cells were arrested in prometaphase with nocodazole, and cells were collected by mitotic shakeoff. Compared with asynchronously growing cells, MCM3 p–TP/SP phosphorylation slightly increased during mitosis (Fig. S5A and B). We also noted although total MCM5 and MCM7 levels remained constant, the amount of MCM5 and MCM7 associated with MCM3 increased modestly during mitosis (Fig. S5A and B). These experiments indicate that MCM3 is phosphorylated at Ser-112 during mitosis and correlates with assembly of hexameric MCM2–7 complexes during mitosis despite high cyclin B–CDK1 kinase activity.

We therefore evaluated whether phosphorylation of MCM3 within MCM2–7 complexes is important for hexameric complex formation during M phase. MCM3 immunoprecipitates were treated with phosphatase and washed, and the remaining MCM3-bound proteins were analyzed. Strikingly, dephospho-

Fig. 1. MCM3 is a CDK2 and CDK1 substrate in vitro. (A) Murine MCM3 or a MCM3 mutant devoid of all CDK consensus sites was incubated with active CDK1/2 and [γ-32P]ATP. MCM3 phosphorylation was assessed by SDS/PAGE followed by autoradiography. In all kinase reactions, negative controls included incubation of MCM3-WT with mock-purified CDK complexes (–). (B) Same as A except that a double-SP, S112A/S611A, and a double-TP, T464A/T719A, MCM3 mutant were analyzed. (C) (Upper) MCM3 phosphorylation was assessed with cyclin A–CDK2 or with cyclin A–CDK2–p21 complexes. (Lower) Before adding MCM3, cyclin B–CDK1 complexes were incubated with roscovitine.

Fig. 2. MCM3 is phosphorylated at Ser-112, Ser-611, and Thr-719 by CDK1. Tryptic peptide maps of MCM3-WT or mutant MCM3 phosphorylated by CDK1 are shown.
ylation of MCM3 complexes prepared from asynchronous or nocodazole-arrested cells significantly reduced binding of MCM3 to MCM5 and MCM7 (Fig. S5B, lanes 1 and 2), suggesting that phosphorylation of one or more MCMs regulates MCM2–7 complex formation in asynchronous and mitotic cells.

To interrogate directly the role of MCM3 phosphorylation in MCM2–7 assembly, we determined the ability of MCM3 mutants to associate with other MCMs. Mutation of all CDK-dependent MCM3 phosphorylation sites significantly inhibited binding to MCM5 in asynchronous and mitotic cells, again implicating MCM3 phosphorylation in MCM2–7 assembly during mitosis (Fig. 4A). To assess precisely which sites are essential for association with other MCMs, we generated single-, double-, and triple-point mutations of MCM3 SP/TP residues. This analysis revealed that mutation of Ser-112 to alanine inhibited binding of MCM3 to MCM2, MCM5, and MCM7 (Fig. 4B and C). Collectively, these results suggest that MCM3 phosphorylation at Ser-112 regulates its incorporation into hexameric MCM2–7 complexes.

Because the binding of Xenopus MCM3 to chromatin can be blocked independently of CDT1 and CDC6 by an inhibitor of serine/threonine kinases, 6-DMAP (2, 14, 22), it can be inferred that phosphorylation of one or more MCMs is necessary for chromatin loading. Therefore, we compared chromatin loading of MCM3-S112A with that of MCM3-WT. Chromatin fractionation of asynchronous NIH 3T3 cells revealed significantly less MCM3-S112A in chromatin-bound fractions compared with MCM3-WT (Fig. 4D and Fig. S6B), suggesting that Ser-112 phosphorylation and binding to other MCMs promote MCM3 chromatin loading. Consistent with these data, abolishing Ser-112 phosphorylation inhibited MCM3 association with both soluble and chromatin-associated MCM5 (Fig. 4E). Even when the levels of chromatin-bound S112A were normalized to MCM3-WT, S112A still interacted less efficiently with MCM5 (Fig. 4E), indicating that Ser-112 phosphorylation promotes binding to other MCMs on both chromatin-bound and soluble MCM2–7 complexes. Given the significant reduction in MCM3 binding to other MCMs, residual binding of S112A to chromatin likely reflects interaction with nonreplication proteins (23–25).

When considered collectively, the data provided suggest that Ser-112 phosphorylation during mitosis promotes MCM3 incorporation into a mature MCM complex followed by chromatin loading during the subsequent G1 phase. To determine the impact of loss of Ser-112 phosphorylation on MCM3 chromatin loading after mitosis, NIH 3T3 cells were arrested with nocodazole, and chromatin loading of MCM3 was assessed after nocodazole release. In this system, early G1 phase occurs 2–4 h after release, whereas G1–S transition takes place 8–10 h after release (26). In contrast to MCM3-WT, which loaded 3 h after nocodazole release, MCM3-S112A failed to load onto chromatin within the same time frame (Fig. 4F). These results suggest that MCM3 phosphorylated at Ser-112 in M phase is loaded onto chromatin in the following G1 phase.

To ascertain whether mammalian MCM2–7 complexes that are synthesized and phosphorylated in the previous cell cycle are functionally relevant in the subsequent G1, nocodazole-arrested NIH 3T3 cells were released into medium containing cycloheximide to inhibit new protein synthesis. Cycloheximide did not inhibit mitotic exit 3 h after nocodazole release (Fig. S6A). Furthermore, members of MCM2–7 loaded onto chromatin, and MCM3 interacted with MCM5, 3 h after release in the presence of cycloheximide (Fig. 5A and B). These results strengthen the notion that mammalian MCM3 protein previously phosphorylated in mitosis assembles into MCM2–7 complexes and loads onto chromatin in the following G1 phase.

Finally, to unravel the biological significance of Ser-112 phosphorylation, we knocked down endogenous MCM3 in U2OS cells with human-specific MCM3 shRNAs and expressed murine MCM3 that is not recognized by the shRNAs (Fig. 5C). MCM3 knockdown promoted the enrichment of cells with a 4 N DNA content indicative of a mitotic arrest (Fig. 5D). Importantly, expression of MCM3-WT rescued cell cycle arrest, whereas expression of MCM3-S112A did not (Fig. 5E). We also noted that MCM3 knockdown reduced accumulation of other MCMs in both soluble and chromatin-associated fractions (Fig. 5C and D and Fig. S6 C and D). Levels of MCM5 were restored by ectopic expression of MCM3-WT but not by MCM3-S112A, demonstrating that loss does not result from shRNA off-target effects (Fig. 5C). Therefore, stability of other MCMs may depend on binding to MCM3 and be indirectly regulated by Ser-112 phosphorylation. Failure to restore completely MCM5/6 levels and the 4 N arrest with MCM3-WT likely reflect transfection efficiencies (~50%) of cells that were infected with virus encoding shRNAs. Collectively, these results indicate that MCM3 Ser-112 phosphorylation is critical for continued cell cycle progression.
Discussion

The data presented reveal a role for CDK1-dependent phosphorylation of MCM3 in the regulation of MCM complex assembly. Our data suggest that phosphorylation of Ser-112 by CDK1 promotes its incorporation into MCM2–7 complexes, permitting subsequent loading of MCM3 onto chromatin in assembly.
cycling cells. It is likely that MCM3 phosphorylated at Ser-112 only contacts one or two other MCMs in the hexameric complex and that loss of Ser-112 phosphorylation results in the dissociation of MCM3 from the hexamer. Binding of Ser-112-phosphorylated MCM3 to other MCMs, for instance, is reminiscent of the association between phosphorylated Stat1α and MCM5 (24, 27). In addition, our results indicate that because Ser-112 phosphorylation regulates MCM3 association with other MCMs, it is also important for loading of MCM3 onto chromatin. Future experiments will need to assess directly the implication that loading of MCM3 onto chromatin and binding to other MCMs are coupled events. Because Ser-112 is conserved from S. cerevisiae (corresponding to Ser-167) to humans, the function of this phosphorylation event may be evolutionarily conserved.

The significance of Ser-112 phosphorylation is highlighted by experiments wherein endogenous MCM3 knocked down with concurrent expression of MCM3-S112A results in cell cycle arrest with a 4 N DNA arrest, indicating that Ser-112 phosphorylation is necessary for mitotic progression. We infer from these experiments that assembly and chromatin loading of MCM2–7 are necessary for mitotic exit and subsequent G1 entry. Therefore, a plausible hypothesis is that mitotic exit into G1 requires turning off an undefined mitotic checkpoint, which senses when MCM2–7 loads on origins of replication in late M phase. Consistent with this notion, knockdown of endogenous MCM3 and expression of MCM3-S112A or MCM3-4QA promoted the appearance of aneuploid cells with >4 N DNA content, suggestive of mitotic defects (Fig. S5D and Fig. S7A). Alternatively, although not mutually exclusive, failure to phosphorylate Ser-112 may compromise helicase function, resulting in a delay in S phase progression and induction of an intra-S phase checkpoint that is ultimately manifested by a G2 arrest.

The fact that MCM3 binds to other MCMs in nocodazole-arrested cells suggests that the MCM2–7 complex assembles during mitosis, a period of high cyclin B–CDK1 kinase activity. Paradoxically, CDK1 kinase activity in M phase also promotes dissolution of pre-RCs by inactivation of other pre-RC components and prevents loading of MCM2–7 onto chromatin. The fact that MCM2–7 assembles in M phase indicates that unknown functions of soluble hexameric MCM2–7 complexes exist during mitosis (Fig. S8). Indeed, both ORC2 and MCM3 can localize to centrosomes, and depletion of both genes leads to appearance of multinucleated cells with disorganized microtubules indicative of mitotic defects (28, 29).

Alternatively, CDK1-dependent assembly of MCM2–7 in mitosis may keep soluble hexameric complexes formed to facilitate loading of MCM2–7 onto origins after inactivation of cyclin B in late M phase. We therefore propose a model whereby MCM3 Ser-112 phosphorylation by CDK1 is essential for assembly of soluble MCM2–7 during mitosis and for subsequent loading of MCM3 onto chromatin in the following G1 phase (Fig. S8). Because MCM3 interacts with MCM5 and loads onto chromatin after mitotic release into G1 whereas MCM3-S112A does not, it is likely that there may be other kinases that phosphorylate MCM3 to promote MCM2–7 assembly once cyclin B is degraded as cells exit mitosis.

Similarly, cells can exit G0 and assemble pre-RCs without CDK1 function. However, this cell cycle transition is longer, and MCM2–7 chromatin loading occurs in late G1. Given that the G0–G1 transition is intrinsically different from the M–G1 transition, it is possible that alternate MCM2–7 modifications promote assembly and loading of newly synthesized MCM2–7 onto origins after reentry from G0. For example, a redundant kinase may function during the G0–G1 progression, although the nature of the kinase is unclear given that no other kinase tested targeted Ser-112.

Our data reveal an important function of mitotic CDKs in the licensing of origins. It is worth noting that of all known CDKs, only CDK1 appears to be essential for embryonic development and embryonic cell cycles (30–32). MCM3 Ser-112 phosphorylation may represent a mechanism whereby mitotic CDK1 communicates with the DNA replication machinery that assembles during G1 phase. However, because MCM3 is thought to be part of the putative replicative DNA helicase, MCM3 Ser-112 phosphorylation may also be important in S phase.

Consistent with this notion, MCM3 knockdown reduced the percentage of cells in S phase. However, neither MCM3-WT nor S112A rescued S phase defects possibly because of incomplete rescue of MCM3 and as a result, incomplete rescue of MCM5/6 levels in those experiments. In contrast, acute expression of MCM3-S112A promoted both the enrichment of cells in S phase and the appearance of cells with a 3 N/BrdU negative DNA content (n = 3, P < 0.05 for 3 N/BrdU−) suggestive of an arrest in S phase. Modest dominant-negative defects were likely caused by inherent high levels of endogenous MCM3 (33). Future efforts by necessity will address possible functions of Ser-112, as well as Ser-611 and Thr-719 phosphorylation, in S phase including DNA helicase activity and release of MCM2–7 from replication forks.

The functional significance of phosphorylation of MCM3 at Ser-611 and Thr-719 remains ambiguous and is under investigation. It is important to consider, however, that both MCM2 and MCM4 are also substrates of checkpoint kinases, ATM/ ATR, and pre-RC components are required for replication checkpoint signaling (33, 34). Interestingly, mouse Thr-719 (human Thr-722) is in close proximity to two ATM-dependent phosphorylation sites of MCM3, Ser-725 and Ser-732 (35). Although the functions of these phosphorylation events are unknown, it is tempting to hypothesize that Thr-719 phosphorylation by CDK1 and Ser-725/Ser-732 phosphorylation by ATM execute similar biological outputs.

Methods

Cell Culture Conditions and Transfections. NIH 3T3 and U2OS cells were grown in DMEM with 1% antibiotics and 10% FBS (Gemiini). Transient expression of plasmids encoding FLAG-MCM3 was achieved by using Lipofectamine Plus (Invitrogen) with expression vectors encoding the appropriate cDNAs.

Construction of MCM3 Mutants. MCM3 mutants were generated by site-directed mutagenesis with the QuikChange mutagenesis kit (Stratagene) according to the manufacturer’s instructions. Mutants were confirmed by sequencing.

Chromatin Fractionation. After trypsinization, cells were lysed in CSK− buffer [10 mM Pipes (pH 7), 100 mM NaCl, 300 mM sucrose, 3 mM MgCl2, 0.5% Triton X-100]. Lysates were incubated on ice for 20 min and cleared by low-speed centrifugation (1,000 × g) for 5 min. The soluble fraction (supernatant) was recovered. The chromatin pellet was washed once with CSK− buffer and once with DNS I buffer and digested with RNase I (Promega) to release chromatin-bound proteins.

Western Blot Analysis. The following antibodies were used: MCM2 (559542; BD PharMingen), MCM3 (N-19; Santa Cruz Biotechnology), MCM5 (clone 33; BD Transduction Laboratories), MCM7 (clone 0.N.194; U.S. Biological), p-TpS (P-Thr-Pro-101), which cross-reacts with some phosphoserine-proline motifs; Cell Signaling), FBX4 (26), and CREB (Cell Signaling). Phospho-Ser-112-specific antibodies were generated by immunizing rabbits with peptides based on p-S112 MCM3 (YenZym Antibodies).

Purification of Active Cyclin–CDK Complexes. SF9 cells were infected with cyclin–CDK viruses and lysed in Tween 20 buffer [50 mM Hepes (pH 8), 150 mM NaCl, 2.5 mM EGTA, 1 mM EDTA, 0.1% Tween 20]. Ten microliters (corresponding to 2 × 106 cell equivalents) of cleared lysates was used for purification of cyclin–CDK complexes. FLAG-D1 complexes were purified by using M2-agarose (Sigma) or with a cyclin D1 antibody (17-13G). Cyclin E–CDK2 and cyclin A–CDK2 were purified by using a CDK2 polyclonal antibody (RCC). Complexes were washed three times in Tween 20 buffer and twice in CDK kinase buffer [50 mM Hepes (pH 8), 10 mM MgCl2, 1 mM DTT, 2.5 mM EGTA, 20 μM ATP, 10 mM β-glycerophosphate, and 1 mM NaF]. GST-cyclin B–CDK1

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complexes were purified with GSH–Sepharose (Amersham) and eluted in kinase buffer.

**CDK Kinase Assays.** Bacterially expressed GST-MCM3 was purified with GSH–Sepharose and eluted in kinase buffer. One microgram of GST-MCM3 was mixed with cyclin–CDK3 complexes purified from Sf9 cells. Kinase reactions were initiated by the addition of 10 μCi of [γ-32P]ATP and were incubated at 30°C for 5–30 min (with linear incorporation kinetics). Labeled products were separated by SDS/PAGE before autoradiography.

**In Vivo 32P Labeling.** NIH 3T3 cells were contact-inhibited and serum-starved in 0.1% FBS for 24 h. Trypsin-treated cells were released from G0 into medium separated by SDS/PAGE before autoradiography.

8084 were washed once with TBS and lysed in Nonidet P-40 lysis buffer [50 mM Tris (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholic acid]. MCM3 was precipitated, separated by SDS/PAGE, and visualized by autoradiography.

**Two-Dimensional Tryptic Peptide Mapping.** 32P-labeled MCM3 was separated by SDS/PAGE and transferred to a PVDF membrane. Pieces of membranes containing MCM3 were excised and subjected to trypsin digestion. Equivalent counts per minute of tryptic peptides were loaded on TLC plates and analyzed by electrophoresis in pH 3.5 buffer in the first dimension and ascending chromatography in the second dimension by using an HTE-7000 apparatus (CBS Scientific). TLC plates were subjected to autoradiography as indicated.

**Gene Silencing.** pGP2 shRNA vectors targeting human MCM3 were purchased from Open Biosystems. U2OS cells were infected with lentiviruses encoding MCM3 shRNA; infected cells were selected with puromycin and transfected with murine FLAG-MCM3 cDNAs. Alternatively, knockdown of human MCM3 and expression of murine MCM3 were achieved by cotransfection of U2OS cells with shRNA and cDNA vectors followed by puromycin selection. Human MCM3 shRNAmir target sequences are as follows (sense–antisense, with antisense in bold): 29, CCGTATGGCCTGAATGTTA–TAAACATTACGGAATCCAGGA; 30, ACCACAGATGACCCACACCTTTA–TAAAGGGGATACGTGCGGC; 54, ACCACACAAAAACATTTAA–TTTACATGGGTTTTGTTGGG.

**FACS.** Cells were pulsed with 10 μM BrdU for 45 min. Adherent cells were collected by trypsinization and pooled with dead, floating cells. Cell cycle profiles were determined by propidium iodide (PI) or PI-BrdU staining.

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Supporting Information

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Assembly of MCM2–7 by MCM3 phosphorylation

Fig. S1. Schematic of cyclin-dependent kinase (CDK) phosphorylation sites within MCM3. (A) Consensus CDK phosphorylation sites of mouse MCM3 and summary of sites targeted by CDKs in vitro. Conserved Walker A, Walker B, and arginine finger motif (R) are also noted. (B) Alignment of mouse (Mm), human (Hs), Xenopus (Xl; GenBank Accession no. NM_001087943), and Saccharomyces cerevisiae (Sc; GenBank Accession no. NP_010882) MCM3 was performed with ClustalW. Amino acids conserved in all orthologs are highlighted in yellow, and murine Ser-112 is underlined. (C) The CDK1-dependent phosphorylation site of MCM3, murine Thr-719 (corresponding to human Thr-722), is in close proximity to two other reported ATM-dependent phosphorylation sites of murine MCM3, Ser-725, and Ser-732.
Fig. S2. Cyclin D-dependent kinases do not phosphorylate MCM3 above background levels. (A) Murine MCM3-WT or a MCM3 quadruple mutant, in which all four CDK consensus sites were mutated to alanine, were incubated with cyclin D1–CDK4 complexes in the presence of [γ-32P]ATP. MCM3 phosphorylation was assessed by SDS/PAGE followed by autoradiography. Negative controls included incubation of MCM3-WT with mock-purified CDK4 complexes (−), i.e., CDK4 complexes purified with normal rabbit serum. (B) Same as in A except that MCM3 was also incubated with kinase-dead cyclin-D1–CDK4-T172A complexes. Phosphorylation of RB by the same complexes is included as positive controls for D1-K4 complexes. (C) Controls for quality of MCM3 as a substrate included phosphorylation of MCM3 by cyclin A–CDK2 and cyclin A–CDK1.
Fig. S3. MCM3 is phosphorylated at Ser-611 by cyclin A–CDK2. Tryptic peptide maps of in vitro phosphorylated WT-MCM3 and of mutant MCM3 phosphoproteins by cyclin A–CDK2 are shown. Phosphorylated MCM3 was separated by SDS/PAGE and transferred to an Immobilon-P membrane. Membrane slices containing phosphorylated MCM3 were digested with trypsin, and tryptic peptides were resolved by sequential pH 3.5 electrophoresis (left to right) and ascending chromatography (bottom to top). Peptide A corresponding to phosphorylated Ser-611 is designated.
Fig. S4. MCM3 is phosphorylated at Ser-611 by cyclin E–CDK2. Same as in Fig. S3 except that cyclin E–CDK2 kinase complexes were used for tryptic peptide mapping of MCM3.
Phosphorylation of Ser-112 is cell cycle-regulated. (A) FLAG-MCM3 expressed in asynchronous or nocodazole-arrested NIH3T3s was precipitated and probed with the indicated antibodies. (B) FLAG-MCM3 precipitates from asynchronous or nocodazole-arrested NIH3T3s were treated with alkaline phosphatase (+Ptase) and washed with lysis buffer. The remaining MCM3-bound proteins were detected by Western blot analysis. (C) MCM3 was precipitated from NIH 3T3 cells released from G0 and probed for phospho-SP/TP. Cyclin A levels were measured to monitor cell cycle reentry.
Fig. S6. Phosphorylation of MCM3 on Ser-112 contributes to chromatin loading of MCM. (A) NIH 3T3 cells were released from a nocodazole block into medium containing DMSO or 100 μg/ml cycloheximide, and mitotic exit was assessed by propidium iodide (PI) staining 3 h after release. (B) NIH 3T3 cells expressing either MCM3-WT or MCM3-S112A were released from a nocodazole block into medium containing DMSO or 100 μg/ml cycloheximide, and chromatin loading of MCM3 was assessed by direct Western blotting. (C) U2OS cells were infected with lentiviruses encoding human-specific MCM3 shRNAs. Infected cells were transfected with empty vector or murine FLAG-MCM3. Efficiency of MCM3 knockdown and MCM5/6 levels were assessed by direct Western blots 8 days after infection and 3 days after transfection. (D) U2OS cells transfected with the indicated shRNAs were separated into soluble and chromatin-enriched fractions and blotted for MCM2, 3, and 7.
Fig. S7. Expression of phosphorylation-deficient MCM3 has modest dominant-negative activity. (A) NIH 3T3 cells stably expressing vector, MCM3-WT, or nonphosphorylatable MCM3-Q4A were arrested in G0 by contact inhibition followed by a 24-h incubation in 0.1% FBS. DNA content was analyzed by propidium iodide (PI) staining, and MCM3 expression was determined by direct Western blotting. (B) U2OS cells were transfected with the indicated constructs; cells were analyzed by PI-BrdU staining and direct Western blotting 24 h after transfection.
Fig. S8. Proposed significance of MCM3 Ser-112 phosphorylation. CDK1-dependent phosphorylation of MCM3 at Ser-112 during mitosis promotes assembly of soluble hexameric MCM2–7 complexes, which are subsequently loaded onto chromatin in late M and early G1. High levels of Geminin combined with inactivation of CDC6/CDT1 prohibit reloading of MCM2–7 onto origins of replication during late S and early M. Paradoxically, inactivation of cyclin B–CDK1 in late M and early G1 is necessary for pre-replicative complex (pre-RC) formation via ORC-, CDC6-, and CDT1-dependent recruitment of the MCM2–7 complex onto chromatin. Phosphorylation of MCM3 at Ser-112 by CDK promotes the formation of soluble MCM2–7 complexes during mitosis to allow for efficient reloading of MCM2–7 onto chromatin during late M and early G1, a period of low kinase activity. Alternatively, although not mutually exclusively, soluble Ser-112-phosphorylated hexameric MCM2–7 complexes may perform unknown functions during mitosis.