The Fission Yeast Minichromosome Maintenance (MCM)-binding Protein (MCM-BP), Mcb1, Regulates MCM Function during Prereplicative Complex Formation in DNA Replication*{5}

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Background: MCM-BP is a novel binding partner of the MCM complex; the mechanisms by which MCM-BP functions and associates with MCM complexes are not well understood.

Results: Genetic analysis showed that mcb1ts mutants exercise defective regulation of prereplicative MCM complex formation during DNA replication.

Conclusion: Mcb1 regulates MCM function during prereplicative complex formation in DNA replication.

Significance: This study presents the first evidence of MCM-BP function during prereplicative complex formation.

The minichromosome maintenance (MCM) complex is a replicative helicase, which is essential for chromosome DNA replication. In recent years, the identification of a novel MCM-binding protein (MCM-BP) in most eukaryotes has led to numerous studies investigating its function and its relationship to the MCM complex. However, the mechanisms by which MCM-BP functions and associates with MCM complexes are not well understood; in addition, the functional role of MCM-BP remains controversial and may vary between model organisms. The present study aims to elucidate the nature and biological function of the MCM-BP ortholog, Mcb1, in fission yeast. The Mcb1 protein continuously interacts with MCM proteins during the cell cycle in vitro and can interact with any individual MCM subunit in vivo. To understand the detailed characteristics of mcb1ts, two temperature-sensitive mcb1 gene mutants (mcb1ts) were isolated. Extensive genetic analysis showed that the mcb1ts mutants were suppressed by a mcm5+ multicopy plasmid and displayed synthetic defects with many S-phase-related gene mutants. Moreover, cyclin-dependent kinase modulation by Cig2 repression or Rum1 overproduction suppressed the mcb1ts mutants, suggesting the involvement of Mcb1 in pre-RC formation during DNA replication. These data are consistent with the observation that Mcm7 loading onto replication origins is reduced and S-phase progression is delayed in mcb1ts mutants. Furthermore, the mcb1ts mutation led to the redistribution of MCM subunits to the cytoplasm, and this redistribution was dependent on an active nuclear export system. These results strongly suggest that Mcb1 promotes efficient pre-RC formation during DNA replication by regulating the MCM complex.

Genome integrity depends on successful and faithful DNA replication, which relies on the concerted activity of multiple replication proteins. The temporal and spatial regulation of DNA replication and the cell cycle control system ensure a single round of replication of chromosome DNA during every cell cycle. The initiation of DNA replication in all eukaryotes involves the assembly of a prereplicative complex (pre-RC) at the replication origins in G1 phase and the subsequent activation of the pre-RC to the preinitiation complex (pre-IC) at the onset of S-phase. The replication origins are recognized by the origin recognition complex and become a platform for the recruitment of Cdc6 (called Cdc18 in fission yeast) and Cdt1-bound double hexamers of the minichromosome maintenance (MCM) complex to form the pre-RC (reviewed in Ref. 1). Upon entry into S-phase, this complex is activated by the S-phase-specific kinase, Dbf4/Drf1-dependent kinase. The Mcm2–7 complex serves as a platform to recruit Cdc45 and GINS, thereby converting the pre-RC into the pre-IC. Dbf4/Drf1-dependent kinase phosphorylates several of the Mcm2–7 proteins and triggers the recruitment of Cdc45 and GINS to form the Cdc45-MCM-GINS complex. Cdc45-MCM-GINS complex formation then induces the helicase activity of the Mcm2–7 complex, promoting the unwinding of the double-stranded DNA at the origin of DNA replication (reviewed in Ref. 2).

The Mcm2–7 hexamer complex is an evolutionarily conserved DNA helicase, which is essential for both the initiation of chromosome DNA replication and elongation (3, 4). Mcm2–7

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2 The abbreviations used are: pre-RC, prereplicative complex; pre-IC, preinitiation complex; MCM, minichromosome maintenance; MCM-BP, MCM-binding protein; HU, hydroxyurea; MMS, methylmethane sulfonate; CPT, camptothecin; DBD, DNA-binding domain; AD, activation domain; DSB, double-stranded DNA break; CDK, cyclin-dependent kinase; GINS, Tetrameric complex composed of Sld5, Psf1, Psf2, Psf3 (Go-Ichi-Ni-San).
proteins are members of the AAA+ ATPase family of proteins and share a region of homology that encompasses the ATPase motif, referred to as the MCM box (1, 5). The MCM box harbors distinct motifs, including Walker A, Walker B, and an arginine finger. The canonical MCM complex consists of the six subunits of Mcm2–7 and is assembled by the binding of one MCM arginine finger to the P-loop within the Walker A motif of another MCM subunit to form the ATP binding site. This hexameric ring is formed by two subcomplexes; the heterodimer is formed by Mcm3 and Mcm5 (Mcm3-5), and the trimeric MCM core complex is formed by Mcm4, Mcm6 and Mcm7 (Mcm4–6–7). Mcm2 connects these two subcomplexes to form the Mcm2–7 hexamer complex (6–8). Mcm2–5 is thought to act as the gate of the ring structure, the site at which the ring structure opens to encircle DNA (9).

Although a great deal of evidence indicates the importance of the Mcm2–7 complex in DNA replication, there are still unanswered questions concerning the functional role(s) of the MCM proteins. For example, the relationship between the Mcm4–6–7 core helicase and the larger Mcm2–7 hexamer is not understood. It is also unclear why MCM proteins are abundant and exceed the number of replication origins. In fission yeast, a reduction in MCM protein levels causes genome instability due to replication fork collapse and DNA damage (10, 11). In human cells, excess chromatin-loaded MCM complexes are important under conditions of replicative stress, where they activate dormant origins to ensure that DNA replication continues when the replication forks stall (12, 13). Mice expressing hypomorphic Mcm2 (Mcm2<sup>iresc</sup>creERT<sup>2</sup>) or Mcm4 (Mcm4<sup>Chuas</sup>) show lower levels of Mcm2–7 loading onto DNA, exhibit replicative stress even under unchallenged conditions, and have a high incidence of cancer (14, 15). In addition, some MCM subunits appear to play additional roles that are independent of DNA replication (16–18).

Although the primary focus of the study of MCM proteins has been to identify the function of the canonical MCM complex, several MCM-related complexes have also been characterized (19). Two additional MCM family members, Mcm8 and Mcm9, which contain an MCM box, were identified in higher eukaryotes (20–23). Mcm8 and Mcm9 work downstream of the Mcm9, which contain an MCM box, were identified in higher eukaryotes (20–23). Mcm8 and Mcm9 work downstream of the Mcm9, which contain an MCM box, were identified in higher eukaryotes (20–23).

In fission yeast, Mcb1<sup>ts</sup> (the fission yeast MCM-BP ortholog) is an essential gene, and its deletion results in gradual cell cycle arrest with a cdc (cell division cycle) phenotype. Overexpression of the Mcb1 protein or Mcb1 inactivation in temperature-sensitive Mcb1<sup>ts</sup> mutants induces DNA damage and G<sub>2</sub> checkpoint activation (29, 30). In human cells, the depletion of MCM-BP also leads to centrosome amplification and abnormal nuclear morphology, which may be due to G<sub>2</sub> DNA damage checkpoint activation (31). The loss of <i>A. thaliana</i> ETG1 leads to reduced DNA replication, activation of the G<sub>2</sub> checkpoint, and reduced sister chromatid cohesion (28, 32). The depletion of human MCM-BP also leads to reduced sister chromatid cohesion (32). The <i>Xenopus</i> MCM-BP appears to play a role in unloading MCM complexes from chromatin after DNA synthesis (27); however, the depletion of human MCM-BP not only increases the levels of chromatin-associated MCM proteins at the end of S-phase but also leads to a similar increase in soluble levels of MCM proteins throughout S-phase (31), suggesting multiple functions of MCM-BP in DNA replication. The human MCM-BP and <i>A. thaliana</i> ETG1 are largely nuclear throughout the cell cycle (28, 31). The fission yeast Mcb1 is widely distributed in the cytoplasm and nucleoplasm and is bound to chromatin (29); however, <i>Xenopus</i> MCM-BP is imported into the nucleus just before the dissociation of Mcm2–7 from chromatin near the end of S-phase. These differences in the localization of MCM-BP may affect other functions associated with the MCM complex proteins. Furthermore, human MCM-BP interacts with Dfp4, the regulatory component of the Dbf4/Drfl-dependent kinase, as well as with MCM complex components, suggesting that MCM-BP may affect DNA replication, at least in part by regulating MCM phosphorylation by Dbf4/Drfl-dependent kinase (33). Thus, the functions of MCM-BP in DNA replication have been extensively studied; however, the mechanisms by which MCM-BP functions and associates with MCM complexes are not well understood, and the functional role of MCM-BP is controversial and may vary among model organisms.

To address these remaining questions, the functional roles of MCM-BP were examined by genetic analysis of temperature-sensitive mutants in fission yeast. Tight genetic links between mcb1<sup>ts</sup> and mcm5<sup>ts</sup> were identified. The mcb1<sup>ts</sup> mutants displayed synthetic defects with many S-phase-related gene mutants, and the temperature sensitivity of mcb1<sup>ts</sup> mutants was suppressed by CDK modulation upon Cig2 repression or Rum1 overexpression, suggesting the involvement of Mcb1 in pre-RC formation during DNA replication. In fact, S-phase progression was delayed in mcb1<sup>ts</sup> mutant cells, and loading of Mcm7 onto the replication origins during pre-RC formation was reduced. Furthermore, mcb1<sup>ts</sup> mutations caused most MCM proteins to exit the nucleus, which was partially rescued by mcm5<sup>ts</sup> overexpression. All of these results strongly indicate the importance of Mcb1 protein in promoting efficient pre-RC formation. This is the first report showing the involvement of MCM-BP in pre-RC formation during DNA replication. Under “Discus-
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tion,” we shall discuss the role of MCM-BP in regulating MCM function(s) during DNA replication in the context of these results.

**EXPERIMENTAL PROCEDURES**

**Strains, Growth Media, and Genetic and Molecular Methods**—The fission yeast strains and plasmids used in this study are listed in supplemental Tables S1 and S2, respectively. Standard growth media and the general biochemical and genetic methods used for fission yeast were described previously (34). Fission yeast cultures were grown at 30 °C in YES medium (0.5% yeast extract, 3% glucose, and supplements) unless indicated otherwise. Geneticin (G418, 100 μg/ml; Sigma), hygromycin (150 μg/ml; Roche Applied Science), or nourseothricin (clonNAT, 200 μg/ml; Werner Bioagents) was added as required. Hydroxyurea (HU; Sigma), methylmethan sulfonate (MMS; Wako), and campothecin (CPT; Sigma) were used at the indicated concentrations. For the induction of expression from the nmt1 promoter, cells were grown at 30 °C in the presence of 5 μM thiamine to repress the nmt1 promoter until mid-log phase and then washed twice with fresh medium and further incubated for 20 h. Tetrad dissection was performed using a Singer Instrument micromanipulator system.

To create the mcb1 gene mutants, the QuikChange site-directed mutagenesis method (Stratagene) was used to mutate the indicated site(s) on a plasmid. All mutations were confirmed by DNA sequencing. The designed mutations were introduced into the wild-type strain using PCR to insert a 5× FLAG epitope at the C terminus and mark the allele with the kanMX6 gene, as described by Krawchuk and Wahls (35). Introduction of the designed mutations into the mcb1 gene on the wild-type chromosome was confirmed by colony PCR, followed by direct sequencing of the PCR product. The expression of the mutated Mcb1 protein tagged with the 5× FLAG epitope was confirmed by Western blotting using an anti-FLAG M2 antibody (Sigma). Exchange of the kanMX6 marker in existing strains with natMX6, which gives rise to resistance to the antibiotic clonNAT, was performed as described by Sato et al. (36).

**Cell Cycle Synchronization**—Yeast strains carrying the cold-sensitive nda3-3K3111 mutation in the β-tubulin gene (37) were synchronized in M phase by incubation for 4 h at 20 °C before being released at the permissive/restrictive temperature. Cell cycle progression was followed by flow cytometry.

**Spotting Assay**—To test the response to temperature, HU, CPT, or MMS, the fission yeast strains were grown on YES plates at 25 °C for 2–3 days. The cells were serially diluted (5-fold) and then spotted onto YES plates containing 5–10 μM CPT, 5–10 mM HU, or 0.005% MMS. The plates were then incubated at the indicated temperatures for 3–6 days.

**Flow Cytometry**—Cells were fixed in 70% ice-cold ethanol overnight and then rehydrated in 50 mM sodium citrate. RNA was removed with 100 μg/ml RNase at 37 °C for 2 h prior to staining with 20 μg/ml propidium iodide as described previously (38, 39). Flow cytometry was performed using a BD Biosciences FACSCalibur instrument and Macintosh BD CellQuest™ software.

**Fluorescence Microscopy**—Cells expressing Mcm2-GFP, Mcm3-GFP, Mcm4-GFP, Mcm5-RFP, Mcm6-GFP, Mcm7-GFP, Rad22-YFP, or Rhp54-GFP (grown in EMM5S medium at the indicated temperatures) were harvested and suspended in Milli-Q water. The cells were then treated with DAPI (Dojindo) at room temperature to visualize DNA. The GFP, RFP, YFP, and DAPI signals were detected using a fluorescent microscope (BX51; Olympus). Images were obtained using a CCD camera (DP71; Olympus) and processed using Photoshop (Adobe) software.

**Yeast Two-hybrid Assay**—The Matchmaker two-hybrid system 3 (Clontech) was used for the yeast two-hybrid assay according to the manufacturer’s instructions. The indicated proteins were fused to the Gal4 DNA-binding domain (Gal4-DBD) on the pGBK7 plasmid or to the Gal4 activation domain (Gal4-AD) on the pGAD-GH or pGAD424 plasmid and expressed in the AH109 reporter strain of *Saccharomyces cerevisiae*.

**In Vitro Pull-down Assay and Semi-in Vitro Pull-down Assay**—Full-length *mcb1* cDNA was cloned into pET28a for expression as a T7-His$_{6}$-tagged protein. pRSFduet-1-sw16 (a gift from J. Nakayama) was used for expression of His$_{6}$-tagged Swi6 as a negative control. Full-length mcms2–7 cDNAs were cloned into pGEX-KG vectors for expression as GST-tagged fusion proteins. T7-His$_{6}$-tagged Mcb1 and His$_{6}$-tagged Swi6 were expressed in *Escherichia coli* KRX (Promega) and purified using nickel-nitrilotriacetic acid beads (Qiagen). GST-tagged fusion proteins (GST and GST-Mcm2–7) were expressed in *E. coli* KRX and purified using glutathione-Sepharose (GE Healthcare). For the *in vitro* pull-down assay, GST-tagged fusion proteins specifically bound to glutathione-Sepharose were incubated with purified T7-His$_{6}$-tagged Mcb1 or His$_{6}$-tagged Swi6 for 1 h at 4 °C. To test the interaction, T7-His$_{6}$-tagged Mcb1 or His$_{6}$-tagged Swi6 was pulled down by GST-tagged proteins. For the semi-*in vitro* pull-down assay, *E. coli* soluble crude lysates expressing the T7-His$_{6}$-Mcb1, T7-His$_{6}$-Mcb1L254P, or T7-His$_{6}$-Mcb1L363P protein, respectively, were used instead of purified T7-His$_{6}$-Mcb1.

**Preparation of Cell Lysates and Western Blot Analysis**—Fission yeast whole-cell extracts were prepared by the trichloroacetic acid (TCA) method. Briefly, the cell pellets (2 × 10$^{8}$ cells) were suspended in 100 μl of 20% TCA and disrupted with glass beads using Micro Smash (MS-100; Tomy Seiko). Cell lysates were collected, and beads were washed twice in 100 μl of 5% TCA. Cell pellets were obtained by centrifugation at 4,000 rpm for 10 min at 4 °C. Then 2× SDS sample buffer was added, followed by 2 μl Tris until the color of the suspension turned from yellow to blue. Samples were boiled at 95 °C for 5 min and cleared by centrifugation at 4,000 rpm for 10 min at 4 °C. Alternatively, whole-cell extracts were prepared using the boiling method, as described previously (40). The proteins in the extracts were separated by SDS-PAGE and subsequently subjected to Western blot analysis using the chemiluminescence system (LAS4000mini, GE Healthcare) or the Odyssey® infrared imaging system (LI-COR Biosciences). The antibodies were as follows: anti-HA monoclonal antibody (HA-probe (F7), Santa Cruz Biotechnology, Inc. (Santa Cruz, CA)), anti-GFP monoclonal antibody (anti-GFP, Roche Applied Science), anti-RFP monoclonal antibody (anti-RFP, Abcam), anti-FLAG monoclonal antibody (anti-FLAG M2, Sigma), anti-Mcm2 anti-Mcm2...
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polyclonal antibody (a gift from H. Masukata) (41), anti-Mcm4 polyclonal antibody (a gift from H. Nishitani) (42), anti-Mcm5 polyclonal antibody (a gift from H. Masukata) (41), anti-Mcm6 polyclonal antibody (a gift from H. Masukata) (41), anti-Mcm7 polyclonal antibody (a gift from H. Masukata) (43), anti-tubulin monoclonal antibody (TAT-1) (44), anti-His6 monoclonal antibody (anti-His6,Clone His-2), Roche Applied Science), and anti-GST polyclonal antibody (GST-Z-5, Santa Cruz Biotechnology, Inc.).

Immunoprecipitation—The spheroplast method was used. Yeast cell extracts were prepared for immunoprecipitation, as described previously (45).

The glass beads method was also used. Cells (1–2 \times 10^8 cells) were suspended in 100 μl of buffer A (50 mM HEPES-KOH, pH 7.5, 300 mM KCl, 0.05% Tween 20, 0.005% Nonidet P-40, 2 mM NaF, 0.4 mM Na_3VO_4, and 2 μM β-glycerophosphate) supplemented with the necessary inhibitors (1× Complete protease inhibitor mixture (Roche Applied Science), 1 mM PMSF, 0.2 mM 4-amidinobenzylsulfonyl fluoride hydrochloride (Sigma)), and 1× protease inhibitor mixture (Sigma)). The cells were disrupted with glass beads using the Multi Beads Shocker (Yasui Kikai). Glass beads were washed in 100 μl of buffer B (buffer A + 1× complete protease inhibitor mixture (Roche Applied Science)). The samples were centrifuged at 13,000 rpm for 15 min at 4 °C, and the cleared supernatants were then used for immunoprecipitation with anti-FLAG M2 affinity gel (Sigma). After rotation for 2 h at 4 °C, the antibody beads were washed with 500 μl of buffer B and buffer C (buffer B + 1× complete protease inhibitor mixture (Roche Applied Science) and 0.2 mM 4-amidinobenzylsulfonyl fluoride hydrochloride (Sigma)). SDS sample buffer was added, and the samples were boiled at 95 °C for 5 min.

Chromatin Immunoprecipitation (ChIP) and Real-time PCR—The ChIP assay and subsequent real-time PCR analysis were conducted according to the method of Fukuura et al. (46).

Immunoprecipitation for Mcm7-3HA was conducted with Dynabeads M-280 anti-mouse IgG beads (Invitrogen) conjugated overnight with an HA monoclonal antibody at 4 °C. Immunoprecipitation for Orp4 was conducted with Dynabeads M-280 anti-rabbit IgG beads (Invitrogen) conjugated overnight with an anti-Orp4 antibody (a gift from H. Masukata) (47) at 4 °C. DNA from whole-cell extracts and immunoprecipitation samples was then subjected to real-time PCR using SYBR Green in a LightCycler (Roche Applied Science). Four sets of primers were used for amplification: ars3002-F (5'-CCAATATTAAATAAGCAAT-3') and ars3002-R (5'-TGAAATTGTTGTT-TTGAGAGC-3') for AT2080; and nonARS-70-F and nonARS-70-R for non-ars1 (47). The percentage recovery was calculated as follows: immunoprecipitated DNA/total DNA \times 100.

Isolation of mcb1 Temperature-sensitive Mutants—Temperature-sensitive mutants were generated and identified as described by Kato et al. (48) with the following changes. Genomic DNA from a mcb1-5FLAG-kanMX6 strain was used as the starting template. The mcb1-5FLAG-kanMX6 construct was amplified using Ex-Taq polymerase (Takara) in the presence of the increased amounts (2-fold) of dNTPs to increase the chance of base misincorporation. Yeast cells transformed with the PCR product were selected on YES plates supplemented with 100 μg/ml G418 (Sigma) and examined for sensitivity to high temperature (36.5 °C). Mutations were identified by sequence analysis.

Isolation of Spontaneous Suppressors of the mcb1^{1-254P} mutant—Spontaneous suppressors of mcb1^{1-254P} were isolated as follows. The mcb1^{1-254P} cells were grown on YES plates at 25 °C (permissive temperature) for 1 day and then incubated at 34 °C (restrictive temperature) for several additional days. The colonies that spontaneously appeared were picked for further analysis.

Cloning of ded1↑—The ded1↑ gene was cloned by complementation for the cold-sensitive defects and temperature-resistant effects of the suppressors 1 and 2, using the fission yeast genomic library obtained from the National BioResource Project (49). Sequencing of the plasmid clones isolated from the genomic library indicated the presence of the same gene, ded1↑, on each plasmid. The ded1↑ locus of suppressors 1 and 2 was amplified by PCR and sequenced, and the mutations in the ded1 genes of both suppressors were characterized.

RESULTS

Mcb1 Is Constitutively Expressed and Continuously Interacts with MCM Proteins—C-terminally 5× FLAG- or GFP-tagged Mcb1 (mcb1-5FLAG or mcb1-GFP, respectively) was constructed to replace the wild-type copy in the genome. The mcb1-5FLAG or mcb1-GFP cells showed normal growth, indicating that the tagged copies were functional. We have confirmed with these strains that Mcb1 is an abundant protein and that protein abundance of Mcb1 is not cell cycle-regulated, findings that agree well with the previous report (data not shown) (29).

Immunoprecipitation and/or mass spectrometry studies in humans, Xenopus, and fission yeast showed that MCM-BPs (hMCM-BP for human, xMCM-BP for Xenopus, and Mcb1 for fission yeast) interact with all MCM complex subunits, except Mcm2 (26, 27, 29, 30). Therefore, MCM-BP was thought to replace Mcm2 within the MCM complex (26); however, in A. thaliana, AtMcm2 was found to co-purify with ETG1, the A. thaliana MCM-BP ortholog, by mass spectrometry analysis (28). To understand this discrepancy, confirmation of the interaction between Mcb1 and MCMs in fission yeast was sought using co-immunoprecipitation studies. Mcb1-5FLAG was immunoprecipitated, and the immunoprecipitate was blotted for most MCM proteins. As shown in Fig. 1A, Mcb1-5FLAG co-precipitated MCM6 and MCM7. In another experiment, Mcb1-5FLAG co-precipitated MCM6-GFP and MCM2, although Mcb1-5FLAG co-precipitated MCM2 at much lower levels than MCM6-GFP (Fig. 1B). In later experiments, Mcb1-5FLAG co-precipitated all six subunits of the MCM complex (Fig. 7A) (data not shown). To investigate whether the interaction between Mcb1 and MCMs is cell cycle-dependent, the interaction between Mcb1 and MCMs was analyzed in synchronized cells. The nda3-KM311 mcb1-5FLAG mcm7-3HA cells were synchronized in M phase and then released, and progression was followed every 20 min (Fig. 1, C and D). Immuno-
precipitation of Mcb1-5FLAG from each sample at the indicated time points showed that Mcb1-5FLAG continuously interacts with Mcm7-3HA and Mcm6 independently of the cell cycle phase (Fig. 1E). These results indicate that the Mcb1 protein is constitutively expressed and continuously interacts with MCM proteins throughout the cell cycle.

*Mcb1 Can Interact with Any of the Individual MCM Subunits*—To understand the interactions between Mcb1 and each of the MCM complex subunits (Mcm2–7), the interaction between Mcb1 and Mcm2–7 was assessed by a yeast two-hybrid assay (Fig. 2A). Mcb1 associated with all of the Mcm2–7 subunits, whereas no interaction between Mcb1 and the vector control or between the vector control and Mcm2–7 was detected. The interaction between Mcb1 and Mcm2–7 was further confirmed by *in vitro* pull-down analysis using bacterially purified recombinant proteins (Fig. 2B). The GST-tagged fusion proteins (GST, GST-Mcm2, GST-Mcm3, GST-Mcm4, GST-Mcm5, GST-Mcm6, and GST-Mcm7) bound to glutathione-Sepharose were incubated with purified T7-His<sub>6</sub>-tagged Mcb1 (T7-His<sub>6</sub>-Mcb1) or His<sub>6</sub>-tagged Swi6 (His<sub>6</sub>-Swi6). Swi6 is one of the fission yeast HP1 (heterochromatin protein 1) homologs and is used as a negative control in this assay. T7-His<sub>6</sub>-Mcb1 interacted with all GST-Mcm2–7, whereas no interaction between T7-His<sub>6</sub>-Mcb1 and GST alone or between His<sub>6</sub>-Swi6 and GST-Mcm5 was detected. These results show that the Mcb1 protein has the ability to interact with any individual MCM subunit.

**Isolation of mcb1 Temperature-sensitive Mutants**—Because it appeared unlikely that disrupting Mcb1 function by temperature-sensitive *mcb1* alleles would have an indirect effect on the normal MCM complex, we tried to isolate temperature-sensitive *mcb1* mutants in fission yeast. The *nda3-KM311 mcb1-5FLAG mcm7-3HA* cells grown in YES medium at 28 °C were incubated at 20 °C for 4 h to induce arrest in M phase and released at 28 °C (time 0). Aliquots of cultures taken at the indicated time points were analyzed by flow cytometry and immunoprecipitation. D, the DNA content of the *nda3-KM311 mcb1-5FLAG mcm7-3HA* cells released from M phase block was analyzed by flow cytometry. S, S-phase. E, soluble lysates of *nda3-KM311 mcb1-5FLAG mcm7-3HA* cells were prepared at the indicated time points and immunoprecipitated as described in A, followed by immunoblotting for Mcm7-3HA with anti-HA antibody, for Mcm6 with an anti-Mcm6 antibody, or for Mcb1-5FLAG with an anti-FLAG antibody.
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A

B

FIGURE 2. Mcb1 interacts with any of the individual MCM subunits. A, two-hybrid interactions between Mcb1 and Mcm2–7. The interaction of Gal4-DBD-Mcb1 in the AH109 strain (when combined with the Gal4-AD-Mcm2–7 plasmids) was tested in the yeast two-hybrid assay. The interactions were monitored on SC–Trp–Leu (SC–WL; non-selective) medium and SC–Trp–His–Ade (SC–WLHA; selective) medium. For vector and Mcb1, the parts of Fig. 7 were used in this figure. B, interactions between bacterially expressed Mcb1 protein and Mcm2–7. T7-His6-tagged Mcb1 protein (T7-His6-Mcb1), His6-tagged Swi6 protein (His6-Swi6), and GST-tagged Mcm2–7 proteins (GST-McmX) were produced in E. coli. Purified GST or GST-tagged Mcm2–7 proteins, which are specifically bound to glutathione-Sepharose, were mixed with purified T7-His6-Mcb1 protein or His6-Swi6 protein and pulled down by glutathione-Sepharose. The GST-pull-down samples were separated on 8% SDS-polyacrylamide gels and blotted with an anti-His6 antibody to detect T7-His6-Mcb1 or His6-Swi6 and with an anti-GST antibody to detect GST-tagged proteins.

struct was amplified by error-prone PCR and introduced into wild-type cells. From the ~1,000 G418-resistant transformants, two mcb1 mutants that showed sensitivity to high temperature (mcb1ts) were isolated. To identify the mutated sites, the mutant genes in the two mcb1ts mutants were isolated by PCR amplification, and the sequences were determined. A single nucleotide change was identified at the 254th or 363th codon, both of which cause substitutions from the conserved hydrophobic leucine residue to proline. These two amino acids (Leu-254 and Leu-363) are highly conserved among MCM-BP orthologs and are located in the C-terminal domain of Mcb1 (Fig. 3A and supplemental Fig. S1). To confirm that the L254P or L363P substitution resulted in the phenotype observed in the two mcb1ts mutants, the L254P or L363P mutation was introduced into wild-type cells using the PCR-based tagging method to place a 5× FLAG epitope at the C terminus and mark the allele with the kanMX6 gene. The resulting G418-resistant haploid transformants, designated mcb1L254P-5FLAG or mcb1L363P-5FLAG, produced the temperature-sensitive phenotypes. Therefore, we concluded that a single amino acid substitution (L254P or L363P) resulted in the phenotypes observed in the two mcb1ts mutants. These two strains, mcb1L254P-5FLAG and mcb1L363P-5FLAG, and mcb1L254P-SFLAG (mcb1L254P) and mcb1L363P-SFLAG (mcb1L363P), were used for further analysis.

The mcb1L254P and mcb1L363P cells were incapable of colony formation at 32 °C (Fig. 3B). The mcb1L254P cells were temperature-sensitive, even at 30 °C, but the mcb1L363P cells were not. Therefore, further analysis focused on mcb1L254P, although mcb1L363P is also partially included in this study. Moreover, the mcb1L254P and mcb1L363P cells were sensitive to S-phase stress agents, such as MMS, HU, and CPT. HU depletes the dNTP pool and inhibits DNA synthesis. CPT traps topoisomerase I on DNA and interferes with DNA replication (50). MMS alkylates the template DNA. Both MMS and CPT induce the formation of double-stranded DNA breaks (DSBs) when the replication fork passes damage sites, whereas HU causes fork stalling. However, there was no sensitivity to UV-mediated DNA damage (data not shown). These results show that Mcb1 is important for surviving replication stress.

The mcb1L254P cells showed cdc phenotypes, with an elongated cell shape and 2C DNA content (Fig. 3C) (data not shown). A small percentage of cells (±2–5%) showed an abnormal nuclear phenotype, such as abnormal DNA segregation, cut (cell uniformed tone) phenotypes, and abnormal seption (Fig. 3C). These phenotypes suggest that the mcb1L254P cells most likely suffer DNA damage. Actually, the mcb1L254P cells accumulated spontaneous DNA damage, as recognized by Rad22 (supplemental Fig. S2, C and D); showed Rad3 and Chk1 dependence for viability at 30 °C (supplemental Fig. S2A); and induced the phosphorylation of Chk1 kinase at the permissive temperature as well as at the restrictive temperature (supplemental Fig. S2B), suggesting activation of the Chk1-dependent DNA damage checkpoint. These results are consistent with the result reported by Li et al. (30), and Ding and Forsburg also conclude that overexpression of Mcb1 triggers cell cycle arrest in a manner that depends upon Rad3 and Chk1 (29).
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Furthermore, the viability of the \textit{mcb1}\textsubscript{L254P} mutant was examined at the restrictive temperature. Mid-log phase unsynchronized cultures of \textit{mcb1}-SFLAG and \textit{mcb1}\textsubscript{L363P}-SFLAG cells were shifted up to 36 °C for variable periods of time. Then variable numbers of cells were plated and incubated at 25 °C. The viability of the \textit{mcb1}\textsubscript{L254P} cells decreased markedly after 4 h of incubation at 36 °C, and no viable cells remained after 8 h of incubation (supplemental Fig. S3, A and B). The phenotype was also observed in a single \textit{mcb1}\textsubscript{L254P} cell grown at 36 °C (supplemental Fig. S3C). The cell invariably stopped growing after 2–4 divisions (4–16 cells) and showed an elongated cell shape.

**Genetic Interaction between \textit{mcb1} and DNA Replication Factors**—Combining two mutations in different genes results in synthetic lethality or enhanced temperature sensitivity when the corresponding gene products function in the same pathway. To explore the genetic relationship between Mcb1 and other replication factors, double mutants carrying \textit{mcb1}\textsubscript{L254P} and a mutation in a component of the pre-RC, the pre-IC, or the DNA polymerases were constructed. The \textit{mcb1}\textsubscript{L254P}, \textit{cdc19-P1/mcm2} and the \textit{mcb1}\textsubscript{L254P}, \textit{swi7-H4} (Polα) double mutants exhibited more severe sensitivity than the parental strains, whereas the sensitivity of \textit{mcb1}\textsubscript{L254P}, \textit{sld3-10} did not differ significantly from that of single mutants (Fig. 4A). The results of similar experiments are summarized in Fig. 4B. A \textit{mcb1}\textsubscript{L254P}, \textit{nda1-376/mcm2} double mutant could not be created, indicating that the combination of the \textit{mcb1}\textsubscript{L254P} mutation with the \textit{nda1-376/mcm2} mutation results in synthetic lethality or an extremely low viability phenotype (data not shown). The temperature sensitivity of the \textit{mcb1}\textsubscript{L254P}\textsubscript{mcm2} cells was increased by \textit{cde19-P1/mcm2}, \textit{cdc19-M68/mcm4}, \textit{mis5-628/mcm6}, \textit{hsk1-1312}, \textit{nda4-108/mcm5}, \textit{cut5-T401}, \textit{mcm10-5}, and \textit{cde27-P11} (Polβ). The temperature sensitivity of the \textit{mcb1}\textsubscript{L254P} cells was slightly increased by \textit{orp5-H19}, \textit{orp1-H5}, \textit{swi7-H4} (Polα), \textit{goa1-U53/cdc45}, \textit{pol3-ts13}, and \textit{cde6-121} (Polβ). By contrast, the temperature sensitivity of the \textit{mcb1}\textsubscript{L254P} cells was not affected by \textit{cde8-K46}, \textit{hsk1-89}, \textit{sna41-928/cdc45}, \textit{sld3-10}, \textit{cde2-33}, \textit{psf3-1}, or \textit{cde20-M10} (Polε) (Fig. 4B). These results suggest that Mcb1 functions in processes that involve the pre-RC components Nda1/Cdc19/Mcm2, Cdc21/Mcm4, and Mis5/Mcm6; the pre-IC components Hsk1, Nda4/Mcm5, and Mcm10; and the DNA polymerase component Cdc27/Polβ.

**Overproduction of Mcm5 Suppresses the Temperature Sensitivity of \textit{mcb1} Mutants**—To identify factors that functionally interact with the Mcb1 protein, a screen for fission yeast genes able to suppress the temperature sensitivity of \textit{mcb1}\textsubscript{L254P} cells was conducted using a multicopy plasmid. The fission yeast genomic library (pTN-L1 genomic library from the National BioResource Project) (49) was introduced into \textit{mcb1}\textsubscript{L254P} cells, and transformants that were less sensitive to high temperature were selected. The plasmids recovered from these less sensitive clones contained the \textit{mcb1} or the \textit{mcm5} gene. The product of the \textit{mcm5} gene, the Mcm5 protein, is one of the subunits of the MCM complex. A serial dilution assay showed that overexpression of Mcm5 suppressed the temperature sensitivity of \textit{mcb1}\textsubscript{L254P} cells at 34 °C but not at 36 °C (Fig. 4C). Complete suppression was achieved only by the introduction of a multicopy plasmid expressing the \textit{mcb1} gene. This result prompted us to investigate the specificity of this suppression by introducing multicopy plasmids expressing other members of the MCM complex. As shown in Fig. 4D, overproduction of Mcm2, Mcm3, or Mcm4 did not suppress the temperature sensitivity of \textit{mcb1}\textsubscript{L254P} cells at all, but overproduction of Mcm6 or Mcm7 did slightly suppress the temperature sensitivity at 30 °C. Similar results were obtained with \textit{mcb1}\textsubscript{L363P} cells (data not shown). These results indicate the close genetic relationship between \textit{mcb1} and some MCM genes, especially the \textit{mcm5} gene. These findings suggest that some MCM functions are impaired by the \textit{mcb1}\textsubscript{L254P} mutation.

**CDK Modulation Suppresses \textit{mcb1} Mutants**—To better understand the defects of the \textit{mcb1}\textsubscript{L254P} mutant, a screen for spontaneous suppressors of the \textit{mcb1}\textsubscript{L254P} mutant able to grow at the restrictive temperature was conducted. Three independent suppressors that could grow even at 34 °C, here called suppressors 1, 2, and 3, were isolated (Fig. 5A). In addition to their ability to support cell growth at a high temperature, suppressors 1 and 2 showed cold-sensitive phenotypes at 20 °C, but suppressor 3 did not (Fig. 5A). To search for the responsible gene, multicopy plasmids were isolated by complementation in suppressors 1 and 2 using the fission yeast genomic library. The sequencing of the clones isolated from the genomic library revealed the presence of the same gene, \textit{ded1}, on each plasmid (Fig. 5B). \textit{Ded1} is a general translation factor that is functionally homologous to RNA helicases from budding yeast.
peratures.

Dilution and incubated at permissive (25 °C) or restrictive (34 and 36 °C) temperatures. In addition to the mcm7 defects with swi7-H4 cells harboring pTN-L1-

mcb1 swi7-H4 mcb1-H4-SFlag swi7-H4 mcb1-SFlag

rad26 cells harboring pTN-L1-

mcm2 swi7-H4 mcb1-SFlag

mcb1-SFlag, swi7-H4, mcb1

mcb1-SFlag

mcb1-SFlag

mcm7  

mcb1-SFlag

mcm7-L254P shows strong synthetic defects with swi7-H4 (+), and mcb1-L254P showed no or little synthetic defects with swi7-H4 (−). B, summary of the growth of the double mutants. The growth of the double mutants was examined as in (B). +++, strong synthetic defects; +, weak synthetic defects; −, no synthetic defects. C, gene dosage suppression of the temperature sensitivity of mcb1-L254P cells. Aliquots of mcb1-L254P, leu1−12 cells harboring the pTN-L1-rad26 (negative control), pTN-L1-mcb1”, or pTN-L1-mcm5” were spotted onto EMM plates after 5-fold serial dilution and incubated permissive (25 °C) or restrictive (34 and 36 °C) temperatures. D, gene dosage suppression of the temperature sensitivity of mcb1-L254P by other components of the MCM complex. Aliquots of mcb1-L254P cells harboring pTN-L1-rad26” (negative control) or pTN-L1-mcm2”, -mcm3”, -mcm4”, -mcm5”, -mcm6”, or -mcm7” were spotted onto EMM plates (after 5-fold serial dilution) and incubated at the indicated temperatures. Introduction of a multicopy plasmid expressing mcm5” plasmid, plasmids expressing mcm6” or mcm7” showed weak suppression of the temperature sensitivity of mcb1-L254P cells.

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FIGURE 4. Genetic interaction between mcb1" and the DNA replication components. A, growth of double mutants. 5-Fold serial dilutions of cells with the indicated genotypes were spotted onto YES plates and incubated at the indicated temperatures. The alleles used were mcb1-L254P, cdc19-P1/mcm2, swi7-H4/pola, and sls3–10. The mcb1-L254P showed strong synthetic defects with swi7-H4 (+), and mcb1-L254P showed no or little synthetic defects with swi7-H4 (−). B, summary of the growth of the double mutants. The growth of the double mutants was examined as in (B). +++, strong synthetic defects; +, weak synthetic defects; −, no synthetic defects. C, gene dosage suppression of the temperature sensitivity of mcb1-L254P cells. Aliquots of mcb1-L254P, leu1−12 cells harboring the pTN-L1-rad26” (negative control), pTN-L1-mcb1”, or pTN-L1-mcm5” were spotted onto EMM plates after 5-fold serial dilution and incubated permissive (25 °C) or restrictive (34 and 36 °C) temperatures. D, gene dosage suppression of the temperature sensitivity of mcb1-L254P by other components of the MCM complex. Aliquots of mcb1-L254P cells harboring pTN-L1-rad26” (negative control) or pTN-L1-mcm2”, -mcm3”, -mcm4”, -mcm5”, -mcm6”, or -mcm7” were spotted onto EMM plates (after 5-fold serial dilution) and incubated at the indicated temperatures. Introduction of a multicopy plasmid expressing the cig2” gene reversed the effect of the ded1” mutant. Aliquots of wild-type, suppressor 1, and suppressor 2 cells harboring the pTN-L1-rad26” (negative control) or pTN-L1-ded1” were spotted on EMM plates (after 5-fold serial dilution) and incubated at the indicated temperatures. E, deletion of the cig2” gene rescues the temperature-sensitive phenotype of the mcb1-L254P mutant. 5-Fold serial dilutions of wild-type, cig2Δ, mcb1-L254P, SFLAG, and mcb1-L254P-SFLAG cig2Δ cells were spotted on YES medium and incubated at 25 and 32 °C for 3–6 days. A mcb1-L254P and cig2Δ double mutant grew even at 32 °C. F, a multicopy plasmid expressing the rum1” gene can suppress the temperature sensitivity of mcb1-L254P. Aliquots of mcb1-L254P cells harboring the pTN-L1-rad26” (negative control) or pTN-L1-rum1” were spotted onto EMM plates (after 5-fold serial dilution) and incubated at the indicated temperatures. Introduction of a multicopy plasmid expressing rum1” partially suppressed the temperature-sensitive phenotype of mcb1-L254P at both 30 and 32 °C.

and Drosophila. A ded1” gene was characterized as sum3”, moc2” or slh3” in independent studies (51–53). The ded1” locus of suppressors 1 and 2 was amplified by PCR and sequenced, and mutations in the ded1” genes were detected in both suppressors of the temperature-sensitive phenotype of the mcb1-L254P mutant. A, isolation of spontaneous suppressors of the mcb1-L254P mutant. 5-Fold serial dilutions of cells with the indicated genotypes, including the three spontaneous suppressors of mcb1-L254P (suppressors 1, 2, and 3), were spotted onto YES plates and incubated at the indicated temperatures. Suppressor 1 and 2 suppressed the temperature-sensitive phenotype of mcb1-L254P cells and showed the additional cold-sensitive phenotypes. B, a multicopy plasmid expressing the ded1” gene suppressed the cold sensitivity of suppressors 1 and 2. Aliquots of wild-type, suppressor 1, and suppressor 2 cells harboring the pTN-L1-rad26” (negative control) or pTN-L1-ded1” were spotted on EMM plates (after 5-fold serial dilution) and incubated at the indicated temperatures. C, schematic representation of the domain (amino acids 171–393), a DEXDc (DEAD-like helicase superfamily) domain (amino acids 403–533) is indicated by lines. The positions of the mutated sites in suppressor 2 (S1-mcb1, A490S) and 2 (S2-mcb1, I404T) are indicated. D, the epitope expression of the cig2” gene reversed the effect of the ded1” mutant. Aliquots of wild-type, suppressor 1, and suppressor 2 cells harboring the pREP41 vector or pREP41-cig2” were spotted onto EMM plates (after 5-fold serial dilution) and incubated at the indicated temperatures. E, deletion of the cig2” gene rescued the temperature-sensitive phenotype of the mcb1-L254P mutant. 5-Fold serial dilutions of wild-type, cig2Δ, mcb1-L254P, SFLAG, and mcb1-L254P-SFLAG cig2Δ cells were spotted on YES medium and incubated at 25 and 32 °C for 3–6 days. A mcb1-L254P and cig2Δ double mutant grew even at 32 °C. F, a multicopy plasmid expressing the rum1” gene can suppress the temperature sensitivity of mcb1-L254P. Aliquots of mcb1-L254P cells harboring the pTN-L1-rad26” (negative control) or pTN-L1-rum1” were spotted onto EMM plates (after 5-fold serial dilution) and incubated at the indicated temperatures. Introduction of a multicopy plasmid expressing rum1” partially suppressed the temperature-sensitive phenotype of mcb1-L254P at both 30 and 32 °C.
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Sors, indicating that mutations in the ded1 genes are responsible for both suppressors. Both suppressors contain a single amino acid change in the ded1 gene (Fig. 5C). Suppressor 1 contained an alanine to serine mutation at position 490 (A490S), and suppressor 2 contained an isoleucine to threonine mutation at position 404 (I404T). Ded1 is an ATP-dependent RNA helicase, the sequence of which contains DNA motifs such as the DEAD-box helicase, the DEAD-like helicase superfamily (DEXDc), and the helicase superfamily C-terminal domain (HELICc). Both mutations are located within the putative HELICc motif.

One of the features of Ded1 is its influence on B-type cyclin Cig2 expression. It is reported that ded1 mutants reduce the translation of Cig2 and suppress defects in pre-RC formation during DNA replication. Consequently, increased expression of Cig2 prevents the ded1 mutants from suppressing such defects, and deletion of the cig2+ gene (cig2Δ) suppresses mutants that show defects in pre-RC formation (52, 54–56). Indeed, the introduction of a multicopy plasmid overexpressing cig2+ restored the temperature sensitivity of suppressors 1 and 2 (Fig. 5D). Furthermore, double mutants of mcbl1254p and cig2Δ grew well at 32 °C (Fig. 5E).

Cig2 promotes the onset of S-phase by activating CDK (55). By contrast, Rum1 inhibits the CDK activity of the Cdc2 kinase, thereby inhibiting the start in the G1 phase (57–59). Because Cig2 repression results in the suppression of the temperature-sensitive phenotype of the mcbl1254p mutant, the effect of Rum1 overexpression was tested. As shown in Fig. 5F, overexpression of Rum1 resulted in the suppression of the temperature-sensitive phenotype of the mcbl1254p mutant at 32 °C. CDK modulation by Cig2 and Rum1 suppresses defects caused by mutations in pre-RC components (Fig. 9). This suppression is specific for pre-RC formation during S-phase because mutations in pre-RC components are not suppressed by CDK modulation (56, 60, 61). A possible explanation is that an extended G1 phase upon transient inhibition of CDK provides time to assemble the pre-RC in mutants that show defects in pre-RC formation (55). Furthermore, the mcbl1254p mutant could partially suppress the HU sensitivity of the cdc18 mutant (data not shown). This result suggests some defects of MCM function in mcbl1254p mutant, because mutations in the MCM complex are known to suppress the HU sensitivity of the replication checkpoint mutants (62). All of these genetic results strongly indicate that the mcbl1254p mutant has some defects in pre-RC formation, suggesting that Mcb1 has an important function in pre-RC formation.

Because suppressor 3 did not show any additional phenotype (Fig. 5A), whole-genome sequencing was performed and showed that the genomic region containing the mcms5+ gene was amplified 3 times (data not shown). This finding is consistent with gene dosage suppression of the temperature sensitivity of mcbl1254p by the mcms5+ plasmid (Fig. 4C). These results further strengthen the tight genetic link between mcbl+ and mcms5+.

Accumulation of Rhp54 Nuclear Foci in mcbl1254p Cells—Following activation of the DNA damage response, DNA repair takes place. Homologous recombination is the major pathway for the repair of DSBs. In this pathway, Rad52 is recruited to sites of DNA damage, where it mediates Rad51 filament formation on the single-stranded tail of the DSBs. Then Rad51 performs a homology search followed by DNA strand exchange with the donor strand, a process mediated by Rad54/Rhp54 (54). It was recently reported that an abundance of pre-RCs is important for the late step of recombination, in which Rhp54 functions (54). A link between the pre-RC and the late step of recombination was supported by the finding that the cdc18-K46 and mcms6-S1 mutants were hypersensitive to MMS and CPT and that the fraction of cells exhibiting Rhp54 foci remained large, even after release from MMS treatment, in both mutants. The mcbl1254p mutant was sensitive to MMS and CPT (Fig. 3B). Although MMS-induced Chk1 activation and Rad22 (fission yeast Rad52 ortholog) focus formation occurred normally (supplemental Fig. 5A and B), the number of cells exhibiting Rhp54 foci after release from MMS treatment increased in the mcbl1254p mutant to a level similar to that observed in the cdc18-K46 and mcms6-S1 mutants (supplemental Fig. 5B). These results support the idea that there are defects in pre-RC levels in the mcbl1254p mutant.

The mcbl4 Mutants Show Defects in S-phase Progression—Based on the genetic observations described above, defects in DNA replication were carefully examined in mcbl1254p cells. To define the role of the Mcb1 protein during DNA replication, S-phase progression was examined in mcbl1254p cells. The nda3-KM311 mcbl1-SFLAG and nda3-KM311 mcbl1-L254P-SFLAG cells were synchronized in M phase and then released either at 28 or 36 °C (block and release by the nda3-KM311 mutant). The DNA content, as analyzed by FACS, showed that S-phase progression in mcbl1-SFLAG was slightly but reproducibly delayed relative to that in mcbl1-SFLAG cells both at 28 and 36 °C (Fig. 6, A and B). It appears that the progression of S-phase, once initiated, did not differ much at 28 and 36 °C. Thus, it is possible that mcbl1254p mutation more specifically affects the initiation stage of S-phase. Furthermore, to follow the progression of S-phase, the septation index of these cells was monitored as shown in Fig. 6, A and B. The peak of the septation index in fission yeast coincides with S-phase during cell cycle progression. The mcbl1254p-SFLAG cells had a much lower septation index than mcbl1-SFLAG cells (Fig. 6, C and D). At the permissive temperature, the septation index of mcbl1-SFLAG cells peaked 40 min after release (76.9%) and rapidly decreased afterward, indicating that S-phase progression was normal. The septation index of mcbl1254p-SFLAG cells also peaked at 40 min, but the peak was only 32.5% (Fig. 6C). From the profile shown in Fig. 6C, S-phase progression in mcbl1254p-SFLAG cells seemed to continue until much later, up to 120 min after release (16.2% in mcbl1254p-SFLAG cells compared with 3.2% in mcbl1-SFLAG cells). When the release temperature was shifted to 36 °C, the cell cycle progression of mcbl1-SFLAG cells became faster, and the septation index peaked 20 min after release (63.1%) (Fig. 6D). Release at the restrictive temperature did not result in any significant differences in the FACS profile compared with release at the permissive temperature (Fig. 6, A and B); however, the septation index profile of mcbl1254p-SFLAG cells barely changed, peaking at 40 min (30.6%) and continuing until 120 min after release (16.3%) (Fig. 6D). Similar results were obtained with mcbl1163p-SFLAG cells (data not shown). Although we cannot rule out the possibility that mcbl1254p mutant cells show defects in release from M phase.
arrest, these results strongly suggest a defect in S-phase progression.

Thus, loading of MCM proteins onto the replication origins during the initiation of DNA replication was investigated in mcb1<sup>L254P</sup> mutants. For this purpose, ChIP analysis was performed to monitor the chromatin binding of Mcm7-3HA to early firing origins (ars2004 and ars3002), a late firing origin (AT2080), and non-origin (non-ars1) both in mcb1<sup>-5FLAG</sup> and mcb1<sup>L254P-5Flag</sup> cells (Fig. 6E and supplemental Fig. S6A). The mcb1<sup>L254P</sup> mutation markedly decreased Mcm7-3HA binding to the replication origin, whereas the binding of Orp4, a component of the origin recognition complex, was not decreased (Fig. 6E and supplemental Fig. S6B). This result indicates that Mcb1 function is important for the efficient loading of MCM complexes onto the replication origins.

The mcb1<sup>ts</sup> Mutations Impair the Interaction of Mcb1 with the MCM Complex—Judging from the phenotypes of the mcb1<sup>ts</sup> mutants described above, we speculated that the interaction of Mcb1 with the MCM complex was affected in the mcb1<sup>ts</sup> mutants. To test this possibility, MCM complex formation and the interaction between Mcb1 and the MCM complex were examined in the mcb1<sup>ts</sup> mutants. As expected, the interaction between the mutant Mcb1 proteins (Mcb1<sup>L254P</sup> and Mcb1<sup>L363P</sup>) and the MCM complex was markedly attenuated.

Using the mcb1<sup>-5FLAG</sup>, mcb1<sup>L254P-5Flag</sup>, and mcb1<sup>L363P-5FLAG</sup> strains, Mcb1-5FLAG immunoprecipitation was performed at permissive (25 °C) and restrictive temperatures (36 °C). Mcb1-5FLAG immunoprecipitated all MCM proteins tested both at 25 and 36 °C (Fig. 7A, lanes 6 and 14). The amount of Mcm2 co-precipitated with Mcb1 was considerably lower in the mcb1<sup>L254P-5Flag</sup> strain compared to the wild-type strain.
less than that of other MCM proteins (Fig. 7A, lanes 6 and 14). The interaction between Mcb1 and the MCM proteins was detectable in both mcb1^{L254P} and mcb1^{L363P} cells but was markedly decreased, even at 25 °C (Fig. 7A, lanes 7 and 8). There was no significant change in the interaction between Mcb1 and MCMs in mcb1^{L254P} or mcb1^{L363P} cells at 36 °C (Fig. 7A, lanes 15 and 16). As shown earlier, Mcb1 interacted with all of the subunits of the MCM complex in a yeast two-hybrid assay and in an in vitro pull-down assay (Fig. 2, A and B). Next, we tested whether the interaction between Mcb1 and the MCM complex is affected in the mcb1^{L254P} and mcb1^{L363P} mutants. Interestingly, neither the Mcb1^{L254P} nor Mcb1^{L363P} protein could interact with any of the subunits of the MCM complex in the yeast two-hybrid assay and pull-down assay (Fig. 7, B and C).
These results suggest that the temperature-restricted phenotypes of the mcb1ts mutants are not simply caused by the altered interaction between the Mcb1 and MCM proteins.

Localization of the MCM Protein Is Compromised in mcb1ts Mutants—In fission yeast, Mcm2–7 proteins are constitutively localized in the nucleus throughout the cell cycle (5). Mutations that disrupt MCM complex formation, such as mcm4ts, cause all of the MCM subunits to exit the nucleus (63). Therefore, the effect of the mcb1ts gene mutations on the nuclear localization of MCM subunits was studied. The localization of all of the MCM complex subunits was observed in wild-type cells or mcb1ts mutants at both permissive and restrictive temperatures using GFP or RFP (GFP for Mcm2, -3, -4, -6, and -7; RFP for Mcm5) (Fig. 8A). Mcm2-GFP showed clear nuclear localization at 25 °C in mcb1-5FLAG, mcb1L254P-5FLAG, and mcb1L363P-5FLAG cells; however, at the restrictive temperature (34 °C), nuclear localization of Mcm2-GFP diminished, and cytoplasmic staining increased in both mcb1L254P-5FLAG and mcb1L363P-5FLAG cells (Fig. 8A). Similar observations were made in mcb1L254P-5FLAG and mcb1L363P-5FLAG cells using Mcm4-GFP, Mcm5-RFP, Mcm6-GFP, and Mcm7-GFP, whereas Mcm3-GFP showed no real change (Fig. 8A). The GFP or RFP signals observed in mcb1ts mutants at the restrictive temperature were not completely excluded from the nucleus because there was residual signal in the nucleus. Afterward, we tested whether these phenotypes could be suppressed by a multicopy plasmid containing mcm5+, which can partially suppress mcb1ts mutants as shown in Fig. 4C (Fig. 8B). Multicopy plasmids containing mcm5+, mcb1ts as a positive control, or ded1ts as a negative control were transformed into mcm2-GFP mcb1L254P-5FLAG and mcm6-GFP mcb1L254P-5FLAG cells. As expected, the mislocalization of Mcm2-GFP and Mcm6-GFP observed above was suppressed by the introduction of a multicopy plasmid containing mcm5+. These results indicate that the mislocalization of MCM proteins is partially rescued by the overexpression of mcm5+. Next, we examined whether these phenotypes were reflected in targeting to the nucleus, retention in the nucleus, or both.

MCM Proteins Are Actively Exported from the Nucleus in mcb1ts Mutants—The loss of MCM proteins from the nucleus in mcb1ts mutants may reflect protein turnover and a failure to import newly synthesized molecules, or it could indicate a defect in protein retention within the nucleus. Steady-state levels of MCM proteins were unchanged in cells arrested by mcb1ts mutations (Fig. 8D). Because there was no evidence of any change in protein levels, the role of nuclear export in the redistribution of MCM proteins in mcb1ts mutants was examined. The crn1ts gene encodes a nuclear export receptor (64). To determine whether MCM relocalization requires active nuclear export, the crn1ts and mcb1ts genes were simultaneously inactivated. If MCM proteins require crn1-dependent active export, they should be trapped in the nucleus under these restrictive conditions.

The crn1ts mutant was used for this experiment. A temperature-sensitive crn1 allele (crn1-11R) (65) was combined with the mcb1L254P mutant. Interestingly, Mcm2-GFP and Mcm6-GFP themselves remained in the nucleus at the restrictive temperature (Fig. 8C). These results suggest that an active nuclear export system is required for the relocalization of MCM proteins when Mcb1 is inactivated. The localization of any one of the MCM proteins is influenced by the activity of all other members of the complex. When MCM function is abrogated, MCM subunits are removed from the nucleus via an active nuclear export system (63). Taking all this into consideration, these results suggest that MCM functions are abrogated in the mcb1L254P mutant.

DISCUSSION

The functions of MCM-BP during DNA replication have been extensively studied in several organisms; however, the mechanisms by which MCM-BP functions and associates with MCM complexes are not well understood, and there seems to be no unified view concerning the shared common function of MCM-BP across species. Therefore, the present study aimed to elucidate the molecular mechanisms underlying MCM-BP function in fission yeast through biochemical and genetic analyses. The results showed that the fission yeast MCM-BP, Mcb1, plays an important role as a regulator of MCM function in pre-RC formation during DNA replication.

Although Mcb1 is a novel binding partner of the MCM complex in fission yeast, the nature of the interaction between Mcb1 and MCM proteins is not yet clear. MCM proteins can be present in three forms: free monomers, subcomplexes, or a complete hexamer complex. In fission yeast (as in humans and Xenopus), Mcb1 interacts with all MCM subunits, except Mcm2 (29, 30). By contrast, ETG1 (AtMCM-BP) interacts with all MCM complex subunits in Arabidopsis. Immunoprecipitation analysis showed that Mcb1 interacted with all MCM subunits, including Mcm2, whereas the amount of Mcm2 co-precipitated by Mcb1 was much lower than that of any other MCM. Two methods of preparing crude cell extracts for immunoprecipitation were tested, the spheroplast method and the beads method; only a very small amount of Mcm2 was immunoprecipitated by Mcb1-5FLAG using either method (Figs. 1B and 7A). Recently, Ding and Forsburg reported that an interaction between Mmc2 and Mcb1 can be observed when Mcb1 is overexpressed, suggesting that this interaction may be weak but not completely lacking (29). Our result is consistent with this observation. Furthermore, the results showed that the Mcb1 protein could bind to any individual subunit of the MCM complex and that the relative affinity of Mcb1 for Mcm2–7 in the immunoprecipitation analyses was variable. Therefore, the interaction profiles described above predict that Mcb1 might form complexes with the individual MCMs and/or subcomplexes in addition to the complete MCM complex. Further studies using in vitro reconstitution of the MCM complex will be required to clarify these points.

Two temperature-sensitive mcb1ts genes were used for genetic analysis of the mcb1ts gene. The mcb1ts alleles showed a cdc phenotype with an elongated cell shape and 2C DNA content. Similarly, temperature-sensitive mutants of fission yeast mcm genes arrested the cell cycle with a cdc phenotype and showed the classic checkpoint-dependent arrest characteristic of other replication mutants. These mcb1ts cells were sensitive to MMS, HU, and CPT. Li et al. (30) recently reported the identification of additional mcb1ts alleles that do not display these sensitivities but share other phenotypes with the muta-
tions identified here. The difference in these two results may relate to the temperature utilized for the experiment rather than allele-specific differences in phenotype. There was a marked reduction in the interaction between Mcb1L254P and Mcm2–7 in vivo, and the interaction between the Mcb1L254P protein and individual MCM complex subunits was almost abolished both in the yeast two-hybrid assay and in the in vitro pull-down assay. These reductions in the in vivo interaction between Mcb1 and Mcm2–7 were observed even at the permissive temperature, suggesting that the reduced level of the interaction is still sufficient to support the viability of mcb1L254P cells at the permissive temperature; however, the viability of mcb1L254P mutant cells was much lower than that of the wild-type cells, even at the permissive temperature, which is probably due to the
reduced interaction between Mcb1 and the MCMs. Indeed, there were defects in S-phase progression and in the loading of Mcm7 onto the replication origins, even at the permissive temperature, in mcb1<sup>L254P</sup> cells. The in vivo interaction between Mcb1 and Mcm2–7 did not change dramatically, even when cells were cultured at the restrictive temperature, whereas the genetic analyses indicated that the functions of MCM in the mcb1<sup>L254P</sup> mutant are abrogated at the restrictive temperature.

Genetic analyses showed that the temperature sensitivity of mcb1<sup>L254P</sup> is suppressed by overexpression of the mcm5<sup>L254P</sup> gene, indicating the close genetic relationship between mcb1<sup>L254P</sup> and mcm5<sup>L254P</sup>. The suppression of mcb1<sup>L254P</sup> temperature sensitivity by CDK modulation (by the repression of Cig2 or by the overexpression of Rum1) strongly suggests that Mcb1 is involved in pre-RC formation during DNA replication because cig2 deletion or Rum1 overexpression extends the period of G1, during which pre-RC assembly occurs and specifically suppresses the mutants that show defects in pre-RC formation (52, 54–56) (Fig. 9). Based on these observations, defects in DNA replication were carefully examined in mcb1<sup>L254P</sup> cells. The mcb1<sup>L254P</sup> cells showed a clear delay in the initiation stage of S-phase compared with wild-type cells at both the permissive temperature and the restrictive temperature, suggesting that mcb1<sup>L254P</sup> cells have defects in the initiation of DNA replication. ChIP analysis of Mcm7 protein showed that the level of loading of Mcm7 onto a replication origin was significantly reduced in mcb1<sup>L254P</sup> cells relative to that in wild-type cells, even at the permissive temperature, suggesting inefficient pre-RC formation in mcb1<sup>L254P</sup> cells. These results indicate that Mcb1 plays an important role in efficient pre-RC formation by supporting the loading of the MCM complex onto the replication origin (Fig. 9).

Recently, Maki et al. (54) reported the importance of abundant pre-RC for survival against replication stress, which causes DSB. The mcm6–S1 mutation impairs the interaction with Cdt1 and decreases the binding of MCM proteins to the replication origins. In mcm6–S1 mutants, the abundance of pre-RC is reduced, and Rhp54 foci accumulate in the nucleus. The requirement of Mcb1 for proper pre-RC formation was also confirmed by the similarity of the phenotypes observed in mcb1<sup>L254P</sup> to those observed in mcm6–S1 mutants. The mcb1<sup>L254P</sup> mutant displayed sensitivity to MMS and CPT<sup>B</sup>, both of which cause the formation of DSBs followed by collapse of the replication fork at the damage sites. The sensitivity to these drugs suggests impaired DSB repair in the mcb1<sup>L254P</sup> mutant. Similarly, the mcb1<sup>L1245P</sup> mutant showed increased numbers of Rhp54 nuclear foci after release from MMS treatment, which is consistent with the phenotypes described above. In addition, the mcb1<sup>L1245P</sup> mutant exhibited spontaneous Rhp54 foci at a semipermissive temperature without MMS treatment and showed increased levels of Rhp54 foci after MMS removal. These phenotypes are quite similar to those of the mcm6–S1 mutant and the cdc18<sup>ts</sup> mutant (cdc18<sup>K46</sup>). The cdc18<sup>ts</sup> is specifically required for the recruitment of the MCM complex onto chromatin during pre-RC assembly.

Although the function of Mcb1 is important in pre-RC formation during replication as described above, ChIP analysis showed that Mcb1 binding to replication origins was not observed during S-phase progression (data not shown). This is consistent with a report by Ding and Forsburg (29). In this case, how does Mcb1 support MCM complex loading onto the replication origins? MCM localization analysis showed that the mcb1<sup>L1245P</sup> mutation leads to redistribution of MCM subunits to the cytoplasm and that this redistribution depends on an active Cig2Δ and overexpression of rum1<sup>B</sup>. CDK modulation does not have any effect on mutations at the other stages of replication.

**FIGURE 9. Typical suppression of pre-RC mutants by CDK modulation.** Many mutants of the pre-RC genes, such as cdc19–P1/mcm2, nda1–376/mcm2, cdc18–K46, cdc21–M68/mcm4, mss5–268/mcm6, and orp1–4/orc1, can be suppressed specifically by modulation of CDK activity, which is caused by cig2Δ and overexpression of rum1<sup>B</sup>. CDK modulation does not have any effect on mutations at other stages of replication.
stittuatively localized in the nucleus, and this nuclear localization requires complete MCM complex assembly. When MCM function is abrogated, MCM subunits are removed from the nucleus via an active nuclear export system (63), strongly suggesting that Mcb1 is important for the regulation of MCM function and/or the complex formation of MCM complex.

How does Mcb1 regulate the function of the MCM complex? In nearly all species, the bulk of the MCMs are constitutively located in the nucleus throughout the entire cell cycle. Budding yeast is a special case because the MCM proteins cycle in and out of the nucleus during a single cell cycle, so that the bulk of the proteins are present only in the nucleus during S-phase. It is possible that Mcb1 functions to regulate MCMs at the level of chromatin association to prevent binding or activation outside of S-phase. This could be a regulatory function that is not needed in budding yeast, which has no MCM-BP ortholog. By contrast, the Mcb1 protein appears to bind nonspecifically to the MCM subunits, including Mcm2, as reported recently (29), and the mcblts mutation leads to the redistribution of the MCM subunits to the cytoplasm in a nuclear export system–dependent manner. These two findings raise the possibility that Mcb1 could serve a chaperone-like function by affecting the pool of MCM proteins that are not assembled into an intact MCM complex. Therefore, by combining the findings of a previous study (29) and those of this study, we propose the following model (supplemental Fig. S7). Mcb1 traps free MCM subunits or subcomplexes in the nucleus so that the cell avoids a situation in which unassembled MCM proteins bind to other factors or interfere with the function of the intact MCM complex. This provides a mechanism through which the cell can assemble intact hexameric MCM complexes in the nucleus and maintain the correct stoichiometry of the individual subunits. Mcb1-containing MCM complex, which lacks Mcm2, might play an important role for this kind of regulation. In the case of Mcb1 overexpression, the presence of excess Mcb1 weakens the stability of MCM complex and increases the occurrence of unassembled MCM subunits or subcomplexes (29). These unassembled MCM proteins might be prone to be exported by a crm1–dependent nuclear export system. The unassembled MCM proteins, which cannot be trapped in mcblts mutants, interfere with the function of the intact MCM complex, leading to redistribution of MCM subunits to the cytoplasm by the nuclear export system.

Our preliminary results showed that Mcb1ts protein is more enriched in chromatin fractions than wild-type Mcb1 protein during early S-phase (data not shown), suggesting the possibility that the association of Mcb1 with chromatin prevents the loading and/or function of MCM complex. This is consistent with the model in which Mcb1 antagonizes MCM function (29). Further analyses will be required to clarify these points.

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REFERENCES


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