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Abundance of Prereplicative Complexes (Pre-RCs) Facilitates Recombinational Repair under Replication Stress in Fission Yeast*

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Background: Assembly of many prereplicative complexes (pre-RCs) is important for DNA replication and surviving replication stress.
Results: A reduction of pre-RCs caused accumulation of Rhp54/Rad54 foci when progression of replication was hindered.
Conclusion: An abundance of pre-RCs facilitates recombinational repair under replication stress.
Significance: This study provides a link between pre-RCs and DNA recombination in S phase.

Mcm2–7 complexes are loaded onto chromatin with the aid of Cdt1 and Cdc18/Cdc6 and form prereplicative complexes (pre-RCs) at multiple sites on each chromosome. Pre-RCs are essential for DNA replication and surviving replication stress. However, the mechanism by which pre-RCs contribute to surviving replication stress is largely unknown. Here, we isolated the fission yeast *mcm6-S1* mutant that was hypersensitive to methyl methanesulfonate (MMS) and camptothecin (CPT), both of which cause forks to collapse. The *mcm6-S1* mutation impaired the interaction with Cdt1 and decreased the binding of minichromosome maintenance (MCM) proteins to replication origins. Overexpression of Cdt1 restored MCM binding and suppressed the sensitivity to MMS and CPT, suggesting that the Cdt1-Mcm6 interaction is important for the assembly of pre-RCs and the repair of collapsed forks. MMS-induced Chk1 phosphorylation and Rad22/Rad52 focus formation occurred normally, whereas cells containing Rhp54/Rad54 foci, which are involved in DNA strand exchange and dissociation of the joint molecules, were increased. Remarkably, G_1 phase extension through deletion of an S phase cyclin, Cig2, as well as Cdt1 overexpression restored pre-RC assembly and suppressed Rhp54 accumulation. A *cdc18* mutation also caused hypersensitivity to MMS and CPT and accumulation of Rhp54 foci. These data suggest that an abundance of pre-RCs facilitates a late step in the recombinational repair of collapsed forks in the following S phase.

A faithful replication of chromosomal DNA is essential for maintaining genetic information through proliferation for all living cells. A failure in this process leads to genome instability, resulting in cell death or a predisposition to cancer in multicellular organisms. The minichromosome maintenance (MCM)[5] complex, composed of Mcm2–7 subunits, is an evolutionarily conserved DNA helicase that is essential for both initiation and elongation of DNA replication (1, 2). Prior to entry into S phase, MCM complexes are loaded onto replication origins, the chromosomal loci where origin recognition complexes (ORCs) are present (3). Both Cdt1 and Cdc6 (called Cdc18 in fission yeast) are required for the loading of MCM onto the origins to form prereplicative complexes (pre-RCs). Cdt1 forms a stoichiometric complex with MCM, and the Cdt1-MCM complex binds to the Cdc6-ORC complex that is preassembled on origin DNA (4–8). Mcm2–7 form double hexameric rings at the origin with a head-to-head configuration (5, 6) consistent with the idea that the MCM helicase is the key player in bidirectional replication from an origin. After entry into S phase, additional factors, including Cdc45 and the Go-Ichi-Ni-San (GINS) complex, are recruited to pre-RCs, leading to the initiation of replication (9–11). To prevent multiple rounds of replication in a single cell cycle, the assembly of the pre-RC is strongly prohibited after entry to S phase through multiple mechanisms, including cell cycle-dependent transcriptional regulation and the ubiquitin-mediated degradation of Cdt1 and Cdc6/Cdc18 (12–19).

Genome-wide studies in fission and budding yeasts have identified hundreds of replication origins on which pre-RCs are assembled (20, 21). However, DNA replication initiates from only a subset of these origins. Neither the efficiency nor the timing of replication initiation is identical between the origins in a nucleus. In fission yeast, the origins are used at most once every two cell cycles, and many operate only once every five cell cycles (22). The origins that support a high incidence of repli-

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[5] The abbreviations used are: MCM, minichromosome maintenance; RC, replicative complex; MMS, methyl methanesulfonate; CPT, camptothecin; ORC, origin recognition complex; ARS, autonomously replicating sequence; DSB, double strand break; HU, hydroxyurea; HR, homologous recombination; SDSA, synthesis-dependent strand annealing; YE, yeast extract; EMM, *Edinburgh* minimal medium; BIR, break-induced replication.

cation initiation in early S phase are called early origins, whereas those that do not are called late or dormant origins. However, essentially all of the types of origins exhibit autonomously replicating sequence (ARS) activity when present on an episomal plasmid. In animals, a set of origins that fire in early S phase differs across development and between cell types (23), showing plasticity in the choice of origins. It appears that the regulation of origin firing is important for the normal development of humans as recent studies have found that mutations in CDT1, CDC6, or ORCs cause Meier-Gorlin syndrome (24–26), which is a rare autosomal recessive genetic condition, the primary clinical hallmarks of which include small stature, small external ears, and small or absent patellae.

The progression of replication forks is challenged by different types of structural and chemical alterations in DNA (27, 28). Methyl methanesulfonate (MMS) treatment of cells produces N^7-methylguanine and N^3-methyladenine, which physically impede fork progression, resulting in the formation of DNA double strand breaks (DSBs) (29). A classical anticancer drug, camptothecin (CPT), stabilizes DNA-topoisomerase I complexes by preventing the religation step of the topoisomerase I reaction, resulting in single-stranded nicks (30). These nicks are converted to DSBs when replication forks encounter them. In contrast to the DSBs created by DNA endonucleases, those that are formed in MMS- or CPT-treated cells appear to be one-ended DSBs in which the second end of the DSB is missing. Another type of replication inhibitor, hydroxyurea (HU), blocks fork progression through a different mechanism. Instead of causing DNA lesions, HU primarily blocks fork progression by decreasing dNTP levels via the inhibition of ribonucleotide reductase (31). Different structural constraints imposed by different replication stresses result in the activation of diverse responses. The proper response to replication stress is crucial for pursuing faithful replication of the whole genome.

An increasing body of evidence has shown that the MCM complex plays an important role in surviving replication stress. Reduced expression levels of or hypomorphic mutations in MCM cause hypersensitivity to replication stress, elevated rates of gross chromosomal rearrangement, and a predisposition to cancer in animals (32–37). Furthermore, the fragility of the *FRA3B* region, the most common fragile chromosome site in human lymphocytes, has been shown to rely on a lack of replication initiation (38), underlining the importance of multiple pre-RCs within a given interval of a chromosome. Under the stressed condition, dormant origins near DSBs become activated to ensure that the entire region of the chromosome is replicated (39, 40). However, how the replication fork from the dormant origin affects the repair of DSBs is largely unknown.

Homologous recombination (HR), which is performed by the Rad52 epistasis group of proteins in yeasts, is the major pathway for the repair of DSBs produced by the collapse of replication forks (41). Rad52 and Rad54 function at the early and late steps in HR, respectively. Rad52 mediates Rad51 nucleoprotein filament formation on the single-stranded tails of DSBs (42–48). The breast cancer susceptibility gene product BRCA2 is a recombination mediator like Rad52 in yeasts (49, 50), implicating the recombinational repair mechanism in tumor suppression. The Rad51 nucleoprotein filament performs a homology search and DNA strand exchange with the donor strand (51, 52). Rad54, a member of the SWI/SNF chromatin-remodeling complex, exhibits many biochemical properties and acts at multiple stages during recombination (53, 54). Rad54 stimulates the Rad51-mediated strand exchange reaction (55). Intriguingly, Rad54 also dissociates the joint molecule by branch migration (56). This apparent antirecombination activity may displace the invading DNA strand from the donor strand following a portion of repair synthesis, stimulating the synthesis-dependent strand annealing (SDSA) mode of DSB repair (54, 57).

To gain insights into the repair of collapsed replication forks, we isolated the fission yeast *mcm6-S1* mutant that was hypersensitive to both MMS and CPT. The overexpression of Cdt1 or Cdc18 suppressed the sensitivity, suggesting that the assembly of a large number of pre-RCs is important for the repair of collapsed replication forks. Consistent with this, the *mcm6-S1* mutation impaired the interaction of Mcm6 with Cdt1 and decreased the number of pre-RCs formed. Although checkpoint activation and the formation of nuclear foci containing Rad22 (the Rad52 homolog in fission yeast) were induced normally in response to MMS treatment, *mcm6-S1* cells accumulated nuclear foci containing Rhp54 (the Rad54 homolog in fission yeast), indicating a specific defect in the late step of HR. Importantly, the overexpression of Cdt1 or the extension of G_1 phase through the deletion of an S phase cyclin, Cig2, suppressed the Rhp54 accumulation. Furthermore, a mutation in the MCM loader Cdc18 also caused hypersensitivity to MMS and CPT and Rhp54 accumulation. These data suggest that the assembly of many pre-RCs facilitates the late step in the recombinational repair of collapsed forks. We propose a model in which the forks converging at one-ended DSBs facilitate the late step in SDSA by providing a second DSB end.

EXPERIMENTAL PROCEDURES

Fission Yeast Strains and Media—The yeast strains used in this study are listed in Table 1. Yeast media were prepared, and standard genetic procedures were conducted as described previously (58). Yeast transformation was performed using the lithium acetate method (59). Centrifugal elutriation was performed as described previously (60).

Yeast Two-hybrid Assay—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 activation domain on the pGADT7 plasmid or the GAL4 DNA-binding domain on the pGBKT7 plasmid and expressed in the AH109 reporter strain of *Saccharomyces cerevisiae*. The transformants harboring both the pGADT7 and the pGBKT7 plasmids were selected on synthetic dextrose minimum medium lacking tryptophan and leucine (SD-WL). Medium lacking tryptophan, leucine, histidine, and adenine (SD-WLHA) was used to identify positive interactions.

PCR-mediated Mutagenesis of mcm6 Gene—The pTN579 plasmid containing the *mcm6-kan* construct was created as follows. To introduce the BglII and EcoRI sites just after the stop codon of the *mcm6*$^+$ gene, the flanking regions were amplified from the pTL-mcm6 plasmid, which harbors the *mcm6*$^+$ ORF with a 0.2-kb downstream region, by PCR using the mcm6-c

TABLE 1
Fission yeast strains used in this study

Strain	Genotype
TNF34	*h*$^-$
TNF35	*h*$^+$
TNF154	*h*$^-$, *leu1–32*
TNF256	*h*$^-$, *cds1::kanMX6*
TNF421	*h*$^+$, *chk1–9Myc2HA6His:ura4*$^+$
TNF598	*h*$^-$, *mcm4-c84:kanMX6*
TNF752	*h*$^-$, *mcm6-S1:kanMX6*
TNF623	*h*$^-$, *leu1–32, chk1::ura4*$^+$
TNF851	*h*$^-$, *leu1–32, mcm6-S1:kanMX6*
TNF1416	*h*$^-$, *cdt1–9Myc*
TNF1982	*h*$^-$, *cdt1–9Myc, mcm6-S1:kanMX6*
TNF1158	*h*$^-$, *chk1::kanMX6*
TNF1990	*h*$^+$, *chk1–9Myc2HA6His:ura4*$^+$, *mcm6-S1:kanMX6*
TNF3075	*h*$^-$, *cig2::hphMX6*
TNF3078	*h*$^-$, *cig2::hphMX6, mcm6-S1:kanMX6*
TNF3087	*h*$^-$, *rad22-GFP:hphMX6*
TNF3217	*h*$^-$, *rad22-GFP:hphMX6, mcm6-S1:kanMX6*
TNF3597	*h*$^-$, *cig2::hphMX6, chk1::kanMX6*
TNF3718	*h*$^+$, *rhp54::kanMX6*
TNF3945	*h*$^-$, *smt0, leu1–32, rhp54-GFP:hphMX6*
TNF3946	*h*$^-$, *smt0, leu1–32, mcm6-S1:kanMX6, rhp54-GFP:hphMX6*
TNF3851	*h*$^-$, *rhp54-GFP:hphMX6*
TNF3858	*h*$^-$, *rhp54-GFP:hphMX6, mcm6-S1:kanMX6*
TNF4029	*h*$^-$, *rhp54-GFP:hphMX6, cig2::hphMX6*
TNF4030	*h*$^-$, *rhp54-GFP:hphMX6, cig2::hphMX6, mcm6-S1:kanMX6*
TNF4035	*h*$^+$, *rhp54-GFP:hphMX6*
TNF4037	*h*$^-$, *smt0, leu1–32, rhp54-GFP:hphMX6, rhp51::kanMX6*
TNF4038	*h*$^-$, *smt0, leu1–32, rhp54-GFP:hphMX6, rad22::kanMX6*
HM253	*h*$^-$, *cdc18-K46*
TNF4168	*h*$^-$, *cdc18-K46, rhp54-GFP:hphMX6*

(5′-GTAGATGGAGTACCAGCGGGAGACC) and mcm6-BgRI 5′-CCGAATTCGAGATCTTAGTTCGGAACATCGCCATTGC) primer pair and the mcm6-RI (5′-CGAATTCGGTTATAAAAGATTCGTAACGATCTCTTATAAG) and Rev (5′-AACAGCTATGACCATG) primer pair. The position of the restriction sites created is underlined. The amplified fragments were mixed and reamplified using the mcm6-c and Rev primers. A 0.3-kb MluI-BamHI restriction fragment containing the BglII and EcoRI sites was introduced between the MluI and BamHI sites of pTL-mcm6, creating pTN576. DNA sequencing of pTN576 confirmed no additional mutations. A 0.4-kb XhoI-SmaI fragment from pTN576 was introduced between the XhoI and the SmaI sites of pBluescriptII KS+ (Stratagene, Agilent Technologies, Santa Clara, CA), creating pTN577. A 1.4-kb BglII-EcoRI fragment containing the *kanMX6* gene from pFA6a-kanMX6 (59) was introduced between the BglII and the EcoRI sites of pTN577, creating pTN578. A 1.7-kb BglII-SmaI fragment containing *kanMX6* and the *mcm6* downstream region from pTN578 was introduced between the BglII and the SmaI sites of pTL-mcm6, creating pTN579. A 4.2-kb region of pTN579 that contained the *mcm6-kan* construct was amplified using AmpliTaq polymerase (Roche Applied Science) in the presence of 0.5 mM $MnCl_2$ to increase the chance of base misincorporation. Yeast cells transformed with the PCR product were selected on YE plates supplemented with 100 μg/ml G418 (Nacalai Tesque, Kyoto, Japan) and examined for sensitivity to MMS, CPT, and HU (Sigma). The *cig2* gene was replaced with *hphMX6* by a PCR method using the pFA6a-hphMX6 plasmid (61), and the *cig2* deletion strain was selected on the medium supplemented with 100 μg/ml hygromycin B (Wako, Osaka, Japan).

Fluorescence Microscopy—The *rad22-GFP* and *rhp54-GFP* strains were constructed as described previously (59). Yeast cells expressing Rad22-GFP or Rhp54-GFP were harvested and suspended in Milli-Q water. The cells were treated with Hoechst 33342 (Dojindo, Kumamoto, Japan) at room temperature to visualize DNA. The GFP and Hoechst 33342 signals were detected using a fluorescence microscope (Axiovert 135M, Zeiss) with a 100× objective (numerical aperture, 1.4). Images were obtained using a charge-coupled device camera (Retiga-Exi, QImaging, Surrey, British Columbia, Canada) and processed using IPLab (Scanalytics, Rockville, MD) and Photoshop (Adobe) software.

Chromatin Immunoprecipitation—Chromatin immunoprecipitation (ChIP) analysis was performed as described previously (62). Rabbit antiserum raised against Mcm6, Mcm2, or Orc4 was used (63). To amplify ars2004, 5′-CGGATCCGTAATCCCAACAA and 5′-TTTGCTTACATTTTCGGGAACTTA oligonucleotide primers were used in PCR. To amplify ars2060, 5′-TTCAGGGCTCAAAGTTAGAAAAATCAAGT and 5′-CCCGAAATTGCACGGATAGTATAATT were used. To amplify the non-ars (*adl1*) region, 5′-AAATATGGCGATCCAGGAGATG and 5′-GCTTAACGTGCGCACAGACA were used.

Western Blotting—In total, 1.0×10^8 yeast cells were collected, washed with 300 μl of 10% trichloroacetic acid (TCA), frozen using liquid nitrogen, and stored at −80 °C before use. The cells were then resuspended in 150 μl of 10% TCA. After the addition of an equal volume of acid-washed glass beads to the cell suspension, the cells were disrupted using a bead beater (Micro Smash MS-100, TOMY, Tokyo, Japan), and the cell lysate was recovered using centrifugation. The glass beads were washed with 250 μl of 5% TCA. The total cell lysate was incubated on ice for 30 min, and the pellet was recovered after centrifugation at 2,300 × *g* for 10 min at 4 °C. The pellets were suspended in 200 μl of elution buffer (1 M Tris base, 5% β-mercaptoethanol, 2% SDS, 10% glycerol, 62.5 mM Tris-HCl (pH 6.8), and 0.005% bromophenol blue). The lysate was boiled for 2 min and centrifuged at 13,000 × *g* for 10 min at 25 °C. The supernatant was recovered and applied to SDS-PAGE. Immunostaining was performed essentially as described previously (62). To detect Chk1-HA, a mouse monoclonal antibody against the HA tag (16B12, Abcam, Cambridge, MA) (1:2,000) and peroxidase AffiniPure goat anti-mouse IgG (heavy + light) (catalog number 115-035-146, Jackson ImmunoResearch Laboratories, West Grove, PA) (1:10,000) were used as the primary and secondary antibodies, respectively. To detect Rhp54-GFP, Cdt1-Myc, and Mcm6, full-length *Aequorea victoria* polyclonal antibody (632460, Clontech) (1:1,500), a rabbit polyclonal anti-Myc tag antibody (Medical and Biological Laboratories, Nagoya, Japan) (1:1,000), and affinity purified anti-Mcm6 rabbit antibody (1:20), respectively, were used as the primary antibodies. Peroxidase AffiniPure goat anti-rabbit IgG (heavy + light) (catalog number 111-005-003, Jackson ImmunoResearch Laboratories) (1:10,000) was used as the secondary antibody. The blots were developed using SuperSignal West Pico Chemiluminescent Substrate or Femto Maximum Sensitivity Substrate (catalog number 34080 or 34095, ThermoScientific, Kanagawa, Japan) and exposed to RX-U films (Fujifilm, Tokyo, Japan).

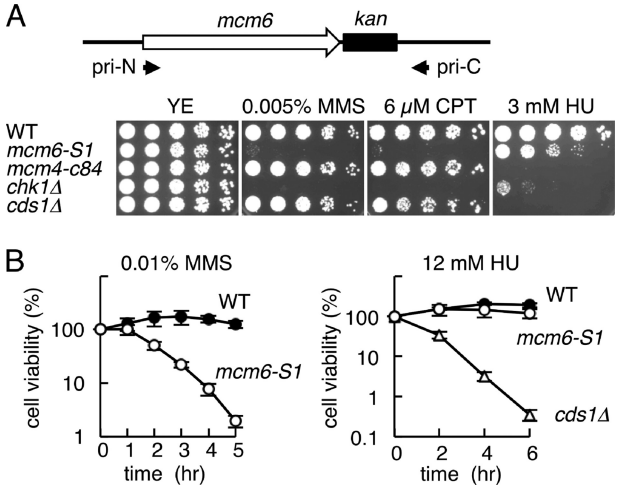

FIGURE 1. ***mcm6-S1* mutant is hypersensitive to MMS and CPT.** *A*, isolation of the *mcm6-S1* mutant. The *mcm6*$^+$*-kan* construct in the pTN579 plasmid is illustrated. The *arrowheads* indicate the positions of the pri-N (5′-GGGGACAGGCCAACTGTAAGTATGCC) and pri-C (5′-GGGTTTCTACGGTATGCAGTGAGG) primers. Log phase cultures of the wild type (WT), *mcm6-S1*, *mcm4-c84*, *chk1*Δ, and *cds1*Δ cells (TNF34, TNF752, TNF598, TNF1158, and TNF256, respectively) in EMM were serially diluted 5-fold with distilled water and spotted onto YE supplemented with MMS, CPT, or HU at the indicated concentrations. *B*, after exposure to MMS or HU, aliquots of the cultures were withdrawn at the indicated time points, diluted with distilled water, and plated onto YE. The plates were incubated for 3–5 days at 30 °C. The cell viability relative to the 0-h time point is shown. The data represent the average ± S.D. ($n = 3$).

Immunoprecipitation—In total, ~5 × 10^8 cells were collected 40 min after the incubation in EMM following the centrifugal elutriation. The cells were washed with ice-cold water and then with 1 ml of lysis buffer (50 mM Tris-HCl (pH 8.0), 140 mM NaCl, 1 mM EDTA, 0.1% sodium deoxycholate, 10% glycerol, 1 mM DTT, and 1 mM PMSF), and then the cells were suspended in 0.2 ml of lysis buffer. After the addition of 5 μl of protease inhibitor mixture (Sigma) to the cell suspension, cells were disrupted with acid-washed glass beads by a bead beater (Micro Smash MS-100, TOMY) for 30 s four times with ≥1-min intervals on ice. After the addition of 20% Triton X-100 to a final concentration of 1%, whole cell extracts were obtained by centrifugation at 20,440 × *g* for 10 min. The protein concentration was determined by the method of Bradford using a Bio-Rad protein assay kit. 1 mg of whole cell extract was incubated at 4 °C for 2 h with protein A magnetic beads (Invitrogen Dynal) that were conjugated with affinity-purified anti-Mcm6 rabbit antibodies or with the mock beads. The beads were washed three times with 400 μl of lysis buffer supplemented with 1% Triton X-100 and then suspended in 40 μl of Laemmli buffer (62.5 mM Tris-HCl (pH 6.8), 25% glycerol, 2% SDS, 0.01% bromophenol blue, and 5% β-mercaptoethanol).

RESULTS

Isolation of mcm6-S1 Mutant That Is Hypersensitive to MMS and CPT—To study the role of MCM in surviving the replication stress that causes fork collapses, we isolated *mcm6* mutants of fission yeast that were hypersensitive to MMS. The *mcm6-kan* construct was created on a plasmid, amplified by error-prone PCR using pri-N and -C primers (Fig. 1*A*), and introduced into yeast cells. From the ~700 G418-resistant transformants, the *mcm6-S1* mutant that exhibited hypersensitivity to MMS was isolated. To examine the specificity of replication stress, cells were plated on YE medium supplemented with MMS, CPT, or HU (Fig. 1*A*). Both MMS and CPT cause the formation of DSBs when the replication fork passes damage sites, whereas HU causes fork stalling. As expected from previous studies (64), a deletion of the damage checkpoint kinase Chk1 resulted in hypersensitivity to MMS and CPT, whereas the deletion of the replication checkpoint kinase Cds1 did not affect the sensitivity to these agents compared with wild type, demonstrating that treatment with MMS and CPT initiates the damage checkpoint pathway. The *mcm4-c84* mutant in which 84 C-terminal residues were eliminated is hypersensitive to HU but not to MMS or CPT (62). In contrast to the *mcm4-c84* mutant, the *mcm6-S1* mutant was hypersensitive to MMS and CPT, suggesting that the repair of collapsed forks is impaired in the *mcm6-S1* mutant. *mcm6-S1* cells were only slightly sensitive to chronic exposure to HU. This may be due to DSBs that are formed when cells are exposed to HU for a long period of time (65, 66). Remarkably, ≥90% of *mcm6-S1* cells became inviable after 4 h of treatment with an acute dose of MMS, although no such effect was observed in response to HU treatment (Fig. 1*B*). These results show that Mcm6 is important for surviving the replication stress that creates DSBs.

Suppression of Hypersensitivity to MMS or CPT of mcm6-S1 Cells by Overexpression of Cdt1 or Cdc18—To obtain insights into the mechanism through which Mcm6 contributes to surviving replication stress, we searched for genes that suppressed the MMS sensitivity of *mcm6-S1* cells when present on multicopy plasmids. The yeast genomic library was introduced into *mcm6-S1* cells, and the transformants that were less sensitive to MMS were selected. We found that the plasmids recovered from these less sensitive clones contained the *cdt1*$^+$ or the *cdc18*$^+$ gene, both of which are involved in the loading of MCM complexes onto the replication origins. A serial dilution assay showed that the overexpression of Cdt1 or Cdc18 suppressed the sensitivity of *mcm6-S1* cells to MMS and CPT (Fig. 2*A*). However, neither of the genes suppressed the sensitivity of *chk1*Δ cells, indicating a specific suppression of the *mcm6-S1* mutant by Cdt1 and Cdc18.

mcm6-S1 Mutation Impairs Interaction of Mcm6 with Cdt1—To examine the physical interaction between Mcm6 and the MCM loaders Cdt1 and Cdc18, we performed yeast two-hybrid assays (67). When the proteins interact, the *HIS3* and *ADE2* reporter genes are transcribed, allowing growth on selective SD-WLHA plates that lack histidine and adenine (Fig. 2*B*). The yeast cells expressing Mcm6 and Cdt1 from the pGADT7 and pGBKT7 vectors, respectively, grew well on SD-WLHA plates. However, those expressing Mcm6 and Cdc18 only grew on nonselective SD-WL plates, suggesting that Mcm6 binds directly to Cdt1 but not to Cdc18. The suppression of *mcm6-S1* cells by Cdc18 overexpression may be due to the increased assembly of Cdc18-ORC, which in turn promotes the loading of MCM-Cdt1 onto origins. To see whether the *mcm6-S1* mutation affects Mcm6 interaction with Cdt1, we first performed DNA sequencing to identify the *mcm6-S1* mutation sites and found three base substitutions: one was silent, whereas the rest caused amino acid changes in the acidic domain of the C-terminal region (Fig. 2*C*; E824G and D825N). Unlike wild type

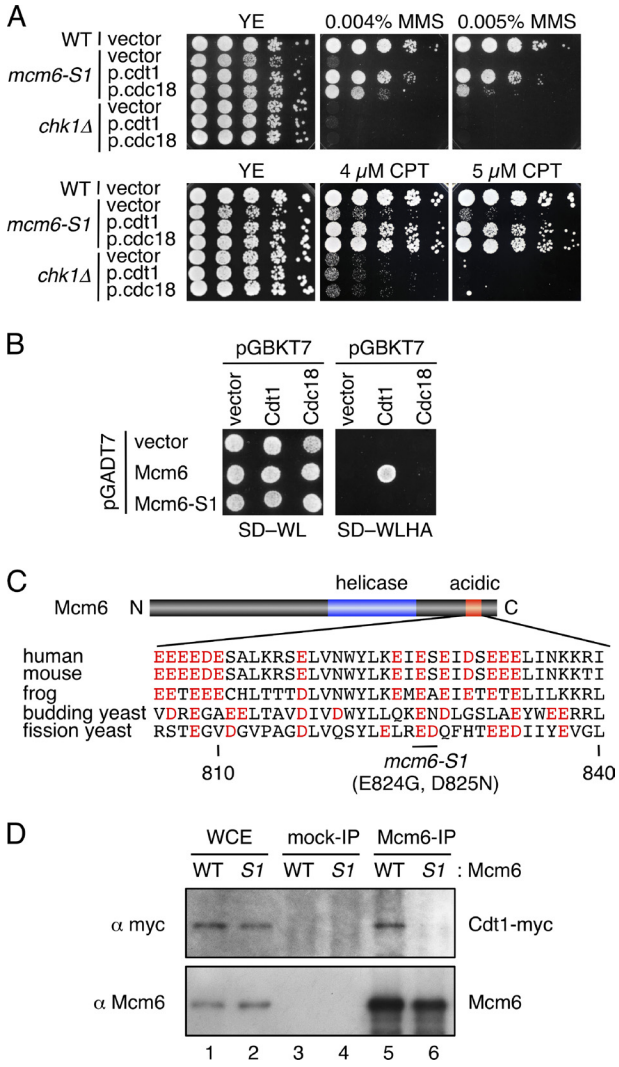

FIGURE 2. **Genetic and physical interactions between Mcm6 and Cdt1.** *A*, overexpression of Cdt1 or Cdc18 suppresses the sensitivity of *mcm6-S1* cells to MMS or CPT. WT, *mcm6-S1*, and *chk1*Δ cells (TNF154, TNF851, and TNF623, respectively) were transformed with the indicated plasmid (pXB940B (*vector*), pTN626 (*p.cdt1*), or pTN627 (*p.cdc18*)), grown to log phase in EMM, serially diluted 5-fold, and spotted onto YE plates supplemented with the indicated concentrations of MMS or CPT. The plates were incubated for 3–5 days at 30 °C. *B*, two-hybrid interactions between Mcm6 and Cdt1. The budding yeast strain AH109 was transformed with the pGBKT7 vector and its derivatives expressing Cdt1 (pTN666) or Cdc18 (pTN716) in combination with the pGADT7 vector and its derivatives expressing wild type Mcm6 (pTN676) or mutant Mcm6-S1 (pTN737). The transformants were grown in SD-WL medium to log phase, spotted onto SD-WL and SD-WLHA plates, and incubated for 3 days at 30 °C. *C*, the *mcm6-S1* mutation sites. The relative positions of the helicase and the acidic domains of Mcm6 are illustrated. The acidic domains of Mcm6 from different organisms are aligned. Acidic residues are shown in *red*. The *mcm6-S1* mutant contained one silent mutation (C2406A; position 1 is "A" of the first ATG) and two transition mutations (A2471G and G2473A) that resulted in the amino acid changes indicated below the alignment. *D*, coimmunoprecipitation of Mcm6 and Cdt1-Myc from yeast extracts. WT and *mcm6-S1* (*S1*) cells (TNF1416 and TNF1982, respectively) that express the Myc-tagged Cdt1 protein from the original genomic locus (68) were collected at 40 min after centrifugal elutriation to obtain G_1 cells. 0.5 μg of whole cell extract (*WCE*) and 4 μl each of the mock and the Mcm6 immunoprecipitates (*mock-IP* and *Mcm6-IP*) (see "Experimental Procedures") were applied to 10% SDS-PAGE (acrylamide:bisacrylamide, 59:1), transferred onto an Immobilon-P transfer membrane (Millipore), and immunostained with anti-Myc to detect Cdt1-Myc (*top panel*). The same membrane was then restained with anti-Mcm6 antibodies (*bottom panel*).

Mcm6, the mutant Mcm6-S1 did not bind to Cdt1 in the two-hybrid assay (Fig. 2*B*), suggesting that the *mcm6-S1* mutation impairs the Mcm6-Cdt1 interaction. To determine the effect of the *mcm6-S1* mutation on the interaction with Cdt1 in fission yeast cells, we immunoprecipitated Mcm6 from the yeast extracts prepared from the strains that express Myc-tagged Cdt1 (68). Cdt1-Myc and Mcm6 were detected by immunostaining using anti-Myc and anti-Mcm6 antibodies, respectively (Fig. 2*D*). In the case of wild type, Cdt1-Myc was specifically detected in the Mcm6 immunoprecipitate but not in the mock immunoprecipitate (Fig. 2*D*, *lanes 3* and *5*). In the case of *mcm6-S1*, however, Cdt1-Myc was hardly detected in the Mcm6 immunoprecipitate (Fig. 2*D*, *lane 6*). These data show that the *mcm6-S1* mutation impairs the interaction between Mcm6 and Cdt1.

Role of Mcm6-Cdt1 Association in Formation of Pre-RCs—To test whether the *mcm6-S1* mutation affects pre-RC assembly, we performed ChIP assays to evaluate the binding of Mcm6 and Mcm2 to the replication origins (21, 69). Relatively small G_2 cells were collected by centrifugal elutriation, and then they were incubated in the presence of HU to obtain early S phase cells. The accumulation of cells with 1c DNA content was confirmed by fluorescence-activated cell sorter (FACS) analysis (Fig. 3*A*). The percentage of DNA recovered in precipitates was determined using quantitative PCR. In wild type cells, the enrichment of Mcm6 binding to an efficient early origin, ars2004, and a late/dormant origin, ars2060 (70, 71), was specifically observed in early S phase ($t = 80$ and 100 min) (Fig. 3*B*, *top panels*). In *mcm6-S1* cells, however, Mcm6 binding to these origins was decreased as compared with the wild type. It should be noted that the anti-Mcm6 antibodies used here were raised against the N-terminal region of Mcm6 (from amino acids 43 to 458) (69). Thus, it is unlikely that the *mcm6-S1* (E824G and D825N) mutation greatly reduces the antibody affinity to the Mcm6 protein. The decreased binding to origins was not limited to Mcm6; Mcm2 binding to ars2004 and ars2060 was also decreased in *mcm6-S1* cells (Fig. 3*B*, *bottom panels*). In contrast to Mcm proteins, similar levels of specific binding to ars2004 were observed for Orc4 in wild type and *mcm6-S1* cells (Fig. 3*C*). Importantly, the overexpression of Cdt1 rescued pre-RC assembly in *mcm6-S1* cells (Fig. 3*D*). These data implicate the Mcm6-Cdt1 association in the loading of Mcm2–7 onto replication origins. Consistent with a limited number of pre-RCs, the doubling time of *mcm6-S1* cells was increased compared with that of wild type cells (*mcm6-S1*, 3.46 ± 0.09 h; wild type, 2.65 ± 0.04 h in EMM at 30 °C).

Chk1 Activation and Rad22 Focus Formation in mcm6-S1 Cells—When DSBs are formed, the Chk1 kinase is phosphorylated, which is dependent on the upstream checkpoint kinase Rad3, the ataxia telangiectasia and Rad3-related (ATR) kinase homolog, and relays the checkpoint signal to downstream targets (72). To determine whether the *mcm6-S1* mutation affects the checkpoint response to MMS, HA-tagged Chk1 expressed in yeast cells was separated by SDS-PAGE and immunostained using an anti-HA antibody (Fig. 4*A*). In wild type cells, a slowly migrating band corresponding to the phosphorylated form of Chk1-HA was observed after the treatment of cells with 0.01% MMS for 4 h. A similar level of Chk1 phosphorylation was

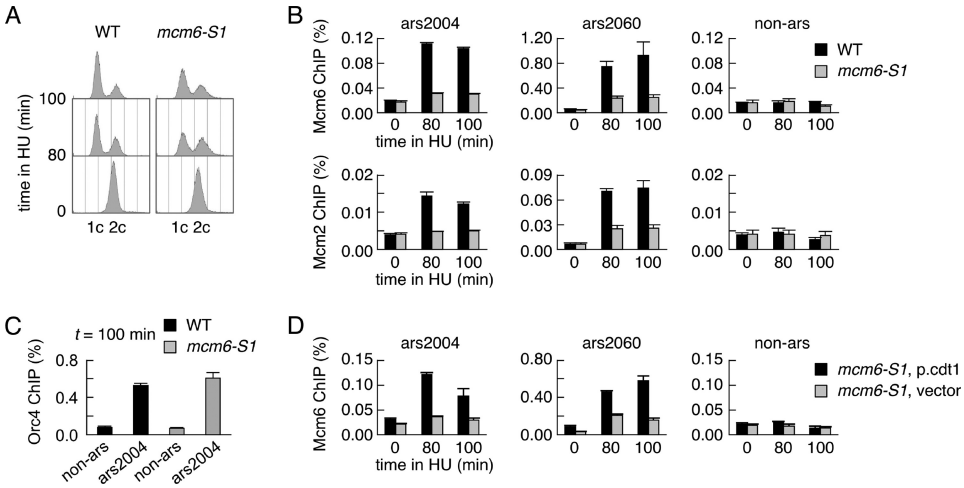

FIGURE 3. ***mcm6-S1* mutation impairs pre-RC assembly.** *A*, the DNA contents of the cells were examined by FACS analysis. Small G_2 cells of WT and *mcm6-S1* strains (TNF34 and TNF752, respectively) were collected by centrifugal elutriation, and cells were released into fresh EMM containing 10 mM of HU to block cell cycle progression in early S phase. *B*, ChIP was performed using anti-Mcm6 or anti-Mcm2 serum. The percentage of the recovered DNA relative to the input was determined by quantitative PCR. Mcm6 binding to ars2004, ars2060, and non-ars (*adl1*) is indicated in the *upper panels*, and Mcm2 binding is shown in the *lower panels*. The data represent the average ± S.E. ($n = 3$). *C*, using anti-Orc4 serum, the specific binding of Orc4 to a replication origin in early S phase ($t = 100$ min) was detected in WT and *mcm6-S1* cells. The data represent the average ± S.E. ($n = 2$). *D*, the overexpression of Cdt1 increased pre-RCs in *mcm6-S1* cells. Mcm6 binding to replication origins was examined in *mcm6-S1* (TNF851) cells harboring the pTN626 (*p.cdt1*) plasmid or an empty vector, pXB940B. The data represent the average ± S.D. ($n = 3$) of the three PCR measurements.

observed in *mcm6-S1* cells, suggesting that the checkpoint activation in response to DSBs occurs normally. A small fraction of Chk1 appeared to be activated even in the absence of MMS, suggesting that a limited number of replication initiation events increase the chance of spontaneous fork collapse.

HR is the major pathway for the repair of DSBs. Rad52 forms nuclear foci at DSB sites and mediates Rad51 assembly onto single-stranded DNA. The focus formation of Rad52 appears to be one of the initial events of HR because it occurs independently of Rad51 (43, 73). To determine whether the *mcm6-S1* mutation affects the early step of HR, we used cells expressing GFP-tagged Rad22 (the Rad52 homolog in fission yeast) from its native chromosomal locus and observed the nuclear foci of Rad22-GFP using fluorescence microscopy. As reported previously, cells containing one or two foci were occasionally observed even in the absence of MMS in wild type cells (74). Half of these appear to be due to recombination between tandem repeats of the rDNA genes (74). However, cells containing ≥3 Rad22-GFP foci appeared only after MMS treatment, and the number of foci gradually decreased after the release from MMS and disappeared completely by 14 h (Fig. 4*B*), suggesting that multiple foci are formed during the repair process. The multiple foci containing Rad22-GFP were similarly formed in the *mcm6-S1* mutant and the wild type, suggesting that the number of pre-RCs does not affect the early step of recombination. However, the disappearance of the Rad22 foci after release from MMS was slightly but significantly delayed in the *mcm6-S1* mutant compared with the wild type, suggesting a partial defect in the dissociation of Rad22-GFP from the damage sites.

Accumulation of Rhp54 Nuclear Foci in mcm6-S1 Cells—Rad54 stimulates Rad51-mediated DNA strand exchange and is also capable of dissociating the joint molecules by branch migration (54). To examine the focus formation of Rhp54 (the Rad54 homolog in fission yeast), we tagged Rhp54 with GFP at the C terminus and expressed the fusion protein from its original chromosomal locus. Compared with the *rhp54*Δ strain, the *rhp54-GFP* and the wild type strains exhibited no sensitivity to MMS or CPT (Fig. 5*A*), indicating that Rhp54-GFP is fully functional. Similar to Rad22-GFP, cells containing ≥3 Rhp54-GFP foci appeared only in the presence of MMS (Fig. 5, *B* and *D*). Importantly, the multiple Rhp54-GFP foci disappeared in the absence of Rad22 or Rhp51 (the Rad51 homolog in fission yeast), although similar levels of the Rhp54-GFP protein were detected using immunoblotting (Fig. 5, *B* and *C*), indicating that the localization of Rhp54 to the damage sites occurs in a Rad22- and Rhp51-dependent fashion. In the *mcm6-S1* mutant, the fraction of cells that contained ≥3 Rhp54-GFP foci was increased 2-fold compared with wild type after MMS treatment, and it remained at elevated levels even after the removal of MMS (Fig. 5*D*). A prominent accumulation of the Rhp54 foci as compared with the Rad22 foci suggests that the *mcm6-S1* mutation specifically impairs a late step of recombinational repair in which Rhp54 functions.

To determine whether the accumulation of Rhp54-GFP foci in the *mcm6-S1* mutant was due to the weak interaction with Cdt1, Cdt1 was overexpressed (Fig. 5*E*). Although Cdt1 overexpression showed no obvious effects on the formation of Rhp54 foci in wild type cells, it reduced cells containing ≥3 Rhp54-GFP foci to close to the wild type level in the *mcm6-S1* mutant, suggesting that the Mcm6-Cdt1 interaction is important for a late step of recombinational repair. A recent study showed in budding yeast that although ORC and Cdc6 were dispensable Mcm2–7 and Cdt1 were involved in break-induced replication (BIR), which is the mode of recombination utilized to repair a DSB when homology is restricted to one side of a DSB (75). Thus, the Mcm6-Cdt1 interaction might affect Rhp54 via BIR rather than pre-RC assembly. To address this hypothesis, we evaluated whether the deletion of an S phase cyclin, Cig2, would suppress the accumulation of the Rhp54-GFP foci. Because the

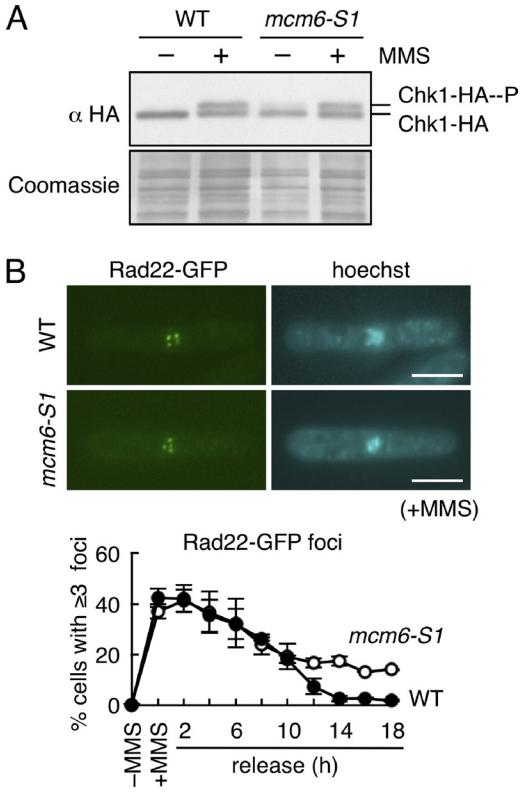

FIGURE 4. **Chk1 activation and formation of Rad22 foci in *mcm6-S1* mutant.** *A*, Chk1 phosphorylation. Extracts were prepared from WT and *mcm6-S1* cells expressing HA-tagged Chk1 (TNF421 and TNF1990, respectively) before and after treatment with 0.01% MMS for 4 h. The extracts were separated by 8.0% SDS-PAGE and transferred onto an Immobilon-P transfer membrane (Millipore). The Chk1-HA protein was detected using an anti-HA antibody, and the blot was also stained with Coomassie Brilliant Blue. *B*, the formation of Rad22-GFP foci. WT and *mcm6-S1* cells (TNF3087 and TNF3217, respectively) expressing Rad22-GFP were treated with 0.01% MMS for 4 h, washed with 10% sodium thiosulfate to neutralize MMS, and released into MMS-free EMM. *Scale bar*, 5 μm. The percentage of the cells containing ≥3 Rad22-GFP foci is shown in the graph. At least 200 cells were examined by fluorescence microscopy in each measurement. The data represent the average ± S.D. ($n = 2$).

cig2 deletion extends the period of G_1 during which pre-RC assembly occurs (76), it should suppress Rhp54 focus accumulation if pre-RCs are important for this process. First, we confirmed that the *cig2* deletion restored the Mcm6 binding to a replication origin, ars2004, in the *mcm6-S1* mutant (Fig. 5*F*). We also found that the *cig2* deletion specifically suppressed Rhp54 focus accumulation in the *mcm6-S1* mutant (Fig. 5*G*). Furthermore, the *cig2* deletion suppressed the sensitivity of *mcm6-S1* cells to MMS and CPT (Fig. 5*H*). However, the *cig2* deletion did not suppress the sensitivity of *chk1*Δ cells, indicating the specific suppression of the *mcm6-S1* mutant phenotype.

Because Mcm2–7 are required for both the initiation and elongation phases of replication, it is formally possible that the *mcm6-S1* mutant has some defect in the elongation phase. However, if the decreased number of pre-RCs we observed rather than a possible defect in the elongation causes the *mcm6-S1* phenotypes, a mutation in the factors specifically required for pre-RC assembly should cause the same phenotype. In fact, we found that a temperature-sensitive mutant of an MCM loader, Cdc18 (13) (the Cdc6 homolog in fission yeast), was hypersensitive to MMS and CPT at a semipermissive temperature of 33 °C (Fig. 6*A*). Accumulation of Rhp54 foci was also observed in the *cdc18-K46* mutant at 33 °C (Fig. 6*B*). These data suggest that an abundance of pre-RCs facilitates a late step of recombination repair in which Rhp54 functions.

DISCUSSION

In eukaryotes, the number of pre-RCs exceeds the number of actual replication initiation events. Pre-RC formation is essential not only for DNA replication but also for surviving replication stress. However, the mechanism through which an abundance of pre-RCs affects the repair of collapsed forks is largely unknown. Here, we isolated the *mcm6-S1* mutant that was hypersensitive to MMS and CPT. We found that the hypersensitivity was suppressed by the overexpression of Cdt1 or Cdc18/Cdc6. The *mcm6-S1* mutation impaired the interaction of Mcm6 with Cdt1 and reduced MCM binding to replication origins. The overexpression of Cdt1 recovered the MCM binding to origins, implicating the Mcm6-Cdt1 interaction in pre-RC assembly. Although MMS-induced Chk1 activation and Rad22/Rad52 focus formation occurred normally, the number of cells exhibiting Rhp54/Rad54 foci increased in the *mcm6-S1* mutant. The deletion of an S phase cyclin, Cig2, and Cdt1 overexpression suppressed the accumulation of Rhp54 foci. Furthermore, we found that a mutation in an MCM loader, Cdc18, caused hypersensitivity to MMS and CPT and accumulation of Rhp54 foci. These data suggest that pre-RC assembly prior to the entry into S phase facilitates the late step of recombinational repair in which Rhp54 functions.

It remains unclear which subunit of Mcm2–7 is the critical Cdt1 binding partner for pre-RC assembly. We showed an interaction between Mcm6 and Cdt1 using yeast two-hybrid and coimmunoprecipitation assays; the Mcm6-Cdt1 interaction was impaired by the *mcm6-S1* mutation that contained alterations in the acidic residues of the C-terminal region (E824G and D825N). Consistent with our findings, a recent study has shown that the acidic C-terminal region of human MCM6 adapts a winged helix-turn-helix fold and serves as the CDT1-binding domain (77). The human MCM6-CDT1 interaction is impaired by an alanine substitution at the acidic residue Glu-757 of MCM6 (77), which corresponds to one of the altered residues in the *mcm6-S1* mutant (*i.e.* Glu-824) (Fig. 2*C*), indicating an evolutionarily conserved mode of interaction between Mcm6 and Cdt1. Our ChIP analysis showed that Mcm6 and Mcm2 binding to the replication origins was decreased by the *mcm6-S1* mutation regardless of whether the origin was early or late/dormant. Importantly, Cdt1 overexpression suppressed the *mcm6-S1* defect in pre-RC assembly. Therefore, it appears that the Mcm6-Cdt1 interaction is critical for the loading of Mcm2–7 complexes onto replication origins.

In addition to pre-RC assembly, Mcm2–7 and Cdt1 appear to play some role in BIR in budding yeast (75). Although it is not known whether the Mcm6-Cdt1 interaction is essential for BIR, it is possible that a defect in BIR rather than a reduced number of pre-RCs causes the hypersensitivity of *mcm6-S1* cells to the replication stress. However, this scenario seems unlikely because although Cdc6 (the homolog of fission yeast Cdc18) is dispensable for BIR (75) we found that the sensitivity of *mcm6-S1* cells was suppressed not only by Cdt1 but also by Cdc18 overexpression.

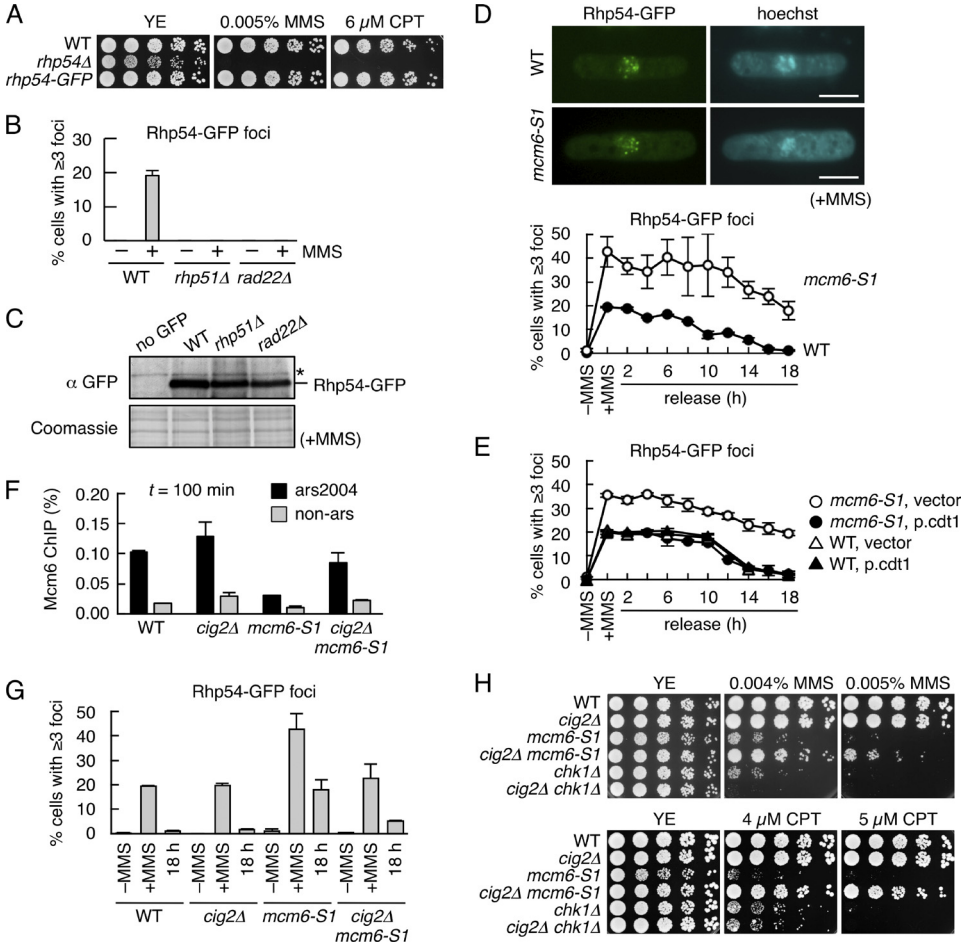

FIGURE 5. ***mcm6-S1* mutation accumulates cells containing Rhp54-GFP foci after MMS treatment.** *A*, log phase cultures of WT, *rhp54*Δ, and *rhp54-GFP* (TNF35, TNF3718, and TNF4035, respectively) cells in YE medium were serially diluted 5-fold and spotted onto YE plates supplemented with the indicated concentrations of MMS or CPT. *B*, log phase cultures of WT, *rhp51*Δ, and *rad22*Δ (TNF3945, TNF4037, and TNF4038, respectively) cells expressing Rhp54-GFP were treated with 0.01% MMS for 4 h. At least 200 cells were counted at each time point. The percentage of the cells exhibiting ≥3 Rhp54-GFP foci is shown. The data represent the average ± S.D. ($n = 2$). *C*, the extracts were prepared from WT, *rhp54-GFP*, *rhp54-GFP rhp51*Δ, and *rhp54-GFP rad22*Δ cells after treatment with MMS, separated by 5.0% SDS-PAGE, and subjected to immunostaining of Rhp54-GFP using anti-GFP antibodies. An *asterisk* indicates a nonspecific species. The blot was also stained with Coomassie Brilliant Blue. *D*, the Rhp54-GFP foci in WT and *mcm6-S1* cells (TNF3851 and TNF3858, respectively) are shown. The number of Rhp54-GFP foci per nucleus was higher than the number of Rad22-GFP foci in both WT and *mcm6-S1* cells. *Scale bar*, 5 μm. The data in the graph represent the average ± S.D. ($n = 2$). *E*, Cdt1 overexpression suppressed the accumulation of Rhp54-GFP. Rhp54-GFP foci were detected in WT and *mcm6-S1* cells (TNF3945 and TNF3946, respectively) harboring the empty vector pXB940B or pTN626 (*p.cdt1*). The cells were examined at the indicated time points. The data in the graph represent the average ± S.D. ($n = 2$). *F*, a *cig2* deletion restored pre-RC assembly in the *mcm6-S1* mutant. The binding of Mcm6 to ars2004 and non-ars (*adl1*) in early S phase ($t = 100$ min) was examined by ChIP assay using WT, *cig2*Δ, *mcm6-S1*, and *cig2*Δ *mcm6-S1* cells (TNF34, TNF3075, TNF752, and TNF3078). The data for WT and *mcm6-S1* were duplicated from Fig. 3*B*. The data represent the average ± S.E. ($n \geq 2$). *G*, a *cig2* deletion suppressed the accumulation of Rhp54-GFP foci. Rhp54-GFP foci were detected in WT, *cig2*Δ, *mcm6-S1*, and *cig2*Δ *mcm6-S1* cells (TNF3851, TNF4029, TNF3858, and TNF4030, respectively). The data represent the average ± S.D. ($n = 2$). *H*, the *cig2* deletion suppressed the sensitivity of *mcm6-S1* cells to MMS and CPT. Log phase cultures of WT, *cig2*Δ, *mcm6-S1*, *cig2*Δ *mcm6-S1*, *chk1*Δ, and *cig2*Δ *chk1*Δ (TNF34, TNF3075, TNF752, TNF3078, TNF1158, and TNF3597, respectively) cells in YE were serially diluted 5-fold and spotted onto YE plates supplemented with the indicated concentrations of MMS or CPT. The plates were incubated for 3–5 days at 30 °C.

Furthermore, the *cdc18-K46* mutant was hypersensitive to MMS and CPT. We also found that the deletion of an S phase cyclin, Cig2 (76), restored pre-RC assembly in the *mcm6-S1* mutant and suppressed the hypersensitivity to MMS and CPT. Therefore, an abundance of pre-RCs appears to be important for surviving the replication stress that causes DSB formation.

How do pre-RCs affect cellular responses to replication stress? We propose a model in which an abundance of pre-RCs facilitates the late step in the SDSA mode of recombination (57) (Fig. 7). When the replication fork encounters sites of MMS- or CPT-induced damage, DSBs are produced. Although the exact nature of DSBs resulting from the drug treatment has not been clarified, the formation of DSBs triggers the damage checkpoint response, including phosphorylation of the Chk1 kinase (78). Once replication protein A (RPA) binds to single-stranded tails of the DSB, Rad22 is recruited to the damage sites and forms nuclear foci (43). Rad22 promotes Rhp51/Rad51 loading onto the single-stranded DNA (46–48). It appears that the number of pre-RCs barely affects these early responses to the fork collapse because the phosphorylation of Chk1 and Rad22 focus formation were induced by MMS similarly in the wild type and *mcm6-S1* strains. *In vitro* studies have shown that Rad54 enhances Rad51-mediated DNA strand exchange, and Rad54 also displaces the invading strand from the joint molecules (53, 54). Consistent with these roles of Rad54 in late steps in

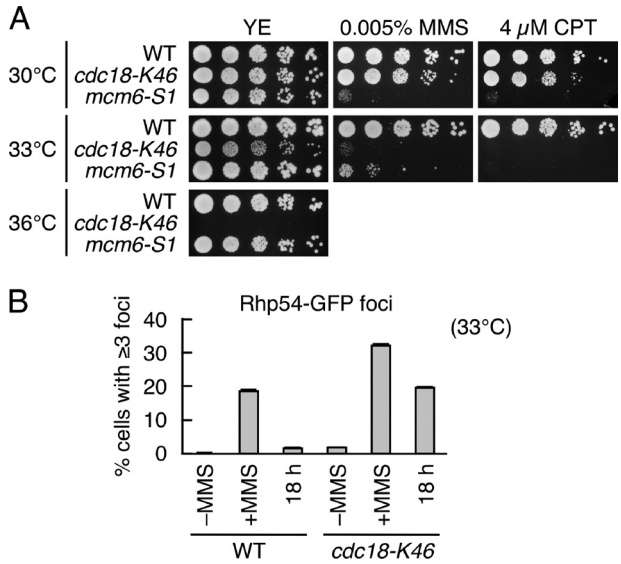

FIGURE 6. ***cdc18-K46* mutant is hypersensitive to MMS and CPT and accumulates Rhp54-GFP foci.** *A*, the *cdc18-K46* mutant is hypersensitive to MMS and CPT. WT, *cdc18-K46*, and *mcm6-S1* (TNF34, HM253, and TNF752) cells were grown at 30 °C and then spotted onto YE plates supplemented with the indicated concentration of MMS or CPT. The plates were incubated at the indicated temperatures. *B*, accumulation of Rhp54-GFP foci in the *cdc18-K46* mutant. Rhp54-GFP foci were observed using WT and *cdc18-K46* strains (TNF3851 and TNF4168, respectively) as described in Fig. 5*G* except that the cells were incubated at the semipermissive temperature of 33 °C. The data represent the average ± S.D. ($n = 2$).

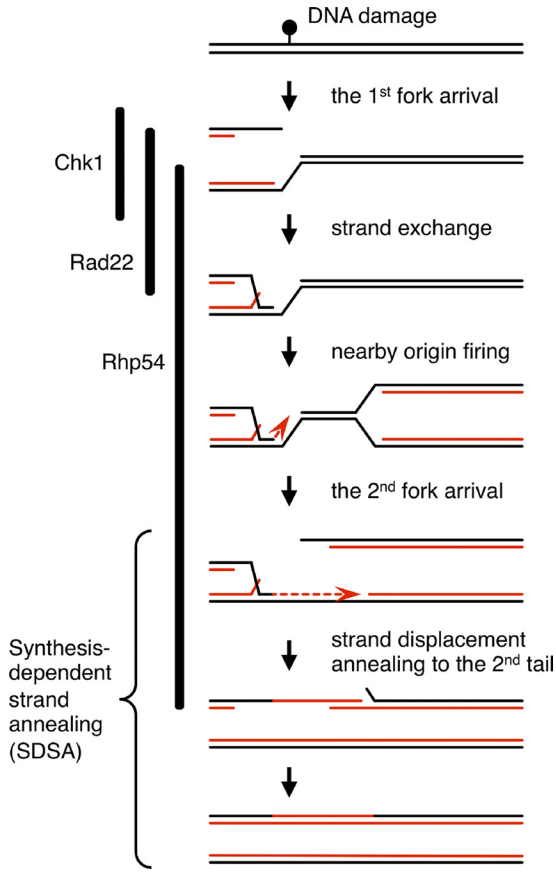

FIGURE 7. **Model showing that abundance of pre-RCs facilitates late step of recombinational repair of collapsed replication forks.** The DNA damage caused by MMS or CPT is converted to a one-ended DSB when a replication fork reaches the damage site. DSBs trigger the damage checkpoint response, including the phosphorylation of Chk1. Replication protein A (RPA) binding to the single-stranded tail of the DSB recruits Rad22. The recruitment of Rhp54 is dependent on Rad22 and Rhp51. Rhp54 stimulates DNA strand exchange mediated by Rhp51 at the early step in recombination. However, in the late steps, Rhp54 dissociates the joint molecules, which is important for the SDSA mode of DSB repair to occur. The arrival of the second fork at the damage site provides the needed second tail to which the first tail anneals. The assembly of many pre-RCs allows the formation of the second fork, thereby facilitating the late step in the recombinational repair of collapsed replication forks.

recombination, we observed that the formation of Rhp54 nuclear foci was completely dependent on Rad22 and Rhp51. Remarkably, in the *mcm6-S1* mutant, the fraction of cells exhibiting Rhp54 foci after MMS treatment increased and remained at high levels even after release from MMS. Because Cdt1 overexpression and *cig2* deletion restored pre-RC assembly and suppressed the accumulation of Rhp54 foci, it appears that an abundance of pre-RCs is important for the late step in recombination in which Rhp54 functions. A link between pre-RC and the late step in recombination was supported by the finding that the *cdc18-K46* mutant was hypersensitive to MMS and CPT and accumulated Rhp54 foci. It is assumed that the second tail of a DSB appears only when the second fork coming from a nearby origin arrives at the damage site (Fig. 7). Following strand invasion, repair synthesis, and strand displacement from the donor strand catalyzed by Rhp54, the first tail of a DSB is able to anneal to the second tail. This annealing reaction in the SDSA mode of DSB repair may allow Rhp54 to leave the damage site. The delay in the disappearance of Rad22 foci after release from MMS observed in the *mcm6-S1* mutant might be due to the partial retention and/or reassembly of Rad22 on the first tail during futile rounds of strand displacement and invasion. Consistent with the idea that the converging forks facilitate DSB repair, recent studies in mammals have shown that Rad51-dependent homologous recombination repairs collapsed forks without an apparent restart of the forks (79). Although further studies are required to understand the detailed mechanisms of the repair of collapsed forks, our data provide evidence that an abundance of pre-RCs facilitates the late step in fork repair.

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