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Minichromosome Maintenance Proteins Interact with Checkpoint and Recombination Proteins To Promote S-Phase Genome Stability▽†

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The minichromosome maintenance (MCM) complex plays essential, conserved roles throughout DNA synthesis: first, as a component of the prereplication complex at origins and, then, as a helicase associated with replication forks. Here we use fission yeast (*Schizosaccharomyces pombe*) as a model to demonstrate a role for the MCM complex in protecting replication fork structure and promoting recovery from replication arrest. Loss of MCM function generates lethal double-strand breaks at sites of DNA synthesis during replication elongation, suggesting replication fork collapse. MCM function also maintains the stability of forks stalled by hydroxyurea that activate the replication checkpoint. In cells where the checkpoint is activated, Mcm4 binds the Cds1 kinase and undergoes Cds1-dependent phosphorylation. MCM proteins also interact with proteins involved in homologous recombination, which promotes recovery from arrest by ensuring normal mitosis. We suggest that the MCM complex links replication fork stabilization with checkpoint arrest and recovery through direct interactions with checkpoint and recombination proteins and that this role in S-phase genome stability is conserved from yeast to human cells.

Replicative stress is a major source of mutations that contribute to chromosome instability and the initiation of cancer (5, 26). A primary defense against genome instability is S-phase checkpoints, which recognize DNA damage, arrest the cell cycle, and preserve essential replication structures (10, 38, 62). In response to S-phase agents such as the ribonucleotide reductase inhibitor hydroxyurea (HU), replication forks stall and activate checkpoint kinases (ATR-Chk1 in mammals and Rad3-Cds1 and Mec1-Rad53 in the yeasts *Saccharomyces pombe* and *Saccharomyces cerevisiae*, respectively) (10, 38, 62). The primary function of this replication checkpoint is to retain the assembled replisome at stalled forks during arrest: checkpoint defects lead to catastrophic replication fork collapse and cell lethality. However, recent studies have shown that proteins directly involved in DNA replication as well as recombination are required for cells to repair DNA damage, restart replication, and recover from arrest and must interact with the checkpoint to facilitate these functions (10, 38, 62).

The minichromosome maintenance (MCM) complex is an essential replicative helicase that consists of six related subunits (MCM2 to MCM7) and is required for the initiation and elongation phases of DNA synthesis (reviewed in reference 22). The MCM helicase is therefore centrally positioned to monitor and maintain genome stability at the replication fork. In budding yeast, the use of degron alleles of MCM subunits, which degrade the protein upon a shift to high temperature, has demonstrated that MCM function is required for fork progression throughout S phase as well as for synthesis to resume after forks have stalled in HU (36). In cells lacking the Rad53 protein kinase or its S-phase activator Mrc1, treatment with HU results in excessive DNA unwinding, catastrophic replication fork collapse, and an inability to restart synthesis (12, 15, 21, 55, 73). Under these conditions, MCM proteins remain chromatin bound, but the leading and lagging strands of the replication fork become uncoupled (12, 34, 55). In metazoans, MCM subunits are phosphorylated by the ATM/ATR kinases (17, 33, 67, 82), suggesting that the MCM complex may be a target or an effector of the replication checkpoint.

However, MCM proteins may also promote S-phase genome stability through checkpoint-independent roles (reviewed in references 3 and 22). Although cellular levels of MCM proteins are estimated as 10- to 40-fold excess over the number of replication origins, yeast temperature-sensitive *mcm* mutants (*mcm-ts*) that partially reduce MCM protein levels exhibit increased recombination, chromosome loss, and checkpoint sensitivity (32, 40, 41). In addition, increased expression or amplification of MCM genes is associated with many types of human cancers that are characterized by genomic instability (reviewed in reference 39) and the mutation or the dysregulation of MCM subunits can induce skin carcinoma and mammary carcinoma in mouse models (30, 68). These results suggest that the MCM complex plays a central role in protecting genome stability during S phase.

The stabilization of replisome structure and its recovery from replication arrest also depend upon proteins involved in homologous recombination (HR). In budding yeast, the Rad51 strand exchange enzyme and the Sgs1 recombination helicase are required to maintain DNA polymerase ε at stalled replication forks (16). Sgs1 is additionally involved in checkpoint

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activation (6). In fission yeast, the SpRad22 (ScRad52) recombination protein is recruited to nuclear foci immediately as cells are released from HU arrest (48). Although fission yeast *rad22* and *rhp51* (*rad51*) mutants are proficient for activation of the replication checkpoint, they still show sensitivity to HU (48, 84). Together, these results suggest that components of the replisome interact with recombination proteins to maintain and restart replication forks.

Previously, we and others have shown that most fission yeast *mcm-ts* mutants accumulate approximately 2C DNA content, but undergo a lethal, checkpoint-dependent arrest in late S phase or G_2 (18, 23, 40, 41). In contrast, a degron allele of *mcm4ts* blocks cells at or near replication initiation (42). Here, we use these different *mcm* alleles to examine the consequences to cells of inactivating MCM function during S phase in the absence or the presence of forks stalled by HU. We demonstrate that the loss of MCM function generates DNA breaks, cell cycle arrest, and a loss of viability similar to that observed in mutants that undergo replication fork collapse. Consistent with these results, we find that Mcm4 interacts with the checkpoint protein kinase Cds1 and undergoes Cds1-dependent phosphorylation in cells treated with HU. This result suggests that MCM proteins act to maintain replication fork structure both during normal S phase and during S-phase arrest.

Our data additionally suggest that MCM function is required for proper recovery from replication arrest induced by HU. We observed an interaction between the MCM complex and the HR protein Rhp51 (Rad51). Although Rhp51 and other HR proteins are not required to activate the replication checkpoint, to maintain fork structure in HU, or to restart DNA synthesis, we find that the loss of HR function results in chromosome missegregation following release from HU-induced replication arrest (this work; 48). We suggest that MCM proteins modulate replication fork progression, arrest, and restart and that they couple these functions with the repair of DNA damage to protect S-phase genome stability. Our analysis in fission yeast is complemented by studies of mammalian cells, suggesting that the role of the MCM complex in S-phase genome stability is conserved.

MATERIALS AND METHODS

Yeast strains and media. *S. pombe* strains (Table 1) were constructed and maintained according to standard procedures (53). Cells were treated with 15 or 20 mM HU (Sigma) for the indicated times or with 0.0025% methyl methanesulfate (Sigma) or 5 μU/ml bleomycin (Sigma) for 1 h or were irradiated with 100 Gy using a ^{60}cobalt source. Asynchronous cultures or cultures synchronized by nitrogen starvation (53) were shifted to the restrictive temperature (36°C) for 4 h; cells pulse-labeled with bromodeoxyuridine (BrdU) were shifted to the restrictive temperature for 3.5 h and then incubated with 200 μg/ml BrdU (Sigma) for an additional 30 min at 36°C.

Cell culture and short interfering RNA (siRNA). HeLa cells were grown in Dulbecco's modified Eagle's medium containing 10% calf serum (Invitrogen), with appropriate antibiotics. HeLa cells were synchronized in G_1/S with 2 mM thymidine (Sigma) for 18 h and then released into S phase for 3 to 4 h in the absence of added DNA damage. Cells were blocked in early S phase with 2.5 mM HU for 18 h.

For siRNA experiments, synthetic RNA duplexes were transfected into HeLa cells to a final concentration of 20 nM using Oligofectamine (Invitrogen) according to the protocol provided by Dharmacon. siRNA duplexes used were CGACAGCTAGAGTCATTAA for MCM4 and ATCGGATTGTGAAGA TGAA for MCM7, synthesized by Integrated DNA Technologies, Inc. Pools of four additional siRNA duplexes against MCM4 or MCM7 were obtained from Dharmacon. The siRNA negative control duplex was from Invitrogen. Relative

TABLE 1. Yeast strains used in this study

Strain	Genotype	Source
FY7	*h⁻ 972*	Our stock
FY72	*h⁻ ade6-M210 ura4-D18 leu1-32*	Our stock
FY243	*h⁻ cdc19-P1 ade6-M210 ura4-D18 leu1-32*	Our stock
FY261	*h⁺ ura4-D18 leu1-32 ade6-M216 can1-1*	Our stock
FY364	*h⁻ cdc21-M68 leu1-32 ura4-D18 ade6-M210 can1-1*	Our stock
FY522	*h⁻ cdc22-M45 ade6-M210 leu1-32 ura4-D18*	Our stock
FY641	*h⁻ cdc6-23 ura4-D18 leu1-32 ade6-M210*	Our stock
FY865	*h⁻ Δcds1::ura4⁺ ura4-D18 leu1-32*	Our stock
FY866	*h⁺ Δcds1::ura4-D18 leu1-32*	Our stock
FY961	*h⁺ mis5-268 ura4-D18 leu1-32 ade6-M210 can1-1*	Our stock
FY969	*h⁻ cdc27-K3 ura4-D18 leu1 ade6-M210*	Our stock
FY986	*h⁺ hsk1-1312 ura4-D18 leu1-32 ade6-M216*	Our stock
FY999	*h⁺ hsk1-1312 Δcds1::ura4⁺ ura4-D18 leu1-32*	Our stock
FY1106	*h⁺ Δrad3::ura4⁺ ura4-D18 leu1-32 ade6-M210*	Our stock
FY1167	*h⁻ mcm4-HA::leu1⁺ ura4-D18 leu1-32 ade6-M210*	Our stock
FY1201	*h⁻ mcm7-98 ura4-D18 leu1-32 ade6-M210*	Our stock
FY1209	*h⁺ Δrad22::[hisG ura4⁺ hisG] ade6-M210 ura4-D18 leu1-32*	A. Pastink
FY1313	*h⁻ cdc17-K42 leu1-32 ade6-M210 can1-1*	Our stock
FY1374	*h⁻ cdc19-P1 Δrad22::[hisG ura4⁺ hisG] ade6-M210 leu1-32 ura4-D18*	This study
FY1375	*h⁺ cdc21-M68 Δrad22::[hisG ura4⁺ hisG] ade6-M210 leu1-32 ura4-D18*	This study
FY1390	*h⁻ smt-0 Δrhp51::ura4 ura4-D18*	Our stock
FY1413	*h⁻ cdc21-M68*	Our stock
FY1507	*h⁻ smt-0 Δrhp51::ura4⁺ cdc21-M68 ade6-M210 ura4-D18*	This study
FY1617	*h⁻ cdc21-M68 Δcds1::ura4⁺ ura4 leu1 ade6-M210*	Our stock
FY1884	*h⁻ smt-0 Δrhp51::ura4⁺ ade6-M210 leu1-32 ura4-D18*	This study
FY1963	*h⁻ cdc21-M68 chk1::ura4⁺ ura4-D18*	Our stock
FY2234	*h⁺ Δtel1::ura4⁺ ade6-210 ura4-D18 leu1-32*	Our stock
FY2317	*h⁺ leu1-32::hENT1-leu1⁺*(pJAH29) *his7-366::hsv-tk-his7⁺*(pJAH31) *ura4-D18 ade6-M210*	31
FY2356	*h⁻ smt-0 Δrhp51::ura4⁺ cdc21-HA::leu1⁺ ade6-M210 leu1-32 ura4-D18*	This study
FY2423	*h⁺ cdc21-HA::leu1⁺ cds1::ura4⁺ ade6-M210 leu1-32 ura4-D18*	Our stock
FY2514	*h⁺ leu1-32::hENT1-leu1⁺*(pJAH29) *his7-366::hsv-tk-his7⁺*(pJAH31) *cdc21-M68 leu1-32 ura4-D18 ade6-M210*	This study
FY2728	*h⁺ Δrad3::ura4⁺ Δtel1::ura4⁺ ade6-M210 leu1-32 ura4-D18*	This study
FY2878	*h⁻ rad22:YFP:kanMX4 ade6-M210 ura4-D18 leu1-32*	G. Freyer
FY2887	*h⁻ mcm4 (cdc21-M68)-ts-td::ura4⁺ ade6-M210 leu1-32 ura4-D18*	This study
FY2906	*h⁺ rad22-YFP:kanMX4 cdc21-M68 leu1-32 ura4-D18*	This study
FY3201	*h⁻ cdc19-P1 Δrad32::kanmx ade6-M210 leu1-32 ura4-D18*	This study
FY3203	*h⁻ cdc19-P1 Δrhp51::ura4⁺ ade6-M210 leu1-32 ura4-D18*	This study
FY3204	*h⁻ cdc21-M68 Δrad32::kanmx ade6-M210 leu1-32 ura4-D18*	This study
FY3396	*h⁺ cdc21-M68-ts-dg::ura4⁺ ura4-D18*	This study
FY3650	*h⁺ Δcds1::ura4 Δrhp51::ura4 ura4-D18 leu1-32*	This study
FY3651	*h⁻ smt-0 Δcds1::ura4 Δrhp51::ura4 ura4-D18*	This study
JBY235	*h⁻ mcm4-HA::leu1⁺ cds1-13myc::kanmx leu1-32 ura4-D18*	This study
JBY267	*h⁻ cds1-13myc::kanMX ade6-M210 leu1-32 ura4-D18*	This study

viability was determined as the percentage of viable cells at 48 h compared to the percentage of viable cells at the time of transfection (0 h).

Immunofluorescence. Spread nuclei were prepared from fission yeast cells according to previous procedures (29). Briefly, cells were spheroplasted in phosphate-buffered saline (PBS) with 0.5 mg/ml zymolyase 20T (Seikagaku) and 0.5 mg/ml lysing enzymes (Sigma). Cells were then washed in MES [(2-*N*-morpholino)ethane sulfonic acid] buffer (0.1 M MES, 1 M sorbitol, 1 mM EDTA, 0.5 mM $MgCl_2$, pH 6.4), resuspended in 100 μl MES plus 360 μl 4% paraformaldehyde, and poured onto Colorfrost microscope slides (Thermo Fisher Scientific). Slides were rinsed with 0.4% Photo-Flo (Kodak) and fixed with low heat.

S. pombe chromatin fibers were prepared by a modification of a protocol used to visualize yeast artificial chromosome chromatin fibers. The protocol generates fibers of approximately 1.8 kb/μm (65). Cells were spheroplasted in zymolyase mix (1 M sorbitol, 60 mM EDTA, 100 mM sodium citrate, pH 7, 0.5 mg/ml zymolyase 20T, 100 mM β-mercaptoethanol), and then spheroplasts were air dried onto Colorfrost slides. Fibers were stretched by pipetting 50 μl lysing solution (50 mM Tris-Cl, pH 7.4, 25 mM EDTA, 500 mM NaCl, 0.1% [wt/vol] Nonidet P-40, 1% [wt/vol] sodium dodecyl sulfate [SDS], 3 mM β-mercaptoethanol) onto the spheroplasts, waiting 1 to 3 min, and then holding the slides vertically to drain the liquid. Chromatin fibers were fixed in 4% formaldehyde in PBS for 20 min, rinsed in PBS, and then heat fixed before being immunostained with the indicated antibodies.

Slides were blocked in PBS containing 5% calf serum and 0.05% Tween 20 for 1 h at room temperature. Primary antibody staining was carried out overnight at 4°C in a humid chamber. Antibodies against phosphorylated histone H2AX (phospho-H2AX; Millipore) and BrdU (Becton Dickinson) were used at 1:50. Immunostaining for BrdU was performed as described previously (29), with sequential immunostaining with phospho-H2AX antibodies. Anti-Rhp51 antibodies (14) and antihemagglutinin (anti-HA) antibodies (16B12; Covance) were used at 1:100. Primary antibodies were detected by using Alexa Fluor 488 or Alexa Fluor 594 secondary antibodies (Invitrogen) at 1:500 in PBS with 5% calf serum. DNA was counterstained with 4′,6-diamidino-2-phenylindole (DAPI).

For staining by terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling, spread *S. pombe* chromosomes were labeled with dUTP-digoxigenin as described previously (4) and then immunostained with an anti-digoxigenin antibody (Invitrogen) and detected by incubation with an anti-digoxigenin secondary antibody coupled to rhodamine (Invitrogen). Slides were immunostained sequentially with antibodies to detect terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling and phospho-H2AX.

HeLa cells used for indirect immunofluorescence experiments were grown on glass coverslips. Cells on coverslips were permeabilized with PBS that contained 0.1% Triton X-100 for 7 min and then fixed with 3.7% formaldehyde in PBS for 15 min. Cells were then incubated with blocking buffer (PBS with 5% bovine serum and 0.5% Triton X-100) for 30 min. Stretched chromatin fibers were prepared from HeLa cells as described previously (7); cells collected by trypsinization were resuspended in 75 mM KCl and pipetted onto Colorfrost slides. Slides were incubated in lysis buffer (25 mM Tris-Cl, pH 7.5, 0.5 M NaCl, 1% Triton X-100, 0.5 M urea) for 10 min and then lifted slowly out of the buffer and fixed with 3.7% formaldehyde in PBS for 15 min. Chromatin fibers generated by this method are approximately 2 kb/μm (49). HeLa cells and chromatin fibers were immunostained overnight at 4°C in a humid chamber with antibodies against phospho-H2AX (Millipore), MCM7 (Santa Cruz), and Rad51 (Santa Cruz), each used at 1:100 dilution; BrdU (Becton Dickinson) used at 1:50 dilution; or cleaved caspase 3 (Cell Signaling) used at 1:500 dilution. Primary antibodies were detected with Alexa Fluor 594 or Alexa Fluor 488 secondary antibodies used at 1:500. DNA was counterstained with DAPI.

Images were visualized and acquired using a Leica DMR microscope and Improvision software or with a Leica 6000 microscope and Leica FW4000 software.

Flow cytometry. *S. pombe* cells were fixed in 70% ethanol and then treated with RNase in 50 mM sodium citrate. Cells were resuspended in Sytox green dye (Invitrogen) at 1:2,500 in 50 mM sodium citrate as described previously (2) for both flow cytometry and examination of nuclear morphology.

HeLa cells were collected by trypsinization and then fixed with 70% ethanol for 30 min. Cells were then washed in PBS, treated with RNase in PBS, and then resuspended in PBS with 4 μg/ml propidium iodide.

Cells were analyzed on a FACScan cytometer (Becton Dickinson) by using CellQuest software.

Viability assays. *S. pombe* cells were grown asynchronously at the permissive temperature for 16 h, and then HU was added for 4 h. For each condition, 5,000 cells were counted and plated for absolute viability. The remaining cells were then washed and transferred to the same original volume of medium, either with or without HU, at the permissive or restrictive temperature. For each condition, 5,000 cells were plated to assess relative viability. Colonies were counted after 4 days of growth on yeast extract plus supplements (YES) solid medium at the permissive temperature. Cellular DNA content was analyzed by flow cytometry as described above.

Immunoprecipitation and immunoblotting. We prepared soluble protein lysates from fission yeast cells by vortexing cells with glass beads in B88 lysis buffer (20 mM HEPES pH 7.0, 50 mM potassium acetate, 5 mM magnesium acetate, 100 mM sorbitol, 0.1% Triton X-100) to which 2 mM dithiothreitol, protease inhibitor cocktail (Sigma), 50 mM sodium orthovanadate, and 50 mM β-glycerophosphate were added (2). A total of 0.5 to 1.0 mg protein was used per immunoprecipitation. Proteins were analyzed by 10% SDS-polyacrylamide gel electrophoresis (PAGE), followed by immunoblotting with anti-HA (16B12; Covance), anti-myc (9e10), antitubulin (4A1) (31), and anti-Rhp51 antibodies (14), each at a 1:1,000 dilution. Antibodies were covalently coupled to protein A beads as described previously (2).

To assay the depletion of MCM protein and the coprecipitation of MCM proteins with Rad51, HeLa cells were lysed in modified CSK buffer [10 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid), pH 6.8, 100 mM NaCl, 300 mM sucrose, 1 mM $MgCl_2$, 1 mM EGTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 10 U/ml aprotinin, 50 μM NaF, 50 μM β-glycerophosphate, 0.5% Triton X-100] for 20 min at 4°C. Lysates were centrifuged at a low speed for 5 min and then analyzed by 10% SDS-PAGE and immunoblotting with anti-MCM4 (Becton Dickinson), anti-MCM7 (Santa Cruz), anti-PCNA (Santa Cruz), or anti-Rad51 (Santa Cruz) antibodies (all used at 1:1,000).

Mcm4 mobility shift. Whole-cell protein extract was prepared with modifications from Foiani et al. (21a). Cells were disrupted with glass beads by vortexing in 10% trichloroacetic acid, washed with acetone, and resuspended in Laemmli buffer. A total of 150 μg of protein was analyzed by 8% SDS-PAGE, followed by immunoblotting with anti-MCM4 antibodies (66). For phosphatase treatment, soluble protein lysates (described above) were incubated for 20 min at 30°C with lambda protein phosphatase (New England Biolabs). Reaction products were analyzed by 8% SDS-PAGE, followed by immunoblotting with anti-MCM4 (66) and anti-HA antibodies (16B12; Covance).

RESULTS

***mcm-ts* mutants cause irreversible DNA damage.** Although MCM activity is crucial throughout S phase, most *mcm-ts* mutants appear to complete bulk DNA replication (18, 23, 40). However, these *mcm-ts* mutant cells activate a Chk1-dependent checkpoint and arrest irreversibly in late S phase or G_2 (41, 47), raising the possibility that the cells accumulate DNA damage during replication termination or in specific chromosomal regions. To test this idea, we prepared spread nuclei from wild-type and *mcm-ts* mutant cells grown at permissive and restrictive temperatures and immunostained for phosphorylated histone H2A (phospho-H2A), a marker for DNA damage. In mammalian cells, the histone H2A variant H2AX is rapidly and specifically phosphorylated in response to DNA double-stranded breaks (DSBs) and this signal spreads to regions of flanking chromatin megabases away from the break sites (64). A phosphorylation-specific H2AX antibody (phospho-H2AX) recognizes a phospho-SQE amino acid sequence in the carboxyl terminus of the H2AX protein that is highly conserved in other species (64, 69), including the *S. pombe* histone H2A homologues Hta1 and Hta2 (54). Phospho-H2A foci in yeast form in response to chemical agents that cause DNA DSBs, localize to DNA break sites, and are dependent on checkpoint protein kinases, indicating that they also mark DSBs (54, 83) (see Fig. S1A, S1B, and S1C in the supplemental material).

Wild-type cells and *mcm4ts* cells grown asynchronously at the permissive temperature contain very little DSB damage: we detected phospho-H2A foci in only a small fraction of spread nuclei, and there were <5 foci per nucleus (Fig. 1A and data not shown). In contrast, we observed extensive DNA damage in elongated, arrested *mcm4ts* cells that had been held at the

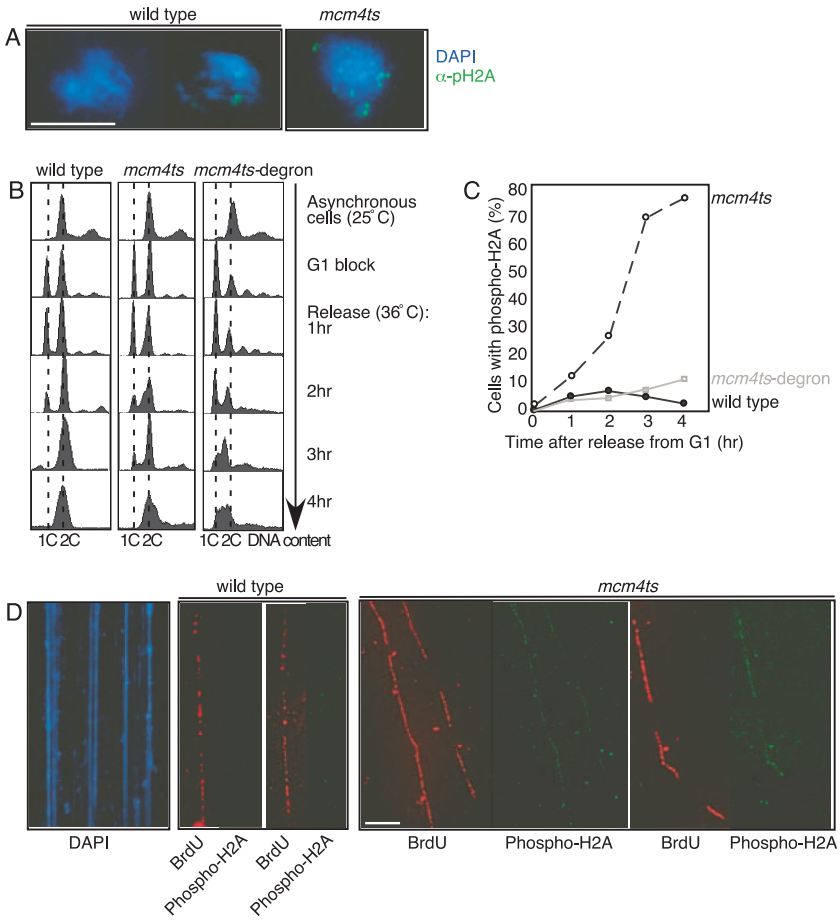

FIG. 1. *mcm-ts* mutants generate DNA damage during replication. (A) *mcm4ts* cells generate DNA damage. Spread nuclei prepared from wild-type (FY72) and *mcm4ts* (FY364) cells shifted to the restrictive temperature (36°C) for 4 h were immunostained for phospho-H2A (green) to mark DNA breaks and counterstained with DAPI (blue) to detect the DNA. α-pH2A, anti-phospho-H2A antibody. Scale bar, 10 mM. Two examples of wild-type nuclei are shown; while most nuclei did not contain phospho-H2A foci, a small number of S-phase nuclei displayed a few phospho-H2A foci. (B and C) DNA damage in *mcm4ts* cells requires S-phase progression. Wild-type (FY72), *mcm4ts* (FY364), and *mcm4ts-degron* (FY2887) cells grown at 25°C were blocked in G_1 by nitrogen starvation and then released into S phase at 36°C. At 1-h intervals, (B) DNA content was measured by flow cytometry, and (C) DNA damage was examined by immunostaining spread nuclei for phospho-H2A. (D) DNA damage in *mcm4ts* cells corresponds to sites of new synthesis. Wild-type (FY2317) and *mcm4ts* (FY2514) cells were shifted to the restrictive temperature for 3.5 h and then pulse-labeled with BrdU for 30 min. Stretched chromatin fibers were then prepared from these cells and visualized with DAPI (left). Chromatin fibers were immunostained with antibodies to BrdU and phospho-H2A to detect sites of replicating DNA and DNA breaks, respectively (right). Scale bar, 10 μm (approximately 18 kb).

restrictive temperature for 4 h, with more than 80% of the spread nuclei containing >20 phospho-H2A foci per nucleus (Fig. 1A). In addition, the *mcm4ts* mutant cells also displayed nuclear foci of the DNA repair proteins Rhp51 and Rad22 (the fission yeast homologues of Rad51 and Rad52) (see Fig. S1D and S1E in the supplemental material; data not shown), which have previously been used as molecular markers for DNA damage in fission yeast (20, 48), supporting the conclusion that DNA breaks occur in these cells.

We also examined other mutants affecting the MCM complex, and additional replication mutants, for the formation of DNA damage at the restrictive temperature. All mutants analyzed contained Rhp51 foci, which mark single-stranded breaks and DSBs (13, 60, 76). Mutations affecting subunits of DNA polymerase δ (*cdc6* and *cdc27*) and the *cdc22* ribonucleotide reductase mutant, all of which retain viability upon a shift to high temperature, displayed few phospho-H2A foci. In contrast, *mcm-ts* mutants and the DNA ligase *cdc17* mutant all contained extensive phospho-H2A foci, indicating DNA DSBs, and suffered severe losses of viability. Thus, phospho-H2A foci correlate with inviability (see Fig. S1D in the supplemental material; Table 2). Together, these data suggest that stability of the MCMs in the replisome is important for cell viability following replication arrest.

DNA damage in *mcm-ts* cells requires replication progression. While *mcm-ts* alleles complete bulk DNA synthesis (18, 23, 40, 41), a degron allele of *mcm4ts* blocks initiation, resulting in limited or no DNA synthesis (42). To determine whether the lethal DNA damage in *mcm-ts* cells occurred as a result of defects in initiation or replication elongation, we compared phospho-H2A focus formation in spread nuclei from wild-type, *mcm4ts*, and *mcm4ts-degron* cells following release from G_1/S arrest into S phase at the restrictive temperature. We observed a dramatic accumulation of phospho-H2A foci in *mcm4ts* cells

TABLE 2. DNA damage in fission yeast replication mutants[a]

Characteristic or mutation	Strain	Wild-type gene function	Arrest point	Relative viability (%)	% Spread nuclei with foci at 36°C		
					Phospho-H2A	Rhp51	Phospho-H2A and Rhp51
Wild type	FY72				4	4	4
mcm2 (*cdc19*)	FY243	MCM complex: replicative helicase	Late S	16.3	84	84	84
mcm4 (*cdc21*)	FY364		Late S	29.5	94	94	94
mcm6 (*mis5*)	FY961		Late S	18.8	75	75	75
mcm7	FY1201		Late S	15.2	85	85	85
cdc6	FY641	DNA polymerase	Late S	111.1	17	38	17
cdc17	FY1313	DNA ligase	Late S	29.9	90	90	90
cdc22	FY522	Ribonucleotide reductase	Early S	102.3	17	55	17
cdc27	FY969	DNA polymerase	Late S	131.3	10	46	10

[a] Yeast replication mutants that arrest irreversibly at the restrictive temperature generate phospho-H2A foci. Cultures were grown to early log phase at 25°C and then shifted to 36°C for 4 h. Arrest points were determined by flow cytometry and analysis of cell morphology (data not shown). At 0 and 4 h after shift to 36°C, aliquots of cells were plated and incubated at the permissive temperature. Relative viability was determined as the number of viable colonies from the 4-h time point compared to the number at the 0-h time point. Spread nuclei prepared from arrested cells were immunostained for phospho-H2A and Rhp51. At least 300 spread nuclei per strain were examined.

as they progressed through S phase, and these persisted in cells that arrested (Fig. 1B and C). In contrast, the *mcm4ts-degron* cells displayed few phospho-H2A foci, and the inefficient DNA synthesis coupled with cell elongation resulted in apparent delayed and incomplete S-phase progression by flow cytometry. This observation suggests that the DNA damage in *mcm4ts* cells occurs during replication elongation, which would be consistent with replication fork collapse. In agreement with this suggestion, the phospho-H2A foci observed in *mcm4ts* cells were suppressed by the addition of HU (<5% of cells with foci [data not shown]), which delays cells in early S phase with replication initiated from only a subset of origins and active forks stabilized by the checkpoint. In comparison, a low percentage of wild-type cells exhibited a few phospho-H2A foci during S phase, but these disappeared as the cells completed S phase (Fig. 1C), suggesting that low levels of spontaneous DNA damage or fork collapse may occur during replication but that such damage is normally recognized and repaired.

Genome-wide replication fork breakage can be monitored using pulsed-field gel electrophoresis gels that detect fragmented DNA (for an example, see reference 51). However, we found that the majority of chromosomes prepared from *mcm-ts* cells at the restrictive temperature did not enter pulsed-field gel electrophoresis gels (41; data not shown), suggesting that they contain topologically complex chromosomal structures, such as replication bubbles or other S-phase intermediates that impair electrophoretic mobility. The same result has also been observed for other replication mutants that arrest S-phase progression (for an example, see reference 81). Although replication fork breakage at specific sites can be monitored by two-dimensional gel electrophoresis, the DNA breaks in *mcm-ts* cells occur late in S phase and are unlikely to be associated with replication origins or other defined sequences. Therefore, as an alternative method to examine DNA breaks associated with replicating DNA in *mcm4ts* mutants, we pulse-labeled cells with BrdU and then prepared stretched chromatin fibers which we costained for BrdU, phospho-H2A, and DAPI (Fig. 1D). The BrdU incorporation pattern on fibers from both wild-type and *mcm4ts* cells was discontinuous and punctate, suggesting that DNA synthesis had initiated from multiple, discrete origins (Fig. 1D). For chromatin fibers prepared from wild-type cells pulse-labeled with BrdU (Fig. 1D), just 9% of BrdU-labeled fibers displayed phospho-H2A staining, indicating that the chromatin-stretching procedure itself and the short incubation with BrdU did not cause DNA DSBs. In contrast, 63% of BrdU-stained chromatin fibers prepared from *mcm4ts* cells also exhibited extensive phospho-H2A staining and these damage foci overlapped with regions of BrdU incorporation (Fig. 1D). These results suggest that *mcm-ts*-induced breaks are associated with regions of newly synthesized DNA, consistent with the model that they result from replication fork collapse. Importantly, these fibers are chromatinized and fully proteinated; the DNA break sites may be held together by protein-protein interactions, such as those suggested for the recombination complex Mre11-Rad50-Nbs1 (45), or may represent DSBs in the process of repair.

MCM proteins are required for replication restart after arrest. These observations suggest that one function of the MCM complex is to protect replication fork structure during normal S phase. Because MCM subunits can be phosphorylated by checkpoint kinases (17, 33, 67, 82), we considered the possibility that the MCM complex also contributes to fork stability during replication arrest. To test this idea, we treated wild-type, *mcm4ts*, or *mcm4ts-degron* mutant cells with HU, which stalls replication forks and activates the replication checkpoint. We next inactivated the Mcm4 subunit by shifting to the restrictive temperature while maintaining HU arrest. We then asked whether this treatment generated DNA DSBs and whether the cells could recover from the arrest (Fig. 2A). Wild-type cells with normal Mcm4 function arrested in HU with a primarily 1C DNA content, and the cells completed S phase and resumed cycling upon release from HU (Fig. 2B and D). Few phospho-H2A foci were observed at either the permissive or the restrictive temperature, consistent with our previous observations that suggest limited, spontaneous DNA damage can occur as a normal part of S-phase progression (Fig. 2C; see Fig. S1 in the supplemental material). In a manner similar to that of wild-type cells, *mcm4ts-degron* cells also arrested in HU, with few phospho-H2A foci (Fig. 2B and C). However, upon a shift to the restrictive temperature, we observed that *mcm4ts-degron* cells generated numerous phospho-H2A foci, even though the cells were maintained in HU and

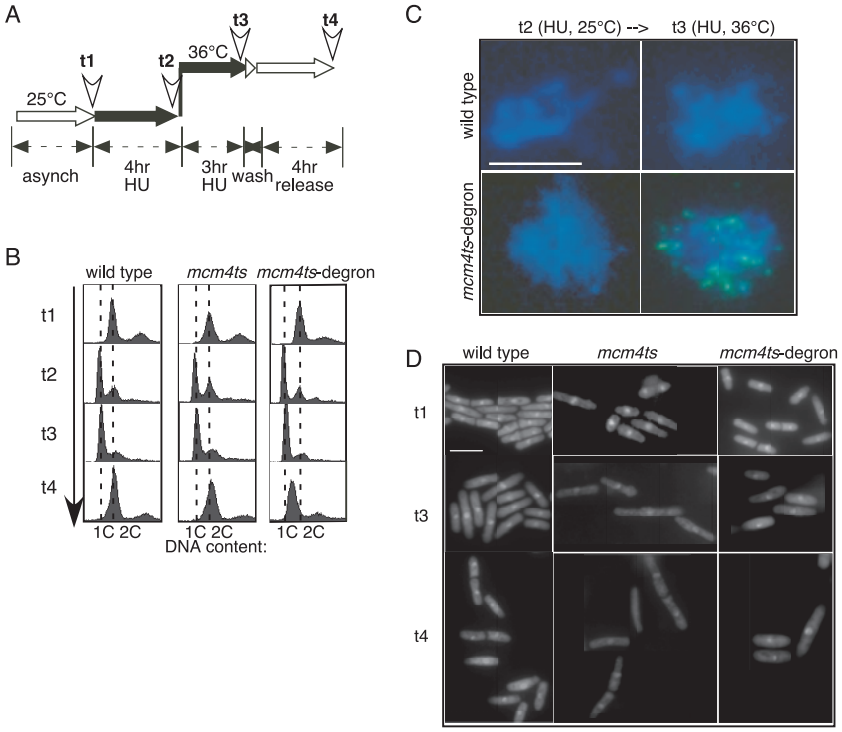

FIG. 2. MCM proteins are required for recovery from HU. (A) Schematic of the experimental protocol. Wild-type (FY72), *mcm4ts* (FY364), and *mcm4ts-degron* (FY2887) cells were grown asynchronously (white arrows) and then arrested in HU for 4 h (black arrows). Cells were shifted to the restrictive temperature of 36°C and then released from HU. Samples were taken at time points t1, t2, t3, and t4. (B) Cellular DNA content was analyzed by flow cytometry. All strains contained approximately 1C DNA content in HU. The wild-type and *mcm4ts* cells, but not the *mcm4ts-degron* cells, completed bulk synthesis upon release from HU. (C) Inactivation of Mcm4 resulted in DNA breaks in HU. Wild-type and *mcm4ts-degron* mutants were arrested in HU at 25°C (t2) and shifted to 36°C while maintained in HU (t3). DNA DSBs were detected by phospho-H2A immunostaining as described in the legend to Fig. 1. (D) Cellular morphology was examined by staining with Sytox green to detect the DNA. Scale bars, 10 μm.

remained arrested (Fig. 2B and C). This result suggests that the loss of Mcm4 function after replication forks have formed and stalled causes extensive DNA damage. Upon release from HU at the restrictive temperature, the *mcm4ts-degron* mutants showed little additional DNA accumulation and no sign of division, consistent with the essential role for the MCM complex throughout DNA replication (36).

Unexpectedly, we found that *mcm4ts* cells blocked in HU and then shifted to a high temperature could recover from arrest, enter mitosis, and remain viable, with an increase in cell number similar to that of the wild type (178.7% ± 11.3% [mean ± standard deviation] relative viability for *mcm4ts* compared to 178.0% ± 6.4% for the wild type) (Fig. 2A). This result was surprising because *mcm4ts* mutants shifted asynchronously to the restrictive temperature, or released from nitrogen starvation to the restrictive temperature, accumulate phospho-H2A foci and lose viability (Fig. 1). The *mcm4ts* mutant contains a single amino acid substitution (Leu238 to Pro), a relatively modest lesion that may cause loss of function at a high temperature through a conformational change in the protein (42). We hypothesized that activation of the replication checkpoint during HU arrest might rescue the defect associated with *mcm4ts* by stabilizing the Mcm4ts mutant protein.

Mcm4 is a target of the replication checkpoint. We therefore tested whether Mcm4 interacts with the Cds1 checkpoint kinase and whether Mcm4 is a target of Cds1-dependent phosphorylation. We found that Mcm4 and Cds1 coimmunoprecipitate in the presence of HU (Fig. 3A). We also observed a mobility shift of the Mcm4 protein in extracts from HU-treated cells (Fig. 3B to E). This shifted band could be collapsed by phosphatase treatment, suggesting that it represents the phosphorylation of Mcm4 (see Fig. S2A in the supplemental material). Identical results were observed using a Δ*cds1* strain with untagged Mcm4 (FY865) (data not shown). Although we observed the Mcm4 mobility shift in both wild-type and *mcm4ts* strains, it did not occur in *mcm4ts-degron* strains, even under permissive conditions. Interestingly, the *mcm4ts-degron* mutant lost viability in HU, prior to any temperature shift, suggesting that it has a defect in the replication checkpoint response even at the permissive temperature (see Fig. S2B, S2C, and S2D in the supplemental material).

Consistent with the possibility that Mcm4 is a target of the replication checkpoint, we found that the Mcm4 mobility shift depends upon Cds1 (Fig. 3E) as well as on the Hsk1 (ScCdc7) kinase (see Fig. S2B, S2C, and S2D in the supplemental material). Hsk1 is required for full Cds1 activation (75) and is itself a potential Cds1 target (72). Simple overexpression of Cds1 was unable to rescue *mcm4ts* inviability (data not shown), indicating that activation of the checkpoint is required. These data suggest that Mcm4 undergoes Cds1-dependent phosphorylation that may contribute to replication checkpoint function

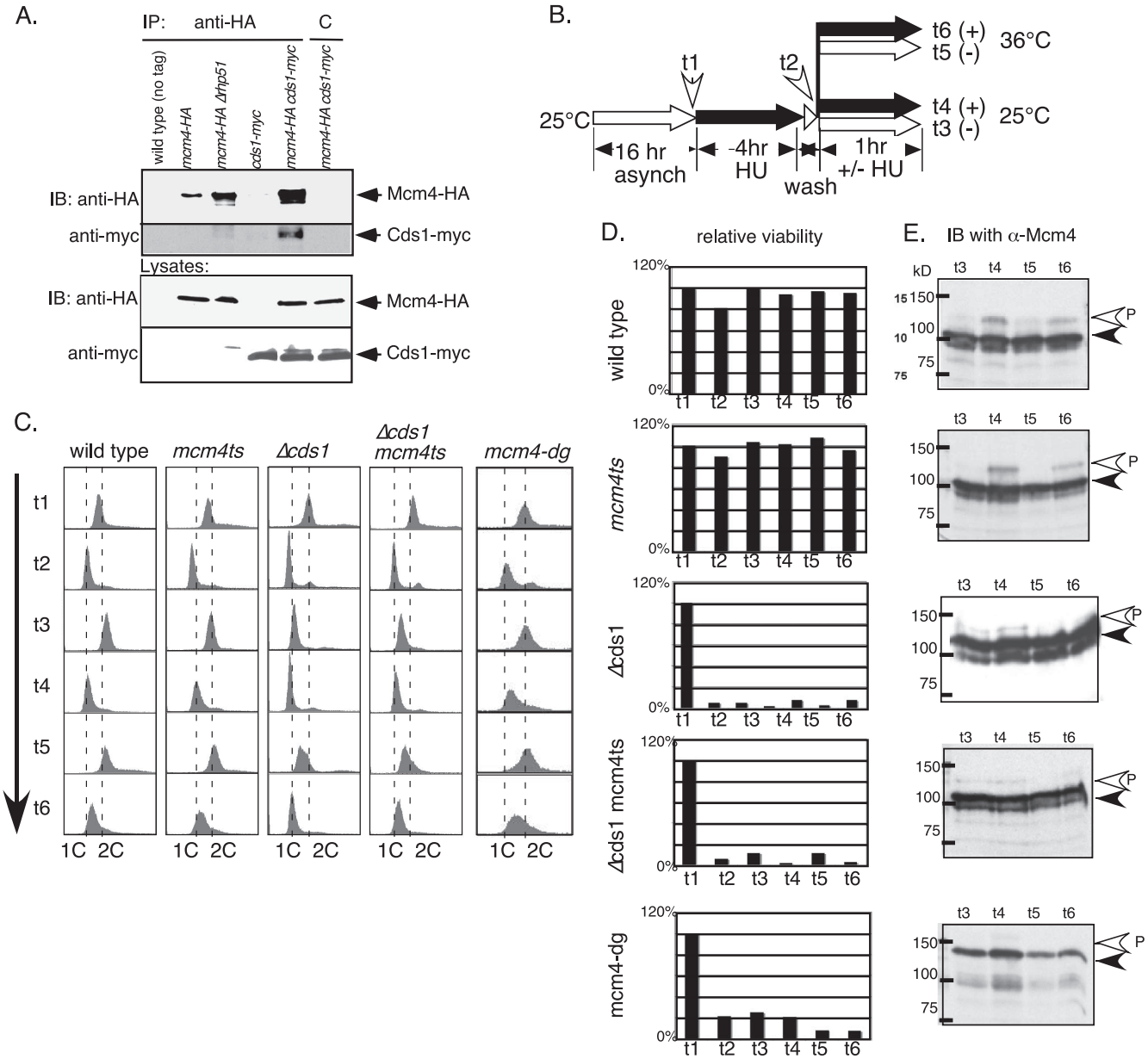

FIG. 3. Cds1-dependent phosphorylation of Mcm4. (A) Mcm4 coimmunoprecipitates Cds1. Protein lysates prepared from wild-type (FY72), *mcm4-HA* (FY1167), *mcm4-HA Δrhp51* (FY2356), *cds1-myc* (JBY267), and *mcm4-HA cds1-myc* (JBY235) strains incubated with 15 mM HU for 4 h were immunoprecipitated (IP) with anti-HA antibodies or control (C) antibody (antitubulin); immunoprecipitates were then immunoblotted (IB) to detect Cds1-myc and Mcm4-HA. (B). Schematic of the experimental protocol. Cells were grown asynchronously (white arrow) and arrested in HU for 4 h (black arrow). Cells were then released into medium with or without HU, either at 25°C or 36°C. Samples were taken at time points t1, t2, t3, t4, t5, and t6. (C) Cellular DNA content was analyzed in wild-type (FY7), *mcm4ts* (FY1413), Δ*cds1* (FY2423), Δ*cds1 mcm4ts* (FY1617), and *mcm4ts-degron* (FY3396) cells by flow cytometry. (D) Relative viability of the strains in panel C was determined as described in Materials and Methods. (E) Mcm4 gel mobility shift in HU. Trichloroacetic acid-precipitated lysates from the strains indicated in panel C were immunoblotted (IB) for Mcm4 protein with anti-Mcm4 antibody (α-Mcm4). The shifted (phosphorylated [P]) form of Mcm4 is indicated by the white arrowhead. The Mcm4-degron protein exhibits reduced mobility relative to wild-type Mcm4 because of the large N-terminal degron tag. Strain FY2423 contains Mcm4HA, but identical results were observed using FY866, which has untagged Mcm4 (data not shown).

and further imply that the Mcm4ts protein is stabilized through Cds1-dependent modification in HU.

MCM proteins link checkpoint activation to recovery. In prokaryotes, it is well established that replication forks that arrest or break can restart and resume synthesis through recombination (reviewed in references 27 and 50). However, evidence for a similar pathway in eukaryotic cells remains controversial. We observed that *mcm-ts Δrhp51* and *mcm-ts Δrad22* double mutants were unable to grow at intermediate temperatures that were permissive for either single mutant (Table 3). This observation suggests that HR is required to repair the fork-dependent DNA breaks in *mcm-ts* cells or that

TABLE 3. Genetic interactions between mutants of the MCM complex and DNA repair mutants[a]

Strain	Genotype	Phenotype
FY1507	*mcm4ts* Δ*rhp51*	Slow/reduced growth at 29°C, 32°C, and 34°C
FY1375	*mcm4ts* Δ*rad22*	Slow/reduced growth at 29°C, 32°C, and 34°C
FY3203	*mcm2ts* Δ*rhp51*	Slow/reduced growth at 29°C, 32°C, and 34°C
FY1374	*mcm2ts* Δ*rad22*	Slow/reduced growth at 29°C, 32°C, and 34°C
FY3204	*mcm4ts* Δ*rad32*	No genetic interaction
FY3201	*mcm2ts* Δ*rad32*	No genetic interaction

[a]HR mutants decrease the ability of *mcm4ts* to grow at semipermissive temperatures. Double mutant strains were compared against the single mutants for growth on solid rich medium (YES) incubated at 25°C, 29°C, 32°C, and 34°C for 4 days.

HR proteins cooperate with the replication machinery to protect replication fork structure. Given that both MCM proteins and HR proteins are predicted to act at or near replication forks, we tested for physical association of these proteins. Intriguingly, we found that Rhp51 (ScRad51) coimmunoprecipitated with Mcm4 from cells treated with HU when both proteins were expressed at endogenous levels (Fig. 4A; see Fig. S3 in the supplemental material). The interaction between Rhp51 and Mcm4 was still observed when lysates were pretreated with DNase (data not shown), indicating that it was not an indirect effect of the association of both proteins with the same DNA fragments. In contrast, little or no Rhp51 bound to Mcm4 in asynchronous cultures (Fig. 4A) or in cells released synchronously from a G_1 block into S phase in the absence of HU (see Fig. S3 in the supplemental material). It remains a possibility that Rhp51 and Mcm4 do associate during normal S phase in the subset of cells that generate Rhp51 or phospho-H2A foci, which may reflect sites of spontaneous DNA damage. We also observed that Mcm4 coimmunoprecipitates overexpressed Rad22-yellow fluorescent protein (YFP) from cells treated with HU (data not shown), suggesting that the MCM complex may interact with repair complexes. These interactions could link fork protection and replication restart with recovery from arrest. Therefore, we examined the dynamics of recovery from replication arrest in recombination mutants in more detail.

When cells are treated with HU, replication forks stall, leading to Cds1 activation and replication arrest. In the Δ*cds1* mutant, this checkpoint cannot be activated to stabilize fork structure and the forks collapse, leading to rapid loss of viability. This is also the case in Δ*rad3* mutants, because the Rad3 kinase is required for Cds1 phosphorylation and activation. In contrast, while the Δ*rhp51* and Δ*rad22* recombination mutants are sensitive to growth on solid medium that contains HU, they display only a modest loss of viability during continuous HU exposure (48, 84) (Fig. 4B and data not shown). The viability loss in Δ*rhp51* and Δ*rad22* cells occurs after 3 to 4 h in HU, which corresponds to the time point when wild-type cells begin to adapt to the drug and resume cell cycle progression (Fig. 4B). Because the loss of viability in HR mutants is modest compared to that in checkpoint mutants and occurs at a later time point, we infer that HR proteins have a distinct, nonessential role in recovery from HU. We next assessed phospho-H2A focus formation in checkpoint and recombination mutant cells that were blocked in HU and then released. Nearly all of the Δ*cds1* and Δ*rad3* checkpoint mutant cells exposed to HU contained substantial numbers of phospho-H2A foci (Fig. 4C), consistent with previous evidence that replication forks collapse in the absence of replication checkpoint activation (48, 56, 73). In the recombination mutant cells, however, a much lower percentage of spread nuclei displayed phospho-H2A foci and there were fewer foci per nucleus (<5 foci/nucleus) than in the checkpoint mutant cells (>20 foci/nucleus) (Fig. 4C). These results suggest that replication forks do not collapse in HR mutants, leading us to conclude that HR proteins are not required for replication fork protection during HU-induced arrest.

We also examined recruitment of the HR proteins themselves by using a Rad22-YFP fluorescent fusion protein described previously (20, 48) as well as by using indirect immunofluorescence to detect Rad22 and Rhp51 (Fig. 4D and E). In asynchronous cultures, a low frequency of wild-type cells displayed nuclear foci of Rhp51 and Rad22 (Fig. 4A and data not shown), a result similar to what we observed for phospho-H2A; these foci may represent sites of spontaneous DNA damage or stalled replication forks in S-phase cells (37). However, the percentage of cells with Rad22 or Rhp51 foci was increased in cells arrested in HU and released. In asynchronous cultures, 8.3% ± 0.57% of cells displayed one Rad22-YFP focus, while fewer than 1% of cells exhibited more than one Rad22-YFP focus (Fig. 4D). After 2 h in HU, 24% ± 2.0% of cells displayed at least one Rad22-YFP focus. By 4 h in HU, when nearly all of the cells were elongated and wild-type cells were starting to adapt and reenter the cell cycle, 82.0% ± 3.2% of cells exhibited at least one Rad22-YFP focus (Fig. 4D). Nuclear foci of Rhp51 were detected in cells treated with HU with timing similar to that for Rad22-YFP (Fig. 4E). While just 6.0% ± 2.8% of spread nuclei prepared from cells from asynchronous cultures displayed Rhp51 foci, 18.5% ± 2.1% of spread nuclei prepared from cells incubated with HU for 2 h and 82.0% ± 2.8% of spread nuclei prepared from cells incubated with HU for 4 h contained multiple Rhp51 foci (Fig. 4C). Consistent with results of previous reports (44, 48), we found that the localization of recombination proteins to nuclear foci did not occur immediately upon treatment with HU but occurred during the recovery stage, consistent with the timing of viability loss in HR mutants (Fig. 4B).

To investigate how recombination contributes to survival of replication arrest, we blocked cells in HU for 4 h, released the cells into fresh medium without HU, and assayed DNA content, cell morphology, viability, and DNA damage during arrest and recovery. Wild-type cells elongated and arrested with a ~1C DNA content on treatment with HU and completed bulk DNA replication and retained normal cell morphology and viability when released from HU (Fig. 5A). Similar results were observed for *mcm4ts* cells (Fig. 3C and data not shown) as described above. In contrast, Δ*rad3* checkpoint mutant cells were unable to restrain cell division in the presence of HU, resulting in immediate chromosome missegregation and cell lethality; Δ*cds1* mutant cells initially arrested due to activation of the Chk1-dependent checkpoint, but then they also divided in the presence of DNA damage (8, 48) (Fig. 5). Significantly, we observed that 78% and 77% of the missegregation events in

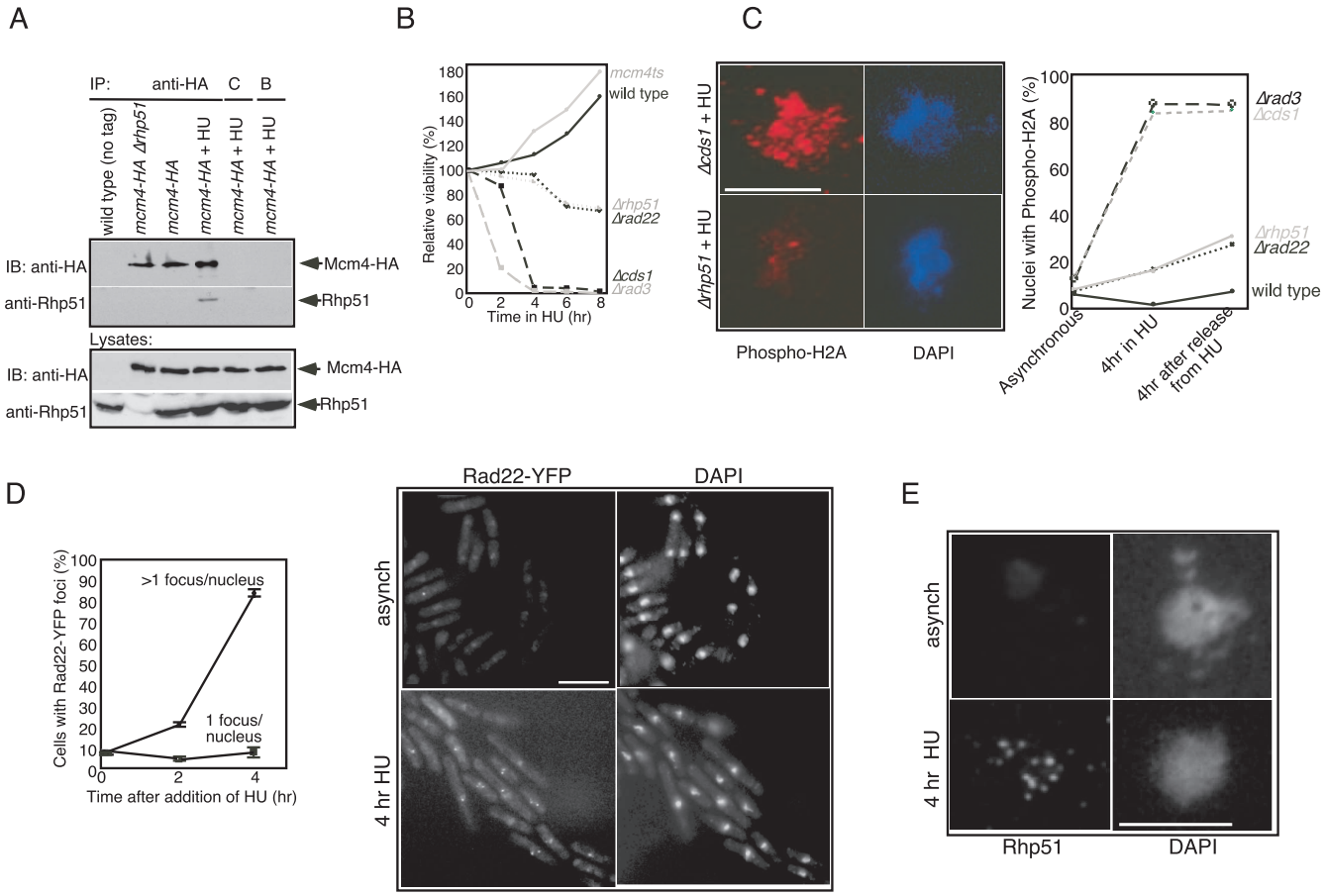

FIG. 4. Recombination proteins in the HU response (A) Mcm4 coprecipitates Rhp51 in HU. Protein lysates prepared from wild-type (FY72), *mcm4-HA* Δ*rhp51* (FY2356), and *mcm4-HA* (FY1167) cells untreated or treated with 15 mM HU for 4 h (+HU) were immunoprecipitated (IP) with anti-HA antibodies, an irrelevant control antibody (lane C), or protein A beads only (lane B). Immunoprecipitates and whole-cell lysates were analyzed by 10% SDS-PAGE, followed by immunoblotting (IB) with antibodies to detect Mcm4-HA (anti-HA) and Rhp51 (anti-Rhp51). (B) HR mutants display a modest loss of viability in HU compared to checkpoint mutants. Wild-type (FY72), *mcm4ts* (FY364), Δ*cds1* (FY865), Δ*rad3* (FY1106), Δ*rad22* (FY1209), and Δ*rhp51* (FY1884) strains were grown in liquid cultures at the permissive temperature (25°C) in the presence of 15 mM HU. Aliquots of cells were plated to YES medium at the indicated time points and incubated at 25°C for 4 days; relative viability was calculated as the number of colonies at each time point relative to the number at the zero time point. (C) HR mutants generate fewer phospho-H2A damage foci than checkpoint mutants, suggesting that most replication forks are stabilized. Asynchronous cultures of wild-type (FY72), Δ*cds1* (FY865), Δ*rad3* (FY1106), Δ*rad22* (FY1209), and Δ*rhp51* (FY1884) cells grown at 25°C were treated with 15 mM HU for 4 h and then released into fresh medium without HU. At the indicated time points, spread nuclei were prepared and immunostained for phospho-H2A. Phospho-H2A focus formation in the Δ*rad3* mutant is due to activity of the Tel1 protein kinase, which acts redundantly with Rad3 (54, 83) (see Fig. S1 in the supplemental material). (D) Rad22-YFP foci form in cells recovering from HU-induced arrest. Asynchronous (asynch) cultures of wild-type (FY72) and Rad22-YFP (FY2878) cells were incubated with 20 mM HU for 4 h at 25°C. At 0, 2, and 4 h after the addition of HU, Rad22-YFP localization was examined and quantitated in live cells (FY2878). (E) Rhp51 localization was assayed by anti-Rhp51 immunostaining of spread nuclei prepared from wild-type cells (strain FY72) treated with HU for 0, 2, or 4 h. The percentage spread nuclei that contained Rhp51 nuclear foci is indicated in the text. DNA was counterstained with DAPI. Scale bar, 10 μM.

Δ*rad3* and Δ*cds1* mutant cells, respectively, displayed the "cell untimely torn" (cut) phenotype, where the cells divided before replication was completed. Cell division was confirmed by immunostaining for tubulin to detect the mitotic spindles (data not shown).

In contrast, the Δ*rhp51* and Δ*rad22* cells elongated and arrested in HU and completed bulk DNA synthesis upon release from HU (Fig. 5B), consistent with the findings of a recent report (48). These results indicate that the recombination mutant cells were competent to activate the checkpoint as well as to resume replication during recovery. However, we found that approximately 25% of HR mutant cells missegregated chromosomes in the subsequent mitosis (Fig. 5B). In contrast to the checkpoint mutants, the main type of chromosome missegregation in HR mutants was unequal division of chromosomes or chromatids (82% of Δ*rhp51* cells and 79% of Δ*rad22* cells), suggesting a defect in spindle attachment, sister chromatid cohesion, or segregation. Interestingly, Meister et al. (48) reported that the deletion of *rhp51*$^+$ allowed some Δ*cds1* cells to complete bulk DNA replication, suggesting that one function of HR proteins during replication arrest and recovery is the resolution of nonproductive DNA structures that form as a consequence of replication fork stalling and collapse. We observed that Δ*cds1* Δ*rhp51* double mutants were heterogeneous in colony size. During acute HU exposure and release, the double mutant was as inviable as the Δ*cds1* single mutant

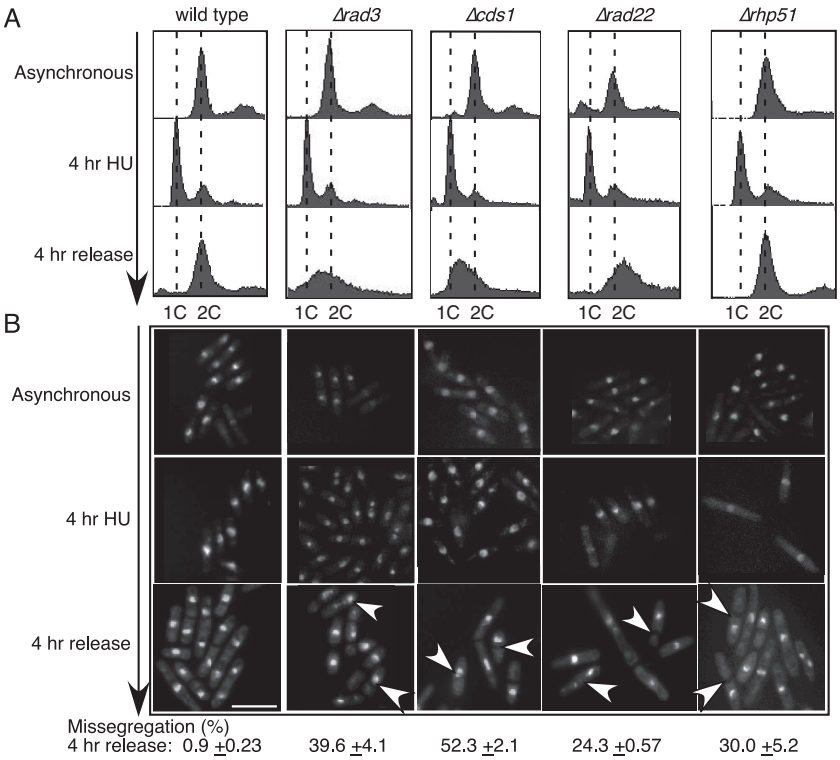

FIG. 5. HR proteins have a role in recovery from HU distinct from that in the checkpoint. Asynchronous cultures of wild-type (FY72), Δ*cds1* (FY865), Δ*rad3* (FY1106), Δ*rad22* (FY1209), and Δ*rhp51* (FY1884) cells grown at 25°C were treated with 15 mM HU for 4 h, then released into fresh medium without HU, and grown for another 4 h at 25°C. Cells were analyzed for (A) DNA content by flow cytometry and (B) nuclear morphology by staining with Sytox green. Arrowheads indicate examples of chromosome missegregation events (detailed in the text); averages and standard deviations were calculated from three independent experiments. Scale bar, 10 μM.

alone, but during chronic exposure, the double mutant was more sensitive to HU, indicating that continued lack of *rhp51* reduces the fitness of the Δ*cds1* mutant, consistent with independent effects on recovery (see Fig. S4 in the supplemental material).

Down-regulation of human MCM proteins by siRNA results in DNA damage and cell death. MCM proteins are highly overexpressed in many human cancers and have also been implicated more directly in cancer etiology (63, 77). We therefore tested whether MCM proteins promote S-phase genome stability in human cells by using siRNA to reduce the levels of MCM4 and MCM7 proteins in HeLa cells (Fig. 6A). Cells that were mock transfected continued to grow and divide, with a 265.0% ± 60.2% increase in cell viability by 48 h. In contrast, cells transfected with MCM siRNA exhibited a modest loss of viability, with just 41.6% ± 19.3% (MCM4) or 53.2% ± 13.1% (MCM7) of the cells remaining viable after 48 h. In experiments where MCM protein levels were not as strongly reduced, cell growth was still inhibited relative to that of cells treated with either mock siRNA or nontargeting control siRNA, although the overall viability of the population of cells did not decrease (data not shown). This result is consistent with observations in yeast that cells with a modest reduction in MCM protein levels still undergo bulk DNA replication, whereas complete depletion of MCM proteins is needed to prevent DNA synthesis (24, 41). In our experiments, MCM4 or MCM7 siRNA did not substantially alter the distribution of G_1, S, and G_2/M cells as analyzed by flow cytometry (Fig. 6B), although a higher proportion of cells displayed abnormal nuclear morphology, such as multinucleate cells or micronuclei (about 30% of cells treated with MCM4 siRNA or MCM7 siRNA compared to about 10% of mock-treated cells) (Fig. 6C). We also found that the siRNA oligonucleotides used to reduce MCM7 levels also partially decreased levels of MCM4, suggesting that loss of function of one of the MCM subunits may affect the stability of other components of the MCM complex. We conclude that the loss of MCM function in HeLa cells, as in yeast, leads to changes in chromosome structure or cell cycle progression that result in cell inviability.

To determine whether the loss of viability resulted from DNA damage, we assessed the focus formation of phosphorylated histone H2AX (phospho-H2AX), the mammalian counterpart to yeast phospho-H2A (64). While just 3.0% ± 1.0% of mock-treated cells contained phospho-H2AX foci after 48 h (Fig. 6D), 78.0% ± 2.8% of cells treated with MCM4 siRNA and 75.0% ± 3.0% of cells treated with MCM7 siRNA contained phospho-H2AX foci (Fig. 6D). To investigate whether this DNA damage localized to replication sites, we pulse-labeled siRNA-treated cells with BrdU, prepared stretched chromatin fibers, and immunostained for Rad51 (the Rhp51 homologue) and BrdU (Fig. 6E). We found that 67% of BrdU-stained chromatin fibers from cells treated with MCM siRNA also displayed Rad51 foci and that the Rad51 foci overlapped with BrdU foci. In contrast, just 8% of BrdU-stained chroma-

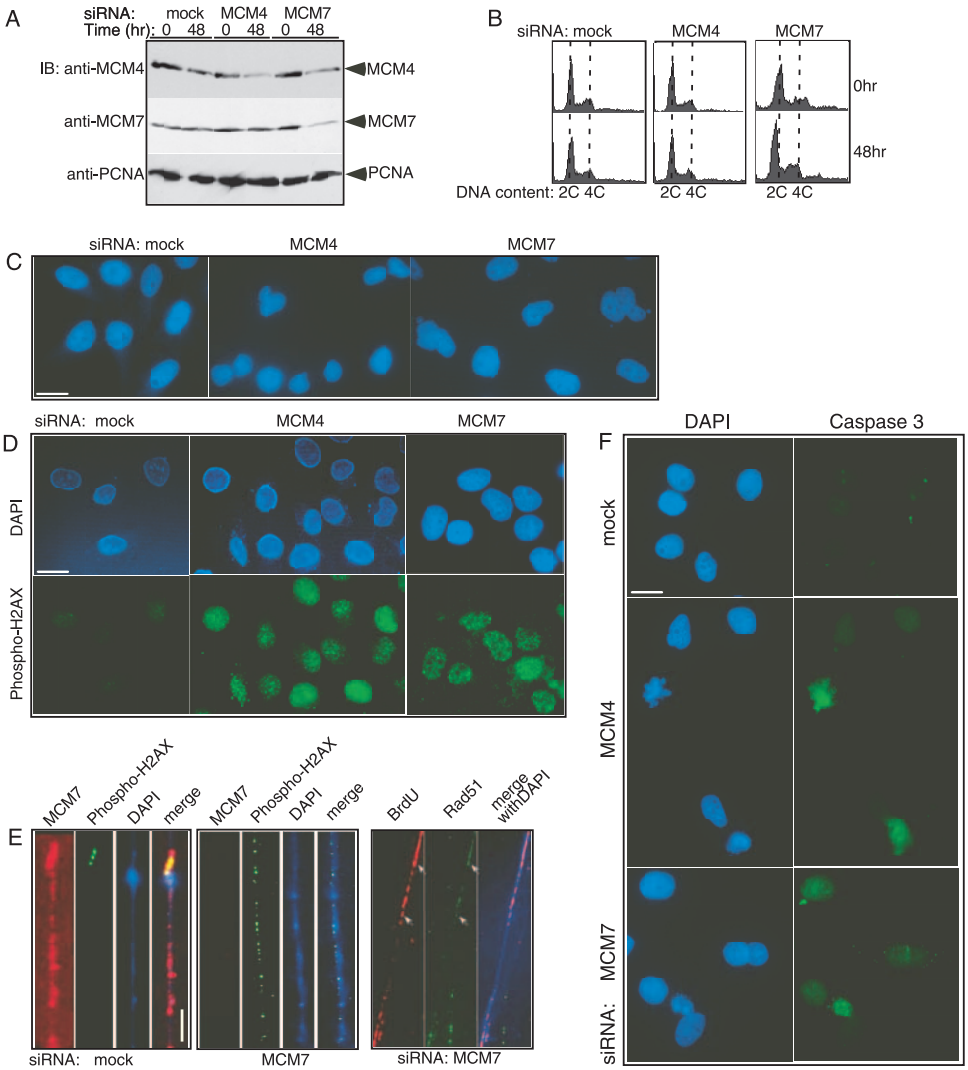

FIG. 6. siRNA against MCM4 or MCM7 in HeLa cells generates DNA damage and cell inviability. HeLa cells were transfected with buffer only (mock) or with siRNA against MCM4 or MCM7, and the cells were analyzed at 0 and 48 h after transfection. (A) Proteins were analyzed by 10% SDS-PAGE, followed by immunoblotting (IB) with antibodies to detect MCM4 or MCM7, and subsequently immunoblotted with anti-PCNA antibody as a loading control. (B) DNA content was assessed by flow cytometry. HeLa cells mock treated or treated with MCM4 siRNA or MCM7 siRNA for 48 h were stained with (C) DAPI to visualize the DNA, and (D) phospho-H2AX and DAPI to detect DNA damage. (E) The DNA damage correlates with loss of MCM localization and corresponds to sites of new DNA synthesis. Stretched chromatin fibers prepared from HeLa cells 48 h after siRNA treatment were immunostained for MCM7 and phospho-H2AX and counterstained with DAPI (left); chromatin fibers prepared from cells pulse-labeled with BrdU for 1 h were immunostained for BrdU and Rad51 and counterstained with DAPI. Scale bar, 10 μm (approximately 20 kb). (F) MCM siRNA results in cell death by apoptosis. Cells mock treated or treated with siRNA against MCM4 or MCM7 were immunostained for cleaved caspase 3 and counterstained with DAPI. Scale bars, 10 μm.

tin fibers from mock-treated cells also contained Rad51 foci. We suggest that as in yeast, the loss of MCM function in HeLa cells is associated with DNA breaks at sites of DNA synthesis. Moreover, the DNA damage resulted in cell death by apoptosis, as cells treated with siRNA against MCM4 or MCM7, but not mock-treated cells, stained for cleaved caspase 3, a marker for activation of the apoptotic pathway (Fig. 6F). We observed similar phenotypes of DNA damage and cell death by using a pool of four additional siRNA duplexes directed against either MCM4 or MCM7, but not with a nontargeting siRNA duplex (data not shown), indicating that the phenotypes we observed are due to specific depletion of these proteins. These findings support a role for the MCM proteins in S-phase genome stability that is conserved from yeast to human cells.

Interactions between MCM proteins and Rad51 in human cells. In fission yeast cells treated with HU, MCM proteins interact with the Rad51 homologue Rhp51 (Fig. 5) and this association may be important for cell survival of replication arrest. To test whether this interaction is conserved in human cells, we examined whether endogenous MCM proteins can coimmunoprecipitate with Rad51 in HeLa cells treated with HU. We found that the interaction between MCM proteins and Rad51 in HeLa cells was not specific to cells treated with HU; it also occurred in asynchronous cultures, as reported recently (70), as well as in cultures of cells released synchro-

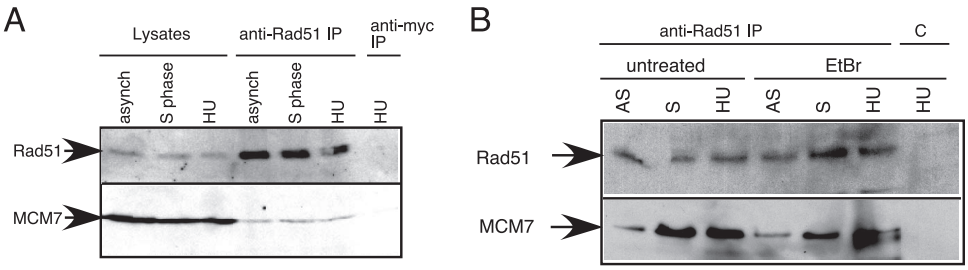

FIG. 7. MCM proteins interact with Rad51 in HeLa cells. (A) Whole-cell lysates prepared from asynchronous cultures of HeLa cells (asynch), cells blocked in 2 mM thymidine for 18 h and then released into S phase for 3 h (S phase), and cells blocked in early S phase with 2.5 mM HU for 18 h (HU) were immunoprecipitated with anti-Rad51 antibodies or with an irrelevant, control antibody (anti-myc). The anti-myc immunoprecipitation (IP) was performed on cells blocked in HU. Rad51 and associated proteins were analyzed by 10% SDS-PAGE, followed by immunoblotting to detect Rad51 and Mcm7. (B) Coimmunoprecipitation of MCM7 with Rad51 from HeLa cells is not dependent on DNA. Anti-Rad51 antibodies immunoprecipitate Rad51 and MCM7 from both untreated HeLa cell lysates and lysates that were treated with ethidium bromide (EtBr). AS, asynchronous cells; S, cells released from thymidine block into S phase; HU, cells arrested in hydroxyurea. C, control IP (anti-myc).

nously into S phase from a thymidine block (Fig. 7A). Furthermore, the association between Rad51 and Mcm7 was still observed when immunoprecipitations were carried out in the presence of ethidium bromide (Fig. 7B) or DNase (data not shown), indicating that the interaction was due to protein-to-protein associations and not mediated by DNA. However, while we observed nuclear MCM7 localization in S-phase cells both in the absence and the presence of HU, Rad51 nuclear foci were found primarily in S-phase cells that had been treated with HU (data not shown). Thus, while MCM proteins interact with Rad51/Rhp51 in both human and yeast cells, the interaction in human cells does not appear to be driven by activation of the replication checkpoint. However, it is possible that the interaction between MCM proteins and Rad51 in untreated cells might reflect a role in response to spontaneous S-phase DNA damage or stalled replication forks that may occur more frequently in the human genome.

DISCUSSION

MCM proteins promote replication fork stabilization and recovery. In response to DNA damage or blocks to DNA replication, checkpoint activation promotes replication fork arrest and also stabilizes fork structure so that the leading and lagging DNA strands and associated proteins remain coupled (12, 15, 34, 46). In this study, we analyze the role of the MCM complex in maintaining genome stability both during normal S phase and in response to replication fork stalling and checkpoint activation caused by treatment with HU. Using fission yeast as a model system, we provide evidence for distinct steps in the recovery process. Once the replication checkpoint is activated, MCM proteins are required to maintain the integrity of arrested replication forks. MCM proteins are also needed for cells to resume synthesis following S-phase arrest. We and others have identified a distinct role for recombination proteins in the recovery process (this work; 1, 48). Here we show that recombination is important for accurate chromosome segregation in the subsequent mitosis, and we suggest that recombination repairs DNA breaks that form spontaneously during replication fork arrest and restart. Thus, the checkpoint, MCM activity, and recombination define separable events that contribute to S-phase genome stability and the survival of replication arrest.

Fission yeast *mcm-ts* mutants undergo lethal Chk1-dependent arrest even though cells appear to complete bulk DNA synthesis (23, 40, 41, 47). Previously, we showed that wild-type MCMs are delocalized from the chromatin and lost from the nucleus when *mcm-ts* mutants are shifted to the restrictive temperature (61). Here, we demonstrate that the inactivation of *mcm-ts* also generates extensive DNA breaks during S phase identified by extensive phospho-H2A staining, and we observed similar results for human cells where MCM function is inhibited by siRNA. That the breaks are linked to active DNA synthesis and replication forks is suggested because the breaks are not observed in a *mcm4ts-degron* mutant that blocks replication elongation and because the DNA damage foci are found in regions of newly synthesized DNA. Thus, we propose that the loss of MCM function causes replication fork breakdown that generates DNA DSBs. This damage may be limited to certain regions of the genome or to regions of replication termination, and genome-wide analysis will be required to distinguish where it occurs.

Interestingly, while the DNA damage in *mcm-ts* mutants activates a DNA damage checkpoint and results in the recruitment of recombination proteins, such as Rhp51 and Rad22, to nuclear foci, the DNA breaks apparently remain unrepaired. In contrast, in prokaryotes, broken replication forks are a substrate for repair by recombination, which reassembles the replisome at the fork and promotes restart of synthesis (reviewed in references 27 and 50). One possibility is that the amount of DNA damage generated in *mcm-ts* mutants is extensive and may exceed the repair capacity of the cell. In support of this model, we observed that the overproduction of Rad22 in *mcm-ts* cells improved cell viability; however, the overproduction of Rad22 also caused a delay in cell cycle progression, suggesting that fewer DNA breaks were formed at any given time (J. M. Bailis and S. L. Forsburg, unpublished data).

MCM proteins have checkpoint-dependent and -independent roles in genome stability. We also find that MCM proteins contribute to fork stability and repair during replication arrest induced by HU. While cells blocked in HU display little

or no DNA DSBs, as assessed by phospho-H2A focus formation, extensive DNA breakage results when MCM function is subsequently lost from stalled forks using the *mcm4ts-degron* allele. This result indicates that the MCM complex is required to maintain fork stability in HU arrest, even after the replication checkpoint kinase Cds1 has been activated. Unexpectedly, we found that the Mcm4ts (nondegron) protein retained its function following HU arrest, correlating with Cds1-dependent phosphorylation. In contrast, the *mcm4ts-degron* strain is HU sensitive even at the permissive temperature and shows reduced modification of the Mcm4ts-degron protein. Together, these data raise the possibility that Mcm4 is a Cds1 substrate. Two motifs for Cds1 family kinases have been identified, RXX-S/T (58) and S/T-F (71), and both motifs exist in the N terminus of SpMcm4. In vertebrates, MCM subunits are substrates of the checkpoint protein kinases ATR and ATM (17, 33, 67, 82). Consistent with a role for checkpoint kinases in regulating MCM proteins, in *S. cerevisiae rad53* or *mrc1* mutants, MCM proteins are delocalized from the replication fork in HU (16). Taken together, these data suggest the MCM complex is a proximal effector of the Cds1 checkpoint, is required for replication fork stability, and may be a direct Cds1 substrate.

An alternative possibility is that *mcm4ts* mutants recover from HU because all replication origins have already fired. In this case, Mcm4 might normally regulate the timing of replication origin firing and no additional origins would need to initiate after the cells are released from HU. However, preliminary data suggest that some origins that normally fire early do not fire in *mcm4ts* (D. D. Luche and S. L. Forsburg, unpublished data). Moreover, when the number of active origins is reduced in budding yeast, such as by mutation of the Cdc7 kinase or MCM subunits, cells can still complete S phase, although more slowly (9, 19, 28). In addition, our comparison of *mcm4ts* mutants and the *mcm4-degron* mutant shifted to the restrictive temperature suggests that the damage does not occur during replication initiation (Fig. 1).

In many respects, *mcm-ts* mutants resemble Δ*cds1* mutants treated with HU: both result in the formation of phospho-H2A foci and the loss of viability, suggesting that both cause replication fork collapse (Fig. 1 and 4) (11, 48, 57). However, there are important differences. Although Δ*cds1* mutants treated with HU activate Chk1 and the DNA damage checkpoint, cell cycle arrest is transient (11) and the cells undergo a lethal mitosis without completing S phase (11, 48, 57) (Fig. 4). Moreover, Δ*cds1* cells in HU retain the capacity to resume synthesis, because at least some cells can complete bulk DNA replication if *rhp51*$^+$ is deleted (48). In contrast, *mcm-ts* mutants undergo Chk1-dependent arrest that is irreversible (23, 41, 47) and the arrest is not suppressed by the deletion of *rhp51*$^+$ or *rad22*$^+$ (J. M. Bailis and S. L. Forsburg, unpublished data). These data suggest that while Cds1 activity is essential to protect the replication fork, possibly in part by stabilizing the MCM proteins, MCM proteins may play additional downstream roles in recovery and reentry into the cell cycle that are Cds1 independent.

Interactions between MCM proteins and recombination during recovery. We found that fission yeast MCM proteins interact with Rhp51 specifically during HU arrest, while human MCM proteins associate with Rad51 both in the presence and the absence of HU (70; this work), suggesting that one such additional role in recovery may be the recruitment of recombination factors to forks that break during recovery. An analysis of Δ*rhp51* or Δ*rad22* mutants following release from HU suggests that cells are proficient to resume DNA synthesis, but defective in chromosome segregation. While the precise defect has not been determined, this result is reminiscent of those of recent studies of budding yeast that suggest recombination proteins, such as the MRN complex and Rad52, are required to tether broken ends together to facilitate proper segregation in mitosis (35, 45) and suggests that the role of HR proteins in HU recovery may be a structural function linked to replication fork restart that is required for proper chromosome segregation. In support of this idea, *S. cerevisiae* Rad51 is needed to maintain the association of DNA polymerase ε with the replisome during arrest (6).

However, recombination could also promote recovery by resolving DNA intermediates. While Sp Δ*rhp51* mutants complete bulk DNA replication after release from HU, as assessed by flow cytometry (48; this work), the chromosomes fail to enter pulsed-field gels (52), suggesting that they contain nonlinear DNA intermediates. While at least some of these intermediate structures are associated with DSBs (this work), they do not activate a G_2/M damage checkpoint. Although a similar phenotype was reported for other recombination mutants, such as *rad60* (52) and *smc6* (1), those mutants may not have activated the checkpoint because the cells arrested prior to mitosis. Thus, while the DNA damage in HR mutants does not activate a checkpoint leading to G_2/M-phase arrest, the breaks may cause uneven separation and segregation of the DNA during mitosis. It remains to be determined whether the role of HR proteins in recovery represents a requirement for active recombination or a structural role in maintaining chromosome integrity as observed in DSB repair mutants in budding yeast (35, 45). Alternatively, DNA breaks in Δ*rhp51* cells may not be in a chromatin context that can be recognized by the checkpoint. This model is supported by evidence that some Δ*rhp51* Δ*cds1* cells can restart replication and suggests that recombination proteins may function both to repair damaged forks and to resolve DNA intermediates formed when forks stall (reviewed in references 10 and 38).

The number of Rhp51 and Rad22 foci that we observe in cells treated with HU is far fewer than the predicted number of active forks after early firing of origins in HU (21), consistent with the possibility that recombination proteins only associate with or accumulate at a subset of replication forks or that damaged forks are assembled into repair centers as suggested for multiple DSBs in *S. cerevisiae* (43). We suggest that the interaction of Rhp51 with MCM proteins during replication fork arrest and recovery may be important to maintain fork stability during replication restart. However, in *mcm-ts* cells, the recruitment of Rhp51 to DNA breaks may instead reflect an MCM-independent attempt to repair forks that have already broken but cannot be processed appropriately. Thus, we speculate there are two forms of HR protein recruitment during S phase: fork recovery, via MCM proteins, and DSB repair, which is MCM independent.

MCM proteins may also contribute to an analogous function as Swi1 and Swi3, which have been proposed to form a fork protection complex (57). We observed that fission yeast *mcm2ts* is synthetically lethal with mutants of *swi1* (W. P. Dolan and S. L. Forsburg, unpublished data). In *S. cerevisiae*,

MCM proteins physically associate with the Swi1 homologue, Tof1 (55), and with Mrc1, a Cds1-adaptor protein which localizes to replication forks and also functions as a replication processivity factor (55, 59, 74, 79). In HU-treated Δ*mrc1* or Δ*tof1* cells, replication forks do not collapse, but the MCM helicase becomes separated from the polymerase on the chromatin as the leading and lagging DNA strands become uncoupled (16, 34, 78). Although Mrc1 contributes to checkpoint activation, the replisome decoupling in *mrc1* mutants is distinct from the irreparable fork collapse of *rad53* (or Sp *cds1*) checkpoint mutants (16, 79). Because MCM proteins interact with both Mrc1 and Cds1/Rad53, we suggest that the MCM complex contributes to S-phase genome stability at multiple levels.

The dysregulation of checkpoint, replication, and repair pathways is strongly linked with the capacity for proliferation and development of cancer (reviewed in references 25 and 80). The MCM complex has recently been used as a molecular marker for malignant cells and has also been proposed as a target for cancer therapy (reviewed in reference 25). The central role of the MCMs is further supported by the recent identification of an allele of Mcm4 linked to mouse mammary adenocarcinoma (68). Additionally, overexpression of the Mcm7 subunit alone can accelerate oncogene-induced tumorigenesis (30). It will be important to determine the mechanisms by which interactions between the MCM, checkpoint, and recombination proteins modulate fork arrest and restart with repair and to regulate whether S-phase arrest is survivable or lethal to cells.

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