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Smc5/6 Is Required for Repair at Collapsed Replication Forks

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In eukaryotes, three pairs of structural-maintenance-of-chromosome (SMC) proteins are found in conserved multisubunit protein complexes required for chromosomal organization. Cohesin, the Smc1/3 complex, mediates sister chromatid cohesion while two condensin complexes containing Smc2/4 facilitate chromosome condensation. Smc5/6 scaffolds an essential complex required for homologous recombination repair. We have examined the response of smc6 mutants to the inhibition of DNA replication. We define homologous recombination-dependent and -independent functions for Smc6 during replication inhibition and provide evidence for a Rad60-independent function within S phase, in addition to a Rad60-dependent function following S phase. Both genetic and physical data show that when forks collapse (i.e., are not stabilized by the Cds1\CHK2 checkpoint), Smc6 is required for the effective repair of resulting lesions but not for the recruitment of recombination proteins. We further demonstrate that when the Rad60-dependent, post-S-phase Smc6 function is compromised, the resulting recombination-dependent DNA intermediates that accumulate following release from replication arrest are not recognized by the G2/M checkpoint.

The eukaryotic structural-maintenance-of-chromosome (SMC) proteins form heterodimers that are the cores of several evolutionarily conserved multisubunit protein complexes. An Smc1/3 complex mediates sister chromatid cohesion during both mitosis and meiosis and is known as cohesin. Chromosome condensation requires two related Smc2/4-based complexes known as condensins (17). Smc5 and Smc6 form a complex along with six non-Smc components (Nse1 to Nse6). An additional protein, Rad60, has been shown to associate with the Smc5/6–Nse1-6 core complex nonstoichiometrically. In Schizosaccharomyces pombe, Smc5, Smc6, and Nse1-4 complex components are essential and loss of function results in chromosomal fragmentation that requires passage through S phase. Hypomorphic smc6, nse1, nse2, nse3, and nse4 mutants are all defective in homologous recombination repair (HRR) (5, 14, 16, 27, 33, 39, 45, 54). Similar observations have been made for Saccharomyces cerevisiae, where compromising the Smc5/6 complex function interferes with ribosomal DNA (rDNA) segregation and sister chromatid exchange (11, 19, 53). In contrast, S. pombe Nse5 and Nse6 are nonessential. nse5 and nse6 null mutants grow slowly and exhibit repair defects similar to those of the smc5-6 complex hypomorphic mutants (46).

Of the non-SMC components, Nse1 contains a RING domain typical of ubiquitin E3 ligases, although its substrates and activity remain unidentified. Nse2 is a SUMO E3 ligase and directs the sumoylation of multiple substrates, including S. pombe Smc6 and S. cerevisiae and human Smc5. Nse3 is a member of the MAGE (melanoma antigen-encoding gene) superfamily and, like Nse4, is a conserved protein of an unknown function (2, 33, 45, 47, 50, 56). The core subunit components localize to the nucleus together with the noncore component Rad60, which has a C-terminal ubiquitin-like domain. Upon S-phase checkpoint activation by the ribonucleotide reductase inhibitor hydroxyurea (HU), Cds1CHK2 kinase phosphorylates Rad60, which is then excluded from the nucleus (5).

The Smc5/6 complex function was first characterized by genetic analysis of S. pombe smc6, formerly known as rad18 (14, 27, 54). smc6-X and smc6-74 are two separation-of-function mutants that are DNA damage sensitive and are defective in HRR pathways but are proficient in the essential function at all temperatures. Conditional mutants (e.g., smc6-T2) exhibit similar but temperature-dependent sensitivities to DNA damage and lose viability over several generations at 35°C. All the S. pombe conditional mutations map to the hinge region (50) (Fig. 1A). The smc6-X mutation (R706C) also maps close to the hinge. In contrast, the smc6-74 mutation (A151T) maps to the highly conserved arginine finger (25) close to the ATP binding site in the N-terminal globular domain, suggesting that it might affect DNA-dependent ATP binding/hydrolysis. (ATP binding site mutations are lethal [14, 54].) smc6-74 mutant cells have previously been shown to be proficient in DNA damage checkpoint initiation following UV-C irradiation but do not maintain checkpoint arrest in the presence of unrepaired damage (16, 54). Intriguingly, smc6-74 damage sensitivity, but not smc6-X sensitivity, is suppressed by overexpression of Brc1, a 6-BRCT (BRCA1 C-terminal) domain protein (54). Brc1 has homology to S. cerevisiae Esc4/Rtt107 and similarities with human Pax2 transactivation domain-interacting protein (PTIP), both of which function in DNA repair during S phase (20, 48, 49). Brc1-dependent smc6-74 suppression is dependent on structure-specific nucleases (Ssl1/4 and Mus81/Eme1) and homologous recombination (HR), and we have interpreted this to suggest that smc6-74 cells are defective in a step of recombination that can be bypassed or resolved by alternative mechanisms (51).

Previous work characterizing the smc6-X mutant HRR re-

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response showed that at low UV doses, survival is rescued by concomitant rhp51\textsuperscript{RAD51} deletion (27). A similar phenomenon was observed for the S. pombe RecQ helicase mutant rqh1\textsuperscript{-d} (26). During HRR in G2, Rqh1 and Top3 act in a manner consistent with double Holliday junction (HJ) dissolution (7, 8). In vitro, the human RecQ helicase BLM and Top3 converge the two HJs and decatenate the resulting hemicatenoid (55). Because RecQ helicases have been implicated in the regulation of recombination at stalled or collapsed replication forks and since functions of the Smc5/6 complex have been indirectly ascribed to S phase, we examined the role of Smc6 during replication arrest due to nucleotide (deoxynucleoside triphosphate [dNTP]) depletion promoted by HU.

We show that replication intermediates are normal in smc6\textsuperscript{-d} mutants both in the presence and in the absence of replication arrest in checkpoint-proficient cells. During replication arrest...
TABLE 1. Strains

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MATERIALS AND METHODS

Genetics and cell biology techniques. Strains were constructed by standard genetic techniques (36) and are listed in Table 1. The smc6-6 MYC strain, constructed by one-step integration (3), has a wild-type generation time at all temperatures and no sensitivity to DNA-damaging agents, such as ionizing radiation (IR) and UV, but is slightly sensitive to higher doses of HU (7.5 mM as opposed to 10 mM for the wild type). Replication was inhibited by the addition of 10 mM HU, and cells were grown at 30°C unless otherwise indicated. The protocols for checkpoint measurements, cell scoring, centrifugal elutriation, and fluorescence-activated cell sorter (FACS) analysis are all described in reference 13.

Microscopy. For Smc6 visualization, an in vivo chromatin binding assay (22) was employed. Anti-Smc6 (50) was used at 1:60 and anti-MYC at 1:200. To visualize asynchronous and HU-blocked cells under the same coverslip, Triton X-100-extracted cells were washed twice in 1 mM CaCl2-Tris-buffered saline (TBS) and HU-blocked cells incubated in 1 mM CaCl2-TBS containing 2 μg/ml Texas Red G5-1 lectin. Cells were washed in CaCl2-TBS and fixed in methanol-acetone before mixing HU-treated/untreated cells and subjecting them to anti-body staining. Live-cell imaging of Rad22-GFP foci was carried out in minimal media (EMM2) at room temperature. To determine the percentages of cells with Rad22 foci, >300 nuclei were scored for each sample.

in the absence of Cds1^{chk2} (when replication forks rapidly collapse in the presence of HU [29]), a phenomenon associated with dissociation of the replicative polymerases (Pol) (10, 21, 32), replication intermediates are stable in the smc6 mutant cells compared to those in smc6^+ cells. Despite the fact that a similar phenomenon is observed in recombination Y84-6 (34), smc6 mutants are proficient in recruiting HR proteins to the chromatin when forks collapse, suggesting that the Smc5/6 complex is involved in later stages of HR under these circumstances. Consistent with this, we show that Smc5/6-dependent functions are required for effective HR-dependent repair of lesions resulting from HU-induced replication arrest and that the HR-dependent DNA structures that accumulate in smc6 mutants are not recognized by the G2 DNA damage checkpoint. We also show that, unlike Rad60, which is subjected to nuclear exclusion during S-phase arrest, Smc6 functions during, as well as upon release from, HU arrest and is chromatin bound in HU-treated cells.
ChIP. Chromatin immunoprecipitation (ChIP) was performed using a protocol modified from that described in reference 52. Anti-Smc6 was used at 1:15, and anti-MYC was used at 1:100. Antibodies were captured with G-protein Dynabeads. Efficiency of immunoprecipitation (IP) was monitored by Western blot analysis of input and IP samples. The relative amounts of PCR products were quantified by real-time quantitative PCR (qPCR) on 5 μl of each sample, using Quantitect SYBR green PCR master mix (QIAGEN). Enrichments (n-fold) were calculated as previously described (9). Briefly, since the number of molecules doubles in each cycle, enrichment (n-fold) was calculated according to the formula 2^{ΔCt–ΔCtcontrol}, in which ΔCt is the difference between the number of cycles required to go above background in input and IP samples and ΔCtcontrol is calculated from parallel input and IP samples from unstained strands or beads only or from nonspecific antibody controls. Since enrichment is calculated from the difference in the cycles, it is independent of the copy number of the genomic locus. Two or three sets of primer pairs were tested for the three positions within the rDNA repeat (the replication fork barrier [RFB], the autonomously replicating sequence ars3001, and the 17S gene), and a representative pair was used. Primers corresponding to the ade6 locus, ars3005 and ars2004 and control regions, 10 kb and 20 kb telomere proximal, respectively, were used to amplify unique loci. Primer sequences are available upon request. qPCR reactions were carried out in duplicate and ChIP data averaged over two or three independent experiments.

### RESULTS

**smc6 mutants are HR defective in response to replication inhibition.** smc6-X and smc6-74 are sensitive to the inhibition of DNA replication by HU (Fig. 1B). smc6-74 was consistently less sensitive than smc6-X, and HU sensitivity was suppressed in smc6-74, but not in smc6-X, by overexpression of brc1. This is consistent with the previously reported suppression of methyl methanesulfonate (MMS) and UV sensitivity (51, 54). Overexpression of brc1 slightly sensitizes smc6+ cells to HU. Thus, survival is dependent on a balance between Smc6- and Brc1-dependent pathways. The difference between the two hypomorphic smc6 mutants suggests that they have distinct defects in responding to HU, as we have previously observed for MMS resistance (51). We therefore further characterized both smc6 mutants in parallel.

Since Smc6 has been shown to function in HRR and, at low UV doses, sensitivity is rescued by concomitant rhp51RAD51 deletion (27), we examined the roles of Smc6 and HRR during replication inhibition by HU. Double mutants with rhp51Δ and rhp55Δ null alleles are not more sensitive than the single mutants, consistent with a function for Smc6 in the HR pathway. Figure 1C shows epistasis between smc6-X and rhp51 (the most sensitive pair) (Fig. 1C, middle panel) and smc6-74 and rhp55-d (the least sensitive pair) (Fig. 1C, bottom panel). Similar results were obtained with smc6-X rhp55-d and smc6-74 rhp51-d (data not shown). smc6 mutants are more sensitive than the recombination mutants but are partially rescued by concomitant deletion of recombination genes. This suggests that stalled replication forks in the smc6 mutants are the target of illegitimate lethal recombination events or that Smc6 is required at a late stage of legitimate HR in response to HU. In the second eventuality, the HR-dependent structures that accumulate in smc6 mutants would be lethal but, in the absence of HR, an alternative pathway could repair the initial structure.

In *S. pombe*, Rhp55Rad55 acts upstream of Rhp51Rad51 in parallel to a second recombination mediator, Swi5 (1). Like loss of rhp51Rad51, loss of rhp55Rad55 rescues smc6 mutant sensitivity to HU. Loss of swi5 does not (Fig. 1D). This is reminiscent of the requirement for the Rhp55/57 complex for Brcl-mediated suppression of the MMS sensitivity of smc6-74, which is also independent of Swi5 (16). A similar pattern of suppression is observed for the *S. pombe* RecQ helicase mutant *rhl1*-d (18, 35; also unpublished data) and for rad60-1 (35). In contrast, the sensitivities of nse5 and nse6 mutants (nonessential components of the Smc5/6 complex) to UV are suppressed by rhp51-d and also weakly by both swi3-d and rhp55-d (46).

* S. pombe * Rad22Rad55 is required for all recombination. We have consistently observed that smc6 mutants are more sensitive in rad22ΔRad55 mutant backgrounds than the single mutants (Fig. 1C, top panel). However, because rad22ΔRad55 mutant cells are slow growing and rapidly acquire suppressor mutations in *fbh1* (38, 44), further epistasis analysis is not feasible.

Replication forks arrested in HU are stabilized by activation of the Cds1CHK2-dependent intra-S-phase checkpoint. In the absence of Cds1CHK2, forks “collapse” (29), a process thought to involve the dissociation of the replicative apparatus from nascent DNA ends (10, 21, 32). Collapsed forks recruit recombination proteins (30, 34), and this is suggested to help restart replication. This requirement for recombination during replication resumption would predict that loss of recombination functions would increase cds1ΔCHK2 mutant sensitivity to low doses (1 to 2 mM) of HU. A slight increase in sensitivity is indeed seen in recombination cds1ΔCHK2 double mutants (Fig. 1C). In contrast, smc6 cds1ΔCHK2 double mutants are much more sensitive than either of the single mutants (Fig. 1C). Loss of recombination genes *rhp51rad51* and *rhp55rad55* partially rescues *smc6 cds1ΔCHK2* double-mutant sensitivity. This again suggests either that smc6 mutants are defective at a late stage of HR or that in smc6 mutants, some collapsed replication forks are illegitimately processed by HR. The latter scenario would result in lethal HR-dependent intermediates. The rescue of viability is not at the level seen in *rhp51-d cds1-d* cells, suggesting that the Smc5/6 complex also has functions independent of HR in tolerating the HU arrest of replication forks.

**smc6 mutants are defective in processing collapsed forks.** To establish how smc6 mutants affected recombination at stalled and collapsed forks, we used 2-D gel electrophoresis to visualize replication intermediates (31) from the intergenic region of the rDNA (Fig. 2). This region contains ars3001 and RFBs that ensure unidirectional rDNA replication. In both asynchronous cultures and HU-arrested cells, the replication intermediates observed in smc6 mutants were similar to those from smc6+ cells and replication forks were stably maintained at the RFBs, as judged by the occurrence of pause spots on the large Y arc. The X spike (recombination intermediates) was slightly increased in smc6-X cells both with and without treatment. Notably, no aberrant structures are seen in smc6-74. Consistent with previous analysis (24), we also observed the loss of Swi1/Swi3-dependent pause sites in the presence of HU (in the asynchronous cultures, two pause sites are visible on the Y arc, and only one is visible in HU-blocked cells). These data indicate that in the smc6 mutants, replication forks were stably maintained when stalled both specifically at the replication fork barriers and randomly due to dNTP depletion.

We next examined a role for Smc6 at collapsed replication
forks. In *cds1<sup>CDS1</sup>* mutants treated with HU, replication forks collapse (29). This leads to the loss of replication intermediates (bubble and large Y arcs) and the accumulation of small Y molecules and a cone-shaped signal resulting from molecules migrating similarly to double-Y- and/or X-shaped structures (31, 34, 43). Consistently, we see neither the bubble arc nor large Y structures and no pause sites in HU-treated *cds1<sup>CDS1</sup>* cells. (No cone-shaped signal was detected using this probe, but this signal was visible when a shorter HindIII-KpnI fragment lacking the RFBs was used as a probe, suggesting that the pause sites are particularly unstable [data not shown].) Surprisingly, bubble arcs, large Y, and pause structures are clearly present in DNA from HU-treated *smc6*—*ars3001* (Fig. 1C), and the accumulation of small Y molecules and a cone-shaped signal resulting from molecules (bubble and large Y arcs) and the accumulation of small Y structures is preserved and the X spike increases, consistent with an increase in recombination intermediates.

*smc6* mutants lose viability upon entry into mitosis. In HU-treated cells, replication forks are stably maintained due to the activation of the intra-S-phase checkpoint. Checkpoint-defective *cds1<sup>CDS1</sup>* null cells rapidly lose viability during an HU-induced S-phase arrest but do not enter mitosis because the DNA damage checkpoint is activated by the structures that arise as forks collapse, and the cells elongate due to the checkpoint delay (29). In contrast, recombination mutants, such as *rhp51-d*, also elongate in response to HU but are not specifically sensitive to a transient inhibition of replication. This would be consistent with a requirement for HR over the small proportion of forks that collapse, the majority of forks would be consistent with a requirement for HRR at only the intra-S checkpoint. We assayed the viability of *smc6* and *smc6*—*cds1<sup>CDS1</sup>* double mutants during a transient HU exposure. *smc6-X* and *smc6-74* mutants start to die 4 to 6 h after exposure (Fig. 3A). At 6 h, wild-type and *smc6* mutant cells overcome the replication block, have completed bulk DNA synthesis to obtain a 2N DNA content (Fig. 3B), and start entering mitosis. Thus, unlike *Cds1<sup>CDS1</sup>*, Smc6 is required when cells resume/complete replication rather than to maintain stalled replication forks. In *smc6*—*cds1<sup>CDS1</sup>* double mutants, where the intra-S-phase checkpoint defect results in the collapse of the majority of forks, viability is lost with the same kinetics as that seen for *cds1<sup>CDS1</sup>* null cells. These data suggest that in checkpoint-proficient cells, Smc6 is required at a specific subset of stalled forks which are not stable and collapse.

**Rad22<sup>Rad22</sup>** focus formation is normal in *smc6* mutants. Previous work (30, 34) has shown a temporal separation of replication and recombination. HU-arrested cells have low levels of recombination foci. Upon release from arrest, approximately 20% of cells transiently acquire one or two foci. In order to further investigate the role of Smc6 in the resumption of replication, we monitored recombination protein foci both during HU arrest and upon release from HU. We assayed the occurrence of Rad22<sup>Rad22</sup> foci in HU-treated *smc6*—*ars3001* and *smc6*—*ars3001* mutant cells. After 4 h in HU, only ~2% of *smc6*—*ars3001* cells displayed Rad22<sup>Rad22</sup> foci (Fig. 4A and B) and Rhp51<sup>Rhp51</sup> (data not shown) foci. *smc6-X* and *smc6-74* cells showed similar levels (4% and 2%, respectively) (Fig. 4B). Thus, in the *smc6*
mutants, stalled replication forks do not lead to an increase in recombination foci.

Ninety minutes after release from HU arrest at room temperature, ~20% of smc6+/H11001 smc6 cells have one or two Rad22 Rad52 foci. Similar percentages of smc6- and smc6-74 cells acquired foci upon release from HU arrest (Fig. 4A and B). Focus numbers peaked at 90 min and had mostly disappeared by 120 min, before cells entered mitosis at ~150 min (Fig. 4B). Focus loss and mitotic entry were both slightly delayed in smc6- cells. Thus, the functions defective in smc6 mutants are not required for recombination foci upon release from HU.

When replication forks collapse in checkpoint-defective cds1CHK2 null cells, the majority of nuclei contain multiple recombination foci (34). We found that cds1CHK2 null cells and cds1CHK2 smc6 double mutants exposed to HU acquired multiple Rad22RaRad52 foci that remained after release (Fig. 4C). The cells remain checkpoint arrested and do not progress into mitosis during the course of the experiment (Fig. 4D). This shows that the functions defective in smc6 mutants are not required to form recombination foci in response to replication fork collapse.

smc6 mutants have a checkpoint maintenance defect. The kinetics of mitotic entry in smc6 mutants following release from an HU arrest is similar to that seen for smc6+ (Fig. 5A). This is consistent with previous work showing that Smc5/6 complex mutants are proficient in the activation of the G2 DNA damage checkpoint (11, 16, 19, 46, 53). However, in contrast to smc6+/H11001 cells, smc6 mutant cells exhibit high levels of morphologically aberrant mitosis; the chromosomes missegregate or fail to separate before septation, leading to a cut phenotype (Fig. 5A and B). This indicates a failure to maintain the Chk1-dependent G2 DNA damage checkpoint even though DNA repair is not complete. Consistent with a dependence on HR for this incomplete repair, the appearance of cut cells is suppressed by deletion of recombination genes (rhp55 RAD55 [Fig. 5A] and rhp51 RAD51 [not shown]). A similar suppression of the cut phenotype is seen when brc1 is overexpressed in smc6-74 cells (Fig. 5C). This is consistent with the suppression of the checkpoint maintenance defect after UV treatment (54).

Smc6 is required for viability when replication is inhibited. Recent work has demonstrated that rad60-1 hypomorphic mutant cells accumulate Rhp51RaRad51-dependent recombination intermediates upon release from HU and that, like those that accumulated in smc6 mutants, such structures do not generate a checkpoint arrest and thus result in aberrant mitoses (35). Rad60 associates with the Smc5/6 complex but unlike the core
subunits is excluded from the nucleus in response to HU following its phosphorylation by Cds1 CHK2 (5). Rad60 is thus likely to be required only after release from HU but not during arrest in HU. Consistent with this, Miyabe et al. used a temperature-sensitive rad60 mutant (rad60-1) to demonstrate that loss of the Rad60 function during HU arrest did not result in viability loss (35).

Because Smc6 remains in the nucleus during an HU arrest, we wished to ascertain whether Smc6 is required for chromosome integrity during this arrest in addition to its common role in recombination. FIG. 4. Recombination focus formation is normal in smc6 mutants. (A) Rad22Rad52 foci (live-cell imaging) in HU and upon release. rad22-GFP cells were arrested for 4 h (10 mM HU) at 30°C and released at 18°C. In smc6+, smc6-74, and smc6-X, the Rad22Rad52 signal was diffuse in the nucleus in HU, but cells with one or two nuclear foci became visible upon release. (B) Quantification of Rad52Rad52 foci and mitotic cells upon HU release. Foci formed with similar kinetics in smc6+, smc6-X, and smc6-74 cultures, peaking at ∼90 min after release, and disappeared prior to mitosis. (C and D) In contrast, smc6 cds1CHK2 double mutants, like cds1CHK2 null cells, showed multiple nuclear Rad22Rad52 foci during HU treatment (examples are shown in panel C) and these remained for the duration of the experiment after release (D).
requirement with Rad60 upon resumption of cell cycle progression following HU withdrawal. We thus arrested smc6<sup>-/-</sup>, rad60<sup>-/-</sup>, and a temperature-sensitive smc6 mutant (smc6-T2) (50) in HU at 36°C (inactive Rad60 or Smc6), followed by release at 22°C (permissive for rad60<sup>-/-</sup> and smc6-T2). We compared the results to those for a regimen in which the same mutants were arrested in HU at 22°C and released at 36°C (Fig. 6). Inactivating Smc6 during HU blocking led to the appearance of aberrant mitotic cells, which were not seen when Rad60 was inactivated during the HU block, and approximately 55% viability compared to that of smc6<sup>-/-</sup> (rad60<sup>-/-</sup> was 88% viable). As expected, inactivation of either Smc6 or

FIG. 5. smc6 mutants have a checkpoint maintenance defect. Cells were arrested for 4 h (10 mM HU) at 30°C, and cell cycle progression was monitored (diamonds, mitotic index; open squares, septation index) following release at 30°C. (A) smc6<sup>+/</sup>, smc6-X, and smc6-74 cultures all delayed mitosis for ~90 min, indicating that the G2/M checkpoint is intact but smc6-X and smc6-74 cultures accumulate aberrant mitotic figures and “cut” cells (closed squares) (examples are shown in panel B). Under the same conditions, few “cut” cells were seen in rhp55-d, rhp55-d smc6-74, or rhp55-d smc6-X cultures. Thus, in smc6 mutants, recombination-dependent structures cause mitotic aberrations. These structures are not recognized by the G2/M checkpoint. (C) Overexpression of brc1 suppresses the accumulation of “cut” cells in smc6-74. Left, smc6-74 cells with an empty vector control (pREP41); right, smc6-74 cells with pREP41brc1. Symbols are as defined in panel A.
Rad60 immediately after HU release caused the appearance of aberrant mitotic cells in both cultures (Fig. 6) and loss of viability (rad60-1 was 48% viable and smc6-T2 was 51% viable). Consistent with smc6-T2 taking at least two cell cycles to start to lose viability (50), incubation at the nonpermissive temperature but not in regimen A (in which cells were HU blocked at the nonpermissive temperature and released into the permissive temperature), indicating that the Rad60 function is required only when the cells resume replication. In contrast, smc6-T2 accumulated aberrant mitotic cells in both regimen A and regimen B, suggesting that the Smc6 function is required both when replication is inhibited (Rad60 is not required) and when replication resumes upon release (a common requirement with Rad60). wt, wild type. (D) Viabilities observed after treatment with regimens A, B, and C or after incubation for 5 h at 36°C expressed as percentages of the smc6+ value.

**FIG. 6.** Smc6 is required in HU and upon release. smc6+, rad60-1, and smc6-T2 cells were incubated in HU (10 mM, 2.5 h) at the nonpermissive temperature for smc6-T2 and rad60-1 (36°C) and released into fresh media at the permissive temperature (22°C) (A), incubated in HU (10 mM, 4.5 h) at the permissive temperature (22°C) and released into fresh media at the nonpermissive temperature for smc6-T2 and rad60-1 (36°C) (B), or incubated in HU (10 mM, 4.5 h) at the permissive temperature and released at the same temperature (22°C) (C). Cell cycle progression following release (time zero) was monitored by septation index, and the percentages of aberrant mitotic or “cut” cells were scored. smc6+, rad60-1, and smc6-T2 entered mitosis with similar kinetics. rad60-1 accumulated aberrant mitotic cells only in regimen B (in which cells were released into the nonpermissive temperature) but not in regimen A (in which cells were HU blocked at the nonpermissive temperature and released into the permissive temperature), indicating that the Rad60 function is required only when the cells resume replication. In contrast, smc6-T2 accumulated aberrant cells in both regimen A and regimen B, suggesting that the Smc6 function is required both when replication is inhibited (Rad60 is not required) and when replication resumes upon release (a common requirement with Rad60). wt, wild type. (D) Viabilities observed after treatment with regimens A, B, and C or after incubation for 5 h at 36°C expressed as percentages of the smc6+ value.

**Smc6 localizes to the nucleolus and accumulates after HU.** Smc5/6 complex components localize to the nucleus (5, 33, 53, 54, 56). We used an in situ chromatin binding assay to establish the subnuclear localization of Smc6 in untreated, S-phase-arrested, and DNA-damaged cells (22). In asynchronous cultures, all cells showed a background diffuse nuclear staining and Triton X-100-insoluble nuclear foci that were not visible after DNase I treatment. The foci did not colocalize with Swi6 in heterochromatin (centromeres, MAT loci, and telomeres) or with Taz1 at telomeres but did colocalize with the nucleolar component Gar2 (Fig. 7A). Smc6 had a more compact distribution than Gar2, which could be indicative of a distinct localization within the nucleolus. HU treatment significantly increased focus intensity but did not change the pattern of localization (Fig. 7B). In order to confirm the increases in Smc6 focus intensity, asynchronous and HU-blocked cells (cell wall-stained with Texas Red-conjugated lectin) were observed under the same coverslip (Fig. 7C). The intensity of the Smc6
nuclear signal was increased in HU-blocked cells compared to that in asynchronous cells. However, incubation in the lectin staining buffer led to the distribution of Smc6 throughout the nucleus rather than restricted to nucleolar foci, suggesting that the subcellular localization of Smc6 is sensitive to the method of sample preparation. The increase in intensity was independent of cell wall staining, as a parallel experiment with the asynchronous cells marked with Texas Red-conjugated lectin also showed the HU-blocked cells to have a more intense nuclear staining (data not shown).

Similar increases in intensity were seen in replication mutants cdc23-M36 (MCM10), cdc20-M10 (Pol e), cdc1-P7 (Pol δ), cdc6-23 (Pol δ), cdc22-M45 (RNR), cdc17-K42 (DNA ligase 1), and cdc24-M38 (RFC and PCNA interacting factor) at the restrictive temperature (Fig. 7D and data not shown). Notably, no increase was seen in cdc21-M68 (MCM4) at the restrictive temperature (data not shown), suggesting that either replication initiation is required or loss of the MCM replicative helicase does not lead to an increase in nuclear Smc6. By Western blot analysis, this increase in Smc6 is due to protein relocalization, as total protein levels do not change (Fig. 7E).

Since these replication mutants arrest with a 2C DNA content (42), we used IR to activate the G2 DNA damage checkpoint. No change was observed after IR (not shown), demonstrating that the increased focus intensity correlated with replicative stress. Localization of Smc6 to the nucleolus could indicate a compartmentalization of Smc6 away from Rad60 (cytoplasmic in HU) but is also consistent with a requirement for Smc6 at the ~150 rDNA repeats, as has been previously described for S. cerevisiae (53).

Smc6 is associated with chromatin. Our data suggest that Smc6 is required both during HU arrest and upon release from arrest. To ascertain whether Smc6 associated with chromatin within the rDNA or other loci, we performed ChIP assays. These assays employed qPCR with primers at three positions within the rDNA repeat (RFB, ars3001, and the 17S gene) and five unique loci (ars3005, 10 kb telomere proximal to ars3005, ars2004, 20 kb centromere proximal to ars2004, and the ade6 ORF) (Fig. 8). In contrast to S. cerevisiae Smc6, which was reported to be enriched at repeat sequences, including rDNA and telomeres (53), Smc6 was modestly enriched at all loci, both rDNA and unique, compared to negative controls (beads only or nonspecific antibody). Consistent with the microscopy results, HU treatment modestly but reproducibly increased enrichment. Similar data were obtained using anti-MYC ChIP and smc6-13MYC cells normalized to anti-MYC ChIP from an untagged strain. Thus, Smc6 is chromatin associated, and this increased in response to inhibition of replication.

**DISCUSSION**

**Function for Smc5/6 at collapsed replication forks.** We have examined the role of Smc6 during replication arrest due to dNTP depletion promoted by HU. While smc6 mutants are sensitive to HU, we show by using 2-D gels that HU-arrested replication intermediates are normal in smc6 mutants when checkpoints are intact. Upon transient HU exposure, smc6 mutant cells lose viability when the cells are overcoming the block, have a 2C DNA content, and are starting to enter mitosis. A significant proportion of smc6 mutant cells, but not smc6-6 cells, exhibit cut phenotypes, suggesting the persistence of unrepaired DNA structures during mitosis that impede chromosome segregation. These data suggest that, rather than being required for stabilization of stalled replication forks, the Smc5/6 complex is required either for the resumption of replication at or for the repair of a subset of forks that are unstable and have collapsed.

We examined the requirement for Smc6 at collapsed forks by arresting the checkpoint-defective mutant cts1-d in HU. One important function of the Cds1 CHK2-dependent inter-S-phase checkpoint in yeast is to stabilize stalled replication forks and prevent them from collapsing (29). Fork collapse is thought to involve the movement of replicative helicases ahead of the site of DNA incorporation (21) and the dissociation of DNA polymerases from nascent strands (10, 32). A stable fork is capable of rapid resumption when dNTP synthesis resumes. A collapsed fork, however, requires processing to restore the ability to replicate. It is not known precisely how this occurs, but a role for HR proteins is proposed in most models. We observed that when forks collapse in HU-treated cts1-CHK2 cells, replication intermediates remain stable in smc6 mutants, while they decay in smc6-6 cells. This phenomenon has recently been noted for rhp51ΔadΔ mutants. However, this common phenotype was not a consequence of an inability to associate recombination proteins with chromatin when replication forks collapse, because we observed that focus formation was normal. Together, these data indicate that Smc6 functions to regulate aspects of HR-dependent DNA processing in response to fork collapse.

**Regulation of recombination at HU-arrested forks.** A role for the Smc5/6 complex in HRR in response to a variety of DNA damaging agents has been well documented. We show here that the HU sensitivities of smc6 mutants can be suppressed by deletion of recombination gene rhp51 or rhp55 but not by deletion of rad22 or swi5. This is similar to the suppression of smc6-X at low UV doses by concomitant rhp51ΔRAD51 deletion (27). Such suppression can be interpreted in two ways:
Smc6 could have an antirecombinogenic role, with stalled forks in the smc6 mutants becoming targets for illegitimate and lethal recombination events, or alternatively, the smc6 mutants may be defective in a late stage of HR and accumulate lethal recombination intermediates.

To distinguish between these possibilities, we assayed the formation of recombination foci. If the Smc5/6 complex acts to suppress recombination at stalled replication forks, then smc6 mutants arrested in HU are predicted to have increased levels of Rad22 foci and recombination intermediates. While for smc6-X cells, cells with recombination foci (4%, compared to 2% in smc6+/H11001 cells [Fig. 4]) and X-shaped DNA structures increased slightly (Fig. 2), the effect is not dramatic and no increase was observed in smc6-74 cells, suggesting that this is not the major defect in these cells.

It is not clear why HRR would be required at replication forks stalled by dNTP depletion, as there is no apparent lesion to be repaired, but HRR mutants are sensitive to HU, and this has led to the idea that HRR is required for a small proportion of stalled forks that become unstable and collapse. Collapsed forks are known to recruit recombination proteins (30, 34). While few recombination foci are seen in cells arrested in HU, cells with recombination foci transiently become visible upon release, suggesting a separation of replication and recombination (30, 34) and consistent with the time when smc6 mutant cells lose viability. This leads us to propose that the Smc5/6 complex is required to regulate a late stage of recombination for the subset of stalled forks that become unstable and collapse.

Smc6 mutants accumulate aberrant DNA structures not recognized by the damage checkpoint. We examined a role for Smc6 during release of checkpoint-proficient cells from HU. Following release from HU arrest, smc6 mutants complete bulk DNA synthesis and enter mitosis with kinetics similar to that of smc6+ cells. However, mitosis is highly aberrant in a significant proportion of mutant cells. The aberrant mitoses were suppressed by deletion of rhp51rad51 (35) or rhp55rad55 (Fig. 5), indicating that HR-dependent DNA structures that are not recognized by the G2 DNA damage checkpoint accumulate, and this is again consistent with a requirement for Smc6 during a late stage in recombination at the subset of stalled forks, which become unstable and collapse. A similar phenomenon was recently reported for rad60 mutants (35). Rad60 is an Smc5/6 complex-interacting protein that is excluded from the nucleus in HU-arrested cells. Our data predict that Rad60 reenters the nucleus upon HU release to participate with the Smc5/6 complex in a late repair event. In the absence of this function, DNA structures that are not visible to
the checkpoint accumulate and result in mitotic catastrophe. Thus, Smc5/6 and Rad60 are required for the proper resolution of HR intermediates.

A similar role has been proposed for RecQ helicases (7, 8), and in vitro, the human BLM-Top3 complex is able to convert the two HJs and decatenate the resulting hemicatenane and in vitro, the human BLM-Top3 complex is able to convert the two HJs and decatenate the resulting hemicatenane. Thus, Smc5/6 and Rad60 are required for the proper resolution of HR intermediates.

The accumulation of “checkpoint-silent” late recombination structures in rhql-d defective cells is consistent with the observation that expression of the bacterial resolvase RusA partially complemented the UV and HU sensitivities and the associated aberrant mitoses of rhql-d (12). Recently, a similar suppression of the UV sensitivity of nse6-d (null for a nonessential Smc5/6 complex component) was reported (46). However, while deletion of rhp51 efficiently suppressed the UV sensitivity of nse6-d, rhp55-d did not, and overexpression of Brcl (see below) sensitized nse6-d to UV. Since wild-type cells are similarly sensitized to HU (Fig. 1) by Brcl overexpression, this suggests that there are both separate and overlapping requirements for the essential and nonessential Smc5/6 components in repair processes.

The DNA damage sensitivity of smc6-74, but not smc6-X, is efficiently suppressed by overexpression of the multiple-BRCT-domain protein Brcl (54). We show here that overexpression of Brcl also suppresses the sensitivity of smc6-74 to inhibition of replication by HU, and this correlates with a decrease in cut cells upon entry into mitosis (Fig. 1B and 5). Overexpression of Brcl had no effect on the HU sensitivity of smc6-X. Thus, either the more severe defect in smc6-X cannot be efficiently suppressed or smc6-74 and smc6-X cause the accumulation of distinct structures late in the recombination process. Both of these hypomorphic smc6 alleles are synthetically lethal with brcl loss, which itself also results in an S-phase-specific DNA repair defect (51, 54). Taken together, these data suggest that the Smc5/6 and Brcl pathways overlap in processing at least some aberrant DNA structures that can arise as a result of replication stress. The balance between these functions is important, as overexpression of brcl sensitized smc6-74 cells to HU.

Genome-wide function for Smc5/6 following replication fork collapse. The accumulation of Smc6 at foci within the nucleolus (Fig. 7) and, in S. cerevisiae, efficient rDNA segregation (53) may reflect a specific function for Smc5/6 confined to the rDNA. However, genome-wide DNA fragmentation in conditional alleles of Smc5/6 complex members and the lack of complementation by episomal rDNA suggest that while the rDNA provides a specific readout of Smc5/6 defects (probably because of the intimate link between replication and recombination at this repetitive locus), Smc5/6 functions are globally important as opposed to rDNA specific (39, 53). Such a notion is also consistent with the observation that, while the rDNA is restricted to one chromosome in S. pombe, the HR-mediated repair of IR-induced strand breaks is equally defective for all chromosomes in the smc6 mutants and that defects in the segregation of all chromosomes are observed in the aberrant mitoses following irradiation or treatment with HU.

We found that, without treatment, Smc6 was equally enriched at the rDNA and unique loci. The association of Smc6 with all loci was modest, but similar profiles were seen with two different antibodies for ChIP (Fig. 8), including anti-Myc against an epitope-tagged Smc6. This is in contrast to observations made by Torres-Rosell et al. (53), who found Smc6 to be enriched at repetitive sequences, including rDNA and telomeres in S. cerevisiae (53), though the use of different methodologies to quantify the ChIP signal may bias their study toward repetitive sequences. Indeed, a recent genome-wide analysis using ChIP-on-Chip concluded that Smc5/6 is associated at other specific loci, including centromeres (4). Our data do not exclude such a distribution for Smc6 in S. pombe, as we have examined only a limited number of loci and we may not have covered more extensively enriched regions. Alternatively, we cannot rule out the possibility that there are real differences

FIG. 9. Model for the Smc5/6 complex function. Cds1Chk2 activity stabilizes stalled replication forks, and replication complexes remain associated. Forks collapse (replication complexes are dissociated), which can lead to the generation of a polar double-strand break. Restoration of the fork then occurs through break-induced replication (BIR) initiated by HR-dependent strand invasion of the intact template and subsequent HJ resolution (15). Rad22Rad52 and other recombination proteins (not shown) associate with the damaged chromatin at collapsed forks independently of Smc5/6, but the complex is required with Rad60 at a later stage for HR repair and/or restoration of replication (bottom right). Both smc6-74 and smc6-X are defective in this process (indicated by bars). Fork resetting without Rhp51Rad51 dependent HR occurs by multiple mechanisms, some of which require Smc5/6 (top right, shown in gray) or Brcl. In smc6-74 mutants, Brcl-dependent resetting of unstable forks can still occur inefficiently, whereas in smc6-X mutants, this process is nonfunctional (indicated by bar and ?X). Overexpression of brcl could thus bypass the requirement for smc6-74 but not for smc6-X.
in Smc5/6 localization between the two yeasts. A genome-wide analysis of *S. pombe* and other species would be required to resolve this.

After HU treatment, the levels of Smc6 were modestly but reproducibly increased at all loci. This was most noticeable at the 17S region of the rDNA. Thus, in contrast to Rad60, which is excluded from the nucleus in response to HU, Smc6 is chromatin bound in response to inhibition of replication, and this may reflect a separate requirement for Smc5/6 to act at stalled replication forks.

**Model.** We and others have provided evidence that Smc5/6 functions genome wide when forks collapse. HR proteins associate with collapsed forks independently of the Smc6 functions defective in the hypomorphic mutants (Fig. 4), but these Smc6 functions are necessary for the processing of collapsed replication forks (Fig. 2), for normal mitosis, and for optimal cell survival (Fig. 1 and 5). Smc6 is assigned to the HRR pathway by epistasis (27) and presumably participates in DNA repair and/or the HR-dependent restoration of DNA replication. However, the two smc6 hypomorphic mutants analyzed are more sensitive to HU exposure than recombination mutants and are also partially rescued by loss of recombination genes (Fig. 1). This suggests that the function of Smc5/6 in the repair/resumption of collapsed replication forks has several aspects, only some of which are HR dependent. This is consistent with the fact that complete loss of the Smc5/6 complex function is lethal because of global genome fragmentation associated with DNA replication (39, 53). In contrast, HR-deficient cells remain viable, demonstrating that Smc5/6 coordinates DNA-processing functions during replication in addition to HR.

We propose that Smc5/6 with Rad60 plays a late role in resolving as-yet-undefined DNA structures associated with DNA repair by HR of both canonical DNA damage and collapsed replication forks (Fig. 9). Our results suggest that these structures can arise at unstable replication forks from both HR-dependent events (partial suppression of smc6 mutant sensitivity by concomitant deletion of HR genes) and HR-independent events (loss of HR is not lethal). In addition to the Rad60-dependent late role in the resolution of recombination intermediates after fork collapse, we also propose that Smc5/6 functions independently of Rad60, possibly in resetting unstable forks.

It remains unclear whether the two hypomorphic alleles analyzed here define distinct roles for Smc6 or whether the ability of brcl overexpression to suppress the sensitivity of *smc6-74* but not of *smc6-X* is a result of differential penetrances in the same process. For example, it is possible that in *smc6-74* mutants, BrCl-dependent resetting of unstable forks can still occur inefficiently, whereas in *smc6-X* mutants, this process is nonfunctional. Overexpression of *brcl* could thus bypass the requirement for *smc6-74* but not for *smc6-X*. Overexpression of BrCl cannot suppress null mutations in Smc5/6 complex genes (our unpublished data).

**Conclusions.** The genetic analysis of Smc5/6 complex components demonstrates that the complex has multiple functions in DNA metabolism. We provide evidence that one “late” function of Smc5/6 complex, in association with Rad60 (35), is to resolve structures established by the processing of collapsed replication forks by HR proteins. The BrCl/nuclease-dependent pathway(s) can alternatively resolve a subset of these structures. These data predict that the highly conserved human Smc5/6 complex participates in pathways that prevent diseases related to genome instability by coordinating DNA repair responses with DNA replication and cell cycle progression.

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