Mus81, Rhp51(Rad51), and Rqh1 Form an Epistatic Pathway Required for the S-Phase DNA Damage Checkpoint

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The S-phase DNA damage checkpoint slows the rate of DNA synthesis in response to damage during replication. In the fission yeast *Schizosaccharomyces pombe*, Cds1, the S-phase-specific checkpoint effector kinase, is required for checkpoint signaling and replication slowing; upon treatment with the alkylating agent methyl methane sulfonate, *cds1Δ* mutants display a complete checkpoint defect. We have identified proteins downstream of Cds1 required for checkpoint-dependent slowing, including the structure-specific endonuclease Mus81 and the helicase Rqh1, which are implicated in replication fork stability and the negative regulation of recombination. Removing Rhp51, the Rad51 recombinase homologue, suppresses the slowing defect of *rqh1Δ* mutants, but not that of *mus81Δ* mutant, defining an epistatic pathway in which *mus81* is epistatic to *rhp51* and *rhp51* is epistatic to *rqh1*. We propose that restraining recombination is required for the slowing of replication in response to DNA damage.

INTRODUCTION

Checkpoints are signaling cascades important for cells to properly respond to DNA damage by coordinating repair with cell cycle progression. Cells use checkpoints at several critical points in the cell cycle to minimize mutation and maintain viability in the presence of damaged DNA (Kastan and Bartek, 2004). Checkpoint activation prevents entrance into S phase in the presence of DNA damage in G1, prevents entrance into mitosis in the presence of damaged or unreplicated DNA, and reduces replication rate in the presence of damaged template during S phase. The mechanism of the G1 and G2 DNA damage checkpoints are well described (Lukas *et al.*, 2004). However, mechanisms used to slow replication in response to DNA damage during S phase are less clear. Two mechanisms have been invoked for replication slowing in response to DNA damage: reduced replication fork rate and reduced origin firing (Tercero and Diffley, 2001; Merrick *et al.*, 2004). Prevention of origin firing requires factors to act on origins located far from sites of DNA damage and thus represents an *in trans* mechanism, whereas reduced fork rate likely represents an *in cis* mechanism in which factors act locally at the site of DNA damage to slow the affected fork. Slowing in response to DNA damage in higher eukaryotes seems to be a combination of both direct action on replication forks and reduced origin firing (Seiler *et al.*, 2007).

In the fission yeast *Schizosaccharomyces pombe*, the well-established S-phase DNA damage checkpoint signaling cas-

cade includes Rad3, the central checkpoint kinase and homologue of the metazoan ATR kinase, and Cds1, homologue of the Rad53 and Chk2 effector kinases (Lindsay *et al.*, 1998; Rhind and Russell, 1998). However, the checkpoint targets underlying replication slowing are not well understood (Figure 1F). Replication slowing in metazoans is catalyzed by parallel pathways acting downstream of the central checkpoint kinases (Falck *et al.*, 2002; Henry-Mowatt *et al.*, 2003). One pathway depends upon Chk2/Cds1 negative regulation of Cdc25-dependent origin firing; another is dependent upon the Mre11–Rad50–Nbs1 (MRN) recombinational repair complex, which may reflect direct regulation of replication fork progression (Falck *et al.*, 2002). Only when both MRN and Cdc25 mediated pathways are compromised do mammalian cells display a complete failure of the S-phase DNA damage checkpoint similar to ATM mutants (Falck *et al.*, 2002). In *S. pombe*, Cdc25 is not required for the S-phase DNA damage checkpoint (Kommajosyula and Rhind, 2006). However, members of the MRN complex are required (Chahwan *et al.*, 2003; Kommajosyula and Rhind, 2006). The lack of Cdc25 involvement in the S phase DNA damage response suggests either that regulation of origin firing is not required for replication slowing or that origins are regulated in a manner independent of Cdc25.

Replication fork response to DNA damage involves fork stalling, recombination and DNA repair (Michel *et al.*, 2004). Recombination allows for error-free repair of damaged DNA through strand exchange between homologous sequences. In fission yeast, this exchange is catalyzed by the central mitotic recombinase Rhp51, homologue of bacterial RecA and budding yeast Rad51 (Muris *et al.*, 1993). Rhp51 is loaded onto the 3'-end of single-stranded DNA by the mediator Rad22, homologue of Rad52. Rhp51 then forms a nucleoprotein filament on single-stranded DNA (ssDNA), which is stabilized and regulated by the additional Rhp51 mediators Rhp54, Rhp55, Rhp57, Sfr1, and Swi5 (Raji and Hartsuiker, 2006). Single-fiber analysis shows the Rad51 re-

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Abbreviations used: IR, ionizing radiation; MMS, methyl methane sulfonate.

combinase and its paralogue XRCC3 are required for slowing of replication fork progression in response to cisplatin and UV in mammalian cells (Henry-Mowatt *et al.*, 2003).

Although beneficial, recombination must be tightly regulated for cells to properly respond to DNA damage and fork stalling. One mechanism for controlling recombination involves the RecQ helicase Rqh1, which is implicated in negatively regulating recombination. Cells lacking Rqh1 display phenotypes attributed to inappropriate, ectopic recombination including hyper-recombination, sensitivity to replication arrest and sensitivity to UV-induced DNA damage (Doe *et al.*, 2000). The DNA damage sensitivity displayed by *rqh1Δ* cells is alleviated by disrupting recombination (Hope *et al.*, 2005). The structure-specific endonuclease Mus81 is also implicated in negatively regulating recombination. Like the helicase mutants, *mus81Δ* mutants display a mutator phenotype and sensitivity to hydroxyurea and UV (Boddy *et al.*, 2000; Kai *et al.*, 2005). Consistent with our results summarized below, the DNA damage sensitivity of *mus81Δ* and synthetic lethality of the *mus81Δ rqh1Δ* mutant is not rescued by removing recombination (Doe and Whitby, 2004).

The hallmark of the S-phase DNA damage checkpoint in the fission yeast *S. pombe* is slowing of replication in response to DNA damage (Lindsay *et al.*, 1998; Rhind and Russell, 1998). With this in mind, we used cell-cycle synchronization and flow cytometry to measure bulk replication slowing in response to DNA damage. We show that several proteins in addition to the checkpoint kinases are required for replication slowing in fission yeast: the Rqh1 helicase, the Mus81 endonuclease, and the Sfr1 mediator. We show that the slowing defects displayed by *rqh1Δ* and *sfr1Δ* strains are suppressed by abrogating recombination, but that the defects displayed by *mus81Δ* and *cds1Δ* mutants are not. Previous experiments using these mutants have shown the importance of recombinational control in proper repair of DNA lesions after replication (Laursen *et al.*, 2003). Our data illustrate the importance of proper control of recombination in response to DNA damage during replication itself.

MATERIALS AND METHODS

Strain Construction and Maintenance

Yeast strains were grown in yeast extract with supplements (YES) or Edinburgh minimal media (EMM) at 25 or 30°C and manipulated using standard methods (Forsburg and Rhind, 2006). Yeast strains are listed in Table 1. Strains containing the *cds1* overexpression cassette were grown in EMM supplemented with 15 μM thiamine to repress *nmf1* transcription. Thiamine was washed out 16 h before G1 elutriation. The *rad51::natMX6* allele was constructed using a polymerase chain reaction (PCR)-based targeting method replacing the open reading frame of *rhp51* with the nourseothricin resistance gene *natMX6* (Bahler *et al.*, 1998; Sato *et al.*, 2005). We targeted *rhp51* using PCR product amplified from the pCR2.1-nat plasmid using the following primers, 5'-ctaactcttcttttataataataaaa-aactcttttcaattccagaatagtgataattctgtcttaacaagttatcgatcccggttaattaa and 5'-cacatacatatctatcttacaactcatccatagaaattgcgaataataaataaagaacgatactaaataa-tgaattcgcagctgtttaaac.

G1 Synchronization

To follow cells through S phase, we used the *cdc10-m17* temperature-sensitive allele to arrest cells in G1 and synchronously release them into S. Simply synchronizing cells by extended *cdc10-m17* arrest and release proved problematic. A 3-h arrest prevents synchronized cells from replicating quickly upon release. Therefore, G1 cells were synchronized using a combination of *cdc10-m17* arrest and centrifugal elutriation that allow us to synchronize cells in G1 that replicate quickly upon release. Cultures were grown to OD₆₀₀ 1.5 at 25°C followed by incubation at 35°C for 2 h to arrest a subpopulation of cells in late G1. The smallest synchronized G1 cells were then collected by centrifugal elutriation. We estimate that the cells recovered were arrested at the Cdc10 execution point for only 30 min. Collected cells were immediately released to 25°C and treated with 0.03% methyl methane sulfonate (MMS) (M4016; Sigma-Aldrich, St. Louis, MO), 10 mM hydroxyurea (H8627; Sigma-Aldrich), 1–9 μg/ml bleomycin (B2434; Sigma-Aldrich), 200-Gray ionizing

radiation using a Faxitron RX-650 (10 Gray/min starting at 5 min after elutriation) or untreated. At 20-min intervals for 3 h after elutriation, 0.5 ODs of cells were pelleted and resuspended in 70% ethanol. Fixed cells were stored at 4°C until processed for flow cytometry.

Isolated Nuclei Preparation for Flow Cytometry

Whole cells arrested using *cdc10* temperature-sensitive alleles or treated with hydroxyurea display significant background fluorescence when analyzed by flow cytometry. The background increases during the arrest because cells continue to elongate. To reduce this background and improve the signal-to-noise ratio in our experiments, we adapted a protocol to analyze isolated nuclei (Carlson *et al.*, 1997). Fixed cells were pelleted and washed with 1 ml of 0.6 M KCl. Cells were spheroplasted by resuspension in 1 ml of 0.6 M KCl containing 0.5 mg/ml Zymolyase 20T and 1.3 mg/ml lysing enzyme (L1412; Sigma-Aldrich) and incubated at 37°C for 30 min. Spheroplasts were pelleted, washed with 1 ml of 0.1% Triton X-100, 0.1 M KCl, and finally washed with and resuspended in 1 ml of 20 mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 8.0. 20 μl of RNase A (10 mg/ml) was added to each preparation followed by overnight incubation at 37°C. Spheroplasts were pelleted, left in buffer, and chilled to 0°C. Spheroplasts were disrupted and nuclei released using a Branson Sonifier 450 set to 0.6 power for 10 s and a chilled microtip. 300 μl of sonicated cells was mixed with 300 μl of 2 μM Sytox Green (S7020; Invitrogen, Carlsbad, CA) in 1× phosphated-buffered saline (PBS) and analyzed on a FACScan flow cytometer (BD Biosciences, San Jose, CA).

S-Phase Progression Analysis

S phase progression was quantitated using CellQuest software version 3.3 (BD Biosciences). The 1C unreplicated value was determined by measuring DNA content of freshly elutriated samples, and the 2C fully replicated value was determined by measuring DNA content from later time points for untreated cultures and asynchronous, prearrested samples. Mean values for S-phase peaks after release were compared with 1C and 2C values. S-phase progression was quantitated using the following equation: % progression = (C – A)/(B – A), where A = 1C, B = 2C, and C = mean histogram value. Error bars represent the SE of the mean for strains in which three or more experiments were performed. For strains for which two experiments were performed, error bars represent the variance. The error bars may be smaller than data symbols used. See Figure 7 for the number of experiments performed for each individual strain.

In Vitro Kinase Assay

G1 cells were synchronized as described above and kinase activity measured as described previously (Lindsay *et al.*, 1998; Kai *et al.*, 2005). 0.5 ODs of cells were fixed for flow cytometry, and 5 ODs of cells were pelleted and frozen in liquid nitrogen. Pellets were thawed on ice in 200 μl of ice-cold lysis buffer (150 mM NaCl, 50 mM Tris-HCl, pH 8.0, 5 mM EDTA, pH 8.0, 10% glycerol, 1% NP-40, 50 mM NaF, 5 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, and 1 mM Na₃VO₄) and broken by vortexing with silica bead for 15 min at 4°C. Crude anti-Cds1 antibodies (a kind gift from T. Wang) were conjugated to protein A-Sepharose beads by constant mixing at 4°C for 1 h in lysis buffer, washed in ice-cold lysis buffer, and resuspended as a 1:1 slurry in lysis buffer. Crude cell lysates were cleared by brief centrifugation (3000 × g; 4 min; 4°C); cleared lysate protein concentration determined by BCA Protein Assay kit (23225; Pierce Chemical, Rockford, IL). Protein concentration was normalized within each experiment in a final volume of 200 μl. Twenty microliters of the 1:1 antibody-protein A bead slurry was added to each sample, and mixtures were incubated with constant mixing at 4°C for 2 h. Beads were washed twice with ice-cold lysis buffer, twice with ice-cold 1× kinase buffer (5 mM HEPES, pH 7.5, 37.5 mM KCl, and 2.5 mM MgCl₂), and resuspended in 10 μl of 1× kinase buffer.

To each sample, 10 μl of 2× kinase buffer (10 mM HEPES, pH 7.5, 75 mM KCl, and 5 mM MgCl₂), 0.5 μl of 10 μCi/μl [γ -³²P]dATP, 2 μl of 1 mM dATP, and 5 μl of 1 mg/ml myelin basic protein [M1891; Sigma-Aldrich]) were added, mixed and incubated at 30°C for 15 min. Reactions were stopped by addition of 20 μl of 4× protein loading buffer (40% glycerol, 200 mM Tris-HCl, pH 6.8, and 4% SDS) and boiled for 5 min at 95°C. Reactions were allowed to cool to room temperature, and beads were pelleted. Twenty microliters of the supernatants was run on a 15% SDS-polyacrylamide gel, Coomassie stained to visualize myelin basic protein target, dried for 30 min at 90°C under vacuum, and exposed to a phosphorimager screen for 6–72 h. Screens were developed on a FLA-5000 PhosphorImager, and bands were quantitated using FujiFilm ImageGauge software (FujiFilm, Tokyo, Japan). For comparison between strains and independent experiments, kinase activity was normalized to percentage of G1 cells at time 0 (G2 cells treated with 0.03% MMS neither septate nor replicate and do not contribute to measured Cds1 activity), and values were normalized to kinase activity in MMS-treated wild-type cells 120 min after elutriation.

Table 1. Strains used in this study

Strain	Genotype ^a	References ^b
yFS105	h+ leu1-32 ura4-D18	
yFS162	h- leu1-32 ura4-D18 ade6-210 cdc10-m17	
yFS260	h- leu1-32 ura4-D18 cdc10-m17 rad3::ura4	
yFS356	h+ leu1-32 ura4-D18 ade6-210 cdc10-m17 swi1::kanMX6	Noguchi et al. (2003)
yFS543	h+ leu1-32 ura4-D18 ade6-210 cdc10-m17 cds1::ura4	
yFS544	h- smt0 leu1-32 ura4-D18 ade6-M210 cdc10-m17 rhp51::ura4	Muris et al. (1993)
yFS545	h- leu1-32 ura4-? cdc10-m17 rql1::ura4	Stewart et al. (1997)
yFS547	h+ leu1-32 ura4-? D18 cdc10-m17 rhp54::ura4	Muris et al. (1996)
yFS548	h- leu1-32::pBF162 (nmt1:GSTcds1 leu1) ura4-? cdc10-m17 nmt1:pyp3 (KanMX6) mik1::ura4 rad3::ura4	Rhind and Russell (2001)
yFS549	h- ura4-D18 cdc10-m17 rhp55::ura4	Khasanov et al. (1999)
yFS550	h-smt0 leu1-32 ura4-D18 his3-D1 cdc10-m17 sfr1::ura4	Akamatsu et al. (2003)
yFS551	h-smt0 leu1-32 ura4-D18 his3-D1 arg3-D1 cdc10-m17 swi5::ura4 rhp57::his3	
yFS552	h-smt0 leu1-32 ura4-D18 his3-D1 arg3-D1 cdc10-m17 rhp57::his3	Tsutsui et al. (2000)
yFS553	h+ leu1-32 ura4-D18 his3-D1 cdc10-m17 swi5::his3	Akamatsu et al. (2003)
yFS554	h-smt0 leu1-32 ura4-D18 his3-D1 cdc10-m17 sfr1::ura4 swi5::his3	
yFS557	h+ leu1-32 ura4-? cdc10-m17 rql1::ura4 sfr1::ura4	
yFS558	h+ leu1-32 ura4-? cdc10-m17 rql1::ura4 rhp54::ura4	
yFS559	h+ leu1-32 ura4-? ade6-m210 cdc10-m17 rql1::ura4 rhp55::ura4	
yFS560	h- leu1-32 ura4-D18 ade6-? cdc10-m17 mus81::kanMX6	Boddy et al. (2000)
yFS561	h+ leu1-32 ura4-? arg3-D1 cdc10-m17 rql1::ura4 rhp57::his3	
yFS562	h+ leu1-32 ura4-D18 his3-D1 cdc10-m17 dmc1::ura4	Fukushima et al. (2000)
yFS563	h+ leu1-32 ura4-? cdc10-m17 rql1::ura4 rhp54::ura4 rhp55::ura4	
yFS564	h- leu1-32 ura4-? arg3-D1 cdc10-m17 rql1::ura4 rhp54::ura4 rhp57::his3	
yFS565	h- leu1-32 ura4-D18 his3-D1 cdc10-m17 rql1::ura4 swi5::his3	
yFS566	h+ leu1-32 ura4-D18 ade6-? his7-366 cdc10-m17 rhp51::natMX6	
yFS567	h-leu1-32 ura4-D18 his3-D1 cdc10-m17 swi5::his3 cds1::ura4	
yFS568	h+ leu1-32 ura4-D18 his3-D1 cdc10-m17 sfr1::ura4 swi5::his3 cds1::kanMX6	
yFS569	h+ leu1-32 ura4-D18 his7-366 cdc10-m17 rql1::ura4 rhp51::natMX6	
yFS570	h-leu1-32 ura4-D18 his3-D1 cdc10-m17 rql1::ura4 swi5::his3 cds1::kanMX6	
yFS571	h-leu1-32 ura4-D18 his3-D1 cdc10-m17 rql1::ura4 sfr1::ura4 swi5::his3	
yFS572	h+ leu1-32 ura4-D18 ade6-? his3-D1 cdc10-m17 rql1::ura4 rhp55::ura4 rhp57::his3	
yFS573	h-leu1-32 ura4-D18 his3-D1 cdc10-m17 rql1::ura4 dmc1::ura4 cds1::kanMX6	
yFS574	h+ leu1-32 ura4-D18 his3-D1 cdc10-m17 dmc1::ura4 cds1::kanMX6	
yFS575	h+ leu1-32 ura4-D18 his3-D1 cdc10-m17 rql1::ura4 dmc1::ura4	
yFS576	h+ leu1-32 ura4-D18 his3-D1 cdc10-m17 dmc1::ura4 rhp51::natMX6	
yFS577	h+ leu1-32 ura4-D18 his3-D1 cdc10-m17 rql1::ura4 rhp51::natMX6 cds1::kanMX6	
yFS578	h-leu1-32 ura4-D18 ade6-? his3-D1 cdc10-m17 sfr1::ura4 rhp51::natMX6	
yFS579	h-leu1-32 ura4-D18 ade6-210 his3-D1 cdc10-m17 rhp51::natMX6 mus81::kanMX6	
yFS580	h-leu1-32 ura4-D18 cdc10-m17 mus81::kanMX6 dmc1::ura4	
yFS581	h+ leu1-32 ura4-D18 his3-D1 cdc10-m17 mus81::kanMX6 swi5::his3	
yFS582	h+ leu1-32 ura4-D18 his3-D1 cdc10-m17 sfr1::ura4 dmc1::ura4	
yFS583	h+ leu1-32 ura4-D18 cdc10-m17 rhp51::natMX6 cds1::kanMX6	
yFS584	h+ leu1-32 ura4-D18 cdc10-m17 mus81-T239A::myc (kanMX6)	Kai et al. (2005)
yFS588	h-smt0 leu1-32 ura4-D18 his3-D1 cdc10-m17 sfr1::ura4 rhp51::natMX6 cds1::kanMX6	
yFS589	h-smt0 leu1-32 ura4-D18 his3-D1 cdc10-m17 sfr1::ura4 dmc1::ura4 cds1::kanMX6	
yFS590	h+ leu1-32 ura4-D18 his3-D1 cdc10-m17 swi2::ura4	
yFS592	h-leu1-32 ura4-D18 his3-D1 cdc10-m17 swi5::his3 swi2::ura4	
yFS593	h-smt0 leu1-32 ura4-D18 his3-D1 cdc10-m17 sfr1::ura4 swi5::his3 swi2::ura4	
yFS594	h-smt0 leu1-32 ura4-D18 his3-D1 cdc10-m17 sfr1::ura4 swi2::ura4	
yFS595	h-ura4-D18 his3-D1 cdc10-m17 rql1::ura4 swi2::ura4	
yFS599	h-leu1-32 ura4-D18 his3-D1 cdc10-m17 mus81::kanMX6 swi2::ura4	
yFS600	h-leu1-32 ura4-D18 ade6-? his3-D1 cdc10-m17 rhp55::ura4 rhp57::his3	
yFS601	h+ leu1-32 ura4-D18 his3-D1 cdc10-m17 rad22::ura4	Doe et al. (2004)
yFS602	h+ leu1-32 ura4-D18 his3-D1 cdc10-m17 sfr1::ura4 rhp54::ura4 rhp57::his3	
yFS603	h-leu1-32 ura4-D18 ade? his3-D1 cdc10-m17 mus81::kanMX6 rad22::ura4	
yFS604	h-leu1-32 ura4-D18 his3-D1 cdc10-m17 rhp54::ura4 rhp57::his3	
yFS621	h+ leu1-32 ura4-D18 cdc10-m17 swi1::kanMX cds1::ura4	
yFS622	h+ leu1-32 ura4-D18 his3-D1 cdc10-m17 sfr1::ura4 swi1::kanMX	
yFS664	h+ leu1-32::pBF260 (nmt1:GSTcds1KD leu1) ura4-? cdc10-m17 nmt1:pyp3 (KanMX6) mik1::ura4 rad3::ura4	
yFS665	h+ leu1-32 ura4-D18 his3-D1 cdc10-m17 mrc1::kanMX6	
yFS666	h-leu1-32::pBF162 (nmt1:GSTcds1 leu1) ura4-? cdc10-m17 nmt1:pyp3 (KanMX6) mik1::ura4 rad3::ura4 mrc1::kanMX6	
yFS667	h+ leu1-32 ura4-D18 his3-D1 cdc10-m17 eme1::ura4	

^a *ura4-?* indicates the allele is *ura4-294* or *ura4-D18*; *ade6-?* indicates the allele is *ade6-210* or *ade6-216*.

^b yFS105, yFS162, and yFS260 are laboratory stocks; the rest of the strains were built for this study. References are for critical alleles in the strains.

Figure 1. Replication slowing in response to DNA damage is checkpoint dependent. (A) S-phase flow cytometry histogram stacks comparing wild-type (yFS162) and *rad3Δ* (yFS260) strains. G1 cells were synchronized by *cdc10-ts* arrest and elutriation and released in the presence or absence of 0.03% MMS. Cells were fixed every 20 min, and nuclear DNA content was measured by flow cytometry. (B) Population progression through S phase is plotted over time by measuring the shifting of the mean of S phase peaks from unreplicated 1C toward fully replicated 2C values. (C) Replication kinetics represented by plotting the extent of replication at 140 min after elutriation, described as Rep₁₄₀. (D) Cds1 activity was measured with an in vitro immunoprecipitation-kinase assay using myelin basic protein as a substrate. (E) Quantitation of kinase assays. *n* = 14 for wild type; *n* = 1 for *rad3Δ*; error bars represent the standard error of the mean. (F) The S-phase DNA damage checkpoint requires the upstream checkpoint sensor kinase Rad3, homolog to metazoan ATR, and the S phase-specific transducer kinase Cds1, homolog to Chk2. Downstream players in replication slowing and the mechanism(s) required for slowing have not been defined for fission yeast.

RESULTS

Flow Cytometric Quantitation of the S-Phase DNA Damage Checkpoint

To measure S-phase DNA damage checkpoint-induced replication slowing, cultures were synchronized in G1 using a combination of transient *cdc10-ts* arrest and centrifugal elutriation. G1 synchronized cells were released at permissive temperature in the presence or absence of the DNA-damaging agent MMS. We used flow cytometry to measure nuclear DNA content and follow populations of cells as they progressed through S phase from unreplicated 1C content toward fully replicated 2C content. To reduce cytoplasmic background, flow cytometry was performed upon isolated nuclei.

Data collected by flow cytometry was analyzed as histogram stacks shown in Figure 1A. The untreated wild-type population in the left-hand stack displays a distinct shift from 1C toward 2C around 80 min after release, whereas MMS-treated cells display no such dramatic shift but a slow progression from 1C toward 2C with time. As reported previously, cells lacking Rad3, the central checkpoint kinase in fission yeast, replicate quickly both in the presence and

absence of DNA damage, confirming that the checkpoint is required for replication slowing in response to DNA damage (Figure 1A, right; Lindsay *et al.*, 1998; Rhind and Russell, 1998).

Measuring mean population peak shifting from 1C toward 2C nuclear DNA content over time allowed us to generate replication progression plots shown in Figure 1B. Unreplicated cells containing 1C DNA content were assigned a value of 0, whereas replicated cells containing 2C DNA content were assigned a value of 1. Wild-type strains slowed replication by ~40% in the presence of MMS (Figure 1B, closed vs. open diamonds), whereas the *rad3Δ* strain did not (Figure 1B, closed vs. open triangles). To illustrate the difference between wild-type and *rad3Δ* strains, the extent of replication at a single time point, 140 min after release, designated Rep₁₄₀, is shown for both strains under both conditions (Figure 1C). Cds1, the S-phase-specific effector kinase, and Mrc1, the S phase checkpoint mediator protein required for Rad3-dependent Cds1 phosphorylation and activation, are also required for DNA damage-induced replication slowing (Figure 4D and Supplemental S3A; Lindsay *et al.*, 1998; Rhind and Russell, 1998). Moreover, replication

Figure 2. Cds1 activity is necessary but not sufficient to promote replication slowing. (A) Replication kinetics in wild-type cells (yFS162) treated with 0.03% MMS or 200 Gy of IR were assayed as described in Figure 1. (B) Cds1 kinase activity in wild-type cells (yFS162) treated as described in A. (C) Cells cycle progression of wild-type cells (yFS105) irradiated as described in A was followed by monitoring septation. (D) Replication kinetics in wild-type cells (yFS162) treated with a range of bleomycin concentrations. (E) Cds1 kinase activity in the cells from (D) were assayed as in Figure 1. Plots represent the mean $\pm$ SEM of between two and five independent experiments done for each condition except for 9 μ g/ml bleomycin for which only a single experiment was conducted. (F) Replication kinetics in wild-type cells (yFS162) treated with a range of MMS concentrations and displayed as Rep₁₄₀.

slowing observed in all mutants is checkpoint dependent (Figure 7 and Supplemental S3B and C).

Checkpoint Signaling Is Not Sufficient to Produce Slowing
DNA damage during S phase induces checkpoint signaling and activates the effector kinase Cds1 as measured by in vitro kinase assay (Lindsay *et al.*, 1998). Using an in vitro kinase assay to quantitate the activity of Cds1 immunoprecipitated from cell lysates, we confirmed that untreated wild-type cells display slightly increased, periodic kinase activity during S phase and strong activity when exposed to MMS during replication (Figure 1, D and E). *rad3Δ* strains failed to slow replication and displayed no increased Cds1 activity in unperturbed S phase or in response to DNA damage during replication (Figure 1, D and E).

Although Cds1 activation correlates with replication slowing, exposure to ionizing radiation (IR) during S phase,

which induces Cds1 signaling, does not induce slowing (Rhind and Russell, 1998). Therefore, we suspected that Cds1 signaling was not sufficient for slowing. We tested for sufficiency by comparing S-phase slowing and Cds1 signaling responses to IR, bleomycin, and MMS during S phase. Exposing cells to 200 Gray IR or 1 μ g/ml IR-mimetic bleomycin, dosages capable of producing a mitotic delay, induces Cds1 kinase activity, but not slowing (Figure 2, A and B). Bleomycin titration experiments show that 9 μ g/ml produced Cds1 kinase activity equivalent to that in MMS-treated cells but this activity was still not sufficient to induce a replication slowing response (Figure 2, D and E). Therefore, simply activating Cds1 is not sufficient to cause slowing of replication.

Alternatively, in vitro Cds1 activity measured in response to bleomycin treatment may be qualitatively different from that produced in response to MMS treatment and may not

be capable of producing replication slowing. To test whether generic Cds1 kinase activity was capable of producing a slowing response, we measured the effect of overexpressing Cds1 on replication progression. Overexpression of Cds1 induces Cds1 kinase activity in the absence of upstream checkpoint signaling and induces both S phase and G2/M phase checkpoint responses (Brondello *et al.*, 1999; de Bruin *et al.*, 2008). We overexpressed Cds1 from the strong, thiamine-regulated *nmt1* promoter in a *rad3Δ* background so that Cds1 activity could be ascribed to overexpression and not checkpoint signaling. To prevent a Cds1-induced G2 arrest, we used a genetic background in which the G2 arrest is overridden by deletion of *mik1*, a Cdc2-inhibitory tyrosine-kinase activated by Cds1, and overexpression of Pyp3, a Cdc2 activating tyrosine-phosphatase that can constitutively drive the G2/M transition (Rhind and Russell, 2001). Cds1 overexpression induces Cds1 kinase activity in the *rad3Δ* mutant with no significant increase in activity when cells are treated with MMS (Figure 3A). Overexpressing Cds1 did not cause replication slowing nor did MMS-treatment of the *nmt1:cds1 rad3Δ* cells when Cds1 is repressed. However, MMS treatment of Cds1-overexpressing cells caused robust replication slowing (Figure 3B). The slowing displayed is kinase-dependent because overexpression of a kinase-dead allele of *cds1* failed to induce MMS-dependent replication slowing (Figure 7). Bleomycin treatment did not induce slowing upon Cds1 overexpression, further suggesting that replication slowing requires a high density of DNA lesions (Figure 3B). Although slowing is normally dependent upon the mediator protein Mrc1 (Supplemental Figure S3A), slowing upon Cds1 overexpression is not, suggesting that Mrc1's main role in the checkpoint is the activation of Cds1 (Figure 3C). These results show that, although checkpoint signaling through Cds1 is necessary for slowing replication in response to DNA damage, Cds1 activity alone is not sufficient.

Rqh1, *Sfr1*, and *Mus81* Are Required for the S-Phase DNA Damage Checkpoint

Given that the recombinational repair complex MRN is required for replication slowing (Chahwan *et al.*, 2003), we wished to assay other proteins involved in recombination. We conducted a survey of additional mutants involved in recombination. We found three strains, *rqh1Δ*, *sfr1Δ*, and *mus81Δ*, that do not slow replication in response to DNA damage (Figure 4A). In addition to other mutants discussed below, we found a number of strains that display normal replication slowing in response to DNA damage, including *swi6Δ*, *clr4Δ*, *swi10Δ*, *srs2Δ*, and *tel1Δ*. Rqh1 is a DNA helicase implicated in replication fork stability and in negative regulation of recombination in response to replication stress (Murray *et al.*, 1997). Sgs1, the Rqh1 homologue in budding yeast, has been shown previously to be involved in replication slowing in response to MMS, although the replication slowing phenotype of *sgs1Δ* cell is much weaker than the of *rqh1Δ* cells (Frei and Gasser, 2000). Sfr1 is a recently discovered mediator of the Rhp51 recombinase (Akamatsu *et al.*, 2003). Mus81 is a structure specific endonuclease required for meiotic recombination and regulation of recombination in response to replication stress (Boddy *et al.*, 2000; Gaillard *et al.*, 2003; Kai *et al.*, 2005). Recent work supports a role for Mus81 in replication-dependent recombination between sister-chromatids (Roseaulin *et al.*, 2008). *mus81Δ* strains do not slow replication in response to DNA damage; *rqh1Δ* and *sfr1Δ* strains display a strong defect with minor residual checkpoint-dependent slowing (Figure 4A and Supplemental S3B).

Figure 3. Cds1 overexpression induces MMS-dependent replication slowing. (A) Asynchronous cultures of *nmt1:GST:cds1 rad3Δ* (yFS548) cells were grown without thiamine for 15 h to induce *cds1* expression before a 4-h treatment with 0.03% MMS. *cds1* transcript levels were determined by Northern blot, normalized against *adh1* transcript levels in each sample, and then normalized to *cds1* levels in the repressed culture. Cds1 kinase activity in these cultures was measured as in Figure 1. (B) Overexpression of Cds1 kinase activity causes slowing only in the presence of MMS. G1 synchronized *nmt1:GST:cds1 rad3Δ* (yFS548) or *nmt1:GST:cds1 rad3Δ mcr1Δ* (yFS666) cells were treated $\pm$ 0.03% MMS or 9 μ g/ml bleomycin and assayed for replication kinetics as described in Figure 1.

To determine whether Mus81's role in replication slowing depends on the known phosphorylation of Mus81 by Cds1, we assayed a *mus81* point mutant for its ability to slow replication. Mus81-T239A disrupts the ability of Cds1 to bind and phosphorylate Mus81 and causes hyperrecombination upon hydroxyurea-induced replication arrest (Kai *et al.*, 2005). We observe no defect in replication slowing in response to DNA damage in *mus81-T239A* cells, indicating that T239-dependent phosphorylation by Cds1 is not required for Mus81's role in replication slowing (Figure 4A). Mus81 forms a heterodimer with Eme1, which is required for in vitro endonuclease activity (Boddy *et al.*, 2001). Similar to Mus81, Eme1 is required for replication slowing (Figure 7).

Figure 4. Replication slowing requires the DNA helicase Rqh1, the mitotic Rad51-mediator Sfr1, and the structure-specific endonuclease Mus81. (A) *rqh1Δ* (yFS545), *sfr1Δ* (yFS550), *mus81Δ* (yFS560), and *mus81-T239A* (yFS584) replication kinetics were assayed as described in Figure 1. (B) Cds1 activity in the cultures shown in (A) was assayed as in Figure 1. Plots represent two independent experiments for *mus81Δ*, *mus81-T239A* and *rqh1Δ* and a single experiment for the *sfr1Δ* and *rad3Δ* mutants.

Rqh1, *Sfr1*, and *Mus81* Are Not Required for *Cds1* Activation

Because Cds1 activity is necessary for replication slowing in response to DNA damage, it was important to determine whether *rqh1Δ*, *sfr1Δ*, and *mus81Δ* mutations influenced checkpoint signaling. Both *rqh1Δ* and *sfr1Δ* strains displayed strong MMS-induced Cds1 signaling (Figure 4B). In contrast, *mus81Δ* displayed 30–50% reduction in Cds1 kinase activity (Figure 4B). However, *mus81-T239A* shows a reduction in Cds1 kinase activity similar to *mus81Δ* but slows replication normally. Thus, reduced kinase activity is not sufficient to cause the slowing defect observed in the *mus81Δ* mutant (Figure 4B). These results suggest that Rqh1, Sfr1 and Mus81 act downstream of, or parallel to, the checkpoint kinase Cds1.

Recombinases Are Not Required for Replication Slowing

In contrast to the *sfr1Δ* mutant slowing defect, *rhp51Δ* mutants slow like wild-type (Figure 5A). Rhp51, the *S. pombe* Rad51/RecA homologue, is the central mitotic recombinase required for the majority of homologous recombination events that occur in vegetative cells. We found that the first *rhp51Δ*

strain we tested failed to slow (Supplemental Figure S1E). However, this phenotype is due to an unlinked modifier (Willis and Rhind, unpublished data). We deleted *rhp51* in a wild-type background and found no slowing defect (Figure 5A). We used this allele for all our subsequent assays and crosses.

To test for functional overlap between the mitotic recombinase Rhp51 and the related meiotic recombinase Dmc1, *dmc1Δ* and *dmc1Δ rhp51Δ* mutants were tested. Both the single and double mutants slowed normally in response to MMS (Figure 7 and Supplemental S1A). To show that recombination is not required for checkpoint signaling, *in vitro* kinase assays were performed comparing *rhp51Δ* to wild-type strains. *rhp51Δ* mutants displayed wild-type Cds1 activity upon exposure to DNA damage during replication (Figure 5C). Thus, the recombinases are not required for replication slowing or to produce DNA structures required to activate the checkpoint.

In addition to the Rhp51 recombinase, we tested whether the central Rhp51 mediator, Rad22, was required for slowing. Rad22 is the fission yeast homologue to budding yeast Rad52, which is responsible for loading Rhp51 onto 3' end of ssDNA (Kim *et al.*, 2002). Some recombination events in fission yeast such as single-strand annealing are Rad22 de-

Figure 5. Recombination is not required for replication slowing or S-phase DNA damage checkpoint signaling, but abrogation of recombination suppresses the defects of the *rqh1Δ* and *sfr1Δ* mutants. (A) *rhp51Δ* (yFS566), *swi5Δ* (yFS553), and *swi1Δ* (yFS356) replication kinetics were assayed as described in Figure 1. (B) Replication kinetics of *sfr1Δ rhp51Δ* (yFS578), *sfr1Δ swi5Δ* (yFS554), *sfr1Δ swi1Δ* (yFS622), *rqh1Δ rhp51Δ* (yFS569), *rqh1Δ swi5Δ* (yFS565), *mus81Δ rhp51Δ* (yFS579), and *mus81Δ swi5Δ* (yFS581) strains. (C) Cds1 kinase activity in wild-type (yFS162), *rhp51Δ* (yFS566), *sfr1Δ* (yFS550), *swi5Δ* (yFS553), *sfr1Δ swi5Δ* (yFS554), and *rad3Δ* (yFS260) strains was assayed as described in Figure 1.

pendent but Rhp51 independent (Osman *et al.*, 2000). *rad22Δ* strains slowed normally indicating that neither Rhp51-dependent nor -independent recombination events are required for slowing (Figure 7 and Supplemental S1A).

The *sfr1Δ* and *rqh1Δ* Slowing Defects Are Recombinase Dependent

Although Rqh1, Sfr1, and Mus81 are required for replication slowing in response to DNA damage, homologous recom-

bination itself is not. Surprisingly, Rhp51 is required for the *sfr1Δ* and *rqh1Δ* strains to display a slowing defect, because *sfr1Δ rhp51Δ* and *rqh1Δ rhp51Δ* double mutants slow in response to DNA damage (Figure 5B). Additionally, deletion of *dmc1* also rescues the slowing defects of *sfr1Δ* and *rqh1Δ* mutants (Figure 7 and Supplemental S1B). Dmc1 has been characterized as a meiotic recombinase (Fukushima *et al.*, 2000). However, microarray transcriptional profiling has shown Dmc1 to be expressed in vegetative cells (Rustici *et al.*, 2004). The suppression of the *sfr1Δ* and *rqh1Δ* slowing defects by *rhp51Δ* or *dmc1Δ* is checkpoint dependent because triple mutants harboring a deletion of *cds1* fail to slow replication (Figure 7 and Supplemental S3C). Unlike the *sfr1Δ* and *rqh1Δ* slowing defects, recombination is not required for the *mus81Δ* defect. *mus81Δ rhp51Δ* and *mus81Δ dmc1Δ* strains still display the *mus81Δ* nonslowing phenotype (Figure 5B, and Supplemental S1B). In addition, *mus81Δ rad22Δ* double mutants also display the *mus81Δ* nonslowing phenotype, indicating that neither Rhp51-dependent nor -independent recombination events are required for *mus81Δ* cells to replicate quickly through damaged DNA (Figure 7 and Supplemental S1D).

The Swi2-Swi5 Complex Is Required for Defects Displayed by *sfr1Δ* and *rqh1Δ* Mutants

Because the *sfr1Δ* mutant displayed a failure to slow, we tested whether its partner Swi5 is also required for slowing. Sfr1 and Swi5 have been shown to act as a heterodimer to

promote Rhp51-dependent recombination in vegetative cells. In vitro, this complex is able to load Rhp51 and Dmc1 onto ssDNA to promote strand-exchange reactions (Haruta *et al.*, 2006). Surprisingly, *swi5Δ* strains slowed like wild-type in a checkpoint-dependent manner indicating that Swi5 is not required for slowing in response to DNA damage (Figure 5A and Supplemental Figure S3B). Furthermore, *swi5* deletion suppressed the *sfr1Δ* slowing defect (Figure 5B). This result indicates that, like Rhp51 and Dmc1, Swi5 is required for the quick replication through damaged DNA displayed by *sfr1Δ* mutants. Like *sfr1Δ*, the *rqh1Δ* mutant defect is suppressed by *swi5* deletion (Figure 5B). Also, the *rqh1Δ sfr1Δ* double mutant failed to slow and the *rqh1Δ swi5Δ sfr1Δ* triple mutant slowed like wild-type (Figure 7 and Supplemental S1C). Similar to the lack of suppression of the *mus81Δ* slowing defect by *rhp51Δ* or *dmc1Δ*, *swi5Δ* had no effect on the failure of *mus81Δ* to slow (Figure 4B).

The Rhp51 mediator Swi5 forms two independent protein complexes by binding to either Sfr1 or Swi2. The Swi2-Swi5 complex is required for efficient mating-type switching, whereas the Swi5-Sfr1 complex promotes homologous recombination repair (Akamatsu *et al.*, 2003). With only the *sfr1Δ* mutant displaying a slowing defect, and this phenotype being Swi5 dependent, we tested what role Swi2 played in Sfr1-dependent replication slowing. *swi2Δ* single mutants slow normally and *swi5Δ swi2Δ* double mutant also slowed, indicating that the Swi5-Swi2 complex is not required for replication slowing in response MMS (Figure 6A). Similar to

Figure 6. The Swi5-Swi2 complex is required for the defect displayed in the *sfr1Δ* and *rqh1Δ* mutants. (A) *swi2Δ* (yFS590) and *swi5Δ swi2Δ* (yFS592) replication kinetics. (B) *sfr1Δ swi2Δ* (yFS594), *rqh1Δ swi2Δ* (yFS595), *mus81Δ swi2Δ* (yFS599), and *sfr1Δ swi5Δ swi2Δ* (yFS593) replication kinetics.

swi5Δ, deletion of *swi2* suppresses the slowing defects of *sfr1Δ* and *rql1Δ* but has no effect on the *mus81Δ* defect (Figure 6B).

Replication Fork Protection Is Required for *sfr1Δ* Slowing Defects

To pause stably in the absence of deoxyribonucleotide, forks require Cds1 and the Fork Protection Complex composed of Swi1 and Swi3 (Noguchi *et al.*, 2004). To determine whether fork stability plays a role in slowing in response to DNA damage, we tested the role of Swi1. *swi1Δ* cells slow normally, indicating the Fork Protection Complex is not required for the checkpoint (Figure 5A). However, Swi1 is required for *sfr1Δ* strains to fail to slow, much as Rhp51 is (Figure 5B). Suppression of the *rql1Δ* and *mus81Δ* strains by *swi1Δ* could not be determined because *mus81Δ swi1Δ* and *rql1Δ swi1Δ* are synthetic lethal (Willis, unpublished data; Noguchi *et al.*, 2004).

Suppression of Slowing Defects Does Not Act by Modulation of Checkpoint Signaling

Exposure to lower concentrations of MMS produces less slowing in wild-type strains, suggesting that increased checkpoint signaling may lead to increased replication slowing (Figure 2F). *swi5Δ* mutants display slightly increased checkpoint signaling over wild type (Figure 5C). Increased checkpoint signaling strength may be related to increased replication slowing and could be responsible for the slowing displayed in the *sfr1Δ swi5Δ* double mutant. However, suppression of the *sfr1Δ* defect does not correlate with increased signaling because the *sfr1Δ* and *sfr1Δ swi5Δ* strains displayed a similar level of Cds1 kinase activity in response to MMS (Figure 5C).

The Role of Other Rhp51 Mediators in Replication Slowing

Given the importance of the Rhp51 mediator Sfr1 for replication slowing, we tested whether additional Rhp51 mediator mutants were also required for slowing. *rhp54Δ*, *rhp55Δ*, and *rhp57Δ* mutants all slow normally (Figure 7 and S2A). To rule out redundant functions among the three mediators, double mutants were constructed and tested. All double mutants slowed replication well (Figure 7 and Supplemental S2A). These data indicates that disruption of Rhp51 mediator activity does not necessarily impact cells' ability to slow replication in response to DNA damage.

DNA damage sensitivities displayed by the DNA helicase mutant *rql1Δ* are recombination dependent and alleviated by disruption of *rhp51* or the recombinase mediators *swi5* and *rhp55* (Hope *et al.*, 2005). Individually, mutation of mediators *rhp54*, *rhp55*, or *rhp57* on an *rql1Δ* background did not suppress the *rql1Δ* slowing defect. However, *rql1Δ* mutants containing deletions for any two of the three recombinase mediators did slow (Figure 7 and Supplemental S2B). These data show that inhibition of recombination, either by deletion of *swi5* or the disruption of several other mediators, prevents the *rql1Δ* slowing defect. A similar relationship was established between these mediators and Sfr1 (Supplemental Figure S2B).

Slowing of Replication Does Not Correlate with Resistance to DNA Damage

The genetic interactions between recombinational repair genes and checkpoint-dependent slowing of replication raise the issue of whether slowing directly contributes to resistance to DNA damage. To investigate the relationship between slowing and resistance, we assayed MMS sensitivity in various slowing and nonslowing strains. We found no

correlation between the ability to slow replication in response to DNA damage and sensitivity to MMS (Figure 8). Both wild-type and the *cds1Δ* mutant, strains that slow and fail to slow, respectively, tolerate acute exposure to MMS. Conversely, the *rhp51Δ* and *rql1Δ* mutants are both sensitive to MMS exposure; yet, the *rhp51Δ* strain is still able to slow replication in response to MMS. These results show that slowing of replication is not necessary for cell to repair DNA damage and suggest that the choice between slowing and not slowing may instead reflect the choice between alternative repair pathways.

DISCUSSION

To investigate how fission yeast slows replication in the presence of DNA damage, we used a flow-cytometric assay of S-phase progression to determine the genetic requirements for this response. We found several proteins involved in recombination to be required for replication slowing in response to DNA damage: the Mus81 endonuclease, the Rqh1 helicase, and the Sfr1 mediator. We found recombination itself not to be required for slowing. However, recombination is involved in the regulation of slowing in response to DNA damage as demonstrated by the epistatic relationship between the slowing phenotype displayed by *rhp51Δ* mutants and the nonslowing phenotype displayed by *rql1Δ*, *sfr1Δ* and *mus81Δ*.

Three Epistasis Groups Regulate Replication Slowing

Our genetic analysis identified three epistasis groups with respect to S-phase DNA damage checkpoint-regulated slowing of replication (Figure 9). Group I includes the *cds1Δ* and *mus81Δ* mutants, both of which display a nonslowing phenotype not suppressed by removing recombination. Group II includes the *rhp51Δ* and *dmc1Δ* recombinase mutants, the *swi2Δ*, *swi5Δ*, *rhp54Δ*, *rhp55Δ*, and *rhp57Δ* mediator mutants and the *swi1Δ* Fork Protection Complex mutant, all of which slow replication in response to DNA damage. Group III consists of the *sfr1Δ* and *rql1Δ* mutants, which display a nonslowing phenotype similar to that of group I except that it is dependent upon recombination. Group I is epistatic to group II because double mutants between members of each group display the group I nonslowing phenotype. Group II is epistatic to group III because double mutants between members of these groups display the group II slowing phenotype. It should be noted that these epistasis groups are simply formal genetic relationships and do not imply any biochemical interactions. For example, Cds1 and Mus81 clearly have different biochemical roles in the checkpoint, but null mutants have the same slowing phenotype and the same epistatic interactions with *rhp51*; therefore, they are formally in the same epistasis group. Moreover, although the genes in groups I and III have the same phenotypes, they can be placed in separate groups because they have opposite epistatic interactions with the genes in group II.

Evidence for Reduction of Fork Rates as the Primary Mechanism for Slowing in Fission Yeast

Consistent with the involvement of recombination proteins in the checkpoint, our Cds1 overexpression results suggest the checkpoint acts locally at forks to slow replication. A priori, the checkpoint could slow bulk replication by either reduction of fork rates or inhibition of origin firing. Regulation of origin firing requires factors to act *in trans* to prevent origins from firing that may be physically distant from the DNA damage activating the checkpoint. In contrast, regulation of replication fork progression

Figure 7. Summary of replication slowing data for all strains discussed. Rep_{140} , the mean value for the 140 time point of each time course, in the presence or absence of MMS is displayed; the number of independent experiments is in parentheses after each strain name. Error bars represent the SEM for strains in which three or more experiments were performed, or the variance for strains for which only two experiments were performed. B1 indicates presence of the *nmt1*-repressor, thiamine.

could be local in nature, acting *in cis* with the DNA damage affected fork, changing the behavior of that fork to produce slowing; alternatively, it could be *trans* in nature, affecting the rate of all forks, regardless of whether they have encountered damage. Our observations that DNA damage and checkpoint signaling are each necessary but not sufficient to induce slowing are more consistent with an *in cis* fork model. An *in trans* model predicts that all checkpoint-activating damage, and bleomycin-induced damage in particular, would cause slowing. However, titration of bleomycin achieved strong

checkpoint signaling, equivalent to that induced by MMS without causing replication slowing (Figure 2, D and E).

We suspect that the difference in response to MMS and bleomycin is due to the quantitative differences between the lesions induced (Figure 2D; Rhind and Russell, 1998). MMS (0.03%) and 200 J/m² UV are each expected to produce tens of thousands of adducts leading to a lesion density of close to one every 500 base pairs (Sedgwick, 1975; Courcelle *et al.*, 2006). Therefore, replication forks would frequently encounter damage and even minor slowing at each lesion could add up to a significant effect on bulk replication. By contrast,

Figure 8. Slowing of replication in response to DNA damage does not correlate with resistance to DNA damage. Survival in response to acute exposure to 0.03% MMS in G1 synchronized cultures of wild-type (yFS162), *cds1Δ* (yFS543), *rhp51Δ* (yFS566), and *rql1Δ* (yFS545) cells. Cells were plated on YES after the indicated times and viability assayed by counting colonies after 4 d. Strains capable of slowing replication are indicated by +, and strains which fail to slow replication in response to MMS are indicated by a -.

200-Gray IR and 1 $\mu\text{g}/\text{ml}$ bleomycin are each expected to produce only ~ 15 double-strand breaks in a fission yeast genome (Povirk *et al.*, 1977; Prise *et al.*, 1998). Even if each break permanently arrests two replication forks, such treatment would reduce the number of forks by only 5%, a reduction that would be undetectable with currently available techniques.

To further discriminate between *cis* and *trans* effects on slowing, we measured slowing upon overexpression of Cds1. Because Cds1 overexpression produces kinase activity capable of inducing a G2/M arrest and checkpoint transcription in the absence of DNA damage (Brondello *et al.*, 1999; de Bruin *et al.*, 2008), we determined whether Cds1 overexpres-

sion could produce slowing in the absence of DNA damage. To exclude Rad3-dependent effects on Cds1 activity we overexpressed Cds1 on a *rad3Δ* background. Cds1 overexpression results in the accumulation of Cds1 kinase activity but failed to induce damage-independent slowing, suggesting that kinase activity alone is insufficient to induce replication slowing (Figure 3A). However, *rad3Δ* strains overexpressing Cds1 do slow in the presence of MMS, showing that overexpressed Cds1 activity is sufficient to produce slowing, but only in the presence of appropriate DNA damage (Figure 3, A and B). Similarly, *rad3Δ mrc1Δ* double mutants overexpressing Cds1 also slow but only in response to DNA damage. Consistent with our *in vivo* data, high concentration of Cds1 *in vitro* stimulates Cds1 autophosphorylation and kinase activity (Xu *et al.*, 2006). We propose that upon Cds1 overexpression, active Cds1 is able to interact with replication forks independently of the upstream checkpoint components Rad3 and Mrc1. The overexpression of a kinase-dead allele of Cds1 has no effect, suggesting that Cds1 has no kinase-independent role in the checkpoint (Figure 7). We interpret these results to mean that Cds1 must be catalytically active at individual forks as they encounter damage for the forks to slow. Moreover, because Cds1 is activated in these experiments by overexpression and is not influenced by Rad3-dependent checkpoint signaling, these results cannot be affected by any potential qualitative difference in Cds1 signaling in response to MMS- and bleomycin-induced DNA damage. We believe our results are inconsistent with *in trans* models of either global fork slowing or global origin inhibition.

Our Cds1 overexpression experiments also confirm the conclusion from previous work that Rad3 acts through Cds1 in the S phase DNA damage checkpoint (Lindsay *et al.*, 1998; Martinho *et al.*, 1998; Brondello *et al.*, 1999). These results show that Cds1 kinase activity is sufficient for replication slowing and suggest that Rad3 has no Cds1-independent role in the checkpoint. Conversely, that normally expressed Cds1 shows no genetic or biochemical activity in the absence of Rad3, suggests that Cds1 normally has no Rad3-independent function.

An alternate model to explain the failure to slow bulk replication displayed by the *rql1Δ*, *sfr1Δ*, and *mus81Δ* mutants is that fork slowing is the primary mechanism of the checkpoint in wild-type cells but that the failure to slow in checkpoint mutants is due to an abnormal increase in origin firing, not a decrease in fork slowing. Deregulation of origin firing would allow for more origins to fire than normal, producing more forks in the presence of damage and leading to fast bulk-S-phase progression even though individual forks are slowed. This model is motivated by the observation that in budding yeast, MMS causes a reduction in fork rates independent of checkpoint activity (Tercero and Diffley, 2001). However, several observations suggest that deregulated origin firing is not the cause of the failure to slow seen in fission yeast checkpoint mutants. First, even if all dormant origins were to fire, it would only increase the total number of forks by approximately threefold (Heichinger *et al.*, 2006)—not enough to compensate for the $>2\text{-h}$ S phase delay caused by MMS treatment. Second, the requirement for the three recombinational regulators for slowing and the genetic interaction between *rql1Δ*, *sfr1Δ* and *rhp51Δ* suggest that suppression of slowing involves strand exchange between nascent chromatids. Third, if slowing were produced by a combination of origin firing inhibition and slowing of forks (either in a local or global manner) then overexpression and activation of Cds1 would be expected to produce a partial slowing phenotype, which it does not. Together, all

Figure 9. An epistatic pathway and genetic model for replication slowing in fission yeast. (A) Three epistasis groups have been identified with respect to S-phase DNA damage checkpoint catalyzed slowing of replication. See text for details. (B) A model for checkpoint regulation of replication forks in response to DNA damage. See text for details.

of these observations suggest that failure to slow in mutants that disrupt the S-phase DNA damage checkpoint is caused by an increase in the rate of replication fork progression. Nonetheless, because our flow cytometry assay measures only bulk replication, we cannot rule out more complicated possibilities. A rigorous demonstration of fork slowing and its abrogation in checkpoint mutants will require direct measurement of fork rates, which has not yet been reported in fission yeast.

A Model for the Regulation of Replication Slowing in Response to DNA Damage

We propose the following model for checkpoint-dependent replication slowing in which replication fork progression at sites of damage is regulated by a balance between the checkpoint, which promotes replication slowing at lesions, and recombination, which facilitates fast replication through damage (Figure 9). We imagine that leading-strand lesions would have a much greater affect on bulk replication speed, because lesions on the lagging strand can be easily bypassed by repriming. By default, replication forks traverse the genome quickly and bulk replication is completed in ~20 min, even in the presence of DNA damage (Figure 1). Replication slowing requires Cds1 checkpoint kinase activation and the Mus81 endonuclease, which promote slowing independent of recombination. However, replication slowing does require negative regulation of recombination by Rqh1 and Sfr1. In particular, recombination interferes with replication fork slowing when Rqh1 or Sfr1 are absent by allowing forks to replicate quickly through damaged DNA (Figure 9). In this model, upon encountering DNA damage, replication forks serve as both the essential substrates for checkpoint activation and as the direct targets of the checkpoint (Tercero *et al.*, 2003). It is possible that the decision to replicate damaged DNA slowly or quickly is an entirely local decision—that checkpoint activation at a single fork is sufficient to cause that fork to slow and may not significantly effect the rest of the cell. Alternatively, it may be necessary to have a threshold level of checkpoint signaling in the cell for any fork to slow, thereby limiting replication slowing to situations in which multiple forks are encountering damage (Shimada *et al.*, 2002). Distinguishing these two possibilities will require techniques that can assay the slowing of individual replication forks.

The biological function of such a model is unclear, especially because we find no correlation between ability to slow replication and resistance to MMS (Figure 8). Several extremely sensitive mutants slow well, whereas others not sensitive fail to slow. We attribute the DNA damage sensitivity in our strains to a DNA damage repair defect in G2 and speculate that any benefit from slowing replication is masked by the greater importance of the G2 repair. This lack of correlation between slowing and resistance has been observed in mammalian cells, as well. However, human patients that lack the S phase DNA damage checkpoint are predisposed chromosome rearrangements and early-onset cancers, suggesting that the checkpoint may prevent genome instability associated with DNA repair (Tauchi *et al.*, 2002).

The Roles of Rqh1 and Recombination in Regulating Replication Slowing

We propose that the Rqh1 helicase promotes replication slowing in response to DNA damage by negatively regulating recombination. In the absence of Rqh1-dependent inhibition, recombination could facilitate quick replication of a damaged template by template switching (Branzei and

Foiani, 2007). Recombination could also catalyze polymerase bypass of DNA damage without strand exchange occurring. Recently, the bacterial recombinase RecA has been proposed to allow bypass of DNA damage by repriming on the leading strand after leading and lagging strand polymerases become uncoupled (Heller and Mariani, 2006; McInerney and O'Donnell, 2007).

The quick replication through DNA damage also requires Swi1, a member of the Fork Protection Complex, and Swi2, a Rhp51 mediator, both of which are involved in Rhp51-mediated mating-type switching, but not in general Rhp51-mediated recombinational repair (Akamatsu *et al.*, 2003; Noguchi *et al.*, 2004). These results suggest that the role of Rhp51 in the checkpoint is more closely affiliated with its mating-type switching function, which may require fork stabilization, than its general recombination function.

The Rhp51-Mediator Sfr1 and Its Relationship with the Swi2-Swi5 Complex

Disruption of *sfr1* does not have the same consequences for replication slowing as deletion of the other recombinase mediators, therefore Sfr1 must serve a unique function, presumably as a heterodimer with Swi5 because it has no characterized role independent of Swi5. Our genetic results suggest that Sfr1 acts to negatively regulate recombination. It is possible that Sfr1-Swi5 can both positively and negatively regulate recombination and that its negative role functions in the checkpoint. Alternatively, Sfr1 may play no direct role in the checkpoint. Instead, it may indirectly inhibit Swi2-Swi5 by simply sequestering Swi5, thereby preventing Swi5-Swi2 complex formation. This model assumes that the Swi2-dependent, mating-type switching function of Swi5 is involved in replication slowing, not the Sfr1-dependent, recombination-repair function (Akamatsu *et al.*, 2003, 2007). This mechanism would provide a simple explanation why only a single Rhp51 mediator, Sfr1, would be required for slowing, whereas others are not.

Mechanisms for Fast Replication through Damaged DNA in the Absence of Recombination

The mechanisms of recombination-dependent DNA damage bypass discussed above do not explain the quick replication displayed by the *rhp51Δ cds1Δ* or *rhp51Δ mus81Δ* double mutants. In these cells, DNA lesions blocking fork progression must be dealt with in an Rhp51-independent manner. One possibility might be replication by translesion synthesis polymerases. However, we see no evidence for involvement of translesion synthesis in the quick replication of *cds1Δ* cells. A quadruple mutant lacking Cds1 and the three defined translesion synthesis (TLS) polymerases η , ζ , and κ (Kai and Wang, 2003) still fails to slow in response to MMS treatment, indicating that TLS activity is not required for fast replication in the absence of the checkpoint (Willis, unpublished data). Alternatively, fork collapse and reassembly, termed break-induced replication, could account for quick replisome movement along damaged template and bypass of DNA damage. Break-induced replication can occur in both an Rhp51-dependent and independent manner (Signon *et al.*, 2001; Davis and Symington, 2004; Cortes-Ledesma *et al.*, 2007) and *cds1Δ* mutants display increased fork instability upon replication arrest (Noguchi *et al.*, 2003). However, fork stabilization per se, dependent on the Fork Protection Complex protein Swi1, is not required for slowing (Figure 5A). Finally, Rhp51-independent leading-strand repriming, in the absence of fork collapse, could allow *rhp51Δ cds1Δ* and *rhp51Δ mus81Δ* cells to replicate damaged DNA quickly.

Comparison of S-Phase DNA Damage Checkpoints in Fission Yeast, Budding Yeast, and Vertebrates

DNA damage induced slowing of replication is a widely conserved checkpoint response. However, the details of the response differ between fission yeast, budding yeast, and metazoa. The checkpoint in vertebrates seems to involve two parallel mechanisms: one mechanism regulating origin firing and one mechanism involving fork slowing and recombination. Contribution of these two mechanisms to replication slowing seems to differ with differing to damaging agents and doses (Falck *et al.*, 2002; Merrick *et al.*, 2004). In response to *cis*-platin and UV, the response seems to be largely fork dependent and to require recombination proteins (Henry-Mowatt *et al.*, 2003). In addition, both Mus81 and BLM, a mammalian Rqh1 homolog, are required for the regulation of replication forks in response to aphidicolin-induced replication stress (Shimura *et al.*, 2008). In contrast, low doses of IR trigger an origin-based response (Merrick *et al.*, 2004), and higher doses of IR may affect fork progression in addition to origin firing (Falck *et al.*, 2002). In budding yeast, replication is slowed in response to different DNA damaging agents, including MMS and bleomycin (Andrews and Clarke, 2005). Both reduction in origin firing and checkpoint independent fork slowing contribute to slowing in response to MMS (Tercero and Diffley, 2001). In contrast to budding yeast and metazoa, fission yeast slows replication only in response to MMS and not the double-strand break producing agents IR and bleomycin. We attribute this slowing to direct regulation of replication fork progression. The fact that fission yeast seems to regulate primarily replication forks makes it an excellent model organism for the exploration of replication fork regulation by the S-phase DNA damage checkpoint.

In conclusion, our data show that regulation of recombination plays an important downstream role in the S phase DNA damage checkpoint. Involvement of helicases, endonucleases, recombinases, and recombinase mediators in the S phase DNA damage checkpoint implicates recombination as an important factor in regulating the slowing of replication in response to DNA damage and further strengthens the notion that the replication slowing we observe is primarily produced by a coordinated response at replication forks. Slowing is likely due to a rapid and local response at replication forks encountering damage through the regulation of recombinational exchange between replicating sister chromatids.

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