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Roles of *SGS1*, *MUS81*, and *RAD51* in the repair of lagging-strand replication defects in *Saccharomyces cerevisiae*

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Abstract

Yeast cells lacking the *SGS1* DNA helicase and the *MUS81* structure-specific endonuclease display a synthetic lethality that is suppressed by loss of the *RAD51* recombinase. This epistatic interaction suggests that the primary function of *SGS1* or *MUS81*, or both genes, is downstream of *RAD51*. To identify *RAD51*-independent functions of *SGS1* and *MUS81*, a synthetic-lethal screen was performed on the *sgs1 mus81 rad51* triple mutant. We found that mutation of *RNH202*, which encodes a subunit of the hetero-trimeric RNase H2, generates a profound synthetic-sickness in this background. RNase H2 is thought to play a non-essential role in Okazaki fragment maturation. Cells lacking *RNH202* showed synthetic growth defects when combined with either *mus81* or *sgs1* alone. But, whereas the loss of *RAD51* had little effect on *rnh202 sgs1* double mutants, it strongly inhibited the growth of *rnh202 mus81* cells. These data indicate that the primary function of *SGS1*, but not *MUS81*, is downstream of *RAD51*. *SGS1* must have some *RAD51*-independent function, however, since the growth of *rnh202 mus81 rad51* cells was further compromised by the loss of *SGS1*. Consistent with these results, we show that *rnh202* cells display a sensitivity to DNA-damaging agents that is exacerbated in the absence of *RAD51* or *MUS81*. These data support a model in which defects in lagging-strand replication are repaired by the Mus81 endonuclease or through a pathway dependent on Rad51 and Sgs1.

Keywords

Sgs1; Mus81; Rad51; Recombination; DNA repair

Introduction

DNA helicases are key components in DNA metabolism. In the yeast *Saccharomyces cerevisiae*, loss of the *SGS1* DNA helicase results in genome instability, including increased rates of mitotic and meiotic recombination, gross chromosomal rearrangements, and chromosome loss (Watt et al. 1995, 1996; Sinclair et al. 1997; Yamagata et al. 1998; Chakraverty and Hickson 1999; Myung et al. 2001; Onoda et al. 2001; Hickson 2003). In addition, *sgs1* mutants are sensitive to high levels of DNA-damaging agents such as ultraviolet light (UV), methylmethane sulfonate (MMS) and hydroxyurea (HU) (Yamagata et al. 1998; Mullen et al. 2000) presumably because of their effects on DNA replication. Mutations in the human homologs of *SGS1* result in Bloom, Werner, or Rothmund–Thomson syndromes, which are all associated with a predisposition to cancer (Ellis et al. 1995; Yu et al. 1996; Kitao et al. 1999). All of these proteins share a 700 aa DNA helicase domain homologous to *E. coli* RecQ and possess an equally large N-terminal domain. Sgs1, like BLM, and Rqh1 in *S. pombe*, interacts physically with DNA topoisomerase III (Top3) (Gangloff et al. 1994; Wu et al. 2000).

SGS1 is required for UV- and MMS-induced heteroallelic recombination and, like *rgh1*⁺, *SGS1* is known to act in a *RAD52*-dependent recombination pathway (Murray et al. 1997; Gangloff et al. 2000; Ui et al. 2005). Both BLM and Sgs1 show physical interactions with the Rad51 recombinase (Wu et al. 2001). The role of *SGS1-TOP3* in recombination is consistent with the fact that *top3* homozygous diploids are incapable of undergoing meiosis unless meiotic recombination is prevented (Gangloff et al. 1999). Similarly, the slow-growth phenotype of *top3* strains is relieved in cells lacking any of the *RAD52* epistasis genes that are required for homologous recombination (HR). Such interactions are also observed in *S. pombe* where the inviability of *top3* strains is relieved by eliminating some HR functions (Gangloff et al. 2000; Maftahi et al. 2002; Oakley et al. 2002; Shor et al. 2002) and the UV sensitivity of *rgh1* cells is suppressed in strains lacking HR (Laursen et al. 2003).

Several *sgs1* synthetic-lethal screens have been employed to identify genes acting in parallel or redundant pathways (Mullen et al. 2001; Tong et al. 2001; Ooi et al. 2003). *MUS81* and *MMS4* were identified using this screen and were shown to encode a conserved heterodimeric structure-specific endonuclease (Boddy et al. 2001; Kaliraman et al. 2001; Mullen et al. 2001; Bastin-Shanower et al. 2003; Ciccio et al. 2003; Fu and Xiao 2003; Ogrunc and Sancar 2003; Whitby et al. 2003). For simplicity, we hereafter refer to this enzyme as the *MUS81* endonuclease. Recent studies have shown that *mus81 sgs1* synthetic-lethality can be suppressed by eliminating HR. Specifically, *mus81 sgs1* cells are viable in the absence of *RAD51*, *RAD52*, *RAD54*, *RAD55*, or *RAD57* (Fabre et al. 2002; Bastin-Shanower et al. 2003). *Rad51* is a well-understood component of the HR machinery. As the eukaryotic homolog of RecA, Rad51 binds DNA as a helical filament to promote HR (Paques and Haber 1999). The viability of *sgs1 mus81 rad51* cells suggests that the lethality of cells lacking Mus81-Mms4 and Sgs1-Top3 is due to the accumulation of toxic recombination intermediates (Fabre et al. 2002).

To identify DNA repair factors that require the *RAD51SGS1 MUS81* pathways, we carried out a synthetic-lethal screen using the *sgs1 mus81 rad51* triple mutant. One of the genes we isolated was *RNH202*, which encodes one of the subunits of RNase H2 (Jeong et al. 2004).

Most species have two classes of RNases H, RNase HI/H1 and RNase HII/H2 (Ohtani et al. 1999), and both enzymes specifically target the RNA portion of RNA-DNA/DNA or RNA/DNA duplex (Ohtani et al. 1999; Qiu et al. 1999; Jeong et al. 2004). Whereas bacterial RNases H2 are active as single polypeptides (Itaya 1990; Chapados et al. 2001), RNase H2 of yeast consists of a heterotrimeric protein complex encoded by *RNH201*, *RNH202*, and *RNH203* (Jeong et al. 2004). Rnh202 and Rnh203 are required in this complex to activate the catalytic activity of Rnh201 (Jeong et al. 2004). Although RNase H1 is important for the development of higher cells (Filippov et al. 2001; Cerritelli et al. 2003), loss of both RNases H in single-celled eukaryotes does not result in severe growth defects (Ray and Hines 1995; Arudchandran et al. 2000). However, loss of RNase H2 results in a higher sensitivity to DNA-damaging agents than does loss of RNase H1 (Arudchandran et al. 2000). It is thought that the primary role of RNase H2 is to process Okazaki fragments by removing RNA primers in cooperation with a pathway involving Rad27/FEN1 and Dna2 (Qiu et al. 1999; Chen et al. 2000; Bae et al. 2001).

We analyzed the role of RNase H2 in a variety of genetic backgrounds. Epistasis analysis in the *rnh202* background indicates that the main function of *MUS81* is independent of *RAD51*. Also, consistent with the idea that RNase H2 functions in parallel with Rad51 and Mus81, we show that the loss of any RNase H2 gene in the *rad51* or *mus81* backgrounds exacerbates their sensitivity to DNA-damaging agents. These results shed light on the overlapping functions of *SGS1* and *MUS81* and indicate that RNase H2 plays a critical role in lagging-strand replication and genome stability.

Materials and methods

Yeast strains and plasmids

Standard procedures were used for mating, sporulation, and tetrad dissection (Rose et al. 1990). All experimental procedures were carried out at 30 °C. Yeast strains used in this study are listed in Table 1. Plasmid pNJ1568 [*CEN-RAD51-SGS1-URA3-ADE3*] was constructed by subcloning a SacI fragment containing the *RAD51* gene into the SacI site of pJM500 (Mullen et al. 2001).

Isolation of synthetic-lethal interactors with *rad51 sgs1 mus81*

Strain MIY1789 (*MATa mus81-10::KAN sgs1-20::HGR rad51::HIS3 pNJ1568 [CEN-RAD51-SGS1-URA3-ADE3]*) and MIY1790 (*MATa* strain of MIY1789) were subjected to ethylmethanesulfonate (EMS) mutagenesis as described below. Cells were grown until saturation and they were resuspended at a concentration of 2×10^9 cells/ml in 1.5 ml of 0.1 M NaPO₄, pH (7.0). From this suspension, 0.7 ml was removed and diluted with 1 ml of 0.1 M NaPO₄. Fifty microliters of EMS was added to this cell suspension, mixed by vortexing, and incubated at 30°C with gentle shaking. Cells were removed at several time points, neutralized with 5% sodium thiosulfate, and aliquots were plated on 1% yeast extract/2% peptone/2% dextrose (YPD) plates. Viabilities of the cells were calculated by comparison with untreated controls, and cells that retained 30% viability were spread on YPD plates for screening. Approximately, 27,000 colonies were screened for colony color and all mutagenized colonies were replica-plated onto YPD and synthetically complete medium containing 1 mg/ml of 5-FOA. The colonies that were viable on YPD but inviable on 5-FOA were picked and confirmed by streaking onto 5-FOA plates. To eliminate false positives, these candidates were transformed with pMI1585 [*CEN-RAD51-SGS1-LEU2*] and the transformants were streaked onto 5-FOA plates. The candidates that were complemented by pMI1585 on 5-FOA were judged to be authentic synthetic-lethal mutants, and wild-type (wt) copies of the genes were cloned from a library. The candidates were transformed with a yeast genomic DNA library, 5-FOA^r colonies were identified, and the plasmid DNA was purified from yeast cells. *E.coli* XL1-Blue was used to transform and amplify the plasmid and the insert DNA was sequenced. To confirm the complementation by these plasmids, the original candidates were transformed with these plasmids and viability on 5-FOA medium was confirmed.

Determination of sensitivity to DNA-damaging agents in liquid culture

Cells were grown to mid-log phase in YPD at 30°C in a roller drum. The specific drugs were added to the indicated concentrations and the cells were then grown at 30°C for an additional 3.5 h with shaking. Following dilution of the cells to an appropriate density, they were spread on YPD plates. In the case of MMS-treated cells, the culture was first neutralized with 5% sodium thiosulfate before they were spread on plates. The plates were then incubated at 30°C for 3 days, and viabilities were determined by comparing the number of colonies obtained from treated and untreated cells.

Determination of sensitivity to UV irradiation

Sensitivities of the various mutant cells to UV irradiation were determined following UV exposure on YPD plates, which is described as follows. Cells were grown to early log phase in YPD at 30°C in a shaking water bath. An appropriate number of the specific cells were spread on YPD plates and irradiated with the indicated amount of UV light using a Stratagene (Stratagene). Plates were transferred to 30°C incubator immediately after irradiation and incubated for 3 days before counting the colonies. Viability was calculated by dividing the number of the colonies obtained following UV treatment by that obtained without irradiation.

Results

A synthetic-lethal screen with the *rad51 sgs1 mus81* triple mutant identifies *RNH202*

The ability of *sgs1 mus81* synthetic-lethality to be suppressed by the deletion of *RAD51* (Fabre et al. 2002; Bastin-Shanower et al. 2003) suggests the presence of alternative repair pathways. In order to identify genes in this alternative pathway, a synthetic-lethal screen was performed. We constructed a *rad51 Δsgs1 Δmus81Δ* triple mutant strain that also carried the *ade2* and *ade3* markers. This strain was transformed with pNJ1568, which contains the *SGS1*, *RAD51*, *ADE3*, and *URA3* genes (Fig. 1a). In this strain background, the *ADE3* gene imparts a red color to the colonies (*ade2 ADE3*), which can be used as a visual marker for the presence of the plasmid. Loss of pNJ1568 results in viable *rad51 Δsgs1 Δmus81Δ* colonies that are white. However, cells that acquire a mutation that is synthetically lethal with this combination of mutations (*rad51 Δsgs1Δ-mus81Δ*) will be unable to survive in the absence of pNJ1568 and remain red on non-selective plates (Fig. 1a). Mutations leading to the red colony-color phenotype were confirmed as potential synthetic-lethal candidates based on the inability of these cells to form colonies on plates containing 5-FOA which selects against the *URA3* gene on pNJ1568 (Boeke et al. 1987).

Following mutagenesis with EMS, approximately 27,000 colonies were screened and potential mutants were identified. To eliminate false positives in which the *URA3* marker gene on pNJ1568 had integrated into the genome, FOA^s candidates were transformed with plasmid pMI1585 [*SGS1/RAD51/LEU2*] and rechecked for viability on 5-FOA plates. A total of three mutants were obtained, which satisfied these criteria. The wild-type copies of the mutant genes were cloned by complementing the synthetic-lethal phenotype of the original mutants with a yeast genomic library. Mutations in two genes, *SLX1* and *SLX4*, were isolated as expected. These genes, like *SLX5* and *SLX8*, were previously isolated in *sgs1Δ* synthetic-lethal screens (Mullen et al. 2001; Tong et al. 2001; Ooi et al. 2003) and were shown to remain inviable even in the absence of *RAD51* (Fabre et al. 2002; Bastin-Shanower et al. 2003). The failure to isolate mutations in *SLX5* and *SLX8* in the current screen may have been due to the slow growth phenotype of these mutants.

The third mutant isolated in the screen (MIY2070) contained a mutation in *RNH202* (Fig. 1b). When the *rnh202-1* allele was recovered from strain MIY2070 and sequenced, it was found to contain a G to A transition at nucleotide 522. This resulted in a stop codon at amino acid 174 of Rnh202. Rnh202 is a subunit of the RNase H2 complex that is essential for RNase H activity (Jeong et al. 2004). Boeke and colleagues have previously shown that yeast cells lacking any of the genes encoding RNase H2 are synthetically sick with mutations in *SGS1* (Ooi et al. 2003).

To understand the relationship between *RAD51*, *SGS1*, *MUS81*, and *RNH202* in more detail, we examined how well the synthetic lethality of this quadruple mutant was complemented by *RAD51*, *SGS1*, *MUS81*, and/or *RNH202*. To do this, strain MIY2070 was transformed with plasmid-borne copies of these genes and the transformants were streaked onto plates containing 5-FOA to select against pNJ1568. As shown in Fig. 1b, the *RNH202* gene alone fully complemented the growth defect of this strain. Similarly, the combination of *RAD51* and *SGS1* complemented the strain (Fig. 1c). As expected, *RAD51* had no effect when transformed alone (Fig. 1c). However, *SGS1* did not effectively complement the growth defect when transformed alone (Fig. 1c). These results indicate that *SGS1* requires *RAD51* for activity. Surprisingly, *MUS81* alone was sufficient to complement this strain, and the combination of *MUS81* and *RAD51* together provided no additional growth advantage (Fig. 1d).

We had previously interpreted the suppression of *sgs1 mus81* synthetic lethality by *rad51* as an indication that the primary functions of *SGS1* and *MUS81* lie downstream of *RAD51*.

Moreover, the viability of this triple mutant implied the existence of pathways that bypass the requirement for all of these genes (illustrated in Fig. 1e). The results of Fig. 1b-d suggest a somewhat different model in which only *SGS1* requires *RAD51* function. In the absence of RNase H2, cell viability appears to rely on this *SGS1-RAD51* pathway or on a *RAD51*-independent pathway involving *MUS81* (Fig. 1f). We note that such a model does not necessarily rule out the existence of minor pathways in which *SGS1* and *MUS81* overlap directly.

Genetic interactions between *RAD51*, *SGS1*, *MUS81*, and *RNH202*

In order to analyze the interactions between *SGS1*, *MUS81*, *RAD51*, and each of the genes encoding RNase H2 subunits, we constructed strains bearing complete deletions of each gene. We then isolated strains containing all combinations of these mutations by crosses (Fig. 2a). The quadruple mutant *sgs1mus81 rad51rnh202* was found to be viable but grew very slow. Because the original *rnh202-1* strain was inviable, rather than slow-growing, we suspected that expression of the residual protein from the *rnh202-1* allele might have exacerbated the growth defect of cells lacking *SGS1*, *RAD51*, and *MUS81*. However, expression of the *rnh202-1* allele in the *rnh202* quadruple-mutant background was not lethal (data not shown). We conclude that the *sgs1mus81 rad51 rnh202* strain displays a synthetic interaction and that the inviability of the original isolate on media containing 5-FOA was due to additional background mutations or to selective pressure to retain the complementing plasmid (pNJ1568) in the presence of 5-FOA.

We next examined the doubling times of each of the mutants (Fig. 2a). Loss of *RNH202* in any of the *rad51*, *sgs1*, or *mus81* single mutant backgrounds resulted in a synthetic growth defect; each double mutant grew more slowly than either single mutant (Fig. 2a). This suggests that RNase H2 acts in a pathway parallel to Rad51, Sgs1, or Mus81.

Although *mus81rnh202* (DT=147 min) and *rad51rnh202* (DT=156 min) were found to be moderately slow growing, the *mus81 rad51 rnh202* strain (DT=200 min) had a substantial growth defect. Thus, in the absence of *RNH202*, *MUS81* and *RAD51* appear to act in separate, or parallel, pathways. A similar conclusion is drawn when one considers the effect of eliminating *RAD51* in the *sgs1rnh202* (DT = 174 min) or *mus81rnh202* (DT = 147 min) backgrounds. The resulting triple mutant strains show either a mild effect [*sgs1rnh202 rad51* (DT = 179 min)] or a severe effect [*mus81rnh202 rad51* (DT = 200 min)]. These results confirm that *MUS81* acts independently from *RAD51* and that *SGS1* and *RAD51* have an epistatic interaction consistent with the model of Fig. 1f. It should be emphasized that this model does not require that the *RAD51* and *MUS81* gene products act on the same substrate or that they act directly in the same repair pathway.

To address whether *RAD51* and *MUS81* have overlapping functions in the repair of exogenous DNA damage, we tested the sensitivity of the single and double mutants by spotting them in serial dilutions onto plates containing methyl methanesulfonate (MMS). As shown in Fig. 2b, the growth of *mus81* and *rad51* single mutants was slightly inhibited on media containing either low or moderate concentrations of MMS. However, at a low concentration of MMS, *mus81 rad51* double mutants grew more poorly than either single mutant, and they were unable to form colonies in the presence of moderate levels of MMS. The sensitivity of a *rad51* mutant to UV-induced DNA damage was similarly exacerbated by the loss of *MUS81*. The *mus81* mutant is only weakly sensitive to UV (Interthal and Heyer 2000; Mullen et al. 2001), and a small increase in UV sensitivity was observed in a *rad51* background (Fig. 2c). It should be noted that this increase in UV sensitivity was most apparent at low UV doses. These results confirm the overlapping nature of the *RAD51* and *MUS81* pathways.

The severe sickness of the *sgs1rad51 mus81rnh202* mutant suggests that cells lacking *SGS1*, *RAD51*, and *MUS81* are dependent on the RNase H2 complex. To confirm this idea, we isolated heterozygous *sgs1rad51 mus81* diploid strains containing a deletion of one copy of either *RNH201*, *RNH202*, or *RNH203*. Following tetrad dissection and germination on YPD plates, we observed that quadruple mutants were similarly slow growing regardless of which RNase H2 gene was disrupted (data not shown). We conclude that *sgs1rad51 mus81* cells require an intact RNase H2 complex for good growth.

Sensitivity of RNase H2 mutants to DNA-damaging agents

To determine whether RNase H2 plays a role in DNA damage avoidance, we examined the sensitivities of RNase H2 mutants to a variety of DNA-damaging agents including UV light. The sensitivity of several single-mutant strains was measured following continuous exposure to camptothecin (CPT), hydroxyurea (HU), and MMS, using the spot-dilution assay (Fig. 3a). The sensitivity of *mus81*, *sgs1* and *rad51* to these treatments is consistent with previous studies (Interthal and Heyer 2000; Mullen et al. 2000, 2001; Chang et al. 2002; Vance and Wilson 2002; Bastin-Shanower et al. 2003). A *rad27* mutant was included for comparison because of its known role in Okazaki fragment processing. As shown in Fig. 3a, the *rnh201*, *rnh202*, and *rnh203* single mutants did not show significant sensitivity to continuous exposure to CPT, HU, or MMS. In addition, these mutants did not show sensitivity to growth at 37°C (data not shown). Although a small increase in the sensitivity of some *rnh201* mutants to continuous exposure to HU has previously been reported, it was noted that there were clear differences between various strain backgrounds with respect to this phenotype (Arudchandran et al. 2000). Interestingly, the *rnh201*, *rnh202*, and *rnh203* mutants displayed a weak sensitivity to UV exposure that was equal to that of *rad51* and *rad27* mutants (Fig. 3b). We conclude that the RNase H2 complex is important for resistance to UV-induced DNA damage.

Sensitivity of the *rad51 rnh202* mutant to DNA-damaging agents

To further analyze the role of RNase H2 in DNA damage tolerance, we examined its relationship to the recombinational repair pathways using epistasis tests. We first examined the sensitivity of the *rad51 rnh202* double mutants to continuous exposure to CPT, HU, and MMS (Fig. 4a). Although the *rad51* single mutant grew poorly on plates containing MMS, the *rad51 rnh202* double mutants failed to form colonies at all (Fig. 4a). Thus, *rnh202* exacerbates the growth of *rad51* cells. Under conditions of continuous exposure to CPT and HU, we could not detect a difference between *rad51* and *rad51 rnh202* cells due to their extreme sensitivity. However, differences in their sensitivity were observed following acute exposure. In this assay, cells were exposed to various concentrations of CPT, HU, or MMS for a fixed duration and the viabilities of the cells were calculated after they were spread on YPD plates (Fig. 4b-e).

In the case of CPT, the *rad51 rnh202* double mutants showed the same sensitivity as the *rad51* mutant under all but with the highest CPT concentration (Fig. 4b). This result is consistent with the indispensable role of HR in the repair of CPT-induced double-strand breaks (Nitiss and Wang 1988) and suggests that RNase H2 does not play a role in resistance to CPT-induced damage. On the other hand, loss of *RNH202* exacerbated the sensitivity of *rad51* cells to HU, MMS, and UV. For example, the sensitivity of the double mutant to HU was twofold greater than that of the *rad51* single mutant (Fig. 4c), and it was up to sevenfold more sensitive to MMS (at 0.01% MMS; Fig. 4d). Most striking was the tenfold increase in the sensitivity of the *rad51 rnh202* double mutants over the entire range of UV doses (Fig. 4e). These results provide strong evidence that *RNH202* and *RAD51* act in parallel pathways to provide resistance to HU-, MMS-, and UV-induced DNA damage.

To test the generality of this result, we measured the DNA damage sensitivity of the *rad51 rnh201* double mutants. When examined under identical conditions, we observed no difference

between the sensitivities of the *rad51 rnh201* and *rad51 rnh202* double mutants to CPT, HU, MMS, and UV (data not shown). On the basis of this result, we conclude that *rad51* cells depend on the intact RNase H2 complex for optimal resistance to DNA damage.

Sensitivities of *mus81 rnh202* and *sgs1 rnh202* mutants to DNA-damaging agents

The *mus81 rnh202* and *sgs1 rnh202* double mutants were subjected to continuous exposure to CPT, HU, and MMS. We saw no effect due to the deletion of *RNH202* in the *sgs1* background and noted only minor effects due to the deletion of *RNH202* in the *mus81* background. However, the *mus81 rnh202* mutant was found to be significantly more sensitive to HU than either of the single mutants (Fig. 5a). This suggests that deletion of *RNH202* may exacerbate the sensitivity of *mus81* cells to some types of DNA damage.

We next examined the viability of these strains following acute exposure. Deletion of *RNH202* in the *mus81* background increased its sensitivity threefold to CPT (Fig. 5b) and up to twofold to HU (Fig. 5c); this occurred under conditions in which the *rnh202* single mutant showed no significant effect of these drugs (Figs. 5b & c). This treatment also revealed a slight increase in the sensitivity of *sgs1 rnh202* cells to HU compared to either single mutant. In contrast, we saw no significant increase in the sensitivities of *mus81 rnh202* and *sgs1 rnh202* double mutants to MMS or UV irradiation (Fig. 5d and e). We again tested the generality of these results by examining the sensitivities of *mus81 rnh201* and *sgs1 rnh201* double mutants to these treatments. As before, we saw no difference between the *rnh201* and *rnh202* versions of these double mutants (data not shown). Taken together, these results suggest that RNase H2 and Mus81 act in parallel pathways to maintain the resistance to CPT- and HU-induced DNA damage.

RNase H2 activity is required for resistance to DNA damage

Given that *rnh201*, *rnh202*, and *rnh203* mutants are known to behave identically (Ooi et al. 2003; Jeong et al. 2004), and that all three subunits are required for RNase H2 activity (Jeong et al. 2004), we considered it likely that it was the loss of RNase H2 activity, and not a novel activity, that was responsible for exacerbating the DNA damage sensitivity of *rad51*, *sgs1*, or *mus81* cells. To confirm this, we examined strains carrying point mutations in the catalytic site of Rnh201 for defects in resistance to UV exposure. The D39A and D155A mutations have previously been shown to eliminate RNase H2 activity (Chapados et al. 2001; Jeong et al. 2004). We therefore created these two changes individually within the *RNH201* gene and integrated these mutant alleles into the genome of an *rnh201* null strain. As expected, both mutant strains showed the same phenotypes as those of *rnh201*, *rnh202*, and *rnh203* deletions strains with respect to DNA damage sensitivity (Fig. 6). Specifically, these strains showed little, if any, sensitivity to continuous exposure to CPT, HU, or MMS (Fig. 6a), but they were as sensitive as an *rnh201* null mutant for UV sensitivity (Fig. 6b). These results strongly indicate that the loss of RNase H2 activity is responsible for the genetic interactions between mutations in *RNH202* and those in *RAD51*, *SGS1*, or *MUS81*.

RNase H2 defects are not mediated through telomere effects

Two hybrid studies have identified an interaction between Rnh201 and Rif2 (Jeong et al. 2004), a known Rap1-binding protein that interacts with the C-terminus of Rap1 (Wotton and Shore 1997). Rap1 binds telomere sequences and negatively regulates telomere length (Smogorzewska and de Lange 2004). Given the role of Rap1 and HR in mediating telomere length, we suspected that the synthetic defects may be due to effects on telomere length, perhaps through the binding of RNase H2 to Rif2. When examined by southern blotting, the length of telomeres from *rnh201*, *rnh202*, and *rnh203* mutants was essentially unchanged from that found in WT cells (data not shown). Any small differences were found to be less than the lengthening found in *dna2-2* cells (Formosa and Nittis 1999) or the shortening found in *rfa2-*

$\Delta N40$ cells (Schramke et al. 2004). Thus, the role of RNase H2 is likely to be limited to its role in lagging-strand replication as opposed to any indirect roles in telomere maintenance.

Discussion

One of the main findings of this study is that the *MUS81* endonuclease acts primarily in a *RAD51*-independent pathway for DNA repair. This conclusion is based on survival after exogenous DNA damage, as well as growth inhibition in the absence of *RNH202* that presumably represents the repair of spontaneous DNA damage. A role for *MUS81-MMS4* in recombination was implied from its functional overlap with *SGS1-TOP3* (Mullen et al. 2001). In the yeasts, there is good evidence that the RecQ-Top3 complex functions in the HR pathway (Wu et al. 1998, 2001; Gangloff et al. 1999; Onoda et al. 2001; Maftahi et al. 2002; Oakley et al. 2002; Shor et al. 2002; Laursen et al. 2003; Hope et al. 2005). The notion that *MUS81* might act downstream of *RAD51* was further suggested by the suppression of *mus81 sgs1* synthetic-lethality by *rad51* (Fabre et al. 2002; Bastin-Shanower et al. 2003). This phenotype appears to be similar to the suppression of *srs2 sgs1* synthetic-lethality (Gangloff et al. 2000; Maftahi et al. 2002) in which both genes are known to act in the HR pathway (Ira et al. 2003). Another curious similarity between *mus81* and *top3* mutants is that their sporulation defects can be suppressed by eliminating recombination (Gangloff et al. 1999; Kaliraman et al. 2001). But unlike *srs2*, *sgs1*, or *top3* mutants which show a hyper-recombination phenotype (Wallis et al. 1989; Rong et al. 1991; Watt et al. 1996), *mus81* and *mms4* mutants exhibit wild-type mitotic recombination rates based on a variety of assays (Interthal and Heyer 2000; Ira et al. 2003; Odagiri et al. 2003). One explanation for these genetic results is that *MUS81* and *SGS1* do not act on similar substrates, but that defects in *MUS81* function somehow lead indirectly to the *RAD51*-dependent accumulation of toxic recombination intermediates that must be resolved by *SGS1*. Our current sense of these overlapping pathways is presented in Fig. 7a.

The *RAD51*-independent function of *MUS81* appears to be conserved between *S. cerevisiae* and *S. pombe*. Using the same spectrum of DNA-damaging agents, it has been shown that *mus81+* acts in a *rad22+* (the *RAD52* homolog of *S. pombe*) -dependent pathway but *rhp51+* -independent pathway for DNA repair (Doe et al. 2004). Our studies have extended this observation to include spontaneous DNA damage in the *rnh202* background. Although we did not test the role of *RAD52* in the *MUS81-MMS4* pathway here, it has previously been shown that *RAD52* is epistatic to *mms4* for UV- and MMS-induced DNA damage in *S. cerevisiae* (Odagiri et al. 2003). Thus, the primary function of *MUS81* appears to be restricted to the same subset of HR pathways in the two yeasts. This conclusion is noteworthy given the differences between the two yeast systems. With respect to spontaneous recombination between heteroalleles, for example, budding yeast *rad51* mutants exhibit a slight hypo-recombinant phenotype (Rattray and Symington 1994), while *S. pombe* *rhp51* mutants are hyper-recombinant (Doe et al. 2004). Moreover, *rhp51* mutations are unable to suppress the synthetic lethality of *rqh1mus81* double mutants (Doe and Whitby 2004) suggesting that *rqh1+* and *mus81+* have significant functional overlap that is independent of *rhp51+*.

A second finding of this study is that RNase H2 plays a crucial role in Okazaki fragment processing that is revealed in cells lacking *MUS81* and *SGS1*. RNase H2 is thought to function in Okazaki fragment processing by removing the RNA primer; however, its role is complicated by redundancies in this process such as the activity of Rad27 (Qiu et al. 1999; Rydberg and Game 2002). It has been shown that an RNase H2 mutant is slightly more sensitive to DNA-damaging agents than an RNase H1 mutant, and while the transcription from *RNH201* is increased in S- and late G2/M-phases, transcription of *RNH1* is stable throughout the cell cycle (Arudchandran et al. 2000). This has led to the suggestion that RNase H1 works as a housekeeping enzyme while RNase H2 acts during DNA replication and during repair of DNA

damage in S- and G2/M (Arudchandran et al. 2000). However, RNase H2 cannot be essential in lagging-strand replication given that *rnh201* mutants are relatively healthy and Rad27/FEN1 is known to play a role in primer removal together with Dna2 and RPA (Bae et al. 2001; Kao and Bambara 2003). Genetic evidence suggests that RNase H2 may act together with Rad27 to remove primers. An *rnh201rad27* double mutant is viable but less healthy than either single mutant (Qiu et al. 1999) (and unpublished data), indicating that RNase H2 functions in a pathway that is redundant with Rad27. It has also been suggested that in yeast the initial nick in the RNA primer may be made by RNase H2 and the second one by Rad27 (Rydberg and Game 2002).

Functional genomics approaches have previously revealed interactions between genes required for lagging-strand replication and *SGS1*. For example, loss of *RAD27* or any one of the RNase H2-encoding genes result in synthetic sickness with mutations in *SGS1* (Tong et al. 2001; Ooi et al. 2003). A synthetic-lethal interaction was also identified between *RAD27* and *MUS81* (Tong et al. 2001; Ooi et al. 2003). Therefore, it is not surprising that we identified an allele of *RNH202* in our screen, as well as synthetic sickness in the *sgs1 rnh202* and the *mus81 rnh202* double mutants. However, in our analysis we find that the growth of both of these strains relies on *RAD51*.

These results appear to disagree with those of Boeke and colleagues who previously examined the genetic interactions between *SGS1*, *RAD51*, and the RNase H2 genes *RNH201*, *RNH202*, and *RNH203* (Ooi et al. 2003). Although there is agreement on the synthetic sickness between *sgs1* and *rnh202*, we find that *rad51* exacerbates, rather than suppresses, this synthetic sickness (Ooi et al. 2003). We showed by complementation that the weak *rad5-535* mutation found in our W303-derived strains (Fan et al. 1996) was not responsible for this difference (unpublished result). At present, we are unable to reconcile these results and suggest that the discrepancy may be due to other differences in the strain backgrounds.

It is not clear how the three genetic pathways described in Fig. 7a compensate for the loss of RNase H2. However, the molecular models presented in Fig. 7b can account for a majority of our findings. We hypothesize that the half-life of RNA primers on lagging-strand replication products increase in an RNase H2 mutant, thereby increasing the load on the alternative processing machinery which includes *RAD27/DNA2*. This may create a long-lived intermediate in which displaced RNA primers could isomerize with the nascent DNA of adjacent Okazaki fragments. This isomerization is likely to be *RAD51*-independent and would create an unstable product that is a substrate for *MUS81* cleavage. As shown in pathway#1, removal of the 3'-flap by *MUS81* would allow processing to begin again and permit *RAD27/DNA2* to properly excise the displaced primer. In pathway#2, the 3'-flap invades homologous sequences in the sister chromatid in a *RAD51*-dependent process. Following repair synthesis and primer removal, a pair of Holliday junctions are established and they are resolved by *SGS1-TOP3*. Consistent with earlier models (Fabre et al. 2002), we hypothesize a minor *RAD51*-dependent pathway (#3) for *MUS81*. In this case, displacement of the extended strand would create an even larger 3'-flap that would be a substrate for *MUS81*.

The observation that strains with defective lagging-strand replication rely on HR was previously made in strains lacking *RAD27* (Symington 1998; Tong et al. 2001). Curiously, we find that loss *RNH202* in a *rad51* background exacerbates the growth of the cells exposed to multiple types of DNA damage. Although the mechanism for DNA damage sensitivity of RNase H2 mutants is not understood, it is possible that exogenous damage creates increased numbers of Okazaki fragments, thus increasing the requirement for RNase H2 in these cells. An alternative idea is that these agents induce replication fork collapse that is repaired by *RAD51*-dependent break-induced replication (BIR). It has been shown that DNA synthesis during BIR involves a different set of proteins than those used in S-phase replication (Wang

et al. 2004). It will be interesting to test whether RNase H2 plays a special role during this process.

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Fig. 1.

A synthetic-lethal screen with *rad51 sgs1 mus81* identifies *RNH202*. **a** Schematic representation of the screen. The indicated triple mutant strain (MIY1789), which carries the plasmid pNJ1568 and the four indicated genes, was mutagenized with EMS and screened for strains that could not grow on medium containing 5-FOA. **b** Complementation analysis of strain MIY2070 (= MIY1789 *rnh202-1*). *RNH202* was subcloned into pRS415 (vector) and transformed into strain MIY2070. Independent transformants carrying the indicated gene, or the indicated untransformed strain, were streaked onto plates containing 5-FOA and incubated at 30°C for 3 days. **c** As above, but complementation was performed with *SGS1* and/or *RAD51*. **d** As above, but complementation was performed with *MUS81*, or *MUS81* and *RAD51*. **e** Schematic representation of the simplest relationship between *RAD51*, *SGS1*, and *MUS81* based on the suppression of *sgs1 mus81* synthetic-lethality. **f** The simplest interpretation of the data shown in panels (b)–(d).

Fig. 2.

Genetic interactions between *RAD51*, *SGS1*, *MUS81*, and *RNH202* reveal a functional overlap between *MUS81* and *RAD51*. **a** Strains of the indicated genotype were grown in liquid YPD medium at 30°C and doubling times were determined. The average value that is obtained from at least two experiments along with the standard deviation is shown. **b** Cells of the indicated genotype were spotted in tenfold serial dilutions onto YPD plates containing the indicated concentration of MMS or no drug. Cells were allowed to grow for approximately 3 days before they were photographed. **c** Cells of the indicated genotype were spread onto YPD plates and exposed to the indicated doses of UV. The number of viable colonies was determined following 3 days of growth at 30°C and is presented as a percentage of the un-irradiated sample. Error bars that are not visible are smaller than the symbol for the corresponding data point

Fig. 3.

The *rnh201*, *rnh202*, and *rnh203* single mutants show a similar sensitivity to UV exposure. **a** Cells of the indicated genotype were spotted in tenfold serial dilutions onto YPD plates with or without CPT, HU, or MMS. Cells were grown for 3 days at 30°C and photographed. **b** Cells were assayed for UV sensitivity as described in Fig. 2c

Fig. 4. Sensitivity of the *rad51 rnh202* double mutant to DNA-damaging agents. **a** Sensitivity to continuous DNA damage. Cells were treated as in Fig. 3a except that the plates containing drugs were incubated for 5 days before being photographed. **b** Sensitivity to CPT. **c** Sensitivity to HU. **d** Sensitivity to MMS. **e** Sensitivity to UV irradiation. The strains in panels (**b**)–(**d**) were grown in liquid culture and exposed to each drug for 3.5 h before an appropriate volume was removed and spread on YPD plates. The cultures in panel (**e**) were treated as described in Fig. 2c. Viability was determined from the number of colonies obtained following 3 days of growth at 30°C and is presented as a percentage of the untreated culture

Fig. 5. Sensitivities of the *mus81 rnh202* and the *sgs1 rnh202* double mutants to DNA-damaging agents. **a** Sensitivity to continuous DNA damage. Cells were treated as in Fig. 3a except that the plates containing drugs were incubated for 5 days before being photographed. **b** Sensitivity to CPT. **c** Sensitivity to HU. **d** Sensitivity to MMS. **e** Sensitivity to UV irradiation. The strains in panels (**b**)–(**e**) were treated as in Fig. 4

Fig. 6.

Active-site and *rnh201* null strains are equally sensitive to DNA damage. **a** Sensitivity to continuous DNA damage was assayed as in Fig. 3a. Note that the suppressor colonies present in the *mus81* culture were not reproducibly observed. **b** Sensitivity to UV irradiation was assayed as in Fig. 2c.

Fig. 7.

Models of *SGS1*, *MUS81*, and *RAD51* function. *a* *RNH202* is shown mediating a repair pathway that runs parallel to three others. Pathway#1 is defined by *MUS81* and is *RAD51*-independent. Pathway#2 is defined by *SGS1* and *RAD51*. *MUS81* may have a minor role downstream of *RAD51* and is presented as pathway#3. **b** In this model, RNA primers are retained on an unknown fraction of Okazaki fragments. If the hybrid is long lived, it could become displaced by DNA polymerase and isomerize with the nascent DNA to produce a superfluous 3'-flap. This substrate could be cleaved by *MUS81* endonuclease in a *RAD51*-independent pathway, allowing the hybrid to reform. Subsequently, the primer could be removed by a repair polymerase, in combination with Rad27 and/or Dna2. Alternatively, the 3'-end may be recombinogenic and feed into a *RAD51*-dependent pathway. The major pathway (#2), involves the formation of double Holliday junctions and resolution by *SGS1*-*TOP3*. A minor pathway (#3, in brackets) may involve *RAD51*-mediated strand invasion, partial extension, and strand displacement to produce a larger 3'-flap that is repaired by *MUS81* and ultimately by *RAD27* and/or *DNA2*.

Table 1

Strains used in this study

Strain	Genotype	Reference or source
W303-1a	<i>MATa ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100</i>	Thomas and Rothstein (1989)
W303-1b	<i>MAT-α ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100</i>	Thomas and Rothstein (1989)
MIY1789	<i>MATa ade2-1 ade3::hisG ura3-1 his3-11,15 trp1-1 leu2-3,112 mus81-10::KAN sgs1-20::HGR rad51::HIS3 can1-100 + pNJ1568 (SGS1/RAD51/URA3/ADE3)</i>	This study
MIY1790	<i>MATa ade2-1 ade3::hisG ura3-1 his3-11,15 leu2-3,112 lys2 mus81-10::KAN sgs1-3::TRP1 rad51::HIS3 can1-100 + pNJ1568 (SGS1/RAD51/URA3/ADE3)</i>	This study
MIY2070	<i>MATa ade2-1 ade3::hisG ura3-1 his3-11,15 trp1-1 leu2-3,112 mus81-10::KAN sgs1-20::HGR rad51::HIS3 can1-100 rnh202-1 + pNJ1568 (SGS1/RAD51/URA3/ADE3)</i>	This study
JMY332	<i>MATa ade2-1 ade3::hisG ura3-1 his3-11,15 trp1-1 leu2-3,112 sgs1-3::TRP1 can1-100</i>	Mullen et al. (2001)
JMY380	<i>W303-1a mus81-10::KAN</i>	Mullen et al. (2001)
HKY1039-4D	<i>W303-1b rad51::HIS3</i>	Hannah Klein
HKY619-3C	<i>W303-1a rad27::TRP1</i>	Hannah Klein
MIY1900	<i>W303-1a rnh201::NAT</i>	This study
MIY1901	<i>W303-1b rnh201::NAT</i>	This study
MIY1902	<i>W303-1a rnh202::NAT</i>	This study
MIY1903	<i>W303-1b rnh202::NAT</i>	This study
MIY1904	<i>W303-1a rnh203::NAT</i>	This study
MIY1905	<i>W303-1b rnh203::NAT</i>	This study
MIY2071	<i>MATa ade2-1 ade3::hisG ura3-1 his3-11,15 trp1-1 leu2-3, 112 sgs1-20::HGR rnh202::NAT can1-100</i>	This study
MIY2072	<i>MATa ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 rad51::HIS3 rnh202::NAT can1-100</i>	This study
MIY2073	<i>MATa ade2-1 ade3::hisG ura3-1 his3-11,15 trp1-1 leu2-3,112 mus81-10::KAN rnh202::NAT can1-100</i>	This study
MIY2074	<i>MATa ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 sgs1-3::TRP1 rad51::HIS3 can1-100</i>	This study
MIY2075	<i>MATa ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 mus81-10::KAN rad51::HIS3 can1-100</i>	This study
NJY1787	<i>MATa ade2-1 ade3::hisG ura3-1 his3-11,15 trp1-1 leu2-3,112 mus81-10::KAN sgs1-20::HGR rad51::HIS3 can1-100</i>	This study
MIY2076	<i>MATa ade2-1 ade3::hisG ura3-1 his3-11,15 trp1-1 leu2-3,112 sgs1-20::HGR rad51::HIS3 rnh202::NAT can1-100</i>	This study
MIY2077	<i>MATa ade2-1 ade3::hisG ura3-1 his3-11,15 trp1-1 leu2-3,112 mus81-10::KAN rad51::HIS3 rnh202::NAT can1-100</i>	This study
MIY2078	<i>MATa ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 mus81-10::KAN sgs1-20::HGR rad51::HIS3 rnh202::NAT can1-100</i>	This study
MIY2079	<i>MATa ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3, can1-100 rnh201::NAT, LEU2::rnh201-D39A</i>	This study
MIY2080	<i>MATa ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3, can1-100 rnh201::NAT, LEU2::rnh201-D155A</i>	This study