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Epistasis analysis between homologous recombination genes in *Saccharomyces cerevisiae* identifies multiple repair pathways for Sgs1, Mus81-Mms4 and RNase H2

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Abstract

The DNA repair genes *SGS1* and *MUS81* of *Saccharomyces cerevisiae* are thought to control alternative pathways for the repair of toxic recombination intermediates based on the fact that *sgs1Δ mus81Δ* synthetic lethality is suppressed in the absence of homologous recombination (HR). Although these genes appear to functionally overlap in yeast and other model systems, the specific pathways controlled by *SGS1* and *MUS81* are poorly defined. Epistasis analyses based on DNA damage sensitivity previously indicated that *SGS1* functioned primarily downstream of *RAD51*, and that *MUS81* was independent of *RAD51*. To further define these genetic pathways, we carried out a systematic epistasis analysis between the *RAD52*-epistasis group genes and *SGS1*, *MUS81*, and *RNH202*, which encodes a subunit of RNase H2. Based on synthetic-fitness interactions and DNA damage sensitivities, we find that *RAD52* is epistatic to *MUS81* but not *SGS1*. In contrast, *RAD54*, *RAD55* and *RAD57* are epistatic to *SGS1*, *MUS81* and *RNH202*. As expected, *SHU2* is epistatic to *SGS1*, while both *SHU1* and *SHU2* are epistatic to *MUS81*. Importantly, loss of any RNase H2 subunit on its own resulted in increased recombination using a simple marker-excision assay. RNase H2 is thus needed to maintain genome stability consistent with the *sgs1Δ rnh202Δ* synthetic fitness defect. We conclude that *SGS1* and *MUS81* act in parallel pathways downstream of *RAD51* and *RAD52*, respectively. The data further indicate these pathways share common components and display complex interactions.

Keywords

Recombinational repair; *SGS1*; *MUS81*; RNase H2

1. INTRODUCTION

Homologous recombination is crucial to the repair of DNA double-strand breaks (DSBs) in living cells [1–3]. HR is an evolutionarily conserved process that uses templated information to repair DSBs in an error-free manner. In humans, HR plays a significant role in maintaining genome stability and tumor suppression [4,5].

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An early step of HR involves homology search and DNA strand invasion. This reaction requires a subset of the *RAD52* epistasis group genes including *RAD51*, *RAD52*, *RAD54*, *RAD55* and *RAD57*. Rad51 is the eukaryotic RecA homolog that catalyzes homologous search and DNA strand exchange [6] with the help of mediator proteins Rad52 and Rad55-Rad57 complex [7–9]. Rad52 accelerates the removal of RPA from ssDNA by Rad51 [10]. Rad52 is capable of annealing homologous ssDNA [11], which is thought to be required for synthesis-dependent strand annealing (SDSA), single-strand annealing (SSA) and the second-end capture step of the DSB repair [12–14]. Rad54 function is diverse: Rad54 stabilizes the Rad51 presynaptic filament [15,16], it stimulates DNA strand invasion mediated by Rad51 *in vitro* [17,18], it dissociates Rad51 from heteroduplex DNA to load DNA polymerase [19], and it has branch-migration activity on Holliday junctions [20,21]. Rad55 and Rad57 are Rad51 paralogs in *S. cerevisiae* and these proteins exhibit mediator activity by forming a heterodimer that accelerates Rad51-mediated recombination *in vitro* [8,22] together with Rad52.

Consistent with the essential role of Rad51 in strand invasion, *rad51Δ* mutants display dramatic reductions in most HR reactions. However, some gene conversion events are known to occur in the absence of Rad51. For example, deletion of *RAD52* reduces the efficiency of spontaneous gene conversion between inverted repeats by 3,000-fold, whereas deletion of *RAD51* reduces it only 4-fold [23]. *RAD51* is not essential in ectopic gene conversion [24], and yeast is capable of RAD51-independent DSB repair resulting in interchromosomal gene conversion [25]. Therefore, budding yeast exhibits both Rad51-dependent and Rad51-independent recombination. Moreover, Break-Induced Replication (BIR) includes both Rad51-independent [26,27] and Rad51-dependent events [28,29]. Taken together with the existence of *RAD52*-independent recombination pathways in budding yeast [30], it is clear that HR pathways have not been completely defined.

SHU1, *SHU2*, *CSM2*, and *PSY3* (referred to as the *SHU* genes) were isolated in a *top3* suppressor screen [31] and loss of any one of these genes suppresses various defects in *sgs1Δ* or *top3Δ* mutants. *SHU* gene products are thought to form a multimeric complex involved in recombinational repair [31].

Recombination intermediates are potentially toxic and must be dissolved or displaced. The human BLM-TOPOIII -RMI1-RMI2 complex catalyzes the dissolution of recombination intermediates containing double-Holliday junctions (dHJs) *in vitro* [32–34]. The corresponding *S. cerevisiae* complex Sgs1-Top3-Rmi1 [35,36] has the same activity [37]. Mutations in *SGS1*, *TOP3* or *RMI1* cause similar phenotypes including sensitivities to DNA damaging agents, hyper-recombination, and synthetic lethality with mutations in *MUS81* and *SRS2* [35–40]. In addition, *sgs1Δ*, *top3Δ* and the catalytic point mutant *TOP3^{Y356F}* cells accumulate MMS-induced recombination intermediates based on two-dimensional (2D) gel electrophoresis of DNA replication products [41,42]. Many of the phenotypic defects of *sgs1*, *top3* and *rmi1* cells including the synthetic lethality of *sgs1Δ mus81Δ* double mutants are suppressed by deletion of *RAD51*, *RAD52*, *RAD54*, *RAD55*, or *RAD57* [38,40,43]. These results strongly suggest that *SGS1*, *TOP3* and *RMI1* are important for resolving recombination intermediates in living cells.

S. cerevisiae *MUS81* and *MMS4* were identified in a synthetic-lethal screen using an *sgs1Δ* mutant [39]. Mus81 and Mms4 form a structure-specific endonuclease that cleaves replication fork-like structures, nicked-Holliday junctions, D-loops and 3'flaps [43–47]. The *mus81Δ* mutant cells accumulate recombination intermediates during replication as detected by 2D gel electrophoresis [48–50]. Based on DNA damage sensitivity, *MUS81* and *MMS4* are known to function downstream of *RAD52* in *S. cerevisiae* and *RAD22* in *S. pombe* [51,52].

RNase H2 appears to play a minor role in DNA replication by acting redundantly with Rad27/FEN1 and Dna2 to remove RNA primers from Okazaki fragments [53,54]. In contrast, new results suggest that RNase H2 plays a more direct role in DNA repair by removing mis-incorporated ribonucleotides [55,56]. Interestingly, the loss of *SGS1* and any of the three genes encoding RNase H2 (*RNH201*, *RNH202*, and *RNH203* in budding yeast) results in synthetic-fitness defects [55,57]. Such a result is consistent with the idea that the loss of RNase H2 creates a need for recombinational repair.

Previous epistasis analysis indicated that *SGS1* acts primarily downstream of *RAD51* and *MUS81* was independent of *RAD51* [55]. To further characterize the recombinational repair pathways that are utilized by *SGS1*, *MUS81* and *RNH202*, we performed additional epistasis tests between these genes and a large set of HR genes (*RAD52*, *RAD54*, *RAD55*, *RAD57*, *SHU1*, *SHU2*). Our results indicate that *MUS81* functions primarily downstream of *RAD52*, whereas *SGS1* and *RNH202* are independent of *RAD52*. Interestingly, the phenotypes of *SGS1*, *MUS81*, and *RNH202* and the synthetic-sickness of *sgs1Δ rnh202Δ* double mutants are suppressed by deletion *RAD54*, *RAD55*, and *RAD57* suggesting a complex interaction between these two sets of genes. In addition, *SHU2* (but not *SHU1*) is epistatic to *SGS1*, *SHU1* is epistatic to *RNH202*, and both *SHU1* and *SHU2* are epistatic to *MUS81*. Finally, deletion of any of three RNase H2 genes results in an increase of recombination frequency strongly suggesting that lesions created in the absence of RNase H2 require Sgs1 for repair. These results allow us to propose a model that further defines the recombinational repair pathways distinguished by *SGS1* and *MUS81*.

2. MATERIALS AND METHODS

2.1 Media and yeast strains

Standard procedures were used for mating, sporulation, and tetrad dissection [58]. To generate *shuΔ* mutants, random spore analyses were performed as described previously [59]. All experimental procedures were carried out at 30°C. Yeast strains used in this study are listed in Table 1. All strains are *RAD5+* derivatives of W303 unless noted otherwise. The weak *rad5-535* mutation found in some W303-derived strains did not account for any MMS sensitivity under the conditions of low (<0.03%) MMS sensitivity used here. In all experiments, multiple meiotic segregants of the same genotype were found to behave similarly.

2.2 Growth rate determination

The OD₆₀₀ of cultures in exponential growth phase in YPD was determined every hour for 7 hr, and doubling times were determined. Three isolates were analyzed for each strain.

2.3 Sensitivity to DNA damaging agents

To measure sensitivity to DNA damaging agents, spot assays were performed as reported previously [55]. OD₆₀₀ = 3 cell suspensions were made and serial 1:10 dilutions were spotted onto indicated plates with or without DNA damaging agents. Plates were photographed after 3 days.

2.4 Recombination assay

The recombination assay was performed essentially as described previously [35,60]. The loss of three different marker genes (*ADE2* and *URA3* integrated independently at rDNA loci and *CAN1* integrated at *LYS2* locus) were measured as follows. Loss of *ADE2* was examined by growing the cells in YPD broth supplemented with adenine, plating them on YPD plates for single colonies, and incubating for 3 days. The marker loss rates for *ADE2* were determined by dividing the number of red colonies by the total number of colonies on

the plates. For the loss of *URA3*, the same number of cells were plated on –URA and non-selective plates and incubated for 3 days. The number of colonies on –URA plate was subtracted from the number of colonies on non-selective medium plate and that number was divided by the total number of colonies on non-selective plate. To measure the loss of *CAN1*, about 400 cells were plated on non-selective plates and 100-fold more cells were on canavanine plates. The colonies growing on canavanine were counted and designated as *can1* mutants having lost *CAN1* through recombination. Thus, the recombination frequency at the *LYS2* locus was determined by dividing the number of colonies on the canavanine plate by the total number of plated cells that was derived from the number of colonies on the non-selective plate.

3. RESULTS

3.1 *MUS81* functions in a *RAD52*-dependent pathway whereas RNase H2 does not

Previous epistasis analysis indicated that *SGS1* functions primarily in a *RAD51*-dependent pathway whereas *MUS81* was independent of *RAD51* [55]. For example, loss of *RAD51* had little effect on the doubling time (DT) of an *sgs1Δ rnh202Δ* double mutant (179 vs 174 min), but it severely inhibited a *mus81Δ rnh202Δ* double mutant (200 vs 147 min). It was also concluded that *RNH202* functioned independently of *RAD51*, *SGS1* and *MUS81* since the doubling time of the *mus81Δ sgs1Δ rnh202Δ rad51Δ* quadruple mutant was significantly slower than the *mus81Δ sgs1Δ rad51Δ* strain (271 vs 202 min).

To further examine the relationships between other *RAD52* epistasis group genes and *SGS1*, *MUS81* and *RNH202*, we crossed strains bearing complete deletions of each gene to create double, triple and quadruple mutants. We first examined the relationships among the *rad52Δ*, *sgs1Δ*, *mus81Δ* and *rnh202Δ* mutants. Surprisingly, we found that the *sgs1Δ mus81Δ rad52Δ rnh202Δ* quadruple mutant is inviable. Since *sgs1Δ mus81Δ* synthetic lethality was previously shown to be suppressed by deletion of *RAD52*, we conclude that some portion of *SGS1* and/or *MUS81* must function downstream of *RAD52*. The lethality of the *sgs1Δ mus81Δ rad52Δ rnh202Δ* quadruple mutant is therefore consistent with the idea that *RNH202* functions independently of *RAD52*.

To test this hypothesis, we examined the doubling times of several *rad52Δ* mutants (Fig. 1A). Although the growth rate of a *rad52Δ* single mutant was not significantly increased by the additional deletion of *MUS81* (147 vs 142 min), the *rad52Δ* growth defect was exacerbated by deletion of *SGS1* (197 vs 142 min), which is consistent with the result reported previously [61]. In addition, we observed a severe synthetic growth defect in the *rad52Δ rnh202Δ* double mutant (234 vs 142 min). Based on these results, *RAD52* is epistatic to *MUS81* and independent of *SGS1*. It had been reported that *RAD52* is epistatic to *MMS4* [51] therefore our result is consistent with theirs. We also conclude that *RNH202* functions in a pathway parallel to *RAD52* due to severity of the *rad52Δ rnh202Δ* synthetic growth defect and the synthetic lethality of the *sgs1Δ mus81Δ rad52Δ rnh202Δ* mutant.

We next examined the sensitivities of each of the strains to DNA damaging agents. This was determined by continuous exposure to camptothecin (CPT), hydroxyurea (HU), and MMS using the spot-dilution assay (Fig. 1B). For each drug, the *rad52Δ* strain was found to be more sensitive than any other single mutant. In addition, the *rad52Δ* and *rad52Δ mus81Δ* strains showed the same level of sensitivity to each of the DNA damaging agents. Thus, *MUS81* functions primarily in the same pathway as *RAD52* consistent with the above epistasis analysis based on doubling time. Since *MUS81* is known to function downstream of HR [51,52], these results suggest that Mus81 acts to resolve recombination-intermediates in the Rad52 pathway. In contrast to *rad52Δ mus81Δ* epistasis, *rad52Δ sgs1Δ* and *rad52Δ rnh202Δ* mutants displayed an additive effect. The double mutants grew more slowly than

their respective single mutants on plates containing CPT, HU or MMS (Fig. 1B). This is especially noticeable in the case of *rnh202Δ*, which eliminated the viability of *rad52Δ* cells on CPT and HU. This analysis indicates that a portion of *SGS1* acts in a pathway parallel to *RAD52*. In contrast, *RNH202* appears to function exclusively in a pathway parallel to *RAD52*. This latter result is again consistent with the results of the doubling time analysis.

Figure 1C uses a schematic to summarize the above results in terms of the relationships between *RAD51* and *RAD52*, and the genes *SGS1*, *MUS81* and *RNH202*. Previous work indicated that *SGS1* functioned downstream of *RAD51* whereas *MUS81* and *RNH202* did not [55]. Thus, *RAD51-SGS1* is shown to form a recombinational repair pathway parallel to the pathway composed of *RAD52-MUS81*. Because neither *RAD51* nor *RAD52* is epistatic to *RNH202*, it is shown to function in parallel to *RAD51-SGS1* and *RAD52-MUS81*.

3.2 The synthetic growth defect of *sgs1Δ rnh202Δ* is partially suppressed by deletion of *RAD54*, *RAD55*, or *RAD57*

To further define these pathways, we searched for genetic interactions between other *RAD52* epistasis group genes and *SGS1*, *MUS81* and *RNH202*. *RAD54* is thought to function upstream of *SGS1* and/or *MUS81* due to the fact that *sgs1Δ mus81Δ* synthetic lethality is suppressed by deletion of *RAD54* [40,43]. Our analyses of *RAD54* revealed that the synthetic sickness between *sgs1Δ* and *rnh202Δ* is partially suppressed by deletion of *RAD54* (Fig. 2A). That is, whereas the doubling time of *sgs1Δ rnh202Δ* was 182 min, the *rad54Δ sgs1Δ rnh202Δ* triple mutant grew faster (167 min). This result is consistent with the results of Ooi and Boeke [57], and the idea that *RAD54* acts upstream of *SGS1* and/or *RNH202*. In contrast, the synthetic growth defect of *mus81Δ rnh202Δ* was exacerbated by deletion of *RAD54*, rather than being suppressed (Fig. 2A: 150 vs 168 min). Our interpretation is that *Mus81* and *RNase H2* function differently in the repair that requires *Rad54*. We next examined the relationships with *RAD55*. In general, we observed similar results to the *RAD54* analysis. The synthetic growth defect of *sgs1Δ rnh202Δ* (DT=182 min) was partially suppressed by deleting *RAD55* (DT=168 min), and the synthetic growth defect of *mus81Δ rnh202Δ* (DT= 150 min) was not suppressed but was exacerbated (DT=161 min). Suppression of the *sgs1Δ rnh202Δ* growth defect was also observed by deleting *RAD57*. We note that suppression of *sgs1Δ rnh202Δ* synthetic sickness was most effective when *RAD57* was deleted. Again, no suppression of the *mus81Δ rnh202Δ* synthetic growth defect was observed by deleting *RAD57* (DT=155 min).

As reported previously, *sgs1Δ mus81Δ* synthetic lethality can be suppressed by deletion of any one of these *RAD* genes [38,40,43]. This fact and our results suggest that the *sgs1Δ rnh202Δ* defect lies downstream of these recombination factors. In contrast, deletion of these *RAD* genes failed to suppress the synthetic growth defects of *mus81Δ rnh202Δ* mutants. Thus, the *mus81Δ rnh202Δ* defect appears to lie upstream of these factors.

To further examine the differences between these recombinational repair pathways, we tested the sensitivity of single and double mutants by spotting serial dilutions of them onto plates containing DNA damaging agents. Figure 2B shows the results of spot assays of *rad54Δ* derivatives. Surprisingly, deletion of *SGS1* partially suppressed the sensitivity of *rad54Δ* to CPT and HU, whereas it did not suppress the sensitivity of *rad54Δ* to MMS. Similarly, deletion of *RNH202* suppressed the sensitivity of *rad54Δ* to CPT and HU, but not MMS. In contrast, deletion of *MUS81* did not significantly affect *rad54Δ*'s sensitivity to any of these agents.

Similar results were obtained in the analyses of *rad55Δ* and *rad57Δ* derivatives as shown in Figures 2C and D. Deletion of *SGS1* and *RNH202* suppressed the sensitivities of *rad55Δ* and *rad57Δ* to CPT and HU to various degrees, but did not affect their sensitivities to MMS. As

above, deletion of *MUS81* did not have a significant affect on the sensitivities of *rad55Δ* or *rad57Δ* to these drugs.

Figure 2E summarizes the above results and highlights the similarities between *sgs1Δ* and *rnh202Δ*. For example, deletion of *SGS1* or *RNH202* resulted in the same general pattern of suppression of DNA damage sensitivity in *rad54Δ*, *rad55Δ* and *rad57Δ* mutants. *MUS81* behaved differently as its deletion suppressed only the HU sensitivity of *rad57Δ*. As described above, deletion of *RAD54*, *RAD55* or *RAD57* partially suppressed the growth defect of *sgs1Δ rnh202Δ* mutants, but could not suppress the growth defect of *mus81Δ rnh202Δ* cells. One interpretation of these results is that the functions of RNase H2 and Sgs1 are partially overlapping upstream of *RAD54*, *RAD55*, and *RAD57*. In addition, it suggests that Sgs1 and RNase H2 contribute to the toxicity of agents that arrest replication forks in the absence of these HR factors.

3.3 Synthetic lethality between *rnh202Δ* and *top3Δ* reveals an overlapping function between RNase H2 and Top3-Rmi1 complex

Since Sgs1 is thought to function together with Top3 and Rmi1 to resolve recombination intermediates [35,36,41,42], we examined whether *top3Δ* and *rmi1Δ* displayed a synthetic lethal/sickness interaction with *rnh202Δ*. A *top3Δ* mutant was crossed to an *rnh202Δ* mutant and sporulated prior to dissection of ascospores. As shown in Figure 3A, *top3Δ rnh202Δ* double mutants were inviable. Haploid *rmi1Δ* and *rnh202Δ* strains were similarly mated and dissected. Shown in Figure 3B are the results from six *rmi1Δ rnh202Δ* spores that were obtained from five tetrads. Two of these double mutants were inviable and, although four others germinated on the YPD plate, they could not be passaged. These synthetic-lethal interactions suggest that Top3-Rmi1 and RNase H2 act in parallel pathways similar to Sgs1 and RNase H2.

3.4 Genetic interactions between *SHU1* and *SHU2* and the downstream genes *SGS1*, *MUS81* and *RNH202*

SHU1 and *SHU2* are known to function in recombination together with *RAD52* upstream of *SGS1* and *TOP3* [31]. To further characterize these recombinational pathways, we searched for genetic interactions between *SHU1* and *SHU2* and these downstream genes. Strains bearing complete deletions of each gene were created, and double or triple mutants were obtained from genetic crosses. As shown in Figure 4, deletion of *SHU1* in the *sgs1Δ* background resulted in a slow-growth phenotype (160 vs 142 min). In contrast, deletion of *SHU2* had no effect on *sgs1Δ* (137 vs 142 min). These results suggest that *SGS1* functions primarily downstream of *SHU2*, but not *SHU1*. Deletion of *SHU1* and *SHU2* had no effect on the growth rate of *mus81Δ* cells. However, deletion of *SHU2* in the *rnh202Δ* background resulted in a slow-growth phenotype (155 vs 137 min) and deletion of *SHU1* in did not. Thus, in contrast to *SGS1*, these results suggest that *RNH202* functions downstream of *SHU1* but not *SHU2*. Finally, the synthetic growth defect of *sgs1Δ rnh202Δ* was suppressed by deletion of *SHU2* (153 vs 174 min), but was exacerbated by a deletion of *SHU1* (184 vs 174 min). These results are consistent with the idea that *SGS1*, *MUS81*, and *RNH202* act in parallel pathways. Although these pathways are downstream of both *SHU1* and *SHU2* they display somewhat different dependencies on *SHU1* and *SHU2*.

3.5 Loss of RNase H2 increases recombination frequency

To determine whether loss of RNase H2 affects genome stability through recombination, a strain was used to assay recombination frequencies in the genome. The strain K1875 carries two selectable markers (*ADE2* and *URA3*) independently integrated at the rDNA loci and the third marker (*CAN1*) integrated at the *LYS2* locus [60]. In all cases the marker is flanked by direct repeats, and it is known that they are most often lost by excision recombination that

results in *ade2*, *ura3*, or *can1* phenotypes. As shown in Figure 5, loss of any one of RNase H2 subunits resulted in an increase in recombination frequency for all the markers *ADE2*, *URA3* and *CAN1*. Compared to wt cells, all the *rnh* mutants displayed a 3 to 4-fold increase in marker excision rate for all the loci. Thus, the loss of RNase H2 causes hyper-recombination phenotype. As control, we determined recombination rates in *sgs1* Δ mutants and confirmed that loss of Sgs1 results in a 5 to 6-fold increase in marker excision rate at all the loci as reported previously [35]. Loss of Mus81 did not affect the marker excision rate at *LYS2*, and resulted in a small (1.4 – 1.9 fold) increase at the rDNA. Taken together, we conclude that RNase H2 is important for genome stability, presumably due to the inability to remove mis-incorporated ribonucleotides.

4. DISCUSSION

One of the major findings of this study is that *MUS81* acts primarily in a *RAD52*-dependent pathway for DNA repair, whereas *SGS1* is primarily independent of *RAD52*. Previously we found that *SGS1* primarily functioned in a *RAD51*-dependent pathway whereas *MUS81* primarily acted independently of *RAD51* [55]. By taking these results into account, we propose that there are at least two major recombinational repair pathways distinguished by *RAD51-SGS1* and *RAD52-MUS81*. *MUS81-MMS4* has functional overlap with *SGS1-TOP3-RMI1* in recombinational repair [39], however there are differences between them. For example, *sgs1* Δ , *top3* Δ , and *rmi1* Δ mutants have been reported to exhibit a hyper-recombination phenotype [62–64], whereas *mus81* Δ and *mms4* Δ mutants have been reported to display wild-type levels of mitotic recombination [51,65,66]. One interpretation of the current results is that the similarities between *RAD51-SGS1* and *RAD52-MUS81* derive from their overlapping functions in recombinational repair, and their differences derive from the fact that the proteins act on different substrates. Previous studies have indicated that *RAD52* is epistatic to *MMS4*, which encodes a subunit of the Mus81/Mms4 complex, and that *RAD22* (a *RAD52* homolog) is epistatic to *MUS81* in *S. pombe* [51,52]. Our result is consistent with these earlier reports and indicates that this epistatic relationship is conserved in yeasts. In addition, it has been reported that *SGS1* functions together with *RAD52* under certain circumstances [38,67,68], however the relative roles of the *RAD52* epistasis genes had not been tested in a comprehensive manner. Our systematic analyses of these genetic interactions provide new information on the functions of these multiple recombinational repair pathways.

These analyses indicate that RNase H2 functions outside of the *RAD52*-dependent pathway and that it is partly epistatic to *RAD51*. This conclusion is based on the severe growth defect of *rad52* Δ *rnh202* Δ cells and the exacerbation of the *sgs1* Δ *rnh202* Δ growth phenotypes by deletion of *RAD52*. Further, loss of *RAD51* had a small negative effect on *rnh202* Δ cells, and no effect on the growth of *sgs1* Δ *rnh202* Δ cells [55]. This suggests that repair in the absence of RNase H2 relies greatly on *RAD52*.

Interestingly, the loss of *RAD54*, *RAD55*, or *RAD57* partially suppressed the synthetic growth defects of *sgs1* Δ *rnh202* Δ cells. This result is consistent with the earlier finding that loss of *RAD51* partially suppressed the growth defects of *sgs1* Δ *rnh* Δ cells [55,57]. We previously failed to observe suppression of the growth defects of *sgs1* *rnh202* Δ cells by deleting *RAD51* [55,57]. We cannot explain this difference other than to suspect that there were differences in the strain backgrounds that were used in the two studies. The current data suggest that the synthetic sickness of *sgs1* Δ *rnh202* Δ double mutants is due to the need for Sgs1 to repair lesions that arise in the absence of RNase H2. The source of these lesions appears to be single ribonucleotides that are mis-incorporated during DNA synthesis since a major role of RNase H2 is to eliminate these ribonucleotides [56]. In the absence of Sgs1

and RNase H2, toxic recombination intermediates arise due to the action of *RAD54*, *RAD55*, *RAD57* and *RAD51* [55,57].

We found that the DNA damage sensitivities of a subset of *RAD* mutants were suppressed by deleting *SGS1* or *RNH202* (Fig. 2B–E). Interestingly, similar levels of suppression were obtained by deleting *SGS1* or *RNH202*, suggesting that they were behaving similarly. But, RNase H2 has functions that are clearly independent of Sgs1-Top3-Rmi1, so this is unlikely to be the correct interpretation. The *rad54Δ*, *rad55Δ*, and *rad57Δ* mutants are sensitive to CPT and HU due to the need for HR-dependent repair of replication forks stalled by these compounds. Perhaps the suppression of their drug-sensitivities is due to the effect of the *sgs1Δ* and *rnh202Δ* mutations on fork movement. For instance, the loss of Sgs1 or RNase H2 may result in lesions that slow the rate of fork movement or stall forks under conditions that are less toxic than those arising due to CPT or HU treatment.

In contrast to the results with *RAD54*, *RAD55* and *RAD57*, growth rates indicate that *SGS1* is epistatic to *SHU2*, *RNH202* is epistatic to *SHU1* and *MUS81* is epistatic to both *SHU1* and *SHU2*. These differences are interesting because the *SHU* genes are thought to function as a complex [31]. Although we are currently unable to establish a mechanistic explanation for this result, we hypothesize that these differences reflect the dependency of the *RAD51-SGS1*, *RAD52-MUS81* and RNase H2 pathways on *SHU1* and *SHU2* and thus reflect the differences in the substrates that Shu1 and Shu2 act on in each pathway.

Finally, RNase H2 was found to suppress genome instability. All three RNase H2 mutants displayed a hyper-recombination phenotype using an assay system that employs three related reporter constructs. We suggest that this phenotype is directly related to the role of RNase H2 in removing ribonucleotides mistakenly incorporated into the genome. In its absence, the ribonucleotides may block the progress of replication forks leading to fork arrest or collapse. These damaged forks can presumably be restarted through an Sgs1-Top3-Rmi1-dependent recombination pathway. Thus, we suggest that loss of RNase H2 creates a need for recombinational repair due to an increase in the rate of stalled or collapsed replication forks.

Figure 6 presents a model summarizing the relationship between two major recombinational repair pathways. The left-hand pathway consists of Rad52-54-55-57, Shu1-2, and Mus81-Mms4, while the right-hand pathway consists of Rad51-54-55-57, Shu1-2, and Sgs1-Top3-Rmi1. The sizes of circles enclosing the protein names reflect the apparent importance of each protein that derived from the results of doubling times. The right-hand pathway appears to depend equally on Rad51, Rad54, Rad55 and Shu2. The Rad57 in the right-hand pathway is a little smaller whereas Shu1 is much smaller. The left-hand pathway depends primarily on Rad52 and Rad54 and somewhat less on Rad55 and Rad57. By taking previous and current studies into account, we suggest that bypass of a given pathway can occur by way of crosstalk between these two pathways indicated by the “shake-hands” symbol between Rad51 and Rad52. To account for the suppression results in Fig. 2B–D, we propose that both the Sgs1-Top3-Rmi1 and the RNase H2 complexes function upstream of recombination. A possible role for Sgs1-Top3-Rmi1 here is in the 5'-end resection of DNA ends to generate the 3'-single stranded overhangs [69]. We note that a positive role for RNase H2 upstream of recombination is currently unknown. However, it is possible that RNase H2 functions similarly. For example, RNase H2 may process DNA lesions that contain ribonucleotides to generate a single-strand break so that they can be acted upon by the HR machinery.

In this study, we have examined the roles of the recombinational repair pathways distinguished by *SGS1*, *MUS81* and *RNH202*. Clearly, a full understanding of the pathways

will require additional studies. However, our systematic genetic analyses provide insight into the composition of the pathways and provide a framework to understand their genetic interactions.

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Figure 1. Genetic interactions between *RAD52*, *SGS1*, *MUS81*, and *RNH202*

A. Strains of the indicated genotypes were grown in liquid medium at 30°C and the doubling times were examined to determine genetic interactions between *RAD52* and other DNA repair genes. **B.** Cells of the indicated genotype were spotted in ten-fold serial dilutions onto YPD plates containing the indicated concentrations of DNA damaging drug or no drug. Photographs were taken after 3 days. **C.** Schematic representation of the simplest relationship between *RAD51*, *RAD52*, *SGS1*, *MUS81* and *RNH202*.

A

Figure 2A

E

<i>SGS1</i>	Suppression of <i>rad54</i> , 55, 57 CPT ^s by <i>SGS1</i> deletion	Suppression of <i>rad54</i> , 55, 57 HU ^s by <i>SGS1</i> deletion	Suppression of <i>rad54</i> , 55, 57 MMS ^s by <i>SGS1</i> deletion
<i>rad54Δ</i>	+	+	-
<i>rad55Δ</i>	++	++	-
<i>rad57Δ</i>	++	+++	-

<i>RNH202</i>	Suppression of <i>rad54</i> , 55, 57 CPT ^s by <i>RNH202</i> deletion	Suppression of <i>rad54</i> , 55, 57 HU ^s by <i>RNH202</i> deletion	Suppression of <i>rad54</i> , 55, 57 MMS ^s by <i>RNH202</i> deletion
<i>rad54Δ</i>	+	-	-
<i>rad55Δ</i>	++	+	-
<i>rad57Δ</i>	++	++	-

<i>MUS81</i>	Suppression of <i>rad54</i> , 55, 57 CPT ^s by <i>MUS81</i> deletion	Suppression of <i>rad54</i> , 55, 57 HU ^s by <i>MUS81</i> deletion	Suppression of <i>rad54</i> , 55, 57 MMS ^s by <i>MUS81</i> deletion
<i>rad54Δ</i>	-	-	-
<i>rad55Δ</i>	-	-	-
<i>rad57Δ</i>	-	+	-

Figure 2. Genetic interactions between *RAD54*, *RAD55*, *RAD57*, and *SGS1*, *MUS81*, and *RNH202*
A. The doubling times of each of the mutants were determined as described in Figure 1. **B.** Spot assay with single mutants and *rad54Δ* derivatives. Assay was performed as described in Fig. 1B. **C.** Spot assay with *rad55Δ* derivatives. **D.** Spot assay with *rad57Δ* derivatives. **E.** Summary of suppressions of drug sensitivities of *rad54Δ*, *rad55Δ*, and *rad57Δ* mutants by deletion of *SGS1*, *RNH202*, or *MUS81*. This summarizes the results of the spot assays shown in Fig. 2B–D and reveals the similarities between *sgs1Δ* and *rnh202Δ* on the drug sensitivities of *rad54Δ*, *rad55Δ* and *rad57Δ* mutants. One plus implies 10-fold better growth compared to the drug sensitivity of the corresponding *rad54/55/57* single mutants. Note that deletion of *SGS1* or *RNH202* resulted in the same general pattern of suppression of DNA damage sensitivity in *rad54Δ*, *rad55Δ* and *rad57Δ* mutants.

A *top3* X *rnh202*

<i>rnh202</i>	<i>top3</i> <i>rnh202</i>	<i>top3</i> <i>rnh202</i>	<i>top3</i>	<i>rnh202</i>
a	a	a	a	a
<i>top3</i> <i>rnh202</i>	wt	wt	<i>rnh202</i>	wt
α	a	α	α	a
<i>top3</i>	<i>rnh202</i>	wt	<i>top3</i>	<i>top3</i>
α	α	α	α	α
wt	<i>top3</i>	<i>top3</i> <i>rnh202</i>	<i>rnh202</i>	<i>top3</i> <i>rnh202</i>
a	α	a	a	α

B *rmi1* X *rnh202*

<i>rnh202</i>	wt	<i>rmi1</i> <i>rnh202</i>	<i>rnh202</i>	<i>rmi1</i> <i>rnh202</i>
a	a	α	α	α
<i>rmi1</i>	<i>rmi1</i>	<i>rmi1</i>	<i>rmi1</i> <i>rnh202</i>	<i>rmi1</i> <i>rnh202</i>
α	α	a	α	α
<i>rmi1</i> <i>rnh202</i>	<i>rnh202</i>	wt	wt	wt
α	a	a	a	a
wt	<i>rmi1</i> <i>rnh202</i>	<i>rnh202</i>	<i>rmi1</i>	wt
a	α	α	a	a

Figure 3. Synthetic-lethal interactions between *TOP3*, *RMI1* and *RNH202*
A. Tetrads from a cross between strains JMY372 (*top3*Δ) and MIY1903 (*rnh202*Δ) were dissected and incubated for 4 days at 30°C. Subsequent analysis revealed that small colonies are *top3*Δ mutants and all dead spores are *top3*Δ *rnh202*Δ double mutants. **B.** Tetrads from a cross between strains JMY1918 (*rmi1*Δ) and MIY1903 (*rnh202*Δ) were dissected and incubated for 4 days at 30°C. Subsequent analysis revealed that both two dead spores and four small colonies are *rmi1*Δ *rnh202*Δ double mutants. Note that four *rmi1*Δ *rnh202*Δ spores were germinated but could not be passaged.

Figure 4. Genetic interactions between *SHU1*, *SHU2* and *SGS1*, *MUS81*, and *RNH202*
 The doubling times of each of the mutants were determined as described in Fig. 2. Note that synthetic-fitness phenotype of *sgs1Δ rnh202Δ* is substantially suppressed by loss of *SHU2*.

Figure 5. Loss of RNase H2 causes an increase of recombination frequency in a whole genome Recombination reporter strains were constructed that contained the indicated genotypes in addition to three different marker genes flanked by direct repeats: *ADE2* and *URA3* integrated independently at rDNA loci, and *CAN1* integrated at the *LYS2* locus. The frequency with which these markers were lost from the genome was determined as described in the Materials and Methods. Shown are the average recombination frequencies of three experiments $\pm$ SD. Also shown is the change in recombination frequency relative to WT.

Figure 6. A model representing the relationships between *RAD51*, *RAD52*, *RAD54*, *RAD55*, *RAD57*, *SHU1*, *SHU2* and *SGS1*, *MUS81* and *RNH202*

The model proposes that there are two major recombinational repair pathways consisting of Rad52-54-55-57, Shu1-2, and Mus81-Mms4 (left), and Rad51-54-55-57, Shu1-2, and Sgs1-Top3-Rmi1 (right). These pathways are derived from the results of genetic interaction analyses shown in Fig. 2A–D and Fig. 4. Based on the results that the drug sensitivities of *rad54Δ*, *rad55Δ* and *rad57Δ* were suppressed by deletion of *SGS1* or *RNH202* as shown in Fig. 2B–E, we propose that both Sgs1-Top3-Rmi1 and RNase H2 complexes function upstream of recombination as indicated at the top of the model.

Table 1

Strains used in this study.

Strain	Genotype	Reference or source
HKY579-10A	<i>MATa ade2-1 ura3-1 his3-11, 15 trp1-1 leu2-3, 112 can1-100</i>	Hannah Klein
HKY580-10D	<i>MATa ade2-1 ura3-1 his3-11, 15 trp1-1 leu2-3, 112 can1-100</i>	Hannah Klein
JMY332	<i>MATa ade2-1 ura3-1 his3-11, 15 trp1-1 leu2-3, 112 sgs1-3::TRP1 can1-100 rad5-535</i>	[39]
JMY380	<i>MATa ade2-1 ura3-1 his3-11, 15 trp1-1 leu2-3, 112 mus81-10::KAN can1-100 rad5-535</i>	[39]
HKY614-10B	<i>MATa ade2-1 ura3-1 his3-11, 15 trp1-1 leu2-3, 112 rad52::TRP1 can1-100</i>	Hannah Klein
HKY624	<i>MATa ade2-1 ura3-1 his3-11, 15 trp1-1 leu2-3, 112 rad54::HIS3 can1-100</i>	Hannah Klein
HKY597-2C	<i>MATa ade2-1 ura3-1 his3-11, 15 trp1-1 leu2-3, 112 rad55::LEU2 can1-100</i>	Hannah Klein
HKY598-8B	<i>MATa ade2-1 ura3-1 his3-11, 15 trp1-1 leu2-3, 112 rad57::LEU2 can1-100</i>	Hannah Klein
JMY372	<i>MATa ade2-1 ura3-1 his3-11, 15 trp1-1 leu2-3, 112 top3-2::HIS3 can1-100 rad5-535</i>	[35]
JMY1918	<i>MATa ade2-1 ura3-1 his3-11, 15 trp1-1 leu2-3, 112 rmi1-10::KAN can1-100 rad5-535</i>	[35]
MIY0614	<i>MATa ade2-1 ura3-1 his3-11, 15 trp1-1 leu2-3, 112 shu1::KAN can1-100</i>	This study
MIY0650	<i>MATa ade2-1 ura3-1 his3-11, 15 trp1-1 leu2-3, 112 shu1::hphMX4 can1-100</i>	This study
MIY0632	<i>MATa ade2-1 ura3-1 his3-11, 15 trp1-1 leu2-3, 112 shu2::hphMX4 can1-100</i>	This study
MIY0640	<i>MATa ade2-1 ura3-1 his3-11, 15 trp1-1 leu2-3, 112 shu2::KAN can1-100</i>	This study
MIY1903	<i>MATa ade2-1 ura3-1 his3-11, 15 trp1-1 leu2-3, 112 rnh202::NAT can1-100 rad5-535</i>	[55]
MIY2071	<i>MATa ade2-1 ade3::hisG ura3-1 his3-11, 15 trp1-1 leu2-3, 112 sgs1- 20::hphMX4 rnh202::NAT can1-100</i>	[55]
MIY0944	<i>MATa ade2-1 ade3::hisG ura3-1 his3-11, 15 trp1-1 leu2-3, 112 sgs1- 20::hphMX4 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>	[55]
MIY0945	<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
NJY1612	<i>MATa ade2-1 ura3-1 his3-11, 15 trp1-1 leu2-3, 112 rad52::TRP1 sgs1- 20::hphMX4 mus81::KAN can1-100</i>	This study
MIY0929	<i>MATa ade2-1 ura3-1 his3-11, 15 trp1-1 leu2-3, 112 rad52::TRP1 sgs1::hphMX4 can1-100</i>	This study
MIY0930	<i>MATa ade2-1 ura3-1 his3-11, 15 trp1-1 leu2-3, 112 rad52::TRP1 mus81::KAN can1-100</i>	This study
MIY0931	<i>MATa ade2-1 ura3-1 his3-11, 15 trp1-1 leu2-3, 112 rad52::TRP1 rnh202::NAT can1-100</i>	This study
MIY0932	<i>MATa ade2-1 ura3-1 his3-11, 15 trp1-1 leu2-3, 112 mus81::KAN rad52::TRP1 rnh202::NAT can1-100</i>	This study
MIY0933	<i>MATa ade2-1 ura3-1 his3-11, 15 trp1-1 leu2-3, 112 sgs1::hphMX4 rad52::TRP1 rnh202::NAT can1-100</i>	This study
VCY1471	<i>MATa ade2-1 ura3-1 his3-11, 15 trp1-1 leu2-3, 112 sgs1-3::TRP1 rad54::HIS3 mus81::KAN can1-100</i>	This study
MIY0934	<i>MATa ade2-1 ura3-1 his3-11, 15 trp1-1 leu2-3, 112 rad54::HIS3 sgs1-3::TRP1 can1-100</i>	This study
MIY0936	<i>MATa ade2-1 ura3-1 his3-11, 15 trp1-1 leu2-3, 112 rad54::HIS3 mus81::KAN can1-100</i>	This study
MIY0938	<i>MATa ade2-1 ura3-1 his3-11, 15 trp1-1 leu2-3, 112 rad54::HIS3 rnh202::NAT can1-100</i>	This study
MIY0941	<i>MATa ade2-1 ura3-1 his3-11, 15 trp1-1 leu2-3, 112 rad54::HIS3 sgs1-3::TRP1 rnh202::NAT can1-100</i>	This study
MIY0942	<i>MATa ade2-1 ura3-1 his3-11, 15 trp1-1 leu2-3, 112 rad54::HIS3 mus81::KAN rnh202::NAT can1-100</i>	This study
MIY0943	<i>MATa ade2-1 ura3-1 his3-11, 15 trp1-1 leu2-3, 112 rad54::HIS3 sgs1-3::TRP1 mus81::KAN rnh202::NAT can1-100</i>	This study
JMY1441	<i>MATa ade2-1 ura3-1 his3-11, 15 trp1-1 leu2-3, 112 rad55::LEU2 sgs1- 3::TRP1 can1-100</i>	This study

Strain	Genotype	Reference or source
MIY0948	<i>MATa ade2-1 ura3-1 his3-11, 15 trp1-1 leu2-3, 112 rad55::LEU2 mus81- 10::KAN can1-100</i>	This study
MIY0950	<i>MATa ade2-1 ura3-1 his3-11, 15 trp1-1 leu2-3, 112 rad55::LEU2 rnh202::NAT can1-100</i>	This study
MIY0952	<i>MATa ade2-1 ura3-1 his3-11, 15 trp1-1 leu2-3, 112 rad55::LEU2 sgs1- 3::TRP1 mus81-10::KAN can1-100</i>	This study
MIY0953	<i>MATa ade2-1 ura3-1 his3-11, 15 trp1-1 leu2-3, 112 rad55::LEU2 sgs1- 3::TRP1 rnh202::NAT can1-100</i>	This study
MIY0954	<i>MATa ade2-1 ura3-1 his3-11, 15 trp1-1 leu2-3, 112 rad55::LEU2 mus81- 10::KAN rnh202::NAT can1-100</i>	This study
MIY0955	<i>MATa ade2-1 ura3-1 his3-11, 15 trp1-1 leu2-3, 112 rad55::LEU2 sgs1- 3::TRP1 mus81-10::KAN rnh202::NAT can1-100</i>	This study
VCY1516	<i>MATa ade2-1 ura3-1 his3-11, 15 trp1-1 leu2-3, 112 rad57::LEU2 sgs1- 3::TRP1 mus81::KAN can1-100</i>	This study
MIY0956	<i>MATa ade2-1 ura3-1 his3-11, 15 trp1-1 leu2-3, 112 rad57::LEU2 sgs1- 3::TRP1 can1-100</i>	This study
MIY0958	<i>MATa ade2-1 ura3-1 his3-11, 15 trp1-1 leu2-3, 112 rad57::LEU2 mus81::KAN can1-100</i>	This study
MIY0960	<i>MATa ade2-1 ura3-1 his3-11, 15 trp1-1 leu2-3, 112 rad57::LEU2 rnh202::NAT can1-100</i>	This study
MIY0963	<i>MATa ade2-1 ura3-1 his3-11, 15 trp1-1 leu2-3, 112 rad57::LEU2 sgs1- 3::TRP1 rnh202::NAT can1-100</i>	This study
MIY0964	<i>MATa ade2-1 ura3-1 his3-11, 15 trp1-1 leu2-3, 112 rad57::LEU2 mus81::KAN rnh202::NAT can1-100</i>	This study
MIY0965	<i>MATa ade2-1 ura3-1 his3-11, 15 trp1-1 leu2-3, 112 rad57::LEU2 sgs1- 3::TRP1 mus81::KAN rnh202::NAT can1-100</i>	This study
MIY0685	<i>MATa ade2-1 ura3-1 his3-11, 15 trp1-1 leu2-3, 112 sgs1-20::hphMX4 shu1::KAN can1-100</i>	This study
MIY0763	<i>MATa ade2-1 ura3-1 his3-11, 15 trp1-1 leu2-3, 112 sgs1-20::hphMX4 shu2::KAN can1-100</i>	This study
MIY0745	<i>MATa ade2-1 ura3-1 his3-11, 15 trp1-1 leu2-3, 112 mus81-10::KAN shu1::hphMX4 can1-100</i>	This study
MIY0825	<i>MATa ade2-1 ura3-1 his3-11, 15 trp1-1 leu2-3, 112 mus81-10::KAN shu2::hphMX4 can1-100</i>	This study
MIY0705	<i>MATa ade2-1 ura3-1 his3-11, 15 trp1-1 leu2-3, 112 shu1::KAN rnh202::NAT can1-100</i>	This study
MIY0777	<i>MATa ade2-1 ura3-1 his3-11, 15 trp1-1 leu2-3, 112 shu2::hphMX4 rnh202::NAT can1-100</i>	This study
MIY0725	<i>MATa ade2-1 ura3-1 his3-11, 15 trp1-1 leu2-3, 112 sgs1-20::hphMX4 rnh202::NAT shu1::KAN can1-100</i>	This study
MIY0766	<i>MATa ade2-1 ura3-1 his3-11, 15 trp1-1 leu2-3, 112 sgs1-20::hphMX4 rnh202::NAT shu2::KAN can1-100</i>	This study
MIY0748	<i>MATa ade2-1 ura3-1 his3-11, 15 trp1-1 leu2-3, 112 mus81-10::KAN rnh202::NAT shu1::hphMX4 can1-100</i>	This study
MIY0698	<i>MATa ade2-1 ura3-1 his3-11, 15 trp1-1 leu2-3, 112 mus81-10::KAN rnh202::NAT shu2::hphMX4 can1-100</i>	This study