Resection Activity of the Sgs1 Helicase Alters the Affinity of DNA Ends for Homologous Recombination Proteins in *Saccharomyces cerevisiae*

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ABSTRACT The RecQ helicase family is critical during DNA damage repair, and mutations in these proteins are associated with Bloom, Werner, or Rothmund-Thompson syndromes in humans, leading to cancer predisposition and/or premature aging. In the budding yeast *Saccharomyces cerevisiae*, mutations in the RecQ homolog, *SGS1*, phenocopy many of the defects observed in the human syndromes. One challenge to studying RecQ helicases is that their disruption leads to a pleiotropic phenotype. Using yeast, we show that the separation-of-function allele of *SGS1*, *sgs1-D664Δ*, has impaired activity at DNA ends, resulting in a resection processivity defect. Compromising *Sgs1* resection function in the absence of the *Sae2* nuclease causes slow growth, which is alleviated by making the DNA ends accessible to *Exo1* nuclease. Furthermore, fluorescent microscopy studies reveal that, when *Sgs1* resection activity is compromised in *sae2Δ* cells, *Mre11* repair foci persist. We suggest a model where the role of *Sgs1* in end resection along with *Sae2* is important for removing *Mre11* from DNA ends during repair.

RPAIR of DNA damage is essential for maintenance of genomic integrity. When DNA repair is compromised, mutations and genetic alterations that are the hallmarks of cancer cells can occur. One group of proteins important for repair of DNA damage is the RecQ family of DNA helicases. In humans, mutations in three of the five RecQ-like proteins (BLM, WRN, RTS) are associated with heritable cancer predisposition diseases and/or premature aging (Bloom, Werner, or Rothmund-Thomson syndromes, respectively). In the budding yeast *Saccharomyces cerevisiae*, the RecQ helicase, *Sgs1*, when mutated, exhibits many aspects of the phenotype associated with alterations in its human homologs such as genomic instability and premature aging (reviewed in Bernstein *et al.* 2010).

Sgs1 and its homologs function in the homologous recombination (HR) DNA repair pathway. When a double-strand break (DSB) occurs, the broken DNA ends are bound by the *Mre11-Rad50-Xrs2* (MRX)/*MRE11-RAD50-NBS1* (MRN) complex in yeast and humans, respectively (hereafter, the yeast nomenclature is written first and the human protein second). Subsequently, *Sae2*/CtIP, together with MRX/MRN, initiate resection of the DNA ends, thus preventing the binding of the *Ku70-Ku80* heterodimer and inhibiting nonhomologous end joining (NHEJ) (Mimitou and Symington 2010; Foster *et al.* 2011; Langerak and Russell 2011). *Sae2* is essential to remove covalent adducts, such as *Spo11*, from DNA ends, but its function in resection initiation at endonuclease-induced DSBs can be bypassed by downstream nucleases. The absence of *Mre11* leads to a greater delay in resection initiation at endonuclease-induced DSBs than that observed for *Sae2* due to the role of the MRX complex in recruiting *Sgs1*, *Dna2*, and *Exo1* to DNA ends (Mimitou and Symington 2010; Shim *et al.* 2010). Subsequent long-range resection of the 3' overhangs can then occur by one of two pathways: the first utilizes the *Sgs1*/BLM helicase in conjunction with the endonuclease

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Dna2/DNA2 and the second utilizes the 5′–3′ double-stranded DNA exonuclease *Exo1*/hEXO1 (Gravel *et al.* 2008; Mimitou and Symington 2008; Zhu *et al.* 2008; Cejka *et al.* 2010; Niu *et al.* 2010). Next, if both DNA ends are engaged, a double Holliday junction is formed, which can be “dissolved” by the *Sgs1*/BLM helicase and the *Top3*/TOP3 α topoisomerase with the accessory protein(s) *Rmi1*/RMI1-RMI2 (Wu and Hickson 2003; Wu *et al.* 2006; Singh *et al.* 2008; Xu *et al.* 2008; Cejka and Kowalczykowski 2010).

Whereas NHEJ requires little or no resection, HR requires processing for *Rad51*-mediated homology search and strand invasion. Regulating resection of DNA ends plays an important role in repair pathway choice (Jasin and Rothstein 2013). Blocking DNA resection, which stabilizes DNA ends, affects the efficiency and accuracy of how the DNA is repaired. For example, inhibiting resection leads to *de novo* telomere addition (Chung *et al.* 2010; Lydeard *et al.* 2010).

Since *Sgs1* functions during multiple processing steps in HR, elucidating its specific role at these different steps has been challenging. Consistent with this notion, complete disruption of *SGS1* leads to a pleiotropic phenotype such as extreme sensitivity to DNA-damaging agents, meiotic defects, gross chromosomal rearrangements, increased sister-chromatid exchanges, and premature aging. To circumvent this pleiotropy, separation-of-function alleles of *SGS1* have been utilized to pinpoint its specific role at different processing steps (Lu *et al.* 1996; Miyajima *et al.* 2000; Mullen *et al.* 2000; Weinstein and Rothstein 2008; Bernstein *et al.* 2009; Hegnauer *et al.* 2012).

We previously described one *sgs1* allele, *sgs1-D664 Δ* , which behaves like an *sgs1 Δ* mutant in largely suppressing the slow growth observed in *top3 Δ* and *rmi1 Δ* cells as well as *top3 Δ* sensitivity to DNA damage (Bernstein *et al.* 2009). The *sgs1-D664 Δ* mutant also accumulates DNA damage-induced X-structure replication intermediates to the same extent as an *sgs1 Δ* (Bernstein *et al.* 2009). However, in many respects the *sgs1-D664 Δ* strain exhibits a wild-type (WT) *SGS1* phenotype. This allele lacks many of the synthetic interactions observed in *sgs1 Δ* cells (such as with *srs2 Δ* , *mus81 Δ* , and *rrm3 Δ*) and does not exhibit increased recombination rates in marker loss assays or sensitivity to DNA-damaging agents. These results lead us to suggest a model where the *Sgs1* repair of replication intermediates is separable from its role in creating DNA substrates that *Top3* and *Rmi1* normally resolve.

To dissect *Sgs1*-specific functions at different recombination steps, we studied the effect of the *sgs1-D664 Δ* mutant during DSB repair. Here we found that *sgs1-D664 Δ* has a specific genetic interaction with *SAE2*, a gene needed for resection of DSB ends. This genetic interaction led us to ask whether *sgs1-D664 Δ* has a resection defect. We find that *sgs1-D664 Δ exo1 Δ* mutants exhibit a resection processivity defect. Furthermore, we find that the genetic interaction between *sgs1-D664 Δ* and *sae2 Δ* is suppressed by disruption of *KU70* or by overexpression of *EXO1*. These results suggest that the resection processivity defect observed in *sgs1-D664 Δ*

cells alters the affinity of DNA ends for HR proteins. Consistent with this notion, *sgs1-D664 Δ sae2 Δ* cells exhibit persistent *Mre11* foci at DSB sites.

Materials and Methods

Strains, plasmids, and media

The strains used in this study are listed in [Supporting Information, Table S1](#). They are isogenic with W303 and derived from the *RAD5+* strains W1588-4C and W5909-1B (Thomas and Rothstein 1989; Zhao *et al.* 1998; Bernstein *et al.* 2011). Standard procedures were used for making crosses, tetrad dissection, and yeast transformation (LiOAc method) (Sherman *et al.* 1986). The media were prepared as described, except twice the amount of leucine was used (Sherman *et al.* 1986). The *sgs1-D664 Δ* allele was assayed by PCR (primers: 5′ CTG ATC TAG AGT TGA TAG ACA GC and 3′ GGC ACT GAT CAT CTC AGG AG) and *NcoI* restriction digestion where *SGS1* gives rise to a 750-bp PCR product and *sgs1-D664 Δ* gives rise to 350- and 400-bp fragments).

Spore-size analysis

Analysis of the individual spore colonies from tetrads was performed using the Dissection Reader software developed by John Dittmar in the Rothstein Lab for use with the publically available ImageJ program. The Dissection Reader script can be downloaded at the Rothstein Lab website at http://www.rothsteinlab.com/tools/apps/dissection_reader. An average of 6–25 spores were analyzed for each genotype, and standard deviations were calculated. Wild-type spores were set to one, and the mutants were analyzed relative to wild-type growth.

Recombination assay

The interhomolog *leu2 Δ BstEII* and *leu2 Δ EcoRI* recombination assay in diploid cells was performed as described previously (Alvaro *et al.* 2007).

Single-strand annealing assay

Strains were grown to mid-log phase in lactate medium. Galactose was then added to the medium for 1 hr to induce *I-SceI* expression, and *I-SceI* was switched off by addition of glucose. Samples were collected hourly from 0 to 6 hr after galactose addition. Genomic DNA was extracted, digested with *NheI* and *EagI*, and then subsequently analyzed by DNA blot using an *ADE2* probe as previously described (Mimitou and Symington 2008). The experiments were performed in duplicate (representative gels are shown in Figure 2 and Figure 4).

Single-strand DNA intermediate analysis by alkaline electrophoresis

Alkaline electrophoresis of *StyI*/*BstXI*-digested genomic DNA was performed as previously described (White and

Haber 1990; Mimitou and Symington 2008). Assays with the *rad51Δ sgs1-D664Δ* and *rad51Δ exo1Δ sgs1-D664Δ* mutants were performed once, and the other strains were analyzed at least two times.

Growth assays

Yeast were grown in YPD overnight at 30° to early log phase, and equal numbers of cells were fivefold serially diluted onto plates containing YPD or YPGal/Raf and were photographed after 1–2 days of growth at 30°.

Fluorescent microscopy

Cells were grown overnight at 30° in 5-ml cultures of SC medium with adenine (100 mg/ml) and harvested for microscopy as previously described (Lisby *et al.* 2001). An *Mre11*-YFP construct was introduced into wild-type, *sae2Δ*, *sgs1-D664Δ*, *yku70Δ*, *sae2Δ sgs1-D664Δ*, and *sae2Δ sgs1-D664Δ yku70Δ* cells and was visualized before and after 40 Gy of ionizing radiation (IR) using a Nikon TiE inverted live-cell system with a ×100 oil immersion objective (1.45 numerical aperture), a Photometrics HQ2 camera, and a motorized Prior Z-stage. Stacks of 11, 0.3-μm sections were captured using the following exposure times: 60 msec (differential interference contrast) and 1000 msec (*Mre11*-YFP). Subsequently, the images were deconvolved using Elements imaging software (Nikon). All images were processed and enhanced identically, and experiments were performed in triplicate unless otherwise noted.

I-SceI induction

Cells harboring the GAL-I-SceI-expressing plasmid (pWJ1089) were grown to early log phase in SC medium with 2% raffinose lacking histidine. Two percent galactose was added to the medium for 2 hr at 30°, and subsequently expression of I-SceI was inhibited by addition of 1% glucose. Immediately after inhibiting I-SceI expression and 4 hr later, the cells were visualized as described above except that the DSB was visualized using the red fluorescent protein (RFP) channel (600 msec).

Results

sgs1-D664Δ sae2Δ double mutants are synthetically sick

Previously, we showed that a separation-of-function allele of *SGS1*, *sgs1-D664Δ*, is proficient for homologous recombination, but is deficient in replication-associated repair (Bernstein *et al.* 2009). Here we compare the *sgs1-D664Δ* strain to an *sgs1Δ* strain for several well-characterized genetic interactions and biochemical reactions. Since *Sgs1* functions at multiple processing steps during HR, it is not surprising that it exhibits genetic interactions with many proteins and pathways. For example, an *sgs1* null mutation confers a synthetic lethal or sick phenotype when combined with at least 60 different mutations (McVey *et al.* 2001; Mullen *et al.* 2001; Tong *et al.* 2001, 2004; Pan *et al.* 2004, 2006). These

proteins are involved in many different cellular processes such as homologous recombination, DNA replication, sister-chromatid cohesion, DNA silencing, sumoylation, or ubiquitination (McVey *et al.* 2001; Mullen *et al.* 2001; Tong *et al.* 2001, 2004; Pan *et al.* 2004, 2006). Using tetrad analysis to create single and double mutants, we assayed *sgs1-D664Δ* for synthetic sick/lethal interactions with mutations of other HR genes. In contrast to *sgs1Δ*, *sgs1-D664Δ* does not exhibit a synthetic sick/lethal interaction with disruption of the MRX complex (*mre11Δ*, *rad50Δ*, *xrs2Δ*) or the DNA repair genes *mus81Δ*, *rad27Δ*, *srs2Δ*, or *top1Δ* (Figure 1) (Bernstein *et al.* 2009). However, to our surprise, this separation-of-function allele does exhibit a synthetic sick phenotype with disruption of one HR gene, *sae2Δ* (Figure 1A). We also examined the synthetic interaction of *sgs1Δ* or *sgs1-D664Δ* combined with five additional mutations—*dun1Δ*, *msh2Δ*, *rad1Δ*, *tof1Δ*, and *uaf30Δ*—which were previously reported to exhibit a synthetic growth defect with *sgs1Δ* (Onoda *et al.* 2004; Pan *et al.* 2004, 2006; Tambini *et al.* 2010; Park *et al.* 2012). None of these synthetic interactions were observed with *sgs1Δ* or *sgs1-D664Δ* in our strain background (data not shown).

Sgs1-D664Δ is expressed at lower levels than the wild-type *Sgs1* protein (Bernstein *et al.* 2009). To confirm that the specific genetic interaction between *sgs1-D664Δ* and *sae2Δ* is not simply a result of decreased *Sgs1-D664Δ* expression, we assayed *Sgs1*-AR1 and *Sgs1*-AR2 mutants, which also exhibit decreased protein expression (Bernstein *et al.* 2009), for synthetic interaction with *sae2Δ* (Figure S1). Importantly, we did not observe a synthetic growth defect between *sgs1-AR1Δ* or *sgs1-AR2Δ* mutants combined with *sae2Δ*. Therefore, the genetic interaction between *sgs1-D664Δ* and *sae2Δ* is specific to the *sgs1-D664Δ* allele and not to its decreased expression.

Interhomolog recombination is not increased in *sgs1-D664Δ* strains

In an earlier study using several different marker loss assays, we showed that *sgs1-D664Δ* does not increase recombination rates (Bernstein *et al.* 2009). Since disruption of either *SGS1* or *SAE2* increases interhomolog recombination rates (Alvaro *et al.* 2007), we asked whether *sgs1-D664Δ* would similarly exhibit an increase in interhomolog recombination. In this assay, interhomolog recombination rates are calculated by measuring the rate of Leu⁺ prototrophs created by recombination between two *leu2*-heteroalleles in a diploid strain (Figure S2). We find that, unlike an *sgs1Δ*, *sgs1-D664Δ* cells do not exhibit increased interhomolog recombination (Figure S2). Thus, in contrast to complete loss of *SGS1*, the *sgs1-D664Δ* allele does not increase recombination in any assay that we have tested.

sgs1-D664Δ cells exhibit a defect in end resection during single-strand annealing

The *sae2Δ sgs1-D664Δ* synthetic interaction focused our attention on whether the defective process in *sgs1-D664Δ*

Figure 1 Cells with *sgs1-D664Δ* have a synthetic sick interaction when combined with *sae2Δ*. (A) Yeast with *sgs1-D664Δ* or *sgs1Δ* were mated to *sae2Δ*, *mre11Δ*, *rad50Δ*, or *xrs2Δ* haploid cells. The diploids were sporulated and the meiotic products analyzed by tetrad analysis. Pictures of representative spores from a single tetrad are shown. The size of the spore colonies were quantitated relative to WT (set at one), and their sizes are plotted on the graph with standard deviations shown. (B) Summary of synthetic interactions for *sgs1-D664Δ* with HR mutant genes. The synthetic interactions marked with an asterisk (*) were previously reported (Bernstein *et al.* 2009).

mutant cells is DNA end resection. During an early HR step, the *Sae2* endonuclease functions with the MRX complex to initiate resection of DSB ends, creating short 3' overhangs (Lengsfeld *et al.* 2007; Mimitou and Symington 2008; Zhu *et al.* 2008). Subsequently, *Sgs1* in conjunction with the nuclease *Dna2* or, alternatively, *Exo1*, perform long-range resection of DSBs (Gravel *et al.* 2008; Mimitou and Symington 2008; Zhu *et al.* 2008; Cejka *et al.* 2010; Niu *et al.* 2010). To monitor end resection, we performed a single-strand annealing (SSA) assay, where two *ade2* hetero-alleles are separated by *TRP1* and plasmid sequences (Figure 2A) (Mimitou and Symington 2008). To create a DSB, one of the *ade2* alleles has a unique site for the rare cutting endonuclease, I-SceI. Expression of the I-SceI enzyme is dependent on galactose addition to the medium (*GAL-I-SceI*). *RAD51* is also disrupted in these strains to prevent gene conversion and force repair of the DSB by SSA. First, we analyzed the cells for formation of Ade⁺ recombinants (Figure 2B). Qualitatively, fewer spontaneous white Ade⁺ recombinants were formed in *rad51Δ exo1Δ sgs1-D664Δ* on rich medium (YPD), and viability was reduced when I-SceI was induced by yeast peptone with galactose and raffinose (YPGal/Raf). To analyze these mutants for a resection defect, we performed genomic blot analysis. As previously observed, *rad51Δ* and *rad51Δ sgs1Δ* cells repair the DSB after I-SceI induction, while a *rad51Δ exo1Δ sgs1Δ* triple mutant does not form the SSA product, even after 6 hr of recovery (Figure 2B) (Mimitou and Symington 2008). These results are consistent with the notion that *Sgs1* and *Exo1* function in two alternative pathways to resect DSB ends (Mimitou and Symington 2008; Zhu *et al.* 2008). Furthermore, smeared

cut products form in the triple mutant caused by the short-range resection activity of *Sae2* and MRX, leading to incremental loss of 50–100 nucleotide fragments (Figure 2B) (Mimitou and Symington 2008; Zhu *et al.* 2008). Examination of the *sgs1-D664Δ* strain shows that, similar to a *rad51Δ* strain, *rad51Δ sgs1-D664Δ* cells efficiently create the SSA repair product (Figure 2B). In contrast, the *rad51Δ exo1Δ sgs1-D664Δ* triple mutant forms very minimal amounts of the SSA repair product after 6 hr (Figure 2B), indicating that the *sgs1-D664Δ* mutation confers a DNA end resection defect. Note that we do not observe the same banding pattern for cut DNA products in *rad51Δ exo1Δ sgs1-D664Δ* as is seen in *rad51Δ exo1Δ sgs1Δ* strains (Figure 2B). This difference may be due to more extensive resection in the *sgs1-D664Δ* strain, which results in the cut fragments becoming more smeared.

sgs1-D664Δ has a resection processivity defect

To more closely monitor the kinetics of single-strand DNA (ssDNA) formation in *sgs1-D664Δ* mutant strains, we analyzed a DSB generated by the *HO* endonuclease (regulated by the *GAL1-10* promoter, *GAL-HO*) at the yeast mating-type (*MAT*) locus (White and Haber 1990). Formation of ssDNA can be monitored on alkaline-denaturing gels by the ability of restriction enzymes to act on *StyI* (S) or *BstXI* (B) sites adjacent to the break site (Figure 3A). Once the DNA is rendered single-stranded by resection, it is no longer able to be digested by the enzymes, resulting in a ladder of higher-molecular-weight products visualized using a 3' strand-specific probe. As before, *RAD51* was disrupted to prevent gene conversion and allow the accumulation of

Figure 2 In the absence of Rad51, *sgs1-D664Δ* *exo1Δ* cells display a resection defect. (A) Schematic of the SSA assay used with two *ade2* heteroalleles with an intervening *TRP1* gene. A unique I-SceI cut site is inserted in one of the *ade2* alleles. When I-SceI is conditionally expressed, SSA occurs and the repair products lead to *ADE2*+ *trp1*- recombinants. The sizes of the bands generated by restriction digestion of genomic DNA are indicated below each schematic. (B) Yeast strains containing the SSA assay diagrammed with the indicated genotypes were grown in rich medium (YPD) to early log phase (0.5 OD₆₀₀) and then fivefold serially diluted onto rich medium (YPD) or medium containing galactose and raffinose (YPGal/Raf), which induces expression of the I-SceI enzyme from a galactose-inducible promoter. Plates were photographed after 3 days of growth at 30°. The same cells were analyzed by DNA blotting to detect the cut fragments and the *ADE2*+ repair product from 0 to 6 hr after I-SceI cutting.

ssDNA intermediates. As seen in the first four panels of Figure 3B, up to 10 kb of ssDNA can be detected in *rad51Δ*, *rad51Δ sgs1Δ*, *rad51Δ exo1Δ*, and *rad51Δ sgs1-D664Δ* strains after DSB induction by HO. However, when both *Sgs1* and *Exo1* resection pathways are blocked in a *rad51Δ exo1Δ sgs1Δ* triple mutant (Figure 3B, slide 5), the DSB fragment persists and only the 1.6-kb product (r1) can be readily detected, indicating that resection tracts are <1600 nt (Mimitou and Symington 2008). In contrast, in a *rad51Δ exo1Δ sgs1-D664Δ* strain (Figure 3B, panel 6), there is an accumulation of the r2 intermediate, and there are no intermediates larger than r3, showing that this mutant cannot support resection beyond 5 kb distal to the DSB, indicating a processivity defect. The inability of the *rad51Δ exo1Δ sgs1-D664Δ* strain to resect beyond 5 kb explains the failure to accumulate the SSA product, which requires removal of >7 kb between the *ade2* repeats (Figure 2B).

Synthetic interaction of *sgs1-D664Δ* *sae2Δ* is attributable to the resection defect of *sgs1-D664Δ*

Recently, it was shown that the inviability of *sgs1Δ sae2Δ* double-mutant cells is suppressed by *YKU70* disruption or

by overexpression of *EXO1*, suggesting that one of the roles of the Ku70–Ku80 complex is to regulate access of nucleases to DNA ends, especially *Exo1* (Mimitou and Symington 2010). It is thought that Ku complex binding promotes DSB repair via NHEJ by limiting the amount of end processing. However, once *Sae2* is activated by CDK, it functions with the MRX complex to initiate resection, removing Ku from ends (Huertas *et al.* 2008; Mimitou and Symington 2010; Langerak *et al.* 2011). To determine whether the resection defect conferred by *sgs1-D664Δ* is responsible for the synthetic interaction observed in the *sae2Δ sgs1-D664Δ* double mutant, we assayed whether *yku70Δ* would suppress its slow growth. Indeed, disrupting *YKU70* alleviates the slow growth observed in the *sae2Δ sgs1-D664Δ* mutant (Figure 4A). Disruption of a downstream NHEJ gene, the DNA ligase *DNL4*, does not suppress the synthetic growth defect in *sae2Δ sgs1-D664Δ* cells (Figure 4B) as it does in *sgs1Δ sae2Δ* cells (Mimitou and Symington 2010). These results suggest that NHEJ itself is not toxic in this genetic background, but rather the improper processing of the DNA ends is responsible for the synthetic interaction observed between *sgs1-D664Δ* and *sae2Δ*. Consistent with this idea,

Figure 3 When *RAD51* is disrupted, *sgs1-D664Δ exo1Δ* cells exhibit a resection processivity defect. (A) Schematic of the mating-type locus on chromosome III before and after an HO-induced DSB. When *RAD51* is disrupted, the formation of ssDNA is favored and can be monitored by alkaline electrophoresis of *StyI* (S)/*BstXI* (B)-digested genomic DNA (see text for discussion). The ssDNA products, designated, r1–r7, can be observed by DNA blot hybridization with a 3' strand-specific probe. (B) Cells with the indicated genotypes were analyzed by DNA blot for the r1–r7 ssDNA fragments 0, 0.5, 1, 1.5, 2, 3, and 4 hr after induction of HO endonuclease.

overexpression of *Exo1* largely suppresses the synthetic growth defect observed in *sae2Δ sgs1-D664Δ* double mutants (Figure 4C), similar to that observed for *sae2Δ sgs1Δ* cells (Mimitou and Symington 2010). Furthermore, deletion of *EXO1* leads to *sae2Δ sgs1-D664Δ* lethality (Figure 4D), as reported previously for *sgs1Δ sae2Δ* (Mimitou and Symington 2008), again suggesting that the defect in the *sgs1-D664Δ* separation-of-function mutation is reduced end resection.

To determine if *sae2Δ sgs1-D664Δ* double mutants are deficient in end resection, we utilized the SSA assay described in Figure 2A to monitor formation of Ade⁺ recombinants. Qualitatively, fewer spontaneous white Ade⁺ recombinants were formed in *sae2Δ sgs1-D664Δ rad51Δ* on rich medium (YPD), and viability was reduced when I-SceI was induced by galactose (YPGal/Raf), consistent with the notion that the combination of *sae2Δ* and *sgs1-D664Δ* results in a resection defect (Figure 4E). To further investigate the resection defect, we assayed the *sae2Δ rad51Δ* and *sae2Δ sgs1-D664Δ rad51Δ* strains by DNA blot and observed a delay in resection initiation and SSA product formation (Figure 4F). The delay in product formation is not as severe as what we observe in an *sgs1-D664Δ exo1Δ rad51Δ* triple mutant, which is defective for long-range resection, but the persistence of the cut fragments suggests that the *sae2Δ sgs1-D664Δ rad51Δ* cells have a resection initiation defect.

Persistent *Mre11* foci form at DSB sites in an *sgs1-D664Δ sae2Δ* double mutant

In most assays, *mre11Δ* is epistatic to *sae2Δ*; therefore, it is surprising that *sgs1-D664Δ* exhibits a synthetic growth defect with *sae2Δ*, but not with *mre11Δ*, *rad50Δ*, or *xrs2Δ* (Figure 1). Previous studies showed that *Mre11* persists longer at DSBs in the *sae2Δ* mutant (Lisby *et al.* 2004; Clerici *et al.* 2006), raising the possibility that the resection initiation defect of the *sae2Δ sgs1-D664Δ* strain results in retention of the MRX complex at DNA ends, causing the subsequent slow growth. To explore this notion, we introduced a tagged version of *Mre11* (*Mre11-YFP*) into WT, *sgs1-D664Δ*, *sae2Δ*, and *sae2Δ sgs1-D664Δ* strains. The cells were exposed to 40 Gy of IR, which leads to approximately four DSBs per cell (Ma *et al.* 2008) and does not result in decreased viability or slow growth of WT, *sgs1-D664Δ*, or *sae2Δ* cells (Figure S3). After DNA damage, we analyzed *Mre11-YFP* recruitment to DNA damage sites using fluorescent microscopy. We find that both *sae2Δ* and *sae2Δ sgs1-D664Δ* cells form more spontaneous *Mre11* foci when compared to WT (Figure S4; $P \leq 0.025$ and $P \leq 0.005$, respectively). Following IR, both WT and *sgs1-D664Δ* cells exhibit an increase in *Mre11* foci that are mostly gone after 2 hr (Figure 5). In contrast, *sae2Δ* cells exhibit a high percentage of cells with an *Mre11* focus, which only gradually decrease

Figure 4 The slow growth of *sae2Δ sgs1-D664Δ* cells is suppressed by *yku70Δ* or by Exo1 overexpression. (A and B) Yeast strains with the indicated genotypes were fivefold serially diluted onto rich medium, grown at 30° for 1 day, and photographed. (C) WT or *sgs1-D664Δ sae2Δ* cells were transformed with an empty plasmid or one that expressed Exo1. The respective strains were fivefold serially diluted onto minimal medium to maintain a plasmid (SC-TRP), grown at 30° for 2 days, and photographed. (D) Spore colony size of the strains of the indicated genotypes. The spore colony size relative to WT was calculated and plotted with standard deviations. The *exo1Δ sae2Δ sgs1-D664Δ* spores did not generate viable colonies. (E) Yeast strains containing the SSA assay diagrammed in Figure 2A with the indicated genotypes were grown in rich medium (YPD) to early log phase (0.5 OD₆₀₀) and then fivefold serially diluted onto rich medium (YPD) or medium containing galactose and raffinose (YPGal/Raf), which induces expression of the I-SceI enzyme from a galactose-inducible promoter. Plates were photographed after 3 days of growth at 30°. (F) The *sae2Δ* and *sae2Δ sgs1-D664Δ* cells were analyzed by DNA blot for resection products and cut fragments were diagrammed as in Figure 2A.

over time [Figure 5; 0.2 hr compared to 2 hr ($P \leq 0.05$) or 4 hr ($P \leq 0.005$)]. In the *sae2Δ sgs1-D664Δ* double mutant, the percentage of cells with an *Mre11* focus remains high throughout the time course following IR (Figure 5; $P > 0.1$ comparing 0.2–2 or 4 hr). We were unable to analyze later time points due to the lethality caused by IR in the *sae2Δ sgs1-D664Δ* cells. These results reveal that the *Mre11* foci that form at DSB sites in the *sae2Δ sgs1-D664Δ* cells are persistent. Perhaps the synergistic interaction between these two mutants could be due to the inability to remove *Mre11* from the DSB ends or a block at this processing step.

To monitor directly *Mre11* recruitment at a site-specific DSB, we analyzed *Mre11* localization at a fluorescently tagged DSB site, a unique I-SceI endonuclease cut site inserted into the *URA3* locus on chromosome V (Figure 6) (Lisby *et al.* 2004). This site is adjacent to a multiple tandem

array of Tet repressor binding sites (112xtetO). The localization of the cut site is revealed by expression of the TetR fused to a monomeric red fluorescent protein, which binds to the TetO. Using this system, we monitored *Mre11*-YFP localization to the cut site after DSB induction for 2 hr by galactose addition and then monitored cells 4 hr after I-SceI enzyme expression was shut off with glucose (Figure 6). Less *Mre11* is seen at the DSB site 4 hr after DSB induction for WT and for *sgs1-D664Δ* and *sae2Δ* mutant cells (Figure 6; $P \leq 0.025$ in WT and *sgs1-D664Δ* and $P \leq 0.005$ in *sae2Δ*). In contrast, we find that more *Mre11* is recruited to the break site in *sae2Δ sgs1-D664Δ* cells and that it remains associated even 4 hr after removing the inducer (Figure 6; $P \leq 0.1$). These results support the notion that the synergistic interaction between *sgs1-D664Δ* and *sae2Δ* is due to the inefficient removal of *Mre11* from DSB ends.

Figure 5 Persistent Mre11 foci form in response to IR in *sae2Δ sgs1-D664Δ* double mutants and is alleviated by disrupting *YKU70*. Cells expressing Mre11-YFP were analyzed in WT, *sgs1-D664Δ*, *sae2Δ*, *sae2Δ sgs1-D664Δ*, *yku70Δ*, or *sae2Δ sgs1-D664Δ yku70Δ* after exposure to 40 Gy of IR for 0.2–4 hr. For each time point, a single Z-stack is shown; white arrowheads indicate a focus. Each experiment was done in triplicate with a total of 200–650 cells analyzed with standard errors plotted.

Persistent Mre11 foci in a *sae2Δ sgs1-D664Δ* mutant are reduced by *YKU70* disruption

Our data suggest that inefficient removal of Mre11 from DSB ends in *sae2Δ sgs1-D664Δ* leads to a slow-growth phenotype (Figure 4, Figure 5, and Figure 6). Since disruption of *YKU70* alleviates the slow growth of *sae2Δ sgs1-D664Δ* mutant cells (Figure 4A), we asked whether the persistent Mre11 foci are still observed in *sae2Δ sgs1-D664Δ* cells after

deletion of *YKU70*. We find that, in *sae2Δ sgs1-D664Δ yku70Δ* triple mutants, the percentage of cells with an Mre11-YFP focus decreases over time (from ~50–25%) in contrast to *sae2Δ sgs1-D664Δ* double mutants, where persistent Mre11 foci are observed (Figure 5; $P \leq 0.05$ and $P \leq 0.005$ comparing *sae2Δ sgs1-D664Δ yku70Δ* cells at 0.2–2 and 4 hr, respectively). However, despite the resolution of Mre11 foci in the triple mutant, there are still more Mre11 foci observed at all time points when compared to WT (Figure 5). In contrast, *yku70Δ* alone results in fewer Mre11 foci after initial IR exposure (0.2 hr; Figure 5). Thus, these results suggest that loss of Ku70 binding allows other nucleases to stimulate removal of Mre11 from DSB ends.

Discussion

The RecQ family of DNA helicases has many different functions during HR. Importantly, mutations in the human RecQ-like genes (BLM, WRN, and RTS) lead to diseases whose defining characteristics are phenocopied by the yeast homolog *Sgs1*. One of the challenges of studying these proteins is the diverse phenotype associated with their disruption. For example, disrupting *Sgs1* leads to mitotic and meiotic defects, chromosomal instability, increased cross-overs, telomere alterations, and premature aging (reviewed in Bernstein *et al.* 2010). Therefore, it is not surprising that *Sgs1* functions during many different stages of HR, including resection of DSB ends; formation of D-loops and branch migration; and, finally, resolution of double Holliday junction. To begin to understand the role that *Sgs1* plays in DSB repair, we isolated a separation-of-function allele, *sgs1-D664Δ*, which largely suppresses *top3Δ* slow growth, but is not sensitive to DNA-damaging agents like an *sgs1* null (Bernstein *et al.* 2009). Further studies showed that this allele results in a deficiency in replication-associated repair, which is distinct from its broader role in recombinational repair (Bernstein *et al.* 2009).

Here we first focused on the synthetic genetic interactions of *sgs1-D664Δ* compared to *sgs1Δ*. Although *sgs1Δ* mutations show synthetic growth defects with a large number of HR genes, *sgs1-D664Δ* specifically exhibits a synthetic effect with *sae2Δ*, but not any other HR genes (Figure 1). Since *Sae2* is involved in DNA end resection, we next examined the kinetics of the appearance of the substrates and products of direct repeat recombination after induction of a DSB in *sgs1-D664Δ* cells. In the absence of *Rad51*, the *sgs1-D664Δ* allele, when combined with *exo1Δ*, slows long-range resection and results in decreased product formation (Figure 2B). Similarly, *sgs1-D664Δ exo1Δ rad51Δ* triple mutants also have a resection processivity defect as revealed by the reduced kinetics of ssDNA formation (Figure 3B). We suspect that the slow growth observed in the *sgs1-D664Δ sae2Δ* double mutant is due to this resection defect for two reasons. Namely, its slow growth is alleviated by eliminating Ku or overexpressing *Exo1* (Figure 4, A and B), which is similar to the rescue of the synthetic lethality of

Figure 6 Mre11 foci persist in *sae2Δ sgs1-D664Δ* double mutants at an inducible DSB site. An I-SceI cut site integrated at the *URA3* locus on chromosome V adjacent to 112 copies of the Tet repressor-binding site (112xtetO) was analyzed (Lisby *et al.* 2004). Recruitment of Mre11-YFP to the cut site was monitored in strains expressing a GAL-I-SceI plasmid either immediately (0 hr) or 4 hr after I-SceI expression was inhibited by the addition of glucose. The experiment was done in duplicate, and the results are quantitated in the graph with standard error plotted.

an *sgs1Δ sae2Δ* double mutant (Mimitou and Symington 2010). The direct repeat assay shown in Figure 2A requires the resection of >7 kb of ssDNA to form recombinants. We find less product formation and see the persistence of cut fragments (Figure 4F) and also observe qualitatively fewer recombinants in an *sgs1-D664Δ sae2Δ rad51Δ* triple mutant compared to the respective double mutants with *rad51Δ* (Figure 4E). Our results suggest a model where the MRX complex and Sae2 normally initiate resection; however, in the absence of SAE2, Sgs1 can now function in resection initiation as well as long-range resection. All of these observations strengthen the notion that Sgs1-D664Δ slows resection.

The Sgs1-D664Δ protein is the first Sgs1 mutant identified to uncouple the role of Sgs1 in DNA end resection from its other functions during DNA repair. This allele could therefore be used as a tool to specifically study the role of Sgs1 during DNA resection. Importantly, the *sgs1-D664Δ exo1Δ* double mutant slows resection without affecting growth or the hyper-recombination phenotype normally associated with an *sgs1Δ* cell. Previously, we found that *sgs1-D664Δ* cells accumulate replication intermediates, namely X structures, by two-dimensional pulse-field gel electrophoresis (Bernstein *et al.* 2009). Perhaps the resection defect observed in the *sgs1-D664Δ* cell is responsible for the accumulation of X structures observed in this mutant. Although it is possible that the *sgs1-D664Δ* is a dominant protein, our preliminary results suggest that it is recessive since a diploid *SGS1/sgs1-D664Δ* cell behaves like a wild-type *SGS1/SGS1* with respect to suppression of *top3Δ* slow growth as well as resolution of X structures. Due to its proximity to the acidic regions, this *sgs1* mutant could disrupt a specific protein–protein interaction or even a post-

translational modification. In our preliminary studies, we have not observed any defects in its other protein–protein interactions to date (*i.e.*, Rmi1), and mutation of aspartate 664 to an alanine results in no detectable phenotype (Bernstein *et al.* 2009). Therefore, it is likely that this allele alters the structure of Sgs1, which specifically inhibits its role in resection.

It was surprising that the *sgs1-D664Δ* mutant allele showed only a specific synthetic sick interaction with *sae2Δ* but not with disruptions in the components of the MRX complex, since they are all members of the same epistasis group (Figure 1A) (Mimitou and Symington 2009). One explanation for this behavior is that Sgs1 normally works in conjunction with Sae2 to remove the MRX complex during end processing or to facilitate efficient DNA end processing. When SAE2 is absent, then Sgs1 can assist in resection initiation and removal of proteins from the DNA ends that might inhibit long-range resection. This includes Mre11 but could also be other proteins as well. The phenotype of the *sgs1-D664Δ* mutant suggests that the joint resection activities of Sgs1 and Sae2 are required for this removal. When Sae2 is present, we do not expect that Sgs1 normally assists in resection initiation and Mre11 removal from DSB ends. Therefore, the role for Sgs1 in resection initiation occurs only in the absence of Sae2 or the Mre11 nuclease. Consistent with this notion, resection is reduced in the *mre11-H125N sgs1Δ* mutant to a greater extent than the single mutants (Mimitou and Symington 2010; Shim *et al.* 2010). Due to lethality of the *sae2Δ sgs1Δ* mutant we could not assess Mre11 persistence at DSBs in the absence of both Sae2 and Sgs1. However, we find that Mre11 foci persist both after IR and at a site-specific DSB in a *sae2Δ*

sgs1-D664Δ double mutant (Figure 5 and Figure 6). Furthermore, enabling end resection by eliminating *YKU70* allows other nucleases (e.g., *Exo1*) to process the DSB ends to remove *Mre11* in *sae2Δ sgs1-D664Δ* mutants (Figure 5). Therefore, *Sgs1* can be thought of as a “back-up” to *Sae2* during DNA resection initiation. Taken together, our results suggest that the combined resection activities of these two proteins are important to remove *Mre11* from DSB ends and for efficient DNA end processing.

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GENETICS

Supporting Information

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Resection Activity of the Sgs1 Helicase Alters the Affinity of DNA Ends for Homologous Recombination Proteins in *Saccharomyces cerevisiae*

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Figure S1 *sgs1-AR1* and *sgs1-AR2* cells do not exhibit a synthetic interaction when combined with *sae2*Δ. Yeast with *sgs1-AR1*Δ or *sgs1-AR2*Δ were mated to *sae2*Δ haploid cells. The diploids were sporulated and the meiotic products analyzed by tetrad analysis. The size of the spore colonies were quantitated and their size relative to WT (set at one) are plotted on the graph with standard deviations shown.

Figure S2 *sgs1-D664Δ* cells do not exhibit increased inter-homologue recombination rates. Diploid strains containing *leu2-ΔEcoRI* and *leu2-ΔBstEII* alleles were monitored for formation of *LEU2*⁺ recombinants in WT, *sgs1Δ*, and *sgs1-D664Δ* strains and plotted with standard deviations. WT and *sgs1-D664Δ* are not significantly different ($p \leq 0.1$).

Figure S3 Growth of WT, *sgs1-D664*Δ, and *sae2*Δ cells relatively unaffected by 40 Gy IR. WT, *sgs1-D664*Δ, *sae2*Δ, and *sae2*Δ *sgs1-D664*Δ cells were grown to early log-phase and five-fold serially diluted onto YPD medium. The treated plate was exposed to 40 Gy IR and incubated for 24-36 hours at 30°C.

Figure S4 More spontaneous Mre11 foci are observed in *sae2Δ* and *sae2Δ sgs1-D664Δ* cells. Cells expressing Mre11-YFP were analyzed in WT, *sgs1-D664Δ*, *sae2Δ*, and *sgs1-D664Δ sae2Δ* for formation of spontaneous Mre11 foci. For each strain, a single Z-stack is shown and the white arrowheads indicate a focus. Each experiment was done in triplicate with a total of 200-300 cells analyzed with standard errors plotted.

Table S1 Strains and plasmids

Name	Description
W1588-4A	<i>MATα ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 RAD5</i>
W5909-1B	<i>MATa ADE2 TRP1 lys2Δ</i>
W7644	<i>MATa/α sae2::kanMX/SAE2 sgs1::HIS3/SGS1</i>
W7645	<i>MATa/α sae2::kanMX/SAE2 sgs1-D664Δ/SGS1</i> <i>leu2ΔEcoR1::URA3::leu2ΔBstell</i>
W7348	<i>MATa/α mre11::LEU2/MRE11 sgs1::HIS3/SGS1</i>
KBY31	<i>MATa/α mre11::LEU2/MRE11 sgs1-D664Δ/SGS1</i>
W7634	<i>MATa/α xrs2::URA3/XRS2 sgs1::HIS3/SGS1</i>
KBY29	<i>MATa/α xrs2::URA3/XRS2 sgs1-D664Δ/SGS1</i>
W7636	<i>MATa/α rad50::URA3/RAD50 sgs1::HIS3/SGS1</i>
KBY30	<i>MATa/α rad50::URA3/RAD50 sgs1-D664Δ/SGS1</i>
LSY1709-9D	<i>MATa rad51::LEU2 ade2-n::TRP1::ade2-I-SceI lys2::GAL-I-SceI</i>
W8823-4C	<i>MATa sgs1::hphMX4 rad51::LEU2 ade2-n::TRP1::ade2-I-SceI lys2::GAL-I-SceI</i>
LSY1983-16B	<i>MATa rad51::LEU2 ade2-n::TRP1::ade2-I-SceI lys2::GAL-I-SceI sgs1::hphMX4</i>
LSY1983-32B	<i>MATa rad51::LEU2 ade2-n::TRP1::ade2-I-SceI lys2::GAL-I-SceI exo1::HIS3</i> <i>sgs1::hphMX4</i>
LSY2090-5B	<i>MATa rad51::LEU2 ade2-n::TRP1::ade2-I-SceI lys2::GAL-I-SceI sgs1-D664Δ</i>
LSY2090-4D	<i>MATa rad51::LEU2 ade2-n::TRP1::ade2-I-SceI lys2::GAL-I-SceI exo1::HIS3</i> <i>sgs1-D664Δ</i>
LSY2172-24C	<i>MATa rad51::LEU2 ade3::GAL-HO</i>
LSY2172-17C	<i>MATa rad51::LEU2 sgs1::hphMX4 ade3::GAL-HO</i>
LSY2173-25D	<i>MATa rad51::LEU2 exo1::HIS3 ade3::GAL-HO</i>
LSY2208-4B	<i>MATa rad51::LEU2 sgs1::D664Δ ade3::GAL-HO</i>
LSY2179-11B	<i>MATa rad51::LEU2 exo1::HIS3 sgs1::hphMX4 ade3::GAL-HO</i>
LSY2208-3C	<i>MATa rad51::LEU2 sgs1-D664Δ exo1::HIS3 ade3::GAL-HO</i>
W9208-10A	<i>MATa yku70::LEU2</i>
W5927-20A	<i>MATα sgs1-D664Δ</i>
W4318-1D	<i>MATa sae2::KanMX bar1::LEU2</i>
W9208-17C	<i>MATα yku70::LEU2 sgs1-D664Δ leu2ΔEcoR1::URA3::leu2ΔBstell</i>
W9208-17D	<i>MATa sae2::KanMX yku70::LEU2 leu2ΔEcoR1::URA3::leu2ΔBstell</i>
W9208-19A	<i>MATa sgs1-D664Δ sae2::KanMX</i>
W9208-2A	<i>MATa sgs1-D664Δ sae2::kanMX yku70::LEU2</i>
KBY126-14D	<i>MATα dnl4::URA3</i>
KBY126-8B	<i>MATα dnl4::URA3 sgs1-D664Δ MRE11-YFP RAD52-RFP</i>
KBY126-2C	<i>MATa dnl4::URA3 sae2::KanMX</i>
KBY126-18A	<i>MATa dnl4::URA3 sae2::kanMX sgs1-D664Δ MRE11-YFP RAD52-RFP</i>
KBY42-8D	<i>MATa bar1::LEU2 MRE11-YFP RAD52-RFP</i>

KBY48-4B	<i>MATα sae2::kanMX MRE11-YFP RAD52-RFP</i>
KBY48-11A	<i>MATα sgs1-D664Δ MRE11-YFP RAD52-RFP</i>
KBY48-4D	<i>MATα sae2::kanMX sgs1-D664Δ MRE11-YFP RAD52-RFP</i>
KBY64-13D	<i>MATα sae2::kanMX MRE11-YFP ura3::3xURA3-TetOx112 I-SceI (ura3-1) TetR-mRFP (iGL119w) pWJ1089</i>
KBY64-14B	<i>MATα sgs1-D664Δ MRE11-YFP ura3::3xURA3-TetOx112 I-SceI (ura3-1) TetR-mRFP (iGL119w) pWJ1089</i>
KBY64-5A	<i>MATα MRE11-YFP ura3::3xURA3-TetOx112 I-SceI (ura3-1) TetR-mRFP (iGL119w) pWJ1089</i>
KBY69-13B	<i>MATα sgs1-D664Δ sae2::kanMX MRE11-YFP ura3::3xURA3-TetOx112 I-SceI (ura3-1) TetR-mRFP (iGL119w) pWJ1089</i>
KBY89-2B	<i>MATα rad51::LEU2 ade2-n::TRP1::ade2-I-SceI lys2::GAL-I-SceI</i>
KBY89-14B	<i>MATα rad51::LEU2 sae2::KanMX ade2-n::TRP1::ade2-I-SceI lys2::GAL-I-SceI</i>
KBY89-3D	<i>MATα rad51::LEU2 sgs1-D664Δ ade2-n::TRP1::ade2-I-SceI lys2::GAL-I-SceI</i>
KBY89-9B	<i>MATα rad51::LEU2 sae2::KanMX sgs1-D664Δ ade2-n::TRP1::ade2-I-SceI lys2::GAL-I-SceI</i>
KBY80-22C	<i>MATα ku70::HIS3 MRE11-YFP RAD52-RFP</i>
KBY80-8C	<i>MATα ku70::HIS3 sae2::kanMX sgs1-D664Δ MRE11-YFP RAD52-RFP</i>
pSM502	<i>pRS424-EXO1, 2-micron, T7 promoter, TRP1, AMP^R</i>
pRS424	<i>pRS424, 2 micron, T7 promoter, TRP1, AMP^R</i>
pWJ1089	<i>GAL-NLS-I-SceI, CEN, KAN^R, HIS3</i>

All yeast strains are *RAD5* derivatives of the W303 background (Thomas and Rothstein, 1989) W1588 (Zhao et al., 1998) and only the relevant genotype is shown. The strains are listed in the order they appear in the text.

SUPPLEMENTAL REFERENCES

- Thomas, B.J., and R. Rothstein. 1989. Elevated recombination rates in transcriptionally active DNA. *Cell*. 56:619-630.
- Zhao, X., E.G. Muller, and R. Rothstein. 1998. A suppressor of two essential checkpoint genes identifies a novel protein that negatively affects dNTP pools. *Mol. Cell*. 2:329-340.