Resection activity of the Sgs1 helicase alters the affinity of DNA ends for homologous recombination proteins in *Saccharomyces cerevisiae*

Kara A. Bernstein*,§, Eleni P. Mimitou†, Michael J. Mihalevic§, Huan Chen†, Ivana Sunjaeric*, Lorraine S. Symington†, Rodney Rothstein*,‡

Departments of *Genetics & Development and †Microbiology & Immunology, Columbia University Medical Center, New York, NY 10032-2704
§Department of Microbiology and Molecular Genetics, University of Pittsburgh School of Medicine, Pittsburgh, PA 15213
†Current address: Molecular Biology Program, Memorial Sloan-Kettering Cancer Center, New York, NY 10065
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Correspondence: Rodney Rothstein, Dept of Genetics & Development, Columbia University Medical Center, 701 West 168th Street, HHSC 1608, New York, NY 10032

email: rothstein@columbia.edu
Tel: +1 212-305-1733
Fax: +1 212-923-2090
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 man, leading to cancer pre-disposition and/or premature aging. In the budding yeast, *Saccharomyces cerevisiae*, mutations in the RecQ homologue, *SGS1*, phenocopy many of the defects observed in the human syndromes. One challenge to studying RecQ helicates 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.
Repair of DNA damage is essential for maintenance of genomic integrity. When DNA repair is compromised, mutations and genetic alterations can occur that are the hallmarks of cancer cells. 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 hereditable 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 homologues such as genomic instability and premature aging [Reviewed in (BERNSTEIN et al.)].

Sgs1, and its homologues, 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 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 non-homologous end joining (NHEJ) (FOSTER et al. 2011; LANGERAK and RUSSELL 2011; MIMITOU and SYMINGTON 2010). 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
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’ over-hangs can then occur by one of two pathways: the first utilizes the Sgs1/BLM helicase in conjunction with the endonuclease Dna2/DNA2 and the second utilizes the 5’-3’ double-stranded DNA exonuclease Exo1/hEXO1 (Cejka et al. 2010; Gravel et al. 2008; Mimitou and Symington 2008; Niu et al. 2010; Zhu et al. 2008). 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/TOP3α topoisomerase with the accessory protein(s) Rmi1/RMI1-RMI2 (Cejka and Kowalczykowski 2010; Singh et al. 2008; Wu et al. 2006; Wu and Hickson 2003; Xu et al. 2008).

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 pleiotrophy, separation-of-function alleles of SGS1 have been utilized to pinpoint its specific role at different processing steps (Bernstein et al. 2009; Hegnauer et al. 2012; Lu et al. 1996;
We previously described one sgs1 allele, sgs1-D664Δ, that behaves like an sgs1Δ mutant in largely suppressing the slow growth observed in top3Δ and rmi1Δ cells as well as top3Δ sensitivity to DNA damage (Bernstein et al. 2009). The sgs1-D664Δ mutant also accumulates DNA damage-induced X-structure replication intermediates to the same extent as an sgs1Δ (Bernstein et al. 2009). However, in many respects the sgs1-D664Δ strain exhibits a wild-type SGS1 phenotype. This allele lacks many of the synthetic interactions observed in sgs1Δ cells (such as with srs2Δ, mus81Δ, rrm3Δ) 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-D664Δ mutant during DSB repair. Here we find that sgs1-D664Δ has specific genetic interactions with SAE2, a gene needed for resection of DSB ends. This genetic interaction led us to ask whether sgs1-D664Δ has a resection defect. We find that sgs1-D664Δ exo1Δ mutants exhibit a resection processivity defect. Furthermore, we find that the genetic interaction between sgs1-D664Δ and sae2Δ is suppressed by disruption of KU70 or by over-expression of EXO1. These results suggest that the resection processivity defect observed in sgs1-D664Δ cells alters the affinity of DNA ends for HR proteins. Consistent with this notion, sgs1-D664Δ sae2Δ cells exhibit persistent Mre11 foci at DSB sites.
MATERIALS AND METHODS

**Strains, plasmids and media:** The strains used in this study are listed in Supplemental Table 1. They are isogenic with W303 and derived from the *RAD5*+ strains W1588-4C and W5909-1B (BERNSTEIN *et al.* 2011; THOMAS and ROTHSTEIN 1989; ZHAO *et al.* 1998). 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-D664Δ* 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 750 bp PCR product and *sgs1-D664Δ* gives rise to a 350 and 400 bp fragments).

**Spore size analysis:** Analysis of the individual spore colonies from tetrads was performed using the Dissection Reader software developed 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 www.rothsteinlab.com/tools/apps/dissection_reader. An average of 6-25 spores were analyzed for each genotype and standard deviations calculated. Wild-type spores were set to one and the mutants were analyzed relative to wild-type growth.
**Recombination assay:** The inter-homologue leu2ΔBstEII and leu2ΔEcoRI 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 one hour to induce I-SceI expression and I-SceI was switched off by addition of glucose. Samples were collected hourly from 0-6 hours 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 and representative gels shown in Figures 2 and 4.

**ssDNA intermediate analysis by alkaline electrophoresis:** Alkaline electrophoresis of StyI/BstXI-digested genomic DNA was performed as previously described (Mimitou and Symington 2008; White and Haber 1990). 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°C to early log phase and equal numbers of cells were five-fold serially diluted onto plates containing YPD or YPGal/Raf and were photographed after one to two days of growth at 30°C.
**Fluorescent Microscopy:** Cells were grown overnight at 30°C in 5 ml cultures of SC with adenine (100 mg/ml) and harvested for microscopy as previously described (Lisby et al. 2001). An Mre11-YFP construct was introduced into WT, 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 100X oil immersion objective (1.45 numerical aperture), a Photometrics HQ2 camera and motorized Prior Z-stage. Stacks of 11 0.3µm sections were captured using the following exposure times: differential interference contrast (60 ms) and Mre11-YFP (1000 ms). 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 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 hours at 30°C and subsequently expression of I-SceI was inhibited by addition of 1% glucose. Immediately after inhibiting I-SceI expression and four hours later the cells were visualized as described above except that the DSB was visualized using the RFP channel (600 ms).

**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; PAN et al. 2006; PAN et al. 2004; TONG et al. 2001; TONG et al. 2004). 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; PAN et al. 2006; PAN et al. 2004; TONG et al. 2001; TONG et al. 2004). 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. 2006; PAN et al. 2004; PARK et al. 2012; TAMBINI et al. 2010). 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 (Supplemental Figure 1). Importantly, we do 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.

**Inter-homologue 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 inter-homologue recombination rates (ALVARO et al. 2007), we asked whether sgs1-D664Δ would similarly exhibit an increase in inter-homologue recombination. In this assay, inter-homologue recombination rates are calculated by measuring the rate of Leu⁺ prototrophs created by recombination between two leu2-heteroalleles in a diploid strain (Supplemental Figure 2). We find that, unlike an sgs1Δ, sgs1-D664Δ cells do not exhibit increased inter-homologue recombination (Supplemental Figure 2). 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 (SSA): The *sae2Δ sgs1-D664Δ* synthetic interaction focused our attention on whether the defective process in *sgs1-D664Δ* mutant cells is DNA end resection. During an early HR step, the Sae2 endonuclease function 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 ([Čejka et al. 2010; Gravel et al. 2008; Mimitou and Symington 2008; Niu et al. 2010; Zhu et al. 2008](#)). To monitor end resection, we performed a single-strand annealing assay (SSA), where two *ade2* hetero-alleles are separated by *TRP1* and plasmid sequences (Figure 2A) ([Mimitou and Symington 2008](#)). To create a double-strand break (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 is induced by galactose (YPG/R). 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 hours 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 nt DNA fragments [Figure 2B; (MIMITOU and SYMINGTON 2008; ZHU et al. 2008)]. Examination of *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 hours (Figure 2B), indicating that the *sgs1*-D664Δ mutation confers a DNA end resection defect. Note, 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 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 *Styl* (S) or *Bst*XI (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 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, panel 5), the DSB fragment persists and only the 1.6-kb product (r1) can be readily detected indicating resection tracts are less than 1,600 nt (IMITOU 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).

The 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 over-expression of EXO1 suggesting that one of the roles of the Ku70-Ku80 complex is to regulate access of nucleases to DNA ends, especially Exo1 (IMITOU 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; LANGERAK et al. 2011;IMITOU and SYMINGTON 2010). To determine whether the resection defect conferred by sgs1-D664Δ is responsible for the synthetic interaction observed in 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
Disruption of a down-stream NHEJ gene, the DNA ligase \textit{DNL4}, does not suppress the synthetic growth defect in \textit{sae2\Delta sgs1-D664\Delta} cells (Figure 4B) like \textit{sgs1\Delta sae2\Delta} 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 \textit{sgs1-D664\Delta} and \textit{sae2\Delta}. Consistent with this idea, over-expression of \textit{Exo1} largely suppresses the synthetic growth defect observed in \textit{sae2\Delta sgs1-D664\Delta} double mutants (Figure 4C) similar to that observed for \textit{sae2\Delta sgs1\Delta} cells (Mimitou and Symington 2010). Furthermore, deletion of \textit{EXO1} leads to \textit{sae2\Delta sgs1-D664\Delta} lethality (Figure 4D), as reported previously for \textit{sgs1\Delta sae2\Delta} (Mimitou and Symington 2008), again suggesting that the defect in the \textit{sgs1-D664\Delta} separation-of-function mutation is reduced end resection.

To examine if \textit{sae2\Delta sgs1-D664\Delta} double mutants are deficient in end resection, we utilized the SSA assay described in Figure 2A to monitor formation of \textit{Ade}^+ recombinants. Qualitatively, fewer spontaneous white \textit{Ade}^+ recombinants were formed in \textit{sae2\Delta sgs1-D664\Delta rad51\Delta} on rich medium (YPD) and viability was reduced when I-\textit{SceI} is induced by galactose (YPG/R), consistent with the notion that the combination of \textit{sae2\Delta} and \textit{sgs1-D664\Delta} results in a resection defect (Figure 4E). To further investigate the resection defect, we assayed the \textit{sae2\Delta rad51\Delta} and \textit{sae2\Delta sgs1-D664\Delta rad51\Delta} 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 \textit{sgs1-D664\Delta exo1\Delta rad51\Delta} triple mutant, which is defective for long-range resection, but the persistence of the cut fragments suggests that the \textit{sae2\Delta sgs1-D664\Delta rad51\Delta} 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 Mre11 persists longer at DSBs in the sae2Δ mutant (Clerici et al. 2006; Lisby et al. 2004), 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 wild type (WT), sgs1-D664Δ, sae2Δ, and sae2Δ sgs1-D664Δ strains. The cells were exposed to 40 Gy of IR which leads to approximately 4 DSBs per cell (Ma et al. 2008) and does not result in decreased viability or slow growth of WT, sgs1-D664Δ or sae2Δ cells (Supplemental Figure 3). 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 (Supplemental Figure 4; p ≤ 0.025 and p ≤ 0.005 respectively). Following IR, both WT and sgs1-D664Δ cells exhibit an increase in Mre11 foci that are mostly gone after two hours (Figure 5). In contrast, sae2Δ cells exhibit a high percentage of cells with an Mre11 focus, which only gradually decrease over time [Figure 5; 0.2 hr compared to 2 hrs (p ≤ 0.05) or 4 hrs (p ≤ 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 hr to 2 or 4 hrs). 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 $sae2\Delta sgs1-D664\Delta$ 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 (112x$tetO$). The localization of the cut site is revealed by expression of the TetR fused to a monomeric red fluorescent protein (mRFP), which binds to the TetO. Using this system, we monitored Mre11-YFP localization to the cut site after DSB induction for 2 hours by galactose addition and then monitored cells 4 hours after I-$SceI$ enzyme expression was shut off with glucose (Figure 6). Less Mre11 is seen at the DSB site 4 hours after DSB induction for WT and for $sgs1-D664\Delta$ and $sae2\Delta$ mutant cells (Figure 6; $p \leq 0.025$ in WT and $sgs1-D664\Delta$ and $p \leq 0.005$ in $sae2\Delta$). In contrast, we find that more Mre11 is recruited to the break site in $sae2\Delta sgs1-D664\Delta$ cells and it remains associated even 4 hours after removing the inducer (Figure 6; $p \leq 0.1$). These results support the notion that the synergistic interaction between $sgs1-D664\Delta$ and $sae2\Delta$ is due to the inefficient removal of Mre11 from DSB ends.

**The persistent Mre11 foci in a $sae2\Delta sgs1-D664\Delta$ mutant are reduced by $YKU70$ disruption:** Our data suggest that inefficient removal of Mre11 from DSB ends in $sae2\Delta sgs1-D664\Delta$ leads to a slow growth phenotype (Figures 4 to 6). Since disruption of $YKU70$ alleviates the slow growth of $sae2\Delta sgs1-D664\Delta$ mutant cells (Figure 4A), we
asked whether the persistent Mre11 foci are still observed in \textit{sae2Δ sgs1-D664Δ} cells after deletion of \textit{YKU70}. We find that in \textit{sae2Δ sgs1-D664Δ yku70Δ} triple mutants, the percentage of cells with an Mre11-YFP focus decreases over time (from approximately 50\% to 25\%) in contrast to \textit{sae2Δ sgs1-D664Δ} double mutants, where persistent Mre11 foci are observed (Figure 5; \( p \leq 0.05 \) and \( p \leq 0.005 \) comparing \textit{sae2Δ sgs1-D664Δ yku70Δ} cells at 0.2 hrs to 2 and 4 hrs, 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, \textit{yku70Δ} alone results in fewer Mre11 foci after initial IR exposure (0.2 hrs; 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 homologue Sgs1. One of the challenges of studying these proteins is the diverse phenotype associated with their disruption. For example, disrupting Sgs1 leads to both mitotic and meiotic defects, chromosomal instability, increased crossovers, telomere alterations, and pre-mature aging [Reviewed in (BERNSTEIN et al.)]. 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 dHJs. To begin to understand the role that Sgs1 plays in DSB repair, we isolated a separation-of-function allele, \textit{sgs1-}
$D664\Delta$, which largely suppresses $top3\Delta$ 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 $sgs1-D664\Delta$ compared to $sgs1\Delta$. Although $sgs1\Delta$ mutations show synthetic growth defects with a large number of HR genes, $sgs1-D664\Delta$ specifically exhibits a synthetic effect with $sae2\Delta$, 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 $sgs1-D664\Delta$ cells. In the absence of Rad51, the $sgs1-D664\Delta$ allele, when combined with $exo1\Delta$, slows long-range resection and results in decreased product formation (Figure 2B). Similarly $sgs1-D664\Delta$ $exo1\Delta$ $rad51\Delta$ 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 $sgs1-D664\Delta$ $sae2\Delta$ double mutant is due to this resection defect for two reasons. Namely, its slow growth is alleviated by eliminating Ku or over-expressing Exo1 (Figures 4A and 4B), which is similar to the rescue of the synthetic lethality of an $sgs1\Delta$ $sae2\Delta$ double mutant (MIMITOU and SYMINGTON 2010). The direct repeat assay described in Figure 2A requires the resection of more than 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 $sgs1-D664\Delta$ $sae2\Delta$ $rad51\Delta$ triple mutant compared to the respective double mutants with $rad51\Delta$ (Figure 4E). Our results suggest a model where the MRX complex and Sae2 normally initiate
resection; however, in the absence of \textit{SAE2}, Sgs1 can now function in resection initiation as well as long-range resection. All of these observations strengthen the notion that Sgs1-D664\(\Delta\) slows resection.

The Sgs1-D664\(\Delta\) 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 \textit{sgs1-D664\(\Delta\) exo1\(\Delta\)} double mutant slows resection without affecting growth or the hyper-recombination phenotype normally associated with an \textit{sgs1\(\Delta\)} cell. Previously, we found that \textit{sgs1-D664\(\Delta\)} cells accumulate replication intermediates, namely X-structures, by 2D-pulse-field gel electrophoresis (\textit{BERNSTEIN et al.} 2009). Perhaps the resection defect observed in the \textit{sgs1-D664\(\Delta\)} cell is responsible for the accumulation of X-structures observed in this mutant. Although it is possible that the \textit{sgs1-D664\(\Delta\)} is a dominant protein, our preliminary results suggest that it is recessive since a diploid \textit{SGS1/sgs1-D664\(\Delta\)} cell behaves like a wild-type \textit{SGS1/SGS1} with respect to suppression of \textit{top3\(\Delta\)} slow growth as well as resolution of X-structures. Due to its proximity to the acidic regions, this \textit{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 (\textit{BERNSTEIN et al.} 2009). Therefore, it is likely that this allele is altering the structure of Sgs1 that specifically inhibits its role in resection.

It was surprising that the \textit{sgs1-D664\(\Delta\)} mutant allele only showed a specific synthetic sick interaction with \textit{sae2\(\Delta\)} 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 only occurs 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 Shim refs 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 (Figures 5 and 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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FIGURE LEGENDS

FIGURE 1. Cells with \textit{sgs1-D664\Delta} have a synthetic sick interaction when combined with \textit{sae2\Delta}. (A) Yeast with \textit{sgs1-D664\Delta} or \textit{sgs1\Delta} were mated to \textit{sae2\Delta}, \textit{mre11\Delta}, \textit{rad50\Delta}, or \textit{xrs2\Delta} 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 \textit{sgs1-D664\Delta} with HR mutant genes. The synthetic interactions marked with an (*) were previously reported (\textit{Bernstein et al.} 2009).
FIGURE 2. In the absence of Rad51, sgs1-D664Δ exo1Δ cells display a resection defect. (A) Schematic of the single-strand annealing (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 panel. (B) Yeast strains containing the SSA assay diagramed with the indicated genotypes were grown in rich medium (YPD) to early log phase (0.5 OD$_{600}$) and then five-fold serially diluted onto rich medium (YPD) or medium containing galactose and raffinose (YPG/R), which induces expression of the I-SceI enzyme from a galactose-inducible promoter. Plates were photographed after three days of growth at 30°C. The same cells were analyzed by DNA blotting to detect the cut fragments and the ADE2+ repair product from 0 to 6 hours after I-SceI cutting.

FIGURE 3. sgs1-D664Δ exo1Δ rad51Δ 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 hours after induction of HO endonuclease.

FIGURE 4. The slow growth of sae2Δ sgs1-D664Δ cells is suppressed by yku70Δ or by Exo1 over-expression. (A and B) Yeast strains with the indicated genotypes were five-fold serially diluted onto rich medium, grown at 30°C for one 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 five-fold serially diluted onto minimal medium to maintain the plasmid (SC-TRP), grown at 30°C for two 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 diagramed in Figure 2A with the indicated genotypes were grown in rich medium (YPD) to early log phase (0.5 OD600) and then five-fold serially diluted onto rich medium (YPD) or medium containing galactose and raffinose (YPG/R), which induces expression of the I-SceI enzyme from a galactose-inducible promoter. Plates were photographed after three days of growth at 30°C. (F) The sae2Δ and sae2Δ sgs1-D664Δ cells were analyzed by DNA blot for resection products and cut fragments diagrammed as in Figure 2A.

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 hours. For each time point, a single Z-stack is shown and white arrowheads indicate a focus. Each experiment was done in triplicate with a total of 200-650 cells analyzed with standard errors plotted.

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 hours) and 4 hours 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.
FIGURE 1

A

Relative colony growth

WT

sgs1-D664Δ

sae2Δ

sgs1-D664Δ sae2Δ

WT

sgs1-D664Δ

mre11Δ

sgs1-D664Δ mre11Δ

WT

sgs1-D664Δ

rad50Δ

sgs1-D664Δ rad50Δ

WT

sgs1-D664Δ

xrs2Δ

sgs1-D664Δ xrs2Δ

B

<table>
<thead>
<tr>
<th>Gene tested</th>
<th>Function of gene during HR</th>
<th>Synthetic interaction with sgs1-D664Δ</th>
</tr>
</thead>
<tbody>
<tr>
<td>mre11Δ</td>
<td>Resection of DSBs</td>
<td>No</td>
</tr>
<tr>
<td>mus81Δ</td>
<td>Endonuclease</td>
<td>No</td>
</tr>
<tr>
<td>rad27Δ</td>
<td>5'-3' exonuclease, 5' flap endonuclease</td>
<td>No</td>
</tr>
<tr>
<td>rad50Δ</td>
<td>Resection of DSBs</td>
<td>No</td>
</tr>
<tr>
<td>sae2Δ</td>
<td>Resection of DSBs</td>
<td>Yes</td>
</tr>
<tr>
<td>srs2Δ</td>
<td>DNA helicase disrupts Rad51 filaments</td>
<td>No</td>
</tr>
<tr>
<td>top1Δ</td>
<td>Topoisomerase I</td>
<td>No</td>
</tr>
<tr>
<td>xrs2Δ</td>
<td>Resection of DSBs</td>
<td>No</td>
</tr>
</tbody>
</table>
FIGURE 4

A

WT  
sgs1-D664Δ  
yku70Δ  
sae2Δ  
sgs1-D664Δ yku70Δ  
sae2Δ yku70Δ  
sae2Δ sae1-D664Δ  
sae2Δ yku70Δ sae1-D664Δ

B

WT  
sgs1-D664Δ  
dnl4Δ  
sae2Δ  
sgs1-D664Δ dnl4Δ  
sae2Δ dnl4Δ  
sae2Δ sae1-D664Δ  
sae2Δ yku70Δ sae1-D664Δ

C

Plasmid

WT  2µ-Exo1  Empty

sae2Δ  2µ-Exo1  Empty

sae2Δ sae1-D664Δ  2µ-Exo1  Empty

D

Relative spore size

WT  exo1Δ  sae2Δ  sae1-D664Δ  exo1Δ  sae2Δ  exo1Δ  sae1-D664Δ

E

rad51Δ  sae2Δ  rad51Δ  sae2Δ rad51Δ  sae2Δ rad51Δ  sae2Δ rad51Δ  sae2Δ rad51Δ

YPD

F

Hours after cut:

repairs product

cut fragment

rad51Δ  sae2Δ  sae2Δ sae1-D664Δ

0 1 2 3 4 5 6

9 kb 5 kb 4 kb 2.3 kb 1.7 kb
FIGURE 5

Hrs after IR: 0.2 hr 1 hr 2 hr 4 hr

WT

sgs1-D664Δ

sae2Δ

sgs1-D664Δ

yku70Δ

sae2Δ

sgs1-D664Δ

yku70Δ

% cells with an Mre11-YFP focus after irradiation (40 Gy)

Hrs after IR: 0.2 1 2 4

WT sgs1-D664Δ sae2Δ

sae2Δ

sgs1-D664Δ

yku70Δ

sae2Δ

sgs1-D664Δ

yku70Δ