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Characterization of Mre11/Rad50/Xrs2, Sae2, and Exo1 in DNA End Resection

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by

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Characterization of Mre11/Rad50/Xrs2, Sae2, and Exo1 in DNA End Resection

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Eukaryotic cells repair DNA double-strand breaks (DSBs) through both non-homologous and homologous recombination pathways. The initiation of homologous recombination requires the generation of 3' overhangs, which are essential for the formation of Rad51 protein-DNA filaments that catalyze subsequent steps of strand invasion. Experiments in budding yeast show that resection of the 5' strand at a DSB is delayed in strains lacking any components of the Mre11/Rad50/Xrs2 (MRX) complex [1]. In meiosis, a specific class of hypomorphic mutants of *mre11* and *rad50* (Rad50S) are completely deficient in 5' resection and leave Spo11 covalently attached to the 5' strands of DNA breaks [2].

Similar to *mre11S* and *rad50S* mutants, *sae2* deletion strains fail to resect 5' strands at meiotic DSBs and accumulate covalent Spo11 adducts [3; 4]. In addition, Sae2 and MRX were also found to function cooperatively to process hairpin-capped DNA ends *in vivo* in yeast. *sae2* and *mrx* null strains show a severe defect in processing these structures and accumulate hairpin-capped DNA ends [5; 6]. The Longhese laboratory has also shown that Sae2 deletion strains show a delay in 5' strand resection, similar to *rad50S* strains [7]. Recently, Bettina Lengsfeld in our laboratory demonstrated that Sae2 itself possesses nuclease activity and that MRX and Sae2 act cooperatively to cleave single-stranded DNA adjacent to DNA hairpin structures [8]. *In vitro* characterization of Sae2 showed that the central and N-terminal domains are required for MRX-independent nuclease activity and that the C-terminus is required for cooperative activities with MRX. Sae2 also acts independently of MRX as a 5' flap endonuclease on branched structures *in vitro*.

Our studies investigate whether MRX, Sae2, and Exo1 function cooperatively in DNA resection using recombinant, purified proteins *in vitro*. We developed assays utilizing strand-specific Southern blot analysis to visualize DNA end processing of model DNA substrates using recombinant proteins *in vitro.* Our results demonstrate that MRX and Sae2 cooperatively resect the 5' end of a DNA duplex together with the Exo1 enzyme, supporting a role for these factors in the early stages of homologous recombination and repair.

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Chapter 1: Introduction

DNA Damage and Homologous Recombination

DNA double strand breaks (DSBs) in chromosomal DNA constantly occur *in vivo* as a result of natural cellular processes such as collapsed replication forks or free radical assault (Rothstein, 2000). External factors such as ionizing irradiation (IR) or chemical compounds can also cause breaks that must be repaired to prevent genome instability. Maintenance of genome stability requires efficient repair of DNA double-strand breaks. Eukaryotic organisms repair DSBs through error-prone non-homologous end joining (NHEJ) or less error-prone homologous recombination (HR).

During meiosis, homologous recombination contributes to genetic diversity from one generation to the next by gene conversion or reciprocal crossing over events. Gene conversion is the transfer of genetic material from one chromosome to the homologous complement without changing the sequence of the donor chromosome. Crossing over is the exchange of genetic material between chromosome pairs.

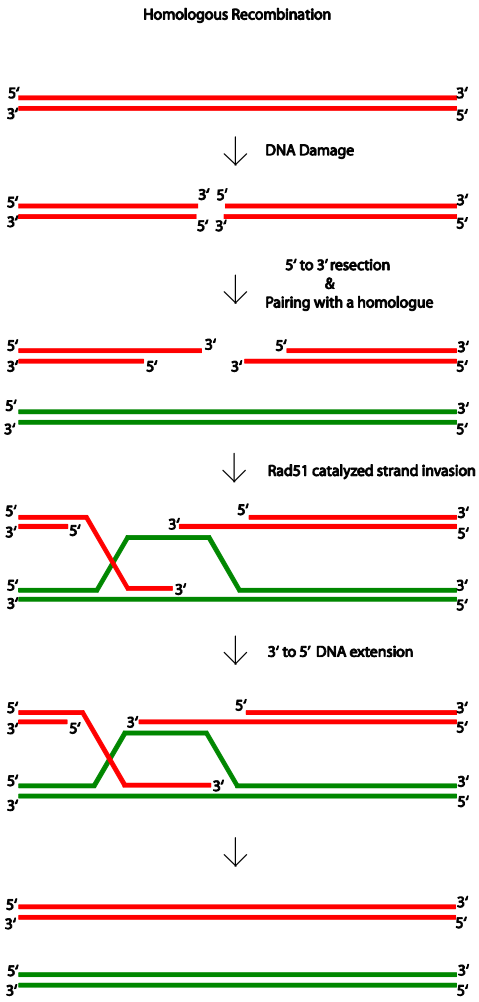


Figure 1: Homologous recombination is a relatively error-free method of DNA repair and the preferred pathway in *S. cerevisiae.* After a DSB, the 5' DNA strand is resected, leaving a 3' DNA overhang. Rad51 filaments form on the 3' overhang and invade the homologous chromosome. In synthesis-dependent strand annealing, as shown here, the DNA is extended through the break and the strands are ligated, resolving the DNA break. (Adapted from Krogh and Symington, 2004)

Mechanisms of Repair

Eukaryotic organisms repair DNA DSBs through two major pathways. Non-homologous end joining (NHEJ) is the more prevalent pathway in mammalian cells and involves direct ligation of the broken ends. Homologous recombination requires DNA synthesis using a sister chromatid or a homolog as a template to repair the break site.

In *S. cerevisiae*, homologous recombination (HR) is the dominant pathway of DNA repair, and most of our knowledge about HR comes from studying this organism. Homologous recombination requires several conserved repair factors including Rad51, Rad52, Rad54, Rad55, Rad57, and the Mre11/Rad50/Xrs2 (MRX) complex. These proteins are all members of the Rad52 epistasis group and are essential for repairing DNA breaks caused by ionizing irradiation (IR) [9](Fig. 1).

HR initiates with the resection of the 5' DNA strand at a DSB, forming a 3' single-stranded DNA overhang that can be several hundred nucleotides to thousands of nucleotides long [10]. Resection is a slow process that can take up to 4 hours *in vivo.* The Haber laboratory discovered that physiological resection occurs at about 4.5 bp per hour [11]. During resection, Replication Protein-A (RPA) binds and stabilizes the 3' overhang and removes secondary structures in the DNA. The RPA is displaced by Rad51 with the help of Rad52 [12; 13]. Rad55 and Rad57 have also been shown to aid in the loading of Rad51 onto the 3' DNA overhang. The Rad51-coated 3' DNA overhang invades a DNA duplex with homologous sequence, creating a D-loop structure. In

vegetatively growing cells this is usually a sister chromatid, while the target sequence in meiosis is always a homologous chromosome. The D-loop structure pairs with the non-invading 3' strand, and usually both 3' strands are extended by DNA synthesis. The remaining gaps are then ligated leaving a double Holliday Junction intermediate that is resolved leaving either crossover or noncrossover products [14]. Alternatively, the newly-synthesized strand is extended from the donor and ligated to the original broken end in a process known as Synthesis-Dependent Strand Annealing (SDSA) or "break-copy."

DSB formation in Meiosis

DSBs are created in meiosis to promote the formation of crossover intermediates, which are necessary for meiotic progression and for the genetic diversity generated by sexual reproduction. In *S. cerevisiae,* the Spo11 protein catalyzes the formation of the DSBs [15] (Fig. 2). Spo11 is a meiosis-specific topoisomerase II-like protein that is essential for meiotic DSB formation. Like topoisomerase II, Spo11 makes a covalent attachment to the 5' strands of DNA at a DSB site. When the active site tyrosine is mutated, Spo11 cannot create DSBs *in vivo* [16]. In addition to Spo11, the meiosis-specific factors Mei4, Rec107, Rec102, Ski8, Rec104, and Rec114 are required for DSB formation as well as the general DSB repair factors Mre11, Rad50, Xrs2, and Sae2 [17].

Unlike topoisomerase II, Spo11 remains covalently attached to the 5' DNA strand, and must be removed for recombination to proceed. *rad50S*, *mre11S*, and *sae2* deletion strains are unable to remove Spo11 adducts from the DNA [2; 3; 18] . This result suggests that the MRX complex and Sae2 are involved in processing Spo11 adducts after the DSB is induced. Further evidence demonstrated that Mre11 localizes to preferred Spo11 cleavage sites in the 5' noncoding regions in genes [19]. Mre11 remains bound to the cleavage site in strains defective in 5' to 3' resection, but is removed in strains defective for the later steps of strand invasion, suggesting a role in resection [14].

Repair and Recombination in Vegetative Cells

Mitotic recombination occurs in response to random DSBs and can also be initiated by DNA endonucleases such as HO [9]. HO nuclease stimulates gene conversion by cleaving a 24 bp sequence within the MAT mating-type locus. The cut leaves 4 bp 3' overhangs at the DSB. The ends are processed with the same factors that process DSBs created by ionizing radiation (IR). Similar to meiosis, *S. cerevisiae rad50*, *mre11*, and *xrs2* null strains exhibit delayed resection after cleavage by HO endonuclease at the *MAT* locus in vegetatively growing cells [10].

MRX is only partially required for mitotic DSB repair. MRX deletion strains show a delay in resection, but a complete loss of resection in vivo is only seen with simultaneous deletion of the 5' to 3' exonuclease Exo1 and the helicase Sgs1 (Mimitou,

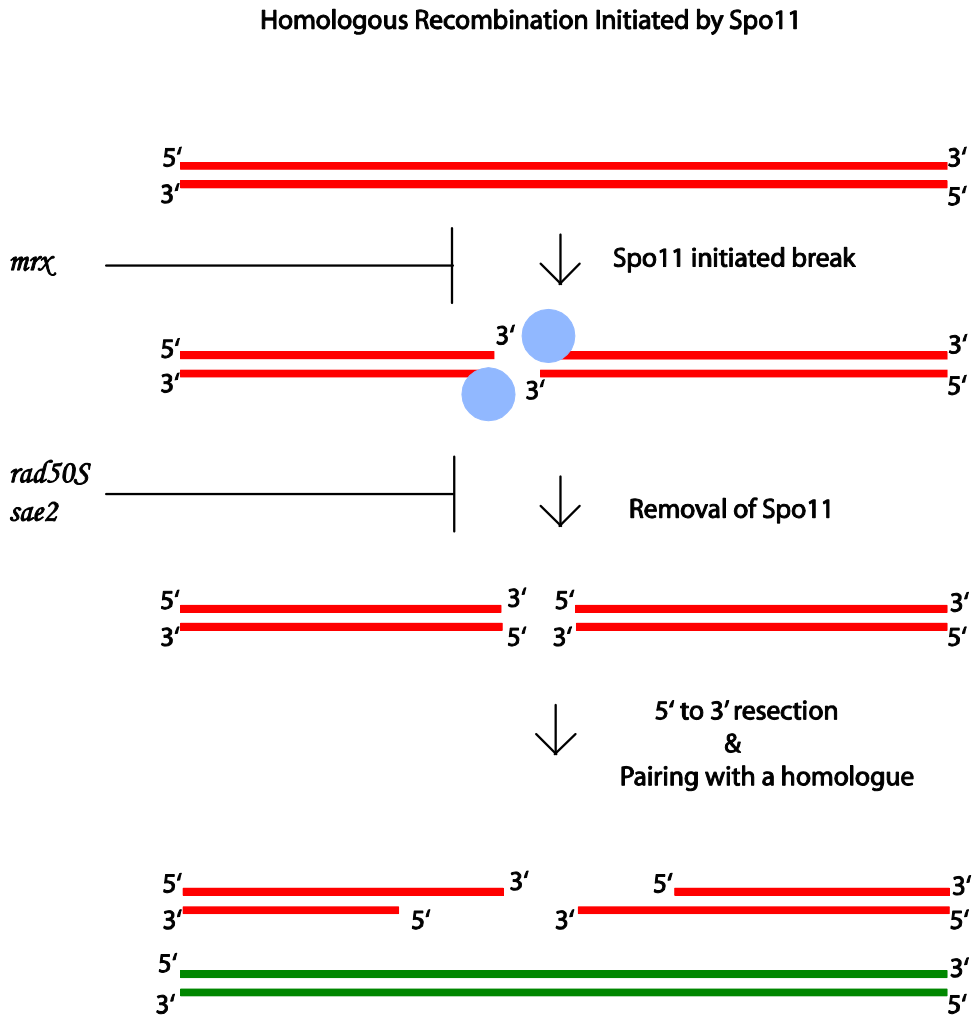


Figure 2: Spo11 initiates homologous recombination by causing a double-stranded break in meiosis. *mrx* null strains fail to initiate the double-strand breaks. *rad50S and sae2* null strains fail to remove Spo11 from the 5' strand of the double-strand break.

2008). The delay in resection is rescued and IR sensitivity is suppressed in *mre11* null strains when Exo1 is over expressed [20]. In addition, *exo1* null strains containing a nuclease-deficient mutant of Mre11 (H125N), are still resistant to IR. A double null strain has severe sensitivity to IR. Additional deletions of any of the Rad52 epistasis proteins, Sae2, or the addition of a Rad50S mutation is synthetic lethal [21].

MRX

HR requires 5' DNA end resection to create 3' single-stranded (ss) DNA at a DSB site. Several proteins have been implicated in this process in *S. cerevisiae* and often have overlapping functions in recombination and repair. The MRX complex is one of the first protein complexes to associate at the DNA break after damage and appears to have a role in DNA end resection [22].

S. cerevisiae proteins Mre11, Rad50, and Xrs2 form a complex that functions in homologous recombination, nonhomologous end joining (NHEJ), and checkpoint signaling after DNA damage (Fig. 3). Mre11 and Rad50 are conserved throughout all organisms. Xrs2 is conserved in eukaryotes and is a functional homolog of mammalian Nbs1.

Mre11 homologs in all organisms share conserved phosphoesterase motifs. Purified Mre11 proteins from humans, budding yeast, bacteria, and thermophilic

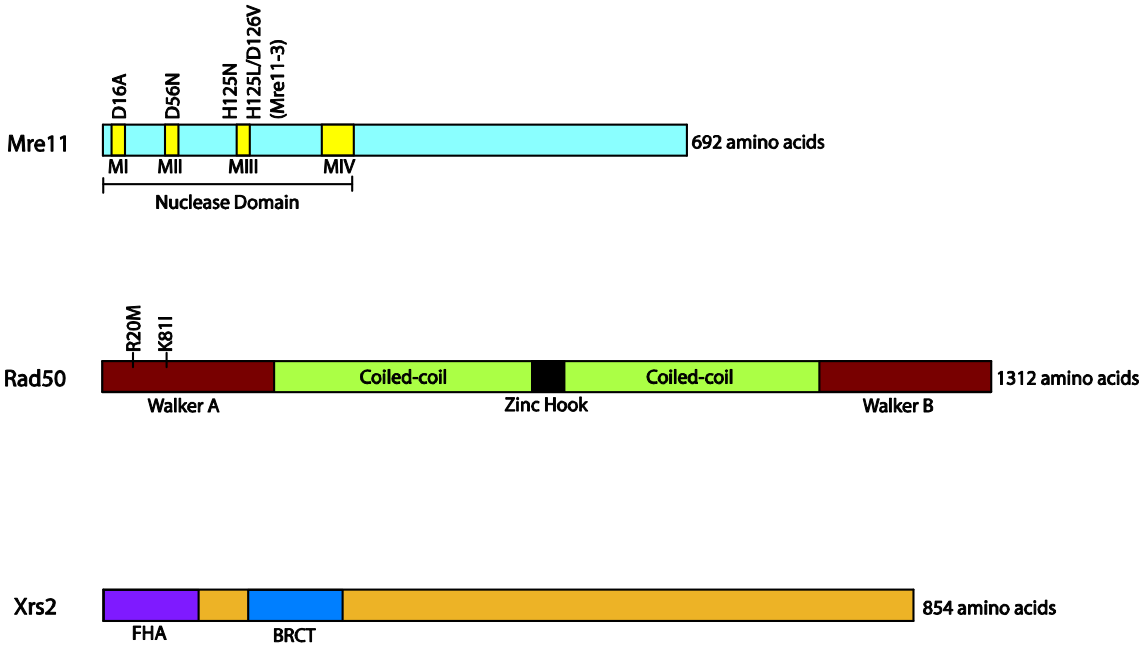


Figure 3: Mre11 is an exonuclease and an endonuclease. It contains a N-terminal nuclease domain. Rad50 is an ATPase that folds intramolecularly by associations in the Walker A and Walker B motifs. Xrs2 contains an N-terminal FHA domain and a BRCT domain that mediate protein-protein interactions. (Adapted from Symington 2002)

Archaea exhibit manganese-dependent 3' to 5' exonuclease activity and ssDNA endonuclease activity [23; 24; 25; 26]. D16A, D56N, P84S, N113S, H125N, P162S, and H213Y mutations in the conserved phosphodiesterase motifs in *S. cerevisiae* Mre11 abolish nuclease activity *in vitro*, although only some of the nuclease-deficient mutants form stable complexes.

Rad50 is also conserved in all organisms and consists of a long coiled-coil region flanked by the nucleotide-binding domains, Walker A and Walker B (Fig. 3). Rad50 forms a homodimer mediated by a central zinc hook motif [27]. The Walker A and Walker B domains function as an ATPase when they are brought together intramolecularly and are stabilized by ATP. The exact roles of the ATP-binding domains and coiled-coils in Rad50 are not yet understood, but are thought to be important for DNA end tethering. The human Mre11/Rad50/Nbs1 complex has also been shown to perform limited unwinding of DNA ends in vitro [28], and this was shown to be important for its role in checkpoint signaling [29].

Xrs2 is conserved among eukaryotes and is homologous to Nbs1 in fission yeast and mammals. Both proteins contain phosphopeptide binding domains. The Fork head Associated (FHA) domain is located on the N-terminus and is followed by a Breast Cancer Carboxy-Terminal (BRCT) repeat domain (Fig. 3). Xrs2 and Nbs1 function in signaling pathways in response to DNA damage, and their role in DSB resection has yet to be elucidated.

The most intriguing protein in the MRX complex with respect to DSB resection is Mre11, which possesses highly-conserved phosphoesterase motifs (Fig. 3). However, the 3' to 5' exonuclease polarity observed *in vitro* with Mre11 proteins from numerous organisms is incompatible with the direction of the physiological resection reaction. Mre11 also exhibits endonuclease activity and is capable of cleaving hairpin structures [28; 30; 31]. It is possible that Mre11 endonuclease activity plays a more relevant role in DNA end resection.

S. cerevisiae, mre11, *rad50*, and *xrs2* null strains are defective in meiotic resection, display delayed resection in vegetative cells, and are highly sensitive to DNA damaging agents [14]. During meiosis, the Mre11 nuclease-deficient mutant H125N is unable to remove Spo11 attached to the 5' strand of the DNA break, which stalls resection [32]. *In vitro* assays have demonstrated that the yeast Mre11-H125N and human Mre11-H129N do not exhibit nuclease activity [28; 32]. However, clean breaks or breaks that generate 3' hydroxyl and 5' phosphate groups do not appear to require Mre11 nuclease activity for resection [33].

Wild-type haploid *S. cervisiae* cells are 100 times more sensitive to IR in the G_1 phase compared to the G_2 phase of the cell cycle, suggesting a strong preference for sister chromatids in recombination repair. IR sensitivity in *mre11* deletion strains is equivalent in G_1 phase and G_2 phase, however, demonstrating that MRX plays an important role in homologous recombination between sister chromatids [34]. Mitotic cells display a hyper-

recombination phenotype in the absence of Mre11, Rad50, and Xrs2 due to the lack of sister chromatid exchange and higher frequency of inter-homolog exchange [35; 36].

Exo1

The 5' to 3' exonuclease Exo1 is another potential candidate enzyme involved in DNA end resection. Exo1 is induced during meiosis and has roles in mismatch repair, post replication repair, telomere maintenance, and the processing of stalled replication forks [37; 38; 39; 40]. *exo1* mutant strains display delayed resection after HO endonuclease breaks, although homologous recombination is eventually completed with high fidelity [33; 38].

Several studies suggest that Mre11 and Exo1 have redundant functions in resection. Over expression of Exo1 in strains lacking Mre11, Rad50, or Xrs2 abrogates the resection defect [41]. The double mutant *exo1 mre11* also shows extreme sensitivity to IR. *exo1* and *mre11-H125N* double mutants show a greater resistance to IR than the *mre11* mutant strain by itself [21; 42].

In the event of DNA damage, eukaryotic cells initiate checkpoints to pause or abort cell cycle progression. After the lesion is repaired, the checkpoint is released and mitosis proceeds. *S. cerevisiae* strains lacking Mre11 have an active G_2/M checkpoint; however, the checkpoint is released prematurely [42]. The *mre11- H125N* strains release the checkpoint at a similar rate as wild type cells, suggesting Mre11 3' to 5' exonuclease

activity is not involved in DNA damage signaling [20; 32]. Strains lacking Mre11, Rad50, or Xrs2 are sensitive to hydroxy-urea, suggesting a defect in the S-phase checkpoint [43].

Sae2

An additional protein that appears to be involved in DNA end resection is Sae2. Sae2 was identified as a gene required for meiotic recombination and repair [3; 4]. Genetic screens were performed in the presence or absence of Spo11 to identify genes necessary for the early stages of meiotic recombination. Additional studies demonstrated that *sae2* null strains were unable to remove Spo11 adducts covalently bound to the 5' DNA strand of a DSB, similar to the separation of function mutant *rad50S* [2] (Fig. 2).

In vegetatively growing yeast cells, Sae2 localizes to DNA breaks after localization of MRX, but coincides with MRX dissociation [44]. Sae2 does not require MRX or Tel1 to localize or associate with the DNA break [22]. In *sae2* deletion strains, MRX localizes at the break efficiently, but release from the break is delayed [45], similar to *rad50S* strains. Conversely, Sae2 removal from the break is delayed in *mre11* deletion strains and strains expressing the nuclease-deficient mutant *mre11-H125N* [22]. Overexpression of Sae2 accelerates the removal of MRX from DNA breaks. Overall, the

localization data is consistent with a role for MRX and Sae2 in DSB end processing. If the processing is not initiated, the proteins remain localized at the DSB.

Genetic assays *in vivo* also determined that Sae2 and the MRX complex function together in DNA repair. When a site-specific break was induced between inverted repeats in the yeast chromosome, duplications of the DNA between the repeats increased 10-fold in strains lacking *sae2* or containing *mre11-H125N*, or *rad50- K81I* (Rad50S) mutations, suggesting Sae2 and MRX are working together to repair DSBs, possibly by removing hairpin structures formed at the inverted repeats [46] (Fig. 4). Rattray et al. suggest that the 3' single-stranded DNA at a break might form a hairpin by utilizing small inverted repeats to fold back on itself. If the hairpin is not removed, the 3' end of the hairpin will prime replication and duplicate the region within the repeats, which was specifically observed in *mrx* and *sae2* mutant strains (Fig. 4).

The role of MRX and Sae2 in removing hairpin-capped ends was further demonstrated by Lobachev and Resnick (Fig. 5) Inverted repeats of 320 bp were inserted into one homologue of the LYS2 gene. The homologous chromosome contained a copy of the LYS2 with the 5' portion of the gene deleted. The inverted repeats form cruciform structures in vivo that must be processed correctly during replication to avoid large gene amplifications. The first step in processing leads to an MRX-independent cut at the base of the hairpin followed by MRX and Sae2-dependent removal of the hairpin. Once the hairpin is removed, homologous recombination with

the truncated LYS2 gene can proceed, leaving a functional gene. Deletions of Mre11, Rad50, Xrs2, or Sae2 were shown to lead to stabilization of the hairpin-ended chromsome fragments and to gene amplification [5].

Sae2 is also necessary for efficient DSB repair through the single-strand annealing pathway (SSA). If a DNA break occurs between direct repeats, the ends are processed back to the repeats and reannealed, resulting in deletion of the intervening sequence. *S. cerevisiae* strains lacking Sae2 or any of the MRX components leads to weak end-bridging and/or resection and, therefore, reduced SSA [47]. Strains expressing the *rad50S* (K81I) mutant display a slight defect in processing and can be rescued by over-expressing Sae2, suggesting Rad50 K81I might limit the ability of Sae2 to bind to MRX efficiently [48].

Recently, Bettina Lengsfeld in our lab purified the recombinant Sae2 protein and characterize its *in vitro* activities through extensive biochemical assays. She first found that Sae2 does bind double-stranded DNA in an MRX-independent manner. Deletion of amino acids 20-173 abolished DNA binding as did a Sae2 mutant containing the missense mutation G270D. This mutant was identified in a genetic screen for Sae2 alleles deficient in mitotic recombination (A. Rattray, pers. comm.) A mutant mimicking Tel1 phosphorylation (5D) had comparable DNA binding activity compared to the wild type protein, suggesting phosphorylation is not necessary for DNA binding [49].

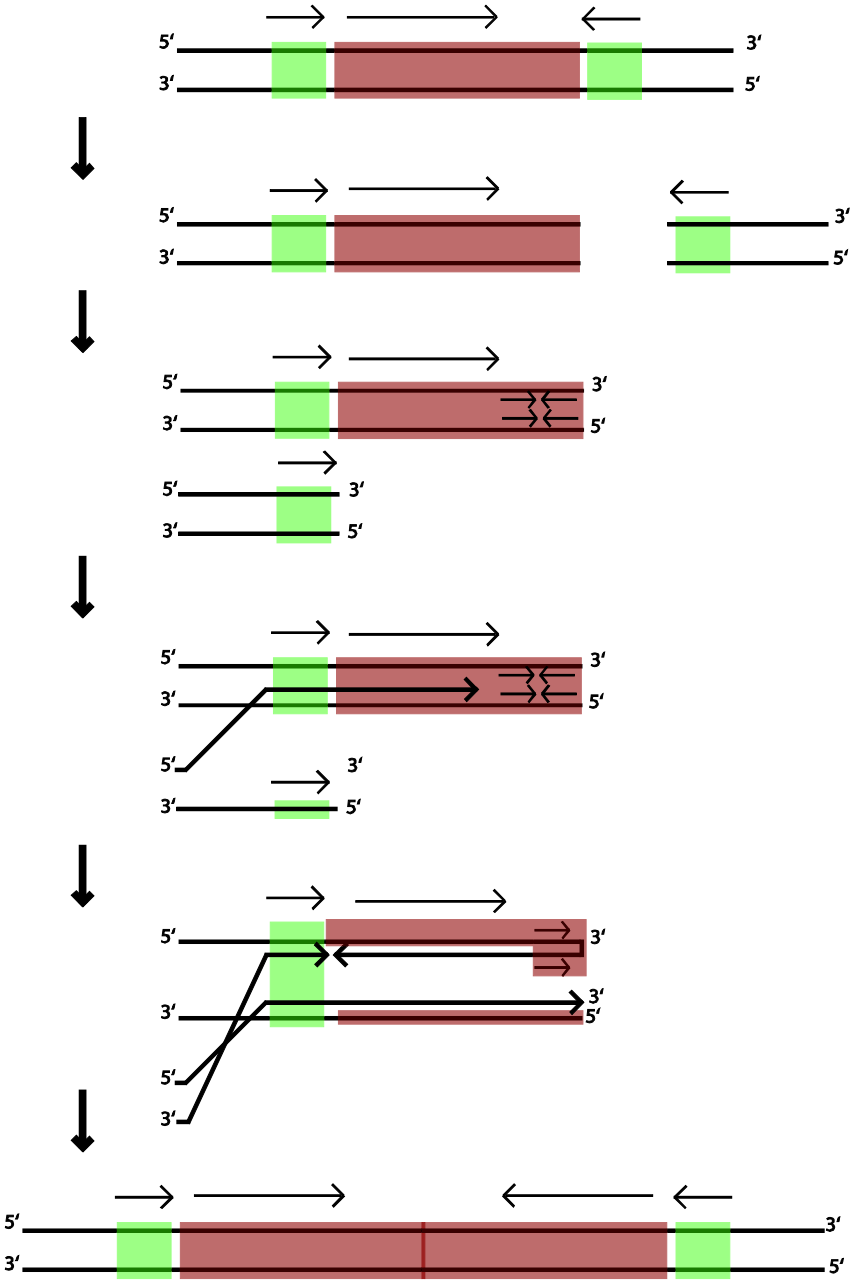


Figure 4: Formation of DSB between inverted repeats can lead to gene amplification. Fold back of the 3′ DNA end due to small inverted repeats can prime DNA synthesis. (Adapted from Rattray et al., 2006).

The recombinant Sae2 protein unexpectedly displayed both MRX-dependent and independent endonuclease activity. Recombinant Sae2 demonstrated MRX-independent endonuclease activity on single-stranded DNA and branched oligonucleotide substrates. However, MRX stimulated Sae2 endonuclease activity on hairpin structures. Contrary to prediction, Sae2 did not cleave the hairpin at the tip, but at the base of the hairpin where the single stranded DNA meets the double-stranded duplex. Mutant analysis revealed the N-terminal deletion (20-173), C-terminal deletion (250-345), missense mutation G270D, and phosphorylation restricted mutant 5A were unable to cleave the hairpin structure. Conversely, the Tel1 phosphorylation mimic (5D) was able to cleave the hairpin comparable to wild type [49] (Fig. 6). The evidence suggests Sae2 may work with MRX to process DNA ends after a DSB. We hypothesize that MRX unwinds the DNA end after a break and Sae2 cleaves the 5' strand at the single-stranded/double-stranded DNA junction to initiate resection.

MRX, Sae2, Exo1, and Sgs1 in DNA Resection

The Symington laboratory developed an assay to monitor resection via single-strand annealing (SSA) in a *rad51* null stain that eliminates homologous recombination. SSA requires resection to uncover homologous sequences that can be annealed together and repaired. Constructs were developed that contained a repeat of the *ade2* gene, one

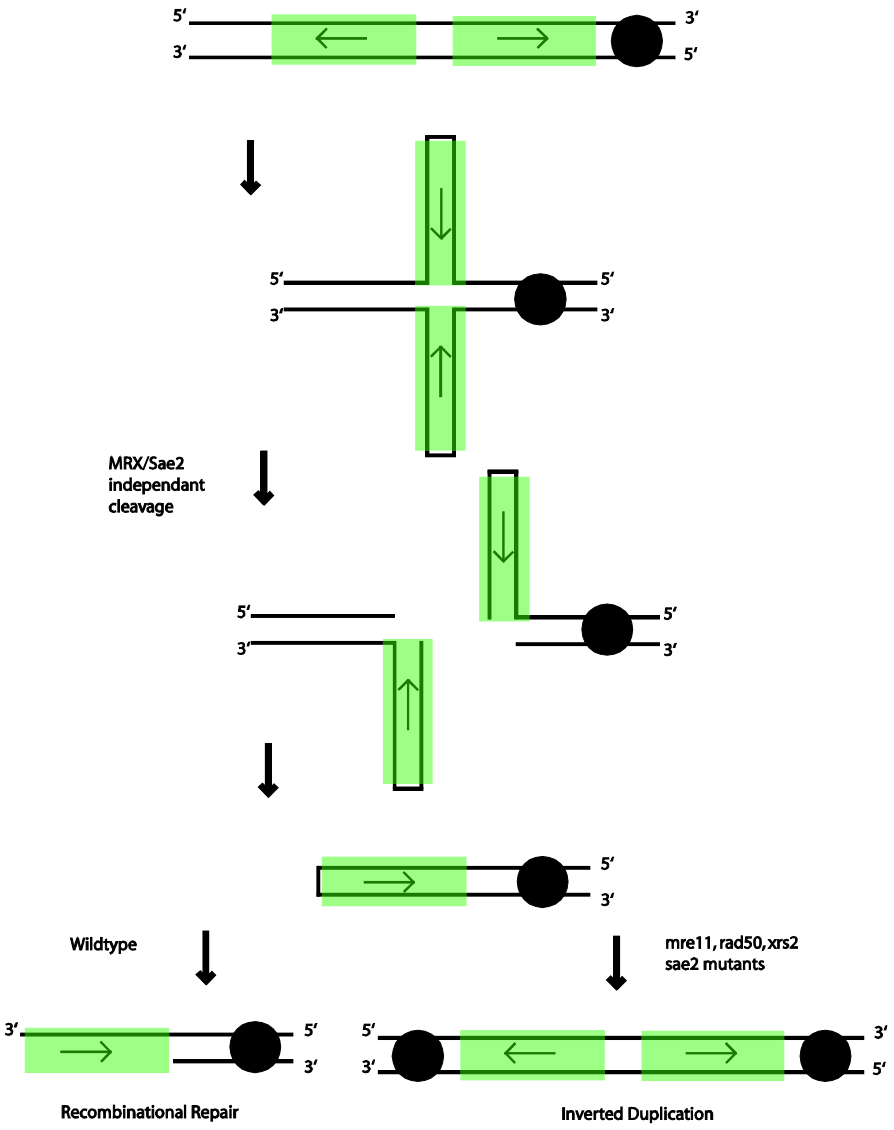
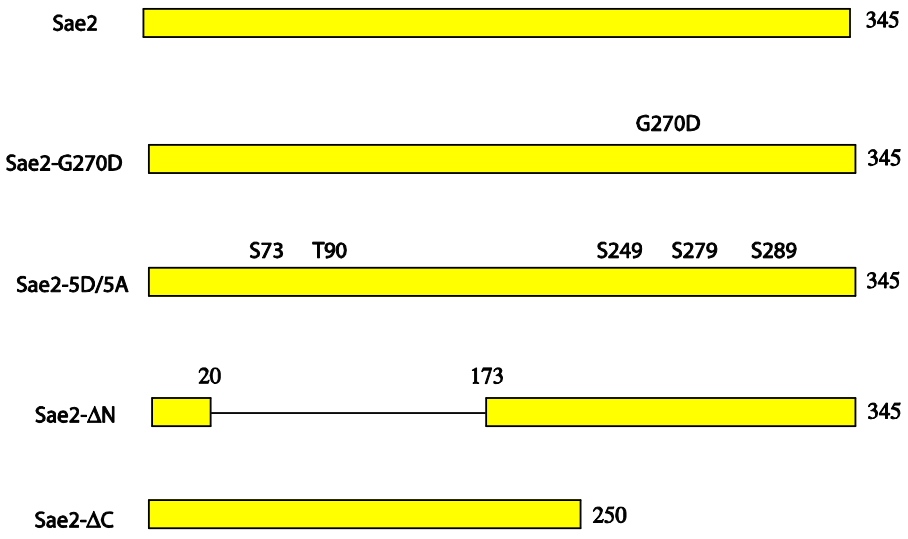


Figure 5: *Lys conversion assay*: 320bp inverted repeats were embedded into the LYS2 gene. Inverted repeats form a cruciform structure which is cleaved at the base in a MRX and Sae2 independent manner. It is likely that MRX and Sae2 process the hairpin-capped end to allow homologous recombination with the *lysΔ-5′* gene, creating a functional gene. (Adapted ftom Lobachev, 2002)

of which had a l-Sce1 restriction site. The strain also contained a GAL1p-l-SCE1 construct to control expression of the l-Sce1 restriction enzyme. After expression of the l-Sce1 restriction enzyme, by plating on YPRaf/Gal media, cells repair the site-specific break by SSA, resulting in a deletion of one of the *ade2* genes and reconstitution of wild-type ADE2. Kinetics of the SSA were analyzed by Southern blot analysis of genomic DNA digested with *Nhe1* and *Eag1* (Mimitou, 2008) (Fig. 7). When compared to *rad51* strains, *exo1/rad51* double mutants exhibited slightly delayed resection and a reduction of the final product. In *rad51/sae2* strains, DSBs persisted longer than in *rad51* strains. However, SSA products were still visible with wild-type kinetics. The *rad51/exo1/sae2* triple mutant exhibited an extreme defect in SSA, suggesting a defect in resection. Southern blot analysis was conducted on the samples using a probe complementary to the site of the DSB. e*xo1 rad51* strains were similar to *rad51* strains in the intiation of single-stranded DNA; however, the single-stranded product persisted. *sae2/rad51* strains demonstrated a delay in single-stranded DNA formation, suggesting a role in rate of the initiation of resection. A small amount of single-stranded product was visible, revealing that Exo1 can process DNA in the absence of Sae2 (Mimitou, 2008).



	DNA binding	ssDNA nuclease activity	hairpin nuclease activity	in vivo hairpin processing	MMS survival
Sae2	+++	+++	+++	+++	+++
Sae2-G270D	-/+	-/+	-	-	+
Sae2-5D	+++	+++	+++	+	+
Sae2-5A	++	++	-	-	+
Sae2-ΔN	-	-	-	-	+
Sae2-ΔC	++	+++	-	-	+

Figure 6: (A) Schematic representation of wild-type Sae2, Sae2-G270D, Sae2-5D, Sae2-5A, and Sae2-ΔN, and Sae2-ΔC genes. (B) *In vitro* and *in vivo* phenotypes of Sae2 and Sae2 mutants. (Adapted from Lengsfeld 2007)

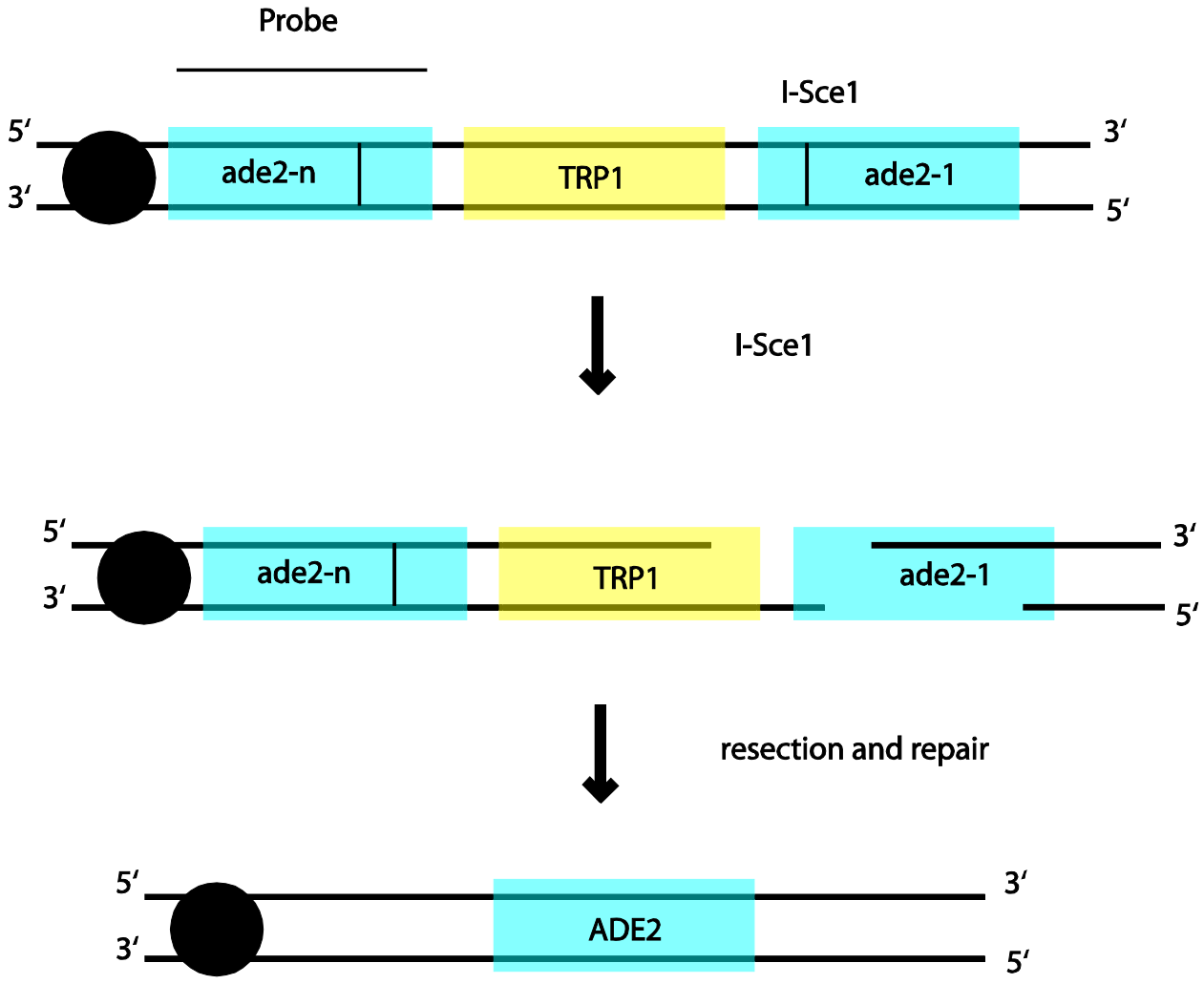


Figure 7: Contructs contain a repeat of the *ade2* gene, one of which had a I-*Sce1* restriction site. The strain also contained a GAL1p-I-SCE1 gene to control expression of the I-Sce1 restriction enzyme. After expression of I-*Sce1* restriction enzyme, by plating on YPRaf/Gal media, cells will repair the site specific break by SSA annealing, resulting in a deletion of one of the *ade2* genes and conversion to wild-type ADE2. Kinetics of the SSA were analyzed by Southern blot analysis of genomic DNA digested with Nhe1 and Eag1. (Adapted from Mimitou, 2008)

Hypothesis and Goals

Genetic analysis has identified many of the key genes involved in DSB processing in *S. cerevisiae*; however, the mechanism by which resection occurs has yet to be elucidated. Mutations in each of the components of the MRX complex, Sae2, and Exo1 display delayed 5' strand resection when deleted from the genome, although HR is completed with high fidelity. Sae2 and MRX are also necessary to remove Spo11 adducts from the 5' strand of a meiotic DSB to allow for proper resection. Recent *in vitro* analysis demonstrates Sae2 is an endonuclease, and along with fellow endonuclease MRX processes specific DNA structures such as single stranded DNA adjacent to a DNA duplex. Mre11 also has exonuclease activity, but the 3' to 5' polarity is inconsistent with physiological 5' to 3' resection. Furthermore, the expression of the nuclease-deficient mutant *mre11-H125N* combined with the deletion of *sae2* does not abolish resection in *S. cerevisiae,* suggesting the role of another enzyme(s).

The 5' to 3' exonuclease Exo1 is an excellent candidate for an enzyme involved in DSB resection. Double mutants lacking both Exo1 and Mre11 show extreme sensitivity to IR. Furthermore, over-expression of Exo1 in strains lacking *mre11, rad50*, or *xrs2* rescues the resection delay and suppresses the IR sensitivity of these strains.

Based on this evidence, it is hypothesized that resection occurs in two distinct steps in *S. cerevisiae*. First, MRX and Sae2 initiate resection, which is closely followed by

extensive resection by Exo1. It is proposed that MRX unwinds the DNA end at the break and allows Sae2 to cleave the 5' strand. Exo1 follows resection initiation and extends the 5' DNA strand resection. The goal was the biochemical characterization of MRX, Sae2, and Exo1 in DNA end resection. I used recombinant purified proteins to reconstitute resection *in vitro* and to determine the roles of the MRX complex, Sae2, and Exo1.

Chapter 2: Materials and Methods

Protein Expression

MRX complex

S. cerevisiae Mre11 and *mre11-3* expression constructs were created using Bac-To-Bac Baculovirus Expression Systems (Invitrogen). A construct containing wild-type Mre11 plasmid containing a C-terminal 6Xhistidine tag was digested with BamH1 and Nhe1 and was cloned into pFastBac1 at sites BamH1 and Spe1 creating plasmid pTP391. Plasmid pTP391 was transformed into DH10bac *E. coli* cells to create the bacmid pTP404. The *mre11-3* mutation (H125L and D126V) was cloned into pTP391 from pTP277 at restriction sites PpuM1 and Cla1 creating plasmid pTP483. Plasmid pTP483 was transformed into DH10bac *E. coli* cells to create the bacmid pTP484.

S. cerevisiae Rad50 expression constructs were created using the BD BaculoGold expression system (BDPharmingen). Plasmid pTP843 containing Rad50 with a C-terminal Flag-tag was digested with Nhe1 and Pst1 and ligated with pTP684 digested with Nhe1 and Pst1 creating plasmid pTP847. The *rad50-K81I* expression plasmid was created by mutating pTP847 by Quickchange mutagenesis (Stratagene) using primers

TP1906 and 1907 to create pTP1259. The *rad50-S1205R* expression construct was created by ligating pTP843 digested with Nhe1 and Pst1 into pTP687 to create pTP849.

S. cerevisiae Xrs2 expression constructs were created using Bac-To-Bac Baculovirus Expression Systems (Invitrogen). The N-terminal Flag epitope was removed from Xrs2 by digesting pTP617 with Sal1 and Xba1 and ligating the fragment into pFastBac1 to create pTP622. Plasmid pTP622 was transformed into DH10bac *E. coli* cells to create the bacmid pTP623.

Recombinant baculovirus was prepared from the bacmids or transfer plasmids according to manufacturer's instructions (Invitrogen or BDPharmingen).

MR(X) complexes were coexpressed from baculovirus infection of Sf21 insect cells and harvested after 72 hours of infection. Cell pellets were flash frozen in liquid nitrogen and stored at -80°C.

Sae2

The wild-type Sae2 construct for E. coli expression, pExp566.gck, was a gift from Alison Rattray. Sae2 expressed as described previously by Bettina Lengsfeld [49].

Exo1

S. cerevisiae Exo1 and *exo1-D173A* expression constructs were gifts from the Liskay laboratory and contained C-terminal Flag epitope tags. An N-terminal 6Xhistidine tag was added to the genes by digestion with Nco1 and Kpn1 and ligation into pFastBacHTA (Invitrogen) to create plasmids pTP1281 and pTP1284, respectively. Plasmids pTP1281 and pTP1284 were transformed into DH10bac *E. coli* cells to create bacmids pTP1309 and pTP1310, respectively. Recombinant Exo1 and Exo1 D173A were expressed from baculovirus infection of Sf21 insect cells and harvested after 72 hours of infection. Cell pellets were flash frozen in liquid nitrogen and stored at -80°C.

hSSB1

Wild-type hSSB1, hSSB1-T117A, and hSSB1-T117E proteins were expressed with N-terminal 6Xhistidine tags using expression constructs from constructs received from the Kum Kum Khanna laboratory. Plasmids were transformed into BL21 (EMD Biosciences) *E. coli* cells and given plasmid names pTP1106, pTP1105, and pTP1107, respectively. The *E. coli* strains were grown to an O.D. 600 of 0.8 and induced overnight with 0.1 mM IPTG at 30°C.

Protein Purification

yMR, yMRX, hMRN, and Exo1

All steps were performed at 4°C. Cells were thawed, lysed by homogenization, and sonicated three times for 30 seconds in Ni A buffer (0.5M KCl, 50mM KH_2PO_4, 10% glycerol, 2.5mM imidazole, 20mM β-mercaptoethanol) containing 0.5%Tween-20 and Complete Mini EDTA-free Protease inhibitor cocktail tablets (Roche). The lysate was centrifuged for 1 hour at 35,000 RPM at 4°C. The supernatant was removed and loaded onto a column containing ~ 5 ml Ni-NTA resin (Qiagen). The resin was washed with 50 ml Ni A buffer followed by 50 ml low-salt Ni A buffer (as above except containing 50 mM KCl) and then with 50 ml low-salt Ni A buffer containing 30mM imidazole. The protein was eluted with low-salt Ni A buffer containing 200mM imidazole and applied to a SP-Sepharose column (GE-Healthcare). The resin was washed with 50 ml A buffer (25mM Tris pH 8.0, 100mM NaCl, 10% glycerol, 1mM DTT) and eluted with 10ml 80% B buffer (25mM Tris pH 8.0, 1M NaCl, 10% glycerol, 1mM DTT). The elution was applied to a column containing ~ 1 ml M2 anti-Flag antibody-conjugated agarose resin (Sigma). The resin was washed with 10 ml A buffer, and the protein was eluted with 0.1 mg/ml Flag peptide (Sigma) in A buffer. The peptide was allowed to flow into the column, paused for 30 min, then the elution was continued. The protein was collected in 0.5 ml fractions, aliquoted, flash frozen in liquid nitrogen, and stored at -80 °C.

Sae2

The purification of Sae2 was performed as previously described (Lengsfeld, 2007) except cells were lysed by french press.

Dimeric ATM

Recombinant dimeric ATM was prepared as previously described [29].

MRN

Purification of MRN was performed as previously described [50] except that the protein was further purified using anti-Flag antibody resin. Briefly, the cells were thawed, lysed by homogenization, and sonicated three times for 30 seconds in Ni A buffer. The lysate was centrifuged for 1 hour at 35,000 RPM at 4°C. The supernatant was removed and loaded onto a column containing ~ 5 ml Ni-NTA resin (Qiagen). The resin was washed with 50 ml Ni A buffer followed by 50 ml low-salt Ni A buffer (as above except containing 5mM KCl) and then with 50 ml low-salt Ni A buffer containing 30mM imidazole. The protein was eluted with low-salt Ni A buffer containing 200mM imidazole and applied to a 1 ml Hitrap Q-Sepharose column (GE-Healthcare). The resin was washed with 50 ml of A buffer (25mM Tris pH 8.0, 100mM NaCl, 10% glycerol, 1mM

DTT) and eluted with 10ml 80% B buffer (25mM Tris pH 8.0, 1M NaCl, 10% glycerol, 1mM DTT). The elution was applied to a column containing ~ 1 ml M2 anti-Flag antibody-conjugated agarose resin (Sigma). The resin was washed with 10 ml A buffer, and the protein was eluted with 0.1 mg/ml Flag peptide (Sigma) in A buffer. The peptide was allowed to flow into the column, then paused for 30 min, then the elution was continued. The protein was collected in 0.5 ml fractions, aliquoted, flash frozen in liquid nitrogen, and stored at -80 °C.

hSSB1

All steps were performed at 4°C. hSSB1 wild-type, T117E, and T117A proteins were expressed in BL21 cells (EMD Biosciences). Cells from 1 liter of culture were lysed in Ni A buffer containing 0.1 mg/ml lysozyme, 5 mM EDTA, and Complete Mini EDTA-free Protease inhibitor cocktail tablets. The lysate was centrifuged at 35,000 rpm for 1 hour. The supernatant was applied to Ni-NTA Superflow resin (Qiagen). The resin was washed with Ni A buffer and eluted with Ni A buffer containing 212 mM imidazole. The eluted fractions were loaded onto a 1 ml HiTrap Heparin HP column (GE-Healthcare) and washed with Buffer A. hSSB1 protein was eluted with Buffer B (25 mM Tris pH 8.0, 1 M NaCl, 1 mM DTT, and 10% glycerol). The most concentrated fraction was further purified by gel filtration using a Superdex 200 column (GE). The fractions containing hSSB1 were aliquoted and stored at -80 °C.

GST-p53

All of the GST fusion proteins were expressed in *the E. coli* strain BL21 Codonplus (Stratagene). Cells were subcultured in LB at 30°C and induced with 1 mM IPTG for 4 hours before harvesting. The GST-fusion proteins were purified identically to the GST-Brca1 fragments previously described [51]. The GST-p53 protein was further purified by separation on a Superdex 200 gel filtration column (Amersham) in buffer A.

Proteins were quantified by staining SDS-PAGE visualized by Colloidal staining (Invitrogen). Protein concentrations were determined on the Odyssey system (Licor) against BSA protein standards.

Resection Assay

Reactions were performed in a volume of 10 µl with 25mM MOPS, pH 7.0, 1mM DTT, 50mM NaCl, 10mM $MgCl_2$, and 1mM ATP as indicated. The DNA substrate consisted of pNO1 (TopoGEN) digested with *Sph1*. The plasmid was digested overnight and purified using Micro Bio-Spin 6 Chromatography Columns (Bio-Rad). Combinations of MR(X), Sae2, and Exo1 were incubated with DNA containing reaction mix at 37°C for 30 minutes unless otherwise stated. Reactions were stopped with 0.2% SDS, 10mM EDTA, and 100 ng of proteinase K per reaction and were incubated at 37°C for an additional 15

minutes. Reactions were then incubated for 65°C for 5 minutes. The reaction was separated on a 1% native agarose gel, unless otherwise stated, for 12 hours at 1.6V/cm. The gel was either stained with SYBR-green DNA stain (Invitrogen) or assayed by non-denaturing Southern blot analysis (see below).

Template Plasmids for RNA Probe Construction

A T7 promoter from pETDuet (Novagen) was inserted into pNO1 between restriction sites *EagI* and *Sal1*, creating pTP1372. pTP1372 was digested with *HindIII* and served as the template for production of the RNA probe complementary to the 3' strand. A T7 promoter from pETDuet was also inserted into pNO1 between restriction sites *ClaI* and *HindIII*, creating pTP1408. pTP1408 was digested with *Sph1* and served as the template for production of the RNA probe complementary to the 5' strand.

Non-Denaturing Southern Blot analysis of resection

Resection reactions were separated using native agarose gel electrophoresis on 1% agarose gels which were run at 1.6 V/cm for 12 hours. Gels were soaked in 20XSSC (3M NaCl, 0.3M sodium citrate) with shaking at room temperature, and 3 changes of buffer over 3 hours. Gels were transferred onto Genescreen Plus Transfer Membrane (Perkin Elmer) in 20XSSC for 12 hours by capillary action. DNA was UV crosslinked to the nylon membrane and prehybridized in buffer containing (50% formamide, 5X Denhardt's

solution, 0.5 % SDS, 6XSSC and 0.2 mg/ml salmon sperm DNA). Membranes were probed with a 1 Kb RNA probe complementary to either the 3' or 5' strand of the DNA substrate flanking the *Sph1* restriction site. The probes were internally labeled with [α-^{32}P] CTP (NEN) using the Riboprobe System-T7 (Promega).

Exonuclease Inhibiton Assay

Reactions were performed in 10 µl with 25 mM MOPS, 2mM DTT, 50mM NaCl, and 5mM $MgCl_2$. The exonuclease assays were performed with oligonucleotide TP74 annealed to TP124 with TP74 labeled on the 5' end with [□-^{32}P] ATP using T4 polynucleotide kinase (NEB) [23]. MRN and 6-(4-hydroxyphenyl)-2-thioxo-2,3-dihydro-4(1H)-pyrimidinone (mirin,1) were incubated with DNA-containing reaction mix at 37°C for 30 minutes. Reactions were stopped with 0.2% SDS and 10 mM EDTA, dried, and resuspended in formamide loading buffer. The reactions were resolved in 20% denaturing polyacyralmide gels containing 7.5 M urea, and 1XTBE. Gels were analyzed by phosphorimager (Molecular Dynamics).

hSSB1 and MRN Interaction Assay

MRN containing 50 ng of biotinylated NBS1 was incubated with 5 ul of Streptavidin MagneSphere Paramagnetic Particles (Promega) in 60 ul of buffer A containing 0.1 %

CHAPS for 1 hour at room temperature. Beads were isolated and placed in a fresh 1.5 ml microcentrifuge tube. 130 ng of hSSB1 wild-type, T117E, or T117A protein, in 80 ul of buffer A (25 mM Tris pH 8.0, 100 mM NaCl, 1 mM DTT, 0.1 % CHAPS, and 10% Glycerol), was incubated with the MRN-bound beads for 30 min. at room temperature. The beads were again isolated and washed three times with buffer A containing 0.1 % CHAPS, and the bound protein was eluted with 2X SDS loading buffer. Elutions were run on a 10% SDS-PAGE gel and blotted on to Millipore PVDF immobilon-FL membrane for 8 hours at 200 mA. The membrane was incubated with anti-hSSB1 sheep serum (gift from Kum Kum Khanna) and detected with the Odyssey system (Licor).

ATM Kinase Assay

ATM kinase assays were performed as previously described [29] in kinase buffer: 50 mM HEPES, pH 7.5, 50 mM potassium chloride, 5 mM magnesium chloride, 10% glycerol, 1 mM ATP, and 1 mM dithiothreitol (DTT) for 90 min. at 30°C in a volume of 40ul. 10 ng DNA was used in each reaction with GST-p53 as the phosphorylation substrate.

Chapter 3: The Roles of MRX, Sae2, and Exo1 in 5' to 3' DNA End Resection

Introduction

In *S. cerevisiae*, homologous recombination is the dominant pathway of DNA repair. The MRX complex, Sae2, and Exo1 play critical roles in homologous recombination and have been extensively studied *in vivo*. Removing any of the proteins results in delayed and less efficient resection [7](Longhese, Mimitou, 2008). Double mutants lacking Exo1 and Mre11 show extreme sensitivity to ionizing radiation (IR), while over-expression of Exo1 in strains lacking *mre11, rad50,* or xrs2, rescues the resection defect [41].

In meiosis, *mre11S* and *rad50S* mutants are completely deficient in 5' resection and leave Spo11 covalently attached to the 5' strands of DNA breaks [2]. Similar to *mre11S* and *rad50S* mutants, *sae2* deletion strains fail to resect 5' strands at meiotic DSBs and accumulate covalent Spo11 adducts [3; 4]

Consistent with the *in vivo* evidence that Sae2 and MRX cooperatively process hairpin-capped DNA ends in *S. cerevisiae* [5; 6], recent studies by Bettina Lengsfeld in our laboratory demonstrated that Sae2 and MRX work together to cleave hairpin structures and branched DNA at single-strand/double-strand junctions [49]. Deletion mutants and

missense mutants of Sae2 were also characterized that displayed reduced DNA processing both *in vivo* and *in vitro.*

The Symington laboratory recently showed that resection occurs in two stages. Sae2 and MRX are involved in resection initiation followed by extensive resection by Exo1 (Mimitou, 2008). To demonstrate this, Mimitou et al. examined the genetic requirements for single-strand annealing (SSA), which requires resection to uncover homologous sequences that can be annealed together and repaired. When compared to *rad51* strains, *exo1 rad51* double mutants exhibited delayed resection and a reduction of the final SSA product. In *rad51/sae2* strains, unresected DSBs remained longer than in *rad51* strains. However, SSA products were visible with wild-type kinetics. The *rad51/exo1/sae2* triple mutant exhibited an extreme defect in SSA, suggesting a defect in resection. Southern blot analysis was also conducted on the samples using a probe complementary to the site of the DSB. *exo1 rad51* strains were similar to *rad51* strains in the intiation of single-stranded DNA; however, the single-stranded product persisted longer before product formation. *sae2 rad51* strains demonstrated a delay in single-stranded DNA formation, suggesting a role in the initiation of resection. A small amount of single-stranded product was visible, revealing that Exo1 can process DNA in the absence of Sae2 (Mimitou, 2008).

With the goal of characterizing the mechanism of DSB resection in *S. cerevisiae*, we expressed and purified recombinant MRX complex, Sae2, and Exo1 for biochemical

analysis. We developed an assay to visualize 5' to 3' DNA end resection *in vitro* for the purpose of characterizing DNA processing by MRX, Sae2, and Exo1. Our results indicate all three proteins contribute to 5' DNA processing. MRX and Sae2 work cooperatively to remove a short section of the 5' strand at a DNA break. MRX and Sae2 both stimulate Exo1 5' to 3' exonuclease activity for efficient DNA end resection.

Purification of Recombinant MR, MRX, and Exo1

The goal of our studies is the *in vitro* characterization of 5' to 3' DNA resection using recombinant purified *S. cerevisiae* MRX, Sae2, and Exo1. We used non-denaturing Southern blot analysis to visualize the products of our resection assay. This is an extremely sensitive assay and requires our recombinant proteins to be purified with a high level of homogeneity. To achieve the desired level of homogeneity, we developed more stringent conditions for purifying our MR(X) complexes and Exo1 than previously published. A protein purification strategy for Sae2 to completely eliminate trace levels of nuclease contaminants was already developed by Bettina Lengsfeld in our laboratory [49].

MR(X) complexes were purified by using a combination of affinity and ion exchange chromatography. In addition to the C-terminal 6Xhistidine tag on Mre11, a C-terminal FLAG tag was added to Rad50. The complexes were expressed in insect cells using

baculovirus expression system, and lysates were applied sequentially to Nickel-NTA, SP-Sepharose, and Flag affinity resins. After elution from the Flag resin with 100mM Flag peptide, the complexes were visualized on a 6% SDS-PAGE gel stained with Coomassie blue protein dye (Fig. 8A; 8B).

We obtained *S. cerevisiae* Exo1 and *exo1- D173A* baculovirus expression constructs from the Liskay laboratory. The Exo1 genes contained a C-terminal 6Xhistidine tag, but to create a more stringent purification strategy we added a Flag epitope affinity tag to the N-terminus. The Exo1 proteins were expressed in insect cells, and the lysates were applied stepwise to Nickel-NTA, SP-Sepharose, and Flag affinity resins. After elution from the Flag resin with 100mM Flag peptide the complexes were visualized on a 6% SDS-PAGE gel stained with coomassie blue protein dye (Fig. 8D). Recombinant Sae2 proteins were also purified according to utilizing Bettina Lengsfeld's purification strategy (Fig. 8C).

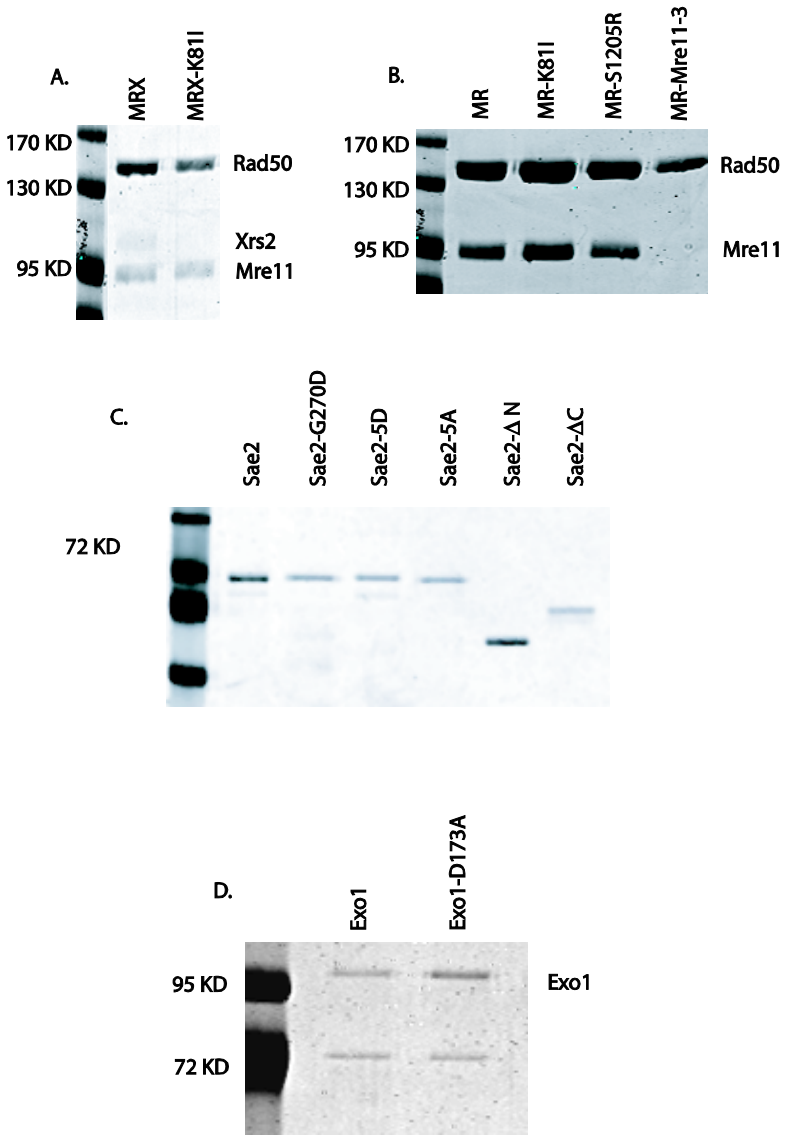


Figure 8: SDS-PAGE gels of recombinant proteins used in this study as visualized with Coomassie blue or Krypton protein stain (Pierce). (A) Coomassie blue stain of MRX and MRX-K81I. (B) Coomassie blue stain of MR, MR-K81I, MR-S1205R, and MR-Mre11-3. (C) Krypton stain of Sae2, Sae2-5A, Sae2-5D, Sae2-G270D, Sae2-ΔC, and Sae2-ΔN. (D) Coomassie blue stain of Exo1 and Exo1-D173A.

MR and Sae2 initiate 5' DNA End Resection

In vivo experiments have demonstrated that *sae2* null strains and separation of function mutants, *mre11S* and *rad50S*, are unable to remove 5' Spo11 conjugates and initiate 5' DNA end resection [2]. *In vitro* results showed a clear functional interaction between MRX and Sae2 and that they work together to process hairpin containing DNA structures [49]. We tested the possibility that MRX and Sae2 work together to process DNA ends at a double strand break.

To test this hypothesis Iincubated MR with Sae2 and a 6.5 Kb linearized double-stranded DNA for 1 hour at 37°C in the presence of $MgCl_2$. The reactions were stopped, and non-denaturing Southern blot analysis was performed with RNA probes either complementary to the 5' strand or the 3' strand (Fig. 9). The results demonstrated that MR is capable of resecting a small amount of the 5' strand of the DNA without the aid of Sae2 (Fig. 10; lane2). However, Sae2 stimulates the MR activity significantly (Fig. 10; lanes 5 and 6). Southern blot analysis proved the DNA end processing was in the 5' to 3' direction (Fig.10). The resection does not appear to be very extensive and we estimate, based on results with these proteins as well as with archaeal homologs of MR, that only about 50-100 nucleotides are being excised. We also tested MRX in our resection assay on a 4.5 Kb DNA substrate. Similar to MR, MRX can initiate resection but, only in the presence of 1 mM ATP (Fig. 11).

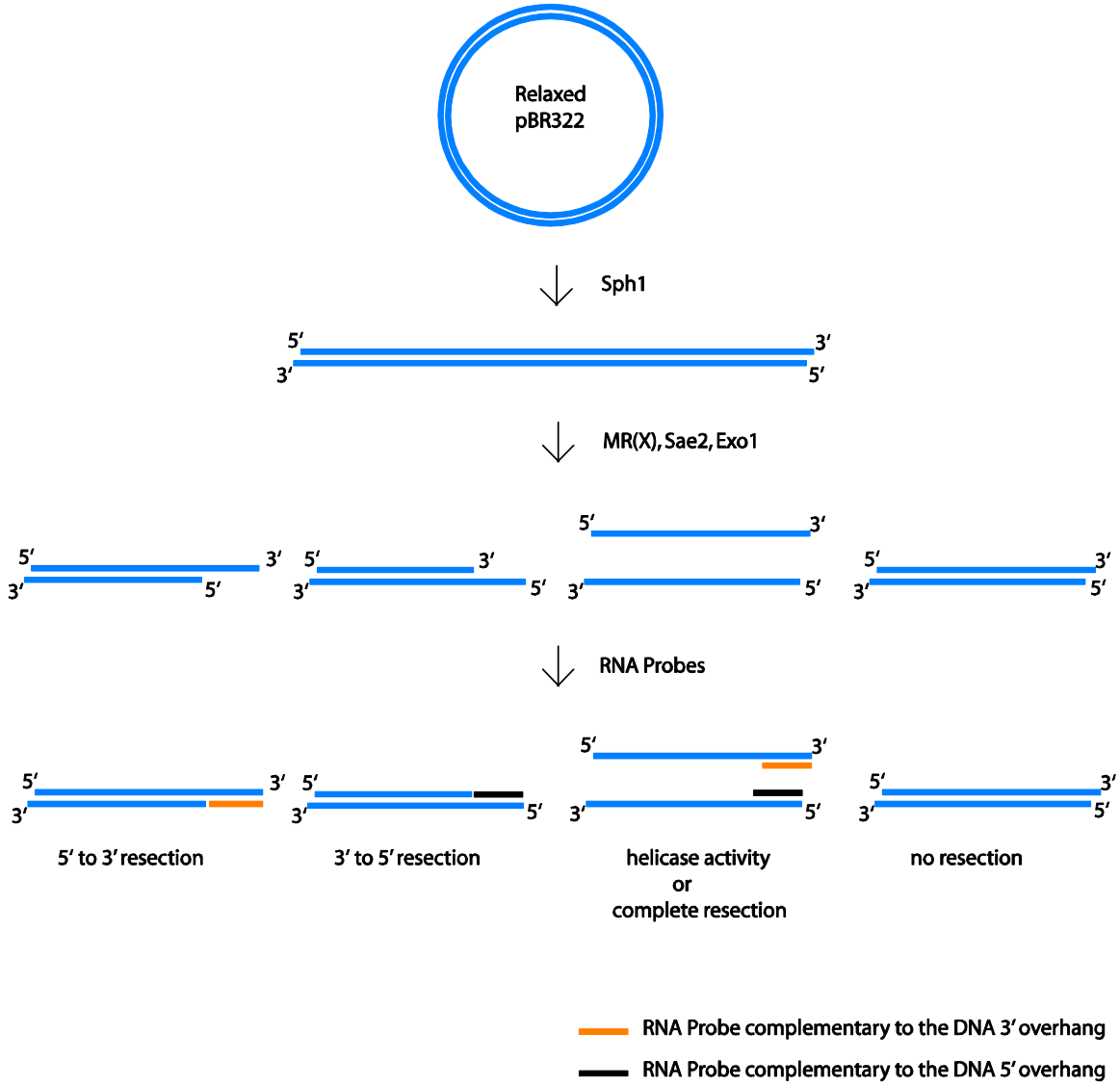


Figure 9: Schematic of the 5' DNA end resection assay. pBR322 was digested with *Sph1* and incubated with MR(X), Sae2, and Exo1, which can create a variety of resected intermediates,as shown. Results are transfered to a nylon membrane and probed with RNA probes complementary to 1 Kb of the 3' or 5' DNA strands at one end of the DNA.

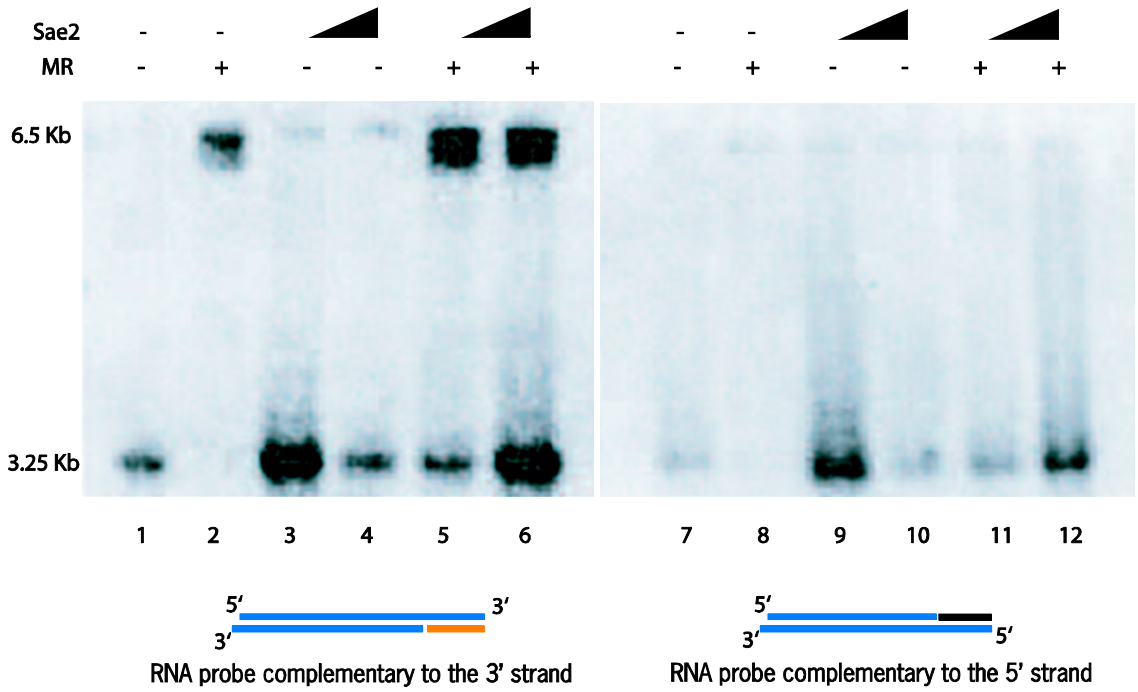


Figure 10: MR (15 nM Mre11) and Sae2 (2 and 4 nM) cooperatively resect the 5′ DNA strand. The proteins were incubated in 5mM $MgCl_2$ with a 6.5 Kb linearized DNA substrate. The reaction was incubated at 37°C for 1 hour. Non-denaturing Southern blot analysis was performed, and the blot was probed with either a 3′ or a 5′ complementary probe labeled internally with [α-^{32}P] CTP.

Sae2 mutants exhibit deficient resection initiation

S. cerevisiae strains expressing the *sae2-ΔC, sae2-ΔN, sae2-G270D, sae2-5A*, and *sae2-5D* mutants exhibit a recombination deficiency at sites of inverted repeats when compared to wild-type Sae2. Among these, *sae-5D* partially rescued recombination. The rest of the mutants did not rescue the recombination defect [49].

Recombinant proteins were incubated with double-stranded oligonucleotide substrates to determine DNA binding. Sae2-ΔC and Sae2-G270D are unable to bind the DNA substrate. The proteins were tested for nuclease activity on hairpin structures as well as double-stranded/single-stranded DNA junctions. Sae2-ΔC, Sae2-ΔN, Sae2-G270D, and Sae2-5A were unable to cleave the hairpin structure. In contrast, the Tel1 phosphorylation mimic Sae2-5D was able to cleave the hairpin comparable to wild type levels [49].

We assayed Sae2 mutants in the presence of MR in our resection assay. Both Sae2-ΔN and Sae2-ΔC showed a severe reduction in resection activity, although these mutants still act cooperatively with MR to create a small amount of resected product. Sae2-G270D displayed an intermediate phenotype, while Sae2-5D had activity comparable to wildtype Sae2 (Fig. 12). These results indicate that all of the mutants exhibit at least partial stimulation of MR, but only Sae2-5D shows activity similar to wild-type Sae2.

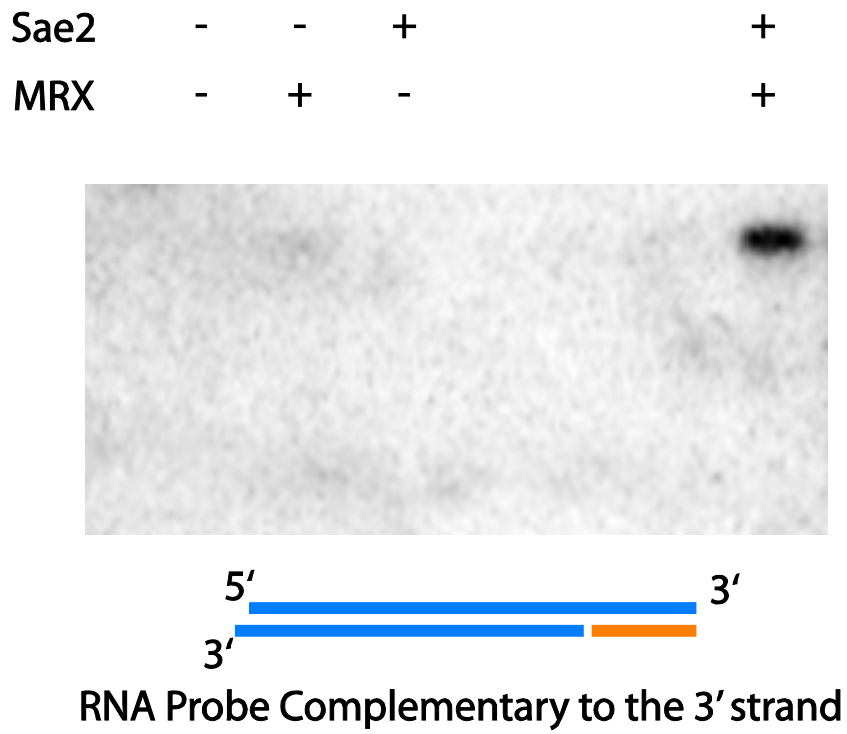


Figure 11: MRX (15 nM Mre11) and Sae2 (2 nM) cooperatively resect the 5′ DNA strand. MR has some resection initiation activity by itself. Resection activity is increased with the addition of Sae2. MRX and Sae2 resect in the 5′ to 3′ direction. The proteins were incubated in 5mM $MgCl_2$ and 1mM ATP with a 4.5 Kb linearized DNA substrate. The reaction was incubated at 37°C for 1 hour. Non-denaturing Southern blot analysis was performed, and the blot was probed with a 3′ complementary probe labeled internally with [α-^{32}P] CTP.

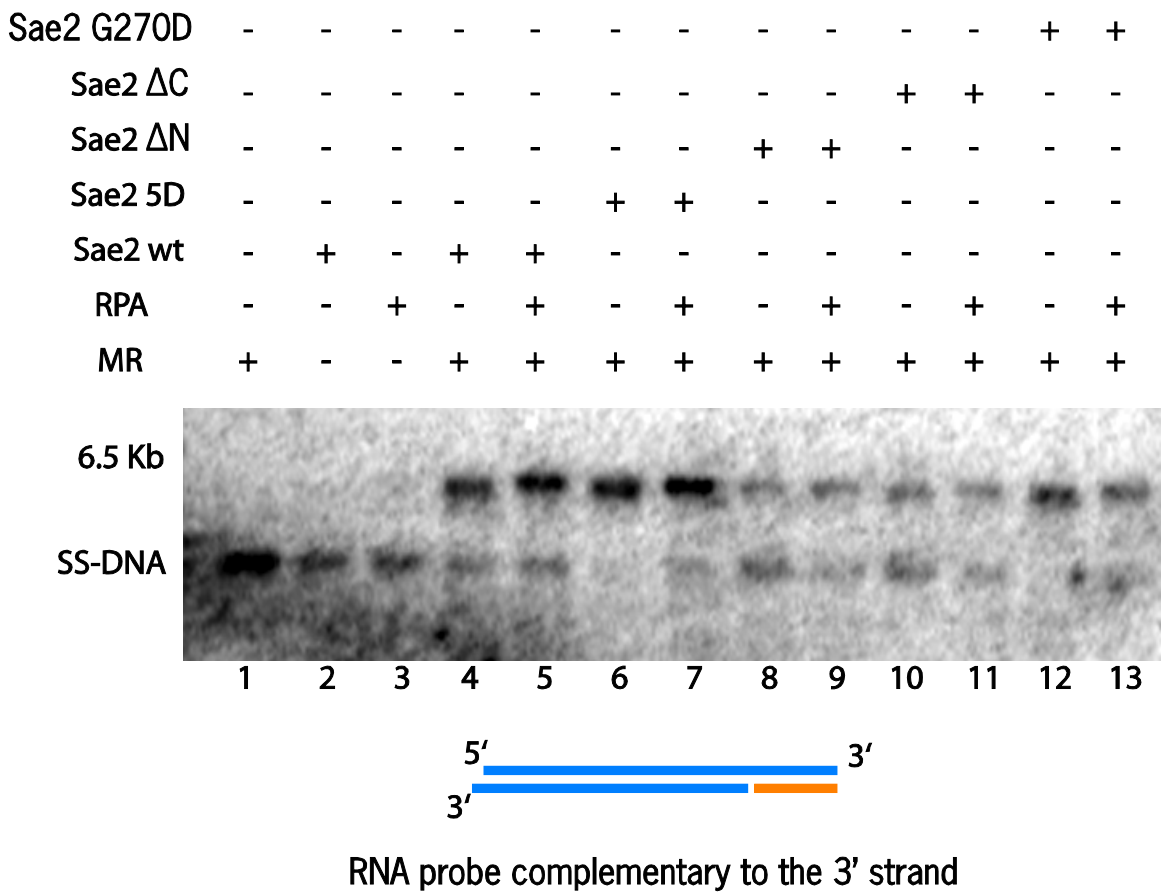


Figure 12: Sae2 mutants inhibit resection initiation. The proteins (15 nM Mre11, 2 nM Sae2 and Sae2 mutants) were incubated in 5mM $MgCl_2$ with a 6.5 Kb linearized DNA substrate. The reaction was incubated at 37 °C for 1 hour. Non-denaturing Southern blot analysis was performed, and the blot was probed with either a 3' or a 5' complementary probe labeled internally with [α-^{32}P] CTP.

MR, Sae2, and Exo1 in 5' DNA End Resection

Although MR and Sae2 are capable of initiating resection, it is clear that additional enzymes are necessary to complete the process. Recent evidence suggests that Exo1 may be the relevant 5' to 3' exonuclease activity involved in resection. Exo1 is induced during meiosis and deficient strains display delayed resection after HO endonuclease breaks, although homologous recombination is eventually completed with high fidelity [33; 38]. In vegetative cells, *exo1* null strains exhibit no delay or deficiency in resection, but *exo1 mre11* and *exo1 sae2* double mutants show an extreme delay and much lower levels of resected DNA products (Mimitou, 2008). To characterize the role of Exo1 in DNA end resection, protein was used in resection assays in combination with MR and Sae2. Results were detected using unlabeled DNA and a sensitive nucleic acid stain (SYBR-green).

These results demonstrated that Exo1 at the concentrations used here (Fig. 13) does not degrade linear DNA. However, DNA degradation was observedin the presence of Exo1 and MR (Fig.13, lanes 7 and 8) as well as Exo1 in combination with Sae2 (Fig.13, lanes 9 and 10). When all the components were included in the assay, degradation increased significantly (Fig.13, lanes11-14). Non-denaturing Southern blot analysis of the resection products showed similar

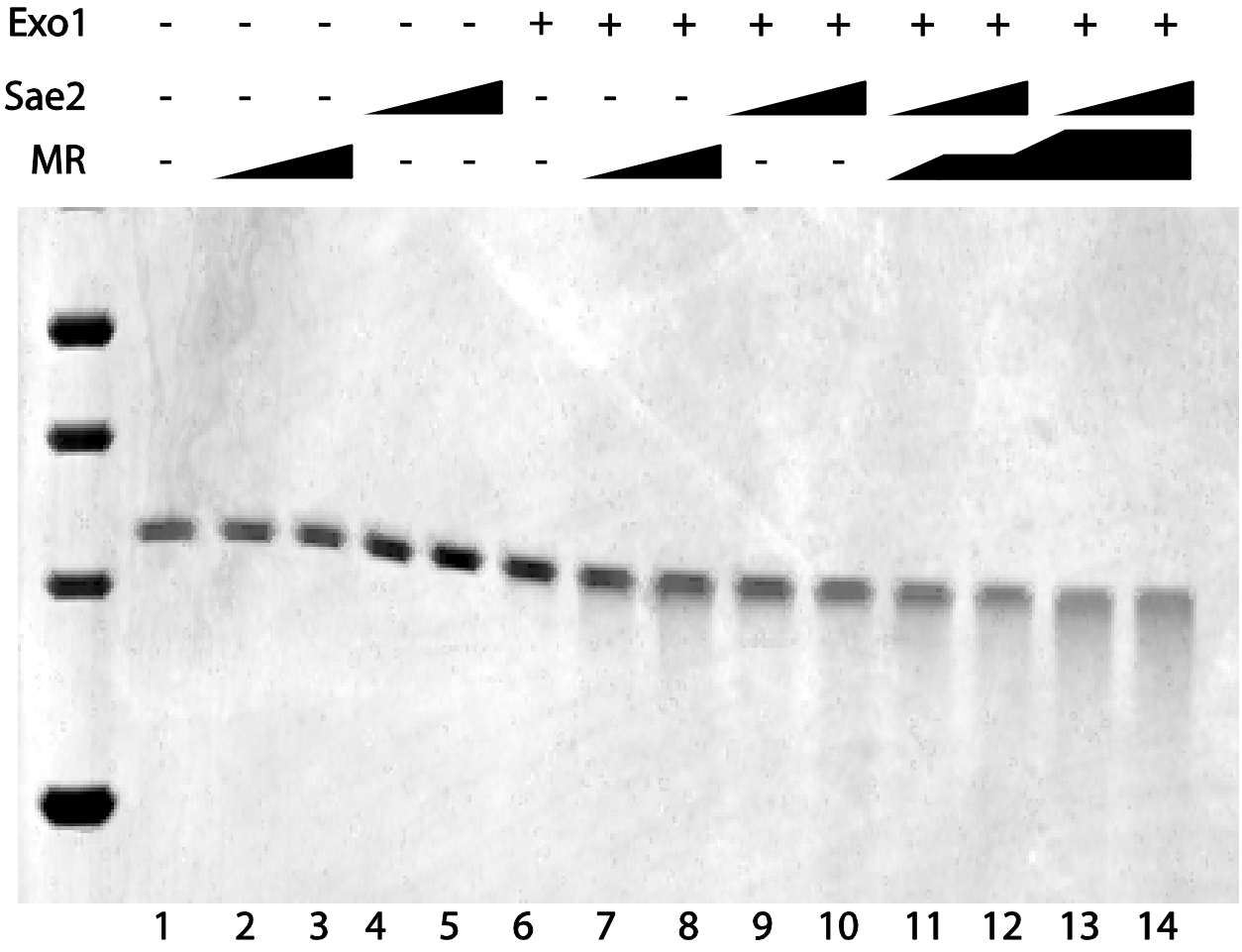


Figure 13: MR (30 and 60 nM Mre11), Sae2 (5 and 10 nM), and Exo1 (5 nM) cooperatively resect linear double-stranded DNA. Protein combinations were incubated as indicated in 10mM $MgCl_2$ with a 4.5 Kb double-stranded DNA substrate. The reaction was incubated at 37 °C for 1 hour. The reactions were separated on a native1% agarose gel and stained with SYBR-Green.

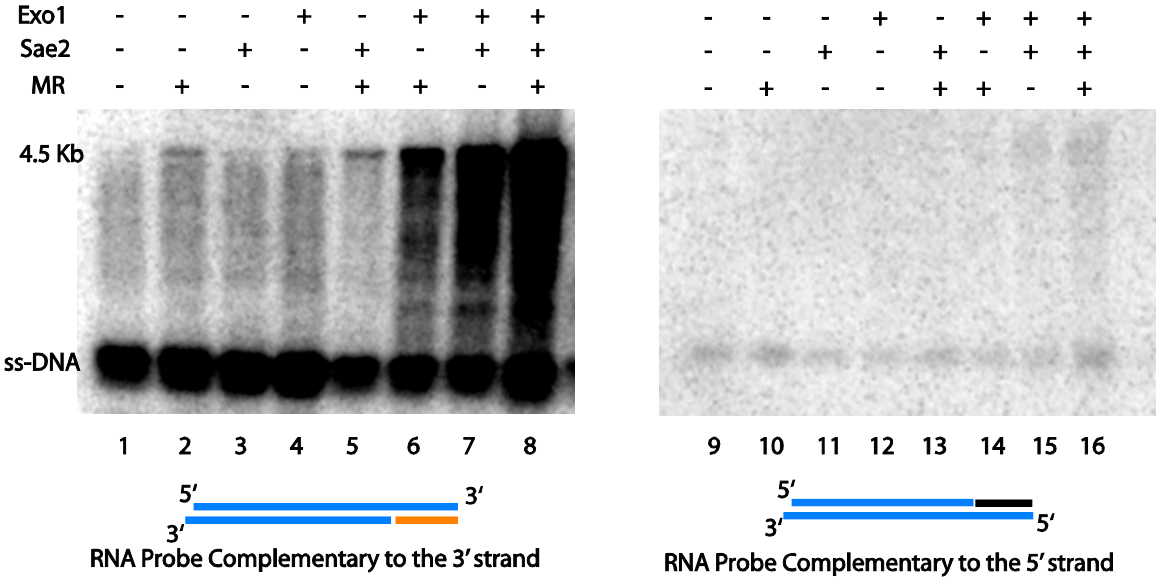


Figure 14: MR (15 nM Mre11), Sae2 (2 nM), and Exo1 (5 nM) cooperatively resect linear double-stranded DNA. Proteins were combined as indicated and incubated in 10mM $MgCl_2$ with a 4.5 Kb double-stranded DNA substrate. The reaction was incubated at 37 °C for 1 hour. Non-denaturing Southern blot analysis was performed, and the blot was probed with either a 3' or a 5' complementary probe labeled internally with [α-^{32}P] CTP.

results (Fig. 14). The presence of either Sae2 or MR stimulates extensive resection by Exo1 (Fig. 14, lanes 6 and 7). When all components were present in the reaction, the extent and efficiency of resection was increased (Fig. 14, lane 8). The results also demonstrated that MR, Sae2, and Exo1 components resect DNA in the physiologically appropriate 5' to 3' direction since we observe very minimal 3' to 5' degradation was observed (Fig. 14, right panel).

Rates of MR, Sae2, and Exo1 in 5' DNA End Resection

Resection is a relatively slow process *in vivo* [11]. The Haber laboratory discovered that physiological resection occurs at about 4.5 Kb per hour. To measure this, they constructed a plasmid with two lacZ genes with a 4.5Kb spacer between the repeats. SSA deletion products were monitored over time to assess the kinetics. Their results demonstrated that SSA deletion products were observed at about 1 hour [11]. When Sae2 or any of the MRX complex components are deleted from the *S. cerevisiae* genome, resection is delayed for up to 1 hour. Results with MR and Sae2 demonstrated that it takes 1 to 3 hours to initiate resection (data not shown). To determine the effect of Exo1 on the rate of resection I assayed MR, Sae2, and Exo1 in our resection assay in increments

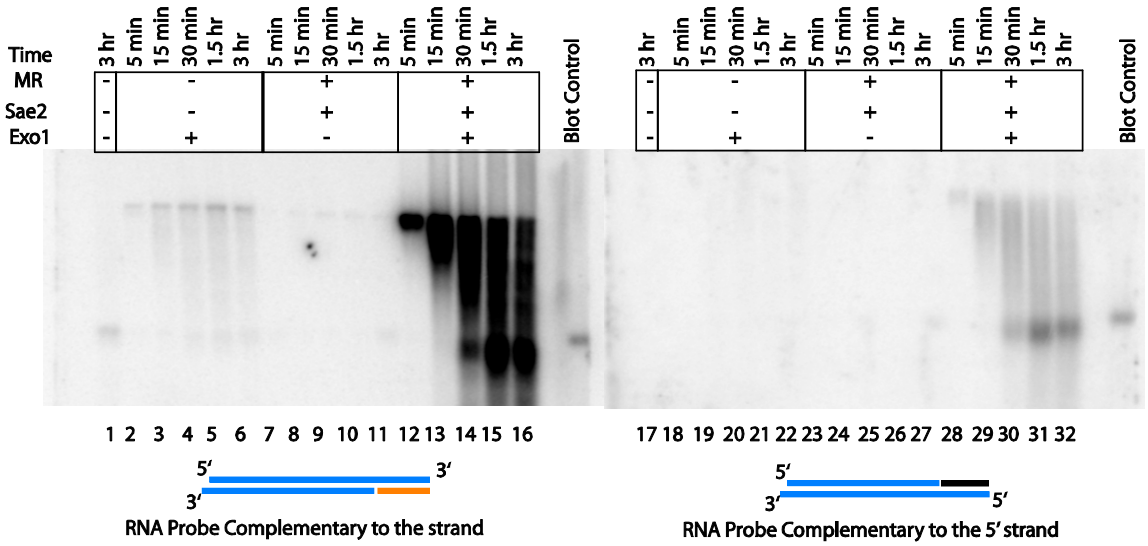


Figure 15: MR (15 nM Mre11), Sae2 (2 nM), and Exo1 (5 nM) cooperatively exhibit extensive resection of the 5′ DNA strand. Exo1 is capable of resecting the 5′ DNA strand by itself. MR and Sae2 initiate resection but fail to process the DNA extensively. MR, Sae2, and Exo1 incubated together resect the 5′ DNA strand extensively. The proteins were incubated as indicated in 10mM $MgCl_2$ with a 4.5 Kb double-stranded DNA substrate. The reactions were incubated at 37 °C for 5 minutes, 15 minutes, 30 minutes, 1.5 hours, and 3 hours. Non-denaturing Southern blot analysis was performed, and the blot was probed with either a 3′ or a 5′ complementary probe labeled internally with [α-^{32}P] CTP.

of 5 minutes, 15 minutes, 30 minutes, 1.5 hours, and 3 hours. Results demonstrate that Exo1 increases the rate of resection dramatically. Resection can be visualized as early as 5 minutes, progresses rapidly in the first 30 minutes, and is nearly complete by 1.5 hours (Fig. 15). Results showed that initial processing with MR and Sae2 begins around 0.5 hours, but is very inefficient (Fig. 15). Exo1 also is capable of resecting DNA ends by itself, although the efficiency is also much reduced (Fig.15).

Sae2 Mutant Analysis with MR and Exo1 in 5' DNA End Resection

The Sae2 mutants were assayed in the presence of MR and Exo1 to determine if the mutations affect the resection process. Resection initiation was reduced in the presence of Sae2-ΔC, Sae2-ΔN, and Sae2-G270D in comparison to wild-type Sae2 (Fig. 16). The component proteins were incubated as previously mentioned except that the reactions were stopped after 30 minutes to look at an early time point in the process. These results demonstrate that with Exo1, the Sae2-ΔC and Sae2-ΔN mutants show a strong defect in resection in the presence of Exo1 only and in the presence of both Exo1 and MR (Fig. 16, lanes 9-16). Sae2-G270D, Sae2-5A, and Sae2-5D display an intermediate deficiency in DNA end resection in the presence of Exo1 and in the presence of Exo1 and MR (Fig. 16, lanes 17-28).

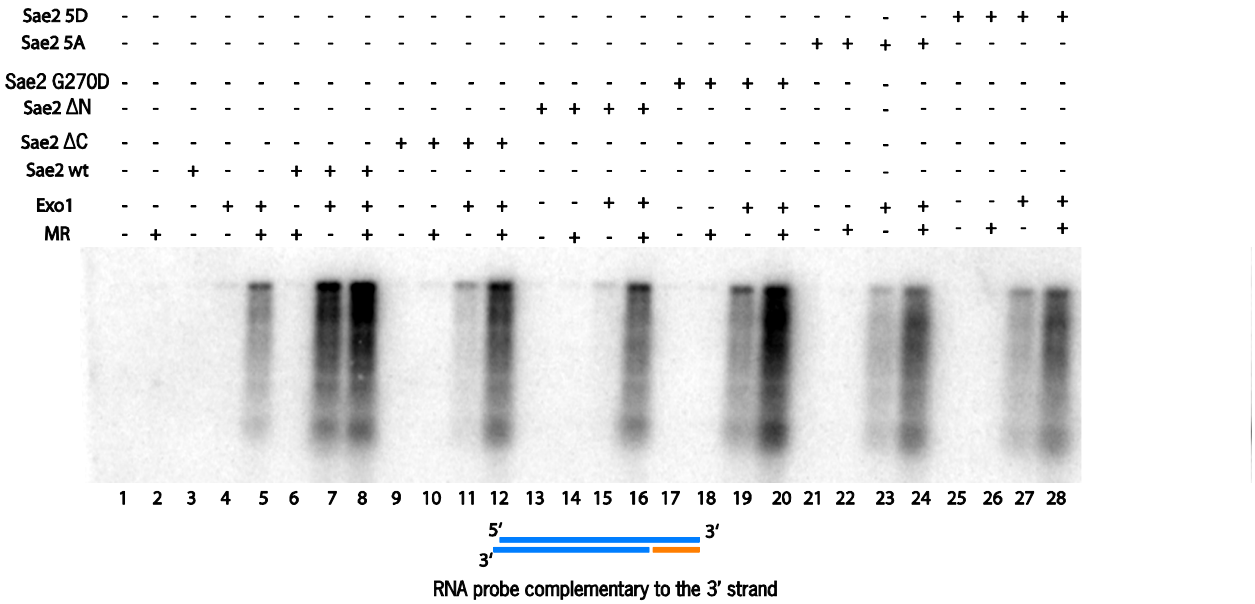


Figure 16: Sae2 ΔN and Sae2 ΔC do not stimulate resection while Sae2 5A, 5D, G270D have partial activity with Exo1. Proteins were incubated as indicated in 10mM MgCl2 with a 4.5 Kb double-stranded DNA substrate. The reaction was incubated at 37 °C for 0.5 hours. Non-denaturing Southern blot analysis was performed, and the blot was probed with a 3' complementary probe labeled internally with [α-^{32}P] CTP. Protein concentrations are consistent with previous levels (15 nM Mre11, 2 nM Sae2, and 5 nM Exo1).

MR Mutant Analysis with Sae2 and Exo1 in 5' DNA End Resection

In meiosis, a specific class of hypomorphic mutants of Mre11 and Rad50 (Rad50S) are completely deficient in 5' resection and leave Spo11 covalently attached to the 5' strands of DNA breaks [2]. The Mre11 nuclease-deficient mutant H125N is also unable to remove Spo11 attached to the 5' strand of the DNA break, which stalls resection [32]. Bettina Lengsfeld in our laboratory previously showed that the Mre11-3 mutant protein is inactive as an exonuclease or endonuclease *in* vitro [49], consistent with previous observations with this mutant [32; 33]. However, Mre11 nuclease activity does not appear to be required for efficient DSB resection *in vivo* in vegetatively growing cells [32]. To test the requirement for Mre11 nuclease activity in DNA processing, we purified the recombinant mutant protein complexes and tested them in our resection assay. The Mre11-3 mutant appears to be able to initiate resection but extensive resection is abolished (Fig. 17 lanes 14-16). However, Mre11-3 does not form a stable complex efficiently. Therefore, we will have to develop a better strategy is needed to improve the stability of Mre11-3 in the complex. Rad50-S1205R displayed the most deficient phenotype with almost complete abrogation of resection (Fig. 17, lanes 11-13). Finally, the Rad50 –K81I mutant exhibits only an intermediate resection deficiency (Fig. 17 lanes (8-10). This data appears to be consistent with previous *in vivo* evidence showing that Rad50S exhibits a subtle resection defect [7] while theS1205R mutant is equivalent to the *rad50* null mutation [52].

MRX with Sae2 and Exo1 in 5' DNA End Resection

To this point we have focused on the MR complex to utilize 5' DNA end resection, in part because MR wild-type and mutant complexes (with the exception of Mre11 nuclease-deficient complexes) are easily expressed and purified. However, in *S. cerevisiae,* MR is associated with a third protein, Xrs2. Xrs2 is conserved among eukaryotes and is homologous to mammalian NBS1. Xrs2 functions in DNA damage signaling but its role in resection has yet to be elucidated. We purified recombinant MRX in order to analyze its function of Xrs2 in our *in vitro* resection assay and visualized the results with non-denaturing Southern blot analysis. Non-denaturing Southern blot analysis demonstrates that MRX is capable of stimulating Exo1 5' to 3' DNA end resection, and that the stimulation is slightly increased with the addition of Sae2 (Fig. 18).

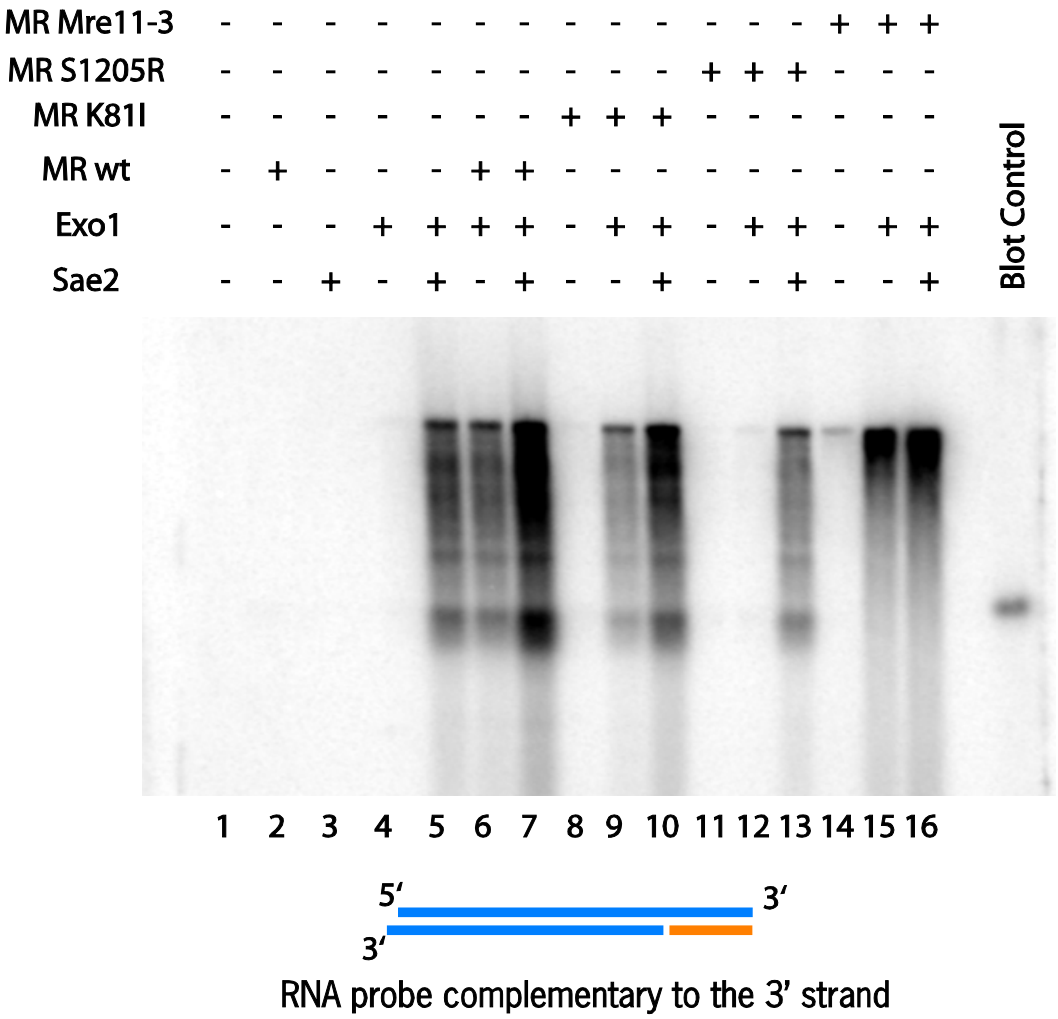


Figure 17: MR S1205R does not stimulate Exo1 resection, while MR Mre11-3 has partial activity with Exo1 and stalls shortly after resection initiation. MR-K81I stimulates Exo1 slightly less than wild-type MR. Proteins were incubated as indicated in 10mM MgCl2 with a 4.5 Kb double-stranded DNA substrate. The reaction was incubated at 37 °C for 0.5 hours. Non-denaturing Southern blot analysis was performed, and the blot was probed with a 3' complementary probe labeled internally with [α-^{32}P] CTP. Protein concentrations are consistent with previous levels (15 nM Mre11, 2 nM Sae2, and 5 nM Exo1).

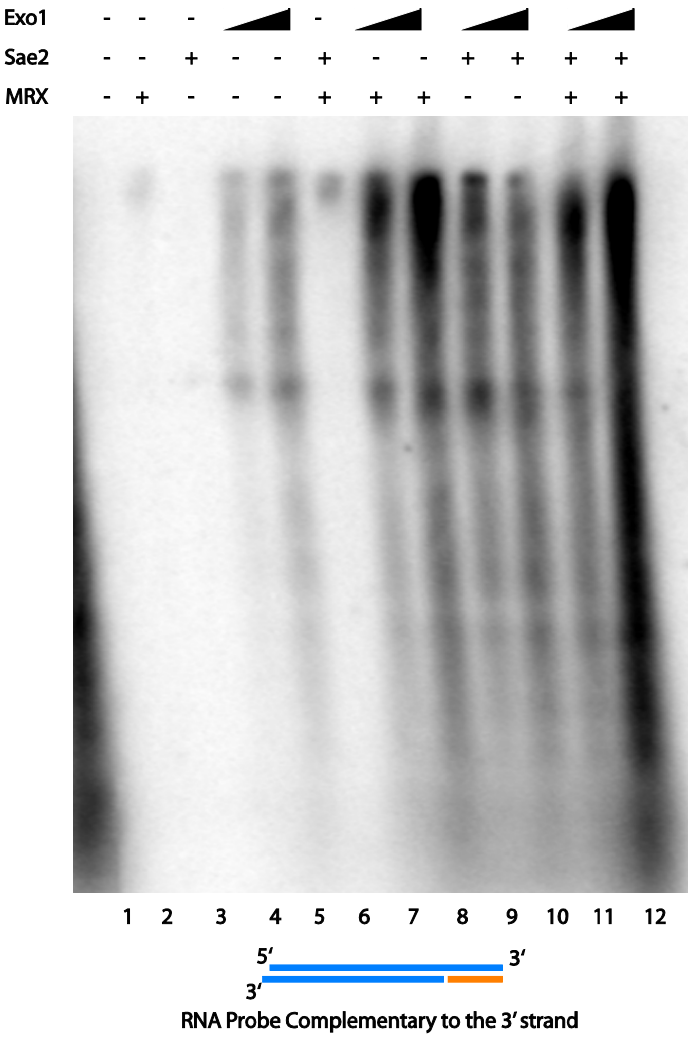


Figure 18: MRX (15 nM Mre11), Sae2 (2 nM), and Exo1 (5 and 10 nM) cooperatively resect double-stranded DNA. MRX and Sae2 can separately stimulate Exo1. The proteins were incubated as indicated in 10mM MgCl2, 1mM ATP, with a 4.5 Kb double-stranded DNA substrate. The reaction was incubated at 37 °C for 1 hour. Non-denaturing Southern blot analysis was performed, and the blot was probed with a 3' complementary probe labeled internally with [α-^{32}P] CTP.

MRX-K81I Does Not Stimulate Sae2 Resection Initiation

Specific mutations in Rad50, termed *rad50S*, fail to remove covalently attached Spo11 from the 5' DNA overhang after cleavage [2; 15]. Similar to *rad50S* mutants, *sae2* deletion strains accumulate Spo11 adducts. We incubated the MRX Rad50S-K81I mutant with Sae2 and Exo1 in our resection assay. Although MRX-K81I can stimulate Exo1 nuclease activity (Fig. 19, lanes 11,12), MRX-K81I is unable to stimulate Sae2 resection initiation (Fig. 19, lanes 5, 10). These results are consistent with *in vivo* evidence demonstrating that MRX-K81I cannot remove Spo11 5' conjugates from a meiotic DSB.

Exo1-D173A does not catalyze extensive resection

Exo1 mutant strains display delayed resection after HO endonuclease breaks, although homologous recombination is eventually completed with high fidelity [33; 38]. We purified recombinant Exo1 nuclease mutant Exo1-D173A to test for the requirement of Exo1 catalytic activity in resection. The Exo1-D173A mutation was previously shown to eliminate Exo1 activity *in vitro* [53]. We incubated Exo1-D173A in our resection assay with MRX and Sae2. Exo1-D173A is deficient in 5' to 3' DNA end resection (Fig. 20). However, Exo1-D173A is still

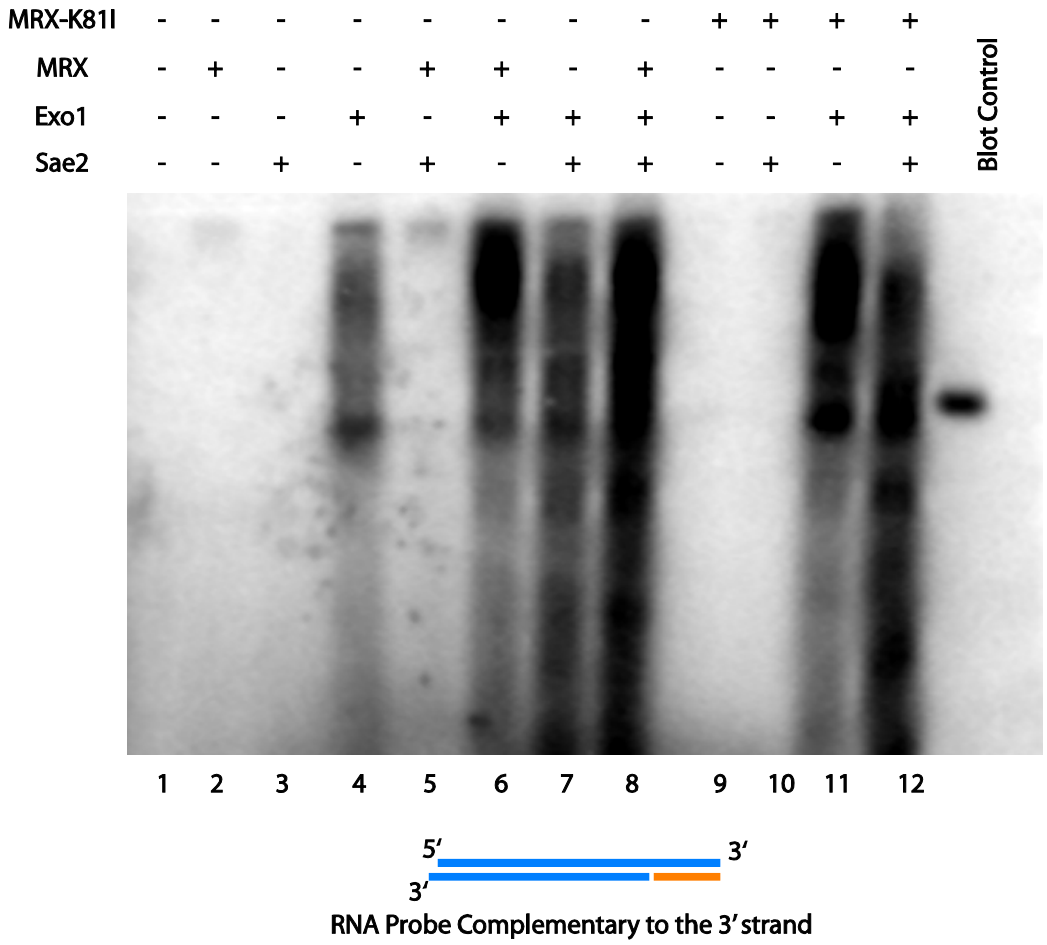


Figure 19: MRX-K81I does not stimulate Sae2-dependent resection initiation. MRX-K81I stimulates Exo1 nuclease activity. Proteins were incubated as indicated in 10mM MgCl2, +/- 1mM ATP, with a 4.5 Kb double-stranded DNA substrate. The reaction was incubated at 37 °C for 1 hour. Non-denaturing Southern blot analysis was performed, and the blot was probed with a 3′ complementary probe labeled internally with [α-^{32}P] CTP. Protein concentrations are consistent with previous levels (15 nM Mre11, 2 nM Sae2, and 5 nM Exo1).

able to stimulate resection initiation in the presence of MRX and Sae2 (Fig. 20, lanes 5, 12). This suggests that Exo1 plays two separate roles in DNA end resection. Initially Exo1 stimulates resection initiation by Sae2 and MRX in an Exo1 nuclease-independent manner. Next, Exo1 resects DNA extensively leaving a 3' strand for Rad51 catalyzed strand invasion.

MR, Sae2, and Exo1 prefer a 3' DNA Overhang in Resection

Sae2 cleaves a 5' flap structure only in the presence of a 3' strand [49]. We tested if the presence of an overhang influenced DNA 5' end resection. The DNA substrate pNO1 was digested with either restriction enzyme Sph1 to generate a 3' overhang, or with restriction enzyme Sal1 to generate a 5' overhang. These results demonstrate that MR, Sae2, and Exo1 prefer a 3' overhang to initiate resection, which we have used in the substrate DNA throughout our experiments (Fig. 21).

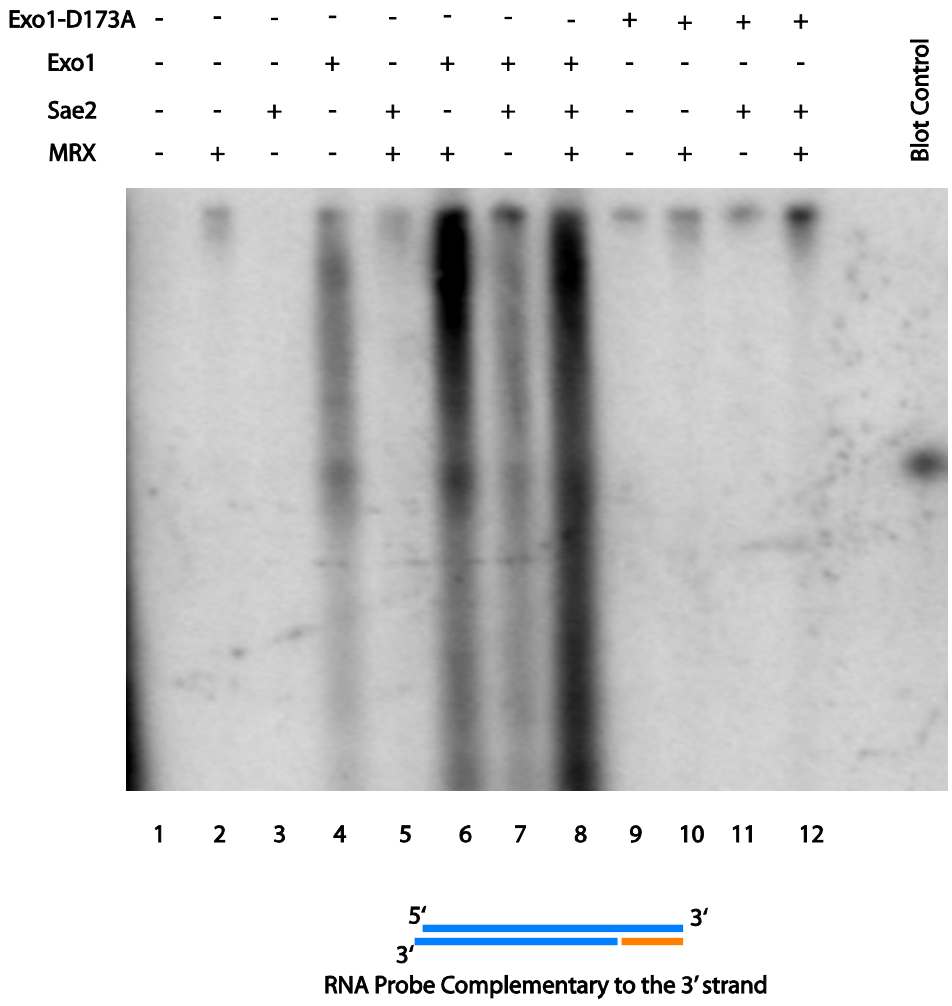


Figure 20: Exo1-D173A does not resect the DNA substrate extensively. Exo1-D173A does increase resection initiation in the presence of MRX and Sae2. The proteins were incubated as indicated in 10mM $MgCl_2$ 1mM ATP with a 4.5 Kb double-stranded DNA substrate. The reaction was incubated at 37 °C for 1 hour. Non-denaturing Southern blot analysis was performed, and the blot was probed with a 3' complementary probe labeled internally with [α-^{32}P] CTP. Protein concentrations are consistent with previous levels (15 nM Mre11, 2 nM Sae2, and 5 nM Exo1).

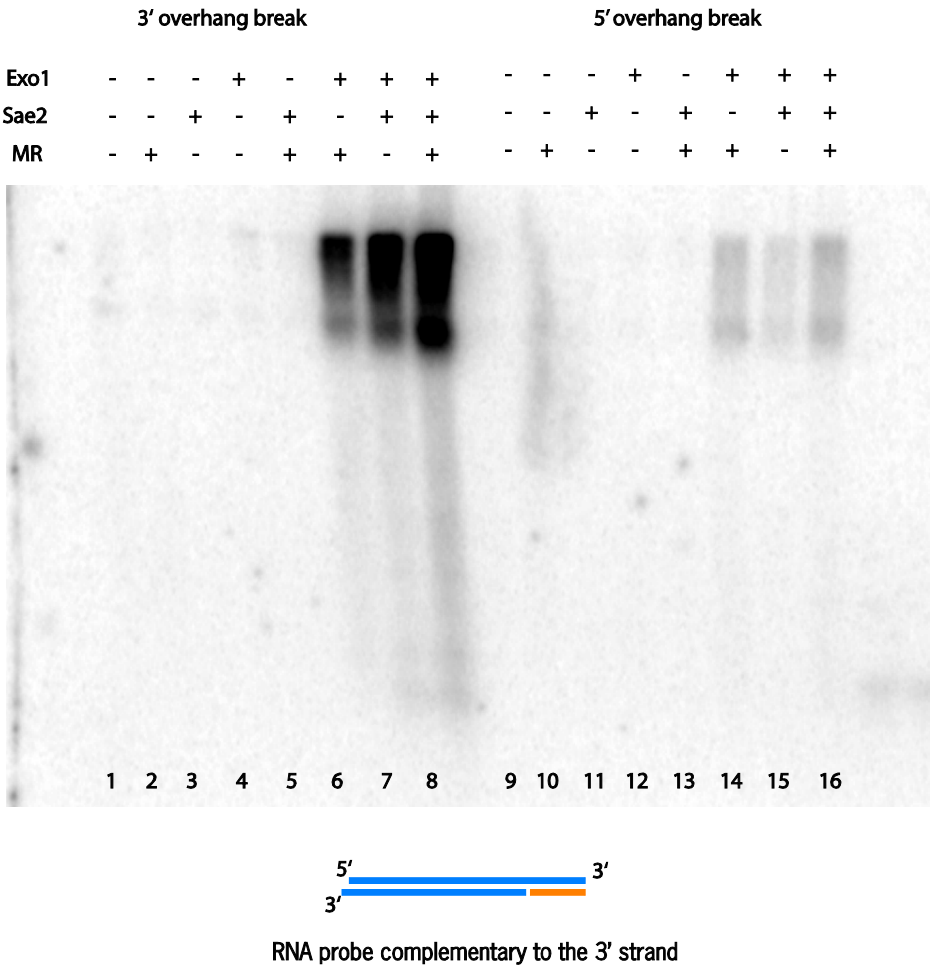


Figure 21: MR (15 nM Mre11), Sae2 (2 nM), and Exo1 (5 nM) prefer a 3' overhang at the site of the break for resection. Resection is dramatically reduced with a substrate containing a 5' overhang. The proteins were incubated as indicated in 10mM MgCl2, 1mM ATP, with a 4.5 Kb double-stranded DNA substrate. The reaction was incubated at 37 °C for 1 hour. Non-denaturing Southern blot analysis was performed, and the blot was probed with a 3',complementary probe labeled internally with [α-^{32}P] CTP.

Conclusion

In *S. cerevisiae,* homologous recombination is the preferred DNA repair pathway to maintain genome stability. Homologous recombination is initiated by 5' to 3' DNA end resection, creating a 3' overhang substrate for Rad51 to initiate strand invasion. The length of this overhang can be a few hundred to several thousand nucleotides, depending on the availability of a homologous target sequence in the genome [10]. Several key protein factors are necessary for DNA end resection *in vivo* including the MRX complex, Sae2, and Exo1 (Mimitou, 2008). However, the processes in which these proteins work have yet to be elucidated.

These results indicate resection occurs in two distinct steps, initiation and extension. First MRX unwinds the end of the DNA at the break and presents the 5' strand to Sae2. Sae2 cleaves the 5' strand leaving a 3' overhang. Exo1 is then able to extensively resect the DNA. The data demonstrates Sae2 and MRX are necessary to initiate resection. The Rad50S mutation K81I is unable to initiate resection, possibly due to an interaction defect with Sae2. In addition, Sae2 requires both the N-terminus and the C-terminus domains for resection initiation.

Exo1 is capable of extensively resecting the 5' DNA strand in the presence of MRX or Sae2. For the most efficient resection MRX, Sae2, and Exo1 must all be present. The rate of resection appears to be slightly faster than the *in vivo* rate of 4.5 Kb per hour. *In*

vitro results indicate resection begins as soon as 5 minutes and the first single-stranded DNA product is observed at 30 minutes. Extensive resection by Exo1 requires an intact nuclease domain and the presence of a 3' overhang. An intact rad50 adenylate kinase active site is also required for resection, suggesting the role of ATP in resection.

Chapter 4: The Role of hSSB1 in ATM Kinase Stimulation *In Vitro*

Introduction:

Resection is the first step of homologous recombination. During 5' to 3' DNA end resection, the single-stranded binding protein, RPA, associates with the 3' single-stranded DNA. RPA removes secondary structure from single-stranded DNA and, along with other factors, RPA helps facilitate Rad51 filament formation. Recently, the Khanna laboratory identified two novel, conserved single-strand DNA binding proteins, hSSB1 and hSSB2 in the human genome located on 12q13.3 and 2q32.3, respectively. Similar to RPA, hSSB1 has a high affinity for single-stranded DNA [54].

To determine the role of hSSB1, human cells were exposed to ionizing irradiation or ultraviolet radiation. Results from the Khanna laboratory demonstrated that hSSB1 accumulates in the nucleus after DNA damage. hSSB1 was also found to rapidly localize to the sites of double-strand breaks caused by IR or single breaks induced by the *SceI* restriction enzyme. hSSB1 localized to these breaks, and chromatin immunoprecipitation showed hSSB1 localized close to the *SceI*- induced break site. This evidence suggests that hSSB1 may be involved in DNA repair [54].

The ATM protein kinase initiates a signaling cascade that is triggered by DNA double-strand breaks. Phosphorylation of ATM targets is essential for DNA damage-induced check point activation (Fig. 22). To determine if hSSB1 affects ATM kinase activity in human cells, Western blot analysis was performed on Ataxia-Telangiectasia (A-T) cells and cells depleted for ATM after IR treatment [54]. The results demonstrated that hSSB1 was not stabilized in the absence of ATM. Furthermore, pull-down assays determined that hSSB1 interacts with ATM between amino acids 772-1102 [54]. *In vitro* kinase assays demonstrated that hSSB1 is phosphorylated by ATM on amino acid threonine 117. A hSSB1-T117A mutant allele was transfected into HeLa cells to determine the role of phosphorylation *in vivo.* After IR treatment, the hSSB1-T117A mutant protein was not stabilized as compared to that of wild-type hSSB1 [54].

ATM phosphorylates p53, Chk2, Chk1, Nbs1, and among many other substrates in response to IR. Cells depleted of hSSB1 were not as efficient in phosphorylation of these substrates, suggesting hSSB1 may play a role in ATM kinase stimulation. The goal of these experiments was to elucidate the role of hSSB1 in ATM kinase activation, which members of our laboratory have previously shown that ATM activation is dependent on the MRN complex and linear DNA *in vitro* [29; 50].

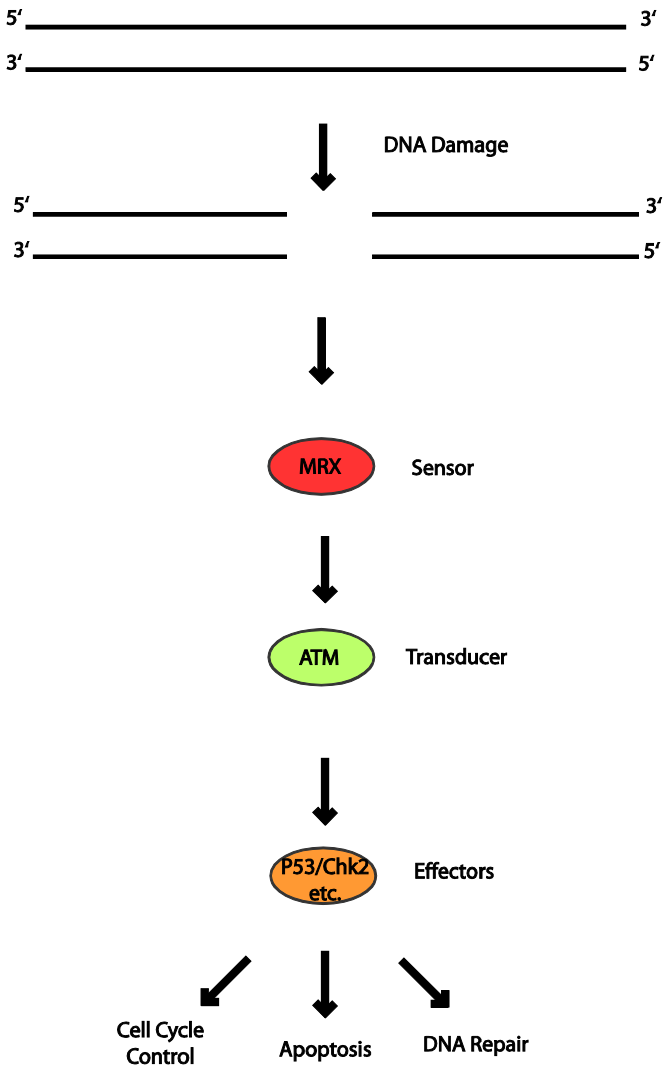


Figure 22: DNA damage activates a damage response which leads to the stimulation of ATM protein kinase by MRN. Activated ATM phosphorylates effector proteins which trigger cell cycle control, apoptosis, or DNA repair.

Results: hSSB1 Purification, Activity, and Interactions

Protein Purification:

To analyze the biochemical role of hSSB1 *in vitro* we purified recombinant hSSB1, hSSB1-T117A, and hSSB1-T117E using an *E. coli* expression system. The proteins contain a C-terminal 6Xhistidine affinity tag and were purified over Ni resin, followed by ion exchange chromatography over Heparin resin, and size exclusion chromatography (Superdex 200). The protein was visualized on a 10% SDS-PAGE gel stained with Coomassie blue protein dye (Fig. 23).

hSSB1 stimulates ATM kinase Activity

Purified recombinant hSSB1 was incubated with recombinant ATM, MRN, p53, and 10 ng of DNA to determine its effect on ATM kinase activity *in vitro*. These results demonstrate that wild-type hSSB1 is unable to stimulate ATM kinase activity (lanes 1 and 2). However, hSSB1-T117E increased ATM phosphorylation of p53 2-fold compared to the effect of wild-type hSSB1 (Fig. 24, lane 6). The stimulation by hSSB1-T117E is still dependent on MRN and DNA (Fig. 24, lanes 7-9).

hSSB1 and MRN Interaction Assay

hSSB1 required MRN to stimulate ATM kinase activity. Therefore we performed binding assays with hSSB1 and MRN to determine if the proteins interact directly. MRN showed a strong interaction with hSSB1 and hSSB1-T117A (Fig. 25, lanes 7 and 9). MRN also displayed an interaction with hSSB1-T117E, although to a lesser extent (Fig. 25, lane8).

Conclusion

hSSB1 is a novel single-stranded binding protein conserved in higher eukaryotes. hSSB1 plays a significant role in the DNA-damage signal transduction pathway [54]. These biochemical data demonstrate a direct interaction between hSSB1 and the MRN complex, and shows that a phosphomimic form of hSSB1 modestly stimulates ATM kinase activity *in vitro*. hSSB1 also promotes Rad51 mediated strand invasion *in vitro,* suggesting a possible role in homologous recombination. It is possible that hSSB1 may also directly affect DNA resection, as suggested by experiments in human cells [54], but further studies must be performed to elucidate the function of hSSB1 in DNA repair and HR.

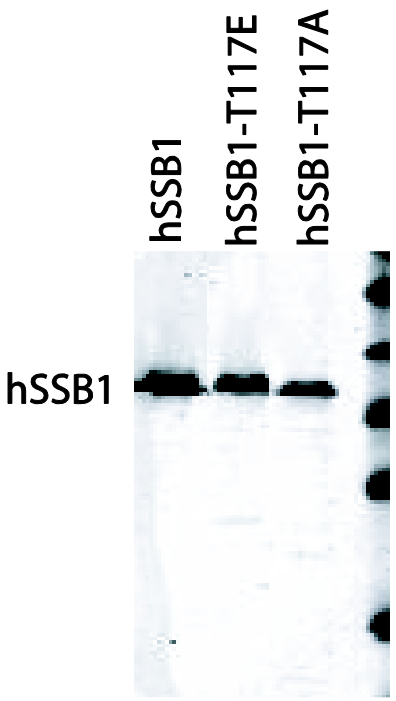


Figure 23: Recombinant hSSB1 wild-type, T117E, and T117A proteins were purified and analyzed on a SDS-PAGE gel which was stained with Coomassie blue.

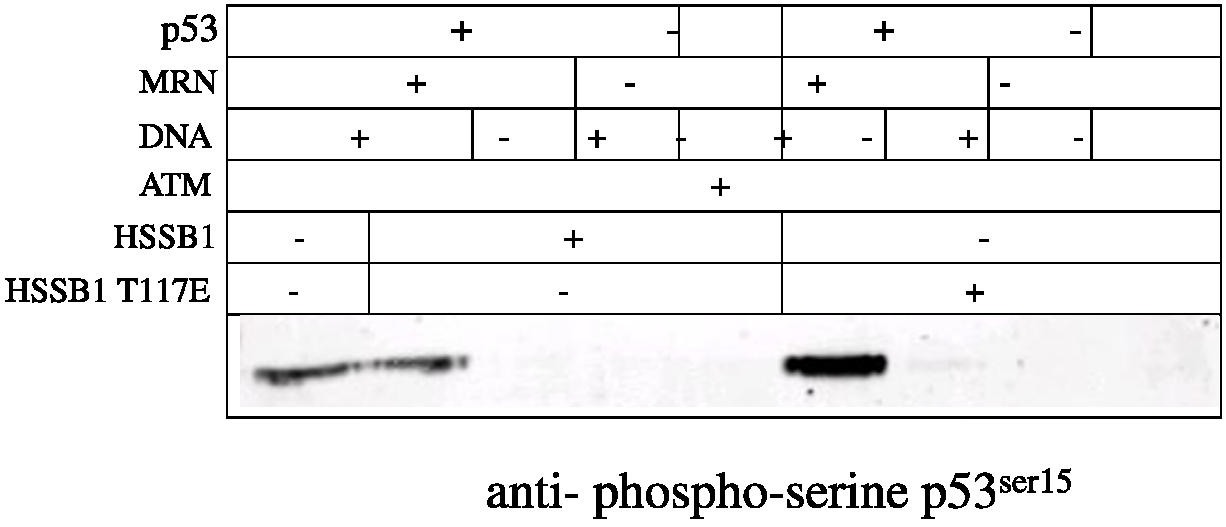


Figure 24: Wild-type hSSB1 does not stimulate ATM kinase activity. hSSB1-T117E stimulates ATM kinase activity by more than 2-fold. Recombinant ATM, MRN complex, GST-p53, and hSSB1proteins were incubated with linear DNA similarly to previously described experiments in our laboratory [29]. ATM kinase activity in this assay was assessed by Western blotting and incubated with antibody directed against phospho-p53^{ser15}.

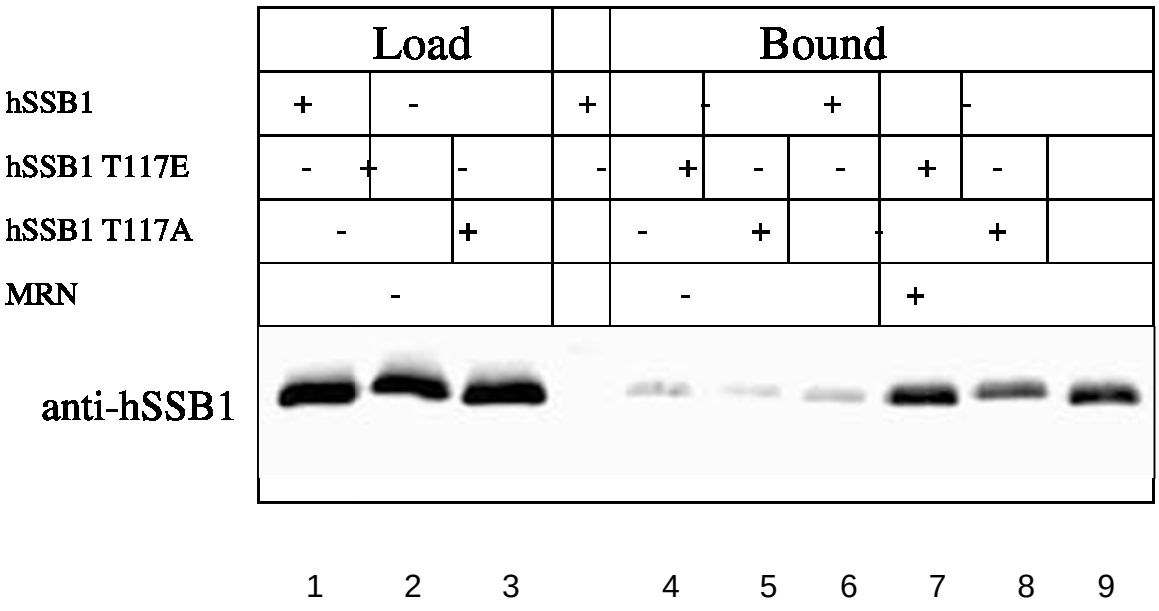


Figure 25: hSSB1 binds to MRN in a purified system. hSSB1-T117E does not bind MRN as strongly as hSSB1 wild-type or hSSB1-T117A. Interactions in this assay were assessed by Western blotting and incubated with antibody directed against hSSB1.

Chapter 5: A Novel Inhibitor of the MRN Complex

Introduction

The MRN complex senses DNA damage and activates the signaling kinase ATM, which is a member of the phosphatidlyinositol-3' kinase–related kinase (PIKK) family. Activated ATM phosphorylates downstream effector substrates Chk2, Brca1, p53, and Nbs1 that regulate cell cycle transitions, DNA repair, and apoptosis. MRN is essential in mammals, which limits studies of the complex. Recently, the Gautier laboratory identified in a forward chemical genetic screen the small molecule 6-(4-hydroxyphenyl)-2-thioxo-2,3-dihydro-4(1H)-pyrimidinone (Mirin) that inhibits the MRN complex [55]. In collaboration with the Gautier laboratory, we helped to characterize the effect of the small molecule on the MRN complex was characterized using purified proteins in kinase and nuclease activity assays *in vitro*.

Results: Mirin Inhibits Mre11 Exonuclease Activity

The screen was planned to capture small molecules from a 10,000 compound DIVERSet library with radiosensitizing and/or chemosensitizing characteristics that inhibit the MRN complex in response to DNA damage. Extracts from *Xenopus laevis*

eggs were used in a cell-free system to perform the screen. DNA damage was simulated by incubating the extracts with plasmid DNA digested with *HaeIII*. Inhibition was monitored by the detection of phosphorylated H2AX peptide by ATM. Mirin was identified using this screen, and the half-maximal inhibitory concentration for inhibition was estimated to be 66uM [55]. Mirin also inhibited autophosphorylation of ATM S1981 and phosphorylation of substrates Nbs1 and Chk2 in the Xenopus egg extract.

To identify the target of Mirin inhibition, Xenopus extracts were depleted of the MRN complex by immunoprecipitation. In cell extracts, Mirin inhibited MRN-dependent ATM kinase activity while MRN-independent ATM kinase activity was not inhibited, suggesting Mirin's mechanism of inhibition functions through the MRN complex (Dupre', 2008). To confirm this finding, Ji Hoon Lee in our laboratory added Mirin to our *in vitro* ATM kinase assay. His results demonstrated that Mirin inhibited dimeric ATM, which requires MRN and linear DNA to stimulate kinase activity [29]. However, monomeric ATM, which does not require MRN to stimulate kinase activity in the presence of manganese, was not inhibited by 50uM Mirin [55]. Thus the effect of Mirin on ATM kinase activity appeared to be specific to MRN.

Next the ability of Mirin to inhibit MRN exonuclease activity was determined. Human Mre11 exhibits manganese-dependent 3' to 5' exonuclease activity *in vitro* [23]. In our biochemical assay we incubated human MRN with Mirin and a ^{32}P-labeled oligonucleotide duplex. The reactions were analyzed on a denaturing sequencing gel.

These results demonstrated that Mre11 exonuclease activity is inhibited by Mirin, but requires 100uM Mirin (Fig. 26). To confirm that inhibition of the exonuclease activity is specific to MRN we performed the nuclease assay with 3' to 5' exoIII. Mirin did not inhibit ExoIII exonuclease activity, confirming Mirin inhibition is specific to MRN (Fig.27). The Gautier laboratory also confirmed that Mirin does not affect the stability of the complex nor the ability for MRN to bind DNA [55]. It is unclear whether inhibition of Mre11 nuclease activity by Mirin is the reason for the inhibitory effect on the ATM kinase assay. No evidence for a role of Mre11 nuclease activity in the ATM kinase assay has been obtained, but a nuclease-deficient Mre11 mutant that is competent for MRN complex formation is not available (Paull, Lee, unpublished observations).

Conclusion

MRN is an essential complex that senses DNA damage and stimulates downstream transducers and effectors. The embryonic lethality of the complex in mice makes it difficult to study the complex *in vivo*. A forward chemical genetic screen using *Xenopus* extracts isolated a small molecule inhibitor that reduced the phosphorylation of an H2AX peptide by protein kinase ATM. These studies demonstrated that Mirin prevents ATM phosphorylation by targeting the MRN complex, thus preventing stimulation of dimeric ATM. Our assays showed that Mirin inhibits Mre11 exonuclease activity and the

Gautier laboratory also showed that it does not disrupt either MRN complex stabilization of DNA binding. Mirin is a possible tool for studies of DNA damage and signaling pathways in mammalian cells by inhibiting MRN and bypassing the problem of embryonic lethality.

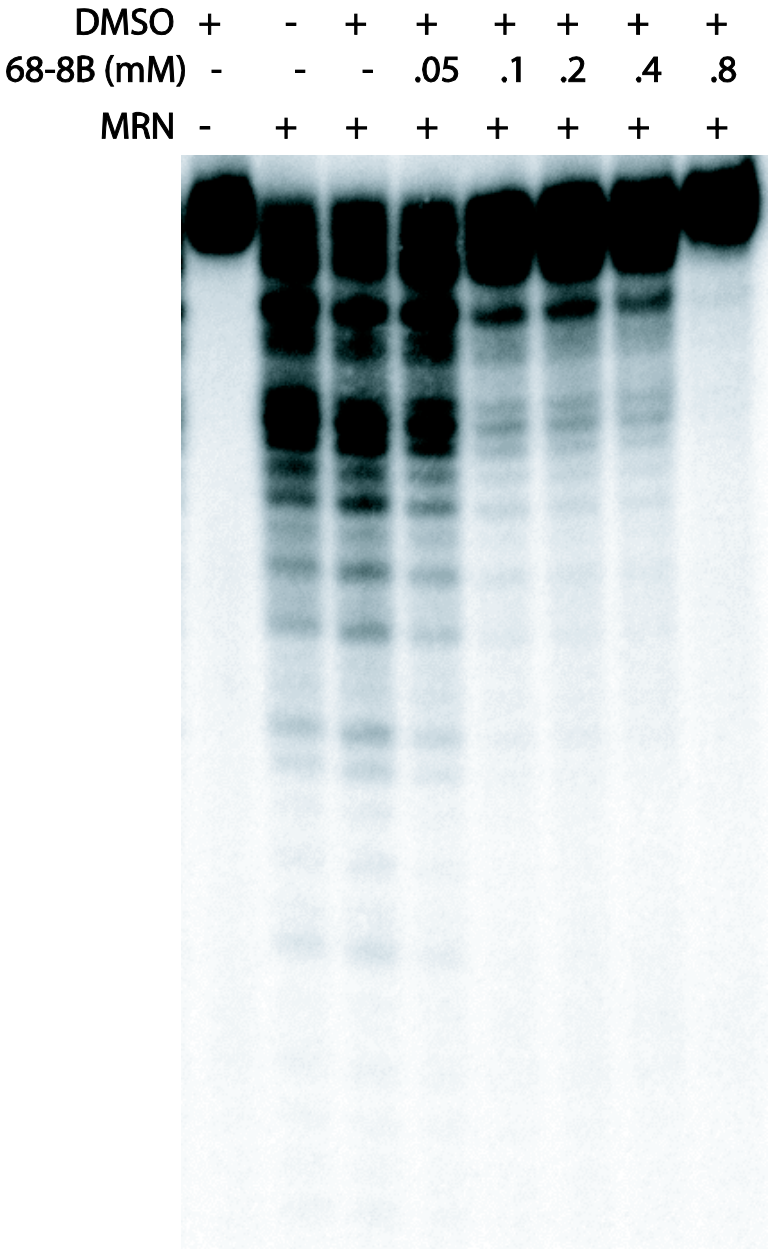


Figure 26: Mirin inhibits MRN (20 nM Mre11) exonuclease activity at a concentration of 100 uM. A ^{32}P-labeled oligonucleotide DNA duplex was incubated at 37°C for 30 minutes with MRN, $MnCl_2$, and Mirin. The reactions were analyzed on a denaturing sequencing gel.

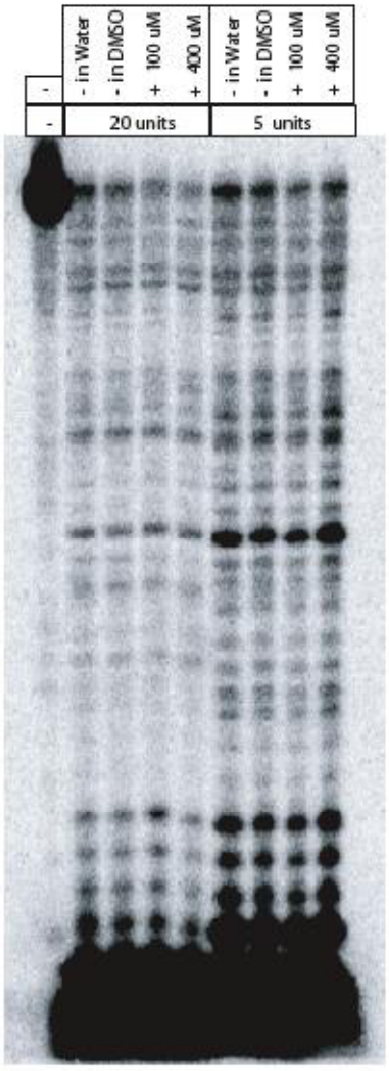


Figure 27: Mirin does not inhibit 3' to 5' exonuclease ExoIII. A ^{32}P-labeled oligonucleotide DNA duplex was incubated at 37°C for 30 minutes with ExoIII, $MnCl_2$, and Mirin. The reactions were analyzed on a denaturing sequencing gel.

Chapter 6: Discussion

MRX, Sae2, and Exo1 in DNA End Resection

In *S. cerevisiae,* homologous recombination is the preferred DNA repair pathway to maintain genome stability. Homologous recombination is initiated by 5' to 3' DNA end resection, creating a 3' overhang substrate for Rad51 to initiate strand invasion. The length of this overhang can be a few hundred to several thousand nucleotides, depending on the availability of a homologous target sequence in the genome [10]. Several key protein factors are necessary for DNA end resection *in vivo* including the MRX complex, Sae2, and Exo1 (Mimitou, 2008). However, the mechanisms for protein functions have yet to be elucidated. The experiments described here utilized purified, recombinant MRX, Sae2, and Exo1 and model DNA substrates *in vitro* to further characterize the process of resection.

MR(X) and Sae2 Initiate DNA End Resection

Bettina Lengsfeld in our laboratory previously characterized novel properties of Sae2 and MRX in biochemical assays *in vitro* [49]. Sae2 was shown to have DNA-binding capacity and exhibits both MRX-dependent and independent endonuclease activity. On oligonucleotide substrates, Sae2 cleaved branched DNA structures independently of

MRX. However, Sae2 required MRX to cleave the base of hairpin structures at the single-strand/double-strand junctions. These results demonstrated that Sae2 and MRX are function together to process hairpin DNA structures, consistent with the requirement for both MRX and Sae2 in the processing of hairpin structures *in vivo* [5; 6].

In the experiments described here we examined the activities of Sae2 and MRX on longer DNA substrates was examined in the absence of hairpins, based on evidence that MRX and Sae2 affect DNA end resection in vegetative budding yeast cells [7; 32]. The Symington laboratory showed that *sae2* strains retain unresected DSBs longer than wild-type strains and also showed a delay in single-stranded DNA formation. This evidence suggests a role in the initiation of resection; however, SSA products were still formed with wild-type kinetics. Thus, Sae2 is clearly not essential for this process (Mimitou, 2008). Strains lacking any of the MRX components also exhibit weak end bridging and/or resection and, therefore, reduced SSA [1; 47]. Strains expressing the *rad50S (K81I)* mutant display a slight delay in resection and are rescued by over-expressing Sae2, suggesting that Rad50 K81I might limit the ability of Sae2 to bind to MRX efficiently [7].

We incubated MR(X) and Sae2 with linear plasmid DNA substrates and found that MR and Sae2 can work together to remove the 5' strand of a double-stranded DNA end. MRX also functioned cooperatively with Sae2 to process the DNA end but only in the presence of ATP. However, these results showed that MR(X) and Sae2 do not appear to have extensive resection capacity. The process appeared to only cleave a small number

of nucleotides from the 5' strand, as demonstrated by the apparent lack of change in the mobility of the substrate. These results are similar to recent results from our laboratory that characterize the resection activity of Mre11/Rad50 homologs from *Archaea.*

In thermophilic *Archaea*, Mre11 and Rad50 are expressed from an operon that also contains a helicase, HerA, and a 5' to 3' exonuclease, NurA. Ben Hopkins in our laboratory expressed and purified all of the recombinant proteins from the *Pyrococcus furiousus* genome and characterized resection using these complexes *in vitro.* These results showed that *Pyrococcus furiousus* Mre11/Rad50 strongly stimulates the 5' to 3' resection of DNA ends by the HerA/NurA complex. *Pyrococcus furiousus* MR also exhibited a very limited 5' strand resection activity in magnesium that removed 15 to 55 nucleotides of the 5' strand and was not observed with an Mre11 nuclease-deficient mutant (Hopkins and Paull, submitted). These results are similar to our *S. cerevisiae* studies that demonstrate DNA end processing by MR in $MgCl_2$, and stimulation of more extensive resection by another enzyme. In our situation that enzyme is Exo1.

Sae2 Mutants Demonstrate Reduced Resection

Several deletion and point mutants of Sae2 were used to determine the role of Sae2 in the initial steps of resection. Sae2-G270D failed to resolve hairpin-capped DNA ends

in a random mutagenesis screen (Rattray, personal communication). Sae2-5A and Sae-5D are Tel1/Mec1 phosphorylation sites (S73, T90, S249, T279, and S289) where the threonines/serines are mutated to alanine or aspartate, respectively. Sae2-5D was designed to mimic phosphorylation, whereas Sae2-5A blocks phosphorylation at the Tel1/Mec1 sites. Sae2-ΔC is a truncation of the C-terminus (251-345) and Sae2-ΔN is the truncation of the N-terminus between amino acids 21-172.

Previous studies demonstrated endonuclease defects with several of Sae2 mutants [49]. Sae2-G270D and Sae2-ΔN do not interact with DNA in the presence or absence of MRX. Additionally, neither mutant was able to process DNA hairpins, and neither showed endonuclease activity on branched DNA substrates *in vitro.* The mutants also demonstrated a decrease in survival after MMS treatment and did not process hairpin-capped DNA ends *in vivo*. Sae2-5A and Sae2-ΔC exhibited a slight reduction in DNA interactions. The mutants were partially deficient in ssDNA endonuclease activity but were unable to process hairpins with MR(X) *in vitro*. MMS survival was reduced with Sae5A and Sae2-ΔC, and they were defective in hairpin processing *in vivo*. The Sae2-5D mutant exhibited similar DNA-binding and endonuclease activity *in vitro* as wild-type Sae2. However, Sae2-5D did exhibit DNA binding and nuclease activity comparable to wild-type Sae2 levels. Although Sae2-5D showed reduced MMS survival and hairpin processing activity *in vivo,* but was the only mutant to process hairpins *in vivo* [49] (Fig.28).

To further characterize Sae2 we tested the resection activity of the Sae2 mutants *in vitro* was tested in our linear plasmid DNA resection assay. Compared to wildtype Sae2, only Sae2-5D demonstrated similar amounts of resection initiation activity. Sae2-G270D had very slight resection activity, while Sae2-ΔC and Sae2-ΔN were completely defective. Although Sae2-5D does exhibit repair defects *in vivo*, it is capable of complementing MRX in our hairpin and ssDNA nuclease assays *in vitro*. Therefore, these results are generally consistent with previously published data and suggest that Sae2 endonuclease activity is important for the initial 5' strand resection event catalyzed by MR(X) and Sae2. The ability of Sae2 to cleave 5' flaps on branched DNA structures suggests a mechanism by which MR or MRX opens the DNA duplex, as shown previously with human MRN [28], and Sae2 cleaves the 5' strand at the single-stranded/double-stranded junction. We have also observed MR(X) has the ability to cleave a small amount of nucleotides from the 5' strand by itself. It is possible that MR(X) unwinds the DNA and can perform the endonuclease cut inefficiently in the absence of Sae2.

	DNA binding	ssDNA nuclease activity	hairpin nuclease activity	in vivo hairpin processing	MMS survival	Resection Initiation in vitro	Resection Extension in vitro
Sae2	+++	+++	+++	+++	+++	+++	+++
Sae2-G270D	-/+	-/+	-	-	+	+	++
Sae2-5D	+++	+++	+++	+	+	+++	+
Sae2-5A	++	++	-	-	+	NA	+
Sae2-ΔN	-	-	-	-	+	-	-
Sae2-ΔC	++	+++	-	-	+	-	-

Figure 28: Summary of *In vitro* and *in vivo* phenotypes of Sae2 and Sae2 mutants. DNA binding was measured in a gel mobility shift assay. ssDNA and hairpin nuclease activity assays were measured by oligonucleotide substates consisting of a branched or hairpin substrate respectively. *In vivo* hairpin processing was measured by growth on nutrient media after our LYS2-conversion assay. MMS survival was measured by growth on media containing MMS. Resection initiation and extension were measured by non-denaturing Southern blot analysis on a 4.5 kb DNA substrate.

Sae2, MR(X), and Exo1 in DNA End Resection

Although we have demonstrated that MR(X) and Sae2 are able to process DNA ends in a physiologically compatible 5' to 3' direction, the rate is inefficient compared to *in vivo* experiments and appears to stall immediately after resection initiation. The Symington laboratory recently showed that resection occurs in two stages in *S. cerevisiae*. Their data suggests that Sae2 and MRX are involved in an early, limited resection of the 5' strand, followed by extensive 5' to 3' resection by Exo1 (Mimitou, 2008). Exo1 is induced during meiosis and has roles in mismatch repair, post replication repair, telomere maintenance, and processing of stalled replication forks [37; 38; 39; 40]. *S. cerevisiae* strains deleted for Exo1 exhibit delayed resection after HO endonuclease breaks [33; 38]. Several studies suggest that Exo1 and MRX have semi-redundant functions in DNA damage. An *exo1* and *mre11* double mutant showed extreme sensitivity to IR suggesting a redundant role in DNA end resection [21]. Additionally, over-expressing Exo1 in *mrx* null strains abolished the resection defect [41]. To test the contribution of Exo1 to resection *in* vitro, we expressed and purified recombinant Exo1 and added it to our resection assay containing MR and Sae2. Results were obtained by direct visualization of the DNA and by non-denaturing Southern blot analysis. These results demonstrated that Exo1 5' to 3' exonuclease activity is stimulated by the addition of MR and Sae2. Surprisingly, we found that MR and Sae2 could each independently stimulate Exo1, but the most efficient degradation was seen with all four proteins present. Thus, Exo1 works

cooperatively with either MR or Sae2 to resect DNA in a 5' to 3' direction. The redundancy of MR and Sae2 with Exo1 is consistent with *in vivo* data, showing that, in the absence of Sae2, Exo1 is capable of resecting DNA, although to a lesser extent (Mimitou, 2008). *rad50*, *mre11*, and *xrs2* null strains also exhibit delayed resection after DNA cleavage by HO endonuclease at the *MAT* locus in vegetatively growing cells; however, homologous recombination is still completed [10].

Purified recombinant MRX was tested in the resection assay. Similar to MR, MRX was capable of stimulating DNA resection by Exo1, and further increased resection in the presence of Sae2 and Exo1 together. In addition, MRX exhibited increased resection in the presence of ATP. Based on this evidence, we hypothesize that ATP is possibly responsible for a conformational change that alters the position of Xrs2 in relation to MR and allows for more efficient resection. ATP does not stimulate resection when incubated with MR, but instead inhibits resection activity, perhaps due to the lack of an Xrs2-dependent conformation change in the presence of ATP.

In Vitro Resection is Consistent with Physiological Rates

Resection is a slow process that may require up to 4 hours *in vivo.* The Haber laboratory discovered that physiological resection of DSBs occurs at about 4.5 Kb per hour. To measure this rate, a plasmid was constructed with two lacZ genes and a 4.5 Kb

spacer between the repeats. A DSB was created by an HO endonuclease cut between the repeats. Single-strand annealing deletion products, which require resection, were monitored to assess the kinetics of the process over time. Their results demonstrated that SSA deletion products were first observed at about 1 hour [11]. We compared the resection kinetics of Exo1 alone, MR and Sae2, and Exo1 together with both MR, and Sae2 by utilizing our non-denaturing Southern blot analysis on a 4.5 Kb plasmid substrate. We observed that resection with MR, Sae2, and Exo1 could be visualized as soon as 5 minutes. Resection continued until the first completely single-stranded product was observed at 30 min. Single-stranded resection products continued to accumulate between 30 minutes and 1.5 hours, which is generally consistent with the *in vivo* evidence. We also observed the resection with Exo1 by itself although it was much less efficient. MR and Sae2 initiated resection, and reaction products could be observed at 45 min. but did not appear to increase for the remainder of the 3 hours. These results are consistent with physiological results demonstrating that Sae2 is capable of initiating resection, and that Exo1 is able to complete resection on its own to a lesser extent (Mimitou, 2008). It is evident that MR, Sae2, and Exo1 are necessary for more efficient resection both *in vitro* and *in vivo*.

Sae2 Mutant Analysis with MR and Exo1

To further characterize the role of Sae2 in the resection process we added the Sae2 mutants to the resection assay containing MR and Exo1. Sae2-ΔC did not show any activity in resection initiation with MR. However, in combination with Exo1, Sae2-ΔC showed slight resection activity and, with the addition of MR, Sae2-ΔC showed increased activity over MR and Exo1 alone. Sae2-ΔN was almost completely inactive in stimulating Exo1 when combined with Exo1 alone and or in combination with MR. This is consistent with Sae2-ΔN not working cooperatively with MR on hairpin oligonucleotide substrates *in vitro* or on cruciform substrates *in vivo* [49]. Sae2-G270D when combined with Exo1 had comparably less resection activity than wild-type Sae2. However, Sae2-G270D showed similar resection activity as wild type Sae2 when combined with Exo1 and MR. This evidence suggests Sae2 –G270D is capable of helping MR stimulate Exo1; however, this mutant is inefficient in stimulating Exo1 by itself in the semi-redundant pathway that is independent of MR. Sae2- 5A and Sae2-5D both have reduced resection activity compared to wild-type Sae2 in the presence of Exo1 and in the presence of Exo1 and MR. Although *in vivo* evidence shows Sae2-5D has increased sensitivity to MMS and is unable to process hairpin-capped ends, this mutant behaves similarly to wild-type Sae2 in biochemical hairpin cleavage and resection initiation assays [49]. Therefore, the inefficiency of Sae2-5D in the resection assay when combined with Exo1 was unexpected. MBP-Sae2-5D is toxic to *S. cerevisiae* strains transfected for

the cruciform processing assay (unpublished data), suggesting possible that Sae2-5D is not an appropriate mimic of Sae2 phosphorylated by Tel1/Mec1.

Sang Eun Lee's laboratory transfected Sae2 mutants into budding yeast and assayed DNA end resection. Lee and colleagues measured the amount of single-stranded DNA exposed after an HO-induced break, as well as the amount of RPA present after the cleavage. His results demonstrate that Sae2-ΔC, Sae2-ΔN, and Sae2-5A all show reduced RPA binding and single-strand DNA formation in comparison to wild-type Sae2, suggesting a resection defect. Sae2-5D exhibited a slight defect in single-stranded DNA formation and RPA aggregates at the DSB (Lee, 2008; in preparation). These results are consistent with the *in vitro* resection data demonstrating defects in resection with Sae2-ΔC, Sae2-ΔN, and Sae2-5A.

MR Mutants in DNA End Resection

Several Mre11 and Rad50 mutants exhibit resection deficiencies in *S. cerevisiae.* The separation of function mutants (*rad50S*) are unable to cleave Spo11 from the 5' end of a DSB in meiosis [2]. Strains expressing the Rad50S mutant *rad50-K81I* also show an intermediate SSA deficiency that can be rescued by over-expressing Sae2, indicating that *rad50S* mutants may be deficient in Sae2/MRX interactions [48].

Similar to *rad50s* mutants, the nuclease-deficient mutant Mre11- H125N is also deficient in Spo11 removal from a DSB [32]. In addition, Mre11-H125N and the human Mre11 equivalent, H129N are deficient in 3' to 5' exonuclease activity and hairpin cleavage in biochemical assays *in vitro* [23; 25; 31]. However, Mre11-H125N lacks a resection defect on clean DSBs after HO cleavage [33], suggesting that Mre11 nuclease activity is not essential for resection.

We expressed and purified MRX complexes containing the nuclease-deficient mutant Mre11-3, the ATPase-deficient mutant Rad50-S1205R, and the Rad50S mutant Rad50-K81I, and tested each in our DSB resection assay. MRX- Mre11-3 was capable of initiating resection in the presence of Exo1 and in the presence of Exo1 and MR; however, the extent of resection was significantly reduced. This result suggests that Mre11 nuclease activity may play a role in the processivity of resection. The MRX-Mre11-3 complex may also remain associated with the DNA end and inhibit Exo1 from migrating down the DNA. In contrast, the MRX-Rad50-S1205R mutant was completely deficient in stimulating resection activity. This mutant is equivalent to human MRN-S1205R, that is unable to unwind DNA substrates [52] suggesting a possible reason for the deficient resection. The MRX-Rad50-K81I mutant exhibited an intermediate level of resection. Furthermore, MRX-K81I did not exhibit the initial Sae2-dependent resection product, although extensive resection was still observed in the presence of Exo1. Therefore, Rad50-K81I reduced efficiency may be due to reduced binding to Sae2 or to

inefficient presentation of unwound ends suitable for Sae2 cleavage. Additional experiments need to be performed to characterize these mutants and identify what aspect of Mre11 and Rad50 functions are acting in resection.

Exo1-D173A Inhibits Extensive Resection

To confirm that Exo1 is responsible for the activities we observed, we purified recombinant nuclease-deficient mutant Exo1-D173A, based on previous studies with this enzyme in mismatch repair [53]. We incubated Exo1-D173A in our resection assay with MRX and Sae2, and found that Exo1-D173A is completely deficient in extensive 5' to 3' DNA end resection, as expected. However, Exo1-D173A was still able to stimulate a low level resection initiation in the presence of MRX and Sae2, suggesting Exo1 plays two separate roles of Exo1 in DNA end resection. Initially Exo1 stimulates resection initiation by Sae2 and MRX in an Exo1 nuclease-independent manner. Next, Exo1 resects DNA extensively, leaving a 3' overhang for Rad51 catalyzed strand invasion.

MR, Sae2, and Exo1 Prefer a 3' Overhang to Initiate Resection

In vitro experiments have shown Sae2 cleaves a 5' DNA strand at a single-stranded/double-stranded junction. The 5' strand cleavage requires the presence of the

unwound 3' strand [49]. If Sae2 is the responsible nuclease for the initiation of 5' resection, it would likely require an unwound or unpaired 3' strand to aid in the 5' strand cleavage. To test this hypothesis we digested our resection substrate with *Sph1* or *Sal1* which leave 3' and 5' overhangs, respectively. Our results clearly demonstrated that the cooperative reaction with MR, Sae2, and Exo1 prefers a 3' overhang to initiate resection. We need to perform further experiments to clarify this observation. Breaks do occur *in vivo* that create 5' overhangs. It is unlikely organisms repair these breaks through two separate pathways. The human MRN complex exhibits ATP-dependent DNA unwinding activity [28] that could potentially unwind the DNA strands to create enough of a 3' single-strand to begin resection initiation. Therefore we plan to repeat the overhang polarity experiment with MRX in the presence of ATP. One possibility is that MRX may be much less sensitive to the overhang polarity compared to MR.

Future Work

In conclusion, we have characterized the process of 5' to 3' resection biochemically using DNA repair enzymes implicated in this process in budding yeast *in vivo*. The process of resection occurs in to distinct steps. Resection is initiated by MRX and Sae2 with the help of Exo1. Previous biochemical evidence suggests Sae2 is the endonuclease responsible for resection initiation and requires a 3' overhang for the initial cut [49]. I

hypothesize that MRX may unwind the DNA end in an ATP-dependent manner to create a 5' and 3' single-stranded/double stranded junction. Sae2 than may cleave the 5' DNA strand, leaving a 3' overhang preferred by Exo1. Exo1 then may extensively processes the DNA with the help of Sae2 and MRX. Rad51 associates with the 3' overhang to catalyze strand invasion and homologous recombination.

Resection is a complicated process that requires many protein components. Although MRX, Sae2, and Exo1 all contribute to DNA resection, these proteins are not essential to cell viability in budding yeast, suggesting that additional, redundant components are involved. Recently the Symington laboratory showed that the Sgs1 helicase is required for resection in the absence of Exo1 and Sae2. An *sgs1 exo1 sae2* triple null strain was completely deficient in SSA annealing, demonstrating a lack of extensive resection (Mimitou, 2008). An SGS1 wild-type gene rescued the SSA deficiency, but the Sgs1-K706A helicase mutant was still deficient in SSA, demonstrating that helicase activity is necessary for resection. In *sgs1* and *exo1* strains, extensive resection products were delayed. A *sgs1/exo1* null strain failed to show any extensive resection products; however, a band with slightly increased mobility was visible, indicating an initial resection cut. The initial resection cut appeared to be 50-100 nucleotides from the end of the 5' strand and the product of an endonuclease cut (Mimitou, 2008). Finally *sgs1/exo1/*Ptet-SAE2 strains were developed to conditionally knockout Sae2. When Sae2 was depleted in this strain, the DNA remained unprocessed,

suggesting that Sae2 is involved in the initial processing cut (Mimitou, 2008). We plan to express and purify recombinant Sgs1 and incubate it in our resection assay to further characterize the process.

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