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The *Saccharomyces cerevisiae* Sae2 Protein Promotes Resection and Bridging of Double Strand Break Ends*

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When eukaryotic chromosomes undergo double strand breaks (DSBs), several evolutionarily conserved proteins, among which the MRX complex, are recruited to the break site, leading to checkpoint activation and DNA repair. The function of the *Saccharomyces cerevisiae* Sae2 protein, which is known to work together with the MRX complex in meiotic DSB processing and in specific mitotic DSB repair events, is only beginning to be elucidated. Here we provide new insights into the role of Sae2 in mitotic DSB repair. We show that repair by single strand annealing of a single DSB, which is generated by the HO endonuclease between direct repeats, is defective both in the absence of Sae2 and in the presence of the hypomorphic *rad50s* allele altering the Rad50 subunit of MRX. Moreover, *SAE2* overexpression partially suppresses the *rad50s* single strand annealing repair defects, suggesting that the latter might arise from defective MRX-Sae2 interactions. Finally, *SAE2* deletion slows down resection of an HO-induced DSB and impairs DSB end bridging. Thus, Sae2 participates in DSB single strand annealing repair by ensuring both resection and intrachromosomal association of the broken ends.

DNA double strand breaks (DSB[3](s)) are a particularly dangerous form of DNA damage, because failure to repair these lesions can lead to loss of genetic information by deletions, duplications, translocations, and missegregation of large chromosome fragments (1). DSBs can arise by failures in DNA replication and by exposure to environmental factors, such as ionizing radiations and genotoxic drugs. However, they also play an important role as intermediates in meiotic and mitotic crossing over, V(D)J recombination and yeast mating type switching.

When DSBs occur, many proteins are recruited to the break sites and serve both to promote a checkpoint response and to initiate DNA repair that can occur through non-homologous end joining or homologous recombination (HR) (2). Whereas non-homologous end joining implies recombination between sequences with little or no homology, HR involves exchange of genetic information between homologous DNA sequences and is the major DSB repair process in *Saccharomyces cerevisiae*.

HR initiates with a DSB (3, 4), whose 5′-ends resection leaves 3′-ended single-stranded DNA (ssDNA) tails. Then, depending on the position of the homologous partner, on the initiation event and on the length of the homology region in the recombinant molecules, HR may occur by different mechanisms, including double strand break repair, synthesis-dependent strand annealing, and break-induced replication (2, 5). Moreover, when a DSB occurs between direct repeats, its repair is primarily achieved by a particular kind of HR named single strand annealing (SSA). SSA requires DSB resection to generate long 3′-ended single-stranded tails that can anneal with each other when resection is sufficient to uncover the duplicated sequences. Single-stranded tails are then removed by nucleases, and the resulting gaps/nicks are filled in by DNA repair synthesis and ligation, resulting in deletion of one repeat and the intervening region (6).

Mitotic HR involves several proteins, including Rad51, Rad52, Rad54, Rdh54/Tid1, Rad55, Rad57, Rad59, and the ssDNA binding complex replication protein A (2). Consistent with a role for Rad52 in providing the single strand annealing activity (7, 8), Rad52 is essential for initiation of most HR events, including SSA, whereas Rad51, Rad54, Rad55, and Rad57 are dispensable for SSA-mediated DSB repair (9).

Different functions in DSB repair can be envisaged also for a protein complex known as MRN (Mre11-Rad50-Nbs1) in mammals and MRX (Mre11-Rad50-Xrs2) in *S. cerevisiae*, which has been shown to localize at meiotic and mitotic DSBs almost immediately after their formation (10, 11). The *S. cerevisiae mrx* null mutants are severely impaired in meiotic recombination (4, 12) and hairpin-capped DSB repair (13) and exhibit decreased frequencies of ionizing radiation-induced sister chromatid and interhomologue recombination (14). Moreover, they markedly retard the kinetics of DSB repair by SSA, although they do not prevent its completion (15).

The Mre11 subunit of MRX has both exonuclease and endonuclease activities (16–18) that likely function in DSB repair. However, whereas point mutations in the Mre11 phosphoesterase domain result in accumulation of unresected meiotic DSBs and sporulation failure *in vivo* (19), most *S. cerevisiae mre11* nuclease defective alleles cause only a mild sensitivity to ionizing radiations and do not seem to affect either HO-induced DSB resection or ionizing radiation-induced sister chromatid recombination (14, 19–21). Moreover, budding yeast Mre11 associates with a DSB only transiently (11), whereas resection of an HO-induced DSB can proceed for several hours until repair occurs (22). This indicates that the functions of MRX in mitotic DSB repair might be partially independent of its nuclease activity, which is instead essential in the single strand endonucleolytic removal of Spo11 from meiotic DNA ends (12, 19).

Consistent with this hypothesis, the MRX complex has been shown to cooperate with Rad52 in holding the two ends of the broken chromosome together independently of its nuclease activity (23, 24). Moreover, it promotes the assembly of DSB-specific cohesion domains, thus contributing to maintain association between sister chromatids during DSB repair (25–27). According to these functions, MRX structure resembles that of the SMC (structural maintenance of chromosome) proteins that

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[3] The abbreviations used are: DSB, double strand break; HR, homologous recombination; SSA, single strand annealing; ssDNA, single-stranded DNA; I-SceI, intron SceI endonuclease; YEPD, yeast extract peptone dextrose; YEP+raf+gal, yeast extract peptone + raffinose + galactose; GFP, green fluorescent protein.

are required for sister chromatid cohesion (28). The MRX complex associates with DNA ends through the Mre11 protein and the globular domain of Rad50, with the coiled-coil arms of Rad50 extending outwards and interacting with an MRX complex on the other site of the break through Cys-Xaa-Xaa-Cys motifs (29).

The *S. cerevisiae* Sae2 protein is known to work together with the MRX complex at least in meiotic DSB processing and in the repair of mitotic hairpin-capped DSBs. In fact, *sae2* null mutants show meiotic recombination defects that are undistinguishable from those of hypomorphic *rad50s* and *mre11s* mutants (30–33). Moreover, similarly to *rad50s* and *mre11s* mutants, *sae2*Δ cells are both hypersensitive to the alkylating agent methyl methanesulfonate and defective in mitotic hairpin-capped DSB repair (13, 34).

Sae2 has neither known homologues in other species nor obvious motifs, and its function is only beginning to be elucidated. The lack of Sae2 does not affect the overall recombination rate when a DSB is induced between two inverted repeats (35). Nonetheless, *SAE2* deletion delays Rad52 recruitment at sites of DNA damage after γ-irradiation or I-SceI-induced DSBs (11) and increases the frequency of repair events that lead to the duplication of inverted repeats (35) and to palindromic gene amplification (36). Because similar duplications of inverted repeats have been observed in the repair of an HO-induced DSB with only one end homologous to the donor (37), Sae2 may be involved in ensuring that both DSB ends participate in HR.

To provide new insights into the Sae2 function in mitotic DSB repair, we have investigated whether the absence of Sae2 affects resection and SSA repair of a single DSB generated by the site-specific HO endonuclease. We show that Sae2 contributes to both resection and intrachromosomal association of DSB ends, thus ensuring efficient DSB repair.

EXPERIMENTAL PROCEDURES

Yeast Strains and Media—Strains YMV80 and YMV45 were kindly provided by J. Haber (Brandeis University, Waltham, MA) and were used to disrupt the *SAE2* gene and to replace *RAD50* with the *rad50s* K81I allele (12) or to integrate the *GAL-SAE2* fusion at the *URA3* locus. YMV45 and YMV80 are isogenic to YFP17 (*MAT*Δ::*hisG hml*Δ::*ADE1 hmr*Δ::*ADE1 ade1 lys5 ura3-52 trp1 ho ade3*::*GAL-HO leu2*::*cs*) except for the presence of a *LEU2* fragment inserted, respectively, 4.6 or 25 kb centromere-distal to *leu2*::*cs* (38). *SAE2* and *MRE11* disruptions were also performed in strains yJK37.2 (*MAT*Δ *hml*Δ *hmr*Δ *can1 lys5 ade2 leu2 trp1 ura3 his3 ade3*::*GAL-HO VII*::*TRP1-HO LacI-GFP*::*URA3 LacO*::*KanR*), yJK40.6 (*MAT*Δ *hml*Δ *hmr*Δ *can1 lys5 ade2 leu2 trp1 ura3 his3 ade3*::*GAL-HO VII*::*TRP1-HO LacI-GFP*::*URA3 LacO*::*LYS5 LacO*::*KanR*), and yJK98.9 (*MAT*Δ *hml*Δ *hmr*Δ *can1 lys5 ade2 leu2 trp1 ura3 his3 ade3*::*GAL-HO VII*::*TRP1-HO LacI-GFP*::*URA3 LacO-GFP*::*LYS5 LacO*::*LEU2*) (23), kindly provided by D.P. Toczyski (University of California, San Francisco, CA). Strain yJK40.6 was also used to replace *RAD50* with the *rad50s* K81I allele.

SAE2 and *MRE11* deletions were performed as described previously (34). To generate strains carrying three copies of the *GAL-SAE2* fusion integrated at the *URA3* locus, the YMV80 strain was transformed with ApaI-digested plasmid pML508, carrying the entire *SAE2* coding region fused to the *GAL1* promoter. The YMV80 derivative *SAE2-HA* and *GAL-SAE2-HA* strains, carrying the fully functional *SAE2-HA* allele at the *SAE2* chromosomal locus, or the *GAL-SAE2-HA* allele at the *URA3* chromosomal locus, respectively, were constructed as described previously (34). To generate *rad50s* mutants, YMV80 and yJK40.6 cells were transformed with MscI-digested plasmid pML533, carrying the first 666 bp of the *RAD50* coding region with the K81I mutation. Accuracy of all disruptions was verified by PCR, whereas *GAL-SAE2* and *GAL-SAE2-HA* integrations were verified by Southern blot analysis.

Standard yeast genetic techniques and media were according to Ref. 39. Cells were grown in YEP medium (1% yeast extract, 2% bactopeptone, 50 mg/liter adenine) supplemented with 2% glucose (YEPD) or 2% raffinose (YEP+raf) or 2% raffinose and 2% galactose (YEP+raf+gal).

Plasmids—To give rise to plasmid pML508, a fragment spanning from position +1 to position +1162 from the *SAE2* translation initiation codon was amplified by PCR using yeast genomic DNA as a template and oligonucleotides PRP559 (5′-CGC GGA TCC ATA TGG TGA CTG GTG AAG AAA ATG-3′) and PRP560 (5′-AAC TGC AGC TGG TAA GTT AGG TGT CAT TTG-3′) as primers and was then cloned together with a 700-bp EcoRI-BamHI fragment containing the *GAL1* promoter, into the EcoRI-PstI sites of plasmid YIplac211 (40). To give rise to plasmid pML533, a 1051-bp fragment containing the promoter and the first 666 bp of the coding region of the *rad50s* K81I allele was amplified by PCR using oligonucleotides PRP706 (5′-CGG AAT TCC CGA TAG TAC TTC CAC TTA CAA TAC-3′) and PRP707 (5′-CGG AAT TCC ATT GCT TTC GAT CTG TCT TTA TCC-3′) as primers and yeast genomic DNA from MJL1699 strain (kindly provided by N. Kleckner, Harvard University, Cambridge, MA) as a template and was then cloned into the EcoRI site of plasmid YIplac128 (40). The presence of the mutation was verified by nucleotide sequence analysis.

Other Techniques—Synchronization experiments, protein extract preparation, and Western blot analysis were performed as previously described (41). DSB formation and repair were detected by Southern blot analysis using an Asp718-SalI fragment containing part of the *LEU2* gene as a probe (38). DSB end resection at the *LEU2* locus was analyzed on alkaline-agarose gels as described in Ref. 42, using a single-stranded probe complementary to the unresected DSB strand. This probe was obtained by *in vitro* transcription using Promega Riboprobe System-T7 and plasmid pML514 as a template. Plasmid pML514 was obtained by cloning the KpnI-ClaI fragment containing part of the *LEU2* gene in the KpnI-ClaI sites of pGEM-7Zf plasmid (purchased from Promega).

RESULTS

The Lack of Sae2 Impairs DSB Repair by SSA—We examined the effects caused by lack or excess of Sae2 on SSA repair by using YMV80 derivative strains (38), carrying either a deletion of *SAE2* or a *GAL1-SAE2* fusion integrated at the *URA3* locus. In these strains, a single DSB can be generated by expressing the site-specific *HO* endonuclease gene from a galactose-inducible *GAL1* promoter. The normal HO recognition sites at *MAT*, *HML*, and *HMR* loci are deleted, whereas an HO target sequence on chromosome III is flanked on either side by homologous sequence repeats, consisting of the 3′-end of the *LEU2* gene. One of these repeats is adjacent to the HO cut site (*leu2*::*cs*), whereas the other repeat (*his4*::*leu2*) is located 25 kb centromere distal from the break site (Ref. 38; see also Fig. 1*A*). Because of the presence of homologous sequence repeats on either side of the HO cut site, the DSB in the YMV80 derivative strains has been reported to be mainly repaired by a Rad52-dependent SSA pathway (38). In fact, the ends of the DSB are degraded by 5′–3′-exonucleases until complementary sequences flanking the break are exposed and can be annealed. Subsequent ligation of the two chromosomal fragments repairs the break, concomitantly abolishing the HO recognition sequence and one of the repeats (38).

Because the ability to repair the HO-induced DSB is influenced by the cell cycle stages (43), we arrested all cell cultures in mitosis and kept them blocked by nocodazole treatment during break induction by galactose addition. We then monitored the kinetics of HO-cut formation and its subsequent repair by Southern blot analysis. As depicted in

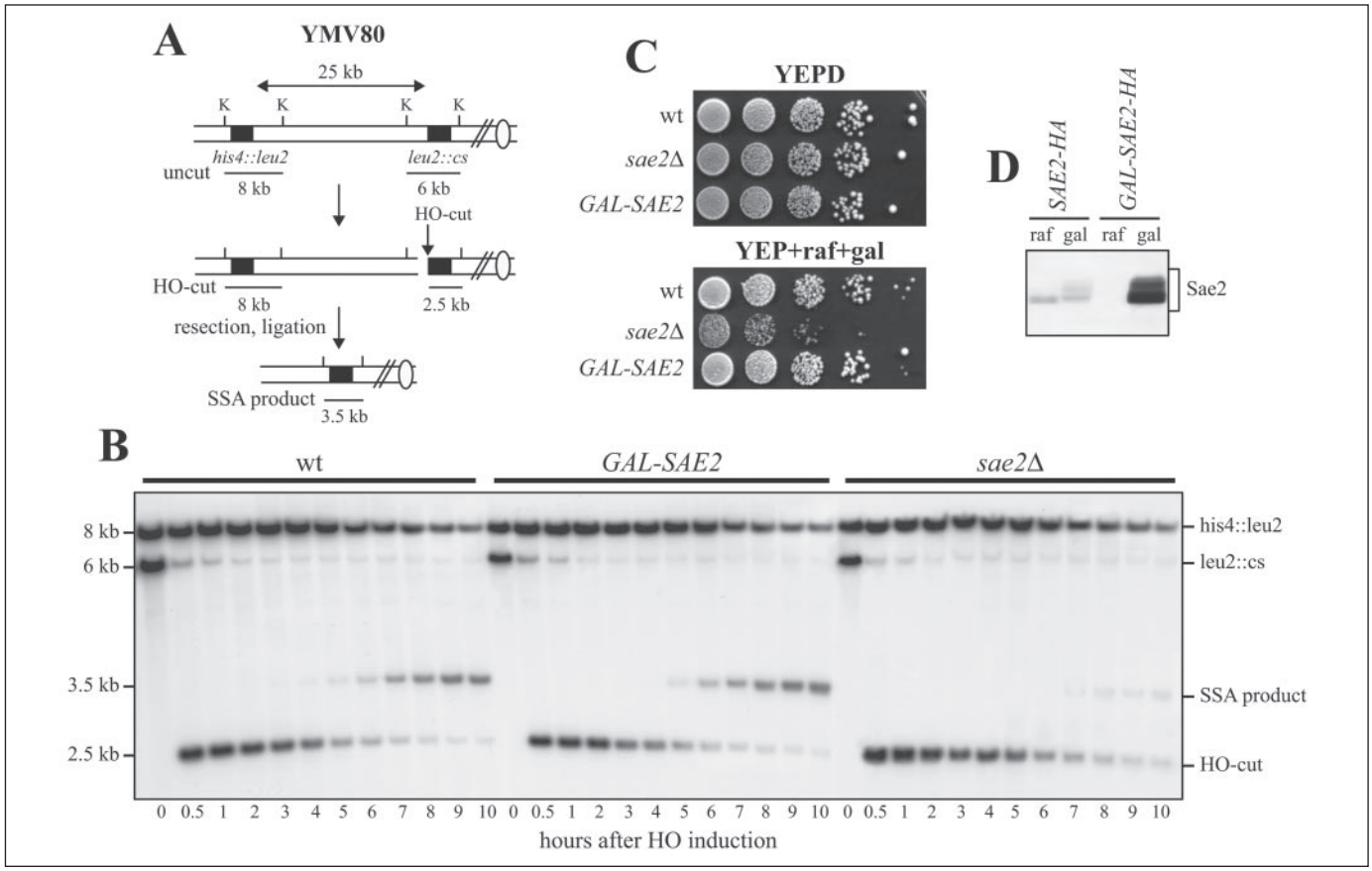

FIGURE 1. **SSA repair of an HO-induced DSB in YMV80 *sae2*Δ and *SAE2* overexpressing cells.** *A*, map of the YMV80 chromosome III region containing the HO-cut site flanked by homologous *LEU2* sequence repeats (*his4*::*leu2* and *leu2*::*cs*) that are depicted as *black boxes* and are 25-kb apart (38). Hybridization with a Asp718-SalI *LEU2* probe of KpnI-digested YMV80 genomic DNA run on a native agarose gel reveals two fragments, 8- and 6-kb long (*uncut*) in the absence of HO-cut, whereas the HO-induced DSB results in the disappearance of the 6-kb species and concomitant formation of 2.5-kb fragments (*HO-cut*) that can be eventually repaired by SSA, leading to a final product of 3.5-kb (*SSA product*). The *vertical lines* indicate the restriction sites recognized by KpnI (*K*). *B*, HO-cut formation and SSA in YMV80 derivative strains. Exponentially growing YEP+raf cell cultures of wild type YMV80 and isogenic *sae2*Δ and *GAL-SAE2* strains, all carrying the HO-cut site as depicted in *A* and a galactose-inducible *HO* gene, were arrested in mitosis with nocodazole and then transferred to YEP+raf+gal in the presence of nocodazole at time zero. KpnI-digested genomic DNA prepared from cell samples collected at the indicated time points were subjected to the Southern blot analysis described in *A*. *C*, serial dilutions of YMV80 and isogenic *sae2*Δ and *GAL-SAE2* strains were spotted on YEPD and YEP+raf+gal plates that were incubated at 30 °C for 3 days. *D*, cell cultures of YMV80 derivative strains carrying either the *SAE2-HA* allele at the *SAE2* chromosomal locus or the *GAL-SAE2-HA* fusion at the *URA3* locus, exponentially growing in YEP+raf (*raf*), were shifted to YEP+raf+gal (*gal*) for 5 h. Protein extracts were subjected to Western blot analysis with anti-hemagglutinin antibodies.

Fig. 1*A*, hybridization with a *LEU2* probe of KpnI-digested YMV80 genomic DNA run on a native agarose gel reveals 8- and 6-kb DNA fragments in the absence of HO cut. HO-induced DSB formation results in decrease of the amount of 6-kb species and concomitant appearance of 2.5-kb fragments. The break can be eventually repaired by SSA, leading to a final product of 3.5-kb and disappearance of both the 2.5- and 8-kb fragments. As shown in Fig. 1*B*, HO-cut induction was rapid and efficient in all cell cultures, because the *leu2*::*cs* sequence was cut in more than 90% of cells within 30 min after galactose addition. Then, the 3.5-kb SSA repair products started to accumulate in both wild type and *SAE2* overexpressing cells ~5 h after HO induction, concomitantly with the decrease of both the 2.5- and 8-kb fragments. SSA products were instead detectable only at very low levels even 10 h after HO induction in *sae2*Δ cells (Fig. 1*B*).

Consistent with defective DSB repair by SSA in the absence of Sae2, only 10% of *sae2*Δ cells were able to form colonies on galactose-containing plates (HO expression on), whereas cell viability was unaffected in wild type and *GAL-SAE2* cells under the same conditions (Fig. 1*C*). Thus, Sae2 is required for efficient repair of the HO-induced DSB by SSA, which appears to be unaffected by *SAE2* overexpression, indicating that increasing Sae2 levels (Fig. 1*D*) is not sufficient to accelerate this process in wild type cells.

The lack of Sae2 severely reduced SSA-dependent recombination events also in YMV45 derivative strains (Fig. 2), where SSA repair of HO-induced DSBs occurs very rapidly (38). In fact, the distal *leu2* sequence in these strains is located only 4.6 kb away from the HO recognition site at *leu2*::*cs* (Ref. 38, see also Fig. 2*A*). When cell cultures of these strains were arrested in mitosis and kept blocked by nocodazole treatment during break induction, SSA products accumulated in wild type cells already 90 min after HO induction (Fig. 2*B*). Moreover, they became detectable in *sae2*Δ cells ~150 min after galactose addition (Fig. 2*B*), but their amount was much lower than in wild type, thus confirming a critical role for Sae2 in SSA repair. Consistent with the faster DSB repair by SSA in YMV45 *sae2*Δ cells compared with YMV80 *sae2*Δ cells, 50% of YMV45 *sae2*Δ cells were able to form colonies on galactose-containing plates (Fig. 2*C*).

SAE2 Overexpression Partially Suppresses the SSA Defects of rad50s Mutants—In contrast to the *mrx* null alleles, which are unable to generate meiotic DSBs, *sae2*Δ cells display the same meiotic recombination defects as the hypomorphic *rad50s* and *mre11s* alleles (30–33), allowing to hypothesize that the Rad50s and Mre11s mutant proteins might specifically impair MRX-Sae2 interaction. We then asked whether *rad50s* mutant cells were defective in SSA. After construction of a *rad50s* K81I mutant (12) in the YMV80 background, cell cultures were arrested in

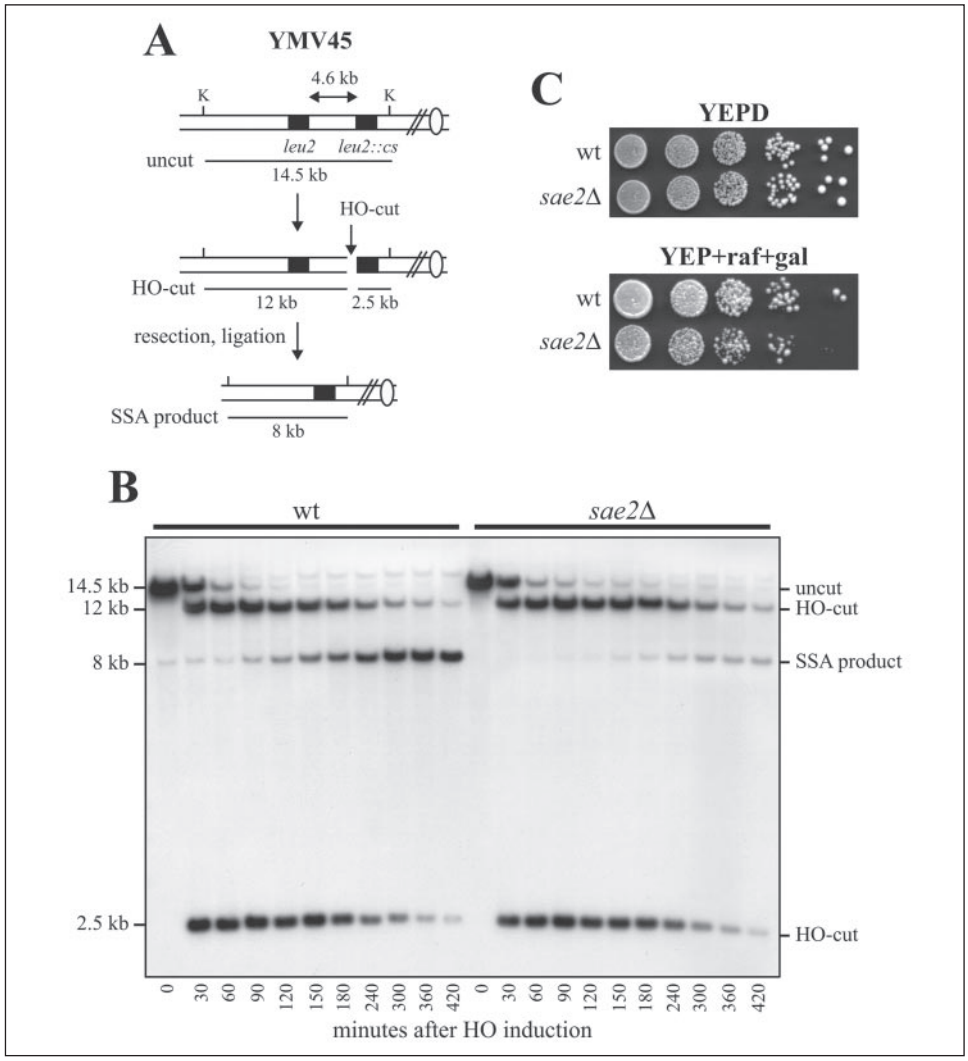

FIGURE 2. **SSA repair of an HO-induced DSB in YMV45 *sae2*Δ cells.** *A*, map of the YMV45 chromosome III region where the HO-cut site is flanked by homologous *LEU2* sequence repeats (*black boxes*) that are only 4.6 kb apart (38). After digestion of genomic DNA with KpnI, DNA separation on a native agarose gel and hybridization with the *LEU2* probe, a 14.5-kb fragments (*uncut*) is detected in the absence of HO-cut, whereas HO-induced DSB formation results in generation of 12- and 2.5-kb fragments (*HO-cut*) that can be eventually repaired by SSA, generating a final product of 8.0-kb (*SSA product*). *B*, HO-cut formation and SSA repair in YMV45 derivative strains. Exponentially growing YEP+raf cell cultures of wild type YMV45 and its *sae2*Δ derivative strain, both carrying the HO-cut site as depicted in *A* and a galactose-inducible *HO* gene, were arrested in mitosis with nocodazole and transferred to YEP+raf+gal in the presence of nocodazole at time zero. KpnI-digested genomic DNA prepared from cell samples collected at the indicated time points were subjected to Southern blot analysis as described in *A*. *C*, serial dilutions of YMV45 and isogenic *sae2*Δ strain were spotted on YEPD and YEP+raf+gal plates that were incubated at 30 °C for 3 days.

mitosis and kept blocked by nocodazole treatment during break induction by galactose addition. Southern blot analysis was then used to follow the kinetics of DSB repair by SSA. As shown in Fig. 3*A*, SSA products were barely detectable in *rad50s* cells even 8 h after HO induction, similarly to what observed in isogenic *sae2*Δ cells (Fig. 1*B*). Accordingly, *rad50s* cell survival on galactose containing plates was reduced to the same extent as in *sae2*Δ cells (Fig. 3*B*).

If the *rad50s* SSA defects were because of altered MRX ability to interact with Sae2, Sae2 overproduction might be expected to suppress it. This was indeed the case, because SSA products in *GAL-SAE2 rad50s* cells treated as above became detectable and started to accumulate, although to a lower extent than in wild type, ~5 h after HO induction (Fig. 3*A*). In agreement with partial suppression of *rad50s* SSA defects by *SAE2* overexpression, *GAL-SAE2 rad50s* cells showed increased cell survival after HO induction on galactose-containing plates compared with *rad50s* cells (Fig. 3*B*).

The Lack of Sae2 Slows Down Resection of an HO-induced DSB—Because the HO break can undergo repair by SSA only once 5′–3′-resection has uncovered both direct repeats, allowing their subsequent annealing and ligation, SSA repair defects in *sae2*Δ cells might be because of impairments in the generation of 3′-ended ssDNA. We therefore used denaturing gel electrophoresis and Southern blot analysis with a single-stranded *LEU2* probe to monitor both HO cut at *leu2*::*cs* and the extent of resection, as depicted in Fig. 4*A*. When these analyses were performed on the same cell cultures shown in Fig. 1, initiation of DSB resection turned out to be delayed in YMV80 *sae2*Δ cells (Fig. 4*B*). In fact, 3′-ended resection products (named r1–r6) could be detected in these cells ~1 h later than in isogenic wild type cells.

The *sae2*Δ DSB resection defects seemed to only partially account for the reduced SSA efficiency. In fact, the amount of SSA products was very low for at least 10 h after HO induction in YMV80 *sae2*Δ cells (Fig. 1*B*), whereas the 3′-ended ssDNA products were detectable in the same cells already 2–3 h after galactose addition and remained stable for at least 10 h (Fig. 4*B*). Conversely, the amount of resection products decreased in the isogenic wild type cells (Fig. 4*B*) concomitantly with the appearance of SSA products (Fig. 1*B*). Moreover, DSB resection in the *sae2*Δ YMV80 derivative strain seemed to proceed for at least 25 kb and to uncover the homologous *LEU2* sequence (*his4*::*leu2*) located 25 kb away from the HO-cut site. In fact, the amount of the 8-kb *his4*::*leu2* DNA fragment started to decrease ~6–7 h after HO induction in both wild type and *sae2*Δ YMV80 cells (Fig. 1*B*). Because SSA products were dramatically reduced in the same *sae2*Δ cells compared with wild type, the disappearance of the 8-kb DNA fragment in the absence of Sae2 was likely because of DSB resection proceeding long enough to uncover the homologous *his4*::*leu2* sequence.

We also monitored the extent of DSB ends resection in the YMV45 cell cultures shown in Fig. 2, where the distal *leu2* sequence is located only 4.6 kb away from the HO-cut site (Fig. 2*A*). As shown in Fig. 4*C*, the

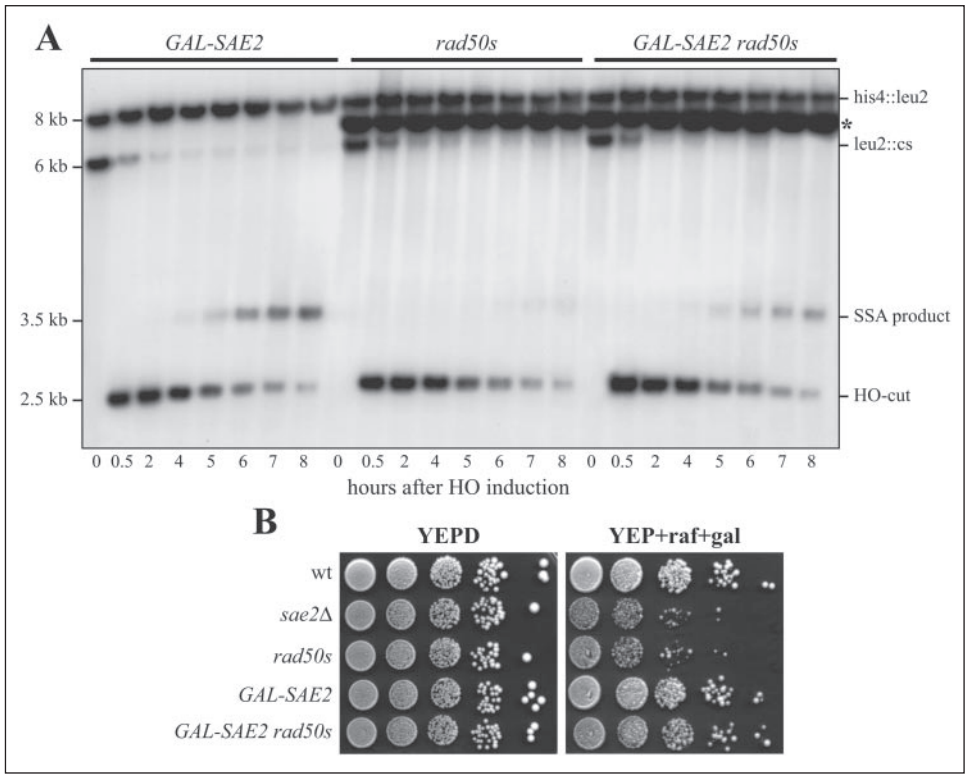

FIGURE 3. **The SSA defects of a *rad50s* mutant are partially suppressed by *SAE2* overexpression.** *A*, HO-cut formation and repair in YMV80 derivative strains. Exponentially growing YEP+raf cell cultures of *GAL-SAE2* YMV80 and isogenic *rad50s* and *GAL-SAE2 rad50s* strains, all carrying the HO-cut site as depicted in Fig. 1 and a galactose-inducible *HO* gene, were arrested in mitosis with nocodazole and then transferred to YEP+raf+gal in the presence of nocodazole at time zero. KpnI-digested genomic DNA prepared from cell samples collected at the indicated time points were subjected to the Southern blot analysis described in the legend to Fig. 1. * denotes the *rad50s::LEU2* construct integrated at the *RAD50* locus that is detected by the probe. *B*, serial dilutions of YMV80 and isogenic *sae2*Δ, *rad50s*, *GAL-SAE2*, and *GAL-SAE2 rad50s* strains were spotted on YEPD and YEP+raf+gal plates that were incubated at 30 °C for 3 days.

ssDNA resection products (named r1–r4) at *leu2::cs* started to accumulate in YMV45 *sae2*Δ cells ~30 min later than in isogenic wild type cells under the same conditions. As expected, the resection products started to decrease in wild type cells (Fig. 4*C*) concomitantly with accumulation of SSA products (Fig. 2*B*). Resection products remained instead stable until the end of the experiment in *sae2*Δ YMV45 cells, which showed very low efficiency of SSA product formation, similarly to what observed in the *sae2*Δ YMV80 strain. Thus, although the lack of Sae2 slows down DSB resection, its effects on DSB repair by SSA likely reflect impairment of other Sae2 functions besides those involved in the generation of 3′-ended tails.

Sae2 Contributes to the Maintenance of Intrachromosomal Association between the Ends of a Broken Chromosome—DSB repair needs the DNA ends flanking the DSB site to remain adjacent to each other, and this requires the MRX complex (23, 24). We therefore asked whether the lack of Sae2 might affect repair of DSBs by impairing not only their resection but also the intrachromosomal association of their ends. We used strains where the DNA proximal to the HO break could be visualized by binding of a LacI-GFP (green fluorescent protein) fusion protein to multiple repeats of the LacI repressor binding site, LacO, integrated at a distance of 50 kb on either side of an HO cleavage site on chromosome VII (Ref. 23 and Fig. 5*A*). To determine the separation efficiency of two unlinked LacO arrays, we used also a strain where the arrays were located both 50 kb telomere-proximal to the HO site on chromosome VII and on chromosome III (Ref. 23 and Fig. 5*C*). Both strains also expressed galactose-inducible HO endonuclease and the LacI-GFP fusion protein, the binding of the latter to the LacO arrays allowing their visualization. To ensure that the broken chromosomal fragments did not attempt recombination, the endogenous HO sites at the *MAT*, *HML*, and *HMR* loci on chromosome III were deleted in both strains (23).

Cultures of the original wild type and isogenic *sae2*Δ derivative strains were arrested in mitosis and kept blocked by nocodazole treatment during break induction by galactose addition. As shown in Fig. 5*A*, the majority of wild type cells containing two LacO arrays flanking the HO site showed a single LacI-GFP spot in both uninduced and break-induced conditions throughout the experiment, because of their ability to hold the broken ends together. In contrast, 33% of the otherwise isogenic *sae2*Δ cells showed two LacI-GFP spots at both 3 and 6 h after HO induction (Fig. 5*A*), with an ~3-fold increase compared with uninduced conditions (Fig. 5*A*, time zero). To rule out the possibility that loss of *SAE2* caused appearance of two LacI-GFP foci by disrupting chromosome structure or sister chromatid cohesion in nocodazole-arrested cells, we performed the same analysis also on isogenic strains carrying a single LacO array 50 kb telomere-proximal to the HO site. As shown in Fig. 5*B*, 90% of *sae2*Δ cells with the unique LacO array showed a single LacI-GFP spot even 3 and 6 h after DSB induction. Thus, the appearance of two LacI-GFP spots in *sae2*Δ cells carrying two LacO arrays flanking the HO site was primarily because of impairment in DSB ends association. In any case, the latter strain did not mimic the wild type and *sae2*Δ control strains carrying two unlinked LacO arrays, which showed ~75% of cells with two LacI-GFP foci in both uninduced and break-induced conditions (Fig. 5*C*), suggesting that some bridging of DSB ends takes place also in the absence of Sae2.

As previously reported, also the MRX complex is involved in bridging DSB ends (Refs. 23 and 24 and Fig. 5). Interestingly, the amount of cells containing two LacO arrays flanking the HO site and showing two LacI-GFP spots after HO-induced DSBs was higher in the *mre11*Δ strain than in the otherwise isogenic *sae2*Δ strain or in the *rad50s* strain that might be specifically impaired in MRX-Sae2 interaction (Fig. 5*A*). However, although appearance of two LacI-GFP foci in a subset of *sae2*Δ cells seemed to be primarily because of defective intrachromosomal DSB ends association (see above), defects other than those affecting bridging of DSB DNA ends appear to contribute to generate two LacI-GFP foci in

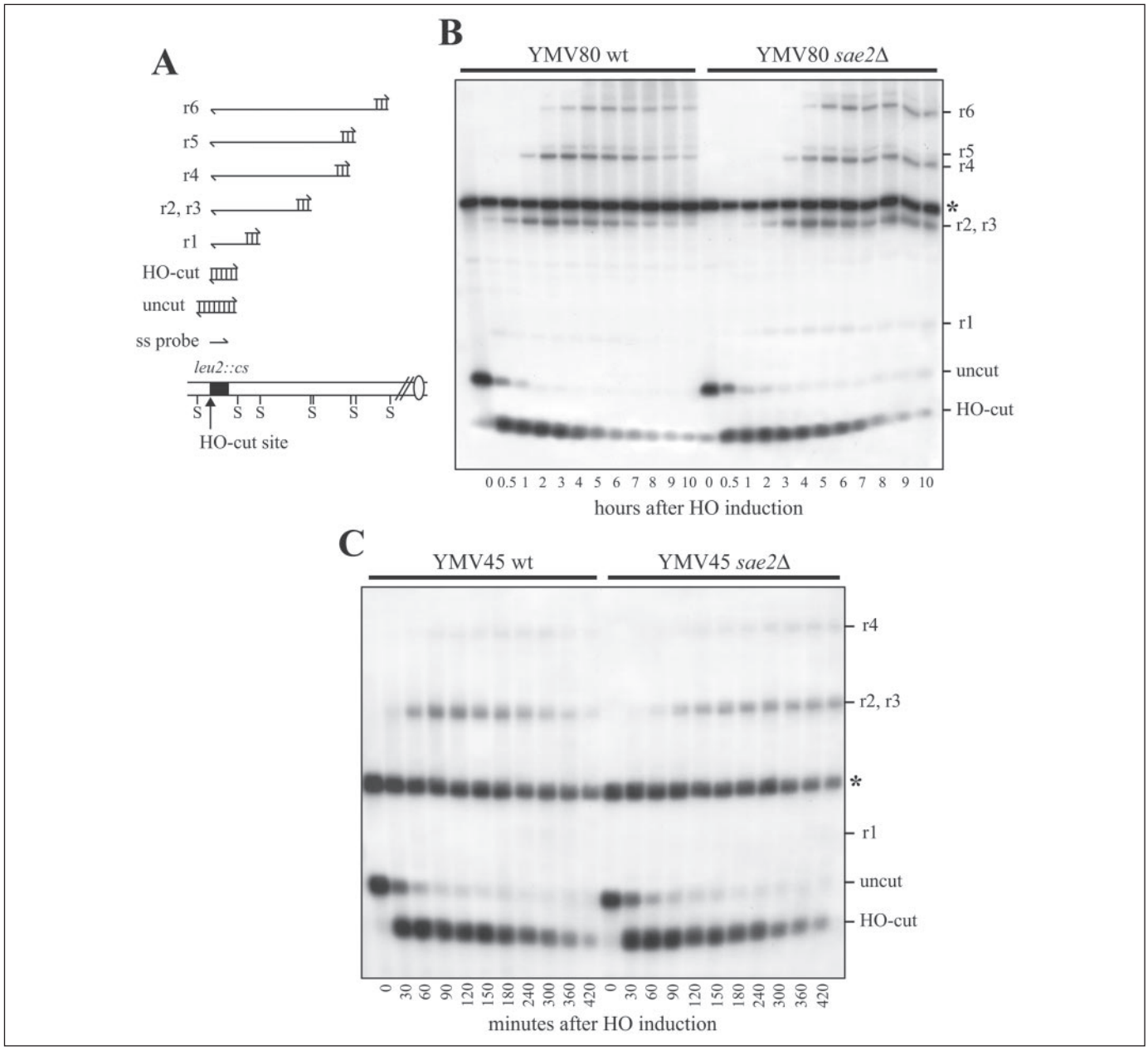

FIGURE 4. **Effects of Sae2 lack on DSB ends resection.** *A*, schematic representation of the method used to detect DSB formation and 5′–3′-resection in the region immediately centromere-proximal to the *leu2*::*cs* DSB site in YMV80 and YMV45 derivative strains. Southern blot analysis with a single-stranded probe specific to the *LEU2* sequence (see "Experimental Procedures") of SspI-digested genomic DNA run on an alkaline-agarose gel reveals a 1.5-kb SspI fragment (*uncut*) in the absence of HO-cut. After HO-cut, the size of this fragment is reduced to 1.1 kb (*HO-cut*), whereas 5′–3′-resection past the SspI sites eliminates cutting at these sites and thus produces larger SspI fragments detected by the probe. Bands labeled r1 through r6 are the products of resection through SspI sites located 2.2, 5.0, 5.2, 8.3, 9.1, and 14.1 kb centromere-proximal to the HO-cut site. *B*, HO-cut and ssDNA formation in YMV80 derivative strains. Genomic DNA prepared from samples taken at the indicated times from the same cell cultures analyzed in Fig. 1 was digested with SspI, separated on alkaline-agarose gel and analyzed as described in *A*. * denotes the distal unresected *his4*::*leu2* SspI fragment that is detected by the probe. *C*, HO-cut and ssDNA formation in YMV45 derivative strains. Genomic DNA prepared from samples taken at the indicated times from the same cell cultures analyzed in Fig. 2 was digested with SspI, separated on alkaline-agarose gel and analyzed as described in *A*. * denotes the distal unresected *leu2* SspI fragment detected by the probe.

*mre11*Δ cells. In fact, a significant amount of *mre11*Δ cells with a single LacO array exhibited two LacI-GFP spots in both uninduced and break-induced conditions (Fig. 5*B*), likely because of impairment in sister chromatid cohesion that has been reported to be defective in *mre11*Δ cells (26, 27).

As shown in Fig. 5*A*, the frequency of *sae2*Δ *mre11*Δ cells containing two LacO arrays flanking the HO site and showing two LacI-GFP spots after HO-induced DSBs was similar to that observed in otherwise isogenic *mre11*Δ cells. Thus, Sae2 and MRX seem to act in the same pathway with respect to intrachromosomal association maintenance.

DISCUSSION

Although Sae2 has been implicated together with the MRX complex in the repair of meiotic DSBs by allowing the endonucleolytic removal of Spo11 from the break sites (30), its function in the cellular response to DSBs during a mitotic cycle is only beginning to be elucidated. Interestingly, Sae2 loss of function has been shown to impair the repair of hairpin-capped DSBs (13) and to increase the percentage of DSB repair events that lead to inverted repeat duplication, without affecting the overall recombination rate (35, 36),

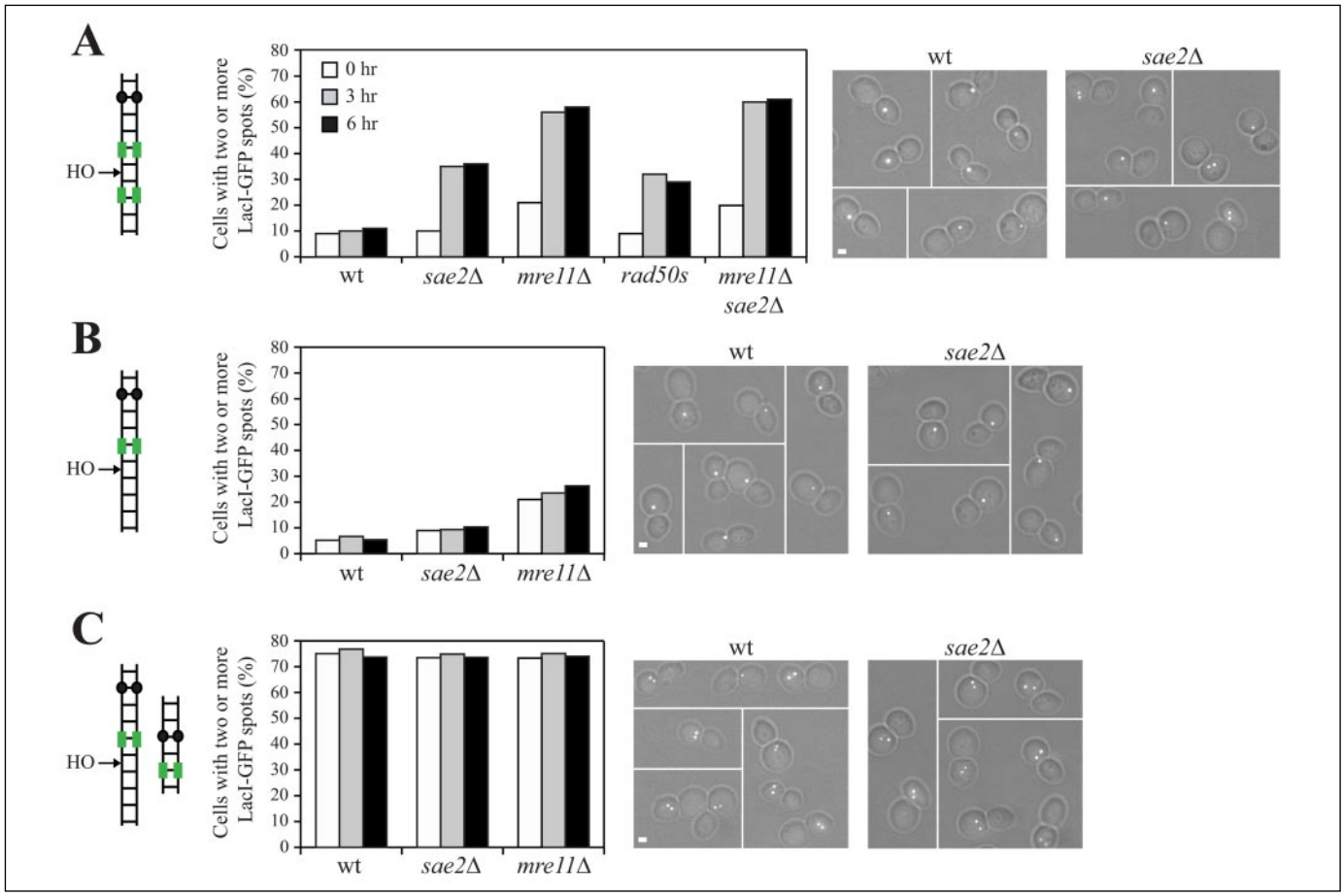

FIGURE 5. **Sae2 contributes to DSB end bridging.** Wild type, *sae2*Δ, *mre11*Δ, *rad50s*, and *mre11*Δ *sae2*Δ strains expressing LacI-GFP and carrying its target sequence LacO (see text for details) and the *GAL-HO* construct were arrested with nocodazole (time zero) and transferred to YEP+raf+gal to induce HO expression in the presence of nocodazole to maintain the cell cycle arrest. As indicated on the *left side* of the figure, where two vertical lines connected by horizontal bars represent sister chromatids with *black circles* indicating the centromeres, strains carried either two LacO arrays (*green boxes*) at 50 kb on either side of the HO-cut site on chromosome VII (*A*), or a single LacO array 50 kb telomere-proximal to the HO-cut site (*B*), or one LacO array 50 kb telomere-proximal to the HO site on chromosome VII and a second unlinked LacO array on chromosome III (*C*) (23). Cell samples taken at the indicated times after HO induction were analyzed with a fluorescence microscope to determine the fraction of cells in each sample that contained LacI-GFP foci separated by >0.5 μm (graphs). The separation distance between foci was measured for 500 cells/sample. Representative pictures of the corresponding wild type and *sae2*Δ cells exhibiting GFP-LacI foci 3 h after HO induction are shown on the *right side* of each panel. *Scale bar* is equal to 1 μm.

suggesting that Sae2 may be involved in ensuring that both DSB ends participate in HR.

Based on the notion that a DSB between two direct repeats stimulates recombination by SSA (2, 5), we have studied the effects of Sae2 loss of function on the repair of an HO-induced DSB occurring between two direct repeats. We show that Sae2 is required to ensure efficient DSB repair by SSA. In fact, SSA repair of HO-induced DSBs is severely impaired in *sae2*Δ cells, although not completely abolished.

Because also the *mrx*Δ mutants show reduced efficiency of DSB repair by SSA (15), Sae2 may influence the SSA mechanism by modulating MRX functions. In contrast to *mrx*Δ mutants that are unable to generate meiotic DSBs (4, 12), both *sae2*Δ and hypomorphic *rad50s* mutants allow meiotic DSB formation but do not allow their resection (30–33), suggesting that the Rad50s protein variant might specifically impair MRX-Sae2 interactions. In agreement with this hypothesis, we found that the SSA defects of *sae2*Δ cells are undistinguishable from those of a *rad50s* mutant. Importantly, high levels of Sae2, which are not able to restore SSA in a *rad50*Δ mutant,[4] partially suppress *rad50s* SSA defects and cell death after an HO-induced DSB. This suggests that Sae2 may play some roles in regulating MRX activity and that the Rad50s amino acid substitutions, which all map to a region on the surface of the protein that is different from both the catalytic and the DNA binding domains (44), may cause loss of Sae2 functions, possibly by disrupting Rad50-Sae2 interaction.

We also examined the molecular mechanisms underlying the reduced efficiency of SSA in the absence of Sae2. Because the HO break within direct repeats can undergo SSA repair only once the 5′–3′-resection has uncovered both homologous sequences, the lack of Sae2 may affect DSB resection and the subsequent repair through SSA. We found that the resection of the HO-induced DSB is slowed down in the absence of Sae2, indicating that Sae2 is involved in generating 3′-ended ssDNA.

Because *mrx*Δ mutants slow down DSB resection (15), Sae2 may affect processing of the 5′-DNA ends by altering MRX functions. However, the lack of Sae2 does not seem to affect MRX complex formation (45), whereas several data suggest that it likely impairs MRX nuclease activity. In fact, both *sae2*Δ cells and *mre11* nuclease-defective mutants are impaired in repairing hairpin-capped DSBs (13) and in resecting meiotic DSBs (19, 30–33). Moreover, both mutants show defective Mre11 dissociation from unprocessed meiotic DSBs (10) and from DNA lesions induced by γ-rays (11), suggesting that Mre11 release from DSBs requires its nuclease activity and that the lack of Sae2 may freeze

[4] M. Clerici, D. Mantiero, G. Lucchini, and M. P. Longhese, unpublished observations.

Mre11 at the DNA-damaged sites by altering its nuclease function. Because Rad50 has been proposed to undergo an ATP-driven conformational switch that is well suited to prepare DNA ends/hairpins for nucleolytic cleavage by Mre11 (44), lack of Sae2 may affect Mre11 nuclease activity either directly or by altering Rad50 conformational switch. However, some of the nuclease-defective *mre11* mutants do not seem to be significantly affected in mitotic DSB resection (14, 19–21), suggesting that Sae2 may impair this process also by altering MRX functions other than its nuclease activity.

In any case, although the resection products in *sae2*Δ cells appear 30–60 min later than in wild type, they accumulate and remain stable for several hours, suggesting that the slowing down of DSB resection cannot be the sole cause of SSA defects. Indeed, we show that Sae2 is also required for intrachromosomal bridging of DSB ends, which have been previously shown to remain associated until the break is repaired by the action of the MRX complex (23, 24). In fact, *SAE2* deletion causes dissociation of broken ends in a significant subset of cells by acting in the same pathway of the MRX complex (Fig. 5). Because some DNA repair options are lost when DNA ends do not remain adjacent to each other, this raises the possibility that the reduced bridging of the DSB ends observed in *sae2*Δ cells may account for the poor efficiency of SSA in repairing HO-induced lesions. Interestingly, Sae2 does not appear to be involved in ensuring sister chromatid cohesion (Fig. 5), which instead requires the MRX complex (26, 27). The MRX-dependent intrachromosomal association has been shown to occur independently of Mre11 nuclease activity but to require the Cys-Xaa-Xaa-Cys zinc-hook motifs located in the coiled-coil region of Rad50, which in turn does not seem to be involved in ensuring sister chromatid cohesion (46). Therefore, the lack of Sae2 might specifically affect the MRX function in DSB end bridging.

Altogether, our data indicate that Sae2 participates in maintaining genome stability by ensuring the efficiency of DSB repair, possibly by modulating a subset of MRX functions. Finding Sae2 functional homologues in other species will be a challenging issue for future research and will increase our overall knowledge of the mechanisms preserving genetic integrity in eukaryotic cells.

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The Saccharomyces cerevisiae Sae2 Protein Promotes Resection and Bridging of Double Strand Break Ends

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