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Distinct Roles of Mus81, Yen1, Slx1-Slx4, and Rad1 Nucleases in the Repair of Replication-Born Double-Strand Breaks by Sister Chromatid Exchange

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Most spontaneous DNA double-strand breaks (DSBs) arise during replication and are repaired by homologous recombination (HR) with the sister chromatid. Many proteins participate in HR, but it is often difficult to determine their *in vivo* functions due to the existence of alternative pathways. Here we take advantage of an *in vivo* assay to assess repair of a specific replication-born DSB by sister chromatid recombination (SCR). We analyzed the functional relevance of four structure-selective endonucleases (SSEs), Yen1, Mus81-Mms4, Slx1-Slx4, and Rad1, on SCR in *Saccharomyces cerevisiae*. Physical and genetic analyses showed that ablation of any of these SSEs leads to a specific SCR decrease that is not observed in general HR. Our work suggests that Yen1, Mus81-Mms4, Slx4, and Rad1, but not Slx1, function independently in the cleavage of intercrossed DNA structures to reconstitute broken replication forks via HR with the sister chromatid. These unique effects, which have not been detected in other studies unless double mutant combinations were used, indicate the formation of distinct alternatives for the repair of replication-born DSBs that require specific SSEs.

Double-strand breaks (DSBs) are among the most harmful DNA lesions. Failure to repair DSBs is often associated with apoptosis, aging, and cancer in metazoans and can lead to different types of genome instability in all organisms, including high mutation frequency, chromosome rearrangements, or chromosome loss. As a consequence, cells have developed a variety of specialized and complex mechanisms for DSB repair, defined as nonhomologous end joining (NHEJ) and homologous recombination (HR). In contrast to NHEJ, which works preferentially in nondividing cells at the G_1 stage of the cell cycle, HR is the major DSB repair mechanism occurring at the S/G2 phase. In particular, HR is responsible for the repair of breaks that are associated with DNA replication (1). Understanding the mechanisms of HR and the proteins that catalyze these reactions is therefore central to our understanding of cell proliferation and associated pathological states and diseases.

A key step in HR is the resolution of crossed-stranded DNA structures formed during DNA strand exchange (28, 41, 53). D-loops formed by Rad51-mediated DNA strand exchange may lead to the formation of double Holliday junctions (HJs), which can be resolved in two ways (17, 28): (i) by dissolution catalyzed by the Sgs1/BLM-Top3 helicase-topoisomerase complex (54); (ii) by endonucleolytic cleavage mediated by structure-selective endonucleases (SSEs) (28, 32, 35, 38). Four conserved SSEs, Mus81-Mms4, Slx1-Slx4, Yen1, and Rad1-Rad10, have been identified in *Saccharomyces cerevisiae*, and their biochemical activities have been studied extensively (15, 20, 21, 31). The *in vivo* roles of Mus81-Mms4, Slx1-Slx4, and Yen1, however, remain to be determined. One complication has been their apparent functional overlap and the presence of alternative pathways by which recombination intermediates can be resolved.

Mus81-Mms4 (Mus81-Eme1 in *Schizosaccharomyces pombe*) is a conserved member of the XPF family of heterodimeric nucleases (9) that is required for maturation of recombination intermediates that lead to meiotic crossovers in different organisms (6, 30, 47) and for efficient DNA repair in mitotic cells and after replicative stress (14, 27). Mus81-Mms4 preferentially cleaves D-loops, 3′-flap structures, and nicked HJs (16, 23, 33, 39, 50). Mutations in Mus81-Mms4 are synthetically lethal with mutations in the Sgs1-Top3-Rmi1 complex, which is involved in dissolution of double Holliday junctions (4, 7, 17). The fact that this synthetic lethality is suppressed by a defect in HR (*rad51*, *rad52*, *rad55*, *rad57*, *rad54*) (4, 17) provides genetic evidence that Mus81-Mms4 cleaves a recombination-dependent joint molecule (29). Slx1-Slx4 preferentially cleaves 5′-flap structures in yeast (12, 21), but the human counterpart has been proposed to have HJ resolvase activity (3, 18, 37, 48). In *Drosophila melanogaster* and *Caenorhabditis elegans*, Slx1-Slx4 is required for meiotic crossover formation (44, 46, 55). Interestingly, Slx1-Slx4 was defined by mutations leading to synthetic lethality with *sgs1* (36) in a screen in which *mus81* and *mms4* were also obtained, but the lethality was not rescued by *rad51*Δ mutations. As a consequence, despite its *in vitro* activities, it appeared unlikely that Slx1-Slx4 had a sole function in HR. Moreover, Slx4 seems to provide a platform for the association of various SSEs, including MUS81-EME1 in humans and the nucleotide excision repair (NER) endonuclease Rad1-Rad10 (18, 19), adding a further complication to understand the specific *in vivo* role of Slx4. Rad1-Rad10, on the other hand, is a 3′-flap endonu-

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TABLE 1 Yeast strains used in this study

Strain	Genotype	Reference or source
WSR-7D	*MAT***a**-*inc trp1-1 ura3-1 ade2-1 his3-11,15 can1-100 ade3*::*GAL-HO leu2*::*SFA1*	This study
WSR-M81	WSR-7D *mus81*Δ::*HphMX4*	This study
WSR-Y1	WSR-7D *yen1*Δ::*KanMX4*	This study
WSR-M81Y1	WSR-7D *mus81*Δ::*HphMX4 yen1*Δ::*KanMX4*	This study
WSR-M81DD	WSR-7D *bar1*Δ::*HphMX4 mus81-dd ura3-1 or ura3*Δ::*loxP*	This study
WSR-S4	WSR-7D *slx4*Δ::*NatMX6*	This study
WSR-S4M81	WSR-7D *slx4*Δ::*NatMX6 mus81*Δ::*HphMX4*	This study
WSR-S4Y1	WSR-7D *slx4*Δ::*NatMX6 yen1*Δ::*KanMX4*	This study
WSR-S4M81Y1	WSR-7D *slx4*Δ::*NatMX6 mus81*Δ::*HphMX4 yen1*Δ::*KanMX4*	This study
WSR-S1	WSR-7D *slx1*Δ::*NatMX6*	This study
WSR-R1	WSR-7D *rad1*Δ::*KanMX4*	This study
WSR-S1R1	WSR-7D *slx1*Δ::*NatMX6 rad1*Δ::*KanMX4*	This study
WSR-S4R1	WSR-7D *slx4*Δ::*NatMX6 rad1*Δ::*KanMX4*	This study
WSR-R1M81	WSR-7D *rad1*Δ::*KanMX4 mus81*Δ::*HphMX4*	This study
WSR-S1M81	WSR-7D *slx1*Δ::*NatMX6 mus81*Δ::*HphMX4*	This study
WSR-S1R1M81	WSR-7D *slx1*Δ::*NatMX6 rad1*Δ::*KanMX4 mus81*Δ::*HphMX4*	This study
WSR-Y1R1M81	WSR-7D *yen1*Δ::*KanMX4 rad1*Δ::*KanMX4 mus81*Δ::*HphMX4*	This study
WSR-S1Y1R1M81	WSR-7D *slx1*Δ::*NatMX6 yen1*Δ::*KanMX4 rad1*Δ::*KanMX4 mus81*Δ::*HphMX4*	This study

clease that, aside from its role in NER, participates in the single-strand annealing repair pathway of DSBs, and it also has *in vitro* SSE activity (40, 45).

Recently, the *S. cerevisiae* Yen1 and human GEN1 proteins were identified and shown to resolve HJs by a symmetrical cleavage mechanism similar to that mediated by the bacterial RuvC resolvase (31, 42). Single *yen1*Δ mutants show no or very subtle defects in either mitotic or meiotic recombination in *S. cerevisiae*, and *Schizosaccharomyces pombe* lacks a Yen1 ortholog. However, *S. cerevisiae mus81*Δ *yen1*Δ double mutants display additional sensitivities to DNA-damaging agents and show reduced levels of crossovers compared to the single mutants, suggesting that Mus81-Mms4 and Yen1 might have possibly overlapping functions in DSB repair (5, 29, 49). Consistent with this, overexpression of human GEN1 can rescue the meiotic phenotype of an *S. pombe mus81* mutant (34).

To assay the *in vivo* role of SSEs in HR as the main mechanism of DSB repair associated with replication, we have taken advantage of an *in vivo* assay that assesses the repair of a specific replication-born DSB by sister chromatid exchange or recombination (SCE or SCR). Using physical and genetic analyses, we show that ablation or inactivation of the catalytic domains of Mus81-Mms4 and Yen1, as well as ablation of Slx4 and Rad1, but not of Slx1, leads to a clear and specific decrease of SCR that is not observed in spontaneous general HR in budding yeast. Our work suggests that these nucleases act *in vivo* on specific DNA intermediates generated during the repair of replication-born DSBs and provides evidence for an independent function of these SSEs in DSB repair that may relate to the resolution of specific DNA intermediates.

MATERIALS AND METHODS

Strains and plasmids. Yeast strains used in this study are listed in Table 1. All strains were made in the W303 (*RAD5*$^+$) genetic background. Mutants were obtained by gene replacement with the KanMX4, NatMX6, or HphMX4 cassettes as described and confirmed by PCR and Southern analyses. The *mus81-dd* (*mus81-D414A,D415A*) mutation was transplaced to the genome by exact gene replacement using *mus81-D*::*URA3*$^{K.\ lactis}$ and transformation with a linear fragment containing *mus81-dd*. Plasmid pRS316TINV containing the 24-bp mini-HO site inserted at the EcoRI site of one of the inverted *LEU2* repeats was described previously (10, 24). Plasmids pAG414GPD-Yen1^{E193AE195A}-HA and pAG414GPD-Yen1-HA, containing different alleles of *YEN1* under the control of the *GPD1* promoter, were generated by Gateway cloning (Invitrogen) using pAG414GPD-ccdB-HA (2) as the destination vector in a similar way as described previously (5). Plasmid pWDH800 (GST-Mms4/HIS10-FLAG-Mus81) containing Mus81-Mms4 under the *GAL1,10* promoter was generated by insertion of a *TRP1* marker into an NcoI site of plasmid pWDH595 (15). Plasmid pWDH815 GST-Mms4/HIS10-FLAG-*mus81-D414A,D415A*, carrying the catalytically deficient form of Mus81, was generated by site-directed mutagenesis using specific primers oIWSD374 (5′-TAGTTGAAAGAAAAAGGCTAGCCGCTTT-3′) and olWDH375 (5′-TCCCTTATACTTAAAGCTAAAGCGGCTA-3′).

Genetic analysis of recombination. Recombination frequencies are the median values of fluctuation tests performed with six independent yeast colonies each from each transformant analyzed. For every genotype, the fluctuation test was repeated three times with three different yeast transformants. The final frequency shown for each genotype corresponds to the mean value of the three median frequencies obtained from the tests. For the analysis of HO-mediated DSB recombination mid-log-phase yeast cells carrying the HO gene under the control of *GAL1* were obtained from SC–3% glycerol, 2% lactate liquid cultures and split into halves. One-half was maintained in liquid SC–3% glycerol, 2% lactate (no HO expression), and the other was cultured in SC–2% galactose for 5 h for transient expression of HO, before performing fluctuation tests. In all cases doxycycline was used to avoid *leu2-HOr* expression, because we previously found that HO cuts more efficiently under these conditions (25). Recombinants were selected on SC-leu-ura containing 2% glucose.

Physical analysis of sister chromatid recombination. Sister chromatid recombination kinetic assays were carried out essentially as described previously (10, 24).

RESULTS

Mus81-Mms4 and Yen1 have independent roles in the repair of replication-born DSBs by SCR. To assay the impact of Mus81-Mms4 and Yen1 in SCR induced by a replication-born DSB, we used pRS316-TINV, a plasmid containing the inverted TINV system carrying a 24-bp mini-HO site (Fig. 1A) (24). We previously showed that HO endonuclease cleaves this site preferentially in one strand, leading to a replication-induced DSB (10). Due to the low efficiency of HO cleavage at this site, the DSB occurs in only

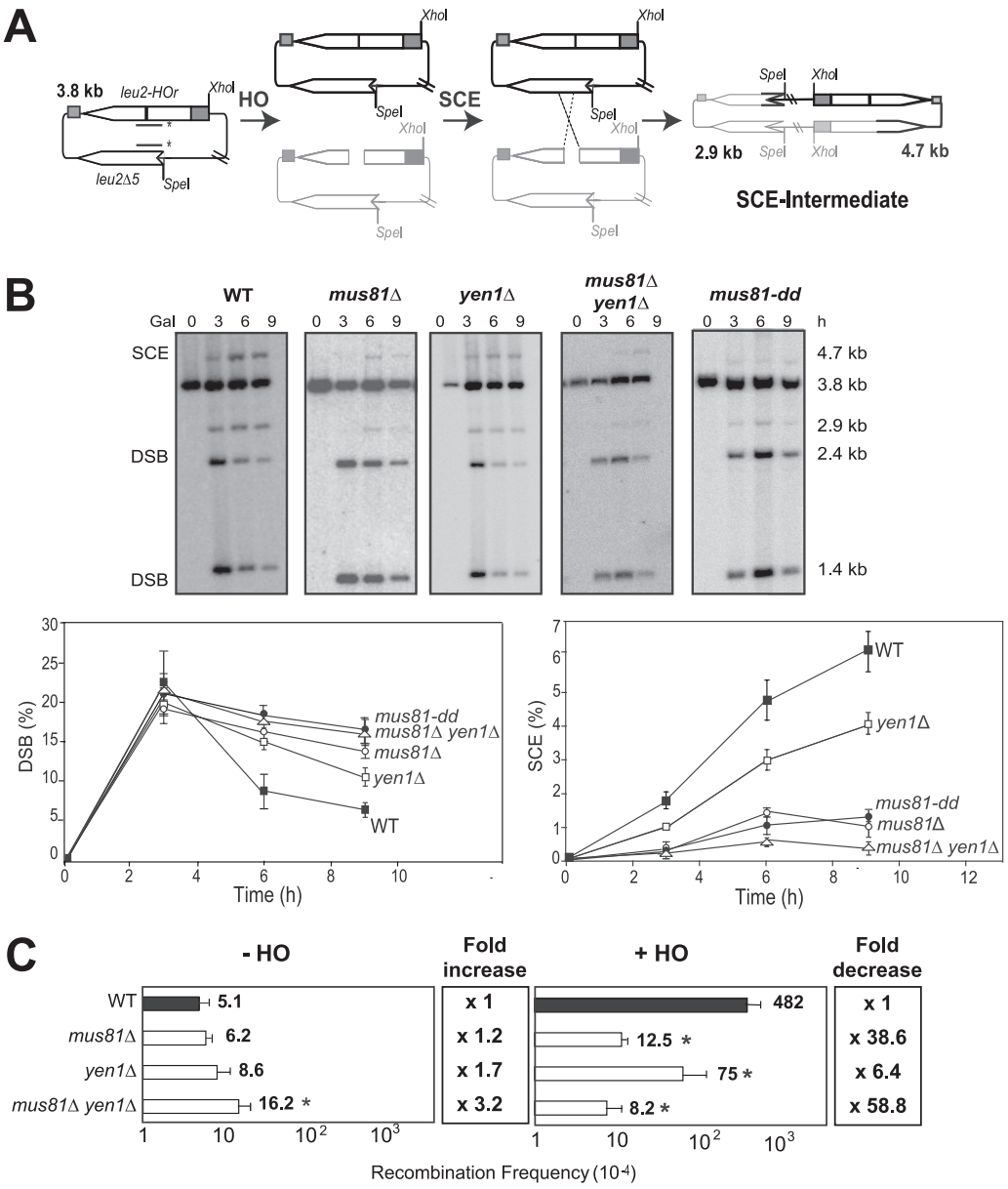

FIG 1 Molecular analysis of the effects of *mus81* and *yen1* mutations in SCE. (A) Schemes of plasmid pRS316-TINV and the intermediates produced by SCE after HO cleavage. Sizes of the XhoI-SpeI bands detected with the *LEU2* probe (line with asterisks) are indicated. SCR intermediates physically detected correspond to an unstable dicentric plasmid that it is not recovered as a final product in Leu$^+$ recombinant colonies. (B) Kinetics and quantification of DSBs and SCE intermediates after different times of HO induction in galactose in the following strains: wild type (WT; WSR-7D), *mus81*Δ (WSR-M81), *yen1*Δ (WSR-Y1), and *mus81*Δ *yen1*Δ (WSR-M81Y1) and the catalytic mutant *mus81-dd* (WSR-M81DD). A representative Southern analysis is shown for each genotype analyzed. Quantification of DSBs (1.4-kb and 2.4-kb bands) and SCE (4.7-kb band) was calculated relative to the total DNA of each lane. Averages and standard deviations (bars) of at least three independent experiments are shown for each time point and genotype. (C) Effects of *mus81*Δ and *yen1*Δ in spontaneous recombination (-HO) and DSB-induced SCE (+HO) frequencies, as determined with Leu$^+$ recombinants, using the inverted repeat system TINV after 5 h of HO activation with 2% galactose, working with the same strains as in panel B. Each value represents the average of three median values obtained from three different fluctuation tests, each performed with 6 independent colonies from three different transformants for each genotype. Asterisks indicate statistically significant differences compared to wild type according to Student's *t* test ($P < 0.001$).

one chromatid, which may then be repaired by HR with the intact sister. We previously showed that the measurement of unequal SCR with the inverted repeat in the sister chromatid is a reliable measurement of SCR, as a valid and direct measurement of equal SCR (24). Genetically, the TINV system can detect Leu$^+$ recombination events, which occur mainly by recombination with the sister chromatid, even though other mechanisms can also occur at a lower efficiency (25). However, spontaneous recombination, which can be initiated in different ways and at different sites, may occur at high frequencies by multiple intrachromatid events, including reciprocal exchange, gene conversion, and break-induced replication (BIR) plus single-strand annealing (SSA) events (41). Therefore, DSB-induced Leu$^+$ events can be taken as an approximate genetic measurement of SCR, whereas spontaneous Leu$^+$

events measure different types of recombination events, many of which do not involve the sister chromatid (24).

To determine the effect of *mus81*Δ and *mms4*Δ on SCR, we first performed a physical analysis of DSB formation and SCR in the TINV system. We performed a kinetic analysis of SCR in a time frame of 9 h under HO induction (Fig. 1B). DSBs accumulated to similar levels in the wild type, *mus81*Δ and *yen1*Δ single mutants, and the *mus81*Δ *yen1*Δ double mutant. However, DSB levels remained high for longer periods in the mutants than in the wild type, suggesting a lower capacity to repair them. Direct analysis of SCR by quantification of the 4.7-kb SCR band revealed that SCR was significantly impaired in both mutants. SCR impairment was stronger in *mus81*Δ than in *yen1*Δ cells, with a further but relatively small additional defect observed in the double mutant.

For the genetic analysis of Leu$^+$ recombinants, DSBs were induced for only 5 h to avoid saturation of recombinants, as previously described (25). As shown in Fig. 1C, whereas spontaneous recombination was unaffected in the single mutants, a slight 3-fold increase was observed in the *mus81*Δ *yen1*Δ double mutant. This might have been a consequence of either replication failures or the SCR defect itself, given that a lower capacity to use the sister chromatid as repair template choice could channel repair into other recombination events. DSB-induced SCR was significantly decreased in both the *mus81*Δ (38.6-fold) and *yen1*Δ (6.4-fold) single mutants and in the double mutant (58.8-fold) with respect to the wild type. The difference between double *mus81*Δ *yen1*Δ and single *mus81*Δ mutants, however, was not statistically significant (Student's *t* test, $P = 0.087$). Altogether, these results indicate an inability of the mutant cells to promote efficient DSB repair by SCR.

Yen1 and Mus81-Mms4 can replace the function of one another if overexpressed. Although our results suggested that the double mutant *mus81*Δ *yen1*Δ showed a stronger reduction in genetic SCR products than the single mutant *mus81*Δ, the difference was not statistically significant to allow the conclusion that Yen1 can substitute for the function of Mus81 in SCR. This contrasts with the results reported for DNA damage sensitivity or mitotic recombination between homologs, in which only double mutant combinations reveal a phenotype for *yen1*Δ (5, 29). To assay the level of functional overlap of both proteins in SCR, we determined the capacities of Mus81-Mms4 and Mus81dd-Mms4 to substitute for the role of Yen1 (where Mus81dd indicates the catalytic mutant *D414A,D415A*). For this, we overexpressed the wild-type Mus81-Mms4 and the catalytically inactive Mus81dd-Mms4 in wild-type and *yen1*Δ cells as well as in *mus81*Δ controls and analyzed SCR. Physical analysis of SCR showed that, whereas Mus81-Mms4 overexpression complemented the SCR defect of both *yen1*Δ and *mus81*Δ strains, Mus81dd-Mms4 overexpression did not (Fig. 2A; see also Fig. S1 in the supplemental material). Notably, Mus81dd-Mms4 overexpression decreased SCR in both wild-type and *yen1*Δ cells, consistent with Mus81dd-Mms4 interfering with endogenous Mus81.

Genetic analysis of Leu$^+$ recombination events revealed that spontaneous recombination was not significantly affected in any of the strains analyzed (Fig. 2B). Consistent with the physical analysis, the defect in HO-induced SCR Leu$^+$ events in *mus81*Δ cells was complemented by overexpression of Mus81-Mms4 but not by Mus81dd-Mms4. Furthermore, Mus81-Mms4 overexpression, but not Mus81dd-Mms4 overexpression, suppressed the SCR defect of *yen1*Δ strains, raising the levels of Leu$^+$ recombination to wild-type levels. Mus81dd-Mms4 overexpression did not affect the levels of Leu$^+$ recombinants in wild-type and *yen1*Δ cells, which may be explained based on the genetic assay measuring other recombination events in addition to SCR. Altogether, these results suggest that the catalytic activity of Mus81-Mms4 can replace the loss of Yen1 function.

As overexpression of Mus81-Mms4 can replace Yen1 in the SCR assay, we wondered whether the reciprocal was also the case. For this, we overexpressed Yen1 and its catalytically inactive Yen1-ee form in *mus81*Δ strains as well as in the wild type and *yen1*Δ control. Physical analysis of SCR showed that overexpression of the active form of Yen1, but not of inactive Yen1-ee, suppressed the *mus81*Δ mutant defect (Fig. 3A). Interestingly, the overexpression of Yen1-ee in wild-type and *mus81*Δ cells did not result in a decrease of SCR levels, as it occurred with Mus81dd-Mms4 overexpression in wild-type and *yen1*Δ cells. This difference can be explained by the distinct relevance of both endonucleases for SCR, as the defect of *yen1*Δ cells is weaker than that of *mus81*Δ (Fig. 1B), or by the ability of high overexpression of Mus81dd to mimic the *mus81*Δ phenotype. Consistently, as shown in Fig. 3B, no effect was observed in spontaneous Leu$^+$ recombination in any of the strains tested, with the exception of *mus81*Δ. This may be consistent with the fact that Yen1-ee retains DNA binding activity and when overexpressed can displace the wild-type Yen1 from the repair site (5). However, while the frequency of Leu$^+$ SCR events after Yen1-ee overexpression had no effect in wild-type, *mus81*Δ, or *yen1*Δ strains, the overexpression of catalytically active Yen1 elevated the frequency of Leu$^+$ recombinants to wild-type levels in both *yen1*Δ and *mus81*Δ strains (Fig. 3B). This result indicates that the role of Yen1 in SCR is mediated by its nuclease activity and that overexpression of Yen1 can compensate for loss of Mus81-Mms4 function in SCR as a mechanism of repair for replication-born DSBs.

Slx4 is required for SCR. Next, we assayed whether Slx4 was also required for the repair of replication-born DSBs by SCR. Physical analysis of the TINV inverted repeat system showed that the levels of DSBs reached in different *slx4*Δ mutant combinations were the same as in wild-type cells after 3 h of HO induction but remained higher during the time course, consistent with a repair deficiency. Importantly, SCR was heavily impaired in the single *slx4*Δ mutants (Fig. 4A). The levels were equally low in double and triple mutant combinations of *slx4*Δ with *mus81*Δ and *yen1*Δ. Therefore, we conclude that Slx4 is required for SCR independently of the presence or absence of Yen1 and/or Mus81-Mms4.

The genetic analysis showed that *slx4*Δ has a minor impact (2-fold increase above wild-type levels) on spontaneous Leu$^+$ recombination levels in the same TINV system (Fig. 4B). The same levels were obtained in double mutants with *yen1*Δ and *mus81*Δ, as well as in the triple mutant lacking the three SSEs, implying that their roles are independent. HO-induced Leu$^+$ recombination was reduced 7- to 8-fold in the single *slx4*Δ mutant and the *slx4*Δ *mus81*Δ and *slx4*Δ *yen1*Δ double mutants, consistent with a defect in SCR. Interestingly, *slx4*Δ suppressed the strong defect in generating Leu$^+$ recombinants observed in *mus81*Δ (38.6-fold for *mus81*Δ [Fig. 1C] and 7.4-fold for *mus81*Δ *slx4*Δ [Fig. 4B]). However, no effect on hydroxyurea (HU) sensitivity and only a slight suppression in methyl methanesulfonate (MMS) sensitivity were observed (see Fig. S2 in the supplemental material). Triple mutants *yen1*Δ *mus81*Δ *slx4*Δ showed the same strong reduction in HO-induced Leu$^+$ recombinants (56.7-fold) as the double *yen1*Δ

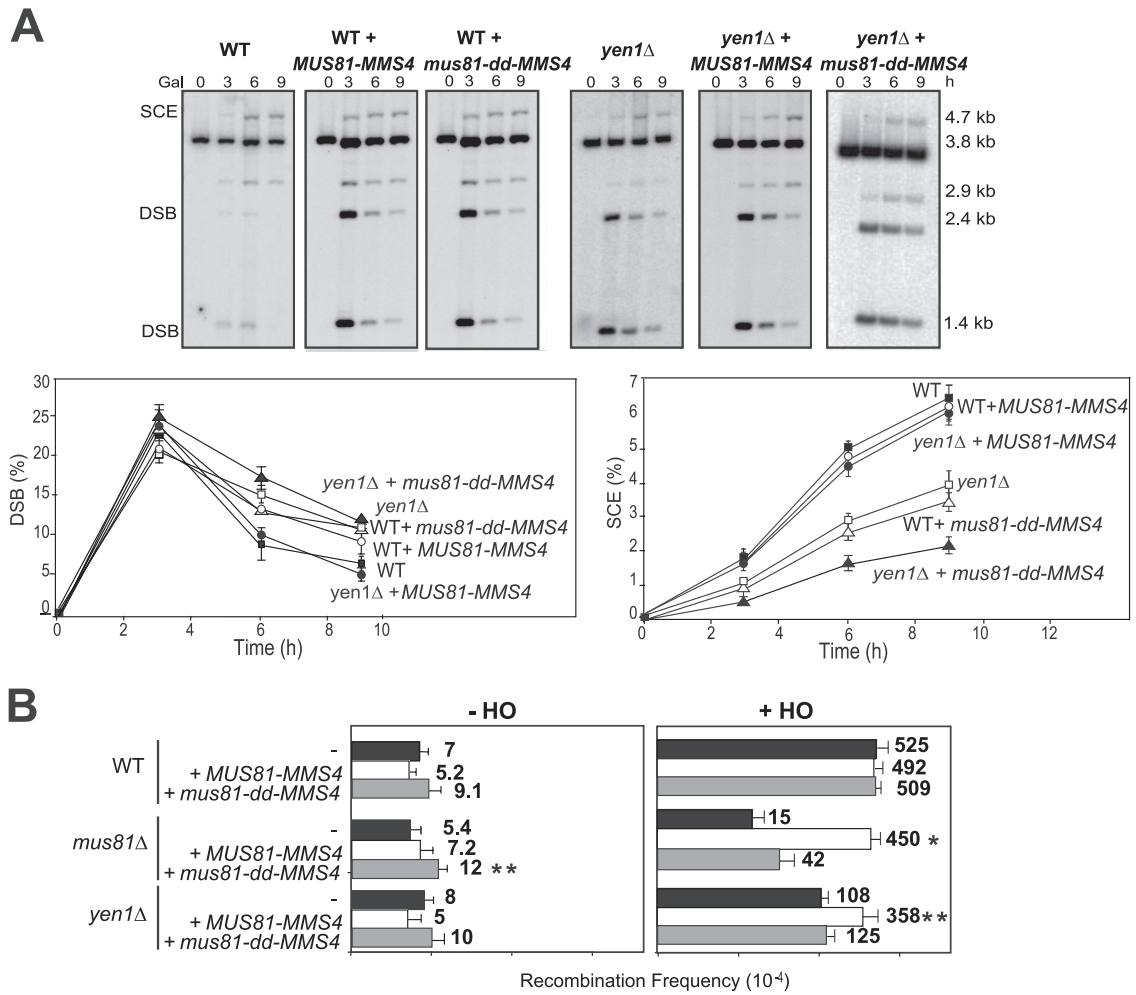

FIG 2 Genetic and physical analyses of the effects of *MUS81-MMS4* overexpression in SCR in *yen1*Δ strains. (A) Physical analysis of the effects of *MUS81-MMS4* overexpression in DSB-induced SCE. Kinetics of DSBs and SCE intermediates in isogenic wild-type (WSR-7D), *mus81*Δ (WSR-M81), and *yen1*Δ (WSR-Y1) strains transformed with pWDH800 carrying the active heterodimer *MUS81-MMS4* or the catalytically inactive heterodimeric *mus81-dd-MMS4* under the control of the *GAL1,10* promoter. (B) Effects of *MUS81-MMS4* overexpression on spontaneous recombination (-HO) and DSB-induced SCE (+HO) frequencies in the TINV inverted repeat system. Wild-type (WT) and mutant strains were transformed with empty vector pRS314 (-) or with pWDH800 carrying either active heterodimeric *MUS81-MMS4* or catalytically inactive heterodimeric *mus81-dd-MMS4* under the control of the *GAL1,10* promoter. Asterisks indicate statistically significant differences between the strains carrying either the active *MUS81-MMS4* or the catalytically inactive *mus81-dd-MMS4* and the strains with the empty vector, according to Student's *t* test (*, $P < 0.001$; **, $P < 0.005$). Other details for the experiment were those described for Fig. 1.

*mus81*Δ (compare Fig. 4B and 1C), while MMS and HU sensitivities were higher in the triple mutant (see Fig. S2). These results are consistent with the physical analysis, which showed that Slx4 is required for the repair of replication-born DSBs via SCR but does not uncover an additional SCR pathway beyond those defined by Mus81-Mms4 and Yen1.

To determine whether overexpression of Yen1 and Mus81-Mms4 suppressed the SCR defect of *slx4*Δ, we determined the effect on SCR by genetic analysis. As shown in Fig. 5, the spontaneous increase in recombination of *slx4*Δ cells was reduced by overexpression of either Yen1 or Mus81-Mms4. Accordingly, the same occurred in the double and triple mutants, suggesting that Yen1 and Mus81-Mms4 facilitate repair with alternative donors. However, when DSB-induced SCR was analyzed, overexpression of Yen1 suppressed the defects of single, double and, to a lesser extent, triple mutants. These results showed that Yen1 overexpression can almost completely suppress the effects provoked by the absence of Mus81-Mms4 or Slx4 (Fig. 5). Mus81-Mms4 overexpression had little or no effect on single *slx4*Δ or double *mus81*Δ *slx4*Δ mutants. A partial rescue of the defect of the *yen1*Δ *slx4*Δ double and *mus81*Δ *yen1*Δ *slx4*Δ triple mutants was observed, as expected from the capacity of Mus81-Mms4 overexpression to suppress *yen1*Δ defects (Fig. 2B). These data showed that overexpression of Yen1, but not of Mus81-Mms4, can suppress the SCR defect in *slx4*Δ mutants.

Rad1, but not Slx1, is required for repair of replication-born DSBs by SCR. Slx4 may work as a platform for the action of SSEs, such as Rad1-Rad10 (XPF-ERCC1), or together with Slx1 as a protein complex with a function in DNA junction cleavage (18, 19). Although there is no evidence yet that this happens in yeast, it was important to determine whether Slx4 action on SCR could be due to a possible function as a Slx4-Slx1 resolvase, and whether Rad1, which has been reported to have *in vitro* cleavage activity on HJs (26), could have a role in SCR. As can be seen in Fig. 6A,

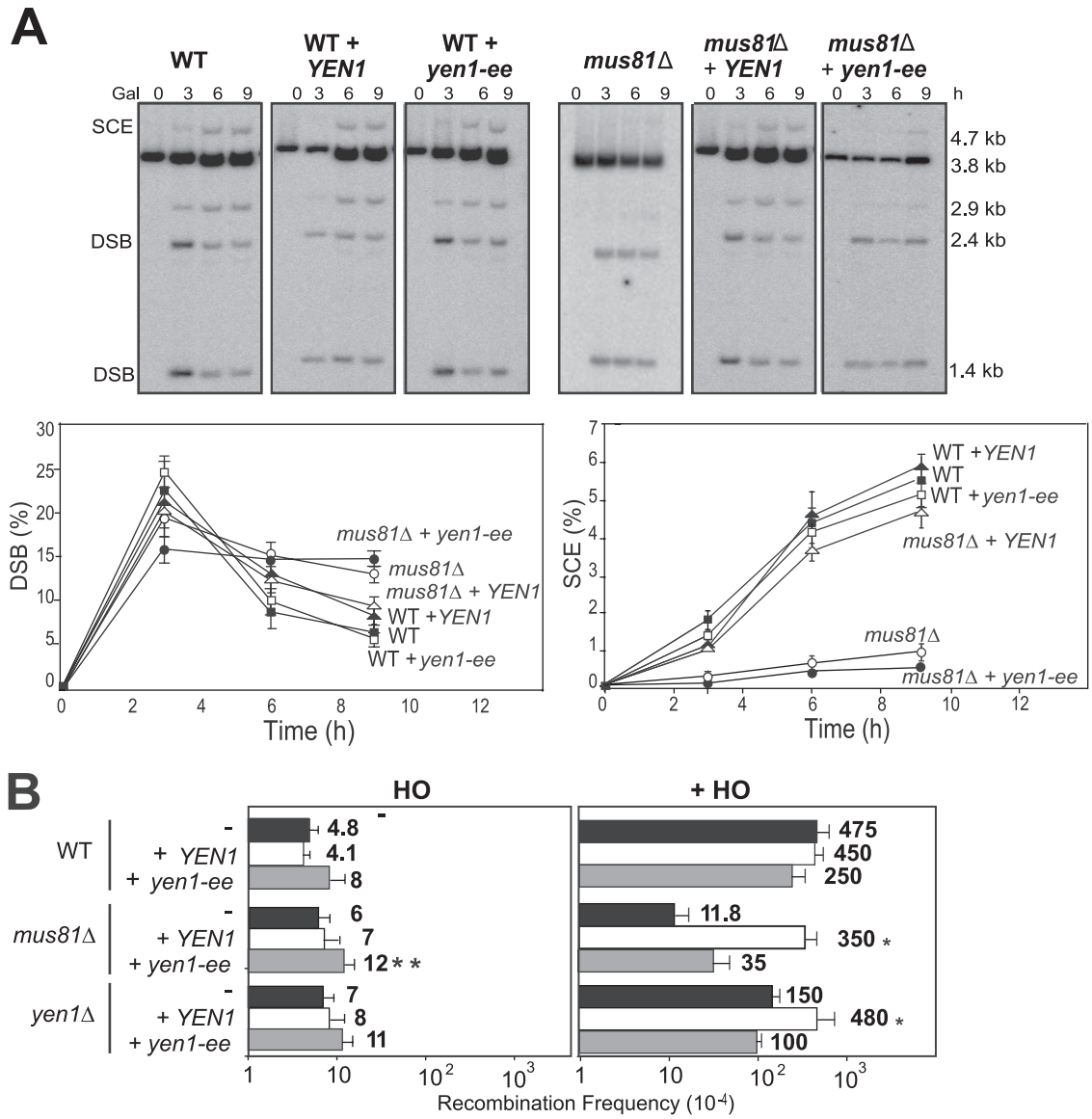

FIG 3 Genetic and physical analyses of the effects of *YEN1* overexpression in SCR in *mus81*Δ strains. (A) Physical analysis of the effects of Yen1 overexpression in SCE. Wild-type (WT) and *mus81*Δ strains were transformed with empty vector pAG414GPD-ccdB-HA (-) or the vector carrying either the active *YEN1* or the catalytically inactive *yen1*$^{E193A/E195A}$ (*yen1-ee*) allele. Averages and standard deviations (bars) of at least three independent experiments are shown for each time point and genotype. (B) Genetic analysis of the effects of Yen1 overexpression on spontaneous recombination (-HO) and DSB-induced SCE (+HO) frequencies in the TINV inverted repeat system. Wild-type, *mus81*Δ, and *yen1*Δ strains were transformed with empty vector pAG414GPD-ccdB-HA (-) or vector carrying either the active *YEN1* or the catalytically inactive *yen1*$^{E193A/E195A}$ (*yen1-ee*) allele under the control of the *GPD1* promoter. Asterisks indicate statistically significant differences between the strains carrying either the active *YEN1* or the catalytically inactive *yen1*$^{E193A/E195A}$ (*yen1-ee*) and the strains with the empty vector, according to Student's *t* test (*, $P < 0.001$; **, $P < 0.005$). Other details for the experiment were those described for Fig. 1.

physical analysis showed that the DSB accumulation kinetics was essentially similar in wild-type, *rad1*Δ, and *slx4*Δ *rad1*Δ cells but slightly enhanced in *slx4*Δ cells. Importantly, SCR levels were clearly diminished in *rad1*Δ and to similar levels in *slx4*Δ *rad1*Δ cells, and further in *slx4*Δ cells (Fig. 6A). However, *slx1*Δ cells did not show the enhanced DSB accumulation kinetics and decreased SCR kinetics of *slx4*Δ, while the *slx1*Δ *rad1*Δ strain showed similar DSB and SCE kinetics to those of *rad1*Δ cells (Fig. 6B). These data indicate that Rad1, but not Slx1, has a role in SCR. The physical analysis was extended to double mutant combinations of *slx1*Δ and *rad1*Δ with *mus81*Δ, and revealed that double and triple mutants accumulated DSBs above the wild-type levels. SCR was reduced in all mutant combinations to levels comparable to *mus81*Δ, with the lowest being those of *mus81*Δ *rad1*Δ and *mus81*Δ *slx1*Δ *rad1*Δ (Fig. 6C). These results confirmed that while Rad1 has an effect on SCR as prominent as that of Mus81, Slx1 does not have a major role in this process, since *slx1*Δ mutants only displayed a very mild decrease in SCR when combined with the absence of Mus81 and Rad1.

Genetic analysis of Leu$^+$ recombinants showed that spontaneous recombination was not significantly affected in *slx1*Δ or *rad1*Δ single mutants or in double mutant combinations with *mus81*Δ,

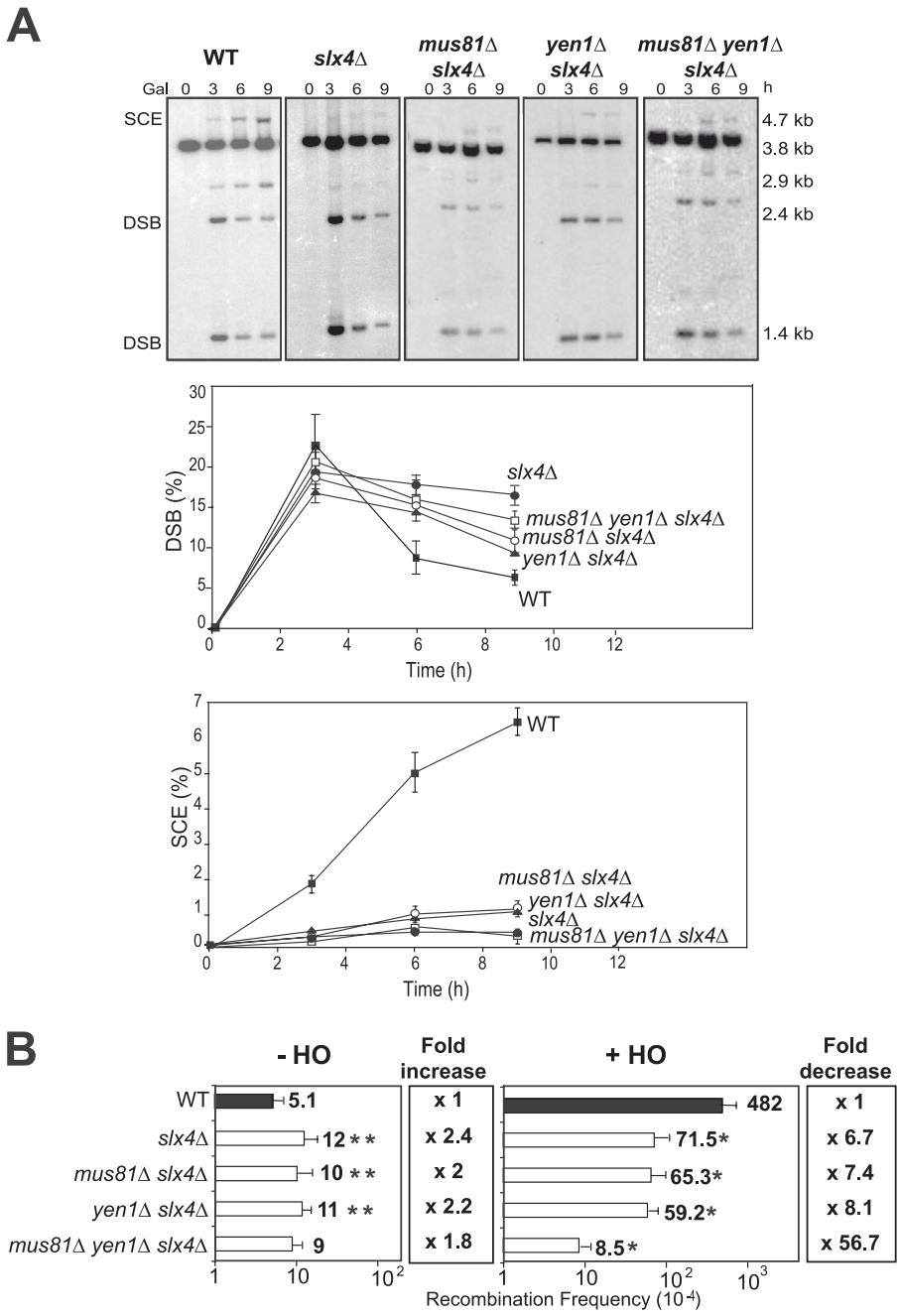

FIG 4 Effects of *slx4*Δ in SCR. (A) Physical analysis of the effects of *slx4*Δ in different genetic backgrounds in DSB-induced SCR. Kinetics and quantification of DSBs and DSB-induced SCE intermediates in isogenic wild-type (WT; WSR-7D), *slx4*Δ (WSR-S4), *slx4*Δ *mus81*Δ (WSR-S4M81), *slx4*Δ *yen1*Δ (WSR-S4Y1), and *slx4*Δ *mus81*Δ *yen1*Δ (WSR-S4M81Y1) strains are shown. (B) Genetic analysis of the effects of *slx4*Δ on spontaneous recombination (-HO) and DSB-induced SCE (+HO) frequencies in the TINV inverted repeat system. Asterisks indicate statistically significant differences compared to wild type according to Student's *t* test (*, $P < 0.001$; **, $P < 0.005$). Other details for the experiment were those described for Fig. 1.

*yen1*Δ, or each other, except in the case of *rad1*Δ *mus81*Δ (Fig. 7). Consistent with the physical analysis, HO-induced SCR was unaffected in *slx1*Δ cells but significantly diminished in *rad1*Δ (3.3-fold). This effect was the same in double, triple, and quadruple combinations with *mus81*Δ, *slx1*Δ, and/or *yen1*Δ. Interestingly, *mus81*Δ was epistatic to *rad1*Δ, whereas *yen1*Δ caused a slightly greater decrease in SCR (Fig. 7), consistent with the results shown in Fig. 1 and with MMS and HU sensitivities (see Fig. S2 in the supplemental material). Altogether, the results indicate that whereas Slx1 has no detectable role in SCR, Rad1 functions in SCR mainly in the same pathway as Mus81. Interestingly, Slx1 was required for SCR in the absence of Rad1. This suggests that Slx1 may cleave the same substrates (or their processing products) as Rad1 in SCR, whether in junction processing or in cleavage of putative short 5′-end flaps that can be generated during strand exchange by the invading heterologous HO site sequences. Fur-

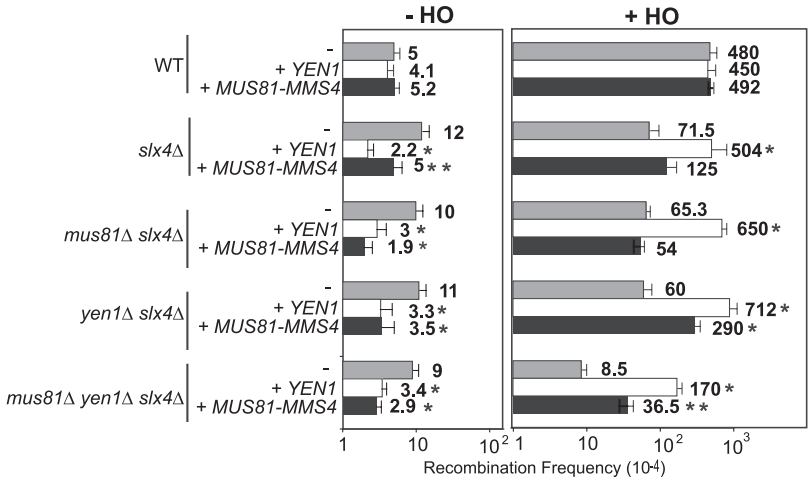

FIG 5 Analysis of genetic interactions between *MUS81*, *YEN1*, and *SLX4*. Genetic analysis of the effects of overexpression of Mus81-Mms4 and Yen1 in *slx4*Δ strains with or without *mus81*Δ or *yen1*Δ on spontaneous recombination (-HO) and DSB-induced SCE (+HO) frequencies in the TINV inverted repeat system are shown. WT, wild type. Asterisks indicate statistically significant differences between the strains expressing either active *MUS81-MMS4* or active *YEN1* and the strains with the empty vector, according to Student's *t* test (*, $P < 0.001$; **, $P < 0.005$). Other details for the experiment were those described for Fig. 1.

ther studies on the role of Slx1 nuclease are required to establish its role *in vivo*. However, in contrast to the idea that Slx1 and Slx4 act together in a resolvase complex, our study reveals that Slx4 has a more central role in SCR, likely in junction processing, that is independent of Slx1.

DISCUSSION

Using genetic and physical analyses, we found that the repair of a specific replication-born DSB by SCR is dependent upon three endonucleases, Mus81-Mms4, Yen1, and Rad1, as well as Slx4. Deletion of any of these activities led to a specific and independent impairment of SCR. In contrast, *mus81*Δ, *yen1*Δ, and *slx4*Δ single mutants showed no defects in general spontaneous recombination. Slx1, on the other hand, did not play a major role in SCR in our assay, unless Rad1 was absent, which suggests that Slx1 may cleave the same substrates or their processing products as Rad1. Studies with the wild-type and catalytically inactive forms of Yen1 and Mus81-Mms4 overexpressed in different mutant background combinations of *mus81*Δ, *yen1*Δ, and *slx4*Δ suggest that each SSE can process the replication-born recombination intermediates that accumulate in the absence of the other two SSEs, albeit to different extents. The three proteins, and in particular the recently discovered Yen1 resolvase, have independent and nonredundant functions in the repair of replication-born DSBs via SCR, acting on different SCR intermediates, one processed by Yen1 and the other by Mus81-Mms4, together with Slx4. Importantly, the fact that *slx1*Δ has no effect in SCR indicates that the contribution of Slx4 does not occur in the context of the Slx1-Slx4 complex, which suggests that in yeast both proteins may not function as a resolvase unit. This is consistent with the proposed role of Slx4 as a platform for other nucleases, including Rad1-Rad10 (18).

The *in vivo* impact of mutations in different HR genes on the general mechanism of DSB repair is usually determined in meiotic assays, in which HR events between homologous chromosomes are initiated by Spo11-mediated DSB formation (35), or in vegetative assays that examine either spontaneous or DSB-induced HR between homologous chromosomes or between ectopic DNA repeats (40). Despite the essential contributions of these assays to our understanding of the *in vivo* functions of HR proteins, the large variety of mitotic HR events and the availability of alternative DSB repair pathways, such as the double Holliday junction pathway, synthesis-dependent strand annealing (SDSA), break-induced replication (BIR), single-strand annealing (SSA), SCR, etc., can limit our capacity to discern the *in vivo* role for many HR proteins. In other words, some factors involved in recombinational repair cannot be linked to a detectable effect in most mitotic HR assays. A role for cohesins and Smc5-6 proteins, for example, has been demonstrated *in vivo* only by studying SCR induced by replication-born DSBs (10, 13). The SCR assay used here is sensitive enough to be able to differentiate between the roles of various SSEs, which has not been possible in other general repair and HR assays unless double mutants were analyzed (5, 29).

The physical and genetic assay used in this study relies on a single-stranded break that leads to a DSB during DNA replication. This leads to a situation in which a spontaneous DSB occurs naturally as a consequence of replication fork collapse. Reestablishment of the interaction between the break end and the sister chromatid (SCR) is the major mechanism for repair of the break and restoration of the replication fork (11, 24, 25). Regardless of whether a DSB occurs at the replication fork as a consequence of its collapse or distal to the fork after the fork has passed the lesion (and therefore regardless of whether the break is single ended or double ended), DNA strand invasion into the sister chromatid is needed to generate the D-loop intermediate (Fig. 8). The Mus81-Mms4 endonuclease may process such D-loops or other junctions that have not yet been ligated (nicked HJ, nicked dHJ) (15, 45), which explains why *mus81* mutants show SCR defects. This is consistent with the requirement for Mus81-Eme1 in *S. pombe* for the repair of DSBs originating from a nick (43). Yen1 has a weak but significant effect on SCR as detected both at the physical and genetic level. To date, this represents the only evidence showing an effect of *yen1*Δ single mutants on DNA repair and recombination. Based on the biochemical specificity of Yen1 (31), it is possible that Yen1 acts after Mus81-Mms4 on HJs that have been ligated. At the physical level, the effect is better observed in a *rad5-G535R* mutant background (see Fig. S3 in the supplemental material),

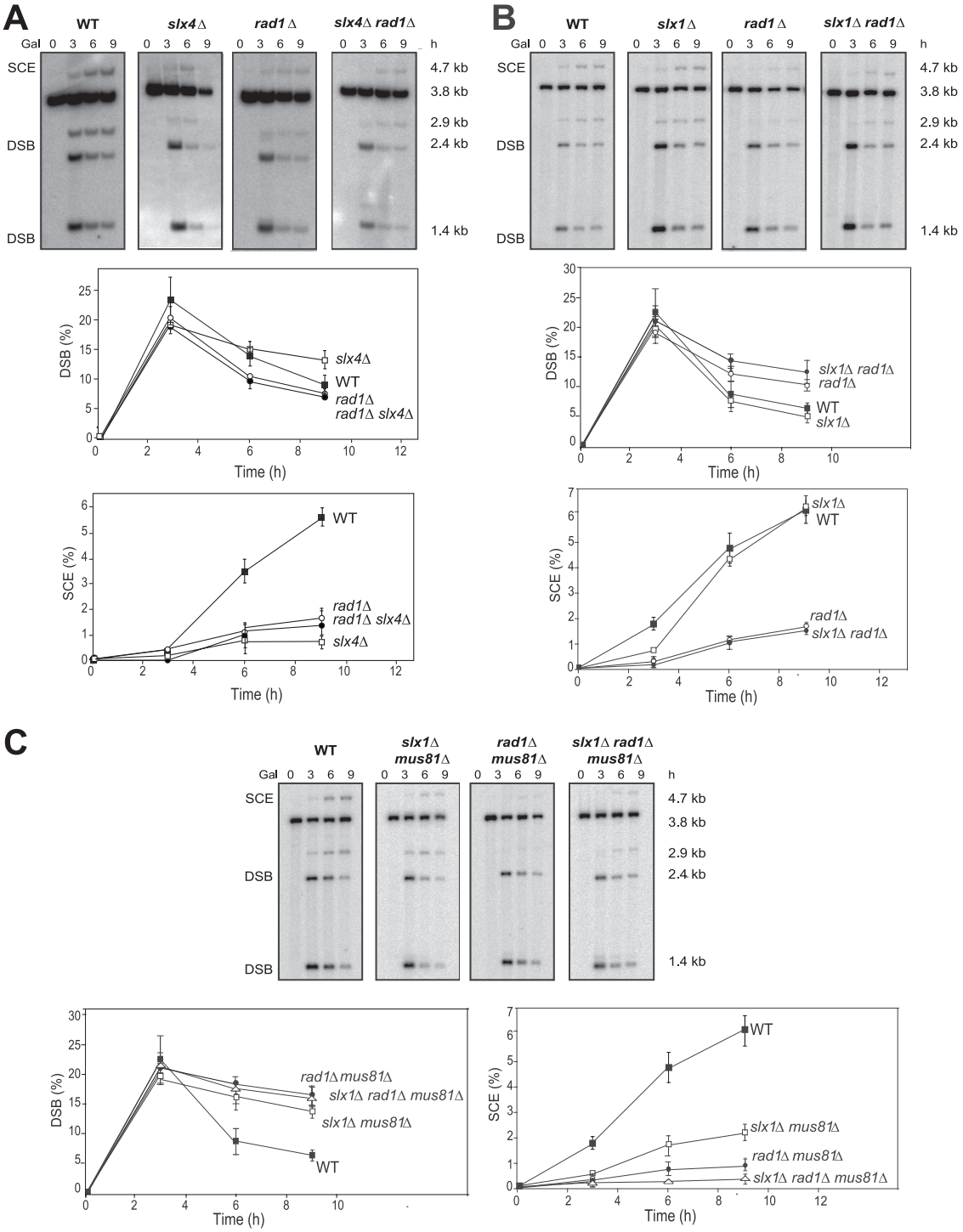

FIG 6 Physical analysis of SCR in *rad1*Δ and *slx1*Δ cells. Physical analysis of DSB formation and SCR in various mutants: (A) *slx4*Δ (WSR-S4), *rad1*Δ (WSR-R1), and *slx4*Δ *rad1*Δ (WSR-S4R1); (B) *slx1*Δ (WSR-S1), *rad1*Δ (WSR-R1), and *slx1*Δ *rad1*Δ (WSR-S1R1); (C) *mus81*Δ. WT, wild type. Other details for the experiment were those described for Fig. 1.

carrying a point mutation in the ATPase domain of the postreplicative repair gene *RAD5* that confers a weak DNA repair defect, consistent with a role of Yen1 in the resolution of an intermediate that arises during replication.

It has recently been shown that depletion of both *MUS81* and *GEN1* reduces the high levels of cytologically detectable sister chromatid exchanges found in $BLM^{-/-}$ human cell lines (52), implying a role of SSEs in crossover HR. However, the increased level of sister chromatid exchanges in $BLM^{-/-}$ human cells, as well as in yeast *sgs1* cells (8, 22, 51), may alternatively be explained

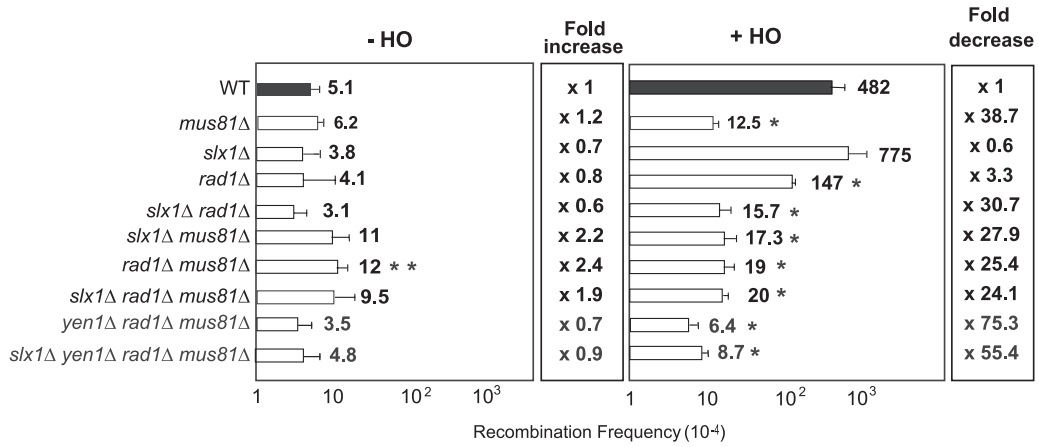

FIG 7 Genetic analysis of the effects of *rad1*Δ and *slx1*Δ on Leu$^+$ recombinants generated by the TINV system. Frequencies of spontaneous and HO-induced Leu$^+$ recombinants are shown for *slx1*Δ and *rad1*Δ mutants, as well as double, triple, and quadruple mutant combinations with *mus81*Δ and *yen1*Δ. WT, wild type. Other details for the experiment were those described for Fig. 1.

by an increased number of DNA breaks that arise in BLM-defective cells during replication. Such breaks presumably would not be observed if they were mediated by SSEs (17). The present results therefore provide important new insights by unambiguously confirming that the SSE proteins assist in the repair of broken replication forks via SCR, rather than acting to suppress DSB formation. The biochemical properties of Yen1/GEN1 (31) suggest an interesting possibility, that Yen1 may cleave specific HJs formed when the double-ended DSB occurs in the lagging versus the leading strand (Fig. 8). Our data indicate that Yen1 and Mus81-Mms4 are required to process SCR intermediates, but they cannot easily interchange their roles, unless overexpressed. Our results therefore confirm the previously reported *in vivo* roles of Yen1 and GEN1 in HR in yeast and mammals (29, 52), and they extend the conclusions further to assign a specific *in vivo* role for each SSE.

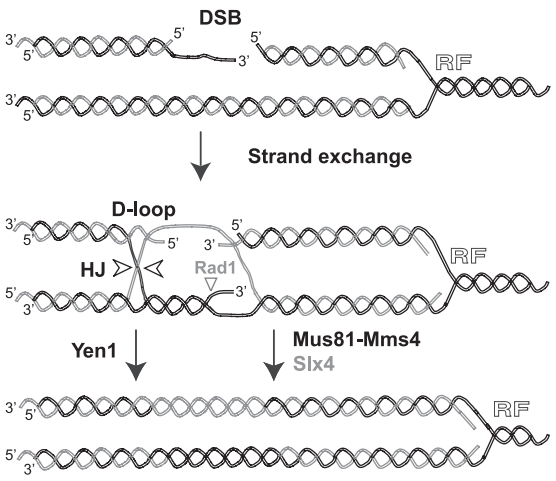

FIG 8 Mus81-Mms4 and Yen1 define two different resolution pathways for the repair of replication-born DSBs by SCR. A nick can lead to a DSB during replication regardless of whether it occurs in the leading or lagging strand (the lagging strand is shown here). Repair by strand invasion and DNA synthesis with the sister chromatid lead to the formation of a D-loop and/or HJ, which would result in SCR following incision by either Mus81-Mms4 (epistatic to Slx4 in this scenario) or Yen1. Rad1 would likely be required to cleave the single-strand tail generated by the heterologous short HO site after strand invasion in our assay, but a role in junction cleavage cannot be discarded. RF, replication fork.

SCR is also strongly reduced in *slx4*Δ cells, as detected by physical kinetic analysis (Fig. 4), confirming a relevant role for Slx4 in SCR. Interestingly, the physical effect is as strong as in *mus81*Δ mutants, but not the genetic end point effect (Leu$^+$ recombinants) (Fig. 1 and 4). This difference between the physical and genetic end point analyses was expected, because molecular assays are more sensitive and genetic end point assays are not reflective of repair kinetics. The *slx4*Δ *mus81*Δ double mutant showed suppression of the much stronger *mus81*Δ defect, whereas the triple mutant lacking the three SSEs showed the low recombination and SCR efficiency of the double *mus81*Δ *yen1*Δ mutants. These results indicate that in DSB-induced SCR, Slx4 plays an important role and possibly functions to inhibit Yen1 or Mus81 action. Thus, we wondered whether the absence of Slx4 could rescue the inviability of *mus81*Δ *sgs1*Δ double mutants, by allowing Yen1 to resolve the toxic recombination intermediates accumulated in the absence of Mus81 and Sgs1 (17). However, this does not seem to be the case, as the *mus81*Δ *sgs1*Δ *slx4*Δ triple mutant was also inviable (see Fig. S4 in the supplemental material). It has been proposed that Slx4 may serve as a platform for the action of the Slx1 SSE, which functions with Slx4 as a heterodimer, as well as other SSEs, such as MUS81-EME1 in humans and Rad1-Rad10 in *S. cerevisiae* (18, 19). Interestingly, our data indicated that yeast Slx1 has no role in SCR unless Rad1 is absent in the cell. This result argues against the possibility that yeast Slx1 and Slx4 form a protein complex responsible for HJ resolution in SCR in a wild-type scenario. Additionally, it suggests that the role of Slx4 in SCR may be exerted as part of a platform for other SSEs. In contrast, the *rad1*Δ mutant showed a clear defect in SCR (Fig. 6). One possibility is that Rad1-Rad10 functions in SCR by using the Slx4 platform. However, given the role of Rad1 nuclease in cleaving overhanging DNA structures formed during recombination (40), it is difficult to establish whether the effects observed in this study indicate a specific role of Rad1-Rad10 in junction cleavage. Alternatively, the Rad1-Rad10 major contribution could be to remove the short nonhomologous tail of the mini-HO site after DNA strand invasion, allowing priming of DNA synthesis and comple-

tion of the recombination event (Fig. 8). Either way, further studies are required to determine at which SCR step Rad1 acts. However, the epistatic relationship of *rad1*Δ with all other mutations tested in HO-induced SCR events (Fig. 7) can be explained without the need of invoking a role for *rad1* in junction resolution. This is clear from the results showing a similar frequency of HO-induced SCR events in *rad1*Δ *mus81*Δ *yen1*Δ cells (6.4×10^{-4} [Fig. 7]) and *yen1*Δ *mus81*Δ cells (8.2×10^{-4} [Fig. 1C]), suggesting that Rad1 participates in a common HR intermediate regardless of whether the recombination event is resolved by Yen1 or by Mus81 (Fig. 8).

Interestingly, only overexpression of Yen1 has a significant effect on *slx4*Δ mutants (Fig. 5). No effect was observed by overexpression of Mus81-Mms4 in *slx4*Δ cells, as suppression of the HR defect observed in *mus81*Δ *slx4*Δ and *mus81*Δ *yen1*Δ double mutants could be explained by the capacity of Mus81-Mms4 overexpression to complement or suppress the *mus81*Δ or the *yen1*Δ mutation (Fig. 1 and 2). This suggests that Mus81-Mms4 and Slx4 functions are not interchangeable and likely involve different types of DNA junction substrates. In mammals, it has been shown that SLX4 immunoprecipitates contain a HJ resolvase activity in association with Slx1 (3, 18, 37, 48), but analysis in yeast has shown that HJ cleavage appears biologically insignificant and that the complex is most active on 5′-flap structures (12, 21).

The fact that the *slx4*Δ *mus81*Δ double mutant (Fig. 4) shows higher levels of SCR than single mutant *mus81*Δ cells (Fig. 1) suggests that the presence of Slx4 at the junction substrate prevents the access or function of Yen1 at the D-loop. Mus81-Mms4 together with Slx4 would prevent Yen1 action. In their absence, Yen1 could access the junction, yielding partial suppression of the *mus81*Δ phenotype.

In summary, this work shows that Yen1 on the one hand and Mus81-Mms4 and Slx4 on the other hand cleave different intercrossed DNA structures to reconstitute broken replication forks via HR with the sister chromatid, with Rad1 having a role in cleavage of either the junction or the 3′-end overhanging tail (Fig. 8). This unique effect, which has previously been detected only in double mutants by measuring general HR events or sensitivity to DNA-damaging agents (5, 29), indicates that distinct alternatives for the repair of replication-born DSBs require specific, nonoverlapping functions of Mus81-Mms4, Slx4, Yen1, and Rad1-Rad10.

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We declare that we have no conflict of interest.

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AUTHOR CORRECTION

Correction for Muñoz-Galván et al., "Distinct Roles of Mus81, Yen1, Slx1-Slx4, and Rad1 Nucleases in the Repair of Replication-Born Double-Strand Breaks by Sister Chromatid Exchange"

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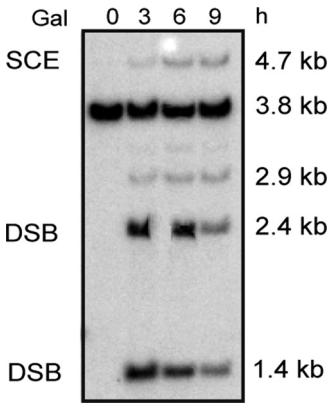
Volume 32, no. 9, p. 1592–1603, 2012, https://doi.org/10.1128/MCB.00111-12. Page 1596, Fig. 2A: The representative Southern image uploaded for WT + *mus81-dd-MMS4* was incorrect. By mistake the image uploaded was the same one used to illustrate the WT + *MUS81-MMS4* control. The corrected image of the WT + *mus81-dd-MMS4* Southern blot is shown below. We apologize for this unintentional error, which did not impact the results or conclusions of the study.



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