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The Slx5-Slx8 Complex Affects Sumoylation of DNA Repair Proteins and Negatively Regulates Recombination▽†

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Recombination is important for repairing DNA lesions, yet it can also lead to genomic rearrangements. This process must be regulated, and recently, sumoylation-mediated mechanisms were found to inhibit Rad51-dependent recombination. Here, we report that the absence of the Slx5-Slx8 complex, a newly identified player in the SUMO (small ubiquitin-like modifier) pathway, led to increased Rad51-dependent and Rad51-independent recombination. The increases were most striking during S phase, suggesting an accumulation of DNA lesions during replication. Consistent with this view, Slx8 protein localized to replication centers. In addition, like SUMO E2 mutants, *slx8*Δ mutants exhibited clonal lethality, which was due to the overamplification of 2μm, an extrachromosomal plasmid. Interestingly, in both SUMO E2 and *slx8*Δ mutants, clonal lethality was rescued by deleting genes required for Rad51-independent recombination but not those involved in Rad51-dependent events. These results suggest that sumoylation negatively regulates Rad51-independent recombination, and indeed, the Slx5-Slx8 complex affected the sumoylation of several enzymes involved in early steps of Rad51-independent recombination. We propose that, during replication, the Slx5-Slx8 complex helps prevent DNA lesions that are acted upon by recombination. In addition, the complex inhibits Rad51-independent recombination via modulating the sumoylation of DNA repair proteins.

The maintenance of genome stability is critical for cell survival and for the proper development of an organism. It requires a network of genes that must be coordinated during various DNA metabolic processes. In the budding yeast *Saccharomyces cerevisiae*, the *SLX5* (or *HEX3*) and *SLX8* genes are among the guardians of genomic stability. Originally identified as genes required for the viability of cells lacking Sgs1 (the homolog of human BLM and WRN), both *SLX5* and *SLX8* were subsequently shown to be required for the viability or fitness of many other strains with mutations that affect genomic integrity (29, 31, 40). Particularly, these genes exhibit extensive interactions with genes involved in replication or replication fork stability, such as *RAD27*, *POL32*, *ELG1*, and *DBF2*, suggesting a role for these genes in replication and/or repair (31). Consistent with this view, the deletion of *SLX5* or *SLX8* leads to a 150- to 200-fold increase in gross chromosomal rearrangement and a 4-fold increase in spontaneous mutation rates (48). These findings point to the importance of Slx5 and Slx8 in the maintenance of genome stability.

Based on biochemical and genetic evidence, Slx5 and Slx8 proteins function as a complex (29, 47, 48). Several recent studies suggest that the Slx5-Slx8 complex participates in the sumoylation pathway, which entails the addition of a small ubiquitin-like modifier (SUMO) to the target proteins. Sumoylation requires the sequential action of E1, E2, and E3 enzymes; while only a single E1 and a single E2 exist in previously studied organisms, multiple E3s have been found and are thought to confer substrate specificities (19). It was shown previously that mutations in Slx5 or Slx8, as well as mutations in several proteins involved in the sumoylation pathway, can restore transcription in a *mot1-301* transcriptional regulator mutant (45). The same study showed that *slx5*Δ and *slx8*Δ mutants are synthetic lethal or sick with mutations in SUMO (*SMT3*), SUMO E1 (*AOS1*/*UBA2*), SUMO E2 (*UBC9*), and two SUMO E3s (*SIZ1* and *SIZ2*). These genetic data are consistent with findings in earlier reports that Slx5 interacts with SUMO in two-hybrid assays (13, 43). All of these results support a role for the Slx5-Slx8 complex in sumoylation. However, it is not clear if such a role is specific to the transcriptional functions of the complex or if it is related to the functions of the complex in genome stability.

Sumoylation and the reverse process, desumoylation, are tightly linked with genomic stability. The mutation of the enzymes or the regulators of the sumoylation and desumoylation pathway (called the SUMO pathway for simplicity) can lead to sensitivity to DNA-damaging agents, recombination defects, and the disruption of chromosomal structures (reviewed in references 19 and 36). In higher eukaryotes, defects in the SUMO pathway can lead to cancer and developmental abnormalities (reviewed in reference 36). Recent studies, including several using proteomic and genomic approaches, revealed a dozen SUMO substrates, such as the replicative clamp (proliferating cell nuclear antigen [PCNA]) and the central recom-

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bination protein Rad52, involved in various DNA metabolism processes (35, 36). Further examination of the effects of sumoylation has revealed several mechanisms by which the SUMO pathway can regulate genome stability. Among these mechanisms, two entail the regulation of homologous recombination during replication. In one, the Siz1-dependent sumoylation of PCNA recruits the antirecombinase Srs2, which can disassemble Rad51 filaments from single-stranded DNA (ssDNA) and thus disfavors homologous recombination (reviewed in reference 36). In the other, the SUMO E3 Mms21 mediates sumoylation to counteract the accumulation of Rad51-dependent recombinogenic structures at damaged replication forks (2).

While both the aforementioned regulatory mechanisms target Rad51-dependent recombination processes, it is unclear if any regulation is imposed on Rad51-independent recombination processes, such as single-strand annealing (SSA) and a subset of break-induced replication (BIR) pathways. SSA refers to the annealing of two 3′ ssDNA tails containing complementary sequences; this reaction is catalyzed by the strand annealing activity of Rad52 and can be facilitated by Rad59 when the repeat length is short (reviewed in references 11 and 22). During BIR, ssDNA from a break site anneals with a homologous sequence and a DNA replication fork is established. Although BIR is efficiently mediated by Rad51, it may also occur in the absence of Rad51. Both SSA and BIR are useful for the repair of damaged chromosomes or the restarting of replication forks, yet they can be mutagenic by generating deletions, amplifications, and translocations (reviewed in references 11 and 22). Therefore, it is conceivable that both SSA and BIR require regulation to minimize their deleterious effects, and such regulation would be critical for genomic stability.

In this report, we show that the absence of the Slx5-Slx8 complex leads to an increase of both Rad51-dependent and -independent recombination. While such increases may be explained by a higher incidence of DNA lesions during replication, our results suggest that the Slx5-Slx8 complex confers an additional negative regulation of Rad51-independent recombination and modulates the sumoylation of several enzymes in this pathway. Our results also suggest that the regulation of Rad51-independent recombination by the Slx5-Slx8 complex is particularly important for the stability of repetitive sequences and for the maintenance of extrachromosomal DNA.

MATERIALS AND METHODS

Yeast strains, plasmids, primers, and genetic manipulations. *S. cerevisiae* strains and plasmids are listed in Table S1 in the supplemental material; where applicable, a single representative of each genotype is listed. Primer sequences are available upon request. The synthetic lethal screen against *mlp1*Δ and *mlp2*Δ was described by Zhao and Blobel (49). Standard yeast protocols were used. To determine whether a particular mutation could suppress the clonal lethality of *slx5*, *slx8*, and *ubc9* mutants, we analyzed 20 to 66 tetrads, except in the case of *rad18*Δ, in which 12 tetrads were analyzed.

Yeast live-cell imaging and fluorescence microscopy. Cells were processed for microscopy and images were captured as described previously (26), except that exposure times for fusion proteins were as follows: cyan fluorescent protein (CFP)-Pol30, 0.5 s; Fob1-yellow fluorescent protein (YFP) and Rad52-CFP, 1 s; Rad52-YFP, 1.5 s; and Slx8-YFP, 3 s. To examine the incidence and duration of Rad52 foci, we performed time lapse microscopy using a microscope setup identical to one previously described (25), except with a BioPrecision 4-by-4-grid-encoded stage to allow the automatic tracking of multiple *x-y* positions. The incidence and duration of foci were determined by tracking individual cells in Openlab (Improvision, Lexington, MA) and logging the number of complete S phases, as well as the exact times of the appearance and disappearance of individual Rad52 foci. At least 50 10-min intervals were monitored for time lapse analyses, and foci lasting for less than one interval were entered as lasting for 5 min. The incidence was defined as the number of foci formed divided by the total number of complete S phases. In this measurement, a Rad52 focus was counted as one event regardless of how long it lasted. Statistical analyses of focus frequency and incidence and ribosomal DNA (rDNA) bridge frequency were carried out using a chi-squared test, while the significance of focus duration was determined using a two-tailed Student *t* test. For frequency analyses, approximately 250 to 500 cells of each strain were inspected.

Recombination assays. The frequency of *ADE2* marker loss from rDNA was measured using a modification of the protocol described previously (8). Briefly, four separate isolates of each strain containing the *ADE2-CAN1* marker integrated into the rDNA were grown in synthetic complete medium at 30°C for 2 days. Cells were diluted in water, and appropriate dilutions were plated onto synthetic complete medium for total cell counts and onto Can-Arg to select for *CAN1* marker loss. After 3 days of growth, colonies were counted and the Can-Arg plates were replica plated onto synthetic complete medium lacking Ade and back onto Can-Arg to determine the frequency of the loss of both the *CAN1* and the *ADE2* markers. For determining the frequency of BIR events, we followed the protocol described by Davis and Symington (4). To measure the marker loss at the *FLO1* open reading frame, we followed the protocol described by Verstrepen et al. (44). The loss of *URA3* at *leu2* repeats was assayed using the method of Smith and Rothstein (38). The significance of all assays was determined by a one-tailed Student *t* test.

Protein extraction, detection, and quantification. To detect total sumoylated proteins from crude extracts or to examine the sumoylation of recombination proteins, we used protocols described by Zhao and Blobel (49). In brief, to examine the sumoylation of recombination proteins, each protein was tagged with a tandem affinity purification (TAP) tag (containing a ProA module) or a Myc tag at its C terminus in its chromosomal locus. The strains with the tagged proteins were checked for growth and DNA damage sensitivity, and they did not exhibit any detectable defects, indicating the functionality of the tagged proteins. Cells were grown to early to mid-log phase in yeast extract-peptone-dextrose (YPD) medium at 30°C, and then 0.3% methyl methanesulfonate (MMS) was added for 2 h at 30°C before harvest. Treating cells with 0.3% MMS for 2 h was shown previously to maximally induce Rad52 and PCNA sumoylation (15, 33, 35). Protein extracts were prepared under denaturing conditions to prevent the copurification of the associated proteins. The TAP- or Myc-tagged proteins were purified via binding to immunoglobulin G beads or to anti-Myc beads (Sigma), respectively, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred onto nitrocellulose membranes by protein blotting. The membranes were first probed with anti-ProA antibody (Sigma) and then stripped and probed with anti-SUMO antibody (49). No detectable bands were present on anti-SUMO blots in control experiments using untagged strains.

To detect total sumoylated proteins in cells, crude protein extracts from wild-type cells and *slx8*Δ mutants were made and protein concentrations were determined by using the protein assay reagent (Cytoskeleton). Equal amounts of protein from both strains were separated by SDS-PAGE and transferred onto a nitrocellulose membrane by protein blotting. The membrane was first stained by amido black to confirm the equal loading and then was probed with anti-SUMO antibody.

To quantify protein bands, X-ray films were scanned and band intensity was measured using either lane scan analysis of the whole lane (Metamorph) or area quantification analysis of specific bands (ImageGauge). To estimate the percentage of sumoylated Rfa1, Rfa2, Rad52, and Rad59 proteins, the intensity of each sumoylated protein band was divided by that of unmodified protein bands, and this ratio was normalized to that for the wild-type strain. At least two blots were analyzed for each protein, and the averages were recorded.

Other procedures. The removal of the 2μm plasmid from cells was performed with the method of Tsalik and Gartenberg (42). 2μm levels were measured using the protocol described by Chen et al. (3). For each genotype, at least three strains were examined. The differences between 2μm levels in wild-type and mutant strains were tested for significance using a one-tailed Student *t* test. Cell cycle arrest and DNA content analysis were carried out as described by Zhao and Rothstein (50). Briefly, early- to mid-log-phase cells were arrested with 5 μg of α-factor/ml and grown in YPD at 30°C for 3 h. Cells were washed three times in alpha-factor-free YPD, and samples were collected at each time point and fixed for fluorescence-activated cell sorting to determine DNA contents.

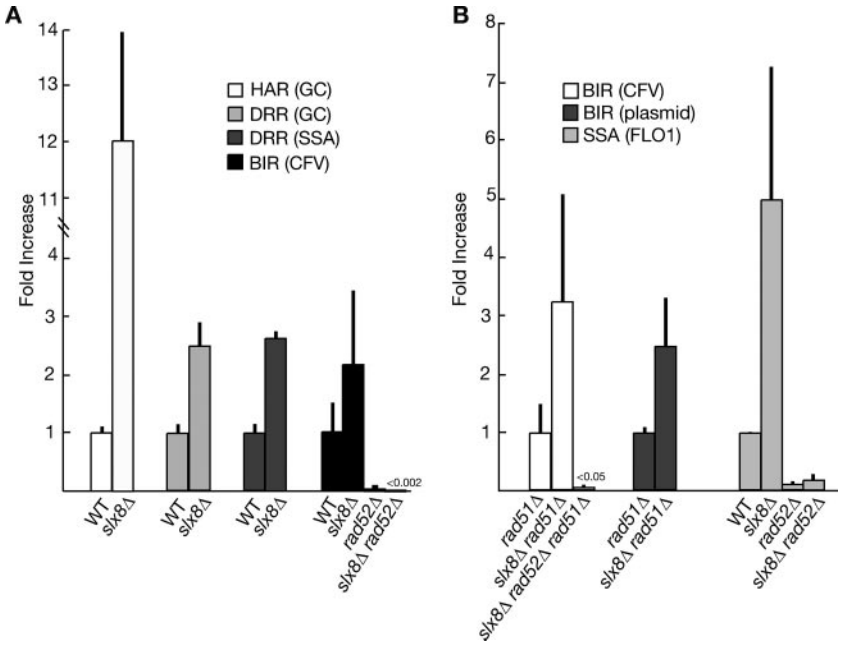


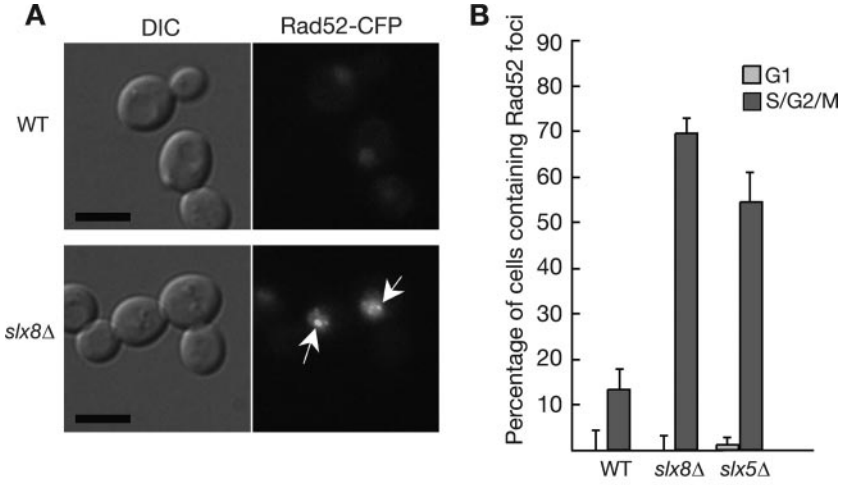
FIG. 1. Rad51-dependent and -independent recombination frequencies increase in *slx8*Δ mutants. (A) Elevated frequencies of heteroallelic recombination (HAR), direct-repeat recombination (DRR), and Rad51-dependent BIR in *slx8*Δ cells. Direct-repeat recombination events were separated into Rad51-dependent events (gene conversion [GC]) and Rad51-independent events (SSA) according to whether the intervening *URA3* gene was lost. The BIR assay examines the retention of a chromosome fragment vector (CFV). WT, wild type. (B) Elevated frequencies of Rad51-independent BIR and SSA in *slx8*Δ mutants. BIR frequencies in a *rad51*Δ background were measured in two assays: one measured the retention of a chromosome fragment vector, and the other measured the retention of a plasmid vector (plasmid). The *FLO1* SSA assay measured the simultaneous loss of *URA3* and the decrease in size of a PCR fragment encompassing the *FLO1* locus. Note that the frequency of BIR as measured by the retention of a chromosome fragment vector in the *rad51*Δ strain was about 10-fold less than that in the wild-type strain but that the frequency of BIR as measured by the retention of a plasmid vector in the *rad51*Δ strain was similar to that in the wild-type strain.

RESULTS

Both Rad51-dependent and -independent recombination events increase in *slx8*Δ strains. Both *slx5*Δ and *slx8*Δ were identified in a screen for increased frequency of spontaneous Rad52 recombination foci by using a yeast gene deletion library (D. A. Alvaro, M. Lisby, and R. Rothstein, unpublished data). To understand further the role of the Slx5-Slx8 complex in spontaneous recombination, we used several assays to examine how the absence of this complex affects recombination. Since Slx5 and Slx8 have been shown previously to function as a complex and mutations of these proteins result in identical defects for the phenotype examined so far (29, 47, 48), our analyses focused mostly on Slx8. First, we examined Rad51-dependent recombination pathways, namely, gene conversion and the primary BIR pathway. *slx8*Δ leads to a 2.5- or 12-fold increase in gene conversion between two *leu2* alleles on the same chromosome or on homologs, respectively (Fig. 1A; also see Fig. S1 in the supplemental material). In addition, using a BIR assay that requires Rad51 for the majority of recombination events (4) (see Fig. S1 in the supplemental material), we found an increase in the BIR frequency in *slx8*Δ cells of about twofold compared to that in wild-type cells (Fig. 1A). Thus, the absence of Slx8 leads to an increase in Rad51-dependent recombination.

Next, we examined Rad51-independent recombination, namely, a secondary BIR pathway and SSA. To assay Rad51-independent BIR, we measured BIR frequencies in *rad51*Δ backgrounds by using the assay described above. In addition, we carried out another assay that measures plasmid retention and is not dependent on Rad51 (17). We found that *slx8*Δ led to an approximately threefold increase in Rad51-independent BIR in both assays, and the increase depended on Rad52 (Fig. 1B). To examine the effect of *slx8*Δ on SSA, we used two assays. In one, SSA occurs between two different *leu2* alleles and results in a functional *LEU2* gene with the concomitant loss of the intervening *URA3* marker (38) (see Fig. S1 in the supplemental material). In the other, SSA leads to the loss of a *URA3* marker inserted in the *FLO1* open reading frame and a concomitant decrease in the size of the PCR fragment corresponding to the *FLO1* locus due to recombination between copies of an ~100-bp sequence repeated 18 times in *FLO1* (44). We found that *slx8*Δ led to three- and fivefold increases in SSA frequency in these two assays, respectively, and both increases were completely dependent on Rad52 (Fig. 1 and data not shown). Thus, the absence of Slx8 also leads to an increase in Rad51-independent recombination. All together, these results show that both Rad51-dependent and -independent recombination events increase in *slx8*Δ mutants. One explanation for the increase in recombination frequency is that the absence of Slx8 leads to a higher incidence of spontaneous DNA lesions. Therefore, we used cell biological methods to examine this possibility.

The higher incidence of Rad52 foci in *slx8*Δ cells is linked to the potential role of Slx8 in replication. First, we examined the



C

Strains	Incidence of Rad52 foci in S phase (± SE)	Duration of Rad52 foci (min)
WT	47% (± 8.6)	34.4 ± 20.0
*slx8*Δ	117% (± 5.4)	372.5 ± 100.0
*slx5*Δ	94% (± 11.7)	218.8 ± 53.1

FIG. 2. The incidence and duration of Rad52 foci are increased in *slx5*Δ and *slx8*Δ cells. (A and B) Higher percentages of *slx8*Δ and *slx5*Δ cells than of wild-type cells contain Rad52 foci at a given time. Rad52-CFP-containing wild-type (WT), *slx5*Δ, and *slx8*Δ cells from which 2μm had been removed were processed for microscopy. Representative images are shown in panel A. Rad52 foci are indicated by arrows. Scale bars, 5 μm. The percentages of cells containing foci and the binomial standard errors (error bars) are plotted in panel B. (C) The incidence and duration of Rad52-YFP foci, as calculated at 50 or more time points for each cell, increased in *slx8*Δ and *slx5*Δ strains. The incidence was calculated as the number of foci formed divided by the total number of S phases completed. The mean duration times and standard errors (SE) of the means are shown.

percentages of cells containing Rad52 foci at a given time. We found that while 15% of wild-type cells contained Rad52 foci, 70% of *slx8*Δ cells and 55% of *slx5*Δ cells had Rad52 foci (Fig. 2A and B). Similar to those in the wild-type, almost all Rad52 foci in the mutants were found in budded cells (Fig. 2B). High percentages of focus-containing cells at a given time may reflect an increased incidence of repair events and/or long-lasting repair processes. If spontaneous DNA lesions occurred more frequently and were repaired by recombination in *slx8*Δ and *slx5*Δ strains, we would expect to see Rad52 foci form more often in these cells. To examine how frequently cells formed Rad52 foci (i.e., the incidence of Rad52 foci), we monitored wild-type and *slx8*Δ cells over time by time lapse microscopy. In these experiments, fluorescent images of cells growing on medium-containing slides were captured every 10 min for 8 h. The G_1 cells started with no Rad52 foci; however, when they traversed S phase, 47% of wild-type cells exhibited a Rad52 focus, while nearly every *slx8*Δ (or *slx5*Δ) S-phase cell produced at least one Rad52 focus and quite often multiple Rad52 foci (Fig. 2C). As the S-phase length for these mutants was similar to that for wild-type cells (see Fig. S2 in the supplemental material), the increased incidence of Rad52 foci in these mutants likely reflects increased DNA lesions. The time lapse experiments also revealed that the time between the appearance and the disappearance of a Rad52 focus (i.e., the duration of Rad52 foci) in *slx8*Δ and *slx5*Δ cells was much longer than that in wild-type cells: while the mean duration of Rad52 foci in the wild type was 34 min, those in the *slx8*Δ and *slx5*Δ strains were 373 and 219 min, respectively (Fig. 2C). These observations are consistent with the idea that more spontaneous DNA lesions occur in *slx8*Δ and *slx5*Δ mutants during S phase. In addition, the long-lasting Rad52 repair foci suggest a defective repair process or increased usage of a slower recombination process, such as BIR (27).

To investigate further if the increase in spontaneous DNA lesions occurring at S phase in *slx8*Δ mutants reflects a function of the Slx5-Slx8 complex during replication, we examined whether this complex localizes with replication foci, which were recently shown to mark active replication centers in yeast and can be visualized by green fluorescent protein-tagged PCNA or DNA polymerase (21). We found that Slx8-YFP formed foci mostly during S/G_2 phase and that nearly all of the S-phase Slx8 foci localized with PCNA foci (Fig. 3A and B), suggesting that Slx8 is present at replication centers. This possibility is consistent with the extensive genetic interactions between the Slx5-Slx8 complex and proteins involved in replication (30, 31, 40). Therefore, it is likely that the higher incidence of Rad52 foci in *slx8*Δ mutants stems from a defect in replication.

Interestingly, not all replication centers contained Slx8 foci, suggesting that the complex may play prominent roles at specific genomic loci. An examination of the relative localization of Slx8 and various nuclear markers revealed that 50% of Slx8 foci localized with Nop1, a nucleolar marker (Fig. 3C). As the nucleolus occupies up to about a quarter of the nucleus (37), the frequency of Slx8 foci in the nucleolus cannot be explained by random distribution; rather, it indicates the enrichment of Slx8 foci in this nuclear compartment. The nucleolus contains 100 to 200 repeats of the rRNA genes, or rDNA. The rDNA

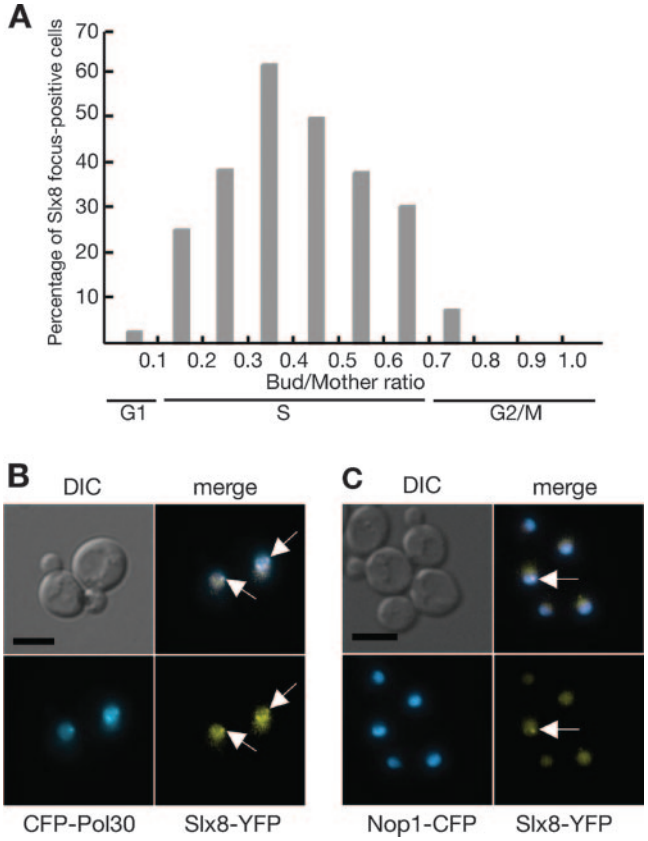


FIG. 3. Slx8 foci often colocalize with replication foci or reside in the nucleolus. (A) Slx8 foci are present predominantly in S/G_2-phase cells. The percentage of cells containing Slx8-YFP foci at each cell cycle stage was plotted. The majority of cells with Slx8 foci had a bud/mother ratio of 0.2 to 0.6, corresponding to S/G_2 phase (25). (B) Slx8 foci colocalize with replication foci as marked with CFP-Pol30; colocalization is indicated by arrows. (C) A subset of Slx8-YFP foci localize to the nucleolus, which is marked by Nop1-CFP. Representative images are shown, and Slx8 foci are indicated by arrows. DIC, differential interference. Scale bars, 5 μm.

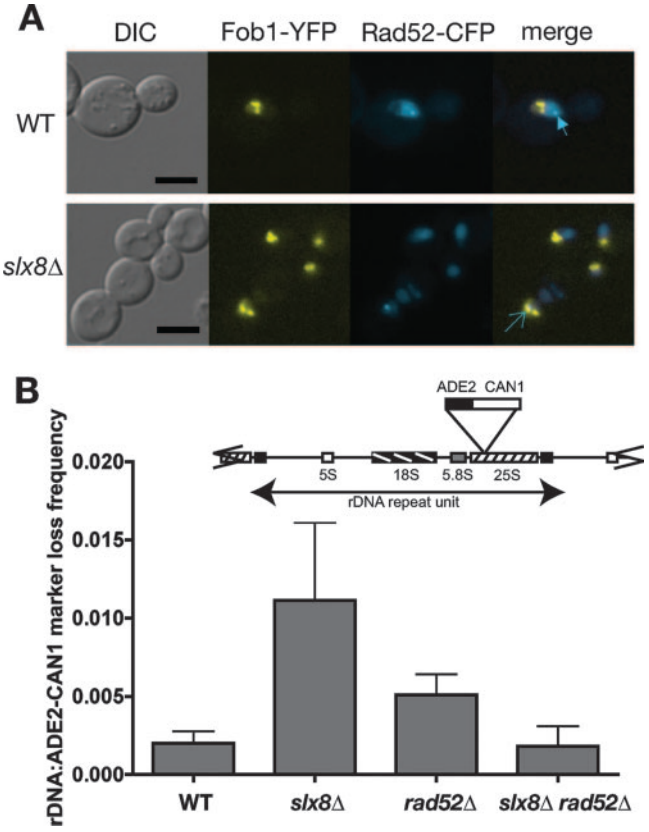


FIG. 4. Slx8 is required for rDNA maintenance. (A) Rad52 foci localize to rDNA in *slx8*Δ cells. Representative images of wild-type (WT) and *slx8*Δ cells containing Rad52-CFP and Fob1-YFP are shown. Arrows with closed arrowheads indicate Rad52 foci that are outside rDNA, which is marked by Fob1-YFP. Arrows with open arrowheads indicate Rad52 foci that are localized with rDNA. DIC, differential interference. Scale bars, 5 μm. (B) The frequency of rDNA recombination in *slx8*Δ cells is elevated. A schematic diagram of the rDNA recombination substrate is shown (top panel). The mean and standard deviation of the frequencies of Can^+ Ade^- colonies are shown.

loci undergo programmed replication fork stalling, a process associated with recombination between the repeats. To test if Slx8 affects rDNA recombination, we first examined Rad52 foci in rDNA. The absence of Slx8 led to a dramatic increase of Rad52 foci located inside or adjacent to rDNA: 5% of Rad52 foci in wild-type cells occurred inside or adjacent to rDNA, while 35% of Rad52 foci in *slx8*Δ mutants were localized inside or adjacent to rDNA (Fig. 4A; also see Fig. S3 in the supplemental material). In addition, an increase in rDNA recombination was observed using an assay that examines the simultaneous loss of the *ADE2* and *CAN1* markers inserted into an rDNA repeat (Fig. 4B) (8). *slx8*Δ strains exhibited about a sixfold increase in marker loss at the rDNA locus, and such an increase depended on Rad52 but not Rad51 (Fig. 4B and data not shown). Taken together, these results support the notion that the Slx5-Slx8 complex plays a prominent role in preventing recombination in rDNA.

Clonal lethality and increased levels of 2μm in *slx8*Δ (or *slx5*Δ) mutants are suppressed by the removal of genes involved in the Rad51-independent BIR pathway. While higher levels of spontaneous DNA lesions can provide some explanation for the increased recombination frequencies in *slx8*Δ mutants, the *slx8*Δ mutation appeared to have a rather unique effect on Rad51-independent pathways, such as SSA, the secondary BIR pathway, and rDNA recombination. This finding prompted us to investigate whether Slx8 has underlying roles in regulating these pathways that cannot be explained simply by increases in the amount of spontaneous DNA lesions. During the course of our study, we observed that *slx8*Δ and *slx5*Δ strains exhibited clonal lethality (also called the nibbled-colony phenotype), which is characterized by clonal growth defects in sectors of a colony and is caused by increased levels of an extrachromosomal, double-stranded DNA plasmid, 2μm (16, 18). We found that the clonal lethality of *slx8*Δ and *slx5*Δ strains was suppressed by removing 2μm from the cells and that the reintroduction of 2μm into *slx8*Δ and *slx5*Δ strains restored the nibbled-colony morphology (Fig. 5A and data not shown). In addition, 2μm levels in *slx8*Δ and *slx5*Δ strains were about sixfold higher than those in wild-type strains (Fig. 5B and data not shown). Since recombination, particularly BIR,

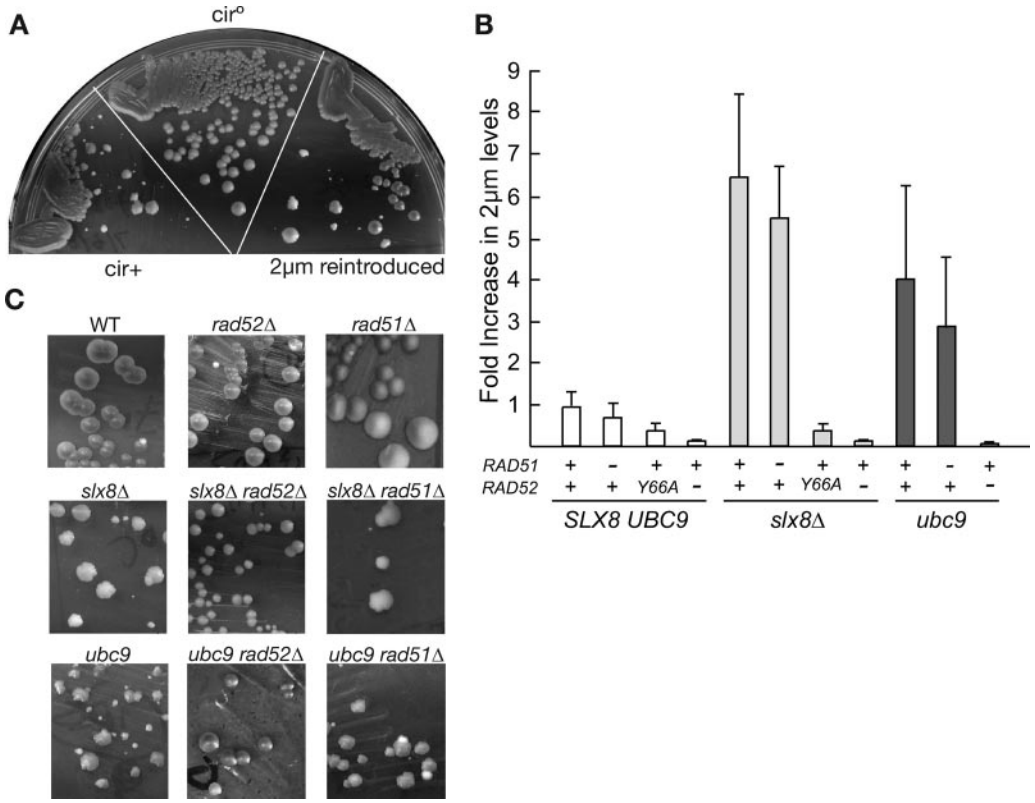


FIG. 5. Clonal lethality and increased 2μm levels in *slx8*Δ and *ubc9* mutants are suppressed by *rad52*Δ but not *rad51*Δ. (A) The clonal lethality of *slx8*Δ cells is rescued by the removal of the 2μm plasmid. *slx8*Δ mutants carrying 2μm (cir^+) give rise to heterogeneously sized, nibbled colonies. The removal of the 2μm plasmid (cir^0) leads to homogenously sized, smooth colonies. The reintroduction of 2μm plasmids into *slx8*Δ cells results in heterogeneously sized, nibbled colonies. (B) Increased levels of 2μm in *slx8*Δ and *ubc9* mutants are suppressed by *rad52*Δ and *rad52-Y66A* but not by *rad51*Δ. Shown are the means and standard deviations of 2μm levels relative to that in the wild-type strain. +, present; −, absent. (C) The clonal lethality of *slx8*Δ and *ubc9* strains is rescued by *rad52*Δ but not by *rad51*Δ. WT, wild type.

can lead to DNA amplification, we asked if the removal of different recombination pathways could suppress the increased 2μm levels and, subsequently, the clonal lethality of *slx8*Δ and *slx5*Δ strains.

We found that the deletion of *RAD52*, but not *RAD51*, completely suppressed the clonal lethality of *slx8*Δ and *slx5*Δ strains (Fig. 5C and data not shown), suggesting that recombination subpathways requiring Rad52, but not Rad51, are responsible for increased 2μm levels in these strains. Consistent with this notion, the deletion of other genes specific for Rad51-dependent recombination, such as *RAD55*, *RAD54*, and *RAD57*, did not suppress the clonal-lethality phenotype (Fig. 6B and C). In addition, we found that an allele of *RAD52* (*rad52-Y66A*) that is proficient for Rad51-dependent gene conversion but defective for Rad51-independent processes (23) could also rescue the clonal lethality of *slx5*Δ and *slx8*Δ strains (Fig. 6C). Furthermore, *rad52-Y66A* or *rad52*Δ, but not *rad51*Δ, suppressed the increased level of 2μm in *slx8*Δ strains (Fig. 5B).

The suppression of clonal lethality is rather unique to the Rad51-independent pathway, as the removal of gene products involved in other known DNA repair pathways, such as the DNA replication-damage checkpoint (*mec1*), postreplication repair (*rad6* and *rad18*), nonhomologous end joining (*yku70*), and nucleotide excision repair (*rad1*), did not eliminate this

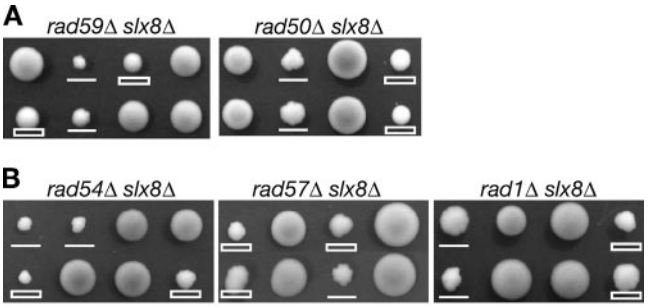


FIG. 6. The clonal lethality of *slx8*Δ and *slx5*Δ strains is suppressed by mutations affecting the Rad51-independent BIR pathway. (A and B) Representative tetrads from diploid strains heterozygous for the indicated genes are shown. *slx8*Δ spore clones (underlined) and double mutants that contain *slx8*Δ and *rad54*Δ, *rad57*Δ, or *rad1*Δ (marked with rectangles) are similarly heterogeneously sized and nibbled, while *slx8*Δ *rad50*Δ and *slx8*Δ *rad59*Δ spore clones (marked with rectangles) are smooth. (C) Summary of genes that can or cannot suppress the clonal lethality of *slx8*Δ and *slx5*Δ strains.

defect (Fig. 6C). The simplest explanation of these results is that Slx8 negatively affects Rad52-dependent, Rad51-independent recombination. This notion is consistent with the increased level of these recombination events as described above.

We further examined which Rad51-independent recombination pathway is responsible for the clonal lethality of *slx8*Δ and *slx5*Δ strains. We found that the removal of gene products known to be required for the Rad51-independent BIR pathway, including Rad59 and the Mre11-Rad50-Xrs2 complex (reviewed in reference 28), completely suppressed the clonal lethality of *slx8*Δ and *slx5*Δ strains (Fig. 6A and C). On the other hand, the deletion of Rad1, a nuclease required to remove heterologous sequences during SSA but not required for Rad51-independent BIR, did not suppress clonal lethality (Fig. 6B). These results suggest that Rad51-independent BIR, but not SSA, is likely to be the cause of clonal lethality. However, they do not completely exclude the possibility that the Slx5-Slx8 complex regulates SSA, because Rad1 is not required for SSA when no heterologous sequences are present. Additionally, clonal lethality, which is related to increased 2μm levels, may not be a good indicator for SSA, which normally results in deletions.

Taken together, these genetic results support the notion that the Slx5-Slx8 complex negatively regulates Rad51-independent BIR and subsequently prevents the overamplification of 2μm DNA, precluding clonal lethality. Considering the similarities in replication and chromatin packaging of 2μm plasmids and chromosomes, the increased level of chromosomal BIR in *slx8*Δ mutants may also indicate a related effect on Rad51-independent recombination throughout the cell.

The Slx5-Slx8 complex participates in the SUMO pathway and regulates the sumoylation of RPA, Rad52, and Rad59. The Slx5-Slx8 complex genetically interacts with multiple proteins in the SUMO pathway (45). Besides the previously reported interactions, we found two *slx8* alleles in a synthetic lethal screen against the simultaneous deletion of genes for Mlp1 and Mlp2, two nuclear pore complex components that anchor the desumoylating enzyme Ulp1 (49, 51). We also found that *slx8*Δ or *slx5*Δ is synthetic lethal with *ulp1NΔ338*, which lacks a nuclear pore complex localization domain, and with a SUMO E3 mutant, *mms21-11* (see Fig. S4A in the supplemental material). Importantly, these genetic interactions do not depend on the presence of 2μm (see Fig. S4B in the supplemental material). All of these genetic interactions involving enzymes in the SUMO pathway strongly support the notion that the Slx5-Slx8 complex participates in the SUMO pathway. While a previous report failed to detect a decrease in sumoylated proteins in *slx8*Δ or *slx5*Δ cells (45), we found that the levels of sumoylation of several proteins in the low-molecular-weight range in *slx8*Δ and *slx5*Δ strains either increased or decreased compared to those in the wild type (Fig. 7A and data not shown). This result demonstrates that the absence of the Slx5-Slx8 complex leads to changes in global sumoylation patterns, which has been reported previously for other sumoylation mutants (20, 49).

Further evidence for the involvement of the Slx5-Slx8 complex in the SUMO pathway is that, like *slx5*Δ and *slx8*Δ, the mutation of several enzymes and regulators of the SUMO pathway leads to increased 2μm levels and subsequent clonal lethality (3, 6, 51). Moreover, in a *ubc9* mutant as in *slx8*Δ and *slx5*Δ cells, *rad52*Δ, but not *rad51*Δ, can suppress the increased 2μm levels and the clonal lethality (Fig. 5B and C). These results suggest that the effect of the Slx5-Slx8 complex on Rad51-independent recombination is linked with the role of the complex in sumoylation.

To test this idea, we examined whether known recombination proteins are sumoylated and if their sumoylation is regulated by the Slx5-Slx8 complex. Although sumoylated species of most proteins are hard to detect due to low abundance and desumoylation during extractions (19), the sumoylation of proteins involved in DNA metabolism (e.g., PCNA and Rad52) has been shown to be stimulated after cells are treated with DNA-damaging agents, such as the alkylating agent MMS (2, 15, 33, 35). Therefore, we investigated the sumoylation of recombination proteins, in both Rad51-dependent and -independent pathways, in the presence and absence of MMS. To examine the sumoylation of the endogenous proteins, each protein was tagged with a TAP tag (containing a ProA module) at its C terminus in its chromosomal locus. The purified TAP-tagged proteins were examined by protein blotting using anti-ProA antibody to detect the unmodified proteins and anti-SUMO antibody to detect the sumoylated forms. We found that proteins specifically required for the Rad51-dependent pathway (Rad51, Rad54, Rad55, and Rad57) and those shared by both pathways (the Mre11-Rad50-Xrs2 complex) were not sumoylated under either condition (data not shown). However, Rad52, Rad59, and two subunits of the single-stranded binding protein replication protein A (RPA), Rfa1 and Rfa2, all of which play important roles in Rad51-independent recombination, were sumoylated after MMS treatment (0.3% MMS for 2 h) (Fig. 7B). In each case, multiple sumoylated forms of the protein were detected: monosumoylated proteins appeared as protein bands approximately 20 kDa larger than the unmodified protein on anti-SUMO blots, while poly- or multisumoylated proteins appeared as ladders of protein bands above the monosumoylated proteins (Fig. 7B). Consistent with the results in a previous report, sumoylated Rad52 appeared at low levels before MMS treatment and increased dramatically after cells were treated with MMS (35; also data not shown). Sumoylated Rfa1, Rfa2, and Rad59 could also be detected at low levels after cells were treated with a low concentration of MMS (0.03%) but were not clearly detectable before MMS treatment (data not shown). Significantly, the sumoylation levels of all these proteins in *slx8*Δ and *slx5*Δ strains after treatment with 0.3% MMS were reduced (Fig. 7B). The effect was most dramatic for the largest subunit of RPA, Rfa1, of which only 3% of the sumoylated form remained in *slx8*Δ and *slx5*Δ strains. The reduction in the sumoylated species of the other proteins ranged from 30 to 60%.

The effect of *slx8*Δ and *slx5*Δ on the sumoylation of these proteins was rather specific, since *slx8*Δ and *slx5*Δ mutations did not lead to a reduction in the sumoylation of Smc5, a known SUMO target in DNA repair (49) (Fig. 7C). Conversely, the sumoylation of Rfa1, Rfa2, and Rad52 was not affected by the deletion of the gene for Siz1, a SUMO E3 responsible for sumoylating most substrates in yeast (20) (Fig. 7D). The regulation of the sumoylation of these proteins by the Slx5-Slx8 complex may provide a molecular mechanism for the

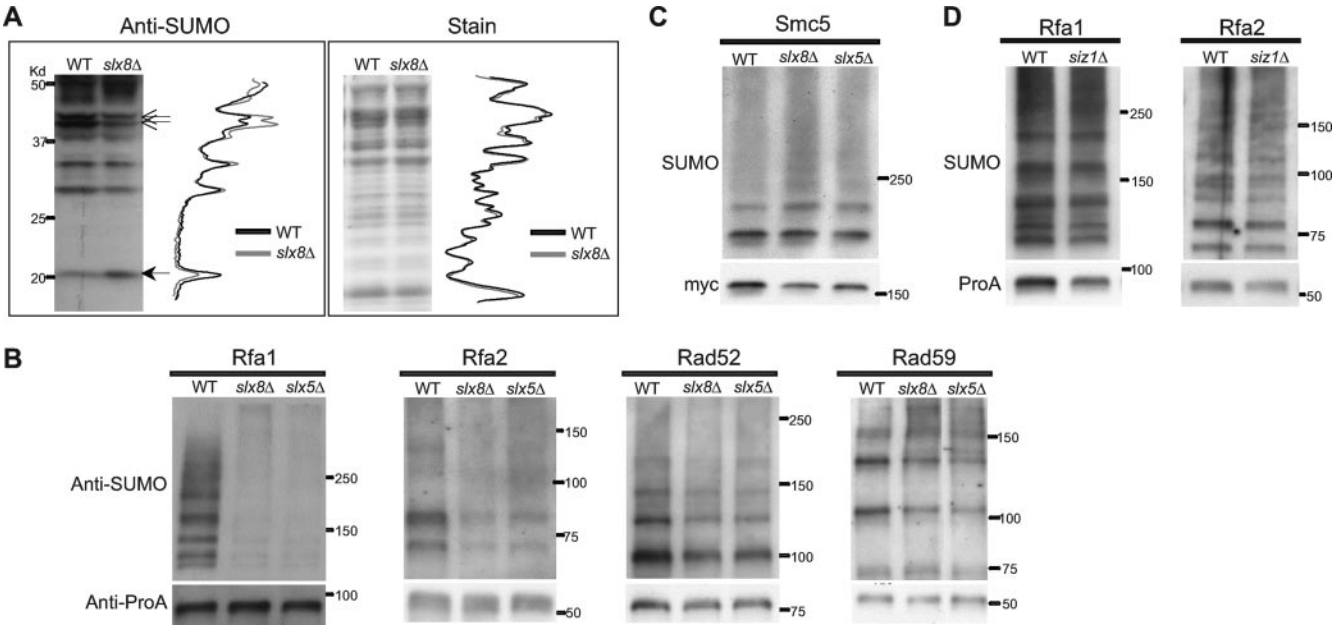


FIG. 7. The Slx5-Slx8 complex affects the sumoylation of Rfa1, Rfa2, Rad52, and Rad59. (A) *slx8*Δ affects the sumoylation of several proteins. Equal amounts of proteins from the whole-cell extracts of wild-type (WT) and *slx8*Δ strains were separated by 4 to 20% SDS-PAGE and subjected to protein blotting. Sumoylated proteins were detected by using anti-SUMO antibody. The portion of the blot that contains distinct sumoylated protein bands is shown. Arrows with open arrowheads indicate bands with decreased intensity in *slx8*Δ cells, and the arrow with the closed arrowhead indicates a sumoylated band with increased intensity in *slx8*Δ cells compared to that in wild-type cells. The same portion of the blot stained with amido black is shown on the right. The band intensities of the protein blot and the stained blot were quantified and are displayed at the right of each blot. (B) The sumoylation of each indicated protein (tagged with TAP) in wild-type (WT), *slx8*Δ, and *slx5*Δ strains was examined by immunoprecipitation followed by protein blotting as described in Materials and Methods. Mid-log-phase cultures were treated with 0.3% MMS for 2 h to facilitate the detection of sumoylated proteins. The membranes were first probed with anti-ProA antibody to detect the unmodified proteins (bottom lanes) and then stripped and probed with anti-SUMO antibody to detect sumoylated proteins (upper lanes). Sumoylated proteins represent a very small fraction of the whole protein population and were therefore not detected or very weakly detected by anti-ProA antibody. Numbers on the right indicate molecular mass markers. (C) The sumoylation of Smc5-Myc in *slx5*Δ and *slx8*Δ strains is not reduced. Experiments were done as described for panel B, except Smc5 was tagged with a Myc tag. (D) The sumoylation of Rfa1 and Rfa2 is not affected by *siz1*Δ. Experiments were done as described for panel B, except wild-type and *siz1*Δ strains were examined.

function of the Slx5-Slx8 complex in regulating recombinational repair.

DISCUSSION

The regulation of various recombinational repair pathways is critical for genomic stability. Here, we showed that the Slx5-Slx8 complex was present at replication centers and that its absence led to the accumulation of DNA lesions that were repaired by recombination, yielding an increased incidence of Rad52 foci. In addition, our observations on the specific effects of this complex on 2μm levels and on Rad51-independent recombination at chromosomal loci suggest that the Slx5-Slx8 complex has additional roles in negatively regulating Rad51-independent recombination. Furthermore, we demonstrated that the Slx5-Slx8 complex participated in the SUMO pathway and that it affected the sumoylation of several key proteins required in Rad51-independent pathways. Our results suggest that the Slx5-Slx8 complex plays multiple roles in genomic stability, at least one of which is to participate in replication and another of which is to negatively regulate Rad51-independent recombination, likely via modulating the sumoylation of key components of that pathway.

Our results show that the Slx5-Slx8 complex regulates the sumoylation of RPA, Rad52, and Rad59, as well as the recombination pathways they initiate. These results are best explained by a model in which the Slx5-Slx8-dependent sumoylation of RPA, Rad52, and Rad59 disfavors SSA and Rad51-independent BIR. Although unproven, this model not only explains our findings but also agrees with previous data about these proteins. All three subunits of RPA interact with Rad52 and the Rad52-Rad59 complex, and unmodified RPA can stimulate the ssDNA annealing activity of these protein entities (5, 14, 34, 39). Perhaps different sumoylation states of these proteins modulate ssDNA annealing reactions by affecting the interactions among these proteins or with DNA. For example, sumoylation may hinder the interaction between RPA and Rad52 or Rad59. Alternatively, sumoylation may diminish the ssDNA annealing capacity of Rad52 or Rad59. In either case, the sumoylation of these proteins may disfavor ssDNA annealing and subsequently the Rad51-independent pathways. This disfavoring may occur when replication forks stall or are damaged, particularly at repetitive sequences where the local concentration of complementing ssDNA is relatively high. Interestingly, multiple sumoylated forms of each protein were detected. This finding may indicate that the proteins were modified by either SUMO chains on one lysine residue, a single SUMO on different lysine residues, or the combination of both. A more complex band pattern for Rfa1 or Rfa2 on

SUMO blots may also indicate the combined modification of the protein by SUMO and other modifiers, as both proteins were shown to be phosphorylated after DNA damage (1). It will be interesting to determine how these different types of modification collaborate in regulating each protein's functions.

Multiple sumoylation events in this process may be collaborative, and several recent studies have shown that sumoylation often targets multiple components of protein complexes, providing a buffering mechanism for biochemical reactions (13, 32, 46). However, it is possible that only a subset of sumoylation events play dominant roles. It is interesting that blocking Rad52 sumoylation does not result in a dramatic recombination phenotype and, notably, does not affect SSA (35). This result implies that multiple sumoylation events are required to regulate recombinational repair or that Rad52 sumoylation is not the main manifestation of Slx5-Slx8-mediated regulation. We favor the latter, since Rad52 sumoylation is least affected by *slx5*Δ and *slx8*Δ while Rfa1 sumoylation is most dramatically affected and since, importantly, the Slx5-Slx8 complex can sumoylate RPA in vitro (S. Brill, personal communication). Rfa1 plays an important role in Rad51-independent recombination, since mutations of *RFA1* affect SSA and type II recombination at the telomere, which is a Rad51-independent BIR event (10, 38). Thus, it is likely that the sumoylation of Rfa1, in an Slx5-Slx8-dependent manner, is a critical event in regulating this process. Rfa1 is a hub for many interacting proteins involved in replication and repair and is subject to other modifications, such as phosphorylation (1, 7). Therefore, it is an intriguing possibility that, like that of PCNA, the differential modification of Rfa1 may be part of the code for choosing which repair pathway to use. Future work is needed to address these possibilities by mapping and mutating the sumoylation sites of Rfa1, Rfa2, and Rad59 and determining the effect of each sumoylation event on BIR and SSA, as well as other repair processes.

While BIR and SSA are important repair pathways, they are also major sources of genomic rearrangements, such as gene amplifications, deletions, and translocations (reviewed in references 12 and 28). Perhaps both positive and negative regulatory circuitries are needed to ensure that these pathways are activated at the right time and place. We suggest that the role of the Slx5-Slx8 complex in regulating these recombination pathways complements the two known SUMO pathways that regulate Rad51-dependent recombination via the Siz1 and Mms21 SUMO E3s. It is interesting that we found Slx8 located at rDNA and affecting both its stability and segregation (Fig. 4; also see Fig. S5 in the supplemental material). It was also shown previously that the Smc5-Smc6 complex, which contains Mms21, is located at rDNA and that Mms21 sumoylation activity is required for nucleolar integrity and for proper rDNA segregation as well (24, 41, 49). In addition to regulating rDNA integrity, the Slx5-Slx8 complex affects the stability of the *FLO1* gene and the extrachromosomal plasmid 2μm, which resembles repetitive DNA during its rolling-circle replication mode (9). Taken together, these results point out the importance of sumoylation in genomic stability, with more prominent roles at repetitive sequences. Since repetitive sequences make up a large percentage of the human genome and many mutations arise from recombination at repeats, these pathways may be more important in human genome integrity. Our studies of yeast provide a foundation to examine the roles of the Slx5 and Slx8 homologs in the maintenance of the human genome as well as their potential roles in disease prevention.

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