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RAD51-independent inverted-repeat recombination by a strand-annealing mechanism

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Abstract

Recombination between inverted repeats is *RAD52* dependent, but reduced only modestly in the *rad51Δ* mutant. *RAD59* is required for *RAD51*-independent inverted-repeat recombination, but no clear mechanism for how recombination occurs in the absence of *RAD51* has emerged. Because Rad59 is thought to function as an accessory factor for the single-strand annealing activity of Rad52 one possible mechanism for spontaneous recombination could be by strand annealing between repeats at a stalled replication fork. Here we demonstrate the importance of the Rad52 single-strand annealing activity for generating recombinants by showing suppression of the *rad52Δ*, *rad51Δ rad52Δ* and *rad52Δ rad59Δ* inverted-repeat recombination defects by the *rfa1-D228Y* mutation. In addition, formation of recombinants in the *rad51Δ* mutant was sensitive to the distance between the inverted repeats, consistent with a replication-based mechanism. Deletion of *RAD5* or *RAD18*, which are required for error-free post-replication repair, reduced the recombination rate in the *rad59Δ* mutant, but not in wild type. These data are consistent with *RAD51*-independent recombinants arising by a faulty template switch mechanism that is distinct from nascent strand template switching.

1. Introduction

Homologous recombination (HR) is an important repair mechanism to eliminate DNA double-strand breaks (DSBs) and to bypass lesions that block the replicative polymerases during DNA synthesis. The *RAD52* epistasis group genes (*MRE11*, *RAD50*, *RAD51*, *RAD52*, *RAD54*, *RDH54*, *RAD55*, *RAD57*, *RAD59* and *XRS2*) encode the proteins responsible for HR, although some participate in other DNA repair processes as well [1]. Of this group, the *rad52Δ* mutant has the most severe recombination defect, while the *rad51Δ* mutant is still proficient for some types of HR. Rad52 functions as a mediator for the Rad51 strand exchange protein by facilitating nucleation of Rad51 on RPA-coated single-stranded DNA (ssDNA) [2–6]. The Rad51 nucleoprotein filament catalyzes homologous pairing and invasion of a donor duplex to initiate DNA repair synthesis. In addition, Rad52 has a potent ssDNA annealing activity that is thought to be important for Rad51-independent recombination events that occur by the single-strand annealing (SSA) mechanism [7,8].

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Spontaneous recombination (recombination during normal growth with no inducing agent) most likely occurs during S phase as a mechanism to restart replication when progression of the DNA polymerase is blocked by a DNA lesion or bound protein. HR between sister chromatids is normally genetically silent, but can be detected between misaligned repeats using mutant alleles (heteroalleles) of a selectable gene to allow selection for recombinants that arise during growth of a culture [1]. Gene conversion between direct repeats is *RAD51* dependent, but deletion of one repeat and the intervening DNA can occur in the absence of *RAD51* [9–12]. DSB-induced deletion between direct repeats can occur by the SSA mechanism, involving resection of the DNA ends, annealing of the exposed complementary sequences, flap trimming and ligation [1]. Because the SSA mechanism lacks a strand invasion step it is Rad51-independent, but requires Rad52-catalyzed strand annealing. This requirement for Rad52 can be alleviated by a mutation in the *RFA1* gene (*rfa1-D228Y*) that results in lower levels of the mutant replication protein A (RPA) complex [13,14]. RPA-coated ssDNA is refractory to strand annealing and the Rad52 protein overcomes this barrier *in vitro* [8]. Smith and Rothstein suggested that the reduced level of the $RPAD^{228Y}$ complex bound to ssDNA allows spontaneous annealing, thus bypassing the requirement for Rad52-promoted annealing [13].

Inverted-repeat recombination assays were developed with the expectation that SSA is unlikely to be an interfering mechanism. Although spontaneous recombination between inverted repeats is reduced by 50 to 3,000-fold in the *rad52Δ* mutant, only a 5 to 10-fold reduction is observed in the *rad51Δ* mutant [15–18]. We identified the *RAD59* gene by its requirement for *RAD51*-independent recombination between inverted *ade2* heteroalleles [19]. The *rad51Δ* and *rad59Δ* single mutants both exhibit modest decreases in the rate of recombination between inverted repeats, while the *rad51Δ rad59Δ* double mutant is very defective, similar to the *rad52Δ* mutant [18,19]. Rad59 is homologous to the N-terminal DNA binding and multimerization domain of Rad52, and interacts directly with Rad52 [20,21]. Although Rad59 exhibits ssDNA-annealing activity *in vitro*, unlike Rad52 it cannot overcome the inhibitory effect of RPA on ssDNA annealing and is thought to augment the activity of Rad52 [20,22,23]. The *rad59Δ* mutant exhibits decreased efficiency of SSA, particularly when the direct repeats are short [20,24,25]. Additional studies suggest Rad59 might be important to counteract the negative effect of Rad51 on Rad52-mediated strand annealing [26,27].

The minimal requirement for *RAD51*, and dependence on *RAD52* and *RAD59*, suggests spontaneous inverted-repeat recombination can occur without Rad51-catalyzed strand invasion. DSB-induced recombination between chromosomal inverted repeats requires *RAD51*, consistent with a Rad51-catalyzed strand invasion step for DSB initiated events, and suggests that *RAD51*-independent spontaneous events do not initiate from DSBs [17]. Thus, we considered the possibility that spontaneous *RAD51*-independent events occur by a strand annealing mechanism during DNA synthesis to account for the requirement for *RAD52* and *RAD59*. In support of this hypothesis, we show the inverted-repeat recombination defect of *rad52Δ* mutants is suppressed by the *rfa1-D228Y* mutation. Furthermore, increasing the distance between the repeats reduced the rate of *RAD51*-independent recombination and altered the spectrum of products, consistent with a replication-based mechanism. In addition, we show the error-free mode of post-replication repair plays a minor role in the formation of inverted-repeat recombinants.

2. Materials and methods

2.1. Yeast strains

All yeast strains are derived from W303, corrected for the *rad5-535* mutation by crossing to generate either *RAD5* or *rad5::URA3* derivatives as appropriate [28] (Table 1). The *ade2*

inverted-repeat recombination reporter (*ade2*-IR) (Figure 1A) was described previously [18,19]. The *ade2::hisG-URA3-hisG* cassette replaces *ade2-1* in strain B404-3C as described in Rattray and Symington [18]. A Ura$^-$ derivative of B404-3C was first selected on 5-fluoorotic acid (5-FOA) for loss of *URA3* before construction of any of the strains described in this study. The *ade2*-IR strains used are *ade2::hisG*, with one exception (*rad18*, LSY2002-2B). The strains containing the *rfa1-D228Y* allele were derived from crosses with a 5-FOA resistant isolate (LSY2236) of strain U859 (a gift from the Rothstein Lab). Determination of the presence of the *rfa1-D228Y* allele by PCR and restriction digestion has been described [13].

A second inverted-repeat substrate (*ade2*-IR-*Hph*), was constructed as follows. The plasmid pAG32 [29] was used to provide a functional Hygromycin B resistance gene and other sequences that have low homology to the rest of the *Saccharomyces cerevisiae* genome. Using the method of Longtine *et al*. [30] primers were designed to amplify the majority of plasmid pAG32 and direct the insertion of this PCR product between the *ade2* alleles in strain LSY2002-9D. The primers used were:

SP-trp+hphexpand1: 5'-GCAGAACCGAGGATAGCGCTACGTCAGGATTCGAGGTCGGCGCGCCAGATCTGTTTAGCTTGCCTTGTCC-3' and AP-trp+hphexpand1: 5'-GAACTAGTGGATCTTTTATGCTTGCTTTTCAAAAGGCCAATCTGCTCTGATGCCGCATAGTTAAGCCAGC-3'. The DNA was amplified using the Phusion DNA polymerase (Finnzymes) under the two-step conditions described by the manufacturer.

Successful targeting was verified by growth of the transformants on Hygromycin B (300μg/mL) containing media and failure to grow on minimal medium lacking tryptophan. Transformants with the correct structure were confirmed by PCR and Southern blot analysis of digested genomic DNA. *rad* mutations were introduced to the strain with the *ade2*-IR-*Hph* by crosses (Table 1).

2.2. Measurement of recombination rates

The method of Lea and Coulson [31] was used to determine recombination rates. Briefly, strains were grown 2–4 days on YPAD (1% yeast extract; 2% bacto-peptone; 2% dextrose; 10mg/L adenine) plates. Nine colonies of similar size were suspended in water, diluted and plated on synthetic complete (SC) or SC-Ade medium. The plates were monitored and counted 2 days after the first colonies were visualized. Rates were determined from the median frequency of Ade$^+$ recombinants. The mean rate shown is from at least three independent trials for each strain and error bars indicate standard deviations. The student's unpaired t-test was used to determine whether differences between strains were significant. The recombination rates shown in Figures 1 and 2 are lower than the rates presented in Figure 3 because the amount of adenine used to supplement YPAD medium was increased from 5 mg/L to 10mg/L during the course of these studies and we have found lower recombination rates when strains are grown on medium with higher adenine concentration. The relative rates for wild type and mutants are the same under both growth conditions.

2.3. Distribution of Recombinants

Strains were plated on YPAD and allowed to grow for 3–5 days. The colonies were then replica plated onto SC-Ade to select Ade$^+$ recombinants. Independent Ade$^+$ isolates were grown in 5mL overnight YPAD cultures and used to prepare genomic DNA. The DNA was digested with *Nde*I, *Pst*I, or *Bgl*I (New England BioLabs), electrophoresed on a 1% agarose gel and then transferred to nylon membrane. The blots were probed with radioactively labeled DNA capable of annealing to *ADE2*, *TRP1*, or *HphMX4* sequences and developed by

phosphorimaging. Alternatively, inversions and non-inversions were scored by PCR using a primer that anneals to *his3* sequences upstream of the *ade2-5'Δ* allele, and primers of opposite orientation that anneal to the *TRP1* sequence between the repeats. Differences in the distribution of events were determined using Fisher's exact test.

3. Results

3.1. The *RAD52* single-strand annealing activity is important for spontaneous recombination between inverted repeats

An *ade2* inverted-repeat (*ade2*-IR) substrate was used to determine the rate of Ade^+ recombinants (Figure 1A) [18]. The *ade2-5'Δ* donor allele has a deletion of the first 174 nucleotides, including the promoter sequences; the recipient allele, *ade2-n*, is present in an inverse orientation relative to *ade2-5'Δ* [18]. The *ade2-n* allele has a +2 frameshift at the *Nde*I restriction endonuclease recognition site and is transcribed from the native *ADE2* promoter. The repeats are separated by 1.9 kb of DNA, including a functional copy of the *TRP1* gene, integrated at the *HIS3* locus of chromosome XV.

Our previous studies had shown a 3,000-fold decrease in the Ade^+ recombination rate in the *rad52Δ* mutant. If the *rad52Δ* defect was due to the role of Rad52 in promoting strand annealing then we expected that suppression of the strand-annealing defect should restore the production of Ade^+ recombinants. The *rfa1-D228Y* mutation was previously identified as a suppressor of the spontaneous direct-repeat recombination defect of the *rad1Δ rad52Δ* double mutant, and the *rfa1-D228Y* single mutant exhibits a hyper-recombination phenotype for spontaneous deletions between direct repeats [13]. Subsequent studies showed that *rfa1-D228Y* suppresses the requirement for *RAD52* in DSB-induced SSA between direct repeats [14]. The RPA^{D228Y} complex is less abundant than wild type RPA suggesting there is less RPA^{D228Y} bound to ssDNA to prevent spontaneous annealing, thus bypassing the requirement for Rad52-promoted annealing [13].

The recombination rate of the *rfa1-D228Y* mutant was equivalent to wild type; however, a 66-fold increase in recombination was observed for the *rfa1-D228Y rad52Δ* compared with *rad52Δ* (P=0.0001) (Figure 1B). To confirm that the suppression of the *rad52Δ* recombination defect by *rfa1-D228Y* is due to the role of Rad52 in ssDNA annealing and not the mediator function, the recombination rate was determined for the *rad51Δ rad52Δ rfa1-D228Y* triple mutant. As anticipated, the *rfa1-D228Y* mutation also resulted in a significant suppression of the *rad51Δ rad52Δ* recombination defect (P=0.01). Thus, the SSA activity of Rad52 appears to be critical for recombination of the *ade2*-IR substrate and this requirement is alleviated by the *rfa1-D228Y* mutation. In the *rad59Δ* mutant most recombination occurs by a *RAD51*-dependent mechanism and in this case RPA is expected to play a positive role by enabling Rad51 to more effectively bind to ssDNA. Consistent with this, the *rfa1-D228Y rad59Δ* showed a small, but statistically significant decrease in recombination compared to *rad59Δ*(P=0.014) (Figure 1B). The *rfa1-D228Y* mutation caused a significant increase in the recombination rate of the *rad52Δ rad59Δ* mutant (P=0.025), suggesting the primary role for Rad59 in the formation of Ade^+ recombinants is in strand annealing together with Rad52.

By physical analysis Ade^+ recombinants were classified as gene conversion events (no inversion of *TRP1*) or inversion events. The inversions include events in which the wild type *Nde*I site is present in both repeats, and events in which the wild type *Nde*I site is transferred from *ade2–5'Δ* to *ade2-n* (Figure 1A). A few aberrant recombination events with three copies of the *ade2* locus were recovered from the *rad51Δ rad52Δ* and *rad52Δ* mutants (classified as "other" in Figure 1C); these were not characterized further. Dornfeld and Livingston previously described similar types of events in a *rad52* mutant [15]. Recombinants analyzed from the *rad52Δ*, *rad59Δ*, *rad51Δ rad52Δ* and *rad52Δ rad59Δ*

mutants showed a bias towards gene conversion events compared with the wild type distribution, however, this difference is only significant for the *rad51Δ rad52Δ* and *rad52Δ rad59Δ* mutants ($P<0.05$) (Figure 1C). Ade^+ recombinants analyzed from all of the *rfa1-D228Y* derivative showed a bias towards inversion events, with a significant change in the distribution for the *rad51Δ rad52Δ rfa1-D228Y* and *rad52Δ rad59Δ rfa1-D228Y* strains, compared with *rad51Δ rad52Δ* and *rad52Δ rad59Δ* ($P<0.005$). The distribution of events recovered from the *rad52Δ rfa1-D228Y* strain is not significantly different to the *rad51Δ rad52Δ rfa1-D228Y* and *rad52Δ rad59Δ rfa1-D228Y* strains. These results are consistent with inversions resulting from a strand annealing mechanism.

3.2. Increasing the distance between *ade2* heteroalleles alters the repair mechanism in *rad51Δ* mutants

Increasing the distance between the *ade2* repeats would be expected to inhibit template switching because of the requirement to uncouple leading and lagging strand synthesis over a longer distance [32]. Thus, we expected the rate of recombination in the *rad51Δ* mutant to decrease with increased distance between the repeats, whereas *RAD51*-dependent homology searching is expected to be less sensitive to the distance between the repeats. A new reporter, *ade2*-IR-*Hph*, was made by replacing the *TRP1* gene and some intervening sequences with most of the pAG32 plasmid, containing the gene for hygromycin resistance (*Hph*), increasing the distance between the *ade2* repeats by 3.1-kb (Figure 2A). The rates of recombination for *ade2*-IR-Hph and *ade2*-IR were the same in the wild-type background ($P=0.07$), but there was a 9-fold decrease in the Ade^+ recombination rate in the *rad51Δ* mutant for the *ade2*-IR-*Hph* compared with wild type (Figure 2B). For the *ade2*-IR substrate the difference between wild type and *rad51Δ* is 5-fold. Thus, extending the distance between *ade2–5'Δ* and *ade2–n* by 3.1-kb increased the dependence on *RAD51* by an additional 2.6-fold ($P=0.03$).

Analysis of the recombination products recovered from the *rad51Δ* mutant with the *ade2*-IR showed a bias towards inversion events, while inversions and conversions were recovered in equal numbers from the wild-type strain (Figure 2C). The Ade^+ recombinants recovered from the wild-type strain with the *ade2*-IR-*Hph* reporter showed the same distribution as events from the *ade2*-IR. However, products from the *rad51Δ* mutant with the *ade2*-IR-*Hph* reporter showed a significant alteration in the distribution of events compared with the Ade^+ products recovered from *ade2*-IR ($P=0.0007$) (Figure 2C). These results are consistent with the mechanism of *RAD51*-independent recombination changing as the distance between the repeats increases.

3.3. Post-replication repair plays a minor role in inverted-repeat recombination

Post-replication repair (PRR) is responsible for the bypass (tolerance) of DNA lesions that stall the replicative polymerases resulting in ssDNA gaps at the fork and behind the replication fork. The Rad6-Rad18 ubiquitin ligase, which is essential for the PRR pathway, monoubiquitinates proliferating cell nuclear antigen (PCNA) to promote translesion DNA synthesis [33–37]. Subsequent polyubiquitination by the Ubc13-Mms2-Rad5 ubiquitin conjugating enzyme complex directs repair through the error-free PRR pathway, which is proposed to occur by template switching between nascent strands [38–42].

To investigate whether *RAD51*-independent inverted-repeat recombination involves a template-switching mechanism we determined the Ade^+ recombination rate in *rad5Δ* and *rad18Δ* derivatives. All of the original W303-derived strains with the *ade2*-IR reporter carried the *rad5–535* allele [18,19]; these strains were remade to be *RAD5* and the recombination rates determined (note, all strains used in Figures 1 and 2 are *RAD5*). The spontaneous Ade^+ recombination rates in the wild type, *rad51Δ*, *rad52Δ* and *rad59Δ RAD5*

derivative were similar to the strains containing *rad5–535* described previously [18,19]; thus, the *rad5-535* mutation did not significantly alter the *ade2*-IR recombination rate in wild type or the mutants tested. The distribution of recombinants recovered from the *RAD5* derivatives was also the same as the *rad5-535* strains analyzed previously.

The Ade$^+$ recombination rate of the *rad5Δ* and *rad18Δ* single mutants did not significantly differ from wild type (P=0.19 and 0.12, respectively), suggesting PRR does not contribute to recombinants in strains that have wild-type HR functions (Figure 3A). If the error-free PRR pathway contributes to *RAD51*-independent recombination we would predict a decrease in Ade$^+$ recombinants in the *rad18Δ rad51Δ* and *rad5Δ rad51Δ* double mutants. The *rad18Δ rad51Δ* and *rad5Δ rad51Δ* double mutants displayed small (2–2.5 fold), but statistically significant, decreases in recombination rates compared to *rad51Δ*(P=0.0006 and P=0.005, respectively); however, the rates were 100-fold higher than the *rad51Δ rad59Δ* double mutant (P=0.0001) [19]. Thus, *RAD5* and *RAD18* have minor roles in *RAD51*-independent recombination compared with *RAD59*. The *rad18Δ rad59Δ* strain showed an 8-fold decrease in recombination compared with *rad59Δ*(P=0.0002), while the *rad5Δ rad59Δ* was 4-fold decreased compared to *rad59Δ*(P=0.006), indicating a more important role for *RAD5* and *RAD18* in the *RAD51*-dependent pathway than the *RAD51*-independent pathway.

Physical analysis of the Ade$^+$ recombinants from the *rad5Δ* and *rad18Δ* strains showed a slight bias towards recovery of inversion events, similar to the *rad51Δ* mutant, and the *rad51Δ rad5Δ* and *rad51Δ rad18Δ* double mutants exhibited the same distribution of events as the *rad51Δ* mutant (Figure 3B).

4. Discussion

HR between direct repeats and inverted repeats shows some variation in the requirement for genes in the *RAD52* epistasis group due to the different mechanisms that can be used to generate products. Gene conversion between direct repeats (maintaining both repeats and the intervening DNA) requires *RAD51* and *RAD52*, whereas deletion of one of the repeats and intervening DNA is *RAD51*-independent [9,11,12]. The deletion events are thought to occur by *RAD52*-dependent SSA [43]. In contrast, recombination between inverted repeats cannot occur by a simple annealing mechanism, yet *rad51Δ* mutants show a less profound spontaneous recombination defect than *rad52Δ* mutants [16–18]. Furthermore, the residual recombination events that occur in *rad51Δ* mutants are *RAD59*-dependent, and Rad59 is thought to augment the ssDNA annealing activity of Rad52 [19,20,23,25]. The other unusual feature of *RAD51*-independent recombination of inverted repeats is the high percent of products with an inversion of the intervening DNA [18]. Because DSB-induced recombination between chromosomal inverted repeats requires *RAD51*, we considered the possibility that spontaneous *RAD51*-independent recombination occurs by a strand annealing mechanism that switches template strands during replication, similar to the template switch models recently proposed for repeat-induced rearrangements [40,44,45]. The faulty template switch was proposed to explain the formation of acentric and dicentric giant palindromes at an inverted repeat, but aborted replication initiated by the same mechanism could generate Ade$^+$ recombinants between inverted repeats and explain the high frequency of inversions observed (Figure 4).

When replication of the leading strand is stalled by spontaneous base damage the replicative helicase continues to unwind generating ssDNA at the fork [32]. We suggest the blocked nascent leading strand dissociates from its template strand and pairs with homologous ssDNA. In the case of direct repeats, annealing can occur on the same template strand, or the sister nascent strand, to generate duplications or deletions [40], but for inverted repeats the only available homology is on the other template strand (Figure 4). Presumably the lagging

strand polymerase is hijacked and the unequal sister alignment is used to restart synthesis of the nascent leading strand. After synthesis of a short tract we suggest the leading strand is displaced from the lagging strand template and returns to the original pairing configuration between template and nascent strands. This mechanism could bypass the leading strand lesion and give rise to a heteroduplex DNA (hDNA) intermediate if DNA synthesis had proceeded across the wild type sequence of the *ade2-5'Δ* repeat; gene conversion could then result from repair of the hDNA, or segregation at the next S-phase. Furthermore, if DNA synthesis extended from *ade2-5'Δ* through the DNA separating the inverted repeats to *ade2-n* then a large loop mispair would form after strand realignment. Repair of the loop could give rise to an inversion of the DNA between the repeats, the most common class of recombinants recovered from the *rad51Δ* mutant [18]. Thus, one possible explanation for the importance of Rad52 and Rad59 for inverted-repeat recombination is to catalyze annealing between the unpaired primer strand and alternative template strand (first template switch), or in the re-establishment of canonical pairing (second template switch).

Because *rfa1-D228Y* suppresses the *rad52Δ* SSA defect observed in direct-repeat recombination assays we rationalized that if inverted-repeat recombination involves a strand-annealing step then *rfa1-D228Y* should also suppress *rad52Δ* in the *ade2*-IR assay [13,14]. The *rad52Δ* recombination rate was increased 66-fold by the *rfa1-D228Y* allele supporting the hypothesis that single-strand annealing is critical for inverted-repeat recombination (Figure 1). Furthermore, the *rfa1-D228Y* mutation partially suppressed the recombination defects of the *rad51Δ rad52Δ* and *rad52Δ rad59Δ* mutants suggesting the defect of the double mutants is due to loss of Rad52 annealing activity. In addition, the *rfa1-D228Y* mutation changed the distribution of recombination events from the gene conversion bias of the *rad52Δ* and *rad59Δ* strains to more inversion events. This result suggests strand annealing generates inversions. The *rfa1-D228Y* and *rad59Δ rfa1-D228Y* mutants did not exhibit a hyper-recombination phenotype indicating the increase in recombination is specific to the *rad52Δ* background and is not due to increased recombinogenic lesions caused by the mutant RPA complex.

The small but significant decrease in Ade^+ frequency for the *rad59Δ rfa1-D228Y* double mutant could result from reduced Rad51 strand exchange activity. The *rfa1-D228Y* mutant exhibits a 6-fold decrease in the rate of recombination between allelic sequences in diploids, indicating the Rad51-dependent strand invasion is defective in the presence of the RPA^{D228Y} complex [13]. RPA functions to remove secondary structures from ssDNA enabling Rad51 filament formation once Rad52 nucleates Rad51 and this may explain the decreased efficiency of *RAD51*-dependent recombination [46].

Paek et al. [45] did not find a decrease in the frequency of rearrangements or formation of dicentrics in the *rad52Δ* mutant in their assay system, whereas Rad22/Rad52 was required for the faulty template switch described by Mizuno et al. [44]. One difference between these systems is the amount of homology between the repeats. The natural repeats that are the substrate for template switching measured by Paek et al. [45] are shorter and more diverged than the *RTS1-ura4* repeats utilized by the Carr group [44], or the *ade2* repeats used here. The Rad52 annealing function might be more effective with longer stretches of homology. Alternatively, the Rad52-dependent function might be more important for the second template switch, which is not required to generate products in the system described by Paek et al [45]. In contrast to our finding, the *rad51Δ* mutant exhibited a reduction in acentric/dicentric products in the study by Mizuno et al. [44]. They proposed the displaced primer strand at a fork stalled by a protein barrier invades the other unreplicated repeat using HR functions. By contrast, we suggest the Rad51-independent events occur by annealing between repeats when replication is uncoupled exposing ssDNA regions.

While Rad52 and Rad59 are proposed to anneal complementary ssDNA exposed at the inverted repeat, a variety of proteins could be responsible for the other hypothetical steps in the template switch. Rad18 and Rad5 were investigated since both are required for error-free PRR and the Rad5 helicase activity could potentially contribute to nascent strand displacement. Previous studies reported elevated frequencies of spontaneous gene conversion using allelic and direct repeat substrates in *rad18Δ* and *rad5Δ* mutants, presumably because ssDNA gaps that are substrates for PRR are channeled to HR [47]. Neither the *rad5Δ* nor *rad18Δ* mutant demonstrated hyper-recombination in the *ade2*-IR assay, suggesting there is little competition with PRR for substrate when HR proteins are present, or that an increase in use of the HR pathway is offset by a minor role for Rad5 and Rad18 in Rad51-dependent recombination of inverted repeats. The Ade^+ rate was decreased 8-fold in the *rad18Δ rad59Δ* double mutant compared with *rad59Δ* (Figure 3). This might be due to a minor role for Rad18 in the Rad51-dependent pathway, as suggested previously by the accumulation of branched DNA structures following methyl methane sulfonate treatment of the *sgs1Δ* mutant [38]. Alternatively, because sumoylation of PCNA is increased in the *rad18Δ* mutant it is possible that Srs2 is more efficiently recruited to stalled replication forks and disrupts Rad51 filaments resulting in down regulation of Rad51-dependent recombination [48–50].

Seeking further evidence for a replicative recombination mechanism, we designed a substrate in which the repeats were further apart expecting to decrease the rate of *RAD51*-independent recombination. The increased distance did not change the Ade^+ rate in the wild-type strain, suggesting *RAD51*-dependent recombination is unaffected by the proximity of the repeats; however, recombination of the *ade2*-IR-*Hph* reporter was reduced by an additional 2.6-fold in the *rad51Δ* mutant (Figure 2B). Physical analysis revealed a significant decrease in the number of inversion events recovered from the *rad51Δ ade2*-IR-*Hph* strain, whereas the distribution of events was the same for both inverted repeats in the wild-type strain (Figure 2C). The interpretation of these results is that *rad51Δ* mutants use a mechanism that is more adversely affected by the increased distance between heteroalleles than wild type. The reduced number of inversions is consistent with the faulty template switch model for *RAD51*-independent recombination because more DNA would need to be synthesized from the lagging template strand before displacing the leading nacent strand and annealing to the original template strand. It is possible that once highly processive replication is initiated on the opposite template then large chromosomal palindromes are the primary outcome and such events would be inviable and not be detected using the *ade2*-IR system. At this point no analysis for acentric/dicentric chromosome formation has been conducted so those outcomes remain a formal possibility in the *ade2*-IR system. We predict these events would be too rare to detect by Southern blot hybridization and the anticipated large inverted repeat would be difficult to detect by PCR because of the formation of snapback structures during the denaturation and annealing steps.

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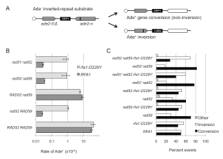

Figure 1. The *rfa1-D228Y* mutation partially suppresses the *rad52* recombination defect

A. Schematic of the *ade2*-IR substrate and Ade$^+$ products formed by gene conversion (non-inversion) or by inversion of the *TRP1* locus. The inversion events can have the wild type or mutant *Nde*I site within the *ade2–5'Δ* allele and are not distinguished here. Recombination rates ($\times 10^{-6}$) events/cell/generation are the mean of 3–4 independent trials. **C**. Distribution of events scored by physical analysis charted as the percentage of events examined (28–32 independent Ade$^+$ events were scored for each strain). "Other" events are complex banding patterns that appear to result from an additional copy of *ade2*; these were not investigated further.

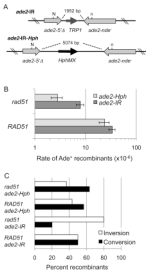

Figure 2. *ade2*-IR-*Hph* reporter to evaluate the role of distance between *ade2* repeats on recombination rates

A. Schematic of the *ade2*-IR-*Hph* substrate compared with *ade2*-IR. The distance between the repeats is increased from 1.9 kb to 5 kb. **B.** Recombination rate $\times 10^{-6}$ events/cell/generation reported as the mean of 3–4 trials. WT refers to wild type. **C.** Distribution of events scored by physical analysis charted as the percentage of events examined (30 independent Ade^+ events were scored for each strain).

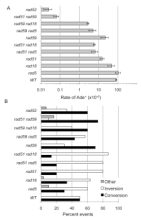

Figure 3. PRR plays a minor role in inverted-repeat recombination

A. Recombination rates $\times 10^{-6}$ events/cell/generation are the mean of 4–7 trials. **B.** Distribution of events scored by physical analysis charted as percentage of events examined (30 independent events from each strain were analyzed). The *rad51Δ* single mutant, and *rad5Δ rad51Δ*, *rad18Δ rad51Δ* and *rad51Δ rad59Δ* double mutants show a distribution significantly different to the wild type (P=0.03, 0.03, 0.005 and 0.004 respectively).

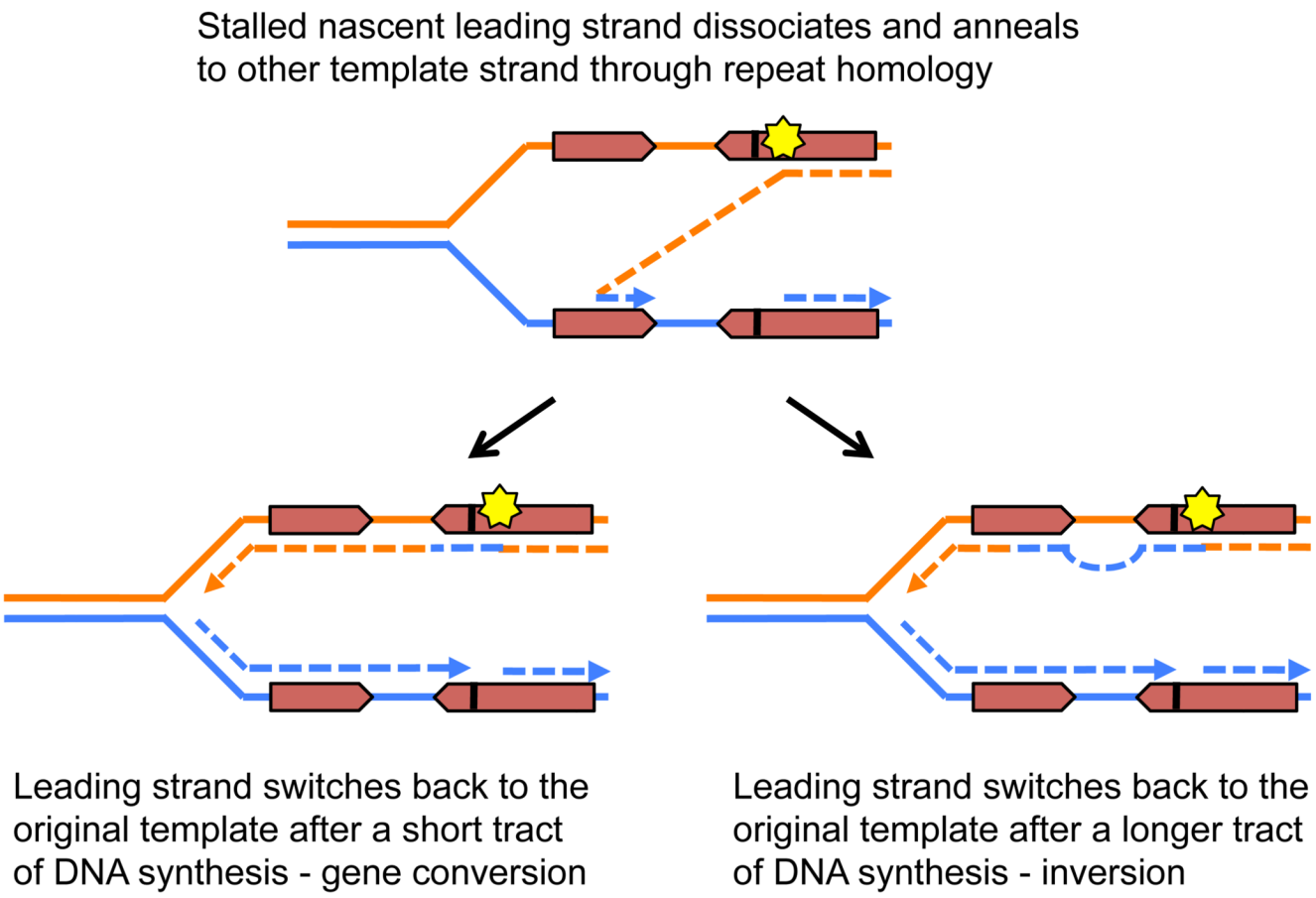

Figure 4. Faulty template-switch mechanism for inverted-repeat recombination

The model proposes stalling of the nascent strand, primer dissociation and annealing to the opposite parental strand via one of the repeats. The unequal pairing alignment is used to restart synthesis of the blocked nascent strand by providing an alternative template. A second template switch is required to restore canonical pairing between the leading nascent and template strands. The leading strand lesion has been bypassed and replication is restarted. The NdeI fill-in is indicated by a vertical black bar; white arrow, *ADE2*; blue and orange lines, ssDNA; dashed lines indicate nascent strands; the yellow star denotes spontaneous damage on the leading template strand.

Table 1

Yeast strains

Strain	Relevant genotype*	Source
U859/LSY2235	*MAT**a** rfa1-D228Y sup4+::HIS3+ rad5-535 ade2-1*	[13]
B404-3C	*MAT**a** his3::ade2-5'delta-TRP1-ade2-n::his3 ade2::hisG rad5-535 rad51::HIS3 rad59::LEU2*	[19]
HKY578-6B	*MATα rad5::URA3 ade2-1*	H. Klein
HKY1331-5D	*MATα rad18::LEU2 ade2-1*	H. Klein
LSY2001-41B	*MATα his3::ade2-5'Δ-TRP1-ade2-n ade2::hisG rad5::URA3*	This study
LSY2001-46A	*MATα his3::ade2-5'Δ-TRP1-ade2-n ade2::hisG rad5::URA3 rad51::HIS3*	This study
LSY2001-49A	*MAT**a** his3::ade2-5'Δ-TRP1-ade2-n ade2::hisG rad5::URA3 rad59::LEU2*	This study
LSY2002-9D	*MAT**a** his3::ade2-5'Δ-TRP1-ade2-n ade2::hisG*	This study
LSY2002-6D	*MATα his3::ade2-5'Δ-TRP1-ade2-n ade2::hisG rad51::HIS3 rad59::LEU2*	This study
LSY2002-16C	*MAT**a** his3::ade2-5'Δ-TRP1-ade2-n ade2::hisG rad59::LEU2*	This study
LSY2002-2B	*MATα his3::ade2-5'Δ-TRP1-ade2-n ade2::hisG rad18::LEU2*	This study
LSY2002-2C	*MAT**a** his3::ade2-5'Δ-TRP1-ade2-n ade2::hisG rad51::HIS3*	This study
LSY2002-10A	*MAT**a** his3::ade2-5'Δ-TRP1-ade2-n ade2::hisG rad18::LEU2 rad59::LEU2*	This study
LSY2185-9D	*MATα his3::ade2-5'Δ-TRP1-ade2-n ade2::hisG rad52::KanMX6*	This study
LSY2204-8B	*MAT**a** his3::ade2-5'Δ-TRP1-ade2-n ade2::hisG rad51::HIS3 rad52::KanMX6*	This study
LSY2249-1	*MAT**a** his3::ade2-5'Δ-HphMX4-ade2-n ade2::hisG*	This study
LSY2253-23C	*MAT**a** his3::ade2-5'Δ-HphMX4-ade2-n ade2::hisG rad51::HIS3*	This study
LSY2263-1C	*MATα his3::ade2-5'Δ-TRP1-ade2-n ade2::hisG rfa1-D228Y*	This study
LSY2263-3A	*MAT**a** his3::ade2-5'Δ-TRP1-ade2-n ade2::hisG rfa1-D228Y rad59::LEU2*	This study
LSY2263-6B	*MAT**a** his3::ade2-5'Δ-TRP1-ade2-n ade2::hisG rfa1-D228Y rad52::KanMX6*	This study
LSY2263-4D	*MATα his3::ade2-5'Δ-TRP1-ade2-n ade2::hisG rad52::KanMX6 rad59::LEU2*	This study
LSY2263-4A	*MATα his3::ade2-5'Δ-TRP1-ade2-n ade2::hisG rfa1-D228Y rad52::KanMX6 rad59::LEU2*	This study
LSY2439-11B	*MAT**a** his3::ade2-5'Δ-TRP1-ade2-n ade2::hisG rfa1-D228Y rad51::HIS3 rad52::KanMX6*	This study

*All strains are *leu2-3,112 trp1-1 ura3-1 can1-100 RAD5* unless otherwise indicated [28].