# Spontaneous and double-strand break repair-associated quasipalindrome and frameshift mutagenesis in budding yeast: role of mismatch repair.

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**ABSTRACT**

Although gene conversion (GC) in *Saccharomyces cerevisiae* is the most error-free way to repair double-strand breaks (DSBs), the mutation rate during homologous recombination is 1000 times greater than during replication. Many mutations involve dissociating a partially-copied strand from its repair template and re-aligning with the same or another template, leading to -1 frameshifts in homonucleotide runs, quasipalindrome (QP)-associated mutations and microhomology-mediated interchromosomal template switches. We studied GC induced by HO endonuclease cleavage at *MAT*$\alpha$, repaired by an *HMR::KI-URA3* donor. We inserted into *HMR*::*KI-URA3* an 18-bp inverted repeat where one arm had a 4-bp insertion. Most GCs yield *MAT::KI-ura3::QP+4* ($Ura^-$) outcomes, but template-switching produces $Ura^+$ colonies, losing the 4-bp insertion. If the QP arm without the insertion is first encountered by repair DNA polymerase and is then (mis)used as a template, the palindrome is perfected. When the QP+4 arm is encountered first, $Ura^+$ derivatives only occur after second-end capture and second-strand synthesis. QP+4 mutations are suppressed by mismatch repair (MMR) proteins Msh2, Msh3, and Mlh1, but not Msh6. Deleting Rdh54 significantly reduces QP mutations only when events creating $Ura^+$ occur in the context of a D-loop but not during second-strand synthesis. A similar bias is found with a proofreading-defective DNA polymerase mutation (*pol3-01*). DSB-induced mutations differed in several genetic requirements from spontaneous events. We also created a +1 frameshift in the donor, expanding a run of 4 Cs to 5 Cs. Again, $Ura^+$ recombinants markedly increased by disabling MMR, suggesting that MMR acts during GC but favors the unbroken, template strand.

## INTRODUCTION

Faithful DNA replication is essential for the survival of all organisms (Cox *et al.* 2000; Courcelle *et al.* 2004). In humans, the accumulation of mutations has profound implications for health. Increased mutability is associated with cancer proneness, immune deficiency, premature aging and neurological and developmental impairments (Cortez 2019; Brown and Freudenreich 2021; D'Amico and Vasquez 2021; Caldecott 2022). Mutations and genomic rearrangements promote cancer, affect reproductive success and can lead to human genetic diseases.

Mutations that reshape genomes derive from many sources. Many frequent mutations arise in repetitive DNA sequences, where where dissociation or slippage of the DNA polymerase copying a repair template results in annealing of short, sometimes imperfect repeated sequences and subsequently generates genomic rearrangements or mutations (Lovett 2004, 2017; Malkova and Haber 2012; Anand *et al.* 2014). This class of mutagenesis, called "template-switching", is intrinsically different in mechanism from other types that result from DNA miscoding, from physical damage to the bases of the DNA template or from alterations in nucleotide pools. Genetic and biochemical analyses have defined many cellular processes that promote or deter mutagenesis by base damage; in contrast, much less is known about the cellular pathways that affect template-switch-derived mutagenesis.

Template-switching can occur in imperfect inverted repeat sequences, known as "quasipalindromes", where DNA polymerase copies one arm of the palindromic region but then anneals to its own newly copied DNA to complete synthesis of the second arm. Mutations at QP sites ("QPM") are often found as hotspots of mutation in viruses, bacteria, yeast and other genetic model systems (reviewed in (Ripley 1982; Lovett 2017)). Evidence for template-switch mutations at QP sites during evolution can also be deduced from genomic sequences (Bissler 1998; Noort *et al.* 2003; Löytynoja and Goldman 2017; Abraham and Hazkani-Covo 2021;

Walker *et al.* 2021; Löytynoja 2022).

To study template-switch mutagenesis at QP sites, we previously developed specific reporters in the *lacZ* gene of the bacterium *Escherichia coli* that revert to $Lac^+$ by a template-switch reaction in an 18-bp quasipalindrome. These specific mutational reporter strains allowed us to deduce that DNA exonucleases ExoI and ExoVII protect *E. coli* cells from template-switching and that these mutations occur during DNA replication (Seier *et al.* 2011, 2012; Laranjo *et al.* 2017). In addition, we have defined several mutagens for QPM including DNA replication inhibitors HU (and azidothymidine, as well as DNA/protein crosslink promoting agents, including formaldehyde, 5-azacytidine and fluoroquinolone antibiotics (Seier *et al.* 2011, 2012; Laranjo *et al.* 2018; Klaric *et al.* 2020).

Here, using a similar approach, we examine QP mutagenesis (QPM) in the budding yeast *Saccharomyces cerevisiae.* We examined events arising spontaneously, presumably during DNA replication, and those that occur during the DNA synthesis that is required for the repair of a chromosomal double-strand break (DSB) by gene conversion. Budding yeast is an ideal genetic system to study eukaryotic QPM, since much is known about DNA replication and repair, and it is easy to generate large populations to measure mutation frequencies. QPM was first discovered by Sherman and colleagues ((Hampsey *et al.* 1988) in which a mutational hotspot in the *S. cerevisiae CYC1* (cytochrome oxidase) gene occurred at a 7 bp QP site; therefore, this template-switching mechanism clearly operates in budding yeast.

We have developed a sensitive system to detect mutations arising during DSB-mediated gene conversion in budding yeast (Hicks *et al.* 2010). A site-specific DSB is created rapidly and synchronously by expression of galactose-inducible HO endonuclease, cleaving the *MAT*$\alpha$ locus (Figure 1A). The DSB is repaired by copying homologous sequences at the heterochromatic *HMR* donor, located 100 kb distally on the same chromosome arm, into which

is embedded a *Kluyveromyces lactis URA3* gene (*KI-URA3*). *KI-URA3* is silenced by the heterochromatic structure within *HMR* but is expressed when these sequences are copied into the *MAT* locus, replacing the Y$\alpha$ sequences with Y**a**::*KI-URA3* (Figure 2A). Nearly all cells in the population complete this gene conversion repair event in one cell cycle. Ura$^-$ mutations that arise during the switching process can be easily recovered by plating cells on 5-fluoroorotic acid (FOA). Mutations proved to be 1000 times more frequent than spontaneous mutations of *MAT::KI-URA3* (Hicks *et al.* 2010) That these mutations arose during the repair event could be demonstrated by un-silencing *HMR*, using the Sir2 inhibitor, nicotinamide; when *HMR::KI-URA3* is expressed the Ura$^-$ cells become Ura$^+$, since the *HMR* donor, harboring a functional *KI- URA3* gene, is unaltered. About 60% of the mutations arising during gene conversion are single base-pair substitutions, but many of the mutants have sequence length changes and many of these appear to have involved the dissociation of the partially copied DNA strand from its template, followed by reannealing at a microhomology. Among these events are -1 (but rarely +1) frameshift (FS) changes in homonucleotide runs, intragenic deletions, and quasipalindrome-mediated events.

Quasipalindrome-mediated changes can be difficult to recognize, since they have imperfect inverted repeats that can be separated by a variable number of base pairs. Therefore, we sought to study these events by creating a specific QP that would be mutated preferentially during gene conversion. We developed an assay to examine this QP as well as an assay to characterize a +1 frameshift (FS) mutation in a homonucleotide run, in different genetic backgrounds defective in mismatch repair, DNA synthesis or recombination. We find that there are some significant differences in the genetic requirements for QP mutagenesis in spontaneous and DSB-induced events.

**RESULTS**

**Design:** To measure quasipalindrome-associated mutations accompanying gene conversion, we started with a *sir3*Δ derivative of strain WH50 that can undergo gene conversion by creating a DSB at *MAT*α and using *HMR::KI-URA3* as a donor (Hicks *et al.* 2010; Tsaponina and Haber 2014) . Deleting *SIR3* removes the heterochromatic state at *HMR* making *HMR* more representative of the yeast genome, which is largely euchromatic. The donor sequence, *HMR*::*KI-URA3,* consists of the *KI-URA3* coding sequence that replaces the *HMR Ya1* coding sequence but uses its promoter, while also removing the HO cleavage site (Figure 1B). The *KI-URA3* sequence was modified to contain a QP composed of inverted 18-bp repeats plus a 4-bp insertion in either the left repeat (QP7) or the right repeat (QP8) (Figure 1C and 1D). The 4-bp insertion creates a frameshift so that *KI-ura3-QP* is expressed in a *sir3*Δ background but is phenotypically Ura$^-$. Upon induction of *GAL::HO,* cells efficiently repair the DSB at *MAT* by gene conversion, using *HMR::KI-ura3-QP* as the donor. The great majority of cells remain Ura$^-$ and retain the 4-bp insertion within the QP sequence; however, in a small fraction of repair events, the 4-bp insertion is lost (i.e. the quasipalindrome is perfected) and cells become Ura$^+$. Ura$^+$ cells arose at a frequency of 6.7 or 2.1 x $10^{-6}$ for QP7 or QP8, respectively; the three-fold difference is statistically significant (Table 1). We note that all of the events producing Ura$^+$ revertants occur during the single cell cycle in which the DSB at *MAT* is repaired by gene conversion; hence, the frequencies that we observe are in fact the rates of QP mutation accompanying repair.

To confirm that the Ura$^+$ recombinants were corrections of the QP frameshift within the inverted repeats, we sequenced *MAT*-specific and *HMR*-specific PCR products from approximately 20 independent Ura$^+$ colonies. From the QP7 strain 20 colonies had the 4-bp insertion precisely deleted at the *MAT* locus while retaining the unchanged QP7 sequence at *HMR*. For the QP8 strain 22 colonies were examined and 19 had precise deletions of the 4bp insertion in QP8 at *MAT* with no changes to the QP at *HMR*. The remaining 3 colonies had the 4bp deleted from

QP8 at *HMR* with no changes to *MAT::KI-ura3-QP*. The fact that QP8 at *MAT* retained the 4-bp in these 3 colonies suggests that the loss likely occurred during the gene conversion event itself, as a pre-existing 4-bp deletion at *HMR* would have led to a duplication of the 4-bp deletion at *MAT*. Our sequencing results for this and later experiments are summarized in Supplemental Table S1.

Inserting the QPs to *KI-ura3* did not affect the ability of yeast to carry out gene conversion as the overall viability after gene conversion was comparable to a strain without the QPs. Figure 3 compares the number of colonies grown on galactose plates relative to glucose plates. Similarly, none of the mutant backgrounds that we discuss later caused a significant decrease in viability (Figure 3).

The frequency of $Ura^+$ events arising during homologous recombination is much higher than the spontaneous rate of QP reversion. We tested for spontaneous correction to $Ura^+$ at the *HMR::KI-ura3-QP* sequence in the *sir3*$\Delta$ strains that allow gene expression of *KI-ura3* mutants embedded within *HMR*. The frequencies of spontaneous $Ura^+$ mutation were assayed in strains that were deleted for *GAL::HO* since leaky expression of the endonuclease could otherwise contribute to background levels. The spontaneous frequencies were approximately $3.5 \times 10^{-9}$ for both QP7 and QP8 (Table 1), about 1000 times lower than the HO-induced frequencies for both QP7 and QP8. We sequenced each QP at *HMR* from 10 colonies that spontaneously became $Ura^+$ and found that all of them precisely deleted the 4-bp sequence from the QP.

In a similar fashion we assayed a specific +1 frameshift (FS) reversion event arising during DSB repair. In our original study we had identified a run of 4 Cs in the *KI-URA3* sequence that was a hotspot for the deletion of a single C (-1C). By adding another C at this site (nucleotide 605 in the coding sequence) we presumed that we would create a hotspot where the loss of one C from a homonucleotide run of now 5 Cs would restore the open reading frame. The frequency of

HO-induced FS reversion ($7.5 \times 10^{-6}$) was > 300 times higher than the spontaneous frequency (Tables 1 and 2). DNA sequencing of *MAT*-specific and *HMR*-specific PCR products from 21 independent colonies showed that all of them had deleted a C from the *MAT* locus and retained the extra C in the *HMR* locus. Similarly the extra C was deleted from the *Kl-ura3-FS* at *HMR* in 10 $Ura^+$ colonies that were spontaneously derived.

**Effect of mismatch repair genes on QP and FS mutagenesis:** The mismatch repair gene *MSH2* plays a number of roles in DSB repair (Modrich and Lahue 1996; Kolodner and Marsischky 1999; Harfe and Jinks-Robertson 2000; Oh and Myung 2022). The MutSβ heterodimer, Msh2-Msh3, acts with MutLα (Mlh1-Pms1) to recognize and correct heteroduplex DNA containing small insertion/deletions. In contrast, the MutSα (Msh2-Msh6) heterodimer, again acting with MutLα, is responsible for the mismatch correction of single base pair heterologies or insertion/deletion of single bases. MutSα and MutSβ have partially overlapping activities. The Msh2-Msh3 complex also facilitates the removal of 3' nonhomologous flaps from DSB repair intermediates, independent of its interaction with the MutL proteins (Sugawara *et al.* 1997; Lyndaker and Alani 2009). Deletion of Msh2 or Msh3 caused a > 40-fold increase in the spontaneous rate of $Ura^+$ QP mutations for both QP7 and QP8 (Figure 4) and an even larger increase (> 400-fold) in FS mutations (Figure 4). Sequencing of the QPs at *HMR* from $Ura^+$ *msh3*Δ gene convertants showed that all 10 QP7 and 10 QP8 strains had lost the 4bp insertion. Deleting *MSH6* had little effect on either QP orientation, a result that is consistent with Msh2-Msh6 recognizing only small distortions in the DNA helix. In contrast, *msh6*Δ still caused a 25-fold increase in spontaneous FS mutations. These results suggest that both Msh2-Msh3 and Msh2-Msh6 can suppress spontaneous -1 frameshifts associated with a 5-bp homonucleotide run.

During HO-induced DSB repair, both *msh2*Δ and *msh3*Δ caused a significant increase in both

QP and FS mutations, though these increases (3.5 - 8 fold) were much less profound than the effects on spontaneous events (Figures 4 and 5). In contrast, *msh*6Δ had no significant effect, even on the FS reversion (Figure 5). This result suggests that there are intermediates of mismatch correction during normal DNA replication that may be distinct from those in DSB repair, where Msh6 does not seem to participate in -1 deletions (see Discussion).

We confirmed that the Ura$^+$ recombinants arising during DSB repair in *msh2*Δ strains were the result of the specific correction of the QP insertions or the +1 frameshift. We PCR-amplified and sequenced the QPs from approximately 20 Ura$^+$ colonies from the *msh2*Δ QP7 and QP8 strains. From the QP7 strain 22 colonies had precise deletions of the 4-bp insertion and the QP sequence at *HMR* remained unchanged. From the QP8 strain 23 colonies had clean deletions of the QP at *MAT* while remaining unchanged at *HMR*. With respect to the FS mutation, 17 of 20 colonies had a C deleted from *mat*::*Kl-ura3* and remained unchanged at *HMR*. One colony contained a C-deletion at *HMR* making it *HMR*::*Kl-URA3* while acquiring *Kl-ura3-FS* at *MAT*, similar to our observations from the wildtype QP8 colonies described earlier. A second colony acquired a short, 31-bp homeologous sequence from *ura3-52* which is a product of an interchromosomal event using *ura3-52* on a different chromosome as a template and which replaced the region containing the C insertion (shown in Supplemental Table 1). Such interchromosomal template switches between *HMR::Kl-URA3* and a 72% identical *Saccharomyces ura3 gene* on a different chromosome have been documented previously (Hicks *et al.* 2010; Tsaponina and Haber 2014) at rates that are compatible with one such event being recovered in this study. (Tsaponina and Haber 2014). No ICTS events were observed among the revertants of the QP mutations.

The last Ura$^+$ FS revertant contained an ambiguous sequence that appears to be the result of a strain containing both a *MAT*::*Kl-URA3* and a *MAT*::*Kl-ura3-FS* sequence where one copy was corrected and one was not. As we selected Ura$^+$ colonies, this outcome should not have

resulted from a heteroduplex formed at *MAT* that was not corrected but after the subsequent DNA replication yielded one $Ura^+$ and one $Ura^-$ outcome. It is more likely that an "**a**-like" cell created by switching *MAT*α to *MAT*::*KI-URA3* may have mated with a *MAT*α cell that switched to *MAT*::*KI-ura3-FS* after mating.

Mlh1-Pms1 is recruited by Msh2-Msh3 or Msh2-Msh6 and creates a nick in the DNA, in preparation for nucleolytic removal of mismatches (Rogacheva *et al.* 2014; Reyes *et al.* 2015) We examined the role of Mlh1 in the QP and FS strains. Like *msh2*Δ, *mlh1*Δ mutants had a highly significant increase in the level of spontaneous mutation for QP7, QP8 and FS strain compared to the wild type (Figures 4 and 5). Among the DSB-induced events, *mlh1*Δ again behaved similarly to *msh2*Δ, with a 6-fold increase in $Ura^+$ recombinants. Thus, both QP and FS correction during DSB repair require both Msh2-dependent recognition and Mlh1-dependent processing. Again, the much larger effects of *mlh1*Δ on spontaneous mutations suggest differences in the way the QP and FS mutations are processed during DNA replication and in repair.

**Effect of DNA replication mutations on QP and FS mutagenesis during DSB repair:** Our understanding of QP mutagenesis requires that the partly-copied DNA strand dissociate from its template in order that the end can anneal to itself, allowing the perfection of the palindrome in the newly-copied DNA but leaving the donor sequence unaltered (Figure 2). With this in mind, we surveyed strains with mutations in a number of polymerase components such as Pol2, Pol3, Pol32, Rev3, and Rad30. Pol3 is the catalytic unit of DNA polymerase δ; the *pol3- 1* mutant is defective in 3' to 5' exonucleolytic error correction. Our previous study had shown that *pol3-01* dramatically reduced template switching events while increasing missense mutations (Hicks *et al.* 2010) An increase in single-nucleotide variants is expected in the absence of proofreading, but the decrease in template switching suggested that the mutant polymerase may dissociate less often from its template, as shown in an *in vitro* study (Jin *et al.* 2005). Here, the *pol3-01*

derivative of QP7 showed a significant 4-fold decrease relative to wild type; however, this reduction was not seen in the QP8 strain (Figure 5). The different effect of *pol3-01* may reflect the important difference in the origin of $Ura^+$ events in the two QP orientations (see Discussion). Spontaneous mutagenesis measured with QP7 was also reduced by *pol3-01* (Figure 4). The *pol3-01* mutation significantly *increased* FS reversion during DSB repair, suggesting that its defect in proofreading a -1 deletion may outweigh any effect on its processivity (Figure 5). The *pol3-01* mutation also led to a 20-fold increase in spontaneous FS reversions.

*Pol2-04*, a proofreading-defective mutation of DNA polymerase ε, showed a small but significant increase in frequency in the QP7 strain relative to the wild type after gene conversion (1.6x), but had no significant effect on QP8 or FS reversion (Figures 4 and 5). There were no significant effects among spontaneous events as well.

Pol32 is a nonessential subunit of Polδ that is important for break-induced replication where there is extensive DNA synthesis after strand invasion (Lydeard *et al.* 2007) In our DSB-induced assay *pol32*Δ significantly decreased the number of frameshift events (3.1-fold relative to wild type) whereas it slightly increased the number of QP8 corrections (1.6x). In addition to being part of Polδ, Pol32 along with Pol31, also forms a complex with Rev3 and Rev7 as part of Polζ (Johnson *et al.* 2012; Makarova *et al.* 2012). Rev3 is the catalytic component of Polζ and is responsible for error-prone damage repair. Examination of the *rev3*Δ mutant showed no significant difference from wild type indicating that the effects of *pol32*Δ occur largely through its association with Polδ (Figures 4 and 5). Rad30 is DNA polymerase η, a translesion repair polymerase. The *rad30*Δ mutant showed no effect on the QP or frameshift mutations in the DSB-induced assays nor in spontaneous events.

**Effect of *rdh54*Δ affecting template switching on QP and FS mutagenesis:**

Previously we surveyed genes to assess their role in generating ICTS events between *HMR*::*KI*-

*ura3* and the *Saccharomyces ura3-52* allele (Tsaponina and Haber 2014). Among deletions of genes known to be involved in homologous recombination, only one - *RDH54* – had a highly selective effect on template switching. An *rdh54*Δ strain had no marked defect in simple gene conversions — i.e., replacing *MAT*α by *MAT::KI-ura3* – but was > 50x decreased for ICTS events (Tsaponina and Haber 2014). Deleting *RDH54* also markedly reduced template jumps in a break-induced replication assay (Anand *et al.* 2014). Here we show that QP7 but not QP8 events are reduced by *rdh54*Δ; but FS events were not reduced by deleting *RDH54* (Figures 4 and 5). The differences between the two QP orientations is again significant and suggests that Rdh54's role is linked to the structure of the recombination intermediates distinguishing QP7 from QP8 (see Discussion). Spontaneous events detected with QP8 were also modestly reduced by *rdh54*Δ.

**DISCUSSION**

From our analysis of quasipalindrome mutagenesis and frameshift reversion we conclude that there are significant differences between spontaneous and DSB repair-associated events, but there are also notable differences depending on the orientation of the QP relative to the direction of DNA synthesis. Previous studies from *E. coli* have shown that the rate of QPM is elevated on the leading strand of the normal replication fork because of single-strand DNA binding protein recruitment of exonucleases that abort QP mutagenesis on the lagging strand (Seier *et al.* 2011; Laranjo *et al.* 2017) In the absence of exonucleases, lagging strand QPM is more frequent, presumably because the opening of the DNA ahead of the DNA polymerase allows the template strand to form hairpin structures that should facilitate QPM.

One of the distinctive features of *MAT* switching is that the *MAT-Z* end of the DSB ends is perfectly matched with the *HMR::KI-URA3* donor sequence, while the other end terminates in a ~700-bp nonhomology that must be removed before second-end DNA synthesis can be accomplished (Figure 2A). Thus, in QP7 the 4-bp insertion in the hairpin is distal to the direction

of first-strand repair synthesis (Figure 2B), while in QP8 the DNA polymerase encounters the QP arm carrying the +4 insertion first (Figure 2C). The polarity of strand synthesis accounts for some of the different effects of mutations that we find between QP7 and QP8. Differences in the composition of the normal replication fork and the machinery involved in DSB repair most likely accounts for differences in the dependence of spontaneous and DSB-induced QPM on various replication and repair factors. *HMR* is flanked by a known origin of replication on the left side, but we do not know the predominant direction of DNA replication across this region.

In theory, mismatch repair removing the 4-bp insertion could occur within a hairpin formed on the template strand before the region is copied during repair (Figure 2B). However, this would revert the *HMR::KI-ura3-QP7* region, whereas we find that the donor sequence almost always remains unchanged and the $Ura^+$ sequence is mainly found at the recipient locus. We did find four such reversion events creating *HMR::KI-URA3* (3 wildtype QP8 events and 1 *msh2*Δ FS event), but in those instances, the sequence transferred to *MAT* still contained *MAT*::*KI-ura3-QP8* or *MAT*::*KI-ura3-FS*, suggesting that reversion of the QP occurred during the repair event itself. We previously observed such changes in the donor sequence in interchromosomal template switches (Tsaponina and Haber 2014)

In QP7, first-strand repair synthesis can form a hairpin such that the dissociation of the partially copied strand will permit the formation of the partial cruciform intermediate that will allow this strand to eliminate the insertion (Figure 2B-2). This intermediate presumes that the two donor strands do not themselves reanneal but remain in a D-loop. We imagine that the D-loop is kept open by binding of single-strand binding protein complex, RPA, to the displaced donor strand. RPA appears to play a role in strand invasion as well as in facilitating Rad51 binding to ssDNA, possibly through stabilizing the displaced ssDNA (Wang and Haber 2004). After DNA polymerase reaches the end of the palindrome (Figure 2B-3), there must be a second template switch to re- align the sequences such that repair synthesis can continue into the *HMR-X* region

that will allow it to – yet again – dissociate so that the second end can be captured at *MAT* and initiate second- strand synthesis (Figure 2B-4 and -5). However, before displacement and second-end capture, the newly copied strand, with its perfected palindrome, apparently anneals to its template strand, creating heteroduplex DNA that can be recognized and mismatch repaired by the MutSβ-MutLα complex. We surmise that mismatch repair is highly directional, favoring the complete template strand over the elongating repair strand (Figure 6A). This directionality of MMR will restore the 4-bp insertion on the newly copied strand and eliminate $Ura^+$ outcomes. Hence we find a very significant increase in $Ura^+$ recombinants when Msh2, Msh3 or Mlh1 are deleted. Because Msh6 does not recognize 4-bp insertions, the rate of $Ura^+$ in *msh6*Δ is not elevated relative to the wild type control.

Although *msh2*Δ, *msh3*Δ and *mlh1*Δ also elevate the rate of $Ura^+$ events in QP8, the rates of these events and of the wildtype are all significantly lower than for QP7. This difference reflects the fact that first-strand repair synthesis cannot produce a corrected $Ura^+$ strand (Figure 2C-2), as the formation of a transient partial cruciform and copying itself will lead to the insertion of a second 4-bp segment. Thus, creating a *KI-URA3* sequence with QP8 can only occur during second-strand synthesis, when the template is not the *HMR* donor locus, but the first-strand copy that is captured by the second end of the DSB (Figure 2C-3). Dissociation of the partially-copied second strand and realignment on the first-strand template will create a heteroduplex that again can be recognized by Msh2-Msh3, and not Msh6 (Figure 2C-5). Again, MMR is likely to favor correction of the heteroduplex in favor of the "intact" strand and hence $Ura^+$ events will be eliminated. In the absence of mismatch repair, the heteroduplex will persist and cells will only become $Ura^+$ after a round to DNA replication that will create a $Ura^+/Ura^-$ sectored colony. Because the rate of QP events is low, it has not been possible to identify sectored colonies directly.

The much larger effect of *msh2*Δ, *msh3*Δ and *mlh1*Δ on spontaneous frequencies relative to those induced during *MAT* switching may arise from limits to mismatch discrimination during DSB repair. During first-strand synthesis, capacity for removal of mutations exists only when they are paired in heteroduplex with their templates and - because of D-loop migration - the newly replicated strand may be extruded as a single-strand shortly after synthesis, and so lacks a partner for mismatch repair. During second-strand synthesis, it may be impossible to discriminate mutant vs parental information. During normal semiconservative synthesis, there is likely a larger window to discriminate and remove mismatched nucleotides from the newly replicated strand than what occurs during DSB repair.

While a deletion of RDH54 did not cause a significant decrease in gene conversion, Rdh54 was important for the correction of the 4-bp insert in the QP7 palindrome but not in the QP8 palindrome. Previous work concluded that Rdh54 antagonizes the action of Rad54 in the formation of displacement loops (D-loops) and that it does not play a role in the subsequent steps that extended the D-loop by DNA synthesis (Piazza *et al.* 2019) . In vitro, Rdh54 acts before the formation of the D-loop but not after the D-loop is formed (Shah *et al.* 2020). Furthermore, a DNA foot-printing assay showed that Rdh54 causes a decrease in the length of D-loops (Shah *et al.* 2020). It was proposed that Rad54 creates the heteroduplex DNA with Rad51 and that Rdh54 attenuates this by creating a roadblock to the formation of long heteroduplex DNA. Another study using smaller DNA templates was able to observe that Rdh54 is important for the removal of Rad51 from the D-loop which allowed extension of the D-loop by new DNA synthesis (Keymakh *et al.* 2022).

Our data showed that *rdh54*Δ caused a decrease in the QP7 strain but not in the QP8 strain. On the surface it would appear that Rdh54 plays a role in the extension step since the QPs lie in the nonhomologous region of *HMR* rather than in the *Z* region common to both *MAT* and *HMR* where a D-loop would initially form. One might conclude that Rdh54 acts

after movement of the D-loop since the QP sequences start 671-bp from the 3' end of the invading strand. However, Rdh54 may still exert its function during strand invasion if D-loops form in the *KI-ura3* sequences during cycles of dissociation and new strand invasion (Smith *et al.* 2007; Ruiz *et al.* 2009; Anand *et al.* 2014). The shorter D-loops made in wildtype strains would favor template switching compared to the longer D-loops formed in *rdh54*Δ mutants.

Furthermore, we suggest that the lack of inhibition in QP8 reflects the different DNA intermediates where mutagenesis is proposed to occur. In QP7, the dissociation of the partially copied strand takes place in the context of a migrating D-loop, whose length, stability and topological state are affected by Rdh54 (Petukhova *et al.* 2000; Piazza *et al.* 2019; Shah *et al.* 2020; Keymakh *et al.* 2022). In QP8, mutagenesis appears to occur in a simpler context, where the first-strand ssDNA has been captured by the second end, which then uses the first strand as a template; this occurs in the absence of a competing complementary strand that is present in the migrating D-loop (Figure 6B). The different effects of deleting Rdh54 in these two contexts supports these models of mutagenesis.

We offer a similar explanation for the different effect of *pol3-01* in QP7 and QP8: in the context of a migrating D-loop the greater processivity of *pol3-01* will lead to a lower rate of new strand dissociation, whereas in QP8, *pol3-01* apparently does not change the rate of dissociation. Another explanation for differential Pol effects on QP mutations may be caused by the superior ability of Pol $\delta$ over Pol $\varepsilon$ to catalyze strand displacement synthesis (Burgers and Kunkel 2017). 3' extension DNA synthesis from an internally paired QP structure may require the displacement of the parental strand template, a reaction more readily catalyzed by Pol $\delta$ in vitro (Burgers and Kunkel 2017). The nonessential Pol32 subunit of Pol has been proposed to promote strand displacement *in vivo* (Budd *et al.* 2006) and to make Pol $\delta$ more processive *in vivo* (Garbacz *et al.* 2018). For spontaneous QP mutagenesis detected with either QP7 and QP8 , there are

indications of effects by Pol32, Rdh54 and proofreading mutants in Pol  but further work will be required to substantiate this.

In frameshift (-1C) mutagenesis, we again find differences in the roles of various repair proteins in spontaneous and repair-associated events.  Here, both Msh3 and Msh6 appear to contribute to the suppression of spontaneous events, presumably along with Msh2, and Mlh1 is also implicated. In both situations, *pol3-01* leads to a significant increase in mutations, attributable to its defect in proofreading, but *pol2-04* does not.

**METHODS**

**Strains**: All strains (Supplemental Table S2) are derivatives of WH050 (*ho hml*Δ::*ADE1 MAT*α *hmr::KI-URA3 ade1 leu2 lys5 trp1::hisG ura3-52 ade3::GAL::HO*) that was altered by introducing *sir3*Δ::HPHMX (TOY7). The QP consists of 18bp inverted repeats (GTGCTTCCTTGGATGTAC) with a 4bp insertion (GTGCTTCC**AATA**TTGGATGTAC) in either the left (QP7) or right (QP8) repeat. The QPs replace the wild type sequence CTCTTGACGTTCGTTCGACTGATGAGCTAT in the *KI-URA3* open reading frame. The frameshift mutation consists of a C inserted into a run of 4 Cs at the start of the sequence CCCCAGGTGTAGGTTTAGAC in the coding sequence. Mutations were introduced by repairing a Cas9-induced DSB within *KI-URA3* (Anand *et al.* 2017).  The DSB was repaired using a synthetic 500bp dsDNA containing the pertinent sequence alterations. Deletion mutations were introduced using the KANMX drug cassette (*msh2*Δ, *msh3*Δ, *msh6*Δ, *pol32*Δ, *rdh54*Δ, *rev3*Δ and *rad30*Δ) or the NATMX cassette (*mlh1*Δ). The *pol3-01* and *pol2-4* alleles were created using Cas9 to create a DSB within *POL3* or *POL2* that was repaired by using a ssDNA oligonucleotide containing the requisite sequence changes (Gallagher *et al.* 2020). We also determined the frequencies of spontaneous mutations using derivatives deleted for *GAL::HO*. These strains were constructed by transforming the strains with a PCR product from *ADE3* that converted

*ade3::GAL::HO* to *ADE3*.

**Assays**: To assay spontaneous events, single colonies grown on plates containing 5-FOA were inoculated into YEPD media and grown for ~18 hours or until saturation. Cultures were then diluted 1:10 with fresh media and incubated for 2 hours to resume growth. Cultures were then washed twice with sterile water by centrifugation then concentrated in sterile water. A fraction of the resulting resuspension was serially diluted and spotted onto YEPD plates to quantify the total cells in the sample. The remaining sample was plated on media plates lacking uracil. YEPD plate colonies were counted after 2 days and uracil-lacking plate colonies after 4 days. The frequencies of reversion events were then calculated. For strains with particularly low reversion frequencies, some assays were performed with 15mL initial cultures. The entire culture was collected after ~18 hours of growth and then assayed as described, proceeding directly to the wash step.

For assaying DSB-induced gene conversion events, cells were grown in 5- FOA to minimize the growth of pre-existing $Ura^+$ mutants, which was especially a problem in strains lacking mismatch repair genes that have an elevated level of spontaneous mutations. Strains were grown in 5-FOA for 2 days, washed twice with sterile water by centrifugation, briefly sonicated to break up clumps of cells, diluted and plated onto 1) selective medium lacking uracil and containing galactose and 2) rich medium (YPD). Colonies were counted after 3 to 5 days and the frequencies were determined. Cells were also plated on dextrose-containing plates lacking uracil to assess background levels of mutation correction. When rich medium was used in place of 5-FOA spontaneous events occurred at a significant level in certain strains in the *msh2*Δ *msh3*ti or *mlh1*tΔ backgrounds which may be due to leaky repression of the *GAL::HO* gene.

In the wild type and in most of the mutants this background level was low relative to the induced levels. These ranged from 0.14% to 9.3% relative to the induced levels. The exceptions were

the frameshift mutants in the *msh2*Δ, *msh3*Δ and *mlh1*Δ backgrounds which had background levels that were respectively 32%, 11% and 42% when compared to the induced levels. For the values reported in Table 2 the background levels were subtracted from the induced levels.

The overall gene conversion levels for the wildtype and mutant strains were determined by growing the cells in YEP-lactate medium and then plating the cells on YEPD and YEP-galactose plates at the appropriate dilutions for calculating the survival frequencies (Figure 3).

**Sequencing**: The removal of the +4 bp insertion in the QPs or one C from *KI-ura3*-FS at *MAT::KI-URA3* was confirmed by PCR amplifying *MAT::KI-URA3* using a *MAT* primer (CGGGGAAACTGTATAAAACTTCC) upstream of *KI-URA3* and a *MAT*-distal primer (CATTTGTCATCCGTCCCGTATAG), and sequencing the PCR products. A primer specific to *HMR*-distal (CTTTATCGCAGTAGAAAGACATATT) was used with the same *KI-URA3*-upstream primer for sequencing *HMR::KI-ura3* sequences.

**DATA AVAILABILITY STATEMENT**: Strains and plasmids are available upon request. The authors affirm that all data necessary for confirming the conclusions of the article are present within the article, figures, and tables.

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**CONFLICT OF INTEREST:** The authors declare no conflict of interest.

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## FIGURE LEGENDS

**Figure 1.** A. DSB-induced gene conversion of *MAT*α to *MAT::KI-ura3*. Galactose-induced expression of HO endonuclease creates a DSB at *MAT*α that initiates gene conversion using *HMR::KI-URA3* as a donor template. B. *KI-URA3* replaces *HMR* Y**a**1 and possesses a quasipalindrome (QP) or frameshift (FS) mutation rendering it Ura$^-$. Gene conversion leads primarily to Ura$^-$ colonies in which the QP or FS is copied into *MAT*, but Ura$^+$ colonies arise by template switching removing the +4-bp insertion in the QPs or by replication slippage causing a correction of the FS. C. The quasipalindromes QP7 and QP8 consist of 18bp inverted repeats plus a 4-bp AATA insertion (blue, upper case). The +1 FS mutation consists of C inserted into a run of C's (blue). D. The locations of the QP and FS mutations are shown on the *KI-ura3* open reading frame.

**Figure 2.** Removal of the 4 bp insertion in the quasipalindromes. **A**. In the Synthesis-Dependent Strand Annealing (SDSA) model, a DSB initiates DNA resection leaving 3' single stranded tails. The 3' tail strand in the Z region invades the donor sequence (*HMR*) forming a D-loop (1). DNA synthesis begins and the D-loop migrates to the left while displacing newly synthesized DNA (2). The displaced newly copied strand engages in second-end capture in which it anneals to

the resected single stranded tail from the left side after which nonhomologous sequences at the 3' end of the ssDNA tail are clipped off (3). The second strand of the repaired *MAT* locus is then filled in (4). **B**. In the QP7 strain the resected DNA from *MAT*-Z invades *HMR*-Z, creating a D-loop, and initiates DNA synthesis (1). As the polymerase passes through the first 18-bp repeat and enters the second repeat a hairpin forms (2). DNA synthesis continues using the first half of the QP, thus not incorporating the 4-bp insertion (red triangle) (3). The newly-synthesized strand then realigns with the donor to complete copying the donor into the *MAT*-X (4). As SDSA progresses the newly synthesized strand is displaced and anneals with the resected DNA from *MAT*-X and completes repair (5). C. In the QP8 strain the 3' tail from *MAT*-Z strand invades *HMR*-Z and primes synthesis through QP8 (1). In the SDSA model the invading strand unwinds and is displaced from HMR (2). This allows it to anneal to the ssDNA from *MAT*-X that is formed as resection proceeds leftward from the DSB (3). Extension of this DNA to the QP sequences allows a hairpin to form and DNA synthesis to continue without incorporating the 4bp insertion (4). The final product contains a heteroduplex that can be resolved by mismatch repair or by DNA replication and mitotic segregation (5).

**Figure 3.** Viability of strains with QPs undergoing gene conversion. Gene conversion was induced by plating cells on YEP-galactose. Graphs show the survival of strains relative to the number of colonies on YEPD plates.

**Figure 4.** Spontaneous frameshift and QP-associated mutagenesis. Spontaneous frequencies of QP correction were measured by first integrating *KI-URA3* at the *HMR* locus in a *sir3*Δ background to allow expression. The QP7, QP8 or FS mutations were introduced to make the strains auxotrophic for uracil. Cells were cultured in rich media (YPD), appropriately diluted and plated on YPD and on selective medium lacking uracil to assay spontaneous correction to $Ura^+$. Values were normalized to the WT frequencies (WT=100). Asterisks indicate statistical significance below p=0.005 using the Mann-Whitney test. Error bars represent the standard

error of the mean.

**Figure 5**. DSB-induced frameshift and QP-associated mutagenesis. Ura$^+$ reversion associated with quasipalindromes, QP7, QP8, or the frameshifting during gene conversion was assayed by inducing a DSB at *MAT* using the HO endonuclease. *Gal::HO* was induced by plating cells on galactose-uracil medium as described in the Materials and Methods section. The rates at which QP7, QP8 or the frameshift were removed, giving rise to Ura$^+$ colonies, were plotted relative to WT (WT=100). Asterisks indicate statistical significance below p=0.005 using the Mann-Whitney test. Error bars represent 95% confidence intervals.

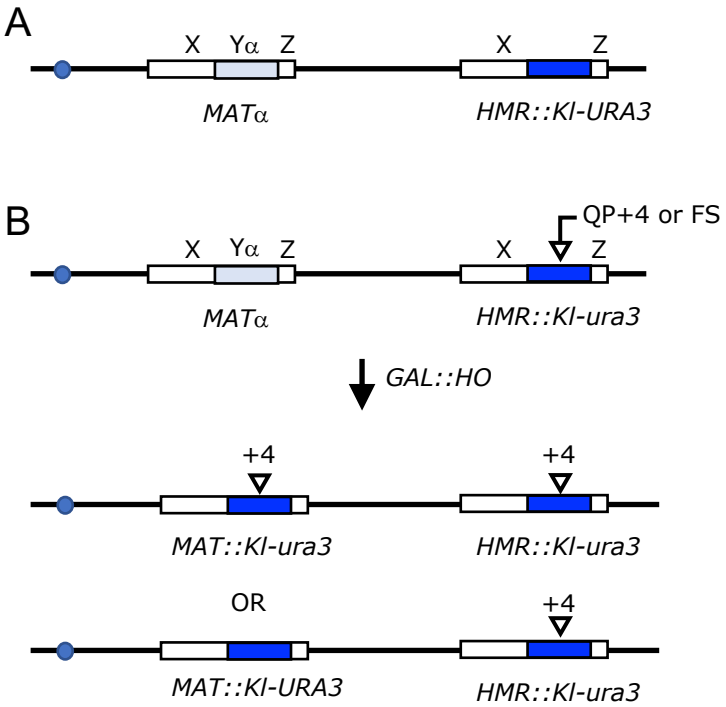
**Figure 6**. Rdh54 is important for mismatch repair of mutations in QP7 but not QP8. A D-loop containing a heteroduplexed DNA intermediate is preferentially repaired in favor of the unbroken template strand (A). In the absence of Rdh54 QP mutations in the context of a D-loop (detected by the QP7 reporter) are less likely to occur (B). Replication after strand displacement and annealing that gives rise to QP associated mutations (detected by QP8) is not affected by Rdh54, presumably because it occurs in the ssDNA gap rather than a D-loop (C).

**Table 1.** Values for DSB-induced Ura$^+$ reversion obtained by the indicated mutational reporters.

| DSB Induced | | | | | | |
|---|---|---|---|---|---|---|
| Genotype | Mean Frequency ± SEM (x10$^{-6}$) | | | Fold Change from WT | | |
| | QP7 | QP8 | FS | QP7 | QP8 | FS |
| WT | 6.7 ± 0.4 | 2.1 ± 0.2 | 7.5 ± 0.7 | 1 | 1 | 1 |
| *msh2Δ* | 56.2 ± 14.1 | 7.5 ± 3 | 52.7 ± 11 | 8.4 | 3.5 | 7.0 |
| *msh3Δ* | 44.4 ± 7.3 | 22.5 ± 5.5 | 30.7 ± 3.4 | 6.6 | 10.7 | 4.1 |
| *msh6Δ* | 5.6 ± 0.5 | 1.7 ± 0.3 | 7.0 ± 1.7 | 0.8 | 0.8 | 0.9 |
| *mlh1Δ* | 40.6 ± 6.3 | 11.2 ± 1 | 49.1 ± 10.1 | 6.1 | 5.3 | 6.5 |
| *pol2-04* | 10.5 ± 0.7 | 3.0 ± 0.2 | 11.3 ± 0.5 | 1.6 | 1.4 | 1.5 |
| *pol3-01* | 1.9 ± 0.2 | 2.9 ± 0.7 | 12.5 ± 0.5 | 0.3 | 1.4 | 1.7 |
| *pol32Δ* | 4.8 ± 0.5 | 3.4 ± 0.2 | 2.3 ± 0.2 | 0.7 | 1.6 | 0.3 |
| *rev3Δ* | 6.7 ± 0.7 | 1.7 ± 0.2 | 6.6 ± 0.9 | 1.0 | 0.8 | 0.9 |
| *rad30Δ* | 7.3 ± 1 | 3.0 ± 0.5 | 7.8 ± 1.1 | 1.1 | 1.4 | 1.0 |
| *rdh54Δ* | 3.0 ± 0.3 | 2.2 ± 0.2 | 4.7 ± 1 | 0.4 | 1.0 | 0.6 |

**Table 2.** Values for spontaneous Ura$^{+}$ reversion obtained by the indicated mutational reporters.

| Spontaneous | | | | | | |
|---|---|---|---|---|---|---|
| Genotype | Mean Frequency ± SEM (x10$^{-9}$) | | | Fold Change from WT | | |
| | QP7 | QP8 | FS | QP7 | QP8 | FS |
| WT | 3.7 ± 0.7 | 3.4 ± 1 | 18.6 ± 9.1 | 1 | 1 | 1 |
| *msh2Δ* | 239 ± 44.7 | 110 ± 52.2 | 9410 ± 1280 | 64.6 | 32.4 | 505.9 |
| *msh3Δ* | 164 ± 63.9 | 85.3 ± 29.4 | 5940 ± 1080 | 44.3 | 25.1 | 319.4 |
| *msh6Δ* | 4.8 ± 2.6 | 1.2 ± 0.5 | 508 ± 139 | 1.3 | 0.3 | 27.3 |
| *mlh1Δ* | 509 ± 174 | 673 ± 580 | 22100 ± 4730 | 137.6 | 197.9 | 1188.2 |
| *pol2-04* | 4.9 ± 1.6 | 1.9 ± 0.7 | 8.0 ± 2.7 | 1.3 | 0.6 | 0.4 |
| *pol3-01* | 1.3 ± 0.5 | 8.5 ± 5.2 | 414 ± 55.2 | 0.4 | 2.5 | 22.3 |
| *pol32Δ* | 64 ± 58.4 | 1.2 ± 0.7 | 2.2 ± 0.9 | 17.3 | 0.4 | 0.1 |
| *rev3Δ* | 3.5 ± 1.1 | 10.2 ± 8.3 | 125 ± 110 | 0.9 | 3.0 | 6.7 |
| *rad30Δ* | 5.2 ± 1.8 | 2.1 ± 1.1 | 20.3 ± 14.4 | 1.4 | 0.6 | 1.1 |
| *rdh54Δ* | 7.1 ± 3.4 | 0.7 ± 0.2 | 9.4 ± 4.3 | 1.9 | 0.2 | 0.5 |

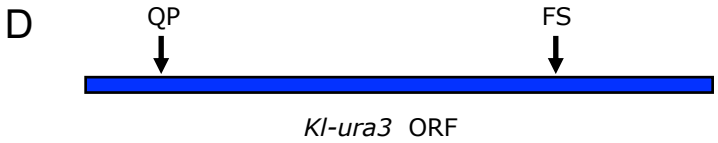


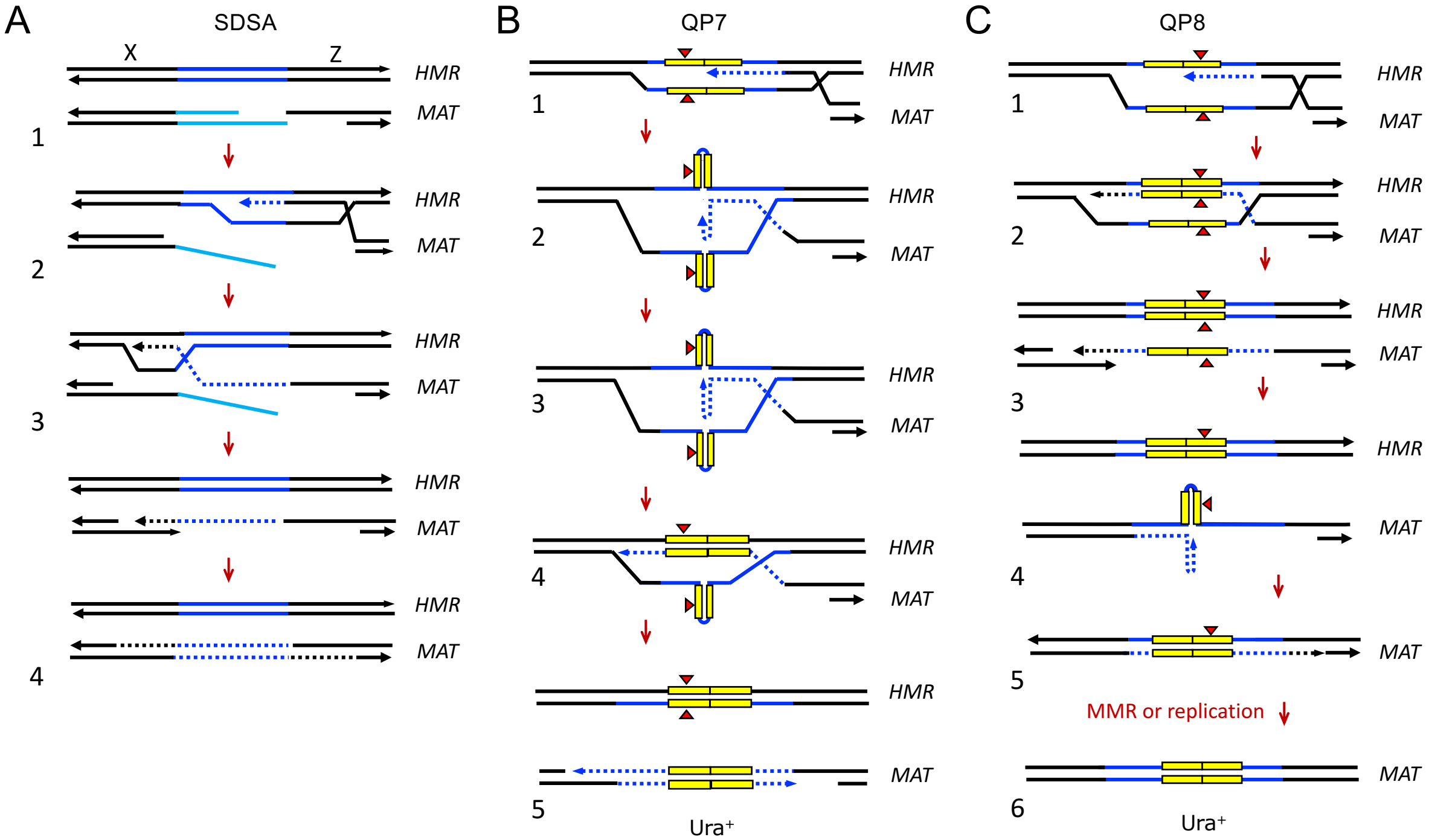

C

QP7 gtgcttccAATAttggatgtacgtacatccaaggaagcac

QP8 gtgcttccttggatgtacgtacatccaaTATTggaagcac

FS gtttgattggctaatcatgaCccccaggtgtaggtttaga




A
SDSA
X
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HMR
MAT
1
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3
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B
QP7
HMR
MAT
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QP8
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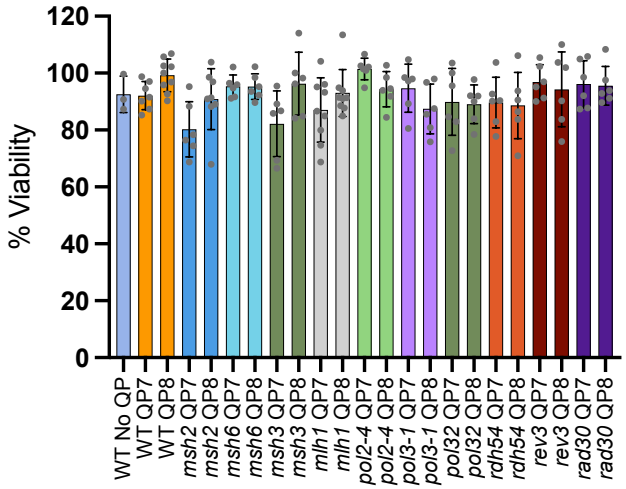
MMR or replication
6
Ura+


% Viability
120
100
80
60
40
20
0
WT No QP
WT QP7
WT QP8
msh2 QP7
msh2 QP8
msh6 QP7
msh6 QP8
msh3 QP7
msh3 QP8
mlh1 QP7
mlh1 QP8
pol2-4 QP7
pol2-4 QP8
pol3-1 QP7
pol3-1 QP8
pol32 QP7
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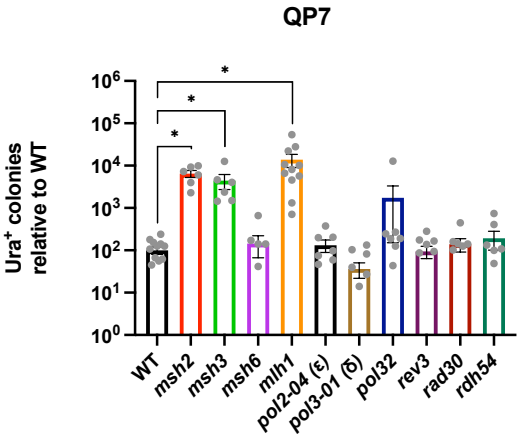
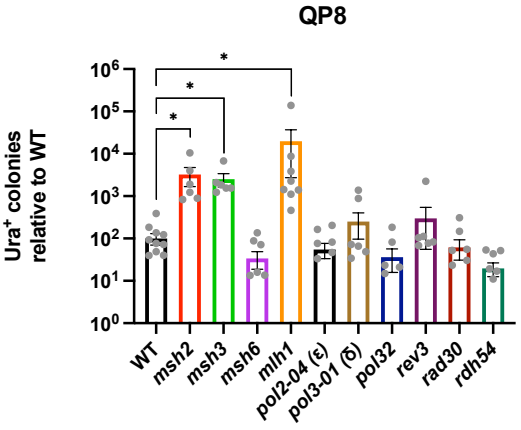
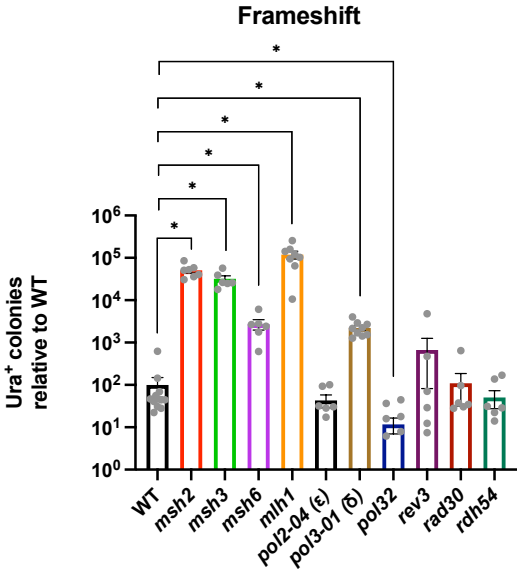
rad30 QP8

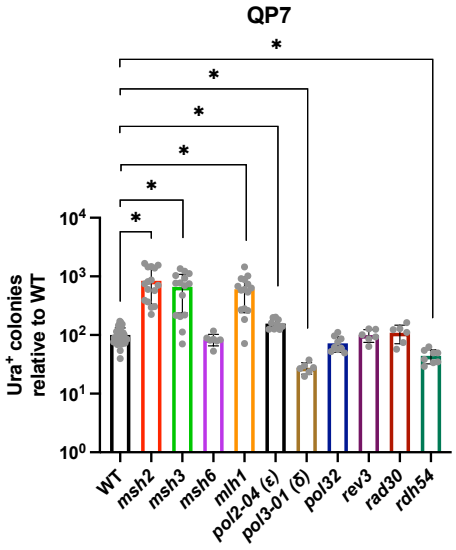
Spontaneous events

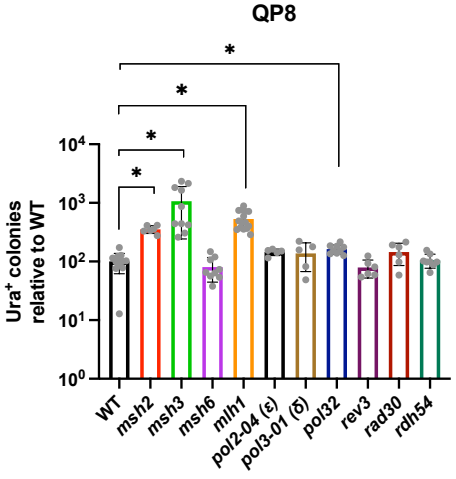


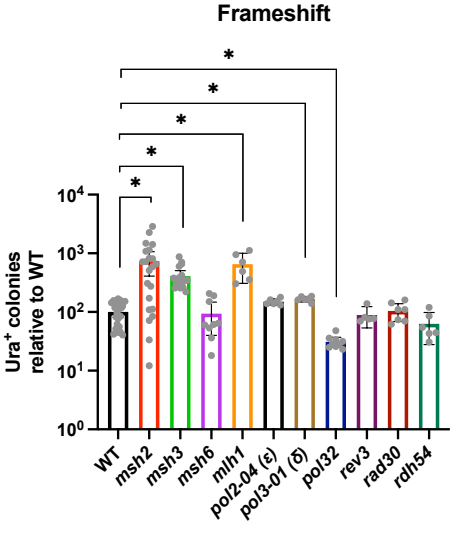



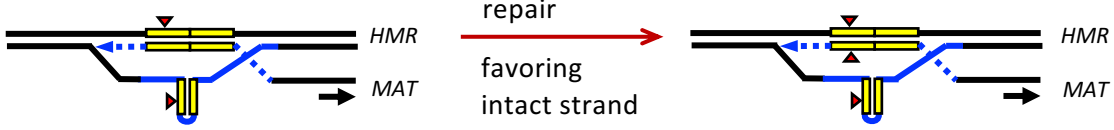
DSB-induced events



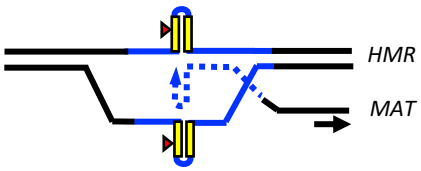



A




B

Rdh54 promotes QP events in this 3-strand structure




Rdh54 does not affect QP events in this 2-strand intermediate