**Mispair specificity resulting in AT to GC and CG to TA substitutions on both DNA strands in *psf1-1* cells**

In the *POL3* strain with the *URA3* coding sequence replicated as a lagging strand (OR1), T to C substitution was almost 12-fold more frequent than A to G substitution, while the G to A versus C to T ratio was 5 (Figure 4A, upper panel), which is consistent with Pol δ specificity and data obtained by others [51].

As expected, when the *URA3* reporter was inserted in the inverse orientation (OR2) and its coding sequence was replicated as the leading strand, A to G and C to T substitutions slightly dominated over T to C and G to A, respectively (Figure 4A, lower panel). These ratios were significantly different from those observed in the other *URA3* orientation (OR1) presented above ($p$=0.002 and $p$<0.0001, Table S4) and were consistent with the model in which Pol ε performed the majority of DNA synthesis on the leading strand. However, the ratios were inverted in the *psf1-1* mutant strain with the OR2 *URA3* orientation: the T to C versus A to G substitution ratio increased from 0.8 to >11, and the G to A versus C to T ratio increased from 0.4 to 1.1 (Figure 4A and B, lower panel), with $p$ values <0.0001 and 0.0171, respectively (Table S4).

**Mispair specificity resulting in GC to TA substitutions on both DNA strands in *psf1-1* cells**

In our analysis, G to T dominated over C to A in *POL3* cells in both the OR1 and OR2 orientations of *URA3* (where the coding sequence was replicated as the lagging and leading strands, respectively). However, the ratios were significantly different ($p$=0.0003) in the two orientations, with higher domination of G to T in OR2 cells (C to A versus G to T ratio of 0.13) and only a two-fold difference in OR1 (C to A versus G to T ratio of 0.56) (Figure S1A upper panel and Table S5). Importantly, a similar two-fold higher G to T mutation rate compared with C to A was observed in *URA3* OR1 in the *msh6*Δ background in a study describing mismatch repair activity on the lagging DNA strand [86]. A later study conducted in the *msh2* background showed an orientation bias for GC to TA substitutions with similar rates of C to A events in both OR1 and OR2 and much higher rates of G to T events in OR2 compared with the OR1 orientation of the reporter gene [54]. Moreover, given the location of the closest ARS, these substitutions were attributed to C•dT mispairs formed on the lagging strand. Because the coding sequence of *URA3* in the OR2 position is replicated as the leading strand, these mispairs are observed as G to T substitutions. These observations explain our results showing low C to A versus G to T ratios in *POL3* cells with *URA3* in the OR2 position, as shown in Figure S1A. Importantly, in *psf1-1* mutant cells with an OR1 orientation of *URA3*, we observed a large increase in G to T substitution rates (7.8-fold), resulting in a significant decrease in the C to A versus G to T ratio (Figure S1A and B, upper panel and Table S5). This result reflects increased rates of C•dT mispairs during replication of the leading strand by Pol δ in the *psf1-1* mutant in *URA3*-OR1 cells, which equates to the situation in which *URA3* is in the OR2 orientation and Pol δ replicates the lagging strand. In parallel, the C to A versus G to T ratio increased in *psf1-1* cells with *URA3* in the OR2 position (Figure S1A and B, lower panel and Table S5), which resulted from a slight increase in C•dT mispairs produced by Pol δ replication on the leading strand. Together, these results indicate a role for Pol δ in leading strand replication in the *psf1-1* mutant, resulting in significant changes in the ratios of specific mispairs.

**L612M Pol δ -specific mispair specificity resulting in GC to TA substitutions observed on both DNA strands in *psf1-1* cells**

In the *pol3-L612M* strain with the *URA3* coding sequence replicated as the lagging strand (OR1), our results showed a 2-fold preference for the C to A substitution over the G to T substitution, which was significantly different ($p$=0.0002) from the 0.56-fold ratio observed in *POL3* cells (Figure S1A and C, upper panel and Table S5). This phenomenon resulted mainly from the almost 5-fold higher rates of C to A changes ($0.92\times10^{-6}$ in *pol3-L612M* compared with $0.19\times10^{-6}$ in *POL3*) and only 1.4-fold higher rates of G to T substitutions ($0.46\times10^{-6}$ compared with $0.34\times10^{-6}$). Importantly, in the *pol3-L612M psf1-1* strain with *URA3* in OR1, the C to A versus G to T ratio was reduced from 2 in *pol3-L612M* to 0.89 ($p$<0.0001) (Figure S1C and D, upper panel and Table S5).

In *pol3-L612M* with *URA3* in OR2 with replication of the coding sequence as the leading strand, G to T domination was stronger than in the *POL3* strain (C to A versus G to T ratio decrease from 0.13 to <0.02) due to an 8-fold increase in G to T rates ($8.68\times10^{-6}$ in *pol3-L612M* compared with $1.09\times10^{-6}$ in *POL3*), while C to A changes were not detected in *pol3-L612M* (a single C to A event would represent a 1.6-fold rate increase) (Figure S1A and C, lower panel and Table S5). Interestingly, in *pol3-L612M psf1-1 URA3* OR2 cells, the mispair ratio was <0.01, with no significant difference compared with *pol3-L612M* (Figure S1C and D, lower panel and Table S5).

These results are consistent with previous work [52], in which the C to A versus G to T ratio was 1.8 in *pol3-L612M* cells while the combination of *pol3-L612M* with the *pol2-16* mutation (inactivating the catalytic activity of Pol ε) reduced the ratio to 1. Therefore, we again concluded that the increase in C to A substitutions resulted from C•dT mispairs generated by Pol δ during leading strand replication in *psf1-1* cells.

**G to T mutation hotspots in *pol3-L612M* and *pol3-L612M psf1-1* cells**

In the *pol3-L612M* mutant, we also observed hotspots of substitutions at positions 679 and 706 ($1.66\times10^{-6}$ when the reporter was in OR2) (Figure S2C, Table S3). These hotspots were previously reported in *pol3-L612M* cells [54,88]. In *URA3* OR1, this type of substitution was absent (Figure S2C, Table S3). The contribution of G to T changes at other sites was also 15-fold higher in strains with *URA3* in OR2 than in OR1, with mutation rates of $7.02\times10^{-6}$ and $0.46\times10^{-6}$, respectively (Figure S2C), $p<0.0001$ (Table S7). These results are consistent with the mutational signature of Pol δ L612M characteristic for the lagging DNA strand.

In *pol3-L612M psf1-1* cells with the reporter gene in the OR1 orientation, we observed a significant relative increase in G to T substitution at 679/706 (Figure S2C and D; $p<0.0001$, Table S6). Similar effects were observed for these substitutions in general at all sites, including hotspots (Figure S2C and D; $p<0.0001$, Table S7). This finding again reflected an increase in C•dT mispair rates occurring during replication of the leading strand by Pol δ in *psf1-1 URA3*-OR1.

***pol3-L612M*-specific deletions**

Previous work has assigned single nucleotide deletions in *URA3* characteristic of L612M Pol δ namely, deletion of T at 201-205 and at 255-260 in OR1 and deletion of A at 174-178 in OR2 [89]. In that study, this type of error constituted almost one-third of all observed mutations. In our analysis, deletions constituted 1.1 - 4% of the total mutation events in *pol3-L612M* mutants and 1.3 - 5.3% of the total mutation events in *POL3* strains. Consequently, when compared to previously published data [89], we did not observe an important contribution of T deletions in *pol3-L612M* cells at positions 201-205, 255-260 or at other sites in the *URA3* OR1 mutation spectrum (Table S3). In total, only 5 of 176 events were deletions, which occurred at a rate of $1.15\text{x}10^{-6}$ of 176 events (two deletions in the TTTTTT run at position 255-260 and three deletions at other sites) (Table S3). In strains with *URA3* in OR2, we found only one minus T event (outside of homopolymeric runs) among 172 events analyzed, representing a rate of $0.18\times10^{-6}$ (Table S3). Similarly, we found no minus A events at hotspots in *pol3-L612M* cells; at other sites, they were formed at a rate of only $0.37\times10^{-6}$ in OR2 and $0.23\times10^{-6}$ in OR1 orientation of the reporter gene (Table S3).

The difference between our results and those obtained previously for *pol3-L612M* might be explained by the observation that in our study MMR was partially inactivated by deletion of the *MSH6* gene, while in the cited study *MSH2* was deleted. The MMR system operates by two protein complexes, MutSβ composed of Msh2-Msh3 (correction of small and large insertions and deletions) and MutSα composed of Msh2-Msh6 (correction of mismatched bases and single-base insertions or deletions) [86]. Deletion of the *MSH2* gene results in a much stronger increase in mutagenesis rates than deletion of *MSH6* [5,87]. Therefore, given that we expected a strong increase in mutagenesis rates in the double replication mutant *psf1-1 pol3-L612M* (Table S3), to better visualize the replication errors in our study, we chose to delete the *MSH6* gene. Since Msh6 plays only a minor role in the repair of insertion/deletion mispairs [87], the observed differences in single deletion contributions to total mutagenesis in *msh2*Δ and *msh6*Δ can be explained by different extents of MMR defects.

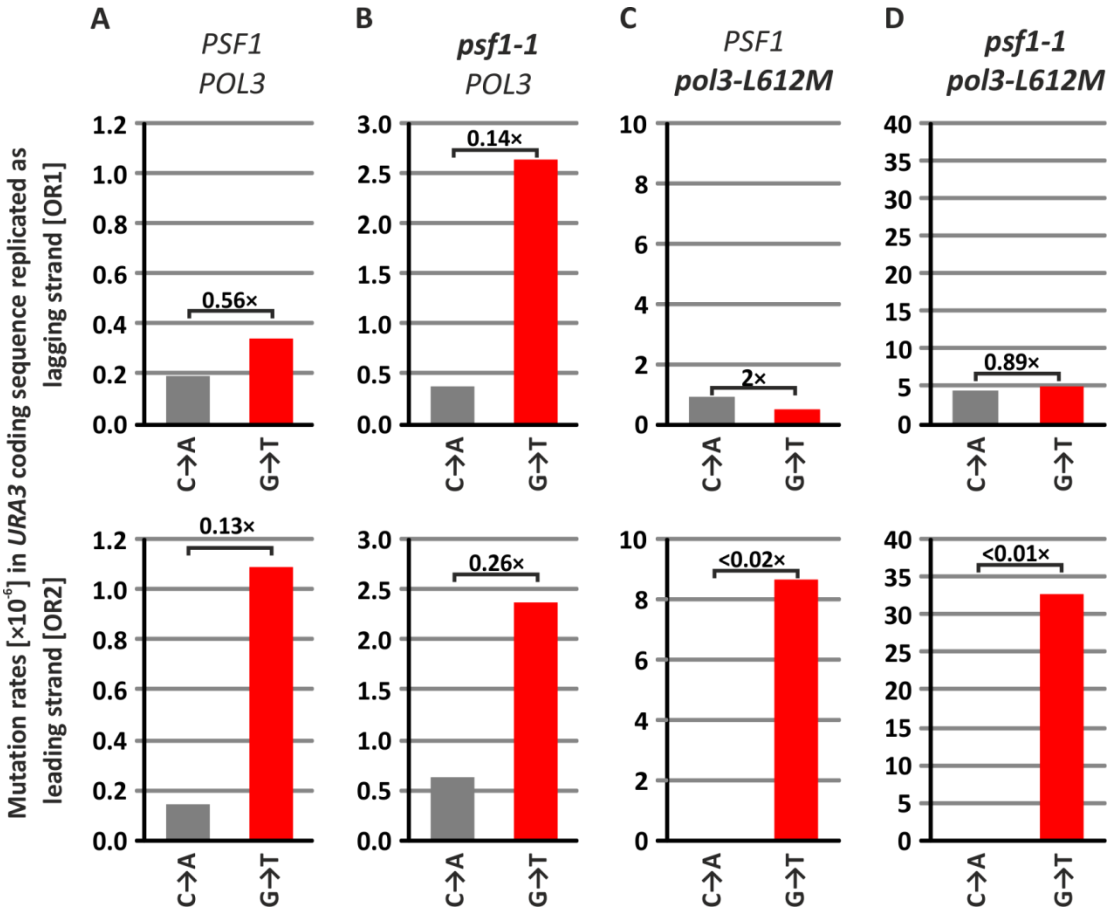


**Figure S1.** Mutation rates calculated for specific substitutions [5-FOA$^R$×10$^{-6}$] in strains with *psf1-1* and/or *pol3-L612M* mutations in the *rev3*Δ *msh6*Δ background. Details of the mutation spectra are shown in Table S3. The statistical analysis is shown in Table S5.

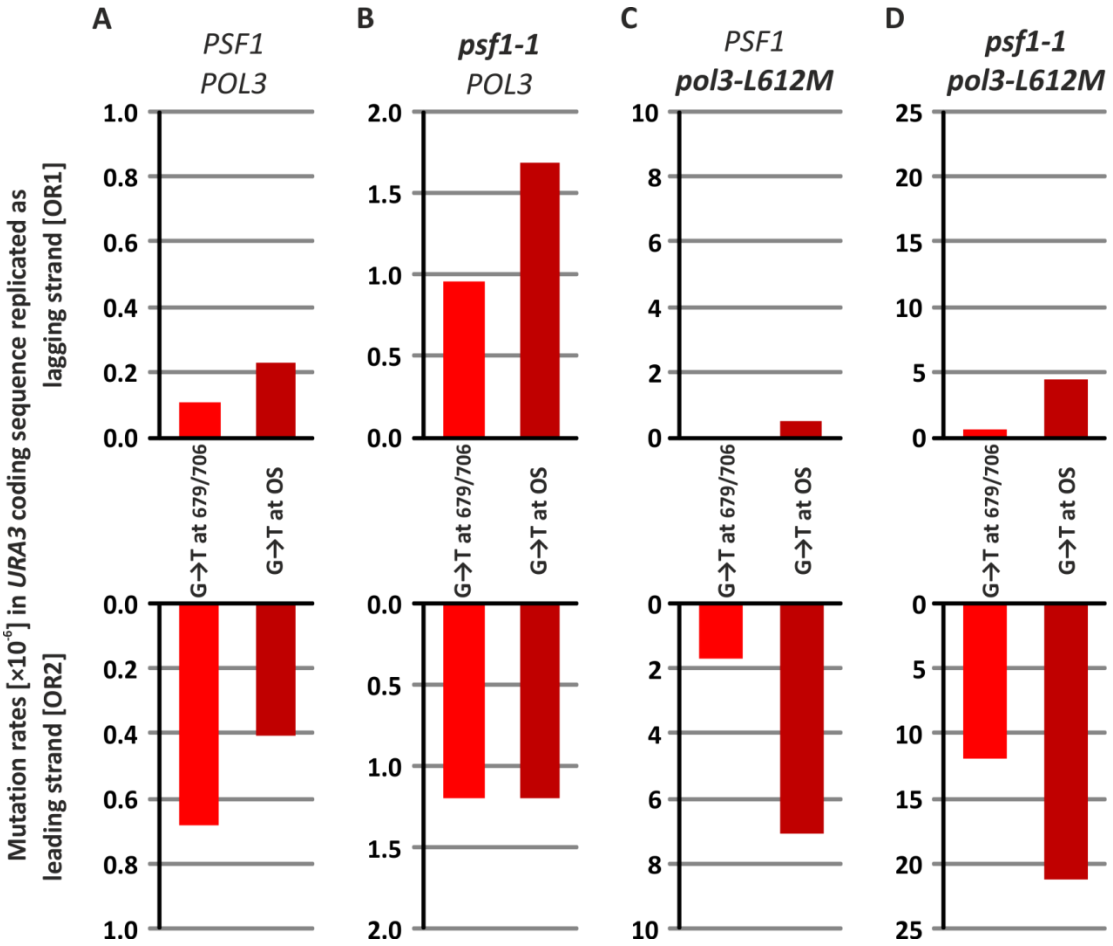


**Figure S2.** Mutation rates calculated for G to T substitutions including the *pol3-L612M*-characteristic hotspot [5-FOA$^R$×10$^{-6}$] in strains with *psf1-1* and/or *pol3-L612M* mutations in the *rev3*Δ *msh6*Δ background. Details of the mutation spectra are shown in Table S3. The statistical analysis is shown in Table S6 and Table S7.

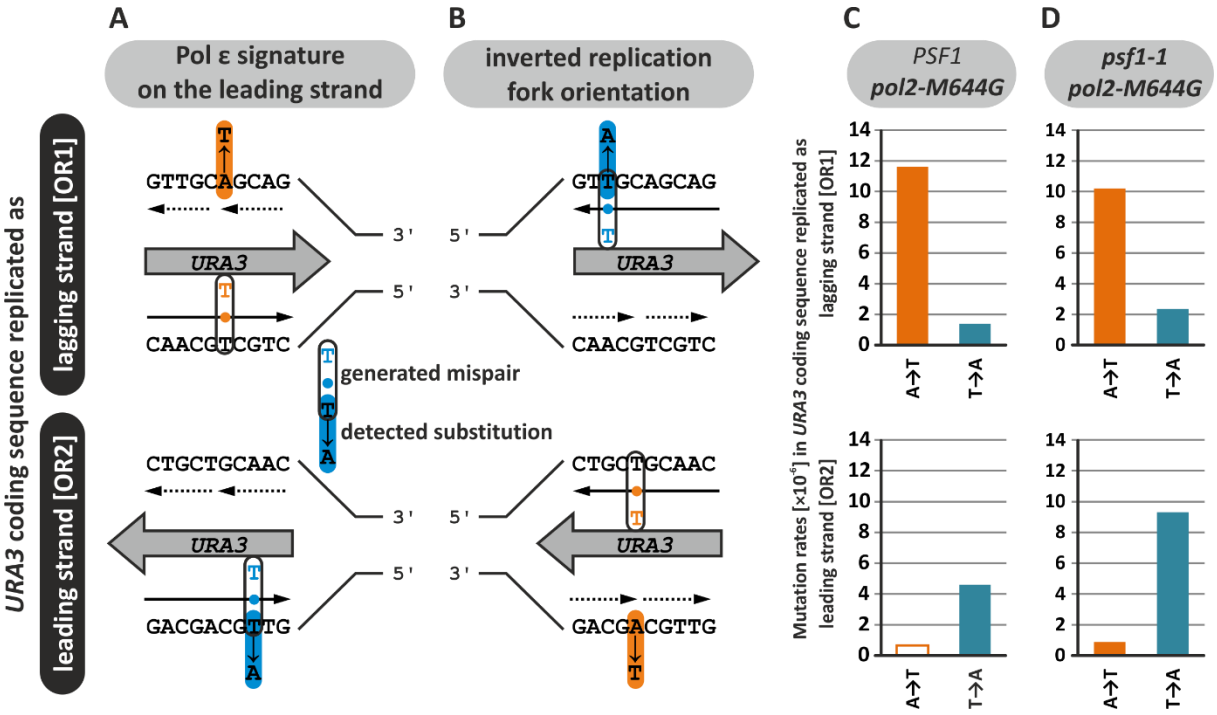


**Figure S3.** Mutation rates calculated for specific substitutions [5-FOA$^R$×10$^{-6}$] in strains with *pol2-L612M* or *pol2-L612M psf1-1* mutations in the *rev3*Δ *msh6*Δ background. Open bar indicates mutation rate that would be observed if a single event was detected. Details of the mutation spectra are shown in the associated Data in Brief paper by Dmowski *et al*.

**Table S1.** Yeast strains used in this study.

| Strain | Relevant genotype | Description | Source |
|---|---|---|---|
| **YTAK001** | *agp1::URA3-OR1* | | [57] |
| **Y467** | *agp1::URA3-OR1 rev3Δ* | *REV3* disruption in YTAK001 | This work |
| **Y473** | *agp1::URA3-OR1 rev3Δ PSF1* | *PSF1-LEU2* derivative of Y467 | This work |
| **Y485-3** | *agp1::URA3-OR1 rev3Δ PSF1 msh6Δ* | *MSH6* disruption in Y473 | This work |
| **Y485-4** | *agp1::URA3-OR1 rev3Δ PSF1 msh6Δ* | *MSH6* disruption in Y473 | This work |
| **Y479-1** | *agp1::URA3-OR1 rev3Δ psf1-1* | *psf1-1-LEU2* derivative of Y467 | This work |
| **Y479-3** | *agp1::URA3-OR1 rev3Δ psf1-1* | *psf1-1-LEU2* derivative of Y467 | This work |
| **Y495-3** | *agp1::URA3-OR1 rev3Δ psf1-1 msh6Δ* | *MSH6* disruption in Y479-1 | This work |
| **Y497-8** | *agp1::URA3-OR1 rev3Δ psf1-1 msh6Δ* | *MSH6* disruption in Y479-3 | This work |
| **YTAK002** | *agp1::URA3-OR2* | | [57] |
| **Y468** | *agp1::URA3-OR2 rev3Δ* | *REV3* disruption in YTAK002 | This work |
| **Y474** | *agp1::URA3-OR2 rev3Δ PSF1* | *PSF1-LEU2* derivative of Y468 | This work |
| **Y486-2** | *agp1::URA3-OR2 rev3Δ PSF1 msh6Δ* | *MSH6* disruption in Y474 | This work |
| **Y486-5** | *agp1::URA3-OR2 rev3Δ PSF1 msh6Δ* | *MSH6* disruption in Y474 | This work |
| **Y480-1** | *agp1::URA3-OR2 rev3Δ psf1-1* | *psf1-1-LEU2* derivative of Y468 | This work |
| **Y480-2** | *agp1::URA3-OR2 rev3Δ psf1-1* | *psf1-1-LEU2* derivative of Y468 | This work |
| **Y498-6** | *agp1::URA3-OR2 rev3Δ psf1-1 msh6Δ* | *MSH6* disruption in Y480-1 | This work |
| **Y499-2** | *agp1::URA3-OR2 rev3Δ psf1-1 msh6Δ* | *MSH6* disruption in Y480-2 | This work |
| **SNM12** | *pol3L612M agp1::URA3-OR1* | | [54] |
| **Y469** | *pol3L612M agp1::URA3-OR1 rev3Δ* | *REV3* disruption in SNM12 | This work |
| **Y477-1** | *pol3L612M agp1::URA3-OR1 rev3Δ PSF1* | *PSF1-LEU2* derivative of Y469 | This work |
| **Y477-2** | *pol3L612M agp1::URA3-OR1 rev3Δ PSF1* | *PSF1-LEU2* derivative of Y469 | This work |
| **Y491-3** | *pol3L612M agp1::URA3-OR1 rev3Δ PSF1 msh6Δ* | *MSH6* disruption in Y477-1 | This work |
| **Y492-1** | *pol3L612M agp1::URA3-OR1 rev3Δ PSF1 msh6Δ* | *MSH6* disruption in Y477-2 | This work |
| **Y481-1** | *pol3L612M agp1::URA3-OR1 rev3Δ psf1-1* | *psf1-1-LEU2* derivative of Y469 | This work |
| **Y481-2** | *pol3L612M agp1::URA3-OR1 rev3Δ psf1-1* | *psf1-1-LEU2* derivative of Y469 | This work |
| **Y481-3** | *pol3L612M agp1::URA3-OR1 rev3Δ psf1-1* | *psf1-1-LEU2* derivative of Y469 | This work |
| **Y501-3** | *pol3L612M agp1::URA3-OR1 rev3Δ psf1-1 msh6Δ* | *MSH6* disruption in Y481-1 | This work |
| **Y502-3** | *pol3L612M agp1::URA3-OR1 rev3Δ psf1-1 msh6Δ* | *MSH6* disruption in Y481-2 | This work |
| **Y503-3** | *pol3L612M agp1::URA3-OR1 rev3Δ psf1-1 msh6Δ* | *MSH6* disruption in Y481-3 | This work |
| **Y638-1** | *POL3/pol3L612M agp1::URA3-OR1/agp1::URA3-OR1 rev3Δ /rev3Δ PSF1/psf1-1 MSH6/msh6Δ* | Diploid strain | This work |
| **Y638-2** | *POL3/pol3L612M agp1::URA3-OR1/agp1::URA3-OR1 rev3Δ /rev3Δ PSF1/psf1-1 MSH6/msh6Δ* | Diploid strain | This work |
| **Y649** | *pol3L612M agp1::URA3-OR1 rev3Δ psf1-1 msh6Δ* | Segregant of Y638-1 | This work |
| **Y652** | *pol3L612M agp1::URA3-OR1 rev3Δ psf1-1 msh6Δ* | Segregant of Y638-1 | This work |
| **Y654** | *pol3L612M agp1::URA3-OR1 rev3Δ psf1-1 msh6Δ* | Segregant of Y638-2 | This work |
| **Y655** | *pol3L612M agp1::URA3-OR1 rev3Δ psf1-1 msh6Δ* | Segregant of Y638-2 | This work |
| **Y656** | *pol3L612M agp1::URA3-OR1 rev3Δ psf1-1 msh6Δ* | Segregant of Y638-2 | This work |
| **SNM24** | *pol3L612M agp1::URA3-OR2* | | [54] |
| **Y470** | *pol3L612M agp1::URA3-OR2 rev3Δ* | *REV3* disruption in SNM24 | This work |
| **Y478-1** | *pol3L612M agp1::URA3-OR2 rev3Δ PSF1* | *PSF1-LEU2* derivative of Y470 | This work |
| **Y478-2** | *pol3L612M agp1::URA3-OR2 rev3Δ PSF1* | *PSF1-LEU2* derivative of Y470 | This work |
| **Y493-1** | *pol3L612M agp1::URA3-OR2 rev3Δ PSF1 msh6Δ* | *MSH6* disruption in Y478-1 | This work |
| **Y494-1** | *pol3L612M agp1::URA3-OR2 rev3Δ PSF1 msh6Δ* | *MSH6* disruption in Y478-2 | This work |
| **Y482-1** | *pol3L612M agp1::URA3-OR2 rev3Δ psf1-1* | *psf1-1-LEU2* derivative of Y470 | This work |
| **Y482-2** | *pol3L612M agp1::URA3-OR2 rev3Δ psf1-1* | *psf1-1-LEU2* derivative of Y470 | This work |
| **Y482-4** | *pol3L612M agp1::URA3-OR2 rev3Δ psf1-1* | *psf1-1-LEU2* derivative of Y470 | This work |
| **Y504-4** | *pol3L612M agp1::URA3-OR2 rev3Δ psf1-1 msh6Δ* | *MSH6* disruption in Y482-1 | This work |
| **Y505-4** | *pol3L612M agp1::URA3-OR2 rev3Δ psf1-1 msh6Δ* | *MSH6* disruption in Y482-2 | This work |
| **Y507-6** | *pol3L612M agp1::URA3-OR2 rev3Δ psf1-1 msh6Δ* | *MSH6* disruption in Y482-4 | This work |
| **Y639-1** | *POL3/pol3L612M agp1::URA3-OR2/agp1::URA3-OR2 rev3Δ /rev3Δ PSF1/psf1-1 MSH6/msh6Δ* | Diploid strain | This work |
| **Y662** | *pol3L612M agp1::URA3-OR2 rev3Δ psf1-1 msh6Δ* | Segregant of Y639-1 | This work |
| **Y663** | *pol3L612M agp1::URA3-OR2 rev3Δ psf1-1 msh6Δ* | Segregant of Y639-1 | This work |

**Table S2.** Primers used in this study.

| Primer | Sequence 5'-3' |
|---|---|
| Rev3_UPTEF | CAATACAAAACTACAAGTTGTGGCGAAATAAAATGTTTGGAAATGAGATCTGTTTAGCTTGCC |
| Rev3_DN TEF | ATAACTACTCATCATTTTGCGAGACATATCTGTGTCTAGATTATTCGAGCTCGTTTTCGACAC |
| msh6UTEF | CAGATAAGATTTTTTAATTGGAGCAACTAGTTAATTTTGACAAAGCCAATTTGAACTCCAAAAGATCTGTTTAGCTTGCC |
| msh6DTEF | CAACGACCAAAACTTTAAAAAAAATAAGTAAAAATCTTACATACATCGTAAATGAAAATATTCGAGCTCGTTTTCGACAC |
| Rev3-R4 | TGACCACTCACATGGCGCTTTG |
| Rev3 A | AATTCTGCCAATCTATTTGATCTTG |
| nat1UO | ACCGGTAAGCCGTGTCGTCAAG |
| Rev3-F4 | AAAGGGCGAGCACAACTACTAC |
| Rev3 D | CACCAGATAGAGTTTTGAACGAAAT |
| nat1DO | GCTTCGTGGTCGTCTCGTACTC |
| MSH6-UO | TAAAGTCGCTGGAGTAGG |
| msh6up2 | GAATCCTTGGAGGAAGAC |
| HPH-UO | ACAGACGTCGCGGTGAGTTCAG |
| MSH6-DO | TCAAGCACCATCCTCAAG |
| msh6dw 2 | CCCATTCTTGCCCAAGATGC |
| HPH-DO | TCGCCGATAGTGGAAACCGACG |
| URA3F393 | AACGAAGGAAGGAGCACAGAC |
| URA3R412 | CCGAAATTCCTGGGTAATAAC |

**Table S3.** Mutation rates calculated for specific mutation types in the *URA3* sequence in the *rev3*Δ *msh6*Δ background.

| Type | OR1 | | OR2 | | *psf1-1* OR1 | | *psf1-1* OR2 | | *pol3-L612M* OR1 | | *pol3-L612M* OR2 | | *psf1-1 pol3-L612M* OR1 | | *psf1-1 pol3-L612M* OR2 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Transitions** | 103[a] | **1.39**[b] | 37 | **0.57** | 82 | **4.33** | 78 | **4.03** | 161 | **36.93** | 112 | **20.70** | 554 | **154.93** | 326 | **159.24** |
| T→C total | 23 | **0.31** | 5 | **0.08** | 16 | **0.84** | 11 | **0.57** | 53 | **12.16** | 2 | **0.37** | 128 | **35.80** | 28 | **13.68** |
| *T→C at 97*[c] | *7* | ***0.09*** | *1* | ***0.02*** | *7* | ***0.37*** | *0* | ***0.00*** | *24* | ***5.51*** | *0* | ***0.00*** | *51* | ***14.26*** | *7* | ***3.42*** |
| *T→C at OS*[d] | *16* | ***0.22*** | *4* | ***0.06*** | *9* | ***0.47*** | *11* | ***0.57*** | *29* | ***6.65*** | *2* | ***0.37*** | *77* | ***21.53*** | *21* | ***10.26*** |
| A→G | 2 | **0.03** | 6 | **0.09** | 0 | **0.00** | 0 | **0.00** | 0 | **0.00** | 18 | **3.33** | 4 | **1.12** | 37 | **18.07** |
| C→T total | 13 | **0.18** | 18 | **0.28** | 12 | **0.63** | 32 | **1.65** | 1 | **0.23** | 85 | **15.71** | 44 | **12.31** | 218 | **106.48** |
| *C→T at 310* | *4* | ***0.05*** | *11* | ***0.17*** | *9* | ***0.47*** | *19* | ***0.98*** | *1* | ***0.23*** | *43* | ***7.95*** | *28* | ***7.83*** | *128* | ***62.52*** |
| *C→T at OS* | *9* | ***0.12*** | *7* | ***0.11*** | *3* | ***0.16*** | *13* | ***0.67*** | *0* | ***0.00*** | *42* | ***7.76*** | *16* | ***4.47*** | *90* | ***43.96*** |
| G→A total | 65 | **0.88** | 8 | **0.12** | 54 | **2.85** | 35 | **1.81** | 107 | **24.54** | 7 | **1.29** | 378 | **105.71** | 43 | **21.00** |
| *G→A at 764* | *18* | ***0.24*** | *2* | ***0.03*** | *16* | ***0.84*** | *6* | ***0.31*** | *45* | ***10.32*** | *2* | ***0.37*** | *132* | ***36.92*** | *9* | ***4.40*** |
| *G→A at OS* | *47* | ***0.64*** | *6* | ***0.09*** | *38* | ***2.00*** | *29* | ***1.50*** | *62* | ***14.22*** | *5* | ***0.92*** | *246* | ***68.80*** | *34* | ***16.61*** |
| **Transversions** | 54 | **0.73** | 87 | **1.35** | 67 | **3.53** | 65 | **3.35** | 8 | **1.84** | 54 | **9.98** | 65 | **18.18** | 88 | **42.98** |
| G→T total | 25 | **0.34** | 70 | **1.09** | 50 | **2.64** | 46 | **2.37** | 2 | **0.46** | 47 | **8.68** | 18 | **5.03** | 67 | **32.73** |
| *G→T at 679/706* | *8* | ***0.11*** | *44* | ***0.68*** | *18* | ***0.95*** | *23* | ***1.19*** | *0* | ***0.00*** | *9* | ***1.66*** | *2* | ***0.56*** | *24* | ***11.72*** |
| *G→T at OS* | *17* | ***0.23*** | *26* | ***0.40*** | *32* | ***1.69*** | *23* | ***1.19*** | *2* | ***0.46*** | *38* | ***7.02*** | *16* | ***4.47*** | *43* | ***21.00*** |
| C→A | 14 | **0.19** | 9 | **0.14** | 7 | **0.37** | 12 | **0.62** | 4 | **0.92** | 0 | **0.00** | 16 | **4.47** | 0 | **0.00** |
| T→G | 9 | **0.12** | 4 | **0.06** | 1 | **0.05** | 0 | **0.00** | 1 | **0.23** | 0 | **0.00** | 8 | **2.24** | 3 | **1.47** |
| A→C | 2 | **0.03** | 1 | **0.02** | 0 | **0.00** | 0 | **0.00** | 0 | **0.00** | 3 | **0.55** | 7 | **1.96** | 7 | **3.42** |
| A→T | 2 | **0.03** | 0 | **0.00** | 4 | **0.21** | 0 | **0.00** | 1 | **0.23** | 0 | **0.00** | 8 | **2.24** | 4 | **1.95** |
| T→A | 1 | **0.01** | 3 | **0.05** | 5 | **0.26** | 5 | **0.26** | 0 | **0.00** | 4 | **0.74** | 7 | **1.96** | 7 | **3.42** |
| G→C | 1 | **0.01** | 0 | **0.00** | 0 | **0.00** | 0 | **0.00** | 0 | **0.00** | 0 | **0.00** | 1 | **0.28** | 0 | **0.00** |
| C→G | 0 | **0.00** | 0 | **0.00** | 0 | **0.00** | 2 | **0.10** | 0 | **0.00** | 0 | **0.00** | 0 | **0.00** | 0 | **0.00** |
| **Indels** | 9 | **0.12** | 8 | **0.12** | 7 | **0.37** | 6 | **0.31** | 7 | **1.61** | 6 | **1.11** | 9 | **2.52** | 14 | **6.84** |
| ΔA | 3 | **0.04** | 2 | **0.03** | 2 | **0.11** | 0 | **0.00** | 1 | **0.23** | 2 | **0.37** | 1 | **0.28** | 3 | **1.47** |
| *ΔA at 174-178* | *0* | ***0.00*** | *0* | ***0.00*** | *0* | ***0.00*** | *0* | ***0.00*** | *0* | ***0.00*** | *0* | ***0.00*** | *0* | ***0.00*** | *0* | ***0.00*** |
| *ΔA at OS* | *3* | ***0.04*** | *2* | ***0.03*** | *2* | ***0.11*** | *0* | ***0.00*** | *1* | ***0.23*** | *2* | ***0.37*** | *1* | ***0.28*** | *3* | ***1.47*** |
| ΔT | 1 | **0.01** | 2 | **0.03** | 0 | **0.00** | 1 | **0.05** | 5 | **1.15** | 1 | **0.18** | 0 | **0.00** | 4 | **1.95** |
| *ΔT at 201-205* | *0* | ***0.00*** | *0* | ***0.00*** | *0* | ***0.00*** | *0* | ***0.00*** | *0* | ***0.00*** | *0* | ***0.00*** | *0* | ***0.00*** | *1* | ***0.49*** |
| *ΔT at 255-260* | *0* | ***0.00*** | *1* | ***0.02*** | *0* | ***0.00*** | *0* | ***0.00*** | *2* | ***0.46*** | *0* | ***0.00*** | *0* | ***0.00*** | *0* | ***0.00*** |
| *ΔT at OS* | *1* | ***0.01*** | *1* | ***0.02*** | *0* | ***0.00*** | *1* | ***0.05*** | *3* | ***0.69*** | *1* | ***0.18*** | *0* | ***0.00*** | *3* | ***1.47*** |
| ΔC or ΔG | 0 | **0.00** | 0 | **0.00** | 0 | **0.00** | 0 | **0.00** | 1 | **0.23** | 1 | **0.18** | 6 | **1.68** | 2 | **0.98** |
| ≥2 deletions | 2 | **0.03** | 3 | **0.05** | 2 | **0.11** | 1 | **0.05** | 0 | **0.00** | 0 | **0.00** | 0 | **0.00** | 0 | **0.00** |
| single insertions | 3 | **0.04** | 1 | **0.02** | 2 | **0.11** | 4 | **0.21** | 0 | **0.00** | 2 | **0.37** | 2 | **0.56** | 5 | **2.44** |
| ≥2 insertions | 0 | **0.00** | 0 | **0.00** | 1 | **0.05** | 0 | **0.00** | 0 | **0.00** | 0 | **0.00** | 0 | **0.00** | 0 | **0.00** |
| **TOTAL** | 166 | **2.25** | 132 | **2.05** | 156 | **8.23** | 149 | **7.69** | 176 | **40.37** | 172 | **31.78** | 628 | **175.63** | 428 | **209.06** |
| **95% CI** | | **1.4**<br>**2.8** | | **1.7**<br>**2.9** | | **4.8**<br>**9.9** | | **6.3**<br>**9.9** | | **28.5**<br>**55.5** | | **23.1**<br>**38.2** | | **174.3**<br>**267.6** | | **208.2**<br>**327.3** |

[a] Number of events identified for given classes.

[b] Mutation rates [5-FOA$^R$×10$^{-6}$] for specific mutation types are shown in boldface.

[c] Specific hotspot positions in the *URA3* coding sequence are indicated.

[d] OS – Other Sites.

**Table S4.** The statistical analysis of the mutation spectra is presented in Figure 4. *p* values were calculated using Fisher's exact test.

| **T→C vs A→G**<br>**G→A vs C→T** | *PSF1 POL3*<br>**OR2** | ***psf1-1*** *POL3*<br>**OR1** | *PSF1* ***pol3-L612M***<br>**OR1** | ***psf1-1 pol3-L612M***<br>**OR2** |
|---|---|---|---|---|
| *PSF1 POL3* **OR1** | **0.002** | 0.0778 | **0.0007** | ND[a] |
| | **<0.0001** | 0.7703 | **<0.0001** | ND |
| ***psf1-1*** *POL3* **OR2** | **<0.0001** | >0.9999 | ND | ND |
| | **0.0171** | **<0.0001** | ND | ND |
| *PSF1* ***pol3-L612M*** **OR2** | **0.0007** | ND | **<0.0001** | **<0.0001** |
| | **<0.0001** | ND | **<0.0001** | **<0.0001** |
| ***psf1-1 pol3-L612M*** **OR1** | ND | ND | **<0.0001** | **<0.0001** |
| | ND | ND | **<0.0001** | **<0.0001** |

[a] ND – not determined

**Table S5.** The statistical analysis of the mutation spectra is presented in Figure S1. *p* values were calculated using Fisher's exact test.

| **G→T vs C→A** | *PSF1 POL3*<br>**OR2** | ***psf1-1*** *POL3*<br>**OR1** | *PSF1* ***pol3-L612M***<br>**OR1** | ***psf1-1 pol3-L612M***<br>**OR2** |
|---|---|---|---|---|
| *PSF1 POL3* **OR1** | **0.0003** | **0.0001** | **0.0002** | ND[a] |
| ***psf1-1*** *POL3* **OR2** | **0.0255** | **0.0059** | ND | ND |
| *PSF1* ***pol3-L612M*** **OR2** | **<0.0001** | ND | **<0.0001** | >0.2298 |
| ***psf1-1 pol3-L612M*** **OR1** | ND | ND | **<0.0001** | **<0.0001** |

[a] ND – not determined

**Table S6.** Statistical analysis of data showing the contribution of substitutions at specific hotspots to the total mutagenesis is presented in Figure 5 and Figure S2. *p* values were calculated using Fisher's exact test.

| **T→C at 97**[a]<br>**C→T at 310**<br>**G→A at 764**<br>**G→T at 679/706** | *PSF1 POL3* **OR2** | ***psf1-1*** *POL3* **OR1** | *PSF1* ***pol3-L612M*** **OR1** | ***psf1-1 pol3-L612M*** **OR2** |
|---|---|---|---|---|
| *PSF1 POL3* **OR1** | 0.0649 | 0.8556 | **<0.0001** ↑ | ND[d] |
| | **0.0072** [2][b] | **0.0362** ↑[c] | **0.0140** ↓ | ND |
| | **<0.0001** [1] | 0.8051 | **<0.0001** ↑ | ND |
| | **<0.0001** [2] | **0.0026** ↑ | **<0.0001** ↓ | ND |
| ***psf1-1*** *POL3* **OR2** | **0.0441** ↓ | **<0.0001** [1] | ND | ND |
| | 0.0881 | **<0.0001** [2] | ND | ND |
| | 0.0867 | **<0.0001** [1] | ND | ND |
| | **<0.0001** ↓ | **0.0227** [2] | ND | ND |
| *PSF1* ***pol3-L612M*** **OR2** | **0.0037** ↓ | ND | **<0.0001** [1] | **<0.0001** ↑ |
| | **<0.0001** ↑ | ND | **<0.0001** [2] | **<0.0001** ↑ |
| | 0.7325 | ND | **<0.0001** [1] | **0.0002** ↑ |
| | **<0.0001** ↓ | ND | **<0.0001** [2] | 0.4058 |
| ***psf1-1 pol3-L612M*** **OR1** | ND | ND | **<0.0001** ↓ | **<0.0001** [1] |
| | ND | ND | **<0.0001** ↑ | **<0.0001** [2] |
| | ND | ND | **<0.0001** ↓ | **<0.0001** [1] |
| | ND | ND | **<0.0001** ↑ | **<0.0001** [2] |

[a] Substitutions at specific hotspots are color-coded.

[b] The *URA3* orientation with a higher contribution of substitutions at specific hotspots is shown in brackets: [1] – OR1, [2] – OR2.

[c] For *psf1-1* and *pol3-L612M* mutants, an increase ↑ or decrease ↓ of substitutions at specific hotspots compared with the *PSF1 POL3* strain, is shown; for *psf1-1 pol3-L612M* mutants, an increase ↑ or decrease ↓ of substitutions at specific hotspots compared with the *pol3-L612M* strain, is shown.

[d] ND – not determined

**Table S7.** The statistical analysis of data showing the contribution of specific substitutions to the total mutagenesis is presented in Figure 5 and Figure S2. *p* values were calculated using Fisher's exact test.

| **T→C**[a]<br>**C→T**<br>**G→A**<br>**G→T** | *PSF1 POL3* **OR2** | ***psf1-1*** *POL3* **OR1** | *PSF1* ***pol3-L612M*** **OR1** | ***psf1-1 pol3-L612M*** **OR2** |
|---|---|---|---|---|
| *PSF1 POL3* **OR1** | **0.0003** [1][b] | 0.1473 | **<0.0001** ↑ | ND[d] |
| | 0.0623 | 0.8880 | **<0.0001** ↓ | ND |
| | **<0.0001** [1] | 0.2386 | **<0.0001** ↑ | ND |
| | **<0.0001** [2] | **<0.0001** ↑[c] | **<0.0001** ↓ | ND |
| ***psf1-1*** *POL3* **OR2** | 0.0830 | 0.0524 | ND | ND |
| | **0.0135** ↑ | **<0.0001** [2] | ND | ND |
| | **<0.0001** ↑ | **<0.0001** [1] | ND | ND |
| | **<0.0001** ↓ | 0.6270 | ND | ND |
| *PSF1* ***pol3-L612M*** **OR2** | **0.0049** ↓ | ND | **<0.0001** [1] | **<0.0001** ↑ |
| | **<0.0001** ↑ | ND | **<0.0001** [2] | 0.1141 |
| | 0.2045 | ND | **<0.0001** [1] | **<0.0001** ↑ |
| | **<0.0001** ↓ | ND | **<0.0001** [2] | **<0.0001** ↓ |
| ***psf1-1 pol3-L612M*** **OR1** | ND | ND | **<0.0001** ↓ | **<0.0001** [1] |
| | ND | ND | **<0.0001** ↑ | **<0.0001** [2] |
| | ND | ND | 0.4867 | **<0.0001** [1] |
| | ND | ND | **<0.0001** ↑ | **<0.0001** [2] |

[a] Specific substitution types are color-coded.

[b] The *URA3* orientation with a higher contribution of specific substitutions is shown in brackets: [1] – OR1, [2] – OR2.

[c] For *psf1-1* and *pol3-L612M* mutants, an increase ↑ or decrease ↓ of specific substitutions compared with the *PSF1 POL3* strain, is shown; for *psf1-1 pol3-L612M* mutants, an increase ↑ or decrease ↓ of specific substitutions compared with the *pol3-L612M* strain is shown.

[d] ND – not determined

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