Supporting Information

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ATGACAAATT CAAAAGAAGA CGCCGACATA GAGGAGAAGC ATATGTACAA TGAGCCGGTC ACAACCCTCT TTCACGACGT TGAAGCTTCA 1 A CAAACACACC ACAGACGTGG GTCAATACCA TTGAAAGATG AGAAAAGTAA AGAATTGTAT CCATTGCGCT CTTTCCCGAC GAGAGTAAAT 91 181 GGCGAGGATA CGTTCTCTAT GGAGGATGGC ATAGGTGATG AAGATGAAGG AGAAGTACAG AACGCTGAAG TGAAGAGAGA GCTTAAGCAA AA AAA AA AA GATTGC CCTTGG AA A AA C ICTTTT CATTGGTTTA TCCACACCTC TGACCAACGC CGGCCCAGTG 271 AGACATATTG GTATC ACTATTGG TA CA AAAA A 361 GGCGCTCTTA TATCATATTT ATTTATGGGT TCTTTGGCAT ATTCTGTCAC GCAGTCCTTG GGTGAAATGG CTACATTCAT CCCTGTTACA AAT CA A $\overline{\mathbf{T}}$ TCCTCTTTCA CAGTTTTCTC ACAAAGATTC CTTTCTCCAG CATTTGGTGC GGCCAATGGT TACATGTAT AA G A A TAGTGT GTTGGCCAA GTCATTCAAT TTTGGACGTA CAAAGTTCCA CTGGCGGCAT GGATTAGTAT TTTTTC 541 TTTGCCC AAC AAA AAA AAA AA AA TACG AA A A A A A A A A GTGAATTCGA GTTCTGGGTC GCTTCCATCA AAGTTTTAGC CATTATCGGG A T GTTCCCTGTC AAATA 631 ATTATCACAA TAATGAACTT CA CAA AC TTTCTAATAT GAAACCCAGG AC A AA 811 CCAGGTATAA TATCTAAGGA TAAAAACGAA GGGAGGTTCT TAGGTTGGGT TTCCTCTTTG ATTAACGCTG CCTTCACATT TCAAGGTACT AAA AAA AA ÂA AA AA AA AA A T TÃ Igaagct gcaaacccca gaaaatccgt tccaagagcc atcaaaaaag ttgttttccg ТÂ GTATCACTGC TGG 901 GAACTAGTTG TATCTTAACC АААААА AAAAA G A GCTCTCTATT ATTCATTGGA CTTTTAGTTC CATACAATGA CCCTAAACTA ACACAATCTA CTTCCTACGT TTCTACTTCT TTCTACATTG 1081 CCCTTTATTA TTGCTATTGA GAACTCTGGT ACAAAGGTTT TGCCACATAT CTTCAACGCT GTTATCTTAA CAACCATTAT TTCTGCCGCA C A A C C C C A C 1171 AATTCAAATA TTTACGTTGG TTCCCGTATT TTATTTGGTC TATCAAAGAA CAAGTTGGCT CCTAAATTCC TGTCAAGGAC CACCAAAGGT AAA A A 1261 GGTGTTCCAT ACATTGCAGT TTTCGTTACT GCTGCATTTG GCGCTTTGGC TTACATGGAG ACATCTACTG GTGGTGACAA AGTTTTCGAA AA AA AA TA C ΤA AA ČAA CA A T TTTTTTGCAT GGTTATTTAT CTCAATCTCG CACATCAGAT TTATGCAAGC TTTGAAATAC A TA TGGCTATTAA ATATCACTGG TGTTGCAGGC AA 1441 CGTGGCATCT CTCGTGACGA GTTACCATTT AAAGCTAAAT TAATGCCCGG CTTGGCTTAT TATGCGGCCA CATTTATGAC GATCATTATC 1531 ATTATTCAAG GTTTCACGGC TTTTGCACCA AAATTCAATG GTGTTAGCTT TGCTGCCGCC TATATCTCTA TTTTCCTGTT CTTAGCTGTT 1621 TGGATCTTAT TTCAATGCAT ATTCAGATGC AGATTTATTT GGAAGATTGG AGATGTCGAC ATCGATTCCG ATAGAAGAGA CATTGAGGCA 1711 ATTGTATGGG AAGATCATGA ACCAAAGACT TTTTGGGACA AATTTTGGAA TGTTGTAGCA TAG

Fig. S1. Spectrum of spontaneous can1 mutations in the msh6 pol3-R696W strain containing pPOL3. Mutations (bold text) are displayed above the coding sequence of CAN1 (plain text). Nucleotides are numbered from the start of the CAN1 ORF. The data are summarized in Table 1 and Fig. 4D.



Fig. S2. (continued)

C. Wild-type Pol δ (S-phase dNTP concentrations)



Fig. S2. (continued)

E. R696W Polo (dNTP concentrations three-fold higher than estimated for S-phase)



Fig. S2. Spectra of mutations generated by wild-type Pol δ and Pol δ -R696W in vitro at the indicated dNTP concentrations. Base substitutions and deletions (open triangles) are displayed above the *lacZ* sequence. Insertions are shown under the *lacZ* sequence. Detectable mutations are shown in black, bold text. Silent mutations are shown in plain, gray text. The region corresponding to nucleotides –210 to –85 contains no sites at which mutations can be detected. Sequencing of wild-type Pol δ synthesis products revealed no mutations in this region. Silent mutations in this region are depicted in the Pol δ -R696W spectra. In addition to the point mutations depicted in *A*–*E*, the following large deletions were found: wild-type Pol δ (100 μ M dNTPs) –177 to 127; wild-type Pol δ (S-phase dNTPs) –96 to 123, –83 to 117, and 148–228.



Fig. S3. Incorporation of noncanonical dNTPs by wild-type Pol δ (WT) or Pol δ -R696W (RW). Reactions were performed with 2.5 nM Pol δ , 24 nM Cy5-labeled DNA substrate, 40 mM Tris-HCl (pH 7.8), 125 mM NaAc, 8 mM MgAc, 1 mM DTT, 0.2 mg/mL BSA, 4% (wt/vol) polyethyleneglycol 8000, and the indicated dNTP at 100 μ M for 50 s at 30 °C. The DNA substrate is shown below the gel, with the first templating base highlighted in yellow. Reaction products were separated on a 12% (wt/vol) polyacrylamide gel under denaturing conditions. The arrows indicate the position of the unextended primer. dHAPTP, 2'-deoxy-6-hydrox-ylaminopurine 5'-triphosphate; dXTP, 2'-deoxyanthosine 5'-triphosphate.



Fig. 54. Western blot analysis of proteins that modulate RNR activity. Immunoblots were conducted using whole-cell extracts from a wild-type strain containing pPOL3 (POL3), a *pol3-R696W* strain containing pPOL3 (RW), and a wild-type yeast strain without pPOL3 (WT) using the indicated primary antibodies. Experimental and loading control blots in each panel are from the same gel. A wild-type strain treated with the DNA damaging agent methyl methanesulfonate (MMS), which activates the DNA damage checkpoint, was used as a control. (*A*) *pol3-R696W* strains have reduced levels of RNR inhibitor Sml1. (*B*) *pol3-R696W* strains do not show detectable Rad53 phosphorylation.

| Genotype* | Can^{R} mutation rate (×10 ⁻⁷) [†] | Fold change [‡] |
|---|---|--------------------------|
| WT [§] | 2.3 (1.6–2.7) | |
| WT + pPOL3 [§] | 2.6 (2.1–3.8) | |
| WT | 2.7 (2.3–3.2) | |
| WT + pPOL3 | 2.6 (1.9–4.0) | 1 |
| $msh6\Delta$ | 38 (32–41) | 14 |
| $msh6\Delta + pPOL3$ | 35 (24–44) | 13 |
| pol3-R696W + pPOL3 [§] | 54 (44–65) | 21 |
| <i>pol3-R696W</i> + pPOL3 | 59 (48–70) | 23 |
| $pol3-R696W msh6\Delta + pPOL3$ | 1,500 (850–1,900) | 580 |
| $dun1\Delta + pPOL3$ | 3.8 (2.9–4.6) | 1.5 |
| $sm/1\Delta + pPOL3$ | 5.5 (4.9–6.6) | 2.1 |
| $crt1\Delta + pPOL3$ | 5.8 (4.8–7.0) | 2.2 |
| $dun1\Delta sml1\Delta crt1\Delta + pPOL3$ | 7.3 (5.5–11) | 2.8 |
| $pol3-R696W dun1\Delta + pPOL3$ | 3.7 (2.1–5.3) | 1.4 |
| $pol3-R696W \ sml1\Delta + pPOL3$ | 61 (50–71) | 23 |
| $pol3-R696W dun1\Delta sml1\Delta + pPOL3$ | 35 (30–45) | 13 |
| $pol3-R696W dun1\Delta sml1\Delta crt1\Delta + pPOL3$ | 97 (88–110) | 37 |
| $pol3-R696W dun1\Delta + pDUN1 + pPOL3$ | 57 (48–71) | 22 |
| WT + pPOL3.GST [§] | 2.9 (2.4–3.9) | 1 |
| WT + pPOL3.GST-5DV [§] | 15 (12–18) | 5.2 |
| WT + pPOL3.GST-R696W [§] | 230 (200–270) | 79 |
| WT + pPOL3.GST-5DV,R696W [§] | 1,200 (1,100–1,500) | 410 |
| $dun1\Delta + pPOL3.GST$ | 3.9 (2.8–4.4) | 1.3 |
| $dun1\Delta + pPOL3.GST-R696W$ | 9.7 (7.5–10) | 3.3 |

| Table S1. | Effect of MMR, proofreading, and RNR metabolism deficiencies upon the mutator |
|-----------|---|
| phenotype | of haploid strains expressing pol3-R696W |

*All haploid yeast strains used for mutation rate measurements are derived from 1B-D770. Full genotypes are described in Table S4. The majority of strains have *LEU2* integrated downstream of *CAN1*. [†]Values are medians for at least 18 cultures. The 95% confidence limits are shown in parentheses.

^{*}Fold change in the mutation rate relative to the wild-type control.

[§]These strains have an unmodified CAN1 allele.

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Table S2. Mutator effects of the *pol3-R696W* allele in MMR-proficient and MMR-deficient diploid strains

| Can ^R mutation rate (×10 ⁻⁷) [†] | Fold change ⁴ |
|---|---|
| 2.3 (2.0–3.0) | 1 |
| 22 (17–26) | 9.6 |
| 41 (31–56) | 18 |
| 470 (280–630) | 200 |
| | Can ^R mutation rate (×10 ⁻⁷) [†] 2.3 (2.0–3.0) 22 (17–26) 41 (31–56) 470 (280–630) |

The Can^{R} mutation rates were measured in diploid strains with a single copy of *CAN1* (Fig. 1A).

*Full genotypes are described in Table S4.

 $^{\rm T}{\rm Mutation}$ rates are medians for at least 18 cultures. The 95% confidence limits are shown in parentheses.

[‡]Fold change in the mutation rate relative to the wild-type diploid.

| | R696W Polð (threefold S-phase dNTP concentration) | |
|-----------------------|---|-------------------------|
| | No. | ER (×10 ⁻⁵) |
| Base substitutions | 172 | 86 |
| Transitions | 100 | 68 |
| A→G (A-dCTP) | 1 | 2.7 |
| G→A (G-dTTP) | 55 | 190 |
| C→T (C-dATP) | 16 | 41 |
| T→C (T-dGTP) | 28 | 68 |
| Transversions | 72 | 28 |
| A→C (A-dGTP) | 0 | <3.8 |
| A→T (A-dATP) | 33 | 73 |
| C→A (C-dTTP) | 17 | 55 |
| C→G (C-dCTP) | 0 | <5.0 |
| G→C (G-dGTP) | 3 | 9.4 |
| G→T (G-dATP) | 10 | 30 |
| T→A (T-dTTP) | 9 | 35 |
| T→G (T-dCTP) | 0 | <3.0 |
| Frameshifts | 47 | 18 |
| Minus 1 | 45 | 17 |
| Plus 1 | 2 | 0.74 |
| Large deletions | 0 | |
| Total | 219 | |
| lacZ mutant frequency | | 0.084 |

Table S3. Fidelity of in vitro synthesis by Polô-R696W at dNTP concentrations threefold higher than estimated for S-phase *pol3-R696W* cells containing pPOL3

The data are based on analysis of 179 *lacZ* mutants, 82 of which contained more than one mutation. Only phenotypically detectable *lacZ* mutations are listed. The error rate (ER) for individual mutation types were calculated as described in *Materials and Methods*. Silent mutations were excluded from the ER calculation. The location of individual mutations in the *lacZ* sequence is shown in Fig. S2. The background mutation frequency for unfilled M13mp2 gapped substrate was 0.0016.

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Table S4. Yeast strains used in this study

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| Strain | Relevant chromosomal mutation* | Plasmid | CAN1 allele | Genotype |
|--------------------|---|---------------------|------------------------------|---|
| Haploid strains us | ed to produce data | 1 | | |
| 1B-D770 | WT . | _ | CAN1 | MATa ade5-1 lys2-Tn5-13 trp1-289 his7-2 leu2-3,112 ura3-4 |
| TM20 | WT | pPOL3 | CAN1 | MATa ade5-1 lys2-Tn5-13 trp1-289 his7-2 leu2-3,112 ura3-4 [GAL1-POL3] |
| TM19 | pol3-R696W | pPOL3 | CAN1 | MATa ade5-1 lys2-Tn5-13 trp1-289 his7-2 leu2-3,112 ura3-4 pol3-R696W [GAL1-POL3] |
| TM30 | WT | — | CAN1::Kl.LEU2 | MATa ade5-1 lys2-Tn5-13 trp1-289 his7-2 leu2-3,112 ura3-4 CAN1::Kl.LEU2 |
| TM42 | WT | pPOL3 | CAN1::Kl.LEU2 | MATa ade5-1 lys2-Tn5-13 trp1-289 his7-2 leu2-3,112 ura3-4 CAN1::Kl.LEU2 [GAL1-POL3] |
| TM43 | $msh6\Delta$ | pPOL3 | CAN1::Kl.LEU2 | MATa ade5-1 lys2-Tn5-13 trp1-289 his7-2 leu2-3,112 ura3-4 CAN1::Kl.LEU2 msh6∆::HygB [GAL1-POL3] |
| TM34 | pol3-R696W | pPOL3 | CAN1::Kl.LEU2 | MATa ade5-1 lys2-Tn5-13 trp1-289 his7-2 leu2-3,112 ura3-4 CAN1::KI.LEU2 pol3-R696W [GAL1-POL3] |
| TM36 | pol3-R696W msh6 | pPOL3 | CAN1::Kl.LEU2 | MATa ade5-1 lys2-Tn5-13 trp1-289 his7-2 leu2-3,112 ura3-4 CAN1::KI.LEU2 pol3-R696W msh6∆::HyqB [GAL1-POL3] |
| TM52 | dun1∆ | pPOL3 | CAN1::Kl.LEU2 | MATa ade5-1 lys2-Tn5-13 trp1-289 his7-2 leu2-3,112 ura3-4 CAN1::KI.LEU2 dun14::KanMX [GAL1-POL3] |
| TM51 | sml1 Δ | pPOL3 | CAN1::Kl.LEU2 | MATa ade5-1 lys2-Tn5-13 trp1-289 his7-2 leu2-3,112 ura3-4 CAN1::KI.LEU2 sml1Δ::HvgB [GAL1-POL3] |
| TM69 | crt1∆ | pPOL3 | CAN1::Kl.LEU2 | MATa ade5-1 lys2-Tn5-13 trp1-289 his7-2 leu2-3,112 ura3-4 CAN1::KLLEU2 crt1A::KanMX [GAL1-POL3] |
| TM70 | $dun1\Delta \ sml1\Delta$ $crt1\Delta$ | pPOL3 | CAN1::KI.LEU2 | MATa ade5-1 lys2-Tn5-13 trp1-289 his7-2 leu2-3,112 ura3-4 CAN1::KI.LEU2 dun15::KanMX sml15::HygB crt15::NatMX [GAL1-POL3] |
| TM56 | pol3-R696W dun1A | pPOL3 | CAN1::Kl.LEU2 | ATa ade5-1 lys2-Tn5-13 trp1-289 his7-2 leu2-3,112 ura3-4 |
| TM58 | pol3-R696W sml1∆ | pPOL3 | CAN1::Kl.LEU2 | MATa ade5-1 lys2-Tn5-13 trp1-289 his7-2 leu2-3,112 ura3-4 CAN1::KI.LEU2 pol3-R696W sml1∆::HygB [GAL1-POL3] |
| TM59 | pol3-R696W dun1∆ sml1∆ | pPOL3 | CAN1::KI.LEU2 | MATa ade5-1 lys2-Tn5-13 trp1-289 his7-2 leu2-3,112 ura3-4 CAN1::KI.LEU2 pol3-R696W dun1∆::KanMX sml1∆::HygB [GAL1-POL3] |
| TM68 | pol3-R696W dun1∆ sml1∆ crt1∆ | pPOL3 | CAN1::Kl.LEU2 | MATa ade5-1 lys2-Tn5-13 trp1-289 his7-2 leu2-3,112 ura3-4 CAN1::KI.LEU2 pol3-R696W dun1∆::KanMX sml1∆::HygB crt1∆::NatMX [GAL1-POL3] |
| TM71 | pol3-R696W dun1∆ | pPOL3 + pDUN1 | CAN1::KI.LEU2 | MATa ade5-1 lys2-Tn5-13 trp1-289 his7-2 leu2-3,112 ura3-4 CAN1::Kl.LEU2 pol3-R696W dun1∆::KanMX [DUN1] [GAL1-POL3] |
| TM72 | WT | pPOL3.GST | CAN1 | MATa ade5-1 lys2-Tn5-13 trp1-289 his7-2 leu2-3,112 ura3-4 [GAL1-GST-POL3] |
| TM73 | WT | pPOL3.GST-5DV | CAN1 | MATa ade5-1 lys2-Tn5-13 trp1-289 his7-2 leu2-3,112 ura3-4 [GAL1-GST-POL3-5DV] |
| TM74 | WT | pPOL3.GST-R696W | CAN1 | MATa ade5-1 lys2-Tn5-13 trp1-289 his7-2 leu2-3,112 ura3-4 [GAL1-GST-POL3-R696W] |
| TM75 | WT | pPOL3.GST-5DV,R696W | CAN1 | MATa ade5-1 lys2-Tn5-13 trp1-289 his7-2 leu2-3,112 ura3-4 [GAL1-GST-POL3-5DV,R696W] |
| TM76 | WT | pPOL3.GST | CAN1::Kl.LEU2 | MATa ade5-1 lys2-Tn5-13 trp1-289 his7-2 leu2-3,112 ura3-4 CAN1::KI.LEU2 dun1∆::KanMX [GAL1-GST-POL3] |
| TM77 | WT | pPOL3.GST-R696W | CAN1::Kl.LEU2 | MATa ade5-1 lys2-Tn5-13 trp1-289 his7-2 leu2-3,112 ura3-4 CAN1::KI.LEU2 dun14::KanMX [GAL1-GST-POL3-R696W] |
| Diploid strains us | ed to produce data | | | |
| TM63 | WT | _ | CAN1::Kl.LEU2/ can1::loxP | MATa/MATα ade5-1/ade5-1 lys2-Tn5-13/lys2-InsE _{A14} trp1-289/ trp1-289 his7-2/his7-2 leu2-3,112/leu2-3,112 ura3-4/ura3-52 can1Δ::loxP/CAN1::KI.LEU2 |
| $TM44 \times TM34$ | POL3/pol3- R696W | — | CAN1::Kl.LEU2/ can1::loxP | MATa/MATα ade5-1/ade5-1 lys2-Tn5-13/lys2-InsE _{A14} trp1-289/ trp1-289 his7-2/his7-2 leu2-3,112/leu2-3,112 ura3-4/ura3-52 can1Δ··loxPICAN1··K1 EU2 PO 300-13-R696W |
| TM45 × TM43 | msh6∆/msh6∆ | _ | CAN1::Kl.LEU2/ can1::loxP | MATa/MATα ade5-1/ade5-1 lys2-Tn5-13/lys2-InsE _{A14} trp1-289/ trp1-289 his7-2/his7-2 leu2-3,112/leu2-3,112 ura3-4/ura3-52 can1Δ::loxP/CAN1::KI.LEU2 msh6Δ::HygB/msh6Δ::KanMX |

Table S4. Cont.

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| Strain | Relevant chromosomal mutation* | Plasmid | CAN1 allele | Genotype |
|--------------------|--|---------|------------------------------|--|
| TM45 × TM36 | POL3/pol3- R696W msh6∆/ msh6∆ | _ | CAN1::KI.LEU2/ can1::loxP | MATa/MATα ade5-1/ade5-1 lys2-Tn5-13/lys2-InsE _{A14} trp1-289/ trp1-289 his7-2/his7-2 leu2-3,112/leu2-3,112 ura3-4/ura3-52 can1∆::loxP/CAN1::KI.LEU2 POL3/pol3-R696W msh6∆::HygB msh6∆::KanMX |
| TM64 | WT | pPOL3 | CAN1::Kl.LEU2/ can1::loxP | MATa/MATα ade5-1/ade5-1 lys2-Tn5-13/lys2-InsE _{A14} trp1-289/ trp1-289 his7-2/his7-2 leu2-3,112/leu2-3,112 ura3-4/ura3-52 can1Δ::loxP/CAN1::KI.LEU2 [GAL1-POL3] |
| TM44 × TM34 | POL3/pol3- R696W | pPOL3 | CAN1::Kl.LEU2/ can1::loxP | MATa/MATα ade5-1/ade5-1 lys2-Tn5-13/lys2-InsE _{A14} trp1-289/ trp1-289 his7-2/his7-2 leu2-3,112/leu2-3,112 ura3-4/ura3-52 can1Δ::loxP/CAN1::KI.LEU2 POL3/pol3-R696W [GAL1-POL3] |
| TM46 × TM34 | pol3-R696W/ pol3-R696W | pPOL3 | CAN1::Kl.LEU2/ can1::loxP | MATa/MATα ade5-1/ade5-1 lys2-Tn5-13/lys2-InsE _{A14} trp1-289/ trp1-289 his7-2/his7-2 leu2-3,112/leu2-3,112 ura3-4/ura3-52 can1Δ::loxP/CAN1::KI.LEU2 POL3/pol3-R696W [GAL1-POL3] |
| Strains used in co | onstruction of diploid s | trains | | |
| E134 | WT | — | CAN1 | MATα ade5-1 lys2::InsE _{A14} trp-289 his7-2 leu2-3,112 ura3-52 |
| ТМ39 | WT | — | can1∆::loxP | MAT α ade5-1 lys2::InsE _{A14} trp-289 his7-2 leu2-3,112 ura3-52 can1 Δ ::loxP |
| TM46 | pol3-R696W | pPOL3 | can1∆::loxP | MATα ade5-1 ys2-Ins E_{A14} trp-289 his7-2 leu2-3,112 ura3-52 can1 Δ ::loxP pol3-R696W [GAL1-POL3] |
| TM44 | WT | pPOL3 | can1∆::loxP | MATα ade5-1 lys2-lnsE _{A14} trp1-289 his7-2 leu2-3,112 ura3-52 can1Δ::loxP [GAL1-POL3] |
| TM45 | $msh6\Delta$ | _ | can1∆::loxP | MATα ade5-1 lys2-InsE _{A14} trp1-289 his7-2 leu2-3,112 ura3-52 can1Δ::loxP msh6Δ::KanMX |

Details of strain construction are provided in *Materials and Methods*. *WT, wild type.