Supporting Information

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SI Text

The Rate of Spontaneous Mutation in the *pol3-R696W* Mutants Is Estimated to Be Incompatible with Life. We observed >65- and >200-fold increases in the rate of Can^r mutation in two independently constructed *pol3-R696W*/*pol3-R696W* diploids (Table 1), which translates to at least a 6,000- and 11,000-fold increase in the rate of mutation at a single *CAN1* allele over the wild-type mutation rate. Although we do not observe the same ≥10,000-fold mutator effect in haploids, this could be explained by the previously proposed idea that the majority of mutagenesis occurs in a hypermutable fraction of cells (1). In haploids, this fraction is largely killed by lethal mutations, and thus, any mutations that occur in our reporter gene in these cells are excluded from the analysis. According to this idea, the measurements done in diploid cells provide a more accurate estimate of the mutator effect of the *pol3-R696W* allele, although it could be to some extent affected by the death of the hypermutable fraction as well. As calculated previously (2), ~10,000-fold increase in the rate of spontaneous mutation in yeast is expected to result in the generation of one lethal mutation per haploid genome per replication. Although this is a rough estimate, it predicts that the haploid *pol3-R696W* strains rescued by a low-level *POL3* expression are at the edge of survival and experience a level of mutagenesis close to a maximum that could be tolerated by haploid cells. This strongly suggests that the increase in mutagenesis that would occur in the absence of the wild-type *POL3* is incompatible with life in haploids and that the *pol3-R696W* strains, indeed, die from the mutation catastrophe. For the *pol3-R696W*/*pol3-R696W* diploids containing pPOL3, the observed Can^r mutation rate of $\sim 5 \times 10^{-6}$ translates to a rate of $\sim 2.2 \times 10^{-3}$ per single *CAN1* allele. Just a 10-fold decrease in the fidelity of DNA synthesis (2.2×10^{-2} errors per single allele per replication) would bring the rate of simultaneous inactivation of both alleles up to $\sim 5 \times 10^{-4}$. When extrapolated from the *CAN1* gene to the whole genome containing ~1,100 essential genes (3), this is close to approximately one inactivating mutation per essential gene per replication. Thus, it is likely that the *pol3-R696W*/*pol3-R696W* diploids reach the lethal level of mutagenesis when pPOL3 is lost, although it is impossible to measure directly.

Materials and Methods

Strains and Plasmids. All *Saccharomyces cerevisiae* strains used in this study are isogenic to the haploid strains E134 (*MAT*α *ade5-1 lys2::InsE$_{A14}$ trp-289 his7-2 leu2-3,112 ura3-52*) or 1B-D770 (*MAT***a** *ade5-1 lys2-Tn5-13 trp1-289 his7-2 leu2-3,112 ura3-4*) (4). The diploid strain PSD93 was derived from E134 by transformation with YEpHO (5), and the diploid strain E134 × 1B-D770 results from a cross of E134 and 1B-D770, as described previously (4). All SNPs and cancer-associated mutations were introduced into plasmid-borne yeast *POL3* and *POL2* genes by using a QuikChange site-directed mutagenesis kit (Stratagene) (primers are listed in Table S5). To generate diploid strains heterozygous for the *pol3* or *pol2* mutations, one chromosomal copy of the corresponding gene was replaced with the mutant alleles using the integration–excision procedure described previously (6). The presence of the mutations was confirmed by PCR amplification of the *POL3* or *POL2* genes followed by DNA sequencing or restriction endonuclease digestion of the product (primers and enzymes listed in Table S5). In the resulting strains, the variant alleles are expressed from the natural promoter at their normal chromosomal location. Haploid *pol3* or *pol2* mutants were obtained by sporulation of the heterozygous diploids followed by tetrad dissection. To rescue the lethality of *pol3-R696W* mutants, pBL336 plasmid (2μ ori *TRP1 GAL1-POL3*, also called pPOL3 in this report) (7) was introduced into the heterozygous *pol3-R696W*/*POL3* diploids before sporulation, and dissections were performed on synthetic complete medium lacking tryptophan (SC-TRP) containing 3% raffinose and 0.02% galactose. Homozygous *pol3-R696W*/*pol3-R696W* diploids carrying pPOL3 were obtained by mating *pol3-R696W* [pPOL3] haploids of the opposite mating type. All SNPs and cancer-associated mutations were constructed using PSD93 as starting material. The construction of *pol3-R696W* haploid and diploid mutants was also repeated using the E134 × 1B-D770 diploid as a starting material. Unless otherwise indicated, the results obtained with PSD93-derived and E134 × 1B-D770-derived strains were indistinguishable, and the mutation rate data for the two strain backgrounds were combined. For the pPOL3-containing strains, all construction steps were done on media to select for the plasmid and drive expression of *POL3* (SC-TRP with 3% raffinose and 0.02% galactose). Disruptions of the *REV3* and *POL3* genes were created by transformation with a PCR-amplified *KanMX* cassette flanked by a sequence homologous to the upstream and downstream regions of *REV3* or *POL3* as described (8). Plasmids pBL335 and pBL341 for overproduction of GST-tagged yeast Pol δ (9) were kindly provided by Peter Burgers (St. Louis, MO).

Measurement of Spontaneous Mutation Rates. The rate of forward mutation to Can^r and the reversion of *lys2::InsE$_{A14}$* and *his7-2* frameshift mutations were measured by fluctuation analysis as described previously (4). The Can^r mutagenesis assay scores a wide variety of mutations that inactivate the *CAN1* gene, including base substitutions, frameshifts, complex mutations, and rearrangements (10–12). The *lys2::InsE$_{A14}$* and *his7-2* alleles contain polyA tracts that are restored to the correct reading frame by −1 or +1 events, respectively (4, 13). Unless otherwise indicated, pPOL3-containing strains were grown in SC-TRP with 2% glucose. The experiments shown in Fig. 3 were conducted in SC-TRP with 2% glucose, 3% raffinose, or the indicated concentration of galactose supplemented to a total of 3% sugar with raffinose.

Plasmid Loss Assay. Colonies grown on SC-TRP to select for pPOL3 were resuspended in water and diluted, and single cells were plated on yeast extract/peptone/dextrose/adenine/uracil (YPDAU) media (1% yeast extract, 2% glucose, and 1% peptone media supplemented with 60 mg/L adenine and 62.5 mg/L uracil). After 2 days of growth, colonies were replica-plated from YPDAU to synthetic complete (SC) and SC-TRP. The frequency of plasmid retention was calculated by dividing the number of Trp$^+$ colonies by the total number of colonies growing on SC. Reported frequencies were calculated by combining colony counts from three to five experiments.

Overproduction and Purification of Pol δ. Plasmid pBL335-pol3-R696W was constructed by gap repair in yeast. The plasmid backbone of pBL335 and the *pol3-R696W* allele were amplified by PCR, and the yeast strain 1B-D770 was cotransformed by the PCR products. The plasmids were then isolated from yeast and used to transform the yeast strain BJ2168 (9). The presence of the *pol3-R696W* allele on the plasmid in the resulting strain was verified by sequencing. Polδ and Polδ-R696W were overproduced in BJ2168 and purified as previously described (9), except that the SPEX 6870 Freezer Mill (SPEX SamplePrep) was used to disrupt yeast

cells and NaAc was used in place of NaCl in the purification buffers. The concentration of NaAc was twice as high as that of NaCl in the previously published purification procedure (9).

DNA Polymerase and Exonuclease Assays. The oligonucleotide substrates for the polymerase and exonuclease reactions were created by annealing a Cy5-labeled primer strand (5′-Cy5-CAGCACCACAAACCATACAAAAACA-3′) to a template strand (5′-GCCATTATCGGGTTTCTAATATACTGTTTTTG TATGGTTTGTG-GTGCTG-3′) by incubation at 85°C for 2 min in the presence of 150 mM NaAc followed by slow cooling to room temperature. DNA polymerase reactions contained 40 mM Tris-HCl (pH 7.8), 125 mM NaAc, 8 mM MgAc, 1 mM DTT, 0.2 mg/mL BSA, 4% polyethylene glycol 8000, 25 nM oligonucleotide substrate, and 100 μM each dNTPs. Reactions were performed at 30°C; incubation time and enzyme concentration are indicated in Fig. 2. Reactions were stopped by placing the tubes on ice and adding 10 μl of formamide loading dye. Exonuclease activity was assayed under the same conditions with the omission of 4% polyethylene glycol and dNTPs. The products were separated by electrophoresis in an 18% denaturing polyacrylamide gel and detected and quantified using the Typhoon imaging system and ImageQuant software (GE Healthcare).

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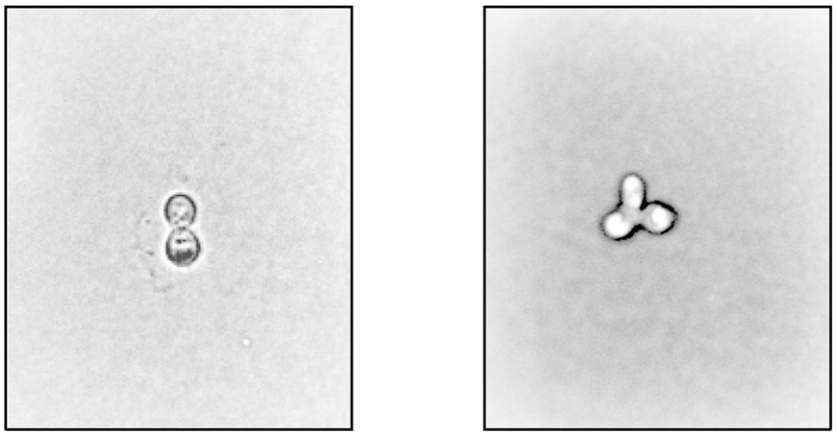

Fig. S1. Haploid cells lacking active Polδ do not form microcolonies. Spores obtained after tetrad dissection of the *POL3/pol3-D762A,D764A* diploid were incubated for 3 days at 30°C. All *pol3-D762A,D764A* spores from 18 dissected tetrads were unable to complete cell division and displayed either dumbbell (~90% of cells) or a trilobe (~10% of cells) terminal morphology. Images of a representative dumbbell (*Left*) and three-lobe (*Right*) cells were taken under 40× magnification with a brightfield microscope.

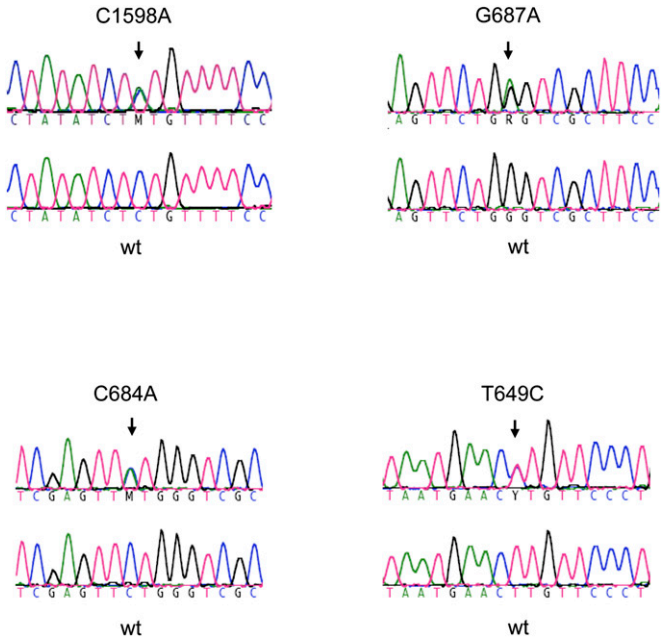

Fig. S2. Examples of DNA sequence analysis of spontaneous *can1* mutants of *pol3-R696W/pol3-R696W* diploids. For each of the four mutants shown, the upper electrophoregram shows the analysis of the *can1* mutant, and the lower electrophoregram shows the corresponding wild-type sequence. The mutation sites are indicated by arrows. The mixed-signal designations are as follows: M, C+A; R, G+A; and Y, C+T. Similar to the four examples shown, all *can1* mutations listed in Table 2 and Table S3 were found in a heterozygous state.

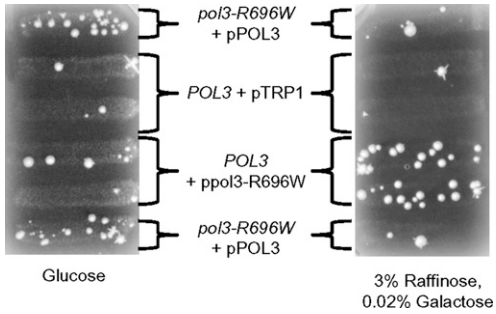

Fig. S3. Qualitative analysis of mutagenesis in strains expressing the *POL3* or *pol3-R696W* alleles from a galactose-inducible promoter. Haploid strains were streaked on SC-TRP media with the carbon source indicated in the figure and grown for two days at 30°C. The cells were then replica plated to SC medium lacking arginine and containing 60 mg/L L-canavanine (SC-ARG+CAN). Only cells that acquired a mutation in the *CAN1* gene can give rise to colonies on this medium. The SC-ARG+CAN plates were photographed after 5 days of incubation at 30°C. In strains containing the chromosomal *pol3-R696W* allele and the galactose-inducible wild-type *POL3* on a plasmid, the mutator phenotype was suppressed by the addition of galactose (streaks 1 and 6). In contrast, in strains containing the wild-type chromosomal *POL3* allele and the galactose-inducible *pol3-R696W* on a plasmid, mutagenesis was increased by the addition of galactose (streaks 4 and 5). The wild-type strain containing a control vector was plated for comparison (streaks 2 and 3).

Table S1. The rate of Can^r mutation in haploid Pol δ and Pol ε mutants and their *msh6* derivatives (× 10^{-7})

Genotype	*MSH6*	*msh6ΔkanMX*
wild type	1.8 (1.5–2.1)	16 (12–30)
pol3-R511H	1.9 (1.5–2.4)	41 (33–50)
pol3-K855H	1.4 (1.2–1.9)	14 (10–16)
pol3-K1084Q	1.3 (1.1–2.0)	25 (18–33)
pol2-F709I	1.4 (1.0–2.3)	16 (13–23)
pol2-E1582A	1.5 (1.2–1.6)	16 (15–20)

The values are medians for at least nine cultures. The 95% confidence limits are given in parentheses.

Table S2. Effects of *pol3-R696W* allele on the rate of Lys$^+$ and His$^+$ reversion in haploid and diploid cells

Genotype	pPOL3	Lys$^+$ ($\times 10^{-7}$)	His$^+$ ($\times 10^{-8}$)
POL3	+	2.9 (2.3–3.2)	<2
pol3-R696W	+	12 (9.5–16)	11 (5.9–12)
POL3/POL3	–	2.2 (2.0–2.6)	0.5 (0.3–0.9)
POL3/pol3-R696W	–	3.9 (3.3–5.7)	1.4 (0.9–2.2)
POL3/POL3	+	3.2 (1.2–6.8)	<3
POL3/pol3-R696W	+	2.8 (2.3–3.7)	<3
pol3-R696W/pol3-R696W	+	280 (230–470)	200 (160–290)
pol3ΔKanMX/pol3-R696W	+	35 (20–49)	nd

The values are medians for at least nine cultures. The 95% confidence limits are given in parentheses. nd, not determined.

Table S3. DNA sequence context of *can1* mutations listed in Table 2 and the resulting amino acid changes

Position	Sequence	Nucleotide change	Amino–acid change	Number of mutations*
648	ATG AAC TTG	C → A	N →K	1
649	AAC TTG TTC	T → C	L → L†	1
670	TAC GGT GAA	G → A	G → S	1
671	TAC GGT GAA	G → A	G → D	2
684	GAG TTC TGG	C → A	F → L	1
687	TTC TGG GTC	G → A	W → stop	1
718	ATC GGG TTT	G → A	G → R	1
732	ATA TAC TGT	C → A	Y → stop	1
1018	ATT GGA CTT	G → A	G → R	3
1240	TTC CTG TCA	C → A	L → M	1
1261	GGT GGT GTT	G → A	G → S	1
1426	ATG CAA GCT	C → T	Q → stop	1
1598	ATC TCT ATT	C → A	S → Y	1
1676	GAT GTC GAC	T → C	V → A	1

The *can1* mutants analyzed by DNA sequencing were selected after growth of the *pol3-R696W/pol3-R696W* diploid containing pPOL3 on the glucose-containing medium. Chromosomal DNA was isolated from ten independent mutants obtained during fluctuation analysis, and the *CAN1* locus was amplified by PCR. The PCR products were cleaned with the QIAquick PCR purification kit (Qiagen) and sequenced using primers 5′-GGACGTACAAAGTTC-CACTG and 5′-CAATGACCCTAAACTAACAC. The sequencing covered nucleotides 618–1773 of *CAN1*.
*All recovered mutations were identified in the heterozygous state (Fig. S2).
†This synonymous mutation was recovered in a mutant with additional mutations in *CAN1* (Table 2).

Table S4. Alignment of Region III in the catalytic subunits of human and yeast B family polymerases

Polymerase	Amino acid	Region III motif*
Hs Pol delta	688	GRQLALKVSANSVYGFTGAQ
Hs Pol zeta	2641	ARQLGLKLIANVTFGYTSAN
Hs Pol alpha	944	IRQKALKLTANSMYGCLGFS
Hs Pol epsilon	803	SLQLAHKCILNSFYGYVMRK*
Sc Pol delta	695	GRQLALKISANSVYGFTGAT
Sc Pol zeta	1080	NKQLALKLLANVTYGYTSAS
Sc Pol alpha	938	IRQQALKLTANSMYGCLGYV
Sc Pol epsilon	818	SLQLAHKVILNSFYGYVMRK

*Light gray, largely conserved; dark gray, fully conserved.

Table S5. Primers and confirmation strategies for polymerase mutations

Mutation	Site-directed mutagenesis primers*	Sequencing primer	Digestion strategy
pol3-R511H	CGATAGTGAAACAAGACACAGGTTGGCCGTTTACTG; CAGTAAACGGCCAACCTGTGTCTTGTTTCACTATCG	CCGTGCAAAGGCGCTAAAGGTG	*Mbo*II site lost
pol3-R696W	GAGATGTTTTAAATGGTTGGCAATTGGCTTTGAAG; CTTCAAAGCCAATTGCCAACCATTTAAAACATCTC	GGATGAATTAATAAGTGGTAG	*Acc*I site lost
pol3-K855H	GAATAAAGTTTTACACAAAATTTTAATTGAAAGAAATGTAGATGG; CCATCTACATTTCTTTCAATTAAAATTTTGTGTAAAACTTTATTC	CCTGACAAGTTTGACAAGTTGGACC	na
pol3-K1084Q	GCGGGTTAAGGTTCAAAAAGAGCTGCAGG; CCTGCAGCTCTTTTTGAACCTTAACCCGC	GTGAAATCCATTAAAATTAACACAGGC	na
pol2-F709I	CATTACAAAATGAGACTATTCCCAACAAAAACAAG; CTTGTTTTTGTTGGGAATAGTCTCATTTTGTAATG	TTGGACAGATTTCCCTTCCA	na
pol2-E1582A	CTACTAAATTAAAAGCAGAAAGAGGTCTGCAG; CTGCAGACCTCTTTCTGCTTTTAATTTAGTAG	GAAATGAAGGATCTTTCAATGGCGG	na

*Highlighted letters indicate codons that differ from wild type. na, not applicable.