Supplemental Experimental Procedures

***S. cerevisiae* strains.** All *S. cerevisiae* strains in this study (listed in Table S1) were derived from the S288C background. *S. cerevisiae* cells were grown at 30°C in YPD (1% yeast extract, 2% Bacto Peptone and 2% dextrose) or in drop out media (0.67% yeast nitrogen base without amino acids, 2% dextrose, and the appropriate amino acid drop out mix). Strains were constructed using standard gene disruption protocols. Transformation of DNA fragments for recombination or plasmids was done using the lithium acetate transformation method.

The strain RDKY8260 (*MATa ura3-52 leu2Δ1 trpΔ63 his3Δ200 hom3-10 lys2::InsE-A10 exo1::hphNT1 pol30::HIS3* [pRDK900, *POL30*]), used for screening of *POL30* alleles, was generated by transforming RDKY7884 with pRDK900 followed by deletion of the genomic *POL30* locus by recombination with a PCR amplified *POL30*-targetting *HIS3* cassette. Mutations were introduced at the *POL30* genomic locus using *Sac*I (New England Biolabs) and *Sna*B1 (New England Biolabs) digested pRDK1743-pRDK1754 (Table S2) with the *LEU2* gene integrated upstream of the *POL30* promoter as a marker for integration (as done previously (Lau et al., 2002)), in both RDKY5964 and RDKY7884. Transformants were selected for on leucine drop out plates and confirmed by PCR. The presence of the *pol30* point mutation and no additional mutations was verified by sequencing of the final strains.

Construction of a *pol30* mutation library. To generate a library of *pol30* mutations the *POL30* gene was amplified from pRDK837 with primers 5'-GCTGCGCGTAACCACCAC-3' and 5'-CGACAGGTTTCCCGACTG-3' using Taq polymerase (New England Biolabs), which lacks proofreading exonuclease activity, for 15 cycles under standard conditions in 50 independent

reactions. These reactions were pooled and purified using a PCR clean up kit to remove the primers (Qiagen). The pooled mutagenized *POL30* fragments were cloned into pRS314 by *in vivo* gap repair by transformation into RDKY8260 along with *Ale*I (New England Biolabs)-digested and gel-purified pRS314. Transformants containing the gap-repaired plasmids were selected on tryptophan drop out plates and replica plated on 5-fluoroorotic acid (5-FOA) containing plates to select for loss of pRDK900 using the plasmid shuffle technique as previously described (Lau et al., 2002; Umezu et al., 1998).

Mutation screen. The colonies resulting from the above mutagenesis protocol were replica plated onto both minus threonine and minus lysine drop out plates to test for an increased mutator phenotype in the *hom3-10* and *lys2::InsE-A10* frameshift reversion assays respectively as well as onto arginine drop out plates containing 60 mg/L canavanine to test for increased mutation rates in the canavanine resistance forward mutation assay (Amin et al., 2001; Marsischky et al., 1996). The plates were incubated at 30°C for 3 days, after which plates were screened for papillation, indicating a mutator phenotype. Colonies exhibiting a mutator phenotype were streaked for single colonies from the original 5-FOA plate, and were retested in the three mutator assays by patch tests. Colonies containing plasmid borne *pol30* alleles that caused an increased mutator phenotype were used to inoculate liquid cultures, genomic DNA was isolated from these cultures using the Gentra Puregene yeast/bact kit (Qiagen) and the plasmids were rescued by transforming the *S. cerevisiae* genomic DNA into *E. coli*. The resulting plasmids (listed in Table S2) were purified using a Qiagen miniprep kit and sequenced to identify the *pol30* mutation. All plasmids were then retransformed into RDKY8260 and retested in the three mutator assays to confirm the mutator phenotype. Strains with plasmids

causing mutator phenotypes were transformed with pRDK1020 containing wild-type *EXO1* or pRS315 control plasmid and tested for complementation of the mutator phenotype by *EXO1*.

In this screen, approximately 8,000 colonies were screened, of which 227 colonies had a mutator phenotype in the *lys2::InsE-A10* and *hom3-10* frameshift reversion assays. 35 of the 227 colonies were confirmed to have a mutator phenotype when retested. Plasmids from these 35 colonies were isolated, sequenced, reintroduced into the *exo1Δ pol30Δ* double mutant strain, and retested to ensure that the mutator phenotype was not due to an unrelated mutation acquired during screening resulting in the verification of 18 *pol30* alleles that cause a mutator phenotype (Table 1). Eight *pol30* alleles contained multiple mutations resulting in multiple amino acid changes; in these cases each mutation was independently generated by site directed mutagenesis and tested to determine which mutation caused the mutator phenotype (Table 1). Five mutations isolated in this screen were previously identified in other screens for *pol30* alleles that caused defects in MMR that were not dependent on an *exo1Δ* mutation (Chen et al., 1999; Lau et al., 2002), and one mutation, *pol30-E143K* (mislabelled as *pol30-E143S* in Amin et al. 2001), was not isolated here but was previously isolated in a genome-wide screen for MMR mutations dependent on an *exo1Δ* mutation (Amin et al., 2001). Wild-type *EXO1* on a single copy plasmid complemented 13 of the *pol30* alleles and did not complement 5 *pol30* alleles (*pol30-K13E, L50S, F144L*; *pol30-V23A*; *pol30-S49P*; *pol30-F207, K217E*; and *pol30-S222P*); these latter mutations appear to cause a general MMR defect and were not studied further. This yielded 14 *pol30* mutations that caused single amino acid substitutions (13 identified in this screen and the previously isolated *pol30-E143K* (Amin et al., 2001)) that resulted in a mutator phenotype that was largely or completely dependent on an *exo1Δ* mutation (Table 1). Eleven of

these 14 *pol30* alleles were verified to cause the same phenotype when present at the chromosomal locus, 1 *pol30* allele (*pol30-C30R*) at the chromosomal locus caused a mutator phenotype that was no longer dependent on an *exo1Δ* mutation and 2 were not tested at the chromosomal locus (Table 1).

Site directed mutagenesis. Site directed mutagenesis was used to independently generate *pol30* mutations in pRDK837 in the event that the *pol30* mutant allele isolated in the above screen contained more than one mutation (Table 1, Table S2). Site directed mutagenesis was also used to generate the *pol30* mutations in pRDK902 used for integration of the mutations at the genomic locus (pRDK1743-pRDK1754, Table S2), pRDK930 for bacterial expression and purification (pRDK1763-pRDK1767, Table S2) and pRDK833 for low copy versus high copy plasmid studies (pRDK1755-pRDK1762, Table S2). Site directed mutagenesis was carried out utilizing either a GeneArt Site Directed mutagenesis kit (Invitrogen) or an XL-Quick Change site directed mutagenesis kit (Agilent) according to manufacturer's instructions. Incorporation of the desired mutation and lack of additional mutations were confirmed by sequencing.

Mutation rate analysis. Fluctuation analysis was used to determine the mutation rate for the *hom3-10* and *lys2::InsE-A10* frameshift reversion assays and the *CAN1* forward mutation assay for the indicated yeast strains as previously described (Amin et al., 2001; Marsischky et al., 1996). Each rate was determined using at least 14 independent cultures.

Protein purification. Wild-type PCNA, Msh2-Msh6, Mlh1-Pms1 and RFC-Δ1N used in this study were purified as previously reported (Bowen et al., 2013). The mutant PCNA proteins

(PCNA-C81R, PCNA-E143K, PCNA-C22Y, PCNA-K217E, PCNA-K13E, PCNA-L68S and PCNA-F254L) were overexpressed using plasmids pRDK931-pRDK932 and pRDK1763-pRDK1767 (Table S2) transformed into One Shot BL21 (DE3) pLysS *E. coli* (Invitrogen). 500 mL of bacterial culture were grown to an OD between 0.4 -0.8 and induced with isopropyl β-D-thiogalactopyranoside (IPTG) at a final concentration of 1 mM for 6 hours. The cells were harvested, lysed and the PCNA purified as previously described (Bowen et al., 2013) with all volumes scaled for purification from 500 mL of bacterial culture and with the following modifications. The pooled hydroxyapatite fractions were dialyzed into Buffer D [25 mM TRIS pH 7.5, 1 mM DTT, 0.5 mM EDTA, PICD and PICW] containing 200 mM NaCl. The final HiTrap™ (GE Healthcare) Q FF column was run as previously described except Buffer D was used instead of Buffer C. The final pooled Q column fractions were dialyzed into Buffer D containing 100 mM NaCl for storage.

Size exclusion chromatography. Purified PCNA was chromatographed on a Sepax SRT SEC-300 sepharose size exclusion column at a concentration of 2.88 μM in 25 mM Tris pH 8.0, 0.1 mM EDTA, 300 mM NaCl containing buffer on an Agilent 1200 series HPLC system with a 20 μL injection loop and fractions were collected. Elution time was monitored by UV absorption and by analyzing fractions by SDS-PAGE followed by silver staining. Stokes radii were calculated by running size exclusion standards (BioRad) with known Stokes radii and using the standard curve to calculate Stokes radii from PCNA elution times.

Surface plasmon resonance. The PCNA interaction with Msh2-Msh6 was determined using surface plasmon resonance with a BiacoreT100. Purified wild-type PCNA and PCNA mutants

were dialyzed into PBS buffer using slide-A-Lyzer cassettes (Pierce) and biotinylated using the Amersham ECL Protein Biotinylation Module (GE Healthcare). The PCNA surface was generated by binding 400 RU (resonance units) of biotinylated wild-type PCNA in channel 2 and 400 RU of biotinylated mutant PCNA proteins into channels 3 and 4 of a Biacore SA streptavidin chip. Channel 1 was left empty for background subtraction. To determine binding, RU was monitored as Msh2-Msh6 protein at 100 nM was flowed over the PCNA chip at a 20 µL/min flow rate for 100 sec followed by 300 sec of buffer flow. The PCNA surface was regenerated with a 20 sec injection of 0.1% SDS at 15 µL/min.

***In vitro* endonuclease assay.** The *in vitro* endonuclease assays were performed exactly as described previously (Smith et al., 2013). The endonuclease reactions contained 100 ng of supercoiled circular pRS425 substrate DNA, 9 nM RFC-Δ1N and 30 nM of either wild-type or mutant PCNA in the presence of 35 nM wild-type Mlh1-Pms1 as indicated in individual experiments. After termination of the reactions, the DNA samples were electrophoresed through an 0.8% agarose gel, the gel was stained with ethidium bromide, extensively destained and then the bands were quantified using a BioRad ChemiDoc XP imaging system. The amount of nicked circular DNA formed was determined relative to that formed in the presence of wild-type PCNA and Mlh1-Pms1, which was considered 100% nicking.

Cell imaging studies. Imaging and analysis of Mlh1-Pms1 GFP foci was carried out as previously described using 2 independent strain isolates in each experiment (Hombauer et al., 2011; Smith et al., 2013).

Statistical analysis. 95 % confidence intervals were calculated for all fluctuation tests. Mann-Whitney tests were performed to report the two-tailed p-values for comparisons between rates. (http://vassarstats.net/utest.html).

Supplemental References

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