Supporting Information

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SI Materials and Methods

Strains and Cell Growth. *Saccharomyces cerevisiae* strains were grown on standard media, either yeast extract/peptone/dextrose (YPD) or complete supplement mixture (CSM) medium (US Biological) lacking specific amino acids to select for plasmid markers. *Escherichia coli* strains were grown in either Luria Broth (LB) consisting of 10 g of tryptone, 5 g of yeast extract, 5 g of sodium chloride, and 50 mg of thymine per liter of media or Terrific Broth (TB) consisting of 12 g of peptone, 2.31 g of monopotassium phosphate, 12.54 g of dipotassium phosphate, 24 g of yeast extract, and 4 mL of glycerol per liter of media containing antibiotics as indicated in individual experiments. Plasmids were typically maintained and propagated in the *E. coli* strains XL1-Blue MRF' (Agilent) or TOP 10F' (Life Technologies). Overproduction of proteins in *E. coli* was performed using the *E. coli* strains BL21 CodonPlus (DE3) RIL (Stratgene) or OneShot BL21 (DE3) pLysS (Invitrogen). Overproduction of proteins in *S. cerevisiae* was performed in the *S. cerevisiae* strains RDKY1293 *MATα, ura3-52, leu2-1, his3-200, pep4::HIS3, prb1-1.6R, can1*, RDKY2418 *MATα, ura3-52, leu2-1, his3-200, pep4::HIS3, prb1-1.6R, can1, msh2::hisG, msh6::hisG* or BJ2168 *MATa ura3–52, trp1–289, leu2–3, 112, prb1–1122, prc1–407, pep4–3* (1–3). The plasmids used for protein overproduction have either been described in published studies or were constructed for the present study and are described under *Plasmid Construction*.

Plasmid Construction. Two plasmids were constructed for use in overproduction studies. A derivative of the Msh2–Msh6 expression plasmid pET11Msh2–Msh6 (4) expressing the Msh2–Msh6–F337A protein, called pRDK1694, was constructed by site-directed mutagenesis using a GeneArt kit (Invitrogen) and the mutagenic oligonucleotides 5′-ggattgtattgtctttttcaaaaagggtaag**GC**ctttgaattatatgaaaaggatgcattattg and 5′-caataatgcatcctttttcatataattcaaag**GC**cttaccctttttgaaaaagacaatacaatcc (mutant nucleotides indicated in uppercase bold). In addition, a pRS425 *LEU2 GAL1-10-EXO1-HIS_6FLAG* expression vector called pRDK1575 was constructed in three steps. First, an XhoI to SalI fragment consisting of the pRS425 *LEU2 GAL1-10* backbone from pRDK274 (3) was isolated and joined to a purified NcoI to XhoI fragment from pRDK379 (5) containing the *EXO1* gene starting from the NcoI site at the *EXO1* ATG codon and ending at an XhoI site downstream of the *EXO1* stop codon, substituting the *EXO1* gene for the *RFA2* gene present in pRDK274 and eliminating the pRS425 polylinker SalI site; this plasmid was called pRDK1695. Then, a His_6FLAG tag was fused to the *EXO1* coding sequence by in vivo recombination. This recombination event was accomplished by generating a fragment from pFA6a-6HisFLAG (6) by PCR using the primers 5′-CGGCAGTCAGATCTATCTCCTTGCTTTCCCAATTTGTTTATAAAGGTAAAgggggttctcatcatcatca (uppercase specifies the C-terminal sequence of *EXO1* and lowercase specifies the N-terminal sequence of the *HIS_6FLAG* region of pFA6a-6HisFLAG) and 5′-GTGAGCGCGCGTAATACGACTCACTATAGGGCGAATTGGGTACCGGGCCCcgcttatttagaagtggcgc (uppercase specifies the pRS425 sequence starting at the polylinker ApaI site and lowercase the species sequence of pFA6a-6HisFLAG downstream of the stop codon of the *HIS_6FLAG* sequence) and co-transforming it into the *S. cerevisiae* strain RDKY1293 along with pRDK1695 DNA that had been linearized at the ApaI site in the pRS425 polylinker and then rescuing plasmids from the transformants into *E. coli*. The resulting plasmid encoded the sequence GGSHHHHHHGMASMTGGQQMGRDYKDDDDK-VPGSSVVE after the last amino acid of Exo1, followed by a TGA stop codon and the downstream 23 nucleotides from pFA6a-6HisFLAG before the start of the pRS425 sequence. Finally, an internal AatII to BglII segment of *EXO1* was excised and swapped for the same *EXO1* fragment from pRDK834 (5) to eliminate a mutation that occurred during the construction process, resulting in the Exo1-His_6FLAG expression plasmid pRDK1575. Both of the expression plasmids were sequenced to ensure they did not contain any mutations.

Protein Purification. All of the proteins used in the described studies were expressed from plasmid expression vectors in either *E. coli* or *S. cerevisiae*. Typical yields ranged from 100 μg to 500 μg per L of expressing cells. All of the protein preparations were confirmed to be greater that 95% pure as judged by SDS/PAGE followed by staining the resulting gels with Coomassie Blue.

Purification of Exo1. The Exo1-His_6FLAG overexpressing strain RDKY7494 (RDKY1293 transformed with pRDK1575 *GAL1-10-EXO1-HIS_6FLAG, LEU2*) was grown overnight at 30 °C in 40 mL of −leucine minimal dropout medium supplemented with 2% (wt/vol) glucose. The expression of Exo1was induced by adding the starter culture to 2 L of –leucine minimal dropout medium supplemented with 2% (wt/vol) galactose and 0.1% glucose resulting in a starting $A_{600} = 0.3$. When an $A_{600} = 2.0$ was reached (~17 h of growth), the cells were harvested by centrifugation, yielding 10.8 g of cells. The cells were resuspended in Buffer A_{500} consisting of Buffer A [50 mM Tris, pH 7.5, 10% (vol/vol) glycerol, 2 mM β-mercaptoethanol, protease inhibitor mixture PIC D (final concentrations of 1 mM phenylmethanesylfonyl fluoride [PMSF], 1 μg/L chymostatin, and 1 μg/L pepstatin A), protease inhibitor mixture PIC W (final concentrations of 1 mM benzamadine, 0.5 μg/L bestatin, 1 μg/L aprotinin, and 1 μg/L leupeptin)] containing 500 mM NaCl and lysed by nine passes through a microfluidizer (Microfluidics). The lysate was then clarified by centrifugations at 16,000 RPM in a Sorval SA600 Rotor at 4 °C for 1 h. The supernatant was then loaded onto a 5-mL HISTrap column (GE Healthcare) that had been equilibrated with 15 mM imidazole in buffer A_{500}, and the column was washed with 25 mL of 15 mM imidazole in buffer A_{500} and then 35 mL of 30 mM imidazole in buffer A_{500}. Then, the proteins were eluted with a 50-mL gradient from 30 to 300 mM imidazole in buffer A_{500}. Peak fractions containing the Exo1 were pooled and diluted with Buffer A to reduce the salt concentration to 250 mM. The protein was then loaded onto a 3.5-mL Anti-FLAG M2 Affinity Gel (Sigma) column, which was then washed with 17 mL of Buffer A_{200} containing 1 mM ATP and 5 mM $MgCl_2$ to remove contaminating Hsp70 protein. The column was then washed with 35 mL of Buffer A_{200}, and then the bound proteins were eluted with 17.5 mL of 200 μg/mL FLAG peptide in Buffer A_{200}. The fractions containing Exo1 were pooled and stored as small aliquots at −80 °C. The entire purification procedure, starting with harvesting the cells, was completed in 16 h.

Purification of Mlh1–Pms1. *S. cerevisiae* Mlh1-Pms1 was overexpressed in *S. cerevisiae* and purified according to a previously published procedure using an overexpression strain consisting of RDKY1293 transformed with the plasmids pRDK573 *Gal1-10-MLH1 TRP1* and pRDK1099 *Gal1-10-PMS1-FLAG LEU2* (7, 8).

Purification of Msh2–Msh6. *S. cerevisiae* wild-type and Msh2–Msh6–Δ2–251 were overexpressed in *E. coli* BL21 (DE) RIL transformed with either pET11Msh2-Msh6 or a derivative of pET11Msh2-Msh6 containing a deletion of *MSH6* codons 2 through 251 called pRDK1145 and purified according to a previously published procedure (4, 9). The Msh2–Msh6–F337A protein was similarly

overexpressed using a derivative of pET11Msh2-Msh6 containing a mutation causing the Msh6-F337A amino acid substitution (pRDK1694; this study) and purified following this procedure with two modifications: (*i*) The mutant protein was purified from 5 L of culture; and (*ii*) the Msh2–Msh6 containing fractions from the SP Sepharose column were diluted with Buffer B [25 mM Tris, pH 8.0, 10% (vol/vol) glycerol, 5mM DTT, 1 mM EDTA, 0.02% Igepal (Nonidet P-40), protease inhibitor mixture PIC D, and PIC W] to a final salt concentration of 100 mM NaCl, loaded onto a 10-mL single-stranded DNA Cellulose column, and then the column was washed with 100 mL of Buffer B containing 100 mM NaCl and eluted with a 150-mL gradient from 100 to 600 mM NaCl.

Purification of Msh2–Msh3. *S. cerevisiae* Msh2–Msh3 was expressed in *S. cerevisiae* RDKY2418 transformed with the expression plasmids pRDK354 *GAL1-10-MSH2 URA3* and pRDK1596 *GAL1-10 MSH3-FLAG LEU2* and the growth protocol described under Purification of Mlh1–Pms1 (1). The Msh2–Msh3 was then purified by sequential chromatography on HiTrap Heparin, FLAG Antibody, and HiTrap Q columns essentially as described for the purification of Mlh1–Pms1.

Purification of PCNA. *S. cerevisiae* PCNA was overexpressed in *E. coli* OneShot BL21 (DE3) pLysS (Invitrogen) freshly transformed with the expression vector pT7/PCNA (10). Two liters of culture were grown in LB containing 100 μg/mL Ampicillin and 34 μg/mL Chloramphenicol at 37 °C until the cell density reached A_{600} = 0.4, and then expression of PCNA was induced with 1 isopropyl β-D-thiogalactopyranoside (IPTG) at a final concentration of 1 mM for 3 h. The cells were harvested, lysed, and ammonium sulfate and Polyethylenimine precipitated according to a previously published procedure (11). PCNA was purified according to a previously published procedure (10) except with the following three modifications: (*i*) The pooled fractions from the hydroxyapatite column was dialyzed into Buffer C [25 mM Tris, pH 7.5, 10% (vol/vol) Glycerol, 1 mM DTT, 0.5 mM EDTA, PIC D and PIC W] containing 200 mM NaCl; (*ii*) the dialysate was loaded onto a 1-mL HiTrap Q FF column (GE Healthcare), washed with 10 mL of Buffer C containing 300 mM NaCl, and eluted with 8 mL of gradient from 300 to 700 mM NaCl in Buffer C; and (*iii*) the Q column fractions were dialyzed into Buffer C containing 100 mM NaCl.

Purification of DNA Polymerase δ. *S. cerevisiae* DNA Polymerase δ was overexpressed in *S. cerevisiae* using the expression plasmids pBL335 and pBL341 (12), which were freshly transformed into RDKY1293. Three liters of cells were processed according to a previously published procedure (12), with the following two modifications: (*i*) Lysis was completed with 10 passes through a microfluidizer; and (*ii*) the fractions incubated with the PreScission protease were loaded onto a 1-mL HiTrap Capto SP ImpRes column (GE Healthcare) and washed with 10 mL of 150 NaCl, and the protein was eluted using a 15-mL gradient from 150 to 1,000 mM NaCl.

Purification of replication factor C. *S. cerevisiae* replication factor C (RFC) was overexpressed in *S. cerevisiae* BJ2168 containing the expression plasmid pBL420 (2). Three liters of cells were processed according to a previously published procedure (2), except for the following four modifications: (*i*) Cells were harvested after 17.5 h of induction with 2% (wt/vol) galactose; (*ii*) cells were lysed by seven passes through a microfluidizer; (*iii*) the flow rate for the 2.2-mL PCNA column was 0.5 mL/min; and (*iv*) a 1-mL HiTrap SP FF column (GE Healthcare) was used for the final column, and the RFC was eluted from the column with a 15-mL gradient from 100 to 500 mM NaCl.

Purification of RFC-Δ1N. *S. cerevisiae* RFC-Δ1N was overexpressed in *E. coli* BL21 Codon Plus (DE3) RIL that was freshly transformed with the expression plasmid pBL481 (13). Two liters of cells were grown in TB containing 50 μg/mL each of Ampicillin and Chloramphenicol at 37 °C until the cell density reached an A_{600} = 3.0, and then expression of RFC-Δ1N was induced with IPTG at a final concentration of 0.4 mM for 4.5 h. The RFC-Δ1N was then purified according to a previously published procedure (13), except with the following three modifications: (*i*) The cells were resuspended in HEG_{200} with 0.2 mg/mL Lysozyme for 30 min before sonication; (*ii*) the volume of the PCNA column was 2.2 mL; and (*iii*) the final column was a 1-mL HiTrap SP FF column (GE Healthcare).

Purification of replication protein A. *S. cerevisiae* replication protein A (RPA) was overexpressed in the *S. cerevisiae* strain RDKY7062 (RDKY1293 transformed with the pRDK273 *GAL1-10-RFA1 URA3*, pRDK274 *GAL1-10-RFA2 LEU2*, and pRDK275 *GAL1-10-RFA3 TRP1* expression plasmids) and purified from 3 L of culture according to a previously published procedure (3), except with the following four modifications: (*i*) Thirty microliters of cells were grown in –leucine –uracil –tryptophan minimal dropout media with 2% (wt/vol) glucose until the culture reached a cell density of A_{600} = 1.00, and then these cells were used to inoculate three 1-L cultures of –leucine –uracil –tryptophan minimal dropout medium containing 2% (wt/vol) galactose; (*ii*) the cells were lysed with seven passes through a microfluidizer; (*iii*) after the DNA Cellulose column, the protein was purified on a 1-mL HiTrap DEAE FF column (GE Healthcare) that was eluted with a 10-mL gradient between 100 and 1,000 mM NaCl; and (*iv*) Nonidet P-40 was omitted from all buffers.

Construction of DNA Substrates. DNA substrates were constructed following previously described methods using previously published mutant derivatives of pBluescript SK+, which are listed in Fig. 1 (9, 14, 15). The substrates were constructed by annealing the combinations of mutant single-stranded circular pBluescript SK+ DNAs and linearized, denatured mutant double-stranded pBluescript SK+ DNAs indicated in Fig. 1. Double-stranded pBluescript SK+ plasmid DNA was purified using a Plasmid Maxi Kit (Qiagen) from a 100-mL overnight culture of *E. coli* XL1-Blue MRF' or TOP 10F' containing a pBluescript SK+ mutant that was grown for 14 h in LB media containing 100 μg/mL Ampicillin. Single-stranded circular pBluescript SK+ DNA was prepared from *E. coli* XL1-Blue MRF' or TOP 10F' containing a pBluescript SK+ mutant using the helper phage rescue method described by the manufacturer (Stratagene). Fifty micrograms of double-stranded plasmid DNA was digested with 150 units of NaeI or AflIII in 140-μL reactions for 2 h at 37 °C. Heteroduplex substrates were constructed according to a previously published procedure (14), except with the following two modifications: (*i*) The denatured DNA was neutralized by adding unbuffered 2 M Tris·HCL to a concentration of 400 mM; and (*ii*) the DNA mixtures were annealed for 2 h at 65 °C. Then, the heteroduplex DNA was purified as follows. The heteroduplex substrate DNA mixture was digested with 660 units of ExoV (RecBCD) in a 1.8-mL reaction for 1 h according to the manufacturer's instructions (NEB) to degrade contaminating single-stranded circular and double-stranded linear DNA. The DNA was then bound to an 0.8-mL BND Cellulose column (Sigma) that was then washed with 4 mL of 300 mM NaCl in 10 mM Tris, pH 8.0, and 1 mM EDTA (TE Buffer), and the bound DNA was eluted with 4 mL of 800 mM NaCl in TE Buffer. The eluted DNA was then concentrated by ethanol precipitation and stored at −20 °C in TE Buffer.

Electron Microscopy. Excision products for analysis by electron microscopy were prepared as described above except for the following three modifications: (*i*) The glycogen was omitted from the phenol extraction and ethanol precipitation step; (*ii*) four 20-μL reactions were pooled together before the phenol and ethanol precipitation step; and (*iii*) the excision products were resuspended in the 10-mM Tris elution buffer provided with the Qiagen DNA extraction kit. The DNA samples were incubated with 1 μg of *E. coli* SSB (USB) in a 20 mM Hepes, pH 7.5, 100 mM

NaCl buffer for 15 min at room temperature, followed by fixation with 0.3% glutaraldehyde for 5 min, at room temperature. The samples were then passed over a 2-mL column of 6% agarose beads (ABT) previously equilibrated with TE buffer, pH 7.4, to eliminate unwanted salts and unbound proteins, and fractions enriched for DNA–protein complexes were collected. Aliquots were mixed with a buffer containing spermidine and adsorbed onto copper grids coated with a thin carbon film glow-charged shortly before sample application. After adsorption of the samples for 2–3 min, the grids were washed with double distilled water and dehydrated through a graded ethanol series from 25% to 95%. Following air-drying, the grids were rotary shadowcast with tungsten at 10^{-7} torr. Samples were examined in an FEI T12 transmission electron microscope equipped with a Gatan 2k × 2k SC200 CCD camera at 40 kV. Adobe Photoshop software was used to arrange images into panels for publication.

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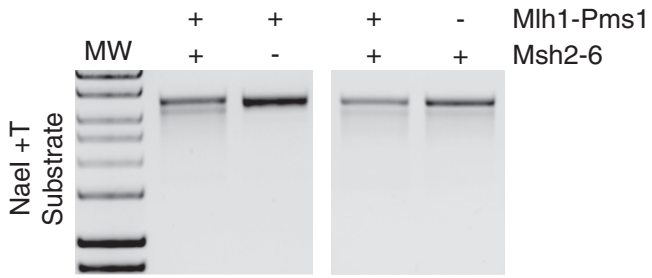

Fig. S1. Excision on the +1 (+T) substrate containing a 5′ nick at the NaeI site is not effected by the presence or absence of Mlh1–Pms1. Excision reactions were performed with the +T substrate containing a 5′ nick at the NaeI site in reactions containing Exo1, PCNA, RFC-Δ1N, RPA without DNA polymerase δ, and either with or without Msh2–Msh6 or with or without Mlh1–Pms1 as indicated for 3 h. The reaction products were analyzed by agarose gel electrophoresis without prior digestion with PstI. MW indicates size standards. The reactions with or without Msh2–Msh6 on the left are from Fig. 5*C* and are repeated here for comparison purposes.