Supporting Information

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SI Text

SI Materials and Methods. ***Protein and DNA preparations.*** Protein preparations used in this study are shown in Fig. S1, and unless specified otherwise purity in each case exceeded 95%. Recombinant human MutSα and MutLα, were isolated from baculovirus-infected Sf9 cells according to Blackwell et al. (1). Replication factor C (RFC) was isolated from HeLa cell extracts (purity >90%) as described (2). *Saccharomyces cerevisiae* RFCΔN (yRFCΔN), lacking the N-terminal ligase homology domain (residues 2–273) of the large subunit was expressed in *Escherichia coli* and isolated as described (2, 3). Proliferating cell nuclear antigen (PCNA) was prepared from *E. coli* harboring plasmid pT7/hPCNA and purified by published methods (2, 4, 5).

PCNA with a N-terminal protein kinase recognition motif and a His_6-tag (PK–PCNA) was overexpressed in *E. coli* from vector pHKEp (6), kindly provided by Mike O'Donnell (Rockefeller University). PK-PCNA was purified using Ni-NTA and Mono Q chromatography. Briefly, BL21(DE3) LysS cells were transformed with the pHKEp vector, and cell paste (30 g) obtained from an IPTG-induced culture. The cell pellet was resuspended and sonicated in 20 mM Tris-HCl, pH 7.6, 0.5 M NaCl, 5 mM imidazole. Soluble material was loaded onto a 15 mL Ni-NTA column, washed with Tris-HCl, pH 7.6, 0.5 M NaCl, 50 mM imidazole, and bound protein then eluted with 0.5 M imidazole in this buffer. PK-PCNA (~90% pure) was further purified by chromatography on a 1 mL MonoQ column (GE Healthcare), which was eluted with a 100 to 700 mM NaCl gradient in 20 mM Tris-Cl, pH 7.6, 0.5 mM EDTA. PK–PCNA eluted at 450 mM NaCl.

A synthetic peptide (KRRQTSMTDFYHSKRRLIFS) containing the p21 C-terminal PCNA interaction element (7) was purchased from AnaSpec. A scrambled sequence peptide (YDRSKLRTQSHRSFKTIMRF) was employed as control.

The circular 3′ G-T heteroduplex contained a strand break 128 bp (8) 3′ to the mismatch (as viewed along the shorter path). The corresponding control was an otherwise identical A–T homoduplex. Supercoiled closed circular G–T heteroduplex and control A–T homoduplexes were prepared from f1MR65 and f1MR66 (9) by ligation in the presence of ethidium bromide (1 molecule/6.4 bp) to result in a negative superhelical density (σ) of approximately −0.12, approximately twice that of plasmid DNA isolated from a 37 °C *E. coli* culture (10). A $d(T)_{10}$ sequence was introduced into the viral strand of bacteriophage f1MR1 (11) between positions 5501 and 5502 to yield f1MR74; $d(A)_{10}$ was introduced at the same location in the viral strand of f1MR3 and f1MR1 (11) to yield f1MR75 and f1MR76, respectively. A 5′ G-T heteroduplex containing a strand break 128 bp 5′ to the mismatch and a $d(T)_{10}$ sequence on both strands was prepared from f1MR74 and f1MR75 using methods described previously (12). A corresponding control 5′ A–T homoduplex was prepared from f1MR74 and f1MR76. Relaxed closed circular heteroduplex DNAs were prepared from nicked substrates by ligation at 37 °C with T4 DNA ligase (New England Biolabs, 4 cohesive end units/10 μL reaction) in 20 mM Tris-HCl, pH 7.6, 1.5 mM ATP, 5 mM $MgCl_2$, 125 mM NaCl, 0.05 mg/mL BSA, 1 mM glutathione. 200 (13) or 202 (1) bp linear homoduplex DNAs were synthesized by PCR using f1MR23 or f1MR1 as described, and purified according to Blackwell et al. (1)

Western blot analysis. Rabbit anti-yRFCΔN antibodies were raised against the native protein and purified on protein A-Sepharose. Samples containing PCNA or yRFCΔN were separated by electrophoresis in the presence of SDS on a 4–20% Criterion gel (BioRad) and transferred onto a PVDF membrane. The membrane was blocked for 1 hr at room temperature with 0.01 M Tris-HCl, pH 8.0, 0.15 M NaCl, 1 mM EDTA, 0.1% Triton X-100 containing 5% milk solids (all washes were performed in this buffer). After incubation with anti-yRFCΔN for 1 hr at room temperature, the membrane was washed, and then incubated for 1 hr with peroxidase conjugated goat anti-rabbit antibodies (Amersham). Data were visualized using the ECL + system (Amersham). The membrane was then blocked for 1 hr as described above, incubated with rabbit anti-PCNA (Abcam), and processed as described above.

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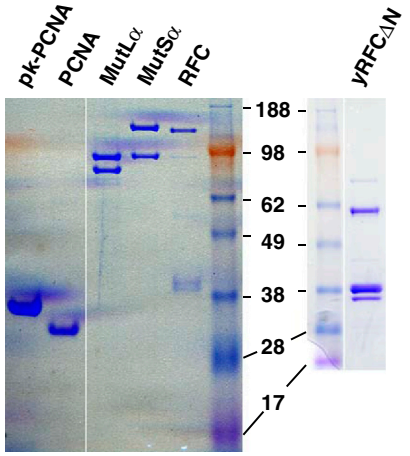

Fig. S1. Protein preparations used in the study. Proteins were isolated as described in *Materials and Methods*. Approximately 0.5–1 μg of each preparation was analyzed on a 10% SDS polyacrylamide gel, which was stained with Coomassie Blue.

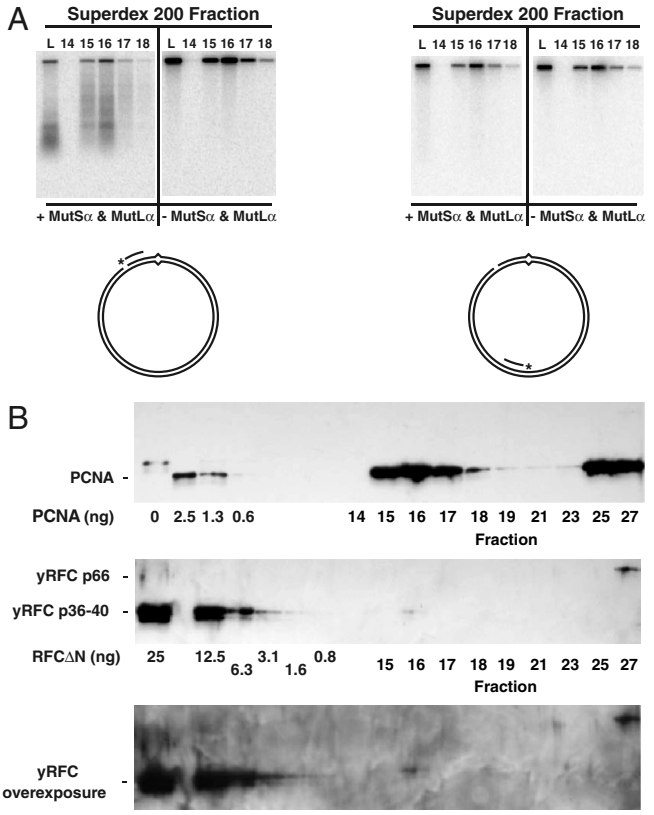

Fig. S2. Heteroduplex-loaded PCNA and MutSα are sufficient to activate MutLα endonuclease. (*A*) Ten μL of the indicated Superdex 200 fractions (Fig. 1; *L* corresponds to column load) were assayed for endonuclease activity in 20 μL reactions under mismatch-dependent conditions (*Materials and Methods*) in the absence or presence of 390 fmol MutSα and 460 fmol MutLα. Endonuclease products were scored by alkaline gel electrophoresis and hybridization using ^{32}P-labeled oligonucleotide probes complementary to nicked (*Left*) or continuous (*Right*) heteroduplex strands. Phosphorimager quantification of the results from the left panel is shown in Fig. 1*B*. (*B*) Standards containing known amounts of PCNA and yRFCΔN (*Left*) as well as 20 μL samples of selected Superdex 200 fractions (*Right*, see Fig. 1) were analyzed by Western blot (*Materials and Methods*). The lower photo shows overexposure of the yRFC portion of the transfer. These results indicate that residual yRFCΔN in the 10 μL samples of column fractions 15–18 used for endonuclease determination ranged from less than 0.4 ng to 0.9 ng (Fig. 1*C*, arrow bracket). As can be seen, p36–40 subunits of yRFCΔN were not detected in the included fractions such as fraction 27, which is shown. The reason for this is unclear, but may indicate instability of yRFCΔN to dissociation under column conditions.

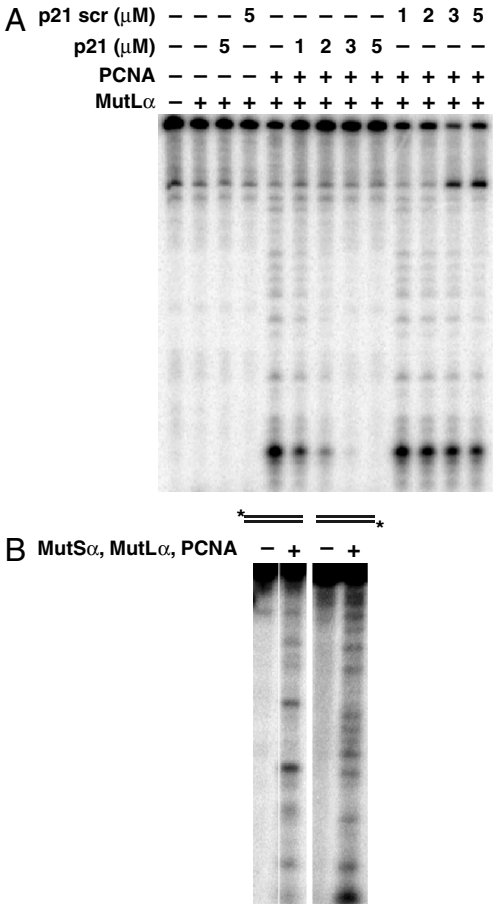

Fig. S3. (*A*) PCNA activation of MutLα in the presence of Mn^{2+} is inhibited by p21 peptide. Reactions (*Materials and Methods*) contained 30 mM KCl, 0.5 mM ATP, 1 mM $MnSO_4$, 200 bp homoduplex DNA (5′-^{32}P labeled on one strand), MutLα, and PCNA as specified. Reactions were supplemented as indicated with a peptide containing the PCNA interaction motif of p21 (p21) or a scrambled sequence peptide (p21 scr). (*B*) Distribution of incision sites on the two strands of linear homoduplex DNA. Reactions (*Materials and Methods*) containing proteins as indicated were performed as in Fig. 2*B* using 202 bp linear homoduplex in the presence of 60 mM KCl and 5 mM $MgCl_2$. Asterisks indicate location of 5′-^{32}P label.

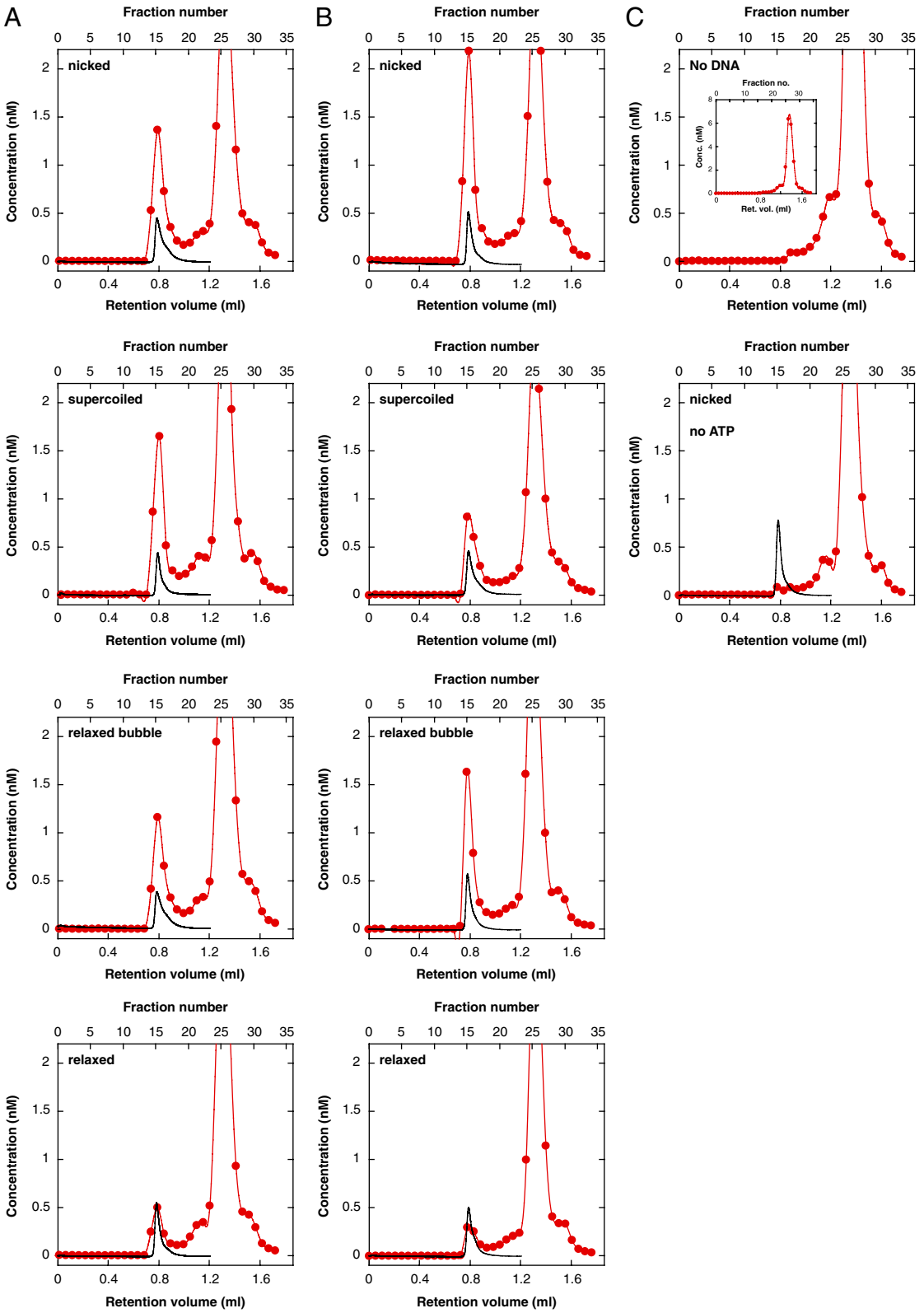

Fig. S4. Gel filtration assay of PCNA loading. [^{32}P]PK-PCNA was loaded onto 3′ nicked, supercoiled, relaxed-bubble, or relaxed G–T heteroduplexes as indicated using yRFCΔN (*A*) or native human RFC (*B*), and clamp–DNA complexes scored by gel filtration on Superdex 200 (*Materials and Methods*). Panel *C* illustrates control experiments in which [^{32}P]PK–PCNA loading reactions in the presence of yRFCΔN were performed in the absence of DNA (*Upper*) or in the presence of nicked heteroduplex DNA but in the absence of ATP.

		Endonucleolytic incision (fmol/10 min)				
Homoduplex	Strand	PCNA MutLα	PCNA yRFCΔN MutLα	PCNA MutLα MutSα	yRFCΔN MutLα MutSα	PCNA yRFCΔN MutLα MutSα
 relaxed-bubble	C (closed)	< 0.2	< 0.2	< 0.2	< 0.2	6.6
	V (closed)	0.4	0.3	0.3	0.5	6.7

Fig. S5. MutLα incision of bubble-containing relaxed DNA is MutSα-dependent. Bubble-containing A–T DNA was incubated with proteins as indicated (*Materials and Methods*) and incision of viral (V) and complementary (C) DNA strands quantified as desribed in Fig. 3. Note incision dependence on MutSα (compare columns 4 and 7).