Supporting Information

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SI Materials and Methods

Stability Assays. Purification of heterotrimers was performed as described in *Materials and Methods*, except that maltose binding protein (MBP)-tag cleavage by tobacco etch virus (TEV) protease was not performed. Samples of both heterotrimeric species were either flash frozen in liquid nitrogen and stored at −80 °C immediately after purification or incubated at various temperatures for various lengths of time (*Results*). Following incubation at different conditions, samples were examined by analytical gel filtration on a Superdex 200 10/300 GL column to determine the extent of heterotrimer reassembly.

Purification of PCNA Homotrimers and Other Proteins Required for Activity Assays. Wild-type (WT) proliferating cell nuclear antigen (PCNA), pcna-79, pcna-90, and pcna-7990 homotrimers were purified similarly to the heterotrimers, but with a single affinity chromatography step. Homotrimeric wild-type PCNA was overexpressed from a pETDuet-1 vector that contained only His-tagged wild-type PCNA in the first cloning site, and purified over a nickel- nitrilotriacetic acid (Ni-NTA) column followed by gel filtration on a Superdex 200 16/60 column. Homotrimeric pcna-79, pcna-90, and pcna-7990 were overexpressed from pETDuet-1 vectors that contained only MBP-tagged mutant PCNA in the second cloning site and purified over an amylose column followed by gel filtration on a Superdex 200 16/60 column. Fractions were pooled and concentrated, TEV protease was added and incubated as described in *Materials and Methods*, and a second gel filtration step was performed to isolate pcna-79, pcna-90, or pcna-7990 trimers with no MBP tags. *Saccharomyces cerevisiae* DNA polymerase δ (Pol δ), flap endonuclease 1 (FEN1), DNA ligase I (Lig1), replication factor C (RFC) and replication protein A (RPA) were purified as described previously (1–4).

RFC ATPase Assays. A primer-template construct was created for PCNA loading, by annealing oligo C13 (GGTAACGCCAGGGTTTTCCCAGTC) to oligo O1 (GCATCGCTCACTGGCCGTCGTTTTACAACGTCGTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTGACTGGGAAAACCCTGGCGTTACCTTTTTTTTTT). The ends of the oligo were not blocked to allow PCNA to fall off and be reloaded. Standard 30-μL assays contained 20 mM Tris·HCl pH = 7.8, 1 mM DTT, 100 μg/mL BSA, 8.3 mM $MgAc_2$, 20 μM ATP, 33 nM [γ-^{32}P]ATP (New England Nuclear) (3,000 Ci/mmol), 75 mM NaCl, 400 fmol of primer-template construct, 200 fmol of RFC, and 1.6 pmol of PCNA trimers.

Assays were incubated at 30 °C for the indicated times. The 7-μL aliquots were removed and placed in precooled 43 μL stop solution: 1 M perchloric acid, 1 mM sodium phosphate. A total of 10 μL was removed to quantify total activity. To the remaining 40 μL, 95 μL of precooled 20 mM ammonium molybdate was immediately added and the tube kept on ice. A total of 140 μL water-saturated isopropyl acetate was added, the tube was mixed vigorously, and then centrifuged for 1 min at 1,000 × *g* at 4 °C. A total of 20 μL was removed from the organic phase to quantify hydrolyzed γ-phosphate. Quantification was performed by spotting samples on filter paper, exposing to storage phosphor overnight, and analyzing on a phosphoimager.

Cross-Linking Assays. For cross-linking, PCNA trimers were purified to homogeneity in amine-free buffer. A total of 15 μL of PCNA at 5 μM was incubated on ice for 2 h with 0.5 μL of disuccinimidyl suberate (DSS) at 1.56 mM (PCNA:DSS ratio of 1:10). Cross-linking was stopped by the addition of 1 μL of 1 M Tris pH = 7.5. Products were separated on SDS/PAGE.

For cross-linking after loading by RFC, standard assays were similar to ATPase assays detailed above, except for the following reagents: 0.8 mM ATP, 15 pmol of primer-template construct, 7.5 pmol of RFC, and 75 pmol of PCNA. Loading was stopped by adding 1 μL of 0.5 M EDTA to 9-μL aliquots. DSS was added at 1:10 ratio as above.

FEN1 Flap Cleavage Assays. Oligo C115 (TTTTTTTTTTTTTTTACGACGTTGTAAAACGACGGCCAGTGAGCG) was radioactively labeled at the 5′ end using T4 Polynucleotide Kinase (Fermentas) in the presence of [γ-^{32}P]ATP (NEN), and purified on a Micro Bio-Spin 30 gel filtration column (Bio-Rad). The labeled oligo, along with oligo C13, were annealed to template oligo (ACGCGCGCTCACTGGCCGTCGTTTTACAACGTCGTGACTGGGAAAACCCTGGCGTTACCCAACT), which is biotinylated at both 5′ and 3′ ends. Streptavidin was added in excess to block the ends of the substrate, so that PCNA will not slide off after being loaded by RFC (1). Standard 40-μL assays contained 20 mM Tris·HCl pH = 7.8, 1 mM DTT, 100 μg/mL BSA, 8.3 mM $MgAc_2$, 0.5 mM ATP, 150 mM NaCl, 100 fmol of oligonucleotide substrate, 200 fmol of RFC, 400 fmol of PCNA trimers, and 200 fmol of FEN1. The substrate was preincubated with PCNA and RFC for 2 min at 15 °C for PCNA loading. FEN1 was added, and the reactions were incubated at 15 °C for the indicated times. Aliquots were quenched at each time point by mixing with formamide loading buffer [95% (vol/vol) formamide, 20 mM EDTA, 0.05% xylene cyanol, 0.05% bromophenol blue] at a 1:1 volume ratio. Samples were heated to 95 °C for 5 min before gel electrophoresis. Products were analyzed by electrophoresis on a 7 M urea, 14% (vol/vol) polyacrylamide gel. The gels were dried, exposed to a storage phosphor screen (GE Healthcare), and subjected to PhosphoImager (FujiFilm) analysis. Results were quantified using ImageJ software (National Institutes of Health).

For FEN1 assays with double-flap substrate, conditions were similar except oligo C13T was used instead of C13. This oligo has an additional T nucleotide at its 3′ end, creating a substrate with a 15-nt 5′ flap and a 1-nt 3′ flap.

Nick Translation Assays. Oligonucleotide C13 was radioactively labeled at the 5′ end using T4 Polynucleotide Kinase (Fermentas) in the presence of [γ-^{32}P]ATP (NEN), and purified on a Micro Bio-Spin 30 gel filtration column (Bio-Rad). The labeled oligo, along with blocking oligo SKrc14, were annealed to template oligo O1, which is biotinylated at both 5′ and 3′ ends. Streptavidin was added in excess to block the ends of the substrate, so that PCNA will not slide off after being loaded by RFC. Standard 30-μL assays contained 20 mM Tris·HCl pH = 7.8, 1 mM DTT, 100 μg/mL BSA, 100 mM NaCl, 8.3 mM $MgAc_2$, 0.5 mM ATP, 0.1 mM of each dNTP, 100 fmol of oligonucleotide substrate, 100 fmol of RFC, 200 fmol of PCNA trimers, 200 fmol of Pol δ, and with or without 200 fmol of FEN1. The substrate was preincubated with PCNA and RFC for 1 min at 30 °C for PCNA loading. Reactions were started by adding Pol δ with or without FEN1 in a mix, and incubated at 30 °C for the indicated times. Aliquots were quenched at each time point by mixing with formamide loading buffer as described for FEN1 assays. Samples were heated to 95 °C for 5 min before gel electrophoresis. Products were analyzed by electrophoresis on a 7 M urea, 12% (wt/vol) polyacrylamide gel. The gel was dried, exposed to a storage phosphor screen, and analyzed as described for FEN1 assays.

Processivity Assays. Oligonucleotide C13 was radioactively labeled at the 5′ end as described above and annealed to single-stranded

Bluescript SKII(+) DNA. The 30-μL assays contained 20 mM Tris·HCl pH = 7.8, 1 mM DTT, 100 μg/mL BSA, 8.3 mM $MgAc_2$, 1 mM ATP, 0.1 mM of each dNTP, 100 mM NaCl, 200 fmol of template plasmid, 40 pmol of RPA, 200 fmol of RFC, 400 fmol of PCNA trimers, and 5 fmol of Pol δ (polymerase to PCNA-loaded primer-template ratio of 1:40, to achieve single-hit criteria). The template plasmid was preincubated with RPA, PCNA, and RFC for 1 min at 30 °C for RPA coating and PCNA loading. Pol δ was then added, and the reactions were incubated at 30 °C for the indicated times. Products were analyzed by electrophoresis on a 2% (wt/vol) denaturing alkaline agarose gel, in the presence of 0.5 μg/mL ethidium bromide. A 100-bp ladder was also run on the gel, and photographed under UV illumination. The gel was dried, exposed to a storage phosphor screen and analyzed on a PhosphorImager. Finally, the UV picture and the autoradiograph were overlaid to position the ladder onto the autoradiograph and assess the sizes of the radioactive products.

1. Ayyagari R, Gomes XV, Gordenin DA, Burgers PMJ (2003) Okazaki fragment maturation in yeast. I. Distribution of functions between FEN1 AND DNA2. *J Biol Chem* 278(3):1618–1625.
2. Harrington JJ, Lieber MR (1994) Functional domains within FEN-1 and RAD2 define a family of structure-specific endonucleases: Implications for nucleotide excision repair. *Genes Dev* 8(11):1344–1355.
3. Gomes XV, Gary SL, Burgers PM (2000) Overproduction in Escherichia coli and characterization of yeast replication factor C lacking the ligase homology domain. *J Biol Chem* 275(19):14541–14549.
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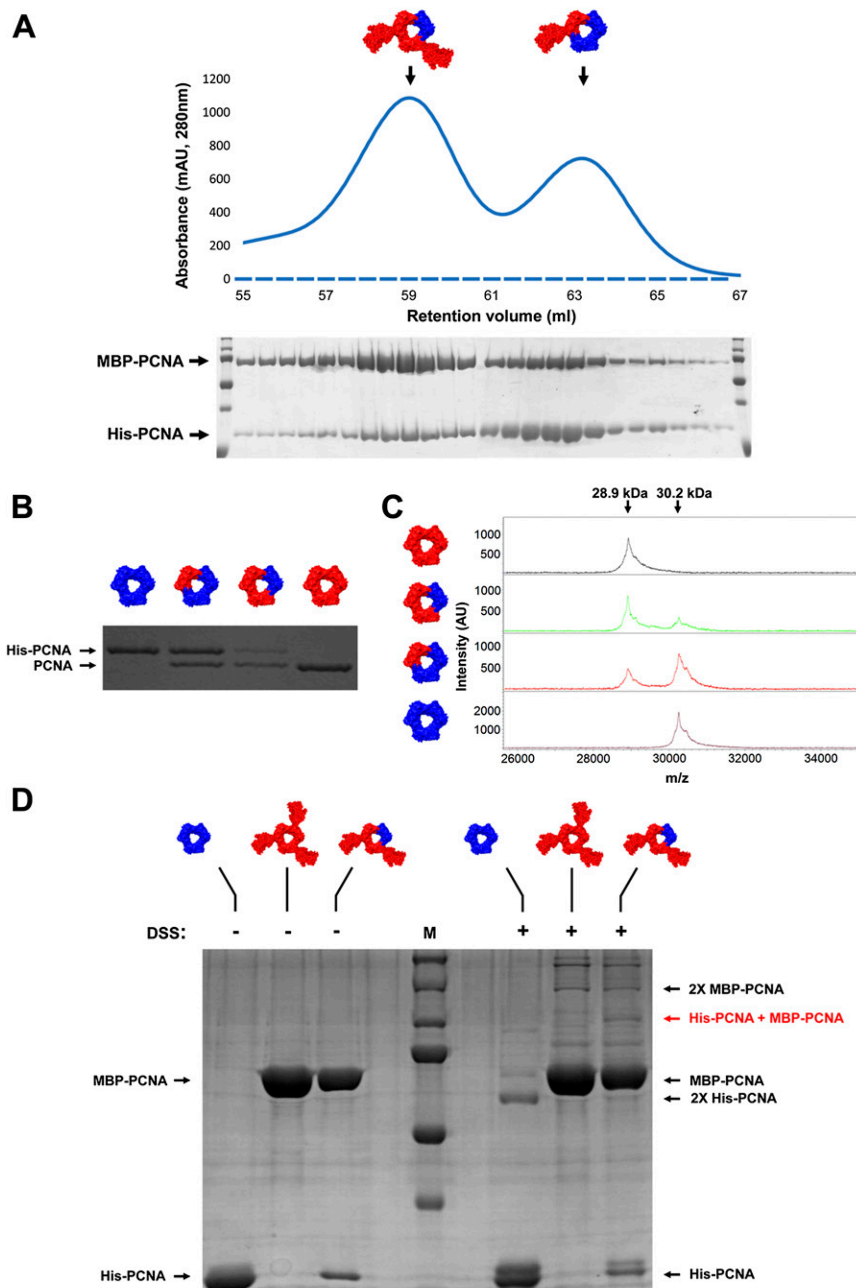

Fig. S1. Purification and validation of PCNA heterotrimers. (*A*) Separation of two heterotrimeric PCNA species, obtained by tandem Ni-NTA and amylose affinity chromatography, by gel filtration chromatography on a Superdex 200 column. (*Upper*) Chromatogram depicting the separation, including schematic images of the trimer species corresponding to each peak. The dashed blue line below the chromatogram indicates the fractions collected from this run. (*Lower*) SDS/PAGE analysis of the collected fractions. (*B*) SDS/PAGE analysis of purified PCNA trimers after MBP-tag cleavage. (*C*) MALDI-TOF mass spectrometry reveals two peaks at the expected molecular weights for PCNA (after MBP-tag cleavage) and His-tagged PCNA. The heterotrimer samples contain peaks of both sizes at approximately the expected ratios. (*D*) Cross-linking of neighboring monomers in PCNA trimers. WT PCNA, pcna-7990 and the WT_1:7990_2 heterotrimer (before MBP-tag cleavage) were cross-linked with disuccinimidyl suberate (DSS) where indicated. A product of ~100 kDa, corresponding to cross-linking between His-tagged PCNA and MBP-tagged PCNA, is visible only in the presence of the heterotrimeric PCNA. M, size marker.

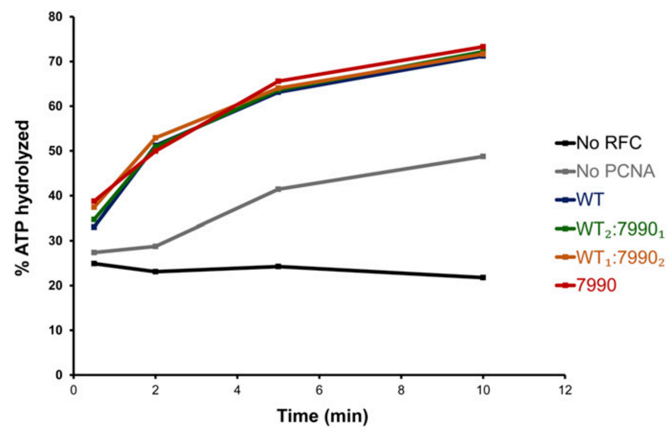

Fig. S2. PCNA-dependent ATPase activity of RFC. RFC was incubated with an unblocked oligonucleotide-based primer-template construct, [γ-^{32}P]ATP and various PCNA trimers (*SI Materials and Methods*). Stimulation of RFC ATPase activity in the presence of PCNA and DNA indicates successful loading. Representative results are shown.

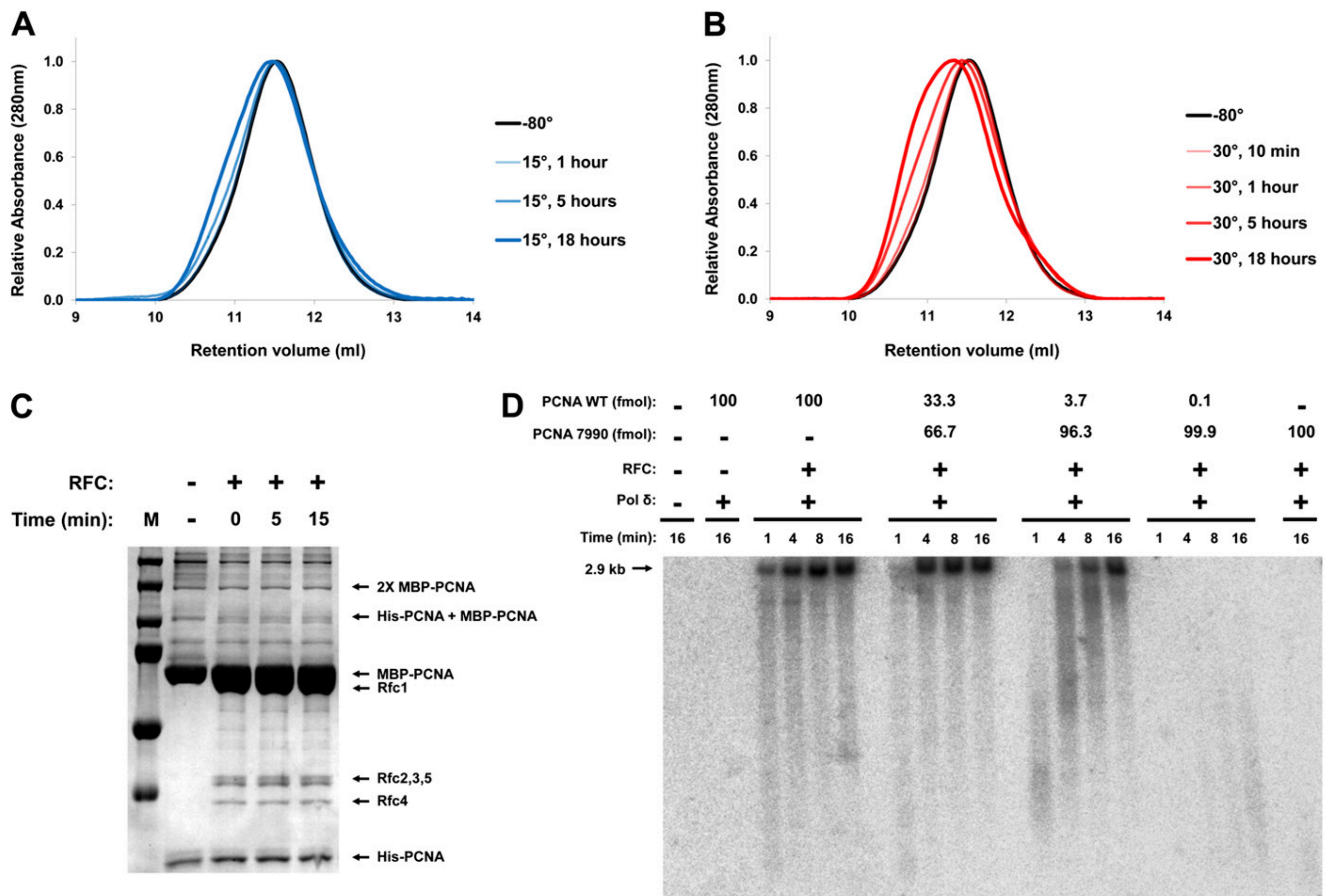

Fig. S3. Stability of PCNA heterotrimers. (*A* and *B*) The WT_1:7990_2 heterotrimer was purified as described but without MBP-tag cleavage to allow detection of trimer reassembly. After incubation for various lengths of time (as indicated) at 15 °C (*A*) or 30 °C (*B*), the samples were analyzed by gel filtration on a Superdex 200 column. As the initially homogenous population of heterotrimers with two MBP tags undergoes reassembly and gains increasing amounts of trimers with zero, one, or three MBP tags, the chromatogram gains additional peaks on both sides of the central peak and broadens. The maximal peak height has been normalized in all chromatograms, so that peak width represents trimer reassembly. Note that such peak broadening represents only minor reassembly—significant reassembly would lead to the appearance of new peaks at significant distance from the central peak. (*C*) Cross-linking of PCNA heterotrimers after loading by RFC. WT_1:7990_2 heterotrimer was purified as described but the MBP tags were not cleaved. We separately verified that the presence of MBP tags does not interfere with loading by RFC using an RFC ATPase assay. The heterotrimer was incubated with RFC, an appropriate DNA construct, and ATP, in conditions similar to RFC ATPase assays described above (Fig. S2). The loading reaction was allowed to proceed for the indicated times before it was stopped and disuccinimidyl suberate (DSS) was added. Changes in the pattern of cross-linking products over time may indicate reassembly of the heterotrimer, but no significant change is visible. M, size marker. (*D*) Functional examination of PCNA stability using Pol δ assay. Assay was performed as described in Fig. 3*A*, but mixtures of WT and pcna-7990 homotrimers were used in different ratios as indicated. Products were analyzed by agarose gel electrophoresis and autoradiography. The arrow denotes the fully replicated, 2.9-kb plasmid. Combinatorially, 3.7% is the maximum expected amount of WT homotrimers in case of full reassembly of the WT_1:7990_2 trimer.

Fig. S4. Stimulation of Pol δ and full Okazaki fragment maturation, by heterotrimers containing various PCNA mutants. (*A*) Stimulation of Pol δ by WT PCNA, pcna-7990, and WT:7990 heterotrimers. The assay is described in Fig. 3*A*. Products were analyzed by agarose gel electrophoresis and autoradiography; the arrow denotes the fully replicated 2.9-kb product, which was quantified and averaged with three additional independent assays to produce Fig. 3*B*. (*B*) Stimulation of Okazaki fragment maturation by WT PCNA, pcna-7990, and WT:7990 heterotrimers. The assay is described in Fig. 5*A*. Products were analyzed by agarose gel electrophoresis and autoradiography; the arrows denote the fully replicated nicked plasmid and the covalently closed plasmid. The latter product was quantified and averaged with two additional independent assays to produce Fig. 5*B*.

Fig. S5. Pol δ and FEN1 activity in different conditions and with various PCNA mutants. (*A*) Stimulation of Pol δ by WT PCNA, pcna-79, and WT:79 heterotrimers. The assay is identical to that described in Fig. 3*A*. Products were analyzed by agarose gel electrophoresis and autoradiography; the arrow denotes the fully replicated 2.9-kb product. (*B*) Effects of different PCNA mutants on FEN1 activity. Assays were performed as described in Fig. 3*C*. Flap cleavage activity was examined in the absence of PCNA or in the presence of 10 different trimer species, including all combinations of WT PCNA with pcna-79, pcna-90, or pcna-7990. Reactions were analyzed by urea-PAGE and autoradiography and the percentage of substrate cleaved by FEN1 was quantified. (*C*) FEN1 assay was performed as described in Fig. 3*C*, but with 150 mM KCl instead of NaCl. (*D*) FEN1 assay was performed as described in Fig. 3*C*, but with a double-flap substrate instead of a single, 5′ flap substrate. This substrate is identical to that used in all other FEN1 assays, except for a single unhybridized nucleotide at the 3′ end of the upstream oligo (*SI Materials and Methods*).

Fig. S6. Nick translation by Pol δ and FEN1 in the absence of PCNA or in the presence of 10 different trimer species, including all combinations of WT PCNA with pcna-79, pcna-90, or pcna-7990. Assay was performed as described in Fig. 4*A*. Reactions were analyzed by urea-PAGE and autoradiography.

Fig. S7. Okazaki fragment maturation in the presence of high concentrations of PCNA. Assay was performed as described in Fig. 5*A*, but with 1,000 or 2,000 fmol of PCNA trimers, whereas 200 fmol of Pol δ, FEN1, and Lig1 were added where indicated.

PCNA: - WT WT WT_2:7990_1 WT_1:7990_2 7990

RFC: - - + + + +

Pol δ: - + + + + +

Time (min): 5 5 0.5 1 2 5 0.5 1 2 5 0.5 1 2 5 0.5 1 2 5

1500 nt
1200 nt
1000 nt
900 nt
800 nt
700 nt
600 nt
500 nt
400 nt
300 nt
200 nt
100 nt

Fig. S8. Pol δ processivity in the presence of different PCNA trimers. To measure true polymerase processivity, Pol δ was diluted to a concentration where single-hit criteria are met (*SI Materials and Methods* for details). Products were analyzed by alkaline agarose gel electrophoresis and autoradiography.