Supplementary information

Experimental Procedures

Adenovirus

Adenovirus construction and infections were performed as described previously (Bi *et al.*, 2005). Essentially cDNAs encoding GFP-Polκ(WT) and GFP-Polκ(FF567-568AA) were subcloned into pAC-CMV. The resulting shuttle vectors were co-transfected into 293T cells with the pJM17 plasmid to generate recombinant adenovirus as described previously (Bi *et al.*, 2005). H1299 cells were routinely infected at ~50% confluence with 2 x 10^9 pfu/ml of adenovirus.

Co-immunoprecipitation of EGFP-REV1 and FLAG-Polκ

Human 293T cells were transfected with pEGFP-hREV1 and pcDNA3.1(+)-FLAG-Polκ, using Lipofectamine 2000 according to manufacture's protocol. The cells were harvested 48 hr after transfection and disrupted in cell lysis buffer (20 mM Hepes, pH 7.6, 150 mM NaCl, 1 mM EDTA, 10% glycerol, 1mM dithiothreitol, 0.1% Tween-20, 1 mM PMSF, 10 μg/ml leupeptin, 5 μg/ml pepstatin A) by three freeze-and-thaw cycles. The cell lysates were centrifuged at 15,000 *g* for 10 min and incubated with protein G-Sepharose beads (Sigma) for 30 min at 4°C to eliminate the non-specific binding of proteins to the beads. After a brief centrifugation (500 g for 30 sec), the supernatants were incubated with anti-REV1 antibody for 10 hr. The antigen-antibody complex was immobilized on protein G Sepharose beads and the beads were washed five times in lysis buffer. The bound proteins were eluted by boiling in 1x sample buffer and subjected to SDS-PAGE and Western blotting with anti-FLAG or anti-REV1 antibody.

Co-immunoprecipitation of PCNA and GFP-Polκ

H1299 cells were plated in 10 cm culture dishes and infected with adenovirus as described above. After genotoxin treatment, the cells were washed twice with PBS (10 ml/wash) then lysed by addition of 1 ml ice-cold CSK buffer to the monolayer. The cells were scraped from the plate and transferred to microcentrifuge tubes. Cell lysates were centrifuged for 10 seconds at 10,000 g and the supernatants (containing cytosolic

and nucleosolic proteins) were removed and frozen at -80°C. The chromatin-containing pellets were washed in 1 ml CSK then fixed by re-suspension in 1 ml PBS containing 1% formaldehyde. After 10 minutes, the formaldehyde fixation was terminated by the addition of 1 M glycine to give a final concentration of 0.1 M. The fixed chromatin fractions were washed twice in 1 ml of PBS, resuspended in 0.5 ml CSK, and sonicated using three 10 second pulses at 30% maximum output. Pulses of sonication were separated by a 10 second intervals on ice to prevent excessive heating. The sonicated chromatin samples were clarified by centrifugation at 10,000 g for 5 minutes. Supernatants containing sheared chromatin were removed and normalized for protein concentration and analyzed directly by SDS-PAGE and immunoblotting (for 'input' proteins). Alternatively, GFP-Polκ was immunoprecipitated overnight at 4°C using 2 mg of a rabbit polyclonal antibody against GFP (Invitrogen) or non-specific rabbit IgG (Santa Cruz) as a negative control. To recover immune complexes, 25 µl Protein A/G beads were added to each sample for 4 hrs. The beads were collected by brief centrifugation and washed 3 times with 1 ml of 20 mM Tris (pH 7.5, 0.1 M NaCl, 0.1 mM EDTA, and 0.05% Tween-20 (15 minutes per wash). The washed immune complexes were boiled in protein loading buffer for 25 minutes to reverse the formaldehyde cross-links prior to separation on SDS-PAGE.

Fluorescence microscopy

H1299 cells were plated in 4-well chamber slides, grown to 30% confluence, and then infected with GFP-Polκ adenovirus vectors. To visualize GFP-Polκ fluorescence, the cells were fixed with 4% paraformaldehyde for 10 minutes, washed with PBS, then DAPI-stained and mounted with Vectashield solution (Vector Laboratories). Slides were imaged and analyzed using a Delta Vision Image Restoration Microscopy System (dv1301421, Applied Precision).

Figure Legends

Supplementary Fig. 1 Yeast two-hybrid analyses. *A*, Effect of Polη FF707-708AA substitution on REV1-binding. *B*, REV1-interacting regions in Polι and Polη.

Supplementary Fig. 2 Effect of FF567-568AA substitutions on the REV1-binding activity of Polκ. EGFP-hREV1 and FLAG-Polκ(WT) or FLAG-Polκ(FF567-568AA) were transiently overproduced in 293T cells, immunoprecipitated with anti-REV1 antibody, and immunoblotted with anti-REV1 antibody or anti-FLAG antibody. I, input fractions; P, pellet fractions. A five-excess of bound fraction was applied to the gel, compared to the input fraction.

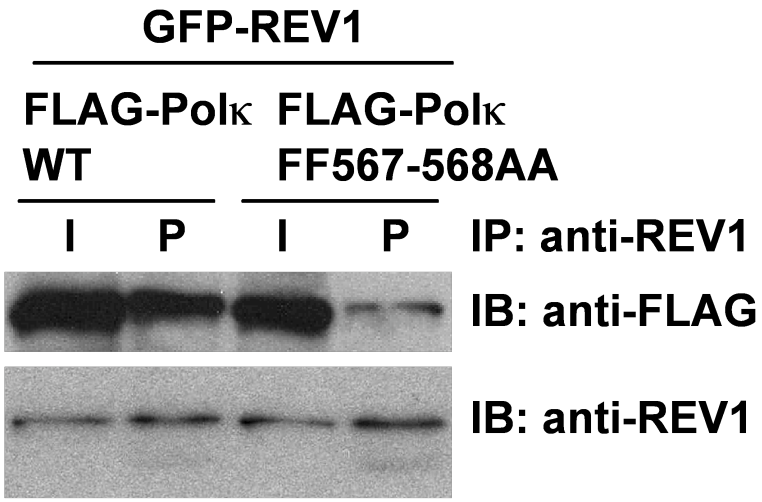
Supplementary Fig. 3 Effects of FF567-568A substitutions on the subcellular distribution and PCNA-binding activity of Polκ. A. Replicate cultures of H1299 cells were infected with AdGFP-Polκ(WT) or AdGFP-Polκ(FF567-568AA). After 24 hr, GFP-Polκ-expressing cells were treated with 600 nM BPDE for 4 hr (or were left untreated for controls). The resulting cells were separated into chromatin and soluble fractions as described under 'Experimental Procedures', and analyzed by SDS-PAGE and immunoblotting with antibodies against PCNA and GFP. (Note that the left and right panels of the figure are from the same immunoblot and represent the same exposure time of the ECL membrane. Non-relevant intervening lanes were excised to generate the final image). *B*, The chromatin fractions of GFP-Polκ(WT) or GFP-Polκ(FF567-568AA)-expressing cells (described in *A* above) were immunoprecipitated with an anti-GFP antibody. To ensure specific immunoprecipitation by the anti-GFP antibody, parallel incubations were performed using non-specific IgG as a negative control (data not shown). The resulting immune complexes were resolved by SDS-PAGE and analyzed by immunoblotting with antibodies against PCNA and GFP. *C*, H1299 cells growing on chamber slides were infected with AdGFP-Polκ(WT) or AdGFP-Polκ(FF567-568AA), then treated with 600 nM BPDE for 4 hr (or left untreated for controls), as described in *A*. The resulting cells were fixed with formaldehyde and GFP-Polκ distribution was analyzed by fluorescence microscopy as described under 'Experimental Procedures'.

Immunoprecipitation assay

Human 293T cells were transfected with pEGFP-hREV1 and pcDNA3.1(+)-FLAGPol□, using transfectamine 2000 according to manufacture's protocol. The cells were harvested 48 hr after transfection and disrupted in cell lysis buffer (20 mM Hepes, pH 7.6, 150 mM NaCl, 1 mM EDTA, 10% glycerol, 1mM dithiothreitol, 0.1% Tween-20, 1 mM PMSF, 10 mg/ml leupeptin, 5 mg/ml pepstatin A) by three freeze-and-thaw cycles. The cell lysates were centrifuged at 15,000 x*g* for 10 min and incubated with protein G-Sepharose beads (Sigma) for 30 min at 4°C to eliminate the non-specific binding of proteins to the beads. After a brief centrifugation (1000 rpm for 30 sec), the supernatants were incubated with anti-REV1 antibody for 10 hr. The antigen-antibody complex was immobilized on protein G Sepharose beads and the beads were washed five times in lysis buffer. The bound proteins were eluted by boiling in 1x sample buffer and subjected to SDS-PAGE and Western blotting with anti-FLAG or anti-REV1 antibody.

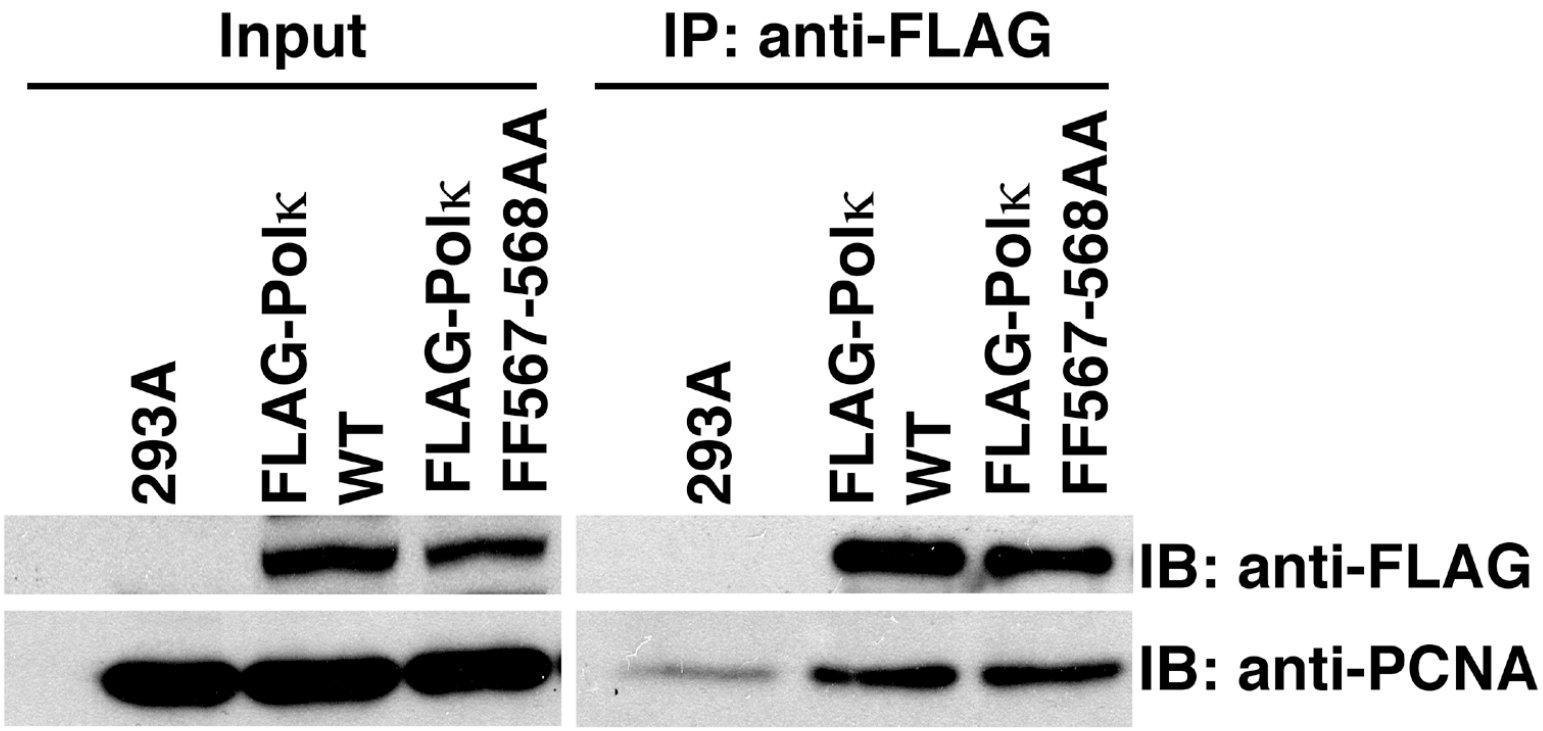
Figure legend

EGFP-hREV1 and FLAG-Pol were transiently overproduced and immunoprecipitated with anti-REV1 antibody and immunoblotted with anti-REV1 antibody or with anti-FLAG antibody. I, input fractions; P, pellet fractions. A five-excess of bound fraction was applied to the gel, compared to the input fraction.


GFP-REV1
FLAG-Polκ WT
FLAG-Polκ FF567-568AA
I
P
I
P
IP: anti-REV1
IB: anti-FLAG
IB: anti-REV1

Immunoprecipitation assay

Human 293A cells were transfected with pEGFP-hREV1 and pcDNA3.1(+)-FLAGPol□, using transfectamine 2000 according to manufacture's protocol. The cells were washed twice with ice-cold PBS and then harvested into RIPA buffer (1xPBS, 1%NP-40, 0.5% sodium deoxycholate, 0.1%SDS, 1mM PMSF, 1mM sodium orthovanadate, and protease inhibitor (Nacalai)). The cell lysates were centrifuged at 13,000 x*g* for 60 min and incubated with protein A/G PLUS-Agarose (SANTA CRUZ) for 2h at 4°C to eliminate the non-specific binding of proteins to the beads. After a brief centrifugation (3000 rpm for 60 sec), the supernatants were incubated with anti-FLAG antibody and protein A/G PLUS-Agarose for 12 hr. The antigen-antibody complex was immobilized on protein A/G PLUS-Agarose and the beads were washed four times in PBS. The bound proteins were eluted by boiling in 1x sample buffer and subjected to SDS-PAGE and Western blotting with anti-FLAG or anti-PCNA antibody.


Input
IP: anti-FLAG
293A
FLAG-Polκ WT
FLAG-Polκ FF567-568AA
293A
FLAG-Polκ WT
FLAG-Polκ FF567-568AA
IB: anti-FLAG
IB: anti-PCNA