Supplementary Methods

Plasmids. The bacterial expression plasmid pRSET-B(hpol λ) encoding human pol λ WT with an N-terminal hexahistidyl sequence has been described previously (Ramadan *et al*, 2003). Site-directed mutagenesis was performed to obtain various pol λ mutants using *PfuTurbo*® DNA Polymerase (Stratagene) according to the manufacturer's instructions and pairs of mutagenic oligonucleotides (Microsynth) for the following sequences: S167A: 5`-GC TTCAGACAGCCCTT**GCT**CCTCCTCCTCCTCCC-3`; S177A: 5`-CCCACCAGGCCTGTG **GCT**CCTCCCCAAAAGGC-3`; S230A: 5´- GGGAGATTGTGAGCCT**GCC**CCAGC CCCTGCTGTCC-3`; T553A: GGCCGAGTGCTGCCC**GCT**CCCACTGAGAAGGATG-3` (changed codons are in bold). To generate expression constructs for mammalian cells, PCRs were performed with pRSET-B(hpol λ) WT and mutant constructs as template, using Phusion™ High-Fidelity DNA Polymerase according to the manufacturer's instructions and the following oligonucleotides: pol λ-up: 5`-CCG*AAGCTT*ACCACCATGGATCCCAGGGT ATCTTG-3` (HindIII); pol λ-myc-down: 5`-CCG*TCT AGA*TCACAGATCCTCTTCTGAGA TGAGTTTTTGTTCCCAGTCCCGCTCAGCAGG-3` (XbaI); pol λ-HA-down: 5`-CCG *TCTAGA*TCATGCGTAGTCAGGCACGTCGTAAGGATACCAGTCCCGCTCAGCAGG-3` (XbaI; the restriction sites used for further cloning are in italics, the respective myc- and HA-tag sequences are underlined). PCR products with C-terminal myc- or HA-tag were cloned via the HindIII and XbaI restriction sites into pcDNA3 (Invitrogen). To generate constructs for retroviral transduction, PCR was performed with pcDNA3 constructs as template and the following oligonucleotides: pol λ-up: 5`-CCG*AAGCTT*ACCACCATGGATCCCAGGGTAT CTTG-3` (HindIII); pol λ-myc-down: 5`-GAG*GCGGCCGC*TCACAGATCCTCTTCTGAG-3` (NotI); pol λ-down: 5`-GAG*GCGGCCGC*TCACCAGTCCCGCTCAGC-3`(NotI). PCR products with C-terminal myc-tag or untagged were cloned via the HindIII and NotI restriction sites into pLPCX (Clontech). pGEX2T-suc1 and baculoviruses encoding recombinant human cyclin A and Cdk2 were provided by H. P. Nasheuer (Galway, Ireland). The mammalian expression plasmid for HA-Ubiquitin was a gift from D. Bohmann (Rochester, USA).

Proteins. 2 l of transformed *E. coli* BL21(DE3) cells were grown at 37°C to an OD_{600} of 0.6 in LB medium supplemented with 100 μg/ml ampicillin. Expression of human His-pol λ WT and mutant proteins was induced by incubation with 1 mM IPTG for 3 h and cells were pelleted. All further purification steps were carried out at 4°C. Cells were resupended in 60 ml buffer A (50 mM Tris-Cl pH 7.5, 10% (v/v) glycerol, 0.05% (v/v) NP-40, 1 mM PMSF, 1

µg/ml pepstatin, 1 µg/ml bestatin, 1 µg/ml leupeptin) supplemented with 500 mM NaCl and disrupted with a French press. Insoluble material was pelleted by centrifugation for 30 min at 48,000 g and the supernatant was diluted to a final concentration of 100 mM NaCl with buffer A. In case of His-pol λ WT, T553A and 4xA mutant, the extract was loaded onto 15 ml phosphocellulose equilibrated in buffer A with 100 mM NaCl and rolled for 2 h at 4°C. After intensive washing with buffer A with 100 mM NaCl, elution was performed with buffer A with 500 mM NaCl (4 x 15 ml). The pooled eluate was diluted with an equal volume of buffer B (50 mM Tris-Cl pH 7.5, 150 mM NaCl, 10% (v/v) glycerol, 0.05% (v/v) NP-40, 1 mM PMSF, 1 µg/ml pepstatin, 1 µg/ml bestatin, 1 µg/ml leupeptin) and adjusted to 10 mM imidazol. 0.5 volumes were loaded onto a 1 ml HisTrap™ HP column (GE Healthcare) equilibrated with buffer B and 10 mM imidazol using an ÄKTApurifier™ (GE Healthcare). After washing the column with 5 ml buffer B with 10 mM imidazol, elution was performed using a gradient from 10 to 500 mM imidazol in buffer B and fractions containing His-pol λ were pooled. The pool was diluted 1:3 with buffer A, and loaded onto a 1 ml HiTrap™ Heparin HP column (GE Healthcare) equilibrated with buffer A with 50 mM KCl. The column was washed with 5 ml buffer A with 100 mM KCl and eluted using a gradient from 100 mM to 1 M KCl in buffer A as well as a final step with buffer A with 2 M KCl. Final fractions containing purified His-pol λ protein were dialyzed to buffer C (20 mM Tris-Cl pH 7.5, 20% (v/v) glycerol, 100 mM NaCl, 1mM DTT) and stored in liquid nitrogen. In case of His-pol λ S167A, S177A, S230A and all triple mutants, *E. coli* extract was loaded directly onto a 1 ml HisTrap™ HP column and eluted as described above. No additional purification steps were performed for these mutants. Recombinant human Cdk2 and cyclin A were expressed and purified as described (Schub *et al*, 2001).

Transfection. All transfections were performed by calcium-phosphate precipitation. For expression analysis, 100 mm plates with 293T or U2OS cells were transfected with 5 µg of the indicated pcDNA3 construct. For ubiquitination analysis, 5 µg of HA-Ubiquitin plasmid and 5 µg of the respective pcDNA3 construct were used for co-transfection of 100 mm plates with 293T cells. Retroviral transduction of *Poll*$^{-/-}$ MEFs was performed utilizing the Phoenix Eco retroviral producer cell line (ATCC # SD 3444) and pLPCX constructs according to the manufacturer's instructions. Transduced MEFs were selected in the presence of 1 µg/ml puromycin, dihydrochloride (Calbiochem) and pooled. For stable transfection of 293T cells, 100 mm plates were transfected with 5 µg of linearized pLPCX constructs and selected in the presence of 0.5 µg/ml puromycin, dihydrochlorid. Isolated clones of resistant cells were harvested and grown independently.

RT-PCR. Total RNA was isolated from cultured cells with TRIZOL® Reagent (Invitrogen) and RT-PCRs were performed with QIAGEN® OneStep RT-PCR Kit (QIAGEN), both according to the manufacturer's instructions. 150 ng total RNA and the following oligonucleotides (Microsynth) were used: pol λ-myc: 5`-GACAAGTGGAGGGCCCTGGGC-3`; 5`-CTCTTCTGAGATGAGTTTTTG-3`; hypoxanthin-guanin-phosphoribosyltransferase (Hprt): 5`-TTTGCTGACCTGCTGGATTAC-3`; 5`-TTCCAGTTTCACTAATGACAC-3`.

DNA polymerase assays (trichloroacetic acid assay). Pol λ activity on poly(dA)/oligo$(dT)_{10:1}$ was assayed in a final volume of 25 µl containing polymerase buffer (50 mM Tris-Cl pH 7.5, 0.25 mg/ml BSA, 1 mM DTT), 0.8 mM $MnCl_2$, 20 µg/ml poly(dA)/oligo$(dT)_{10:1}$ and 20 µM [^{3}H]-dTTP [5 Ci/mmol]. All reactions were incubated for 30 min at 37°C and the DNA was precipitated with 10% trichloroacetic acid. Insoluble radioactive material was determined by scintillation counting.

**5`-deoxyribose-5-phosphate lyase assay.** A 33 mer oligonucleotide containing uracil at position 16 (dRPU-33: 5`-CTGCAGCTGATGCGCUGTACGGATCCCCGGGTA-3`; Microsynth) was annealed to a complementary 34 mer (dRPU-34: 5`-GTACCCGGGGATCC GTACGGCGCATCAGCTGCAG-3`). Incorporation of a radioactively labelled nucleotide at the 3` end of the uracil containing strand was achieved by incubating the double-stranded oligonucleotides with Klenow Fragment (3`→ 5`exo-; New England Biolabs) in the presence of [α-^{32}P]-dCTP. The dRP lyase assay was essentially performed as previously described (Garcia-Diaz *et al*, 2001).

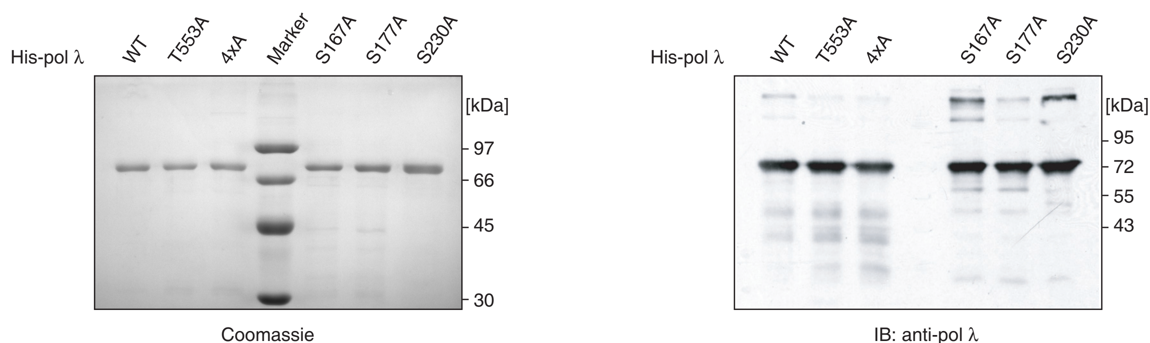
Supplementary References

Garcia-Diaz M, Bebenek K, Kunkel TA, Blanco L (2001) Identification of an intrinsic 5'-deoxyribose-5-phosphate lyase activity in human DNA polymerase lambda: a possible role in base excision repair. *J Biol Chem* **276**: 34659-34663

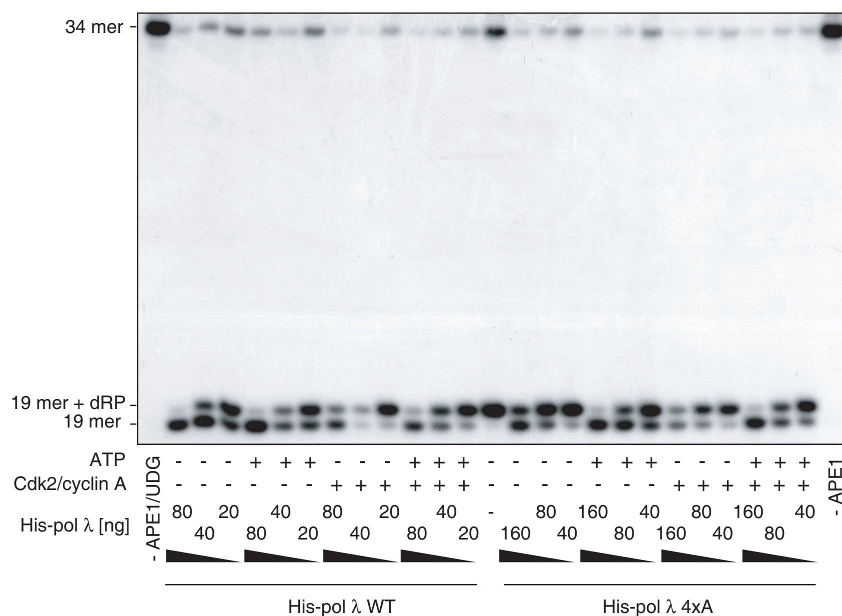
Ramadan K, Maga G, Shevelev IV, Villani G, Blanco L, Hübscher U (2003) Human DNA polymerase lambda possesses terminal deoxyribonucleotidyl transferase activity and can elongate RNA primers: implications for novel functions. *J Mol Biol* **328**: 63-72

Schub O, Rohaly G, Smith RW, Schneider A, Dehde S, Dornreiter I, Nasheuer HP (2001) Multiple phosphorylation sites of DNA polymerase alpha-primase cooperate to regulate the initiation of DNA replication in vitro. *J Biol Chem* **276**: 38076-38083

Supplementary Figures



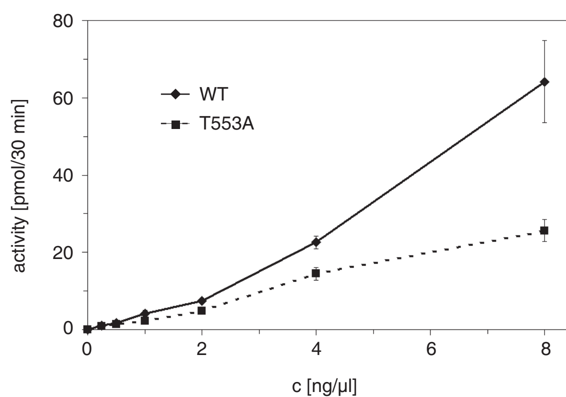
Supplementary Fig S1. Purification of pol λ WT or mutants and antibody affinity. Human His-pol λ WT, single mutant and 4xA protein was expressed in *E. coli* and purified by chromatography to near homogeneity. 2 µg of each protein preparation were separated by SDS-PAGE followed by Coomassie staining (left panel) or immunoblot analysis (right panel). IB, immunoblot; 4xA, quadruple mutant; WT, wild-type.



Supplementary Fig S2. dRP lyase activity of phosphorylated pol λ. For phosphorylation, His-pol λ WT or 4xA protein was incubated with human Cdk2/cyclin A in the presence of ATP. In control reactions, His-pol λ proteins were incubated in the absence of Cdk2/cyclin A, ATP or both. For the dRP lyase assay, a 34 mer double-stranded oligonucleotide containing a uracil residue at position 16 of the 3`-[^{32}P]-labeled strand was treated with human UDG and human APE1 to create a dRP-containing substrate. In control reactions, the template was incubated only with UDG (-APE1) or without any of the two enzymes (-APE1/UDG). Further removal of the dRP moiety was analyzed by incubation of phosphorylated and non-phosphorylated His-pol λ WT or 4xA with this substrate, followed by separation on a sequencing gel and autoradiography. APE1, AP endonuclease 1; Cdk2, cyclin-dependent kinase 2; dRP, 5`-deoxyribose-5-phosphate; 4xA, quadruple mutant; UDG, uracil DNA glycosylase; WT, wild-type.

Supplementary Figures

Supplementary Fig S3. Influence of pol λ phosphorylation on protein levels. (**A**) 293T cells were transfected with constructs encoding human pol λ-HA WT or T553A and protein levels were determined 2 days after transfection by immunoblot with the indicated antibodies. (**B**) 293T cells were transfected with pLPCX vector or constructs encoding untagged human pol λ WT or T553A and protein levels determined by immunoblot. HA, haemagglutinin; IB, immunoblot; WT, wild-type.



Supplementary Fig S4. Pol λ DNA polymerase activity. Different amounts of His-pol λ WT or T553A were incubated with poly(dA)/oligo(dT)$_{10:1}$ in the presence of 0.8 mM $MnCl_2$ and 20 µM [^{3}H]-dTTP, the resulting products were analyzed by scintillation counting (as described in Supplementary Methods). The activities are given as mean value +/- stdev of two independent replicates. c, concentration; WT, wild-type.

Supplementary Figures

Supplementary Fig S5. Results presented for pol λ T553A were also observed for pol λ 4xA (compare Fig 2-4). (**A**) U2OS cells were transfected with constructs encoding human pol λ-myc WT or 4xA and protein levels were determined by immunoblot with the indicated antibodies. *Poll*$^{-/-}$ MEFs were stably transduced with constructs encoding pol λ-myc WT or 4xA. (**B**) 293T cells were transfected with constructs encoding human pol λ-HA (left panel) or untagged (right panel) pol λ WT or 4xA and protein levels were determined by immunoblot. (**C**) 293T cells were transfected and analyzed by immunoblotting as described in (**A**). Semiquantitative RT-PCR was performed in a linear range of detection using primers specifically amplifying mRNA encoding pol λ-myc. As a control, Hprt mRNA was amplified. (**D**) 293T cells were transfected as described in (**A**). 40 h after transfection, cells were incubated in medium containing 50 μM CHX for the indicated time. As a control, cells were incubated with DMSO alone for 36 h. Protein levels were determined by immunoblot with the indicated antibodies. (**E**) 293T cells were transfected as described in (**A**). 1 day after transfection, cells were exposed for 16 h to various concentrations of MG132 and protein levels were analyzed by immunoblot. (**F**) 293T cells were co-transfected with constructs encoding HA-Ubi, pol λ-myc WT or 4xA and 1 day after transfection, cells were treated for 16 h with 10 μM MG132. Pol λ proteins were immunoprecipitated from denatured extracts with anti-myc antibody, rabbit IgG was used as a control. Input (left panel) and immunoprecipitation experiment (right panel) are shown. (**G**) 293T cells stably expressing pol λ-myc WT or 4xA were synchronized 1 day after transfection at the G1/S phase boundary by double blocks with 2 mM thymidine and harvested immediately or released in fresh medium for the indicated time. Unsynchronized control cells were harvested at time point 0 h. Synchronization and release was verified by flow cytometric analysis of DNA content (top panel). Protein levels were analyzed by immunoblotting with the indicated antibodies (lower panel). (**H**) 293T cells were transfected as described in (**A**). The transfected cells were synchronized 1 day after transfection in G2/M phase by incubation with 300 ng/ml nocodazole, harvested immediately or released in fresh medium for the indicated time. Unsynchronized control cells were harvested at time point 0 h. Analysis was performed as described in (**G**). The asterisks indicate nonspecific bands. C, control; CHX, cycloheximide; Δt, time; HA, haemagglutinin; HA-Ubi, HA-Ubiquitin; Hprt, hypoxanthin-guanin-phosphoribosyltransferase; IB, immunoblot; IgG, immunoglobulin G; IP, immunoprecipitation; Noco, Nocodazole; 4xA, quadruple mutant; WT, wild-type.