Supporting Information

Markkanen et al. 10.1073/pnas.1110449109

SI Materials and Methods.

Chemicals. Deoxynucleotides were purchased from Sigma. Labeled $\gamma[^{32}P]$ ATP was purchased from Hartmann Analytic. All other reagents were of analytic grade and purchased from Fluka, Sigma, or Merck.

Antibodies and Proteins. Antibodies (AB) against Pol λ (polyclonal rabbit) were raised in rabbits and affinity purified. Mule and ARF AB were from Bethyl Laboratories and the tubulin AB was from Sigma-Aldrich. The goat polyclonal AB against MutYH, rabbit polyclonal AB against GST, and rabbit polyclonal AB against c-Myc were from Santa Cruz. Mouse monoclonal AB against Fibrillarin and rabbit polyclonal AB against Histone 1 were from Abcam. Recombinant human Pol λ WT and mutants were expressed and purified according to Wimmer et al. (1). Recombinant HECT domain was purified according to Parsons et al. (2).

DNA Substrates. All oligonucleotides were purified on polyacrylamide denaturing gels. The 39-mer oligonucleotide (5′ TAC AAC CAA GAG CAT ACG ACG GCC AGT GCC GAA TTC ACA 3′) was purchased from Microsynth, and the 72-mer oligonucleotide containing 8-oxo-G at position 33 (5′ GTA TTA GAT ATT CGG GAG GTT GGG CGC CGG CG**X** TGT GAA TTC GGC ACT GGC CGT CGT ATG CTC TTG GTT GTA 3′, where X stands for 8-oxo-G) was from Purimex.

RNAi Interference. The Mule siRNA (Hs_HUWE_2) was from Qiagen. The siRNA sequence to target ARF was as follows: 5′-GAACAUGGUGCGCAGGUUCTT-3′ (3). The siRNA against MutYH (5′- UCA CAU CAA GCU GAC AUA UCA AGU ATT -3′) was from Microsynth.

Plasmids for the Expression of Pol λ WT and Ubiquitination-Deficient Mutants. Site-directed mutagenesis was performed to obtain various Pol λ mutants using *PfuTurbo*® Pol (Stratagene) according to the manufacturer's instructions. The following primer pairs (Microsynth) were used: K27R 5′ tgctgatgcatcatcaaaagtacttgcaaagattcctaggagggaagagg 3′ and K273R 5′ ggccaaagcctacagtgttcagggagacaagtggagggccctgggctatgcc 3′ on the pRSETB-Polλ (WT) construct and mutations were confirmed by sequencing.

RT-PCR. Total RNA was isolated from cultured cells with TRIZOL® Reagent (Invitrogen) and RT-PCRs were performed by using QIAGEN® OneStep RT-PCR Kit (QIAGEN). Both TRIZOL® Reagent and QIAGEN® OneStep RT-PCR Kit were used according to the manufacturer's instructions. For RT-PCR analysis, 150 ng total RNA and the following oligonucleotides (Microsynth): Pol λ-myc: 5′-GACAAGTGGAGGGCCCTGGGC-3′→ 5′-CTCTTCTGAGATGAGTTTTTG-3′. L28 primers served as a loading control.

Cells and Whole-Cell Extracts. Human HeLa and T24 cells were purchased from American Type Cell Culture, HEK 293T cells were a gift from R. Santoro (University of Zürich, Switzerland), the T24 cells were a gift from M. Stucki (University of Zürich, Switzerland), and the mouse embryonic fibroblast Pol $\lambda+/+$ and $-/-$ were as described (4). HEK 293T cells stably transfected with myc-Pol λ WT or myc-Pol λ 4A mutants were obtained as described (1). Cells were grown under standard conditions.

Whole-cell extracts for Fig. 2 (*A*)–(*D*) were prepared by Tanaka's method (5) were prepared as previously outlined in ref. 2.

All other whole-cell extracts were prepared by the addition of lysis buffer (10 mM Hepes, pH 7.9, 500 mM KCl, 340 mM sucrose, 10% glycerol, 0.75% Triton X-100, 1 mM DTT, 2 mM PMSF, 1 μg/mL bepstatin, 1 μg/mL leupeptin, and 1 μg/mL pepstatin) to the cells, followed by scraping and incubation of the lysate for 15 min on ice. The cell lysate was sonicated at 4 °C for 30 s and centrifuged at 10,000 × *g* for 10 min. The supernatant was collected, aliquoted, and stored at −80 °C.

Extracts for single-nucleotide incorporation assays were prepared as described in ref. 4.

Cell fractionation was performed as follows: Cells were washed 3 times with PBS, and the pellets were vigorously resuspended and lysed 20 min in 1 volume buffer A (10 mM Hepes, pH 7.9, 100 mM KCl, 1.5 mM $MgCl_2$, 0.34 M sucrose, 10% glycerol, 0.01% Triton X-100, 1 mM DTT, 1 mM PMSF, 1 μg/mL pepstatin, bestatin, and leupeptin) under constant shaking at 4 °C. Nuclei were collected by 5 min centrifugation at 1,700 × *g* at 4 °C. The supernatant was further clarified by 15 min centrifugation at 20,000 × *g*, the supernatant of which yielded the cytoplasmic fraction. Nuclei were resuspended in 1 volume buffer B (10 mM Hepes, pH 7.9, 3 mM EDTA, 0.2 mM EGTA, 1 mM DTT, 1 mM PMSF, 1 μg/mL pepstatin, bestatin, and leupeptin) under constant shaking for 20 min at 4 °C. Centrifugation for 5 min at 2,000 × *g* yielded the nuclear fraction as supernatant. The chromatin pellet was resuspended in 0.5 volumes of buffer A, sonicated 5 times 30 s in a sonication bath (Diagenode), and centrifuged for 5 min at 20,000 × *g*. The supernatant of this step yielded the chromatin fraction. Equal protein amounts of each fraction were analyzed by SDS-PAGE.

Western Blot Analyses. Western blot analyses were performed according to standard procedures and visualized by using the Odyssey image analysis system (Li-cor Bioscience).

H_2O_2 Treatment of T24 Cells. T24 cells were grown to 100% confluency in normal DMEM + 10% FCS and left to arrest in G0 for 5 d. Fourteen hours prior to treatment, they were seeded 1:3 in order to reinitiate cycling. After treatment with 0.5 mM H_2O_2 in DMEM + 10% FCS for 45 min, the cells were washed with 1× PBS, released into fresh medium, and harvested at the indicated time points performing cell fractionation.

1. Wimmer U, Ferrari E, Hunziker P, Hubscher U (2008) Control of DNA polymerase lambda stability by phosphorylation and ubiquitination during the cell cycle. *EMBO Rep* 9:1027–1033.
2. Parsons JL, et al. (2009) Ubiquitin ligase ARF-BP1/Mule modulates base excision repair. *EMBO J* 28:3207–3215.
3. Boldogh I, et al. (2001) hMYH cell cycle-dependent expression, subcellular localization and association with replication foci: evidence suggesting replication-coupled repair of adenine:8-oxoguanine mispairs. *Nucleic Acids Res* 29:2802–2809.
4. Maga G, et al. (2007) 8-oxo-guanine bypass by human DNA polymerases in the presence of auxiliary proteins. *Nature* 447:606–608.
5. Tanaka M, Lai JS, Herr W (1992) Promoter-selective activation domains in Oct-1 and Oct-2 direct differential activation of an snRNA and mRNA promoter. *Cell* 68:755-767.

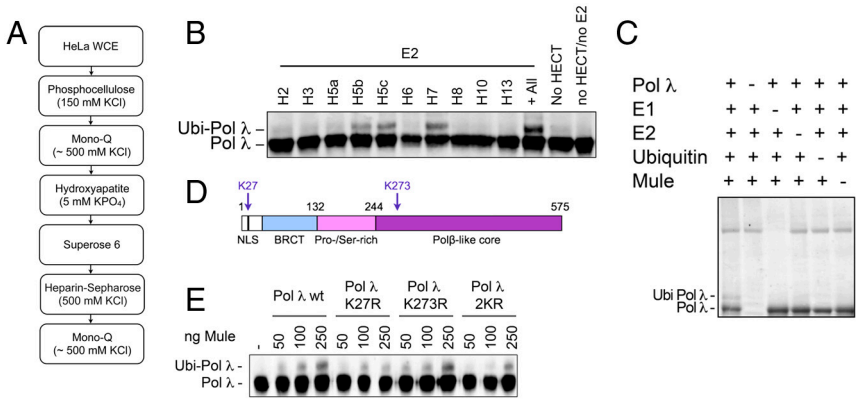

Fig. S1. Identification of Mule as an E3 ubiquitin ligase for DNA polymerase λ and determination of ubiquitinated residues on DNA polymerase λ. (*A*) Purification scheme for the isolation of E3 ubiquitin ligase from crude HeLa cell extracts. (*B*) E2 specificity of the recombinant HECT domain of Mule. (*C*) Identification of in vitro ubiquitinated Pol λ. In vitro ubiquitination reactions with recombinant Pol λ were analyzed by SDS-PAGE and Coomassie stained; the band labeled "Ubi-Pol λ" was excised and subjected to MS/MS analysis. (*D*) Schematic presentation of Pol λ showing the ubiquitinated residues identified by MS/MS. (*E*) Titration of the purified HECT domain of Mule in the presence of recombinant Pol λ WT or its ubiquitination-site mutants K27R and K273R.

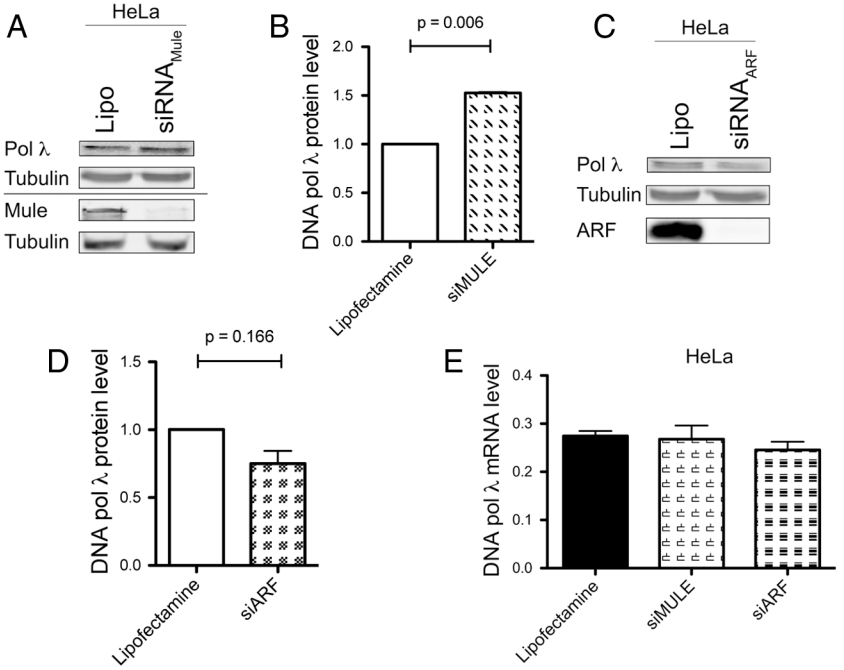

Fig. S2. Identification of Mule as an E3 ubiquitin ligase regulating cellular levels of DNA polymerase λ. (*A*) Effect of siRNA-mediated Mule knockdown on Pol λ levels in HeLa cells, analyzed by Western blotting. (*B* and *D*) Quantification of protein levels shown in *A* and *C* (three independent experiments each) showing mean + SD and *p* values obtained from one-sample *t* tests performed on the data. The Pol λ signal was normalized to tubulin. Error bars for *B* are too small to be discernible in this presentation. (*C*) Effect of siRNA-mediated ARF knockdown on Pol λ levels in HeLa cells, analyzed by Western blotting. (*E*) Quantification of mRNA levels by RT-PCR from the experiments shown in *A* and *C* from three independent experiments, showing mean + SD.

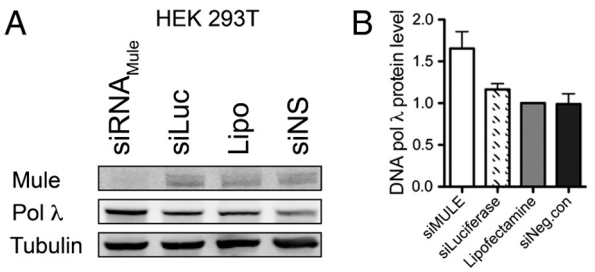

Fig. S3. Analysis of siRNA controls. (*A*) Effect of siRNA-mediated Mule knockdown and siRNA against luciferase (siLuc), Lipofectamine only (Lipo), or nonspecific siRNA (siNS) on cellular Pol λ levels, analyzed by Western blotting. (*B*) Quantification of protein levels shown in *A*, showing mean + SD of two independent experiments. The Pol λ signal was normalized to tubulin and the Lipofectamine control.

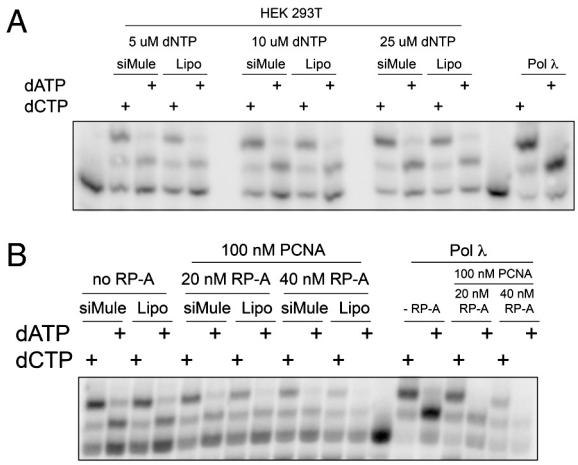

Fig. S4. Titration of dNTPs, RP-A, PCNA, and cell extracts for 8-oxo-G single-nucleotide incorporation assays. (*A*) Titration of dATP and dCTP for single-nucleotide incorporation opposite 8-oxo-G performed with HEK 293T cell extracts. (*B*) Titration of RP-A and PCNA for single-nucleotide incorporation opposite 8-oxo-G performed with HEK 293T cell extracts.

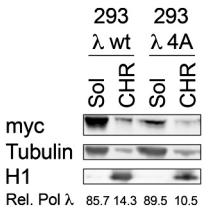

Fig. S5. Subcellular fractionation of HEK cells stably transfected with myc-Pol λ WT or 4A mutant. Fractionation of HEK cells stably transfected with myc-Pol λ WT or 4A mutant into soluble and chromatin-bound fractions. Sol = soluble fraction, CHR = chromatin-bound fraction. The Pol λ signal was normalized to tubulin (for the soluble fraction) or to histone 1 (for the chromatin-bound fraction), and the percentage of the total cellular Pol λ pool is indicated at the bottom of each column.