Supporting Information

Johnson et al. 10.1073/pnas.1206052109

SI Materials and Methods

Plasmids for Protein Expression. All proteins were expressed in yeast by cloning the WT or mutated ORFs downstream from the *GAL:PGK* promoter in various plasmids as described previously (1). Proteins were expressed either as native proteins or as fusions with a GST tag or a FLAG tag, as indicated. A prescission protease cleavage site is present C-terminal to each affinity tag to direct removal of the tag after affinity purification. The Rev3(1-1443) and Rev3(1-1475) truncations were made by amplification of the corresponding regions by PCR, and the F1476A Y1477A and C1468S mutations were generated by oligonucleotide-mediated mutagenesis. All PCR-generated regions and mutations were confirmed by sequencing. GST-Pol31 was expressed from plasmid pBJ1244 as described previously (2). To coexpress GST-Pol31 and Pol32 from a single plasmid, the GAL:PGK:GST-Pol31 PvuII cassette from pBJ1244 was cloned into pBJ1180 (2), generating pBJ1524. Native Rev3/Rev7 was expressed from the dual-expression plasmid pBJ1362, and the FLAG-Rev3/Rev7 was expressed from plasmid pBJ1462.

Protein Purifications. WT Rev3/Rev7-containing yeast Polζ was purified as described previously (3). For the protein purification experiments shown in Fig. 1*C*, expression plasmids pBJ1524 and pBJ1362 were transformed into yeast strain YRP654, and cells were grown as described previously (1). Cells were induced with 2% galactose for 7 h. Then 6 g of cell paste was resuspended in 2.5 volumes of 1× CBB containing 200 mM NaCl, 175 mM $NH_4(SO_4)_2$, 0.1% Triton X-100, 10% (vol/vol) β-mercaptoethanol, and a complete set of protease inhibitors (Roche) and lysed with a French press. Clarified extract was obtained by centrifugation for 30 min at 330,000 × *g*. Cell extract was slowly passed over a 200-μL glutathione Sepharose fast-flow column, followed by equilibration in 1.5 volumes of 1× GBB_{150}. Proteins were eluted by overnight treatment with prescission protease. The harvested supernatant was concentrated in a Microcon protein concentrator (Amicon), and proteins were resolved by 10% SDS/PAGE. Protein bands were excised from the gel, treated with trypsin, and analyzed by MALDI-TOF mass spectrometry at the University of Texas Medical Branch Mass Spectrometry Core. Protein matches with >90% confidence are shown.

To purify the four-subunit Rev3/Rev7/Pol31/Pol32 complex (Polζ-d) (Fig. 2 *A* and *B*) to near homogeneity, expression plasmids pBJ1524 and pBJ1462 were transformed into yeast strain YRP654, cells were grown as described previously (1), and protein expression was induced by 2% galactose for 18 h. Then 80 g of frozen cells was resuspended in 3 volumes of 1× CBB as above but omitting Triton X-100, and lysed in a BioSpec BeadBeater with cooling. After lysis, polymin P was added to the extract at a final concentration of 0.04%, and the extract was incubated on ice with stirring for 15 min before centrifugation at 10,000 × *g* to remove cell debris and precipitated DNA. Clarified extract was prepared by further centrifugation of the supernatant at 100,000 × *g* for 40 min. Proteins were precipitated by the addition of 0.28 g of ammonium sulfate/mL of extract and were pelleted by centrifugation. The resulting pellet was resuspended in 30 mL of 1× GBB containing 200 mM NaCl and protease inhibitors (complete without EDTA; Roche) and dialyzed overnight against 100 volumes of 1× GBB. The protein sample was then passed slowly over a 1-mL Glutathione Sepharose Fast-Flow column (GE Healthcare), washed with 10 volumes of 1× GBB_{200}, and equilibrated in 1× GBB_{150}. Proteins were eluted by incubation with 1× GBB_{150} containing 40 mM glutathione and 0.01% Nonidet P-40. Eluted proteins were subsequently rocked for several hours with 0.5 mL anti-FLAG M2 agarose (Sigma-Aldrich). The matrix was washed with 10 volumes of 1× GBB_{250} and then equilibrated in 1× GBB_{150}. The column was washed with 10 column volumes of 1× GBB_{150} containing 8 mM MgOAc and 1 mM ATP before final eqilibration in 1× GBB_{150} containing 0.01% Nonidet P-40 and 5 mM DTT. Protein was eluted by treatment with 20 units of prescission protease (GE Healthcare) overnight at 4 °C. The eluted protein was concentrated to 100 μL and run on a Superdex 200 PC3.2/30 Gel Filtration column (GE Healthcare) equilibrated in GBB_{150} containing 0.01% Nonidet P-40 and 5 mM DTT. Pooled fractions were then incubated with 100 μL of glutathione Sepharose fast flow to remove residual prescission protease and concentrated, and aliquots were frozen at −70 °C.

For experiments to examine the role of the Rev3 C terminus for binding Pol31 (Fig. 3), yeast cells harboring GST-Pol31/Pol32 or GST-Pol31 alone, and the various mutant Rev3 expression plasmids, were grown in a similar manner and broken using a French press. Cell extracts were treated as above, but proteins were eluted from the glutathione Sepharose matrix by prescission protease.

Plasmids and Yeast Strains for UV Survival and UV Mutagenesis. For genetic studies, a 10.1-kbp fragment from the original *REV3* clone pJA6 (4) was cloned into a derivative of the *CEN ARS LEU2* plasmid YCpLac111 (5) lacking the HinDIII, SphI, and PstI sites, generating plasmid pBJ1372. Subsequently, the EcoRI/PstI fragments harboring the Rev3(1-1475) and Rev3(1-1443) deletions or the F1476A Y1477A or C1468S mutations were used to replace the WT fragment in pBJ1372, generating plasmids pBJ1374, pBJ1375, pBJ1663, and pBJ1714, respectively. For determination of UV-induced $CAN1^S$ to $can1^r$ forward mutations, plasmids were transformed into *rev3Δ* yeast strain YREV3.14. UV-induced reversion of the *arg4-17* allele was examined using yeast strain YREV3.38, a *rev3Δ* derivative of strain CL1265-7C (*MAT**a** arg4-17 leu2-3,-112 his3-Δ1 trp1 ura3-52*).

The Pol31 K358E mutation (*sdp5-15* allele) was made by the quick-change method. A 2.3-kb MluI/PstI fragment containing the *POL31* gene was cloned into the integration vector YIpLac211, which contains the *URA3* gene (5). Subsequently, an MfeI/SnaBI fragment containing the *pol31 K358E* mutation was used to replace the WT *POL31* fragment, resulting in plasmid pBJ1706. This plasmid was used to generate the K358E (*sdp5-15*) mutation in genomic *POL31* by integration/excision. Plasmid pBJ1706 was digested with MfeI and transformed into *rev3Δ* yeast strains YREV3.14 and YREV3.38. $Ura3^+$ integrants harboring the *pol31 K358E* mutation were subcloned onto media containing 5-fluoroorotic acid to select for recombinational excision of the plasmid DNA sequences. The presence of the *pol31 K358E* (*sdp5-15*) allele in each strain was confirmed by DNA sequencing of PCR fragments generated from yeast genomic DNA. The *rev3Δ* strain also carrying the genomic K358E mutation in *POL31*, YREV3.193, was used to examine UV induced $CAN1^S$ to $can1^r$ forward mutations, and YREV3.223 was used to examine *arg4-17* to $ARG4^+$ reversion.

UV Survival and UV Mutagenesis. Cells were grown to mid-logarithmic phase in synthetic complete media lacking leucine (SC-leu), washed with water, and sonicated to disperse clumps when necessary. Cell suspensions were diluted and spread onto SC-leu plates for viability determinations and SC plates lacking arginine but containing canavanine (SC-arg+can) for determination of

CAN1^S to *can1*^{r} mutation frequencies, or onto SC plates lacking arginine (SC-arg) for determination of *arg4-17* to *ARG4*$^+$ mutation frequencies. The plates were UV-irradiated at 1 J/m^2/s for various times to reach the desired dose, and plates were incubated in the dark for 3–4 d before colony counting.

DNA Polymerase Assays and DNA Substrates. DNA polymerase assays were carried out as described previously (1). The standard 5-μL reaction contained 10 nM DNA substrate, 25 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 10% glycerol, 0.1 mg/mL BSA, 1 mM DTT, and 25 μM of each of dGTP, dATP, dTTP, and dCTP. DNA substrates consisted of a ^{32}P-radiolabeled 32 nucleotide primer (5′-GTTTT CCCAG TCTCG ACGAT GCTCC GGTAC TC-3′) annealed to a 75-mer DNA template (5′-AGC TAC CAT GCC TGC CTC AAG AAT TCG TATTAT GCC TAC ACT GGA GTA CCG GAG CAT CGT CGT GAC TGG GAA AAC-3′) containing nondamaged TT or containing either a *cis-syn* TT dimer (CPD) or a (6-4) TT photoproduct at the underlined position. The 75-mer abasic (AP) site template was of identical sequence except that the underlined sequence was 5′-AX-3′, where X denotes the AP site. Assays contained 1 nM DNA Polζ or Polζ-d and were performed at 30 °C for 10 min. Reactions were terminated by the addition of 30 μL of 95% formamide containing 0.03% bromophenol blue and 0.03% xylene cyanol. Terminated reactions were denatured by heating to 95 °C for 3 min before resolving DNA synthesis products on 12% polyacrylamide Tris-borate-EDTA gels containing 8 M urea. Gels were dried, and reaction products were visualized by phosphorimaging scanning analysis.

Steady-State Kinetic Analysis of Primer Extensions. For primer extension assays, the DNA substrate consisted of an oligonucleotide primer in which the 3′ terminal base (A or G) is paired opposite the 3′ T of a nondamaged TT sequence, a TT (CPD) or (6-4) TT photoproduct, or an AP site. The standard 5 μL DNA polymerase assay was used, except that only a single deoxynucleotide was added at concentrations ranging from 0.05 to 2500 μM. dATP was used in assays containing the nondamaged TT, TT (CPD), and (6-4) TT photoproduct substrates, and dTTP was used for assays containing the AP site substrate. Reactions contained 0.5 nM DNA polymerase and were carried out at 30 °C for 5 min. Reaction products were resolved and visualized as above. Kinetic parameters and nucleotide incorporation efficiencies were determined from gel band intensities of the substrates, and products were quantitated using ImageQuant software (Molecular Dynamics). The observed rate of deoxynucleotide incorporation opposite the next template base (primer extension) was plotted as a function of dNTP concentration, and the data were fit by nonlinear regression to the Michaelis–Menten equation describing a hyperbola, $v = (k_{cat} \times [dNTP]/(K_m + [dNTP])$. The k_{cat} and K_m steady-state parameters were obtained from the fit and were used to calculate the efficiency of nucleotide incorporation (k_{cat}/K_m) opposite the next template base, which also reflects the efficiency of primer extension.

1. Johnson RE, Prakash L, Prakash S (2006) Yeast and human translesion DNA synthesis polymerases: Expression, purification, and biochemical characterization. *Methods Enzymol* 408:390–407.
2. Zhuang Z, et al. (2008) Regulation of polymerase exchange between Polη and Polδ by monoubiquitination of PCNA and the movement of DNA polymerase holoenzyme. *Proc Natl Acad Sci USA* 105:5361–5366.
3. Acharya N, Johnson RE, Prakash S, Prakash L (2006) Complex formation with Rev1 enhances the proficiency of yeast DNA polymerase ζ for mismatch extension and for extension opposite from DNA lesions. *Mol Cell Biol* 26:9555–9563.
4. Morrison A, et al. (1989) *REV3*, a *Saccharomyces cerevisiae* gene whose function is required for induced mutagenesis, is predicted to encode a nonessential DNA polymerase. *J Bacteriol* 171:5659–5667.
5. Gietz RD, Sugino A (1988) New yeast-*Escherichia coli* shuttle vectors constructed with in vitro mutagenized yeast genes lacking six-base pair restriction sites. *Gene* 74: 527–534.

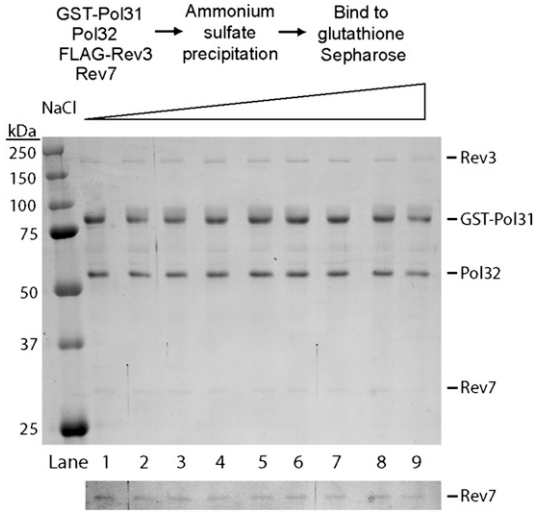

Fig. S1. High salt resistance of the Polζ-d complex. Extract from yeast cells expressing GST Pol31/Pol32 and FLAG Rev3/Rev7 was bound to glutathione Sepharose. The bound complex was washed with buffer containing increasing amounts of NaCl. After each wash, an aliquot of the glutathione Sepharose beads was resuspended in SDS/PAGE loading buffer, and bound proteins were resolved by 10% SDS/PAGE analysis. Lane 1, GST-Pol31/Pol32 Rev3/Rev7-bound beads equilibrated in 150 mM NaCl. Lanes 2–9, aliquots of beads after washing with 300–1,000 mM NaCl, in 100-mM increments. The association between Rev3/Rev7 and Pol31/Pol32 was not disrupted after washes with up to 1 M salt. The poor visibility of the Rev7 band in these GST pull-down experiments is related to the limited amounts of Rev3/Rev7 relative to GST-Pol31. (*Lower*) The region of the gel containing Rev7 is enhanced to better visualize the Rev7 band.

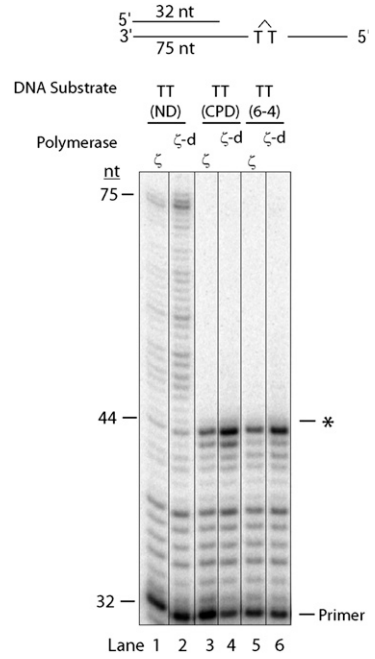

Fig. S2. DNA synthesis by Polζ versus Polζ-d on DNA templates containing no damage (ND), a CPD, or a (6-4) photoproduct at the TT sequence. DNA synthesis was assayed at 30 °C for 10 min under standard polymerase assay conditions with 1 nM Polζ or Polζ-d. The DNA substrate is shown at the top, where the TT represents either a nondamaged TT sequence or the presence of a TT CPD or a (6-4) TT photoproduct. DNA synthesis product sizes are shown on the left, and the asterisk on the right indicates the position of the 3′ T of the CPD or the 6–4 photoproduct. Odd lanes contain Polζ, and even lanes contain Polζ-d.

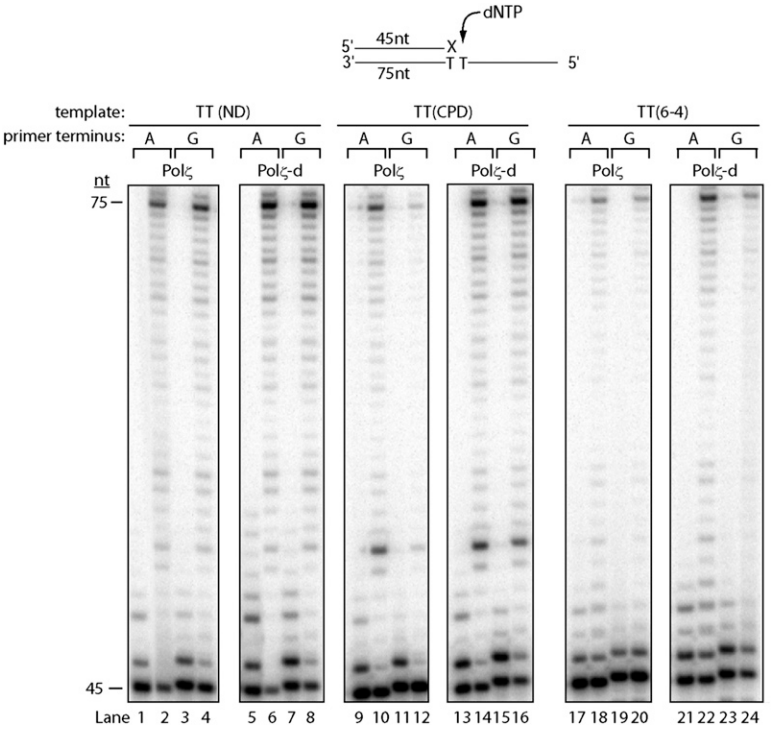

Fig. S3. Primer extension by DNA Polζ vs. Polζ-d from an A or a G opposite the 3′T of undamaged DNA or DNA containing a CPD or a (6-4) photoproduct. DNA synthesis was assayed at 30 °C for 10 min under standard polymerase assay conditions with 1 nM Polζ or Polζ-d. The DNA substrate is shown at the top, where X represents a 3′ primer terminal A or G nucleotide. DNA synthesis product sizes are shown on the left. Odd lanes contain only dATP (25 μM), and even lanes contain 25 μM each of dATP, dGTP, dCTP, and dTTP.

Table S1. Stoichiometry of Polζ-d subunits

Polζ-d subunit	Molecular weight, Da	Relative protein mass in complex, %	Total signal from SDS/PAGE, %*	Stoichiometry†
Rev3	172,965	58.2	54.1	0.93
Pol31	55,395	18.6	17.3	0.93
Pol32	40,309‡	13.6	15.6	1.15
Rev7	28,754	9.7	11.6	1.20
Total	297,419	—	—	—

*Results are the average of three lanes containing 0.5 μL, 1 μL, and 2 μL of Polζ-d complex.
†Stoichiometry was calculated by dividing the percentage of each protein determined from SDS/PAGE anaylsis by its expected relative mass.
‡In SDS/PAGE, the Pol32 protein migrates at a higher molecular weight than expected.

Table S2. Catalytic efficiency of extension by Polζ and Polζ-d from the correct (A) or incorrect (G) nucleotide placed opposite the 3′T on undamaged DNA, the 3′T of a *cis*-syn TT dimer or of a (6-4) TT photoproduct, and from an A or a G placed opposite an AP lesion

Template:primer	Polζ	k_{cat}, min^{-1}	K_m, μM ($\times 10^{-1}$)	Efficiency, k_{cat}/K_m	Relative increase in efficiency by Polζ-d
T:A	Polζ	0.11 ± 0.01	0.072 ± 0.031	15.3	—
	Polζ-d	0.34 ± 0.03	0.061 ± 0.021	55.7	3.6
T:G	Polζ	0.17 ± 0.01	0.093 ± 0.019	18.3	—
	Polζ-d	0.36 ± 0.02	0.043 ± 0.009	83.7	4.6
3′T (CPD):A	Polζ	0.24 ± 0.03	0.8 ± 0.3	3.0	—
	Polζ-d	0.45 ± 0.02	0.9 ± 0.2	5.0	1.7
3′T (CPD):G	Polζ	0.20 ± 0.006	1.4 ± 0.2	1.4	—
	Polζ-d	0.55 ± 0.03	0.8 ± 0.1	6.9	4.9
3′T (6-4):A	Polζ	0.15 ± 0.006	1.4 ± 0.2	1.1	—
	Polζ-d	0.47 ± 0.05	2.1 ± 0.8	2.2	1.9
3′T (6-4):G	Polζ	0.24 ± 0.02	0.9 ± 0.2	2.7	—
	Polζ-d	0.47 ± 0.01	0.9 ± 0.05	5.2	1.9
AP:A	Polζ	0.16 ± 0.01	1.1 ± 0.4	1.5	—
	Polζ-d	0.35 ± 0.02	0.7 ± 0.1	5.0	3.3
AP:G	Polζ	0.25 ± 0.03	3 ± 1	0.8	—
	Polζ-d	0.65 ± 0.04	1.7 ± 0.4	3.8	4.8