SUPPLEMENTARY DATA TO:

A four-subunit DNA polymerase ζ complex containing Pol δ accessory subunits is essential for PCNA-mediated mutagenesis

Alena V. Makarova, Joseph L. Stodola, and Peter M. Burgers*

Department of Biochemistry and Molecular Biophysics, Washington University School of Medicine,
St. Louis, MO 63110, USA

Running Title: A four-subunit Pol ζ complex in mutagenesis

*corresponding author:
TEL: (314) 362-3872
FAX: (314) 362-7183l
E-mail: burgers@biochem.wustl.edu

Materials and Methods

Strains.

The protease deficient yeast strain FM113 (a haploid version of BJ2168; *MAT***a**: *leu2–3,112, pep4–3, prb1–1122, prc1–407, trp1–289, ura3–52*), Pol32 deficient strain PY117 (*MAT***a**: *his3-11, 15, leu2–3,112, nuc1*Δ*::LEU2, pep4–3, pol32*Δ*::HIS3, prb1–1122, trp1–*Δ*, ura3–52*) and Rev1 deficient strain PY201 (*MATα: arg4-17, his3*Δ*-1, leu2-3, 112, trp1, ura3-52, rev1::HISG*) were used for overexpression of Pol ζ_4 and Pol ζ_2 complexes. Strain PJ69-4A (MAT**a**: gal4-Δ, gal80-Δ, his3-Δ200, leu2-3, 112, LYS2::GAL1-HIS3, GAL2-ADE2, met2::GAL7-lacZ, trp1-901, ura3-52) was used for two-hybrid analysis.

Plasmids

The plasmids used in this study are listed in Supplementary Table 1. The pBL813 plasmid encodes *REV3* and *REV7* genes optimized to yeast codon usage. *REV3opt* and *REV7opt* genes were chemically synthesized by GenScript (NJ). Mutations in *REV3* gene were obtained by site-directed mutagenesis using the Quick Change mutagenesis kit (Stratagene). Plasmids and sequences are available upon request.

Supplementary Table 1. Plasmids

name	plasmid	description	reference
pBL346	pRS425-GAL-POL31-POL32	expression of Pol31 and Pol32 under GAL1-10	this work
pBL347	pRS425-GAL-HIS-POL31-POL32	expression of 7xHIS-Pol31 and Pol32 under GAL1-10	this work
pBL348	pRS425-GAL-HIS-POL31-POL32$^{\Delta PIP}$	expression of 7xHIS-Pol31 and Pol32 under GAL1-10	this work
pBL811	pRS426-GAL-GST-REV3	expression of wild type GST-Rev3 under GAL1-10	(28)
pBL811-A1	pRS426-GAL-GST-REV3$^{C1401S, C1417S}$	expression of cysA-GST-Rev3 under GAL1-10	this work
pBL811-A2	pRS426-GAL-GST-REV3$^{C1401A, C1417A}$	expression of cysA`-GST-Rev3 under GAL1-10	this work
pBL811-B	pRS426-GAL-GST-REV3$^{C1449S, C1473S}$	expression of cysB-GST-Rev3 under GAL1-10	this work
pBL812	pRS424-GAL-REV7	expression of Rev7 under GAL1-10	this work
pBL813	pRS426-GAL-GST-REV3opt-REV7opt	expression of wild type GST-Rev3 and Rev7 under GAL1-10 using codon-optimized genes	this work
pBL816	pGBT8-BD-REV3	yeast two hybrid plasmid encoding for wild type Rev3 in a frame with GAL4 DNA binding domain	this work
pBL816-A	pGBT8-BD-REV3 $^{C1401S, C1417S}$	yeast two hybrid plasmid encoding for cysA-Rev3 in a frame with GAL4 DNA binding domain	this work
pBL816-B	pGBT8-BD-REV3$^{C1449S, C1473S}$	yeast two hybrid plasmid encoding for cysB-Rev3 in a frame with GAL4 DNA binding domain	this work
pBL817	pACT2-AD-REV7	yeast two hybrid plasmid encoding for Rev7 in a frame with GAL4 activation domain	this work
pBL364	pACT2-AD-POL31	yeast two hybrid plasmid encoding for Pol31 in a frame with GAL4 activation domain	(16)
pBL391	pACT2-AD-POL32	yeast two hybrid plasmid encoding for Pol32 in a frame with GAL4 activation domain	(16)

Expression and purification of four and two subunit Pol ζ complexes

Stoichiometric and nonstoichiometric Pol ζ_4 and Pol ζ_2 complexes were produced in strains BJ2168, PY117 or PY201 and purified as described previously with modifications (26). Galactose induction was performed at $OD_{660} \geq 3$ and cells were grown for another 10-12 h. 500 - 800 g of cells were resuspended in 3x lysis buffer (150 mM Hepes (pH 7.8), 900 mM KCl, 90 mM K_2HPO_4/KH_2PO_4 (pH 7.8), 6% glycerol, 7.5 mM sucrose, 0.15% Tween 20, 0.03% Nonidet P40, 6 mM DTT, 30 μM pepstatin A, 30 μM leupeptin, 7.5 mM benzamidine) and disrupted in a blender with dry ice. All further steps were carried out at 0–4 °C. When the powder was dissolved, PMSF was added to the suspension to 0.5 mM and glycerol was adjusted to final 8%. To precipitate nucleic acids 45 ml of 10% Polymin P was added per one liter of lysate and the mixture was stirred for 20 min. After preclearing of lysate at 18,000 rpm for 25 min, 0.31 g/ml ammonium sulfate was added to the supernatant and the mixture was stirred for another 20-30 min. The pellet was collected at 18,000 for 20 min and dissolved in 750-1200 ml of buffer A1 (50 mM Hepes (pH 7.4), 300 mM KCl, 30 mM K_2HPO_4/KH_2PO_4 (pH 7.4), 8% glycerol, 2.5 mM sucrose, 0.05% Tween 20, 0.01% Nonidet P-40, 2 mM DTT, 8 μM pepstatin A, 8 μM leupeptin, 2 mM benzamidine, 0.5 mM PMSF) and gently agitated with 2.5 ml of glutathione sepharose

beads (GE Healthcare) for 2 hours. The beads were packed into a disposable BioRad column and washed with 200 ml of buffer A1, followed by 200 ml of A2 (30 mM Hepes (pH 7.8), 200 mM KCl, 30 mM K_2HPO_4/KH_2PO_4 (pH 7.8), 8% glycerol, 2.5 mM sucrose, 0.05% Tween 20, 0.01% Nonidet P-40, 1 mM DTT, 5 mM $MgCl_2$, 1 mM ATP, 2 μM pepstatin A, 0.5 mM PMSF), and buffer A3 (30 mM Hepes (pH 8), 100 mM KCl, 30 mM K_2HPO_4/KH_2PO_4 (pH 8), 8% glycerol, 2.5 mM sucrose, 0.05% Tween 20, 0.01% Nonidet P-40, 1 mM DTT, 2 μM pepstatin A, 0.5 mM PMSF). Proteins were eluted by 4-5 stepwise washes with 2 ml of buffer A3 containing 30 mM of reduced glutathione. Fractions were combined and digested overnight at 4 °C with PreScission protease. The proteins were diluted 2-fold with buffer B0 (30 mM Hepes (pH 7.4), 5% glycerol, 2.5 mM sucrose, 1 mM DTT, 0.01% E10-C12 detergent) and loaded onto a 1-ml heparin agarose column. After washing the column with buffer B1 (30 mM Hepes (pH 7.4), 150 mM KCl, 10 mM K_2HPO_4/KH_2PO_4 (pH 7.4), 5% glycerol, 2.5 mM sucrose, 1 mM DTT, 0.01% E10-C12), the proteins were eluted by step-gradient with buffer B2 (30 mM Hepes (pH 7.4), 750 mM KCl, 20 mM K_2HPO_4/KH_2PO_4 (pH 7.4), 5% glycerol, 2.5 mM sucrose, 1 mM DTT, 0.01% E10-C12). Full stoichiometric Pol ζ_4 was purified as described above, except that a metal-chelate affinity chromatography was performed instead of heparin column step. Fractions with PreScission protease-digested protein were diluted 2-fold with buffer E (30 mM Hepes (pH 7.4), 200 mM KCL, 20 mM K_2HPO_4/KH_2PO_4 (pH 7.4) 5% glycerol, 2.5 mM sucrose, 1 mM DTT, 0.01% E10-C12, 0.5 mM PMSF) containing 10mM imidazole and incubated with 2 ml of Ni-NTA agarose beads (Qiagen) for 45 min. The beads were packed into a column, washed with 400 ml of buffer E with 20 mM imidazole and eluted with buffer E containing 200 mM imidazole. All final preparations were dialyzed against buffer D (30 mM Hepes (pH 7.4), 200 mM NaCl, 8% glycerol, 1 mM DTT).

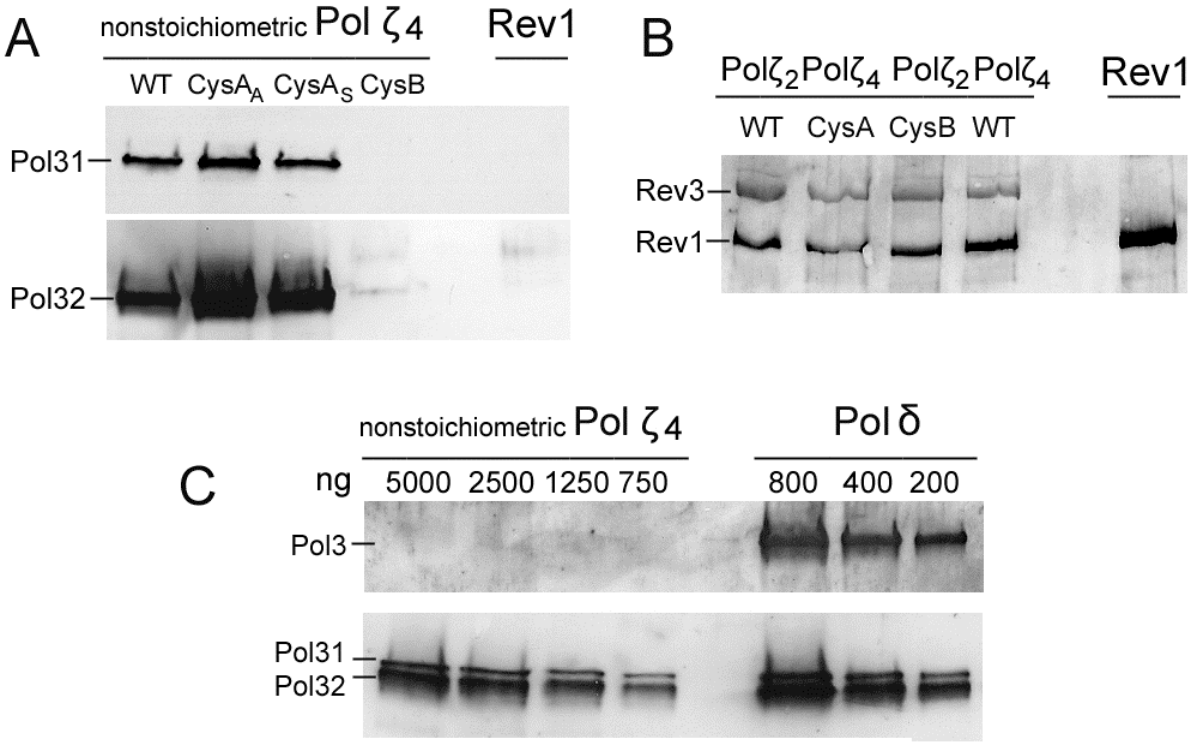

Figure S1: Characterization of composition of stoichiometric and nonstoichiometric Polζ_4 and Polζ_2 complexes preparations by Western analysis. **(A)** Detection of Pol31 and Pol32 in nonstoichiometric Polζ_4 complexes purified from cells without Pol31-Pol32 overexpression. 5 μg of each protein was loaded per lane. $CysA_A$-Polζ_4 contains a double mutation in CysA motif (CC1401,1417AA), $CysA_S$-Polζ_4 variant harbours a CysA mutation (CC1401,1417SS). The right lane had 5 μg of purified Rev1, and shows the lack of Pol31 and Pol32 in the preparation. **(B)** Detection of Rev1 in purified preparations of Polζ_4 and Polζ_2 complexes. The Western blots contained ~600 ng of the indicated complexes, and the right lane contained 80 ng of Rev1 as control. The observed Rev3 signal is due to crossreactivity of Rev3 with the anti-Rev1 antibodies. **(C)** Detection and estimation of amount of Pol3 and Pol31-Pol32 subunits in purified preparation of nonstoichiometric Polζ_{4-} (left lanes) and in Pol δ (right lanes).

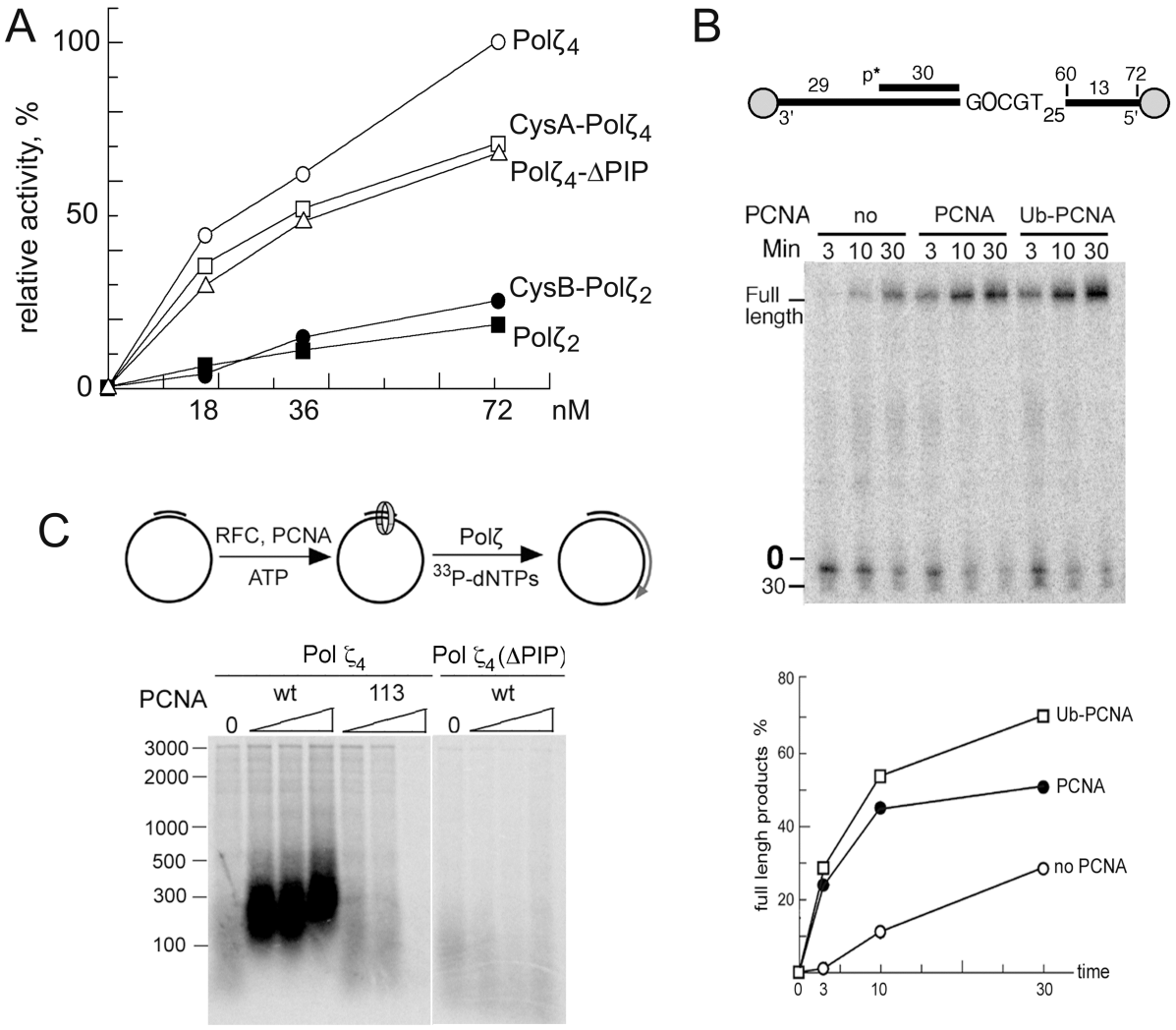

Figure S2: DNA polymerase activity of Polζ$_4$ and Polζ$_2$ complexes. **(A)** Basal DNA polymerase activity of Polζ$_4$, Polζ$_2$, CysA-Polζ$_4$, CysB-Polζ$_2$ and Polζ$_4$ -ΔPIP on activated calf thymus DNA. **(B)** Stimulation of translesion bypass activity of Polζ$_4$ by ubiquitinated and nonubiquitinated PCNA on oligonucleotide substrate with an abasic site. For these experiments we used a split version of PCNA, that is fully functional in vivo, but allows for the facile addition of ubiquiting at position 164 (1). **(C)** Stimulation of DNA polymerase activity of Polζ$_4$ -ΔPIP and Polζ$_4$ by wild type PCNA and by mutant pcna-113 on primed circular bluescript SKII DNA.

Supplementary Reference

47. Freudenthal, B.D., Gakhar, L., Ramaswamy, S. and Washington, M.T. (2010) Structure of monoubiquitinated PCNA and implications for translesion synthesis and DNA polymerase exchange. *Nat Struct Mol Biol*, 17, 479-484.