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

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ABSTRACT

DNA polymerase ζ (Pol ζ) plays a key role in DNA translesion synthesis (TLS) and mutagenesis in eukaryotes. Previously, a two-subunit Rev3–Rev7 complex had been identified as the minimal assembly required for catalytic activity *in vitro*. Herein, we show that *Saccharomyces cerevisiae* Pol ζ binds to the Pol31 and Pol32 subunits of Pol δ, forming a four-subunit Pol ζ_4 complex (Rev3–Rev7–Pol31–Pol32). A [4Fe-4S] cluster in Rev3 is essential for the formation of Pol ζ_4 and damage-induced mutagenesis. Pol32 is indispensible for complex formation, providing an explanation for the long-standing observation that *pol32Δ* strains are defective for mutagenesis. The Pol31 and Pol32 subunits are also required for proliferating cell nuclear antigen (PCNA)-dependent TLS by Pol ζ as Pol ζ_2 lacks functional interactions with PCNA. Mutation of the C-terminal PCNA-interaction motif in Pol32 attenuates PCNA-dependent TLS *in vitro* and mutagenesis *in vivo*. Furthermore, a mutant form of PCNA, encoded by the mutagenesis-defective *pol30-113* mutant, fails to stimulate Pol ζ_4 activity, providing an explanation for the observed mutagenesis phenotype. A stable Pol ζ_4 complex can be identified in all phases of the cell cycle suggesting that this complex is not regulated at the level of protein interactions between Rev3-Rev7 and Pol31-Pol32.

INTRODUCTION

DNA polymerase ζ (Pol ζ) is a B-family DNA polymerase participating in DNA translesion synthesis (TLS) and plays a predominant role in both spontaneous and damage-induced mutagenesis in all eukaryotes (1–3). Pol ζ bypasses a variety of DNA lesions and readily extends mismatched primer-template termini (4,5). Pol ζ was initially identified as a heterodimeric complex of the catalytic Rev3 subunit with the accessory Rev7 subunit that is also required for DNA polymerase activity (6). Mutations in *REV3* or *REV7* result in a severe decrease of induced mutagenesis. The *rev3Δ* and *rev7Δ* strains are also spontaneous antimutators, suggesting that Pol ζ acts to bypass naturally occurring damage or other structural blocks (7–9). Deficiency in the Rev3 catalytic subunit leads to embryonic lethality in mice (10). In humans, alterations in Pol ζ expression are associated with cancer, chromosome instability and cisplatin resistance (11).

All four eukaryotic B-family DNA polymerases, Pol α, δ, ε, and ζ, contain two conserved cysteine-rich metal-binding motifs, CysA and CysB, in the C-terminal domain (CTD) of their catalytic subunits [reviewed in (12,13)]. The four cysteine residues of CysA form a classical zinc ribbon motif. In the case of Pol δ, where the role of both CysA and CysB in metal binding has been studied most extensively, the four-cysteine motif of CysB coordinates a $[4Fe\text{-}4S]^{2+}$ cluster (14). However, the other catalytic subunits have also been shown to bind [4Fe-4S] clusters. Indeed, expression of the CTD of Rev3 in *Escherichia coli* also indicated the presence of a [4Fe-4S] cluster in this domain (14). In Pol δ, the [Fe-S] cluster is required for stable binding of Pol3 to its second subunit Pol31 (14,15), which in turn binds to Pol32 (16–18). The CysB motif of the catalytic subunit of Pol α also coordinates interactions with its second subunit (19,20). Therefore, an arrangement analogous to that determined for Pol δ may also hold for Pol α and for Pol ε.

In contrast to the three replicative DNA polymerases, interactions between the Rev7 subunit of Pol ζ with the catalytic subunit Rev3 have been mapped to the N-terminal region of human Rev3 rather than its CTD (6,21). The possibility then exists that the [4Fe-4S]-containing CTD of Rev3 might provide interactions with other factors that function in mutagenesis. Indeed, two recent articles report on the interaction between Rev3 and Pol31. One interaction study was carried out in

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E. coli with the critical CTD of Rev3 (22), whereas the second study reported the purification of a four-subunit Pol ζ complex from yeast (23). Herein, we also report on the isolation and functional characterization of a four-subunit Pol ζ enzyme (Pol ζ_4) and extend these previous studies by showing that the novel interactions with Pol31 and Pol32 are essential for proliferating cell nuclear antigen (PCNA)-mediated TLS. Mutation of the PCNA-binding domain (PIP) of Pol32 attenuates TLS, in accordance with a decrease in mutagenesis in the *pol32-ΔPIP* mutant (24). Furthermore, deletion of the non-essential *POL32* gene results in a failure to form a complex of Pol31 with Rev3–Rev7, suggesting a logical explanation for the mutagenesis defect of *pol32Δ* mutants (16). Altogether our data suggest that the formation of Pol ζ_4 complex is critical for the TLS function of Pol ζ *in vitro* and *in vivo*.

MATERIALS AND METHODS

Strains and plasmids

All yeast strains are listed in Supplementary Data. Plasmids are listed in Supplementary Table S1.

Enzymes

Saccharomyces cerevisiae Pol δ was expressed in yeast and purified as described previously (25). The replication protein A (RPA), replication factor C (RFC), PCNA and pcna-113 of *Saccharomyces cerevisiae* were expressed and purified from *E. coli* (26,27). Pol ζ_4 (Rev3–Rev7–Pol31–Pol32), Pol ζ_2 (Rev3–Rev7) and their mutant forms were produced in protease-defective strain FM113 or in *pol32Δ* derivative strain PY117, or in *rev1Δ* strain PY201, and purified as described previously with several modifications (28). The detailed protocol is described in Supplementary Data.

Yeast two-hybrid analysis

Indicator strain PJ69-4A was co-transformed with plasmids containing *REV3-GAL4* DNA BD fusion genes (pBL816, pBL816A and pBL816B), and plasmids encoding for *REV7* (pBL817), *POL31* (pBL364) and *POL32* (pBL391) fused to *GAL4* activation domain (AD) or with empty vector pACT2. Transformants were grown on -His plates for 5 days to score protein–protein interactions as growth.

GST-pull down

Yeast cells transformed by plasmids encoding for *GST-REV3, REV7, POL31* and *POL32*, all under control of the GAL1-10 promoter, were grown in 125 ml of selective medium containing 2% raffinose to $O.D_{660} = 0.5$. Protein expression was induced by 2% galactose, and cells were grown for another 8 hours. Cells were collected, resuspended in lysis buffer (50 mM Hepes (pH 7.4), 200 mM NaCl, 5% glycerol, 1 mM DTT, 0.1% Tween 20, 0.01% NP40, 10 μM pepstatin A, 10 μM leupeptin, 2.5 mM benzamidine, 0.5 mM PMSF) and lysed by vortexing with glass beads on ice. Cell lysates were clarified by centrifugation, and 0.8 ml of yeast extract containing 1 mg of protein was incubated with 40 μl of glutathione sepharose beads (GE Healthcare) for 1 h. Beads were washed six times with wash buffer (50 mM Hepes (pH 7.4), 800 mM NaCl, 5% glycerol, 1 mM DTT, 0.1% Tween 20, 0.01% NP40, 1 μM pepstatin A, 0.5 mM PMSF) and boiled for 2 min in 80 μl of 2× sodium dodecyl sulfate (SDS) sample buffer.

Cell cycle analysis and exposure to DNA-damaging agents

Cells containing *GST-REV3* on plasmid pBL813 were grown in 125 ml of selective medium with 2% raffinose to $O.D_{660} = 0.5$ without galactose induction. They were arrested in G1 phase by α-factor (20 μg/ml for 2 h), in G2/M phase with nocodazole (15 μg/ml for 2 h) and in S phase by hydroxyurea (200 mM for 90 min). Then cells were treated with 4NQO (1 μg/ml) or methylmethane sulfonate (0.05%) for 30 min at 30°C. The cells from 200 μl of culture were fixed, stained with propidium iodide and DNA content was measured by flow cytometry. The remaining cultures were harvested, and extract preparation and GST-pull down were performed as described earlier.

Western blot and antibodies

Western blot analysis was performed to detect the presence of GST-Rev3, Rev7, Pol3, Pol31, Pol32 and Rev1 proteins in purified Pol ζ preparations and after pull-down experiments. To detect the Rev1, Rev3 and Rev7 proteins, rabbit polyclonal antisera were raised against purified yeast Rev1 and Pol ζ_2. GST-Rev3 was detected with anti-GST antibody (ab9085, Abcam). Rabbit anti-Pol3, -Pol31 and -Pol32 antibodies were immunopurified. Detection was carried using alkaline phosphatase–conjugated secondary antibody (Sigma) and a BCIP/TNBT chromogenic substrate (Sigma).

DNA polymerase and translesion synthesis assays

Three different assays were used. (i) Measurement of basal DNA polymerase activity: This measures polymerase activity on activated calf thymus DNA, for 45 min at 30°C, as described (29). (ii) DNA replication assay on circular ssDNA: The assay on primed ssDNA (pSKII) was performed as described previously (24). The reactions containing 5 nM of 3 kb circular ssDNA, 500 nM RPA, 3 nM RFC and 10 nM of Pol ζ were incubated at 30°C for 50 min with increasing PCNA as shown in legends to figures. (iii) *In vitro* DNA translesion bypass assay: Sequences of the 107-nt template (with or without a model abasic site) and the primer are given in Supplementary Data. The standard 20 μl reaction contained 40 mM Tris–HCl, pH 7.8, 0.2 mg/ml bovine serum albumin, 8 mM Mg acetate, 120 mM NaCl, 100 μM each dNTPs, 0.5 mM ATP, 10 nM DNA, 15 nM RPA, 30 nM PCNA, 3 nM RFC and 10 nM Pol ζ. The DNA was preincubated with RPA, RFC and PCNA for 30 sec at 30°C, and the reaction was started by addition of Pol ζ and incubated at 30°C. Reactions were stopped with 15 mM ethylenediaminetetraacetic acid and 0.5% SDS and analyzed on a 12% polyacrylamide 7 M urea gel.

Table 1. Damage-induced mutagenesis efficiency of *REV3* mutants

REV3	Spontaneous (10^{-6})	Survival (%)	Induced (10^{-6})
WT	3.1 ± 0.2	56 ± 10	183 ± 30
Δ	2.5 ± 0.5	23 ± 3	1.5 ± 1
cysA	4.7 ± 2	58 ± 4	168 ± 10
cysB	2.0 ± 0.3	12 ± 4	6 ± 2

See 'Materials and Methods' section for details.

Quantification was done by either phosphorimaging of the dried gel (^{32}P) or fluorescence imaging on a Typhoon system.

Damage-induced mutagenesis assays

The *rev3Δ* strain BY4741 (*rev3::KanMX4*) contained empty vector or plasmid pBL811 (GST-*REV3*) or mutants of *REV3* as shown in Table 1. Strains were grown for 2 days to saturation in selective minimal media. The cells were washed with sterile water and 2×10^7 cells plated on selective plates, with or without 80 μg/ml canavanine and either irradiated or not irradiated with 30 J/m^2 of UV light. The plating efficiencies and the percent of UV survival were measured on plates without canavanine. Spontaneous frequencies to canavanine resistance were measured on unirradiated canavanine plates, and UV-induced frequencies to canavanine resistance were measured on irradiated canavanine plates. Colonies appearing after 3 days of growth at 30°C were counted. Frequencies of mutation to canavanine resistance were corrected for the UV survival percentage. The experiments were carried out on three independent cultures, and in duplicate, and the results are presented in Table 1.

RESULTS

The [4Fe-4S] cluster is required for the interaction of Rev3 with the Pol31 subunit of Pol *δ*

The CTD of Pol3 shows strong sequence homology with that of Rev3, particularly in a region C-terminal of the CysB motif (Figure 1A), suggesting the possibility of an interaction between Rev3 and the Pol31–Pol32 subunits of Pol δ. To test this, we performed a yeast two-hybrid analysis using full-length Rev3 as bait (Figure 1B). We co-expressed *REV3,* fused to the *GAL4* DNA BD, together with either *REV7*, as positive control, or with *POL31* or *POL32* fused to the *GAL4* AD, or empty vector. Significant interaction signals were obtained between Rev3 and Rev7 and between Rev3 and Pol31. No interaction between Rev3 and Pol32 was detected by this assay. Importantly, double mutations from cysteine to serine in the CysB motif (*rev3-CC1449,1473SS*), which ligands the [4Fe-4S] cluster, abrogated the Rev3-Pol31 interactions without affecting the Rev3–Rev7 signal. In contrast, double mutations from cysteine to serine in the CysA motif (*rev3-CC1401,1417SS*), did not significantly decrease the Rev3–Pol31 signal (Figure 1B). These data suggest that Pol31 binds to Rev3 through the CysB region, and an intact iron–sulfur cluster is required for interaction. This is the same binding specificity as observed between Pol3 and Pol31 (14).

We next analyzed these interactions by pull-down experiments using GST-Rev3 trapping. We overexpressed *GST-REV3* and *REV7* and assayed for Rev3–Rev7–associated factors by glutathione chromatography (Figure 1C). Significant levels of Pol31 and Pol32 were detected, when compared with controls (Figure 1D, lane 3 vs. 1 and 2). When *POL31* and *POL32* were also overexpressed, a strong interaction signal was detected (lane 4). However, when the same experiment was carried out in a *pol32Δ* strain, Pol31 was undetectable after affinity co-purification (lane 7 vs. lane 3). This defect was rescued by providing back overexpressed *POL32* (lane 8). These data strongly suggest the existence of a four-subunit Rev3–Rev7–Pol31–Pol32 complex called Pol ζ_4. Importantly, unlike Pol δ, in which a Pol3–Pol31 complex is a stable assembly (30), Pol32 is required to stabilize the interactions between Rev3 and Pol31. These important differences in polymerase complex stabilities between Pol δ and Pol ζ explain why *pol32Δ* mutants are viable, but defective for mutagenesis (16).

In agreement with the yeast two-hybrid experiments, we found that Pol31 and Pol32 fail to bind the CysB mutant of GST-Rev3, independent of overexpression (Figure 1E, lanes 5 and 8). In contrast, the CysA mutant of GST-Rev3 pulled down Pol31–Pol32 with the same efficiency as wild-type (compare lane 3 with 4 and 6 with 7).

Rev3-cysB mutant is defective for mutagenesis

Our model suggests that the four-subunit form of Pol ζ is involved in mutagenesis and predicts that mutations disrupting this complex result in a defect in mutagenesis. We measured UV damage-induced mutagenesis in the CysA and CysB mutants of *REV3,* using a forward mutation assay to canavanine resistance (Table 1). Mutations in the CysB motif that are predicted to disrupt iron–sulfur cluster binding disrupt Rev3–Pol31 interactions (Figure 1B and E), which are almost completely defective for damage-induced mutagenesis, although the observed residual signal is higher than that of a *rev3Δ* mutant. However, double cys->ser, or double cys->ala mutations in the CysA motif that should disrupt metal binding to the zinc-ribbon motif show no damage-induced mutagenesis phenotype. Our genetic analysis of the CysA and CysB mutants is in complete agreement with a similar analysis reported recently by Baranovskiy *et al.* (22).

Purification and characterization of two forms of Pol ζ: Pol ζ_2 and Pol ζ_4

To obtain a Pol ζ_4 complex containing an intact [4Fe-4S] cluster, we overexpressed all four genes from galactose-inducible promoters (Figure 1C) and modified the purification protocol of Pol ζ that was described previously (28). Overexpression was carried out in a *rev1Δ* strain to eliminate trace contamination of the purified preparation with Rev1 (see below). The modified procedure made use of two affinity purification tags, an N-terminal GST tag on Rev3 and an N-terminal His_7

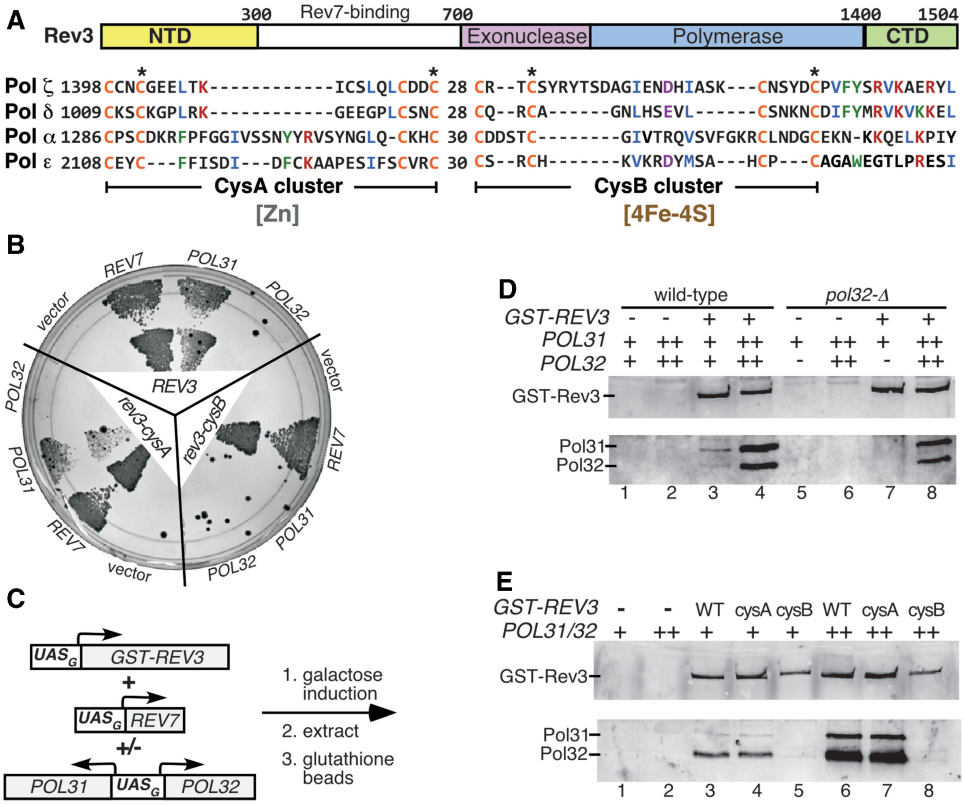

Figure 1. Interaction of Pol ζ catalytic subunit Rev3 with Pol31 and Pol32. (**A**) Domain organization of *S. cerevisiae* Rev3 and alignment of the CTDs of B-family DNA polymerases. The second and fourth residues of each cysteine-rich cluster were mutated in *REV3* to create the CysA (CC1401,1417SS or CC1401,1417AA) and CysB (CC1449,1473SS) mutants. (**B**) Yeast two-hybrid analysis. *REV3*, *rev3-cysA* or rev3-cysB was fused to the *GAL4* DNA-binding domain. *REV7*, *POL31* or *POL32* was fused to the *GAL4* AD; empty vector pACT2 was the negative control. Analysis was in two-hybrid indicator strain PJ69-4A. Cells were grown on His-selective medium. (**C**) Scheme for overexpression of *GST-REV3*, *REV7*, *POL31* and *POL32*, and affinity pull down of complexes. (**D**) Pull down of Pol31 and Pol32 with GST-Rev3. GST-Rev3-Rev7 complex was overexpressed alone or together with Pol31–Pol32 subunits in either wild-type or *Δpol32* yeast. Cell extracts were incubated with glutathione sepharose beads and washed extensively. GST-Rev3 and Pol31 and Pol32 were detected by western analysis. -, gene deleted; +, native level; ++, overexpression. (**E**) Analysis of the interaction between Pol31–Pol32 and GST–Rev3 mutants by GST-pull down. Details are as in (D).

tag on Pol32. First, the extract, after an ammonium sulfate precipitation step, was subjected to glutathione-affinity chromatography. The resistance of the Pol ζ_4 complex to ammonium sulfate precipitation indicates that the interaction between Rev3–Rev7 and Pol31–Pol32 is very strong and specific. This procedure yielded a preparation that was slightly substoichiometric for Pol31–Pol32 (~80–90% in three purifications). Next, after cleavage of the GST-tag by rhinoviral 3C protease, the complex was further purified by Ni-chelate affinity chromatography with ~100 % stoichiometry (Figure 2A). The Pol32-His_7 tag did not influence the activity of the Pol ζ_4 complex (data not shown).

In agreement with the yeast two-hybrid analysis and pull-down experiments, Pol31 and Pol32 were present in affinity-purified preparations of Pol ζ with mutations in the CysA cluster (Rev3-CC1401,1407SS or Rev3-CC1401,1407AA) but not in the purified preparation of Pol ζ sample with mutations in the CysB cluster (Rev3-CC1449S,1473SS) (Supplementary Figure S1A). The Pol ζ_2 complex was purified from a *pol32Δ* strain, and in agreement with the pull-down data in Figure 1D, this two-subunit complex lacks any detectable level of Pol31 by Coomassie staining after sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and by western analysis (Figure 2A).

Overexpression of *REV3* and *REV7* in wild-type yeast without concomitant overexpression of *POL31* and *POL32* yielded affinity-purified preparations that were severely substoichiometrical for Pol31 and Pol32, with abundances ranging from 3 to 15% (Figure 2A). We had previously noted that different Pol ζ preparations were quite variable in activity, but because of the extreme difficulty in purifying the enzyme and the very low yields, it had not been feasible to investigate those issues further at that time (28). We now think that the variations in activity were due to the variable presence of low levels of Pol31–Pol32 that escaped detection. With improved expression and purification methodologies and increased yields, we re-investigated the protein composition of our purified preparations. First, because Rev1 is known to interact with Pol ζ through Rev7 (31), we probed Pol ζ preparations for the presence of Rev1 by western analysis. Both Pol ζ_2 and Pol ζ_4 complexes, as well as all preparations of Pol ζ mutants, contain similar levels of Rev1 (~1–2% compared with Rev3,

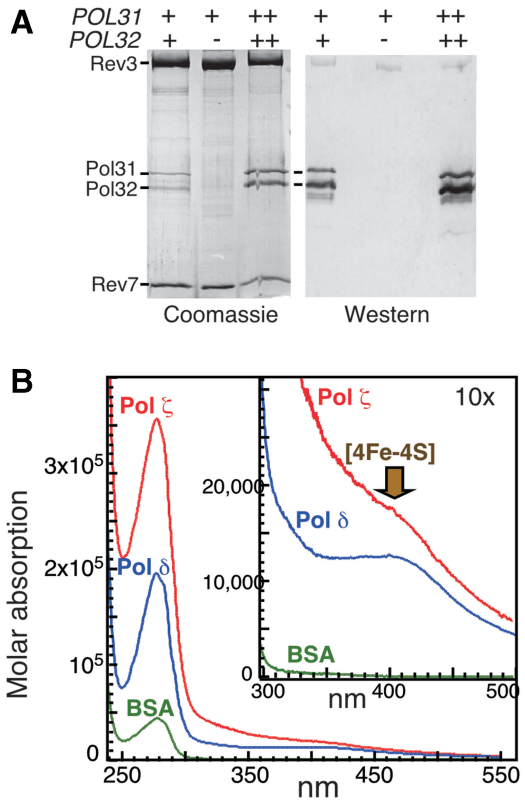

Figure 2. Purification of Pol ζ_2 and Pol ζ_4. (**A**) Subunit composition of substoichiometric Pol ζ_4, Pol ζ_2 and stoichiometric Pol ζ_4 complex. Pol ζ preparations were analyzed by Coomassie blue staining following SDS-PAGE and by western analysis, probed with a mixture of Pol31 and Pol32 antibodies, as indicated. *POL31* and *POL32* were expressed at endogenous levels (+), overexpressed (++) or absent from cells (-). (**B**) UV-VIS spectra of Pol ζ_4, Pol δ and bovine serum albumin. Spectra were collected at ~0.3 to 1 mg/ml protein and recalculated to molar absorptions. Absorption maximum due to the presence of [4Fe-4S] cluster in proteins is indicated.

Supplementary Figure S1B). Therefore, we have purified Pol ζ_2 and Pol ζ_4 from a *rev1Δ* mutant strain without loss of complex stability, indicating that Rev1 is not required for the formation of the Pol ζ_4 complex (data not shown). Second, because Pol31 interacts with the catalytic subunit of Pol δ, we investigated the possibility of the presence of Pol3 by western analysis. However, none of the Pol ζ preparations contained Pol3 at detectable levels (detection limit is ~0.1%), suggesting that Pol31 binds either Pol3 or Rev3, but not both catalytic subunits (Supplementary Figure S1C). Therefore, we conclude that our current forms of Pol ζ_4 and Pol ζ_2 contain the expected subunits without contamination by other proteins that may function in TLS and mutagenesis.

Expression in *E. coli* of the entire CTD of Rev3, containing both CysA and CysB motifs, yielded a yellow-brown preparation that after reduction by dithionite was converted into an electron spin resonance (EPR) active form with the spin signal of that of a $[4Fe\text{-}4S]^{1+}$ cluster (14). This suggests that, like Pol3, Rev3 has a $[4Fe\text{-}4S]^{2+}$ cluster. Indeed, similar to Pol δ, purified Pol ζ_4 has a UV-spectral signature that is indicative of the presence of an iron–sulfur cluster (Figure 2B). Unfortunately, we were unable to obtain sufficiently high quantities of the CysB mutant form to query whether the iron–sulfur cluster was eliminated in the mutant, but on the basis of the strong sequence homology between Pol3 and Rev3 CTD, we accept this as a likely explanation.

Pol31 and Pol32 are essential for functional interactions between PCNA and Pol ζ

The presence of Rev7 is required for DNA polymerase activity of Rev3 (6). We measured basal DNA polymerase activity of Pol ζ preparations on activated DNA in the absence of PCNA. The presence of the Pol31 and Pol32 subunits in Pol ζ_4 enhanced the activity 5- to 10-fold compared with the Pol ζ_2 preparations, which were either obtained by purification from a *pol32Δ* strain or by mutation of the CysB motif in *REV3* (Supplementary Figure S2A).

To determine the role of PCNA in TLS by Pol ζ, we used an oligonucleotide-based replication system with defined template damage. The substrate is incubated with RPA to coat the ssDNA regions, and PCNA is loaded by RFC and ATP. To prevent sliding of PCNA off the DNA, biotin-streptavidin bumpers are added to the 5′- and 3′-termini of the template (Figure 3A). We first assayed the replication by Pol ζ_2 on an undamaged template-primer. Pol ζ_2 activity on this template was much less efficient compared with the Pol ζ_4 complex (Figure 3B). In addition, the presence of PCNA had no detectable effect on DNA replication by Pol ζ_2. Because of the robust activity of Pol ζ_4 on this DNA substrate, PCNA stimulation could not be detected under these conditions. However, PCNA stimulation of Pol ζ_4 on undamaged DNA was readily detected using primed single-stranded plasmid DNA substrates (Supplementary Figure S2C).

To study the role of PCNA in DNA damage TLS, we used the oligonucleotide assay system with a model abasic site at the +2 position after the primer terminus. We observed that Pol ζ_2 readily extended the primer by one nucleotide but did not insert a nucleotide opposite the abasic site, and PCNA did not enhance this activity (Figure 3C). In contrast, the Pol ζ_4 complex bypassed the abasic site damage even in the absence of PCNA. Remarkably, a dramatic stimulation of damage bypass synthesis was detected in the presence of PCNA. These data indicate that formation of the Pol ζ_4 complex is essential for both efficient damage bypass in the absence of PCNA and stimulation of Pol ζ-mediated TLS in the presence of PCNA. Therefore, we conclude that functional interactions between Pol ζ and PCNA require its Pol31 and Pol32 subunits. However, ubiquitination of PCNA did not significantly enhance TLS by Pol ζ_4 (Supplementary Figure S2B). This is consistent with a model in which ubiquitination of PCNA exerts its TLS-promoting activity through Rev1 (32).

The observation that interactions with Pol31–Pol32 enhanced the PCNA-dependent activity of Pol ζ raised the possibility that the PCNA-binding motif is localized in the Pol31 or Pol32 subunit. Previously, we have identified a C-terminal PCNA-binding motif in Pol32

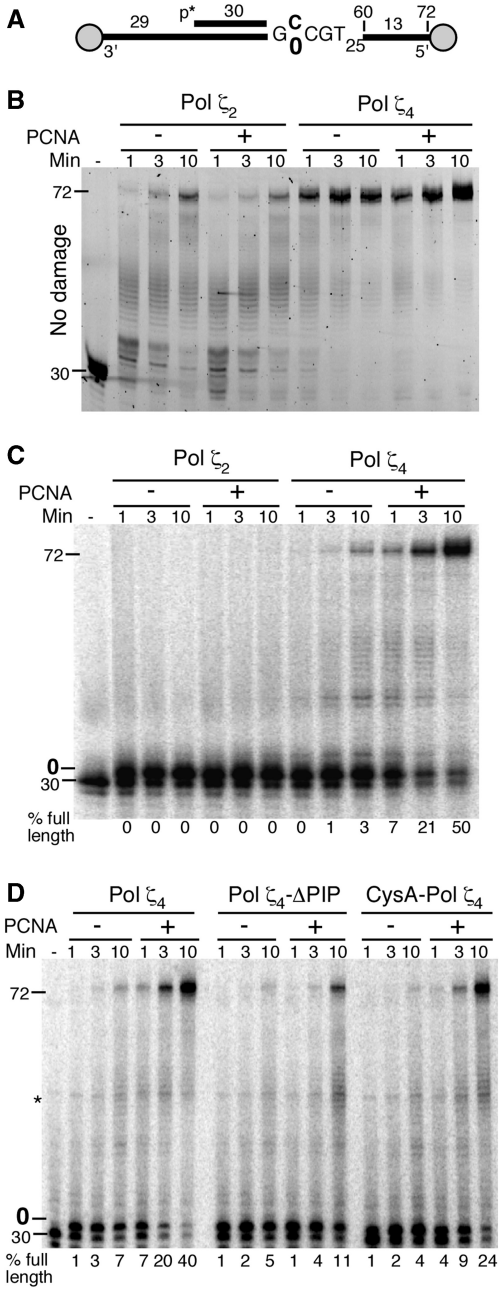

Figure 3. PCNA-mediated translesion activity of Pol ζ_2 and Pol ζ_4. (**A**) A schematic diagram of the oligonucleotide substrate. The template is a 102-mer with streptavidin-biotin blocks at the 5′ and 3′ ends. The template at the +2 position is either a C (in (B)) or an abasic site, indicated as a '0' (in (C) and (D)). The 72-mer products represent full extension of the 30-mer primer to the end of the template. PCNA (30 nM) was added where indicated in (B–D). See Materials and Methods for details. (**B**) Time course of reactions of Pol ζ_2 and Pol ζ_4 on undamaged template DNA. (**C**) Time course of translesion synthesis by Pol ζ_2 and Pol ζ_4 on an abasic site (0) template. (**D**) Stimulation by PCNA of the DNA polymerase activity of Pol ζ_4, Pol ζ_4-ΔPIP and Pol ζ_4-CysA on template DNA with an abasic site. Asterisk indicates an impurity in the radiolabeled primer.

(24). Deletion of this motif only marginally affected processive DNA replication by Pol δ; however, the *pol32-ΔPIP* mutant showed a substantial reduction in the efficiency of damage-induced mutagenesis, particularly at higher loads of DNA damage. We purified a mutant Pol ζ_4 containing a truncated form of Pol32 that lacks its PCNA-binding motif (Pol ζ_4-ΔPIP). Although the basal activities of Pol ζ_4 and Pol ζ_4-ΔPIP were comparable, PCNA stimulation of the mutant complex was substantially reduced (Figure 3D and Supplementary Figure S2A and S2C). We conclude that the PCNA BD of Pol32 contributes to the functional interaction between Pol ζ_4 and PCNA.

DNA replication by Pol δ requires an intact CysA motif, as CysA mutants are severely compromised for PCNA-dependent replication (14). In contrast, the CysA mutant form of Pol ζ_4 demonstrated close to wild-type basal DNA polymerase activity (Supplementary Figure S2A). Although its TLS activity was slightly reduced (~60% of wild-type), PCNA stimulated this TLS activity to the same degree as it did wild-type Pol ζ_4 (Figure 3D). This lack of a strong *in vitro* phenotype is consistent with the lack of a damage-induced mutagenesis phenotype of the same CysA mutations in yeast (Table 1).

The *pol30-113* mutant of PCNA shows severe defects in damage-induced mutagenesis, without affecting the efficiency of a proper DNA damage response through PCNA ubiquitination at Lys164 (27,33). *Pol30-113* has mutations at Glu113 and Leu151 near the monomer–monomer interface of PCNA. Previously, we showed that this mutant form of PCNA was defective for PCNA-mediated TLS *in vitro* (27). With our increased knowledge of the assembly state of Pol ζ, we assume that the previous preparations of Pol ζ contained low levels of Pol31–Pol32 that drove the observed PCNA stimulation. Indeed, the stoichiometrical Pol ζ_4 complex was unable to perform processive replication with pcna-113 (Supplementary Figure S2C).

The Pol ζ_4 complex is stable throughout the cell cycle

To test whether the formation of the Pol ζ_4 complex is subject to either cell cycle or DNA damage control, we prepared synchronized cell populations and determined co-purification of Pol31 and Pol32 with GST-Rev3 on gluthathione sepharose beads. For this experiment, we used the *GST-REV3* expression plasmid, however, omitted galactose induction to maintain Rev3 at low levels. Under the same growth conditions, this construct fully complemented the mutagenesis defect of a *rev3Δ* mutant (data not shown). *POL31* and *POL32* were not overexpressed in these experiments. Cells were arrested in G1 phase with α-factor, in S phase with hydroxyurea and in G2/M phase with nocodazole. About 80–95% of cells were arrested in the appropriate phase of the cell cycle in our experiments (Figure 4A). Synchronized cells were also treated with MMS or 4NQO to induce the DNA damage response. After affinity purification on glutathione beads, the presence of Rev7, Pol32 and Rev1 was monitored by western analysis (Figure 4B). The data indicate that Pol ζ can exist as a four-subunit complex in all phases of the cell cycle. Furthermore, treatment with DNA-damaging agents did not alter the formation or stability of the complex. Interestingly, Rev1 association with Pol ζ is highest in G2 phase. This study addressed the question

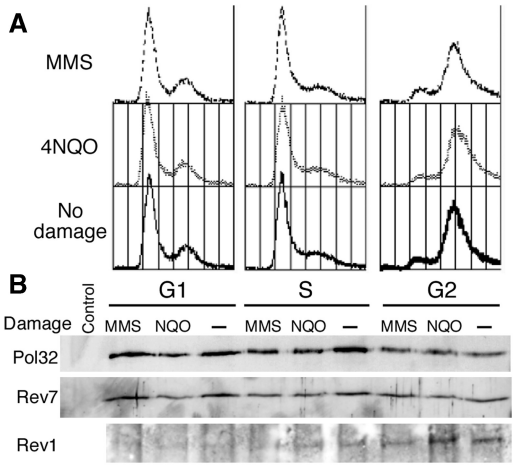

Figure 4. Stability of Pol ζ_4 during the cell cycle. (**A**) Fluorescence-activated cell sorting analysis of the DNA content of cells. Cells expressing low levels of *GST-REV3* and *REV7*, and *POL31* and *POL32* at native levels, were arrested in G1, S, or G2 phase, followed by treatment with MMS or 4NQO. (**B**) Extracts were prepared from arrested cells, and Pol32, Rev7 and Rev1 were detected by western analysis after GST-Rev3 pull down with glutathione sepharose beads. Control: Western analysis of extracts made from cells lacking GST-Rev3 and subjected to glutathione affinity purification.

whether the four-subunit complex, or its stability, is regulated at the level of posttranstional modification, and we found it is not, but we cannot exclude the possibility of cell cycle–specific transcriptional regulation of Rev3.

DISCUSSION

Pol ζ is a low-fidelity, B-family DNA polymerase and the sixth eukaryotic DNA polymerase to be described (6). The original article described a form of Pol ζ that was overexpressed in yeast, and all subsequent studies, including those from our laboratory, used forms that were also purified from yeast overexpression systems (5, 28,30). Therefore, it is likely that these forms contained low, variable levels of Pol31 and Pol32 in the preparations. Our previous observations that TLS by Pol ζ is stimulated by PCNA likely originated from the use of preparations that contained such low levels of Pol31–Pol32, which we now know varied from 3 to 15% over the years. Coupled with the fact that Pol ζ_2 has much lower basal polymerase activity than Pol ζ_4 (Supplementary Figure S2A and Figure S3), the latter species would have contributed more to the observed activity than considerations of abundance suggest. This also explains the variability in activity of different Pol ζ preparations that we remarked on several years ago (28).

Previously, we have shown that the catalytic subunits of the yeast B-family DNA polymerases contain an $[4Fe\text{-}4S]^{2+}$ cluster, coordinated by the CysB motif in their CTDs, and we and others have suggested that all B-family polymerases are similarly organized (14,20). However, a comparison between the architecture of Pol δ and Pol ζ reveals some interesting differences that may underlie their different functions in the cell. Both Pol3 and Rev3 bind Pol31 through their CysB motif as mutations in this motif abrogate binding, while mutations in the CysA motif do not. However, Pol3 forms a stable complex with Pol31 alone (34), but Rev3 does not (Figure1D). As a result, *pol32Δ* mutants are viable, but they are defective for damage-induced mutagenesis (16,35). Furthermore, transformation studies with plasmids containing specific DNA damage show that *pol32Δ* is defective for the bypass of abasic site damage similar to *rev3Δ*, but proficient for the bypass of thymine dimers, which is Pol η dependent (36). This is consistent with Pol32 functioning as an integral part of the Pol ζ complex.

CysA mutations in *POL3* are lethal, most likely because the mutant form of Pol δ is severely defective for PCNA-mediated processive replication (14). However, the analogous mutations in the CysA motif of *REV3* show no defect in mutagenesis [Table 1, (22)] nor is the mutant polymerase defective for PCNA-mediated processive replication (Figure 3D). Functional interactions of Pol δ with PCNA is imparted by multiple potential PCNA-binding motifs in the various subunits of Pol δ (14,24,37–40). In Pol ζ_4, PCNA interacts through the consensus PIP box in the extreme C-terminus of Pol32 as deletion of this motif reduces TLS *in vitro* (Figure 3D). This *POL32* mutant also has a reduced efficiency in damage-induced mutagenesis (24). The residual PCNA stimulation observed *in vitro* and mutagenesis *in vivo* suggests that Pol ζ_4 contains additional PCNA interaction motif(s). The striking difference in CysA phenotype between Pol δ and Pol ζ_4 suggests a different positioning of the PCNA clamp in relation to this motif in these enzymes. Consistent with this, mutations in PCNA differentially affect its interactions with Pol δ versus Pol ζ. A pcna-113 mutant functions as a processivity clamp for Pol δ, although its activity is somewhat reduced (27); however it is defective with Pol ζ_4 (Supplementary Figure S2C). This provides a rational explanation for the mutagenesis defect in this mutant.

The formation and stability of the Pol ζ_4 complex was unaffected by the cell cycle or by exposure to DNA-damaged agents (Figure 4). This result suggests that Pol ζ-mediated mutagenesis can occur throughout the cell cycle. However, other factors, for example, Rev1 and PCNA, show cell cycle and/or DNA damage control, and overall pathway control is likely mediated through those factors. Ubiquitination of PCNA is a key switch in this pathway, and both damage-induced mutagenesis as well as spontaneous mutagenesis in response to replisome dysfunction is dependent on ubiquitination of PCNA (27,41,42). The Rev1 protein, considered to be the scaffold onto which the mutasome assembles through binding of ubiquitinated PCNA on one hand and Pol ζ on the other hand, is most highly expressed in G2 phase (43). Indeed, it has been shown that PCNA ubiquitination and mutagenesis can be restricted to the G2 phase of the cell cycle (44,45). We found that Rev1 association with Pol ζ_4 is also highest during G2 phase (Figure 4). Therefore we suggest that the regulation of Pol ζ_4-dependent mutagenesis is likely mediated by the formation of multisubunit complexes of higher order, for example

with Rev1 and ubiquitinated PCNA, but not through the assembly of the Pol ζ_4 complex. Finally, the cell cycle kinase *CDC7/DBF4* promotes the efficiency of UV mutagenesis (46).

As stated before, two other groups have recently reported that Rev3 interacts with Pol31 and Pol32. The article by Baranovskiy *et al.* reported the co-purification from *E. coli* of the CTD of human Rev3 together with human Pol31 and Pol32 (22). Although this approach did not permit functional polymerase studies, it allowed these authors to probe the relevance of the CysA and CysB motifs for complex formation. In agreement with our results in Figure 1E, CysB mutations, but not CysA mutations, abrogated complex formation. Similarly, their genetic studies of the CysA and B motifs in yeast yielded analogous results to ours (Table 1). The second article by Johnson *et al.* reported the isolation of a Pol ζ_4 complex from a yeast overexpression system and is in accord with ours when all four genes are overexpressed (23). However, our conclusion that Pol32 is required for stable complex formation between Rev3 and Pol31 is at variance with their study. These authors reported the purification of a three-subunit Rev3-Rev7-Pol31 complex from a strain that overexpressed just the *REV3*, *REV7*, and *POL31* genes, and based on this purification concluded that Pol32 was not required for complex formation. However, this three-subunit preparation was purified from a wild-type yeast strain rather than a *pol32Δ* strain and was highly non-stoichiometric containing predominantly the Pol31 polypeptide, to which the purification tag was fused. Given the low levels of Rev3 in this preparation, and the close migration of Pol31 and Pol32 by SDS-PAGE, low levels of Pol32 may have escaped detection. Unfortunately, a more sensitive western analysis with Pol32 antibodies was not used as a detection method in this study. We think that these are important considerations, because our study indicates that Pol32 is absolutely required for complex formation and thereby provides a logical explanation for the long-standing observation that *pol32Δ* strains are defective for damage-induced mutagenesis.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online: Supplementary Text, Supplementary Table 1, Supplementary Figures 1 and 2 and Supplementary Reference [47].

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Conflict of interest statement. None declared.

REFERENCES

1. Morrison,A., Christensen,R.B., Alley,J., Beck,A.K., Bernstine,E.G., Lemontt,J.F. and Lawrence,C.W. (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.
2. Lawrence,C.W. and Maher,V.M. (2001) Eukaryotic mutagenesis and translesion replication dependent on DNA polymerase zeta and Rev1 protein. *Biochem. Soc. Trans.*, **29**, 187–191.
3. Gibbs,P.E., McGregor,W.G., Maher,V.M., Nisson,P. and Lawrence,C.W. (1998) A human homolog of the *Saccharomyces cerevisiae* REV3 gene, which encodes the catalytic subunit of DNA polymerase zeta. *Proc. Natl Acad. Sci. USA*, **95**, 6876–6880.
4. Guo,D., Wu,X., Rajpal,D.K., Taylor,J.S. and Wang,Z. (2001) Translesion synthesis by yeast DNA polymerase zeta from templates containing lesions of ultraviolet radiation and acetylaminofluorene. *Nucleic Acids Res.*, **29**, 2875–2883.
5. Haracska,L., Prakash,S. and Prakash,L. (2003) Yeast DNA polymerase zeta is an efficient extender of primer ends opposite from 7,8-dihydro-8-Oxoguanine and O6-methylguanine. *Mol. Cell. Biol.*, **23**, 1453–1459.
6. Nelson,J.R., Lawrence,C.W. and Hinkle,D.C. (1996) Thymine-thymine dimer bypass by yeast DNA polymerase zeta. *Science*, **272**, 1646–1649.
7. Lemontt,J.F. (1971) Mutants of yeast defective in mutation induced by ultraviolet light. *Genetics*, **68**, 21–33.
8. Lawrence,C.W., Das,G. and Christensen,R.B. (1985) REV7, a new gene concerned with UV mutagenesis in yeast. *Mol. Gen. Genet.*, **200**, 80–85.
9. Larimer,F.W., Perry,J.R. and Hardigree,A.A. (1989) The REV1 gene of *Saccharomyces cerevisiae*: isolation, sequence, and functional analysis. *J. Bacteriol.*, **171**, 230–237.
10. Wittschieben,J., Shivji,M.K., Lalani,E., Jacobs,M.A., Marini,F., Gearhart,P.J., Rosewell,I., Stamp,G. and Wood,R.D. (2000) Disruption of the developmentally regulated Rev3 gene causes embryonic lethality. *Curr. Biol.*, **10**, 1217–1220.
11. Lin,X., Trang,J., Okuda,T. and Howell,S.B. (2006) DNA polymerase zeta accounts for the reduced cytotoxicity and enhanced mutagenicity of cisplatin in human colon carcinoma cells that have lost DNA mismatch repair. *Clin. Cancer Res.*, **12**, 563–568.
12. Tahirov,T.H., Makarova,K.S., Rogozin,I.B., Pavlov,Y.I. and Koonin,E.V. (2009) Evolution of DNA polymerases: an inactivated polymerase-exonuclease module in Pol epsilon and a chimeric origin of eukaryotic polymerases from two classes of archaeal ancestors. *Biol. Direct*, **4**, 11.
13. Johansson,E. and Macneill,S.A. (2010) The eukaryotic replicative DNA polymerases take shape. *Trends Biochem. Sci.*, **35**, 339–347.
14. Netz,D.J., Stith,C.M., Stumpfig,M., Kopf,G., Vogel,D., Genau,H.M., Stodola,J.L., Lill,R., Burgers,P.M. and Pierik,A.J. (2011) Eukaryotic DNA polymerases require an iron-sulfur cluster for the formation of active complexes. *Nat. Chem. Biol.*, **8**, 125–132.
15. MacNeill,S.A., Moreno,S., Reynolds,N., Nurse,P. and Fantes,P.A. (1996) The fission yeast Cdc1 protein, a homologue of the small subunit of DNA polymerase delta, binds to Pol3 and Cdc27. *EMBO J.*, **15**, 4613–4628.
16. Gerik,K.J., Li,X., Pautz,A. and Burgers,P.M. (1998) Characterization of the two small subunits of *Saccharomyces cerevisiae* DNA polymerase delta. *J. Biol. Chem.*, **273**, 19747–19755.
17. Sanchez Garcia,J., Ciufo,L.F., Yang,X., Kearsey,S.E. and MacNeill,S.A. (2004) The C-terminal zinc finger of the catalytic subunit of DNA polymerase delta is responsible for direct interaction with the B-subunit. *Nucleic Acids Res.*, **32**, 3005–3016.
18. Baranovskiy,A.G., Babayeva,N.D., Liston,V.G., Rogozin,I.B., Koonin,E.V., Pavlov,Y.I., Vassylyev,D.G. and Tahirov,T.H. (2008) X-ray structure of the complex of regulatory subunits of human DNA polymerase delta. *Cell Cycle*, **7**, 3026–3036.

19. Mizuno,T., Yamagishi,K., Miyazawa,H. and Hanaoka,F. (1999) Molecular architecture of the mouse DNA polymerase alpha-primase complex. *Mol. Cell. Biol.*, **19**, 7886–7896.
20. Klinge,S., Nunez-Ramirez,R., Llorca,O. and Pellegrini,L. (2009) 3D architecture of DNA Pol alpha reveals the functional core of multi-subunit replicative polymerases. *EMBO J.*, **28**, 1978–1987.
21. Hara,K., Hashimoto,H., Murakumo,Y., Kobayashi,S., Kogame,T., Unzai,S., Akashi,S., Takeda,S., Shimizu,T. and Sato,M. (2010) Crystal structure of human REV7 in complex with a human REV3 fragment and structural implication of the interaction between DNA polymerase zeta and REV1. *J. Biol. Chem.*, **285**, 12299–12307.
22. Baranovskiy,A.G., Lada,A.G., Siebler,H.M., Zhang,Y., Pavlov,Y.I. and Tahirov,T.H. (2012) DNA polymerase delta and zeta switch by sharing accessory subunits of DNA polymerase delta. *J. Biol. Chem.*, **287**, 17281–17287.
23. Johnson,R.E., Prakash,L. and Prakash,S. (2012) Pol31 and Pol32 subunits of yeast DNA polymerase delta are also essential subunits of DNA polymerase zeta. *Proc. Natl Acad. Sci. USA*, **109**, 12455–12460.
24. Johansson,E., Garg,P. and Burgers,P.M. (2004) The Pol32 subunit of DNA polymerase delta contains separable domains for processive replication and proliferating cell nuclear antigen (PCNA) binding. *J. Biol. Chem.*, **279**, 1907–1915.
25. Fortune,J.M., Stith,C.M., Kissling,G.E., Burgers,P.M. and Kunkel,T.A. (2006) RPA and PCNA suppress formation of large deletion errors by yeast DNA polymerase delta. *Nucleic Acids Res*, **34**, 4335–4341.
26. Henricksen,L.A., Umbricht,C.B. and Wold,M.S. (1994) Recombinant replication protein A: expression, complex formation, and functional characterization. *J. Biol. Chem.*, **269**, 11121–11132.
27. Northam,M.R., Garg,P., Baitin,D.M., Burgers,P.M. and Shcherbakova,P.V. (2006) A novel function of DNA polymerase zeta regulated by PCNA. *EMBO J*, **25**, 4316–4325.
28. Garg,P., Stith,C.M., Majka,J. and Burgers,P.M. (2005) Proliferating Cell Nuclear Antigen Promotes Translesion Synthesis by DNA Polymerase zeta. *J Biol Chem*, **280**, 23446–23450.
29. Bauer,G.A., Heller,H.M. and Burgers,P.M.J. (1988) DNA polymerase III from Saccharomyces cerevisiae. I. Purification and characterization. *J. Biol. Chem.*, **263**, 917–924.
30. Murakumo,Y., Ogura,Y., Ishii,H., Numata,S., Ichihara,M., Croce,C.M., Fishel,R. and Takahashi,M. (2001) Interactions in the error-prone postreplication repair proteins hREV1, hREV3, and hREV7. *J Biol. Chem.*, **276**, 35644–35651.
31. Wood,A., Garg,P. and Burgers,P.M. (2007) A ubiquitin-binding motif in the translesion DNA polymerase Rev1 mediates its essential functional interaction with ubiquitinated proliferating cell nuclear antigen in response to DNA damage. *J. Biol. Chem.*, **282**, 20256–20263.
32. Amin,N.S. and Holm,C. (1996) In vivo analysis reveals that the interdomain region of the yeast proliferating cell nuclear antigen is important for DNA replication and DNA repair. *Genetics*, **144**, 479–493.
33. Murakumo,Y. (2002) The property of DNA polymerase zeta: REV7 is a putative protein involved in translesion DNA synthesis and cell cycle control. *Mutat. Res.*, **510**, 37–44.
34. Burgers,P.M. and Gerik,K.J. (1998) Structure and processivity of two forms of *Saccharomyces cerevisiae* DNA polymerase delta. *J. Biol. Chem.*, **273**, 19756–19762.
35. Huang,M.E., Rio,A.G., Galibert,M.D. and Galibert,F. (2002) Pol32, a subunit of *Saccharomyces cerevisiae* DNA polymerase delta, suppresses genomic deletions and is involved in the mutagenic bypass pathway. *Genetics*, **160**, 1409–1422.
36. Gibbs,P.E., McDonald,J., Woodgate,R. and Lawrence,C.W. (2005) The relative roles in vivo of *Saccharomyces cerevisiae* Pol eta, Pol zeta, Rev1 Protein and Pol32 in the bypass and mutation induction of an abasic site, T-T (6-4) photoadduct and T-T cis-syn cyclobutane dimer. *Genetics*, **169**, 575–582.
37. Zhou,J.Q., He,H., Tan,C.K., Downey,K.M. and So,A.G. (1997) The small subunit is required for functional interaction of DNA polymerase delta with the proliferating cell nuclear antigen. *Nucleic Acids Res.*, **25**, 1094–1099.
38. Zhang,P., Mo,J.Y., Perez,A., Leon,A., Liu,L., Mazloum,N., Xu,H. and Lee,M.Y. (1999) Direct interaction of proliferating cell nuclear antigen with the p125 catalytic subunit of mammalian DNA polymerase delta. *J. Biol. Chem.*, **274**, 26647–26653.
39. Reynolds,N., Warbrick,E., Fantes,P.A. and MacNeill,S.A. (2000) Essential interaction between the fission yeast DNA polymerase delta subunit Cdc27 and Pcn1 (PCNA) mediated through a C-terminal p21(Cip1)-like PCNA binding motif. *EMBO J.*, **19**, 1108–1118.
40. Acharya,N., Klassen,R., Johnson,R.E., Prakash,L. and Prakash,S. (2011) PCNA binding domains in all three subunits of yeast DNA polymerase delta modulate its function in DNA replication. *Proc. Natl Acad. Sci. USA*, **108**, 17927–17932.
41. Hoege,C., Pfander,B., Moldovan,G.L., Pyrowolakis,G. and Jentsch,S. (2002) RAD6-dependent DNA repair is linked to modification of PCNA by ubiquitin and SUMO. *Nature*, **419**, 135–141.
42. Stelter,P. and Ulrich,H.D. (2003) Control of spontaneous and damage-induced mutagenesis by SUMO and ubiquitin conjugation. *Nature*, **425**, 188–191.
43. Waters,L.S. and Walker,G.C. (2006) The critical mutagenic translesion DNA polymerase Rev1 is highly expressed during G(2)/M phase rather than S phase. *Proc. Natl Acad. Sci. USA*, **103**, 8971–8976.
44. Daigaku,Y., Davies,A.A. and Ulrich,H.D. (2010) Ubiquitin-dependent DNA damage bypass is separable from genome replication. *Nature*, **465**, 951–955.
45. Karras,G.I. and Jentsch,S. (2010) The RAD6 DNA damage tolerance pathway operates uncoupled from the replication fork and is functional beyond S phase. *Cell*, **141**, 255–267.
46. Pessoa-Brandao,L. and Sclafani,R.A. (2004) CDC7/DBF4 functions in the translesion synthesis branch of the RAD6 epistasis group in *Saccharomyces cerevisiae*. *Genetics*, **167**, 1597–1610.
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.

SUPPLEMENTARY DATA TO:

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

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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).

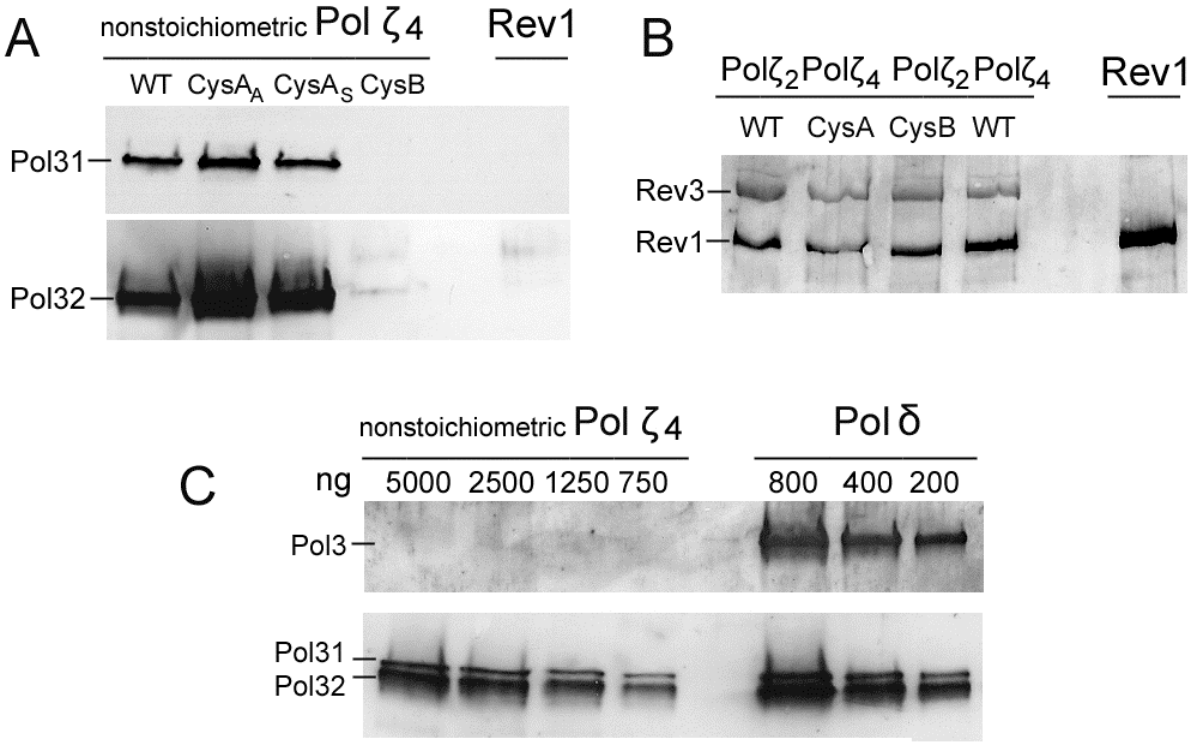

Figure S1: Characterization of composition of stoichiometric and nonstoichiometric Polζ_4 and Polζ_2 complexes preparations by Western analysis. **(A)** Detection of Pol31 and Pol32 in nonstoichiometric Polζ_4 complexes purified from cells without Pol31-Pol32 overexpression. 5 μg of each protein was 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).

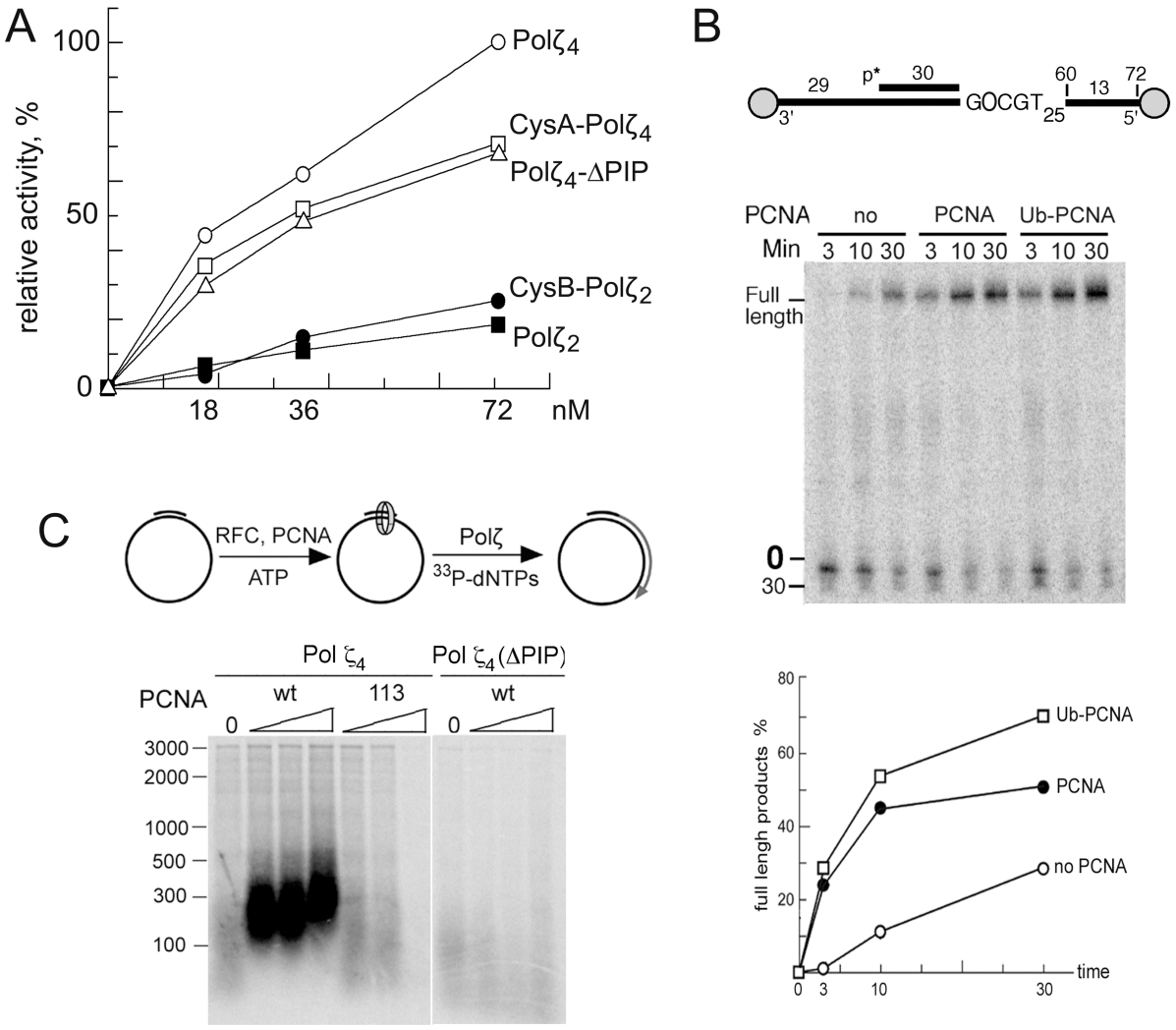

Figure S2: DNA polymerase activity of Polζ$_4$ and Polζ$_2$ complexes. **(A)** Basal DNA polymerase activity of Polζ$_4$, Polζ$_2$, CysA-Polζ$_4$, CysB-Polζ$_2$ and Polζ$_4$ -ΔPIP on activated calf thymus DNA. **(B)** Stimulation of translesion bypass activity of Polζ$_4$ by ubiquitinated and nonubiquitinated 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.