Proficient Replication of the Yeast Genome by a Viral DNA Polymerase

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ABSTRACT

DNA replication in eukaryotic cells requires minimally three B-family DNA polymerases: Pol α, Pol δ and Pol ε. Pol δ replicates and matures Okazaki fragments on the lagging strand of the replication fork. *Saccharomyces cerevisiae* Pol δ is a three-subunit enzyme (Pol3-Pol31-Pol32). A small C-terminal domain of the catalytic subunit Pol3 carries both iron-sulfur cluster and zinc binding motifs, which mediate interactions with Pol31, and processive replication with the replication clamp PCNA, respectively. We show that the entire N-terminal domain of Pol3, containing polymerase and proofreading activities, could be effectively replaced by those from bacteriophage RB69, and carry out chromosomal DNA replication in yeast with remarkable high fidelity, provided adaptive mutations in the replication clamp PCNA were introduced. This result is consistent with the model that all essential interactions for DNA replication in yeast are mediated through the small C-terminal domain of Pol3. The chimeric polymerase carries out processive replication with PCNA *in vitro*, however, in yeast, it requires an increased involvement of the mutagenic translesion DNA polymerase ζ during DNA replication.

Replication of genomic DNA during each cell cycle requires the action of replicative DNA polymerases. In order to ensure faithful transmission of genomic information from the parent to the daughter cells, these polymerases must work efficiently and with very high fidelity (1). The eukaryotic replicative DNA polymerases are members of the B-family polymerases, which are classified as such according to the structure of their catalytic domain (2-5). Three B-family DNA polymerases participate in DNA replication. The current model is that Pol ε replicates the leading strand of the replication fork, whereas Pol α-primase initiates Okazaki fragments on the lagging strand that are elongated and matured by Pol δ (6). However, a recent study suggests that Pol δ may also carry out substantial DNA synthesis of the leading strand (7). A fourth B-family enzyme, Pol ζ is required for translesion synthesis in response to DNA damage, which results in the bulk of mutagenesis in eukaryotes (8), and also participates in replication past structural blocks when replication forks stall (9).

B-family DNA polymerases are ubiquitous; they are found in eukaryotes, bacteria, archaea, and in both bacterial and eukaryotic DNA-based viruses (10). All B-family enzymes contain three large domains: the polymerase domain with the palm, finger, and thumb sub-domains, the 3'-5' exonuclease domain, which is active in some but not all members of the family, and a structural N-terminal domain (NTD) of unknown function. However, the cellular eukaryotic members of the family are structurally more complex in that they are multi-subunit enzymes, and furthermore, they uniquely contain an additional, small C-terminal domain (CTD) in the polymerase subunit that mediates interactions with these accessory subunits (10,11).

The CTDs of the four eukaryotic enzymes are highly conserved suggesting a common structure for each of these enzymes. The crystal structure of the CTD from Pol α shows an elongated, bilobal form, in which the two lobes are connected by a three-helical bundle (11). Each lobe contains four conserved cysteines (Fig. 1A). The N-terminal 4-cysteine lobe binds zinc, while the C-terminal 4-cysteine lobe of all four enzymes has been proposed to ligand an iron-sulfur cluster in the $[4Fe\text{-}4S]^{2+}$ coordination state (12). The CTDs of Pol α, Pol δ, and Pol ε each bind to distinct B subunit subunits (11-13), which show both sequence and structural conservation (11,14,15). Pol ζ has appropriated the

Pol31 B subunit from Pol δ to elaborate its 4-subunit assembly (Rev3-Rev7-Pol31-Pol32) (16-19).

In order to better understand how the multi-subunit structures of eukaryotic replicative DNA polymerases are intricately tied to their function, we have used the lagging strand polymerase Pol δ as a model. This polymerase complex in budding yeast consists of the catalytic subunit Pol3 and the accessory subunits Pol31 and Pol32 (20). Interactions between Pol3 and Pol31 occur through the Pol3 CTD and require an intact iron-sulfur cluster (12). Pol31 then binds the third subunit Pol32 to form the complete heterotrimeric polymerase complex (20). This architecture is conserved in other organisms (15,21), except for the presence of an additional small, regulatory subunit in fission yeast and in mammals (22,23). Pol δ alone is a low-processivity enzyme, replicating only a few nucleotides before dissociating from DNA. This problem is overcome through interactions between Pol δ and the replication clamp proliferating cell nuclear antigen (PCNA) (24). This donut-shaped homotrimeric protein is loaded onto DNA primer-termini by the ATP-dependent Replication Factor C (RFC) complex, and DNA-bound PCNA recruits Pol δ and increases the processivity of the enzyme (25), so that it can replicate hundreds of nucleotides in a single DNA-binding event. PCNA-dependent polymerase processivity is vital to efficient genomic DNA replication. Pol δ mutants that are defective for interactions with PCNA exhibit *in vitro* processivity defects that, if severe, are associated with lethality in yeast (12,26,27).

We were interested in understanding better what activities of Pol δ are required in order to replicate the budding yeast genome. Since the catalytic polymerase and exonuclease activities of Pol δ are conserved in viral and bacterial members of the B-family, we hypothesized that the essential factors enabling Pol δ to act in a eukaryotic setting are the ability to bind its accessory subunits and PCNA. In order to determine whether the Pol3 catalytic core or merely its catalytic activities are required for lagging strand replication, we created a chimeric polymerase subunit by replacing the Pol3 catalytic core domains with those from the structurally homologous bacteriophage RB69 DNA polymerase. Rb69 and T4 are closely related bacteriophages that use a polymerase processivity model similar to Pol δ, containing a homotrimeric clamp and an ATP-dependent clamp loader (gp45 and gp44/62, respectively) (28).

Fusing the 104 kDa RB69 polymerase to the 13 kDa CTD of Pol3 is sufficient to form a three-subunit polymerase complex with Pol31 and Pol32 in yeast. The processivity of this polymerase complex is stimulated by PCNA, but was compromised as compared to Pol δ. We obtained more robust stimulation of this engineered form of Pol δ when we introduced two adaptive mutations in PCNA, and this genetic arrangement confers growth in yeast with the fusion polymerase as only source of Pol δ. Remarkably, when we eliminated fidelity-lowering contributions made by the mutagenic Pol ζ, the fidelity of the engineered Pol δ was only a few-fold lower than that of the native enzyme.

EXPERIMENTAL PROCEDURES

Yeast strains, proteins, and genetic techniques – Strains were derived from PY227 by integration of the appropriate gene deletion cassettes. PY227 (*MATα his3Δ-1 leu2-3,112 trp1-Δ ura3-52 pol3Δ::KANMX4* + pBL304 (*POL3 URA3*)); PY236 (PY227 but *leu2::pBL248-rb2 (LEU2, pol30-rb2 (pol30-Q29H,K31R*))); PY237 (PY236 but *rev3Δ::NATMX4*), PY238 (PY236 but *rad30Δ::HIS3*), PY239 (PY236 but *rev3Δ::NATMX4 rad30Δ::HIS3*). They were transformed with pBL309 (*POL3* in pRS314 (*CEN6 ARSH1 TRP1*)), or pBL326 (RbPol(1-896)-*POL3*(981-1097) fusion under control of the attenuated *ADH1* promoter, in pRS424 (TRP1 2μm ori)) plasmids, or vector, with Trp selection, and then passed over 5-FOA media to eject complementing plasmid pBL304.

Pol δ, Rb69 DNA polymerase (RbPol), PCNA, RFC, RPA, FEN1, and DNA ligase I were purified as described (2,51,52). In order to obtain RbPol δ, yeast strain BJ2168 (*MAT**a** ura3-52 trp1-289 leu2-3,112 prb1–1122 prc1-407 pep4-3*) was transformed with plasmids pBL341 (2μm ori *URA3 GAL1-POL31 GAL10-POL32*) and pBL325 (2μm ori *TRP1 GAL1*-[GST-3C-RbPol(1-896)-*POL3*(981-1097) fusion]). Growth and galactose induction and extract preparation was as described, and RbPol δ was purified by glutathione-affinity purification and, following removal of the GST tag with rhinoviral 3C protease, by MonoS chromatography analogously to described for Pol δ (52).

DNA damage sensitivity assays were carried using standard protocols. Fluctuation analyses to determine spontaneous mutation rates were carried out in triplicate with 15-20

independent cultures, and analyzed by the median (53).

Identification of PCNA suppressor mutants – The *POL30* gene in pBL249 (*POL30* in pRS315 (*CEN ARS TRP1*)) was PCR-mutagenized as described (54). The library was transformed into PY227 containing both pBL304 and pBL326, and plated onto SC-Leu media, and after 2 days of growth, replica-plated onto SC-Leu plates containing 5-fluoroorotic acid (5-FOA), to evict the pBL304 plasmid. Plasmid DNA was isolated from positive colonies and re-applied to the same screen. The pBL249 isolates from the second screen that allowed yeast growth without pBL304 were sequenced. The most robust suppressor *pol30-rb1* carried six non-synonymous mutations (F12Y, D17A, Q29H, K31R, I52M, I100T). Each mutation was separately reverted back to wild-type and loss of suppression assessed. From this analysis, we determined that the Q29H mutation was essential for suppression, and K31R increased suppression. Therefore, *pol30-rb2* contains only the Q29H and K31R mutations.

DNA replication assays – Assays contained 20 mM Tris-HCl pH 7.8, 1 mM DTT, 100 μg/ml bovine serum albumin, 8 mM magnesium acetate, 0.5 mM ATP, 100 μM each of dCTP, dGTP, and dTTP, 10 mM of [α-^{32}P]dATP, 100 mM NaCl, 3.5 nM single-stranded bluescript DNA, singly primed (at positions 592-621) either with a 30-mer DNA primer or 5'-RNA_8DNA_{22} primer, 400 nM RPA, and PCNA or pcna-rb2 as indicated. PCNA was loaded onto the primed DNA by incubation with 7 nM RFC at 30 °C for 1 min prior to reaction initiation. Reactions were initiated by addition of 7 nM Pol δ or RbPol δ. In the assays in Fig. 2D, 7 nM FEN1 and 14 nM DNA ligase I were added together with the polymerase. Aliquots were taken at various time points and stopped with 50 mM EDTA and 0.2% SDS, final concentration. Reactions were either resolved on a 1% alkaline agarose gel (Fig. 2B) or a 1% agarose gel containing 0.5 μg/ml ethidium bromide. Gels were dried and documented by PhosphorImager analysis (GE Healthcare). Alternatively, 1 ml of 10% trichloroacetic acid was added to stopped replication samples. After 10 min on ice, the mixture was filtered over a GF/C filter, The filter was washed twice with 2 ml of 1M HCl and 0.05 M sodium pyrophosphate, rinsed with ethanol, dried, and counted in counting fluid in a liquid scintillation counter. All assays were carried in duplicate or triplicate, and either representative gels are presented or standard errors are shown (Fig. 2C).

RESULTS AND DISCUSSION

Designing the Rb69-Pol3 polymerase fusion gene – Bacteriophage T4 expresses a replication elongation apparatus consisting of a B-family DNA polymerase, a homotrimeric replication clamp gp45, which is the ortholog of eukaryotic PCNA, and an ATP-dependent clamp loader. While extensive biochemical and genetic DNA replication studies are available for the T4 system (29,30), we focused our attention on the highly related bacteriophage Rb69, because its DNA polymerase has been the subject of detailed structural characterization (3,31). Rb69 DNA polymerase can efficiently substitute for T4 DNA polymerase in faithfully replicating the T4 genome (32). The closest eukaryotic homologue to these bacteriophage enzymes is Pol3, the catalytic subunit of Pol δ. T4 and Rb69 DNA polymerase (Rb-Pol) not only carry out high-fidelity DNA replication, but are also responsible for the proper maturation of Okazaki fragments during phage DNA replication. The latter activity is allocated solely to Pol δ in eukaryotic cells (33). Fig. 1A shows a structural comparison between Rb69-Pol and aa95-985 of the 1097aa yeast Pol3 (3,34). The structures of both enzymes were solved in a complex with template-primer and a base-paired dNTP. The Pol3 structure comprises the structured NTD and the exonuclease and polymerase domains, but lacks the unstructured N-terminal tail and its CTD. Only the structure of the Pol α CTD has been solved, and it serves as a structural model for this domain in the other B-family DNA polymerases (Fig. 1A)(11).

We decided to fuse Rb69-Pol (1-896), which lacks only the C-terminal 7aa that mediate interactions with its gp45 clamp (35), to the CTD (981-1097) of Pol3 (Fig. 1A and 2A). This CTD comprises a putative PCNA-binding motif (996-1005) (27), and the two 4-cysteine cluster metal binding sites, starting at aa1009 (12). The fusion gene is designated as *pol3-69* and the resulting three-subunit variant of Pol δ as RbPol δ. First, we established that the fusion polypeptide contained the necessary determinants for expressing a stable 3-subunit enzyme in yeast, which it does (Fig. 2A). Preliminary biochemical studies showed that the fusion enzyme showed processive replication with PCNA, but much less so than wild-type Pol δ (see below). Therefore, it was not surprising that the *pol3-69* fusion gene failed to complement the lethality of

a *pol3-Δ* mutant (Fig. 1B). We reasoned that the fusion polypeptide might show compromised binding to PCNA, since the PCNA binding motifs are located close to the fusion point. We therefore tested whether we could select for PCNA mutations that might ameliorate the processivity defect and allow growth of *pol3-69*. A yeast *pol3-Δ* strain containing both *POL3* and *pol3-69* on separate plasmids was transformed with a heavily mutagenized *POL30* library, encoding PCNA. Transformants were replica-plated onto 5-FOA media, which evicted the wild-type *POL3* plasmid, enforcing viability of the *pol3-69* mutant for cell growth. We isolated two PCNA suppressor mutants of which only one, designated *pol30-rb1,* showed robust growth. The pcna-rb1 mutant carried six amino acid changes. By subsequent elimination analysis, we determined that the Q29H mutation was essential for suppression of lethality, while the additional K31R mutation increased the efficiency of suppression to that of the *pol30-rb1* suppressor containing all six mutations (Fig. 1B and data not shown). These two mutations are localized adjacent to each other on the outer rim of the PCNA donut, close to the interaction pocket of many PCNA-interacting proteins (Fig. 1C). All further studies were carried with this double mutant, which we designate as *pol30-rb2*.

Biochemical activities of RbPol δ – We next investigated the replication properties of RbPol δ with either wild-type PCNA or the double mutant, pcna-rb2 (Fig. 2A). While wild-type PCNA stimulated the replication activity of RbPol δ (Fig. 2B, compare lanes 6,7 with 5), it did not replicate as efficiently as Pol δ. The defect was somewhat suppressed at higher concentrations of PCNA (Fig. 2B, lanes 8,9; Fig. 2C), consistent with an impaired stability of the the DNA-PCNA-RbPol δ complex. Significantly, the mutant pcna-rb2 clamp largely suppressed this processivity defect, allowing more rapid DNA synthesis at lower concentrations than wild-type PCNA did (Fig. 2B,C). Rb69 DNA polymerase itself showed no processive DNA synthesis with either wild-type PCNA or pcna-rb2.

In addition to the elongation of Okazaki fragments, another essential function of Pol δ is the maturation of these fragments (36). During this process, Pol δ coordinates with the flap endonuclease FEN1 to remove a 7-10 nt RNA primer and replace it with DNA during a process called nick translation in order to generate a DNA-DNA nick that can be sealed by DNA ligase I. In our biochemical assay, the polymerizing complex encounters an 8 nt RNA primer when it has completely replicated around the 3 kb DNA circle as shown in Fig. 1D. The RNA is degraded by iterative steps of Pol δ-mediated strand displacement synthesis of 1-2 ribonucleotides, followed by FEN1 cutting of the emerging 5'-flap (37). Finally, after all RNA has been degraded, DNA ligation is mediated by DNA ligase I. With wild-type Pol δ and PCNA, this reaction is essentially complete after 3 min, and substituting pcna-rb2 did not affect the kinetics (Fig. 2D). In contrast, Rb-Pol δ only completed replication and subsequent Okazaki fragment maturation when the suppressor pcna-rb2 was present, and not with wild-type PCNA. These data suggest that the lethality of the *pol3-69* fusion mutant may result not just from inefficient elongation of replication, but perhaps even more from the inability to perform efficient Okazaki fragment maturation, with the suppressor mutant *pol30-rb2* largely overcoming these deficiencies.

Fidelity defects associated with Rb69 polymerase activity – Having established that the suppressor pcna-rb2 largely restored processive functionality to RbPol δ *in vitro*, we next asked which potential defects were associated with the genome being replicated by RbPol δ. While the *pol3-69* fusion allele showed robust growth at 30 ˚C, it was cold-sensitive for growth at 15 ˚C (Fig. 3A). In addition, the strain was sensitive to the replication inhibitor hydroxyurea (Fig. 3A), but not to the topoisomerase inhibitor camptothecin, which induces double stranded breaks (data not shown). However, the mutant was significantly more sensitive to UV irradiation than wild-type *POL3*.

We combined the *pol3-69* allele with a deletion of *REV3*, the catalytic subunit of Pol ζ, and/or with a deletion of *RAD30*, which encodes Pol η. Pol ζ is responsible for the bulk of damage-induced mutagenesis in the cell (8,38), and Pol η mediates mostly error-free bypass of pyrimidine dimers (39). While defects in these damage-response mechanisms showed a slight increase in damage sensitivity, it was not profound, suggesting that no specific pathway was inactivated in *pol3-69*.

Despite being responsible for the replication of a relatively small genome, Rb69 DNA polymerase shows a remarkably high replication fidelity (40). We determined whether this high fidelity phenotype was preserved in yeast, using the *CAN1* gene as a target for forward mutagenesis. In the *pol3-69*

mutant, canavanine-resistant mutations occurred at an 8-fold increased rate compared to wild-type. However, defects in the stability of replication complexes can induce the recruitment of Pol ζ, which results in an increased accumulation of mutations (9,41-43). This process is called DRIM (defective replisome-induced mutagenesis). DRIM is under analogous genetic control as damage-induced mutagenesis, requiring also the Rev1 protein, which acts as a scaffold for assembling the mutasome (41,44). Therefore, we repeated the fluctuation analysis in a *rev3Δ* strain, defective for Pol ζ. Indeed, the *pol3-69 rev3Δ* mutant showed a strongly reduced mutator phenotype, only ~3-fold higher than that of *rev3Δ*. An analysis of the spectrum of mutations obtained showed that by far the largest class of mutations in the *pol3-69* single mutant are GC→CG transversion mutations that are a classical signature of Pol ζ-and Rev1-dependent activity (Table 1)(45-47). Indeed, they are not observed in the *pol3-69 rev3Δ* double mutant. Other types of mutations that are substantially enhanced in *pol3-69* compared to *pol3-69 rev3Δ* are AT→TA transversions and complex mutations, also consistent with Pol ζ- and Rev1-dependent activity (41,47). When the mutation spectrum of the *pol3-69 rev3Δ* strain is compared to that of *POL3 rev3Δ* (42,43,48), substitution mutations in all classes are somewhat enhanced, but the largest increase attributable to RbPol δ are in deletion formation.

Half of the mutants in *pol3-69 rev3Δ* are due to intermediate size deletions (11-64 nt) between direct repeats, 4-8 nt in length (Table I). These deletions are caused by primer misalignment during lagging strand replication by RbPol δ. When Pol ζ is functional, the rate of formation of these deletions is not significantly altered, suggesting that the misaligned primer does not provoke a TLS response by Pol ζ. Interestingly, the same 4-8 nt direct repeats that cause deletion formation in *pol3-69*, induce duplications in a *rad27Δ* strain that is defective for FEN1 flap endonuclease, and therefore compromised in Okazaki fragment maturation (49).

Our analysis has shown that the catalytic polymerase and domains of Pol δ can be substituted with those from a bacteriophage DNA polymerase, provided adaptive mutations are made in PCNA. The N-terminal domain is structurally conserved in all B-family DNA polymerases, and in archaea serves a specific function in the recognition of template uracil residues (50). The function of the NTD in other organisms remains to be determined, but our analysis shows that this NTD does not specify organism-specific essential functions.

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FOOTNOTES

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TABLE I. Spectra of spontaneous mutations in *pol3-69* mutants. [a]Rates and Confidence intervals (C.I.) are from Fig. 3b; [b]spectra from WT and *rev3Δ* are composite from (42,43,48). [b]Complex are defined as multiple changes within 10 nt. [c]One duplication between direct repeats.

	WT	***rev3Δ***	***pol3-69***		***pol3-69 rev3Δ***	
Mutations	**rate**	**rate**	**No**	**rate**	**No.**	rate
Base Substitutions						
GC→AT	4.4	3.3	5	15	11	8
AT→GC	2.1	1.4	1	3	2	1.4
GC→TA	4	1.3	2	6	4	2.7
GC→CG	3	0.5	24	74	0	<0.7
AT→CG	0.8	<0.5	2	6	0	<0.7
AT→TA	0.5	<0.5	9	28	6	4
Indels						
+1	0.7	0.3	0	<3	1	0.7
-1	2.6	1	3	9	3	2.1
-2	1.0	2.0	1	3	3	2.1
Deletions between short direct repeats	<0.5	<0.5	5	15	24	16
Complex[b]	1.5	<0.5	6	18	0	<0.7
Other[c]	-	-	1	3	0	<0.7
Total[a]	20.5	11	58	179	54	37
95% C.I.	17-24	9-17		148-217		36-49

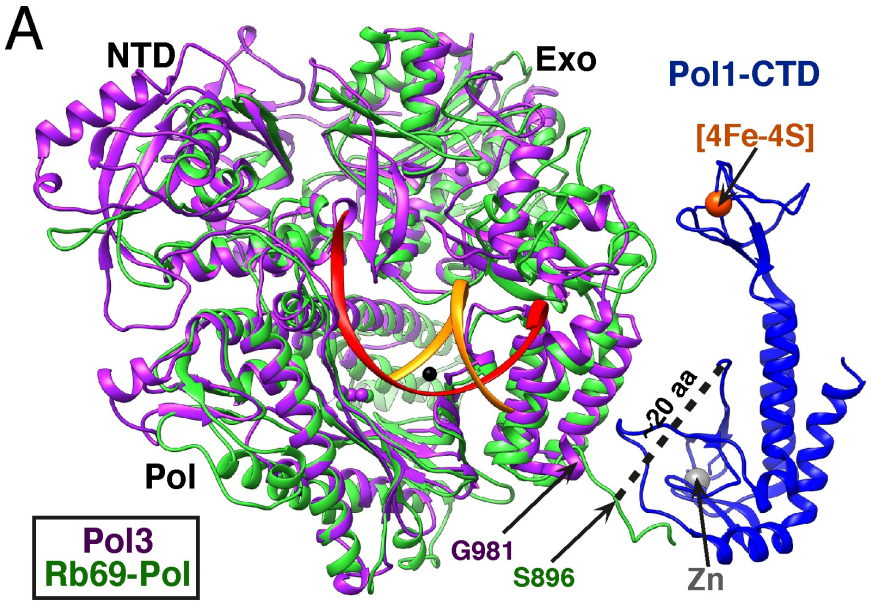
FIGURE LEGENDS

Figure 1. Creating RbPol δ. ***(A)*** Structural alignment of yeast Pol3 (PDB: 3IAY, purple) and Rb69 (PDB: 1RG9, green), both in a ternary complex with DNA (template in red, primer in orange) and dNTP (3,34). The three main domains are the N-terminal domain (NTD), the exonuclease domain (Exo) and the polymerase domain (Pol). Also shown is the portion of the CTD of yeast Pol1 (PDB: 3FLO) that is conserved with the Pol3 CTD (~1005-1080) (11). The proposed localization of the Zn and [4Fe-4S] metal centers within Pol3 is indicated, although in the Pol α-CTD structure both centers contain Zn. RB69-Pol(1-896) was fused to Pol3(981-1097). No structural model exists for the ~20 aa of Pol3 (dashed line) separating the two structural domains. ***(B)*** Serial ten-fold dilutions of *pol3Δ* strain PY227 containing three plasmids: pBL304 (*URA3, POL3*), pBL309 (*TRP1, POL3*) or pBL326 (*TRP1, pol3-69*), and pBL249 (*LEU2, POL30 or pol30-rb1 [F12Y,D17A,Q29H,K31R,I52M,I100T] or pol30-rb2 [Q29H,K31R]*). Growth on 5-FOA media *versus* SC media indicates that the *pol3-69* fusion allele supports growth, but only when the *POL30* suppressors are present. ***(C)*** Location of the *pol30-rb2* suppressor mutations (in red) within PCNA (PDB: 1PLQ) (55). Amino acids in the inter-domain connector loop (IDCL) and C-terminus that interact with a human Pol32 peptide are shown in black (56).

Figure 2. Replication activity of RbPol δ. ***(A)*** Top panel, schematic of interactions within RbPol δ. RbPol3 subunit interacts with Pol31 through its [4Fe-4S] cluster. Pol31 interacts with Pol32. Interaction with PCNA is supported through motifs in the Zn-ribbon of RbPol3 and at the C-terminus of Pol32. Lower panel, SDS-PAGE analysis of purified polymerase complexes. RbPol3 co-purifies with stoichiometric levels of Pol31 and Pol32. ***(B)*** Alkaline agarose gel electrophoresis of replication products with purified proteins as indicated. Schematic is shown. Singly primed ssSKII DNA was coated with RPA; PCNA or pcna-rb2 was loaded with RFC and ATP. Reactions initiated by addition of Pol δ or RbPol δ together with dNTPs. ***(C)*** PCNA titration; replication assays were performed as in **B**, with indicated proteins for 60 sec. Incorporation of [α-^{32}P] dNTPs determined by scintillation counting. Activity is represented relative to that of Pol δ with saturating PCNA. ***(D)*** Okazaki fragment maturation assay; replication products were resolved on agarose gel containing 0.5 μg/ml ethidium bromide. Replication assays were performed as in **B**, except for addition of both FEN1 and DNA ligase I along with polymerase and dNTPs upon reaction initiation. Labels at left indicate positions of nicked double-stranded DNA and closed circular double-stranded DNA. The latter has a high mobility in an ethidium bromide-containing gel.

Figure 3. Damage-sensitivity and fidelity phenotypes of the *pol3-69* mutant. ***(A)*** Sensitivity of the *RbPol3 POL30/pol30-rb2* strain to low-temperature growth and to DNA damaging agents. Serial ten-fold dilutions of strains PY236 (*REV3 RAD30*), PY237 (*rev3Δ*), PY238 (*rad30Δ*), or PY239 (*rev3Δ rad30Δ*), containing either *POL3* or *pol3-69*. All strains contain *pol30-rb2* integrated into the chromosome (in the *LEU2* locus) HU, hydroxyurea. ***(B)*** Spontaneous forward mutation rates (with 95% confidence intervals) to canavanine resistance, of PY236 and PY237, containing either *POL3* or *pol3-69*.

Figure 1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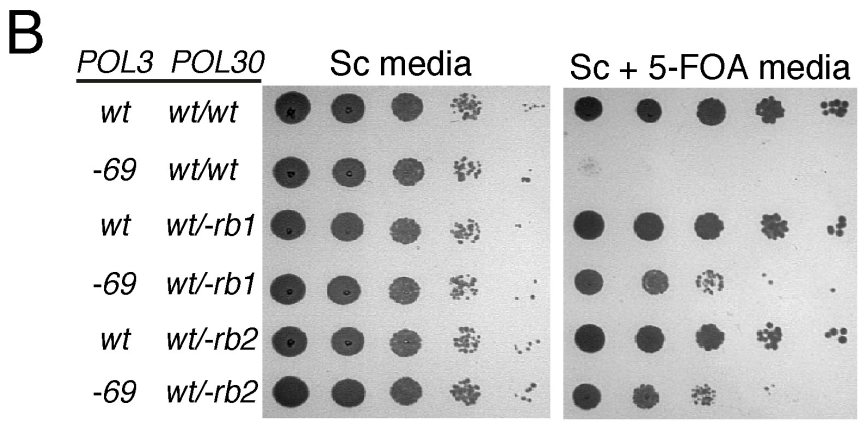
Exo
Pol1-CTD
[4Fe-4S]
20 aa
Pol
Pol3
Rb69-Pol
G981
S896
Zn


B
POL3 POL30
Sc media
Sc + 5-FOA media
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-69 wt/wt
wt wt/-rb1
-69 wt/-rb1
wt wt/-rb2
-69 wt/-rb2

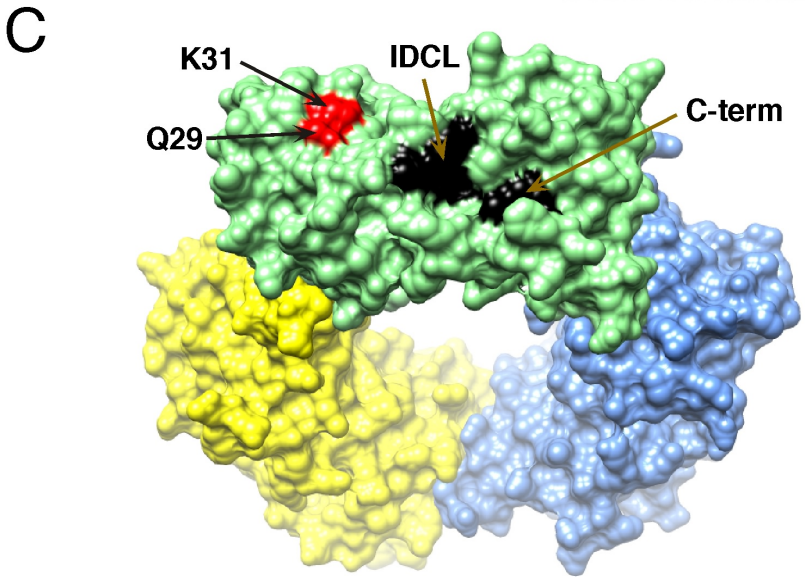
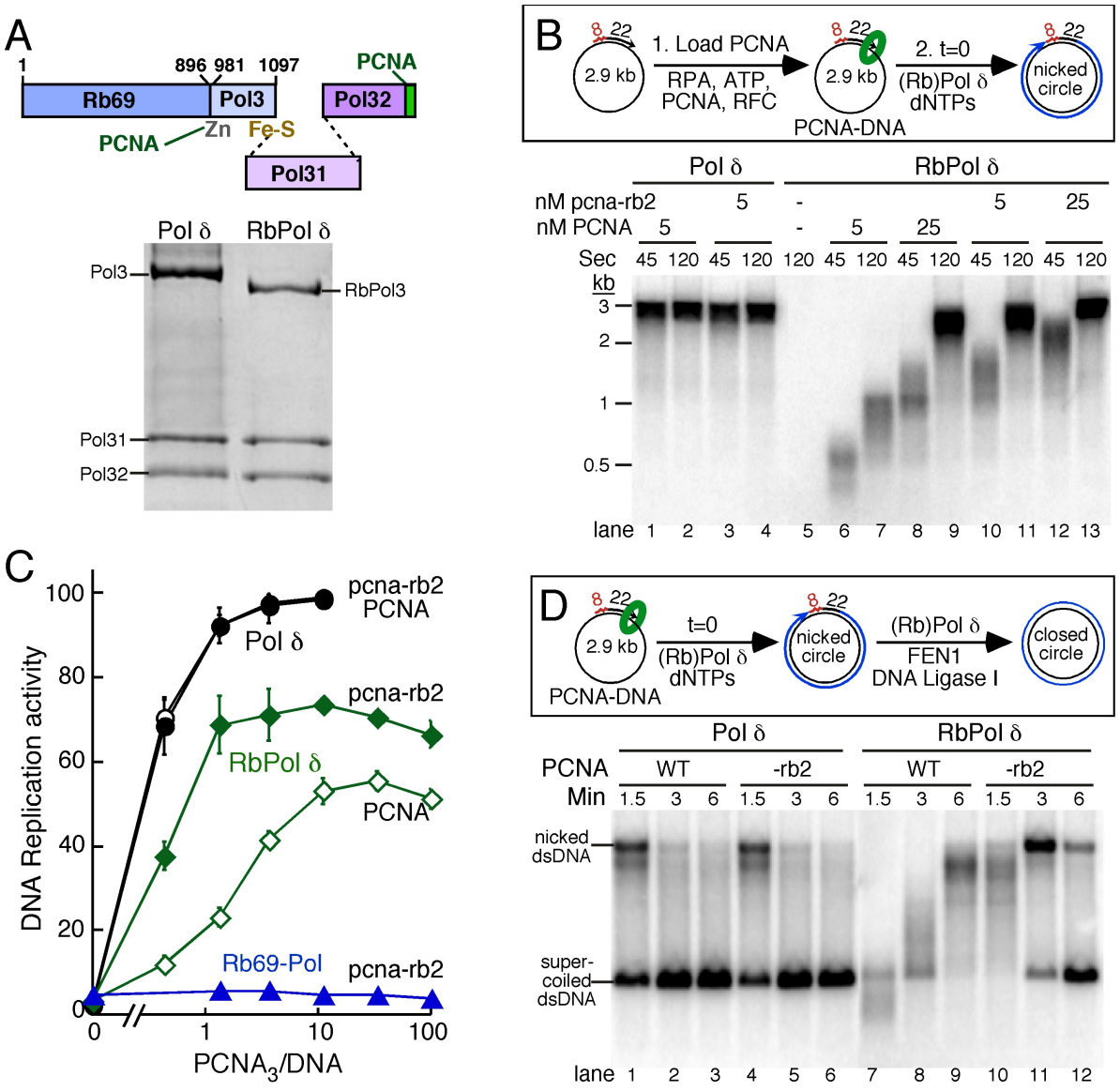

C
K31
IDCL
Q29
C-term

Figure 2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Rb69
Pol3
Pol32
PCNA
PCNA
Zn
Fe-S
Pol31
Pol δ
RbPol δ
Pol3
RbPol3
Pol31
Pol32
B
22
2.9 kb
1. Load PCNA
RPA, ATP,
PCNA, RFC
PCNA-DNA
2. t=0
(Rb)Pol δ
dNTPs
nicked
circle
Pol δ
RbPol δ
nM pcna-rb2
nM PCNA
Sec
kb
3
2
1
0.5
lane 1 2 3 4 5 6 7 8 9 10 11 12 13
C
DNA Replication activity
100
80
60
40
20
0
pcna-rb2
PCNA
Pol δ
pcna-rb2
RbPol δ
PCNA
Rb69-Pol
pcna-rb2
0
1
10
100
PCNA3/DNA
D
22
2.9 kb
PCNA-DNA
t=0
(Rb)Pol δ
dNTPs
nicked
circle
(Rb)Pol δ
FEN1
DNA Ligase I
closed
circle
Pol δ
RbPol δ
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dsDNA
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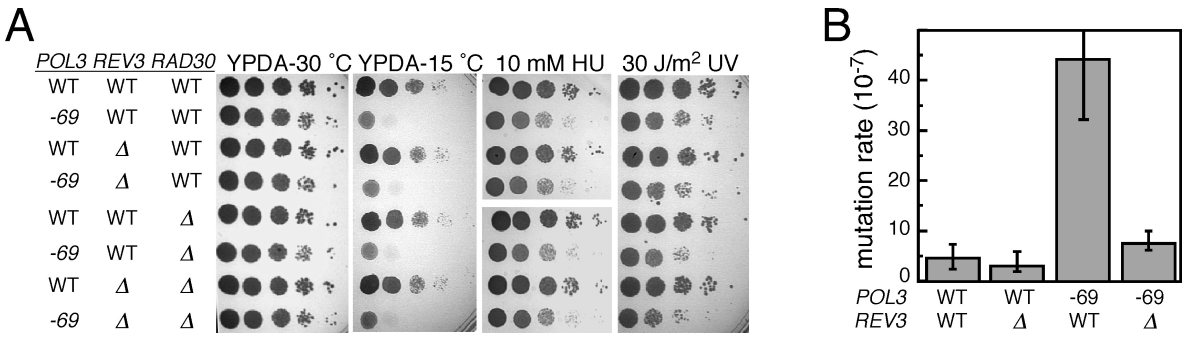
dsDNA
lane 1 2 3 4 5 6 7 8 9 10 11 12

Figure 3


A
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YPDA-15 ˚C
10 mM HU
30 J/m2 UV
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-69 WT WT
WT Δ WT
-69 Δ WT
WT WT Δ
-69 WT Δ
WT Δ Δ
-69 Δ Δ
B
mutation rate (10-7)
0
10
20
30
40
POL3 WT WT -69 -69
REV3 WT Δ WT Δ