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A novel variant of DNA polymerase ζ, Rev3ΔC, highlights differential regulation of Pol32 as a subunit of polymerase δ *versus* ζ in *Saccharomyces cerevisiae*

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Abstract

Unrepaired DNA lesions often stall replicative DNA polymerases and are bypassed by translesion synthesis (TLS) to prevent replication fork collapse. Mechanisms of TLS are lesion- and species-specific, with a prominent role of specialized DNA polymerases with relaxed active sites. After nucleotide(s) are incorporated across from the altered base(s), the aberrant primer termini are typically extended by DNA polymerase ζ (pol ζ). As a result, pol ζ is responsible for most DNA damage-induced mutations. The mechanisms of sequential DNA polymerase switches *in vivo* remain unclear. The major replicative DNA polymerase δ (pol δ) shares two accessory subunits, called Pol31/Pol32 in yeast, with pol ζ. Inclusion of Pol31/Pol32 in the pol δ/pol ζ holoenzymes requires a [4Fe–4S] cluster in C-termini of the catalytic subunits. Disruption of this cluster in Pol ζ or deletion of *POL32* attenuates induced mutagenesis. Here we describe a novel mutation affecting the catalytic subunit of pol ζ, *rev3ΔC*, which provides insight into the regulation of pol switches. Strains with Rev3ΔC, lacking the entire C-terminal domain and therefore the platform for Pol31/Pol32 binding, are partially proficient in Pol32-dependent UV-induced mutagenesis. This suggests an additional role of Pol32 in TLS, beyond being a pol ζ subunit, related to pol δ. In search for members of this regulatory pathway, we examined the effects of Maintenance of Genome Stability 1 (Mgs1) protein on mutagenesis in the absence of Rev3–Pol31/Pol32 interaction. Mgs1 may compete with Pol32 for binding to PCNA. Mgs1 overproduction suppresses induced mutagenesis, but had no effect on UV-mutagenesis in the *rev3ΔC* strain, suggesting that Mgs1 exerts its inhibitory effect by acting specifically on Pol32 bound to pol ζ. The evidence for differential regulation of Pol32 in pol δ and pol ζ emphasizes the complexity of polymerase switches.

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Conflict of interest statement
The authors declare that there are no conflicts of interest.

Keywords

Polymerase ζ; Pol32; Mgs1; Translesion DNA synthesis; Polymerase switch; UV mutagenesis

1. Introduction

Cellular DNA is under constant attack by exogenous and endogenous mutagens. Resulting lesions, if unrepaired [1,2], can block cell division when replicative DNA polymerases are unable to incorporate nucleotides across from the damaged sites. This causes activation of DNA damage tolerance mechanisms to prevent irreversible replication fork collapse and to finish replication of the genome [3]. The DNA damage tolerance pathway includes predominantly error-free recombinational damage avoidance and translesion synthesis (TLS), which often is a source of mutations [4,5]. While TLS machinery deals with the lesion, the replication fork can restart downstream to allow for continuation of replication [6].

During TLS, replicative DNA polymerases yield the damaged template to specialized polymerases which incorporate nucleotides across from the altered base(s) [7–9]. Most prominent in TLS are the low fidelity Y-family polymerases η, κ, ι, and Rev1 [10,11], but in some cases insertion is accomplished by X-family, A-family, or B-family DNA polymerases [5,12]. Then there is extension of the aberrant primer terminus, achieved by the inserter or another polymerase. Most frequently this extension is accomplished by the error-prone B-family polymerase ζ (pol ζ) [4,12–14]. Once the lesion is bypassed, there is a return to synthesis by replicative polymerases or filling of the gap between the bypassed lesion and a downstream restart site, by pol ζ itself or by replicative polymerases [5,6].

TLS events can have opposing effects on mutagenesis. Some TLS polymerases are tailored to bypass specific types of lesions and incorporate predominantly the correct base, *i.e.* the base that should have been incorporated by the replicative polymerase in the absence of damage. Historically, this is called error-free bypass because the action of these polymerases suppresses induced mutagenesis. However, the number of lesions greatly exceeds the number of polymerases. Therefore most lesions are primarily bypassed by the addition of an incorrect base. This so-called error-prone TLS is highly mutagenic. This process is carried out by a complex of proteins composed of replicative pols, TLS pol ζ, Rev1, and monoubiquitylated proliferating cell nuclear antigen (PCNA) [4,9,15,16].

One critical event during TLS in eukaryotes is the physical switch between the polymerases. Details of how it actually occurs *in vivo* are not clear. Currently it is thought that it occurs *via* the two-step insertion–extension mechanism, proposed on the basis of experiments in yeast (Fig. 1A) [11,12,17]. Upon damage, PCNA is monoubiquitylated at K164 [18] and there is a switch from replicative pol δ (or pol ε) to another polymerase (predominantly Y-family pol) which inserts a nucleotide across from the lesion. Rev1 acts as an indispensable scaffold protein and, when necessary, a deoxycytidyl transferase inserting “C” opposite the lesion. Then there is a switch to pol ζ which performs extension from this aberrant terminus. If an error was made during bypass, the action of pol ζ allows the altered sequence to remain in the nascent DNA strand sequence, leading to a mutation. Malfunction of this pathway

abolishes induced mutagenesis. The signals involved, aside from ubiquitylation and probably deubiquitylation of PCNA, are unknown [19,20].

Pol ζ is responsible for most induced point mutations and roughly half of spontaneous mutations [9,21]. It synthesizes DNA *in vitro* with low fidelity and produces a characteristic mutational signature [22], found in mutation spectra *in vivo* [2,23,24]. Part of the signature is attributed to template switches [25]. Pol ζ is the only TLS polymerase essential for viability in mice, suggesting it is required for tolerance of endogenous DNA damage during development. In yeast, deletion of *REV3* is not lethal but causes growth retardation in strains with elevated levels of abasic sites [26]. Loss of the catalytic subunit of pol ζ or Rev1 results in elevated rates of large deletions [24,25] and gross chromosomal abnormalities [27]. Therefore, while error-prone TLS is etiologic in most environmentally induced cancers, its absence can also contribute to genome instability and cancer [13,28,29]. Pol ζ can also contribute to cancer cell resistance to the chemotherapeutic agent cisplatin [30].

Pol ζ was long thought to be composed of only Rev3 and Rev7 [31]. We discovered that the C-terminal domain (CTD) of the human catalytic subunit of pol ζ binds two accessory subunits of pol δ, p50/p66, and predicted that human pol ζ is a four-subunit complex (See Table 1 for nomenclature of human and yeast DNA polymerase subunits) [32]. Four-subunit human pol ζ was later purified from human cells and possessed polymerase activity superior to the two-subunit enzyme [33]. Yeast pol ζ can also stably exist as a four-subunit enzyme, containing the catalytic subunit Rev3, accessory subunit Rev7, and Pol31/Pol32 [34–36]. In this complex, Pol32 binds to Pol31, and Pol31 binds to the CTD of catalytic subunit Pol3 [37–39]. The existence of shared subunits between replicative and TLS pols was the basis for the proposal of an additional mechanism of switching between pol δ and pol ζ through an exchange of the catalytic subunits on Pol31/Pol32 bound to PCNA [32]. In this scenario (Fig. 1B), pol δ stalling at a lesion signals for monoubiquitylation of PCNA. Then the catalytic subunit Pol3 dissociates (and/or is degraded [40]) and Rev3/Rev7 is recruited to Pol31/Pol32 left at the site of the lesion. This mechanism provides an easy, yet unproven, possibility for a switch back to Pol3 for processive synthesis if necessary (more in Section 4, Discussion). In this model, pol δ plays a role in TLS by regulating the entire switch process.

It is believed that based on the structure of another B-family member pol α and a low resolution EM structure of pol ζ, that both Pol3 and Rev3 contain a CTD attached by a flexible linker [39,41]. Both polymerases contain a FeS cluster in this domain [42], which is required for binding to Pol31/Pol32 [32,34,35]. In addition, when the C-terminal tail of Rev3 past the metal binding sites is removed there is no binding to Pol31, suggesting that not only is the cluster necessary for binding but the region of the CTD downstream of it is also [34]. In yeast when the FeS cluster of pol ζ is disrupted, there is a severe reduction of mutagenesis comparable to the complete absence of Rev3 ([32,34], present study). It is possible that this cluster plays a structural role or that the switch is regulated by oxidation–reduction reactions [43]. To better understand how polymerase switches occur in yeast, we created several mutants affecting the CTD of Rev3 (Fig. 2A). Intriguingly, even though disruption of the cluster nearly eliminated induced mutagenesis, a mutant that lacked the entire CTD and thus the whole platform for interaction with Pol31/Pol32, exhibited robust mutagenesis at low doses of UV irradiation and residual mutagenesis at higher doses. The

purpose of this study was to characterize this C-terminal truncation mutant, *rev3ΔC*, and use it as a tool to probe our understanding of polymerase switches *in vivo*.

We found that UV-induced mutagenesis in this mutant still requires the presence of Pol32 but becomes independent of regulation by Maintenance of Genome Stability 1 (Mgs1), whose overproduction suppresses mutagenesis in strains with normal pol ζ [44]. We conclude that Pol32 plays a role in mutagenesis beyond its function as subunit of pol ζ and that Mgs1 is capable of regulating UV-induced mutagenesis only when the Pol31/Pol32 binding platform in Rev3 is intact.

2. Materials and methods

2.1. Materials

Most mutagenesis studies were done in the *Saccharomyces cerevisiae* strain 8C-YUNI101 (*MAT***a** *his7-2 leu2-3,112 ura3-Δ bik1::ura3-29RL trp1-1*$_{UAG}$ *ade2-1*$_{UAA}$) [45] and its derivatives. Mutagenesis studies on the deletion of *MGS1* were done in a derivative of the strain BY4742 (*MATα his3Δ1 leu2Δ0 lys2Δ0; ura3Δ0*) (Life Technologies, USA). Extracts for western blotting were prepared from transformants of the protease-deficient strain BJ2168 (*MATa prc1-407 prb1-1122 pep4-3 leu2 trp1 ura3-52 gal2*) [46]. Plasmids used are described in the next section. Mouse anti-GST, goat HRP-conjugated anti-mouse, and donkey HRP-conjugated anti-goat antibodies were from Genscript (Piscataway, NJ). Goat anti-human actin antibody (cross-reacts with yeast actin) was from Santa Cruz Biotechnology (Santa Cruz, CA). Super Signal West Femto Chemiluminescent Substrate detection kit was from Thermo Scientific (Dubuque, IA). The 1× complete EDTA-free protease inhibitor cocktail was from Roche (Indianapolis, IN). The Immobilon PVDF membrane was from Millipore (Billerica, MA). QuikChange Site Directed Mutagenesis kit was from Agilent Technologies (USA). All other chemicals were reagent grade and were purchased from Sigma–Aldrich (St. Louis, MO) or Fisher Scientific (Atlanta, GA).

2.2. Creation of mutants for this study

All mutant strains used in this study are listed in Table 2. The plasmid pRevLCav2-rev3ΔC is a deletion derivative of integrative plasmid pRevLCav2 [45] created by PCR of the plasmid region flanking the deletion and ligation, where *rev3ΔC* encodes for Rev3 lacking the C-terminus (amino acids 1381–1504). Site directed mutagenesis with the QuikChange kit was further used on pRevLCav2-rev3ΔC to create pRevLCAV2-rev3ΔC-DD, encoding for a catalytically dead Rev3. We used a standard integration–excision protocol to integrate the mutant alleles into the genome and replace endogenous *REV3* [45], creating the *rev3ΔC* and *rev3ΔC-dd* mutant stains, respectively (Table 2). These plasmids were linearized with *Sna*BI before transformation. 8C WT and *rev3ΔC* strains were transformed with the *Bst*EII-linearized plasmid YIp128-GAL-MGS1 (kindly provided by H. Ulrich) to create *WT + MGS1↑* and *rev3ΔC + MGS1↑* strains, respectively, with integration of the *GAL-MGS1* cassette into the *LEU2* locus. *rev3ΔC pol32Δ* and *rev3ΔC rev1Δ* were created by replacing *POL32* and *REV1*, respectively, with KanMX cassettes in the *rev3ΔC* strain. Strains for overexpression of *rev3ΔC* and *REV1* (Table 2) were obtained by transformation of 8C-YUNI101 *rev3Δ* and 8C YUNI101, respectively, with derivatives of the multicopy plasmid

pRS425-GALGST [47] containing the appropriate *GST-REV3* or *GST-REV1* allele under the control of a galactose-inducible promoter (plasmids with wild-type genes were constructed by N. Sharma and P. Shcherbakova, personal communication). The derivatives of p425-GALGST-REV3 plasmid with mutations affecting metal binding site of Rev3 were created by gap repair in yeast *in vivo* and are first described in [32]. The plasmid for overexpression of *rev3ΔC* was created by the same method. All plasmids and site-directed muta-genesis primer sequences are available upon request.

2.3. Determination of survival and induced mutagenesis frequencies

Yeast strains were typically grown for two days at 30 °C in 5 mL of yeast extract peptone dextrose medium with 60 mg/L adenine and uracil (YPDAU) with shaking. Cells were pelleted at 1000 × *g* in a Beckman Model TJ-6 centrifuge for 2 min and re-suspended in 1 mL of sterile water. Cells were diluted 200,000-fold and 100 μL aliquots plated on Synthetic Complete (SC) medium; 50–100 μL of undiluted cells were plated on SC medium supplemented with 60 mg/L of L-canavanine (Can). Plates were irradiated with 0, 20, 40, or 60 J/m^2 of UV light. After three days of growth at 30 °C, colonies on SC plates were counted and survival was calculated by dividing the number of colonies at each UV treatment by the number of colonies without exposure (independently for each strain). After five days of growth, colonies on Can plates were counted and the mutant frequency was calculated by dividing the number of colonies on Can plates at each UV dose by the number of colonies on the SC plate at the same dose (SC colony count was first multiplied by dilution factor) as described in [48]. The induced Can^r mutant frequency was calculated by subtracting the spontaneous frequency (without treatment) from the mutant frequency for each UV-light dose [48]. All data points are averages of at least two independent trials with duplicates of each sample in each trial. Error bars in Figs. 2–5 represent standard deviation.

Strains for overexpression studies of *MGS1* contained a *GAL1* promoter upstream of *MGS1* integrated at the *LEU2* locus. Mutagenesis studies in these strains were performed as described above with the following modifications. These strains were grown for two days in 4.5 mL SC-raffinose medium (no glucose, 3% raffinose) and were induced with 0.5 mL sterile 20% galactose for 2.5 h prior to plating.

Experiments with overexpression of *rev3ΔC* and *REV1* (Table 2) were done as described above except the transformants were grown in SC-raffinose lacking leucine (-leu) to select for the presence of a plasmid, induced for 2.5 h with galactose, and plated with appropriate dilutions on SC-leu and SC-leu-Can plates.

2.4. Preparation of yeast extracts and western blot

For the analysis of levels of soluble Rev3 variants, we used the overexpression conditions because natural endogenous levels of Rev3 are very low. Transformants of protease-deficient strain BJ2168 with appropriate variants of pRS425-GALGST-REV3 plasmids (Sections 2.1 and 2.2) were grown in 12.5 mL SCGL-leu medium overnight. The following day 62.5 mL of SCGL-leu was added. The third day, 62.5 mL of YPGLA medium was added and cells were allowed to grow for 2.5 h. Finally, cells were induced with 2.5 g galactose for 4 h, collected, and flash frozen. Yeast extracts were prepared in buffer

containing 50 mM Tris–HCl (pH 7.5), 300 mM NaCl, 1 mM EDTA, 10% sucrose, 10 mM β-mercaptoethanol, 1 mM PMSF, and 1× complete EDTA-free protease inhibitor cocktail. The volume of buffer (in μL) equals the millgrams of wet cells multiplied by 2.68. Yeast cells overproducing Rev3 were thawed, mixed with 500 μL of 0.5-mm glass beads, and lysed with a Disruptor Genie™ (six cycles, two min each) at 4 °C. The lysates were cleared by centrifugation using a MIKRO 200R centrifuge at 8000 × *g* for 15 min and then 10,000 × *g* for 10 min, all at 0 °C.

For western blot analysis, proteins were separated on an 8% SDS-PAGE Laemmli gel at 200 V, followed by a 1 h, 20 V transfer to an Immobilon PVDF membrane at 4 °C. Mouse anti-GST and goat HRP-conjugated anti-mouse secondary antibodies were used to detect the GST-Rev3 fusion protein. Goat anti-human actin antibodies, which cross-react with yeast actin, and donkey anti-goat HRP-conjugated secondary antibodies were used to detect actin as the loading control. The blot was developed using the Super Signal West Femto Chemiluminescent Substrate detection kit.

3. Results

3.1. Robust mutagenesis at low UV doses and substantial, residual levels of mutagenesis at higher UV doses in the rev3ΔC strain

UV-induced mutagenesis is an effective readout for TLS in yeast. Pol ζ is required for all induced mutations and deletion of *REV3* or *REV7* results in complete loss of UV-induced mutagenesis. To better understand the role of Rev3, its subunits, and accessory proteins in TLS we examined parameters of UV-induced mutagenesis in several mutants affecting different parts of the protein (Fig. 2A, Table 3). The C-terminus of Rev3 contains two cysteine-rich metal binding sites, CysA and CysB [49]. CysA forms a zinc finger (ZnF) and CysB coordinates a FeS cluster [32,42]. In the current study, a novel mutation of *REV3* was created that encoded a protein lacking the entire CTD, thus completely removing both metal binding sites and the platform for binding to Pol31/Pol32 [32,34]. It will be further referred to as *rev3ΔC* (Fig. 2A). We used the popular canavanine-resistance forward mutation assay, where mutations of various types in the *CAN1* gene in yeast confer resistance to the toxic drug canavanine [50].

Mutagenesis was measured in various *rev3* mutants after exposure to increasing doses of UV irradiation (Table 3). Disruption of the Zn finger motif of Rev3 had no effect on survival or mutagenesis, as shown previously [32]. In contrast, disruption of the FeS cluster alone or both metal-binding sites resulted in severe reduction of mutagenesis and a drastic decrease in survival, similar to the catalytically inactive enzyme, Table 3. These results are consistent with literature[32,34,35]. Intriguingly, we found that at low UV doses *rev3ΔC* showed robust mutagenesis levels comparable to WT Rev3. At higher UV doses, it retained residual mutagenesis even though it lacked the necessary elements (see above) to bind Pol31/Pol32, including the FeS cluster. Residual mutagenesis was at intermediate dose about 60% and at highest dose about 10% of WT levels. The observed mutant frequencies in treated and untreated cultures are shown as an illustration of the magnitude of UV-induced mutagenesis (Table 3).

3.2. rev3 mutations do not exert a dominant negative phenotype in UV-induced mutagenesis

The fact that strains with Rev3ΔC and Rev3-FeS, both lacking the binding platform for Pol31/Pol32, show different phenotypes was intriguing. To further characterize these mutants, we tested whether *rev3* mutations lead to a dominant negative effect in strains with WT Rev3. We found that the presence of *rev3ΔC*, *rev3-FeS*, or *rev3-dd* on the expression plasmids (Section 2) in the wild type strain (8C-YUNI101, see Table 2 and Section 3.3 for description of *rev3-dd* mutant) did not affect UV-induced mutagenesis (Fig. 2B). Therefore, all *rev3* mutants examined were not dominant negative and equally unable to compete with WT pol ζ.

3.3. Residual mutagenesis in rev3ΔC strains is dependent on the catalytic activity of Rev3

Two aspartates in the invariant DTD motif in region I of active site of all B-family DNA polymerases are involved in catalysis and their substitution to alanines results in catalytic dead enzymes [9]. To rule out the possibility that another polymerase is recruited to assist Rev3ΔC and is responsible for the mutagenesis seen in the *rev3ΔC* strain, a mutant was created that was both catalytically inactive and lacked the CTD of *REV3* (*rev3ΔC-dd)*. This mutant was hypersensitive and UV-immutable, similar to the mutant with only catalytically inactive *rev3-dd* (Fig. 3A). This demonstrates that the residual mutagenesis seen in *rev3ΔC* strains is dependent upon the catalytic activity of Rev3.

It is formally possible that the Rev3ΔC protein is more stable than the wild-type (WT) holoenzyme and thus, being more abundant, can participate in UV-induced mutagenesis despite missing subunits. However, we did not detect any substantial differences in Rev3 protein levels in the soluble fraction of extracts of the different *rev3* mutant and WT strains by Western blot (Fig. 3B). Furthermore, artificial overexpression of *rev3ΔC* from a galactose-inducible promoter did not significantly increase survival or levels of mutagenesis in *rev3ΔC* strain (Fig. 3C).

3.4. Residual mutagenesis in the rev3ΔC strain is dependent on Pol32, monoubiquitylation of PCNA, and REV1

The third subunit of replicative DNA polymerase δ (Pol32) and the monoubiquitylation of the processivity clamp PCNA are known to be required for UV-induced mutagenesis in yeast [18,51,52]. Accordingly, in strains with the PCNA K164R variant that cannot be ubiquitylated, we observed decreased survival and suppressed UV-induced mutagenesis in both WT and *rev3ΔC* strains (Fig. 4A). Also deletion of *POL32* eliminated UV-induced mutagenesis and decreased survival in the WT strain. Interestingly, we found that although rev3ΔC lacks the domain needed for binding Pol31 and thus Pol32, Pol32 is still required for mutagenesis in *rev3ΔC* strain (Fig. 4A).

The Y-family DNA polymerase Rev1 binds to the Rev7 subunit of pol ζ and to the processivity factor PCNA. These interactions could facilitate mismatch extension by pol ζ during TLS. [53]. Rev1 is thought to act as a scaffold for recruitment of Y-family pol η and pol ζ to stalled replication forks and therefore is indispensable for UV-induced mutagenesis [4]. Deletion of *REV1* reduced survival and eliminated UV-induced mutagenesis in both our

WT and *rev3ΔC* strains (Fig. 4B). It is possible that Rev1 is the only anchor holding Rev3ΔC to PCNA, since this variant of Rev3 has lost the platform for interaction with Pol31/Pol32 and thus one major mode of interaction with PCNA. In this case, an increase in the concentration of Rev1 could elevate the chances of this backup interaction and increase mutagenesis. To test this, the effects of overproduction of Rev1 were investigated. Overexpression of *REV1* from a multicopy plasmid under the control of a galactose-inducible promoter did not affect survival and only slightly increased induced mutagenesis at high UV doses. However, it increased mutagenesis to the same degree in both WT and *rev3ΔC* strains (Fig. 4C).

3.5. Mgs1 is a negative regulator of pol ζ in UV mutagenesis

It is known that overexpression of the ATPase *MGS1* lowers survival and severely decreases MMS-induced mutagenesis in cells with WT Rev3 [44]. We found the same effect for UV-induced mutagenesis (Fig. 5A). Interestingly and unexpectedly, overproduction of Mgs1 had no effect on survival or the residual levels of mutagenesis seen in *rev3ΔC* (Fig. 5A). *MGS1* deletion had no effect on UV sensitivity or induced mutagenesis in WT or *rev3ΔC* strains, consistent with the literature ([54], Fig. 5B).

4. Discussion and conclusions

DNA polymerase switching is essential for TLS. Recently it was found that there is a much more intimate relationship between replicative and TLS polymerases than had been imagined: human and yeast pol δ shares two genuine subunits with pol ζ [36,43]. Based on this, we proposed that a switch can occur directly from pol δ to ζ on the platform of two shared subunits (Fig. 1B, see Table 1 for nomenclature) [32]. In this scenario, the catalytic subunit p125 dissociates when pol δ is stalled by a lesion, leaving p50/p66 bound to PCNA. Rev3/Rev7 is recruited, binds to p50/p66, and bypasses the lesion. It was hypothesized that the signaling here may involve a change in the redox state of the FeS clusters of Pol3 and Rev3. It is possible that the resulting conformation change is recognized by the proteolytic machinery responsible for the removal and degradation of the catalytic subunit of pol δ [40]. The data in the current work emphasize the role of pol δ in initiation of pol switches in mutagenic TLS and indicate that putative subunit exchange is consistent with only a fraction (ranging from 0% to 90%, depending on the dose) of UV light induced mutations.

In this study we examined a novel mutation affecting pol ζ, *rev3ΔC*, which provides new insight into the regulation of pol ζ during TLS. It has been shown that mutation of the FeS cluster in the CTD of Rev3 severely decreases UV-induced mutagenesis [32]. Intriguingly, the *rev3ΔC* mutant allele that encodes for a protein with a deletion of the entire CTD (Fig. 2A) has a much milder effect (Table 3, Figs. 2B and 3B), despite the fact that this critical FeS cluster as well as a downstream region required for interaction with Pol31/32 are absent [34,35]. We show here that neither mutant can act in a dominant negative fashion in the presence of WT Rev3 (Fig. 2B). This means that the different phenotypes of the two mutants cannot be explained by different effects of the two proteins on the TLS complex – in the presence of WT pol ζ they are both excluded from any transactions. The *rev3ΔC* yeast strain shows robust mutagenesis at low UV doses and substantial mutagenesis at higher doses. This mutagenesis is dependent upon the catalytic activity of pol ζ (Fig. 3A). This

intermediate effect on induced mutagenesis resembles effects of rev3 truncation mutants that retained the FeS cluster but lacked binding to Pol31/Pol32 [34]. The effect indicates that the mechanisms of TLS might be somewhat different at low and high doses of mutagens and depend on the level of DNA damage.

We decided to further characterize the unexpected effect of our *rev3ΔC* mutation and use it as a tool to probe the mechanisms of TLS. It is known that deletion of any of the genes encoding for Rev3, Rev7, Y-family pol Rev1, or Pol32 results in loss of all induced mutagenesis [43]. We showed here that mutagenesis is abolished when *rev3ΔC* is combined with an additional mutation causing a defect in the catalytic site of Rev3; therefore *in vivo* the Rev3ΔC/Rev7 polymerase is catalytically active and is responsible for mutation generation. In further experiments we found that mutagenesis in *rev3ΔC* strains is dependent on monoubiquitylated PCNA, consistent with the data from literature for WT pol ζ (Fig. 4A).

For a long time it has not been well-understood why a subunit of pol δ, Pol32, is required for TLS. Now it is clear that Pol31/Pol32 are also subunits of Pol ζ, though they appear to interact differently in comparison to the interaction with the catalytic subunit of pol δ, as discussed in [35]. This observation led to a simple explanation for why Pol32-deficient cells are immutable: because Pol32 is a subunit of pol ζ. This is consistent with the fact that the FeS-less pol ζ variant lacks binding to Pol31/Pol32 and confers immutability. However, the data presented here suggest that the scenarios of polymerase switches are more elaborate and complex.

We have found that even though the protein in the *rev3ΔC* mutant lacks the region required for binding to Pol31/Pol32, the strain is still quite proficient in UV-induced mutagenesis. The effect of *rev3ΔC* is recessive, because it could be seen only when no WT *REV3* was present in the genome (Fig. 2B). This is consistent with observations that two-subunit pol ζ (Rev3 and Rev7) is active *in vitro*, albeit less active than four-subunit pol ζ[33,35]. Thus, Pol32 is not critical for elementary pol ζ function. Despite the fact that the *rev3ΔC* mutant most likely is not utilizing Pol32, we found that mutagenesis in the *rev3ΔC* strain is absolutely dependent on Pol32. This strongly suggests that the immutability of *pol32Δ* strains at least partly reflects an additional role of Pol32 in TLS related to pol δ because the loss of this subunit of pol δ prevents UV-induced mutagenesis independent of its binding to Rev3. This phenomenon of cross-talk between replicative and TLS pols better fits the initial step of switch model depicted in (Fig. 1B). It appears that Pol32-less pol δ is unsuitable for a proper switch to pol ζ. This is consistent with the fact that the *pol3-13* mutation, leading to a change of one cysteine involved in FeS binding in the CTD of pol δ, leads to suppression of UV mutability [55] and (Stepchenkova and Pavlov, unpublished data).

It is generally assumed that pol δ is involved only in lagging DNA strand replication [56]. However, TLS events occur by same mechanism on both DNA strands in yeast system with damaged plasmid [57]. In this context it is interesting that deletion of *POL32* eliminates all mutagenesis, suggesting that Pol32 is required for TLS events on both strands. Our data suggest that pol ζ is active without Pol32 and that the effect of *pol32Δ* is partly due to it being part of pol δ. If pol ε is fully responsible for the whole leading strand, then it is

difficult to explain why missing components of pol δ attenuate mutagenesis on this strand. This supports the idea that pol δ is also involved in leading strand replication [58] or that the polymerase switch involves a complex event: pol ε switching to pol δ and then to pol ζ. Another possibility is that pol δ is involved in mutagenesis by taking over synthesis from pol ζ or filling the gaps resulting from re-initiation of DNA synthesis after TLS downstream of pol ζ [5,6].

A recent paper suggested that Rev7 of pol ζ can bind to Pol32 *in vitro*, which would indicate that Rev3ΔC could hold on to this interaction [39]. However, this interaction appears to be weak because Rev7 was not pulled down with Pol32 by tagged Pol31 [34]. Furthermore if Rev7 were sufficient for binding to Pol32, we would expect to see substantial levels of mutagenesis in the pol ζ FeS mutant. This is not observed. Finally, mutations in *POL31* abolishing the interaction between Pol31 and Pol32 lead to UV-immutability, despite the fact that Pol32, *per se*, is untouched [37].

Another important member of the TLS machinery is Rev1. It interacts with Rev3 *via* Rev7 subunit [59]. Mutagenesis in *rev3ΔC* is dependent on Rev1 which indicates that pol ζ with truncated Rev3 is recruited by Rev1, a scaffold protein during regular TLS. It is likely that the Rev1 interaction with pol ζ is the reason that we see intermediate mutagenesis in *rev3ΔC*. WT pol ζ can contact the processivity clamp PCNA through both Rev1 and Pol31/Pol32, therefore there is robust TLS [60]. It is possible that *rev3-FeS* is unable to bind not only Pol31/Pol32 but also Rev1 due to steric hindrance caused by the absence of the FeS cluster in the CTD of pol ζ. Rev3ΔC lacks the CTD necessary for binding to Pol31/Pol32 but can still maintain its contact with Rev1, which is sufficient for supporting some TLS functions and for mutagenesis at low doses but confers partial defect at higher doses. To explore this hypothesis, we tested whether overproduction of Rev1 would increase UV-induced mutagenesis in *rev3ΔC* strains but found no such influence (Fig. 4C). Therefore, simple increase of Rev1 levels cannot compensate for the lack of subunits. It appears that chromosomal *REV1* is sufficient to fulfill the demand of the Rev1 protein after UV irradiation in both WT and *rev3ΔC* strains.

In most of our experiments, the genetic control of mutagenesis in the *rev3ΔC* strain was very similar to the WT strain. However we found one modulator of TLS, encoded by the gene *MGS1* that behaved differently. Overproduction of Mgs1 severely reduces MMS-induced mutagenesis [44] and eliminates UV-induced mutagenesis (Fig. 5A) in yeast, suggesting that it can act as a negative regulator of TLS. However we found that overproduction of Mgs1 had no effect on mutagenesis in the *rev3ΔC* strain.

Mgs1 is an enigmatic regulator of TLS, an ATPase that plays a role in maintaining genomic stability in yeast by an unknown mechanism. It is thought that Mgs1 helps maintain proper DNA topology [61] and it is targeted to sites of replication stress through interactions with monoubiquitylated PCNA [44,62]. The human homolog of Mgs1, Werner interacting protein 1 (WRNIP1), stimulates pol δ *in vitro* and binds the catalytic subunit p125, p50, and p12 [63]. Therefore it is plausible that Mgs1 binds pol ζ as well as pol δ.

Deletion of *MGS1* abrogates the growth defect of *pol32*Δ, *pol3*Δ*ct*, and *pol31* mutant cells, suggesting that in the context of damage sensitivity Mgs1 is an effector of Pol δ [44,64]. It was also previously proposed that Mgs1 could compete with Pol32 for binding to PCNA, as overproduction of Mgs1 reduced the yeast two-hybrid interaction between Pol32 and Ub-PCNA [44]. Therefore, it is possible that Pol32 could be knocked off of PCNA by Mgs1, resulting in lack of mutagenesis. However, we show here that over-expression of Mgs1 has no negative effect on the *rev3ΔC* strain. This result suggests that in the context of UV-induced mutagenesis Mgs1 exerts its inhibitory effect on mutagenesis by acting specifically on Pol32 bound to pol ζ, not pol δ. This is the first time that Mgs1 and pol ζ have been implicated to functionally interact. This result also argues against the idea that the interaction between pol ζ and Pol32 is achieved through Rev7 binding to Pol32 (see above). If that were the case, Rev3ΔC would still be bound to Pol32 and Mgs1 would compete with it for binding, thus decreasing UV-induced mutagenesis in that strain. Consistent with the data in the literature, we found that deletion of *MGS1* had no effect on induced mutagenesis ([54], Fig. 5B), which is also true for the *rev3ΔC* strain (Fig. 5B). Mgs1 at normal physiological levels therefore may not play an active role in induced mutagenesis.

This raises the question of why Mgs1 preferentially affects involvement in mutagenesis of pol ζ and not pol Δ, since both contain Pol32. As mentioned, the nature of these interactions is not identical. It was shown that Pol3 can form a stable complex with Pol31 alone, but Rev3 cannot [35]. A recent EM structure of pol ζ may also give a clue to this differential binding nature [39]. Both pol Δ and pol ζ contain catalytic and regulatory modules in their structures. However, there is flexibility between the two in pol δ, whereas four subunit pol ζ appears to be more rigid. When Mgs1 displaces Pol32 of pol δ, the flexibility may result in only Pol32 being temporarily displaced instead of the whole enzyme dissociating from PCNA. Because pol ζ is rigid, Mgs1 competition with Pol32 results in the whole polymerase being removed from PCNA.

It has also been suggested that pols δ and ζ interact with PCNA differently. This was shown most clearly with a mutation affecting in the monomer–monomer interface, *pol30–113* [23]. Yeast strains with this mutation show no growth defects or sensitivity to the replication inhibitor HU, suggesting that this PCNA variant is sufficient for replication. However, these cells are UV-immutable indicating defective TLS. *In vitro pol30–113* is an effective (albeit less than WT) processivity clamp for Pol δ, but not for Pol ζ [23].

In conclusion, this study shows that pol ζ can function in TLS *in vivo* despite the absence of its CTD, which serves as a platform for binding to Pol31/Pol32. The necessity of Pol32 in this mutant strain highlights the importance of pol δ integrity in TLS since Pol32 is required even when not a member of pol ζ. Furthermore, we have shown a novel inhibitory effect of the ATPase Mgs1 specifically on the four-subunit pol ζ. Both pol δ and pol ζ have Pol32 as a subunit, but use it for somewhat different transactions. It is possible that Mgs1 can directly compete with Pol32 bound to pol ζ for binding to PCNA and decrease induced mutagenesis, but does not compete with Pol32 of pol δ.

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Abbreviations

CTD	C-terminal domain
FeS	[4Fe–4S] iron sulfur
Mgs1	Maintenance of Genome Stability 1
ZnF	zinc finger

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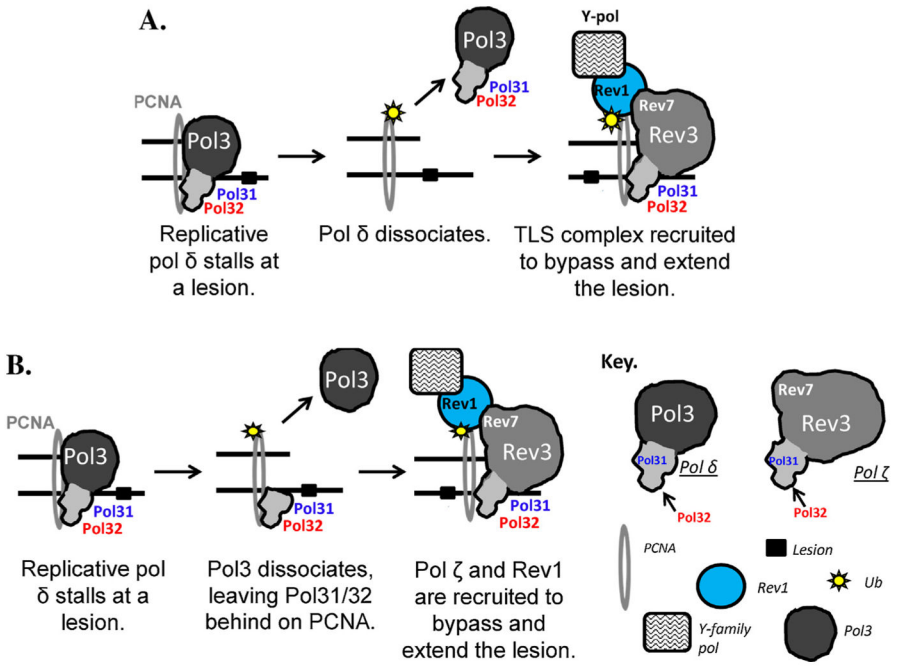

Fig. 1.

Models of polymerase switching during TLS. (A) Classic two-event polymerase switch model. There is a switch from pol δ to a TLS complex composed of a Y-family pol such as Rev1 or pol η and pol ζ. After insertion of a nucleotide opposite the lesion by a member of this complex, pol ζ extends the primer. Rev1 also acts as a scaffold. (B) Illustration of the switch from pol δ to pol ζ in the new variant of polymerase switch model utilizing the exchange of Pol31/Pol32 subunits. The catalytic subunit of pol δ (Pol3) dissociates from the DNA and Rev3/Rev7 binds to the Pol31/Pol32 still left on DNA. Other steps are the same as in A.

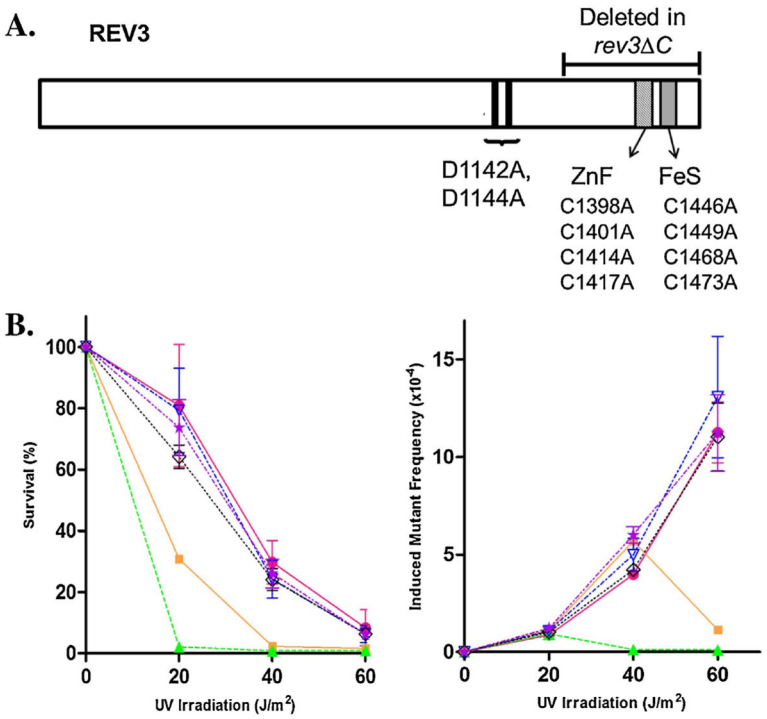

Fig. 2.

Effects of *rev3* mutations on UV mutagenesis alone and in the presence of wild-type *REV3*. (A) Schematic view of Rev3 variants used in this study. (B) WT strains additionally expressing mutant variants of Rev3 exhibit WT levels of survival and mutagenesis. Strains possess WT *REV3* in the chromosomal location and mutant rev3 on an expression plasmid. WT + vector (pink ●), *rev3ΔC* + vector (orange ■), *rev3-FeS* + vector (green ▲), WT + *rev3-dd*↑ (blue ▽), WT + *rev3ΔC*↑ (black ◇), WT + *rev3-FeS*↑ (purple ★).

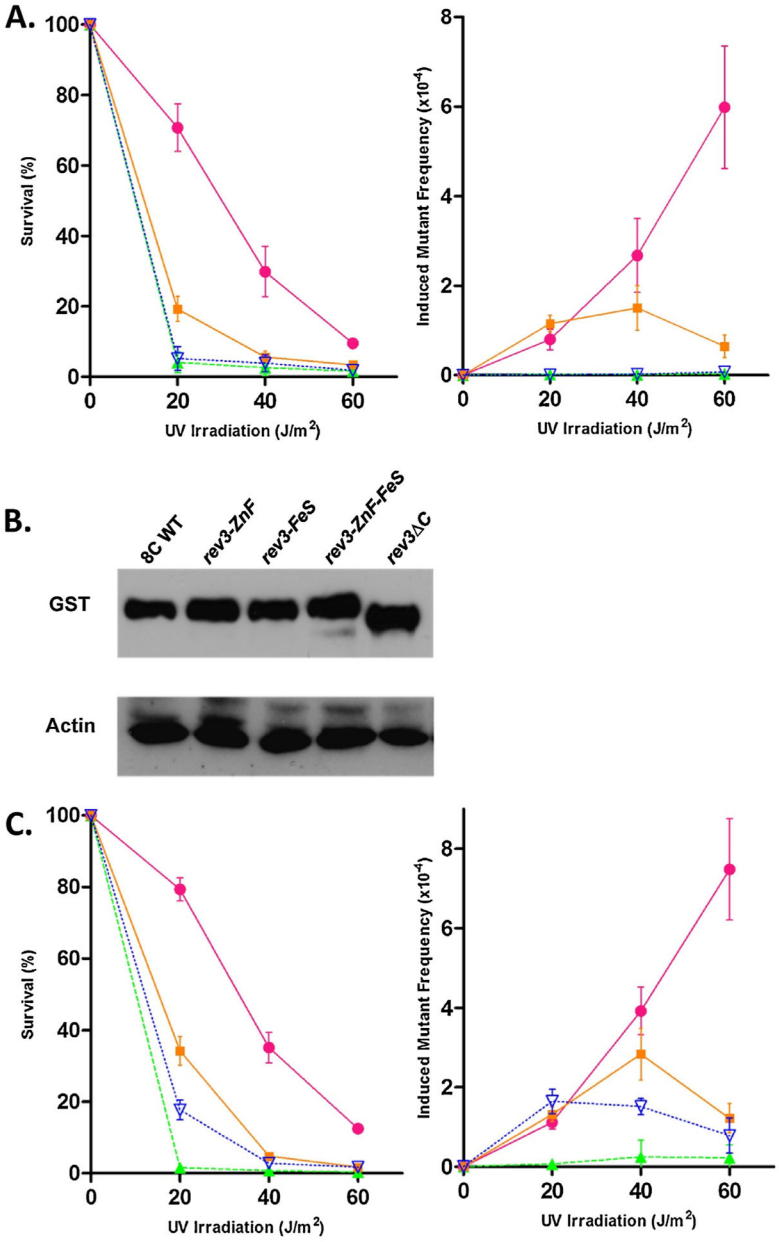

Fig. 3.

Induced mutagenesis in the *rev3ΔC* strain is dependent on the catalytic activity of Rev3 and is not increased by overproduction of Rev3ΔC. (A) The *rev3ΔC-dd* mutant was hypersensitive and immutable, demonstrating that mutagenesis in *rev3ΔC* is dependent on the catalytic activity of Rev3. 8C WT (pink ●), *rev3-dd* (green ▲), *rev3ΔC* (orange ■), *rev3ΔC-dd* (blue ▽). (B) Western blot analysis of the overproduced CTD mutants of Rev3. There was only a slight increase in the levels of rev3ΔC over WT Rev3. (C) Artificial, robust overexpression of *rev3ΔC* over WT has no effect on mutagenesis. WT + vector (pink ●), *rev3Δ* + vector (green ▲), *rev3ΔC* + vector (orange ■), *rev3Δ* + *rev3ΔC*↑ (blue ▽).

Data were collected and analyzed as described in Section 2.3 (three independent trials). All strains are described in Table 2.

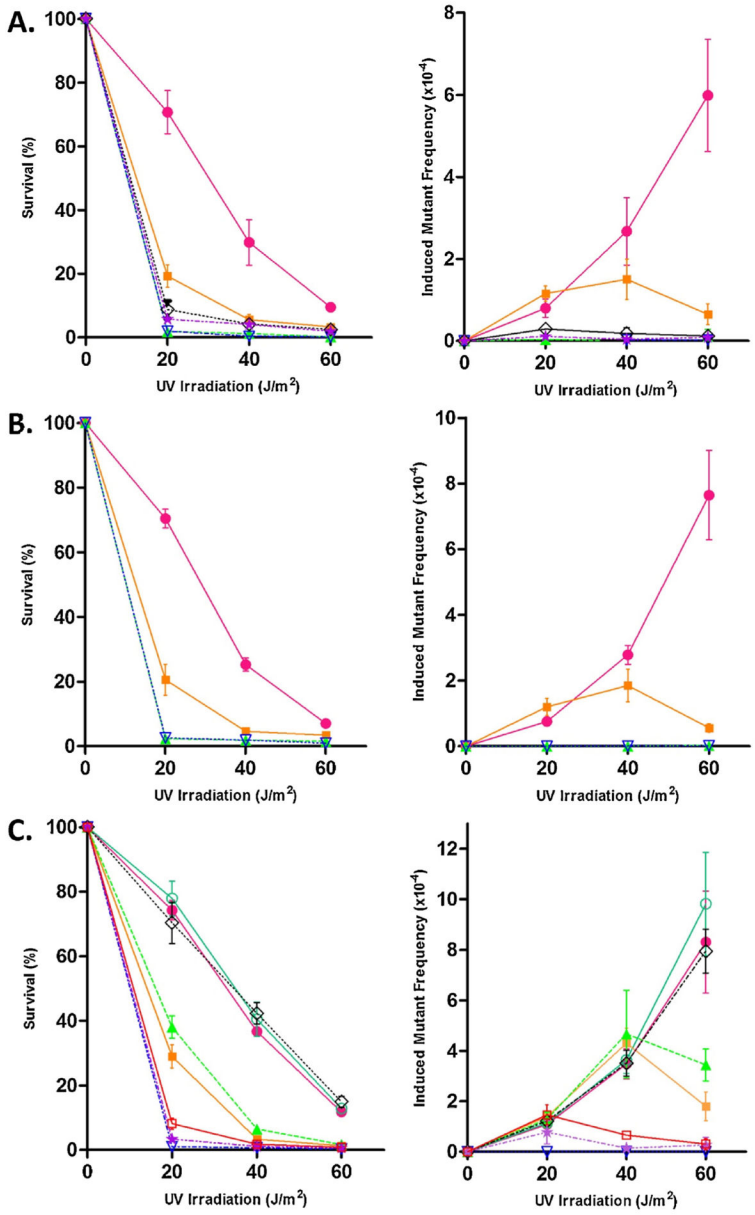

Fig. 4.

UV induced mutagenesis in the *rev3ΔC* and WT strains is under similar genetic control. (A) Survival and mutagenesis of WT and *rev3ΔC* strains are both dependent upon monoubiquitylation of PCNA and the presence of Pol32. 8C WT (pink ●), *rev3ΔC* (orange ■), *pol30-K164R* (green ▲), *rev3ΔC pol30-K164R* (blue ▽), *pol32Δ* (black ◇), *rev3ΔC pol32Δ* (purple ★). (B) Survival and mutagenesis are also dependent upon the presence of Rev1. 8C WT (pink ●), *rev3ΔC* (orange ■), *rev1Δ* (green ▲), *rev3ΔC rev1Δ* (blue ▽). (C) Overexpression of exogenous Rev1 does not elevate mutagenesis at high doses of UV light in WT and *rev3ΔC* strains. WT + vector (pink ●), WT + *REV1↑* (light teal ○), *rev3ΔC* + vector (orange ■), *rev3ΔC* + *REV1↑* (green ▲), *rev1Δ* + vector (blue ▽), *rev1Δ* + *REV1↑* (black ◇), *rev3-FeS* + vector (purple ★), *rev3-FeS* + *REV1↑* (red □). Data were collected

and analyzed as described in Section 2.3 (three independent trials). All strains are described in Table 2.

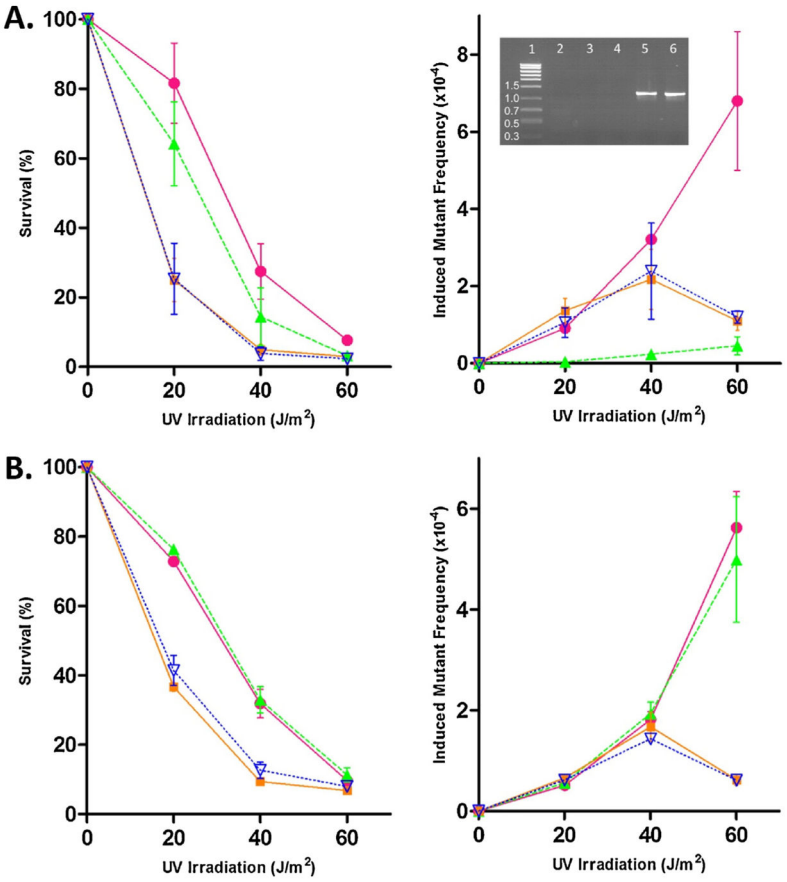

Fig. 5.

*rev3*Δ*C* truncation mutant is insensitive to suppression of UV-induced mutagenesis by overexpression of *MGS1*. (A) Overexpression of *MGS1* suppresses mutagenesis only in the WT strain. 8C WT (pink ●), *rev3*Δ*C* (orange ■), *MGS1*↑ (green ▲), *rev3*Δ*C MGS1*↑ (blue ▽). (A) insert: PCR analysis confirms the correct integration of an *MGS1*expression cassette in WT and *rev3*Δ*C* strains. The forward primer had homology to the plasmid backbone sequence and the reverse primer had homology to the beginning of the *MGS1* gene; these primers amplify a region of about 1.3 kb. Lanes **1** – DNA ladder, **2** – blank PCR sample (no DNA added), **3** – 8C WT DNA, **4** – *rev3*Δ*C* DNA, **5** – WT + *MGS1*↑ DNA, **6** – *rev3*Δ*C* + *MGS1*↑ DN(A). (B) Deletion of Mgs1 has no effect on mutagenesis in WT and *rev3*Δ*C* strains. 8C WT (pink ●), *rev3*Δ*C* (orange ■), *mgs1*Δ (green ▲), *rev3*Δ*C mgs1*Δ (blue ▽). Data were collected and analyzed as described in Section 2.3 (three independent trials). All strains are described in Table 2.

Table 1

Nomenclature for yeast and human Pol δ and Pol ζ.

Organism	Subunit	Gene	Protein
Polymerase δ			
Yeast	Catalytic	*POL3*	Pol3
	B	*POL31*	Pol31
	C	*POL32*	Pol32
Human	Catalytic	*POLD1*	p125
	B	*POLD2*	p50
	C	*POLD3*	p66
	Small 4th	*POLD4*	p12
Polymerase ζ			
Yeast	Catalytic	*REV3*	Rev3
	Accessory	*REV7*	Rev7
	B, C	*POL31*, *POL32*	Pol31, Pol32
Human	Catalytic	*REV3L*	p353
	Accessory	*REV7*	P30
	B, C	*POLD2*, *POLD3*	P50, p66

Table 2

Description of mutant strains used in this study.

	Relevant genotype[a]	Principal defect or combination	Comments
REV3 mutants	*rev3-dd*	Catalytically inactive (D1142A, D1144A)	pCAV2 plasmid was subjected to site directed mutagenesis in the Dr. M. Diaz laboratory (NIEHS) and when mutation was transferred in genomic location of 8C-YUNI101, yeast strain was UV immutable (personal communication)
	rev3-FeS	Disruption of FeS cluster binding site (C1446A, C1449A, C1468A, C1473A)	This site was called MBS2 and mutant first described in [32]
	rev3ΔC	Absence of Rev3 C-terminal domain (amino acids 1381–1505)	This study
Other mutants	*pol30-K164R*	PCNA (encoded by *POL30* gene) variant that cannot be ubiquitylated (K164R) [18]	Plasmid to create this mutant described in [23]
	*pol32*Δ	Absence of Pol32 due to deletion of *POL32* gene, leads to immutability [51]	Disruption by kanMX cassette
	*rev1*Δ	Absence of Rev1 due to deletion of *REV1* gene	Disruption by kanMX cassette
	*mgs1*Δ	Absence of Mgs1 due to deletion of *MGS1* gene	Disruption by kanMX cassette
	MGS1↑	Overexpression of *MGS1* under the GAL1 promoter (GAL1–MGS1 integrated at *LEU2*)	Constructed in this work by integration of the plasmid YIp128-GAL-MGS1, as described in [44]
Double mutants	*rev3ΔC-dd*	Combination of *rev3ΔC* and *rev3-dd*	This study
	*rev3ΔC pol32*Δ	Combination of *rev3ΔC* and *pol32*Δ	This study
	*rev3ΔC rev1*Δ	Combination of *rev3ΔC* and *rev1*Δ	This study
	*rev3ΔC mgs1*Δ	Combination of *rev3ΔC* and *mgs1*Δ	This study
	rev3ΔC MGS1↑	Overexpression of *MGS1* in the *rev3ΔC* strain	This study
	rev3ΔC pol30-K164R	Combination of *rev3ΔC* and *pol30-K164R*	This study
Strains with plasmids[b]	WT + vector	8C WT strain with pRS425-GALGST empty vector	This study
	WT + *REV1*↑	*REV1* overexpression in WT strain with pRS425-GALGST-*REV1*	This study
	WT + *rev3-dd*↑	*rev3-dd* overexpression in WT strain with Yep181-*rev3-dd*	This study
	WT + *rev3ΔC*↑	*rev3ΔC* overexpression in WT strain with pRS425-GALGST-*rev3ΔC*	This study
	WT + *rev3-FeS*↑	*rev3-FeS* overexpression in WT strain with pRS425-GALGST-*rev3-FeS*	This study
	*rev3*Δ + vector	*rev3*Δ strain with pRS425-GALGST empty vector	This study
	*rev3*Δ + *rev3ΔC*↑	*rev3ΔC* overexpression in *rev3*Δ strain with pRS425-GALGST-*rev3ΔC*	This study
	rev3ΔC + vector	*rev3ΔC* strain with pRS425-GALGST empty vector	This study
	rev3ΔC + *REV1*↑	*REV1* overexpression in *rev3ΔC* strain with pRS425-GALGST-*REV1*	This study
	rev3-FeS + vector	*rev3-FeS* strain with pRS425-GALGST empty vector	This study
	rev3-FeS + *REV1*↑	*REV1* overexpression in *rev3-FeS* strain from pRS425-GALGST-*REV1*	This study
	*rev1*Δ + vector	*rev1*Δ strain with pRS425-GALGST empty vector	This study

	Relevant genotype[a]	Principal defect or combination	Comments
	*rev1*Δ + *REV1*↑	*REV1* overexpression in *rev1*Δ strain from pRS425-GALGST-*REV1*	This study

[a] All mutants except *mgs1*Δ were created in the 8C-YUNI101 background (Section 2).

[b] Description of plasmids is in Section 2.2.

Table 3

rev3ΔC strain shows robust mutagenesis at low doses of UV irradiation and retains residual mutagenesis at high doses.

Strain	UV treatment (J/m^2)	Percent survival[a]	Mutant frequency (×10^{-6})[b]	Induced mutant frequency (×10^{-6})[b]
8C WT	0	100.0 ± 6.7	1 ± 0.4	–
	20	69.2 ± 0.9	87 ± 12	86 ± 12
	40	23.8 ± 3.0	299 ± 42	298 ± 42
	60	7.4 ± 7.0	701 ± 137	700 ± 137
rev3-dd	0	100.0 ± 8.8	0.8 ± 0.6	–
	20	4.6 ± 0.0	1.7 ± 1.7	1 ± 1
	40	3.9 ± 0.0	1.9 ± 1.2	1 ± 1
	60	2.0 ± 0.1	7.5 ± 5.4	7 ± 5
rev3ΔC	0	100.0 ± 8.6	0.8 ± 0.2	–
	20	20.6 ± 1.3	130 ± 9	129 ± 9
	40	4.8 ± 1.7	168 ± 33	167 ± 33
	60	2.7 ± 0.6	65 ± 15	64 ± 15
rev3-FeS	0	100.0 ± 15.8	0.8 ± 0.2	–
	20	6.7 ± 0.4	38.8 ± 18.5	38 ± 18
	40	4.1 ± 0.1	7.4 ± 3.6	7 ± 3
	60	2.5 ± 0.1	13.1 ± 11.0	12 ± 11

[a] Values are mean ± SD in %.

[b] Values are mean ± standard deviation (SD).

Data are averages of four independent trials.