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A novel function of DNA polymerase ζ regulated by PCNA

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DNA polymerase ζ (Polζ) participates in translesion DNA synthesis and is involved in the generation of the majority of mutations induced by DNA damage. The mechanisms that license access of Polζ to the primer terminus and regulate the extent of its participation in genome replication are poorly understood. The Polζ-dependent damage-induced mutagenesis requires monoubiquitination of proliferating cell nuclear antigen (PCNA) that is triggered by exposure to mutagens. We show that Polζ contributes to DNA replication and causes mutagenesis not only in response to DNA damage but also in response to malfunction of normal replicative machinery due to mutations in replication genes. These replication defects lead to ubiquitination of PCNA even in the absence of DNA damage. Unlike damage-induced mutagenesis, the Polζ-dependent spontaneous mutagenesis in replication mutants is reduced in strains defective in both ubiquitination and sumoylation of Lys164 of PCNA. Additionally, studies of a PCNA mutant defective for functional interactions with Polζ, but not for monoubiquitination by the Rad6/Rad18 complex demonstrate a role for PCNA in regulating the mutagenic activity of Polζ separate from its modification at Lys164.

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Introduction

Cellular DNA is continuously damaged by endogenous and exogenous genotoxicants. Although cells possess specialized repair pathways that remove various types of damage, DNA damage occurs throughout the cell cycle including S phase. The replication machinery, therefore, occasionally encounters damaged DNA templates, which creates obstacles for DNA replication. Structural studies show that replicative DNA polymerases have active sites that precisely accommodate the four correct and geometrically equivalent Watson–Crick base pairs to allow efficient and accurate synthesis (Kunkel, 2004). Bulky lesions cannot be readily accommodated in the active sites of these polymerases, which likely accounts for the replication block. The last several years have seen the discovery of DNA polymerases that have the ability to bypass lesions in template DNA that block replicative DNA polymerases (Prakash *et al*, 2005). These include Polζ, Polη, Polι, Polκ, and REV1 present in human cells. These DNA polymerases have a remarkably low fidelity when copying undamaged DNA *in vitro*, which is thought to reflect the lower selectivity of their active sites (Kunkel *et al*, 2003).

The existence of several DNA polymerases with very low fidelity suggests that their participation in genome replication needs to be carefully regulated. The mechanisms that regulate the access of translesion synthesis (TLS) DNA polymerases to the primer terminus are not clear. Recent studies in yeast *Saccharomyces cerevisiae* and in human cells suggest that post-translational modification of proliferating cell nuclear antigen (PCNA), an accessory factor of replicative DNA polymerases, is important for the switch from the replicative polymerase to a TLS polymerase (Hoege *et al*, 2002; Stelter and Ulrich, 2003; Haracska *et al*, 2004; Kannouche *et al*, 2004). PCNA is a homotrimeric complex that encircles DNA and functions as a processivity factor for DNA polymerases. Three types of post-translational modification have been shown to occur at a single lysine residue of PCNA, Lys164: monoubiquitination by the Rad6/Rad18 ubiquitin conjugation/ligase complex, subsequent Lys63-linked polyubiquitination by Mms2/Ubc13 and Rad5, and Ubc9- and Siz1-mediated SUMO conjugation. Elegant genetic experiments in *S. cerevisiae* with a mutant that has arginine substituted for Lys164 of PCNA have demonstrated that these three modifications label PCNA to endow different functions. Monoubiquitination of Lys164 is required for TLS *in vivo*. Mms2/Ubc13 and Rad5-mediated polyubiquitination is required for the error-free pathway of post-replicational DNA repair. Consistent with this, ubiquitination of PCNA is induced by exposure to DNA-damaging agents in both yeast and human cells. In contrast, sumoylation of PCNA during the S phase of the cell cycle reduces DNA damage tolerance, thus counteracting the effects of the ubiquitination.

While several low-fidelity DNA polymerases exist in eukaryotic cells, the TLS activity of Polζ is responsible for nearly all mutations induced by DNA-damaging agents *in vivo*. Yeast and mammalian Polζ is comprised of two subunits encoded by the *REV3* and *REV7* genes. In the absence of either gene product, DNA damage-induced mutagenesis is severely decreased or completely abolished (Lawrence, 2002). DNA damage-induced mutagenesis *in vivo* also requires the Rev1 protein, a deoxycytidyl transferase that likely acts in conjunction with Polζ. While the catalytic activity of Rev1 normally functions during TLS, particularly during abasic site bypass,

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most forms of TLS require its organizing function (Lawrence, 2002; Gibbs *et al*, 2005). In contrast, the DNA polymerase activity of Polζ is essential for mutagenic lesion bypass, since point mutations in its polymerase active site abolish DNA damage-induced mutagenesis (Pavlov *et al*, 2001b). Biochemical studies have shown that Polζ is a template-directed DNA polymerase that can synthesize, with limited efficiency, past several types of DNA lesions that block DNA synthesis by normal replicative DNA polymerases (Nelson *et al*, 1996; Guo *et al*, 2001). The TLS activity of Polζ is stimulated by PCNA (Garg *et al*, 2005). The main function of Polζ in TLS appears to be the extension from nucleotides incorporated opposite DNA lesions by other DNA polymerases. Polζ efficiently extends mismatched primer termini as well as those containing a terminal nucleotide opposite a noncoding or helix-distorting lesion (Johnson *et al*, 2000; Guo *et al*, 2001; Haracska *et al*, 2001b; Simhadri *et al*, 2002). This has provided the basis for the 'two-polymerase' lesion bypass model, wherein nucleotide incorporation opposite a lesion by a replicative or one of the TLS polymerases is followed by extension by Polζ (Lawrence and Maher, 2001; Bresson and Fuchs, 2002; Prakash and Prakash, 2002). However, Polζ also replicates undamaged DNA with very low fidelity. The average base substitution error rate for Polζ is $\sim 10^{-3}$, much higher than that for homologous B family polymerases α, δ and ε (Zhong *et al*, 2006). Therefore, in addition to the role of Polζ as an extender in TLS, it could potentially also contribute to the generation of substitution mutations if it is allowed to copy undamaged DNA *in vivo*. Polζ is required for the majority of spontaneous mutations in wild-type strains (Lawrence, 2002), which could at least partly reflect frequent misinsertion of nucleotides by this polymerase.

In addition to spontaneous mutagenesis in wild-type strains and DNA damage-induced mutagenesis, Polζ is also responsible for the increase in mutation rate caused by defects in nucleotide excision repair (e.g., Harfe and Jinks-Robertson, 2000), base excision repair (Xiao *et al*, 2001), postreplicative DNA repair (e.g., Broomfield *et al*, 1998), homologous recombination (e.g., Harfe and Jinks-Robertson, 2000), overproduction of 3-methyladenine DNA glycosylase (Glassner *et al*, 1998), as well as the increase in mutation rate associated with double-strand break repair (Holbeck and Strathern, 1997) and with high levels of transcription (Datta and Jinks-Robertson, 1995). In many, or, possibly, all of these cases, the Polζ-dependent mutagenesis likely reflects the function of this polymerase in the error-prone bypass of endogenous DNA damage.

Here we demonstrate that participation of Polζ in genome replication can be promoted not only by DNA damage but also by a variety of defects in the components of normal DNA replication machinery. We show that such defects lead to an increase in Polζ-dependent spontaneous mutagenesis and increased susceptibility to DNA damage-induced mutagenesis. We also show that both ubiquitin and SUMO modification of PCNA at Lys164 contribute to Polζ-dependent mutagenesis provoked by DNA replication defects. Finally, we show that a specific PCNA mutation (*pol30-113*) affecting amino-acid residues near the monomer–monomer interface of PCNA prevents Polζ-dependent spontaneous and UV-induced mutagenesis, but not Rad6/Rad18-dependent mono-ubiquitination of PCNA. Remarkably, this mutant PCNA is defective for functional interactions with Polζ. These results provide the first combined *in vivo* and *in vitro* evidence that PCNA has a direct role in regulation of Polζ-dependent mutagenic replication distinct from its ubiquitination by Rad6/Rad18, and identify a structural element in PCNA important for this second function.

Results

The mutator phenotype of DNA replication mutants is dependent on Polζ

A variety of mutations in the *S. cerevisiae* genes encoding replicative DNA polymerases and their accessory factors have been described. Many of these mutations result in slow or temperature-sensitive DNA replication and/or impaired interactions between the replisome components. These defects usually bring about a spontaneous mutator phenotype. Examples of such spontaneous mutators include strains with defects in the catalytic and accessory subunits of Polα, Polδ, and Polε (e.g., Longhese *et al*, 1993; Shcherbakova *et al*, 1996; Tran *et al*, 1999; Pavlov *et al*, 2001b; Gutierrez and Wang, 2003), and the polymerase accessory proteins PCNA and RFC (e.g., Ayyagari *et al*, 1995; Chen *et al*, 1999; Xie *et al*, 1999). These mutator phenotypes could be ascribed to reduced fidelity of the replicative polymerases, or, alternatively, to the activation of low-fidelity replication mechanisms triggered by the defects in the normal replication machinery. Based on preliminary genetic studies, we have hypothesized that the majority of spontaneous mutations in such mutants result from the recruitment of Polζ for DNA replication. The spontaneous mutator phenotype of two DNA replication mutants, *pol2-1* and *pol3-Y708A*, is dramatically reduced when Polζ is inactivated (Shcherbakova *et al*, 1996; Pavlov *et al*, 2001b). The *pol2-1* and *pol3-Y708A* mutations affect the catalytic subunits of the two major replicative polymerases, Polε and Polδ. To investigate whether a wide variety of defects in the replication machinery can trigger the recruitment of Polζ to the primer terminus, we have analyzed the effects of Polζ inactivation on the mutator phenotype of several replication mutants.

The *pol1-1* mutation impairs the interaction between the catalytic and the primase subunits of Polα and causes temperature sensitivity (Lucchini *et al*, 1988). The *pol3t* mutation results in a single amino-acid change in the vicinity of the polymerase active site of Polδ (Tran *et al*, 1999). This mutation leads to temperature sensitivity and increased frequency of deletions between repetitive sequences (see references in Tran *et al*, 1999), suggesting defective interaction of the polymerase with the template DNA. The *dpb3* mutation is a deletion of the gene encoding the third subunit of Polε. We observed that all these mutations confer a spontaneous mutator phenotype, in accordance with the previously published data. In all cases, deletion of the *REV3* gene encoding the catalytic subunit of Polζ eliminated most of the spontaneous mutator effect (Figure 1; Supplementary Table I). The extent to which the mutator phenotype was dependent on Polζ, varied from ∼57% in *pol1-1* to ∼80% in *pol2-1* and *pol3t* to >90% in *pol3-Y708A* and *dpb3* mutants. This is consistent with the idea that a fraction of mutations could result from errors made by the defective replicative polymerases themselves (discussed in Pavlov *et al* (2001b); see also Niimi *et al* (2004), for an example of a *pol1* mutation that

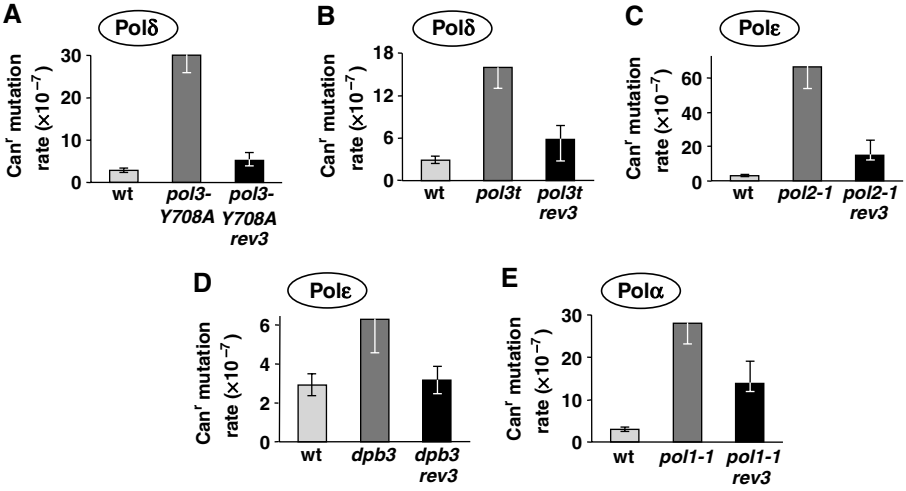

Figure 1 Effect of *rev3* mutation on the spontaneous mutator phenotype of DNA replication mutants. The diagrams show the rates of Can^r mutation in the *REV3*$^+$ and *rev3* variants of *pol3-Y708A* (**A**), *pol3t* (**B**), *pol2-1* (**C**), *dpb3* (**D**), and *pol1-1* (**E**) strains. The DNA polymerases affected by the mutations are indicated on top of each diagram. All data are from Supplementary Table I and are medians and 95% confidence limits for at least 18 independent determinations. On each graph, the upper confidence limit is not shown for the strongest mutator strain.

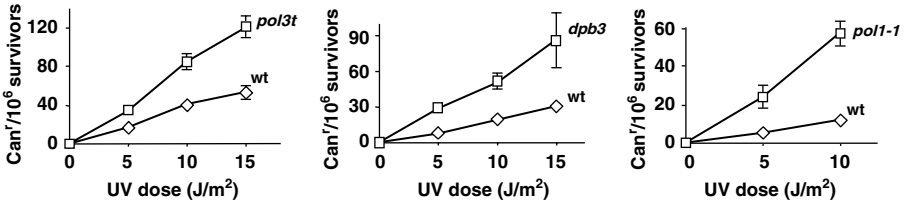

Figure 2 Effect of DNA replication mutations on UV-induced mutagenesis. The data are average frequencies of UV-induced Can^r mutants for three independent determinations. Standard errors are shown where the size of the error bar exceeds the size of the plot symbol.

greatly elevates spontaneous mutagenesis due to frequent DNA synthesis errors by Polα). Interestingly, a strong anti-mutator effect of the *rev3* deletion was observed in all DNA replication mutants for *CAN1* and *TRP1* reporters that score various forward mutations in the *CAN1* gene and reversion of a base substitution mutation, respectively. At the same time, the rate of frameshift mutations, which is slightly elevated in the replication mutants, was minimally affected by Polζ inactivation (Supplementary Table I). This suggests that, while the majority of mutagenesis in the DNA replication mutants is mediated by Polζ, frameshifts are generated through a Polζ-independent mechanism. In agreement with these results are recent *in vitro* studies showing that Polζ rarely makes insertion/deletion errors (Zhong *et al*, 2006).

The Polζ-dependent mutator phenotype of replication mutants suggests that the contribution of Polζ to genome replication in these mutants may be higher than in wild-type strains. As Polζ plays a key role in the generation of mutations by many DNA-damaging agents, we hypothesized that the increased participation of Polζ may result in a higher susceptibility of the Polα, Polδ, and Polε mutants to damage-induced mutagenesis. To test this, we have measured the frequency of UV-induced mutation to canavanine resistance (Can^r) in *pol1-1*, *pol3t*, and *dpb3* mutants. UV mutagenesis was elevated in all of the mutants tested (Figure 2). This elevation was dependent on Polζ: no UV mutagenesis was seen in the double *pol1-1 rev3*, *pol3t rev3*, and *dpb3 rev3* mutants (data not shown).

Polζ-dependent spontaneous mutator phenotype of DNA replication mutants is promoted by ubiquitin and SUMO conjugation to PCNA

Polζ-dependent damage-induced mutagenesis requires monoubuquitination of PCNA at Lys164 (Stelter and Ulrich, 2003; Haracska *et al*, 2004). We aimed to determine whether modification of PCNA at this residue is also important for the recruitment of Polζ in response to defects in the replicative DNA polymerases. We replaced the wild-type *POL30* gene encoding PCNA with a mutant *pol30-K164R* allele in the *pol3-Y708A* and *pol2-1* mutants that show the strongest Polζ-dependent mutator phenotype. The *pol30-K164R* mutation dramatically reduced the spontaneous mutator phenotype of the replication mutants (Figure 3; Supplementary Table I). Moreover, the mutation rate in the *pol30-K164R* and *rev3* derivatives of the replication mutants was similar. This suggests that, as in the case of DNA damage, the recruitment of Polζ in response to replication defects is fully dependent on modification of PCNA at Lys164.

The Lys164 residue in PCNA serves as an acceptor for both ubiquitin and SUMO conjugation (Hoege *et al*, 2002). Therefore, the effect of the *pol30-K164R* mutation on the Polζ-dependent spontaneous mutator phenotype of replication mutants may reflect the importance of ubiquitination, sumoylation, or both types of PCNA modification for the recruitment of Polζ in response to replication defects. DNA damage-induced Polζ-dependent mutagenesis depends entirely on monoubiquitination of PCNA and is not influenced

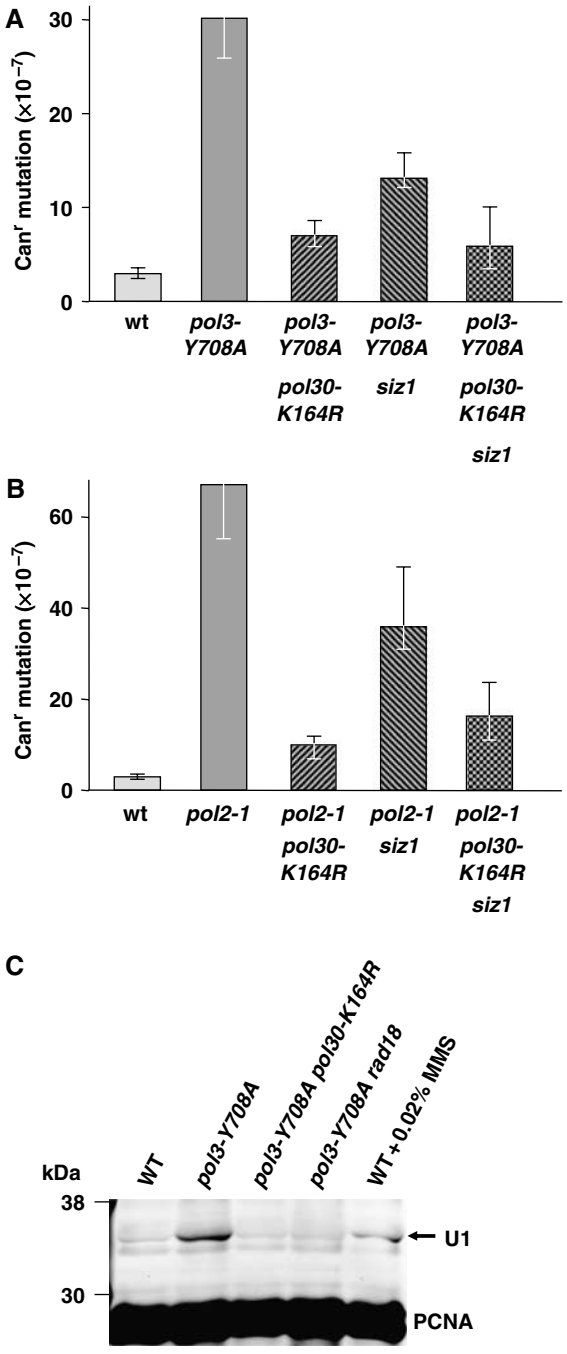

Figure 3 Polζ-dependent spontaneous mutagenesis in DNA replication mutants is promoted by post-translational modification of PCNA. Diagrams (**A**) and (**B**) show the effects of *pol30-K164R* and *siz1* mutations on the spontaneous mutator phenotype of *pol3-Y708A* and *pol2-1* strains, respectively. The data are rates of Can^r mutation from Supplementary Table I and are medians and 95% confidence limits for at least 18 independent determinations. (**C**) Detection of monoubiquitinated PCNA in whole-cell extracts of the *pol3-Y708A* strain by Western blot analysis. The positions of unmodified and monoubiquitinated (U1) PCNA are indicated. The MMS treatment (last lane) was for 1 h. The low-intensity band at U1 position in the untreated wild-type strain likely represents cross-reacting protein species, since it is also seen in *pol30-K164R* and *rad18* strains.

by defects in sumoylation (Stelter and Ulrich, 2003; Haracska *et al*, 2004). To determine whether sumoylation of PCNA plays any role in the spontaneous Polζ-dependent mutagenesis in replication mutants, we studied the effects of *siz1* mutation on the mutation rate in *pol3-Y708A* and *pol2-1* strains. The *SIZ1* gene encodes a SUMO ligase that is essential for sumoylation of PCNA at Lys164. Deletion of the *SIZ1* eliminated ~50% of the spontaneous mutator effect of the *pol3-Y708A* and *pol2-1* mutations (Figure 3; Supplementary Table I). Although Lys164 is the primary sumoylation site on PCNA, sumoylation also occurs on Lys127 (Hoege *et al*, 2002). In addition, Siz1 has other cellular targets besides PCNA. To examine whether sumoylation of PCNA at Lys164 or other functions of Siz1 are important for the spontaneous mutagenesis in replication mutants, we measured the mutation rate in *pol3-Y708A* and *pol2-1* strains in which both Siz1 and the acceptor Lys164 residue were absent (*siz1 pol30-K164R* background). We did not observe any additional reduction in mutation rate in the double *siz1 pol30-K164R* mutants in comparison to the single *pol30-K164R* mutants (Figure 3; Supplementary Table I). This indicates that the *siz1* mutation reduces spontaneous mutagenesis in the DNA replication mutants exclusively because of the defect of sumoylation of PCNA at Lys164.

While sumoylation of PCNA normally occurs in the S phase of the cell cycle (Hoege *et al*, 2002), little if any ubiquitination of PCNA is observed unless the cells are exposed to DNA-damaging agents or a replication inhibitor hydroxyurea (HU) (Hoege *et al*, 2002; Kannouche *et al*, 2004; Papouli *et al*, 2005). On the other hand, the genetic data (Figure 3A and B) suggested an important role for ubiquitination of Lys164 in Polζ-dependent spontaneous mutagenesis in DNA replication mutants. This led us to propose that replication perturbations caused by the intrinsic replisome defects lead to constitutive ubiquitination of PCNA. Indeed, protein species corresponding to monoubiquitinated PCNA was readily detected in extracts of replication mutants even without treatment with replication-blocking agents (Figure 3C; Supplementary Figure 1). This band was absent in extracts of *pol30-K164R* and *rad18* derivatives of the replication mutants, indicating that the modification was Rad18 dependent and occurred specifically at Lys164.

The pol30-113 mutation confers a defect in Polζ-dependent spontaneous and UV-induced mutagenesis

Aiming to identify additional functions or structural features of PCNA important for Pol ζ-dependent spontaneous mutagenesis triggered by replication defects, we analyzed the effects of the *pol30-113* mutation on the spontaneous mutation rate in *pol3-Y708A* and *pol2-1* strains. The *pol30-113* allele was initially identified in a screen for PCNA mutants sensitive to methylmethane sulfonate (MMS) (Amin and Holm, 1996). The mutant contains a double amino-acid change (E113G, L151S) at the monomer–monomer interface of the PCNA trimer (Figure 4A). This mutation reduced the spontaneous mutation rate in both replication mutants (Figure 4B; Supplementary Table I), albeit to a smaller extent than the *pol30-K164R* mutation did (Figure 3; Supplementary Table I). The double *pol3-Y708A pol30-113* and *pol2-1 pol30-113* mutants retained ~40 and ~30% of the Polζ-dependent mutator effect of the *pol3-Y708A* and *pol2-1* mutations, respectively.

We next addressed the question whether the function of PCNA affected by the *pol30-113* mutation is also important for DNA damage-induced mutagenesis. Interestingly, the *pol30-*

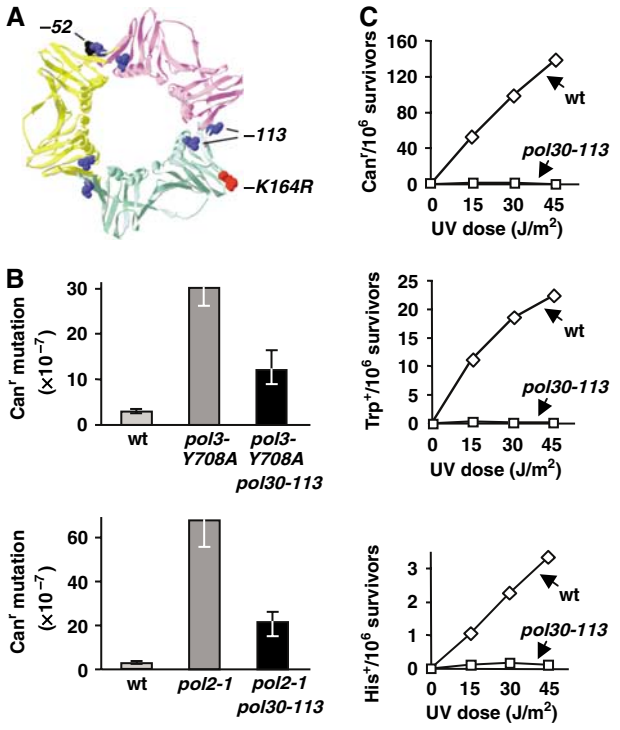

Figure 4 The *pol30-113* mutant is defective in Polζ-dependent spontaneous and UV-induced mutagenesis. (**A**) Location of the *pol30-K164R*, *pol30-113*, and *pol30-52* mutations on the PCNA structure. The three monomers of PCNA trimer are shown in yellow, magenta, and blue ribbon. The amino-acid residues affected by the mutations are shown in space fill mode. (**B**) Effect of *pol30-113* mutation on the spontaneous mutator phenotype of *pol3-Y708A* (top) and *pol2-1* (bottom) strains. The data are from Supplementary Table I and are medians and 95% confidence limits for at least 18 independent determinations. (**C**) Effect of the *pol30-113* mutation on the frequency of UV-induced Can^r mutants (top), Trp$^+$ revertants (middle), and His$^+$ revertants (bottom). The data are the average for at least three independent determinations.

113 strains showed a complete deficiency in UV-induced mutagenesis (Figure 4C), reminiscent of the UV-mutability defect caused by the *pol30-K164R* mutation that prevents ubiquitination of PCNA (Stelter and Ulrich, 2003; Haracska *et al*, 2004).

The pcna-113 is defective in functional interaction with Polζ

In order to understand the nature of the defect in the PCNA variant encoded by *pol30-113*, we studied its properties in well-defined biochemical assays. We compared the properties of pcna-113 with those of an other subunit interface mutant, pcna-52 (S115P), which displays cold-sensitive growth, hypersensitivity to HU and DNA-damaging agents, and is defective for damage-induced mutagenesis (Ayyagari *et al*, 1995).

A gel filtration analysis showed that pcna-113 has a minor defect in trimer stability, consistent with the position of the mutated residues at the subunit–subunit interfaces (Figure 5A). The phenotype of the *pol30-113* strain suggests that the trimerization defect of pcna-113 is minimal *in vivo*. The mutant has no detectable growth defect at normal (30°C) or low (13°C) temperature, and is not hypersensitive to the replication inhibitor HU (data not shown). This indicates that pcna-113 is fully capable of supporting chromosomal DNA replication and is, therefore, predominantly a trimer *in vivo*.

Considering the minor trimerization defect *in vitro*, we anticipated that the function of pcna-113 would be impaired at low concentrations, but that this defect could be suppressed at a higher concentration of the mutant clamp. Therefore, we carried out all our studies at varying PCNA concentrations in order to separate a possible specific defect from the known general defect in trimer stability. As an example of this type of analysis, we measured processive replication by Polδ of the 7.3 kb long single-stranded M13mp18 template DNA, which requires stable interaction with PCNA. About 10-fold higher concentrations of pcna-113 than of the wild-type clamp were required for full stimulation of processive synthesis (Figure 5B). In contrast, the predominantly monomeric mutant pcna-52 failed to fully stimulate processive synthesis, even at several 100-fold higher concentrations of pcna-52.

Next, we investigated the ability of pcna-113 to be ubiquitinated by the Rad6/Rad18 E2/E3 complex. Recently, we have established an *in vitro* PCNA ubiquitination system that faithfully reproduces the *in vivo* specificity, for example, mutant pcna-K164R is not ubiquitinated (Garg and Burgers, 2005); Figure 5C). This system also revealed that only PCNA encircling DNA is ubiquitinated, consistent with the notion that in the cell only PCNA present at stalled forks is ubiquitinated. At a clamp concentration of 25 nM, ubiquitination of pcna-113 was less efficient than that of wild-type (Figure 5C). However, this defect was suppressed at clamp levels of 75 nM or higher. Pcna-52 that is not able to associate stably with DNA, due its severe trimerization defect, was defective for ubiquitination at all concentrations. As the *pol30-113* yeast strain shows no apparent DNA replication defect *in vivo*, we expected this strain to possess primarily trimeric PCNA that could be efficiently ubiquitinated. Indeed, a Western blot analysis of whole-cell extracts demonstrated that monoubiquitination of PCNA is readily induced by treatment with MMS in the *pol30-113* mutant, similar to the isogenic wild-type strain (Figure 5D). In accordance with previous studies (Hoege *et al*, 2002), ubiquitination detected in this assay was specific to Lys164, as no induction of ubiquitination was seen in the *pol30-K164R* strain. These data suggest that the mutagenesis defect of pcna-113 does not stem from a defect in ubiquitination, and therefore, we asked whether pcna-113 was defective as a cofactor for a TLS DNA polymerase.

PCNA is a required cofactor for Polζ in TLS *in vitro* (Garg *et al*, 2005). Unexpectedly, we previously found that monoubiquitination of PCNA does not alter its functional interaction with Polζ, that is, PCNA and ubiquitinated PCNA were equally effective in activating TLS by Polζ (Garg and Burgers, 2005). We measured TLS by Polζ on an oligonucleotide substrate containing UV dimers induced in a oligo(dT)$_{25}$ template by irradiation at 254 nm. The substrate also contained biotin–streptavidin bumpers that prevent PCNA from sliding off the DNA onto which it has been loaded by replication factor C (Figure 5E). Bypass synthesis by Polζ on this UV-damaged template was PCNA dependent, and this bypass was abolished when pcna-113 replaced PCNA. Because neither Polδ nor Polη were proficient in TLS of this type of UV-damage, we next investigated bypass of a model abasic site by these enzymes (Figure 5F and G). Bypass replication of the abasic site by Polδ required PCNA. Pcna-113

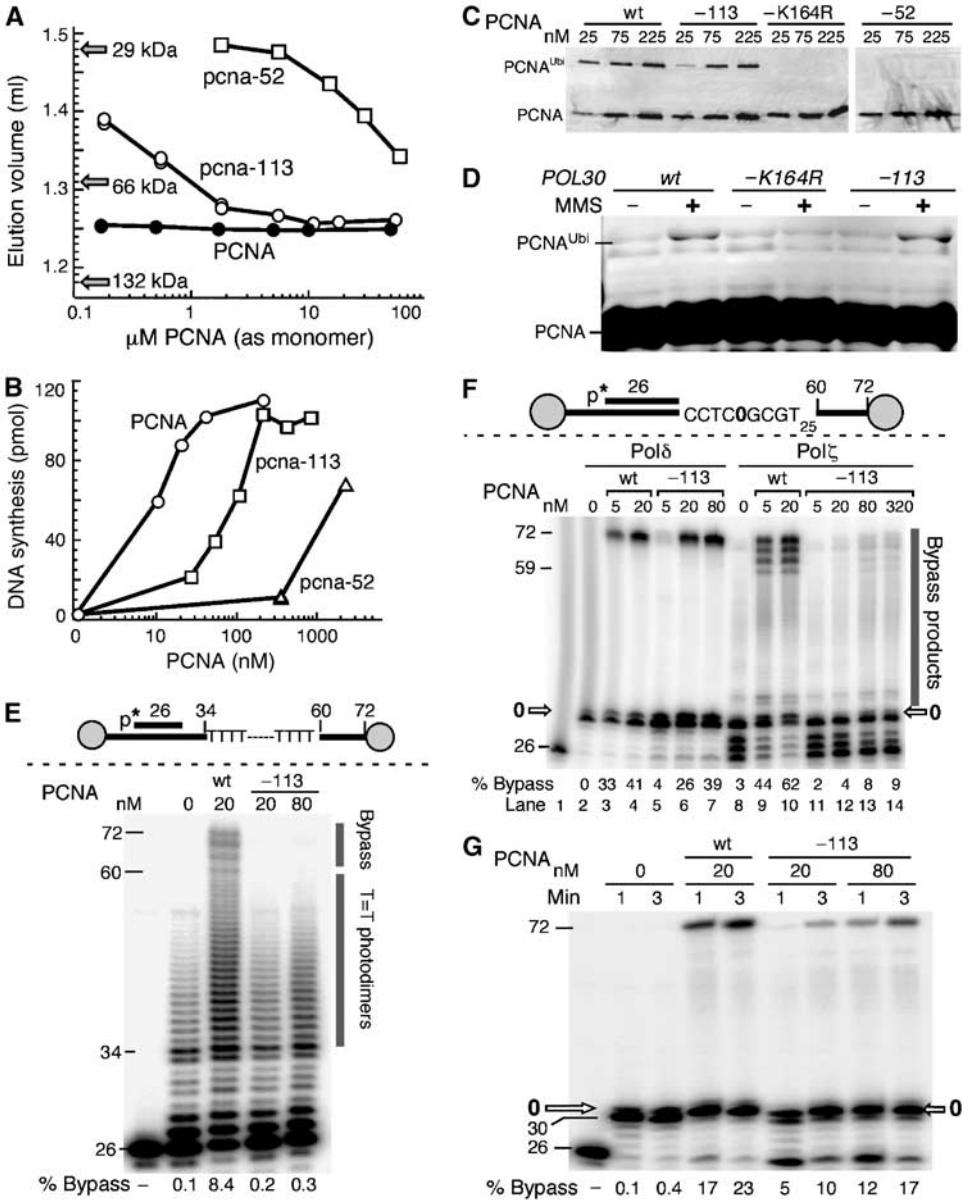

Figure 5 Pcna-113 shows a general trimerization defect and a specific Polζ interaction defect. (**A**) Gel filtration analysis of wild-type PCNA, pcna-52, and pcna-113 on a superose 6 column was carried out as described (Ayyagari *et al*, 1995). The data for pcna-52 were taken from that study. (**B**) Replication of SS mp18 DNA by Polδ with wild-type and mutant PCNA was as described in Materials and methods. (**C**) PCNA ubiquitination was carried in an assay containing ubiquitin, E1, Rad6/Rad18, multiple-primed SS DNA, RFC, and the indicated concentration of wild-type or mutant PCNA as described (Garg and Burgers, 2005). A Western analysis with antibodies to PCNA was used for detection. (**D**) Monoubiquitination of PCNA *in vivo* in the wild-type strain E134 and its *pol30-K164R* and *pol30-113* mutants was analyzed by Western blot with whole-cell extracts and antibodies to PCNA (see legend to Figure 3C for more details). (**E**) TLS on UV-irradiated (1000 J/m^2) template/primer V9/C12-4 by Polζ with the indicated concentrations of PCNA or pcna-113 was for 5 min at 30°C. Bypass products are defined as those proceeding past the irradiated dT_{25} stretch. (**F**) Bypass of an abasic site on template/primer V9AP2/C12-4 by Polδ or Polζ with the indicated concentrations of PCNA or pcna-113 was for 5 min at 30°C. (**G**) Bypass of an abasic site on template/primer V9AP2/C12-4 (see panel F) by Polη with the indicated concentrations of PCNA or pcna-113 was for 1 or 3 min at 30°C. See Materials and methods for details on all TLS assays.

was equally efficient in promoting bypass synthesis by Polδ, provided the mutant clamp was present at the higher concentration of 80 nM (Figure 5F, compare lanes 5–7 with 3–4). However, this is not the case for TLS by Polζ. While 5 or 20 nM wild-type PCNA promoted very efficient TLS (lanes 9 and 10), even 320 nM pcna-113 very poorly activated TLS by Polζ (compare lane 8, no PCNA and 3% bypass, with lanes 11–14, increasing pcna-113 and maximally 9% bypass). Pcna-113 was also defective in stimulating processive DNA replication by Polζ on undamaged DNA (not shown). Therefore, we conclude that pcna-113 is defective for functional interaction with Polζ, and suggest that the *in vivo* mutagenesis phenotype may be the result of this biochemically demonstrated defect.

To investigate whether the interaction defect with pcna-113 is specific for Polζ, we also determined its functionality with Polη, another TLS polymerase that requires PCNA for efficient bypass synthesis (Haracska *et al*, 2001a). While the rate of TLS by Polη past an abasic site was somewhat attenuated with pcna-113, it was still ~70% that of wild-type, indicating

that pcna-113 showed near full functionality with this DNA polymerase, as it did with Polδ (Figure 5G).

Discussion

Polζ is the major contributor to mutagenesis induced by a wide variety of DNA-damaging agents (Lawrence, 2002). There is also substantial evidence that Polζ is responsible for the majority of mutations induced by endogenous DNA damage (e.g., Glassner *et al*, 1998; Harfe and Jinks-Robertson, 2000). It has been hypothesized that Polζ could participate in DNA replication not only when DNA is damaged but also to facilitate extension at forks blocked for any reason, such as unedited terminal mismatch, hairpin structure, or refractory DNA sequence (Lawrence and Maher, 2001). However, the involvement of Polζ in replication of undamaged DNA has not been previously demonstrated. Here, we show that defects in the normal DNA replication machinery promote participation of Polζ in chromosomal DNA replication, which results in an increased mutation rate. We further demonstrate that both ubiquitin and SUMO modification of PCNA contribute to Polζ-dependent spontaneous mutagenesis in response to DNA replication defects. We also show that DNA replication mutants with elevated levels of spontaneous Polζ-dependent mutagenesis display an increased susceptibility to DNA damage-induced mutagenesis.

The discovery of a plethora of low-fidelity TLS polymerases has raised an issue of regulating the access of these polymerases to the primer terminus during replication. The TLS polymerases make, on average, one error per every 100–1000 nucleotides during copying of an undamaged DNA template *in vitro*. This is 100–1000-fold more than replicative DNA polymerases make under the same conditions (Kunkel *et al*, 2003). It is obvious that uncontrolled participation of the TLS polymerases in replicative DNA synthesis would present a threat to genome stability. Current evidence suggests that TLS polymerases cannot freely access the primer terminus during normal DNA replication. For example, under conditions of Polη overproduction, no increase in spontaneous mutagenesis that could be attributed to DNA synthesis by Polη was seen (Pavlov *et al*, 2001a). The mechanisms regulating participation of the multiple DNA polymerases in DNA replication could possibly include intrinsic differences in substrate preferences between different polymerases, regulation of protein–protein interactions at the replication fork by post-translational modification of DNA replication proteins, such as PCNA, as well as other mechanisms. Our results suggest that defects in the normal DNA replication machinery create a situation where access of Polζ to the primer terminus is permitted. One or both of the following factors may trigger the recruitment of Polζ to the primer terminus. First, destabilization of the replisome may lead to frequent dissociation of the replicative DNA polymerase from the primer/template, particularly in regions where polymerization is difficult due to secondary structures or other challenging DNA sequences. This would create many unextended primer termini that could be used by Polζ. Second, slow DNA replication may provide a signal for post-translational modification of PCNA that would result in the polymerase switch, similarly to the proposed mechanism of polymerase switch at replication forks stalled at sites of DNA damage (Ulrich, 2004). Our data on the induction of PCNA ubiquitination in the absence of DNA damage in DNA replication mutants and the absolute requirement of Lys164 residue in PCNA for their Polζ-dependent spontaneous mutator phenotype support the second idea. However, the mechanisms that bring Polζ to the primer terminus in the case of a defective replisome are not exactly the same as in the case of DNA damage.

Our results indicate that both ubiquitination and sumoylation of PCNA contribute to spontaneous mutagenesis in strains with replication defects, with ~50% of Polζ-dependent mutations being eliminated when sumoylation of Lys164 is absent because of a *SIZ1* deletion. At the same time, Polζ-dependent UV-induced mutagenesis relies entirely on monoubiquitination of PCNA and is not affected by defects in sumoylation at all (Stelter and Ulrich, 2003). The existence of more than one pathway promoting mutagenesis in response to DNA replication defects could be explained if we assume that there are distinct mechanisms that impede progression of replication forks in the DNA replication mutants. These could possibly include spontaneous dissociation of the mutant replicative polymerase from the primer terminus, increased polymerase stalling at a difficult DNA structure, or polymerase stalling or dissociation at sites of endogenous DNA damage. Some of these events could serve as a signal for ubiquitination of PCNA, and others could trigger a sumoylation-dependent mutagenesis pathway. Alternatively, sumoylation of PCNA could promote mutagenesis indirectly. Sumoylation has been suggested to inhibit homologous recombination in the S phase of the cell cycle (Haracska *et al*, 2004; Papouli *et al*, 2005; Pfander *et al*, 2005). Some of the aberrant replication intermediates arising in the DNA replication mutants could conceivably be processed in an error-free way by homologous recombination. In this case, the processing of these intermediates would be channeled more into Polζ-dependent mutagenic replication in *SIZ1*$^+$ strains and more into a recombinational pathway in *siz1* mutants, and the latter would explain the reduction of spontaneous mutagenesis in *siz1* strains (Figure 3). In contrast to these results, mutagenesis in response to DNA damage was unaffected by the *SIZ1* mutation that eliminates sumoylation of PCNA at Lys164. (Stelter and Ulrich, 2003). Thus, while the role of monoubiquitination of PCNA in Polζ-dependent DNA damage-induced mutagenesis is well established (Stelter and Ulrich, 2003; Haracska *et al*, 2004), our results provide evidence that sumoylation of PCNA can play a role in regulating spontaneous mutagenesis by Polζ.

The properties of the *pol30-113* mutant described here indicate that the region near the monomer–monomer interface of the trimeric PCNA is critical for promoting mutagenic Polζ-dependent replication. Recent studies have suggested that the ability of PCNA to encircle DNA is necessary for its monoubiquitination by the Rad6–Rad18 complex (Garg and Burgers, 2005). In accordance with this, pcna-52, which has a single amino-acid change at the monomer–monomer interface and is a monomer rather than trimer *in vitro*, can not be ubiquitinated (Garg and Burgers, 2005). Both Glu113 and Leu151 affected by the *pol30-113* mutation are located near the monomer–monomer interface (Figure 4A). Thus, deficiency in UV-induced and Pol ζ-dependent spontaneous mutagenesis in the *pol30-113* strains could potentially result from the reduced stability of the pcna-113 trimer, and a

subsequent defect in Rad6/Rad18-dependent monoubiquitination. However, the trimerization defect *in vitro* is much less pronounced in pcna-113 than in pcna-52 (Figure 5A). This parallels the *in vivo* phenotypes of the corresponding yeast mutants: *pol30-52* strains display slow growth, cold-sensitivity, and sensitivity to replication inhibitor HU characteristic of a severe defect in DNA replication (Ayyagari *et al*, 1995). The *pol30-113* strains grow normally at all temperatures and are not sensitive to HU (our unpublished data), suggesting that DNA replication is fully proficient in these strains. Therefore, the defect in pcna-113 trimerization *in vivo* is mild if it exists at all. Consistent with this, no significant defect in ubiquitination of PCNA upon exposure to MMS is seen *in vivo* in the *pol30-113* mutant (Figure 5D). This, in turn, agrees with our *in vitro* studies of pcna-113 showing that, under conditions when it forms a relatively stable trimer, very little defect exists in ubiquitination by Rad6/Rad18.

The minor defects observed in processive replication by Polδ and in lesion bypass by Polδ or Polη with pcna-113 could well be result of the minor defect in trimerization seen with this mutant *in vitro*. However, pcna-113 is essentially completely defective, at all concentrations, in promoting TLS by Polζ of abasic sites as well as UV damage, thereby providing a biochemical explanation for the complete defect in UV-induced mutagenesis displayed by *pol30-113*. The amino acid residues affected by the *pol30-113* mutation mark a novel region in PCNA that plays an essential role in functional interaction with Polζ. Interestingly, recent studies showed that a G178S substitution in PCNA, located within 5 Å of Leu151 mutated in *pol30-113*, also leads to UV immutability (Zhang *et al*, 2006). Which novel motif in Rev3 and/or Rev7 mediates interactions with PCNA remains to be established and so does the issue whether pcna-113 is in addition defective for interaction with other factors required for mutagenesis. However, this motif in PCNA is not required for TLS *per se*, because bypass synthesis by the Y-class Polη was minimally affected by the mutations in pcna-113 (Figure 5G). It is a more reasonable assumption that Polη, because of the presence of its consensus PCNA-binding motif, interacts with motifs in the carboxy-terminus and the inter-domain connector loop of PCNA (Majka and Burgers, 2004). In addition, Polη possesses ubiquitin-binding domains that mediate its interaction with ubiquitinated PCNA (Bienko *et al*, 2005; Plosky *et al*, 2006).

Current evidence suggests that the function of Polζ in DNA damage-induced mutagenesis is conserved in yeast and human cells. In yeast, inactivation of Polζ causes a complete defect or a severe reduction in mutagenesis induced by almost any DNA-damaging agents that have been tested (Lawrence, 2002). Likewise, in human cells, UV mutagenesis was also shown to require Polζ (Gibbs *et al*, 1998; Li *et al*, 2002). Our results suggest the possibility that human Polζ, like its yeast homolog, may contribute to genome instability when the normal replication machinery is defective. This is particularly important given the fact that polymorphisms in a variety of DNA replication genes have been found in humans (http://www.genome.utah.edu/genesnps/). In addition, mutations in DNA replication genes have been found in human cancers. In further studies, it will be important to determine whether human cells with such replication defects have an elevated rate of Polζ-dependent spontaneous mutation and increased susceptibility to DNA damage-induced mutagenesis.

Materials and methods

Yeast strains and plasmids

All *S. cerevisiae* strains used in this study are isogenic to E134 (*MATα ade5 lys2*::*InsE*$_{A14}$*trp1-289 his7-2 leu2-3,112 ura3-52*; Shcherbakova and Kunkel, 1999). The *pol3-Y708A*, *pol3t*, *pol2-1*, *pol1-1*, and *rev3*::*LEU2* mutants were constructed as described previously (Pizzagalli *et al*, 1988; Shcherbakova *et al*, 1996; Kokoska *et al*, 1998; Pavlov *et al*, 2001b). The presence of the mutations was confirmed by the sensitivity of *pol3-Y708A* mutants to 100 mM HU, by temperature sensitivity of *pol3t* and *pol1-1* mutants, by the slow growth and spontaneous mutator phenotype of *pol2-1* mutants and by UV immutability of *rev3*::*LEU2* mutants. Deletions of the *DPB3*, *SIZ1*, and *RAD18* genes were constructed by transformation with a PCR fragment carrying a selectable *kanMX* cassette (Wach *et al*, 1994) flanked by short sequence homology to *DPB3*, *SIZ1*, or *RAD18*. The disruptions were confirmed by PCR analysis. To construct the *pol30* mutants, the mutations were made by site-directed mutagenesis in plasmid pCH1572 (Amin and Holm, 1996), using a QuickChange site-directed mutagenesis kit from Stratagene. The wild-type chromosomal *POL30* gene was replaced with the mutant alleles as described previously (Amin and Holm, 1996). The presence of the mutations was confirmed by DNA sequence analysis.

Enzymes

The *pol30-113* mutations were introduced into the *Escherichia coli* expression vector pBL228 containing the *POL30* gene by site-directed mutagenesis as described above, and pcna-113 was overproduced in *E. coli* and purified as described (Eissenberg *et al*, 1997). All other proteins were purified as described previously (Garg and Burgers, 2005; Garg *et al*, 2005).

Measurement of the spontaneous mutation rate and UV-induced mutant frequency

The rate of forward mutation to Can^r was measured by fluctuation analysis as described previously (Shcherbakova and Kunkel, 1999). For UV-induced mutagenesis, yeast strains were grown to stationary phase in liquid YPAD medium and plated after appropriate dilutions onto synthetic complete medium for viability count, onto medium lacking histidine or tryptophan to monitor reversion of *his7-2* (−1 frameshift) and *trp1-289* (nonsense) mutations, or onto complete medium containing L-canavanine (60 mg/ml) and lacking arginine for Can^r mutant count. The cells were irradiated with 254 nm UV light immediately after plating and incubated to allow visualization of colonies. The mutant frequency was calculated by dividing the revertant or Can^r mutant count by the viable cell count.

Immunoblot analysis of yeast extracts

Yeast strains were grown to logarithmic phase in liquid YPAD medium. For analysis of MMS-treated cells, MMS was present in the medium at a concentration of 0.02% during the last hour of the culture growth. The cells were collected by centrifugation, resuspended in an equal volume of lysis buffer containing 150 mM NaCl, 50 mM Tris–Cl, pH 7.4, 1 mM EDTA, 10 mM β-mercaptoethanol, and protease inhibitors (Roche Diagnostics), and disrupted by vortexing with an equal volume of 0.5-mm glass beads (BioSpec Products, Inc.). The lysate was then incubated with 0.2% Triton X-100 and 0.1% SDS for 10 min on ice, and cell debris was pelleted by centrifugation. The extracts were loaded onto 12% polyacrylamide gel (Invitrogen) in a loading buffer containing 8 M urea, subjected to electrophoresis and transferred to a nitrocellulose membrane (GE Healthcare). The blots were probed with rabbit polyclonal antibodies to yeast PCNA and goat anti-rabbit secondary antibodies conjugated to Alexa Fluor 680 (Molecular Probes). The bands were visualized using Odyssey infrared imaging system (LI COR).

DNA replication assays

The 30 μl mp18 replication assay contained 40 mM Tris–HCl, pH 7.8, 8 mM $MgAc_2$, 0.2 mg/ml of bovine serum albumin, 1 mM dithiothreitol, 100 μM each of dATP, dCTP, and dGTP, and 25 μM of [^{3}H]dTTP (100 c.p.m./pmol dNTP), 0.5 mM ATP, 75 mM NaCl, 4% w/v polyethylene glycol 8000, 100 ng of singly primed single-stranded mp18 DNA (40 fmol of circles), 10 pmol of RPA, 100 fmol of

RFC, 100 fmol of Polδ, and wild-type or mutant PCNA as indicated. Incubations were at 30°C for 4 min.

The linear oligonucleotide template-primers for TLS assays were prepared as described (Garg *et al*, 2005). They contained a biotin at both ends, to which a two-fold molar excess of streptavidin was added. The templates are: V9, 5′-Bio-CCTTTGCGAATTCT$_{25}$GCGGCT CCCTTCTTCTCCTCCCTCTCCCTTCCCT$_{30}$-Bio; and V9AP2, 5′-Bio-CCTTTGCGAATTCT$_{25}$GCG0CTCCCTTCTTCTCCTCCCTCTCCCTTCC CT$_{30}$-Bio (where 0 indicates a tetrahydrofuran moiety), and the primer is C12-4, 5′-AGGGAAGGGAGAGGGAGGAGAAGAAG. After hybridization, the V9/C12–4 template/primer was irradiated with 1000 J/m^2 of UV light at 254 nM. TLS assays (20 μl) contained 40 mM Tris–HCl pH 7.8, 0.2 mg/ml bovine serum albumin, 8 mM $MgAc_2$, 100 μM each dNTPs, 0.5 mM ATP, 100 mM NaCl, 100 fmol of DNA substrate (the primer was 5′-labeled with ^{32}P), 1 pmol of RPA, the indicated concentrations of wild-type or mutant PCNA, and 200 fmol of RFC. After preincubation at 30°C for 30 s to allow PCNA loading, reactions were started with the addition of the appropriate DNA polymerase (200 fmol). Reaction products were processed as described (Garg *et al*, 2005), and analyzed on a 12% polyacrylamide/7 M urea gel. Gels were quantitated using a STORM phosphoimager and ImageQuant software (Molecular Dynamics).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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