

# Contributions of ubiquitin- and PCNA-binding domains to the activity of Polymerase $\eta$ in *Saccharomyces cerevisiae*

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## ABSTRACT

**Bypassing of DNA lesions by damage-tolerant DNA polymerases depends on the interaction of these enzymes with the monoubiquitylated form of the replicative clamp protein, PCNA. We have analyzed the contributions of ubiquitin and PCNA binding to damage bypass and damage-induced mutagenesis in Polymerase  $\eta$  (encoded by *RAD30*) from the budding yeast *Saccharomyces cerevisiae*. We report here that a ubiquitin-binding domain provides enhanced affinity for the ubiquitylated form of PCNA and is essential for *in vivo* function of the polymerase, but only in conjunction with a basal affinity for the unmodified clamp, mediated by a conserved PCNA interaction motif. We show that enhancement of the interaction and function in damage tolerance does not depend on the ubiquitin attachment site within PCNA. Like its mammalian homolog, budding yeast Polymerase  $\eta$  itself is ubiquitylated in a manner dependent on its ubiquitin-binding domain.**

## INTRODUCTION

Replicative bypass of DNA damage by translesion synthesis (TLS) is essential for the proliferation of cells in the presence of genotoxic agents, but also to overcome lesions caused by the spontaneous decay of DNA in an aqueous environment (1,2). Replicative DNA polymerases are poorly equipped for TLS, because their catalytic centers are optimized for an unperturbed primer-template junction. Most organisms, therefore, harbor specialized damage-tolerant polymerases with more relaxed active sites that can accommodate a variety of lesions in the

template strand (3–5). Conserved in both prokaryotes and eukaryotes, the Y-family of DNA polymerases comprises a group of enzymes dedicated to TLS (6). While the genome of budding yeast encodes only two Y-family polymerases, Pol $\eta$  and Rev1, at least four different enzymes with varying specificities and preferences for different types of lesions are available to human cells.

One inevitable consequence of their ability to synthesize across DNA lesions is the overall reduced fidelity of TLS polymerases, even on undamaged templates (7). The replicative bypass of DNA lesions therefore contributes significantly to the mutagenic effect of DNA-damaging agents (8), and it is not surprising that the activity of the damage-tolerant polymerases is tightly regulated in order to prevent the accumulation of unwanted mutations due to unrestrained activity of these error-prone enzymes. In eukaryotes, the principal mechanism of damage-induced activation of TLS polymerases involves the sliding clamp, proliferating cell nuclear antigen (PCNA). PCNA acts as a processivity factor for replicative polymerases, but also as a binding platform for a large number of other proteins involved in the coordination of events at the replication fork (9). Many of these proteins, including some of the Y-family polymerases, bind PCNA via a conserved motif, termed PCNA interacting peptide (PIP) (10). Covalent modification of PCNA by ubiquitin or SUMO was found to modulate the properties of the clamp and to regulate access of alternative sets of accessory factors to the site of replication (11–16). Specifically, genetic studies indicate that PCNA monoubiquitylation at lysine (K) 164 by the ubiquitin-conjugating enzyme (E2) Rad6 and the ubiquitin ligase (E3) Rad18 is a prerequisite for the activation of all damage-tolerant polymerases in yeast (15), and preferential interactions of TLS polymerases Pol $\eta$ , Pol $\kappa$ , Pol $\iota$  and Rev1 with the modified form of the clamp have now been demonstrated in mammalian cells

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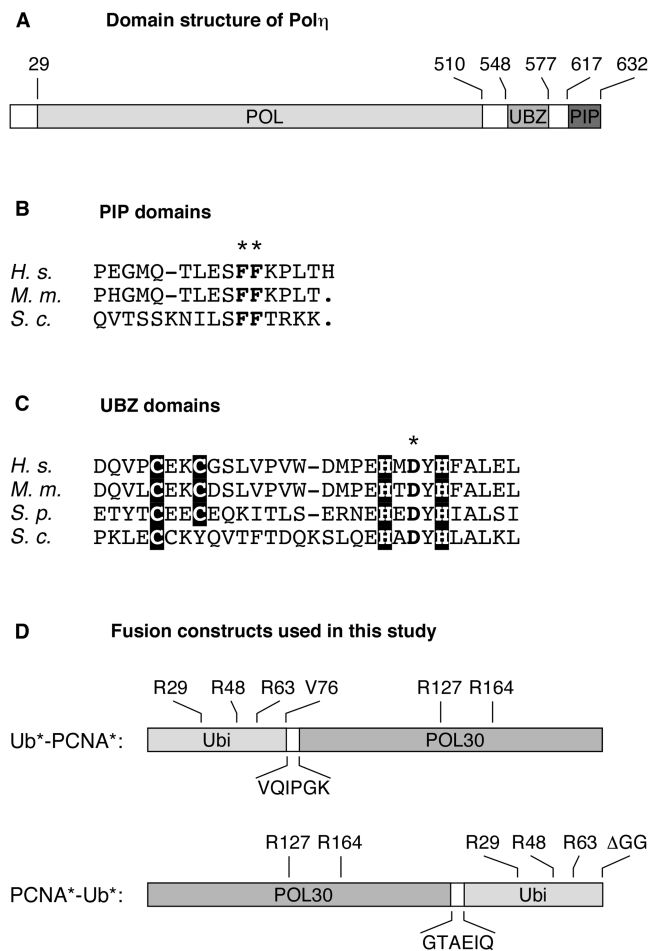
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(12,16–20), providing strong support for a model that explains the recruitment of damage-tolerant polymerases to stalled replication forks by means of PCNA ubiquitylation.

The identification of two novel types of ubiquitin-binding domains (UBDs), termed UBM and UBZ (18), has recently uncovered the molecular basis for the observed enhancement of PCNA binding by ubiquitylation. These motifs are present in one or two copies in all the mammalian Y-family polymerases and were found to directly bind ubiquitin *in vitro*. As they reside outside the catalytic domains, they do not affect polymerase activity of the TLS enzymes, but rather act by modulating their interactions and localization (18,20). Interestingly, they are also responsible for the ubiquitylation of the polymerases themselves, a phenomenon whose significance is not yet understood, but is characteristic for other ubiquitin-binding proteins as well (18–20).

Pol $\eta$  is a Y-family polymerase conserved in yeast and mammals, with a strong preference for cyclobutane pyrimidine dimers (CPDs) induced by ultraviolet (UV) radiation (21,22). It inserts the correct nucleotides opposite a thymine CPD, but is less efficient and highly inaccurate in the bypass of other lesions (23–26). In humans, defects in Pol $\eta$  result in a hereditary disease, Xeroderma pigmentosum variant (XP-V), involving photosensitivity and a predisposition to skin cancer (22,27). PCNA stimulates the activity of Pol $\eta$  without enhancing its processivity, and a PIP domain at the carboxy (C)-terminus of the protein (Figure 1A, B) was found to be required for interaction with the clamp and function *in vivo* (28). A UBZ domain was identified in the C-terminal region of human Pol $\eta$  (Figure 1A, C) and shown to convey ubiquitin binding and recruitment to damage-induced nuclear foci (18,20). Expression of a Pol $\eta$  allele carrying a point mutation in the UBZ domain failed to fully complement the UV sensitivity of XP-V cells without affecting polymerase activity, indicating that an intact UBZ domain is required for Pol $\eta$  function *in vivo* (18).

In Pol $\eta$  and Pol $\kappa$ , the UBZ domains are characterized by a mononuclear Zinc finger (UBZ) of the C<sub>2</sub>H<sub>2</sub> or C<sub>2</sub>HC type, respectively (18). The corresponding sequence within budding yeast Pol $\eta$ , encoded by the *RAD30* gene (29,30), differs considerably from that of the mammalian homolog, and one of the conserved cysteine residues is replaced by a tyrosine (Figure 1C). Moreover, we were unable to detect binding of a recombinant C-terminal fragment comprising the putative UBZ domain and the PIP motif, or of full-length Rad30 from total yeast cell lysate, to free ubiquitin or ubiquitin chains (our unpublished results). We have therefore examined the relevance of the putative UBZ domain for physical interactions with the modified clamp and biological function of Pol $\eta$  in *Saccharomyces cerevisiae*. We show here that the domain fulfills the expected function in yeast, but only in conjunction with an intact PIP domain. In addition, we have characterized the requirements for ubiquitylation of Pol $\eta$  itself, and we provide evidence that the exact molecular arrangement of ubiquitin and PCNA is not critical for recruitment of Pol $\eta$  and effective damage bypass *in vivo*.



**Figure 1.** Structure of Pol $\eta$  and the constructs used in this study. (A) Domain structure of Pol $\eta$ , indicating the positions of the polymerase domain (POL), the UBZ domain and the PCNA-interacting peptide (PIP), numbered according to the budding yeast protein. (B) Sequence alignment of Pol $\eta$  PIP domains from human (H. s.), mouse (M. m.) and budding yeast (S. c.). A dash indicates a gap in the alignment. The PIP\* mutant was generated by changing the two conserved phenylalanine residues (indicated by an asterisk) to alanine. This change is known to disrupt the interaction with PCNA without affecting the overall structure of the protein (28). (C) Sequence alignment of Pol $\eta$  UBZ domains. Conserved cysteine and histidine residues are shaded in black. The conserved aspartate residue indicated by an asterisk was changed to alanine in order to create the UBZ\* mutant in analogy to the mammalian protein (18). (D) Structure of the linear fusions of ubiquitin and PCNA used for analysis of protein–protein interactions and *in vivo* function. Mutations that prevent ubiquitin polymerization, conjugation and processing are indicated above and sequences of the linker peptides below each construct.

## MATERIALS AND METHODS

### Yeast strains and plasmids

Standard procedures were followed for the cultivation and manipulation of *S. cerevisiae*. Unless otherwise noted, yeast strains (*rad30::HIS3*, *rad5::HIS3*, *rad18::TRP1*, *rad6::HIS3*, *rev1::URA3*, *rev3::KanMX* and appropriate combinations) are derived from DF5 and have been described previously (31). For genetic analysis of mutants

in the *RAD30* gene, a *rad30* or *rad30 rad5* deletion strain was transformed with shuttle vectors based on YIplac128 (32), which carried the *WT* or mutated *RAD30* open reading frame (ORF) under the control of the endogenous *RAD30* promoter, for integration into the *LEU2* locus. The empty vector YIplac128 served as a negative control. The *RAD30* vectors were constructed by inserting a PCR product comprising the ORF (SmaI-PstI) flanked by 500 bp of *RAD30* upstream region (SacI-SmaI) and a transcription terminator derived from pGBT9 (Clontech, PstI-SphI). Mutations in the UBZ (D570A) and/or PIP motif (FF627/628AA) were introduced by PCR-based mutagenesis. All constructs were sequenced throughout the ORF. For two-hybrid analysis, the ORFs were subcloned into the vectors pGAD424 and pGBT9 (Clontech). In addition, a truncated version of *RAD30* encoding amino acids 538–632 was constructed. The ORFs of *pol30(K127/164R)*, ubiquitin and the fusion constructs shown in Figure 1D were assembled by PCR and cloned (Ub\*-PCNA\*: BamHI-PstI; PCNA\*-Ub\*: SmaI-PstI) into the two-hybrid vectors, into pQE30, and into YIplac128 under control of the *POL30* promoter, in analogy to the expression vector for PCNA described previously (15). For detection of Pol $\eta$  on western blots, a sequence encoding the 9myc-epitope was appended to the *RAD30* ORF as described (31). For *in vivo* pull-down experiments, a sequence encoding His<sub>7</sub>-tagged ubiquitin was generated by PCR and cloned (BamHI-PstI) into an episomal plasmid based on YEplac181 or YEplac195 (32), under control of the *CUP1* promoter (428 bp of upstream sequence, SacI-BamHI) and the transcription terminator from pGBT9 (PstI-SphI).

### Two-hybrid analysis

Analysis of protein–protein interactions in the two-hybrid system was performed in PJ64-4A, as described previously (32). Selection for the presence of the bait and prey plasmids was carried out on synthetic medium lacking tryptophan and leucine, and positive interactions were scored by growth on medium further lacking histidine or histidine and adenine.

### Measurements of UV sensitivities and UV-induced mutation frequencies

UV sensitivities were determined by plating defined numbers of cells onto YPD medium, irradiation at 254 nm in a UV crosslinker (Stratalinker 2400 by Stratagene), incubation in the dark for 3 days, and colony counting. UV-induced mutation frequencies in the *CAN1* locus were measured similarly by comparing the numbers of *can1<sup>R</sup>* mutants at a given UV dose, selected on synthetic medium without arginine containing 30  $\mu$ g/ml canavanine, with the numbers of survivors on YPD exposed to the same UV dose. Standard deviations were calculated from triplicate measurements.

### *In vivo* analysis of yeast proteins

Total cell lysates were prepared as described (33). PCNA and the fusion constructs were detected by western blot with an anti-Pol30 antibody (15). For detection of Pol $\eta$

modification, strains bearing 9myc-tagged *RAD30* (*WT*, *rad6*, *rad18* or *rad30 mutants*) were transformed with an episomal vector encoding His<sub>7</sub>-tagged ubiquitin (see above). Cultures were grown in selective medium containing 0.1 mM CuSO<sub>4</sub>. Pull-downs on Ni-NTA agarose from total lysates were performed under denaturing conditions as described previously (11,15), and the Rad30 protein was detected by western blot using a monoclonal anti-myc antibody (Santa Cruz Biotechnology). Detection of phosphoglycerate kinase (PGK) with a monoclonal antibody (Molecular Probes) served as loading control.

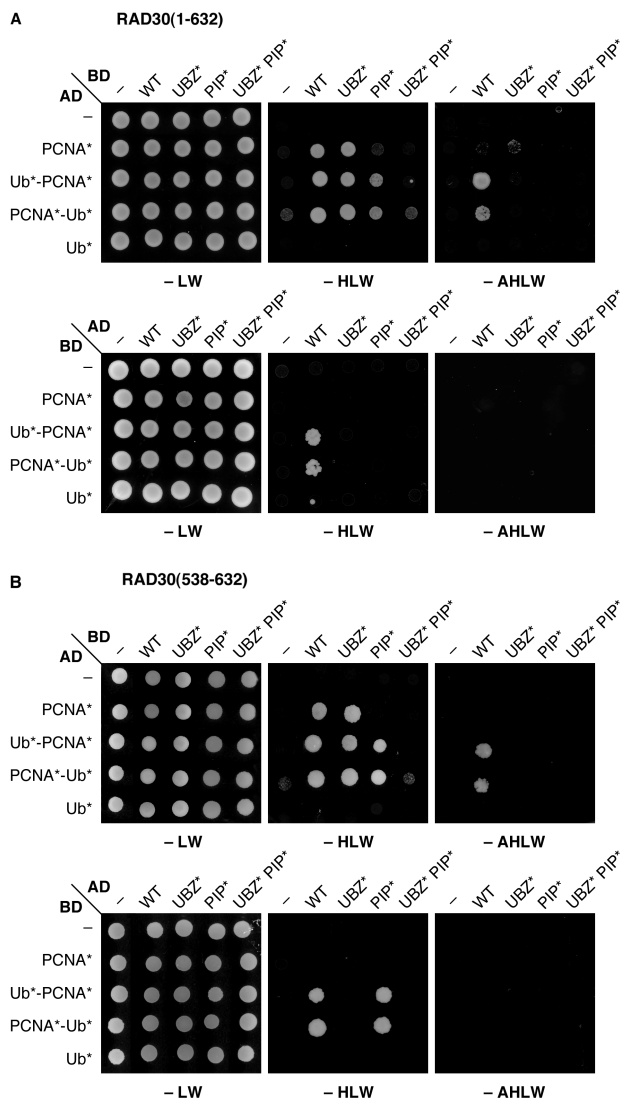
### *In vitro* analysis of protein interactions

Recombinant PCNA\*, Ub\*-PCNA\* and PCNA\*-Ub\* were produced in *E. coli* by expression from pQE30 with an N-terminal His<sub>6</sub>-epitope. Proteins were purified by chromatography on Ni-NTA agarose and dialyzed against a buffer containing 25 mM Tris-HCl, pH 7.5, 50 mM NaCl and 10% glycerol. Gel filtration analysis was performed at 2  $\mu$ M PCNA on a Superdex 200 column as described (34). *In vitro* pull-down assays with GST fusion proteins were performed by immobilizing the GST-PCNA fusion protein (13) on glutathione Sepharose, incubation with the respective binding partner for 30 min at 30°C in phosphate-buffered saline with 0.05% Triton X-100, washing three times with the same buffer and eluting bound material by boiling in SDS sample buffer.

## RESULTS

### UBZ and PIP motifs jointly contribute to interaction of yeast Pol $\eta$ with ubiquitylated PCNA

In order to analyze the interactions between Pol $\eta$  and ubiquitylated PCNA in the two-hybrid system, we constructed linear fusions of ubiquitin to the amino (N)- and the C-terminus of PCNA, respectively (Figure 1D). The lysine residues within PCNA and the ubiquitin moiety known to be involved in ubiquitin or SUMO conjugation were mutated to arginine to prevent further modification, and the C-terminus of ubiquitin was altered in order to prevent processing by isopeptidases or conjugation to other proteins. Using isolated ubiquitin (Ub\*) and PCNA (PCNA\*) carrying the same mutations as controls, these constructs were examined for binding to wildtype (*WT*) Rad30 or variants in which the PIP and/or the UBZ motif were inactivated by point mutations (Figure 1B, C). When PCNA\* was fused to the *GAL4* activation domain (AD) and Rad30 to the DNA-binding domain (BD), interaction depended on an intact PIP domain in Rad30 and was strong enough to activate the *HIS3*-, but not the *ADE2* reporter in the two-hybrid system (Figure 2A, upper panel). Stronger signals with both reporter genes were observed with the two fusion constructs Ub\*-PCNA\* and PCNA\*-Ub\*. These interactions were affected by both PIP and UBZ domains. Mutation of either resulted in weakening of the interaction to the level of the interaction with PCNA\*, and mutation of both completely abolished the signal. Reversal of the orientation in the two-hybrid system resulted in overall



**Figure 2.** Physical interactions between Rad30, PCNA and ubiquitin. Interactions were monitored in the two-hybrid system, based on fusions to the *GAL4* activation (AD) and DNA-binding (BD) domains. Presence of the respective constructs was ensured by selection on plates lacking leucine and tryptophane (-LW), and interactions were scored by growth on plates lacking histidine or histidine and adenine (-HLW, -AHLW). (A) Interaction of Rad30 (*WT*) and the indicated mutants with PCNA\*, ubiquitin (Ub\*) and the linear fusions shown in Figure 1D. PCNA\* and Ub\* were mutated in the same manner as the fusion constructs. (B) same as in (A), but using an N-terminally truncated version of Rad30, comprising amino acids 538–632.

weaker signals: interaction was only visible with the fusions and depended on both UBZ and PIP (Figure 2A, lower panel). Very similar results were obtained with a C-terminal domain of Rad30 comprising the UBZ and PIP motifs (Figure 2B).

Notably, interaction with ubiquitin alone or a fusion of ubiquitin to an unrelated protein such as GFP (data not shown) was never observed in our system. Taken together, these findings suggest that the UBZ domain of Pol $\eta$  from budding yeast does contribute to ubiquitin binding, but this association is unlikely to be strong and may be effective only in conjunction with a basal affinity of Rad30

for PCNA, mediated by the PIP motif. Naturally, neither the two-hybrid system nor the linear fusions of ubiquitin and PCNA fully reflect the physiological arrangement of the interaction partners, which makes quantitative statements problematic. Nevertheless, it is noteworthy that the exact position of ubiquitin on PCNA was irrelevant in this context, such that UBZ-dependent enhancement of PCNA binding was detectable even though ubiquitin was not attached to its natural position, K164.

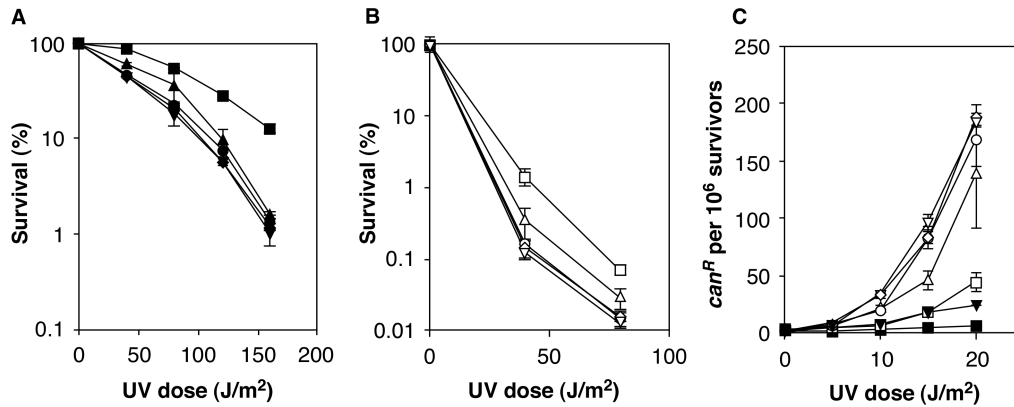
### The UBZ domain is required for Pol $\eta$ function in translesion synthesis

Having obtained evidence for a contribution of the UBZ domain to ubiquitin binding, we analyzed the consequences of mutations in this domain for the function of Rad30 in DNA damage tolerance. Since the Rad30 protein, like human Pol $\eta$ , is largely selective for the bypass of UV-induced CPDs, we used UV radiation as a damaging agent. Mutation of the UBZ domain resulted in a UV sensitivity close to that of a *rad30* deletion or a mutant in the PIP motif (Figure 3A). Similar results were obtained in a *rad5* background, where overall UV sensitivities are exacerbated due to the abolishment of error-free damage avoidance (Figure 3B). In this background, the *rad30(PIP\*)* mutant displayed a substantial, but not complete loss of function, which is consistent with previous results that have demonstrated a somewhat milder phenotype for modification by point mutations as compared to deletion of the PIP motif in this background (28). These results demonstrate a contribution of the Rad30 UBZ domain to resistance towards UV-induced DNA damage.

An alternative way of assessing the function of *RAD30* in lesion bypass is the measurement of damage-induced mutation frequencies in a strain that is dependent on TLS for damage bypass because the error-free damage avoidance pathway has been inactivated. MacDonald *et al.* have shown that in a *rad5* background, deletion of *RAD30* results in a massive increase in UV-induced reversion frequencies (29), presumably due to the processing of lesions by error-prone, Pol $\zeta$ - and/or Rev1-dependent TLS in the absence of error-free, Pol $\eta$ -dependent bypass. This provides a sensitive tool to monitor the contribution of *RAD30* to error-free TLS. When we determined forward mutation frequencies in the *CAN1* locus in response to UV irradiation (Figure 3C), we found that *rad30(UBZ\*)* afforded mutation frequencies comparable to those of the *rad30* deletion, consistent with a complete loss of function, whereas *rad30(PIP\*)* retained some residual activity. Again, these data provide evidence for an absolute requirement of the UBZ domain in yeast Pol $\eta$ .

### Linear fusions of ubiquitin and PCNA can partially substitute for loss of PCNA ubiquitylation

In order to determine whether the linear fusions between ubiquitin and PCNA reflected the physiological situation of naturally ubiquitylated PCNA to any degree, we examined their properties *in vivo* and *in vitro*. Figure 4A shows that the fusion proteins did not rescue the lethality of a *pol30*



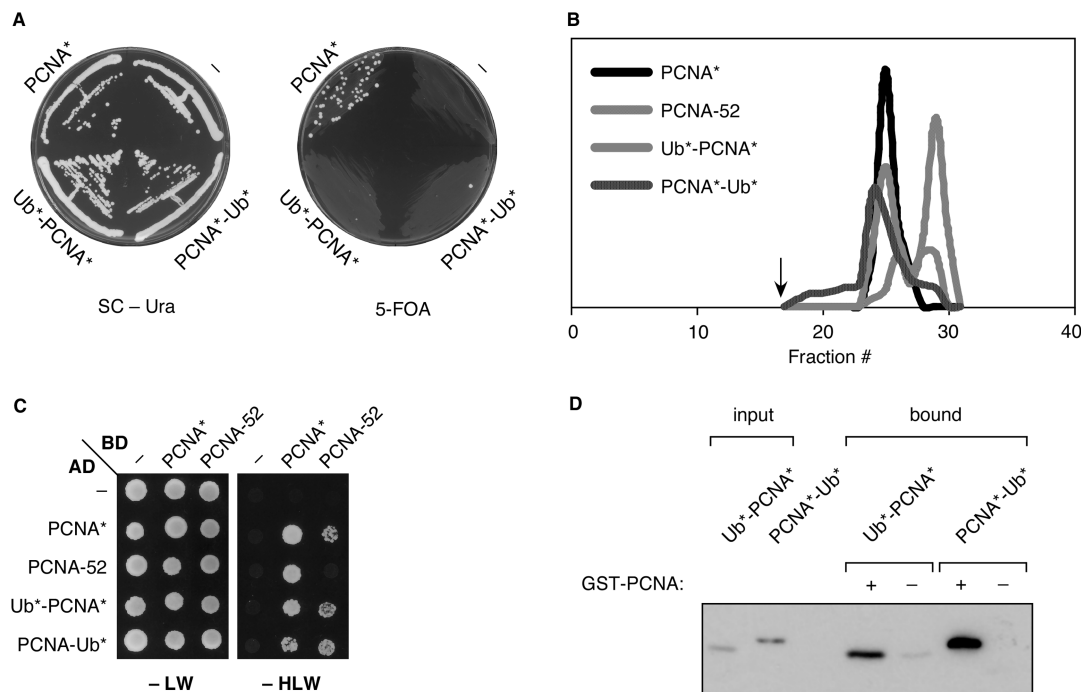
**Figure 3.** The Rad30 UBZ domain is required for Pol $\eta$ -dependent error-free bypass of UV damage in yeast. (A) Effect of UBZ and PIP domain mutations on sensitivities towards UV radiation. Strains bearing a *rad30* deletion were complemented with *WT RAD30* or mutants in the UBZ and/or PIP domain on integrative vectors. The empty vector served as control. (B) same as in (A), but using a *rad5 rad30* deletion background. (C) UV-induced forward mutation frequencies in the *CAN1* locus. Constructs used: *RAD30* (squares), *rad30*, i.e. empty vector (inverted triangles), *rad30(UBZ\*)* (circles), *rad30(PIP\*)* (triangles), *rad30(UBZ\* PIP\*)* (diamonds). Filled symbols indicate *RAD5*, open symbols a *rad5* background.

deletion, indicating that vital functions of PCNA, such as promoting DNA replication, are affected by attaching ubiquitin to its N- or C-terminus. However, gel filtration analysis of the purified proteins indicated that both Ub\*-PCNA\* and PCNA\*-Ub\* exist as relatively stable homotrimers in solution, suggesting that the failure to support viability is not caused by a lack of trimerization (Figure 4B). Two-hybrid and *in vitro* pull-down assays showed that the fusion proteins can interact not only with themselves, but also with native PCNA\* (Figure 4C, D). Thus, coexpression of the fusions with endogenous PCNA should result in the formation of mixed trimers, which could potentially take over the function of physiologically ubiquitylated PCNA in a strain unable to ubiquitylate endogenous PCNA, such as a *rad18* mutant. We, therefore, expressed the two constructs, Ub\*-PCNA\* and PCNA\*-Ub\*, under the control of the *POL30* promoter from an integrative vector in a *rad18* strain (Figure 5A). As a control, we expressed unmodified *pol30(K127/164R)* (PCNA\*) to the same level, as it has previously been shown that overexpression of *POL30* or *pol30(K164R)* suppresses the damage sensitivity of *rad18* mutants to some extent (11). We found that fusion of ubiquitin to the N- or C-terminus of PCNA significantly enhanced its ability to suppress the UV sensitivity of a *rad18* mutant, whereas it had no effect in a *WT* background (Figure 5B). As expected, suppression was incomplete, consistent with the notion that the fusion proteins cannot be further ubiquitylated, which eliminates the error-free, polyubiquitin-dependent pathway of damage avoidance. In order to demonstrate that the observed suppression was in fact due to TLS, we reexamined UV sensitivities in a *rad18* mutant that additionally carries deletions of the genes encoding the TLS polymerases, *REV1*, *REV3* and *RAD30*. In this mutant (*rad18  $\Delta$ TLS*), expression of the fusion constructs had no effect on survival of DNA damage, indicating that Ub\*-PCNA\* and PCNA\*-Ub\* indeed exert their influence by means of the TLS polymerases. The enhanced

UV sensitivity observed in the *rad18  $\Delta$ TLS* strain compared to *rad18* may be due to functions of *REV3* independent of PCNA ubiquitylation (34,35). Taken together, these results indicate that linear fusions of ubiquitin and PCNA can partially substitute for the loss of endogenously ubiquitylated PCNA in DNA damage tolerance, and they suggest that the constructs used for interaction studies reflect the physiological situation to some degree.

#### Pol $\eta$ is ubiquitylated *in vivo* in a *RAD6*- and *RAD18*-independent manner

Ubiquitylation of UBD-containing proteins in a UBD-dependent manner has been observed previously (36,37), and mammalian Pol $\eta$ , Pol $\tau$  and Rev1 were all found to be ubiquitylated *in vivo* (18,19). For detection of yeast Pol $\eta$ , we appended a 9myc-epitope to the protein's C-terminus (Figure 6A). On western blots, a faint band of higher molecular weight suggested a modified form. As shown in Figure 6B, Rad30<sup>9myc</sup> was detected in Ni-NTA pull-downs performed under denaturing conditions from cells expressing His-tagged ubiquitin, suggesting that the modification was indeed monoubiquitin. We were interested to see whether ubiquitylation of Pol $\eta$  required any of the same conjugation factors that operate on PCNA, i.e. Rad6 and Rad18 (11). Figure 6B shows that the levels of modified Rad30<sup>9myc</sup> were unchanged in the *rad18* mutant and only marginally reduced in *rad6*, suggesting that either a different E2-E3 pair is responsible for the modification, or that the UBZ domain functions in an autocatalytic manner and affords modification independent of any additional E3 and not entirely relying on Rad6 as an E2. Relevance of the UBZ domain for Rad30<sup>9myc</sup> modification was confirmed by a significant reduction in ubiquitylation of the UBZ\* mutant (Figure 6C). In contrast, interaction with PCNA was dispensable, because modification of the PIP\* mutant was comparable to that of *WT* Rad30<sup>9myc</sup> (Figure 6C). Moreover, we did not observe an effect of DNA damage, suggesting that



**Figure 4.** Properties of permanently ubiquitylated PCNA. (A) Fusions of PCNA to ubiquitin cannot rescue the lethality of a *pol30* deletion. The linear fusion constructs of ubiquitin and PCNA shown in Figure 1D or a construct for expression of *pol30(K127/164R)* (PCNA\*) under control of the *POL30* promoter or an empty vector ("−") were integrated in a *pol30* strain expressing *WT POL30* on a centromeric plasmid bearing the *URA3* marker. Growth on plates containing 5-fluoroorotate (5-FOA) indicates viability after loss of the plasmid. The rare colonies arising from Ub\*-PCNA\* and PCNA\*-Ub\* on 5-FOA were analyzed by western blot and found to have maintained expression of PCNA despite loss of the *URA3* marker. (B) Gel filtration analysis shows that Ub\*-PCNA\* and PCNA\*-Ub\* form homotrimers in solution with a stability approaching that of native PCNA\*. A non-lethal allele defective in trimerization (PCNA-52) (47) was analyzed for comparison. Proteins were produced in *E. coli* with an N-terminal His<sub>6</sub> epitope. An arrow indicates the void volume of the column. (C) Interaction of the PCNA constructs used in (B) in the two-hybrid system. (D) Subunit exchange between GST-PCNA and His<sub>6</sub>-tagged Ub\*-PCNA\* or PCNA\*-Ub\* *in vitro*. GST-PCNA was immobilized on glutathione Sepharose and incubated with the His<sub>6</sub>-tagged fusion protein, and bound material was analyzed by western blot (anti-His).

ubiquitylation of Pol $\eta$  in *S. cerevisiae* may not be regulated via the DNA damage response (Figure 6B). Thus, the physiological significance of this modification is still unclear.

## DISCUSSION

Our analysis of the UBZ motif of budding yeast Pol $\eta$  has revealed several parallels to the corresponding domain in its mammalian ortholog, but we have also made observations that will help generalize our understanding of UBZs in eukaryotic Y-family polymerases.

### Ubiquitin binding by the UBZ domain

We have shown that, despite its variation in primary sequence, the UBZ domain from yeast Pol $\eta$  is functional in ubiquitin binding and contributes to an enhanced association of the polymerase with ubiquitylated PCNA. However, neither full-length yeast Pol $\eta$  nor its UBZ domain alone exhibited significant affinity for isolated, unconjugated ubiquitin. This situation differs from the mammalian system, where UBZ domains were first isolated by means of interaction with free ubiquitin in a two-hybrid screen, and the UBZ of Pol $\eta$  promoted binding to ubiquitin agarose in the context of the

full-length polymerase (18). This suggests that the interaction of the yeast UBZ domain with ubiquitin might be weaker or more transient, possibly due to its divergent sequence, thus requiring a PIP-mediated basal affinity for PCNA as an obligatory interaction partner. In mammalian Pol $\eta$ , the relative contributions of PIP and UBZ domains to binding of ubiquitylated PCNA have not been analyzed. In human Pol $\eta$ , however, inactivation of the functionally related UBM domains had a much more severe effect on the affinity to a PCNA-ubiquitin fusion than mutation of the PIP domain (18), and in the Rev1 protein, constitutive damage-independent association with PCNA at replication foci was found to require an N-terminal BRCT domain (38), whereas the damage-induced enhancement of binding to the ubiquitylated clamp was mediated by the UBM domains (19). Thus, it appears that UBZs may vary substantially in their affinities to ubiquitin and might, therefore, not always function autonomously in their recognition of ubiquitylated target proteins.

### Function of the UBZ domain in TLS

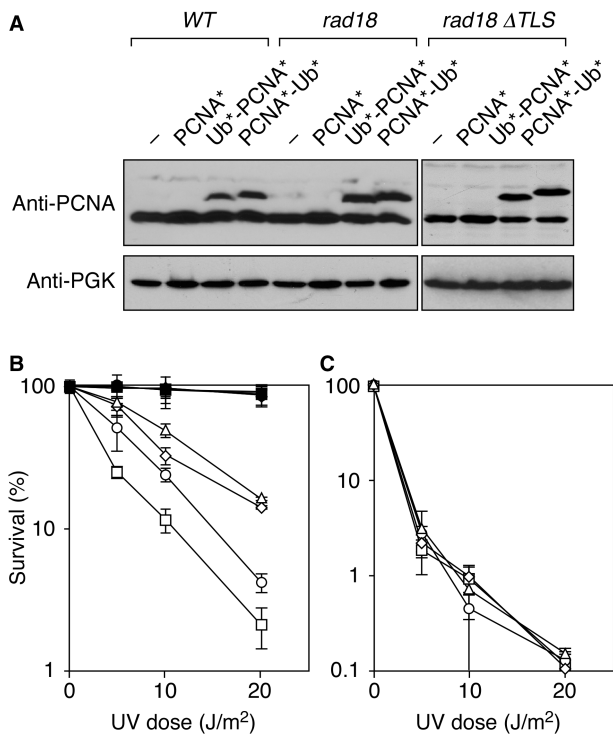
The single amino acid substitution introduced into the UBZ motif to abolish ubiquitin binding does not prevent the interaction of the mutated protein with unmodified

PCNA in the two-hybrid system, and it is unlikely that this situation would be different in the case of chromatin-bound PCNA. Thus, the functions of the PIP and the UBZ motifs appear to be separable. Yet, analysis of damage sensitivities and mutation frequencies demonstrates that both domains are absolutely required

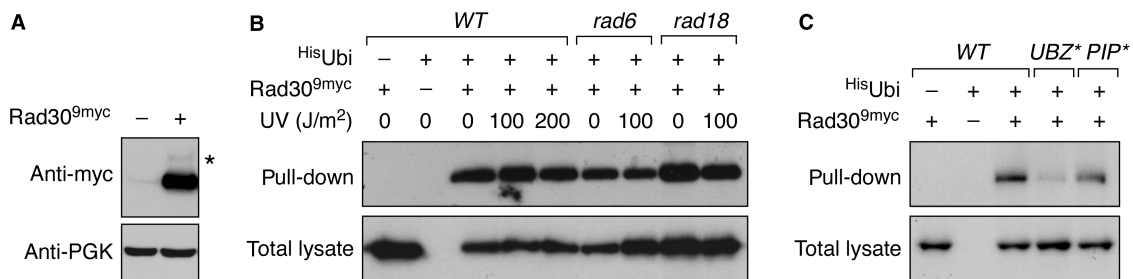
for function in damage bypass and prevention of damage-induced mutagenesis in yeast Pol $\eta$ . Phenotypic analysis of human Pol $\eta$  was more ambiguous with respect to the relative importance of each motif (18). This absolute requirement for both PCNA and ubiquitin binding resembles the phenotype of yeast *rev1* mutants in the BRCT and the UBM domains, which are both inactive with respect to TLS and damage-induced mutagenesis (19,38). Thus, analysis of the yeast proteins in the context of damage bypass can prove valuable when loss-of-function mutants in some of the mammalian Y-family polymerases are poorly accessible to genetic studies.

### Flexibility in the site of ubiquitin attachment

Our study has shown that monoubiquitin attached to the N- or C-terminus of PCNA can partially overcome the need for PCNA ubiquitylation at its physiological site, K164, in mediating UBZ-dependent interaction with Pol $\eta$  in the two-hybrid system and also in terms of biological function in the context of TLS. Precedents for the functionality of linear fusion constructs have been reported for targets of ubiquitin as well as SUMO conjugation (39–42), but in many of these cases more than one lysine within the target protein can naturally serve as acceptor for the modifier. Given that both *in vivo* and *in vitro* PCNA is ubiquitylated exclusively at K164 (11,43), a redundancy in the positioning of the modifier is surprising. In molecular terms, simultaneous interactions involving both the UBZ domain and the PIP motif, which is known to interact with a region involving the interdomain connector loop of PCNA (44,45), must therefore involve significant flexibility either on the side of Pol $\eta$  itself or in the linkage between the C-terminus of ubiquitin and K164 of PCNA. Thus, in the case of PCNA, the ubiquitin acceptor site might reflect an arbitrary preference of the conjugating enzymes rather than a condition for functionality of the modified clamp. Considering that the fusion proteins cannot substitute for PCNA with respect to its essential function in DNA replication, however, K164 might also be one of a limited



**Figure 5.** *In vivo* function of permanently ubiquitylated PCNA. (A) Detection of PCNA by western blot (anti-Pol30) in total lysates from cells expressing the constructs described in the legend to Figure 4 in addition to endogenous PCNA. Detection of phosphoglycerate kinase (PGK) was used as loading control. (B) UV sensitivities of the strains shown in (A): “–”, i.e. empty vector (squares), PCNA\* (circles), Ub\*-PCNA\* (triangles), PCNA\*-Ub\* (diamonds). Filled symbols indicate *WT* host strains, open symbols a *rad18* background. (C) UV sensitivities in the *rad18 rad30 rev1 rev3 (rad18 ΔTLS)* background, labeled as in (B).



**Figure 6.** Budding yeast Pol $\eta$  is modified by monoubiquitin. (A) Detection of Rad30<sup>9myc</sup> in a total cell lysate by western blot (anti-myc). A band of higher molecular weight, which might represent ubiquitylated Rad30<sup>9myc</sup>, is indicated by an asterisk. (B) Monoubiquitylation of Rad30 does not depend on *RAD6*, *RAD18* or UV damage. Ubiquitylated Rad30<sup>9myc</sup> protein was detected by western blot (anti-myc) after isolation of total ubiquitylated proteins under denaturing conditions from a strain bearing His-tagged ubiquitin. Strains with untagged Rad30 or untagged ubiquitin were used as controls. The top panel shows the ubiquitylated Rad30<sup>9myc</sup> protein isolated by pull-down of His-ubiquitin, while the bottom panel shows the levels of Rad30<sup>9myc</sup> in the corresponding total cell extracts. (C) Monoubiquitylation of Rad30 depends on the UBZ\*, but not on the PIP\* domain mutant. A 9myc-epitope was appended to the mutant *rad30* alleles in the strains used in Figure 3, and ubiquitylated Rad30<sup>9myc</sup> was isolated and detected as in (B).

number of sites within the PCNA molecule that allows modification without disrupting vital interactions with other cellular factors.

### Autoubiquitylation of ubiquitin-binding proteins

Finally, ubiquitylation of Pol $\eta$  appears to be conserved across the species and is not unexpected in light of the UBD-mediated modification of many other monoubiquitin-binding proteins, such as components of the endocytic pathway (36,39,46). We have now shown that, in the case of Pol $\eta$ , this phenomenon is largely independent of the conjugation factors responsible for the modification of PCNA or interaction with the clamp, but strongly dependent on the UBZ domain itself, as in mammalian Pol $\eta$ . This suggests either an involvement of a different set of E2 and E3 enzymes, or possibly an autocatalytic action of the UBD. In the latter scenario, the modification could result from interaction of the UBD with an activated ubiquitin moiety loaded onto an E2 (or potentially an E1) molecule, which would circumvent the need for a specific E2–E3 pair. It is yet unclear whether this reaction would be an inevitable consequence of the interaction between a UBD and activated ubiquitin and thus an irrelevant side reaction, or whether modification of UBD-containing proteins can serve as a regulatory strategy of blocking their own ubiquitin-binding properties in an intramolecular fashion (39). The inability of ubiquitylated mammalian Pol $\eta$  to bind ubiquitylated PCNA may suggest the latter (18); however, we did not observe any change in the extent of yeast Rad30 modification in response to DNA damage. Considering that the majority of Pol $\eta$  in yeast and mammals is not modified at any given time, it is difficult to imagine an effective regulatory scenario by modification of the polymerase. Likewise, the apparent absence of polyubiquitylated species argues against a proteolytic function of Pol $\eta$  modification. In order to gain insight into the relevance of this process, it will be necessary to identify the sites of modification and analyze the consequences of the relevant mutations.

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