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Saccharomyces cerevisiae polymerase #*

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## The unusual UBZ domain of *Saccharomyces cerevisiae* polymerase $\eta$

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### Abstract

Recent research has revealed the presence of ubiquitin-binding domains in the Y family polymerases. The ubiquitin-binding zinc finger (UBZ) domain of human polymerase  $\eta$  is vital for its regulation, localization, and function. Here, we elucidate structural and functional features of the non-canonical UBZ motif of *S. cerevisiae* pol  $\eta$ . Characterization of pol  $\eta$  mutants confirms the importance of the UBZ motif and implies that its function is independent of zinc binding. Intriguingly, we demonstrate that zinc does bind to and affect the structure of the purified UBZ domain, but is not required for its ubiquitin-binding activity. Our finding that this unusual zinc finger is able to interact with ubiquitin even in its apo form adds support to the model that ubiquitin binding is the primary and functionally important activity of the UBZ domain in *S. cerevisiae* polymerase  $\eta$ . Putative ubiquitin-binding domains, primarily UBZs, are identified in the majority of known pol  $\eta$  homologs. We discuss the implications of our observations for zinc finger structure and pol  $\eta$  regulation.

### Keywords

polymerase eta; zinc finger; ubiquitin; DNA damage; translesion synthesis

## 1. Introduction

The genomes of living cells are constantly exposed to a variety of DNA damaging agents that range from endogenously produced reactive metabolic intermediates to exogenous chemical agents and radiation [1]. In spite of cellular DNA repair mechanisms, replication-blocking lesions can persist in the DNA. Replication of such damaged DNA is accomplished by the use of different mechanisms of DNA, such as translesion synthesis (TLS) [2]. TLS, the process in which specialized DNA polymerases directly replicate the damaged DNA, is

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### Conflict of interest

The authors declare that there are no conflicts of interest.

carried out by multiple nonessential DNA polymerases. Most of them are members of the Y family [3], and many are optimized for the bypass of distinct cognate lesions.

Polymerase (pol)  $\eta$  is a Y family polymerase whose ability to accurately and efficiently bypass UV radiation-induced cyclobutane pyrimidine dimers (CPDs) [4–7] is important for the avoidance of UV-induced skin cancers. Patients lacking a functional pol  $\eta$  suffer from a syndrome known as Xeroderma pigmentosum variant (XP-V), which is characterized by an increased incidence of cancer, hypermutability, and sensitivity to UV-induced DNA lesions [8,9]. Less deleterious mutations in the *XPV gene* encoding pol  $\eta$  may also predispose patients to melanoma [10]. In addition to UV lesions, pol  $\eta$  is also implicated in the replication of naturally occurring structured regions of DNA [11] and is able to bypass a variety of lesions *in vitro* [12–18]. However, it displays similarly low fidelity ( $10^{-2}$  to  $10^{-3}$ ) in the replication of both damaged and undamaged DNA templates [19,20].

The catalytic activity of pol  $\eta$  resides in its N-terminal domains, which share sequence homology with the other Y-family TLS polymerases [3]. Pol  $\eta$  also includes a Polymerase Associated Domain (PAD), sometimes called the Little Finger, which participates both in DNA binding and in several specific protein-protein interactions [21–24]. Pol  $\eta$ 's recruitment to the DNA is mediated by a C-terminal region of 100 to 200 amino acids, which includes a nuclear localization sequence (NLS), PCNA-interacting regions, and a ubiquitin-binding zinc finger domain (UBZ) (Supplementary Data Fig. S2A) [25–28].

The UBZ was first recognized as a putative C2H2 zinc finger motif located near the C-terminus of *S. cerevisiae* pol  $\eta$  [9,29]. Human pol  $\eta$  contains a similar motif, which was the first UBZ shown to mediate a physical interaction with ubiquitin [30,31]. UBZ motifs have since been identified in several other proteins, including the Y family TLS polymerase  $\kappa$  ( $\kappa$ ), human Rad18, and WRNIP1/Mgs1 [25,32,33]. Although its UBZ domain is required for the normal cellular localization of human pol  $\eta$  [27,34,35], the function and significance of the UBZ in pol  $\eta$  remain to be clarified. Some studies report that truncations of human pol  $\eta$  lacking the UBZ sequence are unable to protect cells from DNA damage [27] and are associated with XPV [36], but a more recent study argues that similar truncated forms of human pol  $\eta$  are functional in TLS [37].

The current model for UBZ function is that the UBZ's interaction with ubiquitin promotes pol  $\eta$  function by increasing the polymerase's affinity for mono-ubiquitinated PCNA [26,38,39], although new evidence points to an additional role for the UBZ which is independent of ubiquitinated PCNA [40]. PCNA ubiquitination at K164 occurs particularly, though not exclusively, in response to DNA damage [41–43], and is required in human cells to increase pol  $\eta$ 's residence time in nuclear foci [44]. Genetic studies in yeast show that TLS is dependent on PCNA modification at K164 [42]. PCNA ubiquitination does not increase the catalytic efficiency of the TLS polymerase [45], cause allosteric changes in PCNA structure, or directly interfere with PCNA's interaction with the replicative polymerase [46]. Thus, it is thought that the effect of PCNA ubiquitination on TLS is primarily to increase PCNA's affinity for the TLS polymerase relative to other PCNA-binding factors.

The structure of the UBZ domain from human pol  $\eta$  was determined by NMR to be a classical  $\beta\beta\alpha$  zinc finger, interacting via the exposed face of its C-terminal  $\alpha$ -helix with the canonical hydrophobic patch of ubiquitin [47]. A single zinc ion is coordinated tetrahedrally by the side chains of the two histidines and two cysteines that make up the signature C2H2 motif [31]. In its structure and mode of interaction, the UBZ domain of human pol  $\eta$  is distinctly different from most other ubiquitin-binding zinc fingers, such as the NZF, ZnF-UBP, and RUZ domains [48–52]. Notably, the ubiquitin-binding CCHC-type zinc finger of

NEMO displays an architecture and ubiquitin-binding region similar to the human pol  $\eta$  UBZ domain [33]. Both zinc coordination and ubiquitin binding are needed for UBZ function in human pol  $\eta$ , as DNA damage tolerance can be impaired by mutations affecting either zinc-coordinating (C638A and H564A) or ubiquitin-interacting residues (D562A and F655A) within the UBZ domain of human pol  $\eta$  [25,26,44,53].

In *Saccharomyces cerevisiae* pol  $\eta$  (encoded by *RAD30*), the UBZ can enhance pol  $\eta$ 's affinity for ubiquitin-PCNA fusions, as detected by yeast two-hybrid assay [38,54], and can mediate a direct interaction with ubiquitin [55]. However, research into the UBZ's function in pol  $\eta$  is complicated by the presence of an unusual, non-canonical C2H2 zinc finger sequence within the UBZ motif in the *S. cerevisiae* pol  $\eta$  homolog. Whereas the canonical C2H2 zinc finger sequence is CxxC...Hxxx(x)H, the sequence of the UBZ from *S. cerevisiae* polymerase  $\eta$  is CC...HADYH. Although there are two cysteine residues, they are positioned adjacent to one another, such that only one of their side chains is available for zinc coordination. It has thus been unclear whether zinc coordination is required for UBZ function in *S. cerevisiae* pol  $\eta$ .

Here, we have undertaken a study to elucidate the roles of zinc coordination and ubiquitin binding in the function of the UBZ motif of *S. cerevisiae* pol  $\eta$ . We performed a comprehensive alignment 60 putative UBZ motif sequences from 79 unique pol  $\eta$  homologs, and describe the distribution of putative UBZ and UBM sequences in pol  $\eta$  homologs from a broad variety of species. Among all these putative UBZ sequences, the *S. cerevisiae* sequence is unique in lacking a canonical C2H2 zinc finger sequence. Characterization of *S. cerevisiae* pol  $\eta$  mutants confirms the importance of the UBZ motif, and implies that its function is independent of zinc binding but correlates with its ability to bind ubiquitin. We show that zinc binds to and affects the structure of the purified UBZ domain, suggesting that it is a true zinc finger. However, we demonstrate that the UBZ of *S. cerevisiae* pol  $\eta$  is able to interact with ubiquitin even in the absence of a zinc ion.

## 2. Materials and Methods

### 2.1 Strains and plasmids

The strain used for the experiment shown in Figure 2B is a BY4741/BY4742 derivative strain constructed by mating of yeast deletion project strains 14255 and 6430. All other UV sensitivity experiments use derivatives of W1588-4C (*MATa leu2-3,112 ade2-1 can1-100 his3-11,15 ura3-1 trp1-1 RAD5*), a W303 strain with wildtype *RAD5* sequence [56]. Deletion of *RAD30* was constructed by gene replacement using PCR-amplified *rad30::KanMX* from the *Saccharomyces* Genome Deletion Project strain 4255. To produce the TEV-ProA-7His tagged Rad30 fusion protein, the tag cassette was amplified from pYM10 [57] and inserted by homologous recombination to replace the stop codon of *RAD30*. See Table 1 for additional information on strains.

The plasmids pEGUh6 [58] and pEGUh6-RAD30 [59], of which the latter expresses 6His-Rad30 from the GAL10 promoter, were the kind gifts of Zhigang Wang. Roger Woodgate and John McDonald generously provided the plasmid pJM96 (*RAD30* cloned into pRS415), which expresses Rad30 from its native promoter [60]. Mutants were constructed by site-directed mutagenesis using QuikChange, and are listed in Supplemental Table 1.

The construct for production of the human pol  $\eta$  UBZ domain was previously published [31]. A DNA sequence including to the UBZ domain of *S. cerevisiae* polymerase  $\eta$  (encoding amino acid residues 538–609) was cloned (using the primers 5'-CGCGGATCCACTACCAGCTCGAAAGCTG -3' and 5'-AAACAACAATCTTT

TTTCCCGAAAGAAAG-3') into the BamH1 and Xho1 sites of the pET28aPB vector (the kind gift of Thomas Schwartz) to produce an N-terminally 6His-tagged yeast UBZ peptide.

PJ69-4A was used for yeast 2-hybrid analysis, transformed with plasmids described previously, which express GBD and GAD fusions of Rad30, Ub\*-Pol30\*, or Pol30\*-Ub\* [61]. (The Pol30 protein, product of *POL30*, is the monomeric subunit of the homotrimer PCNA.) In addition, *rad30* mutants for yeast 2-hybrid analysis (both H568L,H572L and C552R,C553R) were constructed by QuikChange mutagenesis (Stratagene) of the *RAD30* plasmids.

## 2.2 Sequence analyses

Alignments were made using T-Coffee [30] and ClustalW2. BLAST and PSI-BLAST were used to identify homology in the non-redundant protein database (NCBI) [62,63]. Identification of UBZ and UBM motifs in pol  $\eta$  homolog sequences was performed as follows. To identify UBZ motifs, the known and predicted protein sequences (listed in Supplemental Table 2) were first searched for the presence of either of two small signatures: CxxC or HxxxH. Among these sequences, we defined as putative UBZ motifs those sequences which fit at least one of the following patterns: CxxC...HxxxH; CC...HxxxH; ZxxZ...HxxxH (where Z is either H or C); CxxC...ZxxxZ; HxDxHxxxx $\phi$  (where  $\phi$  is a hydrophobic residue). To identify putative UBM motifs, we searched for the motif  $\phi$ xxx $\phi$ xxxLPxx $\phi$  (where  $\phi$  is a hydrophobic residue). Although this pattern excludes some putative UBM motifs, it was chosen to minimize false positives.

## 2.3 UV treatment

The *RAD30* gene (encoding pol  $\eta$ ) was expressed under the control of its endogenous promoter from a low-copy vector, pJM96. This expression allowed the wild-type *RAD30* gene to fully rescue the UV sensitivity of a *rad30* null yeast strain [60]. Cultures were grown to saturation for 3 days at 30°C, diluted in water to approximately 6 colony forming units per  $\mu$ l, and 100  $\mu$ l samples were spread on minimal media plates (multiple plates were used for each culture to increase the number of colonies counted). Within 30 minutes, plates were irradiated using a G15T8 UV lamp (General Electric) at 254 nm with 1 J/m<sup>2</sup> per second for varying amounts of time. After irradiation, plates were kept in the dark at 30°C for 3 days before colonies were counted. The data shown are averages of at least three independent cultures for each strain, and error bars represent standard error.

## 2.4 Immunoblotting

Whole cell extracts were prepared by trichloroacetic acid precipitation [57]. Protein samples were separated on 7.5% or 4–12% SDS-polyacrylamide gels, transferred to a polyvinylidene difluoride membrane (Immobilon-P; Millipore), and probed with appropriate antibodies. ProA-tagged protein was detected using rabbit peroxidase anti-peroxidase (PAP) antibody diluted 1:5,000 (Sigma); the 6His tag was detected using mouse anti-His (Qiagen). Blots were visualized using HRP-conjugated goat anti-mouse or anti-rabbit secondary antibody (Pierce) and SuperSignal West Dura Extended Duration Substrate (Pierce) or SuperSignal West Femto Maximum Sensitivity Substrate (Pierce).

## 2.5 Yeast two-hybrid analysis

Analysis of protein-protein interactions by the two-hybrid system was performed in PJ64-4A, using bait and prey plasmids described previously [61], as well as plasmids carrying two *rad30* (pol  $\eta$ ) mutants, H568L,H572L, and C552R,C553R, which were constructed by site-directed mutagenesis. Bait or prey plasmids carrying POL30\* (KK127/164RR), POL30\*-ubiquitin\*, or ubiquitin\*-POL30\* fusions were paired with prey

or bait plasmids carrying the WT *RAD30*, H568L,H572L mutant, or C552R,C553R mutant. Selection for the presence of both bait and prey plasmids was performed on synthetic medium lacking leucine and tryptophan (-LW); positive interactions were identified by growth on medium lacking histidine as well (-HLW), and, for greater stringency, on medium lacking histidine and adenine as well (-AHLW).

## 2.6 UBZ domain purification

The human pol  $\eta$  UBZ domain was purified as described previously [31]. The *S. cerevisiae* pol  $\eta$  UBZ domain was over-expressed and purified from *E. coli* as an N-terminal His<sub>6</sub>-tagged fusion protein, and the tag was subsequently cleaved by Precision Protease. The protein construct was expressed from pET28aPB as: MGSSHHHHHH SLEVLFGQPGSTSSKADEKTPKLECKYQVTFDQKALQEADYHLALK LSEGLNGAEESKNLSFGKRLLF. Expression of the UBZ construct was induced for 2 hours at 30°C by 1 mM IPTG in media supplemented with 50  $\mu$ M zinc sulfate. Cells were lysed by French press. Lysates were treated with DNase (Sigma) and RNase (Qiagen). His-tagged protein was purified using Ni-NTA slurry (Qiagen). The eluted fraction was dialyzed using 3500 MW Snakeskin dialysis tubing (Pierce) before addition of Precision Protease to cleave off the tag. The digested protein was again mixed with 1 ml Ni-NTA resin to separate the untagged protein from the tag and from other Ni-binding proteins. After addition of 50  $\mu$ M zinc sulfate, the non-binding fraction was concentrated to 2 ml. Gel filtration by Superdex 75 column was used to further purify the UBZ protein, which was eluted in 10 mM HEPES pH 7.7, 200 mM NaCl or in 50 mM sodium phosphate, 100 mM KCl.

## 2.7 Ubiquitin Purification

Ubiquitin (yeast or human) was over-expressed in *Escherichia coli* BL21(DE3) STAR cells (Invitrogen, Carlsbad, CA). <sup>15</sup>N-labeled ubiquitin was grown in M9 minimal media and unlabeled protein was grown in LB. Bacterial cells were induced at 20 °C with 1 mM IPTG. Ubiquitin was initially purified by a Ni<sup>2+</sup>-NTA column, followed by thrombin digestion to remove the N-His<sub>6</sub> tag. Thrombin was removed with a benzamidine column and the N-His<sub>6</sub> tag by a second Ni<sup>2+</sup>-NTA column, followed by further purification using size-exclusion chromatography (Superdex 75, GE Healthcare, Piscataway, NJ).

## 2.8 Colorimetric PAR metal-binding assay

Specified samples were treated with EDTA by addition of excess EDTA followed by EDTA removal by buffer exchange using Zeba Spin desalting columns according to manufacturer's instructions (Pierce). Other samples were assayed as prepared, since the prep involved addition of zinc followed by gel filtration column to remove unbound zinc. Samples were assayed in 50 mM sodium phosphate pH 7.6, 100 mM potassium chloride. Protein samples (40  $\mu$ L) were made up at several concentrations in the 10 to 50  $\mu$ M range. Protein concentrations were determined by BioRad Protein Assay. Each protein sample was digested by incubation at 60°C for 30 minutes with Proteinase K to release bound metal ions. Following digestion, an equal volume of a freshly made solution of 0.2 mM 4-(2-pyridylazo)resorcinol (PAR) was mixed with each sample, and the absorbance at 490 nm was measured by a Beckman Coulter DU530 Spectrometer. *S. cerevisiae* Rev1 UBM1, a ubiquitin-binding domain that does not bind zinc [64], was used as a negative control. The UBZ domain of human pol  $\eta$  was used as a positive control.

## 2.9 Circular Dichroism

Circular dichroism experiments were performed at 25° C on an AVIV 62Ds spectropolarimeter. A 6  $\mu$ M sample of the yeast UBZ domain [Rad30(538–609)] or human UBZ domain in a buffer containing 25 mM phosphate, 100 mM KCl, TCEP, pH=7 was

added to a quartz cuvette. Wavelength scans between 200 and 300 nm were recorded for the protein alone, and an additional scan was performed following the addition of 25  $\mu$ M EDTA. Next, a saturating amount of zinc sulfate was added, and a final scan was taken.

## 2.10 NMR Titrations

NMR experiments were performed on a 600 MHz Varian INOVA NMR spectrometer at 25° C. All NMR samples were prepared in a buffer containing 25 mM phosphate, 100 mM KCl, and 10% D<sub>2</sub>O.

In order to probe the binding of ubiquitin and the yeast UBZ domain, unlabeled ubiquitin was titrated into an 0.4 mM sample of <sup>15</sup>N-labeled pol  $\eta$  UBZ domain. The reverse titration was also performed, in which unlabeled pol  $\eta$  UBZ domain was titrated into a 0.4 mM sample of <sup>15</sup>N-labeled ubiquitin.

To test the effects of EDTA on the human and yeast UBZ/ubiquitin interaction, a 15N HSQC was obtained on an 0.3 mM sample of ubiquitin (yeast or human). A 1:1 ratio of (yeast or human) UBZ domain was added into the sample, and another HSQC was acquired. Next, a saturating amount of EDTA was added, and a final HSQC spectrum was obtained. NMR data were processed by NMRPIPE [65] and analyzed with XEASY/CARA [66].

## 3. Results

### 3.1 UBZ motif sequence conservation among pol $\eta$ homologs

The non-canonical zinc finger of the UBZ motif in *S. cerevisiae* pol  $\eta$  precludes the kind of zinc coordination seen in the human homolog (Fig. 1A; Supplementary Data Fig. S1)[31]. Therefore, the structure of the UBZ domain in *S. cerevisiae* pol  $\eta$  may differ from more typical UBZ domains, possibly with functional consequences for the regulation of TLS. To gain insight into the significance of this variation of the UBZ sequence in pol  $\eta$ , we examined the protein sequences of 79 unique pol  $\eta$  homologs (Supplementary Table 2). UBZ motifs were identified in 57 homologs (72%), three of which contain two tandem UBZ motifs (*Drosophila melanogaster*; *Aedes aegypti*; and *Culex quinquefasciatus*). Alignment of these 60 putative UBZ sequences (Supplementary Data Fig. S1), summarized in Figure 1A, reveals in-depth information about the sequence conservation of this highly conserved motif. For instance, several positions that are not conserved with respect to amino acid identity are nonetheless conserved with respect to amino acid properties, making the UBZ motif several amino acids longer than was previously recognized. The only case in which the cysteines were not conserved was in the UBZ motif from *S. cerevisiae* pol  $\eta$ . Other significant departures from the consensus sequence, shown in Figure 1A, include the loss, in two species, of a conserved aspartate residue (D570 in *S. cerevisiae*) that is important for ubiquitin-binding in both yeast and human pol  $\eta$  homologs [25,31,61].

### 3.2 Mutations affecting the UBZ domain of pol $\eta$ in *S. cerevisiae*

To examine the effects of *S. cerevisiae* pol  $\eta$ 's unusual UBZ sequence on polymerase  $\eta$  function, we initially made two mutants of *rad30* (encoding pol  $\eta$  in yeast), which were intended to disrupt zinc coordination. One produces a mutant protein in which both cysteines of the C2H2 zinc finger motif are replaced by arginines (C552R,C553R). In the other mutant, both histidines are replaced by leucines (H568L,H572L). We assayed the ability of each mutant to rescue the UV sensitivity associated with the *rad30* null yeast strain. While the *rad30* null yeast strain is significantly more sensitive than the wild type to killing by UV radiation, expression of the C552R,C553R mutant confers wild-type survival of UV (Fig. 1B). In contrast, the H568L,H572L mutant is associated with a severe defect in UV survival, making it nearly as sensitive as the *rad30* null strain (Fig. 1C). Simultaneous

substitution of leucines for both histidines is required to cause this effect, as single substitutions of leucine for either H568 (H568L) or H572 (H572L) cause only a mild increase in UV sensitivity (Fig. 1C). It is interesting that the two double mutations of the C2H2 zinc finger (H568L,H572L and C552R,C553R) are associated with such different UV survival phenotypes, as either one would be expected to prevent zinc coordination.

The phenotypic effects of these mutants were further compared with those of other *rad30* mutations, each of which results in one or more amino acid substitutions in the PAD or C-terminal regions of pol  $\eta$  (Supplementary Table 1 and Supplementary Data Fig. S2 and Fig. S3), including two other mutations that affect the UBZ outside of the C2H2 motif (Fig. 1D and 1E). These mutations are associated with a range of UV-sensitive phenotypes, but the majority are very mild in contrast to the dramatic sensitivity of the H568L,H572L mutant (Supplementary Data Fig. S3).

To determine if the H568L,H572L mutant Rad30 protein is expressed similarly to the wildtype, immunoblotting was used to compare the abundance of soluble wildtype and H568L,H572L mutant pol  $\eta$  using either of two different epitope-tagged versions of the protein. As shown in Figure 2, the abundance of the mutant pol  $\eta$  in whole cell extracts is equivalent to that of the wild-type protein in both cases (Fig. 2A and C).

To address the possibility that the UV sensitivity associated with the H568L,H572L mutation is a dominant negative phenotype, the mutant was expressed in a wild-type strain background. As shown in Figure 2D, this did not result in increased UV sensitivity, demonstrating that the phenotype associated with H568L,H572L is recessive. We conclude that the defect caused by the H568L,H572L mutation is a loss of function. Taken together, these observations suggest that the non-canonical zinc finger motif within the UBZ of *S. cerevisiae* pol  $\eta$  is functionally important, but also imply that its function does not require zinc coordination by the C2H2 residues.

### 3.3 Effects of UBZ mutations on ubiquitin interaction

Residues of the human pol  $\eta$  UBZ domain forming the outward face of the  $\alpha$ -helix are primarily involved in ubiquitin binding [31]. If the structure of the UBZ domain of *S. cerevisiae* pol  $\eta$  is similar to that of the human UBZ domain [31], then both the histidines (H568, H572) and tyrosine (Y571) would be located on the helix proximal to the ubiquitin interaction surface, while the two cysteines (C552 and C553) would not be directly involved in the interaction. Hence, the partial loss of function caused by the single residue mutations at H568 and Y571 (Fig 1C and 1D) could result from partially destabilizing the domain's interaction with ubiquitin, while the H568L,H572L double mutation could cause a more severe defect in ubiquitin binding.

We tested the ability of both the H568L,H572L and C552R,C553R mutant proteins to interact with ubiquitin-PCNA fusions, using a yeast two-hybrid assay as described previously [38,61]. As shown in Figure 3, the H568L,H572L mutation significantly weakens pol  $\eta$ 's interaction with a ubiquitin-PCNA fusion protein and also slightly weakens the interaction with unmodified PCNA. In contrast, the C552R,C553R double mutant is similar to the wildtype. Thus, for these two mutants, we observed a correlation between a loss of DNA damage tolerance and the UBZ's functional interaction with ubiquitin. Additionally, a fractionation assay [67] was used to compare the chromatin association pattern of the H568L,H572L mutant with that of wildtype pol  $\eta$  (Supplementary Data Fig. S5). While differences were observed between wild-type and mutant pol  $\eta$  proteins, they were difficult to interpret, as this assay did not detect changes in pol  $\eta$  localization in response to DNA damage.

The observation that the H568L,H572L mutant protein has a reduced affinity for ubiquitin supports the hypothesis that the UV survival defect of cells carrying only the mutant pol  $\eta$  results directly from the mutant UBZ's reduced binding for ubiquitin. Taken together with the observations that the C552R,C553R mutant pol  $\eta$  (Fig. 3) interacts normally with ubiquitin, the data support the model that the ubiquitin binding activity of the UBZ domain of *S. cerevisiae* pol  $\eta$  is independent of C2H2-mediated zinc coordination.

### 3.4 The *S. cerevisiae* pol $\eta$ UBZ domain can bind a zinc ion

In light of its unusual sequence and putative zinc independence, we asked whether the non-canonical UBZ domain of *S. cerevisiae* pol  $\eta$  is a “zincless finger,” a domain similar in structure and function to a zinc finger, but which does not coordinate a zinc ion [68–70]. To assay zinc binding, the UBZ domains from both *S. cerevisiae* pol  $\eta$  (residues 538–609) and human pol  $\eta$  (residues 628–662, as previously described [31]) were expressed and purified to homogeneity from *E. coli*. A colorimetric assay, using the metal-binding compound 4-(2-pyridylazo)resorcinol, was then used to measure the concentrations of metal ions present in both native and EDTA-treated protein preparations, and metal-to-protein molar ratios were determined. As shown in Figure 4A, approximately equimolar concentrations of metal and protein are detected in the native preparations of both human and wildtype yeast UBZ peptides. EDTA treatment of these peptides removed the associated metal ions. As expected, significantly lower metal-to-protein ratios are associated with a non-metal-binding control peptide (the UBM2 domain of *S. cerevisiae* Rev1), and with the H568L,H572L mutant UBZ peptide. This assay demonstrates that the wild-type UBZ domain of *S. cerevisiae* pol  $\eta$ , like the human UBZ domain, is associated with a metal ion.

To determine whether the bound metal ion influences the domain's structure, circular dichroism spectroscopy was used to monitor the secondary structure of both the *S. cerevisiae* and human UBZ domains. As shown in Figure 4B and C, both the *S. cerevisiae* and human UBZ domains contain secondary structural elements indicative of folded domains. The CD spectra were measured in the presence and absence of the metal-chelating agent EDTA. As expected for a zinc finger, addition of EDTA resulted in changes to the CD spectrum of the human UBZ domain, implying a loss of secondary structure. These changes were reversed by subsequent addition of excess zinc to the EDTA-treated protein, demonstrating that addition of zinc ions is sufficient to promote refolding of the domain. Intriguingly, similar results were observed for the *S. cerevisiae* UBZ domain, suggesting that the non-canonical UBZ of *S. cerevisiae* pol  $\eta$  can coordinate a zinc ion in a manner that promotes the folding of the domain. Thus, the non-canonical UBZ of *S. cerevisiae* pol  $\eta$  is a zinc-binding domain with distinct zinc-bound and zinc-free structures, more similar to a zinc finger than to a “zincless” finger.

### 3.5 Effect of EDTA on ubiquitin binding

It has previously been assumed that the ubiquitin-binding function of the UBZ domain of *S. cerevisiae* pol  $\eta$  is zinc-dependent, an assumption that underlay the proposal that the UBZ domain has an additional, zinc-independent function [71]. To address this issue, we used NMR titration assays to detect the UBZ's interaction with ubiquitin, and to test the effect of a metal-chelating agent on this interaction. In this assay,  $^1\text{H}$ - $^{15}\text{N}$  HSQC spectra are obtained for a labeled, purified protein before and after addition of a 1:1 molar ratio of its putative interaction partner. If the two proteins physically interact, the NMR resonances of each protein are perturbed by addition of the other.

We first confirmed the interaction between the purified UBZ domain of *S. cerevisiae* pol  $\eta$  and *S. cerevisiae* ubiquitin in the presence of zinc.  $^1\text{H}$ - $^{15}\text{N}$  HSQC spectra were obtained for labeled yeast ubiquitin before and after addition of a 1:1 molar ratio of unlabeled UBZ

domain. Several residues of ubiquitin were either attenuated or perturbed by addition of the *S. cerevisiae* UBZ domain (Figure 5), and the majority of them are located on the same highly conserved, hydrophobic, concave surface of ubiquitin (centered on residue 144) that interacts with the UBZ domain of human pol  $\eta$  [31]. These results imply that the architecture of the interaction is similar in human and *S. cerevisiae*, in spite of the latter's unusual UBZ sequence.

To determine whether the UBZ domain is able to interact with ubiquitin independently of a bound metal ion, NMR titrations were then performed in the presence of the chelating agent EDTA for both the yeast and human UBZ/ubiquitin protein pairs. For each species,  $^1\text{H}$ - $^{15}\text{N}$  HSQC spectra were obtained for  $^{15}\text{N}$ -labeled ubiquitin in the presence and absence of the UBZ domain. As shown in Figure 5, ubiquitin resonances were perturbed by addition of UBZ domains in both cases, indicating that both yeast and human UBZ domains bind ubiquitin in the presence of zinc (and the absence of EDTA). Next, a saturating amount of EDTA was added to each protein pair to chelate any metal ions, followed by the acquisition of another HSQC spectrum. As shown in Figure 5D, the resonances of human ubiquitin returned to the original position, indicating that chelation of zinc disrupted the human ubiquitin/UBZ interaction. By contrast, the spectrum of *S. cerevisiae* ubiquitin remained perturbed in the presence of EDTA (Fig. 5B). As the yeast ubiquitin does not return to the unbound state, this observation suggests that the UBZ domain of *S. cerevisiae* pol  $\eta$  binds to ubiquitin in a zinc-independent manner.

### 3.6 Ubiquitin-binding domains in pol $\eta$ homologs from different species

To gain an evolutionary perspective on the importance and variations of ubiquitin binding in pol  $\eta$  homologs, we further analyzed the sequences of the pol  $\eta$  homologs in which the UBZ motif sequence is either degenerate or absent. In *Ciona intestinalis* and *Cryptosporidium muris*, the sequence is not conserved at a key aspartate residue (Fig. 1A), which is required for ubiquitin binding in both yeast and human pol  $\eta$  homologs [25,31,61]. Interestingly, the *C. intestinalis* pol  $\eta$  homolog also contains a sequence homologous to a ubiquitin-binding motif (UBM), suggesting the possibility that the putative UBM may functionally substitute for the UBZ in this species. The UBM is the ubiquitin interaction motif typically found in two other Y family polymerases, Rev1 and pol  $\iota$  [25].

Putative UBM motifs, shown in Figure 6A, are also observed in pol  $\eta$  homologs from six additional species, all of which lack recognized UBZ motifs (*Arabidopsis thaliana* [72], *Oriza. sativa*, *Caenorhabditis elegans*, *Ricinus communis*, *Ostreococcus lucimarinus*, and *Schistosoma mansoni*). Notably, four of these seven homologs with putative UBMs are found in plants. Two of the putative UBMs not found in plants (*S. mansoni* and *C. elegans*) may not function in ubiquitin binding, as their primary sequences are most similar to the UBM1 of *S. cerevisiae* Rev1, which does not interact with ubiquitin [64]. Even in those pol  $\eta$  homologs with more canonical UBM sequences, the UBM may participate in a different mechanism of pol  $\eta$  regulation from that mediated by the UBZ domain, since the UBM has a slightly different interaction with ubiquitin than that of the UBZ domain [64].

In thirteen of the pol  $\eta$  homologs examined (17%), neither UBM nor UBZ motifs were identified. As many of these are only predicted sequences, errors in gene assembly may account for the failure to identify UBZ or UBM motifs in some of these species. Indeed, six of these sequences are unusually short for pol  $\eta$  homologs (under 550 amino acids), possibly indicating that some predicted sequences are incomplete. Even if predicted pol  $\eta$  sequences are correct in these species, other un-recognized ubiquitin-binding motifs may be present. Alternatively, an additional protein may mediate the interaction, or ubiquitin may not play the same role in pol  $\eta$  regulation in these species. The absence of recognized putative ubiquitin-binding motifs was not limited to any particular phylogenetic group, though it is

interesting that no putative UBZs or UBMs were found in any of the five homologs from trypanosomes (Fig. 6B).

Phylogenetic distribution of the UBZ motif in pol  $\eta$  homologs is non-random, as shown in Figure 6B. Among the pol  $\eta$  homolog sequences examined, UBZ motifs are found in all 13 vertebrate sequences, 8 of 9 arthropod sequences, and 31 of 32 fungal sequences (including the irregular sequence of *S. cerevisiae*). Double tandem UBZ motifs are observed in 3 of the 8 insect species. In contrast, UBZ domains are not present in any of the five trypanosome pol  $\eta$  sequences, nor were they found in any of the seven sequences from photosynthetic organisms. The latter observation suggests the possibility that pol  $\eta$  may be regulated differently in organisms that are constantly exposed to UV irradiation because of their need for sunlight.

#### 4. Discussion

We have undertaken a detailed genetic and biophysical analysis of the UBZ domain from *Saccharomyces cerevisiae* polymerase  $\eta$ . Characterization of pol  $\eta$  mutants confirmed the importance of the UBZ motif to pol  $\eta$ , and implied that UBZ function is independent of zinc-binding, but correlates with ubiquitin-binding activity. We therefore asked whether the UBZ of *S. cerevisiae* pol  $\eta$  could be a “zincless” finger; however, we found that zinc binds to and affects the structure of the purified UBZ domain, suggesting that it is a zinc finger. We further demonstrated that the UBZ of *S. cerevisiae* pol  $\eta$  is able to interact with ubiquitin even in the absence of a zinc ion. Thus, the UBZ domain of *S. cerevisiae* pol  $\eta$  represents a rare example of a zinc finger which is functional even in its apo form.

While this work was in progress, other studies have characterized four additional mutations affecting the UBZ of *S. cerevisiae* pol  $\eta$  [38,55,61,71]. Like the H568L,H572L mutant protein, the D570A and L577Q mutations are defective in both UV survival and ubiquitin binding; in contrast, the C552R,C553R double mutant and the H568A,H572A double mutant are proficient in both respects [38,61,71,73]. The differences between the H568A,H572A mutant protein [71] and our H568L,H572L mutant is likely due to destabilization of the domain’s interaction with ubiquitin by the additional bulk of the leucine residues.

A normal C2H2 zinc finger provides two cysteines and two histidines to satisfy the tetrahedral coordination requirements of the zinc ion. In the UBZ of *S. cerevisiae* pol  $\eta$ , one cysteine and two histidines are available to coordinate a metal ion. However, it remains unclear what molecule provides the fourth coordination site. One possibility is that another amino acid side chain plays this role [74]. In *S. cerevisiae* pol  $\eta$ , one potential candidate is Q556. An alternative possibility is that the zinc ion is coordinated by three amino acids and a water molecule [75]. Evidence from mutant forms of other zinc finger proteins demonstrates that three amino acids can be sufficient for zinc binding. One example is the CCHC-type zinc finger of NEMO, in which a mutant NEMO (C417F) lacking one of the zinc-coordinating cysteines is nonetheless capable of binding zinc with a 1:1 stoichiometry and with a  $K_d$  (0.7  $\mu$ M) similar to that reported for the wild type (0.3  $\mu$ M) [76]. However, the conservation of the cysteines in all of the other pol  $\eta$  UBZ motifs examined in this study implies that both cysteines are generally required for UBZ function. It is likely that zinc coordination is a prerequisite for the ubiquitin interacting activity of the UBZ domains in most other pol  $\eta$  homologs.

What is it about the UBZ of *S. cerevisiae* pol  $\eta$  that allows the ubiquitin interaction to occur even in the absence of zinc? Because both the yeast and human UBZ domains interact with ubiquitin’s canonical hydrophobic patch ([31] and Figure 5) and both require the UBZ

domain's conserved aspartate residue (D570 in *S. cerevisiae*), we propose that the ubiquitin-interacting face of the UBZ may be quite similar between *S. cerevisiae* and human, but there may be significant differences in the structural core of the domain. It has been previously proposed [71] that the yeast UBZ's zinc-independent function is due to a zinc-independent  $\alpha$ -helix. The results from the CD experiments (Fig. 4) showed that the UBZ domain from *S. cerevisiae* pol  $\eta$  becomes substantially less structured upon removal of zinc, though the  $\alpha$ -helical character is not entirely abolished. The observation that the *S. cerevisiae* UBZ peptide is able to bind ubiquitin in its less-structured apo state is reminiscent of the activity of intrinsically disordered proteins, such as UmuD and UmuD' [77]. Like the UBZ domain, UmuD' also mediates the interaction between TLS polymerases and the replication machinery [78,79].

In three distinct ways, our findings emphasize the functional importance of the UBZ's interaction with ubiquitin for pol  $\eta$  function in *S. cerevisiae*. First, the phenotypes of the UBZ mutants presented here add to the growing evidence that ubiquitin binding correlates with the UBZ's role in promoting pol  $\eta$ -mediated TLS. Second, the observation of ubiquitin binding by *S. cerevisiae* pol  $\eta$ 's UBZ domain in the presence of EDTA demonstrates that ubiquitin binding is a zinc-independent function of *S. cerevisiae* pol  $\eta$ 's UBZ domain. Therefore, we need not posit an additional, unknown, zinc-independent function. Third, the broad conservation of putative ubiquitin-binding domains, primarily UBZs but also some UBMs, among the majority of pol  $\eta$  homologs from diverse origins suggests that pol  $\eta$ 's interaction with ubiquitin is important for its regulation in many species.

In those pol  $\eta$  homologs in which putative ubiquitin-binding motifs have not been identified, there may be unrecognized ubiquitin-binding domains. Alternatively, there may be no need for an interaction with ubiquitin in these species. For example, if one biologically relevant function of the UBZ domain in pol  $\eta$  is to enhance the interaction with (ubiquitinated) PCNA, it may be that in such species, pol  $\eta$ 's direct interaction with PCNA is sufficient for its recruitment to the DNA. This would no doubt affect the set of conditions under which pol  $\eta$  is active. Interestingly, the pol  $\eta$  homolog from *Trypanosoma cruzi* [80], which has no recognized UBZ or UBM, has a canonical PIP box motif, which is likely to bind unmodified PCNA with significantly higher affinity than do the non-canonical PIP box motifs found in many Y family polymerases, including yeast and human pol  $\eta$  proteins [81]. Significant differences have been observed in PIP-PCNA complex structures among the human Y family polymerases  $\kappa$ ,  $\eta$  and  $\iota$  [81]; variation in the architecture and affinity of PCNA binding may also exist among pol  $\eta$  homologs.

To conclude, we present an in-depth characterization of the structure and function of the non-canonical UBZ motif of *S. cerevisiae* polymerase  $\eta$ . We find that it represents a rare zinc-binding domain, which is structurally altered by zinc binding, but can perform its ubiquitin-binding function with or without the metal ion. In spite of its unusual sequence and zinc-independent ubiquitin-binding activity, our findings suggest that the *in vivo* function of pol  $\eta$ 's UBZ motif in *S. cerevisiae* involves ubiquitin binding and does not fundamentally differ from the function of more canonical UBZ domains of pol  $\eta$  homologs from other species.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## References

1. Friedberg, EC.; Walker, GC.; Siede, W.; Wood, RD.; Schultz, RA.; Ellenberger, T. DNA Repair and Mutagenesis. Washington, DC: ASM Press; 2006.
2. McCulloch SD, Kunkel TA. The fidelity of DNA synthesis by eukaryotic replicative and translesion synthesis polymerases. *Cell Res.* 2008; 18:148–161. [PubMed: 18166979]
3. Ohmori H, Friedberg EC, Fuchs RP, Goodman MF, Hanaoka F, Hinkle D, Kunkel TA, Lawrence CW, Livneh Z, Nohmi T, Prakash L, Prakash S, Todo T, Walker GC, Wang Z, Woodgate R. The Y-family of DNA polymerases. *Mol Cell.* 2001; 8:7–8. [PubMed: 11515498]
4. Johnson RE, Prakash S, Prakash L. Efficient bypass of a thymine-thymine dimer by yeast DNA polymerase, Pol  $\eta$ . *Science.* 1999; 283:1001–1004. [PubMed: 9974380]
5. Carty MP, Glynn M, Maher M, Smith T, Yao J, Dixon K, McCann J, Rynn L, Flanagan A. The *RAD30* cancer susceptibility gene. *Biochem Soc Trans.* 2003; 31:252–256. [PubMed: 12546696]
6. Hendel A, Ziv O, Gueranger Q, Geacintov N, Livneh Z. Reduced efficiency and increased mutagenicity of translesion DNA synthesis across a TT cyclobutane pyrimidine dimer, but not a TT 6-4 photoproduct, in human cells lacking DNA polymerase  $\eta$ . *DNA Repair (Amst).* 2008; 7:1636–1646. [PubMed: 18634905]
7. Gueranger Q, Sary A, Aoufouchi S, Faili A, Sarasin A, Reynaud CA, Weill JC. Role of DNA polymerases  $\eta$ ,  $\iota$  and  $\zeta$ , in UV resistance and UV-induced mutagenesis in a human cell line. *DNA Repair (Amst).* 2008; 7:1551–1562. [PubMed: 18586118]
8. Masutani C, Kusumoto R, Yamada A, Dohmae N, Yokoi M, Yuasa M, Araki M, Iwai S, Takio K, Hanaoka F. The XPV (xeroderma pigmentosum variant) gene encodes human DNA polymerase  $\eta$ . *Nature.* 1999; 399:700–704. [PubMed: 10385124]
9. Johnson RE, Kondratik CM, Prakash S, Prakash L. *hRAD30* mutations in the variant form of xeroderma pigmentosum. *Science.* 1999; 285:263–265. [PubMed: 10398605]
10. Di Lucca J, Guedj M, Lacapere JJ, Fargnoli MC, Bourillon A, Dieude P, Dupin N, Wolkenstein P, Aegerter P, Saiag P, Descamps V, Lebbe C, Basset-Seguin N, Peris K, Grandchamp B, Soufir N. Variants of the xeroderma pigmentosum variant gene (*POLH*) are associated with melanoma risk. *Eur J Cancer.* 2009; 45:3228–3236. [PubMed: 19477635]
11. Betous R, Rey L, Wang G, Pillaire MJ, Puget N, Selves J, Biard DS, Shin-ya K, Vasquez KM, Cazaux C, Hoffmann JS. Role of TLS DNA polymerases  $\eta$  and  $\kappa$  in processing naturally occurring structured DNA in human cells. *Mol Carcinog.* 2009; 48:369–378. [PubMed: 19117014]
12. Kusumoto R, Masutani C, Iwai S, Hanaoka F. Translesion synthesis by human DNA polymerase  $\eta$  across thymine glycol lesions. *Biochemistry.* 2002; 41:6090–6099. [PubMed: 11994004]
13. Pollack M, Yang IY, Kim HY, Blair IA, Moriya M. Translesion DNA Synthesis across the Heptanone-Etheno-2'-Deoxycytidine Adduct in Cells. *Chem Res Toxicol.* 2006; 19:1074–1079. [PubMed: 16918247]
14. Haracska L, Prakash S, Prakash L. Replication past O(6)-methylguanine by yeast and human DNA polymerase  $\eta$ . *Mol Cell Biol.* 2000; 20:8001–8007. [PubMed: 11027270]
15. Haracska L, Yu SL, Johnson RE, Prakash L, Prakash S. Efficient and accurate replication in the presence of 7,8-dihydro-8-oxoguanine by DNA polymerase  $\eta$ . *Nat Genet.* 2000; 25:458–461. [PubMed: 10932195]
16. Vaisman A, Masutani C, Hanaoka F, Chaney SG. Efficient translesion replication past oxaliplatin and cisplatin GpG adducts by human DNA polymerase  $\eta$ . *Biochemistry.* 2000; 39:4575–4580. [PubMed: 10769112]
17. Minko IG, Washington MT, Prakash L, Prakash S, Lloyd Translesion RS. DNA synthesis by yeast DNA polymerase  $\eta$  on templates containing N2-guanine adducts of 1,3-butadiene metabolites. *J Biol Chem.* 2001; 276:2517–2522. [PubMed: 11062246]
18. Choi JH, Pfeifer GP. The role of DNA polymerase  $\eta$  in UV mutational spectra. *DNA Repair (Amst).* 2005; 4:211–220. [PubMed: 15590329]

19. Johnson RE, Washington MT, Prakash S, Prakash L. Fidelity of human DNA polymerase  $\eta$ . *J Biol Chem*. 2000; 275:7447–7450. [PubMed: 10713043]
20. Matsuda T, Bebenek K, Masutani C, Hanaoka F, Kunkel TA. Low fidelity DNA synthesis by human DNA polymerase- $\eta$ . *Nature*. 2000; 404:1011–1013. [PubMed: 10801132]
21. Trincao J, Johnson RE, Escalante CR, Prakash S, Prakash L, Aggarwal AK. Structure of the catalytic core of *S. cerevisiae* DNA polymerase  $\eta$ : implications for translesion DNA synthesis. *Mol Cell*. 2001; 8:417–426. [PubMed: 11545743]
22. Masutani C, Araki M, Yamada A, Kusumoto R, Nogimori T, Maekawa T, Iwai S, Hanaoka F. Xeroderma pigmentosum variant (XP-V) correcting protein from HeLa cells has a thymine dimer bypass DNA polymerase activity. *Embo J*. 1999; 18:3491–3501. [PubMed: 10369688]
23. Kanao R, Hanaoka F, Masutani C. A novel interaction between human DNA polymerase  $\eta$  and MutL $\alpha$ . *Biochem Biophys Res Commun*. 2009; 389:40–45. [PubMed: 19703417]
24. Jung YS, Liu G, Chen X. Pirh2 E3 ubiquitin ligase targets DNA polymerase  $\eta$  for 20S proteasomal degradation. *Mol Cell Biol*. 30:1041–1048. [PubMed: 20008555]
25. Bienko M, Green CM, Crosetto N, Rudolf F, Zapart G, Coull B, Kannouche P, Wider G, Peter M, Lehmann AR, Hofmann K, Dikic I. Ubiquitin-binding domains in Y-family polymerases regulate translesion synthesis. *Science*. 2005; 310:1821–1824. [PubMed: 16357261]
26. Plosky BS, Vidal AE, de Henestrosa AR, McLenigan MP, McDonald JP, Mead S, Woodgate R. Controlling the subcellular localization of DNA polymerases  $\iota$  and  $\eta$  via interactions with ubiquitin. *Embo J*. 2006; 25:2847–2855. [PubMed: 16763556]
27. Kannouche P, Broughton BC, Volker M, Hanaoka F, Mullenders LH, Lehmann AR. Domain structure, localization, and function of DNA polymerase  $\eta$ , defective in xeroderma pigmentosum variant cells. *Genes Dev*. 2001; 15:158–172. [PubMed: 11157773]
28. Bienko M, Green CM, Sabbioneda S, Crosetto N, Matic I, Hibbert RG, Begovic T, Niimi A, Mann M, Lehmann AR, Dikic I. Regulation of translesion synthesis DNA polymerase  $\eta$  by monoubiquitination. *Mol Cell*. 37:396–407. [PubMed: 20159558]
29. Gerlach VL, Aravind L, Gotway G, Schultz RA, Koonin EV, Friedberg EC. Human and mouse homologs of *Escherichia coli* DinB (DNA polymerase IV), members of the UmuC/DinB superfamily. *Proc Natl Acad Sci U S A*. 1999; 96:11922–11927. [PubMed: 10518552]
30. Notredame C, Higgins DG, Heringa J. T-Coffee: A novel method for fast and accurate multiple sequence alignment. *J Mol Biol*. 2000; 302:205–217. [PubMed: 10964570]
31. Bomar MG, Pai MT, Tzeng SR, Li SS, Zhou P. Structure of the ubiquitin-binding zinc finger domain of human DNA Y-polymerase  $\eta$ . *EMBO Rep*. 2007; 8:247–251. [PubMed: 17304240]
32. Bish RA, Myers MP. Werner helicase-interacting protein 1 binds polyubiquitin via its zinc finger domain. *J Biol Chem*. 2007; 282:23184–23193. [PubMed: 17550899]
33. Cordier F, Grubisha O, Traincard F, Veron M, Delepierre M, Agou F. The zinc finger of NEMO is a functional ubiquitin-binding domain. *J Biol Chem*. 2009; 284:2902–2907. [PubMed: 19033441]
34. Plosky BS, Vidal AE, Fernandez de Henestrosa AR, McLenigan MP, McDonald JP, Mead S, Woodgate R. Controlling the subcellular localization of DNA polymerases  $\iota$  and  $\eta$  via interactions with ubiquitin. *Embo J*. 2006; 25:2847–2855. [PubMed: 16763556]
35. Sabbioneda S, Green CM, Bienko M, Kannouche P, Dikic I, Lehmann AR. Ubiquitin-binding motif of human DNA polymerase  $\eta$  is required for correct localization. *Proc Natl Acad Sci U S A*. 2009; 106:E20. author reply E21. [PubMed: 19240217]
36. Broughton BC, Cordonnier A, Kleijer WJ, Jaspers NG, Fawcett H, Raams A, Garritsen VH, Stary A, Avril MF, Boudsocq F, Masutani C, Hanaoka F, Fuchs RP, Sarasin A, Lehmann AR. Molecular analysis of mutations in DNA polymerase  $\eta$  in xeroderma pigmentosum-variant patients. *Proc Natl Acad Sci U S A*. 2002; 99:815–820. [PubMed: 11773631]
37. Acharya N, Yoon JH, Hurwitz J, Prakash L, Prakash S. DNA polymerase  $\eta$  lacking the ubiquitin-binding domain promotes replicative lesion bypass in humans cells. *Proc Natl Acad Sci U S A*.
38. van der Kemp PA, de Padula M, Burguiere-Slezak G, Ulrich HD, Boiteux S. PCNA monoubiquitylation and DNA polymerase  $\eta$  ubiquitin-binding domain are required to prevent 8-oxoguanine-induced mutagenesis in *Saccharomyces cerevisiae*. *Nucleic Acids Res*. 2009; 37:2549–2559. [PubMed: 19264809]

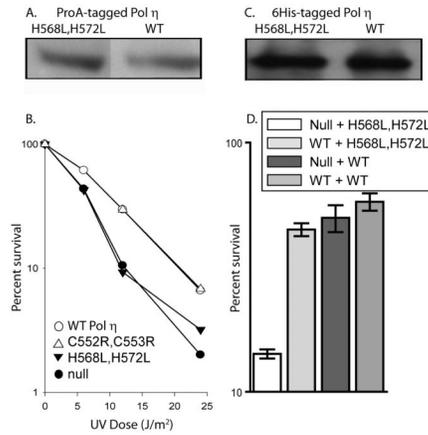
39. Masuda Y, Piao J, Kamiya K. DNA replication-coupled PCNA mono- ubiquitination and polymerase switching in a human *in vitro* system. *J Mol Biol.* 396:487–500. [PubMed: 20064529]
40. Schmutz V, Janel-Bintz R, Wagner J, Biard D, Shiomi N, Fuchs RP, Cordonnier AM. Role of the ubiquitin-binding domain of Pol  $\eta$  in Rad18-independent translesion DNA synthesis in human cell extracts. *Nucleic Acids Res.*
41. Hoege C, Pfander B, Moldovan GL, Pyrowolakakis G, Jentsch S. RAD6- dependent DNA repair is linked to modification of PCNA by ubiquitin and SUMO. *Nature.* 2002; 419:135–141. [PubMed: 12226657]
42. Stelter P, Ulrich HD. Control of spontaneous and damage-induced mutagenesis by SUMO and ubiquitin conjugation. *Nature.* 2003; 425:188–191. [PubMed: 12968183]
43. Terai K, Abbas T, Jazaeri AA, Dutta A. CRL4(Cdt2) E3 ubiquitin ligase monoubiquitinates PCNA to promote translesion DNA synthesis. *Mol Cell.* 37:143–149. [PubMed: 20129063]
44. Sabbioneda S, Gourdin AM, Green CM, Zotter A, Giglia-Mari G, Houtsmuller A, Vermeulen W, Lehmann AR. Effect of proliferating cell nuclear antigen ubiquitination and chromatin structure on the dynamic properties of the Y-family DNA polymerases. *Mol Biol Cell.* 2008; 19:5193–5202. [PubMed: 18799611]
45. Nikolaishvili-Feinberg N, Jenkins GS, Nevis KR, Staus DP, Scarlett CO, Unsal-Kacmaz K, Kaufmann WK, Cordeiro-Stone M. Ubiquitylation of proliferating cell nuclear antigen and recruitment of human DNA polymerase  $\eta$ . *Biochemistry.* 2008; 47:4141–4150. [PubMed: 18321066]
46. Freudenthal BD, Gakhar L, Ramaswamy S, Washington MT. Structure of monoubiquitinated PCNA and implications for translesion synthesis and DNA polymerase exchange. *Nat Struct Mol Biol.* 17:479–484. [PubMed: 20305653]
47. Bomar MG, Pai MT, Tzeng SR, Li SS, Zhou P. Structure of the ubiquitin-binding zinc finger domain of human DNA Y-polymerase  $\eta$ . *EMBO Rep.* 2007; 8:247–251. [PubMed: 17304240]
48. Alam SL, Sun J, Payne M, Welch BD, Blake BK, Davis DR, Meyer HH, Emr SD, Sundquist WI. Ubiquitin interactions of NZF zinc fingers. *Embo J.* 2004; 23:1411–1421. [PubMed: 15029239]
49. Reyes-Turcu FE, Horton JR, Mullally JE, Heroux A, Cheng X, Wilkinson KD. The ubiquitin binding domain ZnF UBP recognizes the C-terminal diglycine motif of unanchored ubiquitin. *Cell.* 2006; 124:1197–1208. [PubMed: 16564012]
50. Penengo L, Mapelli M, Murachelli AG, Confalonieri S, Magri L, Musacchio A, Di Fiore PP, Polo S, Schneider TR. Crystal structure of the ubiquitin binding domains of rabex-5 reveals two modes of interaction with ubiquitin. *Cell.* 2006; 124:1183–1195. [PubMed: 16499958]
51. Lee S, Tsai YC, Mattera R, Smith WJ, Kostelansky MS, Weissman AM, Bonifacino JS, Hurley JH. Structural basis for ubiquitin recognition and autoubiquitination by Rabex-5. *Nat Struct Mol Biol.* 2006; 13:264–271. [PubMed: 16462746]
52. Pai MT, Tzeng SR, Kovacs JJ, Keaton MA, Li SS, Yao TP, Zhou P. Solution structure of the Ubp-M BUZ domain, a highly specific protein module that recognizes the C-terminal tail of free ubiquitin. *J Mol Biol.* 2007; 370:290–302. [PubMed: 17512543]
53. Acharya N, Yoon JH, Gali H, Unk I, Haracska L, Johnson RE, Hurwitz J, Prakash L, Prakash S. Roles of PCNA-binding and ubiquitin-binding domains in human DNA polymerase  $\eta$  in translesion DNA synthesis. *Proc Natl Acad Sci U S A.* 2008; 105:17724–17729. [PubMed: 19001268]
54. Parker JL, Bielen AB, Dikic I, Ulrich HD. Contributions of ubiquitin- and PCNA-binding domains to the activity of Polymerase  $\eta$  in *Saccharomyces cerevisiae*. *Nucleic Acids Res.* 2007; 35:881–889. [PubMed: 17251197]
55. Pabla R, Rozario D, Siede W. Regulation of *Saccharomyces cerevisiae* DNA polymerase  $\eta$  transcript and protein. *Radiat Environ Biophys.* 2008; 47:157–168. [PubMed: 17874115]
56. Zhao X, Muller EG, Rothstein R. A suppressor of two essential checkpoint genes identifies a novel protein that negatively affects dNTP pools. *Mol Cell.* 1998; 2:329–340. [PubMed: 9774971]
57. Knop M, Siegers K, Pereira G, Zachariae W, Winsor B, Nasmyth K, Schiebel E. Epitope tagging of yeast genes using a PCR-based strategy: more tags and improved practical routines. *Yeast.* 1999; 15:963–972. [PubMed: 10407276]

58. Wu X, Braithwaite E, Wang Z. DNA ligation during excision repair in yeast cell-free extracts is specifically catalyzed by the CDC9 gene product. *Biochemistry*. 1999; 38:2628–2635. [PubMed: 10052932]
59. Yuan F, Zhang Y, Rajpal DK, Wu X, Guo D, Wang M, Taylor JS, Wang Z. Specificity of DNA lesion bypass by the yeast DNA polymerase  $\eta$ . *J Biol Chem*. 2000; 275:8233–8239. [PubMed: 10713149]
60. McDonald JP, Levine AS, Woodgate R. The *Saccharomyces cerevisiae* RAD30 gene, a homologue of *Escherichia coli* *dinB* and *umuC*, is DNA damage inducible and functions in a novel error-free postreplication repair mechanism. *Genetics*. 1997; 147:1557–1568. [PubMed: 9409821]
61. Parker JL, Bielen AB, Dikic I, Ulrich HD. Contributions of ubiquitin- and PCNA-binding domains to the activity of Polymerase  $\eta$  in *Saccharomyces cerevisiae*. *Nucleic Acids Res*. 2007; 35:881–889. [PubMed: 17251197]
62. Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res*. 1997; 25:3389–3402. [PubMed: 9254694]
63. Schaffer AA, Aravind L, Madden TL, Shavirin S, Spouge JL, Wolf YI, Koonin EV, Altschul SF. Improving the accuracy of PSI-BLAST protein database searches with composition-based statistics and other refinements. *Nucleic Acids Res*. 2001; 29:2994–3005. [PubMed: 11452024]
64. Bomar MG, D'Souza S, Bienko M, Dikic I, Walker GC, Zhou P. Unconventional ubiquitin recognition by the ubiquitin-binding motif within the Y family DNA polymerases  $\iota$  and Rev1. *Mol Cell*. 37:408–417. [PubMed: 20159559]
65. Delaglio F, Grzesiek S, Vuister GW, Zhu G, Pfeifer J, Bax A. NMRPipe: a multidimensional spectral processing system based on UNIX pipes. *J Biomol NMR*. 1995; 6:277–293. [PubMed: 8520220]
66. Bartels C, et al. The program XEASY for computer-supported NMR spectral analysis of biological macromolecules. *J. Biol. NMR*. 1995; 6:1–10.
67. Donovan S, Harwood J, Drury LS, Diffley JF. Cdc6p-dependent loading of Mcm proteins onto pre-replicative chromatin in budding yeast. *Proc Natl Acad Sci U S A*. 1997; 94:5611–5616. [PubMed: 9159120]
68. Doublet S, Bandaru V, Bond JP, Wallace SS. The crystal structure of human endonuclease VIII-like 1 (NEIL1) reveals a zincless finger motif required for glycosylase activity. *Proc Natl Acad Sci U S A*. 2004; 101:10284–10289. [PubMed: 15232006]
69. Mayorov VI, Rogozin IB, Adkison LR, Gearhart PJ. DNA polymerase  $\eta$  contributes to strand bias of mutations of A versus T in immunoglobulin genes. *J Immunol*. 2005; 174:7781–7786. [PubMed: 15944281]
70. Struthers MD, Cheng RP, Imperiali B. Design of a monomeric 23-residue polypeptide with defined tertiary structure. *Science*. 1996; 271:342–345. [PubMed: 8553067]
71. Acharya N, Brahma A, Haracska L, Prakash L, Prakash S. Mutations in the ubiquitin binding UBZ motif of DNA polymerase  $\eta$  do not impair its function in translesion synthesis during replication. *Mol Cell Biol*. 2007
72. Anderson HJ, Vonarx EJ, Pastushok L, Nakagawa M, Katafuchi A, Gruz P, Di Rubbo A, Grice DM, Osmond MJ, Sakamoto AN, Nohmi T, Xiao W, Kunz BA. *Arabidopsis thaliana* Y-family DNA polymerase  $\eta$  catalyses translesion synthesis and interacts functionally with PCNA2. *Plant J*. 2008; 55:895–908. [PubMed: 18494853]
73. Pabla R, Rozario D, Siede W. Regulation of *Saccharomyces cerevisiae* DNA polymerase  $\eta$  transcript and protein. *Radiat Environ Biophys*. 2007
74. Auld DS. Zinc coordination sphere in biochemical zinc sites. *Biometals*. 2001; 14:271–313. [PubMed: 11831461]
75. McCall KA, Huang C, Fierke CA. Function and mechanism of zinc metalloenzymes. *J Nutr*. 2000; 130:1437S–1446S. [PubMed: 10801957]
76. Cordier F, Vinolo E, Veron M, Delepierre M, Agou F. Solution structure of NEMO zinc finger and impact of an anhidrotic ectodermal dysplasia with immunodeficiency-related point mutation. *J Mol Biol*. 2008; 377:1419–1432. [PubMed: 18313693]

77. Simon SM, Sousa FJR, Mohana-Borges RS, Walker GC. Regulation of *E. coli* SOS mutagenesis by dimeric intrinsically disordered umuD gene products. 2007 In Press.
78. Sutton MD, Opperman T, Walker GC. The Escherichia coli SOS mutagenesis proteins UmuD and UmuD' interact physically with the replicative DNA polymerase. Proc Natl Acad Sci U S A. 1999; 96:12373–12378. [PubMed: 10535929]
79. Godoy VG, Jarosz DF, Simon SM, Abyzov A, Ilyin V, Walker GC. UmuD and RecA directly modulate the mutagenic potential of the Y family DNA polymerase DinB. Mol Cell. 2007; 28:1058–1070. [PubMed: 18158902]
80. de Moura MB, Schamber-Reis BL, Passos Silva DG, Rajao MA, Macedo AM, Franco GR, Pena SD, Teixeira SM, Machado CR. Cloning and characterization of DNA polymerase  $\eta$  from *Trypanosoma cruzi*: roles for translesion bypass of oxidative damage. Environ Mol Mutagen. 2009; 50:375–386. [PubMed: 19229999]
81. Hishiki A, Hashimoto H, Hanafusa T, Kamei K, Ohashi E, Shimizu T, Ohmori H, Sato M. Structural basis for novel interactions between human translesion synthesis polymerases and proliferating cell nuclear antigen. J Biol Chem. 2009; 284:10552–10560. [PubMed: 19208623]



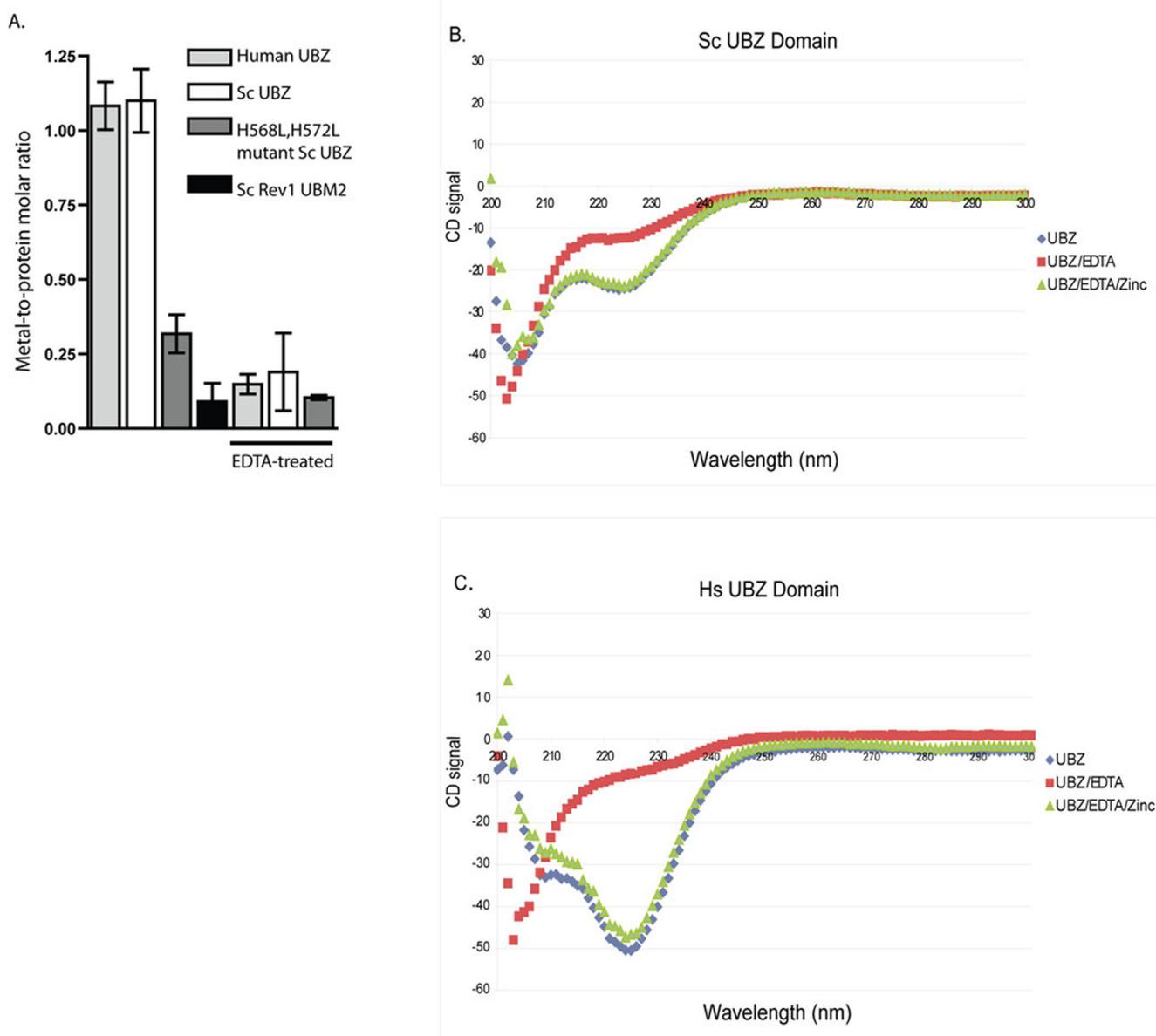
represent sites of mutations made in *S. cerevisiae* pol  $\eta$  for this study. The genes listed are: *Homo sapiens* pol  $\eta$ , NP\_006493; *Mus musculus* pol  $\eta$ , NP\_109640; *Drosophila melanogaster* pol  $\eta$ , AAF51794; *Cryptosporidium muris* pol  $\eta$ , XP\_002142930; *Ciona intestinalis* pol  $\eta$ , XP\_002128588; *Saccharomyces cerevisiae* pol  $\eta$ , EDN60746. **B. through E.** In a *rad30* null background (RWY15), effect on UV sensitivity of mutant pol  $\eta$  proteins expressed from a low-copy plasmid under the *RAD30* native promoter. Error bars represent standard error. **B.** C552R,C553R double mutant (triangles). **C.** H568L,H572L double mutant (open circles), causes greater UV sensitivity than either H568L (black triangles), or H572L (open triangles, overlapping with wildtype). **D.** Y571A. **E.** Rad30–548.



### Figure 2. Characterization of the pol $\eta$ H568L,H572L mutant

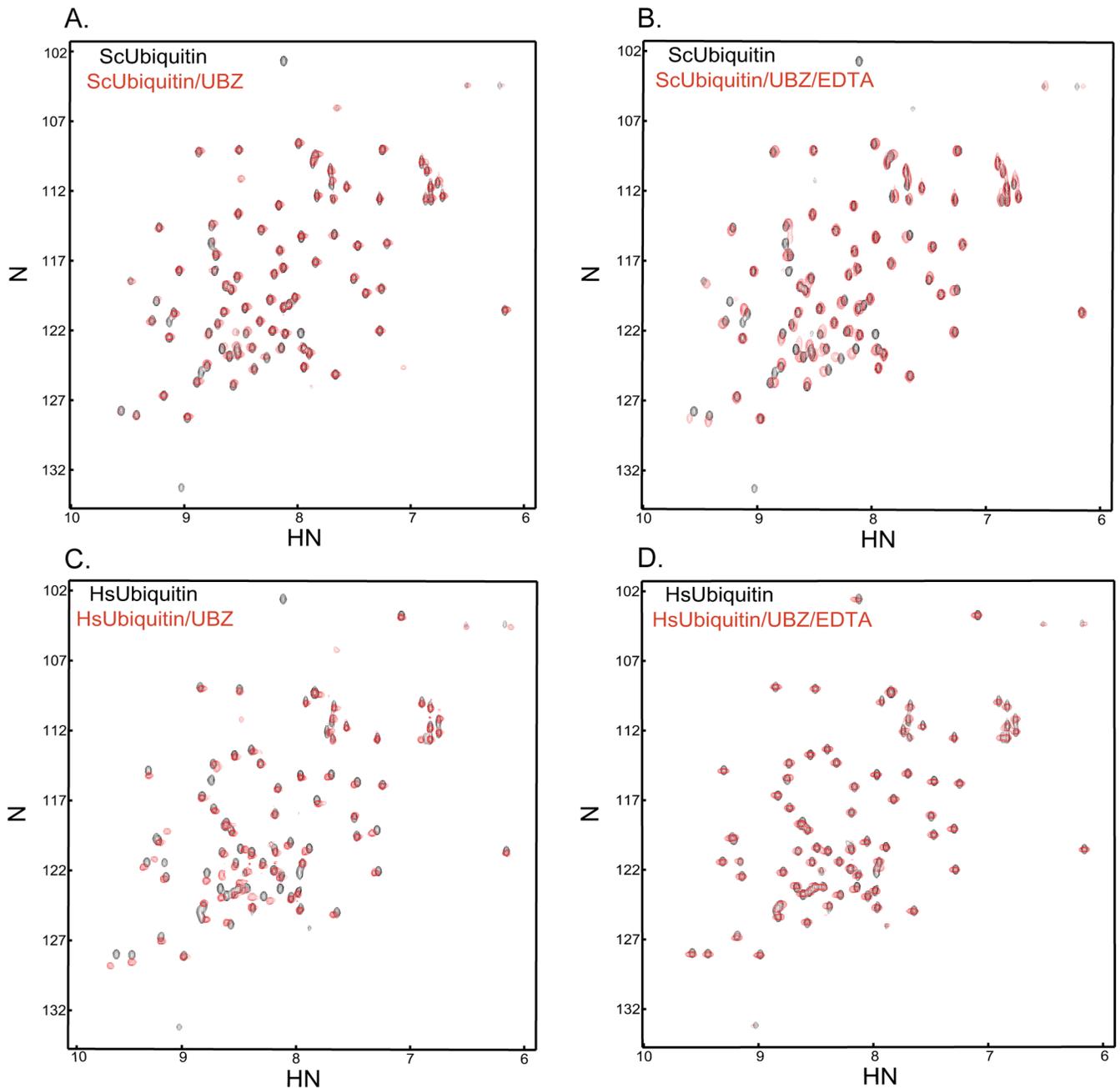
**A.** Expression of ProA-tagged H568L,H572L double mutant and wildtype pol  $\eta$  proteins. Immunoblot (peroxidase anti-peroxidase) showing expression of the –TEV-ProA-His tagged H568L,H572L mutant pol  $\eta$ , left lane, compared with the wildtype, right lane. Equal amounts of total protein were loaded in each lane. **B.** Phenotypes caused by expression of 6His-tagged wildtype and mutant pol  $\eta$  proteins in *rad5rad30* background (C552R,C553R and WT overlap). **C.** Expression of 6His-tagged H568L,H572L double mutant and wildtype pol  $\eta$  protein is compared by anti-His immunoblot. Equal amounts of total protein were loaded in each lane. **D.** H568L,H572L phenotype is recessive. Percent survival after exposure to 30  $J/m^2$  UV. Plasmid-born H568L,H572L mutant protein in *rad30* (white) or wildtype background (pale grey) is compared with plasmid-born wildtype pol  $\eta$  in *rad30* null (black) or wildtype background (dark gray).





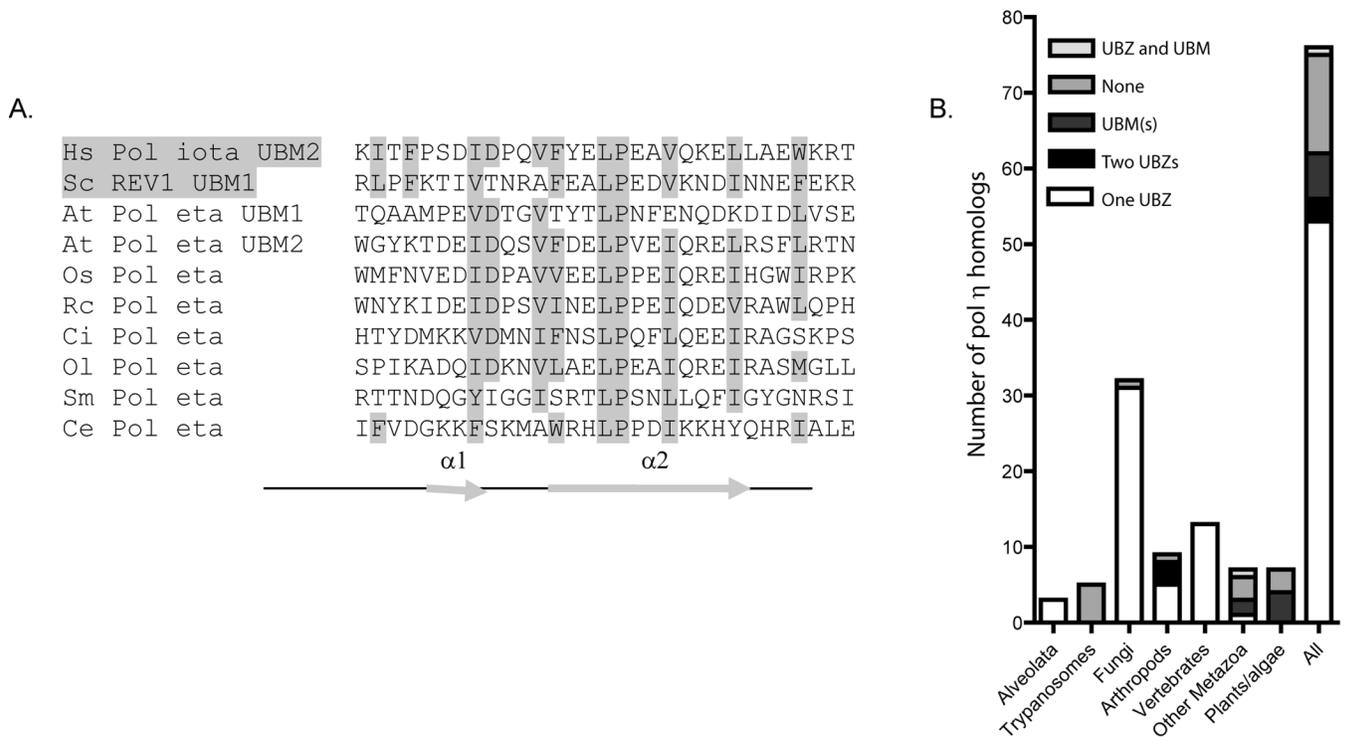
**Figure 4. UBZ binds zinc**

**A**, PAR colorimetric assay was used to measure the concentration of divalent metal cations in each protein prep. Metal to protein ratios were estimated using the BioRad Protein Assay to determine protein concentrations. **B**, CD spectra of human pol  $\eta$  UBZ domain alone (blue), with EDTA (red), and with zinc sulfate (green). **C**, CD spectra of *S. cerevisiae* pol  $\eta$  UBZ domain alone (blue), with EDTA (red), and with zinc sulfate (green).



**Figure 5. Effect of EDTA on *S. cerevisiae* and human UBZ/ubiquitin interaction**

**A**, HSQC spectrum of  $^{15}\text{N}$ -labeled *S. cerevisiae* ubiquitin alone (black) and with *S. cerevisiae* UBZ domain (red). **B**, HSQC spectrum of  $^{15}\text{N}$ -labeled *S. cerevisiae* ubiquitin alone (black) and with *S. cerevisiae* UBZ domain (red) in the presence of EDTA. **C**, HSQC spectrum of  $^{15}\text{N}$ -labeled human ubiquitin alone (black) and with human UBZ domain (red). **D**, HSQC spectrum of  $^{15}\text{N}$ -labeled human ubiquitin alone (black) and with human UBZ domain (red) in the presence of EDTA.



**Figure 6. The putative ubiquitin-binding domains of pol  $\eta$  homologs**

**A.** Sequence alignment of putative UBMs from pol  $\eta$  homologs, compared with two known UBMs. Conserved residues are highlighted. The genes listed are: *Homo sapiens* (Hs) pol  $\iota$ , AF245438; *Saccharomyces cerevisiae* (Sc) Rev1, NP\_014991; *Arabidopsis thaliana* (At) pol  $\eta$  CAC94893; *Oryza sativa* (Os) pol  $\eta$ , BAD87579; *Ricinus communis* (Rc) pol  $\eta$ , XP\_00252815; *Ciona intestinalis* (Ci) pol  $\eta$ , XP\_002128588; *Ostreococcus lucimarinus* (Ol) pol  $\eta$ , ABO98773; *Schistosoma mansoni* (Cm) pol  $\eta$ , XP\_002574021; *Caenorhabditis elegans* (Ce) pol  $\eta$ , BAE7270;. The secondary structural elements for the UBM2 of human pol  $\iota$  are indicated below the alignment [2]. The UBMs of *O.sativa* and *A.thaliana* were previously recognized [3]. It should be noted that there may be two tandem UBMs in the pol  $\eta$  homologs of several of these species; however, only the most highly conserved of the motifs are shown here. **B.** Distribution of putative ubiquitin-binding domains among pol  $\eta$  homologs. Pol  $\eta$  homologs were divided among 5 categories: Those containing exactly one UBZ motif; those containing only putative UBM(s); those containing two UBZ motifs; those containing both a putative UBM and a UBZ motif; and those in which neither a UBM nor a UBZ domain was recognized. For the complete list of species and locus IDs, see Supplementary Table 2.

[1] M.G. Bomar, M.T. Pai, S.R. Tzeng, S.S. Li and P. Zhou Structure of the ubiquitin-binding zinc finger domain of human DNA Y-polymerase eta, EMBO Rep 8 (2007) 247–251.

[2] M.G. Bomar, S. D'Souza, M. Bienko, I. Dikic, G.C. Walker and P. Zhou Unconventional ubiquitin recognition by the ubiquitin-binding motif within the Y family DNA polymerases iota and Rev1, Mol Cell 37 408–417.

[3] H.J. Anderson, E.J. Vonarx, L. Pastushok, M. Nakagawa, A. Katafuchi, P. Gruz, A. Di Rubbo, D.M. Grice, M.J. Osmond, A.N. Sakamoto, T. Nohmi, W. Xiao and B.A. Kunz Arabidopsis thaliana Y-family DNA polymerase eta catalyses translesion synthesis and interacts functionally with PCNA2, Plant J 55 (2008) 895–908.

**Table 1**

## Yeast strains used in this study

Strain	Genotype	Source
RWY10	<i>MATa leu2Δ1 his3Δ1 met5Δ0 ura3Δ0 rad5::kanMX rad30::kanMX</i>	This study
W1588-4C	<i>MATa leu2-3,112 ade2-1 can1-100 his3-11,15 ura3-1 trp1-1 RAD5</i>	Zhao et al (1998)
RWY13	<i>MATa leu2-3,112 ade2-1 can1-100 his3-11,15 ura3-1 trp1-1 RAD5 RAD30-TEV-ProA-7His::HIS3MX</i>	This study
RWY15	<i>MATa leu2-3,112 ade2-1 can1-100 his3-11,15 ura3-1 trp1-1 RAD5 rad30::KanMX</i>	This study
PJ69-4a	<i>MATa trp1-901 leu2-3,112 ura3-52 his3-200 gal4Δ gal80ΔLYS2::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ</i>	James et al (1996)