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The unusual UBZ domain of *Saccharomyces cerevisiae* polymerase η

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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 η is vital for its regulation, localization, and function. Here, we elucidate structural and functional features of the non-canonical UBZ motif of *Saccharomyces cerevisiae* pol η. Characterization of pol η 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 η. Putative ubiquitin-binding domains, primarily UBZs, are identified in the majority of known pol η homologs. We discuss the implications of our observations for zinc finger structure and pol η regulation.

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 carried out by multiple non-essential 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) η 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 η suffer from a syndrome known as xeroderma pigmentosum variant (XPV), 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 η may also predispose patients to melanoma [10]. In addition to UV lesions, pol η 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 η resides in its N-terminal domains, which share sequence homology with the other Y family TLS polymerases [3]. Pol η 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 η'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 *Saccharomyces cerevisiae* pol η [9,29]. Human pol η 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 κ (κ), human Rad18, and WRNIP1/Mgs1 [25,32,33]. Although its UBZ domain is required for the normal cellular localization of human pol η [27,34,35], the function and significance of the UBZ in pol η remain to be clarified. Some studies report that truncations of human pol η 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 η are functional in TLS [37].

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Table 1
Yeast strains used in this study.

Strain	Genotype	Source
RWY10	*MATα leu2Δ1 his3Δ1 met5Δ0 ura3Δ0 rad5::kanMX rad30::kanMX*	This study
W1588-4C	*MATa leu2-3,112 ade2-1 can1-100 his3-11,15 ura3-1 trp1-1 RAD5*	Zhao et al. [55]
RWY13	*MATa leu2-3,112 ade2-1 can1-100 his3-11,15 ura3-1 trp1-1 RAD5 RAD30-TEV-ProA-7His::HIS3MX*	This study
RWY15	*MATa leu2-3,112 ade2-1 can1-100 his3-11,15 ura3-1 trp1-1 RAD5 rad30::KanMX*	This study
PJ69-4a	*MATa trp1-901 leu2-3,112 ura3-52 his3-200 gal4Δ gal80Δ LYS2::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ*	James et al. [81]

The current model for UBZ function is that the UBZ's interaction with ubiquitin promotes pol η 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 η'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 η was determined by NMR to be a classical ββα zinc finger, interacting via the exposed face of its C-terminal α-helix with the canonical hydrophobic patch of ubiquitin [31]. 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 η is distinctly different from most other ubiquitin-binding zinc fingers, such as the NZF, ZnF-UBP, and RUZ domains [47–51]. Notably, the ubiquitin-binding CCHC-type zinc finger of NEMO displays an architecture and ubiquitin-binding region similar to the human pol η UBZ domain [33]. Both zinc coordination and ubiquitin binding are needed for UBZ function in human pol η, 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 η [25,26,44,52].

In *S. cerevisiae* pol η (encoded by *RAD30*), the UBZ can enhance pol η's affinity for ubiquitin–PCNA fusions, as detected by yeast two-hybrid assay [38,53], and can mediate a direct interaction with ubiquitin [54]. However, research into the UBZ's function in pol η is complicated by the presence of an unusual, non-canonical C2H2 zinc finger sequence within the UBZ motif in the *S. cerevisiae* pol η homolog. Whereas the canonical C2H2 zinc finger sequence is **C**xx**C**. . .**H**xxx(x)**H**, the sequence of the UBZ from *S. cerevisiae* polymerase η is **CC**. . .**H**ADY**H**. 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 η.

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 η. We performed a comprehensive alignment 60 putative UBZ motif sequences from 79 unique pol η homologs, and describe the distribution of putative UBZ and UBM sequences in pol η 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 η 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 η 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 Fig. 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 wild-type *RAD5* sequence [55]. 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 [56] and inserted by homologous recombination to replace the stop codon of *RAD30*. See Table 1 for additional information on strains.

The plasmids pEGUh6 [57] and pEGUh6-RAD30 [58], 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 [59]. Mutants were constructed by site-directed mutagenesis using QuikChange, and are listed in Supplementary Table 1.

The construct for production of the human pol η UBZ domain was previously published [31]. A DNA sequence including to the UBZ domain of *S. cerevisiae* polymerase η (encoding amino acid residues 538–609) was cloned (using the primers 5′-CGCGGATCCACTACCAGCTCGAAAGCTG-3′ and 5′-AAACAACAATCTTT TTTCCCCGAAAGAAAG-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* [60]. (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) [61,62]. Identification of UBZ

and UBM motifs in pol η homolog sequences was performed as follows. To identify UBZ motifs, the known and predicted protein sequences (listed in Supplementary 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; HxDxHxxxxxϕ (where ϕ is a hydrophobic residue). To identify putative UBM motifs, we searched for the motif ϕxxxϕxxxLPxxϕ (where ϕ 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 η) was expressed under the control of its endogenous promoter from a low-copy vector, pJM96. This expression allowed the wildtype *RAD30* gene to fully rescue the UV sensitivity of a *rad30* null yeast strain [59]. Cultures were grown to saturation for 3 days at 30 °C, diluted in water to approximately 6 colony forming units per μl, and 100 μl samples were spread on minimal media plates (multiple plates were used for each culture to increase the number of colonies counted). Within 30 min, plates were irradiated using a G15T8 UV lamp (General Electric) at 254 nm with 1 J/m^2 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 [56]. 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 1:5000 (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 [60], as well as plasmids carrying two *rad30* (pol η) 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 η UBZ domain was purified as described previously [31]. The *S. cerevisiae* pol η UBZ domain was over-expressed and purified from *Escherichia coli* as an N-terminal His6-tagged fusion protein, and the tag was subsequently cleaved by Precision Protease. The protein construct was expressed from pET28aPB as: MGSSHHHHHH SLEVLFQGPGSTTSSKADEKTPKLECCKYQVTFT-DQKALQEHADYHLALKLSEGLNGAEESSKNLSFGEKRLLF. Expression of the UBZ construct was induced for 2 h at 30 °C by 1 mM IPTG in media supplemented with 50 μM 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 μM 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 *E. coli* BL21(DE3) STAR cells (Invitrogen, Carlsbad, CA). ^{15}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^{2+}-NTA column, followed by thrombin digestion to remove the N-His_6 tag. Thrombin was removed with a benzamidine column and the N-His_6 tag by a second Ni^{2+}-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 μL) were made up at several concentrations in the 10–50 μM range. Protein concentrations were determined by BioRad Protein Assay. Each protein sample was digested by incubation at 60 °C for 30 min 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 [63], was used as a negative control. The UBZ domain of human pol η 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 μM 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 μM 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_2O.

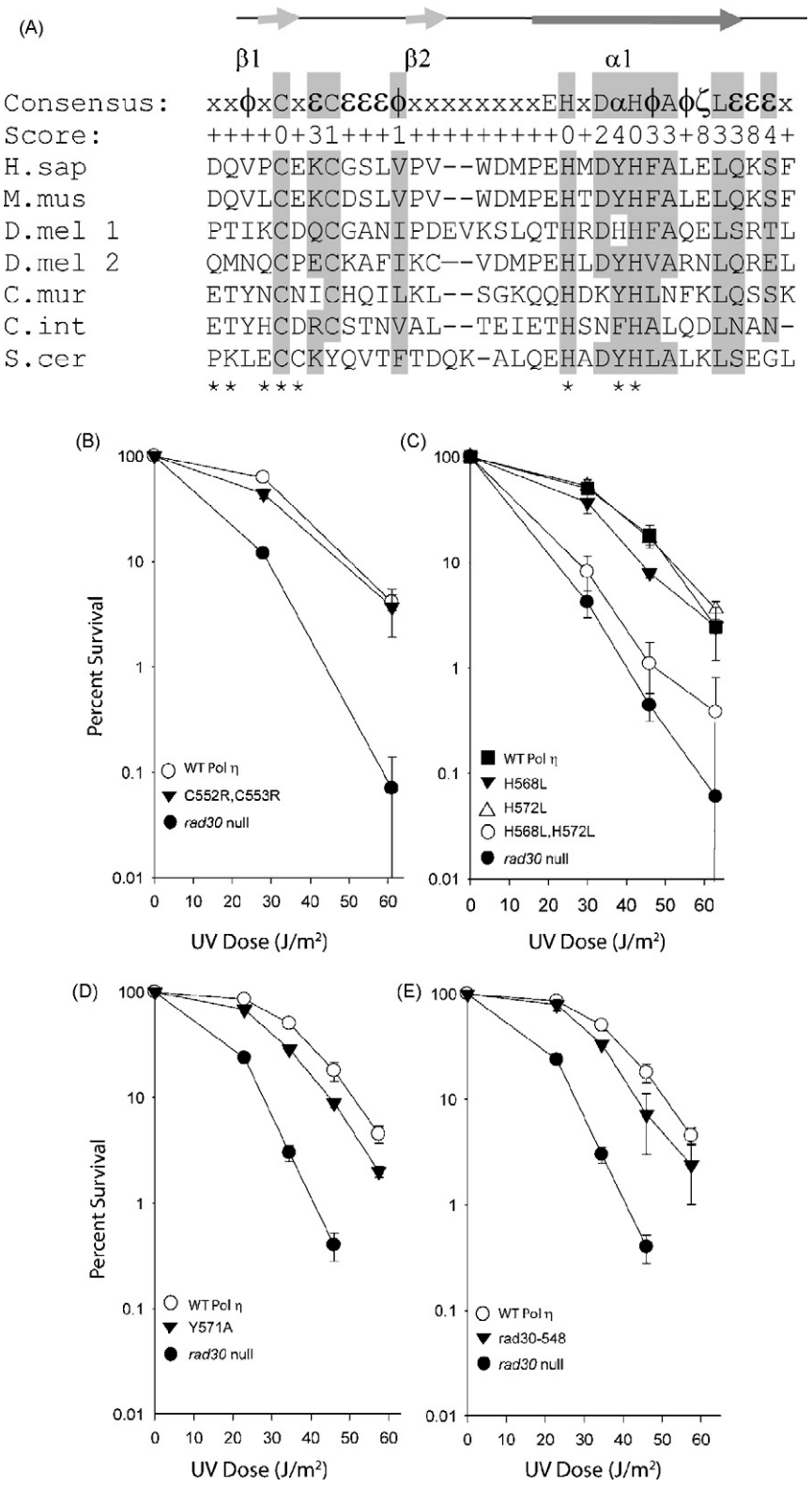

Fig. 1. The non-canonical UBZ domain of *S. cerevisiae* pol η. (A) UBZ sequence alignment. The secondary structural elements of the UBZ from human pol η are indicated above the sequences [31]. UBZ motifs from several pol η sequences are aligned with a consensus sequence derived from alignment of 60 UBZ motifs in pol η homologs (Fig. S1). Highly conserved residues are highlighted. In the consensus sequence, α indicates position of an aromatic residue; ϕ indicates a hydrophobic residue; ζ indicates a charged residue; ε indicates a hydrophilic residue. Consensus scores indicate the number of these sequences which differ from the consensus at each position, with zero indicating the highest conservation (no sequences diverge at the indicated position) and plus (+) indicating the lowest conservation (10 or more sequences diverge at the indicated position). Asterisks represent sites of mutations made in *S. cerevisiae* pol η for this study. The genes listed are: *Homo sapiens* pol η, NP_006493; *Mus musculus* pol η, NP_109640; *Drosophila melanogaster* pol η, AAF51794; *Cryptosporidium muris* pol η, XP_002142930; *Ciona intestinalis* pol η, XP_002128588; *Saccharomyces cerevisiae* pol η, EDN60746. (B–E) In a *rad30* null background (RWY15), effect on UV sensitivity of mutant pol η 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.

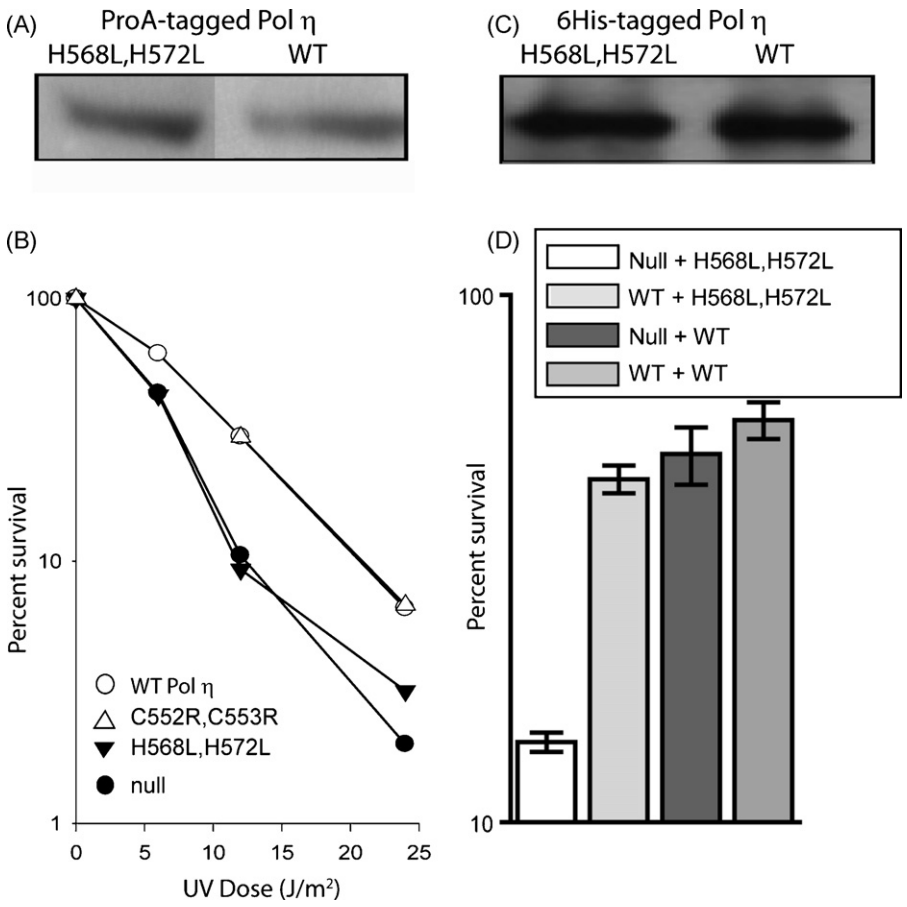

Fig. 2. Characterization of the pol η H568L,H572L mutant. (A) Expression of ProA-tagged H568L,H572L double mutant and wildtype pol η proteins. Immunoblot (peroxidase anti-peroxidase) showing expression of the -TEV-ProA-His tagged H568L,H572L mutant pol η, left lane, compared with the wildtype, right lane. Equal amounts of total protein were loaded in each lane. (B) Phenotypes caused by expression of 6His-tagged wildtype and mutant pol η proteins in *rad5rad30* background (C552R,C553R and WT overlap). (C) Expression of 6His-tagged H568L,H572L double mutant and wildtype pol η 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 η in *rad30* null (black) or wildtype background (dark gray).

In order to probe the binding of ubiquitin and the yeast UBZ domain, unlabeled ubiquitin was titrated into a 0.4 mM sample of ^{15}N-labeled pol η UBZ domain. The reverse titration was also performed, in which unlabeled pol η UBZ domain was titrated into a 0.4 mM sample of ^{15}N-labeled ubiquitin.

To test the effects of EDTA on the human and yeast UBZ/ubiquitin interaction, a 15 N HSQC was obtained on a 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 [64] and analyzed with XEASY/CARA [65].

3. Results

3.1. UBZ motif sequence conservation among pol η homologs

The non-canonical zinc finger of the UBZ motif in *S. cerevisiae* pol η 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 η 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 η, we examined the protein sequences of 79 unique pol η 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 quinquefacietus*). Alignment of these 60 putative UBZ sequences (Supplementary data Fig. S1), summarized in Fig. 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 η. Other significant departures from the consensus sequence, shown in Fig. 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 η homologs [25,31,60].

3.2. Mutations affecting the UBZ domain of pol η in S. cerevisiae

To examine the effects of *S. cerevisiae* pol η's unusual UBZ sequence on polymerase η function, we initially made two mutants of *rad30* (encoding pol η 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 wildtype to killing by UV radiation, expression of

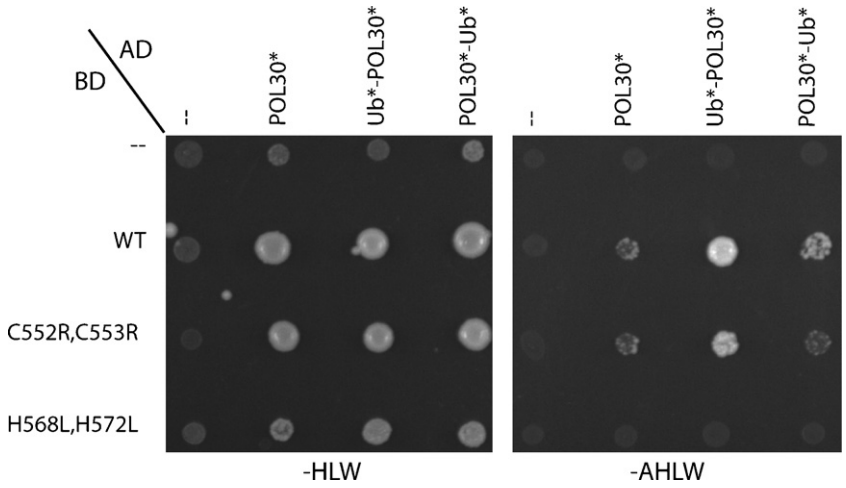

Fig. 3. Interaction of mutant and wildtype pol η with ubiquitin-PCNA by yeast two-hybrid analysis. Mutant (H568L,H572L or C552R,C553R) and wildtype forms of pol η were expressed as fusions to the Gal4 DNA-binding domain (BD), while POL30*, ubiquitin*-POL30*, and POL30*-ubiquitin* fusions were expressed as fusions to the Gal4 activation domain (AD). The presence of the constructs was confirmed by growth on selective medium (-LW, not shown). Growth on plates lacking histidine (-HLW) selects positive interactions, and stronger interactions also allow growth on plates lacking both histidine and adenine (-AHLW). Vectors expressing only Gal4 BD or AD were used as negative controls.

the C552R,C553R mutant confers wildtype 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 η (Supplementary Table 1 and Supplementary data Figs. S2 and S3), including two other mutations that affect the UBZ outside of the C2H2 motif (Fig. 1D and E). 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 η using either of two different epitope-tagged versions of the protein. As shown in Fig. 2, the abundance of the mutant pol η in whole cell extracts is equivalent to that of the wildtype 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 wildtype strain background. As shown in Fig. 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 η 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 η UBZ domain forming the outward face of the α-helix are primarily involved in ubiquitin binding [31]. If the structure of the UBZ domain of *S. cerevisiae* pol η 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 D) 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 [38,60]. As shown in Fig. 3, the H568L,H572L mutation significantly weakens pol η'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 [66] was used to compare the chromatin association pattern of the H568L,H572L mutant with that of wildtype pol η (Supplementary data Fig. S5). While differences were observed between wildtype and mutant pol η proteins, they were difficult to interpret, as this assay did not detect changes in pol η 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 η results directly from the mutant UBZ's reduced binding for ubiquitin. Taken together with the observations that the C552R,C553R mutant pol η (Fig. 3) interacts normally with ubiquitin, the data support the model that the ubiquitin binding activity of the UBZ domain of *S. cerevisiae* pol η is independent of C2H2-mediated zinc coordination.

3.4. The S. cerevisiae pol η 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 η is a "zincless finger," a domain similar in structure and function to a zinc finger, but which does not coordinate a zinc ion [67–69]. To assay zinc binding, the UBZ domains from both *S. cerevisiae* pol η

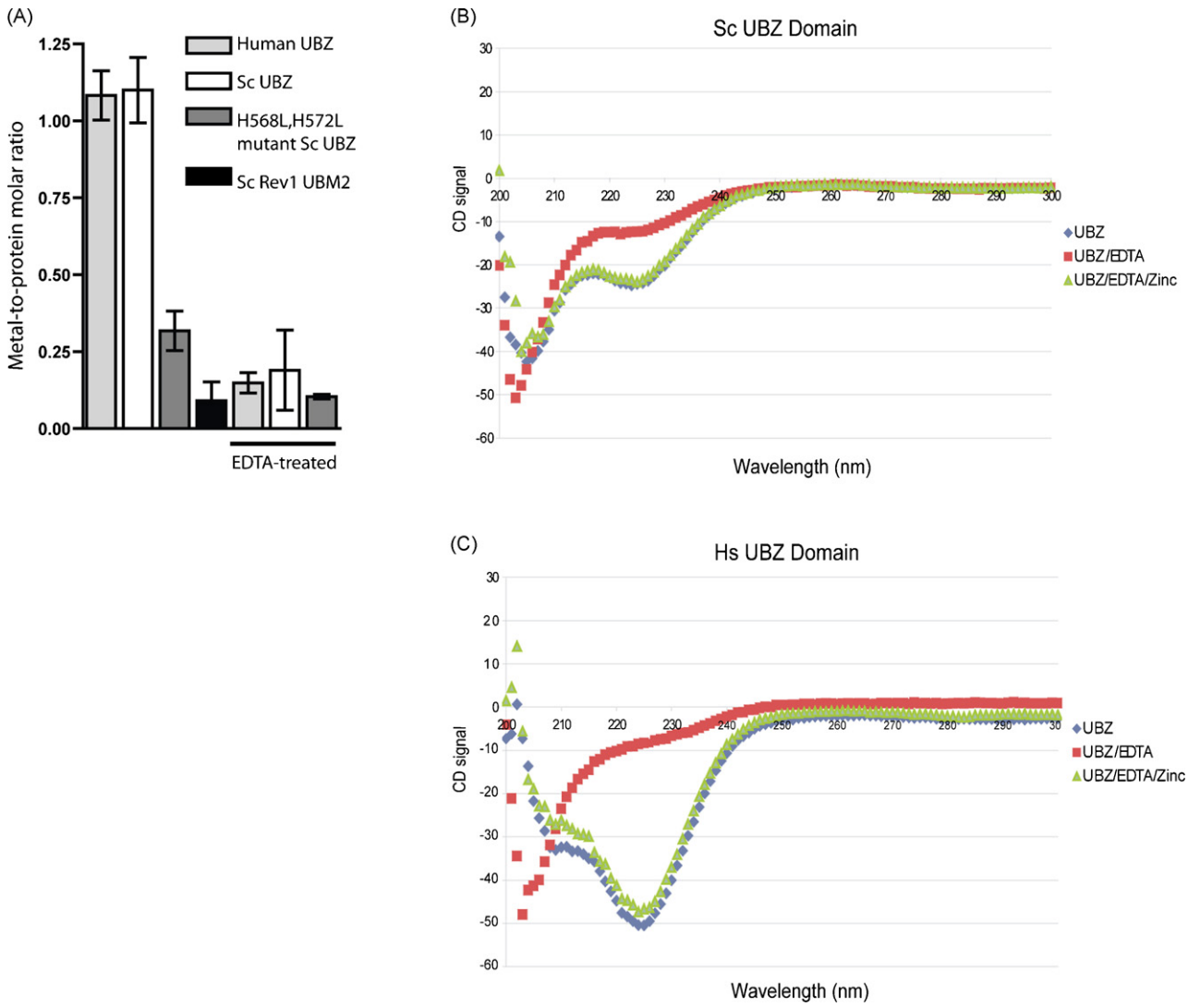

Fig. 4. UBZ binds zinc. (A) PAR colorimetric assay was used to measure the concentration of divalent metal cations in each protein preparations. Metal-to-protein ratios were estimated using the BioRad Protein Assay to determine protein concentrations. (B) CD spectra of human pol η UBZ domain alone (blue), with EDTA (red), and with zinc sulfate (green). (C) CD spectra of *S. cerevisiae* pol η UBZ domain alone (blue), with EDTA (red), and with zinc sulfate (green). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

(residues 538–609) and human pol η (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 Fig. 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 wildtype UBZ domain of *S. cerevisiae* pol η, 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 Fig. 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 η can coordinate a zinc ion in a manner that promotes the folding of the domain. Thus, the non-canonical UBZ of *S. cerevisiae* pol η 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 η is zinc-dependent, an assumption that underlay the proposal that the UBZ domain has an additional, zinc-independent function [70]. To address this issue,

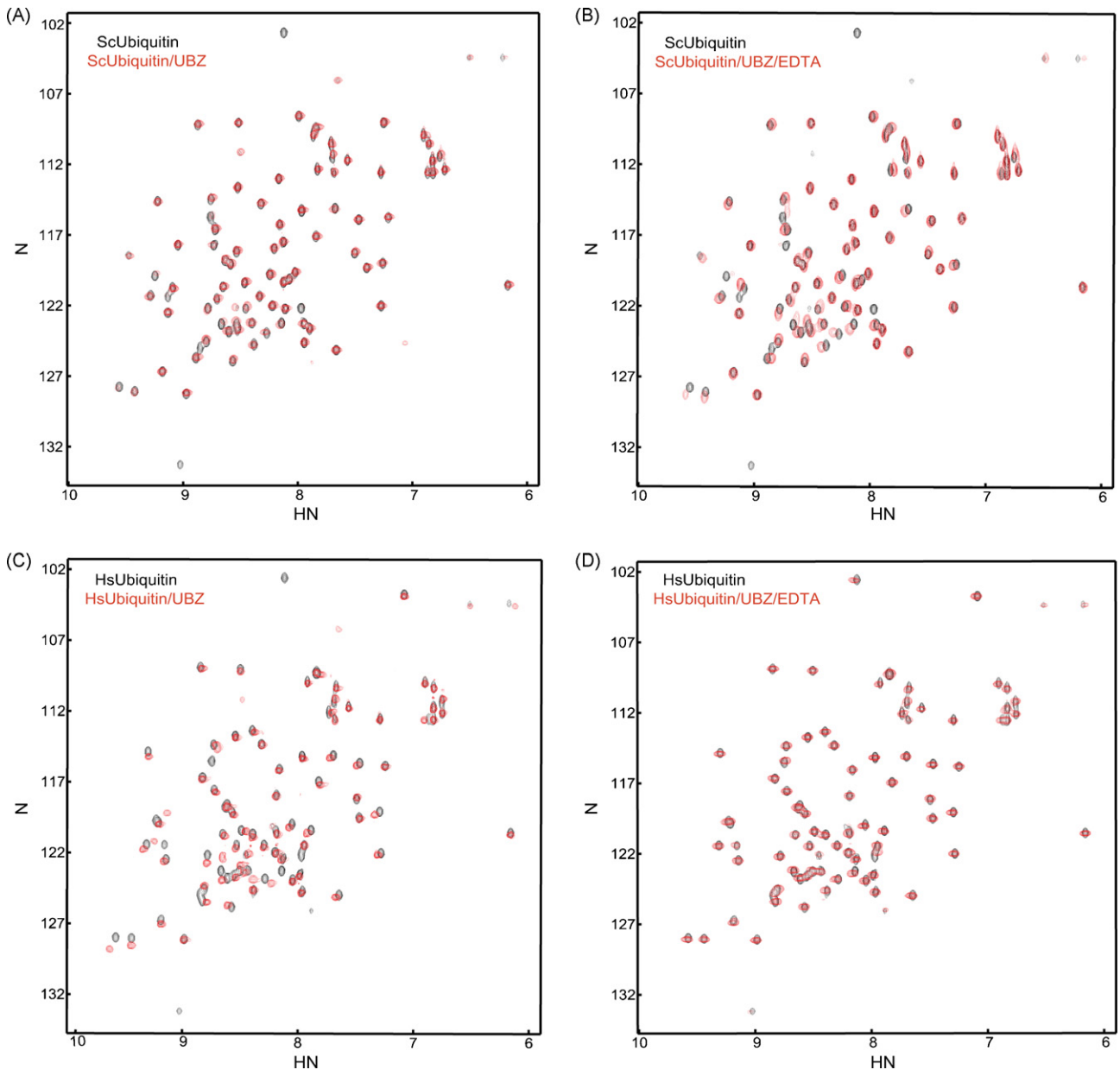

Fig. 5. Effect of EDTA on *S. cerevisiae* and human UBZ/ubiquitin interaction. (A) HSQC spectrum of ^{15}N-labeled *S. cerevisiae* ubiquitin alone (black) and with *S. cerevisiae* UBZ domain (red). (B) HSQC spectrum of ^{15}N-labeled *S. cerevisiae* ubiquitin alone (black) and with *S. cerevisiae* UBZ domain (red) in the presence of EDTA. (C), HSQC spectrum of ^{15}N-labeled human ubiquitin alone (black) and with human UBZ domain (red). (D) HSQC spectrum of ^{15}N-labeled human ubiquitin alone (black) and with human UBZ domain (red) in the presence of EDTA. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

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}H–^{15}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 η and *S. cerevisiae* ubiquitin in the presence of zinc. ^{1}H–^{15}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 (Fig. 5), and the majority of them are located on the same highly conserved, hydrophobic, concave surface of ubiquitin (centered on residue I44) that interacts with the UBZ domain of human pol η [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}H–^{15}N HSQC spectra were obtained for ^{15}N-labeled ubiquitin in the presence and absence of the UBZ domain. As shown in Fig. 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 Fig. 5D, the resonances of human ubiquitin returned to the original position, indicating that chelation of zinc

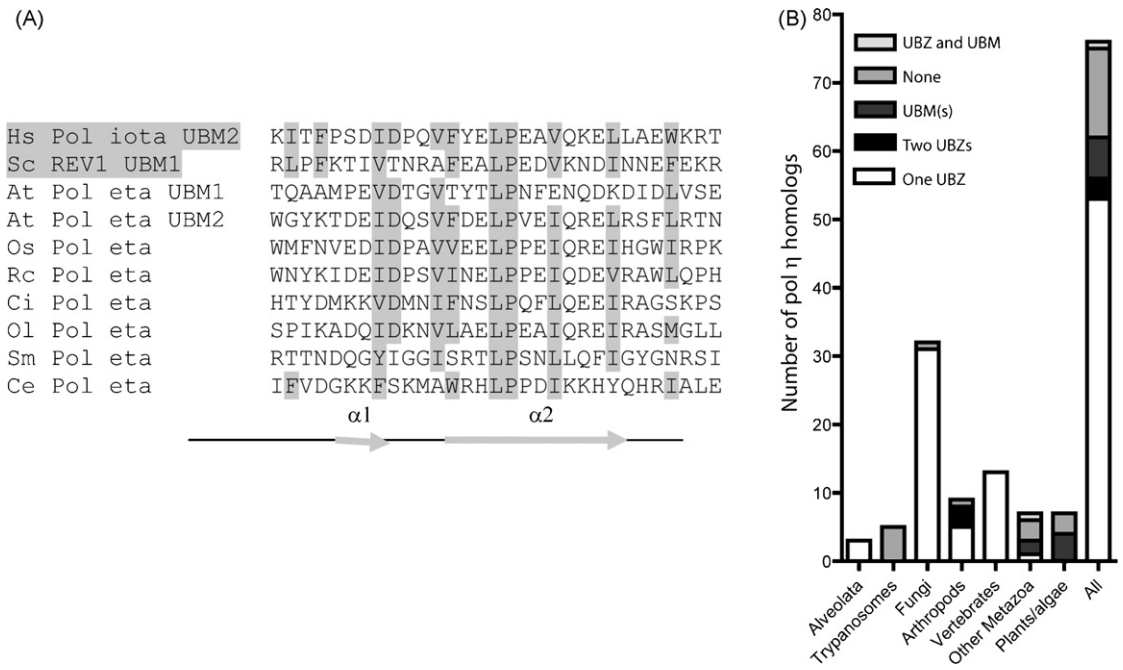

Fig. 6. The putative ubiquitin-binding domains of pol η homologs. (A) Sequence alignment of putative UBMs from pol η homologs, compared with two known UBMs. Conserved residues are highlighted. The genes listed are: *Homo sapiens* (Hs) pol ι, AF245438; *Saccharomyces cerevisiae* (Sc) Rev1, NP_014991; *Arabidopsis thaliana* (At) pol η CAC94893; *Oryza sativa* (Os) pol η, BAD87579; *Ricinus communis* (Rc) pol η, XP_00252815; *Ciona intestinalis* (Ci) pol η, XP_002128588; *Ostreococcus lucimarinus* (Ol) pol η, ABO98773; *Schistosoma mansoni* (Cm) pol η, XP_002574021; *Caenorhabditis elegans* (Ce) pol η, BAE7270. The secondary structural elements for the UBM2 of human pol ι are indicated below the alignment [63]. The UBMs of *O.sativa* and *A. thaliana* were previously recognized [71]. It should be noted that there may be two tandem UBMs in the pol η 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 η homologs. Pol η homologs were divided among five 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.

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 η binds to ubiquitin in a zinc-independent manner.

3.6. Ubiquitin-binding domains in pol η homologs from different species

To gain an evolutionary perspective on the importance and variations of ubiquitin binding in pol η homologs, we further analyzed the sequences of the pol η 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 η homologs [25,31,60]. Interestingly, the *C. intestinalis* pol η 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 ι [25].

Putative UBM motifs, shown in Fig. 6A, are also observed in pol η homologs from six additional species, all of which lack recognized UBZ motifs (*Arabidopsis thaliana* [71], *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 [63]. Even in those pol η homologs with more canonical UBM sequences, the UBM may participate in a different mechanism of pol η regulation from that mediated by the UBZ domain, since the UBM has a slightly different interaction with ubiquitin than that of the UBZ domain [63].

In 13 of the pol η 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 η homologs (under 550 amino acids), possibly indicating that some predicted sequences are incomplete. Even if predicted pol η sequences are correct in these species, other unrecognized ubiquitin-binding motifs may be present. Alternatively, an additional protein may mediate the interaction, or ubiquitin may not play the same role in pol η 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 η homologs is non-random, as shown in Fig. 6B. Among the pol η 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 5 trypanosome pol η sequences, nor were they found in any of the seven sequences from photosynthetic organisms. The latter observation suggests the possibility that pol η 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 *S. cerevisiae* polymerase η. Characterization of pol η mutants confirmed the importance of the UBZ motif to pol η, 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 η 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 η is able to interact with ubiquitin even in the absence of a zinc ion. Thus, the UBZ domain of *S. cerevisiae* pol η 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 η [38,54,60,70]. 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 [38,60,70,72]. The differences between the H568A,H572A mutant protein [70] 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 η, 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 [73]. In *S. cerevisiae* pol η, one potential candidate is Q556. An alternative possibility is that the zinc ion is coordinated by three amino acids and a water molecule [74]. 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 μM) similar to that reported for the wildtype (0.3 μM) [75]. However, the conservation of the cysteines in all of the other pol η 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 η homologs.

What is it about the UBZ of *S. cerevisiae* pol η 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 Fig. 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 [70] that the yeast UBZ's zinc-independent function is due to a zinc-independent α-helix. The results from the CD experiments (Fig. 4) showed that the UBZ domain from *S. cerevisiae* pol η becomes substantially less structured upon removal of zinc, though the α-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' [76]. Like the UBZ domain, UmuD' also mediates the interaction between TLS polymerases and the replication machinery [77,78].

In three distinct ways, our findings emphasize the functional importance of the UBZ's interaction with ubiquitin for pol η 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 η-mediated TLS. Second, the observation of ubiquitin binding by *S. cerevisiae* pol η's UBZ domain in the presence of EDTA demonstrates that ubiquitin binding is a zinc-independent function of *S. cerevisiae* pol η'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 η homologs from diverse origins suggests that pol η's interaction with ubiquitin is important for its regulation in many species.

In those pol η 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 η is to enhance the interaction with (ubiquitinated) PCNA, it may be that in such species, pol η'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 η is active. Interestingly, the pol η homolog from *Trypanosoma cruzi* [79], 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 η proteins [80]. Significant differences have been observed in PIP-PCNA complex structures among the human Y family polymerases κ, η and ι [80]; variation in the architecture and affinity of PCNA binding may also exist among pol η homologs.

To conclude, we present an in-depth characterization of the structure and function of the non-canonical UBZ motif of *S. cerevisiae* polymerase η. 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 η's UBZ motif in *S. cerevisiae* involves ubiquitin binding and does not fundamentally differ from the function of more canonical UBZ domains of pol η homologs from other species.

Conflict of interest

The authors declare that there are no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.dnarep.2010.08.001.

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