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Ubiquitin mediates the physical and functional interaction between human DNA polymerases η and ι

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ABSTRACT

Human DNA polymerases η and ι are best characterized for their ability to facilitate translesion DNA synthesis (TLS). Both polymerases (pols) co-localize in 'replication factories' *in vivo* after cells are exposed to ultraviolet light and this co-localization is mediated through a physical interaction between the two TLS pols. We have mapped the polη-ι interacting region to their respective ubiquitin-binding domains (UBZ in polη and UBM1 and UBM2 in polι), and demonstrate that ubiquitination of either TLS polymerase is a prerequisite for their physical and functional interaction. Importantly, while monoubiquitination of polη precludes its ability to interact with proliferating cell nuclear antigen (PCNA), it enhances its interaction with polι. Furthermore, a polι-ubiquitin chimera interacts avidly with both polη and PCNA. Thus, the ubiquitination status of polη, or polι plays a key regulatory function in controlling the protein partners with which each polymerase interacts, and in doing so, determines the efficiency of targeting the respective polymerase to stalled replication forks where they facilitate TLS.

INTRODUCTION

Most types of DNA damage block the progression of a replication fork. To circumvent these blocks, cells recruit specialized DNA polymerases to facilitate translesion DNA synthesis (TLS) past the damaged DNA, thus allowing completion of genome duplication (1–3). While many human DNA polymerases (pols) have some capacity to promote TLS (4), the most proficient TLS enzymes belong to the Y-family of DNA polymerases (5). Polη, the best-characterized Y-family DNA polymerase, is defective in humans with the sun-sensitive cancer-prone *xeroderma pigmentosum* variant (XP-V) syndrome (6,7). Polη can replicate efficiently and with high accuracy through ultraviolet (UV)-induced cyclobutane pyrimidine dimers (CPDs) (8–10). Polη-deficient XP-V cells manifest high levels of cellular mutagenesis after exposure to UV radiation (11), indicating that polη normally prevents UV-induced mutations and cancer. It has been postulated that in the absence of a functional polη, other low-fidelity pols facilitate TLS of CPDs with mutagenic consequences (2). The most likely candidates are Y-family pols ι and κ and the B-family polζ (12,13).

Structural studies (10,14–19) have shown that compared with replicative polymerases, TLS polymerases share a more open catalytic site. As a consequence, most Y-family polymerases display low-fidelity DNA synthesis when copying undamaged DNA (20,21). The regulation of their activities in a living cell is, therefore, critical to maintain genomic stability.

The current working hypothesis postulates that when the cell's replication machinery is stalled at damaged DNA site, the replicative polymerase is replaced by a TLS polymerase in a process called 'polymerase switching' (5,22). In eukaryotic cells, such replacement is mediated by the proliferating cell nuclear antigen (PCNA)

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processivity factor, which is recruited to the stalled fork. All four human Y-family polymerases (polη, polι, polκ and Rev1) have been shown to interact directly with PCNA (23–27). PCNA is also subject to a DNA damage-dependent monoubiquitination event that helps targeting of polη to the stalled replication forks (28,29). PCNA monoubiquitination occurs at K164 via Rad6, a E2-ubiquitin-conjugating enzyme and Rad18, a E3-ubiquitin ligase (30). Polη has a higher affinity for monoubiquitinated PCNA than unmodified PCNA suggesting that ubiquitination of PCNA helps target polη to stalled replication forks (28,29). The non-covalent association of polη with ubiquitin (and monoubiquitinated PCNA) is mediated via its Ubiquitin-binding-zinc-finger (UBZ) motif (31,32). Mutations within the UBZ block the interaction with ubiquitin and reduce the ability of polη to accumulate into damage-induced foci, or so-called 'replication factories' (31). Like polη, polι, polκ and Rev1 also interact with ubiquitin (26,31,33). Polι and Rev1, however, contain structurally different ubiquitin-binding motifs termed 'UBMs' (26,31,33,34). Similar to polη UBZ mutants, mutations in the polι or Rev1 UBMs not only block the interaction with ubiquitin but also inhibit the accumulation of the TLS polymerases into replication factories (26,31,33).

In addition to a non-covalent interaction with ubiquitin through their respective UBZ and UBMs, both polη and polι can be covalently monoubiquitinated at specific residues in the respective enzyme (31). The sites of ubiquitination in polι are currently unknown. However, recent studies have indicated that polη can be monoubiquitinated at four separate lysine residues near its C-terminus (K682, K686, K694 and K709) (35). Monoubiquitination of polη plays an important regulatory function, as it precludes an interaction with PCNA (35). Interestingly, monoubiquitinated polη is de-ubiquitinated upon DNA damage, thereby allowing an interaction with PCNA at stalled replication forks, when the TLS activity of polη is most needed (35).

Polη and polι have also been shown previously to physically interact and co-localize into replication factories at sites of DNA damage (36), although the kinetics with which the two polymerases reside in these replication factories differs (37). The region within polη and polι responsible for the physical interaction has been loosely mapped to their respective ~200 C-terminal residues (25,36). We were interested in mapping the sites of the polη–ι interaction more precisely, so as to potentially begin to elucidate the structural basis for the interaction, as has recently been reported for the polη-Rev1 interface (38,39). We report here that these interactions occur via the respective UBZ and UBMs of polη and polι. Rather than a direct UBZ–UBM interaction, we present evidence that the polη–ι interaction is actually mediated through ubiquitin. Thus, the monoubiquitination status of pols η and ι is likely to determine which protein partner(s) the respective polymerase interacts with and how efficiently it is recruited to replication factories at sites of DNA damage where they facilitate TLS.

MATERIALS AND METHODS

Saccharomyces cerevisiae two-hybrid vectors and interaction analysis

Two-hybrid vectors carrying full-length human polι, polη, PCNA or ubiquitin, were described earlier (25,33,36). Vectors expressing variants of human polι or polη were either generated by site-directed mutagenesis, or gene synthesis of the mutant allele as a service provided by Genscript Inc. (Piscataway, NJ, USA) and subsequently sub-cloned into the original expression vector (Supplementary Table S1). Interactions between proteins were demonstrated *in vivo* using the *Saccharomyces cerevisiae* two-hybrid Matchmaker III system (Clontech, Palo Alto, CA, USA). pACT2, pGADT7, pGBKT7 and various derivatives were co-transformed into the *S. cerevisiae* strain AH109. Transformants were selected on DOBA-Trp-Leu plates. Colonies were subsequently replica plated on DOBA-Trp-Leu-His-Ade plates, to confirm the activation of the reporter genes.

Escherichia coli expression vectors and protein purification

Full-length His-tagged human polι was expressed in the *Escherichia coli* strain RW644 (40) from plasmid pJM868 (41). Plasmids expressing polι variants F507S (pJRM97), P511R (pJRM102), P680A (pJRM86) and P692R (pJRM108) were generated by sub-cloning the desired synthesized allele (Genscript) into pJM868 (Supplementary Table S2). Wild-type His-polι and mutant variants were purified on Ni^{2+}-charged nickel-nitrilotriacetic acid His-Bind Resin (Qiagen, Valencia, CA, USA) as recommended by the manufacturer. The eluate containing polι was dialyzed in buffer A (20 mM sodium phosphate pH 7.3, 10 mM sodium chloride, 10% glycerol, 10 mM 2-mercaptoethanol) and applied to an HP Q-Sepharose column (GE Healthcare, Piscataway, NJ, USA). Polι was eluted in a step gradient of NaCl and the polι-containing fractions were aliquoted and stored at −80°C.

Fluorescent vectors, transfection and foci formation assay

The fluorescent construct carrying full-length wild-type polι (peCFP-C1-polι) was described earlier (36). Derivatives carrying F507S (pJRM23), or P511R (pMGB9) in polι, or polι-Ub (pJRM128) were generated by sub-cloning the desired synthesized allele (Genscript) into peCFP-C1-polι wt (Supplementary Table S3). The fluorescent constructs were transfected into transformed MRC5 fibroblasts (TurboFectin 8.0) according to the manufacturer's protocol (Origene, Rockville, MD, USA). Twenty hours after transfection, cells were irradiated at 7 J/m^2 and incubated for a further 6 h. Fixation of cells was carried out as described earlier (36). Fluorescence images of cell nuclei were acquired on a Zeiss Axiophot2 microscope (Carl Zeiss) equipped with an Orca ER CCD camera (Hamamatsu) using Simple PCI software. Images were captured by excitation at 436 nm and detection of CFP emission at 480 nm. At least 200 nuclei were analyzed for each cell line and treatment in 2−5 independent experiments.

In vitro transcription/translation of proteins

In vitro transcription/translation of polη, polι (wild type and variants), PCNA or ubiquitin, was performed using a TNT-Quick Coupled Transcription/Translation System (Promega, Madison, WI, USA) according to the manufacturer's instructions. The expression vectors encoding polη (pAVR65), PCNA (pAVR18), ubiquitin (pBP129), polι wt (pAR110), polι_F507S (pNEO155), polι_P511R (pJRM65), polι_P680A (pJRM64) were added separately to the reaction mixtures and incubated for 90 min at 30°C in the presence of [^{35}S] methionine (Perkin Elmer, Waltham, MA, USA). Reaction products were analyzed directly by SDS–PAGE and used in the far-Western assay.

Far-Western analysis

Purified His-tagged polι proteins or K63-linked Ub-chains were separated by 4–20% SDS–PAGE (Invitrogen, Carlsbad, CA, USA) and transferred to nitrocellulose membranes (Invitrogen). Membranes with His-polι proteins were incubated at 4°C overnight with ^{35}S-labeled polη, PCNA or ubiquitin and membranes with K63-linked ubiquitin chains with ^{35}S-labeled polι. Following incubation, membranes were washed three times at 4°C, dried briefly and scanned with a FujiFilm FLA-5100 phosphoimager. The amount of loaded protein was verified by staining membranes with Ponceau S (Sigma, St Louis, MO, USA).

Model building

The images of the murine UBM1 and human UBM2 structures in complex with ubiquitin were generated using Pymol (The PyMOL Molecular Graphics System, Schrödinger, LLC) with PDB files 2KWV and 2KHW, respectively.

FLAG pull-down assay

Mammalian expressing constructs carrying full-length wild-type polι (pJRM46) or polι-Ub chimera (pJRM140) and polη (pJRM56) were generated by sub-cloning the desired synthesized allele (Genscript) into pCMV6AN-DDK and pCMV6AN-HA vectors, respectively (Origene) (Supplementary Table S3). Constructs were transfected into HEK293T cells using Turbofectin 8.0 according to manufacturer's instructions (Origene). Twenty-four hours after transfection, cells were harvested and lysed. The presence of FLAG- and HA-tagged proteins in cell extracts was verified using Western blot. For the pull-down assay, respective cell extracts were incubated overnight at 4°C with EZview Red ANTI-FLAG M2 Affinity Gel (Sigma-Aldrich, St. Louis, MO, USA), washed three times and analyzed directly by SDS–PAGE and Western blot.

RESULTS

Identification of a region in polη involved in binding polι

We previously reported that human polη and polι physically interact through their C-termini (36). In particular, the last 230 amino acids of polη are sufficient to interact with polι (25). To more precisely determine the amino acid residues involved in the polη–polι interaction, we first used a yeast two-hybrid approach. As shown in Figure 1, only cells expressing the polη construct with a deletion between residues S587-L641 failed to grow on selective medium. Interestingly, this deletion contains the N-terminal part of the polη UBZ domain (Figure 2A), consistent with the idea that an intact UBZ domain is required for the polη and polι interaction.

To investigate this hypothesis, we then generated base substitutions in the UBZ domain of full-length polη and assayed their ability to interact with polι in the two-hybrid assay. Polη variants with a double C635A/C638A substitution, or individual C635A, C638A or D652A substitutions eliminated the interaction with polι. The inability of these mutants to interact with polι is specific, as similar to wild-type polη, they retained their ability to interact with PCNA (36) (Figure 2B). In contrast, and as reported earlier (33), the polη H654A UBZ mutant lost its ability to interact with ubiquitin, but still retained its ability to interact with polι.

Identification of regions in polι that interact with polη

Having identified a region in polη that appears necessary for the interaction with polι, we were interested in identifying the reciprocal region in polι that interacts with polη. As wild-type polι cannot interact with the C635A/C638A UBZ polη mutant, we hypothesized that if we were able to identify a suppressor mutation in polι that gained an ability to interact with the UBZ mutant, then the polι 'suppressor' would most likely be a compensatory mutation at, or close to, the polι–polη interface. To identify such a suppressor, we randomly mutagenized the activating domain plasmid expressing full-length polι and screened for colonies in the two-hybrid assay that were able to interact with the C635A/C638A polη mutant. Several interacting clones were identified and one carrying a single nucleotide mutation that leads to a P692L substitution in polι was chosen for further study (Supplementary material for experimental details). The polι P692L mutant is fully functional and interacts with the C635A/C638A double mutant and the C635A, C638A and D652A single polη UBZ mutants, as well as wild-type polη (Figure 2B).

Proline 692 is located in the center of polι's UBM2 motif (31), raising the intriguing possibility that polη and polι might interact through their respective UBZ and UBM2 motifs. To test this hypothesis and potentially identify additional residues in polι's UBM2 involved in the polη–polι interaction, we made additional substitutions at several highly conserved residues in polι's UBM2 motif (Figure 3B) and assayed their ability to interact with polη in addition to PCNA, or ubiquitin, as controls (Figure 3C). Growth of the yeast strains was determined after 4 and 6 days of incubation at 30°C and compared with the growth of the wild-type polι construct to give a qualitative idea of the protein–protein interactions. Most mutants appear to be correctly folded, since like wild-type polι, they gave a positive interaction with PCNA after 4–6 days of growth (Figure 3C). The

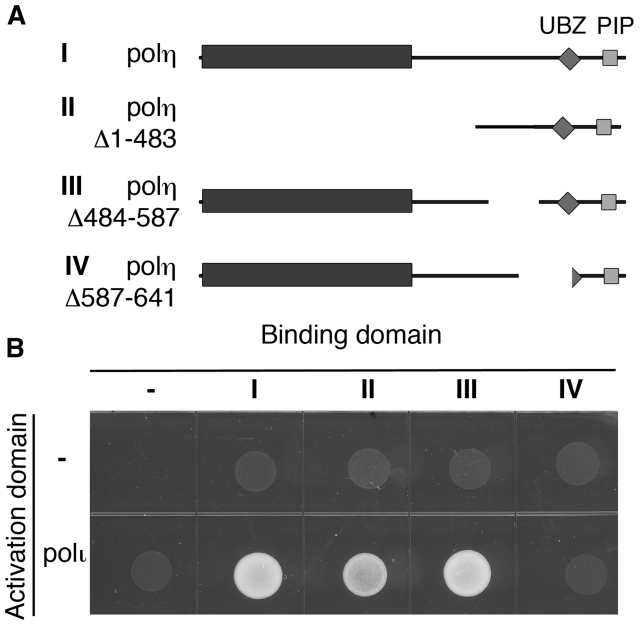

Figure 1. Mapping the region in polη that interacts with polι using a yeast two-hybrid assay. (**A**) Cartoon of polη deletion constructs. The dark gray rectangle is the catalytic core of polη, the UBZ motif is indicated as a gray diamond and the PCNA-interacting motif (PIP-box) is indicated as a light gray box. (**B**) Yeast two-hybrid assay showing the interaction between full-length polι and deletion alleles of polη. Deletion mapping reveals that the interaction with polι is localized to a region containing the UBZ domain of polη. *Saccharomyces cerevisiae* strain AH109 was co-transformed with pACT2-polι wild type (pAR116) and (I) pGBKT7-polη wild type (pAVR65), or (II) pGBKT7-polη_Δ1-483 (pAVR45), or (III) pGBKT7-polη_Δ484-587 (pAVR51), or (IV) pGBKT7-polη_Δ587-641(pAVR52). Images were taken after 4 days of incubation at 30°C.

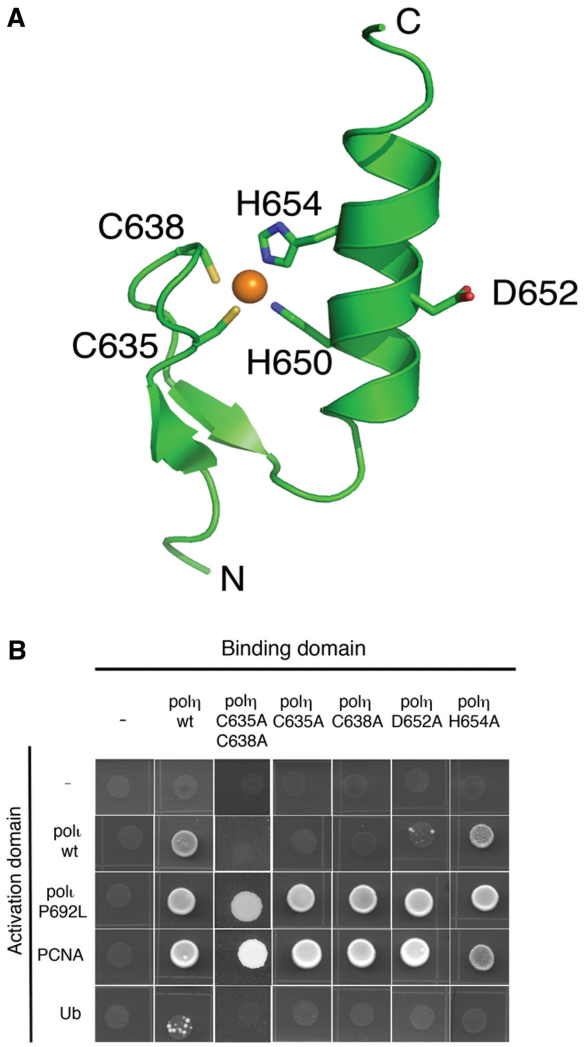

Figure 2. Analysis of the polη UBZ residues that are responsible for the interaction with polι. (**A**) Structure of the human polη UBZ domain (PDB: 2I5O) with key residues used in the analysis are highlighted. Zn^{2+} is indicated as a bronze sphere. (**B**) Yeast two-hybrid assay showing the effect of mutating polη UBZ residues on their ability to interact with polι. Wild-type polι is unable to interact with polη C635A, C638A and D652A substitutions, whereas the polι P692L substitution facilitates an interaction with the various UBZ mutants. Images were taken after 4 days of incubation at 30°C.

main exception was the V687A/F688A construct, which interacted poorly with PCNA, even after 6 days of incubation. P680A also appeared to have a somewhat reduced ability to interact with PCNA, as it took 6 days to observe good growth with this mutant, compared with 4 days for the wild type and other mutants. As expected, given their location in the UBM2 motif, many of the polι substitutions disrupted the ability of the mutant to physically interact with ubiquitin (Figure 3C). Interestingly, and in support of the notion that the UBM2 motif is the region in polι that interacts with polη, many of the UBM2 mutants that had reduced or no interaction with ubiquitin were also unable to interact with polη (Figure 3C), including P680A, I683A/D684A, L691A/P692A, Q696A and E698A. Our data, therefore, identify polι UBM2 as a region within polι that interacts with both ubiquitin and polη. However, these interactions are not necessarily dependent upon each other since in a previous study (33), we identified P692R in UBM2 as a substitution that selectively disrupts polι's interaction with ubiquitin, whilst retaining its ability to interact with polη [Figure 3C; (33)].

Polι has two UBMs (31) and given that UBM2 appears to be important for polι to interact with both ubiquitin and polη, we wanted to determine what effect, if any, substitutions in polι's UBM1 (Figure 3A) might have on the ability of the protein to interact with ubiquitin and polη. We focused on two substitutions: P511R, which would be analogous to the ubiquitin-binding-deficient, but polη-binding-proficient P692R mutant in UBM2, and F507S, as this is a naturally occurring single nucleotide polymorphism (SNP) found in ~3% of humans (http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs=321 8786). Interestingly, both UBM1 substitutions interacted with ubiquitin, yet both showed a reduced ability to interact with polη (Figure 3C).

Far-Western analysis of polι mutants

To confirm the altered protein–protein interactions observed in the yeast two-hybrid assay, we performed 'far-Western' analysis of the interactions (Figure 4). We first determined the ability of the polι mutants to interact

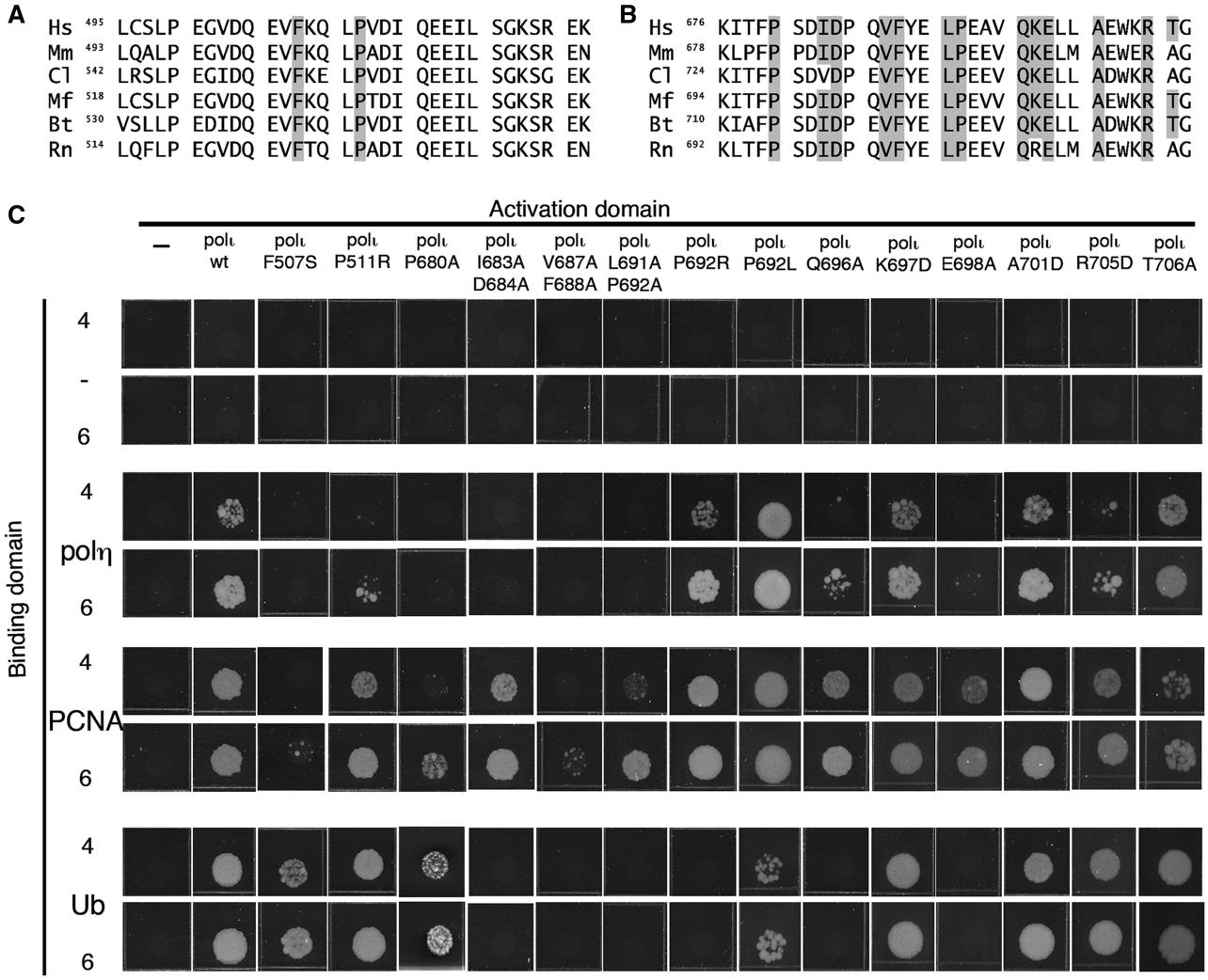

Figure 3. Analysis of polι UBM1 and UBM2 residues that are responsible for the interaction with polη. (**A**) Sequence alignment of UBM1 and (**B**) UBM2. Conserved residues mutated in the analysis are shaded gray. The aligned polι proteins are from the following mammals: Hs, *Homo sapiens*; Mm, *Mus musculus*; Cl, *Canis lupus*; Mf, *Macaca fascicularis*; Bt, *Bos taurus*; Rn, *Rattus norvegicus*. (**C**) Yeast two-hybrid analysis of the interactions between polι UBM1 and UBM2 mutants and polη, PCNA and ubiquitin (Ub). *Saccharomyces cerevisiae* strain AH109 was transformed separately with the GAL4-AD expression vectors pACT2 (control), pACT2-polι wild type (pAR116), pACT2-polι carrying various point mutations in UBM1 or UBM2 as indicated in combination with each one of the following GAL4-BD expression vectors: pGBKT7 and pGBKT7-polη_wild type (pAVR65), pGBKT7-PCNA (pAVR18) and pGBKT7-Ub (pBP129) as indicated. Several colonies from each transformation were grown overnight at 30°C in selective medium, and a sample was spotted on to a DOBA-Trp-Leu-His-Ade plate and incubated at 30°C for 6 days. Four and six represent days of growth at 30°C.

with polη (Figure 4A). In general, the results were consistent with the yeast two-hybrid analysis, with polι F507S, P511R and P680A all exhibiting a reduced ability to interact with polη (~25–45% of the wild-type protein).

We then compared the mutant polι's ability to bind to PCNA (Figure 4B). Again, the data confirmed the two-hybrid analysis. P511R, which exhibited good growth after 4 days of incubation in the two-hybrid assay, also showed a strong interaction with PCNA (similar to wild-type polι). F507S and P680A, which exhibited delayed growth with PCNA in the two-hybrid assay, also interacted less efficiently with PCNA in the far-Western assays (~50–75% of that observed with wild-type polι: Figure 4B).

Finally, we assayed for an interaction with free ubiquitin and K63-linked ubiquitin chains (Figure 4C and D). As expected from the two-hybrid assay, P511R showed a strong interaction with free ubiquitin, whereas P680A, which took longer to reveal an interaction with ubiquitin in the two-hybrid assay, exhibited the weakest interaction with ubiquitin (~40% of wild type). F507S also exhibited a reduced ability to interact with free ubiquitin (~50% of wild-type levels), but nevertheless retained its normal capacity to interact with K63-linked poly-ubiquitin chains (Figure 4D).

Location of polι residues within UBM1 and UBM2 implicated in interacting with polη

The solution structures of human UBM2 (34) and murine UBM1 (42) have previously been determined. The locations of the human polι UBM1 and UBM2 mutants

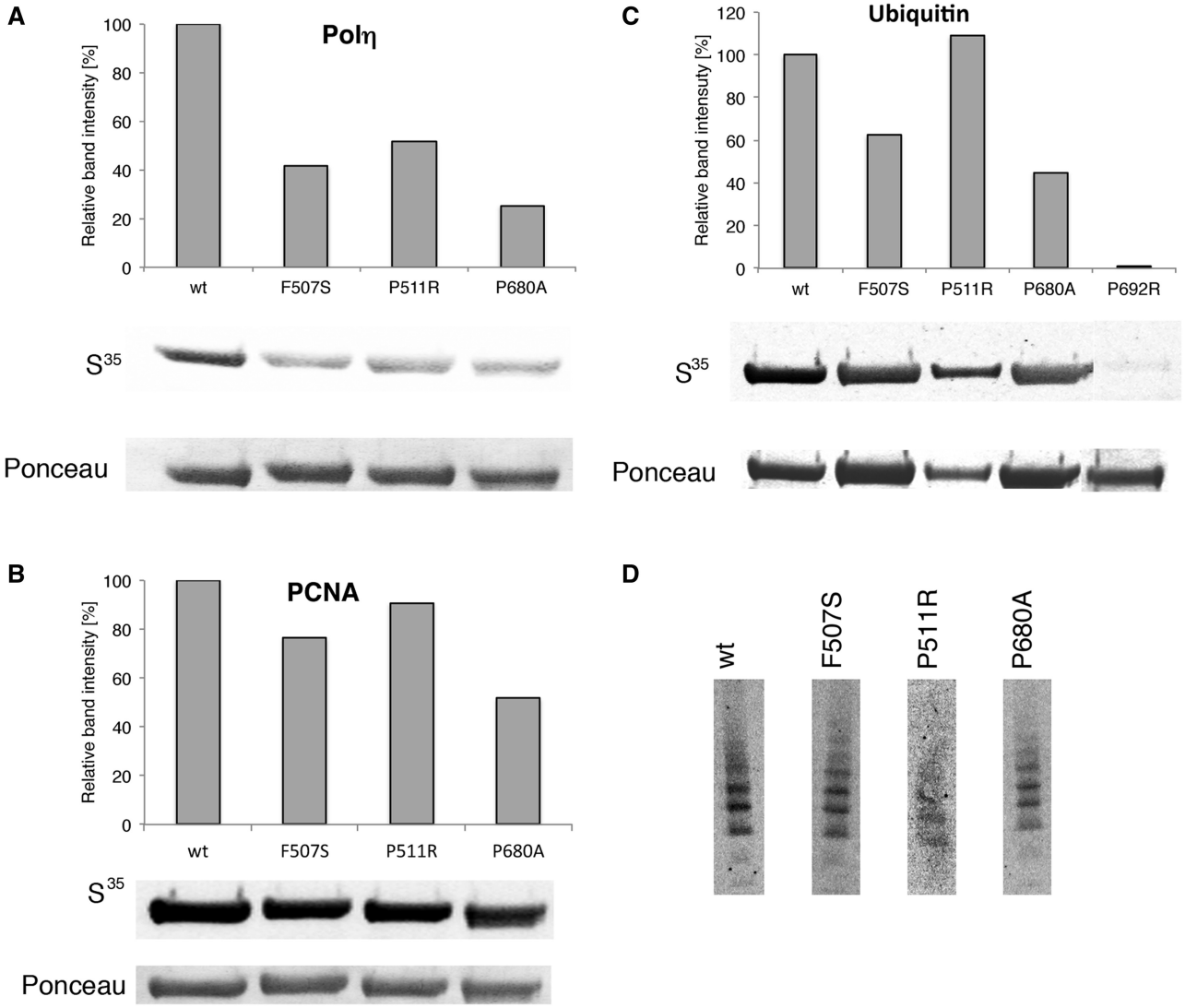

Figure 4. *In vitro* far-Western assay verifying the interactions between polι UBM1 (F507S and P511R) and UBM2 (P680A) substitutions with polη, PCNA, ubiquitin and K63-linked ubiquitin chains. Purified His-tagged wild-type polι and the indicated mutants were separated by SDS–PAGE, transferred to nitrocellulose and incubated with *in vitro* translated ^{35}S-labeled polη (**A**), PCNA (**B**) and ubiquitin (**C**). Densitometric analysis of far-Westerns (top panels) compares the strength of interaction with wild-type polι and mutants with the ^{35}S-labeled proteins; the ^{35}S band intensities (middle panels) were normalized to their respective Ponceau-stained bands (bottom panels). (**D**) Wild-type polι and UBM mutants interact with K63-linked Ub chains; 15 μg of K63-linked ubiquitin chains (Boston Biochem) were separated by SDS–PAGE, transferred onto nitrocellulose and incubated with ^{35}S-labeled wild-type polι or the indicated UBM mutant.

studied here are shown in Figure 5. The two UBM1 mutants (F507S and P511R) are located at the interface between polι and ubiquitin (Figure 5A). From a structural point of view, it is hard to reconcile that these mutants retain their ability to interact with ubiquitin, unless the interaction is mediated through the intact UBM2 motif (see below for further discussion).

The polι UBM2 mutants fall into three classes (Figure 5B). The main class consists of mutants that simultaneously affect binding to polη and ubiquitin. These mutants are colored yellow in Figure 5B and are clustered at the interface between polι and ubiquitin. The second class of UBM2 mutant (K697D, A701D and R705D) retains the ability to interact with both polη and ubiquitin. These residues are colored blue in Figure 5B and are located on the outside surface of the long α-helix 1 of UBM2. The third and final class of UBM2 mutant exhibits split phenotypes/properties. For example, P692R (colored red in Figure 5B) is completely defective in binding ubiquitin, yet has a near normal ability to bind polη [Figures 3C and 4C; (33)]. This observation is also hard to reconcile from a structural point of view, unless the interaction with polη is mediated through the intact UBM1 motif.

Our finding that many mutants in polι UBM2 are simultaneously defective in binding ubiquitin and polη despite possessing an intact UBM1 indicates that the primary binding site for both proteins *in vivo* is the polι UBM2 motif. However, our observation that a single mutation in UBM2 (P692R) blocks the interaction with ubiquitin, but not polη, suggests that polη can also interact with polι via UBM1. This suggestion is supported by the finding that the polι F507S UBM1 mutant is unable to interact with polη

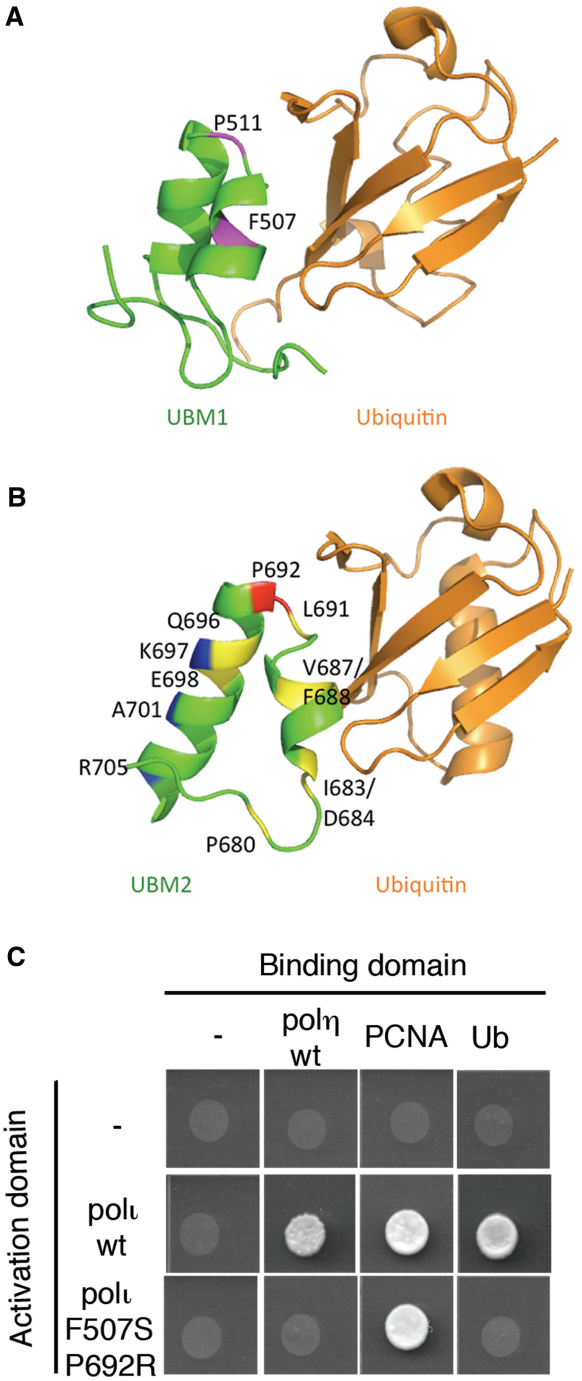

Figure 5. Polι interacts with polη through its UBM domains. Ribbon diagrams show the structure of the UBMs (green) interacting with Ubiquitin (bronze). **(A)** Localization of UBM1 mutants. The model of human UBM1 was generated based upon the closely related murine UBM1 structure (PDB 2KWV). Residues that impair the interaction with polη are highlighted in purple. **(B)** Localization of UBM2 mutants. The human UBM2-ubiquitin structure was generated using PDB 2KHW. Residues that simultaneously disrupt the interaction with ubiquitin and polη are highlighted in yellow. Residues that do not impair the interaction with ubiquitin or polη are highlighted in blue. The P692 residue, which when changed to Arg selectively disrupts the interaction with ubiquitin, is highlighted in red. **(C)** A two-hybrid assay demonstrating that the F507S/P692R UBM1-UBM2 double mutant does not interact with polη or ubiquitin, whilst retaining its ability to interact with PCNA. Yeast strain AH109 was transformed separately with the GAL4-AD expression vectors pACT2, pACT2-polι wild type (pAR116) and pACT2-polι F507S/P692R (pJRM142) in combination with one of the following GAL4-BD expression vectors: pGBKT7, pGBKT7-polη wild type (pAVR65), pGBKT7-PCNA (pAVR18) and pGBKT7-Ub (pBP129) as indicated. Several colonies from each transformation were grown overnight at 30°C in selective medium, and a sample was spotted on to a DOBA-Trp-Leu-His-Ade plate and incubated at 30°C for 6 days.

and leads to the prediction that a double mutant in both UBM1 and UBM2 would be unable to bind ubiquitin or polη. Indeed, as shown in Figure 5C, the polι F507S/P692R (UBM1/UBM2 double mutant) is unable to interact with either protein in the two-hybrid assay, yet exhibits a strong interaction with PCNA.

Reduced accumulation of polι into replication factories in UBM1 mutants unable to interact with polη

It has previously been reported that upon DNA damage, polι accumulates into damage-induced foci (36,37) that are believed to represent subcellular 'replication factories' (5). The number of damaged-induced polι foci drops significantly in polη-deficient XP-V cells, leading to the hypothesis that polη is required to physically target polι into replication factories (36). However, because of their defect in polη, XP-V cells are blocked in S-phase after UV-irradiation (43), and the lack of accumulation of polι into foci might simply result from the indirect consequence of delayed post-replication repair and altered cell cycle signaling, rather than a direct, physical role for polη in targeting polι into replication factories. We tested this hypothesis directly in cells expressing wild-type polη by assaying the ability of polι mutants that are unable to interact with polη to accumulate into replication factories. To do so, we generated eCFP-tagged polι-fusions (36) with single missense mutations in UBM1 (F507S or P511R) as these mutants exhibited a significantly reduced ability to interact with polη, whilst retaining the ability to interact with ubiquitin and compared foci formation to the wild-type eCFP-tagged polι (Figure 6). In these experiments, ~12% of undamaged cells and 30% of UV-irradiated cells exhibited foci formation when transfected with wild-type polι. In contrast, when cells were transfected with the polι UBM1 mutants they exhibited very limited foci formation (<5% of cells), even after being exposed to UV irradiation. We attribute this phenotype to the reduced ability of the polι UBM1 mutant to physically interact with polη. However, in the case of polι F507S, we cannot exclude the possibility that its slightly reduced ability to interact with PCNA (Figures 3 and 4), may also contribute to its inability to accumulate into replication factories *in vivo* (25).

The interaction between polι–polη is mediated via ubiquitin

Our current studies have shown that in addition to facilitating the interaction with ubiquitin and ubiquitinated PCNA, the respective UBZ and UBMs in polη and polι are required for a physical and functional interaction between the two TLS polymerases. However, it is unclear if these protein–protein interactions are direct or indirect. For

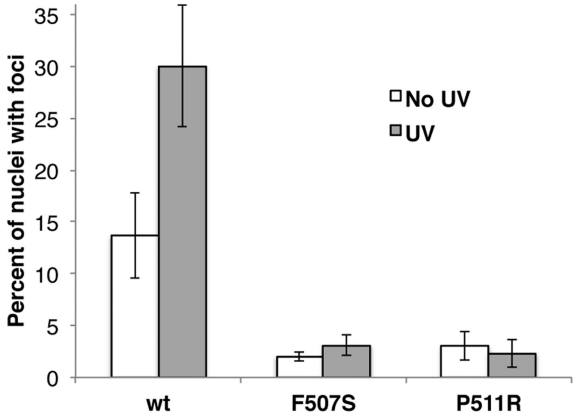

Figure 6. Polι UBM1 mutants (F507S and P511R) do not localize into DNA damage-induced foci. MRC5 human cells were transfected with plasmids encoding eCFP-polι wild type (peCFP-C1-polι), eCFP-polι_F507S (pJRM23) and eCFP-polι_P511R (pMGB9). Twenty hours after post-transfection, the cells were irradiated with UV (7 J/m^2). After 6 h, cells were fixed and the presence of foci was examined. The histogram represents the mean number of cells with foci. Error bars are the standard deviation calculated after counting 200 cells in 2–5 independent experiments with each construct.

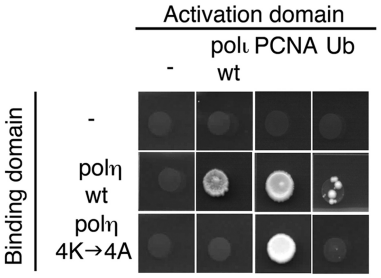

Figure 7. Interaction between polι and polη depends on polη ubiquitination. Yeast two-hybrid analysis of interactions between polη carrying four lysine point mutations (K682A, K686A, K694A and K709A) and polι, PCNA and ubiquitin. Yeast strain AH109 was transformed separately with the GAL4-AD expression vectors pACT2, pACT2-polι wild type (pAR116) and pACT2-PCNA (pAVR17) and pACT2-Ub (pBP127) in combination with each one of the following GAL4-BD expression vectors: pGBKT7 and pGBKT7-polη wild type (pAVR65), pGBKT7-polη 4K→A (pMGB4) as indicated. Several colonies from each transformation were grown overnight at 30°C in selective medium, and a sample was spotted on to a DOBA-Trp-Leu-His-Ade plate and incubated at 30°C for 6 days. The polη 4K→A mutant is unable to interact with polι, suggesting that the interaction is between polι and monoubiquitinated polη.

example, both polymerases are monoubiquitinated *in vivo* (31,35) and it is plausible that the interaction between the two polymerases is mediated by a monoubiquitinated form of each enzyme binding to the UBZ or UBM of its partner.

It has previously been shown that polη can be monoubiquitinated at four different lysine residues (K682, K686, K694 and K709) located near its C-terminus and that mutant forms of polη, in which the four-lysine residues have been changed to alanine (4K→A), cannot be monoubiquitinated *in vivo* (35). To test the hypothesis that polι might interact with monoubiquitinated polη we introduced the 4K→A substitutions into our polη two-hybrid vector and assayed for an ability to interact with polι, PCNA and ubiquitin. As shown in Figure 7, the 4K→A polη mutant retains its ability to interact with PCNA, yet has completely lost its ability to interact with either ubiquitin or polι. Our observations, therefore, support the hypothesis that polι interacts with a monoubiquitinated form of polη via its UBMs.

The monoubiquitination sites in polι are currently unknown, so it is not possible to perform the reciprocal experiments in which monoubiquitination of polι is blocked. To circumvent this obstacle, we instead constructed a chimeric protein in which ubiquitin is fused to the C-terminus of polι (polι-Ub) (Figure 8A). The ubiquitin moiety lacks the terminal glycine residues (G75/G76) and cannot be covalently linked to another substrate. A similar chimeric construct was previously reported for polη and used as a model for monoubiquitinated polη (35). Interestingly, like wild-type polι, the polι-Ub chimera exhibited a strong interaction with polη, but was unable to interact with ubiquitin, presumably because the ubiquitin moiety of the chimera occupies polι's UBM2, thereby precluding any further interactions with free ubiquitin (Figure 8A). To prove that the interaction between polη and polι-Ub is dependent upon the fused ubiquitin moiety, we made an I44A substitution in ubiquitin. The I44 residue is normally located at the center of the interface between ubiquitin and polη's UBZ (32), and the I44A substitution abolishes the interaction between polη and polι-Ub (Figure 8A). The I44A mutation in ubiquitin also perturbs the interaction with polι's UBM2 (34,42) and the I44A substitution in polι-Ub allows the chimera to once again interact with ubiquitin via its UBM2 (Figure 8B). As noted earlier, mutations in both UBM1 and UBM2 completely abolish the ability of the mutant polι to interact with polη and ubiquitin (Figure 5C). While the UBM1 and UBM2 substitutions in the polι-Ub chimera blocked its ability to interact with ubiquitin, it did not preclude an interaction with polη (Figure 8A). Together, these observations provide support for the hypothesis that the interaction with polη's UBZ is mediated through the ubiquitin moiety fused at the C-terminus of polι.

Interestingly, a strong interaction between polη and the polι-Ub chimera was apparent after 2 days growth, compared with 4 days required for wild-type polι, suggesting that polη has a tighter affinity for polι-Ub than with wild-type polι. Indeed, a physical interaction between polη and polι has proven historically difficult to demonstrate in traditional 'pull-down' experiments with extracts from human cells [Figure 8C; (36)]. However, in experiments where FLAG-tagged polι was expressed in human HEK293T cells and a portion (~10%) of the protein is clearly ubiquitinated, we were able to pull down small amounts of polη (Figure 8B, track 2). Furthermore, the amount of polη pulled-down increased significantly in the presence of polι-Ub. (Figure 8B, track 4). Unlike polη, where ubiquitination inhibits an interaction with PCNA (35), the polι-Ub chimera showed no diminished capacity to interact with PCNA, indicating that ubiquitination of polι does not preclude an interaction with PCNA (cf. Figure 8C, tracks 2 and 4).

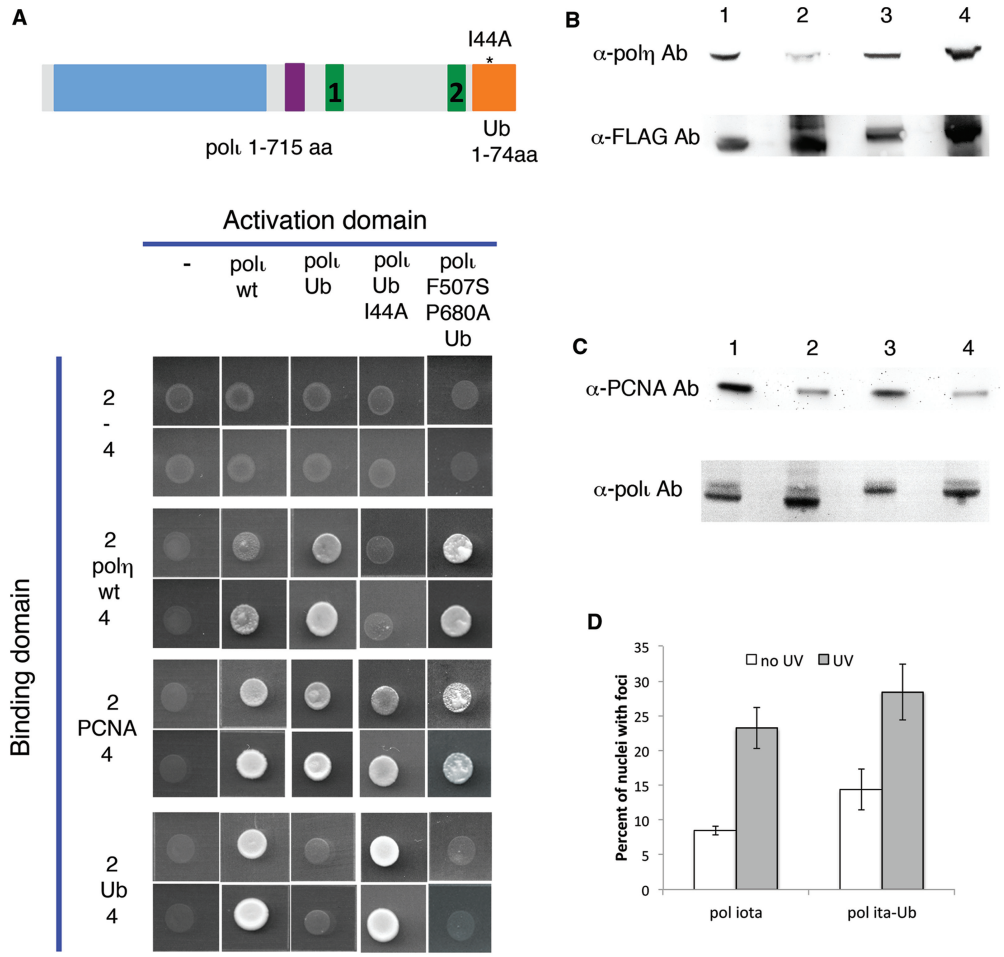

Figure 8. Interactions between a polι-Ub chimera and polη. (**A**) Cartoon of the polι-Ub chimera with the I44A substitution indicated. Yeast two-hybrid analysis of interactions between polι, polι-Ub, polι-Ub-I44A, and polι-F507S-P680A-Ub and wild-type polη, PCNA and ubiquitin (Ub). *Saccharomyces cerevisiae* strain AH109 was transformed separately with the GAL4-AD expression vectors pACT2 (control), pACT2-polι wild type (pAR116), pACT2-polι-Ub (pJRM127), polι-Ub_I44A (pJRM150) and polι-F507S/P680A-Ub (pJRM151) as indicated, in combination with each of the following GAL4-BD expression vectors: pGBKT7 and pGBKT7-polη_wild type, (pAVR65), pGBKT7-PCNA (pAVR18) and pGBKT7-Ub (pBP129) as indicated. Several colonies from each transformation were grown overnight at 30°C in selective medium, and a sample was spotted on to a DOBA-Trp-Leu-His-Ade plate and incubated at 30°C for 4 days. Images were taken after 2 days of growth (2) or 4 days of growth (4). (**B**) FLAG-pull-down assay demonstrating interactions between polι and polη (lane 2), and polι-Ub and polη (lane 4). Extracts from HEK293T cells transfected with plasmids encoding FLAG-tagged wild-type polι (pJRM46) or a polι-Ub fusion (pJRM140) and HA-tagged wild-type polη (pJRM56) were incubated overnight at 4°C with 20 µl of EZview Red ANTI-FLAG M2 Affinity Gel, washed three times and analyzed directly by SDS–PAGE and Western blot with respective antibodies. Lanes 1 and 3 represent 10% of corresponding extracts used for each pull-down reaction. (**C**) FLAG-pull-down assay demonstrating the strength of interactions between PCNA and polι (lane 2), or polι-Ub (lane 4). Extracts from HEK293T cells transfected with plasmids encoding FLAG-tagged wild-type polι (pJRM46) or a polι-Ub fusion (pJRM140) were incubated overnight at 4°C with 20 µl of EZview Red ANTI-FLAG M2 Affinity Gel, washed three times and analyzed directly by SDS–PAGE and Western blot with respective antibodies. Lanes 1 and 3 represent 10% of corresponding extracts used for each pull-down reaction. (**D**) MRC5 human cells were transfected with plasmids encoding eCFP-polι wild type (peCFP-C1-polι) and eCFP-polι-Ub (pJRM128). Twenty hours after post-transfection, the cells were irradiated with UV (7 J/m^2). After 6 h, cells were fixed and the presence of foci examined. The histogram represents the mean and standard deviation calculated after counting 200 cells from three independent experiments with each construct.

We have previously shown that an interaction between polι and polη is required for polι to accumulate into replication factories (Figure 6), and our observations above indicate that the interaction between polη and polι is strengthened when polι is ubiquitinated (Figure 8A and B). We therefore hypothesized that the polι-Ub chimera might accumulate into replication factories more efficiently than the wild-type protein. As seen in Figure 8D, this proved to be the case, as we observed a 2-fold increase in the number of undamaged cells exhibiting eCFP-polι foci and similar levels of damage-induced foci. We note that this is in contrast to a ~3-fold decrease in the number of cells exhibiting GFP-polη-Ub foci (35). Thus, the effect of ubiquitination of polι at its C-terminus is opposite to that of polη. Rather than hindering re-localization, ubiquitination at the C-terminus of polι actually increases its sub-cellular re-localization.

DISCUSSION

It has been known for over a decade that polη and polι physically interact (36), and the regions responsible for the

interaction were previously loosely mapped to the C-terminal ~200 amino acids of each protein (25,36). Although the two polymerases clearly co-localize at sites of DNA damage, the kinetics of their re-localization differs, suggesting that the two polymerases are not tightly associated in a living cell (37). Our studies begin to shed light on how such an interaction is facilitated and regulated. We identified the regions responsible for the polη–ι interaction as their respective UBZ and UBMs (Figures 1–3). Polη is known to be monoubiquitinated *in vivo* (31,35) and we considered the possibility that the physical interaction between the two polymerases might be mediated though the monoubiquitinated form of the polymerases and their respective UBZ or UBMs. To test this hypothesis, we generated a mutant polη (4K→A) that cannot be monoubiquitinated. Interestingly, the mutant polη protein was completely defective in its ability to interact with polι. Monoubiquitination of polη, therefore, appears critical for the interaction with polι.

The fact that we observe an interaction between wild-type polη and polι in the two-hybrid assays suggests that at least a fraction of human polη is likely to be subject to monoubiquitination in the yeast cells used for the *in vivo* two-hybrid assay. Furthermore, if monoubiquitination is a prerequisite for the interaction, how do we explain that we observe an interaction with the *in vitro* translated proteins in the far-Western assays? The answer lies in the fact that a significant fraction of the radiolabeled polη and polι synthesized in the coupled transcription-translation assay is also concomitantly ubiquitinated *in vitro* (Supplementary Figure S1). Thus, the data presented are entirely consistent with the hypothesis that the preferred partner for polι is a monoubiquitinated form of polη.

We identified the region in polη responsible for the interaction with polι as its UBZ (Figures 1 and 2). As polι is also known to be monoubiquitinated *in vivo* (31), we hypothesized that the preferred partner of polη might actually be a ubiquitinated form of polι. To test this hypothesis, we generated a chimera in which the N-terminus of ubiquitin was fused to the C-terminus of polι. The mutant chimera lacked the two C-terminal glycine residues, and therefore only allows for non-covalent interactions. The chimera interacts avidly with polη in the two-hybrid assays and this interaction was dependent upon I44 of ubiquitin (in the polι-Ub chimera) (Figure 8A). When expressed in human HEK293T cells, the polι-Ub chimera was able to 'pull-down' considerably more polη than wild-type polι (Figure 8B). We therefore conclude that the preferred partner for polη is indeed, a ubiquitinated form of polι. The mobility of the 'pulled-down' polη suggests that it is the non-ubiquitinated polη. That being the case, it appears that the interaction between polη and polι is enhanced when either polη (Figure 7), or polι (Figure 8B), is ubiquitinated. Based upon our observations presented here, it appears that polη and polι can interact in a variety of ways through ubiquitinated forms of either protein via their respective UBZ or UBMs (Figure 9).

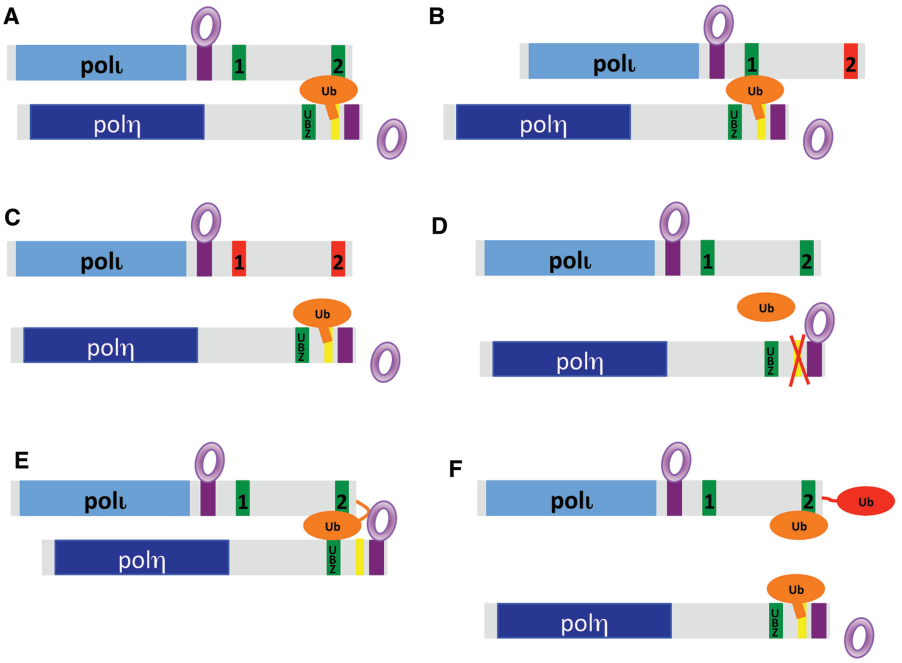

Figure 9. Cartoon explaining how the various interactions between polι, polη and PCNA can be modulated by ubiquitin. The polymerases are indicated as a rod with functional domains/motifs colored as follows: catalytic domain of polι, light blue; catalytic domain of polη, dark blue; PIP-box, purple rectangle; PCNA, purple disk; wild-type UBM1/UBM2/UBZ, green rectangle; mutant UBM1/2, red rectangle; wild-type ubiquitin, orange ellipsoid; I44A Ubiquitin mutant, red ellipsoid. **(A)** polι interacts with ubiquitinated polη predominantly via UBM2. Polι can still bind PCNA via is PIP-box, but ubiquitinated polη is unable to bind PCNA (35); **(B)** when UBM2 is unavailable, polι can potentially interact with ubiquitinated polη via UBM1; **(C)** polι cannot interact with ubiquitinated polη when both UBMs are mutated; **(D)** mutation of polη's natural ubiquitination sites blocks the interaction between polη and polι: **(E)** the polι-Ub chimera binds to the UBZ of polη. Both polymerases are able to interact with PCNA; **(F)** the I44A mutation in the polι-Ub chimera inhibits the interaction between polι and polη, but allows for an interaction between ubiquitin and UBM2.

The functional importance of the polη–ι interaction is clearly demonstrated by the fact that mutants of polι that are unable to interact with polη exhibit reduced accumulation into replication factories (Figure 6). Conversely, the polι-Ub chimera, which exhibits a tighter interaction with polη shows an enhanced accumulation into replication foci (Figure 8D).

Given the complex set of protein–protein interactions that polη and polι are known to participate in (5,35), it is reasonable to predict that the ubiquitination status of the pols allows a cell a variety of ways to regulate the formation of TLS complexes. For example, mono-ubiquitination of polη is known to inhibit an interaction with ubiquitinated PCNA (35), but as shown here, it enhances its interaction with polι. Upon DNA damage, polη is de-ubiquitinated and this will lead to a reduced ability to interact with polι, but a concomitant increased ability to interact with ubiquitinated PCNA. This might explain why the polymerases exhibit different sub-cellular mobility in a living cell (37).

In summary, we have shown here that the physical and functional interaction between pols η and ι occurs between ubiquitinated forms of either polymerase via their respective UBZ or UBMs. We see no reason to exclude the possibility that similar protein–protein interactions might occur between the various TLS pols (not polη and polι exclusively) and monoubiquitinated repair proteins, or the monoubiquitinated TLS pols and repair enzymes containing UBZ or UBMs, thereby enabling the TLS pols to be efficiently targeted to sites of DNA damage where they can facilitate TLS, or possibly channeled into an ever-growing myriad of different repair pathways, such as nucleotide excision repair, homologous recombination and intra-strand crosslink repair, in which they are known to participate (5).

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online: Supplementary Tables 1–3 and Supplementary Figure 1.

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