The ubiquitin-binding domain of DNA polymerase η directly binds to DNA clamp PCNA and regulates translesion DNA synthesis

DNA polymerase eta (Polη) is a unique translesion DNA synthesis (TLS) enzyme required for the error-free bypass of ultraviolet ray (UV)-induced cyclobutane pyrimidine dimers in DNA. Therefore, its deficiency confers cellular sensitivity to UV radiation and an increased rate of UV-induced mutagenesis. Polη possesses a ubiquitin-binding zinc finger (ubz) domain and a PCNA-interacting-protein (pip) motif in the carboxy-terminal region. The role of the Polη pip motif in PCNA interaction required for DNA polymerase recruitment to the stalled replication fork has been demonstrated in earlier studies; however, the function of the ubz domain remains divisive. As per the current notion, the ubz domain of Polη binds to the ubiquitin moiety of the ubiquitinated PCNA, but such interaction is found to be nonessential for Polη’s function. In this study, through amino acid sequence alignments, we identify three classes of Polη among different species based on the presence or absence of pip motif or ubz domain and using comprehensive mutational analyses, we show that the ubz domain of Polη, which intrinsically lacks the pip motif directly binds to the interdomain connecting loop (IDCL) of PCNA and regulates Polη’s TLS activity. We further propose two distinct modes of PCNA interaction mediated either by pip motif or ubz domain in various Polη homologs. When the pip motif or ubz domain of a given Polη binds to the IDCL of PCNA, such interaction becomes essential, whereas the binding of ubz domain to PCNA through ubiquitin is dispensable for Polη’s function.

Y-family DNA polymerases (Pols) are a group of nonessential enzymes that play an imperative role during translesion DNA synthesis (TLS) (1, 2). They protect the stalled replication fork due to DNA lesion blockage from collapsing, prevent double-strand breaks and maintain an unperturbed cell cycle. The budding yeast possesses two Y family DNA pols —Polη and Rev1; whereas in humans, in addition to these, Polι and Polκ also replicate efficiently through distorting DNA lesions, albeit with low fidelity and low processivity (3). Timely recruitment of specific TLS polys to a specific lesion site and their regulated activity determine the stability of a cell’s genome. Deciphering underlying mechanisms by which TLS polys gain access to the template–primer junction and take over synthesis from the replicative pol is crucial to understand the dynamic behavior of the replication fork during trans lesion DNA synthesis (4).

In Saccharomyces cerevisiae and humans, genetic and biochemical studies have indicated that PCNA plays a pivotal role in the pol exchange process (5, 6). The TLS polys gain access to the replication fork by physically interacting with PCNA, which is mediated by 1 to 2 highly conserved PCNA interacting protein (pip) motif(s) present mostly in the non-catalytic region of the pols (7). Since TLS DNA polymerases are distributive in DNA synthesis, physical binding to PCNA increases their nucleotide incorporation efficiency on both undamaged and damaged DNA substrates without affecting their processivity (1). A pip motif consists of a consensus sequence of eight amino acids QxxhxxFF(or YF/FY/FL), where x is any amino acid, and h is any hydrophobic residue. PCNA interaction motifs have been mapped in all the Y-family polymerases from S. cerevisiae and humans, except in Rev1 (4). While a single pip motif has been mapped in ScPolη and HsPolη, two pip motifs have been identified in human Pols-η and -κ (8–11). Mutational inactivation of the ScPolη pip motif α21SKNILSFα28 abolishes its interaction with PCNA (11). Thereby, S. cerevisiae strains harboring F627A and F628A mutations in Polη exhibit enhanced UV sensitivity and UV-induced mutagenesis. In contrast to ScPolη, human Polη possesses two functional pips (G37STDISSF44 and 701MQTLESF708) at the C-terminal domain (8). Both the pips are functionally redundant and can substitute for one another. Only in the absence of both these motifs, HsPolη’s interaction with PCNA on DNA, stimulation of its DNA synthetic activity,
Role of ubz domain

and colocalization with PCNA get abolished. In addition to the pip motif, several highly conserved ubiquitin (Ub)-binding domains (UBDs) have been identified in the C-terminal regions of Y-family pols, which regulate their TLS function (12). While Polη carries a single Zn²⁺-binding UBD, i.e., ubz; Rev1, Pol-ι, and -κ possess two such motifs but without having Zn²⁺-binding ability (ubm). As PCNA gets monoubiquitinated via Rad6-Rad18 ubiquitination conjugating system during TLS (13–16), it was proposed that the TLS polymerases bind to the ubiquitin moiety attached on PCNA through UBDs, and UBD-Ub-PCNA interaction is indispensable for the recruitment of TLS pols to PCNA. However, our subsequent mutational analysis in Sc- and Hs-Polη ruled out such a possibility, as mutations in the C₂H₂ motif or complete deletion of ubz domain had no perceivable effect on UV sensitivity or UV-induced mutagenesis (8, 17, 18). Thus, it was suggested that ubiquitin-binding on PCNA via its ubz domain is not a prerequisite for Polη to gain access to PCNA at the stalled replication site; rather, it may just function as a protein–protein interaction domain. Later studies reported that ubz binding to ub-PCNA increases Polη’s retention time in the replication foci (19–21).

Since the pip motif of Polη is an essential structural component for TLS activity, all Polη homologs should possess such PCNA interaction motifs. To look for the conservation of the pip motif and its function in Polη across the kingdom, 77 Polη amino acid sequences from the different genera were aligned. Interestingly, the C-terminal amino acid sequence of Polη showed maximum variations, and some of the members lacked either pip motif or ubz domain (Table 1 and Fig. S1). They were classified into three distinct categories based on the presence or absence of pip motif and ubz domain. Out of 77, 34 Polηs from species of fungi (11 genera), animals (22 genera), and a single plant genus belong to category-I that harbor both pip motif and ubz. A stable complex of Polη with CaPCNA was resolved, an early major elution peak at about 1.2 ml corresponding to a complex of Polη-CaPCNA (25, 26). The purified wild-type CaPolη protein (1–640 aa, ~70 kDa) was mixed with CaPCNA in a 1:1 M ratio and allowed to resolve in analytical size-exclusion chromatography. For comparison, CaPCNA alone was also passed through the column, and its elution profile was recorded. As reported earlier also, CaPCNA (~90 kDa) eluted as a trimer with an elution volume of ~1.7 ml (29). However, when the mixture of CaPolη-CaPCNA proteins was resolved, an early major elution peak at about ~1.2 ml corresponding to a complex of Polη-PCNA and two smaller peaks at ~1.7 ml and 2.4 ml elution volumes corresponding to free proteins were observed. The shifting of CaPCNA from 1.7 ml to 1.2 ml elution volume suggested that CaPCNA directly interacts and coelutes with CaPolη by forming a stable complex in solution and may regulate Polη’s activity; hence warrants a detailed investigation.

The direct interaction of CaPolη with CaPCNA implicated the involvement of yet unidentified pip motif or domain of CaPolη in PCNA binding. Four putative noncanonical pip sequences: Seq-1 (390SFVISNLF397), Seq-2 (479CVLIREFL486), Seq-3 (521SLTISKFV528), and Seq-4 (547EHEI4KLF554) in and around the PAD of CaPolη were recorded. As reported earlier also, CaPCNA (~90 kDa) eluted as a trimer with an elution volume of ~1.7 ml (29). However, when the mixture of CaPolη-CaPCNA proteins was resolved, an early major elution peak at about ~1.2 ml corresponding to a complex of Polη-PCNA and two smaller peaks at ~1.7 ml and 2.4 ml elution volumes corresponding to free proteins were observed. The shifting of CaPCNA from 1.7 ml to 1.2 ml elution volume suggested that CaPCNA directly interacts and coelutes with CaPolη by forming a stable complex in solution and may regulate Polη’s activity; hence warrants a detailed investigation.

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### Table 1

**Classification of Polyn from various organisms based on the presence or absence of Pip motif and UBZ domain**

<table>
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<th>Sl. No.</th>
<th>Rad30 (Polη)</th>
<th>Organisms</th>
<th>No. of amino acids</th>
<th>Accession No.</th>
<th>PIP</th>
<th>UBZ</th>
<th>Type of organisms</th>
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<td>1</td>
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**Category-I DNA polymerase eta (with both Pip motif (/s) and UBZ domain)**

**Category-II DNA polymerase eta (with only Pip motif)**

**Category-III DNA polymerase eta (with only UBZ domain)**
the constitutive Ubz domain of CaPolη of S. cerevisiae was determined. Polη of S. cerevisiae respectively; therefore, double deletant the constitutive Ubz domain of CaPolη from UV-induced DNA damages (17, 25, 26), to further strengthen our observations, we concluded that the ubz domain of CaPolη is essential for TLS activity. To ascertain the function of ubz domain, two ΔD626A and H628A, H628A of CaPolη mutants were generated and their ability to suppress UV sensitivity of Polη-deficient S. cerevisiae strains was examined. Both the CaPolη mutants did not confer any cellular protection to UV radiation, and growth was inhibited drastically (Fig. 2B). The complementation analysis was reconfirmed by estimating the cell survivability upon UV exposure; where about 85 to 95% of cells expressing wild-type Polη conferred resistance, only ~10 to 35% Δrad30Δ cells harboring CaPolη mutants survived after 32 J/m² of UV treatment (Fig. 2C, i). Similarly, UV-induced mutation rate was estimated by counting the number of canavanine-resistant colonies of various S. cerevisiae strains harboring CaPolη constructs. The Δrad30Δ strain or strains harboring truncated or ubz mutants of CaPolη were highly mutagenic as more number colonies (~4000 per 10⁷ cells) grew as canavanine-resistant, as opposed to the strains expressing wild-type Polη (~750 per 10⁷ cells; Fig. 2C, ii). This result suggested that the ubz domain of CaPolη plays an essential role in the error-free bypass of UV-induced lesions and possibly in PCNA interaction. Subsequently, ITC and GST-pull-down assays were carried out to determine the role of ubz in PCNA binding. About 10 μM of each mutant CaPolη 1 to 601 aa, D626A, and H624A, H628A proteins were placed in the calorimetric cell, and the change in the heat was monitored after each CaPCNA injection (Fig. 2D). Our ITC assays revealed no significant net heat change when PCNA was titrated against the mutants of Polη suggesting no detectable interaction between the proteins. Similarly, the GST-pull-down assay authenticated the ITC results where wild-type GST-Polη pulled down PCNA from the mixture, whereas the mutant Polη proteins failed to pull down PCNA (Fig. 2E compare lane 3 with 6 and 9). From these observations, we concluded that the ubz domain of CaPolη directly binds to PCNA, and this interaction is essential for its in vivo activity.

**Ubz domain of CaPolη is essential for CaPolη’s function in S. cerevisiae and interaction with PCNA**

The above results indicated that the C-terminal portion of CaPolη containing two putative pip sequences: Seq-3 and Seq-4, and one ubz domain could be involved in PCNA interaction. To map the precise location of PCNA binding, another deletant CaPolη 1 to 601 aa was constructed that lacks the last 39 residues encompassing the ubz domain but retains Seq-3 and Seq-4 (Fig. 2A). Similar to CaPolη 1 to 512 aa, Polη 1 to 601 aa also conferred significant growth retardation at 16 to 32 J/m² of UV irradiation in both Δrad30Δ and Δrad30Δrev3Δ S. cerevisiae strains implying that both Seq-3 and Seq-4 are not essential for CaPolη’s TLS activity in the cell and are unlikely to be involved in PCNA interaction (Fig. 2B). Moreover, it suggested the critical role of the C-terminal 39 amino acids comprising the ubz domain of CaPolη in TLS activity. To ascertain the function of ubz domain, two ΔD626A and H624A, H628A of CaPolη mutants were generated and their ability to suppress UV sensitivity of Polη-deficient S. cerevisiae strains was examined. Both the CaPolη mutants did not confer any cellular protection to UV radiation, and growth was inhibited drastically (Fig. 2B). The complementation analysis was reconfirmed by estimating the cell survivability upon UV exposure; where about 85 to 95% of cells expressing wild-type Polη conferred resistance, only ~10 to 35% Δrad30Δ cells harboring CaPolη mutants survived after 32 J/m² of UV treatment (Fig. 2C, i). Similarly, UV-induced mutation rate was estimated by counting the number of canavanine-resistant colonies of various S. cerevisiae strains harboring CaPolη constructs. The Δrad30Δ strain or strains harboring truncated or ubz mutants of CaPolη were highly mutagenic as more number colonies (~4000 per 10⁷ cells) grew as canavanine-resistant, as opposed to the strains expressing wild-type Polη (~750 per 10⁷ cells; Fig. 2C, ii). This result suggested that the ubz domain of CaPolη plays an essential role in the error-free bypass of UV-induced lesions and possibly in PCNA interaction. Subsequently, ITC and GST-pull-down assays were carried out to determine the role of ubz in PCNA binding. About 10 μM of each mutant CaPolη 1 to 601 aa, D626A, and H624A, H628A proteins were placed in the calorimetric cell, and the change in the heat was monitored after each CaPCNA injection (Fig. 2D). Our ITC assays revealed no significant net heat change when PCNA was titrated against the mutants of Polη suggesting no detectable interaction between the proteins. Similarly, the GST-pull-down assay authenticated the ITC results where wild-type GST-Polη pulled down PCNA from the mixture, whereas the mutant Polη proteins failed to pull down PCNA (Fig. 2E compare lane 3 with 6 and 9). From these observations, we concluded that the ubz domain of CaPolη directly binds to PCNA, and this interaction is essential for its in vivo activity.

**Ubz domain of Polη is sufficient for PCNA interaction**

Unlike ScPolη and human Polη, where the ubz domain is dispensable, in CaPolη, ubz is essential for TLS activity. To understand whether any sequence or structural differences of the ubz domains in these Polηs have an impact on functional differences, we used the solution structure of the ubz domain of human Polη as a template and determined the structure of the ubz domain of both Sc- and Ca-Polη by computational
Figure 1. Identification of PCNA interaction region in CaPol. A, amino acid alignment between Ca- and Sc-Pol was carried out by EMBOSS Needle; identical amino acids are marked by * and similar amino acids were marked by symbols. All the important motifs and domains are underlined, including Motif I to V, PAD, ubz and pip. Putative pip sequences were labeled as Seq-1 through Seq-4 in the C-terminal domain. B, size-exclusion chromatograms of CaPCNA (green) and a mixture of CaPol-PCNA (red); CaPCNA elutes with the peak fractionation at ~1.7 ml and a complex of CaPol-PCNA displayed a peak at ~1.2 ml. C, putative pip sequences of CaPol were compared with the already identified pip motifs of other Y-family pols. D, line diagram showing various domains of Polη and a C-terminal deletant CaPol (1–512). E, determination of binding kinetics of wild type or truncated CaPol (1–512) with CaPCNA by ITC. In each panel, the upper half shows the measured heat exchanges during each PCNA protein injection. The lower half of each panel shows the enthalpic changes as a function of the molar ratio of the proteins where PCNA was considered as a trimer. F, UV sensitivities of S. cerevisiae strains. Cells
Role of ubz domain

Table: 2

<table>
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<tr>
<th>Parameters</th>
<th>CaPol versus CaPCNA</th>
<th>Ubz peptide versus CaPCNA</th>
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<th>ScPol versus ScPCNA</th>
<th>Ubz peptide versus ScPCNA</th>
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modeling (Fig. 3A). While human and C. albicans Polηs possess a canonical $\text{C}_2\text{H}_2-Zn^{2+}$-binding domain, ScPolη ubz is noncanonical as the second cysteine residue is replaced by glutamine. Although there is hardly any similarity in the primary sequences among these ubz domains except for the positions of cysteines and histidines that coordinate the metal cofactor, they adopt a classical superimposable $\text{C}_2\text{H}_2$ zinc finger structure characterized by a $\beta$-fold comprised of two short antiparallel strands and a carboxyl-terminal $\alpha$-helix. The two cysteines are located on the fingertip made by the two $\beta$-strands, and the two histidines are located on the $\alpha$-helix. The $\alpha$-helix shows the maximum sequence identity and is found to be involved in interaction with ubiquitin (32). Our structure predictions did not reveal any differences in the ubz structures; however, the in vivo TLS role of ubz at least in CaPolη is found to be distinct from the other two Polηs suggesting the role of ubz to be evolved differently in different species. Since the ubz domain of CaPolη is necessary for its TLS function and PCNA interaction, we wanted to determine whether the ubz domain is sufficient for PCNA binding. A 29-mer ubz peptide (607-KTKKCSRCKLSVDDPVEHA IAMDLNS-635) and its corresponding mutant peptide (607-KTKKCSRCKLSVDDPVEHNYH IAMDLNS-635) and its corresponding mutant peptide (607-KTKKCSRCKLSVDDPVEHNYH IAMDLNS-635) were synthesized, and their interaction with CaPCNA was validated by ITC analysis. While PCNA titration against the wild-type ubz peptide taken in the calorimetric cell resulted in the release of heat, it did not show any sign of binding with the mutant peptide as no net energy change was detected (Fig. 3B, i and ii). The kinetic parameters upon the binding of wild-type ubz peptide with PCNA were determined as $\Delta H = -80 \text{kcal/mol}$, $\Delta G = -6.42 \text{kcal/mol}$, and $K_D = 19.8 \mu\text{M}$ (Table 2). As the binding affinity of ubz peptide toward PCNA is about two-fold less than that of the full-length Polη, the contribution of neighboring residues of ubz domain for the stable interaction with PCNA appears to be substantial. Next we examined whether the ubz peptide can inhibit the binding of PCNA to Polη. An equimolar mixture of the ubz peptide and PCNA was injected into the calorimetric cell having CaPolη to examine the interference of peptide on the interaction between PCNA and Polη by ITC. As shown in Figure 3B, iii, PCNA~ubz peptide mix did not bind to CaPolη as the profile showed no heat exchange. This result suggested that ubz peptide and CaPolη completely inhibit each other’s binding to PCNA and most likely both the ligands bind to the same surface of PCNA. In a similar assay, we also found that ScPolη and ubz peptide derived from CaPolη make stable complexes individually with ScPCNA with about equal affinities ($K_D = 15.6 \mu\text{M}$), but a mixture of ScPCNA and ubz peptide excluded from binding to ScPolη (Fig. 3C and Table 1). These results suggested that irrespective of the sources of PCNA, ubz peptide interacts with them with equal affinities, and ubz peptide blocks the binding of Polη to PCNA, most probably by forming a ubz-PCNA complex that prevented PCNA from binding to Polη. It also indicates that the interaction of ubz peptide and Polη with PCNA is mutually exclusive.

Ubz domain mimics pip motif in binding to the interdomain connecting loop (IDCL) of PCNA

The PCNA interaction motif of the ligand occupies the hydrophobic pocket formed by the IDCL that connects the two globular domains of a PCNA protomer (33, 34). Most of the structural characterization studies suggest that the pip motif forms a $3_{10}$ helix that snugly fits into the hydrophobic pocket of PCNA. To map the binding site of ubz peptide in PCNA, we used two available CaPCNA mutant proteins CaPCNA-79 and CaPCNA-90. In PCNA-79, two key hydrophobic residues L126 and I128 of the interdomain connecting loop were mutated to alanine, whereas in PCNA-90, the extreme C-terminal tail possesses P253A and K254A mutations. Most of the interacting proteins bind to any of these two regions of a trimeric PCNA ring (4). Our ITC analyses revealed that ubz peptide binds to PCNA-90 but not to PCNA-79. Kinetic parameters suggested the interaction between ubz and PCNA-90 to be an exothermic reaction, and the equilibrium dissociation constant was determined to be in the range of 39 μM which is about two-fold higher than that of the ubz-CaPCNA complex (Fig. 3D). To further strengthen our finding, a GST pull-down assay was carried out to pull down PCNA from an equimolar mixture of GST-CaPolη with wild-type or mutants of CaPCNA proteins. While GST-CaPolη was able to pull down most of the wild-type PCNA and PCNA-90 proteins from the solution (compare lane 1 with 3, and 7 with 9), it failed to bind PCNA-79 (compare lane 4 with 6) (Fig. 3A). Both the assays of genomic rad30Δ and rad30Δrev34 deletion S. cerevisiae strains harboring YEP-ADH1p or YEP-ADH1p-ScPolη or YEP-ADH1p-CaPolη or YEP-ADH1p-CaPolη (1–512) or YEP-ADH1p-CaPolη F485LA486A plasmids from an overnight SD-Ura culture were serially diluted and spotted onto SD-Ura plates. The culture plates were irradiated with the indicated doses of UV radiation, covered with aluminum foil, incubated at 30°C for 3 days, and then photographed. ITC, isothermal calorimetry.
Role of ubz domain

Figure 2. Ubz domain of CaPolη is essential TLS activity. A, a line diagram showing the deletion and site-directed mutations in the ubz domain of CaPolη. B, rad30Δ S. cerevisiae cells harboring YEP-ADH1p or YEP-ADH1p-ScPolη or YEP-ADH1p-CaPolη or YEP-ADH1p-CaPolη (1–601) or YEP-ADH1p-CaPolη D626A or YEP-ADH1p-CaPolη (H624A, H628A) mutation plasmids from an overnight SD-Ura culture were serially diluted and spotted onto SD-Ura plates. The culture plates were irradiated with indicated doses of UV radiation, covered with aluminum foil, incubated at 30°C for 3 days, and then photographed. C, appropriate dilutions of rad30Δ deletion S. cerevisiae strains harboring the above-mentioned mutations were plated onto SD medium for viability determinations (i) and onto SD lacking arginine but containing canavanine for mutagenesis assay (ii). UV irradiation was done as indicated. Following UV irradiation, plates were incubated in the dark, and colonies were counted after 3 to 5 days. Experiments were performed in triplicate, and the average was plotted. D, determination of binding kinetics of truncated CaPolη (1–601) (i) or D626A (ii) or H624A, H628A (iii) Polη mutants with PCNA by ITC. In each
Role of ubz domain

suggested that the hydrophobic cavity formed by the IDCL of PCNA is the interaction site of CaPolη, mediated by its ubz domain.

Species-specific role of pip motif and ubz domain of Polη in TLS

Since ubz of CaPolη functionally mimics the pip motif of ScPolη, to understand the species-specific roles of these two critical motifs during TLS, an array of hybrid Polη constructs were generated and analyzed (Fig. 4). The pip box sequence of ScPolη (KQVTSSKNI16FSTTRKSTOP) was fused just before the termination codon in wild-type and mutant CaPolη orfs to generate CaPolη−ScRad30pip, CaPolη D626A−ScRad30pip, and CaPolη H624A, H628A−ScRad30pip. The chimeric CaPolη UBZΔ−ScRad30pip (1−618 aa) was also generated by fusing the ScPolη pip sequence immediately after the catalytic domain; therefore, it does not retain its ubz domain but possesses the pip motif from ScPolη. Similarly, a C-terminal fragment containing ubz amino acid sequences from CaPolη (PKL ECSRCKLSVDDVP5EHNDYHIAMDL5KNNHSTOP) was fused to the catalytic domain of ScPolη to generate a chimeric ScPolη CTDΔ−CaRad30ubz that mimics with the C-terminal domain of CaPolη, lacking the pip sequence. As shown in Figure 4, A and B; F627A, F628A mutations in the pip box of ScPolη abrogated its TLS function and displayed equal UV sensitivity with rad30Δ strain but H568A, H572A mutations in ubz of ScPolη did not affect survival and restored wild-type level of UV sensitivity to the rad30Δ strain (compare sectors i and iii with ii and iv). Thus, while the pip motif of ScPolη plays a critical role in TLS, the ubz of ScPolη is nonessential, and this result further authenticates our earlier reports (15, 17). However, as shown here and in Figure 3B, the ubz mutants of CaPolη are defective in conferring UV resistance (sectors v–vii). Interestingly, the carboxyl-terminal fusion of ScRad30-pip sequence to these mutants rescued the UV sensitive phenotype in the rad30Δ strain. At 16 to 32 J/m² of UV, a significant level of growth was observed for rad30Δ strain expressing CaPolη D625A−ScRad30pip or CaPolη H624A, H628A−ScRad30pip or CaPolη ubzΔ−ScRad30pip chimeras than the vector control alone suggesting that presence of ScRad30 pip motif alone is sufficient to suppress UV susceptibility, and ubz domain is dispensable (compare sectors x–ii with i). Interestingly, a similar fusion of ScRad30-pip to wild-type CaPolη conferred slightly better resistance to UV irradiation than the rad30Δ strain expressing CaRad30 (compare sectors x with ix). Although the presence of ubz of CaPolη is essential for its TLS function, the fusion of this domain alone to the catalytic domain of ScPolη (ScPolη CTDΔ−CaRad30 ubz) partially rescued the UV-sensitive phenotype of rad30Δ deletion strain (sector xiv, up to 16 J/m²). At a higher dose of UV radiation (32 J/m²), ubz fused ScPolη failed to carry out efficient TLS. We obtained almost similar results when we tested these hybrid Polηs in rad30Δrev3Δ, yet another Polη-deficient S. cerevisiae strain (Fig. S3B). Overall, these results suggest that a ubz domain can substitute for a pip motif in CaPolη for TLS activity but not the other way in ScPolη. Probably, not so conserved flanking sequences in Polη might be playing a role in deciding whether ubz can function as a pip motif or not.

Functional analyses of ubz domain of CaPolη in C. albicans

In our earlier studies, we reported that the roles of CaPolη in genome stability, genotoxins-induced filamentation and azole drug tolerance by C. albicans are due to its translesion DNA synthesis activity, while its TLS-independent functions play a pivotal role in serum-induced morphogenesis and amphotericin B resistance. C. albicans cells harboring CaPolη with catalytically inactive mutations (D168A, E169A) exhibited similar phenotypes as the rad30ΔΔ strain (26, 27). To get insights into the cellular role of ubz in Polη, various C. albicans rad30ΔΔ strains possessing either wild-type or ubz deletion or point mutations of CaPolη expressed under the methionine regulated MET3 promoter were subjected to UV sensitivity and in vivo TLS-mediated genomic stability assay (Fig. 5). The C. albicans strains possessing truncated CaPolη 1 to 601 aa or H624A, H628A mutations exhibited similar sensitivity as the vector control (Fig. 5A). Only the knockout strain expressing wild-type CaPolη survived to a higher dosage of UV irradiation (16−32 J/m²). Thus, like in S. cerevisiae, to carry out translesion DNA synthesis even in C. albicans, CaPolη requires both the catalytic and the ubz domains. Further these strains were exposed to sublethal dose of UV radiation and allowed to repair the damage by growing them on fresh media. Since TLS activity of Polη is required to bypass DNA lesions to prevent replication fork collapse and accumulation of DNA breaks, any strains failing to conduct TLS will accumulate fragmented longer chromosomal DNA. Alkali agarose gel electrophoresis of total genomic DNA isolated at various time points of recovery revealed that an equal amount of genomic DNA degradation was found in each cell type prior to recovery (Fig. 5B, lanes 1, 5, 9, and 13). The longer the duration of the recovery period, the more was the accumulation of larger DNA fragments, and a lesser amount of smaller chromosomal DNA fragments were accumulated in the cells expressing wild-type than expressing ubz domain deletion or its site-directed CaPolη mutants (lanes 2−4, 6−8, and 14−16). This result suggested that C. albicans expressing CaPolη was proficient in carrying out UV-inflicted DNA lesion bypass than the cells possessing Polη ubz mutants, and this could be because of the inability of the CaPolη ubz mutants to interact with CaPCNA for efficient lesion bypass. To further strengthen our result, the
Role of ubz domain

Figure 3. Direct interaction of ubz of CaPolƞ with PCNA. A, sequence alignment and a superimposable model structures of ubz of Human (Hs, cyan), S. cerevisiae (Sc, purple) and C. albicans (Ca, green) Polƞs. Secondary structures are highlighted. Human Polƞ ubz structure 3WUP was used as a template for modeling. Zn^{2+} (orange) coordinated to the C_{2}H_{2} motif is also shown. B, determination of the binding kinetics of ubz peptide (i) or ubz mutant peptide (ii) of CaPolƞ mutants with PCNA by ITC. An interaction of a mixture of CaPCNA and ubz peptide with CaPolƞ injection was also determined (iii). In each panel, the upper half shows the measured heat exchanges during each PCNA (i and ii) or CaPolƞ (iii) protein injection. The lower half of each panel shows the enthalpy changes as a function of the molar ratio of the two proteins where PCNA was considered as a trimer. C, determination of binding kinetics of ScPolƞ with ScPCNA (i) or ubz peptide with ScPCNA (ii) mixture of ScPCNA and ubz peptide with ScPolƞ (iii) by ITC. In each panel, the upper half shows the measured heat exchanges during each PCNA (i and ii) or CaPolƞ (iii) protein injection. The lower half of each panel shows the enthalpy changes as a function of the molar ratio of the two proteins where PCNA was considered as a trimer.
requirement of ubz domain of CaPol in maintaining genomic stability was determined by estimating the loss of functionality of URA3 gene upon 5-fluoroorotic acid (5-FOA) treatment with and without UV irradiation. First, we generated a heterozygous URA3/ura3− in Polη-deficient C. albicans strain, and the loss of heterozygosity (LOH) was estimated through a 5-FOA resistance assay (Fig. 5C). We found that without UV irradiation, the rate of LOH in various rad30Δ C. albicans strains remained unaltered (5–8 per 5 × 10⁵ cells), even it was the same in wild-type Polη expressing cells when exposed to UV. However, the number of FOA-resistant colonies increased by ∼three-fold in the cells expressing ubz deletion and H624A, H628A mutants of Polη (16–25 per 5 × 10⁵ cells) upon UV exposure.

Morphogenesis plays an important role in C. albicans pathogenesis. Certain gene knockout strains locked in either round or hyphal structures are avirulent in developing systemic fungal infections in animal models. Filamentation in C. albicans is induced by elevated temperatures, serum, spider media, DNA damaging agents, and other specific nutrients.

**Figure 4. Differential UV sensitivity conferred by various chimeric Polηs.** A, a line diagram showing the various fusion constructs of CaPolη. As CaPolη intrinsically lacks a pip motif, pip sequence from ScPolη was fused to wild-type and ubz mutants of CaPolη. Similarly, the C-terminal domain of ScPolη was replaced with the C-terminal domain of CaPolη in ScPolηΔC−CaRad30ubz. TLS efficiency of these transformants is highlighted. B, cells of genomic rad30Δ S. cerevisiae strains harboring vector alone (YEP-ADH1p) or ScPolη or ScPolη pip (F627A,F628A) mutant or ScPolη H568A,H572A or CaPolη 1 to 512 aa or CaPolη 1 to 601 aa or CaPolη D626A or CaPolη H624A,H628A or CaPolη−ScRad30pip or CaPolη H624A,H628A−ScRad30 pip or CaPolη D627A−ScRad30pip or CaPolη UBZA−ScRad30pip or ScPolη CTDΔ−CaRad30ubz plasmids from an overnight SD-Ura culture were serially diluted and spotted onto SD-Ura plates. The culture plates were irradiated with the indicated doses of UV radiation, covered with aluminum foil, incubated at 30°C for 3 days, and then photographed.
Figure 5. Functional analyses of ubz domain of CaPoln in C. albicans. A, cells of rad30ΔΔ C. albicans strain expressing wild-type or mutants of CaPoln (1–601 aa and H624A, H628A) were grown overnight, serially diluted and spotted on SD without Methionine and Uracl plates, and irradiated with indicated UV dosages, incubated at 30 °C for 3 days, and imaged. B, various strains of C. albicans were exposed to 16 J/cm² UV, followed by recovery, and the cells were harvested at different time points. Genomic DNA was isolated and allowed to resolve in an alkaline agarose gel. After neutralization, DNA fragments were visualized by ethidium bromide staining. Vector control (lanes 1-4), CaPoln (lanes 5-8), CaPoln 1 to 601 aa (lanes 9-12) and CaPoln H624A, H628A (lanes 13-16). C, FOA-resistant colonies were estimated for various strains of C. albicans as indicated as a measure of LOH upon UV exposure. D, microscopic images of serum-induced germ tube formation by Poln deficient cells containing WT and ubz mutants of CaRad30 constructs. E, cells with or without germ tube and chained cells were counted. F, length of germ tubes by various cells was measured.
Role of ubz domain

(27). C. albicans rad30Δ or its catalytically inactive mutant strains exhibited reduced serum-induced germ tube formation (26, 27). To decipher the associated role of ubz domain in morphogenesis, germ tube development assay was carried out (Fig. 5, D–F). The auxotrophic BWP17 strain of C. albicans does not undergo filamentation due to the absence of functional URA3 gene; however, an ectopic expression of URA3 by its integration into the genome results in germ tube formation induced by serum (35). First, we treated C. albicans rad30ΔΔ strains harboring either wild-type or mutations in the ubz domain of CaPolη to 10% fetal bovine serum (FBS) for 1 h before checking their cellular morphology. Except for the strain expressing wild-type Polη, where 97% of C. albicans cells developed germ tubes, only ∼50% of cells from other ubz defective strains developed germ tubes; that also with substantially reduced lengths (Fig. 5, E and F). Consequently, more round cells were counted in C. albicans cells without the functional ubz domain. These results suggest that CaPolη without ubz domain exacerbates morphological defects induced upon serum addition. Taking it all together, we conclude that the ubz domain of CaPolη plays a pivotal role in TLS-mediated genome stability and morphogenesis in C. albicans.

Two modes of PCNA interaction in three categories of Polη

From the above results and our earlier observations, it is evident that while S. cerevisiae and human Polη gain access to PCNA via a pip motif, CaPolη that intrinsically lacks a pip motif interacts with PCNA through its ubz domain for the TLS activity. Thus, for S. cerevisiae and human Polη where a pip motif is present, ubz becomes dispensable; and for C. albicans Polη where pip is naturally absent, the role of ubz becomes imperative. To understand the evolutionarily conserved role of the ubz domain of Polη, we selected a few other members of Polη from category III that lack canonical PCNA interaction motif at the carboxyl-terminal tail. We expressed wild-type Polη from S. pombe, Neurospora crassa, and Aspergillus nidulans in rad30Δ S. cerevisiae strain for complementation analysis (Fig. 6A). Unlike CaPolη, Nc- and An-Polη failed to complement the function of ScPolη, whereas SpPolη (872 aa) partially complemented and supported the growth even at a higher dosage of UV. However, the deletion of the ubz domain of SpPolη (1–682 aa) completely abolished resistance to UV; thus, similar to CaPolη, the ubz domain of SpPolη is essential for its TLS activity (compare sectors v and vi with i). To strengthen the spot assay, we estimated the colony formation units when cells expressing either SpPolη or SpPolη ubzΔ were exposed to UV radiation. The S. cerevisiae cells expressing wild-type SpPolη formed more colonies upon treatment with UV than the cells expressing ubz deletion mutant of SpPolη, which is similar to a Polη-deficient strain (vector control, Fig. 6B). Although we could not find complementation by Nc- and An-Polηs in rad30Δ S. cerevisiae strain, the results obtained from ubz mutational analyses of Ca- and Sp-Polηs clearly supported the indispensable conserved role of ubz of category III Polη in TLS. Considering earlier studies and this study, we propose two modes of Polη recruitment to PCNA, mediated either by pip box or ubz domain in three categories of Polη (Fig. 6, C and D). For category I and II Polηs, pip motif plays an essential role, and ubz/ubm, if present becomes dispensable, whereas in category III Polηs, as they lack pip motif intrinsically, ubz domain interaction with the PCNA becomes critical. While in category I, ubz may interact indirectly with PCNA mediated by the ubiquitin moiety, in category III Polηs, ubz directly binds to the IDCI of PCNA similar to the pip motif of category I and II Polηs.

Discussion

To gain access to the replication fork, whether, during replication or translesion DNA synthesis, DNA polymerase needs to interact with PCNA, a step that is considered to be essential (1, 3). Therefore, cells harboring a DNA polymerase that is either catalytically inactive or defective in PCNA binding or completely deficient in that specific DNA polymerase functions exhibit exactly similar phenotypes. In that context, all the DNA polymerases must possess a dedicated PCNA interaction motif or domain, which is usually found at their regulatory carboxyl-terminal portions. Interestingly, this study serendipitously identified a group of Polηs, mostly belonging to fungal species that differed from the other two categories by not possessing a canonical pip motif in their extreme C-terminal tails. Therefore, this study was designed to decipher the underlying mechanism of PCNA interaction by this new set of Polηs belonging to category III and to propose possible modes of PCNA interaction that exist in these three categories of Polηs.

Although the multiple sequence alignment suggested lack of a pip motif in category III Polηs, by mutational analyses we first ensured the absence of any such pip motif in CaPolη, a member from category III. Further, by carrying out extensive genetic and biochemical analyses of various CaPolη mutants, we revealed that the ubz domain is necessary and sufficient for both physical and functional interaction with PCNA. Additionally, although Polη from S. pombe partially complemented ScPolη, analyses of its ubz mutant further supported the significance of this domain in TLS function. Thus, we proposed that where pip motif is naturally absent, the ubz domain of Polηs could play a critical role in PCNA interaction. In contrast to category III Polηs, the representative studies on Polηs from categories I and II suggested the indispensable nature of the pip motif in PCNA interaction and for their TLS activities, thereby the existence of two distinct modes of PCNA interactions among Polηs mediated either by pip or ubz domains is possible (8, 17, 18, 24). While ubz is present in category I, our multiple sequence alignment of plant Polηs suggested an absence of ubz domain in category II Polη. However, an earlier report predicted the presence of ubm-like motifs in AtPolη, although these motifs hardly show any similarity with the other identified ubms in Polη, Polη, and Rev1 (12, 24). Ubms that do not coordinate zinc are usually absent in Polηs; therefore, whether so-called ubm-like motifs in AtPolη can even bind to ubiquitin requires further investigation (Fig. S1B, ii). Moreover, Polηs from protists such as Trypanosoma, Leishmania, Caenorhabditis elegans of category II also lack ubz completely, but they have pip-like motifs at the
Figure 6. Evolutionarily conserved role of ubz domain. A, the rad30Δ S. cerevisiae strains harboring vector alone or YEP-ADH1p-ScPolŋ or YEP-ADH1p-CaPolŋ or YEP-ADH1p-AnPolŋ or YEP-ADH1p-SpPolŋ or YEP-ADH1p-SpPolŋ ubzΔ (1–628) or YEP-ADH1p-NcPolŋ plasmids from an overnight SD-Ura culture were serially diluted and spotted onto SD-Ura plates. The culture plates were irradiated with indicated doses of UV radiation, covered with aluminum foil, incubated at 30 °C for 3 days, and then photographed. B, the colony formation units of Polŋ deficient cells expressing either full-length SpPolŋ or SpPolŋ ubzΔ (1–628 aa) when exposed to UV radiation. *Student t test p value, p ≥ 0.05 when compared between wild-type SpPolŋ and SpPolŋ ubzΔ (1–628 aa) or the vector control. * Student t test p value, p ≥ 0.05 when compared between wild-type SpPolŋ and the vector control. C, the two proposed modes of PCNA interaction by the three categories of Polŋ. If pip motif is present in a given Polŋ, it becomes the primary site of PCNA interaction. If pip is absent, ubz of Polŋ becomes the primary site. D, a cartoon diagram of two modes of PCNA interaction mediated by either pip box or ubz domain of three categories of Polŋ. A trimeric ring of PCNA and each monomer has been shown in orange, blue, and green colors. The catalytic domain of Polŋ has been shown as “hand shape,” pip as a circle, and ubz as a cylinder located in the C-terminal tail.
Role of ubz domain

C-terminal domain. Since the mutations in the \( C_2H_2 \) motif of ScPol\( \eta \) and HsPol\( \eta \) exhibited either no or weak phenotypes when compared with their pip mutants and ubz is absent in category II Pol\( \eta \), the role of ubz becomes dispensable for the TLS function in category I and Pol\( \eta \) (8, 17, 18). This discrepancy of the role of ubz among Pol\( \eta \) posits two pertinent issues: (1) why does the ubz domain of category I Pol\( \eta \) fail to bind to the IDCL of PCNA, and (2) how does ubz domain of category III Pol\( \eta \) interact with the IDCL of PCNA? An earlier study reported that the binding affinity of pip peptide sequences from ScPol\( \eta \) and HsPol\( \eta \) with their respective PCNAs ranges from 1.6 to 11 \( \mu M \) (36), which is somewhat similar to the binding affinity between ubz peptide and PCNA (~15 \( \mu M \)) but about ten-fold more than that between ubiquitin and ubz of Pol\( \eta \) (~80 \( \mu M \)) (32, 37, 38). Such a high affinity between pip of DNA polymerase and PCNA is ideal and needed for efficient and processive DNA synthesis (4). Therefore, a pip motif of Pol\( \eta \) may be the preferred site of interaction over the indirect ubz binding to PCNA via ubiquitin for a stable binding and efficient DNA synthesis. Even though the binding affinity of the pip and UBZ peptide sequences to the IDCL region of PCNA is fairly similar, it could be possible that in the context of a full-length Pol\( \eta \) protein, the binding affinity of the pip to PCNA might be greater than that of the UBZ domain. In fact, we find the binding affinity of full-length CaPol\( \eta \) toward PCNA is at least two-fold higher than that of the ubz peptide sequence; it also signifies the importance of the neighboring residues surrounding these motifs in PCNA binding as well. Moreover, our analyses of hybrid Pol\( \eta \) constructs derived from the fusion of various C-terminal regions of Sc- and Ca-Pol\( \eta \) yet again suggested the importance of neighboring residues close to pip or ubz domain to have a decisive role in their PCNA interaction. Thus, it could be possible that in category I Pol\( \eta \), the pip motif preferably binds to the IDCL with high affinity, which in turn leads to a conformational change at the C-terminal domain to preclude binding of ubz/ubm to the IDCL of PCNA; however, the binding of ubm/ubz to the ubiquitin moiety of ub-PCNA still can occur, although such interaction is not a prerequisite and nonessential for TLS activity. However, in category III Pol\( \eta \) such as in Ca- and Sp-Pol\( \eta \) since the pip motif is absent, the ubz domain could directly access the IDCL of PCNA. This is also supported by the fact that the fusion of the C-terminal domain of CaPol\( \eta \) to the catalytic core of ScPol\( \eta \) led to a reduced UV sensitivity of the \( S.\ cerevisiae\ rad30\Delta \) strain (Fig. 4B, sector xiv). Thus, even in \( S.\ cerevisiae\ ) Pol\( \eta \), the ubz domain can bind to PCNA provided the pip is completely absent in the C-terminal domain. Unlike the pip motif that forms a flexible \( 3_{10} \) helix and gets stabilized into the hydrophobic pocket of the IDCL in PCNA trimer, ubz is structurally more organized, consisting of two antiparallel \( \beta \)-strands and an \( \alpha \) helix (4, 32). Since the D626A CaPol\( \eta \) mutant did not bind to PCNA and failed to suppress the UV sensitivity of \( S.\ cerevisiae\ rad30\Delta \) strain, the \( \alpha \)-helix of CaPol\( \eta \) may be the site of PCNA interaction. However, the precise mode of binding of the \( \alpha \)-helix of ubz of CaPol\( \eta \) to the IDCL of PCNA requires further structural investigation. Nevertheless, several cellular proteins such as ubiquitin, PD1P38, Rad18, and WRNIP1/Mgs1 were found to be binding to the ubz of Pol\( \eta \) (32, 39–41). Thus, ubz is undoubtedly a protein–protein interaction module, and our mutational analysis of Ca- and Sp-Pol\( \eta \) evidently supported the certain species-specific direct role of the ubz domain of Pol\( \eta \) in PCNA interaction.

Additionally, this study finds the critical role of the ubz domain of CaPol\( \eta \) in other cellular activities. \( C.\ albicans\ ) is a human pathobiont that exists in several morphological forms and causes superficial to invasive systemic fungal infections in immune-suppressed individuals. Switching its morphology from oval-shaped to pseudohyphal to hyphal structures in response to environmental niche is considered to be one of the virulence determinants of \( C.\ albicans\ ). In our earlier reports, we found the role of catalytic activity of CaPol\( \eta \) in genome stability, filamentation, and antifungal drugs resistance in \( C.\ albicans\ ) (25–28). Herein, we found the ubz domain of CaPol\( \eta \) playing a similarly important role as the catalytic domain of CaPol\( \eta \) in TLS, LOH, and germ tube formation. Since ubz peptide-PCNA mixture failed to show any interaction with Pol\( \eta \), blocking of PCNA-CaPol\( \eta \) interaction either by ubz peptide or any other similar small molecule targeting IDCL of CaPCNA can be explored further for translational implications.

This is the first report to provide conclusive evidence to suggest the essential and direct role of the ubz domain of Pol\( \eta \) in PCNA interaction, in TLS, as well as in other cellular activities. Most probably, it is the position of the ubz domain that determines whether it will interact directly or via ubiquitin to PCNA. Nevertheless, both will make a topological link of Pol\( \eta \) to the chromosomal DNA. This study not only has settled a long-standing controversy over the function of the ubz domain of Pol\( \eta \) but also revealed two modes of PCNA interaction by the various groups of Pol\( \eta \). We conclude that the ubz domain is not a mere protein–protein interaction domain; rather, it is an essential regulatory domain at least in \( C.\ albicans\ ) Pol\( \eta \) and possibly in other category III Pol\( \eta \), which can be targeted to develop therapeutics against pathogenic fungi.

Experimental procedures

Oligonucleotides, peptides, strains, and growth conditions

The oligonucleotides used in this study were procured from Integrated DNA Technologies (IDT). Ubz and its mutant peptides were synthesized with 98% purity and procured from China peptides Co Ltd. The wild-type strains of \( S.\ cerevisiae\ ) EMY74.7 and \( C.\ albicans\ ) SC5314 and BWP17 and their derivatives used for the study are given in Table 3. \( C.\ albicans\ ) and \( S.\ cerevisiae\ ) strains were grown in YPD media with or without DNA damaging agents and on various synthetic dropout media as required.

Generation of Rad30 constructs

The cloning strategy for CaRad30 and ScRad30 has already been described (25, 26). A similar Pfx DNA polymerase-based PCR approach was used for the cloning of other fungal Rad30s. A 30-cycle PCR reaction was carried out using primers NAP349 (5'-CGG GGG ATC ACA ATA TGC CGC TCT CCC CAG AAC C-3') and NAP350 (5'-GGC CGG ATC CTC ATC CAG AAC C-3')
CAAC ACG TAA GTT G-3'), for AnRad30; and NAP343 (5'-CCG GGG ATC AAC CAA AAA GC-3') and NAP344 (5'-GGC GGG ATC TTC ATG TTT CAT AAA CAG CAT ATC G-3') for generating ScRad30. As N. crassa Rad30 contains a single intron within the orf, using its cDNA as a template and NAP438 (5'-CCG GGG ATC AAC CAA AAA GC-3') and NAP347 (5'-CCG GGG ATC AAC CAA AGT TAA TAG GCA AAA GC-3') and NAP348 (5'-CCG GGG ATC AAC CAA AGT TAA TAG GCA AAA GC-3') primers NcPol η orf was amplified. To generate truncated Rad30 orfs, NAP03 and NAP08 (5'-CCG GGG ATC ACC TAC ATG TTT CAT AAA CAG CAT ATC G-3') and NAP343 and NAP345 (5'-CCG GGG ATC AAC CAA AGT TAA TAG GCA AAA GC-3') were used for amplification. The PCR products were cloned in pUC19-CaPCNA template. The PCNA sequence of CaPCNA was used as a template for generating Truncated CaPCNA mutants. The PCR products were cloned at the unique XbaI site of the CaPCNA orf construct.

**Table 3**
List of *S. cerevisiae* and *C. albicans* strains used in the study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source/Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>EMY74.7 (S. cerevisiae)</td>
<td>MATa his3Δ1 leu2-3 leu2-112 trp1Δ ura3-52 (derived from DBY747: his3Δ1 leu2-3 leu2-112 ura3-52 a)</td>
<td>(17)</td>
</tr>
<tr>
<td>YR30.2</td>
<td>rad30Δ</td>
<td>(17)</td>
</tr>
<tr>
<td>YR30.13</td>
<td>rad30Δ revΔ</td>
<td>(35)</td>
</tr>
<tr>
<td>SC5314</td>
<td>Wild-type C. albicans</td>
<td>(26)</td>
</tr>
<tr>
<td>BWP17 (C. albicans)</td>
<td>ura3::imm434/ura3::imm434,iro1::iro1::imm434,his1::hisG/ his1::hisG, arg4/arg4</td>
<td>(35)</td>
</tr>
<tr>
<td>CNA11</td>
<td>rad30Δ</td>
<td>This study</td>
</tr>
<tr>
<td>CNA26</td>
<td>rad30Δ, MET3p-URA3</td>
<td>This study</td>
</tr>
<tr>
<td>CNA27</td>
<td>rad30Δ, MET3p-CaRad30-URA3</td>
<td>This study</td>
</tr>
<tr>
<td>CNA28</td>
<td>rad30Δ, MET3p-CaRad30 1-610 aa-URA3</td>
<td>This study</td>
</tr>
<tr>
<td>CNA35</td>
<td>rad30Δ, MET3p-CaRad30 H624A,H628A-URA3</td>
<td>This study</td>
</tr>
</tbody>
</table>

**Generation of CaPCNA mutant constructs**

Inverse PCR was carried out using the primer set NAP224 (5'-GGC GGG ATC AAC CAA AAA GC AAC GTG AAT ATT CTT TAA AAT TA... (17)

**Generation of BWP17 homozygous rad30ΔΔ strain and expression of various mutants of CaPol η**

The BWP17 *C. albicans* strain is an auxotrophic isogenic derivative of SC5314. Deletion of the RAD30 gene in BWP17 was carried out as in the SC5314 strain of *C. albicans* (26). Briefly, two RAD30 deletion cassettes with a nourseothricin marker (pNA1389 and pNA1451) were generated, containing different lengths of upstream sequences but the same downstream sequence to facilitate an efficient homologous expression systems either with amino-terminal GST or carboxyl-terminal His-tagged CaPCNA mutants, similar to CaPCNA expression constructs (29, 34).
Role of ubz domain

knockout. Two successive rounds of transformation, followed by curing of the nourseothricin marker by maltose, resulted in the homozygous deletion of *C. albicans* strain CNA11. To express wild-type CaRad30, CaRad30 (1–601), and CaRad30 H624A, H628A mutants under repressible MET3 promoter, various constructs were integrated into the RP10 locus of CNA11 strain, and the transformants were selected by URA3 marker, and their expression was induced in the absence of methionine in synthetic dropout media.

Bioinformatics analysis

Seventy-seven Polη amino acid sequences from various organisms were taken from the NCBI database and aligned using the t-coffee multiple alignment tool. Further, they were segregated into three groups based on homology and presence or absence of critical pip/ubz motifs and again aligned to get final alignments. The amino acid sequence of *C. albicans* Polη was aligned with that of ScPolη by using pairwise sequence alignment tool, EMBOSS Needle (http://www.ebi.ac.uk/Tools/psa/emboss_needle/) to map the pip motif. Ubz model structures for CaRad30 and ScRad30 were generated by using FFAS (http://ffas.burnham.org/ffas-cgi/cgi/ffas.pl) online modeling database. The ubz domain of HsPolη with PDB ID 3WUP was used as a template for modeling. Secondary structure information was derived by observing the generated model.

Purification of recombinant proteins

PCNA protein expression was carried out in *E. coli* BL21-DE3 strain as described earlier (33, 34), whereas wild-type and truncated CaPolη was purified in YRP654 protease deficient *S. cerevisiae* strain (42).

Physical interaction by ITC

All the purified proteins and purchased peptides were dia-lyzed overnight against 1 l of buffer containing 20 mM HEPES (pH 7.4) and 150 mM NaCl at 4 °C to ensure complete removal of DTT and glycerol from the protein storage buffer, which could affect the heat exchange while they interact. ITC assays were performed using a Malvern MicroCal PEAQ-ITC calorimeter. Before the experiment, the cell and the syringe were thoroughly washed with water, followed by cell rinsing with a buffer. A control run was carried out to make sure that the buffer is not participating in heat change where the cell was filled with 300 μl of buffer and concentrated CaPCNA or ScPCNA protein (200 μM) in the syringe. ITC was performed using various Polηs or UBZ peptide or UBZ mutant peptide in the sample cell and CaPCNA or ScPCNA or a mixture of CaPCNA+ WT UBZ peptide in the syringe. Twenty-five injections of 1.5 μl of protein from the syringe were given at intervals of 120 s with an initial delay of 120 s at 25 °C. The data were analyzed to determine the various kinetic parameters using a single-site binding model provided in the ITC analysis software package. The experiments were repeated twice for the positive interactions, whereas for the negative interactions, those were repeated multiple times at various concentrations of protein or peptide in the cell to confirm that there is no binding.

GST pull-down assay

GST-fused wild-type or mutant Polη proteins bound to glutathione sepharose beads were mixed with 0.5 μg of His-tagged wild-type or mutant CaPCNA, and a pull-down experiment was carried out using a standardized protocol, described previously (43). Then the beads were thoroughly washed thrice with ten volumes of equilibration buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM dithiothreitol, 0.01% NP-40, and 10% glycerol). Finally, the bound proteins were eluted with a 50 μl SDS loading buffer. Various fractions were resolved on a 12% SDS-PAGE, followed by Western blot using an anti-His antibody (Catalog no. H-1029, Sigma-Aldrich) in 1:1000 dilution. After washing thrice, the blot was incubated with an anti-mouse secondary antibody (Catalog no. A-9044, Sigma-Aldrich). Then it was developed after three washes.

Size-exclusion chromatography

For size-exclusion chromatography, about 10 μg of purified proteins (CaPCNA and CaPolη) was incubated together at room temperature for 60 min, loaded onto a Superdex 200 PC3.2/30 column preequilibrated with a buffer containing 50 mM HEPES, pH 7.5, 150 mM NaCl and 10% glycerol. Chromatography was performed on an AKTA pure M system (GE Healthcare) at a flow rate of 0.05 ml/min at 4 °C, and the absorbance was monitored at 280 nm.

UV sensitivity and UV mutagenesis

The rad30Δ and rad30Δ rev3Δ genomic knockout strains of *S. cerevisiae* harboring either wild-type or various mutant Rad30s were grown in dropout medium lacking Uracil (SD-Ura) to maintain selection for the plasmid. Knockout strains with empty vector YEpplac195-ScADH1p (2μ, URA3) were also grown as a control. Cultures were grown up to the mid-logarithmic phase, washed with water, and resuspended to a density of 2 × 10⁸ cells/ml. Desired dilutions of cells were spread onto the surface of SD-Ura plates for viability determination and onto SD-Arg plates containing canavanine for mutagenesis assays (17). UV irradiation was carried out with different doses of UV. After UV irradiation, the plates were incubated in the dark, and colonies were counted after 3 days.

Filamentation test

Overnight grown culture of the different strains of *C. albicans* cells was inoculated in a synthetic dropout medium lacking methionine but containing 10% FBS and incubated at 37 °C for 1 h. Cells were observed under a Leica microscope with 40× magnification. A total number of 100 cells were counted for each strain to calculate the percentage of germ tubes and one-three chained cells in three independent experiments. Similarly, the measurement of germ tubes (n = 23) length was performed for each strain using ImageJ software. All the graphs were made using the GraphPad Prism 8.0 software.
Alkaline agarose gel electrophoresis

The total genomic DNA was isolated from different strains of *C. albicans* and resolved by alkali agarose gel electrophoresis as described earlier (44). Briefly, an equal number of cells were exposed to 16 J/m² UV, and further, they were allowed to recover in fresh media. After subsequent recovery at various time points 0, 6, 12, and 24 h, cells were harvested. Cells were lysed by glass beads and genomic DNA was extracted by removing the impurities by phenol: chloroform: isoamyl alcohol treatment. A 0.7% agarose gel was prepared in 50 mM NaCl and 4 mM EDTA, and the gel was pre-soaked in alkali electrophoresis buffer (30 mM NaOH and 2 mM EDTA) overnight at room temperature prior to running. An equal volume of DNA was loaded for each sample into the wells and allowed to separate at 0.25 V/cm until the dye migrated to 60% of the length of the gel. Agarose gel was neutralized in 500 ml of 0.5 M Tris-HCl, pH 8.0 at room temperature for 1 h, and finally, the gel was stained with ethidium bromide, and the image was captured in a Chemidoc Imaging system (Bio-Rad).

5′ FOA-mediated loss of heterozygosity assay

*C. albicans* cells expressing different forms of Polη were grown in nonselective liquid medium (YPDU) to an OD₆₀₀ of ~1, and the serial dilutions of their cells (10⁵, 5 x 10⁴, 10⁴, etc.) were spread onto SD+5′-FOA (1 μg/μl) and YPDU plates. After plating, one of the sets of plates was exposed to 16 J/m² UV and wrapped in aluminum foil. All the plates were incubated for 3 to 4 days at 30 °C. FOA-resistant colonies were counted, and some of the resistant colonies were picked up and restreaked on SD-Ura, SD-Arg, and SD-His plates to ascertain the genotypes.

Data availability

All data presented is contained within this manuscript.

Supporting information—This article contains supporting information.

Acknowledgments—We thank Mr Sitendra Prasad Panda for his technical assistance and our laboratory colleagues for their thoughtful discussion.

Author contributions—N. A. conceptualization; K. M., P. K., S. K. P., and J. K. S. formal analysis; N. A. writing—original draft; N. A., P. K., S. K. P., and J. K. S. writing—review and editing.

Funding and additional information—CSIR-Senior Research Fellowships to K. M., P. K., and J. K. S. are highly acknowledged. S. K. P. thanks ILS for his PhD fellowship. This work was supported by the intramural core grant from ILS, and intramural research funds from DBT and DST India on various occasions during the study.

Conflict of interest—The authors have no conflicts of interest to declare regarding the publication of this article.

Abbreviations—The abbreviations used are: FBS, Fetal bovine serum; IDCL, Interdomain connecting loop; ITC, Isothermal calorimetry; PAD, Polymerase associated domain; pic, PCNA interacting motif; Pol, DNA polymerase; Rad30 (Polη), DNA polymerase eta; ubz, Ubiquitin zinc finger; UDB, Ubiquitin-binding domain; XVP, Xeroderma pigmentosum variant.

References

Role of ubz domain


