Supporting Online Material for

Ubiquitin-Binding Domains in Y-Family Polymerases Regulate Translesion Synthesis

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Supporting online material

Material and Methods

Yeast two-hybrid system

Sequences corresponding to Ub and Ub-I44A with deletions of the two last glycines, were subcloned in pYTH9 (*1*), creating fusion proteins with the Gal4 DNA-binding domain. Deletions of the two last glycines prevent Ub or Ub-I44A conjugation to other targets in yeast cells thus facilitating non-covalent interactions with Ub as a bait. These vectors were introduced into Y190 yeast strain and human fetal brain, spleen, thymus and kidney cDNA libraries (Clontech) were screened as described (*1*).

Antibodies, plasmids, cells and immunofluorescence

Antibodies used were: anti-HA (12CA5, Roche), anti-FLAG (M2 and M5, Sigma), anti-GFP (BD Living Colours), anti-PCNA (PC10, Santa Cruz Biotechnology), anti-Ub (P4D1, Santa Cruz Biotechnology) (all mouse monoclonal) or anti-polη raised in rabbits against the full-length protein (*2*) and rabbit polyclonal anti-polι (*3*) kindly provided by Roger Woodgate.

The expression construct encoding human wild-type polη was generated by polymerase chain reaction (PCR) and inserted into pcDNA3-N-FLAG. Similarly, a cDNA encoding full-length *Xenopus* polι was subcloned into pEGFP-C3 (Clontech). All deletion and point mutants of polι and polη were generated by PCR. Constructs for bacterial expression of single or both UBMs of polι were generated by PCR and inserted into pGEX-4T-1 (Amersham). The PCNA*-Ub and polη-Ub chimerae were generated by subcloning the cDNAs for a K29A, K48A, K63 mutant of Ub that also lacked the last two glycine residues (*4*), in frame with the 3' terminus of PCNA-K164R and polη subcloned in pcDNA3-His and pcDNA3-N-FLAG, respectively. All constructs were verified by DNA sequencing. Constructs for mammalian expression

of His-PCNA and His-PCNA* were previously described (*2*). Expression plasmids containing mouse FLAG-polι, and human HA-Rad18 were kindly provided by Errol Friedberg and Satoshi Tateishi, respectively.

HEK293T cells were purchased from the American Type Culture Collection (ATCC). XP30RO(sv) (XPV) cells, and their complementation to make XP30RO-pcDNA3.1-polη cells, have been previously described (*5, 6*). Co-expression of wild type and mutant pols in MRC5 human fibroblasts and visualisation by indirect immunofluorescence was performed as described (*6*), but using confocal midsections acquired with a Zeiss LM510meta microscope. XP30RO, XP-V fibroblasts were transfected with indicated cDNAs using Fugene (Roche) and the survival of cells upon UV irradiation was measured by colony-forming ability. HEK293T cells were transfected using the Lipofectamine Transfection Reagent® (Invitrogen) and when indicated, cells were either mock-treated or treated with appropriate stimuli and lysed in indicated lysis buffers.

Biochemical assays

Immunoprecipitation, immunoblotting and *in vitro* pull-down experiments were performed as described (*1,2,4*). Monoubiquitination of endogenous PCNA was induced in HEK293T cells by transfection with HA-Rad18 (the E3 ligase for PCNA) and treatment with 10 mM hydroxyurea (HU) (Sigma) for 16 hours prior to lysis. Cells were then lysed in RIPA buffer 0.1% SDS and treated with 10 units of RNase-free DNase I (Roche) to release the PCNA fraction bound to chromatin.

For ubiquitin-binding assays, HEK293T cells were transfected with the indicated constructs, lysed in 20 mM HEPES (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 25 nM NaF, 1% Triton-X-100, 10% glycerol, 10 μM $ZnCl_2$ and protease inhibitors and incubated overnight at +4°C with either GST-Ub or GST alone coupled

to Glutathione Sepharose™ 4B (Amersham Biosciences). After incubation, the sepharose matrix was washed 4 times with lysis buffer. Bound proteins were then boiled for 5 minutes in SDS sample buffer and analysed by SDS-PAGE and immunobloting. For polyubiquitin pull-down experiments, ubiquitin chains of K48 and K63 type containing a mixture of chains of different length (2-7 Ub moieties/chain; Boston Biochem) were mixed with either GST-UBZ of polη or GST-UBM1,2 of polι in a buffer containing 25 mM Tris pH 7.45, 50 mM NaCl, 0,1% Triton-X-100, 2 mMDTT and 10 µl of $ZnCl_2$.

***In vitro* transcription/translation and primer extension assays**

FLAG-polι was amplified by PCR from pCMV-FLAG-polι using primers contaning XhoI and BamHI sites. The product was cloned into pcDNA3.1(-).0.5 µg of pcDNA3.1(-)-FLAG-polι or pcDNA3-FLAG-polη was added to a Promega rabbit reticulocyte T_NT *in vitro* transcription/translation reaction with ^{35}S-methionine for 2 hours at 30^{o}C. Products were analysed by SDS-PAGE and immunobloting.

FLAG-tagged proteins from 5 µl of each T_NT reaction were purified using M2 anti-FLAG agarose beads (Sigma) according to the manufacturer's instructions. After washing in PBS the beads were mixed with reaction buffer, dNTPs and a labelled 16mer oligonucleotide primer probe annealed to a 30mer template. Samples were then incubated for 10 min at 37^{o}C. The products were resolved by denaturing polyacrylamide gel electrophoresis (8 M urea, 15% acrylamide).

Protein expression, labeling and purification

Uniformly ^{15}N-labeled Ub and Ub-I44A were expressed in BL21D3 Star cells (Invitrogen) in M9 media and purified according to Beal et al. (*7*). GST and GST-tagged proteins were expressed in the same strain in LB media and purified using

glutathione (GSH) beads. The NMR samples were prepared by a final gel filtration step either on a Superdex S75 column (Ub) or a Superdex S200 column (GST and GST-tagged proteins) in 15 mM phosphate buffer (pH 6.15), 125 mM KCl, 2 mM CHAPS, 150 μM PMSF and 0.02% NaN_3. The proteins were concentrated using Amicon Centricons (Millipore) to either 700 μM (Ub) or 3 mM (GST and GST tagged proteins). The concentration of the denatured proteins was determined from UV measurements at 280 nm. For the NMR measurements D_2O was added to a final concentration of 5%.

NMR titrations

All NMR titrations were performed at 30°C as a series of [^{1}H-^{15}N] correlation spectra recorded on Ub and Ub-I44A, which were both labelled with ^{15}N. The spectra were recorded on a 500MHz and on a 750MHz Bruker spectrometer and the chemical shift changes upon titration were monitored. The initial sample concentration of Ub and Ub-I44A was 700 μM. GST, GST-UBM1 and GST-UBM2 were added as small aliquots of a concentrated solution up to a 5-fold molar excess over Ub where the chemical shift changes were almost maximal. The chemical shift change Δδ were calculated as $\Delta\delta = 1/2\ [(\Delta^1H)^2 + (\Delta^{15}N/5)^2]$, where Δ^1H and Δ^{15}N are the chemical shift differences between ^{1}H or ^{15}N signals, respectively, of corresponding amide moieties. The Ub NMR assignment was obtained from BMRB (4769).

Determination of the dissociation constant K_d from titration curve fitting

The chemical shift changes Δδ observed in Ub and Ub-I44A upon titration with GST-UBM1 and GST-UBM2 were analyzed assuming one binding site in Ub. Therefore, at any point in the titration, the Δδ of signals of Ub can be represented as $\Delta\delta = \Delta\delta_{max} + K_d\ (\Delta\delta/[L])$, where $\Delta\delta_{max}$ is the difference in the chemical shift between the free and

fully bound states for a given amide and [L] is the molar concentration of the free ligand. The data fitting included K_d and $\Delta\delta_{max}$ as adjustable parameters.

References for Materials and Methods:

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Supplementary Figure Legend:

Fig. S1 (A) Yeast cells expressing a C-terminal fragment of polι that interacts with either ubiquitin (Ub) or Ub with isoleucine 44 mutated to alanine (Ub*) grow in selective conditions and are stained by β-galactosidase while yeast transfected with an empty vector (bait) do not (left panel). The Total Cell Lysate (TCL) of HEK293T cells containing FLAG-tagged full-length mouse polι was subjected to pull-down assays with GST, GST-Ub and GST-Ub* coupled to sepharose and the bound proteins were analyzed by immunoblotting with anti-FLAG antibodies. Mouse polι bound to GST-Ub or GST-Ub I44A, but not to GST alone (right panel). **(B)** Alignment of representative UBMs. Highly conserved residues are shown on black or grey background. Species abbreviations used: Hs, *Homo sapiens*; Mm, *Mus musculus*; Dm, *Drosophila melanogaster*; Sp, *Schizosaccharomyces pombe*; Sc, *Saccharomyces cerevisiae*. The numbers indicate the position of the first amino acid of each UBM in the sequence. (**C**) Deletion of either UBM1 (ΔUBM1) or UBM2 (ΔUBM2) of polι reduces its binding to Ub, whereas deletion of both UBM domains (ΔUBM1,2) completely abolishes binding of polι to Ub in GST pull-down assays. **(D)** Plot of chemical shift changes Δδ in NMR spectra of Ub and Ub-I44A upon titration with UBM1 (upper panel) or UBM2 (lower panel). Representative binding curves for the residues T9, I13 and Q49 are shown for UBM1 and I13, Q49 and L71 for UBM2.. The K_d values obtained from the individual curves are indicated on the right. (**E**) Alignment of selected UBZ motifs. Highly conserved residues are shown on black or grey background, whereas amino acids involved in Zn-coordination are shown in red. Species abbrevations as in fig. S1B, but additionally: Xl, *Xenopus laevis*; Nc, *Neurospora crassa*; Eg, *Eremothecium gossipy*; An, *Aspergillus nidulans*; Pa,

Podospora anserina. (**F**) Schematical representation of human Y-family polymerases and the presence of UBM or UBZ domains in these polymerases. CD-catalytic domain; BRCT-BRCA1 C-terminal domain. (**G**) K48- or K63-linked polyubiquitin chains are pulled down by UBZ of polη or UBM1,2 of polι fused to GST.

Fig. S2 UBM domains in *Xenopus* polι mediate binding to Ub and are required for accumulation in replication foci. **(A)** Sequence alignment of human and *Xenopus tropicalis* polι. The human sequence (hPolι) is from the NCBI database NP_009126; the *Xenopus tropicalis* sequence is submitted to GenBank under the accession number DQ102380. UBMs are underlined **(B)** EGFP-*Xenopus* polι interacts with GST-Ub in pull-down assays. **(C)** Localization of *X. tropicalis* polι in foci. MRC5 cells were transfected with plasmid containing full-length *X. tropicalis* polι N-terminally tagged with EGFP. The cells were UV-irradiated (7 Jm^{-2}) and fixed 8 hours after UV-irradiation.

Fig. S3 (A) Deletions of UBMs or point mutation in the UBZ domain do not change the enzymatic activity of polι and polη, respectively. Polη is capable of extending the primer to the end of the template resulting in a 30mer product. Polι is an extremely error prone and non-processive polymerase and is only capable of adding a single nucleotide to the primer under reaction conditions used here. (B) *In vitro* binding of GST-UBM1,2 of polι to monoubiquitinated PCNA or PCNA*-Ub chimera. HEK293T cells were transfected with the indicated constructs and lysates were subjected to GST pull-down assays with GST-UBM1,2. Bound proteins were analyzed by immunoblotting with anti-PCNA antibodies. Total cell lysates (TCL) show the level of expression of corresponding proteins. UBM domains bound directly to PCNA*-Ub chimera as well as monoubiquitinated His-PCNA but not unmodified His-PCNA (compare lanes 4 and 5). Small amount of non-ubiquitinated His-PCNA

precipitated with GST-UBM domains due to hetero-trimerization of ubiquitinated and non-ubiquitinated PCNA monomers in the same lysate. **(C)** HEK293T cell lysates (TCL) expressing FLAG-polη or FLAG-polη-Ub chimera were subjected to pull-down assays with Ub-agarose. Bound proteins were detected by immunoblotting (IB) with anti-FLAG antibodies. The arrow indicates monoubiquitinated form of polη.

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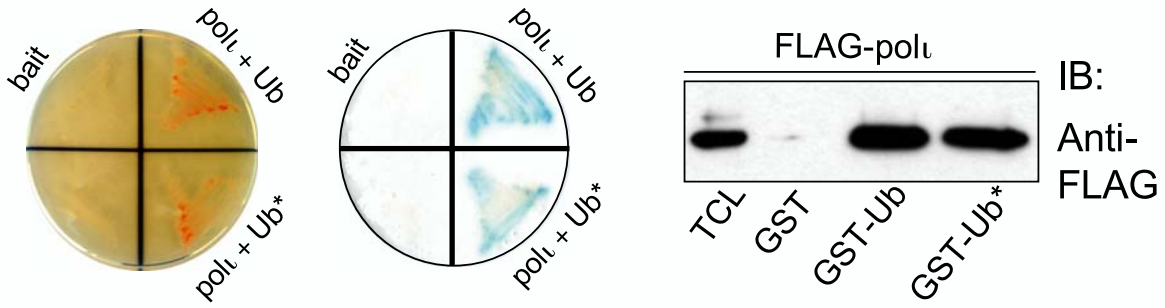
Supplementary Figure 1

A



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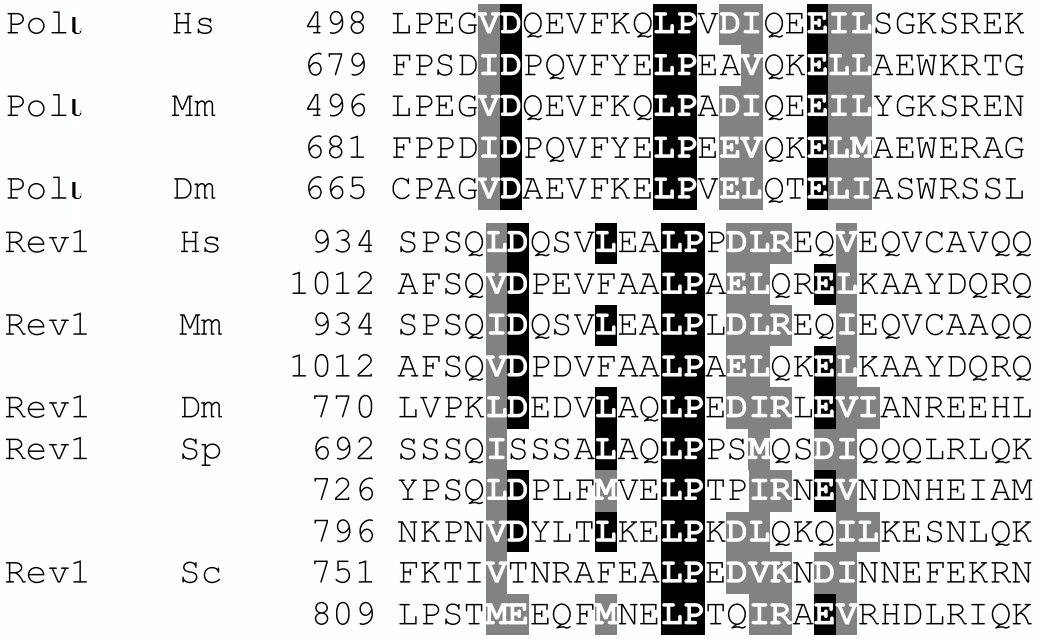

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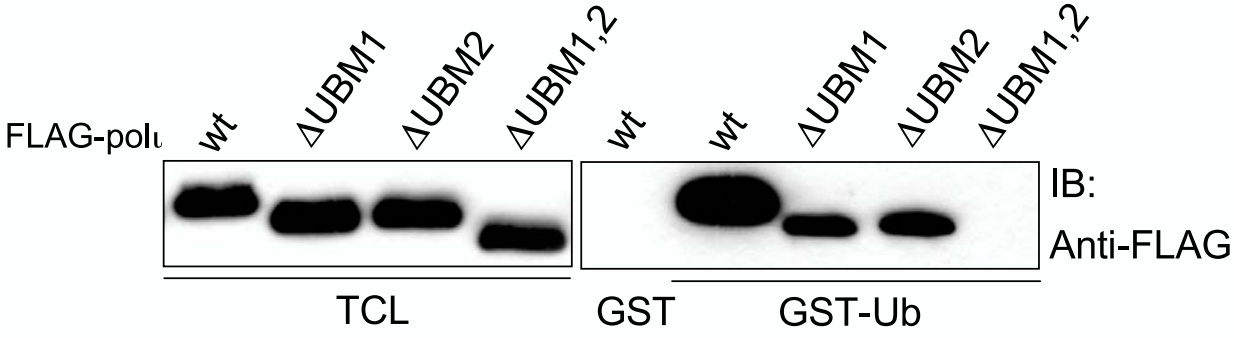

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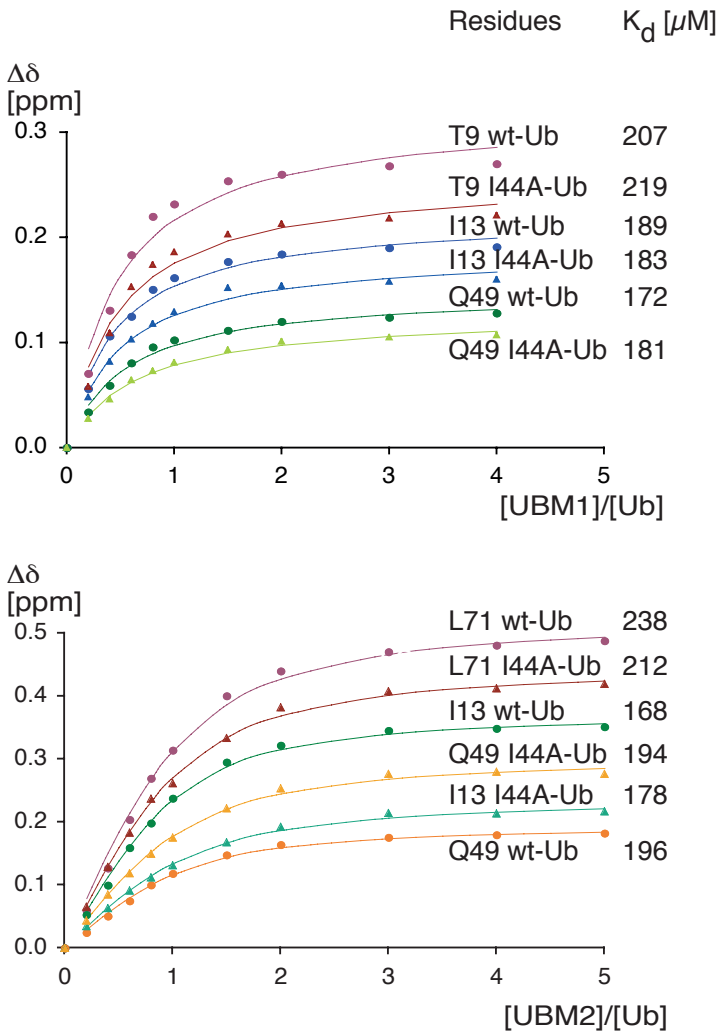
Supplementary Figure 1

D



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Supplementary Figure 1

E

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Pol η    hs    DQVPCEKCGSLVPVW.....DMPEHMDYHFALEL \
Pol η    mm    DQVLCEKCDSLVPVW.....DMPEHTDYHFALEL |
Pol η    xl    DIMCCEKCGLKLLVW.....EIPEHMDYHFAQEL |
Pol η    dm    PTIKCDQCGANIPDEVK...SLQTHRDHHFAQEL |
               QMNQCPECKAFIKCV.....DMPEHLDYHVAKNL | UBZ3
eso1     sp    ETYTCEECEQKITLS.....ERNEHEDYHIALSI |
rad30    nc    ITLTCSRCNASLDSPE....ELQSHQDWHFAKEL |
rad30    eg    AAAACPQCRAPLPSQR....ALQVHLDGHLAQKL |
rad30    sc    PKLECCKYQVTFTDQK....ALQEHADYHLALKL /

Pol κ    hs    QILTCPVCFRAQGCISL..EALNKHVDECLDGPS \
               QALVCPVCNVEQKTSDL..TLFNVHVDVCLNKSF |
Pol κ    mm    QTFICPVCFREQEGVSL..EAFNEHVDECLDGPS |
               RALVCPVCNLEQETSDL..TLFNIHVDICLNKGI |
Pol κ    gg    EIFTCPVCFEEQSSSNL..EEINRHVDECLAGSL | UBZ4
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Pol κ    xl    PEFTCPICFLKESGWDI..ATFNKHIDKCLSGSP |
               QAFICPVCNLRQNTPDL..TAFNRHVDVCLNKGI |
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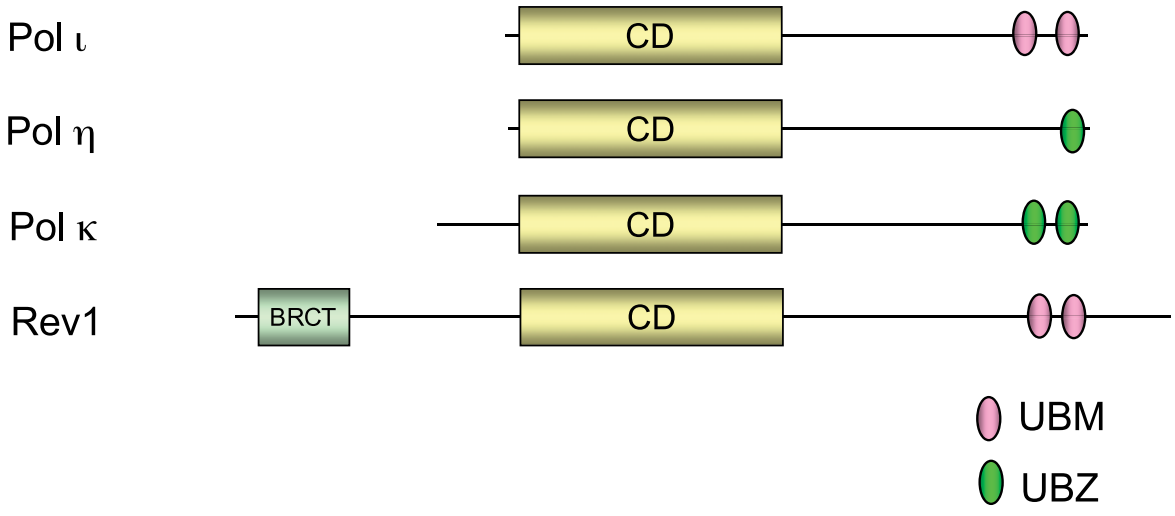
F



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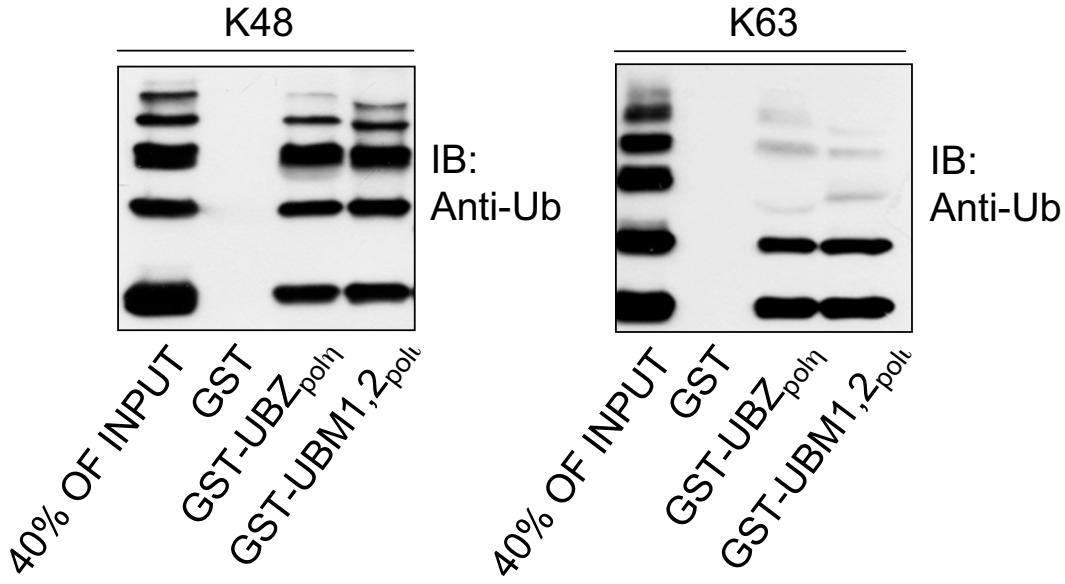
Supplementary Figure 1

G



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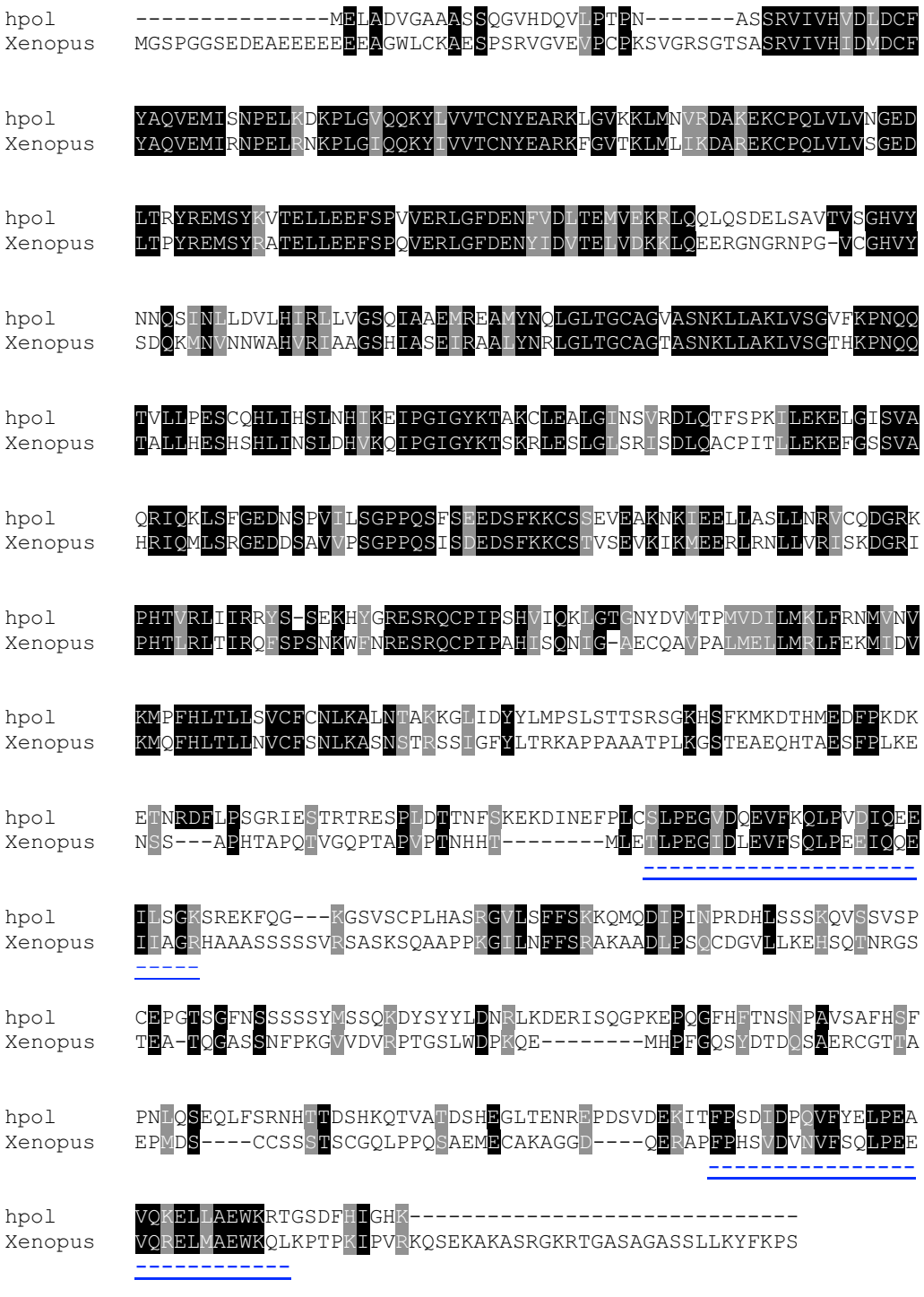
Supplementary Figure 2

A



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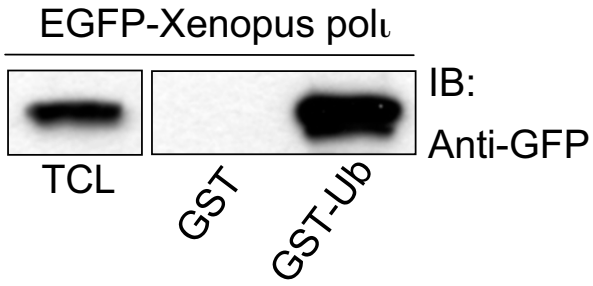
Supplementary Figure 2

B



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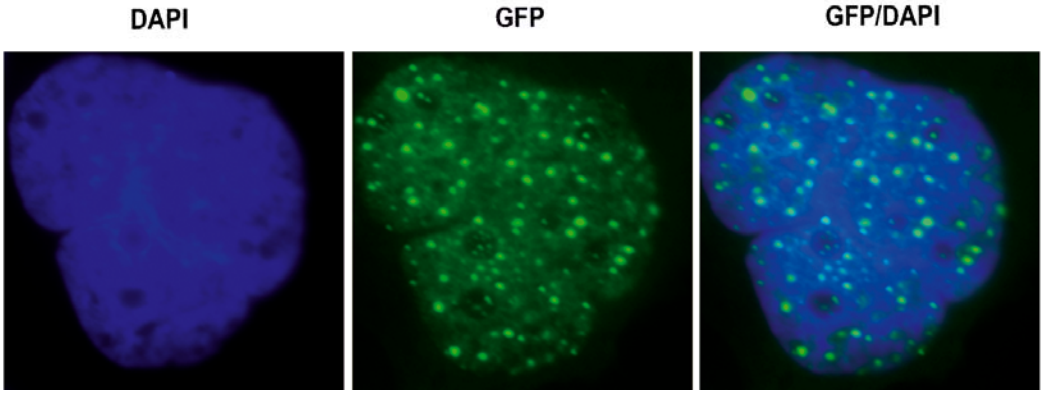

EGFP-Xenopus polι

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Supplementary Figure 3