Supporting Information

Ubiquitin chains bearing genetically encoded photo-cross-linkers enable efficient covalent capture of (poly)ubiquitin-binding domains.

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Materials and Methods

All restriction enzymes used were purchased from New England Biolabs. HisLink™ protein purification resin was purchased from Promega. p-Benzoyl-L-phenylalanine was purchased from Amatek Chemical. BLAK-RAY® longwave ultraviolet lamp was used for irradiation. We were also successful in using a commonly available, battery powered 365 nm LED "flashlight" for di-Ub photo-crosslinking reactions.

Expression Plasmid

Plasmids pET11a-SynUb-His_6, encoding an amber stop codon (TAG) at the position to be targeted for mutagenesis, were created by site directed mutagenesis. The pET11a-SynUb-His_6 mutants were co-transformed with, pSUP-pBpa-2, containing cellular machinery to incorporate p-benzoyl-$_L$-phenylalanine as a genetically encoded photo-crosslinking amino acid at the TAG codon, into BL21(DE3) electrocompetent (EC) cells. To 100 µL of thawed BL21(DE3) EC cells, 1 µL of each plasmid was added. The cells were transferred to a cuvette and electroporated. Cells were recovered with 1 mL of SOC and incubated for 1 h at 37°C. Cells were harvested by centrifugation and 900 µL of SOC was decanted. The cell pellet was resuspended in the remaining 100 µL of SOC and plated on a LB agar plate containing ampicillin (100 µg/mL) and chloramphenicol (35 µg/mL). Plates were incubated overnight at 37°C. The purity of the proteins was confirmed by SDS PAGE (Figure S1).

Protein expression and purification

pET11a-SynUb-His_6 mutants were co-transformed with pSup-pBpa-2 into *E. coli* BL21(DE3) cells. (SynUb is synthetic Ub gene codon optimized for E.coli expression.) A single colony was grown in 5 mL overnight starter culture. The starter culture was inoculated into 100 mL (or 1 L) LB broth (Amp100/Chl35), and the cells grown to an OD600 ~ 0.6 and Bpa added to a final concentration of 1mM and induced with 1 mM IPTG. Bpa is typically dissolved in 1M NaOH, and is stable in all media tested. The cells were then incubated overnight at 37°C. The harvested cells were re-suspended in Ni-NTA binding buffer (50 mM NaH_2PO_4, 300 mM NaCl and 10 mM imidazole, pH 8.5). Lysozyme (1 mg/mL) and PMSF (1 mM) were added to the supension and incubated on ice for 30 min. The cells were sonicated on ice and centrifuged to remove cellular debris. Briefly, a batch mode nickel purification was performed. Lysate was added to HisLink™ protein purification resin which was pre-equilibrated with 1X Ni-NTA bind buffer,

and mixed gently by rotating at 4°C for 1 hr. The resin was washed with 1X Ni-NTA wash buffer (50 mM NaH_2PO_4, 300 mM NaCl and 20 mM imidazole, pH 8.5). Protein was eluted with 1X-Ni NTA elution buffer (50 mM NaH_2PO_4, 300 mM NaCl and 500 mM imidazole, pH 8.5). Purified yields for Ub variants containing Bpa were approximately 2-10mg/L of culture, and variable for the position being mutated. A comparison of the expression yields can be seen in Figure S1. Ub variants not containing Bpa (for Ub dimer assembly) were expressed and purified as detailed elsewhere.[1,2] The UBA2 domain of hHR23A was expressed as GST fusion and purified as previously described[3], the UBA domain of human ubiquilin-1 was expressed and purified as described previously[4], and the tandem-UIM motif of Rap80 was expressed and purified as detailed before.[5]

Assembly of Bpa-containing Ub dimers. K48- and K63-linked diUbs were assembled from recombinant Ub monomers using enzymatic reactions catalyzed by Ub-activating enzyme E1 and linkage specific conjugating E2 enzymes E2-25K (aka UB2K) for K48-linked diUb and Ubc13/Mms2 for K63-linked dimer as detailed previously.[1–3] The reactions were run overnight at 30°C and stopped by adding a few drops of glacial acetic acid. The products (diUbs) were separated from unreacted monomers using size-exclusion or cation chromatography. Ub monomers containing chain-terminating mutations (K48R for K48-linked diUb and K63R for K63-linked diUb) were used in order to control the position of the Bpa-containing Ub in the chain and the chain length. In order to incorporate Bpa in the distal Ub, the corresponding monomer contained two mutations: Bpa at a desired position and K48R or K63R, and the C-terminal His_6 tag was removed to allow activation of this Ub variant by E1.

Photo-crosslinking between monomeric UbBpa-His_6 variants and hHR23A_UBA2(UBA2) or ubiquilin-1 UBA (UBAy).

Photo-crosslinking reactions were performed in 1X PBS buffer, pH 7.4 on ice. 50 µL reactions containing: 10 µg each of UbBpa-His_6 and UBDs (UBA2 or UBAy). Reactions were incubated on ice for at least 1 hr. Half of the reaction was transferred to a chilled 96-well plate on ice and subjected to UV irradiation (λ=365 nm) using a stationary UV lamp for 30 min. Non-exposed reaction samples and UV irradiated samples were observed using an 18% or 20% SDS-PAGE gels and visualized via silver or Coomassie staining.

Photo-crosslinking between Bpa-containing Ub dimers and hHR23A_UBA2 (UBA2), Rap80 tUIM, or Rpn1

Photo-crosslinking reactions with Bpa-containing Ub dimers were performed in 20 mM sodium phosphate buffer, pH 6.8, on ice. 50 µL of buffer typically containing 15 µM diUb and 60 µM of the UBD (UBA2, Rap80 tUIM, or $Rpn1^{391-642}$) were mixed and transferred to a 96-well plate on ice. 10 µL were removed as a non-exposed control, and the remaining solution was subjected to UV irradiation (λ=365 nm) for 35-45 minutes. Non-exposed reaction samples and UV-irradiated samples (10 µL) were analyzed using 15% SDS-PAGE gels and visualized via silver or Coomassie staining.

NMR studies

NMR experiments were performed on a Bruker Avance–III 600 MHz spectrometer equipped with cryoprobe. All experiments were performed at 23°C. The buffer for all NMR samples contained 20 mM sodium phosphate, 0.02% NaN_3 and 7% D_2O, the pH was 6.8.

Amide signal shifts for each residue were quantitated as chemical shift perturbations (CSP) using the following equation:

$$\Delta\delta = \sqrt{{\Delta\delta_H}^2 + \left(\Delta\delta_N / 5\right)^2}\,, \qquad (1)$$

where $\Delta\delta_H$ and $\Delta\delta_N$ are shifts of the ^{1}H and ^{15}N resonances, respectively.

NMR binding experiments were performed by titrating unlabeled Ub or Ub variants into ^{15}N-labeled UBAy (starting concentration 40 μM) and monitoring shifts in UBAy signals using ^{1}H-^{15}N SOFAST-HMQC spectra recorded at each titration step. The dissociation constant, K_d, for Ub binding to UBA was obtained by fitting the measured CSP values ($\Delta\delta$) in UBA to a single-site binding model[3]:

$$\Delta\delta = \Delta\delta_{max}\left\{[P]_t + [L]_t + K_d - \sqrt{\left([P]_t + [L]_t + K_d\right)^2 - 4[P]_t[L]_t}\right\} \Big/ \left(2[P]_t\right). \qquad (2)$$

where $\Delta\delta_{max}$ is the CSP at saturation and $[P]_t$ and $[L]_t$ are the total concentrations of the protein (UBA) and the ligand (WT Ub of Bpa-containing Ub variants), respectively. K_d and $\Delta\delta_{max}$ were treated as global fitting parameters using an in-house Matlab program Kdfit.[3]

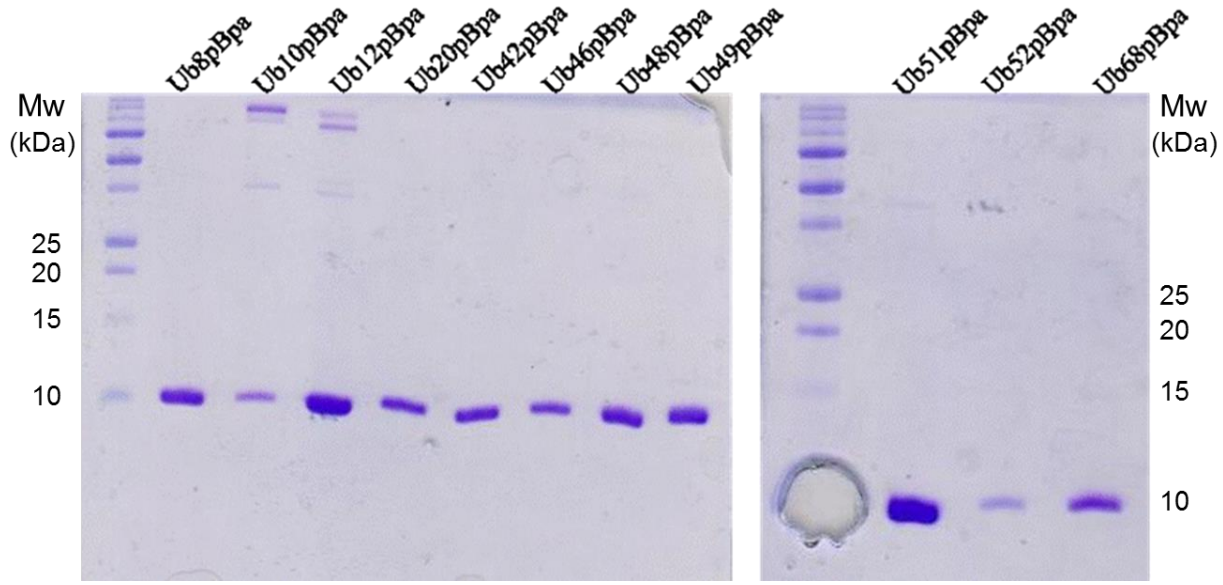


Figure S1. SDS-PAGE gel of UbXpBpa-His_6 mutants. Other successfully purified mutants not shown were Ub9pBpa, Ub11pBpa, and Ub70pBpa.

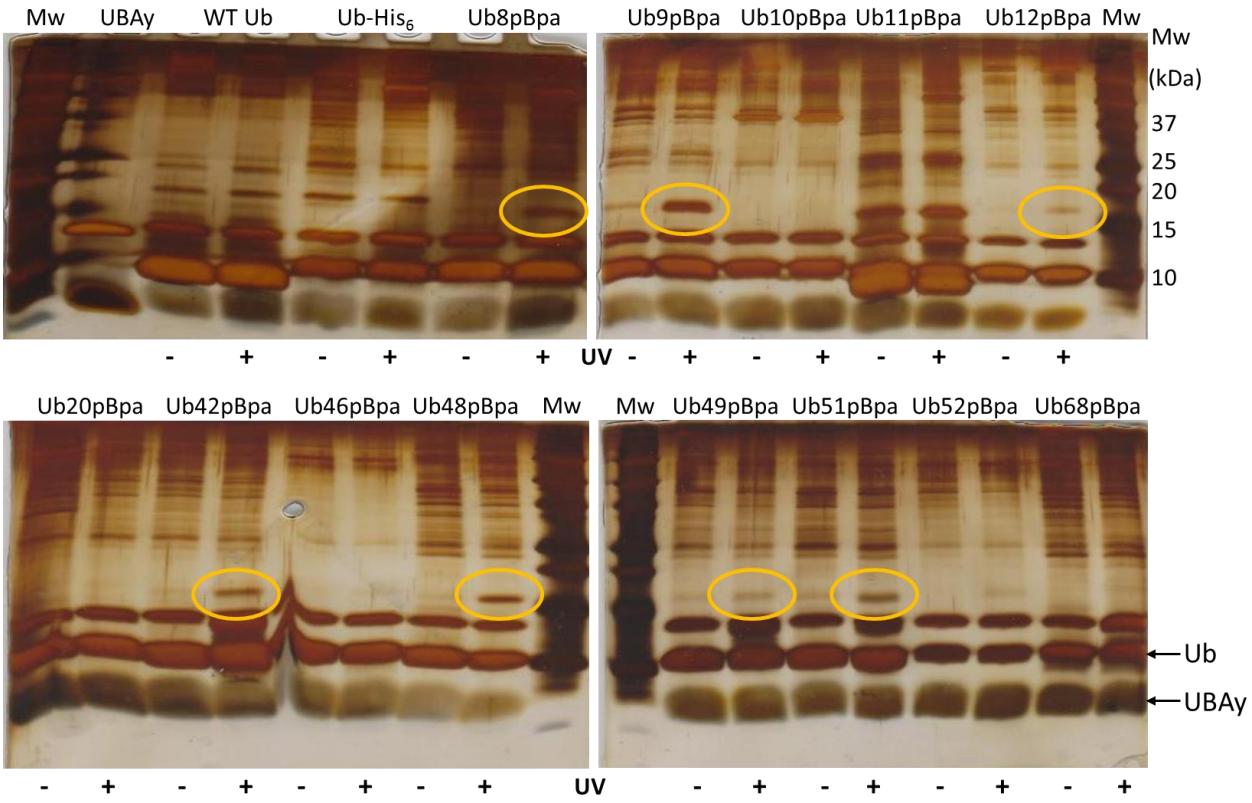


Figure S2. Photo-crosslinking of Bpa-containing monoUb variants (UbXpBpa-His_6 mutants) with the UBA domain of human ubiquilin-1 (UBAy). The bands corresponding to crosslinked products are circled (orange).

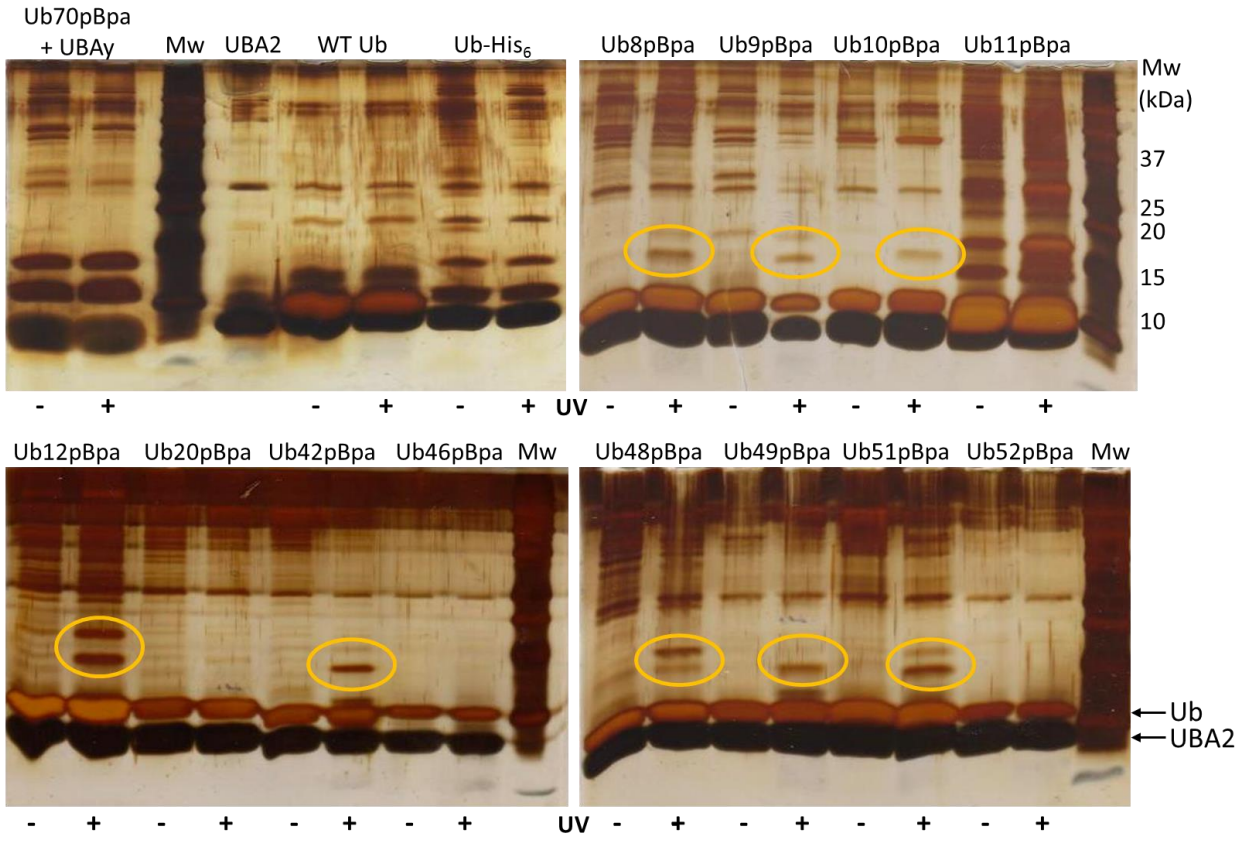


Figure S3. Photo-crosslinking of Bpa-containing monoUb variants (UbXpBpa-His_6 mutants) with the UBA domain (UBAy) from human ubiquilin-1 (lanes 1 and 2 of the top left gel) and the UBA2 domain of hHR23a (all other lanes). The bands corresponding to crosslinked products are circled (orange).

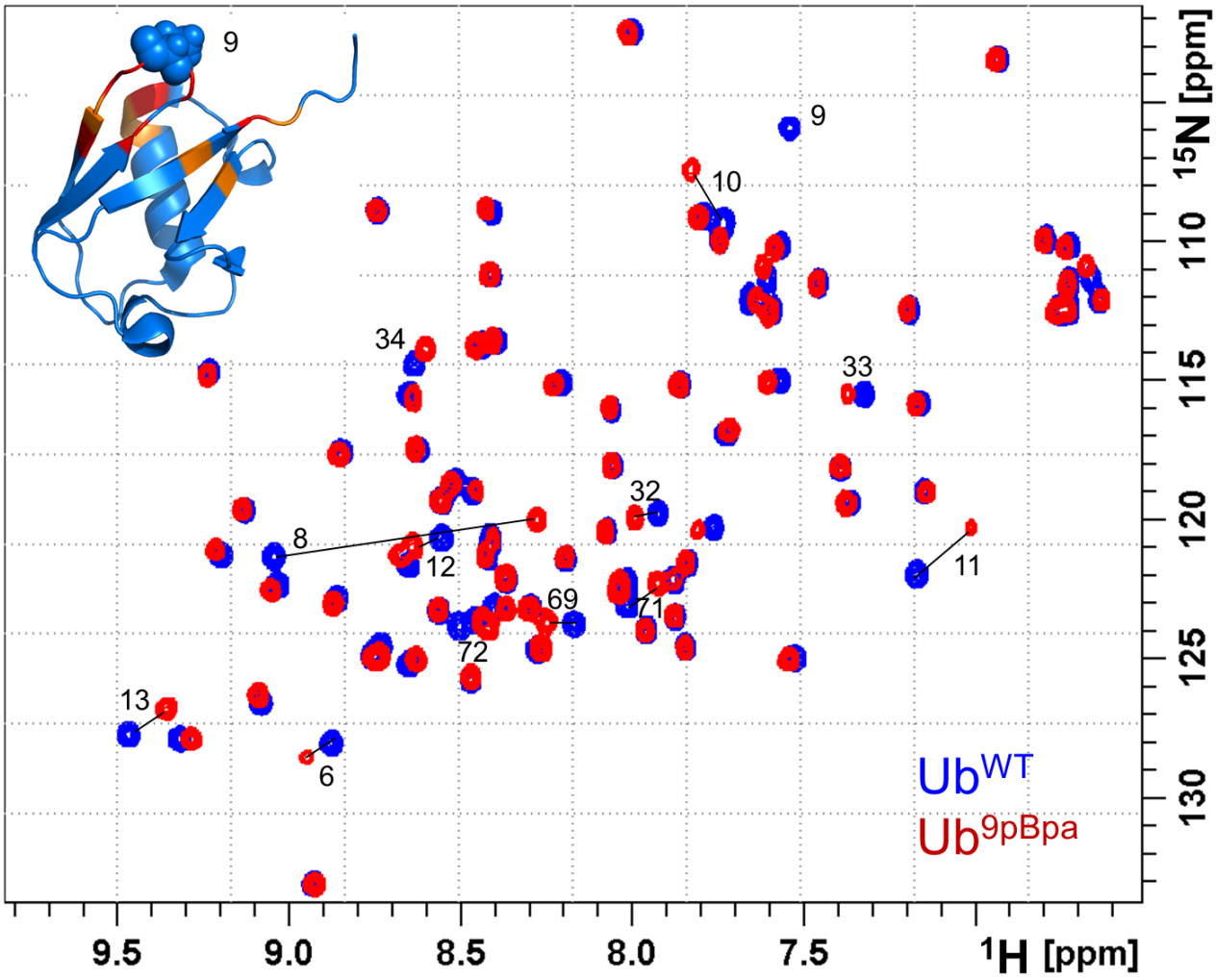


Figure S4. Bpa incorporation at position 9 has little effect on the overall structure of Ub. Shown is overlay of ^{1}H-^{15}N SOFAST-HMQC NMR spectra of WT Ub (blue) and 9pBpa Ub (red) acquired at 25°C at 600 MHz. The proteins were in 20 mM sodium phosphate buffer at pH 6.8 containing 5% D_2O. Significant signal shifts are localized to the indicated residues adjacent to T9 in sequence as well as those located in close proximity to the Bpa. Such shifts are not unexpected as a result of mutation, as well as due to ring current effects on chemical shifts caused by the presence of two aromatic rings in Bpa. The inset shows the structure of Ub with the side chain of T9 in spheres representation, and residues that exhibited amide CSP > 0.1 ppm colored red, 0.1 > CSP > 0.05 ppm colored orange.

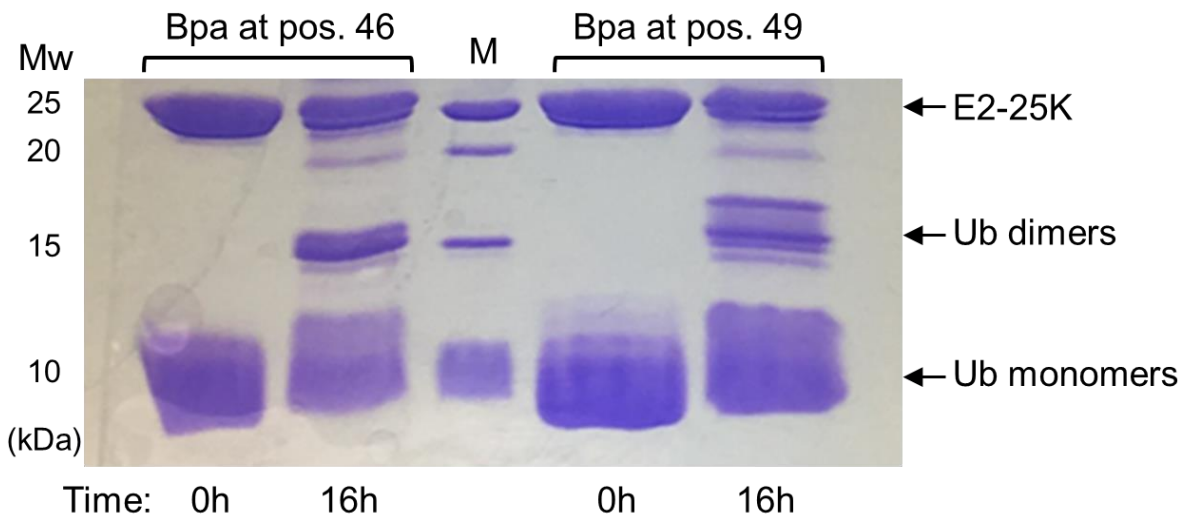


Figure S5. Example SDS PAGE gel (Coomassie stained) of enzymatic assembly of K48-linked diUbs containing Bpa at positions 46 (left lanes) or 49 (right lanes) in the proximal Ub in the overnight reaction. The reaction was catalyzed by E1 and E2-25K; the distal Ub was UbK48R, and the proximal Ub was Ub46pBpa-His_6 or Ub46pBpa-His_6, respectively. The 0 h time point represents the reaction mix prior to adding E1.

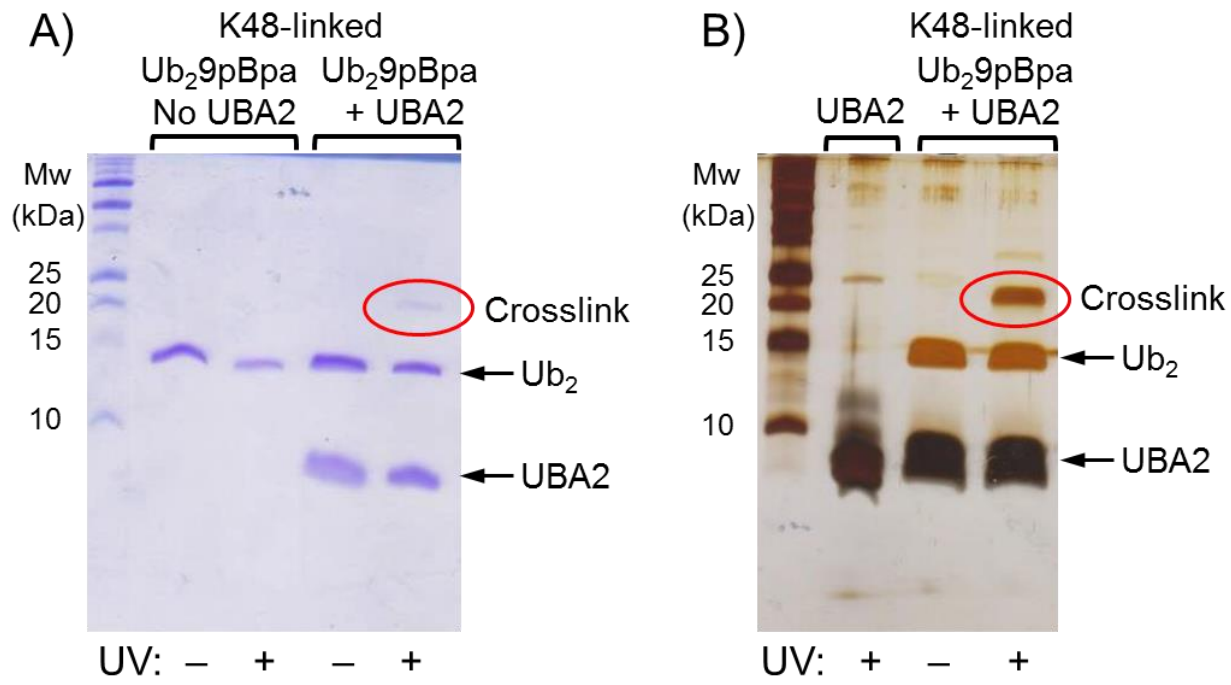


Figure S6. SDS-PAGE gels of photo-crosslinking reaction of K48-linked $Ub_2$9pBpa with UBA2. A) Coomassie stained gel showing a control $Ub_2$9pBpa with no UBA2 and $Ub_2$9pBpa crosslinking with UBA2. ($Ub_2$9pBpa: 23 μM and UBA2: 36 μM). B) Silver-stained gel of the same reaction as in the right two lanes of panel A). The cross-linked product is circled; its expected mass is 24,497 Da. The molecular masses of diUb and UBA2 are 18,924 Da and 5,573 Da, respectively.

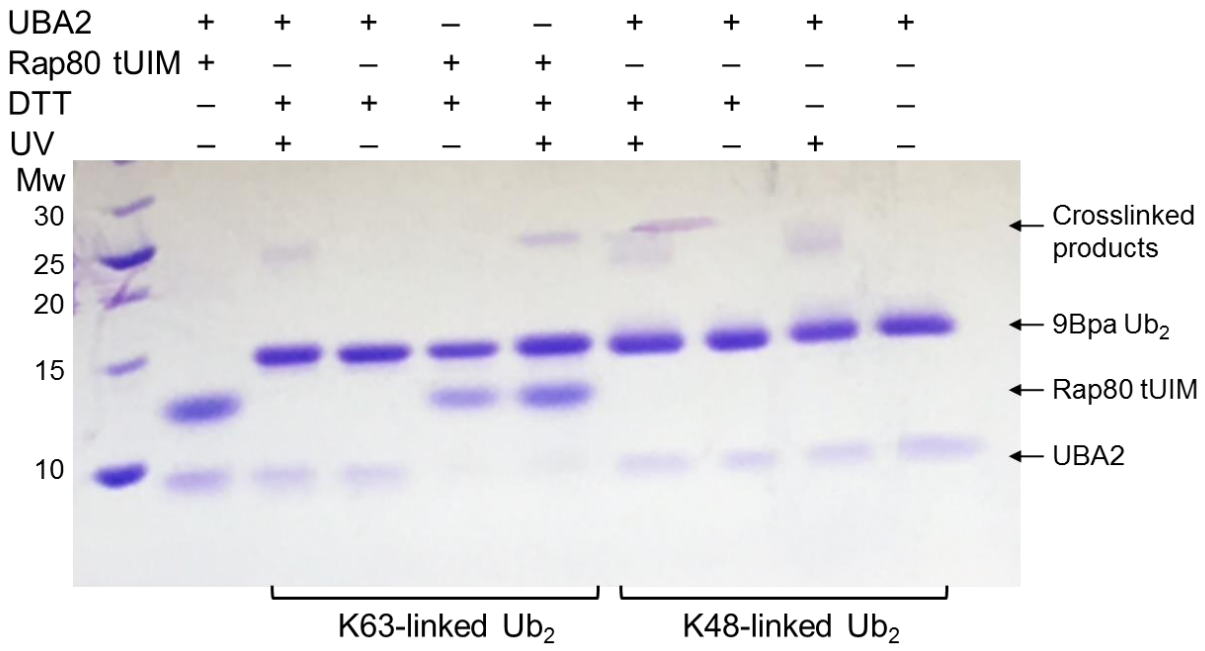


Figure S7. Photo-crosslinking of K48- and K63-linked diUbs having Bpa at position 9 in the distal Ub with hHR23A UBA2 domain and Rap80 tUIM. The molecular masses are in kilodaltons.

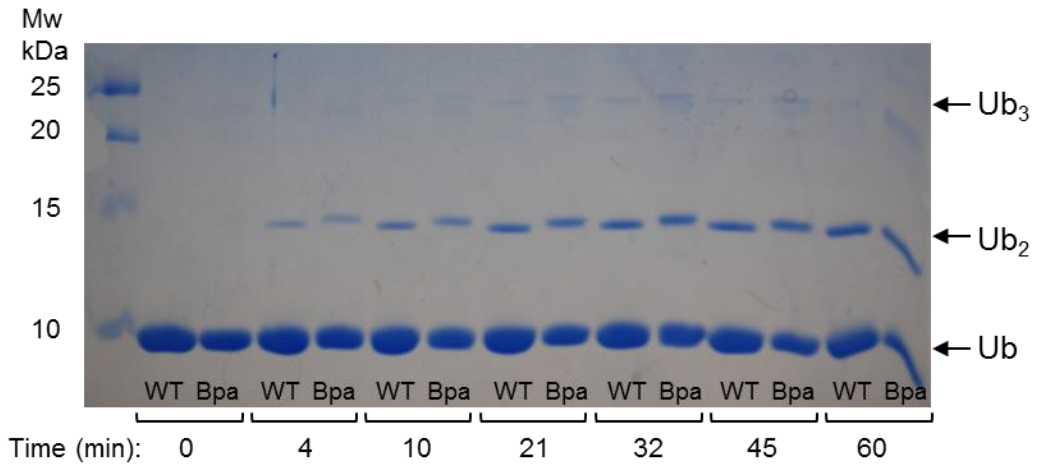


Figure S8. Kinetic comparison of Ub^{WT} and Ub^{9pBpa} as substrates for E1 and E2 enzymes. SDS-PAGE gel (Coomassie) comparing the time course of K48-linked polyUb chain formation reactions for Ub^{WT} and Ub^{9pBpa}, catalyzed by E1 and K48-selective E2-25K enzymes. All the concentrations and other conditions were identical for both proteins (400 μM Ub, 250 nM E1, 15 μM E2-25K, room temperature). These results demonstrate that Ub^{9pBpa} is recognized and treated by the E1 and E2 enzymes similar to Ub^{WT}.

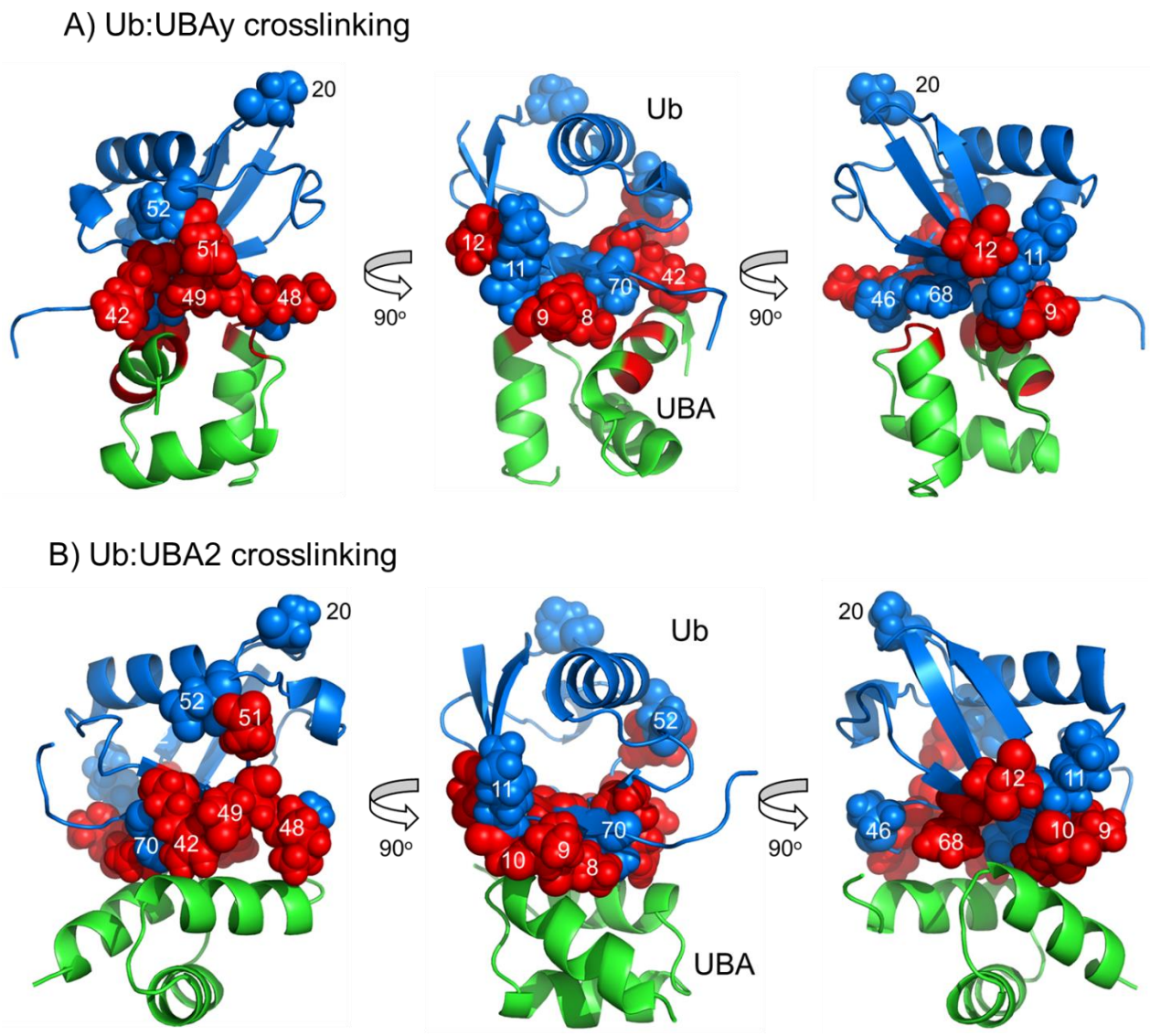


Figure S9. Summary of the results for Ub:UBA crosslinking for various Bpa positions: (A) Structure of Ub:UBAy complex (PDB: 2JY6). (B) Structure of Ub:UBA2 see ref[6]. In both panels, Ub is colored blue and UBA is green. Ub residues mutated to Bpa in this study are shown in sphere representation. Those Bpa positions that showed crosslinking to UBA are colored red, and those that did not crosslink are blue. Residue numbers are indicated.

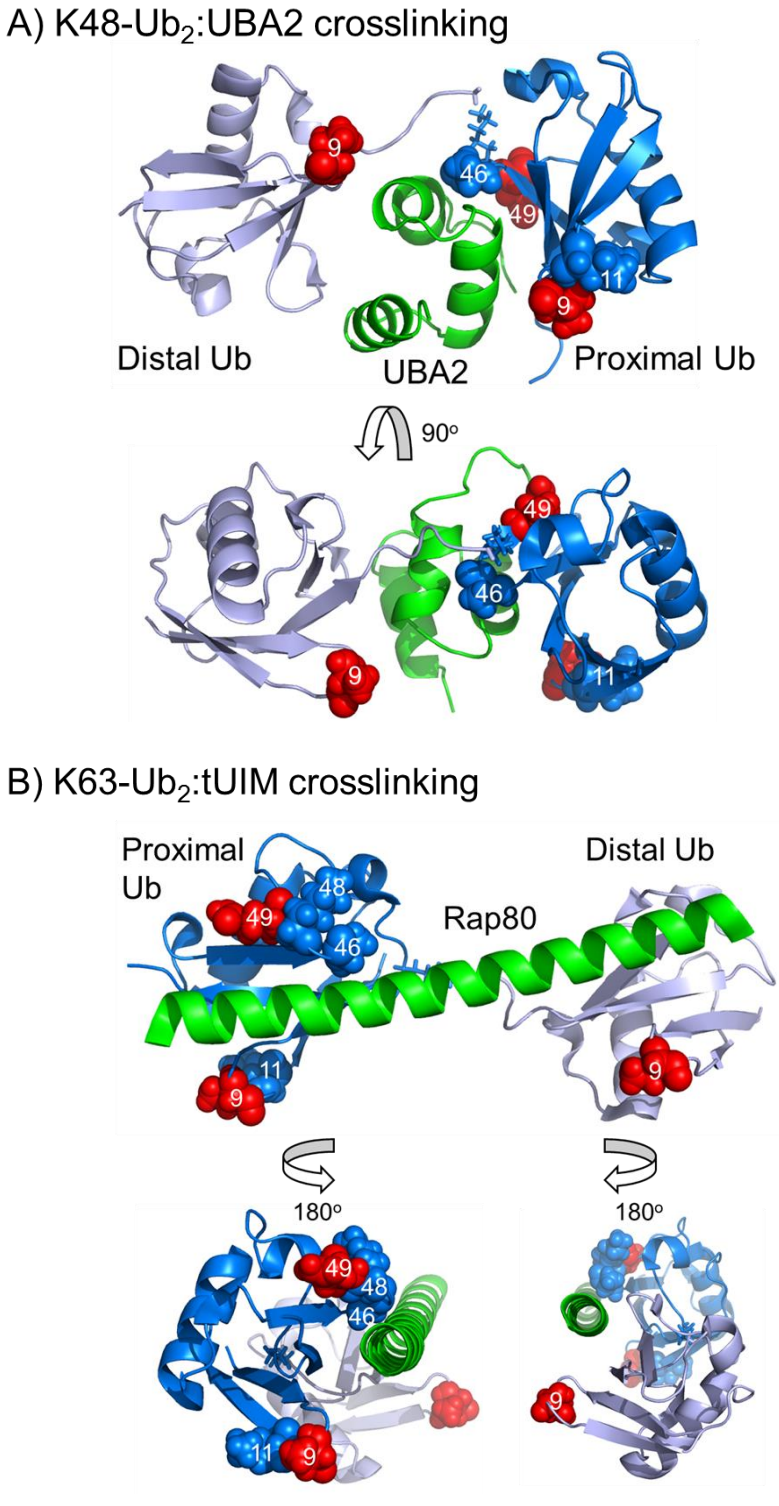
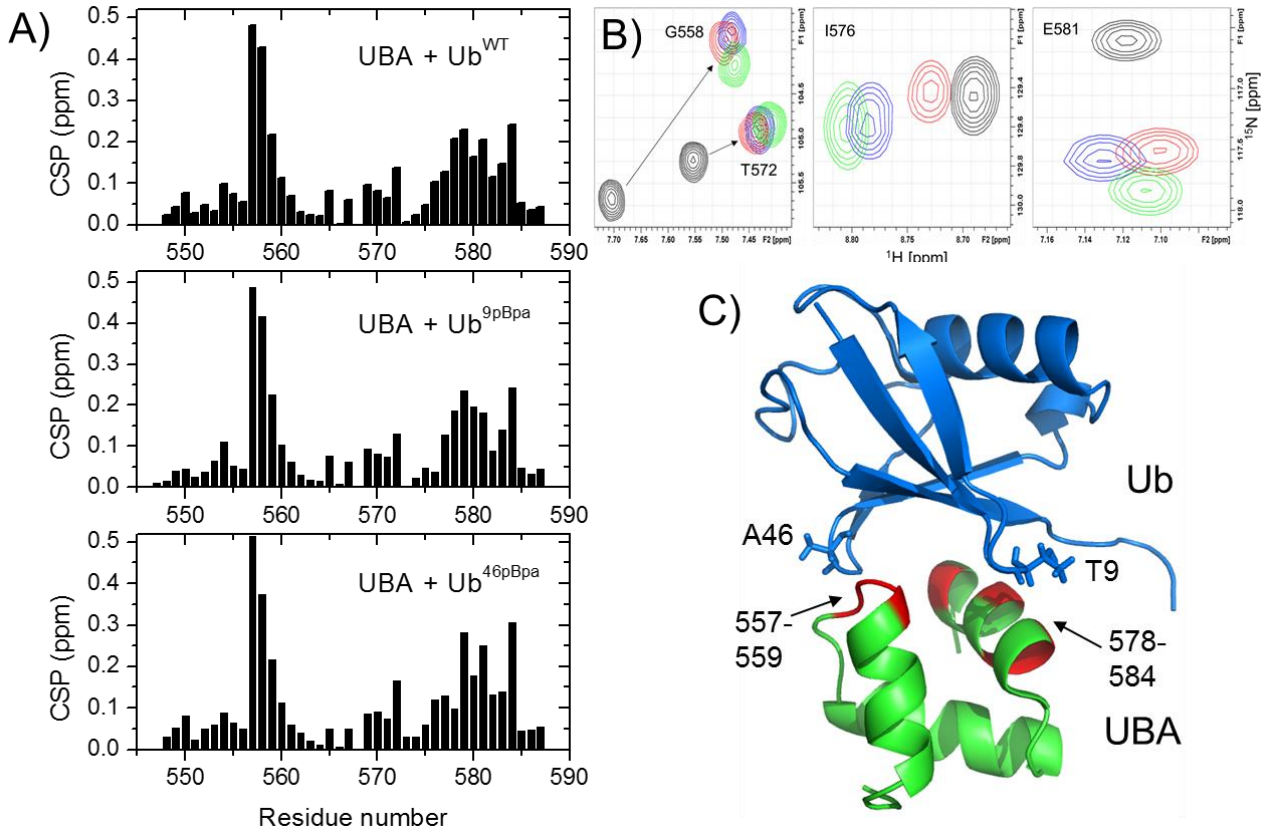
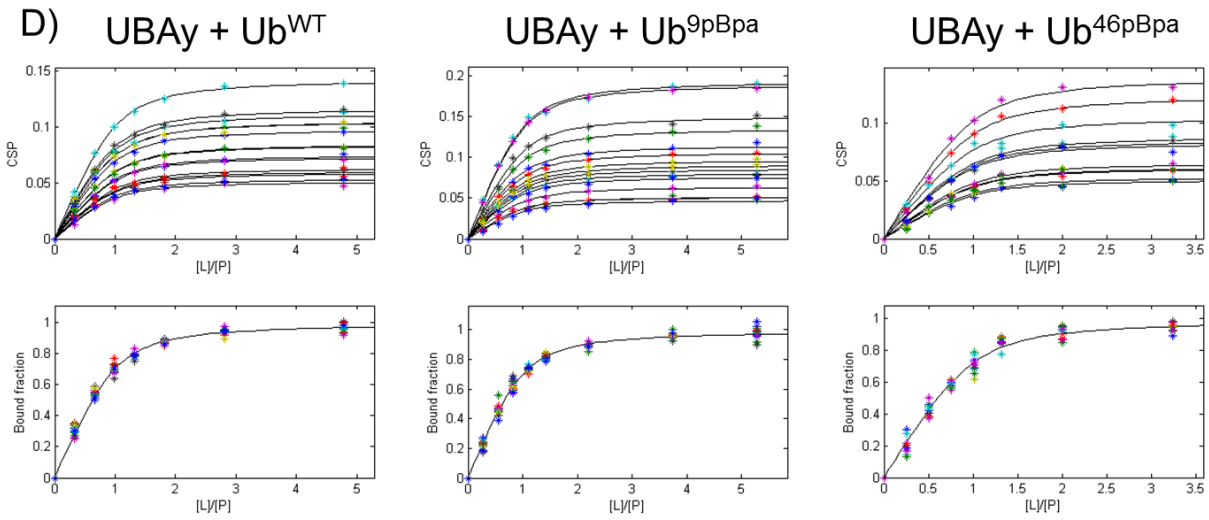


Figure S10. Summary of the results for di-Ub crosslinking with UBA2 and Rap80 tUIM for various Bpa positions: (A) Structure of K48-Ub_2:UBA2 complex (PDB: 1ZO6). (B) Structure of

K63-Ub_2:tUIM complex (PDB: 3A1Q). In both panels, Ub units are colored blue (proximal) or light blue (distal), and the ligand (UBA2 or Rap80) is green. Ub residues mutated to Bpa in this study are shown in sphere representation. Those Bpa positions that showed crosslinking to UBA are colored red, those that did not crosslink are blue. Residue numbers are indicated.





E)

Construct	Ub^{WT}	Ub^{9pBpa}	Ub^{46pBpa}
K_d (μM)	5.1 ± 1.2	5.8 ± 2.1	4.5 ± 2.1

Figure S11. NMR analysis of the effect of Bpa on Ub binding to the UBA domain (UBAy) of human ubiquilin-1 protein. (A) Comparison of residue-specific chemical shift perturbations

(CSP) produced in UBAy upon addition of WT Ub or Bpa-containing Ub variants (9pBpa and 46pBpa) in ca. 3.4:1 molar ratio (Ub:UBAy). (B) Overlay of ^{1}H-^{15}N SOFAST-HMQC spectra of ^{15}N-labeled UBAy alone (black) and at the endpoints of titration with WT Ub (blue), Ub 9pBpa (red), and Ub 46pBpa (green) for select residues (indicated). These spectra show that despite the overall similarity of the CSP patterns (panel A) and the K_d values (panels D-E), there are subtle differences in the local electronic environment of the nuclei in the complex with Ub and Ub variants. (C) Structure of Ub:UBAy complex (PDB: 2JY6; Ub is blue, UBAy is green) with sites of Bpa incorporation (residues T9, A46) shown in stick representation and the UBAy residues that exhibited significant CSPs (>0.15 ppm) upon WT Ub binding colored red and indicated. (D) Representative titration curves for UBAy residues. The lines represent the results of a global fit of all CSPs to a 1:1 binding model (see Materials and Methods). (E) Summary of the Kd values determined from NMR titration experiments (see also panel D).

Table S1. Crosslinking summary of Bpa-containing monoUbiquitin variants with ubiquilin-1 UBA and hHR23A UBA2 domains.

Residue position of Bpa	UBDs	
	Ubiquilin-1 UBA	hHR23A UBA2
Residue 8	+	+
Residue 9	+	+
Residue 10	–	+
Residue 11	–	-
Residue 12	+	+
Residue 20	–	-
Residue 42	+	+
Residue 46	–	-
Residue 48	+	+
Residue 49	+	+
Residue 51	+	+
Residue 52	–	–
Residue 68	–	+
Residue 70	–	–
+ crosslinking; – no crosslinking		

Table S2. Crosslinking summary of K48- and K63-linked ubiquitin dimers with hHR23A UBA2 and Rap80 tUIM.

Domain and residue position of Bpa	UBDs	
	hHR23A UBA2	Rap80 tUIM
Control		
K48-linked Ub_2 (no-Bpa control)	–	–
K48-linked diUbiquitin		
Distal Ub, residue 9	+	+**
Proximal Ub, residue 9	+	+**
Proximal Ub, residue 11	–	–
Proximal Ub, residue 46	–	+**
Proximal Ub, residue 49	+	+**
K63-linked diUbiquitin		
Distal Ub, residue 9	+**	+
Proximal Ub, residue 9	–	+
Proximal Ub, residue 11	–	–
Proximal Ub, residue 46	–	–
Proximal Ub, residue 48	–	–
Proximal Ub, residue 49	–	+
+ crosslinking; +** unexpected; – no crosslinking		

Comments: The results in Table S2 agree with the published structures of UBA2:K48-Ub_2 (PDB ID: 1ZO6) and Rap80:K63-Ub_2 (PDB ID: 3A1Q) complexes. In the UBA2:K48-Ub_2 complex, T9 of both distal and proximal Ubs are positioned close to UBA2, while K11 of the proximal Ub is positioned farther away with its side chain pointing away from UBA2. Q49 of the

proximal Ub is also close to and oriented toward UBA2, whereas A46 of the proximal Ub is in close contact with UBA2 – it is possible that mutation of A46 to Bpa disrupts binding.

A similar picture is observed for the Rap80:K63-Ub_2 complex: T9 of both distal and proximal Ubs are positioned close to tUIM, while K11 and K48 of the proximal Ub are positioned farther away and their side chains are oriented away from tUIM. Q49 of the proximal Ub is also close to and points toward tUIM, whereas A46 of the proximal Ub is in close contact with tUIM – it is possible that mutation of A46 to Bpa disrupts binding.

Table S3. Peptides associated with diUb:UBA2 identified in tryptic digest of cross-linked band.

Protein name	Protein identification probability	Percentage sequence coverage	Peptide sequence	Best Peptide identification probability
Polyubiquitin (Fragment) OS=Hordeum vulgare OX=4513 PE=2 SV=1	99.80%	17.00%	AKIQDKEGIPPDQQR	99.70%
Polyubiquitin (Fragment) OS=Hordeum vulgare OX=4513 PE=2 SV=1	99.80%	17.00%	IQDKEGIPPDQQR	99.50%
Polyubiquitin (Fragment) OS=Hordeum vulgare OX=4513 PE=2 SV=1	99.80%	17.00%	YNIQKESTLHLVLR	99.70%
UV excision repair protein RAD23 homolog A OS=Bos taurus GN=RAD23A PE=2 SV=1	99.30%	4.70%	NENLAANFLLSQNFDDE	99.70%

References

(1) Pickart, C. M., and Raasi, S. (2005) Controlled synthesis of polyubiquitin chains. *Methods Enzym. 399*, 21–36.

(2) Varadan, R., Walker, O., Pickart, C., and Fushman, D. (2002) Structural properties of polyubiquitin chains in solution. *J. Mol. Biol. 324*, 637–647.

(3) Varadan, R., Assfalg, M., Haririnia, A., Raasi, S., Pickart, C., and Fushman, D. (2004) Solution conformation of Lys63-linked di-ubiquitin chain provides clues to functional diversity of polyubiquitin signaling. *J. Biol. Chem. 279*, 7055–7063.

(4) Zhang, D., Raasi, S., and Fushman, D. (2008) Affinity Makes the Difference: Nonselective Interaction of the UBA Domain of Ubiquilin-1 with Monomeric Ubiquitin and Polyubiquitin Chains. *J. Mol. Biol. 377*, 162–180.

(5) Sims, J. J., and Cohen, R. E. (2009) Linkage-Specific Avidity Defines the Lysine 63-Linked Polyubiquitin-Binding Preference of Rap80. *Mol. Cell 33*, 775–783.

(6) Mueller, T. D., Kamionka, M., and Feigon, J. (2004) Specificity of the interaction between ubiquitin-associated domains and ubiquitin. *J. Biol. Chem. 279*, 11926–11936.