Supporting Information

Experimental Section

Nonenzymatic ubiquitination of Rub1

The processing of distal Ub, the attachment of thioester (SR), and its subsequent Alloc protection were performed as previously described.[1, 2] The processing of proximal Rub1 is detailed below.

Production of K48-Lys(Boc) containing Rub1 — E. coli BL21 (DE3) was transformed with plasmids pTXB1-Rub1, containing *rub1* gene from *S. cerevisiae* mutated at codon position 48 to TAG, and pSUP-PylT-PylS, harboring the required tRNA and the cognate tRNA synthetase to incorporate Lys(Boc) as a genetically encoded unnatural amino acid at residue position 48. The ^{15}N-labeled K48-Lys(Boc) Rub1 was expressed by using auto-inducing media with ^{15}N NH_4Cl as the sole source of nitrogen. The expressed protein was purified as described elsewhere[3] followed by buffer exchange with 0.006% (v/v) TFA and subsequent lyophilization.

Protection of K48-Lys(Boc) Rub1 with Alloc groups — To start the reaction, 5 mg of K48-Lys(Boc) Rub1 were dissolved in 675 μL of DMSO, and to this, 25.5 μL of diisopropylethylamine (DIEA) and 112.5 μL of freshly made 40 mg/mL of Alloc-OSu were added. The reaction was incubated for 1-2 hr on a platform rocker. ESI-MS was performed to monitor the complete Alloc protection. The protein was ether precipitated and stored for further reaction.

Boc deprotection of Alloced K48-Lys(Boc) Rub1 — This step was performed as described elsewhere for K48-Lys(Boc) Ub.[1] Briefly, 500 μL of 60% (v/v) TFA was added to K48-Lys(Boc) Rub1 and the reaction was incubated at +4º C in dark for 2-3 hr. The protein was ether precipitated for ligation reaction.

Ligation of Ub and Rub1 — 112.5 μL of DMSO was added to dissolve each monomer separately. The monomers were mixed and added 10 μL of DIEA, 2.5 μL of hydroxysuccinimide (390 mg/mL in DMSO), and 2.5 μL of $AgNO_3$ (57 mg/mL in DMSO). The reaction mixture was thoroughly mixed and incubated at room temperature for 40 hr. The formation of dimer species was monitored by SDS-PAGE. The reaction mixture was ether precipitated.

Deprotection of Alloc groups — The global deprotection of Alloc groups was performed as described elsewhere.[1] Briefly, the ether precipitated ligation mixture was dissolved in 1312.5 μL of DMSO, followed by addition of 750 μL of H_2O, 363.13 μL of 20 mM [Cp*Ru(cod)Cl], and 74.37 μL of neat thiophenol. The reaction mixture was aliquoted into several 200 μL of PCR tubes and incubated at 50º C for 2 hr. The proteins were ether precipitated.

Renaturation and purification of the dimer — This step was performed essentially as described earlier.[1] The purity of the dimer was monitored by SDS-PAGE and ESI-MS.

Nonenzymatic rubylation of Ub and Rub1

Most of the steps in assembly of these dimers were either described earlier[1] or above (Nonenzymatic ubiquitination of Rub1), except for generating a thioesterified Rub1. To attach a SR at the C-terminus of Rub1, 5 mg of Rub1 dissolved in 1 mL of 20 mM sodium phosphate buffer (pH 8) were incubated with 10 mM ATP, 10 mM $MgCl_2$, 100 mM MESNA (sodium 2-mercaptoethane-sulphonate), and 500 nM

Nedd8 E1 for 12 hr at 30º C. The complete attachment of SR was monitored by ESI-MS followed by purification of thioesterified Rub1 using a gel filtration column. The purified protein was buffer exchanged into 0.006% TFA and lyophilized.

NMR experiments

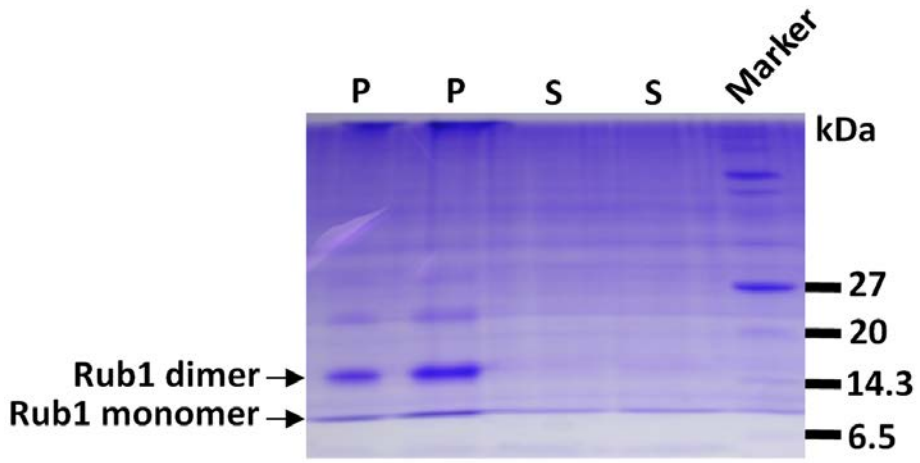
NMR samples were prepared in 20 mM sodium phosphate buffer (pH 6.8) containing 0.02% (w/v) NaN_3 and 5% ^2H_2O. NMR spectra were collected at 22.5ºC on a Bruker Avance III 600 MHz spectrometer equipped with TCI cryoprobe. NMR titration assays were performed by adding increasing amounts of unlabeled UBA2 to ^{15}N-labeled heterodimer. Binding was monitored by ^{1}H-^{15}N SOFAST-HMQC spectra acquired at each step of titration. Chemical shift perturbations (CSPs) in the course of the titration or between the dimer and respective monomers were quantified using the following equation: $CSP = \left[\Delta\delta_H{}^2 + \left(\Delta\delta_N/5\right)^2\right]^{1/2}$, where $\Delta\delta_H$ and $\Delta\delta_N$ are the perturbations for ^{1}H and ^{15}N, respectively.

Mass Spectrometry

All ESI-MS spectra were acquired on JEOL AccuTOF-CS mass spectrometer in a positive electrospray mode. High-resolution mass spectra of m/z 250-2500 were acquired for all samples. The spectra were deconvoluted using MagTran software with a charge range of 2-30 to determine the molecular weight.

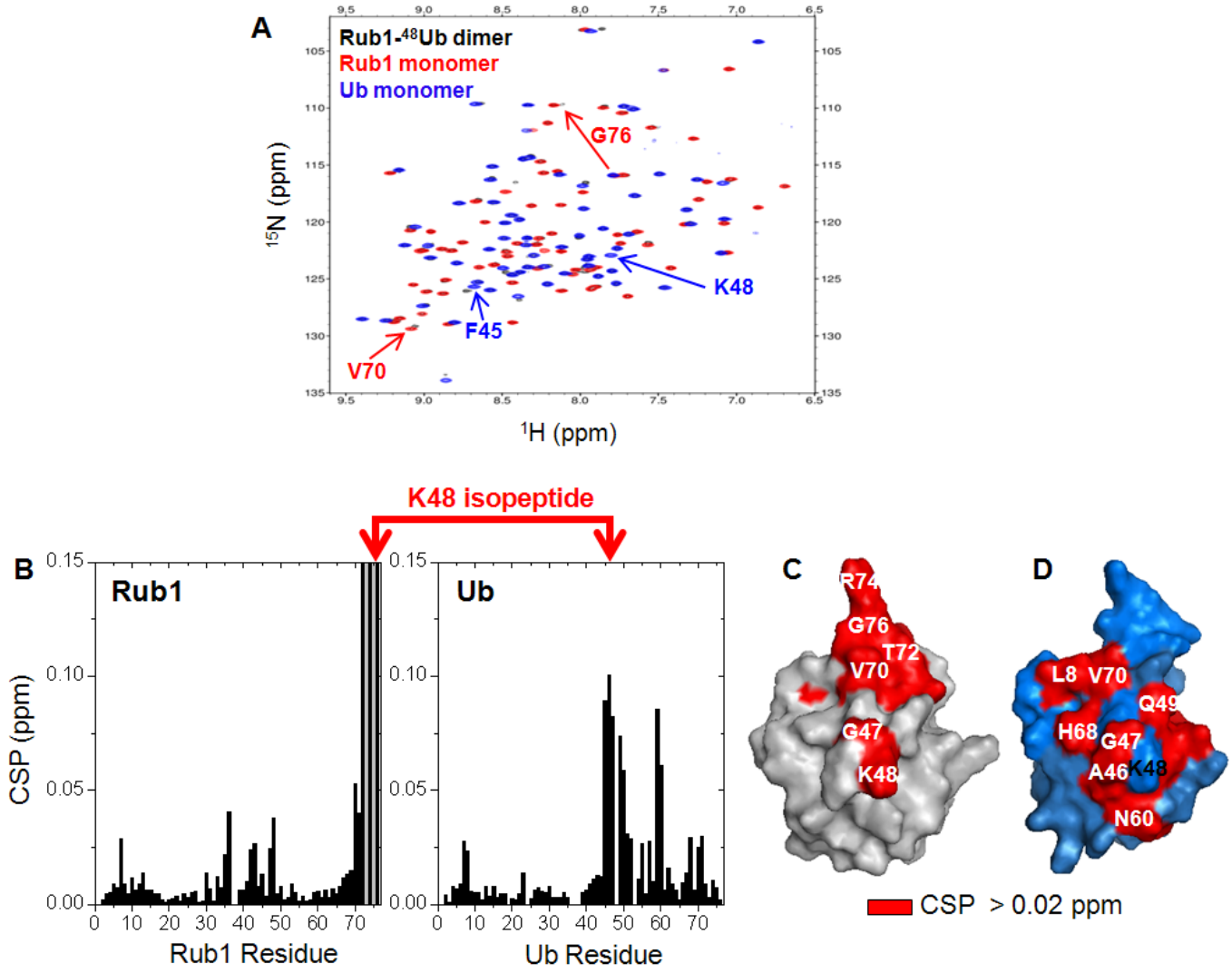
Deubiquitination reactions

Each heterodimer (Ub–^{48}Rub1, Rub1–^{48}Ub, or Rub1–^{29}Ub) was mixed with one of the deubiquitinases in a 50 mM Tris (pH 8.0) buffer containing 50 mM NaCl. The dimer-to-deubiquitinase molar ratio was ~10:1. The reactions were incubated for 16 hr at 30ºC followed by separation on SDS-PAGE and coomassie blue staining.



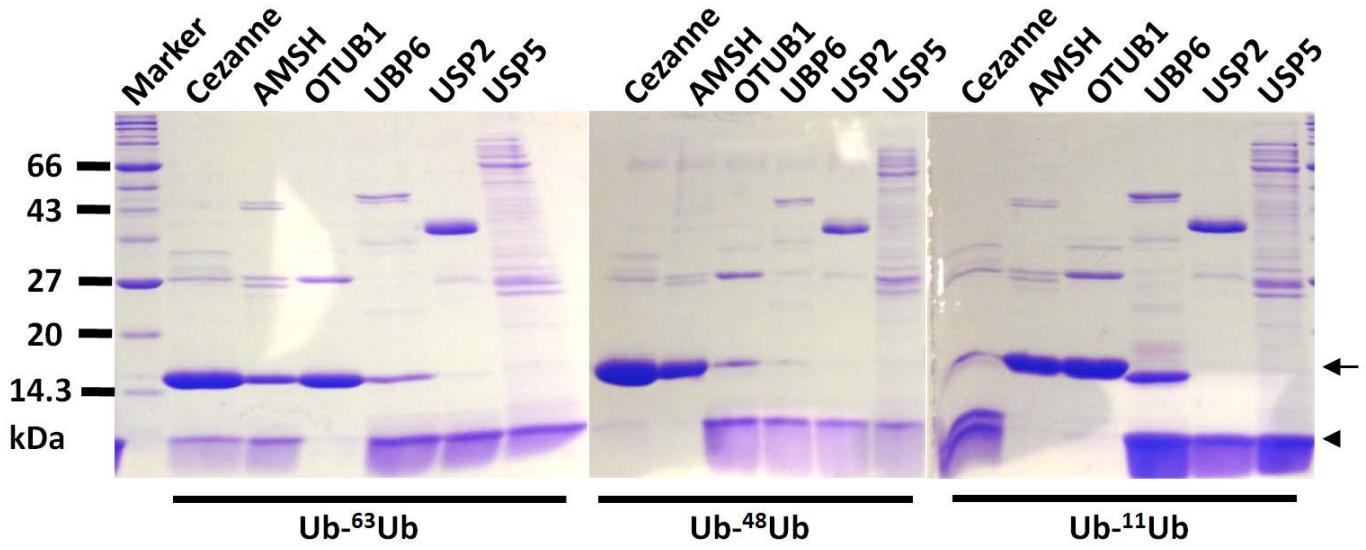
Supporting Figure S1. Nonenzymatic assembly of Rub1-^{48}Rub1 homodimer.

The ligation mixture (after Alloc deprotection) of Rub1-^{48}Rub1 dimer was centrifuged at 13.2 K rpm, and 1% of the pellet (P) and the supernatant (S) fractions were loaded onto a 15% SDS PAGE. The running positions of the monomer and dimer are indicated by the arrows.



Supporting Figure S2. Chemically assembled Rub1–^{48}Ub heterodimer forms interdomain interface similar to that in the enzymatically assembled Rub1–^{48}Ub heterodimer (see Fig 4 in ref [3]).

(A) ^{1}H-^{15}N SOFAST-HMQC spectrum (black) of Rub1–^{48}Ub overlaid with the corresponding spectra of Rub1 (red) and Ub (blue) monomers. Note that K48 of Ub in the heterodimer was not ^{15}N-labeled, hence invisible in the corresponding (black) spectrum. Selected residues showing significant signal shifts are marked with numbers and indicated with arrows. (B) Chemical shift perturbations (CSPs; black bars) and significant signal attenuations (>75%; grey bars) of backbone amides in the heterodimer, due to the formation of the interface, are plotted as a function of the residue number. Residues forming the isopeptide bond are connected by the red arrows. (C-D) The perturbed residues (painted red and indicated) are mapped on the 3D surfaces of (C) Rub1 (grey) and (D) Ub (blue).



Supporting Figure S3. Control cleavage of Ub-Ub homodimers by the deubiquitinases used in this study.

The dimers were incubated for 16 hr in a cleavage reaction with indicated deubiquitinases in a 10:1 molar ratio. The reactions were stopped by adding SDS loading buffer and loaded onto a 15% SDS PAGE. The running positions of monomers and dimers are shown by the arrowhead and the arrow, respectively.

References

[1] C. Castañeda, J. Liu, A. Chaturvedi, U. Nowicka, T. A. Cropp, D. Fushman, *J Am Chem Soc* **2011**, *133*, 17855-17868.

[2] E. K. Dixon, C. A. Castaneda, T. R. Kashyap, Y. Wang, D. Fushman, *Bioorg Med Chem* **2013**, *21*, 3421-3429.

[3] R. K. Singh, S. Zerath, O. Kleifeld, M. Scheffner, M. H. Glickman, D. Fushman, *Mol Cell Proteomics* **2012**, *11*, 1595-1611.