Electronic Supplementary Material (ESI) for Chemical Communications.

Supporting Information

Monitoring Protein Ubiquitination and SUMOylation in real-time by NMR

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Experimental section

Cloning and mutagenesis

Mutants of Ub in pET3a, Ubc13 in pET24d, and MMS2 in pET3a were generated by site-directed mutagenesis using PCR followed by Dpn1 digestion. All the plasmids were confirmed by sequencing. The pET28-Smt3-RanGap1(419-587), pET28-SUMO2, ΔN-364 SENP2, and pET11-AOS1/UBA2 were gifted by Christopher Lima (Sloan Kettering Institute, New York). pET28-UBC9 was acquired from Addgene (25213). The UBC9 construct was used as a template for site-directed mutagenesis to obtain Q130A UBC9 and A131D UBC9. pOPINB-AMSH* was a gift from David Komander (Addgene plasmid # 66712).

Protein expression and purification

All proteins constructs were transformed in BL21 DE3 cells, grown at 37°C (in LB media for unlabeled proteins and M9 media for ^{15}N or ^{13}C labeled proteins), and were induced at an OD 600 of 0.6-0.8 with 1 mM IPTG. The harvested cells were resuspended in lysis buffer (50 mM Tris, 250 mM NaCl, 5 mM BME, 1% glycerol, 0.01% Triton X, pH 7.5), lysed by sonication and centrifuged. GST-RNF38 (residues 387-465) was bound to GSTrap HP columns (GE Healthcare) and eluted with 15 mM reduced glutathione. His tag proteins – E1, Ubc13, and MMS2 – were bound to HisTrap HP columns (GE Healthcare), and the proteins were step eluted with 100 mM-500 mM imidazole. Imidazole elute of E1 was dialyzed in the ion-exchange buffer (50mM Tris, 5mM BME, 0.01% Triton X, pH 8.0), bound to Q FF column (GE Healthcare) and the protein was eluted by gradient elution with increasing salt concentration (0 mM NaCl- 600 mM NaCl). Further purification of all proteins was done by gel filtration chromatography on Superdex 75

16/600 column (GE Healthcare) in SEC buffer (50 mM Sodium Phosphate, 100 mM NaCl, 5 mM BME, pH 6.5).

For purification of Ub and its mutants, cells were resuspended in lysis buffer (50 mM Sodium acetate, 5 mM BME, 0.01% Triton X, pH 4.5), lysed by sonication and centrifuged. The supernatant was passed through the SP column (GE Healthcare) and eluted by a salt gradient (0 mM NaCl- 600 mM NaCl). Further purification was done by gel filtration chromatography on Superdex 75 16/600 column (GE Healthcare) in SEC buffer. For K63-linked di-Ub (^{15}N D77-^{13}C K63A) purification, 1 µM UbE1, 40 µM Ubc13, 40 µM GB1-MMS2, 40 µM GST-RNF38 RING, 200 µM ^{15}N D77 Ub and 200 µM ^{13}C K63A Ub were mixed with reaction buffer (50 mM Sodium Phosphate, 5 mM $MgCl_2$, 3 mM ATP, 0.5 mM DTT, pH 6.5) overnight. The mixture was dialyzed in 50mM Sodium Acetate, 5mM BME, pH 4.5, and centrifuged to remove the precipitate. The supernatant was passed through the SP column (GE Healthcare), and the Ub_2 was separated from unreacted mono Ub by gradient salt concentration (0 mM NaCl- 600 mM NaCl). The enzymes and proteins necessary for SUMOylation reactions were expressed in BL21 (DE3) cells either in the M9 medium for labeled proteins or in LB for unlabelled proteins. Mature SUMO2, E1 (UBA2/AOS1), and E2 (UBC9) were purified with standard Ni NTA affinity and Size-exclusion Chromatography, as mentioned earlier[1]. RanGap1 was cultured at 37℃ to 0.8 OD600, and expression was induced with 0.5 mM IPTG for 4-5 hours. RanGap1 expressed as His-Smt3-RanGap1 chimera. Cells were lysed in 50mM Tris(pH8), 350 mM NaCl and 20 mM imidazole. The lysate was clarified by centrifugation, and the supernatant was used for Ni-NTA affinity purification. Fractions containing RanGap1 were pooled and incubated (at room temperature) with His-ΔN-364 SENP2 (1:1000 molar ratio) to remove the Smt3 tag. Imidazole was removed by dialysis post digestion. His-SENP2 and cleaved His-SMT3

fragments were removed by passing the reaction mixture through Ni NTA beads. The flow-through, which contained the cleaved fragment of RanGap1, was collected and further purified by Size-exclusion Chromatography in PBS. The NMR isotopes used were ^{15}N-NH4Cl (NLM-467-10, Cambridge Isotopes), ^{13}C-Glucose (CLM-1396, Cambridge Isotopes) and ^{15}Nζ-Lysine (NLM-631, Cambridge Isotopes).

Ubiquitination and SUMOylation reactions

For K48-Ub_2 synthesis, Ube2k (40 μM), K48A-Ub (450 μM) and D77-Ub (300 μM) were incubated in 25mM Sodium Phosphate, 0.5 mM DTT, 5 mM MgCl2, 3 mM ATP, pH 6.5 at 37ºC. The reaction was initiated by adding Ube1 (0.3 μM). For K63-Ub_2 synthesis, Ubc13 (10 μM), Mms2 (10 μM), K63A-Ub (450 μM) and D77-Ub (300 μM) were incubated in 50 mM Sodium Phosphate, 0.5 mM DTT, 5 mM MgCl2, 3 mM ATP, pH 6.5 at 37ºC. The reaction was initiated by adding Ube1 (0.3 μM). The SUMOylation reaction was carried out by incubating Ubc9 (10 μM), RanGAP1 (100 μM), K11R-SUMO2 (200 μM) in the reaction buffer with 5 mM ATP, 5 mM $MgCl_2$ in phosphate buffer saline, pH 7.4 at room temperature. The reaction was initiated by adding SAE1/SAE2 (3 μM).

DUB Assay

For DUB assays, the DUB AMSH was incubated in reaction buffer 50 mM Sodium Phosphate, 50 mM NaCl, pH 6.5 buffers for 5min, and then added to purified Ub_2 in the same buffer. Commercially obtained (from R&D systems) 0.5 μM AMSH was added to 150 μM K63 linked diUb (^{15}N D77-^{13}C K63A) in the reaction buffer at 37°C. For cell extract experiments, AMSH in pOPINB vector was transformed in BL21 DE3 cells, grown at 37°C in 100 ml LB media, and induced at an OD 600 of 0.6-0.8 with 1 mM IPTG. The harvested cells were resuspended in 10 ml DUB reaction buffer, lysed by sonication, and centrifuged to pellet cell debris. The cell lysate

was diluted two times, and 3 µl of that was added to 150 µM Ub_2 in the DUB reaction buffer at 37°C.

NMR Experiments

All NMR titration experiments were recorded at 298K on 600 MHz or 800 MHz Bruker Avance III HD spectrometer with a cryoprobe head. 10% (v/v) D_2O was added to the assay samples to constitute the NMR samples. The 2D ^{1}H-^{15}N-HSQCs were collected with 2048x512 time points. The number of scans for each fid was 12, and the total experimental time was 1 hour. The ^{13}CO,^{15}N-edited ^{1}H-detected 1D experiments (Figure S2) were collected with 2048 time points. 640 scans were used for each fid, and the total experimental time was 13 min. The ^{15}N-edited, ^{1}H-detected 1D spectra (Figure S6) were collected with 2048 time points. 640 scans were used for each fid, and the total experimental time was 12 min. For the SUMOylation assays, each 2D ^{1}H-^{15}N-HSQC was performed with 18 scans, and the total experiment time was 90 min. The ^{13}CO,^{15}N-edited ^{1}H-detected 1D experiments were 640 scans, with the experimental time of 20 min. The dead time of all the experiments ranged from 5 to 15 min, which is the time required to insert the sample in the NMR tube, insert the tube in the NMR magnet and start the experiment. All the spectra were processed with Topspin (Bruker), NMRpipe[2], and analyzed with Sparky[3].

Modeling E2~donor-Ub/acceptor-Ub structures

The Ube2k~donor-Ub complex was modeled using the Ube2k (PDB: 1YLA) and Ubc1~donor-Ub structure (PDB: 1FXT). Then acceptor-Ub (PDB: 1UBQ) was docked onto the modeled structure using HADDOCK to create the Ube2k~donor-Ub/acceptor-Ub complex[4,5]. In HADDOCK calculations, the K48 sidechain of acceptor-Ub was constrained to contact the active site cysteine. Rigid body energy minimization generated one thousand initial complex structures,

and the best two hundred lowest energy structures were selected for torsion angle dynamics and subsequent Cartesian dynamics in an explicit water solvent. Default scaling for energy terms was applied. Following the standard benchmarked protocol, the cluster with maximum structures and the highest score was chosen as the representative model. The structure was validated by Verify3D[6], ProSA-web[7], and PDB validation software[8], and details of the analysis is provided in Table S2. The RNF38^{RING}/Ubc13~donor-Ub/Mms2/acceptor-Ub complex was modeled using the Ubc13~donor-Ub/Mms2 structure (PDB: 2GMI), the Ubc13~donor-Ub/TRAF6 structure (PDB: 5VNZ), the Mms2/Acceptor-Ub structure (PDB: 1ZGU) and the Ubc13/RNF38^{RING} structure in UCSF Chimera[9]. All the structures were superimposed using Ubc13 as the reference, and then the redundant structures of Ubc13, donor-Ub, and TRAF6 were removed to obtain the final model. The model was equilibrated for 5ns in the NAMD package[10]. The protein and ions were described with the CHARMM36 force field [11], and water molecules with the TIP3P model. The ionic strength of the solvating solution was 150 mM. The van der Waals interactions were truncated beyond 12 Å. Periodic boundary conditions were imposed in all directions. The temperature of the systems was controlled at 298 K using the Langevin dynamics, and the pressure was kept at 1 atm using the Nose-Hoover Langevin piston method[12,13]. The structure was analyzed by Verify3D[6], ProSA-web[7], and PDB validation software[8], and details of the analysis are provided in Table S2.

References

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Enzymes	**Rate of K48-Ub_2 synthesis**
Ube2k	0.025 nmoles/min
K97E-Ube2k	0.007 nmoles/min
D124R-Ube2k	n. d.
S85N-Ube2k	n. d.
Enzymes	**Rate of K63-Ub_2 synthesis**
UBC13/Mms2	0.021 nmoles/min
UBC13/Mms2/ RNF38	0.060 nmoles/min
UBC13/L46A-Mms2/ RNF38	0.013 nmoles/min
D119A-UBC13/Mms2/ RNF38	n. d.
UBC13/S27A-Mms2/ RNF38	n. d.
UBC13/Mms2/ RNF38 (I44A-acceptor-Ub)	n. d.
Enzymes	**Rate of RanGAP1~SUMO synthesis**
Ubc9	0.070 nmoles/min
Q130A-Ubc9	0.021 nmoles/min
A131D-Ubc9	n. d.

Table S1. The rates of K48-Ub_2, K63-Ub_2, and RanGAP1~SUMO synthesis measured by the different NMR experiments described in the paper. ‘n. d.’ denotes not detectable. Here, the synthesis reaction was impaired strongly, and the product could not be detected.

Ube2k~donor-Ub/acceptor-Ub	
Global quality (Verify 3D, ProSA)	
Verify3D Raw Score	0.18
Verify3D Z-score	-4.49
ProSA Z-score	-6.49
Close contacts and deviations from ideal geometry	
Bond Angles	0.5°
Bond Lengths	0.003Å
Number of close contacts	0
Ramachandran Statistics	
Most Favoured (%)	96.8
Allowed (%)	2.9
Disallowed (%)	0.3
RNF38^{RING}/UBC13~donor-Ub/Mms2/acceptor-Ub	
Global quality (Verify 3D, ProSA)	
Verify3D Raw Score	0.21
Verify3D Z-score	-4.01
ProSA Z-score	-5.90
Close contacts and deviations from ideal geometry	
Bond Angles	1.3°
Bond Lengths	0.007Å
Number of close contacts	0
Ramachandran Statistics	
Most Favoured (%)	92.3
Allowed (%)	5.8
Disallowed (%)	2.0

Table S2. Validation of the Ube2k~donor-Ub/acceptor-Ub and RNF38^{RING}/UBC13~donor-Ub/Mms2/acceptor-Ub models.

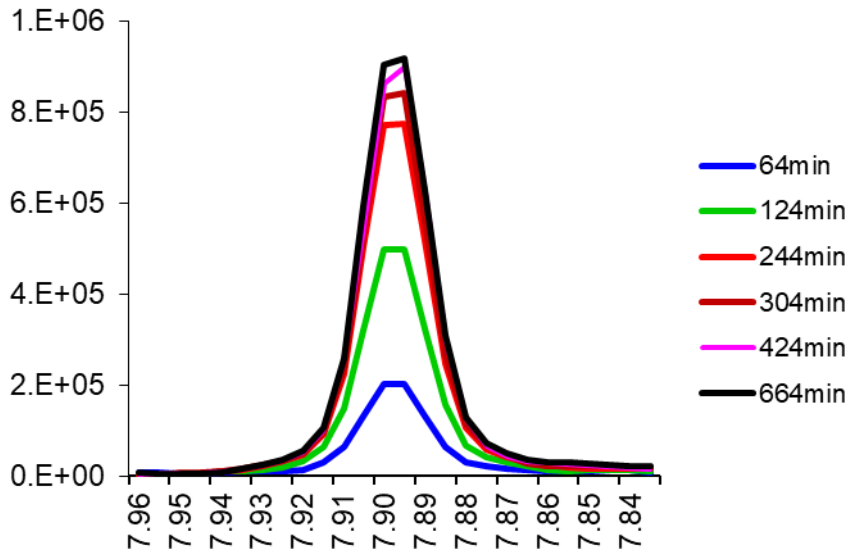

Figure S1. The synthesis of K63-linked Ub_2 in the presence of E3 RNF38 is monitored over time. The cross-section of the new resonance from the new isopeptide bond in the ^{1}H-^{15}N-HSQC is plotted over time.

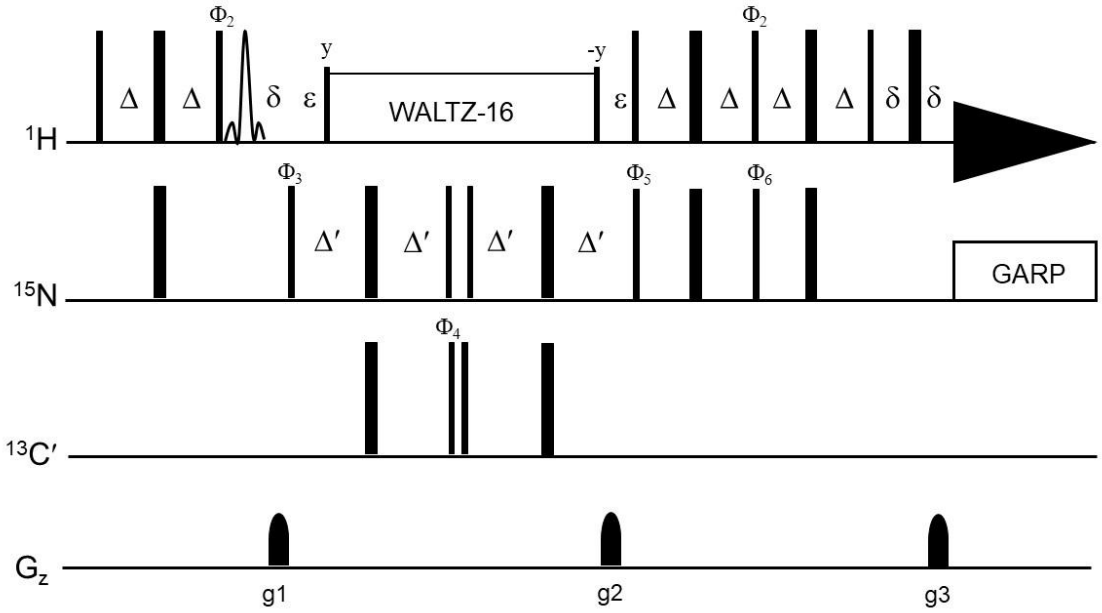

Figure S2. Pulse sequence for ^{13}CO,^{15}N-edited ^{1}H-detected 1D experiment. Unless otherwise indicated, the ^{1}H, ^{15}N, and ^{13}C carrier frequencies were placed at 4.7, 116, and 175 ppm, respectively. The narrow and wide bars represent 90^{o} and 180^{o} pulses, respectively. Unless otherwise indicated, the pulses are applied with phase x. The 90^{o} sine-shaped pulse for water excitation is 1 ms long applied with the RF field of 0.5 kHz. The WALTZ proton decoupling, and the short hard pulse before and after it are accomplished using a RF field of 9.6 kHz. All the ^{13}C pulses are shaped to specifically excite ^{13}CO resonances. The ^{13}C 90^{o} pulses were 0.27 ms long and the 180^{o} pulse was 0.35 ms long. The RF field of ^{15}N GARP decoupling is 1.13 kHz. The delays used are: Δ = 2.3 ms ($1/4J_{NH}$), Δ' = 12 ms ($1/4J_{NCO}$), δ = 0.2 ms and ε = 5.5 ms ($1/2J_{NH}$). The durations of the sine-shaped z-axis pulsed field gradients are 1.0 ms for the gradients. The strength of the gradients are 30 (g1), 40 (g2), and 4.4 (g3) G/cm. The phase cycles employed are Φ_2= {y}, Φ_3= {x, x, x, x, x, x, x, x, -x, -x, -x, -x, -x, -x, -x, -x}, Φ_4= {x, -x}, Φ_5= {x, x, -x, -x}, Φ_6= {-y, -y, y, y}, Φrec = {x, -x, -x, x, x, -x, -x, x, -x, x, x, -x, -x, x, x, -x}.

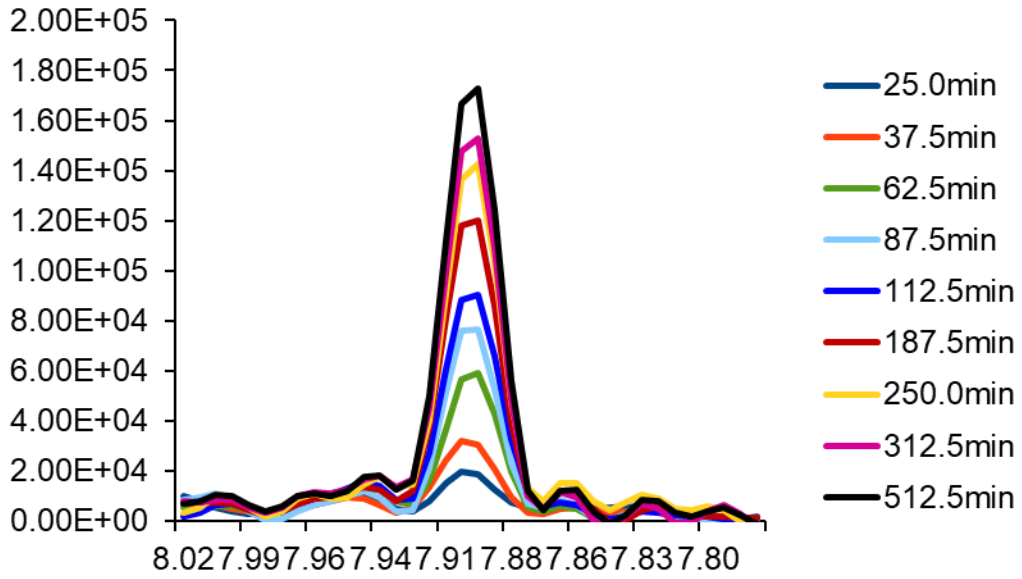

Figure S3. The synthesis of K63-linked Ub_2 is monitored over time. The resonance from the new isopeptide bond in ^{13}CO, ^{15}N-edited, ^{1}H-detected 1D spectra are plotted for different reaction times.

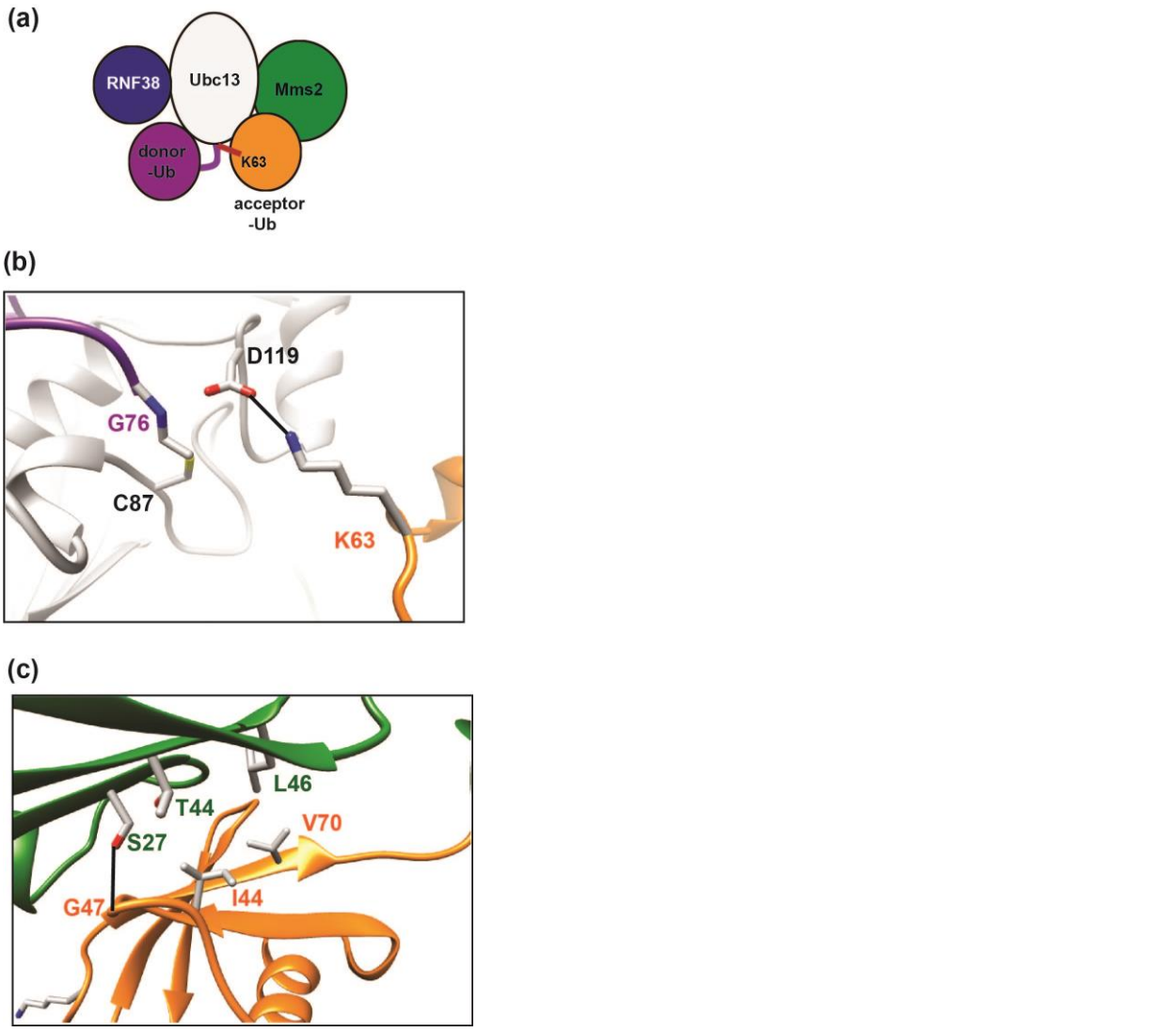

Figure S4. The important intermolecular contacts of acceptor Ub in the complex of (a) $RNF38^{RING}$/UBC13~donor-Ub/Mms2/acceptor-Ub. (b) K63 from the acceptor Ub contacts D119 of Ubc13 at the active site region. Hence, D119 could be crucial in stabilizing the attacking lysine. (c) Mms2 recruits the acceptor Ub and orients it for chain elongation. Interactions between the Mms2 and acceptor Ub are shown. S27 of Mms2 forms a hydrogen bond with the backbone of G47 of acceptor Ub. The residues T44 and L46 of Mms2 form Vander Waals contacts with I44 and V70 of acceptor Ub, respectively.

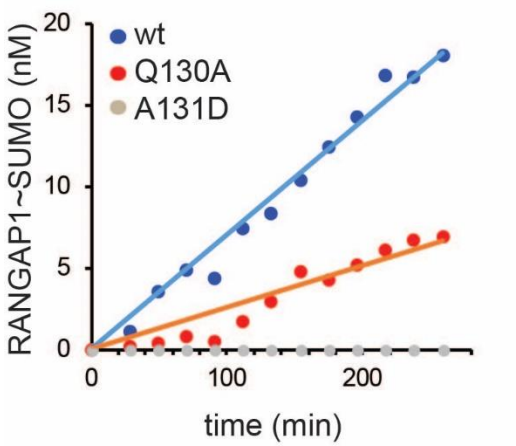

Figure S5. The SUMOylation reaction was carried out using ^{15}N-labeled RanGAP1, ^{13}C-labeled K11R-SUMO2, E1 (Uba1/), E2 (Ubc9), and monitored using the ^{15}N-edited, ^{13}C-edited, ^{1}H-detected 1D experiments. The experiments were repeated for Q130A-Ubc9 and A131D-Ubc9. The early time-points were fit to obtain rates of the reaction.

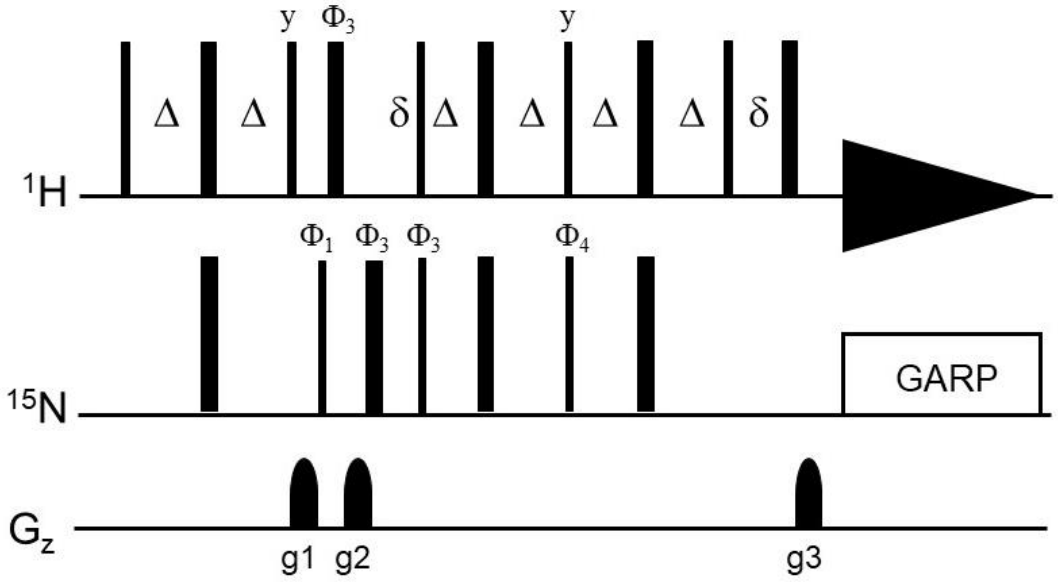

Figure S6. Pulse sequence for the ^{15}N-edited, ^{1}H-detected 1D experiment. Unless otherwise indicated, the ^{1}H and ^{15}N carrier frequencies were placed at 4.7 and 116 ppm, respectively. The narrow and wide bars represent 90^{o} and 180^{o} pulses, respectively. Unless otherwise indicated, the pulses are applied with phase x. The 90^{o} pulses were 0.27 ms long, and the 180^{o} pulse was 0.35 ms long. The RF field of 15N GARP decoupling is 1.13 kHz. The delays used are: Δ = 2.3 ms ($1/4J_{NH}$) and δ = 0.2 ms. The duration of the sine-shaped z-axis pulsed-field gradients is 1.0 ms for the gradients. The strength of the gradients are 25 (g1), 40 (g2), and 4.4 (g3) G/cm. The phase cycles employed are Φ_1= {x, -x}, Φ_3= {x, x, -x, -x}, Φ_4= {y, y, -y, -y}, Φrec = {x, -x, -x, x}.

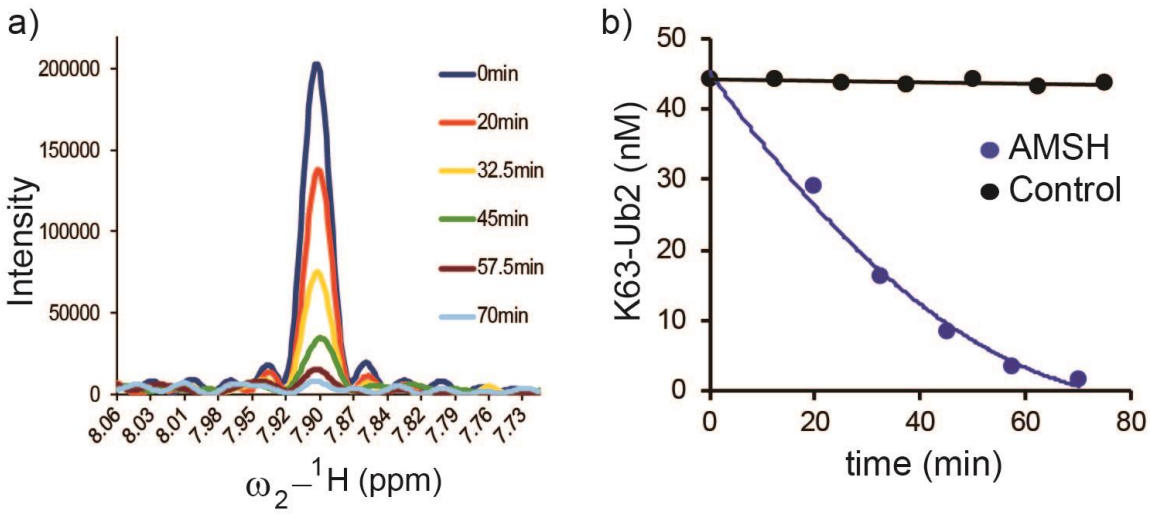

Figure S7. The deubiquitination reaction is monitored by acquiring ^{13}CO, ^{15}N-edited, ^{1}H-detected 1D experiments, and measuring the decrease in intensity of the ^{13}CO-$^{15}N\zeta$-$H\zeta$ resonance due to cleavage of the isopeptide bond. (a) The deubiquitination of K63-linked Ub_2 by the DUB AMSH. (b) The peak integrals in (a) are plotted against time. The control is the same reaction without AMSH.