Supporting Information

The Hydrophobic Patch of Ubiquitin is Important for its Optimal Activation by Ubiquitin Activating Enzyme E1

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Abstract

Protein ubiquitination plays a role in essentially every process in eukaryotic cells. The attachment of ubiquitin (Ub) or Ub-like (UBL) proteins to target proteins is achieved by parallel but distinct cascades of enzymatic reactions involving three enzymes: E1, E2, and E3. The E1 enzyme functions at the apex of this pathway and plays a critical role in activating the C-terminus of ubiquitin or UBL, which is an essential step that triggers subsequent downstream transfer to their cognate E2s resulting in the fidelity of the Ub/UBL conjugation machinery. Despite the central role of the E1 enzyme in protein modification, a quantitative method to measure Ub/UBL activation by E1 is lacking. Here, we present a mass spectrometry-based assay to accurately measure the activation of Ub/UBL by E1 independent of the E2/E3 enzymes. Our method does not require radiolabeling of any components and therefore can be used in any biochemical laboratory having access to a mass spectrometer. This method allowed us to dissect the concerted process of E1-E2-catalyzed Ub conjugation in order to separately characterize the process of Ub activation and how it is affected by select mutations and other factors. We found that the hydrophobic patch of Ub is important for the optimal activation of Ub by E1. We further show that the blockers of the Ub-proteasome system such as ubistatin and fullerenol inhibit Ub activation by E1. Interestingly, our data indicate that the phosphorylation of Ub at the S65 position augments its activation by the E1 enzyme.

Abbreviations

MESNa	Sodium 2-mercaptoethanesulfonate
COSR	C-terminal thioester (–CO–S–CH_2–CH_2–SO_3^-)
EDTA	Ethylenediaminetetraacetic acid
E1	Ubiquitin activating enzyme
E2	Ubiquitin conjugating enzyme
E3	Ubiquitin-protein ligase
Ub	Ubiquitin
UBA	Ubiquitin-associated domain
UBC	Ubiquitin-conjugating enzyme or domain
UBL	Ubiquitin-like protein
NEDD8	Neuronal precursor cell expressed developmentally down regulated gene 8
SUMO	Small ubiquitin modifier
SCF	Skp, cullin, F-box containing ligase complex
GST	Glutathione S-transferase
ATP	Adenosine triphosphate
PPi	Inorganic pyrophosphate
ESI	Electro-spray Ionization
AQUA	Absolute Quantification
UV	Ultraviolet
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis

Supplemental Experimental Procedures

Purification of Ub, UBLs, and E1

Human Ub was expressed in pJY2 cells and purified to homogeneity as described previously[1,2]. The E1 (human UAE1) of Ub was expressed in Rosetta cells and purified to >95% purity using a Ni-column. Human NEDD8 was grown in BL21 (DE3) cells and purified by passing the lysate through a 30 kDa cut-off filter followed by SEC chromatography to reach > 99% purity. The E1 of NEDD8 (APPBPA/UBA3) were expressed in BL21 (DE3) cells and purified by GST-affinity chromatography. Human SUMO-2 was expressed in Rosetta cells and purified by adding glacial acetic acid drop-by-drop until milky precipitates formed. The supernatant was dialyzed and subjected to cation chromatography to get > 99% pure protein. The SUMO E1 (human SEA1/SEA2) was expressed in BL21 (DE3) cells and purified using Ni-column. Small aliquots of all the E1 proteins were frozen at -80 ^{0}C and all experiments were performed with the aliquots thawed only once. All chromatography purifications were performed as per manufacturer's instructions.

Enzymatic phosphorylation of Ub at S65 position

The protocol for phosphorylation of Ub was adapted from[3,4]. Briefly, 2 mM of Ub was incubated with 30 µM of *Tc*PINK1, 10 mM $MgCl_2$, 2 mM DTT in 50 mM Tris-HCl pH 7.5 (total volume 0.3 mL) at room temperature. ATP was added to a final concentration of 10 mM to start the reaction. The phosphorylated Ub was purified from the non-phosphorylated Ub by using high performance cation exchange column (SPHP column from GE Healthcare). The purity and the attachment of phosphate to Ub was verified by ESI-MS and phosphorylation at the S65 position was verified by matching the previously obtained NMR spectrum of S65-phosphorylated Ub.

Activation of Ub/UBLs by E1 enzymes

0.5 mM pure Ub/UBLs (unlabeled or ^{15}N-labeled) was incubated with 0.5 µM of respective E1 at 30 °C along with 10 mM ATP, 10 mM $MgCl_2$, and 0.1 M MESNa in a 20 mM sodium-phosphate buffer at pH 8.0. All concentrations shown above are the final concentrations of the components in a total of 100 µL reaction mixture. Equal amounts of reactions (20 µL) were aliquoted and stopped by adding 4 µL of 0.5 M EDTA. An internal standard of Ub/UBLs was added to a final concentration of 0.5 mM. This standard was identical to the analyzed protein except for the mass,

due to differential isotope labeling, e.g, if ^{15}N-labeled Ub was used as a reactant protein for activation then unlabeled Ub can be used as the internal standard or *vice versa*. The aliquots were buffer exchanged with water (3X) using 3 kDa cut-off filters (Amicon, 0.5 mL volume). 10 µL of samples were mixed with 2 µL of 0.4% (v/v) TFA and injected (3X) into a mass spectrometer in ESI+ flight mode.

Mass Spectrometry

All ESI-MS spectra were acquired on either JEOL AccuTOF-CS mass spectrometer or LTQ Orbitrap 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 combined with S/N of 3 to determine the molecular mass.

***In vitro* ubiquitination reaction**

0.5 mM Ub or its mutants was incubated with 0.5 µM E1, 20 µM E2 (E2-25K or UBC13/MMS2, or UBE2S) in a 50 mM Tris-HCl buffer at pH 8.0 containing 5 mM ATP, 5 mM $MgCl_2$, 1 mM TCEP, phosphocreatine and phosphocreatine kinase. All concentrations shown above are the final concentrations of the components in a total of 100 µL reaction mixture. An equal amount (10 µL) of the reactions were aliquoted at indicated time points and stopped by adding SDS-loading buffer to inactivate the enzymes. Equal amounts of aliquots were loaded onto a 15% SDS-PAGE to separate the proteins. The gels were stained with coomassie blue to visualize the proteins.

NMR studies

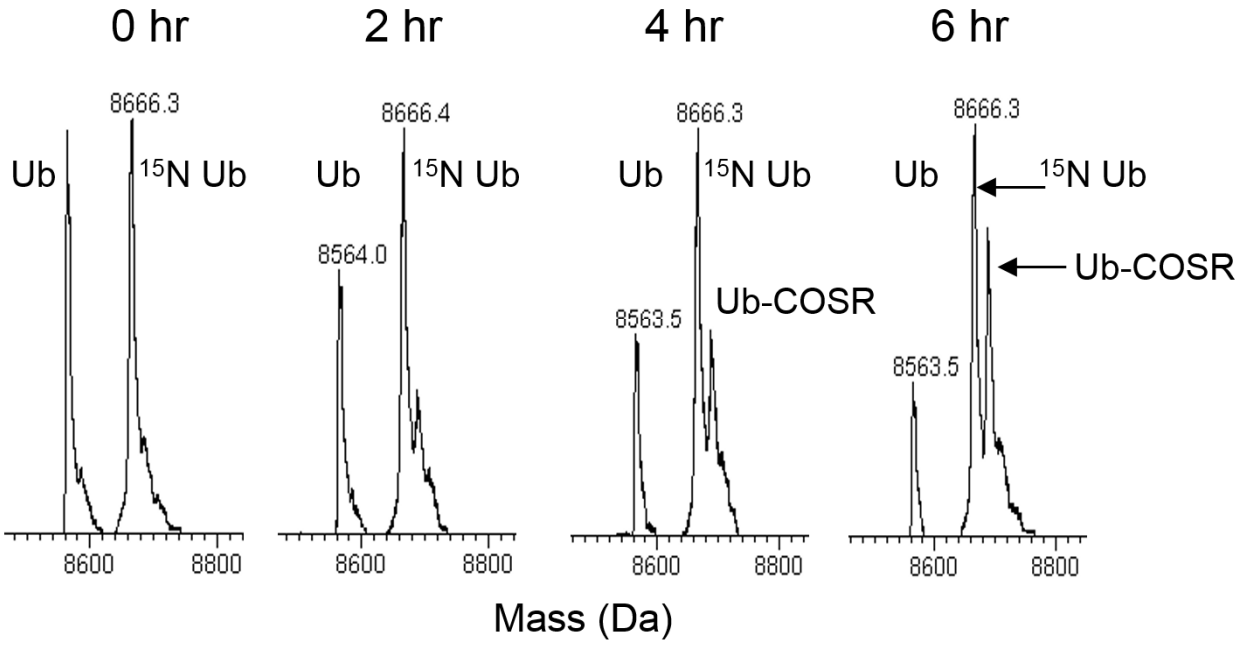
NMR measurements were performed at 22.5 ^{o}C on 600 or 800 MHz Avance III spectrometers (Bruker Biospin, Inc.) equipped with cryoprobes. Proteins samples for the spectra shown in Figure S6 contained 2 mM protein in 160 µL of 20 mM sodium phosphate buffer (pH 6.8) with 10% D_2O and 0.02% NaN_3 in a 5/3 mm NMR tube (New Era Enterprises, Inc.). For Ub:E1 binding studies the proteins samples for Ub:E1 binding assays shown in Figure S3B contained 50 µM ^{15}N Ub or a mixture of 36 µM ^{15}N Ub with 31 µM of either $E1^{WT}$ or $E1^{C632S}$ in a 50 mM sodium-phosphate buffer (pH 8.0), with 7% D_2O and 0.02% NaN in a Shigemi tube. The data were processed in TopSpin and analyzed using Sparky[5] and Matlab.

Supporting Table S1. Initial rates of activation of the indicated reactant proteins by their cognate E1 enzymes

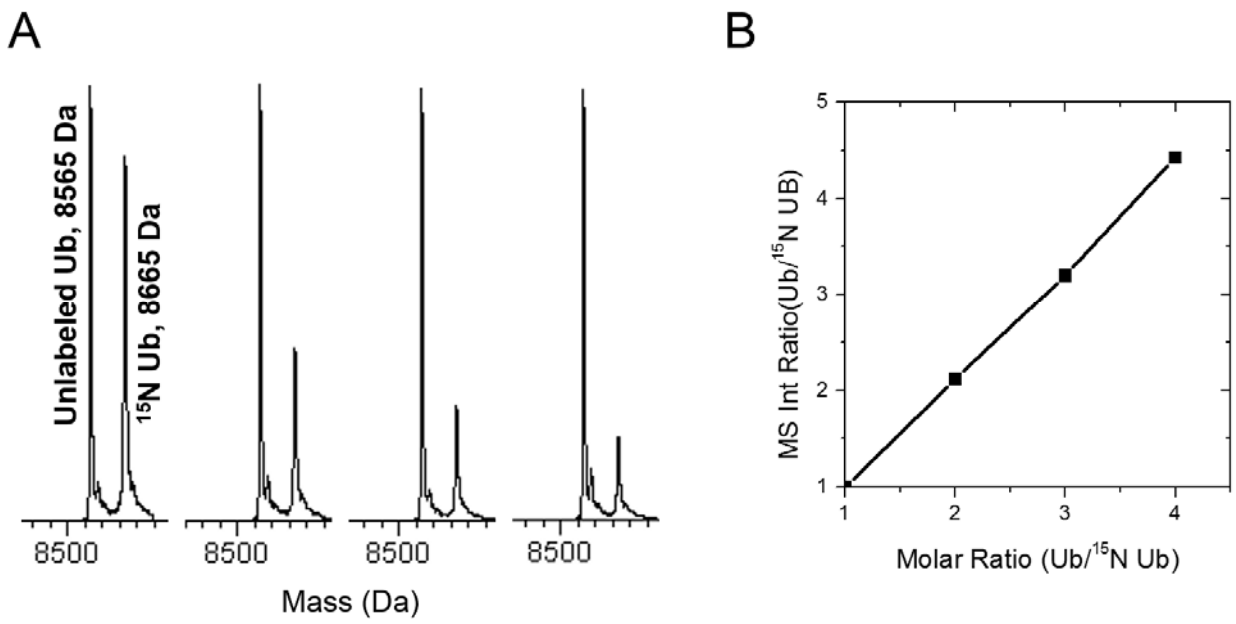
Protein	Initial Rate (nmol/min)
WT Ub	0.270 ± 0.039
G10A Ub	0.228 ± 0.001
E34R Ub	0.256 ± 0.003
Q41N Ub	0.378 ± 0.008
Q49T Ub	0.421 ± 0.007
N60H Ub	0.248 ± 0.008
R72A Ub	0.044 ± 0.005
R72K Ub	0.110 ± 0.004
R74K Ub	0.206 ± 0.004
WT Ub	0.170 ± 0.005
S65D Ub	0.173 ± 0.018
S65E Ub	0.192 ± 0.014
p-S65-Ub	N.D.
WT Ub*	0.171 ± 0.002*
p-S65-Ub*	0.328 ± 0.002*
WT Ub	0.247 ± 0.015
L8A&I44A Ub	0.167 ± 0.001
V70A Ub	0.356 ± 0.003
L8A&I44A&V70A Ub	0.098 ± 0.016
K48-Ub_2	0.257 ± 0.033
K63-Ub_2	0.253 ± 0.011
K48-Ub_3	0.294 ± 0.004
K48-Ub_4	0.313 ± 0.043
WT Ub	0.214 ± 0.046
Ub + ubistatin A	0.057 ± 0.013
Ub + fullerenol	0.042 ± 0.016
NEDD8	0.087 ± 0.002
SUMO-2	0.235 ± 0.003

The initial rate of the reaction (0-2 hr or 0-0.5 hr for the proteins marked with the asterisk) was calculated as $V_0=[S]_0\times\ln(I_0/I_t)/t$, where $[S]_0$ is the (initial) concentration of the substrate at time zero, and I_0 and I_t are the intensities of the MS signals of the substrate at time zero and time t, respectively. The initial rates were averaged over triplicate measurements and are shown as the mean ± standard deviation of the mean.

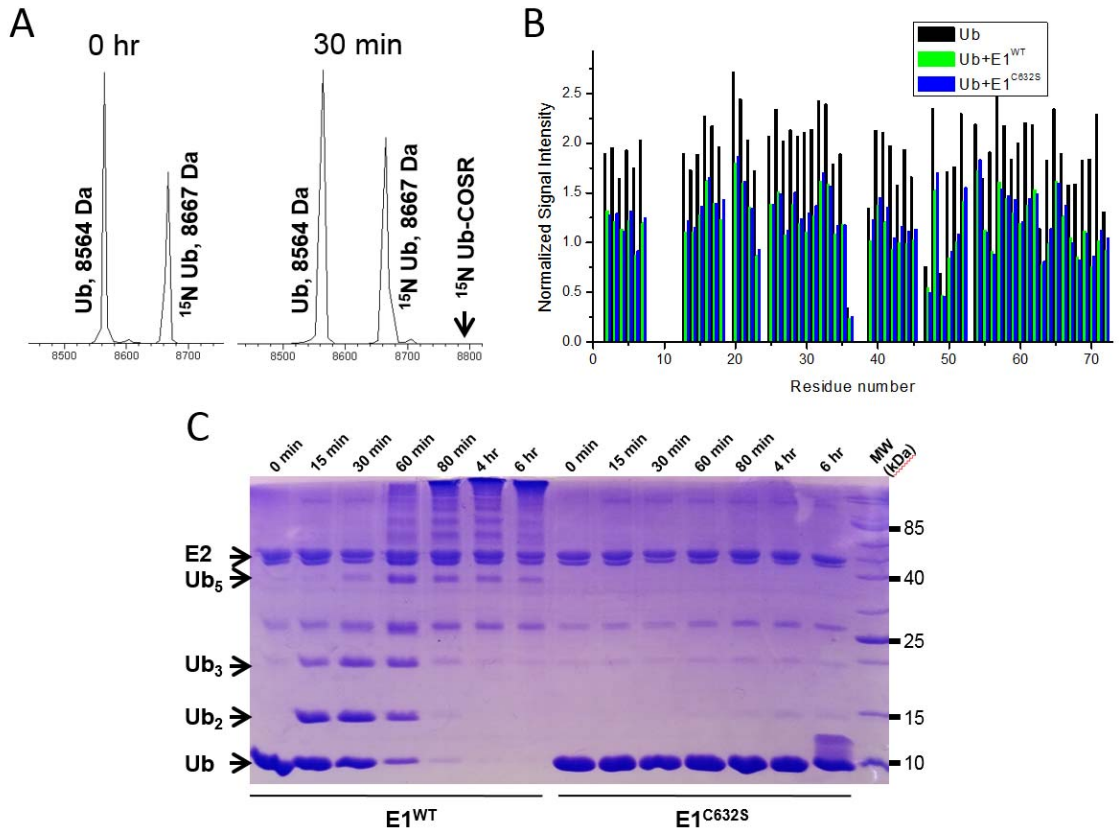
The rates shown here were converted to nanomoles/min by multiplying V_0 by the sample volume. The results in this table are separated into 7 groups. The reactant proteins in each group were activated by using the same aliquots of E1 and at the same time, hence the proteins in each group should be compared with the respective Ub^{WT} for this group, where applicable. 50 nmol of the protein (Ub or UBL) were used to start the reaction for all reactant proteins except for those in groups 2 and 3, where 25 nmol of proteins were used. The activation rates in the presence of ubistatin A or fullerenol (group 5) were derived using the 0h and 4h data points and are averaged over two samples/reactions, each analyzed in triplicates. The errors reported here reflect standard deviations calculated based on the MS analyses of same sample injected three times.



Supporting Figure S1. Activation of unlabeled Ub by the E1 enzyme. ^{15}N-enriched Ub is used here as the internal standard. Unlabeled Ub (8564 Da) was incubated with the E1 enzyme, $MgCl_2$, ATP, and MESNa in a 20 mM sodium phosphate buffer at 30 ^{0}C for 2, 4, or 6 hr. The reactions were stopped by adding EDTA followed by addition of equimolar amount of ^{15}N-labeled Ub (8666 Da). The mixture was buffer exchanged into water containing 0.006% TFA (pH 6.0) and then subjected to ESI+ MS analysis in triplicate. The spectra at each point were deconvoluted using MagTran; a representative spectrum for each point is shown. The formation of the thioester increases the mass by 124 Da.



Supporting Figure S2. Evaluating the accuracy of MS-based quantification of the amount of Ub. Unlabeled Ub was mixed with ^{15}N Ub in a molar ratio of 1, 2, 3, or 4 and injected into the mass spectrometer in ESI+ mode. The MS spectra were acquired (A) and the measured intensity ratios (Ub/^{15}N-Ub) were plotted against the molar ratios of the proteins (B). The ratio was normalized to be 1 for the 1:1 point, and this scaling factor was applied to the rest of the points. Panel B shows the agreement between the ratio of MS signal intensities of Ub and ^{15}N Ub for various molar ratios.

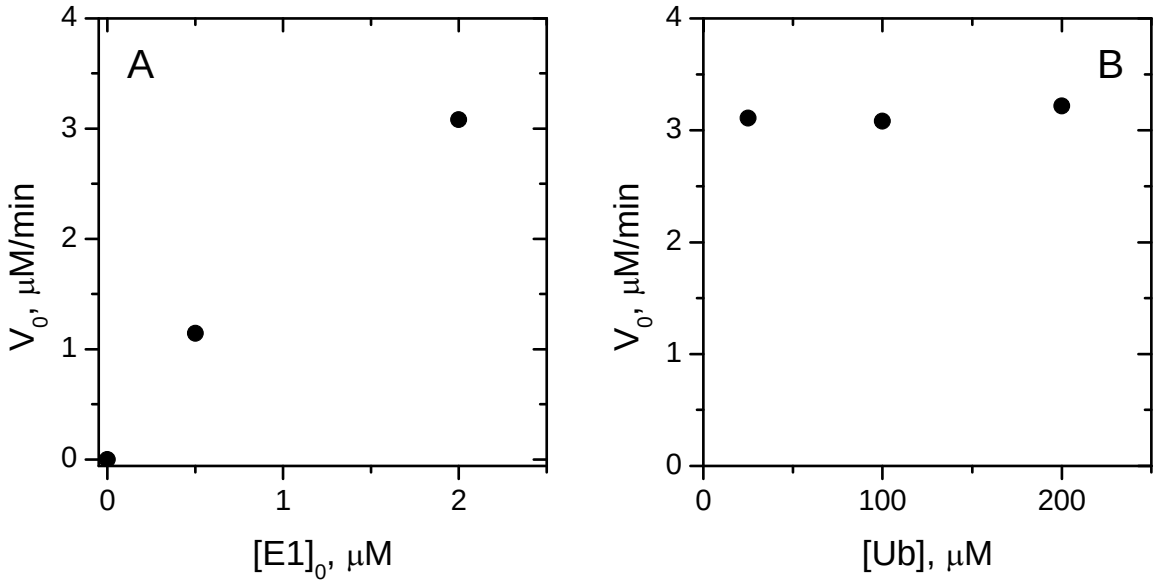


Supporting Figure S3. C632S mutation of the catalytic cysteine dramatically impairs the catalytic activity of E1 while preserving its binding to Ub.

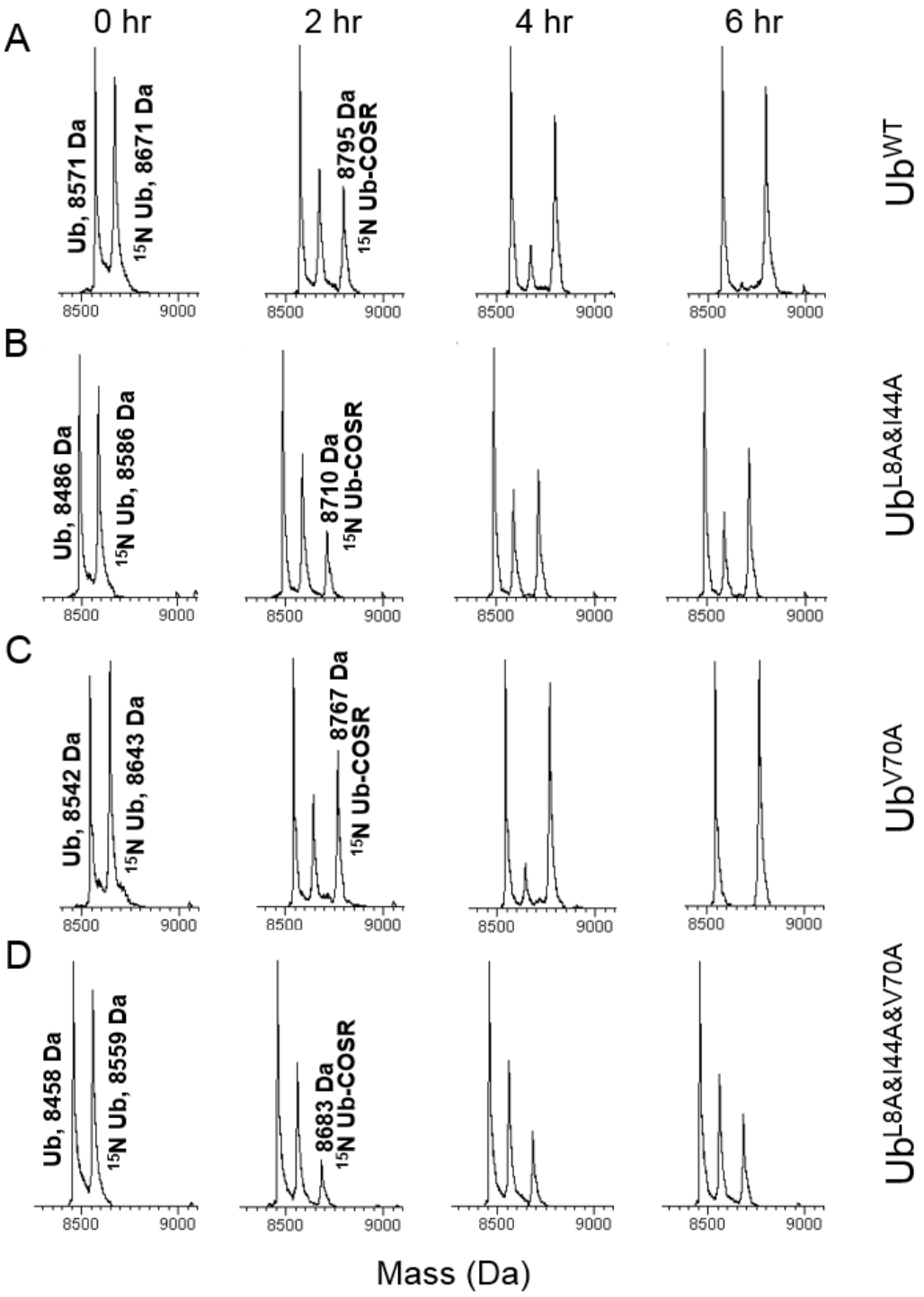
(A) 0.5 mM ^{15}N Ub was mixed with 0.5 μM E1^{C632S} in a 50 mM sodium-phosphate buffer (pH 8.0) containing 10 mM ATP, 10 mM $MgCl_2$, and 0.1 M MESNa. The reaction was stopped at 30 min by adding EDTA. Unlabeled Ub was added as an internal standard marker. The mixture was buffer exchanged into water and injected into ESI+ MS in triplicate. The MS spectra were deconvoluted using MagTran; representative spectra at indicated time points are shown. The expected position of the Ub-COSR signal is indicated by an arrow.

(B) 36 μM ^{15}N Ub was mixed with 31 μM of either E1^{WT} or E1^{C632S} mutant in a 50 mM sodium-phosphate buffer (pH 8.0). Heteronuclear ^{1}H-^{15}N NMR spectra were collected for Ub, Ub+E1^{WT}, and Ub+E1^{C632S}. The NMR signal intensities of the peaks in each spectrum, normalized to the signal of G76 in order to eliminate any concentration/dilution related effects, are plotted against residue numbers as indicated. The decrease in signal intensities upon addition of E1 reflects Ub binding. A similar height of the normalized signal intensities for Ub+E1^{WT} (green bars) and Ub+E1^{C632S} (blue bars) indicates similarity of Ub binding to E1^{C632S} and E1^{WT}.

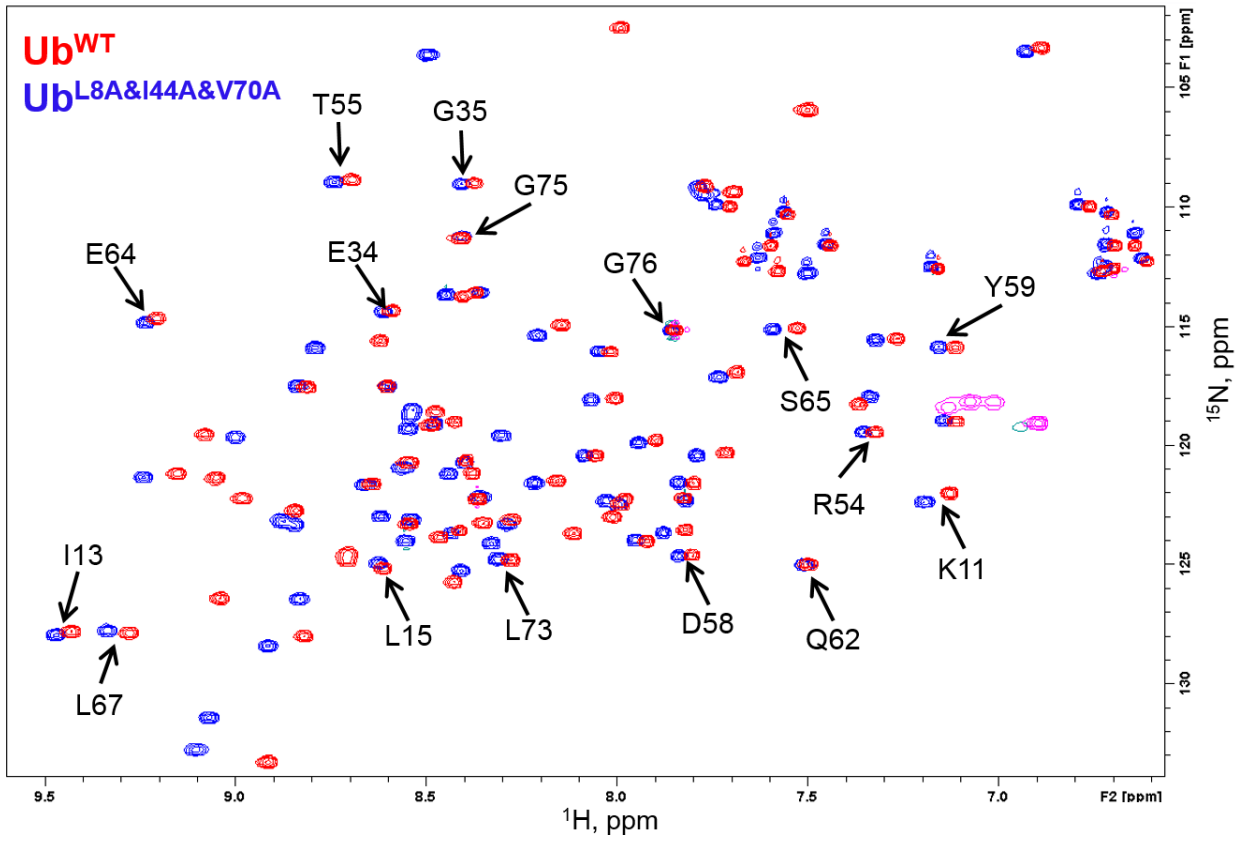
(C) Ub was mixed with E1^{WT} or E1^{C632S} in a standard ubiquitination reaction with UBC13/ MMS2 serving as the E2 enzyme. Equal amounts of reaction mixture were aliquoted at indicated time points and loaded onto a 15% SDS-PAGE. The gel was visualized by coomassie staining. The running position of Ub and formed Ub chains (Ub_2, Ub_3, Ub_5) as well as E2 are indicated by arrows. Molecular weight marker (MW) was loaded onto the last lane of the gel.



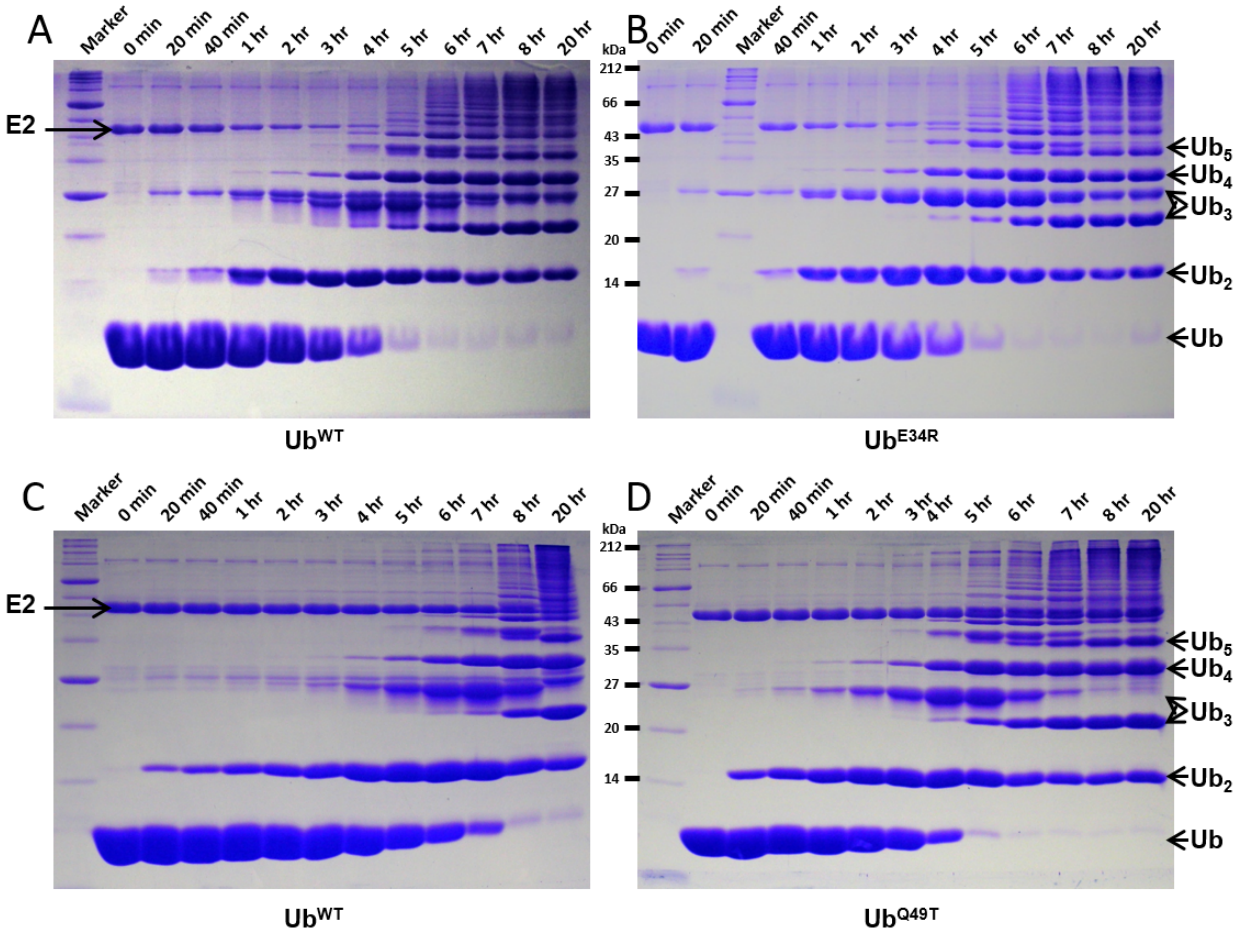
Supporting Figure S4. Assessment of the dependence of the initial rates of WT Ub activation by E1 on the concentrations of E1 (A) and Ub (B). Shown are the initial rates of the reaction determined as detailed in Table S1.



Supporting Figure S5. Activation of ubiquitin and its hydrophobic patch mutants by the E1 enzyme as a function of time. Activation of ^{15}N-enriched Ub^{WT} (A) or the indicated hydrophobic-patch mutants (B-D) by Ub E1 was performed as detailed in the main text (Figure 1 and the Experimental Section). Unlabeled Ub or respective hydrophobic-patch mutants were used as internal standards. The mixture was buffer exchanged into water and then subjected to ESI-MS analysis in triplicate. Shown are representative spectral regions for each point.

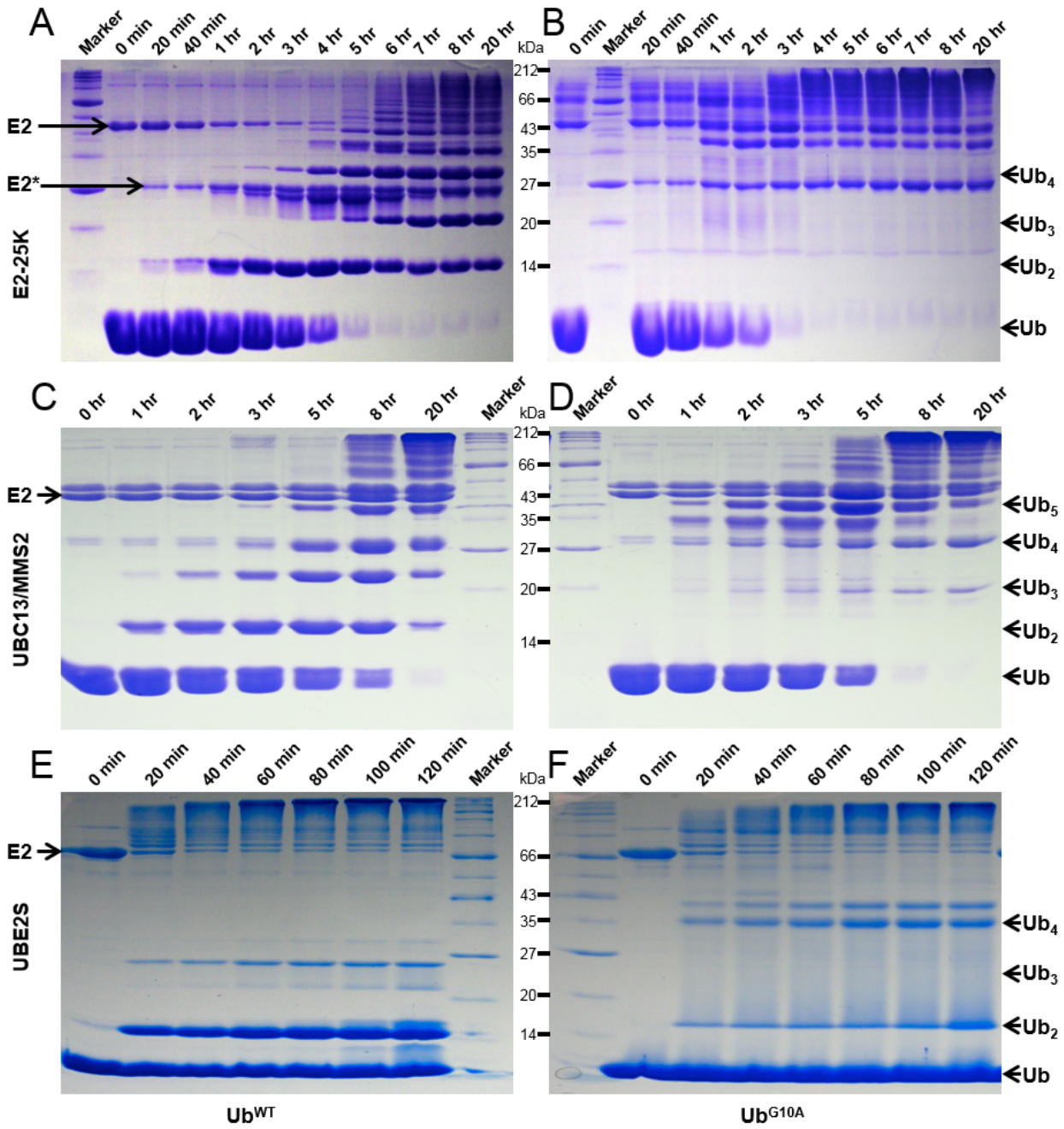


Supporting Figure S6. Overlay of ^{1}H-^{15}N HMQC NMR spectra of WT Ub (red) and L8A&I44A&V70A Ub mutant (blue). Selected residues that have similar chemical shifts in both Ub variants are marked and indicated by arrows.

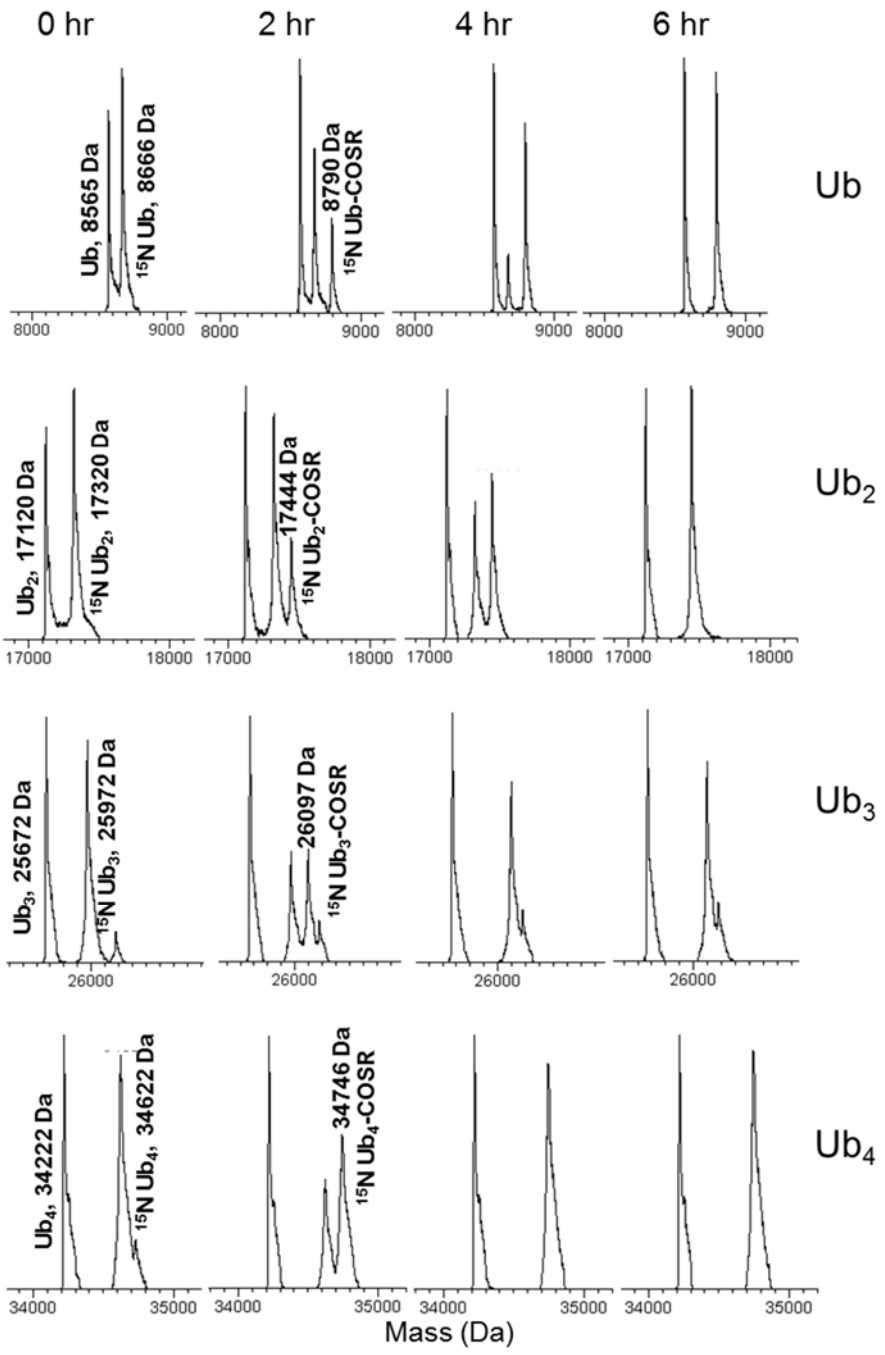


Supporting Figure S7. The E34R and Q49T mutants of Ub polymerize into chains like WT Ub.

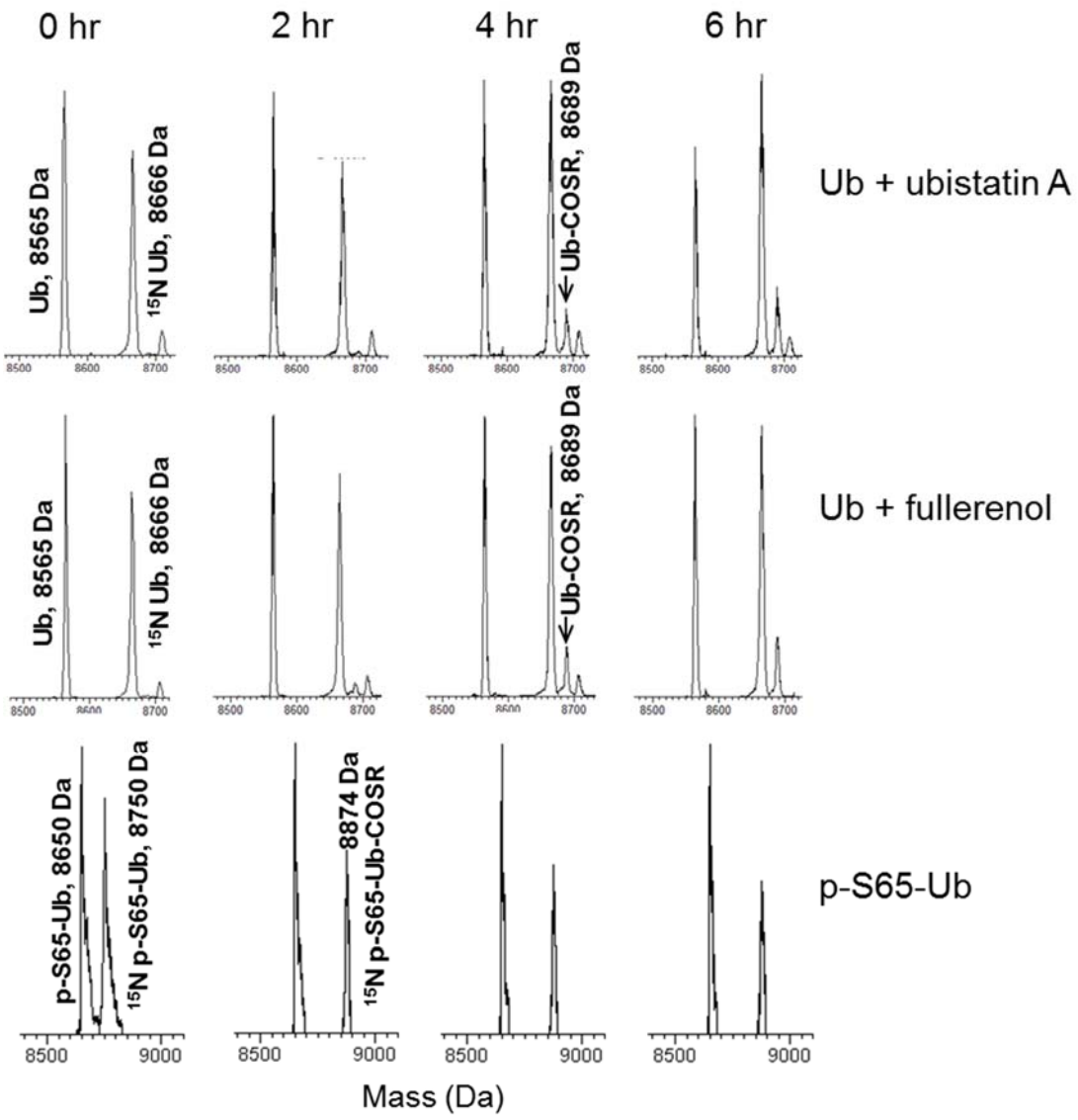
In vitro reactions were set up with either Ub^{WT} (A, C), Ub^{E34R} (B), or Ub^{Q49T} (D) variants in a standard ubiquitination reaction containing E1 and E2-25K. The reactions were aliquoted at indicated time points and added equal amounts of SDS-loading buffer to inactivate the enzymes. Proteins were resolved on 15% reducing SDS-PAGE and stained with coomassie blue to visualize the proteins.



Supporting Figure S8. Comparison of the ability of G10A and WT Ub to polymerize into chains of various linkages. SDS-PAGE gels of *in vitro* polymerization reactions for either Ub^{WT} (left) or Ub^{G10A} (right) catalyzed by linkage-specific E2s: (A-B) E2-25K (K48-specific), (C-D) UBC13/MMS2 (K63-specific), or (E-F) UBE2S (K11-specific). The reactions were performed and analyzed as detailed in the Experimental Section. In upper panels (A-B), E2 indicates the running position of GST-E2-25K (50 kDa) while E2* corresponds to E2-25K and GST (both ~ 25 kDa) resulting from GST-E2-25K cleavage.

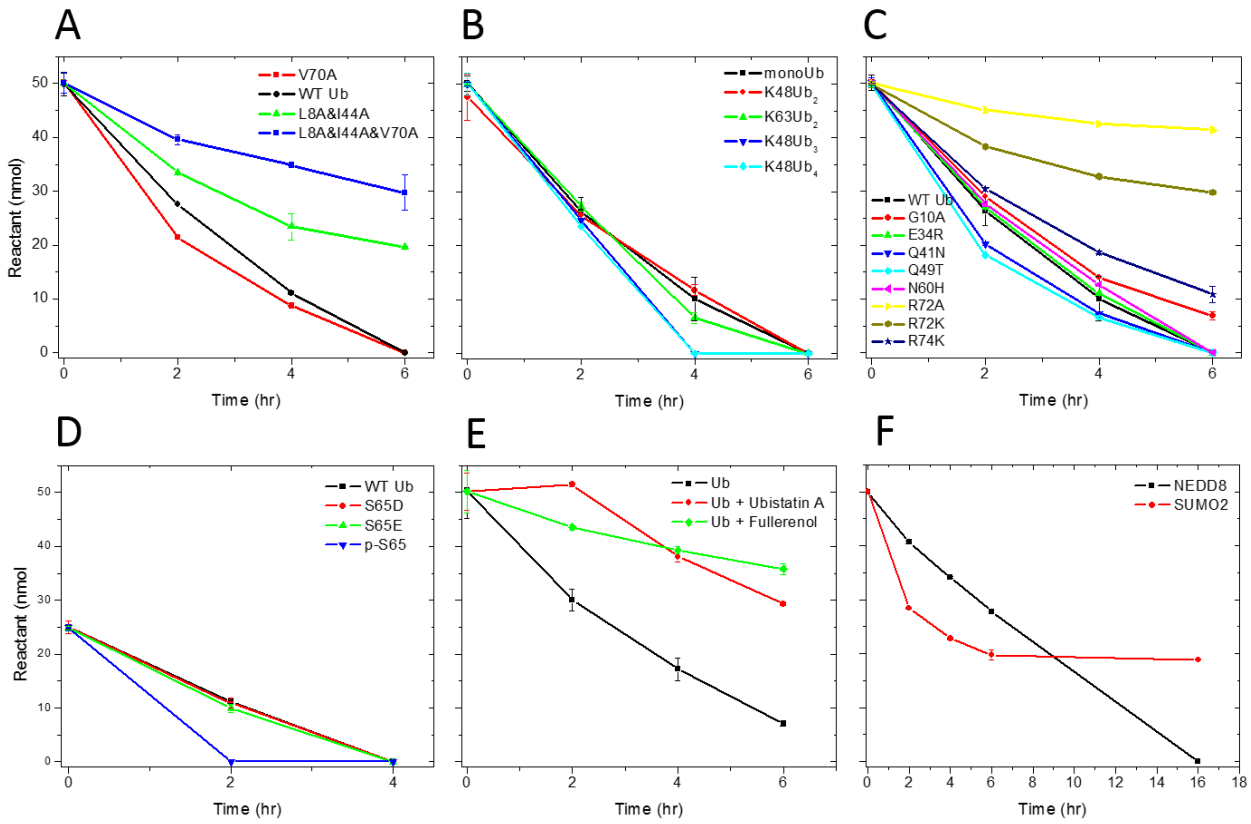


Supporting Figure S9. Preassembled K48-linked Ub chains of various lengths are activated by the E1 enzyme. K48-linked ^{15}N-labeled Ub chains, as indicated, were incubated with the E1 enzyme, $MgCl_2$, ATP, and MESNa in a 20 mM sodium phosphate buffer at 30 ^{0}C for 2, 4, or 6 hr. The reactions were stopped by adding EDTA followed by addition of equimolar amount of unlabeled respective Ub chains, which served as internal standards for MS-based quantitation. The mixture was buffer exchanged into water and then subjected to ESI+ MS analysis in triplicate. A representative spectrum for each points is shown. Line-plots of these data are shown in Figures 2B and S11B.



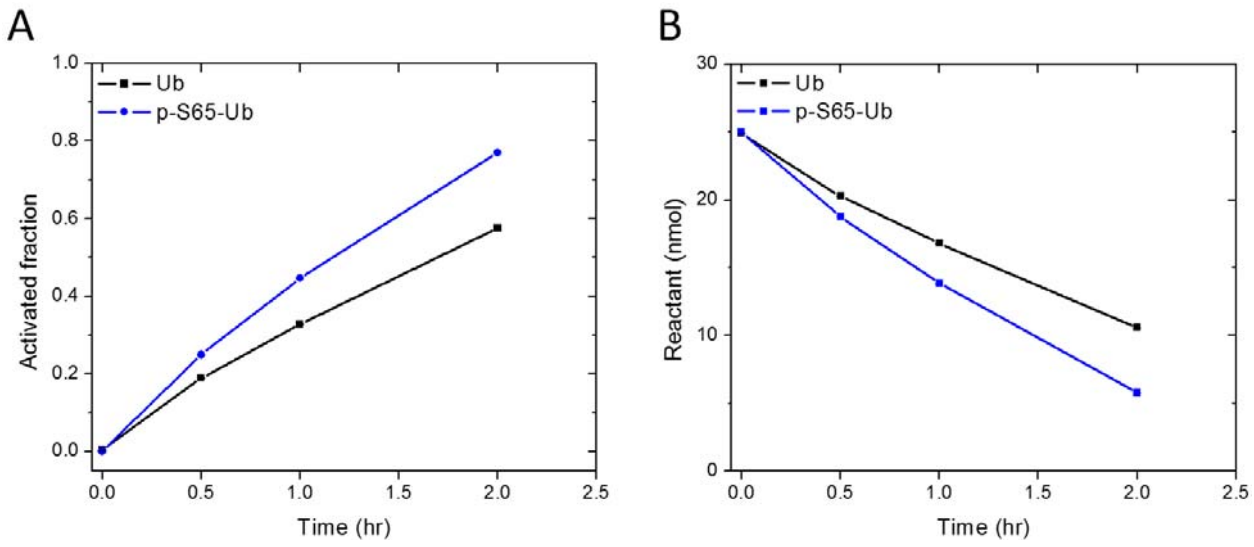
Supporting Figure S10. The effect of inhibitors and phosphorylation on Ub activation by E1.

Top and middle panels: unlabeled Ub^{WT} was mixed with either ubistatin A (upper panel) or fullerenol (middle panel) in a 1:1 molar ratio followed by incubation with the E1 enzyme, $MgCl_2$, ATP, and MESNa in a 20 mM sodium phosphate buffer at 30 ^{0}C for 2, 4, or 6 hr. The reactions were stopped by adding EDTA followed by addition of equimolar amount of ^{15}N-enriched Ub which served as internal standard. The mixture was buffer exchanged into water and then subjected to ESI+ MS analysis in triplicate.
Bottom panel: ^{15}N-labeled Ub phosphorylated at S65 (p-S65-Ub, 8745 Da) was incubated with the E1 enzyme, $MgCl_2$, ATP, and MESNa in a 20 mM sodium phosphate buffer at 30 ^{0}C for 2, 4, or 6 hr. The reactions were stopped by adding EDTA followed by addition of equimolar amount of unlabeled phosphorylated Ub (8650 Da) which served as internal standard. The mixture was buffer exchanged into water and then subjected to ESI+ MS analysis in triplicate.



Supporting Figure S11. Comparison of the kinetics of activation of Ub, its mutants, and the UBLs by E1. Shown is the amount of the unactivated reactant protein as a function of time for (A) hydrophobic patch mutants of Ub, (B) mono- and polyUb, (C) other Ub mutants studied here, (D) p-S65 Ub and its phosphomimics, (E) Ub in the presence of ubistatin A or fullerenol, and (F) NEDD8 and SUMO-2.

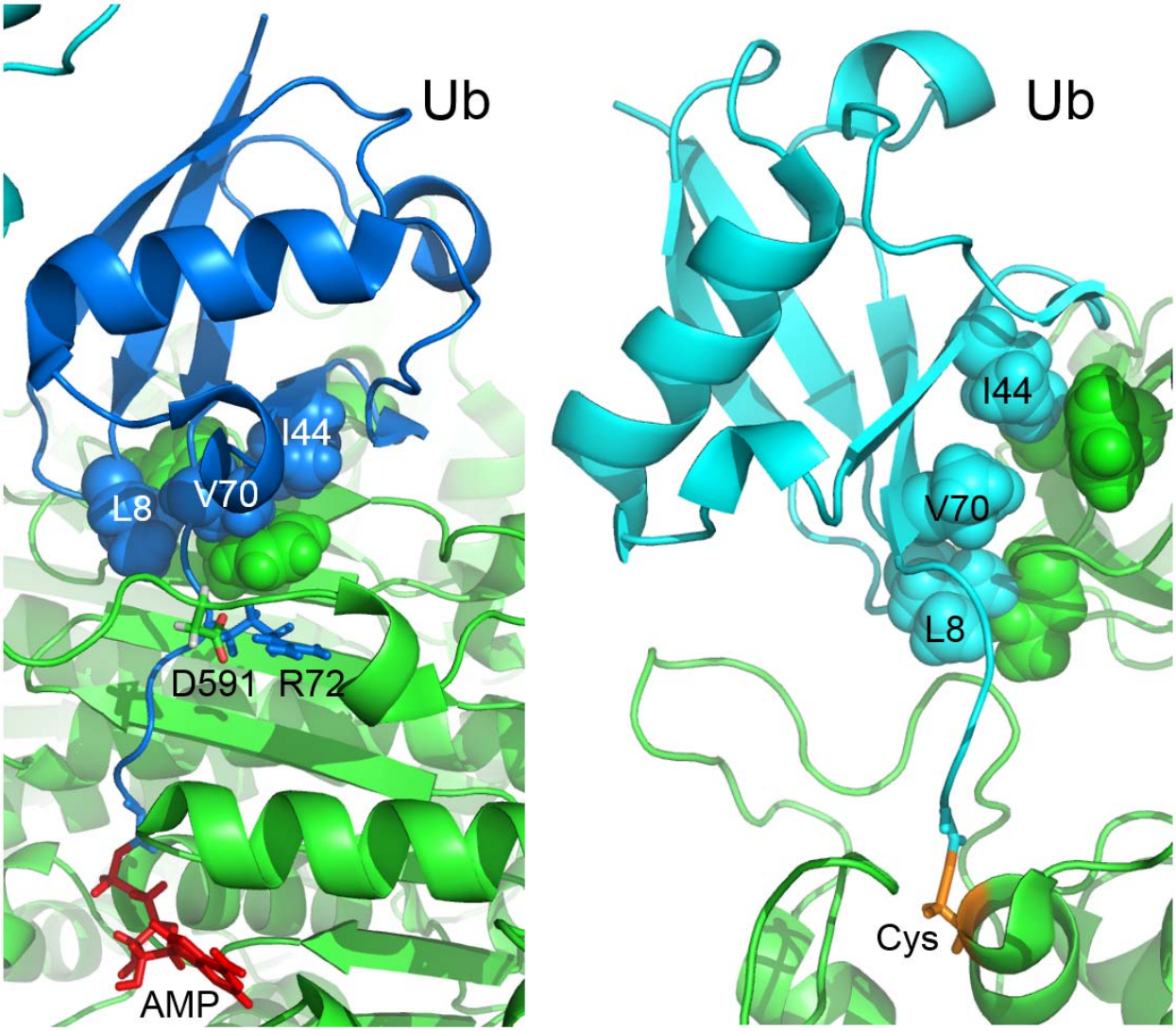
The amount of the reactant protein left at each time point was calculated by multiplying the total amount of the reactant protein in the reaction by the fraction of the reactant protein left at a given time. The error bars indicate the standard deviation of the mean value of triplicate measurements.



Supporting Figure S12. Activation of phosphorylated Ub and Ub^{WT} by the E1 enzyme at earlier points.

(A) Activated fractions of the phosphorylated Ub (blue) and Ub^{WT} (red) at different time points in the reaction (0, 0.5, 1, and 2 hr). The intensities of the MS signals were converted to the thioesterified fraction of the protein as detailed in the Experimental Section (main text).

(B) The remaining amount of (unactivated) reactant proteins (calculated as described in Figure S11) for different time points (0, 0.5, 1, and 2 hr).



Supporting Figure S13. E1-Ub contacts are meditated by Ub's hydrophobic patch residues. Shown are fragments of the co-crystal structure (PDB ID 4NNJ) of E1 with two ubiquitin molecules: (Left) Ub adenylate bound to the adenylation domain and (Right) the other Ub linked via thioester to the catalytic cysteine. Ubs are colored marine (left) and cyan (right), E1 is colored green. Side chains of Ub's hydrophobic-patch residues (L8, I44, V70) are shown as spheres, as well as E1 residues in contact with them. The AMP and the catalytic cysteine are shown in stick representation, colored red and orange, respectively. Also shown on the left panel is the contact between R72 of Ub and D591 of E1, both as sticks. Note the difference in contacts involving Ub hydrophobic patch residues (especially V70) and E1 on the two panels.

References

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