Supporting Information

Non-enzymatic assembly of natural polyubiquitin chains of any linkage composition and isotopic labeling scheme

Carlos Castañeda, Jia Liu, Apurva Chaturvedi, Urszula Nowicka, T. Ashton Cropp, David Fushman

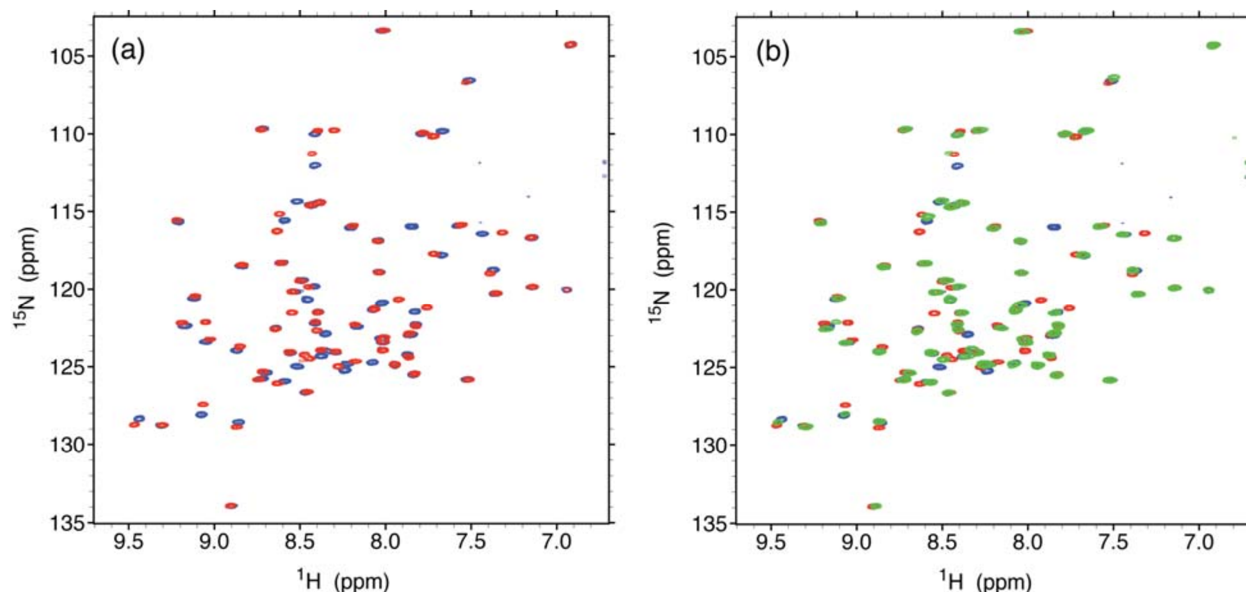

Figure S1. ^{1}H-^{15}N TROSY-HSQC spectra highlighting the importance of domain-specific isotopic labeling for Ub units in polyubiquitin chains. (a) Overlay of the spectra of the distal Ub (red) and the proximal Ub (blue) in all-natural K11-linked Ub_2 showing significant overlap of the signals from the two Ub monomers. (b) Overlay of the spectra of the middle domain in all-natural K11-linked Ub_3 (green), the distal Ub (red) of K11-linked Ub_2 and the proximal Ub (blue) of K11-linked Ub_2. The overlap of the NMR signals from the individual Ub units (reflecting their chemical and spectroscopic identity) would make it essentially impossible to distinguish which signal belongs to which Ub unit if the chain was uniformly isotope-labeled.

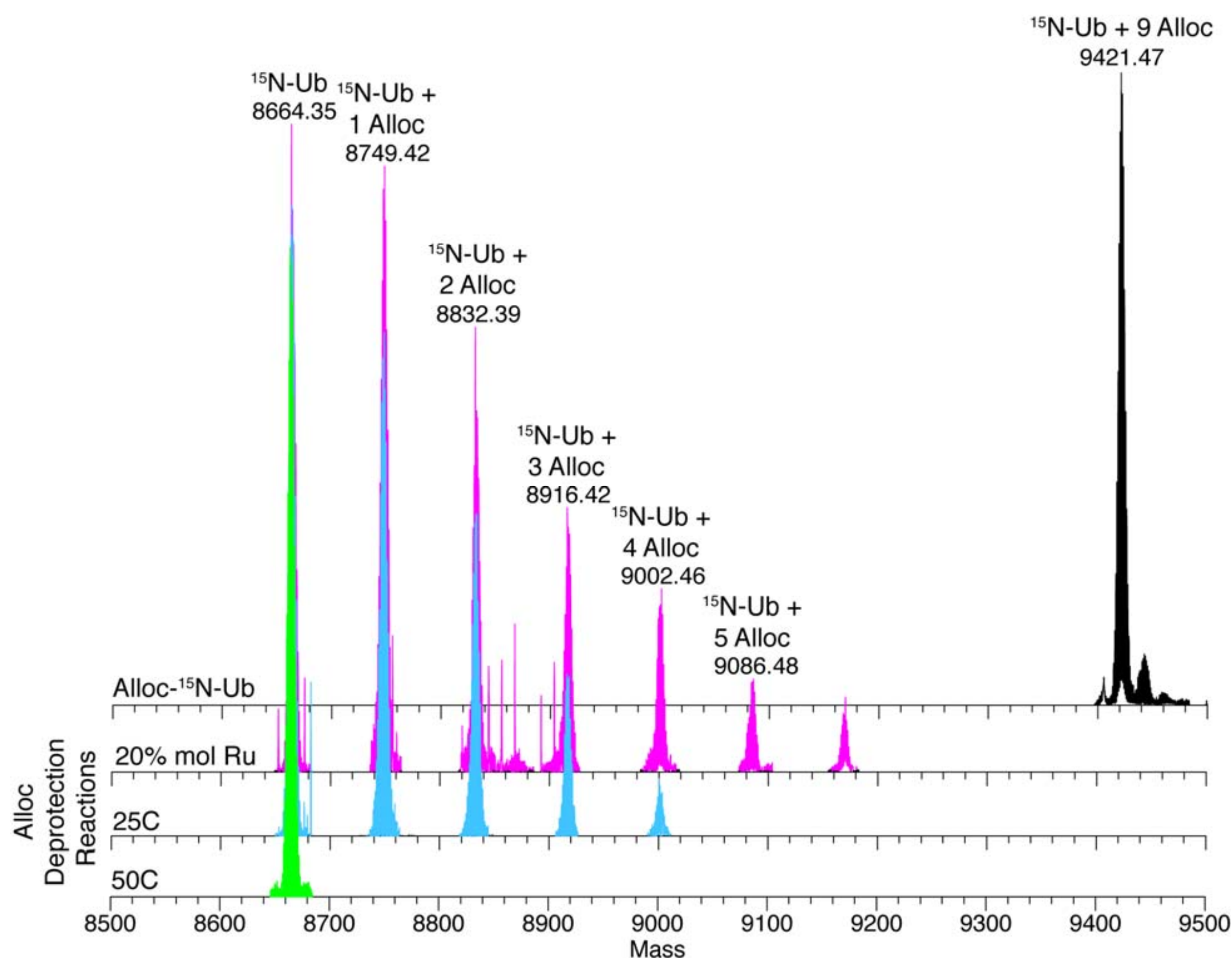

Figure S2. Optimization of Alloc deprotection reactions. In black is the deconvoluted ESI-MS spectrum of ^{15}N-Ub that is fully protected with nine Alloc groups (each Alloc group has a MW of 84 Da). Optimal Alloc deprotection conditions require the use of 50% mol Ru catalyst, 50 eq of thiophenol, 30% H_2O and 50 ºC. After the reaction is incubated for two hours, the protein is fully deprotected (green). If less catalyst (magenta) or lower temperature (sky blue) are used, the Alloc deprotection reaction does not go to completion, resulting in a distribution of multiple Ub species containing a variable number of Alloc groups.

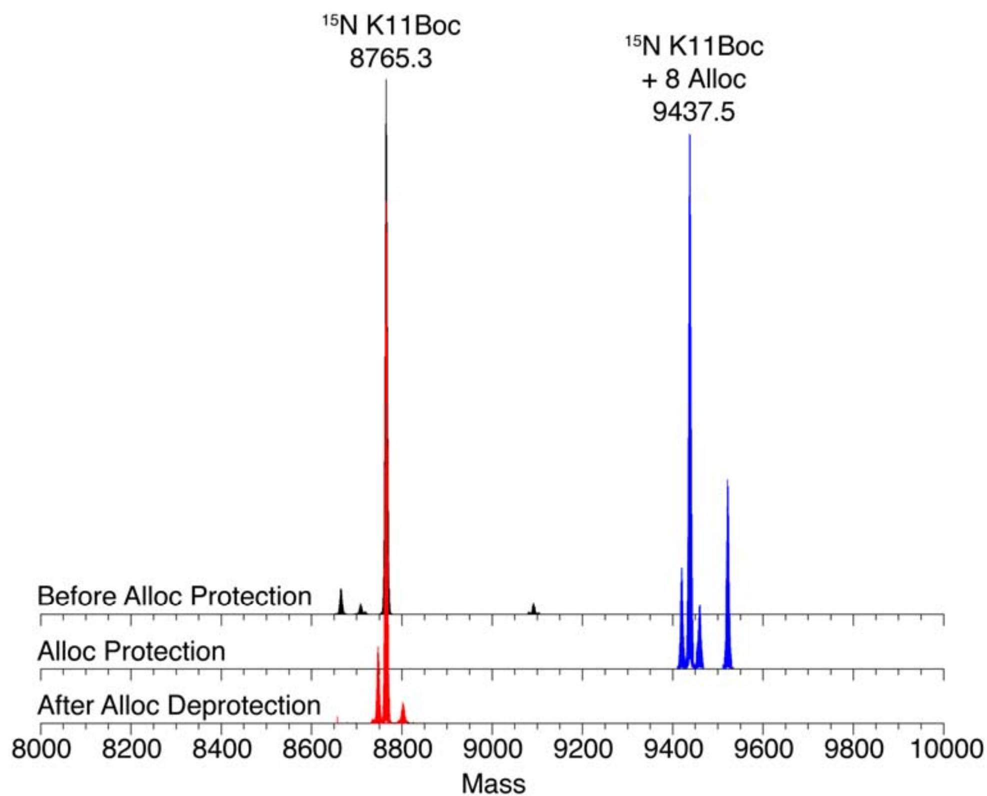

Figure S3. ESI-MS analyses demonstrate that Alloc deprotection does not remove the Boc group on Ub. Shown in black and red are ESI-MS spectra of ^{15}N-labeled K11Boc Ub before Alloc protection and after Alloc deprotection, respectively. Shown in blue is the ESI-MS spectrum of this protein after Alloc protection.

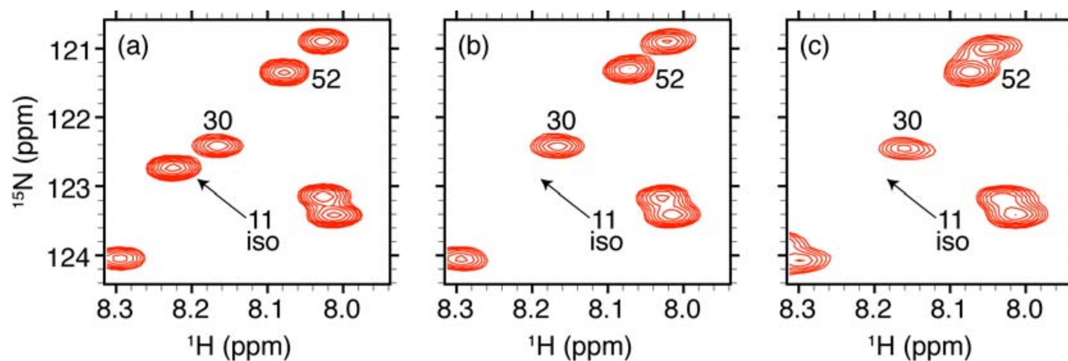

Figure S4. Portion of ^{1}H-^{15}N TROSY spectra of (a) proximal Ub in enzymatically assembled K11-linked Ub_2, (b) proximal Ub in chemically ligated K11-linked Ub_2, and (c) middle Ub in chemically ligated homogeneously K11-linked Ub_3. Arrow points toward the G76-K11 isopeptide signal that is present in the spectrum in (a). This signal is absent in the spectra in (b) and (c) due to the incorporation of unlabeled (^{14}N) Lys(Boc) amino acid at residue 11.

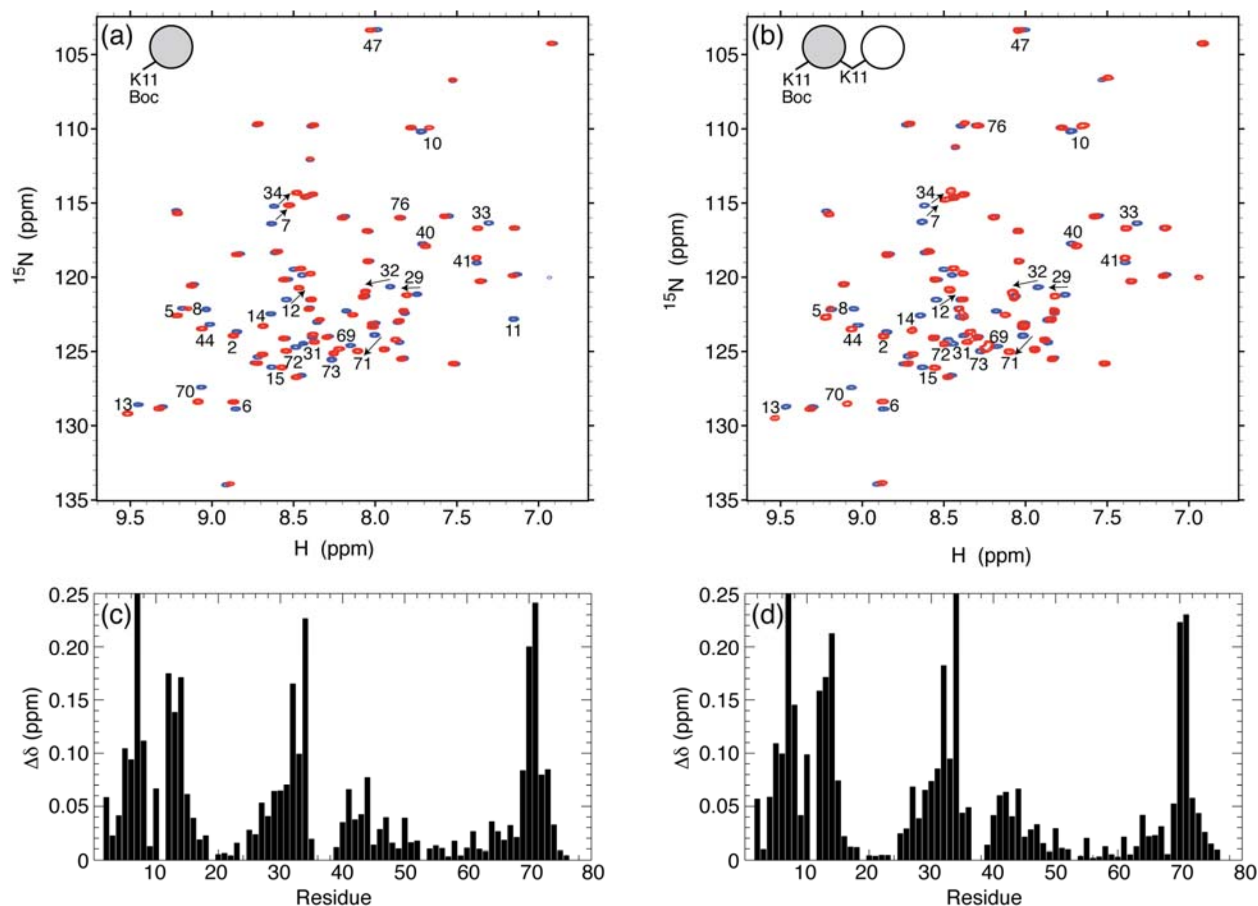

Figure S5. (a) Overlay of ^{1}H-^{15}N TROSY spectra of K11Boc Ub monomer (red) and WT Ub (blue). (b) Overlay of ^{1}H-^{15}N TROSY spectra of the distal K11Boc Ub in K11-linked Ub_2 (red) and of the distal Ub in all-natural K11-linked Ub_2 (blue). (c,d) The spectral differences between residues in (c) K11Boc Ub and WT Ub and (d) distal K11Boc in K11-linked Ub_2 and distal Ub in all-natural K11-linked Ub_2 are quantified as amide chemical shift perturbations (CSPs). The CSPs were calculated as $\Delta\delta = [(\Delta\delta_H)^2 + (\Delta\delta_N/5)^2]^{1/2}$, where $\Delta\delta_H$ and $\Delta\delta_N$ are chemical shift differences for ^{1}H and ^{15}N, respectively.

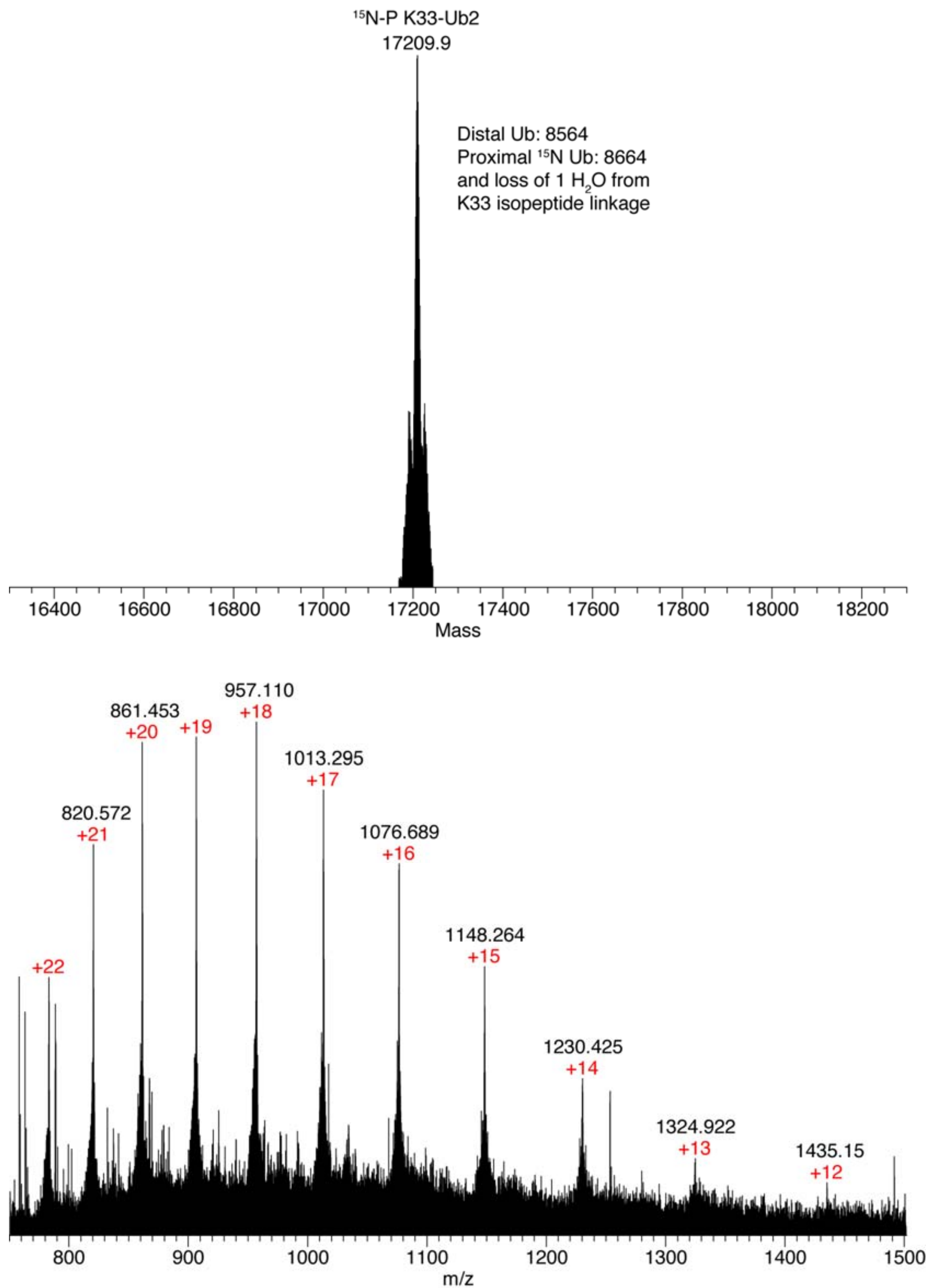

Figure S6. ESI-MS analysis of K33-linked Ub_2 with the proximal Ub ^{15}N-labeled. The expected molecular weight is 17210 Da, resulting from the sum of one unlabeled Ub (8564 Da), one TFA-treated ^{15}N-labeled Ub (8664 Da), and the loss of one water molecule from the K33 isopeptide linkage condensation.

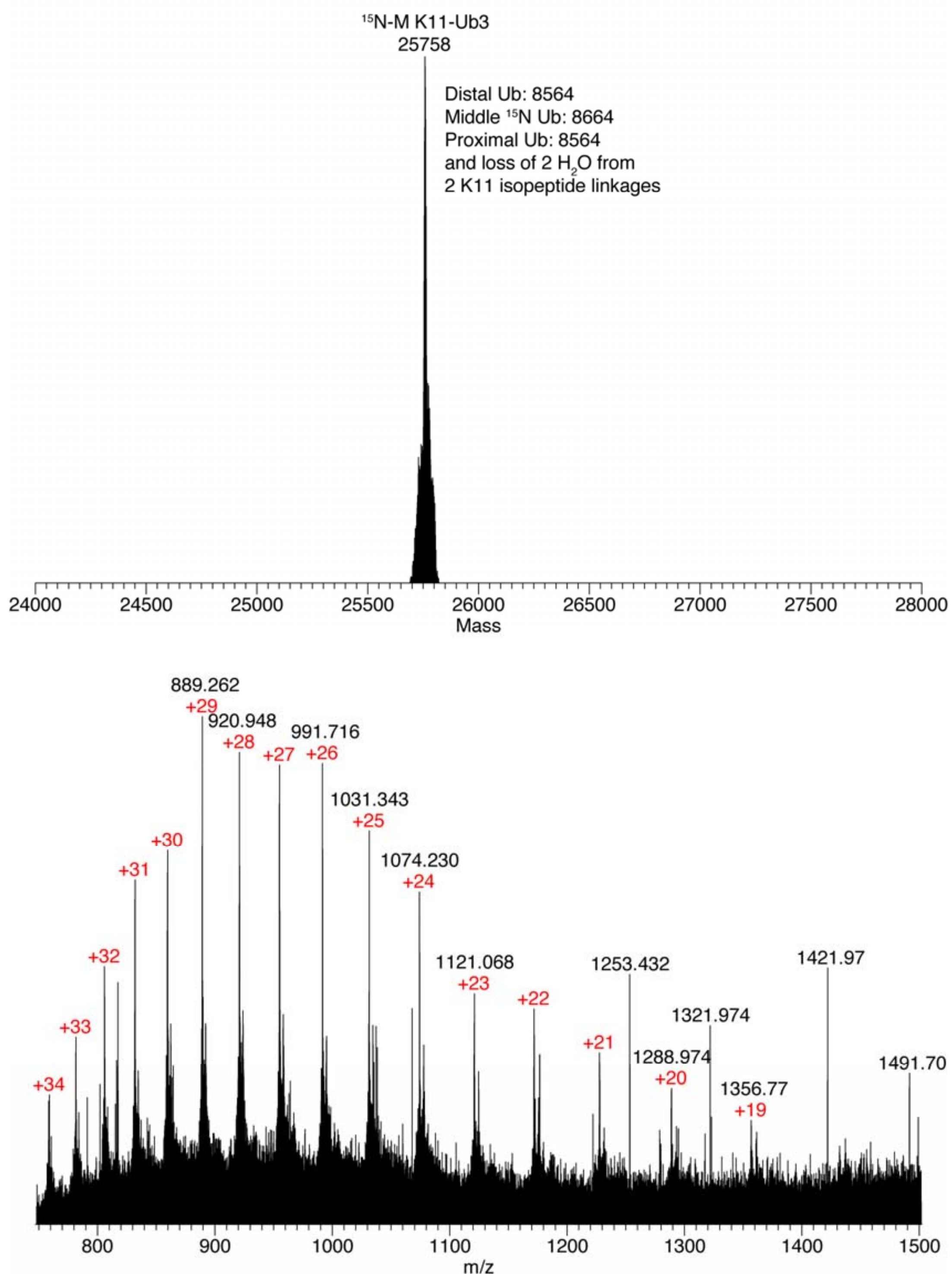

Figure S7. ESI-MS analysis of homogeneously K11-linked Ub_3 with the middle Ub ^{15}N-labeled. The expected molecular weight is 25756 Da, resulting from the sum of two unlabeled Ubs (8564 Da), one TFA-treated ^{15}N-labeled Ub (8664 Da), and the loss of two water molecules from two K11 isopeptide linkage condensations.

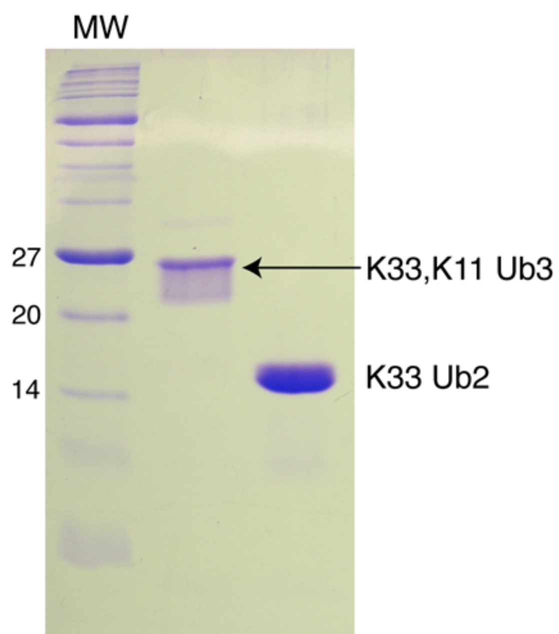

Figure S8. SDS-PAGE gel of purified (post size exclusion chromatography) K33,K11-linked Ub_3 and K33-linked Ub_2. The ESI-MS characterization of the K33-linked Ub_2 is shown in Fig.S9.

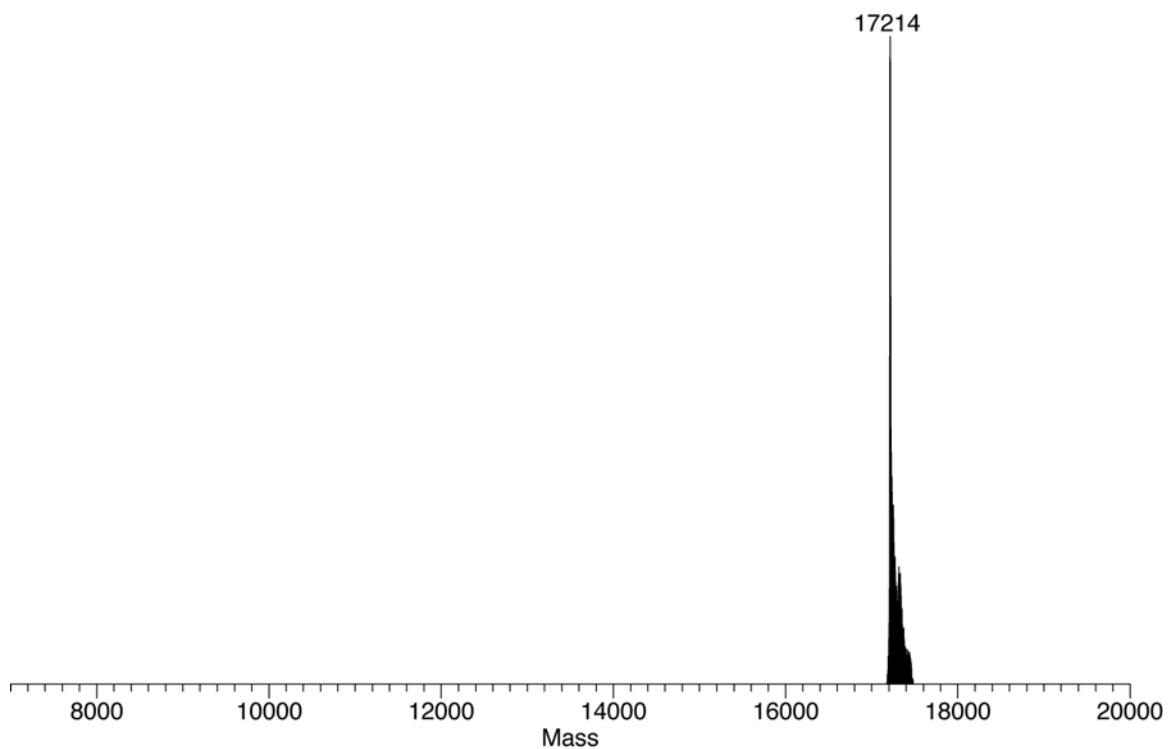

Figure S9. ESI-MS spectrum of purified K33-linked Ub_2 (^{15}N-labeled on the distal Ub). No monomer contamination is evident; no peaks at either 8564 (unlabeled Ub) or 8664 (^{15}N-labeled Ub) are observed.

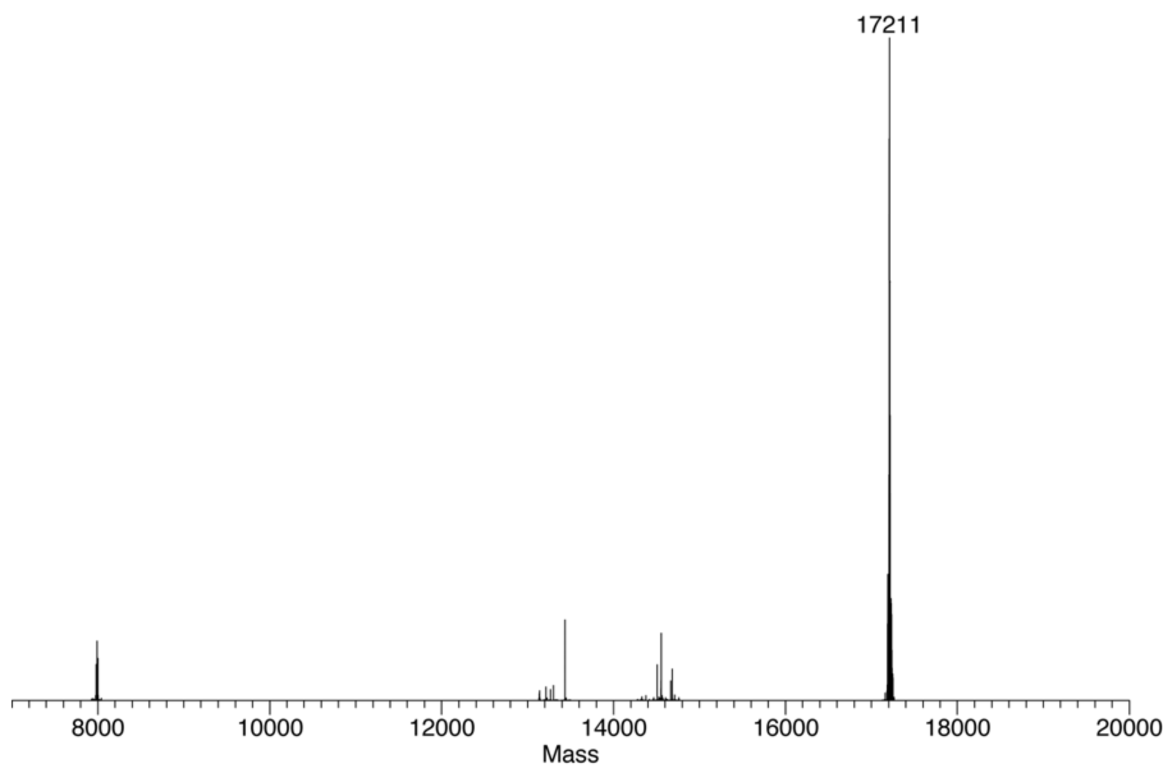

Figure S10. ESI-MS spectrum of purified K11-linked Ub_2 (^{15}N-labeled on the proximal Ub). No monomer contamination is evident; no peaks at either 8564 (unlabeled Ub) or 8664 (^{15}N-labeled Ub) are observed. A shorter mass-range version of this spectrum is shown in Fig. 1c (main text).

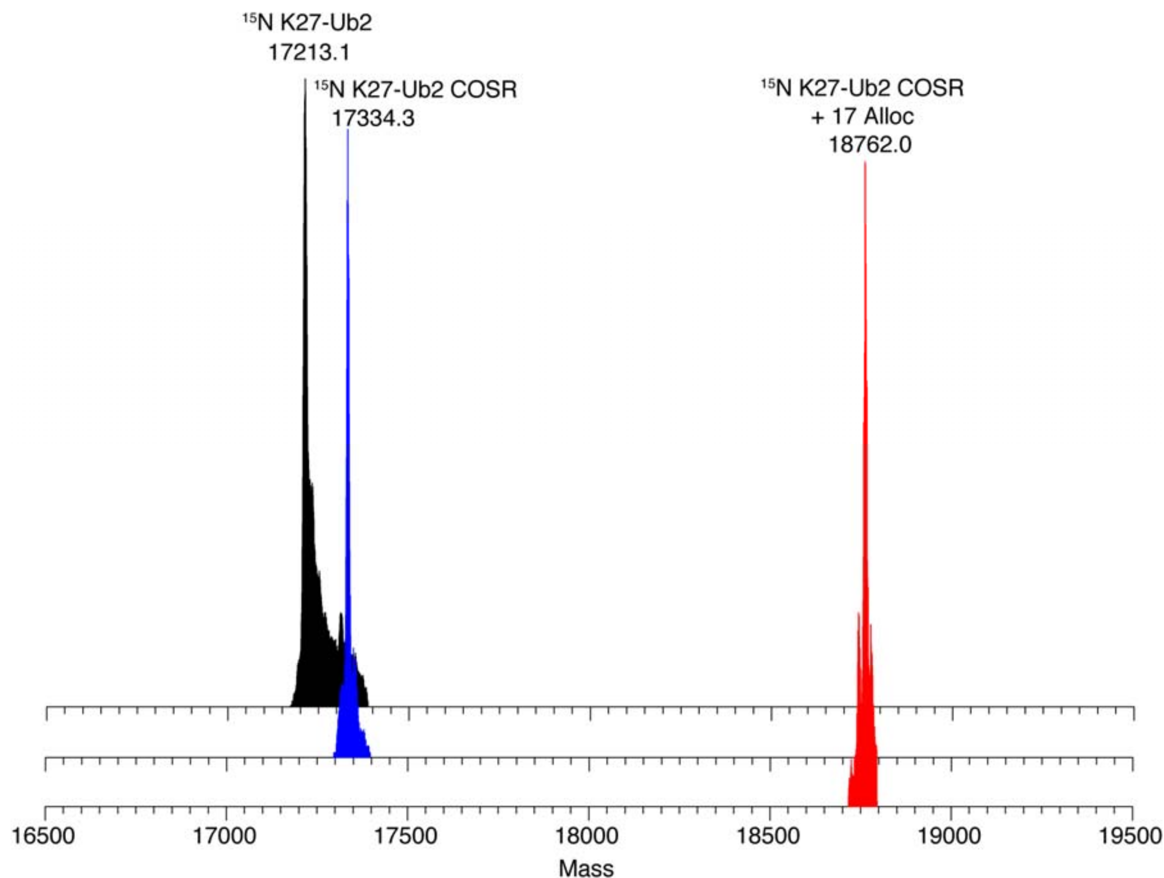

Figure S11. ESI-MS characterization of various steps in preparation of K27-linked Ub_2 (^{15}N-labeled on the proximal Ub) to be used as the distal Ub in the assembly of a Ub_3 chain. The molecular weight of K27-linked Ub_2 is 17211 Da (black). Reaction with E1 and MESNA adds a C-terminal thioester functional group to Ub_2, increasing its molecular weight by ~125 Da to 17335 Da (blue), and fully converts *all* Ub_2s into Ub_2-SR. Alloc protection of K27-linked Ub_2 (red) adds a total of 17 Alloc groups (nine Alloc groups on the distal Ub and eight Alloc groups on the proximal Ub as its K27 is already involved in the isopeptide linkage to the distal Ub). Each Alloc protecting group adds 84 Da.