Orthogonal Thiol Functionalization at a Single Atomic Center for Profiling Transthiolation Activity of E1 Activating Enzymes

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General Methods

LC-MS was carried out with an Agilent 1200 LC-MS system fitted with a Max-Light Cartridge flow cell coupled to a 6130 Quadrupole spectrometer. The solvent system consisted of

0.05 % trifluoroacetic acid (TFA) in H_2O as buffer A, and 0.04 % TFA acid in acetonitrile (MeCN) as buffer B. Protein UV absorbance was monitored at 214 and 280 nm. An Agilent ZORBAX 300SB-C3 5um, 2.1 x 150mm column was employed for proteins unless otherwise stated. Protein MS acquisition was carried out in positive ion mode and total protein masses were calculated by deconvolution within the MS Chemstation software (Agilent Technologies).

Small molecule LC-MS was carried out using the Agilent system with an Agilent ZORBAX Eclipse Plus C18, 4.6 x 100 mm, 3.5 µm column. Variable wavelengths were used and MS acquisitions were carried out in positive and negative ion modes.

Semi-preparative peptide HPLC was carried out on a Dionex Ultimate system with Thermo Biobasic C4 21.2 x 250 mm column at a flow rate of 10 ml min^{-1}.

^{1}H & ^{13}C NMR was carried out with a (Bruker AVANCE II 500 spectrometer). Chemical shifts (δ) are reported in ppm and referenced to residual solvent (MeOH-D_4). J values are in hertz, and the splitting patterns are designated as follows: s, singlet; bs, broad singlet; d, doublet; t, triplet; app. t, apparent triplet; q, quartet; m, multiplet.

All solvents and reagents were purchased from Sigma Aldrich or VWR unless otherwise stated.

Supporting Figures

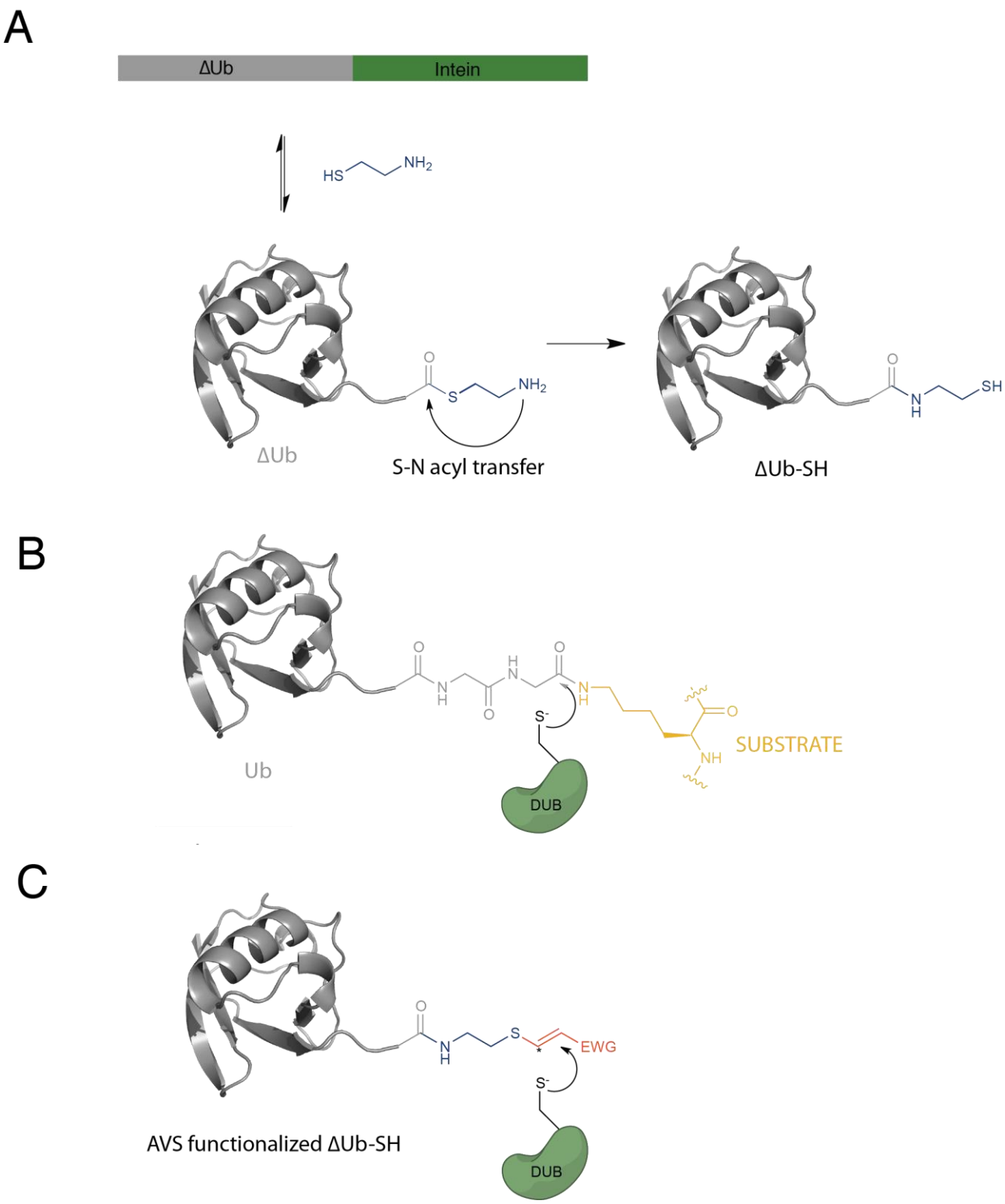


Figure S1. (A) Schematic of Ub_{1-74} mercaptoethyl carboxamide (ΔUb-SH) formation. Ubiquitin missing the C-terminal Gly-Gly motif (residues 1-74; ΔUb) were expressed as an intein fusion and

then subjected to thiolysis with 50 mM cysteamine.[1,2] Transthioesterification followed by an *S*-*N* acyl transfer produced the desired product (ΔUb-SH) with a C-terminal thiol group. (B) The majority of deubiquitinating enzymes (DUBs) hydrolyze Ub from substrate lysine ε-amino groups (orange). The catalytic cysteine in the DUB attacks the C-terminal carbonyl C atom of Ub and forms an acyl-Cys intermediate. Subsequent hydrolysis results in deubiquitination. (C) The semisynthetic strategy used to append the C-terminal thiol onto ΔUb, followed by functionalization with an activated vinylsulfide (AVS), results in the electrophilic centre (*) being nearly spatially equivalent to the carbonyl C atom of full-length Ub. This species would be expected to function as an ABP and covalently label the catalytic cysteine of DUBs like analogous species.

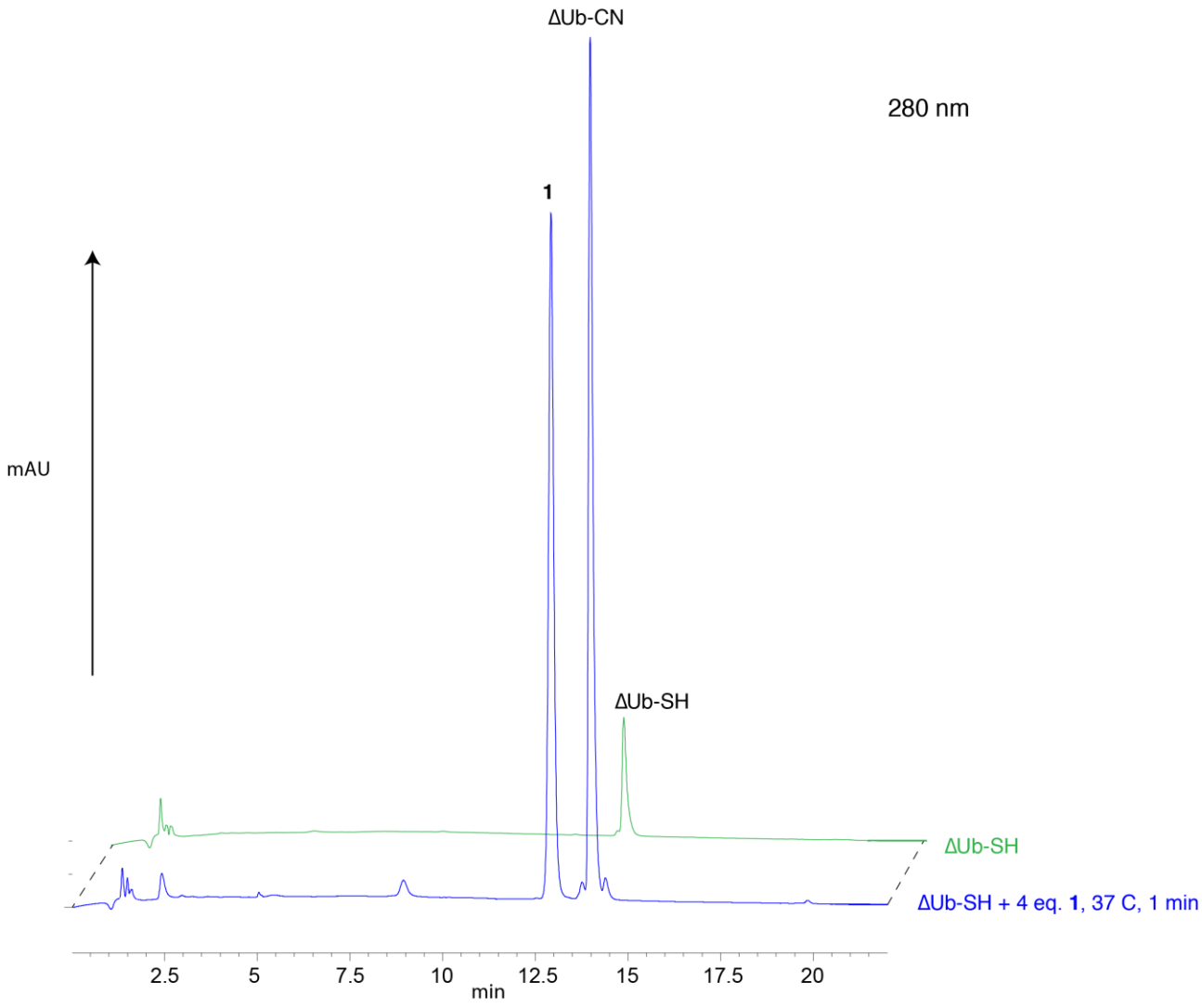
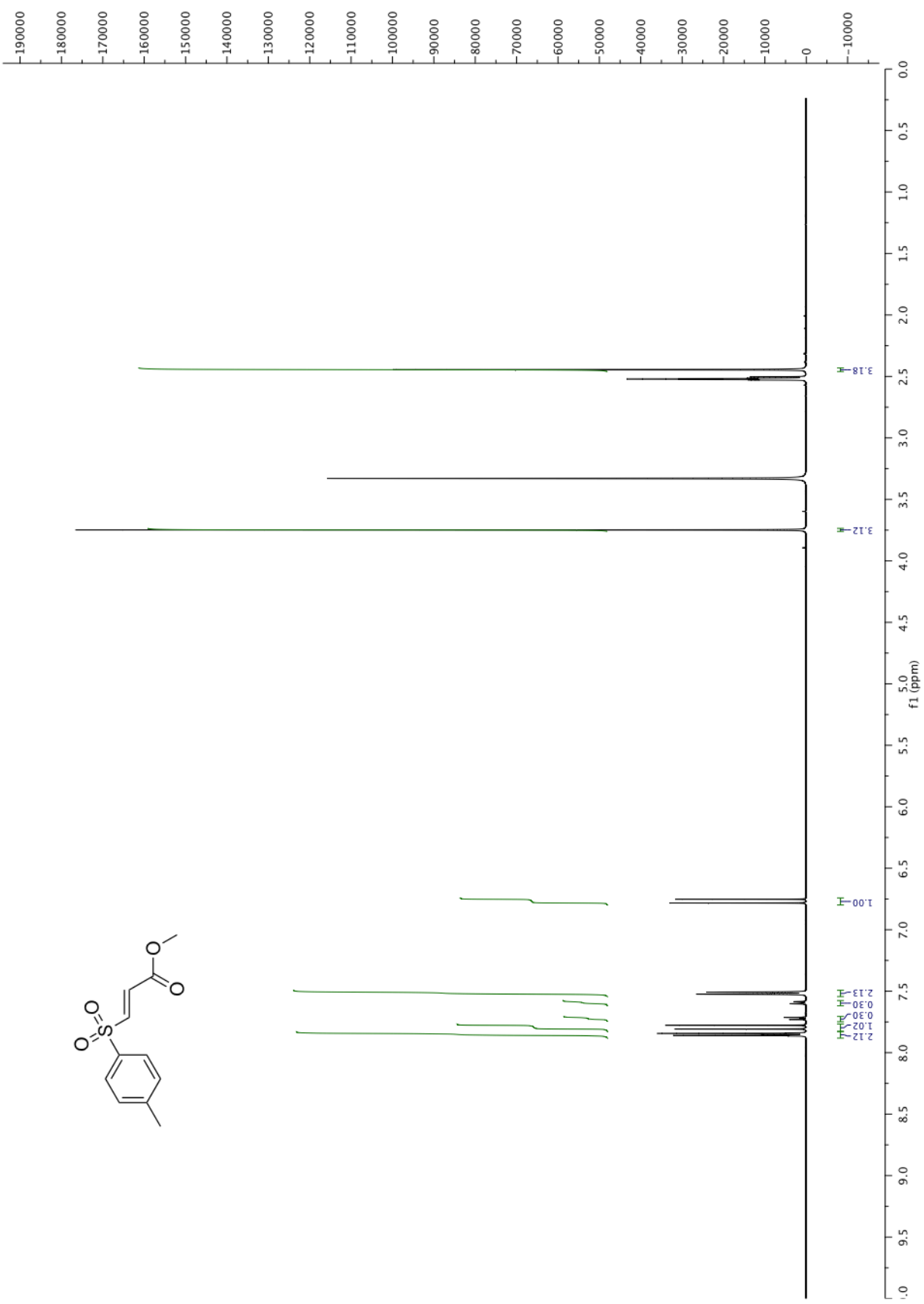


Figure S2. ΔUb-SH (120 µM) was incubated with TDAE **1** (480 µM) in 100 mM pH 8 phosphate buffer at 37 ˚C and the reaction was monitored by LC-MS at 280 nm. ΔUb-SH was analysed before addition of **1** (green). 1 min after addition of **1**, the reaction was quenched with trifluoroacetic acid (TFA) and analysed using identical conditions (blue). ΔUb-SH was quantitatively modified with the major species being ΔUb-CN. The increase in peak area is due to the AVS chromophore.


3.18
3.12
1.00
2.13
0.30
0.30
1.02
2.12
f1 (ppm)

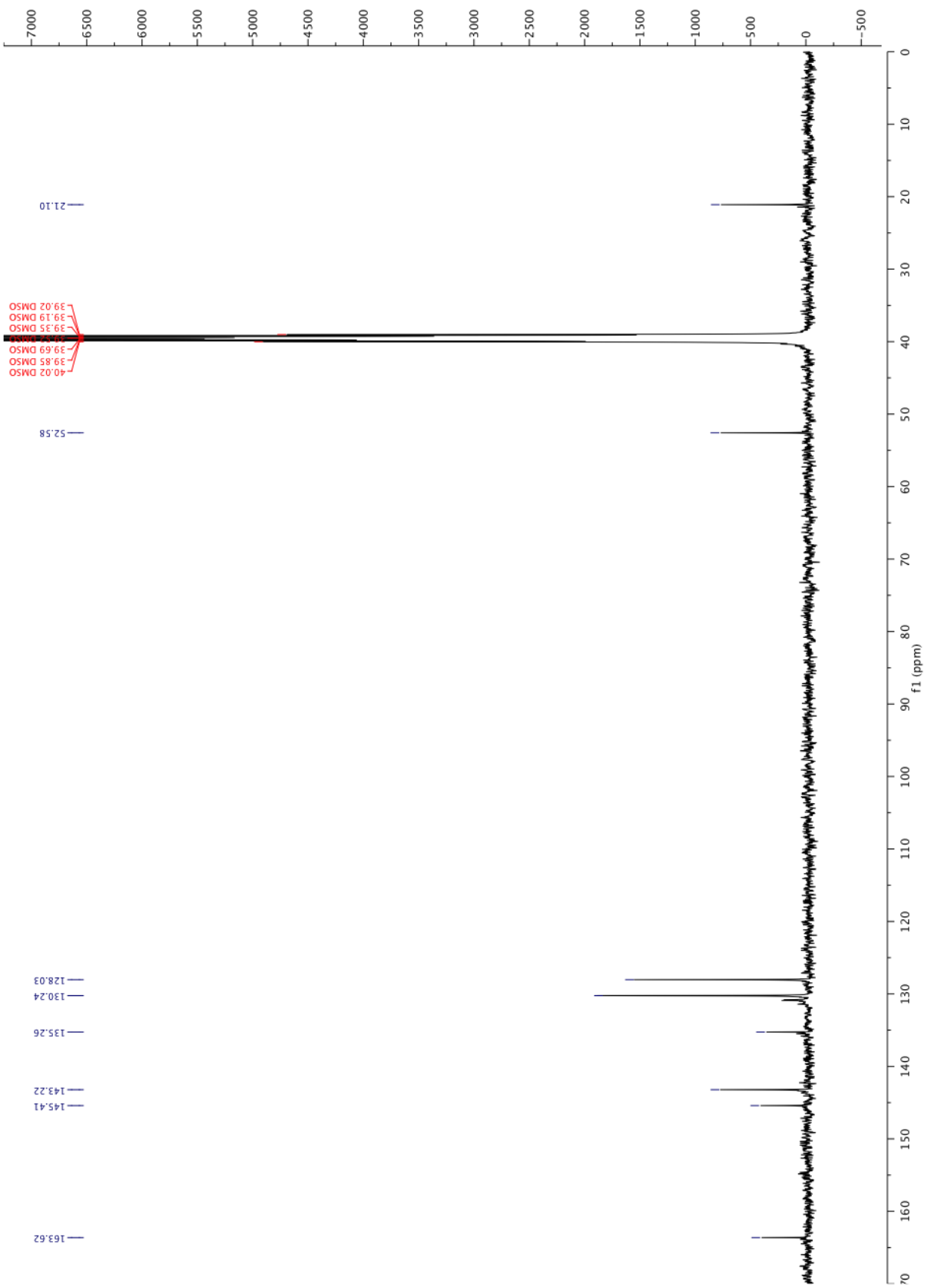
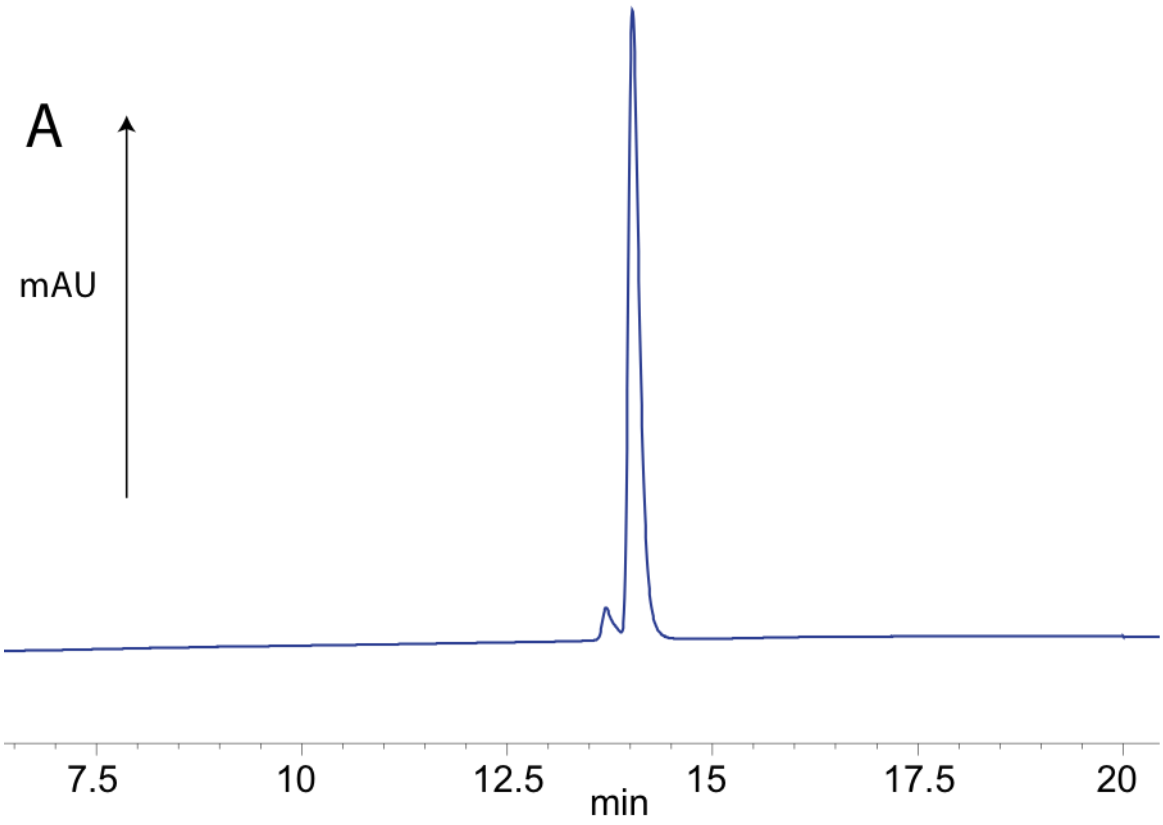

7000
6500
6000
5500
5000
4500
4000
3500
3000
2500
2000
1500
1000
500
0
-500
0
10
20
30
40
50
60
70
80
90
100
110
120
130
140
150
160
170
f1 (ppm)
21.10
39.02 DMSO
39.19 DMSO
39.35 DMSO
39.69 DMSO
39.85 DMSO
40.02 DMSO
52.58
128.03
130.24
135.26
143.22
145.41
163.62

Figure S3. ^{1}H and ^{13}C NMR spectra confirming formation of **2** (*E*)-methyl 3-tosylacrylate as major product. ^{1}H NMR (500 MHz, DMSO): δ 7.85 (ddd, 1.6, 2.0, 8.3 Hz), 7.79 (d, 15.2 Hz), 7.52 (d, 8.5 Hz), 6.77 (d, 15.2 Hz), 3.75 (s, 3H), 2.45 (s, 3H). ^{13}C NMR (126 MHz, DMSO): δ 163.62, 145.41, 143.22, 135.26, 130.24, 128.03, 52.58, 21.10.



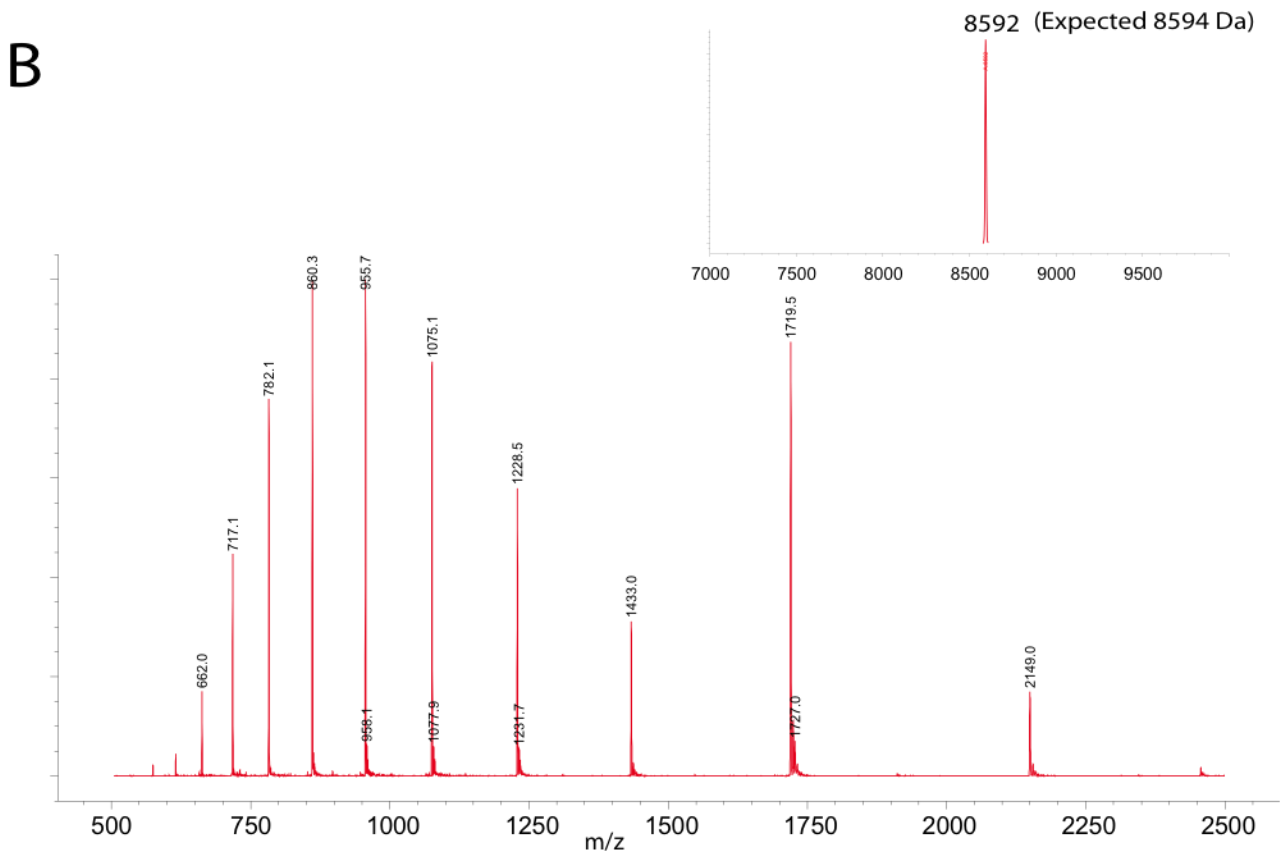


Figure S4. (A) LC-MS characterization of ΔUb-VME* (*cis*) produced by reaction of ΔUb-SH with electron deficient yne **3**. (minor peak at lower t_R corresponds to hydrolysed ΔUb). 4 eq. of **3** were incubated with ΔUb-SH (120 μM) for 1 h in pH 8 phosphate buffer. (B) ESI-MS spectra of ΔUb-VME* Expected mass = 8594 Da; Observed = 8592 Da.

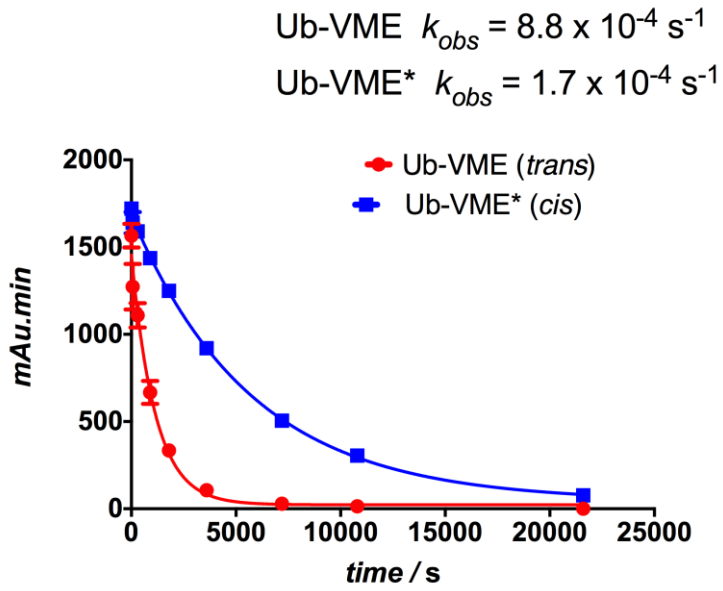


Figure S5. Observed rates for addition of glutathione **4** to ΔUb-VME and ΔUb-VME* at a glutathione concentration of 62.5 mM. Rate of addition to ΔUb-VME was 5-fold higher which was attributed to electronic and steric effects associated with the predominant *cis* and *trans* regioisomers present in ΔUb-VME* and ΔUb-VME respectively.

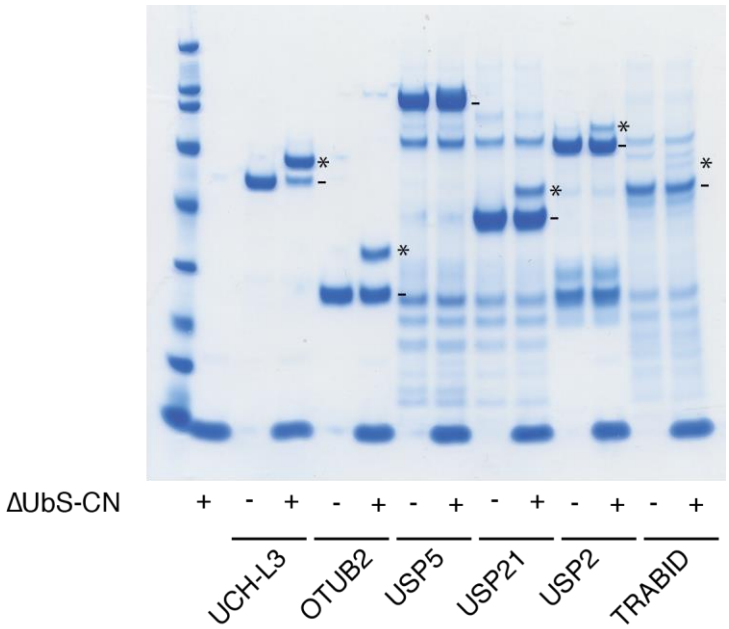


Figure S6. SDS-PAGE analysis of product formation after incubation of ΔUb-SH (20 μM) with 6

different DUBs (5 μM). UCH-L3 belongs to the ubiquitin C-terminal hydrolase (UCH) subfamily; OTUB2 and TRABID belong to the ovarian tumor protease (OTU) subfamily; USP5, USP21 and USP2 belong to the ubiquitin-specific protease (USP) subfamily.[3] Reactions were incubated at 30 °C for 1 hour. DUBs were incubated with and without ΔUb-SH. The unmodified DUB is marked with a hyphen and the DUB covalently modified with ΔUb-SH is marked with an asterisk. SDS sample buffer contained 2-mercaptoethanol at a final concentration of 179 mM, which illustrates redox stability of the modifications.

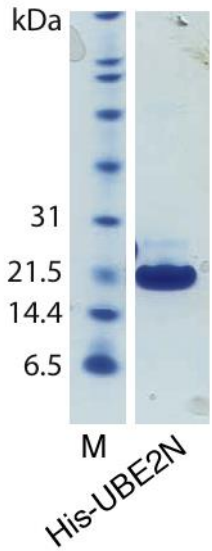


Figure S7. SDS-PAGE analysis of Ni-NTA purified His-UBE2N used in this study. For protein sequence see "Constructs used" section below.

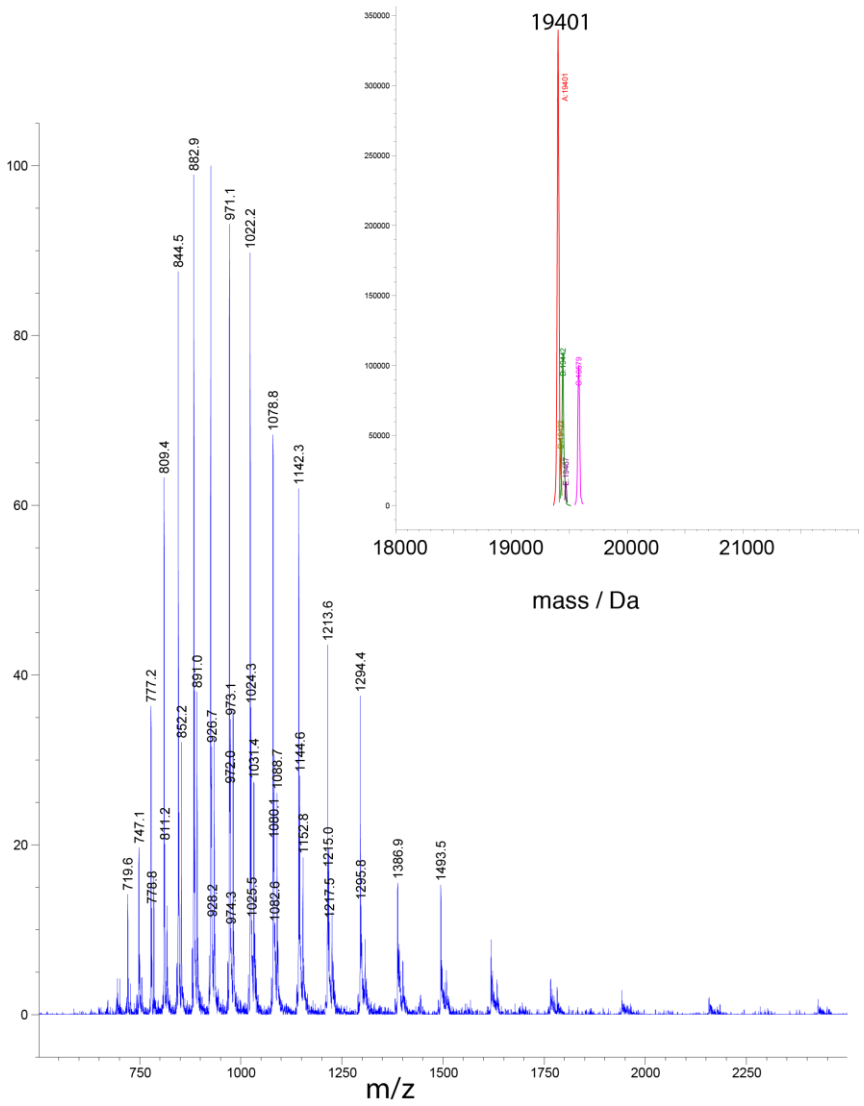


Figure S8. Full ESI-MS spectra for *cis* thiomethyl acrylate-functionalized E2, UBE2N-VME*. (bottom; major product, found: 19401 Da; expected: 19404 Da).

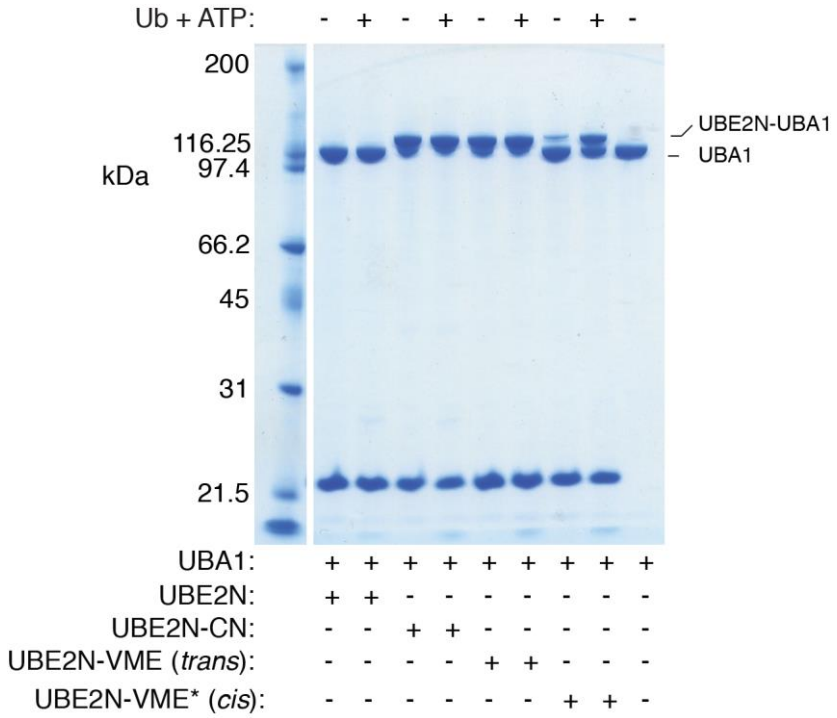


Figure S9. Reducing SDS-PAGE analysis of UBA1 (3 μM) incubation at 30 °C for 3 h with various functionalized UBE2N species (12 μM) in the presence and absence of Ub (2 μM) and ATP (2 mM). Lanes 1 and 2; unmodified UBE2N, lanes 3 and 4, UBE2N-CN; lanes 5 and 6, UBE2N-VME (*trans*); lanes 7 and 8, UBE2N-VME* (*cis*); lanes 9, UBA1 only. UBE2N-CN and UBE2N-VME exhibit similar labelling efficiency suggesting that E1-E2 binding is rate-limiting as UBE2N-VME is 5-fold more reactive UBE2N-CN in model reactions with functionalized ΔUb-SH and glutathione. UBE2N-VME* (*cis*) is significantly less efficient at labelling UBA1 (lanes 7 and 8).

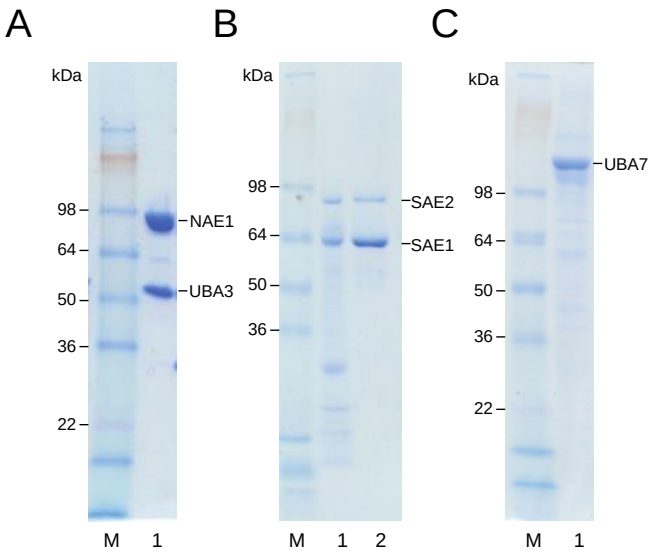
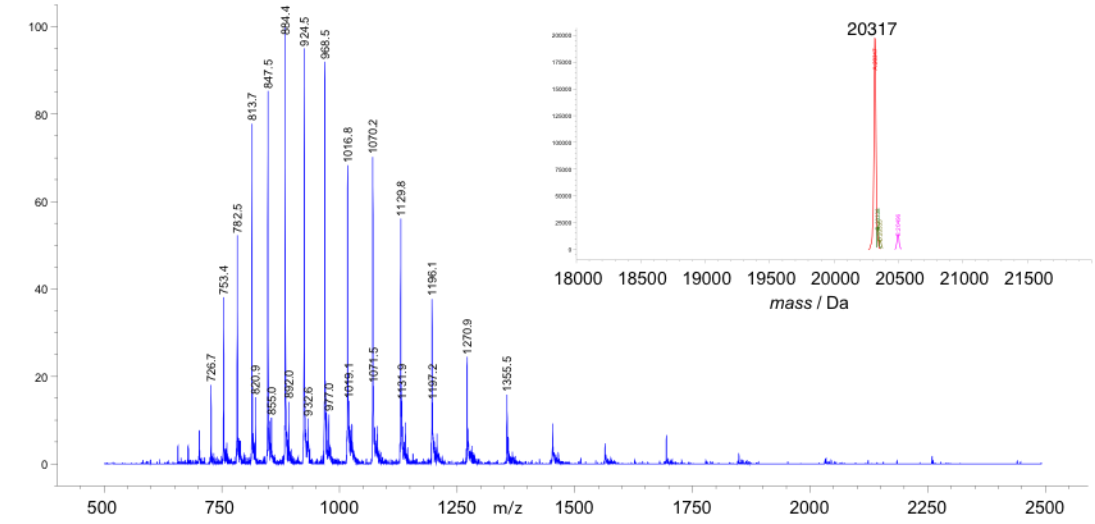
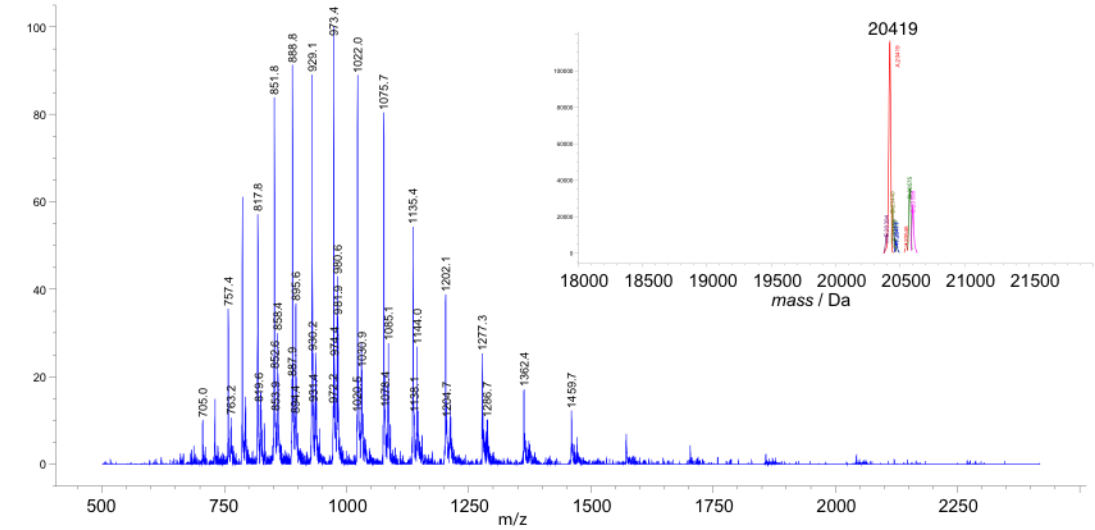


Figure S10. SDS-PAGE analysis of purified recombinant E1s. (A) NAE1-UBA3 (NEDD8); (B) SAE1-SAE2 (SUMO); (C) UBA7 (ISG15).

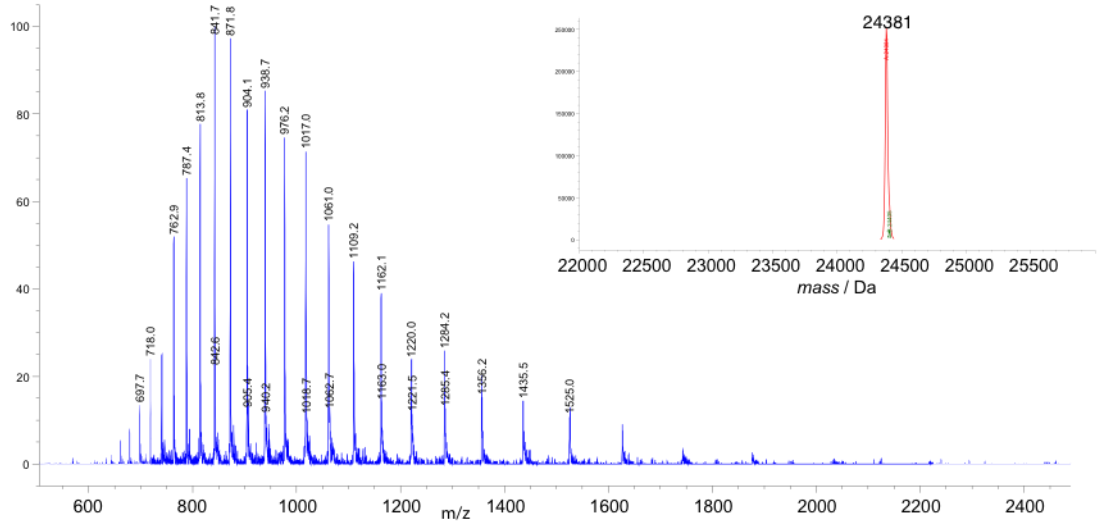
UBE2I



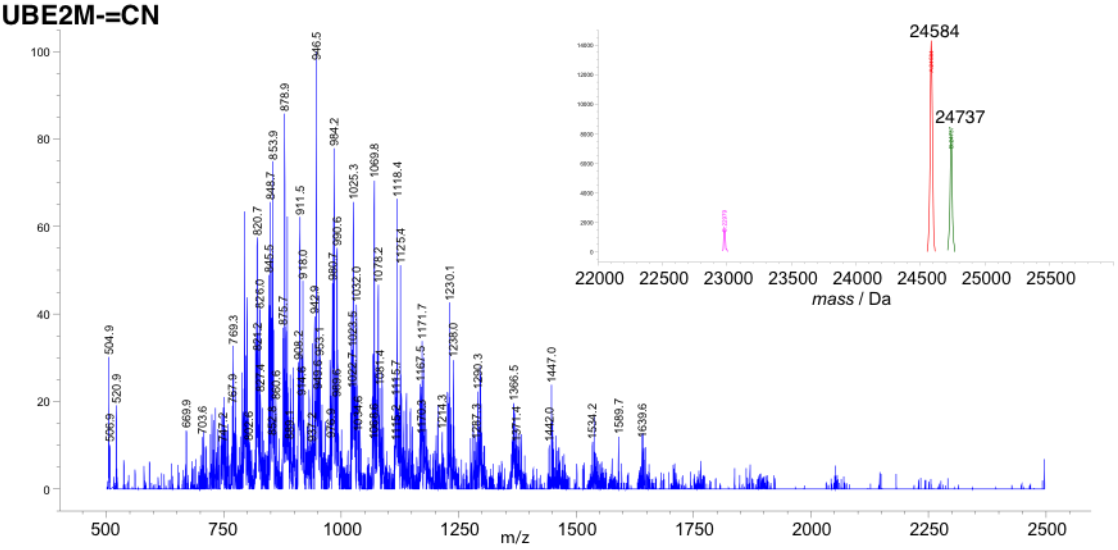
UBE2I-=CN



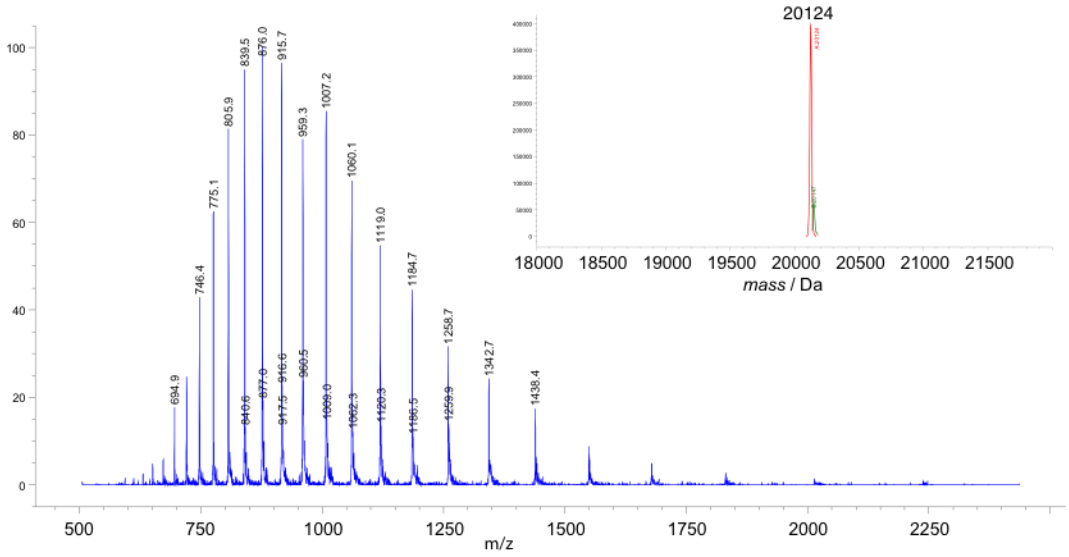
UBE2M



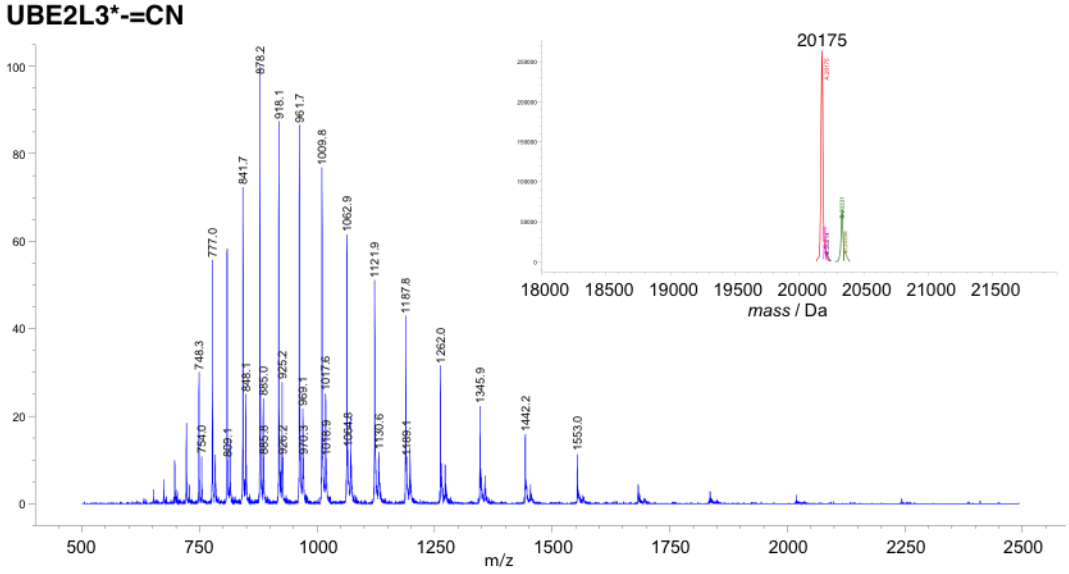
UBE2M-=CN



UBE2L3*



UBE2L3*-=CN



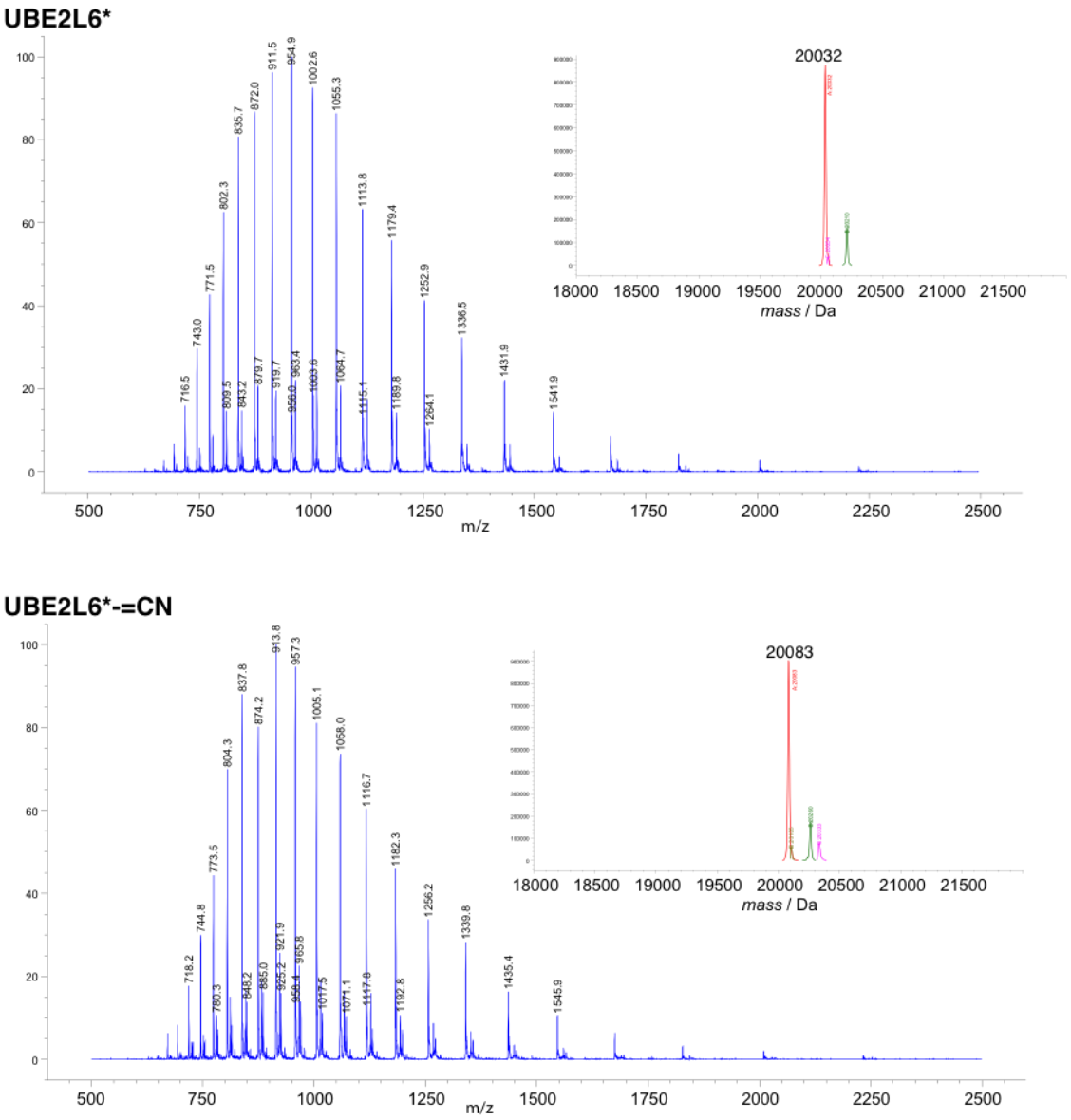


Figure S11. ESI-MS spectra for purified E2s and after functionalization with thioacrylonitrile group. NB. For E2s with multiple cysteines which were overlabeled with **1**, a significant mass signal corresponds to the addition of a single saturated adduct due to the multiplicative effect on the otherwise minor (10%) side product. Formation of the saturated adduct would be expected to be negligible if labelled at 37 °C as observed in model experiments with ΔUb-SH. In the interests of protein stability, labelling was carried out at 25 °C. It was assumed that the saturated adduct was present in equivalent amounts on all cysteines (i.e, ~10 %). N-terminal methionine has been removed by *E. coli* methionyl aminopeptidase. UBE2I, theoretical mass =

20320 Da; observed = 20317 Da. UBE2I-=CN, theoretical mass = 20422 Da; observed = 20419 Da (only 2 of the 4 cysteines are solvent accessible[4]). UBE2M, theoretical mass = 24384 Da; observed = 24381 Da. UBE2I-=CN, theoretical mass = 24585 Da; observed = 24584 Da (only 4 of the 5 cysteines are solvent accessible[5]). UBE2L3**, theoretical mass = 20127 Da; observed = 20124 Da. UBE2L3**-=CN, theoretical mass = 20178 Da; observed = 20175 Da. UBE2L6**, theoretical mass = 20034 Da; observed = 20032 Da. UBE2L6**-=CN, theoretical mass = 20085 Da; observed = 20083 Da.

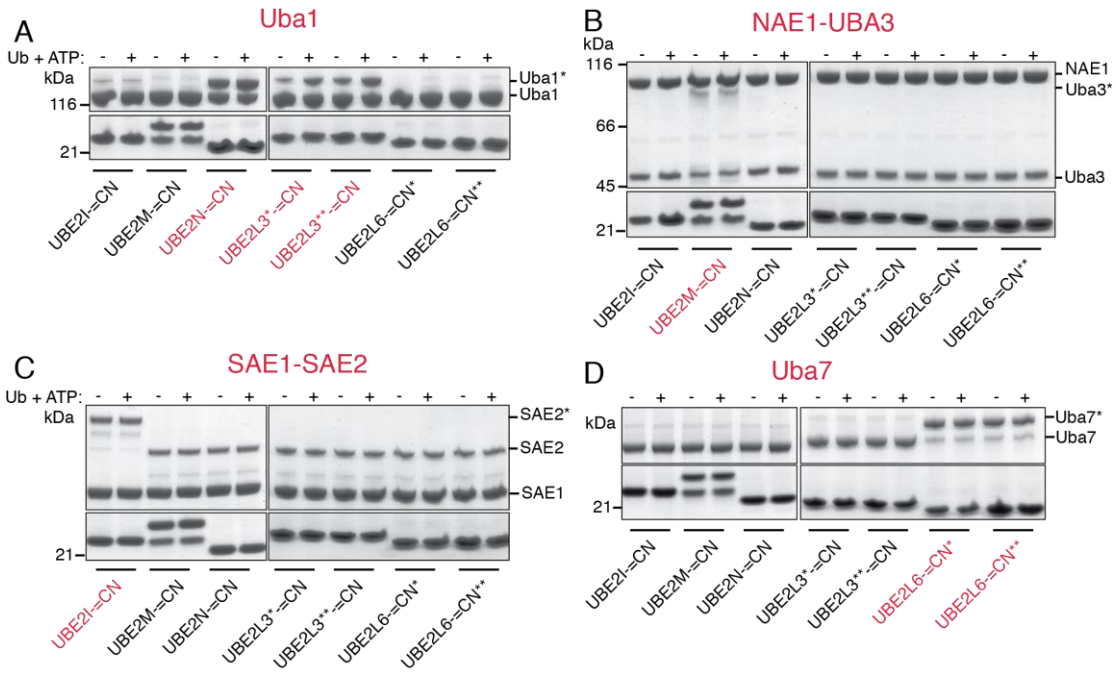


Figure S12. (A) Only E2 probes built on a ubiquitin E2 (UBE2N and UBE2L3) undergo appreciable labelling of the ubiquitin E1, UBA1. UBE2L3 contains 3 cysteines therefore UBE2L3* is a catalytic cysteine-only mutant. UBE2L3** also contains a serine to alanine mutation. This serine is proximal to the installed warhead and we had concerns intramolecular addition could take place. The double band associated with UBE2M is due to E2 degradation. (B) Probe built on the NEDD8 E2 (UBE2M) specifically labels the NEDD8 E1,

NAE1-UBA3. NAE1-UBA3 is heterodimeric and the catalytic cysteine is in the UBA3 subunit. We used wild type UBE2M that contains 5 cysteines. LC-MS analysis of the labelled E2 revealed that only 4 cysteines were labelled. Analysis of deposited crystal structures revealed that one cysteine is not accessible. (C) Probe built on the SUMO E2 (UBE2I) specifically labels the SUMO E1, SAE1-SAE2. SAE1-SAE2 is heterodimeric and the catalytic cysteine is in the SAE2 component. We used wild type UBE2I that contains 4 cysteines. LC-MS analysis of the labelled E2 revealed that only 2 cysteines were labelled. Crystal structure analysis revealed that two cysteines are not accessible. (D) Probe built on the ISG15 E2 (UBE2L6) specifically labels the ISG15 E1, UBA7. UBE2L6* is catalytic cysteine-only mutant. UBE2L6** also contains the described serine to alanine mutation.

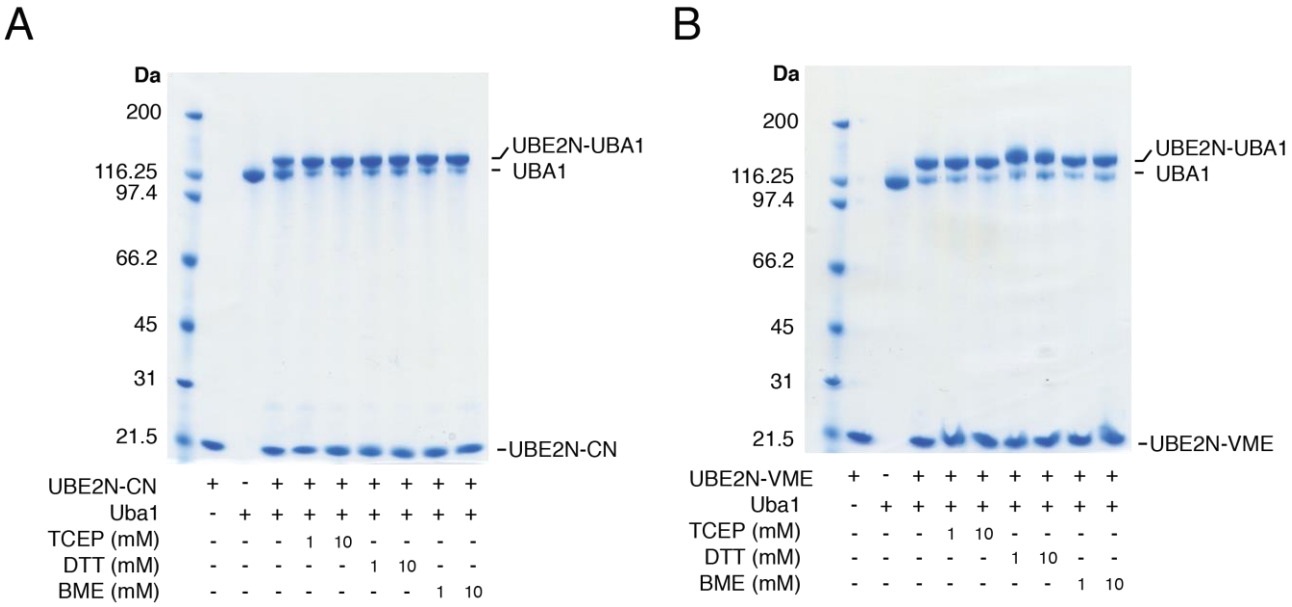


Figure S13. Activity-based labelling of UBA1 with UBE2N-CN (A) and UBE2N-VME (B) (30 °C, 3 h) was compatible with a panel of reducing agents. Presence of at least 10 mM TCEP, DTT or BME did not compromise labelling efficiency of UBE2N-CN or UBE2N-VME. Labelling efficiency was in fact enhanced and was most likely due to reduction of some oxidized cysteine in UBA1.

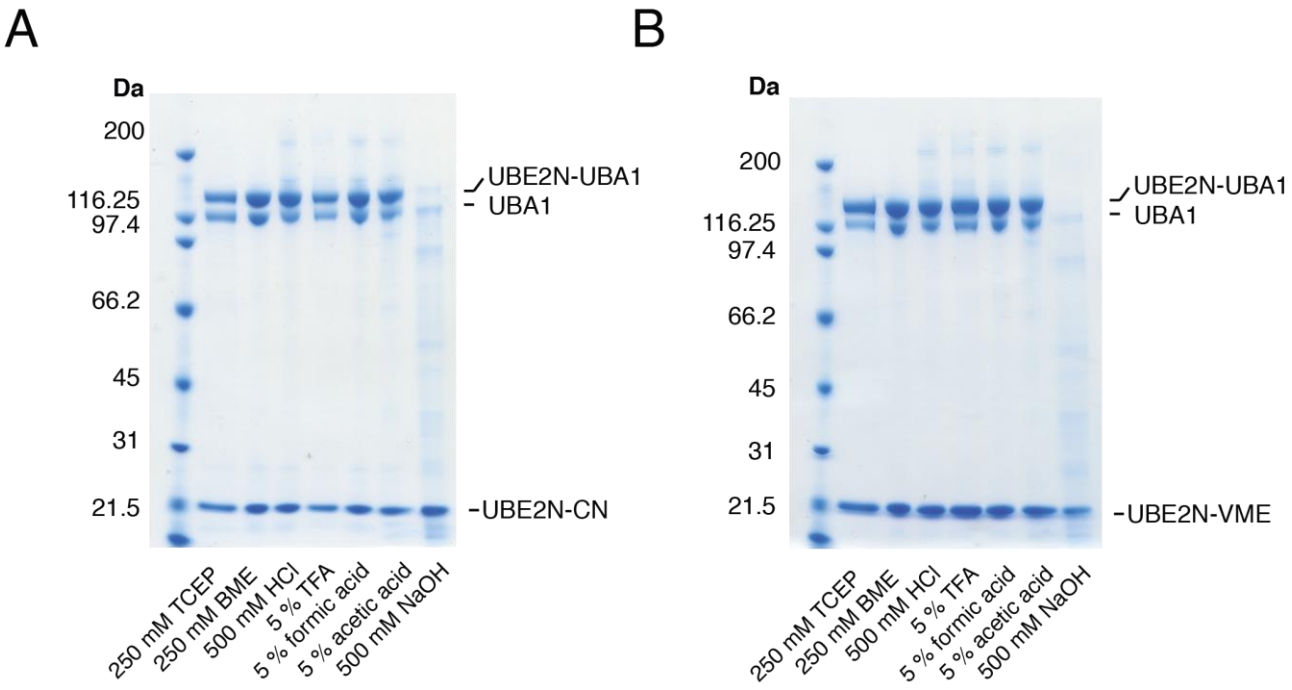
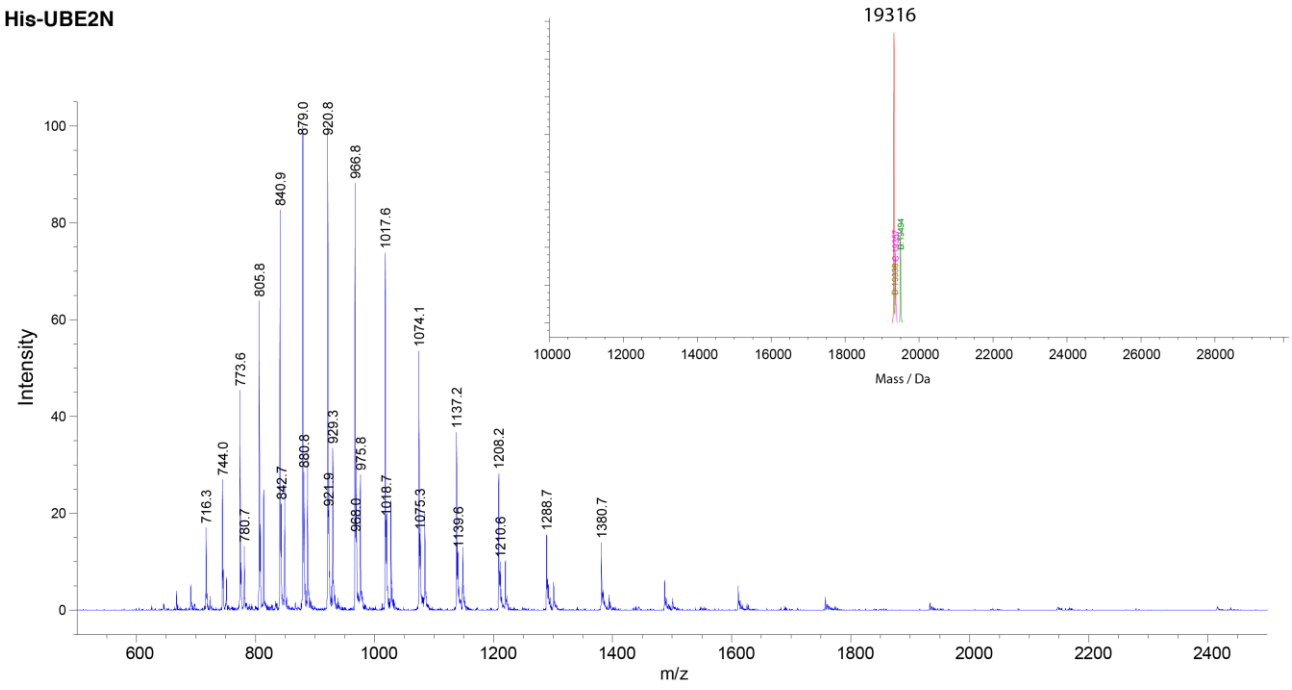
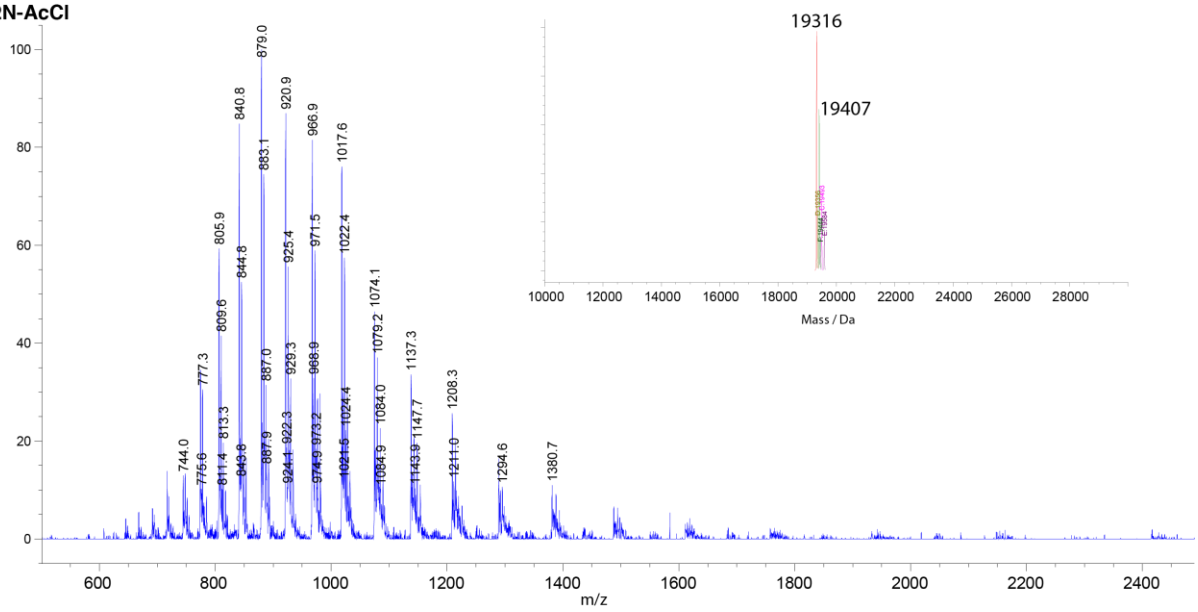


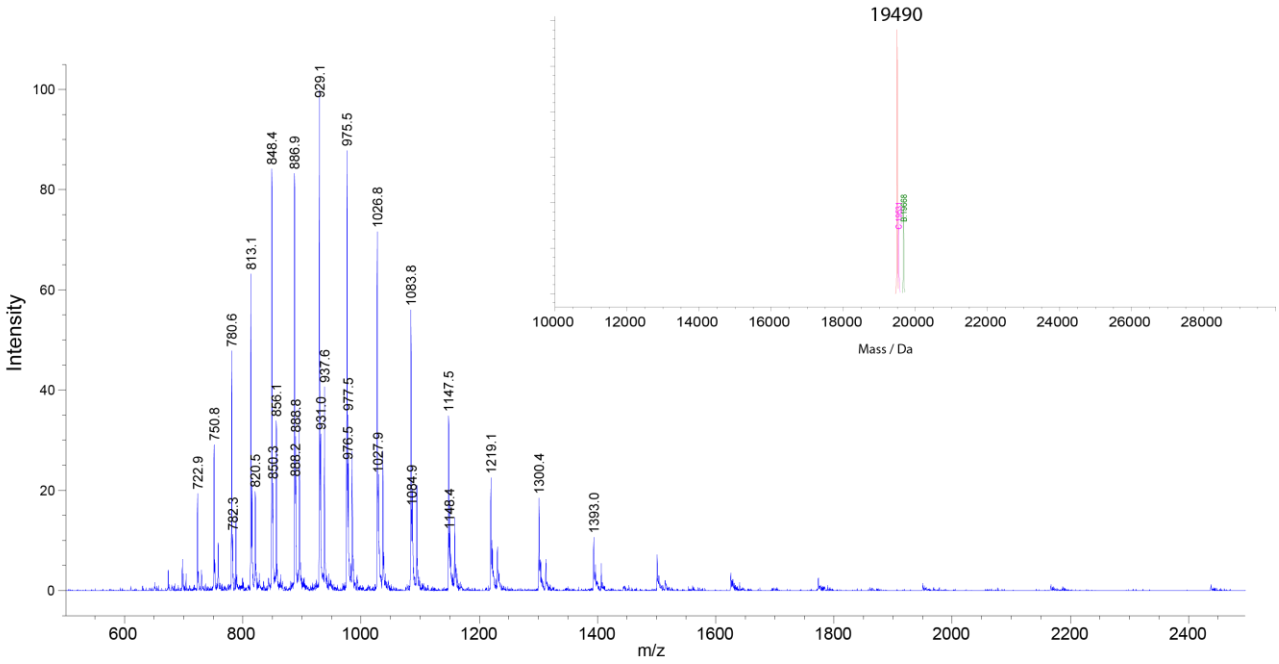
Figure S14. Activity-based labelling of UBA1 with UBE2N-CN and UBE2N-VME was carried out in the absence of reducing agent at 30 °C for 3h. Samples were then subsequently treated with 250 mM TCEP, 250 mM BME, 500 mM HCl, 5 % formic acid, 5 % acetic acid 500 mM NaOH. Samples were then incubated at 23 °C for 2 h prior to SDS-PAGE analysis. The hetero-bisthioether crosslink was stable under all reducing and acidic conditions tested.



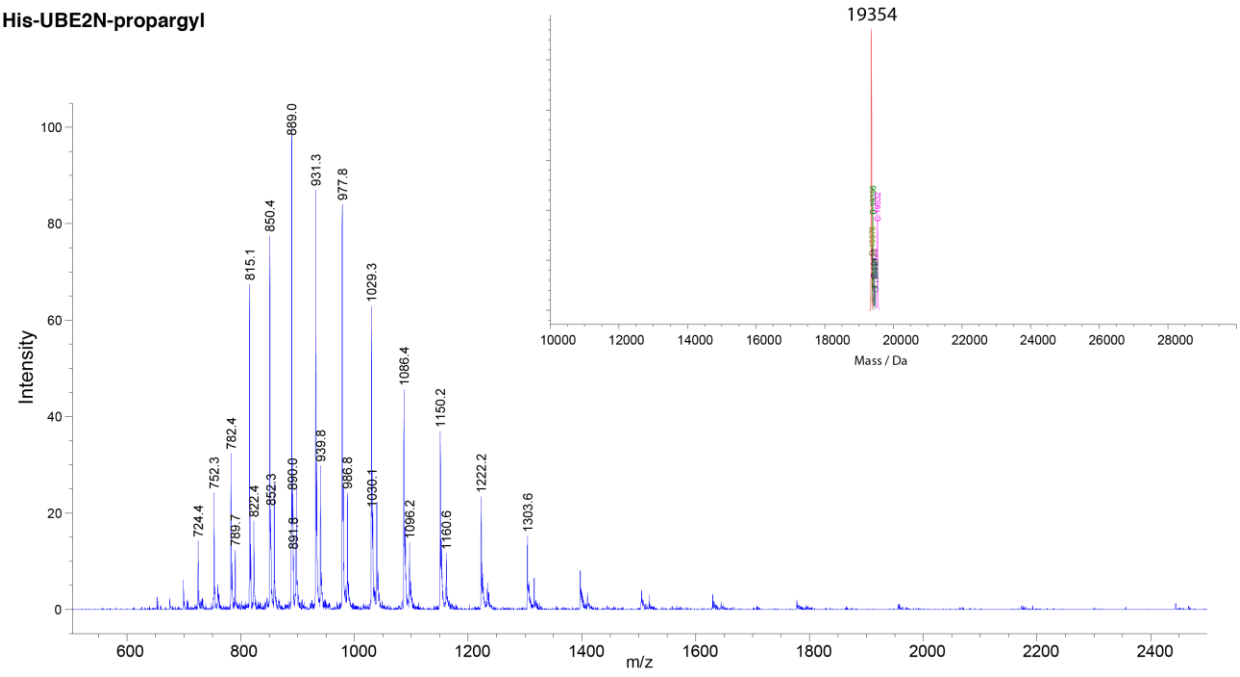
His-UBE2N-AcCl



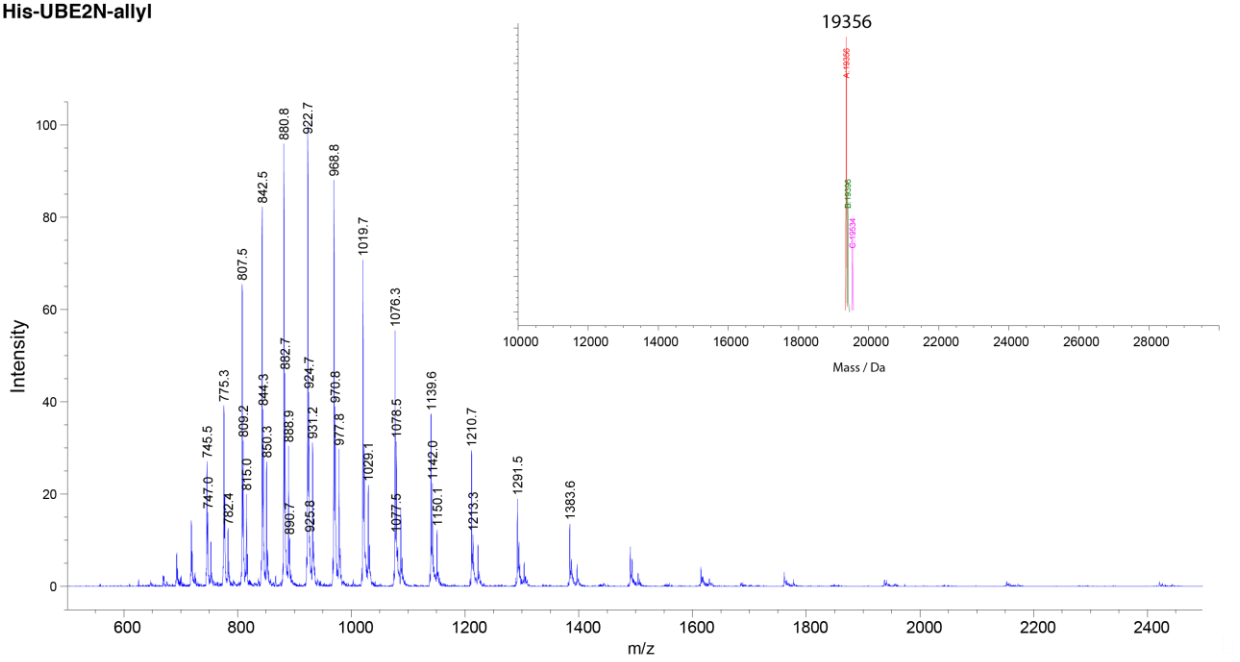
His-UBE2N-BrMal



His-UBE2N-propargyl



His-UBE2N-allyl



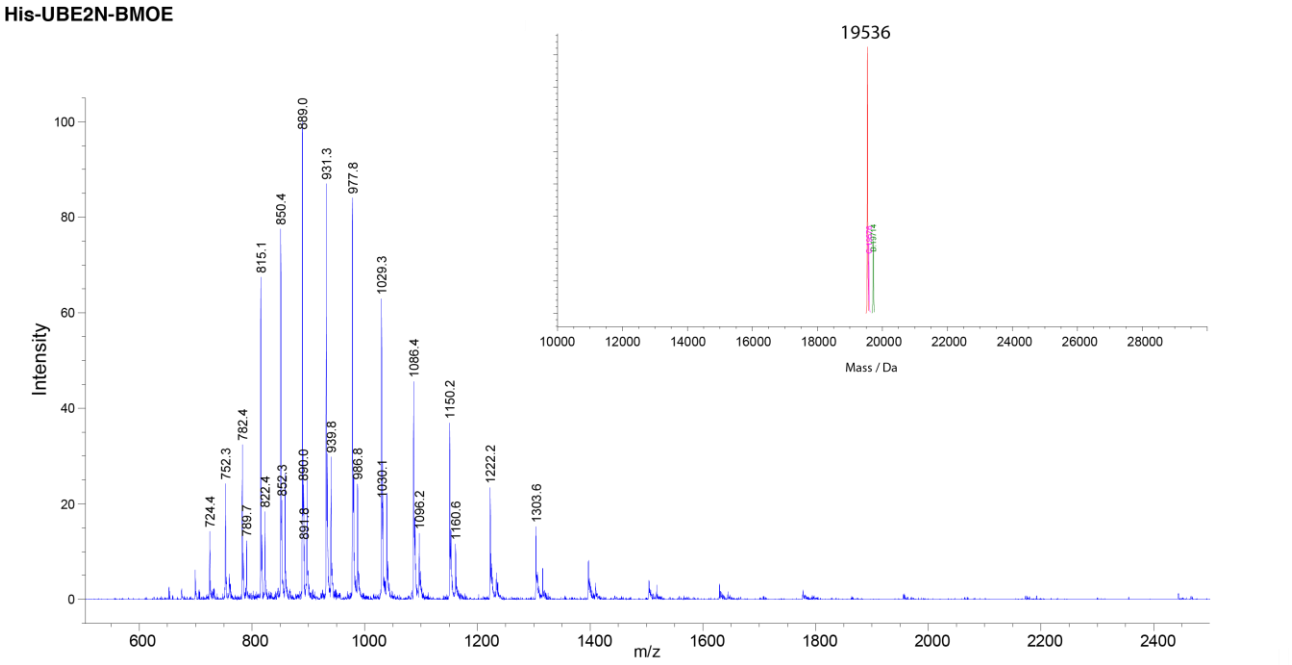
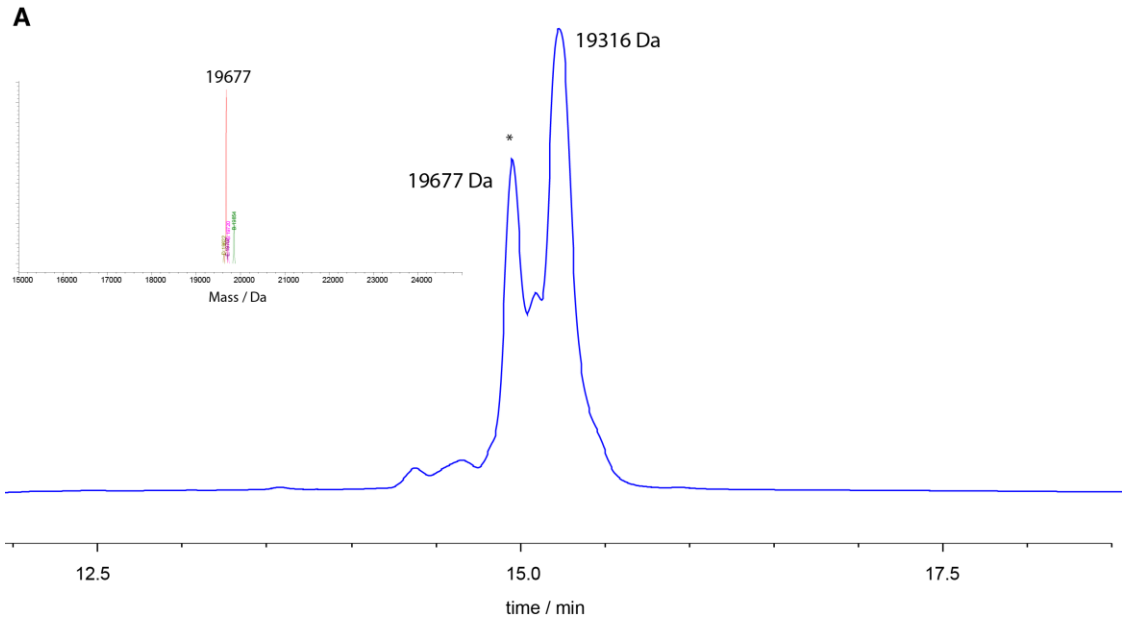
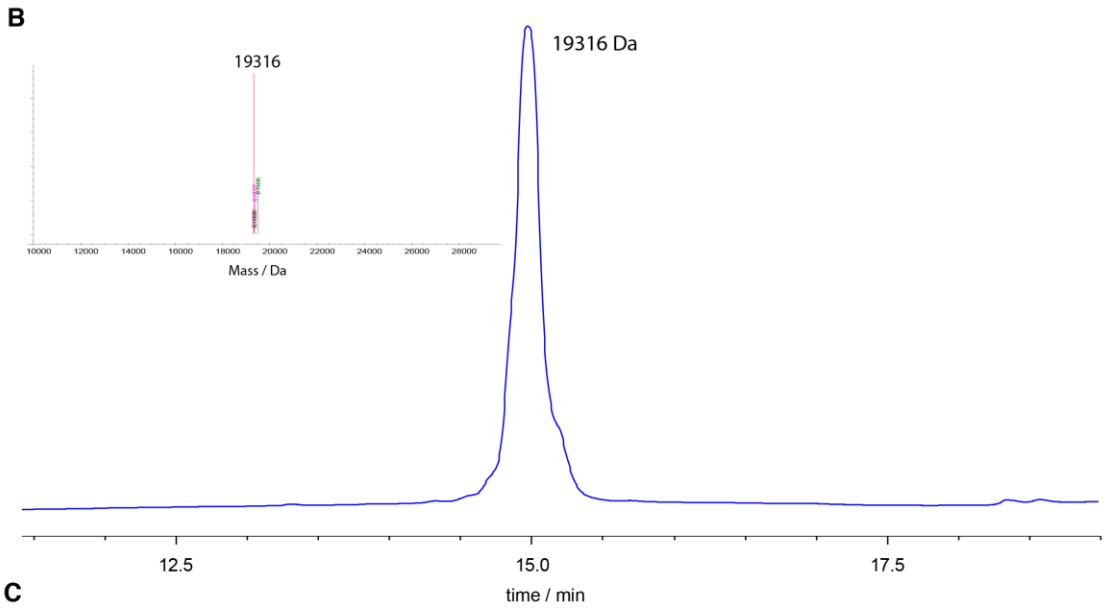
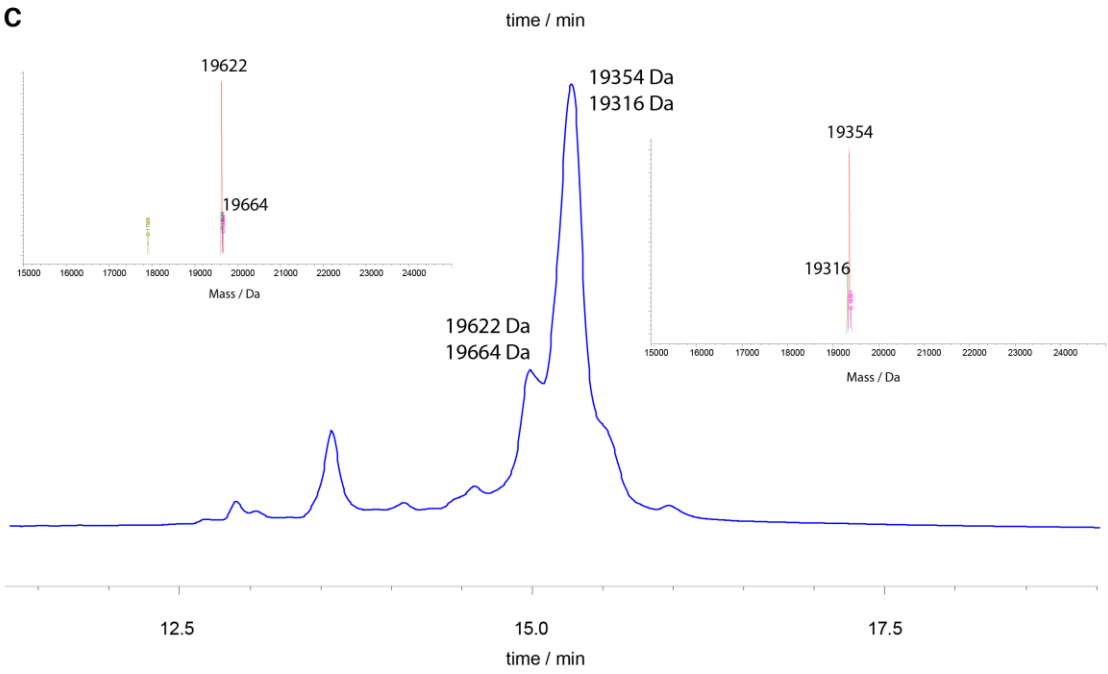


Figure S15. ESI-MS characterisation of commercial/literature cysteine-cysteine cross linkers mono functionalized with His-UBE2N. His-UBE2N, Unmodified His-UBE2N. Observed = 19316 Da; expected = 19320. His-UBE2N-AcCl, His-UBE2N labelled with dichloroacetone (~35 % yield). Observed = 19407 Da; expected = 19410 Da. Conditions could only be developed for formation of ~35% protein bearing a functional electrophile. Extended incubation resulted in chloride elimination. His-UBE2N-BrMal, His-UB2N modified with bromomaleimide. Observed = 19490; expected = 19494. His-UBE2N-propargyl, His-UBE2N was alkylated with propargyl bromide furnishing UBE2N bearing alkyne functionality on the catalytic Cys residue. Observed = 19354 Da; expected = 19358 Da. His-UBE2N-allyl, His-UBE2N was alkylated with allyl bromide furnishing UBE2N bearing alkene functionality on the catalytic Cys residue. Observed = 19356 Da; expected = 19360 Da. His-UBE2N-BMOE, His-UBE2N was modified with *bis*maleimidoethane furnishing UBE2N bearing maleimide functionality. Observed = 19536 Da; expected = 19540 Da.


A
19316 Da
19677
*
19677 Da
Mass / Da
12.5
15.0
17.5
time / min


B
19316
19316 Da
Mass / Da
12.5
15.0
17.5
time / min


C
19622
19664
Mass / Da
19354 Da
19316 Da
19354
19316
19622 Da
19664 Da
Mass / Da
12.5
15.0
17.5
time / min

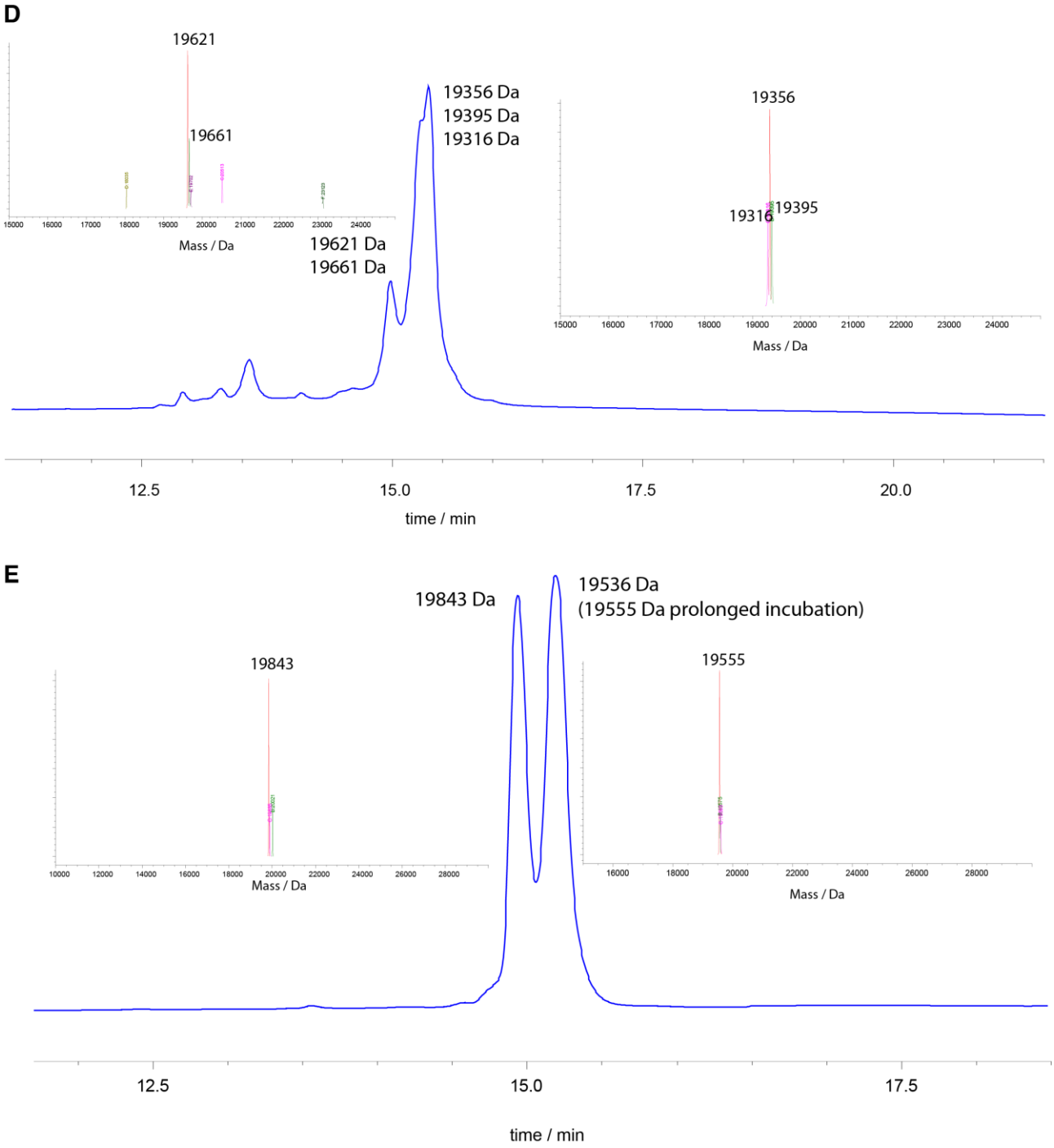


Figure S16. RP-HPLC analysis at 214 nM of electrophile-functionalized E2 (25 μM) after incubation with GSH (62.5 mM) in buffer (100 mM Na_2HPO_4 pH 8, 150 mM NaCl) at 37 °C. Inset(s) are the deconvoluted spectra corresponding to the adjacent UV peak. (A) His-UBE2N-AcCl was incubated for 5 min. 19677 Da peak corresponds to alkylation of GSH with His-UBE2N-AcCl (expected mass = 19680.5 Da). 19316 Da corresponds to unmodified His-UBE2N that could not be quantitatively functionalized with dichloroacetone (see Figure S15). (B) His-UBE2N-BrMal was

incubated for 5 min. 19316 Da peak corresponds to quantitative elimination of the electrophile thereby regenerating unmodified His-UBE2N (expected mass 19320 Da). This is expected to take place in the presence of excess thiol[6]. (C) His-UBE2N-propargyl was supplemented with 2,2-dimethoxy-2-phenylacetophenone (3 yne eq. from 1 mM stock in DMSO), followed by irradiation of the samples at 365 nM for 10 minutes with a UV hand lamp. No thiol-yne addition was observed, only side reactions. 19316 Da peak corresponds to side reaction involving regeneration of His-UBE2N. The His-UBE2N is then observed to undergo oxidative disulfide bond formation with GSH as depicted by the 19622 Da peak (expected mass 19625 Da). (D) His-UBE2N-allyl was reacted under the same conditions used for His-UBE2N-propargyl. Moderate thiol-ene addition was observed as depicted by the 19661 Da species present in the left peak (expected mass 19667 Da). As with the His-UBE2N-propargyl reaction, His-UBE2N is regenerated (19316 Da), presumably by loss of an allyl radical. This then appears to undergo homopropagation[7] with His-UBE2N-allyl as depicted by the 19395 Da peak (expected mass ~19399 Da). (E) After 5 min GSH undergoes addition to His-UBE2N-BMOE as depicted by the 19843 Da peak (expected mass 19847 Da). However, the reaction stalls and does not proceed further. Prolonged incubation (15 h) only results in a hydrolysis product as depicted by the 19555 Da peak (expected mass 19558 Da).

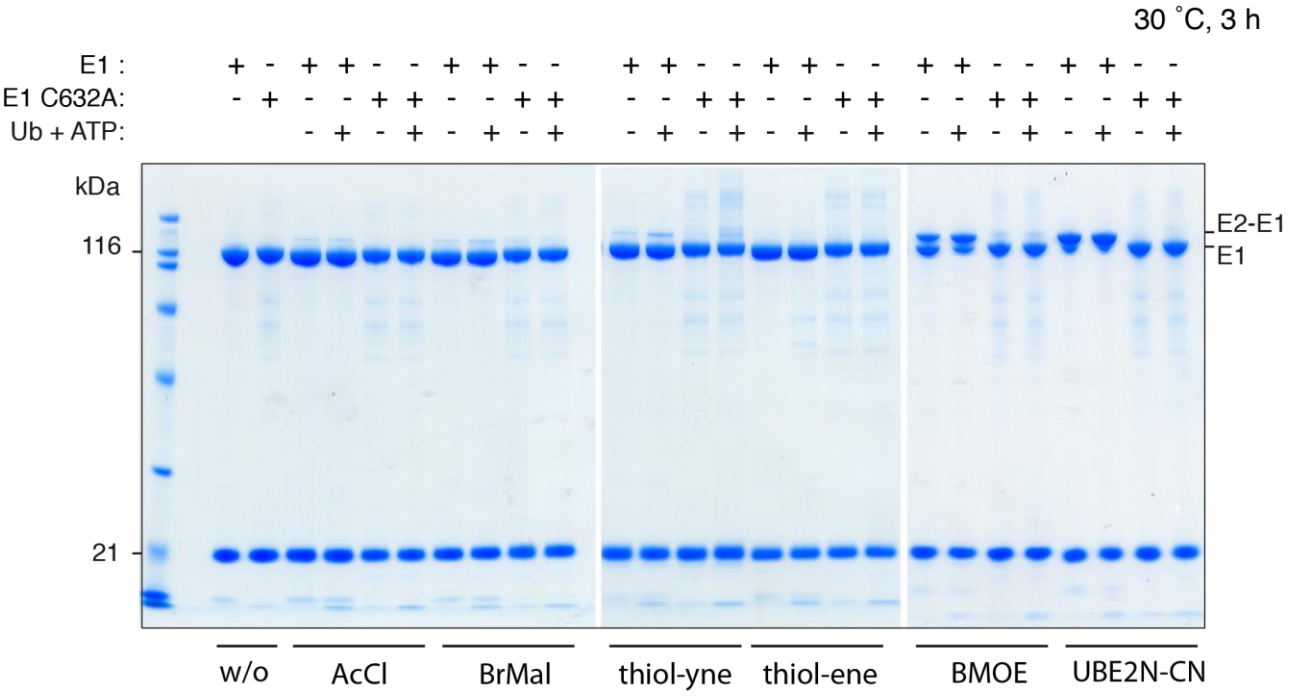


Figure S17. Comparison of commercial and literature cysteine-cysteine cross-linking strategies with the activated vinylsulfide (AVS) approach described herein. E2 (UBE2N), functionalized with a panel of cysteine-reactive moieties, were incubated with wild type or catalytic mutant (C632A) E1. Incubations were also carried out in the presence and absence of Ub and ATP that enhances cysteine-cysteine juxtaposition between E1 and E2. Conditions were consistent with the experiment carried out in Figure 5, panel B.

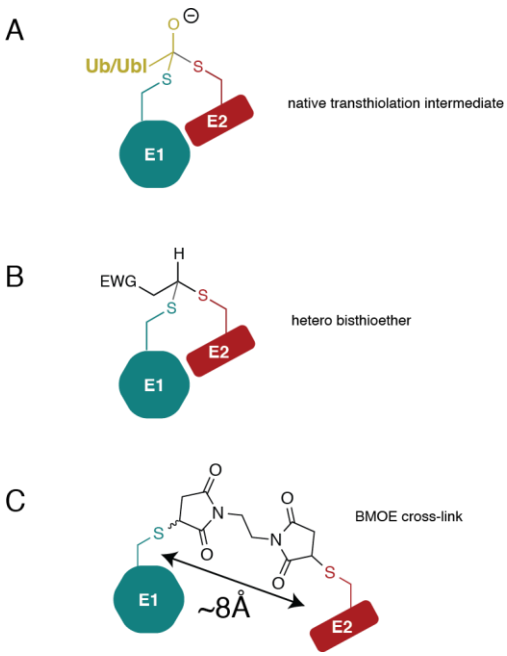


Figure S18. Chemical structures of the stable E1-E2 cross-link formed by cysteine addition to an installed AVS or by use of bifunctional bismaleimidoethane. (A) Structural representation of the native transient, tetrahedral transthiolation intermediate. (B) Hetero-bisthioether formed by use of the AVS warhead approach described herein. Both sulphur atoms are functionalized at a single atomic center forming a tetrahedral species that accurately mirrors the geometry of the native transthiolation intermediate. EWG = electron withdrawing group. (C) The bismaleimidoethane (BMOE) cross-linker displays considerable steric bulk and the cysteine sulphur atoms are linked with an 8 Å extraneous linker.

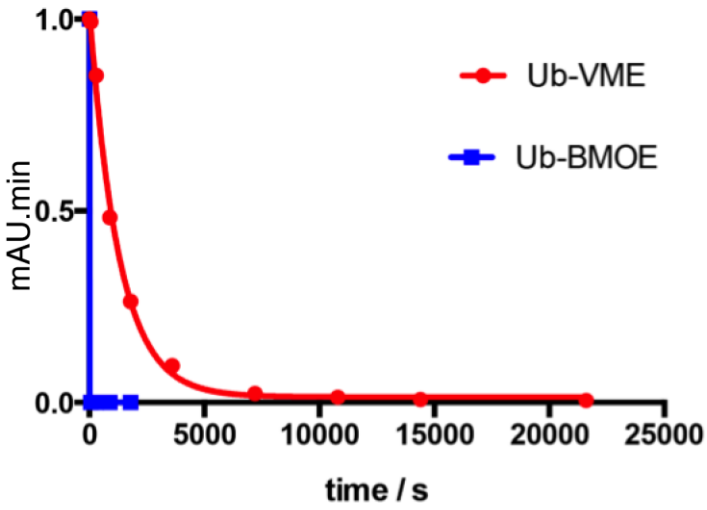


Figure S19. ΔUb-SH was functionalized with bismaleimidoethane to generate the probe ΔUb-BMOE. To compare the kinetics of thiol addition to this commonly employed we attempted to determine an observed rate constant in parallel with ΔUb-VME. As can be seen (blue line), ΔUb-BMOE underwent quantitative addition with GSH at the first time point. Reaction conditions were as those employed in Figure S5.

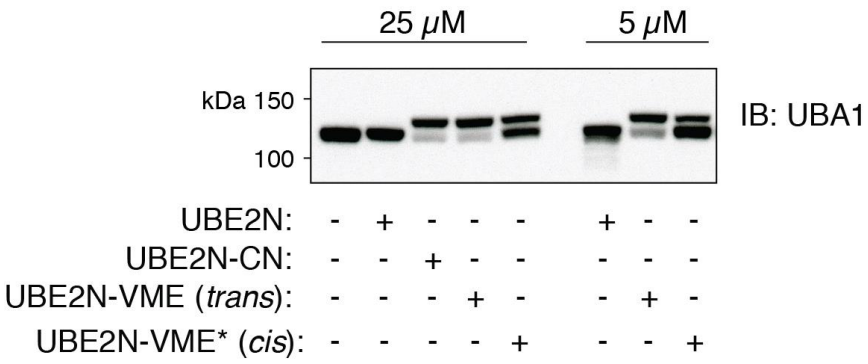


Figure S20. Incubation of various AVS-functionalized UBE2N species with HEK293 proteome obtained by detergent-based lysis. Incubation was at 30 °C for 3 h. Proteome was then resolved by reducing SDS-PAGE and transferred to nitrocellulose membrane and probed with anti-UBA1 antibody. ~140 kDa band is only present when the AVS is installed on UBE2N. Lane 1; proteome only; lane 2, proteome plus UBE2N (25 µM); lane 3, proteome plus UBE2N-CN (25 µM); lane 4, proteome plus UBE2N-VME (25 µM); lane 5, proteome plus UBE2N-VME* (*cis*) (25 µM); lane 7, proteome plus UBE2N (5 µM); lane 8, proteome plus UBE2N-VME (5 µM); lane 9, proteome plus UBE2N-VME* (5 µM). Similarly to the results obtained with probe labelling of recombinant UBA1, UBE2N-CN and UBE2N-VME exhibit comparable labelling efficiency of UBA1. However, the *cis* regioisomer of the thiomethyl acrylate probe, UBE2N-VME*, is significantly less efficient than the *trans* counterpart.

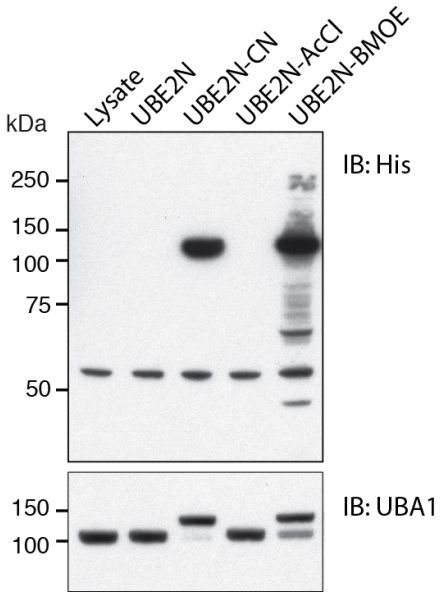


Figure S21. Comparison of E1 labelling efficiency and specificity in HEK293 extracts. The UBE2N-CN probe efficiently labels endogenous UBA1 with no detectable background labelling (lane 3). The UBE2N-AcCl probe (prepared by reaction of UBE2N with dichloroacetone) did not demonstrate detectable labelling despite some very low activity with recombinant E1 (lane 4). This is likely due the haloacetyl group being too reactive which is rapidly quenched by small molecule

thiols such as GSH present in the extract. Probe bearing the maleimide warhead, UBE2N-BMOE, labels endogenous E1. Additional bands were presumed to due be non-activity based labelling. E3s were discounted as UBE2N does not contain a phenylalanine at position 63 that is required for E3 binding.

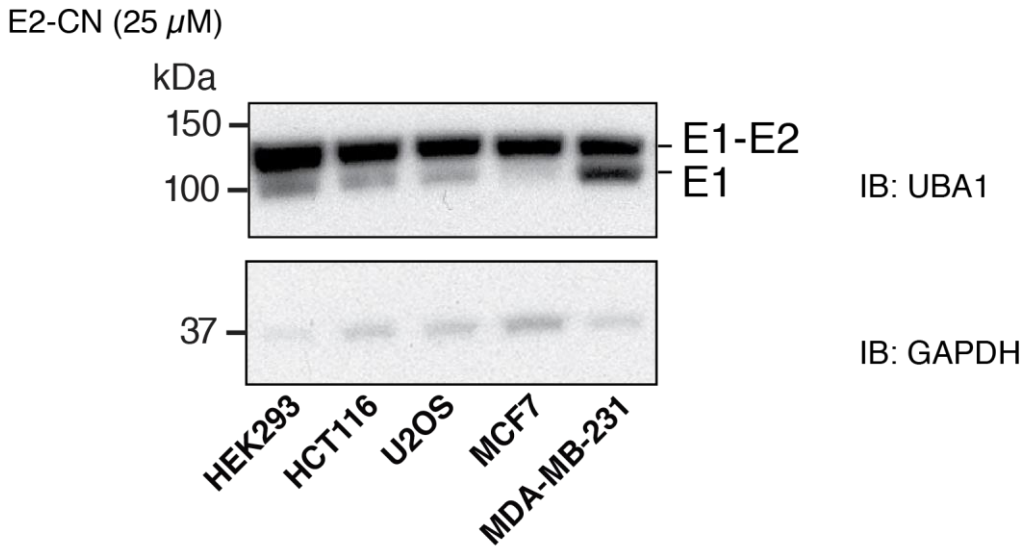


Figure S22. ABPP of endogenous UBA1 across multiple cell lines suggests transthiolation activity between UBA1 and UBE2N is cell-dependent. Immunoblot of cell proteomes after incubation with 25 μM E2 probe (UBE2N-CN) at 30 °C for 3 h. Top, UBA1 immunoblot; bottom, GAPDH loading control. Labelling efficiency of UBA1 in MCF7 line is elevated and in the MDA-MB-231 line it is reduced.

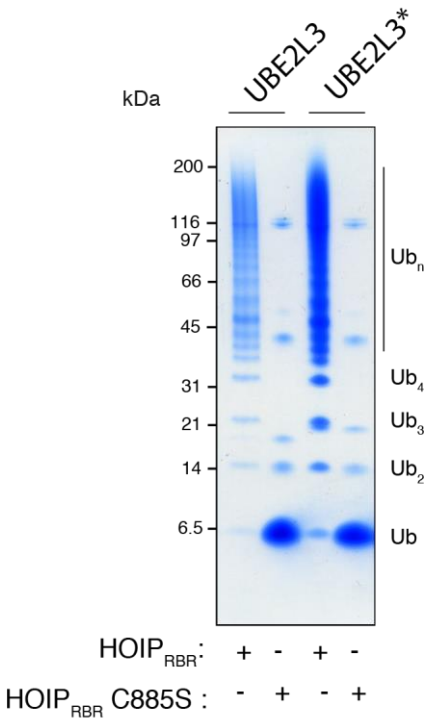


Figure S23. UBE2L3 containing C17S and C137S mutations (UBE2L3*) exhibits slightly enhanced activity in $HOIP_{RBR}$-mediated linear Ub chain assembly. The catalytic C885S mutation ablates $HOIP_{RBR}$ activity. The reaction was carried out in buffer (40 mM Tris pH 7.5, 10 mM $MgCl_2$, 10 mM ATP, 0.6 mM DTT) containing UBA1 (500 nM), E2 (2 μM) and Ub (0.6 mM). Reactions were incubated at 37 °C for 3 h. Samples were then resolved by SDS-PAGE and visualized by coomassie staining.

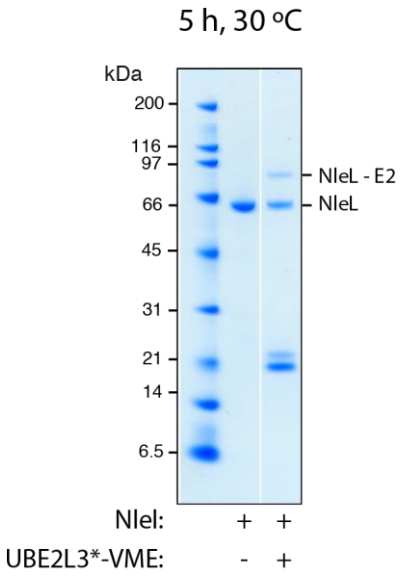


Figure S24. UBE2L3*-VME does label the bacterial E3-like effector protein, NleL.

E2 construct used

> **UBE2N**
MGSSHHHHHHSSGLEVLFQGPGSAGLPRRIIKETQRLLAEPVPGIKAEPDESNARYFHVVIA
GPQDSPFEGGTFKLELFLPEEYPMAAPKVRFMTKIYHPNVDKLGRICLDILKDKWSPALQIR
TVLLSIQALLSAPNPDDPLANDVAEQWKTNEAQAIETARAWTRLYAMNNI

Features :
6His : [5 : 10]
PreScission site : [14 : 21]

> **UBE2L3***
MGSSHHHHHHSSGLEVLFQGPGSMAASRRLMKELEEIRK**S**GMKNFRNIQVDEANLLTWQGLIVPDN
PPYDKGAFRIEINFPAEYPFKPPKITFKTKIYHPNIDEKGQVCLPVISAENWKPATKTDQVIQSLI
ALVNDPQPEHPLRADLAEEYSKDRKKF**S**KNAEEFTKKYGEKRPVD

Features :
6His : [5 : 10]
PreScission site : [14 : 21]

> **UBE2L3****
MGSSHHHHHHSSGLEVLFQGPGSMAASRRLMKELEEIRK**S**GMKNFRNIQVDEANLLTWQGLIVPDN
PPYDKGAFRIEINFPAEYPFKPPKITFKTKIYHPNIDEKGQVCLPVI**A**AENWKPATKTDQVIQSLI
ALVNDPQPEHPLRADLAEEYSKDRKKF**S**KNAEEFTKKYGEKRPVD

Features :
6His : [5 : 10]

PreScission site : [14 : 21]

> **UBE2L6***

MGSSHHHHHHSSGLEVLFQGPGSMMASMRVVKELEDLQKKPPPYLRNLSSDDANVLVWHALLLPDQ
PPYHLKAFNLRISFPPEYPFKPPMIKFTTKIYHPNVDENGQICLPIISSENWKP**S**TKT**S**QVLEALN
VLVNRPNIREPLRMDLADLLTQNPELFRKNAEEFTLRFGVDRPS

Features :
6His : [5 : 10]
PreScission site : [14 : 21]

> **UBE2L6***

MGSSHHHHHHSSGLEVLFQGPGSMMASMRVVKELEDLQKKPPPYLRNLSSDDANVLVWHALLLPDQ
PPYHLKAFNLRISFPPEYPFKPPMIKFTTKIYHPNVDENGQICLPII**A**SENWKP**S**TKT**S**QVLEALN
VLVNRPNIREPLRMDLADLLTQNPELFRKNAEEFTLRFGVDRPS

Features :
6His : [5 : 10]
PreScission site : [14 : 21]

> **UBE2I**

MGSSHHHHHHSSGLEVLFQGPGSMSGIALSRLAQERKAWRKDHPFGFVAVPTKNPDGTMNLMNWEC
AIPGKKGTPWEGGL
FKLRMLFKDDYPSSPPKCKFEPPLFHPNVYPSGTVCLSILEEDKDWRPAITIKQILLGIQELLNEP
NIQDPAQAEAYTIY
CQNRVEYEKRVRAQAKKFAPS

Features :
6His : [5 : 10]
PreScission site : [14 : 21]

> **UBE2M**

MGSSHHHHHHSSGLEVLFQGPGSPEFPGVDSKAAAMIKLFSLKQQKKEEESAGGTKGSSKKASAAQ
LRIQKDINELNLPKTCDISFSDPDDLLNFKLVICPDEGFYKSGKFVFSFKVGQGYPHDPPKVKCET
MVYHPNIDLEGNVCLNILREDWKPVLTINSIIYGLQYLFLEPNPEDPLNKEAAEVLQNNRRLFEQN
VQRSMRGGYIGSTYFERCLK

Features :
6His : [5 : 10]
PreScission site : [14 : 21]

Supplementary Methods

Preparation of ΔUb-SH

ER2566 *E. coli.* cells (NEB) were transformed with *pTXB1-Ub1-74* and recovered with S.O.B. medium (250 µL). The cells were incubated for 1 h at 37 °C and then LB medium (100 mL) containing ampicillin (100 mg mL^{-1}) was inoculated with the recovered cells (200 µL) and the culture was incubated overnight whilst shaking (230 rpm) at 37 □C. LB medium (2 L) containing ampicillin (100 mg mL^{-1}) was inoculated with the overnight culture (60 mL) and incubated whilst shaking (230 rpm) at 37 □C. At $O.D._{600}$ ~0.4, the cells were transferred to a 25 □C incubator and after 30 min the cells were induced with IPTG (0.2 mM). After 5 h the cells were harvested and suspended in 60 ml lysis buffer (20 mM Na_2HPO_4 pH 7.2, 200 mM NaCl, 1 mM EDTA) and frozen. The thawed cells were lysed by sonication on ice and were clarified by centrifugation (39000 x g, 30 min). An empty XK 26/20 column was filled with chitin beads (20 mL) (NEB) and equilibrated with lysis buffer. At 4 □C the clarified lysate was loaded (flow rate:0.5 mL min^{-1}) onto the column using an ÄKTA FPLC system. The column was then washed with lysis buffer (~400 mL) and equilibrated with 60 mL of cleavage buffer (20 mM Na_2HPO_4 pH 7.2, 200 mM NaCl, 50 mM cysteamine, 10 mM TCEP, 1 mM EDTA). The flow was then stopped and the column incubated for 66 h at 23 □C, to allow thiolysis and concomittant S-N transfer. Liberated ΔUb-SH was eluted with elution buffer (20 mM Na_2HPO_4 pH 6, 200 mM NaCl, 1 mM EDTA). The fractions containing ΔUb-SH were pooled and concentrated to ~5 mL using an Amicon Ultra-15 centrifugal filter device (Millipore). The protein was then further purified by semi-preparative RP-HPLC using the Dionex system. A gradient of 10 % buffer A to 80 % buffer B was applied at a flow rate of 10 mL min^{-1} over 30 min (buffer A=0.1% TFA in H_2O, buffer B=0.1% TFA in acetonitrile). Fractions containing ΔUb-SH were pooled and lyophilized.

Synthesis of (E)-methyl 3-tosylacrylate (2)

$PhI(OAc)_2$ (966 mg, 2 mmol) was added to a suspension of methyl methacrylate (2 mmol), sodium arenesulfinate (8.0 mmol), and KI (328 mg, 2.0 mmol) in CH_3CN (8 mL). The reaction mixture was stirred vigorously at room temperature for 1 h under an inert atmosphere. The reaction mixture was quenched by the addition of saturated $Na_2S_2O_3$ aq. (20 mL) followed by a saturated aqueous solution of $NaHCO_3$ aq. (20 mL). Further stirring was followed by extraction with EtOAc (3 x 50 mL). The combined organic phases were washed with saturated NaCl aq. (75 mL) and dried over Na_2SO_4. The organic solvent was removed in vacuo and the crude product was purified by flash chromatography using a Reveleris® X2 flash chromatography system (Grace) (Reveleris® 12g silica column; ethyl acetate (EA):Hexane; 12% to 100% EA gradient elution).

Preparation of ΔUb-CN , ΔUb-VME and ΔUb-VME* (ΔUb-AVS)

ΔUb-SH (3.6 mg, 0.42 μmol) was reconstituted[8] by the addition of DMSO (54 μL). On complete dissolution of ΔUb-SH in DMSO, PBS (1X, 1746 μL) was added to give a final DMSO concentration of 3% (v/v) and a final ΔUb-SH concentration of 233 μM. 4 eq. of **1**, **2** or **3** were then added from a DMSO stock solution and the mixture was then briefly vortexed. The resulting solution was incubated (37°C, 1 hour) and the reaction was monitored by LC-MS. To remove small molecule by-products, the reaction solution was buffer exchanged into MQ water using a pre-packed PD-10 column (GE Healthcare). ΔUb-AVS was eluted at an approximate concentration of 116 μM (based on quantitative conversion of ΔUb-SH.

Determination of ΔUb-AVS concentration was achieved by extrapolation using a standard concentration curve of WT Ub based upon the absorbance of the UV absorbance at 214 nm (Figure S14).

Standard concentration curve of WT Ub

From a 1.66 mM stock solution of Ubiquitin (determined by using A280 0.16 = 1mg/mL)[9] Serial dilution of a 1 mM WT Ub solution (100 mM Na_2HPO_4, pH 8) was then carried out to generate stock WT Ub solutions at concentrations of 5 μM, 10 μM, 30 μM and 50 μM (100 mM Na_2HPO_4, pH 8). 100 μL of each solution was added to 4 μL 25% TFA (in acetonitrile). Samples were then analysed by LC-MS (5 μL injection, see General methods; protein LC-MS) with a gradient of 10% to 75% acetonitrile (flow rate; 0.3 mL/min) over 20 minutes. The peak area of Ub at 214 nm was determined using the Agilent Chemstation software v.4.0. Runs were repeated in duplicate (Fig. S25).

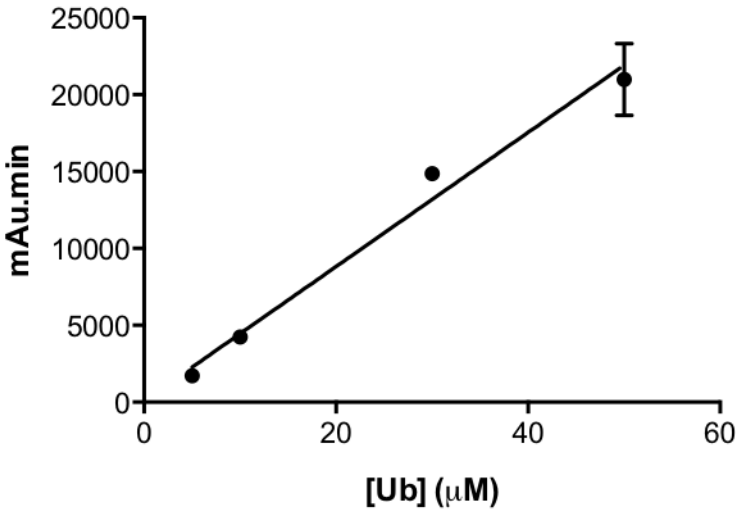


Figure S25. Due to the high UV absorbance of the thioacrylonitrile and thiomethyl acrylate groups at 280 nm present in ΔUb-AVS we could not use the theoretical extinction coefficient of ubiquitin as a means to spectrophotometrically determine the concentration of ΔUb-AVS. To address this we assumed absorbance at 214 nm was approximately the same for ubiquitin and AVS functionalized ubiquitin. Ubiquitin solutions of defined concentration were analysed by RP-HPLC and peaks were integrated. Peak area was plotted against ubiquitin concentration. The concentration of AVS-

functionalized ubiquitin was determined by HPLC peak integration and extrapolation from the standard curve.

Covalent modification of DUBs with ΔUb-CN

100 mM Na_2HPO_4 phosphate buffer, pH 8 and deubiquitinase enzymes (DUBs; UCH-L3 residues 1-103 (93 μM), OTUB2 residues 1-234 (275 μM), His-USP5 residues 1-858 (21 μM), His-USP21 residues 196-565 (25 μM) GST-USP2 residues 1-353 (7 μM), His-TRABID residues 245-697 (18 μM) were individually aliquoted into 1.5 mL microcentrifuge tubes and placed on-ice to give a final DUB concentration of 5 μM (based on a 50 μL reaction volume). The DUB solutions were incubated in an Eppendorf thermomixer (30°C, 400 rpm) for 5 minutes before the addition of ΔUb-CN (8.6 μL, 116 μM in MQ water) was added to each tube to give a final ΔUb-CN concentration 20 μM (in a reaction volume of 50 μL). A control sample of ΔUb-CN (20 μM in 50 μL reaction volume), which contained no DUB, was also prepared and incubated alongside DUB samples. SDS sample buffer contained 2-mercaptoethanol at a final concentration of 179 mM which illustrates redox stability of the modifications. The reaction solution was incubated at 30°C for 1 hour at which time 16 μL of reducing SDS-gel loading buffer (NuPAGE LDS loading buffer (4X), 715 mM 2-mercaptoethanol) was added to each microcentrifuge tube. The quenched reaction solutions were boiled for 1 minute at 100°C and then 15 μL of solution was analysed by SDS-PAGE (NuPAGE Bis-Tris, 4-12%).

Preparation of E2 probes UBE2N-CN, UBE2N-VME and UBE2N-VME*

Preparation of probes built on the E2 UBE2N were prepared as described for ΔUb-SH.

Expression and purification of E1 activating enzymes

His-tagged human UBA1 and UBA7 cDNA were cloned into the pFastBacHTB vector (Life Technologies) with N-terminal 6× His tags. A C632A mutant of UBA1 was made by Quikchange mutagenesis. Dac-tagged[10] NAE1 and UBA3 cDNA were cloned into the pFastBacDual vector (Life Technologies). The recombinant E1s were expressed using the Bac-to-Bac system (Life Technologies). Briefly, the bacmid DNAs were generated in DH10Bac cells, and the resulting baculovirus was generated and amplified in *Spodoptera frugiperda* Sf21 insect cells (Life Technologies). E1s were overexpressed in Sf21 insect cells grown in Insect-Xpress medium (Lonza). Cells were harvested 72 h after infection. For UBA1, UBA1 C632A and UBA7 cells were lysed by sonication in lysis buffer (25mM HEPES, 150mM NaCl, 0.3% Triton X-100, 0.5 mM EGTA, 0.1 mM EGTA, 1 mM DTT, Leupeptin & Pefabloc. The lysate was clarified by ultracentrifugation (40,000 rpm for 1 h). The supernatant was subjected to Ni-NTA affinity chromatography (Qiagen) and washed with wash buffer (25mM HEPES, 250 NaCl, 20 mM imidazole 0.3% Triton X-100, 0.5mM EGTA, 0.1mM EGTA, 1mM DTT). Protein was eluted with imidazole and dialyzed into storage buffer (25mM HEPES pH 7.5, 10% glycerol, 150 mM NaCl, 0.03% Brij35, 1mM DTT). In the case of wt UBA1, the N-terminal His-tag was removed with TEV protease and protease was subsequently depleted by incubation with Ni-NTA resin. For Dac-NAE1-UBA3, cells were lysed by sonication in buffer (40mM Tris pH 7.5, 0.2% Triton X- 100, 0.5mM ETDA, 0.5mM EGTA, 1mM DTT, protease inhibitor). Clarified lysate was incubated with ampicillin sepharose and washed with wash buffer (40mM HEPES pH 7.5 150mM NaCl, 0.03% Brij35) and eluted with wash buffer supplemented with 5 % glycerol and 10 mM ampicilin buffer. Eluted protein was further purified by gel filtration (Superdex 200 16/60, GE Healthcare) using wash buffer as running solvent.

GST-tagged SAE1 and His-tagged UBA2 were cloned into the pET DUET-1 vector and co-expressed protein was expressed in *E. coli* BL21(DE3) cells. Cells were lysed in lysis buffer (50 mM Tris pH 7.5, 150 mM NaCl, 1 mM EGTA 1 mM EDTA, 1 mM Leupeptin, 1 mM DTT, 0.03% Brij-35, 1% Triton-X100). Nickel affinity purification using Ni-NTA resin (Qiagen) was carried out on the clarified lysate and resin was washed with buffer (50 HEPES pH 7.5, 150 mM NaCl, 5 mM imidazole, 0.03% Brij35, 10% glycerol) protein was eluted with elution buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 400 mM imidazole, 0.03% Brij35, 10% glycerol). Protein was subsequently purified using glutathione resin and washed with wash buffer. Protein was then eluted with GST elution buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 10 mM GSH, 0.03% Brij35, 10% glycerol).

Expression and purification $HOIP_{RBR}$ and $HOIP_{RBR}$ C885S

$HOIP_{RBR}$ (residues 607 - 1072) and $HOIP_{RBR}$ C885S were cloned as GST fusions in a pGEX vector. Protein was expressed in BL21(DE3) cells overnight at 16 °C. Clarified lysate was purified against glutathione sepharose 4B (GE Life Sciences). $HOIP_{RBR}$ was cleaved on resin by incubation with Rhinovirus C3 protease and protein was further purified by size exclusion chromatography.

Ubiquitin probes

Ubiquitin vinylsulfone (Ub-VS) was sourced from Boston Biochem. The Ub-Alk probe was prepared as follows. Ub1-75 thioester was prepared as previously described[11] except the Gly76 was deleted producing plasmid *pTXB1-Ub1-75.* Pooled fractions were concentrated and adjusted to pH 8 by the careful addition of 1 N NaOH while mixing. Propargyl amine (250 mM) was then added and the reaction left to incubate at 25 °C for 1 h. Protein functionalization was ~90% as determined by LC-MS and was further purified by semi-preparative HPLC and lyophilized. Probe was folded by

dissolution in denaturing buffer (200 mM Na_2HPO_4 pH 7.5, 6 M guanidinium chloride) and dialyzed overnight against PBS.

Compatibility of probe labeling with reducing agents, acids and bases

Activity-based crosslinking against Uba1 was carried out pro rata as described above with Ubc13-=CN and Ubc13-VME probes but in a reaction volume of 20 µL and in the presence of 1 mM TCEP, DTT and 2-mercaptoethanol (10 mM stock solutions) and 10 mM TCEP, DTT and 2-mercaptoethanol (100 mM stock solutions).

To test the stability of the formed crosslinks, crosslinking reactions with Ubc13-=CN and Ubc13-VME were carried out on a larger scale in a final volume of 140 µL. No additional reducing agent was added and reactions were again incubated for 3 h at 30 °C. 20 µL aliquots of each reaction were then treated with 10 µL of the following: 750 mM TCEP, 750 mM 2-mercaptoethanol, 1.5 M HCl, 15 % TFA (aq.), 15 % formic acid (aq.), 15 % acetic acid (aq.) and 1.5 M NaOH. The treated samples were then incubated for 2 h at 23 °C. Samples were then diluted with 4 X LDS loading buffer + BME and 6 µL was analyzed by SDS-PAGE.

Preparation of E2 probes based on commercial cysteine cross-linkers

1,3-Dichloroacetone (cat no. 168548, Sigma-Aldrich)

His-UBE2N (350 µL, 51.7 µM in 100 mM Na_2HPO_4, 150 mM NaCl, pH 8) was carefully adjusted to pH 7 using 1 M HCl and was subsequently cooled on ice. 1,3-Dichloroacetone (100 mM in DMF) was also cooled on ice and then 0.85 µL was added to the cooled His-UBE2N solution. The reaction was monitored by LCMS. After 30 minutes, 1,3-dichloroacetone (0.5 µL, 100 mM stock in DMF) was added to the solution and kept on ice for a further 30 minutes. LCMS indicated

~50% conversion to the desired monofunctional adduct at which point (to prevent further undesired side reactions) excess 1,3-dichloroacetone was removed by buffer exchange using a disposable PD10 column (Centripure P2, Generon) and then concentrated (Amicon Ultra 0.5 mL centrifugal filters 3K MWCO). Protein concentration was determined by Bradford assay (0.92 $mgmL^{-1}$).

2,3-Dibromomaleimide (cat no. 553603, Sigma-Aldrich)

To His-UBE2N (350 μL, 51.7 μM in 100 mM Na_2HPO_4, 150 mM NaCl, pH 8) was added 1 M Na_2HPO_4, pH 6 (40 μL). The pH was adjusted with 5N HCL (5 μL) to pH 6. 2,3-Dibromomaleimide (4 eq., 0.88 μL, 100 mM in DMSO) was then added to His-UBE2N, followed by end-over-end rotation of the protein solution at room temperature for 1.5 hours. The reaction was monitored by LCMS. On completion, excess 2,3-Dibromomaleimide was removed by buffer exchange using a disposable PD10 column (Centripure P2, Generon) and then concentrated (Amicon Ultra 0.5 mL centrifugal filters 3K MWCO). Protein concentration was determined by Bradford assay (1.08 $mgmL^{-1}$).

Bismaleimidoethane, BMOE (cat no. 22323, Life Technologies)

Bismaleimidoethane (50 mg, Life Technologies) was dissolved in DMSO (250 mM stock solution). To His-UBE2N (250 μL, 51.7 μM in 100 mM Na_2HPO_4, 150 mM NaCl, pH 8) was added 1 M Na_2HPO_4, pH 6 (50 μL). The pH was adjusted with 5N HCL (3 μL) to pH 6. BMOE (4 eq., 6 μL, 10 mM in DMSO) was then added, the solution was carefully mixed and then incubated at room temperature for 1.5 hours with end-over-end rotation. The reaction was monitored by LCMS. On completion, excess BMOE was removed by buffer exchange using a disposable PD10 column (Centripure P2, Generon) and then concentrated (Amicon Ultra 0.5 mL centrifugal filters 3K MWCO). Protein concentration was determined by Bradford assay (1.29 $mgmL^{-1}$).

Propargyl bromide (cat no. P51001, Sigma-Aldrich)

Propargyl bromide (80 % wt. % in toluene, 9.28 M) was dissolved in DMSO (100 mM stock solution). To His-UBE2N (350 μL, 51.7 μM in 100 mM Na_2HPO_4, 150 mM NaCl, pH 8) was added propargyl bromide (50 eq., 9 μL, 100 mM in DMSO). The solution was carefully mixed and incubated at room temperature for 1.5 hours with end-over-end rotation. The reaction was monitored by LCMS. On completion, excess propargyl bromide was removed by buffer exchange using a disposable PD10 column (Centripure P2, Generon) and then concentrated (Amicon Ultra 0.5 mL centrifugal filters 3K MWCO). Protein concentration was determined by Bradford assay (2.08 $mgmL^{-1}$).

Allyl bromide (cat no. A29585, Sigma-Aldrich)

Allyl bromide (11.55 M) was dissolved in DMSO (100 mM stock solution). To His-UBE2N (350 μL, 51.7 μM in 100 mM Na_2HPO_4, 150 mM NaCl, pH 8) was added allyl bromide (50 eq., 9 μL, 100 mM in DMSO). The solution was carefully mixed and incubated at room temperature for 1.5 hours with end-over-end rotation. The reaction was monitored by LCMS. On completion, excess allyl bromide was removed by buffer exchange using a disposable PD10 column (Centripure P2, Generon) and then concentrated (Amicon Ultra 0.5 mL centrifugal filters 3K MWCO). Protein concentration was determined by Bradford assay (2.14 $mgmL^{-1}$).

Recombinant UBA1 crosslinking experiment

UBA1 WT and UBA1 C632A (3 μM) were incubated respectively at 30 °C for 3 hours with various functionalized His-UBE2N species (12 μM) in the presence and absence of Ub (2 μM) and ATP (2 mM) in 50 mM Na_2HPO_4, pH 8, 1 mM TCEP.

Reactions were quenched by the addition of 4X LDS loading buffer (supplemented with

betamercaptoethanol) and samples were analysed by SDS-PAGE (10 % NuPage gel, 1X MOPS-SDS running buffer, 50 minutes) followed by Coomassie staining.

Alkyne/Alkene photoirradiation

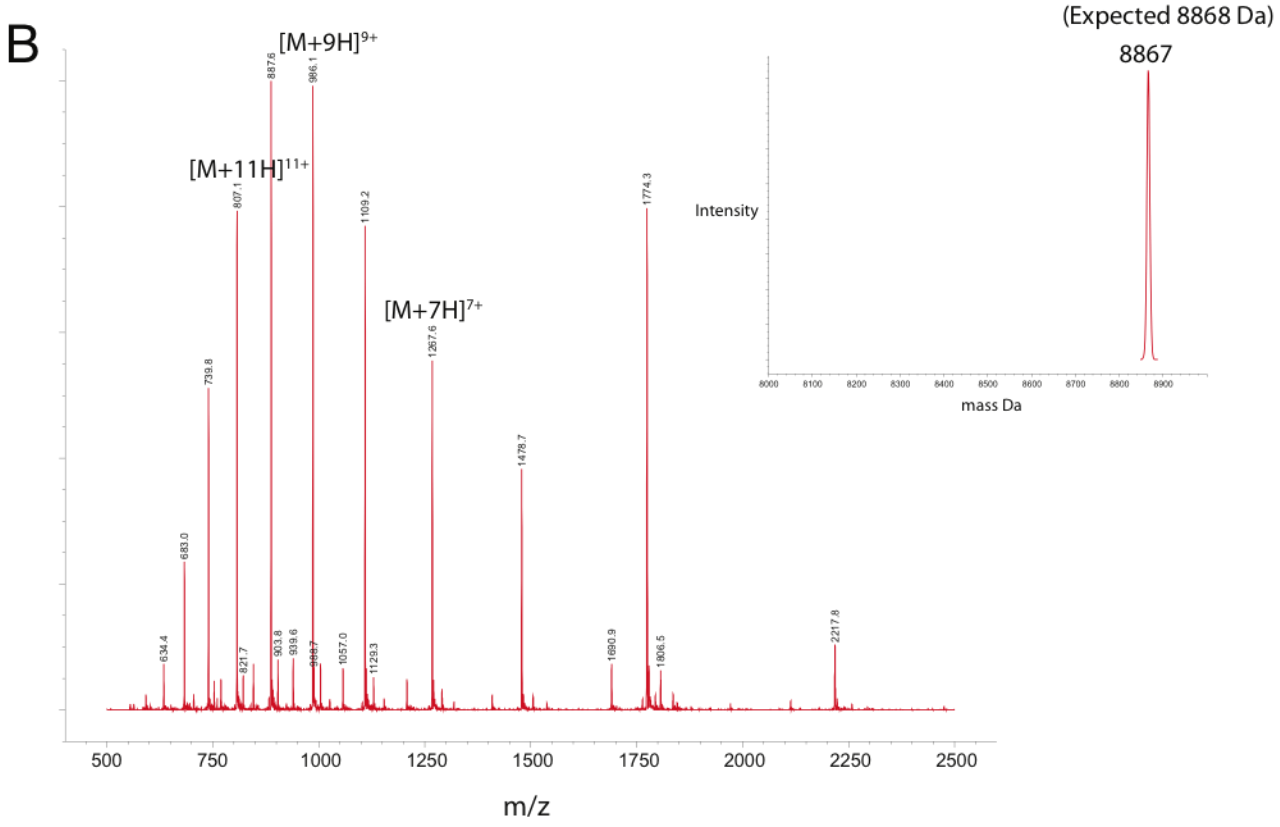
His-E2N-alkyne and -alkene species were prepared and incubated with recombinant WT UBA1 and UBA1 C632A in accordance with previously described protocol (see above) with the following exceptions; The His-E2N-alkyne/alkene solutions were supplemented with 2,2-Dimethoxy-2-phenylacetophenone (3 eq., 2.1 μL, 1 mM stock in DMSO), followed by irradiation of the samples at 365 nM for 10 minutes with a UV hand lamp. The samples were then incubated at 30 °C for 2 hours 50 minutes.

Cell culture and lysis protocol

Cells were cultured in Dulbecco's modified Eagle's medium (DMEM). Cells were supplemented with 10% foetal bovine serum, 2 mM L-glutamine and antibiotics (100 Units ml^{-1} penicillin, 100 $\mu g ml^{-1}$ streptomycin). All cells were cultured at 37 °C in a 5 % CO_2 humidified atmosphere. Cells from one culture flask (75 cm^2, Nunclon^{TM}) at ~90% confluence were washed with Dulbecco's Phosphate Buffered Saline (DPBS) and trypsinised (0.05% Trypsin-EDTA (1X), Gibco) at room temperature. Trypsinised cells from three culture flasks were combined, centrifuged (1200 rpm, 4°C, 3 minutes) and washed with DPBS (repeated once). Cells in cancer cell line panel were harvested by scraping. Cells were snap frozen with liquid nitrogen and stored at -80°C. The combined cells from three culture flasks were lysed with 50 mM Tris pH 7.5, 150 mM NaCl, 1% Nonidet P40 substitute, 0.25% sodium deoxycholate, 1 mM AEBSF, 1mM sodium orthovanadate, 2mM benzamidine, 1 mM sodium fluoride, 1 mM TCEP, Roche complete EDTA free inhibitor cocktail, and benzonase (Sigma-Aldrich) using 200 μL 1.1X lysis buffer per culture flask. The cell

pellet was incubated on-ice for 20 minutes. Lysates were cleared using centrifugation (13200 rpm, 30 minutes) and filtration through 0.45μm spin filters (Millipore) to remove cell debris. For probe labeling experiments against HEK293 proteomes alone, lysates were then diluted approximately 3-fold with 50 mM Tris pH 7.5, 150 mM NaCl, 1mM PMSF, Roche complete EDTA free inhibitor cocktail to bring the Nonidet P40 concentration down to ~0.3% and a final protein concentration of 2.8 mg/mL as determined by Bradford assay (595nm). When probe labeling was carried out across multiple cell types, cells were lysed by sonication in PBS containing 5 mM TCEP. Protein extracts were normalized to 1.5 mg/mL.

Full MS Spectra


B
[M+9H]9+
[M+11H]11+
[M+7H]7+
(Expected 8868 Da)
8867
Intensity
mass Da
m/z

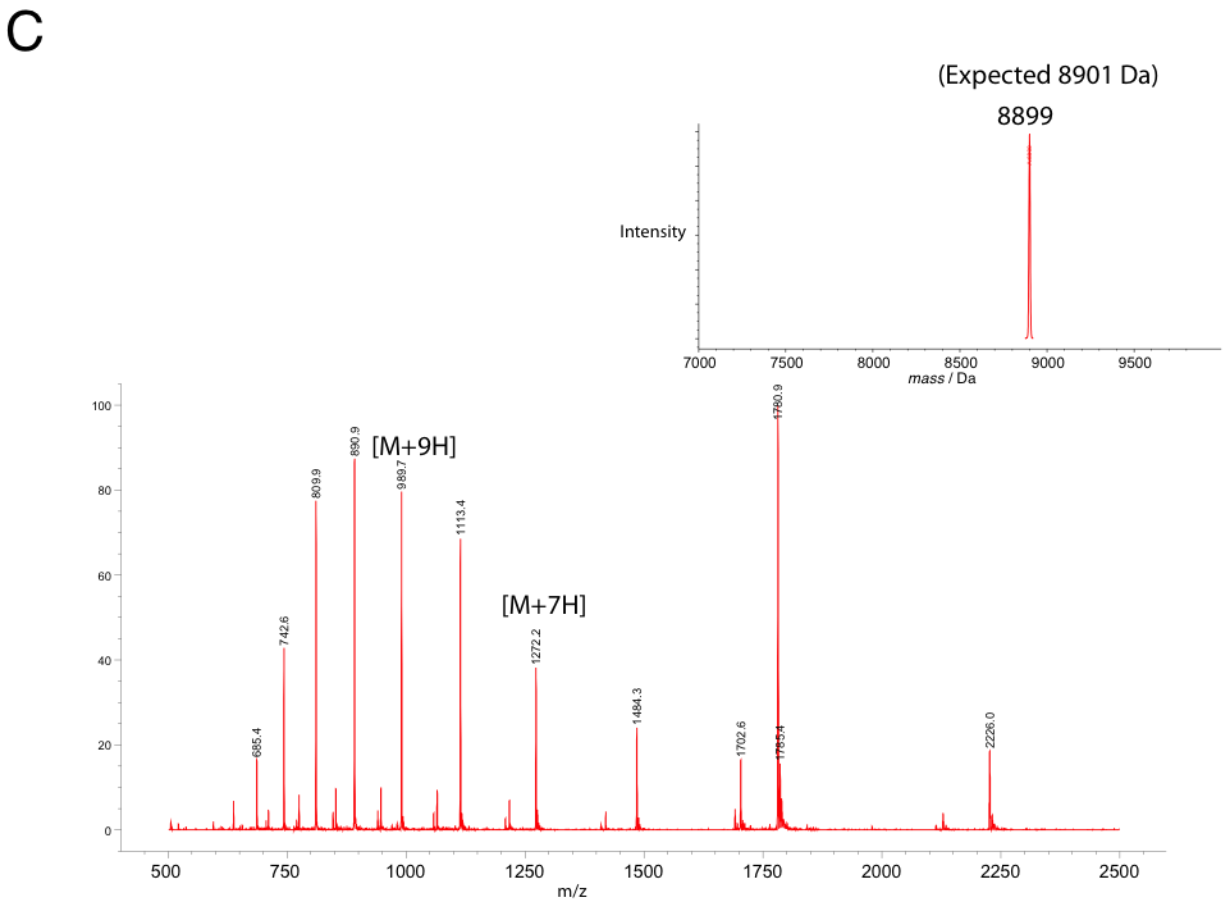

C
(Expected 8901 Da)
8899
Intensity
mass / Da
[M+9H]
[M+7H]
m/z

Figure S26. (B) Full ESI-MS spectrum of hetero-bisthioether adduct of ΔUb-CN after reaction with glutathione **4** (62.5 mM) for 6h at 37 C . Expected mass = 8868 Da; Observed = 8867 Da. (C) Full ESI-MS spectra of glutathione adduct with UbS-VME. Expected mass = 8901 Da; Observed = 8899 Da.

Supplementary References

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