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Supporting Information

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Auxiliary mediated site-specific peptide ubiquitylation

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General Methods

Amino acid (AA) derivatives, coupling reagents and pre-loaded Wang resin were purchased from Novabiochem (San Diego, CA). All other chemical reagents were purchased from Sigma-Aldrich Chemical Company (Milwaukee, WI) or Fischer Scientific (Pittsburgh, PA). Chitin beads for purification of intein-CBD fusion proteins were obtained from New England BioLabs (Ipswitch, MA). Analytical reversed-phase HPLC (RP-HPLC) was performed on a Hewlett-Packard 1100 series instrument with a Vydac C18 column (5 micron, 4 x 150 mm), employing 0.1% TFA in water (A), and 90% CH_3CN, 0.1% TFA in water (B), as the mobile phases. Typical analytical gradients were 0-73%B over 30 min at a flow rate of 1 mL/min. Preparative scale purifications were conducted on a Waters DeltaPrep 4000 system equipped with a Waters 486 tunable detector. A Vydac C18 process column (15-20 micron, 50 x 250 mm) or a semi-preparative column (12 micron, 10 mm x 250 mm) was employed at a flow rate of 30 mL/min, or, 4 mL/min, respectively. ESI-MS analysis was conducted on either a Sciex API-100 single quadrupole spectrometer, or, on a Waters Micromass ZQ 2000. NMR spectra were recorded on a Bruker DPX 400 MHz instrument. All synthetic peptides and ligation products were analyzed by C18 analytical RP-HPLC and ESI-MS. High resolution ESI-FT MS data was obtained in the Angeletti laboratory at the Albert Einstein College of Medicine, Bronx, New York. LTQ ion trap ESI MS analysis was performed at the Proteomics Resource Center, Rockefeller University, New York.

Synthesis of the ligation auxiliary

The ligation auxiliary **1** was prepared over eight steps from vanillin following the procedure reported by Pellois and Muir with one major modification.[1] A terminal step to convert the free carboxylic acid form of the auxiliary to the amide was undertaken in

order to prevent ester formation during its application to the subsequent on-bead alkylation reaction (Chatterjee and Muir, unpublished observations)**.**

4-[4-(1-Amino-2-tert-butyldisulfanyl-ethyl)-2-methoxy-5-nitro-phenoxy]-N-methyl-butyramide (1). Sodium methoxide (0.5 M in methanol, 11.35 mL, 5.68 mmol) was added dropwise over three minutes to a stirred solution of 4-[4-(2-Acetylsulfanyl-1-tert-butoxycarbonylamino-ethyl)-2-methoxy-5-nitro-phenoxy]-butyric acid methyl ester, (2.76 g, 5.68 mmol) in 200 ml of methanol.[1] After an additional 15 minutes of stirring at 25 °C, 2-methyl-2-propanethiol (19.15 mL, 170 mmol) and NaOH (85 mL of 1 N stock, 85 mmol) were added and the resultant mixture was bubbled with a continuous stream of oxygen. The reaction progress was following by LC-MS employing a C18 analytical column at a flow rate of 0.2 mL/min and a gradient of 30-73%B over 30 min (R_t 26 min). After 40 hours, the reaction was quenched by dilution with 500 mL water followed by neutralization with 1 N HCl, and extraction with 4 x 250 mL ethyl acetate. The combined organic phase was dried over magnesium sulfate and the solvent removed *in vacuo* yielding 4-[4-(1-*tert*-Butoxycarbonylamino-2-*tert*-butyldisulfanyl-ethyl)-2-methoxy-5-nitrophenoxyl-butyric acid (1.28 g of crude product). To a stirred solution of the crude product from the previous step (640 mg, 1.23 mmol) in DCM (30 mL) at room temperature were added PyBOP (642 mg, 1.23 mmol) and N,N-diisopropylethylamine (0.633 ml, 3.70 mmol). After an additional five minutes of stirring, methylamine (6.2 mL, 12.4 mmol, 2 M in THF) was added to the mixture and the reaction allowed to proceed for a further 12 h at room temperature. At this stage the solvent was removed *in vacuo* and the residue dissolved in a mixture of TFA (9.5 mL), triisopropylsilane (0.25 mL), and water (0.25 mL). The resulting solution was stirred at room temperature for 1.5 h to effect complete removal of the Boc- protecting group and yield compound **1**. The removal of water by lyophilization yielded crude **1**, which was purified by preparative RP-HPLC (gradient 20-45% B, 60 min). Fractions containing pure **1** were identified by analytical RP-HPLC, combined and lyophilized to obtain 340 mg of pure compound **1** in 28% isolated yield over all four steps (Figure **S1**). ^{1}H NMR (400 MHz, $CDCl_3$): d 7.55 (s, 1H, H_{ar}), 7.29 (s, 1H, H_{ar}), 6.13 (m, 1H, N*H*CH_3), 5.35 (m, 1H, C*H*NH_2), 4.12 (t, 2H, OCH_2), 3.92 (s, 3H, OCH_3), 3.33 (d, 2H, CH_2S), 2.78 (d, 3H, C*H*$_3$NH), 2.41 (t, 2H, CH_2-

CO), 2.15 (m, 2H, CH_2), 1.33 (s 9H, S-tBu). ^{13}C NMR (400 MHz, $CDCl_3$): d 173.90, 154.51, 148.62, 141.78, 124.74, 111.11, 109.78, 69.06, 57.08, 49.06, 42.46, 32.96, 30.12, 26.72, 25.12, 22.29. HRMS (ESI-FT) m/z calculated for $C_{18}H_{29}O_5N_3S_2$ $(M+H)^+$ 432.1622, found 432.1609.

Solid-phase synthesis.

Synthesis of Boc-TKCVTKYTSAK-Wang resin **(2)**. The peptide TKCVTKYTSAK (cH2B) was manually synthesized on a 0.5 mmol scale employing standard 9-fluorenylmethoxycarbonyl (Fmoc-) based N^{α}-deprotection chemistry. Starting with the pre-loaded Fmoc-Lys(Boc)-Wang resin (806 mg, 0.62 mmol/g), each successive amino acid was coupled in 4.4 molar excess. Deprotection of the Fmoc- group was achieved with 20% piperidine in DMF (5 mL, 3 x 5 min). Coupling reactions were undertaken for a minimum of 1 h with a mixture of Fmoc-amino acid (2.2 mmol), 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) (2 mmol) and DIEA (4.4 mmol) in DMF. Coupling efficiency was monitored by the Kaiser test[2] and additional couplings performed until a negative test was obtained. Lys6 in the sequence which is homologous with Lys120 in full-length H2B was orthogonally protected at the ε-NH_2 with the 1-(4,4-dimethyl-2,6-dioxocyclohexylidine)-3-methylbutyl (ivDde) group. Thr1 was coupled as Boc-Thr(tBu)-OH to preclude deprotection of the α-amino group during removal of the ivDde group. The N-terminal residues Thr1 and Lys2 were not incorporated efficiently upon single coupling and required multiple reactions with aggressive coupling reagents such as benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP), or, diisopropylcarbodiimide (DIC) in the presence of 1-hydroxybenzotriazole (HOBt). N^{α}-deprotection times for these residues were also increased to 2 x 20 min followed by 1 x 5 min. Following chain assembly, a test portion of the resin was cleaved with a cocktail consisting of TFA: TIS: H_2O (95:2.5:2.5) for 1 h and the peptide analyzed by C18 analytical RP-HPLC. ESI-MS, **2** calculated m/z 1452 Da (M+ivDde), found 1452 Da.

Synthesis of auxiliary-cH2B peptide **(5)**. The resin bound peptide **2** was treated with 2% hydrazine in DMF (v/v) for 5 x 30 min, followed by a longer incubation for 8 h to effect

removal of the ivDde group from Lys6. The peptidyl resin (550 mg, 0.33 mmol) was then coupled to bromoacetic acid (366.8 mg, 2.64 mmol) with DIC (333 mg, 2.64 mmol) in DMF (4 mL) for 30 min at room temperature. The coupling was repeated once, and a test portion of the resin was cleaved to confirm the correct bromoacetylated peptide **3** had been generated; ESI-MS, **3** calculated m/z $(M+H)^+$ 1367 Da, found 1368 Da. Subsequently, peptidyl resin **3** (250 mg, 0.15 mmol based on initial loading) was swollen in 1.4 mL of DMF and reacted with 1.75 equivalents of auxiliary **1** (112.6 mg, 0.261 mmol) in the presence of DIEA (252.3 mg, 1.95 mmol) and DBU (10.2 mg, 0.07 mmol) for 96 h at room temperature with the exclusion of light. The reaction progress was followed by test cleavages of 5-10 mg of resin after 48 h, 72 h, and 96 h. The entire cH2B-auxiliary-resin **4** was then reacted with 10 mL of a cleavage cocktail consisting of TFA: TIS: H_2O: Anisole (92.5:2.5:2.5:2.5) for 2 h, and the peptide was purified by C18 semi-preparative RP-HPLC to yield 10.2 mg of the cH2B-auxiliary **5** (Figure **S2**). ESI-MS, **5** calculated m/z $(M+2H)^{2+}$ 859.5 Da found 860 Da.

Construction of ubiquitin and SUMO expression plasmids

pHub(1-75). The partial human ubiquitin gene, *ub(1-75)*, was amplified by PCR using the primers Hub13-FP: 5'-GGGAATTCCATATGCAGATCTTCGTGAAGACTC-3' and Hub13-RP: 5'-GAATATATGCTCTTCCGCAACCTCTGAGACGGA-3' with the plasmid pFIR-CMV-ub-IRES-lnGFP (Ho lab, Rockefeller University) as the template DNA. The PCR product was purified (QIAquick kit), digested with *Nde*I and *Sap*I restriction enzymes, and ligated into the identically digested pTXB1 vector (New England BioLabs). The resulting plasmid, pHub(1-75), was sequenced and found to contain the correct insert.

pHASmt3(1-97). DNA encoding the yeast SUMO gene, *smt3*, was amplified by PCR from the plasmid JM11a-4 (Muir lab, Rockefeller University) employing the primer HASmt3-FP: 5'-GGAATTCCATATGTACCCGTATGATGTCCCAGA-3' bearing the *NdeI* restriction site and a sequence encoding the HA epitope (MYPYDVPDYA) and the primer HASmt3-RP: 5'-GGTGGTTGCTCTTCCGCAACCAATCTGTTCTCT-3' bearing the *SapI* restriction site. The purified PCR product was digested with *NdeI* and

SapI and ligated into the identically digested pTXB1 vector. Sequencing of the plasmid pHASmt3(1-97) confirmed the desired sequence.

Overexpression and purification of Ub(1-75)-MES (6) and HA-SUMO(1-97)-MES (9).

E. coli BL21(DE3) cells (Novagen, Madison, WI) transformed with the plasmid pHub(1-75), or, pHA-Smt3(1-97) were grown in 6 L Luria-Bertani medium (100 μg/mL Ampicillin) at 37 °C with shaking at 250 rpm until an OD_{600} 0.6-0.8. Overexpression of the desired proteins was induced by the addition of 0.5 mM IPTG and the cells were grown for an additional 6 h at 25 °C. The cells were then harvested by centrifugation at 10 k*g* for 30 min and the cell-pellet was resuspended in column buffer A (50 mM Tris, 200 mM NaCl, 1 mM EDTA, pH 7.2). The cells were lysed by passage through a French Press and the soluble fraction separated from insoluble cellular debris by centrifugation at 18.5-20 k*g* for 20 min. After filtration through a 0.45 μm filter, supernatants were bound to a 20 mL chitin column, pre-equilibrated with ten volumes of column buffer A, for 2 h at 4 ?C. The resin was washed with 35 column volumes of buffer A, followed by 3 column volumes of column buffer B containing 50 mM Tris, 200 mM NaCl, and 1 mM EDTA, pH 7.5. Ub(1-75) and HA-SUMO(1-97) were cleaved from the respective intein-CBD fusions by incubation with 1.5 column volumes of buffer B containing 100 mM of the sodium salt of 2-mercaptoethanesulfonic acid (MESNa) for 87 h and 42 h, respectively. The eluted Ub(1-75)- and HA-SUMO(1-97)-α-thioesters were subsequently purified by C18 semi-preparative RP-HPLC. For the HA-SUMO(1-97)-α-thioester a gradient of 30-60% B over 45 min was employed (R_t 21 min), while Ub(1-75)-α-thioester was purified employing a gradient of 30-45% B over 45 min (R_t 27.5 min). This yielded 10.2 mg of the Ub(1-75), and 10.6 mg of the HA-SUMO(1-97) thioesters (Figures **S3** and **S4**).

ESI-MS Ub(1-75)-MES calculated m/z $(M+H)^+$ 8632 Da, found 8632 ± 2 Da.

ESI-MS HA-SUMO(1-97)-MES calculated m/z $(M+H)^+$ 12414 Da, found 12414 ± 3 Da.

Expressed protein ligation of the auxiliary-cH2B peptide (5) and Ub(1-75)-MES (6).

In a typical small-scale reaction, purified peptide **5** (1 mg, 0.58 μmol) was dissolved in 70 μL of a buffer containing 300 mM NaP_i, 3 M Gn-HCl, and 50 mM TCEP, pH 7.8 at 4 °C, and incubated for 35 min at 25 °C to completely reduce the mixed disulfide (-S-S-^{t}Bu). To the solution of reduced **5** was then added thioester **6** (1 mg, 0.12 μmol) dissolved in 70 μL of a buffer consisting of 300 mM NaPi, 3 M Gn-HCl, and 100 mM MESNa, pH 7.8 at 4 °C. Upon mixing and cooling to 4 °C the pH of the resultant solution was found to be ~7.5, and the ligation was allowed to proceed with gentle shaking for 5 days. The extent of reaction was checked periodically by withdrawing 1 μL aliquots of the ligation mixture, dilution into 50 μL of 25% B and analysis by C18 analytical RP-HPLC employing a gradient of 0-73% B over 30 min. At the end of 5 days, the ligation product was reduced with TCEP and purified by C18 analytical RP-HPLC employing a gradient of 25-55% B, 30 min (R_t 15.5 min) to yield 0.5 mg of pure **7**. ESI-MS, **7** calculated m/z $(M+H)^+$ 10120 Da, found 10120 ± 2 Da.

Expressed protein ligation of auxiliary-cH2B peptide (5) and HA-SUMO(1-97)-MES (9).

Purified peptide **5** (1 mg, 0.58 μmol) was dissolved in 70 μL of a buffer containing 300 mM NaP_i, 3 M Gn-HCl, and 50 mM TCEP, pH 7.8 at 4 °C, and incubated for 35 min at 25 °C to completely reduce the mixed disulfide (-S-S-^{t}Bu). To the solution of reduced **5** was then added **9** (1.4 mg, 0.11 μmol) dissolved in 70 μL of a buffer consisting of 300 mM NaPi, 3 M Gn-HCl, and 100 mM MESNa, pH 7.8 at 4 °C. Ligation was allowed to proceed with gentle shaking for 7 days and the extent of reaction was analyzed by C18 analytical RP-HPLC employing a gradient of 0-73% B over 30 min. After 7 days, the ligation product was reduced with TCEP and purified by C18 analytical RP-HPLC employing a gradient of 34-47% B, 30 min (R_t 10.1 min) to yield 0.7 mg of pure **10** (Figure **S5**). ESI-MS, **10** calculated m/z $(M+H)^+$ 13900 Da, found 13901 ± 5 Da.

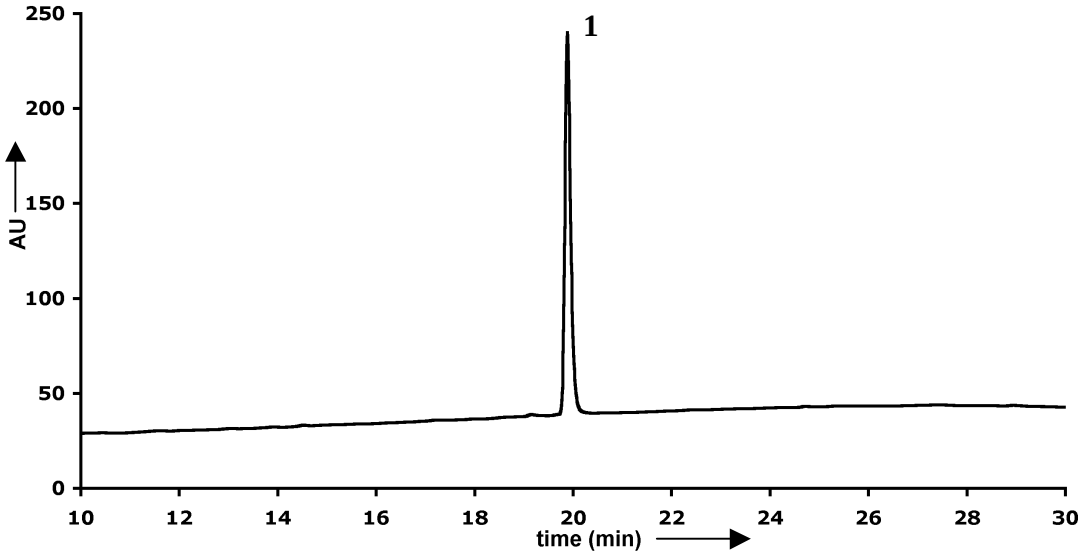
Photolysis of Ub(1-75)-auxiliary-cH2B (7) and HA-SUMO(1-97)-auxiliary-CH2B (10).

For large-scale photolysis, the purified and lyophilized ligation products were taken up in a photolysis buffer (50 mM Tris, 150 mM NaCl, 1 mM DTT, pH 7.5) at a final concentration of 80 μM and mixed by vortexing prior to photolysis. Alternatively, a C18 RP-HPLC fraction corresponding to the ligation product could also be employed in photolysis experiments with no observable changes in the product pattern. A 50-100 μL solution of **7** or **10** was briefly exposed to a He-Cd laser at 325 nm for 3-4 bursts of light of ~5 s duration with vortexing between exposures. The photolyzed ligation products Ub-cH2B (**8**) and HA-SUMO-cH2B (**11**) were analyzed by C18 analytical RP-HPLC for the degree of photolysis and purified away from the photolysis by-products (Figure **S6**).
ESI-MS, **8** calculated m/z $(M+H)^+$ 9793 Da, found 9793 ± 4 Da
ESI-MS, **11** calculated m/z $(M+H)^+$ 13573 Da, found 13573 ± 4 Da.

Ub-cH2B (8) assay with UCH-L3.

Purified **8** was dissolved in 200 μL of assay buffer (50 mM Tris, 150 mM NaCl, 1 mM DTT, pH 7.5) to a final concentration of ~100 μM and reacted with recombinant human ubiquitin C-terminal hydrolase L3 (UCH-L3, Boston Biochem, Boston, MA).[3] Typically, 4.5 μg of UCH-L3 (7 μL of 25 μM stock solution) were incubated in a buffer containing 50 mM Tris, 150 mM NaCl, 12 mM DTT, pH 8.0 for 15-20 min at 25 °C. To the reduced UCH-L3 was then added **8** in 78 μL of assay buffer containing 50 mM Tris, 150 mM NaCl and 1 mM DTT, pH 7.5 at 25 °C. The mixture was mixed by pipetting and incubated for 8 h at 37 °C. A similar control assay was also conducted, which included all the assay components except UCH-L3. The reactions were quenched after 8 h by the addition of 1 μL TFA and analyzed by C18 analytical RP-HPLC employing a gradient of 0-73%B, 30 min, in order to identify the hydrolysis products. ESI-MS, Ub(1-76) calculated $(M+H)^+$ 8565.86 Da, found 8564.03 ± 3.22 Da. The released cH2B peptide was identified by means of an LTQ ion trap mass spectrometer (ThermoFinnigan, Waltham, MA). ESI-MS, cH2B calculated $(M+2H)^{2+}$ 623.33 Da, found 623.30 Da. The correct sequence was further confirmed by MS-MS analysis, which generated 9 b- and 10 y-type ions.

(A)



(B)

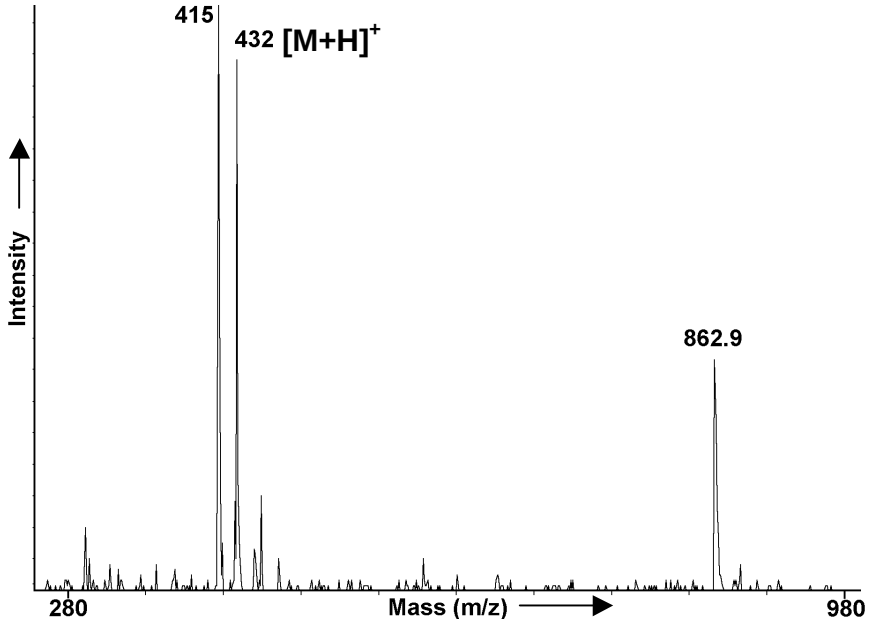
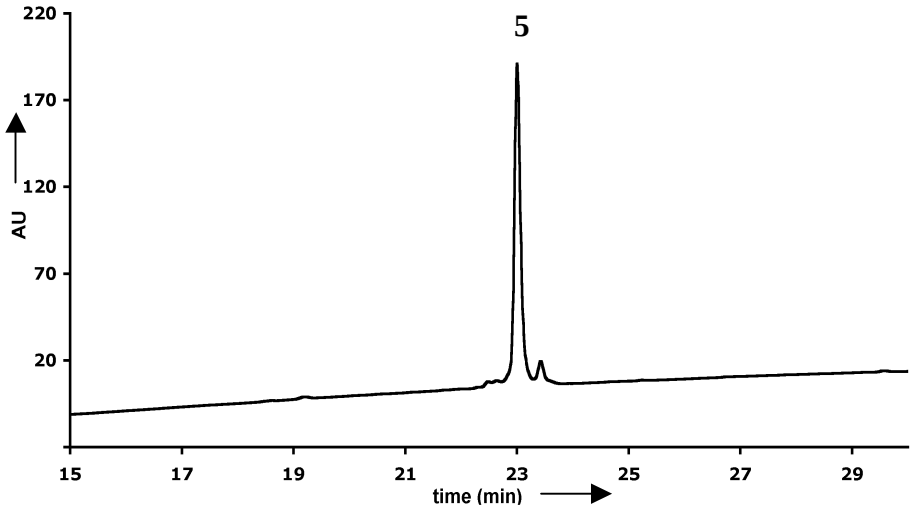


Figure S1. Synthesis and purification of the photo-auxiliary **1**. (A) C18 analytical RP-HPLC chromatogram of purified **1**, gradient was 0-73% B, 30 min. (B) ESI-MS of purified **1**. The ion intensities at 415 Da and 862.9 Da correspond to the photolyzed and dimeric forms of **1**, respectively, that are formed during the ESI process.

(A)



(B)

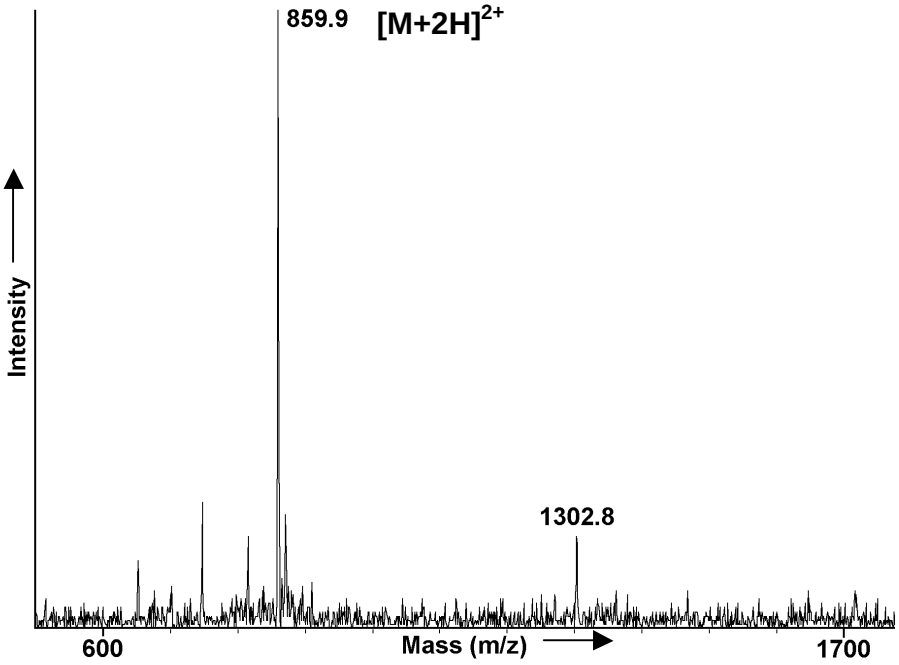
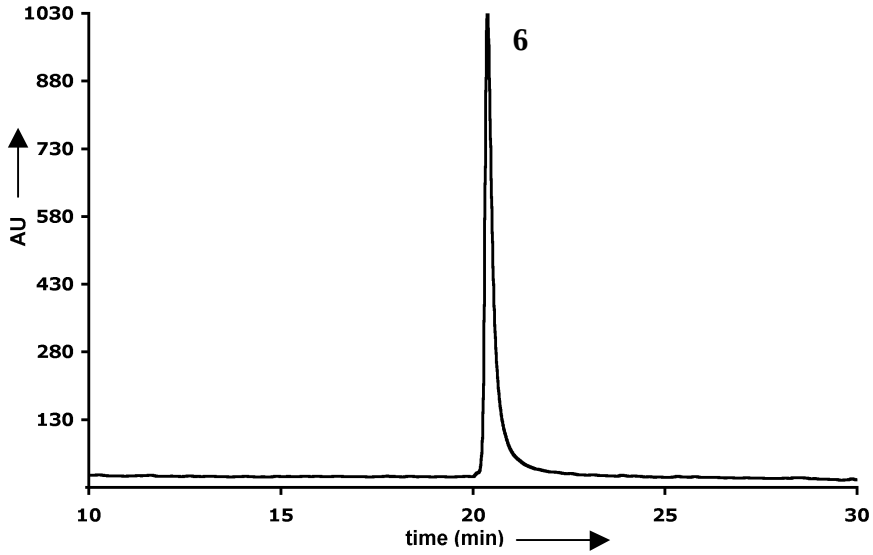


Figure S2. Synthesis and purification of auxiliary-cH2B (**5**). (A) C18 analytical RP-HPLC chromatogram of purified **5**, gradient was 0-73%B, 30 min. (B) ESI-MS of purified **5**. The doubly charged ion of **5** is the predominant species observed. The signal at 1302.8 Da corresponds to cH2B peptide from which the photo-auxiliary is cleaved during the ESI process.

(A)



(B)

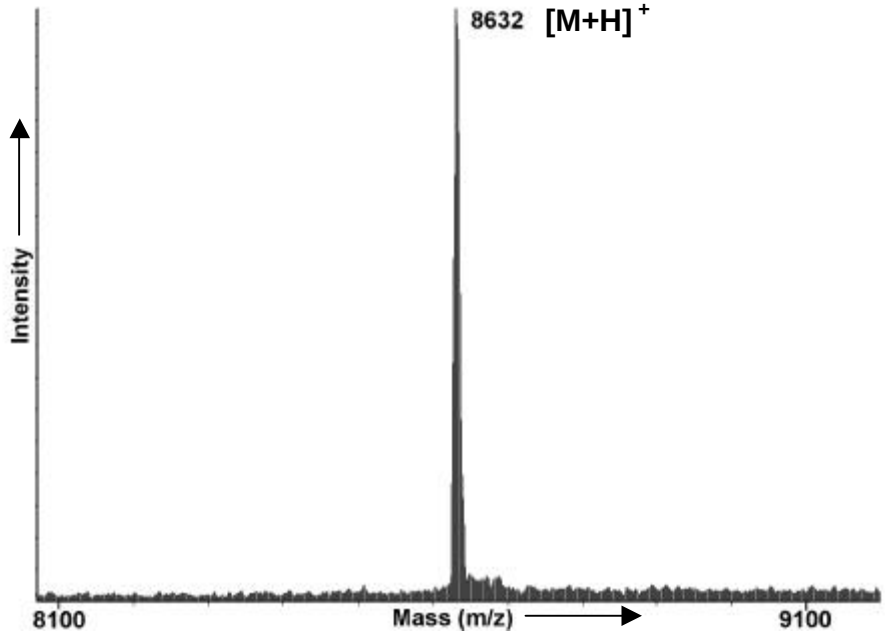
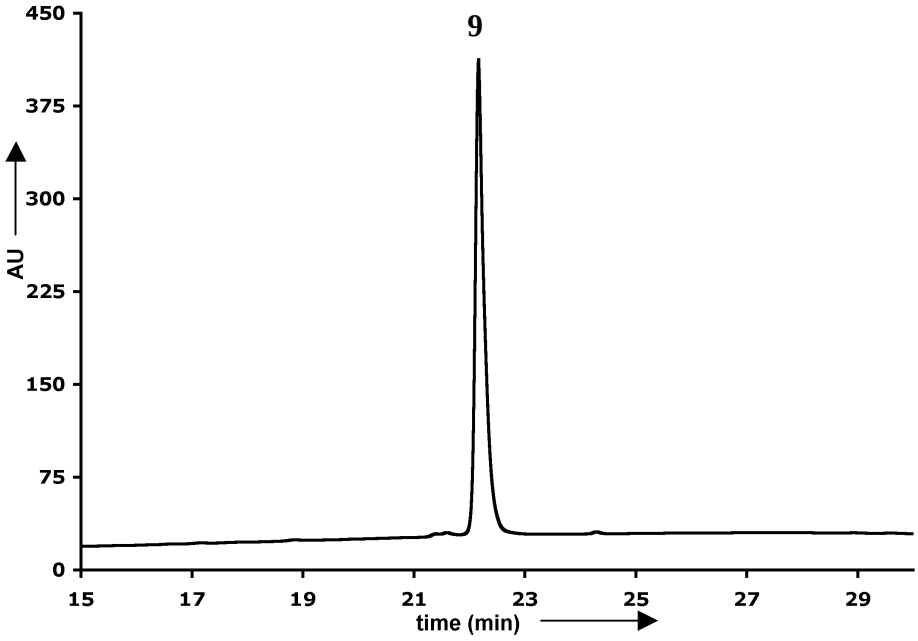


Figure S3. Purification of Ub(1-75)-α-thioester (**6**). (A) Analytical C18 RP-HPLC chromatogram of purified **6**, gradient was 0-73% B, 30min. (B) Corresponding deconvoluted ESI-MS of purified **6**.

(A)



(B)

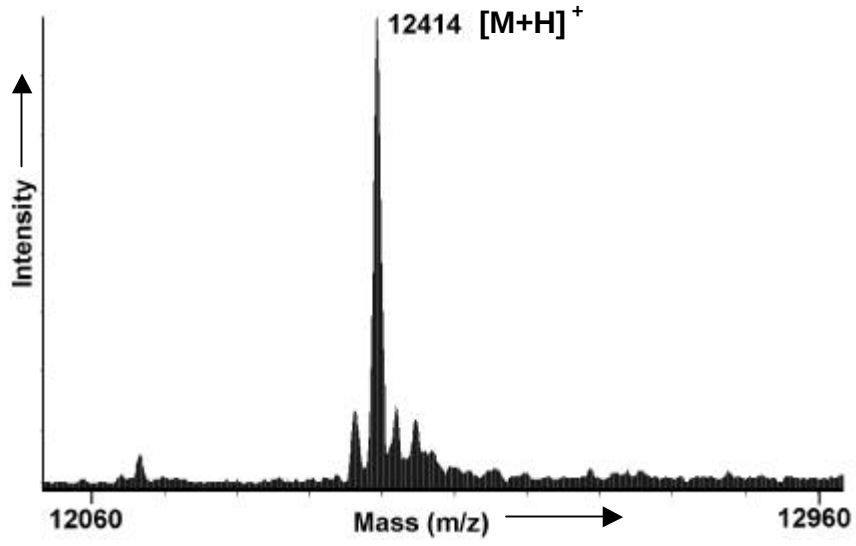


Figure S4. Purification of HA-SUMO(1-97)-α-thioester (**9**). (A) Analytical C18 RP-HPLC chromatogram of purified **9**, gradient was 0-73%B, 30 min. (B) Corresponding deconvoluted ESI-MS of purified **9**.

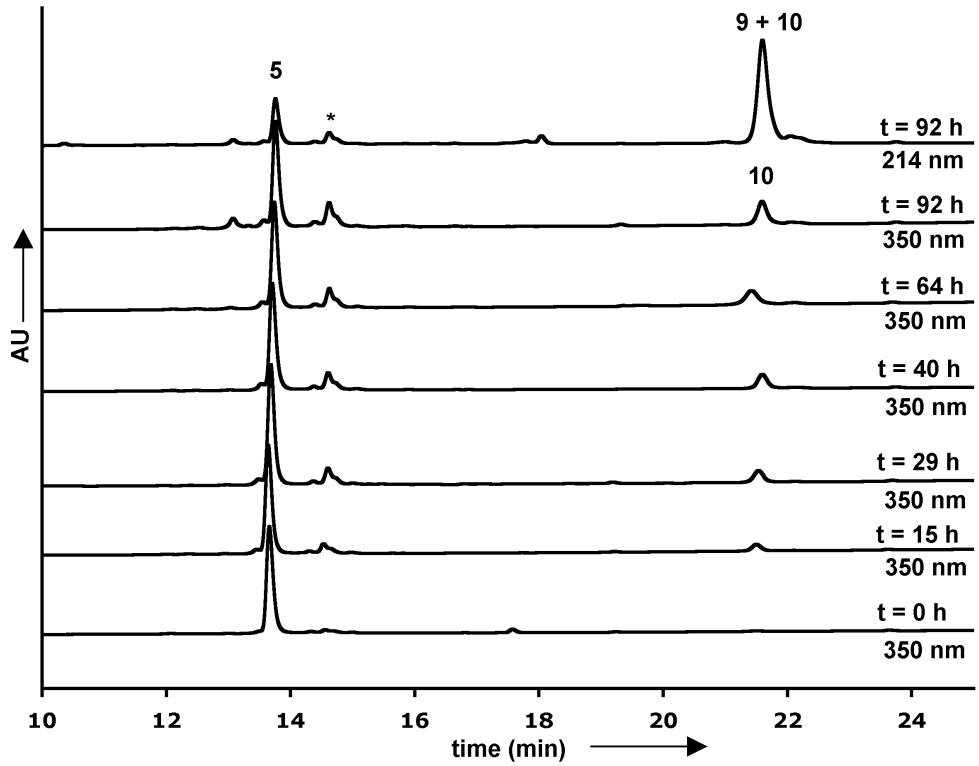


Figure S5. Ligation time course of HA-SUMO(1-97)-MES **9** with auxiliary-cH2B peptide **5**. Formation of the ligation product **10** was followed over 5 days by C18 analytical HPLC with a gradient of 0-73% B, 30 min. Due to the overlap of the thioester and ligation product peaks at 214 nm, the chromatogram at 350 nm is shown for all time-points wherein only **5** and the ligation product **10** (that bears the photo-auxiliary) are visible. The peak denoted by an asterisk corresponds to the unsymmetrical MES disulfide form of **5**, which accumulates over time.

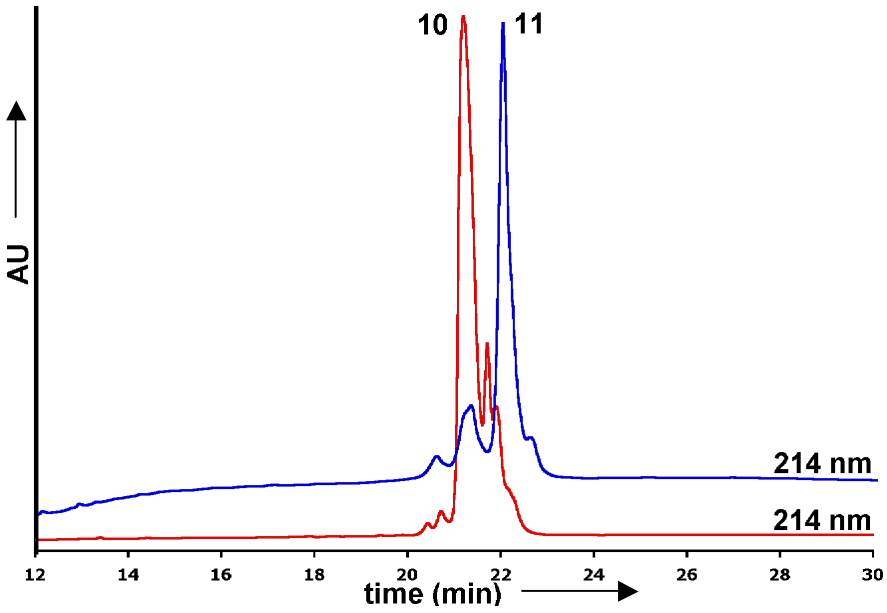


Figure S6. Purification and photolysis of the HA-SUMO(1-97)-auxiliary-cH2B ligation product **10**. In red is the analytical C18 RP-HPLC chromatogram of purified **10**, gradient was 0-73% B, 30 min. The chromatogram of the photolyzed product HA-SUMO-cH2B **11** under an identical elution gradient is shown in blue. The corresponding ESI-MS spectra are reported as Fig 4 in the main text.

References

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[2] E. Kaiser, R. L. Colescott, C. D. Bossinger, P. I. Cook, *Anal. Biochem.* **1970**, *34*, 595-598.

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