

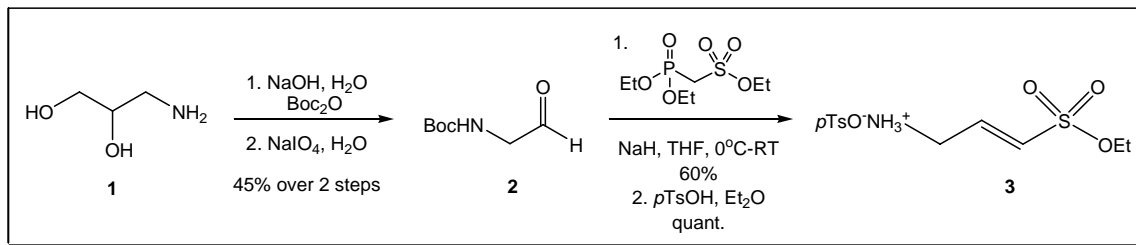
## Supporting Information and Methods

### Ubiquitin C-terminal electrophiles are activity-based probes for identification and mechanistic study of ubiquitin conjugating machinery

Kerry Routenberg Love, Renuka K. Pandya, Eric Spooner, and Hidde L. Ploegh

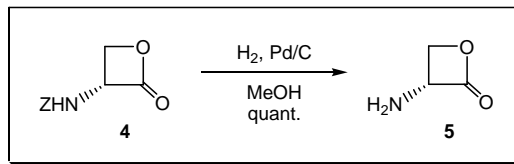
#### Synthesis and Characterization of Electrophilic Glycine Analogs

**General Methods.** All chemicals were reagent grade and used as supplied unless otherwise noted. HPLC-grade, low moisture organic solvents (Dri Solv), including *N,N'*-dimethylformamide (DMF), dichloromethane ( $\text{CH}_2\text{Cl}_2$ ), tetrahydrofuran (THF), methanol (MeOH), and diethyl ether ( $\text{Et}_2\text{O}$ ), were purchased from EMD Chemicals. Analytical thin-layer chromatography was performed on E. Merck silica gel 60  $\text{F}_{254}$  plates (0.25 mm). Compounds were visualized by dipping the plates in a cerium sulfate-ammonium molybdate solution or a ninhydrin solution followed by heating. Liquid flash column chromatography was performed using forced flow of the indicated solvent on EMD Chemicals silica (200-400 mesh).  $^1\text{H}$  NMR spectra were obtained using a 400 MHz spectrometer and are reported in parts per million ( $\delta$ ) relative to  $\text{CDCl}_3$  (7.27 ppm) or MeOD (4.87 ppm, 3.31 ppm).

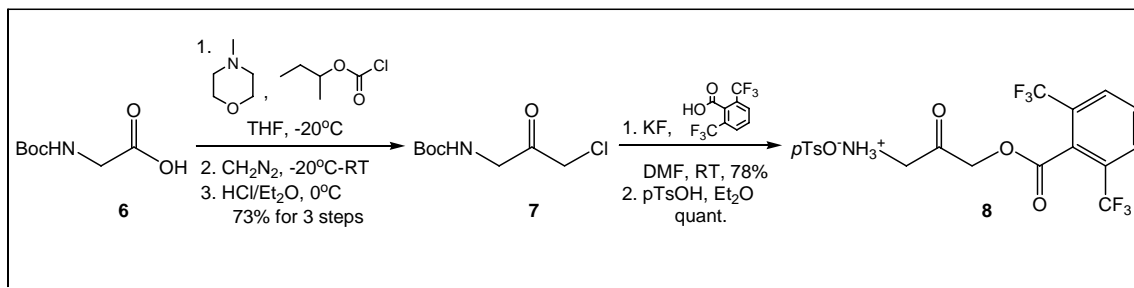


**4-amino-but-1-enyl ethoxysulfonate (3).** *N*-tert-butyloxycarbonyl (Boc) protected glycinal was prepared as previously reported[1] and was subjected to sodium metaperiodate-mediated oxidative cleavage[2] to obtain *N*-Boc-glycinal (2), which was used without further purification. A solution of sodium hydride (258 mg, 10.2 mmol) in THF (60 mL) was cooled to  $0^\circ\text{C}$  before the dropwise addition of triethyl  $\alpha$ -phosphonylmethanesulfonate (2.66 g, 10.2 mmol)[3], followed by a 5 mL rinse of the phosphonate-source vessel[4]. The reaction mixture was stirred for 20 min at  $0^\circ\text{C}$  before adding the above prepared *N*-Boc-glycinal (2) as a neat solid. The reaction mixture was allowed to warm to room temperature and was stirred for 6 h before quenching with water (50 mL) and extracting with ethyl acetate ( $3 \times 50$  mL). The combined organic extracts were dried over  $\text{Na}_2\text{SO}_4$ , filtered and concentrated. The resulting crude residue was purified using flash column chromatography by elution with 25% ethyl acetate/hexanes, yielding 0.8 g (60%) pure *N*-Boc-4-amino but-1-enyl ethoxysulfonate.  $^1\text{H}$  NMR: 6.78 (dt, 1H), 6.34 (d, 1H), 4.82 (br s, 1H), 4.17 (q, 2H), 3.99 (br s, 2H), 1.45 (s, 9H), 1.39 (t, 3H). Removal of the Boc group was accomplished using three equivalents dry *p*-toluenesulfonic acid as described[5] to yield 3 as the sulfonic acid salt

of the deprotected amine. ESI-MS: Calculated;  $[M + H]^+ = 166.05$ . Observed;  $[M + H]^+ = 166.05$ .



**Serine-β-lactone (5).** N-(benzyloxycarbonyl[Z])-D-serine-β-lactone (**4**) was prepared as previously reported[6]. Removal of the Z group was accomplished in quantitative yield by hydrogenation over Pd/C in methanol.  $^1H$  NMR: 4.23 (m, 1H), 4.12 (m, 2H), 3.54 (m, 1H), 1.41 (appd, 1H). ESI-MS: Calculated;  $[2M + H]^+ = 177.09$ . Observed;  $[2M + H]^+ = 177.09$ .



**2,6-trifluoromethylbenzyloxy glycine methyl ketone (8).** Boc-protected glycine (**6**) was converted to the amino acid diazomethyl ketone as previously reported[7] via addition of a cold solution of diazomethane to a mixed anhydride formed by treatment of the protected amino acid with isobutyl chloroformate in the presence of *N*-methylmorpholine. The diazomethyl ketone was easily converted to the chloromethyl ketone (**7**) by addition of a solution of HCl in Et<sub>2</sub>O[8]. *N*-Boc-glycine chloromethyl ketone **7** (430 mg, 2.07 mmol) was treated with potassium fluoride (361 mg, 6.21 mmol) in DMF (20 mL), followed by 2,6-trifluorobenzoic acid (587 mg, 2.28 mmol) overnight at room temperature[9]. After 12 h the reaction was diluted with Et<sub>2</sub>O and ethyl acetate (50 mL each) and washed with water, aqueous NaHCO<sub>3</sub> (sat.) and brine. The organic layer was dried over Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated to yield 0.69 g (78%) of compound **8**, which was used without further purification.  $^1H$  NMR: 7.95 (appd, 2H), 7.76 (appt, 1H), 5.22 (br s, 1H), 4.90 (s, 1H), 4.13 (m, 2H), 1.40 (s, 9H). Removal of the Boc group was accomplished using three equivalents dry *p*-toluenesulfonic acid as described[5] to yield **8** as the sulfonic acid salt of the deprotected amine. ESI-MS: Calculated;  $[M + H]^+ = 330.06$ . Observed;  $[M + H]^+ = 330.06$ .

### Synthesis and Characterization of HAUb-electrophilic Probes

HAUb-electrophilic probes were made using the electrophilic glycine analogs synthesized above as described in the Experimental Procedures. The FPLC purified HAUb-electrophilic probes were characterized by ESI-LC/MS and multi-charged species were observed for each probe. Probe yields were estimated using traces obtained during FPLC purification. HAUbOEtVS (quant. yield) ESI-MS: Calculated;  $[M + 11H]^{11+} =$

937. Observed;  $[M + 11H]^{11+} = 936.7266$ . HAUbLac (50% yield) ESI-MS: Calculated;  $[M + 11H]^{11+} = 931$ . Observed;  $[M + 11H]^{11+} = 931.4602$ . HAUbTF<sub>3</sub>BOK (80% yield) ESI-MS: Calculated;  $[M + 11H]^{11+} = 952$ . Observed;  $[M + 11H]^{11+} = 952.0001$ .

#### **EL-4 Cell Lysate Labeling and Blocking of Labeling with NEM (N-ethylmaleimide)**

30 µg of protein extract (see Methods section for cell culture and lysate harvesting conditions) treated with NEM (10 mM) for 30 min at room temperature. Following this treatment, 0.2 µg of HAUb-electrophilic probe (2.0 µM in each sample) was added and the reactions were incubated for 5 h at room temperature. Control reactions were run for each probe without prior addition of NEM to observe the extent of labeling in 5 h. Reactions were quenched by addition of reducing sample buffer, boiled 10 min and separated by SDS-PAGE (10%). Results were analyzed by immunoblotting with an anti-HA antibody (3F10, Roche).

#### **EL-4 Cell Lysate labeling and Detection of RING Ligase UBAC1 Modification by Gel Shift.**

30 µg EL-4 lysate was incubated with indicated quantities of probe (concentrations are 2.3 µM, 4.6 µM, 9.2 µM, and 18.4 µM) in buffer containing 50mM Tris pH 8.0, 150mM NaCl for 4 hr at room temperature (24°C). Reactions were quenched by addition of reducing sample buffer, resolved by SDS-PAGE analyzed by immunoblot using either a UBAC1 antibody (Novus Biologicals) or a UCH37 antibody (polyclonal rabbit) for comparison.

#### **Plasmid Construction**

The C-terminal residues of ARF-BP1 (amino acids 4012-4374) were subcloned into vector pet28a for expression and purification as an N-terminal His<sub>6</sub> fusion protein using forward primer 5'-ccgcatatgctccggaagaagacatgg and reverse primer 5'-ccggaattcttagccagcccaaagcc. Single, double, and triple cysteine-to-alanine ARF-BP1 HECT domain mutants at residues C4099, C4341, C4367, were generated using site-directed mutagenesis (Stratagene). A truncated version of ARF-BP1 (ARF-BP1 Δ4) (residues 4012-4370) was cloned into pet28a for Ub-thioester assays using the forward primer listed above and the reverse primer 5'-cgcgatccttagccttcagagcactcctg.

The C-terminal residues of Trip12 (amino acids 1629-2040, KIAA0045, Kazusa DNA Research Institute) were subcloned with an N-terminal His<sub>6</sub> tag into the vector pGEX-6P-1 (GE Healthcare) for expression and purification as an N-terminal GST fusion protein using forward primer 5'-cgcgatccggacatcatcatcatcacagcagcggcagagttgcacctagattgg and reverse primer 5'-cgcgatcactcaggaaagatggaacgactg.

The N-terminal residues of Mcl-1 (amino acids 1-327) were cloned with an N-terminal PKA (protein kinase A) recognition sequence into the vector pet16b for expression and purification as an N-terminal His<sub>10</sub> fusion protein using forward primer 5'-ccgcatatggactacaaggacgacgacgacaaggcaggacgtcgatctgttgatccttggcctcaaaagaaacgcgg and reverse primer 5'-cgcgatccctagccaccttctaggtctctac.

Ubiquitin was cloned with an N-terminal PKA (protein kinase A) recognition sequence into the vector pet28a for expression and purification as an N-terminal His<sub>6</sub> fusion protein using forward primer 5'-ccgcatatgcgtcgtgcattctgttgatccatgcagatcttcgtcaagacg and reverse primer 5'-cgcgatcctcatcccccacgcagtc.

#### **Protein Expression and Purification of ARF-BP1 HECT domain and PKA-Mcl-1**

ARF-BP1 HECT domain and FLAG-Mcl-1 containing plasmids were transformed into Rosetta competent cells (DE3(PLysS), Novagen) and cells were plated on LB-agar supplemented with 50 µg/mL kanamycin (ARF-BP1) or 100 µg/mL ampicillin (Mcl-1) and 20 µg/mL chloramphenicol. A single colony for each protein was used to inoculate a 1.5 L liquid culture (LB, Miller) and cultures were grown to late log (OD<sub>600</sub> > 0.8) at 37°C. Cultures were induced with 0.5 mM IPTG at 22°C overnight. Cells were pelleted and washed once with PBS before resuspending in 25 mL lysis buffer (50 mM Tris [pH 8.0], 300 mM NaCl, 10 mM imidazole). DNase was added to a final concentration of 10 µg/mL and the cells were lysed under pressure (1500 psi) using a French press. Cell debris was pelleted and the supernatant was purified using Ni-NTA beads (Qiagen) for His-tag-based purification. Eluted proteins were further purified by gel filtration (Superdex 75 PC 3.2/30, GE Healthcare) using a buffer containing 50 mM Tris (pH 7.5), 150 mM NaCl, 2 mM EDTA, 5 mM MgCl<sub>2</sub>, 2 mM DTT, and 5% glycerol.

#### **Protein Expression and Purification of Trip12 HECT domain**

Trip 12 HECT domain-containing plasmid was transformed into Rosetta competent cells (DE3(PLysS), Novagen) and cells were plated on LB-agar supplemented with 100 µg/mL ampicillin and 20 µg/mL chloramphenicol. A single colony for each protein was used to inoculate a 1.5 L liquid culture (LB, Miller) and cultures were grown to late log (OD<sub>600</sub> > 0.8) at 37°C. Cultures were induced with 0.5 mM IPTG at 22°C overnight. Cells were pelleted and washed once with PBS before resuspending in 25 mL PBS supplemented with DNase (final concentration of 10 µg/mL) and 1 mM MgCl<sub>2</sub>. Cells were lysed under pressure (1500 psi) using a French press. Cell debris was pelleted and the supernatant was purified using Glutathione Sepharose 4B beads (GE Healthcare) for GST-tag-based purification. Eluted proteins were used without further purification.

#### **Protein Expression and Purification of PKA-Ub**

PKA-Ub-containing plasmid was transformed into Rosetta competent cells (DE3(PLysS), Novagen) and cells were plated on LB-agar supplemented with 50 µg/mL kanamycin and 20 µg/mL chloramphenicol. A single colony was used to inoculate 6L liquid culture (LB, Miller) and cultures were grown to late log (OD<sub>600</sub> > 0.8) at 37°C. Cultures were induced with 0.5 mM IPTG at 30°C for 3.5 hr. Cells were pelleted and washed once with PBS before resuspending in lysis buffer (50 mM Tris pH 8.0, 500 mM NaCl, 20 mM imidazole, 10 µg/mL DNase). Cells were lysed under pressure (1500 psi) using a French press. Cell debris was pelleted and the supernatant purified using Ni-Sepharose (High Performance, GE Healthcare) for His-tag-based purification. Eluted proteins were further purified by gel filtration (Superdex 75 PC 3.2/30, GE Healthcare) using a buffer containing 50 mM Tris pH 7.5, 150 mM NaCl, and 5% glycerol.

### **Synthesis of [<sup>32</sup>P]-labeled proteins**

Recombinant proteins (1 mg Ub or 120 ug Mcl-1) bound to Ni-sepharose (High Performance, GE Healthcare) were washed once in HMK buffer (20mM Tris pH 7.5, 100mM NaCl, 12mM MgCl<sub>2</sub>) and incubated with the catalytic subunit of cAMP-dependent Protein Kinase (PKA, New England Biolabs; 50,000 U for Ub or 25,000 U for Mcl-1), and a 10-fold molar excess of ATP in a ratio of 1:100 (Ub) or 1:10 (Mcl-1) γ-[<sup>32</sup>P]-ATP (6000 Ci/mmol, 10mCi/mL, PerkinElmer) to unlabeled ATP, in HMK buffer containing 1 mM DTT in a final volume of either 200 ul (Ub) or 100 ul (Mcl-1) for 3 hours at 25°C. The phosphorylation reaction was terminated by incubation of proteins with HMK stop buffer (10mM sodium phosphate pH 8.0, 10mM sodium pyrophosphate, 10mM EDTA, 1mg/ml bovine serum albumin) for 5 min at 25°C. Proteins were washed 5 times with NET buffer and eluted in 50mM Tris pH 8.0, 250mM NaCl, 250mM imidazole.

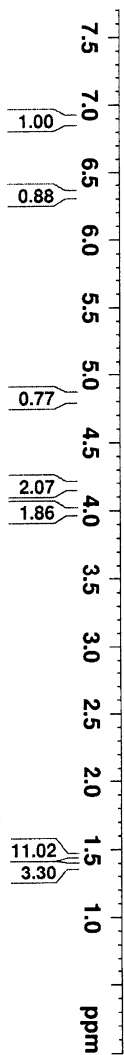
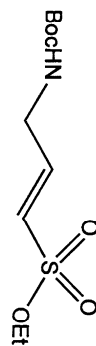
### **Autoubiquitination Assay with GST-Trip12 HECT Domain**

Recombinantly expressed and purified GST-Trip12 HECT domain (10 µg) (see above for plasmid construction, protein expression and purification conditions) was incubated with 100 ng human E1 (Ube1, Boston Biochem), 1 µg UbcH7, and 10 µg Ub with an ATP regenerating system (50 mM Tris [pH 7.6], 5 mM MgCl<sub>2</sub>, 5 mM ATP, 10 mM creatine phosphate, 3.5 U/mL creatine kinase) for 10 min at room temperature. After terminating the reactions with reducing SDS-PAGE sample buffer and boiling 10 min, reaction mixtures were separated by SDS-PAGE (8%) and analyzed by immunoblotting using an anti-Ub antibody (Sigma). Control reactions were run without either E1 or E2.

### **References**

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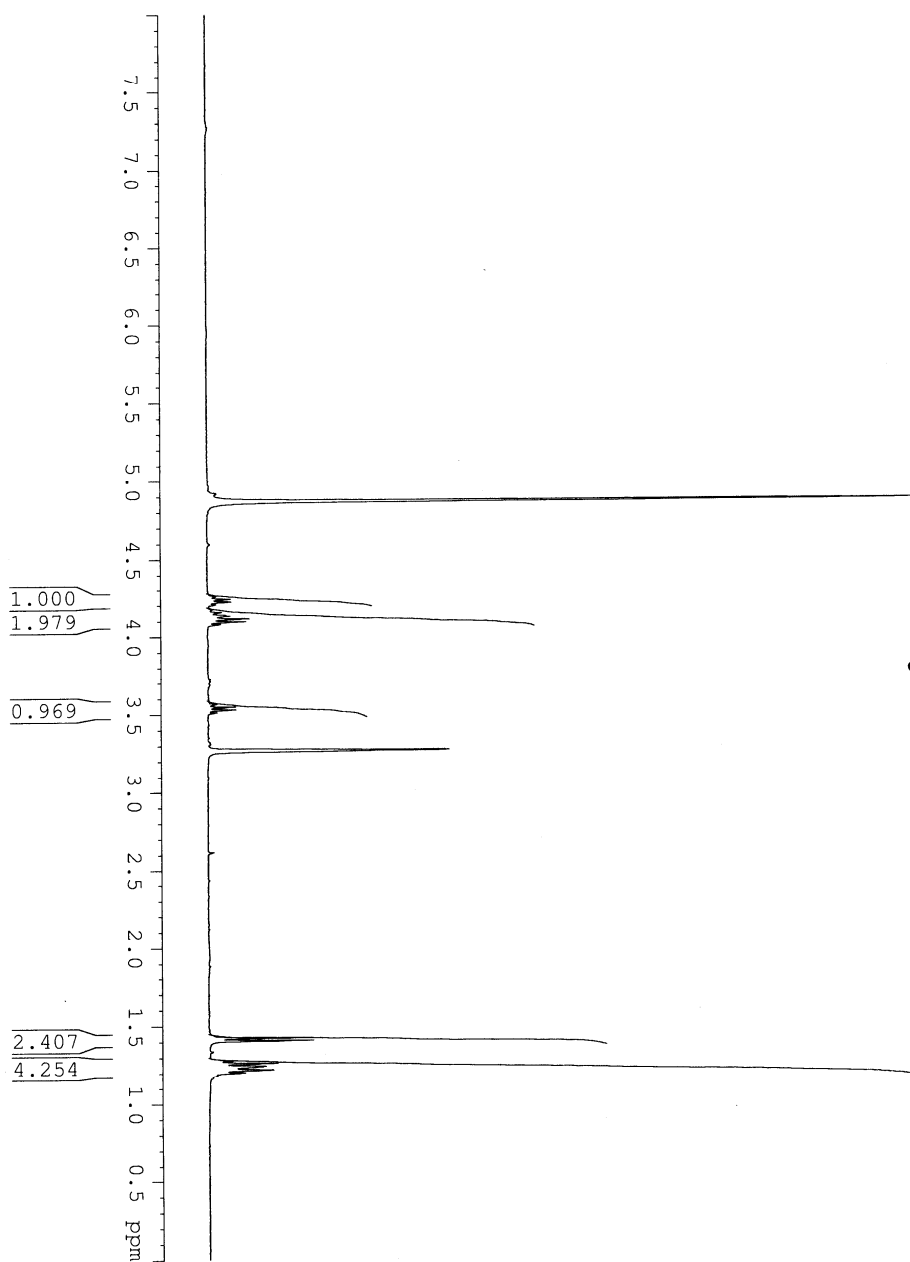
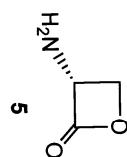
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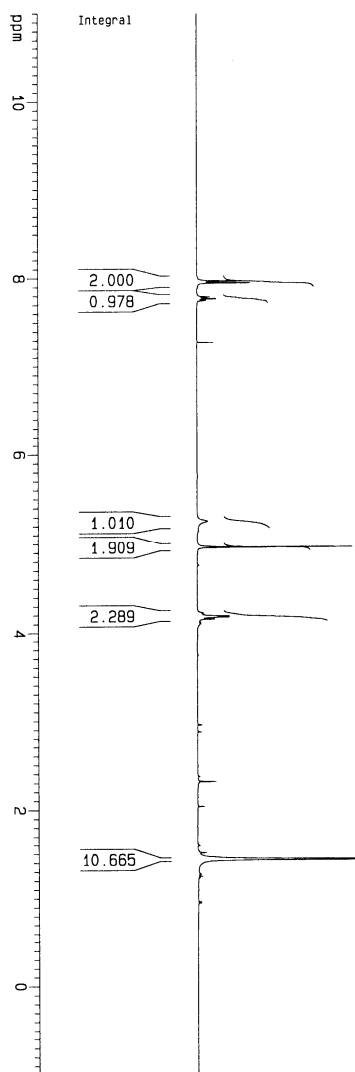
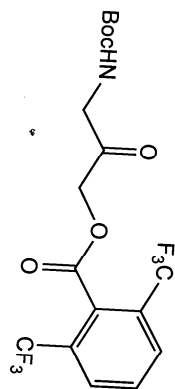
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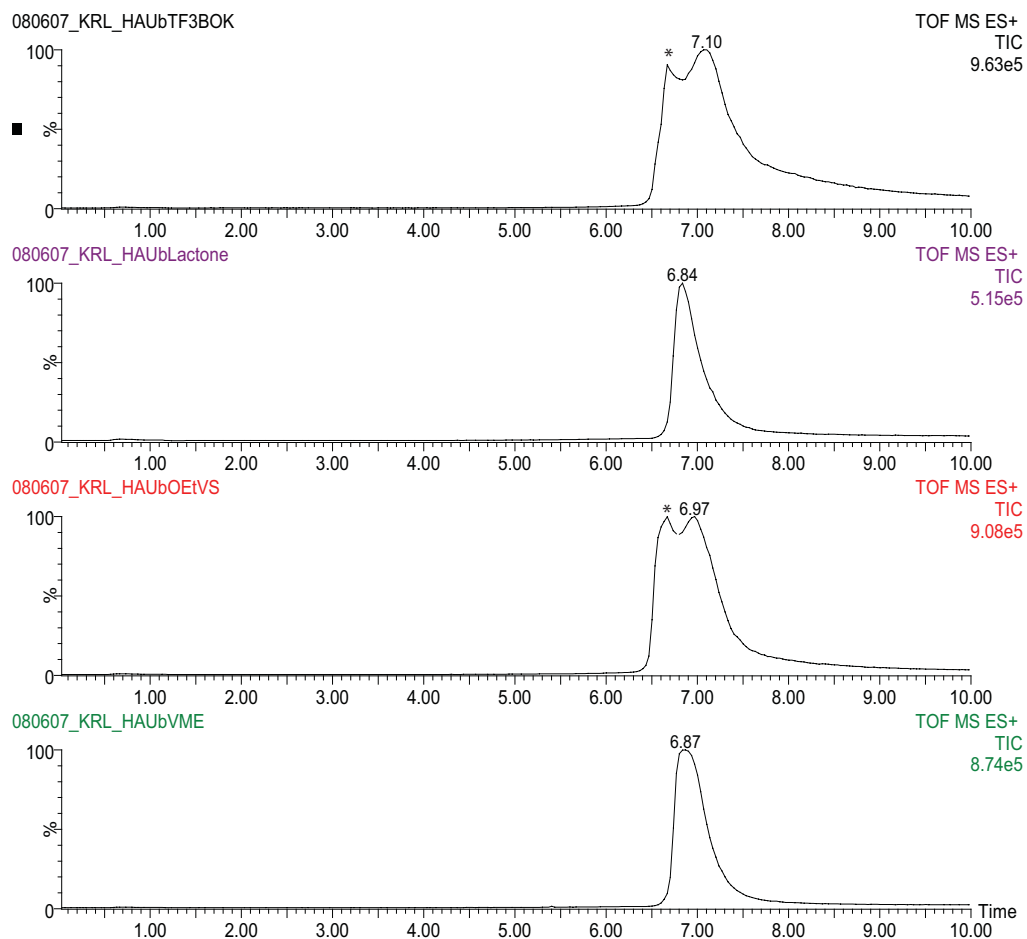
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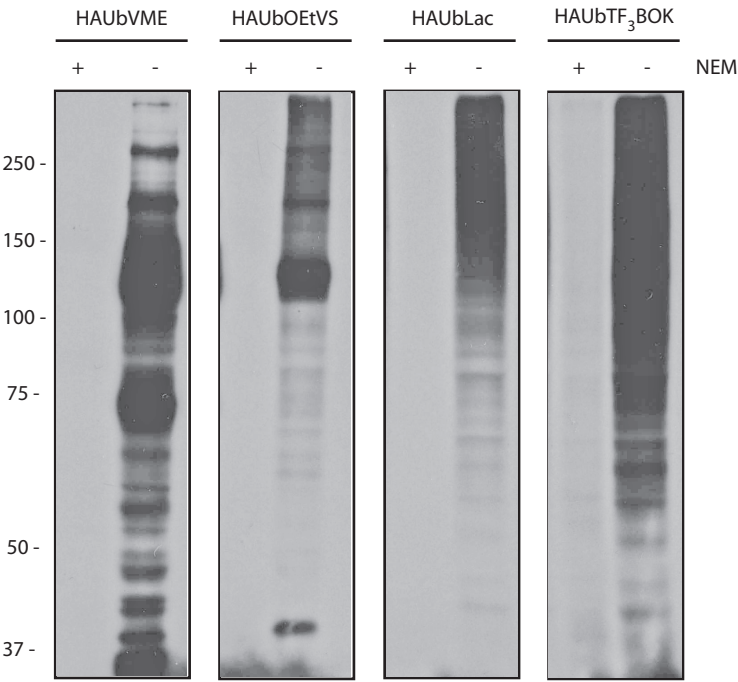
\* This peak corresponds to the loss of the electrophilic warhead from the peptide during LC/MS analysis (Calculated:  $[M + 11H]^{11+} = 921$ ; Observed:  $[M + 11H]^{11+} = 921.6114$ ).

**Supplementary Figure 1.** Labeling of EL-4 cell lysate with Ub electrophilic probes is efficiently blocked by preincubation with NEM (10 mM).

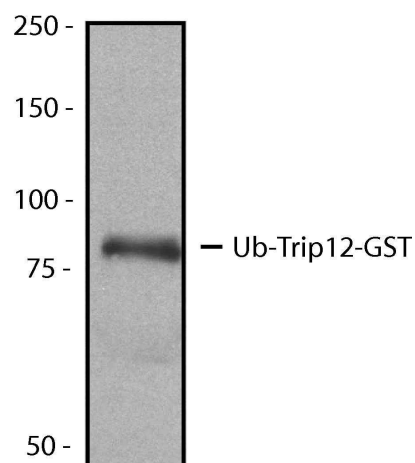
**Supplementary Figure 2.** Autoubiquitination activity of recombinant HECT domain of GST-Trip12. Ubiquitinated Trip 12 HECT domain was resolved by SDS-PAGE (8%) and visualized by immunoblotting with an anti-Ub antibody.

**Supplementary Figure 3.** (a) RING ligase UBAC1, present in EL-4 cell lysate, is not directly modified by HAUbVME, suggesting that RING E3s are retrieved in immunoprecipitations of probe-labeled lysate as part of multi-subunit complexes where 1 or more members binds probe directly. (b) UCH37 (UCH-L5), by comparison, is efficiently and quantitatively labeled when lysate containing this DUB is treated with HAUbVME as seen by a ~10 kD increase in its apparent molecular weight.

Supplementary Figure 1

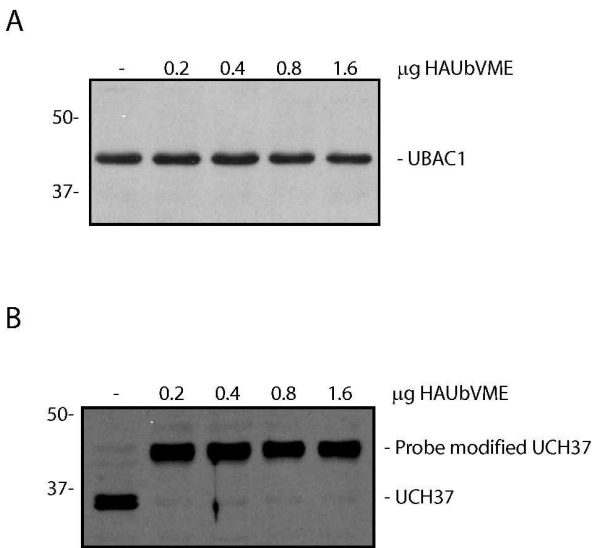


## Supplementary Figure 2



Supplementary Figure 2. Autoubiquitination activity of recombinant HECT domain of GST-Trip12. Ubiquitinated Trip 12 HECT domain was resolved by SDS-PAGE (8%) and visualized by immunoblotting with an anti-Ub antibody.  
95x84mm (600 x 600 DPI)

Supplementary Figure 3



Supplementary Figure 3. (a) RING ligase UBAC1, present in EL-4 cell lysate, is not directly modified by HAUbVME, suggesting that RING E3s are retrieved in immunoprecipitations of probe-labeled lysate as part of multi-subunit complexes where 1 or more members binds probe directly. (b) UCH37 (UCH-L5), by comparison, is efficiently and quantitatively labeled when lysate containing this DUB is treated with HAUbVME as seen by a ~10 kD increase in its apparent molecular weight. 123x164mm (600 x 600 DPI)