ARTICLE

ACS chemical biology

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[†,*]

[†]Whitehead Institute for Biomedical Research, 9 Cambridge Center, Cambridge, Massachusetts 02143. [‡]These authors contributed equally to this work. [§]Current address: Department of Chemical Engineering, Massachusetts Institute of Technology, 77 Massachusetts Ave., Cambridge, MA 02139.

ABSTRACT Protein modification by ubiquitin (Ub) and ubiquitin-like modifiers (Ubl) requires the action of activating (E1), conjugating (E2), and ligating (E3) enzymes and is a key step in the specific destruction of proteins. Deubiquitinating enzymes (DUBs) deconjugate substrates modified with Ub/Ubl's and recycle Ub inside the cell. Genome mining based on sequence homology to proteins with known function has assigned many enzymes to this pathway without confirmation of either conjugating or DUB activity. Function-dependent methodologies are still the most useful for rapid identification or assessment of biological activity of expressed proteins from cells. Activity-based protein profiling uses chemical probes that are active-site-directed for the classification of protein activities in complex mixtures. Here we show that the design and use of an expanded set of Ub-based electrophilic probes allowed us to recover and identify members of each enzyme class in the ubiquitin−proteasome system, including E3 ligases and DUBs with previously unverified activity. We show that epitope-tagged Ub-electrophilic probes can be used as activity-based probes for E3 ligase identification by *in vitro* labeling and activity studies of purified enzymes identified from complex mixtures in cell lysate. Furthermore, the reactivity of our probe with the HECT domain of the E3 Ub ligase ARF-BP1 suggests that multiple cysteines may be in the vicinity of the E2-binding site and are capable of the transfer of Ub to self or to a substrate protein.

The addition of ubiquitin (Ub), a 76-amino-acid polypeptide, or a ubiquitin-like modifier (Ubl) to proteins *via* a primary amino group serves to modulate protein function in a variety of ways. Mono- and multiubiquitination play a critical role in transcriptional activation (*1*), as well as receptor internalization and trafficking (*2*–*4*), while polyubiquitination typically leads to protein degradation by the proteasome and is therefore important for regulation of many cellular functions (*5*). Dysregulation of Ub/Ubl substrate modification has been implicated in a growing number of human diseases, including cancer (*6, 7*) and neurodegenerative disorders (*8*–*11*).

Ub/Ubl's are installed on substrate proteins in a complex cascade involving Ub/Ubl-activating enzymes (E1 enzymes), Ub/Ubl-conjugating enzymes (E2 enzymes), and Ub/Ubl-ligating enzymes (E3 enzymes) (*12, 13*). E1 enzymes activate the C-terminus of Ub/Ubl for nucleophilic attack *via* ATP-dependent thioester formation with an active site cysteine. This activated Ub/Ubl intermediate is then transferred from E1 to E2 as a thioester and is finally installed on a recipient lysine by an E3 ligase, which contains a substrate binding region and ultimately confers specificity. The action of the ligation machinery is balanced by the activity of deubiquitinating enzymes (DUBs) and Ubl-specific proteases (ULPs)—proteases that remove Ub/Ubl from modified substrates, thereby rescuing proteins from proteasomal degradation (*14, 15*). DUBs/ULPs themselves may be key players in disease pathology, as they are known regulators of the cell cycle machinery and can act as ei-

*Corresponding author, ploegh@wi.mit.edu.

Received for review October 17, 2008 and accepted March 3, 2009.

Published online March 3, 2009

10.1021/cb9000348 CCC: $40.75

ther oncoproteins (*16*) or tumor suppressors (*17, 18*). The identification of members of this pathway is important for understanding substrate specificity, for regulation of the Ub−proteasome system, and ultimately for the development of specific inhibitors for possible use in the treatment of disease (*19, 20*).

Activity-based protein profiling (ABPP) has proved an essential complement to *in silico* efforts for the identification of DUBs/ULPs (*14*). Novel members of this class of proteases were previously identified by Ub-based active-site-directed probes (*21*). The recombinant expression of an epitope-tagged Ub fusion protein allowed the installation of electrophilic groups at the C-terminus *via* intein-based chemical ligation. Incubation of these first generation probes with cell lysate led to the isolation and identification of 23 known DUBs by MS/MS analysis (*21*). Additionally, an OTU domain-containing protein was isolated on the basis of probe reactivity and was shown to have DUB-like activity *in vitro*. An ABPP-based approach was also used to identify DUBs and ULPs encoded by both viral (*22, 23*) and bacterial genomes (*24, 25*), as well as eukaryotic pathogens such as *Plasmodium falciparum* (*26*) and *Toxoplasma gondii* (*27*). This strategy proved essential for the identification of the $M48^{USP}$, a DUB within the N-terminal 500 residues of the large tegument protein of the herpes simplex virus 1 (HSV1), which bears no homology to any known host DUBs (*28*). UbVME was confirmed to be a mechanism-based suicide inhibitor as it was crystallized covalently bound to the active site cysteine of UCH-L3 (*29*) and M48 (*28*).

Since earlier campaigns using the C-terminal electrophilic probes identified no more than half of the ~100 proposed mammalian DUBs (*21, 30, 31*), we postulated that incorporation of "warheads" with greater electrophilicity should retrieve a larger number of DUBs. Here we report the synthesis and reactivity of an expanded set of Ub-based chemical probes. Enzymes modified by the probes were isolated from whole cell lysate and identified by tandem mass spectrometry. Although several new DUBs were recovered, including two members of the Josephin-domain subfamily (JOS1 and JOS2) (*32*), we were surprised to recover numerous members of the Ub/Ubl conjugating machinery including a gene product containing a HECT (homologous to E6-AP carboxy terminus) domain but with no reported ligase activity. Previously, only recombinantly expressed and purified E1, E2, or E3 enzymes had shown any reactivity with electrophilic Ub derivatives (*21, 33*). One E3 ligase we identified, ARF-BP1, was particularly intriguing because of recent interest in one of its target proteins, Mcl-1, as a potential tumor marker (*34*). Further characterization of the binding interaction between hemagluttinin (HA)-tagged UbVME and the HECT domain of ARF-BP1 revealed that the probe modified cysteines present in this domain. Mutagenesis of probe-labeled cysteines indicates that several residues in the HECT domain may participate in the transfer of Ub to self or substrate proteins with, as expected, a dominant role in the reaction for the catalytic cysteine.

RESULTS AND DISCUSSION

Synthesis and Characterization of Second Generation HAUb-Electrophilic Probes. Known Ub-based active-site-directed probes were synthesized as previously reported (Figure 1, panel a) (*21*). Three new glycine-based electrophiles were synthesized (see Supporting Information for schemes of chemical syntheses and compound characterization) and installed using an intein-based chemical ligation on a recombinantly expressed HAUb lacking the C-terminal glycine (G76). The product of each ligation reaction was purified using cation-exchange chromatography and characterized by mass spectrometry (LC-ESI-MS; see Supporting Information). These second generation glycine derivatives, including a vinylethoxysulfone (OEtVS), a β-lactone (Lac), and a 2,6-trifluoromethylbenzyloxymethylketone (TF_3BOK), were chosen for increased electrophilicity as compared to compounds synthesized earlier (*21*) and the likelihood to retain specificity for cysteine over serine proteases in reactions with complex mixtures of proteins (*35*). All three HAUb probes should react at the position corresponding to the C-terminal carbonyl group of the G76, as this is the position of conjugation between Ub and peptide substrates. HAUbVME, which is the most reactive first generation probe in retrieval of diverse DUBs, was used as a comparison for probe reactivity.

Profiling and Identification of Enzymes Modified by Newly Synthesized HAUb-Electrophilic Probes in EL-4 and HMLE Cell Lysate. To identify potentially uncharacterized DUBs using an ABPP approach, our new set of probes was used to label whole-cell lysate. The EL-4 mouse thymoma cell line was initially chosen (*21*) because it is known to express a diverse set of DUBs/ULPs (*36*) and was selected for sake of comparison with pre-

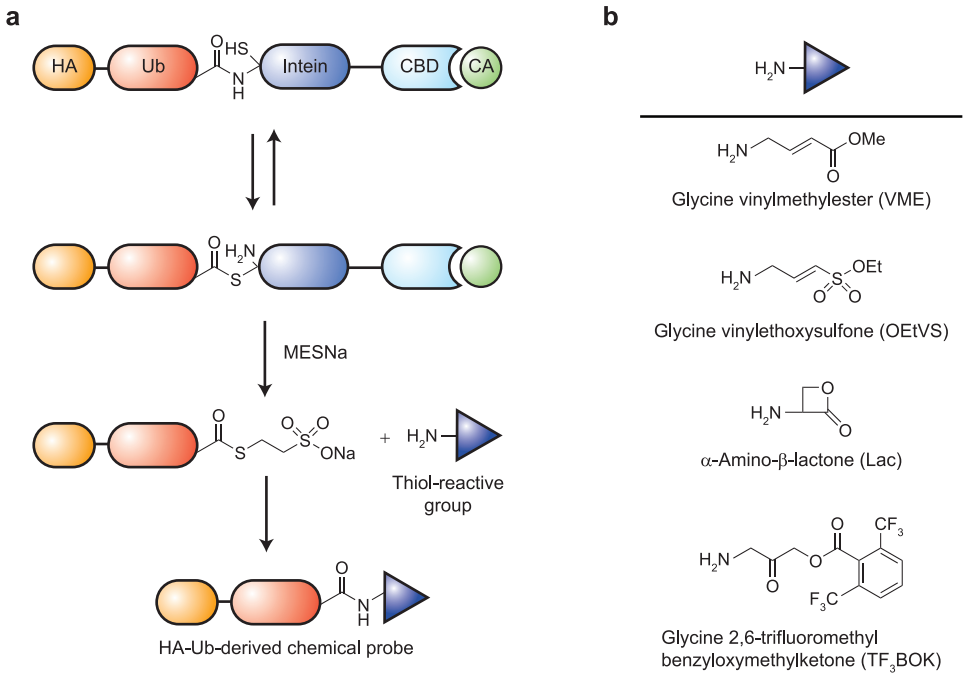


Figure 1. ABPP approach for identifying DUBs, Ub/Ubl activating enzymes, conjugating enzymes, and ligases. a) Synthesis of HAUb-derived probes using an intein-based chemical ligation. Recombinant HA-tagged Ub was expressed as a C-terminal fusion with an intein-chitin binding domain (CBD) for purification and introduction of reactive groups. Addition of β-mercaptoethane sulfonic acid (MESNa) to the chitin agarose (CA) with bound HAUb fusion protein results in elution of a species containing a thioester capable of chemical ligation. b) A table of new glycine-based electrophiles synthesized for attachment to the C-terminus of HAUb. HAUbVME, a first generation probe, was used for comparison of second generation probe reactivity. Figure adapted with permission from ref 14.

vious ABPP experiments. For identification of human orthologs of particular DUBs/ULPs reactive with our HAUb electrophilic probes, we chose the immortalized human mammary epithelial cell line (HMLE) (*37*). Cell lysate was incubated with HAUb-derived probes, and enzymes modified were visualized by anti-HA immunoblotting (Figure 2). Titrations and time courses were conducted to determine the optimal concentrations of each probe and the optimal time for labeling enzymes present in EL-4 cell lysate (data not shown). Significant enrichment of probe-reactive material was observed by immunoblotting of samples incubated with 0.2 μg of each HAUb electrophile per 30 μg of cell lysate for more than 3 h. Extended reaction times or increased quantities of probe beyond 0.2 μg per 30 μg of cell lysate, however, did not affect the number of distinct polypeptides retrieved. HAUb-derived probes have different activities and specificities using otherwise identical labeling conditions and probe concentrations (Figure 2). Furthermore, the pattern of labeled DUBs/ULPs differs between the two cell types; as expected, EL-4 and HMLE cells contain diverse and differentially expressed DUBs/ULPs. In all cases, labeling could be blocked by preincubation of lysate with the alkylating agent *N*-ethylmaleimide (NEM) (Supplementary Figure 1), consistent with active site cysteine modification by the HAUb-derived probes. The increased electrophilicity of our newly synthesized probes did not translate into greater reactivity with proteins in cell lysates, and the first generation HAUbVME probe was still the most broadly reactive (Figure 2). The increased chemical reactivity of these newly synthesized probes likely means they are also more quickly hydrolyzed during the labeling reaction, which may explain their seemingly lower reactivity with cell lysates.

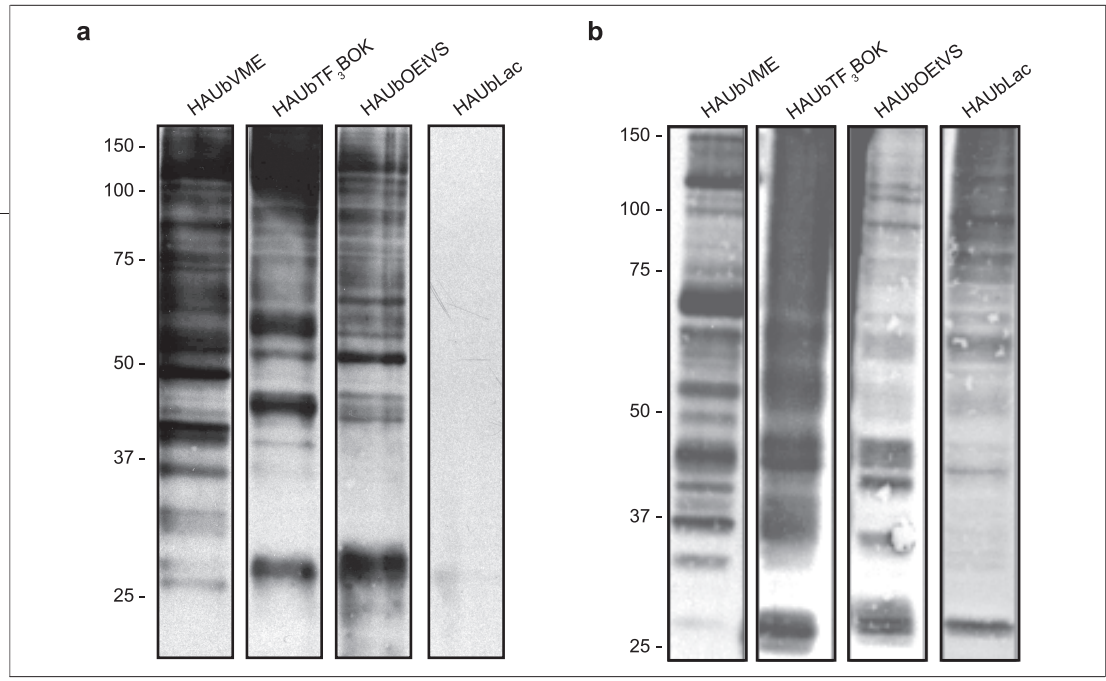


Figure 2. Different HAUb-derived probes show distinct activities and labeling profiles. a) EL4 cell lysates (30 μg) were incubated with 0.2 μg of each HAUb (2 μM) probe for 5 h at RT (22 °C). The labeled proteins were resolved by SDS−PAGE (10%) under reducing conditions and immunoblotted with anti-HA antibody. The HAUbVME probe is the most reactive as visualized by labeling intensity in this immunoblot. Labeling is not observed using HAUbLac at the concentration used. b) HMLE cell lysates (7.5 mg) were incubated with 1 μg of each HAUb probe (0.2 μM) for 5 h at RT (22 °C). A sample of labeled proteins (30 μg protein extract) was resolved by SDS−PAGE (8%) under reducing conditions and immunoblotted with anti-HA antibody. The amount of sample loaded onto the gel and the exposure times of this immunoblot were varied to account for differences in the reactivity and decomposition of the C-terminal electrophiles in each Ub-based probe.

To isolate the proteins modified by each of the HAUb probes, we immunoprecipitated labeled proteins from either EL-4 or HMLE cell lysate using agarose-conjugated anti-HA antibody as previously described (*21*). Precipitated proteins were separated by reducing SDS−PAGE (10%) and visualized by silver staining (data not shown). Polypeptides were excised from the gel, trypsinized, and analyzed by MS/MS. MS/MS data were subjected to database searches using the U.S. NCBI expressed sequence tag (EST) databases. Proteins identified were corrected for nonspecific interactions with the antibody and agarose against untreated, immunoprecipitated cell lysate using the MScomp program (*38*). Twenty-nine DUBs were identified from EL-4 cell extracts, and 23 DUBs and one NEDD8-specific ULP were identified from HMLE cell extracts (see Supplementary Table 1).

We observed selective reactivity with DUBs for each HAUb-electrophilic probe, with HAUbVME being the most diversely reactive probe. $HAUbTF_3BOK$ was the next most DUB-reactive probe and showed significant overlap of labeled species with HAUbVME in EL-4 lysate. Ubiquitin C-terminal hydrolases, including UCH-L1, -L3, and -L4 displayed broad reactivity with all probes in EL-4 lysate, whereas UCH-L3 and -L5 had similar reactivity in HMLE extracts. Several DUBs not previously identified using this ABPP approach were recovered: otubain-domain-containing DUB Cezanne 2 (OTUD-7A) and U2afl-rs1, an uncharacterized peptidase C19 family member with putative DUB activity toward polyubiquitinated peptides, were identified in EL-4 cell lysate using HAUbVME and HAUbLac, respectively. Otubain-domain-containing DUBs OTUD4 (isoform 3) and OTUD5 were identified from HMLE extracts using HAUbVME, as were Josephin domain (JD)-containing DUBs JOS1 and JOS2 (*32*). Josephin-type DUBs represent a new class of DUBs to be identified using a chemical biology approach. Finally, the identification of one ULP, SENP8, a NEDD8 specific protease, in HMLE extracts using HAUbVME was not surprising considering the fact that Ub and NEDD8 contain the same four C-terminal residues and share ~60% sequence identity (*39*). Reactivity of HAUbVME toward a different subset of DUBs, as compared to those identified previously, could be attributed either to altered DUB expression particular to this EL-4 cell culture or to the longer incubation times with probe and increased probe concentrations used here, which allows the retrieval of enzymes that contain more weakly nucleophilic active site cysteines.

Recovery of diverse members of the Ub/Ubl conjugation machinery was also observed (Table 1). Since previous experiments failed to retrieve any such enzymes from cell lysate, this observation was surprising but not altogether unexpected. Most Ub/Ubl conjugating enzymes also contain an active site cysteine that participates in the conjugation of these modifiers to substrate

TABLE 1. Ub/Ubl activating enzymes (E1s), conjugating enzymes (E2s), and ligases (E3s) identified from mouse lymphoma (EL4) and human mammary epithelial cell lysates (HMLE)

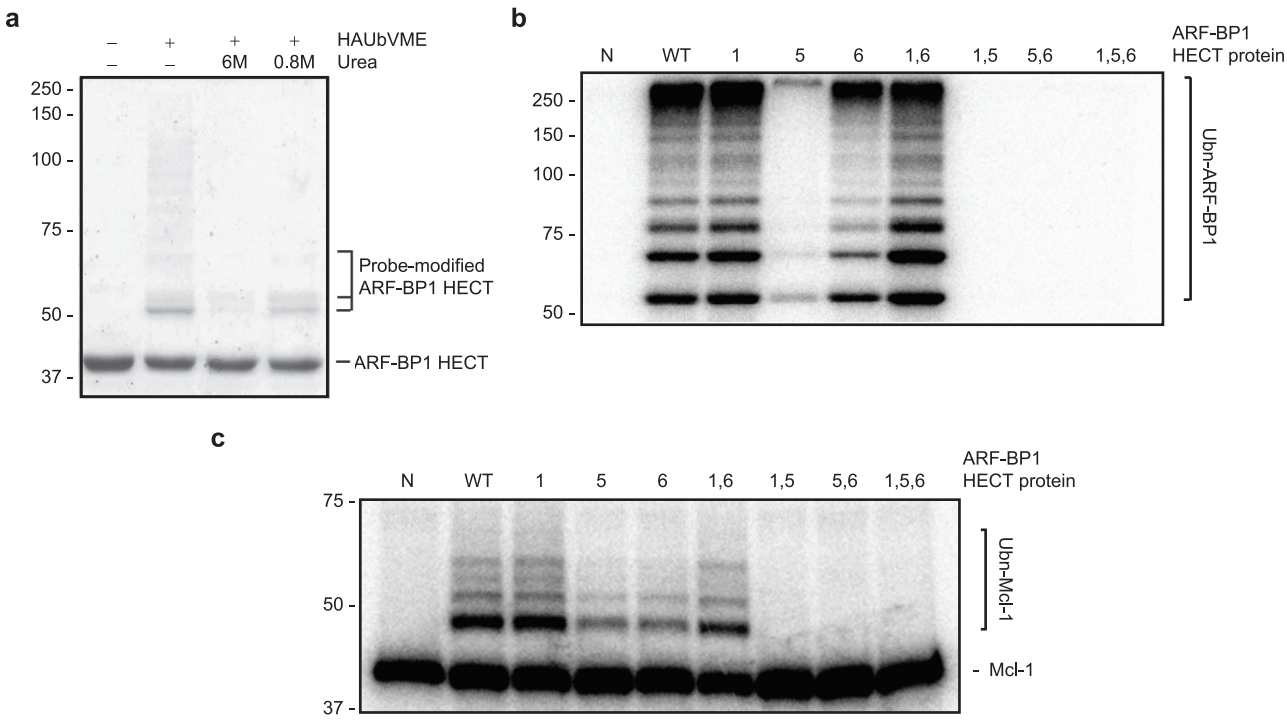
Protein identified	Protein activity	Accession number (NCBI)	Predicted MW (kDa)	HAUb probe used for retrieval
	Identified from mouse lymphoma (EL4)			
UBE1	E1 Ub-activating enzyme	267190	118	TF_3BOK
UBE1-L2	E1 Ub-activating enzyme	27370032	118	TF_3BOK
UBE1 (Chromosome X)	E1 Ub-activating enzyme	148878383	118	TF_3BOK
UBA2	E1 Ubl-activating enzyme	32493410	70.5	TF_3BOK
Aos1	E1 Ubl-activating enzyme	18490720	38.5	TF_3BOK
UBC9	E2 Ubl-conjugating enzyme	54039792	18	TF_3BOK
UBC12	E2 Ubl-conjugating enzyme	46577656	21	TF_3BOK
UBC13	E2 Ub-conjugating enzyme	18017605	17	TF_3BOK
UB2V1	E2 Ub-conjugating enzyme	51702141	16	TF_3BOK
UB2V2	E2 Ub-conjugating enzyme	51702142	16	TF_3BOK
UBE2-L3 (UbcM4)	E2 Ub-conjugating enzyme	54039806	18	TF_3BOK
UBE2S	E2 Ub-conjugating enzyme	53734650	24	TF_3BOK
UBE2O	E2 Ub-conjugating enzyme	50234896	141	TF_3BOK
E6-AP (Ube3A)	HECT E3 Ub ligase	76880500	97.5	VME
Nedd4 (Rsp5)	HECT E3 Ub ligase	32172436	103	OEtVS, TF_3BOK
ARF-BP1 (Mule)	HECT E3 Ub ligase	73915354	482	VME, Lac, TF_3BOK
Trip12	Putative HECT E3 Ub ligase	91932791	224	VME, TF_3BOK
UBAC1 (KPC2)	RING E3 Ub ligase	19527050	45	VME
UBR2	RING E3 Ub ligase	73622074	199	TF_3BOK
CUL-4A	RING E3 Ub ligase	108936014	88	OEtVS
TRAF2	RING E3 Ub ligase	83921633	56	OEtVS
	Identified from human mammary epithelial cell lysates (HMLE)			
UBE1	E1 Ub-activating enzyme	24418865	117.8	VME, OEtVS, TF_3BOK
UBC1	E2 Ub-conjugating enzyme	46577658	22.4	VME, Lac, TF_3BOK
UB2E2	E2 Ub-conjugating enzyme	47606201	22.2	VME, TF_3BOK
UBE2N	E2 Ub-conjugating enzyme	46577660	17.1	VME, Lac, TF_3BOK
UBE2S	E2 Ub-conjugating enzyme	23829978	23.8	TF_3BOK
UBE2C (isoform 4)	E2 Ub-conjugating enzyme	32967289	46.4	TF_3BOK
UBE2T	E2 Ub-conjugating enzyme	73622065	22.5	TF_3BOK
E6-AP (isoform 3, Ube3A)	HECT E3 Ub ligase	19718764	100	VME
ARF-BP1	HECT E3 Ub ligase	73915353	481.6	VME, TF_3BOK
Trip12	Putative HECT E3 Ub ligase	2499839	220.3	TF_3BOK
RN138	RING E3 Ub ligase	74733576	28.2	Lac
CUL-4A	RING E3 Ub ligase	108936013	87.6	Lac
CUL-4B (isoform 2)	RING E3 Ub ligase	121114302	102.2	VME, Lac
SH3R2	Putative RING E3 Ub ligase	122070123	79.2	Lac, TF_3BOK

peptides. Ub/Ubl installation on substrate lysines *via* the E1 to E2 to E3 thioester cascade hinges on reversible interaction of the active site cysteines in these enzymes with the Ub/Ubl C-terminus. In fact, the pK_a of the active site cysteine residue of several E2 enzymes is approximately 2 pH units above that of a free cysteine, likely preventing these residues from reacting nonspecifically with other thiol-reactive groups in the cell (*40*). The increased potency of the newly synthesized HAUb probes should encourage labeling of more weakly nucleophilic cysteine residues; indeed, HAUbTF$_3$BOK was the most reactive probe toward these types of proteins. Representative members of all three classes of conjugating enzymes, including E1s and E2s for both Ub and Ubl's and both HECT and RING domains E3 Ub ligases, were identified in EL-4 and HMLE lysate. One protein we recovered, Trip 12, contains both WWE and HECT domains characteristic of a Ub E3 ligase, but its activity as such had not been verified either *in vivo* or *in vitro*. We expressed the HECT domain of Trip12 (expressed as a GST fusion to improve solubility) and confirmed that it has autoubiquitination activity that is E1/E2-dependent (Supplementary Figure 2) (*41, 42*).

Although active site cysteines capable of being modified by HAUb-electrophilic probes do exist in most classes of conjugating enzymes, some proteins might be retrieved as part of larger multiprotein complexes containing only a single probe-reactive species. This may apply in particular to RING ligases, which do not utilize an active site cysteine but rather activate Ub/Ubl's using two zinc ions coordinated in the active site by eight conserved cysteine and histidine residues (*13*). For example, the RING ligases containing CUL-4A and CUL-4B consist of a multisubunit complex containing the cullin scaffold with C-terminally associated substrate binding proteins and an N-terminally associated RING-domain protein, which recruits the E2-conjugating enzyme to form the active ligase complex (*43*). While the cullin scaffold itself is not capable of reacting with Ub-based electrophiles, reaction of the E2 protein with probe could lead to recovery of these associated scaffold proteins in our immunoprecipitations as performed using nondenaturing conditions. Indeed, this is the case for one example RING E3, UBAC1, identified in EL-4 lysate by immunoblotting but unreactive with HAUbVME as indicated by the presence of a single unmodified species following incubation with probe (Supplementary Figure 3). UBC9 and other Ubl-conjugating enzymes may be retrieved either as a result of cross reactivity with Ub (*44*) under the *in vitro* labeling conditions or as part of a complex with its interacting E1, which in the case of UBC9 was also recovered from EL-4 lysate (Aos1).

HAUb-Electrophilic Probes Are Activity-Based Probes for Ub E3 Ligases. After demonstrating the capability of our panel of probes to identify Ub conjugating machinery, we were interested in validating the utility of these proteins for the mechanistic study of purified enzymes. The HECT Ub E3 ligase that was recovered with the most diverse panel of electrophilic probes using both types of cell lysate was the protein ARF-BP1. ARF-BP1 (Mule) is a 482 kD HECT-domain-containing Ub E3 ligase that has several known substrates: p53 (*45*), Cdc6 (*46*), Mcl-1 (*47, 48*), N-Myc (*49*), C-Myc (*50*), TopBP1 (*51*), and histones (*52*). ARF-BP1 is a key player in both p53-dependent and -independent functions of the ARF tumor suppressor (*45*) and regulates DNA damage-induced apoptosis (*46–48*). ARF-BP1 is interesting not only because its substrates function in multiple pathways but also because a catalytic mutant of ARF-BP1 in which the active site cysteine is mutated to alanine (C4341A) or serine (C4341S) (*50*) reduces but does not abolish ligase activity. We therefore selected this protein for verification of reactivity with HAUbVME and confirmation of enzyme activity as an E3 Ub ligase.

To verify that HAUbVME does indeed bind to the active site cysteine of ARF-BP1, we incubated HAUbVME for 3 h with recombinantly expressed ARF-BP1 HECT domain (residues 4012−4374). The reaction mixture was separated by reducing 10% SDS−PAGE and visualized by Coomassie stain. We observed multiple species, each corresponding to covalent modification of ARF-BP1 (Figure 3, panel a), which appear to occur only in the context of a folded HECT domain; modification of ARF-BP1 by HAUbVME following denaturation in 6.4 M urea is nearly abolished. In contrast, incubation of recombinant DUB enzymes with HAUbVME invariably produces a single modified species (*53*). We identified the nature of the modified species using MS/MS analysis of polypeptides excised from the gel. Three out of the six cysteines within the expressed HECT domain were modified by HAUbVME: C4099, C4341, and C4367 (hereafter called Cys 1, Cys 5, and Cys 6 in reference to their sequential position from the N-terminus of the HECT domain). The modification of Cys 5 was expected, as it was identified as the likely catalytic residue based on multiple sequence alignment (*54*). We reasoned that the



d

ARF-BP1 mutant	Product formed as a % WT (SD) in a 10 min AutoUb reaction	Product formed as a % WT (SD) in a 60 min Mcl-1 Ub reaction
1 (C4099A)	94.03 (22.15)	104.32 (40.39)
5 (C4341A)	8.17 (3.78)	30.04 (7.32)
6 (C4367A)	56.27 (12.69)	40.70 (20.15)
1,6 (C4099A, C4367A)	101.96 (31.15)	73.58 (36.92)
1,5 (C4099A, C4341A)	NP*	NP
5,6 (C4341A, C4367A)	NP	NP
1,5,6 (C4099A, C4341A, C4367A)	NP	NP

NP indicates no product formation was observed for this mutant. *Negligible product detected.

Figure 3. Biochemical analysis of probe-labeled cysteines in ARF-BP1. a) Incubation of HAUbVME with recombinant ARF-BP1 HECT domain covalently labels multiple cysteines as identified using tandem mass spectrometry analysis. A 5 μg sample of recombinant ARF-BP1 HECT domain was incubated with 2.0 μM HAUbVME for 3 h at 22 °C. Labeled species were resolved by 10% SDS—PAGE and detected by Coomassie staining. The labeled peptides were excised from the gel, trypsizined, and subjected to MS/MS analysis. Residues C4099 (Cys 1), C4341 (Cys 5), and C4367 (Cys 6) were modified by probe. Probe labeling is specific for these sites, as HECT domain denatured in 6.4 M urea for 30 min at 30 °C and then diluted with reaction buffer to 0.6 M urea before probe addition no longer efficiently binds HAUbVME. ARF-BP1 HECT domain and HAUbVME retain activity when incubated initially with 0.6 M urea. Ub ligase activity of wild-type (wt) or cysteine-to-alanine mutant ARF-BP1 HECT domains was assayed using (b) [^{32}P]-Ub for autoubiqutination or (c) [^{32}P]-Mcl-1 for substrate ubiquitination. Reaction components contained recombinant UBE1, UbcH7, an ATP regenerating system, and either no ligase (N), WT, or a mutant ligase as indicated. Ligation reaction mixtures were separated by 10% SDS—PAGE and visualized by phosphorimaging. d) Product formation by WT or mutant ARF-BP1 in the autoubiquitination assay or Mcl-1 ubiquitination assay was calculated by averaging the percent wild-type activity quantified using phosphorimaging data from nine independent experiments.

modification of the other two residues should correlate with the ability of these cysteines to contribute to product formation. To investigate this possibility, we expressed and purified ARF-BP1 HECT domain mutants (residues 4012—4374) in which cysteines 1, 5, and 6 were mutated to alanine either singly or in combination.

We then tested the activity of these proteins *in vitro* using one of two assays: autoubiquitination or ubiquitination of the antiapoptotic protein Mcl-1, a substrate of ARF-BP1 (*54*).

When wild-type ARF-BP1 is incubated for 10 min at RT in the presence of recombinant E1, the E2 UbcH7, [^{32}P]-Ub, and an ATP-regenerating system, the E3 efficiently catalyzes polyubiquitination of itself (Figure 3, panel b). Following separation of the reaction mixture on 10% SDS−PAGE, ubiquitinated products are quantified using phosphoimaging (Figure 3, panel d). Mutation of the catalytic cysteine (Cys 5) to alanine greatly reduces activity but does not abolish it; the mutant forms 8.2% wild-type product after a 10 min reaction. Mutation of Cys 6 reduces product formation in the autoubiquitination assay to 56.27% of the wild-type level. Finally, the mutation of Cys 1 alone does not affect activity. The mutation of Cys 1 in combination with either Cys 5 or Cys 6 does dramatically alter autoubiquitination activity: in combination with a Cys 6 mutation, activity is restored; in the context of a Cys 5 mutation, ARF-BP1 fails to ubiquitinate itself. Finally, mutation of all three probe-labeled cysteines (1, 5, and 6) abolishes all activity.

Although we cannot exclude that these cysteine mutations affect binding to UbcH7, these results suggest that mutation of probe-labeled cysteines affects activity of the ARF-BP1 HECT domain. We next asked whether this holds true for ARF-BP1-catalyzed ubiquitination of its substrate protein Mcl-1. In this case, ARF-BP1 is incubated with recombinant E1, the E2 UbcH7, Ub, an ATP-regenerating system, and [^{32}P]-Mcl-1 for 1 h at 37 °C as described (*47*). Reaction mixtures are separated on SDS−PAGE and quantified as described above. Wild-type ARF-BP1 catalyzes multiubiquitination of Mcl-1 as reported (Figure 1, panel c). We observed that the cysteine-to-alanine mutants of ARF-BP1 show a similar pattern of reduced activity as seen in the autoubiquitination assay; however, the relative contributions of cysteines 5 and 6 toward total activity differ in this assay as compared to the autoubiquitination assay. As in the autoubiquitination assay, mutation of Cys 1 has no effect on activity toward Mcl-1. More significantly, mutation of either Cys 5 or Cys 6 reduces activity to a similar extent (30% and 40.7%, respectively) after a 1 h incubation, suggesting that both of these residues participate in ubiquitination of Mcl-1. The pattern of activity of the double mutants in the autoubiquitination assay resembles the pattern for substrate activity: the 1,6 mutant resembles wild-type ARF-BP1, whereas the 1,5 mutant is inactive. Again, the triple mutant lacking cysteines 1, 5, and 6 shows no activity.

Multiple Cysteines in ARF-BP1 Form Thioesters with Ub. The mechanism of catalysis by HECT E3 ligases involves several steps. The E3 enzyme binds Ub-loaded E2, followed by Ub transfer from the E2 catalytic cysteine to the E3 catalytic cysteine. The E3 then catalyzes isopeptide bond formation between Ub and a recipient lysine residue on substrate, which may be the E3 itself, Ub, or another protein. A crucial determinant in isopeptide bond formation is the presence of a conserved phenylalanine located four amino acids from the C-terminus of most HECT E3s (*55*). Truncations that remove this residue abolish Ub transfer to substrate, trapping the E3 with the catalytic cysteine in a thioester linkage to Ub (*55*). We reasoned that if more than one cysteine is capable of receiving Ub from the associated E2, we should be able to detect the presence of multiple Ub-thioesters in an ARF-BP1 HECT domain truncation mutant (ARF-BP1 Δ4) lacking the conserved phenylalanine.

We therefore expressed and purified two versions of the ARF-BP1 HECT domain lacking the terminal four amino acids: wild-type (WTΔ4) and a Cys5 to Ala mutant (C5AΔ4) and assayed these mutants, along with the full-length ARF-BP1 HECT domain (WT FL) for Ub thioester formation. In this assay, ARF-BP1 is incubated with recombinant E1, the E2 UbcH7, Ub, and an ATP regenerating system for 10 min at RT. The reaction is quenched by urea denaturation followed by addition of SDS−PAGE loading buffer with or without β-mercaptoethanol, separated on 10% SDS−PAGE, and analyzed by anti-Ub immunoblot. Under these conditions, WT FL ARF-BP1 efficiently catalyzes autoubiquitination (Figure 4, panel a). In contrast, WTΔ4 ARF-BP1 forms a monoubiquitylated species when the reaction is quenched with reducing sample buffer, indicating that the activity of this protein is compromised by the truncation. The ability of the WTΔ4 ARF-BP1 to form a monoubiquitylated species depends on Cys 5 (Figure 4). Interestingly, quenching the reaction with nonreducing sample buffer preserves multiple Ub-thioesters on WTΔ4 ARF-BP1. We detect the persistence of a single Ub-thioester in the C5AΔ4 ARF-BP1 mutant, which is likely due to Ub binding to a cysteine in the HECT domain. The apparent thioester formation catalyzed by both WTΔ4 and C5AΔ4 ARF-BP1 is E1/E2-dependent (Figure 4, panel b). These results indicate

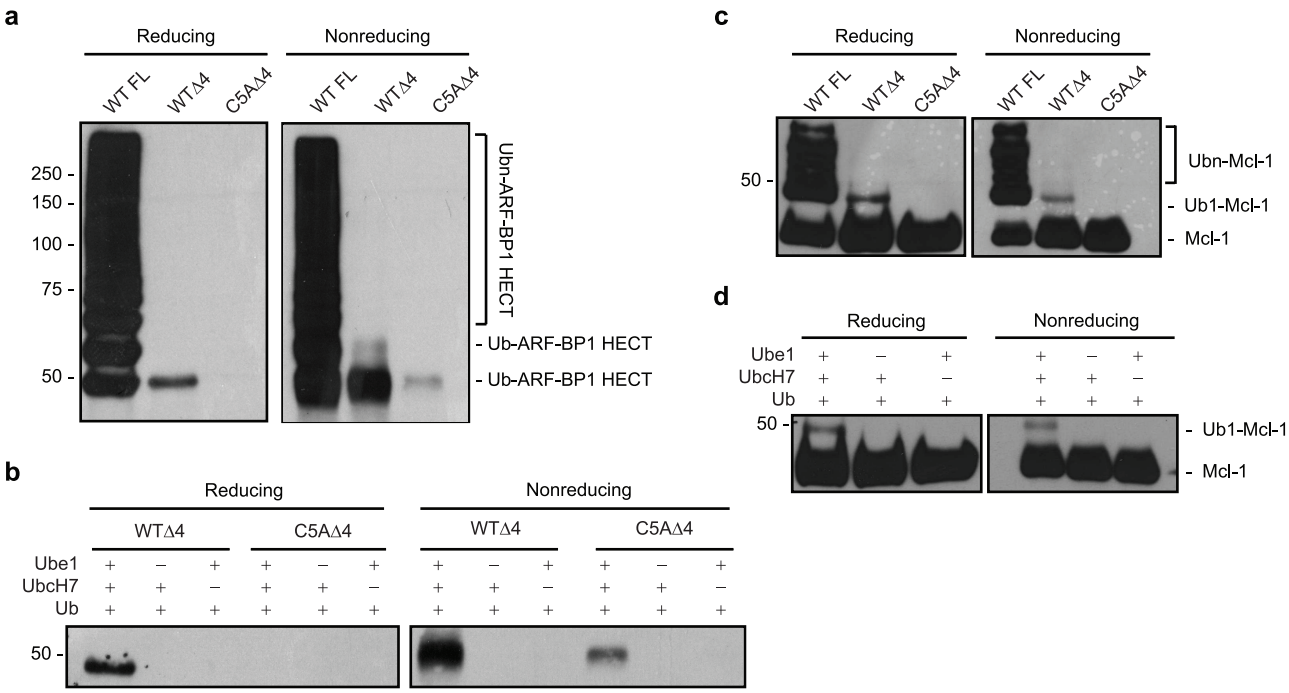


Figure 4. Detection of Ub-thioesters in the ARF-BP1 HECT domain. a) Ub-thioester assay with the indicated ARF-BP1 HECT domain proteins. Purified ARF-BP1 HECT domain proteins were incubated with recombinant E1 and E2 (UbcH7), an ATP regenerating system, and Ub. Reactions were stopped with 4 M urea and either reducing (left panel) or nonreducing (right panel) SDS−PAGE sample buffer, separated by SDS−PAGE, and analyzed by immunoblot with anti-Ub antibody. b) Ub thioester assay with the indicated ARF-BP1 truncation proteins was performed with all necessary components, without UBE1, or without UbcH7. c) Substrate ubiquitination assay using the indicated ARF-BP1 HECT domain proteins and recombinant Mcl-1 as substrate. Reactions contained recombinant UBE1, UbcH7, Ub, an ATP regenerating system, and 1 μg of recombinant Flag-labeled Mcl-1. Reaction mixtures were quenched as above, separated on SDS−PAGE and analyzed by anti-Flag immunoblot. d) Substrate ubiquitination assay using the indicated ARF-BP1 HECT domain with all components, no UBE1, and no UbcH7.

that at least two cysteines in ARF-BP1 simultaneously can form Ub-thioesters and that one of these cysteines is Cys 5.

We predicted that the Δ4 truncation would abolish the ability of ARF-BP1 to ubiquitinate Mcl-1, and indeed we find this to be the case (Figure 4, panel c). WTΔ4 ARF-BP1 catalyzes monoubiquitylation of Mcl-1 in a manner dependent on Cys 5 and the presence of E1 and E2 enzymes (Figure 4, panel d). As this small amount of ligase activity persists after complete denaturation of samples in 4 M urea, it must correspond to covalent lysine modification.

Given that our probes were designed to act as electrophiles capable of reaction with local nucleophiles following specific binding by Ub-reactive proteins, it is likely that the probe's reactivity with cysteines other than the catalytic residue means that these residues are close in proximity to the electrophilic warhead within the tertiary structure of the ARF-BP1 HECT domain. We proceeded to construct a threaded structure based on the structures available for the HECT domains of WWP1 (*56*) and SMURF2 (*57*), the HECT domains with highest homology to ARF-BP1 (>40% identity, data not shown). In our threaded structure, Cys 6 appears to be equidistant to Cys 5 in proximity to the E2 binding site and all three reactive cysteines are along a single face of the domain. We next asked if these three residues from the human ARF-BP1 sequence are conserved across eukaryotes (Figure 5). As expected, Cys 5 is conserved across all species examined, and Cys 6 is conserved within the animal kingdom. Cys 1, the most weakly reactive residue toward our Ub-based electrophiles, is con-

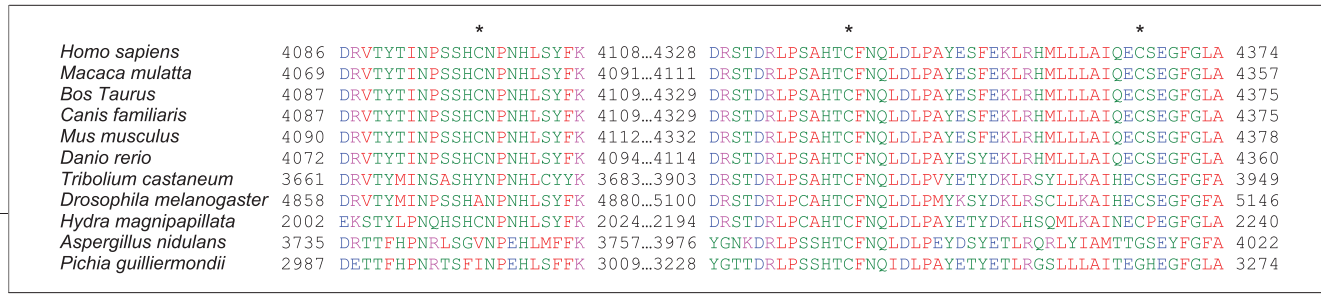


Figure 5. Multiple sequence alignment of ARF-BP1 HECT domain. Sequence conservation of ARF-BP1 HECT domain across diverse eukaryotes. The region depicted represents two portions of ARF-BP1 C-terminus and includes Cys 1 (C4099), Cys 5 (C4341), and Cys 6 (C4367), each marked above with an asterik (*). Residues depicted in red are small or hydrophobic, those depicted in blue are acidic, those depicted in magenta are basic, and the remainders are depicted in green. Alignment made with Clustal W (*61*).

served only through bony fishes (*Danio rerio*) but is also observed in *Hydra*. Altogether, these data link the ability of our probes to identify residues with the potential to react with Ub, due to either catalytic activity or proximity.

Conclusions. The ubiquitin-proteasome system is critical for the regulation of protein function in eukaryotes. Here we extend an earlier effort to allow the isolation and identification of members of all enzyme classes that comprise the ubiquitin-proteasome system. While we continue to find novel enzymes of the DUB family, we have also demonstrated the utility of Ub-based electrophiles as activity-based probes for certain HECT domain E3 ligases. In studying the covalent modification of the HECT domain of E3 ligase ARF-BP1 with HAUbVME, we observed that the probe covalently labels three out of the six cysteines found in the ARF-BP1 HECT domain (C4099 (Cys 1), C4341 (the canonical catalytic residue, Cys 5), and C4367 (Cys 6)). We provide evidence for the formation of multiple thioester-linked Ub molecules during catalysis by wild-type ARF-BP1 HECT domain, supporting the notion that the HAUbVME probe is capable of identifying residues that are competent nucleophiles for Ub tranfer. We are eager to see our hypotheses about the positioning of nucleophilic residues in the ARF-BP1 HECT domain confirmed with structural data. The utility of Ub electrophilic probes as mechanism-based chemical tools to study E3 ligase structure and function will likely provide further insights into the mechanism employed by ARF-BP1 and other HECT E3 Ub ligases.

METHODS

Synthesis and Purification of HAUb-Electrophilic Probes. Electrophilic glycine analogs 4-amino but-1-enyl ethoxysulfonate, serine-β-lactone, and 2,6-trifluoromethylbenzyloxy glycine methyl ketone were synthesized according to literature procedures (*58–60*) (see Supporting Information for synthesis details and compound characterization). $HAUb_{75}$-MESNa (500 μL, ~10−15 mg mL^{-1}), synthesized as previously reported (*21*) from an Ub-intein-chitin domain fusion protein, was treated with 1 mM *N*-hydroxysuccinimide, immediately followed by 0.5 mM of the desired electrophilic glycine analog in 1.1 mL (total volume) of a 1 M $NaHCO_3$ (pH 8.0) solution. These conjugation reactions were incubated overnight at 37 °C. The resulting products were dialyzed and purified as previously reported (*21*). Each purified HAUb-electrophilic probe was characterized by LC/MS using a Michrom Paradigm MS4 HPLC equipped with a Waters Symmetry C8 2.1 mm × 50 mm column using standard reverse-phase gradients. The effluent from the column was mass analyzed using a Waters LCT electrospray time-of-flight mass spectrometer (see Supporting Information for MS characterization).

Preparation of EL-4 and HMLE Cell Lysate and Labeling with HAUb-Electrophilic Probes. EL-4 cells (cultured in DMEM supplemented with 10% IFS and 1% penicillin/streptomycin at 37 °C with 5% CO_2) were harvested and washed once with culture media and once with PBS. Cell pellets were lysed with glass beads as previously reported (*21*). A 30 μg portion of protein extract was incubated with 0.2 μg of each HAUb-electrophilic probe (2 μM, 10 μL total volume per sample) for 5 h at RT. After termination of the reactions with reducing SDS−PAGE sample buffer and 10 min of boiling, the reaction mixtures were separated by SDS−PAGE (10%) and analyzed by immunoblotting with anti-HA antibody (3F10, Roche).

The immortalized, nontransformed HMLE line, expressing the SV40 large-T oncogene and hTERT, was cultured as previously described (*37*) using a 2:1:1 mixture of mammary epithelial growth medium (MEGM, Clonetics), DMEM, and F12 medium supplemented with EGF (10 ng mL^{-1}), insulin (10 μg mL^{-1}), and hydrocortisone (1 μg mL^{-1}). Cells were harvested at a confluence of 90% and washed once with PBS. Cell pellets were
lysed with glass beads as previously reported (*21*). A 7.5 mg portion of cell lysate was incubated with 1 μg of each HAUb-electrophilic probe (0.2 μM) for 5 h at RT. A sample of labeled proteins (30 μg protein extract) was separated by SDS−PAGE (8%) and analyzed by immunoblotting with anti-HA antibody (3F10, Roche).

Anti-HA Immunoprecipitation of Labeled Proteins and Identification by Tandem Mass Spectrometry. EL-4 and HMLE lysate were prepared as above. Then, 7 mg of EL-4 protein extract or 7.5 mg of HMLE protein extract was incubated with 1 μg (0.2 μM) of each HAUb-electrophilic probe for 5 h at RT in 50 mM Tris (pH 8.0), 150 mM NaCl. Anti-HA agarose (3F10 antibody, Roche) was incubated with the samples overnight at 4 °C. The immunoprecipitations were washed extensively with NET buffer (50 mM Tris [pH 7.5], 150 mM NaCl, 5 mM EDTA, 0.5% NP40), and the bound proteins were eluted by boiling for 10 min with reducing SDS−PAGE sample buffer, separated by SDS−PAGE (10%), and visualized by silver stain using standard conditions. Bands were excised, reduced, alkylated and digested with trypsin at 37 °C overnight. The resulting peptides were extracted, concentrated, and injected onto a Waters NanoAcquity HPLC equipped with a self-packed Jupiter 3 μm C18 analytical column (0.075 mm × 10 cm, Phenomenex). Peptides were eluted using standard reverse-phase gradients. The effluent from the column was analyzed using a Thermo LQT linear ion trap mass spectrometer (nanospray configuration) operated in a data-dependent manner. The resulting fragmentation spectra were correlated against the known database using SEQUEST. Bioworks browser was used to provide consensus reports of the proteins identified.

ARF-BP1 Labeling with HAUbVME. Recombinantly expressed and purified ARF-BP1 HECT domain (5 μg) was incubated with HAUbVME (2.0 μM) in 50 mM Tris, 150 mM NaCl, pH 8.0 for 3 h at RT. The specificity of probe labeling was tested by first denaturing the ARF-BP1 HECT domain with 6.4 M urea (30 min incubation at 30 °C) and then diluting the sample to 0.6 M urea before the addition of HAUbVME (2.0 μM). Activity of HAUbVME was tested with ARF-BP1 in 50 mM Tris, 150 mM NaCl, pH 8.0 containing 0.6 M urea to ensure that probe reactivity was not compromised in dilute concentrations of urea. All of the above labeling reactions were quenched by addition of reducing sample buffer, boiled for 10 min, and separated by SDS−PAGE (10%). Labeled and unlabeled species were visualized by Coomassie staining, and bands were excised and treated as above for MS/MS analysis. Modified cysteine residues were identified by the presence of the mass change corresponding to the C-terminus of the VME probe (172.08 Da).

ARF-BP1 Autoubiquitination and Substrate Ubiquitination Assays with Wild-Type and Mutant HECT Domain Proteins. Autoubiquitination activity was tested in a 20 μL reaction by incubating ARF-BP1 HECT domain (10 μg) with 100 ng of human E1 (Ube1, Boston Biochem), 1 μg of UbcH7, and 10 μg of [^{32}P]-Ub (see Supporting Information for plasmid construction, protein expression and purification conditions, and [^{32}P]-labeling) with an ATP regenerating system (50 mM Tris [pH 7.6], 5 mM $MgCl_2$, 5 mM ATP, 10 mM creatine phosphate, 3.5 U mL^{-1} creatine kinase) for 10 min at RT. Substrate ubiquitination activity was tested by adding 1 μg of [^{32}P]-Mcl-1 (see Supporting Information for plasmid construction, protein expression and purification conditions, and [^{32}P]-labeling) to the above reaction containing 100 μg of nonradiolabeled Ub and incubating the mixture for 1 h at 37 °C. A control reaction was run without ARF-BP1 HECT domain but containing all other reaction components for each case. After termination of the reactions with reducing SDS−PAGE sample buffer and 10 min of boiling, reaction mixtures were separated by SDS−PAGE (10%) and analyzed by phosphorimaging. Product formation by ARF-BP1 HECT domain mutants was calculated as a percentage of total (background corrected) [^{32}P] counts attributable to ubiquitinated product in the wild-type HECT domain. The average and standard deviation of nine experiments is shown.

Ub-Thioester Assays. To test ARF-BP1 thioester formation, reaction mixtures (10 μL) were set up in duplicate containing 100 ng of human E1 (Ube1, Boston Biochem), 1 μg of UbcH7, 10 μg of ARF-BP1 Δ4 HECT domain, 10 μg of Ub, 50 μM DTT, and an ATP regenerating system and incubated for 10 min at RT. Reactions were terminated with 10 μL of 8 M urea and incubated 15 min at 30 °C. One set of samples received reducing SDS−PAGE sample buffer, and the other set received nonreducing SDS−PAGE sample buffer. Samples were then boiled for 10 min, separated on 10% SDS−PAGE, and analyzed by immunoblotting with anti-Ub antibody (Sigma). To test the activity of the ARF-BP1 Δ4 proteins against substrate, reactions were set up as above except with 100 μg of Ub and 1 μg of Flag-Mcl-1 and were incubated 1 h at 37 °C. Reactions were terminated as above, separated on 10% SDS−PAGE, and analyzed by immunoblotting with anti-Flag antibody (Sigma).

Acknowledgment: We thank Victor Quesada for software development and help using the MScomp program to analyze our MS results and for the purified E2 UbcH7. HMLE cells were provided by the Weinberg laboratory at the Whitehead Institute for Biomedical Research. cDNA plasmids for ARF-BP1 (Mule) and its substrate Mcl-1 were provided by Fenghe Du and Xiaodong Wang at UT Southwestern Medical Center. K.R.L. was supported by an NIH postdoctoral fellowship (F32 AI63854). R.K.P. is supported by a U.S. Department of Defense Breast Cancer Research Program predoctoral traineeship award (W81XWH-06-1-0789).

Supporting Information Available: This material is available free of charge *via* the Internet at http://pubs.acs.org.

REFERENCES

1. Hicke, L. (2001) Protein regulation by monoubiquitin, *Nat. Rev. Mol. Cell Biol. 2*, 195–201.
2. Hicke, L., Schnell, J., and Stamenova, S. (2005) Regulation of receptor endocytosis by monoubiquitin signals, *FASEB J. 19*, A1689–A1689.
3. Hicke, L., and Dunn, R. (2003) Regulation of membrane protein transport by ubiquitin and ubiquitin-binding proteins, *Ann. Rev. Cell Dev. Biol. 19*, 141–172.
4. Haglund, K., Di Fiore, P. P., and Dikic, I. (2003) Distinct monoubiquitin signals in receptor endocytosis, *Trends Biochem. Sci. 28*, 598–603.
5. Pickart, C. M., and Eddins, M. J. (2004) Ubiquitin: structures, functions, mechanisms, *Biochim. Biophys. Acta 1695*, 55–72.
6. Newton, K., and Vucic, D. (2007) Ubiquitin ligases in cancer: ushers for degradation, *Cancer Invest. 25*, 502–513.
7. Ohta, T., and Fukuda, M. (2004) Ubiquitin and breast cancer, *Oncogene 23*, 2079–2088.
8. Cooper, E. M., Hudson, A. W., Amos, J., Wagstaff, J., and Howley, P. M. (2004) Biochemical analysis of Angelman syndrome-associated mutations in the E3 ubiquitin ligase E6-associated protein, *J. Biol. Chem. 279*, 41208–41217.
9. Davies, J., Sarkar, S., and Rubinsztein, D. (2007) The ubiquitin proteasome system in Huntington's disease and the spinocerebellar ataxias, *BMC Biochem. 8*, S2.
10. Upadhya, S., and Hegde, A. (2007) Role of the ubiquitin proteasome system in Alzheimer's disease, *BMC Biochem. 8*, S12.
11. Lim, K.-L., and Tan, J. (2007) Role of the ubiquitin proteasome system in Parkinson's disease, *BMC Biochem. 8*, S13.
12. Kerscher, O., Felberbaum, R., and Hochstrasser, M. (2006) Modification of proteins by ubiquitin and ubiquitin-like proteins, *Ann. Rev. Cell Dev. Biol. 22*, 159–180.
13. Dye, B. T., and Schulman, B. A. (2007) Structural mechanisms underlying posttranslational modification by ubiquitin-like proteins, *Annu. Rev. Biophys. Biomol. Struct. 36*, 131–150.
14. Love, K. R., Catic, A., Schlieker, C., and Ploegh, H. L. (2007) Mechanisms, biology and inhibitors of deubiquitinating enzymes, *Nat. Chem. Biol. 3*, 697–705.
15. Amerik, A. Y., and Hochstrasser, M. (2004) Mechanism and function of deubiquitinating enzymes, *Biochim. Biophys. Acta 1695*, 189–207.
16. Jensen, D. E., and Rauscher, F. J. (1999) Defining biochemical functions for the BRCA1 tumor suppressor protein: analysis of the BRCA1 binding protein BAP1, *Cancer Lett. 143*, S13–S17.
17. Brooks, C. L., and Gu, W. (2006) P53 ubiquitination: Mdm2 and beyond, *Mol. Cell 21*, 307–315.
18. Trompouki, E., Hatzivassiliou, E., Tsichritzis, T., Farmer, H., Ashworth, A., and Mosialos, G. (2003) CYLD is a deubiquitinating enzyme that negatively regulates NF-κB activation by TNFR family members, *Nature (London) 424*, 793–796.
19. Ciechanover, A., and Schwartz, A. L. (2004) The ubiquitin system: pathogenesis of human diseases and drug targeting, *Biochim. Biophys. Acta 1695*, 3.
20. Nicholson, B., Marblestone, J. G., Butt, T. R., and Mattern, M. R. (2007) Deubiquitinating enzymes as novel anticancer targets, *Fut. Oncol. 3*, 191–199.
21. Borodovsky, A., Ovaa, H., Kolli, N., Gan-Erdene, T., Wilkinson, K. D., Ploegh, H. L., and Kessler, B. M. (2002) Chemistry-based functional proteomics reveals novel members of the deubiquitinating enzyme family, *Chem. Biol. 9*, 1149–1159.
22. Kattenhorn, L. M., Korbel, G. A., Kessler, B. M., Spooner, E., and Ploegh, H. L. (2005) A deubiquitinating enzyme encoded by HSV-1 belongs to a family of cysteine proteases that is conserved across the family herpesviridae, *Mol. Cell 19*, 547–557.

23. Schlieker, C., Korbel, G. A., Kattenhorn, L. M., and Ploegh, H. L. (2005) A deubiquitinating activity is conserved in the large tegument protein of the herpesviridae, *J. Virol. 79*, 15582–15585.
24. Misaghi, S., Balsara, Z. R., Catic, A., Spooner, E., Ploegh, H. L., and Stambach, M. N. (2006) *Chlamydia trachomatis*-derived deubiquitinating enzymes in mammalian cells during infection, *Mol. Microbiol. 61*, 142–150.
25. Catic, A., Misaghi, S., Korbel, G. A., and Ploegh, H. L. (2007) ElaD, a deubiquitinating protease expressed by *E. coli*, *PLoS ONE 2*, e381.
26. Artavanis-Tsakonas, K., Misaghi, S., Comeaux, C. A., Catic, A., Spooner, E., Duraisingh, M. T., and Ploegh, H. L. (2006) Identification by functional proteomics of a deubiquitinating/deneddylating enzyme in *Plasmodium falciparum*, *Mol. Microbiol. 61*, 1187–1195.
27. Frickel, E.-M., Quesada, V., Muething, L., Gubbels, M.-J., Spooner, E., Ploegh, H., and Artavanis-Tsakonas, K. (2007) Apicomplexan UCHL3 retains dual specificity for ubiquitin and Nedd8 throughout evolution, *Cell. Microbiol. 9*, 1601–1610.
28. Schlieker, C., Weihofen, W. A., Frijns, E., Kattenhorn, L. M., Gaudet, R., and Ploegh, H. L. (2007) Structure of a herpesvirus-encoded cysteine protease reveals a unique class of deubiquitinating enzymes, *Mol. Cell 25*, 677–687.
29. Misaghi, S., Galardy, P. J., Meester, W. J. N., Ovaa, H., Ploegh, H. L., and Gaudet, R. (2005) Structure of the ubiquitin hydrolase UCH-L3 complexed with a suicide substrate, *J. Biol. Chem. 280*, 1512–1520.
30. Ovaa, H., Kessler, B. M., Rolen, U., Galardy, P. J., Ploegh, H. L., and Masucci, M. G. (2004) Activity-based ubiquitin-specific protease (USP) profiling of virus-infected and malignant human cells, *Proc. Natl. Acad. Sci. U.S.A. 101*, 2253–2258.
31. Gredmark, S., Schlieker, C., Quesada, V., Spooner, E., and Ploegh, H. L. (2007) A functional ubiquitin-specific protease embedded in the large tegument protein (ORF64) of murine gammaherpesvirus 68 is active during the course of infection, *J. Virol. 81*, 10300–10309.
32. Tzvetkov, N., and Breuer, P. (2007) Josephin domain-containing proteins from a variety of species are active de-ubiquitination enzymes, *Biol. Chem. 388*, 973–978.
33. Hemelaar, J., Borodovsky, A., Kessler, B. M., Reverter, D., Cook, J., Kolli, N., Gan-Erdene, T., Wilkinson, K. D., Gill, G., Lima, C. D., Ploegh, H. L., and Ovaa, H. (2004) Specific and covalent targeting of conjugating and deconjugating enzymes of ubiquitin-like proteins, *Mol. Cell. Biol. 24*, 84–95.
34. Thomadaki, H., and Scorilas, A. (2006) Bcl2 family of apoptosis-related genes: functions and clinical implications in cancer, *Crit. Rev. Clin. Lab. Sci. 43*, 1–67.
35. Powers, J. C., Asgian, J. L., Ekici, O. D., and James, K. E. (2002) Irreversible inhibitors of serine, cysteine, and threonine proteases, *Chem. Rev. 102*, 4639–4750.
36. Borodovsky, A., Kessler, B. M., Casagrande, R., Overkleeft, H. S., Wilkinson, K. D., and Ploegh, H. L. (2001) A novel active site-directed probe specific for deubiquitylating enzymes reveals proteasome association of USP14, *EMBO J. 20*, 5187–5196.
37. Elenbaas, B., Spirio, L., Koerner, F., Fleming, M. D., Zimonjic, D. B., Donaher, J. L., Popescu, N. C., Hahn, W. C., and Weinberg, R. A. (2001) Human breast cancer cells generated by oncogenic transformation of primary mammary epithelial cells, *Genes Dev. 15*, 50–65.
38. The MScomp is a custom script written in visual basic for the comparison of protein sequences identified in tandem mass spectrometry.
39. Whitby, F. G., Xia, G., Pickart, C. M., and Hill, C. P. (1998) Crystal structure of the human ubiquitin-like protein NEDD8 and interactions with ubiquitin pathway enzymes, *J. Biol. Chem. 273*, 34983–34991.
40. Tolbert, B. S., Tajc, S. G., Webb, H., Snyder, J., Nielsen, J. E., Miller, B. L., and Basavappa, R. (2005) The active site cysteine of ubiquitin-conjugating enzymes has a significantly elevated pK_a: functional implications, *Biochemistry 44*, 16385–16391.
41. Park, Y., Yoon, S. K., and Yoon, J. B. (2008) TRIP12 functions as an E3 ubiquitin ligase of APP-BP1, *Biochem. Biophys. Res. Commun. 374*, 294–298.
42. Park, Y., Yoon, S. K., and Yoon, J. B. (2009) The HECT domain of TRIP12 ubiquitinates substrates of the ubiquitin fusion degradation pathway, *J. Biol. Chem. 284*, 1540–1549.
43. Petroski, M. D., and Deshaies, R. J. (2005) Function and regulation of cullin-ring ubiquitin ligases, *Nat. Rev. Mol. Cell Biol. 6*, 9–20.
44. Huang, D. T., Zhuang, M., Ayrault, O., and Schulman, B. A. (2008) Identification of conjugation specificity determinants unmasks vestigial preference for ubiquitin within the NEDD8 E2, *Nature Structural & Molecular Biology 15*, 280–287.
45. Chen, D. L., Kon, N., Li, M. Y., Zhang, W. Z., Qin, J., and Gu, W. (2005) ARF-BP1/Mule is a critical mediator of the ARF tumor suppressor, *Cell 121*, 1071–1083.
46. Hall, J. R., Kow, E., Nevis, K. R., Lu, C. K., Luce, K. S., Zhong, Q., and Cook, J. G. (2007) Cdc6 stability is regulated by the Huwe1 ubiquitin ligase after DNA damage, *Mol. Biol. Cell 18*, 3340–3350.
47. Zhong, Q., Gao, W. H., Du, F. H., and Wang, X. D. (2005) Mule/ARF-BP1, a BH3-only E3 ubiquitin ligase, catalyzes the polyubiquitination of Mcl-1 and regulates apoptosis, *Cell 121*, 1085–1095.
48. Warr, M. R., Acoca, S., Liu, Z. Q., Germain, M., Watson, M., Blanchette, M., Wing, S. S., and Shore, G. C. (2005) BH3-ligand regulates access of Mcl-1 to its E3 ligase, *FEBS Lett. 579*, 5603–5608.
49. Zhao, X. D., Heng, J. I. T., Guardavaccaro, D., Jiang, R., Pagano, M., Guillemot, F., Iavarone, A., and Lasorella, A. (2008) The HECT-domain ubiquitin ligase Huwe1 controls neural differentiation and proliferation by destabilizing the N-Myc oncoprotein, *Nat. Cell Biol. 10*, 643–653.
50. Adhikary, S., Marinoni, F., Hock, A., Hulleman, E., Popov, N., Beier, R., Bernard, S., Quarto, M., Capra, M., Goettig, S., Kogel, U., Scheffner, M., Helin, K., and Eilers, M. (2005) The ubiquitin ligase HectH9 regulates transcriptional activation by Myc and is essential for tumor cell proliferation, *Cell 123*, 409–421.
51. Herold, S., Hock, A., Herkert, B., Berns, K., Mullenders, J., Beijersbergen, R., Bernards, R., and Eilers, M. (2008) Miz1 and HectH9 regulate the stability of the checkpoint protein, TopBP1, *EMBO J. 27*, 2851–2861.
52. Liu, Z. Q., Oughtred, R., and Wing, S. S. (2005) Characterization of E3(histone), a novel testis ubiquitin protein ligase which ubiquitinates histones, *Mol. Cell. Biol. 25*, 2819–2831.
53. Schlieker, C., Korbel, G. A., Kattenhorn, L. M., and Ploegh, H. L. (2005) A deubiquitinating activity is conserved in the large tegument protein of the herpesviridae, *J. Virol. 79*, 15582–15585.
54. Zhong, Q., Gao, W., Du, F., and Wang, X. (2005) Mule/ARF-BP1, a BH3-only E3 ubiquitin ligase, catalyzes the polyubiquitination of Mcl-1 and regulates apoptosis, *Cell 121*, 1085–1095.
55. Salvat, C., Wang, G., Dastur, A., Lyon, N., and Huibregtse, J. M. (2004) The -4 phenylalanine is required for substrate ubiquitination catalyzed by HECT ubiquitin ligases, *J. Biol. Chem. 279*, 18935–18943.
56. Verdecia, M. A., Joazeiro, C. A. P., Wells, N. J., Ferrer, J. L., Bowman, M. E., Hunter, T., and Noel, J. P. (2003) Conformational flexibility underlies ubiquitin ligation mediated by the WWP1 HECT domain E3 ligase, *Mol. Cell 11*, 249–259.
57. Ogunjimi, A. A., Briant, D. J., Pece-Barbara, N., Le Roy, C., Di Guglielmo, G. M., Kavsak, P., Rasmussen, R. K., Seet, B. T., Sicheri, F., and Wrana, J. L. (2005) Regulation of Smurf2 ubiquitin ligase activity by anchoring the E2 to the HECT domain, *Mol. Cell 19*, 297–308.

58. Reddick, J. J., Cheng, J. M., and Roush, W. R. (2003) Relative rates of michael reactions of 2′-(phenethyl)thiol with vinyl sulfones, vinyl sulfonate esters, and vinyl sulfonamides relevant to vinyl sulfonyl cysteine protease inhibitors, *Org. Lett. 5*, 1967–1970.
59. Lall, M. S., Ramtohul, Y. K., James, M. N. G., and Vederas, J. C. (2002) Serine and threonine β-lactones: a new class of hepatitis a virus 3c cysteine proteinase inhibitors, *J. Org. Chem. 67*, 1536–1547.
60. Krantz, A., Copp, L. J., Coles, P. J., Smith, R. A., and Heard, S. B. (1991) Peptidyl (acyloxy)methyl ketones and the quiescent affinity label concept—the departing group as a variable structural element in the design of inactivators of cysteine proteinases, *Biochemistry 30*, 4678–4687.
61. Larkin, M. A., Blackshields, G., Brown, N. P., Chenna, R., McGettigan, P. A., McWilliam, H., Valentin, F., Wallace, I. M., Wilm, A., Lopez, R., Thompson, J. D., Gibson, T. J., and Higgins, D. G. (2007) Clustal w and clustal x version 2.0, *Bioinformatics 23*, 2947–2948.