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Development of Activity-Based Probes for Ubiquitin and Ubiquitin-like Protein Signaling Pathways

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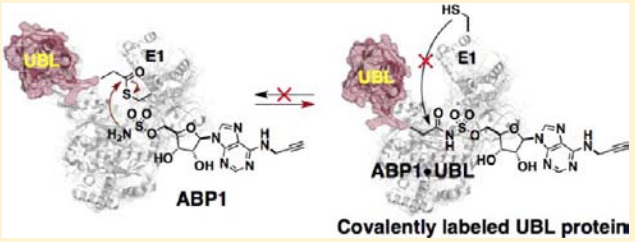
Supporting Information

ABSTRACT: Ubiquitin and ubiquitin-like (UBL) proteins regulate a vast variety of cellular functions. Some UBL proteins are present in all cell types, while others are expressed only in certain cells or under certain environmental conditions. This highlights the central role of UBL systems in regulation of ubiquitous as well as specific cellular functions. UBL proteins share little amino acid sequence identity to each other, yet they share similar 3D shapes, which is exemplified by the β-grasp fold. Central to UBL protein signaling pathways are UBL protein-activating E1 enzymes that activate the C-terminus of UBL proteins for subsequent conjugation to the protein substrates. Due to their critical roles in biology, E1 enzymes have been recognized as emerging drug targets to treat human diseases. In spite of their biological significance, however, methods to discover UBL proteins and to monitor the intracellular activity of E1 enzymes are lacking. Thus, there is a critical need for methods to evaluate the intracellular mechanisms of action of E1 enzyme inhibitors. Here we describe the development of a mechanism-based small-molecule probe, **ABP1**, that can be used to discover and to detect active UBL proteins, and to monitor the intracellular activity of E1 enzymes inside intact cells. The developed probe can also be used to profile the selectivity of E1 enzyme-targeting drugs *in vitro* and inside intact cells.



INTRODUCTION

Ubiquitin and 20 other known ubiquitin-like (UBL) proteins regulate a vast variety of physiological functions by covalently modifying protein substrates, and they serve as important regulators of protein homeostasis.[1] Mis-regulation of UBL systems is known to cause cancers, neurodegenerative disorders, autoimmune diseases, pathogenic infections, and cardiovascular diseases.[2] FDA approval of the proteasome inhibitors bortezomib and carfilzomib, as well as SCF^{DDB1} ubiquitin ligase inhibitors thalidomide, lenalidomide, and pomalidomide, to treat multiple myeloma highlights the therapeutic potential of the ubiquitin system. Other UBL protein systems have also emerged as promising drug targets. For example, a covalent inhibitor of Nedd8 UBL protein activation, MLN4924, is currently undergoing phase 1 clinical trials to treat human cancers.[3]

The covalent conjugation of eukaryotic UBL proteins to protein substrates is controlled by UBL protein-activating E1 enzymes (~8 known), UBL protein-conjugating E2 enzymes (~44 known), and UBL protein E3 ligases (~600 known). Certain UBL proteins such as ubiquitin and SUMO can form poly-ubiquitin and polySUMO chains, adding additional layer of complexity in UBL protein signaling.[4] Isopeptidases (>100 known) cleave UBL proteins from protein substrates, or in certain cases edit branched and linear UBL protein chains, thus antagonizing UBL protein conjugation pathways.[5]

All known UBL proteins range from 8 to 12 kDa in size, except for ISG15 and FAT10 proteins, which are composed of two UBL domains linked through the hinge region and, as a consequence, have molecular weights of 17 and 18 kDa, respectively.[6] The combination of X-ray and NMR structural studies indicates that most known human UBL proteins are characterized by the structurally and evolutionarily conserved β-grasp fold. Although similar in shape, UBL proteins display little amino acid sequence identity to ubiquitin or to each other (<20%) (Figure S1). As a consequence, it is difficult to use bioinformatics methods to discover UBL proteins, and many UBL proteins, such as SUMO proteins, have been discovered serendipitously.[7] Thus, new approaches to discover UBL proteins inside cells are needed.

UBL protein-activating E1 enzymes lie at the apex of the UBL signaling pathways and control the adenosine triphosphate (ATP)-dependent activation of the C-terminus of UBL proteins.[8] E1 enzymes recognize their cognate UBL proteins, activate the carboxyl terminus of UBL proteins, and transfer UBL proteins to the cognate E2 enzymes via the transthiolation reaction. Since the activity of each E1 enzyme regulates the activity of the whole UBL protein-signaling pathway, tools to evaluate the intracellular activity of E1 enzymes are urgently needed. Finally, selective pharmacological inhibitors of E1 enzymes will provide new mechanistic insights on the intracellular functions of UBL signaling pathways and the intracellular effects of E1 enzyme inhibition.[9]

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This paper describes the development of the small-molecule probe **ABP1**, which covalently labels and detects UBL proteins *in vitro* and inside intact cells. The developed **ABP1** serves as an activity-based probe for UBL protein-activating E1 enzymes, enabling the measurement of global activities of E1 enzymes inside intact cells. **ABP1** can also be used as a tool to evaluate the potency and selectivity of E1 enzyme inhibitors *in vitro* and in cells, thus providing a novel tool for drug discovery. During our studies we have discovered that readily prepared *N*-acylsulfamate can serve as a useful pH-cleavable linker for proteomic studies. Other interesting properties of **ABP1** are described.

RESULTS AND DISCUSSION

Design of the Activity-Based Probe ABP1. Ubiquitin and all UBL proteins share a similar biochemical mechanism of activation by E1 enzymes, and this mechanism is well characterized.[10] Initially, ubiquitin-activating enzyme 1 (UBE1) binds ubiquitin and ATP, and forms a ubiquitin·adenosine monophosphate (AMP) adenylate (Figure 1A, step

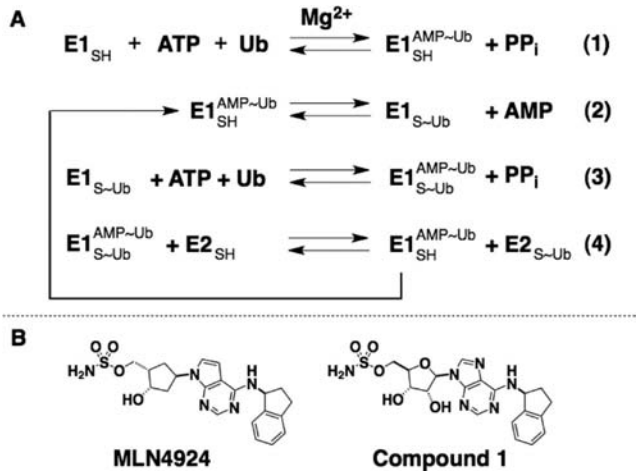

Figure 1. (A) Enzymatic mechanism of ubiquitin activation by UBE1. (B) Chemical structures of Nedd8-activating E1 enzyme inhibitor MLN4924 and a pan-inhibitor of UBL protein-activating E1 enzymes, Compound **1**.

1). Subsequently, the catalytic cysteine of UBE1 undergoes a 1,2-nucleophilic addition to the C-terminus of the ubiquitin·AMP adenylate, forming a binary UBE1~ubiquitin thioester complex (Figure 1A, step 2). The binary UBE1~ubiquitin thioester complex then catalyzes a second round of ubiquitin adenylation, forming a ternary complex of the UBE1~ubiquitin thioester bound to the ubiquitin·AMP adenylate (Figure 1A, step 3). Finally, the resulting ternary complex of UBE1~ubiquitin/ubiquitin·AMP transfers ubiquitin onto the catalytic cysteine of the ubiquitin-conjugating E2 enzyme (Figure 1A, step 4).[11] Nedd8-activating E1 enzyme NAE1/UBA3 shares a similar enzymatic mechanism.[12] Most of the described mechanistic features have also been observed for SUMO-activating E1 enzyme SAE1/UBA2, ubiquitin- and FAT10-activating E1 enzyme UBA6, and ISG15-activating E1 enzyme UBE1L.[13]

The reversibility of each enzymatic step indicates that AMP can bind to the binary E1~UBL protein thioester complex and undergo a reverse reaction to regenerate UBL·AMP adenylate complex, which binds tightly the active site of E1 enzyme with the estimated $K_d \leq 8 \times 10^{-12}$ M in the case of a ubiquitin·AMP/UBE1 complex.[14] The reversibility of this step has been exploited in the development of a mechanism-based inhibitor of NAE1/UBA3, MLN4924, which mimics AMP, binds to the AMP binding site of UBA3~Nedd8 thioester complex, and forms a covalent adduct with Nedd8 (Figure 1B).[15] Subsequent investigations showed that MLN4924 analogue Compound **1** is a nonselective inhibitor of UBL protein-activating E1 enzymes and forms covalent adducts with UBL proteins SUMO1, ubiquitin, Nedd8, ISG15, and GABARAP in the presence of ATP and E1 enzymes *in vitro*, via a similar reaction mechanism (Figure 1B).[9a]

We therefore hypothesized that the Compound **1** scaffold is a good template for an activity-based probe for UBL proteins and their activating E1 enzymes.

Alkyne- or azide-tagged activity-based probes have gained a wide popularity because the alkyne/azide groups are small and nonionic, thus introducing minimal structural and cell-permeability alterations in small-molecule probes.[16] Therefore, we envisioned that the substitution of the indane moiety in Compound **1** with a propargyl group would lead to an activity-based probe for E1 enzymes (Figure 2). The designed **ABP1**

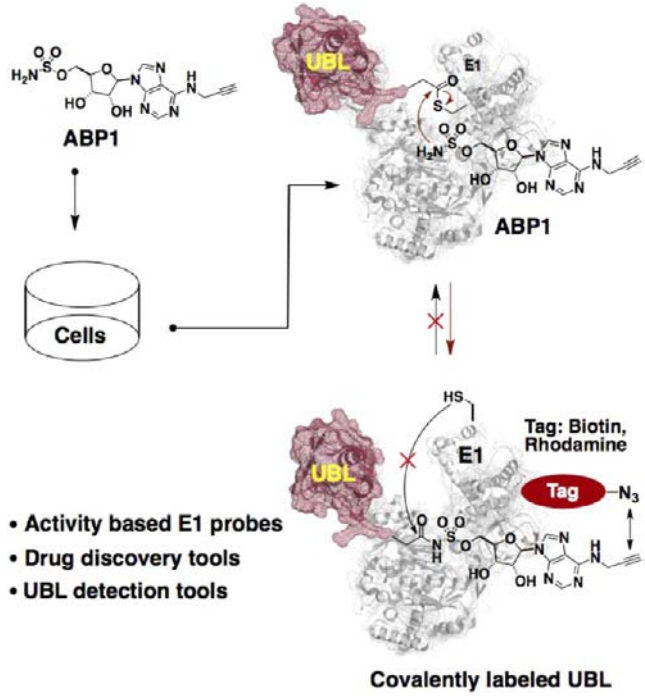

Figure 2. Design and applications of the UBL protein activity-based probe **ABP1**.

probe will bind to the AMP binding site of the binary E1~UBL thioester complex, followed by nucleophilic attack of the sulfamate group in **ABP1** onto the electrophilic thioester of the E1~UBL thioester complex to form a UBL·**ABP1** covalent adduct. This hypothesis is supported by the known high binding affinity of AMP to the UBE1~ubiquitin thioester, with estimated $K_d \approx 27$ nM. **ABP1** structurally resembles AMP and therefore is expected to bind to the AMP binding site of the UBE1~ubiquitin thioester and other E1~UBL thioesters. The proposed mechanism of **ABP1** is similar to those of MLN4924 and Compound **1**.

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Once formed, the alkyne-tagged UBL·**ABP1** adducts can be covalently conjugated to a fluorescent tag or a biotin tag that contains azide functionality via Cu(I)-catalyzed azide−alkyne [3+2] cycloaddition reaction (click chemistry), for visualization and quantification purposes. Because the formation of E1∼UBL thioester is required for the formation of UBL·**ABP1** covalent adduct, we hypothesize that only catalytically active E1 enzymes can produce UBL·**ABP1** covalent adducts. Therefore, the amount of the formed UBL·**ABP1** covalent adducts will serve as a readout of the enzymatic activity of UBL protein-activating E1 enzymes *in vitro* and in cells. Furthermore, we envision that **ABP1** could be used to evaluate the potency and selectivity of mechanistically distinct UBL protein-activating E1 enzyme inhibitors *in vitro* and in cells.[17] Finally, **ABP1** or its analogues may enable the discovery of previously unknown UBL proteins in eukaryotic cells.

ABP1 Forms Covalent Bonds with UBL Proteins. Initially, we chose ubiquitin and the ubiquitin-activating enzyme UBE1 as a model system to test the labeling of UBL proteins with **ABP1** and to develop a protocol to visualize UBL·**ABP1** adducts using click chemistry. The designed **ABP1** was synthesized in four steps using simple synthetic chemistry procedures (Scheme S1). First, we wanted to confirm that **ABP1** forms a covalent adduct with ubiquitin in the presence of UBE1 and ATP. Thus, UBE1, ubiquitin, ATP, and **ABP1** were incubated overnight at room temperature, and the resulting reaction mixture was subjected to electron spray ionization (ESI) MS analysis. The measured value for [M+H] of 8931.67 Da matched well with the expected theoretical value of 8931.92 Da for the ubiquitin·**ABP1** adduct (Figure S2).

The next challenge was to develop a protocol to conjugate biotin or rhodamine tags to the alkyne-bearing ubiquitin·**ABP1** adduct. Our initial attempts to visualize ubiquitin·**ABP1** adduct employing click chemistry protocol were not successful for two reasons. First, the excess of Azide-Fluor-585 reagent (400 μM final concentration) was not separable from the low-molecular-weight ubiquitin·**ABP1** band in SDS-PAGE. This problem was solved by removing the excess of **ABP1** from the reaction mixture using spin-desalting columns, followed by click reaction in the presence of a lower concentration of Azide-Fluor-585 (100 μM).

Second, in-gel fluorescence scanning showed that the efficiency of the conjugation reaction of Azide-Fluor-585 to ubiquitin·**ABP1** adduct under the click reaction conditions was low. We hypothesized that this could be due to the high binding affinity of the ubiquitin·**ABP1** adduct to the active site of UBE1, which makes the alkyne tag of the ubiquitin·**ABP1** adduct inaccessible to Azide-Fluor-585 for covalent conjugation. Therefore, addition of 1% SDS would unfold the E1 enzyme and separate the ubiquitin·**ABP1** adduct from the E1 active site, freeing the alkyne tag for subsequent conjugation with Azide-Fluor-585. Indeed, we found that addition of 1% SDS significantly increased the amount of covalent ubiquitin·**ABP1** adduct formation (Figure S3). For convenience we performed this labeling reaction in the presence of ubiquitin-conjugating E2 enzyme UbcH5a, since we have found that E2 enzyme increases the amount of ubiquitin·**ABP1** covalent adduct during the reaction. The role of E2 enzyme in this reaction will be discussed further in the text. We found that 30 min was the optimal reaction time to covalently label ubiquitin·**ABP1** covalent adduct with Azide-Fluor-585 (Figure S3). When the click chemistry labeling reaction was run for 60 min instead of 30 min, nonspecific labeling of ubiquitin with Azide-Fluor-585 occurred in the absence of UBE1, but in the presence of UbcH5a, ATP, and **ABP1**. Thus, 30 min is the optimal reaction time for click reaction to visualize covalently labeled UBL proteins. With the developed protocol in hand, we were able to visualize the fluorescent ubiquitin·**ABP1** covalent conjugate. The covalent adduct formation was dependent on UBE1 and **ABP1** concentration (Figure 3) and incubation time

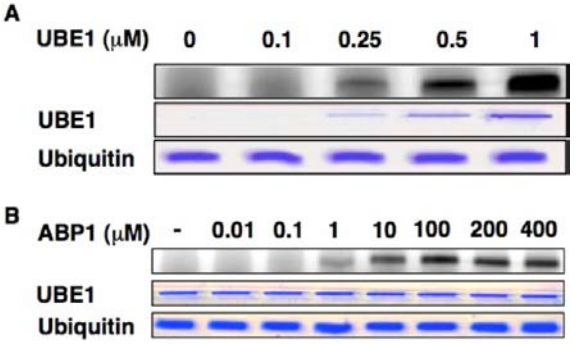

Figure 3. UBE1 and **ABP1** concentration dependence of the ubiquitin·**ABP1** formation. (A) Ub (50 μM), ATP (50 μM), and **ABP1** (400 μM) were treated with different concentrations of UBE1 enzyme, incubated at room temperature for 1 h, subjected to click reaction conditions, and resolved by SDS-PAGE, followed by in-gel fluorescence scanning. Top, in-gel fluorescence scan; bottom, Coomassie stain. (B) UBE1 (1 μM), Ub (50 μM), and ATP (50 μM) were treated with different concentrations of **ABP1**, incubated at room temperature for 1 h, subjected to click reaction conditions, and resolved by SDS-PAGE, followed by in-gel fluorescence scanning. Top, in-gel fluorescence scan; bottom, Coomassie stain.

(Figure S4). Ubiquitin formed a covalent adduct with **ABP1** after 5 min of incubation time and formed saturating amounts after 30 min of reaction time. We did not observe the covalent labeling of ubiquitin in the absence of UBE1 or ATP (Figures 3A and S7).

We hypothesize that a ubiquitin·**ABP1** adduct contains an *N*-acylsulfamate moiety that links the C-terminal Gly[76] of ubiquitin to **ABP1**, and this link is stable in physiological and click chemistry reaction conditions. Extensive biochemical and X-ray crystallography studies on the mechanism of action of MLN4924 and Compound **1** confirm our hypothesis, as **ABP1** is similar in structure to Compound **1**.[9a,15]

Having prepared **ABP1**, we decided to test if we can design structural analogues of **ABP1** with improved UBL detection properties. It has been shown that Compound **1** analogugs were more potent at inhibiting UBE1- and Nedd8-activating enzyme (NAE/UBA3) when the size of the hydrophobic substituent at the exocyclic amine was increased.[18] We therefore prepared two **ABP1** analogues, **ABP2** and **ABP3**,[19] in which the propargyl amine moiety of **ABP1** was replaced with 4-pentyn-1-amine and 5-hexyn-1-amine functionalities, respectively (Figure S5). Under the developed labeling reaction conditions, we observed a slight increase in the covalent labeling of the ubiquitin with **ABP2** probe and a slight decrease in ubiquitin labeling with **ABP3** probe; however, the observed difference was not significant. Therefore, we decided to proceed forward and investigate the chemical properties of **ABP1**.

We tested the generality of **ABP1** for the covalent labeling of UBL proteins. Eight different types of UBL proteins were incubated with their corresponding E1 enzymes, ATP, and **ABP1**. In-gel fluorescence detection showed that the eight

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tested UBL proteins were covalently labeled by **ABP1** (Figure 4A).[20] Similar to ubiquitin, the covalent labeling of Nedd8 and

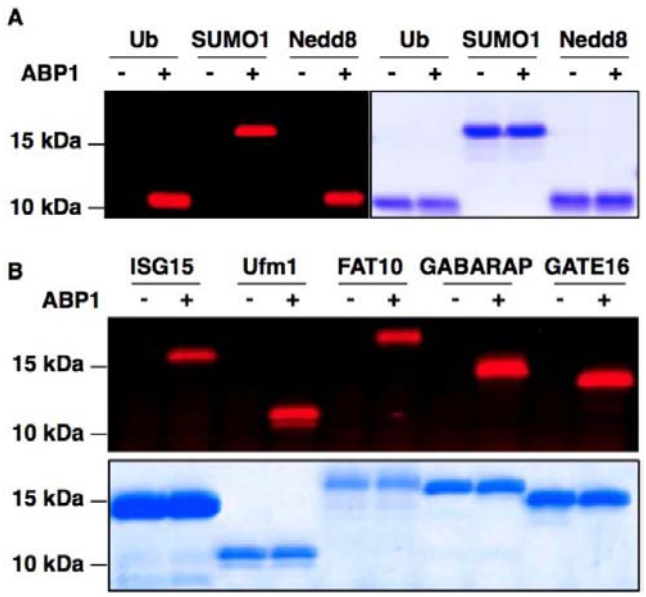

Figure 4. Generality of the UBL·**ABP1** formation. The following UBL/E1 pairs (E1, 1 μM; UBL protein, 50 μM) were incubated with ATP (50 μM) in the presence of **ABP1** (400 μM): (A) Ub-UBE1, SUMO1-SAE1/SAE2, Nedd8-NAE1/UBA3, and (B) ISG15-UBE1L, His_6UFM1-UBA5, FAT10-UBA6, GABARAP-ATG7, and GATE16-ATG7. Reaction mixtures were incubated at room temperature overnight, subjected to click reaction conditions, and resolved by SDS-PAGE, followed by in-gel fluorescence scanning.

Ufm1 was time dependent (Figure S6). Nedd8 formed saturating amounts of a covalent adduct with **ABP1** rapidly after 30 min of reaction time, while the formation of Ufm1·**ABP1** covalent adduct was slower and required >90 min. The observed difference in the UBL·**ABP1** formation kinetics could be due to the different E1 enzyme turnover rates, different rates of nucleophilic attack of the sulfamate group of **ABP1** onto the E1∼UBL thioester, or differences in the inhibition of E1 enzyme with the covalent UBL·**ABP1** adducts. Investigations to distinguish these possibilities are currently in progress, yet overall our results indicate that **ABP1** is a mechanism-based probe not only for canonical E1s (UBE1, SAE1/SAE2, NAE1/UBA3, UBE1L, and UBA6) but also for noncanonical E1s (UBA5 and ATG7).

We subsequently tested whether the formation of UBL·**ABP1** adducts can occur when multiple UBL proteins and E1 enzymes are present in the reaction mixture. These reaction conditions will mimic the intracellular environment, where multiple UBL proteins and their E1 enzymes are present. Under these conditions we observed covalent labeling of four model UBL proteins (ubiquitin, SUMO1, Ufm1, and ISG15) in the presence of their corresponding activating E1 enzymes (UBE1, SAE1/SAE2, UBA5, and UBE1L) (Figure S7). Subsequently, we tested if the covalent labeling of UBL proteins with **ABP1** can occur in the presence of guanosine triphosphate (GTP) as a source of nucleotide triphosphate, instead of ATP. The rationale for this experiment was the relatively high intracellular concentration of GTP, ∼500 μM for GTP versus ∼3 mM for ATP.[21] Interestingly, we observed the formation of UBL·**ABP1** covalent adducts in the presence of GTP when we used a mixture of four model UBL proteins and their cognate E1 enzymes, although a much higher concentration of GTP had to be used (600 μM GTP vs 75 μM ATP, Figure S7). These observations are in agreement with previous results showing that UBE1 can form a UBE1∼ubiquitin thioester complex using GTP as a source of nucleotide triphosphate; however, a much higher concentration of GTP was required compared to ATP.[22] Our results indicate that GTP can promote the formation of UBL·**ABP1** complexes and thus E1∼UBL thioesters *in vitro*.

We subsequently decided to test if **ABP1** can be used to evaluate the specificity of UBL protein activation by a particular E1 enzyme. Since eight known UBL protein-activating E1 enzymes activate 20 known UBL proteins, one of the challenges in the field is to understand why specific E1 enzymes activate unique or multiple UBL proteins.

We tested if UBE1, which activates ubiquitin, can promote the formation of the mismatched Nedd8·**ABP1** covalent complex in the presence of ATP, **ABP1**, and Nedd8. When performing test reactions that contained UBE1 (1 μM), Nedd8 (50 μM), ATP (50 μM), and **ABP1** (400 μM), we did observe the formation of low amounts of Nedd8·**ABP1** complex, but these were not as significant as with the UBE1/ubiquitin pair (Figure S8A,B). On the other hand, Nedd8-activating enzyme NAE/UBA3 promoted the formation of both ubiquitin·**ABP1** and Nedd8·**ABP1** covalent complexes with similar efficiency under similar reaction conditions (Figure S8A).

Thus, our results indicate that UBE1 and NAE/UBA3 can form thioesters with both ubiquitin and Nedd8 proteins, albeit with different efficiencies *in vitro*. This is not surprising, given the high degree of sequence identity (55%) between ubiquitin and Nedd8. Prior work has shown that changes in the intracellular Nedd8:ubiquitin ratio trigger atypical Nedd8 activation by UBE1.[23] Indeed, the current model suggests that, because the intracellular concentration of ubiquitin is much higher than that of Nedd8 (3−10-fold difference depending on the cell line), ubiquitin prevents Nedd8 from being utilized as a substrate by UBE1.[15d]

ABP1 Provides Insights on the Ability of E1 Enzymes To Activate poly-ubiquitin Chains. In 1990, Chen and Pickart reported that Lys^{48}-linked di-ubiquitin could be efficiently activated by the UBE1 enzyme and transferred onto the ubiquitin-conjugating E2 enzyme (E2-25K) *in vitro*.[24] Intrigued by this, we decided to test whether **ABP1** can serve to detect the activation of poly-ubiquitin chains by UBE1 *in vitro*. Commercially available Lys^{48} and Lys^{63}-linked di-ubiquitin and Lys^{48}-linked tetra-ubiquitin chains were incubated with UBE1 in the presence of ATP and **ABP1** and covalently labeled poly-ubiquitin chains were visualized using previously developed reaction conditions. Remarkably, we observed the covalent labeling of not only di-ubiquitin chains but also tetra-ubiquitin chains with **ABP1**, with efficiency similar to that of ubiquitin (Figure 5). In the cases of Ub_2(K63) and Ub_2(K48), we also observed low-intensity fluorescent bands at ∼25 kDa, which corresponds to the impurity of Ub_4(K48) chains in the commercially available Ub_2(K63) and Ub_2(K48) chains (Figure S9). Thus, our results indicate that **ABP1** can detect the activation of poly-ubiquitin chains and the formation of binary UBE1∼polyUb thioester complexes *in vitro*.

Mechanistic Studies of UBL·ABP1 Covalent Complex Formation. **ABP1** forms covalent adducts with UBL proteins by binding at the ATP/AMP binding site of E1∼UBL thioesters, and the resulting UBL·**ABP1** covalent complex,

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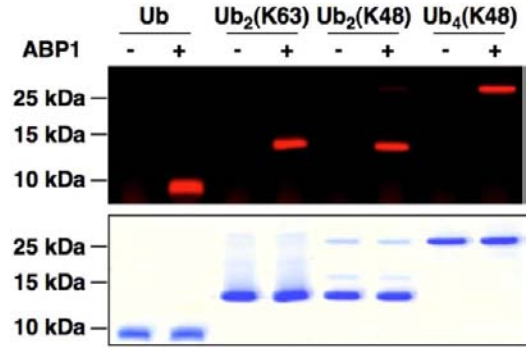

Figure 5. Covalent labeling of poly-Ub chains with **ABP1** was performed as described in Figure 4, except poly-Ub chains (di-Ub chains, 50 μM; tetra-Ub chain, 25 μM) were used as a source of ubiquitin. Top, in-gel fluorescence scan; bottom, Coomassie stain.

which mimics the UBL·AMP adenylate intermediate, binds tightly to the E1 enzyme. Therefore, **ABP1** should inhibit the enzymatic activity of E1 enzymes. This property is expected since **ABP1** is very similar in structure and in its mechanism of action to Compound **1**, a known pan-inhibitor of E1 enzymes. Indeed, we observed the inhibition of substrate poly-ubiquitination in the standard ubiquitination assay where UBE1, ubiquitin-conjugating E2 enzyme UbcH5a, HECT E3 ubiquitin ligase Rsp5, ubiquitin, ATP, and fluorescent GFP-Sic60 substrate protein were incubated in the presence of **ABP1** or Compound **1** as a control (Figure S10). Sic60-GFP protein is a known artificial substrate of Rsp5 that contains a green fluorescent protein (GFP) tag at its C-terminus, which allows convenient monitoring of Sic60-GFP ubiquitiation using in-gel fluorescence imaging.[25]

To test the correlation between the ubiquitin·**ABP1** covalent adduct formation and the inhibition of ubiquitin-activating enzyme, we performed the same ubiquitination assay followed by visualization of the covalent ubiquitin·**ABP1** adducts via click chemistry.

We observed a decrease in the amounts of poly-ubiquitinated GFP-Sic60 protein substrate with the concomitant increase in the amount of formed ubiquitin·**ABP1** adduct (Figure 6). These data further support the notion that **ABP1** is a mechanism-based probe for ubiquitin and UBL protein

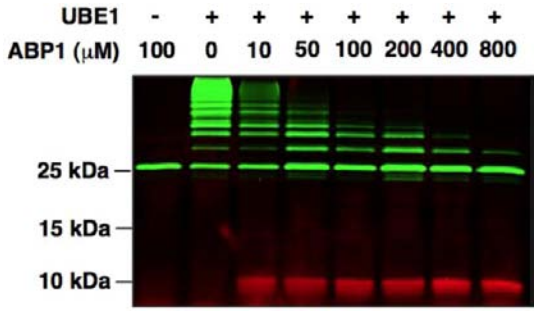

Figure 6. Inhibition of protein ubiquitination correlates with the formation of the ubiquitin·**ABP1** adduct. UBE1 (0.1 μM), UbcH5a (1 μM), Rsp5 (0.5 μM), GFP-Sic60 (0.5 μM), Ub (50 μM), and ATP (50 μM) were treated with different concentrations of **ABP1**, incubated for 1 h at room temperature, and subjected to click reaction conditions. The amounts of GFP-Sic60 and poly-ubiquitinated GFP-Sic60 (green) as well as the amount of ubiquitin·**ABP1** adduct (red) were visualized using in-gel fluorescence scanning.

signaling pathways. The developed **ABP1** also inhibits the activity of SUMO-activating E1 enzyme *in vitro*, and conjugation of another UBL protein, ISG15, to its protein substrates in A549 cells upon interferon-β stimulation (Figures S11 and S12). ISG15 is a critical regulator of anti-mycobacterial and anti-viral immunity in vertebrates,[26] and this is the first described example of pharmacological inhibition of ISG15 conjugation to intracellular proteins. A similar effect was observed for Compound **1** (Figure S12). Taken together, previously published work and our results validate **ABP1** and Compound **1** as scaffolds for the future design of selective inhibitors of UBL protein signaling pathways.

Having established that inhibition of the enzymatic ubiquitination reaction is accompanied by formation of the covalent ubiquitin·**ABP1** adduct, we decided to test the effect of the downstream components of the ubiquitin system on the efficiency of the UBL·**ABP1** adduct formation. We designed two sets of experiments. The first reaction contained UBE1, ubiquitin, and ATP, while the second reaction contained E2 enzyme UbcH5a, E3 enzyme Rsp5, and Sic60-GFP protein substrate as well as UBE1, ubiquitin, and ATP. Both reactions were incubated for 1 h in the presence of increasing concentrations of **ABP1**, and the amount of ubiquitin·**ABP1** adduct was quantified (Figures 7 and S13A–C).

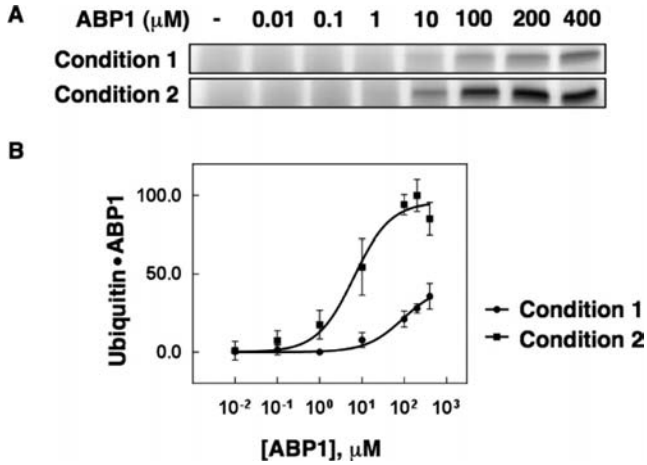

Figure 7. Downstream components of the ubiquitination system increase the formation of the ubiquitin·**ABP1** adduct. (A) Ubiquitin·**ABP1** adduct was formed in two different conditions. Condition 1: UBE1 (0.1 μM), ubiquitin (50 μM), ATP (50 μM), and different concentrations of **ABP1** were incubated at room temperature for 1 h and then subjected to click reaction conditions for visualization purposes. Condition 2: UBE1 (0.1 μM), UbcH5a (1 μM), Rsp5 (0.5 μM), GFP-Sic60 (0.5 μM), ubiquitin (50 μM), ATP (50 μM), and different concentrations of **ABP1** were incubated at room temperature for 1 h and then processed as described for condition 1. (B) Quantification of the amount of ubiquitin·**ABP1** adduct under two different conditions. Each data point is shown as a triplicate.

Interestingly, the amount of ubiquitin·**ABP1** adduct was significantly increased when UbcH5a, Rsp5, and GFP-Sic60 were present in the reaction mixture. Under these experimental conditions, half the amount of ubiquitin·**ABP1** adduct ($K_{1/2}$) was reached at 6.4 ± 0.5 μM **ABP1**. In the absence of UbcH5a, Rsp5, and Sic60-GFP, the amount of ubiquitin·**ABP1** adduct was decreased 11-fold at the same concentration of **ABP1** (6.4 μM). These results suggest that downstream components of

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the ubiquitination machinery increase the amount of the ubiquitin·**ABP1** adduct formation *in vitro*.

Since the ubiquitin-conjugating E2 enzyme, UbcH5a, is directly downstream from UBE1, we hypothesized that UbcH5a may increase the formation of the ubiquitin·**ABP1** adduct. To test this hypothesis, UBE1, ubiquitin, ATP, and **ABP1** were incubated in the presence of increasing concentrations of UbcH5a.

As we expected, the amount of ubiquitin·**ABP1** adduct increased with increasing concentration of UbcH5a (Figure 8A). We validated the generality of the observed phenomena with another E2 enzyme, UbcH7, which increased the formation of the covalent ubiquitin·**ABP1** adduct as well (Figure 8B).

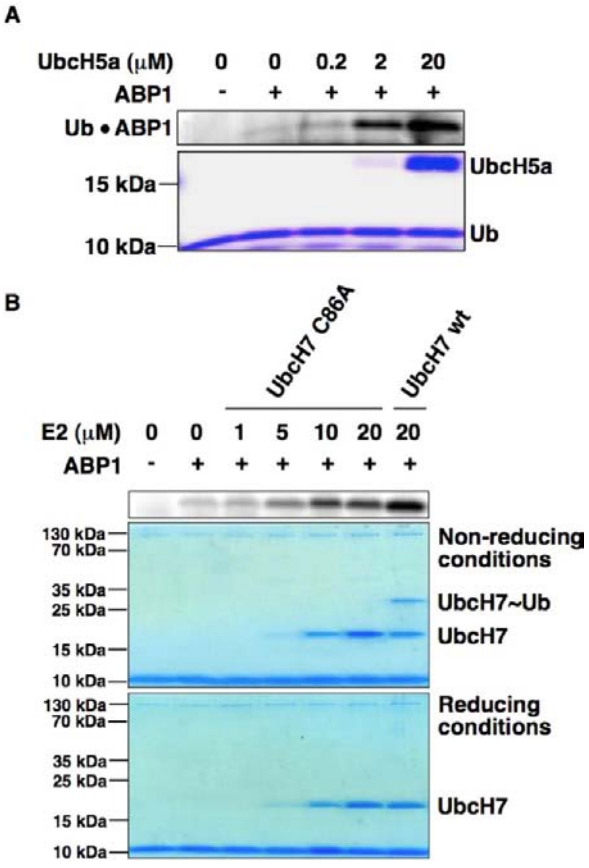

Figure 8. Effect of ubiquitin-conjugating E2 enzymes on the formation of the ubiquitin·ABP1 adduct. (A) Covalent labeling of ubiquitin with **ABP1** was tested in the presence of UBE1 (0.1 μM), ubiquitin (50 μM), ATP (50 μM), **ABP1** (50 μM), and increasing concentrations of UbcH5a. (B) Catalytically inactive UbcH7 promotes the formation of the ubiquitin·**ABP1** adduct. Covalent labeling of ubiquitin with **ABP1** in the presence of UBE1 (0.2 μM), ubiquitin (50 μM), ATP (50 μM), **ABP1** (50 μM), and UbcH7 (20 μM) or in the presence of increasing concentrations of UbcH7 C86A mutant. Top, in-gel fluorescence scan; bottom, Coomassie stain under the nonreducing and reducing conditions.

To show that the observed increase in the formation of ubiquitin·**ABP1** adduct is not due to a chemical reaction between UbcH7~Ub thioester and **ABP1**, we prepared the UbcH7~Ub thioester complex by incubating UBE1, UbcH7, ubiquitin, and ATP for 1 h, followed by the addition of UBE1 inhibitor Pyr41. We have found that EDTA, which is typically used to quench E1 enzyme activity, inhibits click reaction in our conditions most likely by chelating copper; that is why Pyr41 was used. Addition of ABP1 to this UbcH7~Ub-containing reaction mixture did not result in the formation of ubiquitin·**ABP1** adduct (Figure S14). We next asked if catalytic acitivity of E2 enzymes was responsible for the observed phenomena. We prepared the catalytically inactive UbcH7 C86A mutant and tested the formation of the ubiquitin·**ABP1** adduct in the presence of this mutant. Interestingly, we have found that UbcH7 C86A mutant can also promote the formation of the ubiquitin·**ABP1** adduct (Figure 8B). These results indicate that the protein−protein binding interactions between UBE1 and E2 enzymes could be responsible for the observed increase in the ubiquitin·**ABP1** adduct formation. Furthermore, SUMO-conjugating E2 enzyme Ubc9 did not increase the amount of ubiquitin·**ABP1** adduct in our assay, while Nedd8-conjugating E2 enzyme Ubc12, which forms mismatched Ubc12~Ub complex by functionally interacting with UBE1 at high concentrations,[27] produced increased amount of ubiquitin·**ABP1** complex (Figure 9).

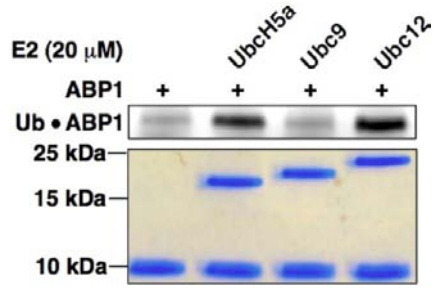

Figure 9. Effect of other UBL-conjugating E2 enzymes on the formation of the ubiquitin·ABP1 adduct. Ubiquitin labeling with **ABP1** was tested in the presence of UBE1 (0.2 μM), ubiquitin (50 μM), ATP (50 μM), and **ABP1** (50 μM) and ubiquitin-, SUMO-, and Nedd8-conjugating E2 enzymes. Top, in-gel fluorescence scan; bottom, Coomassie stain.

Taken together, our observations suggest that E2 enzymes that display protein−protein interactions with UBE1 enhance the amount of ubiquitin·**ABP1** adduct formation, and this can potentially be used as a readout to identify E2 enzymes that bind and/or functionally interact with their cognate E1 enzymes. However, further experiments are required to address how specific E1-E2 interactions promote the formation of the ubiquitin·**ABP1** adduct.

ABP1 Can Measure the Potency and Selectivity of E1 Enzyme Inhibitors *in Vitro*. FDA approval of the proteasome inhibitors bortezomib and carfilzomib to treat multiple myeloma fueled further efforts to discover drugs that target other components of the ubiquitin system. To this end, UBL protein-activating E1 enzymes are emerging drug targets, and the inhibitor of Nedd8-activating E1 enzyme, MLN4924, is currently undergoing phase 1 clinical trials to treat six different types of human cancers.

At present, two assay methods are widely used to measure changes in the catalytic activity of E1 enzymes in the presence of pharmacological inhibitors. The first method is an ATP−PP_i exchange assay, which requires the use of radioactive $^{32}PP_i$.[28] The second method is E1−E2 trans-thiolation assay, which relies on Western blotting.[18]

The first method involves the use of hazardous radioactive materials, while the second method is costly and and labor- and

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time-intensive. Therefore, methods for safe, rapid, and cheap screening of small-molecule inhibitors of E1 enzymes are in critical need. Since the formation of UBL·**ABP1** covalent adduct requires catalytically active E1 enzyme that can form E1∼UBL protein thioester complex, we hypothesized that **ABP1** can be used as a tool to screen for small-molecule inhibitors of E1 enzymes. If an inhibitor inhibits the E1 enzyme, it should also inhibit the formation of the UBL·**ABP1** adduct.

To test this, we pretreated ubiquitin-activating E1 enzyme UBE1 and Nedd8-activating E1 enzyme NAE1/UBA3 with Compound **1**, a pan-inhibitor of E1 enzymes; MLN4924, a selective inhibitor of NAE1/UBA3; and Pyr 41, a selective inhibitor of UBE1 *in vitro*.[29] This was followed by addition of ATP, **ABP1**, and ubiquitin or Nedd8, respectively. Because Compound **1** is a known general inhibitor of E1 enzymes, we expected a dose-dependent inhibition of Nedd8·**ABP1** and ubiquitin·**ABP1** adduct formation. On the other hand, MLN4924 should only inhibit the formation of Nedd8·**ABP1** adduct, while Pyr 41 should only inhibit the formation of ubiquitin·**ABP1** adduct. Indeed, increasing concentrations of Compound **1** led to a decrease in covalent labeling of both ubiquitin and Nedd8 with **ABP1** (Figure 10). As expected,

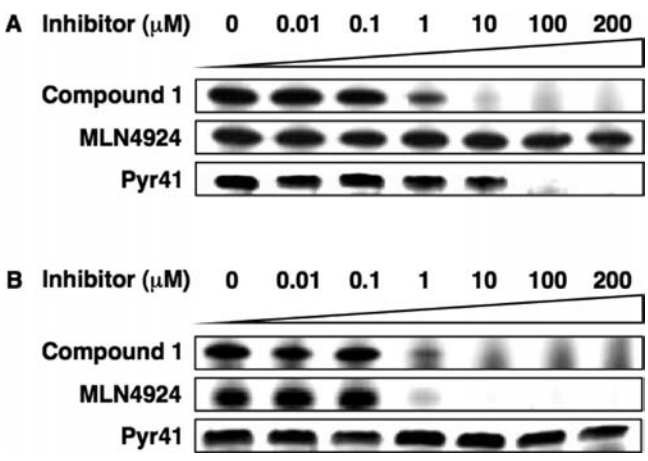

Figure 10. Effect of E1 inhibitors on ubiquitin·**ABP1** formation. (A) UBE1 (0.5 μM), Ub (50 μM), and ATP (50 μM) were treated with different concentrations of E1 enzyme inhibitors and incubated for 1 h at room temperature. **ABP1** (400 μM) was added, and the reaction mixtures were further incubated for 2 h. The reaction mixtures were subjected to click reaction conditions and resolved by SDS-PAGE, and the covalent ubiquitin·**ABP1** adduct was visualized using in-gel fluorescence scanning. (B) NAE/UBA3 (0.5 μM), Nedd8 (50 μM), and ATP (50 μM) were treated with different concentrations of E1 enzyme inhibitors and incubated for 1 h at room temperature, followed by addition of **ABP1** (400 μM) and incubation for 2 h. The reaction mixtures were processed as above.

MLN4924 inhibited the formation of the Nedd8·**ABP1** adduct but did not inhibit the formation of the ubiquitin·**ABP1** adduct. Likewise, Pyr 41 inhibited the formation of ubiquitin·**ABP1** complex, but not the formation of Nedd8·**ABP1** adduct.

It is important to note that Compound **1** and MLN4924 are mechanism-based nucleophilic inhibitors that target electrophilic E1∼UBL thioester complex. Pyr 41, on the other hand, is an electrophilic inhibitor that inhibits ubiquitin-free UBE1, presumably via covalent modification of the catalytic cysteine. Therefore, the developed **ABP1** can successfully be used as a general screening tool to assay E1 enzyme inhibitors that operate via distinct enzyme inhibition mechanisms. The developed method offers a simple, fast, and safe approach to test the potency and selectivity of small-molecule inhibitors of E1 enzymes *in vitro*, which eliminates the need to use radioactive materials as well as the need to use time-consuming Western blotting techniques.

ABP1 Covalently Labels UBL Proteins Inside Intact Cells. Our *in vitro* studies showed that the developed **ABP1** covalently labels UBL proteins *in vitro*, thus suggesting the possibility that **ABP1** can also covalently label UBL proteins in cells. To test this hypothesis, adenocarcinomic human alveolar basal epithelial A549 cells were treated with **ABP1**, followed by cell lysis and conjugation to Azide-Fluor-585. We observed two intensely labeled fluorescent bands with MW ∼15 and ∼10 kDa, which correspond well with the expected molecular weights of several known UBL proteins (Ub, Nedd8, and Ufm1, ∼9 kDa; ISG15 and FAT10, ∼15 kDa) (Figure 11, Table S1). SUMO proteins are the only exception since the molecular weight of these proteins is ∼11 kDa, yet they migrate at 15 kDa apparent molecular weight.

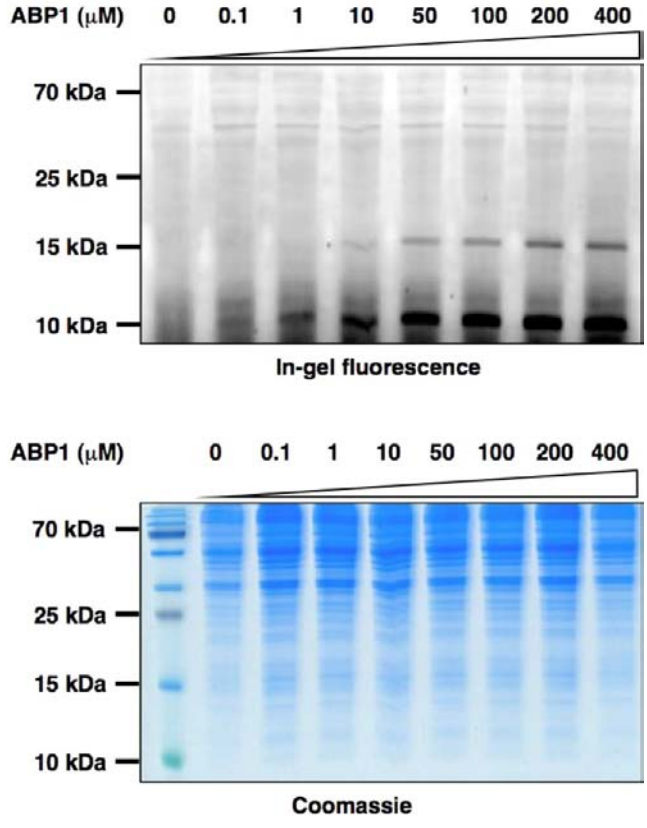

Figure 11. Concentration dependence on UBL·ABP1 formation in live A549 cells. A549 cells were treated with increasing concentrations of **ABP1** for 1 h followed by lysis, conjugation reaction with Azide-Fluor-585 under the click reaction conditions, and in-gel fluorescence detection.

The formation of UBL·**ABP1** adducts in A549 cells was both dose- and time-dependent, with the formation of covalent conjugates observed as fast as 5 min after **ABP1** treatment (Figure S15). We found that treatment of A549 cells with 50 μM **ABP1** for 60 min was sufficient to produce saturating amounts of UBL·**ABP1** adducts. We also decided to use a pull-down assay combined with the Western blotting techniques to visualize covalently labeled UBL proteins with **ABP1**. **ABP1**-

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labeled proteins in A549 cells were conjugated to biotin-azide (PEG_4 carboxamide-6-azidohexanyl biotin) under the click reaction conditions and incubated with streptavidin beads, followed by washes and elution by boiling in 2× laemmli loading buffer. We detected multiple biotinylated protein bands that were not detected by the in-gel fluorescence scanning method (Figure S16). We were also able to detect isolated endogenous ubiquitin in our pull-down experiment using Western blotting techniques (Figure S16).

Encouraged by our preliminary findings, we decided to identify proteins covalently labeled with **ABP1** using proteomic methods. A549 cells were treated with **ABP1** (100 μM) in the presence or absence of **ABP1** competitor Compound **1**, followed by cell lysis, conjugation to biotin-azide, and enrichment with streptavidin-conjugated beads. The streptavidin beads were then washed extensively and subjected to on-bead digestion with trypsin or LysC to identify specifically bound proteins. The resulting peptides were separated by reversed-phase chromatography and analyzed on an LTQ-Orbitrap mass spectrometer. We have identified ubiquitin, SUMO1, SUMO2/3, Nedd8, and Ufm1 present in A549 cells (Table S2). Using a similar protocol we identified UBL proteins (ubiquitin, SUMO1, SUMO2/3, Nedd8, and Ufm1) in HeLa, LNCap, and MCF7 cancer cells derived from different human tissues (Table S3).

It is important to highlight the sensitivity of the developed UBL detection protocol. Recent reports of an absolute quantification of UBL proteins in multiple cell lines showed that the relative concentration of UBL proteins in cells displays up to a 100-fold difference. For example, the absolute concentrations of SUMO1 are as follow: in HeLa cells, 0.7 fmol/mg; in SUMO2/3, 20 fmol/mg; in Nedd8, 11 fmol/mg; and in Ub, 99 fmol/mg.[15d] The fact that we could detect SUMO1 in HeLa cells in the presence of a 100-fold excess of ubiquitin indicates the high sensitivity of **ABP1** and the developed mass spectrometry (MS) protocol for UBL detection.

Together, these experiments suggest that the developed **ABP1** is cell membrane-permeable and can be used to covalently label and detect UBL proteins in intact cells of different origin, as long as the UBL proteins share a common biochemical mechanism of activation, similar to that of ubiquitin.

ABP1 Is a Useful Probe To Evaluate the Selectivity of E1 Enzyme Inhibitors inside Intact Cells. Since UBL protein-activating E1 enzymes (~8 known) are emerging drug targets and regulate critical cell signaling pathways, the evaluation of intracellular selectivity of E1 enzyme inhibitors is critical. To date, intracellular selectivity of inhibitors has been evaluated by measuring total levels of UBL protein conjugation in cells upon treatment of cells with E1 enzyme inhibitors. However, the levels of UBL protein conjugates can be controlled not only by the activity of E1 enzyme but also by the activity of E2 and E3 enzymes and isopeptidases. In addition, emerging evidence shows that a single E1 enzyme can activate multiple UBL proteins. For example, UBA6 activates both ubiquitin and FAT10. Both of these proteins serve as signals for the proteasomal degradation.[30] Therefore, even selective E1 enzyme inhibitors can affect the fate of multiple UBL protein signaling pathways. Thus, more direct interpretation of pharmacological E1 enzyme inhibition is important for both basic research and drug discovery purposes.

In our previous experiments, we have shown that **ABP1** can successfully be used to evaluate the selectivity and potency of E1 enzyme inhibitors *in vitro*, using Compound **1**, MLN4924, and Pyr 41 as model E1 enzyme inhibitors. After proving that **ABP1** is cell permeable and labels endogenous UBL proteins, we hypothesized that **ABP1** could be used to evaluate the intracellular potency and selectivity of E1 enzyme inhibitors. A549 cells were pretreated with different concentrations of Compound **1**, followed by treatment with **ABP1**, cell lysis, and fluorescent detection of UBL·**ABP1** conjugates. We observed that Compound **1** inhibited the labeling of the lower ~10 kDa band in a dose-dependent manner, and there was a slight decrease in the labeling of the upper ~15 kDa band (Figure S17). In a different set of experiments, we pretreated A549 cells with Compound **1**, MLN4924, and Pyr41 to study if ABP1 can measure the selectivity of E1 inhibitors inside cells (Figure 12). Consistent with Figure S17, Compound **1** showed a significant decrease in ~10 kDa bands with a slight decrease in the ~15 kDa band. Unlike Compound **1**, MLN4924 and Pyr41 did not cause significant changes in the fluorescence intensity of ~10 and ~15 kDa bands (Figure 12A). We assumed this is because of the overlapping molecular weights of ubiquitin and Nedd8 as well as an excess of endogenous ubiquitin when compared to endogenous Nedd8.[15d] This prevents us from distinguishing the differences in labeling between these proteins in the case of MLN4924 (Figure 12A).

To measure the intracellular selectivity of Pyr41 and MLN4924, we performed pull-down experiments, in which covalent UBL·**ABP1** adducts in A549 cells were conjugated to biotin-azide, and the resulting biotin tagged UBL·**ABP1** adducts were isolated using streptavidin-conjugated beads. The amount of isolated UBLs was detected via Western-blotting techniques using specific anti-ubiquitin, anti-SUMO2/3, and anti-Nedd8 antibodies (Figure 12B). We observed significant decreases in the amounts of isolated ubiquitin and Nedd8 proteins from A549 cells, which were pretreated with Compound **1**. However, Compound **1** caused a slight decrease in the amount of isolated SUMO2/3, as judged from the Western blot, which may be indicative of the weak inhibitory potency of Compound **1** against SUMO E1 enzyme inside A549 cells. These Western blot results correspond well with the previous in-gel fluorescent results (Figure S17). Treatment of A549 cells with MLN4924 selectively decreased the amount of isolated Nedd8 but did not affect the amount of isolated SUMO2/3 and ubiquitin. Treatment of A549 cells with Pyr41 (50 μM) prior to **ABP1** treatment did not inhibit the amounts of isolated ubiquitin, SUMO2/3, and Nedd8 using our pull-down protocol. This indicates that Pyr41, although selective at inhibiting ubiquitin-activating E1 enzyme *in vitro*, does not inhibit ubiquitin, SUMO, and Nedd8 E1 enzymes in cells at the given concentration. Taken together, our experiments show that **ABP1** and the developed Western blotting technique can be used to deconvolute intracellular targets of mechanistically distinct E1 enzyme inhibitors.

Finally, we measured the changes in UBL protein labeling with **ABP1** upon Compound **1** treatment using semi-quantitative label-free MS analysis, as previously described.[31] A549, HeLa, LNCap, and MCF7 cells were pretreated with Compound **1** or DMSO, followed by **ABP1** treatment, pull-down with streptavidin beads, on-bead trypsin digestion, and MS analysis. Relative quantities of each UBL protein in the four cell lines were calculated by summing the total ion intensity values of each UBL peptide per UBL protein (Figure S18A).

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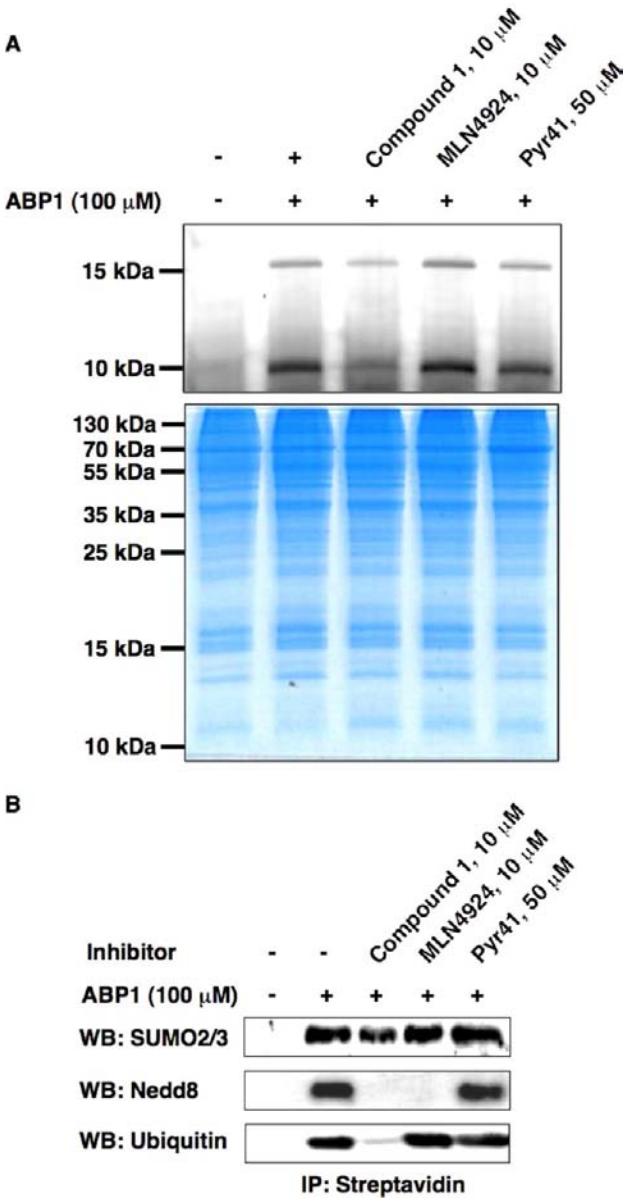

Figure 12. ABP1 can measure the intracellular potency and selectivity of E1 enzyme inhibitors. A549 cells were treated with different E1 inhibitors and incubated for 30 min, followed by the addition of **ABP1** (100 μM) and incubation for an additional 1 h. Cells were lysed, followed by conjugation reaction with rhodamine/biotin-azide using click chemistry reaction conditions. (A) An in-gel fluorescence scan to visualize UBL·**ABP1** covalent adducts. Top, in-gel fluorescence; bottom, Coomassie stain. (B) Biotin-labeled UBL proteins were pulled down with streptavidin beads, followed by Western blotting using UBL antibodies.

Consistent with the in-gel fluorescence data and Western blotting result, Compound **1**-treated A549 cells showed significantly decreased amounts of isolated ubiquitin and Nedd8 compared to control. However, the amount of isolated SUMO was not decreased as much as that of ubiquitin or Nedd8 in A549 and MCF7 cells (Figure S18A). Interestingly, the label-free semiquantitative technique showed that Compound **1** was more potent at inhibiting covalent labeling of SUMO proteins with **ABP1** in HeLa and LNCap cells, indicating that pharmacological potency and selectivity of Compound **1** and most likely its analogues are cell context dependent. These data are in agreement with our in-gel fluorescence data (Figure S18B).

Taken together, our results suggest that **ABP1** can serve as a useful probe to measure the E1 enzyme inhibitor selectivity inside intact cells. Differences in covalent UBL protein labeling can be consistently profiled using in-gel fluorescence, Western blotting, or semiquantitative label-free MS analysis.

ABP1 as an Activity-Based Probe for E1 Enzymes in Cells. UBL protein-activating E1 enzymes are at the apex of UBL protein signaling pathways. Therefore, the activity of E1 enzymes is critical for the activity of the whole UBL protein pathway. The activity of E1 enzymes is dynamically regulated, yet methods to measure the intracellular activity of E1 enzymes are lacking. The distinct advantage of **ABP1** is its cell membrane permeability, which allows the measurement of intracellular activity of E1 enzymes in its native environment.

In the previous experiments, we have shown that **ABP1** forms covalent conjugates with intracellular proteins in a variety of cell lines. Because the formation of UBL·**ABP1** adducts requires the formation of E1∼UBL protein thioesters, we hypothesized that the degree of covalent labeling of UBL proteins with **ABP1** will provide a readout of the intracellular concentration of E1∼UBL protein thioester and therefore the activity of E1 enzymes. If an E1 enzyme is less active, there will be less E1∼UBL thioester, and therefore we should observe less covalent UBL·**ABP1** adduct. To test our hypothesis, we designed an experiment to measure the activity of heterodimeric SUMO-activating E1 enzyme (SAE1/SAE2) inside intact HeLa cells, using **ABP1**.

SAE1/SAE2 is inhibited by H_2O_2 treatment, which causes formation of the disulfide bond between the catalytic cysteines of the SAE2 subunit and the SUMO-conjugating enzyme Ubc9.[32] This leads to the inhibition of protein SUMOylation inside intact cells. Since the catalytic activity of SAE1/SAE2 is essential for the formation of SUMO·**ABP1** covalent adducts, we predicted that cells treated with H_2O_2 should exhibit a decrease in the covalent labeling of the ∼16 kDa band that contains covalent SUMO·**ABP1** conjugates.

To test our hypothesis, we first confirmed that H_2O_2 inhibits protein SUMOylation in HeLa cells, as reported earlier by Bossis et al.[32] HeLa cells were treated with increasing concentrations of H_2O_2, followed by lysis and Western blotting with anti-SUMO1 and anti-SUMO2/3 antibody to detect the levels of SUMO1- and SUMO2/3-conjugated proteins. We observed a dose-dependent decrease in SUMO2/3 conjugates as we increased the concentration of H_2O_2 from 0 to 0.5 mM. When we increased the concentration of H_2O_2 from 0.5 to 5 mM, we observed an increase in the levels of SUMO2/3 conjugates (Figure 13A).

Bossis et al. reported similar results, which were explained by the inactivation of SAE1/SAE2 with low concentrations of H_2O_2 that leads to rapid de-SUMOylation of SUMO2/3 conjugates by SUMO isopeptidases.[32] Accordingly, we observed an increase in the levels of free SUMO2/3 proteins (Figure 13A). At high concentrations of H_2O_2 (>1 mM), both SAE1/SAE2 and SUMO isopeptidases that contain active-site cysteines are inactivated, thereby freezing the dynamics of protein SUMOylation and leading to an apparent increase in the levels of SUMOylated proteins when compared to lower concentrations of H_2O_2. We observed similar effect of H_2O_2 on SUMO1 conjugates in HeLa cells (Figure S19).

Having established the conditions to induce oxidative stress in HeLa cells, we tested if **ABP1** can serve as an activity-based

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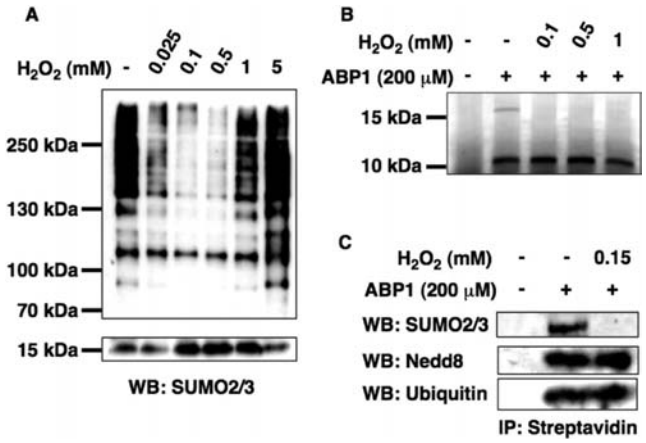

Figure 13. ABP1 is an activity-based probe for UBL protein-activating E1 enzymes. (A) HeLa cells were treated with different concentrations of H_2O_2. The level of SUMO2/3 conjugates was probed with Western blotting using SUMO 2/3 antibodies. (B) HeLa cells were treated with different concentrations of H_2O_2, followed by the addition of **ABP1**. UBL·**ABP1** adducts were visualized via an in-gel fluorescent scanning protocol. (C) HeLa cells were treated with **ABP1** (200 μM) with or without H_2O_2 pretreatment, followed by conjugation to biotin-azide, pull-down with streptavidin beads, and Western blotting with UBL antibodies to detect the amount of isolated UBL proteins.

probe for SUMO-activating enzyme. HeLa cells pretreated with H_2O_2 were treated with **ABP1**, followed by cell lysis, click reaction with the Azide-Fluor-585, and in-gel fluorescence scanning. As we expected, there was a decrease in the fluorescence intensity of ~16 kDa band at 0.1 mM H_2O_2. Therefore, to a first approximation, this indicated decreased formation of SUMO·**ABP1** conjugates in the presence of H_2O_2 and thus SAE1/SAE2 inactivation (Figure 13B). The fluorescence intensity of the lower 10 kDa band (Ub and Nedd8) did not decrease significantly. A visible decrease in fluorescence intensity of the lower ~10 kDa band was observed at 1 mM H_2O_2.

To rule out the possibility that the observed decrease in fluorescence is caused by the degradation of SUMO·**ABP1** adduct by H_2O_2, freshly prepared SUMO1·**ABP1** covalent adduct was treated with H_2O_2 for 20 min *in vitro*, followed by a standard protocol to visualize the covalent adducts. We did not observe any significant decrease in the amount of SUMO1·**ABP1** conjugates, indicating that SUMO1·**ABP1** is stable toward H_2O_2 treatment (Figure S20).

Thus, our initial observations suggest that, at 0.1 mM H_2O_2, SUMO E1 enzyme is inactivated, while ubiquitin and Nedd8 E1 enzymes are still active. To further verify this model, we performed pull-down experiments, in which covalent UBL·**ABP1** adducts in HeLa cells were conjugated to biotin-azide, and the resulting biotin-tagged UBL·**ABP1** adducts are isolated using streptavidin-conjugated beads. The amount of isolated UBLs was detected using Western-blotting techniques using specific anti-ubiquitin, anti-SUMO2/3, and anti-Nedd8 antibodies. As expected, SUMO2/3, Nedd8, and ubiquitin were detected in the absence of H_2O_2 (Figure 13C). In the presence of 0.15 mM H_2O_2, however, we did not detect SUMO2/3 protein in our pull-down experiments. The amounts of isolated Nedd8 and ubiquitin, on the other hand, did not change, indicating that ubiquitin and Nedd8-activating E1 enzymes are active under these conditions. Taken together, our results indicate that **ABP1** is an activity-based probe for E1 enzymes. Importantly, this is the first example of a cell-membrane-permeable activity-based probe for enzymes involved in UBL conjugation pathways.

Studies showed that global levels of protein SUMOylation change under other stresses such as heat, ethanol stress, osmotic stress, and hypoxia.[32] However, investigating these changes in the protein SUMOylation levels is difficult because of the dynamic balance in activities between protein SUMOylation and de-SUMOylation enzymes. This makes it difficult to conclude whether observed changes of SUMOylated proteins are due to changes in the activity of E1, E2, and E3 enzymes for SUMO, or due to the changes in the intracellular activity of SUMO isopeptidases. The developed **ABP1** allows direct measurement of the intracellular activity of E1 enzymes under different physiological conditions. Further experiments, such as stable isotope labeling by amino acids in cell culture (SILAC), will provide a more quantitative and qualitative readout of changes in the degree of covalent labeling of UBL proteins with **ABP1** in response to different physiological conditions. Importantly, the reported Western blotting technique to pull down and detect UBL proteins with **ABP1** provides a complementary approach to MS techniques and requires a simple experimental setup.

ABP1 Is Cleaved from the Covalently Labeled UBL Proteins under Acidic Reaction Conditions. In studies that rely on the use of activity-based probes, biotin-linked proteins are often isolated from complex proteomes using streptavidin beads. However, elution of biotin-tagged proteins from streptavidin beads is difficult due to the high binding affinity of biotin and streptavidin (K_d = 10^{-15} M). Typical elution conditions include boiling streptavidin beads in the presence of biotin (3 mM) and sodium dodecyl sulfate (SDS, >2%). Such harsh elution conditions are not compatible with gel-free MS analysis and lead to the elution of non-specifically bound proteins with only 30% recovery of captured biotinylated proteins.[33]

We have used an on-bead trypsin digestion protocol to show that **ABP1** can covalently detect UBL proteins inside intact cells. While this protocol is well established and effective, there are potential pitfalls associated with it, such as high background signals from digested streptavidin as well as inability to completely distinguish isoforms of UBL proteins such as SUMO2 and SUMO3 using bottom-up proteomics. Therefore, it would be desirable to develop a protocol in which isolated UBL proteins could be cleaved from streptavidin beads under mild reaction conditions and subsequently analyzed using bottom-up or top-down proteomic methods. Since UBL proteins have small MW (<25 kDa), they are suitable candidates for top-down proteomic analysis, which would differentiate closely related protein isoforms.

Significant efforts have been made to develop activity-based probes with chemically cleavable linkers in order to elute target proteins from streptavidin beads under mild reaction conditions.[34] However, the introduction of a cleavable linker group into a small-molecule probe could affect the receptor–probe interaction, the cell membrane permeability, and the labeling efficiency of the probe. Therefore, an ideal activity-based probe should covalently label target proteins and conditionally release covalently labeled protein targets via cleavage of the newly formed covalent bond between the probe and the target protein. Such strategy does not require the incorporation of an additional linker.

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We realized that the UBL·**ABP1** covalent adduct harbors an acidic *N*-acyl sulfamate moiety (calculated pK_a ≈ 3, Table S4), which is formed upon the covalent labeling of UBL proteins with **ABP1**. At physiological conditions (pH 7−8), the NH functionality of the *N*-acylsulfamate is deprotonated and stabilized by the two neighboring electron-withdrawing sulfone and acyl groups (Figure 14A). We have found that the ubiquitin·**ABP1** adduct survives heating at 60 °C in 10 mM hydrazine for 1 h at neutral pH (Figure S21). Even boiling at 95 °C for 5 min in either the presence or the absence of 10 mM hydrazine did not lead to decomposition of the ubiquitin·**ABP1** covalent complex. We therefore hypothesized that, under acidic pH, *N*-acylsulfamate in UBL·**ABP1** will be protonated and subsequently hydrolyzed, liberating the intact UBL proteins for subsequent analysis (Figure 14A).

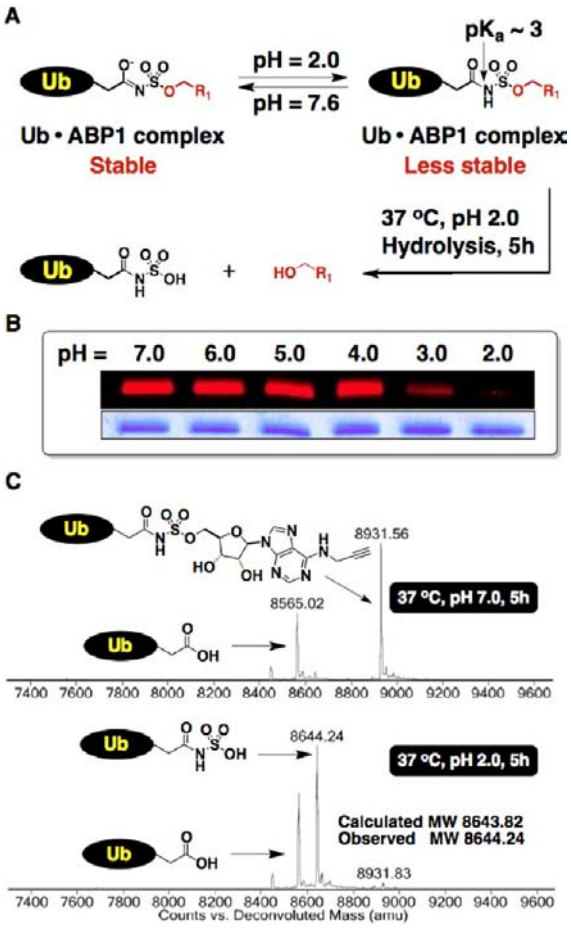

Figure 14. Ubiquitin·**ABP1** covalent adduct can be hydrolyzed under acidic conditions. (A) General scheme of acid-mediated cleavage of ubiquitin·**ABP1** covalent adduct. (B) Ubiquitin·**ABP1** adduct was prepared by incubating UBE1 (1 μM), UbcH5a (1 μM), Rsp5 (0.5 μM), GFP-Sic60 (0.5 μM), Ub (50 μM), ATP (50 μM), and **ABP1** (200 μM) at room temperature overnight at pH 7.6. Excess **ABP1** was removed, and the pH of the reaction mixtures was re-adjusted to 2.0−7.0. The reaction mixtures were further incubated at 37 °C for 5 h, and the pH was re-adjusted to 7.4.The ubiquitin·**ABP1** adducts were visualized using click chemistry conditions and in-gel fluorescence scanning. (C) UBE1 (4 μM), Ub (4 μM), ATP (25 μM), and **ABP1** (25 μM) were incubated at room temperature overnight. Excess amount of **ABP1** was removed, and the resulting reaction mixtures were split into two portions. The first portion was incubated at 37 °C for 5 h at pH 7.0. The second portion was acidified to pH 2.0 and incubated at 37 °C for 5 h. The resulting reaction mixtures were subjected to ESI-MS analysis.

Our hypothesis is supported by previous work, which showed that *N*-acylsulfamates rapidly decompose under acidic conditions.[35] To test this in our system, freshly prepared ubiquitin·**ABP1** adduct was incubated at different pH for 5 h at 37 °C, followed by re-adjustment of pH to 7.4 and the conjugation reaction with the fluorescent Azide-Fluor-585. Coomassie staining showed the presence of ubiquitin in all lanes, suggesting that ubiquitin survived heating at pH 2.0. The disappearance of fluorescent bands at acidic pH indicates that Azide-Fluor-585 is no longer attached to ubiquitin (Figure 14B). A considerable decrease in the fluorescent labeling of ubiquitin was observed at pH 3.0, which is close to the estimated pK_a of the *N*-acylsulfamate. Densitometry analysis of fluorescent bands showed that 69% and 91% of ubiquitin·**ABP1** complex was decomposed at pH 3.0 and 2.0, respectively.

To investigate the mechanism of ubiquitin·**ABP1** hydrolysis, further studies were undertaken. Ubiquitin·**ABP1** adduct was incubated at 37 °C for 5 h at either pH 2.0 or 7.0, followed by direct ESI-MS analysis. Ubiquitin·**ABP1** complex stayed intact after incubation for 5 h at pH 7.0, but was converted mostly into ubiquitin that contained *N*-acylsulfamic acid at its C-terminus after 5 h of incubation at 37 °C at pH 2.0 (Figures 14C and S22). We cannot exclude the possibility that some of the ubiquitin·**ABP1** adduct could be hydrolyzed into ubiquitin, yet the conversion to the *N*-sulfamoylated ubiquitin seems to be predominant, as judged by the relative ratio of reaction product ionization intensities (Figure S22A,C). Thus, our in-gel fluorescence scanning experiments and MS experiments suggest that, under the developed reaction conditions, ubiquitin·**ABP1** adduct undergoes selective and efficient hydrolytic cleavage, a useful property that can be utilized in further proteomic studies.

The utility of pH-cleavable *N*-acylsulfamates for proteomic studies was further examined through direct elution of ubiquitin from streptavidin beads under acidic conditions. The influenza hemagglutinin (HA)-tagged ubiquitin was covalently labeled with **ABP1** in the presence of UBE1 and ATP, followed by covalent conjugation with biotin-azide. Subsequently, the reaction mixture was incubated with streptavidin beads, followed by washes to eliminate any non-specifically bound proteins. The beads were then divided into two portions (Figure 15A).

The first portion was incubated at 50 °C for 2 h at pH 2.0. The resulting supernatant was collected (fraction 1), while the remaining beads were washed thoroughly and boiled in 2× SDS loading buffer for 10 min (fraction 2). The second portion was directly boiled in 2× SDS loading buffer for 10 min (fraction 3). SDS-PAGE followed by silver staining of the three collected fractions showed that there are proteins around the 8 kDa region in fractions 1 and 3, but not in fraction 2. Both 8 kDa bands were immunoreactive with anti-HA antibody, identifying these proteins as HA-tagged ubiquitin (Figure 15B). However, only fraction 3 contained biotin, as detected by streptavidin-HRP in Western blot. These results indicate that, in acidic conditions, covalently labeled ubiquitin was quantitatively eluted from streptavidin beads and did not contain biotin. This supports our hypothesis that the ubiquitin·**ABP1** covalent complex can be cleaved under acidic conditions and can be used to elute **ABP1**-labeled proteins from streptavidin beads for subsequent proteomic studies.

The acid hydrolysis method demonstrated here has several advantages over other conventional methods, such as boiling

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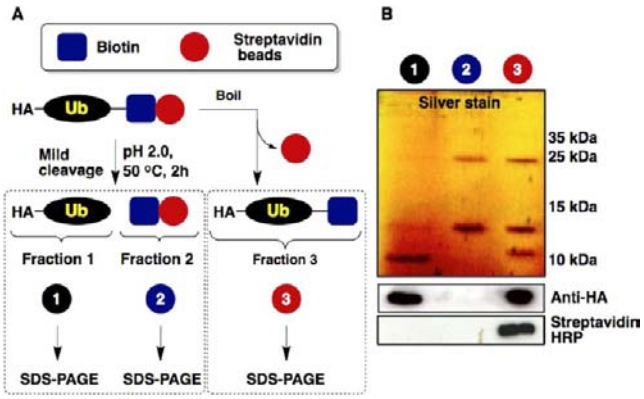

Figure 15. *N*-Acylsulfamates as a useful class of pH-cleavable linkers for chemoproteomic studies. (A) A general scheme to test the utility of *N*-acylsulfamates for proteomic studies. (B) Fraction 1 contains HA-ubiquitin, which does not contain biotin. Fraction 2 does not contain ubiquitin, but contains streptavidin monomers and dimers. Fraction 3 contains biotinylated HA-ubiquitin·**ABP1** complex, as well as streptavidin monomers and dimers. The amount of proteins was evaluated with silver staining and Western blotting.

the streptavidin beads with 2× SDS loading buffer to elute covalently captured proteins for proteomic studies. First, no significant amounts of streptavidin monomers and dimers are eluted from the streptavidin beads under the newly developed conditions, which often hinder the identification of non-abundant protein targets in proteomics studies. Silver staining of fractions 2 and 3 suggests that there are significant amounts of streptavidin monomers and dimers, which are cleaved from streptavidin beads upon boiling (Figure 15B). Second, elution conditions are compatible with gel-free MS analysis, which reduces additional purification steps such as SDS-PAGE and in-gel digestion. These conditions should prevent the loss of non-abundant target proteins. Third, the sulfamic acid attached to the C-terminus after cleavage could be used as a marker to distinguish proteins that were covalently labeled with **ABP1** from non-specifically detected background proteins in MS analysis.

In summary, these experiments confirm that **ABP1** covalently labels ubiquitin *in vitro*, and the resulting covalent bond is conditionally cleavable, facilitating subsequent protein elution from streptavidin beads for proteomic studies. Given that UBL proteins are small in size, the developed elution protocol can conveniently be used for top-down proteomic analysis of UBL proteins or other proteins that are covalently labeled by **ABP1**. To our knowledge, this is the first described example of a self-cleavable activity-based probe that takes advantage of an *N*-acylsulfamate functional group that can be hydrolyzed under acidic conditions, which permits a quantitative elution of covalently captured UBL proteins from streptavidin beads for proteomic studies.

■ CONCLUSION

Ubiquitin and 20 other ubiquitin-like systems ligate ubiquitin and UBL proteins to their protein substrates, thus regulating protein binding, conformation, localization, and concentration. Given the fundamental importance of UBL proteins in biology and human medicine, it is important to have tools to study UBL protein systems. In this regard, several recurrent challenges arise when there is a need to study UBL protein systems.

First, given the pyramidal structure of UBL systems, it is important to have tools to dissect UBL protein/E1 interactions,[23,15d] UBL∼E1/E2 interactions,[13c] E2∼UBL/E3 interactions,[36] E3/substrate interactions,[37] and isopeptidase/substrate interactions.[38] Second, methods to directly measure the enzymatic activity of E1, E2, and E3 enzymes and isopeptidases in cells and *in vivo* will provide the most direct readout on how various stimuli affect the activity of UBL protein conjugating and deconjugating enzymes.[39] Third, selective small-molecule inhibitors for E1, E2, and E3 enzymes and isopeptidases are critically needed for therapeutic purposes and to deconvolute a highly complex network of UBL signaling pathways.[15,40] To this end, we envision that methods to evaluate the intracellular potency and selectivity of pharmacological inhibitors that inhibit E1, E2, and E3 enzymes and isopeptidases will be critically needed. Tools to do so are being successfully developed for protein kinases.[41] Finally, systematic approaches to discover every component of UBL systems need to be developed.[39a] These include both genome and proteome mining tools.

In this paper, we began to address these challenges and focused on proof-of-concept studies to develop a novel chemoproteomic tool to study UBL proteins, their corresponding E1 enzymes, and E1 enzyme inhibitors. We have developed a cell-membrane-permeable small-molecule probe, **ABP1**, that covalently labels UBL proteins *in vitro* and in cells in the presence of E1 enzymes and ATP. Covalently labeled UBL proteins can be conjugated with fluorescent probes or biotin for visualization, isolation, and identification. We have shown that **ABP1** can be used to isolate and identify endogenous ubiquitin, SUMO1, SUMO2/3, Nedd8, and Ufm1 in four different human cancer cell lines derived from different tissues: A549, LNCap, MCF7, and HeLa. The developed protocols and **ABP1** display remarkable sensitivity: for example, endogenous SUMO1 protein, which has low abundance, was detected with **ABP1** in the presence of ∼100-fold excess of endogenous ubiquitin present in HeLa cell lines.

Further studies using **ABP1** showed that UBE1 could activate not only monomeric ubiquitin but also Lys^{48}- and Lys^{63}-linked di-ubiquitin chains as well as Lys^{48}-linked tetra-ubiquitin chains *in vitro*. Whether UBE1 activates poly-ubiquitin chains in physiological conditions remains to be determined. In this regard, **ABP1** can serve as a useful tool for covalent labeling and identification of poly-ubiquitin chains by UBE1 in cells.

We have found that the covalent labeling of ubiquitin with **ABP1** in the presence of UBE1 and ATP is enhanced by the E2 enzymes UbcH5a, UbcH7, and Ubc12, which display functional interactions with UBE1 *in vitro*. SUMO-conjugating E2 enzyme Ubc9 did not cause the equivalent increase in the amount of ubiquitin·**ABP1** conjugate, which indicates that the developed probe can potentially be used to identify functionally relevant E1/E2 enzyme pairs *in vitro*. Whether the same effect is true *in vivo* and is observed for other E1/E2 pairs is currently being investigated. Further mechanistic studies to explain this effect are currently in progress.

The developed **ABP1** can also be used to profile the activity of E1 enzymes in response to pharmacological inhibitors and selectivity of E1 enzyme inhibitors *in vitro* and in cells. This has been shown for the pan-E1 enzyme inhibitor Compound **1** and Nedd8-activating NAE1/UBA3 enzyme inhibitor MLN4924, as well as mechanistically distinct ubiquitin-activating E1 enzyme inhibitor Pyr 41. The developed approach to profile potency and selectivity of E1 enzyme inhibitors *in vitro* offers significant

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advantages over the existing methods, which use either radioactive materials to conduct PP_i exchange assays or Western blotting techniques to monitor UBL~E1/E2 transthiolation reaction. The latter requires E2 enzyme and primary and secondary antibodies; it is also laborious, expensive, and time-consuming.

To profile the selectivity of E1 enzyme inhibitors in cells, we have developed a pull-down protocol, in which the amount of covalently labeled UBL proteins can be detected using Western blotting techniques using specific anti-UBL antibodies. We have shown that Compound **1**, MLN4924, and Pyr41 do not efficiently inhibit the covalent labeling of SUMO2/3 proteins with **ABP1** in A549 cells, which indicates their weak potency for intracellular SUMO E1 enzyme. Furthermore, our experiments show that Compound **1** inhibits intracellular ubiquitin and Nedd8 E1 enzymes, and MLN4924 inhibits intracellular Nedd8 E1 enzyme but not ubiquitin E1 enzyme, while Pyr41 was not effective at inhibiting intracellular ubiquitin, SUMO, or Nedd8 E1 enzymes. In addition to the in-gel fluorescent approach and Western blotting techniques, we used semi-quantitative label-free MS analysis to test the inhibitory potency of Compound **1** in MCF7, LNCap, and HeLa cells. Using this technique, we have found that the pharmacological potency of Compound **1** is cell type specific, since Compound **1** was more potent at inhibiting the covalent labeling of SUMO proteins with **ABP1** in LNCap and HeLa cells than in MCF7 and A549 cells. Subsequent in-gel fluorescence data further confirm these findings.

We showed the potential of **ABP1** as a tool to discover UBL proteins. It is remarkable that many UBL proteins were discovered serendipitously. This is because discoveries of UBL proteins known to date have relied upon sequence homology with known UBL proteins, yet many UBL proteins share little amino acid sequence identity with the ubiquitin or with each other (<20%), which is below the limit of statistical significance.[42] It is speculated that additional UBL proteins may exist.[1c] In this regard, **ABP1** represents a novel proteome-mining tool, which complements the existing bioinformatics approaches to facilitate the discovery of previously unknown UBL proteins in eukaryotes. We have successfully shown the utility of this tool by isolating endogenous UBL proteins from a range of human cell lines. In principle, **ABP1** can potentially be used to detect UBL proteins in cells of different origin, as long as **ABP1** can permeate the membranes of these cells and similar mechanisms of UBL protein activation by E1 enzymes are utilized. In this regard, it is important to note that we have identified ubiquitin fold modifier 1 (Ufm1) protein in A549 and MCF7 cell lines using **ABP1**, which suggests that Ufm1-activating UBA5 enzyme is active in these cells. While ubiquitin, SUMO1−4, and Nedd8 proteins are very well known, Ufm1 is a relatively new UBL protein, which was discovered serendipitously in 2004, and little is known about its physiological functions.[43]

We have shown that the developed **ABP1** can serve as an activity-based probe to measure the activity of E1 enzymes inside intact cells. To this end, we have shown that **ABP1** can be used to directly measure the activity of SUMO E1 enzyme in response to H_2O_2-mediated oxidative stress. Treatment of HeLa cells with H_2O_2 followed by the addition of **ABP1**, cell lysis, conjugation to biotin-azide, streptavidin pull-down, and Western blot detection of the isolated UBL proteins showed that H_2O_2 caused a significant decrease in the amount of isolated SUMO2/3 proteins. At the same time the amount of isolated endogenous ubiquitin and Nedd8 was not decreased, indicating that E1 enzymes that activate ubiquitin and Nedd8 are most likely active under these conditions. Similarly, H_2O_2 treatment decreased the labeling of SUMO proteins with **ABP1**, as judged from in-gel fluorescent scans. Taken together, our experimental data firmly establish **ABP1** as a convenient probe to monitor the intracellular activity of E1 enzymes in cells.

Finally, we have introduced *N*-acylsulfamates as a new class of pH-cleavable linkers for proteomic studies. To isolate proteins that are covalently labeled with **ABP1**, we have developed a new protocol that allows the elution of covalently labeled UBL proteins under mild reaction conditions. The key to this discovery was the realization that the *N*-acylsulfamate moiety in UBL·**ABP1** adduct can be cleaved under mild acidic conditions; thus, **ABP1** serves as a self-cleavable activity-based probe that liberates free UBL proteins for subsequent bottom-up and top-down proteomic studies.[44] To our knowledge, this is the first example of a probe that employs an *N*-acylsulfamate functionality as a pH-cleavable linker for chemoproteomic applications, and **ABP1** is the first example of a self-cleavable activity-based probe. We envision that *N*-acylsulfamates can serve as useful and general pH-cleavable linkers in the fields of activity-based proteomics and biochemistry. *N*-Acylsulfamates are small in size and can be easily introduced into any system by coupling aliphatic alcohols with chlorosulfonamide as a linchpin, followed by coupling of the resulting sulfamate with activated forms of carboxylic acids.

In summary, this paper reports the initial design and characterization of the activity-based probe **ABP1** for UBL signaling pathways, with potential utility for basic research and drug discovery purposes. Further applications and proteomic studies utilizing the self-cleavable *N*-acylsulfamate of **ABP1** will be reported in the near future.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Synthetic procedures, characterization of the synthesized chemical compounds, protocols for biochemical and enzymatic assays, NMR spectra, and supplementary figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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