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Research Article

Facile Preparation of UFMylation Activity-Based Probes by Chemoselective Installation of Electrophiles at the C-Terminus of Recombinant UFM1

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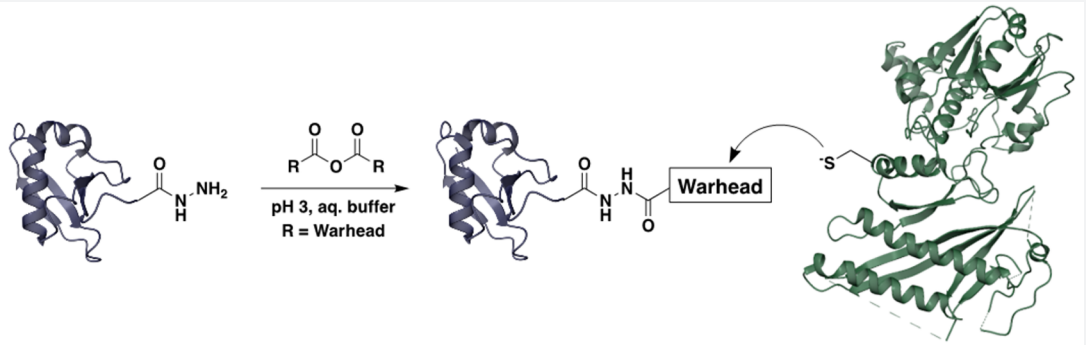

ABSTRACT: Aberrations in protein modification with ubiquitin-fold modifier (UFM1) are associated with a range of diseases, but the biological function and regulation of this post-translational modification, known as UFMylation, remain enigmatic. To provide activity-based probes for UFMylation, we have developed a new method for the installation of electrophilic warheads at the C-terminus of recombinant UFM1. A C-terminal UFM1 acyl hydrazide was readily produced by selective intein cleavage and chemoselectively acylated by a variety of carboxylic acid anhydrides at pH 3, without detriment to the folded protein or reactions at unprotected amino acid side chains. The resulting UFM1 activity-based probes show a range of tunable reactivity and high selectivity for proteins involved in UFMylation processes; structurally related E1s, E2s, and proteases associated with Ub or other Ubls were unreactive. The UFM1 probes were active both in cell lysates and in living cells. A previously inaccessible α-chloroacetyl probe was remarkably selective for covalent modification of the active-site cysteine of de-UFMylase UFSP2 *in cellulo*.

INTRODUCTION

Ubiquitin fold modifier 1 (UFM1) is a small ubiquitin-like protein discovered less than 20 years ago.[1] It shares a β-grasp-fold with ubiquitin (Ub) but differs in its amino acid sequence and function.[2,3] In its mature form, it comprises 83 amino acids with a C-terminal Val−Gly instead of the Gly−Gly motif observed in Ub and other Ubls (Ubiquitin-like proteins). Like other Ubls, UFM1 is attached through an isopeptide bond to Lys residues on its substrate proteins.[4] Numerous enzymes are involved in the UFMylation pathway, and the process is associated with endoplasmic reticulum (ER) homeostasis, ER-phagy, DNA damage response, tumor progression, protein translation, and quality control. However, the exact consequences of dynamic UFM1 attachment and removal remain unclear, in part due to a lack of chemical tools to probe the biology of UFMylation.

Like other Ubls, UFM1 is activated at its C-terminus by ATP and loaded onto the catalytic cysteine of its activating E1 enzyme, UBA5. This is followed by a trans-thioesterification to load UFM1 onto its E2 conjugating enzyme, identified as UFC1, and subsequent transfer to its substrate proteins by the E3 ligase UFL1, with the assistance of adaptor proteins including DDRGK1 and CDK5RAP3.[5] UFMylation is a reversible process with two enzymes, UFSP2 and UFSP1, implicated in de-UFMylation. UFSP1 is thought to be inactive in humans due to a truncated N-terminal region.[6] UFSP2 was previously believed to mediate maturation of pro-UFM1; however, recent experiments in *UFSP2* knockout (KO) cell lines still show the processing of proUFM1 to its mature form and the accumulation of UFMylated proteins.[7] The factors responsible for UFM1 maturation and removal in the absence of UFSP2 remain to be determined.

Most UFMylation target proteins are located in the ER. Recent studies reveal that the UFM1 activating enzyme UBA5

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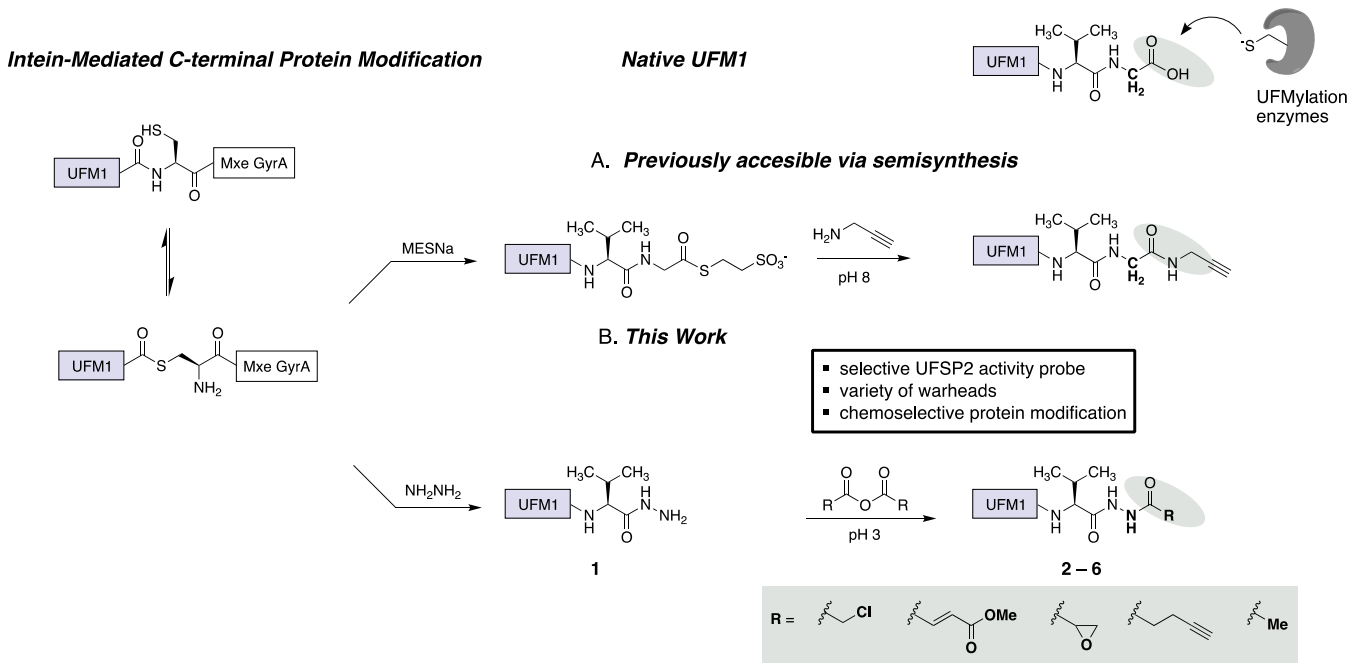

Figure 1. Semisynthesis of C-terminally modified proteins by intein cleavage. (A) Commonly used direct aminolysis of the Gly−MesNa thioester. (B) Selective hydrazide modification with carboxylic acid anhydrides used in this work.

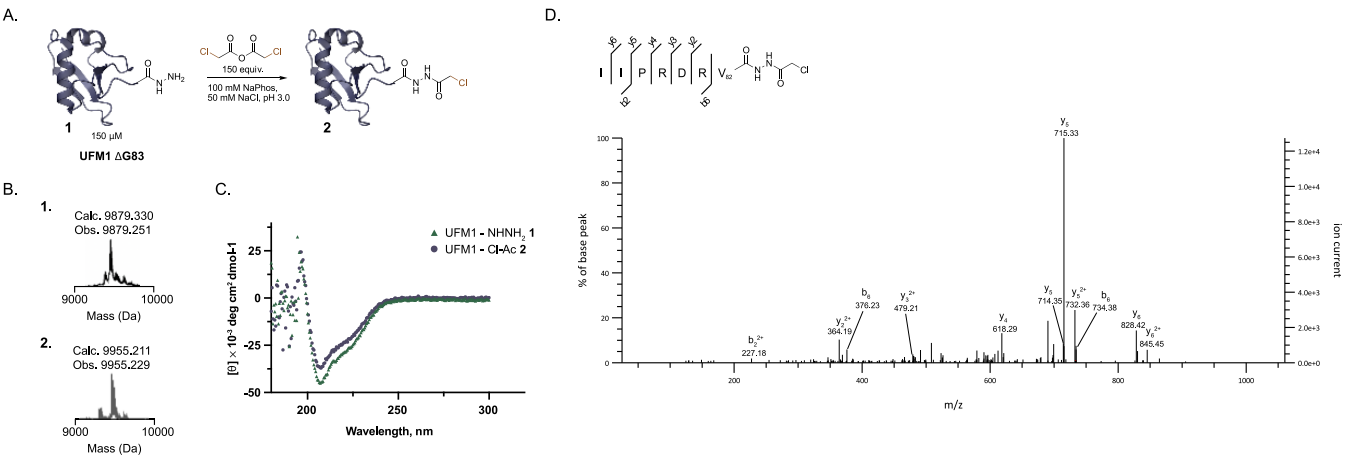

Figure 2. (A) C-Terminal hydrazide UFM1 modification with symmetrical α-chloroacetic anhydride in phosphate buffer at pH 3. (B) HRMS spectra of the starting material **1** and product **2** without prior purification. (C) CD spectra of UFM1-$NHNH_2$ **1** and UFM1-α-Cl-Ac probe **2**. (D) MS/MS spectrum of a C-terminal UFM1 tryptic peptide IIPRDRV bearing hydrazide modified with α-chloroacetyl probe. The precursor ion carried a charge of 5+. Observed m/z = 732.36; theoretical m/z = 732.24. Theoretical parent ion mass 958.56.

is translocated to the ER lumen.[8,9] However, it is still unclear how UFC1 and other enzymes involved are recruited to the ER. Literature reports suggest that there may remain other yet undiscovered enzymes involved,[10] but the paucity of tools to study UFMylation and the incomplete data on the associated enzymes limit further investigations.

In continuation of our interest in both UFM1[11] and affinity probes for Ubl pathways,[12] we sought to develop new activity-based probes (ABPs) for the enzymes involved in UFMylation. Inspired by the work of Ovaa,[13] Ploegh,[14] and others[15] on the generation of C-terminally modified Ub and Ubls bearing electrophilic warheads as powerful tools to investigate and inhibit specific conjugating and deconjugating enzymes, we sought to identify synthetic or semisynthetic UFM1-derived probes that could undergo covalent coupling with proteins involved in UFMylation. The two previously reported synthetic probes are limited to dehydroalanine and propargylamine and require solid-phase peptide synthesis (SPPS), native chemical ligation (NCL), and refolding for their preparation.[16] With the aim of identifying flexible routes to more accessible probes bearing a variety of electrophilic warheads, we sought to employ direct aminolysis of the corresponding thioesters. Unfortunately, the presence of a C-terminal Val−Gly, instead of the Gly−Gly motif found on Ub and almost all other Ubls, complicates this route to electrophilic UFM1 probes. Recently, two UFM1 probes have been obtained via direct aminolysis;[16,17] however, in these instances, the electrophiles were attached to the C-terminal glycine, moving the probes further away, in terms of atomic register, from the desired modification site (Figure 1A). Our own efforts to prepare Val C-terminally modified UFM1 with this approach resulted largely in the formation of hydrolyzed thioesters as the major product. We

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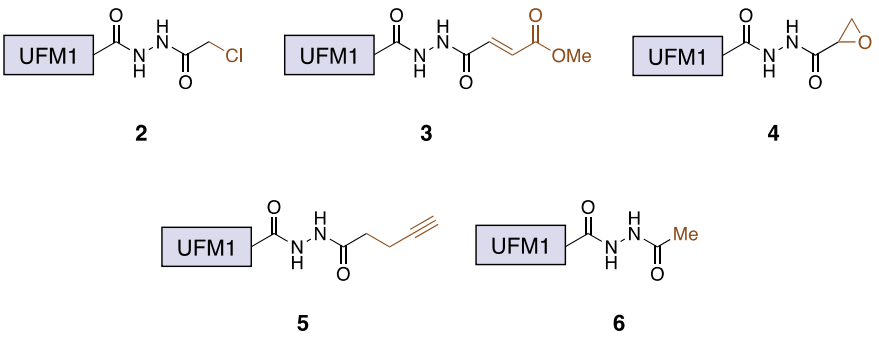

Figure 3. Electrophilic C-terminal probes **2**−**5** and control **6** used in this study.

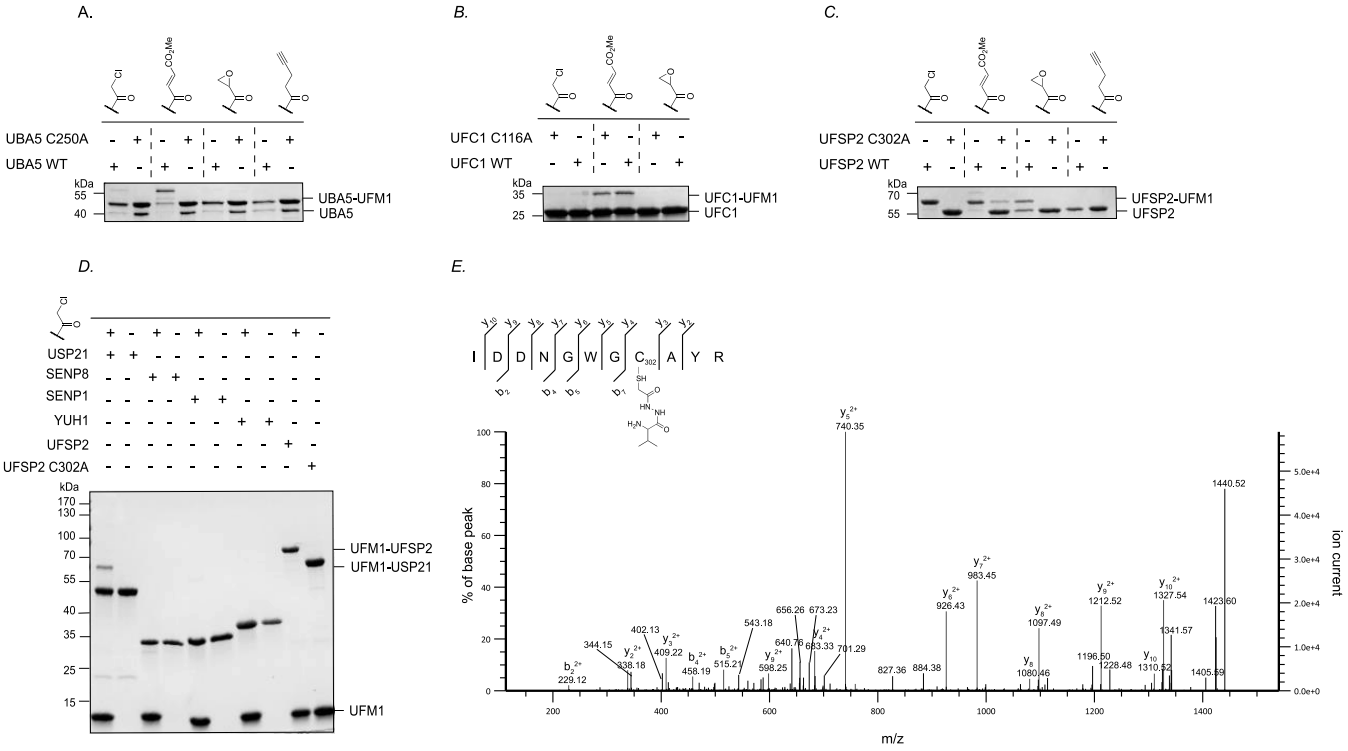

Figure 4. *In vitro* validation of the selectivity of UFM1 C-terminal probes **2**−**5**. Cross-linking with (A) UBA5, UFM1 activating enzyme; (B) UFC1, UFM1 conjugating enzyme, and its catalytically inactive mutant; and (C) UFSP2, UFM1 deconjugating enzyme, and its catalytically inactive mutant. (D) Reactivity of the UFM1 α-chloroacetyl probe *in vitro* **2** with other DUBs: USP21, SENP8, SENP1, YUH1, UFSP2 WT, and UFSP2 C302A. Cross-linking was observed with UFSP2 and USP21. (E) MS/MS analysis: annotated tryptic peptide derived from *in vitro* cross-linking of UFM1 and active-site cysteine of UFSP2. The precursor ion carried a charge of 5+. Observed m/z = 740.35; theoretical m/z = 740.85. Observed parent ion mass 1440.52; theoretical parent ion mass 1440.56. Results shown are representative of at least three independent experiments.

could, however, routinely prepare the C-terminal Val−acyl hydrazide **1** of UFM1 by cleavage of the corresponding intein fusions or thioesters with hydrazine. These acyl hydrazides proved to be excellent starting points for the facile preparation of a wide variety of UFM1-derived ABPs (Figure 1B).

In this manuscript, we report the facile preparation of C-terminal UFM1 acyl hydrazides and their transformation into activity-based probes by chemoselective acylation of the folded proteins at the terminal hydrazide nitrogen. This convenient approach allows the construction of ABPs containing the requisite C-terminal valine residue and is suitable for the installation of numerous electrophilic species including α-halo amides, epoxides, alkynes, and fumarates. The resulting UFM1 derivatives display a range of selectivity in their reactions with cysteine proteases and conjugating enzymes, in a manner dependent on innate interactions with UFM1 as well as on the nature of the electrophile. One of these probes, a novel C-terminal UFM1 α-chloroacetyl derivative, shows both high reactivity and exceptional selectivity for UFSP2, the only known active human de-UFMylating enzyme, in HCT116 cell lysates and in living HEK293T cells.

RESULTS AND DISCUSSION

To prepare semisynthetic UFM1 ABPs, we expressed a fusion of UFM1 (1−82), bearing an N-terminal flag tag, and *Mycobacterium xenopi* GyrA His-tagged intein (*Mxe* GyrA, 83−289) in *Escherichia coli* (*E. coli*) BL21 (DE3). The resulting fusion protein was isolated by Ni-NTA purification, and we reliably obtained expression yields of 120 mg/L cell culture. By following the hydrazinolysis procedure of Cotton et al.,[18] we cleaved the intein with hydrazine and isolated the resulting C-terminal valine acyl hydrazide, which was separated

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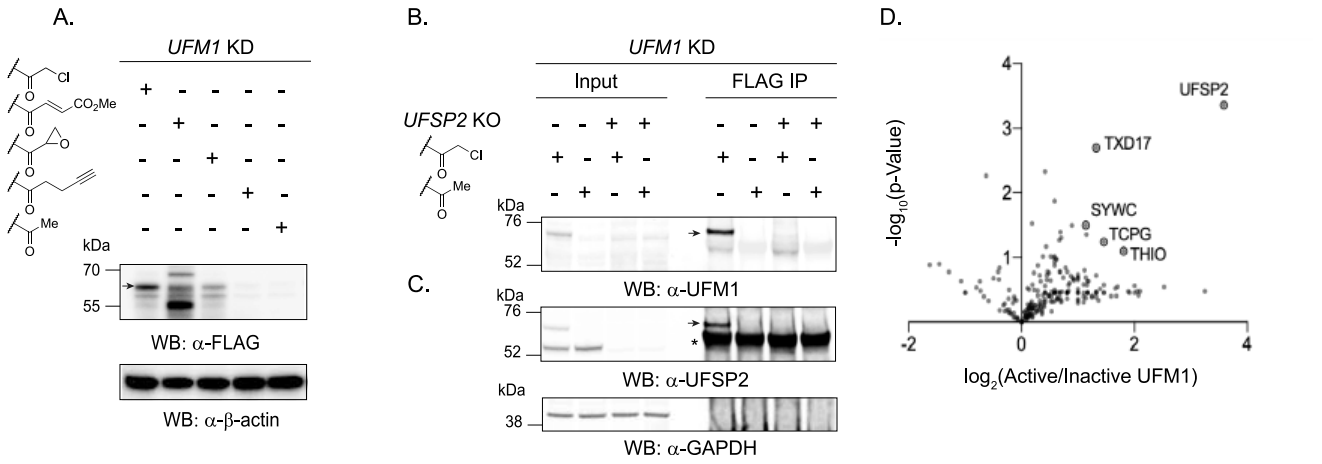

Figure 5. (A) Validation of the reactivity of FLAG-UFM1 C-terminal probes (15 μM) in *UFM1* KD HCT116 whole cell lysates. (B, C) Analysis by Western blot against FLAG. Cell transfection with UFM1 probes **2** and **6** via electroporation: *UFM1* KD HEK293T cells with and without *UFSP2* KO. Subsequent cell lysis and immunoprecipitation on *anti*-FLAG resin. Analysis by Western Blot against (B) UFM1 and (C) UFSP2. * indicates antimouse secondary antibody cross-reactivity with the FLAG-lgG heavy chain used for IP; → indicates UFM1-conjugates. (D) Volcano plot of the most abundant proteins enriched by FLAG immunoprecipitation from HEK293T cells. Results shown are representative of at least 3 of independent experiments.

by reverse Ni-NTA purification. The obtained protein hydrazide **1** retained its folded structure, as evidenced by CD spectrophotometry (Figure 2C).

Based on the widely utilized protocol for the formation of peptide thioesters from acyl hydrazides,[19,20] we anticipated that nucleophilic side chains would be protonated at pH 3, leaving only the terminal hydrazide nitrogen atom as a reactive center.[21,22] In principle, this should leave a single nucleophilic site for a chemoselective acylation reaction, but we could find surprisingly little precedent for acylation of peptide or protein acyl hydrazides. A single, encouraging example was reported in the work of Steitz et al. on the use of peptide hydrazides as purification handles,[23] where they observed the chemoselective formation of a trifluoromethylacetyl hydrazide as a byproduct of resin cleavage. To test our hypothesis, we treated 150 μM UFM1-hydrazide **1** in phosphate buffer at pH 3 and room temperature with 150 equiv of α-chloroacetic anhydride (Figure 2A). Within minutes, we observed clean formation of a single new species **2** (by MALDI MS) with a mass corresponding to a single addition of the α-chloroacetyl group. The modified protein was dialyzed against Tris buffer pH 7.4 to remove excess small molecules. The selective hydrazide modification was further confirmed by HRMS of the intact protein (Figure 2B) as well as tryptic digestion and analysis of the resulting fragments by MS/MS analysis, which established the C-terminal hydrazide as the site of acylation (Figure 2D). The modified protein **2** retained its folded structure, as evidenced by CD spectrophotometry (Figure 2C). α-Chloroacetyl modified UFM1 **2** proved to be stable in PBS for up to 6 days at room temperature (Figure S7).

The simplicity of this modification approach motivated us to expand the scope to other functional groups known to covalently react with cysteines. We tested anhydrides from methyl-fumarate, glycidic acid, pentynoic acid, and acetic acid anhydrides, which all resulted in a single addition and full conversion of UFM1 ΔG83 hydrazide to the acylated product (Figure 3; Figures S1−S6).

α-Chloroacetyl is a commonly used motif in small-molecule cysteine covalent modifiers and undergoes S_N2 reactions with thiols.[24,25] Vinyl methyl ester (VME) is a well-known moiety for ubiquitin and Ubl pathway profiling studies,[14,26] and the related fumarate **3** should be more electrophilic than the common VMEs. Epoxides are known as cysteine-reactive groups that undergo ring-opening upon reaction with nucleophilic thiols.[27,28] Alkynes have been shown to react with deconjugating enzymes,[29,30] although for UFM1, only the propargyl group (UFM1-PA) has previously been tested for UFSP1 and UFSP2 de-UFMylases.[16] UFM1-PA was shown to be inactive with human UFSP1 and required extended incubation to engage with human UFSP2. The acetylated C-terminal UFM1 **6** serves as a negative control. To the best of our knowledge, neither α-chloroacetyl nor epoxide probes—which are commonly used in small-molecule covalent modifiers—have been attached to proteins to generate protein-based ABPs.[15,31]

With the UFM1-derived ABPs in hand, we investigated their reactivity toward isolated samples of the known enzymes of the UFMylation machinery. We recombinantly expressed UFM1 activating enzyme (E1) UBA5, conjugating enzyme (E2) UFC1, de-UFMylating enzyme UFSP2, as well as their active-site inactivated mutants (C→A) as controls. We first tested probes **2**−**5** with UBA5 and its catalytically inactive variant, C250A. α-Chloroacetyl probe **2** and fumarate probe **3** reacted with UBA5, with **3** coupling more extensively (Figure 4A, full gel is shown in Figure S8b). Neither probe displayed any reactivity with the catalytically inactive UBA5 variant, highlighting the selectivity of the probes. Alkynes display well-established reactivity with DUBs and no reactivity with E2s;[29] therefore, we tested the likely more reactive probes **2**−**4** with UFC1 and its catalytically inactive variant, C116A. Probe **3** reacted with UFC1; however, the reaction was unspecific as the catalytically inactive UFC1 variant, C116A, reacted equally efficiently with probe **3** (Figure 4B, full gel is shown in Figure S10). This suggests that fumarate probe **3** is too electrophilic and may couple with a surface exposed cysteine residue. We tested probes **2**−**5** with de-UFMylase UFSP2 and its catalytically inactive variant, C302A. Based on previous literature precedents, we anticipated that alkyne probe **5** would react with the deconjugating enzymes.[31] Probes **2**−**4** showed reactivity toward UFSP2. While methyl fumarate

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probe **3** again reacted unspecifically, α-chloroacetyl **2** and epoxide **4** selectively coupled with wild type UFSP2. In particular, probe **2** was intriguing because it reacted both selectively and efficiently, consuming all of the UFSP2 (Figure 4C, full gel is shown in Figure S12). Pentynoic acid-derived probe **5** showed no reactivity, likely due to the fact that its electrophile is located further away from the native substrate in terms of atomic register.

To further confirm the selectivity of the probes, we screened them against a panel of ubiquitin, NEDD8 and SUMO activating (E1), conjugating (E2s), and deconjugating (DUBs) enzymes that are not involved in the UFMylation pathway. Probes have shown no reactivity toward UBA1 (Figure S8a). α-Chloroacetyl probe **2** and methyl-fumarate probe **3** showed only trace reactivity with Ub K48 chain E2, UBE2K, and did not show distinct reactivity toward other noncognate E2s (see Figures S9 and S10). Further, α-chloroacetyl probe **2** and methyl-fumarate probe **3** showed reactivity toward USP21, a Ub K48 chain protease previously reported to display plasticity in reactivity toward Ubls beyond ubiquitin (NEDD8 and ISG15)[32] (see Figure S11). None of the probes reacted with SENP8 (NEDD8 protease), SENP1 (SUMO protease), or YUH1 (Ub protease) deconjugating enzymes. All cross-linking experiments were performed in triplicate.

The α-chloroacetyl probe **2** proved highly selective toward UFSP2 and showed no distinct reactivity toward other conjugating and deconjugating enzymes (Figure 4D). Thus, we identified the modified cysteine of UFSP2 to be active-site C302. We performed an *in vitro* cross-linking reaction with probe **2**, followed by tryptic digest and analysis of the resulting fragments by MS/MS (Figure 4E).

Encouraged by the specific reactions of the UFM1-derived probes for enzymes known to be involved in the UFMylation pathway, we evaluated their activity in whole cell lysates and living cells. We used wild type HCT116, *UFM1* knockdown (KD) HCT116, and *UFM1* KD HEK293T cell lines, generated using CRISPRi (dCas9-KRAB) (see Figures S13−S15). Cell lysates were incubated with the FLAG-tagged probes at 37 °C. Visualization of the *UFM1* KD experiment by anti-FLAG Western blotting showed intense bands above 55 kDa for α-chloroacetyl probe **2** and fumarate probe **3** (Figure 5A, full gel is shown in Figure S13a). Fumarate probe **3** also showed higher- and lower-molecular-weight bands. This is in line with our biochemical experiments revealing that fumarate probe **3** was nonspecific. In contrast, α-chloroacetyl probe **2** afforded largely a single cross-link with high intensity. Reactions in lysates were performed in triplicate. Based on the molecular mass of 55 kDaA, both UBA5 and UFSP2 could be potential candidates.

To identify the protein that was labeled by probe **2**, we introduced our probe into living HEK293T *UFM1* KD cells using electroporation. A HEK293T cell line with *UFSP2* KO was used for the control experiment. α-Chloroacetyl probe **2** and acetylated UFM1 **6**—which served as a negative control—were nucleofected into the cells, followed by cell lysis and immunoprecipitation using anti-FLAG beads. Visualization of the cross-linked proteins after lysis using Western blot against both UFM1 and UFSP2 gave distinct bands only in the presence of UFSP2 and α-chloroacetyl probe **2**. No bands were observed with acetylated UFM1 **6** or in the absence of UFSP2 (Figure 5B,C, full gels are shown in Figure S15a,b), confirming the specific trapping of UFSP2 with probe **2**. We further verified the efficient reaction of probe **2** with UFSP2 by IP-MS. We used label-free quantification to identify the most enriched proteins using FLAG-IP. Probe electroporation and IP were performed in triplicate. UFSP2, a UFM1 protease in humans, was the most enriched protein identified (Figure 5D). A low-molecular-weight disulfide oxidoreductase, thioredoxin (TXD17), known to have two active-site Cys, was modestly enriched, likely due to unspecific labeling.[33] Additionally, we have confirmed the cross-linking with UFSP2 by the spiking of recombinantly expressed UFSP2 in HCT116 lysates (see Figure S14).

In conclusion, we have developed a new class of UFM1-derived ABPs, including the α-chloroacetyl derivative that shows exceptional selectivity for the de-UFMylase UFSP2. This probe operates equally well in cell lysates or upon electroporation into living cells, which will enable it to be used as a phenotypic tool to interrogate the role of UFSP2. These new probes were easily prepared by site-specific attachment of a variety of electrophilic warheads—many of which were previously inaccessible—to a recombinant, flag-tagged UFM1 C-terminal hydrazide, itself readily produced by cleavage of an intein fusion with hydrazine. Importantly, and in contrast to related approaches employing aminolysis of thioesters, this method is compatible with the sterically hindered C-terminal valine residue required for producing highly specific UFM1 ABPs. We anticipate that this facile, flexible, and selective method for C-terminal functionalization, which is made possible by the unique pK_a of acyl hydrazides, will enable the preparation of other Ub- and Ubl-derived ABPs. The practical simplicity of the method, which requires only simple chemical reagents and operations, will also make it accessible to most biochemistry laboratories.

Safety Statement. No unexpected or unusually high safety hazards were encountered.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acscentsci.2c00203.

Methods, protein sequences, and figures, including characterization by HPLC, MALDI, denaturing PAGE, and Western blots analysis (PDF)

Transparent Peer Review report available (PDF)

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Notes

The authors declare no competing financial interest.

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