Chemoselective placement of unsaturated phosphorus electrophiles into ubiquitin for proximity-induced protein targeting

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ABSTRACT: Mildly reactive electrophiles have emerged as powerful functional groups for developing chemical probes to target pharmacologically important proteins and study key biological mechanisms. With the current manuscript, we introduce unsaturated *N*-alkyl-phosphonamidates as a new and powerful class of chemical warheads that enable proximity-induced labeling and the specific targeting of protein-protein interactions. In contrast to many other electrophilic warheads, these cysteine-selective electrophiles can be incorporated into specific amino acid side on the protein level by a chemoselective Staudinger-phosphonite reaction using unsaturated phosphonites as chemical reactants with azido-containing ubiquitins obtained by auxotrophic expression. Following this protocol, we prepared several ethynyl-*N*-alkyl-phosphonamidate-containing ubiquitins, which undergo proximity-induced labeling with deubiquitinases (DUBs) and other ubiquitin-interacting proteins, even in the presence of high thiol concentrations and cell lysates. Furthermore, we demonstrate that changing the position of the electrophile allows selective targeting, enrichment and distinct functional regulation of DUBs via covalent binding to individual distinct ubiquitin binding pockets as highlighted in the functional regulation of USP5, a DUB that contains multiple ubiquitin binding sites. Our study highlights the prospect of targeting protein-protein interactions with electrophilic protein-based probes by simultaneously enlarging the repertoire of chemical probes beyond purely synthetically derived warheads.

Introduction: In recent years chemical biology has developed a plethora of strategies to investigate and perturb protein structure and function.[1–4] Protein labelling is often achieved by targeting the inherent reactivity of reactive amino acid side-chains.[3,5] Additionally, introducing non-canonical reactive groups into proteins has become increasingly popular.[6] These artificial residues can be used for the selective modification of proteins inside of living cells or even organisms by leveraging biorthogonal reactive groups.[7–9] In addition, functionalities that allow the modified protein to chemically react with other interacting proteins can be incorporated. One possibility to achieve this is the incorporation of a biologically inert functionality that is rendered reactive upon an external stimulus. Most prominent photo-crosslinking groups like benzophenones and diazirines, that liberate a highly reactive carbene upon irradiation with UV-light, have been exploited to study biological processes.[6,10–12] In addition to these external stimulus induced reactivities, significant advances to genetically encode mild bioreactive electrophilic amino acids into proteins have been made in recent years.[13] In this case the reaction with a nucleophilic amino acid side-chain is triggered by proximity. To date functional groups that could be genetically encoded into proteins include alkylbromides[14,15], fluorosulfate[16], arylcarbamates[17] and vinylsulfones.[18,19] With the help of these handles, covalent linkages targeting different nucleophilic amino acids have been achieved. However, the methodology requires an orthogonal tRNA/synthetase pair and often extensive genetic engineering.

On the other hand, these mild electrophiles can be generated using chemical methodologies. Besides the mere chemical synthesis or semi-synthesis of proteins using ligation strategies or advanced methods for solid-phase peptide/protein synthesis, the transformation of an amino acid into an electrophilic group is a valuable approach. In addition to using bis-reactive crosslinkers,[20] or chemo-enzymatic methods,[21] the transformation of cysteine or selenocysteine into dehydroalanine is an frequently employed strategy to access electrophilic moieties on proteins.[22] The drawback of this approach is that the generated electrophile is located close to the amide-

backbone of the protein and therefore less accessible than electrophiles on amino acid side chains. Moreover, depending on the tertiary structure of the protein, the final elimination-step can be incomplete, leading to a heterogeneous product mixture.[23]

One particularly interesting and intensively studied protein for the incorporation of mildly reactive electrophiles is the small, stably folded 76 amino-acid protein ubiquitin (Ub). The attachment of ubiquitin to a target protein, also referred to as ubiquitination, is an important reversible post-translational modification, involved in the regulation of many cellular processes from autophagy and proteasomal degradation to DNA-repair.[24] Protein-ubiquitination is a highly orchestrated, ATP-dependent process catalyzed by a complex cascade involving Ub-activating (E1), -conjugating (E2) and –ligating (E3) enzymes.[25] Thereby, the ubiquitin *C*-terminus is covalently attached to the nucleophilic side chains of lysine via an isopeptide bond. In addition, one ligase that preferentially transfers Ub onto serine/threonine side-chains has been reported.[26] The process of ubiquitination is reversed by deubiquitinating enzymes (DUBs), a large group of proteases that are able to either hydrolyze the isopeptide bond between Ub moieties within a polyubiquitin-chain or between Ub and a substrate protein.[27]

Most DUBs and E1/E2 ligases have a catalytic cysteine, which makes them a suitable target for mild electrophiles. To date a variety of different electrophilic ubiquitin probes have been developed to target these enzymes.[28,29] Reported warheads, including propargylamines, vinylmethylesters, vinylsulfones, and alkyl halides, have been incorporated into the *C*-terminus of monoubiquitin,[30–34] resulting in covalent binding to a DUB. This strategy, however, does not allow to discriminate between different DUB-families that show preferences for certain polyUb chains. To overcome this limitation, diubiquitin-based probes with the warhead positioned either at the *C*-terminus or at the isopeptide linkage have been prepared by means of native chemical ligation or Cu(I)-click chemistry in combination with the incorporation of unnatural amino acids.[35–39] These probes were successfully employed to investigate the linkage specificity of purified DUBs as well as global DUB-profiling. Nonetheless, to the best of our knowledge, no Ub-probes bearing a mild electrophile on an amino-acid side chain have been synthesized so far.

Here, we present a new approach for the selective incorporation of mild electrophiles into specific positions within a protein sequence. Our approach for the first time utilizes unsaturated aliphatic phosphonamidates as such mild electrophiles, which, in combination with the straightforward incorporation of unnatural amino acids by selective pressure incorporation[40], enables the exact placement of warheads into specific side chains within the ubiquitin sequence (Figure 1a).

The obtained electrophilic protein-probes selectively react with recombinant DUBs in a proximity-induced fashion, depending on the position of the phosphonamidate. Moreover, we utilize this strategy for the unprecedented functional modulation of DUB-activity by specific covalent tagging of ubiquitin to given ubiquitin binding sites of the DUB USP5. Finally, we employ ubiquitin-phosphonamidates in the proteome-wide profiling of ubiquitin interactors, where we observe a position-specific reactivity profile.

Design and reactivity of aliphatic phosphonamidates as electrophilic targeting probes: Unsaturated phosphorous electrophiles are important building blocks in organophosphorus chemistry, especially for the synthesis of P-based heterocycles.[41,42] Our group has contributed to this area by introducing unsaturated phosphonites in Cys selective bioconjugation reactions.[43–46] In these studies, we could demonstrate the straightforward incorporation of unsaturated phosphonamidates into various functional synthetic building blocks, including fluorescent labels, payloads for the engineering of antibody-drug-conjugates (ADCs) and cell-penetrating peptides (CPPs). It is important to stress that in all of these studies, we focused on methods enabling high conversion rates to obtain sufficient amounts of functional bioconjugates for biological and pharmacological studies.[47]

While for protein-bioconjugation high reactivity is required, for proteome-wide enzyme targeting[15,16,48,49] a less promiscuous electrophile is needed to ensure the identification of binding partners by proximity induced covalent capture and to prevent non-specific reactions. Based on the ability to fine-tune the electrophilic reactivity of unsaturated P(V)-derivatives by varying the substituents at phosphorus[50], we saw a unique opportunity to engineer a less reactive unsaturated P(V)-derivative as a novel mild Cys-selective electrophile for protein-targeting. In parallel, we envisioned that the chemoselectivity of the Staudinger-reactions[43,51–53], would allow the site-selective placement of this reactive group into proteins, which is impossible for other electrophilic warheads.

In our previous work, we observed that *N*-alkylated vinyl phosphonamidates show only very low conversions in thiol-conjugation reactions at pH 8.5 in aqueous buffers (<20% conversion with reduced glutathione (GSH) after 24 h) in contrast to *N*-phenyl-derivatives.[44] Based on this observation, we hypothesized that the more reactive *N*-alkylated ethynyl-analogue would not only display sufficient reactivity for cysteine upon substrate binding but that this moiety can also be selectively incorporated into protein. For this, we envisioned using well-established auxotrophic expression of alkyl-azide containing amino acids followed by a chemoselective Staudinger-phosphonite-reaction with ethynyl-phosphonites.

We started our study by exploring the reactivity differences between an ethynyl-phosphonamidates containing an aromatic (**1**) and an aliphatic (**2**) *N*-substituent, which we had not investigated before. We incubated compounds **1** and **2** with two equivalents GSH at pH 8.5 (Figure 1b). While the conjugation of phosphonamidate **1** to GSH was complete within approximately 15 minutes, **2** exhibited a more than four times greater half-life (Figure 1c). To our satisfaction, at a physiological pH of 7.4 almost no conjugation was observed for the aliphatic phosphonamidate **2**, which is an important prerequisite to ensure selectivity towards the target.

Expression of azide-containing ubiquitin: Next, we focused on the incorporation of *N*-alkyl-ethynyl-phosphonamidates into proteins. As mentioned before, the incorporation of an alkyl azide-containing amino acid into a protein of interest is key to generate unsaturated phosphonamidate warheads at a later stage. For our intended application to develop electrophilic ubiquitins with an electrophile at specific locations for DUB-targeting, we envisioned azidohomoalanine (Aha) is an optimal unnatural amino acid for the auxotrophic expression, due to its chain length and similarity to methionine in size, structure and polarity. (Figure 1a)

We started with the installation of a C-terminal Aha on *N*-terminally human influenza hemagglutinin epitope peptide (HA)-tagged ubiquitin. Therefore, we removed the *N*-terminal Met and inserted a G76M mutation. For the expression we followed the procedure described by Schneider et al. (For details see Supplementary Information 3.1).[54] Auxotrophic expression usually yielded 2-10 mg protein/L expression medium. To estimate the ratio of Aha to Met incorporation, purified proteins were reduced with TCEP and analyzed via intact protein-mass spectrometry (Figure 1d). Since UbG76M does not interfere with later Staudinger-reactions and can be separated in a later step, a small amount of impurity (5-15%) was tolerated.

Installation of phosphonamidate warheads on ubiquitin: Subsequently, we aimed to convert the aliphatic azide from an Aha-residue into an *N*-Alkyl-ethynyl-phosphonamidate by reaction of ethynyl-phosphonite **3** in aqueous-buffers. To test different reaction conditions, the phosphonite was synthesized as described before[43] and used as a crude in the Staudinger-phosphonite reaction (SPhR). After several rounds of optimization, we were able to achieve 37% conversion of an azide-containing model-peptide (LYR**Aha**AGKAYLG), when reacted with 40 eq. phosphonite **3** for 24 h at 37 °C in basic buffer (AmBiC/MeCN 4:1, pH 8.6; Suppl. Information 4.5). Applying these conditions to the reaction of **3** with Ub76Aha we were not able to achieve significant conversion to the phosphonamidate-analogue. We attribute this result to the lower reactivity of **3** in reactions with alkyl azides in comparison to aryl azides in aqueous conditions.[55,56]

Since our group has previously demonstrated that the Staudinger-phosphonite reaction proceeds smoothly with unprotected peptides containing aryl-azides in polar aprotic solvents[43,44] we changed to DMSO as solvent. Furthermore, ubiquitin is known to dissolve well in DMSO and can be successfully refolded by rapid dilution into physiological buffer.[32] We found that 10 eq. phosphonite **3** are sufficient to achieve >85% conversion after 3 days at 60 °C. (Figure S1) Using these optimized conditions we were able to obtain the phosphonamidate [P(V)] functionalized HA-UbG76P(V) **P1** in high purity and 23% yield after semi-preparative HPLC. (Figure 1d & e) Lyophilized ubiquitin-P(V) was stable at -80 °C for months without any apparent sign of decomposition. The functionalized protein was reconstituted by dissolving the powder in a minimal amount of DMSO (final conc. of 2 mM) followed by rapid dilution with PBS pH 7.4 or ammonium bicarbonate buffer pH 7.9 and subsequent removal of DMSO via spin-filtration. To verify correct refolding of modified ubiquitin, the CD-spectrum of HA-UbG76P(V) **P1** was compared to the non-modified protein HA-UbG76Aha. As the ellipticity did not show major differences, we concluded that the modification procedure does not significantly affect ubiquitin folding (Figure 1f & S2).

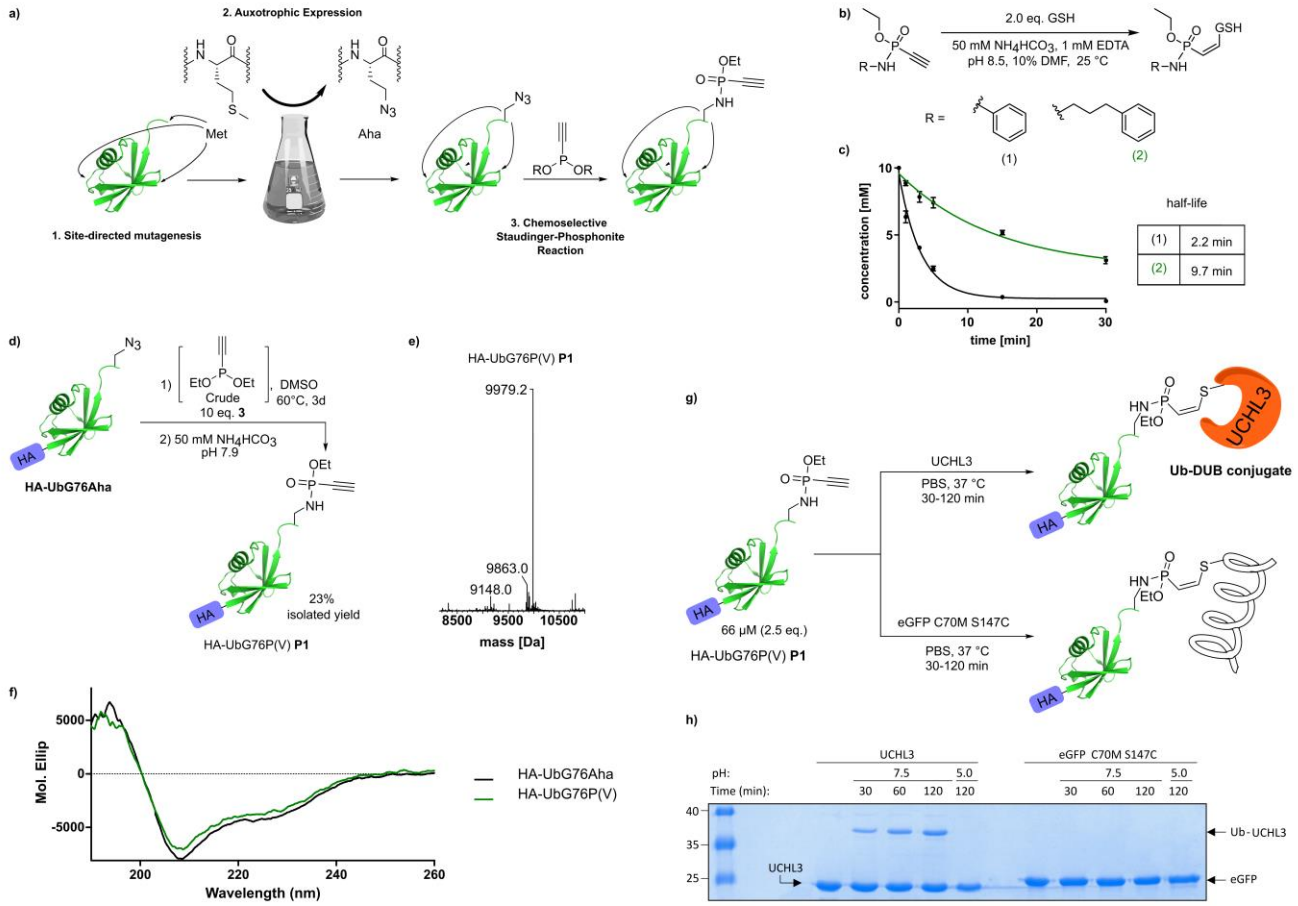

Figure 1. Electrophilic ubiquitin probes based on aliphatic ethynyl-phosphonamidates a) Schematic representation of the semi-synthetic strategy to obtain phosphonamidate functionalized ubiquitin-probes. b) Reaction of phosphonamidates (**1**) and (**2**) with reduced glutathione. c) Half-life of phosphonamidate (**1**) and (**2**) in the presence of red. glutathione in basic buffer. d) Synthesis of HA-UbG76P(V) **P1** via the chemoselective Staudinger-phosphonite reaction. e) Intact protein mass-spectrum of reconstituted **P1**. f) Comparison of the CD-spectra of HA-UbG76Aha and **P1**. g) Reaction scheme of P(V)-modified ubiquitin with UCHL3 and eGFPC70M S147C. h) SDS-gel analysis of the reaction of **P1** (66 µM, 2.5 eq.) with the recombinant deubiquitinating protein UCHL3 and eGFP (each 26.6 µM) containing a single accessible cysteine (PBS pH 7.4, 37 °C, 30-120 min).

DUB labelling using electrophilic ubiquitin probes: With the electrophilic ubiquitin in hand, we probed covalent selective reactions with DUBs. For initial testing, we made use of the DUB UCHL3, a small size cysteine protease, which recognizes and hydrolyzes an amide bond at the *C*-terminal glycine of ubiquitin.[57] We incubated this enzyme with a slight excess (2.5 eq., 66 µM) of our probe in PBS pH 7.5 at 37 °C (Figure 1g). Samples were taken after 30, 60 and 120 minutes and analyzed via SDS-PAGE. Already after 30 minutes a distinct band of the Ub-DUB-conjugate was visible, which increased in intensity over time (Figure 1h). In contrast, performing the same experiment at pH 5.0 did not lead to any product formation. To verify that the reaction between HA-UbG76P(V) and UCHL3 is indeed proximity/affinity induced, we reacted the probe with an eGFP mutant bearing a solvent accessible cysteine (C70M S147C). Using the same conditions as for UCHL3 labeling, no conjugation could be observed (Figure 1h). However, when the reaction time was prolonged to >24 h or the pH was increased to 8.5, the corresponding Ub-GFP adduct was visible in SDS-PAGE and intact protein-MS. (Figure S3 & 3.6).

Since the electrophilic moiety of the HA-UbG76P(V) **P1** extends the *C*-terminal tail relative to wild-type ubiquitin, we envisioned that incorporating the electrophile at position G75 or R74 instead of G76 might alter the conjugation efficiency to the catalytic cysteine. While **P1** (G76) and **P2** (G75) showed similar labeling conversions

with UCHL3, **P3** (R74) showed only poor labeling. In addition, we observed the same trend with the catalytic domain of another DUB USP2-CD (Figure S4). To our delight, the direct comparison to the established DUB-probe UbG75VME showed a similar labeling efficiency for both enzymes (Figure S5). These findings suggest that the ubiquitin *C*-terminus is flexible enough to compensate for some structural difference in its reaction with electrophilic phosphonamidate-probes; however, an extensively truncated protein cannot position the electrophilic warhead correctly in the catalytic site of the DUB, resulting in inefficient protein conjugation.

DUB targeting with specifically positioned electrophile: The enzymatic activity of DUBs is often regulated by conformational changes upon ubiquitin binding.[58] Furthermore, many of the DUBs exhibit ubiquitin linkage and substrate-specificity. Previous studies have shown that linkage specific DUB labeling can be achieved when the electrophile is incorporated at the Ub-Ub isopepide bond.[35–37] Inspired by these discoveries, we investigated whether the site-specific incorporation of a phosphonamidate warhead at K48 or K63 can also enable linkage-specific DUB targeting driven by proximal Ub binding at the S1' site in the absence of the distal Ub. To obtain these probes we constructed K48M or K63M mutants and made use of our auxotrophic expression system to obtain HA-UbK63Aha and HA-UbK48Aha. These ubiquitin variants were reacted with alkyne-phosphonite **3** and purified as described before to obtain **P4** (K63) and **P5** (K48). (Figure S6) In order to test the DUB labeling preference we first chose UCHL3, which efficiently cleaves ubiquitin from small unstructured peptides but lacks an S1' binding pocket.[59] Therefore, it exhibits low hydrolytic activity towards polyubiquitin. Unlike the *C*-terminal mutant **P1,** lysine substituted ubiquitin-phosphonamidates **P4** and **P5** did not react with UCHL3.

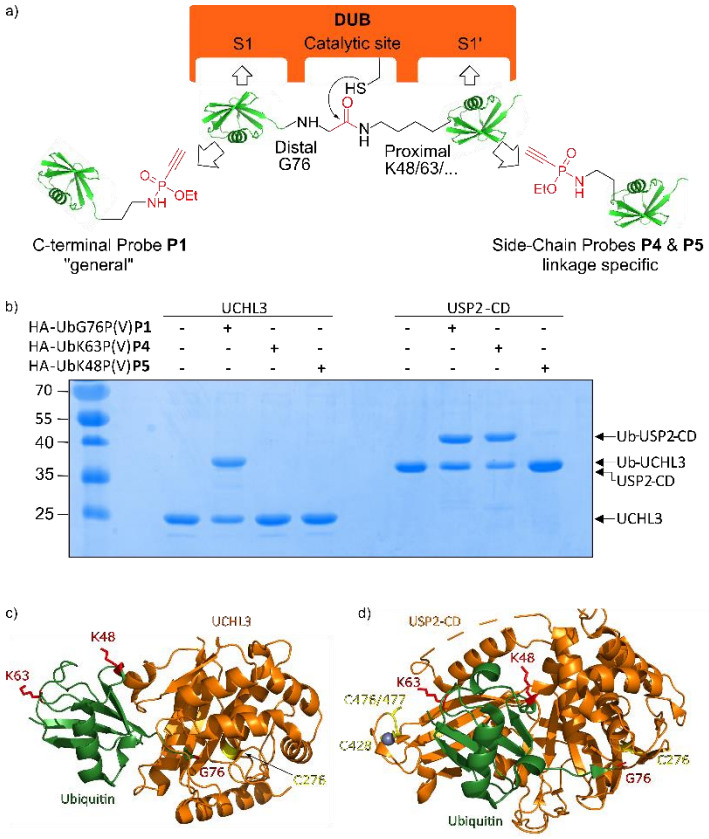

Figure 2. a) Schematic illustration of polyubiquitn recognition by deubiquitinating enzymes via the S1 (distal) and S1' (proximal) binding pockets. b) SDS-PAGE analysis of the reaction between probes **P1**, **P4** or **P5** (each 66 µM, 2.5 eq.) and the DUBs UCHL3 and USP2-CD, respectively. c) Co-crystal structure of UCHL3 (orange) and ubiquitin (green; PDB 1XD3) and d) Co-crystal structure of USP2-CD (orange) and ubiquitin (green; PDB 5XU8). Cysteines are highlighted in yellow, warhead-positions K48, K63 and G76 are highlighted in red.

Next we probed the catalytic domain of USP2 (USP2-CD), a DUB with low linkage specificity, with **P1-5**. While the *C*-terminal probe **P1** and the K63-probe **P4** reacted efficiently with the enzyme, we did not observe any conversion for **P5**. Since K63- and K48-linked polyubiquitins should be equally well recognized by the DUB, we wanted to understand why our K63-derived probe was more reactive towards USP2-CD. To test whether **P4** targets the active site cysteine of USP2-CD we conducted competition experiments with Ub-G75VME (Ub-VME) that contains a vinyl methyl ester as electrophilic warhead on the *C*-terminus of ubiquitin. The enzyme was incubated for 2 h with our probe **P4**, followed by another 2 h with the Ub-VME probe. Additionally, we also performed the reaction in reversed order. In both cases, no double-modification of the DUB could be observed on SDS-PAGE (Figure S7). This implies that modification by one probe impedes labeling by the second probe which could be a result of both probes labelling the catalytic cysteine or occupying the same Ub binding-site. To identify the labeling site, we performed an in-gel trypsin digest of the band corresponding to the probe labeled USP2-CD followed by tandem-MS analysis of the obtained peptides (Supplementary Information 2.16). Of note, the P-N bond of our P(V)-warhead is hydrolyzed under acidic conditions leaving a residual stamp of 134.01 Da on the labeled amino acid. For **P1** we identified the active site cysteine (Cys-276 – counting for the full length protein) to be the major site of labeling, whereas several cysteine residues allocated in the zinc-finger domain of USP2-CD were labeled by **P4** (Cys-476/477 & Cys428). Since USP2-CD only provides a poor S1' binding interface[60], we concluded that HA-UbK63P(V) **P4** occupies the distal S1-site. In this case K63 is positioned in a way, that the phosphorus-electrophile is pointing towards the cysteine residues of the zinc-finger domain, which in the end also results in proximity induced labeling. (Figure 2d & S9) UCHL3 does not contain such a region, which further explains why our K48- or K63-probes did not label this protein (Figure S8). In order to further investigate the ability of Ub-P5 probes to label DUBs via binding to an alternative site other than S1 we applied them in labeling of the 93 kDa large multidomain DUB USP5. This protein is responsible for the hydrolysis of unanchored polyubiquitin chains in the cell. It recognizes at least linear K6-, K29-, K48- and K63 linked chains and has min. four ubiquitin binding sites (S1', S1, S2, S3).[61] Furthermore, USP5 shows a comprehensive interaction with the proximal ubiquitin at the S1' site at which ubiquitin binding is essential for the hydrolysis activity. In addition to probes **P1-P5** we also tested the commercial Ub-VME probe.

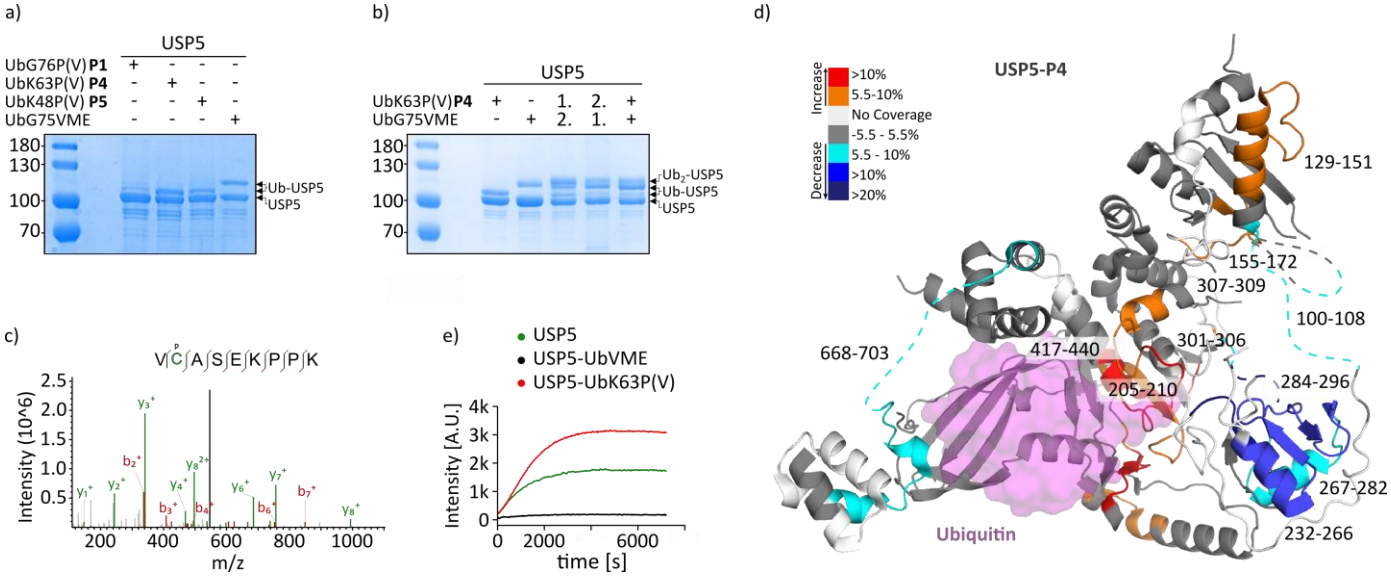

Figure 3. Interaction of USP5 with different electrophilic Ub-probes. a) SDS-PAGE analysis of USP5 reacted with **P1**, **P4**, **P5** and the *C*-terminal probe Ub-VME (3 µM enzyme, 20 µM probe, PBS pH 7.5, 37 °C, 2 h). b) SDS-PAGE analysis of the sequential reaction of USP5 with **P4** and Ub-VME. c) MS/MS-spectrum identifying P(V) labelled Cys-815 obtained from the reaction of USP5 with **P4.** d) Crystal structure of USP5 (PDB: 3IHP) to illustrate the deuterium-exchange (HDX) eperiment. Color-changes indicate regions with altered deuterium incorporation upon labelling with **P4**. USP5 (3 µM) was labeled with 20 µM probe for 2 h. Results were obtained from two technical replicates. e) Enzyme activity of USP5 and USP5 after the reaction with **P4** and Ub-VME, respectively.

Interestingly, the *C*-terminal P(V)-probes (**P1-3**) showed almost no labeling while lysine-substituted probes **P4** and **P5** showed a distinct band of USP5-Ub conjugate (Figure 3a & S10; 20 µM probe, 3 µM enzyme). In addition, Ub-VME was also able to generate a higher-molecular weight species corresponding to the USP5 conjugate. We assume that these observations can be rationalized by structural differences of the *C*-terminal Ub-PA probes and Ub-VME resulting in altered proximity to available cysteines. To our surprise the conjugation product of Ub-VME was detected in a higher molecular weight region than those of Ub-P(V) probes on the denaturing SDS gel. (Figure 3a) We speculated that the difference in migration behavior might be due to a different labeling site resulting in different protein-conformations and changes in electrophoretic mobility. To test this hypothesis, we performed double labeling experiments with **P4** and Ub-VME. (Figure 3b) Independent of the labeling order (**P4** before UbVME, UbVME before **P4** or simultaneous addition) an additional band, most likely belongs to USP5 covalently bound to two ubiquitins (Ub_2-USP5), was present. This indicates that the two probes do not conjugate to the same cysteine and are probably allocated to different binding sites. Furthermore, we observed a more pronounced Ub_2-USP5 band when conjugating **P4** first. To pinpoint the site(s) of labeling formed by the different probes, we performed bottom-up proteomic analysis on the conjugation products. For Ub-VME only the active site Cys-335 was identified to be the targeted amino acid (Figure S11). Opposed to that, we found **P4** to label cysteine residues 793 and 815 (Figure 3c & S11). Cys-815 was also found to be labeled by **P5**; however, the region around Cys-793 was not covered in this analysis, so we cannot make any propositions regarding the modification of this cysteine. Nonetheless, these results show that our lysine-substituted Ub-probes interact with a different region in USP5. This once more highlights the utility of the herein introduced protein-based phosphonamidates as proximity-induced probes targeting cysteines in the environment of their binding-site.

To better understand how our probes interact with the DUB and to investigate if binding induces conformational changes within the DUB we employed hydrogen-deuterium-exchange mass spectrometry (HDX-MS, Supplementary Information 2.17-2.19). In HDX-MS the exchange rate of amide backbone hydrogens in deuterated solvent is measured. Since this process is highly influenced by secondary structure, HDX-MS is a powerful method to monitor protein conformational dynamics. H/D exchange was performed for three different samples: apoUSP5, USP5-UbVME and USP5-**P4** that were prepared similar to the gel-experiments in Figure 3a/b.

Our HDX-MS data for apo-USP5 and USP5-UbVME were consistent with previous reports and our observations that this probe exclusively binds to the distal binding site of USP5.[62] (Figure S12) In line with our previous observations these regions were mostly unaffected in the **P4** treated sample. Instead, H/D-exchange indicated a strong interaction with the ubiquitin diglycine-binding zinc-finger UBP (ZnF-UBP) domain (residues 205-296, Figure 3d). This domain is thought to be of key importance for the recognition of the physiological substrates of USP5 by binding the *C*-terminal diglycine of the extreme distal moiety in a polyubiquitin-chain.[62,63] Moreover, some regions with increased H/D-exchange were determined that were not present in the UbVME treated sample. A higher degree of exchange is a sign for increased flexibility and thus supports our hypothesis that **P4** binding induces a domain rearrangement in the DUB. In contrast, Ub-VME is a *C*-terminally functionalized probe that is not recognized by the ZnF-UBP domain and therefore is unable to induce a structural rearrangement. Previously, it was found that the presence of wt-ubiquitin can increase the hydrolytic activity of USP5 towards Ub-AMC by binding to ZnF-UBP.[64] This should also be the case for the covalently attached **P4.** We compared the catalytic activity of apo-USP5 towards the fluorogenic probe Ub-AMC with the activity of the two probe conjugated USP5 variants. (Figure 3e, Supplementary Information 2.20) The *C*-terminal probe fully inhibited the hydrolytic activity of the enzyme by occupying the S1-site and covalently blocking the active site cysteine. In contrast, **P4** was able to accelerate reaction kinetics drastically, verifying the occupation of the S1'-site.

Profiling interaction partners in cell-lysates: Encouraged by these *in vitro* studies using isolated DUBs, we determined weather ubiquitin-phosphonamidates can be used to specifically target DUBs in a more complex environment such as a cell lysate. We first tested if wild type ubiquitin or reduced glutathione would affect the labeling efficiency of our DUB-probes. Therefore, we repeated the labeling experiments with purified DUBs and analyzed the labeling efficiency by SDS-PAGE. While glutathione concentrations up to 10 mM did not significantly impair labeling, as little as 5 eq. of wild type ubiquitin reduced labeling efficiency by approximately 60%. (Figure S13 & S14) Based on these findings, we concluded that a mild excess of Ubiquitin-P(V)-probes should be used to facilitate optimal labeling.

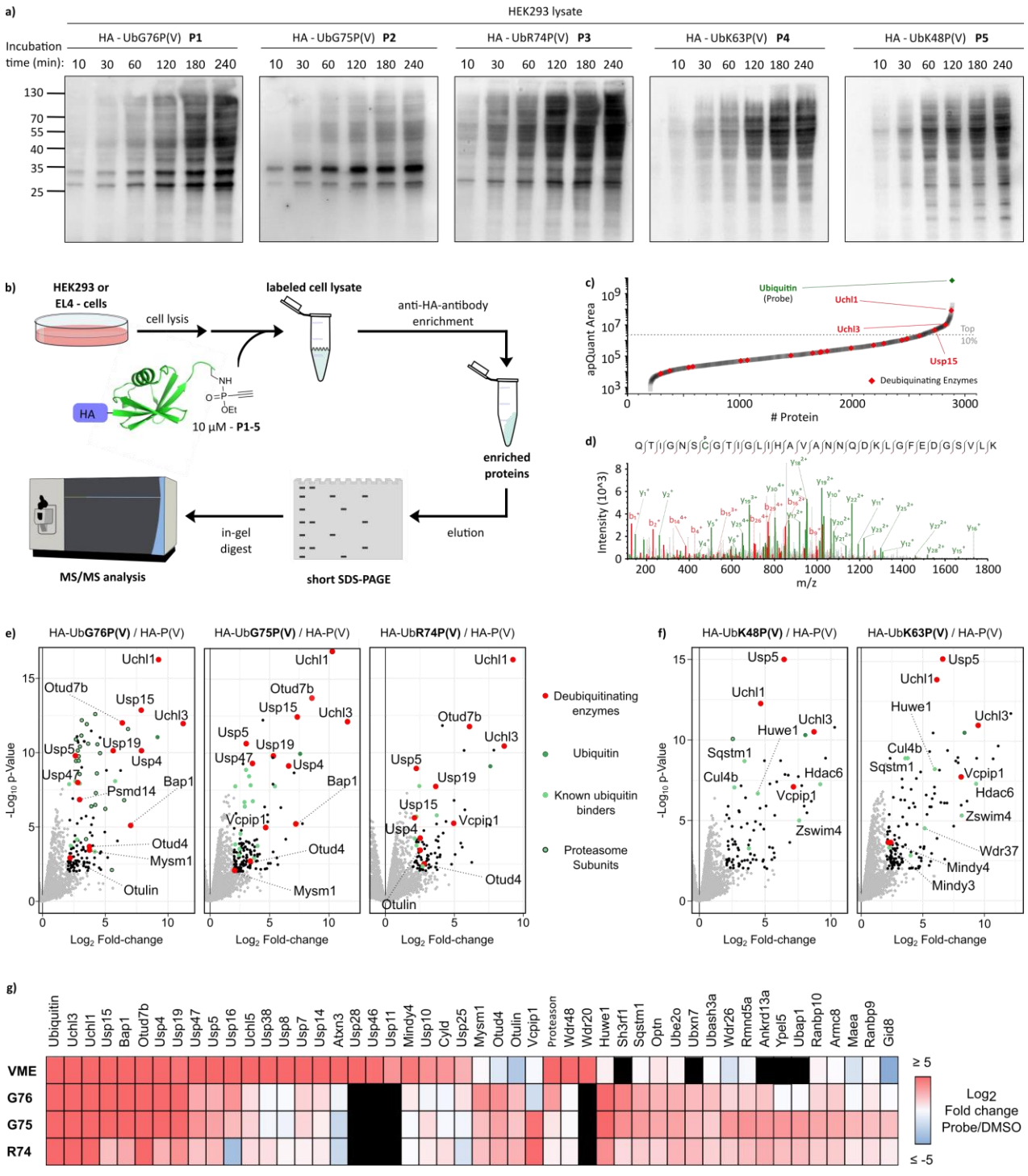

Figure 4: Proteome-wide protein targeting using Ub-phosphonamidates a) Western blot analysis of HEK293 cell-lysate (11 mg/ml) incubated with HA-tagged Ub-probes **P1-5** (30 µM probe, 37 °C). b) Schematic representation of the workflow for mass-spectrometry based investigation of the proteome-wide labelling of ubiquitin-phosphonamidate probes. c) Proteins identified after pull-down from HEK293 lysate using **P1** ranked by intensity. d) MS/MS-spectrum identifying the labelled active-site cysteine of UCHL1. e) Volcano-blots of the pull-down experiments from EL-4 cell lysate using *C*-terminal Ub-P(V) probes **P1-3**. f) Volcano-blots of the pull-down experiments from EL-4 cell lysate using K48/K63 substituted Ub-P(V) probes. All pulldowns were performed at 9 mg/ml protein concentration with 11 µM of the corresponding probe at 37 °C for 2 h. Proteins with a >4-fold enrichment and a p-value <0.01 were considered significant. g) Comparison of the enrichment efficiceny obtained for *C*-terminal phosphonamidate probes **P1-3** and Ub-VME. Only proteins that were significantly enriched (> 4-fold, p-value <0.01) in at least one condition and have a reported ubiquitin interaction were considered.

Having established these reaction conditions, we compared our five different probes **P1-P5** for their ability to label different proteins in the cell-lysate in a time dependent manner. HEK293-lysate (11 mg/ml) was incubated with a probe concentration of 30 µM for up to 4 hours at 37 °C and labeling was visualized by an anti-HA-tag western blot. The labeling intensity did not significantly increase after two hours except for HA-UbG76P(V) **P1** (Figure 4a), whereas every probe showed a different labeling pattern: the *C*-terminal probes **P1** and **P2** showed two prominent bands between 25 and 35 kDa that were not as distinct when using the twice-shortened probe **P3**. Also lysine substituted probes **P4** and **P5** were able to stain a protein around 25 kDa, although they in general showed a more pronounced labeling of proteins larger than 40 kDa.

To obtain more specific information on the identity of the targeted proteins, the HA-tagged probes were used to perform antibody pull-down probes were used to perform pull-down experiments from HEK293 cell lysates followed by analysis using bottom-up proteomics (Figure 4b, Supplementary Information 2.21). For the pull-down of the three *C*-terminal probes **P1-3**, we were able to identify the DUBs UCHL1, UCHL3 and USP15, among the top 10% of the most abundant proteins in our analysis (Figure 4c & S15). To our delight, we were even able to identify the sites of labeling for UCHL1 out of the lysate pull-down, for all three *C*-terminal probes. Next to the active site cysteine (Cys-90, Figure 4d) we also found labeling of Cys-152, which is located in the so called active-site cross-over loop. We propose that this structural feature is involved in substrate selection and therefore likely to react with our warhead installed on one of the terminal amino acids.[65,66] Interestingly, the degree of labeling varied between the three probes, with **P3** being most likely to label the non-catalytic cysteine which correlates with the distance differences measured on the co-crystal structure of ubiquitin and UCHL1 (Figure S16; PDB: 3IFW).

For a more comprehensive analysis of which proteins can be efficiently enriched by our probes, we performed LC-MS/MS analysis of replicate pull-down experiments from EL-4 mouse thymoma cell-lysate. To compensate for the background reactivity of our phosphonamidate-probes we decided to compare the enrichment efficiency to a small HA-tag peptide functionalized with a *C*-terminal phosphonamidate warhead (HA-P(V), Figure S17). This peptide did not show any reactivity towards recombinant DUBs at neutral pH over the course of 8 hours. (Figure S18)

Proteins from pull-down experiments were identified and quantified using MS-Fragger.[67] Relative to the control compound HA-P(V) we were able to enrich 13 DUBs by **P1** (Figure 4e, left volcano-plot). Besides the DUBs also many proteasome subunits were enriched. We rationalize this observation with the fact that some of these proteins such Psmd4 act as ubiquitin receptor. Furthermore, the enriched proteasomal protein Psmd14 acts as a deubiquitinating enzyme. Despite being a metalloprotease, Cys-120 is located next to the active site, so it might be able to react with our warhead. Additionally three E3 ligases (Huwe1, Sh3rf1 and Ube2o) were significantly enriched. The truncated *C*-terminal probe **P2** was only able to enrich 12 DUBs, however Vcpip1 was among the enriched proteins and could not be enriched by **P1** (Figure 4e, middle volcano-plot). In contrast to **P1**, the proteasome subunits as well as the DUB Psmd14 were not enriched. As expected from in-vitro and western-blot data, the two times truncated probe **P3** resulted in only 10 significantly enriched DUBs (Figure 4e, right volcano-plot). Moreover, the enrichment-factor was reduced for most of the enriched DUBs, indicating less efficient labelling. In addition, we performed a gene onthology (GO) analysis of significantly enriched proteins in all three datasets. This revealed a dominant enrichment for ubiquitin associated biological processes, highlighting the utility of our probes (Figure S19).

Next, we applied our lysine-substituted probes **P4** & **P5** to interactor profiling in EL-4 cell lysate. In comparison to the *C*-terminal probes less DUBs were observed to react with both probes. The most prominently enriched DUB for both probes was USP5, for which we could already demonstrate a strong interaction with the ZnF-UBP domain of the recombinant protein. Additionally, UCHL1 and UCHL3 were among the most efficiently enriched proteins. (Figure 4f). Moreover, Vcpip1 (both **P4** & **P5**) and Mindy3 & Mindy4 (only **P4**) could be enriched. One non-DUB protein, HDAC6, was pulled-down very efficiently (enrichment-factor >100) by both **P4** and **P5** but was not enriched by any *C*-terminal probe. This enzyme is known to bind strongly mono- and polyubiquitins by its *C*-terminal ZnF-UBP domain and transport ubiquitinated misfolded proteins to aggresomes.[68]

Finally, we wanted to compare our newly developed probes to a previously established probe. To this end, we also performed the same pulldown with Ub-VME and compared the obtained enrichment-efficiencies to our *C*-terminal probes **P1-3** (Figure 4h). Overall, Ub-VME was able to enrich more DUBs than **P1**-**3**. Especially USP-type DUBs were quite inefficiently enriched. while some DUBs (Mysm1, Otdu4, Otulin & Vcpip1) could be exclusively targeted by our phosphonamidate-based probes. Moreover, Ub-VME did only react with a hand full of ubiquitin

interacting proteins. This is in accordance with the notion that this activity-based probe targets almost exclusively the active site of deubiquitinating enzymes. In contrast, the proximity-induced reactivity of our ubiquitin-PA probes allows targeting a broader range of interactors. Among the ubiquitin-PAs, **P2** was most efficient in enriching ubiquitin-associated proteins other than proteasomal components (16 out of which 11 are E3-ligases or E3-ligase associated).

Conclusion: In summary, we have introduced ethynyl-*N*-alkyl-phosphonamidates as new modalities to engineer protein-based electrophiles. We highlight this concept by incorporating these mildly reactive chemical warheads into specific positions in ubiquitins, combining auxotrophic protein expression with a chemoselective Staudinger-phosphonite reaction of ethynyl phosphonites. We demonstrate that these electrophilic ubiquitins can undergo proximity-induced labeling of a protein binder, resulting in specific conjugation to cysteine residues in deubiquitinases (DUBs), E3-ligases and other ubiquitin binding proteins even in cell lysates. By changing the position of the electrophile within the ubiquitin sequence, we show specific labelling of DUBs and the distinct regulation of their activity, for instance the increased activity of USP5. Moreover, the herein presented Ub-phosphonamidate probes were able to enrich a large portion of DUBs and other ubiquitin-associated proteins, like E3-ligases, from cell lysates in contrast to other purely activity based ubiquitin-based probes.
We strongly believe that this concept will open the door to designing electrophilic protein probes to interrogate protein interaction networks in complex environments and contribute to the development of next-generation biopharmaceuticals for specific targeting. These studies are further explored in our laboratories.

ASSOCIATED CONTENT

1. Supplementary Figures, detailed experimental procedures, materials and methods (PDF)
2. Peptide list of the proteomics analysis of labelled recombinant DUBs. (XLSX)
3. Peptide and protein list of the proteomics analysis of the HEK293 pull-down experiments. (XLSX)
4. List of enriched proteins from the EL-4 pull-down experiments. (XLSX)
5. Gene-Onthology analysis of enriched proteins. (XLSX)

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