

# Chemoproteomic profiling and discovery of protein electrophiles in human cells

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**Activity-based protein profiling (ABPP) serves as a chemical proteomic platform to discover and characterize functional amino acids in proteins on the basis of their enhanced reactivity towards small-molecule probes. This approach, to date, has mainly targeted nucleophilic functional groups, such as the side chains of serine and cysteine, using electrophilic probes. Here we show that ‘reverse-polarity’ (RP)-ABPP using clickable, nucleophilic hydrazine probes can capture and identify protein-bound electrophiles in cells. Using this approach, we demonstrate that the pyruvoyl cofactor of S-adenosyl-L-methionine decarboxylase (AMD1) is dynamically controlled by intracellular methionine concentrations. We also identify a heretofore unknown modification—an N-terminally bound glyoxyl group—in the poorly characterized protein secernin-3. RP-ABPP thus provides a versatile method to monitor the metabolic regulation of electrophilic cofactors and discover novel types of electrophilic modifications on proteins in human cells.**

Post-translational modifications (PTMs) serve to diversify protein structure and function in important ways, including, but not limited to the regulation of protein activity, protein–protein interactions, and protein localization and stability in cells<sup>1</sup>. Proteins are subject to a wide array of chemically diverse PTMs, which have historically been discovered on a case-by-case basis by in-depth investigations of individual proteins<sup>2</sup>. Advances in mass spectrometry (MS) technologies have, however, made it possible to discover novel PTMs with remarkable scope, sensitivity, and structural resolution, leading to an expanded understanding of diverse modification states, such as the N-acetylation/acylation of lysines<sup>3</sup> and electrophilic/oxidative modification of cysteines<sup>4,5</sup>. It is likely, however, that many functionally important PTMs remain to be discovered because they are, as-of-yet, structurally unpredicted and/or occur on proteins of unknown function<sup>3</sup>.

Various approaches have been used to discover and characterize PTMs, including exploiting their often atypical chemical reactivity for covalent tagging and enrichment<sup>6–8</sup>. Activity-based protein profiling (ABPP) also uses chemical probes that react with large classes of proteins based on shared functional and/or structural properties<sup>9</sup>. Such aberrant ‘reactivity’ can originate from conserved amino acid residues that confer special activities to proteins, such as enzymatic catalysis, and, accordingly, ABPP has been applied to characterize diverse enzyme classes in native biological systems<sup>9</sup>. To date, ABPP has principally focused on targeting nucleophilic functional groups<sup>10</sup>, largely because the main twenty proteinogenic amino acids are replete with nucleophilic side chains, but devoid of reactive electrophiles. Proteins, however, also use electrophilic groups for function, which are typically acquired through installation as covalent PTMs<sup>1,2,11</sup> or by binding of exogenous cofactors<sup>12</sup>.

Because electrophilic PTMs define reactive centers that are uncommon and not easily predicted from the primary structures of proteins, it seems likely that additional uncharacterized electrophilic functional groups exist in the human proteome. We reasoned that, by reversing the polarity of activity probes from electrophilic to nucleophilic, ABPP could potentially be adapted

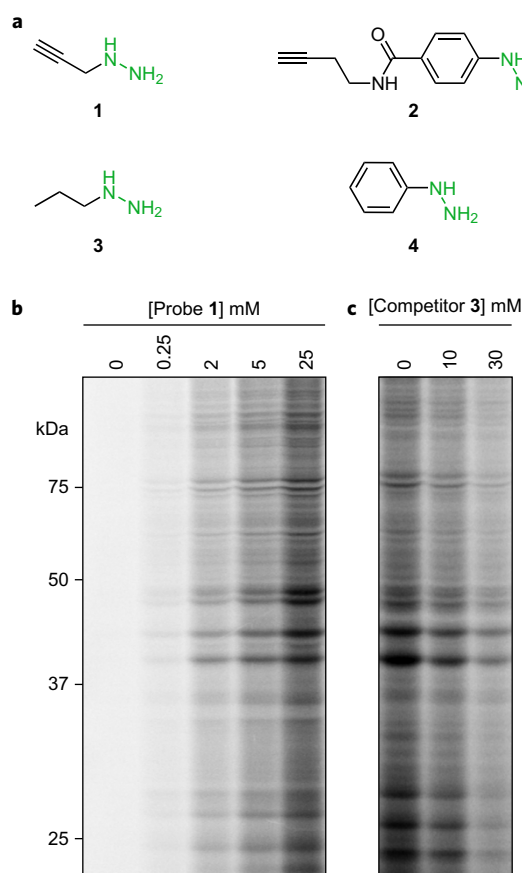
to globally discover protein-bound electrophiles in native biological systems. Here, we show that reverse polarity (RP)-ABPP with clickable hydrazine probes can identify functional electrophilic PTMs on proteins in living cells (*in situ*) and reveal dynamic changes in protein-bound electrophile status in response to metabolic perturbations. We also show that RP-ABPP uncovers structurally novel electrophilic PTMs that occur on conserved residues in proteins of uncharacterized function.

## Results

***In situ* profiling with hydrazine probes in human cells.** Previous studies have used nucleophilic probes to characterize PTMs such as N-linked glycosylation<sup>13</sup> and ADP-ribosylation<sup>14</sup>, but, in these cases, additional chemistry is required (oxidation and exogenous catalysts, respectively) to promote reactions. Nucleophilic probes have also been applied to characterize various protein modifications that are caused by oxidative stress and aging, including direct oxidation of amino acids (for example cysteine sulfenylation<sup>15</sup> and protein carbonylation<sup>16</sup>) and aspartyl and asparaginyl cyclized amino-succinimide modifications<sup>17,18</sup>, respectively. Here, we aimed to complement and advance this past work by developing a chemical proteomic method to discover electrophilic PTMs that are installed into proteins for primary functional purposes. To bias our profiles toward functional electrophilic sites, we elected to capture these sites in living cells (*in situ*) without exposure to exogenous oxidative stressors and to focus on proteins that showed strong (near-complete) reactivity with nucleophile probes in this endogenous setting. For our initial RP-ABPP studies, we selected propargyl hydrazine (probe 1; Fig. 1a) as a prototype nucleophilic probe to capture protein-bound electrophiles in cells, where the hydrazine was intended to serve as a reactive nucleophile and the alkyne as a latent affinity handle for conjugation by Cu(I)-catalysed azide-alkyne cycloaddition (CuAAC, or “click” chemistry)<sup>19</sup> to azide reporter tags for protein detection, enrichment, and identification<sup>20</sup>. We also employed a phenyl hydrazine derivative (probe 2; Fig. 1a) as

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**Figure 1 | RP-ABPP with hydrazine probes in human cells.** **a**, Structures of hydrazine probes (alkyl probe **1** and aryl probe **2**) and corresponding non-clickable analogues or competitors (**3** and **4**); green designates hydrazine reactive groups. **b**, SDS-PAGE analysis of the soluble proteome of HEK293T cells treated with probe **1** (0.5 h), revealing the concentration-dependent labelling of proteins by **1** as measured by CuAAC to a Rh-N<sub>3</sub> tag (fluorescent gel shown in grayscale). **c**, Treatment of HEK293T cells with competitor **3** blocks in a concentration-dependent manner the labelling of proteins by **1** (3 mM, 0.5 h).

a second probe for analysis. The selection of hydrazines for RP-ABPP builds on knowledge that this class of nucleophiles can covalently inhibit enzymes that use both oxidative<sup>21</sup> and electrophilic cofactors<sup>11,22</sup> (Supplementary Fig. 1). We treated HEK293T cells with varying concentrations of probe **1** (0.5 h, 37 °C), lysed cells, and conjugated cell proteomes to a rhodamine-azide (Rh-N<sub>3</sub>) reporter tag. Clear concentration-dependent protein labelling was observed (Fig. 1b and Supplementary Fig. 2) and this labelling was suppressed (Fig. 1c and Supplementary Fig. 2) by increasing concentrations of co-administered non-clickable propyl hydrazine competitor (**3**; Fig. 1a). Protein staining did not reveal any obvious changes in the expression or abundance of proteins in cells treated with probe **1** (Supplementary Fig. 2), and cell viability assays confirmed that the hydrazine probes in the absence or presence of competitor were not toxic to cells under these conditions (Supplementary Methods).

**High-reactivity targets of hydrazine probes in human cells.** Next, we set out to identify proteins modified by probe **1** using multidimensional liquid chromatography-tandem mass spectrometry (LC-MS/MS)<sup>23</sup> combined with SILAC (stable isotope labelling by amino acids in cell culture) methodology<sup>24</sup> (Fig. 2a), where cells were grown in medium containing either natural abundance lysine and arginine ('light') or <sup>13</sup>C- and <sup>15</sup>N-enriched amino acid

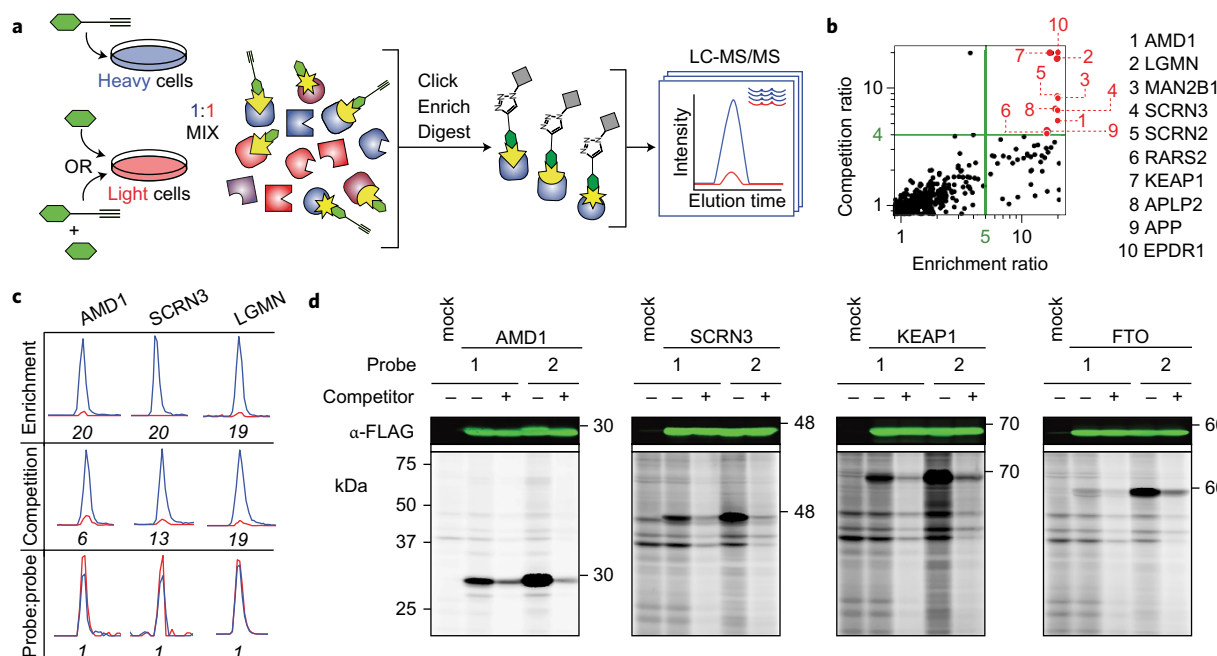
isotopologues ('heavy') and subject to different probe treatment conditions followed by lysis, combination, conjugation to a biotin-azide reporter tag, enrichment by streptavidin chromatography, and quantitative proteomic analysis. MS1 and MS2 data provided information on the relative quantity and identity of enriched proteins, respectively<sup>25</sup>.

Two types of experiments were performed in two different human cell lines (HEK293T cells and the human breast cancer cell line MDA-MB-231): (1) direct enrichment of probe **1**-labelled proteins from heavy cells treated with probe **1** (3 mM for 0.5 h) in comparison with light control cells treated with the non-clickable hydrazine (**3**) at the same concentration; and (2) competition experiments where both heavy and light cells were treated with probe **1**, but light cells were also treated with 10× competitor **3** (Fig. 2a). Proteins both substantially enriched (MS1<sub>heavy:light</sub> ratios > 5) and in competition with by **3** (MS1<sub>heavy:light</sub> ratios > 4) were considered candidate targets of probe **1**. We reasoned that, by requiring both enrichment and competition, we would focus our interpretation on proteins that possess electrophilic modifications capable of near-complete reaction with probe **1** and thereby avoid following up on lower stoichiometry adducts that may originate from minor side reactions with weaker electrophilic groups (for example, sulfenylated cysteines<sup>26,27</sup> or esterified carboxylate side-chains or C-termini<sup>17</sup>). The target list was further refined by requiring that each protein was detected with a minimum of three unique quantified peptides per experiment and by averaging protein ratios across three or more biological replicates per cell line (Supplementary Table 1).

We plotted our competition versus enrichment data (Fig. 2b and Supplementary Table 2), which illustrated four categories of proteins: (1) a lower left quadrant of unenriched, background proteins; (2) a nearly unoccupied (as expected) upper left quadrant that would designate proteins competed by **3**, but not enriched by **1**; (3) a lower right quadrant populated by a substantial number of proteins that were enriched by **1**, but not competed by **3**; and (4) an upper right quadrant, which housed ten proteins that showed strong enrichment and competition and were therefore designated as high-reactivity targets of hydrazine probes (Fig. 2b and Table 1). Representative peptide ratios for three of these high-reactivity proteins from enrichment, competition, and control (where heavy and light cells were treated with equal concentrations of **1**) experiments are shown in Fig. 2c. We also performed analogous experiments with the aryl hydrazine probe (**2**, 1 mM, 0.5 h), which furnished a larger list of high-reactivity proteins (33 in total; Supplementary Fig. 3 and Supplementary Table 3) that contained several of the targets of probe **1** (compared in Supplementary Table 4), as well as additional proteins that may reflect an expanded chemical reactivity for probe **2**.

Unable to predict the chemical properties of all potential probe adducts, we considered the possibility that targets may be lost due to potentially unstable hydrazone adducts that could be generated via capture of certain carbonyl electrophiles. Inspired by previous methods that use reductants to convert labile hydrazones to more stable hydrazides<sup>16</sup>, we performed an enrichment profiling experiment with probe **1** in the presence of 50 mM NaCNBH<sub>3</sub> (added during cell lysis). We did not, however, observe a change or increase in **1**-enriched proteins under these conditions (Supplementary Table 1).

Next, we attempted to validate a representative subset of high-reactivity targets by recombinant expression. Four proteins were selected, of which three (AMD1, SCRIN3 and KEAP1) reacted with both probes **1** and **2** and one (FTO) was preferentially targeted by probe **2**. We confirmed the recombinant expression of each protein in transfected HEK293T cells by western blotting (Fig. 2d) and found that, in each case, treatment of transfected cells with probe **1** or **2**, followed by conjugation to Rh-N<sub>3</sub>, furnished a strong fluorescent band at the appropriate molecular weight that



**Figure 2 | Identification of protein targets of hydrazine probes.** **a**, Schematic for MS-based quantitative (SILAC) proteomics experiments (enrichment and competition) as described in the text. Isotopically heavy and light cells, proteomes, and peptides are depicted in blue and red, respectively. **b**, Quadrant plot of average competition versus enrichment SILAC ratios from quantitative proteomics experiments (left). Probe-1 targeted proteins (upper right quadrant) are highlighted in red and listed to the right of the plot. **c**, Extracted parent ion chromatograms and corresponding heavy/light ratios for representative tryptic peptides of three protein targets of probe 1 quantified in enrichment, competition, and probe 1 vs. probe 1 control experiments. **d**, Western blots (upper) and RP-ABPP data (lower) for hydrazine probe-treated cells expressing the indicated protein targets. The first lane in each panel corresponds to a control transfection ('mock') with the appropriate empty expression vector as described in Supplementary Methods. Molecular weights are indicated.

was absent in mock-transfected control cells (Fig. 2d). Probe labelling of each protein was blocked by treatment with excess non-clickable competitor agents 3 or 4 (Fig. 2d). For FTO, we observed selective reactivity with the aryl probe 2 over the alkyl probe 1 (Fig. 2d), matching the proteomic data obtained for endogenously expressed FTO in cells (Supplementary Fig. 3). These results indicate that hydrazine reactivity is an intrinsic property of the protein targets of probes 1 and 2 that is shared by both the endogenous and recombinant forms of these proteins.

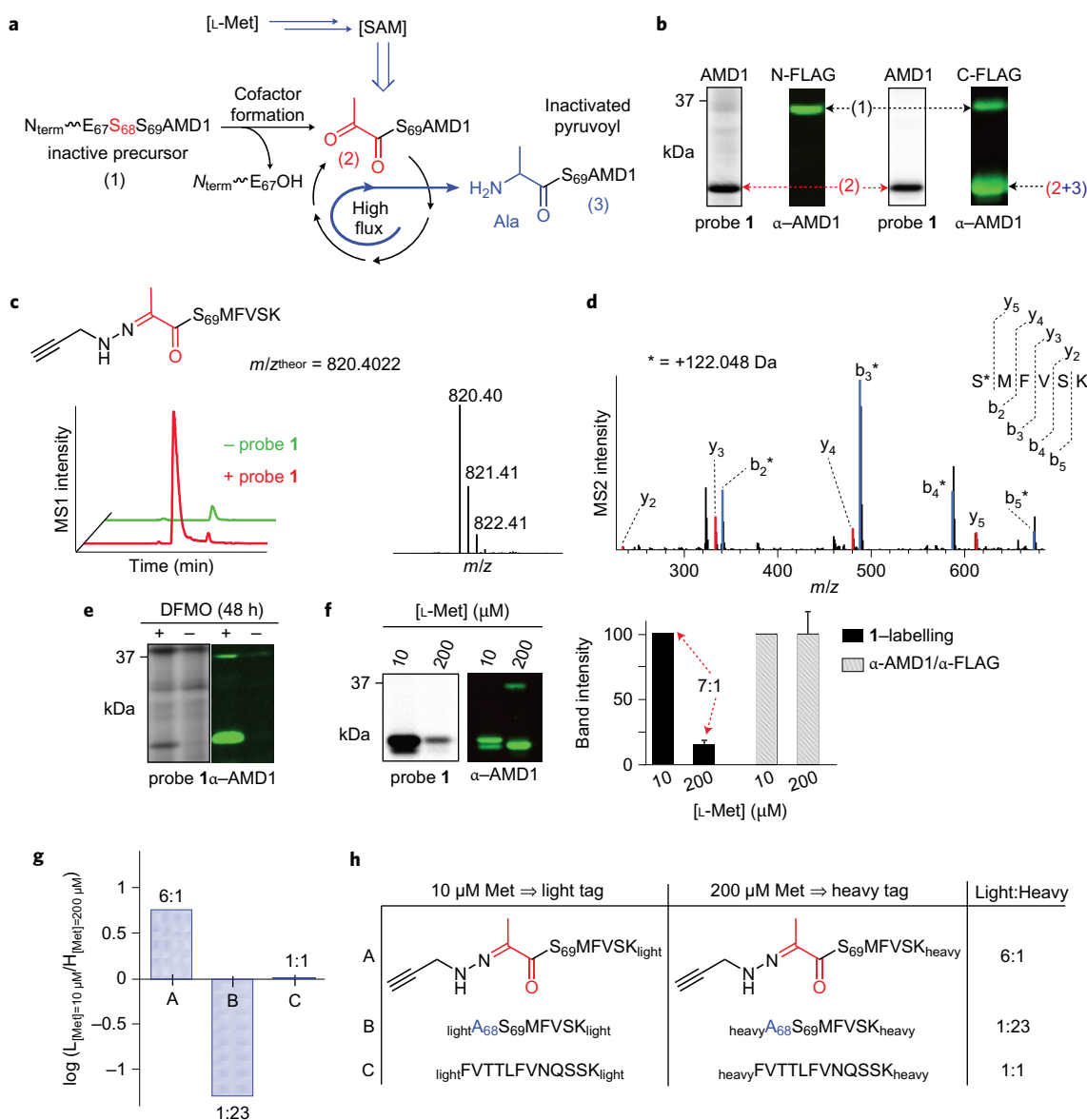
**Monitoring the electrophilic cofactor in AMD1 in cells.** Literature searches revealed that some of the probe targets are known to possess electrophilic PTMs (Table 1). Prominent among these was S-adenosyl-L-methionine (SAM) decarboxylase (AMD1 or AdoMetDC), which employs an N-terminally bound pyruvoyl

group generated from serine to catalyse the rate-limiting step in polyamine biosynthesis<sup>28</sup>. AMD1 can be inhibited by hydrazines and related nucleophiles that target the enzyme's pyruvoyl cofactor<sup>22,29</sup>, which is installed by a putrescine-induced autocleavage of the inactive proenzyme (38 kDa) to generate a processed, catalytically competent enzyme (30 kDa) (Fig. 3a). Blotting with an anti-FLAG antibody detected both the full-length pro-form and processed active form of AMD1 when expressed as a C-terminal FLAG-tagged protein in transfected HEK293T cells, whereas only the larger pro-form was detected for an AMD1 protein bearing the FLAG tag on its N-terminus (Fig. 3b), consistent with the expected processing event that cleaves an N-terminal portion of AMD1. Importantly, for either N- or C-terminally tagged AMD1, strong labelling with probe 1 was observed exclusively for the processed form of these proteins

**Table 1 | High-reactivity protein targets of probe 1.**

Probe targets	Probe reactivity	Function (disease relevance)	Electrophile
1. S-adenosylmethionine (AdoMet) decarboxylase (AMD1/AdoMetDC)	1, 2	Polyamine biosynthesis (cancer)	Pyruvamide*
2. Legumain (LGMN)	1, 2	Cysteine protease (cancer)	Aspartimide†
3. $\alpha$ -Mannosidase (MAN2B1)	1, 2	Glycohydrolase (Mannosidosis)	Unknown
4. Secernin-3 (SCRIN3)	1, 2	N-terminal nucleophile (Ntn) hydrolase‡	Unknown
5. Secernin-2 (SCRIN2)	1, 2		
6. Probable arginine tRNA ligase (RARS2)	1	Protein synthesis (Pontocerebellar hypoplasia 6)‡	Unknown
7. Kelch-like ECH-associated protein-1 (KEAP1)	1, 2	Antioxidant response regulator (cancer)	Unknown
8. $\beta$ -Amyloid-like precursor protein-2 (APLP2)	1	A $\beta$ precursor: amyloid plaques (Alzheimer's disease)	Unknown
9. $\beta$ -Amyloid precursor protein (APP)	1		
10. Ependymin-related protein-1 (EPDR1)	1, 2	Neuron regeneration and memory‡	Unknown
11. Fat mass and obesity-associated Fe(II)- and 2-oxoglutarate (2OG)-dependent dioxygenase (FTO)	2	RNA demethylation (Obesity, growth retardation)	Unknown

\*Enzyme cofactor formed in cells and targeted by nucleophiles<sup>22</sup>; †Required for ligase activity *in vitro*<sup>41</sup>; ‡Predicted function.



**Figure 3 | Functional profiling of the pyruvoyl cofactor of AMD1 by hydrazine probes.** **a**, Shown are three forms of AMD1: inactive proenzyme (38 kDa) (1), catalytically competent N-terminal pyruvoyl-containing enzyme (30 kDa) expected to be probe-reactive (2), and inactivated enzyme bearing an alanine form of the cofactor (3). **b**, Expression and probe 1-labelling profiles of N- (left) versus C-terminal (right) FLAG-tagged AMD1 in transfected HEK293T cells. Forms (1–3) are indicated. **c**, Extracted parent ion chromatograms (left) and corresponding isotopic envelope (right) for the N-terminal pyruvoyl peptide of AMD1 labelled by probe 1. **d**, MS2 spectra of the N-terminal pyruvoyl peptide of AMD1 labelled by probe 1. The b- and y-ions assign the labelled site (\*) to the N-terminal Ser69 residue. **e**, Expression and probe 1-labelling profiles of endogenous AMD1 in HEK293T cells following treatment with DFMO. **f**, Expression and probe 1-labelling profiles of AMD1-transfected cells cultured in media containing 10 versus 200  $\mu\text{M}$  L-methionine (left) and corresponding band intensities for each (right). Results represent average values from three biological replicates, and standard deviations are represented by error bars for the high methionine condition, where band intensities were normalized to the low methionine condition. **g**, From cells generated as in **f**, ratios for AMD1 peptides —probe 1-labelled pyruvoyl (A) and alanine (B) forms of N-terminal peptide, as well as an internal peptide (C)—in experiments comparing cells grown in low (10  $\mu\text{M}$ ) versus high (200  $\mu\text{M}$ ) methionine and compared by modifying corresponding peptides with light and heavy formaldehyde, respectively. Results are from a single experiment representative of two independent biological replicates. **h**, Summary of data in **g**. Results shown are from a single experiment representative of two independently performed experiments.

(Fig. 3b). Next, we treated active human AMD1, expressed and purified from *Escherichia coli*, with probe 1, followed by tryptic digestion of the protein and LC-MS/MS analysis, which identified the N-terminal pyruvoyl modification as the site of 1 reactivity (Fig. 3c,d and Supplementary Fig. 4a). We also observed loss of the unmodified N-terminal pyruvoyl tryptic peptide in 1-treated AMD1 samples (Supplementary Fig. 4b), indicating that the reaction between 1 and the pyruvoyl group of AMD1 proceeded to near-completion. We did not observe evidence of modification of

any other tryptic peptides from AMD1 (Supplementary Fig. 4c,d), supporting that probe 1 reacted specifically with the N-terminal pyruvoyl group.

Difluoromethylornithine (DFMO), an inhibitor of putrescine biosynthesis, has been shown to increase the expression of AMD1<sup>30</sup>, and we confirmed that probe 1 could detect DFMO-induced changes in the endogenous concentration of active AMD1 in cells (Fig. 3e). We also found that lowering the concentration of L-methionine, a biosynthetic precursor for SAM, from that found in standard



culture media (200  $\mu$ M) to physiological serum (10  $\mu$ M)<sup>31</sup>, increased probe 1 labelling of recombinant AMD1 in cells by ~seven-fold with negligible changes in AMD1 expression (Fig. 3f).

A mechanism to explain the regulation of AMD1 activity by SAM concentration has been described for the purified enzyme *in vitro*<sup>32</sup>, where each catalytic event is partitioned such that a small fraction of the pyruvoyl cofactor is inactivated irreversibly to alanine (Ala) instead of being regenerated (Fig. 3a and Supplementary Fig. 5). Consistent with this model being operational in cells, the differences observed in probe 1-labelling of AMD1 in low versus high methionine could reflect changes in the N-terminal structure of AMD1. We tested this premise by culturing AMD1-transfected HEK293T cells in high versus low methionine, treating cells with probe 1, and then enriching AMD1 protein from each cell preparation with anti-FLAG antibodies. The AMD1 protein samples were further purified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), digested in gel with trypsin, and the resulting peptides modified with isotopically heavy or light formaldehyde. Combining heavy and light samples (corresponding to cells grown in high and low methionine), followed by LC-MS/MS analysis, revealed much greater signals for the probe 1-labelled pyruvoyl N-terminal tryptic peptide of AMD1 in low-methionine-treated cells (A in Fig. 3g,h and Supplementary Table 5). Conversely, the alanine form of the N-terminal tryptic peptide of AMD1 was dramatically increased in the high methionine-exposed cells (B in Fig. 3g,h and Supplementary Table 5). We also measured an internal tryptic peptide from AMD1, which revealed no changes in protein expression caused by low versus high methionine exposure (C in Fig. 3g,h and Supplementary Table 5). These data, taken together, indicate that changes in methionine content dramatically alter the fraction of active, N-terminal pyruvoyl-modified AMD1 in cells, such that persistent metabolic flux under high methionine conditions leads to progressive loss of the cofactor and catalytic activity. That probe 1 monitors the post-translational regulation of AMD1 in cells indicates hydrazines can serve not only as inhibitors<sup>22</sup>, but also activity-based probes for this enzyme.

### Discovery of an N-terminal glyoxylyl modification in SCR3.

Our studies with AMD1 confirmed that the hydrazine probes react with electrophilic cofactors of established functionality in enzymes. We therefore extrapolated that additional targets of probes 1 and 2 might possess as-of-yet structurally uncharacterized electrophilic modifications. To address this question, we adapted our chemical proteomic approach to identify the peptide, rather than the whole protein, harbouring the probe-reactive electrophile. This site-specific profiling method, termed isoTOP-ABPP<sup>33</sup>, leverages isotopically differentiated, protease-cleavable biotin-azide tags to enrich and release probe-labelled peptides as mass-differentiated pairs (Fig. 4a). While this approach has been used successfully to identify the sites of reactivity for several probes<sup>34</sup>, in these past instances, the sites all represented natural amino acids. Here, we faced the additional challenge that the sites of probe 1/2 reactivity were expected to be non-natural (for example, post-translationally modified amino acids) and, in most cases, not predictable from the sequences of protein targets.

First, we confirmed that isoTOP-ABPP correctly assigned the site of probe 1 (and 2) reactivity in AMD1 as the N-terminal pyruvoyl modification using both recombinant and endogenous sources of the enzyme (Supplementary Figs 6 and 7). We next selected for analysis Secernin-3 (SCR3), a poorly characterized protein from the target list that reacted with both probes 1 and 2 (Table 1) and is predicted on the basis of sequence to be an N-terminal cysteine nucleophile (Ntn) hydrolase (Fig. 4b)<sup>35</sup>. Based on these predictions, Cys6 of SCR3 would serve as the N-terminal nucleophile responsible for autoproteolytic processing via a mechanism that is shared by other Ntn hydrolases<sup>36,37</sup> (Supplementary Fig. 8); however, to our

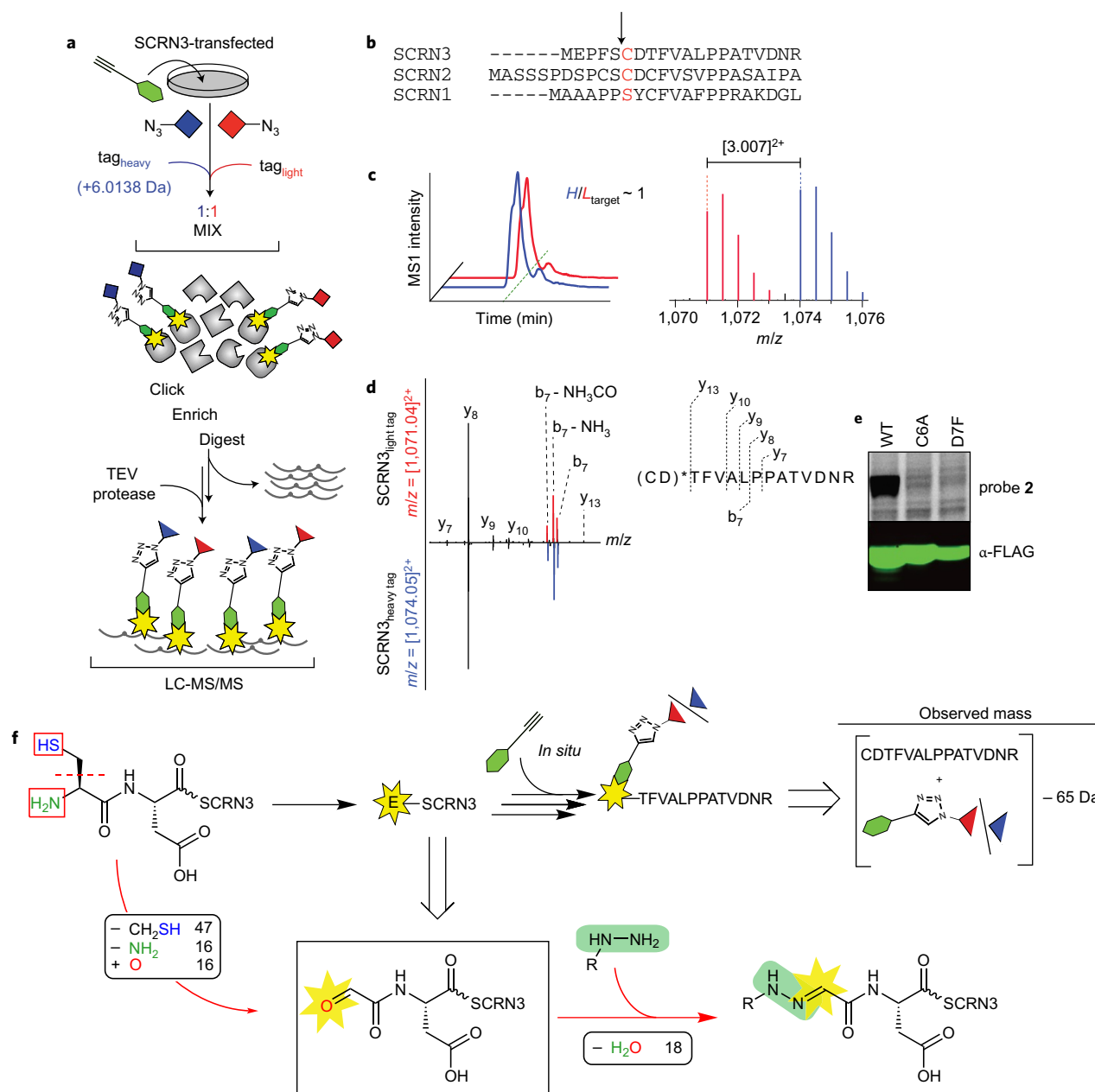
knowledge, SCR3 has not been experimentally verified to undergo this type of N-terminal processing. SCR3-transfected HEK293T cells were treated with probe 2 (1 mM, 0.5 h), lysed, conjugated to a 1:1 ratio of isotopically heavy and light biotin-azide tags, and enriched on streptavidin beads. Enriched proteins were then digested on-bead by sequential proteolysis, first with trypsin to remove unlabelled peptides, followed by TEV protease to release probe-labelled peptides, which were analysed by LC-MS/MS, where authentic probe-labelled peptides were expected to migrate as isotopically differentiated mass pairs with a heavy:light ratio of 1:1. The most abundant probe-labelled peptide pair that conformed to these specifications (Fig. 4c and Supplementary Table 6) was both manually and computationally identified as a half-tryptic peptide spanning from Cys6 to Arg20 of SCR3 based on the pattern of y-ions in the high-resolution tandem mass spectra (Fig. 4d). Importantly, the  $y_{13}$ -ion assigned the probe modification site to one of the first two residues (Fig. 4d), which was supported by mutation of either residue — Cys6 or Asp7 — which abolished probe 2 labelling (Fig. 3e). Similar data were obtained for SCR3-transfected cells treated with probe 1 (Supplementary Fig. 9).

The data accumulated thus far indicated that (i) SCR3 contains a hydrazine-reactive group at or near Cys6, which represented the presumed N-terminus of the protein, as predicted by the activation mechanism for Ntn proteins (Supplementary Fig. 8); and (ii) electrophile formation and subsequent reaction with probe 1 or 2 yielded a net loss of 65 Da from the summated mass of an unmodified N-terminal Cys6–Arg20 peptide combined with the masses of the hydrazine probes (Fig. 4f). The most logical structure conceived to fit these criteria was the hydrazine product of a reaction between hydrazine probes and an N-terminal glyoxylyl group originating from Cys6 (Fig. 4f and Supplementary Fig. 10a,b).

To test our structural predictions, we synthesized a peptide standard for the hydrazone product of a probe-2-glyoxylyl6–Arg20 reaction and then combined the standard with proteomic lysates prepared from probe 2-treated SCR3-transfected HEK293T cells that had been grown in heavy arginine/lysine, such that the peptide standard and probe 2-labelled endogenous SCR3 peptide would be isotopically distinguishable (Fig. 5a). Note that these experiments were performed with probe 2 rather than probe 1 because probe 2 appeared to produce higher product yields with SCR3 (Fig. 2d). The peptide standard and the 2-labelled endogenous SCR3 peptide co-eluted by LC (Fig. 5b) and displayed tandem mass spectra that differed by the predicted 10 Da in the  $y_8$ -ion containing the heavy C-terminal Arg residue, but were identical across the  $b_7$ -ion series lacking this residue (Fig. 5c). These data supported the structural assignment that SCR3 possesses an N-terminal glyoxylyl group originating from the conserved Cys6 residue.

We obtained additional confirmation for the N-terminal glyoxylyl modification of SCR3 by subjecting SCR3-transfected cell lysates to reductive amination with  $(\text{NH}_4)_2\text{SO}_4$ , which furnished the predicted N-terminal glycine peptide (Gly6–Arg20) for the trypsin-digested, affinity-purified SCR3 protein (Supplementary Fig. 11). The parent mass for this peptide and its corresponding b-, but not y-ions also shifted in mass by 1 Da when  $(\text{NH}_4)_2\text{SO}_4$  was substituted with  $^{15}\text{N}$ -enriched  $(\text{NH}_4)_2\text{SO}_4$  (Supplementary Fig. 11).

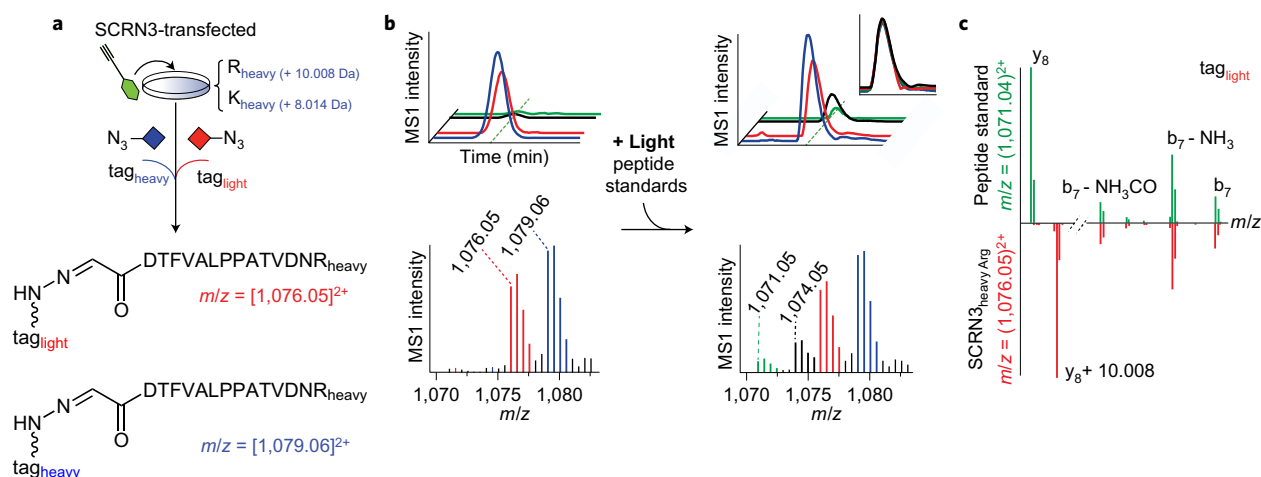
Further analysis of the MS data from SCR3-transfected cells (Supplementary Table 6) identified another prominent probe 2-enriched product that matched the predicted mass for an N-terminal pyruvoyl modification of SCR3 (Supplementary Table 7). This product was detected with ~50% of the ion intensity of the N-terminal glyoxylyl modified SCR3 (Supplementary Fig. 12a). Both the N-terminal glyoxylyl and pyruvoyl forms of recombinant SCR3 were also observed with alkyl probe 1 (Supplementary Table 7), and their relative intensities preserved under different sample preparation conditions (Supplementary Fig. 12b), suggesting that both electrophilic modification states occur for SCR3 *in situ*.



**Figure 4 | Identification of a hydrazine-reactive site in SCRIN3.** **a**, Characterization of probe-labelled peptides using the isoTOP-ABPP method as described in the text. **b**, N-terminal sequence alignment of human secernin proteins. The predicted catalytic residues for the putative Ntn hydrolase activities of secernins are highlighted (red) and the associated cleavage sites of predicted proforms are indicated by the arrow. **c**, Extracted doubly-charged MS1 ion chromatograms (left) and corresponding isotopic envelopes (right) for co-eluting heavy- and light-tagged peptides labelled by probe 2 (blue and red, respectively). **d**, Comparison of high-resolution MS2 spectra generated from light- versus heavy-tagged parent ions (left). The y-ions resolve the modified site (\*) to the N-terminal cysteine and/or adjacent aspartate (right). **e**, Probe 2-labelling and expression profiles of Cys6-to-Ala6 (C6A) and Asp7-to-Phe7 (D7F) mutant SCRIN3 proteins compared with wild-type (WT) SCRIN3. **f**, Reaction scheme (upper schematic) and MS results-based prediction of the structure of the probe 2-SCRIN3 adduct (lower schematic).

In support of the pyruvoyl structure assignment and a model for glyoxyl formation that involves an apparent  $2e^-$  oxidation of the N-terminal Cys residue of SCRIN3 via  $\alpha$ -C $\beta$  bond cleavage and oxidative deamination, we found that SCRIN3-transfected cells grown in media supplemented with L-[ $^{15}N$  $^{13}C$ ]<sub>3</sub>cysteine shifted the parent mass and  $b_7$ -ion series, but not  $y_8$ -ion for the probe 2-labelled pyruvoyl peptide by the expected 3 Da (Supplementary Fig. 12c) and the corresponding glyoxyl peptide by the expected 2 Da (Supplementary Fig. 12d). In contrast, internal peptides that contained a Cys residue shifted the expected 4 Da in parent mass, accounting for all four heavy atoms of the Cys residue (Supplementary Table 8).

As an initial attempt to estimate the fraction of SCRIN3 protein bearing the N-terminal glyoxyl modification, we reacted lysates from probe 2-treated SCRIN3-transfected (and heavy amino acid-labelled) HEK293T cells with aniline to generate an iminium adduct that could be reduced with NaCNBH<sub>3</sub> to a more stable amine product (Supplementary Fig. 13a), following the reactivity trends that have been exploited in bioconjugation approaches<sup>38,39</sup>. We also synthesized an aniline-modified and reduced glyoxyl N-terminal SCRIN3 peptide standard, along with a control standard for an internal SCRIN3 tryptic peptide, and compared the ratios of heavy parent ion peak intensities for the aniline-modified



**Figure 5 | Evidence supporting the structural assignment of an N-terminal glyoxylyl group in SCRIN3.** **a–c**, Heavy Arg/Lys-labelled SCRIN3-transfected cells treated with probe **2**, followed by processing by isoTOP-ABPP, furnishes an isotopically differentiated probe **2**-labelled SCRIN3 peptide pair (red and blue) (**a**) that co-elutes with a light amino-acid-labelled probe **2**-glyoxylyl6-Arg20 standard (also an isotopically differentiated peptide pair; black and green). Inset chromatogram in **b** shows all four traces scaled to the same intensity to show co-elution of endogenous and standard **2**-glyoxylyl6-Arg20 SCRIN3 peptides. Corresponding isotopic envelopes are shown below the chromatograms. **c**, Comparison of high-resolution MS2 spectra for light-tagged standard versus endogenous **2**-glyoxylyl6-Arg20 SCRIN3 peptides (inverted y-axis) distinguished by the expected 10 Da mass shift for  $y_8$ -ions containing the C-terminal Arg residue.

N-terminal and internal peptides with near-equivalent amounts of the corresponding peptide standards (Supplementary Fig. 13b–d). The ratios of the aniline-appended N-terminal and internal peptides compared to their respective standards provided an estimate of  $10 \pm 3\%$  for the N-terminal glyoxylyl-modified SCRIN3. We view this estimate as a lower limit for the fraction of glyoxylyl-modified SCRIN3 in cells, since the probe **2** and aniline reactions with this form of SCRIN3 may proceed with less than 100% efficiency in cell lysates, and it is also possible that, by overexpressing SCRIN3 in transfected cells, we may have saturated the putative endogenous processing system that installs the glyoxylyl modification. At least partly supporting these hypotheses, we estimated that only  $15 \pm 3\%$  ( $n = 4$ ) of the recombinant SCRIN3 possessed an intact Cys6 side chain in transfected cell preparations (Supplementary Fig. 14).

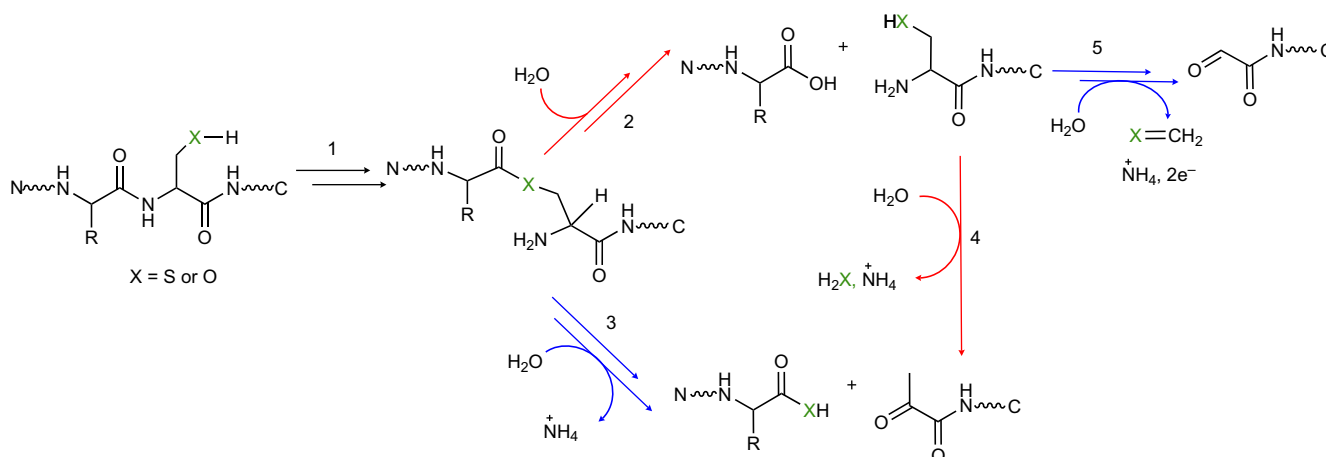
Finally, in initial attempts to assess features required for N-terminal glyoxylyl/pyruvoyl modifications of SCRIN3, we evaluated a variant of SCRIN3 where amino acids 2–5 were deleted and found that this mutant protein was still converted to the glyoxylyl/pyruvoyl form, albeit with an apparently lower efficiency than the wild-type SCRIN3 protein (Supplementary Fig. 15 and Supplementary Table 7). This result indicates that an N-terminal leader sequence is not required for SCRIN3 processing or installation of its N-terminal electrophilic modifications. We also noted that SCRIN3 reacts with hydrazine probes when expressed in mammalian cells, but not *Escherichia coli* (Supplementary Fig. 16). This contrasts with the profile of AMD1, which reacts well with probe **1** or **2** when expressed in *Escherichia coli* (Supplementary Fig. 16). These data are consistent with the established autonomous mechanism of installation for pyruvoyl modification of AMD1 and suggest further that the N-terminal glyoxylyl/pyruvoyl groups in SCRIN3 may require enzymatic machinery of the mammalian host cell for generation. In support of this hypothesis, LC-MS/MS characterization of SCRIN3 expressed and purified from bacteria failed to detect the N-terminal glyoxylyl/pyruvoyl states of the protein, instead revealing only the unprocessed N-terminus containing the initiator methionine residue and the Cys6 N-terminus presumably generated by autocleavage (Supplementary Table 9).

## Discussion

Our results indicate that both known and novel electrophilic modifications on proteins can be profiled in cells using simple hydrazine

probes. That at least some of the sites of hydrazine reactivity represent electrophilic cofactors important for enzyme catalysis indicates that **1** and **2** can be considered authentic ABPP probes. The profiling of dynamic changes in the pyruvoyl modification state of AMD1 by **1** provides a compelling example of how RP-ABPP can be used to evaluate protein function in cellular systems. In this regard, previous studies have shown that heightened methionine concentrations in cells leads to proteasomal degradation of AMD1 by a mechanism postulated to involve conversion of the N-terminal pyruvoyl cofactor to alanine<sup>40</sup>. We did not observe alterations in AMD1 levels in our studies of high versus low methionine, despite substantial changes in the fraction of N-terminal pyruvoyl versus alanine forms of the enzyme, suggesting that catalytic flux-mediated decreases in AMD1 activity can precede or even occur without effects on AMD1 degradation in cells.

Our further discovery of an N-terminal glyoxylyl modification on the poorly characterized protein SCRIN3 underscores the potential of RP-ABPP to illuminate novel structural features of potential functionality in the human proteome, of which we speculate there are others still awaiting excavation. For nucleophilic probes, like the simple hydrazines used herein, however, which have the potential to react with a diversity of electrophilic modifications, structural characterization of these modifications remains a major challenge. In some cases, the electrophilic modification could be inferred from the literature. The lysosomal protease legumain (LGMN), for instance, harbours an active site aspartimide (succinimide) that catalyses peptide ligation reactions *in vitro*<sup>41</sup>. Considering that legumain was a high-reactivity target of probes **1** and **2** and further that succinimide electrophiles are known to react with hydrazines<sup>18</sup> (see Supplementary Fig. 10c for structure and reaction), our findings suggest that the aspartimide cofactor observed previously on purified legumain *in vitro*<sup>41</sup> also exists endogenously, raising the possibility that legumain could function as both a protease and ligase in cells. For proteins like SCRIN3 that possess structurally novel electrophilic modifications, we acknowledge that elucidating their source of reactivity with hydrazine probes may require considerable effort and further methodological advances. We believe that such endeavours are worthwhile, however, especially given that some of the other proteins displaying high reactivity with hydrazine probes have compelling disease relationships (for example,



**Figure 6 | Possible routes for N-terminal processing of SCRIN3 in comparison to the established mechanisms of N-terminal maturation for other protein classes.** The mechanisms for Ntn hydrolases and pyruvoyl-dependent decarboxylases diverge at the transient ester intermediate formed during autoproteolysis (step 1). Hydrolysis yields a catalytic nucleophile for Ntn hydrolases (step 2), whereas active site-directed  $\beta$ -elimination generates an electrophilic pyruvoyl cofactor for decarboxylases (step 3). Detection of a new N-terminus containing an intact side chain, shown as a product of step 2, suggests that formation of pyruvoyl and glyoxylyl groups may occur via steps 4 and 5, respectively, in the N-terminal processing of SCRIN3.

AMD1 (ref. 42) and KEAP1 (ref. 43) with cancer; APPs with Alzheimer's disease<sup>44</sup>; and FTO with obesity<sup>45</sup>).

We are unsure why the aryl hydrazine **2** showed broader proteomic reactivity compared with alkyl hydrazine **1**, but we note that, from a chemical perspective, the aryl hydrazine derivative is likely to form hydrazones of greater stability with respect to hydrolysis<sup>46</sup>, enabling the capture and identification of proteins that possess electrophilic modifications that form otherwise unstable adducts with alkyl hydrazines<sup>11,47</sup>. Probe **2** might also have expanded reactivity towards proteins that deviate from electrophilic chemistry and instead involve, for instance, radical-based mechanisms<sup>21</sup>.

The SCRIN proteins, conserved from bacteria to higher mammals, have three members in humans — SCRIN1–3 — however, only SCRIN2 and SCRIN3 were identified as targets of the hydrazine probes herein. Notably, both of these proteins, as well as their orthologues in other organisms, but not SCRIN1, share an 'SCD' motif that includes the glyoxylyl/pyruvoyl modification site discovered for SCRIN3, suggesting that the N-terminal processing and modification of SCRIN3 may be conserved across SCRIN2/3 proteins, but differ for SCRIN1 (Supplementary Fig. 17). Despite being superficially classified as Ntn hydrolases, SCRIN2/3, based on our data, appear more likely to operate by a distinct catalytic mechanism that involves electrophilic modification of their N-termini. In considering potential functions for SCRIN2/3, we note that formylglycine (fGly)-dependent enzymes, which possess a structurally related aldehyde modification, exploit the hydrated form of this group for nucleophilic attack on substrates to catalyse reactions such as sulfate ester hydrolysis<sup>48</sup>. fGly cofactors, however, are not generated at autoproteolysed N-termini of proteins, but rather installed by  $2e^-$  oxidation of internal cysteine (or serine) residues catalysed by the enzyme sulfatase-modifying factor 1 (SUMF1)<sup>48</sup>. A similar installation mechanism seems incompatible with generating the N-terminal glyoxylyl and pyruvoyl modifications for SCRIN3.

Some common mechanistic features can be found in the activation chemistries of Ntn hydrolases and pyruvoyl-dependent decarboxylases<sup>1,49</sup>. Autoproteolysis occurs via an  $N \rightarrow O$  or  $N \rightarrow S$  acyl shift where nucleophilic side chains attack upstream peptide bonds to form cyclic intermediates that reopen as oxo/thioesters via C–N bond cleavage (step 1 in Fig. 6). Next, their respective active sites direct divergent outcomes (hydrolysis versus  $\beta$ -elimination) to yield catalytic machinery of opposite polarity (steps 2 versus 3, respectively in Fig. 6). N-terminal cysteine-derived

pyruvoyl groups have been proposed to act as the likely cofactor for bacterial reductases<sup>50,51</sup> and can form chemically at engineered N-termini with pyridoxal-5'-phosphate (PLP)<sup>52,53</sup>. However, detection of a cysteine N-terminus, if autoactivated as predicted for Ntn hydrolases, might favour pyruvoyl formation via  $\beta$ -elimination uncoupled from proteolysis (step 4). Self-processing of this type, however, is unlikely to produce the glyoxylyl form of SCRIN3, and, there is no report, to our knowledge, of a cysteine-to-glyoxylyl transformation. This modification can be described as an aldol cleavage coupled to oxidative deamination, perhaps occurring in single or sequential steps (step 5). Both products could emerge, in principle, from transformations catalysed by PLP-dependent enzymes<sup>12</sup>.

The mechanism of formation and consequent function of the N-terminal glyoxylyl/pyruvoyl modifications in SCRIN3 represent important areas for future investigation. That SCRIN3, with its N-terminal modified forms, shares features in common with a subclass of bacterial reductases<sup>51</sup> points to one potential catalytic function that should be further explored. Alternatively, at this stage, we cannot exclude the possibility that the glyoxylyl group, rather than imparting a catalytic activity to SCRIN3, might instead represent a non-catalytic form of the protein that serves a different function. Regardless, the strong conservation of the N-terminal cysteine residue across the SCRIN2/3 protein family argues for a functional role for the modified electrophilic form of this residue.

In summary, our findings demonstrate that the principles of ABPP can be extended to the functional and structural characterization of electrophilic modifications on proteins. We note that proteinaceous electrophilic cofactors, such as pyruvoyl and glyoxylyl PTMs, cannot be easily predicted by analysis of protein sequences, underscoring the value of chemical proteomic methods like RP-ABPP for their *de novo* discovery in native biological systems. That our initial studies, which only profiled two human cell lines, succeeded in identifying a novel N-terminal glyoxylyl modification that appears to be a conserved feature of a poorly characterized class of proteins (the secernins) leads us to speculate that many other proteins in the human proteome may harbour electrophilic groups. We anticipate that the characterization of these groups would benefit from the use of additional types of nucleophilic probes, akin to the diverse sets of electrophilic probes employed in more classical ABPP studies of proteins that use different nucleophilic residues for function<sup>9</sup>. Of course, mapping electrophilic groups will confront



the added challenge of structural characterization of these PTMs, but our studies show how RP-ABPP probes can facilitate this effort (by both enriching these modifications and generating stable adducts with predictable shifts in mass values). Finally, we recognize that, ultimately, one of the most important questions is: what types of functions do electrophilic modifications impart on proteins? To the extent that RP-ABPP probes have the potential to not only characterize, but also inhibit the function of proteins that possess electrophilic modifications, these probes could serve as launching points for more advanced and selective small-molecule inhibitors that modulate electrophile-dependent function for basic and translational research purposes.

## Methods

See Supplementary Information for a detailed Methods section.

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### Author contributions

M.L.M. and B.F.C. conceived the project, designed experiments and composed the manuscript. M.L.M. performed all experiments. L.H. wrote software and J.R.Y. provided advice. M.L.M., L.H., B.E.C. and B.F.C. analysed data. M.L.M., E.J.O. and P.E.D. designed peptide standards. M.L.M. and E.J.O. synthesized peptides. M.L.M. and B.D.H. synthesized probes.

### Additional information

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### Competing financial interests

The authors declare no competing financial interests.