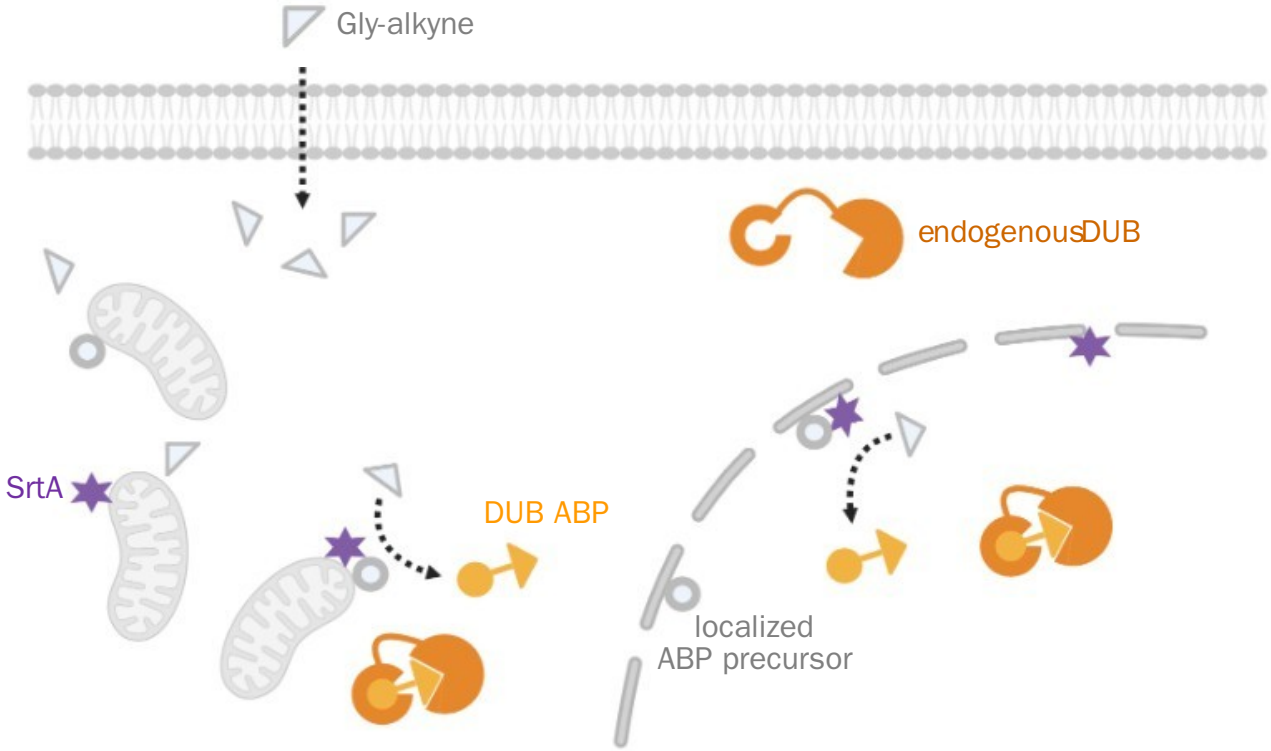
Cellular synthesis of ubiquitin-based probes for spatially resolved deubiquitinase profiling

Jinghao Chen[a], Xinyu Zhang[b], Yang Huang[c], Gang Li[b,*], Yu-Hsuan Tsai[a,*]

[a] *Institute of Molecular Physiology, Shenzhen Bay Laboratory, Shenzhen 518132, China. E-mail: tsai.y-h@outlook.com*
[b] *Institute of Chemical Biology, Shenzhen Bay Laboratory, Shenzhen 518132, China. E-mail: ligang@szbl.ac.cn*
[c] *Key Laboratory of Biocatalysis & Chiral Drug Synthesis of Guizhou Province, Generic Drug Research Center of Guizhou Province, School of Pharmacy, Zunyi Medical University, Zunyi 563000, China*



Activity-based protein profiling (ABPP) has substantially advanced our understanding of deubiquitinases (DUBs). Here, we leverage sortase A–mediated transpeptidation, demonstrate *in cellulo* probe synthesis, and profile nuclear and cytosolic DUBs in mammalian cells.

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[b]*Institute of Chemical Biology, Shenzhen Bay Laboratory, Shenzhen 518132, China. E-mail: ligang@szbl.ac.cn*
[c] *Key Laboratory of Biocatalysis & Chiral Drug Synthesis of Guizhou Province, Generic Drug Research Center of Guizhou Province, School of Pharmacy, Zunyi Medical University, Zunyi 563000, China*

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ABSTRACT

Deubiquitinases (DUBs) orchestrate ubiquitin homeostasis, yet their compartment-specific activities remain underexplored. We developed a genetically encoded strategy to assembly activity-based ubiquitin probes *in cellulo* via sortase A (SrtA)-mediated transpeptidation. Co-targeting SrtA and its ubiquitin substrate to the outer mitochondrial membrane reduced off-target transpeptidation and enabled spatially resolved profiling in live cells. Quantitative proteomics identified distinct sets of cytosolic, nuclear, and cell line specific DUBs in HEK293T and MCF7 cells. This localization-tunable, delivery-free strategy expands the chemical biology toolkit for mapping DUB activity within native cellular environments.

Ubiquitination is a dynamic protein post-translational modification in eukaryotes [1-4]. A set of E1/E2/E3 enzymes catalyze addition of ubiquitin to target proteins, while deubiquitinases (DUBs) are responsible for its removal. As a result, these enzymes maintain the homeostasis of ubiquitination, which are involved in virtually all aspects of eukaryotic biology. In fact, malfunction of DUBs has been associated with many diseases, and a detailed understanding of their cellular function could facilitate the development of new therapeutics [5-8].

Advances in proteomics and activity-based protein profiling have greatly improved our knowledge about DUBs [9]. Different DUB probes have been derived from ubiquitin by attaching a reactive warhead, which is often an electrophile that can react with the catalytic cysteine residue of DUBs [10, 11]. Recently, a new class of ubiquitin-based activity probes equipped with sulfenic acid-reactive warheads was developed to selectively capture the oxidized (sulfenylated) form of DUBs, enabling investigation of redox-dependent DUB regulation under oxidative stress conditions [12]. However, these ubiquitin-based probes are not cell permeable, limiting their applications in cell lysates [13]. To understand the function of DUBs in the relevant cellular setting, a common strategy is to append a cell-penetrating peptide sequence to the DUB probe [14], which has been demonstrated in cellular DUB profiling [15-17]. However, proteins fused with a cell-penetrating peptide are often taken up by cells through endocytosis and trapped in endosomes [18]. It is thus difficult to gauge their cytosolic concentration, which may greatly impact DUB profiling. Moreover, such probes cannot resolve DUB activities in specific subcellular compartments.

Here we attempted to utilize the cellular translation machinery to synthesize DUB probes in cells for protein profiling. Initially, we intended to trap DUBs by genetically incorporating chemical crosslinking amino acids [19] on ubiquitin-derived probes. To validate this hypothesis, three non-canonical amino acids FSY [20], PRO [21] and PEN [22] were incorporated into HA-Ub-TAG-eGFP-FLAG in HEK293T (Fig. S1a) in response to the amber codon TAG via genetic code expansion [23-25]. These amino acids can undergo chemical crosslinking with nucleophilic residues of the interacting proteins. As DUBs cleave the C-terminal amide bond of ubiquitin Gly76, we thought a chemical crosslinking amino acid directly after Gly76 would be in proximity to the catalytic center of DUBs upon substrate recognition and cleavage. Successful crosslinking would lead to higher molecular weight adducts (Fig. S1b). To our disappointment, no crosslinking bands were observed with FSY, PEN or PRO in comparison to the negative control BOC, which contains an inert tert-butyoxylcarbonyl group that cannot undergo chemical crosslinking (Fig. S1c). In all cases, complete cleavage of HA-Ub from the fusion proteins were observed. We then wondered whether slowing down the proteolytic cleavage by mutating Gly75 and Gly76 to alanine residues (Ub*) [26, 27] may enhance the possibility of chemical crosslinking. While cleavage of the double Ala

mutant was slower as indicated by the presence of HA-Ub*-eGFP-FLAG in α-FLAG immunoblotting, no discernible crosslinking bands were detected (Fig. S1d), suggesting a suboptimal spatial arrangement between the warhead and the DUB catalytic cysteine.

This outcome prompted us to adopt sortase A (SrtA)-mediated ligation, a versatile strategy that enables C-terminal protein engineering. Using SrtA-mediated ligation (Fig. 1a), we could fine-tune the C-terminal architecture to assemble a construct highly analogous to the 'gold standard' DUB probe [28, 29], except that Arg72 and Arg74 were mutated to accommodate the SrtA recognition sequence LPXTG at the ubiquitin C-terminus. Indeed, the Lang group previously demonstrated SrtA-mediated ubiquitination using a recombinant Ub variant, Ub(1-70)-LPLTGG, together with a genetically incorporated lysine derivative containing two glycine residues on its side chain [30, 31]. Therefore, if the Ub variant with Arg72Pro and Arg74Thr mutations can still be recognized by endogenous DUBs, the *in cellulo* SrtA-assembled probe should permit efficient trapping of cellular DUBs.

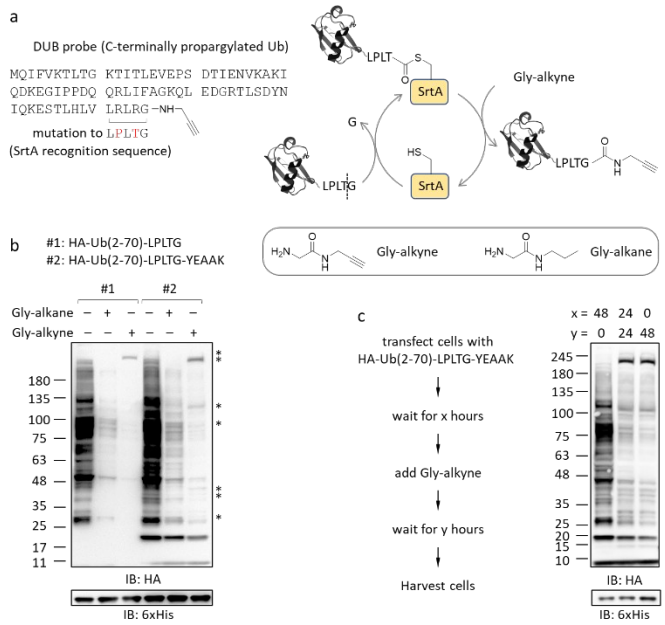

Fig. 1. Design of SrtA-mediated *in situ* synthesis of DUB probes . (a) Molecular basis of SrtA-mediated DUB probe synthesis. A double mutation of Arg72Pro and Arg74Thr was introduced to enable SrtA-catalytic transpeptidation at the C-terminal of ubiquitin. (b) Cytosolic co-expression of HA-Ub(2-70)-LPLTG or HA-Ub(2-70)-LPLTG-YEAAK together with 6xHis-mgSrtA in HEK293T cells for 48 h in the presence or absence of 10 mM Gly-alkane or Gly-alkyne. Bands corresponding to putative cross-linked DUBs are marked with asterisks. (c) Effect of adding Gly-alkyne at different time points on chemical crosslinking efficiency. HEK293T cells were transfected with the plasmids expressing HA-Ub(2-70)-LPLTG-YEAAK and 6xHis-mgSrtA. Cells were first cultured for a defined period (x) without Gly-alkyne, followed by incubation for another peroid (y) in the presence of 10 mM Gly-alkyne.

For verification, we transiently transfected HEK293T cells to express HA-Ub(2-70)-LPLTGG-eGFP-FLAG. Immunoblotting against FLAG showed high cleavage efficiency of HA-Ub(2-70)-LPLTGG from the fusion protein (Fig. S2). Similar to HA-Ub, the cleaved HA-Ub(2-70)-LPLTGG was also utilized by endogenous E1/E2/E3 enzymes for protein ubiquitination (lane 1, Fig. 1b). In contrast, removal of Gly76 prevented the variant from participating in the endogenous ubiquitination system (Fig. S3) but did not affect its recognition by SrtA. We therefore transfected HEK293T cells to co-express HA-Ub(2-70)-LPLTG and 6xHis-mgSrtA, an evolved *Staphylococcus aureus* sortase A variant capable of accepting a single N-terminal glycine as the nucleophile for transpeptidation [32]. In the presence of 10 mM 2-amino-N-(prop-2-yn-1-yl)acetamide (Gly-alkyne), several unique labeling bands were observed in α-HA immunoblotting (asterisks in Fig. 1b), including a prominent high-molecular-weight (> 180 kDa) band. In addition, the labeling intensity was further increased when a short peptide linker was introduced at the C-terminus of the reporter, consistent with the reported effect of the linker in improving transpeptidation efficiency [31]. Interestingly, both the number and intensity of labeled bands were markedly higher in the absence of Gly-alkyne, resembling the pattern observed in cells overexpressing HA-Ub(2-70)-LPLTGG. We reasoned that, in the absence of Gly-alkyne, mgSrtA may mediate transpeptidation between HA-Ub(2-70)-LPLTG and endogenous proteins bearing an N-terminal Gly-Gly motifs. The resulting adducts can then be cleaved by DUBs to afford HA-Ub(2-70)-LPLTGG, which would subsequently serve as a substrate for cellular E1/E2/E3 -mediated ubiquitination (Fig. S4). Upon addition of Gly-alkyne, mgSrtA-catalyzed transpeptidation of HA-Ub(2-70)-LPLTGG linked to proteins to generate the desired DUB probe HA-Ub(2-70)-LPLTG-alkyne, which could then react with DUBs. Indeed, transfected cells first cultured in the absence of Gly-alkyne for 24 h and then supplemented with 10 mM Gly-alkyne for an additional 24 h showed a labeling pattern nearly identical to that of cells continuously cultured with Gly–alkyne for 48 h (Fig. 1c).

To distinguish specific crosslinking signals from background ubiquitination (i.e., endogenous proteins ubiquitinated with HA-Ub(2-70)-LPLTGG), we used 2-amino-*N*-propylacetamide (Gly-alkane) as a control nucleophile in mgSrtA-mediated transpeptidation. This reaction yields a C-terminally propylated ubiquitin variant that can neither crosslink DUBs nor be utilized by endogenous E1/E2/E3 enzymes. Under this condition, multiple bands were still detected, although at lower intensity in comparison to the condition without Gly-alkane, indicating that Gly-alkane could not fully

outcompete endogenous proteins in mgSrtA-mediated transpeptidation.

To improve the signal-to-noise ratio, we next sought to spatially restrict probe assembly by targeting the expressed proteins to specific subcellular compartments. The mgSrtA-mediated DUB probe assembly requires three components (Fig. 1a): a mgSrtA variant, ubiquitin variant bearing Arg72Pro and Arg74Thr mutations, and Gly-alkyne. While Gly-alkyne is chemically synthesized and supplemented into the culture media, the first two components are produced through the cellular translational machinery. Since cytosolic expression of 6xHis-mgSrtA and HA-Ub(2-70)-LPLTG in the absence of Gly-alkyne led to multiple background bands, we hypothesized that confining their subcellular localization would suppress undesired mgSrtA-mediated transpeptidation in the absence of Gly-alkyne [33].

The construct without an additional targeting sequence is expected to reside in cytosol (#1, Fig. 2). To present the probe precursor to the cytosolic face of membranes, we appended targeting sequences at the N terminus (#2-#4) or C terminus (#5, #6). Specifically, the following were employed: N-terminal rabbit CYP2C1 (1-27, UniProt P00180; #2) for endoplasmic reticulum membrane targeting, N-terminal human TOM20 (1-70, UniProt Q15388; #3) or C-terminal human Miro1 (580-618, UniProt Q8IXI2; #6) [34] for outer mitochondrial membrane targeting, N-terminal mouse LCK (1-10, UniProt P06240; #4) for plasma membrane targeting, and C-terminal giantin (3111-3259, UniProt Q14789; #5) [35] for Golgi apparatus membrane targeting.

Co-localizing mgSrtA and the ubiquitin precursor at defined subcellular compartments markedly reduced transpeptidation of endogenous proteins (Fig. 2). Notably, co-expression of HA–Ub(2–70)–LPLTG–YEAAK–Miro1(580–618) and 6×His–mgSrtA–Miro1(580–617) produced a strong signal with minimal background. Miro1 (RHOT1, UniProt: Q8IXI2) is a mitochondrial GTPase with residues 1-592 facing the cytosol, residues 593-615 spanning the outer mitochondrial membrane, and residues 616-618 facing the mitochondrial intermembrane space. The truncated anchor Miro1(580–618) lacks GTPase activity yet provides robust outer-membrane localization. The favorable signal-to-noise ratio persisted across Gly-alkyne concentrations and varying expression ratios of the two fusion constructs (Fig. S5).

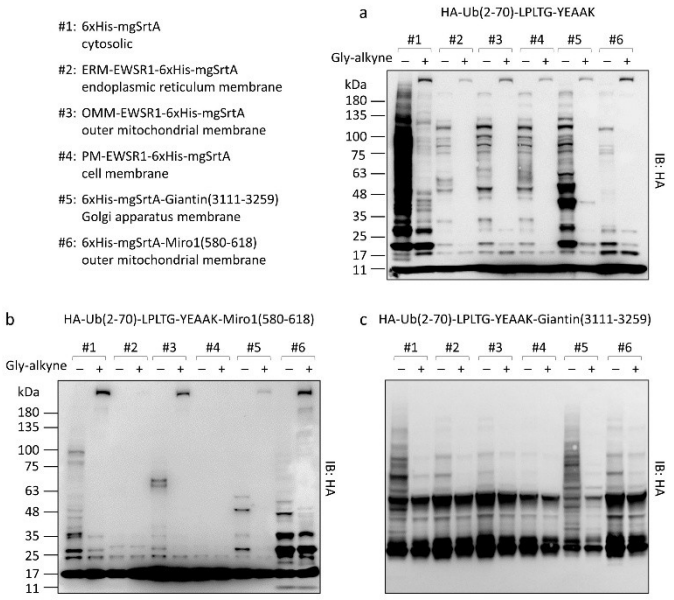

Fig. 2. Effects of protein subcellular localization on SrtA-mediated DUB probe synthesis. HA-Ub(2-70)-LPLTG-YEAAK was expressed in the cytosol (a), on the Golgi membrane (b) or on the outer mitochondrial membrane (c) of HEK293T cells, together with 6xHis-mgSrtA varient(#1-#6) localized to corresponding subcellular compartments. Cells were cultured in the absence or presence of 10 mM Gly-alkyne for 48 hours.

To identify DUBs captured by the *in-situ* synthesized probe, we performed proteome-wide DUB profiling in HEK293T cells using label-free quantitative (LFQ) mass spectrometry [36]. In this system, Gly-alkyne acts as the substrate for mgSrtA, completing transpeptidation at the C terminus of the probe precursor to generate alkyne-bearing activity-based probe inside living cells. A matched control, Gly-alkane, lacks the alkyne handle and therefore does not support covalent crosslinking with DUBs, providing a stringent measure of nonspecific background. Following cell lysis, probe-modified proteins were captured by α-HA agarose (targeting the HA tag on the Ub variant), extensively washed, eluted, digested with trypsin, and analyzed by LC–MS/MS (Fig. 3a). LFQ intensities from biological replicates were normalized and subjected to statistical testing to define significant interactors.

Volcano-plot analysis ($p < 0.05$ and >2-fold change) highlighted proteins enriched in the Gly-alkyne condition as well as singleton proteins that are exclusively present in Gly-alkyne group (Fig. 3b). Integration of significantly changing proteins with singletons yielded eight DUBs confidently assigned as probe targets. Most remaining enriched proteins were housekeeping species commonly observed in affinity-based workflows. The identified DUBs spanned two of five families of the cysteine protease DUBs, including six ubiquitin-specific proteases (USPs) and two ovarian tumor proteases (OTUs), consistent with selective capture of enzymes employing a catalytic cysteine.

A central advantage of this strategy is that the probe is genetically programmed and assembled intracellularly, enabling precise spatial control. To demonstrate this, we redirected SrtA to the inner nuclear membrane by fusing it to lamin A/C, thereby assembling the DUB probe within the nucleus. Under these conditions, proteomic profiling revealed seven DUBs annotated for nuclear localization (while some also function in other compartments) (Fig. 3c). Together, these results establish a robust, localization-tunable platform for spatially resolved DUB activity mapping across organelles, providing a generalizable route to interrogate compartment-specific deubiquitination in native cellular contexts.

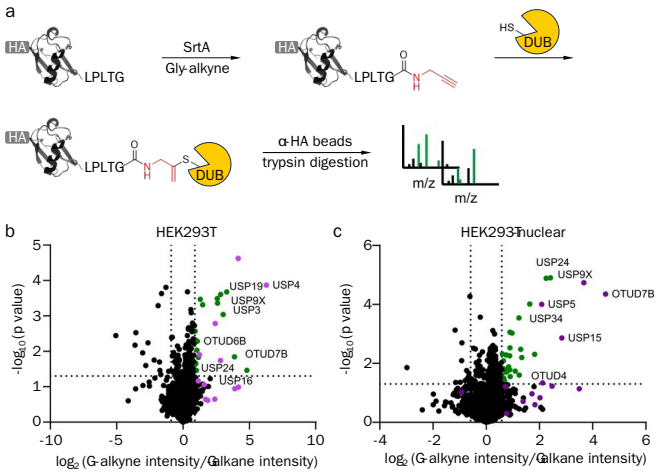

Fig. 3. Proteomic-wide characterization of DUBs in mammalian cells. (a) Schematic overview of the proteomic workflow. HEK293 cells were transfected to co-express HA-Ub(2-70)-LPLTG-YEAAK-Miro1(580-618) and 6xHis-mgSrtA-Miro1(580-618) and cultured in the presence of 10 mM Gly-alkyne or Gly-alkane. After enrichment with α-HA beads and trypsin digestion, the labeled proteome was analyzed by LC-MS/MS. (b, c) Volcano plots showing DUB profiling in HEK293T cells by *in cellulo* synthesized probe localized to the outer mitochondrial membrane (b) or inner nuclear membrane (c). Label-free quantification (LFQ) was performed across three independent biological replicates. A two-sided Student's t-test was applied for statistical analysis. Gly–alkane served as the negative control. Significantly enriched proteins are highlighted in green ($p < 0.05$ and >1.8-fold ion intensity difference in panel b; $p < 0.05$ and >1.5-fold in panel c). Proteins significantly detected in the Gly–alkyne but not in the Gly–alkane group are marked in purple.

The abundance and activity of DUBs vary across different cell types and tissues[37]. To evaluate the versatility of our method, we applied it to profile DUBs in MCF7 cells. In this experiment, both the probe precursor and mgSrtA were anchored to the outer mitochondrial membrane to confine *in-cellulo* probe assembly, resulting in the identification of seven DUBs(Fig. 4a). Notably, four of them (i.e., USP9X, USP3, USP24 and OTUD7B) were also detected in HEK293T cells, indicating a shared core of activities alongside context-specific differences. These differences likely reflect cell type dependent expression, post-translational regulation, and compartment accessibility. To further characterize the captured proteome, we performed gene ontology (GO) enrichment analysis on significantly enriched proteins as well as those identified as singletons. The top five GO terms were identified for molecular function, biological process, and cellular component categories. Enriched molecular functions included thiol-dependent ubiquitin-specific protease activity, cysteine-type endopeptidase activity, and Lys48-specific deubiquitinase activity. Correspondingly, biological processes were enriched in protein deubiquitination and ubiquitin-dependent protein catabolic processes, consistent with the design of our DUB activity profiling strategy(Fig. 4b).

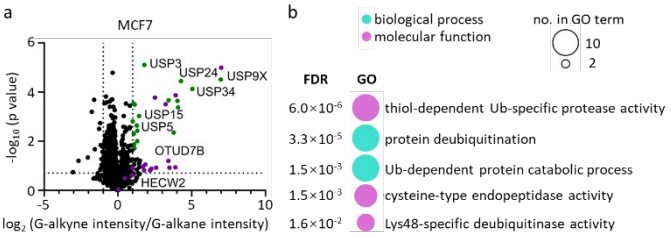

Fig. 4. Proteomic-wide characterization of DUBs in MCF7 cells. (a) Volcano plot of *in situ* DUB probe labeled proteome quantified by label-free quantification (LFQ, n=3 independent biological replicates). A two-sided Student's *t*-test was applied for statistical analysis. Gly-alkane served as the negative control. Significantly enriched proteins are highlighted in green ($p < 0.05$ and >2-fold ion intensity difference). Proteins detected exclusively in the Gly–alkyne group but not in the Gly–alkane group are marked in purple. (b) Gene ontology (GO) enrichment analysis of proteins displaying significant enrichment and those found only as singletons. significantly enriched and singleton proteins. The top five GO terms are shown. Bubbles are colored by GO category, and bubble size is proportional to the number of proteins associated with each term.

In conclusion, SrtA-mediated transpeptidation enables delivery-free, *in cellulo* synthesis of activity-based ubiquitin probes for DUB profiling in live mammalian cells. By co-localizing SrtA and the probe precursor to defined organelles, our genetically encoded system supports compartment-resolved measurements across cell types, revealing both shared and divergent DUB landscapes. The approach is modular, in which the targeting sequences and nucleophiles can be readily exchanged, facilitating extension to additional organelles and primary cells or tissues. Moreover, because cysteine reactivity is redox sensitive, this platform is poised to interrogate redox-dependent regulation of DUB catalysis by coupling organelle-specific probe assembly with controlled oxidative or reductive perturbations and time-resolved labeling. Together, these features establish a versatile, localization-tunable strategy for mapping DUB

activity in native cellular environments and for dissecting how compartmentalization and redox cues shape the ubiquitin system.

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