

Protein-labeling reagents selectively activated by copper(I)

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Abstract

Copper is an essential trace element that participates in many biological processes through its unique redox cycling between cuprous (Cu^+) and cupric (Cu^{2+}) oxidation states. To elucidate the biological functions of copper, chemical biology tools that enable selective visualization and detection of copper ions and proteins in copper-rich environments are required. Herein, we describe the design of Cu^+ -responsive reagents based on a conditional protein labeling strategy. Upon binding Cu^+ , the probes generated quinone methide via oxidative bond cleavage, which allowed covalent labeling of surrounding proteins with high Cu^+ selectivity. Using gel- and imaging-based analyses, the best-performing probe successfully detected changes in the concentration of labile Cu^+ in living cells. Moreover, conditional proteomics analysis suggested intramitochondrial Cu^+ accumulation in cells undergoing cuproptosis. Our results highlight the power of Cu^+ -responsive protein labeling in providing insights into the molecular mechanisms of Cu^+ metabolism and homeostasis.

Main text

Copper is essential for living organisms, and its redox-active property (interconversion of Cu^+ and Cu^{2+}) is exploited as a cofactor of metalloenzymes involved in a diverse biological process, such as aerobic respiration, epigenetic modification, and metabolism.^{1,2} In addition to protein-bound copper ions, recent studies have shown the existence of loosely bound, labile copper ion pools that are implicated in regulation of signal transduction by reversibly binding to protein targets and altering their activities and/or structural properties.^{3,4} Intracellular labile copper ions are normally maintained at a low concentration, but when copper homeostasis is disrupted, labile copper ions accumulate in the cell and generate reactive oxygen species (ROS) through Fenton and Haber–Weiss reactions, which cause oxidative cell damage.^{5,6} In this context, an important copper-mediated cytotoxic mechanism called cuproptosis was recently reported.⁷ Cuproptosis is characterized by intracellular copper accumulation, which induces the aggregation of mitochondrial lipoylated proteins and destabilization of iron–sulfur cluster proteins, leading to proteotoxic stress and cell death.⁷ Such cellular dysfunction caused by dysregulation of copper homeostasis has been found to be associated with various diseases, including Alzheimer's and Parkinson's diseases, cancer, and genetic disorders such as Wilson's and Menke's diseases.^{5,8–10} Therefore, it has become increasingly important to investigate intracellular copper pool dynamics.

Synthetic fluorescent probes that can detect and visualize labile copper ions in living systems provide useful research tools for elucidating the cellular regulation of labile copper ions and related biological functions.¹¹ Several copper-selective chemosensors have been developed for tracking the dynamic changes in copper ions in live cells.^{11–15} Most of these are designed to connect the fluorophore to a copper-specific chelator, which reversibly or irreversibly changes its spectral properties upon copper binding. More recently, a new probe design concept based on copper ion-responsive protein labeling has been reported.^{16–18} This approach typically relies on acyl imidazole reagents designed to have enhanced protein reactivity upon copper binding to the chelator moiety. The activated reagent reacts with surrounding proteins to transfer a fluorescent reporter. Compared with traditional copper-selective chemosensors, this covalent protein modification mechanism provides several advantages: (1) less probe diffusion and thus improved spatial resolution after copper response; (2) an immobilized signal compatible

with cell fixation; and (3) possibility of proteomic analysis of intracellular copper-rich microenvironments. So far, two types of probes based on this concept have been reported: those that are responsive to both Cu^+ and Cu^{2+} , and those selective for Cu^{2+} .^{16,17} However, probes that selectively respond to Cu^+ , the most prevalent oxidation state of intracellular copper ions, have not yet been reported.

Herein, we describe a novel design strategy for creating chemical probes that can be selectively activated in response to labile Cu^+ and thereby covalently anchored on proximal proteins. In our design of Cu^+ -responsive protein labeling reagents (CuRs), a fluorophore and a copper chelator are connected through a latent quinone methide (QM) precursor with a benzyl ether linkage. The C–O bond of the benzyl ether is oxidatively cleaved upon Cu^+ complexation of the reagent, then the highly reactive QM that labels nucleophilic amino acids (e.g., Cys, Lys, and His) of surrounding proteins is released (**Figure 1a** and **Figure S1**).^{19,20} We prepared a series of fluorescein-tethered CuRs to identify their optimal structure (**Figure 1b**). On the basis of previous studies of Cu^+ chemosensors, a tris(2-pyridylmethyl)amine (TPA) or a 2-pyridylmethylbis(2-ethylthioethyl)amine were employed as the chelating moiety ($K_d = 126$ or 1 fM for Cu^+ , respectively).^{21,22} For the QM precursor, 2-fluoromethylphenol (*ortho*-QM type: **O2**, **O3**) and 4-fluoromethylphenol (*para*-QM type: **P2**, **P3**) derivatives were tested. Control CuRs without any chelating group were also synthesized (**O1**, **P1**). The syntheses were carried out as described in the Supporting Information. All of the final compounds were well characterized using nuclear magnetic resonance and high-resolution mass spectrometry.

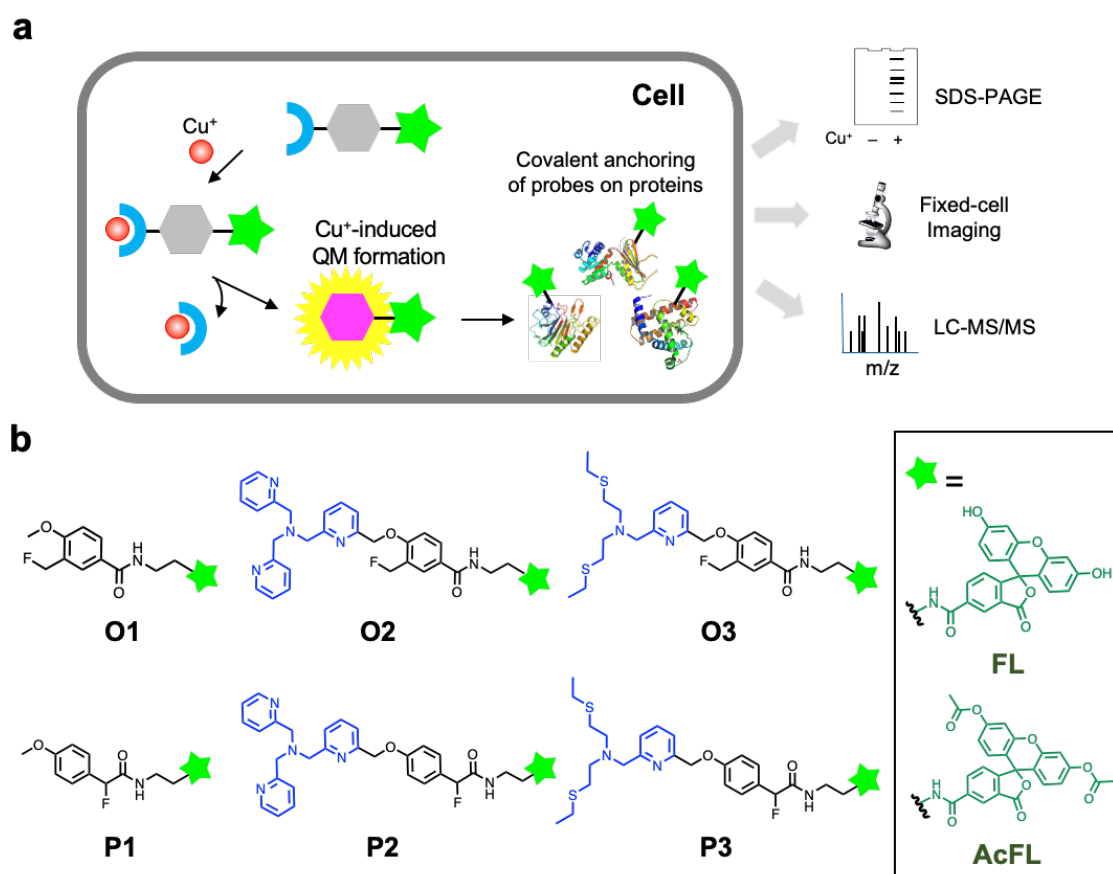


Figure 1 (a) Schematic illustration of Cu^+ -responsive protein labeling in living cells. (b) Molecular structures of Cu^+ -responsive labeling reagents used in this study. QM, quinone methide; FL, fluorescein; AcFL, diacetylated fluorescein; LC-MS/MS, liquid chromatography–tandem mass spectrometry.

We initially confirmed the Cu^+ -catalyzed oxidative cleavage of the benzyl ether and subsequent QM formation for the designed compounds using high-performance liquid chromatography (HPLC). The Boc-protected compound (**P2'**, 1 mM) was incubated in aqueous solution containing Ac-cysteine-OMe (5 mM), a model nucleophile to trap activated QM, and sodium ascorbate (2 mM) in the presence or absence of tetrakis(acetonitrile)copper(I) hexafluorophosphate [$\text{Cu}(\text{MeCN})_4\text{PF}_6$] (1 mM). As shown in **Figure 2a**, upon the addition of Cu^+ , **P2'** was consumed and a new peak appeared in the chromatogram. Mass spectrometry revealed the product to be the cysteine adduct of QM generated by the elimination of the TPA moiety, clearly indicating that the expected reaction had progressed (**Figure 2c**). We also confirmed that the **P2'** activated by Cu^+ can react with His and Lys, which was consistent with the reported reactivity of QM toward

nucleophilic amino acids (**Figure S2**).¹⁹ Cu⁺-mediated reactivity enhancement was also observed for **O2**, while control compounds **O1** and **P1** showed no response to Cu⁺ (**Figure S3**). Notably, **O3** and **P3** gave multiple products resulting from the Cu-promoted oxidation of thioether moieties (**Figure S3**).

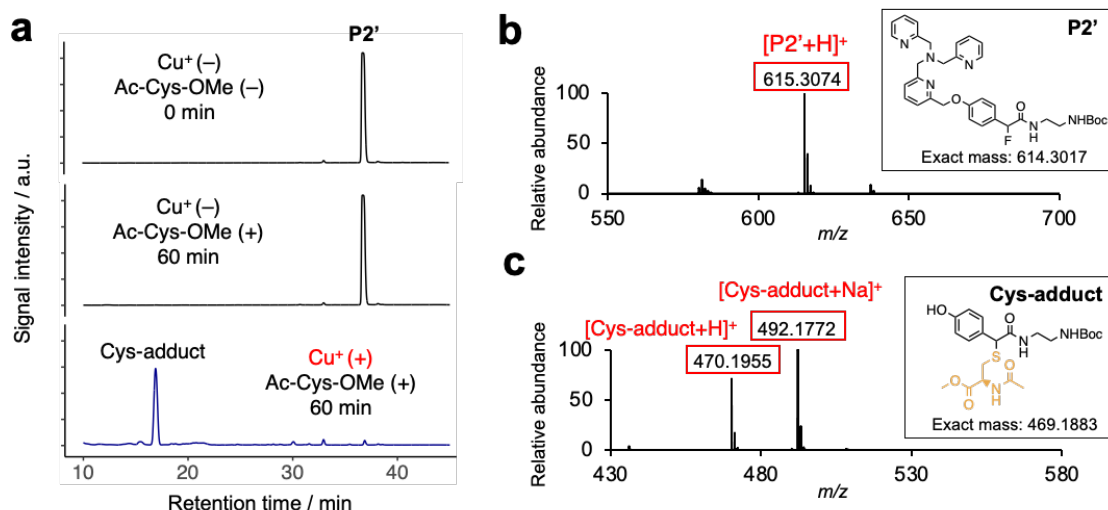


Figure 2 Confirmation of Cu⁺-dependent quinone methide formation. (a) High-performance liquid chromatographs of **P2'** (1 mM) before (0 min) and after (60 min) treatment with Cu⁺ (1 mM) and Ac-Cys-OMe (5 mM) in 50 mM HEPES buffer (pH 7.2)/acetonitrile 1:1 at 25°C. UV detection at 220 nm. (b, c) Mass spectra of the peaks at 37 min (panel b) and 17 min (panel c) in (a).

Next, the Cu⁺-responsive protein labeling capabilities of CuRs were evaluated in vitro using purified bovine serum albumin (BSA) as a model protein substrate. Each CuR (50 μM) was mixed with BSA (10 μM), then Cu⁺ was added (50 μM). After 1 h, the reaction product was analyzed using SDS-PAGE and in-gel fluorescence imaging (the reaction time profiles are shown in **Figure S4**). All chelator-appended CuRs showed increased protein labeling efficiency in the presence of Cu⁺, while negligible fluorescence was observed in the absence of Cu⁺ (**Figure 3a, b**). Control compounds (**FL-O1** and **FL-P1**) did not result in Cu⁺-responsive activation. It was also found the 2-fluoromethylphenol-based compound **FL-O1** had intrinsic reactivity for proteins, causing relatively high background labeling. Among the tested compounds, TPA-based probes **FL-O2** and **FL-P2** had a higher Cu⁺-responsive labeling efficiency than thioether-based probes **FL-O3**

and **FL-P3**. The lower activities of **FL-O3** and **FL-P3** presumably resulted from their deactivation by the Cu^+ -mediated oxidization of the thioether moiety before C–O bond cleavage, as indicated by the HPLC analysis (**Figure S3**). Taken together, we concluded that the structure of **FL-P2** is optimal for Cu^+ -responsive protein labeling in terms of the highest signal-to-noise ratio, and we therefore decided to characterize it further.

We first examined the selectivity of **FL-P2** toward Cu^+ over other biometals. As shown in **Figure 3c**, **FL-P2** exhibited the highest selectivity for Cu^+ among all tested metal ions, including Cu^{2+} , Zn^{2+} , and redox active $\text{Fe}^{2+}/\text{Fe}^{3+}$. We also confirmed that **FL-P2** is more efficiently activated by Cu^+ than a variety of ROS (**Figure 3d**). The less sensitivity toward ROS is important for specific detection of Cu^+ in cells since intracellular copper accumulation often generates a variety of ROS.⁵ Notably, Cu^+ -dependent protein labeling by **FL-P2** proceeded even in the presence of 5–10 mM glutathione, an abundant chelator of intracellular Cu^+ , which suggests its applicability in a cellular context (**Figure S5**).

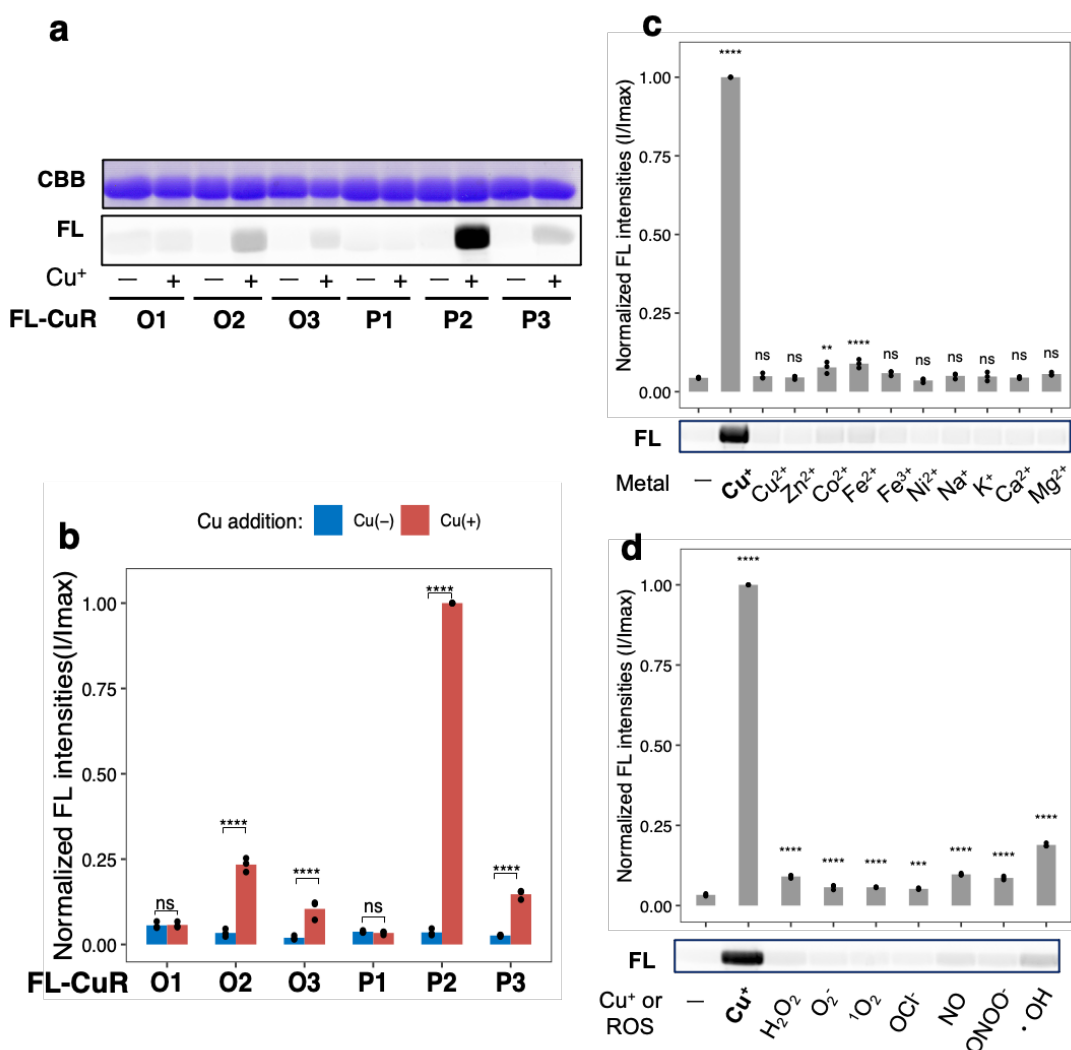


Figure 3 Cu⁺-responsive protein labeling in vitro. (a) SDS-PAGE and in-gel fluorescence analysis of BSA labeling with fluorescein-tethered CuRs (FL-CuRs). The reagent (50 μ M) was added to 50 mM HEPES buffer (pH 7.2) containing BSA (10 μ M). The mixture was incubated at 37°C in the presence or absence of Cu⁺ (50 μ M) for 1 h. (b) Relative fluorescence intensity of proteins labeled by FL-CuRs. (c, d) Selectivity of **FL-P2** for several metal ions (panel c) and ROS (panel d). A mixture of **FL-P2** (50 μ M) and BSA (10 μ M) in 50 mM HEPES buffer (pH7.2) was incubated at 37°C for 1 h in the presence of various metal ions (50 μ M for Cu⁺, Cu²⁺, Zn²⁺, Co²⁺, Fe²⁺, Fe³⁺, and Ni²⁺, and 5 mM for Na⁺, K⁺, Ca²⁺, and Mg²⁺) or ROS/ROS generating reagent (50 μ M). $n = 3$ biological replicates. P-values in (b) were calculated using a two-way ANOVA and Tukey's test by comparing Cu⁺-treated and non-treated samples for each CuR. P-values in (c) and (d) were calculated using a one-way ANOVA and Dunnett's test by comparing metal or ROS-added samples to non-treated control. All values are expressed as independent dots. ns: $p > 0.05$, *: $0.01 < p \leq 0.05$, **: $0.001 < p \leq 0.01$, ***: $0.0001 < p \leq 0.001$, ****: $p \leq 0.0001$. CBB, Coomassie brilliant blue; BSA, bovine serum albumin; CuRs, Cu⁺-responsive protein labeling reagents; ROS, reactive oxygen species.

We subsequently tested whether the Cu⁺-responsive reagent can detect changes in labile copper levels inside cells. For live cell applications, we replaced the reporter moiety of **FL-P2** with diacetyl fluorescein (**AcFL**) to increase cell-membrane permeability (**Figure 1; AcFL-P2**). As expected, the probe was rapidly taken-up by living cells within 5 min, fluoresced upon removal of the acetyl group by endogenous esterases, and was uniformly distributed across the whole cell, making it suitable for spatially unbiased protein labeling and Cu⁺ detection in living cells (**Figure S6**). To demonstrate Cu⁺-responsiveness, we used HeLa cells transiently transfected with copper transporter 1 (CTR1), a membrane protein that selectively imports extracellular copper into cells.²³ The HeLa cells were preincubated in the presence or absence of 5 μ M CuCl₂ for 8.5 h, then treated with **AcFL-P2** for 25 min. Note that the expression level of CTR1 in copper-treated transfected cells was reduced because of the negative feedback of CTR1 in response to intracellular copper accumulation, which is consistent with a previous report (**Figure 4a**).²⁴ In-gel fluorescence analysis showed that many proteins were labeled in the CTR1-transfected cells treated with exogenous copper, whereas the corresponding bands were not detected in the gel when either transfection or copper supplementation was omitted (**Figure 4a**). Control compound **AcFL-P1** exhibited no Cu⁺ response (**Figure S7**). We also found that treatment of the CTR1-overexpressing cells with *N*-methylmaleimide (N-MEM), a thiol alkylation reagent that can release protein-bound copper, caused a remarkable increase in labile Cu⁺, resulting in enhancement of the labeling bands in-gel fluorescence (**Figure S8 and S9**). This suggests that most of the copper ions transported by CTR1 are bound to thiol groups of intracellular metal-buffering substances, such as glutathione and metallothionein.^{25,26} Confocal laser scanning microscopy (CLSM) clearly showed that the strong fluorescent signals derived from the fluorescein-labeled proteins were observed in CTR1-overexpressing cells (**Figure S8**). All of these results clearly demonstrate that **AcFL-P2** is activated and modifies intracellular proteins in response to accumulation of labile Cu⁺ transported into the cell by CTR1.

We next applied **AcFL-P2** to a genetic model of copper dysregulation induced by knockout (KO) of ATP7A, a copper-translocating P-type ATPase that regulates copper export. It is well recognized that a loss of ATP7A function leads to impaired extracellular copper secretion and thus intracellular copper accumulation, ultimately contributing to

the pathogenesis of Menke's disease.¹⁰ ATP7A-KO²⁷ and wild-type (WT) human melanoma Mewo cells were preincubated with the cell-permeable glyoxal-bis(*N*(4)-methylthiosemicarbazonato)-copper(II) (Cu-gtsm) complex (2 μ M) to increase intracellular copper levels, then incubated with **AcFL-P2** for 30 min. The elevated concentration of labile Cu⁺ in ATP7A-KO cells was detected using both SDS-PAGE and CLSM, while WT cells exhibited a weaker signal because ATP7A rapidly excreted excess exogenous copper ions (**Figure 4b, c**). The same result was obtained using an established Cu⁺ chemosensor, validating the Cu⁺-responsiveness of our probe (**Figure S10**). Notably, quantitative imaging analysis could identify differences in the endogenously low basal Cu⁺ levels in the KO and WT cells treated with our probe (**Figure S11**).

Given the unique ability of **AcFL-P2** to covalently attach fluorescent reporters to intracellular proteins in response to labile Cu⁺, it can be used as a histochemical probe to discriminate cells with relatively high labile Cu⁺ levels within a cell population with a heterogeneous genetic background. To demonstrate this idea, we performed a model experiment using a mixed cell culture system in which the cell surface of WT cells was biotinylated and fluorescently labeled with an AlexaFluor647–streptavidin conjugate to distinguish them from non-biotinylated ATP7A-KO cells (**Figure 4d**).²⁸ After treatment with Cu-gtsm and **AcFL-P2**, the mixed cell culture was analyzed using CLSM. As shown in **Figure 4e, f**, stronger fluorescence derived from the fluorescein-modified proteins was observed in ATP7A-KO cells than WT cells. This demonstrated that **AcFL-P2** can selectively label and fluorescently visualize Cu⁺-accumulated cells.

Finally, we characterized the proteins modified with **AcFL-P2** in Cu-gtsm-treated ATP7A-WT and KO cells using proteomics. The labeled proteins were enriched by immunoprecipitation with an anti-fluorescein antibody, digested using trypsin, and analyzed by liquid chromatography–tandem mass spectrometry. Label-free quantification was used in the mass spectrometry analysis to detect the dynamic changes in protein labeling caused by exogenous copper relative to the basal (non-copper-treated) condition (**Figure S12** and **Supplementary Table S1**). Note that the treatment of cells with Cu-gtsm did not cause significant expression changes of most proteins labeled by **AcFL-P2** in either ATP7A WT or ATP7A KO cells (**Figure S13** and **Supplementary Table S2**). We analyzed three independent biological replicates and defined significant labeling of proteins by: fold change (Cu(+)/Cu(–)) ≥ 2 ; number of identified peptides ≥ 2 ; and $p <$

0.05. With this definition, a total of 60 and 547 proteins were identified in WT and KO cells, respectively (**Figure 4g, h, Supplementary Table S1**). The larger number of proteins detected in KO than in WT cells is consistent with the gel- and imaging-based analyses. Gene ontology analysis of the identified proteins provided valuable insights into intracellular Cu^+ metabolism (**Figure 4i**). For example, proteins in extracellular exosomes and vesicles were more enriched in WT cells than other cellular components, suggesting that copper is sequestered and concentrated in these intracellular compartments for subsequent excretion. In contrast, cytosolic proteins were more enriched in KO cells, indicating that abnormal copper efflux results in increased labile copper in the cytosol. Moreover, mitochondrial proteins were more enriched in KO cells than in WT cells, and interestingly, our proteomics dataset contained DLAT, DLST, DLD and PDHA1, which have previously been identified as proteins essential for cuproptosis.⁷ In the proposed mechanism of cuproptosis, excess copper causes aggregation of lipoylated proteins and destabilization of Fe-S cluster proteins, both of which are expressed in mitochondria, resulting in mitochondrial dysfunction and finally cell death. However, direct evidence for Cu^+ accumulation in mitochondria has not yet been demonstrated. In this regard, our proteomic study may suggest that excess copper is enriched in mitochondria, supporting the current cuproptosis mechanism, although further validation is needed.

In summary, we developed a novel molecular probe that is selectively activated in response to labile Cu^+ for labeling proteins in Cu^+ -rich cellular environments. In vitro studies clearly showed its excellent Cu^+ selectivity. The optimized probe was capable of sensing abnormal cellular copper uptake or efflux defects induced by genetic perturbations and converting them into an integrated fluorescent signal. It should be noted that the protein labeling rarely proceeds in the normal cellular state plausibly because of extremely low levels of intracellular labile Cu^+ that can form a complex with the probe to activate it (thermodynamically available intracellular Cu^+ is estimated to be less than 1 fM).^{22,26} Even the distorted copper homeostasis, mM concentration of intracellular glutathione should mask most of Cu^+ in equilibrium. Nevertheless, our probe responded to such a change as the N-MEM treatment experiments revealed. This may be ascribed to that the probe can work relying on the irreversible bond cleavage coupled with Cu^+ -binding events. Therefore, our probe would be suitable for the analysis of cellular events in which labile copper concentrations increase under abnormal conditions of copper

homeostasis. We also demonstrated the applicability of this reagent to selective labeling and fluorescent visualization of Cu-accumulating cells in a mixed cell culture system, as well as to proteomic analysis of proteins associated with Cu⁺ metabolism and Cu⁺-induced cell death at the protein level. These results indicate that this method can detect the elevated Cu⁺ concentration at least single cell-level spatial resolution. On the other hand, the spatial resolution at the subcellular level is not yet defined to date because of the difficulty in measuring the intracellular diffusivity of the probe across organelle membranes and the kinetics of QM formation and subsequent protein labeling in the crowded cellular context. In this regard, the introduction of organelle-targeting motifs into the probe may be useful to enhance spatial resolution in Cu⁺ detection.²⁹ Another limitation of this method is the potential bias in protein labeling due to limited amino acid coverage and interactions with specific proteins. Given these considerations, one should carefully design the experiments and analyze/validate the results provided by this probe.¹⁹ Despite these caveats, our Cu⁺-selective probe offers a unique and powerful tool for selective imaging and analysis of cellular situations with increased Cu⁺ concentrations and thus will shed light on unexplored biological phenomena related to labile-Cu⁺ metabolism. Our ongoing research aims to further refine the probe design to improve its sensitivity toward endogenous levels of labile Cu⁺ and its general applicability to tissues and in vivo systems.

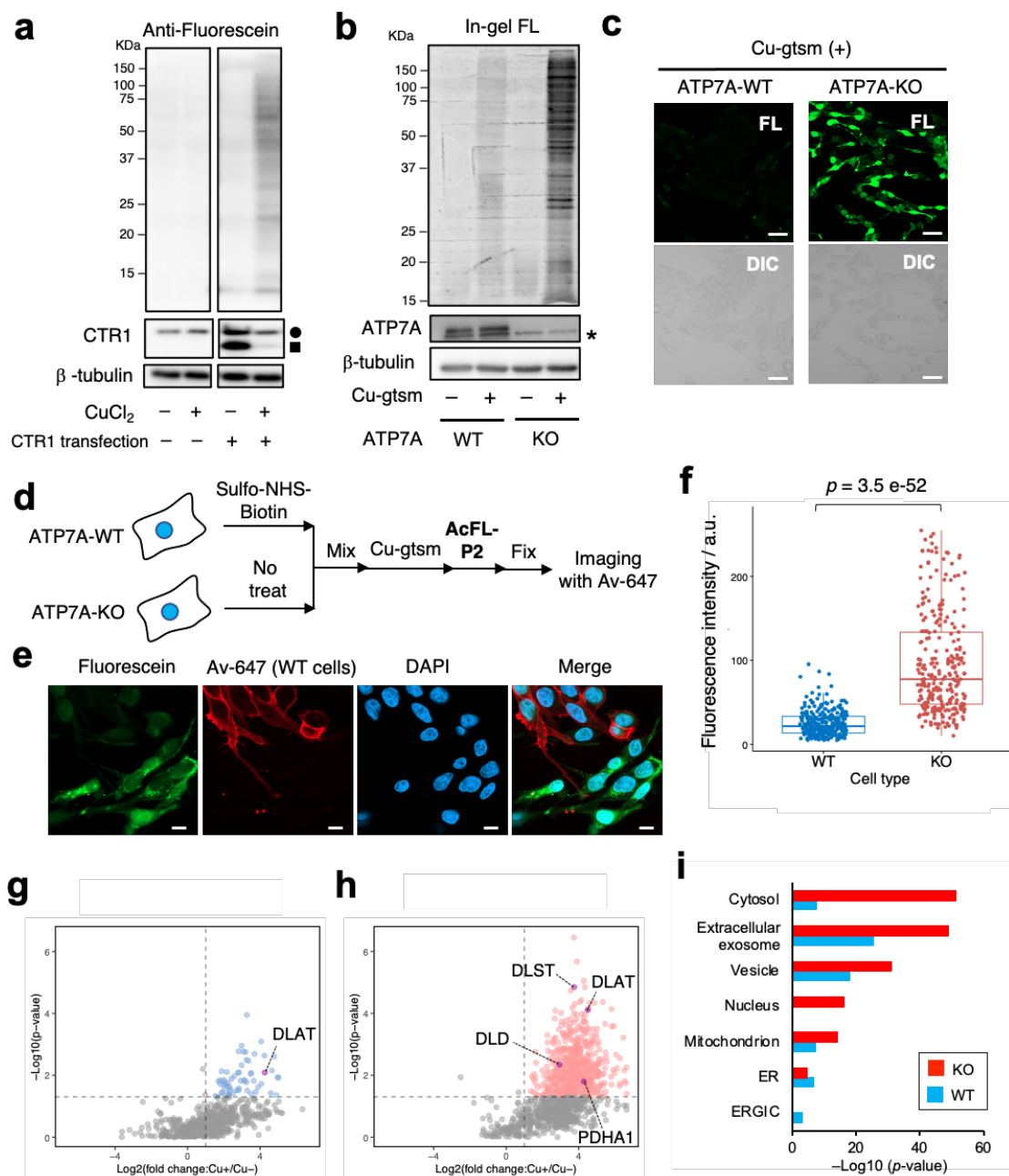


Figure 4 Cu⁺-responsive protein labeling with AcFL-P2 in cells. (a) In-gel fluorescence imaging of copper-responsive labeling in HeLa cells overexpressing CTR1. HeLa cells transfected with a CTR1-expression plasmid were pretreated with CuCl₂ (5 μ M) for 8.5 h, then incubated with AcFL-P2 (5 μ M) for 25 min. ●, mature CTR1; ■, immature CTR1. (b) In-gel fluorescence imaging of labeled proteins in WT and ATP7A-KO Mewo cells pretreated with Cu-gtmsm (2 μ M) for 30 min, then washed with medium and further incubated with AcFL-P2 (5 μ M) for 30 min. The band marked with an asterisk results from non-specific reactivity of the anti-ATP7A antibody. (c) CLSM images of Mewo cells after labeling. Scale bar, 40 μ m. (d) Workflow for Cu⁺-responsive protein labeling in a mixed culture system. (e) CLSM analysis of Cu⁺-accumulated cell-specific labeling. A mixture of ATP7A-KO cells and surface-biotinylated WT cells was treated with Cu-gtmsm (2 μ M) and subsequently incubated with AcFL-P2 (5 μ M) for 30 min. After reaction,

the cells were fixed, treated with an AlexaFluor647–streptavidin conjugate (Av-647), and analyzed using CLSM. Scale bar, 10 μm . (f) Box plot of the fluorescence intensity of cells, measured using a fluorescein channel ($n = 270$). The horizontal line within each box indicates the median. Boxes show interquartile range (IQR). Whiskers show $1.5 \times$ IQR. The p -value was calculated using an unpaired, two-sided Student t -test. (g, h) Volcano plots of proteins identified using Cu^+ -responsive protein labeling in WT (panel g) and ATP7A-KO (panel h) Mewo cells ($n = 3$ biological replicates). Significantly labeled proteins that met our criteria (fold change ($\text{Cu}(+)/\text{Cu}(-)$) ≥ 2 , number of identified peptides ≥ 2 , and $p < 0.05$) in WT and KO cells are shown in blue and red plots, respectively. (i) Gene ontology analysis of significantly labeled proteins in WT (blue) and KO (red) cells. CTR1, copper transporter 1; WT, wild type; KO, knockout; CLSM, confocal laser scanning microscopy; Cu-gtsm, glyoxal-bis($N(4)$ -methylthiosemicarbazonato)-copper(II); DAPI, 4',6-diamidino-2-phenylindole.

ASSOCIATED CONTENT

Data Availability Statement

The data underlying this study are available in the published article and its Supporting Information.

Supporting Information Statement

The Supporting information including detailed experimental procedures, NMR, and MS data is available free of charge.

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Notes

The authors declare no competing financial interest

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