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Chemical Profiling of the Endoplasmic Reticulum Proteome Using Designer Labeling Reagents

Alma Fujisawa,[†] Tomonori Tamura,[†] Yuki Yasueda,[†] Keiko Kuwata,[‡] and Itaru Hamachi*[,†,§]

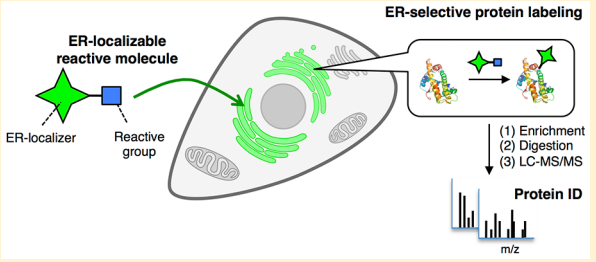
[†]Department of Synthetic Chemistry and Biological Chemistry, Graduate School of Engineering, Kyoto University, Katsura, Nishikyo-ku, Kyoto 615-8510, Japan

[‡]Institute of Transformative Bio-Molecules (ITbM), Nagoya University, Chikusa, Nagoya 464-8602, Japan

[§]Core Research for Evolutional Science and Technology, Japan Science and Technology Agency, Sanbancho, Chiyodaku, Tokyo 102-0075, Japan

Supporting Information

ABSTRACT: The endoplasmic reticulum (ER) is an organelle that performs a variety of essential cellular functions via interactions with other organelles. Despite its important role, chemical tools for profiling the composition and dynamics of ER proteins remain very limited because of the labile nature of these proteins. Here, we developed ER-localizable reactive molecules (called ERMs) as tools for ER-focused chemical proteomics. ERMs can spontaneously localize in the ER of living cells and selectively label ER-associated proteins with a combined affinity and imaging tag, enabling tag-mediated ER protein enrichment and identification with liquid chromatography tandem mass spectrometry (LC-MS/MS). Using this method, we performed proteomic analysis of the ER of HeLa cells and newly assigned three proteins, namely, PAICS, TXNL1, and PPIA, as ER-associated proteins. The ERM probes could be used simultaneously with the nucleus- and mitochondria-localizable reactive molecules previously developed by our group, which enabled orthogonal organellar chemoproteomics in a single biological sample. Moreover, quantitative analysis of the dynamic changes in ER-associated proteins in response to tunicamycin-induced ER stress was performed by combining ER-specific labeling with SILAC (stable isotope labeling by amino acids in cell culture)-based quantitative MS technology. Our results demonstrated that ERM-based chemical proteomics provides a powerful tool for labeling and profiling ER-related proteins in living cells.



INTRODUCTION

The endoplasmic reticulum (ER) is an essential subcellular component responsible for the synthesis, processing, and transport of proteins.[1] Virtually all plasma membrane-associated proteins and secretory proteins are synthesized in the ER and delivered to their final destination through vesicular trafficking.[2] It is increasingly recognized that communication between the ER and other organelles, such as the mitochondria, Golgi apparatus, and endosomes, plays a pivotal role in diverse cellular functions.[3,4] In addition, recent reports suggest that malfunctions of the ER induced by cellular stress are implicated in a variety of diseases, including diabetes, inflammation, and neurodegenerative disorders.[5] Thus, comprehensive characterization of ER-associated proteins should be useful not only as a fundamental biological study but also for understanding the etiology of such diseases. Conventional proteomic studies of the ER have mainly relied on subcellular fractionation with sucrose gradient ultracentrifugation, followed by mass spectrometry (MS) or gel-based analysis.[6−8] However, isolating the intact ER is extremely difficult because of dynamic transitions in ER composition and its complex association with other organelles and cytosolic components in live cells; these factors can result in substantial loss of transiently ER-associated proteins and contamination with other organelles.[7]

Chemoproteomic approaches that do not require any subcellular fractionation are potentially a valuable alternative for circumventing these problems.[9−12] Indeed, proximity biotinylation with ascorbate peroxidase (APEX), a method recently developed by Ting's group, has proven to be a powerful tool for organelle proteomics.[13−16] In this method, APEX is genetically localized to a specific organelle, such as the mitochondria or ER. Therefore, the enzymatic oxidation of the APEX substrate, biotin-phenol, only occurs in a limited space surrounding APEX proteins upon addition of hydrogen peroxide (H_2O_2), such that the targeted organelle's proteins are selectively modified with a biotin affinity tag. The biotinylated proteins can subsequently be enriched with avidin beads and identified by liquid chromatography tandem MS (LC-MS/MS) analysis. Despite the usefulness and robustness of this technique, APEX technology necessitates expression of an exogenous engineered peroxidase and the addition of cytotoxic H_2O_2 to initiate the labeling reaction, which may

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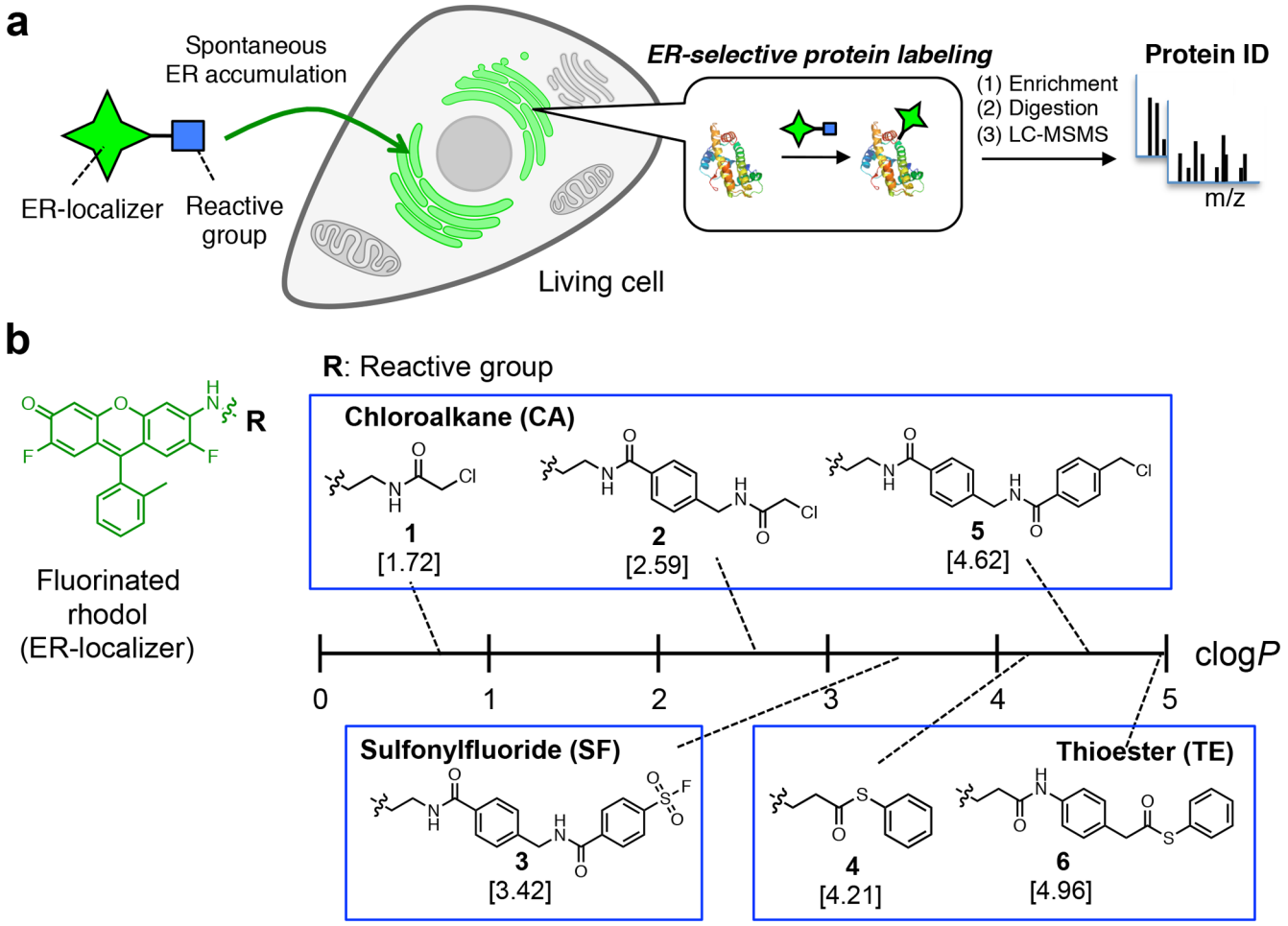

Figure 1. ER-focused chemical proteomics with a set of ER-localizable reactive molecules (ERMs). (a) Schematic of ERM-mediated selective labeling and profiling of ER-associated proteins. (b) Molecular design of ERM **1**−**6** with the different reactive groups. The calculated logP (clogP) value for each of these compounds is shown in brackets.

potentially cause artificial perturbation of natural biological contexts.[17] In contrast, we recently reported mitochondria- and nucleus-focused proteomics using newly developed organelle-localizable reactive molecules (ORMs), which need neither genetic manipulation nor cytotoxic reagents.[18,19] ORMs, composed of an organelle-localizing motif and a reactive group, spontaneously accumulate within the targeted organelle, and the resultant high concentration of ORMs facilitates spatially limited modification of proteins with a detection/purification tag via simple intermolecular reactions; no specific enzyme activity or protein affinity is required. ORM labeling using nuclear- or mitochondria-localizable reactive molecules (NRMs and MRMs) combined with antibody-based enrichment and MS-fingerprinting techniques allows for selective identification of nuclear and mitochondrial proteomes, respectively.

Here, we sought to challenge the current knowledge of ER-focused proteomics, using an ORM strategy that exploited a set of ER-localizable reactive molecules (ERMs) exhibiting a variety of chemical reactivities and amino acid selectivities (Figure 1a). These designer ERMs were observed to selectively localize within the ER of live cells, which allowed spatially restricted chemical modification and identification of ER-associated proteins without biochemical fractionation or genetic manipulation. LC-MS/MS analysis of the labeled proteins validated the ER selectivity of this method. Notably, three proteins were newly assigned as ER-related proteins. We also demonstrated simultaneous usage of three probes, an NRM, MRM, and ERM, in the same cell culture, which highlights the feasibility of applying our chemical fractionation approach to multiple subcellular organelles within a single biological sample. Finally, quantitative MS analysis coupled with one of the ERMs was conducted to analyze the dynamic changes in the ER proteome induced by ER stress.

RESULTS AND DISCUSSION

Molecular Design of ER-Localizable Reactive Molecules. According to the design principle used in our previous studies,[18,19] we prepared a set of ERMs consisting of an ER-localizable motif and a reactive group (Figure 1b). Fluorinated hydrophobic rhodol was chosen as a promising ER localizer.[20] This fluorescent dye is reported to specifically accumulate in the ER, presumably because its hydrophobic and amphipathic properties confer a preference for the environment of the cholesterol-poor ER membranes.[21] Furthermore, rhodol derivatives exhibit excellent photophysical properties that would prove useful for evaluating the subcellular localization and concentrations of ERMs by confocal laser scanning microscopy (CLSM) imaging and spectroscopic in-gel analysis. For the reactive groups, we employed three different electrophiles, chloroalkane (CA; **1**, **2**, and **5**), sulfonyl fluoride

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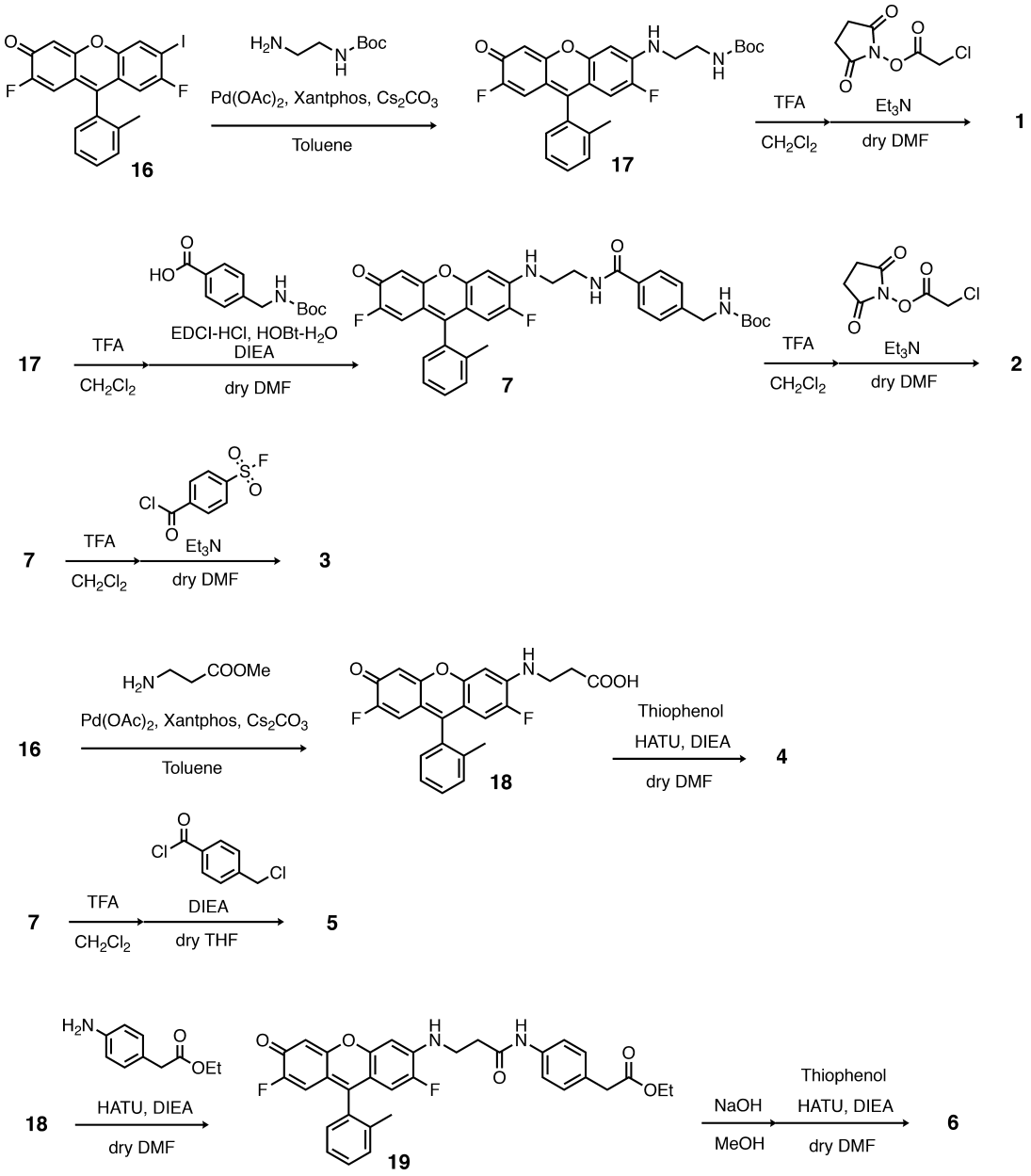

Figure 2. Synthetic schemes for ERM **1**−**6**.

(SF; **3**), and thiophenyl ester (TE; **4** and **6**). We and other groups previously demonstrated that CA and TE preferentially react with the thiol group of cysteines and the amino group of lysines, respectively, while SF shows good reactivity toward both lysines and tyrosines.[18,22−25] Therefore, ERMs equipped with these electrophiles should exhibit distinct amino acid preference, which may result in different protein selectivity and enhance the coverage of proteins in the ER proteome. Given the plausible mechanism of ER-localization with rhodol, the hydrophobicity of the ERMs was deemed likely to be critical for spontaneous accumulation in the ER. Thus, ERM derivatives bearing a benzene-type spacer between the rhodol and the reactive group were also prepared to modulate their hydrophobicity. Figure 1b summarizes the set of ERMs used together with their calculated logP values (clogP) as an indicator of their hydrophobicity. Synthesis of the ERMs was carried out as shown in Figure 2 and in the Supporting Information (SI). ERM **1**, for instance, was synthesized as follows: the core compound **16** was conjugated with a Boc-protected form of the diamine linker through Buchwald−Hartwig cross-coupling. After deprotection of the Boc group, *N*-(chloroacetoxy)succinimide was condensed to yield ERM **1**. All the final compounds were characterized by NMR and high-resolution MS spectroscopy. UV−vis and fluorescence spectra of the representative ERM **6** are shown in Supplementary Figure S1.

Cell Permeability and Localization of the ERMs. We first evaluated the cell permeability and ER localizability of the ERMs (**1**−**6**). HeLa cells were incubated in culture medium containing each of the ERMs (100 nM) and ER-Tracker Red, a conventional ER staining dye, at 37 °C for 15 min, followed by CLSM analysis. It was found that all ERMs except **2** rapidly passed through the cell membrane (<15 min) and spontaneously localized to the ER (Figure 3a and Supplementary Figure S2). The relationship between the ER-selective localization and the hydrophobicity of the ERMs was examined

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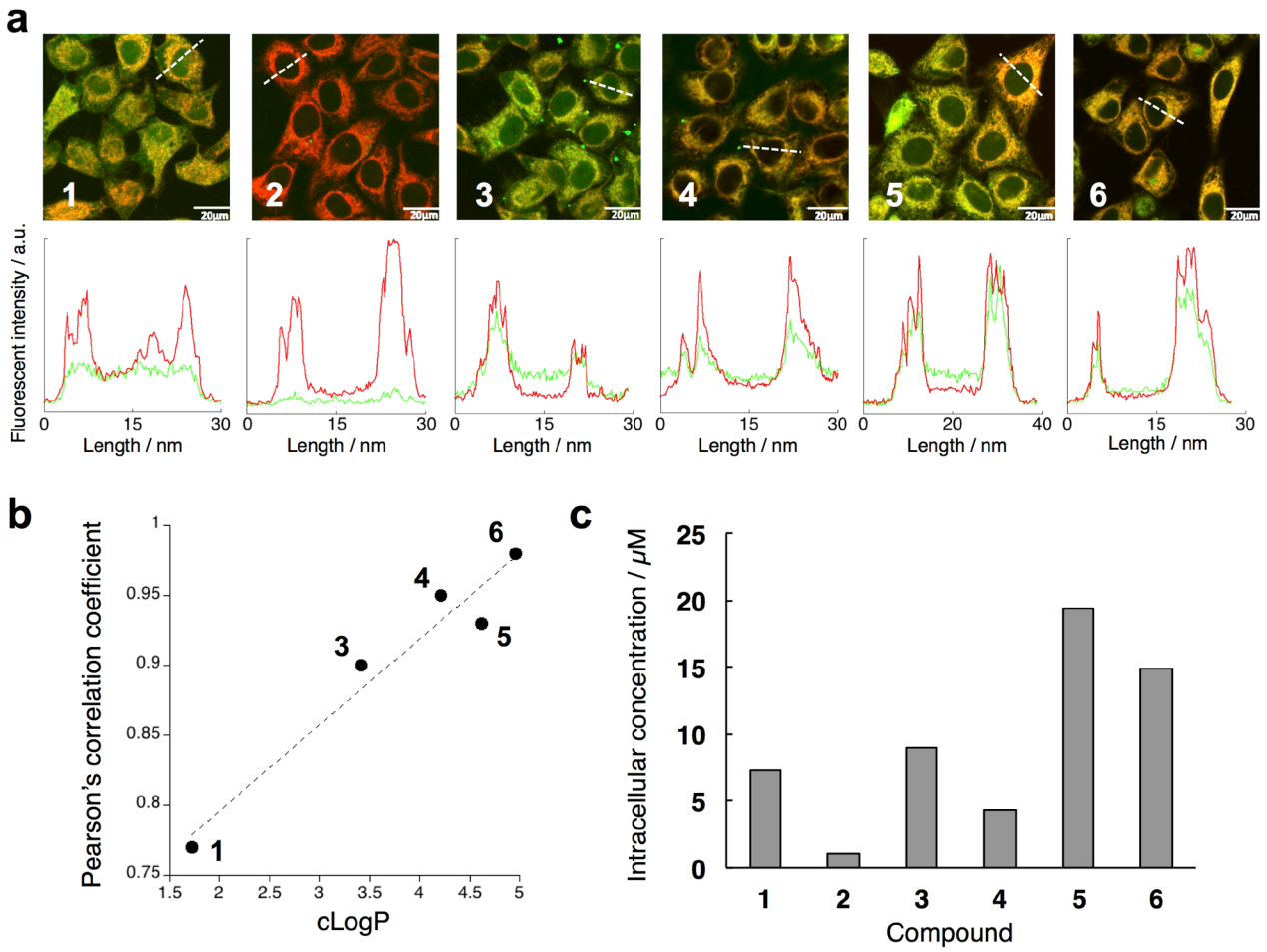

Figure 3. Cell permeability and spontaneous ER localizability of the ERMs **1**−**6**. (a) Representative CLSM merged images of HeLa cells treated with 100 nM of each ERM (green) and ER-Tracker (red) for 15 min. Colocalization areas are in yellow. Line-plot graphs indicate the fluorescence intensity profiles of the ERM (green) and ER-Tracker (red) along the white dotted lines indicated in the CLSM images. Scale bar: 20 μm. (b) Correlation of Pearson's correlation coefficients of the ERM/ER-Tracker signal overlap observed in the CLSM images against the clogP values of the cell-permeable ERMs (**1**, **3**−**6**). (c) Intracellular concentration of each ERM in HeLa cells determined by spectroscopic measurement of rhodol fluorescence.

by plotting the Pearson's correlation coefficients (PCCs) of the ERM/ER-Tracker Red signal overlap against the clogP values of the cell-permeable ERMs (Figure 3b). To our surprise, it was clear that (i) the clogP values were strongly correlated with the ER localization of the ERMs and (ii) the appropriate lipophilicity (clogP > 3.4) was required for ER-specific targeting. Indeed, we noticed that the less hydrophobic ERM **1** (clogP = 1.7) was distributed throughout non-ER regions, such as the cytosol and nucleus. We also found that similar nonspecific distribution occurred in cases where high concentrations of ERM **3**−**6** were used (>1 μM) (Supplementary Figure S3). Thus, 100 nM of ERM (the apparent concentration in the medium) was defined as the optimal concentration for ER proteome-specific labeling.

The ER-selective localization of ERMs was further evaluated by imaging the colocalization of ERM **6** with other organelle markers, including Mito-tracker, Lyso-tracker, and CellLight Golgi-RFP. As shown in Supplementary Figure S4, the fluorescent signal of **6** did not merge with those of Mito-tracker and Lyso-tracker, clearly indicating the ERM does not localize in mitochondria and lysosomes. On the other hand, the image of **6** was partially overlapped with that of the Golgi apparatus. It was considered that this is attributed to the vesicle transport of ERM from ER to the Golgi apparatus during this experiment. Indeed, we confirmed that the colocalization of the ERM in the Golgi apparatus was suppressed by addition of H89, a protein kinase inhibitor that can block ER-to-Golgi trafficking. These indicated that ERMs are precisely localized in the ER at first, and its small portion gradually translocates to the Golgi via an ER-to-Golgi transport system during incubation.

We next quantitatively determined the intracellular concentrations of ERMs by spectroscopic analysis of the lysates prepared from cells treated with 100 nM of each ERM. The concentrations of **1**−**6** per single cell were estimated as shown in Figure 3c. Because the ER comprises about 3−15% of the cellular volume,[26,27] it is deduced that the condensation effect in the ER further raises intraorganellar concentrations by 6.6−33-fold, which would be reasonably expected to facilitate the intermolecular ERM-labeling reactions with proteins in the ER (Supplementary Table S1). Compared with the thioester-type ERM **4** (4.3 μM per cell), the intracellular concentration of the more hydrophobic thioester-type ERM **6** was 3.5-fold higher (14.9 μM), which suggests that probe hydrophobicity is one of the key factors for both localization and condensation of ERM within the ER. Given their excellent ER localizability and

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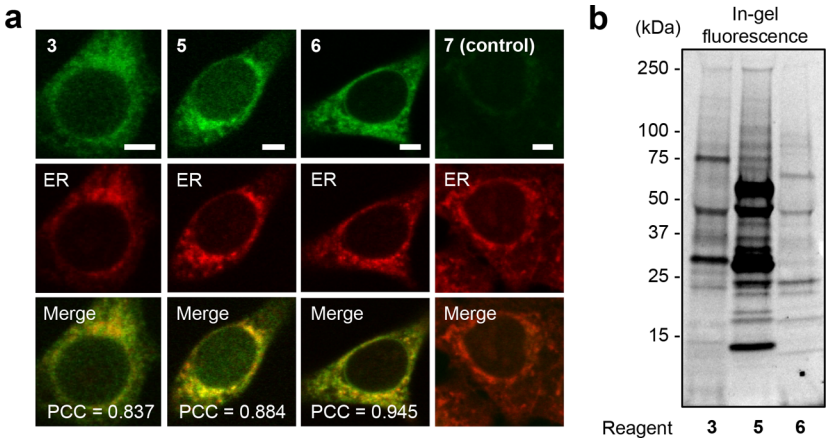

Figure 4. Covalent protein modification in the ER with ERMs. (a) CLSM images of HeLa cells treated with ERMs **3**, **5**, **6**, and **7** (green) and ER-Tracker (red), followed by methanol fixation. Colocalization was quantified using Pearson's correlation coefficient (PCC). Scale bar: 5 μm. (b) SDS-PAGE analysis with in-gel fluorescence imaging. Reaction conditions: HeLa cells were incubated with each ERM (100 nM) in DMEM at 37 °C for 1 h. After cell lysis, the labeled proteins were enriched by immunoprecipitation with anti-rhodol antibody to enhance the fluorescent signal, followed by SDS-PAGE (a 4–12% gradient gel). Optimization of the reaction time with each ERM in living cells is shown in Supplementary Figure S6.

reaction diversity, ERMs **3**, **5**, and **6** were selected for subsequent protein labeling experiments.

Protein Labeling with ERMs. Once the three optimal ERMs (**3**, **5**, and **6**) for selective ER localization had been determined, we next evaluated the reactivity of these ERMs with endogenous ER proteins in HeLa cells using CLSM imaging and gel electrophoresis. After incubation of the cells with each ERM for 1 h, the cells were fixed with cold methanol to remove unreacted ERMs, followed by CLSM analysis. As shown in Figure 4a, strong fluorescent signals from rhodol remained for all three ERMs even after methanol fixation, and more importantly, this fluorescence merged well with the ER-Tracker-based signal.[28] In contrast, no fluorescence was detected for control compound **7**, which lacked a reactive group.[29] This ER-localized fluorescence clearly indicated that the ERMs selectively accumulated within the ER and labeled the ER-localized proteins with the rhodol dye, providing stable fluorescence signal in the ER even after cell fixation. The covalent attachment of rhodol to the ER proteins was additionally confirmed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) followed by in-gel fluorescence imaging (Figure 4b and Supplementary Figure S6). On these gels, many fluorescent bands were detected in all three lanes where each of the ERMs were separately applied to the HeLa cells. Notably, the band patterns for the three ERMs were distinct. These results confirmed covalent bond formation between the ER proteins and the rhodol dye, as well as the different protein selectivity of each ERM.

ER Protein Identification and Assignment of New ER Proteins. Identification of proteins labeled with each ERM was performed with LC-MS/MS analysis after antibody-based enrichment (Figure 5a). We prepared anti-rhodol antibody in-house and confirmed that the antibody was applicable for the enrichment experiments, using isolation of rhodol-modified proteins from cell lysates by immunoprecipitation (IP) (Supplementary Figures S7 and S8). The enriched proteome was then subjected to SDS-PAGE followed by in-gel tryptic digestion, and the resultant peptide fragments were analyzed by nano-LC-MS/MS. The annotation analysis of the cellular compartments occupied by the identified proteins was carried out using the latest UniProtKB, the Gene Ontology Cell Component database, and ProteinCenter Web-based software (Thermo Scientific).[30,31] Figure 5b and Supplementary Data S1 summarize the identified proteins and their localizations. Using ERM **3** as the labeling reagent, 51 proteins were identified, among which seven proteins were assigned as ER proteins (ER ratio: 13.7%) based on the database analysis. Similarly, 19 (among 68) and 28 (among 146) ER proteins were identified by **5** and **6**, respectively. In total, 195 proteins were identified using the three ERMs, which included at least 41 ER proteins (ER ratio: 21%).[32] The relatively low ER ratio observed might have been due to the incomplete database set of ER-associated proteins. Thus, we decided to manually verify the cellular localization of the identified proteins through a literature survey (see Supplementary Data S1). Using this approach, another 43 proteins (22.1%) were reassigned as ER-associated proteins. Consequently, the total ER ratio was improved to 43.1%.[33,34] It should be noted that among the remaining 56.9% not assigned as ER proteins, 46.6% were proteins with cellular component annotations derived from the ER, that is, "membrane", "Golgi", and "extracellular space" (Figure 5b).[35] These data suggest that the latter set of proteins were labeled by the ERMs before being recruited to their final destinations. We further noticed that a considerable number of secretory proteins were included in our data set. This is very reasonable, given that the ER functions as a central hub for vesicle transport and the secretory pathways of proteins in live cells.[2] A similar trend was discussed in a recent study by Ting's group.[15] We therefore next interrogated the obtained proteome for proteins with secretory pathway annotation, which indicated that 92% of the total number of proteins identified by the ERMs were associated with secretory pathways (that is, 180 out of 195 proteins identified in total could reasonably be assigned as ER-related proteins) (Figure 5c). This value is considerably high compared with the known proportion of these proteins in the entire human proteome (51%; 9731 out of 18 962 proteins),[15] which clearly reveals that ER-related secretory proteins were efficiently enriched by our ERM-based method.

We further sought to validate the localization of several of the proteins that did not previously have ER annotation but were identified by ERMs as ER-associated proteins (that is,

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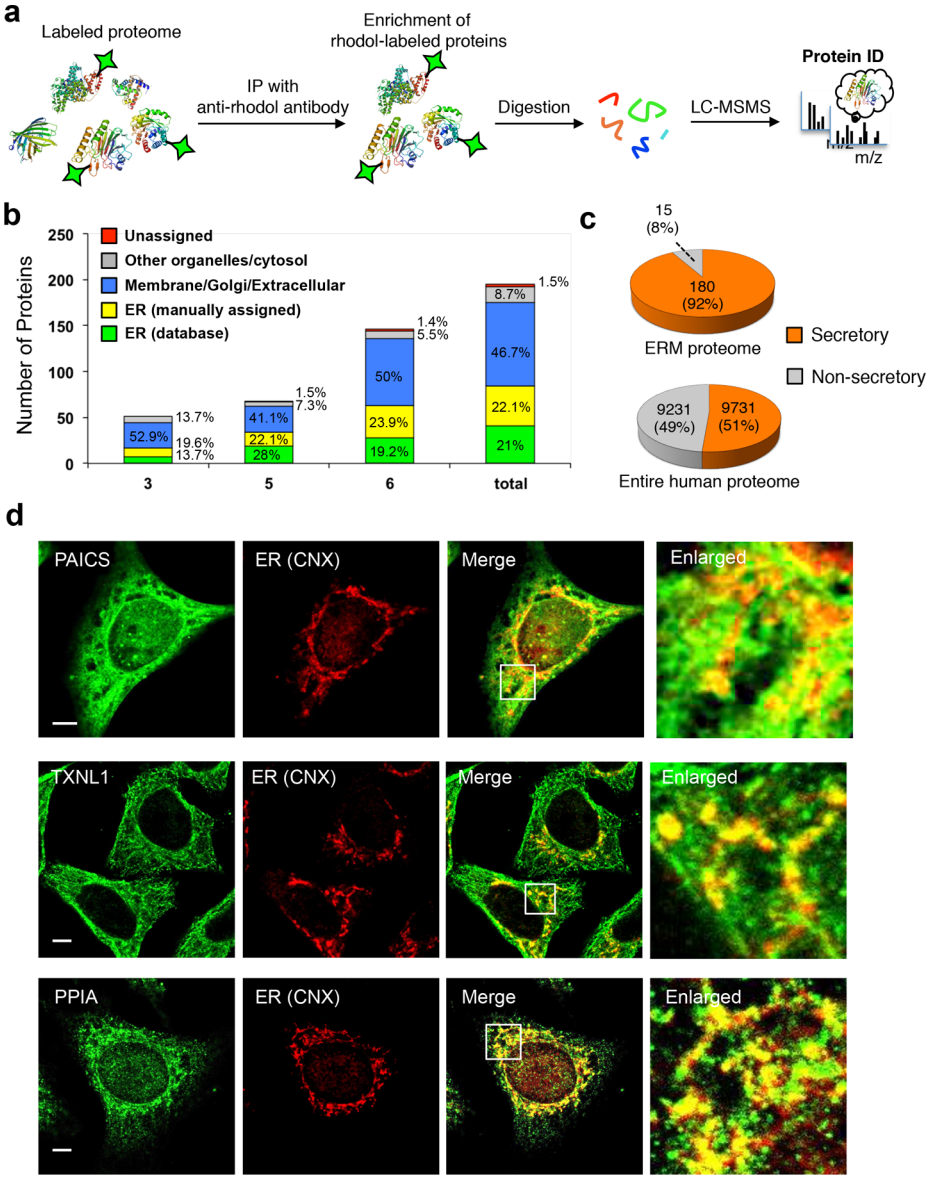

Figure 5. ERM-based identification and profiling of rhodol-labeled proteins in the ER. (a) Schematic of the approach used in this study for protein identification. The rhodol-labeled proteins were enriched by IP with anti-rhodol antibody and then identified by LC-MS/MS analysis. (b) Number of proteins identified in HeLa cells using the ERM-based approach and the ratio of proteins with ER annotations to proteins with other annotations. The identified proteins were classified as "ER", "membrane/Golgi/extracellular", "other organelles/cytosol", or "unassigned" according to the UniProtKB database, the Gene Ontology (GO) cell component annotation terms, and manual literature surveys. Detailed information on the identified proteins is presented in Supplementary Data S1. (c) Pie charts of the percentage of identified proteins with secretory pathway annotation according to the GO database or the Phobius predictive tool (ref 43) among proteins identified with our method (top) and reported for the entire human proteome (bottom). (d) Subcellular localization of the three proteins PAICS, TXNL1, and PPIA, newly assigned to the ER in this study. HeLa cells were fixed and stained with anti-PAICS, anti-TXNL1, and anti-PPIA (green) and anti-calnexin (CNX) (red) to visualize the ER. Colocalization regions are shown in yellow in the merged images. Scale bar: 5 μm.

proteins identified in this study and annotated their localization as Membrane/Golgi/Extracellular in Figure 5b). Four such proteins were identified: multifunctional protein ADE2 (PAICS), thioredoxin-like protein 1 (TXNL1), peptidyl-prolyl cis−trans isomerase A (PPIA), and D-3-phosphoglycerate dehydrogenase (PHGDH). We performed co-immunostaining of these five proteins with the respective antibody and the ER marker protein, calnexin (CNX). As shown in Figure 5d, immunofluorescence signals from PAICS, TXNL1, and PPIA were detected in the ER, suggesting that these proteins are potentially involved in ER function. PAICS is a bifunctional enzyme containing phosphoribosylaminoimidazole carboxylase activity in its N-terminal region and phosphoribosylaminoimidazole-succinocarboxamide synthase activity in its C-terminal region.[36] This enzyme is also a member of the purine biosynthesis pathway. Previous studies indicated that PAICS is associated with extracellular vesicles and the cell membrane, features consistent with an ER-associated protein.[37,38] TXNL1 shows protein-disulfide reductase activity and may be involved in protein folding in the ER.[39,40] PPIA is a chaperone protein that catalyzes cis−trans isomerization of proline imide peptide bonds and is known to be secreted in response to oxidative stress.[41,42] It is therefore reasonable for this protein to pass through the ER. PHGDH did not exhibit good immuno-

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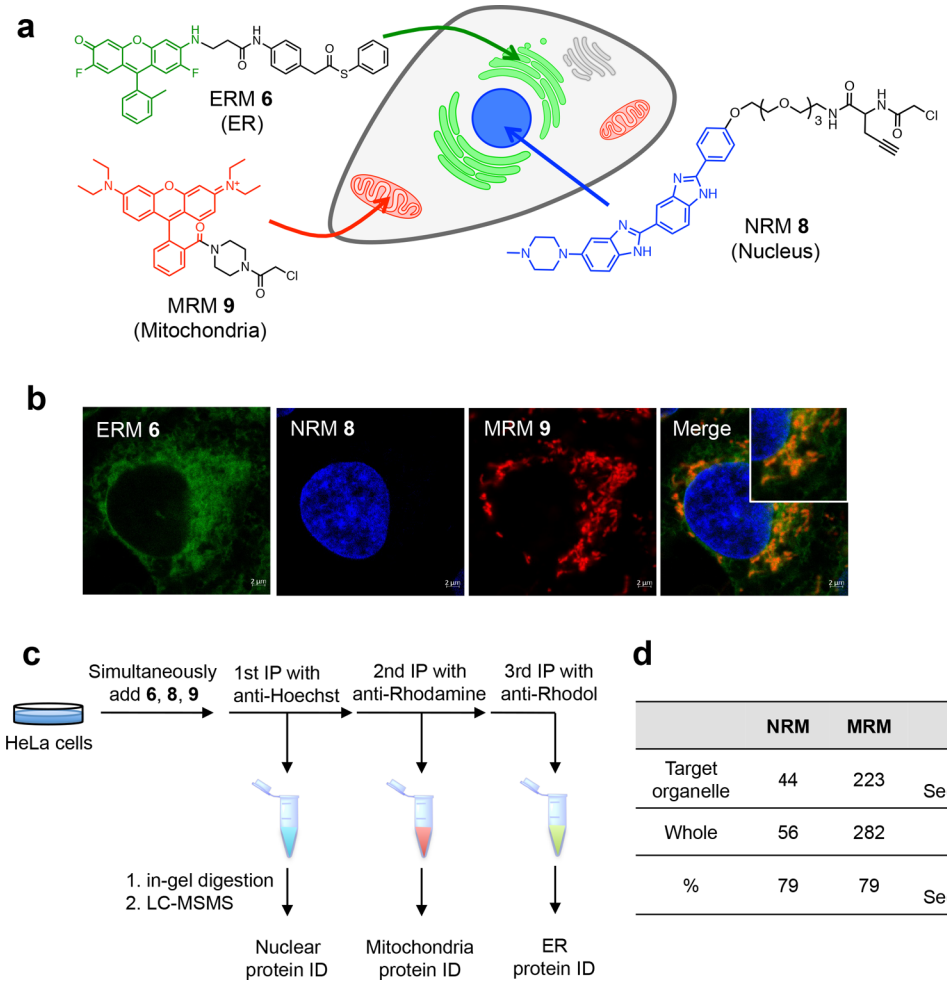

	NRM	MRM	ERM
Target organelle	44	223	ER: 15 Secretory: 32
Whole	56	282	36
%	79	79	ER: 42 Secretory: 89

Figure 6. Simultaneous use of NRM, MRM, and ERM for targeted protein enrichment in HeLa cells. (a) Molecular structures of ERM **6**, NRM **8**, and MRM **9**. The reaction properties of **8** and **9** are reported in refs 19 and 18, respectively. (b) CLSM images of a live HeLa cell concurrently treated with ERM **6** (100 nM, green), NRM **8** (5 μM, blue), and MRM **9** (0.5 μM, red). (c) Workflow of the sequential IP protocol for the enrichment of proteins labeled with each reagent. (d) The cellular locations of the identified proteins were analyzed according to the protein ontology information provided in the UniProt database and from manual literature surveys.

fluorescence overlap, presumably because it represents false positive or it is present in a low amount in the ER (Supplementary Figure S12). Overall, although further investigation regarding the localization of these proteins is required, the results obtained here highlight the usefulness of ERM-based chemical proteomics for identifying new ER-related proteins.

Simultaneous Use of Nuclear-, Mitochondria-, and ER-Localizable Reactive Molecules. We next sought to multiplex the ERMs together with other ORMs such as the Hoechst-based NRMs (nucleus-targeted) and the tetraethylrhodamine-based MRMs (mitochondria-targeted) (Figure 6a).[18,19] These probes were expected to orthogonally localize to their respective target subcellular compartments in the same biological sample and to modify their target organellar proteins with a distinct dye. After cell lysis, the labeled proteins could be enriched by IP using specific antibodies against Hoechst, tetraethylrhodamine, and rhodol and sorted into nuclear, mitochondrial, and ER-associated proteins, respectively. Thus, the chemical labeling-mediated isolation of proteins from each of these three organelles would be achieved without subcellular fractionation.

We first tested the orthogonal localization of NRM, MRM, and ERM in live cells. HeLa cells were simultaneously treated with ERM **6** (0.1 μM), NRM **8** (5 μM), and MRM **9** (0.5 μM) and subsequently analyzed with Zeiss Airyscan super-resolution microscopy. Thanks to the distinct fluorescence wavelength of each of these localization moieties, the nucleus, mitochondria, and ER were clearly and specifically stained by NRM **8**, MRM **9**, and ERM **6**, respectively, demonstrating that these three probes exclusively localize and concentrate within their target organelle even when applied together (Figure 6b and Supplementary Figure S13). We further confirmed that both NRM **8** and MRM **9** did not label proteins in the ER (Supplementary Figure S14).

Encouraged by the imaging data, we proceeded to characterize the labeled proteins by protein enrichment combined with LC-MS/MS analysis. We employed a sequential IP protocol for the selective isolation of tagged proteins (Figure 6c). In the process of optimization for the iterative IP, it was found that anti-tetraethylrhodamin and anti-rhodol antibodies cross-react with Hoechst and tetraethylrhodamine molecules, respectively (Supplementary Figure S15). Thus, to sidestep the cross-reaction, the lysates of labeled cells were successively subjected to IP with anti-Hoechst, anti-

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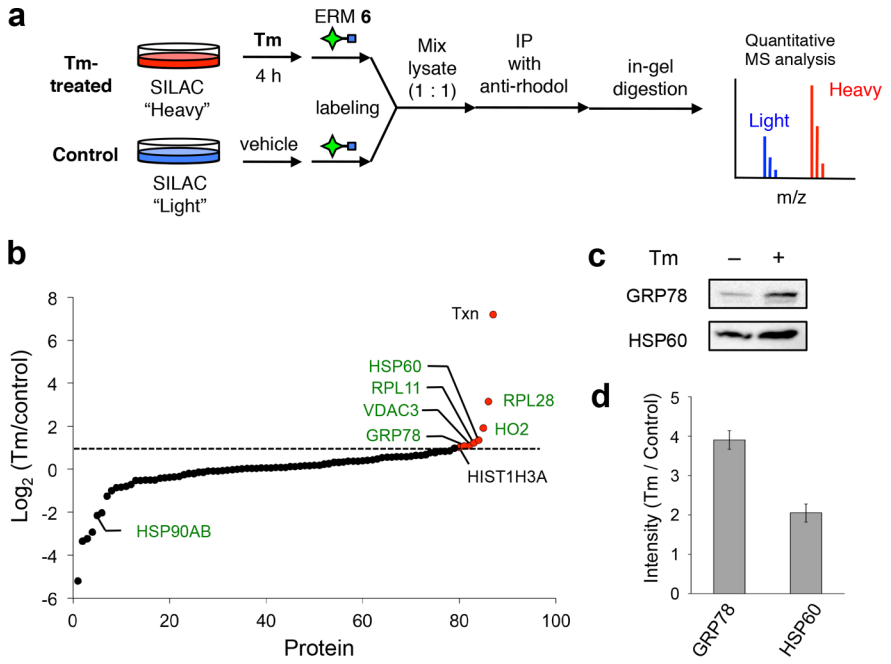

Figure 7. SILAC-based quantitative MS analysis of ERM proteome change induced by ER stress. (a) Design of SILAC experiments coupled with ERM-mediated protein labeling to quantify ER proteome changes in tunicamycin (Tm)-treated HeLa cells. Tm-treated cells were grown in heavy SILAC medium, and nontreated cells were grown in light SILAC medium (control), followed by labeling with ERM **6**. After cell lysis, the lysates from the light and heavy cells were mixed in a 1:1 ratio and the rhodol-labeled proteins were enriched by IP using an anti-rhodol antibody. The enriched proteins were subjected to in-gel tryptic digestion and quantitative LC–MS/MS analysis. (b) Log_2 ratio plot (Tm-treated cells (heavy)/control cells (light)) of the identified proteins. Proteins more than 2-fold upregulated, and the others are shown in red and black in the plot, respectively. Gene names written in green or black letters are proteins assigned as ER-associated proteins (assigned by database and literature) or non-ER proteins, respectively. (c) Western blotting analysis of Tm-induced changes in the expression levels of GRP78 and HSP60. (d) Relative intensities of the Western blotting analysis shown in (c). Data represent the mean ± SD, n = 3.

tetraethylrhodamine, and anti-rhodol in that order, followed by MS fingerprinting analysis. We identified 56 and 282 proteins within the NRM and MRM fractions, and 44 (79%) and 223 (79%) of these proteins were assigned as nuclear and mitochondrial proteins, respectively, which was consistent with results from our previous study on the separate use of this NRM and MRM (Figure 6d and Supplementary Data S2).[18,19] While the number of identified proteins in the ERM fraction was less than when it was separately used, mainly because of protein loss during the iterative IP steps, the ER ratio remained high (42%) (89% had secretory pathway annotation). These results show the potential of this approach for orthogonally tagging and identifying organellar proteins by using multiple organelle-localizable reactive molecules in a single sample.

Quantitative Profiling of ER-Proteins during Tunicamycin-Induced Unfolded Protein Response. ER stress, which is characterized by an increase in unfolded proteins in the ER, results in the activation of specific cellular responses such as the unfolded protein response (UPR) and ER-associated protein degradation (ERAD).[44,45] We applied our ERM-based proteomics approach to examine the dynamic changes in the levels of different ER-associated proteins in response to ER stress. In this experiment, the ERM-based protein tagging strategy was combined with the stable isotope labeling by amino acids in cell culture (SILAC) technology for quantitative analysis of the ER proteins during tunicamycin (Tm)-induced UPR (Figure 7a).[46−48] HeLa cells grown in heavy SILAC media were treated with Tm (2.5 μg/mL) for 4 h, followed by treatment with ERM **6** (100 nM, 1 h), whereas cells grown in light SILAC media were treated with ERM **6** only. The presence of Tm-induced UPR was verified based on the expression of CHOP, a representative UPR marker protein (Supplementary Figure S16).[46,47,49] We also confirmed that the ERM localized in the ER of the Tm-treated cells as well as the normal cells and that the intracellular concentration of the ERM did not substantially change following Tm treatment (Supplementary Figure S17). As shown in Supplementary Figure S18, the fluorescent band patterns on the SDS-PAGE gels were almost identical between the Tm-treated and nontreated cells, indicating that Tm-induced UPR did not largely alter the expression of most ER proteins in HeLa cells in our protocol. The lysates of light- and heavy-labeled cells were mixed in a 1:1 ratio, and the rhodol-tagged proteins were enriched as described above, followed by LC-MS/MS analysis. ERM **6** identified and quantified 87 proteins across three independent biological replicates (Figure 7b and Supplementary Data S3). Among these 87 proteins, 39 proteins (45%) were classified as ER proteins by our annotation analysis, while 84 proteins (97%) were assigned with secretory pathway annotation. Of the identified proteins, six ER proteins were upregulated by more than 2-fold in Tm-treated cells, while only one ER-associated protein (HSP90AB) displayed a significant decrease in the log_2 value (Figure 7b). Notably, three well-known UPR-related proteins, GRP78, HSP60, and HO2, were included in the six upregulated proteins. GRP78 is one of the major UPR marker proteins and is reported to be upregulated under UPR conditions.[50] HSP60 is classically defined as an intramitochondrial chaperone protein, but it was recently revealed that this protein can also be released via the ER/Golgi protein secretory pathway.[51] Indeed, we confirmed that HSP60 surely exists in the ER of HeLa cells and is included in the proteins pull downed by IP using anti-rhodol

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antibody (Supplementary Figure S19). According to previous reports, the expression level of HSP60 slightly increases in response to UPRs.[52,53] We verified the increased expression levels of GRP78 and HSP60 by Western blotting analysis (Figure 7c and d). Given the recent study suggesting the linkage between HO2-mediated reactive oxygen species (ROS) suppression and ER stress, upregulation of HO2 may suppress UPR-induced apoptosis.[54] These results clearly demonstrated that the present ERM-based method can be successfully applied to the sensitive analysis of dynamic ER proteome changes.

CONCLUSIONS

We developed a set of ERMs that spontaneously accumulate in the ER of live cells and selectively label ER-localized proteins with an enhanced concentration effect. ERM-based protein tagging allows the selective enrichment and quantitative profiling of ER-associated proteins without any time-consuming and technically difficult ER fractionation steps. This method needs neither genetic manipulation nor harsh labeling conditions. More importantly, the present study demonstrated that ERMs have the potential to discover unknown ER-related proteins. However, given that ER proteomes of 1190 and 1888 proteins are registered in the UniProt and Gene Ontology databases, respectively, the present ERM-based method still only covers a small fraction of ER proteins. As discussed previously,[18] this limited coverage, in other words, false negatives, may be attributed to (i) biased sub-ER distribution of the rhodol-based ERMs (in the ER-membrane) and/or (ii) failure to label proteins that do not have suitable reactive nucleophilic amino acids on the protein surface. These limitations could be overcome by varying the localization moiety to target different areas within the ER and by employing a variety of reactive groups exhibiting distinct amino acid selectivities. In this regard, the linear relationship we observed between the clogP values of the probes and their ER localization may assist the future rational design of a variety of ER-localizable reagents. We also demonstrated the feasibility of the simultaneous use of three organelle-localizable reactive molecules, which should prove a powerful tool for monitoring proteome changes across multiple organelles in the same cell culture. We believe that the extension of our chemical strategy to other subcellular organelles, such as the lysosome, Golgi apparatus, and peroxisome, will facilitate further elucidation of the complicated organellar protein networks in living cells.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.8b08606.

Supplementary figures and tables, experimental procedures, synthesis and characterization of compounds; Supplementary Data S1−S3; raw data for all LC-MSMS analyses (PDF)
Excel file (XLSX)
Excel file (XLSX)
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AUTHOR INFORMATION

Corresponding Author

*ihamachi@sbchem.kyoto-u.ac.jp

ORCID

Tomonori Tamura: 0000-0003-1648-9296
Itaru Hamachi: 0000-0002-3327-3916

Notes

The authors declare no competing financial interest.

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(33) When the ER ratio is defined by the percentage of peptides being ER proteins (annotated by database and manual assignment), it is improved to 52% (Supplementary Figure S10).
(34) Additionally, two experiments as negative controls, using the biotin-thioester compound as the labeling reagent that cannot localize in the ER or performing the IP without anti-rhodol, showed that the content of the ER proteins decreased to only 17% or 11%, respectively (Supplementary Figure S11).
(35) Given the translocation of ERMs from the ER to the Golgi during the labeling reaction (see Supplementary Figure S4), it may potentially cause undesired labeling reaction in the Golgi apparatus. However, the resultant ERM proteome contains only a limited number of Golgi-localized proteins (6 proteins out of 195 proteins identified in total), which suggests that the labeling predominantly occurs in the ER.
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