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A VCP inhibitor substrate trapping approach (VISTA) enables proteomic profiling of endogenous ERAD substrates

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**ABSTRACT** Endoplasmic reticulum (ER)–associated degradation (ERAD) mediates the proteasomal clearance of proteins from the early secretory pathway. In this process, ubiquitinated substrates are extracted from membrane-embedded dislocation complexes by the AAA ATPase VCP and targeted to the cytosolic 26S proteasome. In addition to its well-established role in the degradation of misfolded proteins, ERAD also regulates the abundance of key proteins such as enzymes involved in cholesterol synthesis. However, due to the lack of generalizable methods, our understanding of the scope of proteins targeted by ERAD remains limited. To overcome this obstacle, we developed a VCP inhibitor substrate trapping approach (VISTA) to identify endogenous ERAD substrates. VISTA exploits the small-molecule VCP inhibitor CB5083 to trap ERAD substrates in a membrane-associated, ubiquitinated form. This strategy, coupled with quantitative ubiquitin proteomics, identified previously validated (e.g., ApoB100, Insig2, and DHCR7) and novel (e.g., SCD1 and RNF5) ERAD substrates in cultured human hepatocellular carcinoma cells. Moreover, our results indicate that RNF5 autoubiquitination on multiple lysine residues targets it for ubiquitin and VCP-dependent clearance. Thus, VISTA provides a generalizable discovery method that expands the available toolbox of strategies to elucidate the ERAD substrate landscape.

**INTRODUCTION** The endoplasmic reticulum (ER) mediates the folding, modification, and deployment of one-third of the cellular proteome. Proteins that fail to fold or lack requisite oligomeric binding partners are degraded through ER-associated degradation (ERAD), a ubiquitin-dependent process that targets substrates to the 26S proteasome for proteolysis (Olzmann et al., 2013; Christianson and Ye, 2014; Ruggiano et al., 2014). A modular network of ERAD machinery coordinates substrate recognition and dislocation (also known as retrotranslocation) from the ER lumen or membrane into the cytoplasm (Carvalho et al., 2006; Christianson et al., 2011). ER-resident E3 ubiquitin ligases mediate substrate ubiquitination, which serves both as a proteosomal targeting signal and a binding interface that facilitates substrate extraction by the homohexameric AAA ATPase VCP (also known as p97) and its associated ubiquitin-binding cofactors (e.g., UFD1L and NPL4C; Olzmann et al., 2013; Ye et al., 2017). VCP-mediated ATP hydrolysis generates the necessary force for the extraction of the ubiquitinated substrate, which is partially unfolded as it is threaded through the VCP central pore (Ernst et al., 2011; Blythe et al., 2017; Bodnar and Raponi, 2017).
A canonical role of ERAD is the clearance of misfolded proteins (i.e., quality control), such as the ΔF508 mutant cystic fibrosis transmembrane conductance regulator in cystic fibrosis and the truncated null Hong Kong (NHK) variant of α-1 antitrypsin in α-1 antitrypsin deficiency (Guerrero and Brodsky, 2012). The identification of disease-associated mutant proteins as ERAD substrates has provided useful tools to study the mechanisms of ERAD (Needham and Brodsky, 2013). A less appreciated role of ERAD is in regulating the levels of endogenous proteins (i.e., quantity control; Hegde and Ploegh, 2010; Stevenson et al., 2016; Qi et al., 2017). For example, ERAD controls the flux through the cholesterol synthesis pathway by facilitating the sterol-regulated degradation of HMG CoA reductase (Song et al., 2005) and squalene monooxygenase (Gill et al., 2011; Foresti et al., 2013). ERAD quantity control has also been implicated in a wide variety of pathological conditions, such as cancer, hepatic steatosis, obesity, diabetes insipidus, and immune system function, through its ability to degrade ER-resident proteins (e.g., HMG CoA reductase, Insig1/2), secreted proteins (e.g., ApoB100, proAVP), and plasma membrane proteins (e.g., KAI1, CD147, pre-B cell receptor, SLC1A5, SLC38A2; Song et al., 2005; Lee et al., 2006; Tsai et al., 2007; Liu et al., 2012; Tyler et al., 2012; Fisher et al., 2014; Jeon et al., 2015; Ji et al., 2016; Shi et al., 2017; To et al., 2017). Thus, by influencing the abundance of ER-resident proteins and secreted proteins, ERAD impacts both cell autonomous and noncell autonomous processes.

Despite the importance of ERAD in protein quantity control, our understanding of the endogenous substrates targeted by ERAD remains limited. This surprising dearth of knowledge is in part due to the lack of generalizable methods to identify endogenous ERAD substrates in human cells. Here, we describe a quantitative proteomics strategy termed VCP inhibitor substrate trapping approach (VISTA) to identify endogenous ERAD substrates.

RESULTS AND DISCUSSION

VCP inhibition traps ubiquitinated NHK in complex with the Hrd1 E3 ubiquitin ligase

A principal function of VCP is to extract ubiquitinated ERAD substrates from the ER into the cytosol for proteasomal degradation (Bagola et al., 2011; Ye et al., 2017). CB5083 is a small-molecule VCP inhibitor that impairs the degradation of several integral membrane ERAD substrates, including CD147 (To et al., 2017), c18orf52 (Bersuker et al., 2018), and overexpressed TCRα-GFP (Anderson et al., 2015). In addition, incubation with CB5083 results in the accumulation of ubiquitinated proteins (Anderson et al., 2015; Gendron et al., 2016). We reasoned that acute pharmacological inhibition of VCP with CB5083 could be exploited to stabilize or “trap” ERAD substrates in a ubiquitinated, membrane-bound form (Figure 1A).

As a first test of the utility of CB5083 to trap ERAD substrates, we examined the impact of CB5083 treatment on the well-characterized luminal ERAD substrate NHK. In a translation shut-off assay, the half-life HA-tagged NHK (NHK-HA) was greatly extended in the presence of CB5083 (Figure 1, B and C), demonstrating that incubation with CB5083 impairs the degradation of a luminal ERAD substrate. Dislocation and ubiquitination of NHK are mediated by the Hrd1 E3 ubiquitin-conjugated S-protein (S-prot) agarose, and SDS lysates analyzed by immunoblotting; Asterisk indicates a USP2cc-reactive band. AP, affinity purification; IP, immunoprecipitation; S-prot, S-protein; Ub, ubiquitinated; endo, endogenous; Em., emetine.

FIGURE 1: VCP inhibition traps the ERAD substrate NHK in complex with the E3 ligase Hrd1.

A) Schematic of VISTA. Pharmacological inhibition of VCP with CB5083 prevents the dislocation of ubiquitinated ERAD substrates. (B) HEK293 cells expressing NHK-HA were incubated with 75 µM emetine and either vehicle or 5 µM CB5083 for the indicated time points. SDS lysates were analyzed by immunoblotting for the indicated targets. (C) The relative levels of NHK-HA (panel B) were quantified and presented as a percentage of the levels at time 0 h ± SEM (n = 3). (D) HEK293 cells stably expressing Hrd1-S were transiently transfected with NHK-HA, treated with vehicle or 5 µM CB5083, subject to affinity purification with S-protein (S-prot) agarose, and SDS lysates analyzed by immunoblotting; Asterisk indicates a USP2cc-reactive band. AP, affinity purification; IP, immunoprecipitation; S-prot, S-protein; Ub, ubiquitinated; endo, endogenous; Em., emetine.

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catalytic core of the deubiquitinating enzyme USP2 (USP2cc; Figure 1D), indicating that this fraction of NHK-HA in complex with Hrd1-S is ubiquitinated. Furthermore, immunoprecipitations of NHK-HA indicate that CB5083 increases the association of NHK-HA with components of the Hrd1 complex, including Hrd1, SEL1L, OS-9, and XTP3-B (Figure 1E). These proof-of-principle experiments demonstrate that CB5083 treatment traps a known ERAD substrate in a ubiquitinated form, in complex with its membrane-embedded degradation apparatus. We also observed that CB5083 treatment resulted in the accumulation of the core glycosylated form of integral membrane protein CD147 (CD147(CG)), an endogenous ERAD substrate (Tyler et al., 2012; To et al., 2017), but it caused only a small increase in the association of CD147(CG) with Hrd1-S (Supplemental Figure S1). This may reflect differences in the mode of interaction between Hrd1 and its luminal and integral membrane substrates. For example, under periods of VCP impairment, an integral membrane substrate may be preferentially released into the surrounding membrane to prevent prolonged occupancy of the Hrd1 ubiquitination complex.

**Global analysis of trapped, ubiquitinated proteins identifies endogenous ERAD substrates in HepG2 liver cells**

To identify endogenous ERAD substrates, VCP inhibition was coupled with quantitative ubiquitin proteomics in a method we refer to as ERAD-VISTA (Figure 2A). Cells were labeled by stable isotope labeling with amino acids in cell culture (SILAC) and treated with vehicle (light) or CB5083 (heavy). Beads conjugated with antibodies that recognize peptides bearing diglycine (diGly)-modified lysine residues (i.e., a tryptic ubiquitin remnant; Kim et al., 2011; Gendron et al., 2016) were used to affinity purify ubiquitin-modified peptides from membrane fractions for proteomic analysis. Consistent with the trapping of ubiquitinated proteins, CB5083 treatment resulted in greater levels of polyubiquitinated proteins in cell lysate and the ER-enriched membrane fraction (Figure 2B). Proteomic analysis of diGly-modified peptides purified from membrane fractions identified a total of 5573 diGly-modified peptides across four independent experiments, corresponding to 478 proteins (Supplemental Table S1). There was some variability in the number of unique diGly peptides identified (Figure 2C and Supplemental Table S1), which may be due to different batches of diGly beads. Experiments 3 and 4 were performed with the same batch of beads and were very similar with respect to the number of unique diGly peptides identified in each experiment (Figure 2C and Supplemental Table S1). diGly-modified peptides with SILAC ratios greater than 2.0 (123 proteins), indicating an accumulation of the ubiquitinated peptide during VCP inhibition, were considered candidate ERAD substrates (Supplemental Figure S2 and Supplemental Table S1). A single diGly modification was identified for the majority of proteins (68.2% for all proteins, 59.2% for proteins with SILAC ratio > 2), but a fraction of the proteins were also observed that contained two diGly modifications (18.8% for all proteins, 20.8% for proteins with SILAC ratio > 2) or three diGly modifications (9.6% for all proteins, 12.5% for proteins with SILAC ratio > 2; Figure 2D). As expected, a large number of ubiquitin diGly peptides were identified, most of which corresponded to K48 and K63 diGly-modified peptides (Figure 2E). In all four experiments, K11, K33, and K48 diGly peptides showed a strong increase, consistent with a role in protein degradation (Figure 2F). In contrast, the amount of K63 diGly peptide was mostly unchanged and the amount of K27 diGly peptide showed a decrease (Figure 2F).

Among the list of candidate substrates (Supplemental Table S1), three bona fide endogenous ERAD substrates were detected: Apolipoprotein B100 (ApoB100; Ginsberg and Fisher, 2009; Stevenson et al., 2016), 7-dehydrocholesterol reductase (DHCR7; Prabhu et al., 2016), and insulin-induced gene 2 (Insig2; Liu et al., 2012). Following CB5083 treatment, increases in the levels of two diGly peptides in ApoB100 (K196, 13.805-fold increase; K2697, 9.249-fold increase), a cluster of three diGly peptides in DHCR7 (K4, 3.602-fold increase; K11, 3.497-fold increase; K13, 3.861-fold increase), and one diGly peptide in Insig2 (K221, 7.27-fold increase) were detected (Figure 2, G–L), suggesting that modification of these lysines by ERAD E3 ligases targets these substrates to the proteasome. Other reported endogenous ERAD substrates may not have been identified due to their low abundance in HepG2 cells or their regulated degradation under specific conditions, such as IP3 receptor degradation following ligand binding (Wojcikiewicz et al., 2009) or HMG CoA reductase degradation following sterol accumulation in ER membranes (Jo and Debose-Boyd, 2010). Although it is unlikely to be a comprehensive list, these data demonstrate the ability of ERAD-VISTA to detect known and candidate endogenous ERAD substrates.

Gene ontology (GO) enrichment analysis of candidate ERAD substrates revealed an expected enrichment in proteins that are known to localize to cell membranes (e.g., ER and plasma membrane) as well as complexes associated with various components of the ERAD network (Figure 3A and Supplemental Table S2). Indeed, analysis of the annotated localizations revealed that 59.2% of the candidate substrates were predicted to be present in, or transit through, the secretory pathway (Supplemental Table S3). A smaller portion of the candidate substrates are annotated as mitochondrial (8.3%), lysosomal (0.8%), and vesicular (1.7%; Supplemental Table S3), indicating a high degree of enrichment in ubiquitinated secretory proteins. We observed an enrichment in proteins involved amino acid transport, protein catabolism, protein folding, and cholesterol and fatty acid biosynthesis (Figure 3B and Supplemental Table S2). This functional diversity reflects the wide array of potential ERAD substrates transiting the early secretory pathway and is consistent with a broad cellular role for ERAD through its regulation of a multitude of targets.

**Degradation of endogenous SCD1 and RNF5 requires VCP, ubiquitin conjugation, and the proteasome**

We next sought to validate select putative ERAD substrates from our candidate list. Two candidate substrates were selected for further analysis. Stearoyl-CoA desaturase (SCD1) is an ER-localized enzyme that catalyzes the production of monounsaturated fatty acids from saturated fatty acids (Ntambi and Miyazaki, 2003). Levels of a diGly-modified lysine in the cytosolic C-terminus of SCD1 increased in response to CB5083 (K341, 10.621-fold increase; Figure 4, A and B). Overexpressed SCD1 in CHO-K1 and HeLa cells as well as endogenous SCD1 in NIH3T3-L1 cells are degraded by the proteasome (Mziat et al., 2000; Kato et al., 2006). However, whether VCP is required for SCD1 degradation and whether endogenous SCD1 is degraded in HepG2 cells remains unknown. Consistent with SCD1 being a direct substrate of VCP, CB5083 treatment increased the amount of VCP associated with S-tagged SCD1 (SCD1-S; Figure 4C). Moreover, the degradation of SCD1 was impaired by CB5083, the proteasome inhibitor MG132, and an inhibitor of the E1 ubiquitin-activating enzyme MLN7243 (Figure 4, D and E). We observed an anti-SCD1 immunoreactive, lower-molecular-weight band that was partially degraded in control cells and exhibited a modest accumulation in the presence of the inhibitors (Figure 4D). This band was depleted by multiple small interfering RNAs (siRNAs) targeting SCD1 (Supplemental Figure S3), confirming that it is a fragment of SCD1, but its functional significance is
unclear at this time. Our data suggest that SCD1 is constitutively degraded by a VCP-dependent ERAD pathway in HepG2 cells. This is similar to the SCD1 yeast orthologue OLE1, which undergoes degradation through an ERAD pathway that requires the VCP orthologue CDC48 (Braun et al., 2002). Future experiments will explore if SCD1 degradation is regulated by the metabolic state of the cell, such as fluctuations in the ratio of unsaturated to saturated fatty acids.
RNF5 ubiquitination activity is required for its degradation. Affinity purification of S-RNF5(4K-R) revealed a ladder of RNF5 bands, with three to four particularly prominent bands that were separated by ∼8 kDa (i.e., the size of ubiquitin) and that increased following CB5083 treatment (Figure 5, C and D). These bands were greatly reduced by incubation with USP2cc, indicating that these represent ubiquitinylated forms of RNF5 (Figure 5, C and D). The ubiquitinated RNF5 species were mostly absent in the C42A mutant RNF5 (Figure 5, C and D). We considered that the small amount of ubiquitinated S-RNF5(C42A) may be due to ubiquitination S-RNF5(C42A) by endogenous RNF5. Indeed, FLAG-HA-RNF5 coprecipitated with S-RNF5, indicating the presence of RNF5 homo-oligomers (Supplemental Figure S4B). To examine the possibility of trans-molecular RNF5 autoubiquitination, we expressed S-RNF5(C42A) in RNF5 knockout (KO) cells generated using CRISPR/Cas9 (Supplemental Figure S5). Similar to the control cells, S-RNF5(C42A) still exhibited a small amount of laddering in the RNF5 KO cells, indicating that the endogenous RNF5 does not contribute to the ubiquitination of S-RNF5(C42A) (Supplemental Figure S5B). Our data are in very good agreement with previous in vitro studies (Matsuda et al., 2001) and suggest RNF5 autoubiquitination is a cis-molecular reaction. The residual ubiquitination of S-RNF5(C42A) must be mediated by an unknown E3 ligase.

Our proteomics results indicate that all four lysines in RNF5 are ubiquitinated and are sensitive to VCP inhibition (Figure 4, F and G, and Supplemental Table S1). To explore the contribution of these lysines to RNF5 degradation we generated constructs harboring lysine-to-arginine substitutions. Although there was a small decrease in the ubiquitination of S-RNF5(K75R) and S-RNF(K86R), all S-RNF5 single lysine mutants were still ubiquitinated (Figure 5E). Therefore, we generated an S-RNF5 construct in which all four lysines were substituted with arginine (4K-R). The 4K-R mutant exhibited a dramatic reduction in ubiquitination (Figure 5, F and G). A very small amount of ubiquitinated S-RNF5(4K-R) was visible, suggesting that RNF5 may either ubiquitinate noncanonical residues (e.g., serine) or one of the lysines in the S-tag. Although the S-tag contains two lysine residues, the ubiquitination of RNF5 was nearly abolished in the 4K-R mutant. This may indicate a structural preference for the
FIGURE 4: Endogenous SCD1 and RNF5 are degraded via a VCP- and ubiquitin-dependent proteasomal pathway.
(A) Diagram of SCD1 protein structure, with detected diGly-modified lysine residue indicated. (B) Log2 SILAC peptide ratios for individual diGly-modified peptides from SCD1. (C) HepG2 cells expressing SCD1-S were treated with vehicle or 5 µM CB5083, subject to affinity purification with S-protein (S-prot) agarose, and SDS lysates analyzed by immunoblotting; n = 3. (D, E) HepG2 cells were treated with 75 µM emetine and 5 µM CB5083, 10 µM MG132, or 10 µM MLN7243 to disrupt various components of ERAD. SCD1 protein stability was assessed via immunoblotting (D) and relative levels quantified (E). Graphical data are expressed as mean ± SEM (n = 3–6 per group). (F) Diagram of RNF5 protein structure, with detected diGly-modified lysine residues indicated. (G) Log2 SILAC peptide ratios for individual diGly-modified peptides from RNF5. (H) HepG2 cells expressing S-RNF5 were treated with vehicle or 5 µM CB5083, subject to affinity purification with S-protein (agarose, and SDS lysates analyzed by immunoblotting) (n = 3). (I, J) HepG2 cells were treated with 75 µM emetine and 5 µM CB5083, 10 µM MG132, or 10 µM MLN7243 to disrupt various components of ERAD. RNF5 protein stability was assessed via immunoblotting (I) and relative levels quantified (J). Graphical data are expressed as mean ± SEM (n = 3–6 per group). TM: transmembrane domain. S-prot, S-protein; Exp., experiment; Em., emetine. Asterisk indicates that the diGly peptide was not detected in that experiment. See also Supplemental Table S1.
function (e.g., fusions of E3 ligases to tandem ubiquitin-binding domains [Mark et al., 2014, 2016] or to ubiquitin [O'Connor et al., 2015]). 4) The method measures changes in substrate ubiquitination rather than steady-state protein levels (e.g., steady-state SILAC [Foresti et al., 2014] or GFP-based global protein profiling [Yen and Elledge, 2008; Yen et al., 2008]), thereby facilitating substrate identification even when only a small fraction is ubiquitinated and degraded. A limitation of ERAD-VISTA is that it relies on diGly ubiquitin proteomics which may impact reproducibility due to stochastic sampling, especially for low-abundance targets (Ordureau et al., 2015). Thus, achieving comprehensive assessments of the ERAD substrate landscape is a challenge. However, depth and coverage may be improved by employing recent improvements in diGly methodologies involving the fractionation of peptides using strong cation exchange chromatography before immunoblotting (Na et al., 2012; Udeshi et al., 2012). It is also important to note that because the diGly approach is specific to diGly-modified lysines, it will not identify ubiquitination on nonlysine residues such as serine (Shimizu et al., 2010).

An additional limitation of ERAD-VISTA is that some CBS083-sensitive ubiquitination events might not target the modified protein for degradation and may instead reflect regulatory ubiquitination, such as the ubiquitin-dependent regulation of protein cluster of 4-lysine residues over the lysines in the N-terminal S-tag. It is notable that S-RNF5(4K-R), despite being no longer ubiquitinated, still coprecipitated ubiquitinated proteins in the presence of CBS083 (Figure 5F). This was in contrast to the inactive S-RNF5(C42A), which did not coprecipitate ubiquitinated proteins (Figure 5C). These results suggest that the S-RNF5(4K-R) mutant uncouples RNF5 catalytic activity and autoubiquitination. This uncoupling mutant could be useful for exploring the functional importance of RNF5 degradation.

In summary, we have developed a new global approach for the identification of endogenous ERAD substrates. This approach identified known (ApoB100, DHC7, and Insig2) and novel (SCD1 and RNF5) substrates. ERAD-VISTA has several important benefits over previous strategies to study ubiquitinated proteins: 1) The method uses endogenous ubiquitin and does not require overexpression of tagged ubiquitin (e.g., his-ubiquitin [Hitchcock et al., 2003; Peng et al., 2003; Kirkpatrick et al., 2005]). 2) The method does not require in-depth knowledge and/or genetic manipulation of the degradation pathway (e.g., proteomic analyses of tagged E3 ligase complexes [Gao et al., 2011; Tan et al., 2011, 2013; Harper and Tan, 2012] or tagged substrate delivery factors [Tyler et al., 2012]). 3) The method does not require expression of chimeric proteins that could affect the function (e.g., fusions of E3 ligases to tandem ubiquitin-binding domains [Mark et al., 2014, 2016] or to ubiquitin [O'Connor et al., 2015]). 4) The method measures changes in substrate ubiquitination rather than steady-state protein levels (e.g., steady-state SILAC [Foresti et al., 2014] or GFP-based global protein profiling [Yen and Elledge, 2008; Yen et al., 2008]), thereby facilitating substrate identification even when only a small fraction is ubiquitinated and degraded. A limitation of ERAD-VISTA is that it relies on diGly ubiquitin proteomics which may impact reproducibility due to stochastic sampling, especially for low-abundance targets (Ordureau et al., 2015). Thus, achieving comprehensive assessments of the ERAD substrate landscape is a challenge. However, depth and coverage may be improved by employing recent improvements in diGly methodologies involving the fractionation of peptides using strong cation exchange chromatography before immunoblotting (Na et al., 2012; Udeshi et al., 2012). It is also important to note that because the diGly approach is specific to diGly-modified lysines, it will not identify ubiquitination on nonlysine residues such as serine (Shimizu et al., 2010). An additional limitation of ERAD-VISTA is that some CBS083-sensitive ubiquitination events might not target the modified protein for degradation and may instead reflect regulatory ubiquitination, such as the ubiquitin-dependent regulation of protein...
complexes (Hoppe et al., 2000; Rape et al., 2001; Ramanathan and Ye, 2012). Thus, the candidate ERAD substrate must be validated with traditional approaches. ERAD-VISTA expands the available toolbox of strategies for probing the ERAD substrate landscape in different cell types and under different conditions (e.g., ER stress).

MATERIALS AND METHODS

Plasmids, antibodies, and reagents
The NHK-HA and Hrd1-S plasmids were previously described (To et al., 2017). The S-RNFS plasmid in a pcDNA3.1(+) backbone was a kind gift from Ron Kopito (Stanford University), and the FLAG-HA-RNFS plasmid in a pcDNA5/FRT/TO backbone was a kind gift from John Christianson (Ludwig Institute for Cancer Research, University of Oxford). S-RNFS lysine-to-arginine and cysteine-to-alanine substitutions were generated by site-directed mutagenesis and confirmed by sequencing. To generate the SCD1-S expression plasmid, SCD1 was PCR amplified from pANT7_cGST-SCD1 (DNASU Plasmid Repository, HsCD00631016) and ligated into a pcDNA3.1(+) vector bearing an in frame C-terminal S-tag.

The primary antibodies used for immunoblotting include anti-S peptide (EMD Millipore), anti-HA (Sigma-Aldrich), anti-ubiquitin (FX2; EMD Millipore), anti-tubulin (Abcam), anti-Hrd1 (Bethyl Laboratories), anti-SEL1L (Santa Cruz), anti-CDD47 (Santa Cruz), anti-VCP (Novus Biologicals), anti-calnexin (Proteintech Group), anti-UBXDB8 (Proteintech Group), anti-AUP1 (Proteintech Group), anti-GAPDH (EMD Millipore), anti-SCD1 (Cell Signaling Technology), and anti-RNFS (Abcam). Anti-OS-9 and anti-XTP3-B were kind gifts from Ron Kopito (Stanford University). Secondary antibodies used were Alexa Fluor 680 goat anti-mouse (Life Technologies) and IRDye 800 goat anti-rabbit (LI-COR Biosciences).

Chemical reagents used include emetine (Sigma-Aldrich), CB5083 (Cleave Biosciences and Cayman Chemical), MLN7243 (Chemietek), MG132 (Selleck Chemicals), and Bortezomib (Cell Signaling Technologies).

Cell culture, transfections, and stable cell line generation
HepG2 cells (American Type Culture Collection) were cultured in Roswell Park Memorial Institute 1640 (RPMI; Thermo Fisher Scientific) or DMEM containing 4.5 g/l glucose and l-glutamine (Corning) supplemented with 10% fetal bovine serum (FBS; Thermo Fisher Scientific and Gemini Bio Products) at 37°C and 5% CO₂. Stable HEK293 cells expressing S-tagged Hrd1 (To et al., 2017) were grown in DMEM supplemented with 10% FBS at 37°C and 5% CO₂.

Cells at 60–80% confluence were transfected with the indicated plasmids using XtremeGENE HP DNA transfection reagent (Sigma-Aldrich) following the manufacturer’s protocols. Depletion of SCD1 in HepG2 cells was accomplished by transfection of SCD1-targeting siRNAs from Sigma-Aldrich using RNAiMAX Lipofectamine reagent (Thermo Fisher Scientific). siRNA sequences targeting SCD1 include 5′-GUAUAGCUGGUGGCUUAA-3′ (siRNA1), 5′-GUAUAGCUGUUUAUGCAAA-3′ (siRNA2), 5′-GACAUAGUUCUCUGGCUU-3′ (siRNA3), and 5′-UGAUGUUGCGCGGACAU-3′ (siRNA4). MISSION siRNA Universal Negative Control #1 (SIC001) was used as the control siRNA.

Differential fractionation
Cultured cells were collected, washed with ice-cold phosphate-buffered saline (PBS), and incubated in hypotonic lysis medium (50 mM Tris-HCl, pH 7.4, 1 mM EDTA) supplemented with 10 mM N-ethylmaleimide (NEM; Thermo Fisher Scientific) on ice for 10 min. Cells were then transferred to a 7-ml chilled glass dounce homogenizer and diced using a tight pestle for 40 strokes. Samples were centrifuged (500 × g, 5 min, two times) to remove unbroken cells. The remaining supernatant was then centrifuged (20,000 × g, 30 min at 4°C) to separate heavy membrane and cytosolic fractions. The resulting pellet (membrane) was then reconstituted to its corresponding cytosolic fraction volume using either HLM buffer (for immunoblotting) or 8M urea lysis buffer (for diGly enrichment, details below). For immunoblotting, SDS was then added to achieve a final detergent concentration of 1% and equal volumes were analyzed.

Affinity purification
Cells were collected and washed twice using ice-cold PBS. Cells were resuspended in immunoprecipitation (IP) lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% digitonin [EMD Millipore]) containing protease inhibitor tablets (Thermo Fisher Scientific) and gently rotated for 30 min at 4°C. Lysates were centrifuged (20,000 × g, 10 min) and soluble protein (supernatant) transferred to new tubes. Supernatant protein concentration was then determined using the BCA assay (Thermo Fisher Scientific) according to the manufacturer’s instructions.

S-protein agarose bead slurry (25 µl bead bed/µg lystate; EMD Millipore) was washed two times with IP lysis buffer followed by one time with IP buffer containing 1% digitonin. Beads were then mixed with equivalent amounts of supernatant (2 h rotating, 4°C), washed three times with IP lysis buffer containing 0.1% digitonin, and proteins eluted with Laemml buffer for immunoblotting. Where indicated, affinity-purified proteins were treated with 1 µg of USP2cc (Boston Biochem) for 1 h at 37°C before elution from the beads.

Immunoblotting
Cells were washed with PBS and lysed in 1% SDS. Protein quantity was determined using a bicinchoninic acid assay (Thermo Fisher Scientific). Normalized cell lysates in Laemmli buffer and the appropriate amino acids: light media—arginine (Arg0) and lysine (Lys0; Sigma-Aldrich) or heavy media—arginine (Arg0) and ¹³C⁶¹⁵N²-l-lysine (lys8; Cambridge Isotope Laboratories). Samples were then processed for diGly immunopurification (Kim et al., 2011). Following a 6 h treatment with DMSO (light) or 5 µM CB5083 (heavy), membrane fractions were collected, solubilized in urea lysis buffer (8 M urea, 50 mM Tris-HCl, pH 8.0, 50 mM NaCl), reduced with 10 mM dithiothreitol (Thermo Fisher Scientific), and alkylated with 25 mM iodoacetamide (Thermo Fisher Scientific). Equal amounts of protein total (5–10 mg) from the membrane fractions were combined, diluted with 50 mM Tris-HCl, pH 8.0, 4 M urea, and digested overnight with 2 µg/ml LysC (Wako Laboratory Chemicals). Proteins were further diluted to 1.6 M urea and digested for 24 h with 10 µg/ml mass spectrometry grade trypsin (Thermo Fisher Scientific).
Digested peptides were desalted via Sep-Pak C18 6-c cartridge (Waters) and lyophilized. Samples were then immunoprecipitated using a PTMScan Ubiquitin remnant Motif (K-ε-GG) Kit (Cell Signaling Technologies) according to the manufacturer’s protocols. Briefly, lyophilized peptides were dissolved in IAP buffer (50 mM MOPS/NaOH, pH 7.2, 10 mM Na2HPO4, and 50 mM NaCl) and cleared by centrifugation at 10,000 x g for 5 min. For each independent experiment, one tube of K-ε-GG antibody bead conjugates were washed four times with PBS, and clarified peptides were incubated with the beads for at least 2 h with gentle agitation. Beads were washed two times with IAP buffer and three times with MilliQ water and eluted twice with 0.1% trifluoroacetic acid. Eluted peptides were desalted using C18 StageTips (Thermo Fisher Scientific), dried using a Speedvac, and resuspended in 0.1% formic acid (Sigma-Aldrich) for analysis by tandem mass spectrometry (LC-MS/MS).

LC-MS/MS analysis
Digested peptides were analyzed by LC-MS/MS on a Q Exactive Orbitrap mass spectrometer (Thermo Fisher Scientific) in conjunction with Proxeon Easy-nLC II HPLC (Thermo Fisher Scientific) and a Proxeon nanospray source at the UC Davis Proteomics Core Facility as described (To et al., 2017). The resulting MS/MS raw spectral data were analyzed using the MaxQuant software platform (version 1.5.1.0; Cox and Mann, 2008), employing the full UniProt human protein sequence database to obtain dGly-modified peptide SILAC ratios. A reversed-protein decoy search strategy was also employed to minimize false discovery rate. All mass spectrometry files are listed in Supplemental Table S2 were based on UNIPROT annotations.

Bioinformatic analyses
GO analysis of candidate ERAD substrates was performed using the Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.8 (Supplemental Table S3; Huang et al., 2009). REVIKO (Supek et al., 2011) was then used to simplify and visualize the GO terms and the Benjamini corrected p values. GO networks were exported from REVIKO and the final networks generated using Cytoscape (Shannon et al., 2003). Protein localization and topology listed in Supplemental Table S2 were based on UNIPROT annotations.

Generation of RNF5 knockout cells
RNF5 knockout lines were generated using the targeting sequence 5′-CGCTCGCAGATTGGCCCTTC-3′ cloned into PX459 (Addgene; plasmid #48139) transfected into HEK293 cells. PX459 without a targeting sequence was transfected as a control. Transfected cells were selected with 1 μg/ml puromycin (Sigma-Aldrich) for at least 1 wk. Clonal cell lines were isolated by limited dilution and screened for knockout by immunoblotting.

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