Understanding the Role of Lipid Modifications in ER Protein Quality Control

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Abstract

Endoplasmic reticulum (ER)-associated degradation (ERAD) is a cellular process responsible for the identification and degradation of misfolded proteins. Defining the mechanisms underlying this process is vital to understanding the pathogenesis of numerous human diseases that result from impaired ER protein quality control. Recent reports indicate that the inhibition of acyl-CoA synthetases with small molecule, triacsin c disrupts ERAD. However, it remains unknown *why* acyl-CoA synthetases are required for ERAD. Palmitoylation, the covalent addition of the fatty acid palmitate to a protein, requires acyl-CoA synthetase activity and can significantly impact protein localization, structure, and physical interactions. Therefore, we hypothesized that palmitoylation of ERAD machinery regulates the identification and degradation of ERAD substrates. To test this hypothesis, we employed copper-catalyzed azide-alkyne cycloaddition to probe for palmitoylated ERAD proteins. Our results reveal palmitoylation of four prominent ERAD proteins: the E2-recruitment factor AUP1, the E3 ligase Hrd1, the rhomboid pseudoprotease Derlin1, and the mannosidase ERMan1, indicating that palmitoylation may regulate ERAD at multiple steps. Consistent with a functional role for palmitoylation in ERAD, we find that treatment the palmitoylation inhibitor 2-bromopalmitate significantly attenuated ERAD. Together, our results identify an unprecedented mechanism of ERAD regulation that will broadly impact our understanding of ERAD-associated diseases.

Introduction

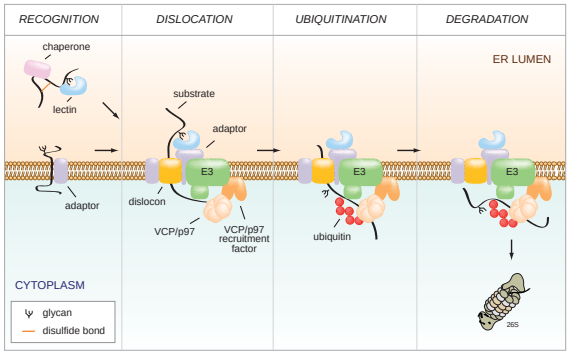


Figure 1. Representation of Endoplasmic Reticulum-Associated Degradation (ERAD). The four major steps of ERAD are: recognition of misfolded proteins' trimmed glycans by ER resident sugar-binding lectins and chaperones; substrate dislocation across the ER lipid bilayer presumably through a proteinaceous pore; polyubiquitination by E3 ubiquitin ligases; and degradation by the 26S proteasome in the cytoplasm.

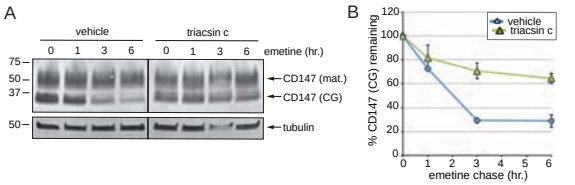


Figure 2. The long chain acyl-CoA synthetase inhibitor triacsin c impairs CD147 degradation. A) CD147 degradation kinetics were analyzed by immunoblotting following a 16-hr triacsin c pretreatment **B)** CD147 levels in panel (A) were quantified using ImageJ and plotted.

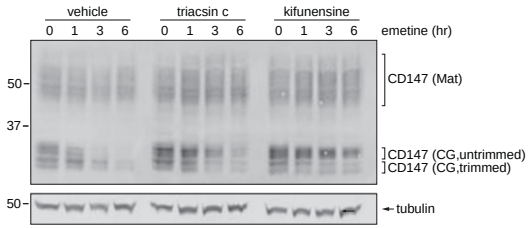


Figure 3. Triacsin c impairs glycan trimming. Immunoblot analysis of CD147 degradation in cells pretreated for 16 hr. with triacsin c or cotreated with the mannosidase inhibitor kifunensine.

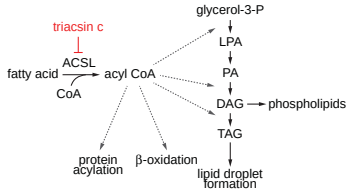


Figure 4. Schematic of the pathways affected by triacsin c. Triacsin c inhibits long chain acyl-CoA synthetases (ACSLs), which are required for the activation of fatty acids that are employed for protein acylation, β-oxidation, and lipid biosynthesis.

Methods and Results

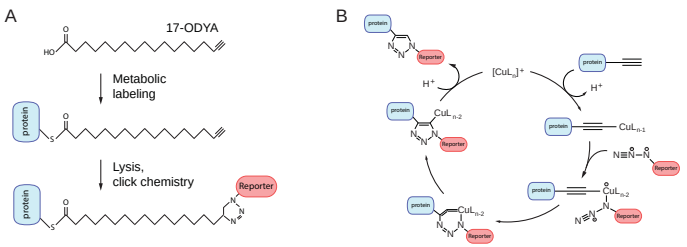


Figure 5. Schematic of the click chemistry method used to detect palmitoylation. A) Cells were pretreated for 8 hr with 17-octadecynoic acid (17-ODYA), an alkyne-containing palmitate analog.**B)** Affinity purified S-tagged proteins were reacted with the fluorescent azide-rhodamine reporter via copper-catalyzed azide-alkyne cycloaddition.

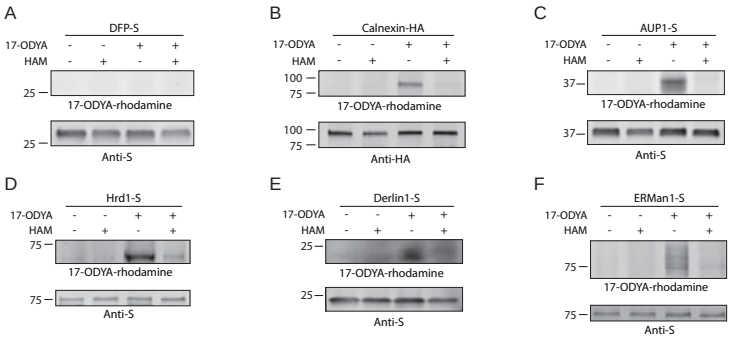


Figure 6. Identification of palmitoylated ERAD proteins. A-F) Affinity purified proteins from cells incubated in the presence or absence of 17-ODYA were reacted with a rhodamine moiety to enable detection of palmitoylation. Complexes were also incubated with hydroxylamine (HAM) to remove the thioester-linked palmitate group and confirm specificity of the signal. Proteins were separated by SDS-PAGE and gels scanned for fluorescence to detect the reporter or analyzed by Western blotting to detect the affinity purified protein. DFP is a non-fluorescent GFP variant used as a negative control, and calnexin is used as a positive control.

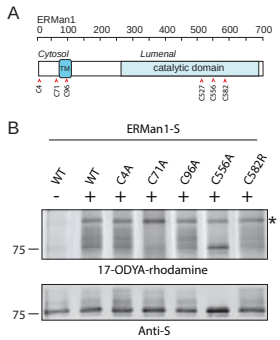


Figure 7. ERMan1 is palmitoylated at cysteine-71. A) ERMan1 domain structure. Cysteine residues are indicated. **B)** ERMan1-S WT and cysteine mutants were affinity purified from cells incubated with 17-ODYA and palmitoylation was detected using click chemistry. *Asterisk indicates nonspecific band.*

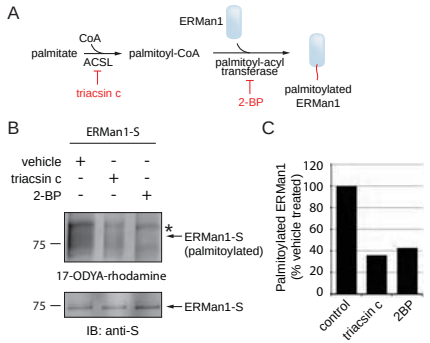


Figure 8. ERMan1 palmitoylation is impaired by triacsin c and 2-BP treatment. A) Illustration of our hypothesis that triacsin and 2-BP inhibit ERMan1 palmitoylation. **B)** ERMan1-S was affinity purified from cells incubated with 17-ODYA and the indicated inhibitors. Palmitoylation was detected by reaction wtih a rhodamine moiety. **C)** The levels of palmitoylated ERMan1 in panel (B) were quantified using ImageJ and plotted.

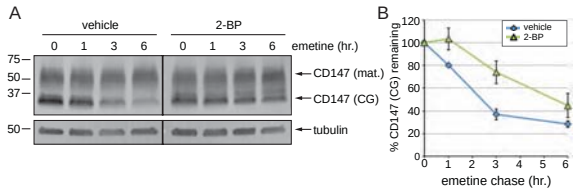
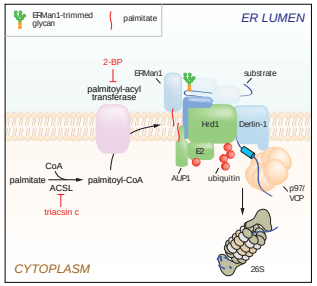


Figure 9. The palmitoylation inhibitor 2-BP impairs CD147 degradation. A) Cells were pretreated for 16hr with 2-bromopalmitate (2-BP) and the degradation kinetics of CD147 analyzed by immunoblotting. **B)** CD147 levels were quantified using ImageJ and plotted.

Conclusions



Fatty acid modification of ERAD factors regulates ER protein quality control.

- Inhibition of long chain acyl-CoA synthetases impairs the glycan trimming step of ERAD
- ERMan1, AUP1, Hrd1, and Derlin1 are palmitoylated ERAD proteins
- ERMan1 is palmitoylated at cysteine-71
- Palmitoylation is required for efficient ERAD

Future Directions

- Continue to probe for palmitoylation of ERAD proteins
- Elucidate the role of palmitoylation in ERAD
- Create mutated, palmitoylation-defective forms of ERMan1, AUP1, Hrd1, and Derlin1 and track degradation of CD147
- Create ERMan1, AUP1, Hrd1, and Derlin1 knockout cell lines using CRISPR/Cas9 to verify ERAD defect and for use in future degradation rescue studies
- Use fluorescence microscopy to localize palmitoylated and mutated, palmitoylation-defective ERAD proteins
- Analyze the protein complexes formed by palmitoylation-defective mutants

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