Lipid disequilibrium disrupts ER proteostasis by impairing ERAD substrate glycan trimming and dislocation

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ABSTRACT The endoplasmic reticulum (ER) mediates the folding, maturation, and deployment of the secretory proteome. Proteins that fail to achieve their native conformation are retained in the ER and targeted for clearance by ER-associated degradation (ERAD), a sophisticated process that mediates the ubiquitin-dependent delivery of substrates to the 26S proteasome for proteolysis. Recent findings indicate that inhibition of long-chain acyl-CoA synthetases with triacsin C, a fatty acid analogue, impairs lipid droplet (LD) biogenesis and ERAD, suggesting a role for LDs in ERAD. However, whether LDs are involved in the ERAD process remains an outstanding question. Using chemical and genetic approaches to disrupt diacylglycerol acyltransferase (DGAT)–dependent LD biogenesis, we provide evidence that LDs are dispensable for ERAD in mammalian cells. Instead, our results suggest that triacsin C causes global alterations in the cellular lipid landscape that disrupt ER proteostasis by interfering with the glycan trimming and dislocation steps of ERAD. Prolonged triacsin C treatment activates both the IRE1 and PERK branches of the unfolded protein response and ultimately leads to IRE1-dependent cell death. These findings identify an intimate relationship between fatty acid metabolism and ER proteostasis that influences cell viability.

INTRODUCTION

As the entry point into the secretory pathway, the endoplasmic reticulum (ER) is host to an extensive cohort of enzymes and chaperones that coordinate the folding, modification, and deployment of a large fraction of the proteome. Failure of secretory proteins to achieve their native structure due to mutations, errors in transcription or translation, protein damage, or inefficient folding can have dire consequences for cellular physiology and has been implicated in the etiology of numerous human diseases (Guerriero and Brodsky, 2012). Incorrect protein folding not only can result in a reduction in protein activity (i.e., loss of function), but it can also lead to the generation of cytotoxic protein aggregates (i.e., gain of function). To ensure the fidelity of the secretory proteome, the ER has evolved a quality control system that detects terminally misfolded and oligomerized proteins and targets them for clearance via a process known as ER-associated degradation (ERAD; Olzmann et al., 2013a; Christianson and Ye, 2014; Stevenson et al., 2016). The cell also responds to perturbations in ER homeostasis by activating the unfolded protein response (UPR; Walter and Ron, 2011; Wang and Kaufman, 2016), a set of signaling pathways that enhance the overall folding capacity of the ER.

ERAD involves a series of spatially and temporally coupled steps that mediate substrate recognition, dislocation (also known as retrotranslocation) across the ER membrane into the cytoplasm, ubiquitination, and targeting to the proteasome for proteolysis (Olzmann et al., 2013a; Christianson and Ye, 2014; Stevenson et al., 2016).
Although the mechanism by which substrates are triaged for degradation is incompletely understood, it is clear that the structure of substrate-conjugated N-linked glycans provides a “molecular code” that plays a determining role in the fate of secretory proteins (Xu and Ng, 2015). During insertion into the ER, the majority of the secretory proteome is modified by covalent attachment of a triantennary glycan moiety (Cherepanova et al., 2016). Progressive trimming by ER-resident mannosidases exposes an α-1,6-linked mannoside, which acts as a signal for ERAD and is recognized by the mannose 6-phosphate receptor homology (MRH) domain of the ER lectin, OS-9, and possibly a second ER lectin, XTP3-B (Sato et al., 2010). These two ER lectins interact with the Hrd1 luminal adaptor SEL1L (Christianson et al., 2008; Mueller et al., 2008; Tyler et al., 2012), facilitating substrate delivery for dislocation. Most models posit that the AAA ATPase VCP (also known as p97) then extracts substrates from proteinaceous pores in the membrane, possibly formed by the E3 ubiquitin ligase Hrd1 (Carvalho et al., 2010; Stein et al., 2014; Baldridge and Rapoport, 2016), the derlin family of proteins (Lilley and Ploegh, 2004; Ye et al., 2004; Greenblatt et al., 2011; Mehnert et al., 2014), or, in some cases, the Sec 61 translocon (Plemper et al., 1997; Scott and Schekman, 2008).

In addition to its role as a protein-folding compartment, the ER functions as a major site of lipid metabolism, mediating the synthesis of important lipids (e.g., phospholipids, sterols, and neutral lipids) and the biogenesis of lipid storage organelles called lipid droplets (LDs; Walther and Farese, 2012; Pol et al., 2014; Hashemi and Goodman, 2015). LDs are ubiquitous, conserved organelles composed of a neutral lipid core (e.g., triacylglycerol [TAG] and sterol esters) encircled by a phospholipid monolayer. Whereas the hydrophobic core of LDs is devoid of proteins, the bounding phospholipid monolayer is decorated with a unique proteome that regulates LD growth, breakdown, and trafficking. LDs function as dynamic repositories of lipids, protecting the cell from fatty acid–induced toxicity (Listenberger et al., 2003) and providing the cell with an “on-demand” source of lipids for membrane biogenesis (Kurat et al., 2009), energy production via β-oxidation (Rambold et al., 2015), and as ligands in lipid signaling pathways (Haemmerle et al., 2011; Tang et al., 2013). Several unexpected roles have also been identified for LDs, such as the regulation of the hepatitis C life cycle (Miyarai et al., 2007; Herker et al., 2010), the sequestration of histones (Cermelli et al., 2006; Anand et al., 2012), and the control of cytosolic inclusion body clearance (Moldavski et al., 2015).

Reports have identified a number of intriguing links between ERAD and LDs. A subset of proteins implicated in ERAD, including UBXD8, UBXD2, VCP, AUP1, and Ube2g2, were identified in proteomic analyses of buoyant, LD-enriched biochemical fractions (Brasaelem et al., 2004; Liu et al., 2004; Hodges and Wu, 2010), and the localization of these proteins to the LD surface was confirmed by fluorescence microscopy (Mueller et al., 2008; Zehmer et al., 2009; Klemm et al., 2011; Spandl et al., 2011; Suzuki et al., 2012; Jo et al., 2013; Olzmann et al., 2013b). This subset of ERAD factors has been implicated in the regulation of LD abundance, size, and clustering (Mueller et al., 2008; Zehmer et al., 2009; Klemm et al., 2011; Spandl et al., 2011; Suzuki et al., 2012; Jo et al., 2013; Olzmann et al., 2013b), but whether these effects on LDs are related to their functions in ERAD remains to be determined. ERAD substrates have also been observed on the LD surface (e.g., ApoB100 [Ohsaki et al., 2006; Suzuki et al., 2012]) and in ER subdomains that are closely juxtaposed to LDs (e.g., 3-hydroxy-3-methylglutaryl-coenzyme A reductase [HMGR; Hartman et al., 2010]). In addition, ER stress induces LD biogenesis (Fei et al., 2009; Vevea et al., 2015), and loss of LDs activates the UPR (Garbarino et al., 2009; Petschnigg et al., 2009; Olzmann and Kopito, 2011; Velázquez et al., 2016).

Indirect experimental evidence supporting a functional role for LDs in ERAD came from studies employing triacsin C, a polyunsaturated fatty acid analogue that inhibits long-chain acyl-CoA synthetases (ACSs; Tomoda et al., 1987; Igal et al., 1997) and blocks LD biogenesis (Fujimoto et al., 2007; Kassan et al., 2013). These studies found that triacsin C impaired the degradation kinetics of several ERAD substrates, including the null Hong Kong (NHK) mutant of α-1 antitrypsin (Klemm et al., 2011), a truncated variant of ribophorin I (Klemm et al., 2011), class I MHC heavy chain (Klemm et al., 2011), and HMGCR (Hartman et al., 2010; Jo et al., 2013). Together these findings led to multiple models of how LDs might be involved in ERAD (Ploegh, 2007; Hartman et al., 2010; Klemm et al., 2011; Suzuki et al., 2012; Jo et al., 2013; Vevea et al., 2015): 1) LD biogenesis is coupled to the dislocation of luminal ERAD substrates via the formation of transient pores in the membrane or the dislocation of integral membrane ERAD substrates via capture in the membrane of an exiting LD, 2) ERAD substrate dislocation and ubiquitination preferentially occur in LD-associated ER subdomains, and/or 3) ERAD substrates are sequestered on the surface of LDs as an intermediate step en route to the proteasome. Although these models are attractive, triacsin C is not a specific inhibitor of LD biogenesis, as it also affects unrelated processes that require activated fatty acids (e.g., de novo phospholipid synthesis; Igal et al., 1997). Moreover, the degradation kinetics of several ERAD substrates was unaffected in a strain of yeast lacking LDs (Olzmann and Kopito, 2011; Nakatsukasa and Kamura, 2016), indicating either that LD formation is not essential for ERAD or that there are unrecognized differences between the ERAD process in yeast and mammalian cells. Thus, the functional relationship between ERAD and LDs remains unresolved.

In this study, we focused our attention on the effect of triacsin C on ERAD and the potential requirement of LDs for ERAD in mammalian cells. Our results demonstrate that, as in yeast (Olzmann and Kopito, 2011; Nakatsukasa and Kamura, 2016), LDs are dispensable for ERAD in mammalian cells. However, our data indicate that triacsin C causes widespread changes in the cellular lipid composition, impairs ERAD substrate glycan trimming and dislocation, and induces the UPR, culminating in cell death. These findings support a fundamental connection between fatty acid metabolism and ER proteostasis.

RESULTS

Inhibition of long-chain acyl-CoA synthetases with triacsin C impairs ERAD pathways

To examine the effect of triacsin C on ERAD, we analyzed the degradation kinetics of a panel of substrates that reflect a range of topologies and use distinct degradation pathways (Figure 1A). The panel included an endogenous ERAD substrate, CD147, which is an asialoglycoprotein that is recognized as an unassembled subunit of an oligomeric complex and is constitutively degraded by a Hrd1/SEL1L pathway (Tyler et al., 2012). We also tested two exogenously expressed mutant substrates: the NHK mutant of α-1 antitrypsin—a soluble, luminal substrate degraded by a Hrd1/SEL1L pathway (Christianson et al., 2008; Hosokawa et al., 2008)—and the ΔF508 mutant cystic fibrosis transmembrane conductance regulator (CFTR ΔF508)—a polytopic integral membrane substrate degraded by multiple E3 ligase pathways (Meacham et al., 2001; Younger et al., 2006; Morito et al., 2008).

To determine the kinetics of triacsin C treatment on ERAD disruption, we performed a time course of triacsin C incubation and
analyzed the degradation of CD147 during emetine translation shutoff (Figure 1, B–D). As expected (Tang, 2004; Tyler et al., 2012), CD147 migrated as two primary species: a high–molecular weight plasma membrane form bearing complex glycans (CD147(mature [Mat.])) and a lower–molecular weight ER form bearing the core-glycan structure (CD147(CG); Figure 1C). CD147(CG) was degraded during the 6-h emetine chase (Figure 1, C and D). Addition of triacsin C at time 0 of the emetine chase had no effect on CD147(CG) degradation (Figure 1, C and D). Increasing stabilization of CD147(CG) was observed as the triacsin C preincubation time was lengthened, with a maximal stabilization occurring after a 16-h triacsin C pretreatment (Figure 1, C and D). Using the 16-h triacsin C pretreatment, we analyzed the degradation kinetics of our full panel of ERAD substrates (Figure 1, E–J). The Hrd1 substrate CD147(CG) was stabilized by triacsin C pretreatment (Figure 1, E and F). Although the majority of newly synthesized CD147 is degraded by ERAD, a small fraction can correctly assemble and mature by trafficking through the Golgi to the plasma membrane (Tang, 2004; Tyler et al., 2012). To account for both fates of CD147, we performed radioactive pulse-chase experiments (Supplemental Figure S1A). Over the 6-h time course of our experiment, no CD147 maturation was detected, and triacsin C pretreatment stabilized CD147(CG). These results indicate that the effect of triacsin C is due to impairment of CD147 degradation rather than maturation. The Hrd1 luminal substrate NHK–green fluorescent protein (GFP) was also stabilized by triacsin C pretreatment (Figure 1, E and F). No secretion of NHK-GFP was observed in this cell line (Supplemental Figure S1B). In contrast to CD147 and NHK-GFP, CFTR∆F508 degradation kinetics was unaffected by the triacsin C pretreatment (Figure 1, I and J). These data demonstrate that treatment with the ACSL inhibitor triacsin C impairs select ERAD pathways.
Inhibition of ACSLs impairs ERAD

Triacsin C does not generally inhibit the ubiquitin-proteasome system or protein secretion.

(D) HEK293 cells expressing TTR-HA were treated with vehicle or 1 µg/ml triacsin C for 16 h. Cells were washed with PBS, and the medium was replaced with serum-free OPTI-MEM containing vehicle or 1 µg/ml triacsin C for the remaining 6 h. Lysates and TTR-HA immunoprecipitated from the media were analyzed by immunoblotting. The levels of TTR-HA in the media were quantified as percentage of the levels in the control sample (n = 3). (F, G) The morphology of the ER, anti-KDEL (green) and the Golgi complex, anti-GM130 (green), in HeLa cells treated with vehicle or 1 µg/ml triacsin C for 16 h was analyzed by immunofluorescence microscopy. Nuclei were stained with DAPI (blue). Scale bar, 10 µm. Mat., mature; CG, core glycosylated; -CHO, deglycosylated. Error bars indicate SEM.

Triacsin C does not generally inhibit the ubiquitin-proteasome system.

Our finding that triacsin C inhibits the degradation of a subset of ERAD substrates suggests that triacsin C treatment does not generally inhibit the ubiquitin-proteasome system (UPS). In agreement with this notion, ubiquitinated proteins accumulated in cells treated with the proteasome inhibitor MG-132, but not with triacsin C (Figure 2A). To assess more directly the effect of triacsin C on the degradation of cytosolic proteins, we used flow cytometry to measure the degradation kinetics of a cytosolic UPS reporter (Figure 2B). This reporter consists of the Venus fluorescent protein fused to a destabilized domain (Venus-DD), a variant FK506-binding domain from FKBP12 that, in the absence of the small molecule shield-1, is misfolded and rapidly degraded via the UPS (Banaszynski et al., 2006; Egeler et al., 2011; Bersuker et al., 2016). Triacsin C had no significant effect on the constitutive degradation of Venus-DD (Figure 2B), indicating that triacsin C does not gener-

ally affect the degradation of cytosolic UPS substrates.

After dislocation, ERAD substrates are deglycosylated by the cytosolic peptide:N-glycanase (PNGase) and cleared by the UPS (Olzmann et al., 2013a; Xu and Ng, 2015). Thus, the presence and accumulation of a deglycosylated form of ERAD substrates reflect inefficient coupling of dislocation with proteasomal degradation. Incubation with the proteasome inhibitor MG-132 during an emetine chase resulted in the accumulation of deglycosylated CD147 (CD147(-CHO)), indicating the buildup of cytosolically dislocated CD147 (Figure 2C). CD147 deglycosylated in vitro by incubation with the glycosidase PNGase F resolved at the same molecular weight as the CD147 band that accumulated in MG-132–treated cells, and no additional lower-molecular weight forms appeared (Supplemental Figure S2), confirming the identity of the CD147(-CHO) species. A portion of CD147 also migrated in a high–molecular weight smear, likely representing ubiquitinated CD147 (Figure 2C). In contrast to MG-132, triacsin C pretreatment solely stabilized CD147(CG); deglycosylated CD147 and ubiquitinated CD147 were absent (Figure 2C). Together, these data indicate that triacsin C impairs ERAD upstream of the proteasome and does not cause a global defect in the UPS.

Triacsin C does not impair protein secretion

Dysregulated lipid metabolism can alter organelle morphology and function (Han et al., 2008; Adeyo et al., 2011; Thibault et al., 2012), and disruptions in ER-to-Golgi trafficking reduce the degradation of some ERAD substrates (Caldwell et al., 2001; Vashist et al., 2001; Taxis et al., 2002). To examine the function of the secretory pathway, we analyzed the secretion of hemagglutinin-tagged transthyretin (TTR-HA), a tetrameric protein that is normally secreted into the serum, where it functions as a carrier of the thyroid hormone thyroxine. Similar levels of TTR-HA were immunoprecipitated from media isolated from cells incubated in the presence or absence of triacsin C (Figure 2, D and E), indicating that triacsin C pretreatment does not affect TTR secretion. Furthermore, the overall morphology of the ER (Figure 2F) and Golgi complex (Figure 2G) remained unperturbed by a triacsin C pretreatment at the resolution of fluorescence deconvolution microscopy. Together, these results indicate that the secretory system remains functionally and morphologically intact after a 16-h triacsin C treatment.

Triacsin C impairs CD147 glycan trimming

Our initial results indicated that triacsin C affects ERAD upstream of the proteasome (Figure 2). To determine more precisely the steps in ERAD that are compromised, we focused our attention on the degradation of the endogenous substrate CD147, which was...
trimming, we separated CD147 on large-format SDS–PAGE gels (Figure 3A). On these larger gels, the variety of CD147 glycoforms becomes evident, and CD147(CG) is resolved as approximately five bands (Figure 3A). Treatment of lysates in vitro with PNGase F collapsed all CD147 forms into a single band of ~29 kDa (Figure 3D), consistent with the conjecture that the variations in the CD147 banding pattern reflect the diversity of CD147 glycoforms.

During the course of an emetine translocation shutoff experiment, the upper CD147(CG) bands were rapidly lost (Figure 3, A and B, vehicle), whereas the lower bands displayed a slight lag period before clearance (Figure 3, A and C, vehicle). These results are consistent with the conversion of CD147(CG) from a slower-migrating, untrimmed form into a faster-migrating, trimmed form before degradation. Treatment with the mannosidase inhibitor kifunensine (Figure 3, A–C, kifunensine) or the glucosidase inhibitor deoxynojirimycin (Supplemental Figure S3) stabilized CD147(CG) in the slower-migrating form, providing evidence that these bands represent an untrimmed form of CD147(CG). It is worth noting that CD147(CG) continued to be degraded in the presence of kifunensine (Figure 3A, kifunensine), albeit at a slower rate, indicating either that glycan trimming is not a strict requirement for CD147(CG) degradation or that kifunensine inhibition of glycan trimming is incomplete. Cotreatment with kifunensine and deoxynojirimycin did not result in additional stabilization (Supplemental Figure S3).

Analysis of CD147(CG) in cells pretreated with triacsin C revealed a significantly reduced rate of CD147(CG) conversion from untrimmed to the trimmed glycoform (Figure 3, A–C, triacsin C), similar to the effect of kifunensine. In contrast, blocking CD147(CG) degradation at a downstream step with the VCP inhibitor CB-5083 resulted in the accumulation of a lower–molecular weight, presumably highly trimmed form of CD147(CG) (Figure 3, A–C, CB-5083). These data suggest that triacsin C impairment in ERAD is caused, at least in part, through inhibition of substrate glycan trimming.

**Triacsin C disrupts CD147 delivery to the Hrd1 dislocation complex**

CD147 is degraded via an ERAD pathway that requires Hrd1, SEL1L, and, to some extent, the lectins OS-9 and XTP3-B (Tyler et al., 2012). The Hrd1 dislocation complex is a membrane-embedded, macromolecular complex (Mueller et al., 2008; Christianson et al., 2012). Several properties of membrane lipids can influence the interactions and functions of membrane-embedded protein complexes.
ACS L inhibition impairs ERAD and ubiquitin. The significance of Hrd1 association with RPN1 (also known as ribophorin I), PGRC1, and EMD is unknown. These proteins are not known to be involved in protein quality control and may represent endogenous substrates of the Hrd1 complex. Several previously reported Hrd1 complex members (UBXD8, AUP1, derlin-1, derlin-2) were not detected in our SILAC experiment, possibly due to their lower abundance. Therefore, we examined the association of these interactors with Hrd1 by immunoblotting of affinity purified S-tagged Hrd1 complexes (Figure 4B). Analysis of the results from both the SILAC (Figure 4A) and immunoblotting (Figure 4B) experiments indicate that few Hrd1 interactions were affected by triacsin C treatment. The core Hrd1 complex, characterized by SEL1L, FAM8A1, XTP3-B, OS-9, and ERLIN2, remained intact after triacsin C treatment. There were minor trends toward increased associations with VCP and ubiquitin, as well as decreased association with Hsp47.
Triacsin C impairs the dislocation of a luminal glycosylated ERAD substrate

Given the effects of triacsin C on CD147 glycan trimming (Figure 3) and association with Hrd1 (Figure 4, C and D), we predicted that triacsin C would affect substrate dislocation. The accumulation of deglycosylated CD147 in response to MG-132 treatment provides one potential method to assess dislocation. However, MG-132 also stabilized CD147(CG), and the appearance of deglycosylated CD147 was minimal and difficult to detect (Figure 2C). Therefore, to assess quantitatively the effects of triacsin C on dislocation, we used a more sensitive and robust fluorescent ERAD dislocation assay based on the reconstitution of split Venus (Figure 4E; Grotzke et al., 2013). In this assay, the N-terminal half of deglycosylation-dependent Venus is fused to the H2-Kβ signal sequence (SS-dgdV1Z), targeting it to the ER lumen (Grotzke et al., 2013). SS-dgdV1Z is glycosylated, recognized as an aberrant protein, and dislocated into the cytosol for degradation (Grotzke et al., 2013). In the presence of MG-132, SS-dgdV1Z accumulates in the cytosol and associates with the C-terminal half of Venus (VZ2), reconstituting the mature fluorescent protein and enabling dislocation to be measured by flow cytometry (Grotzke et al., 2013). Of importance, the fluorescence is deglycosylation dependent (Grotzke et al., 2013), ensuring that any fluorescence detected results from the dislocation of dgdV1Z from the ER lumen into the cytosol.

Incubation of 293T.FluERAD cells stably expressing SS-dgdV1Z and VZ2 with MG-132 resulted in a large increase in Venus fluorescence (Figure 4F, 16.4-fold increase). In agreement with a role for VCP in SS-dgdV1Z dislocation (Grotzke et al., 2013), co-incubation with CB-5083 and MG-132 nearly completely blocked the increase in fluorescence (Figure 4F, 1.6-fold decrease). Similar to the effect of kifunsensine treatment (Figure 4F, 7.6-fold), triacsin C treatment partially blocked the increase in fluorescence in response to MG-132 (Figure 4F, 7.3-fold). Thus, triacsin C significantly reduces the dislocation of a luminal glycosylated ERAD substrate.

Lipid droplets are dispensable for CD147 ERAD

The observation that triacsin C inhibits ERAD (Hartman et al., 2010; Klemm et al., 2011; Jo et al., 2013; Figure 1) is in agreement with a role for LDs in ERAD; however, triacsin C is not a selective inhibitor of LD biogenesis (Figure 5A). Although a selective inhibitor of LD biogenesis has not been identified, ablation of the diacylglycerol acyltransferase (DGAT) enzymes (DGAT1 and DGAT2), which catalyze the final and committed step in TAG synthesis...
DGAT2-/- MEFs exhibited a low amount of LDs under basal conditions, which increased dramatically after a 6-h treatment with 200 µM oleate (Figure 5, B and C), indicating that DGAT2-/- MEFs are still able to generate LDs in response to an oleate challenge, due to the presence of DGAT1. Treatment with either triacsin C or DGAT1i reduced the amount of LDs in non–oleate-treated cells and completely blocked the increase in LD biogenesis in response to oleate (Figure 5, B and C). The levels of the LD protein perilipin-2 (PLIN2) are known to correlate with LD abundance, and, in the absence of LDs, PLIN2 is degraded by the ubiquitin-proteasome system (Xu et al., 2005; Masuda et al., 2006; Takahashi et al., 2016). Analysis of PLIN2 levels and cellular distribution indicate that triacsin C and DGAT1i treatments block oleate-induced increases in PLIN2 levels and PLIN2-immunoreactive LDs (Supplemental Figure S4). Together these data demonstrate that the DGAT2-/- MEFs provide a facile means to acutely manipulate LD biogenesis at an upstream step (i.e., with triacsin C) or a downstream step (i.e., with DGAT1 inhibitor).

As observed in HEK293 cells, CD147(CG) was degraded in DGAT2-/- MEFs during an emetine translation shutoff experiment and was stabilized by a triacsin C pretreatment (Figure 5, D and E). The rate of CD147(CG) degradation was greater in the DGAT2-/- MEFs than in the HEK293 cells (half-life ~25 min vs. ~2 h). DGAT1i pretreatment, despite inhibiting LD biogenesis (Figure 5, B and C), had no effect on the kinetics of CD147 degradation (Figure 5, D and E). These results argue against a requirement for LDs in CD147 degradation and suggest that triacsin C affects ERAD through a mechanism independent of LDs.

Metabolomic profiling reveals global alterations in the cellular lipid landscape of triacsin C–treated cells

To understand the effects of triacsin C on cellular lipid homeostasis, we performed targeted single reaction monitoring (SRM)–based liquid chromatography–tandem mass spectrometry (LC-MS/MS) steady-state lipidomic profiling of >100 lipid metabolites, encompassing a wide array of lipid classes, including neutral lipids, fatty acids, acyl carnitines (ACs), N-acyl ethanolamines, sterols, phospholipids, sphingolipids, lysophospholipids, and ether lipids (Figure 6 and Supplemental Table S3). Among the 118 lipids, 71 exhibited significant changes (p < 0.05) after a 16-h treatment with 1 µg/ml triacsin C for 16 h revealed alterations in 71 lipid species, illustrated as a volcano plot (A) and a heat map organized by lipid class (B). Red text in B indicates a significant change (p < 0.05). (C–K) Quantification showing the relative levels of significantly altered lipids (n = 4 or 5). *p < 0.05, **p < 0.01. White bars, vehicle; black bars, triacsin C. (L) Pathway map depicting the general effects of triacsin C on neutral lipids and phospholipids. DAG, diacylglycerol; FFA, free fatty acid; MAG, monoacylglycerol; NAE, N-acyl ethanolamine; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; TAG, triacylglycerol. “L” before a lipid phospholipid designation indicates lyso-; “e” after a lipid designation indicates an ether lipid; “p” after a lipid designation designates plasmalogen. Error bars indicate SEM.
and C18:0/C18:0/C18:0 TAG; Figure 6, B and C), suggesting that there may be protected pools of TAGs or that some ACSLs that are incompletely inhibited mediate the formation of these specific TAGs (Igal et al., 1997). We also observed an anticipated decrease in AC levels, particularly in C16:0 AC (Figure 6, B and E). Although free fatty acids might be expected to accumulate due to the inhibition of ACSLs and consequent lack of conversion into the CoA intermediate for cellular use, no changes in fatty acid levels were detected (Figure 6B). This may be due to a compensatory efflux of free fatty acids (Igal et al., 1997), which could result in an underestimate of total free fatty acid levels, or increased flux through ACSL enzymes that are not inhibited.

Broad changes in additional cellular lipids were also observed, including decreases in many phospholipids, phospholipid ethers, neutral ether lipids, and lysophospholipid ethers (Figure 6, B–K). The decreases in lipid levels presumably resulted from impairments in synthesis caused by the inability of ACSLs to activate fatty acids, a requirement for conjugation. Particularly striking was the general decrease in nearly all phosphatidylinositol and phosphatidylinositol ether lipids (Figure 6, B, F, and J). This is interesting, given the recent finding that phosphatidylinositol maintains ER homeostasis in yeast by sequestering fatty acids when LD biogenesis is inhibited (Velázquez et al., 2016). Our results suggest that phosphatidylinositol may represent an especially dynamic phospholipid pool that reflects the levels of fatty acid flux.

Several lipid species displayed significant increases, including many lysophospholipids (Figure 6, B, D, and H), which can act as signaling molecules, and several phospholipids (Figure 6, B–K). The increase in some lipids is consistent with the possible increased flux of fatty acids through ACSL enzymes that are not inhibited or are incompletely inhibited by triacsin C. The ratio of phosphatidycholine (PC) to phosphatidylethanolamine (PE) has been implicated in ER homeostasis (Li et al., 2006; Fu et al., 2011; Thibault et al., 2012), and although we observed alterations in PC and PE levels (Figure 6, B and F), the ratio between the two lipid species was relatively unchanged. An increase in ceramides (C16:0 ceramide and C18:0 ceramide) was detected (Figure 6, B and G), which is notable, given their role in cellular stress responses and UPR activation (Volmer and Ron, 2015). Together our results indicate that triacsin C treatment not only affects the levels of neutral lipids sequestered in LDs, but it also causes widespread alterations in the cellular lipid landscape (Figure 6). The levels of several of the altered lipids have been suggested to affect ER homeostasis (e.g., phosphatidylinositol and ceramides).

**Triacsin C activation of the PERK and IRE1 arms of the UPR has opposing effects on cell viability**

Disruptions in ERAD and in lipid homeostasis can activate the UPR (Jonikas et al., 2009; Volmer and Ron, 2015). Inositol-requiring enzyme-1 (IRE1), an ER transmembrane serine/threonine kinase and endonuclease, is a primary mediator of the UPR that splices XBP1 mRNA to enable the translation of the XBP1 transcription factor (Walter and Ron, 2011). Analysis using reverse transcription PCR revealed that incubation with triacsin C induced XBP1 splicing (Figure 7A). The spliced form of XBP1 was detectable at low levels as early as 8 h, and it became much more prominent at 16 and 24 h (Figure 7A). A second arm of the UPR is controlled by the ER-resident kinase PKR-like ER kinase (PERK), which phosphorylates the α subunit of eukaryotic translation-initiation factor 2 (eIF2α). Phosphorylation of eIF2α represses global translation while simultaneously promoting the translation of the ATF4 transcription factor to up-regulate stress-responsive genes such as the prosapoptotic transcription factor C/EBP homologous protein (CHOP; Hiramatsu et al., 2015). To examine the potential effect of triacsin C on PERK induction of stress-responsive genes, we exploited a clonal HEK293 reporter cell line expressing an 8.5-kb CHOP gene fragment fused to GFP (CHOP::GFP; Wang et al., 1998; Novoa et al., 2001). Treatment with tunicamycin, an inhibitor of N-linked glycosylation that induces the UPR, resulted in a robust and rapid accumulation in GFP fluorescence (Figure 7C and Supplemental Figure S5). Treatment with triacsin C also caused an increase in GFP fluorescence but with different temporal dynamics. During the first 8 h, no increase in GFP fluorescence was observed (Figure 7C). This lag period was followed by an increase in GFP fluorescence levels at 16 and 24 h (Figure 7C).

The IRE1 and PERK arms of the UPR play well-characterized protective roles through the induction of genes involved in protein folding and membrane expansion and through the repression of translation (Walter and Ron, 2011). Of note, UPR up-regulation protected yeast from ER trafficking and ERAD defects induced by lipid disequilibrium (Thibault et al., 2012). However, persistent activation of IRE1 or PERK can lead to cell death (Lin et al., 2007; Han et al., 2009). To determine the role of the IRE1 and PERK pathways in the cellular response to triacsin C treatment, we analyzed the effects of the IRE1 inhibitor 4µ8c (IRE1i) and PERK inhibitor GSK2606414 (PERKi). IRE1i completely blocked triacsin C–induced XBP1 cleavage (Figure 7, A and B), and PERKi significantly attenuated the induction of the CHOP::GFP reporter (Figure 7D). Inhibition of PERK increased the amounts of cell death induced by triacsin C at 8, 16, and 24 h (Figure 7E), indicating that PERK plays a predominantly protective role under these conditions. In contrast, inhibition of IRE1 had little effect during triacsin C treatment and increased the amount of cell death at 24 h (Figure 7E). These findings indicate that both the IRE1 and PERK arms of the UPR are induced by triacsin C, but that the outputs of these two signaling pathways have opposing effects on cell viability.

**DISCUSSION**

Although there are several intriguing connections between LDs and ERAD, whether LDs are directly involved in the ERAD mechanism has remained an outstanding question. Our data argue that LD biogenesis is not a fundamental requirement for ERAD. Instead, our results support a model (Figure 7F) in which triacsin C inhibition of ACSLs causes widespread changes in the cellular lipid composition that impair specific steps in ERAD, resulting in disruptions in ER proteostasis, activation of the UPR, and eventual cell death. Thus, dysregulated fatty acid metabolism negatively affects ER homeostasis and protein quality control independently of LDs.

To inhibit LD biogenesis but avoid the broad effects that ACSL inhibition has on lipid homeostasis, we pursued an approach that would disrupt a downstream step in TAG synthesis. To this end, we characterized a combined chemical (DGAT1 inhibition) and genetic (DGAT2-/-) approach to inhibit both of the DGAT enzymes, which are required for the conversion of DAG to TAG and the generation of LDs (Yen et al., 2008; Harris et al., 2011). This strategy enabled acute disruption of LD biogenesis, reducing LD abundance under basal and oleate-stimulated conditions as effectively as triacsin C does. In contrast to triacsin C, disruption of LD biogenesis by inhibiting the DGATs had no effect on the kinetics of CD147 ERAD. These results are consistent with previous analyses of ERAD in yeast models of LD disruption (Olzmann and Kopito, 2011; Nakatsuksa and Kamura, 2016), which together demonstrate that LD biogenesis is not integral to the ERAD mechanism in yeast or mammalian cells. The possibility that LDs may function in the degradation of specific substrates or in ERAD under specific conditions is still worth consideration. For
example, for ApoB100, an extremely large, hydrophobic protein, the association with LDs might provide a specialized ERAD mechanism to reduce aggregation (Ohsaki et al., 2006; Suzuki et al., 2012). LDs may also contribute to ERAD only under particular conditions, such as periods of disrupted proteostasis. Under conditions in which proteasomal capacity is limiting, the LD surface could act as a transient site for the sequestration of ERAD and other UPS substrates (Ohsaki et al., 2006; Vevea et al., 2012).

Our findings are in agreement with previous reports that triacsin C impairs ERAD (Hartman et al., 2010; Klemm et al., 2011; Jo et al., 2013). Indeed, we found that triacsin C inhibited the degradation of two glycosylated Hrd1 substrates—the luminal substrate NHK and the endogenous integral membrane substrate CD147. The highest amount of substrate stabilization required a 16-h pre-treatment with triacsin C, suggesting that ACSL activity is not required acutely during ERAD but instead that ACSL activity is required to establish a particular cellular environment conducive for ERAD. To define more precisely the step in ERAD that is affected by triacsin C, we tested individual steps of ERAD in the context of triacsin C treatment. Our results indicate that the triacsin C–induced defect in protein degradation is upstream of the proteasome and is confined to a subset of ERAD pathways. This conclusion is supported by several findings: 1) ubiquitinated proteins did not accumulate in response to triacsin C, 2) triacsin C did not stabilize a cytosolic UPS substrate, 3) triacsin C affected a subset of ERAD substrates—CD147 and NHK—but not CFTR∆F508, and 4) triacsin C impaired the dislocation of a luminal glycosylated substrate. Moreover, analyses of the glycosylation state of CD147 during degradation indicate that triacsin C treatment impaired CD147 glycan trimming and delivery to the Hrd1 complex, suggesting that the primary impairment in ERAD is due to the failure to expose the trimmed glycan structure necessary for degradation commitment. Our proteomics data indicate that the composition of the Hrd1 complex is largely unaltered in triacsin C–treated cells; however, it is possible that alterations in the ER lipid composition could modulate the structure and/or function of the complex. The enzymes involved in the trimming of CD147’s glycans are unknown, but this step is most likely catalyzed by ER-resident mannosidases ERMan1 and/or EDEM1-3. Disruptions in lipid composition could influence substrate localization to ERMan1-containing ER subdomains (Benyair et al., 2015) or could affect EDEM membrane association, which is known to affect EDEM glycan trimming activity toward certain substrates (Tamura et al., 2011). It is also possible that the inhibition of ACSLs could influence protein acylation, and both calnexin (Lakkaraju et al., 2012; Lynes et al., 2012, 2013) and the ERAD E3 ligase gp78 (Fairbank et al., 2012) have been reported to be palmitoylated. Whether other ERAD factors are regulated by lipid modifications is unknown.

Activation of the UPR initiates signaling pathways with opposing outputs, a protective response that seeks to reestablish ER homeostasis and an apoptotic response that promotes cell death in the face of persistent ER stress (Lin et al., 2007, 2009; Hetz, 2012;
Lu et al., 2014). Consistent with disruptions in ER homeostasis, treatment with triascin C induced XBP1 splicing (IRE1 arm) and CHOP::GFP expression (PERK arm) and eventually caused cell death. Treatment of cells with the UPR inducer tunicamycin causes a rapid and transient up-regulation of IRE1 signaling that is paralleled by a slower increase in apoptotic PERK signaling at later times (Lin et al., 2007). Of interest, in response to triascin C, we see very different temporal dynamics and effects of UPR induction. Both the PERK and IRE1 arms exhibited similar activation kinetics and, after an initial lag period, steadily increased until the end of our experiments. Despite increasing CHOP reporter expression, PERK actions were overall protective in response to triascin C. This finding indicates that CHOP expression alone is not conclusive evidence of a proapoptotic signaling output, consistent with the observation that forced CHOP expression was insufficient to induce cell death (Han et al., 2013). In contrast to PERK, IRE1 signaling appeared to promote cell death, and the inhibition of IRE1 attenuated triascin C–induced apoptosis, possibly by inhibiting excessive regulated Ires-dependent decay (RIDD) of important secretory transcripts (Han et al., 2009) or activation of a JNK apoptotic signaling pathway (Uruno et al., 2000). These results highlight the complex relationship between the UPR and cell death and reveal that the mode of UPR activation (e.g., tunicamycin vs. triascin C) has a profound effect on the ultimate effects of each UPR branch. Alterations in phospholipids can directly induce UPR signaling (Volmer et al., 2013; Volmer and Ron, 2015), and whether the changes in the lipid environment, the defects in ER protein quality control, or both are responsible for triascin C activation of the UPR is unclear. In addition, how the UPR is customized to fit a particular ER stressor is not evident. It is possible that the temporal coordination of individual UPR branches influences the end output (i.e., protection vs. cell death) or that different ER stressors provide a unique “second hit” (e.g., disruptions in lipid homeostasis or depletion in ER calcium pools) that sensitizes cells to IRE1– or PERK-dependent cell death pathways.

Our study reveals an intimate relationship between cellular lipid homeostasis and ER protein quality control. Our findings raise the possibility that certain lipid environments and/or modifications may affect ER proteostasis by regulating specific steps of the ERAD process. It is worth noting that a multitude of diseases, ranging from obesity to neurodegenerative diseases, are associated with altered lipid homeostasis and upregulated UPR (Hetz et al., 2013). In addition, targeting lipid metabolic enzymes to decrease fatty acid availability (e.g., inhibition of FASN) is being actively pursued as a therapeutic strategy for the treatment of cancer (Menendez and Lupu, 2013). In contrast, FASN upregulation associated with metabolic diseases may contribute to ER stress and cell death, and inhibition of FASN might provide therapeutic benefit via relieving ER stress (Hegg et al., 2015).

Materials and Methods

Plasmids, antibodies, and reagents

The pcDNA3.1(-) plasmids for expression of TTR-HA, the null Hong Kong mutant of α-1 antitrypsin (NHK-HA and NHK-GFP), and S-tagged Hrd1 (Hrd1-S) were previously described (Christianson et al., 2008, 2011). The CFTRAp508 plasmid was kindly provided by Doug Cyr (University of North Carolina at Chapel Hill, Chapel Hill, NC). Antibodies employed in this study include anti-CD147 (A-12, G-19, 8D6; Santa Cruz Biotechnology), anti-Hrd1 (A302-946A; Bethyl), anti-HA (HA7; Sigma-Aldrich), anti-S-peptide (EMD Millipore), anti-tubulin (Abcam), anti-glycerolaldehyde-3-phosphate dehydrogenase (EMD Millipore), anti-GFP (Roche), anti-CFTR (University of North Carolina at Chapel Hill, CFTR Antibodies Distribution Program), anti–ubiquitin conjugates (FK2; EMD Millipore), anti-AUP1 (Proteinitech), anti-SEL1L (T-17; Santa Cruz Biotechnology) and anti-KDEL (Enzo). Anti–derlin-1 and anti–derlin-2 antibodies were kind gifts from Yihong Ye (National Institutes of Health, Bethesda, MD). Rabbit polyclonal anti-UBXDB antibodies were generated against a histidine-tagged fragment of UBXDB (amino acids 97–445) by Proteintech Group. All IRDye680- and IRDye800-conjugated secondary antibodies for Western blotting were obtained from LI-COR. Alexa Fluor–conjugated secondary antibodies for immunofluorescence microscopy were obtained from Thermo Fisher Scientific.

Reagents employed in this study include triascin C (Enzo Life Sciences), emetine dihydrochloride hydrate (Sigma-Aldrich), CB-5083 (Anderson et al., 2015; Cleave Biosciences), oleate (S-1-Aldrich), kifunensine (Cyman Chemical), deoxycholic acid (Sigma-Aldrich), MG-132 (Selleck Chemicals), T863 (Sigma-Aldrich), 4µ8C (EMD Millipore), GSK2606414 (EMD Millipore), tunicamycin (Cyman Chemical), and PNGase F (New England Biolabs).

Cell culture and transfections

HEK293, HEK293T, MEF, HeLa, and U2OS cells were cultured in 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. 293T.FuERAD cells stably expressing a split-Venus system for the analysis of the dislocation step of ERAD (Grotzke et al., 2013) were kindly provided by Peter Cresswell (Yale University, New Haven, CT). U2OS cells stably expressing Venus-DO (Bersuker et al., 2016) and HEK293 cells stably expressing the CHOP::GFP reporter were kindly provided by Ron Kopito (Stanford University, Stanford, CA). DGAT2-/- MEF cells were kindly provided by Robert Farese, Jr. (Harvard University, Cambridge, MA). All plasmid transfections were performed using X-tremeGENE HP (Roche) transfection reagent according to the manufacturer’s instructions.

Immunoblotting analysis

Cells were washed extensively in phosphate-buffered saline (PBS) and lysed in 1% SDS. Protein amounts were normalized using a bicinchoninic acid (BCA) protein assay (Thermo Fisher Scientific). Proteins were separated on 4–20% polyacrylamide gradient gels (Bio-Rad) and transferred onto low-fluorescence polyvinylidene fluoride membranes (Bio-Rad). Large-format gel electrophoresis was performed using 10% acrylamide gels made with acrylamide/bis 19:1. Membranes were incubated in 5% nonfat milk in PBS plus 0.1% Tween-20 (PBST) for 30 min to reduce nonspecific antibody binding. Membranes were then incubated for at least 2 h in PBS containing 5% milk or 1% bovine serum albumin (BSA; Sigma-Aldrich) and primary antibodies, followed by incubation for at least 1 h in PBS containing 1% BSA and fluorescence-conjugated secondary antibodies. Immunoblots were visualized on a LI-COR imager (LI-COR Biosciences), and ImageJ (Schneider et al., 2012) was used for quantification.

Immunofluorescence microscopy

HeLa and MEF cells were plated on poly-l-lysine–coated coverslips. Cells were treated the next day, washed with PBS, and fixed at room temperature with 4% paraformaldehyde in PBS for 10 min. Cells were washed three times with PBS and permeabilized with 0.1% Triton X-100 plus 1% BSA in PBS at room temperature for 30 min. Cells were washed three times with 1% BSA in PBS and incubated for 2 h in primary antibodies, washed three times, and incubated for 1 h with Alexa Fluor–conjugated secondary antibodies, BODIPY493/503 (LD staining; Thermo Fisher Scientific), fluorescence microscopy was obtained from Thermo Fisher Scientific.

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and 4′,6-diamidino-2-phenylindole (DAPI; nuclei staining; Thermo Fisher Scientific). Cells were washed three times and mounted using Fluoromount-G (SouthernBiotec). Cells were visualized using a DeltaVision Elite microscope and acquired images deconvolved and analyzed using SoftWoRx. The abundance of LDs per cell was determined by measuring the area of BODIPY493/503-stained LDs per cell using ImageJ (Schneider et al., 2012).

Affinity purifications
HEK293 cells were harvested, washed with PBS, and lysed in immunoprecipitation (IP) buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% digitonin, and protease inhibitor tablets [Thermo Fisher Scientific]) at 4°C for 30 min. Lysates were clarified by centrifugation at 20,000 × g for 10 min. Protein concentrations were measured using the BCA assay. For the affinity purification of S-tagged protein complexes, lysates were loaded onto S-protein agarose beads (EMD Millipore) at a concentration of 25 µl beads per 1 mg of lysate. For endogenous Hrd1 IPs, 2 mg of lysate was incubated with anti-Hrd1 antibodies for 1 h and then loaded onto 25 µl of protein G agarose beads (EMD Millipore). Lysates were incubated with the beads rotating at 4°C for 2 h, washed three times with lysis buffer containing 0.1% digitonin, and eluted in loading buffer.

Radiolabeling and pulse-chase analysis
HEK293 cells plated on poly-L-lysine–coated plates were washed twice with “cold” medium, which lacked -methionine and -cysteine and contained 10% dialyzed FBS, and then starved in this medium for 30 min. Cells were radiolabeled in medium containing 125 µCi/ml 13C6-labeled cysteine/methionine (Easysay Express Protein Labeling Mix 35S; PerkinElmer) for 30 min, washed twice with Hanks’ buffered saline solution, and then chased in complete medium containing 75 µM emetine for the indicated times. Cells were harvested, collected by centrifugation, washed in PBS, and lysed in pulse-chase IP buffer (25 mmol/l 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer, pH 7.4, 150 mmol/l NaCl, 5 mmol/l MgCl2, 1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate detergent, and protease inhibitors). Lysates were cleared by centrifugation at 20,000 × g for 15 min at 4°C and protein concentrations determined using the BCA assay. Lysates were pre-cleared with protein G beads (EMD Millipore). CD147 was immunoprecipitated from lysates by incubation with anti-CD147 antibody (8D6; Santa Cruz biotechnology) for 4 h at 4°C with mixing, followed by incubation with protein G beads (EMD Millipore) for an additional 2 h at 4°C with mixing. Immunoprecipitated proteins were washed thrice with the pulse-chase IP buffer and then separated by SDS-PAGE. Gels were dried and exposed to a Storage Phosphor Screen (GE Healthcare Life Sciences) for 16 h at room temperature. Radioactive signals corresponding to CD147(Mat.) and CD147(CG) were detected using a Typhoon 9400 Molecular Imager (GE Healthcare Life Sciences).

Lipidomic profiling
HEK293 cells were grown to 70% confluence in a 10-cm dish and treated for 16 h with vehicle or 1 µg/ml triacsin C. Cells were washed twice with PBS and harvested, and cell pellets were stored at −80°C. Lipid metabolite extraction and analysis by SRM-based LC-MS/MS was performed as previously described (Benjamin et al., 2013, 2015; Mulvihill et al., 2014). Briefly, nonpolar lipid metabolites were extracted in 2:1:1 chloroform/methanol/PBS supplemented with internal standards C12:0 dodecylglycerol (10 nmol) and pentadecanoic acid (10 nmol). The organic and aqueous layers were collected after separation by centrifugation at 1000 × g for 5 min. The aqueous layer was acidified by addition of 0.1% formic acid and subjected to a second chloroform extraction. The resulting organic layers were combined and mixed, dried down under N2, and dissolved in 120 µl of chloroform. A 10-µl aliquot was analyzed by SRM LC-MS/MS. Metabolites were separated using a Luna reverse-phase C5 column (Phenomenex), and MS analysis was performed on an Agilent 6430 QQQ LC-MS/MS. Quantification of metabolites was performed by integrating the area under the peak, normalized to internal standard values, adjusted based on external standard curves, and expressed as relative levels compared with the control sample.

XBP1 splicing assay
RNA was isolated using TRizol Reagent (Life Technologies) and cDNA generated using the High-Capacity cDNA Reverse Transcription Kit
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