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Establishing the lipid droplet proteome: Mechanisms of lipid droplet protein targeting and degradation

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Abstract

Lipid droplets (LDs) are ubiquitous, endoplasmic reticulum (ER)-derived organelles that mediate the sequestration of neutral lipids (e.g. triacylglycerol and sterol esters), providing a dynamic cellular storage depot for rapid lipid mobilization in response to increased cellular demands. LDs have a unique ultrastructure, consisting of a core of neutral lipids encircled by a phospholipid monolayer that is decorated with integral and peripheral proteins. The LD proteome contains numerous lipid metabolic enzymes, regulatory scaffold proteins, proteins involved in LD clustering and fusion, and other proteins of unknown function. The function of LDs is inherently determined by the composition of its proteome and alteration of the LD protein coat provides a powerful mechanism to adapt LDs to fluctuating metabolic states. Here, we review the current understanding of the molecular mechanisms that govern LD protein targeting and degradation.

1. Introduction

Lipid droplets (LDs) are highly conserved, ubiquitous organelles that function as cellular hubs of lipid metabolism [1–3]. Although the mechanisms of LD biogenesis remain incompletely understood, emerging data indicates that LDs are endoplasmic reticulum (ER)-derived organelles [1–3]. The most widely proposed model suggests that neutral lipids (e.g. triacylglycerol [TAG] and cholesterol esters) are deposited between the leaflets of the ER bilayer forming a “lens” structure, and the LD subsequently buds into the cytoplasm from the outer leaflet of the ER [1–3]. The mature cytoplasmic LDs may remain in contact with the ER and/or associate with other organelles in the cell [4]. LDs serve diverse cellular functions, including sequestering toxic lipids [5,6] and acting as dynamic lipid storage depots that enable rapid mobilization of fatty acids for energy [7,8], membrane biosynthesis [9–11], and lipid signaling pathways [12,13]. Notably, dysregulation of LD homeostasis has been implicated in the pathogenesis of numerous diseases [14,15], including diseases associated with an excess of LDs (e.g. obesity, diabetes, and cardiovascular disease) and with a deficiency of LDs (e.g. lipodystrophy and cachexia).

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LDs have a unique ultrastructure, consisting of a central core of neutral lipids encircled by a phospholipid monolayer that is decorated with integral and peripheral proteins [16]. The functions of LDs are intrinsically connected to the composition of the LD proteome, which contains proteins that mediate lipid synthesis and degradation, integrate nutrient signals, and facilitate LD clustering and fusion [17,18]. Regulated alteration of the LD proteome provides a useful mechanism for homeostatic adaptation of LD functions in response to fluctuations in the cellular demand for lipids and energy. Here, we review the current understanding of the molecular mechanisms that govern LD protein targeting and degradation.

2. Establishing the LD proteome

2.1. Challenges in defining the composition of the LD proteome

An accurate inventory of LD proteins is essential for understanding LD functions. The protein composition of LD-enriched buoyant fractions isolated from many species and cell types has been extensively analyzed by proteomics [19–39]. However, this method yields many false positives due to contamination of buoyant fractions with co-fractionating organelles or membrane remnants, which likely reflects physical associations of LDs with the ER [40–44], mitochondria [7,8,45], peroxisomes [46,47], and early endosomes [48]. Indeed, abundant ER proteins (e.g. BiP, Grp94, and PDI) are common contaminants identified in LD proteomic experiments. The most stringent studies analyzing LD proteomes applied a quantitative protein correlation profiling strategy to identify 111 high confidence LD proteins in oleate-treated Drosophila S2 cells [49] and 35 high confidence LD proteins in yeast grown to stationary phase [20]. However, LD proteins present in other organelles in addition to LDs (i.e. dual localization) may not be designated as LD proteins using this approach.

Efforts to define the LD proteome are also complicated by the heterogeneity of LDs, including differences in LD size, function, organelle association, and lipid composition [50]. This heterogeneity in LDs is lost when LDs are purified in bulk for proteomics analysis. A recent study addressed this limitation by further separating bulk buoyant fractions with additional rounds of differential centrifugation, yielding fractions enriched in LDs that differed in size [51]. Interestingly, different sized LDs exhibited differences in their protein composition, association with co-purifying organelles, membrane phospholipid content, and enzymatic activity for phospholipid synthesis *in vitro* [51]. Heterogeneity in the LD proteome composition arises in part from the ordered LD proteome remodeling that occurs during LD biogenesis, maturation, and degradation. For example, treatment of differentiated 3T3-L1 adipocytes with oleate established a temporal gradient of distinct LD subpopulations marked by members of the perilipin (PLIN) family of LD coat proteins, including small PLIN3 and PLIN4-positive LDs, intermediate PLIN2-positive LDs, and large PLIN1-positive LDs [52]. The sequential recruitment of proteins to emerging LDs has also been observed by time-lapse fluorescence microscopy using fluorescent markers of nascent LDs [53]. While long chain acyl-CoA synthetase 3 (ACSL3) was constitutively present at sites of LD biogenesis in the ER, PLIN2 and PLIN3 were recruited to these sites with a delay following the addition of oleate [53]. Alterations in the metabolic state of the cell can induce

functional specialization of existing LDs through the regulated recruitment of effector proteins, such as the recruitment of the TAG-synthesis enzyme glycerol-3-phosphate acyltransferase 4 [GPAT4] to a subpopulation of LDs for LD growth [40] and the recruitment of Rab18 for LD degradation [54,55]. Differences in LD lipid composition can also impact the composition of the associated proteomes (e.g. cholesterol ester-rich versus TAG-rich LDs [39,56]). Finally, the co-fractionation of ER and other organelles with specific populations of differentially sized LDs [51] raises the possibility that differences in LD proteome composition may determine which LDs associate with other organelles, potentially by facilitating the formation of unique membrane contact sites. Thus, given the heterogeneity of LDs, it is important to note that the associated LD proteome may differ depending on the metabolic state of the cell, the biogenesis stage of the LD, and the LD function.

Despite the complications involved in defining the LD proteome, many proteins identified in buoyant fractions have been confirmed to localize to LDs by fluorescence and/or electron microscopy. General classes of validated LD proteins include, but are not limited to, regulatory scaffold proteins (PLIN1-5), TAG biosynthetic enzymes (ACSL3, GPAT4, 1-acylglycerol-3-phosphate O-acyltransferase 3 [AGPAT3], phosphatidic acid phosphatase [PAP], diacylglycerol O-acyltransferase 2 [DGAT2]), TAG lypolytic machinery (adipose triglyceride lipase [ATGL], comparative gene identification 58 [CGI-58], G0/G1 switch gene 2 [G0S2], hormone-sensitive lipase [HSL]), ubiquitination factors (UBX domain-containing protein 8 [UBXD8], ancient ubiquitous protein 1 [AUP1], ubiquitin-conjugating enzyme E2 G2 [UBE2G2]), and Ras-related proteins (Rab8a, Rab18). Cell types that exhibit large LDs often express additional proteins that mediate LD fusion (Cell death activators CIDEA and CIDEC [FSP27] in adipocytes). A future challenge is to develop novel methods to accurately identify LD proteins in heterogenous populations of LDs and to quantify changes in the composition of the LD proteome under different metabolic states.

2.2. Structural elements involved in protein association with LDs

The ultrastructure of the LD imposes biophysical constraints on the type of protein domains that can associate with LDs. The hydrophobic neutral lipid core of LDs is an energetically unfavorable environment for hydrophilic protein domains. As a consequence, the interior of the LD is devoid of proteins, and integral LD proteins associate with the phospholipid monolayer by adopting monotopic conformations. These conformations can be broadly categorized into two classes: proteins that associate with the membrane through hydrophobic domains (Class I) or amphipathic helices (Class II) [57] (Fig. 1). Although the organization of LD proteins into different classes (Class I and Class II) is a generalization and the actual structures that these proteins adopt within membranes are not known, this terminology provides a useful conceptual framework for discussing how proteins associate with the LD membrane.

LD proteins that contain hydrophobic domains (Class I) frequently adopt a hairpin topology, which is defined by a membrane-embedded internal hydrophobic segment that is flanked by cytosolically-accessible, hydrophilic N- and C- termini. Examples of Class I hairpin proteins that traffic from the ER to LDs include UBXD8 [58,59], AUP1 [60], caveolin-1 [61,62],

DGAT2 [63], and GPAT4 [40]. A common feature of many of these hairpin domains is a conserved proline residue positioned in the center of the hydrophobic region. It has been hypothesized that this proline generates a sharp turn in the protein structure, facilitating the formation of an antiparallel helix or β-strand conformation [64,65]. Consistent with the significance of these proline residues, mutation of a triad of prolines in plant oleosin (often referred to as the “proline knot”) abolished its localization at LDs and confined oleosin to the ER [64–66]. Similarly, mutation of two prolines in the hepatitis C viral core protein [66] or a proline-valine-glycine sequence in AUP1 [51] prevented their trafficking from the ER to LDs. Mutations of the proline knot in oleosin did not impact its topology [64], but mutation of the proline-valine-glycine sequence in AUP1 [51] or the central proline residue in caveolin-1 [67] converted them into single-pass transmembrane proteins, indicating that these residues can play a critical role in determining the final topology. However, a proline within the hydrophobic region is not an absolute requirement for trafficking of Class I proteins to LDs. Mutation of a central proline residue in the hydrophobic regions of AAM-B, ALDI, and CYB5R3 did not affect their LD localization [68]. The hydrophobic regions of these proteins are at their extreme N-termini, raising the possibility that the position of the hydrophobic region determines the structure that these regions adopt as well as the importance of the proline. It should be noted that while the hydrophobic region of Class I LD proteins is generally necessary and sufficient for LD targeting, trafficking of some proteins from the ER to LDs is impacted by flanking positively-charged sequences [60,62]. The significance of these regions is unclear, but they may mediate interactions with specific negatively charged lipid headgroups or proteins in the LD monolayer.

A second major class of LD proteins (Class II) associates with the LD membrane via amphipathic helices. Examples include CTP:phosphocholine cytidylyltransferase (CCT) [69,70], CIDEA [71], the perilipin family of proteins [72], and the antiviral protein viperin [73]. The mechanism by which Class II proteins selectively target the LD membrane, as opposed to other cellular membranes, is an active area of investigation. Current models propose that amphipathic helices within Class II proteins confer the ability to “sense” certain properties of the LD membrane. Indeed, emerging findings suggest that distinct physiochemical properties of organelle membranes can specify the types of proteins motifs that can bind to them [74]. In contrast to the membranes of the plasma membrane and later portions of the secretory pathway, the ER and LD membranes are characterized by low levels of negatively charged phospholipids (e.g. phosphatidylserine) and a high degree of lipid packing defects, caused by the presence of unsaturated fatty acids and the general lack of cholesterol [74]. The amphipathic helix of CCT, the rate-limiting enzyme in phosphatidylcholine synthesis, and its association with membranes have been studied in detail [75]. Consistent with an important role for lipid packing in conferring binding specificity to LD proteins, CCT is activated by binding to membranes that contain high levels of lipids with smaller headgroups (e.g. diacylglycerol and phosphatidylethanolamine), which have a conical shape that increases lipid packing defects and membrane curvature strain [65–68]. In solution, CCT’s amphipathic helix binds to its active site and inhibits its activity [75]. Insertion of the amphipathic helix into a membrane exhibiting packing defects, such as a membrane with low levels of phosphatidylcholine, anchors CCT to the membrane and relieves the helix-mediated autoinhibition of its enzymatic activity [75]. Coupling of

CCT activation to membrane binding provides an elegant feedback mechanism that regulates LD size [69,70,76]. In Drosophila S2 cells, CCT relocalizes from the nucleus to LDs during LD expansion, when levels of phosphatidylcholine in the LD monolayer become limiting [69,70,76]. Binding of CCT to LDs increased synthesis of phosphatidylcholine at the monolayer, which reduced membrane surface tension and prevented fusion between LDs [69,70,76]. In contrast to Drosophila, mammalian cells contain two CCT isoforms, CCTα and CCTβ. CCTα was recruited to the surface of expanding LDs in murine macrophages [69], suggesting that the role of CCT in LD growth is conserved from insects to mammals. However, a recent study found that while CCTα activity was important for LD expansion, CCTα was not recruited to LDs in differentiating 3T3-L1 adipocytes or a wide variety of non-adipocyte cell types incubated with oleate [77]. One explanation that was proposed for these apparent contradictory results is that Drosophila S2 cells have significantly different phosphatidylcholine: phosphatidylethanolamine ratios, resulting in more recruitment of CCT to LDs during LD expansion [77]. Irrespective of the site of CCT action, the subsequent and final step in phosphatidylcholine synthesis is mediated by choline phosphotransferase (hCPT1, Golgi localized) or choline/ethanolamine phosphotransferase 1 (hCEPT, ER and nuclear envelope localized) [78].

Perilipins are another important family of Class II LD proteins that integrate nutrient signals and control lipid storage in LDs [79]. PLIN1 is translated by cytosolic free ribosomes [72,80,81] and thought to be directly inserted into LD membranes. It is generally assumed that all perilipins are translated in the cytosol and directly targeted to LDs, but experimental evidence is lacking. The perilipins can compete with each other for LD localization [79,82], indicating that sites for their insertion and/or association with LDs are limited. A conserved series of 11-mer repeats in the perilipins form an extended amphipathic helix that is sufficient for recruitment to LDs [72]. Mutations in this region that affect the amphipathic nature or helix register significantly reduce LD localization [72]. Most perilipins also contain a C-terminal helical bundle that is analogous to the receptor-binding region of apolipoprotein E [72,83]. This region contains a deep hydrophobic cleft that may bind specific lipids and contribute to LD localization under certain conditions [72,83].

Although hydrophobic hairpins and amphipathic helices are the most common motifs that target proteins to droplets, other mechanisms have been described (Fig. 1). ELMOD2 [84] and SNAP-23 [85] are anchored to droplets through a palmitate group. Since palmitoylation is a reversible, post-translational modification, it possible that the LD localization of these proteins could be controlled by the addition and/or removal of palmitate. Another alternative LD targeting mechanism is utilized by the ATGL activator CGI-58, which employs a unique membrane-embedded motif [86]. Solution state NMR of a CGI-58 peptide embedded in a dodecylphosphocholine micelle indicates that this region of CGI-58 forms a hydrophobic core consisting of two short arms containing critical tryptophan residues, mutation of which abolishes LD localization in cells [86]. Finally, several proteins are recruited to LDs through direct interactions with Class I and II LD proteins. Examples include recruitment of UBE2G2 by the UBE2G2-binding motif of AUP1 [87,88], VCP by the UBX domain of UBXD8 [59,89], and HSL by the phosphorylated N-terminus of PLIN1 [90,91].

2.3. Insertion of LD proteins into the ER

Most, if not all, Class I LD proteins that have been studied exhibit dual ER and LD localization. During LD biogenesis, Class I proteins inserted into the ER have been proposed to traffic to nascent LDs by diffusing within the outer leaflet of the ER membrane, which is likely contiguous with the monolayer of forming LDs [53] (Fig 1). Proteins can also traffic from the ER to mature LDs through membrane bridges that link the two organelles [40]. The canonical pathways for insertion of proteins into the ER membrane [92–95] include: 1) the signal recognition particle (SRP) pathway, which mediates co-translational insertion of proteins bearing N-terminal signal sequences or transmembrane domains through the Sec61 translocon, and 2) the guided entry of tail-anchored proteins (GET) pathway, which mediates posttranslational insertion of proteins bearing C-terminal transmembrane domains. Compared to well-characterized SRP and GET pathway substrates, insertion of Class I hairpin proteins poses a unique biophysical challenge and may require a different set of insertion machinery. However, the insertion of caveolin-1 into rough microsomes *in vitro* and the insertion of heterologously expressed oleosin into the ER in yeast required SRP and Sec61 [61,96], suggesting that at least a portion of Class I LD proteins employ the SRP-Sec61 ER insertion pathway (Fig. 2A).

A novel posttranslational ER insertion pathway was recently discovered for the Class I LD protein UBXD8 [97] (Fig. 2B). *In vitro* translated UBXD8 was inserted posttranslationally into purified rough ER microsomes, independently of both the SRP and GET pathways [97]. In cells, insertion of UBXD8 into discrete ER subdomains was mediated by the soluble peroxisome biogenesis factor PEX19, which bound the hairpin of UBXD8 and functioned in concert with its ER membrane receptor PEX3 [97] (Fig. 2B). No insertion of UBXD8 directly into LDs was observed [97], consistent with the model that Class I LD proteins insert first into the ER and then traffic to LDs. Furthermore, insertion of UBXD8 into ER puncta was unaffected by inhibition of acyl-CoA synthetases with triacsin C, suggesting that these puncta are not nascent LDs. Deletion of PEX19, depletion of PEX3, or disruption of the PEX19-PEX3 interaction impaired UBXD8 ER insertion and resulted in aberrant insertion of UBXD8 into mitochondria [97]. Together, these results provide strong evidence that support the importance of the PEX19-PEX3 pathway for mediating insertion of hairpin proteins such as UBXD8, and potentially other Class I proteins, into the ER. The UBXD8 hydrophobic region is composed of ~20 amino acids that are predicted by bioinformatic algorithms to form a transmembrane helix. Employing a posttranslational insertion pathway could be one mechanism to ensure that proteins are inserted in a hairpin conformation, rather than an ER-restricted, single-pass transmembrane conformation. Such a pathway could also mediate insertion of hairpin proteins into ER subdomains that lack ribosomes and Sec61 translocation machinery (i.e. smooth ER).

The PEX19-PEX3 pathway also mediates the insertion of a subset of peroxisomal proteins and is required for peroxisome biogenesis [98]. Interestingly, UBXD8 ER insertion required PEX19 farnesylation and strong overexpression of a PEX19 farnesylation-defective mutant resulted in mislocalization of UBXD8 to peroxisomes [97]. Given that the PEX19 farnesylation was found to be dispensable for peroxisome biogenesis [99], farnesylation may function as a master switch that controls the sorting of proteins to LDs and not peroxisomes

[97]. However, a recent study found that PEX19 farnesylation is required for the insertion of a subset of peroxisomal membrane proteins, possibly by inducing a conformational change in PEX19 that increases its affinity for its substrates [100]. Thus, while farnesylation is clearly important for UBXD8 targeting [97], the precise role of PEX19 farnesylation in controlling LD and peroxisome protein sorting is unclear. Given that both LDs and peroxisomes are involved in lipid metabolism, the shared PEX19-PEX3 insertion pathway could provide an important mechanism to coordinate biogenesis or functional crosstalk between the two organelles. Since UBXD8 is the only protein known to employ PEX19-PEX3 for insertion into LDs, it will be important to determine if other LD proteins generally employ this pathway. In addition, a new SRP-independent targeting (SND) co-translational insertion pathway was recently discovered in yeast that inserts a broad range of ER proteins, with an intriguing preference for proteins bearing internal hydrophobic domains [101]. However, the role of SND proteins in mammalian cells and in inserting LD proteins is unknown.

2.4. ER-LD protein sorting: Generating asymmetry in contiguous membranes

The insertion of LD proteins into the ER and the diffusion of proteins between LDs and the ER via membrane bridges present the cell with a fundamental protein sorting challenge. This raises the question as to how the proteomes of these two highly connected organelles are established and maintained. While proteins within the ER lumen are spatially segregated from cytosolic LDs, sorting mechanisms likely control the relative distribution of proteins between contiguous ER and LD membranes. The biophysical properties of the ER phospholipid bilayer and the LD phospholipid monolayer, the latter of which is unable to accommodate bitopic and polytopic ER membrane proteins, likely constitute a primary sorting mechanism. The deposition of TAG between the leaflets of the ER membrane, which forms the LD “lens”, alters the bilayer height and would thus generate asymmetry through its inability to accommodate transmembrane domain-containing proteins, due to hydrophobic mismatch (i.e. a difference in the hydrophobic thickness of the membrane bilayer and the length of the hydrophobic portion of a transmembrane portion). As the LD emerges, a protein topology-selective diffusion barrier would be created by the junction between the ER phospholipid bilayer and LD phospholipid monolayer (Fig. 2C). This junction would permit monotopic proteins, such as Class I proteins, to traffic into the phospholipid monolayer of LDs. In contrast, transmembrane proteins that cannot be physically accommodated in the LD phospholipid monolayer would remain by default in the ER phospholipid bilayer. At the junction of other organelles connected by contiguous membranes there are often proteins that regulate the diffusion barriers to maintain the correct proteomes, such as septin 2 at the junction between the plasma membrane and primary cilium [102] and the nuclear pore complex at the junction between the inner and outer nuclear membranes [103]. Seipin is positioned at ER-LD contact sites [41,42,44,104], but whether seipin or another protein acts as a “gatekeeper” to control protein diffusion between the ER and LDs is unknown.

A diffusion barrier model based solely on topology predicts that Class I proteins would equilibrate between the ER and LDs. However, this prediction is inconsistent with the observed distributions of Class I proteins between the two organelles. Some proteins that

traffic through the ER are enriched almost exclusively in LDs (e.g. AAM-B [68,105] and GPAT4 [40]), while others localize to both ER and LDs (e.g. UBXD8 [89]). This asymmetry in protein distribution may be generated by differences in mobility within a particular organelle, which could be imposed by the preference for a binding partner (or lipid region) that is present exclusively at one organelle and not the other (Fig. 2C). For example, the polytopic ER-resident protein UBAC2 binds to UBXD8 in the ER and blocks its trafficking to LD [89]. This indicates that UBAC2 binding functions as a dominant signal for retention of UBXD8 in the ER. Membrane curvature may also facilitate protein segregation between membranes. Membrane curvature is likely increased at the TAG-rich "lens" structure that forms between the leaflets of the ER at sites of LD biogenesis. The specific lipid composition of membranes and/or the presence of lipid packing defects at these sites may constitute a more favorable environment for LD proteins relative to bulk ER. While this mechanism has not been directly addressed, it has been noted that certain LD proteins, such as ACSL3 and truncated fragments of AAM-B (i.e. HPos) and GPAT4 (i.e. livedrop), become concentrated at LD biogenesis sites in the ER [41,53]. Finally, differences in protein stability may also contribute to the asymmetric distribution of proteins between organelles (Fig. 2C). Many LD proteins are strongly stabilized in cells treated with oleate, suggesting that their stability is increased by insertion into LDs. In addition, degradation of some Class I LD proteins in the ER results in their relative enrichment on LDs [106,107].

Thus, emerging findings support a model in which the proteomes of the contiguous ER and LDs is determined by a topology-selective diffusion barrier as well as by differences in protein mobility and stability between the two organelles. It is possible that targeted insertion of LD proteins into specialized ER subdomains (e.g. PEX19-PEX3-mediated insertion of UBXD8) could also contribute to the enrichment of LD proteomes within specific regions of the ER. Given the heterogeneity in LD proteomes (e.g. GPAT4 trafficking to select LDs), there must be mechanisms to target LDs to select LDs or to remove proteins from select LDs.

3. Connections between LDs and the ubiquitin-proteasome system

3.1. Protein degradation: General concepts in protein quality and quantity control

Protein degradation is essential for maintaining the fidelity and appropriate composition of the cellular proteome [108]. Organelles contain specialized pathways (e.g. ER-associated degradation [ERAD] [109–111]) that mediate the recognition of their respective proteomes for degradation. These include quality control pathways that degrade misfolded and damaged proteins (e.g. mutant proteins involved in human diseases) as well as quantity control pathways that degrade correctly folded, functional proteins in order to regulate cellular processes (e.g. metabolic enzymes such as HMG-CoA Reductase in cholesterol biosynthesis) [109–111]. Covalent modification of substrate proteins with a polyubiquitin chain is the canonical signal that targets substrates to the 26S proteasome for proteolytic degradation [112]. In this pathway, sequential enzymatic reactions performed by an E1 ubiquitin-activating enzyme, an E2 ubiquitin-conjugating enzyme, and an E3 ubiquitin-protein ligase (E3 ligase) conjugate ubiquitin to one or more lysines, the N-terminus, or, in seemingly rare cases, serines or threonines in target proteins [112]. Successive conjugation

of ubiquitin to any of the seven lysines (K6, 11, 27, 29, 33, 48, 63) within the preceding ubiquitin forms structurally distinct polyubiquitin chains that have diverse signaling roles, such as in the regulation of protein interactions, trafficking, and enzymatic activity [101]. K48-linked polyubiquitin chains, and in some cases K11 and K63 linkages, target proteins to the proteasome [101]. Hundreds of E3 ligases and associated adaptor proteins provide specificity to the ubiquitin conjugation reaction, mediating the recognition and ubiquitination of distinct sets of substrates [112]. However, the substrates of the vast majority of E3 ligases have not been elucidated.

3.2. Ubiquitin-dependent degradation of LD proteins

Several LD proteins are degraded by the ubiquitin-proteasome system (UPS) under conditions where the flux of lipids into cells and the overall LD abundance are low (e.g. cells cultured in the absence of fatty acid supplementation) (Table 1). Induction of LD biogenesis with oleate increases the stability of many of these proteins, including the perilipins PLIN1 [113] and PLIN2 [82,114], the TAG lipase ATGL [89,115], the LD fusion mediator FSP27 [116,117], and the ATGL inhibitor G0S2 [118]. Linking the stability of the LD proteome to the formation of LDs is a clever mechanism that enables rapid LD biogenesis in responses to fluctuations in intracellular lipid abundance without the need to initiate compensatory gene expression programs. The clearance of lipolytic proteins under low LD conditions may also be a regulatory mechanism that ensures that TAGs are not hydrolyzed when energy stores are low.

The mechanisms that target LD proteins for degradation are mostly unknown. One possibility is that insertion of LD proteins into the LD monolayer “masks” degrons (i.e. degradation signals) that are recognized by the UPS in the absence of a membrane (Fig. 3). These degrons may also become accessible to UPS machinery when molecular crowding forces LD proteins to disassociate from monolayers, for instance during lipolytic degradation of LDs [119]. Consistent with the “masking” model, deletion of PLIN1 stabilized LD-associated PLIN2 and increased its half-life [82,120], indicating that PLIN2 levels are in part controlled through competition with PLIN1 for limited binding sites on the LD monolayer. Mutation of the N-terminal alanines of PLIN2 stabilized the protein in the absence of LDs, leading the authors to propose that these residues constitute an N-end rule degron [82]. However, whether PLIN2 is degraded via the N-end rule degradation pathway remains to be experimentally determined. Alternatively, disordered amphipathic helices in PLIN1 and PLIN2 could function as degrons in the absence of a monolayer. Indeed, a purified amphipathic helix from PLIN1 is unstructured in solution and only gains α-helical structure when incubated with membranes, and mutations that disrupted PLIN1’s binding to LDs significantly accelerated its degradation in yeast [72]. A degron consisting of a consensus motif that binds to the cytosolic/nuclear E3 ligase COP1 (also known as RFWD2) was recently found in ATGL. In this study, COP1 was proposed to regulate ATGL stability by ubiquitinating at least one lysine in the ATGL patatin-like domain [115] Degrons may also be masked by high affinity interactions between proteins at the LD surface. Indeed, binding of ATGL to G0S2 [118] and PLIN1 to CGI-58 [120] impaired the proteasomal clearance of their respective binding partners. Thus, in the absence of LDs, exposed degrons may target LD-associated proteins to the proteasome. However, levels of UBXD8 and

AUP1, proteins that are present at LDs and the ER, are not affected by the presence of LDs [89,119], indicating that the relationship between LD abundance and protein stability does not extend to all LD proteins.

The diffusion of proteins between LDs and the ER through extensive contact sites [76] raises the possibility that degradation of some LD proteins may occur in the ER (Fig. 3). Consistent with this possibility, a recent study in yeast demonstrated that hairpin motifs were sufficient to target proteins for degradation through ERAD [106]. ERAD enables the cytosolic degradation of proteins from the early secretory pathway by mediating their recognition, ubiquitination, and subsequent extraction from the ER membrane by the AAA ATPase VCP (Cdc48 in yeast) [109–111]. Deletion of the yeast, polytopic ERAD E3 ligase Doa10p or its cognate E2 ubiquitin-conjugating enzymes Ubc6p or Ubc7p stabilized the Class I LD protein Pgc1p in the ER [106]. Similarly, expression of a temperature sensitive Cdc48 mutant also stabilized Pgc1p [106]. Two additional Class I LD proteins, Dga1p and Yeh1p, were identified as substrates of Doa10p [106]. Intriguingly, a Pgc1p chimera in which the hairpin region was replaced with the heterologous hairpin region from mammalian GPAT4 was similarly unstable in the absence of LDs and degraded through a Doa10p-dependent pathway [106], suggesting that hairpin conformations are recognized as degrons by Doa10p. Together, these data provide the first evidence that ERAD mediates the degradation of LD proteins. An additional mechanism for regulating early steps in LD formation could be the stabilization of machinery necessary for LD growth and budding at predetermined sites. This idea is supported by a study in mammalian cells which demonstrated that overexpressed Class I LD protein DGAT2, a TAG-synthesis enzyme that functions in LD expansion, is degraded through an ERAD pathway that uses the E3 ligase gp78 [107]. These findings support the hypothesis that degradation of LD proteins by ERAD is a mechanism that maintains the composition of the ER proteome, contributes to the relative enrichment of ER proteins at LDs or sites of LD biogenesis, and degrades LD proteins during lipolysis. How general the role of ERAD is in degrading LD proteins is and whether this pathway of LD protein degradation is regulated remains to be determined.

Ubiquitin-dependent degradation of proteins directly from LDs (Fig. 3) is another mechanism that could regulate the composition of the LD proteome. Interestingly, proteins that function in ubiquitination pathways or that contain ubiquitin binding motifs have been observed on LDs (Table 2), raising the possibility that these proteins interact to form a functional LD-associated ubiquitination complex. Several Class I LD proteins recruit soluble ubiquitination factors to the LD surface; UBXD2 and UBXD8 recruit the membrane extraction factor VCP [59,89], AUP1 recruits the E2 ubiquitin-conjugating enzyme UBE2G2 [87,88], and spartin (also known as SPG20) recruits the E3 ligases AIP4 and AIP5 [121–123]. Some of these proteins are functional components (AUP1, UBE2G2, UBXD2, UBXD8, VCP) [124] of multimeric ERAD complexes in the ER and could have analogous functions in LDs. However, whether these factors contribute to the ubiquitination and/or degradation of LD proteins has not been established. Spartin has been reported to mediate the ubiquitination of PLIN2 by recruiting AIP4, as overexpression of wild-type spartin but not spartin lacking an AIP4-interaction motif (PPXY motif) increased ubiquitination of PLIN2 [121]. Whether AIP4 ubiquitination of PLIN2 requires other ubiquitination machinery in addition to spartin is unknown. A functional ubiquitination complex is

supported by data showing that purified buoyant fractions enriched in LDs contained polyubiquitinated proteins and exhibited activity in an *in vitro* ubiquitination assay [83]. However, buoyant fractions are heavily contaminated with ER, raising the possibility that ubiquitination machinery associated with ER and not LD was the source of the observed activity. Thus, identification of ubiquitinated proteins at LDs and an understanding of how the ubiquitination machinery is regulated are important future research directions.

3.3. A role for LDs in the degradation of non-LD proteins

The presence of ERAD machinery on LDs led to the hypothesis that LDs may be functionally involved in ERAD. Several models have been proposed: i) LDs function as an "escape hatch" that facilitates the dislocation of ERAD substrates from the ER [125], ii) ERAD occurs in LD-associated ER subdomains [126,127], iii) ERAD substrates transiently localize to the LD surface prior to proteasomal degradation [59,128,129]. In agreement with the potential importance of LDs for ERAD, inhibition of LD biogenesis with the long chain acyl-CoA synthetase inhibitor triacsin C stabilized several ERAD substrates in mammalian cells [87,126,127]. However, the degradation kinetics of multiple ERAD substrates were not affected in a yeast strain lacking the neutral lipid synthesis machinery necessary to make LDs [130,131]. Moreover, depletion of LDs in mammalian cells through inhibition of the TAG synthesis enzymes DGAT1 and DGAT2 also did not impair ERAD of the endogenous substrate CD147 [132]. Instead, this study found that triacsin C impaired CD147 glycan trimming [132], an early step in ERAD that results in the generation of degradation signal recognized by ERAD-implicated lectins [133,134]. Other studies have demonstrated that some ERAD substrates accumulate in LD-enriched buoyant fractions when ERAD is compromised, potentially indicating that these proteins traffic through LDs or ER-associated buoyant compartments on route to the proteasome. One such ERAD substrate is HMG-CoA reductase, the rate-limiting enzyme in cholesterol biosynthesis, which is stabilized in buoyant fractions and ER sites juxtaposed to LDs following knockdown of VCP or inhibition of the proteasome [126,127]. Interestingly, an HMG-CoA reductase lysine mutant that was resistant to ubiquitination appeared in the buoyant fraction, suggesting that trafficking into this compartment precedes its extraction by VCP into the cytosol, a step that canonically requires prior ubiquitination of the substrate [126]. Another ERAD substrate is Apolipoprotein B-100 (ApoB), a component of very low density lipoproteins (VLDL) and low density lipoproteins (LDL). ApoB was observed in a crescent-shaped compartment comprised of ER wrapped around one hemisphere of an adjoining LD in cultured hepatocellular carcinoma cells [135]. Knockdown of UBXD8 resulted in the accumulation of ApoB in crescents [59], suggesting a possible role of LD-associated UBXD8 in the degradation of ApoB from the crescent compartment. Thus, although LDs are not fundamentally required for ERAD, a subset of ERAD substrates may transit through compartments that are in close proximity to LDs prior to undergoing proteolysis by cytosolic proteasomes. However, the precise requirement for these compartments in ERAD has not been established.

3.4. Degradation-independent roles for ubiquitination on LDs

Several studies raise the possibility that ubiquitin may have roles on LDs independent of protein degradation [112]. Ubiquitin can mediate interactions with proteins containing

ubiquitin-binding domains (e.g. CUE, UBA, and UIM domains), such as AUP1, which contains a CUE domain [87,136]. AUP1 was also found to be monoubiquitinated and diubiquitinated (or monoubiquitinated on multiple lysines) [87,136]. Interestingly, overexpression of AUP1 was sufficient to induce LD clustering [136]. The clustering phenotype was dependent on the ubiquitination status of AUP1, since overexpression of a CUE domain mutant that was not ubiquitinated did not induce clustering, but fusion of this mutant to ubiquitin at its C-terminus rescued AUP1-induced clustering [136]. Similarly, ubiquitination of Drosophila LD protein CG9186 in Kc167 cells was required for LD clustering induced by CG9186 overexpression [137,138]. A model that explains the clustering phenotype observed in these studies is that ubiquitinated AUP1 or CG9186 promotes the formation of ubiquitin-dependent homo- or hetero-dimers that bridge adjacent LDs. The physiological importance of the clustering phenomenon is unclear, but it has been suggested that LD clustering precedes LD fusion [136,139,140].

LD-associated VCP may also function outside of its well characterized role in ERAD [141]. VCP, and its yeast ortholog CDC48, also mediate the disassembly of ubiquitinated protein complexes [141–143]. Interestingly, depletion of the VCP adaptor UBXD8 from LD increased the association of ATGL with its activator CGI-58 [85], indicating that VCP may function in remodeling protein-protein interactions on droplets. Although ATGL is degraded by the UPS in the absence of LDs, recruitment of VCP to LDs by overexpression of UBXD8 had no effect on ATGL levels, suggesting that UBXD8 and VCP regulate the activity, but not the stability of ATGL [85]. Additional studies are needed to elucidate the role of this important protein at LDs.

4. Autophagy and LDs

4.1. Lipophagy: The selective autophagic degradation of LDs

Macroautophagy is a process that mediates the engulfment of portions of cytoplasm within double-membrane organelles called autophagosomes [144]. These organelles fuse with acidic lysosomes, forming autolysosomes that hydrolyze sequestered cytoplasmic contents [144]. Lipophagy refers to the selective autophagic degradation of LDs, which leads to degradation of LD lipids and proteins. Lipophagy has been recently reviewed [145–148] and is beyond the scope of this review. Here, we will focus on autophagic mechanisms that govern the targeted clearance of select LD proteins.

4.2. Chaperone-mediated autophagy of select LD proteins

Chaperone-mediated autophagy (CMA) mediates the delivery of a subset of proteins bearing a pentapeptide motif (KFERQ or a related sequence) to the lysosome for degradation. In this process, heat shock cognate protein of 70 kDa (hsc70) recognizes and delivers the substrate to the lysosome-associated membrane protein 2A (LAMP-2A), which forms a multimeric complex that transports unfolded substrates into the lysosome lumen for degradation. Mice lacking LAMP-2A exhibit defects in CMA and have profound accumulation of fat in the liver [149,150], suggesting that CMA controls lipid metabolism. Interestingly, CMA motifs were identified in PLIN2 (LDRLQ) and PLIN3 (SLKVQ), and both proteins were degraded in mouse embryonic fibroblasts through a pathway that required hsc70 and LAMP-2A [150].

Furthermore, mutation of the CMA motif in PLIN2 impaired binding of hsc70 to PLIN2 and increased its steady state levels [150]. Disruptions in CMA-mediated degradation of PLIN2 and PLIN3 reduced LD degradation through both ATGL-dependent lipolysis and macroautophagy [150], suggesting that the removal of perilipins by CMA increases lipolysis of lipid stores by ATGL in LDs and increases recognition of LDs by autophagic machinery. This is consistent with a previous finding that PLIN2 can compete with ATGL for binding to LDs [151]. Interestingly, PLIN2 phosphorylation by AMP-activated protein kinase (AMPK) may prime PLIN2 for degradation by CMA, coupling the degradation of this important regulator of LD function to the energy status of the cell [150,152].

5. Perspectives and conclusions

LDs contain a unique proteome that places this organelle at the center of cellular lipid and energy homeostasis. In recent years, many studies have attempted to answer questions regarding how the LD proteome is established and regulated. Bioinformatics approaches have not been successful at predicting which proteins are targeted to LDs and new empirical approaches are still under development. However, general mechanisms based on membrane biophysics and the types of monotopic conformations that can be accommodated within monolayers have now become appreciated. Likewise, the mechanisms that regulate the abundance of LD proteins are complex, likely involving multiple UPS pathways in separate subcellular compartments as well as autophagic pathways. Our understanding of these pathways is still in its infancy and many questions remain as to the nature of the degradation signals presented by the substrates, the identities of the ubiquitination components within the degradation pathways, and the regulation of these pathways in different cell types and under different metabolic states. Ultimately, a comprehensive understanding of how the LD proteome composition is established is essential to elucidate the role of LDs in human disease. This understanding will require extensive research into the regulation of LD protein targeting and clearance.

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Highlights

- Lipid droplet function is inherently connected to its proteome composition
- Proteins target to lipid droplets from the ER and cytoplasm
- Proteins employ multiple mechanisms for lipid droplet association
- A diffusion barrier contributes to lipid droplet protein sorting in the ER
- Lipid droplet proteins can be degraded by the proteasome and by autophagy

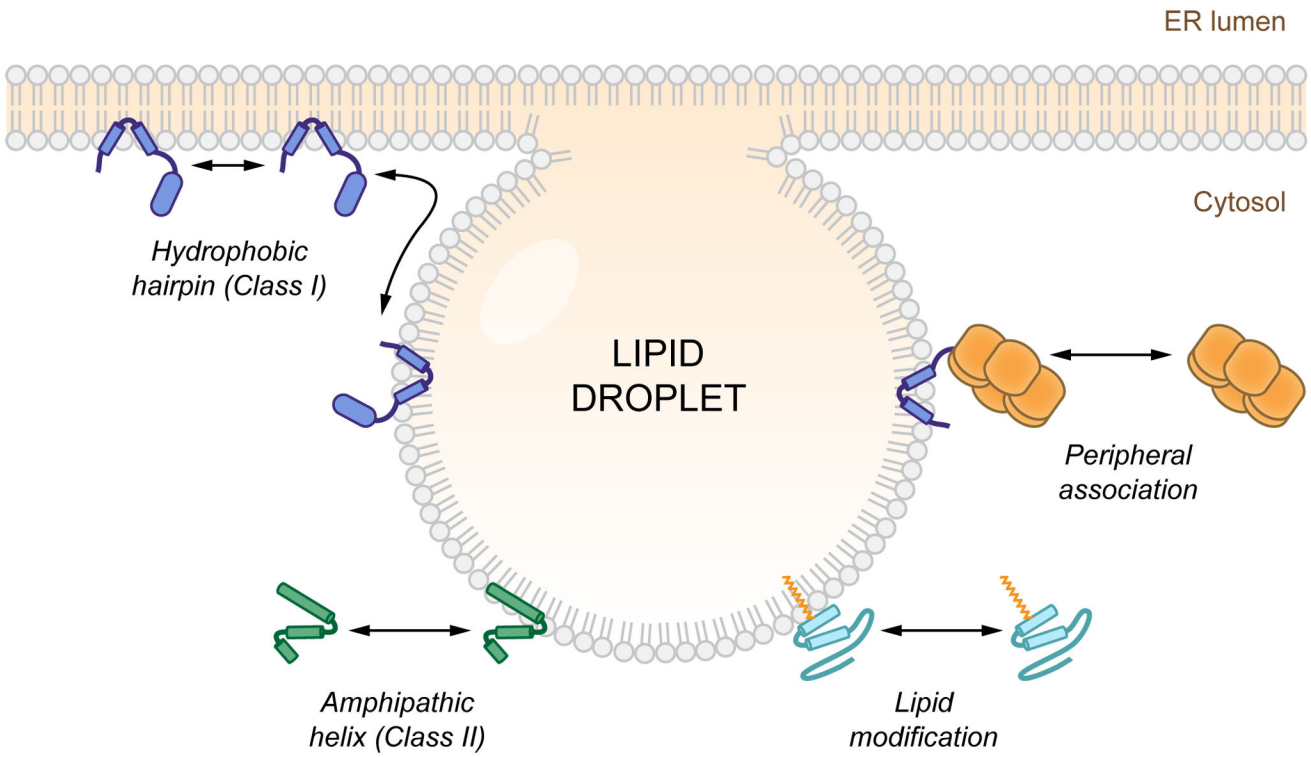

Fig. 1.

The LD proteome: Structural features and targeting pathways. Proteins utilize a variety of mechanisms for association with LDs, including insertion into the membrane via hydrophobic hairpin structures (Class I), amphipathic helices (Class II), association with the membrane via lipid modifications, and indirect recruitment via interactions with integral proteins. Class I proteins insert into the ER and are subsequently trafficked to LDs.

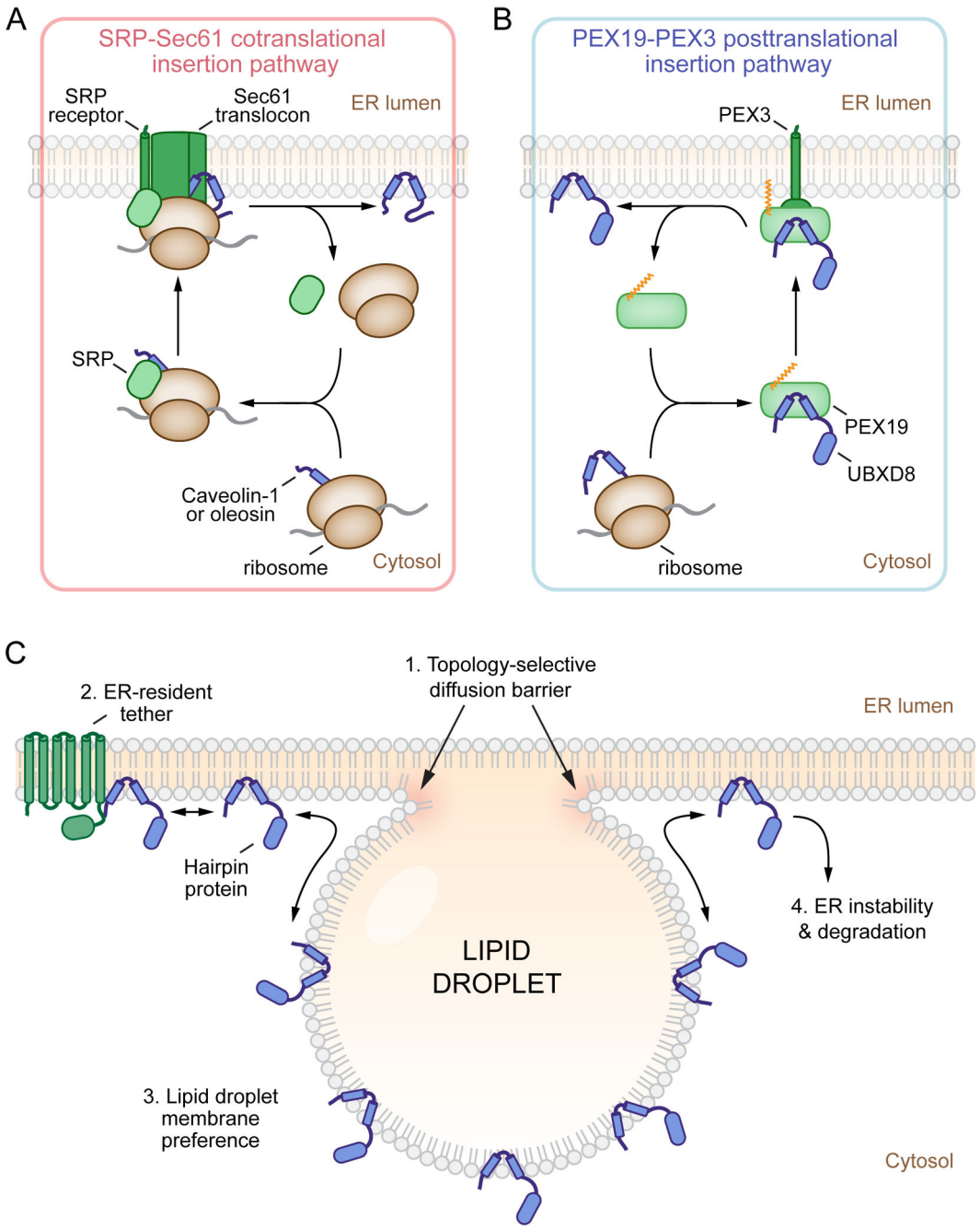

Fig. 2.

Mechanisms of LD protein insertion into the ER and ER-LD protein sorting. (A) Two pathways have been implicated in the insertion of Class I LD proteins into the ER. The canonical SRP-Sec61 pathway (*red box*) mediates the insertion of caveolin-1 and oleosin. In this pathway, SRP recognizes the hydrophobic region as it emerges from the ribosome and translation is stalled. SRP docks with the ER-resident SRP receptor, facilitating ribosome association with the Sec61 translocon, and the proteins are cotranslationally inserted into the ER. (B) The PEX19-PEX3 pathway (*blue box*) mediates the insertion of UBXD8. PEX19

binds the hydrophobic hairpin region of UBXD8. Through association with its receptor PEX3, farnesylated PEX19 then mediates the posttranslational insertion of UBXD8 into discrete ER subdomains. (C) Multiple mechanisms contribute to the sorting of ER and LD proteomes, including *1*) a topology-selective diffusion barrier formed by the junction of the LD monolayer and the ER bilayer membranes, *2*) protein interactions with ER-resident proteins, which function as tethers and impact ER motility, *3*) protein domains that have an inherent preference for the LD monolayer, and *4*) instability in the ER, facilitating enrichment in LDs.

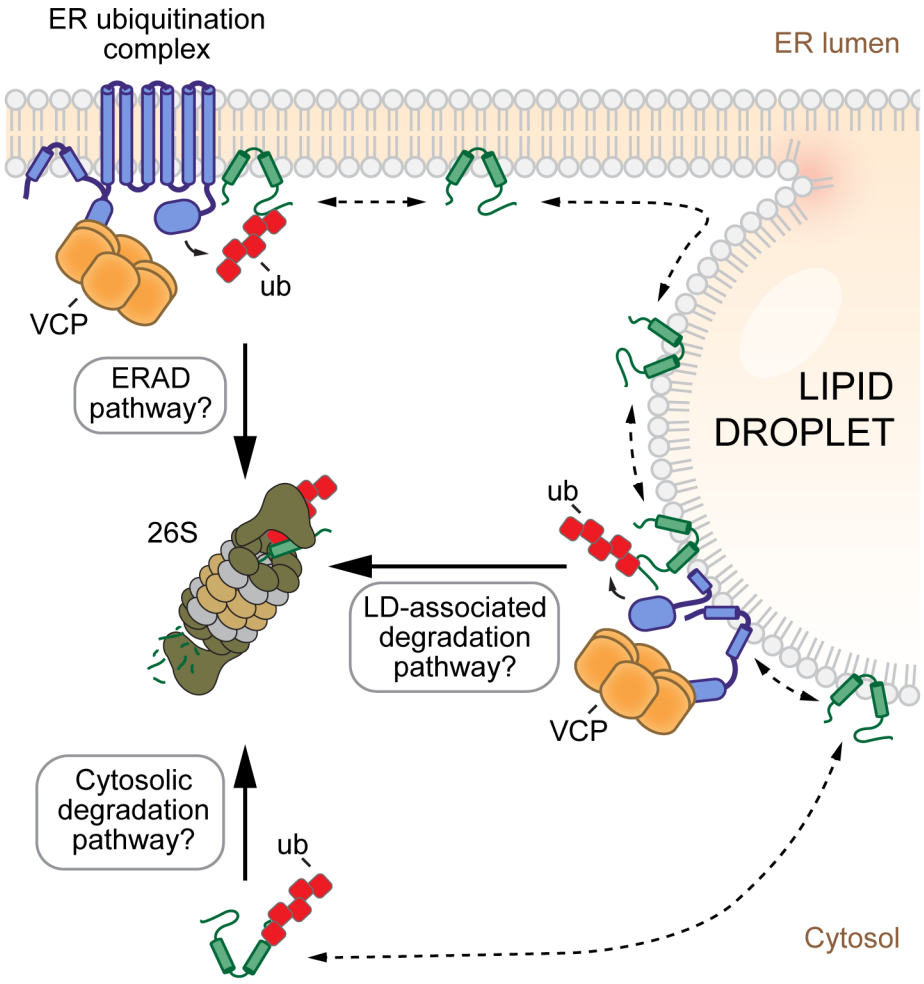

Fig. 3.

Potential routes for LD protein degradation by the ubiquitin-proteasome system. Emerging evidence suggests that LD proteins (*shown in green*) can be degraded by multiple ubiquitin-proteasome pathways under different conditions. In the absence of LDs, LD proteins are likely degraded by both ER ubiquitination pathways (i.e. ERAD) and cytosolic ubiquitination pathways. The presence of LD-localized ubiquitination components suggests the existence of a LD-associated ubiquitination pathway.

Table 1

LD proteins degraded by the ubiquitin-proteasome system

Protein	Function	Cell type	Reference
PLIN1	Scaffold	CHO	[113]
PLIN2	Scaffold	Adipocytes differentiated from MEFs, 3T3-L1 adipocytes, CHO, HeLa, Huh7	[82,114,123,153]
CIDEC (FSP27)	LD fusion	3T3-L1 adipocytes, HEK293T	[116,117]
DGAT2	Diacylglycerol acyltransferase	HEK293, HEK293T, Huh7	[107]
ATGL	Triacylglycerol lipase	HEK293, HepG2, 3T3-L1 adipocytes	[89,115,154]
CGI-58	ATGL activator	COS7	[120]
G0S2	ATGL inhibitor	HeLa, 3T3-L1 adipocytes	[118,155]

Table 2

LD-localized ubiquitination machinery

Protein	Function	Cell type	Reference
AUP1	Ubiquitin binding, UBE2G2 recruitment	A431, Huh7, MDCK, COS7, HeLa, astrocytoma, CHO-7, SV-589	[60,87,88,126,136]
UBE2G2	E2 ubiquitin-conjugating enzyme	COS7, SV-589, CHO-K1	[87,88]
UBXD2	VCP recruitment	Huh7	[59]
UBXD8	VCP recruitment	HeLa, Huh7, NRK	[59,89,97,105]
VCP	AAA ATPase	HeLa, Huh7	[59,89]
Spartin	AIP4/AIP5 recruitment	HeLa	[121–123]
AIP4	E3 ubiquitin-protein ligase	HeLa	[121,122]
AIP5	E3 ubiquitin-protein ligase	HeLa	[122,123]