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Lipid Droplet Biogenesis

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Abstract

Lipid droplets (LDs) are ubiquitous organelles that store neutral lipids for energy or membrane synthesis and act as hubs for metabolic processes. Cells generate LDs de novo, converting cells to emulsions with LDs constituting the dispersed oil phase in the aqueous cytoplasm. Here we review our current view of LD biogenesis. We present a model of LD formation from the ER in distinct steps and highlight the biology of proteins that govern this biophysical process. Areas of incomplete knowledge are identified, as are connections with physiology and diseases linked to alterations in LD biology.

Keywords

biogenesis; endoplasmic reticulum; lipid droplets; neutral lipids; seipin; sterol esters; triglycerides; triacylglycerol

Introduction

Lipid droplets (LDs), organelles of lipid storage, are found in most eukaryotic cells and in some prokaryotes. LDs are found primarily in the cytoplasm, but in some cell types, the nucleus also contains LDs (Ohsaki et al. 2016). These organelles are composed primarily of a neutral lipid core (oil phase), consisting in most cells of triacylglycerols (TGs) and sterol esters (SEs) (see Figure 1).

The neutral lipids serve as lipid reservoirs for energy generation or membrane synthesis. The process of neutral lipid synthesis and storage in LDs is also important for protecting cells from lipotoxicity due to the buildup of excess lipids, such as fatty acids, toxic glycerolipids, and sterols, in cell membranes (Devries-Seimon et al. 2005, Koliwad et al. 2010, Listenberger et al. 2003). LDs also serve as localization sites for some proteins (Kory et al.

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2016), such as enzymes related to LD metabolism (e.g., specific enzymes of neutral lipid synthesis or lipolysis), or, in some instances, for proteins (e.g., histones) that utilize LDs as a platform for storage (Z. Li et al. 2012).

During the past decade, the cell biology of LDs has been a focus of intensive research. It has become clear that LDs are dynamic organelles, integral to cell metabolism, with a fascinating cycle of biogenesis and consumption. Many interesting questions, such as those pertaining to the mechanisms of LD formation, protein targeting to the surfaces of LDs, and the behavior of lipids or proteins during LD consumption, are being investigated. The focus of this article is to review progress in our understanding of LD biogenesis. Other recent reviews highlight additional features of LD cell biology (Barneda & Christian 2017, Gao & Goodman 2015, Kory et al. 2016, Krahrmer et al. 2013a, Roingeard & Melo 2017, Thiam & Foret 2016, Thiam et al. 2013b, Walther & Farese 2012, Welte 2015, Wilfling et al. 2014a).

THE BIOPHYSICS OF OIL DROPLETS IN CELLS

The presence of LDs makes cells exist as emulsions, with the oil or neutral lipid constituting the dispersed phase in the continuous phase of the aqueous cytoplasm. When confronted with excess lipids, such as fatty acids or sterols, cells synthesize neutral lipid oils that then form droplets that are dispersed in the aqueous phase. Because LDs form a separate phase, LD biology in general follows the principles of emulsion physics (Thiam & Foret 2016, Thiam et al. 2013b). One such principle is that emulsions, such as LDs in cells, are metastable; i.e., the emulsion droplets are stable and exist on a timescale that is relevant to cell biology, but they would coalesce to form a continuous oil phase if left to themselves. The driving force for coalescence of LDs is that the system moves to minimize surface tension (i.e., the energy cost for the unfavorable molecular interactions at the interphase between the oil and aqueous medium).

In cells, LD stability is modulated by the presence of lipid or protein surfactants at the interface of the oil and water phases. Surfactants lower the LD surface tension. The major surfactants for LDs constitute a surface monolayer of amphiphilic molecules, such as phospholipids, with the polar head groups oriented toward the aqueous cytosol and the hydrophobic acyl chains oriented toward the neutral lipid cores, thus allowing for preferred molecular interactions of molecules in each phase at the interface. Of the different phospholipids, phosphatidylcholine (PC) appears to be particularly important as an optimal biological surfactant to lower surface tension (Krahrmer et al. 2011, Moessinger et al. 2014, Thiam & Foret 2016, Thiam et al. 2013b). When the levels of surfactants, such as PC, are inadequate, LDs coalesce via fusion to minimize the interface surface and to increase surface coverage by surfactant. This is likely how giant LDs (>2 μm diameter) are formed (Krahrmer et al. 2011), for example, in liver steatosis. Similarly, when lipids with worse surfactant properties, such as phosphatidic acid, accumulate on LDs, they can induce LD coalescence (Fei et al. 2011).

In addition to coalescing, oil droplets in an emulsion can combine to form larger droplets via diffusion of neutral lipids from a smaller droplet to a larger droplet in a process termed Ostwald ripening (Thiam et al. 2013b). In this process, single molecules diffuse from one LD to another through the aqueous solution. Because small LDs have higher surface-to-

volume ratios than do larger LDs (a measure reflected in their Laplace pressure), a larger fraction of their oil molecules is close to the aqueous environment and can diffuse into it. Due to this different likelihood of solubilization for LDs of different sizes, Ostwald ripening results in a net transfer of oil molecules from smaller, less stable LDs to larger, more stable LDs. However, because TGs are only very poorly soluble in water, this process in cells is likely aided by specific protein machinery (discussed below). The combination of two LDs to form a single LD by fusion or diffusion illustrates how LDs behave according to the principles of biophysics and how the cell regulates these processes via proteins.

BIOCHEMISTRY AND GENETICS UNCOVER PROTEINS REQUIRED FOR NORMAL CELLULAR LIPID DROPLETS

Although the principles of emulsion physics apply to LD biology, a central hypothesis that drives the field of LD cell biology is that cells utilize specific protein machinery to catalyze and/or regulate these biophysical processes. Crucial tasks for the field are, thus, to identify the important proteins that govern LD biology and to elucidate their functions and means of regulation. With the goal of identifying such proteins, unbiased screens have played a key role. These include genetic screens, carried out in yeast, fly, and mammalian cells, to identify genes and proteins that are functionally involved in LD biology (Beller et al. 2008, Fei et al. 2008, Gronke et al. 2005, Guo et al. 2008, Z. Liu et al. 2014, Szymanski et al. 2007, Whittaker et al. 2010) and unbiased quantitative assays of proteins for components of LD organelles in these types of cells (Beller et al. 2006, Brasaemle et al. 2004, Cermelli et al. 2006, Currie et al. 2014, Ding et al. 2012, Khor et al. 2014, Kraemer et al. 2013b, Liu et al. 2004, Schmidt et al. 2013, Zhang et al. 2012). Such LD proteomes have been determined by organelle purification and mass spectrometry-based proteomics. Many such studies identified core components of LDs, such as enzymes of lipid synthesis and degradation and structural or regulatory proteins, such as perilipins (Kimmel & Sztalryd 2016). However, due to the challenges of obtaining pure preparations of LDs, combined with the ever-increasing sensitivity of mass spectrometry, many LD proteome lists are populated by protein contaminants. Techniques such as protein correlation profiling have been used in numerous cell types to minimize false positive results and to identify LD proteins that are enriched in the LD fraction (Currie et al. 2014, Kraemer et al. 2013b).

The core LD protein machinery may be similar in many cell types. However, some cell types, such as adipocytes or hepatocytes, are highly specialized cells for storing neutral lipids and have specific proteins for regulating certain LD processes. For example, perilipin1 is found predominantly in adipocytes, where it plays a key role in regulating fasting-induced lipolysis (Brasaemle et al. 2000, Kimmel & Sztalryd 2016, Paul et al. 2008).

OVERVIEW OF LIPID DROPLET FORMATION: CONCEPTUAL STEPS IN THE FORMATION OF AN ORGANELLE

In eukaryotes, LDs are formed de novo from the ER (Choudhary et al. 2015, Jacquier et al. 2011, Kassan et al. 2013). This was long suspected because the enzymes of neutral lipid synthesis are localized in the ER (Buhman et al. 2001), where they generate neutral lipids (TGs and SEs) for LD formation. How, in the next step, cells form emulsion droplets from the synthesized neutral lipid and which proteins function in this biogenesis process are

subjects of considerable investigation. Our focus here is to review the major advances in our understanding of these issues. Because most current knowledge is derived from studying the generation of TG-containing LDs, we focus on this process. It remains to be clarified whether the same concepts will apply broadly to the formation of SE-rich LDs.

To facilitate investigation, it is helpful to break down the process of LD formation into discrete conceptual steps. We propose that the steps follow a conventional and logical model of oil formation and budding into the aqueous cytosol; these steps are illustrated in Figure 2. However, this model is far from validated. Other proposed models of LD formation include a model of diffusion of TGs from the ER into formed LDs contained in a membrane cup (Robenek et al. 2004), a model of LDs budding first into the lumen of the ER (Choudhary et al. 2011), and an ER scission model (Ploegh 2007).

Step 1: Triacylglycerol Synthesis Within the ER—In eukaryotes, neutral lipids, such as TGs or SEs, are synthesized predominantly in the ER (Buhman et al. 2001). TGs were discovered in the early 1800s, and the first observations of TG storage in cells were reported by Richard Altmann as early as the 1890s (Altmann 1894). It was not until approximately 1960 that the biochemical reactions yielding TGs were discovered by Kennedy and coworkers (Weiss et al. 1960). This pathway, termed the *de novo* glycerolipid synthesis pathway, or the Kennedy pathway, is the primary pathway of TG synthesis in most cells. It uses glycerolphosphate and fatty acyl-CoA to generate glycerolipids, such as glycerophospholipids or TGs. A second pathway for TG synthesis, the monoacylglycerol pathway, exists in some cell types, such as intestinal enterocytes and adipocytes, and is involved in the recycling of mono-acylglycerols and diacylglycerols, generated by TG hydrolysis, back to TGs via a reesterification process (Coleman & Haynes 1985). The monoacylglycerol acyltransferase (MGAT) enzymes 1–3 play key roles in this pathway (Cheng et al. 2003, Yen & Farese 2003, Yen et al. 2002).

In either pathway, the final step of TG synthesis is catalyzed by the DGAT (diacylglycerol acyltransferase) enzymes: DGAT1 and DGAT2 (Cases et al. 1998b, 2001; Lardizabal et al. 2001). Thus, these enzymes are central to the process of LD biogenesis. The two DGAT enzymes are an example of convergent evolution at the biochemical level, inasmuch as the enzymes are unrelated in sequence but catalyze the same biochemical reaction. The two enzymes markedly differ in their structures, substrate preferences, subcellular localizations, and physiological roles. DGAT1, a member of the membrane-bound O-acyltransferase gene family (Chang et al. 2011), is localized exclusively to the ER, where it exists as dimers or tetramers that appear to be the functional units (Cases et al. 1998b, Cheng et al. 2001, Shockey et al. 2006). Here, the enzyme encounters its substrates, a fatty acyl-CoA and an acyl acceptor, and catalyzes the esterification reaction to generate a neutral lipid. DGAT1 can esterify a variety of substrates, including diacylglycerol, retinol, monoacylglycerol, and long-chain alcohols (Yen et al. 2005), although TG synthesis appears to be its predominant function. DGAT1 has an important role in detoxifying excess lipids that are taken up from outside the cell (Villanueva et al. 2009). As such, DGAT1 may be important for preventing activation of ER stress pathways due to lipotoxic lipids (discussed below). DGAT2, a member of the DGAT2 gene family (Yen et al. 2008), in contrast, is localized to both the ER and LDs (Kuerschner et al. 2008, Stone et al. 2009, Wilfling et al. 2013). Unlike DGAT1,

DGAT2 does not have multiple transmembrane domains but instead has a hydrophobic sequence, possibly a hairpin, that is embedded in the ER bilayer (Stone et al. 2006, Wilfling et al. 2013). DGAT2 appears to be a major enzyme for TG synthesis from fatty acid substrates derived from de novo lipogenesis (Irshad et al. 2017, Li et al. 2015, Qi et al. 2012, Villanueva et al. 2009, Wurie et al. 2012). In agreement with the different biochemical and cell biological properties of the two enzymes, mice lacking either DGAT1 or DGAT2 have very different phenotypes. DGAT1 knockout mice are viable and have body fat but are lean and resistant to diet-induced obesity, diabetes, and liver steatosis (Chen et al. 2002, Smith et al. 2000). In addition, these mice live on average ~25% longer than control mice (Streeper et al. 2012). Mice lacking DGAT2 die shortly after birth in large part due to a lipid defect in the skin impairing its barrier function (Stone et al. 2004). These mice also nearly completely lack TGs, implicating DGAT2 as a major contributor to mammalian TG synthesis.

Both DGAT enzymes localize to the ER in cells without LDs (Shockey et al. 2006, Stone et al. 2009). The relationship of the localization of these enzymes to TG synthesis within the ER is uncertain. A current model suggests that TG synthesis occurs throughout the ER wherever enzyme substrates are found in excess and that the product of the reaction, TGs, is deposited within the ER bilayer. Either DGAT enzyme localized within the ER appears to generate TGs for initial LD formation (Harris et al. 2011, Li et al. 2015, Wilfling et al. 2013). Studies in plant cells suggested that DGAT1 and DGAT2 may reside in distinct domains of the ER (Shockey et al. 2006). Whether this property is evolutionarily conserved and how this might impact LD formation remain to be determined.

Of note, SEs are also formed in the ER, for example, by the enzymes Are1 and Are2 in yeast (Yang et al. 1996) and by the acyl CoA:cholesterol acyltransferase enzymes in mammals (Anderson et al. 1998, Cases et al. 1998a, Chang et al. 1993, Oelkers et al. 1998). Most LDs contain a mixture of TGs and SEs. However, genetic manipulations in yeast allow for the generation of LDs that contain primarily one or the other neutral lipid (Fu et al. 2014). Compared with studies of TG-containing LDs, there has been relatively less investigation into the mechanisms of formation of SE-containing LDs.

Step 2: Formation of an Oil Lens in the ER Membrane—The second step of LD formation is the formation of a lens of neutral lipids within the ER bilayer. As TG is synthesized and accumulates within the bilayer of the ER, biophysical processes drive lens formation. One model is that TG will nucleate an oil phase to minimize the entropy costs of disrupting the ER bilayer leaflets. The transition to an oil lens in the ER will accommodate newly synthesized TG and will lead to the relative depletion of TG within other spaces of the bilayer leaflet. In agreement with this model, only a limited amount of TG (~3 mol%) can be accommodated in a dispersed form in model bilayers before oil droplets appear (Hamilton et al. 1983).

Whether cells have specific sites for TG lens nucleation and how such sites are determined are unclear. The nucleation model predicts, for instance, that the lipid composition of the ER membrane, its local curvature, and possibly proteins restricting the diffusion of lipids may have a role in lens formation. However, this prediction has not yet been tested comprehensively.

Several studies using biophysical techniques reported data that are consistent with a model of TG lens formation. As noted above, TGs can reach maximally ~3 mol% of lipids in small vesicle membranes before forming a distinct droplet phase (Hamilton & Small 1981, Hamilton et al. 1983). However, another recent study reports that TGs can accumulate in higher concentrations in multilamellar vesicles, and when TGs are found at 5–10 mol%, they tend to form oil domains (Duelund et al. 2013).

ER structure may have an important role in modulating LD formation, although how it might exert effects remains to be determined. The ER is a vast membranous network that is spread throughout the cell and consists of membrane sheets and tubules (Nixon-Abell et al. 2016; Shibata et al. 2006, 2010; Voeltz et al. 2006). These different domains are characterized by different membrane curvatures. Whereas membrane sheets are flat with essentially no curvature, tubules have positive curvature in one principal direction (but not in the other). Moreover, tips of tubules have positive curvature in both principal directions, and junctions between tubules have positive curvature in one principal direction but negative curvature in the other principal direction (Schweitzer et al. 2015). Examples for the different ER structures include the outer nuclear membrane, which is a vast ER membrane sheet, and the dense interconnected network of cytoplasmic ER tubules. Proteins such as reticulons and atlastin (Hu et al. 2009, Voeltz et al. 2006) help to maintain ER tubules and sheets and to form and build junctions of tubules, respectively. For reviews of these proteins, please see Chen et al. (2013), Park & Blackstone (2010), and Shibata et al. (2009).

TG lens formation and subsequent LD formation occur at ER tubules (Jacquier et al. 2011, Kassan et al. 2013), where local curvature may aid TG lens formation by locally disturbing the bilayer order. The relationship of TG lens formation with ER sheets, which lack curvature, is less clear, as is whether lens formation occurs at tubular tips or junctions. What is clear, however, is that perturbations of ER structure can affect LD formation. For example, depletion of proteins that affect ER shape and the balance between sheets and tubules, or the formation of tubular junctions, results in alterations of LD morphologies. For example, depletion of atlastin dramatically affects LD size (Klemm et al. 2013), as does deletion of the protein REEP1 (Falk et al. 2014, Klemm et al. 2013, Renvoise ´ et al. 2016), a protein involved in generating ER tubules. Intriguingly, many ER proteins whose genetic mutations give rise to hereditary spastic paraplegia also result in changes in LD morphology (Blackstone 2012, Eastman et al. 2009, Ito & Suzuki 2009, Renvoise ´ et al. 2016). Hereditary spastic paraplegia appears to be due to ER structural deficiencies that are particularly manifested in large motor neurons. It is unclear whether the LD abnormalities found in cells with some of the mutant proteins are involved in the pathogenesis of hereditary spastic paraplegia or, instead, are markers for the disrupted ER.

Proteins are likely involved in TG lens formation, although this remains to be confirmed. A candidate for such a function is the protein FIT2. FIT2 is an ER protein with multiple trans-membrane domains that has been linked to LD formation (Choudhary et al. 2015, Kadereit et al. 2008). FIT2 binds lipids, such as diacylglycerol and TGs, and FIT2 may be involved in partitioning neutral lipids within the ER for LD formation (Gross et al. 2011). Overexpression of FIT2 in cells results in larger LDs, although levels of TG synthesis are unchanged, suggesting altered partitioning of newly synthesized TGs into LDs (Kadereit et

al. 2008). Loss of FIT2 function in cells results in smaller LDs, possibly with a reduction in TG synthesis (Kadereit et al. 2008). In mice, deletion of FIT2 in intestinal enterocytes results in severe intestinal pathology, including a near absence of LDs and massive intestinal dysfunction (Goh et al. 2015), highlighting the importance of FIT2 in neutral lipid biology of dietary fat intake. These data suggest that FIT2 plays a role in LD formation, possibly at the step of lens formation and/or a TG partitioning step. However, the functional role of FIT2 in LD formation remains to be elucidated.

In addition to FIT2, other proteins may be involved in regulating TG lens formation in the ER. For example, perilipin3 (or Tip47) has been suggested to bind nascent lenses, thus stabilizing them in the earliest steps of LD formation (Bulankina et al. 2009). Other proteins may be involved in determining lens nucleation sites or, alternatively, in preventing TG lenses from forming in specific regions of the ER (Kassan et al. 2013, Wilfling et al. 2014a).

Step 3: Budding and Nascent Lipid Droplet Formation—In a prevailing model of LD formation, when sufficient TG accumulates within the ER bilayer, budding of the droplet into the cytosol occurs. However, it is unclear whether this happens for all LDs or whether some (or indeed all) of them remain connected to the ER. At least in yeast, a substantial fraction of LDs remains physically connected with the ER (Jacquier et al. 2011), whereas in mammalian cells at least some LDs appear to be completely budded from the ER (Wilfling et al. 2013). It is currently unclear what determines the directionality of the budding reaction toward the cytosol and away from the ER lumen, although FIT2 may be important in regulating this directionality (Choudhary et al. 2015). In addition, specific lipids may have a role in LD budding. For instance, dephosphorylation of phosphatidic acid by the yeast enzyme Pah1 to generate diacylglycerol appears to be important for packaging of TGs into LDs (Adeyo et al. 2011).

The process of budding itself can be thought of as a dewetting process, in which a TG lens is formed and converted to a bud on the ER. A gradual increase in the contact angle between the droplet and ER bilayer eventually leads to fission of the bud from the ER when a sufficient angle is achieved. Whether proteins are involved in the scission of LDs from the ER remains to be determined. For a more in-depth discussion of the biophysics of dewetting as it pertains to LD formation, see recent reviews (Thiam & Foret 2016, Thiam et al. 2013b).

Although budding of LDs from the ER is a biophysical process of emulsion formation, proteins are likely involved in mediating or regulating this step. One possibility is that specific ER proteins mark and modulate the sites of LD budding in the ER. Such proteins may act as gatekeepers for the formation of domains with specific phospholipid or protein compositions that facilitate the budding of nascent LDs. Alternatively, these proteins may affect the ER bilayer and emerging LD structure in a way that facilitates the dewetting and budding process.

The protein seipin appears to mark sites that correlate with the initial growth of nascent budded LDs and, therefore, may be involved at sites of initial LD formation. Seipin was originally identified as deficient in a particular form of congenital lipodystrophy (Magre et al. 2001). Subsequently, several screens in yeast revealed that deficiency of the yeast

ortholog for seipin results in abnormal LDs characterized by both giant LDs and numerous very small LDs, depending on the growth conditions (Fei et al. 2008, Szymanski et al. 2007). Seipin is an ER protein with two transmembrane domains and an evolutionarily conserved ER luminal loop (Lundin et al. 2006). Biochemical studies showed that purified seipin appears to form multimers that may organize into a toroid-shaped structure (Binns et al. 2010, Sim et al. 2014). Several recent studies indicated that seipin is involved in the early steps of LD formation. For example, in yeast, seipin is required for normal LD formation in a model system in which LD biogenesis can be induced (Cartwright et al. 2015). In fly cells, endogenous seipin forms patches in the ER, and these patches move rapidly along ER tubules (Wang et al. 2016). During LD formation, some seipin foci encounter sites of TG accumulation, subsequently become colocalized with these TG collections, and enable the initial growth of nascent LDs and their conversion to larger initial LDs (Wang et al. 2016). In the absence of seipin, in *Drosophila* and mammalian cells, a multitude of small TG collections associated with the ER accumulate. Ultrastructural studies, including electron tomography, showed that these TG collections are nascent, budded LDs of 100–200-nm diameter that remain tightly associated with the ER (Binns et al. 2010, Choudhary et al. 2015). These regions of association are characterized by the absence of ribosomes and by regions of increased electron density, suggesting that they represent areas of LD-ER contact sites.

These data indicate that seipin plays a functional role in the initial steps of LD formation either by helping to generate nascent LDs or by facilitating their growth and expansion (Salo et al. 2016, Wang et al. 2016). Supporting a model for seipin regulating these early steps, several groups showed that seipin regulates the protein composition of LDs (Grippa et al. 2015, Wang et al. 2016) and that aberrant proteins can access LDs at early times when seipin is deficient (Wang et al. 2016). Among these proteins are the enzymes of TG synthesis that can stimulate LD expansion (discussed below), contributing to both the formation of LDs that are prone to fusion and the formation of giant LDs (Wang et al. 2016). Seipin deficiency is associated with alterations of phospholipid composition of LDs at late times during formation (Fei et al. 2011, Pagac et al. 2016, Wang et al. 2016, Wolinski et al. 2015), which might explain, in part, the observed abnormalities of phospholipid composition of yeast LDs in seipin deficiency (Fei et al. 2011, Wolinski et al. 2015). Because LDs and the ER stay in contact even when seipin is absent (Salo et al. 2016, Wang et al. 2016), other proteins may be involved in defining the initial budding and growth step of LD formation.

During LD budding, some proteins may gain immediate access to the nascent LDs, but others may be excluded. We recently described a protein marker for nascent LDs that we named LiveDrop (Wang et al. 2016). The LiveDrop marker consists of a membrane-embedded hairpin motif from the GPAT4 protein linked to a fluorescent marker. In our experience, LiveDrop localizes to all nascent LDs at the time of their formation and is more sensitive to detecting nascent LDs than are neutral lipid dyes, such as BODIFY 493/503 (Wang et al. 2016). A similar protein that includes a hydrophobic domain from the protein ALDI also appears to mark nascent LDs (Kassan et al. 2013). Interestingly, although LiveDrop localizes to all nascent LDs, the GPAT4 protein from which LiveDrop is derived does not localize to nascent LDs (Wilfling et al. 2013). This finding suggests that

mechanisms exist to exclude specific proteins from nascent LDs. How this exclusion occurs molecularly is unknown.

Step 4: Lipid Droplet Growth and Expansion via Acquisition of Specific

Proteins—Within minutes to hours after initial LD formation, a subset of these newly formed LDs can be converted to a subpopulation of LDs termed expanding LDs (eLDs) (Wilfling et al. 2013, 2014a). This phenomenon is prominent in and was best described in *Drosophila* cells but can also occur in cultured mammalian cells (Wilfling et al. 2013). The formation of eLDs within cells leads to the existence of two LD populations in cultured cells: initial LDs (iLDs) and eLDs. iLDs are those LDs of 400–800-nm diameter that are formed from the ER. eLDs are iLDs that are converted to a specialized LD, and eLDs are characterized by localized TG synthesis. This TG synthesis is catalyzed by specific isoforms of the TG synthesis pathway, such as GPAT4 and DGAT2, that appear to relocalize from the ER to and around the surfaces of LDs (Kuerschner et al. 2008, Stone et al. 2009, Wilfling et al. 2013). This relocalization occurs through membrane bridges, where the LD surface is continuous with the ER membrane (Wilfling et al. 2013, 2014b).

What triggers the conversion of an iLD to an eLD is unknown. However, under normal cell physiology conditions, the actions of the ARF1 and COP-I/coatamer protein machinery are clearly required for this process (Wilfling et al. 2014b). Initial screens in *Drosophila* cells showed that deficiency of ARF1 or COP-I/coatamer proteins resulted in a striking phenotype of intermediate-sized, dispersed LDs (Beller et al. 2008, Guo et al. 2008). Subsequently, ARF1/COP-I proteins mediate the budding of 60–80-nm nano-LDs from LDs in an *in vitro* system (Thiam et al. 2013a). Furthermore, a portion of the ARF1/COP-I protein localizes specifically to the surfaces of LDs in cells (Wilfling et al. 2014b). A current model is that the LD expansion pathway is triggered by activation of ARF1/COP-I proteins at the surfaces of LDs to form nano-LDs and to remove predominantly phospholipids from the LD surface, thereby increasing surface tension and enabling the modified iLD to interact with the ER. This process then allows for the formation of ER-LD membrane bridges (Wilfling et al. 2014b) and for the migration of TG synthesis enzymes to the surface for LD expansion. Consistent with this model, a recent study implicates TRAPPII (transport protein particle II) and Rab18 as factors in the process downstream of the COP-I proteins (Salo et al. 2016). An alternative model, proposed from studying the requirement of the ARF1/COP-I proteins for targeting of ATGL to LDs, suggests that these proteins are involved in vesicle formation from the ERGIC region of the ER for vesicular targeting of specific proteins to the LD surface (Soni et al. 2009).

Studies of the eLD pathway have been instrumental in our understanding of the mechanisms of protein targeting to LD surfaces. The targeting of proteins to the LD surface provides an unusual circumstance that is different from other membrane targeting events within the cell in that the LD surface exists as a monolayer that covers a neutral lipid oil phase. As such, the biophysical properties of LDs do not allow for targeting of ER proteins with membrane domains that completely span the bilayer, as such targeting would be energetically extremely unfavorable. From the study of enzymes that target eLDs in *Drosophila* cells, we proposed that proteins that target the LD surface fall in one of two major classes (see Figure 3, and for review, see Kory et al. 2016). Class I proteins target the LD surface from the ER bilayer

(Kory et al. 2016). This class is particularly well illustrated by the enzyme GPAT4, which specifically marks eLDs in *Drosophila* cells (Wilfling et al. 2013). GPAT4 and other class I proteins are typically characterized by a membrane-embedded domain that is hydrophobic and possibly adopts a hairpin motif. This hydrophobic domain is embedded in the ER but does not completely span the ER, which thereby allows it to be localized to the ER in the basal condition and to be translocated to the monolayer surface of LDs that are expanding, presumably via ER-LD membrane bridges (Wilfling et al. 2014b). Other likely examples of class I proteins are DGAT2, ATGL, UBXD8, and ACSL3. For UBXD8, the insertion of the hairpin-containing protein initially into the ER bilayer is dependent on the proteins PEX9 and PEX3 (Schrul & Kopito 2016). It remains to be determined whether this is a general membrane targeting pathway for this class of proteins.

A second category, termed class II proteins, target LD surfaces from the cytosol typically through hydrophobic domains, such as amphipathic helices or short, hydrophobic-rich sequences (Kory et al. 2016). An example of this second category is the CCT1 protein, which targets the surfaces of *Drosophila* eLDs via an amphipathic helix in its membrane-binding domain (Krahmer et al. 2011). This amphipathic helix is both necessary and sufficient for targeting of CCT1 to LDs (Krahmer et al. 2011). Studies of CCT1 during eLD formation indicate that CCT1 apparently recognizes PC deficiency at the surfaces of eLDs. Binding of CCT1 to these surfaces activates the enzyme (Cornell & Vance 1987, Krahmer et al. 2011), thereby catalyzing a rate-limiting step of PC synthesis. How CCT1 or other amphipathic helix-containing proteins recognize the surface of eLDs remains to be determined. Another example of class II proteins that bind LD surfaces is the perilipin family proteins (Li et al. 2017). The amount of class II proteins on LD surfaces in particular is determined by binding affinity and macromolecular crowding (Rowe et al. 2016). During lipolysis, when LD surfaces shrink, class II proteins with relatively lower affinity are displaced from the monolayer surface. In contrast, class I proteins, which are likely more deeply embedded in the neutral lipid core of LDs, generally remain bound to LDs.

In addition to these pathways used by the two major classes of proteins targeting LDs, other routes may exist. For instance, lipid anchors could mediate targeting of proteins to LDs. Such a mechanism could be responsible for the recruitment of small Rab GTPases such as Rab18 (Martin et al. 2005) and ELMOD2, a noncanonical Arf-GAP (Suzuki et al. 2015). However, evidence for the biological function of these proteins on LDs is currently sparse. In addition, protein-protein interactions can mediate the recruitment of proteins to LDs. For instance, during *Drosophila* development, the adaptor protein Jabba recruits specific histones for temporary storage on LDs (Z. Li et al. 2012).

THE FORMATION OF GIANT LIPID DROPLETS

Some cells have LDs whose diameters far exceed the 1–2- μm diameter of eLDs; such LDs are often referred to as giant LDs. Such cells include hepatocytes or adipocytes, in which LD diameters can exceed tens of micrometers. These giant LDs are formed by one of two processes (discussed above). The first process is coalescence, in which two LDs almost instantaneously fuse to form a larger LD. An example in which LD coalescence is prominent is PC deficiency. A number of gene depletions in a screen of *Drosophila* or yeast cells that

result in giant LDs appear to have PC deficiency in common (Guo et al. 2008), and genetic depletion of CCT1 or culture conditions that result in PC deficiency also specifically lead to LD coalescence (Krahmer et al. 2011). Coalescence can also be induced by adding agents that raise surface tension and act as poor surfactants. For example, propranolol and some LD dyes induce LD coalescence (Murphy et al. 2010).

A second process that results in the formation of giant LDs is diffusion mediated by Ostwald ripening. This process is much slower than coalescence and is characterized by the gradual enlargement of one LD and the depletion of oil in the adjacent second LD. Although this process would be quite slow for TG exchange between LDs in the absence of proteins, there is evidence that specific proteins facilitate this process in cells such as adipocytes. For example, the protein FSP27/CIDE-C localizes to contact zones between two LDs and facilitates the slow transfer of TGs from a smaller droplet to LDs (Gong et al. 2011), suggesting that FSP27 combines LDs by facilitating TG diffusion. A related protein, CIDE-B, is found in the liver, where it has been implicated in TG secretion (Ye et al. 2009), and may be involved in an analogous diffusion process of TGs from LDs to the ER, where it is used for very low density lipoprotein assembly.

INTERACTIONS OF LIPID DROPLETS WITH OTHER CELLULAR ORGANELLES

As knowledge of LD biology increases, we are beginning to understand the interactions of LDs with other cellular organelles. Far and away the most prominent organelle that interacts with LDs is the ER. As described above, LDs are formed from the ER and often remain in close association with the ER after their budding and formation. Ultrastructural studies from mammalian cells indicate that LDs may sometimes be associated with the ER in a structure that resembles an egg cup (Robenek et al. 2006), with the LD being cradled by a region of ER membrane. In yeast, LDs may be associated with the ER and may remain connected to it after formation as part of specialized ER domains (Jacquier et al. 2011). In nonyeast cells, budded iLDs appear to reconnect with the ER during eLD formation (discussed above; see Wilfling et al. 2013). This reconnection has been shown by electron microscopy to consist of a membrane bridge that spans the cytosolic leaflet of the ER via a continuous bridge with the surface monolayers of LDs (Wilfling et al. 2013). Proteins may be involved in establishing or maintaining these bridges, but the identity of such proteins is unknown. One function of ER-LD bridges appears to be in allowing enzymes of TG synthesis to migrate to LD surfaces (Wilfling et al. 2013). Because DGAT2 is one of these enzymes and DGAT2 has been linked functionally to de novo lipogenesis (discussed above), one function of these ER-LD bridges may be to enable partitioning of de novo lipogenesis to a region of the ER that is linked to TG storage.

LDs may also interact with other organelles. For example, in yeast, LDs associate under specific circumstances with peroxisomes (Binns et al. 2006), possibly to facilitate the β -oxidation of fatty acids. In mammalian cells, LDs sometimes appear to interact closely with mitochondria. This interaction may be regulated metabolically, as activation of the master metabolic kinase AMPK results in dispersion of LDs on stable microtubules, possibly to increase interactions with mitochondria (Herms et al. 2015). The LD-mitochondria interaction is particularly prominent in skeletal or heart muscle, as demonstrated by electron

micrographs (Wang et al. 2013, Zhang et al. 2011). LDs may interact with mitochondria in mammalian cells to facilitate the transfer of fatty acids to mitochondria for β -oxidation under starvation conditions. In support of this possibility, LDs in cultured mammalian cells that are preloaded with labeled fatty acids transfer these fatty acids to mitochondria, apparently for β -oxidation (Rambold et al. 2015), although whether this process requires LD-mitochondria contacts is unclear.

IMPLICATIONS OF LIPID DROPLET FORMATION FOR CELL PHYSIOLOGY

The formation of LDs has many implications for cell physiology. The storage of fatty acids and other lipids in LDs provides important stores of metabolic energy and lipids and lipid precursors for membrane lipid synthesis during cell proliferation or cell remodeling. Illustrating this function, yeast that are cultured to stationary phase with energy substrate depletion store lipids in LDs. When yeast from stationary phase are transferred to fresh medium and proliferation is induced, these cells consume LDs and utilize the lipids from LDs for cell proliferation (Kurat et al. 2009). Intriguingly, some cancer cells accumulate massive amounts of LDs. For example, clear cell carcinoma of the kidney is characterized by vast amounts of LDs in the cytoplasm (Saito et al. 2016, Yu et al. 2013), although it remains to be tested whether these LDs are important for enabling the proliferation of such cells. More investigation is needed to better understand whether LDs provide stores of energy or membrane lipids during cancer cell proliferation.

LD formation also appears to be central to cell physiology in preventing the accumulation of toxic lipids within the ER. The ER is a large cellular organelle that is involved in a multitude of processes, including lipid synthesis and the generation of membrane and secreted proteins. Glycosylation of membrane and secreted proteins also occurs in the ER. For these processes to occur normally, the proper composition of lipids in the ER must be maintained. Thus, when excess simple lipids, such as fatty acids or sterols, accumulate in the ER, mechanisms exist to convert these lipids to more inert neutral lipids for storage in LDs. For example, the ACAT enzymes generate SEs, and the DGAT enzymes generate TGs and other neutral lipids to be stored in LDs. The absence of these enzymes may thus result in abnormal accumulation of lipids in the ER bilayer, which can trigger ER stress and the unfolded protein response. Indeed, deficiency of the ACAT enzymes results in ER stress when cells are loaded with excess cholesterol (Devries-Seimon et al. 2005, Kedi et al. 2009). Similarly, DGAT1 may play such a role in protecting the ER from lipotoxic ER stress. Interestingly, triggering of ER stress in the liver results in upregulation of IRE1 and downstream lipid metabolism targets, including DGAT2, as part of a homeostatic response (Lee et al. 2008). Related to this finding, LDs could protect phospholipid pools by preventing their oxidation, serving as a sink for radical oxygen species (Bailey et al. 2015, Liu et al. 2015).

LDs have also been implicated in modulating pathways of oxidative stress in cells. For example, studies in *Drosophila* show that LDs can limit reactive oxygen species in glial cells and can inhibit oxidation of polyunsaturated fatty acids (Bailey et al. 2015). In contrast, glial LDs have been implicated in the propagation of oxidative stress in *Drosophila* models of neurodegeneration induced by mitochondrial defects (Liu et al. 2015).

Studies in mice and humans highlight the importance of LD formation in normal metabolism and physiology. For example, humans lacking DGAT1 are characterized by a severe congenital diarrhea syndrome that is exacerbated by exogenous fat (Lee et al. 2008). Also, humans treated with inhibitors of DGAT1 develop diarrhea in a dose-related fashion (DeVita & Pinto 2013, Lee et al. 2008). Humans appear to lack expression of DGAT2 in their intestine (Haas et al. 2012), which may make them particularly susceptible to the effects of DGAT1 deficiency.

Additionally, a large number of LD proteins are linked to metabolic disease. As mentioned above, deficiency of FIT2 in the murine small intestine results in severe pathology and intestinal failure that are associated with impaired LD formation, and deficiency of FIT2 in murine adipose tissue results in lipodystrophy (Goh et al. 2015, Miranda et al. 2014). Deletion of seipin in mice results in postnatal lipodystrophy and numerous metabolic abnormalities (Cui et al. 2011, Dollet et al. 2014, Gao et al. 2015, L. Liu et al. 2014). Similarly, homozygous or compound heterozygous mutations of CCT α lead to a lipodystrophy syndrome (Payne et al. 2014). FSP27/CIDE-C deletion results in adipose tissue characterized by many small LDs, rather than one large unilocular LD, and in protection from the insulin resistance that accompanies these morphological changes (Nishino et al. 2008). Another LD protein, PNPLA3, is the major genetic risk factor for liver steatosis (Anstee & Day 2013, Romeo et al. 2008). A variant of PNPLA3 is found in increased concentrations in LD fractions, where it may alter cell physiology in a manner that promotes TG and LD accumulation (He et al. 2010, J.Z. Li et al. 2012, Smagris et al. 2015). The evidence is growing that proteins involved in the formation or maintenance of LDs are of tremendous importance for normal cell physiology and that mutations of specific LD proteins can lead to cell dysfunction, severe metabolic abnormalities, and disease (for reviews, see Carr & Ahima 2016, Greenberg et al. 2011, Kraemer et al. 2013a).

CURRENT QUESTIONS IN LIPID DROPLET FORMATION RESEARCH

In this review, we propose a model of LD formation and describe progress that has been made to understand the molecular aspects of this process. Relative to other cellular organelles, however, an understanding of LD biology is incomplete, and many questions remain. For example, it is unclear how sites of TG synthesis are localized within the ER and how such localization correlates with the sites of LD formation. The model in which TG initially forms lenses within the ER membrane is attractive but is largely untested. How and which proteins participate in TG lens formation and organization are mostly unknown. Additionally, whether proteins are required for the LD budding process is also unknown. How some LDs are selected for the expansion pathway is similarly mostly unknown. The coming years of investigation into LD formation will likely yield many insights into these processes. Advances will come from clues from unbiased screening approaches, such as genetic screens or proteomics, combined with mechanistic studies of cell biology and the biochemistry of candidate proteins. A molecular understanding of how cells utilize proteins to control and regulate the biophysical process of emulsion formation will likely emerge. These advances will provide a multitude of molecular insights affecting our understanding of cell physiology, metabolic diseases, and the basic science underlying oil droplet formation that is central to industrial processes such as seed oil production and biofuels.

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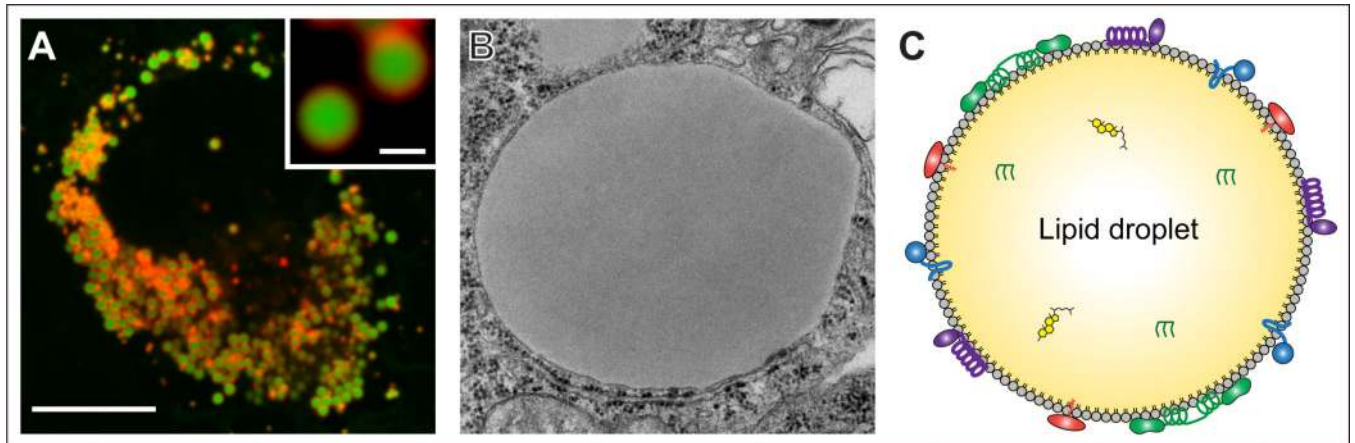


Figure 1.

Lipid droplets (LDs): cellular organelles for neutral lipid storage. (a) Fluorescence microscopy image showing LDs (green, stained with BODIPY 493/503) in mammalian SUM159 cells. This cell was transfected with the LD marker mCherry-LiveDrop (red). (b) Electron micrograph showing a monolayer-bound LD in *Drosophila* S2 cells. The ribosome-studded ER bilayer can be seen in close proximity. Image courtesy of Yi Guo (Mayo Clinic, Rochester, MN). (c) Schematic representation of the structural composition of an LD. Colored objects represent LD surface-bound proteins localized to the phospholipid monolayer. Triacylglycerols and sterol esters are found in the neutral lipid core.

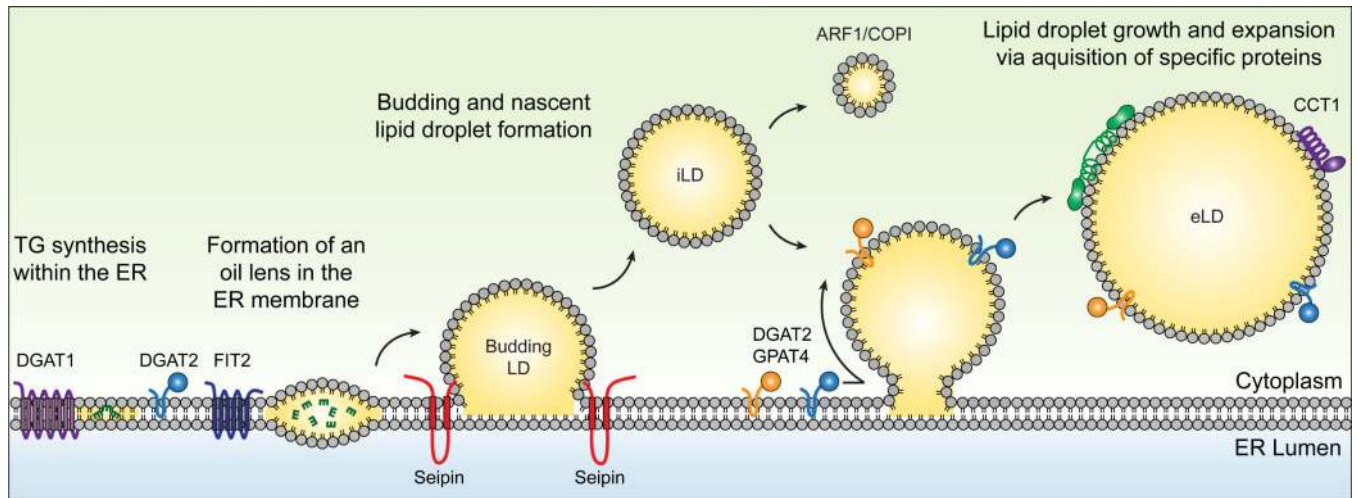


Figure 2. Model of steps of lipid droplet (LD) formation and expansion. Abbreviations: DGAT, diacylglycerol acyltransferase; eLD, expanding LD; iLD, initial LD; TG, triacylglycerol.

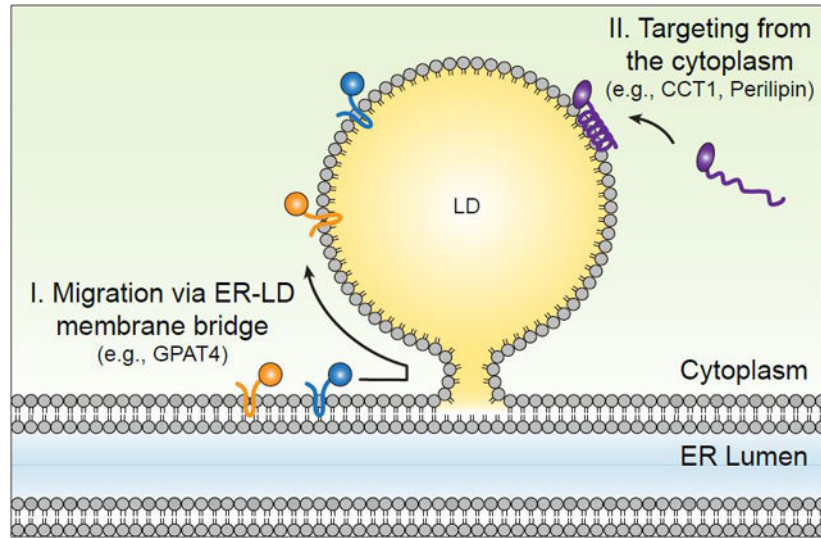


Figure 3. Mechanisms for targeting of proteins to lipid droplet (LD) surfaces during LD formation and growth. Class I proteins, such as GPAT4, are inserted into the ER and from there translocate to the surfaces of LDs either during LD formation or after formation via membrane bridges. Class II proteins, such as CCT1, target from the cytosol via amphipathic helices or other short hydrophobic domains.