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REVIEW

Lipid droplet–membrane contact sites – from protein binding to function

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ABSTRACT

In the general context of an increasing prevalence of obesity-associated diseases, which follows changing paradigms in food consumption and worldwide use of industry-transformed foodstuffs, much attention has been given to the consequences of excessive fattening on health. Highly related to this clinical problem, studies at the cellular and molecular level are focused on the fundamental mechanism of lipid handling in dedicated lipid droplet (LD) organelles. This Review briefly summarizes how views on LD functions have evolved from those of a specialized intracellular compartment dedicated to lipid storage to exerting a more generalized role in the stress response. We focus on the current understanding of how proteins bind to LDs and determine their function, and on the new paradigms that have emerged from the discoveries of the multiple contact sites formed by LDs. We argue that elucidating the important roles of LD tethering to other cellular organelles allows for a better understanding of LD diversity and dynamics.

KEY WORDS: Lipid droplet, Molecular tethers, Contact sites, Protein targeting, Lipid metabolism, Multifunctional lipid droplets

Introduction

Our knowledge of lipid droplet (LD) function has expanded strikingly in the past decades, driven by numerous genome-wide screens of LDs (Box 1), which have identified hundreds of genes involved in LD regulation in various organisms (Guo et al., 2008; Ashrafi et al., 2003; Beller et al., 2008; Fei et al., 2008a). Indeed, because energy homeostasis is a vital requirement, all living organisms can make LDs. The identification of many genes that function in lipid metabolism and beyond has raised many exciting questions, and will help to capture the full view of LD implications in cellular biology.

Structurally, an LD has a neutral lipid core surrounded by a phospholipid monolayer, in which proteins are embedded. In the life cycle of LDs, proteins are dynamically recruited to and dissociate from the LD surface to control LD function; this is achieved by the temporal remodeling of the physical chemistry properties of the LD surface. However, how proteins are targeted to the LD surface still remains elusive.

The LD life cycle encompass its biogenesis from the ER and its degradation by the breakdown of its neutral lipid content according to a currently accepted model, which has been greatly detailed in previous reviews (Pol et al., 2014; Hashemi and Goodman, 2015; Henne et al., 2018; Walther et al., 2017; Thiam and Forêt, 2016), and is depicted in Fig. 1. Briefly, LD biogenesis is based on the generation of a neutral lipid lens within the ER membrane bilayer, which enlarges and buds into the cytoplasm. These steps are essentially controlled by the global and local phospholipid composition of the ER (Ben M'barek et al., 2017; Wang et al., 2018a; Choudhary et al., 2018; Chorlay and Thiam, 2018), which is possibly altered by proteins such as seipin (also known as BSCL2) (Yan et al., 2018; Sui et al., 2018 preprint). Cytoplasmic LDs then undergo cycles of shrinking and refilling that are mediated by interactions with surface-associated lipases or acyltransferases (Hashemi and Goodman, 2015). Depending on the cell type and metabolic conditions, other pathways can be used for degradation of LDs (Fig. 1B), including macrolipophagy (fusion of LD engulfed in autophagosomal vesicles with lysosomes; Singh et al., 2009), microlipophagy (LD entry into invaginated lysosomes; Tsuji et al., 2017), or even LD secretion through the plasma membrane into the extracellular medium (Flaherty et al., 2019), as occurs during milk secretion (Argov-Argaman, 2019). During their life cycle, LDs are, however, not isolated (Schuldiner and Bohnert, 2017; Salo and Ikonen, 2019; Barbosa et al., 2015). They communicate with nearly all other organelles through contact sites that are mediated by protein tethers (Scorrano et al., 2019). The formation of these contact sites with specific organelles also relies on the specific proteins that are bound to the LD surface.

In this Review, we aim to bring together recent discoveries in LD biology obtained from different fields that have led to consider LDs as stress-responsive organelles, beyond their simple role in fat storage. We first summarize the main principles that have emerged with regard to protein targeting to LDs and the interactions they engage in at the LD surface. In light of these findings, we then discuss the implication of recent discoveries that identified membrane contact sites between LDs and organelles, which point to inter-organelle communication as a novel key feature of cellular energy regulation.

Lipid droplet functions – from simple fatty acid storage to generalized stress response

The core of LDs serves as the place for the hydrophobic storage of various molecules (Fig. 2). However, well beyond lipid reservoirs, LDs have functions that are related to the cell stress response, cell protection and molecular folding platforms, as summarized in this section.

Neutralizing lipotoxic fatty acids

A local elevation of free fatty acids in the medium of cultured cells can have detergent-like effects and thus induce membrane lysis. Similarly, cholesterol excess can generate deleterious cytoplasmic crystals (Fabricant et al., 1973; Baumer et al., 2017) or induce membrane rigidity, thereby altering membrane dynamics and signaling properties (Lingwood and Simons, 2010). Esterification

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Box 1. Development of the LD field

Starting in the 1960s from the view of amorphous lipid deposits within the cytoplasm, mostly considered to be a consequence of cell dysfunction, the main organ where lipid accumulated, adipose tissue, had not yet been recognized as being a professional organ orchestrating lipid management. Times began to change when the isolation of adipocytes, bona fide active cells, identified a surprisingly high ability to respond to insulin (Rodbell, 1964). Also, 3T3-derived cell lines (Green and Kehinde, 1974), which spontaneously undergo gene differentiation and adipose conversion highlighted that lipid storage was genetically programmed. Following this, the lipolytic cascade was elucidated, as well as the process of lipase activation at the lipid–cytoplasm interface. Perilipin 1 was identified as the first LD-coating protein (Blanchette-Mackie et al., 1995), and its phosphorylation controls lipase access and anchorage to LDs (Londos et al., 1999). During that time, the main genes encoding the ER enzymes that mediate triglyceride synthesis were cloned (Cases et al., 1998) (note that sterol synthesis enzymes were discovered much earlier, back in the 1960s; for a review on the details on the neutral lipid synthesis enzymes see Buhman et al., 2001). A next step was the elucidation of the LD surface structure, which resolved as a phospholipid monolayer with an original lipid composition distinct from that of the ER membrane (Tauchi-Sato et al., 2002); this finding greatly contributed to the idea that LDs are a cell organelle. As was the case for other organelles, large-scale genetic screens, as well as proteomics and lipidomics were applied to gain insights in the detailed composition and regulation of LDs (Guo et al., 2008; Beller et al., 2008; Cermelli et al., 2006; Brasaemle et al., 2004; Fei et al., 2008b; Szymanski et al., 2007). These efforts identified a number of different proteins, most of them participating in other processes beyond simple lipid storage, and suggested that LDs have extended functions and are highly dynamic (Cermelli et al., 2006; Ding et al., 2012; Athenstaedt et al., 1999; Brasaemle et al., 2004; Fujimoto et al., 2004; Bersuker and Olzmann, 2017; Welte and Gould, 2017; Blouin et al., 2010). In addition, biophysical and reconstitution tools have emerged as useful approaches to elucidate the molecular details underlying LD cell biology (Khandelia et al., 2010; Choudhary et al., 2018; Ben M'barek et al., 2017; Prévost et al., 2018; Čopič et al., 2018; Chorlay et al., 2019).

of fatty acids and an excess of cholesterol channels these molecules into LDs; this is therefore considered a buffering process against lipotoxicity (Unger and Orci, 2000). Furthermore, among fatty acids, an excess of saturated species such as palmitate is more deleterious for cells than unsaturated fatty acids, owing to their lower ability to be esterified into neutral lipids compared to well-tolerated oleate, which is preferentially converted into triglycerides (TGs) (Listenberger et al., 2003; Chitraju et al., 2017). The mechanisms underlying saturated fatty acid toxicity mostly relate to the preferential generation of proapoptotic intermediates, such as ceramides (Listenberger et al., 2003) or desaturated glycerolipids (diacylglycerols or phosphatidic acids), which only poorly support TG synthesis (Piccolis et al., 2019). They are highly relevant for human health, as a chronic diet high in saturated fats increases the risks for developing cardiometabolic diseases or cancer. Thus, it is now recognized in the field of metabolic diseases that LDs, as packaging organelles for fatty acids or cholesterol, serve a protective role at the cellular level. This notion of protective LD sequestration extends to lipid sequestration by adipose tissue, the specialized LD-forming organ, which fulfills a physiological role until it is overwhelmed in aggravating obesity (McGarry, 2002; Unger and Orci, 2000).

Beyond a primary storage function, LD formation can also be observed as a consequence of various stress responses, ranging from hypoxia to thermal stress, or nitrogen deprivation in plants and algae (Fig. 2). In these conditions, the driving force for neutral lipid assembly is not necessarily an excess of fatty acids, but also fatty acids that are produced from membrane phospholipids through the activation of phospholipases to generate diacylglycerols, which are at the crossroad of the polar versus neutral lipid synthesis pathways.

Lipid droplets in oxidative stress and hypoxia

Exposure to oxidative stressors or a reduction in the antioxidant capability of a cell results in an increased formation of LDs, suggesting that they have the potential to buffer reactive oxygen species (ROS) (Liu et al., 2015). Indeed, the connection between LDs and ROS is multifaceted and crucial for cell metabolism (Fig. 2). Under conditions of energy need, LD-derived fatty acids are generally used as substrates for oxidation, which subsequently generates ROS along the mitochondrial electron transport chain for ATP production. However, excessive ROS production can oxidize lipids that contain polyunsaturated chains and more intensively if these are associated with the membrane than with LDs. Indeed, in developing *Drosophila*, LDs that form during oxidative stress in niche glia were found to be crucial to limit the levels of ROS and inhibit the oxidation of polyunsaturated fatty acids (Bailey et al., 2015). In this study, the formation of triglycerides was found to protect phospholipids and render them less susceptible to peroxidative damage, thereby increasing cell survival (Bailey et al., 2015). The interplay between LDs and ROS might be particularly relevant in cancer cells that usually grow under hypoxic conditions (Harris, 2002). Induction of hypoxia-induced factor 1α (HIF1α) is an important cell stress response, and its activation through its downstream effector HIF-induced gene 2 (HIG2; also known as HILPDA) was found to obliterate fatty acid mobilization from LDs by directly inhibiting the adipose tri-glyceride lipase (ATGL; also known as PNPLA2) on the LD surface (Zhang et al., 2017). As an adaptation to hypoxia, such a brake on lipolysis contributes to the reduction of fatty acid oxidation and subsequent ROS production. By lowering fatty acid utilization, hypoxic LD accumulation thus participates in the switch from an oxidative towards a glycolytic-oriented metabolism of cancer cells (Annibaldi and Widmann, 2010). LD accumulation under hypoxic conditions has also been associated with an increased uptake of fatty acids and their channeling to LDs, as suggested by the abolition of hypoxia-dependent LD accretion in mice with knockdown of two fatty-acid-binding proteins (Bensaad et al., 2014).

Lipid droplets as platforms for protein folding and DNA maintenance

A major process in cellular stress adaptation resides in the endoplasmic reticulum (ER) where membrane sensors can trigger the unfolded protein response (UPR). Bidirectional links have been found between ER-dependent protein quality control and LDs (Fig. 2). On the one hand, common UPR inducers such as tunicamycin, which blocks protein glycosylation, can induce LD formation in yeast (Fu et al., 2009), indicating that proteotoxic stress can cause lipid accretion. Similar to what occurs upon the accumulation of misfolded proteins, the UPR can be activated in conditions that cause ER lipid bilayer stress, such as changes in the ratio between phosphatidylethanolamine and phosphatidylcholine (Thibault et al., 2012; Ho et al., 2018), or the degree of fatty acid saturation (Kitai et al., 2013). In yeast, UPR activation can also be triggered by modulating inositol-mediated transcriptional regulation of phospholipid synthesis genes (Moir et al., 2012; Halbleib et al., 2017).

On the other hand, LDs have been proposed to serve as a depository for proteins before their degradation, further highlighting LDs as hotspots for protein quality control (Vevea et al., 2015; Ploegh, 2007). Along this line, a key event in quality control is the regulated protein degradation pathway orchestrated in the ER, referred to as ER-associated degradation (ERAD). Although ERAD

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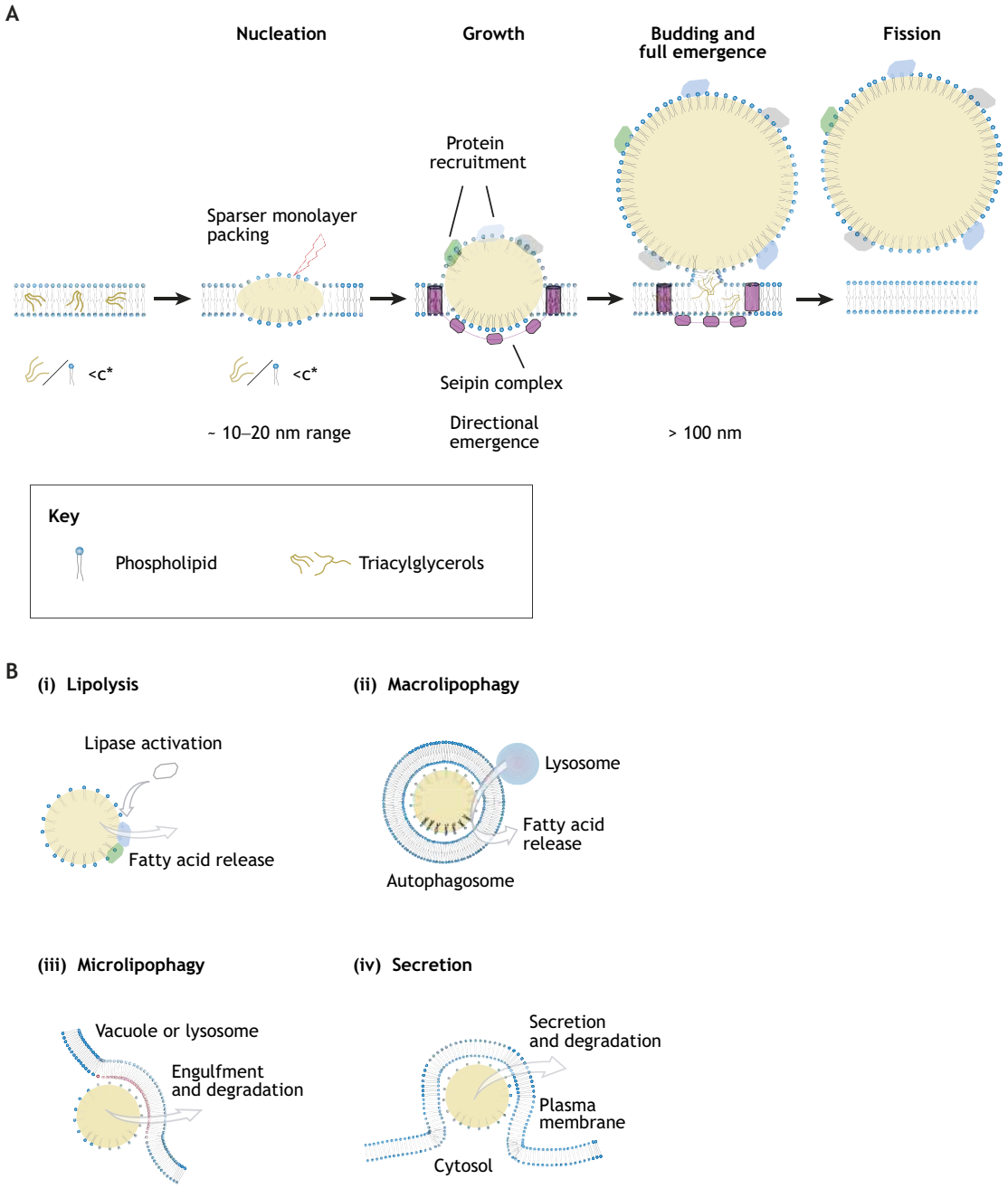

Fig. 1. Lifecycle of LDs. (A) A schematic of currently accepted model for the sequential steps involved in the emergence of a phospholipid monolayer-covered LD from the ER bilayer. Upon an increase in the triglyceride-to-phospholipid ratio (referred to as c*) in the ER bilayer, a neutral lipid 'lens' is nucleated, which grows and emerges directionally as a spherical LD into the cytosol. The approximate size of the lipid core is indicated for each step. The monolayer phospholipid density is probably smaller than that of the ER bilayer. This difference is likely to be recognized by proteins that associate with the LD surface. Particularly, the seipin complex detects the nascent LD and assembles at the ER LD interface. (B) Schematic illustration of the different modes of LD degradation. (i) Lipolysis owing to activation of cytoplasmic lipase at the LD surface results in the release of free fatty acids. Lipophagy involves LD degradation through either (ii) macroautophagy (macrolipophagy; shown here as a LD enwrapped into an autophagosome that subsequently will fuse with a lysosome), or (iii) microlipophagy (shown here as an LD engulfed in an invaginated vacuole or lysosome). Finally, (iv) LD secretion through the plasma membrane is commonly seen in milk production.

can proceed in the absence of LD formation (Olzmann and Kopito, 2011), results obtained from proximity labeling of components of the ERAD machinery led to a reevaluation of the LD proteome (Bersuker et al., 2018; Bersuker and Olzmann, 2017). Among the proteins that are involved in ERAD and can localize to LDs is ancient ubiquitous protein 1 (AUP1), a LD-associated protein that also participates in the extraction of 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMGCoA) reductase from ER membrane for subsequent degradation (Jo et al., 2012). AUP1 depletion not only diminishes sterol-dependent degradation of the reductase, but reduces the degradation of misfolded proteins (Klemm et al., 2011). This protein with a dual function in LD abundance and ER quality control therefore illustrates their intertwined relationship, which still has to be elucidated in more detail.

Related to the storage function of LD, a transient aggregation of proteins on LDs has been reported, indicating they might have a role

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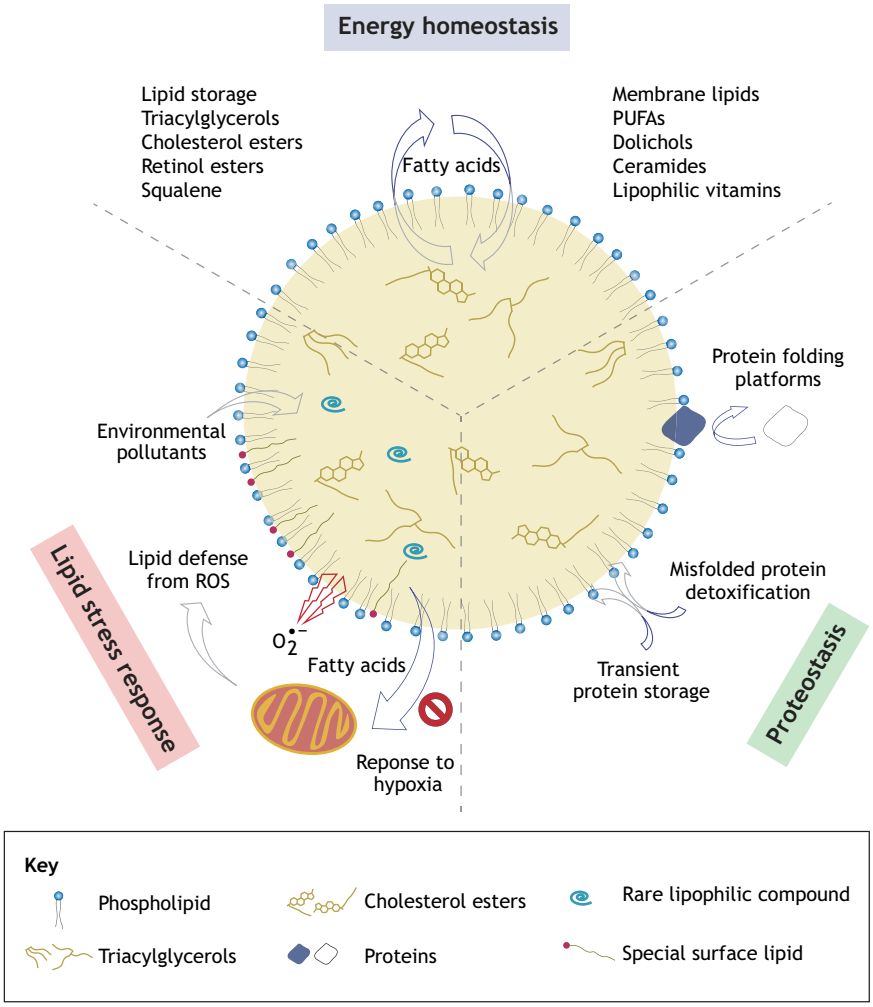

Fig. 2. Diversity of LD functions. Mature LDs have a phospholipid monolayer that surrounds a neutral lipid core, which is mainly composed of triacylglycerols and cholesterol esters. Essential LD functions can be classified into three general aspects that relate to energy homeostasis, namely, energy homeostasis, where LDs act as storage for diverse lipid species, such as dolichols, modified ceramides or polyunsaturated fatty acids (PUFAs), proteostasis (i.e. LDs acting as maturation and/or degradation platforms for proteins) and the lipid stress response, where LDs have a role in the response to hypoxia and lipid defense from ROS, as well as the sequestering of toxic lipophilic molecules including environmental pollutants (see Box 2).

in the temporary deposition of proteins to cope with situations of acute need. In particular, the massive requirement for maternal histone in early embryonic development of *Drosophila* is made possible by their transient storage on LDs (Cermelli et al., 2006; Johnson et al., 2018).

Further highlighting the role of LDs as maturation platforms are nuclear LDs that form by reflux of intraluminal LDs from the ER back to the nucleoplasm, as recently reported (Sołtysik et al., 2019). Indeed, these LDs have been suggested to be involved in gene regulation (Soltysik et al., 2019). Previously, images of nucleic acid ribbons at the LD surface of human mast cells (Dvorak et al., 2003) have been observed, which confirmed nucleic-acid–LD interactions. It is therefore plausible that formation of nuclear LDs or nucleic-acid-associated LDs may modulate gene expression by impacting chromatin structure, but this awaits further investigation. In conclusion, the surface of LDs may modulate the activity of proteins implicated in gene regulation or serve a buffer of soluble proteins.

Proteins targeted to the surface of LDs

The LD surface proteome is diverse, dynamic and highly dependent on cell type and metabolic status (Olzmann and Carvalho, 2018). Several hundred proteins localize to the surface of LDs; these include proteins with identified functions, such as the LD-resident perilipin (Plin) proteins, proteins with thus far unclear functions, and ‘intruder’ proteins, such as the core protein of some flaviviruses. Proteins use various mechanisms and binding motifs to localize to the LD surface (Fig. 3A,B). So far, amphipathic helices (AHs) and hydrophobic helix (HH) domains, which include hairpins, have been identified as the most common of the motifs that target a protein to the LD surface. These proteins are essentially targeted to LDs from the ER or the cytosol (Kory et al., 2016). However, it cannot not excluded that proteins from other organelles, such as mitochondria or Golgi, are also targeted to LDs, for instance through contact sites. Below, we will discuss the possible mechanisms underlying protein localization to LDs.

Localization of AHs to LD surface

Proteins with an AH-binding motif for LD targeting include the acyl-CoA synthetase long chain family member 3, which initiates the neutral lipid synthesis by fatty acid activation. AHs are also found in the most-abundant LD-coating proteins, the perilipins, the first discovered family of LD proteins (Blanchette-Mackie et al.,

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Box 2. LDs as reservoirs for toxic lipophilic molecules

Exposure to toxic molecules present in the environment can cause cellular stress. Since pollutants are generally lipophilic (derived from hydrocarbons), they have a high propensity to target LDs and accumulate therein (Fig. 2). High concentrations of exogenous compounds in fat tissues of mammals or fish are now recognized as a sign of chronic exposure to ecological contaminants. The fat-cell accumulation of polychlorinated biphenyl compounds (PCBs) has been studied in some detail and found to be dependent on initial lipid content (Bourez et al., 2013, 2012). Whereas it cannot be excluded that some molecules could hijack specific cell transport routes, a likely hypothesis to account for the accumulation of toxic compounds in LDs is that they can travel nonspecifically within cell membranes through apposition sites, as free fatty acids do (Blanchette-Mackie and Scow, 1983). The accumulation of exogenous pollutants in LDs further reinforces the notion of LDs serving as sequestration centers, as a mean to preserve other cell organelles. More recently, the list of toxic lipid species found to be sequestered in LD has expanded, including polyprenols such as dolichols (Hoffmann et al., 2017) and unusual ceramides, for instance, the neutral lipid compound acyl-ceramide, synthetized by acyl-CoA synthase 5 (Senkal et al., 2017). Finally and also indirectly related to this aspect, many nano-carriers for drug delivery are designed to mimic the structure of LDs; here, drug molecules are encapsulated into the carrier core in a formulation that enables efficient drug delivery to cellular membranes.

The drug-sequestering properties of LDs are also important in the context of chemotherapy, as, in this case, they reduce therapeutic efficiency. However, considering chemo-resistance in LD-containing tumor cells, it is often difficult to distinguish the impact of LD drug sequestering, which reduces the active concentration, from metabolic advantages that are conferred by the presence of LD themselves, which promote recovering from drug-induced toxic stress (Shyu et al., 2018). In fact, both mechanisms have been reported to contribute to chemo-resistance to the microtubule-targeting drug docetaxel in progestin-responsive breast cancer cells (Schlaepfer et al., 2012).

1995). Perilipins, for which there are five members Plin1 to Plin5, regulate the accessibility of LDs for other proteins and, particularly, the activity of lipases involved in neutral lipid breakdown (Sztalryd and Brasaemle, 2017). All perilipins use an 11-mer AH repeat in their N-terminus for localization to LDs (Brasaemle and Wolins, 2012; Rowe et al., 2016). However, the binding strength of Plin1, Plin2, Plin3 and possibly Plin5, is regulated by other motifs in their C-terminal regions (Ajjaji et al., 2019).

Both the monolayer lipid membrane and protein features regulate binding of AHs to LDs (Fig. 3A). The LD surface can accommodate a lower phospholipid density compared to bilayers (Thiam et al., 2013a,b; Bacle et al., 2017; Prévost et al., 2018). Hence, AHs may more easily detect and bind to phospholipid packing defects present on LDs (Prévost et al., 2018; Thiam et al., 2013b). The possible difference in packing defects between a monolayer and a bilayer could explain how AHs specifically localize to LDs. The ability of AH motifs to specifically recognize LDs versus lipid bilayers also appears to require a tuned balance of the hydrophobic level of an AH (Fig. 3C). This hydrophobic balance depends on (1) the hydrophobicity of the amino acids in the AH, (2) the proportion of hydrophobic amino acids in the AH sequence, and (3) AH length. Increasing the number of bulky hydrophobic residues on the hydrophobic face of AH, such as tryptophan or phenylalanine, will impair its binding specificity to LDs (Prévost et al., 2018; Giménez-Andrés et al., 2018). This loss of specificity is due to the higher free energy of bulky hydrophobic residues in bilayers than that of other amino acids (Wimley and White, 1996). The fraction of hydrophobic bulky residues will thus be a determining factor in conferring an AH with a binding specificity for LDs. In the absence of bulky hydrophobic residues, the ratio between hydrophilic and hydrophobic amino acids may modulate the binding specificity of an AH to LDs (Fig. 3C). In the extreme case of hydrophobicity, an AH will resemble an HH (Fig. 3B) and can efficiently bind to all membranes. In proteins with long AH sequences (100 amino acids or more), as in those found in the 11-mer repeats of perilipins, the overall hydrophobic level can equal that of shorter AHs that are rich in hydrophobic residues. Thus AHs with a weak hydrophobic face may gain specificity to LDs by having an optimal length (Čopič et al., 2018; Giménez-Andrés et al., 2018). Nonetheless, different AHs with overall conserved hydrophobicity may specifically target LDs, but with different on- and off-rates. Finally, the nature of the neutral lipid may be important in being recognized by specific AHs, but this possibility has so far not been well documented.

In addition to the above parameters, the presence of charged phospholipids can help to initiate, increase or stabilize the binding of AHs that bear opposite charges (Fig. 3A). Such a contribution has been reported for the role of phosphatidic acid in controlling the LD-association of proteins that belong to the cell death inducing DFFA-like effector (CIDE) family (CIDE-A and CIDE-C; Barneda et al., 2015; Wang et al., 2018b), which mediate the transfer of neutral lipids between LDs at LD–LD contact sites. The presence of charged phospholipids, such as phosphoinositides, has not been well documented thus far, probably because their low concentration makes detection difficult. For example, phosphatidylinositol 3-phosphate (PI3P) mainly localizes to the ER, but has also been proposed to localize to LDs where it possibly recruits septins (Akil et al., 2016), proteins involved in the control of organelle biogenesis, shape and position (Omrane et al., 2019). Finally, charged residues on the hydrophilic face of AHs could also mediate lateral AH–AH interactions, thereby stabilizing the protein on the LD surface, as has been proposed for Plin4 (Čopič et al., 2018).

Localization of hydrophobic domains to LDs

Instead of AH motifs, many LD proteins have hydrophobic domains, such as hairpins (HPs), HHs or lipid anchors (Fig. 3B). Indeed, mammalian glycerophosphate acyl transferase 4 (GPAT4), which is involved in lipid synthesis, AUP1, which acts in quality control as discussed above, caveolins, which shape membranes, and the oleosins, which coat the LDs of plant seeds, all possess HP motifs. Proteins such as the peroxisomal fatty acyl-CoA reductase 1 may use HHs that are attached parallel to the LD monolayer (Exner et al., 2019) (Fig. 3B). Furthermore, most SNARE components have a single transmembrane hydrophobic helix (TMH) and may localize to the surface of LDs, as proposed for vehicle-associated membrane protein 4 (VAMP4) on milk LDs (Honvo-Houéto et al., 2016; Ding et al., 2012). Most of the proteins that contain HHs, at least for those with HPs, target LDs after their initial incorporation into the ER membrane (Kory et al., 2016; Wilfling et al., 2013; Jacquier et al., 2011); they 'flow' from the ER to LDs through physical bridges between these organelles. The regulatory mechanisms that underpin the relocalization of these proteins to LDs remain unclear. Oleosins have been shown to participate in the extraction of LDs from the ER (Huang and Huang, 2017), and, therefore, are found on almost every single LD. In contrast, GPAT4 localizes to only very few LDs in *Drosophila* cells (Wilfling et al., 2013, 2014). Similarly, the distribution of caveolin 1 in adipocyte LDs is not homogenous (Blouin et al., 2008).

The driving force for the relocalization from the ER to LDs mediated by HPs or HHs is currently unknown. By viewing a TMH as half of a HP domain or a HH, insights on the relocalization

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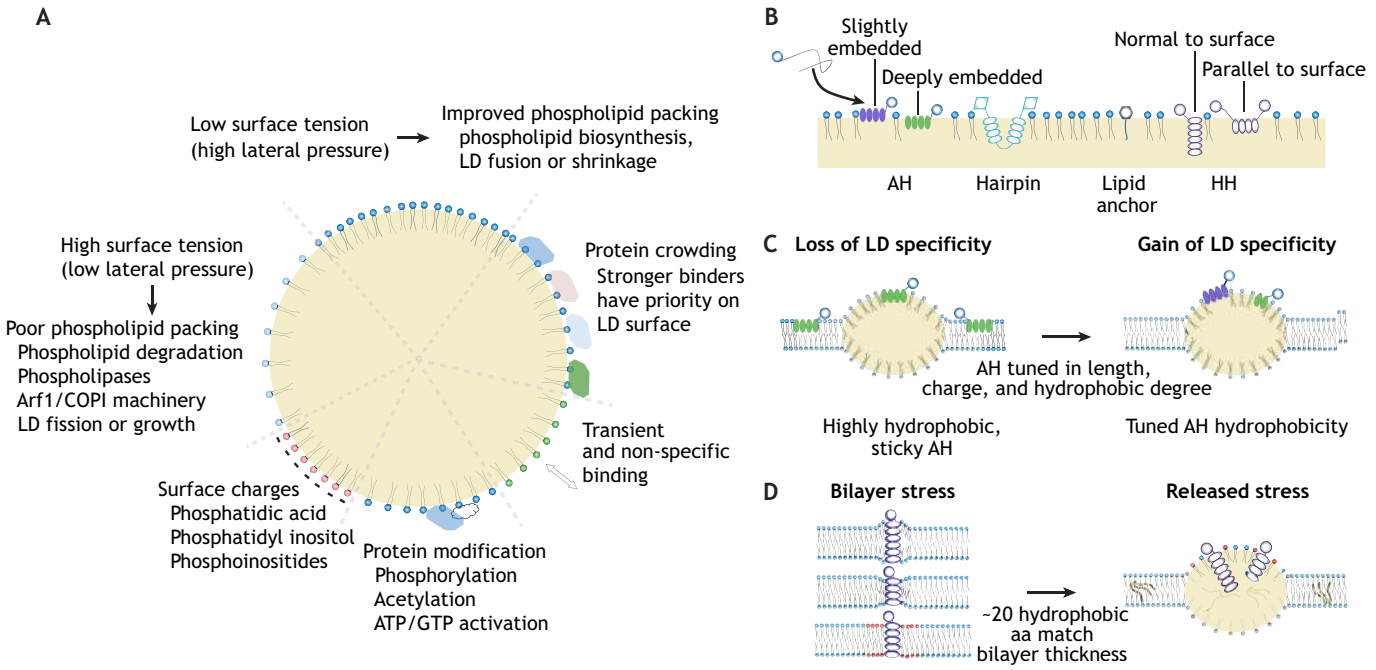

Fig. 3. Principles controlling LD–protein interactions. (A) Overview of the factors that affect LD–protein interactions. LD surface tension is determined by phospholipid packing, which can be modulated by phospholipase activity or phospholipid synthesis. Electrostatic interactions due to the presence of specific surface lipids can also regulate protein binding. Furthermore, post-translational modifications such as phosphorylation can modulate binding strength. Protein crowding also regulates access to LD by preventing transient or non-specific interaction. (B) Schematic representation of interactions of specific protein motifs, such as AH, HP, lipid anchor or HH with LDs. In the AHs shown, green is hydrophobic and mauve is hydrophilic. (C) Preferential binding of phospholipids to LD monolayers as compared to membrane bilayers by AH-containing proteins depends on the AH length, charge and hydrophobicity. The AH is embedded deeper in the monolayer if it has a large hydrophobic component. (D) Illustration of the mechanisms that favor the relocalization of an HH domain from a bilayer (left) to a LD (right). The top and middle panels on the left show an example of a HH domain that generates bilayer stress due to a hydrophobic mismatch; in the bottom panel, the HH favorably clusters with specific lipids (indicated in red). The presence of a forming LD favors the relocalization of the HH from the bilayer to the droplet surface (shown on the right). This targeting is a means to release bilayer stress and is favored by the preferential mixing at the LD surface of specific lipids containing an HH.

mechanism of such motifs to LDs may be obtained by understanding the biophysical basis of TMH segregation in membranes (Phillips et al., 2009). In bilayers, membrane stress is, for example, induced by a mismatch between the TMH length and membrane thickness (Fig. 3D). A small mismatch contributes to the sorting of proteins of different TMH length between the ER, Golgi and plasma membrane, which all have different thicknesses (Rayner and Pelham, 1997; Sharpe et al., 2010), with longer TMHs relocating to the plasma membrane and shorter ones to the Golgi. Because the phospholipid monolayer of a LD directly connects to its hydrophobic core, its phospholipid surface behaves as if it has a much larger membrane thickness than any other organelle; therefore, the localization of TMHs to LDs would alleviate the stress generated by membrane bilayer insertion. Henceforth, the principle of mismatch could favor the relocation of proteins from the ER to LDs. Furthermore, the formation of LDs could be considered as a mechanism to release the ER bilayer stress that is caused by proteins with TMHs longer than the ER bilayer thickness. TMHs of ∼20 amino acids would match the thickness of a bilayer made of the phosphatidylcholine POPC, the major membrane phospholipid. In oleosins, for example, the length of the HP is ∼35 amino acids, which is unfavorable for ER, but not LD, association. Indeed, truncation of the protein enabled its ER relocation (Huang and Huang, 2017). As an alternative to the mismatch mechanism, TMHs may cluster with specific lipids in the ER, forming a 'nano-domain' (Fig. 3D). Owing to specific and favorable dynamic interactions still to be defined, these nano-domains may preferentially move towards forming LDs, or be the site of LD formation. Consequently, TMHs would also preferentially move to LDs. This type of mechanism is reminiscent of protein segregations observed in plasma membrane domains that are enriched in specific lipids. In conclusion, the mechanisms leading to the relocation of hydrophobic domains to LDs are unclear, but answers may come from existing insights into the biophysics of TMHs.

Regulation of protein binding to LDs

The LD surface appears to be permissive to peripheral proteins that are able to bind to bilayer membranes. However, not all of these are found on LDs, suggesting that binding is either regulated or prevented by the protein and lipid coat of LDs. Here, perilipins, which are the most abundant proteins on LDs, or proteins that strongly associate with the LD surface, provide the protein coat barrier. By crowding on the LD surface, these proteins can prevent the non-specific binding of other proteins from the cytosol (Kory et al., 2015). With regard to proteins that arrive at the LD surface from the ER, seipin may act as a lateral protein-diffusion barrier during LD biogenesis (Thiam and Beller, 2017). In accordance with this, deletion of seipin leads to an abnormal relocalization of GPAT4 from the ER to almost all LDs (Pagac et al., 2016; Wang et al., 2016), supporting the idea that it has a gatekeeper function. In agreement, recent ultrastructural analysis of seipin has resolved a ring-like shape oligomer structure in the luminal side of the bilayer at the site of forming LDs (Yan et al., 2018; Sui et al., 2018 preprint) (Fig. 1). In this complex, the TMH of each seipin unit localizes around the forming LD, so that seipin could indeed directly control protein access to LDs from all sides of the ER membrane.

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The LD phospholipid coat, and in particular its packing level, also regulates non-specific protein binding. For example, α-synuclein, which is not a 'genuine' LD protein, localizes to phospholipid-depleted monolayer surfaces, but its binding is prevented by dense phospholipid packing (Thiam et al., 2013b).

Concomitant to the above physical regulation of protein binding to LDs, cells also tune protein levels on LDs by transcriptional means to modulate intracellular concentrations, or by switching their binding capacity (e.g. through GTPase activity or phosphorylation) (Bartz et al., 2007; Guo et al., 2008; Bickel et al., 2009). This is particularly important for proteins that can strongly associate with the LD surface, because if they are highly expressed or if their binding capacity can be efficiently turned on, they would displace other proteins from the LD surface and perturb LD function. By tuning the levels of such proteins, a metabolic control over LDs can be achieved in a tissue-specific manner. For example, in adipocytes that are specialized in long-term lipid storage, Plin1, which strongly associates with LDs, is heavily phosphorylated by protein kinases that are activated in conditions of lipid mobilization (Brasaemle, 2007). This probably induces a conformation change in Plin1 to ensure efficient binding and/or activation of lipases. Also, in these fat cells, the area of the LD surface is minimized by the presence of a central single large droplet filling almost the entire cell volume, which also ensures an optimal control of the LD surface by Plin1 (Suzuki et al., 2011). In contrast, in hepatocytes, Plin1 is not expressed, but instead Plin2 and Plin3 are present, which associates with LD less strongly (Ajjaji et al., 2019), thereby making the LD surface more permissive for other proteins. This might also explain why the surface of these LDs is easily hijacked by some flaviviruses (Barba et al., 1997). This would also be consistent with the presence of numerous dynamic liver LDs with high turnover rates that depend on the circadian rhythm.

In summary, the LD surface appears to be permissive for many proteins, but the regulation of the LD proteome mainly originates from a prevention of non-specific binding to LDs; this is achieved by controlling LD surface accessibility of both proteins and phospholipids, as well as by transcriptional regulation in a cell-specific manner.

LD contact sites with other organelles

Membrane contact sites are recognized as a major route for the intracellular trafficking of proteins and lipids between organelles (Dolgin, 2019). The defining feature of contact sites is organelle proximity, which typically depends on molecular tethers (Scorrano et al., 2019). In the case of LDs, the past few years have brought evidence that LD dynamics greatly relies on specific contact sites with neighboring organelles (Welte and Gould, 2017; Barbosa et al., 2015; Schuldiner and Bohnert, 2017; Wu et al., 2018). On the basis of the different tethers that have been characterized for bilayer–bilayer interactions, distinct types of contacts may be present on LDs (Fig. 4A) as outlined below.

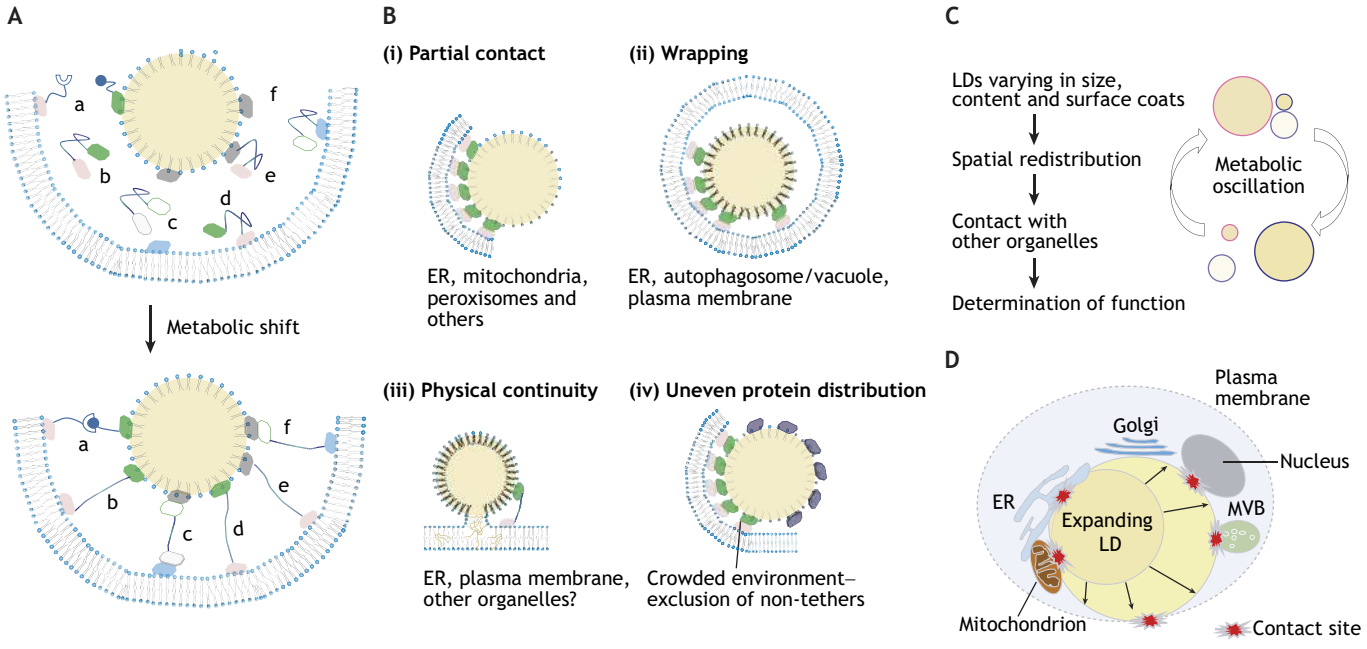

Fig. 4. LD contacts create LD heterogeneity. (A) Metabolic shifts (changes in nutrient availability or lipid mobilization signaling) can induce different types of contact sites to anchor LDs to other organelles through various forms of protein–protein interactions: a, direct anchoring, similar to a ligand–receptor interaction; b, direct binding of a soluble protein to both the bilayer and the monolayer, thereby forming a molecular bridge; c, binding of a soluble protein to proteins presents on the LD and the bilayer; d,e, indirect anchoring mediated by a protein located on either the bilayer (d) or the LD monolayer (e), which binds to the facing organelle; f, a protein located on the bilayer interacts with a protein present on the LD (the opposite situation could also happen). (B) Heterogeneity of LD contact sites. Schematic illustration of the different LD contact sites reported or possible with other organelles. (i) Partial contact. Membrane–droplet adhesion mediated by molecular tethers. (ii) Wrapping. LD–membrane adhesion leads to the full engulfment of LD by the bilayer; such a process would possibly need larger amounts of tethers than for a partial contact. This has been shown to occur in macrolipophagy or milk fat globule secretion. (iii) Physical continuity. The LD monolayer can be continuous with the outer monolayer of the bilayer; this occurs in at sites of LD biogenesis close to the ER and the plasma membrane in eukaryotes and some prokaryotes. In addition, the organelles can be tethered by proteins. (iv) Uneven protein distribution. This is often observed in partial contacts; tethered proteins are enriched at the contact site, while other LD proteins are found outside the contact regions. The LD–protein signal thus appears as a half-moon, suggesting that different activities take place at different location on the LD surface. (C) Depending on the metabolic state, LDs can cluster or be spatially redistributed to establish contacts with other organelles. This redistribution depends on LD size, lipid and protein content, and will ultimately determine the function of a particular LD. (D) The establishment of contact sites between a single LD and several organelles can occur as a consequence of LD expansion (shown in red), and lead to so-called multi-organelle–LD interactions.

Types of contact sites

Anchoring LDs

LDs may simply be 'caught' in the proximity of organelle membranes by proteins that could harbor a LD-recognizing motif, such as an AH, a lipid moiety, a HH anchor or motifs that can detect particular LD lipids. Such types of tethers might include diacyl glycerol-O-acyl transferase (DGAT2), which also resides in the ER and has a C-terminal domain that is sufficient for LD interaction (McFie et al., 2018). Recently, several studies have demonstrated that sortin nexin protein 14 (SNX14) and the nuclear protein Mdm1, which are both embedded into the ER, could control formation and turnover of LDs through tethering them to the ER membrane by using an AH on SNX14 (Datta et al., 2019) and a HH motif on Mdm1 (Hariri et al., 2019). Another LD tether is Plin5, whose C-terminus has been shown to be involved in LD–mitochondria contacts (Wang et al., 2011); here, the protein at the LD surface detects the facing membrane, possibly through recognizable features, such as bilayer charges or curvature.

Heterotypic or homotypic protein dimerization

Interactions among LDs or between LDs and organelles can be mediated by classical protein–protein interactions, the scenario most frequently reported thus far. For instance, in *Caenoerhabditis elegans*, fatty acid transport protein 1 docks LDs to the ER membrane by directly interacting with DGAT2 (Xu et al., 2012). In preadipocytes, Rab18 on the LD surface mediates interaction through SNARE complexes on the ER (Xu et al., 2018; Martin et al., 2005). In addition, members of the vacuolar protein sorting-associated protein 13 (Vps13) family have been shown to be involved in ER–LD contacts, possibly to mediate lipid transfer (Kumar et al., 2018); however, whether Vps13 has a partner on LDs has not yet been resolved. Seipin protein partners have been proposed to participate in the interactions of LDs; these include promethin in mammals (Castro et al., 2019), Ldo proteins in yeast (Teixeira et al., 2018; Eisenberg-Bord et al., 2018) and the LDAP proteins in *Arabidopsis*. Importantly, all these proteins have key roles in LDs biogenesis. Furthermore, in adipocytes, fat specific protein 27 (FSP27), which is present on LDs transdimerizes with the assistance of Plin1 to mediate LD–LD contact and subsequent lipid exchange (Gong et al., 2011; Sun et al., 2013).

Help of a third party

Although this type of tethering is less reported, cytoskeletal elements such as microtubules can also participate I the formation of LD–membrane contact sites (Valm et al., 2017). In cells that contain small LDs, movement along microtubules is key to their association with organelles and has been shown to require the recruitment of Arf1 or kinesin-1 to LDs (Rai et al., 2017; Arora et al., 2016). Interestingly, such a microtubule-mediated interaction has been reported to depend on the metabolic state and is used to redistribute LDs to mitochondria during starvation for optimized fatty acid oxidation (Herms et al., 2015). A comparable tethering mode occurs for the secretion of milk fat globules, which are LDs enwrapped by a surrounding membrane bilayer; here, Plin2 on LDs has been shown to interact with butyrophilin and xanthine oxidase on the plasma membrane (Argov-Argaman, 2019).

One particularity of the LD surface is that it can have a much higher surface tension than bilayer, that is, lower surface phospholipid density (Thiam et al., 2013a,b). The energy barrier that must be overcome for a LD to fuse to a bilayer is therefore likely to be lower than that involving two bilayers. The fusion of a LD to a bilayer involves a lipid bridge, that is, a hemifusion state generated by the continuity of the LD monolayer and the outer leaflet of a bilayer. The hemifusion state may be the underlying basis of an additional type of contact site (Fig. 4B). Such a lipid bridge exists between LD and the ER membrane (Fig. 4B) and can be interrupted after LD budding (Wilfling et al., 2014) (Fig. 1A). A bridge would enable the diffusion of proteins and small molecules without the need for active protein-mediated transfer. LD contact sites may hence involve both a lipid bridge and molecular tethers. Alternatively, a role of the molecular tethers could be to prevent the formation of the lipid bridge, by keeping LDs at optimal distance from a bilayer, and allow communication without content mixing.

Towards understanding LD heterogeneity in the light of LD–organelle contacts

LDs are heterogeneous in terms of their composition, number and distribution in different cells. They frequently appear to cluster during lipogenesis and re-disperse during situations of energy need (Herms et al., 2015) (Fig. 4C). The significance of changes in their intracellular distribution remains poorly understood. Regulation of clustering could be linked to formation or disruption of large LD–LD contact sites. Furthermore, diverse tethering modes may mediate LD–multi-organelle contacts, either separately or concomitantly.

The existence of multi-organelle contacts, as observed by spectral imaging between LDs, peroxisomes, mitochondria and ER, or vacuoles in yeast (Valm et al., 2017), is emerging as a key feature of lipid metabolism and biology (Welte and Gould, 2017; Krahmer et al., 2018; Rambold et al., 2015; Joshi et al., 2018; Hariri et al., 2018; Wang et al., 2018a). The establishment of diverse LD–organelle contact sites has profound consequences and is a source of LD functional heterogeneity. First, some LD proteins can be excluded from these contact sites, whereas others with a function at the contact site, for example, lipid transfer proteins, will be enriched therein (Fig. 4B). Furthermore, the extent of the contact site will define the rates of inter-organelle communication with regard to, for instance, lipid transfer or protein turnover. A striking example is an LD that is completely wrapped by membrane bilayer as observed in lipophagic autophagosomes; this entirely impedes access of any cytosolic proteins to the LD surface (Fig. 4B). A more frequently observed scenario is the 'half-moon like' appearance of partial rings instead of a 'full ring' distribution of fluorescent proteins around the lipid core (Counihan et al., 2011), which likely reflects the exclusion of the probe from LD–contact sites (Fig. 4B). Another example of specific protein enrichment at contact sites is the RAB18-dependent ER–LD contact, which is the hub of active viral assembly during infection with hepatitis C Virus (Dansako et al., 2014; Neufeldt et al., 2018). Such a contact site is pivotal for the relocation of the assembling virion into the ER lumen to enter the secretory pathway. Finally, it is fascinating that the establishment of large or multi-organelle contact sites on LDs can result in a non-homogenous protein distribution on the LD surface. Such a mosaic protein patterning suggests that a single LD could concomitantly perform different tasks, but at different locations on its surface (Fig. 4B).

Identification of new molecular tethers at LD contact sites

The identification of molecular tethers that connect LD to other organelles is a difficult challenge. The recent interesting observation that some mitochondria from mouse brown adipose tissue are present in LD fractions, as purified by differential centrifugation, indicating an mitochondria–LD association, has led to the characterization of a specific mitochondria population with tight

links to LDs (Benador et al., 2018). These so-called 'peridroplet' mitochondria were found to exhibit high efficiency for ATP synthesis from pyruvate, but had a low fatty acid oxidative capacity compared to the bulk of cell mitochondria. This suggested that peridroplet mitochondria might be specialized to generate ATP for esterification of fatty acids, but the multiprotein–lipid assembly at these contact sites remains to be elucidated (Benador et al., 2018). This finding also needs to be considered in light of *in situ* observations in muscle of endurance-trained athletes, a situation of efficient lipid-dependent ATP production, which show close apposition of LDs and mitochondria (Tarnopolsky et al., 2017). Here, mitochondria–LD apposition is likely to promote lipid oxidation rather than lipid synthesis and might involve different molecular complexes at the contact sites. An important protein for metabolic LD–mitochondria interaction is Plin5, also called Oxpat or LD storage protein 5 (Wolins et al., 2006; Dalen et al., 2007). Plin5 is expressed in tissues with a high lipid oxidative metabolism, such as cardiomyocytes, brown and beige fat, liver and skeletal muscle. Its expression is upregulated by exercise (Koves et al., 2012). It is still unknown whether Plin5 acts as a tether between LDs and mitochondria and how it channels fatty acids towards utilization. More generally, we are still lacking a full understanding on the nature of the physical interactions between LDs and others organelles besides mitochondria.

Consequences of LD enlargement

A main feature of LDs is metabolic flexibility during cycles of lipid filling or mobilization upon LD enlargement or shrinkage. Combined sophisticated proteomics and subcellular fractionation approaches have provided a systematic, large-scale view of LD–organelle interactions and their dynamics in cell metabolism (Krahmer et al., 2018). By using support vector machine algorithms to determine protein localization of more than 6000 liver proteins and 15,000 organelle phosphosites (i.e. a phosphorylation event observed in specific organelles) upon LD accumulation in mice that have been triggered by consumption of a high-fat diet, several proteins belonging to the vacuolar protein sorting-associated protein family, including VPS13A and VPS13D were observed to be re-directed from organelle contact sites to LDs. This indicates that orchestration of changes linked to fat accumulation involves the remodeling of the interactions between organelles and metabolically active LDs. Particularly, the observation that proteins residing in the ER (e.g. the putative methyltransferase METTL7A), mitochondria (e.g. ATP synthase subunits), at the plasma membrane (e.g. extended synaptotagmin-2) and Golgi [such as GolginA5 (GOLGA5) or GM130 (also known as GOLGA2)], as well as key signaling lipid metabolism factors (such as protein kinase C isoforms D and E), are dynamically re-distributed during early phases of metabolic reprogramming underlies the importance of enlarging LDs as drivers of inter-organelle contacts. Taken together, these data indicate the *de novo* formation of LD contact sites under prolonged lipid challenge, which could be key in the re-orchestration of cell metabolism in lipid-related dysfunction (Krahmer et al., 2018) (Fig. 4D). This view of a 'connection-dependent' LD metabolism might be particularly relevant in specific cell types, such as adipocytes, where a single prominent LD occupies the center of the cell in the proximity of and undergoing possible multiple contact points with all organelles. Owing to its central LD, the adipocyte cytoplasm is reduced to a small volume with a thickness between the cell membranes of 100 to 200 nm. When growing, the adipocyte LD is likely to exert mechanical force onto the plasma membrane through the cytoplasm. Whether adipocyte LDs behave as mechano-active transducers of lipid store fluctuation, by engaging new inter-organelle contacts, remains to be demonstrated. Reciprocally, the shrinkage of the LD would pull on the LD tethers to generate a spring-like force that propagates to neighboring cells and to the tissue. Such a mechanical signal could be translated into a physiological response. In line with this idea, caveolae, flask-shaped invaginations at the plasma membrane that are abundant in adipocytes, might contribute to the linkage of LDs to the cell surface. Indeed, adipocyte shrinkage has been shown to disrupt caveolae dynamics (Briand et al., 2014), and the main caveolar scaffold membrane protein, caveolin 1, redistributes from plasma membrane to LD in a lipid-dependent manner (Le Lay et al., 2006). These findings point to new research avenues to uncover the still poorly understood aspects of adipocyte lipid regulation, such as understanding how these cells can gauge the level of their lipid stores.

Conclusions

The importance of inter-organelle contact sites on LDs is now being appreciated and further insights into this aspect will likely bring deeper out understanding of LD biology. In particular, there is the need to elucidate how these contact sites are organized at the molecular level, and how changes in lipid composition alter their dynamics. This research will also likely bring new insights into the pathophysiology of human diseases linked to fat excess. We anticipate that they will contribute to a better understanding of the cell signaling defects that characterize these illnesses and thus help to open novel therapeutic avenues.

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Competing interests

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