Hello From The Other Side: Membrane Contact Of Lipid Droplets With Other Organelles and Subsequent Functional Implications

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Abstract: Lipid droplets (LDs) are ubiquitous organelles that play crucial roles in response to physiological and environmental cues. The identification of several neutral lipid synthesizing and regulatory protein complexes have propelled significant advance on the mechanisms of LD biogenesis in the endoplasmic reticulum (ER). Increasing evidence suggests that distinct proteins and regulatory factors, which localize to membrane contact sites (MCS), are involved not only in interorganellar lipid exchange and transport, but also function in other important cellular processes, including autophagy, mitochondrial dynamics and inheritance, ion signaling and inter-regulation of these MCS. More and more tethers and molecular determinants are associated to MCS and to a diversity of cellular and pathophysiological processes, demonstrating the dynamics and importance of these junctions in health and disease. The conjugation of lipids with proteins in supramolecular complexes is known to be paramount for many biological processes, namely membrane biosynthesis, cell homeostasis, regulation of organelle division and biogenesis, and cell growth. Ultimately, this physical organization allows the contact sites to function as crucial metabolic hubs that control the occurrence of chemical reactions. This leads to biochemical and metabolite compartmentalization for the purposes of energetic efficiency and cellular homeostasis. In this review, we will focus on the structural and
functional aspects of LD-organelle interactions and how they ensure signaling exchange and metabolites transfer between organelles.

**Keywords:** lipid droplet, membrane contact sites, membrane biogenesis, interorganellar communication, molecular tether, metabolism.

**Abbreviations:** ACAT, acyl-CoA:cholesterol O-acyltransferases; ACSL3, acyl-CoA synthetase long chain 3; AGAT, acyl-coenzyme A:cholesterol acyltransferase; AGPAT, 1-acylglycerol-3-phosphate O-acyltransferase; AKAP, A-kinase anchoring protein; AMPK, AMP-activated protein kinase; ARHGAP12, Rho GTPase-activating protein 12; ATGL, adipose triglyceride lipase; ATP, adenosine triphosphate; BAT, brown adipose tissue; BiFC, bimolecular fluorescence complementation; BNIP1, BCL2 interacting protein 1; BSCL2, Berardinelli-Seip congenital lipodystrophy type 2; CAMP, cathelicidin antimicrobial peptide; CE, cholesteryl esters; CGI-58, comparative gene identification-58; CM, cytoplasmic mitochondria; CMA, chaperone-mediated autophagy; CMT, Charcot-Marie-Tooth disease; COVID-19, coronavirus disease 2019; DG, Diacylglycerol; DDR, DNA damage response; DFCP1, double FYVE-containing protein 1; DGAT, acyl-CoA:diacylglycerol acyltransferase; dHMN, distal hereditary motor neuropathy; DIAPH1, protein diaphanous homolog 1; DNA, deoxyribonucleic acid; EM, electron microscopy; ER, endoplasmic reticulum; ESCRT, endosomal sorting complexes necessary for transport; FA, fatty acid; FABP, fatty acid binding protein; FATP1, fatty acid transport protein 1; FIT, fat inducing transmembrane; GPAT, glycerol-3-phosphate acyltransferase; GTP, guanosine-5'-triphosphate; HCV, hepatitis C virus; HSL, hormone-sensitive lipase; HSP, Hereditary spastic paraplegia; IMM, inner mitochondrial membrane; INM, inner nuclear membrane; IOGAP1, Ras GTPase-activating-like protein; LAMP1, lysosome-associated membrane glycoprotein 1; LD, lipid droplets; LDAF1, lipid droplet assembly factor 1; LDAM, lipid-droplet-accumulating microglia; Ldo, lipid droplet organization; MBOAT, membrane-bound O-acyltransferases; MCS, membrane contact sites; MCTP, multiple C2 domain-containing transmembrane protein; MFN2, mitofusin-2; MIGA2, mitoguardin-2; MIM, MIT interaction motifs; MIT, microtubule interacting and organelle transport; MLC12, myosin, light chain 12; MTP, microsomal triglyceride transfer protein; MUFA, monounsaturated fatty acid; NAFLD, non-alcoholic fatty liver disease; NPC, nuclear
pore complex; NRZ, NAG-RINT1-ZW10; Nups, nucleoporins; NVJ, nucleus-to-vacuole junction; OMM, outer mitochondrial membrane; ONM, outer nuclear membrane; OPA1, optic atrophy gene 1; ORP2, oxysterol binding protein (OSBP)-related protein 2; PA, phosphatidic acid; PC, phosphatidylcholine; PDM, peridroplet mitochondria; PEX, peroxin; PGC-1α, peroxisome proliferator-activated receptor-gamma coactivator-1-α; PKA, protein kinase A; PKC, protein kinase C; PLIN, perilipin; PM, plasma membrane; PML, premyelocytic leukemia; PMN, piecemeal microautophagy of the nucleus; PPARγ, peroxisome proliferator-activated receptor gamma; PPV, preperoxisomal vesicles; PUFA, polyunsaturated fatty acid; PX, Phox; REEP1, receptor expression-enhancing protein 1; RGS, regulator of G protein signalling; RHD, reticulon homology domain; ROS, reactive oxygen species; SARS-CoV-2, syndrome coronavirus 2; SCAR20, autosomal recessive spinocerebellar ataxia 20; SE, sterol ester; SEPT9, septin-9; SERINC, serine incorporator; SIRT1, sirtuin 1; TG, triacylglycerol; TGN, trans-Golgi network; TORC1, target of rapamycin kinase complex 1; UFA, unsaturated fatty acid; UPR, unfolded protein response; VAMP4, vesicle-associated membrane protein 4; VAPA, vesicle-associated membrane protein-associated protein A; V-ATPase, vacuolar ATPase; VLDL, very-low-density lipoprotein; VPS, vacuolar protein sorting; WAT, white adipose tissue; YAP, Yes-associated protein.

**Highlights**

- Lipid droplets (LDs) are ubiquitous organelles that play vital roles in lipid and energy homeostasis
- Membrane contact sites (MCS) affect the biogenesis, physiology and dynamics of organelles in response to the functional status of the cell
- LDs establish contact sites with various organelles for lipid exchange, and regulation of organelle dynamics and overall stress response
- LD-ER junctions are important for LD generation and mutations in biogenesis factors contribute to disease
- LDs establish interactions with peroxisomes and mitochondria LDs to channel free fatty acids (FFA) toward FA oxidation and support energy production
1. Introduction

1.1. LDs: A historical perspective

Mammalian lipid droplets (LDs), firstly described in the 1880s, remained unstudied for approximately a century. In 1886, Edmund Wilson reported the existence of so-called “oil-drops” in developing sea urchin eggs, but such structures were considered “a purely passive part in the activities of the cell, being either reserve food-matters destined to be absorbed and built up into the living substance, or by-products formed from the protoplasm as waste matters…” [1]. Such discovery was made in a particular meaningful context, in the middle of significant discussion among the scientific community about the lipid-based nature of cell membranes. The sharing and discussion of ideas and opinions about the topic goes back to the 1880s and early 90s [2, 3], but it gained momentum with Overton’s publications in 1895-1899 [4, 5], which essentially led to the general acceptance of the existence of lipid cell membranes with significant permeability to apolar molecules. Later, in 1925, Gorter and Grendel made major advances in the field with their seminal work on the lipid bilayer structure of membranes, rebounding the interest in the lipid field [6]. Between 1940 and 1950, Keith Porter and colleagues also reported the visualization of LDs on the basis of electron microscope (EM) based studies in tissue culture [7, 8], but again they were regarded as inert oil depots.

However, fundamental discoveries and breakthroughs, between 1991 and the early 2000s, led to a rising interest in metabolic diseases, and in particular in the metabolism of intracellular triacylglycerol (TG) and the nature of these "globs of stored fat" with their associated proteins. Insights included the recognition that obesity and diabetes, for instance, could, at least in part, be dictated by significant deleterious alterations of LD metabolism that disrupt the availability of structural lipids for membrane biogenesis, the synthesis and turnover of lipids that contribute to energy homeostasis, and also changes the distribution of lipids with signaling functions. In this review, we will describe the metamorphosis of LDs from unnoticed, static fat bodies to active, dynamic and biologically significant organelles due to their involvement in cell dysfunction and disease.
1.2. Cell Biology Of LDs

The physicochemical nature of membrane lipids is the crucial basis for the lipid assembly into structural and functional membranes. This physical organization allows the cell to perform temporal and spatial segregation of biochemical reactions into tailored microenvironments and organelle subdomains, thus leading to chemical compartmentalization that integrates the intracellular and extracellular information [9, 10].

Lipid metabolism, in general, is highly regulated and different organelles coordinate their functions to achieve homeostasis. The endoplasmic reticulum (ER) constitutes the major site for lipid biosynthesis [11], mitochondria and peroxisomes promote oxidation of free fatty acids (FFA) to generate adenosine triphosphate (ATP) under nutrient deprivation [12], and lysosomes are involved in lipid turnover via breakdown of lipid stores in the form of LDs [13]. These bona fide organelles are evolutionarily conserved, and are mostly recognized by their role in storing and supplying lipids for the purposes of energy homeostasis and membrane synthesis [14-17]. LDs consist of a hydrophobic neutral lipid mixture of TG and cholesteryl esters (CE), encased by a single monolayer of phospholipids with various decorating proteins [14-16].

Although commonly associated as lipid storage depots, these organelles are highly plastic and adjust their metabolism to reflect the abundance of nutrients, energy and growth factors [14, 18]. Conversely, their biogenesis process is tightly regulated to meet the requirements necessary for cellular homeostasis. More recently, LD biogenesis was claimed to be a canonical liquid-liquid phase separation process [19-21], as the segregation and formation of ER subdomains with specific lipid composition enables the flux and packaging of neutral lipids into LDs without compromising ER integrity and the toxic accumulation of neutral lipids in the ER membrane [21]. Significant progresses have now been made in disclosing the molecular mechanisms involved in LD formation, signalling, and interorganellar communication, in part with due to increasingly powerful types of microscopy techniques and various biochemical and genetic tools, which greatly accelerated recent advances in LD dynamics at the system and molecular level.

1.2.1. LD biogenesis in steps: a brief summary
1.2.1.1. Neutral lipids synthesis. The first stage of LD biogenesis is the synthesis of neutral lipids, which takes place in the ER bilayer or at the surface of the LD monolayer [14, 22] (Figure 1). For assembly into nascent apoB-containing lipoprotein particles and CE synthesis, acyl-CoA:cholesterol O-acyltransferases ACAT1 and ACAT2 (Are1 and Are2 in yeast) catalyze the esterification of cholesterol with FAs. A second set of enzymes with acyl-CoA:diacylglycerol acyltransferase (DGAT) activity are responsible for TG generation (mammalian DGAT1 and DGAT2, orthologous to yeast Dga1 and Lro1) [14]. In the budding yeast, Dga1 is transcriptionally controlled by the Target of Rapamycin Kinase Complex 1 (TORC1) downstream effectors Sit4 (the yeast homologue of mammalian protein phosphatase PP2A/PP6) and the transcription factor Sfp1, which shares key functions with the mammalian proto-oncogene MYC in ribosomal protein gene expression and cell growth. Importantly, TORC1 and protein kinase A (PKA) act coordinately to inhibit LD accumulation, in a mechanism involving vacuolar ATPase (V-ATPase) and the plasma membrane protein Pma1 H⁺-ATPase pump [23].

Figure 1

DGAT1 enzyme was first reported in 1960 [24]. It is a part of the superfamily of membrane-bound O-acyltransferases (MBOAT) and possesses three transmembrane domains with a cytosol-facing N-terminus and an ER luminal C-terminal domain [25]. Although the N-terminal domain is not required for TG synthesis, this domain is important for enzyme activity and oligomer formation [26]. DGAT1 is also known for catalyzing the generation of diacylglycerols (DG), waxes and retinyl esters [27]. The existence of a second DGAT enzyme was considered in 2000 after DGAT1⁻/⁻ mice were viable and able to synthesize TG [28]. One year later, DGAT2 was sequenced and cloned was purified from the fungus Mortierella ramanniana [29]. DGAT2 is involved in the bulk of TG synthesis, and DGAT1 and DGAT2 present distinct expression patterns, according to cell type, and play non-redundant functions. Whereas DGAT1-deficient mice are viable and resistant to diet-induced obesity [28], DGAT2⁻/⁻ mice are inviable and this has been associated with altered skin barrier and severe reductions of whole body TG levels [30]. Altogether, changes in DGATs levels have profound effects on TG levels, LD morphology and proteome, and alterations in mitochondrial membrane integrity and functions in response to dietary fat [31].
1.2.1.2. Oil lens formation. Once neutral lipids are synthesized, they locally accumulate between the two leaflets of the ER into nanometer-sized lipid lenses, as predicted in previous molecular dynamic simulations [32] and observed in cells devoid of yeast Fat storage-inducing transmembrane (FITM) proteins Yft2/Scs3 [33]. When TG accumulates to a certain threshold, it will eventually bud off towards the cytosol and nascent LDs emerge from the ER (Figure 1). This biophysical process relies on properties of lipids and is controlled by dedicated protein machinery at LD-generating ER subdomains [34, 35]. Importantly, lipid asymmetry and packing across the ER bilayer, and surface tension and membrane lateral pressure are key factors for LD budding: local lipid remodeling modifies the membrane curvature strain and surface tension to drive LD generation, unidirectionality (towards the cytosol) and size [35].

Evidence for discrete LD-forming ER subdomains supporting LD biogenesis has now been found to rely on membrane contact sites (MCS) (please see section 1.4.1 for more details). In human cells, Seipin and lipid droplet assembly factor 1 (LDAF1) form a complex to mark discrete sites permissive for LD budding [36]. Moreover, ER-shaping proteins also modulate LD assembly, including spastin, atlastin-1, receptor expression-enhancing protein 1 (REEP1) and spartin [37–41]. These proteins likely facilitate the deformation of the ER bilayer to accommodate newly synthetized TG at LD budding sites [42, 43].

In yeast, seipin (Fld1/Sei1) and Nem1 relocalized to similar specific ER subdomains [44]. Yeast Yft2 and membrane-shaping Pex30 are then recruited to form a supramolecular complex required for targeting of yeast TG synthases Dga1 and Lro1 to these nucleation sites, thus stimulating TG synthesis and oil lens formation [44] (Figure 1). By accumulating at these sites, these proteins may aid in nascent LD stabilization and, possibly through protein crowding effects, collectively facilitate bilayer deformation to enable LD budding.

1.2.1.3. Flux of TG and contact sites. To prevent lipotoxic effects and safeguard organellar integrity and budding, vectorial lipid flux from the ER to LDs is tightly regulated to prevent toxic buildup of lipids in the ER [45]. In this case, LD budding is specifically facilitated by phospholipids that lower surface tension of the ER membrane [34, 35, 46]. Dynamic alterations in lipid composition are most likely
determined by lipid lateral segregation (microdomain formation) and sequential action
of distinct lipid-catalyzing enzymes. For instance, neutral lipids accumulate in the ER
and a decrease in LD number is observed in yeast pah1Δ cells [Pah1 generates DG
from phosphatidic acid (PA)] [47], which can be related to the fact that effective
budding of neutral lipids and LD growth are severed when PA accumulates [35, 46]
(Figure 1). Transfer of neutral lipids within LDs is also driven by protein machinery
directly involved in LD biogenesis, factors required for maintenance and regulation of
membranous bridges between the ER and LDs, canonical contact site proteins or
tethering factors.

In mammals, perilipins (PLIN) are some of the most abundant LD-coating proteins
that belong to the so-called “PAT” family proteins [48] (Figure 1). The class generally
shares three domains with varying conservation: the signature PAT domain, an 11-
mer amphipathic repeat segment that is important for targeting, and a more divergent
four-helix bundle with variable effector functions [49, 50]. PLIN1 and PLIN2 are LD-
resident proteins, whereas PLIN3, PLIN4, and PLIN5 are cytosolic or ER enriched
[51]. The major isoform of PLIN1 (PLIN1A), and PLIN2 and PLIN5 preferentially
associate with LDs enriched in TG, whereas shorter isoforms of PLIN1 (perilipins 1C
and 1D) and PLIN4 preferentially associate with CE-containing LDs [51].

How the perilipin family members recognize and target LDs and whether interactions
with the surface phospholipid monolayer direct perilipin targeting remain largely
unanswered questions. PLIN1 is highly expressed in adipocytes and regulates both
lipid storage and lipolysis in a process involving protein kinase A (PKA), and
depending on the metabolic status of the cell [52]. It associates with the LD surface
through its central domain. Both N- and C-terminal domains of PLIN1 are required to
block PKA-associated hormone sensitive lipase (HSL) and non-HSL lipase-mediated
breakdown of LDs (lipolysis) [53]. Genetic ablation of PLIN1 leads to lower adiposity,
presumably due to elevated basal lipolysis [54]. Importantly, PLIN1 also interacts with
Fat-specific protein 27 (FSP27) to facilitate lipid transfer and LD growth [55]. In
oxidative tissues such as skeletal muscle, heart, and brown adipose tissue, lipolysis
is coordinated by PLIN5 [56].

Cytoplasmic perilipin proteins may sense the accumulation of DG and/or TG at the
ER. PLIN3 and PLIN4 bind to nascent droplets at early stages of the biogenesis
process [57-60]. In yeast, PIn1/Pet10, the yeast orthologue of mammalian PLIN3,
contributes to the assembly of LDs and functionally interact with other proteins like seipin and Fit2, suggesting that Pln1 assists LD budding [58]. Notably, a decrease in TG synthesis is associated with altered localization of Dga1 but not Lro1 [58]. Overall, these data suggest that, by accumulating at these sites, mammalian PLIN3/yeast Pln1 may aid in nascent LD stabilization and, possibly through protein crowding effects at LD-forming sites, support bilayer deformation to promote LD budding, while protecting nascent LDs from lipase action by making them impervious to lipolysis [51, 61].

Apart from control of lipolysis, PLINs also participate in the regulation of LD-consuming autophagic processes. Autophagy and lipolysis are both evolutionarily conserved pathways central to cell survival during periods of nutrient deprivation. Both processes are suppressed during conditions of sufficient or excessive nutrient supply (as in the fed state or with obesity) and both mechanisms are increased in response to a limited supply of energy, therefore providing FFAs as fuel for mitochondrial β-oxidation to meet cellular energy requirements. The knockdown of PLIN1 increases the association of both RAB7 and the lysosomal integral membrane protein LAMP-1 with LDs under basal conditions, concomitant with augmented lipolysis. Moreover, the stimulation of β-adrenergic receptors causes no further increases of LAMP-1 recruitment [62]. Unphosphorylated PLIN1 prevents the anchoring of RAB7 onto the surface of LDs, thus blocking lipophagy, and phosphorylation-induced conformational changes in PLIN1 enable RAB7 recruitment to LDs, allowing the association of these organelles with lysosomes [62]. A recent study provides the first evidence that overexpression of PLIN2 protects hepatic LDs from autophagic lipolysis, whereas PLIN2 deficiency reduces droplet content by enhancing autophagic lipolysis in both livers of mice and cultured hepatoma cells [63]. Recent studies have also revealed that, under conditions of limited nutrient availability, chaperone-mediated autophagy (CMA) is the major mechanism for removal of PLIN2 and 3 from LDs [64, 65]. Conversely, the removal of PLIN2 and 3 from LDs via CMA promotes both classical lipolysis and autophagic lipolysis [61]. This indicates that PLIN function is regulated by the autophagic machinery through a native feedback loop.

Seipin is a ubiquitous oligomeric ER transmembrane protein implicated in LD formation [66]. Seipin is a wheel-like undecamer in humans [67], a dodecamer in Drosophila [68] and a nine-subunit homo-oligomer protein complex formed by...
Sei1/Ldb16 in yeast [69]. It plays a role in stabilizing ER-LD contact sites through the generation of a diffusion barrier, which helps to regulate LD surface tension during budding and growth, in part by regulating the profile of acyl chains in phospholipids [70-73] (Figure 1). Seipin also interacts with multiple proteins at ER-LD junctions, which are described in section 1.4.1.1.

**1.2.1.4. Growth & expansion via acquisition of specific proteins.** Once nascent LDs begin their maturation process, they can experience additional growth and expansion (Figure 1) via well-recognized mechanisms that are now described.

**1.2.1.4.1. Flux of neutral lipids through the ER-LD contact sites.** The nature of ER-LD contacts is still a matter of debate. They are morphologically diverse and topologically unique as there is membrane continuity between the ER bilayer and the LD phospholipid monolayer and these connections are mostly stalk-like lipidal bridges [72, 74-76]. This ensures fast diffusion of lipids between the two organelles. LDs can also remain largely embedded into the ER membrane (embedded LD state) in an egg in-a-cup shaped configuration. Nevertheless, the lipid bridge formation mechanisms are still poorly understood, as they seem highly dynamic with significant rearrangements between the ER and LD compartments. Apart from these lipidic connections, several proteins have been reported to work as classical contact site proteins, biogenesis factors and maintenance factors (reviewed in [45, 77] and please refer to section 1.4.1.6).

**1.2.1.4.2. Synthesis of neutral lipids at the LD phospholipid monolayer.** Independently of the ER, the *de novo* generation of TG in mammals occurs at the LD monolayer surface, only in a subset of Glycerol-3-Phosphate Acyltransferase 4 GPAT4-containing expanding droplets (eLD), which are essentially mature LDs growing large due to the relocalization of several TG synthesis isozymes form the ER to LDs, including 1-acylglycerol-3-phosphate O-acyltransferase 2 AGPAT2, DGAT2, GPAT3 and acyl-CoA synthetase long chain family member 3 ACSL3 [75]. Importantly, it is not clear the underlying mechanisms responsible for the formation of two distinct LD subpopulations, the eLDs and iLDs. The latter correspond to smaller LDs that rely only on ER-direct TG synthesis [78].
Arf1/COP-I proteins regulate the morphology of LDs [79]. These proteins localize to LDs and can bud off nano-LDs from cellular LDs. It is proposed that they control the formation of the bridges between ER and LDs and allow key TG synthesis enzymes to relocate from the ER to LDs, possibly by regulating LD surface tension. Importantly, the seipin complex is known to act as a diffusion barrier to control LD surface tension [72] and it was proposed that LDAF1 relocalization to LDs when LD formation ceases may decrease LD surface tension at the contact sites [36]. Of notice, the formation of the fatty acid transport protein 1 FATP1-DGAT2 complex at the ER-LD interface may constitute another layer of sophistication by directing local TG synthesis and possibly facilitate DGAT2 redistribution to LDs to support their growth [80].

1.2.1.4.3. Lipid transfer between LDs and/or ripening. Other mechanisms that contribute to LD growth involve either direct fusion between neighboring LDs, which essentially corresponds to the coalescence of lipid emulsions in the cytosolic aqueous phase, or the Ostwald ripening effect [81, 82]. Both processes aim at reducing the interface surface area with the cytosol, usually leading to bigger and fewer LDs. The earlier process is rather rapid, occurring a millisecond scale, and appears to be relatively rare. LDs can establish a contact bridge with an adjacent LD, forming a membranous pore. Depending on the types of the phospholipid in the LD monolayer, the curvature strain largely drives the fusion of LDs at the contact sites: when the monolayer’s spontaneous curvature is negative, the curvature of the lipids matches the bending, and tension is low. The pore is thermodynamically stable and LDs undergo coalescence, generating one larger LD [82]. In the slower ripening mechanism, bigger LDs acquire lipids from smaller ones, where one droplet shrinks while the other one grows in a process dictated by Laplace pressures differential between the two droplets [81, 82]. Fat-specific protein 27/ cell death-inducing DFFA-like effector C (FSP27/CIDEHC), which interacts with PLIN1, assists in this process [83]. More recently, it was reported that ripening can happen between nearby LDs via the connecting ER bilayer in a mechanism regulated somehow by seipin, possibly by controlling the ER-LD contact site functionality [70].

1.3. LDs in stress response: an overview
As major lipid storage depots, LDs constitute crucial metabolic hubs that integrate the pathways of lipid uptake, storage, exchange, and signaling within the cell, in response to various nutrient and environmental cues. As our current understanding of LD metabolism expands beyond lipid metabolism, there is mounting evidence that LDs, like any other organelle, are heavily implicated in stress response [84]. A key feature of LDs is the ability to buffer excess amounts of lipids and to finely tune their release and mobilization when required, in order to fulfill cellular needs [17, 85, 86]. Due to this buffering capacity, LDs are largely recognized for protecting cells from deleterious lipotoxicity and by sequestering excess or damaged lipids into their neutral lipid core.

As LDs are essential to maintaining lipid homeostasis in the organism, they are mostly present in tissues particularly dedicated to energy storage or lipid turnover, which includes adipose tissue, the intestine and the liver. Besides, they also accumulate in skeletal muscle, the adrenal cortex, macrophages, and mammary glands. It is now recognized that LD biogenesis and breakdown are dynamically engaged in stressed cells to maintain cellular homeostasis [14]. Different tissues undergo cycles of biogenesis and breakdown depending on their metabolic and health status, thus leading to coexisting subpopulations of LDs with different size, number, subcellular localization and composition [60, 82, 87-89]. Importantly, these organelles act as signaling platforms working in cooperation with other organelles, and dynamically change their structure (lipid composition and proteome) and morphology in response to physiological demands [90, 91]. LD accumulation is observed in different contexts, ranging from protection from lipoptoxicity, nutrient starvation, oxidative stress, and other settings involving perturbation of the energetic and redox status [92].

Upon fasting, cells shift their metabolism from glycolysis to FA oxidation to generate energy [93]. For FA oxidation to occur, TGs stored in the cell are hydrolyzed to FAs by cytosolic lipases located at the surface of LDs (lipolysis) and/or by lysosomal lipases (after autophagy) [94]. Selective autophagic breakdown of LDs in lysosomes (designated by lipophagy) is known to contribute to mobilization of TG during starvation [94].

In cells exposed to oxidative stress, LD biogenesis is stimulated to prevent toxic events derived from extensive lipid peroxidation [95-97]. In tumor cells, hypoxia induces LD accumulation involving fatty acid binding protein FABP3/7-mediated FA
uptake, and this is required for cell survival after hypoxia-reoxygenation [98]. In the brain, neuronal hyperactivation is associated with increased lipogenesis and generation of peroxidated lipids [86, 97, 99]. To protect neuronal membranes from these peroxidation reactions, the oxidized lipids produced are exported to and housed in accumulating LDs in astrocytes, which in turn up regulate β-oxidation in mitochondria and induce an overall reactive oxygen species (ROS) detoxification metabolic reprogramming as a means of neuroprotection [100]. Interestingly, FABP7-dependent lipid uptake and LD generation also confers protection from hypoxic injury in astrocytes [101]. Although stimulation of LD biogenesis can have a protective effect in the brain, over-accumulation of LDs in glial cells can prompt neurotoxicity when homeostatic mechanisms gradually decline during aging. In aged mouse and human brains, microglia accumulates LDs and becomes dysfunctional lipid-droplet-accumulating microglia (LDAM), contributing to ROS build-up and inflammation in the brain [102]. This shows that with age, chronic loss of LD homeostasis is likely related to LDAM-mediated pro-inflammatory environment in the brain, which may account for age-associated and genetically defined forms of neurodegeneration [86, 99]. Overall, LDs can confer resistance against oxidative damage, which is required for cell survival in stressful conditions, but chronic accumulation of LDs can have harmful consequences.

Under ER stress pathological conditions, the ER capacity to export proteins and lipids is exceeded and the organelle induces the unfolded protein response (UPR) to cope with imbalance and recovery. This involves a reduction in global protein translation and increases specifically the transcription of genes involved in the ER stress response [103, 104]. Dysfunction of LD metabolism has been associated with induction of ER stress in several human ailments, such as dyslipidemia, atherosclerosis, hepatic steatosis, insulin resistance, heart failure, and inflammation [105]. The close relationship between LD formation and ER stress became evident from seminal studies in yeast showing accumulation of LDs in a specific set of mutants in genes linked to ER function [106, 107]. Deleterious alterations in LD dynamics may also induce ER stress [108-110], suggesting a dynamic crosstalk between these two organelles in stressful conditions. In mammals, accumulation of LDs is often observed when ER stress is induced [111-113]. The protective effect of LDs during ER stress resolution involves the removal of misfolded or damaged proteins and buffering of unwanted or toxic lipids, restoration of ER membrane lipid
homeostasis and regulation of autophagic processes [108-110, 114-117]. As LDs are present in the nucleus, it is not surprising their involvement in the regulation of phospholipid synthesis. In particular, the nuclear regulation of phosphatidylcholine (PC) synthesis [118, 119] is crucial for preservation of membrane trafficking and homeostasis in response to ER stress [120]. Frequently, ER stress leads to the release of calcium (Ca^{2+}) from the ER lumen. Barba et al. have also proposed that LDs could prevent cell death by sequestering free Ca^{2+} and reducing cytosolic Ca^{2+} overload [121].

Several studies have supported a homeostatic role played by LDs in cell cycle progression and deoxyribonucleic acid (DNA) damage response. For instance, leptin-mediated activation of the mTOR can drive intracellular LD accumulation together with increased cell cycle entry and cell proliferation [122]. Caveolin-1-deficient mice showed compromised liver regeneration after hepatectomy, triggered by cell cycle arrest of hepatocytes due to impaired LD accumulation [123]. Kurat et al. showed that Cdk1/Cdc28-dependent activation of a major TG lipase Tgl4 in yeast couples lipolysis to cell cycle progression [124]. Moreover, the sequestration of excess histones can protect Drosophila embryos from DNA damage [118, 119, 125]. In response to genotoxic UV irradiation, genetic ablation of mafr-1, the C. elegans orthologue of Maf1 in humans, results in the activation of the DNA damage response (DDR) pathway, which is associated with intracellular accumulation of LDs [119]. These observations recapitulate previous observations, as loss of Maf1 function causes lipid accumulation in mammalian cells [126, 127] and in other model systems [126, 128, 129]. This suggests that Maf1 control of lipid homeostasis is evolutionarily conserved in the response to genotoxic stress. More recently, it was shown that LD biogenesis is also coupled to alterations in distribution and subcellular localization of transcription factors and chromatin components, and to be the source of lipid ligands for some nuclear receptors [118, 130-132].

The impact of lipid loading on mechanosensing stress by human hepatocytes has also been reported [133]. Chin et al. showed that LDs, when large enough to displace and deform the nucleus, increase nuclear localization of the mechano-sensor Yes-associated protein (YAP) in primary hepatocyte cultures and in cirrhotic livers from non-alcoholic fatty liver disease (NAFLD) patients, and this was associated with altered hepatocyte mechanosensing. It is proposed that LD-associated mechanical signals in hepatocytes act as a mechanical stress akin to underlying matrix stiffness,
thus providing a possible pathway for hepatocyte dysfunction [133]. Nuclear
deformation resulting from large LDs could be potentially associated with genetic
changes, as observed for cancer cells that undergo nuclear deformation and exhibit
genetic instability after pore migration [134].

Yet, at the organismal level, it has become evident that LDs play even broader roles
than previously appreciated. As the biology of LDs is being unearthed, so is their
relevance in several human pathologies. Aberrant LD accumulation in many disease
states is characterized by impaired lipid supply and metabolism, as observed in
obesity, atherosclerosis, and fatty liver disease [105, 135]. Excessive, atypical
storage of lipids influences the metabolic homeostasis of the impacted tissues and
organs. This has also been associated with disturbances in cell signaling, in
regulation of immune cell function, in immunometabolism and in inflammatory
signaling [136]. Some studies have shown that various pathogens tap into host LDs
to ensure the necessary lipid supply, while using them as cellular platforms for viral
maturation and assembly. Common parasites (such as trypanosomes and
*Plasmodium falciparum*), bacteria (such as mycobacteria and *Chlamydia*), and
viruses (such as hepatitis C and dengue) induce LD accumulation and target LDs
during their life cycles [137, 138]. More recently, it was reported that LD accumulation
is required for productive severe acute respiratory syndrome coronavirus 2 (SARS-
CoV-2) replication and production of inflammatory mediators in monocytes derived
from Coronavirus disease 2019 (COVID-19) patients [139].

LDs can act in innate immune response, by controlling the distribution of
antimicrobial peptides, such as Virus inhibitory protein, ER associated, interferon
inducible (Viperin) and Cathelicidin Antimicrobial Peptide (CAMP), and by regulating
cellular immunometabolism in a perilipin-dependent manner [140]. Viperin was
previously shown to be enriched at LD surface and to interact with the Hepatitis C
Virus (HCV) nonstructural protein NS5A to produce an effective antiviral effect [141].
In infected cells, *Toxoplasma gondii* stimulates LD biogenesis and number to
scavenge neutral lipids and promote its replication [142]. Histones can also interact
with LDs and they are at the center of a newly proposed antibacterial
immunomodulatory mechanism. Work in *Drosophila* suggests that the presence of
histones on LDs constitutes an antibacterial mechanism for immune-related
pathways [143-145]. Notably, histone-associated antimicrobial activity was previously
described for many pathogens [146].
To fulfill these roles in stress response, LDs take part of an extensive network with other organelles that is interconnected by membrane-bound compartments [17, 90]. For these interactions with other organelles, LDs have to move within the cell [147]. Previous work showed that LDs attach to components of the cytoskeleton to traffic along the microfilaments and microtubules [148, 149]. Several recent papers showed detailed interactions between LDs and cytoskeletal proteins, including motor proteins such as dyneins [150-152], myosins [149, 153, 154] and vimentin [155, 156]. Examples of well-established stable/dynamic associations include junctions or MCSs between the ER and other organelles including mitochondria, plasma membranes, vacuoles/lysosomes, Golgi, and endosomes [157, 158]. At these MCS, several protein complexes assemble to establish specific functions dedicated to binding, sensing and transfer of molecules and metabolites, and even in engaging in organelle biogenesis and dynamics [158]. Whether physical connections between LD and the other organelles have physiological importance is now a matter of intensive debate, as proteins specific to junctions are being identified and studied in more detail, and genetic tools have been employed to unravel their role and functions within the context of stress.

1.4. LD Membrane Contact Sites: Everything We Do Is Communication

Interorganellar MCSs correspond to domains that exist in close proximity between heterologous membranes, approximately up to 30 nm [158]. At these sites, proteins (termed tethers) attach two organelles together to facilitate lipid and ion exchange. Bipartite or even tripartite organelle membranes can be tethered via protein-protein and protein-lipid interactions, thus supporting close proximity between the organelles while preventing membrane fusion [158, 159]. Because MCS have been implicated in crucial biological processes including nutrient signaling, stress response, apoptosis, lipid and ion exchange and organelle integrity and dynamics [158, 160, 161], their study has gained momentum in the research field. Physical interaction between ER and mitochondria was firstly reported in the 1950s [162, 163]. Since then, powerful omics and microscopy tools approaches have greatly accelerated the identification of MCS protein tethers and regulatory factors at the molecular and system level [164], founding the era of "tether-omics", and we are now appreciating the beauty and complexity of this highly interconnected network of membranes that assemble or
disassemble at MCS in response to various endogenous and exogenous stimuli. The importance of MCS in cellular physiology is highlighted by the existence of mutations in proteins tethering diverse pairs of organelles that have been associated with the progression of neurodegeneration and cancer [158, 160, 161]. Here, we will emphasize the identity and vital roles of LD tethering to other cellular organelles and discuss their importance in different metabolic and pathophysiological contexts.

1.4.1. LD-ER interactions

The ER is a dynamic organelle that serves many roles in the cell, namely protein synthesis, folding and transport, calcium storage, redox homeostasis and lipid metabolism. The ER forms a single continuous membrane that is composed of a network containing multiple subdomains with distinct structures (tubules, sheets and the nuclear envelope) and functions [11, 165]. In addition to being classically associated with the nucleus, the ER has largely interdependent structures that can communicate with other organelles through MCS to support lipid exchange, ion transport and even organelle distribution and division [166]. LDs can establish extensive contacts with many organelles, including the ER [167, 168] (Figure 2). ER-LD contact sites are virtually present in higher eukaryotes and physically associate the ER bilayer to the LD monolayer through a lipid bridge. Early cryo-EM studies have demonstrated the membranous nature of the junction, in which the cytoplasmic leaflet of the ER membrane is continuously connected to the LD surface, forming a cup-shaped structure that enwraps the nascent LD [169, 170]. In yeast and mammalian cells, LDs commonly remain associated to the ER via a lipid junction that is decorated by seipin, a protein that specifically localizes to ER-LD junctions to drive proper LD formation and growth [67-73, 77, 168, 171-175].

Figure 2

1.4.1.1. Seipin

Seipin, encoded by the gene Bernadelli-Seip congenital lipodystrophy type 2 (BSCL2) [66, 176] and SEI1 in yeast [73, 171, 177], is a ubiquitous integral ER membrane protein containing two transmembrane domains, a long luminal loop, and
cytoplasmic N- and C-terminal tails [67]. In yeast, seipin is a large protein complex composed of non-redundant Sei1 and Ldb16 proteins, that form a stable homoooligomer made of nine subunits with a radially symmetric shape [69, 72, 178], whereas in humans it forms a undecamer [67]. Despite multiple topologies [67, 68, 72], seipin is specifically targeted to LD-ER contact sites to facilitate the delivery of TG from ER to LDs [71, 72] (Figure 2). ER-LD tethering seems to be evolutionary conserved to facilitate LD biogenesis [71, 74, 75, 120, 168, 174, 179]. Seipin deficiency provokes defective targeting of proteins to LDs and contributes to ER Ca²⁺ dyshomeostasis and aberrant lipid metabolism involving PA accumulation [72, 172, 180-182]. Moreover, deletion of seipin does not abolish LD biogenesis, but alters the profile of acyl chains in phospholipids, which largely accounts for alterations in LD morphology, characterized by ‘supersized’ LDs or clusters of abnormally small and misshapen LDs that may arise from premature growth [70-73, 171, 174, 175, 181, 183].

Structurally, different domains of the protein are required to fulfil its role in LD biogenesis. Overexpression of a truncated form of seipin lacking the C-terminal part dramatically reduced LD formation in hepatocytes and mouse preadipocyte stem cells [184]. Importantly, both N- and C-terminus of seipin interact with 14-3-3β and seipin-14-3-3β-cofilin-1 interaction seems to be important for actin cytoskeleton remodeling and adipogenesis [185]. Finally, the C-terminus domain of seipin binds to Lipin-1 [186] and PLIN2 during adipogenesis stimulation [187], whereas the core/loop region is crucial for GPAT3/4 interaction [188]. Seipin is preferentially enriched at ER tubules, owing to its highly curved domains that favors and controls LD nucleation [34, 183]. Interestingly, seipin interacts with REEP1, which shapes the ER network in cells, and REEP1, atlastin-1 and the M1 isoform of spastin interact with each other through hydrophobic hairpin domains within the tubular ER to spatially coordinate ER shaping and distribution and microtubule interactions [40]. These ER specific subdomains may be specialized in lipid remodeling, particularly that of PA/DG/TG levels, to support vectorial LD budding, while preventing bulk accumulation of these lipids that otherwise would disrupt ER structure and morphology. Importantly, both human seipin and C. elegans ortholog SEIP-1 are known to be recruited to a tubular ER subdomain enriched in specific polyunsaturated fatty acids (PUFAs), and to tightly associate with a subset of...
LDs at nascent sites [34, 183, 189]. Therefore, LDs heterogeneity may originate from FA-driven targeting of distinct protein ensembles.

Seipin also interacts to and regulates the activity of GPATs, which converts glycerol-3-phosphate and long-chain acyl-CoA to lysophosphatidic acid, a precursor of PA [1]. In mammalian cells, seipin binds to GPAT3 and GPAT4 to modulate LD expansion and adipogenesis [188]. Human seipin, more precisely its β-sandwich structure, can bind to anionic phospholipids [67]. GPAT could then help to control the levels of PA along with seipin at sites of LD biogenesis. Seipin also directly regulates TG synthesis through physical interaction with acyl-coenzyme A:cholesterol acyltransferase 2 (AGAT2) and Lipin-1, key enzymes of the TG biosynthetic pathway [190, 191]. Overall, seipin acts as a scaffold for PA [67] and for many lipid biosynthetic enzymes to fulfill its role. In this context, Molenaar et al. provided evidence for the production of LDs containing retinyl esters in hepatic stellate cells, independently of DGAT1, TG synthesis and seipin [192]. So the role of seipin in LD generation is seemingly dictated by the nature of the neutral lipid to be stored in LDs, and the reason for the observed association of seipin to RE lipid droplets is currently unknown. Although seipin influences the transfer of REs into TAG-filled LDs [192], distinct and specific machineries may exist to catalyze the transfer of neutral lipids into subtypes of LDs, possibly accounting for different subpopulations fulfilling particular functions in a cell-type manner. Through contact sites, seipin also negatively controls sphingolipid homeostasis by targeting serine palmitoyltransferase activity, although the mechanism remains poorly understood [193].

So far, the mechanism of action of seipin posits that the protein scans the ER membrane, using its hydrophobic α-helices, to screen for lipid packing defects corresponding to small oil lenses formed within the ER bilayer in a FIT-dependent manner. Once seipin gets trapped in these specialized ER subdomains permissive to LD biogenesis, it concentrates to mediate local lipid remodeling, by transferring newly synthesized TG from the ER membrane into the growing LDs [68], while facilitating LD budding and growth. Furthermore, the seipin complex, along with its partners at the base of ER-LD junctions, generates a diffusion barrier that promotes the unidirectional flux of TG and uncouples the lipid composition of these ER sites to prevent disruption of the bulk ER morphology and counteract ripening-induced shrinkage of small LDs [70, 72]. Recent experimental and molecular dynamics
simulation studies showed that seipin traps TG and its immediate precursor DG inside its disk-like oligomeric structure [175, 194], and these interactions require both its luminal and transmembrane regions [194]. Consistently, cryo-EM studies of seipin luminal regions showed that each protomer harbors a lipid binding domain and a hydrophobic, membrane-anchored helix that is essential for this function [67, 68].

Apart from cytoplasmic LDs, cells can also generate LDs at the inner nuclear membrane (INM) in a seipin-dependent and independent manner (please see section 1.4.3 for more details).

Additional yeast studies revealed that seipin Sei1 and Nem1 form discrete ER subdomains that are permissive for LD biogenesis [44]. Once they are formed, Sei1-Nem1 marked sites are enriched in DG generated by phosphatidate phosphatase lipin Pah1, possibly by trapping DG in the ER membrane as reported for mammalian seipin [175, 194]. At these sites, FIT proteins, Pex30, and TG synthases (Dga1 and Lro1) are recruited to drive oil lens formation, and prompt TG synthesis to facilitate the emergence of nascent LDs once TG reaches a threshold [44]. Mature LDs can remain associated with seipin/Nem1 puncta in the ER, or expand and grow even bigger as described before. More recently, Ldb16, which forms a complex with Sei1 in yeast, was shown to concentrate TG within the Sei1 ring-shaped complex, while the transmembrane domains of Sei1 recruit more TG into these ER subdomains [195]. These observations highly recapitulate the findings made in human seipin [175, 194].

Missense mutations of human BSCL2 have been linked to lipodystrophy and they are concentrated mainly in the ER luminal region (e.g., L91P and A212P), indicating that this fragment of seipin is essential [196]. Seipinopathy represents a spectrum of hereditary neuropathies caused by BSCL2 mutations. Individuals carrying gain-of-function N88S and S90L mutations display clinical features related to distal Hereditary Motor Neuropathies (dHMN) type V, Silver syndrome, or Charcot-Marie-Tooth disease type 2D (CMT2D) [86], and this has been associated with impaired folding of seipin due to blocked N-glycosylation at these sites, thus promoting severe ER stress and cell death [197]. The BSCL2 R96H mutation has been recently assigned as a novel cause of dHMN, which is characterized by low seipin expression and reduced cellular viability without inducing ER stress [198].

1.4.1.2. Lipid Droplet Organization (Ldo) and Promethin/LDAF1
Recent studies have identified new interacting proteins of the seipin complex that perform regulatory role at the ER-LD contact sites, namely the yeast Lipid droplet organization (Ldo) machinery, Ldo16 and Ldo45, and the corresponding human homologue of Ldo45 promethin/TMEM159/LDAF1 (Figure 2). In yeast, Ldo proteins were recently described as partners of seipin in independent studies [88, 199]. Teixeira et al. performed pull-down assays to identify two splicing isoforms of the same protein, Ldo45 and Ldo16. Both proteins are differentially expressed during growth and fulfill distinct functions. During active growth, Ldo45 expression controls TG accumulation, in part by controlling lipolysis, and influences LD size and morphology [199]. Since the overexpression of Ldo45 promotes aberrant LD clustering, resembling the LD phenotype of mutants lacking seipin, Ldo45 seems to antagonize seipin in expanding LDs [88]. On the other hand, Ldo16 expression remains constant during aging and is required for vacuolar microdomain formation, which in turn is necessary for sterol ester (SE) turnover and efficient lipophagy [199]. In another study, the LD biogenesis factors Pdr16, a lipid transfer protein and Ldo45, was shown to accumulate near the edges of the nucleus-to-vacuole junction (NVJ) containing a specific subpopulation of LDs, and Ldo16 is dispensable for Pdr16 targeting but required for correct LD positioning at the NVJs [88].

In the search for Ldo protein homologues, Castro et al. identified TMEM159/promethin as a protein with strong similarity to Ldo45 [200], and then renamed to LDAF1 [36]. LDAF1 is up-regulated in liver cells under lipid storage conditions [201], and seipin was then identified as an interactor of promethin in human cell lines [200]. Mechanistically, LDAF1 and seipin form a complex where TG accumulates and generates discrete ER sites of LD formation [36]. In oleate-challenged cells, seipin remains localized at ER-LD junctions, but LDAF1 no longer associates with seipin and relocates to the LD surface of growing LDs, using a conserved domain containing four possible membrane-spanning helices that forms a hairpin structure required for localization to both the ER and LDs [36]. These findings suggest that seipin may facilitate TG phase transition in the ER bilayer, while excluding other lipids from the ER, to promote vectorial transfer of TG to the nascent LDs and prevent droplet ripening and profound changes in ER lipid composition [70]. It was also proposed that LDAF1 lowers the energy barrier by decreasing the TG concentration threshold, as LD generation is defective upon LDAF1 ablation [36].
Once TG reaches a threshold, seipin excludes LDAF1 from these sites, which can then move freely onto the monolayer of a nascent LD [36]. Here, LDAF1 may have protein-remodeling properties to control proteome composition and LD growth. Exogenous oleate, which is commonly used to stimulate TAG synthesis and lipid storage in various studies, was used in this work probing the regulatory role of LDAF1 in the context of seipin function in LD budding and size. However, caution is necessary in the interpretation of the physiological relevance of the proposed mechanisms. Under regular growth conditions, LDAF1 was shown to have a dispersed distribution in MCF7 cells, and only when challenged with oleate, LDAF1 levels are increased and the protein is markedly relocalized to the surface of LDs in a circular pattern, which indicates that LDAF1 localization depends on the metabolic status of the cell [200]. Interestingly, absence of seipin also results in loss of stability of LDAF1, similarly to what happens with Ldb16 in the yeast seipin complex [178]. Overall, this suggests that seipin deficiency may actually represent circumstances where both seipin and its interactors are absent.

1.4.1.3. Fat-Storage-Inducing Transmembrane Proteins (FITM)

FITM proteins are members of the evolutionarily conserved family of polytopic ER-localized transmembrane proteins [202, 203] that are enriched at ER-LD junctions during LD biogenesis. Consistent with a role in neutral lipid metabolism, FITM proteins are required for oil lens formation by trapping TG in the ER bilayer [202, 204]. FITM2 is required for adipogenesis in a process controlled by peroxisome proliferator-activated receptor gamma (PPARγ), which in turn controls the accumulation of TG within LDs [202]. Purified FITM2 has the ability to bind both DG and TG in vitro, but does not contribute to TG biosynthesis, suggesting that FIT proteins may play a role in recruiting TG synthases to locally control DG accumulation [204], to promote the formation of TG-enriched LDs and to control LD number and size [202]. How FITM2 proteins fit within this process remains puzzling. Hayes et al. showed that FITM2 proteins could be potential lipid phosphatase/phosphotransferases on the ER luminal side [205], where it may contribute to changes in lipid composition at these ER subdomains, possibly by
regulating DG content [34, 47]. They may actually alter phospholipid composition to adjust the surface tension of the inner leaflet of the ER membrane and collectively regulate droplet budding at these sites. By decreasing DG levels at the cytosolic leaflet of the ER membrane, in the vicinity of the budding site, FITM proteins assist on the unidirectional LD emergence [34]. FITM2 also exhibits acyl-CoA diphosphatase activity [206], which is essential for proper ER membrane homeostasis and maintenance of lipid composition [206]. It is thus not surprising that deletion of FITM2 in adipocytes results in lipodystrophy and insulin resistance [207].

Two FITM2 homologs, Yft2 and Scs3, were identified in yeast to play roles in protein and lipid homeostasis [208]. Prinz’s lab characterized in detail the effects of Yft2 and Scs3 in the LD biogenesis process by showing that lack of these proteins results in stalled LDs that do not bud off towards the cytoplasm, but instead remained embedded within the ER bilayer, thus generating ER tangles [205]. This indicates that these proteins prompt effective budding of nascent LDs from the ER [33, 209, 210]. This is in line with the fact that Yft2, Nem1 and Lro1 come together to LD biogenesis discrete sites when LD production is stimulated [44]. Lack of FITM2 in human 3T3-L1 fibroblasts leads to similar phenotypes, showing conserved function in LD formation [33]. Notably, deletion of FITM2 in C. elegans and mice is lethal [33, 211].

1.4.1.4. Pex30/MCTP

In yeast, Prinz and Carvalho’s labs have recently shown that the seipin complex and Pex30 are enriched at sites of preperoxisomal vesicles (PPV) budding in the de novo peroxisome biogenesis process [212], and also at budding sites for LD growth [42, 43]. Importantly, Pex3 and Pex19, which are involved in peroxisome biogenesis and related to the topogenesis of hairpin-containing membrane proteins, also insert membrane-embedded proteins into the surface of LDs at ER subdomains [213, 214]. For instance, LD resident UBX8 interacts with Pex19 in the cytosol and targets it to ER-localized Pex3 [213]. Collectively, peroxins have pleiotropic effects in LD dynamics.

Pex30 possesses a N-terminal reticulon homology domain (RHD), a hairpin-type membrane domain similar to members of the reticulon family of ER-shaping proteins, and a C-terminal Dysferlin (DysF) domain [212]. It has the ability to tubulate the ER
membrane and contrarily to reticulons, Pex30 localizes to subdomains in the ER where most PPVs form and is excluded from ER exit sites at which COPII-coated vesicles are generated [212]. Importantly, deletion of Pex30 and seipin impairs both LD biogenesis and peroxisome biogenesis, and leads to proliferation of ER convoluted membranes, independently of reticulons. This has been associated with altered phospholipid and neutral lipid profile, suggesting that seipin and Pex30 are required for effective LD budding [42, 43, 183]. Moreover, this process is UPR-independent and is not prevented by abrogation of autophagic processes, indicating that cells can tolerate high levels of lipotoxic stress despite mild defective growth [42].

DysF-containing proteins play a regulatory role in the repair of damaged membranes due to mechanical stress, which is likely attributable to its membrane-shaping properties. Whether Pex30 displays such membrane repair properties [215] should be addressed in future research. In its C-terminal cytosolic domain, Pex30 has a DysF-like domain that could bind DG [43, 216, 217], a feature that is shared with FITM2 [204]. Mechanistically, activation of Pah1 and accumulation of DG at LD biogenesis sites drives Yft2 recruitment at Fld1/Nem1 sites. Once formed, Pex30 localizes to Fld1/Nem1/Yft2-containing ER subdomains, and shape the ER bilayer. This creates a favorable environment for TG producing enzymes to produce TG and form nascent LDs [44]. How membrane-shaping and DG-binding activities are necessary to promote organelle budding along with FIT2 proteins is unclear, but Pex30 could modulate DG distribution locally at sites where LDs and peroxisomes emerge, and it may become more relevant when seipin activity is decreased or absent or even under conditions of impaired cholinephosphate cytidylyltransferase Pct1 function [42]. These observations also imply that the ER segregates into multiple subdomains, with specific protein and lipid composition, in a process dictated, at least in part, by different RHD-containing proteins for the purpose of assuming specific functions: organelle biogenesis at Pex30-marked sites [42, 43, 212, 218, 219], or assembly of the distinct Pex30-containing complexes for regulation of MCS under metabolic stress conditions [220]. Moreover, Pex30 also interacts with Pex28, Pex29, Pex31 and Pex32, as well as Rtn1 and Yop1 [221, 222]. All these proteins have membrane-deforming properties. At PPVs, enriched in seipin and Pex30, reticulons likely regulate the curvature strain to exclude ER matrix proteins and allow PPV budding. Thus, Pex30 probably marks sites where multiple organelles
function in tandem to control the lipid exchange and convey metabolic signals at several MCS. Multiple C2 domain-containing transmembrane proteins, MCTP1 and MCTP2, are also at sites of LD formation in specialized ER subdomains. As functional mammalian homologues of Pex30, they are associated to a subpopulation of seipin-marked LDs, and contain multiple C2 domains and an RHD with different functions [43]. RHDs regulate LD formation, while C2 domains controls LD size, probably by regulating LD-ER contacts [223]. Also, loss of MCTP leads to a significant reduction in LD size and number, reinforcing its role in LD biogenesis [223]. As described for Pex30, MCTP2 resides in ER subdomains permissive to LD and peroxisome biogenesis [43], and its membrane-shaping ability might account for the accumulation of DG (and possibly TG) at sites of LD and peroxisome biogenesis [223]. As for Pex30 [220], MCTPs are present at multiple ER-organelle contact sites, which may be important for the regulation of organelle division, metabolism and integrity [223].

1.4.1.5. Mdm1/Sortin Nexin 14

Along with seipin, FITM and MCTP proteins, mammalian SNX14 (Figure 2) and its yeast counterpart Mdm1 were also identified as ER-LD tethers [224, 225]. SNX14 harbors a phosphatidylinositol 3-phosphate-binding Phox (PX) domain and a regulator of G protein signalling (RGS) domain, and plays a role in ER-LD tethering and LD growth. SNX14 is targeted to ER-LD junctions upon oleate addition and overexpression of SNX14 enhances interorganellar contact sites, whereas its loss reduces ER-LD tethering [224]. The amphipathic helix in the C-terminus, the C-Nexin domain, is required for interaction with LDs in trans [224]. Notably, depletion of SNX14 generates small LDs and few very large LDs, a phenotype observed upon seipin deficiency; nevertheless, seipin and SNX14 are not functionally redundant [224]. In fact and despite high similarity in terms of domain composition, SNX14 lacks the residues for phosphoinositide binding [226], and NVJ contacts have not been reported in mammalian cells. Mutations in SNX14 have been associated with recessive forms of spinocerebellar ataxia associated with intellectual disability (autosomal recessive spinocerebellar ataxia 20, SCAR20). How SNX14 participates in the pathophysiological manifestations of SCAR20 is unknown, but could be related to gross LD morphology defects and impaired metabolism.
In yeast, Mdm1 is targeted to LD biogenesis sites via its N-terminal domain, while a
the PX domain is required for association with the vacuole [225]. The PX-associated
(PXA) domain is necessary for LD recruitment, and collectively these domains
essentially mark a subpopulation of Mdm1-associated LDs at the NVJ [225].
Importantly, Mdm1 interacts with the fatty acyl-CoA synthetase Faa1 via the PXA
domain, which also binds to FAs in vitro [67]. It is likely that Mdm1 participates in FA
activation to support LD biogenesis. More recently, it was shown that Mdm1 prompts
nucleophagic degradation of nucleolar proteins at the NVJ when TORC1 is inactive,
but it was unnecessary for nucleophagic flux [227]. Like Pex30, Mdm1 and many
other tethering proteins bring different organelles in close proximity using different
lipid-binding specificities, thus forming individual LD sub-populations at distinct
MCSs.

1.4.1.6. Other biogenesis factors

Several other proteins have been implicated in LD biogenesis. VPS13 proteins
(mammalian VPS13A and VPS13C and yeast Vps13) act as tethers, but are
potentially involved in interorganellar bulk lipid transfer [228, 229] (Figure 2). RAB18
is a master regulator of lipogenesis and lipolysis in adipocytes [230], and was also
detected with LD-ER contact sites (Figure 2), where it associates with the NAG-
RINT1-ZW10 (NRZ) tethering complex and the Q-SNARES Use1, Syntaxin18 and
BCL2 Interacting Protein 1 (BNIP1) to jointly support LD growth [231, 232]. Although
the Rab18-NRZ complex tethers the ER and LDs, this is dispensable for
maintenance of LD growth in all cell types [233]. A crosstalk between RAB18 and the
double FYVE-containing protein 1 (DFCP1) has also been reported for efficient ER-
LD tethering and LD expansion [234]. Recently, loss of RAB18 was shown to impede
LD breakdown and induce metabolic adaptive changes in autophagic machinery,
namely on ATG2 and ATG9 [235]. Mutations in RAB18 are associated with Warburg
Micro syndrome, a neurodevelopmental disorder defined by microcephaly,
intellectual disability optical atrophy, and hypogonitalism [236, 237]. Whether LD
dysfunction contributes to the disease is currently unknown.

Yeast Ice2, an ER membrane multspanning protein (Figure 2) necessary for
maintenance of cortical ER structure and distribution [238], accumulates at sites in
close proximity to LDs at later stages of growth, where LD biogenesis is stimulated
From a structure point of view, amphipathic helices contained within the cytoplasmic loop of Ice2 can bind to LD surfaces. As reported, Ice2 is important for LD breakdown to supply phospholipids upon growth resumption, and this is associated with relocalization of the DGAT Dga1 to the ER [239]. When Ice2 function is lost, cells display a significant defect when growth resumes, which essentially recapitulates the phenotype of cells lacking LDs. It is thought that in cells devoid of Ice2, the newly generated DG catalyzed by the TG lipases remains on LDs and enters a potentially futile cycle of re-esterification to TG [239]. A more recent study reported a direct interaction between Ice2 and Spo7, the yeast homolog of human TMEM18A, also called NEP1-R1 and member of the yeast Nem1-Spo7 complex, which activates lipin Pah1 (homolog of Lipin1 in humans) [240]. Since Pah1/Lipin channels PA to generate DG, the immediate precursor of TG, Ice2 functions to promote LD biogenesis. Recently, Ice2 was reported to be a full-length homolog of mammalian serine incorporator (SERINC), an evolutionarily conserved family of proteins with 10 transmembrane helices, which play a role in serine assimilation into lipids. However, they are more broadly recognized for their role as potent restriction factors for HIV and other viruses [241]. Whether LD generation plays a role in this context would be an interesting direction to follow.

Of particular relevance, a complex made by ER-localized FATP1 and LD-associated DGAT2 was also observed at the LD-ER interface [80] (Figure 2). Given their roles, they are potentially required for localized TG synthesis at the contact sites to promote LD growth. Finally, in mammalian cells, the oxysterol binding protein (OSBP)-related protein 2- Vesicle-associated membrane protein-associated protein A (ORP2-VAPA) complex also localizes to LD-ER contacts and EM staining actually revealed a rather modest but significant increase of the total length and number of such connections in the ORP2-knockout cells, indicating that ORP2 and VAP partners play a functional role in the dynamic regulation at the LD-ER interface [242] (Figure 2). ORP2 has multiple functions in lipid transfer, steroid hormone biosynthesis, cholesterol and TG metabolism. To fulfill these roles, ORP2 was shown to interact physically with F-actin regulators such as Protein diaphanous homolog 1 (DIAPH1), Rho GTPase-activating protein 12 (ARHGAP12), Septin-9 (SEPT9) and myosin, light chain 12 (MLC12), as well as with Ras GTPase-activating-like protein (IQGAP1) and the Cdc37-Hsp90 chaperone complex to control the activity of serine/threonine kinase 1 Akt [243]. The
functions of ORP2-VAP in the context of LD biogenesis and ER-LD junctions are currently enigmatic.

1.4.2. LDs and Golgi apparatus come closer

The Golgi apparatus is mostly recognized by its role in the anterograde transport of newly synthesized proteins to the plasma membrane and other intracellular organelles, and also in the retrograde traffic from recycling pathways and endocytic routes [244-247]. Importantly, the exit site of the Golgi (trans-Golgi network, TGN) is the major site for sorting proteins and lipids to different cellular sites [248]. Valm et al. have shown that Golgi and LDs can come in close proximity [249], however the identification of bona fide and functional Golgi-LD tethers is currently undefined. A recent preprint identified the vacuolar protein sorting-associated protein 13B (VPS13B), a member of the highly conserved VPS13 family, as a tether that establishes MCS between the TGN and LDs in human cell lines. It requires the C-terminal amphipathic helix to bind to LDs, and the WD40 and VPS13B-PH domains to recognize TGN membranes through RAB6 [250]. VPS13B is required for proper Golgi structure [251] and cargo sorting [252]. In this study, they also showed that VPS13B lipid transfer capacity was necessary to maintain TGN integrity and functions [250]. Some evidence of LD-Golgi interactions came from Krahmer and colleagues [253], who showed co-fractionation of Golgi proteins with LDs in the steatotic liver, which is relevant in the context of NAFLD. They observed that the Golgi apparatus partially wrapped around LDs to establish direct MCS under severe conditions of LD accumulation. In consequence of high hepatic lipid content, very-low-density lipoprotein (VLDL) particle expansion and secretion is enhanced, a hallmark of dyslipidemia [254]. A general decrease in protein secretion during severe steatosis may indicate that TGN integrity and function are somewhat compromised. Excessive accumulation of TG in LDs and impaired hepatocyte lipid metabolism may affect protein sorting and/or maturation during cargo sorting, feeding a vicious cycle of perturbed protein secretion and exacerbated lipid imbalance [255], where VPS13B may well play a role in this process as a lipid transfer protein. Loss-of-function mutations of VPS13B have been associated with pathological alterations in Cohen
disease [256, 257], pointing toward a possible involvement of TGN-LD contact sites into disease mechanisms.

Other proteins have been posed as possible candidates to mediate LD-Golgi interactions, including golgins, but their role remains unexplored in this context [258, 259]. Moreover, ORP5 and ORP8, two members of the OSBP family of sterol sensors and lipid transfer proteins, have been reported to transfer sterols across ER-Golgi MCS in a Sac1-dependent mechanism [260]. ORP5 is enriched at ER-LD MCS and functions in PI(4)P/PS exchange. Deletion of ORP5 promotes an increase in LD size, whereas overexpression of ORP5A, but not of PS- or PI(4)P-binding mutants, had the opposite effect. This indicates that the phospholipid transfer activity is required for targeting and control of LD size [261]. Importantly, mislocalization of trans-Golgi resident proteins to endosomal compartments and cholesterol accumulation in late endosomes and lysosomes was also observed upon ORP5 depletion [261]. Whether they also participate in Golgi-LD contact sites would be an interesting issue to be investigated in future studies.

1.4.3. LD-nucleus contacts

The specific lipid pathways that drive LD biogenesis in the nucleus are now being unravelled, but the origin and physiological significance of perinuclear LDs (nLDs) are still poorly understood [262].

In mammals, LDs associate with premyelocytic leukemia (PML) nuclear bodies and type I nucleoplasmic reticulum. These nLDs harbor DGAT2 and CTP:phosphocholine cytidylyltransferase α, two important enzymes needed for TG and phosphatidylcholine synthesis, respectively [179]. A recent proteomic study provided a complex view of nLD proteome [263], revealing the presence of a novel carboxylesterease 1d (Ces1d/Ces3), which is involved in TG metabolism and VLDL assembly [264]. In hepatocytes, a sub-population of LDs emerge from apolipoprotein B-free luminal LDs and require the ER luminal lipid transfer protein, microsomal triglyceride transfer protein (MTP), and importantly, they accumulate inside the INM under ER stress [120]. Under these conditions, ApoB levels drop [265], but MTP levels remain unaltered [266]. Hence, lack of ApoB-dependent neutral lipid secretion promotes the accumulation of ApoB-free luminal LDs, which grow inside the ER lumen and ultimately disintegrate the INM to facilitate nLD formation [267]. It is then
possible that aberrant nLD formation in hepatocytes is on the basis of NAFLD pathophysiology [268, 269].

Contrarily to hepatocytes, nLDs are formed directly from the INM in U2OS cells, and do not emerge from ER luminal LDs. As observed in yeast, key enzymes needed for TG synthesis, including ACSL3, GPAT3/4, AGPAT2, lipin-1β, and DGAT1/2, were present in the nucleus [270]. Seipin, which is absent in the INM of U2OS cells, is dispensable for nLD biogenesis and actually knockdown of seipin increases connections between nLDs and the INM, and stimulates nLD generation by increasing local PA levels [120]. This in turn favors the recruitment of lipin-1β to the INM to generate de novo nLDs. This posits a new paradigm: seipin establishes contacts sites between LDs and other organelles for lipid exchange, and at the same time it actively regulates the nuclear lipid environment (PA levels) to possibly control the redistribution of enzymes (e.g lipin-1) or transcription factors (e.g PPARγ) that support LD biogenesis [271].

In yeast studies, Barbosa et al. [272] showed that Lro1 moves from the ER to a nuclear subdomain resembling the nucleolus to drive the formation of a TG pool at the INM, indicating that Lro1 activity is responsive to metabolic stress and coupled to cell cycle progression. The INM-associated generation of DG observed at the INM should stimulate Lro1 to produce TG, and this is associated with the formation of nLDs established by seipin-mediated contact sites [74]. TG generated from the INM is packed into mature LDs connected with the outer nuclear membrane (ONM). Consistent with a functional crosstalk between LD metabolism and nuclear homeostasis, cell cycle signals influence Lro1 nuclear targeting and its activity impacts on nuclear morphology. Importantly, the nuclear envelope integrity was compromised upon constitutive LD generation at the INM, indicating that Lro1 activity and distribution at the ER and at the INM subdomain are tightly controlled [272].

Together with the work of Romanauska & Köhler [74], which earlier reported that the INM is tailored for lipid storage, it becomes clear that LD biogenesis is highly dynamic and can be compartmentalized in close association with the function of the organelle where it occurs. In this work, the development of genetically encoded lipid biosensors has allowed for the visualization of specific lipids inside individual, living cells. However, a number of caveats and considerations should be taken into considerations, especially those related to possible inhibitory effects of the biosensor, expression levels of the probe, affinity and...
equilibration of the probe with its lipid, and importantly the specificity of these biosensors using domains of proteins to detect specific lipids [273-275]. A good example is the use of the Q2 domain of the *S. cerevisiae* transcription factor Opi1 and of the yeast Spo20 fragment as a PA sensing domain. Horchani et al. proved that the Spo20 probe responded to small variations in the amount of PA in an unspecific manner, revealing a rather strong affinity not only for PA but also for other anionic lipids [276]. This may represent a limitation as a PA marker, especially when not formally tested or experimentally validated under conditions of altered PA metabolism for instance, and used to explore subtle changes in lipid distribution and dynamics in response to stress. In this study, several proteins involved in TAG synthesis and LD biogenesis, including seipin, were observed in the nuclear face of the INM under conditions of reduced phospholipid synthesis [74]. Although the strategy attenuates phospholipid synthesis (Cds1 inhibition) and favour lipid storage by shunting PA towards neutral lipid synthesis, it is possible that the occurrence of nLDs may represent a pleiotropic effect derived from using such genetic approach and other chemical manipulations (e.g. oleate treatment) that induce LD generation, along with multiple pleiotropic effects on phospholipid composition whose contributions remain unexplored. In fact, nLDs were not obvious in yeast cells under normal growth conditions, but only when challenged with oleate [181]. BiFC analysis revealed that seipin resided in the INM only when overexpressed. Despite the known advantages of using BiFC assay to easily detect protein-protein interactions in living cells, there are some potential problems and critical factors that one should consider for a BiFC assay, especially when proteins are overexpressed [277, 278]. It is possible that overexpression of seipin circumvents local quality control mechanisms in the nucleus, leaving behind a pool of seipin enough to be detected in the nucleus. As observed of cytoplasmic LDs, seipin also forms membranous bridges between the inner nuclear envelope and the forming nLDs. However, absence of bridges in seipin mutant could result from budding of LDs derived from enhanced local TAG synthesis upon oleate treatment [173, 279]. However, key questions arise from these reports. Many lipid biosynthetic enzymes and lipases reside in the INM [74, 272], indicating that the required machinery can
sustain LD biogenesis and turnover in the INM. Shunting PA into TG generation using genetic approaches was sufficient to reconfigure the content and distribution of PA and DG at the INM and drive TG synthesis and storage in nLDs, denoting a remarkable plasticity at the INM [272]. The finding that DG specifically accumulates in the inner leaflet of the nucleus reveals that cells require lipid asymmetry between the leaflets. This suggests that cells have adapted their enzymatic compositions and functions to the specialized requirements of membrane curvature and to particular metabolic contexts, through distinct nuclear lipid organization into microdomains.

Local lipid membrane remodelling per se can have other implications, namely in terms of recruitment and/or binding of proteins to these subdomains, control of nuclear function in spatial proximity with histones and promoters, and other processes relevant for nuclear integrity and shape, including the Nuclear Pore Complex (NPC) assembly. Putting forward this idea, LDs act as reservoirs for a subset of nucleoporins (Nups) from the NPC in yeast [280]. Alterations in redistribution of Nups to LDs was dependent on PA levels, and LD formation affected NPC crowding at the nuclear membrane, with significant consequences on nuclear morphology, nucleo-cytoplasmic transport, nucleolar stability and metabolism. Importantly, Nup dynamics is greatly impaired by knockdown of seipin and CDS1, which alters the surface tension and the lipid composition of the LD monolayer [280]. It is not clear how seipin affect Nup loading onto LDs. This could be related to changes in TG content, or to lipid packing defects created by changes in the ER phospholipid composition that affect loading to LDs [72, 177]. In the absence of seipin, PA accumulates in the ER and blocks adipogenesis [22, 281] and this may be a contributing factor to exclude Nups from the LD surface in these mutants.

Phase-separated multi-molecular assemblies can provide a general regulatory mechanism to describe features of transcriptional control. In addition, lipid-induced segregation in the nucleus may impact on membrane fluidity since it would diminish the lipids’ diffusion rate, the lateral movement and clustering of specific proteins and particularly the required fluidity of the membrane for cell viability and chromosome segregation [10]. As Lro1 no longer localizes to the nucleolar subdomain when nuclear envelope growth occurs [272], it would be interesting to characterize in more detail the interconnection of Pah1 and Lro1 activities in this process and how this impacts on PA and DG turnover. Of particular note, TG accumulation during active cellular growth occurs in the ER and is mediated by Lro1, whereas TG synthesis in
stationary phase (under conditions of cell cycle arrest and nutrient deprivation) is mainly driven by Dga1 [239]. It would be noteworthy to explore if Dga1 is nucleus-targeted and regulated by cell cycle and nutrient signalling, as it happens for Lro1. As several important proteins involved in signalling are associated with lipid domains, they are considered hot spots and hubs. Examples of amphitropic enzymes that respond to lipid polymorphism are protein kinase C (PKC), which is found to transduce signals within the nucleus. Interestingly, the amphitropic enzyme CTP:phosphocholine cytidylyltransferase, which regulates phosphatidylcholine biosynthesis and competes with a pool of DG, precursor of TG, is required for LD expansion [282].

The identification of a feedback inhibition loop controlling lipid synthesis involving the PA-binding transcription factor Opi1 offers another example of metabolic adaptability between the INM and the ER, and comprehensively couples gene expression of phospholipid biosynthetic enzymes to alterations in lipid metabolism [74]. Given the fact that the PA-DG axis constitutes a central hub in phospholipid biosynthesis and lipid storage, these studies open new avenues on how different compartments regulate membrane biogenesis and storage, which is reflected by changes in transcription of key enzymes and alterations in PA, DG and TG levels. This broadly disturbs overall lipid levels and flux within the cell in response to physiological and environmental cues, with far-reaching implications in known human pathologies.

These studies emphasize the capability for de novo lipid synthesis at the INM. The protein machinery and associated factors tailored for LD generation are now expanding, and the importance of compartmentalized LD synthesis at this nuclear subdomain is still enigmatic. However, it has opened up many new possibilities of investigation. Remaining questions concern the asymmetric lipid distribution across the nuclear envelope and maintenance of the identity of the INM and ONM lipid niches, the role of Pah1 and Lro1 and other proteins in establishing the ideal environment to regulate membrane synthesis and lipid storage at specific compartments, the mechanisms that regulate nuclear envelope growth, shape and integrity at these sites, and the relationship to other phospholipid synthesis pathways. Importantly, recent insights into the biogenesis of nLDs in yeast and mammalian cells demand better characterization of how lipid species, including PA and DG, may control the biogenesis of cytoplasmic LDs. In contrast to other organelles, each LD is surfaced by a single layer of phospholipids. Unlike COPII vesicles and
autophagosomes, which involve the activity of large protein machineries dedicated to their biogenesis, the initial steps leading to lipid accumulation may be largely driven by remodeling of local lipid composition (PA, DG) of the perinuclear ER and INM, before enzymes and associated factors become systematically redistributed and come together to control LD budding and growth. Further work is necessary to define the role of lipid turnover and INM lipid composition in the control of cell cycle and other cellular functions, including lipotoxicity. Recently, another piece of work by Romanauska & Köhler showed that changing the balance of cLD/nLD generation allows the INM to prevent accumulation of excessive amounts of unsaturated FAs (UFAs) and nLDs that otherwise could compromise nuclear integrity and genome function and stability [283]. Under normal conditions, the transcriptional repressor Opi1, which controls inositol metabolism and phospholipid synthetic genes, controls nLD and cLD formation. To detoxify UFAs from the INM, cLD formation is specifically stimulated by a transcriptional circuit involving control of Ole1 expression and activity by the transcription factor Mga2 under conditions of inhibited nLD generation, which in turn involves lower seipin activity at the INM or decreased levels of PA [283]. More studies focused on these aspects will provide new insight on the mechanisms of cellular energy storage and will offer a better understanding of human diseases such as cancer, progeria and lipodystrophies. Lipid modulation, replacement or supplementation strategies by changing composition and properties of cell membranes may present a very promising reality to improve and treat such diseases.

1.4.4. LD contacts with peroxisomes

Peroxisomes are ubiquitous organelles that are mostly associated with FA β-oxidation and redox homeostasis, as they house antioxidant enzymes and enzymes responsible for ROS generation. Importantly, peroxisomes can undergo morphological and functional changes, which occur under specific physiological and environmental states [284-287]. Some studies have revealed that LDs and peroxisomes interactions seem to be a common feature in higher eukaryotes [288-290]. In yeast cells grown in oleate, peroxisomes form stable connections with LDs and protrude into LD core to form pexopodia [289]. Such protrusions may correspond to sites of hemi-fusion between the outer leaflet of the peroxisomal membrane and the LDs surface, allowing the
peroxisome inner membrane to extend into the LD core. Enzymes of peroxisomal β-oxidation are selectively enriched in purified LDs, suggesting that pexopodia may constitute places that couple LD lipolysis of the neutral lipid cores to supply peroxisomes with FFA oxidation with the purpose of preventing lipotoxicity and generate energy [291]. Consistent with this idea, the number of pexopodia was greatly decreased and the presence of gnarts, corresponding to structured arrays of accumulated FFAs within the LD core, become more evident in cells with aberrant peroxisomes that are incompetent to metabolize FAs [289]. Coupling LD lipolysis with peroxisomal FA β-oxidation seems to require trafficking of proteins and lipids between these organelles [289], and also other organelles with similar metabolic functions, including the mitochondria [218]. Consistently, a metabolic circuit involving LDs, peroxisomes and mitochondria influence energy expenditure in white adipose tissue (WAT) and brown adipose tissue (BAT). Ablation of LD-resident proteins CIDEA and CIDEC increased energy consumption and insulin sensitivity [292].

Higher levels of lipolysis were correlated with activation of β-oxidation and oxidative phosphorylation in peroxisomes and mitochondria in CIDE-deficient adipocytes. Finally, this crosstalk was mechanistically controlled by the CIDE-ATGL-PPARα pathway [292]. It is possible then that LDs form intimate contact with multiple oxidative organelles, and these interactions work as metabolic niches for FA flux, thus avoiding the need to spill FAs into the cytoplasm [293], as they would cause deleterious changes like lipid peroxidation and loss of organelle function and architecture. Notably, a recent preprint unraveled a functional crosstalk between LDs and peroxisomes with a lifespan-extending effect imparted by ether lipids and monounsaturated fatty acids (MUFA) remodeling and suppression of ferroptosis, an iron-direct oxidative form of programmed cell death caused by lipid peroxidation [294].

The nature of the molecular machinery that connects peroxisomes and LDs come from work of Chang et al., showing that, in mammalian cells the AAA ATPase M1 Spastin, involved in hereditary spastic paraplegia, tethers these organelles along with peroxisomal ABCD1 (a peroxisome surface protein and long chain FA importer) to promote FA interorganelle trafficking [295]. Chang and colleagues further observed that components of the endosomal sorting complexes necessary for transport (ESCRT) III machinery are also required for FA flux from LDs of peroxisomes: M1 Spastin microtubule interacting and organelle transport (MIT) domain can interact
with the MIT Interaction Motifs (MIM) motifs of ESCRT-III subunits IST1 and CHMP1B and recruit them to the surface of LDs [295]. This implies that ESCRT-III subunits may play a specific role in lipid flux at the LD-peroxisome contact sites, potentially by extracting lipids from LDs. In agreement with this, using a mutant Spastin unable to bind IST1/CHMP1B, the authors showed that FA exchange between LDs and peroxisomes was compromised. Importantly, mutant Spastin was still sufficient to tether these organelles together, suggesting that the ESCRT-III machinery seems to play a specific role other than LD-peroxisome tethering, which would be lipid exchange in this case [295]. Some ESCRT-III proteins contain amphipathic helices that could associate with the LD monolayer surface and promote changes in membrane curvature and availability of enzymes to the lipid core [296].

IST1 and CHMP1B subunits polymers form external coats on positively curved membranes in vitro and in vivo, and therefore could assist on LD surface shaping and potentially be involved in FA transfer [297]. Chang et al. also unraveled a role of Spastin in reducing peroxidated lipid accumulation under oxidative stress, but this seems to be circumvented to the tethering capacity, because expression of an autosomal dominant hereditary spastic paraplegia (HSP) patient-derived mutant, Spastin^{K388R}, displayed defective LD-peroxisome tethering ability associated with elevated peroxidated lipid levels [295]. Collectively, Spastin is implicated in FA exchange and redox maintenance, loss of which could be on the basis of the neuropathy features observed in HSP patients. Notably, in Drosophila or C. elegans, loss of Spastin was shown to reduce LD number and TG content [39], showing already some involvement of the protein in LD metabolism.

Apart from participating in FA transfer events, LD-peroxisomes interactions are important for recruiting protein machinery to promote lipolysis during nutrient starvation. Under these conditions, KIFC3, a kinesin implicated in peroxisome movement, facilitates microtubule-dependent movement of peroxisomes to LDs, and PEX5 mediates translocation of Adipose triglyceride lipase (ATGL) onto LD monolayer to stimulate PKA-dependent lipolysis, independently of comparative gene identification-58 (CGI-58) and peroxisomal FA oxidation [298]. How these events are coupled remains to be investigated in future studies. Upon nutrient deprivation, mitochondria did not rapidly migrate to LDs as observed for peroxisomes [298], suggesting that peroxisomes may be recruited first to stimulate ATGL-associated
lipolysis and mobilize TG stores in LDs, before they become accessible for oxidation
to support energy generation at mitochondria, or even help LD growth if excessive
FA release become lipotoxic during fasting-induced lipolysis [299]. Consistent with
this idea, PLIN5 increases LD-mitochondria contact sites and LD accumulation [299],
and blocks ATGL-mediated lipolysis [300]. Furthermore, PLIN5 expression rises in
pancreatic islets under fasting conditions [301]. How these proteins come together to
work coordinately and establish a unique metabolic hub made by mitochondria,
peroxisomes and LDs in fasting conditions remains a fascinating question. Similarly
to ATGL recruitment to LD under fasting conditions, peroxisomal tubular extensions
reportedl
y allow relocalization of the Arabidopsis thaliana lipase SDP1 to LDs during
early seedling growth [290]. These protrusions could also play a role in targeting of
peroxisomal fatty acyl-CoA reductase 1 (Far1) towards LD [302].

As stated, integral membrane peroxin Pex30 accumulation at ER sites is observed in
cells lacking seipin, and both deletion of seipin and Pex30 impairs LDs and
peroxisomes biogenesis processes [42, 43]. It was recently shown that Pex29 directs
Pex30 to the NVJ to regulate a subpopulation of LDs [220]. The fact that Pex30-
containing protein complexes act as focal points from which de novo peroxisomes
and LDs emerge from the ER indicate that local lipid remodeling at specialized ER
subdomains [42, 43] shape ER architecture to favor organelle budding, while allowing
lipid addition and membrane growth for organellar maturation at multiple MCS.
Importantly, different members of the peroxin (PEX) family seems to function with
Pex30 at distinct MCS [220], denoting specialized metabolic and regulatory functions
in the cellular response to environmental cues. However, to what extent these
pathways are connected to each other and the corresponding molecular mechanisms
remain unclear.

1.4.5. LD-lysosomes interplay

Lysosomes are key hubs for integrating signals in response to nutrients and energy.
mTORC1 and AMP-activated protein kinase (AMPK), both of which are important
metabolic regulators, assemble their signal centers on the surface of lysosomes to
form a switch for anabolic and catabolic processes [303]. These organelles play
essential roles in stress responses to nutrient availability and composition, ion
homeostasis and cell death [304]. Moreover, lysosomes indirectly influence mitochondria, which also have pivotal roles in cellular homeostasis, and a decline in the function of these organelles has been associated with the progression of metabolic and age-related diseases [305].

Lysosomes are commonly associated to processes of LD catabolism, involving autophagic degradation of LDs to increase availability to neutral lipid stores and provide free FA required for energy metabolism during starvation [306]. In the liver, bulk autophagy and lipophagy are the main processes contributing to lipid breakdown [307-309]. Apart from providing lipid precursors for β-oxidation in mitochondria and peroxisomes, LDs also support autophagosome biogenesis [114, 310] and establish contacts with light chain LC3-labeled autophagosomes and Lysosome-associated membrane glycoprotein 1 LAMP1-containing lysosomes to promote autophagic LD breakdown [306]. Furthermore, LDs can form associations with autophagosomes and other degradative vesicles by multiple mechanisms involving regulation of lipophagy [13].

Although our current understanding of LD-lysosome contact sites is still embryonic, some studies have provided hints on the nature and functional purposes of these MCS. Using super-resolution microscopy techniques, Zheng et al. have analyzed the dynamics of LDs and lysosomes in mammalian cells and observed that some LDs lie closely together with lysosomes [311]. During yeast sporulation, LDs firstly associate with the vacuolar surface and then clustered near the dumbbell-shaped nucleus at the onset of meiosis II [312]. This redistribution could be a consequence of spatial reorganization of the LD-vacuole contact sites, or LDs are being pulled away by vacuoles, or it occurs due to the disassembly of the cortical ER [313].

The identity of proteins that bridge mammalian LDs and lysosomes remains elusive. Possible candidates include Rab GTPases and membrane transfer and trafficking proteins that may help to regulate and establish such MCSs. In fact, lipid transfer profiles are more prevalent in hepatocyte-starved cells, consistent with observations made in yeast showing that nutritional deprivation results in changes in vacuolar microdomains distribution to support lipophagy. This strengthens the idea that lipophagy is a critical process to regulate energy generation during nutrient scarcity [307].

Previous work has provided a complex view on how subpopulations of LDs may involve lysosomes (vacuole in yeast) during starvation. Following starvation-induced
autophagy, LD biogenesis is stimulated and spatially clustered in close proximity with the mitochondria network in mammalian cells [314], and positioned around NVJ in yeast cells [315] (Figure 3). NVJ is constituted by a protein complex formed by the nucleus-associated Nvj1 and the vacuolar-resident Vac8. These contact sites play crucial roles in many cellular processes, as they enable lipid and ion transport, support LD biogenesis under stress, and facilitate the piecemeal microautophagy of the nucleus (PMN) [315, 316]. Importantly, Mdm1, a NVJ tether, is important for the association of a subpopulation of LDs with the NVJ [315, 317]. This specific droplet sub-population has specific features, including recruitment of Pdr16 (Figure 3), which in turn requires Ldo45 and Ldo16 proteins for proper targeting to LDs [88, 199]. Interestingly, Ldo45 increases TG content and is required to cluster LDs around the NVJ in a Pdr16-dependent manner [88, 199]. These NVJ-associated LDs then enter into vacuoles during the transition to stationary phase, characterized by cell quiescence and nutrient scarcity, to be degraded by Ldo16-assisted lipophagy [318]. Remarkably, this involves a feed-forward mechanism in which SEs within LDs are required for the formation and maintenance of sterol-enriched vacuolar microdomains [203, 319] that allow LD translocation form the perinuclear ER to the vacuole and progression of lipophagy.

**Figure 3**

How LD translocation from the NVJ to the vacuolar Lc domains occurs is not well defined, but it is definitely metabolically controlled, and likely requires signaling events [320] and additional factors other than Mdm1 and Pdr16. Consistent with this idea, Pex29 recruits Pex30 to the NVJ to regulate a subpopulation of LDs [220]. Intriguingly, an Mdm1 variant can drive LD generation when moved away from the NVJ [225], and this process involves fatty acyl-CoA synthetase Faa1 possibly for FA activation. This indicates that Mdm1, like other tethers, is highly versatile and has different requirements to spatially regulate LD formation, depending on the nature of the metabolic or physiological stress.

Since the late endomembrane system involves massive lipid trafficking and exchange through endocytosis and autophagy, it is clear that contacts of degradative vesicles other than lysosomes, such as autophagosomes, autolysosomes, and late endosomes, with LDs can serve potential functions beyond the scope of lipid
breakdown [13]. Nonetheless, the nature of lysosome/vacuole and LD contact sites remains largely unknown and future research will surely shed new light into the cell biology and mechanisms of disease pathogenesis.

1.4.6. LDs and mitochondria in lipid metabolism and energy homeostasis

LDs and mitochondria are essential organelles for the purposes of energy and redox homeostasis, as they are central hubs for direct handover of FAs and oxidative metabolism. Interestingly, each organelle undergoes dynamic and constant morphological fluctuations in response to metabolic stress: they can fuse or divide in context-specific conditions [321, 322], and they maintain dynamic contacts with other membranous compartments of the cell. Close proximity and association between mitochondria with LDs was firstly reported back in 1959 in pig pancreatic cells [323] and has ever since detected in a myriad of cells and tissues, particularly in those with high FA storage and oxidation capacity such as BAT, skeletal muscle, and heart. So far, most studies have focused on mitochondria-LD tethering based on mechanisms that involved some degree of protein-protein interaction. However, it was previously reported that mitochondria-LD associations could be impervious to tryptic digestion and under high ionic strength [324]. This reveals that mitochondria-LD tethering can also be mediated by mechanisms that are independent of protein complexes present at contact sites, most likely involving hemi-fusion or fusion of the outer leaflet of the mitochondrial outer membrane and the phospholipid monolayer of LDs. In agreement with this idea, mitochondria-LD contact sites are sensitive to detergents, pointing out to the membranous nature of these associations [324]. More recently, a cryo-EM study also revealed LD-mitochondrial MCS (16-nm spacing) under starvation, and it was proposed that this would favor FA transfer between the two organelles when lipolysis is activated and mitochondrial FA oxidation becomes stimulated to meet cellular energy needs [325]. Of great interest, this study defines structural phase transitions in LDs that may affect the extension and the nature of the interaction with different organelles in response to the metabolic status of the cell. This would in turn affect the accessibility of these organelles to lipids and its precursors, enzymes or lipid transporters associated with LDs [325].
LD-mitochondria tethering (Figure 4) may also involve the ER to some extent. Ca\(^{2+}\) is an important ion stored in the ER, mitochondria and Golgi that is involved in several processes, including the ER stress response, ROS signaling and cell fate [326]. LDs can work as platforms for Ca\(^{2+}\) signal transduction from the ER to mitochondria. They act as major storage sites for Ca\(^{2+}\) during neurite tube formation [327] and be both sources and sinks of calcium in immune cells [328]. Some calcium-binding proteins were identified in different LD-enriched fractions on proteomic studies [329, 330]. LDs can then act as sources and sequestering sites of calcium, adding complexity to our current understanding of LD dynamics in ion storage and interorganelle function.

**Figure 4**

The morphological and functional nature of LD-mitochondria interactions has been unraveled in the past few decades and has become an intensive area of research due to potential metabolic implications in the context of disease. LD can form complexes with mitochondria in fibroblasts in an *in vitro* system of isolated LD and mitochondria, indicating that proteins at the surface of these organelles mediate such interactions [331]. They also found that yet unknown cytosolic factors favor complex formation between mitochondria and LD. Importantly, they first discussed the possibility of SNARE protein factors in establishing mitochondrial-LD complexes, as depletion of SNARE protein SNAP23, also required for LD homotypic fusion along with Vesicle-associated membrane protein 4 (VAMP4) [332], reduced the number of contact sites between these organelles and impaired mitochondrial \(\beta\)-oxidation [331]. Young and colleagues then reported interactions between SNAP23 and the ASCL1 and VAMP4 in hepatocyte LDs [333]. Although ACSL1 is located on the outer mitochondrial membrane (OMM) and directs FAs into mitochondria for \(\beta\)-oxidation, it is not currently known if these proteins work together.

Using systems-level spectral imaging and high-resolution microscopy, Valm et al. characterized the organelle interatomic network, including interactions of LDs with mitochondria [249]. Nowadays, some individual and a few protein complexes have been identified to mediate these contact sites. An example of a well-established class of tether proteins is the family of perilipins, which are LD-associated proteins that provide physical and metabolic linkage to mitochondria to maintain proper mitochondrial oxidative function in several tissues [299, 334]. PLIN1 is particularly
abundant in adipose tissues, and its expression is almost restricted to WAT and BAT [335]. PKA phosphorylation alters PLIN1 conformation, which allows lipases to access LDs and degrade neutral fat. A consequence of PLIN1 phosphorylation by PKA is that CGI-58 binding to PLIN1 is disrupted, with concomitant CGI-58-mediated activation and recruitment of ATGL [336, 337]. Interestingly, PKA also regulates the phosphorylation status of ATGL and is required for proper induction of lipolysis in response to adrenergic stimulus [338, 339]. Furthermore, PKA-mediated phosphorylation of PLIN1 is also required for HSL redistribution to LDs in rats, but dispensable in humans [340, 341].

Perilipin 5 (PLIN5) also promotes LD-mitochondrial contact sites (Figure 4) in several tissues [56, 299], and PLIN5 deficiency impairs mitochondrial oxidative capacity [342, 343]. This protein is highly abundant in cells with high mitochondrial β-oxidation requirements, including cardiomyocytes, BAT, hepatic cells, and skeletal muscle cells, and its expression increases upon FA elevation [344, 345]. Importantly, PLIN5 is known to modulate LD metabolism by suppressing lipolysis. Overexpression of PLIN5 increased LD accumulation [345, 346], and cardiac depletion of LD stores observed in PLIN5-deficient mice can be abolished using a lipase inhibitor [347]. Mechanistically, PLIN5 interacts with and blocks access of ATGL lipase (Figure 4) and its co-activator CGI-58 to the LD surface, and limits mobilization of PLIN5-coated LD stores [300, 348]. During catecholamine stimulation, PKA-mediated phosphorylation of PLIN5 at serine 155 leads to the formation of transcriptional complexes with peroxisome proliferator-activated receptor-gamma coactivator-1-α (PGC-1α) and sirtuin 1 (SIRT1) that boost lipolysis and mitochondrial FA oxidation [349, 350]. Hence PLIN5 couples lipolysis with transcriptional regulation of mitochondrial proteins to stimulate mitochondrial lipid oxidation.

Members of the perilipin family are most known for their role in LD dynamics, however only PLIN1 and PLIN5 have been implicated in mitochondria-LD interactions, thus revealing functional specialization and unique metabolic adaptations to lipid requirements. Boutant et al. have reported the presence of PLIN1 and PLIN5 in mitochondrial extracts from mice BAT, whose levels were regulated by mitofusin-2 (MFN2). Importantly, MFN2 specifically interacts with PLIN1 to form a protein complex tethering mitochondria to LDs (Figure 4). This interaction requires intact MFN2 GTPase activity, and it is responsive to adrenergic and lipolytic stimuli [351]. Optic atrophy 1 (OPA1), usually located in the inner mitochondrial membrane...
(IMM), is expressed in WAT and BAT during adipocyte differentiation and targeted to LDs where it organizes a supramolecular complex with both PKA and PLIN1 [352]. Interestingly, OPA1 is the A-kinase anchoring protein (AKAP) targeting the pool of PKA responsible for PLIN1 phosphorylation on lipolytic stimulation [352]. It is tempting to conceive that the dual localization of OPA1 to mitochondria and LDs could somehow coordinate the positioning of LDs in association to mitochondria to channel FFAs derived from lipolysis to mitochondria in order to sustain β-oxidation, ATP synthesis and mitochondrial function. OPA1, however, generally localizes to the IMM, which may require some level of maturation before targeting to LDs [353] or additional molecular adaptors for interaction with OMM proteins. Interestingly, MFN2 has been shown to interact with OPA1 [354], so a balance between mitochondrial fusion and fission may dictate adaptive responses involving lipid storage and/or turnover of LDs, and could have foreseeable consequences in the context of disease, as mutations in OPA1 and MFN2 are closely related to human disease [355]. Overall, the existence of an intricate interplay between mitochondrial dynamics and mitochondrial-organellar communication is becoming increasingly evident. Abnormalities of mitochondrial dynamics, common in early stages of the pathological neurodegenerative process and usually associated with deleterious changes in mitochondrial oxidative metabolism and abnormal mitophagy [356], have extended the prevailing view of mitochondrial dynamics well beyond their critical role in mitochondrial stability and interconnectivity, and grounds the idea that mitochondrial network is highly dynamic and responsive to alterations in lipid distribution and trafficking within the cell through MCS.

Using super-resolution microscopy, it was recently shown that PLIN5 is specifically located at the interface between LDs and mitochondria [357, 358] and requires its C-terminus to recruit mitochondria to LDs. Overexpression of PLIN5 induces significant redistribution of LDs around mitochondria with massive increase in LD size. Such expansion was associated with enhanced TG synthesis, regardless lipolytic activation [299, 346]. How PLIN5 couples the expansion of mitochondrial-LD interactions with cellular metabolism is still largely unknown. Importantly, the physiological relevance of PLIN5 overexpression and oleic acid treatment in tethering of LDs and mitochondria has been recently challenged by the work of Pribasing et al.. Applying a 4D time-resolved high-resolution live cell imaging of LDs and mitochondria in COS-7 cells, they showed that insufficient optical
resolution and inherent pitfalls of imaging processing methods may lead to an overestimation of calculated organelle contacts between LD and mitochondria using widely applied image processing routines, and also these commonly genetic and chemical approaches [358]. PLIN5 overexpression promoted LD size enlargement and caused initial mitochondrial fragmentation, where mitochondrial fragments were surrounded by individual or clustered LDs [358]. Incorporation of oleate into neutral lipids is known to increase LD size, and to promote some degree of LD clustering [358]. As a result, the mitochondrial network can passively come closer to LDs due to reduced space between the organelles. In both cases, the number of putative LD-mitochondria contacts was significantly higher, and local association of LDs with mitochondria was more stable when compared to unchallenged COS-7 cells. In fact, these cells exhibited a rather elongated and tubular mitochondrial network, contrarily to what is observed upon PLIN5 overexpression, and much lower association of LDs with the mitochondrial network was observed [358]. Finally and contrarily to PLIN1 (which interacts with MFN2), no definite binding partner(s) at the OMM has been identified so far for PLIN5. It is known that PLIN5 functionally interacts with ATGL and CGI-58 [359], and could potentially form a protein complex to establish mitochondria-LD contact sites.

Another protein that is present in the mitochondria-LD contact sites is DGAT2 (Figure 4). In contrast to the ER-resident polytopic DGAT1, DGAT2 has a hairpin structure that allows it to redistribute between ER and the LDs, to stimulate local TG synthesis and increase in LD size [75]. DGAT2 is present in the ER, LD and promotes redistribution of mitochondria network around LDs [360]. Interestingly, biogenesis of starvation-induced LDs confers protection from lipotoxicity and it required DGAT1, but not DGAT2, indicating that DGAT2 may have functional specificity when associated with mitochondrial membranes in high oxidative capacity tissues [314]. Similarly, Rambold and colleagues reported that nutrient deprivation leads to an increase of LD number associated with mitochondrial elongation and association with LDs [361]. DGAT2 and FATP1 can form heterodimeric complexes at ER-LD junctions to support TG synthesis and packing into LDs. A recent study aiming at identifying DGAT2-interacting partners found some mitochondrial proteins with crucial roles in LD metabolism [362], such as AKAP1 and the acyl-CoA dehydrogenase ACAD9, whose deficiency is associated with a defect in mitochondrial β-oxidation and
episodes of acute liver dysfunction, hypoglycemia, neuronal dysfunction and cardiomyopathy [363]. Whether these proteins make part of a molecular mechanism establishing organelle interfaces with significant impact on LD accumulation and mitochondrial long-chain fat metabolism is currently unknown.

Mitoguardin-2 (MIGA2) is a newly identified OMM protein (Figure 4) that establishes contact sites between mitochondria and LDs in differentiating white adipocytes [364]. The contribution of MIGA2 in TG metabolism was previously reported [365, 366]. Importantly, MIGA2 channels non-lipid precursors into mitochondria and TG generation in the ER, in a VAP-B dependent manner, to then favor lipid packing and storage in LDs. The amphipathic region between amino acids 450 and 550 and the cytoplasmic portion of MIGA2 are required for LD interaction. It is interesting to observe that MIGA2, just like DGAT2 [80], is detected at multiple inter-organelar contact sites, including mitochondria, the ER, and LDs. This puts forward the concept of a spatial control of the TG synthesis pathway by distinct organelle-resident proteins that are brought together at multiple organelle interfaces to coordinate cope under metabolic stress.

Morphological studies described so far have disclosed highly dynamic yet complex interactions between mitochondria and LDs. Consistent with the idea that LDs have unique capabilities to change its number, size, morphology and even undergo liquid-crystalline phase transitions in LDs in response to metabolic fluctuations [325], Benador et al. have recently reported the existence of a distinct peridroplet mitochondria (PDM) population that segregate from the remaining pool of cytoplasmic LDs to specifically promote TG synthesis, instead of mobilizing TG stores as a means to gain access to FAs to fulfill energy demands and obtain membrane lipid precursors [299] (Figure 5). Taking advantage of the contribution of BAT to thermogenesis and energy expenditure and therefore of the bi-directional switch between lipid storage and oxidation, they aimed at defining the role of PDM. The group has provided significant evidence that PDM displayed divergent mitochondrial proteome, bioenergetics and cristae organization when compared to cytoplasmic mitochondria (CM). This population was less motile and exhibited preference for pyruvate oxidation through the TCA cycle over lipid oxidation when compared to CM [299] (Figure 5). Remarkably, PDM had higher levels of ATP synthase and mitochondrial proteins of the electron transport chain associated with higher capacity to produce ATP required for lipid esterification, which in turn
stimulated TG synthesis and LD generation. Moreover, these mitochondria do not fuse or share components with CM, which can be explained by their association with LDs [299]. Although forcing mitochondria to attach to the LD may be sufficient to promote PDM (through overexpression of PLIN5) and enable a segregated mitochondrial population with unique structural and energetic features [299], such functional specialization may require additional factors that are yet to be identified, including the nature and the magnitude of the metabolic stress.

**Figure 5**

It is known that nutrient deprivation leads to an increase in LD number and association with elongated mitochondrial network [361]. Importantly, mitochondrial fusion proteins MFN1 and OPA1 are required for proper mitochondrial-LD tethering and robust mitochondrial oxidation of FAs. Together with the fact that OPA1 is targeted to LDs and interacts with PKA and PLIN1 [352], it is possible that PDM favors lipolysis and FA flux toward mitochondrial FA oxidation. Herms et al. also reported that, under nutrient scarcity, LD-mitochondria contacts increase and LDs supply FA to support mitochondrial FA oxidation in an AMPK-dependent manner. This involved an overall reorganization of the network of detyrosinated microtubules to activate mitochondria, indicating that LD motility is important to rapid cellular adaptation [148]. Later, Nguyen et al. then showed that nutrient starvation stimulated DGAT1-mediated increases in LD mass to prevent lipotoxicity originating from liberated FAs derived from autophagic processes [314]. This was associated with increased number of contacts between LDs and mitochondria, also resembling PDM. Although PDM is associated with LD expansion under starvation conditions, this population of associated mitochondria-LD can fulfill two crucial roles: protection from lipotoxicity associated with autophagy-generated FAs, as proposed by Nguyen et al. [314], and at the same time provide optimal mitochondrial FA oxidation in conditions of metabolic stress as defined by Rambold et al. [361]. Both processes need to be tightly regulated, as uncontrolled release of FA can be highly detrimental to the cell under stress, and cause cellular damage such as oxidative and ER stress. Lastly, elongation of the mitochondrial network is required for efficient mitochondrial FA oxidation and is also observed upon induction of autophagy during fasting in mice muscle and liver, providing another level of sophistication: mitochondria escape from
autophagic degradation and maintain ATP production [367], while expanding the membrane interface to interact with LDs and fulfill the aforementioned requirements to sustain cell viability. Mechanistically, improved pyruvate oxidation by PDM should upregulate malonyl-CoA levels, thereby reduce acyl-CoA entry into these specific mitochondria at PDM and confer this mitochondrial population with functional specialization and unique proteomic identity [299]. For instance, it was reported that mitochondria-derived LDs is prompted to remove excessive uroplakins in urothelial plaques via autophagic processes upon Snx31 ablation [368]. This pathway could possibly represent an inducible, versatile detoxification mechanism to remove damaged proteins in the mitochondria under various forms of stress. Such mechanism was previously observed reported for the removal of apoptotic factors from the mitochondrial outer membrane to LDs during stress [369].

So, how PDM function can be important on systemic lipid metabolism? It is known that mitochondrial-LD contacts increase during exercise and this is associated with increased PGC-1α, which mediates alterations in mitochondrial metabolism and functions, and stimulates PLIN5 expression in skeletal muscle [370]. We envision that PLIN5-stimulated PDM [299] could be important as a ROS scavenging mechanism during periods of acute oxidative stress induced by ROS generation at the mitochondria, as ROS can promote LD formation and PLIN5 confers protection against oxidative stress [371, 372]. Stimulation of endogenous PLIN5 expression during fasting also correlates with increased insulin sensitivity in the skeletal muscle, and the newly generated PLIN5-associated LDs are important to prevent mitochondrial lipotoxicity and dysfunction, suggesting that PDM could be crucial in the modulation of fasting-mediated lipotoxicity [301]. In hepatocytes, PLIN5 ablation contributes to insulin resistance and loss of glycemic control, which was associated with lower mitochondrial-LD contact sites, lower FA oxidation and reduced TG secretion [373]. Remarkably, PLIN5 overexpression inhibits activation of hepatic stellate cells, suggesting a potential role of PLIN5 and PDM in metabo-inflammation [374]. Moreover, fasting or β-agonist treatment also enhances PLIN5 expression and increase mitochondria-LD tethering in the heart and this was also associated with increase in LD content [293]. Similarly, Trevino et al. reported an increase in PLIN5 expression in pancreatic islets under fasting conditions associated with increased levels of serum FFAs, and this is important to stimulate post-prandial insulin secretion, as PLIN5 overexpression greatly enhanced glucose tolerance [375].
Overall, these studies support that PDM may be more relevant in specific metabolic contexts than anticipated, depending on the tissue, and that differential expression of PLIN5 and other tethering factors may reflect those metabolic requirements and adaptations during stress response.

The use of powerful omics and microscopy techniques has expanded the array of proteins and enzymes involved in LD-mitochondria tethering. A pioneer interatomic study performed in yeast also employed a large-scale bimolecular fluorescence complementation (BiFC) assay to search for protein partners between LD and mitochondrial surface proteins [376], and they identified the δ(24)-sterol C-methyltransferase Erg6 and perilipin Pln1 as LD-resident protein mediating such interaction with mitochondria. However, it was not formally tested whether these proteins acts as tethers, although PLIN5 is known to mediate LD-mitochondria associations. In vitro reconstitution of these contacts using isolated organelles revealed that Guanosine-5'-triphosphate (GTP) was required for these interactions [376].

1.4.7. LD-Plasma Membrane (PM) contact sites

LD-PM contact sites have been associated with lipid exchange. In mammary glands, LDs are anchored to the apical PM during milk secretion in a process dictated by PM butyrophilin, the cytosolic xanthine dehydrogenase/oxidoreductase, and PLIN2 [377, 378]. During adipocyte lipolysis, small LDs are in close proximity to caveolae domains, implying that LD-PM contacts somehow influence lipid mobilization, endocytosis or transcytosis. Consistent with a role in lipid flux between these compartments, FATP1, CD36, and FATP4, which are all involved in lipid metabolism, reside at caveolae [379, 380]. Importantly, caveolins were reportedly distributed on the surface of cytoplasmic LDs to modulate local cholesterol metabolism and work as signaling platforms [381-383].

As observed for other tripartite contact sites, a close functional interaction between the LDs, the PM and the ER was reported in Drosophila fat body. Here, there is clear heterogeneity in terms of size and positioning of LDs within the cell: a population of small peripheral LDs (pLDs) adjacent to the PM, and larger LDs dispersed on the cytoplasm [89]. The pLDs appear to be a specialized LD subpopulation that makes direct contact with the PM. Although the fruit fly has no canonical caveolae, there are
PM invaginations in contact with pLDs. Notably, fasting promotes the consumption of pLDs, but the cytosolic pool intracellular LDs is not affected, indicating that this population presents specific features for lipid trafficking [89]. Conversely, inhibition of de novo lipogenesis has the opposite effect targeting the cytoplasmic LD population, which reinforces the idea that pLDs fulfill the aforementioned role.

*Drosophila Snazarus* (Snz), a fly homologue of mammalian SNX14/SNX25, also contains a C-Nexin domain for LD targeting, and stimulates TG storage at the ER-PM contact sites [89]. It is targeted to LD surface at the cell periphery, and co-localizes with ER-PM marker dMAPPER via its non-canonical PX domain, highly resembling yeast Mdm1 [89]. By binding LDs, Snz can contribute to pLD dynamics and turnover. Indeed, flies devoid of Snz are viable with irregular pLD morphology and perturbed TG storage, whereas Snz overexpression in the fat body raises TG stores, improves resistance to nutrient deprivation, and even extends fly lifespan. Altogether, Snz is likely to play a critical role in the interplay between the PM, ER, and LDs with impact on nutrient metabolic stress and longevity [89]. Once again, more studies are now revealing the intrinsic complexity that bring together three and more organelles the integrate signals to fine tune cellular homeostasis, and perform crucial cellular functions, including lipid trafficking, organelle dynamics, and stress response in the plasma membrane.

### 1.5. Conclusions and perspectives

A very interesting era for LD biology is just around the corner and exciting discoveries are eagerly anticipated. Once LDs were finally recognized as *bona fide* organelles, we have now the opportunity to address several questions that still remain unanswered about their biogenesis. Different tissues have distinct energetic requirements, and that influence LD diversity and heterogeneity in size, number and subcellular localization. Is it related to the expression level of specific proteins in different tissues, or it reflects an adaptive response to stress stimuli in a cell and tissue specific manner? Is it possible that different subpopulations of LDs can co-exist within the cell and if so, could they perform discrete functions? It certainly involves dissecting the functions specific to LDs, like lipid storage, ER stress response or antimicrobial resistance, from those involving inter-organelle interaction. In this case of interorganellar communication, it may involve channeling FA derived
from lipolysis or autophagy at the interface with peroxisomes and mitochondria, or segregation of specifically-coated LDs at particular the MCS to be engulfed and be directed to the lysosome to be consumed by resident lipases.

The interplay between LDs and other organelles requires interactions at different levels that are yet elusive. However, we still lack a profound mechanistic understanding of how organelles cooperate with each other in response to physiological and environmental cues. The signaling effectors by the organelles, their release and/or propagation, and their perception by other organelles and LDs are still enigmatic. A major limitation in the study of such interactions is the known dependence of the regulations of interconnected systems and protein-based signaling on a multitude of factors, including anticipated pleiotropic cellular effects imparted by shared roles in distinct cellular processes that potentially interfere with the functional analysis of factors affecting LD-organelle contact sites. Important factors also include the metabolic status of the cell, activity, localization and abundance of proteins, the overall distribution and concentration of particular signaling lipids, and the accessibility of lipid intermediates as building blocks. The need for a parallel analysis of lipid- and protein-based signaling in order to understand complex interconnections at a systems biology level is paramount.

An essential prerequisite is the availability and resolution of methods that enable the simultaneous, comprehensive, unbiased, and quantitative analysis of proteins, lipids, and metabolites from a single sample, rather than separate analysis with unimolecular strategies involving laborious genetic and chemical manipulations. Using high-end multi-omics and cell biology approaches, which combines genetic approaches with state-of-the-art omics techniques and workflows (including aspects related to epigenome, transcriptome, epitranscriptome, proteome, lipidome and metabolome), can be a useful platform for the study of interorganellar interconnectivity regarding energy metabolism. An example is the simultaneous metabolite, protein, lipid extraction (SIMPLEX) procedure, which allow exploring the crosstalk between all molecular classes of metabolites and identifying novel potential molecular mechanisms and entry points in interorganellar communication [384].
New applications and approaches of fluorescence imaging have expanded the possibilities for examining 3D cellular organization at higher resolution, particularly on details on organelle and protein localization [385-387]. However, current microscopy approaches mostly require the use of fluorescent probes, which limits the number and type of molecules that can be imaged at once. These restrictions impede to image specific organelles while still capturing details about their identities, localizations, and structural aspects of the architecture of neighboring organelles or cellular components, therefore hindering unbiased discovery. Therefore, with these methods alone, it is challenging to reconstruct 3D volumes of multiple cells under a variety of conditions, as is required for mapping the dynamic processes involved in subcellular reorganization and association of organelles through MCS. Imaging and image processing workflows are also important to define and characterize in detail organellar interactions with spatiotemporal resolution, both qualitatively and quantitatively. Using the right imaging capabilities and processing algorithms is paramount to best answer a specific biological question for understanding and interpreting its biological relevance. This is an often-neglected and complex task that should follow well-defined and specific criteria [388]. However, currently available methods are limited and difficult to compare due to different experimental setups (e.g. distinct detector systems and microscopy settings, distinct thresholding techniques and different deconvolution and segmentation algorithms, etc). Other techniques such as super-resolution techniques used in the field are even more error-prone: image resolution is inversely correlated with acquisition speed, localization precision is usually much smaller than spatial image resolution, and importantly hardware for ensemble techniques is becoming even more challenging, and image artifacts can be easily propagated by inappropriate mathematical processing of image data. Each of these considerations represents an imaging or a biological trade-off that must be taken into account for any experiment using super-resolution microscopy [389]. Therefore, the development of microscopic techniques, particularly those involving time-resolved high-speed imaging with extended resolution, will allow the capture of multiple modalities from a cell, and will be pivotal to obtain more insights into the dynamics and interactions of LDs with other organelles. This integration of multiple layers of
information will propel the development of tailor-made computational tools for integrative data analysis. A wide variety of bioinformatics, including mathematical arrays, algorithms, statistics, computational skills, artificial intelligence, deep learning, programming, and machine learning techniques, are currently adapting to quantify fluorescence with very high spatial precision, and even combine imaging and omics data into a single workflow for understanding lipid metabolic pathways and interorganellar communication (tether-omics) in various model organisms, and the underlying factors contributing to human disease. Overall, the emergence of these tools will propel new analyses that will identify various new functions for yet unidentified genes, new subcellular localization proteins with regulatory roles and the corresponding signaling pathways. At the intersections of these events, we will uncover new cellular processes and the principles of interorganellar communication and membrane contact sites that involve LD metabolism and dynamics.

It is clear now that LD dysfunctions, particularly LD accumulation, are hallmarks of several diseases, including obesity, diabetes, atherosclerosis, neurodegeneration and cancer. This has drawn attention to the development of therapeutic strategies that target different proteins involved in LD metabolism, including biogenesis effectors and associated factors, and components of lipophagy and lipolysis as potential biomarkers for disease. Several proteins controlling lipid storage are being characterized, and a number of genes encoding LD proteins have been proposed to be potential therapeutic targets, like DGAT in viral infection and obesity, or E2F1 and E2F2, classically recognized as cell proliferation activators, for preventing the progression of NAFLD and the development of hepatocellular carcinoma. However, the contribution of LD contact sites to cellular lipid homeostasis and organismal health is elusive. A key question that remains to be answered is how the disruption of inter-organelle networks leads to metabolic disorders of FA metabolism.

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Figure legends

Figure 1. The LD biogenesis process. TG (depicted in the figure at the nascent LD) synthesis in the ER takes place through the activity of DGAT proteins (Dga1 and Lro1 in yeast). During LD nucleation and oils and lens formation, Seipin (Sei1 in yeast), FITM (Yft2/Scs3 in yeast), MCTP2 (Pex30 is the putative yeast ortholog), ACSL3, Nem1, lipin (Pah1 in yeast), DGAT, and cPLA2α (cytosolic phospholipase A2α) mediate local lipid remodeling involving phosphatidic acid (PA), diacylglycerol (DG) and lysophospholipids. cPLA2α, FITM proteins, PLIN1 and PLIN3 (Pln1 in yeast), lysophospholipids and DG are necessary for efficient vectorial LD budding and emergence. Several factors contribute to further expansion and LD growth, such as phospholipase D (PLD), VPS13A/C, ORPs, Seipin, LDAF1 (Ldo45 in yeast) and PA. Lastly, other biogenesis factors associate to ER-LD contact sites, namely Rab18 and the trimeric NBAS, RINT1 and ZW10 (NRZ) tethering complex, SNAREs, and VPS13/ORPs. The yellow line represents the LD monolayer. Figure was created using BioRender.

Figure 2. Hypothetical scenario of tethering proteins and associated factors that contribute to LD-ER contact sites and functional interactions. Seipin associates with LDAF1 and mark sites for LD formation and budding. Once TG accumulates above a certain threshold, LDAF1 dissociates and redistributes to the LD surface. GTP-bound Rab18 acts in concert with the Double FYVE-containing protein 1 (DFCP1, omitted in the figure due to space limitations) and binds the NRZ-SNARE complex to bring together both organelles. Rab18 can also facilitate dengue virus infection by targeting fatty acid synthase (FAS) to sites of viral replication. It required Rab18 to be GTP-associated. ORP2 FFAT motif is required to interact with ER-associated protein VAP. DGAT2 and the fatty acyl-CoA synthetase FATP1 likely contribute to local TG synthesis and accumulation at LD-ER contact sites. The ER-anchored Ice2 interacts with LDs though its amphipathic helices and concentrates a pool of DG from LDs to the ER to facilitate LD catabolism, while regulating Dga1/DGAT localization to the ER. Snx14 mediates LD-ER tethering via its C-
terminal nexin (CN) domain and is recruited to ER fatty acyl-CoA ligase ACSL3-enriched microdomains. VPS13A/C interact with ER-resident VAP using its FFAT motif, and recruit LDs via its DH-like (DH₃) and PH domain. Figure was created using BioRender.

**Figure 3. Functional interactions between LDs, ER and mitochondria.** LD-ER contact sites (mediated by the seipin complex) are required for proper LD biogenesis. In the yeast model, Ldo proteins Ldo45 (the yeast remote homologue of human LDAF1) and Ldo16 are important to regulate LD morphology, size and proteomic compositions, apart from playing key role in LD breakdown (lipophagy). LD can associate with mitochondria via PLIN5, mostly for lipid exchange and flux of fatty acids (FAs) to sustain mitochondrial fatty acid oxidation and ATP generation. A similar feature is observed at LD-peroxisomes sites, as peroxisomes are hubs for FA β-oxidation. In yeast, nuclear-to-vacuole junctions (NVJ) are stabilized by a complex formed by Nvj1 and Vac8. Here, resides a subpopulation of Pdr16- and Ldo-associated LDs that co-localize with the NVJ tether Mdm1. These NVJ-connect LDs are also enriched with lipid Pah1 under metabolic stress. Upon severe nutrient starvation, these LDs associate with sterol-enriched microdomains (whose formation engages Ldo16) and eventually engulfed by lipophagy at the vacuolar surface, in a process that involves Atg14. PA, phosphatidic acid, DAG, diacylglycerol; TAG, triacylglycerol. Used with permission from [18]: Copyright Clearance Center, 2021, John Wiley and Sons.

**Figure 4. Hypothesized tethers and regulators of LD-mitochondria contact sites.** The C-terminal domain of LD-resident PLIN5 regulates and mediates contacts between the two organelles, but its mitochondrial anchoring partner(s) is(are) currently unknown. The N-terminus of DGAT2 seems to recruit LDs in close proximity to mitochondria, possible by forming dimers, or using its dual localization to mitochondria and LDs. The LD-associated PLIN1 and the mitofusin MFN2 form a complex in primary brown adipocytes (BAT). The MIGA2 C-terminus is anchored to LDs, while the N-terminus targets the mitochondria. In addition, MIGA2 can associate with mitochondria, ER, and LDs to promote adipogenesis. In adipocytes, MIGA2 couples reactions of de novo lipogenesis in mitochondria to stimulate LD biogenesis and glucose-derived TG production in the ER. Figure was created using BioRender.
Figure 5. Distinct subpopulations of mitochondria associated with LDs (peridroplet mitochondria, PDM) display specific bioenergetics, cristae organization, proteome and dynamics when compared to cytoplasmic mitochondria (CM). Both PDM and CM channel free fatty acids and convert them to TG stores within LDs to prevent lipotoxicity. While PDM is more specialized in pyruvate oxidation and foments TG synthesis derived from enhanced ATP synthesis, the CM, on the other hand, can consume free fatty acids via mitochondrial β-oxidation, either to support ATP production or generate heat through uncoupling protein 1/UCP1-mediated and other mechanisms. Figure was created using BioRender.
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5