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Title: Lipid droplet-organelle interactions: emerging roles in lipid metabolism

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Abstract: Cellular homeostasis depends on the precisely coordinated use of lipids as fuels for energy production, building blocks for membrane biogenesis or chemical signals for intra and inter-cellular communication. Lipid droplets (LDs) are universally conserved dynamic organelles that can store and mobilize fatty acids and other lipid species for their multiple cellular roles. Increasing evidence suggests that contact zones between LDs and other organelles play important roles in the trafficking of lipids and in the regulation of lipid metabolism. Here we review recent advances regarding the nature and functional relevance of interactions between LDs and other organelles - particularly the endoplasmic reticulum (ER), LDs, mitochondria and vacuoles - that highlight their importance for lipid metabolism.

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Lipid droplet-organelle interactions: emerging roles in lipid metabolism

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Abstract
Cellular homeostasis depends on the precisely coordinated use of lipids as fuels for energy production, building blocks for membrane biogenesis or chemical signals for intra and inter-cellular communication. Lipid droplets (LDs) are universally conserved dynamic organelles that can store and mobilize fatty acids and other lipid species for their multiple cellular roles. Increasing evidence suggests that contact zones between LDs and other organelles play important roles in the trafficking of lipids and in the regulation of lipid metabolism. Here we review recent advances regarding the nature and functional relevance of interactions between LDs and other organelles - particularly the endoplasmic reticulum (ER), LDs, mitochondria and vacuoles – that highlight their importance for lipid metabolism.

Introduction
Cells have the ability to store metabolic energy in the form of nonpolar or “neutral” lipids in ubiquitous organelles, lipid droplets (LDs). Unlike other organelles, LDs exhibit a unique topology consisting of a hydrophobic core, predominantly of triacylglycerol (TAG) and steryl esters (SE), and coated by a phospholipid monolayer, which solubilizes the LD in the cytoplasm and a set of proteins involved in LD function. In response to metabolic signals, mobilized fatty acids (FAs) and other precursors derived from stored neutral lipids are used for a striking variety of functions, including energy production via β-oxidation, membrane biogenesis for cell growth, protein modification, signalling, and even secretion within lipoproteins. Growth and consumption of LDs can occur via multiple pathways but ultimately both processes depend on the regulated exchange of lipid content between LDs and other organelles within an aqueous cytoplasm. Because LDs are not directly connected to the vesicular transport pathways, the neutral lipids and phospholipids required for their biogenesis must be generated either *in situ* or arrive from other organelles through physical interactions. Here we discuss recent advances and highlight open questions on how contacts between LDs and other organelles are established and how they regulate lipid metabolism. LD biogenesis will not be discussed in detail as it has been comprehensively reviewed elsewhere [1-5].

LD-ER contact sites

While it is widely thought that LDs emerge from the ER membrane, the mechanisms responsible for the initial stages of their formation are not well understood. A popular model proposes that accumulation of neutral lipids between the leaflets of the ER bilayer forms an oil droplet or “lens” that eventually buds towards the cytoplasm [6], although there is still little direct evidence to support it. Whether this budding process is spontaneous or assisted by ER proteins is also not clear [7]. Subsequent growth of LDs may take place by neutral lipid synthesis either on the LD surface or at the ER, or by a “fusion” mechanism that transfers lipid during homotypic LD-LD interaction. Regardless of the pathway, neutral lipid addition to the LD core must be coupled with addition and remodelling of phospholipids, mostly phosphatidylcholine (PC), at the LD surface to enable the coordinated expansion of the organelle.

The close apposition of LDs with the ER is conserved from yeasts to mammals (Figure 2) but the exact nature of this association remains poorly defined. LDs between the two leaflets of the ER have been visualized in cells with defects in apolipoprotein B processing [8], which would be consistent with a physical continuity between the two organelles. A different arrangement was seen by freeze-fracture analysis where both leaflets of the ER bilayer were observed external to the LD and enclosed it tightly [9]. More recently, the existence of ER-LD conduits was supported by high-pressure freezing electron microscopy and tomography analyses depicting direct connections between the LD phospholipid monolayer and the adjacent ER in mammalian cells “loaded” with excess fatty acids [10*,11*]. Generating such a connection is likely to depend upon the generation of negative membrane curvature at the ER-LD interface; the role of specific lipids or proteins has yet to be established, although a role for diacylglycerol (DAG) has been proposed [12]. In yeast where LDs predominantly appear to remain in contact with the ER [13, 14*, 15], protein machinery may be required to stabilize the ER-LD association.

There is increasing evidence in recent years suggesting that ER-LD contacts provide a conduit for the transport of proteins with metabolic or signalling functions on LDs (Figure 1). Such compartmentalization may increase the efficiency of metabolic inter-conversions and limit the diffusion of bioactive lipids within the ER membrane. Early biochemical studies in yeast provided evidence that enzymes involved in neutral lipid synthesis show a dual LD-ER localization [16, 17]. Indeed, some of the best-studied examples include TAG biosynthetic enzymes. TAG synthesis is initiated by two sequential FA acylation reactions on glycerol-3-phosphate, catalysed by GPAT and AGPAT enzymes respectively. The resulting phosphatidate (PA) is dephosphorylated by PA phosphatase (PAP) to DAG, which is then converted by DGATs to TAG. Notably, with the exception of PAP, these enzymes are ER membrane-bound but move to LDs during conditions of increased TAG synthesis. For example, in mammalian cells DGAT2 [18, 19] and acyl-CoA synthetase 3 [11*] target LDs following the addition of FAs. In yeast, re-localization of the DGAT Dga1 enzyme in response to transcriptional induction of TAG synthesis, is energy- and temperature- independent, supporting a diffusion-

based transport mechanism through an ER-LD membrane continuity [14*]. FA loading of Drosophila cells results in the targeting of all four TAG biosynthetic enzymes onto a subpopulation of LDs that expand, while a second class of LDs lacking the enzymes remains constant in size [10*]. Other enzymes that play key roles in LD homeostasis, like the lipases Tgl1 and Tgl3 in yeast [14*, 20] or the mammalian lyso-PC acytransferases LPCAT 1 and 2 that remodel PC on LDs [21], also partition between ER and LDs.

Because these enzymes behave biochemically like integral membrane proteins, their transport from the ER poses the challenge of moving from a phospholipid bilayer with an aqueous lumen (ER) to a monolayer with a hydrophobic lumen (LD). It was proposed that the presence in the enzymes of long hydrophobic domains with a kink, inserted in a hairpin fashion that does not span the entire ER membrane, would allow such a transition [22] (Fig. 1). Consistently, modifying the hydrophobic domains of LD proteins to disrupt their hairpin structure blocks their translocation from the ER [10*, 23, 24]. This mechanism may explain the remarkable conservation of LD targeting between species despite the lack of apparent linear LD-localization signals [25, 26]. However, several proteomic and imaging approaches have now identified additional LD proteins with longer membrane-spanning domains, although their topology in most cases has not been experimentally determined. Whether these proteins are actually resident in an ER domain that contaminates LDs during analysis, or whether other mechanisms to accommodate membrane proteins on LDs exist, remains to be determined.

Another open question is whether phospholipids are also transferred at ER-LD contact sites to support LD surface expansion. The phosphocholine cytidylyltransferase a (CCTα) catalyzes the rate-limiting step in the Kennedy pathway of PC synthesis and targets LDs during FA loading in fly and mammalian cells, although the final step of PC production takes place in the ER [27]. Another pathway that remodels PC acyl chains through LPCAT1 and 2, the Lands cycle, is also active on LDs, but the primary source of the PC substrate may be also derived from the ER [21, 28]. Thus, LD growth probably depends on a phospholipid transport step, which could take place either through lateral diffusion from the ER-LD connections, or via phospholipid transport proteins at the zones of ER-LD proximity. However the specific mechanisms remain largely mysterious. Evidence for a further role of phospholipid metabolism in LD growth has been also provided by genetic studies in yeast [29].

A model for the formation of ER-LD contacts has been recently proposed, based on a mechanism requiring the GTPase Arf1 and the COPI coat complex. These have canonical functions in Golgi to ER retrograde membrane and protein trafficking and previous studies have also documented a role for Arf1/COPI in the LD targeting of adipose triglyceride lipase (ATGL) [30, 31] and TAG biosynthetic enzymes from the ER membrane [32*]. A pool of Arf1/COPI localizes on the LD surface and buds micro-droplets from either artificial or purified cellular LDs [32*, 33*]. Because this process removes phospholipids from the LD monolayer that solubilize the TAG core, it was suggested that the resulting increase in LD surface tension drives the

formation of membrane contacts with the adjacent ER, enabling protein transport to the LD [32*]. Thus, Arf1/COPI may act by modifying the physical properties of the LD.

Additional pathways for ER-LD contact formation are likely to exist, at least in yeast, where COPI mutations do not block the transport of Dga1 to LDs [14*]. Another way in which ER-LD contacts could be regulated is by the assembly of protein-protein complexes at the interface between the two organelles. This has been suggested by studies in *C.elegans* where the DAG acyltransferase DGAT2 on LDs was shown to bind the ER-localized acyl-CoA synthetase FATP1 that generates the FA substrate of DGAT2 [34]. There is growing evidence that neutral lipid biosynthetic enzymes can form complexes to facilitate substrate channeling, although evidence for a general role of protein-protein interactions in tethering LDs to the ER is currently missing.

LD-LD contact sites

LDs tend to cluster together under certain conditions or when specific proteins are over-expressed [35-39]. However, for the most part these observations of changes in LD proximity do not conclusively document direct contact sites between adjacent LDs. Furthermore, the biological importance of this proximity to one another remains largely unknown; one notable exception is the role of Fsp27 (fat specific protein of 27 kDa, also known as Cidec) in mediating formation of the large unilocular LDs present in vertebrate adipocytes (Figure 1). In this case, cellular knockdown studies [40], mouse knockout models [41-43] and a human with biallelic loss-of-function mutations in CIDEC [44], clearly indicate that Fsp27 is required for the formation of a unilocular LD and that, in its absence, adipocytes accumulate smaller multiloculated LDs which permit higher rates of lipolytic fatty acid release (presumably due, at least in part, to the relative increase in LD surface area and hence access of lipases to the LD). This ultimately limits the maximal lipid storage in adipose tissue *in vivo*; instead lipid accumulates in the liver and induces insulin resistance in the absence of Fsp27 [43, 45].

Gong et al [46*] were the first to show that Fsp27 localises to membrane contact sites between abutting LDs, where it appears to form stable ‘contacts’ between the LDs. This enables net unidirectional transfer of neutral lipid from the smaller to the larger LD via a slow (typically over hours) transfer process, for which the term ‘permeation’ has been suggested [2]. The internal pressure difference (which is higher in the smaller LD) provides the ‘driving force’ for this neutral lipid transfer. This process is thought to be distinct from much faster LD coalescence events which have been observed *in vitro* and the *in vivo* relevance of which are still debated; current consensus suggests that coalescence probably does not occur *in vivo*, although depleting surface PC or the addition of certain pharmacological agents can destabilise LDs and promote this type of rapid fusion [47, 48]. LD permeation has also been observed using CARS imaging in differentiating adipocytes, where it was possible to precisely document the additive effect of several ‘permeation’ events on LD volume and measure factors influencing the rate of lipid transfer between LDs [49, 50*]. The latter suggests that this depends primarily on the volume of the smaller LD. Recent studies have suggested that several

additional proteins, including perilipin 1 [51] and Rab8 [52] enhance the rate of lipid transfer mediated by Fsp27, although the mechanistic basis underlying these observations is still unknown. It also remains unclear at this stage whether Fsp27 is involved in the formation of a channel or 'pore' between the LDs, or facilitates lipid transfer by another mechanism.

LD-Mitochondria/peroxisome interactions

Catabolism of LD-derived fatty acids through β-oxidation takes place in mitochondria in metazoans, or peroxisomes in yeasts and plants. Extensive fatty acid trafficking is therefore likely to take place between LDs and these organelles (Figure 1). Increased fatty acid flux from LDs induces mitochondrial biogenesis, highlighting the close interplay between the two organelles [53]. Close association of LDs with mitochondria, or peroxisomes, has previously been described in many cell types. For example, a fluorescence complementation assay in yeast described protein interactions between LDs and mitochondria or peroxisomes [54]. Moreover, in yeast cells using oleate as a carbon source, LDs develop stable contacts with peroxisomes, termed "pexopodia", that were proposed to facilitate peroxisomal fatty acid import and contain several β-oxidation enzymes [55]. In mammalian oxidative tissues that require high fatty acid fluxes for their energy demands, LDs often localize near mitochondria [56]. High levels of perilipin 5, which is most highly expressed in oxidative tissues, result in clustering of mitochondria around LDs in skeletal and heart muscle [57-59]. Close LD-mitochondrial association and proper mitochondrial fusion are required for the distribution and oxidation of lipolytically released fatty acids within the mitochondrial network during starvation [60*]. These observations imply a role of LD-mitochondrial contacts in the channelling of mobilized fatty acids although it is also possible that both organelles contact a common ER subdomain through which fatty acids and other lipids traffic. Protein machinery mediating LD-mitochondrial contacts has, to date, not been described.

Lipid transport between LDs and mitochondria is likely to be bidirectional. In cell types such as adipocytes, hepatocytes or yeast, PC can be synthesized by either the Kennedy pathway or by the methylation of mitochondrial-derived phosphatidylethanolamine (PE), catalysed by PEMT [61]. PEMT localizes to the mitochondrial-associated ER membrane (MAM). In adipocytes, PEMT is found in close proximity to LDs and may use mitochondrial PE for the synthesis of PC on LDs [62]. How these two biosynthetic processes could be coupled remains unknown. An analogous communication may also take place between LDs and peroxisomes as the latter are the sites of ether-linked lipids, which are also present in LDs [63].

LD-Vacuole/lysosome interactions

In addition to the activity of cytosolic lipases, fatty acids can be mobilized from LDs through macroautophagy in mammalian cells. This process is known as lipophagy and involves the sequestration of small, or portions of larger, LDs in the autophagosome [64]. Genetic and pharmacological inhibition of autophagy leads to accumulation of LDs in different cell types. Therefore, proteins that control autophagic flux and are also associated with LDs may specifically control lipophagy. The small GTPase Rab7, whose activity is required for

autolysosome formation, associates with LDs and is required for lipophagy. This association increases during β-adrenergic receptor stimulation and is inhibited by perilipin 1 on the surface of LDs under basal conditions [65]. Moreover, Rab7 activation during nutrient stress also increases the association of Rab7-positive compartments with LDs [66]. Similarly, chromosome 19 open reading frame 80 (C19orf80) associates with LDs during thyroid hormone stimulation and mediates lipophagy in human hepatoma cell lines [67].

In budding yeast, LDs associate with the vacuolar membrane and are incorporated into the vacuole through microautophagy, which requires the core machinery of autophagy [68, 69]. Although both nitrogen starvation and stationary phase induce lipophagy in yeast, vacuolar incorporation of LDs may proceed through different mechanisms. In fact, stationary phase induces the formation of liquid disordered and sterol-rich liquid ordered microdomains on the vacuolar membrane, which are not formed during nitrogen starvation [70]. During stationary phase-induced lipophagy, LDs associate with the sterol-rich liquid-ordered microdomains, which mediate the vacuolar internalization of LDs [69]. Interestingly, lipophagy is required for maintenance of these vacuolar microdomains, probably by providing the sterols stored in LDs [69].

LDs may also contribute to the initiation of autophagy. Recent work in mammalian cells provides evidence that LDs interact with autophagosomes during starvation and TAG is mobilized by the neutral lipase PNPLA5 that localizes on LDs [71]. The resulting DAG is converted into PC via the Kennedy pathway, suggesting a role for TAG in the formation of membrane phospholipids required for autophagosome biogenesis [71]. TAG is also required for autophagosome formation in budding yeast, although the underlying mechanisms remain currently unknown [72].

Conclusion and perspective

Lipid synthesis is highly compartmentalized in eukaryotic cells and therefore regulated contacts between LDs and other organelles are likely to be critical for cellular homeostasis. LD tethering to organelles may result in more efficient channeling of lipid intermediates from LDs explaining, perhaps, why lipolysis-derived FAs are used by cells more efficiently over other sources of FAs. How LD-organelle contacts are established, maintained and regulated in response to metabolic cues remains poorly understood. The unique topology of LDs may dictate novel modes of membrane interactions and the function of specific lipid and protein components awaits further investigation. Moreover, the spatial organization of LDs, and the role of the ER network that contacts most other organelles, in controlling LD-organelle interactions is also not well understood. Although we focused here on LD contacts with four organelles, LDs apparently manifest a much broader repertoire of interactions including caveolae, components of the endocytic machinery and pathogens such as viruses or bacteria. It is likely that future studies on the regulation of LD contacts will lead to a better understanding of lipid metabolism, with important implications for prevalent human diseases such as obesity, diabetes, fatty liver and cancer.

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Figure legends

Figure 1. Topology and roles of key LD-organelle contact sites in lipid metabolism. (a) LD-ER interaction zones. Selective partitioning of lipid metabolic enzymes (in blue) from the ER to the LD phospholipid monolayer through ER-LD membrane "bridges" controls LD homeostasis. These include enzymes of the *de novo* TAG biosynthetic pathway, phospholipid remodelling, TAG mobilization or other. TAG may be also channelled to LDs from the ER via these LD-ER contacts though poorly characterized mechanisms (dashed arrow). Phosphatidylcholine (PC, in green) from the adjacent ER can be added during LD growth via lipid transport proteins or lateral diffusion. (b) LD-LD contact sites are established by CIDE-proteins (e.g. Fsp27) and mediate directional neutral lipid exchange from the smaller to the larger LD. (c) Mitochondrial-LD contacts are thought to channel lipolytically-derived fatty acids (FAs) destined for β-oxidation but their molecular basis remains elusive. Such contacts may also provide mitochondrial-derived phosphatidylethanolamine (PE) for the synthesis of PC destined to coat LDs. MAM: Mitochondrial associated membrane.

Figure 2. Morphology of LD-organelle interactions as visualized by light and electron microscopy. (a) Organelle-LD interactions in budding yeast as visualized by electron tomography of cryofixed/freeze substituted samples. ER, Endoplasmic reticulum; LD, Lipid droplet; M, Mitochondria; N, Nucleus; SV, Secretory vesicles; V, Vacuole. Bar, 100 nm. (b) Electron microscopy of LD-ER contacts in Vero cells. Bar, 1000 nm. (c) LD-ER association as visualized by fluorescence microscopy in budding yeast; LDs, seen in red, localize primarily in contact with the cortical and nuclear ER, in green. Bar, 5 μm. (c) Electron microscopy of LD-mitochondrial contacts in Vero cells. Bar, 1000 nm. Image (a), courtesy of Maja Radulovic and Sepp D. Kohlwein (University of Graz) and Yannick Schwab (EMBL); images (b) and (d), courtesy of Charles Ferguson and Robert Parton (University of Queensland).

Figure 1

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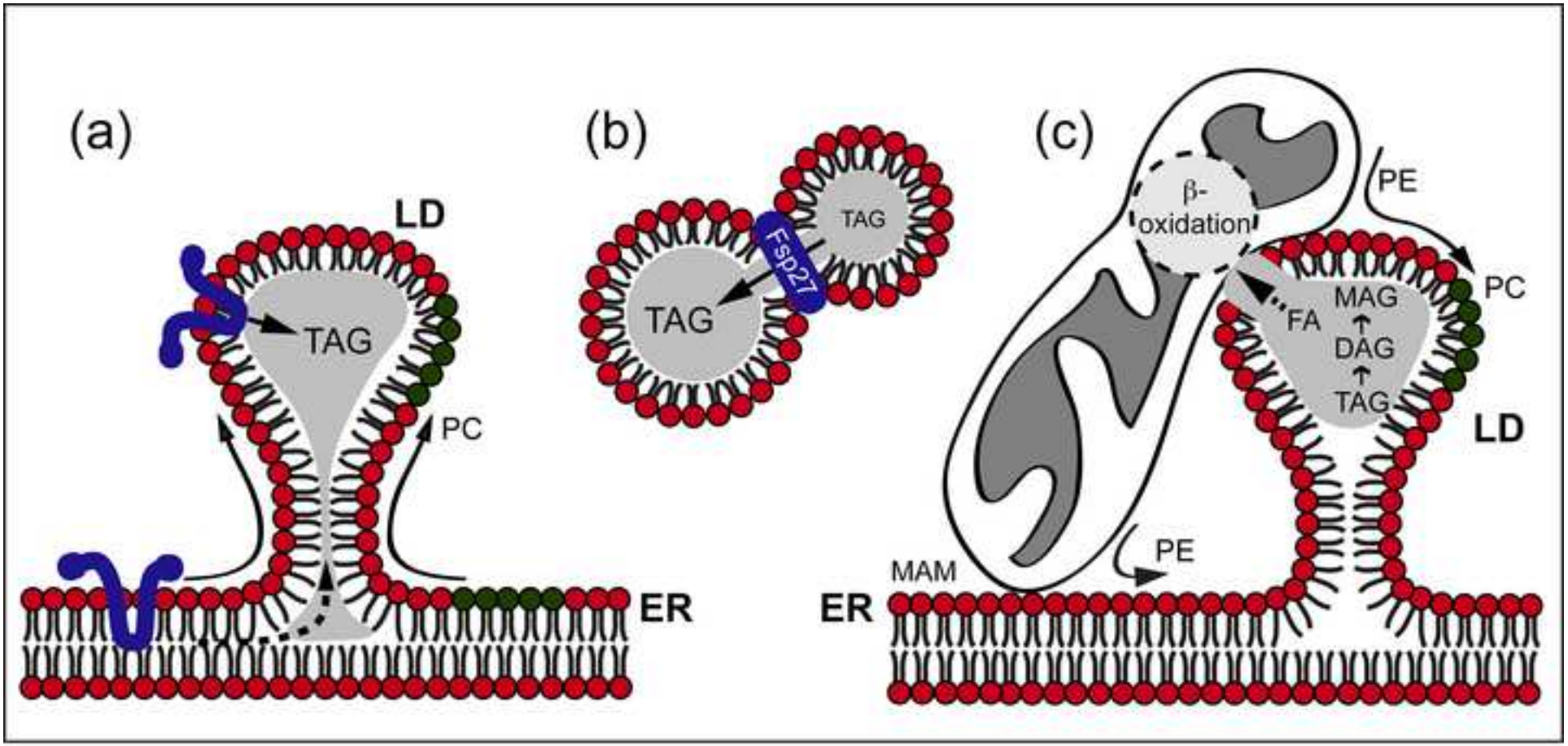

Figure 1

Figure 2

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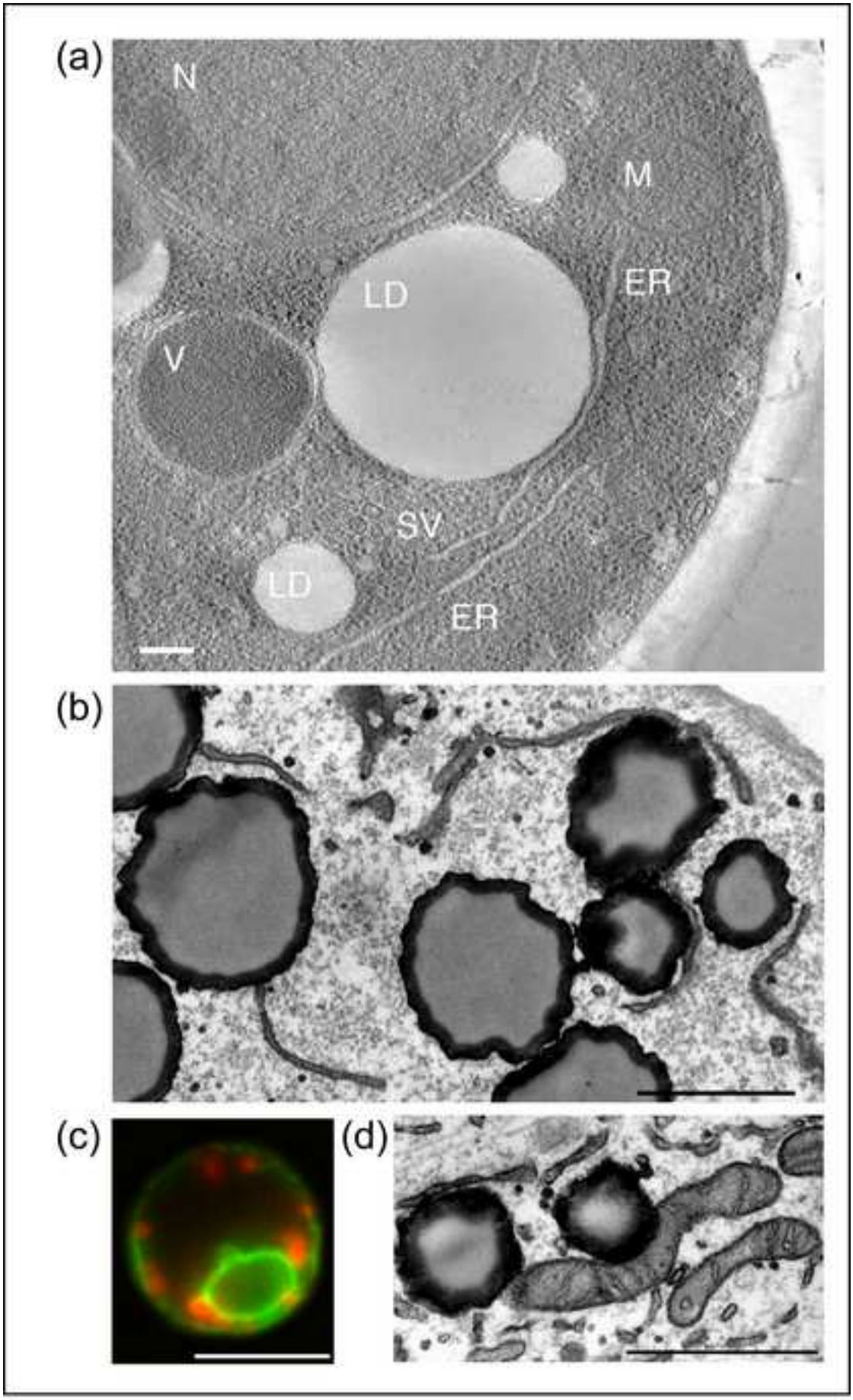

Figure 2