Function of lipid droplet-organelle interactions in lipid homeostasis

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Abstract

Storage of non-polar lipids in ubiquitous eukaryotic organelles, lipid droplets (LDs), prevents the toxic consequences of unesterified fatty acids and provides a lipid reservoir that can be promptly used to satisfy cellular needs under multiple metabolic and physiological conditions. Tight temporal and spatial control of LD biogenesis and mobilization of neutral lipids is essential for the correct channelling of lipid intermediates to their various cellular destinations and the maintenance of cellular homeostasis. These functions are mediated by multiple interactions between LDs and other intracellular organelles that are required for the delivery of stored lipids. Here we review recent advances in the interactions of LDs with the endoplasmic reticulum (ER), mitochondria and vacuole/lysosome.

1. Introduction

The universal ability of eukaryotic organisms to store excess energy from nutrients in the form of non-polar or "neutral" lipids is essential for their survival when food sources are not readily available. At the cellular level, this storage takes place in specialized organelles called lipid droplets (LDs). What sets LDs apart from the majority of other intracellular organelles is their unique topology: they consist of a densely packed hydrophobic core of neutral lipids, mainly triacylglycerol (TG) and steryl esters (SE), and are surrounded by a phospholipid monolayer decorated by LD-associated proteins. During starvation, fatty acids released ("mobilized") from LDs can be used for energy production via β-oxidation in mitochondria or peroxisomes. Moreover, neutral lipids stored in LDs play additional key roles: they can provide essential lipid precursors that are used for membrane biogenesis to support organelle or cell growth and can also act as a "sink" for toxic fatty acids that otherwise are lethal for cells. These functions depend on significant bidirectional intracellular exchange of several lipid species to and from LDs. This poses two major challenges for cells: firstly, as, to our knowledge, LDs are not connected to the general vesicular trafficking pathways, fatty acids and lipid species are probably exchanged exclusively through physical interactions with other organelles; and secondly, given the unique topology of LDs, the nature of these interactions is likely to be distinct from the canonical membrane contact sites (MCSs) that mediate lipid exchange between most other organelles. Indeed, while MCSs normally involve the close apposition of two phospholipid bilayers with an underlying aqueous lumen, LD-organelle interactions consist of a phospholipid monolayer coating a hydrophobic core contacting a bilayer with an aqueous lumen.

Many features of LD biology have been recently discussed in several excellent reviews [1-5]. In this article we will review recent developments on how contacts between the LDs and the ER, mitochondria, peroxisomes or the vacuole/lysosome are established and regulated and elaborate on their functions on lipid metabolism. We focus on these specific organelles because, as discussed below, they are the most relevant for the biogenesis of LDs (ER) and the catabolism of their stored lipid (mitochondria, peroxisomes and vacuole/lysosome).

2. LD-ER contact sites

2.1 Anatomy and functions of LD-ER contact sites

Because the enzymes catalysing the biosynthesis of TGs and SEs (Figure 1) localize to the ER and the production of these neutral lipids is coupled to their storage, it is widely accepted that LDs emerge from the ER. Several recent studies in a diverse set of organisms have provided strong support for this model. However, the mechanisms underlying the early stages of LD emergence from the ER phospholipid bilayer and the nature of the association between the two organelles remain poorly defined. Biophysical studies propose that neutral lipids can be kept at low levels within the phospholipid bilayer, above which they undergo liquid-liquid phase separation ("de-mixing" or "oiling-out"; reviewed in [6]); in vivo such a process could lead to the partitioning of the neutral lipid into the emerging LD that buds towards the cytoplasm. Ultrastructural studies on the nascent LD provide evidence that it forms a "lens" between the two leaflets of the ER bilayer, which would be consistent with a physical continuity between the two organelles [7, 8]. However, a different topology has been observed by freeze fracture studies where both leaflets of the ER appeared external to the LD and surround it in a "egg in a cup" manner [9]. During further expansion of LDs, driven by exogenous supplementation of oleate in fruitfly or mammalian cells, membrane contacts or "bridges" appear to link the LDs to the ER [10, 11] (Figure 2). Mature LDs in budding yeast appear to remain in contact with the ER [12-15]; in higher eukaryotes LDs remain in contact with the ER or dissociate from it [10, 11].

In addition to ultrastructural studies, evidence for a direct connection between the ER and LDs exists from several experiments examining the localization of the enzymes synthesizing neutral lipids during the biogenesis of LDs. Pioneering biochemical studies in budding yeast documented that enzyme activities responsible for neutral lipid synthesis can be detected in both the ER and LDs [16, 17]. Dga1, the enzyme that catalyzes the final acylation step in TG synthesis in yeast is an integral membrane protein that rapidly moves from the ER to the surface of LDs upon transcriptional induction of LD formation [14]. This movement was found to be reversible and independent of new protein synthesis, energy and temperature. In mammalian cells, induction of LD biogenesis by different means (oleate supplementation) also targets DGAT2, the ortholog of Dga1, to LDs [18, 19]. Oleate-loading of fly cells leads to the re-localization of the TG biosynthetic enzymes to a specific pool of LDs that appear to be connected to the ER membrane [11]. Other ER membrane enzymes with roles in LD homeostasis have been also shown to partition dynamically between the two organelles [20-22]. The mechanisms of membrane protein transport through ER-LD contacts have not been clarified. The hydrophobic core of LDs should preclude the insertion of integral membrane proteins with luminal domains, normally present in the ER. It has been suggested that hydrophobic "hairpin" domains, that can only partially cross the ER phospholipid bilayer, could be accommodated on the

4

LD phospholipid monolayer [23] (Figure 2). In agreement with this model, disruption of the predicted "hairpin" topology of LD proteins blocks their translocation from the ER membrane [11, 21, 24, 25]. It should be noted that not all ER membrane proteins that are detected on LDs lack predicted luminal domains [26], so it remains unclear whether alternative modes to accommodate such proteins on LDs exist. A challenging combination of proteomics, immuno-EM and membrane biochemistry to determine protein topology is required to address the complexities of LD protein targeting.

Whether similar to proteins, phospholipids can also traffic at the LD-ER interface remains unclear but has fundamental implications for LD biology. This is because LD phospholipids can critically regulate LD biogenesis: (1) the phospholipid monolayer that coats LDs is essential for solubilizing their hydrophobic core into the aqueous cytosol. Decrease of monolayer phospholipids leads to increased LD surface tension that results in their coalescence and consequently large or "supersized' LDs. Consistently, genetic ablation of the synthesis of phosphatidylcholine (PC), the most abundant LD phospholipid, leads to the formation of supersized LDs in yeast, fly or mammalian cells [27-29]; an additional link between PC metabolism and LD morphology could be due to the fact that both PC and TG share common precursors [30] and thus, in the absence of PC synthesis, excess fatty acids could be re-directed into TG with consequences on LD content and morphology; (2) the phospholipid composition of LDs can also impact their protein composition: in addition to the enzymes that target LDs after inserting to the ER membrane, a second class of proteins associate with LDs directly from the cytosol. These employ amphipathic helices, a motif that separates hydrophobic from polar residues to the two opposite faces of the α-helix, in order to insert into the LD phospholipid monolayer. Accordingly, changes in phospholipid packing on the LD surface lead to the aberrant targeting of LD proteins [29, 31].

Given the continuity at the ER-LD contacts, lateral diffusion of phospholipids through the apparent membrane "bridges" may provide a direct route for this traffic and ensure LD homeostasis when neutral lipids accumulate or are consumed in response to cellular metabolic changes. Seipin, an oligomeric protein that localizes specifically to the LD-ER contacts, and will be discussed in more detail below, could play a key role in this process, but the molecular details remain unknown. Alternatively, but not exclusively, phospholipids could be also synthesized and/or remodelled directly on the LD surface. According to this hypothesis, PC biosynthetic enzymes should be present on LDs. CCT1, the rate-limiting enzyme in the Kennedy pathway for PC synthesis (Figure 1), translocates to and associates with LDs via its amphipathic helix in *D. melanogaster* cells in response to oleate supplementation [29], although whether this translocation takes

place in mammalian cells is currently debated [32]. However, the enzyme catalyzing the following, and final, step in the Kennedy pathway is not present on LDs [21, 29]. Therefore, Kennedy pathway-derived PC likely requires a transport step from the ER in order to reach the surface of LDs. Alternatively, PC on LDs could be produced by the Lands cycle, a pathway that remodels PC acyl chains through acyl-CoA:lysophosphatidylcholine acyltransferases (LPCAT) 1 and 2 and which are active on LDs [21].

The precise nature and composition of the membrane contacts between LDs and the ER remain elusive. However, there is evidence that modulation of the biophysical properties of LDs can control the formation of the contacts. Recruitment of the COP protein coat, that normally operates in the Golgi to ER vesicular transport pathway, on the surface of LDs by its activator Arf1, mediates the removal of surface phospholipids from LDs [33, 34]. It was proposed that by increasing LD surfacing tension this process promotes the attachment of LDs to the ER membrane. Consistently, transport of ER membrane proteins to LDs was found to depend on Arf/COP1 in fly and mammalian cells [34-36].

2.2 Seipin: gatekeeper of LD-ER contacts?

Seipin is an evolutionarily conserved integral membrane protein that localizes to the LD-ER contact site [12, 37, 38]. Recessive loss-of-function mutations in seipin are associated with severe Berardinelli-Seip congenital lipodystrophy type 2 (BSCL2), in which adipose tissue is almost completely absent [39]. The knowledge on how seipin controls LD morphology has increased in the past few years, with results from different model systems suggesting that seipin controls lipid and protein sorting to LDs to establish LD identity and maturation [31, 38, 40-44].

In yeast, seipin (Sei1, formerly known as Fld1) forms a complex with Ldb16, which requires Sei1 self-interaction and the transmembrane helices of both proteins [38]. Deletion of *SEI1* or *LDB16* result in two distinct types of LD morphology abnormalities: small clustered LDs enwrapped in ER or supersized LDs [12, 37, 38]. Although in a complex, mostly at the neck of LDs, overexpression of *SEI1* or *LDB16* cannot compensate for the loss of its partner, but deletion of both genes increase the frequency of cells with supersized LDs over small clustered lipid droplets [38, 41]. Overexpression of human seipin restores LD morphology in *sei1Δ*, *ldb16Δ*, or the double deletion strain, suggesting that during evolution human seipin alone has replaced the function of the Sei1-Ldb16 complex [38]. Interestingly, wild-type LD morphology is restored in *sei1Δ* mutant with the expression of human seipin or seipin neuronal seipinopathy mutants, but

not with most of seipin lipodystrophy mutants, which also fail to localise to LD-ER contact sites [12, 37, 38]. Aberrant LD morphology is also observed in Drosophila and human cells [42, 43], with initial accumulation of small nascent LDs in close proximity to the ER and subsequent formation of giant LDs at later stages during induction of LD biogenesis with oleic acid in seipin-depleted cells [43]. In human cells, a mutation that causes BSCL2 fails to localize to LD-ER contact sites and leads to aberrant LD morphology [42]. Importantly, aberrant LDs in seipin mutants appear to correlate with structural defects at the LD-ER contacts sites: *ldb16Δ* mutants analysed by electron tomography display enlarged ER-LD contact sites, with ER protrusions enwrapping LDs and enlarged ER width not exclusively restricted to the contact site [31]. Similarly, in seipin-knockout human cells, LD-ER contact sites are more heterogeneous, with LDs showing extensive contacts sites or string-like bridges to the ER, or even absent. Moreover, the ER diameter widens when these cells are challenged with fatty acids [42]. Collectively, these data strongly support a role of seipin at LD-ER contact sites in controlling LD morphology.

How does loss of seipin at LD-ER contacts disrupt LD structure? Studies on *sei1Δ* and *ldb16Δ* in yeast have provided important insights. Changes in phospholipid synthesis in these mutants, either by the addition of inositol, or by genetic manipulation of the pathway, resulted in the conversion of the supersized LDs into small clustered LDs [28, 31, 38, 40, 44]. It has been previously reported that PC deficiency, caused by mutations in the phosphatidylethanolamine N-methyltransferase (PEMT) pathway (Figure 1) also results in the formation of supersized LDs [28, 37, 38] and that this can be rescued by induction of the parallel PC biosynthetic route, the Kennedy pathway, by adding its precursor choline. However, choline addition does not rescue the *sei1Δ* and *ldb16Δ* supersized LDs, suggesting that induction of phospholipid synthesis affects their LD structure via a distinct mechanism. Grippa et al (2015) [31] reported that small clustered LDs in *sei1Δ* and *ldb16Δ* cells were significantly enriched in PC and phosphatidylethanolamine (PE), the two most abundant phospholipids, despite the fact that their whole-cell lipid composition was the same as in wild-type cells. LD phospholipid changes were accompanied by severe perturbation of the LD proteome: resident LD proteins were lost while many amphipathic helix-containing proteins, including Pct1, the yeast orthologue of CCT1, targeted ectopically LDs. These results suggest that, in the absence of the Sei1-Ldb16 complex, yeast cells cannot control the sorting of ER phospholipids to LD, which results in abnormal LD morphology and deregulated targeting of amphipathic helix-containing proteins, likely due to defects of phospholipid packing on LDs. Similarly, depletion of seipin in Drosophila and human cells has no major effect in cellular lipid metabolism but results in phospholipid deficiency in giant LDs [43]. Defects in protein targeting to LDs are also observed in seipin-deficient Drosophila and human cells [42, 43].

Another mechanism that could lead to the formation supersized LDs in seipin-deficient cells was recently proposed [45]. Seipin was shown to physically interact with glycerol-3-phosphate acyltransferase (GPAT; Figure 1) to decrease its enzymatic activity both in yeast and humans; overexpression of yeast or human GPATs phenocopy the supersized LD phenotype of seipin-deficient cells, which can be prevented by co-overexpression of seipin. The supersized LD phenotype in seipin deficient human cells can be reverted by knocking down GPAT. Because GPATs catalyse the rate-limiting step in PA synthesis and the latter has been implicated in the formation of supersized LDs [28], it was suggested that high PA levels in the ER is the driver for the seipin phenotype [45].

Seipin is also required for the early stages of LD biogenesis. In yeast, in an inducible *de novo* LD formation system, generation of LDs is delayed in the absence of Sei1, leading to accumulation of neutral lipids within membranes [40]. A short N-terminal segment in Sei1 is specifically involved in LD biogenesis initiation independently of the function of Sei1 in neutral lipid sorting. Similarly, seipin-depleted fly and mammalian cells show defects in the formation of initial LDs during oleate treatment, resulting in an aberrant accumulation of nascent LDs. Seipin-deficient cells have premature targeting and activation of the machinery required for LD expansion on nascent LDs, which results in the formation of giant LDs. Notably, in Drosophila and human cells, seipin shows a highly dynamic ER localization that becomes less mobile once it encounters a nascent LDs, with this interaction promoting its maturation into an initial LD [42, 43]. LDs from seipin-knockout human cells also show increased mobility and aberrant multidirectional trajectories [42].

It remains unclear whether defects in *sei1Δ* or *ldb16Δ* mutants affect the cellular amounts of neutral lipids because different studies have reported opposite results [28, 38, 41]. However, overexpression of *LDB16* in *sei1Δldb16Δ* cells restores TG levels under the conditions that lead to their reduction in this mutant. Since Ldb16 is degraded in *sei1Δ* cells and *LDB16* overexpression cannot compensate for the loss of Sei1 in LD morphology [38], this indicates that Ldb16 likely plays a specific role, independently of Sei1, in TG synthesis [41]. Total TG levels do not seem to be affected in seipin-deficient cells in Drosophila [43], but neutral lipids decrease and lipid delivery is deficient in seipin-knockout human cells [42].

The complexity of seipin mutant phenotypes likely reflects the multiple consequences of disrupting proper LD-ER attachments and the diversity of model systems used to study its function. The role of seipin on membranes at the molecular level remains unknown and

represents a major challenge for the future. Interestingly, seipin is known to form oligomers – of predicted nine or twelve subunits in yeast or humans, respectively – with a disk or toroid configuration [46, 47]. This structural organization may be required for lipid sorting and/or assembly of other factors interacting with seipin at LD-ER contact sites. Since LDs undergo dynamic changes in response to metabolic status in all organisms, the temporal and spatial regulation of seipin activity is likely to be critical for LD formation and function.

2.3 LD-ER contacts and lipid compartmentalization in the ER

There is evidence that the distribution of LD-ER contacts sites is not random and that the protein machinery involved in this process localizes to specific subdomains of the ER. This can be probed, for example, by following the localization of enzymes required for neutral lipids synthesis, an essential event during LD biogenesis. In yeast, Pah1 has been useful in the identification of these ER subdomains [44, 48]. Pah1 is a soluble cytosolic $Mg2+$-dependent phosphatidic acid (PA) phosphatase (PAP) that generates diacylglycerol (DG) used in TG synthesis ([49]; Figure 1). Pah1 activity requires dephosphorylation by the phosphatase complex Nem1-Spo7 [50], which mediates its association with membranes [51-53]. This dynamic association with membranes enables Pah1 to work as a 'molecular switch' to shift lipid intermediates towards TG synthesis when yeast cells exit logarithmic growth and initiate neutral lipid storage to LDs. Upon activation, Pah1 first localizes to nucleus-vacuole junctions (NVJ), an MCS established by the physical interaction of Nvj1 (on the nuclear envelope) and Vac8 (on the vacuole membrane) and is involved in piecemeal microautophagy of the nucleus [54]. Pah1 then moves to the perinuclear membrane subdomain flanking NVJ, where it concentrates on LD-ER contact sites [48]. By keeping Pah1 at the interface between the ER and LDs, cells may restrict the access of other ER phospholipid biosynthetic enzymes to the DG pool formed by Pah1. This could ensure the channelling of DG, a lipid that is at the crossroads of many pathways in the ER, towards storage in LDs and not membrane synthesis. Consistent with this hypothesis, yeast cells unable to make neutral lipids exhibit nuclear and ER membrane proliferation during post-diauxic shift phase, which can be partially rescued by ablation of phospholipid biosynthesis [48]. ER membrane defects of cells lacking LDs during nutrient starvation have been also reported [55, 56].

Despite not formally addressed, several studies in budding yeast suggest that LDs accumulate at the ER subdomain flanking NVJ during stationary phase or other conditions that promote LD biogenesis. Whether this positioning enables the passage of LDs from the nuclear ER to the vacuole, where LDs are degraded at a later stage by

lipophagy (see chapter 4) or it performs another role at the nuclear envelope remains unknown. Moreover, in fission yeast TG-enriched LDs were also localized at the nuclear envelope [57]. Interestingly, loading mammalian cells with fatty acids induces LD clustering around the nucleus and, during starvation, dispersion of LDs to cell periphery is required for LD consumption and β-oxidation [58]. Moreover, complexes of ORP2 (an oxysterol-binding protein (OSBP)-related protein) and VAP (a vesicle-associated membrane protein (VAMP)-associated protein) at LD-ER contact sites favour TG synthesis and inhibit TG hydrolysis in human cells. These outcomes seem to be related to the spatial organization of LDs: overexpression of ORP2-VAP complexes induces the formation of LD clusters close to the nucleus, where TG synthesis may be facilitated; and silencing of the complex enhances TG mobilization, presumably by facilitating the movement of LDs to regions that favour TG hydrolysis [59]. Because mobilized fatty acids can be also used for membrane biogenesis [60], the positioning of LDs within the ER network could be important to support localized organelle biogenesis. One relevant example comes from sporulation (gametogenesis) of yeast cells: during this process, LDs move from the vacuole towards the nucleus where they form clusters. Interestingly, lipolysis from a specific pool of LDs that are in contact with the forming prospore membrane - a double membrane vesicle that sequesters the four meiotic nuclei – is thought to contribute to its expansion and, consequently, spore development, by providing fatty acids and other lipid precursors [61, 62].

The role of Pah1 in LD biogenesis is likely not restricted to TG production. In fact, *pah1Δ* mutants contain significantly fewer LDs despite that their total levels of TG and steryl esters combined together, are similar [63]. Interestingly, deletion of *DGK1* – which encodes for the diacylglycerol kinase that mediates the reverse reaction of Pah1 – suppresses LD defects in *pah1Δ* cells [63-65]. Therefore, the levels of PA and DG at LD-ER contact sites may be critical for LD biogenesis. A number of observations in yeast are consistent with this model: LD biogenesis is induced by an increase in DG levels in cells that lack neutral lipids and express the heterologous LD scaffolding proteins perilipins or oleosin [66]; mutants with increased PA levels show supersized LDs [28]; and fluorescent PA-binding reporters accumulate at LD-ER contact sites in seipin-deficient yeast cells during (or perhaps due to) LD formation [41, 44]. PA-binding reporter accumulation is not observed in *sei1Δldb16Δ* cells expressing human seipin, which suggests that modulation of PA levels at LD-ER contact sites is a conserved function of seipin [41]. However, overexpression of enzymes that metabolize PA fail to reduce the targeting of the PA-binding reporters at LD-ER junctions in sei1Δ cells [41]. Furthermore, accumulation of PA reporters is still observed under growth conditions that favour PA consumption [31], suggesting that their targeting to LD-ER contacts may be independent of PA. Alternatively,

these observations could also be explained by the inaccessibility of this PA pool to PA metabolizing enzymes. Elevated PA levels, caused by the overexpression of *DGK1*, induce the recruitment of the PA phosphatase Pah1 to the nuclear membrane that is in association with the nucleolus [51] and both Pah1 and Nem1 target LD-ER contact sites in seipin mutants at the nuclear membrane [41, 44], further reinforcing the notion of PA accumulation at this subdomain. PA accumulation due to overexpression of *DGK1* leads to expansion of the nuclear/ER membrane [51, 64], and seipin-deficient cells also show ER expansion enwrapping LDs in yeast [13, 31, 44] and LDs surrounded by ER in human cells [42]. These observations highlight the central role of LD biogenesis in ER morphology; the exact origin and nature of these membranes has not been yet identified but their characterization will help define the specific subdomains of the ER involved in LD-ER contact sites and consequently in LD formation.

3. LD-vacuole/lysosome interactions

The relationship between LDs and the vacuole (in yeast) or the lysosome (in higher eukaryotes) is probably best exemplified by the reciprocal interaction of LDs with autophagy. Degradation of LDs by autophagy – a process called lipophagy – is evolutionarily conserved but it proceeds through distinct mechanisms: macroautophagy (in mammals) and microautophagy (in yeast). Following the initial observation that small, and portions of larger, LDs can be sequestered by the autophagosome for degradation through macroautophagy [67] (Figure 3a), many studies have reported an accumulation of LDs due to genetic/pharmacological inhibition of autophagy in different cells types. Reciprocally, increased autophagy leads to clearance of LDs, which may prevent pathologies such as fatty-liver disease [68]. Therefore, in order to obtain a comprehensive understanding of lipophagy, it will be important to characterize the molecular machinery playing a direct role in this process. This includes, for example, proteins that associate with LDs during lipophagy, such as the small GTPase Rab7. Under basal conditions, perilipin 1 on the surface of LDs prevents the association of Rab7 with this organelle. However, β-adrenergic receptor stimulation increases the translocation of Rab7 to LDs, which stimulates lipophagy [69]. Activation of Rab7 during nutrient stress also mediates the physical interaction of LDs with degradative compartments to promote lipophagy [70]. Interestingly, in yeast cells, the Rab7-like protein Ypt7 – a late endosome and vacuolar marker – shows a partial colocalization with LDs and is found in the proteome of isolated LDs. Cells lacking Ypt7 show an increase in the number of LDs and total cellular lipid fatty acid content, and abnormal LD morphology, which is consistent with a role for Ypt7 in LD dynamics. [71]. Similar to Rab7, the chromosome 19 open reading frame 80 (C19orf80) also associates with LDs to promote

lipophagy during thyroid hormone stimulation [72]. Interestingly, autophagy can also fuel the biogenesis of LDs by releasing fatty acids from membranes that can be re-esterified and stored as neutral lipids (Figure 3b, [73]).

Notably, LDs also affect autophagy (Figure 3). Indeed, in mammalian cells, an increase in LD biogenesis, by treating the cells with oleate, enhances autophagy initiation and maturation, with multiple interactions of LDs with autophagic organelles [74]; and, in yeast, autophagy is inhibited at early stages of autophagosome biogenesis in cells defective in neutral lipid synthesis [55, 75]. A role of neutral lipids in autophagy was supported by the identification of lipases involved in the formation of autophagosomes (Figure 3c). In mammalian cells, the neutral lipase PNPLA5 mobilizes TG to generate DG used for PC synthesis that contributes for the formation of membranes for autophagosomes [74]. Similarly, in yeast, the steryl-ester hydrolase Yeh1, the TG lipase Ayr1 and the serine hydrolase Ldh1 (that also displays both steryl-ester hydrolase and weak TG lipase activities) are required for autophagy during nitrogen starvation [55]. Interestingly, LDs are not required for rapamycin-induced autophagy [56, 76]; and in LD-deficient cells, the inhibition of fatty acid synthesis or the modulation of ER phospholipid composition improves starvation-induced autophagy and cell survival. Therefore, during starvation, cells unable to make neutral lipids increase phospholipid synthesis (particularly phosphatidylinositol) to buffer excess fatty acids, and these changes in ER phospholipid composition compromise autophagy [56]. Collectively, these results suggest that LDs may contribute to membrane synthesis used for autophagosome formation and ER homeostasis to ensure normal regulation of autophagy.

In yeast, LDs are degraded in the vacuole, in a process that resembles microautophagy (Figure 4), during nitrogen starvation [77], stationary-phase [78], and lipid-stress-induced ER stress [79]. Although the association of LDs with the vacuole membrane is morphologically similar, these conditions trigger to some extent different types of lipophagy. Indeed, while nitrogen-starvation- and stationary-phage-induced lipophagy requires the core autophagy machinery [77, 78], at least Atg7 is dispensable for lipid-stress-induced lipophagy [79]. Also, stationary-phase lipophagy requires the association of LDs with a sterol-enriched liquid-ordered domain on the vacuolar membrane [78], which is not formed during nitrogen-starvation [80]. Interestingly, this sterol-enriched liquid ordered domain is excluded from NVJ during Pah1 recruitment to this contact site [48], and Nem1 is required for the formation of the domains [78, 80]. This suggests that LD biogenesis and the formation of the domains are linked. The concentration and coordination of their molecular machinery at NVJ may have an impact in stationary-phase lipophagy by controlling the organization of LDs at this perinuclear-ER subdomain and

lateral movement to the vacuole. Interestingly, the disruption NVJ does not hamper but actually modestly increases stationary-phase lipophagy [81]. This may be due to a negative effect of NVJ on the lateral movement of LDs to the vacuole, or a consequence of a compensatory mechanism of other ER-vacuole contact sites in response to loss of NVJ.

The mechanisms responsible for the mobilization of the neutral lipids delivered to the vacuole by lipophagy remain obscure. During nitrogen-starvation induced lipophagy, the lipase Atg15 is responsible for most of this activity, which provides fatty acids for growth when the *de novo* fatty acid biosynthesis is inhibited [77]. Since lipophagy is required for the formation of sterol-enriched liquid-ordered domains in the vacuole – most likely by releasing sterols stored in steryl-esters – and these are absent in Atg15-deficient cells [78], it is likely that this lipase also operates during stationary-phase lipophagy. Cells likely coordinate neutral lipid mobilization because yeast cells lacking Atg15 increase lipolysis by cytosolic TG lipases Tgl3 and Tgl4 [82].

Several aspects of lipophagy require significant further clarification (Figure 4). What is the fate of the lipids degraded in the vacuole/lysosome? Is lipophagy used as an alternative route to provide fatty acids and/or other lipids under metabolic conditions that hamper de novo biosynthesis, as previously suggested [77]? If so, what is the advantage for cells of using lipophagy over lipolysis? Could the type of LD degradation be influenced by their spatial organization in cells? What is the machinery required for lipid export from the vacuole/lysosome and does it involve membrane contact sites? The existence of multiple contact sites between the vacuole and the ER [83], and between the vacuole and mitochondria (vCLAMP; [84, 85]) suggest that this may be the case. Interestingly, vCLAMP depends on the HOPS subunit Vps39 and Ypt7 that, as described above, are involved in LD dynamics [71]. Alternatively, fatty acids may be exported from the vacuole to the cytosol through an unidentified mechanism, involving their conversion into acyl-CoA, followed by trafficking to specific compartments such as the peroxisome (for β-oxidation) or the ER. In addition to fatty acids, other products of vacuolar lipolysis may use similar pathways for lipid recycling. As mentioned above, turnover of SE likely provides ergosterol for the formation of sterol-rich microdomains during stationary-phase lipophagy.

Finally, lipophagy may also play a role in the degradation of protein aggregates: it was shown in yeast that chaperones are recruited to LDs enriched in ubiquitinated proteins, presumably misfolded ER proteins, during lipid stress [79]. Further work is required to test whether this precedes clearance of these specific LDs by lipophagy.

4. LD-mitochondria and LD-peroxisomes interactions

Conversion of fatty acids into acetyl-CoA through β-oxidation in mitochondria (in metazoans) or peroxisomes (in yeast and plants) is one of the fates of lipids stored in LDs. Therefore, a close interplay must take place between LDs and these organelles to ensure the coordination between lipolysis and trafficking of fatty acids to mitochondria or peroxisomes. Indeed, TG lipolysis mediated by adipose triglyceride lipase (ATGL) positively regulates SIRT1 to induce the expression of genes involved in β-oxidation and mitochondrial biogenesis [86, 87]. In a similar mechanism, ATGL promotes mitochondrial function in islet β cells, which is required for glucose-stimulated insulin secretion [88]. Catabolism of LD-derived fatty acids may proceed through contact sites between LDs and mitochondria or peroxisomes. In fact, LDs can be found close to mitochondria in oxidative tissues with high demand of fatty acids for energy production [89]; and, in yeast, stable contacts between LDs and peroxisomes, called 'pexopodia', contain enzymes required for β-oxidation and may presumably facilitate the transfer of fatty acids between these organelles [90]. Interestingly, TG mobilization negatively regulates LD-peroxisome physical interactions in Arabidopsis thaliana [91, 92]. Using bimolecular fluorescence complementation assays in yeast, Pu et al (2011) have provided evidence of physical interactions between LDs and mitochondria or peroxisomes. Since most of the interacting proteins are enzymes or proteins involved in transport, these results suggest a role for these contact sites in metabolism and trafficking. Because β-oxidation occurs in peroxisomes in yeast, these findings indicate that LD-mitochondria contact sites may also be involved in other processes [93]. Furthermore, perilipin 5 promotes the physical association of mitochondria with LDs through its C-terminal region in oxidative tissues, thus controlling mitochondrial fatty acid exposure and utilization [94, 95]. Tight physical interactions between LDs and mitochondria in mouse interscapular brown adipose tissue are further highlighted by the presence of mitochondrial proteins in the proteome of isolated LDs. The levels of some of these mitochondrial proteins change with cold treatment, which may reflect the dynamics of LD-mitochondria interaction for fatty acid utilization in thermogenesis [96].

Recent studies have provided some intriguing insight on the physiological signals that regulate LD-mitochondria associations. Transient LD-mitochondria (kiss-and-run) and stable contacts increase during starvation and depend on microtubule-mediated dispersion of these organelles to the cell periphery, which is required for LD consumption and fatty acid β-oxidation. The spatial organization of LDs (and mitochondria) under these conditions is promoted and maintained by detyrosinated microtubles due to AMPK activation [58]. During starvation, fatty acids derived from lipolysis are transferred to mitochondria in close proximity with LDs while autophagy recycles fatty acids from

membranes to maintain neutral lipid stores (Figure 3b). As LD-mitochondria associations are limited, efficient fatty acid catabolism relies on mitochondrial fusion for a homogenous distribution of fatty acids through the mitochondrial network. To avoid toxic effects due to mitochondrial fusion defects, fatty acids are re-esterified and stored in LDs or exported to the extracellular medium [73]. Consistently, cells deficient in proteins required for mitochondrial fusion show fewer but larger LDs when grown in the presence of oleate [97].

LD-mitochondria interactions are not only required for channelling fatty acids for oxidation but also for proper LD biogenesis and stability. Indeed, PEMT (which methylates PE into PC), DGAT2 (responsible for DG acylation into TG) and MGAT2 (which catalyses the production of DG and is in a complex with DGAT2, Figure 1) are found in mitochondria-associated ER membranes (MAM) in close association with LDs [19, 98, 99]. In fact, a murine DGAT2 mutant that mis-localises to mitochondria but not ER retains the ability to promote TG storage [100]. During adipogenesis in murine cells, mitochondrial-derived PE is used by PEMT to make PC on LDs, contributing to LD growth. PEMT-driven PC synthesis also affects perilipin A association with LDs, which impacts on LD stability by controlling lipolysis [98]. Taken together these observations support a close interaction between, LDs and mitochondria/peroxisomes that mediate bidirectional lipid traffic between these organelles. Whether specific protein machinery exists to tether these organelles and facilitate lipid traffic or whether traffic takes place through a common proximal ER domain, with which these organelles associate, remains unknown.

Fatty acid activation and transacylation.

Fatty acids need to be thioesterified with coenzyme A (CoA), both for their storage in LDs as well as for their utilization by mitochondria and peroxisomes following their release from LDs (Figures 1 and 2). The formation of these acyl-CoAs is mediated by acyl-CoA synthetases (ACS). Previous reviews have comprehensively addressed the role of these enzymes in fatty acid compartmentalization in different tissues [101, 102]. Here we will briefly discuss how ACS might coordinate fatty acid channelling in the context of LD interactions with other organelles.

To our knowledge, there is no evidence of a specific enrichment of ACS in LD-organelle contact sites. In fact, the determination of the subcellular localization of these enzymes has been hampered by organelle contamination in subcellular fractionation studies (e.g., ER contamination of LD fractions) and low expression levels in microscopy analyses [102]. Despite these limitations, examples of associations of ACS with other organelles include LDs [103-106], mitochondria [107, 108], and peroxisomes [109, 110]. The subcellular localization and function of these enzymes may vary between cell types and

be subject to transient associations under specific conditions. For example, this is the case of ASCL3, which localises both to ER and LDs in a cell- and FA-specific manner [105]. ACS on LDs may directly activate fatty acids released during lipolysis or, alternatively, the close proximity promoted by LD-organelle interactions may facilitate fatty acid activation by ACS present on mitochondria and peroxisomes.

Fatty acids can also be directly transferred from phospholipids to DG by phospholipid:diacylglycerol acyltransferases (PDATs), or to sterols, by lecithin:cholesterol acyltransferase (LCATs), in an acyl-CoA independent manner. Fungi, green algae and plants, express several PDATs, and are therefore able to produce TG directly from phospholipids. Lro1 is responsible for the PDAT activity in *S. cerevisiae* and transfers *sn*-2 acyl groups from PE and PC to DG [111-113]. The *C. reinhardtii* PDAT mediates TG synthesis, which is coupled to chloroplast membrane turnover in response to stress [114]. How membrane-derived fatty acids would be channelled to LDs, remains unknown. It also remains to be clarified whether in mammals, and other organisms, that lack apparent PDAT-related proteins, phospholipase activity coupled to ACS-mediated fatty acid activation may replace PDAT function; there is evidence that during stress, mammalian cells induce TG synthesis and LD biogenesis that depends on phospholipase A2 [115]. Finally, both Lro1 and Dga1 in budding yeast, and the human PLA2 PLA2G15 can also acylate ceramide [116-118], and acylceramide produced by DGAT2 can also be stored in LDs [119].

5. Conclusion and perspective

The essential roles of LDs in membrane biogenesis or energy production can be performed only through their regulated release and delivery of lipids to other intracellular organelles. At the same time, LDs receive extensive protein and lipid traffic from the ER and mitochondria, which is critical for their formation and stability. Because this bidirectional cross talk must be precisely coordinated with the metabolic status of cells, the interactions of LDs with other organelles need to be highly dynamic.

In most cell types, LDs are stably connected to the ER, which extends throughout the cell contacting most other organelles. Therefore, the positioning and dynamics of LDs within the ER network could influence their interaction with other organelles. Whether machinery mediating direct contact between LD and mitochondria or peroxisomes, where mobilized fatty acids are catabolized, exists, remains also unknown. This raises also the question of whether mobilized lipids from LDs traffic to these organelles through LD-ER contacts. Several exciting advances have been made in the last few years on seipin; however, details on its precise molecular function at LD-ER contacts are still missing and may

require the development of new *in vitro* assays to follow LD assembly in phospholipid bilayers. Finally, LDs show a much broader spectrum of organelle associations than originally thought, which include "homotypic" LD-LD contacts that control neutral lipid transfer in specialized cell types such as adipocytes, as well as components of the endocytic pathway, the plasma membrane in the form of caveolae, bacterial and viral pathogens and more recently the intra-nuclear compartment. Determining how LD-organelle interactions are brought by and regulated will be an important and stimulating challenge for the future.

6. Acknowledgments

7. References

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Figure legends

Figure 1. Simplified schematic representation of the pathways involved in the synthesis of phospholipids and triacylglycerol. Common steps are represented in gray, the Kennedy pathway in blue, and other phospholipid synthesis pathways in orange. Dashed arrows represent steps presented in yeast but not in humans. Relevant enzymes discussed in the text are highlighted in bold. The orange inset highlights phospholipid synthesis at mitochondria-ER contact sites, and the dashed inset shows fatty acid activation by acyl-CoA synthetases. FA, fatty acid; lyso-PA, lyso-phosphatidic acid; PA, phosphatidic acid; DG, diacylglycerol, TG, triacylglycerol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; Cho, choline; P-Cho, phosphocholine; CDP-Cho, cytidine diphosphate-choline; Etn, ethanolamine; CDP-Etn, cytidine diphosphate-ethanolamine; GPAT, glycerol-3-phosphate acyltransferase; AGPAT, acylglycerophosphate acyltransferase; PAP, phosphatidic acid phosphohydrolase; DGAT, diacylglycerol acyltransferase, CCTα, Choline-phosphate cytidylyltransferase; PEMT, phosphatidylethanolamine N-methyltransferase; PSD, phosphatidylserine decarboxylase, PSS, Phosphatidylserine synthase; ACS, acyl-CoA synthetases.

Figure 2. Model of LD-ER contact site structure and function. Contact sites provide a route for membrane-associated proteins (in red) to translocate from the ER onto the surface of the LDs. This is thought to involve hydrophobic "hairpins" that can only span part of the ER phospholipid bilayer. Contact sites may also allow traffic of PC (in green) or other ER phospholipids by lateral diffusion to the surface of the LD and mediate its coordinated expansion during neutral lipid storage. Seipin, (or the Sei1-Ldb16 complex in yeast), localizes to LD contacts sites and has been proposed to control this process

although molecular details remain unknown. Pah1, a PA phosphatase that generates DG, localizes to LD-ER contacts and may be required for both TG synthesis and LD biogenesis. Whether in addition to its synthesis on the surface of LDs, TG can be also channeled from the ER through LD-ER contacts, remains unknown. SE synthesis is also important for LD biogenesis, but the mechanisms underlying its packaging at LD-ER contacts are unknown.

Figure 3. Interactions of LDs with lysosome/vacuole. (a) Degradation of small or portions of larger LDs by the lysosome involves proteins that associate with LDs during lipophagy, such as Rab7 and C19orf80. (b) Degradation of cellular membranes during autophagy may provide FAs for LDs biogenesis. (c) Neutral lipids stored in LDs may be released by specific lipases, in mammalian and yeast cells, that can be then used for membrane synthesis during autophagosome formation. See chapter 3 for details.

Figure 4. Lipophagy in yeast. In yeast, lipophagy resembles microautophagy. During stationary-phase (Stat-phase), LDs accumulate in an ER subdomain flanking nuclear-vacuole junctions (NVJ) and the membrane of the vacuole is organized in two subdomains. In Stat-phase lipophagy, LDs associate with sterol-rich microdomains for vacuole internalization, which does not happen during lipophagy induced by nitrogen starvation. However, both require the core autophagy machinery. Degradation of LDs in the vacuole likely involves the lipase Atg15, and the ergosterol released may promote the formation of the sterol-rich microdomains. Lipid intermediates released during lipophagy are expected to be channeled to other cellular compartments – through unidentified transporters or organelle contact sites, depicted in blue in the figure – where they may be used for phospholipid synthesis or energy production. Inset: micrograph of a yeast cell in the post-diauxic shift phase, co-expressing ER (green) and vacuolar (red) reporters. Lipid droplets are labelled in blue with monodansylpentane. The outline of the cell is depicted; bar, 2.5 μm.
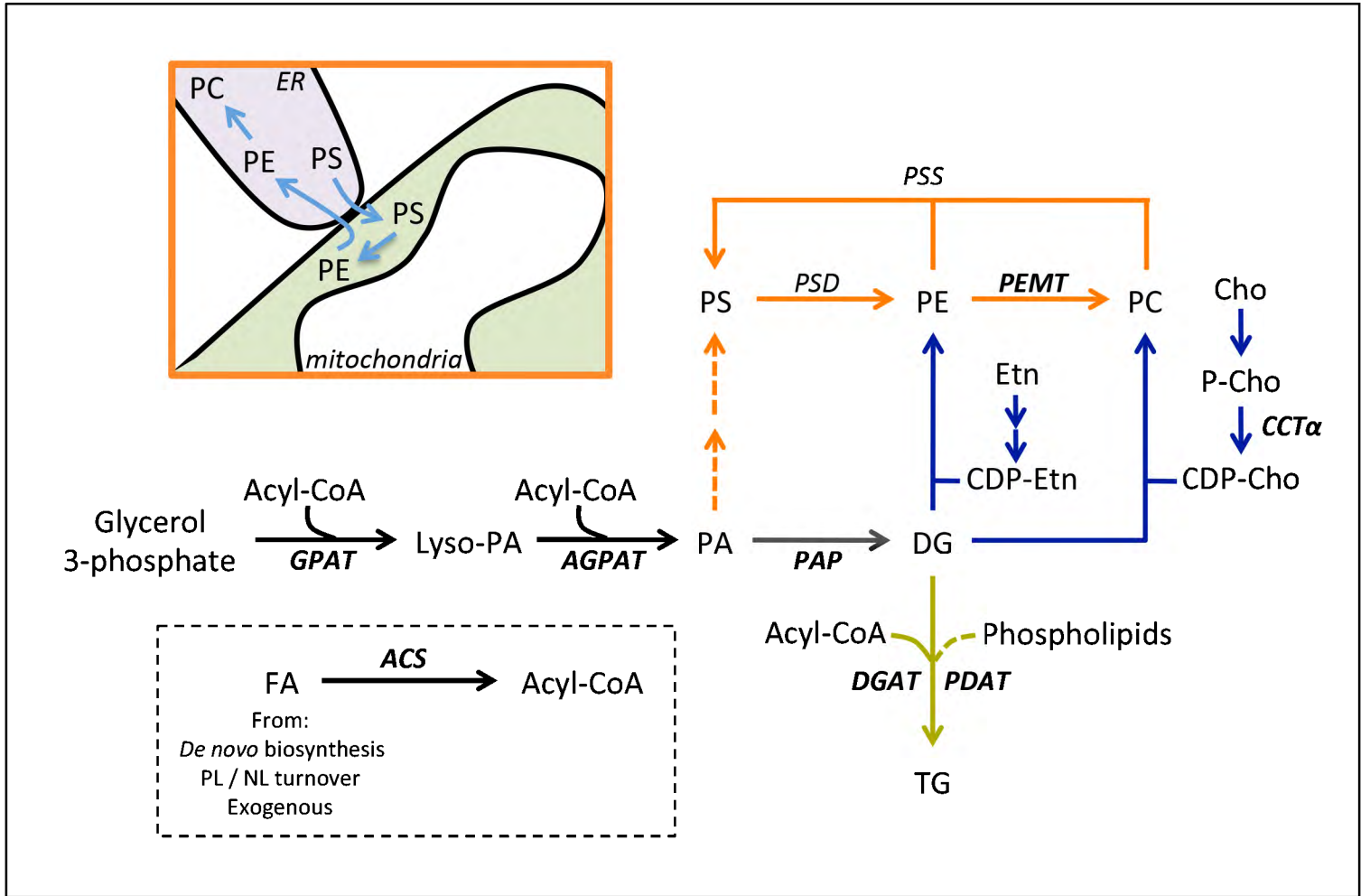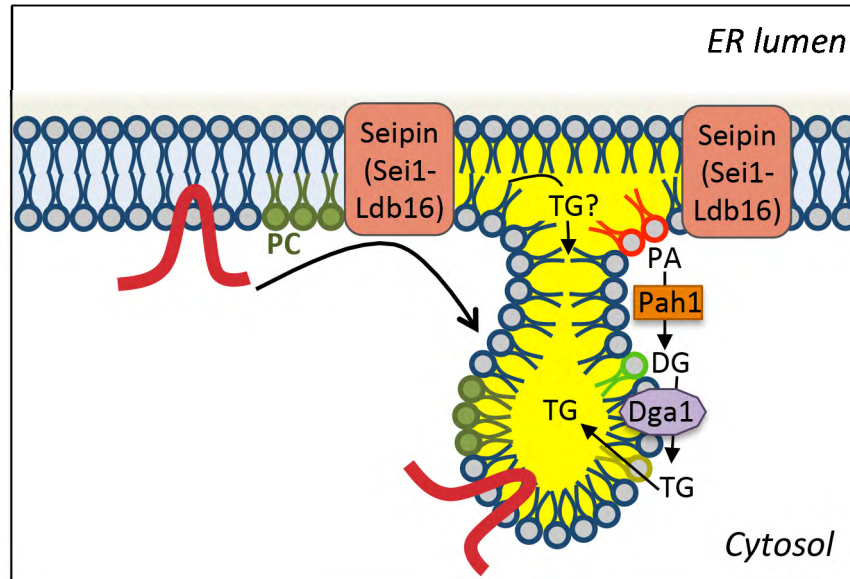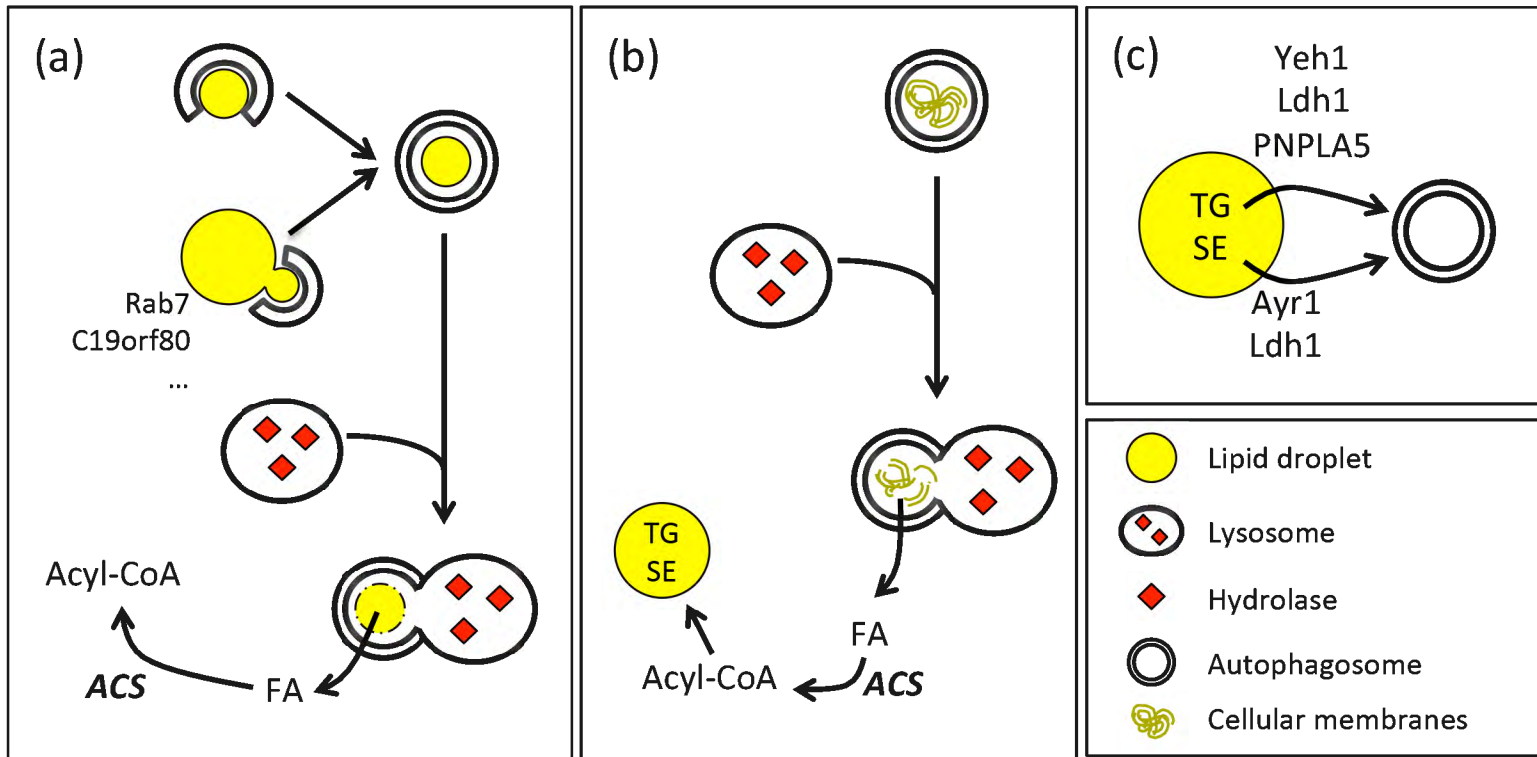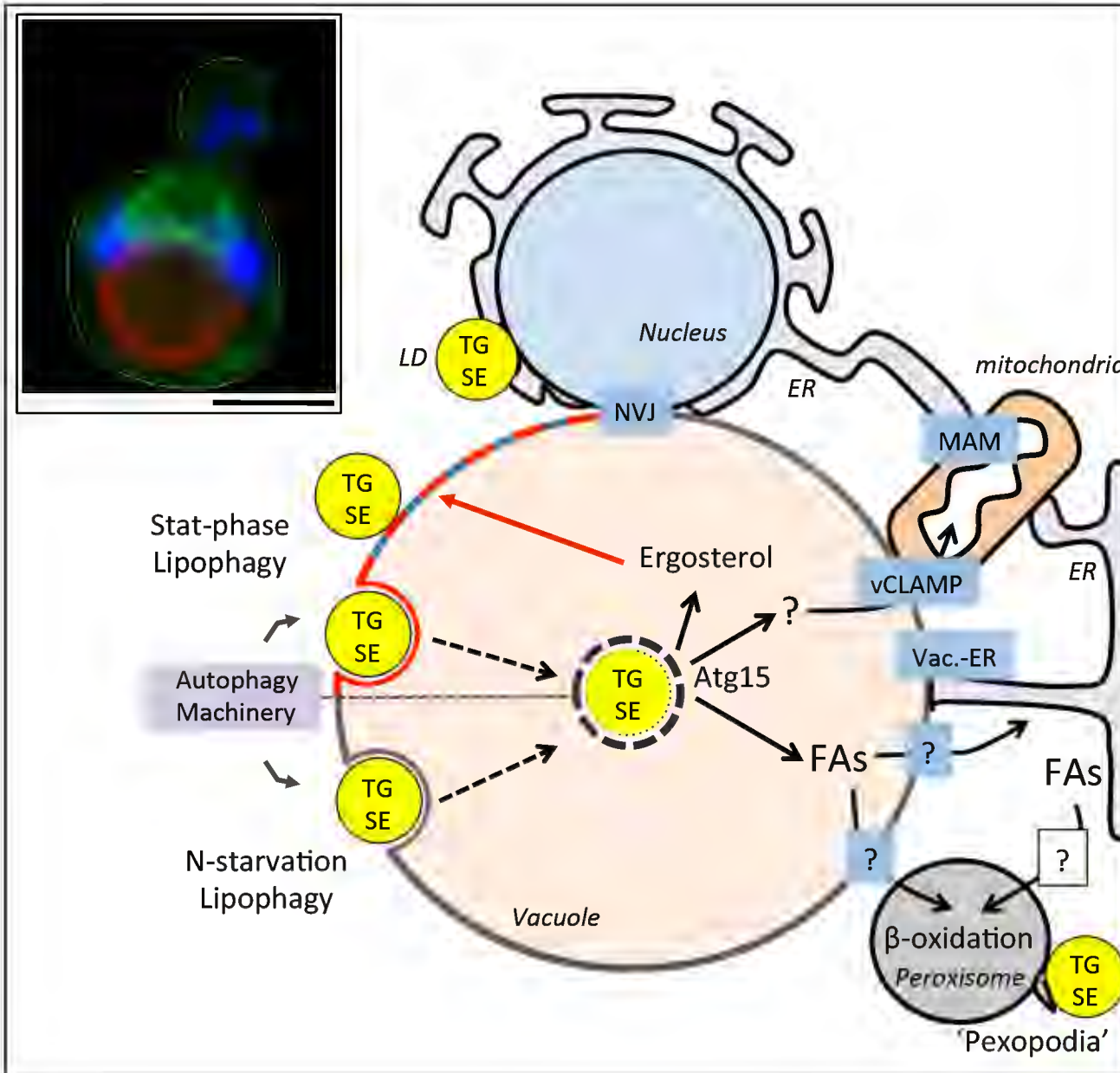
Figure 1

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