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REVIEW

# Emerging functions of the mitochondria–ER–lipid droplet three-way junction in coordinating lipid transfer, metabolism, and storage in cells

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**Over the past two decades, we have witnessed a growing appreciation for the importance of membrane contact sites (CS) in facilitating direct communication between organelles. CS are tiny regions where the membranes of two organelles meet but do not fuse and allow the transfer of metabolites between organelles, playing crucial roles in the coordination of cellular metabolic activities. The significant advancements in imaging techniques and molecular and cell biology research have revealed that CS are more complex than what originally thought, and as they are extremely dynamic, they can remodel their shape, composition, and functions in accordance with metabolic and environmental changes and can occur between more than two organelles. Here, we describe how recent studies led to the identification of a three-way mitochondria–ER–lipid droplet CS and discuss the emerging functions of these contacts in maintaining lipid storage, homeostasis, and balance. We also summarize the properties and functions of key protein components localized at the mitochondria–ER–lipid droplet interface, with a special focus on lipid transfer proteins. Understanding tripartite CS is essential for unraveling**

**Abbreviations**

AD, Alzheimer's disease; AH, amphipathic helix; ALS, amyotrophic lateral sclerosis; APP, amyloid precursor protein; Aβ, amyloid-β; BSCL, Berardinelli–Seip congenital lipodystrophy; BSCL2, Berardinelli–Seip congenital lipodystrophy type 2; CaMKII, calmodulin/$Ca^{2+}$-dependent protein kinase II; CJ, cristae junction; CKI, casein kinase I; CL, cardiolipin; CS, contact sites; CYP27A1, 27-hydroxylase; DFCP1, double FYVE-containing protein 1; DGAT2, diacylglycerol o-acyltransferase 2; ECT, electron transport chain; EM, electron microscopy; ER, endoplasmic reticulum; E-Syts, extended synaptotagmins; FA, fatty acid; FFAT, two phenylalanines in an acidic tract; hMIGA2, human mitoguardin 2; HRP, horseradish peroxidase; IMM, inner mitochondrial membrane; iPS, induced pluripotent stem cells; KDEL, endoplasmic reticulum lumen protein-retaining receptor 1; KO, knockout; LC3, microtubule-associated protein 1A/1B-light chain 3; LD, lipid droplet; LDs, lipid droplets; LTP, lipid transfer protein; LTPs, lipid transfer proteins; LXR, liver X receptor; MAM, mitochondria-associated ER membrane; MFN2, mitofusin 2; MIB, mitochondrial intermembrane space bridging complex; Mic60, mitochondrial contact site and cristae organizing system subunit 60; MICOS, *mitochondrial contact site and cristae organizing system*; MIGA1, mitoguardin 1; MIGA2, mitoguardin 2; OA, oleic acid; OMM, outer mitochondrial membrane; ORD, oxysterol-binding protein-related lipid-binding/transfer; ORP, oxysterol-binding protein-related protein; ORP5, oxysterol-binding protein-related protein 5; ORP8, oxysterol-binding protein-related protein 8; OSBP, oxysterol-binding protein; PA, phosphatidic acid; PC, phosphatidylcholine; PDM, peridroplet mitochondria; PDZD8, *PDZ domain-containing protein 8*; PE, phosphatidylethanolamine; PG, phosphatidylglycerol (we did not define it in the text); PH, pleckstrin homology; PI(3)P, phosphatidylinositol 3-phosphate; PI, phosphatidylinositol; PI3K, phosphatidylinositol 3-kinase; PI4P, phosphatidylinositol-4-phosphate; PIP2, phosphatidylinositol 4,5-bisphosphate; PISD, phosphatidylserine decarboxylase; PISD1, phosphatidylserine decarboxylase 1; PLA, proximity ligation assay; PLIN1, perilipin 1; PS, phosphatidylserine; PSS1, phosphatidylserine synthase-1; PTPIP51, tyrosine phosphatase-interacting protein 51; RXR, *retinoid X receptor*; Sac1, phosphatidylinositol-3-phosphatase SAC1; SAM, sorting and assembly machinery complex; SAM50, sorting and assembly machinery component 50; SE, sterol ester; SNAP23, synaptosome-associated protein 23; Snx14, *sorting nexin 14*; TAG, triacylglyceride; TDP-43, TAR DNA-binding protein-43; TM, transmembrane domain; TOM, multisubunit translocase of the outer mitochondrial membrane complex; TPR, tetratricopeptide repeat domain; VAMP4, *vesicle-associated membrane protein 4*; Vap33, vesicle-associated membrane protein-associated protein of 33 kDa; VAPA, vesicle-associated membrane protein-associated protein A; VAPB, vesicle-associated membrane protein-associated protein B; VPS13A, vacuolar protein sorting-associated protein 13A; VPS13D, vacuolar protein sorting-associated protein 13D; zMIGA2, zebrafish mitoguardin 2.



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**the complexities of inter-organelle communication and cooperation within cells.**

**Keywords:** contact sites; fatty acids; lipid metabolism; lipid transfer proteins; MAM

One hallmark of eukaryotic cells is the presence of physically and functionally specialized membrane-enclosed organelles. Compartmentalization of specific biological processes and biochemical reactions within the organelles allowed life to adapt to new challenging and ever-changing environments. At the same time, cell compartmentalization created the need for organelles and cellular membranes to communicate with each other to coordinate and integrate their multiple activities. Thus, cellular organelles must establish an efficient communication network to balance the flow of information material across the various membranes and react to metabolic cues in coordination to ensure the maintenance of cellular homeostasis.

Vesicle-mediated transport of proteins and lipids between cellular membranes has long been thought to be the main mechanism of inter-organelle communication. However, during the last decade it has become evident that all organelles and cellular membranes are also physically and functionally connected by vesicle-independent transport pathways that allow the transport of signals and metabolites (i.e. lipids and calcium ions) at regions where organelles are in direct contacts. These regions of tiny distances between organelles' membranes (within the range of a few nanometers), where no fusion occurs, have been termed contact sites (CS) and have recently emerged as key mediators of inter-organelle crosstalk [1]. Disruption of their functional and structural integrity has been associated with many pathological conditions and human diseases. Yet, we are just beginning to understand how cellular metabolites are exchanged at CS and how their functions are coordinated and integrated within other communication pathways within the cell.

The existence of CS has been noticed already in the 1950s since cells' internal structures were first visualized by electron microscopy [2,3]. However, only during the last decade, thanks to the growing identification of the molecular components populating these junctions, we have started to unveil the mechanisms regulating CS formation, organization, regulation, and functions. CS are maintained by an ensemble of tethering forces generated by specific multiprotein complexes, often with redundant activity, including lipid transfer proteins (LTPs) or ion (i.e. calcium) channels, which mediate the exchange of lipids or $Ca^{2+}$ between the two juxtaposed membranes. To date, several types of contact sites have been described, and it is now evident that they can be established between all cellular membranes. The endoplasmic reticulum (ER) is the largest membrane-bound organelle that extends throughout the cytosol in the eukaryotic cells, and it performs diverse functions, including lipid synthesis and storage of $Ca^{2+}$. Thus, it is not surprising that the most commonly described CS involve the ER [4].

While vesicular trafficking allows the transport of a great amount of lipids, non-vesicular lipid transport mediated by lipid transfer proteins at CS allows a fast and selective exchange of lipids between the apposed membranes [5]. Moreover, lipid transfer at CS can be temporally and spatially regulated in response to metabolic needs, stress, or environmental changes [6,7]. Because of all these features, non-vesicular lipid transport is currently considered to be a central mechanism to preserve organelles' membrane composition and intracellular lipid homeostasis. Besides, CS have been proposed to be a general and ancient mechanism of inter-organelle connection that even preceded the vesicle-mediated communication [8]. Indeed, the exchange of information via CS is essential for all organelles, although they are linked by vesicular trafficking, but is particularly important for mitochondria and lipid droplets (LDs), which, given their atypical structure (delimited by two lipid bilayer membranes or one phospholipid monolayer, respectively), are largely excluded from the classical vesicular trafficking pathways [9].

Mitochondria, responsible for energy production, and LD, responsible for energy storage, are key gatekeepers of the energy metabolism homeostasis in the cell. Mitochondria are complex and dynamic organelles that constantly move, fuse, and divide within the cell and whose main function is to provide more than 90% of the cellular ATP, but they also play essential roles in the regulation of lipid metabolism, calcium homeostasis, and cell fate. Mitochondria consist of a double membrane system of an outer membrane

(OMM) and an inner membrane (IMM), which are structurally and functionally distinct [10]. The IMM folds into the cristae, unique structures of the mitochondria that house the large protein complexes involved in energy production, such as the electron transfer system (ECT), responsible for oxidative phosphorylation and ATP generation.

On their side, LDs also possess an atypical structure among organelles, as they are formed by a core of neutral lipids (mostly triacylglycerides (TAGs) and sterol esters (SEs)) surrounded by a phospholipid monolayer filled with integral and associated proteins [11]. LDs are vital for the cell as the fatty acids (FAs) stored in their core in the form of neutral lipids, when nutrients are abundant, serve as energy sources in periods of nutritional scarcity. Storing lipids in excess and releasing them upon cellular needs are essential for cells to respond to energy fluctuations and allow cell survival when external nutrients are low. Furthermore, during stress conditions LDs can sequestrate the excess of intracellular lipids in their core and protect the cell against lipotoxicity [12]. Thus, LDs are very dynamic organelles that constantly remodel in response to metabolic changes and are tightly integrated into cell physiology [13]. Their size, number, and composition vary depending on the cell type, nutrient availability, and metabolic state [14].

Mitochondria and LD structure, dynamics, and function are tightly linked to their membrane lipid composition. However, to maintain and modulate their lipid composition in accordance with their own needs, these organelles largely depend on exchanges with the ER, where these lipids are synthesized. For instance, mitochondrial cristae and cristae junctions (CJs), where the cristae are connected to the inner mitochondrial boundary membrane, are rich in two phospholipids, cardiolipin (CL) and phosphatidylethanolamine (PE), which are essential to maintain their morphology and to provide a proper environment for the assembly and function of the ECT proteins [15]. However, mitochondria possess the enzymes necessary to generate PE and CL, but not their precursors, phosphatidylserine (PS) and phosphatidic acid (PA), respectively, which are synthesized in the ER. Therefore, PS, PA, and other phospholipids important to support mitochondrial membranes (phosphatidylcholine [PC] and phosphatidylinositol [PI]) must be imported from the ER at specific subdomains, rich in lipid biosynthetic activity, known as mitochondria-associated ER membranes (MAMs) [16,17]. The recent increasing discovery of LTPs localized at ER–mitochondria contacts is starting to reveal the complexity of the pathways involved in these transfer processes (reviewed in refs [16,18–20]).

Lipid droplets emerge from the cytosolic monolayer of ER membranes, following the biosynthesis and deposition of neutral lipids within the bilayer hydrophobic region [21]. The neutral lipids condense to nucleate a nascent LD, which grows and buds from the ER into a mature LD. LDs often remain connected to the ER membrane via lipidic bridges that may be dynamically disrupted and re-formed. Throughout their lifetime, LDs can grow or shrink in size, depending on the need of the cell, and interact with other organelles. At the end of their life, LDs are consumed by lipolysis or lipophagy. Increasing evidence suggests that contact sites between LD and other organelles, especially with the ER and mitochondria (Fig. 1), play a key role in balancing LD synthesis and turnover, to maintain the complex cellular needs and cellular energy homeostasis [22,23]. Contact sites of LD with the ER supply to the LD the FAs that are stored in their core as neutral lipids (TAG and SE). These FAs can be transferred to the mitochondria to be used for energy production by β-oxidation. Conversely, mitochondria are the source of ATP and other metabolites that can contribute to the growth or expansion of LD. Also, during LD biogenesis and growth, specific lipids (i.e. phospholipids) should be transported from the ER to the growing LD to regulate their size and to maintain a balanced lipid monolayer composition. Several LTPs localized at ER-LD and LD–mitochondria CS have started to be identified [5,24]. However, we are just starting to understand how lipids and lipid precursors are transferred between these organelles.

With the advancement of the technologies to study CS, it has become evident that the complexity of the structure and functions of CS go far beyond the transfer of molecules between two organelles. Multiple layers of evidence emerging from recent studies reveal that CS can be established simultaneously between three or even more organelles: ER–mitochondria–Golgi, mitochondria–ER–lysosomes, ER–LD–peroxisome, and ER–mitochondria–plasma membrane [25–27]. In this review, we are going to describe recent advancements in the newly identified mitochondria–ER–LD contact sites that started to unveil a central function of this three-way inter-organelle association in the regulation of lipid homeostasis in the cell.

## Role of the mitochondria–ER–lipid droplet junction in *de novo* lipogenesis in adipocytes

The existence of close proximity of LD with other organelles in addition to the ER, and especially with

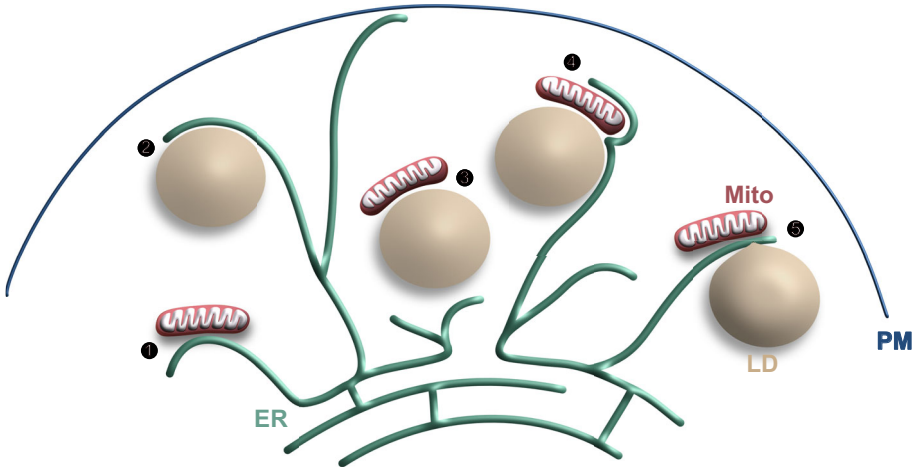


**Fig. 1.** Diagram showing different types of contact sites between ER, mitochondria, and/or LD. Contact sites can be formed between (1) ER and mitochondria; (2) ER and LD; (3) mitochondria and LD; and (4 and 5) ER, mitochondria, and LD in a three-way association. ER, endoplasmic reticulum; Mito, mitochondria; LD, lipid droplet; PM, plasma membrane.

mitochondria, was revealed by multiple studies and in various cell types [28,29]. However, the functional significance of their association has long remained unknown. Mitochondria–LD contacts are very abundant in tissues with high energy requests, such as skeletal muscle, heart, brown adipose tissue, and liver. More recently, they have been shown to be critical sites for the exchange of metabolites between mitochondria and LD and for the regulation of LD consumption and expansion.

Multiple evidence revealed a role of mitochondria–LD contact sites in the transfer of FA from the LD to the mitochondria, as a source for ATP production via their oxidation (reviewed in ref. [30]). This is mostly occurring in conditions where the nutrients are maintained scarce (and cell metabolism switches from glycolysis to FA oxidation) and mitochondria–LD associations are increased [23,31]. Several proteins involved in the formation of mitochondria–LD contact sites (SNAP23-VAMP4, VPS13D, and PLIN1-MFN2) have been shown to contribute to the transport of FA from the LD to the mitochondria [30,32–34].

Contact sites between mitochondria and LD can also function in LD expansion under conditions that promote lipid storage. Benador *et al.* [35] have shown that a subpopulation of mitochondria found highly anchored to the LD, also known as peridroplet mitochondria (PDM), has distinct proteomic and metabolic capabilities that support TAG synthesis and LD expansion in brown adipose tissue. Similarly, a recent work showed that PDM isolated from mouse hepatocytes facilitate fatty acid esterification. Interestingly, the same work also revealed that PDM associated with ER membranes exhibit a specific metabolic profile that favors lipid storage [36]. Indeed, many enzymes of the glycerolipid/Kennedy pathway of lipid synthesis, which is responsible for the synthesis of TAGs and phospholipids, localize to both ER and mitochondria [37], indicating that an intimate association between ER, mitochondria, and LD may exist to favor lipid biosynthesis. For example, DGAT2, one of the acyltransferases involved in the last steps of TAG production, was shown to localize at both the ER–mitochondria and mitochondria–LD interfaces [38]. Thus, a spatial proximity between the enzymes and metabolic intermediates involved in the biosynthesis of TAGs across these organelles may facilitate the exchange of substrates and products to promote its efficient production.

In 2019, a study by Freye *et al.*, combining proteomic, lipidomic, and imaging approaches, revealed that the mitochondrial membrane protein mitoguardin 2 (MIGA2) promotes lipogenesis in white adipocytes and provided the first evidence of the molecular mechanisms that link mitochondria, LD, and ER in differentiating white adipocytes (Fig. 2).

## MIGA2 links mitochondria, ER, and LDs at membrane contact sites to promote *de novo* lipogenesis

MIGA2 protein, originally identified by Tong's group [39], localizes to ER–mitochondria or mitochondria–LD contact sites, thanks to its multiple targeting domains/motifs: an N-terminal transmembrane domain mediates its anchoring to the outer mitochondrial

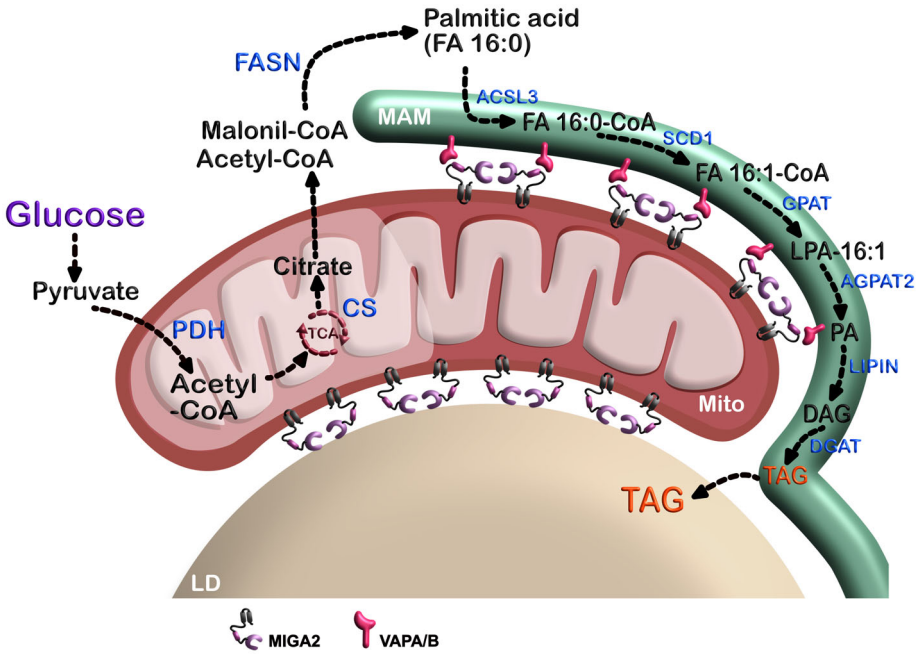


**Fig. 2.** Three-way ER–mitochondria–LD association in *de novo* lipogenesis. MIGA2 mediates the association between ER–mitochondria–LD for *de novo* TAG synthesis [40]. The mitochondrial outer membrane protein MIGA2 bridges mitochondria to the ER via interaction with VAPA/B and mitochondria to the LD via direct interaction with LD. MIGA2 ensures the spatial proximity of the components and intermediates localized/synthesized in different organelles (mitochondria or ER), involved in *de novo* lipogenesis to support triacylglyceride (TAG) synthesis. TAGs are synthesized from glucose through a series of biochemical reactions that involve the conversion of glucose into pyruvate via glycolysis. In the mitochondria, pyruvate is decarboxylated by the pyruvate dehydrogenase (PDH), leading to the formation of acetyl-CoA. This acetyl-CoA combines with oxaloacetate to form citrate in a reaction catalyzed by citrate synthase (CS). The citrate is transported out of the mitochondria into the cytoplasm, where it is cleaved into oxaloacetate and acetyl-CoA. Acetyl-CoA is then carboxylated into malonyl-CoA, the precursor of palmitic acid (FA16:0). FA16:0 is generated by the fatty acid synthase (FASN) and then transported into the ER. FA16:0 undergoes various modifications, including activation and desaturation, respectively, mediated by acyl-CoA synthetase-3 (ACSL3) and stearyl-CoA desaturase (SCD1), to produce FA16:1-CoA. Fatty acyl group from fatty acid 16:1-CoA is esterified to a glycerol by the glycerol-3-phosphate-acyltransferase-3 (GPAT3), forming lysophosphatidic acid (LPA). Thereafter, LPA is converted into phosphatidic acid (PA) via the action of 1-acyl-sn-glycerol-3-phosphate acyltransferase-2 (AGPAT2), which mediates the incorporation of another fatty acyl-CoA such as fatty acid 16:1-CoA into LPA. Finally, TAG is generated from PA via the sequential actions of lipin and diacylglycerol-O-acyltransferases (DGAT1). Lipin dephosphorylates PA to produce diacylglycerol (DAG), and DGAT1 mediates the esterification of a third fatty acyl group to DAG. ER, endoplasmic reticulum; MAM, mitochondria-associated ER membrane; Mito, mitochondria; LD, lipid droplet; TCA, tricarboxylic acid cycle.

membrane; a middle region containing a coiled-coil domain and two phenylalanines in an acidic tract (FFAT) motif binds the ER-anchored VAPA/B proteins to promote the formation of ER–mitochondria CS [40]; and a C-terminal domain is involved in lipid transfer and LD binding at mitochondria–LD contacts [41,42]. MIGA2 is conserved in higher eukaryotes, from *C elegans* (Miga) to humans (MIGA1 and MIGA2 that share ~ 36% sequence identity), as well as its tethering function, since its overexpression increases ER–mitochondria or mitochondria–LD CS in multiple cell types and organisms [40,43].

The binding of MIGA2 to the ER membranes is mediated by its FFAT motif. MIGA1 and MIGA2, proposed to form homo- and hetero-typic complexes on the outer mitochondria membrane, have identical FFAT motifs, although MIGA1 shows low affinity to VAP proteins [43]. The FFAT motif of MIGA2 belongs to a recently reported new subclass of non-conventional FFAT motifs called phospho-FFAT motifs, in which a conserved acidic residue is replaced by a serine/threonine [44]. Interestingly, phosphorylation of MIGA2 FFAT is required for its interaction with VAP proteins and for the formation of Miga-mediated ER–mitochondria CS, uncovering the existence of a novel mechanism to dynamically regulate ER–mitochondria membrane contacts [43,44]. Moreover, studies on *Drosophila* revealed that starvation, previously shown to enhance ER–mitochondria CS, increases *Drosophila* Miga2 phosphorylation and its

interaction with the VAP-equivalent Vps33 protein. They also showed that Miga-Vap33 interaction can promote further phosphorylation of serine/threonine clusters upstream to FFAT, suggesting that this hyperphosphorylation could be an additional mechanism to modulate Miga2 activity. Besides, Xu *et al.* [43] identified casein kinase I (CKI) and calmodulin/$Ca^{2+}$-dependent protein kinase II (CaMKII) as two of the kinases involved in the phosphorylation of Miga2 in the fly retina. However, other kinases are likely to be involved and possibly also phosphatases that could play a key role in regulating Miga2 activity and Miga2-dependent ER–mitochondria CS. Nevertheless, if this is the case, their identity is still unknown. It is possible that Miga2 targeting and function at ER–mitochondria contacts could be regulated by calcium and that local rises in calcium might function as a trigger to control CaMKII activity and further modify the extent of ER–mitochondria contacts through the phosphorylation of Miga2.

MIGA2 binds to the LD surface via its C-terminus segment, whose structure has been recently solved, revealing its ability to dimerize and form a conserved concave surface patch [41,42]. The C-terminal surface patch contains multiple helices, two of them originally proposed to be amphipathic and to mediate LD binding by Freye *et al.* [40], and positively charged residues that when mutated prevent Miga2 binding to LD. Nevertheless, whether and how MIGA2 directly binds LD surface remain unclear. Given the higher curvature of the C-terminal conserved surface in comparison with the relatively flat surface of a LD, it is possible that MIGA2 binds LD through interaction with LD-associated proteins rather than directly interacting with their lipid surface [41]. Thus, additional proteins on LD or mitochondria could be required to establish MIGA2-mediated LD–mitochondria contact sites. Also, it is still unknown whether the conserved surface patch in MIGA2 mediates a specific binding to LD or it could also mediate an association with other cellular membranes, even transiently or in specific cell types. Likewise, whether the binding of LD could induce conformational changes in MIGA2 and influence the exposure of its FFAT for interaction with VAP, or the binding to VAP could either enhance or restrict exposure of the LD-binding sites, is still unknown. Therefore, how MIGA2 interacts with ER and LD membranes, individually or in a three-way mitochondria–ER–LD junction, and how these interactions are regulated by molecular and metabolic cues deserve further investigations.

MIGA2 plays a role in mitochondrial well-being, as its loss of function results in fragmented mitochondria with reduced activities [39] and its overexpression in clustered and hyperfused mitochondria in both fly and mammalian cells [43]. Loss of function of *Drosophila* Miga2 causes neurodegeneration in the eyes, suggesting a role of this protein in the brain, at least in flies [43]. MIGA2 KO mice displayed reduced body weight, suggesting a role for MIGA2 also in regulating fat metabolism [45]. In particular, in white adipocytes, MIGA2 promotes the synthesis of TAG from non-lipid precursors and *de novo* lipogenesis, thus activating their differentiation. This function of MIGA2 is in accordance with its augmented mRNA and protein levels during adipocyte differentiation [40]. Importantly, Freye *et al.*'s findings highlight a specific function of peridroplet mitochondria in fostering LD formation, rather than LD consumption.

## Mitochondria–ER-Associated membranes (MAMs): a key platform for LD biogenesis

Lipid droplet biogenesis has been long considered as an isolated event occurring at the ER membrane. Yet, recent evidence indicates that several metabolic cues, involving additional organelles, can induce LD biogenesis. For instance, LD can form at the vacuole–nucleus junctions in yeast [46] or peroxisome contact sites [47], suggesting that CS could be critical hotspots for LD biogenesis, for example by pooling key enzymes and lipid intermediates. Following the work by Freire *et al.*, a more recent study by Guyard *et al.* [48] uncovered a novel function of MAM in promoting LD biogenesis and growth at sites where the ER interacts with both the mitochondria and the LDs, which they named MAM-LD CS, in HeLa and hepatocytic Huh7 cell lines. Notably, this study revealed that in these cells most of the LDs originate from MAM subdomains specialized in lipid trafficking and characterized by the presence of two lipid transfer proteins (LTPs), ORP5 and ORP8 (Fig. 4).

### ORP5 and ORP8 targeting membrane contact sites

ORP5 and ORP8 belong to the oxysterol-binding protein (OSBP)-related proteins (ORPs) family of lipid transfer proteins, whose common structural feature is the presence of an OSBP-related lipid-binding/transfer (ORD) domain [49]. ORP5 and ORP8 form a subgroup of ORPs as they possess similar ORD domain sequences and are the only ORPs to be directly anchored to the ER, via their C-terminal transmembrane (TM) domain [50,51]. Additionally, like the majority of the ORPs,

ORP5 and ORP8 possess a pleckstrin homology (PH), which is characterized by the presence of a 20-amino acid extended loop [52]. PH domain binds to a non-ER organelle membrane, and in the case of ORP5 and ORP8, it binds the plasma membrane phosphoinositides phosphatidylinositol-4-phosphate (PI4P) and phosphatidylinositol 4,5-bisphosphate (PIP2), whose abundance regulates the recruitment of ORP5 and ORP8 to the plasma membrane [52,53].

ORP5 also possesses a coiled-coil domain, at the N-terminus of the PH domain, which has been shown to be important for binding to the plasma membrane [52] and possibly for ORP5 and ORP8 interaction [48]. However, the precise function of the coiled coil needs to be further investigated. Indeed, these two ORP proteins were initially shown to localize at ER–plasma membrane contact sites by Chung *et al.* and to transfer PS from the cortical ER to the plasma membrane, in counter-exchange with plasma membrane PI4P [53] or PIP2 [52].

Galmes *et al.* [54] then revealed that ORP5 and ORP8 localize also at ER–mitochondria contact site through their interaction with the OMM tether protein PTPIP51. Consistently, expression of PTPIP51 promoted a massive accumulation of ORP5 and ORP8 at the MAM [54], indicating that PTPIP51 may help the recruitment and/or stabilization of ORP5 and ORP8 at ER–mitochondria contact sites. Furthermore, it is important to consider that ORP5 and ORP8 interact and act as a complex [53,54]. A recent study, combining proximity ligation assay (PLA) with 3D segmentation of mitochondria, has revealed that the majority of the endogenous ORP5-ORP8 interactions occur at MAM and < 5% at the cortical ER [55]. Interestingly, co-overexpressed and endogenous ORP5 and ORP8 have similar distribution among the contact sites and in particular were enriched at ER–mitochondria CS [55], emphasizing the idea that these proteins act as a complex and suggesting that other proteins that interact with each other (i.e. E-Syts) at membrane contact sites might behave the same. However, how ORP5 and ORP8 distribution within the contact sites is regulated remains to be elucidated.

Because PTPIP51 mediates the recruitment of ORP5 and ORP8 to MAM [54], it is possible that their localization to ER–mitochondria CS is mainly mediated by the binding to their mitochondrial partners, rather than by the binding to specific lipids, as it occurs at ER–plasma membrane CS. Interestingly, the MAM subdomains where ORP5 and ORP8 localize are closely associated with intra-mitochondrial membrane CS at the CJ level [55]. Indeed, ORP5 and ORP8 were shown to interact with Mic60 and SAM50, which are central components of mitochondrial intermembrane space bridging (MIB) and CJ organizing cristae (MICOS) complex that bridges the IMM and OMM at the level of the CJ. Interestingly, the absence of SAM50 and Mic60 reduced the interaction of ORP5 and ORP8 at MAM [55], suggesting that these ORP5/8 binding partners could also regulate their function at ER–mitochondria CS.

In addition to ER–plasma membrane and ER–mitochondria contacts, ORP5 was found to localize at ER-LD contact sites [56]. Following this study, Guyard *et al.* [48] further unveiled the localization of ORP5 and ORP8 at ultrastructural level showing that ORP5 and ORP8 localize at MAM subdomains that are in contact with both mitochondria and LD forming a three-way MAM-LD CS association. Several layers of experimental evidence show that ORP5 and ORP8 binding to LD is mainly mediated by their ORD domain [48,56]. Also, the ORD domain of ORP5 binds the LD more efficiently than the ORD of ORP8, explaining why the expression of ORP5 induces an expansion of MAM around the LD while ORP8 does not have this "ER wrapping LD" effect. However, when co-expressed with ORP5, ORP8 accumulated at the MAM-LD contacts together with ORP5 [48]. Detailed analysis of the putative structure of the ORD domain of ORP5 together with experimental evidence led to the identification of an amphipathic helix (AH, aa 422–439) as a potential LD-binding site [56]. However, ORP5 AH was able to target the LD only when fused to a transmembrane domain, and the substitution of ORP8 AH by ORP5 AH did not enhance the targeting of ORP8 to LD [56], suggesting that targeting of ORP5 and ORP8 to the LD is far more complex than anticipated and it requires anchoring to the ER. Additionally, it could require the cooperation of different domains within ORP5 and ORP8 proteins, to confer them a specific conformation and allow their binding to specific lipids or proteins on the LD. This seems not to be a unique case, as interestingly, also MIGA2 binding to LD requires both its C-terminal amphipathic segments and the attachment to a membrane anchor [40]. Moreover, the AH sequence of ORP5 is located within the ORD, as predicted from AlphaFold, and supported by the homology with the recently solved structure of ORP8 ORD [57]. This positioning renders the AH less accessible from the exterior, posing challenges for its binding to the surface of LD. Therefore, more studies are required to fully understand how ORP5 and ORP8 bind LD.

## LD biogenesis at MAM

ORP5 and ORP8, despite having distinct binding affinities for LD, have been found to equally promote LD formation at MAM. The functions of ORP5 and

ORP8 in LD biogenesis started to be unveiled with the observation that ORP5 was recruited to MAM subdomains where LD emerged upon fatty acid or oleic acid treatment in HeLa and Hu7h hepatocyte cells [48]. Following this observation, Guyard *et al*. reported that depletion of ORP5 and ORP8 resulted in a significant reduction of newly formed LD.

Ultrastructural analysis of oleic acid-induced LD in HeLa cells uncovered the morphological features of the ER subdomains where LDs originate. These subdomains are characterized by a peculiar electron-dense structure that invaginates within the LD, and it can remain connected to tubular ER or very often also to the mitochondria (Fig. 3). Similar atypical dot or tubular electron-dense structures within LD in contact with mitochondria were also recently noticed, for the first time, in primary cultured mouse hepatocytes [58]. These structures were more easily found in hepatocytes treated with oleic acid or ethanol, although their origin and composition were still unknown. Guyard *et al*. [48] revealed that the electron-dense structures within the LD were composed of ER membrane invaginations by using EM combined with HRP-KDEL staining of the lumen of the ER and proposed that LDs showing these structures in close proximity to mitochondria were those originated from MAM. Notably, in the absence of ORP5 the occurrence of these structures simultaneously with LD and mitochondria was decreased, identifying MAM as local platforms for LD biogenesis and a key role of ORP5/8 in this process [48]. Nevertheless, the specific composition and function of MAM subdomains from which LDs originate remain to be further elucidated. More in-depth morphological and biochemical studies are needed to address what composes these electron densities: Are they just membranous or are they enriched in specific proteins or lipids? Since they are still detectable after long times (16 h) of OA loading, in addition to be involved in LD formation at earlier steps, could they be involved in LD growth and/or turnover at later steps? Are they also occurring at LD CS with other cellular membranes than those of ER and mitochondria? Deciphering the molecular composition and function of these new structures will bring novel insights into our understanding of LD biology and crosstalk with other organelles.

The involvement of MAM in LD biogenesis has been also supported by the parallel finding that LD biogenesis dramatically decreases by perturbing ER–mitochondria contact sites knocking down PTPIP51, and this decrease was even stronger than upon ORP5 or ORP8 depletion. Moreover, LD biogenesis defects induced by PTPIP51 KD cannot be rescued by re-expression of ORP5, indicating a major role of MAM in this process [48]. In the same line, a recent study revealed that hepatic peridroplet mitochondria and MAM are enriched in proteins that support lipid anabolic pathways, such as oleate biosynthesis and LXR/RXR signaling, which facilitates LD formation and/or growth [36]. Therefore, it is reasonable to propose that such proteins may also be important for ORP5- and ORP8-mediated LD biogenesis.

Du *et al*. [56] reported that cells lacking ORP5 have bigger LD than control cells after oleic acid treatment, raising the possibility that in addition to orchestrating LD biogenesis at MAM, ORP5 may also control LD growth at MAM-LD contacts. In support of this hypothesis, the treatment of hepatocytes with oleic acid resulted in an enrichment of ORP5 in both nascent and pre-existent LDs [48]. Oleic acid results in TAG-rich LD; however, LD core is composed of a complex mixture of both TAG and SE containing a variety of FA structure. Therefore, it would be interesting in the future to investigate whether the roles of ORP5/ORP8 at MAM in LD biogenesis are specific to oleate, or if they are reproduced, and eventually enhanced or decreased, in the presence of other types of lipid source (e.g. palmitate and cholesterol).

Several studies have shown that the sites of LD nucleation are physically marked by seipin, an integral ER protein whose mutations result in lipodystrophies (i.e. Berardinelli–Seip congenital lipodystrophy 2) and neurological disorders [59,60]. Seipin localizes to the ER-LD neck in both yeast and mammals, and the absence of seipin from these sites results in alteration of LD biogenesis, morphology, and growth [61–63]. Seipin has been shown to form an undecamer and bind negatively charged phospholipids, especially PA [64]. Thus, it could form a scaffold that helps to locally maintain phospholipid homeostasis [65]. In line with this, it has been shown that seipin regulates the levels of phosphatidylinositol 3-phosphate (PI(3)P) in the ER to promote the lipidation of the nascent LD via DFCP1 [66]. Interestingly, studies in *Drosophila* revealed that the levels of PI(3)P are also modulated by Miga, by regulating phosphatidylinositol 3-kinase (PI3K) activity at MAM [67]. Together, these studies suggest that the lipidation of nascent lipid droplets may occur at MAM. As a matter of fact, Combot *et al*. [68] have recently shown that a pool of seipin resides at MAM in human A431 and 3T3-L1 mouse cell lines. Parallel work by Guyard *et al*. [48] identified seipin at the three-way MAM-LD contact sites and showed that such localization is dependent on ORP5, with which seipin physically interacts (Fig. 4). Thus, it is possible that ORP5 could mediate LD biogenesis by

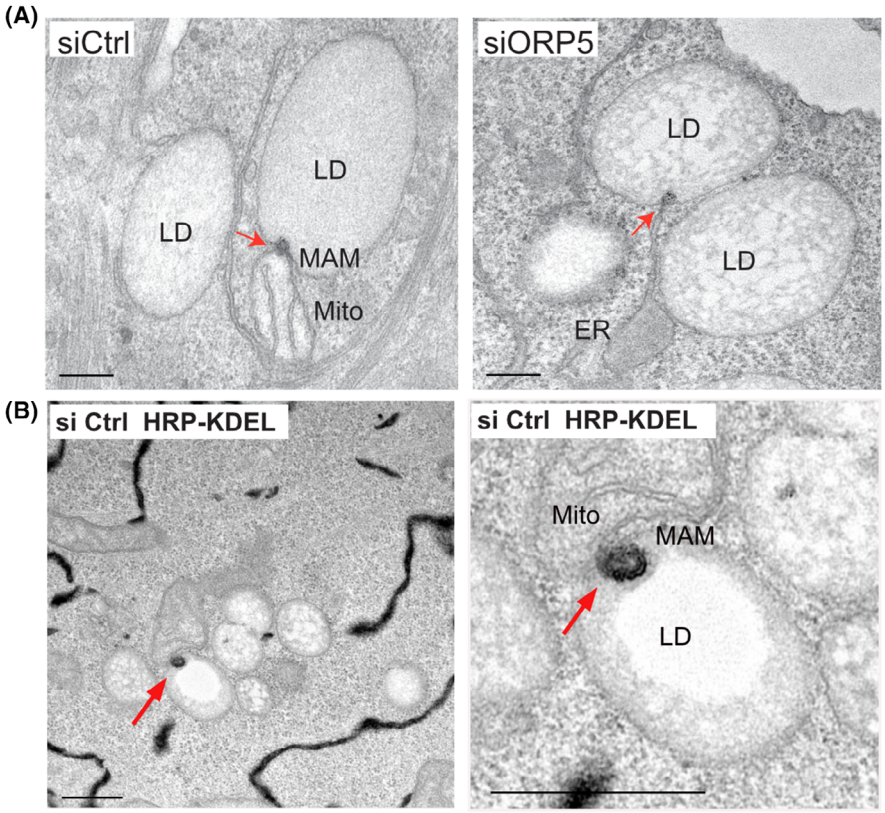


**Fig. 3.** Three-way mitochondria–ER–LD contact sites in HeLa cells. Cells treated with oleic acid for 16 h were analyzed by (A) conventional transmission electron microscopy or by (B) electron microscopy combined with HRP-KDEL staining of the ER (dark precipitate in the ER lumen). (A) Presence of an electron-dense dot structure invaginating into the LD is observed primarily at contact sites with mitochondria in control cells or in direct connection to ER tubules in ORP5-depleted cells (siORP5). (B) In control cells, these structures, when in contact with mitochondria, correspond to MAM, as visualized by the dark signal of the ER. Mito, mitochondria; ER, endoplasmic reticulum; LD, lipid droplet; MAM, mitochondria-associated ER membrane. Panels (A) and (B) are reproduced, with modifications and with permission from the authors/journal, from figure 3C and D in ©2023 GUYARD *et al.*, originally published in the *Journal of Cell Biology* (https://doi.org/10.1083/jcb.202112107), under Creative Commons License (Attribution-Noncommercial-Share Alike 4.0 International).

recruitment of seipin to MAM. Nevertheless, several questions remain to be answered regarding the role of ORP5 and ORP8 in LD biogenesis, in particular, whether the regulation of seipin by these ORPs at MAM is only mediated by their physical interaction or by binding to specific lipids at MAM whose levels can be modulated by ORP5 lipid transfer function. In support of the latter hypothesis, the ORP5 and ORP8 are well known for their role in lipid transfer *in situ*, and they likely possess the ability to edit lipid composition of cellular membranes, impacting the physical properties of the membranes in which they localize [52,53,55]. Notably, the presence of the ORD domain of ORP5 is necessary to rescue LD biogenesis in ORP5-depleted cells [48]. Thus, ORP5 may regulate LD biogenesis by modulating the MAM lipid composition.

Interestingly, Datta *et al.* [69] have shown that the absence of Snx14, an ER protein that stabilizes ER-LD contacts after LD induction with oleic acid, results in an LD phenotype similar to that promoted by the absence of seipin, which consists of a reduced abundance of LD per cell and altered LD morphology with a significant increase in the proportion of very small LD versus large LD. Notably, the effects of Snx14 on LD were seipin-independent [69], suggesting that these proteins may play similar roles in the LD maturation and growth. However, whether Snx14 is involved in LD biogenesis still needs to be confirmed, and whether it localizes at MAM-LD is not known.

Localization of seipin at MAM could also be important for other processes in addition to LD formation. Indeed, Combot *et al.* [68] showed that seipin localizes at MAM to regulate the influx of calcium from the ER to the mitochondria and that such localization is metabolically regulated, being decreased by oleate treatment. Further studies are needed to understand

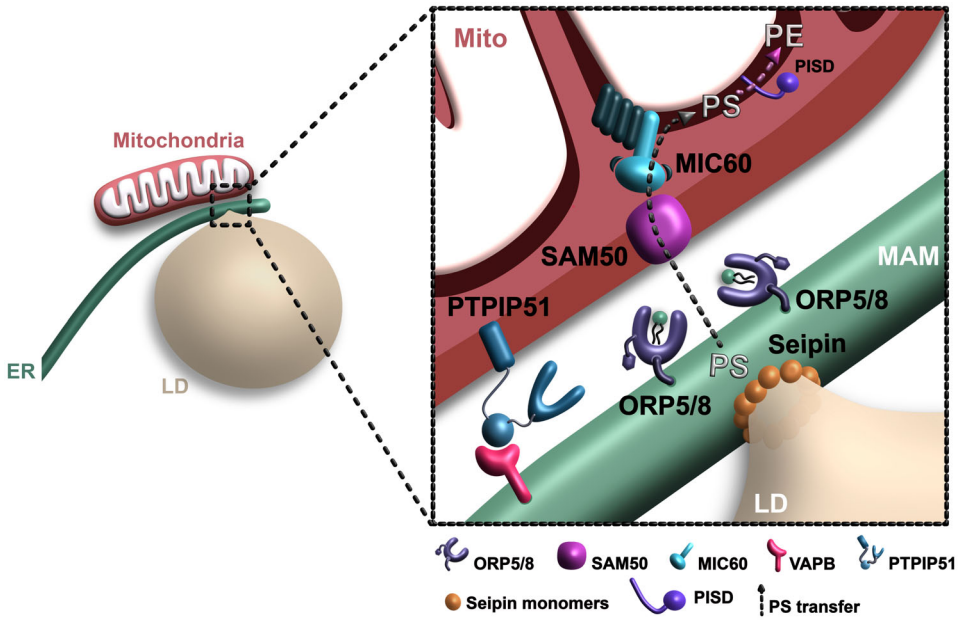


**Fig. 4.** Three-way MAM-LD association and LD biogenesis. The lipid transfer proteins ORP5 and ORP8 localize to MAM where they regulate the transport of PS from ER to mitochondria and orchestrate LD biogenesis. ORP5 and ORP8 cooperate with the mitochondrial proteins SAM50 and MIC60 to mediate the import of PS into mitochondria to be converted into PE in the IMM. Concurrently, these two lipid transfer proteins regulate the recruitment of seipin to MAM enriched in PA to mediate LD biogenesis. Importantly, PTPIP51-mediated ER–mitochondria contact site integrity is crucial for ORP5/8 function in regulating LD biogenesis. ER, endoplasmic reticulum; IMM, inner mitochondrial membrane; LD, lipid droplet; MAM, mitochondria-associated-ER membrane; Mito, mitochondria; PA, phosphatidic acid; PE, phosphatidylethanolamine; PISD, PS decarboxylase; PS, phosphatidylserine.

seipin functions at MAM and its crosstalk with other MAM components including ORP5/ORP8. It would be interesting to address whether the calcium and lipid transport machineries lie within the same MAM microdomains to facilitate and coordinate calcium and lipid fluxes across ER and mitochondria and how they impact LD formation.

## LD turnover at MAMs

In addition to their newly identified function in LD biogenesis and growth, MAM could also be hotspots for the regulation of LD turnover. Although there is still a lack of studies directly exploring such a role of MAM, emerging evidence suggests that LTPs known to localize at ER–mitochondria contact sites are involved in LD turnover. Interestingly, in a recent study Pu *et al.* described an additional function of ORP8 in lipophagy at ER-LD contacts where it interacts with LC3 to regulate LD abundance. They reported that the knockout of ORP8 in HeLa and HEK293 cells, starved or treated with OA, results in increased levels of TAG [70]. Similarly, ORP8 knockdown in starved HeLa cells promoted LD accumulation, suggesting that ORP8 is required for LD catabolism [70]. These findings are in conflict with the studies showing that ORP8 knockdown decreases LD formation after culture in lipoprotein-deficient serum and OA treatment [48]. However, these disparities could reflect the different nutritional states of the cells and culture conditions. It is possible that ORP8 mediates both lipogenesis and lipophagy at MAM-LD in dependence of the metabolic needs of the cell, to regulate the balance between LD synthesis and turnover. Nevertheless, it is also possible that ORP8 plays additional specific functions in lipophagy at other contact sites, such as ER-LD contacts.

PDZD8 is a LTP found to localize at ER–mitochondria, ER–late endosome/lysosome, and mitochondria–ER–late endosome/lysosome CS [71–73]. A recent work reported an accumulation of cholesteryl esters and a defective degradation of LD in the brain of PDZD8-deficient mice, suggesting a role of PDZD8 in promoting clearance of cholesteryl esters from the brain by lipophagy [74]. Kurihara *et al.* [75] have shown that, during lipophagy, PDZD8 promotes cholesterol transfer to LDs, resulting in fusion of LDs with lysosomes. They also showed that the function of PDZD8 in lipophagy in mice and in cells is specific in the brain (for instance, PDZD8-KO mice have

abnormal CE accumulation in the brain but not in the liver), where it is highly expressed, suggesting that lipid transport proteins other than PDZD8 may predominate in other tissues such as liver. Whether mitochondria or MAM could participate in PDZD8-mediated lipophagy is unknown.

Other LTPs localized to ER–mitochondria contact sites could be involved in regulating LD biogenesis and/or turnover. Interestingly, VPS13A, a member of the VPS13 protein family, and a phospholipid transfer protein, was found at both ER–mitochondria CS and ER-LD contacts [76]. Whether these contact sites might correspond to a three-way MAM-LD association has not been investigated yet, but it is a possibility. Loss of VPS13A leads to a decreased LD abundance, during oleate treatment in U-2OS cells [77], and an increased number of LD in MRC-5 lung fibroblasts, through a yet-undetermined mechanism [78]. These findings suggest a role of VPS13A in LD formation and turnover that could be specific to cell types/tissues and dependent on the metabolic cellular needs. Further studies are needed to determine whether and how VPS13A could regulate LD formation and/or consumption at MAM. It would be also interesting to address whether VPS13A could cooperate with ORP5/ORP8 or MIGA2 in regulating lipid storage and homeostasis at the three-way MAM-LD CS.

Recently, the protein spartin, found to localize to both LD and endosomal compartment and to act as lipophagy receptor [79], has been shown to have the ability to transfer phospholipids via its senescence domain [80], and it links LD to autophagy machinery to mediate lipophagy [79]. Interestingly, Wan *et al.* revealed that the role of spartin in LD turnover is dependent on its senescence domain, and therefore, it may require spartin lipid transfer activity [80]. However, whether this process happens at ER–mitochondria–LD contacts remains to be elucidated.

## Non-vesicular lipid transfer at MAM-LD contact sites

Non-vesicular exchange of lipids between ER, mitochondria, and LD is essential to preserve their complex morphologies, dynamics, and functions and, in broader context, to maintain cellular lipid homeostasis. But which lipids are exchanged at the mitochondria–ER–LD CS interface and the underlying molecular and regulatory mechanisms are still unknown. Nevertheless, our knowledge of how lipids are exchanged between ER and mitochondria and ER and LDs (reviewed in ref. [24]), even if still limited, is rapidly increasing and may provide some clues on how lipid metabolic crosstalk is regulated at MAM-LD CS.

Growing evidence suggests that the physical properties of the ER membrane, such as membrane curvature and surface tension, which are determined by phospholipid composition, are critical for proper LD biogenesis [81,82]. Hence, locally editing the MAM phospholipid content, possibly by lipid transfer at MAM-LD CS, could be key to LD biogenesis and expansion.

### Emerging functions of MIGA2 in lipid transfer at mitochondria–LD–ER contacts

Recently, it was shown that MIGA2 has, in addition to its tethering function, a possible lipid transfer activity. Evidence of MIGA2 lipid transfer activity emerged from the work by Kim *et al.* where they reported that MIGA2 from zebrafish (zMIGA2) possesses a large hydrophobic pocket that can accommodate phospholipids and that it can transfer them between liposomes *in vitro*. In this study, zMIGA2 has been shown to transfer PS, but not PA, PC, PE, or cholesterol *in vitro* [42]. Following work by Hong *et al.* revealed the X-ray structure of the human MIGA2 (hMIGA2) and showed that it can transfer several phospholipids and can also bind fatty acids and TAGs. Contrary to the zebrafish ortholog, hMIGA2 was shown to robustly transfer PS, PA, PE, and PC between liposomes *in vitro* and PE and, to some extent, PC between artificial LDs and liposomes [41]. Given that the lipid-binding residues in zMIGA2 and hMIGA2 are highly conserved [42], it is possible that these discrepancies emerge from technical aspects. However, these differences may also be due to possible differences in MIGA2 structure/functions from different species. Despite the *in vitro* evidence that MIGA2 could be a LTP, its role in transferring phospholipids across the ER–mitochondria–LD membranes *in vivo* and its effect in modulating their lipid composition are still unknown (Fig. 5).

Nevertheless, functional studies addressing the effects of MIGA2 in mitochondria and LD in HeLa cells revealed that the absence of MIGA2 affects both mitochondrial morphology and LD formation. Moreover, the lipid transfer activity of MIGA2 is necessary to rescue these phenotypes, highlighting the importance of MIGA2-mediated lipid transfer to preserve mitochondrial and LD integrities [41]. It is possible that MIGA2 may localize at the three-way contact ER–mitochondria–LD to coordinate lipid exchanges between these organelles. For instance, MIGA2 could regulate the transport of phospholipids (i.e. PC/PE or

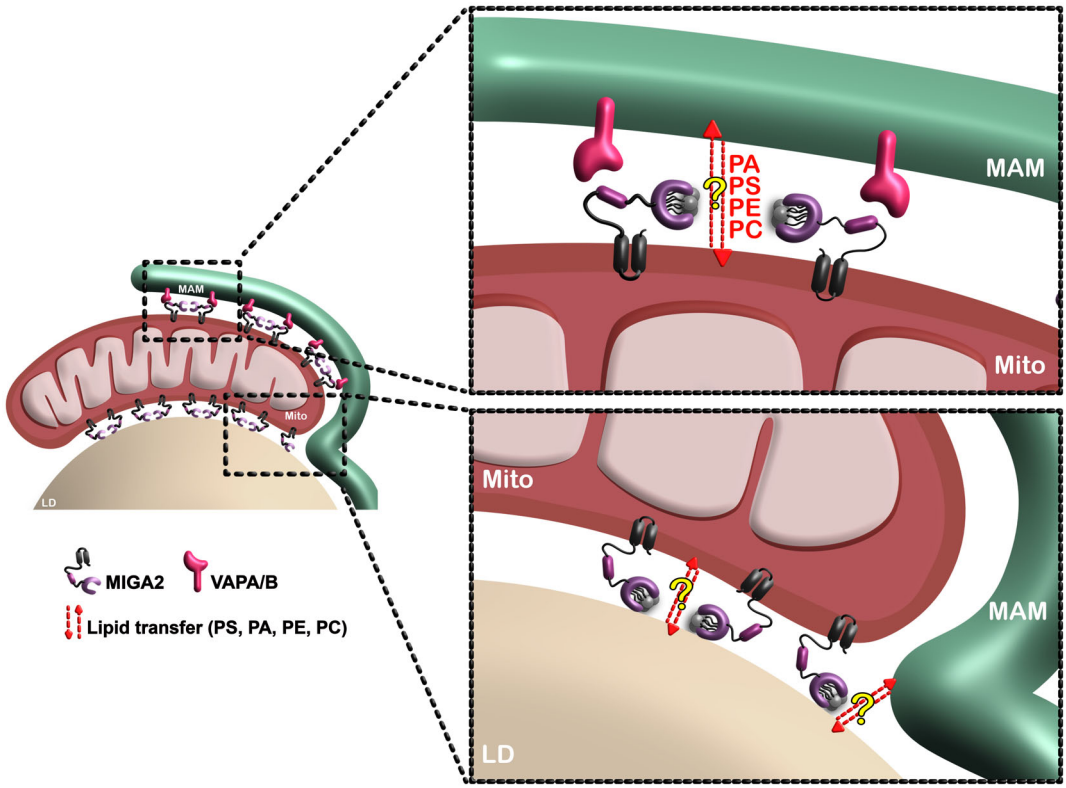


**Fig. 5.** Putative roles of MIGA2 in lipid transport at ER–mitochondria–LD contact sites. Besides its role as tether protein, MIGA2 also possesses the ability to transfer several phospholipids, including PS, PA, PE, and PC, *in vitro* between liposomes. However, whether MIGA2 mediates the transport of these phospholipids *in vivo* is not known. Since the role of MIGA2 in LD homeostasis is just starting to be explored, it would be interesting to understand (1) whether MIGA2 also mediates LD biogenesis at MAM, (2) whether the role of MIGA2 in LD growth is mediated by its tethering function, its lipid transfer function, or both, (3) whether MIGA2 mediates phospholipid transfer *in vivo* what is the direction of transfer from ER to mitochondria, mitochondria to ER, mitochondria to LD, etc., and (4) whether MIGA2 cooperates with other ER or LD proteins to mediate *de novo* lipogenesis and LD growth. ER, endoplasmic reticulum; LD, lipid droplet; MAM, mitochondria-associated ER membrane; Mito, mitochondria; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine.

PA) and fatty acids between LD and mitochondria or the transport of fatty acids to the ER for glycolipid synthesis (Fig. 5).

## Non-vesicular lipid transfer mediated by ORP5/ORP8

ORP5 and ORP8 lipid transfer activity at CS was first described at ER–plasma membrane contacts. Chung *et al.* [53] found that ORP5 and ORP8 function as PS/PI4P exchangers at ER–plasma membrane CS, using the gradient of PI(4)P between the plasma membrane and the ER, generated by the double action of PI4P kinases on the plasma membrane and a phosphatase (Sac1) in the ER, to transport PS from the ER, site of its synthesis, to the inner leaflet of the plasma membrane, where it is maintained at high levels as it contributes to the negative surface charge. The role of ORP5 and ORP8 in lipid transport at ER–plasma membrane contacts was further corroborated by Ghai *et al.* However, challenging the finding from the previous study, they showed that ORP5 and ORP8 bind more strongly plasma membrane $PI(4,5)P_2$, rather than PI(4)P, via their PH domain, and counter-exchange it with PS at ER–plasma membrane contacts [52].

More recently, ORP5 has been shown to additionally localize at ER-LD contacts to mediate LD growth [56]. In order to explain the effect of ORP5 in modulating LD size, the authors proposed that ORP5 could function at ER-LD contact sites to regulate the levels of PI(4)P on LD, by transporting PS from the ER to the LDs and PI(4)P back to the ER, mimicking its well-known PS/PI(4)P counter-exchange function. This hypothesis was supported by an enrichment of PS or PI(4)P around the ER or the LD, respectively, by using specific fluorescent lipid probes and confocal

imaging of individual cells. However, further studies are needed to fully validate this model in cells (and, for instance, at MAM-LD CS) by using biochemical/-biophysical methods and also to address whether ORP5 has the ability to transport other lipids in addition to PS and PI(4)P. Likewise, although recent studies have shown an involvement of the ORD domain in ORP5 targeting LDs [48,56], the identity of the lipids bound by ORD on LDs still remains unknown.

Lately, ORP5 and ORP8 were shown to mediate the transport of PS lipids from the ER to the mitochondria, to fuel mitochondrial PE synthesis, at the ER–mitochondria interface. Hence, ORP5 or ORP8 is necessary to maintain proper morphology of mitochondrial cristae and respiratory activity [54,55]. Notably, ORP5 and ORP8, among other known LTPs localized at ER–mitochondria CS, have the unique feature of not acting as tethers, as neither their overexpression nor their knockdown changes the extension and/or the number of ER–mitochondria contacts [55]. Thus, the alterations of mitochondrial lipid composition (decrease in PE), morphology, and function induced by ORP5 and ORP8 depletion appear to be due to the loss of their lipid transfer activity rather than secondary effects on ER–mitochondria contacts. Interestingly, mitochondrial membranes are poor in phosphatidylinositides as well as in PS (that is synthesized in the ER and rapidly converted in PE on the mitochondrial membranes); therefore, the mechanisms by which ORP5/ORP8 mediate PS (and possibly other lipids) transfer at ER–mitochondria CS are likely different from those occurring at ER–plasma membrane contacts.

Differences in local lipid gradients at CS may play a key role in influencing the specificity and the directionality of lipid transfer mediated by LTPs. The study of Monteiro Cardoso *et al*. indicates that PS can be unidirectionally transferred by ORP5 and ORP8 at ER–mitochondria CS. This is in accordance with a model where the driving force for this transfer results from the concomitant activity of the PSS1 synthase that enriches PS on the donor membrane (MAM) and from the activity of the PS decarboxylase that rapidly converts PS into PE in the acceptor membranes (mitochondria). This suggests that the local synthesis of a specific lipid and its transfer across apposed membranes must be spatially and functionally coupled at ER–mitochondria contacts. An alternative possibility, regarding ORP5/ORP8 function at MAM, is that the local gradient of PS could be used to counter-exchange it with some other mitochondrial lipids that need to be transported back to the ER. Although the ORD domains of ORP5 and ORP8 have been shown to specifically transfer PS, but not PC or PE, between liposomes *in vitro* [55], their ability to transfer other phospholipids, such as PA, PG, or PI, has not been investigated yet (Fig. 6). Also, *in vitro* assays do not reproduce the native protein and lipid environment of cellular membranes, and thus, some transport mechanisms occurring in specific cell locations might not be detected by using such approaches. Thus, further studies are needed to understand whether ORP5 and ORP8 could mediate a counter-transport of PS with other phospholipids (i.e. PE and PA) or sterols (i.e. cholesterol) at ER–mitochondria CS. Since ablation of ORP5 and ORP8 affects LD biogenesis [48], it is reasonable to hypothesize that the transfer of these lipids from MAM to mitochondria and/or from MAM to LD may be involved in the formation and growth of LDs (Fig. 6). Indeed, PA and cholesterol are the precursors of TAGs and cholesteryl esters, the main neutral lipids of the LD core.

Like other ORPs, ORP5 and ORP8 were initially thought to act as sterols, in particular oxysterols, sensors [51]. Oxysterols are a family of bioactive lipids that originate from cholesterol hydroxylation by specific enzymes that not only localize in the ER but also in other organelles, including mitochondria. For instance, 27-hydroxycholesterol and 25-hydroxycholesterol are generated from cholesterol hydroxylation by the mitochondrial enzyme 27-hydroxylase (CYP27A1) [83]. These oxysterols regulate several metabolic pathways, including cholesterol catabolism (to be excreted as bile acids or to be stored in LD), by interacting with membrane receptors, such as LXR [84]. Suchaneck *et al*. [85] have shown using photo-cross-linking assays that ORP5 and ORP8 bind both 25-hydroxycholesterol and cholesterol. Following this study, Du *et al*. [86] investigated whether ORP5 could transfer sterols and showed that the purified ORD domain of ORP5 could transfer dehydroergosterol (cholesterol analog) between liposomes *in vitro* and it has been implicated in the transfer of cholesterol between late endosomes/lysosomes to the ER. Furthermore, several layers of evidence reviewed in ref. [51] indicate that ORP5 and ORP8 can modulate cholesterol metabolism, but whether ORP5 and ORP8 transfer cholesterol and oxysterols at ER–mitochondria or at MAM-LD contacts is still unclear (Fig. 6).

## MAM and mitochondrial protein interplay for lipid transfer at mitochondria–ER–LD contacts

The mitochondrial protein PTPIP51, initially identified as a molecular tether linking ER and mitochondria by interacting with the ER protein VAPB [87,88], contains an atypical TPR domain that transfers PA

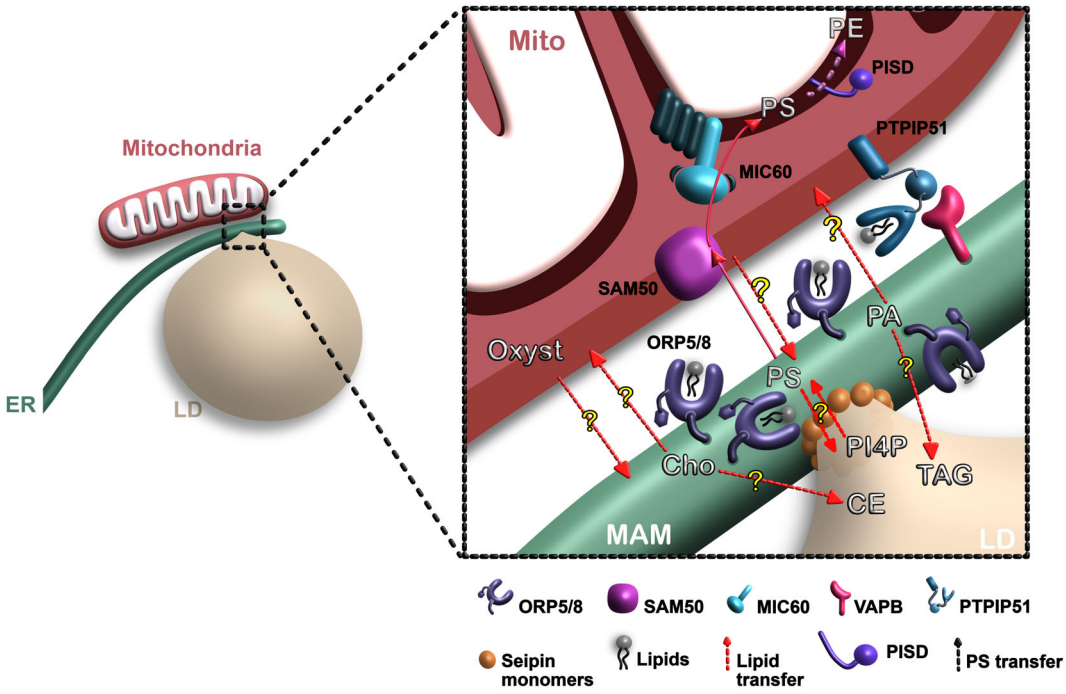


**Fig. 6.** Putative roles of ORP5 and ORP8 in lipid transfer at MAM-LD contact sites. ORP5 and ORP8 are oxysterol sensors, and the ORD domain of ORP5 transfers cholesterol between liposomes *in vitro*. Maintenance of cholesterol homeostasis requires its transport from the ER to the mitochondria, where it is converted into other metabolites, such as oxysterols (e.g. 25-hydroxycholesterol and 27-hydroxycholesterol). Oxysterols are then transported to ER to be further metabolized or to be excreted from the cells. Additionally, transport of cholesterol to MAM could be important for its conversion to sterol esters, since ACAT1, which converts cholesterol into sterol esters, is enriched at MAM. However, whether ORP5 or ORP8 mediates non-vesicular transport of cholesterol, and possibly of oxysterols, *in vivo*, regulating their levels at MAM, and whether this mechanism is involved in LD biogenesis remain to be explored. Du *et al.*'s [56] study proposed that ORP5 mediates counter-exchange of PS with PI4P at ER-LD contact sites to regulate LD growth. However, whether ORP5 and ORP8 could mediate the transfer of other lipids (i.e. PA or cholesterol) to the nascent LD to regulate LD biogenesis at MAM-LD CS is still unclear. Finally, the OMM protein PTPIP51, also required for proper LD biogenesis at MAM, has been shown to transfer PA from the ER to the mitochondria [90]. It is possible that PTPIP51 regulates PA levels at MAM and may cooperate with ORP5 and ORP8, to regulate LD biogenesis. ER, endoplasmic reticulum; LD, lipid droplet; MAM, mitochondria-associated ER membrane; Mito, mitochondria; OMM, outer mitochondrial membrane; PISD, PS decarboxylase.

between liposomes *in vitro* [89,90]. Moreover, depletion of PTPIP51 in HeLa cells results in decreased CL levels, suggesting that PTPIP51 transfers PA from the ER to the mitochondria [90]. Although PTPIP51 function in PA lipid transfer/metabolism needs to be further investigated, it is possible that the dramatic effects of PTPIP51 ablation in decreasing LD biogenesis (see previous section) are due to both its tethering and its lipid transfer functions. Interestingly, ORP5 was found to localize at MAM subdomains in contact with LD enriched in PA [48], suggesting that ORP5 may bind PA or even be involved in its metabolism/transport. Since ORP5 and PTPIP51 interact [54], it is possible that they act concertedly to regulate the levels of PA at MAM and mitochondria and its transfer from the MAM to the LD to sustain their growth (Fig. 5).

The idea that mitochondrial proteins cooperate with MAM LTPs to maintain MAM and mitochondrial lipid composition is further supported by evidence suggesting that ablation of both ORP5 and MIC60, an ORP5/ORP8 binding partner, in HeLa cells has a stronger effect in decreasing PS transport to mitochondria, than ORP5 alone. [55]. Furthermore, ablation of MIC60 alone or together with SAM50 reduces PS transport to mitochondria [55], thus possibly affecting mitochondria and MAM lipid composition and in the last-case scenario affecting lipid droplet biogenesis at MAM. However, MIC60 and SAM50 do not possess direct lipid transfer activity [91–94] and they do not mediate tethering between ER and mitochondria [55], suggesting that their role in PS transfer to the mitochondrial membranes for PE synthesis might be due to

either their known function in bridging the two mitochondrial membranes and thus favoring PS-to-PE conversion or their possible role in stabilizing the ORP5/8 complex at MAM. Another interesting possibility is that mitochondrial large protein insertion complexes such as SAM could be involved in lipid (PS and other lipids as well) scrambling across the mitochondrial outer membrane. A recent study from Vanni's team revealed that multiple mitochondrial complexes which engage in protein insertion, translocation, or assembly into the membrane, including the SAM and TOM complexes, have clear scramblase activity *in silico* [95]. They proposed that lipid rearrangements within and between membrane leaflets could be a general feature of "insertases," as all protein insertion complexes analyzed in their study, including some residing in the ER or MAM, had lipid scrambling activity. Thus, new studies should explore the mechanisms that regulate and couple lipid synthesis, scrambling, transfer, and conversion at CS, especially across the mitochondria–ER–LD junction. Indeed, lipid biosynthesis enzymes, LTPs, and lipid scramblases could cooperate at MAM for the proper and regulated delivery of TAG or CE precursors to the LD during their biogenesis.

Mitofusin 2 (MFN2), another mitochondrial protein that acts as an ER–mitochondria tether [96], was also shown to regulate transport of PS from the ER to the mitochondria in mouse liver tissue [97,98]. Like MIC60 and SAM50, MFN2 does not possess a lipid transfer domain containing a hydrophobic cavity. Therefore, while the role of MFN2 in PS transfer may be explained by its ER–mitochondria tethering function, it cannot be excluded that MFN2 may associate with LTPs or scramblases that mediate the transfer of PS. However, whether MFN2, MIC60, and/or SAM50 play a role in LD biogenesis is not known.

Two recent studies in yeast and mammals reported the presence of the PS decarboxylase enzyme PISD1 in ER subdomains where LD biogenesis occurs [99,100]. In human cells, Kumar *et al*. revealed the existence of a second isoform of PISD that, instead of being localized to the mitochondrial inner membrane as the main isoform, is localized to the ER-derived LD. Notably, depletion of both PISD isoforms (mitochondrial and ER-LD) alters the incorporation of OA into TAG [100]. Gok *et al*. further reported that in yeast, a small pool of Psd1 (ortholog of PISD) can localize on the ER membrane and, under conditions that promote LD biogenesis, on a subset of LD. They also demonstrated that Psd1 plays a conserved role in LD formation as the loss of the ER-localized Psd1 pool perturbed LD formation and morphology [99]. Whether PISD could localize and function at MAM-LD CS, and cooperate with MAM LTPs to regulate LD biogenesis, is still unknown but deserves further investigation. For instance, PISD could regulate levels of PE at the neck of LD, a site of negative curvature, to promote their formation and growth and possibly ORP5/ORP8 (or other LTPs such as MIGA2) could cooperate with PISD by modulating the availability of the PE precursor PS at MAM via its lipid transfer activity. Moreover, PSD-generated PE can be converted into PC that is a major constituent of LD. However, it is still unclear which PE, mitochondrial or ER, is the major substrate for PC synthesis.

## Possible implication of MAM-LD contacts in human diseases

To date, no studies have investigated the direct involvement of MAM-LD contacts in disease mechanisms. However, numerous indirect pieces of evidence suggest that the structure and function of this three-way association could potentially be altered in various human diseases. Loss of function in *BSCL2* gene, which encodes seipin, leads to Berardinelli–Seip congenital lipoatrophy (BSCL) type 2, a rare genetic disorder characterized by a near absence of adipose tissue (body fat) from birth, along with the progressive onset of various metabolic abnormalities, such as insulin resistance [60,101]. The mechanisms by which seipin deficiency results in lipodystrophy are not fully understood, and whether MAM-LD contacts are affected in this disorder is not clear. Nevertheless, studies in animal and cellular models lacking seipin, as well as yeast models, have been crucial to shed some light on the role of seipin and how its absence is a determinant for the development of BSCL [102]. For instance, seipin has been shown to regulate *de novo* LD formation in yeast and LD maturation in *Drosophila* and 3T3-L1 adipocyte cells [63,103]. It has been suggested that the effects of seipin in LD synthesis and maturation may result from its ability to modulate the levels of PA at the ER sites where LD matures [104], by interacting with several ER enzymes involved in PA synthesis and metabolism, such as AGPAT2 and lipin1 [105]. Additionally, as discussed before in this review, seipin regulates $Ca^{2+}$ uptake in the mitochondria, as well as mitochondrial respiration [68]. Altogether, these data suggest that seipin, which localizes and functions at MAM-LD contacts, could simultaneously regulate LD biology, mitochondrial function, and the ER lipid and protein composition. Moreover, BSCL-associated A212P-seipin is less enriched at MAM than seipin WT, emphasizing the functional role of seipin at MAM [68]. Therefore, it would not be surprising if

MAM-LD contact function and structure would be affected in BSCL type 2 disease.

Notably, in addition to lipodystrophy and associated metabolic deficits, BSCL patients also present cognitive impairment, suggesting a role of seipin in the brain [60,106]. Indeed, seipin is highly expressed in the brain and is required for normal brain development [107]. The importance of seipin for brain function is further evidenced by the fact that gain-of-function mutations on *BSCL2* gene result in brain disorders, known as seipinopathies [59]. The *BSCL2* mutations associated with these disorders, particularly N88S and S90L, lead to production of improperly folded seipin protein, which subsequently accumulates in the ER [108]. It is possible that the abnormal accumulation of mutated seipin in the ER may affect MAM-LD structure and function.

Dysfunction in mitochondria and LD has been associated with pathophysiology of many neurodegenerative disorders such as Alzheimer's disease (AD) [109,110]. AD is an age-related neurodegenerative disorder that results in progressive loss of neurons in the cortex and in the hippocampus. AD brains are characterized by extensive neuronal loss, extracellular accumulation of toxic amyloid-β (Aβ) peptides in Aβ plaques, and accumulation of intracellular phosphorylated tau forming neurofibrillary tangles [111]. Based on the discovery that Aβ is the major component of the extracellular plaques and that genetic mutations in the amyloid precursor protein (*APP*), presenilin 1 (*PSEN1*), and presenilin 2 (*PSEN2*) genes result in the accumulation of Aβ and early onset familial dementia, it was proposed that deposition of this protein aggregates in the brain triggers AD pathology [112]. However, the "amyloid cascade hypothesis" cannot explain several facts associated with AD [113]. For instance, before the appearance of Aβ plaques and neurofibrillary tangles, other metabolic alterations have been consistently documented in AD brains, which include mitochondrial dysfunction, oxidative stress, disruption of $Ca^{2+}$ homeostasis, and aberrant lipid homeostasis [114–116]. All these functions are regulated at ER–mitochondria contact sites, suggesting that these contact sites are affected in the early stages of AD [17]. Interestingly, APP and PSENs have been found to localize and function at MAM, and MAM function has been shown to be altered in AD models [117,118]. This has given rise to a new hypothesis regarding the underlying mechanisms of AD, termed "the MAM hypothesis" [119]. However, how MAM malfunction contributes to the pathogenesis of AD remains poorly understood. Another feature of AD brains is the aberrant accumulation of LD. Indeed, lipid accumulation in the brain was documented by Dr. Alois Alzheimer [120], and more recently, abnormal accumulation of LD has been reported in AD animal models, iPS AD-derived astrocytes, and microglia from AD human brains [121–124]. As discussed previously in this review, a subset of LD emerging from MAM and ER–mitochondria contact sites presumably plays a role also in LD growth [40,48]. These findings provide compelling evidence suggesting a potential role of ER–mitochondria–LD contacts malfunctioning in the onset and progression of AD. If confirmed, this hypothesis would provide a mechanistic link to most of, if not all, the apparently unrelated metabolic abnormalities resulting in neurodegeneration.

Like AD, amyotrophic lateral sclerosis (ALS) is a neurodegenerative disorder, with progressive loss of motor neurons, which display mitochondrial dysfunction and disruption of $Ca^{2+}$ homeostasis. Stoica *et al.* [88] have uncovered that these features are associated with disrupted ER–mitochondria contact sites mediated by VAPB-PTPIP51 in familial forms of ALS. Accordingly, mutations (P56S and T46I) in VAPB result in a rare type of dominantly inherited familial amyotrophic lateral sclerosis (ALS8), where the mutated protein tends to aggregate, is non-functional, and is unstable [125,126]. Additional studies have found that mutant forms of TAR DNA-binding protein-43 (TDP-43), which are implicated in familial forms of ALS, can activate glycogen kinase-3β and further contribute to the disruption of the interaction between VAPB and PTPIP51 [88]. More recent observations indicating that reduced interactions between VAPB and PTPIP51 contribute to ALS have been supported by studies conducted on post-mortem human spinal cord samples from ALS patients, using PLA [127]. The authors of these studies found a reduced expression of VAPB protein, which correlated with the decreased VAPB-PTPIP51 interactions, suggesting that the loss of VAPB may contribute to ALS [127]. Consistent with these studies, Markovinovic and Martín-Guerrero *et al.* [128] demonstrated that expression of either VAPB or PTPIP51 restored the interaction between VAPB and PTPIP51, as well as $Ca^{2+}$ signaling in SH-SY5Y cells expressing TDP-43 mutants linked to familial ALS. Overall, these studies reveal a connection between ALS and ER–mitochondria functions. It is noteworthy that changes in lipid metabolism have been observed in ALS and there is growing evidence indicating a link between ALS pathophysiology and LD [129]. However, a definitive association between ALS and LD is yet to be firmly established. Interestingly, Guyard *et al.* [48] have shown that PTPIP51 is required for proper LD biogenesis in HeLa cells, and

PTPIP51 knockdown reduces ER–mitochondria contact sites, suggesting that MAM-LD association may contribute to the pathological mechanisms of ALS.

## Conclusions and perspectives

The discovery of membrane contact sites and the increasing evidence of their widespread diversity and plasticity have dramatically changed our view of how organelles communicate and coordinate their functions within the cell. It is now clear that non-vesicular routes co-exist with vesicular transport systems in the cell and play fundamental roles in most, if not all, cellular processes including transport of key metabolites and signals across organelles and regulation of organelle biogenesis, positioning, division, and degradation in response to metabolic changes, nutrient availability, and stress. Moreover, the existence of close contacts between more than two or even three organelles adds a further layer of complexity in how organelles communicate with each other and suggests a key role of contact sites in spatially and temporally regulating specific metabolic processes across multiple organelles.

The recent identification of the mitochondria–ER–LD three-way junction [40,48], whose emerging functions are presented and discussed here, suggests the importance of contact sites in the compartmentalization of fatty acid metabolism and transport across these organelles, to balance the storage and the release of energy and maintain cellular homeostasis. It also highlights the existence of different subpopulations of mitochondria and MAM, which can adjust and remodel their molecular composition and functions, depending on the organelles they are in contact with, in this case the LD. For instance, peridroplet mitochondria and their associated MAM, in certain cell types and metabolic conditions, appear to have anabolic biosynthetic functions as compared to the cytosolic (catabolic) mitochondria [35,36] and MAM. These discoveries lead to a number of open questions: What are the molecular players and the mechanisms that define the specific mitochondria and MAM subpopulations? Are the proportion in these organelles' subpopulations and their functions different depending on the cell type and on their specific metabolism? Do mitochondria and MAM associated with other organelles, such as lysosomes, have also a peculiar proteome and functions? Are CS with the ER and other organelles also important for LD biogenesis, growth, and/or turnover, and, if yes, to what extent?

Many proteins populate MAM-LD contact sites, including membrane tethers, LTPs, and lipid biosynthesis enzymes, some of them have been discussed here, and many more will likely be identified in future studies. How they function, individually and in coordination, to orchestrate lipid fluxes across mitochondria–ER–LD and modulate these organelles' morphology, protein and lipid composition, functions, and dynamics, is still unclear. The role of MAM as a central regulator of cellular lipid metabolism and storage, which is emerging from recent work, will need to be further studied in multiple cell types and metabolic conditions *in vivo*.

Dysfunctions of lipid metabolism, mitochondria, and MAM, as well as a LD accumulation, have been associated with the pathophysiology of many metabolic and neurodegenerative disorders including AD. However, how such dysfunctions are established and whether they are functionally connected remain uncertain. Uncovering the molecular mechanisms that regulate lipid metabolism and LD formation/turnover at MAM-LD contact sites could represent an important therapeutic target in these diseases.

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