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REVIEW

Phospholipid ebb and flow makes mitochondria go

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Mitochondria, so much more than just being energy factories, also have the capacity to synthesize macromolecules including phospholipids, particularly cardiolipin (CL) and phosphatidylethanolamine (PE). Phospholipids are vital constituents of mitochondrial membranes, impacting the plethora of functions performed by this organelle. Hence, the orchestrated movement of phospholipids to and from the mitochondrion is essential for cellular integrity. In this review, we capture recent advances in the field of mitochondrial phospholipid biosynthesis and trafficking, highlighting the significance of interorganellar communication, intramitochondrial contact sites, and lipid transfer proteins in maintaining membrane homeostasis. We then discuss the physiological functions of CL and PE, specifically how they associate with protein complexes in mitochondrial membranes to support bioenergetics and maintain mitochondrial architecture.

Introduction

Biological membranes give a means of regulated communication, as the lipid bilayer itself acts as a platform for many reactions. The amphipathic nature of lipids underlies the creation of a bilayer; the hydrophobic acyl chains cluster at the middle to get buried, while the hydrophilic head groups face the aqueous surroundings. A diverse array of lipids, including glycerophospholipids, sphingolipids, and sterols, present in different amounts, gives a biological membrane its identity (Harayama and Riezman, 2018). The lipid composition of membranes is also subject to dynamic control in response to extrinsic and intrinsic signals (Atilla-Gokcumen et al., 2014; Köberlin et al., 2015).

The complexity of membranes is illustrated well in the mitochondrion. It consists of two sheaths, the inner mitochondrial membrane (IMM) and outer mitochondrial membrane (OMM), separating two aqueous environments, the matrix and the intermembrane space (IMS). Mechanisms that guarantee the tight coordination of lipid and protein biogenesis and translocation to specific subcompartments are necessary to achieve membrane homeostasis. This review focuses first on the mechanisms that govern mitochondrial membrane composition (the ebb and flow) and second on the numerous ways in which individual phospholipids impact mitochondrial biology (making mitochondria go).

Phospholipid biosynthesis

The mitochondrion harbors biosynthetic machineries needed to produce some phospholipids, such as cardiolipin (CL), phosphatidylethanolamine (PE), phosphatidic acid (PA), and phosphatidylglycerol (PG). Other phospholipids, such as phosphatidylserine (PS), phosphatidylinositol (PI), and phosphatidylcholine (PC), as well as sphingolipids, cholesterol, and many lysophospholipids, it has to recruit from other organelles. This section will focus on the mitochondrion's contribution to the generation of two highly abundant phospholipids in its membranes: CL and PE (Fig. 1).

CL metabolism

CL production. CL is the defining membrane constituent of the mitochondrion, the organelle in which it is made (Fig. 1 A). PA synthesized in the ER or the mitochondrion (Box 1) has to be delivered to the matrix side of the IMM to access Tam41 (TAMM41 in mammals), a mitochondrial cytidine diphosphate (CDP) diacylglycerol (DAG) synthase. Tam41 uses PA and CTP to yield CDP-DAG and pyrophosphate (Tamura et al., 2013). What follows is the committed step of CL biosynthesis, catalyzed by Pgs1 (phosphatidylglycerol phosphate synthase), which forms phosphatidylglycerophosphate (PGP) from CDP-DAG and glycerol-3-phosphate (G3P; Chang et al., 1998a). PGP is dephosphorylated to PG by Gep4 (PTPMT1 in mammals; Osman et al., 2010; Zhang et al., 2011). Crd1 (CL synthase [CLS1] in mammals) catalyzes the condensation of PG and CDP-DAG, generating nascent CL (Chang et al., 1998b; Chen et al., 2006; Houtkooper et al., 2006; Jiang et al., 1997; Tuller et al., 1998). Tam41, Pgs1, and Gep4 are peripherally associated with the matrix-facing leaflet of the IMM, while Crd1 is an integral IMM protein (Baile et al., 2014a).

CL remodeling. Following CL biosynthesis, acyl chain remodeling occurs. This involves deacylation and reacylation steps, leading to fatty acyl chain compositions that are organism and tissue specific. For instance, CL profile is highly varied in the mammalian brain but is more homogeneous in terms of

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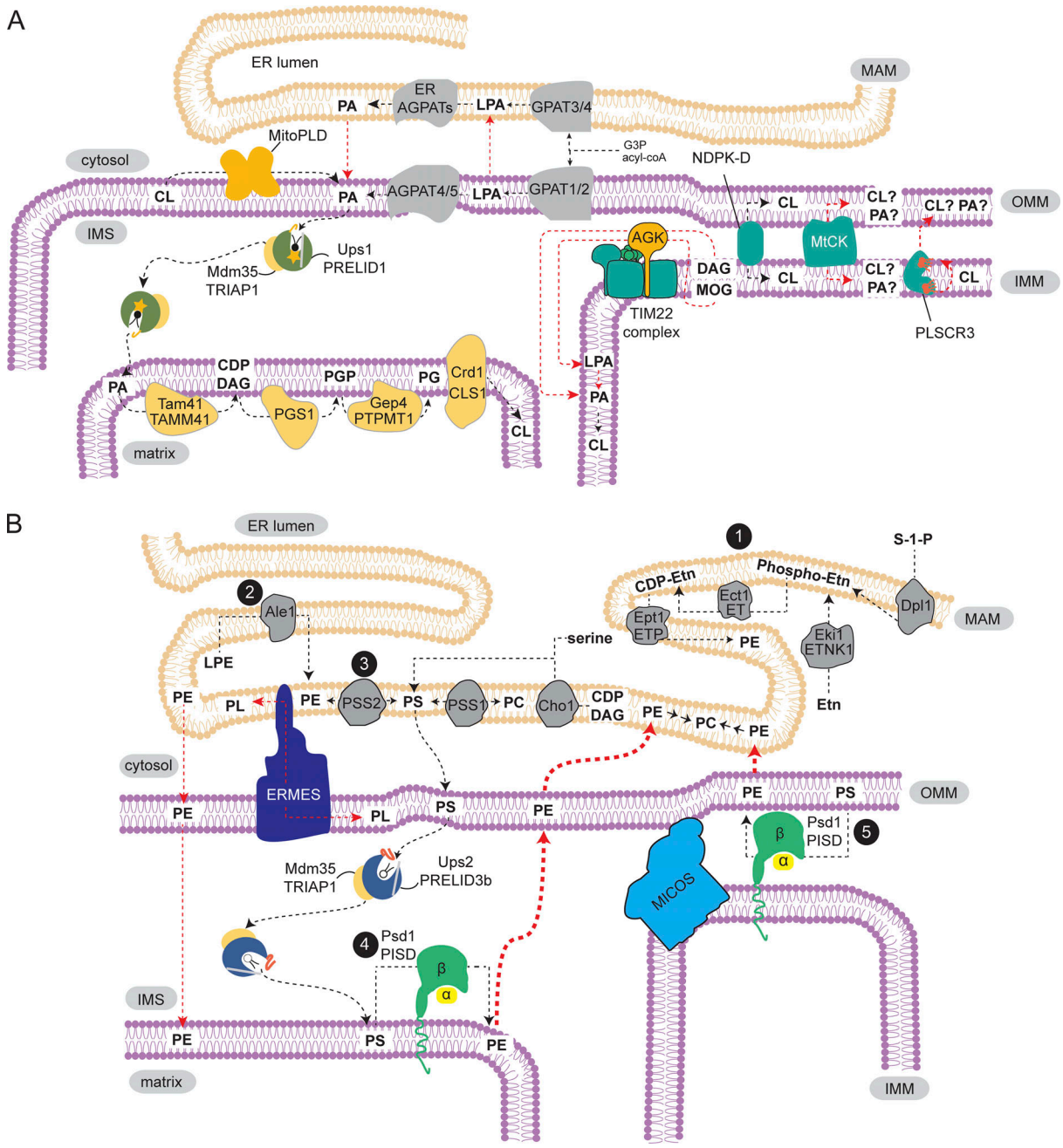

Figure 1. **Biosynthesis of CL and PE. (A)** CL production starts with PA, which can be sourced from multiple pathways (Box 1). PA needs to be trafficked to the matrix side of the IMM, where CL biosynthetic enzymes reside. PA shuttling from the OMM to the IMM is performed by Ups1-Mdm35/PRELID1-TRIAP1. Trafficking of CL or PA between OMM and IMM may also involve proteins at contact sites such as NDPK-D and MtCK. PLSCR3 promotes CL externalization to the OMM. It may transport CL or PA from the IMM to OMM or facilitate CL flip-flop from the inner to outer leaflet of the IMM. **(B)** PE is produced in the ER and mitochondria. ER pathways include the CDP–ethanolamine/Kennedy pathway (1), lysophosphatidylethanolamine acylation in yeast (2), and head group base exchange with PS in mammals (3). In mammals, PS can also be formed via base exchange with PC by PSS2, while in yeast, it is generated from CDP-DAG and serine by Cho1. PS produced in the MAM by PSS1/PSS2/Cho1 is then translocated to the OMM with the help of ER-mitochondrion MCS, such as ERMES in yeast. Ups2-Mdm35/PRELID3b-TRIAP1 transports PS to the IMM, where Psd1/PISD decarboxylates it to PE (4). Tethering of IMM and OMM by MICOS may allow Psd1/PISD to convert PS to PE in the OMM (5). PE made in the mitochondrion is exported to the ER, where it serves as a substrate for PC production. PE from the ER or OMM can also reach the IMM to a limited extent. Unresolved mechanisms are depicted by red arrows. MOG, monoacylglycerol.

molecular species in other tissues, including the heart (Kiebish et al., 2008; Schlame et al., 2005; Tyurina et al., 2014). The phospholipid environment, particularly the balance of linoleoyl (18:2) and oleoyl (18:1) levels, is a key determinant of the CL landscape across tissues (Oemer et al., 2020).

CL remodeling is initiated by a phospholipase, removing an acyl chain from CL to form monolyso-CL (MLCL). In yeast, this is performed by Cld1, which is peripherally localized to the matrix-facing side of the IMM (Baile et al., 2013; Beranek et al., 2009). Cld1 does not have a clear orthologue in metazoans,

Box 1. PA sources

PA, a nonbilayer phospholipid that has a central role in glycerolipid and triacylglycerol metabolism, serves as the precursor for CL. Several pathways lead to PA production, although the contribution of each to CL biosynthesis is not presently known.

For de novo PA generation, the first step is catalyzed by GPATs (G3P acyltransferases) that transfer an acyl group from acyl-CoAs to the *sn*-1 position of G3P, yielding LPA. There are four GPAT isoforms; GPAT1 and GPAT2 are OMM localized (Lewin et al., 2004; Wang et al., 2007; Yet et al., 1993), while GPAT3 and GPAT4 are at the ER (Chen et al., 2008; Nagle et al., 2008). LPA acyltransferases (also called AGPATs) then act on LPA, attaching an acyl group at the *sn*-2 position to form PA. Currently, five LPA acyltransferases are thought to exist in mammals, but there are additional related proteins that display activities toward other lysophospholipids (Bradley and Duncan, 2018; Yamashita et al., 2014). Only AGPAT4 and AGPAT5 are found in mitochondria, specifically the OMM (Bradley et al., 2015; Prasad et al., 2011).

A distinct PA pool may also be generated by mitoPLD, anchored at the OMM. In contrast to classical PLDs that use PC as substrate to produce PA, mitoPLD hydrolyzes CL instead, with the formed PA being important for modulating mitochondrial dynamics (Adachi et al., 2016; Choi et al., 2006). The dihydroxyacetone phosphate pathway housed in peroxisomes, necessary for ether-linked phospholipid synthesis, is another route to get LPA (Hajra and Das, 1996).

PA synthesis inside mitochondria was speculated after the identification of the metazoan-specific acylglycerol kinase (AGK; also called multilipid kinase [MuLK]), a protein associated with the IMM at the IMS side (Vukotic et al., 2017; Waggoner et al., 2004). Originally discovered through its homology with sphingosine kinases, AGK contains a DAG kinase catalytic domain (Bektas et al., 2005; Waggoner et al., 2004). In vitro, it phosphorylates monoacylglycerol and DAG, generating lysophosphatidic acid (LPA) and PA, respectively (Bektas et al., 2005; Waggoner et al., 2004). Its overexpression increased LPA amounts in a PC-3 cell model, implicating it in prostate cancer progression (Bektas et al., 2005). AGK has recently been demonstrated to be a subunit of the TIM22 translocase and independent of its lipid kinase activity is necessary for the assembly of the TIM22 complex and proper import of mitochondrial carriers (Kang et al., 2017; Vukotic et al., 2017). The lipid kinase function of AGK seems to be important for the maintenance of mitochondrial structure and respiration, as well as for apoptotic resistance, but the molecular basis for these observations is unclear, as there were no obvious changes in mitochondrial phospholipid abundance, including LPA, PA, and CL, in the absence of AGK (Vukotic et al., 2017).

but the iPLa$_2$ (Ca^{2+}-independent phospholipases A_2) family has been implicated in CL remodeling (Hsu et al., 2013; Mancuso et al., 2000). MLCL is subsequently reacylated to produce mature CL that typically displays high symmetry and predominantly contains unsaturated acyl groups (Claypool and Koehler, 2012; Schlame et al., 2005). Reacylation of MLCL can be mediated by Taz, an evolutionarily conserved transacylase (Xu et al., 2006). Because MLCL is produced at the IMM inner leaflet, it needs to get access to Taz, a monotopic protein that associates with the IMS-facing leaflets of the IMM and OMM in both yeast and mammals (Claypool et al., 2006; Lu et al., 2016). How this trafficking step happens is unknown. In humans and different model organisms, deficiency in Taz leads to increased MLCL, reduced CL, and aberrant CL acyl chain composition (Acehan et al., 2011; Claypool et al., 2006, 2011; Gu et al., 2004; Houtkooper et al., 2009; Lu et al., 2016; Schlame et al., 2003; Soustek et al., 2011; Valianpour et al., 2005; Vreken et al., 2000; Xu et al., 2006). These are considered hallmarks of Barth syndrome, an X-linked disease caused by *TAZ* mutations (Barth et al., 1999; Bione et al., 1996). Possible regulation of TAZ by the mitochondrial cochaperone DNAJC19, in complex with prohibitins, has also been linked to CL remodeling (Richter-Dennerlein et al., 2014).

Aside from TAZ, mammals have other enzymes that can reacylate MLCL: MLCLAT1 (MLCL acyltransferase 1) and ALCAT1 (acyl-coenzyme A [CoA]/lysocardiolipin acyltransferase-1). MLCLAT1 is a mitochondrial matrix leaflet-associated protein that binds MLCL and utilizes linoleoyl-, oleoyl-, and palmitoyl-CoA as acyl donors (Carpenter et al., 1992; Taylor and Hatch, 2009). ER-localized ALCAT1, however, displays activity toward several lysophospholipids using various acyl-CoA derivatives (Cao et al., 2004; Zhao et al., 2009), and can synthesize CL species that are prone to oxidative damage and are thus detrimental to mitochondrial function (Li et al., 2010). The physiological roles of these alternate pathways in CL metabolism are currently debated, although it is clear that they cannot compensate for the loss of TAZ or its activity.

In yeast, remodeled CL does not have a functional advantage over unremodeled CL in terms of maintaining mitochondrial structure and oxidative phosphorylation (OXPHOS; Baile et al., 2014b; Ye et al., 2014). The physiological function of CL remodeling in mammals warrants further investigation.

PE metabolism

Although there exists extramitochondrial pathways for PE generation (Box 2), the majority of cellular PE is generated in the mitochondrion (Fig. 1 B). ER-made PS is delivered to mitochondria and decarboxylated to PE by Psd1 (PS decarboxylase; PISD in mammals), an IMM-anchored complex consisting of α/β subunits produced after autocatalysis (Birner et al., 2001; Horvath et al., 2012; Tamura et al., 2012b). A second nonconserved Psd isoform, Psd2, is found in the yeast endomembrane system (Gulshan et al., 2010; Trotter and Voelker, 1995). Psd1 generates up to 70% of cellular PE in yeast; deletion of Psd1 and Psd2 confers ethanolamine auxotrophy, allowing growth from PE synthesis via an intact CDP–ethanolamine pathway (Birner et al., 2001; Bürgermeister et al., 2004; Trotter and Voelker, 1995). Mitochondrion-synthesized PE is then exported to the ER for PC production. Intriguingly, in a series of experiments in yeast using IMM-, OMM-, and ER-targeted Psd1, it was shown that the PE that accumulates in mitochondria when Psd1 is in the OMM or ER cannot efficiently reach the IMM (Calzada et al., 2019). Thus, PE can cross the IMS in both directions, but its flux out of the IMM is more robust. As there have been conflicting results as to whether ethanolamine supplementation impacts mitochondrial PE levels and OXPHOS (Baker et al., 2016; Bürgermeister et al., 2004; Chan and McQuibban, 2012), this study also suggests that extramitochondrial PE can indeed access the IMM but cannot fully substitute for the function of mitochondrion-derived PE (Calzada et al., 2019). Genetic ablation of *PISD* in mouse is embryonically lethal, highlighting the importance of the mitochondrial pathway that cannot be compensated for by extramitochondrial PE pools (Steenbergen et al., 2005). PE is crucial for mitochondrial functionality, as

Box 2. ER-housed PE biosynthetic pathways

The CDP–ethanolamine or Kennedy pathway occurring in the bulk ER is a major source of cellular PE in eukaryotes (Hjelmstad and Bell, 1991; Ishidate et al., 1985; Mancini et al., 1999; van Hellemond et al., 1994; Wittenberg and Kornberg, 1953). The initial step is the formation of phosphoethanolamine, either by phosphorylation of ethanolamine by Eki1/ETNK1 (ethanolamine kinase; Lykidis et al., 2001) or by the degradation of sphingosine-1-phosphate by Dpl1 (dihydrosphingosine-1-lysase; Gottlieb et al., 1999; Zhou and Saba, 1998). The second step, considered as rate limiting, is the formation of CDP–ethanolamine, catalyzed by Ect1/ET (CTP/phosphoethanolamine cytidyltransferase), encoded by the *Pcyt2* gene (Nakashima et al., 1997). Disruption of mouse *Pcyt2* has detrimental effects on development and metabolism (Fullerton et al., 2007; Fullerton et al., 2009). Finally, Ept1/ETP (1,2-DAG ethanolamine phosphotransferase) facilitates the transfer of the phosphoethanolamine moiety to CDP-DAG to generate PE (Lykidis et al., 2001; Sundler, 1975; Sundler and Akesson, 1975; Tijburg et al., 1987). A small percentage of cellular PE is derived from acylation of lysophosphatidylethanolamine by Ale1 in yeast (Riekhof et al., 2007) and head base exchange with PS by PSS2 in mammals (Dennis and Kennedy, 1972), which take place in the MAM subcompartment of the ER and bulk ER, accordingly (Stone and Vance, 2000).

demonstrated by OXPHOS defects in the absence of Psd1/PISD and its role in the regulation of mitochondrial dynamics and biogenesis of OMM proteins (Becker et al., 2013; Birner et al., 2001; Böttinger et al., 2012; Calzada et al., 2019; Heden et al., 2019; Tasseva et al., 2013).

The strict mitochondrial residence of Psd1 has been recently challenged by a study suggesting its dual localization to mitochondria and ER in yeast (Friedman et al., 2018). Using a set of chimeric constructs, it was further suggested that in the absence of mitochondrial Psd1, the ER fraction of Psd1 can support cellular PE homeostasis, particularly for ER function, but is not sufficient to maintain mitochondrial morphology and respiration, indicating organelle-specific functions. Additionally, the amount of glycosylated, presumably ER-localized Psd1 is modulated by metabolic conditions; growth on dextrose, a fermentable carbon source, causes enrichment compared with growth on ethanol/glycerol, which necessitates mitochondrial respiration. While highly intriguing, it must be noted that the functionality of the glycosylated Psd1 was not directly demonstrated. Moving forward, it is of paramount importance to provide this information, since the localization of Psd1 at the IMM has served as basis for numerous studies relevant to our current understanding of basic cell biology, including trafficking steps required for phospholipid biosynthesis.

Interorganellar phospholipid transport

Mitochondria are not connected with the rest of the cell's membrane network by classical vesicular routes, which entail long-distance trafficking of vesicles and ultimately fusion with an acceptor membrane. They must therefore receive and export small molecules through nonvesicular transport, prominently occurring at zones of close proximity with other organelles termed membrane contact sites (MCSs). Among the multitude of functions of MCSs between mitochondria and other organelles (Fig. 2 A) is providing a means to tackle the problem of shuttling amphipathic lipids across aqueous environments to their target membranous destinations. Different players at MCSs enable lipid transport, including tethers that bridge two membranes, lipid transfer proteins (LTPs) that extract lipid from a donor membrane and deliver to an acceptor membrane, recruitment proteins that allow accumulation of specific factors, and other regulators (Scorrano et al., 2019). LTPs, some with tethering capacity, possess hydrophobic cavities that allow lipid shuttling or passage (Wong et al., 2019). This is how the mitochondrion acquires precursors for CL and PE and phospholipids such as PC and PI that it cannot synthesize.

ER–mitochondrion exchange

The ER is the major site of lipid generation and supplying lipids to other membrane-bound compartments necessitates an ample amount of bidirectional trafficking. A classic example is the PS–PE–PC pathway (Achleitner et al., 1995). ER-generated PS is trafficked to the IMM to be converted to PE by Psd1. This mitochondria-made PE is then shuttled back to the ER and distributed to other organelles through vesicular pathways. Alternatively, this PE serves as substrate for sequential methylation reactions in the ER to produce PC (Vance, 1990), which stays in the ER or is shuttled to other organelles, including mitochondria.

Mitochondria-associated membranes (MAMs), the ER membrane subset tightly bound to mitochondria, have been linked with a role in lipid exchange (Gaigg et al., 1995; Vance, 1990). Since the characterization of MAMs, identifying protein complexes bridging the two organelles has been of huge interest. In yeast, a mutant screen revealed that an artificial tether connecting ER and mitochondria can complement the absence of Mdm12 (Berger et al., 1997; Kornmann et al., 2009). Analysis of Mdm12 interactors uncovered ER–mitochondria junction components, namely Mmm1, Mdm10, and Mdm34. Together, they build a complex termed ERMES (ER–mitochondria encounter structure; Kornmann et al., 2009; Fig. 2 B).

Individual deletion of ERMES proteins impairs respiratory growth and mitochondrial morphology and reduces ER–mitochondria contacts (Kornmann et al., 2009; Lahiri et al., 2014). ERMES mutants have a lower PS-to-PC conversion rate, decreased CL levels, and synthetic lethality with *CRD1*, pointing to a role for ERMES in ER–mitochondria phospholipid exchange (Kornmann et al., 2009). Conserved SMP (synaptotagmin-like mitochondrial lipid-binding protein) domains found in Mdm12, Mdm34, and Mmm1 form a tubular structure serving as a hydrophobic tunnel proposed to transfer PC and PS (AhYoung et al., 2015; Jeong et al., 2017; Kornmann et al., 2009). However, the notion that ERMES functions directly in phospholipid transport aside from serving as a physical linkage has been challenged. Two groups showed that there is only a subtle difference in steady-state cellular phospholipid levels between WT and ERMES mutants, suggesting that other routes exist. Importantly, these studies also used a radioisotope labeling approach, which demonstrated that the rate of PS transfer from ER to mitochondria is not hampered significantly upon disruption of the ERMES complex (Nguyen et al., 2012; Voss et al., 2012). It is important to note that different steps in the biosynthetic pathway were monitored; Kornmann et al. (2009) measured PS-to-PC conversion, whereas the other groups investigated PS-to-PE conversion. A modified in vitro phospholipid transport assay

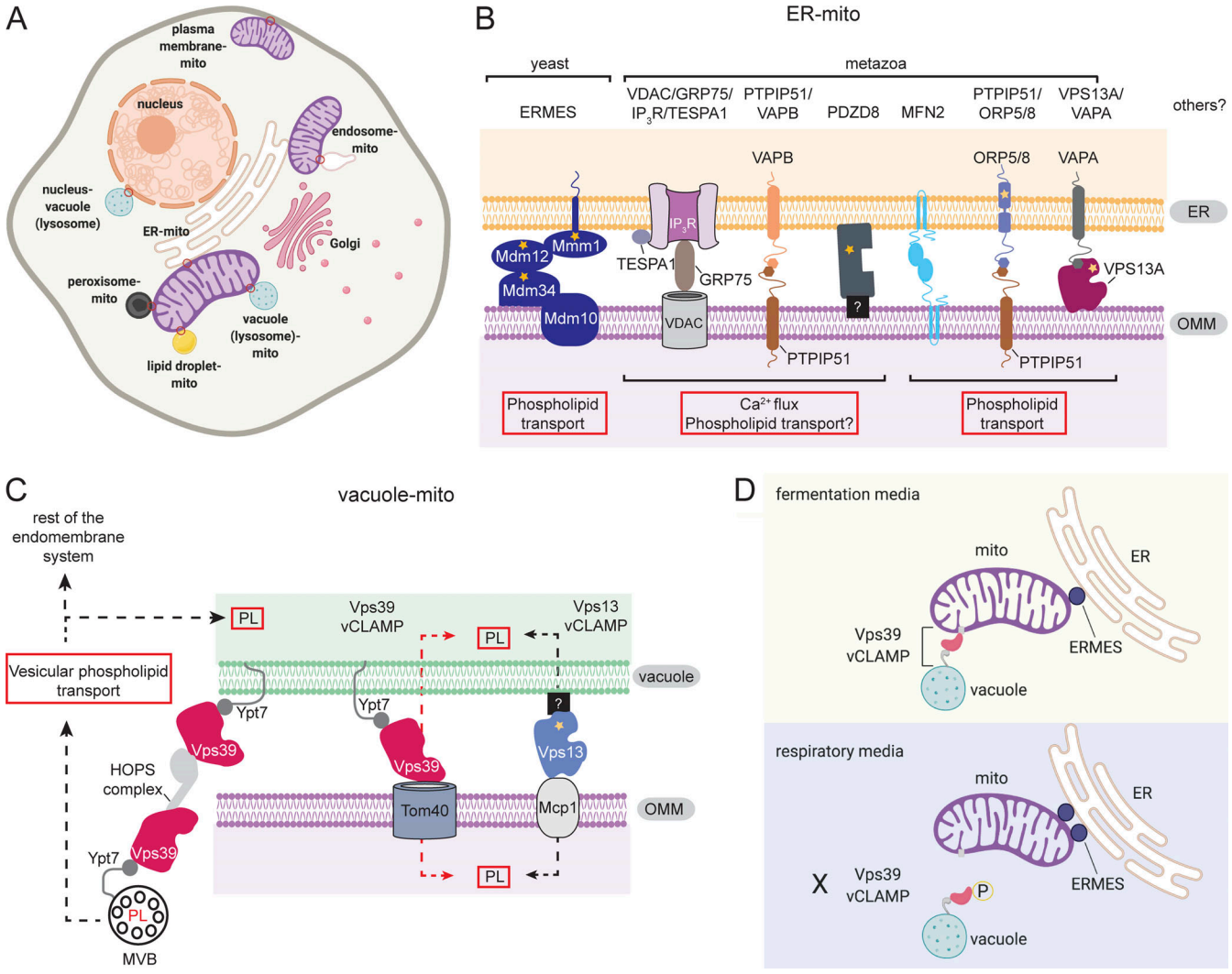

Figure 2. **Mitochondrial membrane contact sites. (A)** The mitochondrion establishes contacts with multiple organelles, which are important for various cellular processes, including phospholipid transport. **(B)** Physical interaction between ER and mitochondria is mediated by the ERMES complex in yeast, whereas a number of ER–mitochondria tethers have been documented in metazoans. SMP lipid-binding domains are present in ERMES, indicating a role in phospholipid shuttling aside from maintaining a close apposition of ER and OMM. ORP5/8 and PDZD8 have ORD and SMP domains, respectively; PDZD8 has not been shown to directly bind/transfer phospholipids. A domain in yeast Vps13 has been shown to possess lipid transport capacity. This domain is conserved in VPS13A and other VPS13 human isoforms. **(C)** Two types of vCLAMP, Vps39/Ypt7/Tom40 and Vps13/Mcp1 (vacuole counterpart unknown), connect vacuole and mitochondrion, allowing phospholipid (PL) exchange. As a component of the HOPS complex, Vps39 is also relevant for endolysosomal trafficking. Vps39 may facilitate vesicular phospholipid transport to the vacuole or to other parts of the endomembrane system, which can in turn supply phospholipids to the mitochondrion. **(D)** Metabolic conditions can regulate mitochondrial contact sites. Respiratory conditions lead to Vps39 phosphorylation that prevents vCLAMP formation, favoring OMM contacts with ERMES. Stars, lipid-binding domains; red arrow, unresolved mechanism. A and D were created with Biorender.

using yeast membrane fractions enriched in both mitochondria and ER was developed to interrogate which step in the PS-to-PC route is impacted in ERMES mutants (Kojima et al., 2016). Using this system, ERMES disruption impairs PS transport to mitochondria but not PE export. Still, PS import is not completely halted, indicating ERMES-independent trafficking routes may exist. Mediators of the translocation of mitochondria-synthesized PE to ER are also unknown.

Crystal structures of yeast Mdm12, Mmm1, and Mdm12–Mmm1 were recently solved (AhYoung et al., 2017; Jeong et al., 2016, 2017; Kawano et al., 2018), revealing that they can form a hydrophobic channel that binds phospholipids. It remains an important goal to get a structure of the tetramer to better elucidate the organization of the intact ERMES complex, if/how it recognizes specific substrates, and how the various subunits cooperate in mechanical tethering and phospholipid translocation.

Because ERMES is not conserved, it is perhaps not surprising that an additional ER–mitochondria MCS has been identified in yeast (Box 3) and other proteins have been suggested to replace its function in metazoans (Fig. 2 B). Early studies focused on mitochondrial Ca^{2+} uptake and physical linkage, as in the case of FIS1/BAP31 (Iwasawa et al., 2011), VDAC1/GRP75/IP_3R/TESPA1 (Matsuzaki et al., 2013; Szabadkai et al., 2006), VAPB/PTPIP51 (De Vos et al., 2012), and PDZD8 (Hirabayashi et al., 2017). Recent investigations highlight the relevance of metazoan

Box 3. An additional ER–mitochondrion contact site in yeast

The EMC (ER membrane complex) was implicated in ER–mitochondrion lipid exchange based on negative genetic interactions with *CHO2*, which codes for an ER-localized PE methyltransferase involved in PC generation (Lahiri et al., 2014). Loss of multiple EMC components retards mitochondrial PS import, decreases PS and PE, and elevates CL and PA amounts, in effect compromising growth (Lahiri et al., 2014). Deletion of several EMC proteins reduces ER–mitochondria contacts, and introduction of an artificial bridge corrects growth defects and PS-to-PE conversion rates (Lahiri et al., 2014). This link to phospholipid metabolism seems to be conserved in mammals (Janer et al., 2016). However, EMC has other roles related to membrane protein biology, including ER-associated degradation and insertion of a subset of single-pass membrane proteins into the ER post- or cotranslationally (Chitwood et al., 2018; Christianson et al., 2011; Guna et al., 2018). Unlike ERMES, no lipid-binding domains have been documented for EMC, arguing against a direct role in lipid transport. Hence, it is conceivable that EMC is significant for membrane insertion of proteins that facilitate lipid exchange and/or that form additional ER–mitochondrion contacts. ER-shaping proteins, which are necessary to maintain tubular ER, have also been suggested to facilitate lipid translocation between ER and mitochondria (Voss et al., 2012).

ER–mitochondria MCSs in phospholipid transport. MFN2, aside from acting as a tether (de Brito and Scorrano, 2008), also transfers PS from ER to mitochondria (Hernandez-Alvarez et al., 2019). It remains to be seen whether this role in lipid homeostasis is related to its function in mitochondrial fusion, as discussed later. ORP5/ORP8, which contain an ORD (oxysterol-binding protein–related domain), display PS transfer capacity and may also be relevant for PS delivery to mitochondria for PE synthesis (Galmes et al., 2016; Rochin et al., 2019 *Preprint*). VPS13A has a conserved domain shown to exhibit bulk lipid transfer activity in its yeast homologue (Kumar et al., 2018).

Vacuole–mitochondrion exchange

vCLAMP (vacuole and mitochondria patch) links mitochondria and vacuole (yeast lysosome; Fig. 2 C). A genome-wide screen identified genes whose deletion results in a higher number of ERMES puncta per cell (Elbaz-Alon et al., 2014), an indication that ERMES is trying to compensate for a related pathway. One of the uncovered ERMES modulators was Vps39, a component of the HOPS (homotypic membrane fusion and vacuole protein sorting) tethering complex (Stroupe et al., 2006). Vps39 associates with vacuoles and mitochondria via the GTPase Ypt7 and the translocase of the outer membrane machinery component Tom40 (Bröcker et al., 2012; Gonzalez Montoro et al., 2018). It is concentrated in defined regions on the vacuolar membrane that are in close proximity to mitochondria, and its overexpression expands vacuole–mitochondria contacts (Elbaz-Alon et al., 2014; Hönscher et al., 2014). Of note is that Vps39 was found to interact with multiple vacuolar transporters, putting forward the idea that vCLAMP can be a site of small molecule transport between vacuoles and mitochondria (Elbaz-Alon et al., 2014).

Vacuole–mitochondria and ER–mitochondria MCS are tightly linked functionally (Elbaz-Alon et al., 2014; Hönscher et al., 2014). Loss of ERMES expands the periphery occupied by vCLAMP, perhaps to improve vacuole–mitochondria tethering when that of ER–mitochondria is compromised, ensuring that transport of essential materials persists (Elbaz-Alon et al., 2014). Vps39 overexpression partially corrects ERMES-related growth defects, suggesting overlapping functions (Elbaz-Alon et al., 2014; Hönscher et al., 2014). A gain-of-function mutation in the endosomal resident Vps13 and overexpression of the mitochondrial proteins Mcp1 and Mcp2 also revert this phenotype (John Peter et al., 2017; Lang et al., 2015; Park et al., 2016; Tan et al., 2013). Mcp1 was determined to be the receptor for Vps13 at the OMM (John Peter et al., 2017). It is worth mentioning that Vps13, with multiple isoforms in mammals, can be recruited to other organelles by specific adaptor proteins (Bean et al., 2018; Kumar et al., 2018). Recent work supports the notion that the Ypt7–Vps39–Tom40 complex and the Vps13–Mcp1 complex are functionally independent MCSs (Gonzalez Montoro et al., 2018). Cells cannot tolerate concurrent ablation of ERMES and Vps39, Vps13, or Mcp1, highlighting MCS interdependence.

Vps39 has a dual role in vCLAMP formation and endolysosomal trafficking. The use of variants with impaired vCLAMP, but not HOPS, revealed that vesicular transport by Vps39 is what allows bypass of ERMES (Gonzalez Montoro et al., 2018). It is thus possible that small molecules need to reach the vacuole first via the endomembrane system before facilitated movement to mitochondria by Vps13, which possesses a lipid transfer module that can act as a bridge for bulk lipid transport (Kumar et al., 2018; Li et al., 2020). In an inducible system, the absence of Vps39 and repression of ERMES led to phospholipid defects, particularly reduced CL and PE levels (Elbaz-Alon et al., 2014), which may reflect a decrease in the vacuole's phospholipid pool due to disrupted vesicular trafficking, not allowing a backup for ERMES. A comprehensive analysis of the consequences on phospholipid flux of ablating vacuole–mitochondria MCS is lacking at the moment.

Vps39 also responds to metabolic conditions to control interorganellar connectivity (Hönscher et al., 2014; Fig. 2 D). Respiratory conditions promote ER–mitochondria contacts while diminishing those between vacuole–mitochondria. Yeast growth in glycerol-containing medium enhances Vps39 phosphorylation that in turn precludes vCLAMP formation. It was demonstrated that Vps39, independent of being a subunit of vCLAMP and HOPS complexes, is critical for ethanolamine-stimulated PE transport from ER to mitochondria, with ethanolamine supplementation promoting Vps39 redistribution to these two organelles (Iadarola et al., 2020). It is uncertain if Vps39-facilitated PE transport also takes place in basal conditions and whether Vps39 possesses PE-binding and transfer activities.

Regulatory proteins, like yeast Lam6 (also known as Ltc1), influence the extent of contact between organelles. Lam6 localizes to multiple MCSs, such as ER–mitochondrion, vacuole–mitochondrion, ER–vacuole, and nucleus–vacuole (Elbaz-Alon et al., 2015; Gatta et al., 2015; Murley et al., 2015). ERMES expansion upon vCLAMP disruption and vCLAMP expansion in the absence of ERMES both necessitate Lam6 (Elbaz-Alon et al., 2015). What controls the distribution of Lam6 and whether it is dynamically regulated by cellular conditions are currently not addressed.

In mammals, lysosome/endosome–mitochondrion contacts have been recently documented (Daniele et al., 2014; Das et al., 2016; Wong et al., 2018). Whether they function in phospholipid exchange remains unknown.

Phospholipid translocation across mitochondrial subcompartments

For the double membrane–bound mitochondrion, there is an asymmetrical distribution of phospholipids between the OMM and IMM, and between two leaflets of a membrane (de Kroon et al., 1997; Zinser and Daum, 1995; Zinser et al., 1991). Regulated intramitochondrial trafficking helps deliver phospholipids to the right sites in order to maintain membrane composition needed to support mitochondrial function.

The Ups/PRELI family: LTPs in the IMS

The Ups/PRELI family, highly conserved in eukaryotes (Dee and Moffat, 2005), was first implicated in mitochondrial function when yeast Ups1 was found to be involved in the processing of Mgm1 (OPA1 in mammals), a regulator of mitochondrial morphology (Sesaki et al., 2006). *UPS2* and *UPS3* that encode other homologues were uncovered as genetic interactors of prohibitins, membrane-bound ring complexes in the IMM (Osman et al., 2009). Loss of Ups1 and Ups2 in yeast results in diminished CL and PE levels, respectively; *UPS3* deletion, however, does not disrupt phospholipid levels, although its overexpression in Ups2-deficient cells improves PE accumulation, indicating partially overlapping function with Ups2 (Osman et al., 2009). In humans, there are four Ups family homologues: PRELID1, PRELID2, PRELID3a, and PRELID3b (Dee and Moffat, 2005). The conserved domain is called PRELID (protein of relevant evolutionary and lymphoid interest domain; Dee and Moffat, 2005; Fox et al., 2004; Punta et al., 2012).

Ups proteins do not harbor canonical IMS-targeting signals, and their efficient import into the IMS is mediated by interaction with Mdm35 (TRIAP1 in mammals), a member of the twin Cx_9C protein family (Potting et al., 2010; Tamura et al., 2010). Complex formation with Mdm35 promotes Ups accumulation in the IMS by shielding them from proteolytic degradation (Potting et al., 2010).

Role of Ups1-Mdm35/PRELID1-TRIAP1 in CL metabolism

CL accounts for ∼20% of total IMM phospholipids, an enrichment relative to CL levels in the OMM (Simbeni et al., 1991). PA generated in the ER or on the mitochondrial surface must traverse membranous and aqueous compartments to have access to the first enzyme of CL biosynthesis residing in the inner leaflet of the IMM (Baile et al., 2014a). Ups1-Mdm35 binds negatively charged phospholipids and specifically shuttles PA across the IMS (Connerth et al., 2012; Fig. 1 A). PA transfer is enhanced by negatively charged phospholipids, especially PA itself, and inhibited by physiological concentrations of CL (Connerth et al., 2012). This suggests a feedback mechanism for the control of CL production by impeding PA trafficking to the IMM when CL is abundant. Although purified Ups1-Mdm35 can transfer PA bidirectionally, high CL levels, as it is in the IMM, compromise Ups1 dissociation from the membrane, making it prone to proteolysis and giving PA transport directionality (Connerth et al., 2012).

PRELID1-TRIAP1, the mammalian homologue of Ups1-Mdm35, also serves as a PA transfer complex in the IMS, with knockdown of either protein impairing CL accumulation in the IMM (Potting et al., 2013). Curiously, TRIAP1 depletion decreases CL to a greater degree than mitochondrial PE in contrast to Mdm35 deletion (Potting et al., 2010), suggesting different regulation of CL and PE homeostasis in yeast and mammals. Downregulation of PRELID1 or TRIAP1 increases susceptibility to apoptosis, linking cellular fitness to mitochondrial CL metabolism (Potting et al., 2013).

Role of Ups2-Mdm35/PRELID3b-TRIAP1 in PE homeostasis

PE, the second most abundant phospholipid in the cell representing 15–25% of total phospholipids (Vance, 2015), is also enriched in mitochondrial membranes. ER-synthesized PS must be presented to the IMS-facing catalytic domain of Psd1. In yeast, Ups2-Mdm35 acts as a PS-shuttling complex (Fig. 1 B) that is particularly critical when yeast are grown in nonfermentable carbon sources (Miyata et al., 2016). Interestingly, in glucose-containing medium, loss of Ups2 only mildly impacts mitochondrial PE accumulation and does not significantly affect cellular levels (Aaltonen et al., 2016; Miyata et al., 2016). This indicates fine-tuning of PE synthesis by metabolic conditions. Importantly, deletion of *UPS2* does not completely prevent Psd1-dependent PE production, supporting the idea that PS has other ways of feeding into Psd1 that are independent of Ups2 (Aaltonen et al., 2016; Miyata et al., 2016). In contrast to its impact on PA flux by Ups1, CL stimulates PS transport by Ups2 (Aaltonen et al., 2016). Complementation experiments indicate that human PRELID3b is the functional orthologue of Ups2 and that PRELID3b-TRIAP1 mediates PS transfer between liposomes (Aaltonen et al., 2016).

Loss of Mdm35 leads to depleted mitochondrial PE levels reminiscent of the loss of Ups2, whereas CL amount is affected to a lesser extent (Potting et al., 2010). This is similar to what was observed for Δ*ups1*Δ*ups2* cells (Tamura et al., 2009). It is possible that CL levels are maintained by PA import that does not rely on Ups1-Mdm35 or that there are additional regulatory mechanisms that respond to reduced PE levels to enhance CL accumulation. For example, Fmp30/Mdm31/Mdm32 were identified to cooperatively reinforce CL production independently of Ups1 in low-PE conditions (Miyata et al., 2017).

Mechanism of lipid transport and selectivity by Ups-Mdm35/PRELID-TRIAP1

Crystal structures of Ups1-Mdm35/PRELID1-TRIAP1 have provided insights into how these partners work together to transport lipid cargo (Miliara et al., 2015; Watanabe et al., 2015; Yu et al., 2015). They were found to be structurally similar to LTPs belonging to the StARkin (kin of steroidogenic acute regulatory protein) superfamily, which include ceramide transfer protein and phosphatidylinositol transfer protein (Miliara et al., 2015). Ups1 is barrel-like, composed of seven antiparallel β-sheets and three α-helices, with a flexible loop (Ω) between β3 and β4 that, together with the C-terminal helix α3, may serve as a lid for regulated lipid passage (Miliara et al., 2015; Watanabe et al., 2015; Yu et al., 2015). The bottom of the pocket contains a positively charged residue, K58, essential for making contact with the PA head group; the Ω-loop and most of the conserved basic and hydrophobic residues lining the cavity wall are critical for

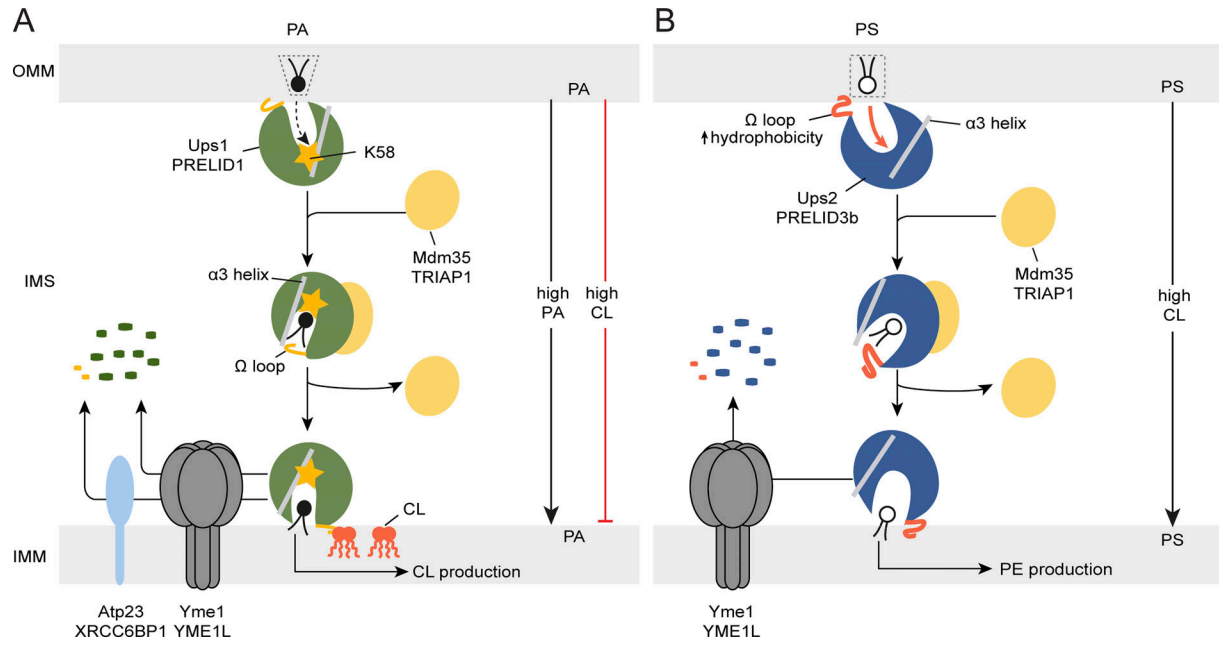

Figure 3. **Phospholipid transport across the IMS.** Ups/PRELI family contributes to PA and PS transport between the OMM and IMM. Once lipid is extracted from the OMM and incorporated into the pocket of the transfer protein, Ups/PRELI associates with Mdm35/TRIAP1 and closes its lid, enabling efficient transport. Upon reaching the IMM, the protein complex dissociates, facilitating lipid release. Ups/PRELI proteins are then subjected to proteolytic degradation. **(A)** A positively charged residue (K58) at the bottom of Ups1/PRELID1's cavity interacts with the PA head group. PA is extracted from OMM in a process that is perhaps facilitated by the cone-shaped structure of PA that prevents tight packing. The high CL concentration in the IMM helps recruit Ups1/PRELID1. PA transport is stimulated and repressed by high PA and high CL levels, respectively. **(B)** PS transport by Ups2-Mdm35/PRELID3b-TRIAP1 permits PE production in the IMM. The greater hydrophobicity of the Ω loop and the α3 helix of PRELID3b aids in PS extraction from OMM, even though its cylindrical structure does not support its extraction. High CL amounts promote PS translocation.

PA extraction from the membrane (Miliara et al., 2015; Watanabe et al., 2015; Yu et al., 2015). Mdm35 wraps around Ups1 at a hydrophobic interface (Miliara et al., 2015; Watanabe et al., 2015; Yu et al., 2015). This explains why dissociation of Mdm35 from the heterodimeric complex improves the efficiency of multiple steps in PA transfer, especially membrane binding, as this exposes the hydrophobic residues in Ups1.

Comparison of the crystal structures of PRELID1 or PRELID3b bound by TRIAP1 yielded information into the lipid specificity of PRELI proteins (Miliara et al., 2019; Fig. 3, A and B). In PRELID1, the Ω-loop and the disordered helix α3 form a more constrained space when compared with PRELID3b. This tighter space is likely more suited to accommodate PA than the bulkier PS. A reverse genetics approach identified amino acids that either broaden lipid substrate recognition or confer lipid specificity to Ups/PRELI proteins (Miliara et al., 2019). Notably, K58 at the bottom of the Ups1 cavity is required for PA selection, whereas the hydrophobicity of the Ω loop and helix α3 is critical for PS membrane targeting and extraction (Miliara et al., 2019).

Role of STARD7 in intramitochondrial PC transport

PC, cylindrical and bilayer forming, is the most abundant phospholipid in cellular membranes (Sperka-Gottlieb et al., 1988; Zinser et al., 1991). In mitochondria, it constitutes 40–50% of total phospholipids (van der Veen et al., 2017). It is exclusively synthesized in the ER by two separate routes: (1) via the CDP–choline pathway, by phosphorylation of choline, binding to CDP, and attachment of CDP–choline to DAG; and (2) by a series of methylation steps with PE as precursor (van der Veen et al., 2017). Normal PC levels are vital for mitochondrial integrity, including the biogenesis of β-barrel and some α-helical OMM proteins, TIM (translocase of the inner membrane) 23–mediated mitochondrial protein translocation, mitochondrial respiration, and cristae morphogenesis (Horibata et al., 2016; Schuler et al., 2015, 2016).

STARD7, which contains a lipid-binding START (StAR-related lipid transfer) domain, catalyzes PC transfer between liposomes (Horibata and Sugimoto, 2010). Intriguingly, STARD7 is dually localized in the cytosol and the mitochondrion. There have been conflicting reports about the submitochondrial localization of STARD7—that is, if it is in the OMM or the IMS (Horibata et al., 2017; Horibata et al., 2016; Saita et al., 2018). Data showing that STARD7 is a substrate of PARL (presenilin-associated rhomboid-like), a protease in the IMM, demonstrates that STARD7 can indeed make its way underneath the OMM (Saita et al., 2018); nevertheless, some may still be anchored at the OMM. IMM-directed, but not cytosol- or OMM-targeted, STARD7 can rescue the respiratory and mitochondrial morphology defects of STARD7-null cells (Saita et al., 2018). Processing of STARD7 by PARL during mitochondrial import exposes an internal sorting signal of negatively charged residues that directs a fraction of mature STARD7 into the cytosol (Saita et al., 2018). However, PARL-mediated cleavage of STARD7 after insertion into the IMM by the TIM23 complex promotes localization of mature STARD7 in the IMS, where it is proposed to act as a PC-shuttling protein between mitochondrial membranes.

Loss of mitochondrial STARD7 in mouse hepatoma cells specifically reduces PC in the IMM and impairs ATP production (Horibata et al., 2016). Still, the mechanisms of PC recognition, binding, and transport by STARD7 are not well elucidated.

Role of IMM–OMM contact sites

MICOS (mitochondrial contact site and cristae organizing system), a hetero-oligomeric complex in the IMM, functions in the generation and maintenance of cristae junctions where it is enriched (Harner et al., 2011; Rabl et al., 2009). Mic60, an integral subunit of MICOS with an IMS-facing domain, was found to interact with several OMM proteins. Thus, it was proposed to establish a tether between mitochondrial membranes (van der Laan et al., 2016) that is suggested to facilitate mitochondrial protein translocation (Horvath et al., 2015) and mitochondrial phospholipid biosynthesis (Aaltonen et al., 2016).

Synthetic lethality screens have been helpful in discovering novel regulators of phospholipid homeostasis (Gohil et al., 2005; Osman et al., 2009). Strong negative genetic interactions between MICOS subunits and CL biosynthetic enzymes (Hoppins et al., 2011), thus suggested a role for MICOS in phospholipid metabolism. In fermentable conditions, Psd1-dependent PE production is only subtly reduced, whereas PE-to-PC conversion is even improved, in the absence of Ups2 (Aaltonen et al., 2016; Tamura et al., 2012a). This points to the existence of Ups2-independent PS transport pathways. The capacity of liposome-reconstituted Psd1 to decarboxylate PS yielding PE in a juxtaposed membrane indicates that it may act in trans and that without Ups2, a close apposition between IMM and OMM may allow Psd1 to act on PS in the OMM, obviating the need for PS to cross the IMS (Fig. 1 B). MICOS's ability to mediate this was tested (Aaltonen et al., 2016). Yeast lacking MICOS have altered mitochondrial ultrastructure, growth defects in the absence of ethanolamine (which fuels the CDP–ethanolamine pathway), and lower PE synthesis and subsequent conversion to PC. These results are consistent with the possibility that MICOS allows PS decarboxylation by Psd1 in the OMM (Aaltonen et al., 2016). They additionally indicate that MICOS-dependent PE production is more important for maintaining PC supply, perhaps by reducing trafficking steps required for its synthesis or instead by promoting PE export from the mitochondrion. However, these do not rule out the possibility that MICOS directly traffics PS, or that it regulates another player in PS flux. Also, deletion of Ups2 in the ΔMICOS background rescues the mitochondrial defects (Aaltonen et al., 2016), possibly by preventing PS accumulation, though the exact mechanism of how PE homeostasis is maintained, albeit at lower levels, in this setting is unclear.

Other proteins enriched at contact sites may provide alternative means of transferring phospholipids between the IMM and OMM (Fig. 1 A). An example is NDPK-D (nucleotide diphosphate kinase isoform D), which can catalyze the exchange of γ-phosphate between nucleotide triphosphates and diphosphates (Milon et al., 2000). It is a peripheral IMS-facing, IMM protein that binds anionic phospholipids, with greatest affinity for CL (Tokarska-Schlattner et al., 2008). Another is MtCK (mitochondrial creatine kinase), which moves phosphocreatine between cellular microcompartments (Wallimann et al., 1992). Their ability to bind negatively charged phospholipids, together with their symmetrical structure with two binding domains that can interact with separate lipid bilayers, led to the idea that they may bridge IMM and OMM (Epand et al., 2007; Tokarska-Schlattner et al., 2008). NDPK-D and MtCK increase the phospholipid transfer rate (Epand et al., 2007), which can be attributed to tethering of liposomes or direct lipid transfer. In HeLa cells, overexpression of WT, but not a CL-binding mutant of NDPK-D, causes a selective increase of CL in the OMM (Schlattner et al., 2013). This enhanced CL externalization is important for apoptotic susceptibility and mitophagy induction (Epand et al., 2007; Kagan et al., 2016; Schlattner et al., 2013; Box 4). Interestingly, NDPK-D–lipid complex formation that is necessary for CL transfer represses phosphotransfer activity, enabling NDPK-D to toggle between its two putative functions (Schlattner et al., 2013). The signal for this switch, particularly if the distribution of CL or other anionic phospholipids between mitochondrial membranes affects NDPK-D binding to OMM, is unknown.

Transbilayer lipid transport

Because of the hydrophilic head group of a phospholipid, its flip-flop across a membrane bilayer is energetically less favorable than lateral translocation. Spontaneous transbilayer phospholipid transport occurs at a tremendously slow rate that is not suitable for supporting physiological processes (Kornberg and McConnell, 1971). Sufficiently robust rates can be achieved through assistance from proteins, such as scramblases, flippases, and floppases, which mediate constitutive or regulated phospholipid movement (Pomorski and Menon, 2016). Scramblases are responsible for rapid phospholipid translocation down the concentration gradient, while flippases and floppases are enzymes that mediate inward or outward active transport of phospholipids, accordingly. However, since pretreatment with protease or sulfhydryl reagents did not largely affect transbilayer lipid movement across the OMM, the contribution of proteins to the transbilayer flux of phospholipids across mitochondrial membranes, which occurs rapidly, has been questioned (Gallet et al., 1999; Janssen et al., 1999).

The sole mitochondrial scramblase characterized to date is mammalian PLSCR3 (phospholipid scramblase 3; Fig. 1 A; He et al., 2007; Liu et al., 2003), a member of the PLSCR family of membrane proteins that catalyze bidirectional phospholipid movement that is generally ATP independent, phospholipid unspecific, and Ca^{2+} responsive (Bevers et al., 1999; Kodigepalli et al., 2015; Zhou et al., 1998). Cells expressing PLSCR3 with a calcium-binding site mutation have reduced proliferation, mitochondrial mass, and membrane potential, leading to defective mitochondrial structure and respiration (Liu et al., 2003). Overexpression of WT or mutant PLSCR3 augments or blunts UV-induced apoptosis, respectively (Liu et al., 2003). These have been attributed to emergent roles for the regulated flux of CL to the OMM in response to mitochondrial damage that can signal the removal of functionally impaired mitochondria by mitophagy or, alternatively, apoptosis (Box 4). PLSCR3 boosts intramembrane scrambling (He et al., 2007); however, it is not obvious whether it speeds up CL translocation to the outer

Box 4. PE, CL, and organellar versus cellular demise decisions

PE is a regulator of autophagy. It is conjugated to the autophagy-related protein LC3 (Atg8 in yeast), and membrane-anchored lipidated LC3 plays a critical role in autophagosome formation (Ichimura et al., 2000; Kabeya et al., 2000; Rockenfeller et al., 2015; Tanida et al., 2004). One source of PE for LC3 lipidation is provided by PISD (Thomas et al., 2018).

CL externalization from IMM to OMM acts as a signal for either mitophagy or apoptosis. CL exposed on the OMM can be bound by LC3, facilitating autophagosome formation and mitophagy-mediated clearance of defective mitochondria (Chu et al., 2013). Perturbed CL metabolism stemming from the absence of TAZ also affects mitophagy (Hsu et al., 2015). Alternatively, externalized CL can recruit and activate caspase-8, leading to extrinsic apoptosis (Gonzalvez et al., 2008). Moreover, accumulation of oxidized CL on the OMM stimulates the release of proapoptotic factors, including cytochrome *c*, to the cytosol after mitochondrial membrane permeabilization (Kagan et al., 2005). It is plausible that the nature of insult and extent of damage are related to the amount of externalized CL and whether or not it is oxidatively modified, which may govern whether apoptosis or mitophagy occurs. Cytochrome *c* release is also regulated by OPA1, which is attributed to its role in modulating cristae structure (Frezza et al., 2006). This further highlights the intricate interplay that exists among intramitochondrial phospholipid dynamics, mitochondrial cristae morphology, and important cellular decisions.

leaflet of the IMM, expediting CL transfer to the OMM by other factors (e.g., MtCK and/or NDPK-D), or whether it moves CL from IMM to OMM. Also, it is unclear why expression of WT or mutant PLSCR3 affects CL biosynthetic rate the same way by increasing CLS1 activity (Van et al., 2007). It will be important to determine whether PLSCR3 can act on other phospholipids like PA and whether distribution of other lipids is disturbed in its absence. To date, there has been no scramblase identified that can mediate PA or PS transfer from outer to inner leaflet of the OMM, trafficking steps required for CL and PE synthesis, respectively. Also, it is not known what moves nascent CL made in the matrix side of the IMM to the IMS side, where remodeling occurs. Importantly, there has not been any mitochondrial scramblase found in yeast. Mitochondrial flippases or floppases, should they exist, have yet to be identified in yeast or mammals.

Physiological functions of mitochondrial phospholipids

The IMM is an extensively folded, protein-dense environment with numerous proteins organized into higher-order structures. In the following sections, we tackle how mitochondrial phospholipids interact with and influence the function of large protein machineries, such as key proteins for energy production and components controlling mitochondrial architecture and dynamics.

Role in bioenergetics

Mitochondrial phospholipids associate with OXPHOS complexes and affect their functionalities (Fig. 4 A, left). CL has been shown to interact with complexes I–V, PC with complex IV, and PE with complexes I–IV (Guo et al., 2017; Lange et al., 2001; Mehdipour and Hummer, 2016; Schwall et al., 2012; Sharpley et al., 2006; Shinzawa-Itoh et al., 2007; Sun et al., 2005; Wu et al., 2016). PE specifically synthesized in the IMM is important for complex III function through its conserved interaction with the Qcr7 subunit (Calzada et al., 2019). Thus, it is likely that maintaining proper phospholipid levels in specific mitochondrial membrane regions is important in supporting distinct mitochondrial protein functions.

CL in particular has a propensity for binding proteins and promoting the formation of macromolecular complexes. CL is crucial for the assembly of respiratory supercomplexes (RSCs), high-molecular weight structures consisting of complexes III and IV in yeast and I, III, and IV in mammals (Althoff et al., 2011; Pfeiffer et al., 2003; Schägger and Pfeiffer, 2001; Zhang et al., 2002; Fig. 4 A, left). RSCs are proposed to assist in electron transfer between individual respiratory chain components and lessen oxidative damage (Acín-Pérez et al., 2008; Lapuente-Brun et al., 2013), although the actual functional advantage of this structure is still under debate (Milenkovic et al., 2017). Additionally, CL preserves the integrity of mitochondrial translocases (Gebert et al., 2009; Malhotra et al., 2017; van der Laan et al., 2007) and stimulates the assembly and/or stabilization of several mitochondrial transporters (Claypool et al., 2008b; Kadenbach et al., 1982; Lee et al., 2015). By interacting with AAC (ADP/ATP carrier) or UCP1 (uncoupling protein 1), CL is also implicated in proton leak control and thermogenesis (Bertholet et al., 2019; Lee et al., 2015).

New evidence suggests that the strong affinity of CL to proteins is also beneficial for CL function. In Barth syndrome patient lymphoblasts and TAZ-knockdown mouse tissues, loss of CL–protein interaction increases CL turnover, which is proposed to contribute to the increased MLCL/CL ratio in this setting (Xu et al., 2016). Moreover, the expression and assembly of OXPHOS complexes induce CL remodeling that consequently enhances CL stability proposed to optimize lipid–protein interactions in the IMM (Xu et al., 2019). These results highlight that the ability of CL to associate with a range of proteins and protein complexes is of mutual benefit; these interactions promote a remodeling process that stabilizes CL, which in turn helps maintain the functionality of a crucially important and yet protein-crowded lipid bilayer (Fig. 4 A, right).

Role in mitochondrial membrane structure

The IMM contains morphologically distinct domains: a flat inner boundary membrane and tubular cristae. Cristae structure is postulated to be critical for OXPHOS function. Recently, it was shown that in yeast, Mdm35-supported transport of PE and CL precursors into the IMM helps drive cristae formation, partially by optimizing CL levels in this membrane (Kojima et al., 2019). The importance of CL in the maintenance of the IMM structure has been suggested by ultrastructure data in a *Caenorhabditis elegans* model of CL depletion (Sakamoto et al., 2012). In yeast, the absence of CL does not globally impact IMM architecture (Baile et al., 2014b); however, mitochondria with abnormal IMM structure, including part-stacked and lamellar cristae, have been reported (Rampelt et al., 2018). Although whole-body deletion of *CLS1* is lethal, adipose tissue–specific *cls1*$^{-/-}$ mice are viable; brown adipose tissue isolated from these mice has aberrant

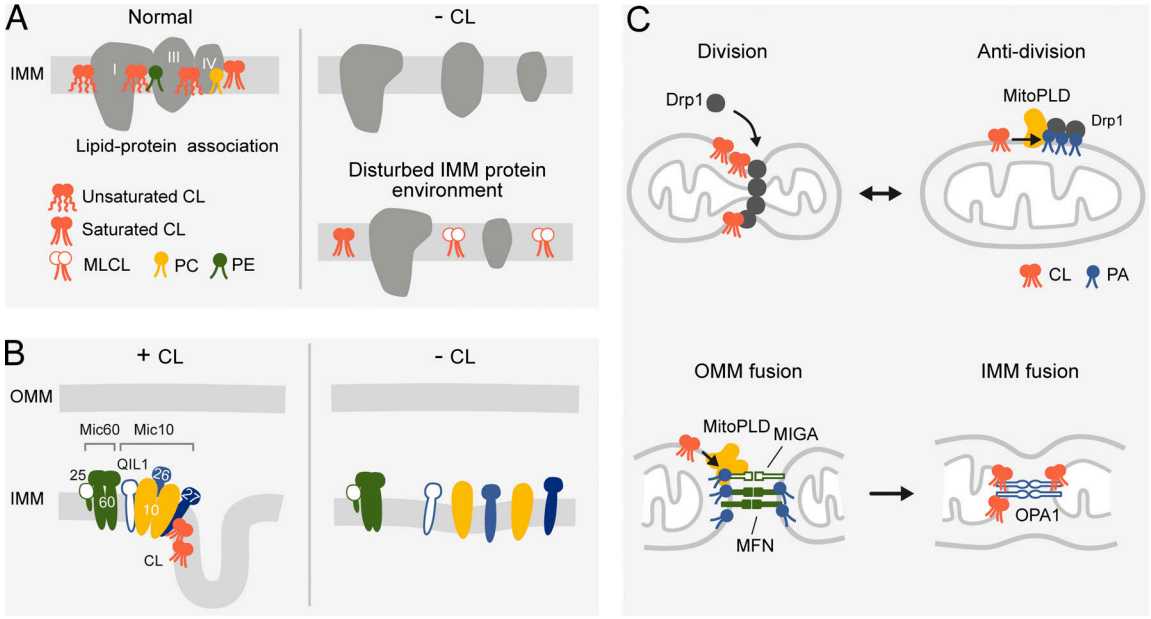

Figure 4. **Physiological functions of mitochondrial phospholipids. (A)** Association of phospholipids and proteins in the IMM impacts stability of both protein complexes and membrane phospholipids. Loss of CL leads to RSC disassembly, while loss of OXPHOS proteins (thus disrupting RSC formation) results in faster CL turnover that is thought to elevate the MLCL/CL ratio. The amount of saturated CL also increases in the absence of OXPHOS complexes that induce CL remodeling. **(B)** CL-supported cristae structure via MICOS integrity. The mammalian MICOS complex is depicted. In the presence of CL, the MIC10 subcomplex, composed of MIC10, QIL1, MIC27, and MIC26, assembles and organizes cristae structure. MIC27 specifically interacts with CL. In the absence of CL, the MIC10 subcomplex is diffused, disrupting cristae morphology. MIC60 subcomplex assembly is independent of CL. **(C)** Phospholipid involvement in mitochondrial dynamics. (Top) DRP1 modulates membrane restriction in response to the local concentrations of CL and PA. DRP1 is recruited to the OMM and oligomerizes in a CL-dependent manner, leading to membrane constriction. It directly interacts with mitoPLD and binds mitoPLD-produced PA, which in turn suppresses division. (Bottom) MFN and MIGA promote OMM fusion where PA locally accumulates, as a consequence of MitoPLD-mediated CL hydrolysis. MIGA directly interacts with mitoPLD. OPA1 forms a dimer and induces IMM fusion at the CL-enriched sites.

cristae structure and mitochondrial dysfunction associated with altered respiratory chain and RSC formation (Sustarsic et al., 2018). *PTPMT1* deletion models also showed that perturbed CL metabolism leads to aberrant mitochondrial morphology (Wolf et al., 2019; Zhang et al., 2011). As discussed below, the underpinnings of how CL supports cristae structure likely stems from a combination of both its physicochemical properties (dimeric, cone-shaped structure combined with negatively charged headgroups) and its functional association with complexes vital for cristae formation and organization.

CL interaction with organizers of cristae morphology

MICOS. MICOS consists of two evolutionarily conserved subcomplexes, Mic60 (also known as mitofilin) and Mic10, which are integrated via Mic19 (Rampelt et al., 2017). The Mic60 subcomplex consists of Mic60 and Mic19 (MIC25 in metazoans), while the Mic10 subcomplex includes Mic10, Mic12 (QIL1 in humans), Mic27, and Mic26 (Rampelt et al., 2017).

Different MICOS subunits play unique roles in supporting the IMM architecture. Mic60, the largest component, is essential for cristae formation and maintenance (John et al., 2005; Rabl et al., 2009; Zerbes et al., 2012). Additionally, it has the capacity to bend membranes in vitro (Hessenberger et al., 2017; Tarasenko et al., 2017). Mic10 forms large oligomers via its two glycine-rich motifs, promoting membrane curvature for cristae junction formation (Barbot et al., 2015; Bohnert et al., 2015). In yeast, Mic10 assembly at cristae junctions is stabilized by CL, RSCs, and other MICOS components; conversely, Mic60 assembly is independent of these factors (Friedman et al., 2015; Rampelt et al., 2018; Fig. 4 B). Interestingly, the effect of Mic27 or Mic60 deletion on MICOS organization is exacerbated by the lack of CL, indicating a role for CL in complex stabilization (Friedman et al., 2015; Rampelt et al., 2018). In relation to this, mammalian MIC27/APOOL specifically binds CL, and this interaction is important for its incorporation into the MICOS complex (Weber et al., 2013).

Aim24, an IMM protein and a binding partner of Mic10, is necessary for MICOS integrity (Harner et al., 2014). Indeed, mitochondria lacking Aim24 have aberrant structure. Aim24 controls CL composition, perhaps through its impact on the levels of the CL remodeling enzyme, Taz, suggesting that a give-and-take relationship also exists between MICOS and CL.

F_1F_O-ATP synthase. The mitochondrial ATP synthase forms dimers located in rows along the highly curved cristae ridges (Hahn et al., 2016). Loss of ATP synthase dimers results in aberrant cristae morphology (Davies et al., 2012; Paumard et al., 2002; Strauss et al., 2008), demonstrating an essential role for proper cristae formation, perhaps by promoting membrane curvature. However, because failure of ATP synthase to dimerize leads to pleiotropic phenotypes (Bornhövd et al., 2006), a direct role in membrane bending still warrants demonstration.

The cryo-EM structure of ATP synthase dimers from *Euglena gracilis* identified tightly bound CL (Mühleip et al., 2019). Importantly, CL-binding sites are found at the rotor-stator and dimer interface and in a peripheral F_O cavity. At the dimer interface, bound CL molecules, coordinated by different subunits, predominantly fill the cavity. Although the necessity of CL for ATP synthase dimerization seems to vary in different model systems (Acehan et al., 2011; Claypool et al., 2008a) and its requirement for normal IMM morphology has not been established (Baile et al., 2014b), it is possible that proper CL levels can improve the efficiency of membrane bending by lowering the energetic cost of this process.

FAM92A1. FAM92A1 is a BAR domain–containing protein that localizes to the matrix leaflet of the IMM (Wang et al., 2019). Absence of FAM92A1 severely distorts mitochondrial morphology and IMM ultrastructure. Through preferential binding with negatively charged phospholipids like CL, FAM92A1 can induce membrane curvature in model membranes. In the IMM, FAM92A1 can potentially cause membrane tubulation upon interaction with CL, but whether CL has a direct regulatory role on FAM92A1 function in vivo is unclear.

Roles in mitochondrial dynamics

Mitochondrial dynamics, characterized by fission and fusion events, are responsible for modulating mitochondrial size and number. Perturbation of the balance of fission and fusion underlies pathologies (Archer, 2013), emphasizing its importance in cellular homeostasis. Studies in the last decade revealed essential roles of phospholipids in mitochondrial dynamics (Fig. 4 C).

Mitochondrial fission. The GTPase DRP1 (dynamin-related protein 1; Dnm1 in yeast) primarily mediates mitochondrial fission. DRP1 is recruited from the cytosol to the mitochondrial surface by binding to OMM adaptor proteins MFF, MID49, and FIS1. DRP1 then forms oligomers, driving membrane constriction and leading to membrane division. CL binding stimulates DRP1 oligomerization and GTPase activity (Bustillo-Zabalbeitia et al., 2014; Francy et al., 2015; Macdonald et al., 2014; Montessuit et al., 2010; Stepanyants et al., 2015). Moreover, DRP1 is recruited to CL-containing membranes and induces membrane tubulation in a CL-dependent manner (Macdonald et al., 2014; Montessuit et al., 2010). Cryo-EM structural analysis of DRP1 helices on nanotubes with specific lipid compositions determined that DRP1 recruitment and activation are more efficient with CL compared with other phospholipids (Francy et al., 2017). Additionally, DRP1 stabilizes the physicochemical properties of CL. Using GTP hydrolysis as energy source, DRP1 induces CL reorganization from a bilayer to a nonbilayer configuration, resulting in constrictions in the membrane poised for division (Stepanyants et al., 2015). In vivo, this entails CL to be translocated to the cytosol-facing leaflet of the OMM, a process not mechanistically understood.

In contrast to CL, PA and phospholipids harboring saturated acyl chains have an "antidivision" effect on the DRP1-mediated machinery (Adachi et al., 2016). DRP1 binds the PA head group, and if saturated acyl chains are enriched in neighboring phospholipids, then its GTPase activity is suppressed, preventing OMM constriction. It is noteworthy that DRP1 directly interacts with mitochondrial phospholipase D (mitoPLD; Adachi et al., 2016), an OMM resident that can convert CL to PA (Choi et al., 2006). This interaction is postulated to modulate the local concentration of CL and PA in the OMM for regulating mitochondrial division (Adachi et al., 2016).

Overexpression of Mdm33, an IMM protein in yeast, fragments mitochondria and leads to lower PE and CL levels (Klecker et al., 2015; Messerschmitt et al., 2003). Thus, Mdm33 is another potential factor that links phospholipid homeostasis and mitochondrial fission.

Mitochondrial fusion

Lipid-regulated OMM fusion. Dynamin-related GTPases also facilitate mitochondrial fusion. At the OMM, this is performed by mitofusins (MFNs; Fzo1 in yeast). Although highly similar in terms of sequence and structure, MFN1 and MFN2 have unique functions; the former is a central component of the mitochondrial fusion machinery, while the latter is involved in mitochondrial tethering before fusion in addition to its role in ER–mitochondrion MCS formation (de Brito and Scorrano, 2008; Rojo et al., 2002). PA produced by mitoPLD-mediated CL hydrolysis at the mitochondrial surface promotes mitochondrial fusion downstream of mitochondrial tethering by MFNs, in contrast to its suppressive effect on DRP1-facilitated division as noted previously. MitoPLD overexpression results in mitochondrial aggregation, implying fusion, while knockdown leads to mitochondrial fragmentation (Choi et al., 2006; Huang et al., 2011). In the absence of MFN1 or MFN1/2, mitoPLD overexpression does not induce aggregation, indicating that the role of PA in mitochondrial fusion may be through the actions of PA on MFNs (Choi et al., 2006).

Other proteins contribute to the fusion process. Mitoguardins (MIGA1/2 in mammals), OMM proteins that form homo- and heterocomplexes, bind, stabilize, and promote mitoPLD dimerization (Zhang et al., 2016). By stimulating mitoPLD function, MIGA1/2 overexpression elevates mitochondrial PA levels and promotes mitochondrial fusion (Zhang et al., 2016). Overexpression of Lipin 1b, a phosphatase that converts PA to DAG, or PA-PLA1 (phosphatidic acid–preferring phospholipase A), which makes LPA from PA, fragments mitochondria (Baba et al., 2014; Huang et al., 2011). Further, elongation of mitochondrial tubules is observed upon PA-PLA1 knockdown (Baba et al., 2014). It is important to note that Lipin 1b and PA-PLA1 are cytosolic proteins that need to be recruited to the OMM. There is evidence indicating that mitoPLD-produced PA is required for the translocation of Lipin 1b to the OMM, and for PA-PLA1–mediated regulation of mitochondrial fusion (Baba et al., 2014; Huang et al., 2011). These results point out that coordination of the mechanisms for regulating local PA amounts at the OMM is crucial for mitochondrial dynamics.

Knockdown of another LPA-synthesizing protein, mitochondrial G3P acyltransferases (GPAT), causes mitochondrial scission in HeLa cells and *C. elegans* (Ohba et al., 2013). It is presently unclear why depletion of PA-PLA1 or mitochondrial GPAT, enzymes that both produce the same lysolipid, have

opposite effects on the mitochondrial network. It is possible that LPA generated by mitochondrial GPAT serves as substrate for PA production by acylglycerolphosphate acyltransferases (AGPATs) and that this may be pro-fusion, but this requires further investigation.

Lipid-regulated IMM fusion. OPA1/Mgm1, a dynamin-related GTPase that regulates cristae architecture (Frezza et al., 2006; Griparic et al., 2004; Harner et al., 2016; Patten et al., 2014) is also essential for IMM fusion. Its direct association with MFN1 coordinates the fusion of IMM and OMM (Cipolat et al., 2004; Meeusen et al., 2006). One gene encodes OPA1/Mgm1, but via a combination of alternative splicing and constitutive and inducible proteolysis, multiple forms are generated, categorized as long (L-OPA1/Mgm1) and short (S-OPA1/Mgm1; MacVicar and Langer, 2016). L-OPA1/Mgm1, anchored to the IMM by a transmembrane domain, is a major player in fusion whereas S-OPA1/Mgm1, lacking a transmembrane domain and residing in the IMS, participates in fusion and division.

OPA1/Mgm1 biogenesis and assembly are controlled by phospholipids. Its import to the IMM is facilitated by the TIM23 complex, whose maintenance and function involves CL (Malhotra et al., 2017; Tamura et al., 2009). In yeast, low CL levels impair both the membrane insertion and proteolytic processing of Mgm1 (Herlan et al., 2004; Sesaki et al., 2006; Tamura et al., 2009), negatively impacting IMM fusion. CL has also been shown to stimulate oligomerization of OPA1/Mgm1, activating GTPase function and enhancing mitochondrial fusion (Ban et al., 2010; DeVay et al., 2009; Faelber et al., 2019; Ge et al., 2020; Meglei and McQuibban, 2009; Rujiviphat et al., 2009; Yan et al., 2020). In contrast to what happens in yeast, L-OPA1 present in one population of liposomes can mediate fusion with protein-free liposomes as long as they contain CL levels thought to mimic those at IMM–OMM contact sites (Ban et al., 2017). When CL levels are low in the protein-free liposomes, L-OPA1 tethers, but does not fuse, the two distinct liposomes. Since OPA1 has a pivotal role in cristae formation that is independent of its GTPase activity (Patten et al., 2014), it is intriguing to speculate that the different OPA1 functions are regulated by local CL levels that vary depending on the membrane engaged.

It is also suggested that PE homeostasis is relevant for fusion, as both S-Mgm1 protein and mitochondrial fusion are decreased when mitochondrial PE levels are low (Chan and McQuibban, 2012; Joshi et al., 2012). Recently, a lipid signaling cascade dependent on the PA phosphatase LIPIN1 was found to modulate mitochondrial PE abundance (MacVicar et al., 2019). mTORC1 inhibition by hypoxia or amino acid starvation leads to LIPIN1 activation, decreasing PA amounts and ultimately mitochondrial PE levels, which then promotes proteolysis by YME1L (MacVicar et al., 2019; Peterson et al., 2011). As YME1L plays a role in OPA1 proteolytic processing and degradation (Mishra et al., 2014; Song et al., 2007), this can be one way mitochondrial PE affects IMM fusion and fission.

Perspectives

Membrane phospholipid composition profoundly affects mitochondrial function. Our knowledge of phospholipid biology is comparatively scant relative to other biomolecules mainly due to a limitation in available techniques for lipid visualization, quantification, and manipulation. Currently, we have a limited picture of the distribution of phospholipids across different mitochondrial compartments. Although there are highly sensitive mass spectrometry–based tools that can provide accurate measurement of phospholipids and information on acyl chain length and saturation, there is a scarcity of methods that can cleanly separate the IMM from the OMM and prevent the inclusion of contaminating ER membranes. With the right tools at hand, it will be interesting to see whether certain phospholipid types are present in distinct regions of the IMM (e.g., inner boundary membrane or different parts of the cristae membrane) or the OMM (e.g., those involved in forming MCS and those that are not). It will also be relevant to determine whether clustering of phospholipids at specific sites occurs and whether this can drive biological processes, such as mitochondrial fusion/fission or site-specific recruitment of protein complexes.

The recent identification and characterization of LTPs and MCS components, coupled with the acquisition of structures for some of these, have broadened and deepened our mechanistic understanding of intramitochondrial and interorganellar phospholipid translocation. Many questions remain, including whether in the wide array of MCSs any specific complex has a direct role in shuttling phospholipids, if there are signals telling the cell to prioritize specific phospholipid transport mechanisms in particular cellular contexts, and how phospholipids flip from one side of the membrane to another. It will be fascinating to see if and how any of these yet-to-be-discovered processes are regulated and harnessed in biologically meaningful ways.

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