

## Review

# Lipid synthesis and transport are coupled to regulate membrane lipid dynamics in the endoplasmic reticulum



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## ABSTRACT

Structural lipids are mostly synthesized in the endoplasmic reticulum (ER), from which they are actively transported to the membranes of other organelles. Lipids can leave the ER through vesicular trafficking or non-vesicular lipid transfer and, curiously, both processes can be regulated either by the transported lipid cargos themselves or by different secondary lipid species. For most structural lipids, transport out of the ER membrane is a key regulatory component controlling their synthesis. Distribution of the lipids between the two leaflets of the ER bilayer or between the ER and other membranes is also critical for maintaining the unique membrane properties of each cellular organelle. How cells integrate these processes within the ER depends on fine spatial segregation of the molecular components and intricate metabolic channeling, both of which we are only beginning to understand. This review will summarize some of these complex processes and attempt to identify the organizing principles that start to emerge. This article is part of a Special Issue entitled Endoplasmic reticulum platforms for lipid dynamics edited by Shamshad Cockcroft and Christopher Stefan.

## 1. Localization of phospholipids versus their synthesizing enzymes

### 1.1. Localization of enzymes for de novo phosphatidic acid biosynthesis

The biosynthetic pathways responsible for the synthesis of various phospholipids were already established between the late 1950s and early 1970s and have been thoroughly summarized in many excellent reviews (e.g. [1,2] (Fig. 1)). These intricate metabolic pathways became increasingly complicated in the genomic era after the identification of multiple genes encoding enzymes that seemingly carry out the same enzymatic reaction. While many of these enzymes are located in the ER, as expected, the localization and membrane topology of some of the lipid synthesizing enzymes remain puzzling. Moreover, as many of the emergent enzymes are identified and classified by sequence homology, it is not always clear which lipid substrates they use within the intact cell. Good examples include the growing family of GPATs (glycero-3-phosphate acyl transferases), which transfer fatty acyl chains to glycerol-3-phosphate (G3P) as the first step in de novo lipid synthesis. GPAT3 and GPAT4 are integral ER-resident membrane proteins, whereas GPAT1 and GPAT2 are localized to the outer mitochondrial membrane [3,4]. Some of these enzymes, such as GPAT4 [5], are critical for triacylglycerol (TG) synthesis and lipid droplet (LD) biogenesis

while others are involved in controlling phospholipid synthesis. However, the exact contribution of these enzymes to these respective pathways is still not all that clear. Similarly, some of the AGPAT (acylglycerophosphate acyl transferase) enzymes that catalyze the acylation of lyso-phospholipids are found in the outer mitochondrial membrane (e.g. AGPAT4 and AGPAT5) [3], whereas others are found in the ER. AGPAT enzymes are mostly believed to use lyso-PA to generate PA in the de novo lipid synthetic pathway, but which isozymes are most important for phospholipid synthesis is not yet known. Interestingly, *Agpat4*  $-/-$  mice show significant decreases in phosphatidylinositol (PI), phosphatidylcholine (PC), and phosphatidylethanolamine (PE) but not in phosphatidic acid (PA), phosphatidylserine (PS), phosphatidylglycerol (PG) or cardiolipin levels in the brain [6]; suggesting that PA made on the outer surface of the mitochondria by this enzyme has to find its way to the ER, where the affected phospholipids are known to be made, and that not all phospholipid synthetic pathways are fed uniformly through the action of this enzyme.

### 1.2. Localization of enzymes required for phospholipid biosynthesis

The location of the enzymes directly involved in phospholipid synthesis is similarly puzzling. For example, PC, which is present in every membrane, is synthesized by either the Golgi-localized CDP-

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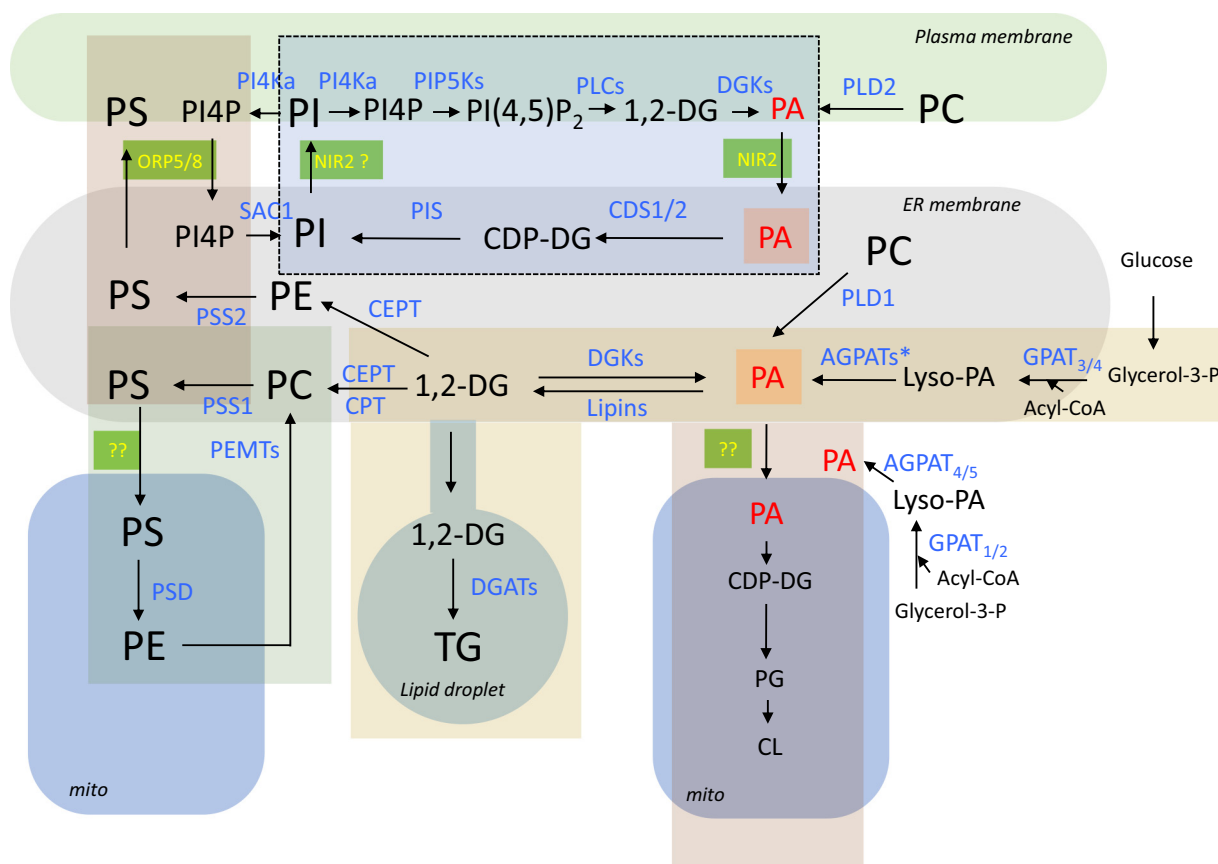
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**Fig. 1.** Enzymatic pathways of lipid synthesis and their membrane localization in a generic mammalian cell. The enzymes are labeled blue and the lipids are black except PA (red) which represents a major hub for several metabolic pathways. Major organelles are designated by the rounded shapes. Metabolic units that have been linked are designated by rectangles of different colors. The dotted lines denote inositol lipids that serve as signaling entities. Lipid transfer proteins working in contact sites are labeled yellow on green background. Note that lipids in the ER can be part of multiple metabolic units and how these are kept separated is one of the important questions yet to be understood. Abbreviations of enzymes: PI4K, PI 4-kinase; PIP5K, PI4P 5-kinase; PLC, phospholipase C; DGK, diacylglycerol kinase; PLD, phospholipase D; Sac1, Sac1 phosphatase; PIS, phosphatidylinositol synthase; CDS, CDP-DG synthase; PSS, phosphatidylserine synthase; CEPT, choline/ethanolamine phosphotransferase; CPT, CDP-choline:1,2-diacylglycerolphosphocholine transferase; PSD, phosphatidylserine decarboxylase; PEMT, Phosphatidylethanolamine *N*-methyltransferase; DGAT, diacylglycerol acyl transferase; AGPATs, 1-Acylglycerol-3-Phosphate O-Acyltransferase (\* denotes that several forms of these enzymes exist in the ER. AGPAT4 and -5 are found in mitochondria); GPAT, Glycerol-3-Phosphate Acyltransferase. Abbreviation of lipids: PI, phosphatidylinositol; PI4P, phosphatidylinositol 4-phosphate; PI(4,5)P<sub>2</sub>, phosphatidylinositol 4,5-bisphosphate; DG, diacylglycerol; PA, phosphatidic acid; PS, phosphatidylserine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; TG, triacylglycerol; PG, phosphatidylglycerol; CL, cardiolipin; CDP-DG, cytidine-diphosphate-diacylglycerol.

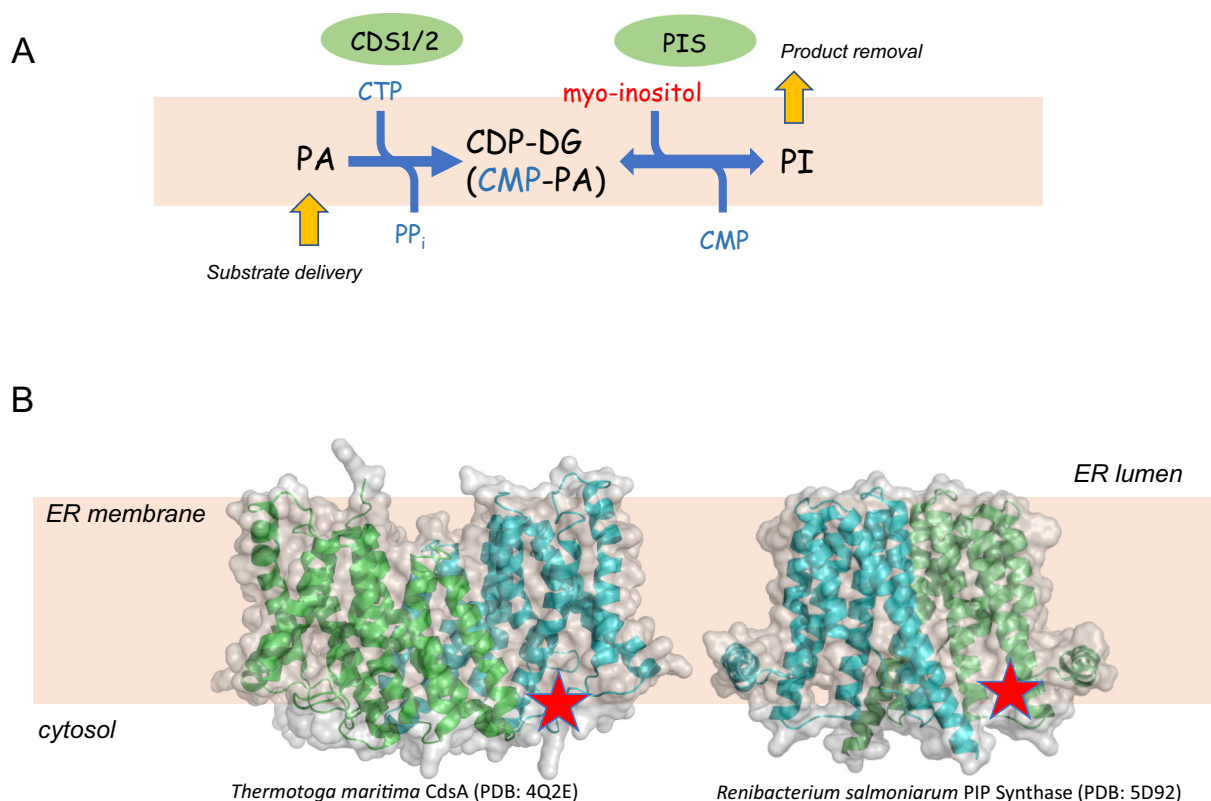
choline:1,2-diacylglycerolphosphocholine transferase (CPT) enzyme or by the ER-localized choline/ethanolamine phosphotransferase (CEPT) enzyme using diacylglycerol (DG) and CDP-choline as precursors [7,8]. However, the enzymes that generate CDP-choline from phosphocholine and CTP (CTP:phosphocholine cytidylyltransferase, or CCT $\alpha$  and CCT $\beta$ ) are soluble, peripheral membrane proteins, which are found in the nucleus in quiescent cells. In response to oleic acid exposure, CCT $\alpha$  associates with intranuclear canalicular membranes and subsequently moves out from the nucleus to associate with LDs and promote LD formation as well as lipid storage [7,9,10]. How PC gets to other membranes from its site of synthesis is far from being understood, although several PC transport proteins have been identified [11].

The PS synthesizing enzymes, phosphatidylserine synthase (PSS)-1 and -2, that are responsible for exchanging the headgroups of PC and PE, respectively, for serine are multi-membrane-spanning proteins localized to the ER [12], but their enzymatic activities have been found highest in mitochondria-associated membranes (MAMs) [13]. MAMs are special membrane compartments where the ER and mitochondrial outer membrane form close contacts and allow non-vesicular lipid transfer. Despite this localization and confined catalytic activity, the bulk of membrane PS is not found in these sites; rather, it is enriched within the inner leaflet of the PM.

The enzymes CDP-diacylglycerol synthase (CDS)-1 and -2 that

generate CDP-diacylglycerol (CDP-DG) from PA are also localized to the ER [14], while a third CDS enzyme, Tam41, is a structurally-distinct peripheral membrane protein that associates with the mitochondrial inner membrane and generates CDP-DG, the precursor of mitochondrial PG and cardiolipin [15,16]. The phosphatidylinositol synthase (PIS) enzyme (also called CDIPT for CDP-diacylglycerol-inositol 3-phosphotransferase) that synthesizes PI from CDP-DG and myo-inositol is unique in that it is not only found in the ER proper but is also located in a highly mobile ER-derived membrane compartment, which appears to make contacts with all other organelles, including the PM [14]. The unique localization of the PIS enzyme requires its enzymatic activity as several point mutations that render the enzyme catalytically inactive eliminate its localization to the rapidly moving compartment [14]. The significance of this localization feature of the enzyme for PI synthesis is not clear at this point nor is it understood what other proteins or lipids are found in this unique membrane structure. At the same time, the distribution of PI among the various subcellular membranes is still very poorly understood.

PE, like PC, is synthesized by the ER-localized enzyme CEPT, but it is also made in the mitochondria via decarboxylation of PS. This latter reaction requires PS transport from the ER to the mitochondria. On the other hand, PE made in the mitochondria can find its way to be converted to PC again within the ER by the PE-methyl transferase (PEMT)



**Fig. 2.** The enzymatic reactions of PI synthesis. (A) The two enzymes that work in tandem are CDS1/2 that convert PA to CDP-DG and, PIS, which converts CDP-DG to PI. Note that the PIS-catalyzed step is reversible and hence, the PI lipid product has to be removed in order to keep the reaction flowing to the right. Yellow arrows show that the direction of the reaction is determined by substrate delivery and product removal. (B) The X-ray structures of bacterial homologues (or ancestors) of the two enzymes are shown with the active site (red star) facing the cytoplasmic aspect of the ER membrane. The structures of the *Thermotoga maritima* CdsA (PDB: 4Q2E) (the closest bacterial homologue of the CDS enzymes) and the *Renibacterium salmoninarum* PIP Synthase (PDB: 5D92) (the closest bacterial homologue of the PIS enzyme) are shown using Pymol for rendering.

enzyme (Fig. 1). A recent studies suggests that mitofusin2 (Mfn2), one of the proteins that plays a role in mitochondrial fusion, facilitates PS availability to the mitochondrial PSD enzyme [17]. Although Mfn2 was found to bind PS, the exact mechanism of how it works remains elusive.

Taken together, just by looking at these enzymatic pathways, it is obvious that lipid transfer between organelles is critical for the metabolic flow required for controlling lipid synthesis. Moreover, compartmentalization or metabolic channeling must exist in order to make sense of the otherwise seemingly futile or redundant enzymatic cascades.

## 2. Most phospholipid synthesizing enzymes are under feed-back inhibition

While the amounts of the enzymes required for phospholipid synthesis are subject to transcriptional regulation, their activities are also directly controlled by the lipid composition of the very membranes to which they are localized. Selected examples of these regulatory processes will be discussed below.

### 2.1. Phosphatidylserine synthesis

It was already recognized 10 years ago that PS synthesis is under strong feedback inhibition [18]. Studies on the cloned and expressed PSS1 and PSS2 enzymes showed that it was PSS2, rather than PSS1, that showed the marked inhibition by the enzymatic product, PS [18,19]. In light of these early studies, it was a surprising development that the *PTDSS1* gene that encodes human PSS1 was found mutated in the rare disease of Lenz-Majewski Syndrome in a manner that removed its end-product inhibition [20]. Cells of these patients display massive PS

synthesis and display PS on the outer leaflet of the PM and secrete large amounts of PS. This is most likely caused by the saturation of the PM-localized PS flippase that maintains the PS asymmetry between the inner and outer leaflets of the PM. Since extracellular PS is critical for the bone calcification process, excessive PS secretion is probably a major factor in the hyperostotic phenotype associated with affected patients. Importantly, cells expressing PSS1 harboring the patient mutations also displayed large amounts of PS on the cytoplasmic leaflet of the ER, a compartment where PS is not detected even in cells over-expressing the wild-type PSS1 enzyme [12]. The accumulation of PS in the cytoplasmic leaflet of the ER is caused by the overburdened non-vesicular PS transport machinery. Curiously, all of the identified PSS1 mutations are found in the luminal side of the multi-membrane spanning PSS1 enzyme based on topology predictions [20]. If the topology predictions are correct, it raises the question of which side of the ER membrane contains the enzyme active site and which part of the PSS1 enzyme is responsible for PS sensing. The corollary of this enzyme regulation is that the rate of PS synthesis is tightly coupled to PS removal from the ER and when this process is impaired, PS synthesis is rapidly inhibited [12].

### 2.2. Phosphatidylinositol synthesis

A different form of regulation is found with the PI synthesizing enzyme, PIS/CDIPT. This enzyme converts CDP-DG to PI using inositol in exchange for CMP. Curiously, the enzyme can also work in the reverse mode and, in the presence of PI and CMP, it will rapidly exchange the inositol headgroup; which explains the rapid [<sup>3</sup>H]-inositol labeling of PI in the presence of CMP [21,22]. For a while, this inositol exchange activity, which is stimulated by Mn<sup>2+</sup> in intact cells [23], was believed

to be separate from the PI synthase, until the cloning of PIS proved that the two activities belong to the same protein [24,25]. Although most of these studies have been done twenty or more years ago, they have important implications for understanding the control of PI synthesis, which is only now being realized. Namely, that the rate of PI synthesis is determined by the relative concentrations of the precursors CDP-DG and inositol as well as the enzymatic product, PI (Fig. 2A). This explains why the strongest stimulus of PI labeling with  $^3\text{H}$ -inositol is the phospholipase C (PLC)-mediated hydrolysis of  $\text{PI}(4,5)\text{P}_2$ , which ultimately yields an increased supply of both CDP-DG and inositol [26]. This, however, also means that PI and PA transport between the ER and the PM is a critical determinant of the PI synthetic rate. This PI/PA exchange is mediated by the Nir2 protein [27–29] (Fig. 1). PIS is an essential gene in yeast [30]; in vertebrates, PIS is required for survival of photoreceptor and lens epithelial cells [31]. There is no structural information available on mammalian PIS enzymes, but in yeast, the active site of the enzyme has been mapped facing the cytoplasm [32]. The X-ray structure of the *Renibacterium salmoninarum* PIP Synthase (the bacterial enzyme synthesizes PI3P from CDP-DG and inositol 3-phosphate) also suggests that the active site is facing the cytoplasm (Fig. 2B).

### 2.3. Phosphatidylcholine synthesis

The rate limiting step in PC biosynthesis is the generation of CDP-choline by the CCT enzymes. The two forms of CCT, CCT $\alpha$  and CCT $\beta$ , are encoded by separate genes, but CCT $\alpha$  is the more widely expressed form. CCT $\alpha$  is a soluble protein that is found primarily in the nucleoplasm, but it rapidly translocates to the nucleoplasmic reticulum, (a tubular membrane network connected to the nuclear envelope) when the relative PC content of membranes decreases (for original citations and more details on CCTs see [33]). CCT $\alpha$  also rapidly associates with LDs upon oleic acid loading, apparently through membrane-binding features that can detect the relatively PC-poor single layer phospholipid coat of LDs [7]. Specifically, membrane-association of CCT $\alpha$  is achieved through an amphipathic helix that somehow senses the ratio of PE/PC lipids present in membranes and membrane-association is important for increasing the enzyme's catalytic activity [34]. The important question raised by these observations is how this localization of CCT $\alpha$  provides PC to the respective membranes given the fact that the final PC synthesizing step is mediated by the ER- and Golgi-localized CEPT and CPT enzymes, respectively.

### 2.4. Phosphatidylethanolamine synthesis

PE is generated by two independent pathways, one of which is analogous to that of PC synthesis, whereby CDP-ethanolamine is generated by the phosphoethanolamine cytidyltransferase (ECT) enzyme, which is the rate-limiting step of this pathway. However, ECT differs from CCT $\alpha$  in that its activity is not controlled by membrane association. The final step of this pathway is the conjugation of CDP-ethanolamine with DG by the ER-localized integral protein, CEPT [35]. The other major pathway of PE synthesis is PS decarboxylation by the phosphatidylserine decarboxylase (PSD) enzyme, which is located in the outer leaflet of the inner mitochondrial membrane. It is notable that yeast also contains another PSD, Psd2, within the ER [36]. Importantly, the PSD enzyme requires the transport of PS from the ER to the mitochondria and further from the outer into the inner mitochondrial membrane. This entire process was found to be ATP-dependent [37]; however, more recent studies have shown that PS transport from the ER to the mitochondria does not require ATP or GTP [38]. While the mechanism responsible for the transport of PS from the ER to the mitochondria is not known, the transport of PS between the outer and inner mitochondrial membranes is mediated by the Ups2-Mdm35 protein complex [39]. As mentioned before, Mfn2 was found to facilitate PS availability to the mitochondrial PSD enzyme [17]. Some of the mitochondrial PE has been shown to exit the mitochondria and is

transported to the ER by a yet unknown mechanism where it can be converted to PC [40,41]. These lipid transport steps between the ER and the mitochondria occur in the MAMs, the contact sites formed between the two organelles, as the efficiency of these transport-coupled enzymatic steps are greatly reduced in yeast cells lacking members of the organelle tethering ERMES (endoplasmic reticulum (ER)-mitochondria encounter structure) complex [39].

## 3. Lipid transport is critical for the control of lipid synthesis

The picture that emerges from the above examples is that the synthesis of phospholipids must be tightly coupled to the transport processes that prevent accumulation of the lipid products at the site of synthesis. It is entirely possible, that the activity of lipid synthesizing enzymes were found highest in membrane preparations that contained contact sites, because the lipid products can be moved away from the ER in such preparations. Lipids can be transported out from the ER by lipid transfer proteins (LTPs) that often, although not always, function at membrane contact sites. Alternatively, lipids can also leave the ER by vesicular transport in the form of complete budded membranes. Non-vesicular lipid transport is much more selective than bulk membrane transport and has received an immense amount of attention in recent years.

### 3.1. Non-vesicular lipid transport at membrane contact sites

Several excellent recent reviews have covered non-vesicular transport extensively [11,42,43]. Therefore, we will discuss this topic only from the standpoint of lipid dynamics at the ER. An early indication that phosphoinositide lipids are important for non-vesicular lipid transport came from studies that identified relatively specific binding of PI4P to several pleckstrin-homology (PH) domains found within LTPs [44,45]. The connection to PI4P was further established, and the critical role of non-vesicular lipid transfer relative to vesicular trafficking was emphasized, by the discovery of the ceramide transfer protein, CERT, and its central importance in supplying ceramide from the ER for sphingomyelin biosynthesis in the Golgi [46]. However, the real breakthrough in understanding the importance of PI4P for non-vesicular lipid transfer came from the realization that OSBP and its yeast orthologue, Osh4/Kes4, use PI4P gradients between the Golgi and ER for driving cholesterol export from the ER to Golgi [47,48]. This gradient is set up by phosphatidylinositol 4-kinase (PI4K) beta (PI4KB and Pik1 in yeast) on the Golgi side, and perhaps different PI4K, PI4K2A functioning in the TGN. In all cases, the Sac1 phosphatase that dephosphorylates PI4P in the ER ensures that PI4P is very low in the ER [48,49]. This counter transport is based on the ability of the lipid transfer domain, which is named ORD in the case of OSBP and its homologues, to bind either PI4P or cholesterol, but not both at the same time, within the same lipid-binding pocket [47]. A recent study found that cholesterol transport from the ER directly to the PM is also mediated by another OSBP-related protein (ORP), ORP2, and suggested that the inositol lipid that is counter transported in the opposite direction is  $\text{PI}(4,5)\text{P}_2$ , rather than PI4P [50].

The importance of PI4P gradients was subsequently shown in the context of PS transport from the ER to the PM. Here, the PI4KA enzyme generates PI4P in the PM and the Sac1 phosphatase facilitates the rapid removal of PI4P within the ER. This process involves ORP5 and ORP8 in mammalian cells and Osh6 and perhaps some close relatives in yeast [51–53]. Due to the tight connection between PS transport and PI4KA function, inhibition of the PI4KA enzyme also halts PS synthesis [12]. In addition, PS has a strong stimulatory effect on the activity of the ER-localized Sac1 phosphatase [54]. The consequence of this regulation is that excess PS production, as in the case in the Lenz-Majewski PSS1 mutations, stimulates Sac1 activity and therefore affects the PI4P gradient across all ER contact sites causing secondary alterations in the transport of other lipids that rely upon the PI4P-driven



countertransport mechanism [12].

The fact that PI4P can be removed from the PM by ORP5 and ORP8 has important implications to the production of the PI(4,5)P<sub>2</sub> within the PM, for which PI4P also serves as a precursor. The fact that PM recruitment of the N-terminal PH domain and adjacent polybasic motifs of ORP5 and ORP8 relies on the levels of both PI4P and PI(4,5)P<sub>2</sub> in the PM ensures that when PI4P or PI(4,5)P<sub>2</sub> levels are low, these LTPs cease working. Alternatively, when PI(4,5)P<sub>2</sub> levels are increasing, ORP8 joins ORP5 and by delivering more PI4P to the ER, they limit the amount of PI4P available for PI(4,5)P<sub>2</sub> production [55]. In addition, ORP8 can directly remove PI(4,5)P<sub>2</sub> from the PM [56]. These findings have highlighted the close connection between the regulation of PI4P and PI(4,5)P<sub>2</sub> levels within the PM and the control of PS transport, which directly informs the rate of PS synthesis.

It has also been suggested that non-vesicular lipid transport at membrane contact sites was important for the control of PI synthesis and its integration into the cyclic model of inositol lipid-based signal transduction. As pointed out above in Section 2.2, PI synthesis in the ER is controlled by the relative abundance of both the substrate, PA (as a precursor of CDP-DG) and the product, PI. It has been known for a long time that the strongest stimulus of PI labeling by either <sup>32</sup>P-phosphate or <sup>3</sup>H-inositol was receptor-mediated activation of PLC [26]. Since PI is consumed in the PM as PLC hydrolyses PI(4,5)P<sub>2</sub> that needs continuous replenishment from PI and PA is also generated in the PM during this process as DG is converted to PA, it has been postulated that a transport of PI from the ER to the PM and PA in the reverse direction was necessary for increased PI synthesis to occur in the ER [26]. As mentioned above, recent studies have found that the LTP Nir2 can fulfill both of these transport functions [27–29] as it is specifically recruited to ER-PM contact sites during PLC activation [57].

Additional mechanisms for PI transfer from the ER to the PM have been suggested by studies characterizing TMEM24, which tethers the ER to the PM and is important for Ca<sup>2+</sup> signaling as well as insulin release from pancreatic beta cells [58,59]. TMEM24 has an synaptotagmin-like mitochondrial-lipid-binding (SMP) domain, a characteristic feature of organelle-tethering proteins that are found in membrane contact sites [60,61]. SMP domains are thought to act as hydrophobic tunnels capable of lipid transport along concentration gradients [62]. The SMP domain of TMEM24 was found to deliver PI from the ER to the PM during glucose-induced secretion [58]. In contrast, the SMP domain of the family of extended synaptotagmins (E-Syt1/2/3) appears to be important for DG transport from the PM to the ER during high Ca<sup>2+</sup>-induced PLC activation [63]. The unique localization of the PIS enzyme not only in the ER proper but in a highly mobile vesicular structure that contacts various membranes, and also spends extended periods (minutes) in repeated contacts with the PM, raised the possibility that PI transfer may also take place in more dynamic contact sites between the PM and this unique organelle that function in addition to the more classical ER-PM contacts discussed above [14].

There is a common theme in these examples, namely the close connection between all these lipid transport processes and PI or its phosphorylated derivatives. The reliance of many lipid transport processes on PI4P gradients really calls for a better understanding of the transport pathway(s) responsible for the redistribution of PI between membranes. The long-known mammalian PI transfer proteins (PITPs) and the Sec14-related proteins in yeast have been the logical candidates to fulfill this function since they have been shown to be able to transport PI (and PC) between membranes *in vitro*. However, it has been more difficult to identify their PI transfer functions *in vivo* within intact cells despite large amount of work devoted to study these proteins. As already mentioned, the Class II PITPs, Nir2 and Nir3 (and their fly orthologue, RdgBc) has been identified as PA/PI lipid exchangers at ER-PM contacts during PLC activation, but it is not clear if that is all that these proteins do since Nir2 was also found to be critical for the maintenance of DG levels in the Golgi [64]. Whether Sec14 or the Class I PITPs work as bona fide PI transfer proteins, however, has been

challenged. It has been suggested that these proteins work by presenting PI to specific PI kinases [65,66] and even function in very specific biological contexts [67]. Most lipid transfer proteins work in membrane contact sites, but there is no reported enrichment of Class I PITPs in such sites, although the TGN localization of PITPβ [68] could reflect localization to ER-Golgi contact sites. It has also been speculated that the ER-derived mobile PIS organelle (see above) that makes ample contacts with the different membranes may be a way PI is delivered to those membranes. Since no membrane fusion has been detected between the PIS organelle with any membranes, the transfer of lipid would still require a PI transfer protein [14].

PITPs have been covered over the years in many excellent reviews (for two recent ones see [69,70]) and will not be discussed in further details.

### 3.2. Lipid regulation of vesicular transport at the ER

The assembly of COPII coat components and their roles in ER membrane budding in the process of Golgi-destined vesicular carrier generation has been elegantly worked out [71]. What is less understood, however, is how lipids contribute to the control of this process. That said, the lipid-modifying enzyme, phospholipase D (PLD) was found to be critical for COPII vesicle formation and cargo exit from the ER [72]. It has also been shown that Sar1, the small GTPase that is first recruited to the ER and marks the site of COPII coat formation, stimulates PLD activity thereby enriching PA at these sites [73]. Sar1 activation, which includes insertion of its N-terminal helix into the ER membrane, recruits the Sec23-Sec24 heterodimer as well as additional coat proteins that function to generate the curvature of the growing bud (for details see [71]). Importantly, a Sec23-interacting protein (Sec23ip, also called p125A), which is also enriched in ER exit sites, is a member of a family of three proteins that possess DDHD domains. (DDHD domains are 180 amino acid protein modules that selectively binds PA and can also be found in the lipid transfer proteins, Nir2/3 [57]). The other two proteins of this family, DDHD1 and DDHD2 are phospholipase A1 (PLA) enzymes capable of generating lyso-PI and lyso-PA, respectively (PLA activity has not been demonstrated for Sec23ip/p125 [74]. That said, Sec23ip/p125 was found to be important in the functional assembly of ER exit sites [75,76] and was shown to depend on both PA and PI4P [77]. Sar1 was also shown to promote the local generation of PI4P, a lipid that promoted Sar1-induced membrane constriction and COPII assembly in an *in vitro* ER-budding assay, presumably through the activity of one class of the four PI 4-kinases (PI4Ks), namely the type II PI4Ks [73]. Another report found that the number of ER exit sites was decreased in cells depleted in another PI4K, the type III PI4K, PI4KA [78], although this effect could be secondary as many changes to cellular lipid levels have been shown to follow PI4KA depletion [79]. These findings suggest that lipid remodeling by lyso-lipid generating enzymes likely plays a role in helping to shape the membrane at ER exit sites. Indeed, a recent study showed that lyso-lipids, and Lyso-PI (16:0) in particular, could rescue *Sec-14* temperature-sensitive mutant yeast strains and that this lipid also increased binding of COPII proteins to membranes as well as lowered the rigidity of reconstituted in giant unilamellar vesicles (GUVs) [80].

Another interesting piece of information related to the ER exit process is the finding that expression of PIS-GFP, but not mutant forms that eliminate enzymatic activity, generate rapidly moving ER-derived vesicles that do not contain any of the classical ER-markers but are enriched in the PIS enzyme itself [14]. This process also requires Sar1 and Arf1 [14]. This dynamic membrane compartment was found to contain other lipid synthesizing enzymes, such as CEPT1 and is regulated by Rab10 [81]. Another report showed that the LYCAT enzyme, which remodels acyl-chains in PI and specifically contributes to the enrichment of PI(4,5)P<sub>2</sub> in arachidonic acid at the sn-2 position, also co-localizes with PIS in this unique membrane structure [82]. The importance of these observations is not clear at present, but they also

point to the possible role of lyso-lipids and lipid remodeling in membrane budding processes.

#### 4. Concluding remarks

The specific lipid composition of membranes is critical for defining organelle identity and the proper assembly and function of protein complexes that work on those organelles. Lipid synthesis and distribution are fine-tuned to maintain the unique lipid composition of membranes. The product inhibition characterized in most lipid synthesizing enzymes makes lipid transport processes not only important for lipid distribution, but also for the dynamic regulation of lipid synthesis. Non-vesicular lipid transfer, often confined to membrane contact sites, provides both the speed and specificity requisite for the intricate control of lipid transport and synthesis. It is also important that non-vesicular lipid transport only samples the cytoplasmic leaflet of the membrane bilayer, which clearly contrasts vesicular transport, which carries both membrane layers. However, it is still not clear whether tethering proteins only form the molecular architecture of membrane contact sites or if they also play direct role(s) in the lipid-transfer process. Tethering proteins that have SMP domains possess the structural features that could serve as hydrophobic lipid tunnels bridging the aqueous phase between two membranes but do not explain how specificity of lipid transport can be achieved. The recent developments pointing to the importance of PI4P gradients in driving many non-vesicular lipid transport processes raise the question of how general this principle is among the various LTPs. Additionally, even for LTPs with defined lipid-binding pockets, there are still outstanding questions concerning cargo specificity and many LTPs may be more promiscuous in their selection of lipid cargoes than currently believed. In fact, it is becoming apparent that the local composition of the membranes between which LTPs operate may ultimately determine what lipid transport processes they support. This possibility places a special emphasis on methods that analyze lipid transfer processes in situ using intact cells, which will complement the in vitro lipid transfer assays already employed by many studies. Another important point to consider is the speed of these processes, which makes it extremely difficult to analyze them inside living cells as a new steady state can be established extremely quickly when lipid changes are induced. These challenges will require improvement of methods as well as a new way of thinking about old problems in order to make significant progress in this exciting research field.

#### Transparency document

The [Transparency document](#) associated with this article can be found, in online version.

#### Declaration of Competing Interest

The Authors declare no conflict of interest related to this work.

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