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REVIEW

Mechanisms of nonvesicular lipid transport

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We have long known that lipids traffic between cellular membranes via vesicles but have only recently appreciated the role of nonvesicular lipid transport. Nonvesicular transport can be high volume, supporting biogenesis of rapidly expanding membranes, or more targeted and precise, allowing cells to rapidly alter levels of specific lipids in membranes. Most such transport probably occurs at membrane contact sites, where organelles are closely apposed, and requires lipid transport proteins (LTPs), which solubilize lipids to shield them from the aqueous phase during their transport between membranes. Some LTPs are cup like and shuttle lipid monomers between membranes. Others form conduits allowing lipid flow between membranes. This review describes what we know about nonvesicular lipid transfer mechanisms while also identifying many remaining unknowns: How do LTPs facilitate lipid movement from and into membranes, do LTPs require accessory proteins for efficient transfer in vivo, and how is directionality of transport determined?

Introduction

Eukaryotic cells contain diverse membranes, each with a characteristic and carefully regulated protein and lipid content. Most membrane proteins are first inserted into the ER and then traffic among cellular compartments in vesicles (Mellman and Warren, 2000). The same is true of lipids—most are synthesized in the ER and are then exchanged between organelles in vesicles (Vance, 2015). Lipids, however, are also moved between organelles by nonvesicular pathways. This type of transport has a number of functions. One is the bulk transfer of lipids sufficient to sustain organelle biogenesis. For example, mitochondria membrane biogenesis requires the import of most lipids (Acoba et al., 2020). Nonvesicular lipid transport also allows cells to change the lipid composition more rapidly and precisely than is possible by vesicular trafficking or during stress conditions when vesicular trafficking is compromised. It can be used to enrich or deplete membranes of particular lipids, either to modulate the lipid composition of a membrane or to regulate levels of signaling lipids.

Nonvesicular lipid exchange within cells could, in theory, occur by two mechanisms. One is lipid diffusion between membranes following hemifusion, where the outer leaflets of two bilayers merge. While hemifusion is thought to occur transiently during membrane fusion, there is no evidence that this mechanism is used to move lipids between cellular compartments that do not fuse. The second type of mechanism is transport by proteins known as lipid transport proteins (LTPs). These proteins have the ability to move lipids between membranes via hydrophobic cavities that shield the lipids from the aqueous environment during transport. There are many families of LTPs, with most eukaryotic cells expressing multiple members of each family (Chiapparino et al., 2016; Wong et al., 2019). Although most of the known transporters function as shuttles that carry single lipid molecules between membranes, others serve as bridges that allow lipids to flow between membranes (Li et al., 2020). Mutations in some LTPs are known to cause diseases (Table 1).

Most LTPs operate at membrane contact sites (MCSs), regions where two organelles are closely apposed. Localization at these sites could serve to speed transport by reducing the distance that LTPs must diffuse as they shuttle lipids between membranes. However, some LTPs do not operate at MCSs, (e.g., STARD4 [Mesmin et al., 2011] and ORP2 [Wang et al., 2019]), and it has been argued that LTP diffusion is not the rate-limiting step of lipid transport (Dittman and Menon, 2017). The enrichment LTPs at MCSs could have functions other than increasing the transport rate, such as facilitating LTP interaction with proteins that modulate lipid transport or restricting lipid exchange to a specific pair of organelles.

This review focuses on our emerging understanding of how nonvesicular lipid transport occurs and identifies important challenges and unanswered questions in the field. It begins by summarizing what we know about the rates and volumes of lipid exchange in cells and the general structural features of LTPs. We then discuss open questions about mechanisms of lipid transport and what drives lipid transport.

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Table 1. **Diseases caused by mutations in LTPs**

LTP	Disease	References
α-Tocopherol transfer protein	Ataxia with isolated vitamin E deficiency (OMIM 277460)	Ouahchi et al., 1995
Microsomal triglyceride transfer protein	Abetalipoproteinemia (OMIM 200100)	Shoulders et al., 1993
Niemann-Pick C1 protein	Niemann-Pick disease type C1 (OMIM 257220)	Carstea et al., 1997
Niemann-Pick C2 protein	Niemann-Pick disease type C2 (OMIM 607625)	Naureckiene et al., 2000
Steroid acute regulatory protein	Lipoid congenital adrenal hyperplasia (OMIM 201710)	Lin et al., 1995
Vps13A	Chorea-acanthocytosis (OMIM 200150)	Rampoldi et al., 2001
Vps13B	Cohen syndrome (OMIM 216550)	Kolehmainen et al., 2003
Vps13C	Early onset Parkinson's disease (OMIM 616840)	Lesage et al., 2016
Vps13D	Spastic ataxia (OMIM 607317)	Gauthier et al., 2018; Seong et al., 2018

OMIM, Online Mendelian Inheritance in Man number.

Volume and rates of nonvesicular lipid transport in cells

Cells have considerable capacity to move some types of lipids between organelles by nonvesicular pathways. This was suggested by early studies on lipid transport from the ER, where most lipids are synthesized, to the plasma membrane. These studies concluded that newly synthesized glycerophospholipids, cholesterol, and glucosylceramides are transferred to the plasma membrane by nonvesicular mechanisms, because lipid transport was not blocked by inhibiting vesicular trafficking and occurred at rates that were too rapid to be explained by vesicular trafficking (Kaplan and Simoni, 1985a; Kaplan and Simoni, 1985b; Sleight and Pagano, 1983; Vance et al., 1991; Warnock et al., 1994). There is also evidence that exogenous sterols, which first enter cells by incorporating into the plasma membrane, are rapidly exchanged between the plasma membrane and other organelles by nonvesicular transport pathways. When the naturally fluorescent sterol, dehydroergosterol (DHE), is added to cells, it equilibrates between the plasma membrane and endocytic recycling compartments in minutes, and it has been estimated that there are ∼10^6 molecules of DHE exchanged between these organelles per second in CHO cells (Maxfield and Mondal, 2006). This is a remarkable volume of transport given that there are ∼3 × 10^8 cholesterol molecules in the plasma membrane of this cell type, suggesting that these cells have the capacity to exchange all the cholesterol in the plasma membrane in ∼5 min. This volume of DHE transport is more than can be explained by vesicular trafficking.

There must also be a substantial amount of nonvesicular lipid transport to such organelles as mitochondria and chloroplasts, which are largely disconnected from vesicular trafficking pathways. This has been termed bulk transport, since the function is to provide sufficient lipid to support membrane expansion (Fig. 1 A). To put in perspective the volume of nonvesicular phospholipid transport required to sustain membrane biogenesis, consider mitochondrial membrane biogenesis in the yeast *Saccharomyces cerevisiae*. It has been estimated that mitochondrial biogenesis requires the transport of ∼20,000 phospholipids per second when this yeast is growing at top speed (doubling every 2 h; Petrungaro and Kornmann, 2019). A similarly high rate of phospholipid transport is required for the maturation of nascent autophagosomes, which can occur in minutes and has been estimated to require the transport of ∼10^8 lipids per cell (Melia et al., 2020).

In addition to bulk transport to support membrane expansion, cells use lower-volume transport in three ways (Fig. 1 B). One way is to support phosphoinositide (PIP) signaling and other signaling that uses lipid messengers. For example, PIP signaling at the plasma membrane requires the movement of phosphatidylinositol (PI) from the ER, where it is synthesized, to the plasma membrane to replenish PIP pools (Pemberton et al., 2020). Transport of PIPs out of the plasma membrane by LTPs, hypothetically, could also serve to attenuate PIP signaling. Nonvesicular lipid transport may also be required to enrich specific lipids in an organelle (Fig. 1 B). For example, nonvesicular transport of phosphatidylserine (PS) from the ER to the plasma membrane is required to enrich PS in the plasma membrane (Chung et al., 2015; Moser von Filseck et al., 2015a). Similarly, nonvesicular cholesterol transport from the ER to the Golgi complex is necessary to maintain cholesterol levels in the Golgi complex (de Saint-Jean et al., 2011; Mesmin et al., 2013). Both of these processes are driven by what has been termed counter-exchanging transport. This and other mechanisms of determining the directionality of lipid transport will be discussed later in this review. A third function for low-volume lipid transport is to regulate membrane organization at membrane contact sites (Fig. 1 B). In yeast, sterol-transporting LTPs at contact sites between the ER and vacuole—an organelle similar to lysosomes in higher eukaryotes—are necessary for the formation of sterol-enriched domains on the vacuole membrane during stress (Murley et al., 2017). More recently, it has been found that lipid trafficking by LTPs at contact sites between the ER and plasma membrane may promote the assembly of lipid nanodomains in the plasma membrane, which is critical for regulating vesicular trafficking to that compartment (Nishimura et al., 2019). It has been suggested that lipid microdomains are found at many contacts sites (King et al., 2020), and their formation may be driven by lipid transport by LTPs at these sites.

While these and other studies show there is significant nonvesicular lipid transport at some MCSs, it is important to

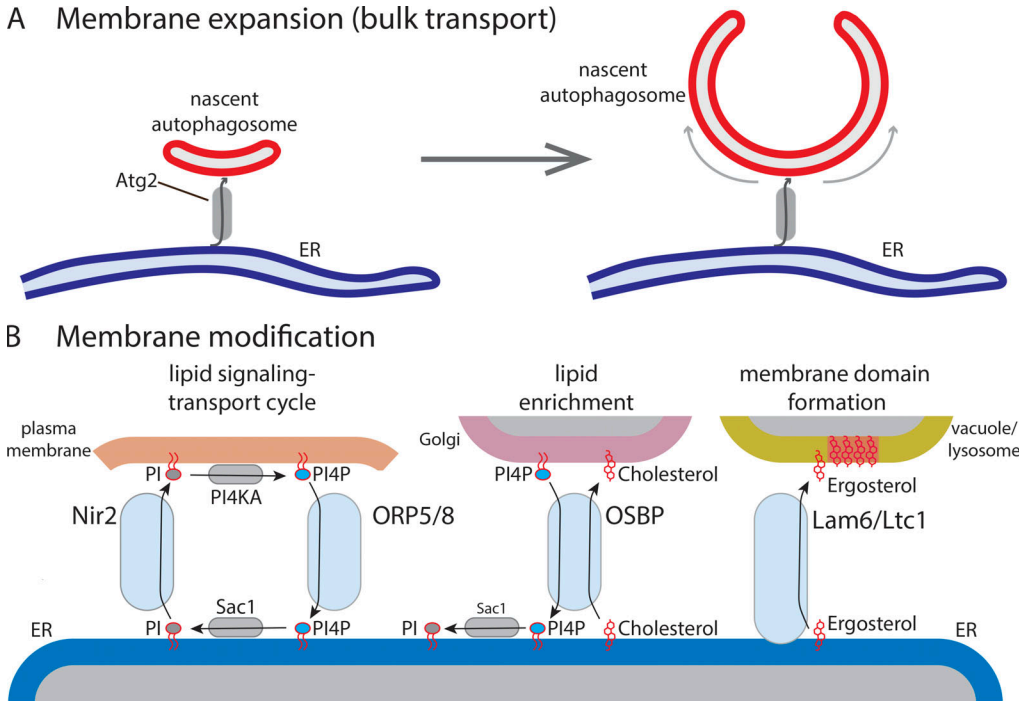

Figure 1. **Functions of nonvesicular lipid transport. (A)** High-volume lipid transport required for membrane expansion (bulk transport). Shown for growth of an autophagosome and also necessary for biogenesis of mitochondria and chloroplasts. **(B)** Types of lower-volume lipid transport. Representative examples of three functions are shown. One is to support lipid-based signaling (left). The protein Nir2 transfers PI from the ER to the plasma membrane, where it is converted to PI4P by the enzyme phosphatidylinositol 4-kinase-α (PI4KA). PI4P can removed from the plasma membrane by ORP5 or ORP8, which bring it to the ER, where it is hydrolyzed by the phosphatidylinositide phosphatase, Sac1. Lipid transport can also regulate the levels of a specific lipid. For example, OSBP uses counter-exchange transport to enrich cholesterol in the Golgi membrane (center). Lipid transport can also support membrane domain formation. For example, Lam6/Ltc1 brings the sterol, ergosterol, to the vacuole in *S. cerevisiae* and supports membrane domain formation there (right; domain in red).

note that, for most MCSs, we still know little or nothing about how much lipid exchange occurs.

Studying nonvesicular lipid exchange in cells

Our knowledge of nonvesicular lipid trafficking in cells is incomplete because following the movements of lipids in cells is challenging. Four types of approaches are used and each has notable limitations (Table 2). One is to use microscopy to follow the trafficking of fluorescent lipids in cells. These lipids, which are added exogenously to cells, contain fluorescent groups, like boron-dipyrromethene, for example (Marks et al., 2005), or are naturally fluorescent, like DHE (Hao et al., 2002). However, boron-dipyrromethene and other fluorescent groups can have biophysical properties that can make their intracellular trafficking and metabolism different from endogenous lipid—they can also perturb cells (Maekawa and Fairn, 2014). New tools for following lipid movements in cells are being rapidly developed that may overcome some of these limitations (Bumpus and

Table 2. **Approaches used to study lipid trafficking in cells**

Approach	How it works	Advantages	Caveats
Fluorescent lipids	-Fluorescent lipids added to cells -Trafficking assessed by microscopy	-Monitor trafficking in real time in live cells	-Fluorescent groups can alter physical properties and metabolism of lipids -Fluorescent lipids may affect cells
Labeling & fractionation	-Labeled lipid precursors added to cells -Cells fractionated	-Allows simultaneous analysis of transport and metabolism -Direct measurement of lipid levels in organelles	-Fractionation challenging -Analysis requires cell destruction
Lipid modification	-Lipid modifying enzyme localized in compartment outside synthesis site -Labeled lipid precursors added to cells	-Does not require fractionation	-Lipid modification must be faster than transport -Modified lipids may affect cells -Requires effective localization of modifying enzyme -Analysis requires cell destruction
Lipid sensors	Fluorescent or luminescent sensors indicate changes in membrane lipid composition	-Monitor trafficking in real time in live cells -Can detect small changes in membrane lipid composition	-Factors other than lipid concentration may affect sensor binding -Sensors may perturb membranes

Baskin, 2018). A second approach (Table 2) is to track lipids from their site of synthesis to other organelles. Radiolabeled or stable isotope–labeled lipid precursors are added to cells, and the transport of newly synthesized lipids is assessed by fractionating cells. This approach was first used in early studies of lipid transport to the plasma membrane (Kaplan and Simoni, 1985a; Kaplan and Simoni, 1985b); however, fractionation can be laborious and it is often difficult to obtain pure fractions. A third approach (Table 2) uses lipid modification in cells to indirectly measure the transport of newly synthesized lipids without the need for cellular fractionation. This approach requires localizing a lipid-modifying enzyme outside the organelle where lipid synthesis occurs. Lipid modification indicates that the newly synthesized lipid has been transported from its site of synthesis to the organelle containing the lipid-modifying enzyme. For example, PS transport from the ER to mitochondria has been estimated by measuring the conversion of newly synthesized PS, which is made in the ER, to phosphatidylethanolamine, which is catalyzed by an enzyme in mitochondria (Vance, 2015). There are a number of caveats to this type of approach: Lipid transport may not be the primary factor determining the rate of lipid modification, modified lipids may affect membrane function, and ensuring that the lipid-modifying enzyme is only, or mostly, active in the desired organelle can be challenging. A fourth approach (Table 2) is to use fluorescent lipid-binding proteins, often called lipid sensors, to measure changes in the lipid content of a membrane, which can occur as a result of lipid transport. These sensors contain a fluorescent protein fused to a protein domain that binds membranes containing a specific lipid (Wills et al., 2018). For example, some pleckstrin homology domains bind membrane containing specific PIP species. Pleckstrin homology domains fused to fluorescent proteins have been used to measure changes in PIP levels in cellular membranes (Várnai et al., 2017). There are important caveats to this approach. One is that the sensor can perturb membranes, and another is that the membrane affinity of some lipid sensors is determined by factors in addition to ligand concentration (Várnai et al., 2017). In addition, membrane binding by a sensor may be affected by changes in the availability of the lipid in a membrane, rather than changes in the lipid concentration of the membrane.

The lipid transport machinery

As noted before, LTPs generally fall into one of two major categories, acting either as shuttles or as bridges to facilitate lipid movement through the cytosol between membranes (Fig. 2 A). Both types of lipid transporter feature a hydrophobic cavity that shields lipids as they transit the cytosol, though the cavity size differs. Shuttles mostly resemble cups in their overall shape, typically accommodating a single lipid moiety within the cup cavity, and often they have a lid that closes over the lipid once it has bound. Cup-like transporters first associate with the donor organelle to select and extract the cargo lipid and then ferry the lipid to and associate with the acceptor membrane, finally inserting the lipid there. In its open form, the lid may facilitate transporter association with organelle membranes, and hence lipid extraction from or deposition to the membrane, or—not mutually exclusive—the lid can play a role in the recognition of cargo lipids. The structure and function of these transporters, including the oxysterol-binding protein (OSBP)–related proteins (ORP/OSH), the StARkin, and some members of the tubular lipid binding proteins (TULIP) superfamily, have been excellently described (Wong et al., 2019). These cup-like transporters usually recognize either a single class of lipid or a limited subset of lipids, consistent with a role for these proteins in tweaking the membrane lipid compositions of different organelles (Fig. 2 B). The most efficient shuttles transfer approximately one phospholipid per second, at least in reconstituted systems (de Saint-Jean et al., 2011; Moser von Filseck et al., 2015a), too slow to yield the high-volume bulk lipid transport required for organelle biogenesis. It is possible that lipid-shuttling LTPs transport at higher rates in cells than they do in vitro. Factors that could boost or regulate LTP transport are rate discussed in the next section.

High-volume bulk lipid transfer could instead be mediated largely by bridge-like transporters, which may be able to transport lipids at significantly higher rates than cup-like LTPs. Previously known only in bacteria, these were recently also found in eukaryotes, and, so far, only a handful have been identified. Whether the endoplasmic reticulum–mitochondria encounter structure (ERMES) complex, which comprises several TULIP modules strung together into a tube and that mediates glycerolipid transport between the ER and mitochondria in yeast, is a shuttle or bridge-like transporter is under active discussion (Kornmann, 2020). Currently, the best studied of the bridge-like transporters are vacuolar protein sorting 13 homologue (Vps13) and autophagy-related protein 2 (Atg2; Chowdhury et al., 2018; De et al., 2017; Kumar et al., 2018; Li et al., 2020; Maeda et al., 2019; Osawa et al., 2019; Valverde et al., 2019), both of which are conserved across all eukaryotes. Most likely, they are evolutionarily related as they share a ∼120-residue chorein-N motif that forms part of the lipid transport module at their very N terminus (Kumar et al., 2018; Muñoz-Braceras et al., 2015; Osawa et al., 2019). They are large proteins long enough to span the 10–30-nm space between apposed membranes at membrane contact sites, allowing lipids to traverse the cytosolic space via a long hydrophobic groove that accommodates many lipids at once (Fig. 2 C). In contrast to the shuttles, these proteins may remain stably associated with both donor and acceptor membrane throughout the transfer process. Lipids are extracted from the donor membrane, then move along the bridge rather than occupying a well-defined binding site, as in cup-like proteins, and finally insert into the acceptor membrane. Vps13 resembles a bubble wand at low resolution, with a stem and a loop at one end, the latter probably corresponding to a predicted C-terminal WD40 (beta-transducin repeat) domain that plays a role in localization (De et al., 2017; Fig. 2 C). ATG2 is smaller, lacking the WD40 (beta-transducin repeat) domain and the corresponding "loop" (Chowdhury et al., 2018). A low-resolution cryo-EM reconstruction of ATG2 revealed a groove running along its entire length that could potentially serve as a track for lipids (Valverde et al., 2019; Fig. 2 C). A higher-resolution reconstruction for a ∼160-kD N-terminal fragment of Vps13 (∼40% of the protein) shows a series of β-strands assembled into a taco-like shell (Fig. 2 C) lined with hydrophobic residues along its entire length (Li

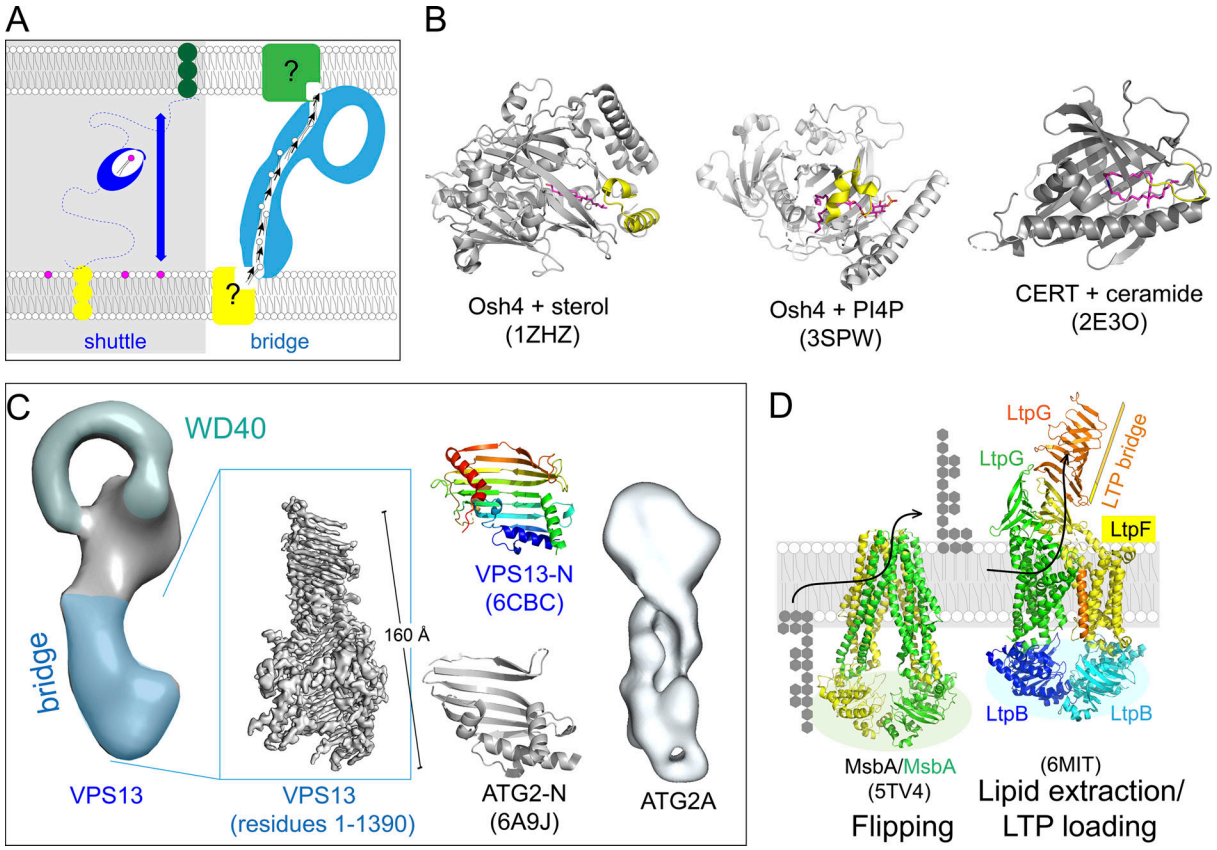

Figure 2. **Lipid transport machinery. (A)** Schematic of a shuttling LTP (left) and a bridge-like LTP (right). A shuttling LTP (blue) extracts lipid monomers from one bilayer and then diffuses to a second bilayer and delivers the lipid. Bridge-like LTPs (light blue) form conduits that allow lipid molecules to flow between membranes. **(B)** Cup-like lipid transport modules with lipid bound. Osh4 can bind either sterol or PI4P (magenta) in the same pocket, with slight rearrangements in the lids (yellow). Osh4 is shown in different orientations. The StART-domain of CERT is shown with ceramide (magenta) bound. Protein Data Bank accession nos. are indicated: 1ZHZ, 3SPW, 2E3O, 6CBC, 6A9J, 5TV4, and 6MIT. **(C)** Bridge-like lipid transporters. From left to right: Intact VPS13 structure at ∼30-Å resolution by negative stain EM (De et al., 2017; courtesy of Y. Skiniotis, Stanford School of Medicine, Stanford, CA); cryo-EM structure of the N-terminal 160 kD of VPS13 showing it forms a tunnel (EMD-21113); ribbons representations of the VPS13 and ATG2 N-terminal fragments, showing they have the same fold; and ∼18-Å resolution cryo-EM structure of intact ATG2. **(D)** The LPS transport system in the inner membrane of *E. coli*, showing the flippase MsbA, which flips LPS from the inner to the outer leaflet of the membrane, and part of the transporter, which features an integral membrane portion that helps to load lipid into the bridge-like portion (indicated). ATPase domains in MsbA and in the LPS transporter are highlighted (light green and light blue).

et al., 2020). Both Vps13 and Atg2 bind to glycerolipids apparently nonspecifically (Kumar et al., 2018; Valverde et al., 2019), with the lipid fatty acyl moieties presumably bound within the taco and hydrophilic headgroups exposed in the solvent. In VPS13, the taco shell is widest at the N-terminal end and narrows toward the C-terminal end, where lipids most likely would be accommodated in a single file as they flow through the protein. Among other roles, both Vps13 and Atg2 play roles in membrane expansion. They are critical for the biogenesis of at least three double-membrane, cup-shaped structures: the yeast pro-spore membrane during sporulation, the acrosome that forms at the tip of spermatids (Da Costa et al., 2020), and the autophagosome (Park and Neiman, 2012; Suzuki et al., 2013b); Vps13A in humans is proposed to play a role in ER–mitochondrial glycerolipid exchange (Kumar et al., 2018).

While we argue here that bridge-like LTPs may transport significantly faster than shuttling transports in cells, it is important to note that, to date, this has not been found in vitro (Kumar et al., 2018; Li et al., 2020; Osawa et al., 2019; Valverde et al., 2019). We consider it likely that these proteins do not function in isolation, but rather as part of lipid transport systems, as considered below. Still unidentified, the additional components have not been included in assays in vitro. In addition, one study (Valverde et al., 2019) shows that an N-terminal fragment of the bridge-forming LTP Atg2 can rescue the full-length protein when 10-fold overexpressed in cells. This fragment likely can function as a shuttle, probably because the Atg2 N terminus is also responsible for Atg2 localization (Tamura et al., 2017). How both shuttling and bridge-forming LTPs work in conjunction with other proteins in cells remains to be determined.

Studies of eukaryotic lipid transfer have focused primarily on the ability of LTPs to move lipids between membranes, either working alone or together with integral membrane proteins, like the vesicle-associated membrane protein–associated proteins (VAPs), which are ER-resident proteins. There has been little investigation of roles for integral membrane proteins or domains as active participants in lipid transfer rather than simply

as scaffolds to localize LTPs. In contrast, LTPs responsible for lipid transport across the periplasm in gram-negative bacteria frequently function as components of systems, including several multispan integral membrane components, which facilitate lipid extraction from or insertion into membranes. For example, lipopolysaccharide (LPS) transport across the periplasm involves a β-strand bridge—comprising the proteins LptC, LptA, and soluble portions of LptD—reminiscent of VPS13, with a groove lined with hydrophobics (Li et al., 2019; Owens et al., 2019; Fig. 2 D). Additionally, the LPS transport system includes an ATP-binding cassette transporter embedded in the inner membrane that selectively loads LPS onto the bridge, ensuring directional flow toward the outer membrane, and, further, an integral membrane protein in the outer membrane, LptD, which facilitates lipid transfer from the inner to the outer leaflet of this membrane (Sperandeo et al., 2017). Collaborations between a bridge-like transporter or a shuttle, respectively, and integral membrane proteins are also observed in the case of the *Escherichia coli* Pql and Mla phospholipid transport systems (Ekiert et al., 2017; Isom et al., 2020).

Similarly, lipid transfer in eukaryotes by bridge-like transporters and some shuttling transporters might also involve integral membrane partners. Indeed, multispan integral membrane proteins were recently discovered to play a role in some instances of cholesterol transfer. Cholesterol transfer from the lumen of lysosomes to the lysosomal membrane has been particularly well characterized and requires Niemann-Pick proteins, NPC1 and NPC2. NPC2 is a cup-like LTP shuttle that solubilizes sterols in the lysosomal lumen and hands them off to the luminal domain of NPC1, which also has a membrane-embedded portion. The luminal domain of NPC1 forms a hydrophobic channel that funnels cholesterol through the lysosomal glycocalyx, a carbohydrate-enriched coating that covers the inside surface of lysosomes. The cholesterol is then handed off to the multipass membrane domain of NPC1, which facilitates membrane insertion (Gong et al., 2016; Li et al., 2016; Winkler et al., 2019). A beautiful recent study revealed mechanistic details of cholesterol transfer from NPC2 to NPC1 and demonstrated that it is pH dependent, which is consistent with the low pH in the lumen of lysosomes, where it occurs (Qian et al., 2020). A similar arrangement has also been observed in hedgehog signaling, except that here membrane-embedded portions of the Patched receptor are thought to be involved in cholesterol extraction from the plasma membrane rather than sterol insertion (Gong et al., 2018; Qi et al., 2018; Zhang et al., 2018). Whether and how significantly the membrane-embedded protein domains accelerate cholesterol transfer remains unknown.

Do eukaryotic glycerolipid transporters, like their bacterial counterparts, work together with partner proteins in the membrane that do more than simply localize them to contact sites? Direct interactions have been reported between both Vps13 and Atg2 and multispan integral membrane proteins (Guardia et al., 2020; John Peter et al., 2017; Tang et al., 2019), and others may yet be identified. Interestingly, the interaction of Vps13 with the mitochondria outer membrane protein, Mcp1, seems to be required for more than Vps13 localization, though how Mcp1 affects Vps13 function remains to be determined (John Peter et al., 2017). The ERMES complex, which is involved in glycerolipid exchange between the ER and mitochondria in yeast, includes a multispan integral membrane protein (Mdm10; Kornmann et al., 2009). These membrane residents might have roles in selecting and/or extracting lipids from membranes, in loading lipids onto LTPs to drive directional transfer, in lipid insertion at the acceptor membrane, or in lipid scrambling or flipping (lipid movement between the two leaflets of a bilayer). Interestingly, there is growing evidence that some LTPs are associated with scramblases, integral membrane proteins that facilitate lipid movement between the leaflets of membrane bilayers. The bridge-forming Atg2 interacts with Atg9 (Guardia et al., 2020), which has recently been shown to be a scramblase and is key to autophagosome membrane expansion (Maeda et al., 2020; Matoba et al., 2020; Orii et al., 2021). The yeast cup-like phosphatidylserine transporter, Osh6, has been reported to work in conjunction with Ist2 (D'Ambrosio et al., 2020), which is homologous to members of the transmembrane protein 16 (TMEM16) family of Ca^{2+}-activated scramblases (Suzuki et al., 2013a). Conceivably, membrane proteins, like scramblases, that associate with LTPs could play a role in accelerating lipid transfer beyond the rates observed for LTPs operating by themselves in vitro.

How do LTPs facilitate transport?

How LTPs facilitate lipid transport between membranes has been studied mostly in the context of cup-like transporters, though many of the same principles may also apply to bridge-like transporters. The basic outline of how these cup-like LTPs function, described in the previous section (Fig. 2 A), has been known for some time, but the mechanistic details are only now beginning to emerge. To understand how LTPs extract lipids from membranes, it is important to consider the energetics of lipid desorption from membranes. Lipids can desorb from membranes spontaneously without the assistance of proteins, but the rates are very slow. For example, the half-time of the exchange of phospholipids between liposomes is tens of hours (Jones and Thompson, 1990; McLean and Phillips, 1981; McLean and Phillips, 1984). This is because there is a high energic barrier to spontaneous lipid movement into the aqueous phase. One study estimated that the desorption free energy of pulling a phospholipid entirely out of a bilayer is ∼63 $kJ \cdot mol^{-1}$ (Grafmüller et al., 2013). LTPs facilitate lipid transfer by significantly lowering the energy of lipid desorption from membrane, because the lipid is no longer desorbed into the aqueous phase, but rather into the hydrophobic cavity of the protein.

The molecular mechanism by which LTPs remove and deliver lipid monomers to membranes remain to be determined; both probably require LTPs to partially insert into membranes or disrupt bilayer organization. Another important question is how the affinity of LTPs for membranes and lipids affects the rate of lipid extraction and delivery to membranes. Since many LTPs contain flexible lid-like domains that shield bound lipids, the opening and closing of these lids may play critical roles in determining lipid extraction and delivery. For example, the lid of the LTP Osh4/Kes1 regulates lipid transport, preventing the

release of one type of cargo but not of a second (Moser von Filseck et al., 2015b).

Given how challenging it is to understand the interactions of LTPs with membranes at a molecular level, molecular dynamics simulations may currently be the best hope of gaining insight. Simulations of membrane binding and phospholipid extraction by two cup-like LTPs revealed that both have domains that penetrate the bilayer upon membrane binding and may help orient the proteins to facilitate lipid extraction (Grabon et al., 2017; Miliara et al., 2019). Unfortunately, in both cases, the phospholipids were not fully extracted during the simulations, which were run for up to 5 µs, and the details of how these LTPs remove phospholipids from membranes remain to be elucidated. LTPs probably capture lipids during what have been termed protrusions (Pfeiffer, 2015), spontaneously occurring excursions of lipid molecules partially out of a membrane (i.e., partial desorption). LTPs may facilitate lipid protrusions from membranes by penetrating or binding the bilayer.

Since the propensity of lipids to protrude from membranes is determined by the biophysical properties of bilayers, such as hydration, curvature, tension, lipid packing, and order, these factors must also affect lipid transport by LTPs. Proteins and lipids at contact sites that determine these properties could have a significant effect on lipid transport. We are just beginning to understand the physical properties of membranes at MCSs. Two recent studies showed that, in yeast, the ER at some ER–plasma membrane contact sites forms peaks with extremely high curvature that could affect lipid exchange at these sites; peak formation requires tricalbins, the yeast orthologues of extended synaptotagmins in mammals (Collado et al., 2019; Hoffmann et al., 2019). Other ER-shaping proteins, the reticulons, have also been proposed to promote lipid exchange at ER–mitochondria contacts (Voss et al., 2012). There are also hints that the lipid composition of the ER at MCSs can differ from the rest of an ER, which could affect lipid transport at these sites. It has been suggested that ER–mitochondria contacts and perhaps other contact sites have raft-like properties (Area-Gomez et al., 2012; Currinn et al., 2016; King et al., 2020). If the membranes at these MCSs are, like rafts, more ordered than the surrounding membrane, this would be expected to inhibit lipid protrusion and reduced transport. There is also some indication that lipid production at contact sites facilitates transport (Kannan et al., 2017; Schütter et al., 2020), perhaps by increasing the frequency of lipid protrusions at these sites. Understanding the biophysical properties of membranes at contact sites and how they modulate lipid exchange remains an important challenge for the future.

How LTPs deliver lipids to membranes is another important question. It is likely that the same factors that affect lipid partitioning from donor membrane into the LTP also affect lipid transfer from the LTP into the receiving membrane. A recent molecular dynamics simulation of sterol exit from a StARkin LTP suggests that it is facilitated by the entry of water into the lipid-binding pocket (Khelashvili et al., 2019). This may be true of other sterol-binding LTPs (Singh et al., 2009), but it remains to be determined whether this mechanism holds for other classes of lipids and types of LTPs.

What are the mechanisms underlying directional transport?

Cup-like LTPs do not require energy to transport lipids between liposomes and can spontaneously equilibrate their cargos between two populations of liposomes; however, in cells, some LTP-mediated lipid transport is directional. A number of mechanisms are employed (Fig. 3). In some cases, directional transfer occurs when a lipid is moved from one membrane to a second, but cannot be returned, either because it is enzymatically altered (Fig. 3 A) or because it becomes complexed with other lipids in the second membrane (Fig. 3 B). For example, the ceramide transfer protein (CERT) shuttles ceramide from the ER to the Golgi complex, where the ceramide is converted to complex sphinoglipids, which CERT cannot transport back to the ER (Hanada et al., 2003). Directional lipid transport by LTPs may also be promoted by lipid synthesis in one of the membranes at an MCS (Fig. 3 C). Phospholipid synthesis in regions of the ER in contact with mitochondria or growing phagophores may promote transport to these organelles (Kannan et al., 2017; Schütter et al., 2020). Directional transport can also be driven by ATP hydrolysis. In *E. coli*, the ATP-binding cassette transporter LptBFG may use ATP hydrolysis to drive LPS out of the inner membrane into a bridge-like domain, and then into the outer membrane (Owens et al., 2019; Figs. 2 D and 3). In other words, the transporter may push lipids into a tube-like domain, which causes directional transport. It is possible that other transporters similarly drive directional transport by using ATP hydrolysis to pull lipids out of a tube-like domain and into a membrane. One such transporter may be the trigalactosyldiacylglycerol in *Arabidopsis thaliana*, which spans the inner and outer chloroplast membrane. This complex is thought to use ATP hydrolysis to drive lipids phospholipids from the ER into chloroplasts (Fan et al., 2015; Roston et al., 2012; Wang et al., 2012). Whether the trigalactosyldiacylglycerol complex forms a tube-like lipid transporter and the role of ATP hydrolysis in driving transport remains to be determined.

Some of the transporters in the ORP/Osh family make use of energy stored in cellular phosphoinositide gradients to redistribute lipids from their sites of synthesis in the ER to other organelles, where these lipids are enriched. One of the best characterized examples is OSBP, which transfers sterol to the Golgi, where cholesterol levels are higher relative to the ER, by counter-exchanging ER-derived sterol for phosphatidylinositol 4-phosphate (PI4P) present at the Golgi (Mesmin et al., 2013). OSBP exchanges its sterol cargo for PI4P at the Golgi because the LTP has a higher affinity for the phosphoinositide. The LTP then carries the phosphoinositide back to the ER, where a resident lipid phosphatase (Sac1) hydrolyzes it to PI to decrease its affinity for the LTP and to allow the LTP to pick up another sterol molecule (de Saint-Jean et al., 2011; Mesmin et al., 2013). Phosphatidylserine transfer from the ER to the plasma membrane is mediated by Orp5/Orp8 (Osh6 in yeast) by using a similar mechanism, exchanging the glycerolipid for PI4P or phosphatidylinositol 4,5-bisphosphate ($PI(4,5)P_2$) present at the plasma membrane (Chung et al., 2015; Ghai et al., 2017; Moser von Filseck et al., 2015a). While it is possible that ORP/OSH proteins are the only family of transporters that operate in this way, the mechanism could be more general, extending to other LTP

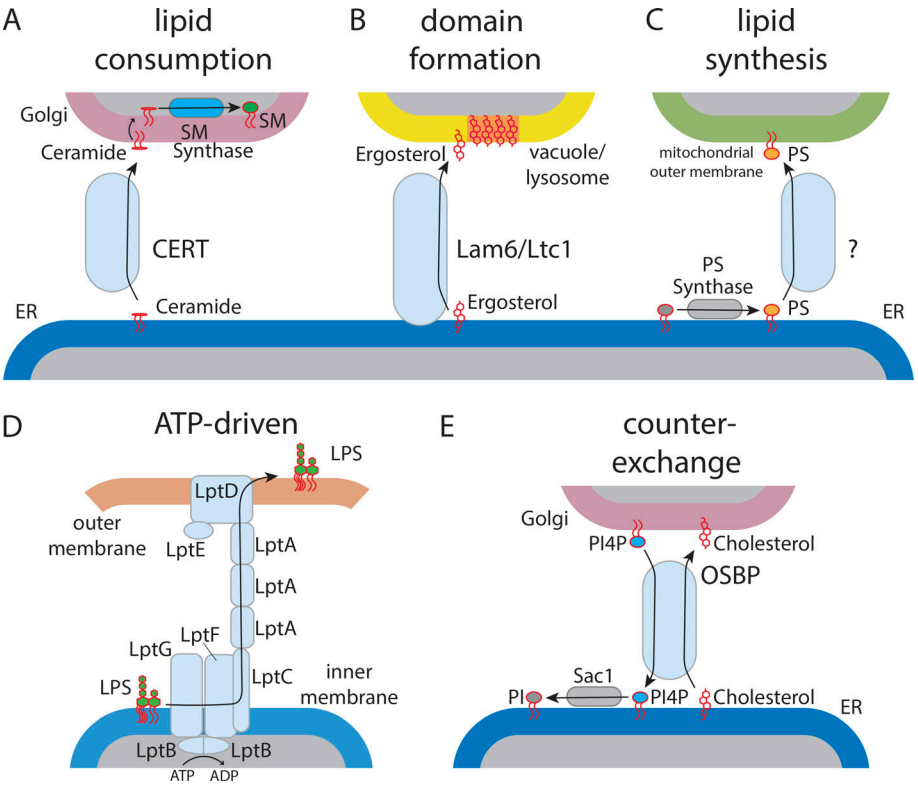

Figure 3. **Examples of five mechanisms of directional transport. (A)** Lipid consumption–driven lipid transport. Ceramide is transported by CERT from the ER to the Golgi, where it is converted to sphingomyelin (SM) and other complex sphingolipids and cannot be returned to the ER by CERT. **(B)** Lipid domain formation can drive lipid transport when a lipid becomes associated with a domain in one of the two membranes. Lam6/Ltc1 brings the sterol, ergosterol, to the vacuole in *S. cerevisiae*, and membrane domain formation probably drives the accumulation of ergosterol (domain in red). **(C)** Lipid synthesis at MCSs can drive lipid transport. PS synthesis at ER–mitochondria contact sites in *S. cerevisiae* promotes PS transport to mitochondria. **(D)** ATP consumption drives LPS transport from the inner to the outer membrane of *E. coli*. **(E)** Counter-exchange transport using the difference in PIP concentration in two membranes to drive the transport of a second lipid. OSBP uses counter-exchange transport to enrich cholesterol in the Golgi membrane.

families as well, and there may be additional, still undiscovered mechanisms by which cup-like LTPs to transport lipids against their gradient. While counterexchange is widely viewed as a mechanism to redistribute ER-derived lipids against a gradient, it may also serve to regulate phosphoinositide levels at the acceptor membrane and may play a role in attenuating or regulating signaling.

The most extreme case of directional transfer occurs during organelle biogenesis, likely facilitated by bridge-like LTPs, such as VPS13 or ATG2. As these LTPs seem to bind glycerolipids nonspecifically and, moreover, simultaneously bind tens of these lipids that must all move concertedly for efficient transport (Kumar et al., 2018; Valverde et al., 2019), it seems unlikely that chemical gradients could drive directionality. Instead, lipids are probably pushed or pulled into bridge-like transporters, perhaps driven by lipid synthesis or ATP hydrolysis. Since LTPs transfer lipids between the cytosolic leaflets of different organelles, they almost certainly operate together with scramblases or flippases that can transfer lipids between the cytosolic and luminal leaflets, since membrane expansion requires both leaflets to grow. It is not known whether these scramblases/flippases must be associated directly with the LTP, as in the LPS system. Discovering the basis of directional transfer by bridge-like proteins, potentially by identifying interacting partners in this process, is an ambitious undertaking for the coming years.

Conclusions and future directions

The last 10 yr have seen tremendous progress in our understanding of nonvesicular lipid transport in cells in both its functions and mechanisms; however, many fundamental questions remain. Our knowledge of the volume and rates of nonvesicular lipid transport at MCSs is still incomplete, primarily because measuring lipid movements in cells remains challenging. A better picture is likely to emerge in the next few years as new lipid sensors and fluorescent lipid analogues are developed and used with rapidly evolving super-resolution microscopy.

A better knowledge of how LTPs promote lipid desorption and delivery to membranes will be critical for understanding how lipid transfer rates in cells are determined. The mechanistic and energetic details are not well understood for any LTP, let alone representatives from all of the various LTP families. Molecular dynamics simulations may lead the way. Another important question is how lipids are moved within tube-like transporters or between LTPs and other proteins. LTPs have largely been studied in isolation, but in cells, their activity is probably determined by proteins that affect the properties of membranes, such as those that deform membranes—lipid metabolizing enzymes and flippases (or scramblases). These proteins may also help to determine the directionality of lipid transport by LTPs.

Understanding how high-volume bulk lipid transport occurs is another important challenge for the field. It seems likely that LTPs transport lipids significantly faster in cells than they do in vitro, but how is not clear. We still have much to learn about the microenvironments formed at MCSs and how they contribute to lipid exchange by LTPs. In the next few years, we are likely to get a better understanding of how LTPs function and work in concert with other proteins that modulate membranes.

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