Lipid transfer proteins: the lipid commute by shuttles, bridges and tubes

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Abbreviations:

CERT – ceramide transport protein, CETP – cholesteryl ester transfer protein, DAG – diacylglycerol, EE – early endosome, ER – endoplasmic reticulum, ERMES – ER-mitochondrial encounter structure, E-Syt – extended-synaptotagmin, FAPP – phosphatidylinositol-four-phosphate adaptor protein, FFAT – 2 phenylalanines in an acidic tract, HDL – high density lipoprotein, LAM – LTP anchored at an membrane contact site, LBP – LPS binding protein, LDL – low density lipoprotein, LE – late endosome, LPS – lipopolysaccharide, LTP – lipid transfer protein, MCE – mammalian cell entry, NMR – nuclear magnetic resonance, NPC – Niemann-Pick type C, OSBP – oxysterol binding protein, ORP – OSBP-related protein , PA – phosphatidic acid, PC – phosphatidylcholine, PE – phosphatidylethanolamine, PH – pleckstrin homology, PI – phosphatidylinositol, PITP – PI transfer protein, PS – phosphatidylserine, RO – replication organelle , SMP – synaptotagmin-like mitochondrial-lipid-binding protein, StAR – Steroidogenic acute regulatory protein, StARD – StAR domain, StART – StAR-related lipid transfer, TGN – *trans*-Golgi network, TULIP – tubular lipid binding protein, VAP – VAMP associated protein, VLDL – very low density lipoprotein.

Abstract

Lipids are distributed in a highly asymmetric fashion in different cellular membranes. Only a minority of lipids achieve their final intracellular distribution by selection into the membranes of transport vesicles. Instead, the bulk of lipid traffic is mediated by a large group of lipid transfer proteins (LTPs), which move small numbers of lipids at a time using hydrophobic cavities that stabilise lipid outside membranes. Despite the first discoveries of LTPs almost 50 years ago, most progress has been made in the last few years, leading to considerable temporal and spatial refinement in our understanding. The number of known LTPs has increased, with exciting discoveries of multimeric assemblies. Structural studies of LTPs have progressed from static crystal structures to dynamic structural approaches that show how conformational changes contribute to lipid handling at a sub-millisecond time-scale. Many intracellular LTPs localise to membrane contact sites, nanoscale zones where an LTP can form either a shuttle, bridge or tube linking donor and acceptor compartments. Understanding how each lipid achieves its final destination at the molecular level allows a better explanation of the range of defects that occur in disease, with therapies being developed to target lipid transfer.

Introduction

Cellular integrity requires separation of its contents from its surroundings. Lipid bilayers form the physical boundary that defines a cell's spatial limits and mediates exchange with the environment. Limiting membranes of many eukaryotic organelles perform similar functions. The lipid composition of each membrane is precisely tailored to these functions[1], with a dedicated system of intracellular lipid traffic to achieve different lipid mixtures. There are also lipid transfer systems outside cells, for such basic functions as scavenging lipids from the environment.

Traffic of membrane vesicles in eukaryotic cells necessarily moves lipids in the secretory pathway. However, lipids must also be supplied to compartments that do not receive vesicular traffic, thus requiring an alternative non-vesicular lipid transport[2]. Even for organelles in the secretory pathway, lipids are trafficked by non-vesicular means. This might have multiple purposes, including maintenance of lipid compositions that cannot be achieved by vesicles, for example low protein concentrations found in phagophore **[G]** membranes[3]. Non-vesicular traffic also allows rapid alterations of lipidome, for example so that the plasma membrane adjusts to environmental changes[4]. Mammalian cells must also correct any non-ideal lipid movement between donor and acceptor compartments caused by vesicular traffic. These situations call for changes in membrane lipids without changes in membrane proteins. The two main lines of experimental evidence for non-vesicular lipid traffic between compartments linked by vesicular traffic[5]: (i) speed - phospholipids and cholesterol move bidirectionally between the endoplasmic reticulum (ER) and the plasma membrane much faster ($t_{½}$=2-5 minutes) than vesicular traffic would allow[6–8]; (ii) chemical or genetic disruption of the secretory pathway has little effect on bulk cellular lipid transport between ER and plasma membrane[7,9–11].

The hydrophobicity of lipids that allows them to form hydrophobic barriers also prevents their movement across the cytoplasm or between cells. Such movement is entropically unfavourable due to the high activation energy required for the initial step of membrane desorption **[G]**[12,13]. The so-called lipid transfer proteins (LTPs) **[G]** were postulated to facilitate transfer lipid components of bilayers across the aqueous phase by decreasing this activation energy[14]. LTPs have since been studied *in vitro* as enhancers of lipid movement between liposomes. To date hundreds of LTPs have been found in all species, from bacteria to animals. The one feature that unites LTPs is that they provide hydrophobic cavities where lipids are at a much lower free energy than if they were left free in aqueous solution. Most LTPs have been found to form a cavity with a hydrophobic lining, like a protein box, the lipid fitting inside (Fig. 1A)[15,16]. Stoichiometry is typically 1 LTP : 1 lipid, which is selected both for its headgroup and for its acyl chain length. This implies a specific interaction of lipid with distinct residues within the cavity. Several LTPs are bispecific, meaning they can bind two lipids with different headgroups. LTPs with cavities move lipids one at a time from donor to acceptor, returning either empty to achieve net lipid traffic, or, as

is the case for many bispecific LTPs, returning with a different lipid to achieve lipid exchange.

This review will build on previous surveys of LTPs[17] to include considerable recent advances in our understanding of how proteins mediate the transfer of the lipid components of membranes. One area of progress is the finding that many are localised to sites where organelles form narrow gaps that are bridgeable by the LTP itself (typically ≤30 nm). These sites of contact between different organelles ("membrane contact sites") allow the anchoring points of the LTP to be static, while the domain with the lipid binding cavity transfers lipid cargo between two organelles. An exciting development is the discovery of LTPs that, as opposed to being box-like, form open bridges or closed tubes that cross between membranes, so that lipid moves in the complete absence of protein movement. Other developments include several ideas on how LTPs impose direction on lipid traffic.

1. Structures and conformation

At the structural level, 27 protein families form hydrophobic cavities that transfer membrane bilayer lipids (Table 1). LTPs in the same family can have quite different ligands, even if they share considerable sequence[18]. In this section, we will briefly describe how LTPs form spaces for hydrophobic lipids.

Box-like lipid shuttles

The archetypal form of LTP resembles a box, with an internal cavity large enough for one lipid molecule**.** Lipid transfer requires the LTP to shuttle between donor and acceptor compartments with several steps: membrane docking, lipid extraction, undocking, cytosolic diffusion (Fig. 1B) and then the reverse steps for deposition. Most box-like cavities have residues that move, equivalent to a lid that opens and closes, however some LTPs such as MlaC in bacteria have no lid, exposing the lipid headgroup to the aqueous environment (Fig. 1A)[19]. Here, we describe the main examples of box-like LTPs.

StARkin-superfamily

The StARkin superfamily contains domains with similar structure to steroidogenic acute regulatory protein (StAR), the founding member of the StAR-related transfer (StART) family. StARkins, by far the largest grouping of LTPs, have an α-β grip with a hydrophobic cavity[20]. StAR was identified first, but the closely related StARD4, is the better understood mechanistically (Fig. 1C, 1D)[21]. Membrane docking by StARD4 is initiated by electrostatic interactions mediated by an electropositive surface patch with anionic membrane lipids. The entrance to the internal cavity, which is near the electropositive patch, is between a long amphipathic α-helix and the so-called Ω1 ("Omega-1") loop[22]. Nuclear magnetic resonance (NMR) shows that the α-helix rotates so that its hydrophobic face engages with the bilayer and the Ω1 loop opens, though it does not embed in the membrane (Fig. 1D)[21]. The movement of these

elements is essential for lipid transfer. The application of NMR to these questions is significant because it reveals conformational changes that take place at the highly relevant, but little explored, time-scale of microseconds to milliseconds during which lipid transfer occurs.

Crystal structures indicate that similar movements occur in phosphatidylinositol (PI) transfer proteins (PITPs), which are bi-specific StARkins, either for PI and phosphatidylcholine (PC) (PITPα/β) or for PI and phosphatidic acid (PA) (PITPNM1, aka Nir2/RdgBα)[23,24]. Compared to StARD4, the entrance to the cavity of PITPs is closed by a combination of a much expanded Ω1 loop called the exchange loop and an elongated extreme C-terminus. When engaged with the membrane, both the exchange loop and the C-terminus move into an open conformation exposing the site for phospholipid binding[23,25]. PITPs have various unique structural elements, including the so-called G-helix near the cavity opening, which moves and unwinds when PITPs are engaged with the membrane[25]. PITPα also illustrates a mechanism often employed by LTPs, whereby a loop of the protein inserts hydrophobic residues into the bilayer to enhance dwell-time during lipid exchange[26].

The StARkin family closest to PITP is the PRELI domain (also known as SLMO in metazoa and Ups in yeast), which is found in the inter-membrane space of mitochondria. PRELIs bind phospholipids such as PA or phosphatidylserine (PS), or both[27–30]. Instead of a G-helix, PRELIs have a shared obligatory small helical subunit (TRIAP1 in humans, Mdm35p in yeast) which binds in a similar position to the G-helix. Membrane docking by PRELI necessitates dissociation of TRIAP1/Mdm35[28,31], indicating the extent of conformational change that accompanies lipid (un-)loading.

Sec14-like PITPs

Sec14 and related proteins (aka CRAL/TRIO) are widespread in all eukaryotes[32–34], typically bi-specific for PI and PC, like StARkin PITPs[35,36]. Sec14 has an all helical structure with no structural homology to StARkins, implying convergent evolution on a common function. The lid of the lipid binding pocket of Sec14p moves substantially (~17.5 Å) during opening and closing, which is regulated by lipid occupancy[15,37,38].

OSBP related proteins

The large family of oxysterol **[G]** binding protein (OSBP)-related proteins (ORPs) are all LTPs, but not all transfer sterol as the name would suggest. They are bispecific, and their one common ligand is a phosphoinositide **[G]**, usually PI4P[39,40]. ORPs are then divided on their second specificity: OSBP and its closest homologues bind sterol, ORP5/8 and their homologues bind PS[41,42], and other ORPs (Osh3p in yeast) likely bind other lipids[43]. As for the phosphoinositide ligand, while many ORPs are specific for PI4P, PI(4,5)P_2 has also been found to be a ligand in two cases: ORP5/8[44] and OSBP[45]. Transfer of phosphoinositides was quite unexpected[46] and is a special case because it provides a relatively simple system to impart directionality on traffic of the second lipid (see section on Counter-transport)[39].

Bridge-like LTPs

Whilst box-like LTPs have a singular access point to their binding cavities, bridge-like LTPs have openings that extend along their length. The extended openings form seams that theoretically allow lipids to slide while the protein remains stationary (Fig. 2). These LTPs have been found in multimers that make continuous elongated lipid transfer modules similar to bridges.

Prokaryotic Lpt

The lipopolysaccharide transport (Lpt) operon of 7 genes (LptA-G) transports lipopolysaccharide **[G]** (LPS) from the inner to the outer membrane of Gram-negative bacteria **[G]**. LPS has up to six fatty acyl chains and a bulky polysaccharide headgroup with >200 sugars. The seven Lpt proteins are organised in two membrane subcomplexes: $LptB_2FGC$ in the inner membrane **[G]**, which modulates LPS insertion and flipping to the periplasmic face; and LptDE in the outer membrane **[G]**, which inserts LPS into the outer leaflet of the outer membrane. In between the membrane subcomplexes sits LptA. This has a "U"-shaped cross-section, the inside surface of which is hydrophobic (Fig. 2Ai)[47]. Domains in LptC and LptD have the same U-shape[48,49], and an in-line complex of $LptCA_nD$ forms a bridge that spans the entire periplasmic gap (~21 nm) between the membrane subcomplexes (Fig. 2Aii). This creates a path for LPS from the start (inner membrane) to the end (outer membrane) of its route.

Putative bridge-like LTPs

Given the presence of a bridge-like complex in prokaryotes, it is appealing to seek eukaryotic counterparts. Tubular lipid binding proteins (TULIPs) are currently the most promising candidate to adopt a multimeric bridge-like form, though as detailed below for both extracellular and intracellular TULIPs, strong evidence for this is lacking and a shuttle mechanism is more widely accepted.

Extracellular TULIPs

TULIPs are elongated cones with extended openings along their length that form seams (Fig. 2Bi)[50]. Like LptA, hydrophobic portions of lipids are protected within a groove-shaped cavity, and hydrophilic headgroups are exposed. Cholesteryl ester transfer protein (CETP) is a TULIP that transfers cholesterol ester from high density lipoprotein (HDL) lipoproteins ("good cholesterol") to (very) low density lipoprotein (LDL/VLDL) ("bad cholesterol")[51], making it an attractive drug target (see also section on LTPs and disease below)[52]. Cholesterol esters have no hydrophilic portion, so they bind only at the base of the groove[53]. Most TULIPs form head-to-head dimers that are highly elongated, shaped like bananas up to 13 nm long (Fig. 2Bii). Electron microscopy of purified CETP and LDL/VLDL suggest that the TULIP dimer can form a bridge between lipoproteins, so that lipids might travel the entire length of the grooves across the dimer, analogous to $LptCA_nD$[51]. However, contradictory evidence

indicates that CETP shuttles lipids like the box-like LTPs, for example antibodies binding the ends of CETP do not inhibit its function[54].

Intracellular TULIPs

Many years after the extracellular TULIPs were discovered, they were shown to have intracellular counterparts in the Synaptotagmin-like Mitochondrial-lipid-binding Protein (SMP) domain family[55–58]. Like extracellular TULIPs, SMP domains mostly dimerise head-to-head (Fig. 2Bii)[56], and they form larger complexes that include head-to-tail linkages (Fig. 2Biii and inset)[57]. This suggests that SMPs might form long lipid bridges as was proposed for CETP[55]. However, the evidence that the end of the tube is the lipid entry point is almost all indirect and based on molecular dynamics simulations[50]. Therefore, as for CETP, the current results suggest that a complex of multiple SMP domains shuttles back and forth across contact sites (Fig. 2Biv). For extended-synaptotagmin-2 (E-Syt2), which transfers a range of glycerolipids between the ER and the plasma membrane, the shuttle mode of action is more strongly supported because the dimer formed by E-Syt2's SMP domains is too short to bridge the gap[56]. An SMP dimer even more likely to act as a shuttle is formed by TMEM24, which is selective for PI over other phospholipids. Here the crystal structure shows that lipid cannot flow across the head-to-head dimer interface[59].

The ER-mitochondrial encounter structure (ERMES) is a complex at ER-mitochondrial contact sites that contains three proteins with SMP domains: Mmm1p, Mdm12p and Mdm34p[55]. The SMP domains combine into interesting complexes: not only head-to-head homodimers like CETP, but also heterotetramers with an Mmm1p dimer sandwiched between Mdm12p monomers (Fig. 2Biii and inset), and Mdm34p may join in to make even larger complexes[57,60]. Individual ERMES SMPs poorly transfer lipids between liposomes, but Mdm12 and Mmm1 in combination transfer lipid very efficiently[61]. This multimeric complex, which may bind as many as six phospholipids at once, has inspired two models that are alternate to LTP shuttles. The first is a static bridge with an interconnected path for lipid to move along (Fig. 2Bv). However, the narrow ("tail") ends of static SMP domains in available crystal structures do not have a hydrophobic path wide enough for lipid to traverse. Secondly, an ingenious suggestion (with no evidence as yet) is that a linear multimeric SMP bridge is not static, but constantly changes aspects of its orientation, with subunits ± bound lipid flipping 180°, to pass lipid between subunits only across head-to-head interfaces, like a chain of fire fighters passing buckets of water (Fig. 2Bvi)[61].

Tube-like lipid conduits

LTPs do not need individual hydrophobic cavities because the cavity can be formed from patches of multiple building blocks. The bacterial MCE domain (for **M**ammalian **C**ell **E**ntry) forms fully enclosed tubes with internal hydrophobic environments separate from the surrounding aqueous environment. EM structures of MCE

complexes show that the domains multimerise is two ways: firstly, they hexamerise to form a disk with a central pore lined by hydrophobic residues (Fig. 2Ci). Secondly, the disks stack up to extend the pores into a hydrophobic tube[62].

MlaD, YebT and PqiB are MCE proteins in the inter-membrane space of bacteria. All three proteins form polymers with six-fold radial symmetry that contain phospholipids, though transfer is yet to be tested[62]. MlaD, which has one MCE domain, forms a single disk that accepts lipids from MlaC, a soluble LTP (Fig. 2Cii). YebT has seven conserved MCE domains that each hexamerise. Together, the 42 YebT domains contribute to seven stacked rings with a hydrophobic central tube (Fig. 2Ciii). PqiB has three MCE domains, which are extended by an α-helical domain. As well as hexamerisation of MCE into disks, the helix forms a six-bundle superhelix with a central hydrophobic pore (Fig. 2Civ). For PqiB this leads to a striking syringe and needle shape (Fig. 2Cv and inset). Lipid import into plastids, endosymbionts descended from cyanobacteria, requires TGD2, a chloroplast MCE protein[63], and this likely forms a similar structure to PqiB, but with only one disk.

Site of action

LTPs were initially thought to be purely cytosolic proteins because their activity was identified in cytosolic extracts[2]. However, moving lipids between two membrane compartments requires that LTPs function at membranes[5]. Therefore, their membrane targeting is an important and regulated aspect of their activity.

Dual membrane targeting domains

To access lipid membranes many LTPs contain domains or motifs that target them to not just one organelle, but two (Fig. 3). Dual targeting ensures that LTPs encounter the source and destination of their ligands. If the two targeting domains/motifs are both exposed, then LTPs tends to localise where both receptors for these domains/motifs are engaged. Since many LTPs can extend up to 30 nm, LTPs with two targeting domains are therefore found at membrane contact sites, places where the gap between two organelles is often less than 30 nm (Fig. 3)[64]. This capacity for dual targeting is a simple explanation for the large proportion of LTPs that is found at contact sites[65]. Many LTPs target the ER. For SMPs, LTPs Anchored at Membrane contact sites (LAMs, which belong to the StARkin superfamily) and some ORPs, ER targeting is irreversible and occurs via transmembrane domains[66,67]. An alternate, reversible means of ER targeting used by many LTPs is binding to the ubiquitous ER integral protein VAMP-associated protein (VAP)[68]. This requires a short FFAT motif (“two phenylalanines in an acidic tract”) which is present in at least four different LTP families (Fig. 3).

Targeting of non-ER membranes by LTPs can be achieved by interaction with proteins, lipids or both at these sites. The most common membrane-targeting domains are pleckstrin homology (PH)-like and C2. For example, a PH-like domain in Lam6p (aka Ltc1p) targets ER-mitochondrial contacts coincident with ERMES[67],

possibly binding one of it subunits or an associated factor[69]. Highly homologous PH domains in OSBP, ceramide transport protein (CERT) and Four-adaptor protein phosphatidylinositol-**f**our-phosphate **a**da**p**tor **p**rotein-2 (FAPP2) bind a combination of phosphoinositide lipids (PI4P and PI(4,5)P_2) and Arf1 GTPase, which are only coincident at the *trans*-Golgi network (TGN). For CERT, the PH domain and its FFAT motif localises it to ER-TGN contacts where it transfers ceramide out of the ER.

LTP targeting can be regulated by post-translational modification. CERT targeting is affected by two different phosphorylations, one of which activates the FFAT, while the other causes autoinhibitory binding of the PH and StARkin domains[70–72]. LTPs and their localisation can also be regulated by Ca^{2+} signalling. E-Syts have three or five C2 domains. When inactive, E-Syts are held in the ER thanks to a hydrophobic segment that forms a hairpin anchor[73]. E-Syts are active when localised to ER-plasma membrane contact sites. E-Syt2/3 are at these sites constitutively because their fifth C2 domain (C2E) binds PI(4,5)P_2 on the plasma membrane in a Ca^{2+}-independent manner[73]. C2E in E-Syt1 requires high Ca^{2+} to bind PI(4,5)P_2, so E-Syt1 concentrates at the ER-plasma membrane contacts only after cell stimulation. The rise in cytosolic Ca^{2+} breaks two auto-inhibitory interactions: C2C+C2E and C2A+SMP, so that after stimulation C2E is finally free to bind PI(4,5)P_2, and SMP can transfer lipid[74]. Cell stimulation typically also activates phospholipase C, which produces diacylglycerol (DAG) at the plasma membrane. This is related to the recruitment of E-Syt1 at the same time, since its SMP domain can traffic DAG from the plasma membrane to ER for re-synthesis of PI(4,5)P_2 (ref. 75).

LTPs without membrane targeting domains

Some LTPs that have no targeting domain/motif detected by bioinformatics still exhibit specific membrane targeting, for example several short OSBP homologues[41,76]. Even LTPs that are diffuse in the cytosol, for example StARD4, must target membranes to acquire lipids, even though the interaction can be hard to detect[77]. Here the interactions may be of a similar form to those that produce tight membrane attachment (protein-protein or protein-lipid), but the affinities are likely to be lower.

Extracellular LTPs

Extracellular LTPs control the distribution of lipids between the environment and cells. Here for consistency we only address LTPs whose ligands are large enough to participate in lipid bilayer formation. One group of such extracellular LTPs moves lipids between different extracellular carriers, such as lipoproteins – see ***Cholesterol traffic between lipoproteins*** (below).

Another major class of LTPs secreted by cells are the Pathogen related (PR) proteins. In some instances these proteins export bound lipid from cells to prevent intracellular accumulation, for example yeast Pry1 binds sterol acetate[78,79]. PR proteins are also important for defence by binding extracellular lipids: plant PR-1 can sequester sterol to suppress growth of sterol-auxotrophic pathogens such as

Phytophthora. Yeast PR proteins can also bind and thereby directly neutralise harmful small hydrophobic compounds such as eugenol[80].

Some extracellular LTPs can salvage lipids from the environment for cellular use. LPS binding protein (LBP) is a widely conserved TULIP that binds bacterial endotoxin **[G]** and signals its presence to the innate immune response. In animals, this works by hand-off of LPS from LBP to CD14 to MD-2 and eventual presentation to toll-like receptor-4 **[G]** (TLR4)[81]. LTPs secreted by plants have many functions that vary from preventing re-uptake of bound lipid[82], to specific receptor binding to stimulate a response[83]. Plant LTPs are ubiquitous human allergens; for example, 15% of the population of Europe and North America are allergic to Bet v 1 protein from birch trees. Once loaded with lipid, such plant LTPs are highly resistant to degradation. Thus, when processed in antigen presenting cells, LTP-lipid complexes may be more allergenic than each separate component[84,85].

Forcing direction of lipid transfer

Cells synthesise most of their lipids in one major site. In eukaryotes, this is the ER; for Gram-negative bacteria lipid synthesis occurs in the inner membrane. Many lipids then are transported up concentration gradients to achieve higher concentrations in their destination compartments, and also highly asymmetric distributions between leaflets, indicating that lipid transport consumes energy. Since LTP domains have no clear way to consume energy, they must be linked indirectly to energy consuming cellular processes.

Direct ATP driven lipid transport

One way of moving lipid up a gradient is linking an LTP to a lipid pump that forces transfer (Fig. 4A). A clear example of this is found in LPS traffic by the LptA–G complex in bacteria such as *E. coli* (Fig. 2Aiii). The inner membrane subcomplexes contain LptB, which is an ATP-binding cassette (ABC) transporter **[G]**. Members of this family use ATP to pump substrates across a membrane, here the substrate being LPS. This pumping then pushes a continuous line of LPS molecules along the rest of the Lpt pathway[86,87], which consists of: (i) LptFG (for extraction from the inner bilayer); (ii) $LptCA_nD$ (bridge-like Lpt, see Fig. 2A); (iii) LptDE (for insertion into the outer membrane). Memorably, LptB filling the pathway from the bottom has been described as a "PEZ Model", calling to mind the sweet dispensers that have been in circulation for over 60 years[88].

LptB is not the only lipid pump involved in lipid export. Human ABCA1 and ABCG1, are phospholipid pumps in the same protein family as LptB. The pumps activate lipids by inducing asymmetry in the bilayer, with excess lipids building up in the exofacial leaflet[89]. ABCA1 loads ApoA-1 with whatever lipids are available (phospholipids and cholesterol) to form nascent high density lipoprotein (HDL) particles, which are lipid bilayer nanodiscs 8-11 nm diameter; ABCG1 subsequently

loads this to allow the discs grow into HDL spheres. Thus, ApoA-1 acts like a non-specific lipid transfer protein[89].

Gradients created by lipid consumption

Once transferred from donor to acceptor compartment, a lipid can be made unavailable for return, for example by enzymatic conversion in the acceptor compartment (Fig. 4B). A convincing example is provided by ceramide transport from the ER by CERT for conversion into sphingomyelin in the TGN[90]. Other examples apply to lipid building blocks supplied to mitochondria. PA and PS are supplied to enzymes of the inner mitochondrial membrane to make cardiolipin and PE respectively, with different PRELI proteins specific for either PA or PS in the intermembrane space[30,31]. The enzymes that make sphingomyelin, PE and cardiolipin are “exceptions to the rule” about the confinement of lipid biosynthetic enzymes to the ER. This has possibly evolved because the lipids they make have unique biophysical properties. Thus, it could be disadvantageous to make them in the “wrong” place. PE synthesis holds a unique place in lipid cell biology, and exemplifies the complexity of lipid biosynthesis and transport mechanisms (see Supplementary Box 1)[91].

Role of membrane effects

Not all the lipids in a bilayer are available for interactions with other cellular components, including with LTPs. This is particularly relevant for cholesterol. Despite high levels of cholesterol in the plasma membrane (30-40%, compared to 5% in the ER), only a small proportion of plasma membrane cholesterol is detectable, *i.e.* is accessible or availabile[92]. In plasma membranes there is a “J-shaped” curve of sterol accessibility. Until a threshold concentration of cholesterol (~25%) is reached, the cholesterol is virtually inaccessible, for example it is not detected by sterol-binding proteins[92]. By comparison, in an ER-like bilayer, the threshold for accessibility is <5%[93]. Such thresholds arise from reversible low affinity interactions of cholesterol with other lipids. Cholesterol binds saturated lipids including sphingomyelin and PC most strongly, and these lipids are enriched in the plasma membrane[40]. Localisation of these interacting partners is thought to drive the intracellular redistribution of cholesterol via LTPs (Fig. 4C)[11]. All membranes have set-points for sterol release depending on which other lipids are present. Specific pools of sterol can be made accessible by removing their partner lipid[92], and conversely sterol can be shielded by increasing saturated lipids. Such shielding happens when glyco- and sphingo-lipids accumulate, which can then induce a build-up of cholesterol[94].

Packing defects are another property of a bilayer that can increase ability of LTPs to penetrate into the membrane. Packing defects occur when lipids have unsaturated acyl chains, or where there is higher curvature, both of which are features of ER tubules[95]. These defects reduce the energy barrier to lipid either leaving or entering the bilayer (Fig. 1B, steps 2 and 3).

Counter-transport of a second lipid

Since 2011, a new concept has been introduced for forcing the direction of lipid traffic : lipid **counter-transport [G]**[39]. The concept applies to bispecific LTPs that transport two ligands, lipid A and lipid B in different directions. A counter-current to move lipid B can develop if there is a mechanism for maintaining a strong gradient of lipid A. An LTP interacting with the membrane where lipid A levels are maintained at a high concentration will load with it. When LTP-lipid A reaches the other compartment and unloads, since lipid A levels are kept very low it will be unlikely to reload with lipid A. Instead, it will swap to its other ligand, lipid B. LTP-lipid B can return to the starting membrane. Here lipid A is high, so after release of lipid B this is unlikely to reload and as before lipid A will load[39]. Thus, a permanent gradient of lipid A forces lipid B in the opposite direction (Fig. 4D). This mechanism of transport has been described as counter-transport similar to antiporter ion transport, however the antiport by LTPs is imperfect and is not essential, because transport of lipid B can still be obtained without lipid A, though the rate for that depends solely on the strength of the gradient for lipid B.

The counter-transport concept was first applied to ORPs where lipid A is PI4P. This phosphoinositide is synthesised from PI by multiple PI 4-kinases located in different compartments of the late secretory pathway[96], and PI4P is hydrolysed back to PI by the PI 4-phosphatase SAC1, which is anchored in the ER[39,97–99]. Both PI 4-kinase and PI 4-phosphatase are essential for counter-transport of “lipid B” out of the ER. Accordingly speed of the counter-transport is determined by the rates of PI4P generation and degradation. Despite neither of these yet being established, there is strong evidence for the counter-transport system from the hijacking of the entire system by viruses to drive cholesterol transport to virally determined membranes (see section “LTPs and disease”)[100]. Specificity for “lipid B” varies between ORPs, and phosphoinositides other than PI4P (“lipid A”) might also drive counter-transport[44,45]. Other examples of counter-transporting LTPs are PITPNM1 (exchanging PA for PI between the ER and plasma membrane)[24,101], and the PRELI protein Ups2p (exchanging PS for PA between the mitochondrial membranes)[102].

ORPs that transfer lipids by counter-transport not only have a hydrophobic cavity to internalise PI4P, they also have PH domains that bind PI4P’s headgroup. This homeostatically adjusts membrane recruitment to co-vary with levels of PI4P substrate[97]. Experimentally, filling the cavity of an ORP with an inhibitor prevents PI4P traffic, leading it to accumulate at its site of synthesis, which therefore enhances membrane recruitment of the inhibited ORP. This explains the long-standing observation that OSBP translocates to the TGN when 25-hydroxycholesterol is added to cells[103]: this soluble oxysterol fills OSBP’s cavity, preventing transfer of PI4P, which accumulates on the TGN and recruits the PH domain more tightly[96]. Since OSBP spans the gap at the TGN-ER contact site via its FFAT motif and PH domain, the unnatural addition of 25-hydroxycholesterol causes it to link the two compartments ever more tightly. In this way, OSBP and its homologues might cause pathology through holding a contact together too tightly even though their absence does not lead the contact to fall apart[104].

For cholesterol, it is not yet known how important counter-transport by ORPs is for traffic from the ER to the plasma membrane. In both human cells and yeast, the overall capacity for non-vesicular traffic of sterol outstrips the amount of traffic needed for cell growth by 3 to 10-fold[105,106]. In yeast lacking the entire ORP family, some aspects of sterol traffic are largely unaffected, but the plasma membrane has a changed structure that radically alters sterol availability[107]. This makes it hard to determine whether ORPs move a significant pool of sterols. One possibility is that counter-current by ORPs drives cholesterol to specific locations, such as the TGN[96], or post-Golgi vesicles[108], reaching and possibly exceeding the local set-points for cholesterol.

LTP itself imposes direction of traffic

The direction of lipid traffic can be controlled by the LTP itself. The most obvious effect is created by lipid cargo inside the cavity, which imparts conformational changes that affect the LTP's external surface. A clear example of this is Osh4p, a yeast ORP[98]. Osh4-PI4P off-loads PI4P into ER-like acceptors much faster than Osh4-sterol offloads sterol. This correlates with the lid of Osh4-PI4P being predicted to be much more mobile than that of Osh4-sterol[98]. The predictions of how the lid of Osh4p behaves were obtained through computer simulations, which provide a way forward even when the biophysical approaches do not currently exist (Box 1). After new techniques are developed for studying these aspects of LTP action, a subsequent challenge will be to marry *in vitro* experiments with *in vivo* observations of LTPs in action[109].

Other effects on the LTP come from the membrane it interacts with. For example, unloading of Osh4p (both Osh4-PI4P and Osh4-sterol) into liposomes that have no sterol is almost non-existent[98]. This indicates that the unloading step, which has been modelled to involve a large release of free energy[110], is highly regulated and needs to be understood in more detail[13]. A preference for particular membrane characteristics, varying from biophysical parameters such as packing to the presence of specific lipids, could allow many LTPs to convert the energetics of membrane differences into lipid gradients (Fig. 4E).

LTP function is affected not only by lipid environment but also by protein partners that are asymmetrically distributed. It was observed that a yeast OSBP homologue interacts with Afg2p, an AAA ATPase **[G]** chaperone[111]. If this type of interaction is distributed asymmetrically between donor and acceptor compartment, it might impose a direction on lipid traffic.

Roles of LTPs beyond lipid traffic

The term LTP applies both to a physiological activity found in living organisms and to a laboratory definition tested by *in vitro* experiments with liposomes. Although scientists are interested in finding the former, the latter is much easier to measure. The question here is whether, just because a protein has a domain capable of lipid

transfer *in vitro*, is this the main protein's function *in vivo*? Here we look at some of the alternative functions for LTPs.

LTPs in cell signalling

Many domains first identified in LTPs are found in large proteins that contain other active domains. Examples are common for Sec14-like domains, which appear with RhoGEF, tyrosine phosphatase and RasGAP domains in TRIO, PTPN9 and neurofibromin-1 respectively. StARkin domains co-occur with Rho-GAP domains in DLC proteins (for "deleted in liver cancer") and in some acyl-CoA thioesterases. In plants, StARkin domains are often found in proteins with transcription factor domains, which have lipid-regulated transcription similar to nuclear steroid receptors **[G]**[18]. It is theoretically obvious how a box-like LTP might signal lipid occupancy by changing its external structure when it is internally occupied by a lipid.

One proposed sensor is OSBP, which binds two phosphatases only when occupied by sterol[111], though more studies are needed. Another proposed sensor is ORP1L, suggested to signal cholesterol levels on endosomes to recruit specific endosomal components for the formation of cholesterol dependent contact sites[112]. However, more recent work suggests that the effects of ORP1L can be explained purely through it bridging endosomes to the ER and transferring for cholesterol between the two[113].

Overall, the few clear cut examples of LTPs as sensors come in large multidomain proteins. Before describing LTPs as "sensors", we should exclude if the downstream responses are induced simply by lipid traffic. One way to do this is to test if an unrelated lipid transfer activity replaces its function[77].

LTPs presenting lipid to other proteins

There are several situations where lipids are passed from one protein to another, for which lipid presentation might be a better description than transfer or traffic. Presentation of LPS by LBP to CD14, then to MD2 and TLR4 in the non-adaptive immune system has already described (see discussion of extracellular LTPs above). For adaptive immunity to lipids, γδ T cells **[G]** recognise pathogen-derived lipids, which are presented by CD1, an MHC-I-like surface molecule on antigen presenting cells. Unlike the peptide binding groove of MHC-I, the groove of CD1 isoforms is hydrophobic and binds lipids. Loading of CD1 with lipids takes place in endosomes and lysosomes, with saposins and other soluble LTPs in the late endosomes(LE)/lysosomal lumen presenting the lipid to CD1[114]. Another LTP has a parallel role: microsomal triglyceride transfer protein (MTTP) in the ER allows CD1 to exit the ER, presumably loading it with endogenous lipid to allow its correct folding, thereby avoiding ER-associated degradation[115]. Saposins along with another endo-lysosomal protein GM2AP, are also "activator proteins" for enzymes that break down glycosphingolipids. Here "activator protein" means that saposin and GM2AP stimulate the enzymes, which have very low activities when mixed with liposomes alone, by presenting the lipids to them[116].

LTPs as lipid modifiers

LTPs have cavities that engulf the hydrophobic part or the entire lipid molecule, providing the opportunity for labile bonds in the lipid to be remodelled, or new groups to be added, thereby generating new lipids. One example is GM2AP, which as well as activating (presenting) glycolipids like GM2, has been shown to hydrolyse PC with the generation of lyso-PC and oleic acid[117]. There may be other examples of enzymes among the different StARkin families in bacteria[118].

LTPs and disease: cholesterol as an example

Altered function of human LTPs is linked to many diseases, too many to address here. Instead, we will consider just one lipid in detail to illustrate all of LTP-related pathology. We look at all aspects of the LTP-mediated traffic of cholesterol (Fig. 5), as this major lipid species traffics by a large number of routes, and excess, aberrant cholesterol deposition is the cause of atherosclerosis, the foremost cause of human death worldwide.

Intracellular cholesterol traffic from site of synthesis

Cholesterol generates membranes that are more impermeable to water by causing tighter packing of the membrane lipids and increases the overall thickness of a membrane by straightening acyl chains[1]. Cholesterol is synthesised mainly in the ER and exported from there to all other membranes, including mitochondria where it can be converted irreversibly to bile acids or steroid hormones. Export is mediated by LTPs such as StARTs, ORPs and LAMs. Possibly because of overlapping specificities, there is redundancy, explaining how defects of individual proteins are not linked to specific diseases. However there are diseases where this pathway is hijacked, so that inhibition of LTPs might be beneficial. Replication of plus-strand RNA viruses requires the proliferation of a specialised replication organelle (RO), which is mostly usurped from the secretory pathway, either the Golgi apparatus[119] or the ER in the case of hepatitis C virus[100]. ROs are double membrane structures that contain high concentrations of cholesterol, which is delivered by ORPs in a counter-current with PI4P. Most viruses hijack cellular PI 4-kinases to their ROs, which then powers delivery of cholesterol across contact sites between the RO and a cholesterol source, typically the ER, but for hepatitis C virus cholesterol can come from endosomes. This explains how molecules that block the internal cavity of ORPs inhibit viral replication[120]. When hijacking cholesterol from endosomes, StARD3 and NPC1 (see below) are also involved in viral replication[100].

Cholesterol traffic out of cells

Cholesterol is reversibly converted to membrane-inactive esterified forms for storage inside cells and for transport between cells. Although all cells can make cholesterol, 80% of total synthesis occurs in the liver, which exports LDL/VLDL particles that

contain >2000 cholesterol molecules each, mainly esters, scaffolded on ApoB. ApoB loading takes place in the ER, where MTTP delivers lipids to it. MTTP is in the vitellogenin family of major yolk sac proteins[121], and like them, it has a massive cavity that can bind many lipids (>30), both polar (phospholipids) and neutral (esters). Lack of MTTP causes abetalipoproteinemia **[G]** because, like CD1, lipoproteins such as ApoB can only escape ER quality control if they are lipidated[115]. Since gut-derived lipoproteins are also loaded by MTTP, lack of MTTP causes malabsorption of dietary lipid. Because of this, patients with familial hypercholesterolemia, where LDL accumulates to toxic levels, can be treated by inhibiting MTTP[122]. Export of cellular free cholesterol also takes place from the plasma membrane to ApoA-1 powered by the phospholipid pump ABCA1, and to HDL powered by ABCG1, as described above[89]. Mutations in ABCA1 cause Tangier disease, with aberrant cholesterol ester deposits.

Cholesterol traffic between lipoproteins

Once cholesterol is secreted in lipoproteins, extracellular LTPs transfer cholesterol between them, notably CETP transfers sterol esters from HDL to LDL and VLDL. Because of many genetic links between CETP function and atherosclerosis, several specific CETP inhibitors that bind in its pocket to prevent lipid transfer have been extensively (and expensively) tested over the past 30 years. These drugs change lipoprotein profiles significantly but have not delivered the expected improvement in clinical outcome. This shows how biomarkers (here blood lipids) may not be valid treatment end-points[52].

Cholesterol traffic to the limiting membrane of LE/lysosomes

Lipoproteins are taken up by endocytosis into lysosomes by processes that do not involve LTPs. This is followed by degradation and release of free cholesterol and distribution of sterol from lysosomes to other membranes, including back to the ER, steps which do require LTPs. Exit of cholesterol and other lipids from lysosomes is inhibited in NPC disease, a rare neurodegenerative disorder caused by mutations in either NPC1 (95% of cases) or NPC2 (5%). NPC2 is an MD-2 like LTP specific for cholesterol, which picks up cholesterol from intraluminal vesicle **[G]** membranes and lipoproteins and delivers it to the lysosomal limiting membrane. NPC2 engages in “hydrophobic hand-off” with the N-terminal domain of NPC1, delivering cholesterol directly to it[123]. NPC1 is a large multi-domain protein, its N-terminus being a cholesterol-specific lipid transfer domain exposed to the lysosomal lumen[124], the rest being a channel related to bacterial resistance-nodulation-division efflux pumps[125]. NPC2 delivers cholesterol directly into NPC1’s N-terminal domain. Lipid is then delivered to the bilayer for export. NPC disease can occur with mutations in either of NPC1’s domains, so their function is linked, but the link is not simple because NPC1 is not a permease for cholesterol[125].

Cholesterol traffic from LE/lysosomes to other compartments

Once in the limiting membrane of LE/lysosomes, LDL-derived cholesterol is destined either for the plasma membrane by vesicular recycling or for the ER by non-vesicular traffic. For the latter step, ORP1L is strongly implicated and it also forms ER-LE/lysosome bridges[113].

Certain cancers are linked to (mis)-handling of cholesterol leaving LE/lysosomes by LTPs, in particular StARD3 (aka MLN64), the gene locus of which is adjacent to ErbB2 in a region often amplified in breast cancer[126]. Unlike StAR and other close relatives in humans, StARD3 has transmembrane domains anchoring it in endosomal membranes. It also has a FFAT-like motif, suggesting that it can shuttle lipid between endosomes and ER. However, its function, and its likely contribution to oncogenic signalling, is not to move sterol out of endosomal compartments. Rather, StARD3 moves cholesterol into endosomes from the ER[127]. This traffic in the "wrong" direction is similar to the action of ORP1L to deliver cholesterol from ER to specialised endosomes containing EGF-receptor (aka ErbB1) to promote endosomal maturation by inward budding of intra-luminal vesicles[128]. Over-expression of StARD3 also affects mitochondria, increasing their cholesterol content[129]. More work is required to show both how this enhances malignancy of breast tumours, and whether StARD3 traffics lipids from LE/lysosomes to mitochondria directly (as well as from ER into endosomes). Perhaps it is unexpected that the same LTP can move the same lipid in different directions on different occasions, but there is good evidence for that in the case of ORP1L[113,128].

High rate cholesterol trafficking to mitochondria is a specialised function of adrenal cortex cells that convert cholesterol to steroids for production of mineralocorticoids and sex hormones. It was long known that loss of enzymes in the conversion pathway causes various combinations of congenital adrenal hyperplasia and sexual development disorders. Mutations at one further locus caused a similar syndrome, leading to the discovery of StAR. Comprising an N-terminal mitochondrial localisation sequence and a cholesterol specific StARkin domain, StAR is required for the first committed step in steroid synthesis in mitochondria[130]. Its mechanism of action is not fully understood, but it seems to act in two phases: firstly on the outer mitochondrial membrane to deliver sterol from the ER, then in the mitochondrial matrix, most likely for inhibition of cholesterol import to the inner mitochondrial membrane to limit overproduction of steroids[131].

Conclusion

The field of lipid traffic is advancing on many fronts. One major development in the discovery of new LTPs is ultrastructural analysis by EM, identifying multimeric LTPs, and showing that something as unremarkable as an α-helix can transfer lipid, so long as it multimerises to form a hydrophobic channel wide enough. Extending this work may identify many more protein modules that act as bridges and tubes particularly

since these have not yet been established in eukaryotes. In future, all the understanding we have of LTPs will be applied increasingly to address very different time-scales that are relevant for non-vesicular lipid transfer: the millisecond range during which LTPs load and unload; the seconds to minute range during which lipids flow between compartments; and the life-time range during which LTPs contribute to health and disease.

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Glossary

Lipid transfer protein (LTP)
A protein that facilitates the movement of a lipid from one membrane to another across a cytoplasmic gap. This review is restricted to hydrophobic molecules large enough to contribute to membrane structure – i.e. bilayer lipids, or their adducts. By this definition, we have excluded proteins such as fatty acid binding proteins and lipocalins that bind and transfer other, smaller, hydrophobic molecules such as fatty acids and hydrophobic vitamins.

Phagophore
The double membrane, also termed isolation membrane, where autophagy initiates. Autophagy-related proteins act on the phagophore to create the autophagosome.

Lipid desorption
The release of a lipid molecule from a membrane bilayer into the aqueous phase. This process requires a high activation energy for highly hydrophobic lipids, such as glycerophospholipids with two acyl chains.

Oxysterol
An oxidised derivative of cholesterol often created by a specific enzyme, implicated in different cellular processes including cholesterol homeostasis, metabolism, and apoptosis.

Phosphoinositide

A phosphatidylinositol lipid that is further phosphorylated on the inositol headgroup. Any of the 3, 4 or 5 positions of the sugar ring can be reversibly phosphorylated to make 7 different phosphoinositides. Each phosphoinositide has a specific biological activity related to the proteins that interact with it.

Lipopolysaccharide (LPS)
Also known as endotoxin, LPS is a component of the outer membrane of Gram-negative bacteria with structural and protective functions. It is also a strong pro-inflammatory molecule in the immune system.

Gram-negative bacteria
Group of bacteria that do not stain with the crystal violet used in the Gram staining method. They have two membranes, with LPS confined to the outer leaflet of the outer membrane. A peptidoglycan cell wall is found in the periplasmic space between the outer and inner (cytoplasmic) membranes.

Inner mitochondrial membrane
Membrane that separates the mitochondrial matrix from the inter-membrane space. This membrane forms cristae and is similar to bacterial inner membranes in composition. The inner mitochondrial membrane hosts many enzymes including the electron transport chain, function of which requires cardiolipin, one of several mitochondrial lipids synthesised in the inner mitochondrial membrane.

Outer mitochondrial membrane
Limiting membrane of mitochondria, containing only low levels of lipids synthesised in the inner mitochondrial membrane. The outer mitochondrial membrane makes functional contacts both with the inner mitochondrial membrane and with other organelles, including the ER.

Endotoxin
See LPS.

Toll-like receptor (TLR)
TLRs are single pass transmembrane proteins expressed on the surface of sentinel cells of the immune system and cycling through endosomes. TLRs recognise structurally conserved molecules in pathogenic organisms and initiate immune responses via intracellular signalling cascades, often after endocytosis.

ATP-binding cassette (ABC) transporter
Membrane embedded proteins containing a AAA ATPase domain (see below), where consumption of ATP is linked to pumping of a small molecule across the membrane. In ABCA1 and ABCG1 the pumped substrate is a phospholipid, movement of which leads to cholesterol flux.

Counter-transport (Counter-current lipid transport)
Lipid transport of two different lipids in opposite directions between two membranes by a single LTP. The LTP shuttles the lipids alternately as it shuttles between the two compartments. This is analogous to counter-transport by antiporter pumps, although these carry out the transport in opposite directions simultaneously and obligatorily.

AAA ATPase
ATPase Associated with diverse cellular Activities proteins couple energy generated by ATP hydrolysis with conformational changes. The variable N-terminus is usually involved in substrate recognition. ATP consumption result in remodelling, so that AAA ATPases can be chaperones, such as Afg2p in yeast, which binds Osh1, or pumps (see ABC transporters)

Nuclear steroid receptors
Soluble intracellular receptors for steroid hormones (cortisol, oestrogen *etc.*) that consist of a steroid binding domain and a DNA binding domain. In response to ligand binding they translocate to the nucleus and regulate transcription.

γδ T cells
T cell subpopulation particularly found in the gut mucosa expressing a T cell receptor made of one γ (gamma) and one δ (delta) chain (as opposed to the majority of T cells, which express αβ). They have a major role in recognising lipid antigens.

Abetalipoproteinemia
Human disorder characterised by dysfunctional absorption of dietary fat caused by autosomal recessive mutations in Microsomal Triglyceride Transfer Protein (MTTP), impairing the gut's ability to synthesise chylomicrons and VLDL from absorbed fat.

Intraluminal vesicle
Endosomes generate intraluminal vesicles by inward budding of the endosomal limiting membrane. When secretory lysosomes fuse with the plasma membrane, intraluminal vesicles are secreted as exosomes.

Box 1. Computer simulations of lipid transport

All LTPs have hydrophobic cavities, indicating that they stabilise lipid after its desorption from a membrane. Yet the way LTPs engage with membranes to stimulate lipid desorption, reducing the energy barrier for lipids to leave bilayers, is poorly understood. This particularly applies to identifying flexible LTP conformations that sculpt the energy pathway for lipid loading and unloading. These intermediates are hard to capture and the time-scale of conformational change is unknown. The fastest lipid transfer observed in living cells is about 10 lipids transferred per second per LTP, *i.e.* 10 each of loading at donors and unloading at acceptors[121]. Prior to obtaining detailed information on (un-)loading in real-time, an alternative option is to model how LTPs are likely to interact with membranes by molecular dynamics computer simulations based on static crystallographic structures. Interesting work on PITPα, which takes up PC or PI to a final position ~3 nm distant from their starting point in the membrane, suggested that the LTP's exchange loop changes conformation upon bilayer insertion[101]. The simulations showed spontaneous lifting of lipid approximately 1 nm out of the bilayer might occur once in a 1 μs time window. This work required $5x10^9$ integrations (2 fs each time frame) of all the atoms of an LTP-bilayer interface, which is at the current limit for computing power. This indicates that we have a long way to go to understand the complete journey to lipid (un-)loading, which occurs over a time-frame of up to 50,000 μs[121].

LEGENDS

Fig. 1: Box-like LTPs with lids undergo conformational shifts to allow lipid (un-)loading.

(A) Box-type LTPs enclose part or all of the lipid ligand in an internal hydrophobic cavity, shielding lipid from the aqueous environment. Box-type LTPs may either expose the hydrophilic head group (left) or have a region analogous to a lid (right) that changes conformation from closed (purple) to open (grey) to allow (un-)loading. Note that inside the cavity, the hydrophilic head group can be either proximal (as shown) to the opening or distal (not shown). (B) Shuttling of an LTP to transfer lipid requires several steps: donor docking, lipid extraction, donor undocking, diffusion, acceptor docking, lipid deposition, acceptor undocking and further diffusion. (C) Two different views of StARD4 showing the β-grip surrounding its binding cavity and the Ω1 loop. Top: cross sections of the crystal structure (PDB: 1JSS). Bottom: cartoon, with green indicating cavity lining and the hydrophobic face of the C-terminal α-helix. (D) In the open, membrane-binding conformation of StARD4, the C-terminal α-helix rotates exposing its hydrophobic face to the membrane, and the Ω1 loop bends away from the cavity creating an entrance.

Fig. 2: Multi-subunit assembly of LTPs.

(A) Individual domains in LptC make a lipid-conducting bridge, along with LptA and LptD. (i) Diagram and crystal structure of LptC (PDB: 3MY2), showing its U-shaped cross-section, which makes a seam along which lipid can slide. (ii) LptC forms end-to-end multimers with similarly folded domains in LptA and LptD. Note that the bridge is helical, twisting about its main axis, but the twist has been omitted in the diagram. (iii) This bridge is preceded by a pump (equivalent to LptB; see also Fig. 4a) pushing lipid molecules into one end of the bridge. (B) Three models for lipid transfer by ERMES. (i) Mmm1p, Mdm12p and Mdm34p are cone-shaped LTPs, possibly with seams running along one side. (ii) Like most TULIPs, all three ERMES SMPs form head-to-head dimers. (iii) The Mmm1p dimer can be capped by Mdm12 subunits to make heterotetramers. **INSET** cryo-electron microscopy images of Mdm12/Mmm1 heterotetramer (image from ref. [132]). (iv)-(vi) Lipid traffic might occur by three routes: (iv) Shuttling, where different cavities in the complex (here shown as a dimer) pick up lipids and shuttle them between membranes. (v) Bridging by a lipid slide, with one continuous seam across three subunit interfaces, one of which is head-head, and two head-to-tail. (vi) Bridging by a multimeric lipid shuttle, here illustrated as a Mmm1p/Mdm12p/Mdm34p trimer, where lipid only ever crosses head-head interfaces, and net movement is facilitated by rotations of the subunits. (C) Different LTP tubes constructed by MCE multimers. (i) MCE domains form discs of six subunits with a hydrophobic central pore. (ii) MlaD has 1 disc that interacts with the shuttle LTP MlaC; (iii) YebT has 7 discs. **INSET** cryo-electron microscopy images of YebT (image from ref. 62); (iv) one MCE domain in PqiB has an α-helix extension that forms a 6-bundle super-coil (here shown as straight for simplicity), which forms a

tube with a central cavity that matches the pore size of MCE domains. (v) PqiB has two other MCE domains, making three overall and the tube. **INSET** images of syringe-and-needle-like PqiB (image from ref. 62).

Fig. 3: Localisations of LTPs.

LTPs of different families, intracellular and extracellular. Many intracellular LTPs target membrane contact sites, with strong targeting indicated by position, and weak targeting indicated by arrows. Intracellular targeting domains are also shown (see Key).

ACBD, Acyl-CoA Binding Domain containing protein; BPI, Bactericidal/Permeability-Increasing protein; CD14, Cluster of Differentiation 14; CERT, CERamide Transfer; CETP, Cholesteryl Ester Transfer Protein; CPTP, Ceramide-1-Phosphate Transfer Protein; DDHD, domains characterised by these conserved residues (for metal ion binding); E-Syt, Extended Synaptotagmin; FAPP2, phosphatidylinositol-Four-phosphate AdaPtor Protein-2; FFAT, two phenylalanines (FF) in an Acid Tract; GLTP, GlycoLipid Transfer Protein; LAM, Lipid transfer protein Anchored at Membrane contact sites; LBP, Lipopolysaccharide-Binding Protein; LNS2, Lipin/Ned1/Smp2 domain; MD2, *myb*-regulated 2; Mdm12, Mitochondrial Distribution and Morphology-12; Mmm1, Maintenance of Mitochondrial Morphology-1; NPC1, Niemann-Pick disease, type C1; nsLTP, Non-Specific Lipid Transfer Protein; Nvj2, Nucleus-Vacuole Junction protein 2; ORP, OSBP-Related Protein; ORP1L, OSBP-Related Protein 1; OSBP, OxySterol Binding Protein; Osh, OSBP Homologue; PDZD8, Phorbol-ester/DAG-type/Zn-finger Domain-containing protein 8; PH, Pleckstrin Homology; PITP, PhosphatidylInositol Transfer Protein; PITPNM, Membrane-associated phosphatidylinositol transfer protein 1; PLTP, PhosphoLipid Transfer Protein; PRELI, Protein of Relevant Evolutionary and Lymphoid Interest; PRY, Pathogen-Related Yeast protein; SEC14, yeast SECretory mutant 14; SFH, Sec Fourteen Homologue; SPLUNC, Short Palate, LUng, and Nasal epithelial Clone; StAR, Steroidogenic Acute Regulatory protein; StARD, StART Domain-containing protein; STARkin, relatives (kin) of StAR; TLR4, Toll-Like Receptor 4; TMEM24, TransMEMbrane protein 24; TULIP, TUbular LIPid-binding.

Fig. 4: Different ways LTPs contribute to creation of lipid gradients

(A) Direct consumption of ATP by LTP co-factors. For example, the LptA-G complex forms a bridge that stretches from the donor membrane to the acceptor membrane. ATP is used by LptB in the donor membrane to pump an LPS lipid into one end of the $LptCA_nD$ bridge, driving the lipid transfer up a gradient. (B) Consumption of lipid in the acceptor membrane. After synthesis in the donor membrane and transfer by an LTP to the acceptor membrane, lipid can be consumed or modified, trapping it in the acceptor membrane so that the LTP cannot return it to the donor. An example is

878 ceramide conversion to sphingomyelin in the TGN after transfer from the ER by
879 CERT. (C) Acceptor membrane acts as a sink. The acceptor membrane contains
880 other lipids that interact with the transferred lipid more strongly than do the lipids of
881 the donor membrane. The amount of total lipid is not reflected in the amount of lipid
882 that is available for traffic. At equilibrium, the concentrations of free lipid in the donor
883 and acceptor are the same, but the concentration of total lipid in the donor is less
884 than in the acceptor. (D) LTPs exchange two different lipids in a counter-current.
885 Energy is consumed to create a gradient of lipid A (green triangle headgroups;
886 mechanism of synthesis and consumption not shown). The LTP transfers this ligand
887 down this gradient (right→left). Where [lipid A] is low, the LTP picks up and transfers
888 lipid B (blue circles). Maintenance of the steep gradient of lipid A drives lipid B in the
889 opposite direction. (E) LTP conformation changes in response to lipid binding,
890 affecting LTP loading or unloading at specific membranes. In this diagram, empty
891 LTP has a preference for a property of pink membranes (left) and the lipid-bound
892 form has a preference for a property of blue membranes (right) leading to net
893 transfer left→right.

894

895 **Fig. 5: Cholesterol transport by LTPs that is associated with pathology.**

896 Red arrows indicate routes of cholesterol traffic that are both mediated by LTPs and
897 involved in human diseases. After synthesis, the bulk of intracellular cholesterol
898 traffic is non-vesicular, mediated by LTPs in the ORP and StARkin families (LAM and
899 StART), along routes such as ER to plasma membrane, and ER to TGN (details not
900 shown). Blue arrows on these routes indicate that no single genetic lesion is linked to
901 a disease of cholesterol traffic, possibly because of redundancy. However, ORPs are
902 key cellular components hijacked by positive strand RNA viruses replicating in virus
903 factories called replication organelles (RO). Cholesterol is imported into ROs by
904 ORPs using a counter-current of PI4P that is created by a PI 4-kinase recruited to
905 the RO, and consumed by SAC1 on the ER. For bulk export of cholesterol from cells
906 into the circulation, cholesterol esters are loaded by microsomal triglyceride transfer
907 protein (MTTP) in the ER into apolipoprotein B (ApoB), which is then secreted via
908 vesicular traffic. LTP function is ascribed to lipid pumps involved in the maturation of
909 HDL. First cholesterol and phospholipids are exported to ApoA-1 by ABCA1 to form
910 nascent HDL (HDL^N), then mature HDL (HDL^M) is formed by further lipid delivered by
911 ABCG1. Lipoproteins in the circulation exchange cholesterol esters via CETP. Most
912 cells acquire cholesterol from circulating lipoproteins, which are endocytosed and
913 trafficked via early endosomes (EE) to late endosomes (LE) and lysosomes (here
914 combined as LE/lysosome for simplicity). Before hydrolysis of endocytosed
915 cholesterol ester can begin, StARD3 and ORP1 on the cytoplasmic face of different
916 classes of endosomes traffic cholesterol from the ER to allow endosomal maturation
917 by the formation of intra-luminal vesicles. After further acidification, cholesterol esters
918 are hydrolysed and large amounts of cholesterol are released. Exit of cholesterol
919 requires hydrophobic hand-off of free cholesterol within the LE/lysosome lumen
920 between NPC2 and NPC1. ORP1 traffics released cholesterol to the ER and
921 StARD3 likely mediates transport to mitochondria. Cholesterol traffic from all

intracellular sources to mitochondria is very high in steroidogenic cells, where StAR, also called StARD1, imports cholesterol to the outer mitochondrial membrane.

Supplementary Box 1. Phosphatidylethanolamine synthesis and the LTP That Never Was

A few major reactions in eukaryotic lipid synthesis take place outside the ER (see Section 3). For cardiolipin (synthesised in the mitochondrial matrix) and sphingomyelin (produced in the exofacial leaflet of the TGN), return of these lipids to the rest of the cell is minimal, requiring flipping back out of these compartments plus intracellular traffic by as yet unidentified LTPs. PE synthesis in mitochondria differs from both of these for two main reasons: (i) the pathway appears redundant, since there is a universal PE synthetic pathway in the ER; and (ii) even though it is redundant and goes outside the ER, under some circumstances this pathway can supply all the needs of extra-mitochondrial membranes not only for PE but also of PC, the cell's most numerous phospholipid, which is formed from PE in the ER. These observations strongly implied that there must be an LTP to return PE from mitochondria to the ER, and this was the basis for some of the earliest and most influential hypotheses about LTPs and non-vesicular lipid traffic[1,2]. This hypothesis has just been overturned by detailed studies of how the PE biosynthetic enzyme works[3].

The mitochondrial enzyme that synthesises PE is PS decarboxylase-1 (Psd1). It is not expressed universally, in animals being restricted to liver cells, but it has been studied most extensively in budding yeast. Mitochondrial PE is made in the inner membrane by Psd1 acting on PS that is imported by an LTP in the inter-membrane space (Ups2p in yeast)[4]. It was presumed that this pool of inner mitochondrial membrane PE was then transported back to the outer membrane and then the ER[1,2]. However, the long-term "well-known" localisation of Psd1, embedded in the inner mitochondrial membrane, missed two subtle alternate possibilities. Firstly, it was shown that the active site of Psd1 enzyme is able to synthesise PE in the outer membrane by reaching across the inter-membrane[4]. The catalytic site of Psd1 is in a globular domain located in the inter-membrane space, attached to the transmembrane domain embedded in the inner mitochondrial membrane by an unstructured linker of 26 residues, long enough to reach across the inter-membrane space[4]. Secondly, and more significantly, a proportion of the Psd1 enzyme has been found not in mitochondria, but in the ER[3]. This proportion is regulated to match demand, rising either when the ER and the bulk of cellular membranes lack PE, or when the need for PE to support mitochondrial matrix function is decreased by switching from oxidative phosphorylation to glycolysis[3]. Thus, one enzyme makes PE in three different membranes, and the postulated PE-specific LTP may never be found.

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Figure 1

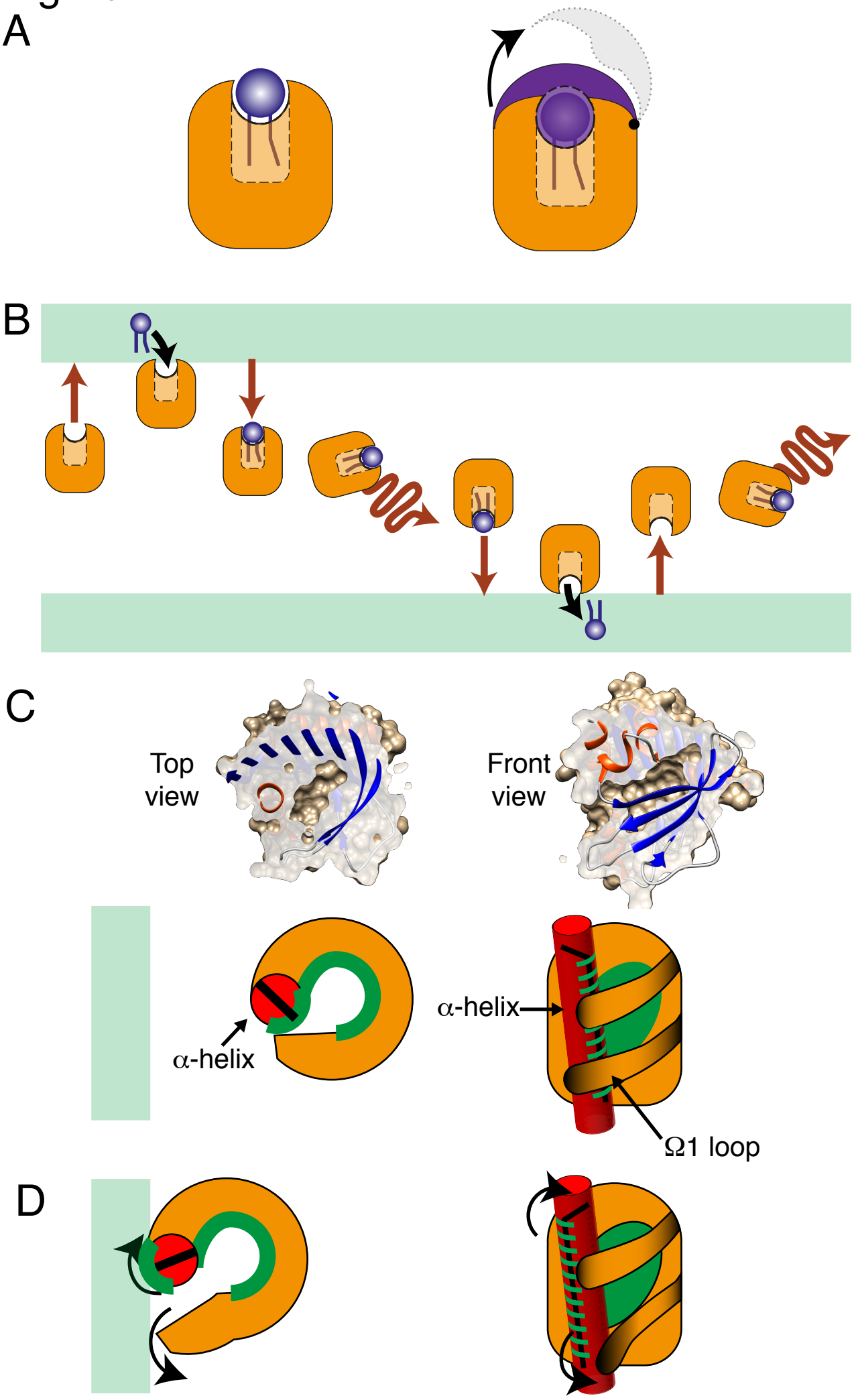
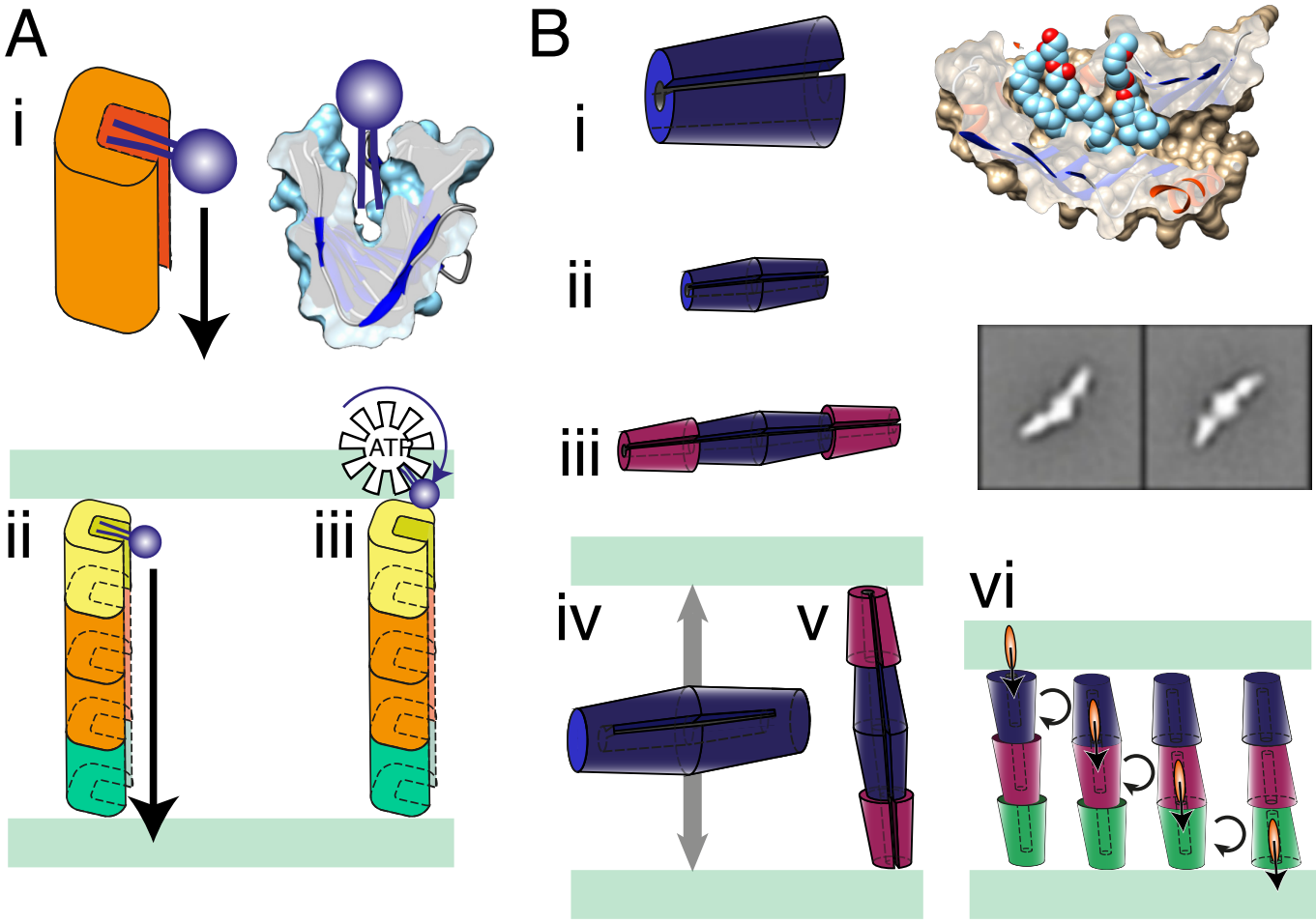


Figure 2


A
i
ii
iii
ATP
B
i
ii
iii
iv
v
vi

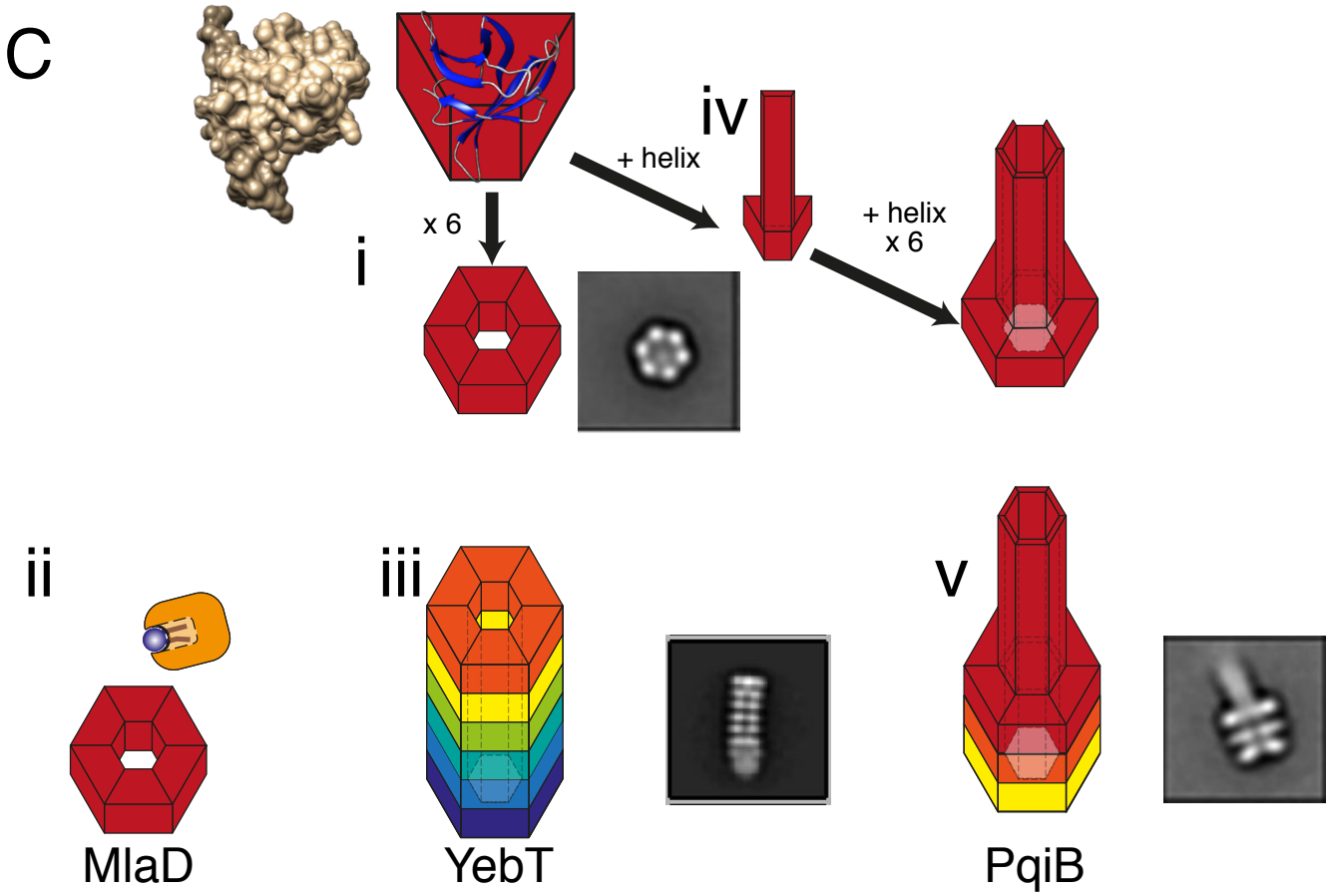

C
i
x 6
iv
+ helix
+ helix
x 6
ii
MlaD
iii
YebT
v
PqiB

Figure 3

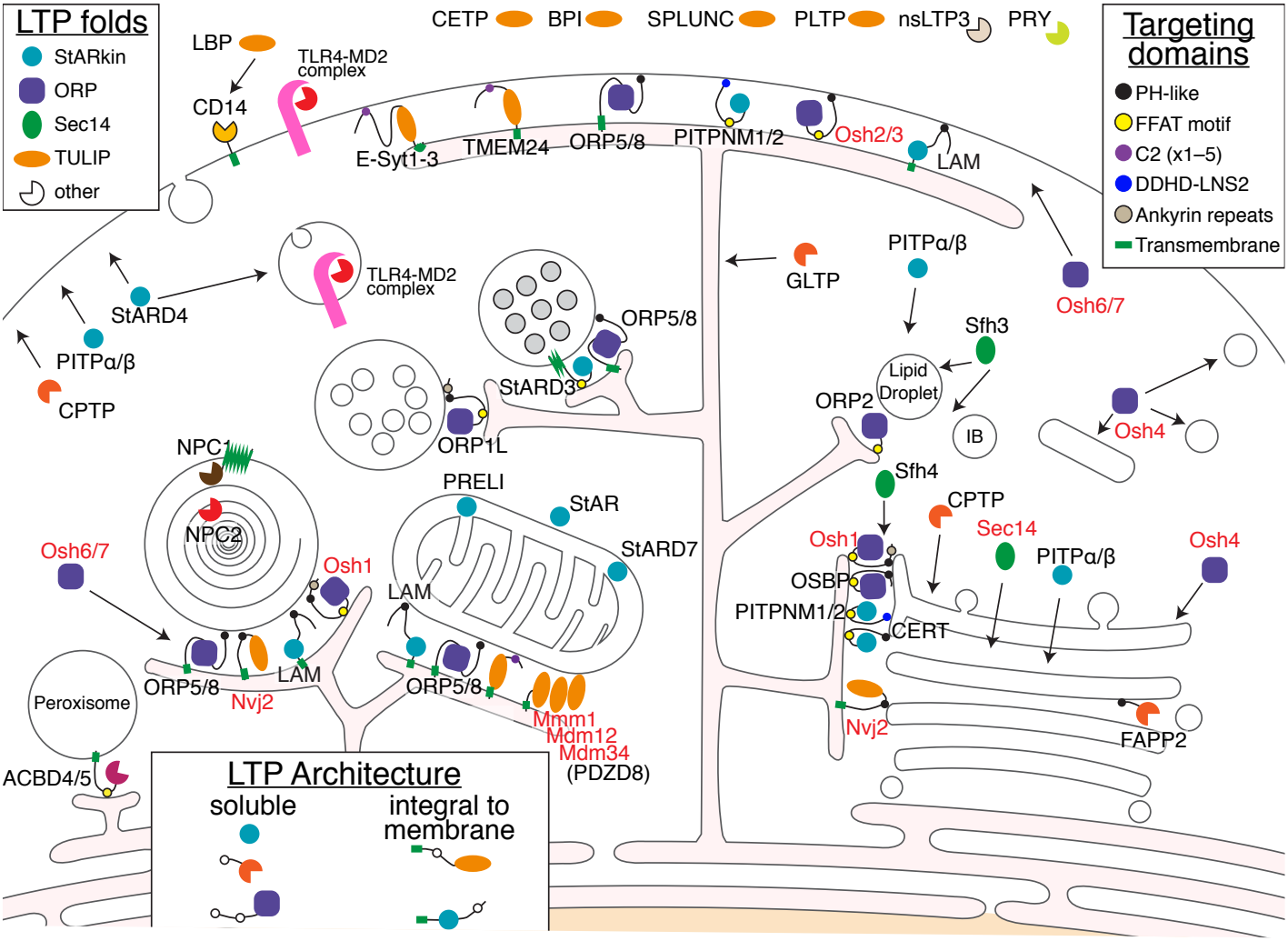

LTP folds
StARkin
ORP
Sec14
TULIP
other
CETP
BPI
SPLUNC
PLTP
nsLTP3
PRY
Targeting domains
PH-like
FFAT motif
C2 (x1–5)
DDHD-LNS2
Ankyrin repeats
Transmembrane
LBP
CD14
TLR4-MD2 complex
E-Syt1-3
TMEM24
ORP5/8
PITPNM1/2
Osh2/3
LAM
GLTP
PITPα/β
Osh6/7
StARD4
PITPα/β
CPTP
TLR4-MD2 complex
ORP5/8
StARD3
ORP1L
Sfh3
Lipid Droplet
ORP2
IB
Osh4
Sfh4
CPTP
Sec14
PITPα/β
Osh4
NPC1
NPC2
PRELI
StAR
StARD7
Osh6/7
Osh1
LAM
Osh1
OSBP
PITPNM1/2
CERT
Peroxisome
ORP5/8
Nvj2
LAM
ORP5/8
Mmm1
Mdm12
Mdm34
(PDZD8)
Nvj2
FAPP2
ACBD4/5
LTP Architecture
soluble
integral to membrane

Figure 4

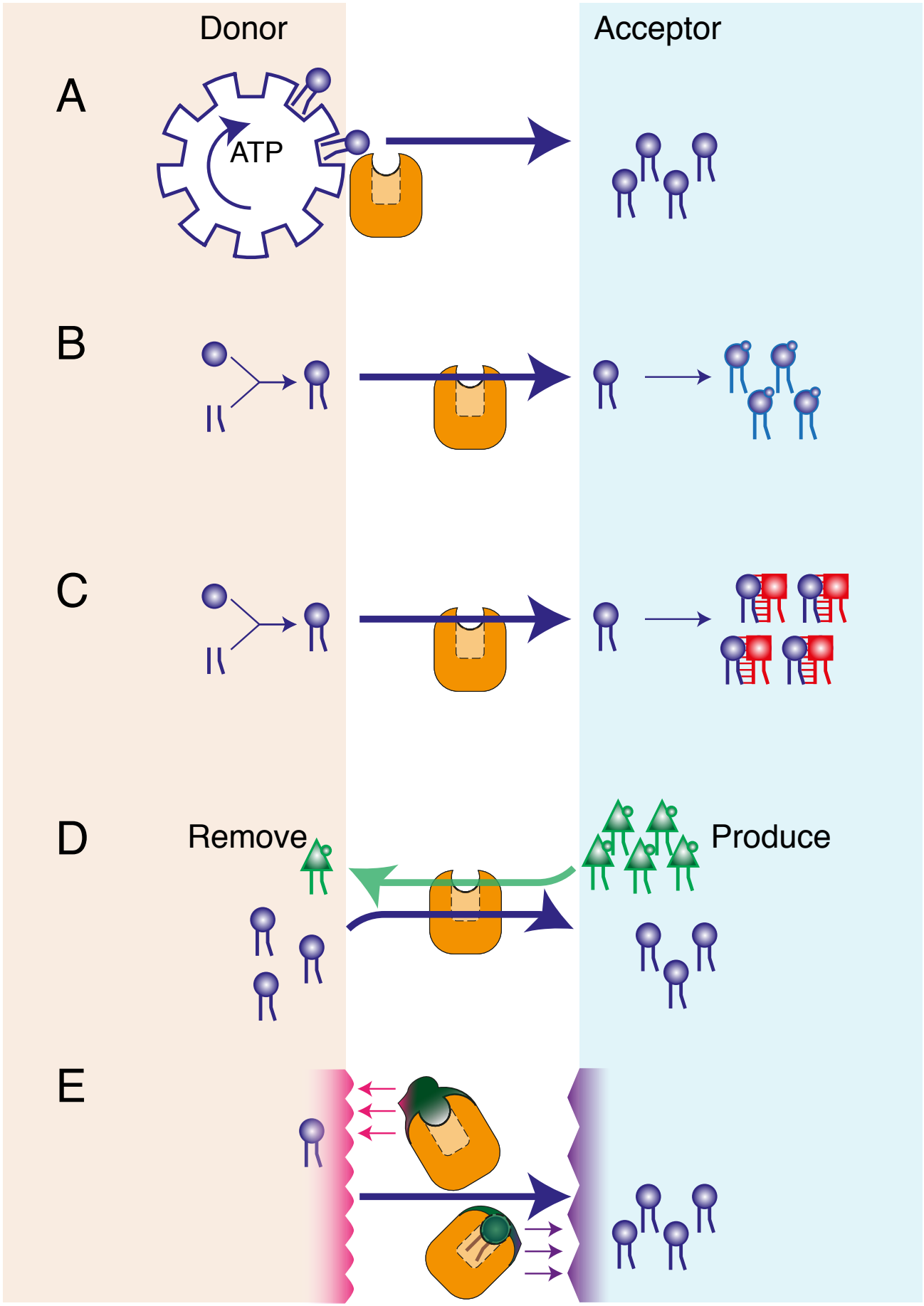
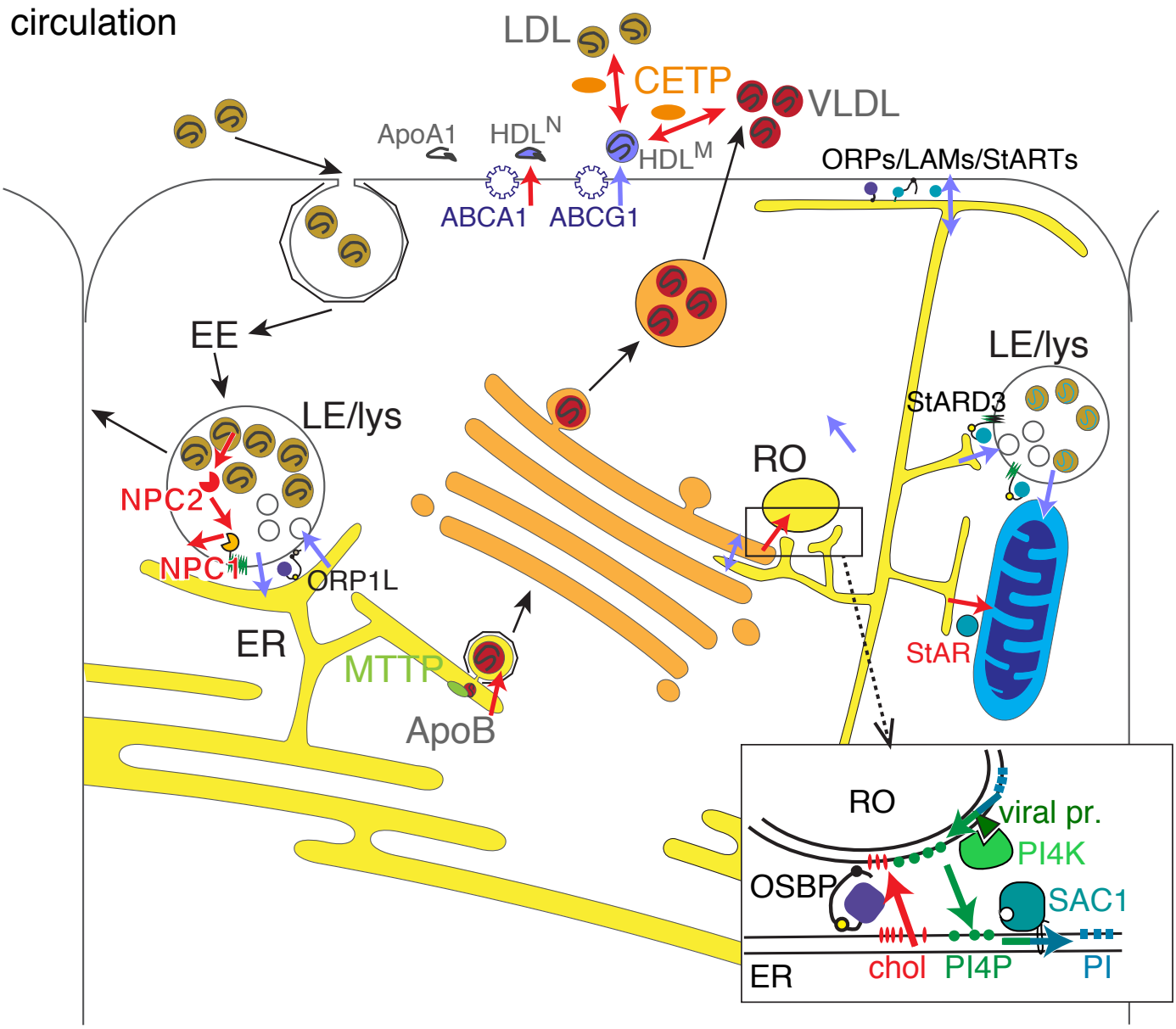


Figure 5


circulation
LDL
CETP
VLDL
ApoA1
HDL^N
HDL^M
ABCA1
ABCG1
ORPs/LAMs/StARTs
EE
LE/lys
NPC2
NPC1
ORP1L
ER
MTTP
ApoB
RO
LE/lys
StARD3
StAR
RO
viral pr.
PI4K
OSBP
SAC1
ER
chol
PI4P
PI