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COMMENTARY

Inner workings and biological impact of phospholipid flippases

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ABSTRACT

The plasma membrane, trans-Golgi network and endosomal system of eukaryotic cells are populated with flippases that hydrolyze ATP to help establish asymmetric phospholipid distributions across the bilayer. Upholding phospholipid asymmetry is vital to a host of cellular processes, including membrane homeostasis, vesicle biogenesis, cell signaling, morphogenesis and migration. Consequently, defining the identity of flippases and their biological impact has been the subject of intense investigations. Recent work has revealed a remarkable degree of kinship between flippases and cation pumps. In this Commentary, we review emerging insights into how flippases work, how their activity is controlled according to cellular demands, and how disrupting flippase activity causes system failure of membrane function, culminating in membrane trafficking defects, aberrant signaling and disease.

KEY WORDS: Golgi complex, P-type ATPase, Flippase, Lipid asymmetry, Phosphatidylserine, Vesicular transport

Introduction

A striking aspect of eukaryotic cell membranes is the uneven distribution of different lipid species across the bilayer, with sphingolipids concentrated in the exoplasmic leaflet and the amino-phospholipids phosphatidylserine (PtdSer) and phosphoethanolamine (PtdEth) mainly restricted to the cytoplasmic leaflet (Op den Kamp, 1979). This lipid asymmetry provides membranes with two distinct surfaces, each with unique adhesive properties. For instance, a high PtdSer concentration in the cytoplasmic leaflet of the plasma membrane is essential for various signaling events mediated by membrane translocation and activation of specific kinases such as protein kinase C (Newton and Keranen, 1994). A low PtdSer concentration in the exoplasmic leaflet of the plasma membrane, by contrast, is crucial for cell survival, as phagocytes recognize and engulf cells by binding to the PtdSer exposed on their surface (Balasubramanian and Schroit, 2003). The dissipation of plasma membrane lipid asymmetry by Ca^{2+}-activated scramblases and exposure of aminophospholipids on the cell surface triggers physiological responses ranging from blood coagulation, myotube formation and sperm capacitation to the clearance of apoptotic cells (Zwaal and Schroit, 1997; Suzuki et al., 2010; Malvezzi et al., 2013). This Commentary focuses on type 4 P-type ATPases (P_4-ATPases) as ATP-fueled phospholipid pumps that create lipid asymmetry by catalyzing unidirectional phospholipid transport across cellular bilayers. As P_4-ATPases stem from an ancient family of cation pumps, much effort is currently aimed at understanding how P_4-ATPases acquired the ability to translocate phospholipids instead of small ions. Another intriguing aspect of P_4-ATPases is their crucial role in vesicular trafficking to and from the plasma membrane. Below, we discuss emerging evidence indicating that unidirectional phospholipid transport catalyzed by P_4-ATPases helps establish the membrane curvature needed to bud vesicles from the trans-Golgi network (TGN), endosomes and plasma membrane. We also describe how P_4-ATPase-catalyzed flippase activity is subject to complex regulatory mechanisms that interconnect the establishment of lipid asymmetry with phosphoinositide metabolism and sphingolipid homeostasis, allowing cells to cross-regulate multiple key determinants of membrane function. At the organismal level, disruption of P_4-ATPase function has been linked to diabetes, obesity, immune deficiency, neurological disorders and a potentially fatal liver disease. Recent insights into the molecular basis of these diseases are also discussed.

Origin and biological relevance of lipid asymmetry

Membrane lipids in eukaryotic cells display non-random distributions among subcellular organelles as well as between the two membrane leaflets of individual organelles. For instance, sphingolipids and sterols form a step gradient along the secretory pathway, with their levels being highest at the plasma membrane and lowest in the endoplasmic reticulum (ER; Fig. 1) (Holthuis and Menon, 2014). This arrangement has important functional consequences. Sterols are rigid and sphingolipids have saturated acyl chains, so both increase acyl chain order and thicken the plasma membrane bilayer to reduce its permeability to solutes (Brown and London, 1998). By contrast, the low sphingolipid and sterol content of the ER results in a loosely packed lipid bilayer that facilitates the insertion of newly synthesized proteins and lipids, thus supporting the biogenic function of this organelle. Moreover, as membrane proteins tend to seek bilayers with a thickness that matches the length of their membrane spans, the sphingolipid and sterol step-gradient might separate proteins with short membrane spans that cycle between the ER and Golgi segregated from proteins with longer membrane spans that cycle between the Golgi and plasma membrane (Ceppi et al., 2005; Munro, 2005; Sharpe et al., 2010).

Superimposed on the sphingolipid and sterol step-gradient are the asymmetric lipid distributions across the bilayers of the TGN, endosomes and plasma membrane, with PtdSer and PtdEth concentrated in the cytoplasmic leaflet and sphingolipids enriched in the exoplasmic leaflet (van Meer et al., 2008). This asymmetry serves a multitude of functions. A tight packing of sphingolipids and sterols in the exoplasmic leaflet is important for membrane stability in circulating blood cells and makes the apical surface of intestinal epithelial cells resistant to bile salts. Conversely, the accumulation of cone-shaped PtdEth in the cytoplasmic leaflet of the plasma membrane and on the surface of endocytic and secretory vesicles might keep these sterol-rich membranes in a fusion-competent state (Holopainen et al., 2000; Alder-Baerens et al., 2006). The high concentration of negatively charged PtdSer on the cytoplasmic surface of the TGN and plasma membrane provides a specific cue for the recruitment of peripheral membrane proteins with polybasic motifs (Kay and Grinstein, 2013). Studies with a genetically encoded PtdSer probe indicate that the bulk of PtdSer in the ER faces the lumen, an

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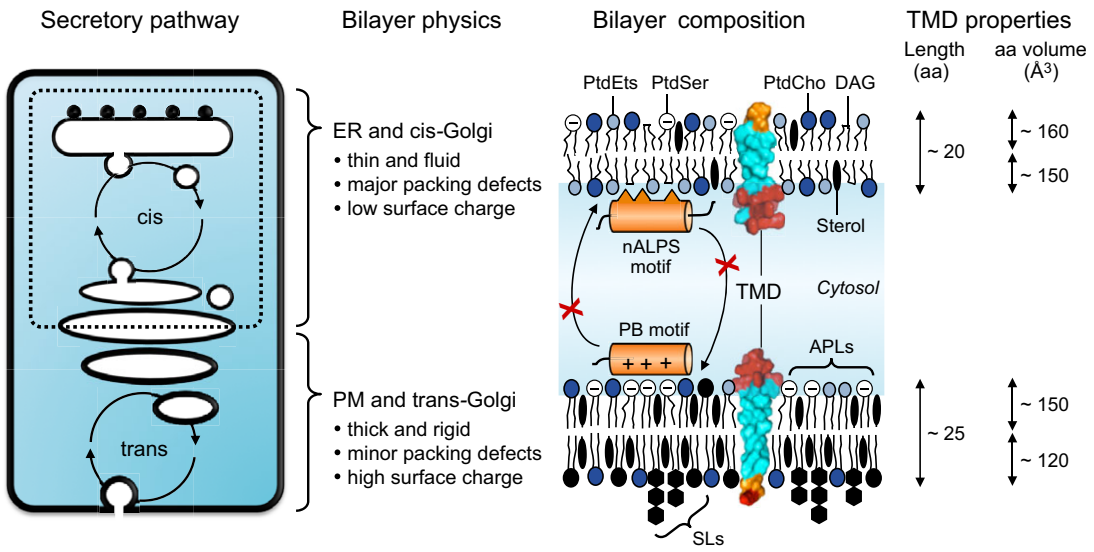

Fig. 1. Membranes of early and late secretory organelles display contrasting lipid compositions and physical properties. Physical membrane properties are influenced by lipid composition. Fluidity is promoted by lipids with short and unsaturated acyl chains, predominantly glycerophospholipids. Thickness is promoted by sphingolipids (SLs), which have long and saturated acyl chains, and by sterols, which order and stretch the acyl chains. Packing defects are promoted by lipids with unsaturated acyl chains and/or small head groups, such as PtdEth or diacylglycerol (DAG). Surface charge is determined by the chemical properties of the lipid head group, which is neutral in PtdCho and negative in PtdSer. The ER has a thin bilayer, loose lipid packing and neutral cytosolic surface charge, adapted for its biogenic function. By contrast, the plasma membrane has a thick bilayer, tight lipid packing and negative cytosolic surface charge, adapted for its barrier function. These contrasting physical properties are not only reflected in the length and geometry of transmembrane domains (TMDs) of ER- and plasma-membrane-resident proteins (Sharpe et al., 2010), but also permit organelle-specific recruitment of peripheral membrane proteins (orange cylinders). Membrane traffic between the ER and plasma membrane passes the Golgi, a polarized multi-cisternal organelle. In the Golgi, both lateral and transbilayer lipid sorting must occur to preserve the unique lipid compositions and features, and, thus, the specialized functions of the ER and plasma membrane. APLs, amino phospholipid translocases; nALPS, neutral Arf lipid packing sensor; PB, polybasic

asymmetric distribution opposite to that of the TGN and plasma membrane (Fairn et al., 2011). The relatively neutral cytosolic surface and loose lipid packing allows the ER to specifically recruit proteins with neutral amphipathic lipid packing sensor (nALPS) motifs, which contain bulky hydrophobic residues that readily insert where there are lipid packing defects (Fig. 1) (Bigay and Antonny, 2012). Sphingolipids are primarily synthesized in the luminal leaflet of trans-Golgi cisternae and delivered to the cell surface by vesicular transport, which explains their asymmetric distribution across the plasma membrane (van Meer et al., 2008). However, transbilayer lipid asymmetry cannot be explained by sidedness of lipid production or breakdown alone and relies, at least in part, on a combination of other principles. These include biophysical properties that dictate the ability of a lipid to cross the bilayer spontaneously (charge or bulkiness of the polar headgroup), retentive mechanisms that trap lipids on one side of the bilayer (packing density and lipid-binding proteins), and the presence of protein catalysts, termed flippases, which hydrolyze ATP to actively translocate specific phospholipids to the cytoplasmic leaflet against a concentration gradient. These activities should not be confused with the ATP-independent bidirectional lipid transporters that operate in biogenic membranes such as the ER (Box 1). The first ATP-fueled flippase described was the aminophospholipid translocase associated with red blood cells, which catalyzes a fast inwards movement of PtdSer and PtdEth across the plasma membrane cells (Seigneuret et al., 1984). Similar flippase activities occur in the plasma membrane of most nucleated cells as well as in the TGN, secretory vesicles and endosomes (Zachowski et al., 1989; Natarajan et al., 2004; Alder-Baerens et al., 2006).

Flippases evolved from a family of cation pumps

The discovery of an aminophospholipid translocase activity in bovine chromaffin granules, termed ATPase II, led to the cloning of a gene referred to as *ATP8A1* (Tang et al., 1996). The corresponding enzyme is homologous to Drs2, a TGN protein in yeast. ATP8A1 and Drs2 are the founding members of a conserved subfamily of P-type ATPases, the P_4-ATPase subfamily (Axelsen and Palmgren, 1998). This subfamily comprises five members in yeast (Drs2, Dnf1, Dnf2, Dnf3 and Neo1) and 14 members in man (for examples, see Table 1). P_4-ATPases catalyze unidirectional transport of specific phospholipid classes from the exoplasmic to the cytoplasmic leaflet and are clearly linked to the establishment of phospholipid

Box 1. ER flippases

The term 'flippase' was originally coined to refer to the bidirectional lipid transporters responsible for equilibrating newly synthesized phospholipids across biogenic membranes, such as the ER (Bishop and Bell, 1985). ER flippases function independently of metabolic energy and promote lipid symmetry by catalyzing a fast scrambling of most phospholipid classes across the bilayer. The identity of ER flippases is not known. Peptides mimicking the α-helices of membrane proteins stimulate phospholipid scrambling in model membranes, suggesting that this activity is not necessarily restricted to one specific protein (Kol et al., 2003a). The rhodopsin-like G-protein-coupled receptor (GPCR) opsin exhibits a constitutive phospholipid scrambling activity that is distinct from its light-sensing function (Goren et al., 2014). Other GPCRs also scramble phospholipids. Thus, GPCRs en route to the plasma membrane might provide the phospholipid scramblase activity that is necessary for the biogenic activity of the ER (Goren et al., 2014). The constitutive scramblase activity of GPCRs is silenced at the plasma membrane. This might be accomplished by the high levels of sterols, which cause a tight packing of the acyl chains through which the polar lipid headgroup has to travel (Kol et al., 2003b). Note that low scramblase activity is a prerequisite for preserving the asymmetric lipid arrangements created by unidirectional P_4-ATPase flippases that operate in late secretory and endosomal orgnanelles.

Table 1. Substrate specificities and biological roles of flippases

Organism	P_4-ATPase	Subunit	Substrate	Localization	Cellular roles	Physiological roles	Key references
H. sapiens[a]	ATP8A1	CDC50A or CDC50B[b]	PtdSer, PtdEth	Golgi,RE, PM	Endosomal trafficking; cell migration	Hippocampus-dependent learning	Bryde et al., 2010; van der Velden et al., 2010; Levano et al., 2012; Kato et al., 2013; Lee et al., 2015
	ATP8A2	CDC50A	PtdSer, PtdEth	Golgi, Disk	Photoreceptor and spiral ganglion cell survival	Visual and auditory functions	Coleman et al., 2009; Coleman et al., 2009; Coleman et al., 2014; Vestergaard et al., 2014
	ATP8B1	CDC50A/B	PtdCho, PtdSer	PM	Apical membrane barrier function	Bile secretion; auditory and airway functions	Paulusma et al., 2006 and 2008; Stapelbroek et al., 2009; Ray et al., 2010; Takatsu et al., 2014
	ATP8B3	CDC50C?	PtdSer	Acrosome	Acrosome development?	Male fertility	Wang et al., 2004; Gong et al., 2009
	ATP9A	–	n.d.	TGN, RE	n.d.	n.d.	Takatsu et al., 2011
	ATP9B	–	n.d.	TGN	n.d.	n.d.	Takatsu et al., 2011
	ATP11A	CDC50A	n.d.	PM, RE	n.d.	n.d.	Takatsu et al., 2011
	ATP11B	CDC50A	n.d.	RE	Golgi-PM trafficking	n.d.	Takatsu et al., 2011; Moreno-Smith et al., 2013
	ATP11C	CDC50A	PtdSer	PM	PtdSer signaling in apoptosis	Red blood cell longevity; B-cell maturation	Yabas et al., 2011; Segawa et al., 2014; Yabas et al., 2014
C. elegans	TAT-1	CHAT-1	PtdSer	PM, LE	PtdSer signaling in apoptosis; lysosome biogenesis	Apoptotic cell clearance during development	Darland-Ransom et al., 2008; Ruaud et al., 2009; Chen et al., 2010; Li et al., 2013
	TAT-2	n.d.	n.d.	n.d.		Post-embryonic growth	Seamen et al., 2009
	TAT-5	n.d.	PtdEth	PM	Sterol metabolism; extracellular vesicle biogenesis	Embryo morphogenesis	Lyssenko et al., 2008; Wehman et al., 2011
D. melanogaster	dATP8B	n.d.	n.d.	PM	Odorant receptor function	n.d.	Ha et al., 2014; Liu et al., 2014
A. thaliana	CG33298	n.d.	n.d.	n.d.	Sterol homeostasis	Wing development	Ma et al., 2012
	ALA1	ALIS1/3/5	n.d.	PM	n.d.	Chilling tolerance	Gomès et al., 2000; López-Marqués et al., 2010
	ALA2	ALIS1/3/5	PtdSer	LE	n.d.	Chilling tolerance	López-Marqués et al., 2012
	ALA3	ALIS1/3/5	PtdSer, PtdEth, PtdCho	Golgi	SV biogenesis	Root tip and trichome development	Poulsen et al., 2008; Zhang and Oppenheimer, 2009
S. cerevisiae	Drs2p	Cdc50p	PtdSer, PtdEth	TGN	SV biogenesis; TGN-endosomal trafficking; sterol homeostasis	Chilling tolerance	Gall et al., 2002; Natarajan et al., 2004; Alder-Baerens et al., 2006; Zhou and Graham, 2009; Muthusamy et al., 2009
	Dnf1p	Lem3p	PtdCho, PtdEth	PM, EE	Endosomal trafficking	Chilling tolerance	Hua et al., 2002; Pomorski et al., 2003
	Dnf2p	Lem3p	PtdCho, PtdEth	PM, EE	Endosomal trafficking	Chilling tolerance	Pomorski et al., 2003; Riekhof and Voelker, 2006
	Dnf3p	Crf1p	PtdCho, PtdEth	TGN	n.d.	n.d.	Alder-Baerens et al., 2006
	Neo1p	–	n.d.	TGN, LE	Endosomal trafficking; growth	n.d.	Hua et al., 2002; Wicky et al., 2004

[a]Physiological roles of human P_4-ATPases as deduced from genetic studies in mice. [b]Human CDC50A, CDC50B and CDC50C are also referred to as TMEM30A, TMEM30B and TMEM30C, respectively. SV, post-Golgi secretory vesicle; PM, plasma membrane; Disk, photo-receptor disk membrane; EE, early endosome; RE, recycling endosome; LE, late endosomes; CL, cardiolipin; LPC, lyso-phosphatidylcholine; LPE, lyso-phosphatidylethanolamine; LPS, lyso-phosphatidylserine; n.d., not defined.

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asymmetry in various cell types and organisms (Table 1). Thus, removal of the P_4-ATPases Dnf1 and Dnf2 abolishes inwards translocation of fluorescent PtdEth and phosphatidylcholine (PtdCho) analogs across the plasma membrane in yeast (Pomorski et al., 2003). Chemical labeling of exoplasmic-leaflet phospholipids and staining with aminophospholipid-specific probes has shown that there is an accumulation of PtdSer and PtdEth on the surface of a *dnf1 dnf2* double deletion mutant, a phenotype that is exacerbated when Drs2 is also removed (Pomorski et al., 2003; Chen et al., 2006). Both Drs2 and Dnf3 are required to sustain aminophospholipid transport and PtdEth asymmetry in yeast post-Golgi secretory vesicles (Alder-Baerens et al., 2006). In purified TGN membranes carrying a temperature-sensitive form of Drs2, the PtdSer flippase activity is abolished at the restrictive temperature (Natarajan et al., 2004), indicating that Drs2 directly contributes to this activity. Indeed, reconstitution of a PtdSer translocase activity with purified Drs2 indicates that the enzyme functions as a PtdSer flippase (Zhou and Graham, 2009). The *Arabidopsis* P_4-ATPases ALA1, ALA2 and ALA3 support plasma membrane flippase activity when expressed in P_4-ATPase yeast mutants (Poulsen et al., 2008). Removal of the P_4-ATPase TAT-1 in *Caenorhabditis elegans* or of ATP11C in mammalian cells causes an increased cell surface exposure of PtdSer, resulting in an aberrant phagocytic clearance of living cells (Darland-Ransom et al., 2008; Segawa et al., 2014). ATP8A2, a P_4-ATPase present in the disc membranes of rod and cone photoreceptors, displays aminophospholipid transport activity when purified and reconstituted in proteoliposomes (Coleman et al., 2009).

Flipping phospholipids is an unexpected activity for a P-type ATPase as most P-type ATPases pump small cations or soft-transitional metal ions across membranes. Prominent examples are the Ca^{2+}-ATPase SERCA, which transports cytosolic Ca^{2+} into the lumen of the sarcoplasmic reticulum of skeletal muscle cells (Inesi et al., 2008), and the Na^+/K^+-ATPase, which generates the electrochemical gradients that are vital to animal cells (Kaplan, 2002). Despite their unusual substrate, P_4-ATPases share a common architecture and substantial sequence similarity with cation-pumping ATPases (Fig. 2A), indicating that they utilize a transport mechanism that rests on the same molecular principles (Kühlbrandt, 2004; Lenoir et al., 2007). How P_4-ATPases adapted this mechanism to flip phospholipids is not well understood. As discussed below, moving such bulky amphipathic substrate molecules across the membrane poses unique mechanistic problems. In addition, P_4-ATPases form heterodimeric complexes with members of the Cdc50 family of membrane proteins (Saito et al., 2004). Although Cdc50 subunits were originally identified in a genetic screen for loss of lipid asymmetry (Kato et al., 2002), their primary function remains to be established. As all flippase reconstitution experiments have been performed with heterodimeric P_4-ATPase complexes (Coleman et al., 2009; Zhou and Graham, 2009), it is unclear whether P_4-ATPases alone are sufficient to translocate phospholipids or whether they rely on a Cdc50 binding partner to accomplish this task. Nevertheless, reverse genetics and structural approaches have begun to reveal the first insights into how flippases operate.

Inner workings of flippases

Giant substrate problem

P-type ATPases are multi-domain membrane proteins that form a phosphorylated intermediate during the transport reaction cycle,

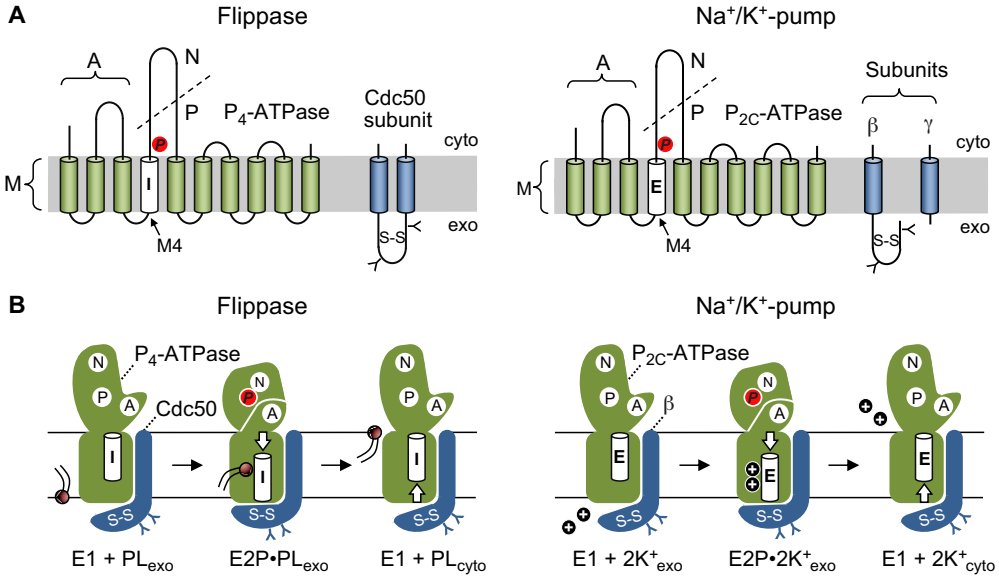

Fig. 2. Architecture and transport cycle of flippases and Na^+/K^+-pumps. (A) Flippases and Na^+/K^+-pumps share a common architecture. Flippases comprise a P_4-ATPase catalytic chain associated with a Cdc50 subunit. Na^+/K^+-pumps comprise a P_{2C}-ATPase catalytic chain associated with β- and γ-subunits. Both the Cdc50 and β-subunit have a bulky N-glycosylated ectodomain with conserved disulfide bridges (S-S). Na^+/K^+-pumps utilize an ion-binding pocket in the center of the helical bundle or M domain. Transmembrane segment M4 harbors a conserved glutamate (E) that binds Na^+ or K^+ ions. In flippases, the residue located at this position is an isoleucine (I). M, transmembrane region; A, actuator domain; N, nucleotide-binding domain; P, phosphorylation domain. The *P* with a red background shows phosphorylation. (B) Cartoons of a flippase and Na^+/K^+-pump illustrating domain reorientation and subunit rearrangements during the transport reaction cycle (see also Box 2). In Na^+/K^+-pumps, the transition from E_1P to E_2P involves a vertical movement of M4, allowing delivery of Na^+ ions bound at the M4 glutamate (E) to the exoplasm and loading of the enzyme with exoplasmic K^+ ions. During transition from E_2 to E_1, M4 moves in the opposite direction to release K^+ ions into the cytosol. In flippases, the isoleucine (I), which is present in the place of the M4 glutamate, is crucial for translocating phospholipid to the cytoplasmic leaflet, presumably by functioning as a hydrophobic gate for the polar headgroup (brown circle). Transition from E_1P to E_2P is accompanied by tighter binding of the ATPase to its subunit, involving a high-affinity interaction with the ectodomain of the subunit. In this way, the subunit might stabilize E_2P to help load the ATPase with luminal substrate (K^+ ions or phospholipid) or serve as a 'lid' to close access to the substrate-binding site from the exoplasm.

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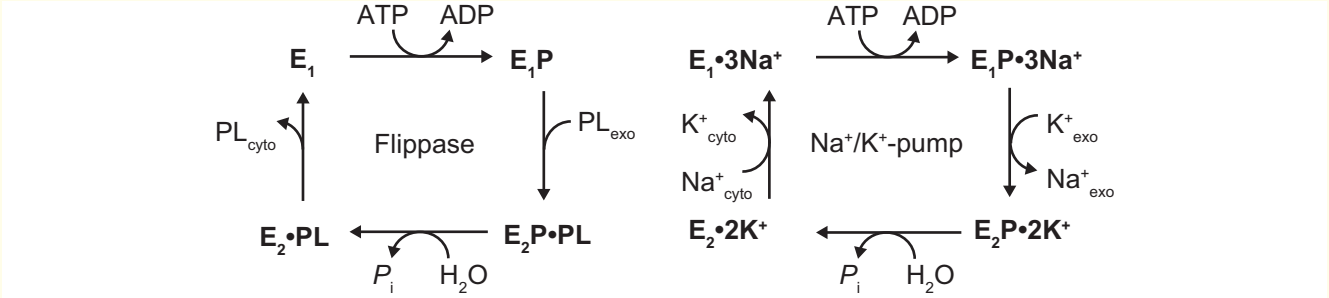
Box 2. P-type ATPase transport reaction cycle

The transport reaction cycle of P-type ATPases comprises four main enzyme conformations, i.e. E_1, E_1P, E_2P and E_2, with P referring to the aspartyl-phosphorylated intermediate of the enzyme (see box figure). The substrate-binding sites are buried inside the M domain, the region of the enzyme that spans the membrane. In E_1, these sites are accessible for substrates from the cytosol – Na^+ ions in the case of the Na^+/K^+-pump and unknown in the case of P_4-ATPase flippases (Lenoir et al., 2007). Substrate binding promotes phosphorylation of the enzyme at a conserved Asp residue in the phosphorylation (P) domain. The phosphate is donated by an ATP molecule, which binds to the nucleotide-binding (N) domain. The side product, ADP, remains briefly associated with the pump. Formation of the E_1P intermediate results in occlusion of the substrates, that is, they become inaccessible from either side of the membrane. The enzyme then releases ADP and relaxes to a lower energy E_2P conformation, whereupon a pathway opens to discharge the substrates to the exoplasmic side. The substrate-binding site now has high affinity for the counter-transported substrates – K^+ ions in the case of the Na^+/K^+-pump and phospholipid (PL) in the case of flippases, which bind from the exoplasmic side (shown on the right). Hydrolysis of the phosphorylated Asp residue, catalyzed by the actuator (A) domain, results in another state with occluded substrates, E_2. Mg^{2+} and inorganic phosphate (P_i) dissociate, and the enzyme reverts to the E_1 state, in which the counter-transported substrates are released into the cytosol. Thus, contrary to the principle by which ions travel through an ion channel, P-type ATPases create and destroy substrate-binding sites at different points in the cycle that are accessible to opposite sides of the membrane. This mechanism allows P-type ATPases to transport a substrate against a gradient, while avoiding leakage of substrate in the opposite direction.



hence the designation 'P-type'. Four main conformations exist, E_1, E_1P, E_2P and E_2, with conformational changes being accompanied by translocation of a substrate across the membrane. Phospholipid transport catalyzed by P_4-ATPases would correspond to the transport of K^+ ions by the Na^+/K^+-pump, as the direction of flipping is from the exoplasmic to the cytoplasmic side. This predicts that the phospholipid substrate in P_4-ATPases binds to the phosphoenzyme intermediate E_2P (Box 2). Consistent with this model, dephosphorylation of the P_4-ATPase ATP8A2 is stimulated by the transported substrates PtdSer and PtdEth (Vestergaard et al., 2014), similar to when K^+ ions activate dephosphorylation of the Na^+/K^+-pump, opposing the action of Na^+ ions, which stimulate phosphorylation. Although these data argue for a high degree of mechanistic similarity between cation pumps and flippases, it is an open question as to how P_4-ATPases translocate a bulky phospholipid that is about 40-times more voluminous than the ions transported by Na^+/K^+- and Ca^{2+}-pumps. This enigma has been referred to as the 'giant substrate problem' (Puts and Holthuis, 2009; Stone and Williamson, 2012). In addition, substrate recognition by P_4-ATPases is complex. Transport is headgroup-dependent, and in some cases is specific for PtdSer and in others is mainly restricted to PtdCho. Transport is also backbone-dependent, directed at glycerophospholipids and excluding sphingolipids (Pomorski et al., 2003; Baldridge and Graham, 2012). Phospholipid transport by P_4-ATPases also imposes another unique requirement. Whereas ion substrates do not have to move in the binding pocket as cation pumps change conformation, phospholipid translocation demands that the substrate physically reorients during the transport process.

Phospholipid translocation pathway models

How do P_4-ATPases meet the structural requirements imposed by phospholipid translocation? Where is the phospholipid-binding site in P_4-ATPases? The conserved anionic and polar residues in the central helices M4, M5 and M6 that make up the substrate binding sites in cation pumps are largely replaced by non-polar residues in P_4-ATPases (Tang et al., 1996). A screen for residues that define phospholipid headgroup specificity in the yeast P_4-ATPases Drs2 and Dnf1 has revealed a series of side-chains that are crucial for substrate recognition. These residues form two clusters outside of the canonical substrate-binding site, one on the exoplasmic membrane face, where substrate is initially selected, and the second near the cytosolic membrane face, where substrate is released (Baldridge and Graham, 2012; Baldridge and Graham, 2013). This suggested that P_4-ATPases use a two-gate mechanism for phospholipid selection and that the phospholipid translocation pathway is unique compared with the canonical pathway used by cation pumps. However, a recent study on the functional consequences of mutating an isoleucine in M4 of the P_4-ATPase ATP8A2 at a position equivalent to the cation-binding glutamate in Ca^{2+}- and Na^+/K^+-pumps uncovered a striking analogy between the roles of these residues in the translocation of substrate (Vestergaard et al., 2014).

M4 serves a central role in the transport mechanism of cation pumps. Crystal structures of the Ca^{2+}-pump indicate that the E_1P to E_2P transition involves a vertical movement of M4, like a pump rod, allowing delivery of Ca^{2+} bound at the M4 glutamate residue to the lumen (Toyoshima, 2009). During the E_2 to E_1 transition of the dephosphoenzyme, M4 moves in the opposite direction to the cytosol. In ATP8A2, a missense mutation of the isoleucine corresponding to the M4 glutamate residue of cation pumps was recently identified as the cause of cerebellar ataxia, mental retardation and disequilibrium (CAMRQ) syndrome (Onat et al., 2013). This M4 isoleucine residue is highly conserved among P_4-ATPases and plays a crucial role in phospholipid translocation. Structural homology modeling and molecular dynamic simulations suggest that the M4 isoleucine and adjacent hydrophobic residues in P_4-ATPases function as a hydrophobic gate that separates the entry and exit sites of the phospholipid. This hydrophobic gate controls the sequential formation and abolishment of water-filled cavities in the central core of the protein, enabling translocation of the phospholipid headgroup, with the acyl chains following passively,

still in the membrane lipid phase (Vestergaard et al., 2014). This model suggests that the pump rod function of M4 is a general feature of P-type ATPases and that movement of the M4 isoleucine in P_4-ATPases is crucial for releasing the phospholipid into the cytoplasmic leaflet during the transformation from E_2 to E_1, likely involving a non-favorable interaction between the hydrophobic side-chain with the polar phospholipid headgroup. Hence, the overall function of M4 in flippases is reminiscent of the role of M4 in cation pumps, with the pump rod moving up and down to bring about translocation of the substrate (Fig. 2B).

Accessory subunits

Besides their unusual substrate, another feature that sets P_4-ATPases apart from most other P-type pumps is their association with an obligatory Cdc50 subunit (Saito et al., 2004). This property is shared by only one other P-type ATPase subfamily, namely the Na^+/K^+- and H^+/K^+-ATPases, which associate with a β- and γ-subunit. Although the γ-subunit is dispensable for function, association with the β-subunit is required for membrane insertion and catalytic activity of X^+/K^+-ATPases (Geering, 2008). With the notable exception of Neo1 and its homologs (see below), association with a Cdc50 subunit is crucial for the stability, ER export and catalytic activity of P_4-ATPases (Saito et al., 2004; Lenoir et al., 2009; Bryde et al., 2010; van der Velden et al., 2010; Coleman et al., 2012). Cdc50 and β-subunits are small in comparison to their P-type ATPase partners and show little if any sequence similarity (Fig. 2A). However, both have extended *N*-glycosylated ectodomains that are stabilized by conserved disulfide bridges (Kato et al., 2002; Geering, 2008; Coleman and Molday, 2011; Puts et al., 2012). Conceivably, Cdc50 proteins and β-subunits adopted similar structures to accomplish analogous tasks.

An early idea was that the β-subunit might be necessary because X^+/K^+-ATPases are unique among cation pumps in catalyzing counter-transport of K^+ ions (Geering, 2008), analogous to the counter-transport of phospholipids catalyzed by P_4-ATPases. Reduction of the disulfide bridges in the ectodomain of the β-subunit by treatment with dithiothreitol (DTT) causes a drop in the K^+ affinity of the pump and impairs its enzymatic activity (Kawamura et al., 1985; Lutsenko and Kaplan, 1993). This phenomenon can be prevented in the presence of K^+ ions, suggesting that the β-subunit helps stabilize the K^+-occluded state of the pump. Consistent with this idea, disruption of interactions between specific residues within the membrane spans of the Na^+/K^+-ATPase results in a shift towards an E1 conformation (Dürr et al., 2009). A crystal structure of the heterodimeric Na^+/K^+-ATPase complex in the K^+-occluded *E*2P state has revealed that the β-subunit is not directly involved in binding or occlusion of extracellular K^+ ions (Shinoda et al., 2009). Instead, it appears that the β-subunit helps promote formation of a K^+-binding cavity. Such a cavity is absent in the corresponding crystal structure of the Ca^{2+}-ATPase, which translocates protons as exoplasmic substrates (Toyoshima and Mizutani, 2004).

Although the Cdc50 subunit is not a crucial determinant of the substrate specificity of P_4-ATPases (López-Marqués et al., 2010; Baldridge and Graham, 2012), it is feasible that the subunit promotes formation of a sizeable phospholipid-binding site, analogous to the role of the β-subunit in X^+/K^+-ATPases. This idea is supported by the observation that the affinity of the Cdc50 subunit for the P_4-ATPase fluctuates during the transport cycle, with the strongest binding occurring at E_2P, the point where the flippase is loaded with phospholipid substrate (Lenoir et al., 2009). Functional analysis of cysteine mutants that disrupt the conserved disulfide bridges in the Cdc50 ectodomain revealed that there is an inverse relationship between subunit binding and flippase activity, suggesting that a dynamic association between subunit and transporter is crucial for the transport reaction cycle of the heterodimer (Puts et al., 2012). An intimate role of Cdc50 subunits in P_4-ATPase-catalyzed phospholipid transport can also be inferred from the isolation of conditional Cdc50 mutants that retain the ability to associate with their P_4-ATPase binding partner, but show loss of function *in vivo* at the non-permissive temperature (Takahashi et al., 2011). Thus, acquisition of Cdc50 subunits might have been a crucial step in the evolution of flippases from a family of cation pumps.

The idea that Cdc50 subunits are an integral part of the P_4-ATPase flippase machinery is challenged by the fact that the yeast P_4-ATPase Neo1p and its mammalian homologs ATP9A and ATP9B lack a Cdc50 partner (Saito et al., 2004; Takatsu et al., 2011). Among the five P_4-ATPases in yeast, Neo1 is unique in that deletion of its gene is lethal (Hua and Graham, 2003). This raises the possibility that Neo1 and its homologs possess an enzymatic activity different from that of other P_4-ATPases and for which it does not require a Cdc50 partner. Direct evidence that Neo1 catalyzes phospholipid transport is lacking. Consequently, how Neo1 executes its essential function remains to be established.

Flippases participate in key biological processes

Vesicular trafficking

An intriguing aspect of P_4-ATPases is that their activity is tightly linked to vesicular trafficking to and from the plasma membrane. In yeast, the plasma-membrane-resident flippases Dnf1 and Dnf2 are required for endocytosis at low temperature (Pomorski et al., 2003), whereas loss of Drs2 flippase activity at the TGN blocks formation of a clathrin-dependent class of post-Golgi secretory vesicles (Chen et al., 1999; Gall et al., 2002; Natarajan et al., 2004). Drs2 is also required for bidirectional vesicular transport between the TGN and early endosomes (Hua et al., 2002; Liu et al., 2008). Furthermore, human ATP8B1 mediates apical protein localization (Verhulst et al., 2010), and *C. elegans* TAT-1 is required for endocytosis and lysosome biogenesis (Ruaud et al., 2009). In *A. thaliana*, ALA3 is essential for the formation of post-Golgi vesicles in actively secreting cells at the plant root tip (Poulsen et al., 2008). Taken together with the trafficking defects found in P_4-ATPase mutant strains of fungal and plant pathogens (Gilbert et al., 2006; Hu and Kronstad, 2010), these data support a fundamental role of P_4-ATPases in vesicle formation at late secretory and endosomal organelles.

In support of a direct role in vesicle biogenesis, P_4-ATPases genetically and physically interact with components of the vesicle coat machinery. In yeast, genetic interactions between Drs2, clathrin heavy chain (Chc1) and Arf1, a GTPase controlling clathrin coat assembly, were detected (Chen et al., 1999). Furthermore, Drs2 physically interacts with both Gea2, an Arf guanine-nucleotide-exchange factor (GEF), and AP-1, a clathrin adaptor protein (Chantalat et al., 2004). Taken together with the observation that inactivation of temperature-sensitive Drs2 rapidly blocks formation of clathrin-coated vesicles *in vivo* (Gall et al., 2002), these findings initially suggested that P_4-ATPases support vesicle formation by facilitating recruitment of the vesicle coat machinery. However, this model appears to be incorrect as AP-1, Gea2p and clathrin efficiently assemble on TGN membranes of a *drs2* deletion strain (Liu et al., 2008). An alternative model postulates that P_4-ATPase-catalyzed phospholipid transport creates a mass imbalance between both membrane leaflets (Fig. 3). The resulting lipid-packaging stress in the cytoplasmic leaflet would

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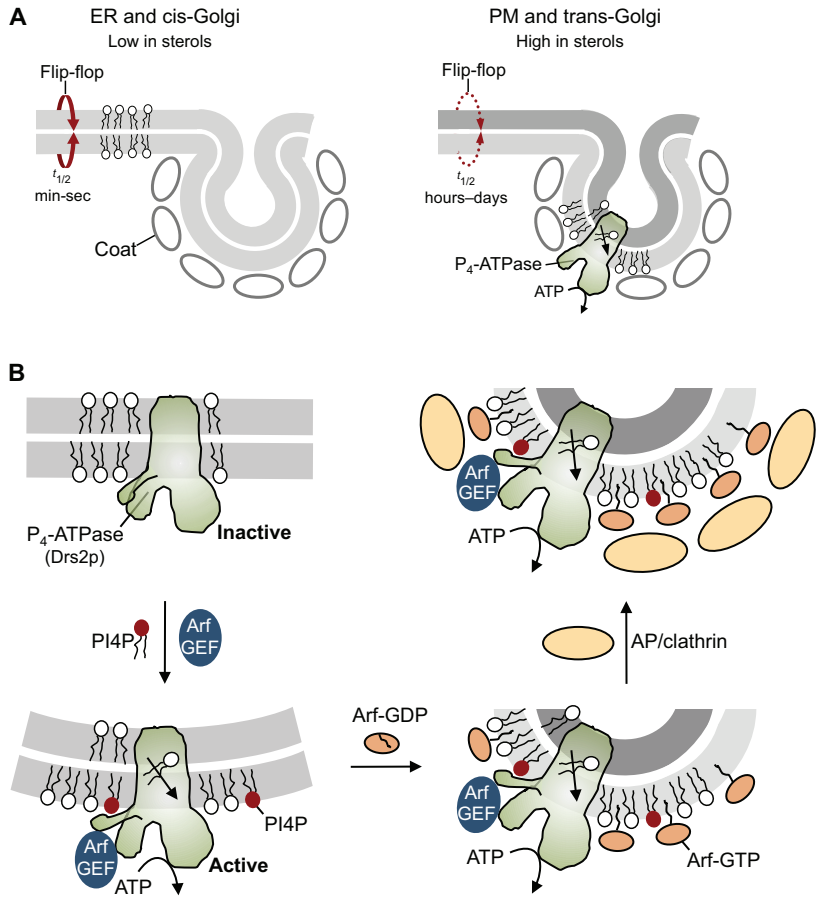

Fig. 3. Role of flippases in vesicle biogenesis. (A) Membrane curvature during vesicle budding requires a selective increase in surface area of the cytoplasmic leaflet. In ER and cis-Golgi membranes, phospholipids can readily cross the bilayer in both directions owing to loose lipid packing (low sterol content). In these flexible membranes, assembly of a protein coat would be sufficient to deform the bilayer into a bud. However, in the TGN and plasma membrane, free flip-flop of phospholipids is constrained owing to high sterol levels. Here, coat assembly might no longer be sufficient to drive vesicle budding, and this process would require assistance of a phospholipid pump whose activity helps to expand the cytoplasmic leaflet at the expense of the luminal one. Selectivity of this unidirectional flippase would prevent destabilization of the bilayer, generate transbilayer phospholipid asymmetry (marked by the light and dark gray membrane leaflets), and help establish a phospholipid environment favourable for coat recruitment. (B) The TGN-resident yeast P_4-ATPase Drs2p is activated by phosphatidylinositol-4-phosphate (PI4P) and the guanine nucleotide exchange factor Arf-GEF. Both activators bind to an auto-inhibitory domain in the C-terminal tail of Drs2p, suggesting a coincidence detection system to control flippase activity in coordination with cargo loading and vesicle biogenesis. Note that Arf-GEF triggers membrane association of the ADP ribosylation factor Arf, a key regulator of clathrin coat assembly. AP, adaptor protein.

then drive inwards-directed membrane bending, thus contributing to the membrane curvature needed to bud small-diameter vesicles (Graham, 2004; Takeda et al., 2014). Coat assembly would then serve to localize the site of vesicle budding rather than to provide the principal driving force. Consistent with this hypothesis, AP-1 and clathrin do not efficiently induce TGN membrane bending in the absence of Drs2 (Chen et al., 1999; Liu et al., 2008). Moreover, the stimulation of plasma-membrane-resident flippase activity results in the formation of endocytic-like vesicles in erythrocytes (Birchmeier et al., 1979; Müller et al., 1994) and accelerates endocytosis in erythroleukemia K562 cells (Farge et al., 1999). As P_4-ATPases exclusively populate membranes of late secretory and endocytic organelles, it is tempting to speculate that they primarily evolved to facilitate vesicle budding from the relatively stiff, sterol-rich bilayers.

There is also evidence that flippases contribute to vesicle biogenesis by enriching specific phospholipids in the cytoplasmic leaflet that help control recruitment of vesicle budding and fission machinery. A recent study has identified the Arf GTPase-activating protein (GAP) Gcs1 as a downstream effector of Drs2 flippase activity in yeast (Xu et al., 2013). Gcs1 contains a variant of the Arf-GAP lipid packing sensor (+ALPS) motif, which harbors a basic amino acid upstream of ALPS that is crucial for membrane association. Site-directed mutagenesis has revealed that the +ALPS variant in Gcs1 senses both curvature and negative charge imparted to the TGN by Drs2-catalyzed PtdSer flippase activity (Xu et al., 2013). A point mutation in Dnf1 that allows it to recognize and flip PtdSer was sufficient to rescue membrane recruitment of Gcs1 and restore vesicular trafficking between the TGN and early endosomes in a *drs2* mutant, demonstrating a crucial role of PtdSer translocation in these pathways (Xu et al., 2013). Analogous to these findings, PtdSer flipped to the cytoplasmic leaflet of recycling endosomes in mammalian cells by ATP8A1 is essential for the recruitment of the membrane fission protein EDH1 (Lee et al., 2015). ATP8A1 depletion impaired PtdSer asymmetry, dissociated EDH1 from recycling endosomes, and generated aberrant endosomal tubules that appeared to be resistant to fission and that were defective in transferrin receptor recycling. ATP8A2, a tissue-specific ATP8A1 paralog, is associated with the neurological disorder CAMRQ. ATP8A2, but not the disease-causative ATP8A2 mutant, rescued the endosomal defects in ATP8A1-depleted cells. This indicates that ATP8A2 is essential for the formation of transport vesicles from recycling endosomes and that defects in this pathway contribute to CAMRQ (Lee et al., 2015). By influencing membrane curvature and lipid composition simultaneously, it appears likely that flippases exert synergistic effects on vesicle biogenesis.

Cell signaling

The ability of P_4-ATPases to transport phospholipids such as PtdSer to the cytoplasmic leaflet generates concentration gradients across the bilayer that can be exploited for signal transduction. Regulated disruption of phospholipid asymmetry at the plasma membrane and exposure of PtdSer play an important signaling role in apoptosis and blood clotting (Zwaal and Schroit, 1997). An early event in apoptosis is the Ca^{2+}-dependent exposure of PtdSer on the outer surface of dying cells, which serves as an 'eat me' signal for macrophages that engulf the cell corpse (Fadok et al., 1992). A direct role of P_4-ATPases in stimulating PtdSer-induced phagocytosis has

been demonstrated for the Drs2 ortholog, TAT-1 in *C. elegans* (Darland-Ransom et al., 2008). Loss of TAT-1 results in increased surface exposure of PtdSer accompanied by random cell loss. This cell clearance occurs through a phagocytic mechanism that is dependent on PSR-1, a PtdSer-binding phagocyte receptor, and CED-1, which is involved in recognition and engulfment of apoptotic cells. Moreover, a recent haploid genetic screen identified the P_4-ATPase ATP11C and its Cdc50 subunit CDC50A as key regulators of PtdSer asymmetry at the plasma membrane of lymphoid cells (Segawa et al., 2014). Intriguingly, ATP11C contains caspase-cleavage sites, and expression of a caspase-resistant form of ATP11C prevented both apoptotic PtdSer exposure and engulfment by macrophages. Conversely, deletion of CDC50A in a T-cell line expressing a permanently active scramblase resulted in constitutive PtdSer exposure and efficient macrophage engulfment *in vivo*, indicating that PtdSer is sufficient as an 'eat me' signal even in living cells (Segawa et al., 2014). Indeed, ATP11C-deficient mice lose a large number of B-cells during differentiation from progenitor B-cells to precursor B-cells in bone marrow, indicating a crucial role for ATP11C in murine B-cell development (Siggs et al., 2011; Yabas et al., 2011).

Cell polarity and migration

P_4-ATPases have also been shown to control polarized growth during cell division of budding yeast (Saito et al., 2007). PtdEth is specifically exposed on the exoplasmic leaflet at polarized sites during the early stage of budding, and disappears in G2 as the apical bud growth switches to isotopic growth. This PtdEth exposure is enhanced by deletion of the plasma-membrane-associated P_4-ATPases Dnf1 and Dnf2, or their Cdc50 partner Lem3, all three of which are needed to translocate PtdEth (Table 1). How PtdEth becomes exposed on the exoplasmic leaflet remains to be established, but sustained PtdEth exposure in a *dfn1 dnf2* or *lem3* mutants causes prolonged apical growth due to a defect in the switch to isotropic bud growth. In the mutant cells, the GTP-bound form of the small GTPase Cdc42, a key-signaling molecule in cell polarity that normally localizes only transiently to the bud tip to mobilize the actin cytoskeleton, remains polarized at the site of PtdEth exposure. These phenotypes can also be mimicked by cell surface immobilization of PtdEth using a PtdEth-binding peptide (Das et al., 2012). Interestingly, the Cdc42 GAPs Rga1 and Rga2 are stimulated by PtdEth. Collectively, these data support a model in which the apical-isotropic switch is triggered by a P_4-ATPase-catalyzed redistribution of PtdEth, which downregulates Cdc42 signaling.

Recent work in mammalian cells has revealed a crucial role for the P_4-ATPase ATP8A1 and its subunit CDC50A in cell migration (Kato et al., 2013). Whereas overexpression of CDC50A induced extensive cell spreading and greatly enhanced cell migration, depletion of either CDC50A or ATP8A1 caused a severe defect in the formation of membrane ruffles, thereby inhibiting cell migration. Depletion of CDC50A affected inwards translocation of both PtdEth and PtdSer, whereas depletion of ATP8A1 only disrupted PtdEth transport, suggesting that cell migration critically relies on the inwards translocation of cell surface PtdEth. Consistent with this idea, cell surface immobilization of PtdEth with a PtdEth-binding peptide or genetic disruption of PtdEth biosynthesis in each case affected remodeling of cortical actin filaments and the formation of membrane ruffles, disrupting cell migration (Kato et al., 2013). How the effector molecules in the migratory machinery are controlled by flippase-mediated changes in the phospholipid distribution across the plasma membrane remains to be established.

Apical barrier function

Progressive familial intrahepatic cholestasis type-1 (PFIC1) is a potentially lethal liver disease caused by mutations in the P_4-ATPase ATP8B1 and characterized by a bile salt secretion defect. ATP8B1 exhibits PtdCho flippase activity (Takatsu et al., 2014) and localizes to the canalicular membrane in liver cells (Paulusma et al., 2006). This membrane also harbors the ABC transporter ABCB4, which is responsible for excreting PtdCho into the bile (Smit et al., 1993), and the bile-salt-export pump ABCB11. Interruption of the enterohepatic circulation of bile salts in PFIC1 patients results in normalization of their hepatobiliary output, indicating that the bile salt transport defect in PFIC1 is not a direct consequence of ATP8B1 dysfunction (Kurbegov et al., 2003). ATP8B1 mutant mice display a dramatic increase in biliary output of canalicular cholesterol. Subsequent studies have shown that the activity of the bile salt pump is crucially dependent on the cholesterol content of the canalicular membrane (Paulusma et al., 2009). The exoplasmic leaflet of this membrane is rich in sphingolipids that are tightly packed with cholesterol to provide maximum resistance against the detergent action of hydrophobic bile salts. By flipping excess PtdCho from the exoplasmic surface towards the cytoplasmic leaflet, ATP8B1 might help preserve this barrier function. Loss of ATP8B1 function would result in lipid scrambling, thereby reducing the lipid ordering in the exoplasmic leaflet and increasing its sensitivity towards hydrophobic bile salts. Increased cholesterol extraction by bile salts reduces the cholesterol content of the bilayer, which in turn impairs the activity of the bile salt pump, causing cholestasis (Paulusma et al., 2006; Paulusma et al., 2009). Moreover, as ABCB4 antagonizes the PtdCho flippase activity of ATP8B1 (Takatsu et al., 2014), an imbalance in PtdCho flip-flop across the canalicular membrane might explain the shedding of membrane protrusions and release of vesicular structures in the canalicular lumen of ATP8B1 mutant mice (Paulusma et al., 2006), a process that might further undermine the bile salt secretion capacity of the liver.

Flippase regulation

Regulation in relation to vesicle biogenesis

As expected from their active participation in a multitude of biological processes, flippases are subject to tight regulation. For instance, the trans-Golgi P_4-ATPase Drs2 in yeast is activated by phosphoinositide-4-phosphate (PI4P) produced by the phosphatidylinositol 4-kinase Pik1 (Natarajan et al., 2009). A phosphoinositide-binding site with preference for PI4P maps to a basic patch of residues within the C-terminal cytosolic tail of the enzyme, and mutation of these residues abrogates Drs2 activity. Moreover, the basic patch overlaps with a binding site for the Arf GEF Gea2, and this interaction also stimulates Drs2 flippase activity. Analogous to regulation of the Ca^{2+} P-type ATPase PMCA1 by calmodulin (Di Leva et al., 2008), the C-terminal tail of Drs2 is an auto-inhibitory regulatory domain, and binding to PI4P relieves the auto-inhibition to stimulate activity (Jacquot et al., 2012; Zhou et al., 2013). Whereas PI4P recruits AP-1, GGA and epsinR adaptors to facilitate assembly of budding clathrin-coated vesicles, Gea2p initiates vesicle biogenesis by promoting membrane recruitment of Arf (Behnia and Munro, 2005; Wang et al., 2007; Wang et al., 2003). Hence, the synergistic activation of Drs2 by PI4P and Gea2 suggests that a coincidence detection system is used to activate phospholipid translocation at sites of vesicle formation at the trans-Golgi (Fig. 3B).

In *C. elegans*, the P_4-ATPase TAT-1 that is involved in budding and tubulation of endosomal and lysosomal organelles is controlled by NUM-1A, a member of the Numb protein family regulating

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endocytosis (Nilsson et al., 2011). In a tissue-specific manner, NUM-1A blocks endocytic recycling by inhibiting TAT-1 translocase activity. Numb proteins bind to the clathrin adaptor α-adaptin and numerous proteins of the epsin15 homology domain family that are involved in both clathrin-dependent and -independent endocytosis (Gulino et al., 2010). Thus, mechanisms to control flippase activity in coordination with vesicle biogenesis appear to be a universal feature among eukaryotes.

Regulation in relation to sphingolipid and sterol homeostasis

A remarkable homeostatic circuit appears to control plasma membrane lipid organization by linking the transbilayer phospholipid asymmetry of the plasma membrane to its sphingolipid content. This circuit relies on a number of protein kinases whose action in this context has been best studied in yeast. First insights into this regulatory network came from a genetic screen to identify positive regulators of the plasma membrane P_4-ATPases Dnf1 and Dnf2, which yielded a closely related pair of flippase kinases termed Fpk1 and Fpk2 (Kato et al., 2002; Nakano et al., 2008). Deletion of Fpk1 and Fpk2 phenocopies mutations in Dnf1 and Dnf2, and *fpk1 fpk2* double mutants are deficient in plasma-membrane-associated flippase activity. Fpk1 itself is under direct control of another kinase, Ypk1, which phosphorylates Fpk1 and inhibits Fpk1 activity (Roelants et al., 2011). Fpk1, in turn, phosphorylates Ypk1 and inhibits its kinase activity. Several kinds of input into this 'tug of war' between Fpk1 and Ypk1 can tip the balance in favor of one of the two kinases. Notably, sphingolipid long chain bases stimulate the kinase Pkh1 (Liu et al., 2005), which phosphorylates and activates Ypk1. Thus, conditions that increase the levels of sphingolipid precursors are expected to tip the balance in favor of Ypk1 and reduce flippase activity. In contrast, complex sphingolipids have been found to stimulate Fpk1 activity (Roelants et al., 2011). Collectively, these and other observations point at a homeostatic mechanism ensuring that sphingolipid levels in the exoplasmic leaflet of the plasma membrane are sensed and corrected by fresh synthesis of precursors in coordination with a P_4-ATPase-catalyzed redistribution of aminophospholipids to the opposite leaflet (Berchtold et al., 2012; Roelants et al., 2011), presumably to maximize impermeability of the plasma membrane.

Outlook

Both genetic and biochemical evidence now firmly establishes that P_4-ATPases, in association with their Cdc50 subunits, are necessary and sufficient for flipping specific phospholipids to help create phospholipid asymmetry in late secretory and endocytic organelles. P_4-ATPase–Cdc50 complexes are strikingly reminiscent of Na^+/K^+-pumps. Mutagenesis and functional assays, in combination with computational studies, have revealed the first insights into how flippases actually operate. However, the role of the Cdc50 subunit in the flippase complex remains ill defined. Do Cdc50 subunits stabilize or form part of the phospholipid translocation channel, or do they primarily control P_4-ATPase-catalyzed flippase activity? And what is the primary activity of P_4-ATPases that lack a Cdc50 partner?

Unexpectedly, a recent study indicates that PtdSer asymmetry in yeast post-Golgi secretory vesicles can be generated in the absence of P_4-ATPase flippases (Mioka et al., 2014). One possibility is that a flippase-like protein of unknown identity acts in conjunction with P_4-ATPases to control PtdSer translocation at the TGN. However, the identification of Osh6 and Osh7 as cytosolic PtdSer transfer proteins that catalyze non-vesicular transport of PtdSer from the ER to the plasma membrane (Maeda et al., 2013) raises an alternative possibility, namely that PtdSer asymmetry at the TGN relies on a combined action of PtdSer flippases and transfer proteins. As lipid traffic in cells is largely independent of vesicular traffic (Holthuis and Menon, 2014), it will be of interest to investigate how the activities of lipid transfer proteins and flippases are coordinated to ensure that each organelle along the secretory pathway acquires an appropriate phospholipid distribution across its bilayer.

Studies in yeast, plants and animals have uncovered a fundamental requirement of flippases in vesicle biogenesis from the TGN, endosomes and plasma membrane. An attractive model is that flippases evolved to facilitate vesicle biogenesis from these organelles by assisting the coat machinery in bending their relatively rigid, sterol-rich bilayers. As a plethora of other membrane modeling proteins has been implicated in vesicle biogenesis (e.g. coat proteins, GTPases, ENTH domain proteins and BAR domain proteins), a key challenge will be to define the precise contribution of flippases to this process. It appears likely that cells exploit the unique ability of flippases to influence membrane curvature and lipid composition simultaneously to accomplish membrane vesiculation. Functional reconstitution of flippases into giant proteoliposomes would offer attractive opportunities to further unravel the underlying molecular principles.

Flippases are subject to tight regulation, involving phosphorylation, protein-binding partners and specific protein–lipid interactions. However, we still do not have a clear picture of how these regulatory mechanisms help coordinate phospholipid translocation to the cytoplasmic leaflet and sphingolipid assembly in the luminal leaflet with sterol-loading at the TGN to ensure the fundamental transition of a bilayer adapted for biogenic activities (the ER) to one specialized in acting as a barrier (the plasma membrane). Future studies will certainly reveal more fascinating insights into the mechanistic properties and biological roles of flippases.

Competing interests

The authors declare no competing or financial interests.

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