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Functions of phospholipid flippases

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Asymmetrical distribution of phospholipids is generally observed in the eukaryotic plasma membrane. Maintenance and changes of this phospholipid asymmetry are regulated by ATP-driven phospholipid translocases. Accumulating evidence indicates that type 4 P-type ATPases (P4-ATPases, also called flippases) translocate phospholipids from the exoplasmic leaflet to the cytoplasmic leaflet of the plasma membrane and internal membranes. Among P-type ATPases, P4-ATPases are unique in that they are associated with a conserved membrane protein of the Cdc50 family as a non-catalytic subunit. Recent studies indicate that flippases are involved in various cellular functions, including transport vesicle formation and cell polarity. In this review, we will focus on the functional aspect of phospholipid flippases.

Keywords: Cdc50/flippase/phospholipid asymmetry/P-type ATPase/vesicle transport.

Abbreviations: AP-1, adaptor protein 1; GAP, GTPase-activating protein; NBD, 7-nitrobenz-2-oxa-1,3-diazol-4-yl; P4-ATPase, type 4 P-type ATPase; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; SV, secretory vesicle; TGN, *trans*-Golgi network.

Eukaryotic plasma membranes have asymmetrical distributions of phospholipids across the bilayer. In this phospholipid asymmetry, sphingolipids and phosphatidylcholine (PC) are distributed in the exoplasmic leaflet, whereas aminophospholipids phosphatidylserine (PS) and phosphatidylethanolamine (PE) are distributed in the cytoplasmic leaflet (*1–3*). It is well known that exposure of PS to the exoplasmic leaflet triggers phagocytosis by macrophages, but it is believed that much remains to be learned about physiological significance of phospholipid asymmetry: what are benefits of maintaining phospholipid asymmetry and what are cellular functions that are regulated by changes in phospholipid asymmetry? Phospholipid asymmetry is dynamically maintained in equilibrium by the transbilayer movement from the exoplasmic to the cytoplasmic leaflet (flip) and that in the opposite direction (flop). 'Flippase' is a collective name for proteins that facilitate phospholipid flipping, and their identification seems to be still on their way. Accumulating evidence indicates that the type 4 P-type ATPase (P4-ATPase) is a flippase. In this article, we call P4-ATPases flippases, and focus on their functional aspect.

Structure and activity of flippases

Identification of flippases

The first evidence for a phospholipid flippase activity was presented in human erythrocyte plasma membrane by Seigneuret and Devaux (*4*). They showed that aminophospholipids, PS and PE, are selectively transported from the outer to the inner leaflet in an ATP-dependent manner. The flippase activity was detected in the plasma membrane of a variety of cells using reporter (*e.g.* spin- and fluorescent-) labeled phospholipids in subsequent studies by several laboratories. They have defined the biochemical properties of flippase: flippase activity requires ATP, is sensitive to N-ethylmaleimide, vanadate and Ca^{2+}, and exhibits a high selectivity for PS (*5*). An ATP-dependent flippase activity was found in chromaffin granules from bovine adrenal glands (*6*), suggesting that an ATP-dependent phospholipid transport also occurs in intracellular organelles. On the other hand, a vanadate- and N-ethylmaleimide-sensitive 115-kDa ATPase was purified from chromaffin granule membranes (*7*) and this 115-kDa ATPase was subsequently shown to be activated by PS (*8*). Similarity of biochemical properties between the identified flippase activity and the purified 115-kDa ATPase led to cloning of the gene that encodes the 115-kDa ATPase (ATPase II, now called ATP8A1) (*9*). The sequence of the gene revealed that the ATPase II is an 1148-amino acid polypeptide homologous to proteins in the P-type ATPase superfamily (*9*). The P-type ATPases are a large group of evolutionarily related pumps that contain a diagnostic phosphorylation motif (DKTGTLT), located in the hydrophilic center of the polypeptide, within which an aspartyl-phosphate intermediate is formed during ATP hydrolysis (*10*). A search of the non-redundant group of database at the National Library of Medicine revealed that one of the most similar proteins to ATPase II was Drs2p of the yeast *Saccharomyces cerevisiae*. Tang *et al.* (*9*) demonstrated that disruption of the *DRS2* gene in yeast abolished the translocation of a fluorescent PS analog (NBD-PS) across the plasma membrane. Although this DRS2-dependent plasma membrane translocation activity was controversial

(see below), ATPase II and Drs2p were the first members of a novel subfamily of the P-type ATPase superfamily, P4-ATPases.

Structure of P4-ATPases

The P4-ATPases, which have been identified only in eukaryotic cells, are major components of phospholipid flippases. To date, studies on P4-ATPases have been reported in *S. cerevisiae*, *Leishmania donovani*, *Arabidopsis thaliana*, *Caenorhabditis elegans*, *Cryptococcus neoformans* and mammals including human. The human genome contains 14 genes encoding P4-ATPases and the *C. elegans* genome contains 6 genes. *Saccharomyces cerevisiae* and *A. thaliana* genomes contain 5 and 12 genes, respectively. In the database, the *Drosophila melanogaster* genome contains at least 6 genes (Fig. 1). P4-ATPases have the same membrane topology as those of other subfamily of P-type ATPases; 10-transmembrane segments, NH_2- and COOH-termini facing to the cytoplasm, and two large intracellular loops: one is between transmembrane segments 2 and 3, and the other is between transmembrane segments 4 and 5 harboring P-type ATPase-specific sequences including a characteristic pattern of conserved residues, most notably the DKTGTLT sequence motif (in which D is the reversibly phosphorylated Asp) and an ATP-binding site (Fig. 2). A distinct feature of this subfamily is that in transmembrane domains 4 and 6, the intrabilayer positions typically occupied by conserved charged and polar amino acids in cation-transport ATPases are replaced with hydrophobic and aromatic amino acids.

Flippase activity of P4-ATPases

Tang *et al.* (*9*) have first cloned a P4-ATPase, ATP8A1 (ATPase II), and presented that in yeast mutant *drs2Δ*, which lacked the P4-ATPase *DRS2* gene, the translocation of NBD-PS across the plasma membrane was impaired significantly (*9*). However, in two subsequent studies, deletion of the *DRS2* gene had no effect on the uptake or distribution of NBD-PS or -PE (*11*, *12*). Considering that Drs2p is primarily associated with the TGN/endosomes rather than the plasma membrane (*13–16*), the observations in the latter studies are more likely. However, the observations in the former study may result from the entrapment of plasma membrane P4-ATPases Dnf1p and Dnf2p in internal membranes in the *drs2Δ* mutant, because the trafficking of them may be defective in the *drs2Δ* mutant ('functions of flippases in membrane trafficking' section). Pomorski *et al.* (*15*) showed that loss of Dnf1p and Dnf2p (*dnf1Δ dnf2Δ*) abolishes the ATP-dependent transport of NBD-PE, -PC and -PS across the plasma membrane. A defect in NBD-PC transport was surprising, but we cannot rule out the possibility that other enzyme(s) catalysing flip of NBD-PC is not properly localized to the plasma membrane or not activated in the *dnf1Δ dnf2Δ* mutant. We should await a biochemical reconstitution study to clarify the substrate specificity of Dnf1p and Dnf2p. Drs2p, Dnf1p and Dnf2p are confined to the plasma membrane when endocytosis is blocked. Taking advantage of this phenomenon, Saito *et al.* (*16*) presented that under conditions of blockade of endocytosis, overexpression of *DRS2* dramatically increased internalization of NBD-PE and -PS across the plasma membrane in the *dnf1Δ dnf2Δ* mutant. These studies clearly showed that P4-ATPases are responsible for flip of fluorescent analogs of phospholipids across the plasma membrane.

Further evidence of the participation of P4-ATPases in flip of phospholipids was provided in the several yeast studies. Natarajan *et al.* (*17*) presented that TGN membranes purified from wild-type cells contained an ATP-dependent flippase that translocates NBD-PS from the luminal to the cytosolic leaflet. A weaker activity was also detected for NBD-PE, but not for NBD-PC. On the other hand, TGN membranes purified from a strain carrying a temperature-sensitive functional mutant *drs2* (*drs2-ts*) lost an ATP-dependent NBD-PS flippase activity under the restrictive condition at 37°C, suggesting that the flippase activity in the TGN membrane is directly coupled with function of Drs2p (*17*). Alder-Baerens *et al.* (*18*) presented that post-Golgi secretory vesicles (SVs) contained an ATP-dependent flippase activity that translocates NBD-PS, -PE and -PC to the cytosolic leaflet. This activity was independent of Dnf1p and Dnf2p but required Drs2p and Dnf3p. Moreover, SVs had an asymmetric PE arrangement that was lost upon removal of Drs2p and Dnf3p. These results suggested that these P4-ATPases are primarily involved in the flippase activity in SVs (*18*). Both studies showed that P4-ATPases are implicated in flippase activity, but did not exclude the possibility that P4-ATPase activities are the secondary effect: the P4-ATPases pump an unidentified ion into the lumen of TGN or SVs that is subsequently coupled to lipid translocation as the ion moves back down its concentration gradient by an unidentified ion/phospholipid symporter.

More recently, two independent studies provided evidence that P4-ATPases directly catalyse phospholipids translocation across membranes (*19*, *20*). Zhou and Graham (*19*) purified Drs2p from yeast and reconstituted it into proteoliposomes. In their studies, in proteoliposomes containing Drs2p, NBD-PS was actively flipped across the liposome bilayer in the presence of Mg^{2+}-ATP, whereas proteoliposomes reconstituted with a catalytically inactive form of Drs2p showed no translocation activity. This flippase activity was observed for NBD-PS, but not for NBD-PC or NBD-sphingomyelin (*19*). These observations suggest that Drs2p directly mediates flippase activity. Coleman *et al.* (*20*) purified ATP8A2 from photoreceptor outer segments by immunoaffinity chromatography. The ATPase activity of purified ATP8A2 was stimulated by PS and to lesser degree by PE but not by PC or other membrane lipids. Purified ATP8A2 reconstituted into liposomes containing NBD-PS flipped NBD-PS from the inner to the outer leaflet of the liposome membranes in an ATP-dependent manner, suggesting that P4-ATPase can directly translocate aminophospholipids across membranes (*20*).

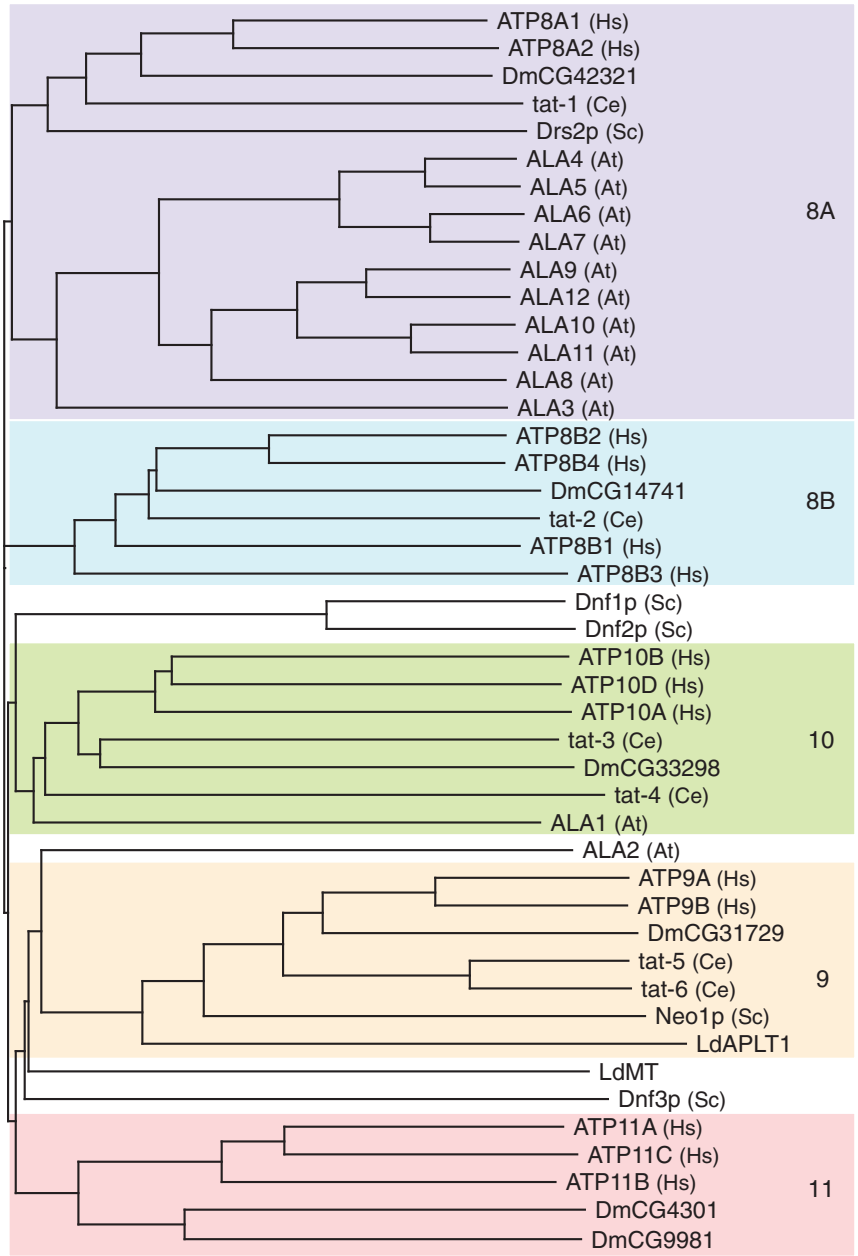

Fig. 1 Phylogenetic tree of P4-ATPases in major model organisms. Multiple sequence alignment of P4-ATPases from *H. sapiens*, *A. thaliana*, *D. melanogaster*, *C. elegans*, *L. donovani*, and *S. cerevisiae* was performed using Clustal X 2.0.1 sequence alignment software. The phylogenetic tree was calculated using Clustal X 2.0.1 program with the neighbor joining method. Database accession numbers: *H. sapiens* (Hs): ATP8A1 (AAI09319), ATP8A2 (NP_057613), ATP8B1 (O43520), ATP8B2 (P98198), ATP8B3 (O60423), ATP8B4 (Q8TF62), ATP9A (O75110), ATP9B (O43861), ATP10A (O60312), ATP10B (O94823), ATP10D (Q9P241), ATP11A (P98196), ATP11B (Q9Y2G3), ATP11C (Q8NB49); *A. thaliana* (At): ALA1 (P98204), ALA2 (P98205), ALA3 (Q9XIE6), ALA4 (Q9LNQ4), ALA5 (Q9SGG3), ALA6 (Q9SLK6), ALA7 (Q9LVK9), ALA8 (Q9LK90), ALA9 (Q9SX33), ALA10 (Q9LI83), ALA11 (Q9SAF5), ALA12 (P57792); *D. melanogaster*: DmCG42321 (ACL83108), DmCG14741 (AAF54749), DmCG33298 (AAS64663), DmCG31729 (AAF53278), DmCG4301 (AAF48606), DmCG9981 (AAF48605); *C. elegans* (Ce): tat-1 (NP_001022894), tat-2 (NP_001023252), tat-3 (NP_499363), tat-4 (NP_495244), tat-5 (NP_001021457), tat-6 (NP_503858); *L. donovani*: LdMT (AAQ82704), LdAPLT1 (AAC19127); *S. cerevisiae* (Sc): Drs2p (P39524), Dnf1p (P32660), Dnf2p (Q12675), Dnf3p (Q12674), Neo1p (P40527).

β-Subunit of flippase, the CDC50 family

Some of the P4-ATPases have been shown to form a complex with a non-catalytic β-subunit of the CDC50 family. The CDC50 protein family has first been identified in yeast. Two independent groups identified Lem3p/Ros3p in yeast genetic screens as a protein, lack of which caused significant reduction of uptake of NBD-PE and -PC across the plasma membrane (*21*, *22*). This phenotype of the *lem3Δ* mutant was very similar to that of the *dnf1Δ dnf2Δ* mutant (*15*, *21*, *22*). Saito *et al.* (*16*) revealed that CDC50 proteins are accessory proteins for P4-ATPases. They demonstrated that Drs2p binds to Cdc50p, while Dnf1p and Dnf2p bind to Lem3p. The yeast third homologue, Crf1p, binds to Dnf3p (*16*, *23*). These interactions are specific as shown by

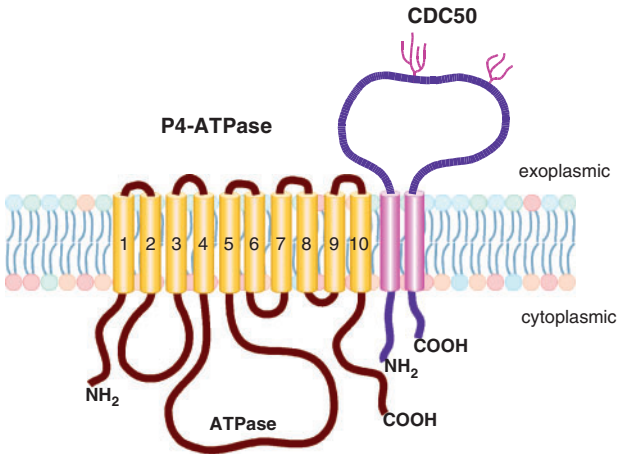

Fig. 2 Predicted topology of P4-ATPase and CDC50 protein. It is unknown how transmembrane helices of CDC50 interact with P4-ATPase. Phospholipids are translocated from the exoplasmic to the cytoplasmic leaflet.

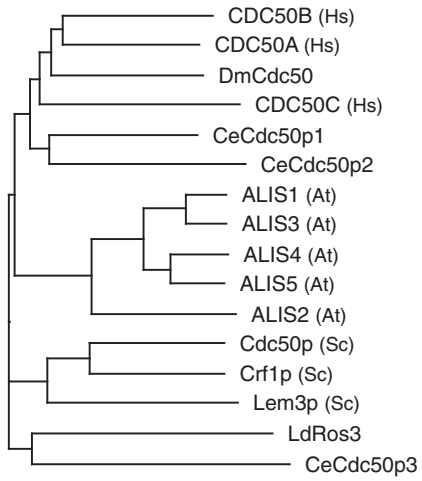

Fig. 3 Phylogenetic tree of the CDC50 protein family in major model organisms. Multiple sequence alignment of CDC50 family members from *H. sapiens*, *A. thaliana*, *D. melanogaster*, *C. elegans*, *L. donovani* and *S. cerevisiae* was performed using Clustal X 2.0.1 sequence alignment software. The phylogenetic tree was calculated using Clustal X 2.0.1 program with the neighbor joining method. Database accession numbers: *H. sapiens* (Hs): CDC50A (Q9NV96), CDC50B (Q3MIR4), CDC50C (EAW79828); *A. thaliana* (At): ALIS1 (Q9LTW0), ALIS2 (Q67YS6), ALIS3 (Q9SLK2), ALIS4 (Q9SA35), ALIS5 (Q8L8W0); *D. melanogaster*: DmCDC50 (AAF48613); *C. elegans*: CeCdc50p1 (Q21844), CeCdc50p2 (Q19635), CeCdc50p3 (Q23151); *L. donovani*: LdRos3 (ABB05176); *S. cerevisiae* (Sc): Cdc50p (P25656), Lem3p (P42838), Crf1p (P53740).

immunoprecipitation and split-ubiquitin assays (*16*, *23*–*25*). The formation of the complexes is essential for the exit from the ER of CDC50 proteins as well as Drs2/Dnf proteins and subsequent proper localization (*16*, *23*).

These findings in yeast have led to the identification and functional studies of CDC50 family proteins in other organisms. To date, studies in yeast, plants, mammals and *Leishmania* parasites have been reported (Fig. 3). CDC50 family proteins are predicted to contain two transmembrane segments and a large, intervening exoplasmic loop that is glycosylated (Fig. 2) (*21*). *Arabidopsis thaliana* contains five members, ALIS1-5 (for ALA-interacting subunit) (*26*). In contrast to yeast CDC50 proteins, ALIS proteins did not display binding specificity to an ALA P4-ATPase: ALIS1, ALIS3 and ALIS5 could interact and function with ALA2 and ALA3 (*27*). In mammals, three CDC50 proteins termed CDC50A-C have been identified (*28*). CDC50A and CDC50B are ubiquitously expressed, while CDC50C is expressed mainly in testis (*28*–*30*). Human ATP8B1 expressed in Chinese hamster ovary cells could exit from the ER and be trafficked to the plasma membrane or lysosomes/late endosomes, only when CDC50A or CDC50B was co-expressed, respectively (*31*). The specificity of CDC50–P4-ATPase interactions in mammalian cells is presently not known. However, based on expression profiles, it has been postulated that ATP8B3 and CDC50C, and ATP8B1 and CDC50A are physiologically relevant binding partners (*29*, *30*, *32*–*35*). In *Leishmania* parasites, a Cdc50 family member LdRos3 is also required for the ER exit of the P4-ATPase LdMT (*36*). The *D. melanogaster* and *C. elegans* genome encodes at least one and three sequences homologous to known members of the CDC50 protein family, respectively, although in both organisms no interactions between P4-ATPases and CDC50 proteins have been described yet. Binding partners of each P4-ATPase are summarized in Table I.

One interesting question is whether all P4-ATPases require β-subunits for their function. In yeast, none of the three CDC50 members form a complex with Neo1p (*16*, *23*). Neo1p may function without a β-subunit or might form a complex with an unidentified β-subunit. Another question is how strictly the specificity of the interaction between P4-ATPases and CDC50 proteins is determined. In all organisms, in which P4-ATPases and CDC50 proteins have been identified, the number of CDC50 proteins is fewer than that of P4-ATPases. In some cases, multiple P4-ATPases share CDC50 proteins: Dnf1p and Dnf2p form a complex with Lem3p (*16*) and ALA2 and ALA3 form a complex with ALIS1, ALIS3 or ALIS5 (*27*). There has been no information about which region in P4-ATPases interacts with CDC50 proteins. Identification and comparison of amino acid sequences of such a region would provide valuable insights into these questions.

Roles of the CDC50 family in flippase activity

CDC50 family proteins are required for the ER export of their partner P4-ATPases, but they are still associated with P4-ATPases after localization to their final destination (*16*, *23*, *27*, *31*, *36*). Do CDC50 proteins function only as a chaperone for P4-ATPases or play a role in catalytic activity of flippases like a β-subunit of Na^+, K^+-ATPases (*37*)? In yeast, a C-terminally HA-tagged version of Lem3p (Lem3p-HA) and some mutant versions of Lem3p did not affect the formation or localization of Lem3p–Dnf1p, but did cause a functional defect,

suggesting that Lem3p plays an important role within the mature Lem3p–Dnf1p complex (*16*, *38*).

Recently, Lenoir *et al*. (*24*) have provided intriguing insights into the role of Cdc50p by analysing purified Drs2p–Cdc50p complex and using a genetic reporter system. The P-type ATPases form an aspartylphosphate intermediate during ATP hydrolysis (*10*). Lenoir *et al*. (*24*) presented that this intermediate phosphoenzyme formation of Drs2p is dependent on association with Cdc50p and that the interaction of Cdc50p with Drs2p is dependent on whether Drs2p can be phosphorylated. The ATPase reaction cycle of Ca^{2+}-ATPases and Na^+, K^+-ATPases has been studied in detail [for a review, see ref. (*10*)]. It consists of two distinct conformations of the ATPases called E1 and E2 with each having a different affinity for the nucleotide and the transported ligands, and phosphorylated intermediate states called E1-P and E2-P. In Ca^{2+}-ATPase, SERCA, mutations that block each step of the reaction cycle have been identified (*39*). Lenoir *et al*. (*24*) created analogous mutations in Drs2p and presented that the mutant trapped in the E2P-conformation ($Drs2^{E342Q}p$) bound with Cdc50p, whereas the mutant trapped in the E1P-conformation ($Drs2^{G341L}p$) did not. Both mutants could not complement the growth defect of the *drs2Δ* mutant at a low temperature, suggesting that both mutants were defective in progressing through the reaction cycle. From these observations, they proposed that the interaction of Cdc50p with Drs2p is crucial for the catalytic mechanism of the P4-ATPase (*24*).

In the reconstitution study of flippase activity with Drs2p, some Cdc50p was reconstituted into the proteoliposomes together with Drs2p (*19*). In their study, although ~40% of the liposomes were estimated to contain one Drs2p molecule, they detected Mg^{2+}-ATP-dependent flip of only 4% of the NBD-PS input, suggesting that only 4% of the liposomes contained an active flippase. Based on mass spectrometric analysis, the number of Cdc50p peptides recovered in the Drs2p-containing proteoliposomes were approximately 1/10 that of Drs2p. These results may suggest that the detected flippase activity was catalysed by Drs2p–Cdc50p complex and that Drs2p alone was not enough to drive flippase activity. A further improved reconstitution experiment will provide more convincing evidence as to whether CDC50 proteins participate in flippase activity.

Functions of flippases in membrane trafficking

Most of our current knowledge about the functions of flippases in membrane trafficking has been derived from studies in budding yeast. Implication of a flippase in membrane trafficking was first described for *DRS2* (*13*). *DRS2* was identified as a mutation that was synthetically lethal with a mutation in the Arf1p small GTPase. Synthetic lethal is that combination of two non-lethal mutations results in growth defects, indicating that these two genes are functionally related and have overlapping functions. Genes that show genetic interaction with flippase genes are summarized in Tables II and III. Arf1p in its GTP-bound form recruits coat proteins for vesicle formation on the membrane surface. COPI and clathrin coats are well characterized as coat proteins regulated by Arf1 (*40*). The *drs2Δ* mutation was synthetically lethal with a mutation in clathrin, but not with that in COPI, and recovery of clathrin-coated vesicles is decreased in the *drs2Δ* mutant (*13*), suggesting that Drs2p is involved in the formation of clathrin-coated vesicles.

Phenotypic analyses of yeast flippase mutants suggested that they are involved in a variety of vesicle transport pathways. The *dnf1Δ dnf2Δ drs2Δ* mutant shows defects in endocytosis at a lower

Table I. Binding partners of flippases.

Organism	P4-ATPase	β-subunit	References
Saccharomyces cerevisiae	Dnf1p, Dnf2p	Lem3p	(*16*, *23–25*)
	Dnf3p	Crf1p	(*16*, *23–25*)
	Drs2p	Cdc50p	(*16*, *24*, *25*)
Arabidopsis thaliana	ALA2, ALA3	ALIS1, ALIS3, ALIS5	(*26*, *27*)
Leishmania donovani	LdMT	LdRos3	(*36*, *88*)
Homo sapiens	ATP8B1	CDC50A, CDC50B	(*31*)

Table II. Genetic interaction with flippases (multicopy suppression[a]).

Mutation	Suppressor	Function	Suppressed phenotype	References
cdc50-11 lem3Δ crf1Δ, *cdc50-162 lem3Δ crf1Δ*	*YPT31, YPT32*	Rab family GTPase	Temperature-sensitive growth	(*23*)
drs2Δ	*SAC1*	Phosphatidylinositol-4-phosphatase	Cold-sensitive growth	(*25*)
lem3Δ	*BEM3*	Cdc42p GTPase-activating protein	Hyperpolarized growth	(*63*)
	RGA1	Cdc42p GTPase-activating protein	Hyperpolarized growth	(*63*)
neo1-69	*DOP1*	Ysl2p-interacting highly conserved protein	Temperature-sensitive growth	(*89*)
myo3Δ myo5-360	*CDC50*	Type I myosin involved in actin patch assembly	Temperature-sensitive growth	(*90*)
dop1-3	*NEO1*	Ysl2p-interacting highly conserved protein	Temperature-sensitive growth	(*89*)

[a]Multicopy suppression is that mutant phenotypes are suppressed by overexpression of a gene on a multicopy plasmid.

Table III. Genetic interaction with flippases (synthetic effect).

Flippase mutation	Interactor	Function	Phenotype	References
cdc50Δ	*erg2Δ*, *erg3Δ*, *erg5Δ*, *erg6Δ*	Enzyme that catalyses late steps in the ergosterol biosynthesis pathway	Synthetic growth defect	(*66*)
	vps1	Dynamin-like GTPase	Synthetic growth defect	(*66*)
	srv2	Adenylyl cyclase-associated protein	Synthetic growth defect	(*66*)
	rgp1	Subunit of GDP/GTP exchange factor for Ypt6p	Synthetic growth defect	(*66*)
	ric1	Subunit of GDP/GTP exchange factor for Ypt6p	Synthetic growth defect	(*66*)
	ypt6	Rab family GTPase	Synthetic growth defect	(*83*)
	chc1-521	Clathrin heavy chain	Synthetic growth defect	(*44*)
	arf1Δ	Small GTPase	Synthetic growth defect	(*44*)
	gga1Δ gga2Δ	Golgi-localized, γ-adaptin ear-containing, Arf-binding protein	Synthetic growth defect	(*44*)
	gsg1 (*trs85*)	Subunit of TRAPP (transport protein particle)	Synthetic growth defect	(*83*)
	pep8	Subunit of the retromer complex	Synthetic growth defect	(*83*)
	gcs1Δ	GTPase-activating protein for Arf1p	Synthetic growth defect	(*44*)
	fpk1Δ fpk2Δ	Flippase kinase	Synthetic growth defect	(*83*)
	ubp3	Deubiquitinating enzyme	Synthetic growth defect	(*83*)
	sec3	Subunit of the exocyst complex	Synthetic growth defect	(*83*)
drs2Δ	*chc1-5*, *chc1-Δ57*, *chc1-521*	Clathrin heavy chain	Synthetic growth defect	(*13*)
	arf1Δ	Small GTPase	Synthetic growth defect	(*13*)
	gea2Δ	Arf GDP/GTP exchange factor	Synthetic cold-sensitive growth	(*56*)
	gga1Δ gga2Δ	Golgi-localized, γ-adaptin ear-containing, Arf-binding protein	Synthetic growth defect	(*44*, *48*)
	apl4Δ	γ-subunit of the clathrin AP-1 adaptor	Synthetic growth defect	(*48*)
	gcs1Δ	GTPase-activating protein for Arf1p	Synthetic growth defect	(*44*)
	erg2Δ, *erg3Δ*, *erg5Δ*, *erg6Δ*	Enzyme that catalyses late steps in the ergosterol biosynthesis pathway	Synthetic growth defect	(*66*)
	pan1-20	Component of the endocytic adaptor complex consisting of Pan1p, End3p, Sla2p and Sla1p	Synthetic growth defect	(*13*)
	sla1Δ	Component of the endocytic adaptor complex consisting of Pan1p, End3p, Sla2p and Sla1p	Synthetic growth defect	(*45*)
	chs6Δ	Component of exomer for the Chs6p transport	Restoration of calcofluor white-sensitive growth	(*48*)
	kes1	Oxysterol-binding protein family	Rescue from cold-sensitive growth	(*67*)
	cho1Δ	PS synthase	Rescue from the sensitivity to a cyclic lipopeptide papuamide B	(*91*)
lem3Δ, *dnf1Δ dnf2Δ*	*psd1Δ psd2Δ*	PS decarboxylase for PE biosynthesis	Synthetic growth defect in the presence of lyso-PE as the sole source of PE	(*64*)
	cho2Δ opi3Δ (*pem1Δ pem2Δ*)	Phospholipid methyltransferase for PC biosynthesis	Synthetic growth defect in the presence of lyso-PC as the sole source of PC	(*65*)
neo1-1	*sec21-1*	γ-Subunit of COPI coatmer	Synthetic growth defect	(*41*)
	ret1-1 (*cop1*)	α-Subunit of COPI coatmer	Synthetic growth defect	(*41*)
neo1-39, *neo1-67*	*arl1Δ*	Arf-like GTPase	Rescue from temperature-sensitive growth	(*42*)

temperature (*15*), but the *cdc50*-ts (temperature sensitive) *lem3Δ crf1Δ* mutant did not at a higher temperature (*23*). The *drs2Δ dnf1Δ* mutant also shows a defect in the transport of alkaline phosphatase to the vacuole (*14*). Neo1p was shown to be involved in the retrograde pathway from *cis*-Golgi to the ER (*41*). Neo1p was also implicated in the transport through the endosomal/TGN membranes, although it remains to be precisely determined in which pathway Neo1p is involved (*42*).

Drs2p was implicated in the formation of a certain class of exocytic secretory vesicles by using electron microscopy (*43*). On the other hand, apparent defects were not observed for the formation of two types of secretory vesicles, a low-density type and a high-density type, by density gradient fractionation in the *cdc50*-ts *lem3Δ crf1Δ* mutant (*23*). Thus, further investigation is required for the involvement of flippase in the exocytic pathway. One prominent phenotype in flippase mutants is that they show a defect in the endocytic recycling pathway (in yeast, it is equivalent to the retrograde pathway from early endosomes to the TGN) (*14*, *23*, *44*). In the *cdc50*-ts *lem3Δ crf1Δ* mutant, a vesicle SNARE protein Snc1p, which is recycled through this pathway, is accumulated in large early endosome-derived structures. Dnf1p and Dnf2p are mainly localized to the plasma membrane, but are also localized to early endosomes through the recycling pathway possibly to perform this function (*16*, *45*). Thus, one important flippase function seems to form a transport vesicle from the early endosome.

Clathrin is recruited to the site for vesicle formation by clathrin adaptors, which tether vesicle cargos to clathrin. A tetrameric clathrin adaptor AP-1 (β1-, γ-, μ1-, δ1-adaptin) is implicated in vesicle formation from early endosomes for transport to the TGN (*44*, *46*, *47*). AP-1 is also implicated in the opposite pathway, vesicle formation from the TGN for transport to early endosomes (*48*). In the latter case, Drs2p is potentially a cargo of AP-1, and its flippase activity is required for the transport of Drs2p itself to early endosomes. Interestingly, the *drs2Δ* mutation does not show synthetic growth defects with a mutation in AP-1 (*44*, *48*). These results could suggest that Drs2p and AP-1 function in the same pathway: Drs2p-mediated lipid flipping might be coupled with AP-1 vesicle formation. However, defects in AP-1 vesicle formation do not account for defective endocytic recycling in the *cdc50Δ* mutant, because the *apl2Δ* mutant (β1 subunit of AP-1) shows normal recycling of Snc1p (*44*). Therefore, another clathrin adaptor seems to be involved in Drs2p-mediated formation of transport vesicles from early endosomes. Such candidates are ENTH/ANTH domain proteins Ent3p and Ent5p that have recently been implicated in the early endosome-to-TGN transport (*49*). The *cdc50Δ* mutation actually shows genetic interaction (synthetic growth defects) with the *ent3Δ ent5Δ* mutations (our unpublished data). Monomeric type clathrin adaptors Gga1p and Gga2p bind to a potential GDP/GTP exchange factor for the Arl1p (Arf-like) small GTPase, Ysl2p, which physically associates with Neo1p (*50*).

As to the mechanism of how flippases promote vesicle formation by transporting phospholipids to the cytosolic surface, two models could be envisioned. One mechanism is that lipid pumping by flippases forms a local membrane curvature according to the bilayer couple mechanism (*51*), and this curvature is recognized by coat proteins (*e.g.* clathrin adaptors) to promote vesicle formation. The other mechanism is that some specific phospholipid pumped by a flippase recruits a coat protein. One candidate phospholipid would be PS, since PS is distinct from PE and PC in its negative charge and since NBD-PS is a preferable substrate of Drs2p in *in vitro* studies (*19*). Currently available data do not discriminate between these two possibilities, but they are not mutually exclusive. In either case, phospholipid flipping by a flippase would possibly recruit a coat protein to membranes. Although it has been shown that AP-1 is normally recruited to the TGN in the *drs2Δ* mutant (*48*), another recruiting mechanism that is redundant with Drs2p might be present on the TGN. In addition, it is possible that AP-1 is localized to the TGN, but not to early endosomes in the *drs2Δ* mutant. This point is technically difficult to be examined, because an early endosome-specific marker is not currently available in budding yeast. Interestingly, AP-1 was diffused into the cytoplasm in the Cdc50p-depleted *gcs1Δ* mutant, which shows severe synthetic defects in endocytic recycling (*44*). Gcs1p is a GTPase-activating protein (GAP) for Arf1p and was shown to be required for recycling of Snc1p (*52*). Diffused localization of AP-1 was not observed in either *cdc50Δ* or *gcs1Δ* single mutant, suggesting that Cdc50p-Drs2p and Gcs1p redundantly function to recruit AP-1 to early endosomes.

Since phospholipid flipping is implicated in inducing membrane curvature, proteins that can induce or recognize membrane curvature would be functionally relevant to flippases. Those proteins functionally relevant to Drs2p would include Arf1p and Ent3p. The amphipathic α-helix of Arf1p-GTP, but not Arf1p-GDP, can induce membrane curvature when it is inserted into the cytosolic leaflet of the bilayer (*53*, *54*). Similarly, the NH_2-terminal α-helix of the ENTH domain of mammalian epsin can induce membrane curvature upon binding of the ENTH domain to phosphatidylinositol-4,5-bisphosphate (*55*). Although it should be examined whether the ENTH domain of Ent3p also has a capability to induce membrane curvature, co-operative functioning between Drs2p and Ent3p or Arf1p for vesicle formation would be an interesting possibility.

One promising approach toward understanding regulation and function of flippases would be identification of interacting proteins. Proteins that show physical interaction with flippases are summarized in Table IV. Gea2p, a GDP/GTP exchange factor for Arf1p, was shown to bind to the COOH terminal region of Drs2p (*56*). Gea2p binding to Drs2p was implicated in stimulation of flippase activity in Golgi membranes (*57*). Gea2p is known to be involved in the retrograde transport from the *cis*-Golgi to the ER or in the intra-Golgi transport (*58*, *59*). However, since

Table IV. Physical interaction with flippases.

Flippase	Interactor	Method	Function	References
Neo1p	Ysl2p	Co-IP	Potential GDP/GTP exchange factor for the Arl1p small GTPase	(*42*)
Drs2p	AP-1	Co-IP (Cross-linking)	Tetrameric clathrin adaptor	(*48*)
	Gea2p	Co-IP, Two-hybrid	GDP/GTP exchange factor for Arf1p	(*56*)
	Rcy1p	Co-IP	F-box protein involved in endocytic recycling	(*23*)
	Sac1p	Pull-down (Cross-linking), Membrane two-hybrid	Phosphatidylinositol-4-phosphatase	(*25*)
Dnf1p, Dnf2p	Fpk1p, Fpk2p	Phosphorylation	Serine/threonine kinase	(*83*)

Drs2p is implicated in endosomal/TGN functions, it has to be clarified which post-Golgi pathway is regulated by this Gea2p–Drs2p interaction. An F-box protein Rcy1p, originally isolated as a protein involved in endocytic recycling (*60*), was co-immunoprecipitated with Drs2p (*23*). Our recent results with recombinant proteins indicate that Rcy1p directly binds to the COOH terminal region of Drs2p (our unpublished results). Interestingly, the *rcy1Δ* mutant phenocopies the *cdc50Δ* and *drs2Δ* mutants, and, importantly, the *cdc50Δ rcy1Δ* double mutant showed no synthetic growth defects, suggesting that Rcy1p functions in the same pathway as Cdc50p–Drs2p. Rcy1p was suggested to be an effector of the Rab family small GTPases Ypt31p/32p (*61*). An interesting question would be whether Rcy1p has regulatory functions for the Drs2p flippase activity or it functions as an adaptor for formation of clathrin-coated vesicles.

Other functions of flippases in yeast

The role of phospholipid asymmetry mediated by flippases on signalling for polarity formation was clarified in budding yeast. PE is specifically exposed on the outer leaflet at polarized sites during the early stage of budding, and disappears in G2 as the apical bud growth switches to the isotropic growth. This PE exposure is enhanced by disruption of *LEM3* or both of *DNF1* and *DNF2* (*62*, *63*). How PE becomes exposed on the outer leaflet during the bud growth is a remaining problem. This PE flipping was shown to regulate Cdc42p small GTPase signalling via GTPase-activating proteins (GAPs) (*63*). The *lem3Δ* and *dnf1Δ dnf2Δ* mutants exhibit prolonged apical growth due to a defect in the switch to isotropic bud growth. In these cells, Cdc42p remains polarized at the bud tip where PE remains exposed on the outer leaflet. Interestingly, the GAP activities of Rga1p and Rga2p, GAPs for Cdc42p, are stimulated by PE and PS. These results propose a model in which the apical-isotropic switch is triggered by phospholipid flipping by Lem3p–Dnf1/2p, and this flipping dissolves the apical membrane domain by GAP-stimulated GTP hydrolysis of Cdc42p.

Yeast is found to be able to uptake lyso-PE and lyso-PC from exogenous culture. The ethanolamine-auxotrophic *psd1Δ psd2Δ* and the choline-auxotrophic *pem1Δ pem2Δ* mutant can use lyso-PE and lyso-PC as a source of PE and PC, respectively. Lyso-PC and lyso-PE taken up into cells are acylated to PC and PE, respectively, by lysophospholipid acyltransferase encoded by *ALE1*. Disruption of *LEM3* or both of *DNF1* and *DNF2* in the *psd1Δ psd2Δ* or *pem1Δ pem2Δ* mutant blocks uptake of radiolabeled lyso-PC or lyso-PE, and inhibits lyso-PC- or lyso-PE-dependent growth, respectively, suggesting that Lem3p–Dnf1/2p flippases function to uptake lyso-PC or lyso-PE (*64*, *65*).

The synthetic lethal screen revealed a strong genetic interaction between *cdc50Δ* and mutations exhibiting defects in the late steps of ergosterol biosynthesis (*66*). In the absence of *ERG3*, which encodes a sterol C-5 desaturase catalysing a late step in the ergosterol biosynthesis, Cdc50p depletion results in strong defects in the endocytic recycling pathway and interestingly causes accumulation of abnormal intracellular actin patch-like structures, suggesting that Cdc50p–Drs2p-regulated phospholipid asymmetry and ergosterol integrity cooperate for endocytic recycling pathway and actin cytoskeleton reorganization (*66*).

The interesting relationship between Cdc50p–Drs2p and Kes1p, one of the oxysterol-binding protein family, was reported (*67*). Loss of function mutations of *KES1* was identified in a screen for suppressors of the cold-sensitive growth defect of *drs2Δ*. *DRS2* disruption causes the Kes1p-dependent increased rate of cholesterol transport from the plasma membrane to the ER, suggesting that Drs2p antagonizes Kes1p activity. Further studies are required to solve how Drs2p regulates the Kes1p activity and its physiological meaning.

Functions of flippase in other organisms

Among mammalian flippases, the most analysed one is ATP8B1. Mutations of ATP8B1 expressing in the apical membrane of many epithelial cells in the gastrointestinal trace and liver are associated with the progressive familial intrahepatic cholestasis type 1 (PFIC1) and benign recurrent intrahepatic cholestasis type 1 (BRIC1), which is characterized by impaired bile salt excretion from the liver into bile and by periodic bouts of cholestasis that leave no liver injury, respectively (*68*). The role of ATP8B1 as PS flippase has been indicated by showing the uptake of NBD-PS in the UPS-1 cell, a CHO-K1 mutant cell line defective in NBD-PS uptake (*31*, *69*). It is also supported by the luminal accumulation of PS in the canalicular membrane of ATP8B1-deficient hepatocytes (*70*) and by the enhanced recovery of PS in bile from

Atp8b1$^{G308V/G308V}$ mutant mice, in which ATP8B1 protein is nearly absent (*71*). From studies using cell lines (*33*, *70*) and ATP8B1-deficient mice (*33*, *71–74*), the hypothetical model of how ATP8B1 deficiency results in cholestasis is as follows. ATP8B1 deficiency leads to lowered PS flipping, and increases the PS concentration in the outer leaflet. This reduces the liquid ordered state of sphingolipid/cholesterol-rich outer leaflet of canalicular membranes. This disturbance of the lipid order state makes the membrane more sensitive to bile salt-mediated extraction of membrane components, including cholesterol, sphingolipids and ectoenzymes such as alkaline phosphatase. The increased extraction of cholesterol may cause the impaired activity of the bile salt export pump, ABCB11, and consequently lead to cholestasis (*68*). Similar to many PFIC1 patients, ATP8B1-deficient mice cause hearing loss. ATP8B1 is localized to the apical region in the stereocilia of the cochlear hair cells in the organ of Corti. The mechanosensory function and integrity of the cochlear hair cells may be critically dependent on ATP8B1 activity (*75*). A flippase-independent function of ATP8B1 was recently proposed (*76*). Blocking of ATP8B1 expression in polarized epithelial Caco-2 cells caused dramatic morphological changes in the apical brush border membrane, without affecting flippase activities on the apical membrane. ATP8B1 may have an ATPase-independent role, for example, by mediating a protein–protein interaction as Drs2p does (*56*).

The mouse ATP8B3 is expressed in the acrosomal region of the head of spermatozoa and has been implicated in the capacitation process (*34*). Recently, a novel murine P4-ATPase that is specifically expressed in the testis, named FetA, was identified. When expressed in the budding yeast, FetA localizes partially to the plasma membrane and increases internalization of NBD-labelled PE and PC. RNA interference suggested that FetA is involved in Golgi morphology and secretory function (*77*).

The analysis based on the expression pattern of reporter transgenes suggest that each of *C. elegans* TAT flippases may have distinct functions and four of them are not essential under regular growth conditions (*78*). TAT-1 functions in maintaining PS asymmetry on the plasma membrane, and its disruption results in removal of affected cells by neighboring phagocytes (*79*). TAT-1 is also required at an early step of endocytosis and at a late step in lysosome biogenesis in specialized cell types of several tissues (*80*). TAT-2 antagonistically controls the growth regulatory function of monomethyl branched-chain fatty acids in intestinal cells (*81*).

Downregulation of ALA1, the first-analysed putative flippase in *Arabidopsis*, causes suffering from chilling sensitivity, although the mechanism how phospholipid flip relates to chilling tolerance should be clarified (*82*). ALA3, which is mostly related to yeast Drs2p, localizes to the Golgi apparatus, and its mutants show severe defects in vesicle production in secreting peripheral columella cells of the root tip (*26*). Although it is shown that ALA3 requires the β-subunit of the CDC50 family such as ALIS1, ALIS3 or ALIS5 (*26*), detailed phenotype comparison of ALA3 and ALIS plant mutants, just not only analysis of their localization, will be utilized for understanding physiological role of these complexes.

Regulation of P4-ATPase activity

What signal and how it regulates phospholipid flippases are interesting issues, which are being clarified about yeast flippases. Recently, yeast novel kinases named flippase kinases 1 and 2 (Fpk1p and Fpk2p) were identified as upstream regulators for Dnf1p and Dnf2p. These were originally identified as a mutation that exhibits synthetic lethality with the *cdc50Δ* mutation. The *fpk1Δ fpk2Δ* double mutant phenocopies the *lem3Δ* or *dnf1Δ dnf2Δ* mutant. Although the *fpk1Δ fpk2Δ* mutations do not affect the subcellular localization of Lem3p–Dnf1/2p, Fpk1p can phosphorylate Dnf1p and Dnf2p to a greater extent than Drs2p (*83*). Their phosphorylation sites by Fpk1/2p and whether their phosphorylation really activates flippase activity of Dnf1/2p remain to be solved.

Identification of the regulator for Fpk1/2p may clarify the upstream signal for Dnf1/2p. Fpk1p was shown to be a physiological substrate of Ypk1p (orthologues of mammalian SGK1), which is implicated in multiple processes such as endocytosis and coupling of membrane expansion to cell wall remodelling (*84*). Ypk1p and its homolog Ypk2p are activated by eisosome-associated Pkh1p and Pkh2p (orthologues of mammalian PDK1) and they downregulate Fpk1/2p. On the other hand, a complex sphingolipid stimulates Fpk1/2p, which phosphorylate the N-terminal non-catalytic domain of Ypk1p, and as a result counteracts the downregulation of Fpk1/2p by Ypk1p. These results suggest a mechanism to balance phospholipid asymmetry and sphingolipid content. Such a crosstalk between sphingolipids and phospholipid flippases was also previously proposed (*85*). Interestingly, Dnf1/2p have a motif similar to the Fpk1p-catalysed phosphorylation site in Ypk1p, and exactly it is phosphorylated by the recombinant Fpk1p (*84*). However, the physiological meaning of this phosphorylation should be examined.

Several lipids are suggested to regulate the activity of phospholipid flippases. Phosphatidylinositol-4-phosphate binds to the C-terminal tail of Drs2p homologous to a split PH domain and this binding is required for Drs2p activity (*57*). Interestingly, phosphoinositide-binding basic residues overlap a Gea2p-binding site (*57*), and recent proteomic analysis revealed the interaction of Drs2p with Sac1p, which predominantly acts as a phosphatidylinositol-4-phosphatase (*25*). As described earlier, *DRS2* and an oxysterol-binding protein-encoding *KES1* exhibit the interesting genetic interaction (*67*). Disruption of *KES1* can suppress a temperature-sensitive *drs2* allele in the absence of Dnf P4-ATPases. Drs2p-dependent PS flippase activity is elevated in TGN membranes from *kes1Δ* cells and additional recombinant Kes1p reduces this activity to the wild-type level. These results suggest that Kes1p antagonizes Drs2p activity. Further analyses will clarify how these complicated interactions of Drs2p with lipids and proteins controls its activity

and vesicle formation. Additionally, a remaining and interesting question is whether the activity of other flippases such as Dnf1/2p is also regulated by some lipids.

Perspectives

Phospholipid asymmetry was first described in the plasma membrane. It was unknown whether internal membranes also show transbilayer phospholipid asymmetry due to the lack of available techniques. Localization study indicated that flippases are localized to early endosomes and TGN membranes in addition to the plasma membrane, suggesting that these membranes show phospholipid asymmetry, which is regulated by flippases. Recent development of a PS-specific probe, the C2 domain of lactadherin, has enabled us to observe PS distribution of internal membranes. Interestingly, in mammalian cells, GFP-Lact-C2 was localized to the plasma membrane and early endosomes, but not to other internal membranes, and this was essentially the case in yeast: GFP-Lact-C2 was localized to the plasma membrane, but not to internal membranes including Golgi, ER, mitochondria and vacuole (*86*; our unpublished data). Because flippases are localized to both the plasma membrane and the Golgi membrane, differential distribution of PS on the cytosolic surface of these membranes suggests that flippase activity is spatiotemporally regulated. P4-ATPases in the plasma membrane may be basically kept in an active state, whereas the activity of those in the Golgi membrane may be strictly regulated: they may become active only when flippase activity is required for vesicle formation. In addition, because phospholipid asymmetry should be dynamically regulated by flip and flop, proteins with floppase activity should also be considered. One candidate is an ABC transporter (*87*), but most of them are localized to the plasma membrane. An unknown phospholipid floppase might be involved in the regulation of phospholipid asymmetry in the Golgi membranes.

One of the most studied functions of flippases is to promote formation of transport vesicles from endosomal/TGN membranes. A next challenge is to reveal molecular mechanisms of how flippase-mediated phospholipid translocation is coupled with vesicle formation. One plausible mechanism is that flippases pump phospholipids to assist vesicle formation by inducing membrane curvature. This membrane curvature should be efficiently recognized and captured by adaptor proteins or their associated proteins; otherwise the membrane curvature would be easily dissolved by rapid lateral diffusion of transported phospholipids. Thus, physical linkage of a flippase with vesicle formation machinery would facilitate efficient formation of a transport vesicle.

Accumulating evidence indicates that flippases are involved in a variety of membrane-related cellular processes. How phospholipid translocation regulates these cellular functions should be revealed in future studies. Flippases could affect shape, fluidity, static electricity and lipid microenvironment of membranes through changes in phospholipid asymmetry. These physicochemical changes in membranes could in turn affect activity, localization and functions of membrane proteins. Thus, one important challenge would be identification and functional analysis of such proteins that are directly regulated by changes in phospholipid asymmetry.

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Conflict of interest

None declared.

References

1. Devaux, P.F. (1991) Static and dynamic lipid asymmetry in cell membranes. *Biochemistry* **30**, 1163–1173
2. Zachowski, A. (1993) Phospholipids in animal eukaryotic membranes: transverse asymmetry and movement. *Biochem. J.* **294**, 1–14
3. Pomorski, T., Hrafnsdóttir, S., Devaux, P.F., and van Meer, G. (2001) Lipid distribution and transport across cellular membranes. *Semin. Cell Dev. Biol.* **12**, 139–148
4. Seigneuret, M. and Devaux, P.F. (1984) ATP-dependent asymmetric distribution of spin-labeled phospholipids in the erythrocyte membrane: relation to shape changes. *Proc. Natl. Acad. Sci. USA* **81**, 3751–3755
5. Daleke, D.L. and Lyles, J.V. (2000) Identification and purification of aminophospholipid flippases. *Biochim. Biophys. Acta* **1486**, 108–127
6. Zachowski, A., Henry, J.P., and Devaux, P.F. (1989) Control of transmembrane lipid asymmetry in chromaffin granules by an ATP-dependent protein. *Nature* **340**, 75–76
7. Moriyama, Y. and Nelson, N. (1988) Purification and properties of a vanadate- and *N*-ethylmaleimide-sensitive ATPase from chromaffin granule membranes. *J. Biol. Chem.* **263**, 8521–8527
8. Moriyama, Y., Nelson, N., Maeda, M., and Futai, M. (1991) Vanadate-sensitive ATPase from chromaffin granule membranes formed a phosphoenzyme intermediate and was activated by phosphatidylserine. *Arch. Biochem. Biophys.* **286**, 252–256
9. Tang, X., Halleck, M.S., Schlegel, R.A., and Williamson, P. (1996) A subfamily of P-type ATPases with aminophospholipid transporting activity. *Science* **272**, 1495–1497
10. Kühlbrandt, W. (2004) Biology, structure and mechanism of P-type ATPases. *Nat. Rev. Mol. Cell Biol.* **5**, 282–295
11. Siegmund, A., Grant, A., Angeletti, C., Malone, L., Nichols, J.W., and Rudolph, H.K. (1998) Loss of Drs2p does not abolish transfer of fluorescence-labeled phospholipids across the plasma membrane of *Saccharomyces cerevisiae*. *J. Biol. Chem.* **273**, 34399–34405
12. Marx, U., Polakowski, T., Pomorski, T., Lang, C., Nelson, H., Nelson, N., and Herrmann, A. (1999) Rapid transbilayer movement of fluorescent phospholipid analogues in the plasma membrane of

endocytosis-deficient yeast cells does not require the Drs2 protein. *Eur. J. Biochem.* **263**, 254–263
13. Chen, C.Y., Ingram, M.F., Rosal, P.H., and Graham, T.R. (1999) Role for Drs2p, a P-type ATPase and potential aminophospholipid translocase, in yeast late Golgi function. *J. Cell Biol.* **147**, 1223–1236
14. Hua, Z., Fatheddin, P., and Graham, T.R. (2002) An essential subfamily of Drs2p-related P-type ATPases is required for protein trafficking between Golgi complex and endosomal/vacuolar system. *Mol. Biol. Cell* **13**, 3162–3177
15. Pomorski, T., Lombardi, R., Riezman, H., Devaux, P.F., van Meer, G., and Holthuis, J.C. (2003) Drs2p-related P-type ATPases Dnf1p and Dnf2p are required for phospholipid translocation across the yeast plasma membrane and serve a role in endocytosis. *Mol. Biol. Cell* **14**, 1240–1254
16. Saito, K., Fujimura-Kamada, K., Furuta, N., Kato, U., Umeda, M., and Tanaka, K. (2004) Cdc50p, a protein required for polarized growth, associates with the Drs2p P-Type ATPase implicated in phospholipid translocation in *Saccharomyces cerevisiae*. *Mol. Biol. Cell* **15**, 3418–3432
17. Natarajan, P., Wang, J., Hua, Z., and Graham, T.R. (2004) Drs2p-coupled aminophospholipid translocase activity in yeast Golgi membranes and relationship to *in vivo* function. *Proc. Natl. Acad. Sci. USA* **101**, 10614–10619
18. Alder-Baerens, N., Lisman, Q., Luong, L., Pomorski, T., and Holthuis, J.C. (2006) Loss of P4 ATPases Drs2p and Dnf3p disrupts aminophospholipid transport and asymmetry in yeast post-Golgi secretory vesicles. *Mol. Biol. Cell* **17**, 1632–1642
19. Zhou, X. and Graham, T.R. (2009) Reconstitution of phospholipid translocase activity with purified Drs2p, a type-IV P-type ATPase from budding yeast. *Proc. Natl. Acad. Sci. USA* **106**, 16586–16591
20. Coleman, J.A., Kwok, M.C., and Molday, R.S. (2009) Localization, purification, and functional reconstitution of the P4-ATPase Atp8a2, a phosphatidylserine flippase in photoreceptor disc membranes. *J. Biol. Chem.* **284**, 32670–32679
21. Kato, U., Emoto, K., Fredriksson, C., Nakamura, H., Ohta, A., Kobayashi, T., Murakami-Murofushi, K., and Umeda, M. (2002) A novel membrane protein, Ros3p, is required for phospholipid translocation across the plasma membrane in *Saccharomyces cerevisiae*. *J. Biol. Chem.* **277**, 37855–37862
22. Hanson, P.K., Malone, L., Birchmore, J.L., and Nichols, J.W. (2003) Lem3p is essential for the uptake and potency of alkylphosphocholine drugs, edelfosine and miltefosine. *J. Biol. Chem.* **278**, 36041–36050
23. Furuta, N., Fujimura-Kamada, K., Saito, K., Yamamoto, T., and Tanaka, K. (2007) Endocytic recycling in yeast is regulated by putative phospholipid translocases and the Ypt31p/32p-Rcy1p pathway. *Mol. Biol. Cell* **18**, 295–312
24. Lenoir, G., Williamson, P., Puts, C.F., and Holthuis, J.C. (2009) Cdc50p plays a vital role in the ATPase reaction cycle of the putative aminophospholipid transporter Drs2p. *J. Biol. Chem.* **284**, 17956–17967
25. Puts, C.F., Lenoir, G., Krijgsveld, J., Williamson, P., and Holthuis, J.C. (2010) A P4-ATPase protein interaction network reveals a link between aminophospholipid transport and phosphoinositide metabolism. *J. Proteome Res.* **9**, 833–842
26. Poulsen, L.R., López-Marqués, R.L., McDowell, S.C., Okkeri, J., Licht, D., Schulz, A., Pomorski, T., Harper, J.F., and Palmgren, M.G. (2008) The *Arabidopsis* P4-ATPase ALA3 localizes to the golgi and requires a beta-subunit to function in lipid translocation and secretory vesicle formation. *Plant Cell* **20**, 658–676
27. López-Marqués, R.L., Poulsen, L.R., Hanisch, S., Meffert, K., Buch-Pedersen, M.J., Jakobsen, M.K., Pomorski, T.G., and Palmgren, M.G. (2010) Intracellular targeting signals and lipid specificity determinants of the ALA/ALIS P4-ATPase complex reside in the catalytic ALA alpha-subunit. *Mol. Biol. Cell* **21**, 791–801
28. Katoh, Y. and Katoh, M. (2004) Identification and characterization of CDC50A, CDC50B and CDC50C genes in silico. *Oncol. Rep.* **12**, 939–943
29. Osada, N., Hashimoto, K., Hirai, M., and Kusuda, J. (2007) Aberrant termination of reproduction-related TMEM30C transcripts in the hominoids. *Gene* **392**, 151–156
30. Xu, P. and Ding, X. (2007) Characterization and expression of mouse Cdc50c during spermatogenesis. *Acta Biochim. Biophys. Sin.* **39**, 739–744
31. Paulusma, C.C., Folmer, D.E., Ho-Mok, K.S., de Waart, D.R., Hilarius, P.M., Verhoeven, A.J., and Oude Elferink, R.P. (2008) ATP8B1 requires an accessory protein for endoplasmic reticulum exit and plasma membrane lipid flippase activity. *Hepatology* **47**, 268–278
32. Folmer, D.E., van der Mark, V.A., Ho-Mok, K.S., Oude Elferink, R.P., and Paulusma, C.C. (2009) Differential effects of progressive familial intrahepatic cholestasis type 1 and benign recurrent intrahepatic cholestasis type 1 mutations on canalicular localization of ATP8B1. *Hepatology* **50**, 1597–1605
33. Paulusma, C.C., de Waart, D.R., Kunne, C., Mok, K.S., and Elferink, R.P. (2009) Activity of the bile salt export pump (ABCB11) is critically dependent on canalicular membrane cholesterol content. *J. Biol. Chem.* **284**, 9947–9954
34. Wang, L., Beserra, C., and Garbers, D.L. (2004) A novel aminophospholipid transporter exclusively expressed in spermatozoa is required for membrane lipid asymmetry and normal fertilization. *Dev. Biol.* **267**, 203–215
35. Gong, E.Y., Park, E., Lee, H.J., and Lee, K. (2009) Expression of Atp8b3 in murine testis and its characterization as a testis specific P-type ATPase. *Reproduction* **137**, 345–351
36. Perez-Victoria, F.J., Sanchez-Canete, M.P., Castanys, S., and Gamarro, F. (2006) Phospholipid translocation and miltefosine potency require both *L. donovani* miltefosine transporter and the new protein LdRos3 in *Leishmania* parasites. *J. Biol. Chem.* **281**, 23766–23775
37. Geering, K. (2001) The functional role of beta subunits in oligomeric P-type ATPases. *J. Bioenerg. Biomembr.* **33**, 425–438
38. Noji, T., Yamamoto, T., Saito, K., Fujimura-Kamada, K., Kondo, S., and Tanaka, K. (2006) Mutational analysis of the Lem3p-Dnf1p putative phospholipid-translocating P-type ATPase reveals novel regulatory roles for Lem3p and a carboxyl-terminal region of Dnf1p independent of the phospholipid-translocating activity of Dnf1p in yeast. *Biochem. Biophys. Res. Commun.* **344**, 323–331
39. Anthonisen, A.N., Clausen, J.D., and Andersen, J.P. (2006) Mutational analysis of the conserved TGES loop of sarcoplasmic reticulum Ca^{2+}-ATPase. *J. Biol. Chem.* **281**, 31572–31582
40. Donaldson, J.G. and Jackson, C.L. (2000) Regulators and effectors of the ARF GTPases. *Curr. Opin. Cell Biol.* **12**, 475–482
41. Hua, Z. and Graham, T.R. (2003) Requirement for Neo1p in retrograde transport from the Golgi complex

to the endoplasmic reticulum. *Mol. Biol. Cell* **14**, 4971–4983
42. Wicky, S., Schwarz, H., and Singer-Krüger, B. (2004) Molecular interactions of yeast Neo1p, an essential member of the Drs2 family of aminophospholipid translocases, and its role in membrane trafficking within the endomembrane system. *Mol. Cell. Biol.* **24**, 7402–7418
43. Gall, W.E., Geething, N.C., Hua, Z., Ingram, M.F., Liu, K., Chen, S.I., and Graham, T.R. (2002) Drs2p-dependent formation of exocytic clathrin-coated vesicles in vivo. *Curr. Biol.* **12**, 1623–1627
44. Sakane, H., Yamamoto, T., and Tanaka, K. (2006) The functional relationship between the Cdc50p-Drs2p putative aminophospholipid translocase and the Arf GAP Gcs1p in vesicle formation in the retrieval pathway from yeast early endosomes to the TGN. *Cell Struct. Funct.* **31**, 87–108
45. Liu, K., Hua, Z., Nepute, J.A., and Graham, T.R. (2007) Yeast P4-ATPases Drs2p and Dnf1p are essential cargos of the NPFXD/Sla1p endocytic pathway. *Mol. Biol. Cell* **18**, 487–500
46. Foote, C. and Nothwehr, S.F. (2006) The clathrin adaptor complex 1 directly binds to a sorting signal in Ste13p to reduce the rate of its trafficking to the late endosome of yeast. *J. Cell Biol.* **173**, 615–626
47. Valdivia, R.H., Baggott, D., Chuang, J.S., and Schekman, R.W. (2002) The yeast clathrin adaptor protein complex 1 is required for the efficient retention of a subset of late Golgi membrane proteins. *Dev. Cell* **2**, 283–294
48. Liu, K., Surendhran, K., Nothwehr, S.F., and Graham, T.R. (2008) P4-ATPase requirement for AP-1/clathrin function in protein transport from the trans-Golgi network and early endosomes. *Mol. Biol. Cell* **19**, 3526–3535
49. Zimmermann, J., Chidambaram, S., and Fischer von Mollard, G. (2010) Dissecting Ent3p: the ENTH domain binds different SNAREs via distinct amino acid residues while the C-terminus is sufficient for retrograde transport from endosomes. *Biochem. J.* **431**, 123–134
50. Singer-Krüger, B., Lasić, M., Bürger, A.M., Hausser, A., Pipkorn, R., and Wang, Y. (2008) Yeast and human Ysl2p/hMon2 interact with Gga adaptors and mediate their subcellular distribution. *EMBO J.* **27**, 1423–1435
51. Sheetz, M.P. and Singer, S.J. (1974) Biological membranes as bilayer couples. A molecular mechanism of drug-erythrocyte interactions. *Proc. Natl. Acad. Sci. USA* **71**, 4457–4461
52. Robinson, M., Poon, P.P., Schindler, C., Murray, L.E., Kama, R., Gabriely, G., Singer, R.A., Spang, A., Johnston, G.C., and Gerst, J.E. (2006) The Gcs1 Arf-GAP mediates Snc1,2 v-SNARE retrieval to the Golgi in yeast. *Mol. Biol. Cell* **17**, 1845–1858
53. Beck, R., Sun, Z., Adolf, F., Rutz, C., Bassler, J., Wild, K., Sinning, I., Hurt, E., Brügger, B., Béthune, J., and Wieland, F. (2008) Membrane curvature induced by Arf1-GTP is essential for vesicle formation. *Proc. Natl. Acad. Sci. USA* **105**, 11731–11736
54. Krauss, M., Jia, J.Y., Roux, A., Beck, R., Wieland, F.T., De Camilli, P., and Haucke, V. (2008) Arf1-GTP-induced tubule formation suggests a function of Arf family proteins in curvature acquisition at sites of vesicle budding. *J. Biol. Chem.* **283**, 27717–27723
55. Horvath, C.A., Vanden Broeck, D., Boulet, G.A., Bogers, J., and De Wolf, M.J. (2007) Epsin: inducing membrane curvature. *Int. J. Biochem. Cell Biol.* **39**, 1765–1770
56. Chantalat, S., Park, S.K., Hua, Z., Liu, K., Gobin, R., Peyroche, A., Rambourg, A., Graham, T.R., and Jackson, C.L. (2004) The Arf activator Gea2p and the P-type ATPase Drs2p interact at the Golgi in *Saccharomyces cerevisiae*. *J. Cell Sci.* **117**, 711–722
57. Natarajan, P., Liu, K., Patil, D.V., Sciorra, V.A., Jackson, C.L., and Graham, T.R. (2009) Regulation of a Golgi flippase by phosphoinositides and an ArfGEF. *Nat. Cell Biol.* **11**, 1421–1426
58. Peyroche, A., Courbeyrette, R., Rambourg, A., and Jackson, C.L. (2001) The ARF exchange factors Gea1p and Gea2p regulate Golgi structure and function in yeast. *J. Cell Sci.* **114**, 2241–2253
59. Spang, A., Herrmann, J.M., Hamamoto, S., and Schekman, R. (2001) The ADP ribosylation factor-nucleotide exchange factors Gea1p and Gea2p have overlapping, but not redundant functions in retrograde transport from the Golgi to the endoplasmic reticulum. *Mol. Biol. Cell* **12**, 1035–1045
60. Wiederkehr, A., Avaro, S., Prescianotto-Baschong, C., Haguenauer-Tsapis, R., and Riezman, H. (2000) The F-box protein Rcy1p is involved in endocytic membrane traffic and recycling out of an early endosome in *Saccharomyces cerevisiae*. *J. Cell Biol.* **149**, 397–410
61. Chen, S.H., Chen, S., Tokarev, A.A., Liu, F., Jedd, G., and Segev, N. (2005) Ypt31/32 GTPases and their novel F-box effector protein Rcy1 regulate protein recycling. *Mol. Biol. Cell* **16**, 178–192
62. Iwamoto, K., Kobayashi, S., Fukuda, R., Umeda, M., Kobayashi, T., and Ohta, A. (2004) Local exposure of phosphatidylethanolamine on the yeast plasma membrane is implicated in cell polarity. *Genes Cells* **9**, 891–903
63. Saito, K., Fujimura-Kamada, K., Hanamatsu, H., Kato, U., Umeda, M., Kozminski, K.G., and Tanaka, K. (2007) Transbilayer phospholipid flipping regulates Cdc42p signaling during polarized cell growth via Rga GTPase-activating proteins. *Dev. Cell* **13**, 743–751
64. Riekhof, W.R. and Voelker, D.R. (2006) Uptake and utilization of lyso-phosphatidylethanolamine by *Saccharomyces cerevisiae*. *J. Biol. Chem.* **281**, 36588–36596
65. Riekhof, W.R., Wu, J., Gijón, M.A., Zarini, S., Murphy, R.C., and Voelker, D.R. (2007) Lysophosphatidylcholine metabolism in *Saccharomyces cerevisiae*: the role of P-type ATPases in transport and a broad specificity acyltransferase in acylation. *J. Biol. Chem.* **282**, 36853–36861
66. Kishimoto, T., Yamamoto, T., and Tanaka, K. (2005) Defects in structural integrity of ergosterol and the Cdc50p-Drs2p putative phospholipid translocase cause accumulation of endocytic membranes, onto which actin patches are assembled in yeast. *Mol. Biol. Cell* **16**, 5592–5609
67. Muthusamy, B.P., Raychaudhuri, S., Natarajan, P., Abe, F., Liu, K., Prinz, W.A., and Graham, T.R. (2009) Control of protein and sterol trafficking by antagonistic activities of a type IV P-type ATPase and oxysterol binding protein homologue. *Mol. Biol. Cell* **20**, 2920–2931
68. Folmer, D.E., Elferink, R.P., and Paulusma, C.C. (2009) P4 ATPases - lipid flippases and their role in disease. *Biochim. Biophys. Acta* **1791**, 628–635
69. Ujhazy, P., Ortiz, D., Misra, S., Li, S., Moseley, J., Jones, H., and Arias, I.M. (2001) Familial intrahepatic cholestasis 1: studies of localization and function. *Hepatology* **34**, 768–775
70. Cai, S.Y., Gautam, S., Nguyen, T., Soroka, C.J., Rahner, C., and Boyer, J.L. (2009) ATP8B1 deficiency disrupts the bile canalicular membrane bilayer structure

in hepatocytes, but FXR expression and activity are maintained. *Gastroenterology* **136**, 1060–1069
71. Paulusma, C.C., Groen, A., Kunne, C., Ho-Mok, K.S., Spijkerboer, A.L., Rudi de Waart, D., Hoek, F.J., Vreeling, H., Hoeben, K.A., van Marle, J., Pawlikowska, L., Bull, L.N., Hofmann, A.F., Knisely, A.S., and Oude Elferink, R.P. (2006) Atp8b1 deficiency in mice reduces resistance of the canalicular membrane to hydrophobic bile salts and impairs bile salt transport. *Hepatology* **44**, 195–204
72. Pawlikowska, L., Groen, A., Eppens, E.F., Kunne, C., Ottenhoff, R., Looije, N., Knisely, A.S., Killeen, N.P., Bull, L.N., Elferink, R.P., and Freimer, N.B. (2004) A mouse genetic model for familial cholestasis caused by ATP8B1 mutations reveals perturbed bile salt homeostasis but no impairment in bile secretion. *Hum. Mol. Genet.* **13**, 881–892
73. Groen, A., Kunne, C., Paulusma, C.C., Kramer, W., Agellon, L.B., Bull, L.N., and Oude Elferink, R.P. (2007) Intestinal bile salt absorption in Atp8b1 deficient mice. *J. Hepatol.* **47**, 114–122
74. Groen, A., Kunne, C., Jongsma, G., van den Oever, K., Mok, K.S., Petruzzelli, M., Vrins, C.L., Bull, L., Paulusma, C.C., and Oude Elferink, R.P. (2008) Abcg5/8 independent biliary cholesterol excretion in Atp8b1-deficient mice. *Gastroenterology* **134**, 2091–2100
75. Stapelbroek, J.M., Peters, T.A., van Beurden, D.H., Curfs, J.H., Joosten, A., Beynon, A.J., van Leeuwen, B.M., van der Velden, L.M., Bull, L., Oude Elferink, R.P., van Zanten, B.A., Klomp, L.W., and Houwen, R.H. (2009) ATP8B1 is essential for maintaining normal hearing. *Proc. Natl. Acad. Sci. USA* **106**, 9709–9714
76. Verhulst, P.M., van der Velden, L.M., Oorschot, V., van Faassen, E.E., Klumperman, J., Houwen, R.H., Pomorski, T.G., Holthuis, J.C., and Klomp, L.W. (2010) A flippase-independent function of ATP8B1, the protein affected in familial intrahepatic cholestasis type 1, is required for apical protein expression and microvillus formation in polarized epithelial cells. *Hepatology* **51**, 2049–2060
77. Xu, P., Okkeri, J., Hanisch, S., Hu, R.Y., Xu, Q., Pomorski, T.G., and Ding, X.Y. (2009) Identification of a novel mouse P4-ATPase family member highly expressed during spermatogenesis. *J. Cell Sci.* **122**, 2866–2876
78. Lyssenko, N.N., Miteva, Y., Gilroy, S., Hanna-Rose, W., and Schlegel, R.A. (2008) An unexpectedly high degree of specialization and a widespread involvement in sterol metabolism among the *C. elegans* putative aminophospholipid translocases. *BMC Dev. Biol.* **8**, 96
79. Darland-Ransom, M., Wang, X., Sun, C.L., Mapes, J., Gengyo-Ando, K., Mitani, S., and Xue, D. (2008) Role of *C. elegans* TAT-1 protein in maintaining plasma membrane phosphatidylserine asymmetry. *Science* **320**, 528–531
80. Ruaud, A.F., Nilsson, L., Richard, F., Larsen, M.K., Bessereau, J.L., and Tuck, S. (2009) The *C. elegans* P4-ATPase TAT-1 regulates lysosome biogenesis and endocytosis. *Traffic* **10**, 88–100
81. Seamen, E., Blanchette, J.M., and Han, M. (2009) P-type ATPase TAT-2 negatively regulates monomethyl branched-chain fatty acid mediated function in post-embryonic growth and development in *C. elegans*. *PLoS Genet.* **5**, e1000589
82. Gomès, E., Jakobsen, M.K., Axelsen, K.B., Geisler, M., and Palmgren, M.G. (2000) Chilling tolerance in *Arabidopsis* involves ALA1, a member of a new family of putative aminophospholipid translocases. *Plant Cell* **12**, 2441–2454
83. Nakano, K., Yamamoto, T., Kishimoto, T., Noji, T., and Tanaka, K. (2008) Protein kinases Fpk1p and Fpk2p are novel regulators of phospholipid asymmetry. *Mol. Biol. Cell* **19**, 1783–1797
84. Roelants, F.M., Baltz, A.G., Trott, A.E., Fereres, S., and Thorner, J. (2010) A protein kinase network regulates the function of aminophospholipid flippases. *Proc. Natl. Acad. Sci. USA* **107**, 34–39
85. Kihara, A. and Igarashi, Y. (2004) Cross talk between sphingolipids and glycerophospholipids in the establishment of plasma membrane asymmetry. *Mol. Biol. Cell* **15**, 4949–4959
86. Yeung, T., Gilbert, G.E., Shi, J., Silvius, J., Kapus, A., and Grinstein, S. (2008) Membrane phosphatidylserine regulates surface charge and protein localization. *Science* **319**, 210–213
87. Daleke, D.L. (2003) Regulation of transbilayer plasma membrane phospholipid asymmetry. *J. Lipid Res.* **44**, 233–242
88. Weingärtner, A., Drobot, B., Herrmann, A., Sánchez-Cañete, M.P., Gamarro, F., Castanys, S., and Pomorski, T.G. (2010) Disruption of the lipid-transporting LdMT-LdRos3 complex in *Leishmania donovani* affects membrane lipid asymmetry but not host cell invasion. *PLoS One* **5**, e12443
89. Barbosa, S., Pratte, D., Schwarz, H., Pipkorn, R., and Singer-Krüger, B. (2010) Oligomeric Dop1p is part of the endosomal Neo1p-Ysl2p-Arl1p membrane remodeling complex. *Traffic* **11**, 1092–1106
90. Misu, K., Fujimura-Kamada, K., Ueda, T., Nakano, A., Katoh, H., and Tanaka, K. (2003) Cdc50p, a conserved endosomal membrane protein, controls polarized growth in *Saccharomyces cerevisiae*. *Mol. Biol. Cell* **14**, 730–747
91. Chen, S., Wang, J., Muthusamy, B.P., Liu, K., Zare, S., Andersen, R.J., and Graham, T.R. (2006) Roles for the Drs2p-Cdc50p complex in protein transport and phosphatidylserine asymmetry of the yeast plasma membrane. *Traffic* **7**, 1503–1517