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Phospholipid flippases: building asymmetric membranes and transport vesicles

Tessy T. Sebastian[*], **Ryan D. Baldridge**[*], **Peng Xu**, and **Todd R. Graham**
Department of Biological Sciences, Vanderbilt University, Nashville TN 37235, Phone: 615 343-1835, Fax: 615 343-6707

Todd R. Graham: tr.graham@vanderbilt.edu

Abstract

Phospholipid flippases in the type IV P-type ATPase family (P4-ATPases) are essential components of the Golgi, plasma membrane and endosomal system that play critical roles in membrane biogenesis. These pumps flip phospholipid across the bilayer to create an asymmetric membrane structure with substrate phospholipids, such as phosphatidylserine and phosphatidylethanolamine, enriched within the cytosolic leaflet. The P4-ATPases also help form transport vesicles that bud from Golgi and endosomal membranes, thereby impacting the sorting and localization of many different proteins in the secretory and endocytic pathways. At the organismal level, P4-ATPase deficiencies are linked to liver disease, obesity, diabetes, hearing loss, neurological deficits, immune deficiency and reduced fertility. Here, we review the biochemical, cellular and physiological functions of P4-ATPases, with an emphasis on their roles in vesicle-mediated protein transport.

1. Introduction

Newly synthesized secretory proteins travel from the endoplasmic reticulum (ER) through the Golgi complex to the *trans*-Golgi network (TGN) where they are packaged into exocytic carriers for delivery to the plasma membrane or extracellular space [1]. As a major sorting station in the secretory pathway, the TGN also segregates proteins destined for the endosomal/lysosomal system from exocytic cargo, thus making an essential contribution to the protein composition of lysosomes and endosomes. In fact, a remarkable number of distinct protein trafficking pathways, mediated by specific types of vesicles, transport proteins between the TGN, plasma membrane and endocytic/lysosomal compartments [2]. The small GTP-binding protein Arf and its ArfGEF and ArfGAP regulators, along with clathrin and its various adaptor proteins are critically important and highly conserved components of the protein trafficking machinery in this system. The Golgi also plays an important role in establishing the appropriate composition and organization of lipids in the plasma membrane and internal organelles. For example, sphingolipids and glycosphingolipids are synthesized in the lumenal leaflet of late Golgi elements and glycerophospholipids are translocated to the cytosolic leaflet by phospholipid flippases to establish an asymmetric membrane structure [3].

[*]Denotes an equal contribution

The type IV P-type ATPases (P4-ATPases) are phospholipid flippases that not only establish membrane phospholipid asymmetry, but are also tightly coupled to vesicle-mediated protein transport in the Golgi and endosomal systems. P4-ATPases were first implicated in vesicular transport through studies in *Saccharomyces cerevisiae* [4–6], but more recent studies in *Arabidopsis thaliani*, *Caenorhabditis elegans* and mammalian tissue culture cells indicate that this function is conserved [7–10]. Precisely how and why phospholipid flippases are coupled to vesicle budding events is uncertain and remains an active area of investigation.

Most flippases in the P4-ATPase family are comprised of a catalytic α-subunit (the P4-ATPase) and a noncatalytic β-subunit in the Cdc50 family of integral membrane proteins [3]. The budding yeast flippase Drs2, for example, associates with Cdc50 and the complex must be formed before the newly synthesized flippase is allowed to leave the ER [11]. This arrangement is well conserved though evolution and several metazoan P4-ATPases are known to have a functional requirement for association with a Cdc50 homolog [12, 13].

In this review, we will trace the lines of evidence supporting the contention that P4-ATPases are phospholipid flippases. The transverse flip of specific phospholipid species from the exofacial to the cytosolic leaflet is an unusual activity for a P-type ATPase as members of this protein family are more famous for their roles in pumping ions or heavy metals across membranes [14]. How the P4-ATPases evolved such a different transport substrate is unclear. Only a single human disease, familial intrahepatic cholestasis, is currently known to result from a P4-ATPase deficiency [15]. However, studies in mice are beginning to illuminate additional physiological roles for mammalian P4-ATPases that will be discussed. We will also describe specific protein transport pathways linked to P4-ATPase activity and a model for how flippases may help establish the membrane curvature required to bud vesicles from Golgi and endosomal membranes. Other emerging topics from studies in budding yeast that will be addressed are regulatory mechanisms controlling flippase activity with connections to sterol, sphingolipid and phosphoinositide metabolism. We will conclude with a discussion of future directions for the phospholipid flippase field.

2. The P4-ATPase and Cdc50 family of proteins

The first P4-ATPase sequence to appear in the literature was Drs2 from budding yeast, although it was initially thought to be a Ca^{++} ATPase because these pumps were the closest homologs known at the time [16]. Soon thereafter, Drs2 homologs with greater similarity appeared in the newly sequenced yeast genome and bovine ATPase II (now known as ATP8A1) was cloned and found to be nearly 50% identical to Drs2 [17]. As additional eukaryotic genomes were sequenced, it became apparent that Drs2 and ATPase II were the founding members of a very large subgroup in the P-type ATPase superfamily. Axelson and Palmgren formalized this relationship in their phylogenetic analysis of the P-type ATPases and the Drs2/ATP8A1-related subgroup was categorized as type IV P-type ATPases (P4-ATPases) [18]. While the Axelson and Palmgren nomenclature is most commonly used, an alternative Transporter Classification (TC) system places the Drs2/ATP8A1-related subgroup in P-type ATPase family 8 [19]. The P4-ATPases are not found in prokaryotes, but are typically represented by multiple members in every sequenced eukaryotic genome.

An example of the phylogenetic relationship of P4-ATPase catalytic subunits from yeast, plants and animals, for which functional studies have been performed, is shown in Figure 1. A unified nomenclature has been established for mammalian P4-ATPases, which are designated ATP8A1 through ATP11C. The human genome expresses 14 members of this family and the mouse genome contains a 15th member, ATP8B5, not found in the human genome [10]. The nomenclature used in model systems is less consistent with six *Caenorhabditis elegans* P4-ATPases named TAT-1 through TAT-6 for *T*ransbilayer

*A*mphipath *T*ransporter, 12 *Arabidopsis thaliani* members named ALA1 through ALA12 for *A*minophospho*L*ipid *A*TPases, and five *Saccharomyces cerevisiae* P4-ATPases named Drs2 (*D*efect in *R*ibosome *S*ynthesis), Neo1 (*neo*mycin resistance) and Dnf1 through Dnf3 for *D*rs2 *N*eo1 *F*amily ATPases. Only three noncatalytic subunits in the CDC50 family are encoded by mammalian (CDC50A, CDC50B and CDC50C), yeast (Cdc50, Lem3 and Crf1) and *C. elegans* (W03G11.2, R08C7.2a (CHAT-1) and F20C5.4) genomes while *Arabidopsis* expresses five (ALIS1 - ALIS5) (Fig 2). The relatively small number of β-subunits relative to α-subunits can be accommodated because not all P4-ATPases appear to require a β-subunit (e.g. Neo1) and several α-subunits can share the same β-subunit in independent heterodimer pairs (Fig 2) [7, 8, 11–13, 20–24].

While no high-resolution structure for a P4-ATPase has been determined, the presence of well-conserved motifs found in all P-type ATPases suggests that they adopt the four-domain structure observed with the Ca^{++}, Na^{+}/K^{+} and H^{+} ATPases that have been crystallized [25]. These include the nucleotide-binding (N), phosphorylation (P), actuator (A) and membrane (M) domains (Fig. 2). Most of the P4-ATPases have relatively long N- and C-terminal cytosolic extensions that may form additional regulatory (R) domains and all of these pumps are predicted to have 10 transmembrane segments composing the membrane domain. The actuator domain folds from cytosolic sequences preceding the first transmembrane segment (TM1) and in the loop between TM2 and TM3, while the P and N domains are formed from the cytosolic loop between TM4 and TM5. The noncatalytic subunits are membrane proteins with two transmembrane segments separated by a large, glycosylated extracellular domain (Fig. 2).

For cation transporters, binding of ATP to the N-domain and the subsequent phosphorylation of an Asp residue in the P domain induces dramatic conformational transitions described as E1 to E2~P in the Post-Albers catalytic cycle [25]. Hydrolysis of the aspartyl-phosphate bond by sequences in the A domain allows the pump to relax back to the E1 conformation to complete this cycle. These conformational changes in the three cytosolic domains drive equally dramatic conformational changes in the substrate-binding membrane domain that pumps ions across the membrane against their electrochemical gradient. While it is likely that the P4-ATPases undergo similar E1–E2 conformational transitions, it is not known how these pumps couple ATP hydrolysis to translocation of phospholipid substrate across the membrane. Flipping of phospholipid has been a controversial activity for a P-type ATPase and so we provide below the lines of evidence that support this contention.

3. Evidence that the P4-ATPases are phospholipid flippases

The plasma membrane of living cells is an asymmetric structure with an uneven distribution of specific phospholipids between the inner (cytosolic) and outer (extracellular) leaflets. PS and PE, for example, are confined almost exclusively to the inner leaflet while PC and sphingolipids are enriched in the outer leaflet. This asymmetry creates different chemical environments on opposing membrane faces important for integral membrane protein function, interaction of soluble proteins with the membrane surface and cell-cell interactions [26]. In contrast, when bilayers are formed spontaneously from lipids suspended in water, the component phospholipids are evenly distributed between the two faces of the bilayer. The hydrophobic core of the membrane provides an energy barrier that prevents transverse movement of the hydrophilic headgroup, and so spontaneous flip/flop of component phospholipids like PC, PS or PE is a rare event in artificial membranes [27]. A low intrinsic rate of spontaneous phospholipid flip/flop allows plasma membrane asymmetry to be maintained with minimal energy input, but how is the ordered plasma membrane structure with PS and PE enriched in the cytosolic leaflet initially established?

The discovery of an energy-driven aminophospholipid translocase (APLT) activity in human erythrocytes provided the first mechanistic insight into how cells create an asymmetric plasma membrane structure [28]. Spin-labeled analogs of PS and PE incorporated into the extracellular leaflet are rapidly transported to the cytosolic leaflet of the erythrocytes, while spin-labeled PC remains in the extracellular leaflet. The selective transport of spin-labeled PS and PE to the cytosolic leaflet by the APLT suggested that this enzyme pumped endogenous PS and PE to establish asymmetry. The energy requirement for flipping the charged lipid headgroup across the hydrophobic membrane core is provided by ATP and vanadate inhibits translocation, providing the first hint that a member of the P-type ATPase family catalyzed this translocase activity. This study provided the first evidence for an APLT activity in human erythrocytes, but reports of an APLT activity in bovine chromaffin granules [29], platelets [30] and yeast [17, 31] followed.

The erythrocyte APLT is also able to flip unlabeled PS and PE to the inner leaflet as measured by changes in the shape of the cells [28, 32]. As described by the bilayer couple hypothesis, an unbalanced increase in the lipid number in one face of the membrane creates curvature in the direction of the leaflet with the greater surface area [33]. Incorporation of exogenous phospholipid into the extracellular face of the bilayer induces a dramatic outward bending of the membrane, and changes the erythrocyte shape from the normal discocyte shape to an echinocytic shape resembling a sea urchin. When a substrate of the APLT is incorporated into the membrane (PS or PE), erythrocytes rapidly revert from echinocytes back to discocytes, and then to crenated stomatocytes as phospholipid is pumped to the inner leaflet. However, if a non-substrate for the aminophospholipid translocase, such as PC, is incorporated into the outer leaflet, the cells retain their echinocyte shape [28, 32]. Importantly, these experiments demonstrated the potential of phospholipid flippases to induce curvature in biological membranes.

The identity of the APLT is still uncertain [34], although ATP8A1 remains the best candidate for the erythrocyte [35] and bovine chromaffin granule flippase [29]. This connection derived from the purification of a novel 115 kDa ATPase from bovine chromaffin granules, called ATPase II at the time, that is dramatically stimulated by PS (and PE to a minor extent) [36, 37]. A similar 110 – 120 kDa Mg^{++}-dependent ATPase purified from human erythrocytes was suspected to be homologous to ATPase II and responsible for the aminophospholipid translocase activity of the erythrocyte plasma membrane [38]. This ATPase was partially purified from human erythrocytes and reconstituted into proteoliposomes, which displayed a PS translocase activity [39]. However, the protein responsible for the reconstituted APLT activity was not identified.

Validation that ATPase II (ATP8A1) is a P-type ATPase occurred when the bovine chromaffin granule enzyme was cloned, a discovery that nucleated a dramatic expansion of the field as additional homologs were uncovered [17]. ATPase II was initially found to be homologous to the yeast protein Drs2, and soon after to ATP8B1 (FIC1)(See section 4-Roles of P4-ATPases in disease)[40]. Disruption of *DRS2* (*drs2Δ)* was reported to cause a defect in PS translocation at the plasma membrane of *S. cerevisiae* [17], although later reports suggested that Drs2 had a negligible impact on this activity [41, 42]. The discovery of protein trafficking defects in *drs2Δ* cells also raised concerns that the influence of Drs2 on the plasma membrane flippase activity was indirect (see section 5-Trafficking).

However, a number of subsequent genetic studies strongly supported a direct role for P4-ATPases in phospholipid translocation. In *S. cerevisiae*, Dnf1 and Dnf2 are required for the plasma membrane flippase activity towards NBD-labeled PC and PE [6]. Drs2 localizes to the TGN, and purified TGN membranes carrying a temperature-sensitive form of Drs2 contain an NBD-PS flippase activity that is inactivated at the nonpermissive temperature

[43]. Secretory vesicles exiting the TGN contain a flippase activity that is Drs2 and Dnf3-dependent [44] and all of the *DRS2* and *DNF* genes contribute to the establishment of membrane asymmetry [6, 45]. The *Arabidopsis* P4-ATPases ALA1, ALA2 and ALA3, and murine Atp8b5 (FetA) can support plasma membrane flippase activity when expressed in the yeast P4-ATPase mutants [8, 46]. In addition, overexpression of ATP8B1 in CHOK1 cells, results in an increased PS translocation at the plasma membrane [22, 47] (although see also [48]) and Atp8b1 deficiency in mice results in disruption of the membrane asymmetry in bile canalicular membranes [49, 50].

The P4-ATPase β subunits were independently identified in genetic screens for loss of membrane asymmetry or phospholipid translocase activity. For example, *LEM3/ROS3* (encoding a member of the Cdc50 family [51]) was identified in a screen for mutants exhibiting a loss of phosphatidylethanolamine asymmetry in *S. cerevisiae* [52], and *lem3Δ* mutants were also found to be deficient in the uptake of edelfosine, a toxic PC analog [53]. More recently, a forward genetic screen for loss of PS asymmetry in *C. elegans* uncovered mutations in a Cdc50 homolog (CHAT-1) as well as TAT-1 [7]. The connection of the Cdc50 family to membrane asymmetry was not immediately clear because it seemed unlikely that these proteins could transport lipid directly as they have only two transmembrane segments and no ATPase domain. However, the Tanaka group linked a series of observations showing similar phenotypes of P4-ATPase mutants and Cdc50 family mutants to discover the functionally essential interaction between P4-ATPases (alpha subunits) and the Cdc50 family members (beta subunits) [11]. In sum, these genetic studies clearly demonstrate that the P4-ATPases and their β subunits are necessary for phospholipid flippase activities detected in several different membranes, and for the establishment of plasma membrane asymmetry. However, it still remained formally possible that these influences of P4-ATPase mutation or overexpression on flippase activity and membrane asymmetry were indirect.

Only recently has biochemical evidence emerged indicating that the P4-ATPases directly catalyze a phospholipid flippase activity. Working independently, the Graham and Molday groups were able to purify two P4-ATPases to near homogeneity, Drs2 and ATP8A2, respectively, reconstitute the P4-ATPases into proteoliposomes and demonstrate *in vitro* flippase activities [54, 55]. These studies provided the first evidence that a P4-ATPase is sufficient to catalyze an ATP-dependent phospholipid flippase activity. In the Drs2 proteoliposomes, a sub-stoichiometric amount of the Cdc50 beta subunit was present, and it is unclear if the flippase activity was catalyzed by the heterodimers in the preparation or if Drs2 can translocate substrate in the absence of Cdc50 [55]. ATP8A2 associates with CDC50A, and the complex was purified by a dual affinity approach using monoclonal antibodies that recognize each subunit. The purified heterodimer retained a robust PS-stimulated ATPase activity and NBD-PS flippase activity in proteoliposomes [56]. However, the ATP8A2 monomer was not assayed to determine if it is capable of flipping phospholipid in the absence of CDC50A.

It is generally accepted that most P4-ATPases associate with a beta subunit and this interaction is required for their mutual export from the ER [8, 11–13, 22, 24]. Whether or not the β-subunit has additional roles in the trafficking, substrate transport, or regulation of flippase activity is still uncertain. Recent evidence with *Arabidopsis thaliana* and human P4-ATPase complexes suggest the beta subunit is not responsible for the substrate specificity or localization of the P4-ATPase heterodimer [13, 21]. However, with both yeast and human complexes, the beta subunit appears to preferentially bind the E2~P form of the P4-ATPases, suggesting an important role in the catalytic cycle [12, 57]. In addition, conditional mutant forms of Cdc50 have been isolated that retain the ability to bind Drs2, but show a loss of function *in vivo* at the nonpermissive temperature. This implies an

important role for the Cdc50-Drs2 interaction with the TGN-endosomal system [58]. By creating a series of chimeras between CDC50A and CDC50B, Coleman et al. found that the TM segments and ectoplasmic domain of CDC50A are critical for association with ATP8A2, and the N-terminal cytosolic domain had a significant influence on catalytic activity [56]. It appears that the β-subunit can modulate the activity of the α-subunit, but whether this is a regulatory interaction or an essential component of the pump is an important open question.

4. Influence of membrane asymmetry and flippases on metazoan physiology

The human diseases progressive familial intrahepatic cholestasis (FIC) and benign recurring intrahepatic cholestasis are caused by mutations in the *ATP8B1* gene [15]. These remain the only known human diseases that are caused by a P4-ATPase deficiency. However, ATP8A2 has been linked to neurological defects of a single patient [59] and a recent genome-wide association (GWAS) study suggests a link between an ATP10D variant and increased risk of myocardial infarction [60]. In addition, studies in mice, *Arabidopsis* and *C. elegans* are indicating critical roles of P4-ATPases in diverse physiological processes.

4A. ATP8A P4-ATPases

By pumping PS to the inner leaflet of the plasma membrane, P4-ATPases create a concentration gradient intrinsic to the membrane that can be used for signal transduction in a manner analogous to how ion gradients established by cation-pumping P-type ATPases are used. Regulated disruption of membrane asymmetry and exposure of PS plays an important signaling role in apoptosis and blood clotting [61]. One of the first events in apoptosis is the Ca^{2+}-dependent exposure of PS on the outer surface of dying cells. The exposed PS is an "eat-me" signal that is recognized by phagocytes such as macrophages, which engulf the cell corpse. In *C. elegans*, the *ATP8A1* ortholog *TAT-1* is critical for maintaining cell surface asymmetry of PS. In animals deficient for *tat-1*, PS is abnormally exposed on the cell surface of non-apoptotic cells, and these living cells are stochastically removed through a mechanism dependent on PSR-1, a PS-recognizing phagocyte receptor, and CED-1, which contributes to recognition and engulfment of apoptotic cells [62]. Thus, TAT-1 appears to function in preventing appearance of PS in the outer leaflet of plasma membrane, and inappropriate exposure of PS on the cell surface may result in removal of living cells by neighboring phagocytes. As described below (section 5A) *tat-1* mutants also exhibit protein trafficking defects in the endocytic pathway.

ATP8A1 is the best candidate for the aminophospholipid translocase in erythrocytes and platelets. Restriction of PS to the inner leaflet in these blood cells may protect against thrombosis as exposure of PS is known to stimulate clotting reactions. A Ca^{2+} influx into activated platelets induces a "scramblase" activity that exposes PS on the extracellular leaflet [61]. Recently, a protein called TMEM16F was linked to a calcium-dependent scramblase activity in a mouse B-cell line [63]. Moreover, mutations in the TMEM16F gene have been found in patients with Scott syndrome, a rare bleeding disorder characterized by the lack of scramblase activity [63, 64].

ATP8A2 is disrupted by a molecularly balanced translocation between chromosomes 10 and 13 in a single patient with severe mental retardation, suggesting that haploinsufficiency of ATP8A2 caused the neurological condition [59]. ATP8A2 is highly expressed in brain, testes and retina, and was also identified as a membrane protein enriched in the disc membranes of photoreceptor outer segment preparations by a proteomic study [54, 59]. Although loss-of-function phenotypes have not been characterized for the visual system, the

observation that ATP8A2 catalyzes ~50% of the total ATPase activity in the outer segment preparations suggests a high demand for PS translocation across the photoreceptor disc membranes [54]. This may be germane to a recent report showing opsin (or rhodopsin) can mediate energy-independent flip-flop of phospholipid when reconstituted into liposomes [65]. In order to maintain an enrichment of PS in the disc cytosolic leaflet, perhaps a robust ATP8A2 activity is needed to offset phospholipid "leak" caused by the high opsin concentration in this membrane.

4B. *ATP8B* P4-ATPases and cholestasis in humans

Mutations in *ATP8B1* cause progressive familial intrahepatic cholestasis type 1 (PFIC1) or a less severe form of the disease called benign recurrent intrahepatic cholestasis type 1 (BRIC) [15]. Patients with PFIC1 suffer from chronic intrahepatic cholestasis that progresses to severe, end-stage liver disease, and often requires liver transplantation during the first or second decade of life [66]. Cholestasis results from a disruption of bile flow from the liver, which normally aids digestion and absorption of lipids in the small intestine. Bile salts are actively secreted into a canalicular lumen surrounded by the apical membranes of adjacent hepatocytes. ATP8B1 localizes to the apical membrane of epithelial cells, including hepatocytes and bile duct epithelial cells (cholangiocytes) [47].

In a mouse model for PFIC1, *Atp8b1* deficiency disturbs canalicular membrane phospholipid asymmetry and decreases the resistance of the canalicular membrane to hydrophobic bile salts, as evidenced by enhanced biliary recovery of PS, cholesterol, and ectoenzymes. *Atp8b1* deficiency also impairs the transport of hydrophobic bile salts into bile. The loss of phospholipid asymmetry of the canalicular membrane may lead to enhanced extraction of cholesterol from the canalicular membrane, which subsequently impairs hepatobiliary transport of bile salts and cause cholestasis [50]. Besides this critical function in the liver, *Atp8b1* is abundantly expressed at the stereociliar membrane and *Atp8b1* deficiency causes hearing loss associated with progressive degeneration of the cochlear hair cells [67].

Atp8b3 is exclusively expressed in the testis and localized in the acrosomal region of mouse sperm cells. Although Atp8b3$^{-/-}$ male mice are fertile, their litter sizes are slightly smaller than wild-type controls and in vitro fertilization rates with Atp8b3$^{-/-}$ sperm is significantly reduced. These sperm also display abnormal PS exposure on the outer leaflet of the plasma membrane [68]. Mouse Atp8b5 (also named as FetA) is also specifically expressed in testis and its intracellular localization implies a role in acrosome formation, a Golgi-derived organelle [46]. The co-expression of Atp8b3 and Atp8b5 in the sperm acrosome suggests that they redundantly function in the acrosome reaction.

The Arabidopsis *ALA3 - ALA12* genes are closely related to the mammalian *ATP8A* and *ATP8B* P4-ATPases. Of this group, only ALA3 has been characterized and plants deficient for this P4-ATPase exhibit a defect in the growth of roots, pollen tubes and the patterning of trichomes [8, 69]. As described below (section 5), ALA3 is implicated in the formation of exocytic vesicles from the Golgi and this may underlie many of the mutant phenotypes. In *C. elegans*, *TAT-2* has a strong influence on viability of worms starved for sterol or monomethyl branched chain fatty acids. The *tat-2* mutations cause hypersensitivity to sterol deprivation [70], but suppress the growth arrest induced by depletion of the branched chain fatty acids [71].

4C. ATP9 P4-ATPases

The ATP9 subgroup contains the least well-understood members of the metazoan P4-ATPase family. Like Neo1 in yeast, ATP9A and 9B can exit the ER in the absence of

CDC50-related β subunits [11, 24]. ATP9A and 9B localize to the TGN, with a portion of ATP9A also localizing to endosomes [24]. However, loss of function phenotypes have not been reported, nor is the substrate of these potential flippases known. The *C. elegans TAT-5* gene appears to be ubiquitously expressed throughout the worm and RNAi knockdown is embryonically lethal [70]. *NEO1* is also an essential gene in *S. cerevisiae* [5], implying that *ATP9/NEO1/TAT-5* orthologs may be essential in all eukaryotes. Therefore, it is surprising that *Arabidopsis* appears to lack a member of this subgroup.

4D. Atp10 P4-ATPases and obesity

Atp10a (also called pfatp and Atp10c) has been implicated in type 2 diabetes associated with obesity. Mice heterozygous for a deletion of chromosome 7 that uncovers the *pink-eyed dilution (p)* locus and extends distally into *Atp10a* display diet-induced obesity, but only if the chromosomal deletion is inherited maternally [72]. Pups inheriting the chromosome deletion from the sire do not gain excessive weight. The Atp10a-deficient mice are hyperinsulinemic, insulin-resistant and have an altered insulin-stimulated response in peripheral tissues [73].

The mode of inheritance of diet-induced obesity suggests that *Atp10a* is only expressed from the maternally inherited chromosome. Both parental *Atp10a* transcripts are initially expressed in muscle and adipocytes, but after 12 weeks on a high fat diet, expression of the maternal allele in muscle tissue dramatically declines [74]. These studies suggest that Atp10a is subject to unique mode of imprinted gene silencing modulated by environmental conditions (high fat diet). The authors of this work suggested that a disruption in the trafficking or expression of Glut4, the insulin-responsive glucose transporter, may underlie the insulin resistance of peripheral tissues in *Atp10a* deficient mice [74].

ATP10D may also been linked to defects in lipid metabolism in mice and humans. A naturally occurring truncated variant of *Atp10d* might be linked to the fat-prone phenotype of C57BL/6 mice [75]. In addition, a recent genome-wide association study linked human *ATP10D* allele polymorphisms to variances in plasma levels of glucosylceramide and ceramide, which are associated with risk of myocardial infarction [60].

ALA1 from *Arabidopsis* appears to be most closely related to the ATP10 subgroup, although ectopic expression of *ALA1* in *Saccharomyces* complements the cold-sensitive growth defect of a *drs2Δ* mutant (homologous to the ATP8A group) [76]. Remarkably, knockdown of *ALA1* expression causes a cold-sensitive growth defect in plants [76]. These results imply a well-conserved role for the P4-ATPases in adaptation to growth at cold temperatures.

4E. Atp11 P4-ATPases and B-cell development

Recent work from two groups has uncovered a critical role for Atp11C in murine B cell development [77, 78]. Interestingly, the mutant mice also develop hepatocellular carcinoma, hyperbilirubinemia, anemia and cholestasis, suggesting important roles in liver homeostasis [77, 78]. In fact, dietary supplementation with cholic acid caused death of the Atp11c deficient mice within a few weeks, supporting an important role of Atp11c in bile export [79]. The extent of genetic overlap between Atp11c and Atp8b1 function has not yet been characterized, nor is the precise influence on B-cell development known.

5. Flippase influence on protein trafficking

P4-ATPases were first linked to protein trafficking when Drs2, and its chaperone, Cdc50, were discovered to genetically interact with Arf [4, 45, 80, 81]. Arf (ADP-ribosylation factor) is a small G-protein that cycles between GTP- and GDP-bound form through the

action of several guanine nucleotide exchange factors (ArfGEFs) and GTPase activating proteins (ArfGAPs). In the GTP-bound form, Arf mediates the binding of adaptor proteins and coat proteins, such as COPI and clathrin, to sites of vesicle formation [1]. The synthetic lethal interaction between Drs2 and Cdc50 with Arf suggested that these proteins were important players in vesicle budding pathways. *drs2Δ* is also synthetically lethal with clathrin temperature-sensitive alleles, but not COPI or COPII mutations, which implicated Drs2 in clathrin-mediated protein trafficking pathways [4].

Drs2 primarily localizes to the *trans*-Golgi network (TGN) and this is one of the sites from which clathrin-coated vesicles bud. The phenotypes exhibited by *drs2Δ* cells are similar to the defects observed in cells deficient in clathrin. Both mutants accumulate enlarged Golgi cisternae and late Golgi enzymes involved in proteolytically processing pro-α-factor are mislocalized in both *drs2Δ* and clathrin mutants. Furthermore, the *drs2Δ* cells are markedly deficient in clathrin-coated vesicles (CCVs) that can be purified from these cells [4]. The screen for mutants *d*efective in *r*ibosome *s*ynthesis that first identified *DRS2* may seem at odds with the roles described here in protein trafficking and membrane asymmetry. However, many mutants that exhibit defects in vesicle-mediated protein transport create membrane stress, which is sensed by the cell integrity pathway to downregulate ribosome production and attenuate translation [82, 83]. The defect in ribosome synthesis only appears after shifting *drs2Δ* cells to cold temperatures (below 20°C) for a few hours [16], while most of the Golgi defects are observed at any temperature [4, 84]. The localization of Drs2 to the TGN and observation that temperature-sensitive for function alleles of *DRS2* (*drs2-12* or *drs2-31*) cause a loss of vesicle formation within 30 minutes of temperature shift imply a direct role for Drs2 in vesicle budding [85].

There are many co-factors involved in the formation, budding and uncoating of clathrin-coated vesicles [86]. Most notably, adaptor proteins are essential in cargo selection and organizing the coat at sites of vesicle formation. In yeast, AP-1 (adaptor protein-1), a heterotetrameric complex composed of γ, β1, μ1, and σ1 adaptin subunits, is recruited by Arf and functions at the TGN and early endosomes. Another set of adaptor proteins, Gga1 and Gga2 (Golgi-localized, Gamma-ear containing, Arf-binding) bud clathrin-coated vesicles from the TGN that are targeted to the late endosome. The AP-3 tetrameric adaptor (composed of δ, β3, μ3 and σ3 adaptin subunits) also appears to bud from the TGN in an Arf-dependent, but clathrin-independent manner [3]. After a clathrin-coated vesicle buds, the yeast auxilin (Swa2) and Hsp70 (Ssa proteins) are required for clathrin disassembly and uncoating (Fig 3) [87, 88].

Drs2 and Cdc50 are required for bi-directional transport between the TGN and the early endosome in pathways mediated by AP-1/clathrin and an F-box protein called Rcy1 (Fig. 3) [5, 20, 89]. The phenotypes associated with loss of Drs2 or AP-1 function within cells are very similar. For example, chitin synthase III (Chs3) is thought to cycle between the TGN and early endosome and is exported to the plasma membrane at certain times in the cell cycle [90]. Mutation of exomer subunit genes, such as *CHS6*, prevents transport of Chs3 to the plasma membrane. But, when *chs6Δ* is combined with either *drs2Δ* or AP-1 subunit mutations, Golgi-endosomal trafficking is disrupted and Chs3 redirects to the plasma membrane and late endosome or vacuole [89, 90]. These results suggest that Drs2, AP-1 and clathrin mediate both anterograde and retrograde transport of this cargo between the TGN and early endosome [89].

Drs2 also appears to cycle between the TGN and early endosomes (Fig 3). Occasionally, some Drs2 molecules find their way to the plasma membrane, but multiple endocytosis signals ensure a quick return to the early endosome and TGN system [91]. When AP-1 is disrupted, wholesale re-routing of Drs2 to the plasma membrane is observed and Drs2 can

be held at this location with an endocytosis block. However, upon lifting the endocytosis block, Drs2 recycles through the endocytic pathway back to the TGN in the absence of AP-1 [89]. The role of AP-1, it seems, is to traffic Drs2 and Chs3 from the TGN to the early endosome, but unlike Chs3, Drs2 does not need AP-1 for the return trip. Because Drs2 is required to support Chs3 trafficking in AP-1 pathways, it seemed possible that the flippase activity may be required to support Drs2's own transport by AP-1. In fact, inactivation of Drs2 activity (by shifting a GFP-tagged temperature sensitive form of Drs2 to the nonpermissive temperature) caused re-routing of Drs2 to the plasma membrane, comparably to what was observed by inactivation of AP-1 [89].

These observations support the idea that the formation of AP-1/clathrin-coated vesicles requires Drs2 flippase activity. Remarkably, however, the Arf-dependent recruitment of AP-1 and clathrin to the TGN is not perturbed in *drs2Δ* cells [89]. Moreover, the enlarged Golgi cisternae that accumulate in *drs2Δ* cells lack budding profiles or highly curved tubular elements [4, 85], even though the coat proteins are present on the membrane. Thus, a simple model that Drs2 facilitates coat recruitment to drive vesicle budding is incorrect. It seems more likely that Drs2 imparts curvature to the membrane by pumping phospholipid to the cytosolic leaflet, thereby producing a surface that the clathrin coat can more easily deform [92].

Drs2 and Cdc50 are also critical for directing cargo from the early endosome back to the TGN in the "Rcy1 pathway" [5, 20] (Fig 3). The F-box protein Rcy1 can form a Skp1-cullin-F-box (SCF^{Rcy1}) complex [93] and Rcy1 is required for the early endosome to TGN trafficking of Snc1, an exocytic SNARE protein that cycles between the early endosome, TGN and plasma membrane [94, 95]. The SCF^{Rcy1} complex is an effector of the Rab proteins Ypt31 and Ypt32, and is proposed to ubiquitinate Snc1 to generate a signal for recycling out of the endosome [96]. Trafficking of Snc1 is unaffected by AP-1 disruption [89] and there is some indication that COPI might influence the Rcy1 pathway, although the vesicle coat acting in this pathway is unclear [97]. The sorting nexin complex Snx4/41/42 and the ArfGAP Gcs1 are also linked to this recycling pathway [81, 98]. The *drs2Δ* and *cdc50Δ* mutations disrupt retrograde trafficking of Snc1 from the early endosome to the TGN comparably to the *rcy1Δ* mutation. In addition, Cdc50 accumulates in the early endosomes of *rcy1* mutants and so Drs2-Cdc50 also appears to travel the Rcy1 pathway back to the TGN [20]. In fact, growth defects associated with *rcy1Δ* can be suppressed by overexpression of Drs2/Cdc50 and Snc1, implying that these proteins are the critical cargos in the Rcy1 pathway [20]. While Rcy1 interacts with Drs2-Cdc50 [20], it is not known if Rcy1 ubiquitinates this flippase to facilitate retrieval or acts by another mechanism.

The *C. elegans tat-1* and *chat-1* mutants (potentially orthologous to Drs2-Cdc50) also show a strong defect in cargo recycling through endosomes [7, 9], indicating that this function in protein trafficking is well-conserved through evolution. TAT-1 and CHAT-1 co-localize within intestinal cells to the plasma membrane (apical and basolateral) along with early/recycling endosomes. In fact, CHAT-1 decorates tubules on recycling endosomes that require RAB-10 for formation, and these tubules are exaggerated in an *rme-1* mutant (Eps15 homology domain (EHD) protein required for tubule scission) [7]. Intestinal cells of the *tat-1* or *chat-1* mutants accumulate large vacuolated structures bearing markers for late endosomes and lysosomes [7, 9]. In addition, several markers for the early and late endosomes, recycling endosomes and late Golgi show aberrant co-localization to aggregated vesicles. A number of proteins that normally recycle from the early endocytic pathway back to the plasma membrane, including human transferrin receptor, human IL-2 receptor a-chain and glucose transporter 1 (GLUT-1), are all trapped in abnormal endosomal intermediates in the mutants [7]. Moreover, the recycling endosome tubular extensions appear to be completely lost in the *tat-1* and *chat-1* mutants [7]. These data suggest that TAT-1 and

CHAT-1 help drive the tubular membrane extensions from recycling endosomes that enrich cargo for delivery back to the plasma membrane.

In addition to bidirectional transport between the TGN and early endosome, and recycling from endosomes back to the plasma membrane, P4-ATPases may also contribute to budding of exocytic vesicles from the TGN [85]. Budding yeast produce at least two classes of exocytic vesicles distinguished by density and cargo [99]. Inactivation of either Drs2 or clathrin prevents formation of the denser class that accumulates when actin assembly is perturbed [85]. It remains unclear if these Drs2-dependent exocytic vesicles actually bud from the TGN or an early endosome, and it is possible that they are analogous to the tubular carriers produced by recycling endosomes in *C. elegans*.

However, phenotypes exhibited by Arabidopsis *ala3* mutants suggest a defect in forming exocytic secretory vesicles directly from the TGN [8]. The peripheral columella cells in the root tip of wild-type plants display TGN cisternae with large, bulbous extensions filled with electron translucent slime polysaccharide. Secretory vesicles containing the slime polysaccharide derived from the Golgi are easily detected in the cytoplasm prior to their fusion to the plasma membrane. In contrast, the *ala3* columella cells have a complete absence of the distended TGN cisternae and a marked deficiency of secretory vesicles in the cytoplasm [8]. ALA3 also plays a critical role in trichome formation on leaves, which is a wonderful model for directional membrane growth and complex cell patterning. The trichome branch elongation defect in *ala3* plants may result from defects in membrane trafficking and/or regulation of the actin cytoskeleton. This is reminiscent of the polarized growth defects reported for *lem3Δ* mutants in budding yeast [100], and the loss of microvilli from the apical membrane of Caco-2 cells deficient for Atp8b1 [48].

Thus far, only Atp8b5 has been shown to have a role in protein trafficking in mammalian cells [46]. RNA interference of Atp8b5 in murine mastocytoma P815 cells causes the distension of Golgi cisternae and abrogates constitutive secretion. Interestingly, these secretory pathway defects are only observed at lower temperatures (33°C), perhaps because other P4-ATPases can compensate for the Atp8b5 deficiency at 37°C [46]. Alternatively, it is possible that Golgi membranes in Atp8b5 deficient cells are more sensitive to changes in membrane fluidity associated with reduced temperature.

In the budding yeast system, there is clear evidence for both overlapping and non-overlapping functions for the P4-ATPases [5]. The defects in bidirectional transport between the TGN and endosomes and exocytosis are observed in *drs2Δ* single mutants, in spite of the presence of four other P4-ATPases in these cells. Thus, Neo1 and the Dnf P4-ATPases cannot replace the critical function of Drs2 in these pathways. However, growth and protein trafficking defects associated with *drs2Δ* become more severe as additional *DNF* genes are knocked out. For example, neither a *drs2Δ* nor a *dnf1Δ* single mutant displays a defect in the AP-3-dependent transport of cargo to the vacuole. However, the *drs2Δ dnf1Δ* double mutant exhibits a substantial defect in sorting AP-3 cargo [5]. Thus, it appears that Drs2 and Dnf1 are interchangeable in this pathway. Neo1 and Dnf1 may also compensate for loss of Drs2 in delivering cargo from the Golgi to the late endosome, which is a GGA-dependent pathway [101–103]. In addition, the *dnf1Δ dnf2Δ* cells show a cold-sensitive endocytosis defect that exacerbated by addition of *drs2Δ* [6]. While cells are viable until all four *DRS2 DNF* genes are knocked out, the single *neo1Δ* mutation is lethal [5]. Thus, Neo1 has an essential role that cannot be replaced by Drs2 or Dnf proteins.

Neo1 appears to localize to both Golgi and endosomes and has been implicated in the COPI-dependent retrograde transport of cargo from the Golgi to the ER [101–103]. For example, the Rer1 protein rapidly cycles between the ER and Golgi, but COPI inactivation causes

mislocalization of Rer1 to downstream compartments [104]. Similarly, Neo1 inactivation of causes the same Rer1 mislocalization phenotype [101]. Additionally, Neo1 has been implicated in GGA-dependent trafficking of cargo from the TGN to the late endosome. GGA is recruited to the TGN membrane by associations with both Arf and the Arf-like protein Arl1, which forms a complex with Mon2 (a large scaffold protein) and Neo1 [102].

6. Regulation of flippase activity

The activity of budding yeast P4-ATPases appears to be tightly regulated and responsive to changes in membrane composition. The first regulatory mechanism to be described came from a genetic screen designed to identify positive regulators of Dnf1 and Dnf2. This screen identified a closely related pair of kinases named flippase protein kinase 1 and 2 (Fpk1, Fpk2; orthologs of $p70^{S6K}$) [105]. The *fpk1Δ fpk2Δ* double mutant exhibits many of the same phenotypes as the *dnf1Δ dnf2Δ* double mutant. Membrane asymmetry is perturbed and the *fpk1Δ fpk2Δ* cells are also deficient in flippase activity at the plasma membrane measured with fluorescent PC or PE derivatives. Protein trafficking defects observed in *fpk1Δ fpk2Δ* cells are consistent with a loss of Dnf1-Lem3 and Dnf2-Lem3 function. Moreover, Fpk1 directly phosphorylates the P4-ATPases in order of substrate preference: Dnf1=Dnf2 > Dnf3 > Drs2 > Neo1 [105]. But how does phosphorylation activate the flippases and what is being “sensed” by Fpk1 and Fpk2?

A recent study has uncovered a regulatory network of protein kinases controlling Fpk activity that is responsive to the sphingolipid composition of the membrane [106]. A nucleating discovery was that Ypk1 (ortholog of serum and glucocorticoid induced kinase (SGK)) directly phosphorylates Fpk1 and inhibits Fpk1 activity. Fpk1, in turn, phosphorylates Ypk1 and inhibits its kinase activity [106]. Several kinds of input into this tug of war between Ypk1 and Fpk1 can tip the balance in favor of one of the kinases. For example, sphingolipid long chain base stimulates Pkh1 (ortholog of human 3-phosphoinositide-dependent kinase (PDK1)) [107], which phosphorylates and activates Ypk1. Thus, conditions that increase sphingolipid long chain base (e.g. heat shock) should tip the balance in favor of Ypk1 and reduce flippase activity. On the other hand, mature glycosphingolipids stimulate Fpk1 activity by an unknown mechanism and should increase flippase activity [106]. These observations imply that a homeostatic mechanism is in place to ensure an appropriate organization of glycerophospholipid and sphingolipid in the plasma membrane.

The flippase activity of Drs2 is also subjected to complex regulatory mechanisms (Fig. 3). Genetic interactions suggested a relationship between Drs2 and phosphoinositol-4-phosphate (PI4P) produced by the Pik1 phosphatidylinositol 4-kinase [108]. Drs2 appears to have no influence on the production of PI4P, but ablation of PI4P by inactivation of Pik1 or destruction by a phosphoinositide phosphatase dramatically reduces Drs2 flippase activity in isolated TGN membranes [109]. A phosphoinositide binding site with preference for PI4P maps to a basic patch of residues within the C-terminal cytosolic tail of Drs2, and mutation of these residues abrogates Drs2 activity. In addition, the basic patch overlaps a binding site for Arf guanine nucleotide exchange factor (ArfGEF) and this interaction also stimulates Drs2 flippase activity [109]. By analogy to the plasma membrane calcium ATPase (PMCA1) regulation by calmodulin [110], the C-terminal tail of Drs2 is likely an autoinhibitory regulatory domain and binding interactions relieve the autoinhibition to stimulate activity. The C-terminal tail of Dnf1 and Dnf2 may also be a regulatory domain as Fpk1 phosphorylation sites (RXSLD) are found in this part of the protein [106].

Genetic studies also implied a relationship between yeast flippases, sterol metabolism and an oxysterol-binding protein called Kes1 (or Osh4)(Fig. 3) [84, 111]. Moreover, recombinant

Kes1 can potently downregulate the flippase activity of Drs2 in isolated Golgi membranes [84]. A recent study suggests that oxysterol binding proteins regulate phosphoinositide metabolism by stimulating Sac1, a PI4P phosphatase [112]. Thus, it is possible that Kes1 downregulation of Drs2 activity is mediated by the destruction of PI4P and the loss of this positive activator. Interestingly, Drs2 is also capable of downregulating Kes1 function [84], perhaps by creating a membrane structure that restricts the ability of Kes1 to extract sterol from the membrane.

Another exciting development from the Tuck laboratory is the observation that the endocytic regulator Numb binds to the C-terminus of an intestine-specific spliced isoform of TAT-1 [113]. This interaction appears to inhibit TAT-1 activity and is responsible for the ability of overexpressed Numb to inhibit endocytic recycling in intestinal cells. Surprisingly, knockdown of phosphatidylserine synthase was able to suppress endocytic recycling defects caused by loss of tat-1 or overexpression of Numb. One interpretation of this observation is that inappropriate PS accumulation in the lumenal leaflet of the *tat-1* mutant endosomes is inhibiting the budding of transport vesicles [113].

7. Concluding remarks and future directions

Based on what is currently known, the primary biochemical activity of the P4-ATPases is the unidirectional translocation of specific phospholipid species across the membrane bilayer. One major cell biological consequences of this activity is the generation of an asymmetric membrane structure with a high concentration of substrate lipids, such as PS and PE, in the cytosolic leaflet. The asymmetric membrane organization can influence the physical properties of the membrane, the binding of peripheral membrane proteins and likely impacts the activity of integral membrane proteins. Moreover, signals can be transduced by regulated disruption of the phospholipid concentration gradient allowing exposure of PS on the outer leaflet, an event linked to recognition of apoptotic cells by macrophages and blood clotting reactions.

A second important consequence of a unidirectional, ATP-dependent flippase activity is an increase in phospholipid number within the cytosolic leaflet relative to the exofacial leaflet. As first described by the bilayer couple hypothesis [114], this imbalance in surface area between the two leaflets should induce bending in the membrane towards the cytosol, a process that is essential to vesicle budding [115]. Indeed, flippases play a critical role in vesicle budding within the Golgi and endosomal systems of yeast, plants and animals. We favor a model whereby P4-ATPase flippase activity helps drive membrane curvature to support vesicle budding (see reference [92] for a more complete discussion of the potential influences of flippases on membrane curvature and vesicular trafficking). Many other types of transport proteins are also proposed to drive membrane curvature, including coat proteins (COPI, COPII and clathrin), small GTP binding proteins (Arf and Sar1), ENTH domain proteins, BAR domain proteins and lipid modifying enzymes [115, 116]. A significant challenge for the future is tease out the relative contributions of each of these membrane remodeling proteins on the vesicle budding process.

While the last decade has produced a dramatic expansion in the literature on P4-ATPases, many fundamental questions remain unanswered. On the biochemical front, how do P4-ATPases recognize and translocate phospholipid across the membrane? While atomic resolution mechanistic insight into substrate recognition and transport by cation transporting P-type ATPases is being achieved [25], it is not known if P4-ATPases transport phospholipid transport by the same mechanism. What is the precise role of the β-subunit in the catalytic cycle of the P4-ATPase? Does the β-subunit form part of the phospholipid translocation pathway or is it regulating the pump activity? It is becoming clear that P4-

ATPases are tightly regulated by phosphorylation and/or binding partners, but how is this regulation achieved and why is it important to modulate flippase activity? On the cellular level, how are the membrane transformation events in the late Golgi system - translocation of glycerophospholipid to the cytosolic leaflet, sphingolipid biosynthesis in the lumenal leaflet, and cholesterol loading - integrated through the regulatory mechanisms described above? The hypothesis that flippases induce curvature in the membrane to support vesicle budding requires formal testing, as does the degree to which the role of flippases in vesicle trafficking is conserved in mammals. Physiological studies of P4-ATPase knockout mice are in their infancy and it seems very likely that genome-wide association studies will continue to identify additional human diseases linked to P4-ATPase deficiency. We have every reason to expect that the next decade will witness an even greater expansion of the P4-ATPase field.

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Highlights

1. Evidence that type IV P-type ATPases (P4-ATPases) are phospholipid flippases.
2. Phospholipid flippases create membrane phospholipid asymmetry.
3. Phospholipid flippases play critical roles in vesicle-mediated protein transport in the secretory and endocytic pathways.
4. P4-ATPases are linked to many disease states.
5. Mechanisms for regulating flippase activity are being uncovered.

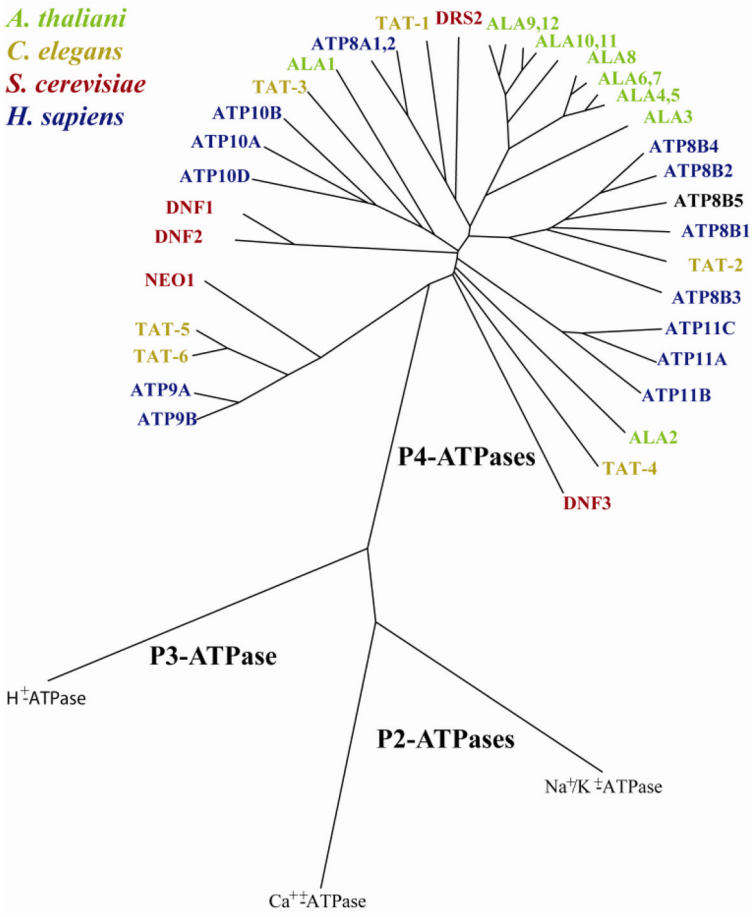
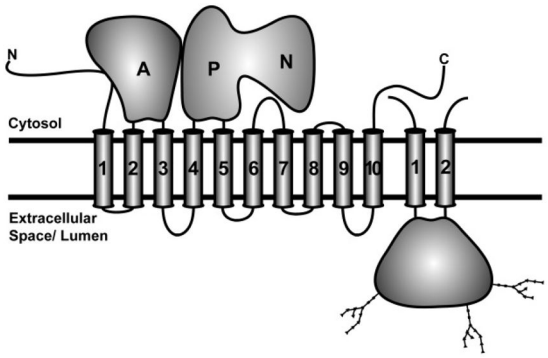


Figure 1.

Phylogeny of P4-ATPases from *Homo sapiens*, *Arabidopsis thaliana*, *Saccharomyces cerevisiae* and *Caenorhabditis elegans*. An alignment was produced from amino acid sequences using EXPRESSO(3DCOFFEE) rooted with the P2- and P3-ATPases with known structures. Phylogenetic distances were calculated using PHYLIP 3.67 and presented using Kitsch drawtree. ATP8B5 is the *Mus musculus* sequence because *Homo sapiens* lacks this P4-ATPase. Accession numbers for sequences used are available upon request.



α subunit	β subunit	Reference
Drs2	Cdc50	[4]
Dnf1	Lem3 (Ros3)	[4]
Dnf2	Lem3 (Ros3)	[20]
Dnf3	Crf1	[20]
Neo1	None detected	
TAT-1	CHAT-1	[10]
ALA2	ALIS1, ALIS3, ALIS5	[21]
ALA3	ALIS1, ALIS3, ALIS5	[11]
ATP8A1	CDC50A (also CDC50B?)	[5, 6]
ATP8A2	CDC50A	[6]
ATP8B1	CDC50A, CDC50B	[22]
ATP8B2	CDC50A, CDC50B	[5, 6]
ATP8B4	CDC50A (also CDC50B?)	[5, 6]
ATP9A	None detected	[24]
ATP9B	None detected	[24]
ATP10A	CDC50A	[24]
ATP10B	CDC50A	[24]
ATP10D	CDC50A	[24]
ATP11A	CDC50A	[24]
ATP11B	CDC50A	[24]
ATP11C	CDC50A	[24]
LdMT	LdRos3	[23]

Figure 2.

P4-ATPases form an α–β heterodimer. The predicted topology of the P4-ATPase (α subunit) and the Cdc50 family protein (β subunit) along with the experimentally defined αβ pairs is shown. A question mark indicates a discrepancy in the cited literature for Cdc50B.

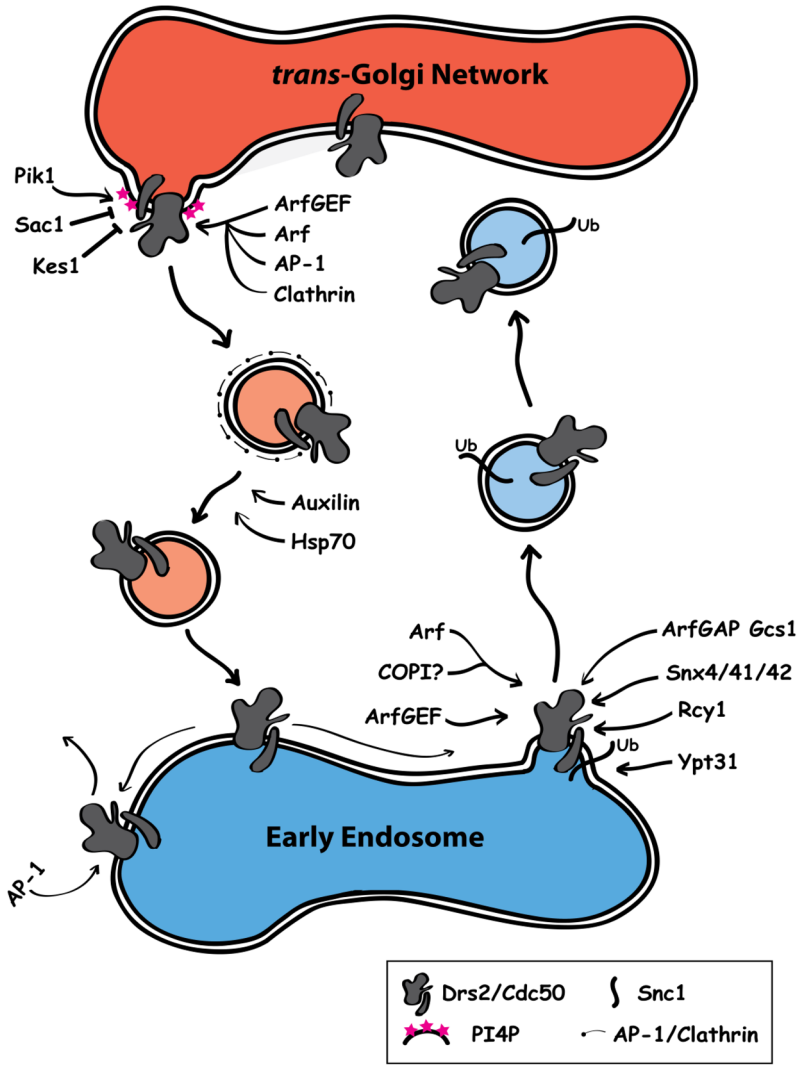


Figure 3.

The role of Drs2-Cdc50 and other factors in the formation and trafficking of transport vesicles between the late Golgi and the early endosome. The flippase activity of Drs2 is proposed to generate membrane curvature at sites of vesicle budding at the TGN. Arf, recruited to these sites by ArfGEF, mediates the recruitment of AP-1 and clathrin to the newly forming vesicle. Vesicle formation is controlled by various factors that regulate Drs2 flippase activity, including phosphatidylinositol-4-phosphate (PI4P), Sac1 (PI4P phosphatase), and Kes1 (oxysterol binding protein). At the early endosome, retrieval of Drs2-Cdc50 and the exocytic SNARE Snc1 back to the TGN is mediated by Rcy1 (F-box protein), an effector of the Ypt31 Rab protein, a sorting nexin complex (Snx4/41/42), and the Arf cycle, in particular the ArfGAP Gcs1.