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Review

P4 ATPases – The physiological relevance of lipid flipping transporters

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ABSTRACT

P4 ATPases are integral transmembrane proteins implicated in phospholipid translocation from the exoplasmic to the cytosolic leaflet of biological membranes. Our present knowledge on the cellular physiology of P4 ATPases is mostly derived from studies in the yeast *Saccharomyces cerevisiae*, where P4 ATPases play a pivotal role in the biogenesis of intracellular transport vesicles, polarized protein transport and protein maturation. In contrast, the physiological and cellular functions of mammalian P4 ATPases are largely unexplored. P4 ATPases act in concert with members of the CDC50 protein family, which are putative β-subunits for P4 ATPases. This review highlights the current status of a slowly emerging research field and emphasizes the contribution of P4 ATPases to the vesicle-generating machinery.
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1. Introduction

Lipid flippases are proteins that catalyze the transport of lipid molecules from the exoplasmic to the cytosolic leaflet of membrane bilayers. Lipid flippases are crucial for maintaining a non-randomized distribution of phospholipids over the two hemi-leaflets in many biological membranes. Already since the early 1970-ties it is known that phospholipids are non-randomly distributed in biological bilayers. In most eukaryotic cells, phosphatidylcholine (PC) and (glycero)sphingolipids are enriched in the exoplasmic leaflet, whereas the aminophospholipids phosphatidylserine (PS) and phosphatidylethanolamine (PE) are largely confined to the cytosolic leaflet of the endomembrane system [1–3]. Furthermore, the less abundant phospholipids, phosphatidylinositol (and derivatives) and phosphatidic acid, are also concentrated in the cytosolic leaflet [4–6]. An exception are the membranes of the endoplasmic reticulum in which the phospholipids are distributed randomly over the two leaflets. Phospholipids tend to equilibrate between the two leaflets of a bilayer (a process termed 'scrambling') at very slow rates (reviewed in [7]). However, due to extensive membrane fusion and budding events in the intracellular trafficking pathways, phospholipid scrambling is accelerated. Scrambling is of physiological importance for e.g. the activation of the blood coagulation, sperm capacitation, and the engulfment of apoptotic cells and can be accelerated by Ca^{2+}-regulated scramblases [8,9]; However, phospholipid randomization also interferes with membrane dynamics and may impair membrane-associated protein structure and function [10]. Thus, maintaining and dissipating the non-random distribution of phospholipids is crucial for normal regulated cell function, and requires the activity of proteins that are able to catalyze the intramembranous transport phospholipids. Lipid flippases and lipid floppases are ATP-dependent proteins that are implicated in the generation and preservation of the non-random distribution of phospholipids (reviewed in [11,12]). Lipid floppases transport lipids from the cytosolic to the exoplasmic leaflet of bilayers, and several members of the ATP-binding cassette (ABC) transporter protein family display such an activity [13]. The role of ABC transporters in lipid flopping will be discussed elsewhere in this issue. In 1984, the first ATP-dependent lipid flippase activity was identified in erythrocyte membranes [14]. In 1989, a second flippase activity was identified in Golgi-derived chromaffin granules from bovine adrenal glands, which would be required for the generation of fusion-competent membrane vesicles [15]. However, the gene(s) encoding these activities, and the nature of the protein(s) remained elusive. In 1996, the cDNA encoding the bovine chromaffin granule flippase, termed ATPaseII and presently known as ATP8A1, was isolated [16].

2. The P4 ATPase subfamily

ATP8A1 and its *Saccharomyces cerevisiae* ortholog Drs2p were identified as the first members of the type 4 subfamily of the P-type ATPase superfamily (abbreviated to P4 ATPase) (Fig. 1). Both proteins were identified as aminophospholipid flippases for their ability to translocate fluorescently-labeled PS (NBD-PS) [16]. P4

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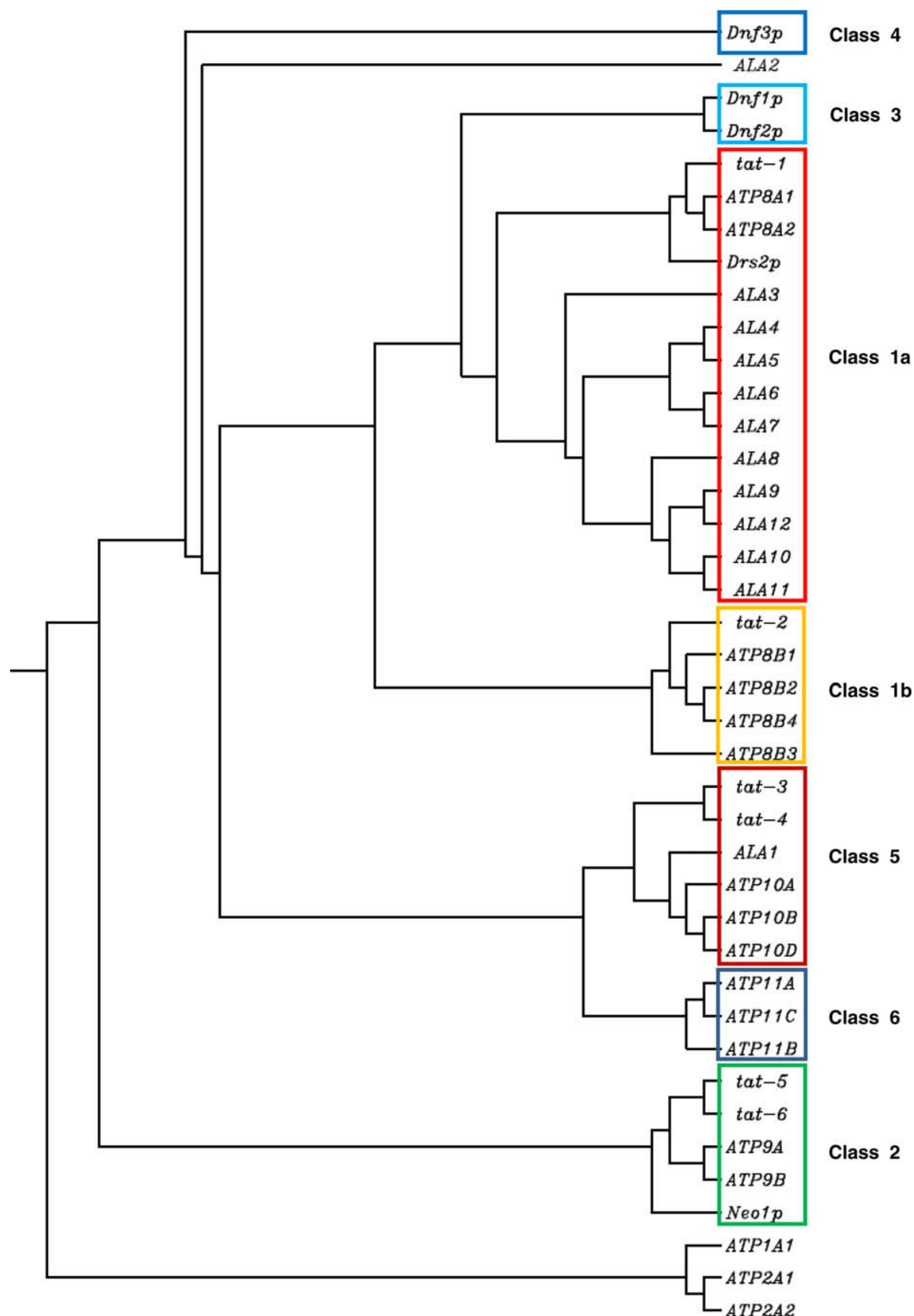

Fig. 1. Phylogenetic analyses of the P4 ATPase protein family of mammalian, *Arabidopsis thaliana*, *S. cerevisiae*, and *C. elegans* using ClustalW sequence alignment software (http://align.genome.jp/clustalw/). The P4 ATPases are subdivided into classes, based on amino acid consensus sequences [17,110]. ALA2 is closest related to Dnf3p (class 4) but does not contain any of the class-specific consensus sequences. Database accession numbers: *C. elegans*: tat-1 (NP_001022894), tat-2 (NP_001023252), tat-3 (NP_499363), tat-4 (NP_495244), tat-5 (NP_001021457), tat-6 (NP_503858); *Arabidopsis thaliana*: ALA1 (P98204), ALA2 (P98205), ALA3 (Q9XIE6), ALA4 (Q9LNQ4), ALA5 (Q9SGG3), ALA6 (Q9SLK6), ALA7 (Q9LVK9), ALA8 (Q9LK90), ALA9 (Q9SX33), ALA10 (Q9LI83), ALA11 (Q9SAF5), ALA12 (P57792); *S. cerevisiae*: Drs2p (P39524), Dnf1p (P32660), Dnf2p (Q12675), Dnf3p (Q12674), Neo1p (P40527). Human: ATP8A1 (P70704), ATP8A2 (P98200), ATP8B1 (O43520), ATP8B2 (P98198), ATP8B3 (O60423), ATP8B4 (Q8TF62), ATP9A (O75110), ATP9B (O43861), ATP10A (O60312), ATP10B (O94823), ATP10D (Q9P241), ATP11A (P98196), ATP11B (Q9Y2G3), ATP11C (Q8NB49). Type 2 (non-P4) ATPase accession numbers: ATP1A1 (P05023), ATP2A1 (O14983), ATP2A2 (P16615).

ATPases are exclusively expressed in eukaryotic cells and are deviant from the other P-type ATPase subfamilies in that they are implicated in the transport of phospholipids rather than in the transport of cations [12,17,18]. Studies in *S. cerevisiae*, *Arabidopsis thaliana*, and *Caenorhabditis elegans* suggest an important role for P4 ATPases in the biogenesis of transport vesicles in the biosynthetic and endocytic pathways. In particular, the mammalian P4 ATPase subfamily is poorly studied. However, inactivation of one member causes severe human disease, while inactivation of some murine P4 ATPases suggests roles in fertility-related disorders, insulin resistance and obesity (reviewed in [19]). Recently, several groups have demonstrated an important chaperone function for members of the evolutionary conserved Cdc50 protein family. It is suggested that CDC50 proteins are β-subunits for P4 ATPases, analogous to the β-subunits for other P-type ATPases such as the Na,K-ATPase, and are important for regulation of trafficking and activity of the P4 ATPase. In this review, we will highlight the present knowledge of this slowly emerging and very exciting family of proteins.

3. P4 ATPases in *S. cerevisiae*

Most of our present knowledge on the cellular physiology of P4 ATPases is derived from studies in the yeast *S. cerevisiae*. Yeast cells express five P4 ATPases i.e. Drs2p, Dnf1p, Dnf2p, Dnf3p, and Neo1p that are pivotal for the biogenesis of intracellular transport vesicles in the biosynthetic and endocytic pathways (reviewed in [20]). Drs2p is the most extensively-studied P4 ATPase. Drs2p-deficient yeast cells have a defect in late Golgi function evidenced by impaired formation of clathrin-coated transport vesicles at the *trans*-Golgi network (TGN) and delayed posttranslational processing and transport of several secretory proteins [21,22]. Drs2p shuttles between the TGN and early endosomes in an AP-1/clathrin-dependent pathway [23]. Drs2p, which physically interacts with AP-1, is not essential for coat recruitment, since in Drs2p-deficient cells both AP-1 and clathrin are normally recruited to the TGN. However, Drs2p ATPase activity, and thus most likely the flipping of phospholipids, is essential for clathrin-coated vesicle formation [23]. Thus, although the initial binding of the AP-1/clathrin coat proteins does not require Drs2p activity, Drs2p-mediated lipid flipping possibly induces membrane curvature which facilitates the binding of additional (coat) proteins and drives the formation of a clathrin-coated vesicle at the TGN or endosome. There are more indications that Drs2p contributes to the vesicle-generating machinery. First, several studies have shown a genetic association between Drs2p and proteins of the trafficking machinery, including ADP-ribosylation factors (recruitment of coat protein complexes), Rab proteins (membrane docking), and proteins of the endocytic recycling machinery [23–26]. Secondly, and in addition to a direct physical interaction with AP-1 [23], Drs2p also interacts with the Arf guanine nucleotide-exchange factor Gea1p [27,28], and with the F-box protein Rcy1 (involved in recycling between endosomes and TGN) [24]. Thirdly, using a proteomics-based approach, Puts et al. [29] have identified nine new Drs2p-interacting proteins of which three proteins are involved in phosphoinositide metabolism. Phosphoinositides play a critical role in the regulation of membrane/protein trafficking in the biosynthetic and endocytic pathways, mainly via binding and activation of downstream target proteins (reviewed in [30]). In mammalian cells, PtdIns(4)P is essential for the recruitment of the clathrin coat protein machinery to the TGN, and thus for the initiation of clathrin-coated vesicle generation [31]. Recently, Natarajan et al. [28] have shown that Drs2p needs to interact with phosphoinositides, and in particular with PtdIns(4)P, to catalyze NBD-PS flipping across isolated TGN membranes. All these observations suggest that Drs2p-catalyzed phospholipid flipping is critical for the vesicle-generating protein machinery at the TGN. In addition, the different subcellular distributions of the newly identified Drs2p-interacting proteins (i.e. Golgi apparatus (GA), ER, plasma membrane, and vacuole) suggest that Drs2p resides in different protein complexes within distinct trafficking pathways [29]. The physiological substrate for Drs2p is presently not known. Drs2p flips NBD-PS and NBD-PE (but not NBD-PC) across isolated membranes of the TGN and of post-Golgi secretory vesicles, suggesting that Drs2p-mediated flipping is headgroup-specific [32,33]. Drs2p-deficient cells display enhanced binding of PS-specific annexin V and are sensitive to the PS-binding drug papuamide B, which suggests that Drs2p mediates the flipping of natural PS at the plasma membrane [34]. Using temperature-sensitive Drs2p mutants (Drs2p-ts), Chen et al. [34] showed that acute inactivation of Drs2p did not result in PS exposure within 4 h after inactivation. In contrast, NBD-PS translocation in isolated Drs2p-ts TGN membranes was inactivated already after 1 h [33], and already within 30 min after inactivation these mutants displayed impaired vesicle formation at the TGN [21]. Thus, plasma membrane exposure of PS in Drs2p-deficient most likely is a consequence of the defect in Drs2p-mediated protein trafficking to the plasma membrane [34]. Furthermore, natural PS seems an unlikely in vivo substrate since PS is not essential for the biogenesis of transport vesicles at the TGN: yeast mutants that completely lack PS synthesis are not impaired in clathrin-coated vesicle generation at the TGN [33]. This does not exclude the possibility that PS is relevant for vesicle generation; In the complete absence of PS, other, low-affinity phospholipids (e.g. PE) may substitute the contribution of PS. Reconstitution experiments of Drs2p into proteoliposomes again indicate that Drs2p is a flippase specific for NBD-PS [35]. The authors did not study NBD-PE flipping in this system.

Less is known about the other four P4 ATPases. Dnf1p and Dnf2p are mainly plasma membrane-associated and cycle between early endosome and plasma membrane and early endosome and TGN [22,36]. Dnf1p and Dnf2p double mutant cells have a defect in the internalization step of fluid-phase endocytosis (to which Dnf2p deletion was the major contributor); this phenotype was aggravated when also Drs2p was deleted [36]. In these triple mutants, receptor-mediated endocytosis is also severely impaired. In addition, Drs2p and Dnf1p double mutants are impaired in the TGN-to-vacuole transport of alkaline phosphatase [22]. The endocytosis defects coincide with impaired ATP-dependent and non-endocytic uptake of NBD-PE, NBD-PC, NBD-PS, and radiolabeled lyso-PC and lyso-PE [36–38]. Uptake of NBD-labeled sphingoid-based lipids was not impaired, which indicates that the translocation defect is specific for glycerophospholipids [36]. Importantly, double and triple mutants exposed small amounts of natural PE and PS in the plasma membrane [34,36]. Although this justifies the speculation that Dnf1p and Dnf2p flip glycerophospholipids to initiate (endocytic) membrane vesiculation, the substrate specificities are not known. In addition, and in contrast to Drs2p, no Dnf1p or Dnf2p binding partners have been identified yet that connect these plasma membrane P4 ATPases to the vesicle-generating machinery.

Dnf3p predominantly localizes to the TGN and early endosomes and, together with Drs2p, in post-Golgi secretory vesicles [22,32,36]. Post-Golgi secretory vesicles of Drs2p-Dnf3p double mutant cells are impaired in the NBD-PS, PE, and PC translocation [32]. NBD-PE flipping in both single mutants is still present. Together with the observation that the asymmetric PE distribution (normally present in wild-type post-Golgi secretory vesicles) is dissipated in Drs2p-Dnf3p double mutant cells, this suggests that PE can be the natural substrate for Drs2p and/or Dnf3p.

Neo1p is the only essential P4 ATPase in yeast. This implicates that, in contrast to Drs2p and Dnfp1-3, there is no functional redundancy with the other P4 ATPases in the Neo1p pathway. Neo1p localizes to the ER and GA and is implicated in retrograde, COPI-dependent transport between GA and ER [20,39]. Neo1p mutant cells exhibit defects in secretory protein transport and protein glycosylation in the GA, phenotypes that have suggested to be secondary to the defect in retrograde transport [39]. In another study, Neo1p localizes to GA and endosomes [40]. In this study, Neo1p mutant cells display defects in receptor-mediated endocytosis, vacuolar protein sorting, and vacuole biogenesis. In contrast to the other four P4 ATPases, there is presently no experimental data to support a lipid flippase function for Neo1p. Interestingly, the *NEO1* gene was named as such because overexpression of Neo1p conferred resistance to neomycin [41]. At present, the relation between neomycin resistance and Neo1p expression is not clear. It is of interest to mention that Goodyear et al. [42] recently showed that neomycin induces PS exposure (associated with membrane blebbing) in the apical membrane of sensory hair cells in the cochlea, as shown by fluorescent annexin V labeling. PS scrambling was caused by neomycin-induced PIP2 clustering which is a known trigger for phospholipid scrambling ([42] and references therein).

Interestingly, when neomycin was washed out, annexin V-labeled PS was rapidly endocytosed and redistributed throughout the cytoplasm in a myosin VI-dependent manner, the latter being a motor protein implicated in endocytosis [43]. In this light, it is of interest to note that deficiency of the mammalian P4 ATPase ATP8B1 causes hearing defects as a result of degeneration of sensory hair cells [44] (see below).

4. P4 ATPases in *Arabidopsis thaliana*

Studies in the plant *A. thaliana* also support a role for P4 ATPases in phospholipid flipping and vesicle biogenesis. The *Arabidopsis* genome encodes 12 P4 ATPases, termed ALA1-ALA12 (for aminophospholipid ATPase) [45,46]. Thus far, only ALA3 has been extensively characterized [47]. ALA3 is an *Arabidopsis* ortholog of Drs2p (42% amino acid identity) and localizes to the GA of peripheral columella cells in the tips of roots and shoots. *Ala3* mutant plants display impaired growth of roots and shoots, which is most likely caused by a defect in the release of the peripheral cell layer (an event required for normal root growth) [47]. Analogous to yeast Drs2p mutants, *Ala3*-deficient peripheral columella cells displayed reduced amounts of a specific class of TGN-derived secretory vesicles. These vesicles contain polysaccharides and enzymes required for cell wall breakdown and release of the peripheral cell layer. *Ala3* mutants also accumulated large vacuolar structures reminiscent of the GA-derived autophagosome-like vacuoles observed in Drs2p mutants [22,48]. ALA3 expressed in yeast Drs2p/Dnf1p/Dnf2p triple mutants stimulated plasma membrane flipping of NBD-PS, PE, and PC (but not of lyso-PLs), and reduced outer leaflet-exposed PS and PE [47,49]. Little is known for the other 11 ALA proteins. *Ala1* is expressed in many different tissues, however the subcellular localization of the protein is not known [45]. ALA1 expression in Drs2p mutant yeast cells complemented the defect in plasma membrane flipping of NBD-PS, which suggests a role for ALA1 in the GA. Furthermore, ALA1 stimulated the flipping of NBD-PS and NBD-PE in reconstituted microsomes. *Ala1* mutant plants displayed impaired growth only at chilling temperatures ($\leqslant$12 °C), however, the relation between this phenotype and a lipid flippase function for ALA1 remains to be demonstrated. ALA2 has recently been localized to the prevacuolar compartments of tobacco epidermal cells; ALA2 expression in yeast Drs2p/Dnf1p/Dnf2p triple mutants stimulated specifically NBD- and natural PS plasma membrane flipping [49].

5. P4 ATPases in *C. elegans*

The nematode *C. elegans* expresses 6 P4 ATPases, termed TAT-1–6 (for transbilayer amphipath transporter), of which most have different splice variants, and of which TAT-6 is a possible pseudogene of TAT-5 [50–52]. TAT-1 shares 40% amino acid identity with Drs2p and is expressed in neurons, muscle, epidermis, and intestine [51]. In the intestinal epithelial cells, a GFP fusion of TAT-1 localized to basolateral and apical membranes and to the endosomal and Golgi system [51,53]. Studies on the role of TAT-1 are not conclusive. Initially, TAT-1 was characterized as an aminophospholipid translocase required for PS exposure during the early stages of apoptosis as *Tat-1* deficient apoptotic cells did not expose PS [52]. However, these findings could not be reproduced by others. Data by Darland-Ransom et al. [53] suggested a function for TAT-1 in the maintenance of the asymmetric distribution of PS in the plasma membrane; *Tat-1* deletion (but not *tat-2* to *tat-6*) coincided with annexin V detectable PS exposure in (non-apoptotic) somatic cells and in germ cells of the gonads. Ruaud et al. [51] presented data that suggest a role for TAT-1 in the early steps of endocytosis in several cell types, including intestinal epithelial cells and oocytes, and in the biogenesis of lysosomes; *Tat-1* mutants displayed impaired endocytic uptake of fluid-phase markers by the intestine and of yolk by oocytes. Furthermore, *Tat-1* mutant intestinal epithelial cells were devoid of a certain class of storage granules (the formation of which requires lysosomal protein-1 (LMP-1)), and accumulate large LMP-1-positive pre-lysosomal vacuoles; this latter observation suggests a defect in a late phase process in the formation of these storage granules. *Tat-1* mutant epidermal cells accumulate large multivesicular bodies, which are intermediates in the biosynthetic- and endocytic (recycling) machinery and in the biogenesis of (endo)lysosomal compartments. Genetic studies in *Tat-2* mutant animals indicate a role for TAT-2 in the regulation of post-embryonic growth of *C. elegans*, predominantly via its activity in intestinal epithelial cells [54]; *Tat-2* depletion overcomes the growth inhibition observed in worms that are deprived from monomethyl branched-chain fatty acids. However, the relation between this phenotype and intestinal TAT-2 activity is presently not known. Whereas *Tat-5* is the only, ubiquitously expressed, essential gene, *Tat-1* to *Tat-4* are not essential for normal growth, development, and reproduction of *C. elegans* [55]. Interestingly, *Tat-2* and *Tat-4* mutants are growth-inhibited when deprived from exogenous sterol sources [55]. *C. elegans* ingests exogenous sterols in the intestine to convert it into 7-dehydroxysterol, which is metabolized to an essential reproductive hormone. It is presently not known what the contribution is of TAT-2 and TAT-4 in sterol metabolism. Although not the most likely explanation, these TAT proteins could mediate direct sterol uptake. Alternatively, TAT-2 and TAT-4 are important for maintaining the asymmetric distribution of phospholipids in biological membranes. *Tat-2* and/or *Tat-4* deficiency may compromise membrane structure, which impairs the activity of (a) sterol transporter(s) in the intestinal epithelial cells. Although both TAT proteins have overlapping expression patterns (including the intestine), the subcellular localization nor substrate specificities are known.

6. P4 ATPases in mammalian cells

The mammalian P4 ATPase subfamily consists of 14 proteins (excluding alternative splice variants) which all have orthologs in mice [12,17–19]). P4 ATPases are expressed in many different tissues, including various parts of the central nervous system ([17,56], and see e.g. Allen Brain Atlas Resources [Internet]. Seattle (WA): Allen Institute for Brain Science. Available from: http://www.brain-map.org. [57]). The first mammalian P4 ATPases that were biochemically studied were bovine and mouse isoforms of chromaffin granular-localized ATP8A1, which upon purification and reconstitution showed the highest activation of ATPase activity in PS-containing micelles [58,59]. However, PS translocation by ATP8A1 has not been demonstrated yet. In contrast to yeast P4 ATPases, the physiological and cellular functions of most mammalian P4 ATPases are largely unexplored. The importance of mammalian P4 ATPases is, however, underscored by a severe human liver disease caused by mutations in the P4 ATPase gene *ATP8B1*. Mutations in *ATP8B1* cause progressive familial intrahepatic cholestasis type 1 (PFIC1) and benign recurrent intrahepatic cholestasis type 1 (BRIC1), two liver disorders which are characterized by an impaired bile flow ([60], and reviewed in [61]). PFIC1 patients develop progressive, end-stage liver disease before adulthood, whereas BRIC1 patients suffer from episodes of disease that evolve and resolve spontaneously. ATP8B1 is expressed in apical membranes of many epithelial cells, including the canalicular membrane of hepatocytes [62,63]. The pathophysiological function of ATP8B1 has been studied in *Atp8b1*-mutant mice in which a severe PFIC1 patient mutation, resulting in a glycine to valine substitution at position 308 (G308V), was introduced ([64] and reviewed in

[19]). This mutation leads to near-absence of the protein, most likely due to the inability of the G308V to exit the ER [65,66]. ATP8B1-deficient canalicular membranes were prone to damage induced by detergent bile salts present in the canalicular lumen, evidenced by enhanced biliary output of canalicular ectoenzymes (alkaline phosphatase, Cd13), cholesterol, and PS [67,68]. The appearance of PS in bile suggests exposure of PS in the exoplasmic leaflet from which it is extracted by bile salts; Hence, this indirectly implies a role for ATP8B1 as a PS flippase. Enhanced cholesterol extraction from these membranes reduced the cholesterol to PC ratio >50% which in turn impaired the activity of several transporter proteins essential in bile formation [67,69]. From these observations it has been hypothesized that ATP8B1 is essential in the protection of the canalicular membrane to the solubilizing capacity of bile salts [69,70]. One of the protective mechanisms of this membrane, which is enriched in sphingolipids en cholesterol, is to maintain a rigid, liquid-ordered, state (reviewed in [71]). ATP8B1 maintains the asymmetric distribution of PS, which guarantees a liquid-ordered, detergent-resistant membrane.

Similar to many PFIC1 patients, ATP8B1-deficient mice suffer from hearing loss [44]. ATP8B1 is expressed in the apical region of the stereocilia of the cochlear hair cells in the Organ of Corti, in which (sound) pressure-induced fluid movements are converted into action potentials. Action potentials are generated by the activation of several ion channels in these stereocilia. ATP8B1 deficiency leads to a progressive degeneration of stereocilia and hair cells, thus affecting mechanotransduction. This phenotype closely resembles the hair cell degeneration induced by neomycin, which coincides with PS exposure (see above) [42]. From this it may be inferred that ATP8B1 deficiency in the apical membrane of hair cells leads to phospholipid scrambling (PS exposure), which causes progressive hair cell degeneration and subsequent hearing loss.

These observations suggest an essential function for ATP8B1 in preserving a stable membrane environment, most likely by maintaining the asymmetric distribution of phospholipids. Indeed, ATP8B1 overexpressed in CHO cells catalyzes enhanced plasma membrane flipping of NBD-PS and, more importantly, of natural PS [69,72]. Still, the cell physiological functions of ATP8B1 are poorly understood. For instance, it is not known whether ATP8B1 is a plasma membrane PS flippase that is directly involved in the maintenance of the asymmetric distribution of PS, or whether its activity is required for the generation of endocytic transport vesicles. In the latter case, ATP8B1 deficiency in hepatocytes would result in impaired (compensatory) endocytosis at the canalicular membrane, which may lead to canalicular membrane expansion and instabilization. In bile of ATP8B1-deficient humans and mice multilamellar and multivesicular structures accumulate, which may be shedded remnants of such an instable membrane [70,73]. Furthermore, in WIF-B9 cells, a polarized hepatoma cell line, GFP-tagged ATP8B1 localized to the apical membrane but also to Lamp-2-positive endosomal/lysosomal (and subapical) compartments, indicative for a role of ATP8B1 in the endomembrane system [72].

Murine ATP8B3 has several splice variants which are implicated in sperm cell acrosome formation and capacitation [74–76]. Capacitation is a cascade of membrane remodelling events that occurs in spermatozoa to prepare them for binding to and penetration of the zona pellucida. Capacitation is associated with PS exposure in the sperm cell head; In contrast to controls, *Atp8b3* knockout sperm cells exposed PS even before capacitation. In addition, *Atp8b3* knockout sperm cells displayed impaired in vitro fertilization, however, these mice did not have significantly reduced litter sizes [74]. The acrosome is a Golgi-derived organelle in the head of the sperm cell that contains digestive enzymes. The acrosome reaction is an exocytic event by which the sperm cell secretes a.o. hydrolases that digest the zona pellucida, in order to fuse with the oocyte. Knockdown of the ATP8B3 isoform, FetA, in a mouse mastocytoma cell line resulted in affected GA morphology and a defect in protein secretion from these cells [76]. Furthermore, the authors demonstrated FetA-catalized flipping of NBD-PE and PC.

Impaired P4 ATPase activity may also cause more general defects in lipid metabolism. For instance, mouse *atp10a* has been implicated in obesity, type 2 diabetes, and non-alcoholic fatty liver disease [77,78]. High-fat diet feeding of ATP10A-deficient mice resulted in hyperglycemia and enhanced insulin levels. These mice also showed affected expression of genes involved in insulin-stimulated glucose uptake, including Glut4. Glut4 is a glucose uptake transporter in myocytes and adipocytes. Upon activation of the insulin signalling pathway by insulin-receptor binding, Glut4 is translocated to the membrane where it mediates the uptake of glucose. The authors suggested that plasma membrane mobilization of Glut4-containing vesicles was affected in ATP10A deficient animals, however, this remains to be demonstrated. ATP10D has also been implicated in lipid metabolism. Of 15 inbred mouse strains only C57BL/6J contained a premature stop codon in the *Atp10d* gene [79]. Interestingly, C57BL/6J mice are predisposed to develop obesity, hyperglycemia, hyperinsulinemia, and hypertension when fed a high-fat diet [80]. However, the relation between these phenotypes and ATP10D deficiency is not known.

Recently, a significant association between the locus containing the *ATP8B4* gene with Alzheimer's disease has been reported [81]. One of the SNPs described localized very close to the *ATP8B4* gene on chromosome 15. This allows the hypothesis that mutations in *ATP8B4*, which is highly expressed in the brain, might play a role in Alzheimer's disease. However, this association remains to be replicated.

Very recently, the close homolog of ATP8A1, ATP8A2 has been biochemically characterized [82]. ATP8A2 is expressed in the retina and testis of mice. In the retina, the protein localizes to the disc membranes of the outer segments of the photoreceptor. The ATPase activity of affinity-purified ATP8A2 was stimulated by PS and PE. Reconstitution of the protein into NBD-labeled phospholipid-containing liposomes demonstrated flippase activity of the protein which was specific for NBD-PS. The authors conclude a role for ATP8A2 in the maintenance of PS asymmetry in the disc membranes of the photoreceptor. What the function of this protein is in the testis and whether ATP8A2 deficiency contributes to human disease is not known.

7. The Cdc50 protein family; β-subunits or chaperones for P4 ATPases?

P4 ATPases physically interact with members of the evolutionary conserved CDC50 protein family, present in a.o. yeast [83], plants [47], mammals [84], and *Leishmania* [85] (Fig. 2). CDC50 proteins are glycosylated 50–60-kDa proteins with two putative transmembrane domains and a large loop that protrudes into the exoplasmic space [83,86]. It is at present not clear whether CDC50 proteins function as β-subunits or as chaperones for P4 ATPases. In case of a β-subunit a stable, permanent interaction occurs whereas a chaperone only interacts transiently with the α-subunit during protein maturation and trafficking. CDC50 proteins structurally resemble the β- and γ-subunits of the Na,K-ATPase, which contribute to protein maturation, trafficking, and catalytic activity [46,87]. A physical P4 ATPase-CDC50 protein interaction is pivotal for exit of both proteins from the ER [49,72,88]. Such a mechanism is highly reminiscent of that for non-phospholipid translocating human P-type ATPases, including the Na,K- and H,K-ATPase [89]. Assembly of the α- and β-subunit of these pumps in the ER is essential for α-subunit maturation, ER exit, and modulation of protein activity [90,91]. Protein sorting signals are present

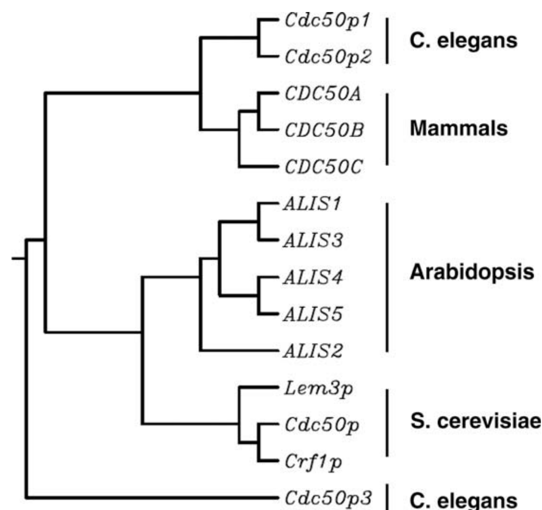

Fig. 2. Phylogenetic analyses of the CDC50 protein family of mammalian, *Arabidopsis thaliana*, *S. cerevisiae*, and *C. elegans* using ClustalW sequence alignment software (http://align.genome.jp/clustalw/). Putative CDC50 sequences from *C. elegans* are derived from the *C. elegans* protein database Wormpep based on sequence comparisons with all other CDC50 sequences (http://www.sanger.ac.uk/Projects/C_elegans/WORMBASE/current/wormpep.shtml). Database accession numbers: CDC50A (361 aa; Q9NV96), CDC50B (351 aa; Q3MIR4), CDC50C (373 aa; EAW79828 (non-reviewed sequence)), ALIS1 (350 aa; Q9LTW0), ALIS2 (343 aa; Q67YS6), ALIS3 (349 aa; Q9SLK2), ALIS4 (353 aa; Q9SA35), ALIS5 (350 aa; Q8L8W0), Lem3p (414 aa; P42838), Cdc50p (391 aa; P25656), Crf1p (393 aa; P53740), *C. elegans* Cdc50p1 (348 aa; Q21844), *C. elegans* Cdc50p2 (361 aa; Q19635), *C. elegans* Cdc50p3 (352 aa; Q23151).

in the both the α- and β-subunits [92,93]. In most epithelia, the Na,K-ATPase localizes to the basolateral membrane [94]. Three Na,K-ATPase β-subunits have been identified, β_1–β_3, of which β_1 and β_3 are associated with basolateral localization of the α-subunit [90,91]. However, α-subunits associated with a β_2-subunit localize to the apical membrane, as is the case in retinal pigment epithelium [95], prostate gland secretory epithelium [96], in the choroid plexus [97], and in polycystic kidney disease which is associated with aberrant expression of the β_2-subunit [98]. This unusual localization is due to the glycosylation status of the β_2-subunit [99]; The Na,K-ATPase β_2-subunit structurally resembles the H,K-ATPase β-subunit (the H,K-ATPase localizes to the apical membrane), in that they both have up till 9 *N*-glycosylation sites while β_1 and β_3 only have 3 *N*-glycosylation sites. This suggests an important function for the β-subunit in sorting of the protein complex. Although it is clear that a CDC50 protein-P4 ATPase physical interaction is pivotal for release of the P4 ATPase from the ER, the contribution of CDC50 proteins to subcellular trafficking, catalytic activity, and/or substrate specificity of the P4 ATPase is not clear.

The CDC50 protein family of yeast has first been described by Kato et al. [83]. Genetic studies suggested a strong correlation between P4 ATPases and CDC50 proteins, in that these mutant cells displayed similar phenotypes [21,22,36,83,100]. Saito et al. [88] were the first to demonstrate that CDC50 proteins were accessory proteins for P4 ATPases. Drs2p assembles with Cdc50p, while Dnf1p and Dnf2p assemble with Lem3p. A third homolog, Crf1p assembles with Dnf3p whereas Neo1p doesn't interact with neither of the three CDC50 homologs. These interactions are specific as shown by immunoprecipitation and split-ubiquitin assays [24,29,88,101].

In contrast to yeast CDC50 proteins, CDC50 proteins in *Arabidopsis*, termed ALIS proteins (for ALA-interacting subunit), did not display a specific preference for an ALA P4 ATPase. In *Arabidopsis*, five ALIS proteins (1–5) are expressed, of which ALIS1 is the most likely physiological binding partner of ALA3 [47]. Lopez-Marques et al. [49] studied the physiological functionality, subcellular localization, and substrate specificities of two P4 ATPases, ALA2 and ALA3, upon co-expression with 3 out of 5 ALIS proteins in Drs2p/Dnf1p/Dnf2p triple mutant yeast cells. When expressed in this triple mutant, both the ALA and ALIS proteins were released from the ER only upon co-expression. Independent of which ALIS protein was co-expressed with ALA2 (subcellular localization: prevacuolar compartment) or ALA3 (subcellular localization: GA), both P4 ATPases always displayed the same physiological functionality, substrate specificity, and subcellular localization. Instead, the P4 ATPases determined the subcellular localization of the different ALIS proteins. These observations indicate that the ALIS proteins do not determine the subcellular localization nor determine the substrate specificities of the P4 ATPase.

Studies in non-polarized mammalian cells do suggest a role for CDC50 proteins in subcellular trafficking of ATP8B1. In mammals, three CDC50 proteins are expressed (excluding alternative splice variants), and are termed CDC50A–C [12,18,84]. Co-expression of ATP8B1 with CDC50A or CDC50B in Chinese hamster ovary cells allowed exit of both proteins from the ER and trafficking to the plasma membrane and endosomal/lysosomal compartments, respectively [72], Folmer et al., manuscript in preparation). Similar to the β-subunits of the Na,K-ATPase [99], the *N*-glycosylation status of CDC50A (four *N*-glycosylation sites) and CDC50B (six *N*-glycosylation sites) may determine subcellular trafficking of P4 ATPases; however, targeting may be determined by the P4 ATPase itself and/or by other P4 ATPase-CDC50 interacting proteins. We anticipate that the three CDC50 proteins serve as accessory proteins for many, if not all, P4 ATPases. CDC50A and CDC50B are ubiquitously expressed while CDC50C is mainly expressed in testis and brain ([84,102,103] and see Allen Brain Atlas Resources available from: http://www.brain-map.org. [57]). The specificity of CDC50–P4 ATPase interactions is presently not known, although, based on overlapping expression profiles, it has been hypothesized that ATP8B3 and CDC50C and ATP8B1 and CDC50A are physiologically relevant binding partners [19,72,74,75,103].

For *C. elegans* no interactions with CDC50 proteins have been described yet. The *C. elegans* genome encodes at least three sequences with considerable homology to known members of the CDC50 protein family (Fig. 2).

One important question is whether CDC50 proteins are involved in the modulation of P4 ATPase activity. Thus far, no conclusive evidence has been presented that rejects this assumption. First, reconstituted, N-terminal-tagged Drs2p (but not of C-terminal tagged Drs2p) catalyzed the flipping of NBD-PS in proteoliposomes ([35]. However, in the Drs2p preparations additional proteins were present, including Cdc50p, which is consistent with a role for stimulating Drs2p-mediated flippase activity. Secondly, Lenoir et al. [104] presented data that Cdc50p is required in the reaction cycle of Drs2p; Cdc50p, but not Lem3p, stimulated Drs2p phosphoenzyme formation; In addition, the affinity of Cdc50p for Drs2p fluctuated, but was the highest during loading of the phospholipid substrate. Thirdly, the reconstituted ATP8A2 preparations (which catalyzed NBD-PS flipping) may have been contaminated with CDC50A [82], which is expressed in outer segments of the photoreceptor [105].

8. How do P4 ATPases drive vesiculation?

From the studies in yeast it is evident that P4 ATPases are pivotal in the formation of transport vesicles by flipping phospholipids from the exoplasmic to the cytosolic face of the bilayer. How does this process drive vesicle formation? The current models are based on the *bilayer couple* hypothesis [106]. The *bilayer couple* hypothesis proposes that if one leaflet of the bilayer expands e.g.

through a local increase in phospholipid or protein concentrations, the coupled leaflet follows, which leads to bending of the bilayer. The resulting membrane curvature can be the initiating event in the generation of vesicles. Farge et al. [107] have shown that the formation of endocytic vesicles could be enhanced when the cytosolic leaflet surface area was increased upon administration of PS and PE but not by lyso-alpha-phosphatidylserine (PS and PE were flipped into the cytosolic leaflet by a plasma membrane flippase, whereas lyso-alpha-phosphatidylserine was not a substrate for this plasma membrane flippase).

A *bilayer couple* hypothesis-based model is applicable for Drs2p-catalyzed clathrin-coated vesicle formation [86,108]. Drs2p mediates the translocation of phospholipids from the exoplasmic- to the cytosolic face of the TGN (or endosome), which results in a positive curvature of the membrane i.e. bending of the membrane towards the cytosol. In this way, Drs2p facilitates coat protein binding and mobilization of the vesicle-generating protein machinery. Similarly, Dnf1p and Dnf2p may be important for generating initial positive membrane curvature at the plasma membrane, thus facilitating coat protein binding and mobilization of the endocytic protein machinery. Alternatively, Dnf1p/Dnf2p may redistribute/concentrate specific phospholipid species, which triggers mobilization or release of the protein budding/fission machinery. The latter model is supported by findings in Lem3p mutant cells, which have a delayed apical-isotropic growth switch at the growing bud tip [109]. The apical-isotropic growth switch is triggered by the release of Cdc42p from the membrane after activation of its GTPase activity by the GTPase activating proteins (GAPs) Rga1p/2p. Rga1p/2p are activated by PS and PE [109]. During early budding stages, PE is locally concentrated in the exoplasmic leaflet at the apical growth site; In the corresponding cytosolic leaflet, Cdc42p is concentrated. The authors propose that Dnf1p- or Dnf2p-Lem3p-mediated PE flipping to the cytosolic leaflet activates GAP-mediated release of Cdc42p, which triggers the growth switch. However, also in this case, enhanced phospholipid concentration in the cytosolic leaflet can drive vesiculation and, as a consequence, release of Cdc42p via endocytosis.

9. Conclusions

From all the published work it is clear that P4 ATPases serve important functions in cellular physiology. First, P4 ATPases are critical initiating determinants of the vesicle-generating machinery and thus of polarized membrane and protein transport. Secondly, P4 ATPases are, either directly or indirectly, important in maintaining an optimal physical state of the (plasma) membrane, which is essential for proper membrane barrier- and membrane protein function.

In yeast, all five P4 ATPases are important in the biogenesis of transport vesicles in several intracellular vesicular transport routes. Also in plants and worms, there is emerging evidence for a role of P4 ATPase in the generation of transport vesicles. In mammalian cells, however, still very little is known about the roles of P4 ATPases in cellular physiology. When extrapolating the findings in yeast, plants and worms, mammalian P4 ATPases may also be implicated in the initiation of vesiculation. A role for mammalian P4 ATPases in vesicle biogenesis along different trafficking pathways would be especially relevant for epithelial cells and neuronal cells. In epithelial cells, for instance hepatocytes and enterocytes, major endocytic, exocytic, and transcytotic transport routes need to be maintained in order to efficiently facilitate excretion, uptake, and/or recycling of numerous substrates. In neuronal cells, extensive and rapid anterograde and retrograde vesicular transport is essential for proper signal propagation. For instance, newly synthesized neurotransmitters need to be transported from the cell body to the axon terminal, released into the synaptic cleft, and cleared from the synaptic cleft by endocytosis (or breakdown). Many P4 ATPases are abundantly expressed in the brain (See Allen Brain Atlas Resources available from: http://www.brain-map.org., and [17]). Also in liver and in the human hepatoma cell line HepG2, many P4 ATPases are expressed ([17], our unpublished results). How all these different proteins contribute to cellular homeostasis is not known. Impairment of functional cellular polarity e.g. by mutations in P4 ATPase genes may cause severe and complex human inherited diseases. One example of complex disease is associated with mutations in *ATP8B1*. Despite the severe liver phenotype, patients also suffer from other less severe phenotypes, including hearing loss, diarrhea, or abnormal sweat electrolyte concentrations. At present, however, it remains to be elucidated how ATP8B1 deficiency results in these different phenotypes. Although it is clear that ATP8B1 deficiency interferes with normal membrane function, a primary role in the vesicle-generating machinery cannot be excluded. It is a major challenge to elucidate the contribution of mammalian P4 ATPases in maintaining cellular homeostasis and to elucidate how P4 ATPase deficiencies contribute to human inherited diseases, such as ATP8B1 disease.

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