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Mammalian P₄-ATPases and ABC Transporters And Their Role in Phospholipid Transport

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Abstract

Transport of phospholipids across cell membranes plays a key role in a wide variety of biological processes. These include membrane biosynthesis, generation and maintenance of membrane asymmetry, cell and organelle shape determination, phagocytosis, vesicle trafficking, blood coagulation, lipid homeostasis, regulation of membrane protein function, apoptosis among others. P₄-ATPases and ATP binding cassette (ABC) transporters are the two principal classes of membranes proteins that actively transport phospholipids across cellular membranes. P₄-ATPases utilize the energy from ATP hydrolysis to flip aminophospholipids from the exocyttoplasmic (extracellular/lumen) to the cytoplasmic leaflet of cell membranes generating membrane lipid asymmetry and lipid imbalance which can induce membrane curvature. Many ABC transporters play crucial roles in lipid homeostasis by actively transporting phospholipids from the cytoplasmic to the exocyttoplasmic leaflet of cell membranes or exporting phospholipids to protein acceptors or micelles. Recent studies indicate that some ABC proteins can also transport phospholipids in the opposite direction. The importance of P₄-ATPases and ABC transporters is evident from the findings that mutations in many of these transporters are responsible for severe human genetic diseases linked to defective phospholipid transport.

1. Introduction

Transport of phospholipids across cell membranes plays a crucial role in many biological processes. Phospholipids, and more specifically glycerophospholipids, are synthesized on the cytoplasmic side of the endoplasmic reticulum (ER) membrane. Half of the newly synthesized phospholipid must be transported to the opposing luminal leaflet in order to maintain a balanced ER membrane bilayer. In contrast, other cell membranes including late Golgi, endosomes and plasma membrane are highly asymmetrical in their bilayer lipid distribution [1, 2]. Phosphatidylserine (PS) and phosphatidylethanolamine (PE) are preferentially found on the cytoplasmic leaflet of these membranes, while phosphatidylcholine (PC), sphingomyelin (SM) and glycolipids are enriched on the extracellular or lumen leaflet. Membrane asymmetry is generated in part by the selective transport of phospholipids across cell membranes [3, 4]. Phospholipid transport is also important in generating lipid imbalance between the two leaflets which contributes to membrane bending and curvature. Lipid asymmetry and imbalance have been implicated in

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such biological processes as phagocytosis, fertilization, membrane budding and vesicle trafficking, modulation of membrane protein function, blood coagulation, cell and organelle shape. The transport of phospholipids across membranes is also essential for lipid homeostasis. For example, cholesterol efflux from cells, formation of bile, nutrient transport, removal of selected toxic compounds from cells, and secretion of pulmonary surfactants require phospholipid transport. Indeed, the importance of phospholipid transport in these and other processes is evident by the finding that many severe human disorders are caused by defects in phospholipid transport [5, 6] [7].

It is well-known that the phospholipid bilayer serves as an energy barrier for the free movement of hydrophilic molecules into and out of cells and subcellular organelles. As a consequence, specific transporters and channels are needed to move hydrophilic molecules across cell membranes. This is also true for amphipathic phospholipids which due to their hydrophilic, charged head group require a transporter or translocase to actively or passively facilitate their movement across membranes. Three classes of proteins have been implicated in the transport or flipping of phospholipids across cellular membranes: scramblases, P₄-ATPases, and ABC transporters (Fig 1).

Scramblases are energy independent, bi-directional lipid transporters. In general they have a broad substrate specificity transporting a wide variety of phospholipids and other membrane lipids across the lipid bilayer. Scramblases are important for dissipating lipid asymmetry generated during the biosynthesis of lipids in the ER and counteracting lipid asymmetry in other cell membranes to facilitate such processes as phagocytosis, apoptosis, fertilization, and blood coagulation. Scramblases in general are poorly characterized although several studies have reported the cloning and characterization of proteins which appear to function as scramblases [8-10].

P₄-ATPases are a class of P-type ATPases which utilizes the energy from ATP hydrolysis to transport or flip aminophospholipids from the exocytosolic (extracellular/lumen) to the cytoplasmic leaflet of cellular membranes. These phospholipid flippases have been implicated in the generation and maintenance of phospholipid asymmetry found in many cellular membranes and several members have been recently linked to severe human disorders [5, 11-17].

ABC transporters comprise a large class of membrane proteins involved in the active transport of a wide variety of compounds across cell membranes. A significant number of ABC proteins are now known to transport phospholipids across cell membranes [6, 7, 18]. Most mammalian ABC transporters function as exporters or floppases transporting substrates from the cytoplasmic to the exocytosolic side of membranes. Recent studies, however, now indicate that some eukaryotic ABC transporters function as importers or flippases, transporting phospholipids and related derivatives from the exocytosolic to the cytoplasmic leaflet of cells [19]. ABC proteins play a crucial role in lipid homeostasis as indicated by the finding that mutations in some ABC transporters are responsible for severe human diseases associated with defective phospholipid transport. ABC transporters may also contribute to membrane lipid asymmetry in specific biological membranes.

A number of excellent reviews have been written on membrane lipid asymmetry and its role in various cellular processes [1, 2, 4, 20, 21]. The purpose of this review is to provide an update on the molecular characterization of mammalian P₄-ATPases and ABC transporters involved in phospholipid transport.

2. P-type ATPase Superfamily

P-type ATPases comprise a large family of ion and lipid transporters (483 unique sequences, Prosite) which undergo reversible phosphorylation of the aspartic acid in the P-type signature sequence DKTGT. Interconversion of four principle conformational states termed E_1 , E_1P , E_2P , E_2 (P denotes phosphorylation) drives ion translocation [22]. P-type ATPases are found in all kingdoms of life and are classified into five families based on ion specificity and sequence (Fig 2A). Type IA are bacterial K^+ pumps, Type IB are heavy metal pumps, Type IIA and Type IIB are Ca^{2+} pumps and Type IIC are Na^+/K^+ or H^+/K^+ pumps. Type IIIA ATPases are specific to H^+ . Type IV and Type V ATPases are only found in eukaryotes. Type IV ATPases have recently been shown to transport phospholipids [11, 12]. The substrate specificity of Type V ATPases is unknown.

An extensive structural account of the reaction sequence for the Ca^{2+} ATPase has described many of the key events which underlie ion pumping [23]. Recent structures of the Na^+, K^+ -ATPase, H^+ -ATPase, and Cu^{2+} -ATPase have advanced our understanding of the similarities and differences between various P-type transporters of different ion specificities [24-26]. Structural information is not currently available for the Type IV and V ATPases.

3. P_4 -ATPases

In humans, there are 14 members of the P_4 -ATPase family which are organized into five subfamilies based on sequence similarity of the catalytic or α -subunit (Fig. 2B, Table 1). Most P_4 -ATPases are known to associate with an accessory or β -subunit known as CDC50 to form a heteromeric complex (Fig 3A). The CDC50 family consists of only three members: CDC50A, CDC50B, and CDC50C suggesting that many P_4 -ATPases can bind the same CDC50 β -subunit. In humans and other primates, the *CDC50C* gene is truncated and may result in a nonfunctional protein [27]. The interaction of various P_4 -ATPases with CDC50 family members have been studied primarily in cells overexpressing these proteins [28-30] (Table 1). To date there are only a few reports showing the direct interaction of mammalian P_4 -ATPases with CDC50 family members in normal tissues and cells [31].

3.1 Role of P_4 -ATPases in lipid asymmetry and vesicle trafficking

Lipid asymmetry is important for a wide variety of different physiological and cellular processes such as vesicle budding, membrane curvature, fertilization, phagocytosis, exocytosis, apoptosis, blood coagulation, regulation of the activities of membrane-associated proteins, intracellular signaling, and the maintenance of membrane integrity and impermeability [14]. One of the most widely recognized function of phosphatidylserine (PS) asymmetry occurs in apoptotic cells where exposure of PS in the outer leaflet of the plasma membrane (PM) serves as an “eat me” signal for phagocytic cells. In *C. elegans*, loss of the PS transporter TAT-1 leads to abnormal exposure of PS resulting in the clearance of neuronal and muscle cells [32].

Nearly all synthesis of new membranes occurs in the endoplasmic reticulum (ER). Lipids synthesized in the cytoplasmic leaflet of the ER are rapidly and symmetrically redistributed by the action of scramblases to ensure an even lipid distribution on both membrane leaflets [33]. Newly synthesized membranes and proteins travel out of the ER by budding of small vesicles which fuse with other membranous organelles such as the Golgi complex. Lipids are sorted and further processed in the Golgi where lipid asymmetry is first generated by the combined action of flippases and floppases. Proteins are also sorted in the trans-Golgi network (TGN) and are exported to the endosomal/lysosomal pathway or sorted into cargo destined for the exocytic pathway. Clathrin dependent trafficking is a major pathway for vesicle transport from the TGN to endosomes. ARFs (ADP ribosylation factors) are GTP-

binding proteins that in their active GTP-bound state regulate protein trafficking by recruiting coat proteins such as clathrin to sites of vesiculation. ArfGEFs (guanine nucleotide exchange factors) and ArfGAPs (guanine nucleotide activating factors) catalyze the exchange of GDP for GTP and increase the rate of Arf GTP hydrolysis, respectively.

Accumulating evidence in yeast and other cells supports a role for P₄-ATPases in vesicle transport and budding, regulating many distinct protein trafficking pathways [34, 35]. Loss of TAT-1 in *C. elegans* results in the accumulation of large intracellular vacuoles derived from the endolysosomal pathway [36]. Loss of TAT-5, in *C. elegans* causes large scale shedding of extracellular vesicles disrupting cell adhesion and morphogenesis [37]. Early studies in yeast showed that knockout of Drs2p cause accumulation of abnormal membrane structures and an inability to form clathrin-coated vesicles [38, 39]. Furthermore, genetic interactions with many members of the clathrin-mediated pathway have been described. Drs2p physically interacts with ArfGEF in the Golgi [40] and binding of ArfGEF stimulates the flippase activity of Drs2p [41]. A model has been proposed for vesicle formation whereby ArfGEFs recruit activated Arfs and bind and activate P₄-ATPase flippase activity thereby concentrating aminophospholipids on the cytosolic leaflet [21]. According to the bilayer couple hypothesis, a small increase in surface area of one leaflet of the bilayer and/or a decrease in the surface area of the opposing leaflet will induce bilayer curvature. Thus, expansion of the cytosolic leaflet resulting from P₄-ATPase catalyzed aminophospholipid transport and the corresponding reduction in the luminal leaflet will initiate localized membrane curvature and membrane budding. Activated-Arfs recruit coat and adaptor proteins to site of vesiculation which target the newly derived vesicles to their subcellular destinations. Evidence for P₄-ATPase involvement in vesicle transport in mammalian cells is not as well understood, however, many pathophysiological features suggest that this is a conserved function for P₄-ATPases across evolution. Additional experimental data is needed to more clearly define the role of P₄-ATPases in mammalian vesicle trafficking mechanisms.

3.2. P₄-ATPase-CDC50 Complex: Domain Structure and Mechanism

P-type ATPases associated with ion transport are integral membrane proteins which form an elongated structure consisting of four domains: a cytosolic A (“actuator”) domain, P (phosphorylation) domain, N (nucleotide binding) domain, and an M (membrane) domain consisting of ten transmembrane helices M1-10 [42]. P₄-ATPases adopt a similar four domain structure based on sequence analysis (Fig. 3A). Short extracellular loops connect each transmembrane helix such that only a small portion of the structure is exposed on the luminal or extracellular surface. The cytoplasmic C-terminal region of P₄-ATPases may be involved in the regulation of phospholipid transport and/or targeting of the protein to specific cellular membranes since it displays the least sequence similarity between the various P₄-ATPases. Furthermore, the C-terminal region has been shown to be a regulatory region in other P-type ATPases [43, 44].

Structural analysis of the P-ATPase ion pumps indicate that large movements of the cytosolic A-, N-, and P-domains drive the conversion of the enzyme between its four principle conformational states [42]. A flexible linker region couples these conformational changes to the M-domain, facilitating ion translocation. The transport cycle is initiated when N-domain binds ATP in the *E*₁ conformation and rapidly phosphorylates the aspartic acid of the P-domain (Fig 3B)[45]. Many P-type ATPases require binding of a transported ion to a high affinity cytoplasmic facing site in the *E*₁ form for phosphorylation by ATP. This is not a requirement for P₄-ATPases [45]. Instead, P₄-ATPases may use a charged amino acid residue as “built-in” ion as a substitute for a cation required for other P-type ATPases [26].

The phosphorylated enzyme exists in two distinct conformational states (Fig 3B). The *E*₁P form can react with ADP producing ATP while the *E*₂P form is resistant to ADP. During the

transition from E_1P to E_2P , the A-domain rotates 90° inserting the TGES (DGET in P_4 -ATPases) motif into the space which was occupied by ADP, positioning it near the phosphorylated aspartate [42]. The consequence of A-domain rotation is the conversion of the high affinity cytoplasmic site to a low affinity extracellular facing site allowing exit of the ion. Transported aminophospholipids activate the dephosphorylation of E_2P for P_4 -ATPases in a manner analogous to the dephosphorylation of the Na^+, K^+ -ATPase by K^+ [45, 46]. Aminophospholipids likely interact with a high affinity extracellular site in the E_2P form of the enzyme similar to K^+ in the Na^+, K^+ -ATPase. This stabilizes the transition state and accelerates the rate of dephosphorylation. Binding of aminophospholipids to E_2P , may trigger further movement of the glutamic acid of the DGET motif in closer proximity to the P-domain allowing it to catalyze dephosphorylation by acting as a base to remove a proton from water which carries out nucleophilic attack on the aspartyl-phosphate. In P_4 -ATPases, mutation of this glutamate stabilizes the E_2P form and addition of PS triggers further E_2P accumulation [45]. In the case of Drs2p, E_2P dephosphorylation seems to be regulated by binding of phosphoinositides [41, 46]. Dephosphorylation converts the enzyme to the E_2 form changing the high affinity extracellular site to a low affinity cytoplasmic site allowing the lipid to leave. The A-domain moves away from the P-domain, returning the protein to the E_1 conformation.

In most P-type ATPases, transmembrane helices M1-6 are thought to form the core catalytic unit of ion transport while M7-10 support the core catalytic unit and may facilitate interactions with β -subunits. M1-6 are more flexible while M7-10 remain fairly rigid throughout the reaction cycle. P_4 -ATPases are unique among P-type ATPases in their ability to transport a substrate much larger than a cation. In other P-type ATPases, ion translocating pathways are not large enough to accommodate a “giant substrate” such as a phospholipid [47].

Recent studies have identified key residues which form a potential phospholipid translocation pathway [45, 48]. In the yeast PC transporter Dnf1 substitution of Phe for Tyr located at the cytosolic membrane interface of M4 alters the specificity of the transporter from PC to PS. Mutation of the equivalent Tyr for Phe in the PS transporter Drs2, essentially eliminates transport. Furthermore, substitution of a Phe in the extracellular loop connecting M3 and M4 reduces PC transport through Dnf1 [48]. Based on this work, it has been proposed that phospholipids are translocated through a non-classical pathway located along the groove of M1, M3, and M4 or alternatively M3, M4, and M5. In the mammalian ATP8A2 mutation of a lysine located in the middle of M5 produces an enzyme which is essentially devoid of PS transport displaying a large reduction in PS affinity relative to wild type [45]. The lysine is located at a “hot spot” for cation binding in the Ca^{2+} - and Na^+, K^+ -ATPases suggesting that lipids are transported through a classical pathway. A recent study based on the Ca^{2+} -ATPase structure has compared the two proposed translocation pathways [49]. However, it is unclear if the pathways are compatible. These studies suggest that the phospholipid could be transported through the membrane with the headgroup forming key interactions with residues along the groove of either M1, M3, and M4 or M3, M4, and M5. The acyl chains would be free to interact with the rest of the lipid environment as it moves through the membrane similar in a concept to a credit card reader. This model potentially alleviates the “giant substrate” problem encountered for P_4 -ATPases [48]. Further studies are necessary to determine if these proposed pathways are compatible and what additional residues are necessary for phospholipid specificity and transport.

Most P_4 -ATPases form heterologous complexes with the CDC50 family of proteins (Table 1). CDC50 proteins are composed of a large glycosylated E (extracellular/lumen) domain containing three or more N-linked glycosylations, a membrane domain composed of two transmembrane helices, and two small cytoplasmic N- and C-terminal segments. In the

Na⁺,K⁺-ATPase, numerous interactions of the M7 and M10 helices are made with the transmembrane helix of the β -subunit. The extracellular domain forms further interactions with the L7/8 loop of the α -subunit [24]. In P₄-ATPases, both the membrane and extracellular domains of CDC50 are required for assembly with the β -subunit [31]. These subunit interactions are clearly necessary for the correct assembly and folding of P₄-ATPase heteromeric complex allowing it to exit the ER [28-31, 50]. However, CDC50 proteins do not appear to be important for subcellular targeting [51]. For many P-type ATPases, complex interactions of β -subunits exert substantial changes on ion translocation kinetics [24]. Further to this, the FXYD family of single spanning membrane proteins modulate the kinetics or affinities for Na⁺ or K⁺ for the Na⁺,K⁺-ATPase in a tissue dependent manner. The CDC50 family has been proposed to behave in a similar manner as a “fusion” of the FXYD and β -subunits of the Na⁺,K⁺-ATPase [14]. In yeast, CDC50 has been shown to bind with higher affinity to the phospholipid-loaded E₂P form of Drs2 suggesting that it could participate directly in lipid flipping [52]. Furthermore, the N-terminal domain of CDC50A has been shown to modulate pump kinetics of ATP8A2 [31]. Recent studies in yeast also revealed that the ectodomain is necessary for subunit specificity [53]. However, ectodomain binding was not sufficient for P₄-ATPase function which required either M1 and the N-terminus or M2 and the C-terminus. In this study, two disulfide bridges in the ectodomain have also been shown to be important for subunit affinity and function. These experiments suggest that the interaction surface of the P₄-ATPase-CDC50 complex is extensive and that many of these subunit interactions are necessary for subunit assembly and optimal phospholipid transport. The precise role of CDC50 proteins in the reaction cycle will require further structural and biochemical investigations.

3.3 ATP8A Subfamily

The first evidence that P₄-ATPases transport aminophospholipids came from studies on the yeast Drs2p where notable deficiencies in the uptake of NBD-labeled PS were reported [54]. ATP8A1, an ortholog of Drs2p was the first P₄-ATPase discovered in mammals and shown to be ubiquitously expressed throughout the body [55](Table 1). Relative to wild-type, the brains of ATP8A1 knockout mice appear morphologically normal despite PS-externalization in the hippocampus. Interestingly, these mice display hyperactivity and marked deficiencies in hippocampus-dependent learning [15]. Pioneering biochemical studies first identified a 120 kDa ATPase isolated from human red blood cells and bovine brain by conventional techniques that was robustly activated by aminophospholipids and inhibited by the phosphate analog vanadate and sulfhydryl-reactive reagents [56-59]. This ATPase was thought to be responsible for the rapid transport of spin labeled PS and PE observed in both of these systems [60, 61]. In red blood cells, phospholipid transport is thought to influence cell shape, inducing the discocyte to echinocyte change [62]. Transport stoichiometry was measured to be one ATP molecule per phospholipid [63]. Reconstitution of the ATPase from red blood cells demonstrated that it was able to catalyze the rapid flipping of PS [64]. Despite these efforts, the identity of the red blood cell ATPase still remains uncertain. Western blotting with highly specific antibodies showed that both red cells and chromaffin granules contain ATP8A1 [65, 66]. Heterologously expressed ATP8A1 from insect cells has many similar properties as the ATPase isolated from natural sources [66, 67]. ATP8A1 has been reported to bind CDC50A [30, 50] although it is possible that it can also bind CDC50B depending on experimental conditions [50]. Splice variants of ATP8A1 differ in their phospholipid selectivity and specific ATPase activity [66]. However, markedly different specific ATPase activities were observed in some studies possibly due to the absence of the appropriate CDC50A β -subunit [67]. Isolation and identification of ATP8A1 from natural sources using highly specific reagents and comparison of its enzymatic properties with earlier studies is necessary to clarify the role of ATP8A1 in these tissues.

ATP8A2 is 67% identical to ATP8A1 in sequence. ATP8A2 has a more restricted expression pattern than ATP8A1 with high level of expression in the retina, brain, spinal cord, and testis [11, 68]. In the retina, it is expressed in both rod and cone photoreceptor cells where it localizes to outer segment disc membranes, the site of phototransduction. CDC50A co-purifies with ATP8A2 from photoreceptors [31]. The ATPase activity of the purified complex is activated by the aminophospholipids PS and PE with a strong preference for PS. Reconstitution of ATP8A2 into liposomes containing NBD-labeled PS demonstrated that it is a PS-translocase [11, 31]. A patient with severe mental retardation and major hypotonia has been reported carrying a balanced translocation of chromosomes 10 and 13 disrupting the coding sequence of the *ATP8A2* gene [68]. Recently, a family with four individuals carrying a missense mutation in ATP8A2 has been reported [69]. These individuals exhibit severe cerebellar ataxia, mental retardation and dysequilibrium syndrome. Additionally, wabblers-lethal mice carry mutations in the *ATP8A2* gene [70]. These mice develop severe neurological abnormalities including ataxia and body tremors due to distal axonal degeneration in spinal neurons which may be a result of disrupted vesicle transport. Wabblers mice die at approximately one month of age. Knockout of both *ATP8A1* and *ATP8A2* results in neonatal lethality suggesting the function of ATP8A1 partially overlaps with ATP8A2. The function of ATP8A2 in photoreceptors is unknown and mutations in *ATP8A2* which cause impaired vision have yet to be found. However, several functions for ATP8A2 in photoreceptors have been proposed. Rhodopsin the major membrane protein of outer segments has recently been shown to act as a phospholipid scramblase [9]. The flippase activity of ATP8A2 may maintain lipid asymmetry necessary for phototransduction in the presence of high rhodopsin concentrations. ATP8A2 may also be involved in outer segment renewal. Using microarray analysis, ATP8A2 expression was found to be downregulated in *Nrl* mice, potentially disrupting phospholipid organization [71]. The retinas of *Nrl* knockout mice only contain cone photoreceptors and large amounts of aberrant phagosomes are present at the RPE-photoreceptor interface. PS is normally localized in the cytoplasmic surface of photoreceptor plasma membranes. However, coinciding with the onset of light and phagocytosis, PS becomes exposed to the extracellular surface at the tips of photoreceptors [72]. This extracellular PS could then serve as an “eat me” signal for the RPE cells triggering phagocytosis. This would require regulation of ATP8A2 activity at the apical tips of the photoreceptor. While this is an intriguing hypothesis, ATP8A2 has yet to be shown in the plasma membrane of rod photoreceptors. ATP8A2 may also be involved in vesicular trafficking in photoreceptors and other neurons. In PC12 and hippocampal neurons, overexpressing ATP8A2 causes enhanced neurite outgrowth providing support for a role of ATP8A2 in vesicle formation or transport [73].

3.4 ATP8B Subfamily

ATP8B1 was the first P₄-ATPase found to be involved in human disease. Mutations in ATP8B1 cause two forms of intrahepatic cholestasis: progressive familial intrahepatic cholestasis (PFIC) and benign recurrent intrahepatic cholestasis (BRIC) [16]. These patients can also experience hearing loss and are predisposed to pneumonia [74, 75]. Cholestasis is a condition where the flow of bile from the liver is blocked. Patients often develop the disorder early in life resulting in cirrhosis within the first decade of life. ATP8B1 mutations can result in a wide spectrum of clinical severity as some patients experience cholestasis intermittently while others do not experience any symptoms [17]. In hepatocytes, ATP8B1 localizes in the canalicular membrane where its activity is thought to be important for the maintenance of membrane integrity and function of ABCB4, an ABC floppase, and consequently bile export [76-78]. ATP8B1 is necessary for apical protein expression and apical membrane organization. The loss of ATP8B1 causes substantial microvilli loss in Caco-2 cells [79]. Degeneration of stereocilia of cochlear hair cells where ATP8B1 is present is observed in knockout mice [74]. ATP8B1 can bind both CDC50A and CDC50B

[29, 30, 50] but in the liver and other tissues, CDC50A is preferentially expressed and likely serves as the β -subunit for ATP8B1 in the native tissue [29, 80]. The lipid substrate that is flipped by ATP8B1 is not fully understood. In UPS-1 cells, ATP8B1 has been shown to be involved in PS translocation. In PFIC patients with severe pneumonia, cardiolipin levels were found to be elevated which correlates with higher levels of lung injury. ATP8B1 was found to bind and translocate cardiolipin via a basic residue containing motif [75]. To clarify the phospholipid substrate specificity of ATP8B1, it would be important to purify ATP8B1 from native tissue and examine its ability to transport various lipids across membranes. However, biochemical studies on purified ATP8B1 seemed to indicate that it was trapped in the E_2P conformation and could not dephosphorylate [50]. It is unclear if this was due to experimental conditions, regulation, or lack of a suitable substrate. In order to determine which lipid species are transported by this important pump it will be necessary to understand what is required for dephosphorylation and completion of the reaction cycle.

To date little is known about ATP8B2 and ATP8B4. ATP8B4 is expressed throughout the brain and may be involved in Alzheimer disease [81]. Both pumps may be able to interact with CDC50A and CDC50B [50], although in the case of ATP8B4 experimental conditions may influence interaction with CDC50B [30]. ATP8B3 and its homolog ATP8B5 in mice are exclusively expressed in the testis where they are known to localize to the acrosomes of spermatids, the organelle which develops in the anterior half of the head of spermatozoa [82-84]. Knockdown of ATP8B5 has a profound effect on Golgi structure and protein secretion [84]. On the basis of cell biology and biochemical studies, ATP8B3 appears to also be a PS pump. ATP8B3 does not seem to interact with either CDC50A or CDC50B [30] however it is possible that it could interact with CDC50C based on expression. Interestingly, disruption of ATP8B3 in mice does not affect the morphology of sperm or the rate of fertilization despite sperm-egg interactions being disrupted *in vitro* [83].

3.5 ATP9, ATP10, and ATP11 Subfamilies

ATP9 is the only subfamily of P_4 -ATPases which do not appear to interact with CDC50 proteins [28]. In transfected cells, ATP9A localized to the both endosomes and the trans-Golgi network (TGN), whereas ATP9B localized only to the TGN. An N-terminal targeting sequence appears to be important for the localization for ATP9B to the TGN. The substrate(s) transported by ATP9A and ATP9B is not currently known. In yeast, loss of Neo1p, an ortholog of the ATP9 members, is lethal. Neo1p localized to endosomes and Golgi where it appears to play a role in endosomal membrane trafficking [85]. Further investigations are needed to determine if the ATP9 subfamily plays a similar role in mammalian cells.

The ATP10 subfamily harbors the least biochemically characterized subfamily of P_4 -ATPases. Members of the subfamily have a longer C-terminus, the function of which is unknown. CDC50A is required for exit of ATP10 proteins from ER [28]. The substrate specificities of ATP10A, ATP10B and ATP10D have not yet been defined. In mice, ATP10A and ATP10D are associated with obesity and diabetes [86-90]. There are also reports that link imprinting mutations and deletions in the region of ATP10A with Angelman syndrome, a form of autism-spectrum disorder [91, 92].

Recent work from two independent labs has shown that ATP11C is important for the proper development of B-cell lymphocytes in mice [13, 93]. ATP11C seems to be required for efficient flipping of PS in B-cells. Intrahepatic hyperbilirubinemia, an accumulation of bilirubin from hepatocellular origin and high serum levels of cholic acid indicative of cholestasis, is also observed in these animals [94]. ATP11A, ATP11B and ATP11C appear to be ubiquitously expressed and only bind CDC50A as its β -subunit [28].

4. ABC Transporters

ATP binding cassette (ABC) transporters are found in all living organisms from bacteria to mammals [95] [96]. They typically use ATP binding and hydrolysis to actively translocate a wide variety of compounds across cell membranes including amino acids, peptides, ions, metabolites, vitamins, fatty acid derivatives, steroids, organic anions, phospholipids, drugs and other compounds. Prokaryotic ABC transporters can function either as exporters or importers. Eukaryotic ABC transporters have been initially thought to only function as exporters, but several studies have now shown that some eukaryotic ABC transporters including the mammalian ABC transporter ABCA4 function as importers translocating substrates from the exocytosomal (extracellular/lumen) to the cytoplasmic side of biological membranes [19, 97, 98].

ABC transporters consist of four principal domains: two transmembrane domains (TMDs) containing multiple membrane-spanning segments that provide a pathway for the translocation of a substrate across the membrane and two cytoplasmic ATP-binding cassettes or nucleotide binding domains (NBDs) that provide the energy for substrate transport. Eukaryotic ABC transporters are typically synthesized as full transporters in which all four domains reside on a single polypeptide chain with a modular organization of TMD-NBD-TMD-NBD or half transporters in two polypeptides each containing a TMD and NBD assemble as homo- or hetero-dimers. Some transporters contain additional domains attached to the TMD or NBD which regulate the activity of these ABC transporters. In prokaryotes, the TMDs and NBDs can reside on individual polypeptide chains or fused together in various arrangements [96].

Eukaryotic ABC transporters typically contain 6 membrane spanning segments per core TMD. NBDs approximately 200 amino acids in length have a number of conserved structural features including Walker A and Walker B motifs found in many ATPases and the ABC signature motif (LSSGQ), D, H and Q loops characteristic of ABC proteins. Structural and biochemical studies indicate that the two NBDs dimerize in a head-to-tail manner with the two ATP molecules present at the dimer interface [99-102].

The human genome is known to contain at least 49 genes that encode ABC proteins [103]. These transporters have been organized into seven subfamily (ABCA-ABCG) based on their amino acid sequence and structural organization (Fig 4A). Two subfamilies ABCE and ABCF, which contain NBDs, but no TMDs, do not function as transporters, but instead are involved in the regulation of protein biosynthesis. Several members of various ABC subfamilies are known to transport specific membrane phospholipids across cell membranes either serving as floppases or flippases or exporting the phospholipid to an acceptor molecule [104].

4.1. General Mechanisms

Crystal structures of several ABC exporters (Sav1866, murine P-glycoprotein, MsbA, TM287/TM288) together with biochemical studies have provided insight into the transport mechanism of ABC proteins [105-109]. The 'alternating-access model' proposed for ABC transporter drug export function can be adapted for lipid translocation [106, 110]. In the absence of nucleotide, the NBDs impose separation in the TMDs, and create a large central cavity towards the cytoplasmic side of the membrane that represents an 'open-inward' conformation. The transport cycle is initiated when a phospholipid(s) enters the substrate binding site from the inner leaflet that is assumed to represent a high affinity binding interaction. Binding of ATP induces a conformational change in the NBDs and significant rearrangement of the TMDs resulting in a central cavity facing the extracellular/lumen side of the membrane. This is termed as the 'outward-closed' conformation for the ABC

transporter, and is similar to that observed for Sav1866 [106]. The polarity of the TMD substrate-binding site then becomes reversed with a concomitant reduction in substrate affinity, and release of the lipid substrate to the outer leaflet. The conformational change in the TMDs may be driven either by ATP binding, in which case hydrolysis of ATP subsequently resets the ABC transporter to the inward-facing conformation or by the energy released on ATP hydrolysis. Alternatively, the transporter may only bind to the polar head group of the phospholipid similar to ion or pore channel mechanisms [111]. This model relegates the acyl chains as being shuttled across the hydrophobic interior of the bilayer as the lipid headgroup is mediated by the substrate binding pocket and transitions between the inner and outer leaflets.

The events that occur at the NBDs are also incompletely understood but two models are generalized to link substrate binding and the catalytic cycle. The 'Switch model' proposes that sequential ATP hydrolysis is required to destabilize the NBDs and the adjacent Walker A motifs and signature motifs in the sandwich dimer move apart before the transport cycle can begin again [112](Fig 4B). ATP binding switches from the dissociated NBD dimer to the closed dimer, reduces the substrate binding affinity, and facilitates substrate translocation *via* corresponding conformational changes in the TMDs. Evidence from electron paramagnetic resonance (EPR) analysis of the NBDs of MsbA, a lipid transporter, shows large conformational changes (10-20 Å) following ATP hydrolysis suggestive of NBD dissociation, and is consistent with the crystal structure conformations of MsbA [107, 113]. In contrast, the 'Constant-contact model' proposes that the NBD dimer is stabilized regardless of nucleotide or substrate content and that each NBD twists about enabling the exchange of nucleotide hydrolysis products (Fig 4C) [114, 115]. This effect occludes ATP in one site while the other site opens to release ADP and P_i. The transition from the inward to the outward facing conformation of the TMDs requires a twisting motion of the NBDs and a rearrangement of the consensus site, which allows binding of the second ATP. The bound lipid substrate may then be released to the outer leaflet of the membrane bilayer. Vanadate trapped complexes in P-gp support an asymmetric effect of nucleotide occluded in one site while the other remains solvent accessible consistent with the opening of one ATP site and the other one closed [116]. A cross-linking study in which the two-halves of the TMDs in Pgp were clamped in the closed conformation displayed high affinity and transport activity of drugs [117], although the open-inward conformation of the Pgp structure showed a 30 Å NBD separation bound to some substrates [105]. A recent crystal structure of the putative drug TM287/TM288 ABC transporter from *T. maritima* presents an inward-facing conformation with contacting NBDs [108], which is uniquely different to the ABC exporter structures of inward-facing conformations with dissociated NBDs [105, 107]. Although, ATP binding and hydrolysis may propagate conformational changes as fulfilled by the constant contact model, the importance of a highly flexible open inward conformation as part of the switch model may also be essential for efficient binding of large size physiological substrates [107, 111].

To date, only one ABC exporter, Pgp, has been resolved with a bound substrate [105]. The TMDs bear hydrophobic, aromatic and polar amino acids where multiple ligands can bind through different interactions with distinct sets of amino acids and provide multiple substrate binding sites, as found in Sav1866 [106]. The TMD cavity of MsbA has a high dielectric environment that stabilizes polar regions and hydrophobic areas in the bilayer [107]. The proposed models account for both lipid and drug efflux. In this case, translocation of lipids may be similar to that of drug substrates but partitions into the outer leaflet of the membrane bilayer, instead of being expelled into the aqueous environment. Alternatively, the transporter may promote dissociation of the lipid from the donor membrane, thereby facilitating its efflux from the cell. Such a case would require the incidental movement of lipids to a region of partial hydrophobicity on the exocytolic face of

the protein. This places the substrate in an environment which energetically favors its binding and removal by an acceptor molecule like a bile salt or a docking protein such as apolipoprotein-A1 (apo-A1) [20]. This mechanism may govern the functionality in several ABCA and ABCG proteins. In the absence of additional ABC transporter structures, predicting how a lipid is flipped and/or effluxed is challenging and requires the rigorous use of homology models, molecular simulation analysis, and biochemical studies.

4.2. ABCA transporters

The A subfamily of ABC transporters consist of 12 members. All are full-length transporters organized in two tandem halves, each with a hydrophobic TMD followed by a cytoplasmic NBD. In addition, these transporters contain two large extracellular domains (ECDs) between the first and second membrane-spanning segment of each TMD [118]. Although the substrates of most ABCA members remain to be identified, disease-associated phenotypes, analysis of knockout mice, and biochemical and cell based studies suggest that most ABCA proteins play a role in phospholipid translocation and cellular homeostasis. Mutations in ABCA1 are known to cause Tangier disease and familial high density lipoprotein deficiency associated with defective cholesterol and phospholipid efflux from cells and a deficiency in the formation of high density lipoprotein (HDL) [119-123]. Defects in ABCA3 are associated with neonatal surfactant deficiency and pediatric interstitial lung disease resulting from abnormal surfactant secretion into the alveoli of the lungs and the formation of abnormal lamellar bodies [124, 125]. Disease mutations in ABCA4 cause autosomal recessive Stargardt macular degeneration and related retinal degenerative diseases associated with defective retinylidene-phosphatidylethanolamine transport [126]. Mutations in ABCA12 are associated with harlequin ichthyosis, a disease arising from defective lipid transport in the skin [127, 128]. ABCA1, ABCA2, and ABCA7 have been also linked in the etiology of Alzheimer's disease [6, 129, 130].

4.2.1 ABCA1—ABCA1 is recognized as the principal protein involved in cholesterol efflux from peripheral tissues in a process known as the reverse cholesterol transport (RCT) [122, 131]. It mediates the efflux of cellular phospholipid and cholesterol to lipid poor apolipoprotein acceptors as a key step in the production of HDL. The loss of ABCA1 function in humans leads to Tangier disease, a disorder associated with severe HDL deficiency and increased risk of atherosclerosis. To date, more than 100 mutations in the gene encoding ABCA1 have been linked to Tangier disease in which patients have absent or low circulating HDL [123]. The inverse relationship between plasma HDL levels and the risk of coronary artery disease has been reported [132], although low HDL per se is not an absolute risk factor [133].

Abca1^{-/-} mice and chickens with dysfunctional ABCA1 also exhibit HDL deficiencies further highlighting a pivotal role of ABCA1 in the RCT pathway [134-136]. Tissue specific deletion of ABCA1 in liver and intestines of mice resulted in an 80% and 30% reduction in plasma HDL, respectively [137, 138], while ablation of ABCA1 in macrophages did not alter HDL levels [139].

ABCA1 expressed in cultured cells, mediates the secretion of PC and cholesterol when lipid-free apolipoprotein apo-A1, an extracellular lipid acceptor in the plasma, is added to the medium [140, 141]. Cell based studies of 15 disease-associated mutations in ABCA1 showed decreased phospholipid, predominantly PC, and cholesterol efflux [123, 142]. Direct interaction between apo-A1 and ABCA1 has been reported in numerous studies using cross-linking, immunoprecipitation, radiolabelling, and biotinylation techniques [143-145]. More recently, Nagao et al reported that initial binding of apo-A1 is mediated through electrostatic interactions with the extracellular domains of ABCA1 [146]. This interaction can be

inhibited by heparin and poly-L-lysine treatments. Dimeric and tetrameric forms of ABCA1 represent the minimal functional unit required for apo-A1 binding and lipidation [147].

HDL constitutes a heterogeneous group of particles differing in size, density, electrophoretic mobility, lipid composition and apolipoprotein content with average diameters of ~12, 10, 7.5, and < 6nm. Electron microscopy studies suggest that the HDL particles are mostly spheroidal [148]. The HDL particles formed by the lipidation of apo-A1 via the functional activity of ABCA1 is an essential step for the formation of HDL that ultimately determines plasma HDL levels. In several studies, the formation of heterogeneous nascent pre- β -HDL was observed during incubation of apo-A1 with Chinese hamster ovary cells and THP-1 macrophages, presumably by the interaction of apo-A1 with cell surface binding sites or through retroendocytosis [149, 150]. In J774 macrophages, in which ABCA1 expression was up-regulated by cAMP, the presence of extracellular apo-A1 induced formation of 7.4-, 9.4, and 11.2-nm sizes of particles. The largest pre HDL particle carried up to 45% of total particle lipids as cholesterol and is bound by 3 apo-A1 molecules in a belt-like conformation [148]. The most abundant lipids in HDL are PC, cholesterol, and SM. The total amount of lipid also increases as the diameter of the HDL particle increases. For instance, ~26 lipid molecules reside with two apo-lipoprotein particles in the 7.4 nm HDL particle whereas ~240 lipid molecules are found along with three apo-A1 molecules in the 11.2 nm particle [148]. Interestingly, the 7.4 nm and 11.2 nm particles contain ~3:1 and 1:1 PC/free cholesterol (mol/mol), respectively. In contrast PS and SM levels remain relatively similar during HDL particle maturation.

Although it is well established that ABCA1 mediates the efflux of cholesterol and phospholipids from cells, the actual substrates transported by ABCA1 are still unclear (Table 1). Several models of ABCA1-mediated cholesterol efflux have been proposed, including a two-step model in which ABCA1 first mediates PC efflux to apo-A1, and this apo-A1 PC complex accepts cholesterol in an ABCA1 independent manner [151]. A concurrent process model in which PC and cholesterol efflux by ABCA1 to apo-A1 are coupled to each other has also been proposed [152]. A third model has been proposed in which ABCA1 generates a specific apo-A1 binding site through PS translocation to the outer leaflet of the plasma membrane with subsequent translocation of PC and cholesterol to apo-A1 [153]. More recently, Vedhachalam et al [150] and Iatan et al [154] advanced new models in which HDL particle maturation is mediated by the lateral heterogeneity of the plasma membrane (Fig 5). In this system, ABCA1-dependent activity creates a PC-rich membrane binding site for apo-A1 on the plasma membrane that has almost 10-fold higher capacity to bind apo-A1 compared with ABCA1. This is called a high-capacity binding site (HCBS) and is required for apo-A1 lipidation. In this model monomeric or oligomeric ABCA1 creates a HCBS population either associated with or excluding ABCA1 [154]. Initial interactions of apo-A1 with ABCA1 stabilize the membrane protein; reduction of intracellular degradation by calpain proteases promotes increased levels and activity of ABCA1 in the plasma membrane. In response to ATP hydrolysis, ABCA1 promotes the transbilayer transport of PC and/or PS from the inner to outer leaflet of the plasma membrane. The initial events consist of binding of a small pool of apo-A1 to ABCA1 at the PM, stimulating the net phospholipid translocation to the exofacial leaflet. ABCA1 facilitates its translocation to the HCBS populations from which apo-A1 desorbs PC, thereby becoming an efficient acceptor of cholesterol. These small PC containing particles may interact successively or simultaneously with different microdomains, including cholesterol rich rafts, allowing the formation of larger heterogeneous cholesterol containing nascent HDL particles. Alternatively, ABCA1 activity leads to unequal lateral packing densities in the two leaflets of the phospholipid bilayer, creating exovesiculated lipid domains, primarily mediated by PS. This would then result in a pool of bound apo-A1 spontaneously solubilizing phospholipids from the exovesiculated domain to create discoidal nascent HDL

particles. The latter effect may be the underlying cause of apo-A1 solubilizing phospholipids and cholesterol from small 20 nm diameter unilamellar vesicles in an *in vitro* system [155]. More studies, however, are needed to definitively identify the lipid substrates transported by ABCA1 and the mechanism by which HDL particles are generated from apo-A1 and ABCA1.

4.2.2 ABCA2—ABCA2 is a 270 kDa protein that shares 50% sequence identity with ABCA1. It is mainly expressed in the central nervous system, ovary and macrophages [156]. *Abca2*^{-/-} mice display a compact myelin sheath phenotype with reduced levels of SM and gangliosides during the early development of mice (Fig 6A). The phospholipids, PE and PS, were slightly decreased compared to the wildtype mice [157]. Recent studies have shown that ABCA2 plays a role in the trafficking of LDL-derived cholesterol and is coordinately expressed with sterol responsive genes including LDLr and HMGCoA synthase [95]. ABCA2 overexpression in neuroblastoma cells lead to a decrease in total, free, and esterified cholesterol levels. ABCA2 overexpression also decreased LDLr receptor mRNA and protein levels and increased its turnover rate. This suggests that ABCA2 may modulate cholesterol homeostasis in neuronal cells and play a regulatory role in cholesterol metabolism. Very few ABC protein encoding genes have been identified that play a role in the trafficking of LDL-derived cholesterol. In HEK293 cells, ABCA2 overexpression does not result in cholesterol efflux to apo-A1, apo-E, or apo-E disks [158] while phospholipid efflux activity remains to be investigated.

4.2.3 ABCA3—ABCA3 is highly expressed in alveolar epithelial type II cells of the lung. In these cells, ABCA3 localizes to the limiting membrane of lamellar bodies, lipid rich organelles associated with the production, storage, and secretion of pulmonary surfactant. Surfactant is a complex mixture of 90% lipids (mostly phospholipids) and 10% surfactant-specific proteins that reduce surface tension of the air-liquid interface and prevents alveolar collapse [159]. ABCA3 is implicated in the translocation of surfactant lipids into lamellar bodies where surfactant is assembled. Mutations in the ABCA3 have been associated with neonatal surfactant deficiency and chronic interstitial lung disease [125]. Uptake of NBD-PC and NBD-PE into ABCA3 LAMP-positive vesicles in A549 cells was demonstrated (Fig 6D)[160]. Matsumura et al showed increased levels of PC, but not PE or cholesterol, in low density vesicles in A549 cells stably expressing the wild-type ABCA3 [159]. Consistent with this observation, Cheong et al showed decreased incorporation of radiolabeled PC, PG, and PE into lamellar bodies of *abca3*^{+/-} mouse [161]. However, a high throughput mass spectrometry lipid profile found a significant decrease in PG and short chain PC in *abca3*^{-/-} mice [162]. The exact molecular contribution of ABCA3 for surfactant production with respect to PC and PG transport remains to be established.

4.2.4 ABCA4—ABCA4 is a 250 kDa ABC transporter having a 50% amino acid sequence similarity with ABCA1/2/7. It is highly expressed in the light sensitive outer segment disc membranes of retinal rod and cone photoreceptor cells [163, 164]. Over 800 mutations in ABCA4 are known to cause Stargardt macular degeneration, a severe retinal degenerative disease characterized by progressive impairment of central vision early in life, presence of lipofuscin deposits in the central retina, and bilateral atrophy of photoreceptor cells and adjacent retinal pigment epithelial (RPE) in the macula [126, 165, 166]. Mutations in ABCA4 have also been reported to cause several related retinal degenerative diseases including cone-rod dystrophy and retinitis pigmentosa and a subset of age-related macular degeneration [167-170].

ABCA4 facilitates the clearance of retinal from rod and cone photoreceptors by functioning as a *N*-retinylidene-phosphatidylethanolamine (*N*-ret-PE) flippase [171]. Photoreceptors are subjected to a high flux of retinal as part of the visual process. In the dark, 11-*cis* retinal

derived from the visual cycle combines with opsin in disc membranes to regenerate rhodopsin and cone opsin in the outer segment disc membranes of rod and cone cells, respectively. Vision is initiated when light photoisomerizes 11-*cis* retinal to all-*trans* retinal within the binding pocket of opsin. All-*trans* retinal is subsequently released from opsin and must be cleared from photoreceptor cells to prevent the formation of potentially toxic compounds. Removal of retinal can occur in part by the direct reduction of all-*trans* retinal to all-*trans* retinol, a reaction catalyzed by the retinol dehydrogenase RDH8 on the surface of photoreceptor disc membranes. All-*trans* retinol is subsequently converted back into 11-*cis* retinal via a series of reactions that comprise the visual cycle in adjacent retinal pigment epithelial (RPE) cells for the regeneration of photopigment [172]. However, all-*trans* retinal can also reversibly react with PE which comprises about 40% of the phospholipid in the disc membrane to form a Schiff-base adduct *N*-ret-PE. A fraction of *N*-ret-PE can be trapped on the lumen leaflet of the disc membrane [173]. ABCA4 functions in the active transport or flipping of *N*-ret-PE from the lumen to the cytoplasmic side of the disc membrane (Fig 6C) [19]. Upon dissociation of *N*-ret-PE, all-*trans* retinal can be reduced to retinol by RDH8 and for entry into the visual cycle, thereby facilitating the complete removal of all-*trans* retinal from disc membranes after photoexcitation.

Support for the role of ABCA4 as a *N*-ret-PE transporter has come from both biochemical studies of ABCA4 and analysis of *abca4*^{-/-} mice. Purified ABCA4 reconstituted into lipid vesicles has a basal ATPase activity which is stimulated 2-5 fold by all-*trans* and 11-*cis* retinal and is dependent on PE [174, 175]. Solid-phase binding studies further demonstrated that *N*-ret-PE binds with high affinity to ABCA4 and is quantitatively released by the addition of ATP [176]. More recently, a direct transport assay has been developed which confirms the function of ABCA4 as an *N*-ret-PE flippase [19]. In this study, ABCA4 reconstituted into liposomes or present in disc membranes was observed to transport *N*-ret-PE from the lumen to the cytoplasmic leaflet of disc membranes. In the absence of retinoids, ABCA4 was also shown to actively flip fluorescent-labeled NBD-PE in the same 'import' direction. ABCA4 is the first mammalian ABC transporter known to function as an importer and more specifically as a phospholipid flippase.

Analysis of *abca4*^{-/-} mice also supports the role of ABCA4 in the clearance of retinal compounds from photoreceptors [177, 178]. Initial studies reported that mice deficient in ABCA4 display elevated levels of all-*trans* retinal, PE, and *N*-ret-PE compared to wild-type (WT) mice consistent with a reduced efficiency of removal of all-*trans* retinal from photoreceptors. A light-dependent accumulated lipofuscin deposits containing the diretinal compound A2E was also observed in RPE cells of *abca4*^{-/-} mice [177, 179]. A similar accumulation of lipofuscin deposits and A2E is also observed in patients with Stargardt disease [178, 180]. More recently, Boyer et al. confirmed the finding that *abca4*^{-/-} mice show a large increase in A2E and lipofuscin compared to WT mice, but in this study the increase in A2E containing lipofuscin deposits in the *abca4*^{-/-} mice was light-independent [181]. These studies suggest that ABCA4 may play a crucial role in removing excess 11-*cis* retinal as well as all-*trans* retinal from photoreceptors via its *N*-ret-PE transport activity. Whether ABCA4 directly transports the 11-*cis* isomer of *N*-ret-PE across the disc membrane or transports the all-*trans* isomer generated through PE-induced chemical isomerization of 11-*cis* retinal in disc membranes remains to be determined.

4.2.5 ABCA7—ABCA7 is a 220 kDa ABCA transporter sharing a ~54% sequence homology to ABCA1 [182]. The highest levels of expression are found in the brain, lung, myelo-lymphatic tissues, kidneys, macrophages, and platelets [183]. Given the homology of ABCA7 to ABCA1, it was predicted that ABCA7 may stimulate cellular phospholipid and cholesterol efflux to apo-A1 or apo-E. Initial studies confirmed ABCA7 expression promoting efflux of PC and SM, but not cholesterol to apo-A1 [184]. Interestingly, ABCA7

overexpression in HEK293 cells regulates cholesterol efflux only to apo-E disks, which resemble pre-HDL, but not lipid free apo-E or lipid-free apo-A1 (Fig 6A) [185]. The potential of ABCA7 to replicate this activity with reconstituted apo-A1 remains to be tested.

Cholesterol and phospholipid efflux levels of bone marrow-derived macrophages from *abca7*^{-/-} mice were similar to that of wild-type mice [186]. However, female *abca7*^{-/-} mice show a reduction in serum cholesterol, decreased HDL levels, and alteration in adipose mass. The basis of the gender specificity is unknown. A connection to an immune disorder was related to ABCA7 when an epitope of Sjogrens syndrome corresponding to a residual protein fragment of ABCA7 was detected in salivary glands of Sjogren's patients [187]. This epitope corresponds to a region from the first extracellular domain of the membrane protein and is homologous to ABCA1, ABCA2, ABCA3, and ABCA4. Sjogrens syndrome is one of a rare number of organ-specific, autoimmune diseases that is characterized by oral and ocular dryness [188]. Since, ABCA7 is expressed in myelo-lymphatic tissues, it may play an important role in lipid homeostasis in cells of the immune system.

Whereas earlier studies suggested a residual phospholipid efflux role for ABCA7, *abca1*^{-/-} macrophages display no detectable apo-AI stimulated phospholipid efflux activities, inconsistent with this notion [189]. In addition, peritoneal macrophages treated with ABCA7 siRNA and from heterozygous *abca7*^{+/-} mice show unaffected apo-A1 phospholipid release [189]. Recent studies point to a central role of ABCA7 in phagocytosis and engulfment of apoptotic cells (Fig 6A)[190-192]. ABCA7 mRNA and protein levels are upregulated during phagocytosis via the SREBP2 pathway, while siRNA knockdown of ABCA7 results in a decreased phagocytic activity and that *abca7*^{+/-} mice exhibit a defective clearance of apoptotic cells. In ABCA1 deficient fibroblasts, both the phagocytic rate and the expression of ABCA7 are increased [190]. In J774 cells and mouse peritoneal macrophages, phagocytic activity was also stimulated two-fold by apo-AI and apo-AII [192]. In contrast, the phagocytic activity is decreased in the peritoneal cavity of *abca7*^{-/-} mice. This suggests that ABCA7 forms a complex with apo-A1/HDL prior to endocytotic internalization for intracellular mediated proteolysis although a defined mechanism is still unclear. Linsel-Nitschke et al proposed that the contact between ABCA7 and apo-A1 might be restricted to specific cellular conditions [189]. In cases of cell migration or phagocytosis, it is probable that ABCA7 participates in lipid efflux activities in macrophages or blood cells. The authors also allude to the high cell surface expression of ABCA7 in kidneys where this organ plays a distinctive role of apo-A1 catabolism [189].

4.2.6 ABCA12—Mutations in ABCA12 underlie three related autosomal recessive disorders: Harlequin ichthyosis (HI), lamellar ichthyosis type 2, and non-bullous congenital ichthyosiform erythroderma [127, 128, 193]. All three diseases represent dyskeratinization disorders in which the skin develops as thick armour plates, but present with different clinical severity. It is possible that disease-causing mutations are reflected on the level of ABCA12 expression, subcellular localization, and biochemical function that correlates with the intensity of the involved disorder. For instance, a missense mutation of ABCA12 N1380S [127, 128] implicated in HI and corresponding to nucleotide binding domain 1 region: N965S (ABCA4) [19] and N935S (ABCA1) [123] have been shown to reduce PE translocation and cholesterol efflux activities in ABCA4 and ABCA1, respectively.

Extracellular lipids are thought to be essential for skin formation. Ultrastructural experiments point to the absence or malformation of lamellar granules, which physiologically contribute to assemble the skin barrier by exporting their lipid content into the extracellular space during the keratinization process [128]. ABCA12 co-localizes with glucosylceramide to lamellar granules on route from the Golgi apparatus to cell periphery [194]. In the epidermis of *abca12*^{-/-} mice, increased levels of ceramides (of various acyl

chain sizes), sphingosine (breakdown product of ceramide) and glucosylceramides were noted with the largest change observed in ceramides (Fig 6E)[195]. However, no differences in total levels of phospholipids (PC, PI, or PE) were noted. In mouse skin fibroblasts, a modest two-fold cholesterol efflux to apo-A1 was enhanced by LXR activation when compared to *abca12*^{-/-} cells [195]. Like ABCA3, the mechanism of lipid uptake into intracellular compartments mediated by ABCA12 is unknown, as there is no characterization of lipid-protein acceptors mediated by ABC transporters within intracellular compartments.

4.3. ABCB transporter and biliary phospholipid translocation

4.3.1 ABCB1/P-glycoprotein—In addition to transporting a broad spectrum of drugs, P-glycoprotein (Pgp) also functions as a phospholipid transporter with little head group specificity. Initial studies by van Hoolvert et al showed the translocation of short chain NBD-PC and NBD-PE by Pgp across the plasma membrane of LLC-PK1 cells [196]. Using cellular fluorescence in conjunction with FACS analysis, Bosch et al demonstrated a decrease in accumulation of the NBD-PC analog in the multidrug resistant cell line (CEM/VBL300) compared to the parental cell line (CEM) [197]. There was concern that short chain lipid analogs may not reflect the physiological relevance of natural long chain lipid transport. This exception was provided by studies of Platelet Activating Factor (PAF), a naturally occurring short-chain PC and a mediator of inflammation in response to various stimuli [198]. In LLC-PK1 cells, [¹⁴C] PAF translocation to albumin was stimulated by Pgp expression and inhibited by Pgp inhibitors, PSC833 and cyclosporin A [199].

Notwithstanding these inferences, the first direct biochemical evidence of Pgp translocase activity was revealed by Romsicki and Sharom [200]. Using purified Pgp reconstituted into proteoliposomes, translocation of NBD-PC, NBD-PE, NBD-PS, NBD-SM, and a variety of NBD-glycosphingolipids was demonstrated in an ATP-dependent manner (Fig 6A)[200, 201]. Both short chain and long chain NBD-lipid analogues were translocated by Pgp, although the long chain lipid analogues were flipped at ~50% levels compared to the short chain lipid probes. This lipid translocation shared many of the fundamental characteristics of drug transport, such as energy dependence and vanadate sensitivity, and drug competition with lipid flippase activity, suggesting that membrane lipids probably followed a similar path as drug substrates through the Pgp membrane protein.

Interestingly, cholesterol exerted distinct effects on basal and verapamil-induced ATPase activity. The basal ATPase activity of Pgp reconstituted in PC vesicles increased over 5 fold in the presence of 20% cholesterol and was shown to be sterol-specific. In contrast the cholesterol effect on verapamil-induced ATPase activity was unspecific and not related to its influence on membrane fluidity and on verapamil membrane affinity [202].

4.3.2 ABCB4—Biliary PC excretion is essential for preventing bile toxicity by the formation of mixed micelles with bile acids and cholesterol. PC excretion is mediated by a PC specific translocase, ABCB4, which shares 76% identity with Pgp. Several transporters expressed on the hepatocyte canalicular membrane play important roles in canalicular bile formation (Fig 6B). Besides ABCB4, ABCB11 secretes bile salts into the canaliculus while the heterodimeric complex ABCG5/ABCG8 mediates the translocation of cholesterol into bile (Fig 6B)[203]. In addition, the P₄-ATPase ATP8B1 mediates the flipping of PS from the lumen to the cytosolic side and is required to maintain the liquid crystalline nature of the canalicular membrane (Fig 6B)[203]. The clinical significance of these transporters are apparent in a condition known as progressive familial intrahepatic cholestasis (PFIC), a disorder characterized by jaundice, growth retardation, and progressive liver damage due to

impaired bile flow. In order of phenotype severity, mutations in ATP8B1, ABCB11, and ABCB4 contribute to PFIC type 1, PFIC type 2, and PFIC type 3, respectively [204].

Abcb4^{-/-} mice develop severe liver disease characterized by a lack of PC in bile [205, 206] while transgenic infusion of human ABCB4 gene into these mice rescue PC translocation activity into bile [207]. Polarized cells transfected with ABCB4 showed increased rates of NBD-PC translocation to the outer apical leaflet when compared to the absence of activity of NBD-PE and NBD-SM [196]. Smith et al demonstrate PC translocation in fibroblasts overexpressing ABCB4 by metabolic labeling with radioactive choline [207]. In this case, PC-transfer protein was used as an acceptor to monitor radioactive PC secretion. In addition, ABCB4 transfected in HEK293 cells demonstrated PC efflux in the presence of taurocholate, a mimic analog of bile salt [208]. The specificity of this assay was surprising because cholesterol efflux into bile salts was also noted along with PC efflux. In the canalicular membrane, the major cholesterol excretion is driven by the activities of the ABCG5 and ABCG8 complex (discussed below). This observation may be a side activity of taurocholate and, perhaps, using the natural lipid acceptor is not feasible as micellar concentrations of bile salts induce cytolysis in cultured cells. More recently, Groen et al illustrated the importance of ATP8B1 as being essential for cellular survival in the presence ABCB4 [209]. Transient expression of ABCB4 in HEK293 cells decreased protein levels and compromised the integrity of the cellular membrane, as noted by the leakage of lactate dehydrogenase into the culture medium. However, co-expression of ATP8B1 and ABCB4 rescued protein expression levels and displayed PC secretion in a bile salt dependent manner [209].

4.4 ABCC1

Members of the ABCC subfamily are full transporters with several members (ABCC1/2/3/6/10) containing an additional TMD on the N-terminus. The prototypical member, ABCC1, has a ubiquitous expression profile and confers resistance to a broad-spectrum of antitumor drugs, glutathione-conjugates, and sulfate-conjugates [210-212]. In membrane vesicle studies, verapamil stimulates transport of GSH via ABCC1 but conjugated oestrogen, oestrone sulfate, and vincristine are efficiently transported without reciprocal translocation of GSH suggestive of a specific allosteric or co-transport role of GSH among different substrates [213-215]. *Abcc1*^{-/-} mice are healthy but have compromised inflammatory responses due to decreased secretion of leukotriene C4, an endogenous substrate of ABCC1 [216]. Red blood cells derived from *abcc1*^{-/-} mice loses the ability to translocate NBD-PC and NBD-PS [217]. Active translocation of NBD-PS in wild-type erythrocyte membranes was inhibited by various antitumor drugs, glutathione-conjugates, and oxidized GSSG, indicating ABCC1 may function as a phospholipid translocase [218]. In addition, LLC-PK1 cells transfected with ABCC1 specifically flipped NBD-GlcCer and NBD-SM [219]. Direct evidence showing that ABCC1 may function as a phospholipid translocase was provided by Huang et al, whereby purified ABCC1 reconstituted into proteoliposomes demonstrated NBD-PC, but not NBD-PE, translocation (Fig 6A)[220]. Interestingly, transport of lipid was enhanced by inclusion of GSH. Further studies are required to assess whether GSH is co-transported or serves as an allosteric modulator in PC transport. In addition, a few drugs including vinblastine and doxorubicin partially inhibited NBD-PC, whereas MK-571, an inhibitor of ABCC1 protein, inhibited lipid translocation [220]. Phospholipid translocation by other homologous ABCC subfamily members remains to be investigated.

4.5. ABCG Transporters

Except for ABCG2, all members of the ABCG family function as cholesterol transporters [7, 221]. This subfamily has five members: ABCG1, ABCG2, ABCG4, ABCG5, and ABCG8.

All members are half transporters in which two polypeptide chains each containing a single NBD domain followed by a TMD assemble as either homo- or hetero-dimers to form a functional complex.

4.5.1 ABCG1—ABCG1 has been demonstrated to promote cholesterol efflux to HDL, but the mechanism by which ABCG1 mediates cholesterol efflux is not well understood. Like ABCA1, ABCG1 displays similar tissue expression profiles and regulatory pathways [222-226]. In contrast to ABCA1, the efflux activity of ABCG1 is relatively nonspecific, as cholesterol efflux can be promoted to synthetic PC vesicles > LDL > HDL2 > HDL3 [223, 227]. Intriguingly, phospholipid efflux was also observed but occurred in the opposite preference to cholesterol acceptors. Targeted disruption of *abcg1* in mice has no effect on plasma lipids but results in massive accumulation of both neutral lipids and phospholipids in hepatocytes and in macrophages within multiple tissues [222]. In cell cultures of HEK293 and HepG2, transfected ABCG1 altered cellular morphology and caspase-3 activation, and PS externalization events were observed [228]. Apparently, benzamil and thyroxine, effective inhibitors of ABCG1 ATPase activity, also decreased annexin V binding indicative of attenuated PS translocation. In addition, PC and SM efflux along with cholesterol has been demonstrated by ABCG1 in HEK293 cells.

Several studies in different cell types have differed on the cellular localization of ABCG1 with minor trafficking to the plasma membrane and the bulk residing in intracellular vesicles [229, 230]. More recently, Tarling and Edwards showed that ABCG1 is specifically associated with endosomal vesicles and, unlike ABCA1, is independent of LXR activation for plasma membrane localization in peritoneal macrophages [231]. Accordingly, this suggests that ABCG1 transfers sterols to the inner leaflet of endosomal vesicles before their fusion to the plasma membrane. This would result in redistribution of these sterols and phospholipids to the outer leaflet of the plasma membrane such that they can desorb in a non-specific manner to multiple acceptors including LDL and HDL.

Studies with *abcg1*^{-/-} *abca1*^{-/-} mice demonstrated that loss of both sterol transporters resulted in an even more striking lipid accumulation phenotype in macrophages and specific tissues than in single knockout mice [232, 233]. Thus, both transporters are important for sterol homeostasis and one cannot complement for loss of the other. Sr-B1 is a transmembrane receptor mediating bidirectional cholesterol flux, depending on concentration gradients, in the plasma membrane [221]. A systematic approach using normal and cholesterol loaded primary peritoneal macrophages from *abca1*^{-/-}, *abcg1*^{-/-}, or *Sr-B1*^{-/-} mice was probed to determine the relative contributions of these transmembrane proteins [234]. A substantial 20% of intracellular cholesterol was mobilized/effluxed by ABCG1, ~35% by ABCA1, ~10% by SR-B1 and 35% by human serum (diffusion). In addition, phospholipid efflux analysis via mass spectrometry and TLC revealed that ABCG1 and ABCA1 effluxed several species of SM and PC, with ABCG1 transporting both SM and PC and ABCA1 preferentially transporting PC [235]. These multiple mechanisms point to the importance of maintaining cholesterol and phospholipid levels for cell maintenance and viability.

4.5.2 ABCG2—ABCG2 forms a homodimer that localizes to the plasma membrane of cells. ABCG2 is highly expressed in normal epithelial cells of the placenta, kidney, and intestine, where it has been suggested to play a role in regulating the absorption, circulation and metabolism of xenobiotics [221]. ABCG2 was shown to promote the exofacial exposure of NBD-PS and endogenous PS, as determined by Annexin V binding, in EPG85-257 human gastric carcinoma and MCF-7 breast cancer cells [236].

4.5.3 ABCG4—ABCG4 is 94% identical to ABCG1 but its tissue expression is restricted with the highest levels found in the brain, spinal cord, and testis [237]. ABCG4 also

promotes cholesterol efflux to HDL [223, 238]. Vaughan et al showed that ABCG4, in addition to ABCG1, act sequentially with ABCA1 to efflux cholesterol and phospholipids and increase the formation of HDL-like particles, although the gel filtration chromatography of HDL particles for the ABCG4 or ABCG1 expressing cells showed some heterogeneity in the cholesterol distribution [239]. These findings raise the possibility that the ABCG1 and ABCG4 cholesterol export pathways remodel nascent HDL particles in slightly different ways. The observation is suggestive of the fact that these transporters can work in tandem where ABCA1 performs initial lipidation of apo-A1 generating HDL which is then used as an acceptor ABCG1/G4 transport of cholesterol.

4.5.4 ABCG5/ABCG8—The transport system contributing major cholesterol secretion into bile is the heterodimeric transporter ABCG5/G8 [240]. Mutations causing a loss of function of ABCG5 or ABCG8 are associated with β -sitosterolemia, a disorder characterized by enhanced accumulation of plant and shellfish sterols [241]. Largely present in intestines and liver, ABCG5 and ABCG8 heterodimers also transfer plant and shellfish sterols obtained from diet. In *abcg5/g8*^{-/-} mice, biliary cholesterol secretion is significantly reduced [242], whereas it is significantly increased in response to overexpression in hepatocytes [243]. Wang et al developed the first biochemical sterol transfer assay expressing recombinant ABCG5/G8 heterodimer using purified proteoliposomes and “inside-outside” Sf9 membrane vesicles. Cholesterol and sitosterol, but not PC, behaved as transport substrates and were transferred in an energy dependent manner (Fig 6B)[226, 238]. Similar studies with ABCA1 would also clarify the preferred substrates transported to apo-A1 as part of the RCT pathway. In addition, a sterol binding/sensing motif (L/MxxLxxL) found in steroid/hormone receptors has been identified in the TMD of ABCG2 and is conserved among other family members, including ABCG5/G8 [244]. It will be of interest to evaluate if sterols/other classes of lipids are modulated by ABC transporters involved/implicated in cholesterol efflux.

5. Apo-E and lipid homeostasis in the brain

The brain is separated from plasma via the blood-brain-barrier and thus operates its own lipoprotein system. The cerebrospinal spinal fluid (CSF) contains lipoproteins whose composition is distinct from that in plasma. Several ABC transporters are expressed in the brain, including ABCA1 and ABCG1 which play important roles in the transfer of phospholipid and cholesterol efflux to apo-E [130]. The major apolipoprotein of HDL-like particles in the central nervous system (CNS) is apo-E which is made by astrocytes (and a few types of neurons), cells associated with the maintenance of neurons. Apo-E is secreted by glial cells and assembled with phospholipids and cholesterol into HDL-sized lipoprotein particles.

Studies on transgenic mice lacking or over-expressing ABCA1 support the view that this ABC transporter contributes to the lipidation of apo-E, although its role in astrocytes remains to be clarified. *Abca1*^{-/-} mice have reduced cholesterol content and size of apo-E in CSF by 24% and reduced cholesterol concentration in the culture medium of astrocytes [245, 246]. Conversely, overexpression of ABCA1 in the mouse brain increased the size of CSF lipoproteins and enhanced the cholesterol to apo-E ratio in lipoproteins from cultured astrocytes [247]. This may signify that in the absence of ABCA1, there is enhanced catabolism of apo-E due to insufficient lipidation. Finally, absence of apo-E enhanced the transcript level of *abca1* in the brain indicating that ABC transporters can compensate for the loss of apo-E-mediated cholesterol release. Astrocyte-secreted human apo-E is present in high density-like lipoproteins of three predominant sizes ranging from 8 to 15 nm in diameter. ABCA1 and ABCG1 are expressed in the brain, in both neurons and glial cells, suggesting that these transporters participate in the formation of apo-E-containing

lipoproteins in the brain. Detailed characterization of phospholipid composition by electrospray ionization mass spectrometry analysis showed that PE was more abundant than PC in the apo-E particles [248]. The significance of this unusual composition remains to be established.

The residual efflux of cholesterol from astrocytes lacking ABCA1 may be mediated by ABCG transporters. Astrocytes from mice lacking both ABCG1 and ABCG4 showed reduced release of cholesterol and desmosterol onto high-density lipoprotein particles [238] and in cultured cerebellar glia, the extent of cholesterol efflux correlated with the expression level of ABCG1, but not of ABCA1 [249]. On the other hand, overexpression of ABCG1 in the CNS did not affect the apo-E content in the brain or the cholesterol efflux from cultured glial cells [250]. Interestingly, increasing the levels of ABCG1 in glial cells enhanced the ability of glia-derived lipoproteins to stimulate axonal growth *in vitro* [251].

In summary, there is good evidence that ABC transporters in astrocytes secrete cholesterol and phospholipids via apo-E containing lipoproteins. These lipoproteins exhibit different properties than those found in plasma, although the molecular mechanisms underlying these differences remain to be investigated.

Conclusions and Future Directions

Over the past decade significant progress has been made in understanding the molecular and cellular basis for phospholipid transport across membranes. P₄-ATPases and some members of the ABC transporter family are the principal membrane proteins that carry out active phospholipid transport. Genetic, biochemical, molecular and cell biology studies using various eukaryotic cell systems including yeast, plants, mice, humans and other organisms point to the crucial role of these lipid transporters in the generation and maintenance of lipid asymmetry, lipidation of plasma proteins, cholesterol efflux, bile production, membrane budding and vesicle trafficking, vision, phagocytosis, apoptosis, membrane protein function and regulation, and membrane curvature among others.

Recent biochemical studies now directly support the function of P₄-ATPases as flippases which actively transport aminophospholipids from the exocytosolic to the cytosolic leaflet of cell membranes. The contributions of CDC50 β -subunits on modulation of the lipid transport activity of P₄-ATPases, protein stability, and cellular localization are becoming more well-defined. Mutagenesis studies in conjunction with improved protein expression systems and molecular modeling based on other P-type ATPases have identified a number of key residues important in the phospholipid translocation pathway and plausible steps in the catalytic transport cycle.

However, many fundamental issues remain to be resolved. Although sequence analysis suggests that all 14 members of the mammalian P₄-ATPase family may function as phospholipid flippases, to date only one member, ATP8A2, has been purified and shown experimentally to actively flip aminophospholipids and several other members have been implicated in aminophospholipid transport on the basis of genetic and cellular studies. A future challenge is to isolate and characterize other P₄-ATPases to more fully understand their phospholipid substrate specificity, define interactions with CDC50 subunit and other cellular proteins, determine the *in vivo* tissue distribution and subcellular localization, and elucidate regulatory mechanisms. Additionally, the role of various P₄-ATPases in such cellular processes as vesicle trafficking, bile secretion, fertilization, lipid asymmetry, phagocytosis and membrane protein structure and function needs further study through the generation and characterization of transgenic and knockout animal models, analysis of protein-protein interactions, and functional studies. Although many of the structural properties of P₄-ATPases appear to resemble other P-type ATPases, it will be important to

determine the high resolution structure of a P₄-ATPase to more fully define phospholipid recognition and transport mechanisms.

ABC transporters are known to transport a diverse set of substrates across cell membranes. Cell-based studies have implicated many ABC transporters in the movement of phospholipids across cell membranes. These include some members of the ABCA, ABCB, ABCC and ABCG subfamilies. In many cases these ABC transporters appear to serve as 'true' exporters in that they transport phospholipids from the cytoplasmic leaflet of a membrane to acceptor proteins or extrude the phospholipid into the extracellular medium. In some instances, however, ABC transporters appear to function as floppases or flippases, translocating phospholipids from one leaflet of the lipid bilayer to the other. The importance of ABC transporters in phospholipid translocation is apparent in the finding that mutations in genes encoding many of these lipid transporters cause severe human diseases.

Despite the emerging importance of ABC transporters in lipid transport and homeostasis, relatively few ABC transporters have been characterized at biochemical level. As a result the information on the phospholipid substrate specificity, transport mechanisms and regulatory properties are limited. Likewise, the high-resolution structures for only a few ABC transporters have been reported to date. Additional structures with and without bound phospholipid substrates and nucleotides will be needed to more fully clarify the mechanism by which ATP binding and hydrolysis promotes phospholipid transport by ABC proteins.

The next decade should witness significant advances in our understanding of phospholipid transport by P₄-ATPases and ABC transporters at a molecular and cellular level. This knowledge should provide increased insight into the role of these transporters in physiological processes and facilitate the search for therapeutic treatments for diseases linked to defects in phospholipid transport.

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Highlights

- Up to date review on phospholipid transport across cell membranes
- Review of the role of P4-ATPases in the flipping of aminophospholipids across cell membranes
- Review of the role of ABC transporters in the efflux and flipping of phospholipids across cell membranes.
- Overview of the role of P4-ATases and ABC transporters in various cellular processes and disease

Fig. 1.

Regulation of lipid asymmetry in biological membranes. The distribution of lipids in biological membranes is regulated by three distinct families of membrane transporters: ABC transporters, P₄-ATPases, and scramblases. Most ABC transporters catalyze the ATP-dependent transport of lipids from the cytoplasmic leaflet of the bilayer to the extracellular (luminal) side of the membrane while P₄-ATPases transport in the opposite direction. ABC transporters can efflux lipids to lipoproteins such as Apo-A1 in the case of ABCA1 or to bile micelles. Recent studies indicate that a few ABC transporters are importers or flippases transporting phospholipids in the same direction as P₄-ATPases. Scramblases are energy independent transporters and act to abolish lipid asymmetry by randomizing lipid distributions. Lipids such as PC, SM, and glycolipids are found in the extracellular or luminal leaflet while the aminophospholipids PE and PS are preferentially on the cytosolic leaflet. Abbreviations used: PC, phosphatidylcholine; SM, sphingomyelin; PE, phosphatidylethanolamine; PS, phosphatidylserine; GSL, glycosphingolipid; Chol, cholesterol.

Fig. 2.

Phylogenetic tree of human P-type and P₄-ATPases. **(A)** There are 36 different genes which encode for P-type ATPases in humans. Type IB are heavy metal pumps, Type IIA and Type IIB are Ca²⁺ pumps, and Type IIC are Na⁺/K⁺ or H⁺/K⁺ pumps. Type IV ATPases transport phospholipids. Type V ATPases have no assigned specificity. Type IA and Type III pumps are not found in humans. **(B)** There are 14 different Type IV ATPases in humans which are organized into five different subfamilies according to sequence. Trees were generated using ClustalX and visualized using the Interactive Tree of Life online tool (<http://itol.embl.de/>).

Fig. 3.

Domain structure, organization, and proposed mechanism of P₄-ATPases. **(A)** P₄-ATPases adopt a four domain structure consisting of cytosolic A (actuator), P (phosphorylation), and N (nucleotide binding) domains as well as a M (membrane) domain consisting of 10 membrane spanning segments and containing the translocation pathway. Absolutely conserved motifs are shown. P-type ATPases are phosphorylated at the aspartic acid of the invariant DKTG motif. The glutamic acid of the DGE motif in the A domain catalyzes dephosphorylation. The CDC50 β -subunit is shown containing four N-linked glycosylations and two disulfide linkages in the large E (extracellular) domain. **(B)** P-type ATPases exist in four primary conformations. Binding of ATP and phosphorylation of the P-domain in the E₁ form converts the enzyme to E₁P. During the E₁P to E₂P transition, the A-domain rotates allowing lipids to bind to an extracellular binding site in the M-domain in the E₂P conformation. Dephosphorylation of E₂P drives the translocation of the lipid through the membrane to the cytoplasmic side. The enzyme is converted back to the E₁ form when the A-domain moves away from the P-domain. The role of CDC50 (green) is unknown.

Fig 4. Predicted two-dimensional topology of lipid ABC transporters and models of ABC transporter mechanism. A. The two transmembrane domains (TMDs) and nucleotide binding domains (NBDs) arrange to form full transporter and are represented by some ABCB members and ABCA members with additional exocytosolic domains. Some ABCC family members are full transporters with an additional N-terminal TMD. Half transporters in ABCB and ABCG subfamilies have different modular organizations of a TMD and NBD. B. A simplified 'Switch Model': In the inward-facing conformation, the NBDs are separated and nucleotide-free. Phospholipid substrate entry into the substrate binding site occurs from the cytosolic leaflet of the membrane bilayer. ATP-dependent dimerization of NBDs progresses to pull the TMDs from an inward- to outward-facing conformation. Phospholipid is translocated to the extracellular side of the membrane and ATP hydrolysis resets the transporter in the inward conformation. C. A simplified 'Constant-contact Model': The two NBDs remain associated in the sandwich dimer form throughout the transport cycle. Inward and outward conformations are directed via subunit conformational changes and the twisting motion of NBDs. ATP hydrolysis alternates with one site hydrolyzing nucleotide that facilitates the binding and hydrolysis in the other site.

Fig 5.

Apo-A1 lipidation via ABCA1 activity within plasma membrane microdomains. Lipid free apo-A1 initially interacts with monomeric or oligomeric ABCA1. The PC flippase activity of ABCA1 creates a High Capacity Binding Site (HCBS) within cholesterol poor microdomains. Phospholipid translocation via ABCA1 may also induce membrane bending of the bilayer to create an exovesiculated binding site. ABCA1 subsequently mediates transfer of apo-A1 to these HCBS populations, from which apo-A1 selectively microsolubilizes PC to become discoidal apo-A1 species. Further microsolubilization of cholesterol and PC generates nascent HDL with two, three, and four molecules of apo-A1.

Fig 6.

A. Overview of ABC transporters involved in lipid efflux. Schematic representation of general ABC transporter localization, known acceptors and direction of transport. Vectorial transport depicted by black arrows at the plasma membrane. Vectorial transport in many ABC transporters and by intracellular ABC transporters has not been firmly established. B. Hepatocytic ABC transporters. Newly synthesized bile salts are effluxed by ABCB11, where they form micelles with PC translocated to the outer leaflet of the canalicular membrane by ABCB4. ABCG5/G8 translocates cholesterol into the bile lipid mixture. The action of PS translocation to the inner leaflet by ATP8B1 maintains the lipid asymmetry at the canalicular membrane. C. Role of ABCA4 in photoreceptor disc membranes. Photoexcitation of disc membranes in photoreceptors causes release of all-trans retinal from rhodopsin. Retinal is reduced in the visual cycle or reacts with PE in disc membranes to form *N*-ret PE. ABCA4 binds and translocates *N*-ret PE from the lumen to the cytosolic side.

of the disc membrane. All-trans retinal then dissociates from *N*-ret PE and retinal is shuttled into the visual cycle. ABCA4 also translocates PE from the lumen to the cytosolic side of the disc membrane. D. Surfactant production in alveolus. In alveolar type II cells, ABCA3 present in lamellar bodies translocates PG, PC, and PE which is then secreted into the epithelial lining of the surfactant. E. Epidermal production in skin. ABCA12 transports GlcCer and Cer into the lamellar body which is then redistributed into the cell periphery. Apo-A1/E, apolipoprotein; HDL High Density Lipoprotein; LDL, low density lipoproteins; SR-B1, Scavenger receptor Class B1 protein; Chol, cholesterol; PC, phosphatidylcholine, PE, phosphatidylethanolamine; PS, phosphatidylserine; Cer, ceramine; GlcCer, glucosylceramide; *N*-Ret PE, *N*-retinyldiene PE; SM, sphingomyelin

Table 1

P₄-ATPase and CDC50 Families

P ₄ -ATPase	β-subunit	Substrate	Expression Pattern	Pathophysiology	References
ATP8A1	CDC50A (CDC50B?)	PS, isoforms differ in PE selectivity	Ubiquitous, high in skeletal muscle, thyroid, spinal cord	Defective hippocampus-dependent learning	[15, 30, 50, 55, 66, 67]
ATP8A2	CDC50A	PS, weak PE transporter	Brain, retina, testis, spinal cord	Neurological, spinal, axonal degeneration (mental retardation?)	[11, 30, 31, 68-70, 73]
ATP8B1	CDC50A, CDC50B	PS, CL?	Ubiquitous, high in small intestine, pancreas	Intrahepatic cholestasis, hearing loss, pneumonia	[16, 29, 30, 50, 74, 75]
ATP8B2	CDC50A, CDC50B	Unknown	Ubiquitous	Unknown	[252]
ATP8B3	Unknown	PS	Testis	Defective sperm-egg interactions	[30, 82, 83]
ATP8B4	CDC50A, (CDC50B?)	Unknown	Moderate levels throughout brain	Alzheimer disease?	[30, 50, 81, 253]
ATP9A	Not detected	Unknown	Ubiquitous, high in brain.	Unknown	[28, 254]
ATP9B	Not detected	Unknown	Ubiquitous, high in testis	Unknown	[28, 254]
ATP10A	CDC50A for ER exit	Unknown	High in brain, pancreas, kidney, lung	Angelman syndrome, obesity, diabetes	[28, 86-89, 91, 92]
ATP10B	CDC50A for ER exit	Unknown	Low expression, brain.	Unknown	[28, 255]
ATP10D	CDC50A for ER exit	Unknown	High in placenta, low kidney, undetectable in other major organs	Obesity?	[28, 90]
ATP11A	CDC50A	Unknown	Ubiquitous, moderate levels in liver, skeletal muscle, ovary	Unknown	[28, 256]
ATP11B	CDC50A	Unknown	Ubiquitous, high levels in kidney, testis, ovary	Unknown	[28, 257]
ATP11C	CDC50A	PS	Ubiquitous, high liver, pancreas, heart	Impaired B lymphocyte differentiation, cholestasis	[13, 28, 93, 94, 258]

Table 2

Mammalian ABC transporters, related lipid substrates, and associated genetic disorders.

Gene	Major sites of expression	Substrates	Modulators	Acceptors	Associated genetic disorders	References
<i>ABCA1</i>	Ubiquitous	PC, PS, Chol, SM,	Cer (stimulates) LacCer (inhibits)	Apo-A1, AII, E,	Tangier disease	[123, 151, 152, 259, 260]
<i>ABCA2</i>	Brain	Chol, PE, PS		LDL		[157, 158]
<i>ABCA3</i>	Lung, brain, heart, pancreas	PC, PG, PE		Surfactant	Neonatal surfactant lung deficiency, chronic interstitial lung disease	[125, 159-162]
<i>ABCA4 (ABCR)</i>	Rod photoreceptors	<i>N</i> -ret-PE, PE	All- <i>trans</i> retinal 11 <i>cis</i> -retinal	(Cytosolic)	Stargardt's disease, cone-rod dystrophy, retinitis pigmentosa, age-related macular degeneration	[19, 126, 167-169, 174, 175]
<i>ABCA7</i>	Myelolymphatic system, brain, kidney, skin	PC, SM, Chol, (PS?)		Apo-AI/Apo-AII, Apo-E disc	(Sjogren's syndrome?)	[184-187]
<i>ABCA12</i>	Skin keratinocytes	Cer, GlcCer			Harlequin ichthyosis, lamellar ichthyosis type 2, non-bullous congenital ichthyosiform erythroderma	[127, 128, 193-195]
<i>ABCB1 (Pgp)</i>	Brain, liver, kidneys, GI, placenta	PC, PS, PE, Chol, SM, GlcCer, PAF	short chain PC (8:0), DHA PC (22:0) (inhibits)			[196, 197, 199-201, 261]
<i>ABCB4</i>	Liver, canalicular membrane, placenta	PC, PE, SM		Bile salts	Progressive familial intrahepatic cholestasis type 3	[196, 205-207, 262]
<i>ABCB11</i>	canalicular membrane	Bile salt			Progressive familial intrahepatic cholestasis type 2	[196, 203]
<i>ABCC1</i>	Ubiquitous	PC, PS, SM, GlcCer	GSH (stimulates)			[217-220]
<i>ABCG1</i>	Ubiquitous	Chol, PS, PC, SM,	Thyroxin (inhibits) Benzamil (inhibits) SM (stimulates)	HDL, LDL, PC vesicle, BSA, cyclodextrin		[231] [223, 227, 228, 235]
<i>ABCG2</i>	Placenta, breast, liver, GI	PC, PS	Chol (stimulates)			[236, 263, 264]
<i>ABCG4</i>	Macrophage, brain, eye, spleen, liver	Chol				[223, 238, 239]
<i>ABCG5/G8</i>	Liver, GI	Plant sterols, Chol		HDL, Bile salts	β -Sitosterolemia	[229, 241, 265]

Apo, apolipoprotein; Cer, ceramide; Chol, cholesterol; DHA, docosahexaenoic acid; LacCer, Lactosylceramide; LDL, low density lipoprotein; GlcCer, glucosylceramide; GSH, glutathione, GI, gastrointestinal tract;; HDL, high-density lipoprotein; PAF, platelet activating factor