Lipid transport by mammalian ABC proteins

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Abstract

ABC (ATP-binding cassette) proteins actively transport a wide variety of substrates, including peptides, amino acids, sugars, metals, drugs, vitamins and lipids, across extracellular and intracellular membranes. Of the 49 human ABC proteins, a significant number are known to mediate the extrusion of lipids from membranes or the flipping of membrane lipids across the bilayer to generate and maintain membrane lipid asymmetry. Typical lipid substrates include phospholipids, sterols, sphingolipids, bile acids and related lipid conjugates. Members of the ABCA subfamily of ABC transporters and other ABC proteins such as ABCB4, ABCG1 and ABCG5/8 implicated in lipid transport play important roles in diverse biological processes such as cell signalling, membrane lipid asymmetry, removal of potentially toxic compounds and metabolites, and apoptosis. The importance of these ABC lipid transporters in cell physiology is evident from the finding that mutations in the genes encoding many of these proteins are responsible for severe inherited diseases. For example, mutations in ABCA1 cause Tangier disease associated with defective efflux of cholesterol and phosphatidylcholine from the plasma membrane to the lipid acceptor protein apoA1 (apolipoprotein A1), mutations in ABCA3 cause neonatal surfactant deficiency associated with a loss in secretion of the

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lipid pulmonary surfactants from lungs of newborns, mutations in \textit{ABCA4} cause Stargardt macular degeneration, a retinal degenerative disease linked to the reduced clearance of retinoid compounds from photoreceptor cells, mutations in \textit{ABCA12} cause harlequin and lamellar ichthyosis, skin diseases associated with defective lipid trafficking in keratinocytes, and mutations in \textit{ABCB4} and \textit{ABCG5}/\textit{ABCG8} are responsible for progressive intrafamilial hepatic disease and sitosterolaemia associated with defective phospholipid and sterol transport respectively. This chapter highlights the involvement of various mammalian ABC transporters in lipid transport in the context of their role in cell signalling, cellular homoeostasis, apoptosis and inherited disorders.

\textbf{Introduction}

The hydrophobic lipid bilayer separates eukaryotic cells from their extracellular environment and further compartmentalizes these cells into distinct organelles. Assembly and maintenance of the various cellular membranes requires translocation of lipids from one leaflet of the bilayer to the opposing leaflet. The plasma membrane, endosomes and lysosomes depend completely on lipid transport and synthesis from other organelles, in particular from the ER (endoplasmic reticulum). The lipid composition of separate leaflets of the membrane bilayer generally show a clear asymmetric arrangement, with the majority of the aminophospholipids PS (phosphatidylserine) and PE (phosphatidylethanolamine), typically present in the inner, cytoplasmic, leaflet, and PC (phosphatidylcholine), SM (sphingomyelin) and glycolipids predominantly, if not exclusively localized in the outer, exoplasmic, leaflet. The plasma membranes, Golgi and endosomal membranes, unlike the membranes of the ER, display high lipid asymmetry [1]. This bilayer asymmetry is less governed by the size, charge and polarity of the headgroup, but more so by ATP-dependent protein translocators. This type of distribution influences important physiological functions such as cell viability, membrane fusion, cell–cell recognition, and protein function and regulation.

Maintenance of lipid asymmetry is accomplished by integral membrane transporters that specifically flip (out-to-in translocation), flop (in-to-out translocation) or scramble lipids across the bilayer (Figure 1). Lipid flippases and floppases are ATP-dependent membrane proteins that maintain transbilayer distribution of phospholipids by translocating specific phospholipid species from the cytoplasmic to the exoplasmic leaflet of the bilayer and vice versa respectively. P4 ATPases are a subclass of P-type ATPases that function as phospholipid flippases, whereas ABC (ATP-binding cassette) transporters have been generally shown to act as lipid floppases [2,3]. In contrast, asymmetric lipid distribution can be undone by the Ca$^{2+}$-dependent bidirectional activities of scramblases that tend to act with a low lipid headgroup specificity.
To date, 49 mammalian ABC transporters have been identified in the human genome. These have been organized into seven subfamilies: ABCA–ABCG. In most cases, these ABC proteins are either full-transporters consisting of a single polypeptide, as exemplified by ABCA1, or half-transporters consisting of homo- or hetero-dimers such as ABCG1 (see Figure 3) [4]. In either case, the ABC transporters contain at least two TMDs (transmembrane domains) with multiple membrane-spanning segments and two NBDs (nucleotide-binding domains). A significant number of mammalian ABC proteins are involved in the transport and regulation of steroids/steroid conjugates and phospho-/glyco-/sphingo-lipids and, as such, they participate in lipid trafficking, lipid asymmetry and lipid homeostasis across cellular membranes [5,6] (Figure 2 and Table 1).

Genetic defects in six ABC transporters that function in lipid trafficking in cells have been linked to a variety of severe human diseases [7] (Table 1). For instance, inherited defects in ABCA1 which mediate the efflux of cholesterol and phospholipids from cells cause Tangier disease [8], defects in ABCA3, a transporter of lipid molecules in the lung, is associated with neonatal surfactant deficiency in newborns [9], mutations in ABCA4, a retinal-PE transporter, are linked to a variety of retinal degenerative diseases including
Stargardt macular degeneration [10], defects in \( \text{ABCG5/ABCG8} \), transporters of nutritional plant sterols, are linked to sitosterolaemia [11], and defects in \( \text{ABCB4} \) are involved in the transport of PC on to bile micelles cause PFIC (progressive familial intrahepatic cholestasis) [12].

This chapter presents a snapshot of the current position of the field by focusing on a varied selection of mammalian ABC transporters, classes of lipids which are transported, and molecular mechanisms underlying lipid transport (Figure 2). The role of lipids after membrane redistribution in response to cellular events and their underlying disorders are also reviewed.

**Mechanism of lipid efflux**

Two general models have been proposed to explain the transport of lipid substrates across membranes: ‘hydrophobic vacuum cleaner’ and ‘flippase’ models [13]. In the hydrophobic vacuum cleaner model, lipid substrates diffuse into the membrane bilayer and are subsequently extruded from a central channel of the transporter into the extracellular space in an ATP-dependent process [14]. This model typically explains the drug-efflux action observed for multidrug-resistance transporters. The flippase model, on the other hand, is used to explain the translocation of lipids, most commonly...
Table 1. Mammalian ABC transporters, lipid substrates and associated human genetic disorders

<table>
<thead>
<tr>
<th>Gene</th>
<th>Tissue expression</th>
<th>Lipids transported</th>
<th>Acceptors</th>
<th>Genetic disorders</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABCA1</td>
<td>Universal</td>
<td>Chol, PS, SM, PC, S1P, 25-OH-chol</td>
<td>Apolipoproteins AI, AII, E, CI, CII, CIII and AIV</td>
<td>Tangier disease, familial hypoalphalipoproteinaemia</td>
<td>[30,83,90]</td>
</tr>
<tr>
<td>ABCA2</td>
<td>Brain</td>
<td>Chol, SM, PS, PE, Cer</td>
<td></td>
<td></td>
<td>[34,52]</td>
</tr>
<tr>
<td>ABCA3</td>
<td>Lung, pancreas, brain, heart</td>
<td>Chol, SM, PC</td>
<td></td>
<td></td>
<td>[9,35,53]</td>
</tr>
<tr>
<td>ABCA4</td>
<td>Retinal photoreceptors</td>
<td>N-retinylidene-PE</td>
<td>(Cytosolic)</td>
<td>Stargardt macular degeneration, cone–rod dystrophy, retinitis pigmentosa, age-related macular degeneration</td>
<td>[54,55,60]</td>
</tr>
<tr>
<td>ABCA7</td>
<td>Brain, skin, myelolymphatic system</td>
<td>Chol, PS, PC, Cer</td>
<td>ApoAI</td>
<td></td>
<td>[36,81]</td>
</tr>
<tr>
<td>ABCA12</td>
<td>Keratinocytes</td>
<td>GlcCer</td>
<td></td>
<td>Harlequin ichthyosis</td>
<td>[80]</td>
</tr>
<tr>
<td>ABCB1</td>
<td>Placenta, brain, liver, kidneys</td>
<td>SM, GlcCer, sphingoid bases, PC, PS, PE, Chol, PAF, corticosteroids, androgens, oestrogens, progestins</td>
<td></td>
<td></td>
<td>[45–48,68]</td>
</tr>
<tr>
<td>ABCB4</td>
<td>Liver, bile, canicular membrane, placenta</td>
<td>PC</td>
<td></td>
<td>PFIC</td>
<td>[12]</td>
</tr>
<tr>
<td>ABCC1</td>
<td>Placenta</td>
<td>LTC4, GlcCer, SM, PC, S1P, GSH, UGT, steroid conjugates</td>
<td></td>
<td></td>
<td>[29,49,78,95]</td>
</tr>
<tr>
<td>ABCG1</td>
<td>Universal</td>
<td>Chol, PC, SM, 7β-OH-chol, 7-oxo-chol</td>
<td>HDL</td>
<td></td>
<td>[40,42,50,96]</td>
</tr>
<tr>
<td>ABCG2</td>
<td>Placenta, breast, liver, gastrointestinal tract</td>
<td>PC, PS</td>
<td></td>
<td></td>
<td>[88]</td>
</tr>
<tr>
<td>ABCG4</td>
<td>Macrophage, brain, eye, spleen, liver</td>
<td>Chol</td>
<td></td>
<td></td>
<td>[29]</td>
</tr>
<tr>
<td>ABCG5/</td>
<td>Liver, gastrointestinal tract</td>
<td>Plant sterols, Chol</td>
<td>HDL bile salts</td>
<td>Sitosterolaemia</td>
<td>[11,44]</td>
</tr>
<tr>
<td>ABCG8</td>
<td></td>
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phospholipids, from the inner to outer leaflet of biological membranes [15]. Additionally, for many lipophilic substrates, the docking of an acceptor protein creates energetically favourably conditions required to promote dissociation of the lipid from the donor membrane, thereby facilitating its efflux from the cell. In this composite model, the transporter relocates the substrate to a region of intermediate hydrophobicity adjacent to the transporter in the extracellular leaflet of the plasma membrane [16]. This places the substrate in an environment which energetically favours its binding and removal by an acceptor molecule/docking protein such as apoA1 (apolipoprotein A1).

Role of ABC transporters in cholesterol homoeostasis and other sterol export

ABCA1 as a cholesterol transporter

Cells in peripheral tissues produce all of the cholesterol needed for cellular homoeostasis. However, the liver is the only organ that is capable of degrading cholesterol. Therefore cholesterol must be transported through the blood to the liver for processing, degradation and secretion into bile, a pathway termed RCT (reverse cholesterol transport). Because cholesterol is a highly insoluble molecule, it must be packaged and transported by special particles in the plasma called lipoproteins. HDLs (high-density lipoproteins) are an essential determinant responsible for movement of most cholesterol from peripheral tissues through the blood back to the liver.

ABCA1, a member of the ABCA subfamily of ABC transporters, mediates the transport of excess cholesterol from cells to HDL apolipoproteins such as apoA1 [17]. Loss of function results in Tangier disease, a rare lipid disorder characterized by very low levels of HDLs and apoA1 [18]. Tangier patients accumulate high concentrations of cholesteryl esters in macrophages and various tissues, including liver, intestine, tonsils, spleen, lymph nodes and neuronal Schwann cells. Accumulation of excess cholesterol causes atherosclerotic cardiovascular disease, and may contribute to the early onset of AD (Alzheimer’s disease) [19] and renal dysfunction [20].

Abca1-knockout mouse models exhibit HDL deficiency and reduced cellular cholesterol efflux activity [21]. On the other hand, overexpression of ABCA1 increases plasma HDL levels and protects against atherosclerosis in animal models [22]. Hepatic deletion of ABCA1 dramatically reduces apoA1 and HDL levels. However, selective knockout of ABCA1 in macrophages has little influence on the plasma concentration of HDL, but leads to excessive accumulation of cholesterol esters. Thus, although ABCA1 in macrophages is not a major determinant of plasma HDL levels, it is a crucial factor in the prevention of excessive cholesterol accumulation in macrophages of the arterial walls.
Interaction of ABCA1 and apolipoproteins
ABCA1 localizes to the plasma membrane and intracellular compartments of cells, where it can facilitate the transport of lipids to either cell-surface-bound or internalized apolipoproteins (Figure 3). Overexpression of ABCA1 increases cholesterol efflux and apoA1 binding to the cell surface.

Direct interactions between apoA1 and ABCA1 have been confirmed in numerous studies using cross-linking, immunoprecipitation, radiolabelling and biotinylation techniques [23–25]. Although cholesterol transport to other lipoproteins such as apoE (apolipoprotein E) may also occur, apoA1 is the most physiologically relevant lipid acceptor for ABCA1 because of its high abundance and its role in forming nascent HDL particles. It contains eight 22-amino-acid and two 11-amino-acid tandem amphipathic α-helical domains. Further examination of apoA1 domain mutants shows that the C-terminal region is important for cross-linking with the large exocytoplasmic domains of ABCA1. This binding is not required for the intrinsic lipid transport activity of ABCA1, as forced overexpression of ABCA1 increases lipid domains on the cell surface in the absence of lipoproteins [26]. Instead, binding appears to facilitate removal of these lipids, perhaps by targeting apolipoproteins to ABCA1-generated lipid domains. Taken together, a two-step model is proposed: (i) apoA1 binds to ABCA1 by different combinations of apoA1 helices with the exocytoplasmic domain of ABCA1; and (ii) apoA1 is lipidated and nascent HDL dissociates [27].

Figure 3. Principal cholesterol-efflux pathway in macrophages
ABCA1 and ABCG1 mediate the transport of cholesterol to the extracellular acceptor particle, apoA1. ApoE and other lipoproteins may also serve as acceptors for ABCA1-mediated cholesterol efflux. Phospholipids and cholesterol are loaded on to surface-bound apoA1 by ABCA1 or HDL by ABCG1. HDL maturation is mediated by lecithin–cholesterol acyltransferase (LCAT, not shown). Oxidized cholesterol derivatives or oxysterols such as 27-hydroxycholesterol or 7-oxocholesterol can serve as ABCA1 or ABCG1 substrates or endogenous ligands of the LXR regulating ABCA1 and ABCG1 transcription.

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However, other studies indicate that only 10% of the cell-associated apoA1 can be cross-linked to ABCA1 [28]. In addition, ABCA1 significantly decreases plasma membrane rigidity independent of its interactions with apolipoproteins. ABCA1 can also transport SM and PC, and function co-ordinately in removing these lipids together with cholesterol from peripheral cells [29]. Taken together, a second model is suggested whereby ABCA1 enriches the outer leaflet with cholesterol and phospholipids, generates high-affinity binding sites for apoA1 and facilitates apoA1 docking and cholesterol uptake without direct contact [30]. This may promote the formation of apoA1-containing exovesicles and solubilization of apoA1-bound phospholipids and cholesterol for incorporation into nascent HDL.

Evidence of apoA1 internalization via receptor-mediated endocytosis also suggests a third model, whereby cholesterol and phospholipids are accumulated internally before secretion [31]. However, recent evidence suggests that this retroendocytotic pathway contributes less than 2% of lipidated HDL, reinforcing the previous models of plasma membrane being the primary site of apoA1 lipidation [32].

Although there is strong evidence that ABCA1 plays an essential role in the efflux of cholesterol from cells, the mechanism by which cholesterol is transported across the lipid bilayer remains to be determined. To date, there is no evidence for the direct binding of cholesterol to ABCA1 or the activation of the ATPase activity of ABCA1 by cholesterol. Furthermore, the transport of cholesterol by purified ABCA1 reconstituted into a non-cellular system such as liposomes has not been reported. The molecular mechanism by which ABCA1 mediates cholesterol transport remains an area of considerable importance in understanding cholesterol transport and metabolism.

**ABCA1 and cell signalling**

The interaction of apolipoproteins with ABCA1-expressing cells activates signalling molecules that modulate ABCA1 expression and lipid transport activity. These include JAK2 (Janus kinase 2), protein kinase C, Cdc42 and other protein kinases [33]. Although it remains to be shown whether these activation steps involve direct binding of apolipoproteins to ABCA1, these findings suggest that apolipoprotein behaves like a ligand capable of interacting with ABCA1 as a receptor and activating intracellular signalling pathways.

Activation of JAK2, a tyrosine kinase, is the initiating step in downstream signalling pathways. Inhibiting JAK2 activity has no effect on the intrinsic lipid transport function of ABCA1, but it does reduce the apolipoprotein binding to ABCA1 required for removal of translocated lipids [33]. The interaction of apoA1 with ABCA1-expressing cells stimulates autophosphorylation of JAK2, thus generating the active form of JAK2 that in turn phosphorylates its target proteins [33]. This suggests that apolipoproteins influence their own interactions with ABCA1 by activating JAK2, which in turn increases
apolipoprotein binding to ABCA1 required for lipid removal. This type of JAK2-mediated feedback mechanism has not been described for any other transporter to date.

**Other ABCA subfamily members**

Overexpression of ABCA2 in CHO (Chinese-hamster ovary) cells reduces the trafficking of LDL (low-density lipoprotein)-derived cholesterol to the ER [34]. ABCA3, which is predominantly expressed in lungs, has also been shown to remove cholesterol in an alveolar cell line [35]. However, direct translocation of cholesterol to apolipoproteins has not been demonstrated for these ABC proteins, so the mechanistic explanation for these observations is not clear.

ABCA7 shows the highest homology with ABCA1 and in fact mimics ABCA1 in mediating the production of HDL from cellular lipid *in vitro*. In induced sterol uptake conditions, a notable increase in mRNA levels of the *ABCA7* gene occurs, whereas cholesterol-depleting conditions result in down-regulation of its expression [36]. ABCA7 generates mostly small cholesterol-poor HDL particles, unlike ABCA1, which forms predominantly cholesterol-rich HDL particles. Deletion of *Abca7* in mouse macrophages does not seem to affect phospholipid or cholesterol efflux to apoA1, whereas inactivation of ABCA1 results in complete absence of efflux [37]. To date, no disease-causing genetic defect has been reported for ABCA7.

It is suggested that ABCA7 plays an important role in lipid transport within defined microenvironments. ABCA7 expression has been detected in murine renal tubules in the apical brush border membrane [37]. The kidney plays a major role in apoA1 catabolism, and expression of other ABC proteins has been demonstrated in this organ. In female *Abca7*-knockout mice, a significant reduction in visceral fat and serum cholesterol concentrations has been reported [38]. *Abca7* expression is also detected during late keratinocyte differentiation and significantly influences ceramide levels. These studies point to a subtle role of ABCA7 in cholesterol and other lipid-transport processes.

**ABCG subfamily**

The human ABCG subfamily contains five half-transporters, i.e. ABCG1, ABCG2, ABCG4, ABCG5 and ABCG8. These are unique transporters in which the NBD is localized on the N-terminal side of the TMD (NBD-TMD). All members of the ABCG subfamily, with the exception of ABCG2/BCRP (breast cancer-resistance protein), function as cholesterol transporters. Four of the five mammalian ABCG subfamily members, namely the homodimers ABCG1–ABCG1 and ABCG4–ABCG4 and the heterodimer ABCG5–ABCG8, have been shown to have a role in transporting sterols across membranes. ABCG1 and ABCG4 have been implicated in lipid efflux, predominantly cholesterol and SM, from peripheral cells to HDL [29,39]. The
ABCG5–ABCG8 heterodimer mediates the translocation of plant sterols as well as cholesterol.

ABCG1 displays the most widespread expression pattern, and it is currently thought to be one of the major regulators of cellular cholesterol content in mammals [30]. ABCG1 displays many similarities to ABCA1, such as tissue expression patterns and regulatory pathways [30] (Figure 3). However, to date, unlike ABCA1, no mutations in ABCG1 have been linked to any human disease. Initial studies demonstrated that transient overexpression of ABCG1 increased the efflux of cellular cholesterol to specific extracellular lipid acceptors that included HDL, LDL, PC vesicles and apoE complexes, but not lipid-free apoA1, as is the case with ABCA1 [40]. This leads to a proposal that ABCA1 and ABCG1 may function to redistribute cholesterol pools to either apoA1 or HDL.

ABCG4 shares 82% amino acid identity with ABCG1, so it is not surprising that they exhibit functional similarities: overexpression of either protein in cultured cells facilitates the efflux of cellular cholesterol to HDL, but not lipid-poor apoA1 [39]. Unlike ABCG1, ABCG4 expression is highly restricted to the brain and the neural layer of the retina [41]. Indeed, a concerted model for cholesterol efflux has been proposed for ABCA1, ABCG1 and ABCG4, in which initial lipidation of apoA1 to nascent HDL is performed by ABCA1 followed by HDL maturation via ABCG1/ABCG4 [42].

The ABCG5 and ABCG8 obligate heterodimers are expressed in the intestines and the liver. They function to limit the absorption of plant sterols found in our diet by transporting these sterols back into the intestinal lumen and by facilitating efficient secretion of plant sterols and cholesterol from hepatocytes into the bile. Mutations in these genes are linked to β-sitosterolaemia, a rare disorder caused by accumulation of cholesterol and plant sterols, eventually leading to premature coronary atherosclerosis [43]. Wang et al. [44] showed that cholesterol and sitosterol are the direct substrates of ABCG5 and ABCG8. Interestingly, acceptors for cholesterol are bile salts and not apolipoproteins or HDL.

**Sterol transport by other ABC proteins**

Active redistribution of cholesterol across the cell membrane by ABCB1 [Pgp (P-glycoprotein)] has been described. Considering the above-mentioned transporters, Pgp is unlikely to be a primary cholesterol transporter. Additionally, ATP-dependent cholesterol trafficking from the plasma membrane to the ER has been reported for Pgp [45]. Some studies suggest that this may be a consequence of its sphingolipid-efflux function, since cholesterol is known to interact with GlcCer (glucosylceramide) [46]. Recently, a variety of other steroids have been shown to be transported by Pgp. Overexpression of Pgp in human colon carcinoma cells reduces intracellular levels of glucocorticoids (cortisol, dexamethasone), mineralocorticoids (aldosterone, corticosterone) and, to a lesser extent, androgens (testosterone,
dihydrotestosterone), oestradiol and progestins [47]. The steroid role of Pgp in endocrine function awaits further clarification.

**Role of ABC proteins in phospholipid transport**

A number of ABC proteins have been shown to transport or flip phospholipids such as PC, PE and PS. Using lipid substrates labelled with the fluorophore 7-nitrobenz-2-oxa-1,3-diazole in conjunction with cellular or reconstituted systems, Pgp (ABCB1), responsible for pleiotropic drug resistance in tumour cells, was found to transport various phospholipid analogues, including PC and PE, as well as the sphingolipid GlcCer from the cytoplasmic to the exoplasmic leaflet of the plasma membrane [47]. Expressed mainly in the liver, ABCB4 is primarily a floppase for PC [48]. Mutations in ABCB4 have been associated with PFIC, a disorder involving liver inflammation and fibrosis [12]. Cholestasis is thought to result from the toxicity of bile in which detergent bile salts are not effectively neutralized by phospholipids, leading to bile canaliculi and biliary epithelium injuries. PC and PS also act as substrates for the other xenobiotic transporter BCRP (ABCG2) and MRP1 (multidrug-resistance protein 1) (ABCC1) [49]. Taken together, these studies support a key role of these ABC proteins in regulating membrane composition, asymmetry, and stability via translocation of key membrane phospholipids across cellular membranes.

ABCA1 and ABCG1 also regulate cellular phospholipid levels. Early evidence suggests that ABCA1 acts as a translocator of PC, PS and PE, whereas ABCG1 transports only PC [25,29,50]. As discussed above, generation of new HDL requires ABCA1-mediated phospholipid efflux to apoA1. More recently, missense mutations of ABCA1 within conserved residues of the Walker A motif and selected disease mutations (in exocytoplasmic domains) demonstrate impaired phospholipid export function, apoA1-binding activity and JAK2 activation [51].

ABCA2, a lysosome-associated protein, is predominantly localized in the brain in the cell bodies of oligodendrocytes. Recently, a correlation between ABCA2 and AD was reported from co-localization studies with amyloid β-peptides and other AD markers. As AD is associated with lipid-transport disorders and abnormal myelination, an indirect link has been proposed for ABCA2 in controlling lipid trafficking from the neuronal cell body to the membrane [52].

ABCA3 is localized in the lamellar bodies of lung alveolar type II cells. Lamellar bodies are densely packed lysosome-like structures which store pulmonary surfactant, a mixture of phospholipids and proteins. Upon secretion from lamellar bodies, pulmonary surfactants coat the airways of the lung with a lipid-rich monolayer thereby reducing the surface tension at the air/liquid interface. Secretion of pulmonary surfactant represents a critical feature in the switch of the lung from an aqueous environment at birth. Abca3-null
mice display low PC content in lamellar bodies of alveolar type II cells [53]. Mutations in Abca3 are associated with fatal surfactant deficiency in newborns [9]. On the basis of these studies, ABCA3 has been implicated in the transport of lipids and pulmonary surfactant metabolism.

ABCA4 is expressed in rod and cone outer segment disks of vertebrate retinal photoreceptors, where it plays an important role in the removal of retinal derivatives following the photobleaching of the photopigment proteins rhodopsin and cone opsin [54]. Photon absorption induces the isomerization of 11-cis-retinal to all-trans-retinal chromophore within the opsin protein (Figure 4A). This is followed by the release of the chromophore into the disc membrane. All-trans-retinal can be directly reduced to all-trans-retinol by retinol dehydrogenase as a key step in the regeneration of 11-cis-retinal as part of the visual cycle. However, a substantial fraction of all-trans-retinal reacts with PE in disc membranes to form the Schiff base adduct N-retinylidene-PE, which can be trapped on the luminal side of the disc membrane. ABCA4 is generally thought to function in the transport of N-retinylidene-PE from the lumen to the cytoplasmic leaflet of the disc membrane [55]. Dissociation of N-retinylidene-PE into all-trans-retinal and PE enables retinol dehydrogenase to reduce all-trans-retinal to all-trans-retinol for entry into the visual cycle. This ensures that all of the retinoid obtained from photoexcitation is removed from disc membranes. In the absence of ABCA4, all-trans-retinal and N-retinylidene-PE in disc membranes react to form fluorescent di-retinoid compounds including A2PE, which, after phagocytosis of photoreceptor outer segments, is hydrolysed to A2E in RPE (retinal pigment epithelial) cells and progressively accumulate as lipofuscin deposits [56] (Figure 4B). A2E and related di-retinoids are toxic, resulting in RPE and photoreceptor cell death and a loss in vision. The accumulation of A2E in RPE cells has been reported for individuals with Stargardt macular degeneration linked to mutations in ABCA4 [57].

Several lines of evidence support the role of ABCA4 as a N-retinylidene-PE transporter. The ATPase activity of detergent-solubilized and reconstituted ABCA4 is activated by retinal in the presence of PE [58,59]. Furthermore, ABCA4 immobilized on an immunoaffinity matrix binds stoichiometric amounts of N-retinylidene-PE and its reduced analogue N-retinyl-PE with an apparent $K_d$ of 4–5 μM [55]. Addition of ATP (or GTP) to ABCA4 containing bound N-retinylidene-PE causes the dissociation of the substrate from the protein. Finally, Abca4-knockout mice display light-dependent elevated levels of PE, N-retinylidene-PE and all-trans-retinal in the outer segments of photoreceptors and A2E in RPE cells, consistent with the role of ABCA4 in the removal of N-retinylidene-PE from disc membranes following photoexcitation [57,60].

ABCA12 is predominantly expressed on the skin and in the stomach and in particular keratinocytes [61]. Mutations in the ABCA12 gene are responsible for harlequin ichthyosis, a rare hereditary disease characterized by skin desquamations over the whole body [61]. A disorder in the keratinization process
Figure 4. Proposed role for ABCA4 in the transport of N-retinylidene-PE and the removal of retinoids from disc membranes of photoreceptors

(A) All-trans-retinal released from rhodopsin after photoexcitation can be directly reduced to all-trans-retinol by retinol dehydrogenase or react with PE to produce a Schiff base conjugate N-retinylidene-PE (N-ret PE). ABCA4 can bind and transport N-retinylidene-PE from the lumen to the cytoplasmic side of the disc membrane. All-trans-retinal derived from the dissociation of N-retinylidene-PE can be reduced to all-trans-retinol for removal from discs by the visual cycle (not shown). (B) Chemical reactions involved in the formation of N-retinylidene-PE and the production of the di-retinoid A2PE in disc membranes in the absence of ABCA4 transport activity. A2PE is subsequently hydrolysed to A2E upon phagocytosis of photoreceptor outer segments by retinal pigment epithelial cells. A2E progressively accumulates as fluorescent lipofuscin deposits in retinal pigment epithelial cells, a characteristic feature of Stargardt macular degeneration.
results in a thick ‘armour’-like scale layer covering the whole body. ABCA12 localizes in the lamellar granules of keratinocytes, where it may play a major role in lipid trafficking.

Eukaryotes primarily express outward directed ABC transporters. However, in the yeast *Candida albicans*, Cdr3p, a subfamily member of ABC proteins, has been found to exhibit an inward-directed phospholipid activity translocating NBD-PE, NBD-PC and NBD-PS [62]. This is in contrast with its related homologues, Cdr1p and Cdr2p. Two other *Saccharomyces cerevisiae* transporters, Aus1p and Pdr11p, facilitate exogenous sterol uptake when sterol biosynthesis is compromised [63]. In humans, ABCA4 is implicated to be an inwardly directed flippase translocating N-retinylidene-PE from the luminal to the cytosolic leaflet of the outer segment disc membranes in the retina [54]. Finally, the presence of CFTR (cystic fibrosis transmembrane conductance regulator) (ABCC7), which is not a true transporter, but a channel, is correlated with an increased uptake of signalling lipids sphingosine 1-phosphate and lysophosphatidic acid [64].

Members of the eicosanoid family of lipid mediators are also substrates for ABC proteins. Leukotriene C4 is effluxed by ABCC1 (MRP1) and ABCC2 (MRP2) [65], whereas ABCC4 (MRP4) is a transporter of PG (prostaglandin) E1 and E2 [66]. ABCC4 may also transport other related eicosonoids such PGA2, PGF2a and thromboxane A2 [66]. The physiological importance of these transporters with respect to eicosanoid secretion remains to be determined.

**Phospholipid- and sterol-transport assays**

Transport of lipids by Pgp has been studied most extensively. A variety of approaches and techniques have been used, including the efflux of lipids from confluent monolayers of transfected kidney cells, fluorescence microscopy, FACS analysis of NBD fluorescence, and fluorescence quenching of reconstituted proteoliposomes. Pgp has been shown to transport short-chain analogues of membrane lipids across the plasma membrane, including C6-NBD and C8-short chains of PC and PE, and the sphingolipids GlcCer and SM.

Van Helvoort et al. [48] showed the translocation of lipids with stable transfectants of pig kidney epithelial (LLC-PK1) cells containing Pgp. To generate C6-NBD-PC and C6-NBD-PE in cytosol, they incubated confluent monolayers of cells and Pgp transfectants with a precursor, C6-NBD-PA (phosphatidic acid), complexed to BSA for 3 h at 15°C (Figure 5A). C6-NBD-PA partitions into the plasma membrane and is dephosphorylated to C6-NBD-diacylglycerol and further converted by cells into C6-NBD-PC and C6-NBD-PE. Vectorial transport of lipids to the cell surface is discriminated by lowering the temperature to 15°C, at which point lipid transport by vesicular processes is blocked. After 3 h, only a small fraction of each lipid was found in apical BSA medium of control cells, whereas 20–25% of both C6-NBD-PC and C6-NBD-PE was found in the apical medium of Pgp-transfected cells.
which suggests that Pgp can translocate both C₆-NBD-PC and C₆-NBD-PE across the apical membrane. This was confirmed by NBD fluorescence microscopy. Translocation was also confirmed by [³H]choline radioactivity. C₆-NBD-PC in apical medium was ³H-labelled, so it was derived from intracellular C₆-NBD-PC and confirms the selective translocation of C₆-NBD-PC by Pgp transfectants. The continuous presence of precursor ensured synthesis of C₆-NBD-PC and C₆-NBD-PE in Pgp cells and was linear over 5 h at 15°C. Translocation of these fluorescence lipids was efficiently blocked by Pgp inhibitors verapamil, PSC833 and by energy depletion, demonstrating a direct involvement for the functional involvement of Pgp. Surprisingly, Pgp also demonstrated broad specificity by translocating C₆-NBD-SM and C₆-NBD-GlcCer (derived from the precursor C₆-NBD-ceramide) and most natural versions of these lipids lacking the NBD moiety.
Short-chain lipid analogues have also been studied with other techniques. Bosch et al. [67] demonstrated by fluorescence microscopy decreased accumulation of NBD-PC and NBD-PE in multidrug resistant cell line (CEM/VBL300) compared with the parental cell line (CEM, human T-lymphoblastic cell). Exogenous NBD-PC, NBD-PE and NBD-PS were added to CEM/VBL 300 and insect cells expressing recombinant Pgp. These lipid compounds are internalized and eventually translocated to the plasma membrane. Dithionite treatment was used to quench NBD fluorescence on the outer leaflet of the plasma membrane. Quantification of internal cellular fluorescence was measured by FACS analysis. Drug-resistant CEM/VBL 300 cells accumulated approximately 10% of the amount of NBD-PE and NBD-PC compared with drug-sensitive CEM cells. No internal accumulation of NBD-PS was found between drug-resistant and drug-sensitive cell lines. Pgp reversal agents (verapamil, cyclosporin A and SDZ PSC 833) both increase accumulation and inhibit efflux of these phospholipids in CEM/VBL 300 cells, but not in CEM cells. The increased accumulation was dose-dependent, and the relative potency of the reversal agents substantiates the evidence that PC and PE, but not PS, behave as substrates for Pgp. However, the possibility of NBD-PS accumulating in mitochondrial membranes after internalization may explain its inaccessibility for Pgp efflux as opposed to physicochemical differences.

The use of dithionite quenching of fluorescently labelled lipids for Pgp in in vitro systems was first employed by Romsicki and Sharom [68]. The distribution of NBD-labelled lipids can be monitored between the inner and outer leaflets of reconstituted proteoliposomes before and after incubation with ATP. Addition of dithionite quenches the NBD lipids partitioned in the outer leaflet and produces a stable baseline. Owing to the inaccessibility of the fluorescently labelled lipids in the inner leaflet of vesicles to dithionite, quantification of lipid distribution can be monitored in the presence of ATP, drugs and inhibitors (Figure 5B). Purified Pgp reconstituted into proteoliposomes was found to flip both short- and long-chain NBD analogues of PC, PS and PE in an ATP-dependent and vanadate-sensitive manner [68]. Similarly, NBD-GlcCer, NBD-galactosylceramide and NBD-SM flipping was also observed by Pgp proteoliposomes and was inhibited by various drugs and modulators in a concentration-dependent manner [69].

The ATPase and transport activities of reconstituted protein are modulated by the lipid environment in which it is embedded. The possibility that cholesterol might serve as a substrate for Pgp was also studied. When NBD-cholesterol was used in the fluorescence-based flippase assay, a transbilayer gradient of the lipid was not observed after dithionite treatment in both PC liposomes and Pgp proteoliposomes [70]. This behaviour arose from the rapid flip–flop of NBD-cholesterol between the inner and outer leaflet portions. A modest decrease in NBD-PC flippase activity was observed at 20–30% cholesterol, but cholesterol itself was not a substrate for Pgp. Cholesterol content, however,
has modest effects on both basal and drug-stimulated ATPase activity of Pgp. In the presence of verapamil, Pgp reconstituted into PC proteoliposomes exhibits a 2.2-fold increase in ATPase stimulation. In the presence of 30% (w/w) cholesterol, the stimulation is a modest decrease to 1.7-fold. In addition, it has no effect on Pgp conformation and neither does Pgp cross-link with \([3,5,6-\text{H}^3]-\text{azi}-5-\alpha\text{-cholestan-3\beta-ol}\), a photoactive cholesterol analogue, showing that it is not a direct substrate for Pgp. However, evidence from whole cell assays support cholesterol translocation by Pgp. Wang et al. [71] reported that in an NIH-G-185 cell line overexpressing Pgp, cholesterol caused a dramatic inhibition of daunorubicin transport, yet had no effect in the parent cell line, suggesting that cholesterol directly interacts with cholesterol transported by Pgp [71]. Gayet et al. [72] found in human VEM acute lymphoblastic leukaemia cells expressing Pgp, the amount of cholesterol increased linearly with the level of resistance to vinblastine, whereas the amounts of total and free cholesterol increased in a non-linear manner.

Although fluorescently labelled lipids have been widely employed to measure Pgp-mediated lipid transport across membranes, such techniques have not been successfully used to study lipid transport for ABCA proteins. For the most part, transport of lipids by this class of ABC transporters has been inferred from cell-based assays or through the phenotypic analysis of knockout mice.

It is unclear whether one can correlate ATPase stimulation as seen with drug transport to identify lipid substrates. Reconstituted ABCG5/ABCG8 and ABCG2 display sterol uptake in reconstituted proteoliposomes in an ATP-dependent and vanadate-sensitive manner. However, the ATPase activity is slightly decreased, rather than stimulated by sterols [44,73]. Although ABCA1 is a well-accepted phospholipid transporter, its ability to transport cholesterol directly is still unclear. First, cholesterol efﬂux from cells with low ABCA1 expression was enhanced by cell culture media which were conditioned by pre-incubation with ABCA1-expressing cells [74]. Secondly, in experiments with photoactivated lipids, ABCA1 was found to bind phospholipids, but not cholesterol, although overexpression of ABCA1 increased efﬂux of photoactive cholesterol [74]. Thirdly, it has been reported that the ATPase activity of ABCA1 shows robust ATPase activity (400–900 nmol/min per mg of protein) when reconstituted into liposomes made of choline headgroups, PC and SM [75]. Addition of cholesterol, \(\beta\)-sitosterol and campesterol mildly suppresses ABCA1 ATPase activity. Glibenclamide, an effective inhibitor of apoA1-dependent cellular cholesterol efﬂux suppresses the ATPase activity of ABCA1 in a dose-dependent manner [75]. These studies suggest that sterols may affect membrane ﬂuidity, which suppresses the ATPase activity of ABCA1. It is not known whether sterol transfer and its lack of stimulation in ATPase activity is an artefact of the systems used or an intrinsic property of the sterol transporters. However, further studies are necessary to reveal a direct biochemical interaction between cholesterol and sterol transporters.
Export of sphingolipid metabolites and S1P (sphingolipid 1-phosphate)

**ABCB1 [MDR1 (multidrug resistance 1)]**
Sphingolipids comprise a vital fraction of membrane lipids in eukaryotic cells. Individual sphingolipids are known to be signalling molecules and are important mediators of survival, stress response and apoptosis. Sphingolipids form a relatively non-fluid membrane which can associate with cholesterol to form microdomains commonly known as lipid rafts. The trafficking of sphingolipids between organelles is also an important regulated process. SM, sphingosine, ceramide and GlcCer are mainly localized in the plasma membranes, but a significant fraction can be found in the Golgi and ER. Among the ABCB subfamily, Pgp is rather promiscuous in its specificity: besides analogues of PC, PE and PS, Pgp translocates short-chain fluorescent sphingolipid substrates across membranes [76]. It has been shown that the ability of Pgp to transport drugs and act as a sphingolipid floppase occurs via the same mechanism [47]. There is also evidence that trafficking of the two sphingolipid species between the Golgi and plasma membrane may be MDR1-dependent [77]. Finally, Pgp also regulates the translocation of GlcCer synthase activity and synthesis of complex glycosphingolipids [69].

**ABCC1 (MRP1)**
Like Pgp, ABCC1 has been reported to translocate short-chain fluorescent cholesterol, SM and GlcCer across membranes [49]. Both Pgp and ABCC1 transport hydrophobic cytotoxic drugs; Pgp transports compounds in an unmodified state. In contrast, ABCC1 mainly transports substrates conjugated to glutathione, glucuronide and sulfate [78].

**ABCA and ABCG members**
*Abca2*-null mice exhibit low SM levels in oligodendrocytes, suggesting a role for ABCA2 in the transport of SM and possibly other sphingolipids [79]. SM is also a substrate of ABCG1, which translocates this lipid to HDL particles [29]. Indeed, other ABCA proteins, including ABCA2, ABCA7 and ABCA12 mediate transport of ceramide [79,80]. In keratinocytes, ABCA7 expression increases significantly during differentiation, coinciding with the accumulation of ceramide, suggesting a link between ABCA7 and ceramide levels [81]. Cultures of keratinocytes obtained from harlequin ichthyosis patients (mutations in the *ABCA12* gene) show abnormality in GlcCer distribution, which is restored by genetic correction of the *ABCA12* gene [80]. Hence, ABCA12 may function as a ceramide transporter, but a direct interaction between sphingolipids and ABCA7 or ABCA12 has not been reported.

S1P exerts a wide range of actions in the nervous system. S1P receptors are highly expressed in cerebellum; Anelli et al. [82] and Sato et al. [83] investigated S1P release from astrocytes. On the basis of glyburide inhibition and
siRNA (short interfering RNA) down-regulation, translocation of S1P appears to be highly dependent on ABCA1 expression [83]. Moreover, S1P release from astrocytes is coupled with HDL formation [83]. Inhibitors of ABCB1 (Pgp) and ABCC1 (MRP1) (cyclosporin A and MK571 respectively) diminish S1P release, suggesting that ABCA1, rather than ABCB1 or ABCC1, is involved in the process [84].

**Transport of pro-apoptotic lipids by ABC transporters**

**Pgp**

Modulation of lipid distribution in cells may also prevent cell death. Cells induced to express Pgp either by drug stimulation or retroviral gene transduction with Pgp cDNA are resistant to cell death induced by a range of death stimuli such as FasL (Fas ligand), TNFα (tumour necrosis factor α), UV irradiation and other factors that activate the caspase apoptotic cascade. Overexpression of Pgp transporters in drug-resistant cells is accompanied by altered membrane content of cholesterol, SM, GlcCer and other glycosphingolipids [85]. Interestingly, ceramide, a precursor of SM, acts as an intracellular signalling molecule during apoptosis and is liberated by apoptotic cascade factors. Pgp promotes SM externalization, resulting in lower levels of SM available for ceramide synthesis on the intracellular side of the membrane [86]. In response to treatment with PSC833, a Pgp inhibitor, SM levels increase together with enhanced sphingomyelinase activity and ceramide synthesis [87]. However, it is unclear whether PSC833 can act in a Pgp-independent manner.

**ABCG2**

ABCG2 (BCRP) has also been suggested to play an important role in cell survival. Trophoblast cells, which typically express BCRP at high levels, undergo loss of plasma membrane symmetry during cell fusion without further progression to terminal phases of apoptosis. Suppression of BCRP expression by siRNA leads to a marked increase in PS externalization followed by accumulation of ceramides and increased apoptosis. This can occur as a result of cell–cell fusion during the differentiation process [88], or in response to activation of the extrinsic apoptosis pathway by TNFα and interferon-γ [89]. However, whereas BCRP silencing was associated with increased susceptibility to ceramide-induced apoptosis and increased intracellular ceramide levels, evidence of ceramide transport by BCRP was not provided.

**Oxidation products of cholesterol transport**

In addition to cholesterol, ABCA1 and ABCG1 may prevent the accumulation of cytotoxic cholesterol-derived oxysterols in cells. Evidence from in vitro studies indicate that oxidized LDLs contain oxysterols such as 25-hydroxy- and 7-oxo-cholesterol, both abundant oxysterols in atherosclerotic plaques, can...
up-regulate the expression of death mediators, including p53, Fas and FasL, promote cytochrome c release and trigger an increase in intracellular calcium concentrations. ABCA1 mediates high-affinity uptake of 25-hydroxycholesterol in membrane vesicles (inside-outside orientation) [90], whereas 7-oxocholesterol is effluxed by ABCG1 macrophages [91]. In addition, oxysterols are also implicated in the cellular response to increased levels of cholesterol by binding to and activating LXR (liver X receptors). LXR function as heterodimers with the RXR (retinoic X receptors) and induce transcription of genes involved in cholesterol catabolism, including ABCA1 and ABCG1 [90] (Figure 3).

**Phagocytosis**
In mammals, PS externalization is associated with phagocytosis. Macrophages deficient in ABCA1 fail to engulf apoptotic cells, whereas forced transfection in non-phagocytic HeLa cells promotes phagocytic behaviour [92]. At the in vivo level, Abca1-null mice exhibit transient accumulation of apoptotic corpses in limb buds that can be seen during embryonic development [92]. Following phagocytosis of apoptotic cells, cholesterol efflux is enhanced in an ABCA1-dependent manner [93]. Indeed, cholesterol efflux may depend on PS externalization, since PS vesicles are able to stimulate efflux. However, annexin, a PS-binding protein, neither competes with apoA1 for ABCA1 nor inhibits cholesterol efflux [94]. Hence the molecular control exerted by ABCA1 in phagocytosis remains uncertain.

**Conclusions**
Lipid-efflux activity of mammalian ABC transporters accomplishes a wide variety and diverse range of activities related to homoeostasis and cytoprotection. The structure and function of these transporters dictate an intimate association with the constituents of the plasma membrane lipid bilayer. Not surprisingly, some ABC transporters show preferential localization to different organelles and, like other domain-associated proteins, their activity is affected by their surrounding lipid environment. Hence they are well placed to regulate the efflux and redistribution of lipids during periods of cellular division, stress and recovery, and, in turn, are regulated by alterations in membrane lipid composition. A number of ABC proteins are also able to transport ceramide and sphingolipids, as well as phospholipids, which play important roles in cellular signalling, differentiation and apoptosis.

There is strong indirect evidence that cholesterol serves as a substrate for several ABC transporters. ABCA1 mediates the transport of cholesterol and phospholipids such as PS from the inner to the outer leaflet of the membrane, but it remains to be shown whether cholesterol and related sterols and phospholipids are transported directly by ABCA1 and ABCG proteins. The involvement of ABCA1 in the reverse cholesterol pathway comprises distinct cellular events, including intrinsic formation of cell-surface lipid domains that interact with apolipoproteins,
solubilization of these lipid domains, direct binding of apoA1 to ABCA1, and activation of signalling molecules. But the complexity and diversity of this process is far from being understood. It is less likely that ABCA7 or ABCB1 play a major role in cholesterol homoeostasis, but this does not exclude a more subtle role in sterol trafficking. Several other species of sterols are also effluxed by ABC transporters, including toxic oxysterols, consistent with the view that ABC proteins function to prevent build-up of toxins and waste products.

Although the majority of ABC proteins transport substrates from the cytoplasmic to the extracellular/lumen leaflet of membranes, several transporters, including Cdr3p, Aus1p, Pdr11p and ABCA4, have been implicated in transport in the reverse direction. Additional biochemical transport assays are needed to confirm the identity of physiological substrates and the direction of transport by various ABC proteins. ABC lipid transporters expressed elsewhere in other organelles, including the Golgi and the ER, perform important functions with respect to distribution of sphingolipids between the organelles. Through regulation of ceramide and SM levels, and the availability of SM for conversion into ceramide by sphingomyelinase, ABC transporters are able to influence cellular sensitivity to apoptotic signals.

Further clarification of the mechanism of transfer of lipid substrates by ABC transporters, and their regulation at both the transcriptional and activity levels by lipid substrates is underway. These studies will enhance further our understanding of the role ABC transporters in a wide variety of cellular processes and functions, and offer further insight into various lipid diseases.

Summary

• **Members of the ABC family of transporters are involved in multiple aspects of lipid transport.** Defective sterol and phospholipid transport are linked to mutations in genes encoding ABCA1 (Tangier disease), ABCA3 (fetal newborn surfactant disease), ABCA4 (Stargardt macular degeneration), ABCA12 (harlequin ichthyosis), ABCB4 (PFIC) and ABCG5/ABCG8 (sitosterolaemia).
• **A concerted model for cholesterol efflux has been proposed for ABCA1, ABCG1 and ABCG4, in which initial lipidation of apoA1 to nascent HDL is performed by ABCA1 followed by HDL maturation via ABCG1/ABCG4.**
• **Ceramide plays a major role in apoptosis.** Increased turnover of ceramide by modulating the plasma membrane pool of SM may allow Pgp-expressing cells to escape apoptosis.
• **Oxysterols may contribute to the regulation of their elimination by inducing LXRs to increase expression of ABCA1 and ABCG1 genes.**
• **Apoptotic cell engulfment and efflux of cellular lipids depend on ABCA1-induced perturbation of PS externalization.**
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