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ATP-binding cassette transporter-2 (ABCA2) as a therapeutic target

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Abstract

The ATP binding cassette transporter ABCA2 is primarily an endolysosomal membrane protein that demonstrates pleiotropic functionalities, coalescing around the maintenance of homeostasis of sterols, sphingolipids and cholesterol. It is most highly expressed in brain tissue and ABCA2 knockout mice express neurological defects consistent with aberrant myelination. Increased expression of the transporter has been linked with resistance to cancer drugs, particularly those possessing a steroid backbone and gene expression (in concert with other genes involved in cholesterol metabolism) was found to be regulated by sterols. Moreover, in macrophages ABCA2 is influenced by sterols and has a role in regulating cholesterol sequestration, potentially important in cardiovascular disease. Accumulating data indicate the critical importance of ABCA2 in mediating movement of sphingolipids within cellular compartments and these have been implicated in various aspects of cholesterol trafficking. Perhaps because the functions of ABCA2 are linked with membrane building blocks, there are reports linking it with human pathologies, including, cholesterolemias and cardiovascular disease, Alzheimer's and cancer. The present review addresses whether there is now sufficient information to consider ABCA2 as a plausible therapeutic target.

Graphical Abstract

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Keywords

ABCA2; transporter; cancer; Alzheimer; cholesterol; sphingolipid

1. Introduction

ATP binding cassette transporters shuttle biological materials into and out of cells in an ordered, energy dependent fashion. Humans are multicellular organisms that have 48 separate genes that code for such transporters and these have been classified into seven distinct families designated by the nomenclature ABCA-G [1]. Within the ABCA family there are twelve examples that are split into two subgroups, where ABCA1 and ABCA2 share homology and represent among the largest of the family (~270kD; [2]). The importance of each of these in human disease [3] has been established through the analysis of multiple SNP's and genetic mutations. Interestingly, *Pubmed* searches for publications that carry ABCA1 in their title have reached approximately 1000, while for ABCA2 the number is presently less than 50. To some degree this reflects the initial publication that reported linkage of ABCA1 mutations with Tangier disease, a cholesterolemia that causes high levels of morbidity and mortality in young adults in isolated island geography [4].

Perhaps the discovery of this linkage has served to catapult much of the work on ABCA1, leaving its family member in need of a relationship with a defined human pathology. In this regard, there is by now a quite mature literature that suggests that ABCA2 may have been overlooked in terms of its importance to human metabolism. Some of the earliest reports detailed overexpression of ABCA2 through an amplification and gene duplication of a region of chromosome 9q34 in a human ovarian cancer cell line with resistance to the anticancer drug estramustine [5], an anti-microtubule chemotherapeutic, structurally similar to estradiol [6] used in the 1990's to treat hormone refractory prostate cancer [7]. Because estramustine is an estrogen-based therapeutic (although its cytotoxic properties are related to antimicrotubule activity [8], this may have been a predication of the involvement of ABCA2 with sterol homeostasis. While there are numerous transporters involved in the efflux of small molecules, and ABCA2 has been shown to enhance sequestration of some agents into lysosomes [9], much of the more recent published material perhaps indicates a broader association of the transporter with CNS and brain function, although this does not preclude other role(s) in oncology. The remainder of this article will detail some of the findings that may serve to invigorate interest in this transporter and its role in cellular homeostasis and human diseases.

2. ABCA2: general information

Initially, both ABCA1 and ABCA2 were co-isolated from embryonic mouse brain tissue [10]. They share the general protein structure of two transmembrane domains (TMD) and two nucleotide-binding domains (NBD) with an intervening highly hydrophobic loop domain (Figure 1).

The *Abca2* gene locus mapped to chromosome 9q34 within a 21kb region [11], containing 48 exons encoding a polypeptide of 2436 residues [12, 13]. The minimal promoter region

mapped 321 bp upstream of the translation start site [14] with the promoter containing consensus sequences for transcription factors HNF-3, c-myc and AP-1. In addition, it has two GC-boxes, encoding overlapping sites for the early growth response-1 (EGR-1) and Sp1 transcription factors [14]. ABCA2 is the largest of the ABC transporters, with a molecular mass of ~270 kD [1]. Full length human ABCA2 was cloned and a detailed tissue distribution studies confirmed high levels in brain, but also described expression patterns in ovary, leukocytes and macrophages [12, 13]. Temporal localization in oligodendrocytes in brain [15] and spinal cord [15] of the CNS, as well as Schwann cells in peripheral nerves [16, 17] has been found. Yeast two-hybrid studies revealed that within the hypothalamus, amygdala, cortex and selected brain stem nuclei ABCA2 colocalized with the orphan G protein-coupled receptor 50. This implies a link between functional aspects of G protein and neurotransmitter signaling strengthening a plausible role for this complex in stress response and energy homeostasis. (GPR50; [18]). Within the cell, fluorescent antibody studies revealed that ABCA2 primarily localizes to intracellular structures such as late endosomes/lysosomes, trans-Golgi and endoplasmic reticulum [19], although there are also indications of some expression at the cell membrane. Isoforms of 1A and 1B of ABCA2 are generated by alternative splicing [20], where exon 1 of isoform 1A encodes 22 amino acids, while that of isoform 1B, located 699 bp upstream contains a coding sequence for 52 amino acids. Both splice variants share similar tissue expression profiles and co-localize with lysosome-associated membrane proteins-1 and -2 (LAMP-1 and -2)[20]. Their specific functions in differentiation and/or development remain to be determined.

Within the A sub-family ABCA2 shares amino acid sequence homology with ABCA1 (50), ABCA7 (44), ABCA3 (43), ABCA4 (40) and ABCA6 (32) [9]. With ABCA1, this includes two cytoplasmic NBD's and two conserved sequences in the N- and C-terminal domains (LLLWKN and VFVNFA, respectively). The LLLWKN (function unknown) sequence is present in all members except ABCA6 and 9 [9]. The VFVNFA motif is critical for apolipoprotein A-I binding and HDL cholesterol efflux in ABCA1 [21], obviously implying a similar function for ABCA2. A lipocalin signature sequence exists downstream of the first NBD and this is present in other proteins including, apolipoprotein D, prostaglandin D synthase, complement component C8 γ -chain and α -1-microglobulin [9]. Lipocalin motifs form a small pocket for binding to hydrophobic compounds, such as lipids and sterols. This is consistent with studies implicating small, hydrophobic compounds, such as estramustine as transport substrates for ABCA2 [22].

3. ABCA2 and cancer drug response

Overall, the expression of ABC transporters has been linked with multidrug resistance phenotypes through the efflux of drugs via ATP-dependent transport. For example, 13 distinct transporters (*ABCA2*, *ABCB1*, *ABCB4*, *ABCB11*, *ABCC1-6*, *ABCC10*, *ABCC11* and *ABCG2*) have been associated with drug transport/resistance [23]. Historically, members of the MDR and MRP subfamilies (*e.g.* *ABCB1*/MDR1 and *ABCC1*/MRP1) have garnered the most attention [1], since their primary functions appear to be with efflux of foreign compounds such as cancer drugs. ABCA2 expression has been measured in cancer cell lines, (especially those with acquired drug resistance), normal and cancer stem cells and in human biopsies from cancer patients undergoing therapy. In most instances correlations

were drawn between the extent of expression and drug response in the samples. The first report [5] was in an ovarian carcinoma cell line with acquired resistance to estramustine (EM). These selected cells amplified a region in chromosome 9q34, resulting in 5-fold increase in ABCA2 expression and an increased rate of efflux of dansyl-labeled EM. The resistance rendered by ABCA2 expression was reversed upon antisense treatment [5]. Resistance to EM and estradiol was also shown in transfected HEK293 cells [12], but not in dominant negative ABCA2 transfected cells [20] and further cross resistance to a free radical initiator, 2,2'-azobis-(2-amidinopropane) (AAPH) [24], a compound structurally unrelated to steroids [25] was also detailed. ABCA2 may mediate the transport and sequestration of damaged lipids into the lysosome, preventing any propagation of chain reactions, where initiation, propagation and termination of lipid peroxides is prevented. ABCA2 was also linked with resistance to mitoxantrone and EM in a small cell lung cancer cell line [26] and treatment with both agents caused accumulation of mitoxantrone, indicating that EM may block its transport, although there was no direct proof that ABCA2 was the mediator of the transport. ABCA2 was also over-expressed in a daunorubicin-resistant promyelocytic leukemia cell line [27]. Overall, ABCA2 has been implicated in resistance to a diverse range of compounds, both sterol and non-sterol. The localization of ABCA2 to intracellular membrane organelles suggest that enhanced ABCA2 levels may protect cells through sequestration of toxic products into lysosomes, where specific examples have been shown [28]. Perhaps post-translational modifications may also influence substrate specificity and these could be both species and organ specific. Recently, Celecoxib has been shown to sensitize tumor cells resistant to the tyrosine kinase inhibitor imatinib. The authors concluded that this reversal was mediated at least in part by inhibition of ABCA2 [29].

Many cancers also have deregulated cholesterol metabolism and it is a critical early precursor for a number of pathways that influence the development and progression of cancer [30]. Hormones can influence cholesterol metabolism in cancer patients [31] and interfering with cholesterol metabolic pathways (*e.g.* HMGCoA R inhibition via statins) has been suggested to be a possible target in the therapeutic management of cancer patients [32]. Lysosomes in cancer cells have been suggested as viable drug targets [33]. Reducing the availability of this essential component of membranes and lipid raft domains may influence survival in rapidly dividing cancer cells. The plausible role of ABCA2 in regulating availability of cholesterol may validate it as a target for drug development.

There is now a better understanding of the importance of cancer stem cells as the arbiters of drug resistance. These cells have many of the properties of normal stem cells that permit them to survive chemotherapy, including high expression of various ABC transporters [34]. Such stem cells can provide a reserve of drug resistant progenitor cells that might repopulate the tumor following chemotherapy. ABC transporter over-expression is not uncommon among normal and malignant hematopoietic stem cells [35]. In AML progenitor cells, ABCA2 expression was significantly enriched in CD34+CD38- cells compared to more differentiated CD34+CD38+ cells [36]. These CD34+CD38- cells have greater malignant and self-renewing properties and such observations concur with the fact that ABCA2 expression has been implicated in leukemic cell multi-drug resistance in both AML [37] and T-ALL [38]. ABCA2 is also prevalent in some pluripotent side populations of lung cancer cells. These cells had high expression of ABCA2, ABCG2, ABCB1 and ABCC1 and

showed resistance to a range of anticancer drugs. These cells also displayed stem cell properties indicative of a quiescent state, but higher replicative potential [39].

There is a growing literature on ABCA2 expression in human biopsies, where it has been described as a potential biomarker of leukemias. In childhood T-cell acute lymphoblastic leukemia, ABCA2 and ABCA3 were expressed at high levels, which upon treatment with methotrexate, vinblastine and doxorubicin was amplified further [38], implying a function in resistance. Since normal brain and bone marrow express high levels, it is unsurprising that greater abundance occurs in their neoplastic counterparts. Specifically, high expression occurs in vestibular schwannomas, a benign intracranial tumor originating from a branch of the acoustic nerve in the PNS [17]. ABCA2 was abundant in histologically defined Antoni type A and type B areas of the biopsies [17]. The transporter was also shown in oligodendrogliomas of the CNS [40], where it outperformed Olig2 as a biomarker for oligodendrogliomas from astrocytic tumors. Most recently, within a group of ALL patients, quantification of the protein levels of ABCA2 and ABCA3 had a prognostic impact on pediatric ALL MDR. These authors deduced the tertiary structure of ABCA2 and using docking analysis revealed a possible compensatory effect between ABCA2 and ABCA3, which they thought might contribute to drug efflux and ultimately, to chemoresistance [41].

4. ABCA2 knockout studies/phenotypes

Two groups have independently generated ABCA2 knockout mice [19, 42]. Our own and Sakai et al observed a distinct shaking phenotype particularly affecting the hind limbs, that we termed “Skittish”. While in general the mouse phenotypes were very similar, there were some apparent differences in myelin sheath morphology and in brain lipid profiles. Our group reported that mice displayed tremor, hyperactivity and abnormal myelin ultrastructure, *i.e.*, compaction of the myelin sheath in the brain and spinal cord that we proposed was the cause of the tremor. In contrast, the Sakai group did not observe abnormalities in the cytoarchitectonic and compact myelin structure or oligodendroglia differentiation. Analysis of brain lipids of mice aged 11 days to 14 weeks revealed differences in the sphingolipid profile. They reported age-related deficiencies in the brains of phosphatidylethanolamine (PE) and phosphatidylserine (PS), sphingomyelin (SM) and a significant (two-fold) accumulation of ganglioside GM1 in ABCA2 knockout mice compared to wild-type littermates. Similar results were obtained in the myelin fraction of ABCA2-null brain. They proposed that ABCA2 functions to modulate the intracellular metabolism of sphingolipids in the brain. These results provide additional evidence *in vivo* that ABCA2 is a key molecule that regulates sphingolipid homeostasis. The appearance of the skittish phenotype prevented the extension of any further cancer related work on the transporter and instead altered focus onto neurological pathologies. Given the extreme nature of the knockout mouse tremors, it would appear that there may be added opportunities for further research with this model.

5. Rationale for studying the endogenous function of ABCA2

Our rationale for studying the endogenous function of ABCA2 in cells involved a consideration of the similarity between its DNA sequence and predicted protein structure compared to ABCA1. ABCA1 is the key molecule involved in reverse cholesterol transport

(RCT) [43]. We hypothesized that ABCA2 was also a key molecule in maintaining intracellular cholesterol homeostasis. However, the subcellular distribution of ABCA1 and ABCA2 differ significantly; whereas ABCA1 is predominantly localized to the plasma membrane compartment [44], ABCA2 is predominantly localized in the late-endosome/lysosomal membrane compartment (LE/LY) [12, 45]. The LE/LY is the terminal endocytic compartment where free cholesterol, liberated from cholesteryl ester molecules contained in low-density lipoprotein (LDL) particles by acid lipase, is distributed to other intracellular compartments in a highly regulated process. This difference in subcellular localization suggested fundamental differences in the roles of ABCA1 and ABCA2 in maintaining intracellular cholesterol homeostasis.

6. ABCA2 is a sterol-responsive gene

The first evidence to demonstrate that ABCA2 expression responded to sterol loading was provided in an experiment involving monocyte to macrophage differentiation *in vitro*. Upon sterol loading with enzymatically degraded LDL (eLDL), ABCA2 expression increased only in differentiated macrophages and not in monocytes [13]; however, the mechanism for the increase in expression or its relevance *in vivo* was not investigated.

Our early studies that investigated a functional role for ABCA2 in cholesterol homeostasis sought answers to the following questions: 1) was endogenous ABCA2 expression coordinately regulated with other sterol-responsive genes; 2) did constitutive overexpression of ABCA2 in cells alter the expression of other sterol-responsive genes; 3) did overexpression of ABCA2 affect the delivery of free-cholesterol to the endoplasmic reticulum for esterification by acyl CoA:cholesterol acyltransferase (ACAT); 4) did overexpression of ABCA2 sequester free cholesterol in the LE/LY upon sterol-loading; 5) did pharmacological agents that sequestered free cholesterol in the LE/LY alter ABCA2 expression; 6) did cells with genetic defects in cholesterol uptake by LDLR (familial hypercholesterolemia fibroblasts) or defects in cholesterol exit from the LE/LY that results in cholesterol sequestration (Niemann-Pick type C1 fibroblasts) alter ABCA2 expression.

To investigate whether ABCA2 was coordinately regulated with other sterol-responsive genes, we measured the mRNA levels of several genes in HepG2 liver hepatocarcinoma cells cultured in lipoprotein-deficient medium (LPDS) and in medium supplemented with exogenous sterols. The mRNA levels of both the Low-density lipoprotein receptor (LDLR) and Hydroxymethylglutaryl coenzyme A synthase (HMGCS) genes are sensitive to changes in the level of cellular cholesterol. Transcription of these genes is increased when cells are cultured in lipoprotein-deficient medium and repressed when exogenous sterols are added to the culture medium. Up-regulation of the LDLR under lipoprotein-deficient conditions facilitates the increased uptake of extracellular LDL (salvage pathway) to restore cellular cholesterol levels. HMGCS is one of a battery of enzymes whose expression is up regulated under sterol-deficient conditions to synthesize cholesterol *de novo* from acetyl CoA precursors to restore cellular cholesterol levels (synthetic pathway). Under lipoprotein-deficient culture conditions we measured increased mRNA levels for ABCA2, LDLR and HMGCS that were repressed upon supplementation of culture medium with exogenous

cholesterol [46]. The results of these studies provided further evidence that ABCA2 was a sterol-responsive gene involved in cholesterol homeostasis.

To determine whether the constitutive overexpression of ABCA2 in cells alters the expression of other sterol-responsive genes, we generated a CHO cell line that constitutively overexpressed human ABCA2 under control of the cytomegalovirus (CMV) promoter control element. The mRNA of the transcription factor, Sterol response element binding protein-2 (SREBP2), is elevated in cells under sterol-depleted conditions and repressed upon sterol loading [47]. SREBP2 regulates the transcription of both the LDLR as well as other enzymes in the cholesterol synthetic pathway, including HMGCS. We hypothesized that constitutive overexpression of ABCA2 in CHO cells in culture medium containing serum (a source of LDL-derived free cholesterol) would mimic sterol-deprivation. Under these conditions, ABCA2-mediated inhibition of cholesterol trafficking from the LE/LY to cholesterol sensors in the endoplasmic reticulum would activate the cholesterol synthetic pathway and sterol-responsive gene expression would be up regulated. In fact, this was what we observed, *i.e.*, both the LDLR and SREBP-2 mRNAs were elevated in CHO cells overexpressing ABCA2 compared to control CHO cells [46]. The results were recapitulated in a study of the human LDLR promoter coupled to the luciferase gene in CHO cells under the same cell culture conditions. These findings indicated that constitutive overexpression of ABCA2 in cells was sufficient to increase the expression of genes involved in cholesterol homeostasis.

7. Progesterone and U18666a increase expression of ABCA2 mRNA

To gain insight into a functional role for ABCA2, we treated cells with agents that are known to modulate cholesterol homeostasis in the LE/LY. Numerous investigators have reported that treatment of cells in culture with either the hormone progesterone or a pharmacological reagent, the cationic amphiphile U18666a, resulted in the sequestration of free-cholesterol in the LE/LY [46, 48–50]. More recent work has elucidated the mechanism of U18666a function in inhibiting cholesterol exit from the LE/LY. When an U18666a-derivative, synthesized to contain an ultraviolet light-sensitive benzophenone moiety was added to CHO cells, exposure to UV light induced the crosslinking of the derivative to the sterol-sensing domain (SSD) of the Niemann Pick Type C1 protein (NPC1), abrogating its activity [51]. We hypothesized that these LE/LY cholesterol-sequestering agents would mimic the cellular physiological effects of ABCA2 in cells and may in fact be modulators of ABCA2 expression. Treatment of CHO cells with either progesterone or U18666a resulted in an elevation of ABCA2 mRNA expression over 2-fold and 3.5-fold, respectively [46]. These results indicated that ABCA2 expression is responsive to hormonal and pharmacological agents that sequester cholesterol in the LE/LY.

8. ABCA2 mRNA expression is elevated in Niemann-Pick type C and Familial Hypercholesterolemia fibroblasts

Niemann-Pick Type C disease is a neurological disorder of adolescents due to an autosomal-recessive mutations in either one or both Niemann-Pick -2 (NPC2) and NPC1 genes, which, like ABCA2, are localized to the LE/LY compartment [52]. Cholesterol exit from the LE/LY

requires the concerted action of both NPC2 and NPC1 to deliver free cholesterol from the lumen to the limiting membrane of the LE/LY, where it traffics to other intracellular compartments, *e.g.*, Golgi apparatus, endoplasmic reticulum, by vesicular and non-vesicular mechanisms [53–55]. Mutant NPC1 fibroblasts have a defect in the delivery of LDL-derived free cholesterol from the LE/LY leading to cholesterol accumulation within this compartment [56, 57]. In addition to cholesterol accumulation, other lipids including sphingomyelin, glycosphingolipids and sphingosine also accumulate in the LE/LY [58, 59]. The expression of the LDLR and the cholesterol synthetic genes are also elevated in mutant NPC1 fibroblasts. Fibroblasts from Familial Hypercholesterolemia (FHC) patients lack functional LDL receptors that are required for LDL uptake from the plasma [60].

We hypothesized that in cultured cells, decreased delivery of LDL-FC to the endoplasmic reticulum in NPC1 fibroblasts or decreased uptake of LDL in FHC fibroblasts would mimic sterol-deprivation and induce cholesterol sequestration and result in activation of ABCA2 expression. Human fibroblasts from normal, FHC and NPC1 patients were cultured in complete medium and following RNA isolation, ABCA2 expression was measured by Northern blot. ABCA2 expression was elevated in NPC1 and FHC fibroblasts compared to normal fibroblasts. These studies were significant in that they represented the first reports that modeled sterol deprivation and ABCA2 expression in cells with genetic defects in cholesterol uptake and exit from the LE/LY. These results are also consistent with the results of experiments described above, where ABCA2 expression was elevated in cells cultured in LPDS containing medium (sterol deprivation).

9. Role of ABCA2 in intracellular cholesterol transport

We discovered that ABCA2 overexpression functioned to sequester cholesterol in the LE/LY compartment. In one set of experiments, we employed a CHO cell line that stably overexpressed ABCA2 [46]. Cells were cultured in lipoprotein-deficient serum (LPDS) to up-regulate LDL receptors and then cultured in increasing concentrations of LDL. Radioactive [¹⁴C]oleate fatty acid was added and the esterification of free cholesterol to [¹⁴C]cholesteryl ester by ACAT in the ER was determined following extraction of lipids, thin layer chromatography (TLC) and autoradiography. In control CHO cells cholesteryl ester formation increased in a dose-dependent fashion while overexpression of ABCA2 reduced the magnitude of cholesteryl ester formation. Measurement of *in vitro* ACAT activity confirmed that the decrease in cholesteryl ester was not due to defects in ACAT activity in ABCA2 overexpressing cells. Filipin, a polyene antibiotic that binds to free cholesterol, decorated the abundant cholesterol in the plasma membrane in control cells, as revealed by ultraviolet fluorescence microscopy. In contrast, in cells treated with U18666a or in CHO cells overexpressing ABCA2 there was reduced plasma membrane staining and increased punctate staining of free cholesterol in the cytosol (filipin positive droplets) that was indicative of the LE/LY.

Since ABCA2 is most highly expressed in the brain, we extended our studies to determine the effects of ABCA2 overexpression on cholesterol metabolism in mouse N2a neuroblastoma cells. In the first set of experiments to measure the effects of ABCA2 on uptake and trafficking of LDL-derived cholesterol back to the plasma membrane, we

replaced the cholesteryl ester in the LDL particle with radioactive [^3H]cholesteryl linoleate ([^3H]CL-LDL). We were able to measure the recycling of cholesterol to the plasma membrane by its efflux to the cholesterol-binding molecule 2-hydroxypropyl- β -cyclodextrin [61]. ABCA2 did not significantly alter cholesterol trafficking to the plasma membrane. These results suggested that following endocytosis of LDL/LDLR by receptor-mediated endocytosis into early endosomes, cholesterol recycling back to the plasma membrane occurred mainly between early endosomes and the endocytic-recycling compartment (ERC). Under these experimental conditions (*i.e.*, short duration culture with [^3H]CL-LDL), cholesterol recycling to the plasma membrane was not sensitive to ABCA2 activity because the free cholesterol did not reach the more mature acidic endosomes of the LE/LY, where it would be sequestered.

As an alternative approach to determine the effects of ABCA2 overexpression on LDL-derived cholesterol recycling to the plasma membrane, we measured the efflux of lipoprotein-derived cholesterol from the plasma membrane to the physiological cholesterol acceptor apolipoprotein E (ApoE) that was added exogenously to the cell culture medium. When control N2a cells and N2a cells overexpressing ABCA2 were cultured for longer periods with [^3H]CL-LDL, we did observe a modest reduction in efflux of free cholesterol from the plasma membrane to ApoE in ABCA2 overexpressing cells. We interpreted from these results that over extended periods of time of cells in culture a greater fraction of cholesterol had trafficked from the ERC to more mature endosomal compartments (LE/LY), where ABCA2 functions in cholesterol sequestration, thereby reducing cholesterol trafficking back to the plasma membrane for efflux to ApoE.

Since non-lipoprotein-derived free cholesterol is recycled from the plasma membrane through the endosomal system through receptor-independent mechanisms, we wanted to determine whether ABCA2 could alter the trafficking of this source of free cholesterol. Our approach was to measure the effect of mobilizing cholesterol at the plasma membrane by reducing its association with the sphingolipid sphingomyelin and measuring free cholesterol trafficking to the plasma membrane for esterification by ACAT. In this experiment we radiolabeled cells overnight to equilibrium with [^3H]cholesterol and then treated cells over several hours with bacterial sphingomyelinase (beta SMase) that selectively hydrolyzes plasma membrane sphingomyelin to ceramide. Increased ceramide levels displace cholesterol from membrane phospholipids and enhance its delivery to the ER for esterification [62, 63]. We observed that under these conditions, ABCA2 overexpression reduced cholesteryl ester formation with increasing duration of beta SMase treatment, compared to control N2a cells [61, 64]. In other experiments we determined the effect of depletion of endogenous ABCA2 by RNAi treatment, beta SMase mobilization of plasma membrane cholesterol, cholesterol trafficking to the ER and cholesterol esterification. We utilized the rat schwannoma cell line, D6P2T that expresses high levels of endogenous ABCA2 and stably expressing a lamin control RNAi and a D6P2T cell that stably expresses a rat ABCA2-specific RNAi to silence ABCA2 expression. In contrast to the ABCA2 overexpression studies, reduction of endogenous ABCA2 by RNAi increased the basal level as well as the beta SMase-treated level of cholesteryl ester formation [64]. These studies indicated that changes in ABCA2 expression level were sufficient to modulate non-lipoprotein-derived free cholesterol trafficking to the ER for esterification. Interestingly,

addition of the oxysterol, 25-hydroxycholesterol (25-HC), increased the mobilization of free cholesterol by forcing its displacement from membrane phospholipids [65, 66] and increased cholesterol esterification in both N2a cells and N2a cells overexpressing ABCA2. Importantly, the magnitude of the increase in cholesteryl ester formation mediated by 25-HC alone could be reduced to the level of control untreated N2a cells by co-culture in 25-HC/U18666a containing medium. These data indicated that a fraction of the 25-HC-released cholesterol trafficked through an U18666a-sensitive compartment, *i.e.*, the LE/LY [64].

10. Basal ABCA2 expression is regulated by general transcription factors

In order to understand the mechanisms responsible for regulating the transcription of the human ABCA2 gene, the basal promoter and *cis*-DNA regulatory control elements have been determined. Over 5 kilobases of the ABCA2 gene containing DNA control elements was analyzed and the basal promoter to 321 base pairs upstream of the ATG (methionine) start codon demonstrated a functional role for two GC-rich boxes containing overlapping early growth response protein-1 (EGR-1) and Sp1 binding sites [14]. When oligonucleotides containing overlapping EGR-1/Sp1 sites were incubated with nuclear extracts from BE(2)-M17 human neuroblastoma cells, binding the Sp1, Sp3 and Sp4 transcription factors was found. When these cells were treated with a protein kinase C activator, phorbol 12-myristate 13-acetate (PMA), inducible expression and binding of the EGR-1 transcription factor to the two GC-boxes was detected. Transfection of Sp1 SP3 or Sp4 expression constructs into *Drosophila* S2 insect cells, which do not express endogenous Sp-factors, induced dose-dependent increases in the expression of the human ABCA2 promoter but transfection of an EGR-1 expression construct alone failed to activate transcription. Co-transfection of an EGR-1 expression construct and an ABCA2 reporter construct into cells resulted in a dose-dependent reduction in expression of the ABCA2 basal promoter. The results from these studies suggested that Sp-factors and Egr-1 factors compete for binding to the GC-boxes in the proximal human ABCA2 promoter to regulate its transcription. This mechanism has also been established for other genes, *e.g.*, SREBP-1a [67]; β (1)-Adrenergic Receptor [68] and Protein-tyrosine phosphatase 1B [69]. Understanding of the regulation of ABCA2 expression may lead to the identification of molecular targets for therapeutic intervention to prevent or ameliorate diseases due to altered expression of ABCA2.

11. Mechanisms for regulation of intracellular cholesterol homeostasis

Cells maintain intracellular free cholesterol levels and distribution within stringent tolerances by several mechanisms [55]: (1) uptake of native LDL (the major extracellular carrier of cholesterol) by LDL receptors and modified LDL (*e.g.* oxLDL) by scavenger receptors; (2) removal of excess intracellular cholesterol by efflux transporters ABCA1 and ABCG1 that function in the lipidation of plasma apolipoprotein acceptors to form high-density lipoprotein particles, HDL or “good cholesterol” and circulation to the liver for excretion in the bile [70]; (3) re-esterification of intracellular free cholesterol by ACAT and storage of the cholesteryl esters in cytoplasmic lipid droplets and (4) sequestration of free cholesterol in the LE/LY subcellular compartment.

Due to its hydrophobic nature, very little cholesterol exists in aqueous compartments in cells and is primarily associated with lipid bilayers of membranes, consisting of phospholipids and sphingolipids or associated with sterol binding proteins. The plasma membrane and endocytic-recycling compartment (ERC) are key physiological buffers that harbor most of the free cholesterol in cells [71]. Net expansion of the free cholesterol pools above a threshold level signals transport mechanisms that deliver free cholesterol to the ER for esterification by ACAT [72]. In fact, the steady-state level of free cholesterol in the plasma membrane is set primarily by the capacity of its association with phospholipids [73, 74]. Phospholipid headgroup and acyl chain composition are the primary determinants for the strength of phospholipid-cholesterol interaction in order of sphingomyelin (SM) > phosphatidylserine (PS) > phosphatidylcholine (PC) > phosphatidylethanolamine (PE) [75].

12. Mechanisms of transport of cholesterol from LE/LY to ER cholesterol sensors

Cholesterol traffics from the LE/LY to the ER by both non-vesicular and vesicular pathways [53–55]. Following the hydrolysis of cholesteryl esters contained in lipoproteins by the action of lipoprotein lipase in acidic endosomal compartments and release free cholesterol, it binds to the Niemann-Pick type C protein-2 (NPC2) within the aqueous compartment of the LE/LY lumen [76]. The cholesterol is directly transferred from NPC2 to the Niemann-Pick type C protein-1 (NPC1) protein and into the limiting membrane of the LE/LY. Members of the oxysterol-binding protein family, *i.e.* oxysterol-binding protein-related protein 1L/ ORP1L, sense the cholesterol content in the LE/LY limiting membrane. The fate of cholesterol at this point moves from the LE/LY to intracellular compartments, *e.g.* from NPC1 compartments to the Trans-Golgi apparatus, by vesicular transport mechanisms involving the SNARE protein complex [53] or directly to the endoplasmic reticulum by non-vesicular transport mechanisms, involving the involving the cholesterol sensor ORP1L [54] or ORP5 [77].

ORP1L functions in the reversible assembly of protein complexes including Rab-7 and RILP proteins that modulate LE/LY vesicle trafficking [54, 78]. ORP1L and NPC1 containing late-endosomes mediate cholesterol export to the ER [78]. Studies suggest that in the non-vesicular transport pathway, the oxysterol binding protein-related protein-5 (ORP5) binds the LE/LY membrane, forming a membrane contact site (MCS) between the LE/LY and ER and mediates desorption of free-cholesterol from the limiting membrane of the LE/LY to the ER membrane. ORP5 can also physically interact with NPC1 to facilitate the transfer of cholesterol to the endoplasmic reticulum membrane [76, 77, 79]. ORP5 is tethered to the ER through its carboxy-terminal domain and binds to the LE/LY membrane by its pleckstrin homology domain (PH) to membrane phospholipids, perhaps phosphoinositide 4 phosphate (PI4P) [80]. ORP5 can bind cholesterol, PI4P and phosphatidylserine (PS) through its oxysterol binding protein related domain (ORD) and recent reports demonstrate that ORP5 can also function in PS/PI4P exchange at ER/plasma membrane MCS [81, 82]. Disassembly of the cholesterol transport protein complex of NPC1, ORP5 and perhaps other proteins may be mediated through the action of the AAA ATPase VPS4/SKD1 upon completion of cholesterol exchange [83].

13. ABCA2 modulates intracellular sphingolipid metabolism in the LE/LY

Recycling of plasma membrane lipids that occurs by constitutive endocytosis and vesicularization of complex glycolipids, complex sphingolipids and cholesterol as well as free cholesterol liberated from LDL after receptor-mediated endocytosis of the LDLR. As depicted in figure 2, following endocytosis of plasma membrane lipids into vesicles, further invagination of endosomal vesicles into intraluminal vesicles and the progressive acidification of the lumen by proton pumps establish distinct compartments, (*i.e.*, early endosome to late endosome/lysosome maturation) favorable to the enzymatic activities (glucocerebrosidase, acid sphingomyelinase, acid ceramidase). Their catalytic activities are restricted to defined acidic pH optima. The results of the enzymatic activities recycle membrane lipid components (salvage pathway) to other intracellular compartments for structural and signaling functions dependent on lipid metabolism [84] (Figure 2). At the acidic pH of LE/LY (~ 5.0 to 5.5), the action of sphingolipid recycling enzymes (salvage pathway), (*e.g.* glucocerebrosidase, acid sphingomyelinase and acid ceramidase modulate the catabolism of the membrane sphingolipids derived from the plasma membrane [85].

The central molecule of sphingolipid metabolism is ceramide, which consists of the sphingoid base sphingosine covalently linked to a fatty acid [86]. Ceramide is produced through the action of *de novo* synthetic pathways as well as through catabolism of more complex sphingolipids in the salvage/recycling pathway in the LE/LY [85]. Ceramide is the precursor for the synthesis of more complex lipids, including sphingomyelin, cerebrosides and complex glycosphingolipids. In the umbrella model both ceramide and free cholesterol, which have small polar head groups and relatively large nonpolar bodies, compete for coverage of their headgroups by phospholipids in the membrane bilayer, to prevent exposure of their nonpolar bodies to aqueous environments [87]. Elevated ceramide levels contained within intraluminal vesicles in the LE/LY displace free cholesterol onto the aqueous free cholesterol acceptor NPC2 protein. NPC2 loaded with free cholesterol then interacts with NPC1 present at the limiting membrane of the LE/LY and transfers free-cholesterol to the limiting membrane of the LE/LY [88].

We hypothesize that the ceramide/sphingosine ratio is a key regulator of the movement of LE/LY cholesterol onto NPC2. Since sphingosine is less effective than ceramide in displacing cholesterol onto NPC2 [89], elevation of sphingosine in the salvage pathway at the expense of ceramide may reduce free cholesterol loading onto aqueous NPC2, resulting in cholesterol sequestration in the LE/LY. Sphingosine is produced by catabolism of membrane sphingolipids to ceramide (*i.e.*, glucocerebrosidase and sphingomyelinase to ceramide) and by the deacylation of the resulting ceramide by acid ceramidase in the LE/LY [90, 91]. Agents that decrease ceramide levels or increase sphingosine in the LE/LY would be expected to decrease free cholesterol loading onto NPC2 for transfer to NPC1 and cholesterol exit, resulting in cholesterol sequestration in the LE/LY. In fact, Niemann-Pick Type C disease has been characterized as a sphingosine storage disease since elevation of sphingosine levels precedes the sequestration of other lipids associated with the disease [92].

We have reported that modulation of ABCA2 expression level alters the cellular sphingolipid profile by electrospray ionization mass spectrometry (ESI/MS/MS). Lipidomics

analysis was performed in control N2a cells, in N2a cells that overexpress human ABCA2, in control D6P2T cells that express high levels of endogenous ABCA2 and in RNAi-treated D6P2T [61, 64]. ABCA2 overexpression in mouse N2a neuroblastoma cells exhibited reduced levels of key ceramide metabolites and a greater than two-fold increase in sphingosine levels. Conversely, depletion of endogenous ABCA2 expression in D6P2T cells exhibited elevated the levels of key ceramide metabolites. In addition, we measured an increase in *in vitro* acid ceramidase activity in microsomes isolated from ABCA2 overexpressing N2a cells. These results suggest that ABCA2 expression may modulate the ceramide: sphingosine ratio necessary for cholesterol loading onto NPC2. In a broader sense, our studies have suggested a convergence between ABCA2 expression, sphingolipid salvage pathway catabolism and cholesterol exit from the LE/LY.

14. Theoretical mechanism of ABCA2 activity in LE/LY in modulation of cholesterol trafficking

It is important to confirm that ABCA2 is a lipid transporter and identify the substrates that are mechanistic in its cholesterol sequestering activity. We hypothesize that ABCA2 modulates cholesterol sequestration in the LE/LY by altering the intraluminal membrane lipid bilayer organization that results in increased acid ceramidase activity in the LE/LY, facilitating ceramide deacylation to sphingosine (Figure 3). At the acidic pH of the LE/LY, enzymes in the sphingolipid salvage pathway are positively charged. In addition, Saposin Activator Proteins (*e.g.* SAP-C, SAP-D) that facilitate salvage pathway enzymatic activity are also positively charged [90]. Electrostatic interactions between the positively charged SAP proteins and anionic phospholipids [*e.g.* bis-monoacylglycerol phosphate (BMP), PS, phosphatidylinositol (PI), phosphatidic acid (PA) phosphatidylglycerol (PG)] mediate salvage pathway enzymatic activity (*e.g.*, glucocerebrosidase, acid sphingomyelinase [91, 93, 94] (Figure 4). In addition, acid ceramidase activity also requires the anionic phospholipid interaction with salvage pathway enzymes and SAP proteins. We hypothesize that ABCA2 may function as an anionic phospholipid transporter, facilitating acid ceramidase activity and elevation of LE/LY sphingosine levels.

Interestingly, elevated sphingosine levels in the LE/LY are associated with depleted calcium levels in the LE/LY by modulating calcium channel activity [95, 96]. Elevated calcium levels in the LE/LY are critical for vesicular budding in the non-vesicular pathway of cholesterol trafficking [92]. Depleted calcium levels in the LE/LY may indirectly result in cholesterol sequestration.

In summary, ABCA2 may function in: (1) the intramembrane transport of anionic phospholipids in the LE/LY; (2) increased salvage pathway conversion of ceramide to sphingosine by acid ceramidase activity, (3) increased LE/LY sphingosine levels that result in cholesterol sequestration.

15. ABCA2 and Alzheimer's disease

ABCA2 has been most strongly linked to Alzheimer's disease (AD). Three independent groups have identified a genetic link to both early-onset and sporadic late-onset AD. The

first group identified a synonymous single nucleotide polymorphism (C to T transition in the third nucleotide position of the aspartate codon, rs908832) (C679T) in exon 14 of ABCA2 [97]. A second independent group confirmed the first finding that rs908832 was linked to AD, although in an ethnicity-dependent manner [98]. A more recent third epidemiological study, employing a meta-analysis of eight polymorphisms associated with AD, identified the same rs908832 SNP as a significant risk factor for AD in certain ethnic populations [99]. How the rs908832 SNP mutation modulates ABCA2 function and AD etiology is unknown.

Our laboratory demonstrated that overexpression of ABCA2 in human embryonic kidney cells was associated with increased expression of genes associated with AD, including the amyloid precursor protein (APP) [24]. We also determined that overexpression of ABCA2 in N2a mouse neuroblastoma cells increased transcription from the human APP promoter and resulted in a shift in the subcellular trafficking and processing of the APP holoprotein to early endosomal compartments that promoted A β production [100]. In contrast, Michaki et al found that knockdown of endogenous ABCA2 in mammalian cells *in vitro*, in a *Drosophila melanogaster* model system and in ABCA2 knockout mice reduced A β production by altering nicastrin maturation, a key component of γ -secretase [101]. As described above, ABCA2 overexpression in N2a cells increases sphingosine levels. We determined that elevated sphingosine directly modulated a protein kinase C signaling pathway that regulated APP transcription at the AP-1 and E-box control sites in the basal APP promoter [102]. These results indicate that ABCA2 is a viable target for continuing studies into its role in Alzheimer's disease pathology.

16. ABCA2 and Cardiovascular disease

Cardiovascular disease is a major risk factor responsible for human mortality. The link between elevated plasma cholesterol and the etiology of cardiovascular disease is unequivocal and reducing circulating plasma cholesterol carried in the LDL particle has been a major pharmacological goal due to its effects on target tissues.

A principal mechanism for initiation and progression of cardiovascular disease involves the cellular response to lipoprotein-derived cholesterol, *i.e.*, low-density lipoprotein (LDL) [103, 104]. The effects of ABCA2 on LDLR expression in N2a cells provides a viable experimental model to study these effects. Steady-state levels of both LDLR mRNA and LDLR protein are decreased when ABCA2 is overexpressed. Immunoprecipitation of biotinylated cell surface proteins indicated that ABCA2 overexpression reduced surface LDLR levels [61]. Measurement of LDL uptake by the LDLR using a fluorescently labeled LDL (DiI-LDL) was also lower in ABCA2 overexpressing N2a cells. Kinetic experiments by metabolic radiolabeling with [35 S]methionine and immunoprecipitation of the LDLR indicated that ABCA2 overexpression decreased LDLR stability (*i.e.*, increased metabolic turnover). These results in neuroblastoma cells are in contrast to those described for stable ABCA2 overexpression in CHO cells and suggest that mechanisms of modulation of LDLR by ABCA2 may be cell type/tissue type dependent. However, such results could be significant given the various pharmacological efforts employed to increase plasma LDL clearance (*e.g.*, antibodies to PCSK9, inhibition of internalization of dietary cholesterol through NPC1L1) [105–107]. Although only modest reductions in plasma cholesterol levels

were measured in ABCA2 knockout mice [19], these experiments were performed on mice fed standard rodent chow and not chow enriched in cholesterol, which may more accurately assess the effects of ABCA2 on modulation of LDL and plasma cholesterol levels. Importantly, ABCA2 overexpression was sufficient to reduce LDLR and therefore may be an attractive target for LDLR therapies (*e.g.*, selective reduction of endogenous ABCA2 to increase LDLR levels and increase plasma LDL clearance).

The macrophage is the major cell type responsible for cholesterol uptake and metabolism associated with the initiation and progression of atherosclerosis in focal areas of the arterial subendothelial space. In advanced atherosclerotic lesions of cardiovascular disease, cholesterol-induced macrophage apoptosis generates cell debris that can promote the formation of the necrotic core of the atherosclerotic plaque [108]. The lipid-rich plaques may become unstable, increasing the potential risk for thrombosis, cardiac arrest and death. Although the mechanisms regulating cholesterol uptake, efflux, esterification and storage in cytoplasmic neutral lipid droplets in macrophages have been well-characterized, a role for ABCA2 in modulating macrophage response to excess sterol loading, free cholesterol trafficking from the LE/LY compartment to the endoplasmic to the ER and apoptosis has not been fully elucidated.

Excess free cholesterol is toxic to macrophages [109–113]. The principal site of cholesterol-induced toxicity in macrophages is the endoplasmic reticulum (ER), through activation of the unfolded protein response (UPR) and initiation of apoptosis [112]. In cell culture, sterol loading of macrophages with acetylated LDL (AcLDL) together with a pharmacological inhibition of ACAT re-esterification of free cholesterol to cholesteryl esters produced an increase of free cholesterol-induced toxicity [113]. Significantly, addition of U18666A or progesterone, inhibitors of cholesterol transport from the LE/LY, reduced cholesterol-induced toxicity. Importantly, macrophages from mice, heterozygous for a loss of function mutation in the Niemann-Pick type C1 (NPC1) gene, exhibited reduced cholesterol trafficking from the LE/LY to the ER and were more resistant to cholesterol-induced apoptosis [112]. This *in vivo* finding suggests that cholesterol trafficking from the LE/LY to the endoplasmic reticulum is a key pathway for modulating cholesterol-induced toxicity in macrophages. ABCA2 may function to modulate cholesterol trafficking from the LE/LY to the ER in macrophages to reduce free cholesterol induced toxicity under conditions of high plasma lipoprotein-derived cholesterol.

A possible mechanistic role for ABCA2 in the etiology of cardiovascular disease was suggested by a study describing the effects of ABCA2 deletion in ABCA2 knockout macrophages generated in LDL receptor knockout mice [114]. They reported diminished lesion size in the aortic root and descending thoracic aorta, and a 3-fold increase in apoptotic cells. While wild-type macrophages developed filipin positive droplets in lysosomal-like compartments, corresponding to free cholesterol accumulation, ABCA2-deficient macrophages displayed an abnormal diffuse distribution of free cholesterol over peripheral regions, as well as an accumulation of neutral sterols in lipid droplets. Finally, in oxLDL-loaded macrophages, a source of lipoprotein-derived cholesterol, apoptosis was increased 2.7-fold. These studies provide additional evidence of a key role for macrophage ABCA2 in

modulating free cholesterol sequestration in the LE/LY that may be important for the initiation and progression of cardiovascular disease.

17. Conclusions & Perspectives

To date, the mechanisms for how ABCA2 modulates cholesterol trafficking by sequestration in the LE/LY have not been elucidated. In this report we have postulated several mechanisms that suggest avenues for further study: (1) what lipid substrates are transported across membranes by ABCA2? (2) Are anionic phospholipids translocated across intraluminal membranes of the LE/LY to modulate the salvage pathway of sphingolipid metabolism? (3) Does ABCA2 modulation of sphingosine levels by the action of acid ceramidase in the LE/LY modulate cholesterol loading onto NPC2 and cholesterol levels at the limiting membrane by NPC1? (4) Does ABCA2 modulate calcium channel function and calcium influx from the cytosol into the LE/LY and thereby modulate cholesterol trafficking by the vesicular pathway to other cellular compartments? The answers to these and other questions may provide support for a mechanistic model of ABCA2 activity and suggest possible targets for therapeutic intervention to ameliorate diseases dependent on ABCA2 function. By necessity, balancing this is the concern that neither agonists nor antagonists of transporter function have previously met with a great deal of pharmaceutical success. While in theory, ABC transporters should prove to be viable drug targets, in practice, there have been few successes, particularly in cancer. One issue is certainly the high degree of homology within the family, particularly regions of functional commonality. Because members of the B and C family were shown to be involved in resistance to a range of anticancer drugs, in the 90's testing of modulators of transporter function were studied in clinical trials. However, clinical efficacy was never realized.

In addition, pleiotropic functionalities have been ascribed to ABCA2 and while there are certainly commonalities, there are few definitive examples of mutations linked with specific disease pathologies. In this regard, associations with neurological and cardiovascular diseases as well as cancer may speak to broad based functions that might be challenging in terms of controlling the therapeutic index of any drug. A successful drug would in all likelihood also need to cross the blood brain barrier, instilling a further series of medicinal challenges. Such issues notwithstanding, the unravelling of ABCA2 structure/function has now provided a springboard to consider the challenges associated with medicinal manipulations of this quite important transporter protein.

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ABCA2 Structural Organization in Membrane Bilayer

Figure 1. ABCA2 Structural Organization in Membrane Bilayer

ABCA2 is synthesized as a single polypeptide consisting of twelve transmembrane (TM) alpha helices that cross the lipid bilayer, spanning the aqueous (luminal) and cytosolic environments. Within the cytosolic compartment, are two nucleotide binding domains (NBD1 and NBD2) that bind and hydrolyze ATP to energize lipid translocation from the between the inner leaflet (IL) and outer leaflet (OL). The NBDs also contain the “ABC signature motif” common to all ATP transporters as well as Walker A and Walker B domains that function in binding hydrolysis of ATP.

Figure 2. Endosomal Maturation and Sphingolipid Salvage

Uptake of plasma membrane sphingolipids and glycolipids from the plasma membrane at coated pits and caveolae fuse with the membranes of early endosomes. Here, the membrane topology reverses as they pinch off intraluminal vesicles, where the lumen is derived from the cytosol. Progressive acidification of endosomes by H^+ /ATP pumps as they mature results in a dramatic decrease in pH to ~4.5 to 5.5 in late-endosomes/lysosomes. This pH is optimal for the activity of sphingolipid salvage pathway enzymes, *e.g.*, Acid sphingomyelinase and Glucocerebrosidase. Catabolism of the complex sphingolipids and glycolipids generate ceramide that displaces free cholesterol from intraluminal membranes onto the soluble cholesterol carrier NPC2, which then transfers the cholesterol to NPC1 on the limiting membrane of the LE/LY. From the limiting membrane cholesterol sensors distribute cholesterol from the LE/LY to intracellular sites including the Golgi apparatus and the endoplasmic reticulum.

Hypothetical Model of ABCA2 Function in LE/LY

Figure 3. Hypothetical Model of ABCA2 Function in the LE/LY

Under reduced ABCA2 expression levels or ABCA2 loss of function (LOF) conditions *e.g.*, ABCA2 RNAi or ABCA2 knockout, the ceramide generated from sphingolipid salvage displaces cholesterol from LE/LY intraluminal vesicles onto soluble NPC2, which then transfers the cholesterol to NPC1 on the limiting LE/LY membrane, where it is distributed to other intracellular sites. Genetic mutations (stable ABCA2 expression under constitutive promoter in cell lines) or pharmacological agents that increase ABCA2 expression (GOF) increase acid ceramidase activity and sphingosine production. Because of its lipid structure, sphingosine is less effective than ceramide in displacing cholesterol onto soluble NPC2 and ultimately transfers to NPC1 on the LE/LY limiting membrane, resulting in cholesterol sequestration in the LE/LY.

Figure 4. Negatively charged phospholipids promote sphingolipid salvage enzymatic activity
On the membranes of LE/LY intraluminal vesicles, the presence of negatively charged phospholipids, *e.g.*, bis-(monoacylglycerol) phosphate (BMP), phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidylglycerol interact with positively charged (at acidic pH) Saposin (C and D) proteins and positively charged (at acidic pH) acid ceramidase and glucocerebrosidase to promote salvage pathway enzymatic activity. The mechanism for the establishment and maintenance of negatively charged phospholipid asymmetry has not been determined, but we theorize that it may be due to ABCA2 translocation of these substrates across the intraluminal membrane bilayer.