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Defective Lipid Transport and Biosynthesis in Recessive and Dominant Stargardt Macular Degeneration

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Abstract

Stargardt disease is a common inherited macular degeneration characterized by a significant loss in central vision in the first or second decade of life, bilateral atrophic changes in the central retina associated with degeneration of photoreceptors and underlying retinal pigment epithelial cells, and the presence of yellow flecks extending from the macula. Autosomal recessive Stargardt disease, the most common macular dystrophy, is caused by mutations in the gene encoding ABCA4, a photoreceptor ATP binding cassette (ABC) transporter. Biochemical studies together with analysis of *abca4* knockout mice and Stargardt patients have implicated ABCA4 as a lipid transporter that facilitates the removal of potentially toxic retinal compounds from photoreceptors following photoexcitation. An autosomal dominant form of Stargardt disease also known as Stargardt-like dystrophy is caused by mutations in a gene encoding ELOVL4, an enzyme that catalyzes the elongation of very long chain fatty acids in photoreceptors and other tissues. This review focuses on the molecular characterization of ABCA4 and ELOVL4 and their role in photoreceptor cell biology and the pathogenesis of Stargardt disease.

Keywords

Stargardt Disease; ABCA4; ABC Transporters; Retinoids; ELOVL4; Elongase; Very long chain fatty acids

1. Introduction

Inherited retinal degenerative diseases are a clinically and genetically heterogeneous group of disorders that constitute a major cause of vision loss in the world population. They are typically characterized by the progressive loss of rod and cone photoreceptor cells often leading to severe blindness [1–5]. To date over 160 genes associated with various retinal diseases have been identified and partially characterized at a genetic and molecular level and the loci of another 42 genes have been mapped (<http://www.sph.uth.tmc.edu/Retnet/>). The heterogeneity of retinal

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degenerative diseases is further highlighted by the finding that different mutations in a given gene can lead to different clinically defined disease phenotypes.

Retinal degenerative diseases are typically classified by their phenotypic characteristics. Two major classes are retinitis pigmentosa (RP) and macular degeneration (MD). RP affects 1 in 3,500 people and is typically characterized by night blindness, progressive loss in peripheral vision and subsequent loss in central vision, often leading to total loss in vision [1,6]. Genetically, RP can be separated into three subtypes: autosomal dominant RP, autosomal recessive RP, and X-linked RP. Each subtype is caused by any of a number of different genetic defects. MD involves the loss in central vision with varying preservation of peripheral vision and can affect people of all ages [7–9]. Early onset or juvenile MD is a set of disorders caused by a mutation in a given gene and therefore they are referred to as monogenic diseases [9,10]. These macular dystrophies are divided into subtypes based on their clinical presentation and include such diseases as Best disease, Stargardt macular degeneration, Doyme honeycomb retinal degeneration also known as Malattia Leventinese, Sorby fundus dystrophy, dominant macular dystrophies and others. Age-related macular degeneration (AMD) is a leading cause of vision loss in the elderly affecting as many as 30–50 million people world-wide [11–15]. It is a complex disease involving both genetic and environmental factors. In recent years, significant progress has been made in identifying genetic variants that increase one's risk for developing AMD. Major contributors are genetic variants that encode immunoregulatory proteins including complement factor H [16–19]. Genetic studies, however, also point to the involvement of other cellular pathways in AMD pathogenesis [20,21].

Most monogenic forms of RP, MD and related inherited retinal degenerative diseases are associated with genes that are expressed in photoreceptor cells or retinal pigment epithelial (RPE) cells where they encode proteins that are critical for photoreceptor structure, function and survival. Specific cellular processes and biochemical pathways implicated in various retinopathies include: phototransduction, visual cycle, photoreceptor structure and morphogenesis, cell adhesion, cellular metabolism, vesicle and protein trafficking, synaptic function, cilium structure and transport, ion and small molecular transport, chaperones, RNA splicing, transcription factors associated with photoreceptor development, protein folding and subunit assembly, posttranslational protein modification, among others. In some instances the function of the protein encoded by disease-associated genes is not known.

Two genes associated with inherited macular degenerative diseases encode proteins that function in the processing of lipids in photoreceptor cells. Mutations in the gene encoding a photoreceptor ABC transporter known as ABCA4 cause autosomal recessive Stargardt macular degeneration and related retinal degenerative diseases [22]. Biochemical studies have implicated ABCA4 in the transport of a retinal phospholipid compound, known as N-retinylidene-phosphatidylethanolamine, across photoreceptor outer segment disc membranes following photoexcitation, thereby facilitating the removal of potentially toxic retinal compounds from photoreceptor cells [23–25]. Mutations in the gene encoding ELOVL4, an elongase enzyme involved in the elongation of very long chain fatty acids, have been linked to autosomal dominant Stargardt-like disease [26]. In this chapter, we review the genetic and biochemical studies linking ABCA4 to lipid transport and ELOVL4 to lipid biosynthesis in photoreceptors and provide insight into molecular mechanisms underlying these forms of Stargardt diseases.

2. Autosomal Recessive Stargardt Disease and ABCA4

2.1 Clinical and Genetic Characteristics of Stargardt Disease

Stargardt disease (STGD1; MIM #248200), first described by the German ophthalmologist Karl Stargardt in 1909, is the most common autosomal recessive, early onset macular dystrophy

with an incidence of 1 in 8,000–10,000 individuals [27–30]. Affected individuals typically experience significant loss in central vision with a marked reduction in visual acuity in their first or second decade of life [31,32]. Progressive decrease in visual acuity generally occurs throughout life with values reaching 20/200 or worse in the final stages of the disease [29]. Stargardt patients also show a delay in dark adaptation and variable loss in color vision [30, 33–35].

Ophthalmoscopic examination of Stargardt patients typically reveals bilateral atrophic changes in the macula associated with the degeneration of photoreceptor cells and underlying retinal pigment epithelium. A characteristic feature of the disease is the level presence of yellow-white flecks around the macula and midperiphery of the retina at the level of the retinal pigment epithelial (RPE) cells (Fig. 1). Histochemical analysis of donor eyes from deceased Stargardt patient revealed significant loss in photoreceptor cells and excessive accumulation of lipofuscin deposits in the RPE cells [36,37]. Lipofuscin deposits are composed of a heterogeneous mixture of oxidized lipids, diretinoids and other components derived from the incomplete degradation of phagocytized photoreceptor outer segments [38,39]. During fluorescein angiography, lipofuscin absorbs blue excitatory light producing the characteristic “dark choroid” appearance in many Stargardt patients [40]. Scotopic electroretinograms (ERGs) characteristic of rod photoreceptor function and photopic ERGs which measure cone responses vary between Stargardt patients. Some individuals show relatively normal full-field ERGs, whereas other patients exhibit significant loss in scotopic and/or photopic ERGs [41–43].

Fundus flavimaculatus is a retinal disease first described by Franceschetti in the 1960's [44]. It is characterized by the symmetrical appearance of yellow-white flecks extending from the macula [37,44]. Extensive clinical and genetic analyses have shown that autosomal recessive Stargardt disease and fundus flavimaculatus are variations of the same disease, typically caused by different mutations in the same gene [28,45,46]. Fundus flavimaculatus is generally used to describe a late onset, more mild form of Stargardt disease [30,47].

The gene for autosomal recessive Stargardt disease was first identified by Allikmets and coworkers in 1997 and shown to encode a novel member of the ABC transporter superfamily [22]. At the same time, the gene for the Rim protein, a high molecular weight, relatively abundant glycoprotein in photoreceptor outer segments first described in the late 1970's [48, 49], was cloned and found to encode for the same ABC transporter [50–52]. This ABC transporter, initially known as ABCR, is now more commonly called ABCA4 for the fourth identified member of the ABCA subfamily of ABC transporters. Stargardt patients from around the world have been screened for mutations in ABCA4. Currently, over 500 different mutations in ABCA4 are known to cause Stargardt disease. These include missense, nonsense, splice-site, frameshift, and small deletion and insertion mutations. Missense mutations, the most common type of mutation, are distributed throughout the protein (see Figure 4).

2.2 Other ABCA4-associated Retinal Degenerative Diseases

In addition to Stargardt disease, mutations in ABCA4 are known to cause two more severe, clinically distinct retinal degenerative diseases, known as cone-rod dystrophy and retinitis pigmentosa. Cone-rod dystrophy is a retinal disorder in which cone photoreceptor cells degenerate before rod cells [53]. Affected individuals experience severe loss in visual acuity and color vision and display significant atrophy of the central retina or macula. This is followed by progressive loss in peripheral vision and night blindness. Mutations in *ABCA4* are a major cause of the autosomal recessive form of cone-rod dystrophy accounting for between 30% and 60% of the cases [54–57]. In some instances Stargardt disease can progress to cone-rod dystrophy [53,58].

Retinitis pigmentosa or RP is a severe retinal degenerative disease associated with night blindness, reduction in peripheral vision and progressive loss in central vision often leading to complete blindness [1,59]. RP patients exhibit attenuated retinal vessels, bone-spicule pigmentation, and a waxy pallor of the optic disk. To date over 45 different genes have been implicated in various forms of RP (<http://www.sph.uth.tmc.edu/Retnet/>). Mutations in *ABCA4* have been linked to a subset of autosomal recessive RP known as RP19 [57,60]. Unlike typical RP, however, affected individuals show an early loss in central vision and atrophy of the macula in addition to the characteristic features of RP.

Mutations in *ABCA4* have also been implicated in some forms of age-related macular degeneration (AMD). In one study a higher number of individuals heterozygous for Stargardt disease mutations were found to have AMD [61,62]. However, the involvement of *ABCA4* in AMD remains controversial since not all studies have found a direct association between Stargardt mutations and AMD [63,64].

It is now generally believed that mutations in *ABCA4* result in a spectrum of related retinal dystrophies, the severity of which depends on a number of factors including the types of mutations and its effect on protein function, age of diagnosis, stage of the disease, and genotypic variations of individuals [58,65–68].

2.3. ABC Transporters

ABC transporters comprise a superfamily of proteins found in essentially all living things including micro-organisms, plants and animals [69,70]. They typically transport a wide variety of compounds across cell membranes using ATP hydrolysis as an energy source. Substrates known to be transported by various ABC transporters include phospholipids, fatty acids, steroids, organic anions, vitamins, metal ions, drugs, amino acids, peptides, and other compounds. Eukaryotic ABC transporters are unidirectional exporters typically translocating substrates from the cytoplasmic side to the extracellular side of the plasma membrane or lumen side of intracellular membranes. Prokaryotic ABC transporters can be either importers or exporters.

ABC transporters are composed of four core domains: two transmembrane domains (TMDs) and two nucleotide-binding domains (NBDs) [69,71,72]. In prokaryotes, these domains exist either on individual polypeptides which associate to form a heterotetrameric complex or fused onto a larger polypeptide in different combinations. In eukaryotes, ABC transporter typically are organized either as full length transporters in which all four domains reside on a single polypeptide chain or half transporters in which two polypeptides each containing a TMD and a NBD associate to form homo- or heterodimers.

The two TMDs form the substrate binding site and pathway for translocation of substrates across the membrane. Each TMD contains multiple membrane spanning segments most often numbering six. The binding and hydrolysis of ATP within the NBDs provide the energy for transport of the substrate across the membrane. NBDs consist of approximately 200 amino acids and contain a number of conserved motifs including Walker A and Walker B motifs found in many ATPases and the ABC signature motif (LSSGQ) which is used to define members of the ABC transporter family [71–73].

The human genome contains 49 ABC transporters (<http://nutrigene.4t.com/humanabc.htm>). These have been organized into 7 subfamilies (ABCA through ABCG) based on similarities in gene organization and sequence [70,74]. A significant number of ABC transporters have been linked to severe human genetic diseases [75–77]. For example, mutations in ABCD1 (ALD) involved in the transport of very long chain fatty acids is associated with adrenoleukodystrophy, defects in (Sterolin1 and 2) which function in the transporter of sterols

have been linked to sitosterolemia, defects in ABCB11 (BSEP) involved in the transport of bile acids cause liver disease, and mutations in ABCC7 (CFTR), a chloride channel expressed in exocrine tissue are responsible for cystic fibrosis.

The ABCA subfamily of ABC transporters consists of 12 members [76]. All members are full-length transporters organized in two tandem halves. Each half contains a TMD consisting of multiple membrane spanning segments followed by a NBD. A characteristic feature of ABCA proteins is the presence of a large glycosylated exocyttoplasmic domain that separates the first membrane spanning segment from a cluster of five transmembrane segments in both the N and C halves of the protein [78]. The sequence identity between members of the ABCA subfamily ranges from 30% to over 70%. Homologues of mammalian ABCA proteins have been identified in eukaryotic and prokaryotic organisms suggesting that ABCA genes have evolved from a common primordial gene [79].

Of the twelve members in the human ABCA subfamily, three ABCA transporters (ABCA1, ABCA3, ABCA12) in addition to ABCA4 have been linked to inherited diseases associated with defective transport of lipids [76]. Mutations in *ABCA1* are known to cause Tangier disease and familial high density lipoprotein deficiency associated with defective cholesterol and phospholipid efflux from cells and a deficiency in the formation of high-density lipoprotein [80–82]. Mutations in ABCA3 cause neonatal surfactant deficiency and pediatric interstitial lung disease resulting from abnormal surfactant secretion into the alveoli of the lungs and the formation of abnormal lamellar bodies [83–86]. Mutations in ABCA12 are associated with harlequin and lamellar ichthyosis diseases of the skin arising from defective lipid transport [87,88]. Other members of the ABCA subfamily have yet to be associated with inherited diseases.

2.4 Expression and Localization of ABCA4

ABCA4 is highly expressed in the vertebrate retina with little or no detectable expression in most other tissues including the lung, kidney, liver, lung and spleen as analyzed by Northern blot analysis [22]. Using highly sensitive real time RT-PCR methods, *ABCA4* expression has been detected in testes and brain [89]. ABCA4 protein and mRNA expression has also been found in the choroid plexus of rats, although the role of ABCA4 in this tissue remains to be determined [90,91].

The retina consists of 5 major neuronal cell types (photoreceptors, bipolar cells, horizontal cells, amacrine cells and ganglion cells) and 1 glial cell type known as Mueller cells (Figure 2A). Within the retina, *ABCA4* gene expression is restricted to the photoreceptor cells as shown by *in situ* hybridization [22]. The photoreceptor cell is highly differentiated with respect to its structure and function (Figure 2A). It consists of five principal regions: the outer segment which functions in the detection of light and its conversion into an electrical signal in a process known as phototransduction; a connecting cilium that joins the outer segment to the inner segment and allows for the passage of specific proteins and other molecules between the inner and outer segment; the inner segment which contains the biosynthetic and metabolic machinery of cell including the mitochondria, endoplasmic reticulum (ER), golgi, transport vesicles, and lysosomes; the cell body which houses the nucleus; and the synaptic region consisting of synaptic vesicles and a ribbon synapse for transmission of electrical signals from the photoreceptors to secondary neurons of the retina. Immunofluorescence labeling and subcellular fractionation studies have localized ABCA4 to the light sensitive photoreceptor outer segment layer that lies adjacent to the retinal pigment epithelium (Figure 2B) [48–50, 92].

The two principal types of photoreceptors in the vertebrate retina are the rod and cone cells. Rod cells are highly sensitive to light and function under dim lighting conditions, whereas cone

cells respond to normal light and are responsible for color vision and high visual acuity. Cone photoreceptors are further divided into subtypes based on their sensitivity to light. Humans have 3 types of cone cells: blue or short wavelength (S) cones; green or middle wavelength (M) cones; and red or long wavelength (L) cones. The human retina contains over 6 million cone cells and 120 million rod cells. The highest density of photoreceptors is found in the central pigmented region of the retina known as the macula. Within the macula there is a specialized cone-rich region called the fovea which is responsible for high visual acuity in humans. The inner cell layers are displaced outward from the central fovea such that light has a more direct path to the cone cells. Rod cells are the major photoreceptor cell type in the peripheral human retina with cone cells sparsely dispersed in a mosaic pattern.

Initial immunolabeling studies suggested that ABCA4 was only present in the outer segment of mammalian rod photoreceptor cells [50,92]. However, subsequent immunofluorescence microscopy and western blotting techniques have clearly shown that ABCA4 is present not only in rod outer segments but also in the outer segments of all human peripheral and foveal cone photoreceptors [93]. The presence of ABCA4 in mammalian rod and cone outer segments is consistent with earlier immunoelectron microscopic studies of Papermaster and colleagues showing that ABCA4 or the Rim protein is present in both cone and rod outer segments of frog photoreceptors [48,94].

Photoreceptor outer segments consist of an organized array of disc-like membranes. In the rod outer segments, the stack of over 1000 closed discs is surrounded by a separate plasma membrane (Figure 3A). Each disc is composed of two flattened membranes or lamellae which are circumscribed by a hairpin loop known as the rim region (Figure 3B). One or more incisures penetrate toward the center of the discs. The continuous disc membrane encloses an intracellular compartment known as the intradiscal space or disc lumen which is topologically equivalent to the lumen of the endoplasmic reticulum. In cone cells, the disc and plasma membranes are continuous resulting in a folded, disc-like organization often with a tapered 'cone-like' appearance. The flattened region of the disc membrane is densely packed with the photopigment protein, rhodopsin in rod cells and cone opsin, also known as iodopsin, in cone cells. The rim region of the discs in rod and cone outer segments contains a distinct set of membrane proteins [95]. Immunoelectron microscopic studies have shown that ABCA4 is confined to the rim region and incisures of rod and cone photoreceptor disc membranes (Figure 3B,C) [48, 50, 94].

The biological significance of ABCA4 localization to the rim region is not known. However, ABCA4 contains two large exocytosolic domains which are unlikely to fit within the intradiscal space between the two flattened membranes of a disc estimated to be 2.5–3.0 nm (Figure 3C). In contrast the intradiscal space within the disc rim is considerably greater with an axial length of about 13.5 nm which can accommodate ABCA4 [96]. The mechanism by which ABCA4 or other rim specific proteins are targeted to the rim region of rod and cone outer segments is not known. The initial finding that ABCA4 is localized to outer segment disc membranes together with its link to Stargardt disease provided the first clue that ABCA4 may play central role in photoreceptor outer segment function [22, 50].

2.5 Structural Features of ABCA4

ABCA4 located on chromosome 1 (position 1p21-p22.1), is most closely related to the *ABCA1* on chromosome 9 (position 9q31.1). Both genes consist of 50 exons and code for proteins of similar size, sequence, and structural organization. Human ABCA4 and ABCA1 contain 2,273 and 2,261 amino acids, respectively, and are 52% identical and 60% similar in amino acid sequence. ABCA4 also shares a high degree of sequence similarity to ABCA7 (49%), ABCA2 (40%), and ABCA3 (39%). Among vertebrates, human ABCA4 is 88–89% identical in sequence to bovine and mouse orthologues and 66% identical to *Xenopus laevis*

ABCA4. Comparative sequence analysis of ABCA4 from various species supports an evolutionary model in which the full-length transporter evolved from the fusion of two distinct half-transporter progenitors [97].

ABCA4 is a relatively abundant membrane glycoprotein in photoreceptor outer segments comprising approximately 2–4% of the total rod outer segment (ROS) membrane protein by weight [48,92,98]. On SDS polyacrylamide gels, it migrates as a single sharp band with an apparent molecular mass of 220–250 kDa in general agreement with its molecular mass of 256 kDa calculated from its amino acid sequence [50].

ABCA4 is organized in two structurally related tandem-arranged halves with each half containing a transmembrane domain (TMD) followed by a nucleotide binding domain (NBD). The topological organization of ABCA4 within the membrane has been predicted using computer algorithms in conjunction with biochemical studies [25,50,78]. Both the N and C halves are predicted to have a single membrane spanning segment followed by a large exocytosolic (extracellular/lumen) domain (ECD), five membrane spanning segments and a nucleotide binding domain (NBD) as illustrated in Figure 4. A similar topological model has been proposed for ABCA1 and other ABCA family members [78,99].

The small 24 amino acid N-terminal segment preceding the first transmembrane segment of ABCA4 is predicted to reside on the cytoplasmic side of the membrane based on the presence of several positively charged lysine and arginine residues and the positive inside rule [100]. ECD1 in the N-half contains 602 amino acids; ECD2 in the C-half of ABCA4 is smaller with 289 amino acids. Both ECDs have multiple N-linked oligosaccharide chains as revealed by site-directed mutagenesis, lectin binding and endoglycosidase analysis supporting the location of these domains on the lumen side of the disc membrane [78]. In addition there are numerous conserved cysteine residues which form intramolecular disulfide bonds. One or more disulfide bonds also links ECD1 to ECD2 in ABCA4 as well as ABCA1 [78,101]. The function of the ECDs is not known although in the case of ABCA1 these domains bind ApoA1 [99]. However, analyses of disease-associated mutations in the ECDs indicate that these domains are important in proper protein folding and stability. To date no proteins have been identified that bind to the ECDs of ABCA4.

Each transmembrane domain (TMD) of ABCA4 consists of a single membrane spanning segment preceding each ECD and a cluster of membrane spanning segments upstream of the NBD (Figure 4). The number and identity of the multi-spanning segments remain to be determined experimentally. However, the total number of transmembrane segments including the initial membrane spanning segment in each TMD most likely numbers six. The two TMDs form the substrate binding site although the specific segments involved in substrate binding remain to be determined.

NBD1 and NBD2 consist of approximately 200 amino acids each and are 37% identical in amino acid sequence. Modeling studies indicates that these NBDs have a similar structural organization as NBDs of ABC transporters of known structure [71–73,102–104]. Each NBD has an L-shape appearance and consists of two subdomains, a RecA-like catalytic domain and a smaller helical subdomain. The RecA-like domain contains the Walker A motif (GxxGxGKS/T where x is any amino acid) and Walker B motif (hhhhD where h is a hydrophobic amino acid). The ABC signature motif (LSSGQ) resides in the helical domain. The Walker A motif, present in many ATPases and GTPases, interacts with the phosphate residues of ATP [105]. The Walker B motif also part of the ATP binding site and contacts the γ -phosphate of ATP. The ABC signature motif is found between the Walker A and Walker B motifs. When the two NBDs dimerize during a transport cycle of ABC transporters, the ABC signature motif of one NBD interacts with the bound ATP on the opposing NBD [71,72].

The NBDs of ABCA4 contain additional structural elements found in other ABC transporters. These include an A-loop with a conserved aromatic amino acid (phenylalanine in NBD1 and tyrosine in NBD2) involved in stacking interactions with the adenine ring of ATP [106,107], flexible Q-loop containing a conserved glutamine and involved in the transmission of conformational changes between the NBDs and the TMD [108], an H-loop with a conserved histidine [109], and a D loop that follows the Walker B motif [103].

Each NBD has been expressed and purified from *E. coli* and shown to display ATP hydrolysis activity [110,111]. Furthermore, the two isolated NBDs interact in vitro in the presence and absence of nucleotide [112].

ABCA4 also contains a stretch of 140 amino acids downstream from NBD2. This C-terminal region is highly conserved between different vertebrates suggesting that it plays an important structural and/or regulatory role. More recently, a highly conserved VFVNFA motif near the C-terminus has been shown to play an essential role in the folding of ABCA4 into a functional protein [113].

2.6 Functional Properties of ABCA4

2.6.1 ATP binding and hydrolysis—The binding of nucleotides to ABCA4 was first shown by photoaffinity labeling using radiolabeled 8-azido ATP [50]. Addition of either ATP or GTP inhibited 8-azido ATP labeling of ABCA4 in ROS membranes indicating that either nucleotide could effectively bind ABCA4. Similar methods have been used to detect the binding of nucleotides to ABCA4 expressed in HEK 293 and COS1 cells [113,114]. The photoaffinity reagents, 8-azido-ATP and 8-azido-ADP preferentially label NBD2 [115].

The ATPase activity of ABCA4 cannot be directly measured in isolated photoreceptor outer segment membranes due to the low enzymatic activity of ABCA4 and the high activity of other ATPases in these preparations [23,116]. To get around this problem, detergent solubilized ABCA4 was purified from either rod outer segment membranes or transfected cells by immunoaffinity chromatography and reconstituted into lipid vesicles for analysis of ATP hydrolysis activity [23,113,114,117]. The reconstituted protein exhibited a low basal ATPase activity. Putative transport substrates were identified by their capacity to stimulate the ATPase activity of ABCA4 in these preparations. Out of over 40 different compounds tested, two physiologically relevant retinoid compounds, all-*trans* retinal and 11-*cis* retinal, stimulated the ATPase activity of ABCA4 several fold above the basal activity [23,117]. The 13-*cis* retinal isomer also activated ABCA4, but other retinoid and aliphatic aldehyde derivatives including retinol, retinyl esters, and nonylaldehyde were ineffective. These studies provided the first indication at a biochemical level that retinoid compounds, known to play a central role in the capture of light by photoreceptors, may serve as the transport substrate for ABCA4 [23].

The ATPase activity of purified and reconstituted ABCA4 requires the presence of phospholipids [23,117]. Basal and retinal stimulated ATPase activity was observed for ABCA4 reconstituted into soybean, brain polar, and native rod outer segment lipids with the highest activities found for ABCA4 reconstituted with rod outer segment lipids known for having a high content (~40 mol %) of phosphatidylethanolamine (PE) [117]. Addition of exogenous PE to brain polar lipids during reconstitution increased the basal and retinal stimulated ATPase activity of ABCA4 to levels comparable to that measured for ABCA4 reconstituted into rod outer segment lipids confirming the importance of PE. ABCA4 reconstituted into PC liposomes exhibited low basal ATPase activity which was not activated by the addition of retinal compounds [23,117]. Taken together, these studies indicate that PE together with retinal strongly stimulates the ATPase activity of ABCA4.

Heterologous expression of ABCA4 has been used to study the effect of mutations in the Walker A motifs (K969M in NBD1 and K1978M in NBD2) on the basal and retinal activated activities of purified and reconstituted ABCA4 [114]. Both single and double Walker A mutants expressed at protein levels comparable to wild-type ABCA4 and were labeled with 8-azido ATP [114,115]. However, the K969M/K1978M double mutant and the K969M single mutant showed little or no basal or retinal stimulated ATPase activity. The K1978M single mutant exhibited basal ATPase activity but no retinal stimulated activity. These studies indicate that NBD1 and NBD2 are both important, but play distinct mechanistic roles. The ATPase activity of ABCA4 containing disease linked missense mutations have also been reported [114]. Basal and stimulated activities were reduced to varying degrees in essentially all disease-linked mutants [113,114].

The ATPase activities of the NBDs expressed and purified from *E. coli* have been measured [110,111]. NBD1 displays a lower ATPase activity than NBD2, but has broader nucleotide substrate specificity. Isolated NBD1 can hydrolyze GTP, CTP and UTP as well as ATP, while NBD2 is specific for ATP.

2.6.2 Retinylidene-Phosphatidylethanolamine Substrate Binding—Retinal reversibly reacts with PE to form the Schiff base conjugate known as N-retinylidene-phosphatidylethanolamine or N-ret-PE (Figure 5). Accordingly, either free all-*trans* retinal or N-ret-PE could be responsible for the increase in ATP hydrolysis observed when all-*trans* retinal is added to PE-containing liposomes reconstituted with ABCA4. Mechanistically, ABCA4 could utilize the energy from ATP hydrolysis to compounds extrude all-*trans* retinal from disc membranes similar to the extrusion of hydrophobic compounds from membranes by multi-drug resistant transporters such as P-glycoprotein [118] although in the reverse direction, i.e. into the cytoplasm of the outer segment. Alternatively, ABCA4 could function as a lipid flippase to actively transport N-ret-PE across the disc membrane similar to the phospholipid flippase activity displayed by other ABC transporters [119–121].

To identify the retinoid substrate that binds to ABCA4, various retinoids were added to ABCA4 immobilized on an immunoaffinity matrix and the bound substrate was identified by HPLC and spectrophotometry [122]. When all-*trans* retinal was added to immobilized ABCA4 in the presence of PE, stoichiometric amounts of N-ret-PE bound to ABCA4 with an apparent K_d of 4–5 μ M. N-retinyl-PE produced by sodium borohydride reduction of the N-ret-PE (Figure 5) also bound tightly to ABCA4 and competed with N-retinylidene-PE for the same binding site [117]. In contrast, all-*trans* retinal in the absence of PE or all-*trans* retinol in the presence of PE failed to bind ABCA4. These studies provide strong evidence for the role of N-ret-PE as the likely physiological substrate for ABCA4.

The Schiff base of N-ret-PE can exist in either a protonated or nonprotonated state (Figure 5). Recent spectrophotometric measurements of N-ret-PE bound to ABCA4 indicate that the nonprotonated form of N-ret-PE binds to ABCA4 (Zhong and Molday, unpublished results).

The effect of ATP and other nucleotides on the binding of N-ret-PE to ABCA4 has been investigated [122]. Addition of ATP (or GTP) to ABCA4 containing bound N-ret-PE resulted in the dissociation of substrate from the protein [113,122]. This is consistent with a mechanism in which N-ret-PE binds to a high affinity binding site, presumably within the TMDs of ABCA4, in the absence of ATP. The binding of ATP results in a protein conformational change which converts the high affinity binding site to a low affinity site and a dissociation of substrate from the transporter. Although these studies support N-ret-PE as the substrate for ABCA4, they do not provide information on the direction of transport or detailed insight into the transport mechanism.

2.6.3 Possible Mechanism of N-ret-PE Transport—The mechanism by which ABC proteins transport substrates across membranes is not fully understood. However, a number of studies support an ATP-switch model [72,123]. In this model, the substrate binds to a high affinity site in the TMDs with the NBDs in an open dimer state. The subsequent binding of ATP causes the NBD dimer to close. This is coupled to a conformational change in the TMDs converting the high affinity substrate binding site to a low affinity site and a dissociation of substrate on the other side of the membrane. ATP hydrolysis destabilizes the NBD closed dimer state. Finally, the dissociation of ADP and phosphate restores the transporter to its high-affinity binding state thereby completing the cycle. Figure 6 shows a conceptual model for the transport of N-ret-PE by ABCA4. In this model both NBD1 and NBD2 are suggested to be involved in ATP binding and hydrolysis as supported by mutagenesis studies although their distinct roles in the transport process remains to be defined [114]. Although the direction of transport has not been determined experimentally, data from *abca4* knockout mice and phenotypic analysis of Stargardt patients are consistent with ABCA4 transporting N-ret-PE from the lumen to the cytoplasmic side of the disc membrane. In this regard, it is important to note the proposed direction of transport of N-ret-PE by ABCA4 from the lumen to the cytoplasmic side of the disc membrane is opposite to the direction of substrate transport for most other mammalian ABC transporters that have been studied.

2.7 Abca4 Knockout Mice

Travis and coworkers produced an *abca4* knockout mouse to investigate the role of ABCA4 in the physiology and pathology of photoreceptors [24,124,125]. Homozygous *abca4* knockout mice showed a relatively normal photoresponse. The rate of rhodopsin regeneration in these mice also appeared normal suggesting that a sufficient amount of all-*trans* retinal is converted to 11-*cis* retinal by the visual cycle for regeneration of rhodopsin. *Abca4* knockout mice, however, show significant light-dependent changes in lipids. The outer segments of the *abca4* knockout mice exposed to cyclic or continuous lighting had elevated levels of all-*trans* retinal, prorogated N-ret-PE, and PE, and decreased levels of all-*trans* retinol and all-*trans* retinal esters relative to age-matched wild-type mice or *abca4* mice reared in the dark. In addition, an accumulation of lipofuscin deposits were found in the RPE cells of *abca4* (−/−) and *abca4* (+/−) mice exposed to continuous or cyclic lighting. Biochemical analysis of the lipofuscin deposits from *abca4* knockout mice show elevated levels of several fluorescent diretinoid compounds, including A2E, a diretinal pyridinium compound known to be a major component of lipofuscin, all-*trans* retinal dimer, and related diretinal compounds [124–129]. Many of these same compounds have been found in lipofuscin deposits from individuals with Stargardt disease and age-related macular degeneration [39,124].

In addition, initial reports indicated that *abca4* knockout mice, like individuals with Stargardt disease, show a delay in dark adaptation consistent with the delayed removal of all-*trans* retinal from outer segments following photobleaching [24,125]. Other studies, however, indicate that *abca4* knockout mice can undergo dark adaptation at a faster rate than wild-type mice under certain conditions [130]. The reasons for the observed differences in dark adaptation in these studies remain to be worked out.

Although initial studies found little or no evidence of photoreceptor degeneration in *abca4* knockout mice, more recent studies indicate that progressive photoreceptor degeneration is evident when *abca4* knockout mice are bred onto an albino (balb/c) background. This may be due an increase in the formation of A2E epoxides or oxiranes [131,132] in the absence of pigmentation. More recently, mice deficient in both *abca4* and RDH8, the principal retinol dehydrogenase in rod and cone outer segments, were studied [133]. These double knockout mice exhibited a marked reduction in clearance of all-*trans* retinal, high levels of lipofuscin deposits, and severe photoreceptor and RPE degeneration at an early age. These studies support

the concept that ABCA4 together with RDH8 play an essential role in the removal of all-*trans* retinal and related retinoid compounds from photoreceptor outer segments following photoexcitation.

2.8 Role of ABCA4 in Lipid Transport and the Visual Cycle

In the dark, the 11-*cis* retinal chromophore is covalently bound to Lys296 of rhodopsin through a protonated Schiff base linkage. Phototransduction is initiated when light isomerizes 11-*cis* retinal to all-*trans* retinal within the binding pocket of rhodopsin to generate the activated state known as metarhodopsin II. This leads to the G-protein activation of phosphodiesterase resulting in a decrease in cGMP concentration, closure of cGMP-gated channels in the plasma membrane, and a hyperpolarization of the photoreceptor cell [134,135]. Following photoexcitation, the rod photoreceptor cell returns to its dark state through the inactivation of rhodopsin and other components of the visual cascade, resynthesis of cGMP by guanylate cyclase, and the reopening of the cGMP-gated channels in the plasma membrane in response to increased levels of cGMP. A similar mechanism occurs in cone photoreceptor outer segments.

In addition, all-*trans* retinal has to be converted back to 11-*cis* retinal for regeneration of rhodopsin. This occurs through a series of reactions known as the visual cycle (Figure 7A) [136–138]. Following the decay of the active state of rhodopsin, hydrolysis of the retinal-opsin Schiff base linkage occurs. Recent X-ray crystallographic studies suggest that all-*trans* retinal is subsequently released from its binding pocket through an opening between transmembrane helices TM1 and TM7 of opsin [139, 140]. All-*trans* retinal can either bind weakly to another hydrophobic site on the surface of rhodopsin or freely diffuse into the lipid bilayer of the disc membrane.

All-*trans* retinal accessible on the cytoplasmic surface of disc membranes can be directly reduced to all-*trans* retinol in a reaction catalyzed by the retinol dehydrogenase RDH8 [141]. All-*trans* retinol is then shuttled to RPE cells by the protein IRBP and subsequently converted back to 11-*cis* retinal through a series of enzymatic reactions for the regeneration of rhodopsin (Figure 7) [137,138]. However, it is known that a fraction of all-*trans* retinal released from rhodopsin reversibly reacts with PE in the disc membrane to form N-ret-PE [142,143]. This compound when trapped on the lumen or intradiscal side of the disc membrane is inaccessible to RDH8 for reduction to all-*trans* retinol. ABCA4 is proposed to function as a lipid transporter, binding N-ret-PE on the lumen side and flipping it to the cytoplasmic side of the disc membrane using ATP hydrolysis as an energy source (Figure 7B). Once on the cytoplasmic side, the Schiff base of N-ret-PE can hydrolyze to form all-*trans* retinal and PE. This enables all-*trans* retinal to be reduced to all-*trans* retinol by RDH8 for entry into the visual cycle. Hence, ABCA4 ensures that all of the all-*trans* retinal and N-ret-PE generated from the photobleaching of rhodopsin or cone opsin is effectively detoxified by reduction to all-*trans* retinol and cleared from photoreceptor cells for conversion to 11-*cis* retinal by the visual cycle.

2.9 Role of ABCA4 in Stargardt Disease

Loss in the function of ABCA4 as an N-ret-PE transporter explains many of the characteristic features of Stargardt disease. These include the presence of A2E-containing lipofuscin deposits, dark choroid, loss in RPE and photoreceptor viability, and delay in dark adaptation. A reduction in ABCA4 activity will result in the accumulation of N-ret-PE and all-*trans* retinal in the disc membrane following photoexcitation as observed in *abca4* knockout mice. N-ret-PE can condense with a second molecule of all-*trans* retinal in photoreceptor outer segments to produce diretinoid compounds including the phosphatidyl pyridinium diretinoid derivative A2PE [39,124,129] (Figure 8A). Following phagocytosis by the RPE cells, the outer segments are digested by lysosomal enzymes of RPE cells. As part of this process, the phosphatidyl

moiety of A2PE is removed by lysosomal enzymes to produce A2E [144]. Since A2E and related diretinoids cannot be readily metabolized, they progressively accumulate in RPE cells as fluorescent lipofuscin deposits as seen in Stargardt patients (Figure 1) and *abca4* knockout mice [24,38,124,126].

The accumulation of A2E in RPE cells has negative effect on RPE cell function and viability. A2E has been reported to act as a detergent to disrupt intracellular membranes, a photosensitizer for the generation of free radicals that can adversely affect DNA and membrane lipids, an inhibitor of RPE phagocytosis and normal RPE degradative functions, and an inhibitor of cholesterol efflux from RPE cells [39,126,145–150]. A2E has also been shown to activate the complement system possibly contributing to the pathogenesis of Stargardt disease as well as age-related macular degeneration [151]. Photoreceptor cells depend on healthy RPE cells for removal of aged outer segments, regeneration of 11-*cis* retinal, and providing the appropriate environment for maintaining viable photoreceptor cells. Accordingly, the degeneration of RPE cells will cause photoreceptors to die resulting in the loss in the vision experienced by Stargardt patients (Figure 8B).

The loss of N-ret-PE transport activity of ABCA4 can also explain the observed delay in dark adaptation observed in Stargardt patients and *abca4* knockout mice. Since N-ret-PE and all-*trans* retinal are in equilibrium, an accumulation of N-ret-PE in disc membranes due to dysfunction of ABCA4 will also result in an increase in free all-*trans* retinal in disc membranes. All-*trans* retinal can reassociate with opsin to form a complex that activates the visual cascade, although less efficiently than the photoactivated rhodopsin [152,153]. This low level of activity can contribute to background noise and a delay in the recovery of photoreceptors to their dark-adapted state. Finally, the initial loss in central vision in patients with Stargardt disease can be rationalized by the high density of photoreceptors in the macular region of the retina and hence the high rate of production and accumulation of A2E compounds in the macula due to loss in ABCA4 activity.

The effect of disease-causing mutations on the level of ABCA4 expression, subcellular localization, and biochemical properties has been studied in transfected HEK293 and COS1 cells. Sun et al. have examined the protein expression levels and basal and retinal activated ATPase activities of 37 disease-associated ABCA4 mutations [114]. One-third of these mutants were expressed at significantly reduced levels presumably due to their instability. These included mutant proteins with small deletions or amino acid substitution which introduced charged amino acid residues in putative transmembrane domains. Mutants which displayed a level of protein expression comparable to wild-type ABCA4 generally showed a reduction or elimination of ATP binding and hydrolysis. Some mutations caused a reduction in both basal and retinal activated ATPase activities whereas others retained basal ATPase activity, but had reduced or no retinal stimulated activity [114]. In a more recent study, the effect of C-terminal deletion mutations on protein expression, N-ret-PE binding, subcellular localization, and basal and retinal stimulated ATPase activity of ABCA4 was examined [113]. The ABCA4D30 lacking the C-terminal 30 amino acids and associated with cone-rod dystrophy [154] showed a significant decrease in protein expression, complete loss in both N-ret-PE binding and retinal-stimulated ATPase activity, and retention in the endoplasmic reticulum indicative of severe protein misfolding. In contrast, the ABCA4D24 mutant lacking the C-terminal 24 amino acids and associated with a mild form of Stargardt disease [155] displayed essentially normal N-ret-PE binding, a small reduction in retinal stimulated ATPase activity and intracellular vesicular localization characteristic of wild-type ABCA4 implying relatively normal protein folding. These results highlight the importance of C-terminus in proper protein folding and functional activity of ABCA4 and contribute to our understanding of how selected mutations in ABCA4 can cause different disease phenotypes [113].

Although disease-linked mutations can cause a reduction in the transport activity of ABCA4, they also can cause the mistargeting of ABCA4 to the outer segment. In one study the targeting of ABCA4 mutants to outer segments was studied in transgenic *Xenopus laevis*. Several disease-associated mutations were retained in the inner segment of photoreceptors suggesting that protein mislocalization as well as diminished function can contribute to the mechanisms underlying ABCA4 associated diseases [156]. Taken together, these studies indicate that the activity and targeting of ABCA4 to outer segments vary for different disease-associated mutations in ABCA4. This can contribute to the wide range of phenotypes displayed by patients with Stargardt disease and other related retinal degenerative diseases arising from mutations in ABCA4.

3. Autosomal Dominant Stargardt-like Disease and ELOVL4

3.1 Clinical and Genetic Characteristics of Autosomal Dominant Stargardt-like Disease

As the name implies, autosomal dominant Stargardt-like disease is phenotypically similar to autosomal recessive Stargardt disease [157–160]. Characteristic features include significant loss in central vision within the first or second decade of life, a progressive reduction in visual acuity, bilateral macular atrophy often associated with yellowish flecks which extend toward the mid-periphery of the retina, accumulation of lipofuscin in the RPE cells, and progressive degeneration of the RPE and photoreceptors cells (Figure 1B). Unlike Stargardt disease, however, Stargardt-like disease is exceedingly rare and inherited in a dominant manner. Genetically distinct autosomal dominant forms of Stargardt-like disease have been mapped to chromosome 6q (*STGD3*; MIM #600110) and chromosome 4q (*STGD4*) [157,161].

The *STGD3* gene was first identified in 2001 by Zhang and coworkers and shown to encode ELOVL4 (elongation of very long chain fatty acid-4), a member of the ELO family of proteins involved in the elongation of fatty acids [26]. Three distinct disease-causing mutations in ELOVL4 have been identified in different families. Mutational analysis of the *ELOVL4* gene in five large Stargardt-like macular dystrophy pedigrees revealed a 5 base-pair deletion which results in a frame-shift and the introduction of a stop codon, 51 codons from the end of the coding region [26]. Subsequently, two single base-pair deletions in *ELOVL4*, 789delT and 794delT, were found in a large Utah pedigree [162]. A third mutation, Y270stop, was found in a Dutch family with dominant STGD [163]. All three mutations result in a truncated protein missing the C-terminal segment of ELOVL4 including a conserved dilysine ER retention signal (KXXXX).

3.2 ELOVL Protein Family

Fatty acids are key components of membrane glycerophospholipids and sphingolipids. Mammalian fatty acids up to 16 carbons in length are synthesized by the cytosolic fatty acid synthase complex [164]. A significant fraction of the resulting fatty acids produced by fatty acid synthase and fatty acids obtained through the diet are further elongated into long-chain or very long-chain fatty acids by membrane associated elongase enzymes in the ER [165,166]. This elongation process typically involves four steps: 1) Condensation of malonyl-CoA with a long chain acyl-CoA catalyzed by β -ketoacyl-CoA synthase to produce β -ketoacyl-CoA with the acyl moiety elongated by 2 carbon atoms and carbon dioxide; 2) the reduction of β -ketoacyl-CoA to β -hydroxyacyl-CoA catalyzed by β -ketoacyl-CoA reductase; 3) dehydration of β -hydroxyacyl-CoA to enoyl-CoA catalyzed by β -hydroxyacyl-CoA dehydrase; and 4) reduction enoyl-CoA to yield the elongated acyl-CoA catalyzed by *trans*-2-enoyl-CoA reductase [166, 167].

A family of mammalian ELOVL enzymes catalyzes the first rate-limiting step in the elongation of very long chain fatty acids [166]. Seven members (ELOVL1-7) have been identified and

shown to share common structural features including five predicted transmembrane segments, a histidine cluster dideoxy binding motif (HXXHH), and a C-terminal dilysine motif (KXXXX) for retention in the ER [168–170]. Each member exhibits different fatty acid substrate specificities with respect to fatty acyl carbon chain length and degree of saturation [166]. ELOVL1, ELOVL3, ELOVL6 and ELOVL7 utilize saturated and monounsaturated fatty acids as the preferred substrates, whereas ELOVL2, ELOVL4 and ELOVL5 are more selective for polyunsaturated fatty acid [166,171–175].

3.3 Localization and Biochemical Properties of ELOVL4

The expression profile of ELOVL4 has been examined by Northern-blotting and RT-PCR amplification [26,176–178]. The highest level of expression is present in the retina with lower expression found in the brain, skin, and testes. Within the retina *ELOVL4* mRNA expression is restricted to the photoreceptor cell layer in monkey and mouse retina as visualized by *in situ* hybridization [26]. The ELOVL4 protein is localized to the ER of rod and cone photoreceptor inner segments and transfected HEK293 cells [179], consistent with the known ER localization of other mammalian elongase enzymes.

ELOVL4, the gene associated with Stargardt-like dystrophy (STGD3), contains six exons and encodes an integral membrane protein of 314 amino acids having a molecular mass of 36.8 kDa [26]. ELOVL4 proteins are highly conserved with the human protein being over 90% identical to mouse and bovine orthologues and 66% identical to the zebrafish protein [176]. Like other ELOVL family members, ELOVL4 contains a dioxy iron-binding motif (HXXHH) and a C-terminal dilysine motif for retention in the endoplasmic reticulum and is predicted to have five membrane spanning segments. In addition, ELOVL4 contains a single, conserved N-linked glycosylation consensus motif (NXT/S). Endoglycosidase and site-directed mutagenesis studies have shown that N20 within this consensus sequence is glycosylated [179]. Figure 9 shows a topological model for the organization of ELOVL4 in a membrane and characteristic structural features. *In vitro* expression studies have further shown that ELOVL4 assembles as a homo-oligomeric complex, although the exact number of subunits within this complex remains to be determined [179].

3.4 Elov14 Knockout and Transgenic mice

Both *Elov14* knockout and knockin mice have been produced in order to gain insight into the mechanism by which mutations in the *Elov14* gene contribute to Stargardt-like disease. Homozygous *Elov14* knockout mice and mice homozygous for the Y270X *Elov14* mutation associated with Stargardt-like macular degeneration exhibited normal prenatal retinal development but died several hours after birth. The cause of death has been linked to defective skin permeability resulting from depletion of epidermal ceramides having very long chain saturated and monounsaturated fatty acids (>C28). This is consistent with the proposed role of ELOVL4 in the elongation of very long chain fatty acids [180,181]. In contrast mice heterozygous for *Elov14* deletion showed reduced *Elov14* RNA expression, but developed normally and had relatively normal ERGs and only minimal morphological abnormalities in their photoreceptors [182,183]. Additionally, no significant retinal degeneration was evident over a period of 11 months.

Transgenic and heterozygous knockin mice expressing disease-associated mutant forms of ELOVL4 were also generated as a model for Stargardt-like disease [184–186]. These Stgd3-knockin mice showed abnormal ERGs and accumulated undigested phagosomes and lipofuscin deposits containing A2E in their RPE cells. The rod and cone photoreceptors were found to undergo degeneration, the extent of which depended on the expression level of the mutant transgene. In addition, biochemical analysis revealed a deficiency in C32–C36 acyl phosphatidyl cholines. These Stgd3-knockin mice are useful animal models for studying the

pathological mechanisms underlying Stargardt-like macular dystrophy and developing potential therapeutic interventions.

3.5 Role of ELOVL4 in Fatty acid Elongation and Stargardt-like Disease

Several lines of evidence point to the role of ELOVL in the elongation of very long chain fatty acids. Various tissues including retina, brain, testes, and skin which express high to moderate levels of *ELOVL4* have been reported to contain glycerophospholipids and sphingolipids containing very long chain fatty acids [187–196]. Photoreceptor outer segments in particular have large amounts of phospholipids with long chain and very long chain polyunsaturated fatty acyl chains [143,197]. Docosahexaenoic acid (22:6n3) or DHA is the most abundant long-chain polyunsaturated fatty acid in these tissues and as a result ELOVL4 was first thought to play a role in the biosynthesis of DHA. However, studies by a number of groups have shown that ELOVL4 is not directly involved in the synthesis of DHA, but instead catalyzes the elongation of fatty acids greater than 26 carbons in length. Furthermore, increasing the levels of DHA in ELOVL4 transgenic mice had no effect on the structure or function of photoreceptors or the extent of photoreceptor degeneration [198]. Agbaga et al. [175] have reported that rat neonatal cardiomyocytes and a human retinal epithelium cell line overexpressing ELOVL4 produce C28 and C30 saturated fatty acids and C28–C38 polyunsaturated fatty acids [175]. These results are consistent with studies of transgenic mice containing a Stargardt-like disease mutation in *Elovl4* [184–186]. These mice with a retinal pathology similar to patients with Stargardt-like disease have a significant deficiency in C32–C36 acyl phosphatidylcholines [186,199]. The role of ELOVL4 in the elongation of very long chain fatty acids is also consistent with studies of *Elovl4* knockout mice which exhibit a defective skin permeability barrier associated with depletion of ceramides containing very long chain fatty acids [180,181,200].

Although these studies point to the role of ELOVL4 in the elongation of very long chain fatty acids, it remains to be determined which fatty acids serve as the preferred substrates for this elongase. ELOVL4 may have a broad substrate specificity, elongating 26:0 and 28:0 saturated fatty acids as well as 26:5n3 and longer polyunsaturated fatty acid [175]. However, the saturated and unsaturated fatty acids which serve as substrates for ELOVL4 remain to be determined experimentally. Such studies will require the availability of a series of very long chain fatty acids together with an ELOVL4 expression system such as has been previously reported [175,179].

Polyunsaturated C28–C36 fatty acids are known to be components of phosphatidylcholine found in the retina. They comprise as much as 10 mol % of the fatty acids of rod and cone outer segment membranes [191,193,201]. However, to date the function of these very long chain fatty acids is not known. Earlier studies have indicated that C28–C36 containing phospholipids bind tightly to rhodopsin [202]. However, it is also possible that these C28–C36 fatty acyl containing phospholipids may produce specialized membrane microdomains that are important in maintaining the structural integrity of the outer segment disc membrane. Phospholipids with very long chain fatty acids may also be important in facilitating the clearance of retinal compounds from disc membranes following photoexcitation thereby preventing the accumulation of potential toxic retinoid compounds in photoreceptors and RPE cells. Defining the role of phospholipids containing C28–C36 fatty acids and determining the fatty acid substrate specificity of ELOVL4 remains an area of research critical for understanding the role of very long chain polyunsaturated fatty acids in photoreceptor outer segment structure and function and the pathogenic mechanisms underlying Stargardt-like macular degeneration.

The dominant inheritance trait of Stargardt-like disease can arise either as a result of haploinsufficiency of ELOVL4 or a dominant negative effect of the mutant protein. Haploinsufficiency has been explored in heterozygous *Elovl4* knockout mice [182,183]. These

mice with decreased expression of *Elovl4* displayed a normal appearance with no evidence of retinal degeneration, indicating that haploinsufficiency is not responsible for retinal degeneration found in Stargardt-like patients with mutations in ELOVL4. The dominant negative mechanism has been examined in cultured cells expressing wild-type and disease-associated mutant ELOVL4 [179,203,204]. Wild-type ELOVL4 localizes to the ER of transfected cells in agreement with the localization of other members of the ELOVL4 family and the presence of the C-terminal di-lysine ER retention motif. In contrast, mutant ELOVL4 lacking the di-lysine ER retention motif accumulated as large aggresome-like deposits within the cells. When wild-type and mutant ELOVL4 were co-expressed in cultured cells, wild-type ELOVL4 co-purified and co-localized with mutant ELOVL4 in aggresome-like inclusions adjacent to the nucleus similar to that observed for singly expressed mutant protein. These studies indicate that the mutant protein has a dominant negative effect on the WT protein localization and possibly its function as an elongase.

4. General Conclusions

Over the past 12 years significant progress has been made in characterizing the molecular properties of ABCA4 and ELOVL4 defining their role in photoreceptor cell biology and the pathogenesis of recessive and dominant forms of Stargardt macular degeneration. Biochemical studies together with analysis of *abca4* knockout mice and patients with Stargardt disease have implicated ABCA4 as a specialized photoreceptor lipid transporter that flips N-ret-PE from the lumen to the cytoplasmic side of disc membranes thereby facilitating the complete removal of potentially toxic retinoid compounds from rod and cone photoreceptor outer segments following photoexcitation. Failure to efficiently remove N-ret-PE and all-*trans* retinal from disc membranes due to the loss or decrease in ABCA4 transport activity results in the elevation of all-*trans* retinal and N-ret-PE and the production of dirutinoid compounds that progressively accumulate in RPE cells upon phagocytosis of outer segments. These dirutinoid compounds induce RPE apoptosis and consequently photoreceptor cell death and a loss in vision as found for individuals with Stargardt disease.

Although the biochemical properties of ABCA4 have been investigated in considerable detail, it will be important to obtain a high resolution structure of ABCA4 in order to gain insight into nature of the substrate binding site and the mechanism of energy dependent transport. It will also be critical to develop biochemical assays to define the direction of ABCA4 mediated substrate transport across disc membranes and cellular mechanisms that specifically direct ABCA4 to photoreceptor disc membranes. Another important unresolved issue is the regulation of ABCA4. Is the activity of ABCA4 as a transporter regulated through posttranslational modification or protein-protein interactions? Finally, another active area of research is the development of therapeutic approaches to slow or eliminate RPE and photoreceptor degeneration in individuals with Stargardt disease and related retinal degenerative diseases linked to mutations in ABCA4.

Mutations in the *STGD3* gene encoding ELOVL4, an enzyme involved in the biosynthesis of very long chain fatty acids, are responsible for a subset of autosomal dominant Stargardt disease. Biochemical studies and analysis of knockout and knockin mice have shown that ELOVL4 is essential for the production of C26 and longer fatty acids. Loss in activity of ELOVL4 through a dominant negative effect of mutant ELOVL4 on the wild-type enzyme results in the inability to synthesize these very long chain fatty acids which are normally found in relatively high amounts in photoreceptor outer segments. It will be important to define the role of these very long chain fatty acids in photoreceptor structure and function.

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Abbreviations

ABC	ATP-Binding Cassette
MD	macular degeneration
RP	retinitis pigmentosa
AMD	age-related macular degeneration
RPE	retinal pigment epithelial
ELOVL	elongation of very long chain fatty acids
ERGs	electroretinograms
TMD	transmembrane domain
NBD	nucleotide-binding domain
ECD	exocyttoplasmic domain
ER	endoplasmic reticulum
N-Ret-PE	N-retinylidene-phosphatidylethanolamine
PE	phosphatidylethanolamine
PC	phosphatidylcholine
N-retinyl-PE	N-retinyl-phosphatidylethanolamine
RDH	retinol dehydrogenase

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Figure 1. Fundus photograph of individuals with autosomal recessive Stargardt Disease (A) and autosomal dominant Stargardt disease (B). Atrophy of the macula and the presence of yellow-white flecks extending from the central retina are evident.

Figure 2.

A. Diagram of a vertebrate retina showing the cell types (left) and the various layers as seen by light microscopy. B. Immunofluorescence micrograph of a mouse retinal cryosections labeled with a monoclonal antibody to ABCA4 (green) [160] and counterstained with DAPI nuclear stain (blue) to show the outer nuclear layer (ONL) and inner nuclear layer [205] and the ganglion cell layer (GCL). ABCA4 is predominantly in the outer segment (OS) layer.

Figure 3.

A. Schematic of the rod photoreceptor cell adjacent to a retinal pigment epithelial (RPE) cell. The five regions (rod outer segment, connecting cilium, rod inner segment, cell body, and synaptic region) are shown along with the location of various subcellular organelles. B. Schematic showing a cutout portion of the rod outer segment with the stack of discs surrounded by a plasma membrane and the location of ABCA4 in the rim and incisure regions of the disc membrane. C. Enlarged schematic of a portion of the disc membrane showing the approximate distances between the various membranes in relation to the location of ABCA4.

Figure 4.

Topological organization of ABCA4 in the disc membrane showing the domain organization (ECD - exocyttoplamic domain; NBD - nucleotide binding domain; TMD - transmembrane domain) and selected missense mutations responsible for Stargardt macular degeneration found throughout ABCA4. Some of the N-linked oligosaccharide chains are shown as a series of hexagons in the ECDs. The NBDs are on the cytoplasmic side of the membrane.

Figure 5.

Formation of N-retinylidene-phosphatidylethanolamine (N-ret-PE) in disc membranes. All-*trans* retinal released from rhodopsin after photoexcitation reacts with phosphatidylethanolamine (PE) in disc membranes to produce the Schiff base adduct N-retinylidene-phosphatidylethanolamine (N-ret-PE). The nonprotonated form is in equilibrium with the protonated form. The Schiff base of N-ret-PE can be reduced with sodium borohydride (NaBH₄) to form the stabilized derivative N-retinyl-PE. Both N-ret-PE (nonprotonated) and N-retinyl-PE bind with relatively high affinity to ABCA4.

Figure 6.

A model for the transport of N-retinylidene-PE across the disc membrane by ABCA4. In the absence of ATP, N-retinylidene-PE on the lumen side of the disc membrane binds to a high affinity site within the transmembrane domain (TMD) of ABCA4 altering the affinity of the nucleotide binding domains (NBDs) for ATP. The binding of ATP to the NBDs causes a dimerization of the NBDs coupled to a conformation change in the TMD that converts the high affinity N-retinylidene-PE substrate binding site to a low affinity site and access of N-ret-PE to the cytoplasmic side of the disc membrane. Dissociation of N-retinylidene-PE from ABCA4 on the cytoplasmic side of the lipid bilayer together with ATP hydrolysis and dissociation of ADP returns ABCA4 to its initial state.

Figure 7.

The role of ABCA4 in the visual cycle. A. All-*trans* retinal released from rhodopsin is reduced to all-*trans* retinol by RDH8 and subsequently shuttled to the RPE cell by IRBP where in a series of reactions it is converted back to 11-*cis* retinal for the regeneration of rhodopsin. Some all-*trans* retinal reacts with phosphatidylethanolamine (PE) to form N-retinylidene-PE (N-ret-PE). Reactions relevant to ABCA4 function are shown in the dashed box and given in more detail below. B. Diagram depicting the proposed role of ABCA4 in the active transport of N-ret-PE from the lumen to the cytoplasmic side of the disc membrane for re-entry into the visual cycle. RDH - retinol dehydrogenase; LRAT - lecithin:retinol acyl transferase; IRBP - interphotoreceptor binding protein; 11-*c*-ral - 11-*cis* retinal; all-*t*-ral - 11-*trans* retinal; all-*t*-rol - all-*trans* retinol; RDH - retinol dehydrogenase

Figure 8.

Proposed mechanism responsible for Stargardt and related retinal degenerative diseases. A. Reactions which lead to the production of A2E, a potential toxic compound. B. Diagram showing how the loss in the function of ABCA4 as a N-ret-PE transporter results in photoreceptor degeneration and a loss in vision. The photoexcitation of rhodopsin results in the production of all-*trans* retinal (Ret) which is in equilibrium with N-retinylidene-PE. The inability to remove N-ret-PE from discs due to defective ABCA4 results in the accumulation of N-ret-PE and all-*trans* retinal. These compounds react to form the diretinoid compound A2PE in disc membranes. After phagocytosis of outer segments by RPE cells, A2PE is hydrolyzed to A2E. This diretinal pyridinium compound together with other fluorophores progressively accumulates in RPE cells as lipofuscin deposits. A2E and other retinoid derivatives cause RPE apoptosis possibly through their action as detergents or light-induced generation of epoxides. Since photoreceptor cells rely on RPE cells for survival, the loss of RPE cells will lead to photoreceptor degeneration and a loss in vision.

Figure 9.

Predicted topological organization of ELOVL4 in the membrane based on SOUSI algorithm. The N-terminal segment contains an N-linked glycosylation site (hexagons) and therefore is on the lumen side of the ER membrane. In addition ELOVL4, like other members of the ELOVL family of elongates contain a histamine cluster dideoxy binding motif [HVVYHH] and a C-terminal dilysine ER retention motif [KAKGD]. The three Stargardt-like disease associated mutations which result in a truncated protein lacking the C-terminus are also shown.