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Expression of ABCA4 in the retinal pigment epithelium and its implications for Stargardt macular degeneration

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Recessive Stargardt disease (STGD1) is an inherited blinding disorder caused by mutations in the Abca4 gene. ABCA4 is a flippase in photoreceptor outer segments (OS) that translocates retinaldehyde conjugated to phosphatidylethanolamine across OS disc membranes. Loss of ABCA4 in Abca4−/− mice and STGD1 patients causes buildup of lipofuscin in the retinal pigment epithelium (RPE) and degeneration of photoreceptors, leading to blindness. No effective treatment currently exists for STGD1. Here we show by several approaches that ABCA4 is additionally expressed in RPE cells. (i) By in situ hybridization analysis and by RNA-sequencing analysis, we show the Abca4 mRNA is expressed in human and mouse RPE cells. (ii) By quantitative immunoblotting, we show that the level of ABCA4 protein in homogenates of wild-type mouse RPE is about 1% of the level in neural retina homogenates. (iii) ABCA4 immunofluorescence is present in RPE cells of wild-type and Mertk−/− but not Abca4−/− mouse retina sections, where it colocalizes with endolysosomal proteins. To elucidate the role of ABCA4 in RPE cells, we generated a line of genetically modified mice that express ABCA4 in RPE cells but not in photoreceptors. Mice from this line on the Abca4−/− background showed partial rescue of photoreceptor degeneration and decreased lipofuscin accumulation compared with nontransgenic Abca4−/− mice. We propose that ABCA4 functions to recycle retinaldehyde released during proteolysis of rhodopsin in RPE endolysosomes following daily phagocytosis of distal photoreceptor OS. ABCA4 deficiency in the RPE may play a role in the pathogenesis of STGD1.

Stargardt disease | retinal pigment epithelium | bisretinoid | lipofuscin | ABCA4

Rhodopsin and the cone-opsin visual pigments are present in the membranous discs of rod and cone outer segments (OS). Upon capture of a photon, the 11-cis-retinaldehyde (11cR-AL) chromophore coupled to a visual opsins is converted to all-trans-retinaldehyde (aR-AL), thereby activating the pigment and triggering visual transduction (1). Shortly thereafter, the bleached pigment dissociates, releasing free aR-AL into the disc bilayer. Here, retinaldehyde combines rapidly and reversibly with phosphatidylethanolamine (PE) to form N-retinylidene-phosphatidylethanolamine (N-ret-PE) in the disc membrane. Here, N-ret-PE has two potential orientations: with its retinylidene-bearing head group facing into the disc lumen or with the head group facing outward into the cytoplasmic space. The retinol dehydrogenase (RDH8) that reduces aR-AL to all-trans-retinol (atROL) as a first step in the regeneration of visual chromophore (2) is located in the OS cytoplasm. Cytoplasmically oriented N-ret-PE upon dissociation becomes a substrate for RDH8. However, N-ret-PE located on the luminal surface is inaccessible by RDH8. To accelerate the reduction of toxic retinaldehydes, OS membranes contain an ATP-dependent transporter called ABCA4, or ATP-binding cassette subfamily A member 4, that flips N-ret-PE from the luminal to cytoplasmic surface of disc membranes (3, 4).

Significance

Recessive Stargardt macular degeneration (STGD1) and a subset of cone–rod dystrophies are caused by mutations in the Abca4 gene. The ABCA4 protein is a flippase in photoreceptor cells that helps eliminate retinaldehyde, a toxic photoprodut of vision. Here we found that ABCA4 is additionally present in the retinal pigment epithelium (RPE) of mice at approximately 1% of its abundance in the neural retina. Genetically modified mice that express ABCA4 in RPE but not in photoreceptors showed partial rescue of both the lipofuscin accumulation and photoreceptor degeneration observed in Abca4−/− mice and in STGD1 patients. These observations suggest that ABCA4 in the RPE prevents photoreceptor degeneration in Abca4−/− mice and possibly in STGD1 patients.

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The authors declare no conflict of interest.

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(A2PE–H₂) or its oxidized form (A2PE). It is thought that following diurnal phagocytosis of distal photoreceptor OS (8, 9), these bisretinoids are converted to the major lipofuscin fluorophore A2E in the acidic environment of RPE phagolysosomes (10). According to this model, the source of retinaldehyde to form N-ret-PE and the various bisretinoids is atRAL released by photobleached rhodopsin and cone opsinns in the OS. A strong prediction of this model is that Abca4−/− mice reared in total darkness should not accumulate bisretinoids, since photobleaching of visual pigments does not occur in the dark. Unexpectedly, Abca4−/− mice maintained in constant darkness accumulated A2E in RPE cells at the same rate as Abca4+/− mice reared under 12-h cyclic light (11). This finding suggests that retinaldehyde released by photobleaching of visual pigments is not the major source of bisretinoids that accumulate as lipofuscin in the RPE.

Another possible source of retinaldehyde for A2E formation in the RPE is the 11cRAL chromophore contained within the visual pigments of phagocytosed rod and cone OS discs. The distal 10% of rod and cone OS are diurnally shed and phagocytosed by the RPE (8, 9). Since the dominant ocular retinoid is 11cRAL coupled to rhodopsin, ~10% of visual retinoids are processed daily by the RPE through phagocytosis of photoreceptor OS. This process occurs at similar rates in mice maintained under cyclic light or constant darkness (12). Retinaldehyde released during the degradation of rhodopsin likely condenses with PE on the luminal surface of endolysosome membrane in RPE cells to form N-ret-PE. Here, we suggest that ABCA4 performs the same function as in photoreceptor OS: ATP-dependent translocation of N-ret-PE from the luminal to cytoplasmic leaflet. This model assumes that ABCA4 is normally present in the endolysosomal membranes of RPE cells. In the current work, we show that ABCA4 is expressed in RPE internal membranes, where it plays a role in preventing the buildup of bisretinoid-containing lipofuscin and preventing photoreceptor degeneration.

Results

The Abca4 Gene Is Expressed in RPE Cells. We used chromogenic in situ hybridization (13) to detect the Abca4 mRNA in human and wild-type (BALB/c) mouse retina sections. As expected, the ABCA4 mRNA was intensely expressed in the photoreceptor outer nuclear layer (Fig. 1A and B). We also observed significant chromogenic labeling of the Abca4 mRNA in RPE cells (Fig. 1A and B). Importantly, no labeling of photoreceptor nuclei or RPE cells was detected by the same probe in sections of Abca4−/− retina (Fig. 1B). We also performed in situ hybridization to detect the ABCA4 mRNA in primary cultured human fetal RPE (hRPE) cells (14), where we observed robust labeling of the Abca4 mRNA (Fig. 1C). Positive and negative control probes confirmed tissue-specific chromogenic reactivity by in situ hybridization assay (SI Appendix, Fig. S1). Finally, by qRT-PCR we quantified the levels of the Abca4 mRNAs in 3-wk-old mouse neural retina separated from the RPE/eyecup, normalizing to 18S rRNA. The Abca4 mRNA level in the wild-type (129/Sv) RPE/eyecup was about 10% of the level in the neural retina sample (SI Appendix, Fig. S2).

To confirm the expression of Abca4 in the RPE, we performed RNA-sequencing (RNA-seq) analysis on RNA extracted from confluent cultures of hRPE cells. This analysis revealed the presence of Abca4 and several other RPE-expressed mRNAs including RPE-specific 65-kDa protein (Rpe65), Bestrophin-1 (Best1), and Lecithin retinol acyltransferase (Lrat). As expected, mRNAs for the photoreceptor proteins rhodopsin (Rho), melanopsin (Opn4), cone M-opsin (Opn1LW), and cone S-opsin (Opn1SW) were not detected (SI Appendix, Table S1). RNA-seq

Fig. 1. (A–C) Abca4 mRNA and protein is expressed in RPE cells. In situ hybridization using the RNAscope assay with an Abca4-specific probe on human cadaveric ocular sections (A), mouse retina sections (B), and hRPE cells in culture (C). Note the intense chromogenic reactivity (red punctate staining, indicated by the black arrows) for Abca4 mRNA in outer nuclear layer (ONL) and inner segments (IS) of the photoreceptor cells and in RPE cells of human (A) and wild-type BALB/c sections (B, Left). This reactivity is absent in Abca4−/− tissue (B, Right). Red punctate staining (white arrows) corresponding to ABCA4 protein is also observed in hRPE cultured cells (C). CC, choriocapillaris; INL, inner nuclear layer. (Scale bars, 20 μm.) (D) Abca4 immunohistochemistry (red fluorescence) on retina sections from pigmented wild-type (129/Sv), Merk−/−, and Abca4−/− mice. Note that ABCA4 immunoreactivity is seen in the RPE and OS of 129/Sv mice and in the RPE but not in the OS (indicated by white asterisk) of Merk−/− mice but is not seen in the retina section from an Abca4−/− mouse. The white arrows indicate retinal detachment. Cell nuclei are stained with DAPI (blue). (Scale bars, 10 μm.) (E) Representative immunoblots for ABCA4 protein using neural retina and RPE/eyecup homogenates loaded as a fraction of one mouse eye per lane, as indicated. The RNAscope assay (A–C) was done with two human cadaveric eyes, three cultured hRPE cells of different donor eyes, and n = 3 mice (5-mo-old) of each genotype; Immunohistochemistry experiments (D) were repeated three times with n = 3 5-mo-old mice per group. The immunoblotting experiment (E) was done in duplicates varying the fraction of the homogenate corresponding to one mouse eye (n = 4 mice for each experiment).
analysis of RPE from fresh bovine eyes also evidenced the Abca4 mRNA, with greatly reduced or absent expression of mRNAs for photoreceptor-specific proteins (SI Appendix, Table S1). These data establish that the Abca4 gene is expressed in RPE cells.

The ABCA4 Protein Is Present in RPE Internal Membranes. We tested for ABCA4 protein expression in RPE cells by immunofluorescence microscopy. Sections of wild-type (129/Sv) retinas showed ABCA4 immunofluorescence in photoreceptor OS and RPE cells, with much greater immunoreactivity in the OS (Fig. 1D). Mer tyrosine kinase is present in RPE cells and is required for phagocytosis of shed OS discs (15). For this reason, Mertk−/− mice exhibit greatly impaired OS phagocytosis and photoreceptor degeneration, which is complete by 2 mo of age (16). To rule out the possibility that the RPE immunoreactivity is due to ABCA4 in phagocytosed OS discs, we performed immunofluorescence microscopy on retina/RPE sections from 5-mo-old Mertk−/− mice. These sections showed ABCA4 immunoreactivity in RPE cells similar to that in wild-type mice but no OS immunoreactivity (Fig. 1D). The persistence of ABCA4 immunoreactivity in RPE cells from fully degenerated Mertk−/− mice, with no OS immunoreactivity, indicates that ABCA4 is endogenously expressed in RPE. As expected, we observed no ABCA4 immunofluorescence in Abca4−/− retina/RPE sections (Fig. 1D).

To estimate the relative amounts of ABCA4 in OS and RPE, we performed semiquantitative immunoblotting on homogenates of isolated retinas and RPE-containing eyecups from wild-type (129/Sv) mice (Fig. 1E). The amount of protein loaded onto the gel was adjusted to yield similar ABCA4 immunoreactivities in the retina and RPE lanes within the linear range of the infrared-fluorescent scanner used to quantitate the blots. The results of this experiment showed that the total ABCA4 in the RPE is ~1% of the total ABCA4 in the neural retina.

ABCA4 Colocalizes with Endolysosomal Markers. A possible role for ABCA4 in RPE cells, consistent with its known function in photoreceptor OS (3), is as an N-ret-PE flipase that helps clear retinaldehyde released during proteolysis of rhodopsin and cone opsins in phagocytosed OS discs. We tested this possibility by looking for colocalization of ABCA4 with proteins of the endolysosomal system. In retina/RPE sections from wild-type (BALB/c) mice we observed colocalization of ABCA4 with lysosomal-associated membrane protein-1 (LAMP1) in the RPE but not in photoreceptor OS (Fig. 2A). As anticipated, LAMP1 but not ABCA4 immunofluorescence was seen in similar retina sections from Abca4−/− mice (Fig. 2A). ABCA4 also colocalized with the early-endosomal protein Rab5 in RPE cells from wild-type (129/Sv) and Mertk−/− mice (Fig. 2B). The use of Mertk−/− mice here ruled out an OS origin of the ABCA4 in RPE cells. ABCA4 was also present in hRPE cells, where it exhibited a granular pattern of immunoreactivity consistent with an internal membrane distribution (Fig. 2C). Here, ABCA4 largely overlapped with caveolin-1 (CAV1) (Fig. 2C), a protein of multiple functions and a marker for RPE phagosomes (17). The presence of ABCA4 in hRPE cells that were never exposed to photoreceptor OS is further evidence that ABCA4 is endogenously expressed in RPE cells. Together, these results suggest that ABCA4 is present in endolysosomal membranes of RPE cells.

Generation of Mice Preferentially Expressing ABCA4 in RPE Cells but Not in Photoreceptors. To study the function of ABCA4 in RPE cells, we prepared a transgene construct containing the mouse Rpe65 promoter upstream of the Abca4 coding region. Injection of DNA from this construct into fertilized mouse oocytes yielded several founder lines, only one of which showed significant ABCA4 expression. We crossed this RPE-Abca4-Tg–expressing mouse line onto the albino Abca4−/− background to yield RPE-Abca4-Tg/Abca4−/− mice for this study. To compare levels of the ABCA4 protein in retinas and RPE from RPE-Abca4-Tg/Abca4−/−, wild-type (BALB/c), and Abca4−/− mice, we performed
quantitative immunoblotting on homogenates from these tissues (Fig. 3A). The levels of ABCA4 in 6-mo-old RPE-Abca4-Tg/Abca4−/− mouse were ~25% of the levels in wild-type RPE, while the levels of ABCA4 protein in retinas from the same mice were negligible (Fig. 3B). Finally, retina sections from 1-y-old wild-type mice showed ABCA4 immunofluorescence in photoreceptor OS and RPE, while sections from age-matched RPE-Abca4-Tg/Abca4−/− mice primarily showed ABCA4 in the RPE. As expected, no ABCA4 immunofluorescence was seen in sections from Abca4−/− mice (Fig. 3C). These data confirm that the Abca4 transgene is expressed mainly in RPE cells.

Reduced Bisretinoid-Lipofuscin Levels in RPE-Abca4-Tg/Abca4−/− Mouse RPE. To determine whether ABCA4 expressed in RPE cells prevents bisretinoid accumulation, we compared levels of several lipofuscin fluorophores in the retina and RPE of 3-mo-old mice (Fig. 4 A–D and SI Appendix, Fig. S3). Levels of A2E (Fig. 4A) were reduced by ~50% in RPE-Abca4-Tg/Abca4−/− vs. Abca4−/− RPE, although the levels of this bisretinoid were still higher in RPE-Abca4-Tg/Abca4−/− than in wild-type RPE. Similarly, levels of atRAL dimer-PE (Fig. 4B), A2E-H2 (Fig. 4C), and A2P-E (Fig. 4D) were all lower in the RPE of RPE-Abca4-Tg/Abca4−/− mice than in the RPE of Abca4−/− mice. Interestingly, the levels of these bisretinoids were similar in retinas from RPE-Abca4-Tg/Abca4−/− and Abca4−/− mice, as is consistent with the negligible expression of ABCA4 in photoreceptors (Fig. 4 B–D). By confocal microscopy of RPE flat mounts, we observed ~30% lower autofluorescence (488-nm excitation) in the RPE of RPE-Abca4-Tg/Abca4−/− mice than in their Abca4−/− littermates (Fig. 4E and SI Appendix, Fig. S4). Here again, autofluorescence was still greater in RPE-Abca4-Tg/Abca4−/− mice than in wild-type mice. Finally, the fraction of lipofuscin granules per 100 μm² of RPE area was approximately one-half that seen in nontransgenic Abca4−/− littermates (8.6% vs. 15.8%) in electron micrograph sections of RPE (Fig. 4 F and G).

Transgenic Expression of ABCA4 in RPE Slows Photoreceptor Degeneration. An important feature of the Abca4−/− phenotype is the slow degeneration of photoreceptors, which leads to visual loss in STGD1 patients. Here we tested whether transgene-mediated expression of ABCA4 in RPE cells affects the photoreceptor survival rate in Abca4−/− mice by counting photoreceptor nuclei in the outer nuclear layer of retina sections. Compared with wild-type mice, 1-y-old nontransgenic Abca4−/− mice exhibited an ~20% reduction in photoreceptor nuclei (Fig. 5) (P < 0.0001). In contrast, RPE-Abca4-Tg/Abca4−/− littermates exhibited only a 10% loss of photoreceptors compared with wild-type mice. Expression of ABCA4 in RPE cells therefore slowed the photoreceptor degeneration seen in Abca4−/− mice by a factor of two.

Discussion

The results presented here establish that ABCA4, known to be present in photoreceptor OS, is also expressed in the RPE. First, we showed that the Abca4 mRNA is present in mouse, human, and bovine RPE by in situ hybridization (Fig. 1 A–C), RNA-seq (SI Appendix, Table S1), and qRT-PCR analysis (SI Appendix, Fig. S2). These data indicate that the Abca4 gene is expressed in RPE cells. Next, we showed that the ABCA4 protein is present in RPE cells by quantitative immunoblotting of wild-type mouse retina and RPE homogenates (Fig. 1E) and by immunohistochemistry on wild-type mouse retina/RPE sections (Fig. 1D). To confirm that the ABCA4 protein in RPE cells is endogenously expressed and not due to phagocytosis of ABCA4-containing OS, we demonstrated ABCA4 immunoreactivity in the RPE of 5-mo-old Mertk−/− retina/RPE tissue sections (Fig. 1D). OS

**Fig. 3.** ABCA4 is expressed in the RPE of RPE-Abca4-Tg/Abca4−/− mice. (A) Representative immunoblots of retina and RPE homogenates from BALB/c, Abca4−/−, and RPE-Abca4-Tg/Abca4−/− mice (all albino) reacted with antisera against ABCA4 or α-tubulin. Total protein load was 10 μg for neural retina and 25 μg for RPE/eyecup homogenates. (B) Levels of ABCA4 protein in Abca4−/− and RPE-Abca4-Tg/Abca4−/− homogenates were normalized to α-tubulin and presented as relative to wild-type BALB/c levels; n = 7 6-mo-old mice per group. (C) Representative confocal images of retinal sections from BALB/c (Left), Abca4−/− (Center), and RPE-Abca4-Tg/Abca4−/− (Right) mice. ABCA4 immunoreactivity (red) in the RPE of Abca4−/− mice is not stained by the ABCA4 antibody. DAPI nuclear staining is shown in blue. (Scale bars, 10 μm.) n = 3 1-y-old mice per group.
phagocytosis by RPE cells is blocked in Mertk−/− mice, and by age 5 mo the retinas from these mice have lost all photoreceptors (15, 16). Finally, we show that primary cultured hfRPE cells, which were never exposed to ABCA4-containing OS, also express ABCA4 (Fig. 2C).

In the RPE, ABCA4 colocalized with three protein markers of the endolysosomal system, LAMP1, Rab5, and CAV1 (Fig. 2). This pattern suggests that ABCA4 is inserted into the membranes of early endosomes where it remains during endolysosomal maturation. In OS disc membranes, ABCA4 has been shown to be a major component of polymorphic lipofuscin granules, which are characteristic of AMD.

Fig. 4. Bisretinoid, autofluorescence, and lipofuscin levels are reduced in the RPE of RPE-Abca4-Tg/Abca4−/− mice. (A–D) Bisretinoids were extracted from retina and RPE homogenates of 3-mo-old albino mice and analyzed by normal-phase HPLC. Representative HPLC chromatograms from RPE/eyecup and neural retina extracts are shown in SI Appendix, Fig. S3. Note the lower levels of all bisretinoids in RPE from RPE-Abca4-Tg/Abca4−/− mice. (A) Total A2E (sum of A2E and iso-A2E) is expressed as picomoles per eye. (B–D) All-trans-retinaldehyde dimer PE (atRAL-Dimer-PE) (B), A2PE-H2 (C), and A2PE (D) are expressed as milli-absorbance units (mAU) per eye. Data are presented as mean ± SD; n = 5 mice per group; ** P < 0.001; *** P < 0.001; n/s, not significant. (E) Representative confocal images of RPE choroid-sclera flat mounts captured using a 488-nm excitation laser and a 500- to 545-nm emission filter. Note the reduced autofluorescence intensity (AF, green) in the RPE-Abca4-Tg/Abca4−/− flat mounts compared with the Abca4−/− RPE flat mounts. RPE cell borders are highlighted by anti-ZO1 staining (blue); nuclei are stained with DAPI (blue); n = 3 or 4 6-mo-old mice per group. (Scale bars, 20 μm.) (F) Representative electron micrographs of RPE cells from 1-yr-old BALB/c (Left), Abca4−/− (Center), and RPE-Abca4-Tg/Abca4−/− (Right) albino mice. Arrows point to polymorphic lipofuscin granules of heterogeneous electron density within RPE cytoplasm. BM, Bruch’s membrane; N, nucleus. (Scale bars, 2 μm.) (G) Fractional lipofuscin granules per 100-μm2 cell area were measured and averaged from 10 adjacent electron microscopy images per eye. Data are presented as mean ± SD; n = 5–9 mice per group; *P = 0.0186; ** P < 0.001.

Fig. 5. Photoreceptors are preserved in RPE-Abca4-Tg/Abca4−/− vs. Abca4−/− mice. (A) Representative retina images from 1-yr-old albino mice acquired by light microscopy. (Scale bars, 20 μm.) (B) Total numbers of photoreceptor nuclei were counted per 100-μm2 cell area. Note the increased number of cells in the ONL of RPE-Abca4-Tg/Abca4−/− mice compared with Abca4−/− mice indicating partial rescue of photoreceptor degeneration. Data are presented as mean ± SD; n = 5–9 mice per group; RPE-Abca4-Tg/Abca4−/− vs. Abca4−/−, **P = 0.0061.
to translocate N-ret-PE from the luminal to the cytoplasmic leaflet (3). The nucleotide-binding domains of ABCA4 are located on the cytoplasmic surface where they have access to cellular ATP. The retinaldehyde reductase RDH8 is also on the cytoplasmic surface where it reduces atRAL to atROL following spontaneous dissociation of N-ret-PE. What role does ABCA4 play in RPE cells? Following phagocytosis, the OS disc packet undergoes generalized digestion in the RPE. Retinaldehydes released during proteolysis of rhodopsin and the cone opsin react with PE on the luminal surface of the RPE endolysosomal membrane to form N-ret-PE. As shown in Fig. 6, we propose that ABCA4 performs a similar function in RPE cells. RDH11, which reduces atRAL and 11cRAL to their respective retinols (18), is on the cytoplasmic side of the ABCA4-containing membranes in RPE cells, similar to RDH8 in the OS disc membranes. OS contain predominantly 11cRAL as a chromophore in the opsin pigments. However, N-ret-PE undergoes both thermal and photoisomerization (19), resulting in a mixture of retinaldehyde isomers. ABCA4 was shown to transport both at- and 11c-N-ret-PE (20). A recent study reported elevated bis (monoacylglycero)phosphate lipids in the RPE of Abca4−/− mice, suggesting endolysosomal dysfunction in these animals (21). In Fig. 6, we present the pathways in RPE cells for recycling of retinaldehydes in the presence or absence of ABCA4. Experimental systems, such as conditional-knockout mouse and induced pluripotent stem cell (iPSC)-derived RPE cells from Stargardt patients, would be appropriate tools to further validate the proposed model.

By quantitative immunoblotting, we estimate that the abundance of ABCA4 in RPE is ~1% of its abundance in the retina (Fig. 1E). Why do photoreceptors require 100-fold more ABCA4 than RPE cells? The cytotoxicity of retinaldehyde is well established (22, 23). The likely role of ABCA4 in photoreceptor OS and RPE cells is to mitigate retinaldehyde toxicity by accelerating its reduction to retinol. The major source of retinaldehyde in RPE cells is chromophore released by visual pigments undergoing proteolysis in phagolysosomes. Only 10% of OS are phagocytosed per day, and only one retinaldehyde is released per visual pigment during OS digestion. Accordingly, the rate of retinaldehyde production is vastly lower in RPE endolysosomes than in photoreceptor OS. This may explain the lower abundance of ABCA4 in RPE cells. Interestingly, while the ABCA4 protein is present in RPE at only ~1% of its level in the retina (Fig. 1E), the Abca4 mRNA is present in RPE at ~10% of its level in the retina (SI Appendix, Fig. S2). This suggests a faster turnover of ABCA4 in the RPE than in the retina, as is consistent with its expression in rapidly turning over endolysosomal membranes. The lower abundance of ABCA4 in the RPE than in the retina does not imply that ABCA4 is less important in the RPE. The observation that A2E accumulates at similar rates in RPE from Abca4−/− mice reared under cyclic light or total darkness (11) suggests that de novo bisretinoid formation within RPE endolysosomes contributes more to lipofuscin buildup than do bisretinoids formed in OS discs during light exposure. Therefore, the clearance of retinaldehydes from RPE phagolysosomes may be more critical for photoreceptor viability than for the clearance of retinaldehydes from OS discs.

The phenotype in albino Abca4−−/− mice includes the accumulation of bisretinoids such as A2E in the RPE, the deposition of fluorescent lipofuscin granules in RPE cells, and slow photoreceptor degeneration (24, 25). To what extent does this phenotype depend on the loss of ABCA4 from RPE cells? We addressed this question by expressing ABCA4 in the RPE of transgenic Abca4−−/− mice. These RPE-Abca4-Tg/Abca4−−/− mice expressed the ABCA4 protein in the RPE at ~25% of the level in wild-type RPE (Fig. 3B). Although retina homogenates from these mice showed a weak band by ABCA4 immunoblotting (Fig. 3A), we observed no labeling of photoreceptor OS by ABCA4 immunohistochemistry (Fig. 3C). This band in retina homogenates probably reflects contamination of the retina with ABCA4-expressing RPE during dissection or ectopically expressed ABCA4 in nonphotoreceptor cells of the retina. ABCA4 expression in the retina is therefore an unlikely factor in the rescue of the Abca4−−/− phenotype RPE-Abca4-Tg/Abca4−−/− mice. Despite the expression of ABCA4 in the RPE at only ~25% of wild-type RPE expression, we observed an ~50% reduction of A2E accumulation in RPE-Abca4-Tg/Abca4−−/− vs. nontransgenic Abca4−−/− mouse RPE (Fig. 4A). We also observed ~50% slowing of photoreceptor degeneration in RPE-Abca4-Tg/Abca4−−/− vs. nontransgenic Abca4−−/−/− mice (Fig. 5). Thus, expression of ABCA4 in RPE cells at ~25% of the

![Fig. 6. Proposed function of ABCA4 in the endolysosomal membranes of RPE.](image-url)
level in wild-type mice yielded ~50% rescue of both lipofuscin accumulation and photoreceptor degeneration. Expression of ABCA4 in the RPE at the wild-type level may deliver greater and possibly complete rescue of RPE lipofuscin accumulation and photoreceptor degeneration.

Interestingly, atRAL-dimer and the phospholipid-conjugated bisretinoids A2PE-H2 and A2PE were present at approximately equal levels in retinas from RPE-Abca4-Tg/Abca4–/– and transgenic Abca4–/– mice (Fig. 4 B–D). Thus, the formation of these retinoid and telethonin products in the retina was unaffected by transgenic expression of ABCA4 in the RPE and may have resulted from the loss of ABCA4 in photoreceptor OS. These observations are further evidence of negligible ABCA4 expression in photoreceptors of RPE-Abca4-Tg/Abca4–/– mice.

The findings presented here have implications for the treatment of Abca4-dependent retinopathies. One treatment approach is gene therapy. If ABCA4 are expressed only in photoreceptor OS, as previously thought, treatment would require efficient transduction of photoreceptors by a recombinant virus containing the 6.8-kb Abca4 coding region. However, photoreceptors are transduced at lower efficiency than RPE cells (26). Thus, RPE cells are more tractable than photoreceptors as targets for gene therapy of Abca4-mediated diseases (27). The best strategy for gene therapy of patients with Abca4-dependent retinopathies may be to target both the RPE cells and photoreceptors. RPE cells are also a second treatment approach is cell transplantation. Here, again, expression of ABCA4 in RPE cells opens therapeutic possibilities. The Abca4 gene defects could be corrected by targeted gene editing in iPSCs derived from a STGD1 patient’s fibroblasts. These cells could then be programed to become functional RPE cells, overcoming some of the long-term possibilities of iPSC-derived RPE cells into the subretinal space of animals and humans (28, 29).

In summary, we have shown that ABCA4 is expressed in RPE cells and that at least part of the ocular Abca4–/– phenotype is caused by the loss of RPE-expressed ABCA4. These observations suggest that RPE cells, in addition to the photoreceptors, should be targeted in rescue approaches to treat Abca4-mediated retinal degenerations.

Methods

Animals. All experiments were performed in accordance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research and University of California, Los Angeles Institutional Animal Care and Use Committee guidelines. Animals were housed in normal cyclic 12-h light/12-h dark conditions and were fed ad libitum. Pigmented wild-type (129Sv) Merk × mice (backcrossed at least five times onto 129Sv) and Abca4–/– mice were used for ABCA4 protein localization studies. Albino wild-type (BALB/c) mice and Abca4–/– mice (on the BALB/c background) were used for in situ hybridization studies. Transgenic mice expressing Abca4 in the RPE on the albino Abca4–/– background (RPE-Abca4-Tg/Abca4–/–) were compared with nontransgenic Abca4–/– littermate controls and BALB/c mice for all other studies. All animals were homozygous for the rpe65 Leu450 variant and were free of the rd8 mutation in the Crumbs homolog-1 gene.

Generation of the RPE-Abca4-Tg/Abca4–/– Transgene. We generated a transgenic construct (SI Appendix, Fig. S5) containing the normal mouse Abca4 coding region downstream of the RPE-specific Rpe65 promoter. Briefly, a 700-bp fragment of the Rpe65 gene containing the promoter, 5′ UTR, and first intron was amplified from pTR4 plasmid and subcloned into the HindIII (5′) and PstI (3′) restriction sites of the pSTEC-1 vector (30, 31). The Rpe65 promoter/intron 1 complex was then subcloned into the 5′ end of the Abca4 cDNA in the pSPORT-Abca4 plasmid using EcoRI (5′) and SalI (3′) restriction sites. The entire transgene (Rpe65-Abca4) was excised from the pSPORT-Abca4 by EcoRI restriction. The construct was sent to the University of California, Los Angeles Transgenic Core facility for fertilized oocyte injection, which resulted in six lines of transgenic mice. Each line was crossed onto the albino Abca4–/– background, and one line was identified as having the most robust RPE-specific expression of ABCA4 by qRT-PCR, immunoblotting, and immunocytochemistry. Primers used for genotyping RPE-Abca4-Tg/Abca4–/– mice were (forward) AGG AAA AGG CAG AAG ATT GCC TTT GTA G and (reverse) TGG GAA AAT GGC ATT CAT GTC GAC.

In Situ Hybridization. Retina sections of eyes from a 52-y-old human donor (generic gift of Gregory Hageman, University of Utah, Salt Lake City), and 84-y-old human eyes (San Diego Eye Bank), human fetal RPE (hRPE) cultured cells (2 to 6 wk in culture), and eyes from 3-mo-old mice were used for in situ hybridization studies. Prior consent was obtained for using the postmortem human donor eyes samples, and the research adhered to the tenets of the Declaration of Helsinki. The assays were done with the RNAscope 2.5 HD Chromogenic Detection Kit (Advanced Cell Diagnostics) according to the manufacturer’s protocol. Tissues were hybridized with target oligo probes (Advanced Cell Diagnostics) for murine or human ABCA4, murine or human RNA polymerase II subunit A probe (Po(III)A2 as a positive control probe), or bacterial dhlydroidopilolate reductase as a negative control probe, followed by amplification steps and chromogenic detection with Fast Red (Advance Cell Diagnostics). Images were collected with a Zeiss Axiohot microscope fitted with a 40× oil-immersion objective lens and a CoolSNAP digital camera (Media Cybernetics). Detailed methods are provided in SI Appendix.

RPE Cell-Culture Immunocytochemistry. A comprehensive protocol for culture of hRPE cells has been previously described (14). After 2 mo in culture, hRPE cells with their associated filters were fixed in 4% formaldehyde/0.1 M phosphate buffer, embedded in agarose (Type XI low gelling temperature; Sigma-Aldrich), and cut into 100-μm sections on a VT1000S vibratome (Leica Microsystems). The sections were blocked with goat or donkey serum and 1% BSA in 1× PBS followed by separate incubation with rabbit anti-ABCA4 (1:100; ab72995; Abcam) and goat anti-caveolin1 (1:100; ab36152; Abcam). The sections were rinsed and incubated in secondary antibodies conjugated with Alexa Fluor dyes (goat anti-rabbit IgG-647 or donkey anti-goat IgG-594; 1:500; Invitrogen). The sections were stained with DAPI nuclear marker (Invitrogen), mounted with 5% n-propyl gallate in 100% glycerol, and imaged with an Olympus Fluoview FV1000 confocal microscope under a 60× oil-immersion objective lens.

Mouse Tissue Immunohistochemistry. Mice under deep isoflurane-induced anesthesia were perfused and fixed in 4% paraformaldehyde/0.1 M sodium phosphate buffer. Eyes were enucleated and immersed in the same fixative overnight after preparation of eyecups and then were infiltrated with 10–30% sucrose for cryoprotection. Eyecups were embedded in cryo-Optical Cutting Temperature embedding medium (OCT; Tissue-Tek) and cut into 10-μm sections. Slides for colocalization studies were blocked with normal goat serum and 1% BSA and were probed overnight with rabbit polyclonal anti-ABCA4 (1:100; ab72995; Abcam), mouse monoclonal anti-Rab5 (1:75; sc-46692; Santa Cruz), and mouse anti-LAMP1 (1:100; ab25630; Abcam) primary antibodies. Slides for ABCA4 expression in albino and transgenic mice were probed with mouse monoclonal anti-ABCA4 [1:1,000; generous gift from Hii Sun (University of California, Los Angeles) and Robert S. Molday (University of British Columbia, Vancouver)] in conjunction with the Mouse-on-Mouse Immunodetection Kit (Vector Labs). Retina sections from pigmented mice were bleached for 30 s using the Melanin Bleach Kit (Polysciences); bleaching was quenched with 50 mM ammonium chloride for 25 min, and sections were washed, and blocked with 1% BSA and 5% goat serum before probing with rabbit polyclonal anti-ABCA4 primary antibody (1:100; Abcam). All sections were washed and labeled with secondary antibodies conjugated Alexa Fluor 647 (1:500; Invitrogen) or DyLight 647 streptavidin (1:150; Vector Labs) for 1 h at RT. Images were obtained with an Olympus Fluoview FV1000 confocal microscope as above.

Immunoblotting. Five-month-old mouse eyes were harvested, and the neural retina was separated from the RPE (eye cup containing RPE/Bruch’s membrane/chorioid) and homogenized in 1× PBS with Halt Protease Inhibitor mixture (Life Technologies). Protein samples were treated with Benzonase nuclease (Sigma-Aldrich) at RT for 1 h and were rehomogenized with 0.5–1% SDS, followed by centrifugation (3,000 × g for 10 min) to collect the supernatant. Protein concentrations were measured using the Micro BCA Protein Assay Kit (Thermo Fisher), and samples were fractionated on 4–12% Bis-Tris gels (Invitrogen). Membranes were blocked with Odyssey blocking buffer (LI-COR Biosciences) followed by incubation in primary antibodies overnight at 4 °C (goat anti-ABCA4 (1:200; sc-21460; Santa Cruz), goat anti-ABCA4 (1:500; EB08615; Everest Biotech Ltd.), and mouse anti-α-tubulin (1:1,000; T9026; Sigma-Aldrich)). Membranes were washed with PBS-Tween,
probed for 1 h at RT with cognate IR-dye-labeled secondary antibodies from Li-COR, and imaged with the CLx Odyssey system (Li-COR). Band intensity corresponding to a known homogenate fraction was determined using the Li-COR application.

Quantitation of A2E in Mouse Eyes. Bisretinoids were extracted by chloroform followed by analysis using HPLC as described previously (33). Briefly, retina and RPE samples were homogenized in 1x PBS, washed with chloroform/ methanol (2:1, vol/vol), and extracted with chloriform (4:3, vol/vol). The organic phase was isolated after centrifugation at 1,000 × g for 10 min, dried under argon, and resuspended in 100 μL of isopropanol. Absorbance units corresponding to the A2E and iso-A2E peaks at 435 nm were converted to picomoles using a calibration curve with authentic standards and published molar extinction coefficient (34).

RPE Flat Mount. Eyes of 6-mo-old mice were enucleated, fixed in 2% paraformaldehyde, 1 M sodium phosphate buffer, NaPO4, pH 7.4, for 30 min at RT, and rinsed and dissected in 0.1 M NaPO4, removing the neurosensory retina and anterior segment to create RPE-choroid-scleral eyecups. To flatten the eyecups, eight leaflets were made with straight cuts using microdissection scissors. The resultant RPE-choroid-scleral flat mounts were permeabilized with 1% Triton X-100, blocked with 1% BSA/5% goat serum, and incubated at 4 °C overnight with rabbit anti-ZO-1 (1:100, Thermo Fisher), mouse anti-ZO-1 antibody with Promega Gold antifade with DAPI (Life Technologies). The eyecups were imaged with the Olympus FV 1000 confocal microscope (60x objective). Autofluorescence was detected by excitation with a 488-nm (argon) laser with a 500- to 545-nm emission filter, and images were quantified using the ImageJ program (NIH) (35).

Light and Electron Microscopy. Light and electron microscopy analyses were done as previously described (36). The total number of photoreceptor nuclei in three adjacent midperipheral visual field locations per eye were averaged and plotted in Microsoft Excel (n = 5–9 animals per group). The fractional lipofuscin granules were measured by obtaining the area (in square micrometers) occupied by lipofuscin granules over the area (in square micrometers) occupied by cytoplasm. Each animal’s fractional lipofuscin granule measurement corresponded to an average of at least 10 adjacent electron microscopy images from one eye (n = 5–9 animals per group). Detailed methods are provided in SI Appendix.

Statistical Analysis. The results are presented as means with SD of a minimum of four to six animals per group unless otherwise specified. Two-group comparisons were performed with Student’s t test using Microsoft Excel; multiple-group comparisons were performed using one-way ANOVA testing with Tukey–Kramer post hoc analyses using JMP Pro12.0 (SAS).

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