

Insights into the Function of Rim Protein in Photoreceptors and Etiology of Stargardt's Disease from the Phenotype in *abcr* Knockout Mice

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Summary

Rim protein (RmP) is an ABC transporter of unknown function in rod outer segment discs. The human gene for RmP (*ABCR*) is affected in several recessive retinal degenerations. Here, we characterize the ocular phenotype in *abcr* knockout mice. Mice lacking RmP show delayed dark adaptation, increased all-*trans*-retinaldehyde (all-*trans*-RAL) following light exposure, elevated phosphatidylethanolamine (PE) in outer segments, accumulation of the protonated Schiff base complex of all-*trans*-RAL and PE (*N*-retinylidene-PE), and striking deposition of a major lipofuscin fluorophore (A2-E) in retinal pigment epithelium (RPE). These data suggest that RmP functions as an outwardly directed flippase for *N*-retinylidene-PE. Delayed dark adaptation is likely due to accumulation in discs of the noncovalent complex between opsin and all-*trans*-RAL. Finally, *ABCR*-mediated retinal degeneration may result from "poisoning" of the RPE due to A2-E accumulation, with secondary photoreceptor degeneration due to loss of the RPE support role.

Introduction

Rim protein (RmP) is a glycoprotein of 210 kDa in the rims of outer segment discs (Papermaster et al., 1978). Outer segments comprise a stack of these flattened membranous structures, which are the sites of photon capture and the reactions of visual transduction. In rods, discs are topologically separate from one another and the plasma membrane. Recently, RmP was purified from bovine retinas and shown to be expressed exclusively in rod outer segment discs (Azarian and Travis, 1997; Illing et al., 1997). Sequence analysis revealed it to be a novel member of the ATP-binding cassette (ABC) transporter family. Most ABC transporters effect ATP-dependent translocation of specific substrates across cellular membranes. The translocated substrate for RmP is unknown.

Mutations in the human *ABCR* gene for RmP are responsible for Stargardt's disease (STGD), a recessive form of macular degeneration with an onset during childhood (Allikmets et al., 1997a; Stone et al., 1998; Lewis

et al., 1999). STGD is characterized clinically by progressive loss of central vision and progressive atrophy of the retinal pigment epithelium (RPE) overlying the macula (Stargardt, 1909). Early in the disease course, patients show delayed dark adaptation but otherwise normal rod function (Fishman et al., 1991). Histologically, STGD is associated with deposition of lipofuscin pigment granules in RPE cells, presumably arising from impaired digestion after phagocytosis of shed distal outer segments (Birnbach et al., 1994; De Laey and Verougstraete, 1995). Degeneration of the RPE occurs subsequently, with photoreceptor degeneration appearing late in the disease. This pathological picture has led to the conclusion that STGD is primarily a defect of the RPE (Lee and Heckenlively, 1999). However, the pattern of early RPE degeneration and preservation of photoreceptors must be reconciled with the observation that RmP is present exclusively in outer segments and not expressed in RPE cells (Azarian and Travis, 1997). Besides STGD, mutations in *ABCR* have been implicated in fundus flavimaculatus (Rozet et al., 1998), recessive retinitis pigmentosa (Cremers et al., 1998; Martinez-Mir et al., 1998), recessive cone-rod dystrophy (Cremers et al., 1998), and nonexudative age-related macular degeneration (AMD) (Allikmets et al., 1997b; Lewis et al., 1999), although the prevalence of *ABCR* mutations in AMD is still uncertain (Stone et al., 1998). Similar to STGD, all four diseases are associated with delayed rod dark adaptation (Alexander and Fishman, 1984; Fishman et al., 1991, 1994; Steinmetz et al., 1993). Lipofuscin deposition in RPE cells is also seen prominently in AMD (Kliffen et al., 1997) and some cases of retinitis pigmentosa (Kolb and Gouras, 1974; Bergsma et al., 1977).

In the current study, we sought to investigate the function of RmP, the cause of delayed dark adaptation, and the mechanism of lipofuscin accumulation in the *ABCR*-mediated diseases. To this end, we generated mice with a null mutation in the *abcr* gene. Analysis of retinas and RPE from these *abcr*^{-/-} mutants revealed a complex phenotype involving physiological, biochemical, and ultrastructural changes. Based on this analysis, we propose a function for RmP that explains the phenotype in *abcr*^{-/-} mice and humans with *ABCR*-mediated recessive retinal degeneration.

Results

Generation of *abcr*^{-/-} Mice that Lack RmP

The targeting construct depicted in Figure 1A was transfected into 129-strain embryonic stem (ES) cells, and a targeting frequency of 2% was observed in clones that survived G418 and gancyclovir selection. Blastocyst injection of six clones resulted in the birth of 17 high-percentage male chimeras. Southern blot analysis of tail-cut DNA from their F1 offspring (Figure 1B) revealed germline transfer of the disrupted locus in three lines, representing three independent targeting events. The BamHI fragments detected in lines *abcr2* and *abcr3* mice were larger than the predicted 18 kb (Figure 1B),

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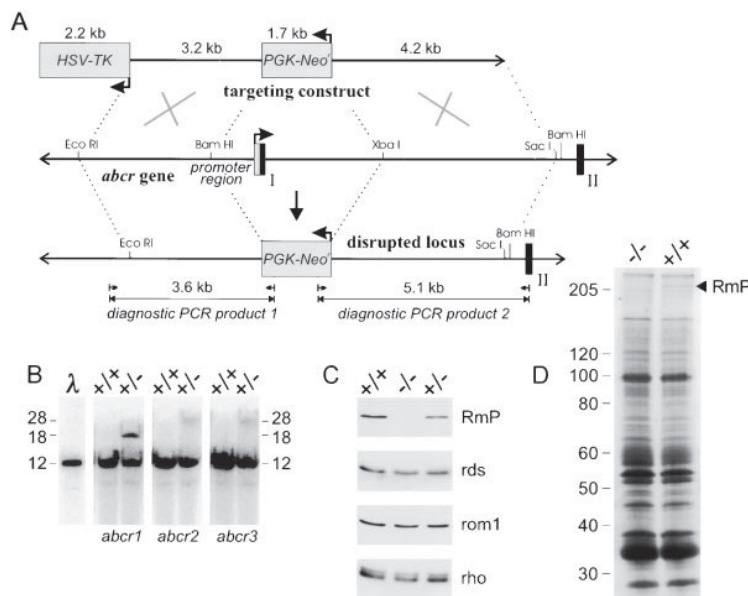


Figure 1. Generation of Mice Homozygous for a Null Mutation in the *abcr* Gene

(A) Targeting strategy for *abcr*. Schematic depictions of the targeting construct, normal *abcr* gene, and resulting disrupted locus. Positions of the neomycin phosphotransferase gene under transcriptional control of the phosphoglycerate kinase promoter (*PGK-Neo*) and herpes simplex thymidine kinase gene (*HSV TK*) are indicated. Successful targeting eliminates a 4.0 kb *Bam*HI-*Xba*I fragment of *abcr* containing the promoter and first exon. The two diagnostic PCR amplification products after successful targeting are indicated.

(B) Southern blot analysis of tail DNA from 16 F1 offspring of the indicated lines. Blots were probed with a genomic fragment from the *abcr* promoter region. Note the presence of an 18 kb *Bam*HI fragment in *abcr*^{+/-} heterozygotes from line *abcr*1. In lines *abcr*2 and -3, the new *Bam*HI fragments are significantly larger, suggesting multiple tandem integrations of the construct. The left lane contains linearized construct DNA (λ). The positions of DNA size standards in kilobases are indicated.

(C) Immunoblot analysis of retinal homogenates from representative wild-type (+/+), *abcr*^{-/-} (-/-), and *abcr*^{+/-} (+/-) mice. Blots were reacted with antisera against the outer segment proteins RmP (Azarian and Travis, 1997), rds/peripherin (rds) (Kedzierski et al., 1999), rom1 (Kedzierski et al., 1999), and rhodopsin (rho) (Azarian and Travis, 1997). Note the absence of RmP and the presence of rds, rom1, and rho immunoreactivity in the *abcr*^{-/-} lane.

(D) Coomassie blue stain of outer segment proteins from wild-type (+/+) and *abcr*^{-/-} (-/-) mice after SDS-PAGE. Equal amounts of purified rod outer segments were subjected to SDS-PAGE and stained with Coomassie. Note the absence of the 210 kDa RmP band and the presence of all other outer segment proteins in the *abcr*^{-/-} lane. The migration positions of protein molecular weight standards in kDa are indicated to the left.

suggesting tandem integration of the construct. Heterozygous sibs from all three lines were inbred to yield *abcr*^{-/-} homozygotes.

We tested for expression of the disrupted gene by nuclease protection analysis of RNA from wild-type and *abcr*^{-/-} retinas. In contrast to samples from control retinas, no transcription products were detected in retina from mice of the three *abcr*^{-/-} lines (not shown). To test for protein expression, we performed immunoblot analysis on retinal homogenates from wild-type, *abcr*^{+/-}, and *abcr*^{-/-} mice from all three lines. No RmP immunoreactivity was detected in samples from *abcr*^{-/-}, and partial immunoreactivity was detected in samples from *abcr*^{+/-} mutants (Figure 1C). We analyzed the same retinal homogenates with antisera against the unrelated outer segment proteins: rds/peripherin, rom1, and rhodopsin. No difference in the levels of these proteins was observed between wild-type and *abcr*^{-/-} mice (Figure 1C). Similarly, we observed no difference in the patterns of total protein from wild-type and *abcr*^{-/-} outer segments except for absence of the 210 kDa RmP band in the mutant (Figure 1D). These results indicate that we knocked out the *abcr* gene and that loss of RmP does not affect the overall protein composition of outer segments.

Delayed Rod Dark Adaptation in *abcr*^{-/-} Mutants

To check for photoreceptor degeneration and delayed dark adaptation, we performed full-field electroretinograms (ERGs) on wild-type and *abcr*^{-/-} mice of different

ages. The ERG records the summation of electrical responses by the retina at the corneal surface to a light flash. The a-wave results from light-induced hyperpolarization of photoreceptors (Granit, 1947). Representative a-waves in dark-adapted mice exposed to five flashes of increasing intensity are shown in Figure 2A. In dark-adapted mice up to 13 weeks, no significant differences were observed between wild-type and *abcr*^{-/-} mice in a-wave maximal amplitude (Rm_{p3}), gain (S), b-wave maximal amplitude (V_{max}), b-wave threshold, or b-wave sensitivity (k). Also, no changes were observed in the cone ERGs of *abcr*^{-/-} mice up to 8 weeks. However, log Rm_{p3} values in 20- to 52-week-old wild-type (n = 9) and *abcr*^{-/-} (n = 8) mice were 2.22 ± 0.18 and 2.02 ± 0.16, respectively (p < 0.05). This 35% mean reduction in a-wave amplitude in *abcr*^{-/-} mice up to 1 year suggests slow ongoing photoreceptor degeneration.

In another study, dark-adapted wild-type and *abcr*^{-/-} mice were exposed to 400 lux illumination for 5 min and then placed in the dark. This light exposure resulted in an approximate 45% photobleach (Figures 3A and 3B). From an intensity series similar to that in Figure 2A, Rm_{p3} values were determined as a fraction of the dark-adapted Rm_{p3} at different time points following the photobleach. We observed significant slowing in the rate of dark adaptation in *abcr*^{-/-} compared to wild-type mice, as shown in Figure 2B. Wild-type animals achieved nearly full dark-adapted sensitivity by 40 min, at which time the mutants only recovered approximately 50% of dark-adapted sensitivity. Based on exponential fits of

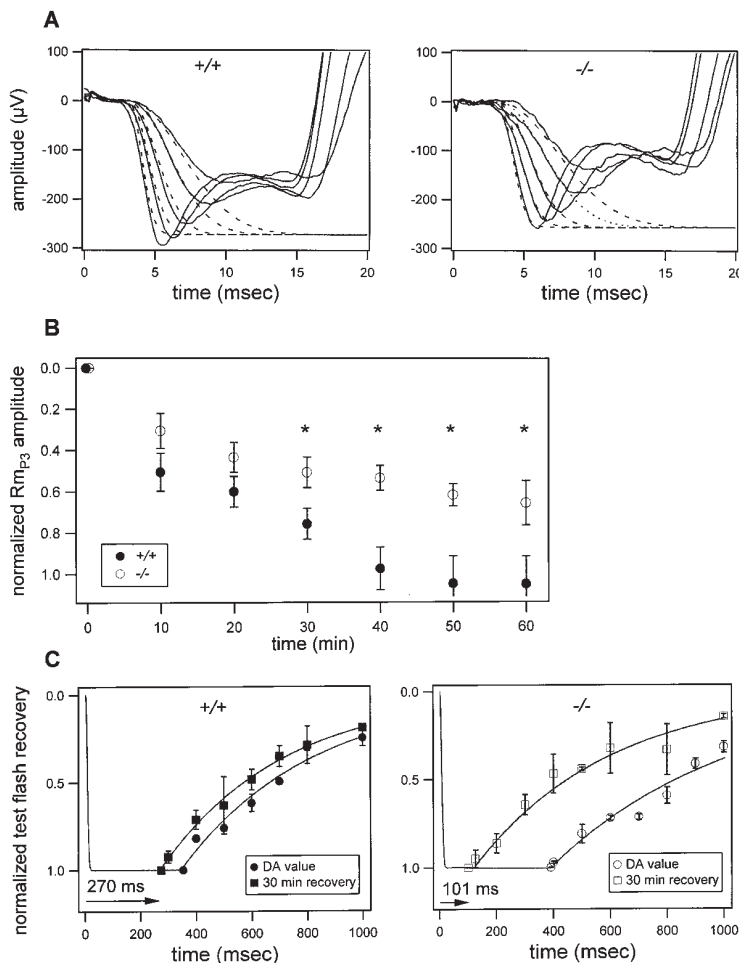


Figure 2. ERG Analysis of Mice

(A) Representative rod a-waves elicited from dark-adapted wild-type (+/+) and *abcr*^{-/-} (-/-) mice with a series of high-intensity flashes (2.1 to 3.4 log scot td-s; 0.3 log unit per step). The dashed curves indicate the best composite fit to a computational model for the activation phase of phototransduction (Hood and Birch, 1993). Data are plotted in microvolts (μV).

(B) Recovery of rod sensitivity in wild-type (+/+) and *abcr*^{-/-} (-/-) mice following 5 min exposure to 400 lux illumination. Data are plotted as the ratio of observed to dark-adapted RmP3 (normalized RmP3 amplitude) plus or minus standard errors at the indicated times. An asterisk (*) above the data points indicates a significant difference between wild-type and mutant (Student's t test, p value < 0.05).

(C) Paired-flash ERG analysis. Each data point shows the difference between the RmP3 value elicited by the probe flash with no prior test flash and the RmP3 value elicited by the probe flash at the indicated time following a test flash of intensity 1.5 log scot td-s (normalized test flash recovery). Circles represent normalized test flash recovery values in dark-adapted wild-type (filled) and *abcr*^{-/-} (open) mice. Squares indicate normalized test flash recoveries at 30 min following a photobleach (5 min at 400 lux) in wild-type (filled) and *abcr*^{-/-} (open) mice. The saturation time (*t*_{sat}) is obtained by extrapolating the normalized test flash recovery back to 1.0 and is indicated for the 30 min photobleach curves by the arrows (wild-type *t*_{sat} = 270 ms; *abcr*^{-/-} *t*_{sat} = 101 ms).

the data from individual mice, the time constants were 23.8 min for wild-type and 50.1 min for *abcr*^{-/-} mice.

A limitation of the ERG is that the latter phase of the photoreceptor response, represented by the a-wave, is obscured by intrusion of the b-wave, which arises from depolarization of second-order retinal neurons (Granit, 1947). A useful technique to circumvent this limitation is the paired-flash paradigm (Birch and Hood, 1995; Lyubarsky and Pugh, 1996; Pepperberg et al., 1997). This method involves stimulation with a briefly saturating test flash, followed at different times by a bright probe flash that causes rapid photoreceptor hyperpolarization. In dark-adapted animals with full rod sensitivity, the refractory period following a test flash is relatively long. With incomplete dark adaptation, rod sensitivity is reduced and the refractory period is shorter. The refractory period, or saturation time (*t*_{sat}), can be determined by extrapolating the normalized test-flash recovery value back to 1.0 (Figure 2C). We performed a paired-flash study on dark-adapted mice following a 5 min 400 lux photobleach. As expected, we observed similar inactivation kinetics in dark-adapted wild-type and *abcr*^{-/-} mice (Figure 2C). However, the recovery time was significantly reduced in *abcr*^{-/-} compared to wild-type mice at 30 min following the photobleach (*t*_{sat} = 270 ms for

wild-type and 101 ms for *abcr*^{-/-} mice). Since the photo-response time is reduced with light adaptation (Tamura et al., 1989; Nakatani et al., 1991), a lower *t*_{sat} value in *abcr*^{-/-} suggests that the rods in these animals perceive a background of "equivalent light." We could simulate the accelerated recovery of sensitivity observed in *abcr*^{-/-} mutants by exposing wild-type mice to 4 lux background illumination during the paired-flash ERG experiment.

Delayed Clearance of All-*trans*-Retinaldehyde in *abcr*^{-/-} Mutants

In an attempt to identify the biochemical cause for delayed rod dark adaptation, we measured the levels of rhodopsin spectrophotometrically in eyecup homogenates from 7- to 8-week-old mice, using the same experimental design as in the ERG section. No significant differences were observed between wild-type and *abcr*^{-/-} mice in dark-adapted levels of rhodopsin or in the rate of rhodopsin regeneration (Figure 3A). The levels of 11-*cis*-retinaldehyde (11-*cis*-RAL) were determined by high performance liquid chromatography (HPLC) analysis on retinal extracts from 16- to 20-week-old dark-adapted mice and age-matched mice at different times following a photobleach. Similar to rhodopsin, we observed no

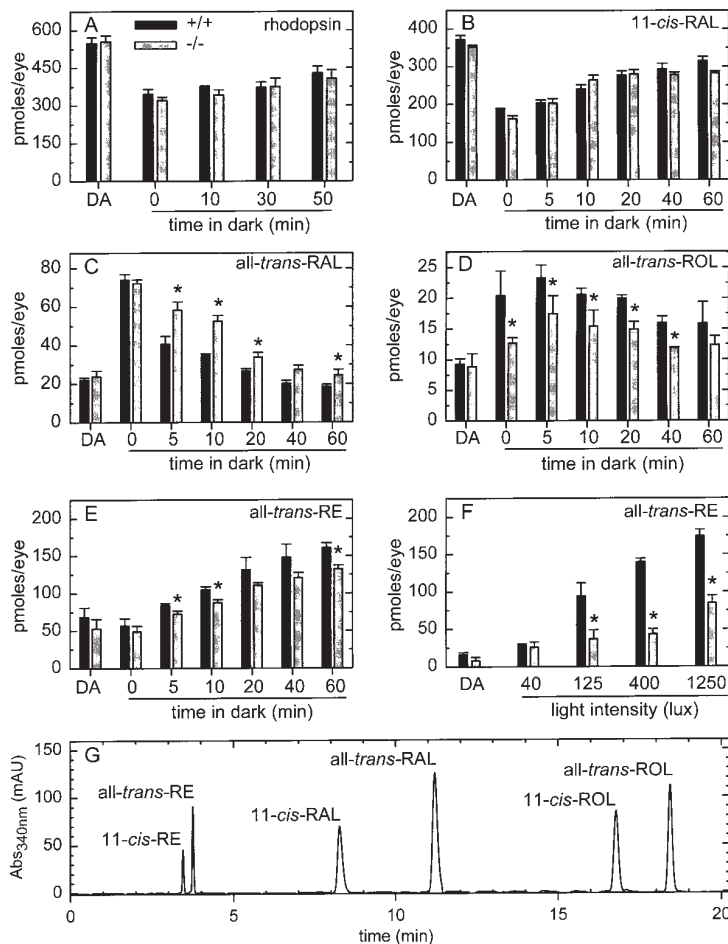


Figure 3. Levels of Rhodopsin and Retinoids in Retina and RPE after Light Exposure

Each data point represents an average value in pmol per eye $\pm$ standard deviation from at least four eyes in dark-adapted (DA) mice at the indicated times in the dark following a partial photobleach. Light conditions were as described in Figure 2 except where noted. Wild-type (+/+; black bars) and *abcr*^{-/-} (-/-; shaded bars).

(A) Level of rhodopsin in eyecups, determined spectrophotometrically.

(B) Level of 11-*cis*-RAL in retinas, by HPLC.

(C) Level of all-*trans*-RAL in retinas, by HPLC.

(D) Level of all-*trans*-ROL in eyecups, by HPLC.

(E) Level of all-*trans*-RE in RPE, by HPLC.

(F) Level of all-*trans*-RE in RPE, by HPLC immediately following 1 hr of light exposure at the indicated intensities.

(G) Representative HPLC chromatogram of retinoid standards detected at wavelength 340 nm. Shown as absorbance units $\times 10^{-3}$ (mAU). An asterisk (*) above a (-/-) data bar indicates a significant difference from wild-type (Student's t test, p value < 0.05).

significant differences between wild-type and *abcr*^{-/-} mice (Figure 3B). The absolute level of rhodopsin in dark-adapted wild-type mice was approximately 30% higher than that of 11-*cis*-RAL, probably due to our extraction of rhodopsin from total eyecups versus dissected retinas for the retinaldehydes. The results of these experiments show that RmP is not required for the synthesis of 11-*cis*-RAL, its uptake into photoreceptors, or regeneration of rhodopsin from bleached pigment.

The levels of all-*trans*-RAL in dark-adapted wild-type and *abcr*^{-/-} mice were also similar (Figure 3C). However, we observed significant elevations in all-*trans*-RAL in *abcr*^{-/-} mice compared to controls for up to 1 hr following a photobleach (Figure 3C). In contrast, we observed significant reductions in the levels of all-*trans*-retinol (all-*trans*-ROL) in *abcr*^{-/-} mice compared to wild-type controls following a photobleach (Figure 3D). Also, the levels of all-*trans*-retinyl esters (all-*trans*-RE) were lower in RPE from *abcr*^{-/-} compared to wild-type mice (Figure 3E). The levels of 11-*cis*-retinol (11-*cis*-ROL) and 11-*cis*-retinyl esters (11-*cis*-RE) in wild-type and *abcr*^{-/-} mice were near the detection threshold and did not appear to change during the course of the experiment. When we exposed dark-adapted mice to continuous light for a full hour, we observed larger differences in the levels of all-*trans*-RE between wild-type and *abcr*^{-/-} RPE at

high light intensities (Figure 3F). Together, these results suggest that clearance of all-*trans*-RAL from discs following a photobleach is partially dependent upon the presence of RmP.

Increased Phosphatidylethanolamine in *abcr*^{-/-} Outer Segments and RPE

Since several ABC transporters have been shown to effect phospholipid transfer (Smit et al., 1993; Decottignies et al., 1998; Zhou et al., 1998; Dogra et al., 1999), we compared levels of the major phospholipid species in extracts of retina, outer segments, and RPE by HPLC analysis. Phosphatidylinositol, cardiolipin, phosphatidylserine, and phosphatidylcholine were each present at similar levels in wild-type and *abcr*^{-/-} mice (Figure 4A). Phosphatidylethanolamine (PE), however, was 1.6-fold higher in extracts of outer segments from *abcr*^{-/-} compared to wild-type mice (Figure 4A). PE was similarly increased in extracts of RPE from *abcr*^{-/-} compared to wild-type mice. The fatty acid profiles of PE from wild-type and *abcr*^{-/-} outer segments were similar, both containing approximately 32 mol% docosahexenoic acid (22:6), 17 mol% oleic acid (18:1), 12 mol% palmitic acid (16:0), 5 mol% stearic acid (18:0), and the remainder undefined polyunsaturated fatty acids. No significant difference in phospholipid levels was observed between

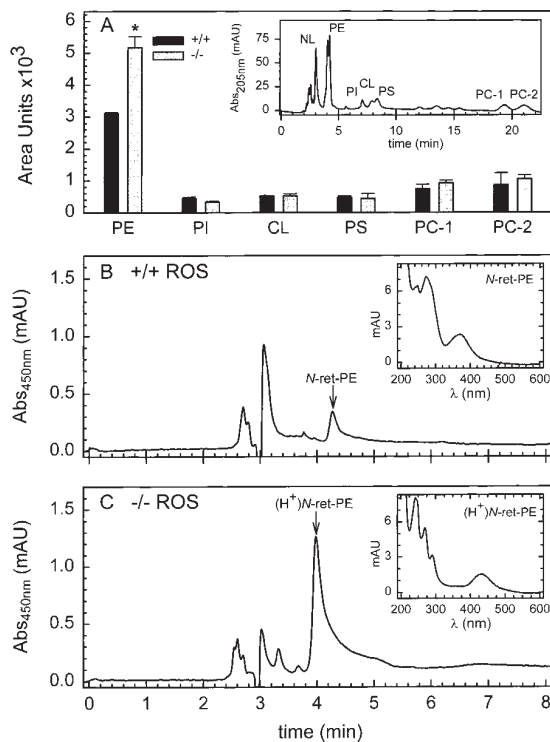


Figure 4. Phospholipids and *N*-retinylidene-PE in Wild-Type (+/+) and *abcr*^{-/-} (-/-) Outer Segments

(A) Major phospholipid species: phosphatidylethanolamine (PE), phosphatidylinositol (PI), cardiolipin (CL), phosphatidylserine (PS), first phosphatidylcholine peak (PC-1), second phosphatidylcholine peak (PC-2). Values are shown in absolute area units of UV absorption at 205 nm $\pm$ standard deviation. Inset shows representative HPLC chromatogram of phospholipids from wild-type outer segments detected at wavelength 205 nm. Early eluting peaks are neutral lipids (NL). Shown as absorbance units $\times 10^{-3}$ (mAU).

(B) HPLC chromatogram from +/+ rod outer segments (ROS) at detection wavelength 450 nm. Peak corresponding to nonprotonated *N*-retinylidene-PE is labeled. Inset shows absorption spectrum for *N*-retinylidene-PE peak. Note the absence of 450 nm *N*-retinylidene-PE absorbance.

(C) HPLC chromatogram from -/- rod outer segments (ROS) at detection wavelength 450 nm. Peak corresponding to protonated (H⁺)*N*-retinylidene-PE is labeled. Inset shows absorption spectrum for (H⁺)*N*-retinylidene-PE peak.

wild-type and mutant extracts prepared from retinas depleted of outer segments.

N-Retinylidene-Phosphatidylethanolamine in *abcr*^{-/-} Outer Segments

It has long been established that PE reacts spontaneously as a primary amine with retinaldehyde to form a Schiff base condensation product called *N*-retinylidene-phosphatidylethanolamine (*N*-retinylidene-PE) (Poincelot et al., 1969; Anderson and Maude, 1970). *N*-retinylidene-PE undergoes a shift in absorption from $\lambda_{\max} = 360$ to 450 nm with protonation of the Schiff base (Anderson and Maude, 1970). To test for the presence of *N*-retinylidene-PE, we performed HPLC analysis on outer segment extracts at detection wavelengths 450 and 360 nm. Figures 4B and 4C show chromatograms at 450 nm for wild-type and *abcr*^{-/-} mice, respectively. Although

the total levels were similar, wild-type mice contained largely nonprotonated *N*-retinylidene-PE by spectral analysis, while *abcr*^{-/-} mice mainly contained the protonated form (Figures 4B and 4C, insets), despite extraction of outer segments under identical conditions. We confirmed this difference in protonation state by demonstrating a spectral shift from $\lambda_{\max} = 450$ to 360 nm upon addition of alkali to the *N*-retinylidene-PE fraction from *abcr*^{-/-} outer segments and a shift from $\lambda_{\max} = 360$ to 450 nm upon addition of acid to the *N*-retinylidene-PE fraction from wild-type outer segments. Compared to the nonprotonated form of *N*-retinylidene-PE, the protonated form had a slightly shorter retention time on the silica column, possibly due to its zwitterionic character (Daemen and Bonting, 1969). Calculations based on the published extinction coefficients for *N*-retinylidene-PE (Anderson and Maude, 1970) and all-*trans*-RAL (Hubbard et al., 1971) showed that nearly all of the all-*trans*-RAL was present as *N*-retinylidene-PE in dark-adapted, wild-type retinas. The 11-*cis* isomer of *N*-retinylidene-PE was undetectable in dark-adapted outer segments, consistent with 11-*cis*-RAL being exclusively complexed with rhodopsin.

Lipofuscin Accumulation in *abcr*^{-/-} Mice

The major fluorescent species of lipofuscin is *N*-retinylidene-*N*-retinylethanolamine (A2-E), a positively charged Schiff base condensation product of two retinaldehydes with ethanolamine (Eldred and Lasky, 1993). The closely related species *iso*-A2-E contains one 13-*cis* isomer of retinaldehyde. *iso*-A2-E probably arises by photoisomerization of A2-E rather than direct condensation of ethanolamine with 13-*cis*-RAL, since this retinoid species is present in vivo at very low levels (Parish et al., 1998). The chromatographic and spectral properties of A2-E and *iso*-A2-E are well defined (Eldred and Katz, 1988; Parish et al., 1998), with a $\lambda_{\max}$ for each of approximately 430 nm. Since lipofuscin accumulation is a feature of several human *ABCR*-mediated diseases, we tested for A2-E and *iso*-A2-E in retina, RPE, and purified outer segments from wild-type and *abcr*^{-/-} mice by HPLC. RPE extracts from 16- to 20-week-old *abcr*^{-/-} mice contained 21 and 10 pmol per eye of A2-E and *iso*-A2-E, respectively (Figure 5). RPE from age-matched control mice had <1 pmol per eye of each isomer. No A2-E or *iso*-A2-E was detected in retinas or purified outer segments from wild-type or *abcr*^{-/-} mice. We confirmed our identification of A2-E by synthesizing it in vitro (Parish et al., 1998) and showing identical chromatographic and spectral properties with those of A2-E isolated from mouse RPE (Figure 5 inset). Further, we performed fast atom bombardment mass spectrometry on synthetic and endogenous A2-E. Both yielded a molecular ion (*m/z*) peak of 592.4, which is in good agreement with the calculated molecular mass of 592.45 for C₄₂H₅₈ON.

Ultrastructural analysis of eyes from 44-week-old mice showed significant accumulation of dense bodies within RPE cells of *abcr*^{-/-} mutants (Figure 6A). These included large oval structures of high electron density, presumably representing melanosomes or melanosomophagosome fusion particles (Feeney-Burns and Eldred, 1983). We also observed numerous smaller structures of variable density, probably representing lipofuscin granules. In many cells, the dense bodies were displaced into the apical processes (Figure 6A). Finally, we

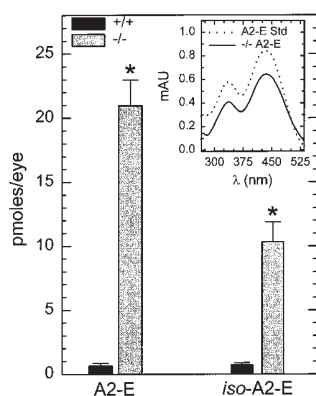


Figure 5. A2-E and iso-A2-E in RPE from Wild-Type and *abcr*^{-/-} Mice

Levels of the two isomers in pmol/eye $\pm$ standard deviation. Inset shows absorption spectra for synthesized A2-E standard (dotted line) and A2-E from *abcr*^{-/-} RPE (solid line). Values for (-/-) mice differed significantly from (+/+) mice (Student's t test, p value < 0.001).

observed thickening of Bruch's membrane, between the RPE and choroid, in *abcr*^{-/-} mice. The photoreceptors and the rest of the retina in *abcr*^{-/-} mutants were indistinguishable from those in wild-type mice (not shown). In particular, both rod and cone outer segments were of normal dimensions and showed good alignment of discs.

Discussion

In this work, we report the generation and phenotypic characterization of mice with a null mutation in the *abcr* gene for RmP. In summary, these mice showed: (1) delayed rod dark adaptation; (2) transient accumulation of all-*trans*-RAL and transient depletion of all-*trans*-ROL and all-*trans*-RE following a photobleach; (3) 1.6-fold increased PE in outer segments; (4) presence of protonated and absence of nonprotonated *N*-retinylidene-PE in outer segments; and (5) dramatically increased A2-E in RPE cells. These observations can all be explained by our model for the function of RmP, as presented below.

Delayed Dark Adaptation

All vertebrates sustain a period of reduced visual sensitivity following light exposure, due to the accumulation of an undefined activating photoproduct (reviewed in

Fain et al., 1996; Leibrock et al., 1998). Prolongation of this reduced-sensitivity state in *abcr*^{-/-} mutants is similar to the clinical pattern in patients with the *ABCR*-mediated retinal diseases, including STGD (Alexander and Fishman, 1984; Fishman et al., 1991, 1994; Steinmetz et al., 1993). During the first 40 min in darkness following a photobleach, rod cells in *abcr*^{-/-} mice behave as if exposed to low-level background illumination (Figure 2). The otherwise normal ERG parameters in dark-adapted *abcr*^{-/-} mice suggest that RmP plays no direct role in visual transduction. Opsin apoprotein has been shown to interact spontaneously with all-*trans*-RAL to form a noncovalent complex that activates the visual transduction pathway with approximately 10⁶-fold greater efficiency than opsin alone and nearly 10% the efficiency of photoactivated rhodopsin (metarhodopsin II) (Surya et al., 1995; Jager et al., 1996; Melia et al., 1997). A likely explanation for the delayed dark adaptation in *abcr*^{-/-} mice is transient accumulation of this "noisy" opsin/all-*trans*-RAL photoproduct. As rhodopsin regenerates, all-*trans*-RAL is displaced by the formation of a covalent complex between 11-*cis*-RAL and opsin, with rod cells returning to normal dark sensitivity. The time courses following a photobleach for the delay in dark adaptation, disappearance of all-*trans*-RAL, and regeneration of rhodopsin (Figures 2 and 3) are consistent with this explanation. The delayed dark adaptation in humans with *ABCR*-mediated retinal disease also may result from transient accumulation in outer segments of the opsin/all-*trans*-RAL product.

Abnormal Phospholipid Composition

Another aspect of the *abcr*^{-/-} phenotype was a 1.6-fold increase in outer segment PE. This represents a large difference in PE mole percentage (54 mol% in *abcr*^{-/-} vs. 34 mol% in wild-type outer segments) according to published values (Fliesler and Anderson, 1983). Phospholipids, especially PE, are rapidly turned over in outer segments by the action of transfer proteins, phospholipases, and acyltransferases. Based on these observations, the turnover of PE may be reduced in outer segments from *abcr*^{-/-} mice. Phospholipids are normally distributed asymmetrically across the disc bilayer, with 70%–80% of PE present in the outer (cytoplasmic) leaflet (Miljanich et al., 1981; Wu and Hubbell, 1993). In *abcr*^{-/-} mutants, the distribution of PE may be more symmetrical, with the excess located preferentially in the inner (intradiscal) leaflet. This is understandable, since the enzymes that effect phospholipid turnover are present in the cytoplasmic compartment and hence can only

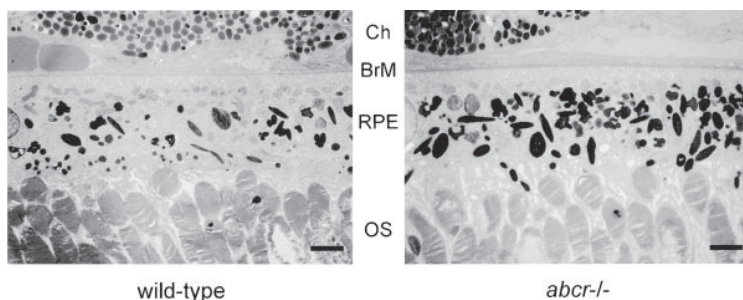


Figure 6. Electron Microscopy of RPE and Retina from 44-Week-Old Mice

Comparison of RPE from wild-type and *abcr*^{-/-} mice. Choroid (Ch), Bruch's membrane (BrM), RPE, and outer segment (OS) layers are labeled. Large dense bodies in wild-type RPE cells are melanosomes. Note the increased number of small dense bodies in *abcr*^{-/-} RPE and the thickening of Bruch's membrane. Size bar, 2 μ m.

act on PE oriented with its headgroup on the extradiscal surface.

Lipofuscin Accumulation and the Mechanism of ABCR-Mediated Disease

An important pathological feature of both STGD and AMD is the accelerated accumulation of lipofuscin in RPE phagosomes (De Laey and Verougstraete, 1995; Kliffen et al., 1997). A2-E, the major fluorophore of lipofuscin (Eldred and Lasky, 1993), was significantly elevated in *abcr*^{-/-} RPE (Figure 5). *N*-retinylidene-PE, a likely precursor of A2-E (Parish et al., 1998), was present at approximately equal levels in outer segments from dark-adapted wild-type and *abcr*^{-/-} mice. This was expected, since the dark-adapted levels of all-*trans*-RAL were similar between wild-type and mutant retinas, and approximately 100% of all-*trans*-RAL was present as *N*-retinylidene-PE. However, *N*-retinylidene-PE was largely protonated in *abcr*^{-/-} mutants and unprotonated in wild-type mice (Figures 4B and 4C). Upon shedding of rod outer segments, the accumulated *N*-retinylidene-PE is sequestered into RPE phagolysosomes where the low pH environment favors the formation of A2-E salts (Eldred and Lasky, 1993). At low concentrations, A2-E inhibits normal proteolysis in lysosomes (Eldred, 1995; Holz et al., 1999). At higher concentrations, A2-E may act as a positively charged lysosomotropic detergent, dissolving cellular membranes and ultimately killing the RPE cell (Eldred and Lasky, 1993). By electron microscopy, we observed accumulations of electron-dense bodies in RPE cells and significant thickening of Bruch's membrane in *abcr*^{-/-} mice (Figure 6A). A similar ultrastructural pattern has been described in RPE from humans with early-stage STGD (Steinmetz et al., 1991; Birnbach et al., 1994). These ultrastructural changes are consistent with the observed accumulation of A2-E in RPE cells and indicate significant lipofuscin deposition. We saw no evidence of RPE cell enlargement or degeneration, pathologic features of advanced STGD (Eagle et al., 1980; Birnbach et al., 1994). Photoreceptor degeneration also occurred slowly in *abcr*^{-/-} mice. In the ABCR-mediated retinal dystrophies, we suggest that photoreceptor degeneration follows the death of RPE cells and results from loss of the RPE support role (reviewed in Steinberg, 1985). This explains the slower photoreceptor degeneration in *abcr*^{-/-} mice compared with the other retinal mutants that act by photoreceptor cell-autonomous mechanisms (reviewed in Travis, 1998).

Another hallmark of STGD and AMD is strikingly early involvement of the central retina, or macula. The perfoveal region of the human macula contains a ring-like area of very high rod density (150,000 mm⁻² compared to ~30,000 mm⁻² in peripheral retina) (Jonas et al., 1992). The fovea itself is populated exclusively with cone photoreceptors. Selective involvement of the macula in STGD may result from the high ratio of rod outer segments to RPE cells in this region. Ring-like deficits in the central visual field and ring-like areas of higher lipofuscin deposition in RPE overlying the macula have been observed in humans (Wing et al., 1978; Lee and Heckenlively, 1999). Thus, foveal cones probably do not contribute A2-E precursor to the macular RPE in the ABCR-mediated diseases. Subsequent degeneration of

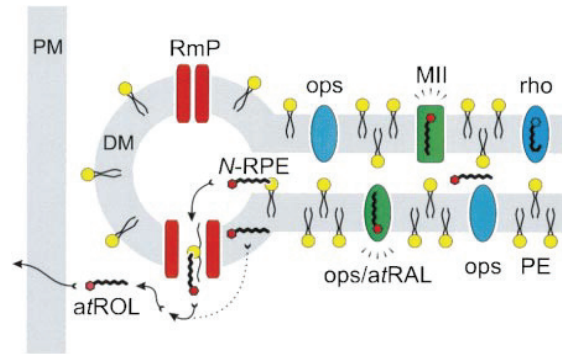


Figure 7. Model for the Function of RmP in Disc Membranes

Schematic drawing of a rod outer segment disc near the rim. Several forms of opsin visual pigment are represented: rhodopsin (rho), which is covalently coupled to 11-*cis*-RAL and has no transducin-stimulating activity; metarhodopsin II (MII), which is covalently coupled to all-*trans*-RAL (atRAL) and has high transducin-stimulating activity; opsin apoprotein (ops), which has very low transducin-stimulating activity; and the noncovalent complex of opsin with all-*trans*-RAL (ops/atRAL), which also has high transducin-stimulating activity. The all-*trans*-RAL released into the disc interior by decay of MII reacts with the primary amine of phosphatidylethanolamine (PE) to form the Schiff base condensation product *N*-retinylidene-PE (*N*-RPE). Also, all-*trans*-RAL combines noncovalently with opsin apoprotein to form the "noisy" ops/all-*trans*-RAL photoproduct. RmP in the disc rim is an *N*-retinylidene-PE flippase, effecting ATP-dependent translocation of *N*-retinylidene-PE to the outer leaflet of the disc membrane (DM). Upon translocation, *N*-retinylidene-PE is hydrolyzed to release all-*trans*-RAL, which is then reduced to all-*trans*-ROL (atROL), indicated by the tandem arrows. All-*trans*-ROL diffuses spontaneously, or is translocated by unknown mechanisms, across the outer segment plasma membrane (PM), where it is taken up and esterified by the RPE cell (not shown). The alternative pathway for clearance of free all-*trans*-RAL is passive diffusion through the disc membrane, indicated by the dotted line.

the foveal cones with loss of central vision may result from rod-mediated "poisoning" of the macular RPE. The peripheral retina is affected later in STGD due to the more favorable ratio of outer segments to RPE cells. Mouse retinas do not contain a macula, more closely resembling a human peripheral retina (Carter-Dawson and LaVail, 1979). In studies of two pedigrees, the ABCR null phenotype in humans was retinitis pigmentosa (Cremers et al., 1998; Martinez-Mir et al., 1998), a blinding disease that predominantly affects the peripheral retina. Thus, early involvement of the less-vulnerable peripheral retina may only be seen with more severe alleles of ABCR.

Model for the Function of RmP

The results presented in this paper can be integrated by the model shown in Figure 7. In brief, we propose that RmP functions as an outwardly directed flippase for protonated *N*-retinylidene-PE. Such a role is consistent with the biology of ABC transporters, since other members of this family are also phospholipid flippases, including MDR2 in mammals (Smit et al., 1993), Yor1p in yeast (Decottignies et al., 1998), MsbA in *Escherichia coli* (Zhou et al., 1998), and CDR1 in *Candida albicans* (Dogra et al., 1999). Recently, it was shown that several retinoids, including all-*trans*-RAL, increased the ATPase

activity of reconstituted RmP (Sun et al., 1999). However, this increase in ATPase activity was only seen when RmP was reconstituted in the presence of PE, suggesting that all-*trans*-RAL reacted with PE to form *N*-retinylidene-PE, the putative substrate for RmP. The proposed *N*-retinylidene-PE flippase activity of RmP may serve two potential functions.

First, it may accelerate dark adaptation by speeding the transfer of all-*trans*-RAL from the disc interior to the cytoplasmic surface following a photobleach. It has not been established on which side of the disc membrane 11-*cis*-RAL and all-*trans*-RAL interact with opsin. However, for all other seven-transmembrane receptors, ligands arrive and depart via the extracellular face. Since the disc interior is topologically equivalent to the extracellular space, we think it likely that all-*trans*-RAL dissociates from opsin into the intradiscal space. The elevated levels of all-*trans*-RAL and reduced levels of all-*trans*-ROL and all-*trans*-RE following a photobleach in *abcr*^{-/-} mutants suggest that RmP functions in a step following photoactivation of rhodopsin but preceding reduction of all-*trans*-RAL. Therefore, all-*trans*-retinol dehydrogenase must act downstream of RmP. Localization of RmP to the disc rim immediately subjacent to the plasma membrane may enhance the efficiency of retinoid recycling by shortening the diffusion path to the RPE. We observed substantial accumulation of retinyl ester above the dark-adapted level in wild-type RPE cells, suggesting significant basal synthesis in response to light exposure. The observed difference in retinyl ester levels between RPE from wild-type and mutant mice after light exposure may reflect impaired ester synthesis due to reduced apical uptake of all-*trans*-ROL. On the other hand, this difference may reflect "sickness" of the RPE due to lipofuscin accumulation. Mutant mice lacking RmP regenerate rhodopsin normally and achieve full dark adaptation, albeit more slowly than in wild-type mice. Thus, an alternative path must exist for the recycling of all-*trans*-RAL, probably involving free diffusion through disc membranes (Groenendijk et al., 1984; Jin et al., 1994). RmP may accelerate the removal of all-*trans*-RAL from the disc interior by the translocation of *N*-retinylidene-PE. In cone outer segments, the intradiscal and extracellular spaces are contiguous, which may explain the absence of RmP from these cells (Allikmets et al., 1997a; Illing et al., 1997; Sun and Nathans, 1997). Also, cones are not used for vision under low-light conditions and thus have no requirement for rapid dark adaptation.

The second, and perhaps more important, function of RmP as a flippase may be to eliminate *N*-retinylidene-PE from the disc interior, thus protecting the RPE from lipofuscin accumulation. Following a significant photobleach, all-*trans*-RAL is present at very high concentrations within the disc, favoring formation of *N*-retinylidene-PE. In *abcr*^{-/-} mutants lacking the RmP flippase activity, protonated *N*-retinylidene-PE may be "entombed" within the disc interior. In normal animals, protonated *N*-retinylidene-PE is translocated to the exterior (cytoplasmic) surface of the disc. Reduction of all-*trans*-RAL and subsequent esterification of the resulting alcohol shifts the equilibrium to favor hydrolysis of the *N*-retinylidene-PE Schiff base.

According to our model for RmP function, photoreceptor degeneration and visual loss in the *ABCR*-mediated diseases is a three-step process. First, protonated *N*-retinylidene-PE accumulates in outer segments due to loss of the flippase activity. Second, isomers of A2-E build up in RPE lysosomes, resulting in progressively impaired digestion of phagocytosed outer segments and ultimate dissolution of cellular membranes. Finally, photoreceptors die due to loss of RPE support functions. Since all-*trans*-RAL is only released following light activation of rhodopsin, a strong prediction of this model is that A2-E accumulation should be reduced or abolished in *abcr*^{-/-} mice raised in total darkness. In a preliminary study, A2-E was virtually absent from the RPE of dark-reared *abcr*^{-/-} mice (N. L. M. and G. H. T., unpublished observation). A corollary of this prediction is that patients with STGD may slow the progression of their disease by avoiding bright light. The etiology of AMD may be similar to that of STGD, with lipofuscin accumulation in RPE cells causing secondary photoreceptor degeneration. Accordingly, the *abcr*^{-/-} knockout mouse may be a good animal model for AMD independent of its genetic cause. A potential therapeutic strategy for both STGD and AMD will be to find agents that inhibit A2-E accumulation in RPE cells.

Experimental Procedures

Generation of *abcr*^{-/-} Mice

A replacement-type targeting construct for *abcr* was assembled by screening a mouse strain 129Sv/J lambda genomic library (Stratagene) with a probe from the 5' region of a mouse RmP cDNA (Azarian and Travis, 1997). A clone of ~18 kb containing the first four exons and promoter region of *abcr* was isolated, restriction mapped, and partially sequenced. A 3.2 kb EcoRI/BamHI fragment from upstream of *abcr* and a 4.2 kb XbaI/SacI fragment from intron I were placed in pBluescript, flanking a 1.7 kb SalI/XhoI fragment of pPGKneobpA containing the neomycin phosphotransferase gene (*Neo*) under control of the mouse PGK promoter. Successful targeting with this construct would result in deletion of a 4.0 kb fragment of *abcr* containing the promoter and first protein-coding exon (Azarian et al., 1998). Finally, a 2.2 kb SalI/XhoI fragment of pMC1tkbpA containing the herpes simplex virus thymidine kinase gene (HSV TK) was cloned into the upstream SalI site of the targeting vector for negative selection. The construct was electroporated into J1 ES cells derived from 129Sv mice. ES cell clones resistant to both G418 and gancyclovir were expanded and analyzed by PCR using primer sets from the regions shown in Figure 1A. After sequencing the diagnostic 3.6 and 5.1 kb amplification products, cells from six clones that had undergone homologous recombination were injected into C57BL/6 blastocysts, resulting in the birth of 17 high-percentage chimeras by coat color. Six male chimeras were bred with C57BL/6 females to check for germline transmission. Three germline-transmitting animals, representing three independent targeting events, were bred with 129SVEV females. Tail-cut DNA from the offspring was analyzed by Southern blot analysis, as described below. Heterozygotes from each line were inbred to yield *abcr*^{-/-} homozygous mutants.

Southern Blot Analysis

DNA from mouse tails was digested with BamHI, separated by electrophoresis in 0.5% agarose, and transferred to Hybond-N+ membranes (Amersham). Blots were hybridized with a 174 bp fragment from the *abcr* promoter region or a 400 bp fragment from the *Neo* gene, both labeled with ³²P by PCR. Autoradiograms were detected on X-ray film (Amersham).

Immunoblot Analysis

Crude preparations of rod outer segments were made from two wild-type and two *abcr*^{-/-} retinas by vortexing in 200 µl of 45%

sucrose, 20 mM Tris-HCl (pH 7.4), 1 mM EDTA, 2 mM MgCl₂, 20 μ M leupeptin, and 2 mM PMSF. Retinas were centrifuged for 5 min at 5000 $\times$ g. The supernatants were collected, diluted with an equal volume of 150 mM NaCl, 20 mM Tris (pH 7.4), 1 mM EDTA, and 2 mM MgCl₂, and recentrifuged. The outer segment pellets were dissolved in Laemmli buffer and separated by SDS-PAGE in a 9% gel, transferred to Immobilon-P membrane (PVDF, Millipore), reacted with the antisera shown in Figure 1C, and detected by ECL (Amersham), following the manufacturer's procedure.

ERG Analysis

Mice were dark adapted for a minimum of 12 hr and anesthetized by intraperitoneal injection of ketamine (200 mg/kg body weight) and xylazine (10 mg/kg body weight). Pupils were dilated by topical application of 1.0% atropine sulfate. In the partial-bleach experiments, anesthetized mice were exposed to 400 lux illumination in a Ganzfeld dome for 5 min. Full-field ERGs were recorded in the Ganzfeld dome using a gold-coil active electrode on the corneal surface, reference electrode in the mouth, and needle-type ground electrode on the tail. The relationship between rod-mediated b-wave and illumination level was estimated by fitting V-log functions with the Michaelis-Menton equation: $V = (I/(I+k))V_{max}$. We determined the sensitivity (k) and the maximum b-wave amplitude (V_{max}) parameters by solving this equation. The leading edge of the a-wave was fit by a computational model of the activation phase of photo-transduction (Hood and Birch, 1993), described by the equation $P3(t) = [1 - \exp(-I \cdot S \cdot (t - t_d))] \cdot Rm_{p3}$ for $t > t_d$, where P3 is the sum of individual rod responses and (i) is flash intensity, (t) is time after flash onset, S is a sensitivity parameter that scales (i), Rm_{p3} is the maximum response, and t_d is a brief delay.

Paired-flash ERG analysis was used to determine the full time course of the massed rod response to a test flash of intensity 1.5 log scot td-s followed at different times by a bright probe flash. The kinetics of rod recovery following the test flash were determined by measuring the probe flash Rm_{p3} at different times in dark-adapted mice and in mice 30 min following a partial photobleach.

Rhodopsin Quantitation

Rhodopsin was determined spectrophotometrically in total eyecup extracts according to the procedure of Saari et al. (1998). Seven- to eight-week-old wild-type and *abcr*^{-/-} mice (both inbred strain 129) were dark adapted for at least 12 hr before the experiment. The light-sensitive absorbance of the detergent extract at 500 nm was measured, and rhodopsin levels were calculated based on a molar extinction coefficient of 40,000 M⁻¹cm⁻¹ and an M_r of 40,000. The time course of rhodopsin regeneration was determined following the same experimental design as described in the ERG section.

SDS-PAGE Analysis of Outer Segment Proteins

Rod outer segments were prepared from 8- to 10-week-old dark-adapted wild-type (hybrid strain B6 $\times$ D2) and *abcr*^{-/-} (hybrid strain 129 $\times$ B6) mice, as described (Tsang et al., 1998). An aliquot of each preparation was analyzed for rhodopsin content spectrophotometrically. Equal amounts (equivalent to nine retinas) were solubilized on ice in Laemmli sample buffer and subjected to SDS-PAGE on a 10% porous gel (Azarian and Travis, 1997), followed by Coomassie staining.

Retinoid Analysis

Experimental Design

Retinoids of the visual cycle were determined in 16- to 20-week-old wild-type (hybrid B6 $\times$ D2) and *abcr*^{-/-} (hybrid strain 129 $\times$ B6) mice. Dark-adapted mice were treated as described in the ERG section and studied at the indicated times. In a separate study, the levels of retinyl esters were determined following 1 hr of constant illumination at 40, 125, 400, or 1200 lux.

Tissue Dissection and Preparation

Retinoids were extracted from whole eyecups (posterior poles containing retinas), whole retinas, and RPE/eyecups (eyecups devoid of retina). The dissected tissues were immediately homogenized on ice in 1 ml PBS (pH 7.0) for analysis of retinyl esters and retinols or in 1 ml 100 mM potassium phosphate buffer (pH 7.0) containing 6.0 M formaldehyde for analysis of retinas (Suzuki et al., 1986).

Extraction of Retinyl Esters and Retinols

An equal volume of absolute ethanol was added to the PBS-containing homogenates. The samples were vortexed and incubated at room temperature for 10 min. Samples were extracted twice with 2 vol of hexane followed by centrifugation for 2 min at 1000 $\times$ g. The organic phases were collected, evaporated under a stream of N₂, and the samples dissolved in 200 μ l of hexane.

Extraction of Retinaldehyde

Two milliliters of methylene chloride was added to the formaldehyde-containing homogenates, followed by incubation at 30°C for 10 min and methylene chloride-hexane extraction.

HPLC Analysis

Retinoids were analyzed by normal-phase HPLC using a Hewlett Packard 1100 series liquid chromatography system with photodiode array detector and a Supelcosil Si column with dioxane/hexane gradient elution. The flow rate was 1 ml/min, and the detection wavelengths were 318 nm (11-*cis*-ROL and -retinyl esters), 325 nm (all-*trans*-ROL and -RE), 340 nm (all retinoids), and 365 nm (retinaldehydes). In addition, spectral data (450 to 210 nm) for all peaks were acquired. Sample peaks were identified spectrally and confirmed by coelution with authentic retinoid standards. Quantitation of sample peaks was performed by area-unit versus concentration-slope coefficients, determined with the authentic retinoid standards immediately before sample analysis.

Phospholipid Analysis

Phospholipids were extracted from rod outer segments, the rest of the retina, and RPE by the method of Bligh and Dyer (1959). The major phospholipid classes were separated on a Microsorb Si column with the mobile phase hexane/2-propanol/ethanol/25 mM potassium phosphate (pH 7.0)/acetic acid (485:376:100:56:0.275, v/v) flowing at 1 ml/min and detected at wavelength 205 nm. Identification of the phospholipids extracted from outer segments, the rest of the retina, and RPE was achieved by comparison of the sample retention times to those of the authentic standards.

Characterization of Fatty Acid Molecular Species

Fatty acids were prepared from retinal phospholipids, derivatized to form phenacyl esters (FAPEs), and analyzed by reverse-phase HPLC, as previously described (Wood and Lee, 1983; Chen and Anderson, 1992). FAPEs were also prepared from standard free fatty acids for sample peak identification.

Analysis of A2-E and iso-A2-E

Extraction of A2-E and iso-A2-E from RPE tissue was performed using a previously described procedure (Reinboth et al., 1997) and analyzed by HPLC using the same conditions as for phospholipids, with a detection wavelength of 430 nm. Spectral data for all eluted peaks were acquired from 200 to 600 nm. An authentic A2-E standard was synthesized (Parish et al., 1998) and used to identify and quantitate A2-E in tissue sample extracts. iso-A2-E was generated by exposure of A2-E to room light and purified by HPLC. Confirmation of synthetic and endogenous A2-E was achieved by fast-atom bombardment mass spectrometry and UV-vis spectroscopy.

Identification of N-Retinyldene-PE

N-retinyldene-PE isoforms were extracted from outer segments and analyzed by HPLC under similar conditions to the phospholipids. Detection wavelengths of 205 nm for phospholipids, 360 nm for unprotonated N-retinyldene-PE, and 450 nm for protonated N-retinyldene-PE were used. To confirm the presence of a Schiff base in the N-retinyldene-PE fractions, eluted peaks were treated with concentrated HCl or 2% KOH in methanol and the samples reanalyzed by HPLC.

Electron Microscopic Analysis

Eyes dissected from 44-week-old wild-type (129 strain) and *abcr*^{-/-} (129 $\times$ B6 hybrid strain) mice were fixed overnight at 4°C in 0.1 M cacodylate buffer (pH 7.4) containing 2.5% formaldehyde, 2.5% glutaraldehyde, and 0.5% picric acid. The fixed tissues were treated with 1% osmium tetroxide and 1.5% potassium ferrocyanide for 1 hr and stained en bloc with 1% aqueous uranyl acetate for 1 hr. The specimens were then dehydrated in ethanol and embedded in

Spurr resin. Eighty nanometer sections were cut on a Reichert Ultratrac S ultramicrotome, collected on Formvar coated copper oval slot grids, and stained with uranyl acetate and lead citrate. The sections were examined using a JEOL 1200EX II Transmission Electron Microscope at 80 kV.

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References

- Alexander, K.R., and Fishman, G.A. (1984). Prolonged rod dark adaptation in retinitis pigmentosa. *Br. J. Ophthalmol.* **68**, 561–569.
- Allikmets, R., Singh, N., Sun, H., Shroyer, N.F., Hutchinson, A., Chidambaram, A., Gerrard, B., Baird, L., Stauffer, D., Peiffer, A., et al. (1997a). A photoreceptor cell-specific ATP-binding transporter gene (*ABCR*) is mutated in recessive Stargardt macular dystrophy. *Nat. Genet.* **15**, 236–246.
- Allikmets, R., Shroyer, N.F., Singh, N., Seddon, J.M., Lewis, R.A., Bernstein, P.S., Peiffer, A., Zabriskie, N.A., Li, Y., Hutchinson, A., et al. (1997b). Mutation of the Stargardt disease gene (*ABCR*) in age-related macular degeneration. *Science* **277**, 1805–1807.
- Anderson, R.E., and Maude, M.B. (1970). Phospholipids of bovine outer segments. *Biochemistry* **9**, 3624–3628.
- Azarian, S.M., and Travis, G.H. (1997). The photoreceptor rim protein is an ABC transporter encoded by the gene for recessive Stargardt's disease (*ABCR*). *FEBS Lett.* **409**, 247–252.
- Azarian, S.M., Megarity, C.F., Weng, J., Horvath, D.H., and Travis, G.H. (1998). The human photoreceptor rim protein gene (*ABCR*)—genomic structure and primer set information for mutation analysis. *Hum. Genet.* **102**, 699–705.
- Bergsma, D.R., Wiggert, B.N., Funahashi, M., Kuwabara, T., and Chader, C.J. (1977). Vitamin A receptors in normal and dystrophic human retina. *Nature* **265**, 66–67.
- Birch, D.G., and Hood, D.C. (1995). Abnormal rod photoreceptor function in retinitis pigmentosa. In *Degenerative Diseases of the Retina*, R. Anderson, J. Hollyfield, and M. LaVail, eds. (New York: Plenum), pp. 359–369.
- Birnbach, C.D., Jarvelainen, M., Possin, D.E., and Milam, A.H. (1994). Histopathology and immunocytochemistry of the neurosensory retina in fundus flavimaculatus. *Ophthalmology* **101**, 1211–1219.
- Bligh, D.G., and Dyer, W.J. (1959). A rapid method for total lipid extraction and purification. *Can. J. Biochem. Physiol.* **37**, 911–917.
- Carter-Dawson, L.D., and LaVail, M.M. (1979). Rods and cones in the mouse retina. I. Structural analysis using light and electron microscopy. *J. Comp. Neurol.* **188**, 245–262.
- Chen, H., and Anderson, R.E. (1992). Quantitation of phenacyl esters of retinal fatty acids by high-performance liquid chromatography. *J. Chromatogr.* **578**, 124–129.
- Cremers, F.P., van de Pol, D.J., van Driel, M., den Hollander, A.I., van Haren, F.J., Knoers, N.V., Tijmes, N., Bergen, A.A., Rohrschneider, K., Blankenagel, A., et al. (1998). Autosomal recessive retinitis pigmentosa and cone-rod dystrophy caused by splice site mutations in the Stargardt's disease gene *ABCR*. *Hum. Mol. Genet.* **7**, 355–362.
- Daemen, F.J., and Bonting, S.L. (1969). Internal protonation in retinylidene phosphatidylethanolamine and the red-shift in rhodopsin. *Nature* **222**, 879–881.
- De Laey, J.J., and Verougstraete, C. (1995). Hyperlipofuscinosis and subretinal fibrosis in Stargardt's disease. *Retina* **15**, 399–406.
- Decottignies, A., Grant, A.M., Nichols, J.W., de Wet, H., McIntosh, D.B., and Goffeau, A. (1998). ATPase and multidrug transport activities of the overexpressed yeast ABC protein Yor1p. *J. Biol. Chem.* **273**, 12612–12622.
- Dogra, S., Krishnamurthy, S., Gupta, V., Dixit, B.L., Gupta, C.M., Sanglard, D., and Prasad, R. (1999). Asymmetric distribution of phosphatidylethanolamine in C-albicans: possible mediation by CDR1, a multidrug transporter belonging to ATP binding cassette (ABC) superfamily. *Yeast* **15**, 111–121.
- Eagle, R.C., Jr., Lucier, A.C., Bernardino, V.B., Jr., and Yanoff, M. (1980). Retinal pigment epithelial abnormalities in fundus flavimaculatus: a light and electron microscopic study. *Ophthalmology* **87**, 1189–1200.
- Eldred, G.E. (1995). Lipofuscin fluorophore inhibits lysosomal protein degradation and may cause early stages of macular degeneration. *Gerontology* **41**, 15–28.
- Eldred, G.E., and Katz, M.L. (1988). Fluorophores of the human retinal pigment epithelium: separation and spectral characterization. *Exp. Eye Res.* **47**, 71–86.
- Eldred, G.E., and Lasky, M.R. (1993). Retinal age pigments generated by self-assembling lysosomotropic detergents. *Nature* **361**, 724–726.
- Fain, G.L., Matthews, H.R., and Cornwall, M.C. (1996). Dark adaptation in vertebrate photoreceptors. *Trends Neurosci.* **19**, 502–507.
- Feeney-Burns, L., and Eldred, G.E. (1983). The fate of the phagosome: conversion to "age pigment" and impact in human retinal pigment epithelium. *Trans. Ophthalmol. Soc. U. K.* **103**, 416–421.
- Fishman, G.A., Farbman, J.S., and Alexander, K.R. (1991). Delayed rod dark adaptation in patients with Stargardt's disease. *Ophthalmology* **98**, 957–962.
- Fishman, G.A., Pulluru, P., Alexander, K.R., Derlacki, D.J., and Gilbert, L.D. (1994). Prolonged rod dark adaptation in patients with cone-rod dystrophy. *Am. J. Ophthalmol.* **118**, 362–367.
- Fliesler, S.J., and Anderson, R.E. (1983). Chemistry and metabolism of lipids in the vertebrate retina. *Prog. Lipid Res.* **22**, 79–131.
- Granit, R. (1947). *Sensory Mechanism of the Retina* (London: Oxford University Press).
- Groenendijk, G.W., De Grip, W.J., and Daemen, F.J. (1984). Rod outer segment membranes: good acceptors for retinoids? *Vision Res.* **24**, 1623–1627.
- Holz, F.G., Schutt, F., Kopitz, J., Eldred, G.E., Kruse, F.E., Volcker, H.E., and Cantz, M. (1999). Inhibition of lysosomal degradative functions in RPE cells by a retinoid component of lipofuscin. *Invest. Ophthalmol. Vis. Sci.* **40**, 737–743.
- Hood, D.C., and Birch, D.G. (1993). Human cone receptor activity: the leading edge of the a-wave and models of receptor activity. *Vis. Neurosci.* **10**, 857–871.
- Hubbard, R., Brown, P.K., and Bownds, D. (1971). Methodology of vitamin A and visual pigments. *Methods Enzymol.* **18C**, 615–653.
- Illing, M., Molday, L.L., and Molday, R.S. (1997). The 220-kDa rim protein of retinal rod outer segments is a member of the ABC transporter superfamily. *J. Biol. Chem.* **272**, 10303–10310.
- Jager, S., Palczewski, K., and Hofmann, K.P. (1996). Opsin/all-trans-retinal complex activates transducin by different mechanisms than photolyzed rhodopsin. *Biochemistry* **35**, 2901–2908.
- Jin, J., Jones, G.J., and Cornwall, M.C. (1994). Movement of retinal animal cone and rod photoreceptors. *Vis. Neurosci.* **11**, 389–399. Erratum: *Vis. Neurosci.* **17**(5), 1994.
- Jonas, J.B., Schneider, U., and Naumann, G.O. (1992). Count and density of human retinal photoreceptors. *Graefes Arch. Clin. Exp. Ophthalmol.* **230**, 505–510.
- Kedzierski, W., Bok, D., and Travis, G.H. (1999). Transgenic analysis of rds/peripherin N-glycosylation: effect on dimerization, interaction with rom1, and rescue of the rds null-phenotype. *J. Neurochem.* **72**, 430–438.
- Kliffen, M., van der Schaft, T.L., Mooy, C.M., and de Jong, P.T. (1997). Morphologic changes in age-related maculopathy. *Microsc. Res. Tech.* **36**, 106–122.
- Kolb, H., and Gouras, P. (1974). Electron microscopic observations

- of human retinitis pigmentosa, dominantly inherited. Invest. Ophthalmol. 13, 487-498.
- Lee, B.L., and Heckenlively, J.R. (1999). Stargardt's disease and fundus flavimaculatus. In Retina-Vitreous-Macula, D.R. Guyer, L.A. Yannuzzi, S. Chang, J.A. Shields, and W.R. Green, eds. (Philadelphia: W.B. Saunders), pp. 978-988.
- Leibrock, C.S., Reuter, T., and Lamb, T.D. (1998). Molecular basis of dark adaptation in rod photoreceptors. Eye 12, 511-520.
- Lewis, R.A., Shroyer, N.F., Singh, N., Allikmets, R., Hutchinson, A., Li, Y., Lupski, J.R., Leppert, M., and Dean, M. (1999). Genotype/phenotype analysis of a photoreceptor-specific ATP-binding cassette transporter gene, *ABCR*, in Stargardt disease. Am. J. Hum. Genet. 64, 422-434.
- Lyubarsky, A.L., and Pugh, E.N., Jr. (1996). Recovery phase of the murine rod photoresponse reconstructed from electroretinographic recordings. J. Neurosci. 16, 563-571.
- Martinez-Mir, A., Paloma, E., Allikmets, R., Ayuso, C., Delrio, T., Dean, M., Vilageliu, L., Gonzalezduarte, R., and Balcells, S. (1998). Retinitis pigmentosa caused by a homozygous mutation in the Stargardt-disease gene *ABCR*. Nat. Genet. 18, 11-12.
- Melia, T.J., Jr., Cowan, C.W., Angleson, J.K., and Wensel, T.G. (1997). A comparison of the efficiency of G protein activation by ligand-free and light-activated forms of rhodopsin. Biophys. J. 73, 3182-3191.
- Miljanich, G.P., Nemes, P.P., White, D.L., and Dratz, E.A. (1981). The asymmetric transmembrane distribution of phosphatidylethanolamine, phosphatidylserine, and fatty acids of the bovine retinal rod outer segment disk membrane. J. Membr. Biol. 60, 249-255.
- Nakatani, K., Tamura, T., and Yau, K.W. (1991). Light adaptation in retinal rods of the rabbit and two other nonprimate mammals. J. Gen. Physiol. 97, 413-435.
- Papermaster, D.S., Schneider, B.G., Zorn, M.A., and Kraehenbuhl, J.P. (1978). Immunocytochemical localization of a large intrinsic membrane protein to the incisures and margins of frog rod outer segment disks. J. Cell Biol. 78, 415-425.
- Parish, C.A., Hashimoto, M., Nakanishi, K., Dillon, J., and Sparrow, J. (1998). Isolation and one-step preparation of A2E and iso-A2E, fluorophores from human retinal pigment epithelium. Proc. Natl. Acad. Sci. USA 95, 14609-14613.
- Pepperberg, D.R., Birch, D.G., and Hood, D.C. (1997). Photosponses of human rods *in vivo* derived from paired-flash electroretinograms. Vis. Neurosci. 14, 73-82.
- Poincelot, R.P., Millar, P.G., Kimbel, R.L., Jr., and Abrahamson, E.W. (1969). Lipid to protein chromophore transfer in the photolysis of visual pigments. Nature 221, 256-257.
- Reinboth, J.J., Gautschi, K., Munz, K., Eldred, G.E., and Reme, C.E. (1997). Lipofuscin in the retina: quantitative assay for an unprecedented autofluorescent compound (pyridinium bis-retinoid, A2-E) of ocular age pigment. Exp. Eye Res. 65, 639-643.
- Rozet, J.M., Gerber, S., Souied, E., Perrault, I., Chatelin, S., Ghazi, I., Leowski, C., Dufier, J.L., Munnich, A., and Kaplan, J. (1998). Spectrum of *abcr* gene mutations in autosomal recessive macular dystrophies. Eur. J. Hum. Genet. 6, 291-295.
- Saari, J.C., Garwin, G.G., Van Hooser, J.P., and Palczewski, K. (1998). Reduction of all-trans-retinal limits regeneration of visual pigment in mice. Vision Res. 38, 1325-1333.
- Smit, J.J., Schinkel, A.H., Oude Elferink, R.P., Groen, A.K., Wagenaar, E., van Deemter, L., Mol, C.A., Ottenhoff, R., van der Lugt, N.M., and van Roon, M.A. (1993). Homozygous disruption of the murine *mdr2* P-glycoprotein gene leads to a complete absence of phospholipid from bile and to liver disease. Cell 75, 451-462.
- Stargardt, K. (1909). Über familiäre, progressive Degeneration in der Maculagegend des Auges. Graefes Arch. Ophthalmol. 71, 534-550.
- Steinberg, R.H. (1985). Interactions between the retinal pigment epithelium and the neural retina. Doc. Ophthalmol. 60, 327-346.
- Steinmetz, R.L., Garner, A., Maguire, J.I., and Bird, A.C. (1991). Histopathology of incipient fundus flavimaculatus. Ophthalmology 98, 953-956.
- Steinmetz, R.L., Haimovici, R., Jubb, C., Fitzke, F.W., and Bird, A.C. (1993). Symptomatic abnormalities of dark adaptation in patients with age-related Bruch's membrane change. Br. J. Ophthalmol. 77, 549-554.
- Stone, E.M., Webster, A.R., Vandenberg, K., Streb, L.M., Hockey, R.R., Lotery, A.J., and Sheffield, V.C. (1998). Allelic variation in *abcr* associated with Stargardt-disease but not age-related macular degeneration. Nat. Genet. 20, 328-329.
- Sun, H., and Nathans, J. (1997). Stargardt's ABCR is localized to the disc membrane of retinal rod outer segments. Nat. Genet. 17, 15-16.
- Sun, H., Molday, R.S., and Nathans, J. (1999). Retinal stimulates ATP hydrolysis by purified reconstituted ABCR, the photoreceptor-specific ATP-binding cassette transporter responsible for Stargardt disease. J. Biol. Chem. 274, 8269-8281.
- Surya, A., Foster, K.W., and Knox, B.E. (1995). Transducin activation by the bovine opsin apoprotein. J. Biol. Chem. 270, 5024-5031.
- Suzuki, T., Fujita, Y., Noda, Y., and Miyata, S. (1986). A simple procedure for the extraction of the native chromophore of visual pigments: the formaldehyde method. Vision Res. 26, 425-429.
- Tamura, T., Nakatani, K., and Yau, K.W. (1989). Light adaptation in cat retinal rods. Science 245, 755-758.
- Travis, G. (1998). Mechanisms of cell death in the inherited retinal degenerations. Am. J. Hum. Genet. 62, 503-508.
- Tsang, S.H., Burns, M.E., Calvert, P.D., Gouras, P., Baylor, D.A., Goff, S.P., and Arshavsky, V.Y. (1998). Role for the target enzyme in deactivation of photoreceptor G protein *in vivo*. Science 282, 117-121.
- Wing, G.L., Blanchard, G.C., and Weiter, J.J. (1978). The topography and age relationship of lipofuscin concentration in the retinal pigment epithelium. Invest. Ophthalmol. Vis. Sci. 17, 601-607.
- Wood, R., and Lee, T. (1983). High performance liquid chromatography of fatty acids: quantitative analysis of saturated monoenoic, polyenoic, and geometrical isomers. J. Chromatogr. 254, 237-246.
- Wu, G., and Hubbell, W.L. (1993). Phospholipid asymmetry and transmembrane diffusion in photoreceptor disc membranes. Biochemistry 32, 879-888.
- Zhou, Z., White, K.A., Polissi, A., Georgopoulos, C., and Raetz, C.R. (1998). Function of *Escherichia coli* MsbA, an essential ABC family transporter, in lipid A and phospholipid biosynthesis. J. Biol. Chem. 273, 12466-12475.