ATP-binding cassette transporter ABCA4 and chemical isomerization protect photoreceptor cells from the toxic accumulation of excess 11-*cis*-retinal

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The visual cycle is a series of enzyme-catalyzed reactions which converts all-*trans*-retinal to 11-*cis*-retinal for the regeneration of visual pigments in rod and cone photoreceptor cells. Although essential for vision, 11-*cis*-retinal like all-*trans*-retinal is highly toxic due to its highly reactive aldehyde group and has to be detoxified by either reduction to retinol or sequestration within retinal-binding proteins. Previous studies have focused on the role of the ATP-binding cassette transporter ABCA4 associated with Stargardt macular degeneration and retinol dehydrogenases (RDH) in the clearance of all-*trans*-retinal from photoreceptors following photoexcitation. How rod and cone cells prevent the accumulation of 11-*cis*-retinal in photoreceptor disk membranes in excess of what is required for visual pigment regeneration is not known. Here we show that ABCA4 can transport *N*-11-*cis*-retinylidene-phosphatidylethanolamine (PE), the Schiff-base conjugate of 11-*cis*-retinal and PE, from the lumen to the cytoplasmic leaflet of disk membranes. This transport function together with chemical isomerization to its all-*trans* isomer and reduction to all-*trans*-retinol by RDH can prevent the accumulation of excess 11-*cis*-retinal and its Schiff-base conjugate and the formation of toxic bisretinoid compounds as found in ABCA4-deficient mice and individuals with Stargardt macular degeneration. This segment of the visual cycle in which excess 11-*cis*-retinal is converted to all-*trans*-retinol provides a rationale for the unusually high content of PE and its long-chain unsaturated docosahexaenoyl group in photoreceptor membranes and adds insight into the molecular mechanisms responsible for Stargardt macular degeneration.

ABC transporters | Stargardt disease | retinoids | photoreceptor degeneration | retinal pigment epithelial cells

The visual cycle plays a crucial role in the removal of all-*trans*-retinal from photoreceptor cells following photoexcitation and its conversion to 11-*cis*-retinal for the regeneration of visual pigments in rod and cone photoreceptor cells (1). Deficient clearance of all-*trans*-retinal and its Schiff-base conjugate *N*-retinylidene-phosphatidylethanolamine (PE) from rod and cone photoreceptor outer segments results in condensation reactions which produce a mixture of bisretinoid products including the pyridinium bisretinoid compound A2PE and its hydrolytic product A2E (2, 3). These bisretinoid compounds accumulate as lipofuscin deposits in retinal pigment epithelial (RPE) cells upon phagocytosis of outer segments and have been implicated in the pathology of a number of retinal degenerative diseases. This is particularly evident in autosomal recessive Stargardt macular degeneration in which mutations in the ATP-binding cassette (ABC) transporter ABCA4 which impair the *N*-retinylidene-PE transport activity of ABCA4 cause a buildup of lipofuscin, atrophy of the central retina, and severe progressive loss in vision (4–8).

In initial studies, *Abca4* knockout mice were reported to show a light-dependent accumulation of all-*trans*-retinal, PE, and *N*-retinylidene-PE in photoreceptors and lipofuscin and A2E in RPE (9). This led to a model in which ABCA4 functions as a lipid transporter flipping the all-*trans* isomer of *N*-retinylidene-PE from the lumen to the cytoplasmic leaflet of photoreceptor

disk membranes to facilitate the removal of all-*trans*-retinal from disk membranes of rod and cone photoreceptor cells following photoexcitation. Recent studies have confirmed that ABCA4 can flip the all-*trans* isomer of *N*-retinylidene-PE and PE from the lumen to the cytoplasmic leaflet of membranes (6, 10). However, the light-dependent accumulation of lipofuscin and A2E in *Abca4* knockout mice has been recently challenged. Boyer et al. (11) have shown that the levels and rates of increase in lipofuscin, including the lipofuscin fluorophore A2E, were similar in dark-reared and cyclic light-reared *Abca4* knockout mice. These results and experiments showing that the addition of 11-*cis*-retinal to metabolically compromised photoreceptors results in the accumulation of lipofuscin-like fluorophores have led to the suggestion that ABCA4 may play a more important role in the removal of 11-*cis*-retinal from photoreceptor outer segments although the mechanism remains to be determined (11).

In this study we show that ABCA4 can transport the 11-*cis* isomer of *N*-retinylidene-PE from the lumen to the cytoplasmic leaflet of membranes. This transport activity together with the chemical isomerization of *N*-11-*cis*-retinylidene-PE to *N*-all-*trans*-retinylidene-PE and reduction of all-*trans*-retinal to all-*trans*-retinol can remove excess 11-*cis*-retinal from disk membranes through the visual cycle, thereby preventing the buildup of potentially toxic retinal and its bisretinoid condensation products. The high content of PE in disk membranes together with the long-chain docosahexaenoyl acid (DHA) group play crucial roles in the trapping of 11-*cis*-retinal and enhancing the rate of chemical isomerization of *N*-11-*cis*-retinylidene-PE to its all-*trans* isomer.

Significance

ABCA4 is an ATP-binding cassette transporter encoded by the gene responsible for Stargardt macular degeneration, an inherited retinopathy associated with severe vision loss. Previous studies have shown that ABCA4 facilitates the clearance of all-*trans*-retinal from photoreceptor disc membranes following photoexcitation. More recent studies implicate excess 11-*cis*-retinal in the etiology of Stargardt disease. In this study we show the ABCA4 can transport the *N*-11-*cis*-retinylidene-phosphatidylethanolamine (PE), the Schiff-base adduct of 11-*cis*-retinal and PE, across membranes. This transport activity together with chemical isomerization of *N*-11-*cis*-retinylidene-PE to its all-*trans* isomer and reduction to all-*trans*-retinol can prevent the accumulation of excess 11-*cis*-retinal and potentially toxic bisretinoid compounds implicated in Stargardt and related retinal degenerative diseases.

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Fig. 1. ATP-dependent retinoid transport activity from proteoliposomes reconstituted with ABCA4 to acceptor DOPE/DOPC vesicles. (A) [3 H]-labeled 11-*cis*-retinal transfer with the addition (arrow) of 1 mM ATP ($\diamond$), AMP-PNP ($\square$), or no addition (*); (B) [3 H]-labeled all-*trans*-retinal with the addition of 1 mM ATP ($\blacktriangle$), AMP-PNP ($\square$), or no addition (*). (C) ATP-dependent retinal transfer activity as a function of 11-*cis*-retinal ($\circ$) and all-*trans*-retinal ($\blacktriangle$). Solid line shows best-fit sigmoidal curve for 11-*cis*-retinal ($K_{0.5}$ of $6.1 \pm 0.4 \mu\text{M}$ and a Hill coefficient of 1.6 ± 0.3) and for all-*trans*-retinal ($K_{0.5}$ of $6.9 \pm 0.4 \mu\text{M}$ and a Hill coefficient of 1.7 ± 0.3). Data are the mean $\pm$ SD for three independent experiments.

Results

ABCA4 Transports both the 11-*cis* and all-*trans* Isomers of *N*-retinylidene-PE. To explore the role of ABCA4 in the clearance of 11-*cis*-retinal from photoreceptors, we first determined if ABCA4 can transport the 11-*cis* isomer of *N*-retinylidene-PE across membranes. This was measured using a biochemical assay which quantifies the ATP-dependent transfer of radiolabeled 11-*cis*-retinal from donor dioleoylphosphatidylethanolamine (DOPE)/dioleoylphosphatidylcholine (DOPC) proteoliposomes reconstituted with ABCA4 to acceptor liposomes, a reaction driven by the ATP-dependent transport or flipping of *N*-11-*cis*-retinylidene-PE across the proteoliposome membrane by ABCA4 (6). The resulting higher content of *N*-11-*cis*-retinylidene-PE on the outer leaflet of the proteoliposome, i.e., the side containing the ATP accessible nucleotide-binding domains of ABCA4, together with dissociation into 11-*cis*-retinal and PE drives the transfer of 11-*cis*-retinal to acceptor vesicles. The ATP-dependent accumulation of 11-*cis*-retinal together with 11-*cis*-retinylidene-PE in acceptor liposomes was similar to that of all-*trans*-retinal isomer indicating that ABCA4 can actively transport both isomers of *N*-retinylidene-PE in an import direction, i.e., the flipping of substrate from the lumen to cytoplasmic leaflet of biological membranes (Fig. 1 *A* and *B*). Both isomers displayed similar sigmoidal curves when the rate of transfer was measured as a function of retinal concentration (Fig. 1C). *N*-11-*cis*-retinylidene-PE exhibited a $K_{0.5}$ of $6.1 \pm 0.4 \mu\text{M}$ and a Hill coefficient of 1.6 ± 0.3 , and the all-*trans* isomer had a $K_{0.5}$ of $6.9 \pm 0.4 \mu\text{M}$ and a Hill coefficient of 1.7 ± 0.3 with the V_{max} for the 11-*cis* isomer modestly higher. Because ABCA4 exists as a monomer (12), the sigmoidal curves imply cooperative transport of two retinoid substrates as part of the mechanism.

Effect of 11-*cis* and all-*trans* Isomers of *N*-retinylidene-PE on the ATPase Activity of ABCA4. Retinal and PE react reversibly to form *N*-retinylidene-PE which binds to ABCA4 and activates its ATPase activity (5, 13, 14). We have further investigated the effect of the 11-*cis* and all-*trans* isomers on the ATPase activity of ABCA4 (Fig. 24). ABCA4 reconstituted into DOPE/DOPC

liposomes had a basal ATPase activity which was stimulated threefold by the 11-*cis*-retinal isomer and twofold by the all-*trans* isomer. Both isomers displayed Michaelis–Menten kinetics with similar apparent K_m values ($\sim 0.23 \pm 0.02$ mM) for ATP in the presence of 40 μ M retinal and showed a similar dependence of ATPase activity on retinoid concentration (Fig. 2B).

We further investigated the possible role of 11-*cis*-retinal in regulating the activity of ABCA4 because it had been reported previously that 11-*cis*-retinal binds with high affinity to the bacterial expressed nucleotide-binding domain 1 (NBD1) of ABCA4 (15). A modest increase in retinal-stimulated ATPase activity was observed at higher (>20 μ M) 11-*cis*-retinal concentrations in the presence of 40 μ M all-*trans*-retinal (Fig. 2C). This increase in activity, however, can be attributed to the enhanced ATPase activation of ABCA4 by the 11-*cis* isomer together with the all-*trans* isomer.

Chemical Isomerization of *N*-11-*cis*-retinylidene-PE to *N*-all-*trans*-retinylidene-PE. Although ABCA4 can flip *N*-11-*cis*-retinylidene-PE from the luminal to the cytoplasmic leaflet of disk membranes, this function alone cannot efficiently remove excess 11-*cis*-retinal from disk membranes because 11-*cis*-retinal is a poor substrate for retinol dehydrogenase (RDH) 8, the only known retinol dehydrogenase in photoreceptor disk membranes (16, 17). However, the 11-*cis* isomer of *N*-retinylidene-PE has been previously reported to undergo chemical isomerization in the dark, although the physiological significance of this reaction has been unclear (18). We further explored this reaction by adding 11-*cis*-retinal to rod outer segment (ROS) vesicles in the dark and measuring its conversion to other retinal isomers by HPLC after reaction with hydroxylamine to form their stable retinoyloxime derivatives (Fig. 3A). Initially, only the syn- and anti-configurations of 11-*cis*-retinaloximes were observed as predominant components. However, after 30 min at 37 °C, ~50% of the 11-*cis*-retinal isomer in ROS was converted to all-*trans*- and 13-*cis*-retinal isomers at a ratio of about 4:1. The depletion of 11-*cis*-retinal occurred with a half-time of 30 min (Fig. 3B). A similar time

Fig. 2. Effect of 11-*cis*- and all-*trans*-retinal on the ATPase activity of reconstituted ABCA4. (A) ATPase activity as a function of ATP. Basal activity (○) and retinal-stimulated activity for the addition of 50 μM all-*trans*-retinal (▲) and 50 μM 11-*cis*-retinal (◇) concentration. (B) ATPase activity of ABCA4 as a function of all-*trans*-retinal (▲) and 11-*cis*-retinal (◇). (C) The effect of increasing 11-*cis*-retinal on the ATPase activity of ABCA4 reconstituted into DOPC/DOPE (PC/PE) or DOPC (PC) vesicles in the presence of 40 μM all-*trans*-retinal. Data are the mean ± SD for three independent experiments.

course was observed when 11-*cis*-retinal was added to liposomes prepared from ROS lipids, indicating that the isomerization reaction was not mediated by proteins. A small amount of 13-*cis*-retinal was also observed when ROS was treated with all-*trans*-retinal (Fig. S14).

To further investigate the role of specific phospholipids in the isomerization reaction, we added 11-*cis*-retinal to liposomes consisting of synthetic DOPC or a DOPE/DOPC mixture at a ratio of 4:6 reflecting the 40% PE content of ROS lipids (19). No

significant isomerization was observed in DOPC liposomes whereas isomerization was observed in DOPE/DOPC liposomes with 50% conversion occurring at 4 h (Fig. 3C and Fig. S1B). This supports previous results indicating that Schiff-base formation between the aldehyde group of 11-*cis*-retinal and the primary amine group of PE is required for isomerization (18). We rationalized that the faster rate of isomerization observed in ROS lipid vesicles relative to DOPE/DOPC vesicles may arise in part from the high content of DHA acyl side chains present in PE of ROS (19). This was investigated by determining the rate of 11-*cis*-retinal isomerization in liposomes consisting of 40% 1-stearoyl-2-docosahexanoylethanolamine (SDPE) and 60% DOPC. Isomerization was considerably faster in SDPE/DOPC vesicles than DOPE/DOPC vesicles with a half-time of 50 min. This indicates that the DHA group significantly enhances the rate of isomerization (Fig. 3C). Other phospholipids may further contribute to the faster rate of *N*-11-*cis*-retinylidene-PE isomerization observed for ROS lipids. This chemical isomerization reaction together with the reversible dissociation of *N*-all-*trans*-retinylidene-PE to all-*trans*-retinal and PE enables all-*trans*-retinal to be reduced to all-*trans*-retinol by RDH8 for reentry into the visual cycle.

Rate of Formation of A2PE in ROS. The loss in retinoid transport activity of ABCA4 as found in *Abca4* knockout mice and mutations responsible for Stargardt disease results in an accumulation of retinal and *N*-retinylidene-PE which initiates a series of reactions that produce bisretinoid compounds including A2PE in photoreceptors and its hydrolysis product A2E in RPE cells (3, 20) (Fig. 4A). For the ABCA4-mediated transport of the *N*-11-*cis*-retinylidene-PE across membranes and *cis*-to-*trans* chemical isomerization to play an important role in the clearance of excess 11-*cis*-retinal from disk membranes, these reactions must be significantly faster than the formation of bisretinoids. We have measured the production of A2PE and other bisretinoids after the addition of excess 11-*cis*- and all-*trans*-retinal to ROS depleted of ATP and NADPH required for ABCA4 and RDH8 activity, respectively (Fig. 4B and Fig. S2). Because A2PE is highly heterogeneous owing to the fatty acyl groups of PE, A2PE was converted to A2E by phospholipase D before analysis by HPLC. A2PE formation from 11-*cis*- and all-*trans*-retinal was similar and considerably slower than ABCA4-mediated transport and isomerization of *N*-11-*cis*-retinylidene-PE, occurring over several days (Fig. 4C). A2PE and retinal dimer formation (21, 22) was also observed when 11-*cis*- or all-*trans*-retinal was added to liposomes consisting of synthetic lipids (Fig. S2B and C).

Discussion

The present study provides a mechanism by which rod and cone photoreceptors prevent the accumulation of potentially toxic 11-*cis*-retinal in excess of that required for the regeneration of visual pigments in rod and cone photoreceptors (Fig. 5). The 11-*cis*-retinal enters the photoreceptor outer segments from the visual cycle and covalently binds to the lysine in the binding pocket of opsin (Lys296 for rhodopsin) to regenerate the visual pigment. Excess 11-*cis*-retinal reversibly reacts with the PE to form *N*-11-*cis*-retinylidene-PE, a portion of which is trapped on the lumen/extracellular leaflet of rod or cone disk membranes. The high content of PE in photoreceptor disk membranes (40% of the total phospholipid) serves as a sink to prevent significant diffusion of 11-*cis*-retinal from disk membranes. ABCA4 expressed in both rod and cone photoreceptors (23) actively transports *N*-11-*cis*-retinylidene-PE across the disk membrane ensuring that at any given time there is a high content of 11-*cis*-retinal isomer on the cytoplasmic leaflet of the disk membrane. *N*-11-*cis*-retinylidene-PE undergoes chemical isomerization to its all-*trans* isomer which can also be transported by ABCA4. All-*trans*-retinal generated by mass action is then reduced to all-*trans*-retinol by RDH8 for reentry into the

Fig. 3. Isomerization of *N*-11-*cis*-retinylidene-PE to its all-*trans* isomer. (A) HPLC chromatograms showing the production of all-*trans*-retinal and 13-*cis*-retinal after the addition of 100 μ M 11-*cis*-retinal to isolated ROS containing 5 μ M unbleached rhodopsin at 37 $^{\circ}$ C after 0 min (Upper trace) and 30 min (Lower trace). Retinoids were converted to their stable retinylloxime derivatives for absorbance (Abs) at 360 nm. (B and C) Decrease in 11-*cis*-retinal as a function of time. (B) The 11-*cis*-retinal was added to either ROS disk membranes or lipids extracted from ROS. The curves were fitted as a single exponential with $t_{1/2} = 28.5 \pm 2.3$ min for ROS disk and $t_{1/2} = 34.8 \pm 3.5$ min for ROS lipid. (C) The 11-*cis*-retinal was added to DOPC (PC), 40% DOPE/60% DOPC (DOPE/PC), or 40% SDPE/60% DOPC (SDPE/PC) vesicles. The curves were fitted as a single exponential with a $t_{1/2} = 56 \pm 5$ min for SDPE/PC and $t_{1/2} = 223 \pm 13$ min for DOPE/PC. SDPE contains a DHA group (22:6) at position 2 of PE. Data are the mean $\pm$ SD for three independent experiments.

PE following chemical isomerization and photoexcitation. ABCA4, also known as the Rim protein, is localized along the rim region of rod and cone outer segment disk membranes (25, 26). This localization is dictated by the exocytosomal domains (ECD) of ABCA4 (27). These domains are too large to fit within the luminal space between the flattened disk membranes but can fit in the space outlined by the rim region (12, 27, 28). It remains to be determined if the transport and clearance of 11-*cis*- and all-*trans*-retinal from photoreceptors is further facilitated by the rim localization of ABCA4.

The mechanism by which ABCA4 transport activity together with chemical isomerization removes excess 11-*cis*-retinal from photoreceptor outer segments provides a molecular rationale for the light-independent accumulation of A2E and lipofuscin recently observed in *Abca4* knockout mice (11). In the absence of ABCA4, 11-*cis*-retinal and its Schiff-base conjugate will accumulate in disk membranes leading to the formation of A2E and other bisretinoids in photoreceptors and lipofuscin deposits in RPE cells following phagocytosis of outer segments. The bisretinoids most likely form from all-*trans* as well as 11-*cis*-retinal isomers due to chemical isomerization of *N*-11-*cis*-retinylidene-PE which can precede bisretinoid formation. Whether the 11-*cis* isomer incorporated into the bisretinoids is stable or undergoes chemical isomerization remains to be determined. In the case of Stargardt disease, bisretinoids and lipofuscin would also form as a result from inefficient clearance of excess 11-*cis*-retinal in the dark.

To date, the transport activity of ABCA4 has not been shown to be regulated as studied here and in a previous report (29). It is possible that ABCA4 is constitutively active to ensure that 11-*cis*-retinal entering the photoreceptor does not accumulate in excess of that required for visual pigment regeneration. No other feedback mechanisms have been identified which contribute to the regulation of 11-*cis*-retinal levels in photoreceptors. If such mechanisms exist, they may work in concert with the ABCA4 transport-isomerization mechanism described here to prevent the accumulation of excess 11-*cis*-retinal in outer segment membranes under normal conditions.

Chemical isomerization of *N*-11-*cis*-retinylidene-PE provides a rationale for the high content of PE and DHA fatty acyl chains in photoreceptor outer segments. The high content of PE acts as a trap to prevent significant diffusion of 11-*cis*- and all-*trans*-retinal from disk membranes to other cells and cellular compartments. The high content of PE is not only required for the Schiff-base formation with retinal but also for the proposed isomerization reaction involving the nucleophile attack by a second PE (18). The DHA fatty acyl group on the PE further increases the rate of isomerization by reducing the activation energy of isomerization, the mechanism of which requires further study. Although the high content of DHA in the retina has been proposed to serve many roles including preservation of photoreceptor structure and function (30–32), a recent study has shown that dietary DHA supplementation also prevents the age-related accumulation of A2E in wild-type and *ELOVL4* transgenic mice that serve as a model of autosomal dominant Stargardt disease (STGD3). This is consistent with a role of DHA in facilitating the removal of excess 11-*cis*-retinal through enhancing the rate of *N*-11-*cis*-retinylidene-PE chemical isomerization.

Collectively, our studies indicate that ABCA4 together with chemical isomerization is important in clearing excess 11-*cis*-retinal from photoreceptors. Loss in function mutations in ABCA4 leads to the accumulation of 11-*cis*- and all-*trans*-retinal and their Schiff-base conjugates. These compounds can condense to form bisretinoid compounds, including A2E which accumulate in RPE cells as lipofuscin deposits. These bisretinoid compounds have been implicated in the degeneration of RPE and photoreceptor cells as evident in Stargardt disease and related retinal degenerative diseases.

Materials and Methods

Materials. Porcine brain polar lipid (BPL), 1,2 dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), 1,2 dioleoyl-sn-glycero-3-phosphocholine (DOPC), and cholesteryl hemisuccinate were purchased from Avanti Polar Lipids. Phospholipase D, ATP, and adenylylimidodiphosphate (AMP-PNP) were purchased from Sigma, CHAPS was from Anatrace, radiolabeled sodium borohydride (NaB^3H_4) was obtained from American Radiolabeled Chemicals, [α - ^{32}P] ATP was from Perkin-Elmer. Organic solvents were HPLC-grade. The 11-*cis*-retinal and all-*trans*-retinal were radiolabeled and stored under N_2 as described (33).

Purification of ABCA4 from ROS. The Rim3F4 monoclonal antibody conjugated to Sepharose 2B was used to isolate ABCA4 from CHAPS-solubilized bovine ROS membranes as described previously (14). Briefly, ROS membranes were solubilized in 50 mM Hepes, pH 8.0, 0.2 mg/mL BPL, 0.002% cholesteryl hemisuccinate, 150 mM NaCl, 1 mM MgCl_2 , 1 mM DTT containing 18 mM CHAPS and protease inhibitor and stirred for 45 min at 4 °C. The supernatant after a 10-min centrifugation at $100,000 \times g$ (TLA110.4 rotor in a Beckman Optima TL centrifuge) was mixed with 50 μL of Rho3F4-Sepharose 2B for 1 h at 4 °C. The matrix was washed six times in the same buffer but at a lower 10-mM CHAPS condition. The protein was eluted three times with 150 μL each at 12 °C over 60 min in the same buffer with 0.2 mg/mL 3F4 peptide. The concentrated eluted protein (20–70 ng of ABCA4/ μL) was promptly used for proteoliposome reconstitution.

Preparation of Acceptor Liposomes and Reconstitution of ABCA4 in Donor Proteoliposomes. Preparation of vesicles for transport was done as previously characterized (6). Briefly, for the preparation of donor and acceptor liposomes vesicles, the designated lipid compositions were prepared in chloroform and dried under N_2 . Donor proteoliposomes lipids DOPC/DOPE/BPL were mixed at a ratio of 6:2:2. The lipids were resuspended in buffer containing 20 mM Hepes pH 8.0, 150 mM NaCl, 2 mM MgCl_2 , 3.5 mM CHAPS at a concentration of 2.5 mg/mL by bath sonication and incubated at room temperature for 3 h. Both donor lipids and ABCA4 (in 10 mM CHAPS and DTT) were mixed to yield a final protein-to-lipid ratio of 1:100 (wt/wt). This condition was set by diluting to achieve a final CHAPS concentration of 6 mM at 4 °C for 1 h. Finally, detergent was removed by dialysis with a minimum of three 1-L changes of buffer containing 10 mM Hepes, pH 8.0, 150 mM NaCl, 2 mM MgCl_2 , and 1 mM DTT. For acceptor liposomes, DOPC and DOPE at a weight ratio of 7:3 were resuspended in 20 mM Hepes pH 8.0, 150 mM NaCl, 2 mM MgCl_2 , and 18 mM CHAPS with 300 mM sucrose, incubated at room temperature for 4 h, and dialyzed as above against 10 mM Hepes, pH 8.0, 300 mM sucrose, 2 mM MgCl_2 , and 1 mM DTT.

[^3H]-Retinal Transfer Assays and ATPase Activity. Transport activity was determined as described previously (6). ABCA proteoliposomes and acceptor liposomes were incubated in 100 μL reaction containing [^3H]-11-*cis*-retinal or [^3H]-all-*trans*-retinal (1.68 kBq and 3.36 GBq/mmol). ATP hydrolysis was measured using [α - ^{32}P] ATP (PerkinElmer Life Sciences) and thin-layer chromatography as described previously with minor modification (14, 34). Briefly, ATP was added in a concentration-dependent manner (while maintaining a final activity 0.2 μCi) or fixed at 1,000 μM [α - ^{32}P] ATP (final activity 0.2 μCi) for assaying retinal isomer stimulation. Retinal isomers were handled at room temperature under dark conditions and in glass test tubes. Appropriate dilution of retinal isomers prepared in ethanol were added to <0.05% of the volume of the ATPase reaction. Reactions in glass test tubes were carried out in 30 μL at 37 °C for 15 min with retinoid or buffer addition. In some assays, ABCA4 was reconstituted into DOPC/DOPE at a ratio of 6:4 by weight to measure any additive effect or synergistic effect of retinal isomer stimulation. The all-*trans*-retinal and 11-*cis*-retinal concentrations were determined under dark conditions in a spectrophotometer ($\epsilon_{380 \text{ nm}} = 42.88 \text{ mM}^{-1} \text{ cm}^{-1}$).

Extraction of Phospholipids from ROS Membranes. Phospholipids were extracted from ROS membranes as previously described (14, 35, 36). Briefly, 25 mg ROS were washed thrice in 10 mM potassium phosphate, pH 7.0, and resuspended in 0.8 mL of buffer. Retinaloxime was produced by adding 0.8 mL of 1 M NH_2OH (neutralized using NaHCO_3) and 4.2 mL of methanol to the membranes and incubating on ice for 10 min. Organic phase extraction was performed thrice with 7.5 mL of chloroform (with 50 mg/mL butylated hydroxytoluene) and 4 mL of water. The organic phase was pooled, washed with 5.6 mL of 0.3 M NaCl and 4.2 mL of MeOH, followed by N_2 evaporation. The lipids were resuspended in 0.2 mL of $\text{CHCl}_3/\text{MeOH}$ (1:1). This was applied to a TLC plate (0.5 mm Sili-cagel) under N_2 and developed in a tank exposed to N_2 , using a solvent phase of hexane/ether (1:1). The retinaloxime migrated near the solvent

front, whereas the phospholipids remained at the origin. The phospholipids were scraped and eluted from Silicagel using $\text{CHCl}_3/\text{MeOH}$ (1:1).

Dark Isomerization of 11-*cis*-retinal. DOPC, DOPC/DOPE, or rod outer segment lipid suspensions were prepared from dry films, obtained by evaporating the organic solvent with nitrogen. In some cases, dark-adapted ROS membranes were used for determining time course of retinal isomerization. Phosphate buffer (60 mM) was added to yield a concentration of 1.25-mM lipids. All-*trans*- or 11-*cis*-retinal was added as a concentrated solution in ethanol at 37 °C in darkness to a final concentration of 100 μM . After incubation, 10 mM hydroxylamine was added, and the retinoids were extracted twice with hexane followed by normal-phase HPLC.

Bisretinoid formation was observed at various time points (10 h, 1 d, 2 d, 3 d, and 7 d). A2PE was analyzed by $\text{CHCl}_3/\text{MeOH}$ (2:1) containing 0.1% trifluoroacetic acid (TFA) extraction and reverse-phase HPLC analysis. Hydroxylamine derivatization was not performed in reverse-phase analysis. Similarly, for A2E conversion, 50- μL aliquots of ROS, OS lipid, or synthetic lipids was added to 0.5 mL buffer containing 30 units of phospholipase D and 40 mM Mops pH 6.5. This mixture was incubated at 37 °C for 2 h and extracted with $\text{CHCl}_3/\text{MeOH}$ (2:1) containing 0.1% TFA. The final sample was redissolved in $\text{CHCl}_3/\text{MeOH}$ (1:1) for reverse-phase HPLC analysis.

HPLC Chromatography. Retinoid oximes were analyzed on a normal-phase HPLC column (Agilent ZORBAX Rx-SIL; 4.6 $\times$ 250 mm, 5 μm) and elution with

a gradient of hexane (A) and hexane/ethyl acetate (9:1) (B): 0–3 min, 100% A, 1.0 mL/min; 3–55 min, 0–100% B, 1.0 mL/min; 55–60 min, 100% A, 1.0 mL/min. Absorbance peaks were identified by comparison with external standards (11-*cis*-retinal and all-*trans*-retinal), and molar quantities per reaction were calculated by comparison with standard concentrations and normalized to total sample volumes.

For quantification of A2E and A2PE, reverse-phase HPLC was carried out using a C18 column (Atlantis 3 μm $\times$ 150 mm, 3.9 mm) and the following gradients of acetonitrile in water (containing 0.1% TFA): 75–90% (0–30 min; flow rate, 0.5 mL/min), 90–100% (30–40 min; flow rate, 0.5 mL/min), and 100% (40–80 min; flow rate, 0.5 mL/min), and monitored at 360, 430, and 490 nm. Fractions corresponding to A2E were collected, and fluorescence emission profile was verified (λ_{ex} = 430/488 nm; λ_{em} = 600–700 nm with a broad maximum seen at $\sim$ 620 nm). All-*trans*-retinal dimer was generated in both PC and PE containing lipid mixtures; however, A2E was seen only in PE containing natural or synthetic lipids.

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