Supplementary Information

ABCA4 is an *N*-retinylidene-phosphatidylethanolamine and phosphatidylethanolamine importer

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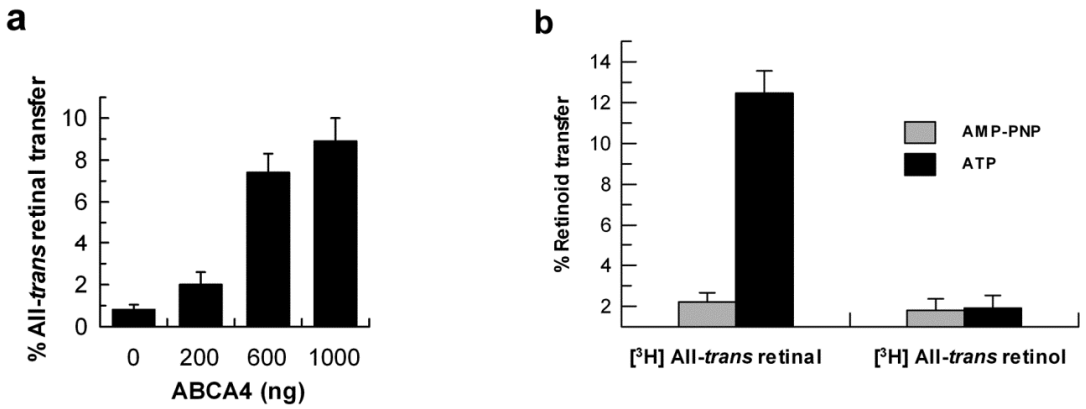
Supplementary Figures S1-S7

Supplementary Tables S1 and S2

Supplementary Methods

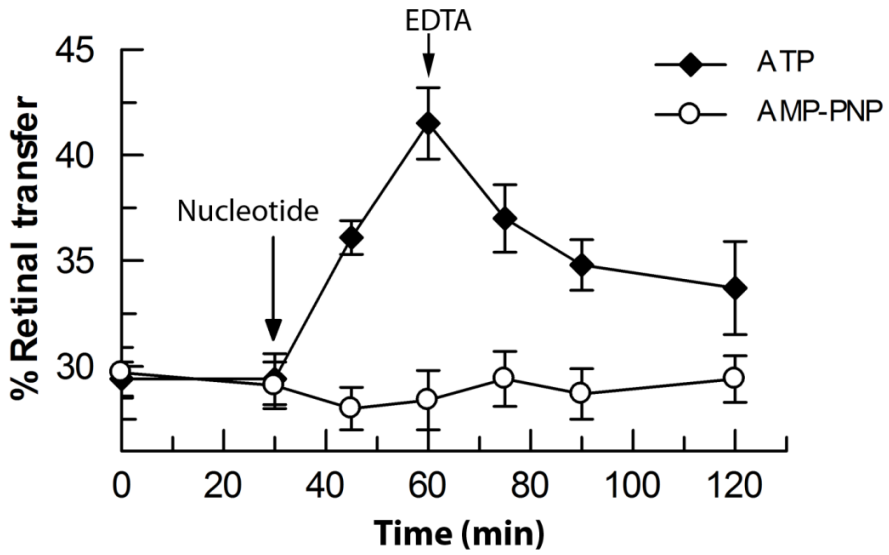
Supplementary References

Supplementary Figure S1. Effect of ABCA4 protein levels and retinoids on ATP-dependent transfer of all-*trans* retinal from donor proteoliposomes to acceptor liposomes.



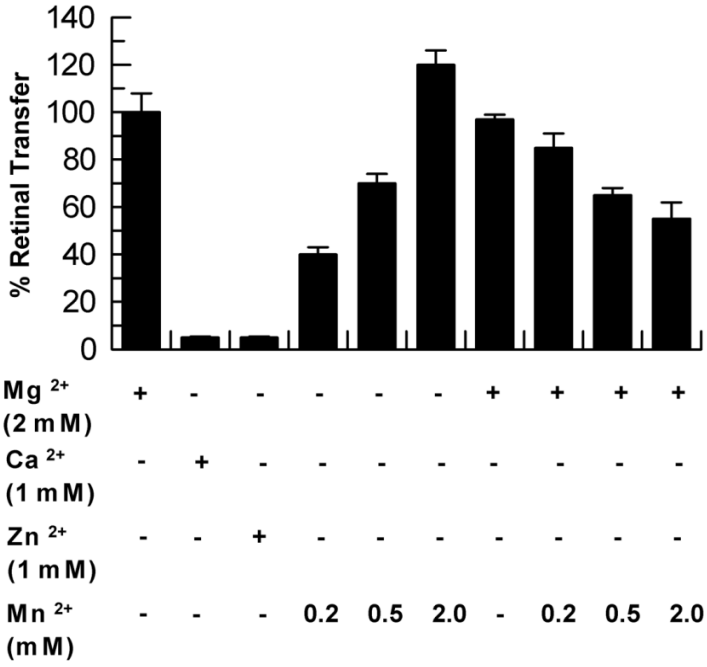
Supplementary Figure S1. ABCA4 was purified from bovine rod outer segments by immunoaffinity chromatography and reconstituted into donor proteoliposomes. (**a**). Effect of increasing amounts of ABCA4 on ATP-dependent [^{3}H] all-*trans* retinal transfer activity. Concentrations of ABCA4 were adjusted such that the same volume of proteoliposomes was used in the assay. Data are plotted as mean with error bars indicating ± s.d. for n =3. (**b**)**.** ABCA4-mediated all-*trans* retinal and all-*trans* retinol transfer activity from donor proteoliposomes to acceptor liposomes. Vesicles were incubated with either 10 μM [^{3}H] all-*trans* retinal or [^{3}H] all-*trans* retinol (1.68 kBq, 3.36 GBq/mmol). Retinoid transfer from donor to acceptor vesicles was measured after the addition of 2 mM ATP or AMP-PNP at 37 °C for 1 h. Data are plotted as mean with error bars indicating ± s.d. for n =3.

Supplementary Figure S2. Effect of EDTA on the ATP-dependent accumulation of retinal in acceptor liposomes.



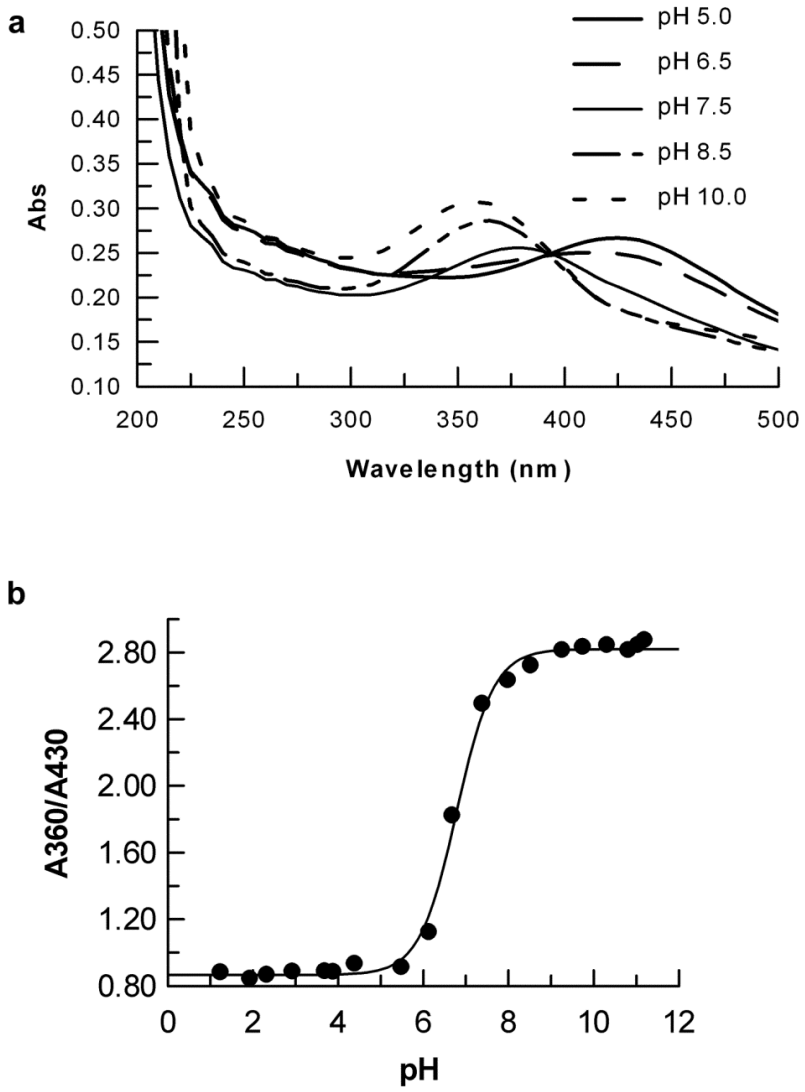
Supplementary Figure S2. Nucleotide (2 mM ATP or AMP-PNP and 3 mM $MgCl_2$) was added to a mixture of ABCA4 containing proteoliposomes and acceptor liposomes after 30 min. At 60 min. 10 mM EDTA was added to chelate Mg^{2+} required for ATP binding and hydrolysis. A decrease in accumulated retinal in the liposomes was observed after the addition of EDTA. AMP-PNP had no significant effect on the accumulation or EDTA-dependent reduction of retinal. Data are plotted as mean with error bars indicating ± s.d. for n =3.

Supplementary Figure S3. Effect of divalent cations Mg^{2+}, Ca^{2+}, Zn^{2+}, and Mn^{2+} on relative all-*trans* retinal transfer from ABCA4 proteoliposomes to acceptor liposomes.



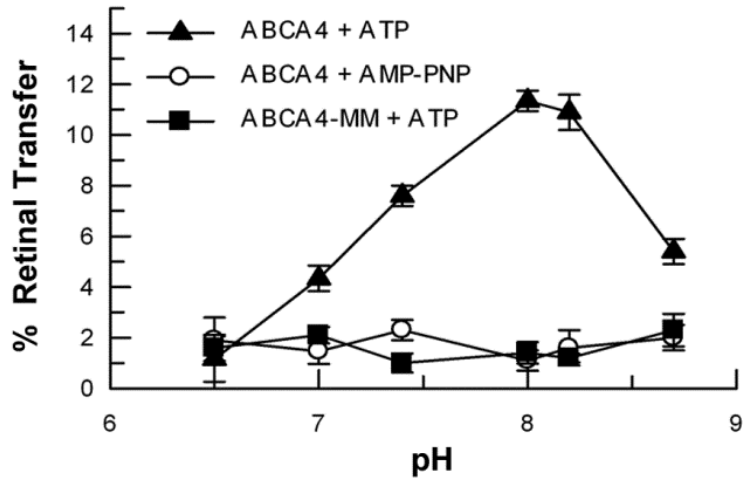
Supplementary Figure S3. Both Mg^{2+} and Mn^{2+} support transfer activity but were inhibitory when mixed together. Zn^{2+} and Ca^{2+} could not substitute for Mg^{2+} and Mn^{2+}. No activity was detected when divalent cations were omitted from the reaction. Data are plotted as mean with error bars indicating ± s.d. for n =3.

Supplementary Figure S4. Determination of pK_a of *N*-retinylidene-PE in a liposome system.



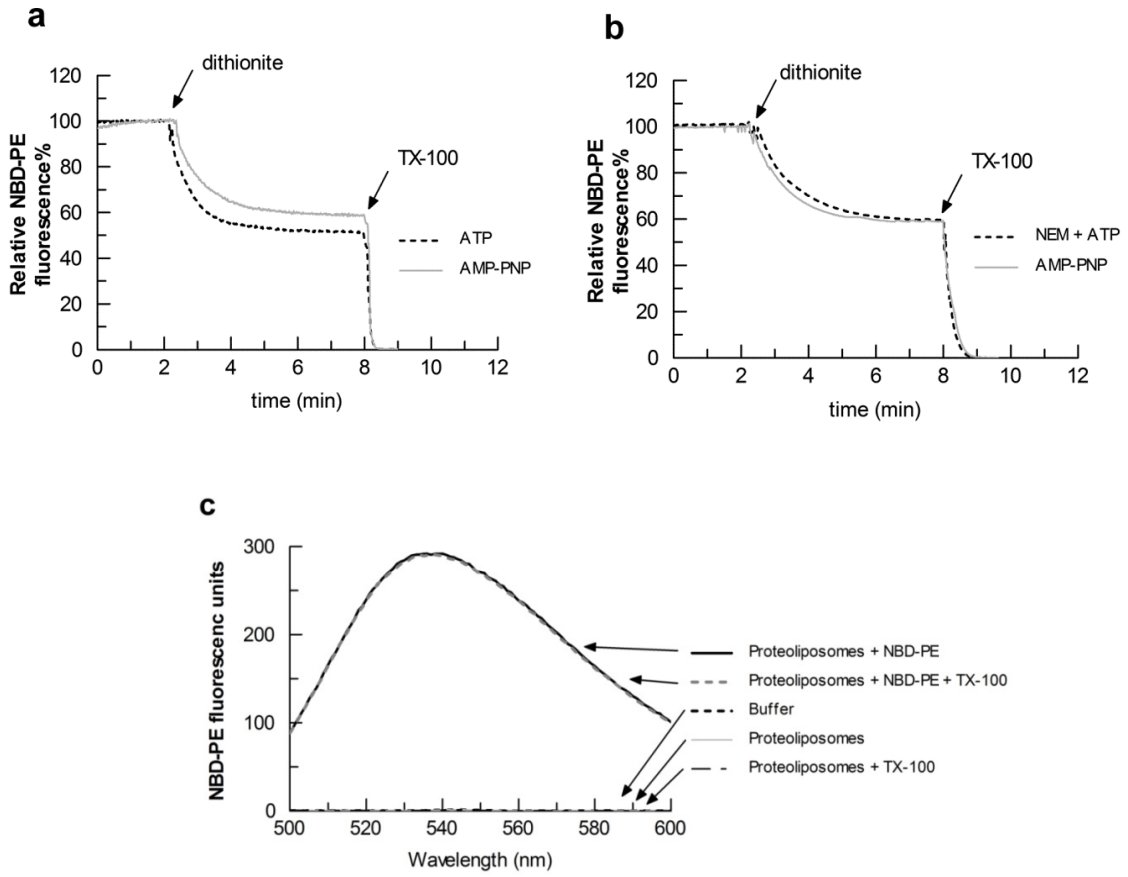
Supplementary Figure S4. DOPC/DOPE vesicles (150 μM) were mixed with 1 μM all-*trans* retinal in 3 ml buffer consisting of 0.5 mM HEPES, pH 8.0, and 1 mM NaCl. The pH was changed by the addition of HCl or NaOH (a) Examples of the absorption spectra of N-retinylidene-PE at various pH values. (b) Titration curve for *N*-retinylidene-PE is shown in which the ratio $A_{360\ nm}$/ $A_{430\ nm}$ of *N*-retinylidene PE is plotted as a function of pH; the curve (solid line) was fitted with the Henderson-Hasselbalch equation yielding a pKa of 6.9.

Supplementary Figure S5. Dependence of retinal transfer on pH.



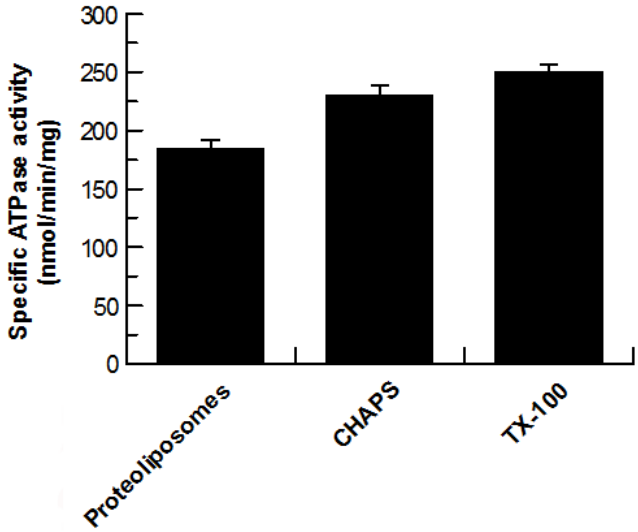
Supplementary Figure S5. Donor proteoliposomes reconstituted with either wild-type ABCA4 or the ATPase-deficient mutant (ABCA4-MM) were mixed with DOPC/DOPE acceptor vesicles at the designated pH in the presence of 10μM [^{3}H]-ATR. ATP or AMP-PNP (2 mM) was added to initiate ATR (Retinal) transfer. % Retinal transfer was measured after 1 h at 37^{o}C. Data are plotted as mean with error bars indicating ± s.d. for n =3.

Supplementary Figure S6. Fluorescence traces used to evaluate the PE flippase activity of ABCA4.



Supplementary Figure S6. ABCA4 was reconstituted into PC liposomes containing 0.6% NBD-labeled-PE. The proteoliposomes were incubated with nucleotides (1 mM ATP or AMP-PNP) for 1 hour at 37^0C to initiate NBD-labeled-PE flipping and subsequently treated with the impermeable reducing agent dithionite (4mM) to bleach NBD-labeled-PE on the outer leaflet of the vesicles. After a stable baseline was obtained 1% Trition X-100 was added to permeabilize the membrane and bleach the remaining NBD-labeled-PE derivatives. (a) Extent of flipping was determined from the difference in fluorescence for the ATP and AMP-PNP treated samples as described in the experimental methods. (b) Control samples in which ABCA4 activity was inhibited by the addition of 10 mM NEM showed no difference between ATP and AMP-PNP. (c) Examples of emission scans at 540 nm for NBD-labeled-PE and proteoliposomes in the presence and absence of Triton-X-100 (TX-100).

Supplementary Figure S7. Orientation of ABCA4 as determined by ATPase activities.



Supplementary Figure S7. The retinal-stimulated ATPase activity of ABCA4 reconstituted in proteoliposomes was determined in the absence (proteoliposomes) and presence of either 15 mM CHAPS or 0.1% Triton X-100 to solubilize or permeabilize the proteoliposomes. The increase in ATPase activity in the detergent-treated samples was used to estimate the total accessibility of ABCA4 to ATP. A 1.25 and 1.34 fold increase in ATPase activity was observed for CHAPS and TX-100 treated samples indicating that as much as 70-80% of ABCA4 is oriented with its NBDs exposed to the outside of the proteoliposomes. Data are plotted as mean with error bars indicating ± s.d. for n =3.

Supplementary Table S1: Efficiency of vesicle separation monitored by fluorescence intensity of NBD-PE after separation of donor from acceptor vesicles [a]

Acceptor Vesicle	Donor vesicle	Treatment	Fluorescence Intensity %
DOPC/DOPE/N-NBD-PE	*DOPC/DOPE*	-	100 ± 1.4
DOPC/DOPE/N-NBD-PE	*DOPC/DOPE*	1 mM ATP	101 ± 0.8
DOPC/DOPE	*DOPC/DOPE/N-NBD-PE*	1 mM ATP	0.7 ± 0.5
DOPC/DOPE	*DOPC/DOPE/N-NBD-PE*	10 µM All-*trans* retinal + 1 mM ATP	0.4 ± 1.1
DOPC/DOPE	*ABCA4/DOPC/DOPE/N-NBD-PE*	1 mM ATP	0.5 ± 1.2
DOPC/DOPE	*ABCA4/DOPC/DOPE/N-NBD-PE*	10 µM All-*trans* retinal + 1 mM ATP	1.2 ± 1.1

[a] For the preparation of labeled vesicles, *N*-NBD-labeled-PE is included at 1 mol % each. Vesicles are composed of DOPC/DOPE (~70/30 mol%). Lipids are solubilized at 18 mm CHAPS in an aqueous buffer containing 10 mM HEPES, pH 7.6, 150 mM NaCl and reconstituted by overnight dialysis. DOPC/DOPE/NBD-labeled-PE vesicles with 10% sucrose and unlabeled DOPC/DOPE vesicles were added to a final concentration of 500 µM each into 2 ml 10 mm HEPES, pH 7.6 ,150 mM NaCl for 1 h at 37 ºC. After ultracentrifugation separation, in 10 mM HEPES, pH 7.6, 75 mm NaCl, 150 mM sucrose, acceptor vesicles were resuspended and fluorescence intensity monitored for NBD-labeled-PE using excitation and emission wavelengths of 467 and 540 nm, respectively with an emission filter of 530 nm. In the last four set of trials, the NBD-labeled-PE fluorophore was reconstituted into lighter donor vesicles and incubated with unlabeled acceptor vesicles. Retinal does not have intrinsic fluorescence and the N-NBD fluorescent label is covalently bound to the amino moiety present in the head group PE. Data is the mean ± s.d. for n=3.

Supplementary Table S2: Analysis of vesicle fusion by fluorescence quenching of N-NBD-PE and N-Rh PE after separation of donor and acceptor vesicles [c]

	Fluorescence quenching %			
vesicles	none	Ca^{2+}	ATP	Triton X-100
N-Rh PE + N-NBD PE	0.3 ± 0.1	2 ± 1.2	4.2 ± 2.1	96 ± 3
ABCA4/N Rh PE + N-NBD-PE	4.2 ± 1.5	4 ± 2.4	4 ± 1.7	100 ± 4

[c] Vesicle-vesicle fusion analysis. Vesicles contained 3 mol% of the indicated fluorescent phospholipid derivative. Fluorescence donor *N*-NBD-PE and acceptor *N*-Rh-PE were incorporated in separate vesicle populations. DOPC/DOPE/N-Rh-PE (67:30:3) vesicles were prepared in CHAPS/PC/10% sucrose buffer and dialyzed while N-NBD-PE and/or ABCA4 vesicles were reconstituted into DOPC/BPL/DOPE/N-NBD-PE (57:20:20:3). Vesicle fusion was monitored with resonance energy transfer. ABCA4 was incorporated in *N*-Rh-PE populations and subjected to 1 mM ATP treatment. Fusion/quenching was initiated by addition of 5 mM Ca^{2+} or 1 % Triton X-100. Ca^{2+} or Mg^{2+} did not produce fluorescence quenching suggesting that fusion events did not occur in these vesicle compositions. Fluorescence quenching of the samples were recorded by excitation at 475 nm and monitored at 530 nm with 2.5 nm band-widths. Any fusion of the vesicles will result in intermixing of the membrane lipids so that the fluorescence donor and acceptor come into close proximity and effect fluorescent quenching. Data is the mean ± s.d. for n=3.

Supplementary Methods

Materials. Porcine brain polar lipid (BLP), 1,2 dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), 1,2 dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), C6-NBD-PE, C6-NBD-PS, C6-NBD-PC, C-12-NBD-PE, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-N-(7-nitro-2-1,3-benzoxadiazol-4-yl) (*N*-NBD-PE), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (*N*-Rh PE) were purchased from Avanti Polar Lipids (Alabaster, AL). ATP, ADP, and AMP-PNP were purchased from Sigma, dithionite was from Fisher, CHAPS was from Anatrace (Maumee, OH), radiolabeled sodium borohydride (NaB^3H_4) was obtained from American Radiolabeled Chemicals, [α^{32}P] ATP was from Perkin Elmer. Organic solvents (chloroform, hexane, methanol) were HPLC grade and water was distilled and deionized. All-*trans* retinal was radiolabeled and isolated as described Garwin and Saari and stored under N_2 gas[61]. Stock solutions of ATP, ADP, and AMP-PNP were adjusted to pH 7.5 with NaOH and filter sterilized. Buffers were degassed, filter sterilized, and light protected prior to usage. Assays and reactions performed with retinoids were light protected. The extinction coefficients for all-*trans* retinal, all-*trans* retinol, *N*-retinyl PE, and all-*trans* retinal oxime were 42.9, 52.8, 37.8, and 59.3 $mM^{-1}cm^{-1}$ [62, 63]. The Rim3F4 and Rho1D4 monoclonal antibodies have been described previously[18,65].

SDS gel electrophoresis and Protein Concentrations. Proteins were separated on 8% SDS polyacrylamide gels and transferred to Immobilon FL membranes for western blotting as previously described[29]. Protein concentration of reconstituted proteoliposomes was estimated on Coomassie Blue-stained SDS-PAGE gels using bovine serum albumin as a standard. Protein concentration of bovine and mice ROS vesicles was measured with the Bradford assay using bovine serum albumin as a standard.

Expression and purification of ABCA4-1D4 from H293T cells. Wild-type (WT) and mutant ABCA4 containing a 9 amino acid 1D4 tag (T-E-T-S-Q-V-A-P-A) in pCEP were expressed in HEK293T cells as previously described [6]. For purification, a cell suspension from two 10-cm dishes was added slowly to 1.0 ml Buffer **B** (50 mM HEPES, pH 7.6, 0.5 mg/ml BPL, 100 mM

NaCl, 1 mM DTT) containing 18 mM CHAPS and protease inhibitor and stirred for 60 min at 4 °C. The supernatant after a 10 min centrifugation at 100,000 ×g (TLA110.4 rotor in a Beckman Optima TL centrifuge) was mixed with 100 µl of Rho1D4-Sepharose 2B for 1 h at 4 °C. The matrix was washed six times in Buffer B containing 10 mM CHAPS and the ABCA4 protein was eluted twice at 12 °C over 30 min in the same buffer with 0.2 mg/ml Rim 1D4 peptide.

Preparation and purification of *N*-retinyl PE. Synthesis of *N*-retinyl PE was carried out under dim light in glassware wrapped in aluminum foil to minimize photoisomerization of retinoids. Freshly prepared solutions of all-*trans*-retinal (29 mg, 0.1 mmol) in 1 ml of methanol and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (100 mg, 0.135 mmol) in 2 ml of chloroform were mixed and incubated at room temperature for 1 hour. The color of the reaction mixture changed from yellow to deep red due to formation of corresponding Schiff base. $NaBCH_3CN$ (31 mg, 0.5 mmol) was added and the reaction mixture was stirred for 12 hours. The color of the reaction mixture gradually changed from deep red to light yellow. Five ml of $CHCl_3$ was added to the reaction mixture and $NaBH_3CN$ was neutralized by addition of 5 µL of water. Organic layer (bottom layer) was collected, concentrated under reduced pressure and the product was purified by flash chromatography ($CHCl_3$: MeOH = 8:1) yielding *N*-retinyl-PE with a 40 % yield. The desired product was contaminated with cis isomers of retinyl-PE (~10% impurities total based on normal phase HPLC with diode array detection at 330 nm). The analytically pure sample was purified by normal phase HPLC purification (gradient elution starting with 100% "A"= 100% $CHCl_3$+0.2% Et_3N (v/v), linear change over the course of 40 minutes to 100% "B" = 100% MeOH +0.2% Et_3N (v/v), followed by 100% B (10 minutes), linear gradient to 100% A (2 minutes) and equilibration of the column with 100% A (15 minutes). ^1H NMR (300 MHz, $CDCl_3$): 10.105 (broad singlet, 2H, $(R_2N\underline{H}_2)^+$); 6.668 (t, j = 11.7 Hz, 1H, a); 6.1-6.4 (m, 4H, a); 5.652 (broad s, 1H, a); 5.348 (s, 4H, a); 5.234 (s, 1H, a); 4.270 (dd, j=67.5 Hz, j=11.7 Hz, 4H, b); 4.106 (d j=42.6 Hz, 2H, b); 4.035 (broad m, 2H, b); 3.785 (broad s, 2H, c); 3.097 (broad s, 2H, d); 2.294 (two overlapping triplets, j=8.0 Hz, 4H, e); 2.2-2.4 (m, 5 H); 1.91-2.05 (m, 18 H); 1.900 (s, 3H); 1.722 (s, 3H); 1.52-1.65 (m, 5H); 1.4-1.5 (m, 3H); 1.2-1.4 (broad s, 56H); 1.038 (s, 9H); 0.8-0.9 (m, 10H). ^{31}P: 0.703 (s). Mass Spec: Sample was dissolved in 80:20:0.2=CH_3CN:H_2O:formic acid and infused directly on Agilent LC/MSD 3D Ion Trap

instrument. MH^+ calculated =1012.77; observed 1012.7; MS/MS on 1012.7 peak yields 408.3; 603.7; 625.7; 723.7; 766.8; 951.9; 978.8; 992.9.

Generation of ABCA4 mutant constructs. Human ABCA4 with a C-terminal 1D4 tag subcloned into a pCEP4 vector (Invitrogen) at its *Not*I/*Asi*SI sites was used as a template for site-directed mutagenesis. Mutations were introduced by overlap-extension PCR using Pfu DNA polymerase and the following mutagenic primers (with introduced mutations shown in bold): G863Af, gagcccct**agcc**gaggaaacg; G863Ar, cgtttcctc**ggc**taggggctc, N965Sf, gcattcctgggccac**agc**ggagctgggaaaacc; N965Sr, ggttttcccagctcc**gct**gtggcccaggaatgc. G863A was constructed with ABCA4-fwd (aatattgcggccgccaccatgggcttcgtgagac) and ABCA4-*Fse*I-rev (gccacagggctcaaaaatct) primers and subcloned into the *Not*I and *Fse*I sites of the ABCA4 construct. N965S was constructed with ABCA4 *Fse*I-Fwd (agatttttgagccctgtggc) and ABCA4-*Sbf*I-rev (ccctggtgctgcacctgc) primers and subcloned into the *Fse*I and *Sbf*I sites. K969M, K1978M, K969M/K1978M previously cloned into ABCA4-pcDNA3 [7] were cut out *via Fse*I and *Sbf*I restriction sites and replaced as inserts into the ABCA4-1D4-pCEP4 vector. The presence of these mutations was confirmed by DNA sequencing.

ATPase activity. ATP hydrolysis was measured using 50 μM [α-^{32}P] ATP and thin layer chromatography as described previously [6]. The all-*trans* retinal concentration was determined spectrophotometrically ($\varepsilon_{383\ nm}$ = 42.88 mM^{-1} cm^{-1}).

Analysis of Retinoid Binding. Solid phase binding assay using [^{3}H]-labeled all-trans retinal was carried out as described previously[29]. All incubations were carried out at 4 °C. Briefly, HEK293T cells transfected with 1D4-tagged ABCA4 were harvested from one dish, centrifuged at 2800×g for 3 min, and resuspended in 0.5 ml solubilization buffer. The supernatant fraction obtained after centrifugation (100,000 g for 10 min) was incubated with 30 μl Rho1D4-Sepharose 2B immunoaffinity matrix pre-equilibrated in column buffer. After 1h, the matrix containing the bound 1D4-tagged protein was washed several times with 0.4 ml column buffer by low speed centrifugation and mixed with 0.25 ml of 10 μM [^{3}H]-labeled all-*trans* retinal (specific activity of 500 dpm/pmol, 2.5 x 10^6 dpm total) in column buffer for 30 min. The matrix was washed several times with 0.4 ml column buffer to remove unbound [^{3}H]-labeled all-trans

retinal and then incubated in the presence or absence of 0.5 mM ATP, for 15 min. The matrix was washed 2 more times with 0.4 ml column buffer before being transferred to Ultrafree-MC (0.45 μm filter) spin column (Millipore). The samples containing the immobilized 1D4 tagged ABCA4 protein was extracted from the matrix with 0.5 ml of ice-cold ethanol for 15 min at room temperature and eluted by centrifugation. Radiolabeled all-*trans* retinal in the ethanol extractions was determined by liquid scintillation counting.

Supplementary References

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