Supplemental Materials

Detailed Methods

Materials: PIP strips (P-6001), Sphingo strips (S-6000), PI(4,5)P2 (P-4524), PI(4,5)P2 ELISA kit (K-4500), PI(4)P Grip (G0402), PI(4,5)P2 Grip (G4501), biotin-PIP2 (C-45B6), fatty acid labeled-bodipy PIP2 (C-45F16a), and FITC conjugated Anti-PIP2 antibody(Z-G045) were from Echelon Biosciences. HRP-conjugated GST antibody was from Sigma. Alexa647-Antibody labeling kit was from Molecular Probes (Cat No. A-20186). Purified recombinant human proteins apoA2 (TP721104) and apoE (TP723016) were from Origene. [^{3}H]-labeled PIP2 (NET895005UC), myo-inositol (NET1177001MC), and cholesterol (NET13900) were from Perkin Elmer. ApoA1 was purified form human plasma[1], and dialyzed against PBS. Recombinant human apoA1 and truncation mutations were prepared as previously described.[2] RAW264.7 cells were from ATCC. Mifepristone ABCA1-inducible BHK cells, as previously described were obtained from Chongren Tang, University of Washington.[3] Mifepristone SR-BI-inducible BHK cells, as previously described[34], were obtained from Alan Remaley, NIH.[4] ABCA1-GFP and the mutant isoform stably transfected HEK cells were as previously described. [5]

Control and target siRNAs were purchased from Origene or Ambion as follows: mouse TMEM55B (Cat. No. SR408149, Origene); mouse PIP4K2A (Cat. No. AM16708, siRNA ID: 150120); human PIP4K2B (Cat. No. AM16708, siRNA ID: 1322); mouse PIP4K2A (Cat. No. AM16708, siRNA ID: 150120); human PIP4K2B (Cat. No. AM51331, siRNA ID: 618); and mouse Abca1 (Cat. No. AM16708, siRNA ID: 7092). Antibodies were purchased from either Santacruz Biotech or Thermofisher: PIP5Kα (Thermofisher, Cat. No. PA5-31141), PIP5Kβ (Thermofisher Cat. No. PA5-46587), TMEM55B (Santacruz Biotech, Cat. No. SC-160875). PI-PLC was purchased from Sigma (Cat. No. P5542).

Protein-lipid overlay assays: The PIP strip and sphingo strip membranes were blocked with 5% milk powder in PBS-Tween for 30 min, and apoA1 was added at 50 µg/ml and incubated at room temperature for 2 hr. The bound protein was detected by using anti human apoA1 goat (Meridian Life Science, #K45252G) antibody and HRP conjugated anti-goat antibody. HRP was visualized using ECL reagent (Pierce) and exposure to x-ray film. Lipids extracted from conditioned media or cells were dissolved in methanol:chloroform:12N HCl (40:80:1) and spotted onto nitrocellulose membranes. After treating with casein blocker (Thermo scientific; #37528), the membranes were incubated with GST-PLCδ-PH (1µg/ml, Echelon Biosciences) to detect PIP2, or with GST-SiDC-3C (1µg/ml, Echelon Biosciences) to detect PI4P. The binding interactions were detected using HRP-conjugated anti-GST antibody (Sigma) and ECL chemiluminescence.

Surface Plasmon resonance: Binding kinetics of PIP2 with different apolipoproteins was analyzed using a Biacore3000 instrument. Either biotinylated apoA1 or biotinylated PIP2 was immobilized on a streptavidin sensor chip (GE Healthcare). The immobilized apoA1 or PIP2 was stable over the course of the experiment and baseline drift was <10 response units (RU)/h after the washing with Hepes buffered saline (HBS) buffer. Different concentrations of apoA1 or PIP2 were injected using the KINJECT procedure at flow-rate of 10 µl/min and dissociation was monitored by injecting HBS buffer. The injections were performed in triplicate for each ligand concentration. For comparing binding kinetics of PIP2 with apoA1, apoA2 and apoE, these proteins were immobilized by covalent coupling on a CM5 sensor chip (GE Healthcare) using EDC-NHS reagents. PIP2 was injected as described above. Corrected response data were fitted with BIAevaluation software version 4.01, and K_d values were calculated.

Fluorescence anisotropy: Increasing concentrations of apoA1 were incubated with 100 nM fatty acid-labeled bodipy PIP2 in a quartz cuvette at 25^{o}C. Relative anisotropy was determined

using polarized filters with excitation at 503 nm and emission at 513 nm in a Perkin Elmer spectrofluorimeter. The K_d was determined as the EC50 by non-linear regression of the log apoA1 concentration. A similar K_d value was obtained using 400 nM PIP2.

Liposome clearance assay: 1,2-Dimyristoyl-sn-glycero-3-phos-phocholine (DMPC) or 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) with other lipids as specified (Avanti Polar Lipids) with or without 5% PIP2 were dissolved in chloroform: methanol (2:1 v/v) and were dried in a stream of nitrogen and placed in vacuum overnight. DMPC or POPC was rehydrated in PBS by five cycles of freeze-thaw and extensive vortexing to form multilamellar vesicles (MLVs) at 5 mg/ml. These MLVs were subjected to apoA1 solubilization assay. Briefly, the MLVs dissolved in Tris-buffered saline-EDTA (pH 7.5) were incubated with human apoA1 at 25^{o}C. MLV solubilization by human apoA1 was monitored by measuring sample turbidity (absorbance) at 325 nm using a plate reader.

Liposome floatation assay. POPC MLVs made with or without 5 mole % PIP2 were incubated at room temperature with apoA1 (20:1, lipid:apoA1 mass ratio) in 30% sucrose and placed at bottom of a sucrose density step gradient and subjected to ultracentrifugation, as previously described.[6] Equal volume aliquots of the top (0% sucrose) and bottom (30% sucrose) fractions were precipitated and analyzed by SDS-PAGE and apoA1 western blot.

ApoA1 cross linking: ApoA1was incubated in the presence or absence of PIP2 or POPS at 1:4 (apoA1:lipid) mole ratio and then incubated with bis(sulfosuccinimidyl) suberate (BS3, Pierce) crosslinker at room temperature for 30 minutes. The reactions were quenched with 1M Tris, pH 8.0 and samples were analyzed by SDS-PAGE and apoA1 western blot.

Cell growth and ABCA1 induction: All cell culture incubations were performed at 37^{o}C in humidified 5% CO_2 incubator. The growth media was Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal calf serum, 100 µg/mL penicillin, 100 µg/mL streptavidin. ABCA1 was induced in RAW26.47 cells by 16-24 hr incubation with 0.3 mM 8Br-cAMP.[7] ABCA1 was induced in BHK cells by 16-24 hr incubation with 10 nM mifepristone.[3] Inducers were included in the media during subsequent assays. ABCA1 expression was confirmed by western blot using the AC10 antibody (Santa Cruz Biotech).

Cholesterol efflux assay: On day 1, cells were plated on 24-well plates at a density of 200,000 to 400,000 cells per well. On day 2, the cells were labeled with 0.5 µCi/ml [^{3}H]cholesterol in DMEM containing 1% FBS. On day 3, the cells when indicated were treated with or without ABCA1 inducers in serum-free DMEM. On day 4 (or day 3 for HEK293 cells and ABCA1 stably transfected cells) the cells were washed and chased for 2-6 hr in serum-free DMEM in the presence or absence of 5 µg/ml apoA1. The radioactivity in the chase media was determined after brief centrifugation to pellet any residual debris. Radioactivity in the cells was determined by extraction in hexane:isopropanol (3:2) with the solvent evaporated in a scintillation vial prior to counting. The percent cholesterol efflux was calculated as 100 × (medium dpm) / (medium dpm + cell dpm).

Inositol lipid efflux: For [^{3}H]myo-inositol labeling, the growth medium was replaced with inositol-free DMEM (including 10% fetal calf serum, 100 µg/mL penicillin, 100 µg/mL streptavidin and 2 mM glutamine) and [^{3}H]myo-inositol was added to a final concentration of 40 µCi/mL for 24 hr followed by ABCA1 induction in serum-free DMEM where indicated. The cells were washed and chased for 4-6 hr in serum-free medium in the presence or absence of 5 µg/ml apoA1. The chase media was collected, centrifuged to remove any cell debris, and acidic lipid fractions containing PIPs were isolated as following the protocol provided by Echelon Bioscience: 1 ml medium was resuspended in 750 µL chloroform/methanol/12N HCl (40:80:1, v/v/v) and incubated for 15 min at RT while vortexing the sample for 1 min every 5 min. After transferring the tube to ice, 250 µL cold chloroform and 450 µL cold 0.1 M HCl was added

followed by 1 min vortexing and centrifugation (6,500 × g, 2 min at 4 °C). The bottom organic phase was transferred to a fresh tube, dried under N_2 gas in a scintillation vial and subjected to scintillation counting.

Inositol lipid reverse transport in vivo. Bone-marrow derived macrophages from C57BL/6 mice were labeled with 40 μci/ml of [^{3}H]myo-inositol for 24h as described above. An aliquot of the cells was extracted in hexane:isopropanol (3:2) to determine total ^{3}H dpm in inositol labeled lipids. ~1.8 x 10^6 dpm of labeled macrophages were injected s.c. into the back of each mouse. 3 days later, plasma was collected, followed by acidic extraction of lipids, resupended in PBS-PS (PBS plus 0.25% Protein Stabilizer, Echelon # K-GS01). This was incubated with PH-PLC δ-GST tagged protein (Echelon). The PIP2 bound to GST tagged protein was separated from other inositol labeled lipids by incubation with glutathione-beads, and after washing the bound PIP2-protein complex was eluted by incubation with 50 mM Tris, 10 mM reduced glutathione, pH = 8.0. The eluate was subjected to scintillation counting. The % efflux to plasma was determined by calculating 100 x PIP2 dpm in total body plasma divided by the injected inositol lipid dpm.

PIP2 cellular reporter assay: RAW264.7 macrophages and ABCA1-inducible BHK cells were transfected with the 2PH-PLCδ-GFP plasmid (Addgene) using Lipofectamine 2000 transfection reagent (ThermoFisher Scientific). The GFP positive colonies were visually identified by epifluorescent microscopy selected and expanded in 1.5 mg/ml G418. RAW264.7 cells and BHK cells were induced to express ABCA1 as indicated. The cells were washed with PBS and visualized by epifluorescent microscopy. Images were taken using the same exposure time. To quantify the percentage of membrane associated PIP2 reporter, one cell at a time, the image was processed using the line tool in Image J software to plot fluorescence profile across each cell. These profiles were analyzed using GraphPad Prism. After background subtraction the area under the curve for each cells was calculated along with the two areas associated with the cell membrane (11 pixels surrounding the edge peak). The % PIP2 reporter associated with the plasma membrane for each cell was calculated as 100 x the sum of two membrane signals divided by the total cell signal.

Cell surface PS, PIP2, and apoA1 binding assays via flow cytometry: Cell surface PS levels were determined by flow cytometry after cell scraping in PBS, re-suspension in Annexin V binding buffer, and incubation with AnnexinV-Cy5 (Biovision) at room temperature for 5 minutes in the dark. Cell surface PIP2 levels were determined by flow cytometry by incubation with Alexa647 or FITC labeled anti-PIP2 antibody (Echelon) in phenol red-free, serum-free, DMEM at room temperature for 30 min. Human apoA1 was labeled with Alexa647 (Molecular Probes) on free amines using a 6:1 mole ratio of dye: apoA1. Alexa647-apoA1 binding was determined by flow cytometry after incubation with cells for 45 minutes at room temperature. All flow cytometry assays were performed on a BD Biosciences LSRFortessa cytometer using the following settings: FITC, Ex: 488 nm, Em:505-525 nm (Filter 515/20); Cy5 and Alexa 647, Ex: 639 nm, Em: 650-670 nm(Filter 660/20). Data was analyzed by Flowjo software and the median relative fluorescent intensities were compared.

PIP2 ELISA: PIP2 was quantified by using the PI(4,5)P2 Mass ELISA kit from Echelon Biosciences, following the protocol provided. Briefly, conditioned media or plasma was extracted using the acidic lipid extraction protocol described above, dried, and resuspended in PBS containing protein stabilizer. Cells were suspended, pelleted, and washed in cold 5% TCA with 1 mM EDTA. Cell neutral lipids were extracted in 1 mL chloroform:methanol (1:2). The pellet containing acidic lipids was extracted in 750 μL chloroform:methanol:12N HCl (40:80:1). 250 μL cold chloroform and 450 μL cold 0.1 M HCl was added to the supernatant. The bottom organic phase was dried, suspended in PBS-PS. Media and cell extracts in PBS-PS were subjected to the PIP2 Mass ELISA assay according the Echelon protocol.

Plasma analyses: 0.5 ml of fresh human plasma (obtained under informed consent in an IRB approved protocol) was separated by fast protein liquid chromatography (FPLC) on a Superose 6 column (Amersham), and 0.5 ml fractions were collected. Total cholesterol and PIP2 levels were measured in freshly prepared mouse plasma or human FPLC fractions using the Cholesterol LiquiColor kit (Stanbio Laboratory) and the PIP2 ELISA assay (described above), respectively. Human HDL was isolated by equilibrium density ultracentrifugation at density between 1.063 and 1.21 g/ml. LC-MS/MS was used for PIP2 profiling in human HDL as previously described.[8] In brief, HDL lipids extracts were rapidly dried under nitrogen flow, suspended in 200 μl methanol/water (70:30), and stored under an argon atmosphere at –20 °C until analysis within 24 hr. 20 μl of the extract was introduced onto a 2690 HPLC system (Waters, Milford, MA) and phospholipids were separated through a C18 column (2 x 50 mm, Gemini 5 μm, Phenomenex, Rancho Palos Verdes, CA) under gradient conditions at flow rate of 0.3 ml/min. A gradient was used by mixing mobile phase A (Methanol/water (70:30) containing 0.058% ammonium hydroxide) and B (acetonitrile/2-propanol (50:50) containing 0.058% ammonium hydroxide) as follows: isocratic elution with 100% A for 1 min, linear gradient to 100% B from 1 to 6 min, kept at 100% B for 10 min and then equilibrated with 100% A for 7 min. The HPLC column effluent was introduced onto a triple quadruple mass spectrometer (Quattro Ultima Micromass, Beverly, MA) and analyzed at negative electrospray ionization in the multiple reaction monitoring (MRM) mode for the targeted PIP2. The MRM transitions used to detect the PIP2 was the mass to charge ratio (m/z) for the molecular anion $[MH]^-$ and the product ion at m/z 79, arising from its phosphate group (i.e. $[MH]^- \rightarrow$ m/z 79).

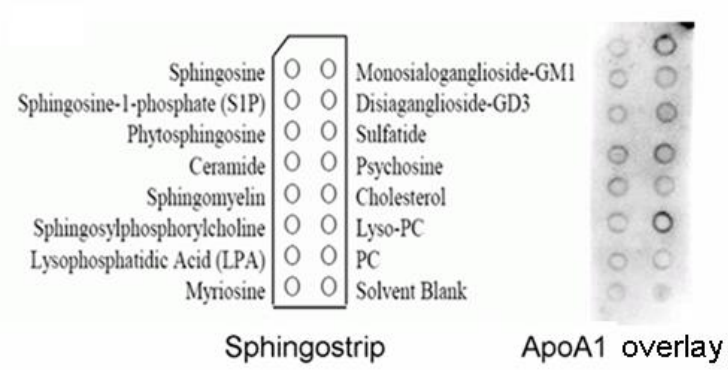
SR-BI mediated PIP2 uptake: Mifepristone SR-BI-inducible BHK cells were treated with 10 nM mifepristone to for 14 hr. 0.5 μCi [^{3}H] PIP2 was dried down and 650 μg (protein) of human HDL was added and incubated for 6 hr at room temperature to absorb PIP2 into HDL. The radiolabeled PIP2- HDL complex at 100 μg/ml final concentration was incubated with cells in serum free media for 4 hr at 37^{o}C. Cellular lipids were extracted and ^{3}H was determined by scintillation counting, and normalized to cellular protein after lysis in 0.2 N NaOH, 0.2% SDS.

siRNA knockdown: Cells (RAW264.7 or HEK293) were plated in either 6 well plates or 24 wells plates(400,000 cells per ml). After a day, media was replaced with OptiMEM reduced serum media; siRNA complexes were prepared in OptiMEM reduced media using Lipofectamine RNAiMAX reagent following manufactures protocol. The final siRNA concentration was 250 nM for RAW macrophages and 150 nM for HEK293 cells. Cells were treated with either control, ABCA1, TMEM55B or PIP5Kα siRNAs for 3 days. Cells were then processed for apoA1 binding, cholesterol efflux, and western blot assays.

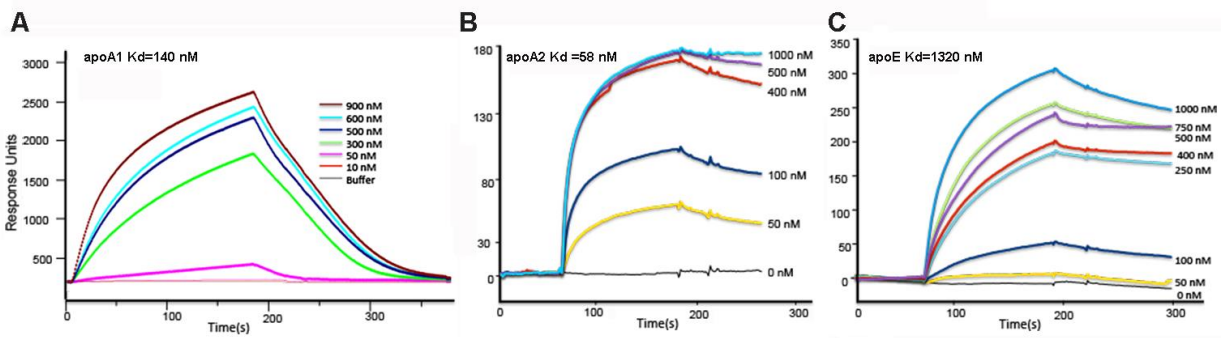
PIP2 supplementation: RAW cells or HEK293 cells were incubated with 20 μg/ml POPC (100%) or POC:PIP2 (90:10%) liposomes for 45 min in serum-free DMEM. This media was removed and cells were washed twice with PBS, followed by either apoA1 binding or cholesterol efflux assay. ApoA1 binding was performed at RT for 45 min, and the apoA1 chase for cholesterol efflux was performed at 37^{o}C for 2 hr.

Statistical analyses: Data are shown as mean ± SD. Comparisons of 2 groups were performed by a 2-tailed t test, and comparisons of 3 or more groups were performed by ANOVA with Bonferroni posttest. All statistics were performed using Prism software (GraphPad).

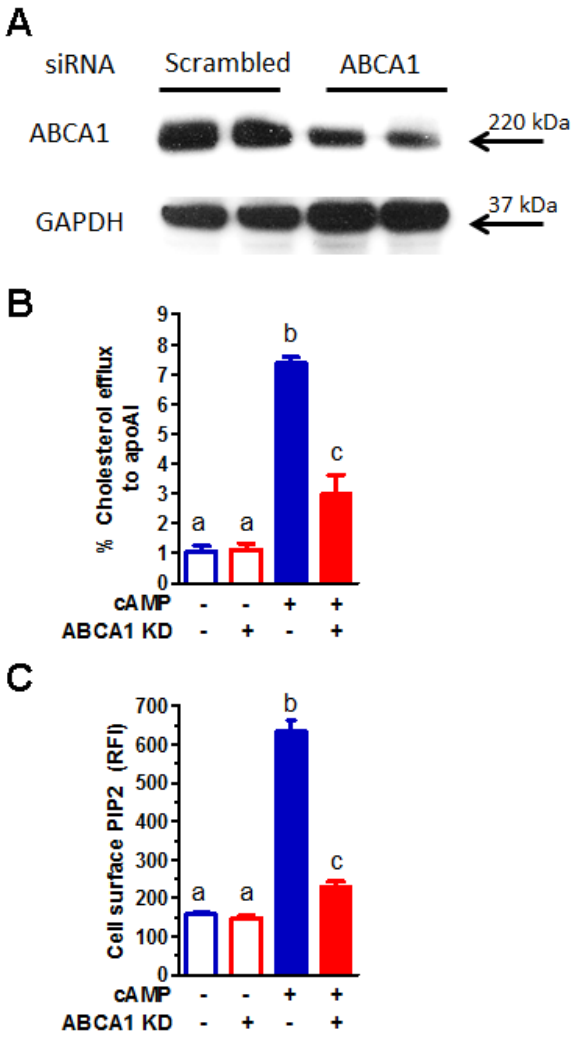
Online Figures:



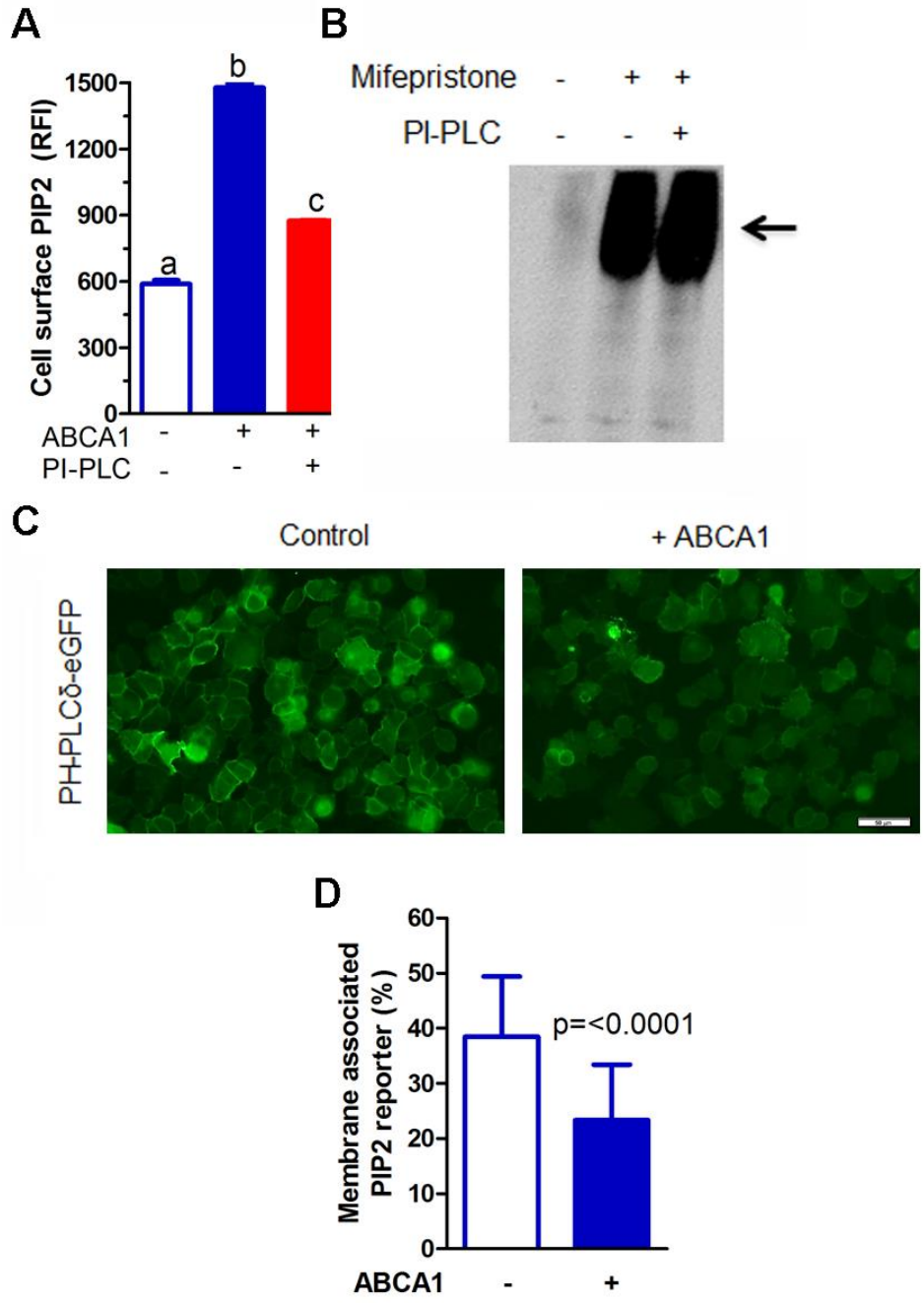
Online Figure I. Lipid-protein overlay assay using the sphingostrip demonstrates that apoA1 does not bind appreciably to various cellular lipids including PC, sphingomyelin, cholesterol, and sphingosine-1-phosphate.



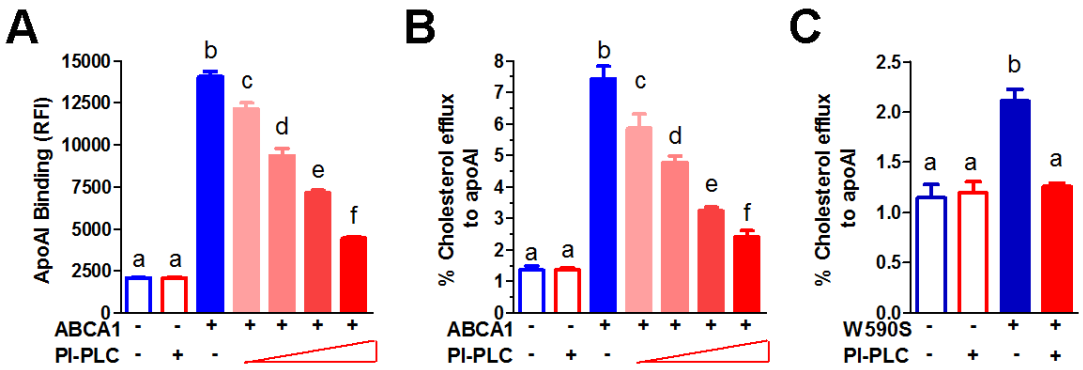
Online Figure II. PIP2 binds to HDL apolipoproteins. SPR assay showing dose-dependent direct binding PIP2 to immobilized apoA1 (**A**), immobilized apoA2 (**B**), and immobilized apoE (**C**). Calculated K_d values are shown for each assay. For apoA1, the K_d for binding to PIP2 was140 nM, with $K_{on}=2.23x10^4$ $M^{-1}s^{-1}$ and $K_{off}=1.93x10^{-3}$ $M^{-1}s^{-1}$, similar to the K_d obtained by fluorescence anisotropy (Figure 1D).



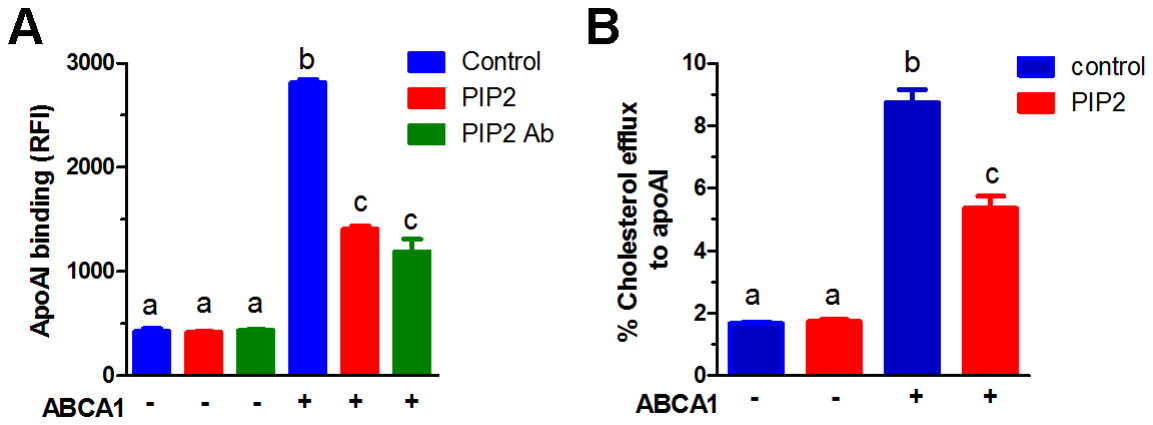
Online Fig III: ABCA1 knockdown decreased PIP2 exposure in cAMP treated RAW264.7 cells. A. Levels of ABCA1 protein in 8Br-cAMP treated RAW264.7 cells treated with either scrambled siRNA or siRNA against ABCA1. GAPDH was used as loading control. RAW264.7 cells were treated with siRNAs for 72 h, protein extracts were prepared and resolved on SDS gels, followed by western blot analysis and probing with anti-ABCA1 or GAPDH antibodies. **B.** % Cholesterol efflux from RAW264.7 cells with or without 8Br-cAMP induction of ABCA1 and without (scrambled siRNA) or with ABCA1 knockdown (KD, +Abca1 siRNA), chased with apoA1 (5 µg/ml) **C.** Cell surface PIP2 assessed by flow cytometry in RAW264.7 cells with or without 8Br-cAMP induction of ABCA1 and without (scrambled siRNA) or with ABCA1 knockdown (KD, +Abca1 siRNA). For panels B and C, mean + SD; different letters show $p<0.001$ by ANOVA Bonferroni posttest, n=3.



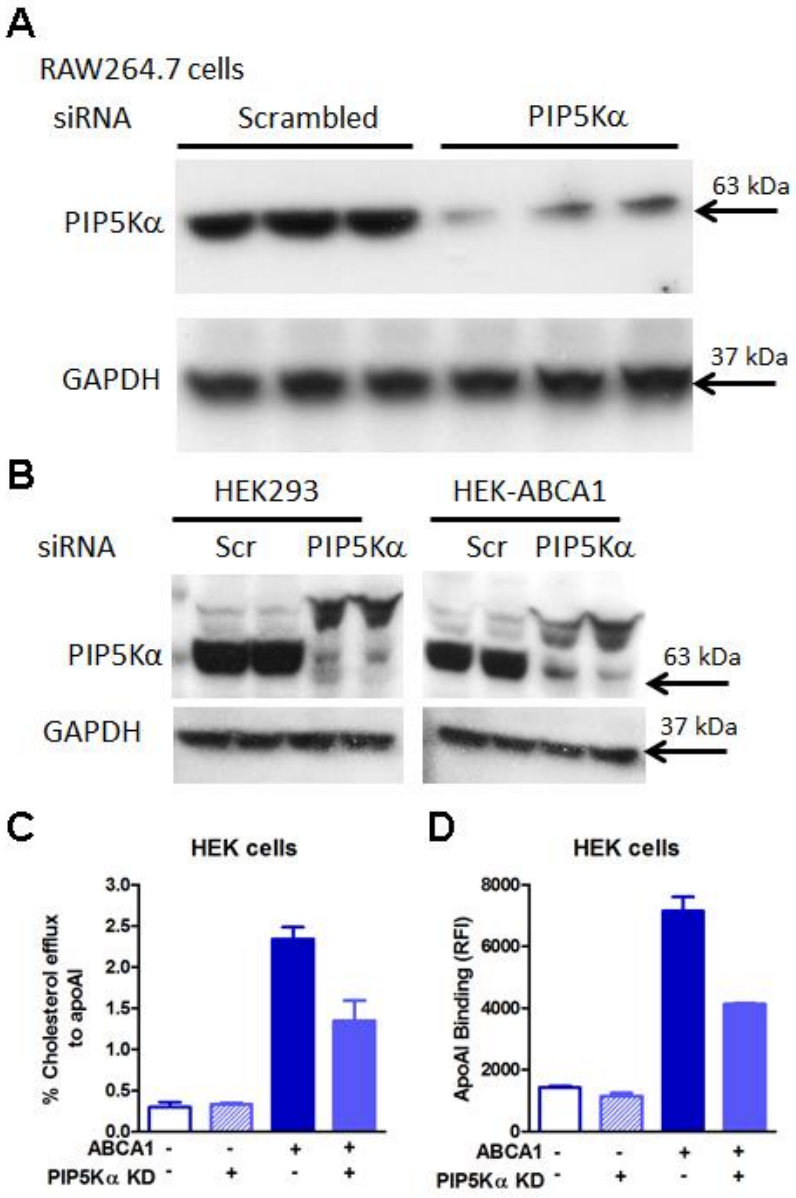
Online Figure IV. ABCA1 flops PIP2 promoting apoA1 binding and cholesterol efflux in BHK cells. A. Cell surface PIP2 assessed by flow cytometry in BHK cells ± ABCA1 induction, which were pretreated ± PI-PLC (RFI, relative fluorescence intensity; mean ± SD; different letters show p<0.001 by ANOVA Bonferroni posttest, n=3). **B**. Western blot of BHK cell extracts showing expression of ABCA1 ± PI-PLC treatment. **C**. ABCA1 expression redistributed the PIP2 reporter, PH-PLCδ-eGFP, away from the plasma membrane in stably transfected BHK cells. **D.** Percentage of PIP2 reporter association with plasma membrane ± ABCA1 induction (n=20 and 28 for control and ABCA1 expressing cells, respectively).



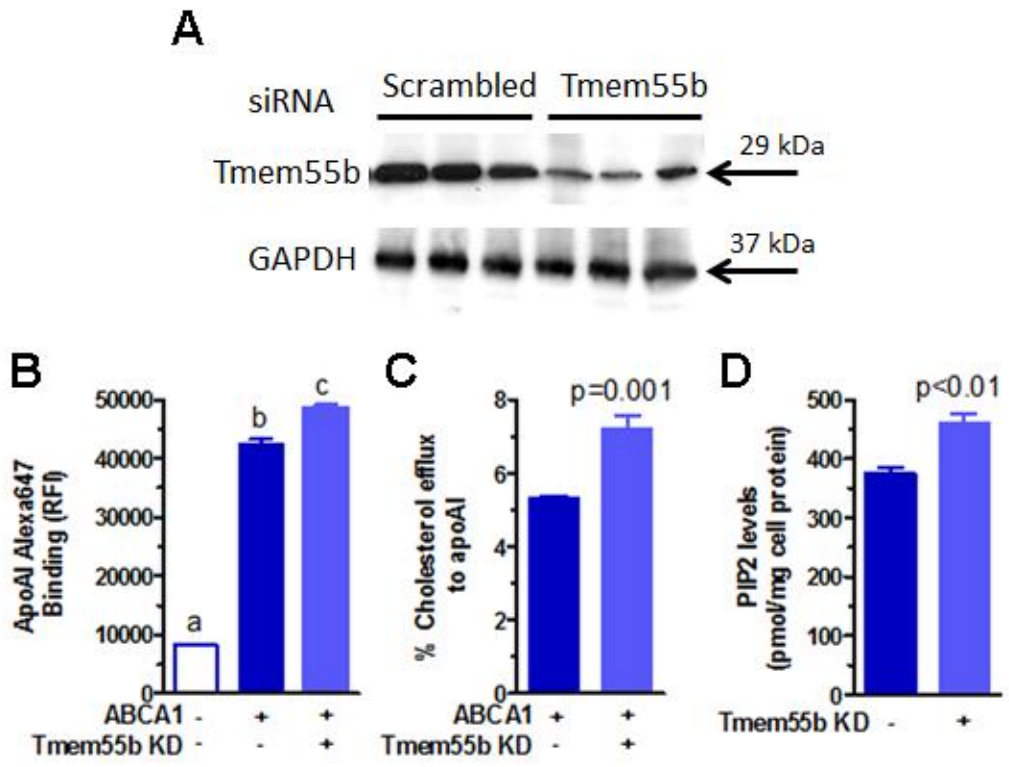
Online Fig V: PIP2 required for ABCA1-mediated apoA1 binding and cholesterol efflux. ApoA1 binding to **(A)** and cholesterol efflux **(B)** from control HEK293 cells or HEK293-ABCA1 cells treated with a PI-PLC dose response (0.4, 0.8, 1.2, and 2 U/ml, with 2 U/ml also used for a control HEK293 cells). **C.** % Cholesterol efflux from HEK293-W590S ABCA1 cells chased with apoA1 (5 µg/ml) ± 2units/ml of PI-PLC. For all panels, mean ± SD; different letters show $p<0.05$ by ANOVA Bonferroni posttest, n=3.



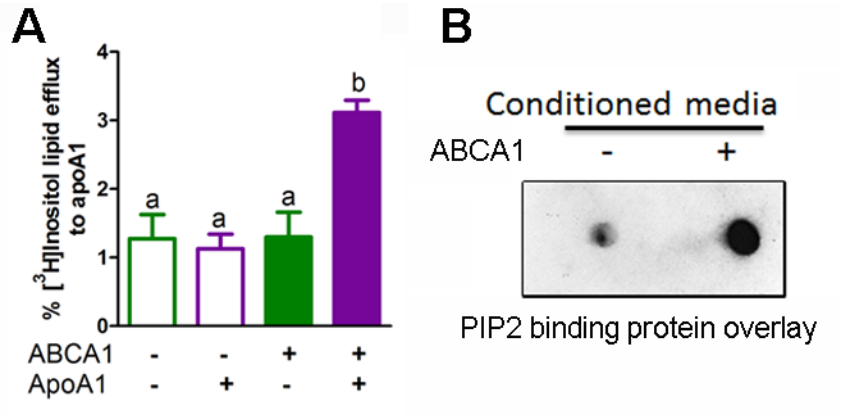
Online Figure VI. Additional controls for Fig. 5. **A.** ApoA1 binding to control and ABCA1 expressing RAW264.7 cells ± exogenous PIP2 pre-incubated with apoA1 prior to adding to cells (PIP2:apoA1, 5:1 mole ratio), or ± PIP2 specific monoclonal antibody (2 μg/ml, PIP2 antibody was added to cells along with apoA1). Cells without ABCA1 expression were used as control. **B.** Cholesterol efflux from control and ABCA1-expressing RAW264.7 cells to apoA1 (5 μg/ml) that was pre-incubated ± exogenous PIP2 (PIP2:apoA1, 5:1mole ratio). For both panels, mean ± SD; different letters show $p<0.01$ by ANOVA Bonferroni posttest, n=3.



Online Fig VII: PIP5Kα knockdown decreased apoA1 cell surface binding and cholesterol efflux: Levels of PIP5Kα protein in RAW264.7 cells **(A)**, HEK293 cells, and ABCA1stably transfected HEK293 cells **(B)** treated with either scrambled siRNA or siRNA against PIP5Kα. GAPDH was used as loading control. Cells were treated with siRNAs for 72 hr, protein extracts were prepared and resolved on SDS gels, followed by western blot analysis and probing with anti-PIP5Kα or GAPDH antibodies. Cholesterol efflux assays **(C)** and apoA1 binding assays **(D)** were performed in HEK293 cells and ABCA1 stably transfected HEK293 cells treated with either scrambled siRNA or siRNA against PIP5Kα. Cholesterol efflux activity was determined via radioactive cholesterol labeling followed by apoA1 chase and apoA1 binding was determined via flow-cytometry.



Online Fig VIII: TMEM55b alpha knockdown increased apoA1 cell surface binding and cholesterol efflux: A. Levels of Tmem55b in RAW264.7 cells treated with either scrambled siRNA or siRNA against Tmem55b. GAPDH was used as loading control. RAW cells were treated with siRNAs for 72 hr, protein extracts were prepared and resolved on SDS gel, followed by western blot analysis and probing with anti-Tmem55b or GAPDH antibodies. RAW cells treated with either scrambled or siRNA against Tmem55b and apoA1 binding **(B)**, cholesterol efflux **(C)**, and PIP2 levels **(D)** were assessed. Mean ± SD, n=3; for panel B different letters show $p<0.001$ by ANOVA Bonferroni posttest,



Online Figure IX. PIP2 is effluxed from BHK cells via ABCA1 to apoA1. A. ABCA1 mediates efflux of [^{3}H]inositol labeled lipids to apoA1 from BHK cells (mean ± SD; different letters show $p<0.001$ by ANOVA Bonferroni posttest, n=3). **B.** PIP2 in apoA1 containing conditioned media from BHK cells with or without ABCA1 expression. PIP2 was visualized by spotting extracted media lipids onto a membrane followed by protein overlay with the tagged PIP2 binding protein GST-PLCδ-PH.

Supplemental References

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