Supporting Information

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SI Materials and Methods

Plasmid DNA, Mutagenesis, and Reagents. ORF clones for human inositol polyphosphate-5-phosphatase E (INPP5E) (BC028032), human ARL2 (BC002530), and mouse ARL3 (BC042941) were purchased from Open Biosystems. ORFs for human ADP-ribosylation factor-like 13B (ARL13B), RAB11A, and phosphodiesterase 6D (PDE6D) were obtained using an in-house–generated retinal cDNA library as a template. Clones for RAB8A were generous gifts from G. Pazour (University of Massachusetts, Worcester, MA). ORFs of these genes were subcloned into pCS2 plasmids with a Myc, FLAG, HA, or GFP tag using PfuUltra II Fusion HS DNA polymerase (Agilent) and standard molecular biology techniques. GST–ARL13B expression plasmid, pGEX6P1–ARL13B, was kindly provided by K. Kontani (University of Tokyo, Tokyo). For tandem affinity purification (TAP), ORFs of INPP5E and PDE6D were inserted into the pSS-FS plasmid, which was generated by inserting 3× FLAG and 2× S tags into the pEGFP-N1 vector (Clontech) after removing the GFP portion. Deletion and substitution mutants were generated by using PfuUltra II polymerase. Small interfering RNAs (siRNAs) were purchased from Dharmacon (ON-TARGETplus SMARTpool or individual siRNA) and transfected with RNAiMAX (Invitrogen) following the manufacturer's recommendations.

TAP and Coimmunoprecipitation. Protein–protein interaction studies were conducted using HEK293T cells grown in DMEM (Invitrogen) supplemented with 10% (vol/vol) FBS (Invitrogen). Stable cell lines expressing FS-INPP5E and FS-PDE6D were generated by cotransfecting pSS-FS-INPP5E and pSS-FS-PDE6D with pCS2-puro into HEK293T cells followed by puromycin selection (1.5 μg/mL; Invitrogen). For TAP, proteins were extracted from twenty 15-cm dishes using lysis buffer (50 mM Hepes pH 7.0, 150 mM NaCl, 0.5% (vol/vol) Triton X-100, 2 mM EGTA, 2 mM $MgCl_2$, 0.5 mM DTT) supplemented with Complete Protease Inhibitor mixture (Roche Applied Science). After centrifugation for clearance, lysates were loaded onto an anti-FLAG affinity gel (M2; Sigma), and bound proteins were eluted with 3× FLAG peptide (100 μg/mL; Sigma). Eluate was loaded onto an S-protein affinity gel (Novagen), and bound proteins were eluted in the elution buffer (50 mM Hepes pH 7.0, 150 mM NaCl, 1% (wt/vol) SDS, 2 mM EDTA). Purified proteins were concentrated by ultrafiltration (Microcon MWCO, 10 kDa; Millipore), loaded onto a 4–12% NuPAGE gel (Invitrogen) for SDS/PAGE, and visualized with SilverQuest Silver Staining kit (Invitrogen). Excised gel slices were submitted to the University of Iowa Proteomics Facility and protein identities were determined by mass spectrometry using a LTQ XL linear ion trap mass spectrometer (Thermo Scientific).

For interaction domain mapping studies, cells were transfected in six-well plates with a total 1.5 μg of indicated plasmids using FuGENE HD (Promega). After 24–30 h of transfection, cell lysates were immunoprecipitated with anti-FLAG (Sigma) or anti-Myc (Santa Cruz) antibodies conjugated to agarose beads for 2 h at 4 °C. Beads were washed in the lysis buffer, and precipitated proteins were analyzed by SDS/PAGE and Western blotting following standard protocols.

GST Pull-Down Assay. HA-tagged INPP5E was produced by using TNT Coupled Reticulocyte Lysate system (Promega) following the manufacturer's instructions. For GST and GST-ARL13B, BL21 (DE3) strain of *Escherichia coli* cells (Invitrogen) containing pGEX4T-2 (GE Healthcare Life Sciences) or pGEX6P1–ARL13B were cultured in Luria-Bertani medium and induced with 0.1 mM isopropyl-1-thio-β-D-galactopyranoside at 15 °C for 16 h. Cells were lysed with BugBuster protein extraction reagent (Novagen) with lysozyme (Sigma) and benzonase (Novagen). After centrifugation for clearance, lysates were incubated with glutathione sepharose resin (GE Healthcare Life Sciences) at 4 °C for 1 h. Glutathione resin was extensively washed with PBST and directly used for GST pull-down assay. In vitro translated HA-INPP5E was diluted in 600 μL of binding buffer (50 mM Hepes pH 7.0, 150 mM NaCl, 2 mM MgCl2, 0.5% (vol/vol) Triton X-100, 2 mM EGTA) and incubated with GST or GST–ARL13B bound beads at 4 °C for 2 h. After extensive washing with the binding buffer, bound proteins were subjected to SDS/PAGE and Western blotting.

Immunofluorescence Microscopy. Localization studies were conducted using hTERT–RPE1 and IMCD3 cells, which were maintained in DMEM/F12 (Invitrogen) supplemented with 10% FBS. Cells were seeded on glass coverslips in 24-well plates and transfected with indicated siRNAs using RNAiMAX (Invitrogen) for 48 h following the manufacturer's instructions for reverse transfection or with plasmid DNAs using FuGENE HD (Promega) for 24 h. For ARL13B rescue experiments, cells were first transfected with siRNA targeting ARL13B 3′ UTR for 24 h, followed by plasmid DNA transfection for another 24 h. Cells were further incubated in serum-free medium for 24 h for ciliation. Cells were fixed first with 4% (wt/vol) formaldehyde in PBS followed by methanol fixation. Samples were blocked with 5% (wt/vol) BSA and 5% (vol/vol) normal goat serum (NGS) in PBST (PBS with 0.1% Triton X-100), and incubated with primary antibodies in the blocking buffer with 1% NGS. Primary antibodies were visualized by secondary antibodies conjugated with Alexa Fluor 488 and Alexa Fluor 568 (Invitrogen). Coverslips were mounted on VectaShield mounting medium with DAPI (Vector Lab), and images were taken with an Olympus IX71 or Zeiss 710 confocal microscope.

Quantitative Real-Time PCR. For quantitative real-time PCR, (qRT-PCR), total RNA was extracted using TRIzol Reagent (Invitrogen) following manufacturer instructions. Quantitative PCR was performed as previously described (1, 2). Briefly, 1 μg of total RNA was used for cDNA synthesis using SuperScript III reverse transcriptase (Invitrogen). Quantitative real-time PCR was performed with iQ SYBR Green Supermix (Bio-Rad) and a Mx3000P QPCR system (Stratagene). RPL19 mRNA levels were used for normalization, and the ΔΔCt method (3) was used to calculate changes in gene expression. The PCR products were confirmed by melt-curve analysis and sequencing. PCR primer sequences are shown in Table S1.

1. Seo S, et al. (2010) BBS6, BBS10, and BBS12 form a complex with CCT/TRiC family chaperonins and mediate BBSome assembly. *Proc Natl Acad Sci USA* 107(4):1488–1493.
2. Seo S, et al. (2011) A novel protein LZTFL1 regulates ciliary trafficking of the BBSome and Smoothened. *PLoS Genet* 7(11):e1002358.
3. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 25(4):402–408.

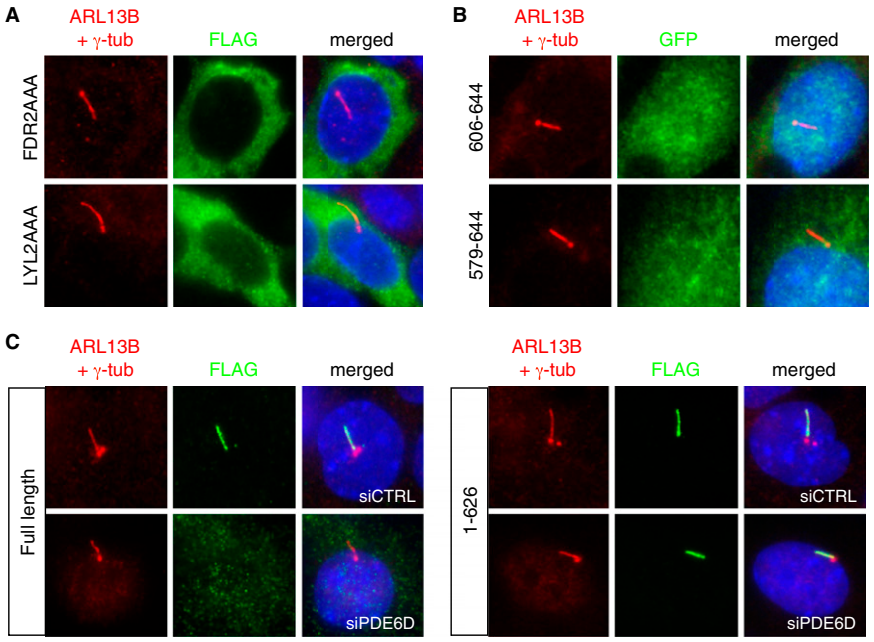

Fig. S1. Localization of INPP5E mutant variants. (*A*) Mislocalization of FLAG-INPP5E, FDR2AAA, and LYL2AAA mutants (green). ARL13B and γ-tubulin antibodies (red) were used to label cilia and centrosomes, respectively. (*B*) Localization of GFP fused to INPP5E C-terminal sequences. Indicated portions of INPP5E C terminus were fused to the C terminus of GFP (green) and their localization was examined by indirect immunofluorescence microscopy using GFP antibodies. (*C*) INPP5E 1–626 localizes to cilia in a PDE6D-independent manner.

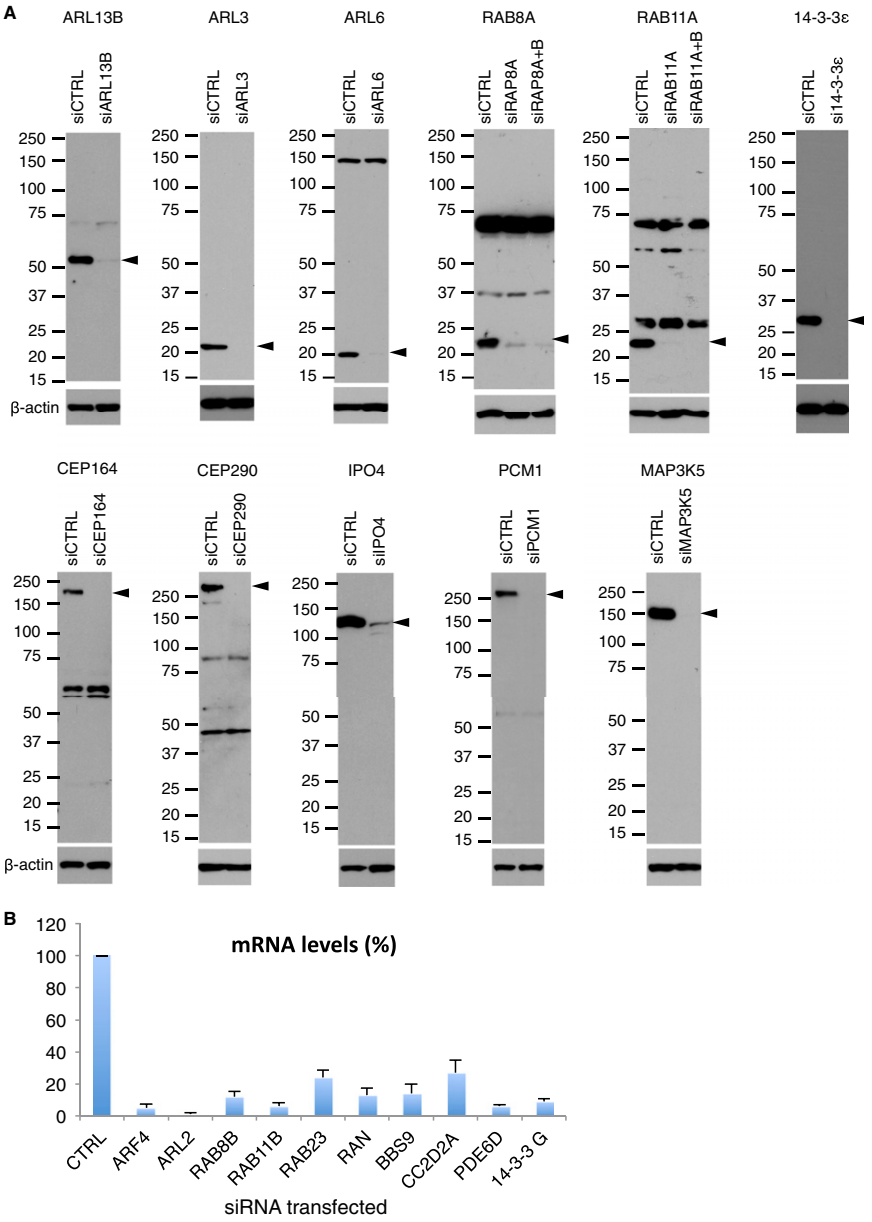

Fig. S2. Verification of siRNA-mediated gene knockdowns by immunoblotting and quantitative PCR. (*A*) RPE1 cells were transfected with either control or indicated siRNAs for 48 h and further incubated in a serum-free medium for 24 h. Cell lysates were analyzed for immunoblotting using indicated antibodies. Black arrowheads denote endogenous target proteins. β-Actin was used as a loading control, and migration of size markers is shown (*Left*). (*B*) mRNA levels were quantified by qRT-PCR in RPE1 cells transfected with indicated siRNAs. Error bars represent SD in duplicate qPCR reactions.

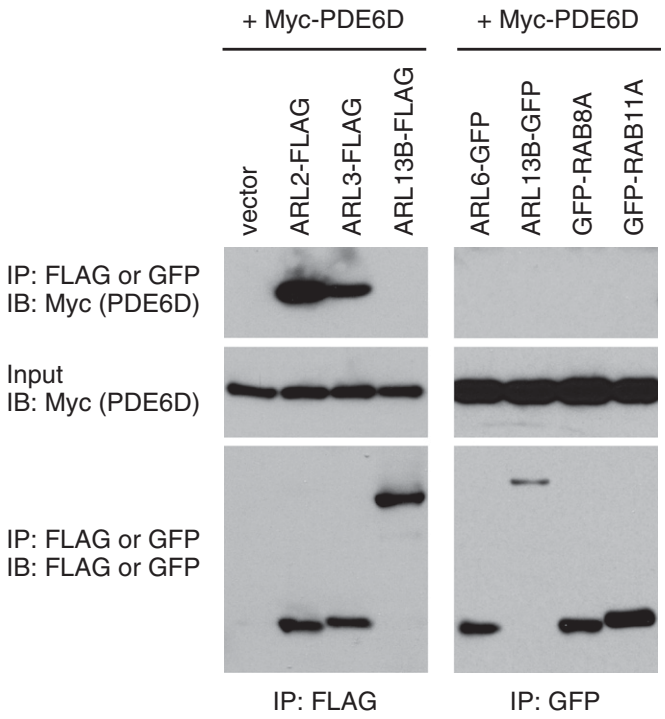

Fig. S3. PDE6D interacts with ARL2 and ARL3 but not with ARL13B. ARL2, ARL3, ARL6, and ARL13B with C-terminal FLAG or GFP tags were cotransfected with Myc-tagged PDE6D into HEK293T cells. Cell lysates were subjected to coimmunoprecipitation analyses using anti-FLAG or anti-GFP antibodies. Interestingly, although RAB8A and RAB11A are known to be prenylated (geranylgeranylated), RAB8A and RAB11A also did not show interaction with PDE6D, suggesting that there is at least some level of specificity in PDE6D binding to prenylated proteins.

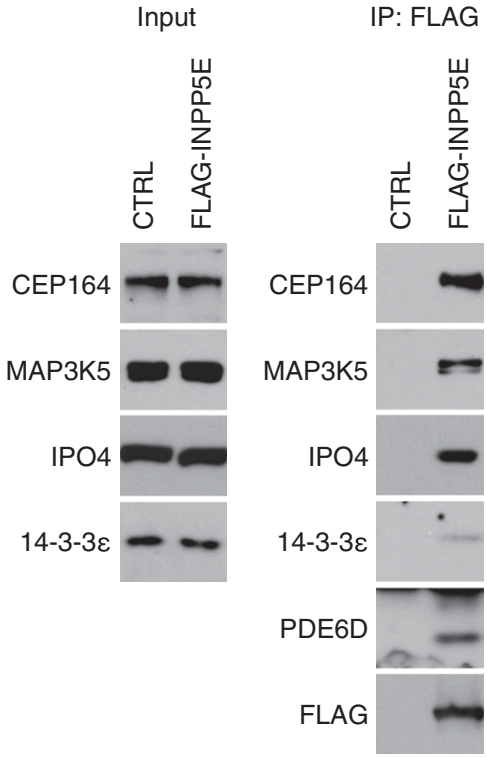

Fig. S4. Confirmation of physical interactions of centrosomal protein 164 (CEP164), MAP3K5, IPO4, 14-3-3ε, and PDE6D.

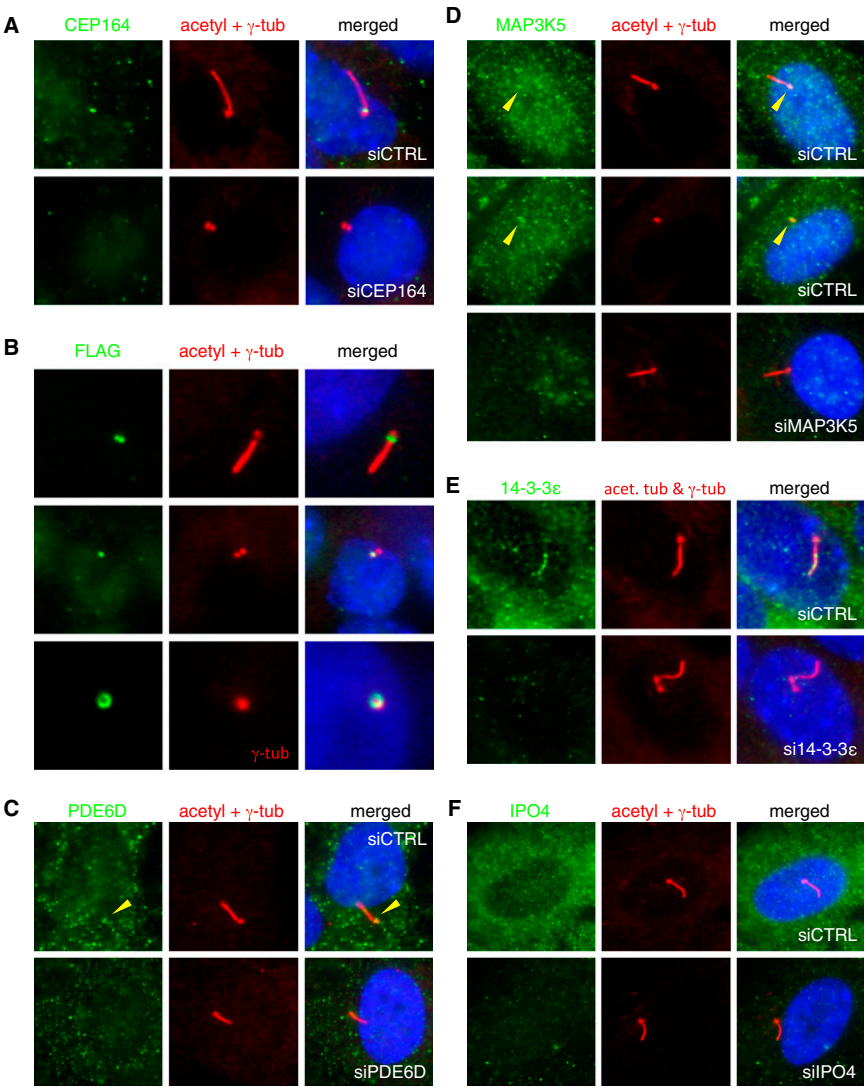

Fig. S5. Localization of INPP5E interacting partners. (*A*) Localization of CEP164. Endogenous CEP164 (green) is detected between the ciliary shaft and centrosomes. Cilia and centrosomes (red) were labeled by acetylated tubulin and γ-tubulin antibodies. (*B*) Localization of FLAG-CEP164 in IMCD3 cells (green). FLAG-CEP164 (green) localizes to the mother centriole in both ciliated and unciliated cells. FLAG-CEP164 often shows a doughnut-shaped staining pattern that is slightly larger than that of γ-tubulin (*Bottom*). Cilia and centrosomes were labeled by acetylated tubulin and γ-tubulin antibodies (red). (*C* and *D*) Both PDE6D (*C*; green) and MAP3K5 (*D*; green) are found throughout the cytoplasm but specific enrichment around the centrosome is evident (yellow arrowheads). (*E*) Ciliary localization of 14-3-3ε. (*F*) Localization of IPO4.

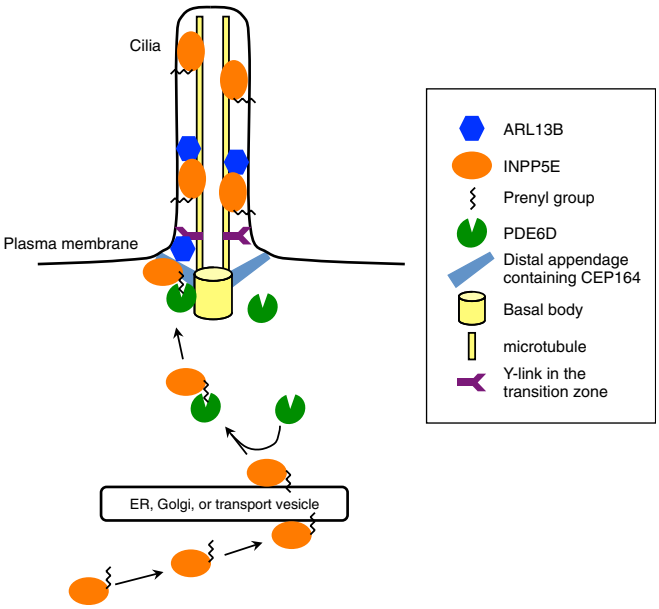

Fig. S6. Model for ARL13B-, PDE6D-, and CEP164-dependent ciliary targeting of INPP5E.

Table S1. Primers used for qRT-PCR

Primer name	Sequence
F-hRPL19	CCGGAAGCTCATCAAAGATGG
R-hRPL19	TTGGCTGTACCCTTCCGCTTA
F-hARF4	GTTTAGTTTGCCTGGGAGCTT
R-hARF4	CTGCTTCTTGCCAAATAGTCG
F-hARL2	TTCAACATCAAGACCCTGGAG
R-hARL2	GCTGTCCACTACCCAGATGAG
F-hBBS9	GGCCCATGACTGAGAGAGAAG
R-hBBS9	CACTCTGTTCTGCAGTGTGACC
F-hCC2D2A	GGCCAAGAAGAAGAAAAGGAA
R-hCC2D2A	CACCTCCTGCTCTATCACTGC
F-hPDE6D	ATCCTGAGGGGCTTCAAACTA
R-hPDE6D	TGCACTTGAGGATTTTCTTGG
F-hRAB8B	TCTCTTCAAGCTCCTGCTGAT
R-hRAB8B	GGTGGAGATGAAGGTGGTGT
F-hRAB11B	GGGACGACGAGTACGACTACC
R-hRAB11B	CTCTCCAGGTTGAACTCGTTG
F-hRAB23	GGTGAGCTCCTGCTCTCATAA
R-hRAB23	TTCAAAGCTGGGTTCTCAGTG
F-hRAN	CCCTTCCTCTGGCTTGCTA
R-hRAN	GGGAGAGCAGTTGTCTGAGC
F-mYwhag	CAGCACCCAAGTCTACTCTGC
R-mYwhag	TCTTCCTAGTGGGTTCCCTGT
F-mRpl19	GCAAGCCTGTGACTGTCCATT
R-mRpl19	GCATTGGCAGTACCCTTCCTC

Other Supporting Information Files

Dataset S1 (XLSX)