

Supporting Online Material

Materials and Methods

Constructs

Full length mouse Htr6 (a gift from Dr. Kirk Mykytyn, Ohio State University) (1) was subcloned into pEF5P-FRT-SEP containing GFP pH sensitive mutant called super-ecliptic pHluorin (SEP) (2) at Htr6's N-terminus to generate pEF5P-FRT-SEP-Htr6 (Htr6^{SEP}); super-ecliptic pHluorin allows detection of only surface-exposed Htr6. The cytoplasmic tail of mouse fibrocystin _{CTS}PKHD1 (1-193) (a gift from Dr. Gregory J. Pazour, University of Massachusetts Medical School) (3) was subcloned into pEF5B-FRT-EGFP to generate pEF5B-FRT-EGFP-_{CTS}PKHD1 (_{CTS}PKHD1^{GFP}). Mouse Sstr3^{GFP} (a gift from Dr. Kirk Mykytyn) (1) and mouse Smo^{YFP} constructs (4) were described previously. Mouse SEPT2 was subcloned from SEPT2-YFP (5) into pEF5P-FRT-LAP to generate pEF5P-FRT-LAP-SEPT2 (SEPT2^{LAP}) using Gateway subcloning (Invitrogen). CEP164^{LAP} and ODF2^{LAP} constructs were kindly provided by Dr. Tim Stearns (Stanford University). RFP-pericentrin PACT domain (PeriCT^{RFP}) (6) was a gift from Dr. Sean Munro (MRC Laboratory of Molecular Biology, Cambridge). GPI^{YFP} and VSV-G^{GFP} were a gift from Dr. Kai Simons (Max Planck Institute, Germany). SEPT2 shRNAs were synthesized, purified (Elimbio) and cloned into pSuper-puro to generate pSuper-puro-shRNA1/2/3. SEPT2 ShRNA1 sequence was described previously (5, 7, 8). SEPT2 shRNA2 and shRNA3 sequences were predicted using siDesign-Center (Dharmacon):

SEPT2 shRNA1: 5'-GGUGAAUAUUGUGCCUGUC-3';

SEPT2 shRNA2: 5'-GCAGGAAAGUAGAGAAUGA-3';

SETP2 shRNA3: 5'-UGAAAGAACUGUCCAGAU-3'.

All shRNAs were subject to BLAST search and found to have at least four or more mismatches from other genes. pSuper-puro vector was generously provided by Dr. Ron Kopito (Stanford University).

Cell culture

Inner medullary collecting duct cells (IMCD3) were maintained at 37°C in 5% CO₂ in air in DMEM/F12 (Invitrogen) containing 10% fetal bovine serum (FBS). Mouse embryonic fibroblasts (MEF) were maintained in high glucose DMEM containing 10% FBS. To induce ciliogenesis, cells were shifted from 10% serum to 0.2% serum for 24 hours.

Stable cell lines, transfections and siRNA

Stable IMCD3 cell line was generated using Flp-InTM system (Invitrogen). Transgene was integrated at a single, predetermined locus in an IMCD3 host cell line using flippase (Flp) recombination target (FRT)/Flp-mediated recombination (Flp-In system). To generate cell lines stably expressing Htr6^{SEP} (^{FI}Htr6^{SEP}), ^{CTS}PKHD1^{GFP} (^{FI}/_{CTS} PKHD1^{GFP}) or SEPT2^{LAP}, Flp-In cells were first generated and verified according to the manufacture's instructions (Invitrogen). Subconfluent Flp-In IMCD3 cells were transiently transfected with pEF5P-FRT-SEP-Htr6, pEF5B-FRT-EGFP-^{CTS}PKHD1 or

pEF5P-FRT-LAP-SEPT2 and split 48 hours later into medium containing 5ug/ml puromycin or 5ug/ml blasticidin (pEF5B-FRT-EGFP-CTS_{PKHD1}). Single cell clones were selected for moderate expression levels. To generate cell lines in which SEPT2 was stably knocked down, subconfluent IMCD3 parental cells were transfected with pSuper-puro-shRNA1, 2, or 3 and split 48 hours later into medium containing 5ug/ml puromycin. Single cell clones were tested for SEPT2 knock down efficiency by immunofluorescence and western blotting for SEPT2. The IFT88^{YFP} stable cell line was previously described (a gift from Dr. David R. Beier) (9). SEPT2 siRNA oligonucleotides (the same sequence as shRNA1) and scrambled oligonucleotides (Dharmacon) have been described previous (5, 7, 8). SEPT2 siRNA and scrambled siRNA oligonucleotides were transiently transfected into cells using RNAi Max (Invitrogen) following the manufacturer's instructions. Cells were shifted from 10% serum to 0.2% serum for 24 hours after transfection of siRNA or plasmids to induce ciliogenesis before fixation. Plasmids were transfected into cells using Fugene HD (Roche) or Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions.

Live cell imaging and FRAP

Cells were grown on collagen-coated imaging dishes at subconfluent density and imaged in phenol red-free DMEM or DMEM/F12 supplemented with 25 mM HEPES using the Marianas system (Intelligent Imaging Innovations) equipped with the MicroPoint fluorescence recovery after photobleaching (FRAP) laser system

(Photonic Instruments). Cells were serum-starved for 24 hours before live cell imaging. Photobleaching experiments were carried out using the FRAP module of the Marianas system. Equivalent laser intensity, repetition, and exposure time were used for FRAP experiments. Bleached regions were accurately positioned along each cilium (Fig. 1 and 4; Fig. S1 and S5), or on the periciliary membrane (Fig. S1). No-neighbors deconvolution was used with a 3D image (Fig. S1A b) and post-acquisition analyses were performed using Slidebook 5.0. Sum (sIF) and mean (mIF) fluorescence intensities were measured using the mask and pencil tools of Slidebook. Fluorescence intensity (FI) in a primary cilium (Fig. 1B and D; Fig. 4B; Fig. S1B and S5B) = $(mIF_{\text{cilia}} - mIF_b) \times \text{Area}$, where mIF_{cilia} is mean fluorescence intensity in the cilium, mIF_b is the mean background fluorescence intensity in a representative region around the cilium and Area is the pixel value of the cilium. Recovery percentages (RP) = $FI_{\text{end}} \div FI_{\text{before}}$ where FI_{before} is the FI in the cilium before photobleaching and FI_{end} is the FI in the cilium at the last time point indicated in each figure. Mobility fraction = $(FI_{\text{bl}'} \div FI_{\text{bl}}) \div (FI_{\text{f}'} \div FI_{\text{f}})$ where $FI_{\text{bl}'}$ is the FI in the bleached region of the cilium after equilibrium and FI_{bl} is the FI in the bleached region of the cilium before photobleaching; $FI_{\text{f}'}$ is the FI in the whole cilium after equilibrium and FI_{f} is the FI in the whole cilium before photobleaching.

Immunostaining, antibodies and imaging

Subconfluent cells were fixed with warm PHEM (60 mM Pipes-KOH, pH 6.9, 25 mM HEPES, 10 mM EGTA, and 1 mM MgCl₂) containing 3% paraformaldehyde (EM

Sciences) and 0.1% TritonX-100 and stained with antisera to SEPT2 (N5N; rabbit polyclonal; a generous gift from Dr. Makoto Kinoshita, Nagoya University, Japan) (5, 7, 8), acetylated α -tubulin (Sigma-Aldrich; mouse monoclonal), α -tubulin (YL1/2; rat monoclonal), Odf2 (Sigma-Aldrich; rabbit polyclonal), polyglutamylated tubulin (GT335; monoclonal; a generous gift from Dr. Carsten Janke, CNRS, France), γ -tubulin (Sigma-Aldrich; mouse monoclonal), GFP (Invitrogen; rabbit polyclonal) and secondary FITC- or rhodamine redX- or Cy5- conjugated F(ab')₂ goat anti-mouse, anti-rabbit or anti-rat IgGs (Jackson ImmunoResearch Laboratories). Samples were mounted in VECTASHIELD mounting medium (Vector Laboratories) and imaged with an inverted Zeiss microscope (Axiovert 200) or an Applied Precision Delta Vision wide field deconvolution system (Fig. 2A a; Fig. S3E) or a Zeiss-Yokogawa spinning disk confocal system (Fig. S2). Images using deconvolution system were captured, deconvolved and one focal plane was shown. Structured illumination was done using DeltaVision OMX SRTM 3D super-resolution imaging system (Jennifer Atkins, Applied Precision; Fig. 2A d; Movie S14, S15). Briefly, raw data were acquired by illuminating samples with a 3 dimensional sinusoidal pattern of light. The illumination pattern was rotated to three angles, and laterally shifted 5 times at each rotation. A total of 15 images were saved from each z-section of 125nm spacing. The raw data stack was fed into the reconstruction algorithm in the softWoRx software, and the super resolution image was returned. Cilia were scored by acetylated α -tubulin staining longer than 1 μ m. The FI of SEPT2 at the base of the primary cilium (Fig. S4C) was calculated by subtracting the background FI in the periciliary region from

the FI at the base of the primary cilium. Diffusion barrier index (Fig. 4D) = $mIF_{\text{cilia}} \div mIF_{\text{b}}$ where mIF_{cilia} is mean FI in the cilium, mIF_{b} is the mean FI in a representative periciliary region. Images were quantified using ImageJ.

Assays for Sonic hedgehog pathway and QPCR

For live cell imaging, MEF cells stably expressing Smo^{YFP} (Fig. 1) or IMCD3 cells expressing Smo^{YFP} (Fig. 4) were grown to subconfluence in 10% serum medium and treated with 100nM SAG or Shh for 24 hours in medium containing 0.2% serum before imaging. SAG was provided by James Chen (Stanford University).

Conditioned medium made from 293T cells expressing full-length Shh was used as the source of Shh as published previously (10). To assess downstream gene induction in IMCD3 parental cells and SEPT2-depleted cells, an equal amount of cells was plated in a 96 well plate and treated with vehicle or 100nM SAG or Shh in medium containing 0.2% serum for 24 hours. Cells were lysed and QPCR was carried out using TaqMan Gene Expression Cells-to-CT kit (Ambion) according to the manufacturer's instructions. Gene induction was normalized using the internal control gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and calculated by comparing the relative values of gene expression level treated with vehicle and SAG or Shh. TaqMan gene expression probes (Applied Biosystems) used were: Mm00494645_m1 (Gli1), Mm00970977_m1 (Ptc1) and Mm99999915_g1 (GAPDH). Cells were plated in triplicate and error bars represent the SD of three independent experiments.

Immunoblotting

Cell lysates was separated by 10% SDS-PAGE and proteins transferred onto Immobilon P membranes (Millipore), and incubated overnight at 4°C in blocking buffer (10 mM Tris HCl, pH 6.8, 150 mM NaCl, 1 mM DTT, 0.1% Tween-20, 2% BSA). Antibodies were diluted in blocking buffer and incubated with the membrane for 2 hours. Blots were subsequently incubated with secondary AlexaFluor680 and IRDye800-conjugated antibodies and scanned in a Li-COR infrared imager. Quantification of protein bands was performed with the Odyssey Infrared Imaging System (LI-COR Biosciences).

Statistical analysis

Two-tailed paired t test was used in Fig. 3C and two-tailed unpaired t test was used in Fig. 4F. The other P values were calculated using on Mann-Whitney two-tailed test in Prism software. Box-whisker plots show the 25th to 75th percentiles (box), 5th and 95th percentiles (whiskers), and median (line within the box). All Plots were generated using OriginPro 8.0.

Supporting Figure Legends

Figure S1: (A) FRAP of the whole cilium 24hrs after serum starvation of IMCD3 cells stably expressing $^{FI}_{CTS}$ PKHD1^{GFP} (a), or transiently expressing $_{CTS}$ PKHD1^{GFP} (b) or Sstr3^{GFP} (c); arrowheads mark one end of the cilium. Scale bars, 5µm. (B) Kinetics

of average (\pm SEM) fluorescence recovery of proteins within the primary cilium (n=8-14). (C) FRAP of Htr6^{SEP} transiently over-expressed in the periciliary membrane of IMCD3 cells; the base of ciliary membrane indicated by PeriCT^{RFP}, and the photo-bleached region indicated by the circle. Scale bar, 2 μ m. Note fluorescence recovery of Htr6^{SEP} in the periciliary membrane (graph), but not in the primary cilium, which was also photo-bleached. (D) FRAP of ciliary membrane proteins following photobleaching part of the cilium represented as heat-map images; dotted lines mark the photobleached/unbleached boundary. Scale bars, 5 μ m. (E) Representative example of kinetics of fluorescence recovery of ciliary membrane proteins in the photobleached region (orange curve), unbleached region (blue curve) and the two regions combined (red curve) of a primary cilium (n=9-14). (F) Summary of the mobile fraction of ciliary membrane proteins photo-bleached in part of the cilium: ^{FI}Htr6^{SEP}, ^{FI}_{CTS} PKHD1^{GFP}, Sstr3^{GFP} expressed in IMCD3 cells, and Smo^{YFP} in MEFs treated with SAG for 24hrs (n=9-14).

Figure S2: (A, B) Confocal images of IMCD3 cells expressing GPI^{YFP} (A) or VSV-G^{GFP} (B) stained for AcTub; scale bars, 10 μ m. Higher magnification of boxed regions are shown as insets; scale bars, 2 μ m.

Figure S3: (A) IMCD3 cells stably expressing _{CTS}PKHD1^{GFP} (^{FI}_{CTS} PKHD1^{GFP}) stained for SEPT2; scale bar, 2 μ m. (B) Fixed IMCD3 cells stained for SEPT2 and AcTub. Arrows mark the base of the primary cilium, and arrowheads mark SEPT2 staining

along, and at the tip of axoneme. Scale bar, 2 μ m. (C) Whole cell lysates of parental IMCD3 cells (parental) and IMCD3 cells stably expressing SEPT2^{LAP} immunoblotted for SEPT2 (upper panel) and GFP (SEPT2^{LAP}, lower panel); SEPT2^{LAP} band intensity is 3.93% of endogenous SEPT2. (D) Upper panel: fixed IMCD3 cells stably expressing SEPT2^{LAP} stained for SEPT2 and ^{Ac}Tub. Scale bar, 10 μ m. Lower panel: boxed region in upper panel is enlarged; arrows mark the base of a primary cilium. Scale bar, 2 μ m. (E) Odf2 and ^{Ac}Tub localization at the cilium of IMCD3 cells stably expressing SEPT2^{LAP}; below, fluorescence intensity profiles of protein staining from the basal body to the tip of cilium. Scale bar, 2 μ m. (F) IMCD3 cells (a) or IMCD cells transiently expressing CEP164^{LAP}(b), ODF2^{LAP}(c), or PeriCT^{RFP}(d) extracted with buffer containing 0.5% TritonX-100 for 1min, fixed and stained for SEPT2 and ^{Ac}Tub. Scale bar, 2 μ m.

Figure S4: (A) IMCD3 cells transfected with scrambled oligos (a) or SEPT2 siRNA (b, c) were serum-starved to induce ciliogenesis, and stained for SEPT2 and ^{Ac}Tub, scale bars, 10 μ m; a' and b' show higher magnifications of the cilium and SEPT2 staining, scale bars, 2 μ m. (B) Distribution of SEPT2 fluorescence intensities at the base of the primary cilium of scramble or siSEPT2 IMCD3 cells (n=53-58); ***p<0.0001. (C) Distributions of cilia length (Top) and SEPT2 fluorescence intensities at the base of the primary cilium (Bottom) of parental IMCD3 cells and different clones of SEPT2 stable knockdown cells (n=8-96). (D) Fixed parental IMCD3 cells, IMCD3 cells stably depleted of SEPT2 using shRNA1, shRNA2,

shRNA3 stained with GT335, an antibody against polyglutamylated tubulin and a marker for the primary cilium. Scale bars, 10 μm .

Figure S5: (A, B) FRAP of the whole cilium of parental IMCD3 (Parental) and ShRNA1 IMCD3 cells transiently expressing $\text{CTS}^{\text{PKHD1}^{\text{GFP}}}$ or $\text{Sstr3}^{\text{GFP}}$ (A; scale bars, 5 μm), and kinetics of average (\pm SEM) fluorescence recovery (B; n = 8-12). (C) SEPT2 and $^{\text{Ac}}$ Tub staining at the primary cilium of parental and ShRNA1 IMCD3 cells transiently expressing Htr6^{SEP} (top panels) or Smo^{YFP} (bottom panels).

Figure S1

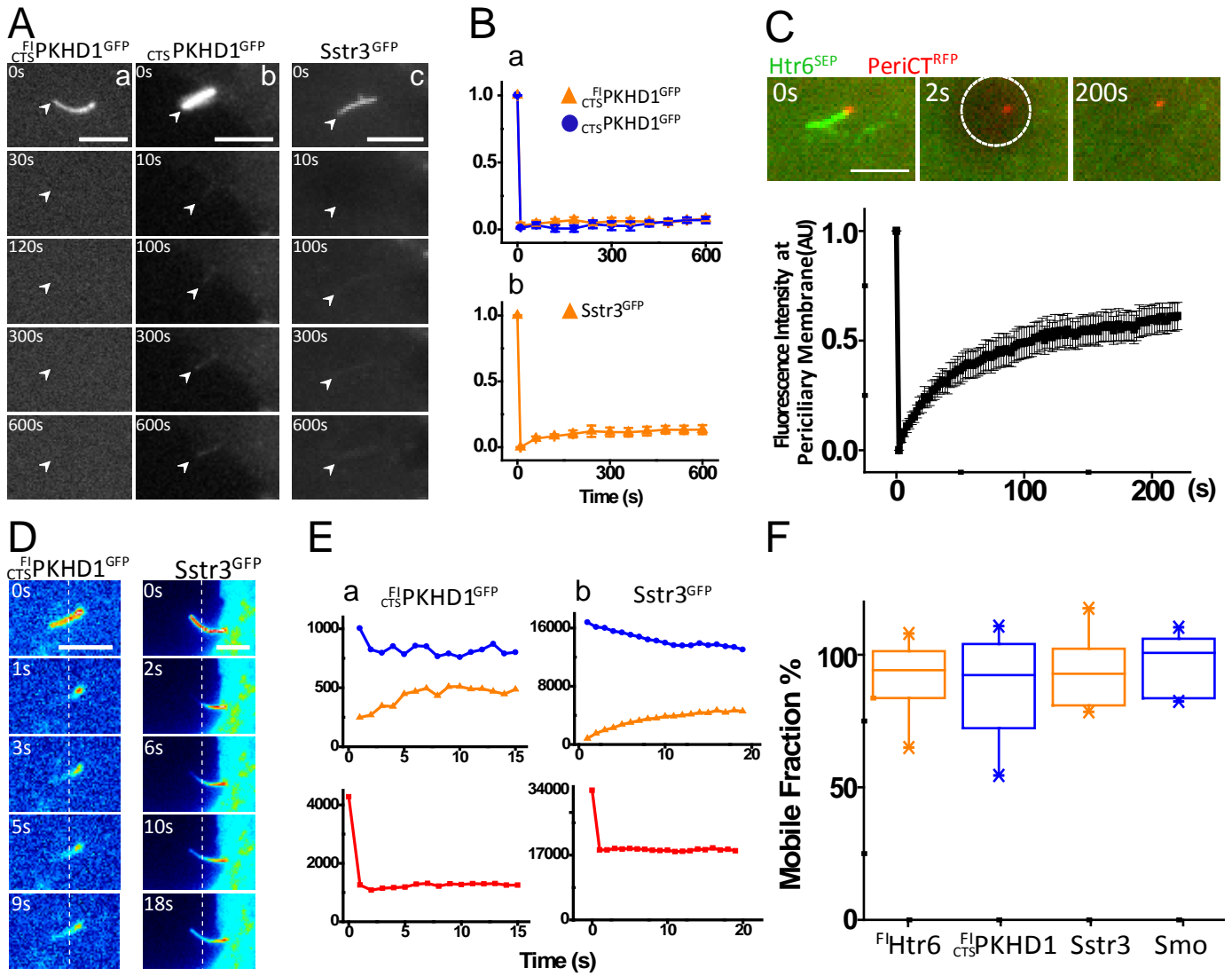
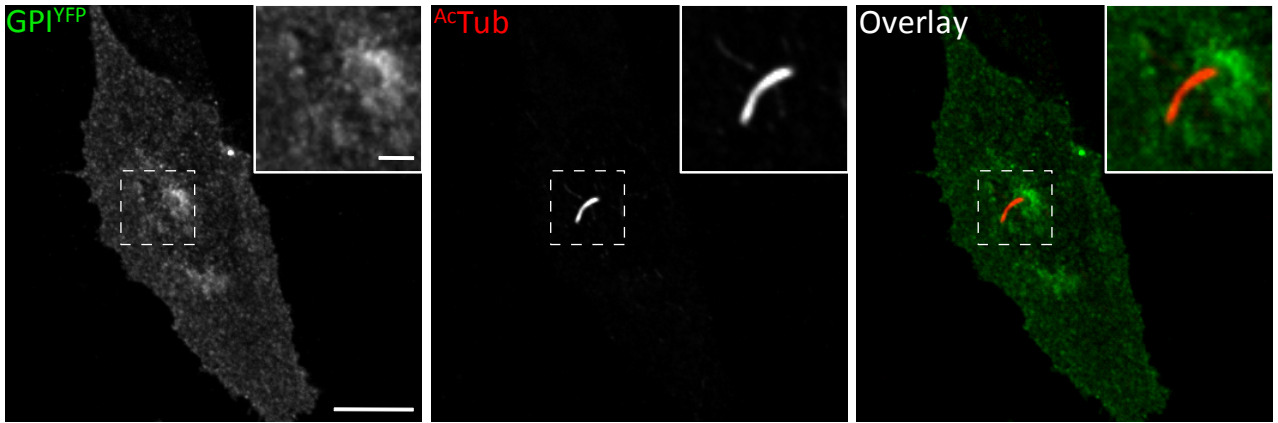


Figure S2

A



B

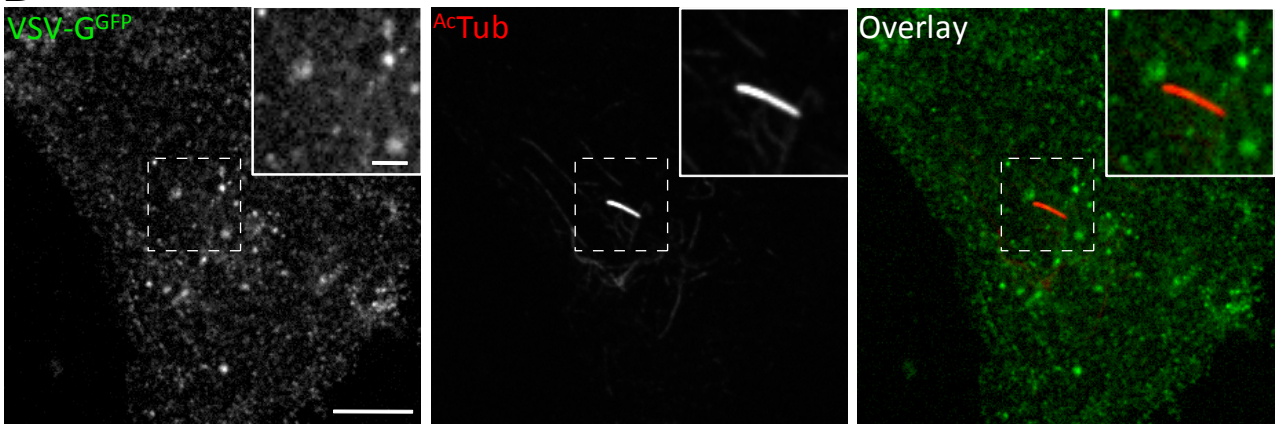


Figure S3

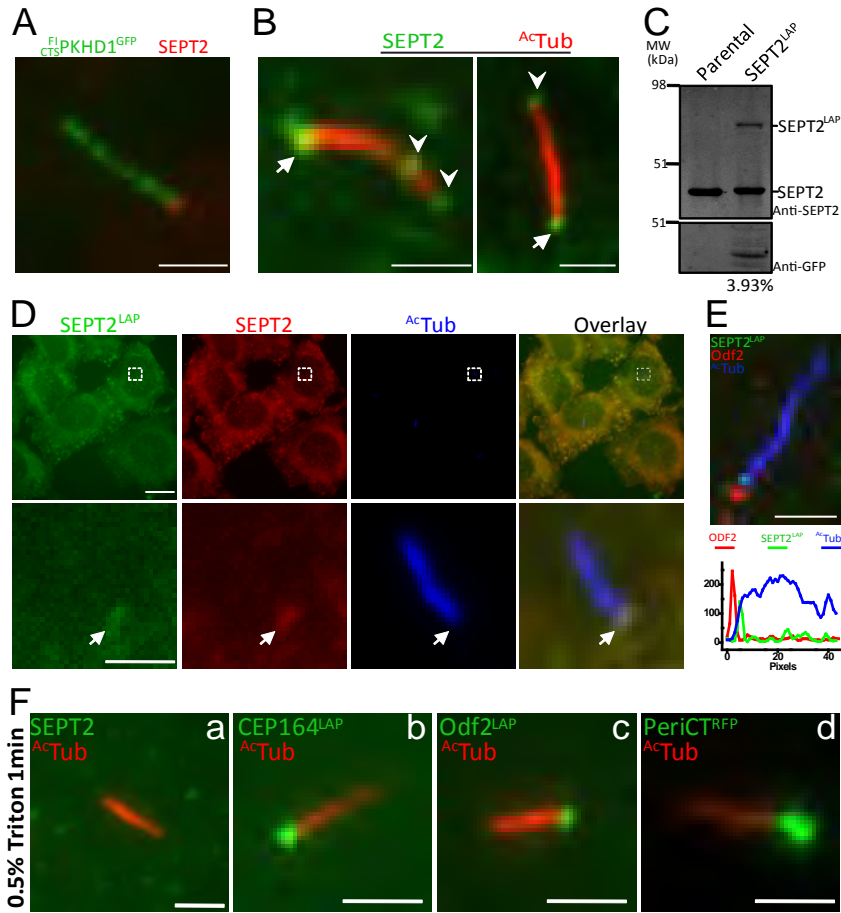


Figure S4

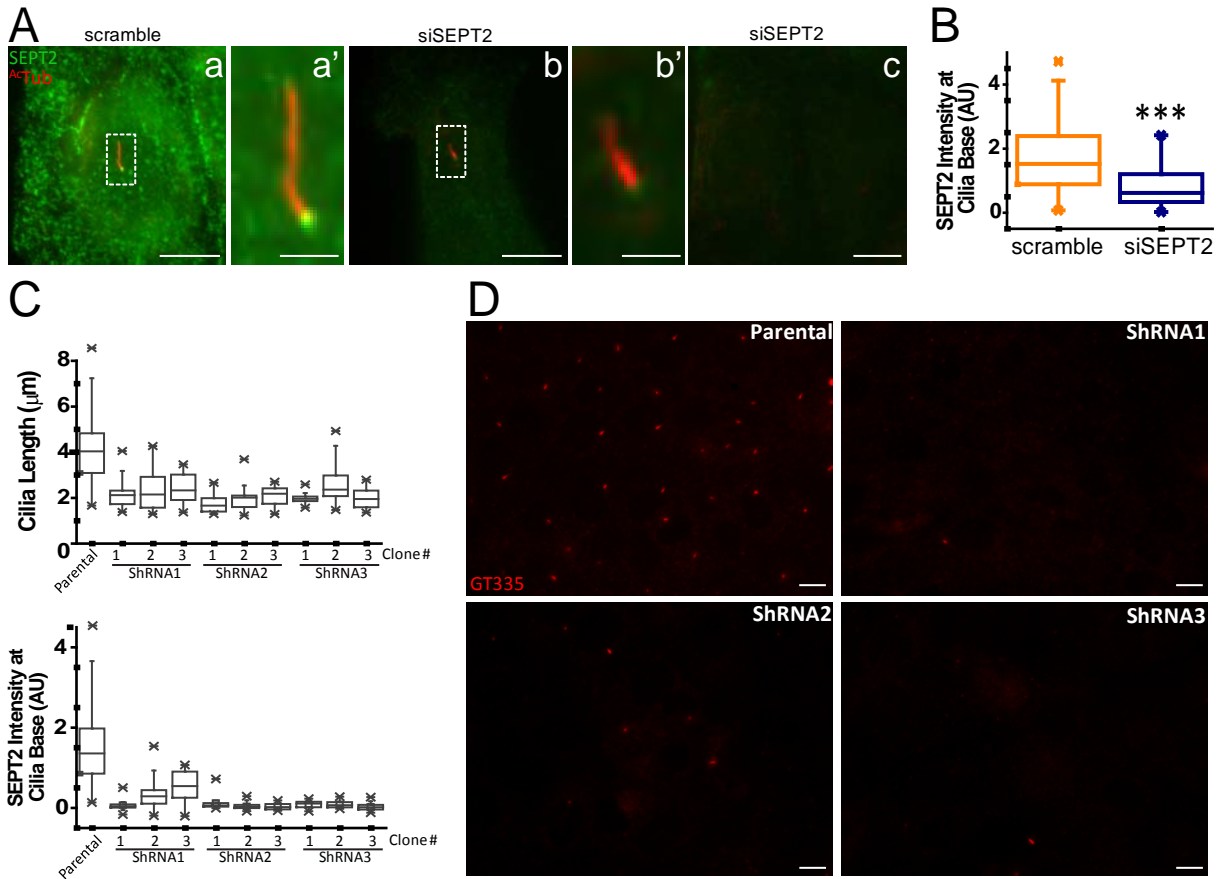
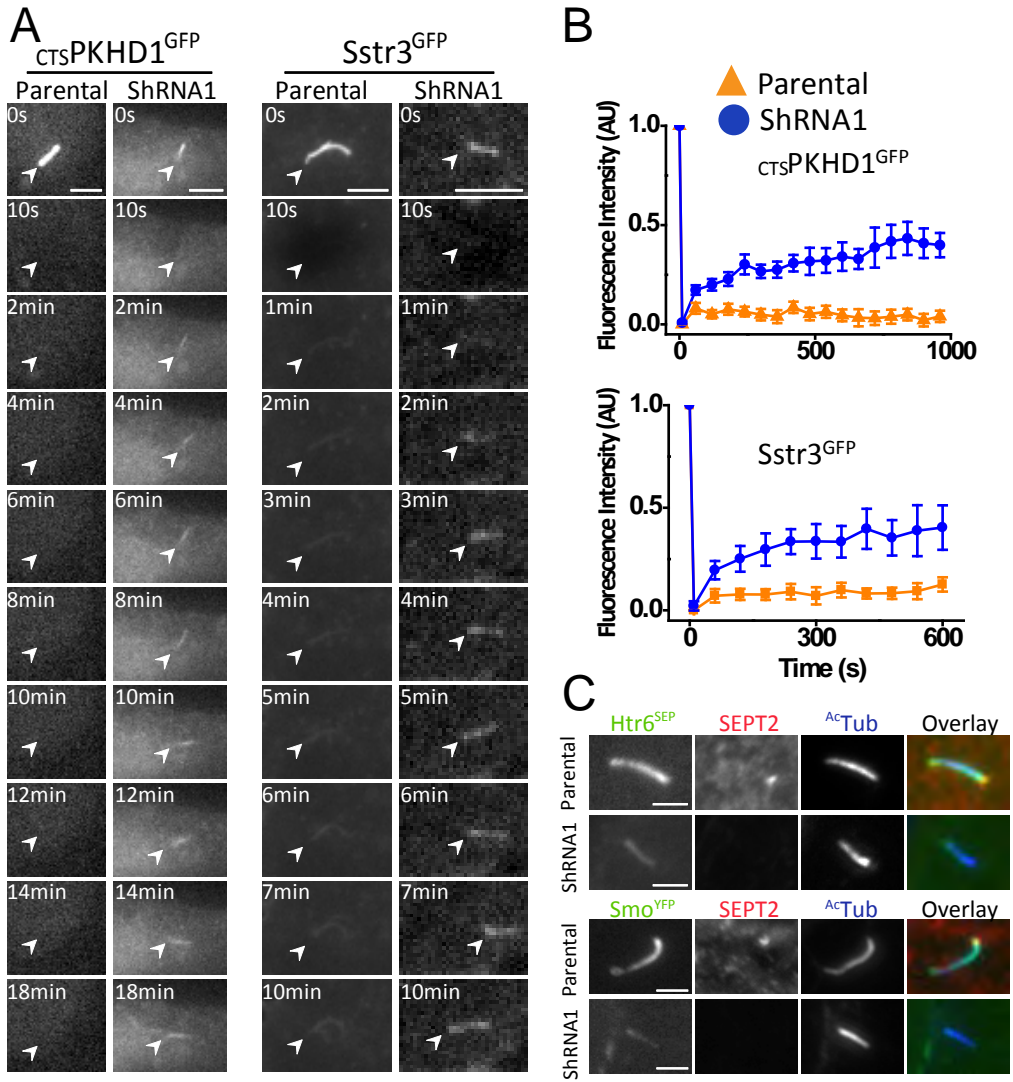


Figure S5



Supporting Tables S1-S3

Table S1: Percent recovery of fluorescence of ciliary membrane proteins in the cilium of IMCD3 cells and MEFs (average \pm SEM).

Proteins	Recovery percentage	n
^{FI} Htr6 ^{SEP}	2.7 \pm 0.9%	12
Htr6 ^{SEP}	5.5 \pm 2.5%	8
^{FI} _{CTS} PKHD1 ^{GFP}	8.1 \pm 2.0%	14
_{CTS} PKHD1 ^{GFP}	7.0 \pm 2.4%	11
Sstr3 ^{GFP}	14.0 \pm 3.1%	8
Smo ^{YFP*}	5.7 \pm 1.3%	10
IFT88 ^{YFP}	61.6 \pm 7.4%	11
Htr6 ^{SEP**}	61.2 \pm 6.2%	6

*: MEFs

** : Periciliary membrane Htr6^{SEP} in Fig. S1C

Table S2: Mobile fraction of ciliary membrane proteins in the cilium of IMCD3 cells and MEFs (average \pm SEM).

Proteins	Mobile Fraction	n
^{FI} Htr6 ^{SEP}	91.2 \pm 3.7%	13
^{FI} _{CTS} PKHD1 ^{GFP}	88.7 \pm 4.9%	14
Sstr3 ^{GFP}	93.9 \pm 4.8%	9
Smo ^{YFP*}	97.3 \pm 3.3%	12

*: MEFs

Table S3: Percent recovery of fluorescence of ciliary membrane proteins in parental and shRNA1 IMCD3 cells (average \pm SEM).

Proteins	Parental cells		ShRNA1 cells	
	Recovery percentage	n	Recovery percentage	n
Htr6 ^{SEP}	5.7 \pm 3.7%	9	34.1 \pm 7.9%	12
CTSPKHD1 ^{GFP}	4.0 \pm 2.7%	9	35.2 \pm 5.3%	12
Sstr3 ^{GFP}	12.6 \pm 3.5%	9	40.4 \pm 10.9%	8
Smo ^{YFP}	7.0 \pm 4.2%	10	51.1 \pm 10.4%	9

Supporting References and Notes

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Supporting Movie Captions

Movie S1: FRAP the whole cilium in IMCD3 cells stably expressing Htr6^{SEP}. Images were captured every 3 s for 10 minutes with an exposure of 500ms.

Movie S2: FRAP of the whole cilium in IMCD3 cells transiently expressing Htr6^{SEP}. Images were captured every 10 s for 10 minutes with an exposure of 500ms.

Movie S3: FRAP of the whole cilium in IMCD3 cells stably expressing _{CTS}PKHD1^{GFP}. Images were captured every 10 s for 10 minutes with an exposure of 500ms.

Movie S4: FRAP of the whole cilium in IMCD3 cells transiently expressing _{CTS}PKHD1^{GFP}. Images were captured every 10 s for 10 minutes with an exposure of 500ms.

Movie S5: FRAP of the whole cilium in IMCD3 cells transiently expressing Sstr3^{GFP}. Images were captured every 10 s for 10 minutes with an exposure of 500ms.

Movie S6: FRAP of the whole cilium in MEFs stably expressing Smo^{YFP}. Cells were treated with 100nM SAG for 24 hours before imaging. Images were captured every 10 s for 10 minutes with an exposure of 500ms.

Movie S7: FRAP of periciliary membrane Htr6^{SEP} in IMCD3 cells transiently over-expressing Htr6^{SEP}. Images were captured every 2 s for 3 minutes with an exposure of 500ms.

Movie S8: FRAP of the whole cilium in IMCD3 cells stably expressing IFT88^{YFP}. Images were captured every 10 s for 9 minutes with an exposure of 500ms.

Movie S9: FRAP of part of the cilium in IMCD3 cells stably expressing Htr6^{SEP}.

Fluorescence is represented as heat-map images. Images were captured every 1 s for

20s with an exposure of 500ms.

Movie S10: FRAP of part of the cilium in IMCD3 cells stably expressing $CTSPKH1^{GFP}$. Fluorescence is represented as heat-map images. Images were captured every 1 s for 12s with an exposure of 500ms.

Movie S11: FRAP of part of the cilium in IMCD3 cells transiently expressing $Sstr3^{GFP}$. Fluorescence is represented as heat-map images. Images were captured every 1 s for 20s with an exposure of 500ms.

Movie S12: FRAP of part of the cilium in MEFs stably expressing Smo^{YFP} . Fluorescence is represented as heat-map images. Images were captured every 3 s for 30s with an exposure of 500ms.

Movie S13: FRAP of part of the cilium in IMCD3 cells stably expressing $IFT88^{YFP}$. Fluorescence is represented as heat-map images. Images were captured every 2 s for 30s with an exposure of 500ms.

Movie S14: 3-dimensional reconstruction (x-volume) of a primary cilium in IMCD3 cells fixed and stained for SEPT2 (green) and $AcTub$ (red). Imaging was done by a super-resolution OMX system utilizing structured illumination (API DeltaVision).

Movie S15: 3-dimensional reconstruction (y-volume) of a primary cilium in IMCD3 cells fixed and stained for SEPT2 (green) and $AcTub$ (red). Imaging was done by a super-resolution OMX system utilizing structured illumination (API DeltaVision).

Movie S16: FRAP of the whole cilium in IMCD3 parental cells transiently expressing $Htr6^{SEP}$. Images were captured every 10 s for 10 minutes with an exposure of 500ms.

Movie S17: FRAP of the whole cilium in shRNA1 IMCD3 cells transiently

expressing Htr6^{SEP}. Images were captured every 10 s for 10 minutes with an exposure of 500ms.

Movie S18: FRAP of the whole cilium in IMCD3 parental cells transiently expressing $CTSPKHD1^{GFP}$. Images were captured every 10 s for 18 minutes with an exposure of 500ms.

Movie S19: FRAP of the whole cilium in shRNA1 IMCD3 cells transiently expressing $CTSPKHD1^{GFP}$. Images were captured every 10 s for 18 minutes with an exposure of 500ms.

Movie S20: FRAP of the whole cilium in IMCD3 parental cells transiently expressing $Sstr3^{GFP}$. Images were captured every 10 s for 10 minutes with an exposure of 500ms.

Movie S21: FRAP of the whole cilium in shRNA1 IMCD3 cells transiently expressing $Sstr3^{GFP}$. Images were captured every 10 s for 10 minutes with an exposure of 500ms.

Movie S22: FRAP of the whole cilium in IMCD3 parental cells transiently expressing Smo^{YFP} . Cells were treated with 100nM SAG for 24 hours before imaging. Images were captured every 10 s for 18 minutes with an exposure of 500ms.

Movie S23: FRAP of the whole cilium in shRNA1 IMCD3 cells transiently expressing Smo^{YFP} . Cells were treated with 100nM SAG for 24 hours before imaging. Images were captured every 10 s for 18 minutes with an exposure of 500ms.

Movie S14 and S15 were made using the reconstruction algorithm in softWoRx software of a 3D structured illumination system. Other movies were made using MPEG4 compression in ImageJ to reduce the size.