Supplemental Experimental Procedures

Antibodies and plasmids. Commercial antibodies for acetylated α-tubulin (clone 6-11B-1, Sigma) and γ-tubulin (clone GTU-88, Sigma) were used. Antibodies to Smo and Ptch1 were gifts from Matthew Scott and Raj Rohatgi (Stanford University). Secondary antibodies for immunofluorescence were from Jackson ImmunoResearch and Invitrogen. The KIF17-mCitrine [S1] and Arl13b-mCherry [S2] plasmids have been described. NUP62-Fv plasmids were constructed by subcloning into NUP62-eGFP3 (EUROSCARF). Rev-Gr-GFP plasmid was a gift from John Hanover (NIH). PalmPalm-mCit was constructed by inserting the N-terminal 20 amino acids of human growth associated protein 43 (GAP43) into the mCit-N1 vector. Tsga14-GFP and Gtl3-GFP were gifts from Wallace Marshal (University of California, San Francisco). GFP-Gli2, IFT20-GFP, IFT88-GFP, and RP2-GFP were gifts from Jeremy Reiter (University of California, San Francisco), Greg Pazour (University of Massachusetts Medical Center), Jeff Martens (University of Michigan), and Ben Margolis (University of Michigan), respectively. Gpr161-mCit plasmid was constructed by subcloning pDONR-GPR161 (DNASU) into mCit-N1.

Cell culture and transfection. Except for IFT88-mCit, all constructs were transiently transfected. NIH 3T3 cells were grown in DMEM (Gibco) supplemented with 10% fetal clone III (Hyclone) and 1% GlutaMAX (Gibco). Transfections were carried out using Trans-IT (Mirus) and cells were fixed or imaged level 24 h later. Cells were serum starved for 24–48 h before experiments for ciliated cells. To construct a cell line stably expressing IFT88-mCit, FRT-IFT88-mCit plasmid was constructed and stably integrated into NIH 3T3 Flp-In cells (Life Technologies) by selection in 150 μg/ml hygromycin B.

Microscopy. Immunofluorescence was performed as described [S3]. Briefly, cells were fixed with 3.7% paraformaldehyde for 10 min, quenched with 50 mM NH_4Cl for 5 min, permeabilized with 0.2% TX-100 for 5 min or 0.1% SDS for 10 min (for staining with anti-γ-tubulin antibody), and blocked in 0.2% fish skin gelatin for 20 min. Fluorescence images were obtained using an inverted epifluorescence microscope (Nikon TE2000-E) with 60x oil immersion objective (N.A. 1.4) and a Photometrics CoolSnap HQ camera.

Microinjection. Cells were microinjected as described [S2] with sulforhodamine B-labeled BSA-NLS (2 mg/ml, Sigma) or rGFP (1 mg/ml, Prospec). Cells expressing either NUP62-Fv-EGFP3 or NUP62-Fv-Cer and Arl13b-mCherry were chosen for analysis.

Supplemental References

S1 Hammond, J. W., Blasius, T. L., Soppina, V., Cai, D., and Verhey, K. J. (2010). Autoinhibition of the kinesin-2 motor KIF17 via dual intramolecular mechanisms. J. Cell Biol. *189*, 1013–25.

S2 Kee, H. L., Dishinger, J. F., Blasius, T. L., Liu, C.-J., Margolis, B., and Verhey, K. J. (2012). A size-exclusion permeability barrier and nucleoporins characterize a ciliary pore complex that regulates transport into cilia. Nat. Cell Biol. *14*, 431–7.

S3 Dishinger, J. F., Kee, H. L., Jenkins, P. M., Fan, S., Hurd, T. W., Hammond, J. W., Truong, Y. N.-T., Margolis, B., Martens, J. R., and Verhey, K. J. (2010). Ciliary entry of the kinesin-2 motor KIF17 is regulated by importin-beta2 and RanGTP. Nat. Cell Biol. *12*, 703–10.