Supplemental Experimental Methods

Materials and reagents.

Chemicals and drugs used in this study were purchased from Sigma-Aldrich (St. Louis, MO, USA) and BioMol (BioMol International LP., USA). Stock concentrations of reagents were made by dilution in solvents (DMSO or H_2O) according to the manufacture protocols and stored at -20° C. The final concentrations of reagents were freshly prepared in DMEM/10%FBS or CO2-independent medium (Invitrogen, Carlsbad, CA, USA), depending on the type of experiment. The final concentrations of reagents were: 100 µM Forskolin, 10 µM 8-Bromo-cAMP (8-Bromoadenosine-3',5'-cyclic monophosphate), 100 µM 1,9-Dideoxy Forskolin, 10 µM Rp-cAMP (Rp-Adenosine-3',5'-cyclic monophosphorothioate), 30 µM Sp-8-Bromo-cGMP, 30 µM Rp-8-Bromo-cGMP, 10 µM and 50 µM H-89 (N-[2-(p-Bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide), 10 µM KT-5720, 50 µM Dantrolene, 30 µM Gd^{3+}, 100 nM Triptolide, 2 µM Thapsigargin, 30 µM Rolipram, 125 µM IBMX. All drug treatments were carried out for three hours before measurements were made.

Calcium Measurements.

To determine basal level of calcium, IMCD cells were cultured on MatTek (MatTek Corp., Ashland, MA, USA) dishes in DMEM/10% FBS to 100% confluency. Confluent IMCD cell (untreated or drug treated) cells were loaded with 5 µM Fura-2 AM (Molecular Probes, Invitrogen, Carlsbad, CA, USA) for 20 min at room temperature, extensively rinsed with CO2-independent medium [1] to remove excess dye, permitted to equilibrate in CO2- independent medium at least 10 minutes and then imaged with dual-wavelength excitation at 340nm and 380nm under an inverted Nikon TE2000-S microscope (PlanFluor 40X NA 1.3 objective) and emission captured at 510 nm. Time-lapse images were captured every 5 sec by IPLab software to a cooled CCD camera (COOLSNAP HQ, Roper Scientific, Tuscon

AZ, USA). Ratiometric image analysis of Ca^{2+} measurements was carried out using ratio imaging tools (RatioPlus plug-in) in ImageJ software (rsb.info.nih.gov/ij).

Flow Measurements.

Confluent IMCD and MEK cells were stimulated by flow (shear stress 0.75 dyne/cm^2) in a laminar flow chamber [2] for 3 hours with or without drug addition to the perfusate. After 3 hours flow samples were collected for measurements of cAMP concentration and for cilium immunostaining. cAMP level and cilia length were determined in ≥3 independent experiments. Calcium imaging under flow induction was performed as described previously [1].

Generation of Cell Lines and Primary Cells.

The IMCD cell line stably expressing IFT88-EYFP was generated as previously described [3]. pSuper RNAi System (pSuperior.retro.puro, OligoEngine.com) was used to generate shRNA mediated knockdown of mouse polycystin-2 gene in IMCD cells. The 19-nt target sequence corresponding to the coding region of mouse polycystin-2 mRNA (GCTCTTCAAATTCATCAAT) was targeted. Following infection, colonies were isolated after selection in 2μg/mL of puromycin and expanded to create monoclonal cell lines. Immunoblotting was done to verify level of polycystin-2 knockdown **(Figure S4A)**. Mouse Embryonic Kidney (MEK) cells were generated as previously reported [1]. To isolate primary bone mesenchymal progenitor cells (BME) frontal, nasal and fronto-premaxilllary bones, with sutural mesenchymal cells preserved were dissected out from 7-9 days old mice, cut into small pieces and digested in serum-free DMEM containing 1 mg/ml Collagenase P (Roche Applied Science, Indianapolis, IN) and 0.02% Trypsin /EDTA at 37Cº for 4 hr. After digestion, cells were harvested by centrifugation for 10 min at 1700 rpm and passed through a 70 μm nylon cell strainer (BD Falcon, Bedford, MA, USA). The resulting cell population was collected and cultured in DMEM containing 10% FBS until 100% confluent.

Small interfering RNA-mediated gene silencing.

AC5, AC6 and GAPDH silencing were performed with Dharmacon SmartPool siRNAs according to manufacture protocol (Thermo Scientific DharmaFECT® General Transfection Protocol, DharmaFect reagent #4, Dharmacon, Lafayette, CO, USA). GAPDH silencing was used as a control to measure the efficiency of transfection. Cells were subjected to WB and IF analysis after 48 hours of incubation with transfection cocktail. Six well plating format was used to seed the cells. 12-mm poly-L-lysine-coated cover slips were placed in 6 wells plate to ensure that WB and immunofluorescence analysis were performed under the same conditions.

Immunofluorescence and Cilium Length Measurements.

IMCD, MEK and BME were cultured on 22-mm poly-L-lysine-coated cover slips in DMEM/10% FBS to 100% confluency. Confluent cells (untreated or drug treated) were fixed in 4% formaldehyde, permeabilized in 0.4% Triton X-100, blocked in 1% BSA and stained with primary antibody against acetylated alpha-tubulin (1:10000) (Sigma, St. Louis, MO, USA). Nuclei were labelled with DAPI (Molecular Probes, Invitrogen, Carlsbad, CA, USA). Immunofluorescence samples were examined for cilia length using a Nikon TE2000-E2 inverted microscope system (Nikon instruments Inc., Melville, NY, USA) with a PlanFluor 40X NA 1.3 oil objective at 1X1 binning. Cilia were measured by using ImageJ (rsb.info.nih.gov/ij) software. Average cilia length and standard deviation were determined by measuring cilium length in ≥ 3 independent experiments ($n \geq 100$).

Immunoblot analysis.

Western blots were carried out using well-established protocols. 10% and 5% acrylamide gels were used for loading polycystin-2 and -1, respectively. To determine levels of PC2 and PC1, antibodies against

PC2 (1:1000) and anti-PC1 (1:5000) [1] were used; α-tubulin (1:2000) (Sigma) and IFT88 were used as loading controls (1:5000) (gift from B.Yoder).

Supplementary Data: Figure Legends

Figure S1 MEK and BME cells increase cilium length in response to second messenger modulation.

(A) MEK cells showed cilium elongation after Ca^{2+} inhibition (30μM Gd^{3+}) or cAMP activation (100μM Forskolin). Scale bar is 5 μm. **(B)** Histograms depict distribution of cilia length in untreated and compound treated MEK cells (n > 100). **(C)** BME cells showed cilium elongation after Ca^{2+} inhibition (30μM Gd^{3+}) or cAMP activation (100μM Forskolin). Scale bar is 5 μm. **(D)** Histograms depict distribution of cilia length in untreated and compound treated BME cells (n > 100). Acetylated α-tubulin was used as a ciliary marker (red).

Figure S2 Cilium length is unaffected by PDE inhibition or modulation of intracellular cGMP levels, does not require translation and is reversible. (associated with Figure 2)

(A) IBMX, a non-specific PDE inhibitor and Rolipram, a specific PDE4 inhibitor, (PDE4 is abundantly expressed in kidney epithelium) had no significant effect on cilium length. **(B)** Elevation (Sp-8-Bromo-cGMP) and competition (Rp-8-Bromo-cGMP) of cGMP showed no significant effect on cilia length compared to non-treated cells. **(C)** Pretreatment of IMCD cells with cycloheximide, a protein synthesis inhibitor, had no effect on rapid cilium elongation induced by cAMP activator (Forskolin) and Ca^{2+} blocker (Gd^{3+}). **(D)** Confluent IMCD, Gd^{3+}- treated, cells (30μM Gd^{3+}) followed by addition of EGTA at equimolar with Gd^{3+} concentration (30μM) showed return to untreated cilium length ($pK_{d\ Ca^{2+}\text{-EGTA}}$= 6.4; $pK_{d\ Gd^{3+}\text{-EGTA}}$= 12.9). Error bars indicate mean ± s.d.; *P<0.005. **(E)** Effect of PKA inhibition on forskolin-induced cilium length change. **(F)** Cilia length changes after simultaneous addition of Thapsigargin and 8-Br-cAMP. Acetylated α-tubulin (red); Scale bar is 5 μm. **(G)** AC5 and AC6 are

expressed in primary cilia of IMCD cells. Visualization of the AC5 and 6 by IF revealed a punctuate distribution throughout the ciliary axoneme. AC5/6 silencing by siRNAs abrogate adenylate cyclases staining in cilia (AC5 1:2000, AC6 1:500; Abcam). **(H)** Western blot shows level of AC6 and GAPDH in untreated or siRNA AC6 and siRNA GAPDH treated IMCD cells; α-tubulin was used as a loading control. **(I)** Silencing of AC5 or AC6 prevents cilium lengthening in response to Gd^{3+} but not PKA activation by 8-Br-cAMP. Acetylated α-tubulin (red); scale bar, 5 μm.

Figure S4 Reduction of polycystins in IMCD^{PC2KD} and MEK^{PC1mut} cells do not generate a fluid shear Ca^{2+} response and have lower baseline Ca^{2+} levels.

(A) Immunoblot analysis showed the level of PC2 knockdown in IMCD^{PC2KD} cells; Clone #2 was used in experiments; α-tubulin was used as a loading control. **(B)** Immunoblotting showed the absence of full-length PC1 in MEK^{PC1mut} cells; IFT88 was used as a loading control. **(C)** Untreated cells subjected to fluid flow (shear stress 0.75 dyne/cm^2) (light grey bars – IMCD, red bars – IMCD^{PC2KD}) underwent a significant increase in transient Ca^{2+} response. Cells deficient for PC2 via stable shRNA do not respond low shear stress. Arrows indicate the start of flow. **(D)** Cells derived from PC1 mutant embryonic kidney do not generate a Ca^{2+} response under fluid shear stress (dark grey bars – MEK^{wt}, red bars – MEK^{PC1mut}). **(E)** Steady-state levels of intracellular Ca^{2+} were reduced in IMCD^{PC2KD} in comparison to wildtype IMCD cells. After treatment of Gd^{3+} or forskolin, wildtype IMCD reduced steady-state Ca^{2+} but not in IMCD^{PC2KD} cells. (light grey bars – IMCD, hatched bars - IMCD^{PC2KD}). **(F)** Baseline Ca^{2+} was lower in MEK^{PC1mut} cells. After treatment of Gd^{3+} or forskolin, wildtype MEK^{wt} reduced steady-state Ca^{2+} but not in MEK^{PC1mut} cells. (dark grey bars – MEK, hatched bars – MEK^{PC1mut}). Error bars indicate mean ± s.d.; n=3.

Movie S1 Movement of IFT88 in a live untreated IMCD-IFT88-EYFP cell as depicted in Figure 4B.

Movie S2 Movement of IFT88 in live untreated IMCD-IFT88-EYFP cells. (associated with Figure 4)

Movie S3 Movement of IFT88 in live IMCD-IFT88-EYFP cells treated for 3 hours with 30 μM Gd^{3+} as depicted in Figure 4B.

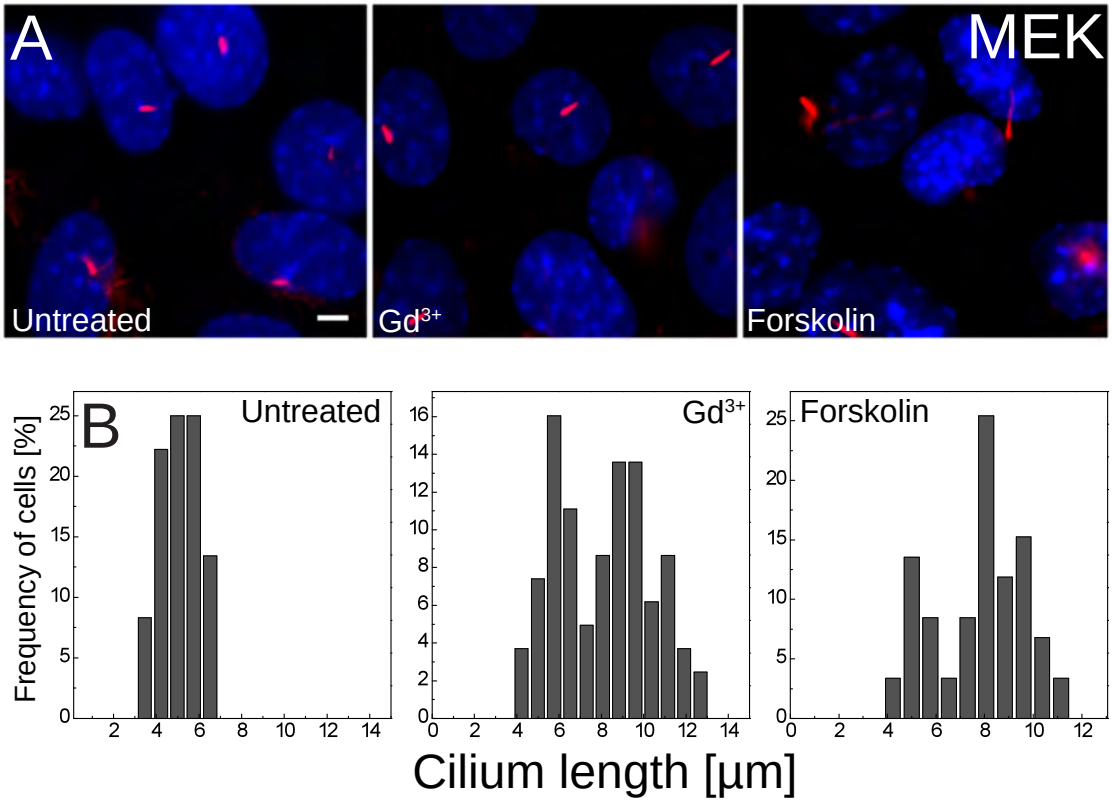
Movie S4 Movement of IFT88 in live IMCD-IFT88-EYFP cells treated for 3 hours with 30 µM Gd^{3+}. (associated with Figure 4)

Movie S5 Movement of IFT88 in live IMCD-IFT88-EYFP cells treated for 3 hours with 100 µM Forskolin as depicted in Figure 4B.

Movie S6 Movement of IFT88 in live IMCD-IFT88-EYFP cells treated for 3 hours with 100 µM Forskolin. (associated with Figure 4)

Supplemental References

1. Nauli, S.M., Alenghat, F.J., Luo, Y., Williams, E., Vassilev, P., Li, X., Elia, A.E., Lu, W., Brown, E.M., Quinn, S.J., Ingber, D.E., and Zhou, J. (2003). Polycystins 1 and 2 mediate mechanosensation in the primary cilium of kidney cells. Nat Genet *33*, 129-137.
2. Allen, J.W., Johnson, R.S., and Bhatia, S.N. (2005). Hypoxic inhibition of 3-methylcholanthrene-induced CYP1A1 expression is independent of HIF-1alpha. Toxicol Lett *155*, 151-159.
3. Tran, P.V., Haycraft, C.J., Besschetnova, T.Y., Turbe-Doan, A., Stottmann, R.W., Herron, B.J., Chesebro, A.L., Qiu, H., Scherz, P.J., Shah, J.V., Yoder, B.K., and Beier, D.R. (2008). THM1 negatively modulates mouse sonic hedgehog signal transduction and affects retrograde intraflagellar transport in cilia. Nat Genet *40*, 403-410.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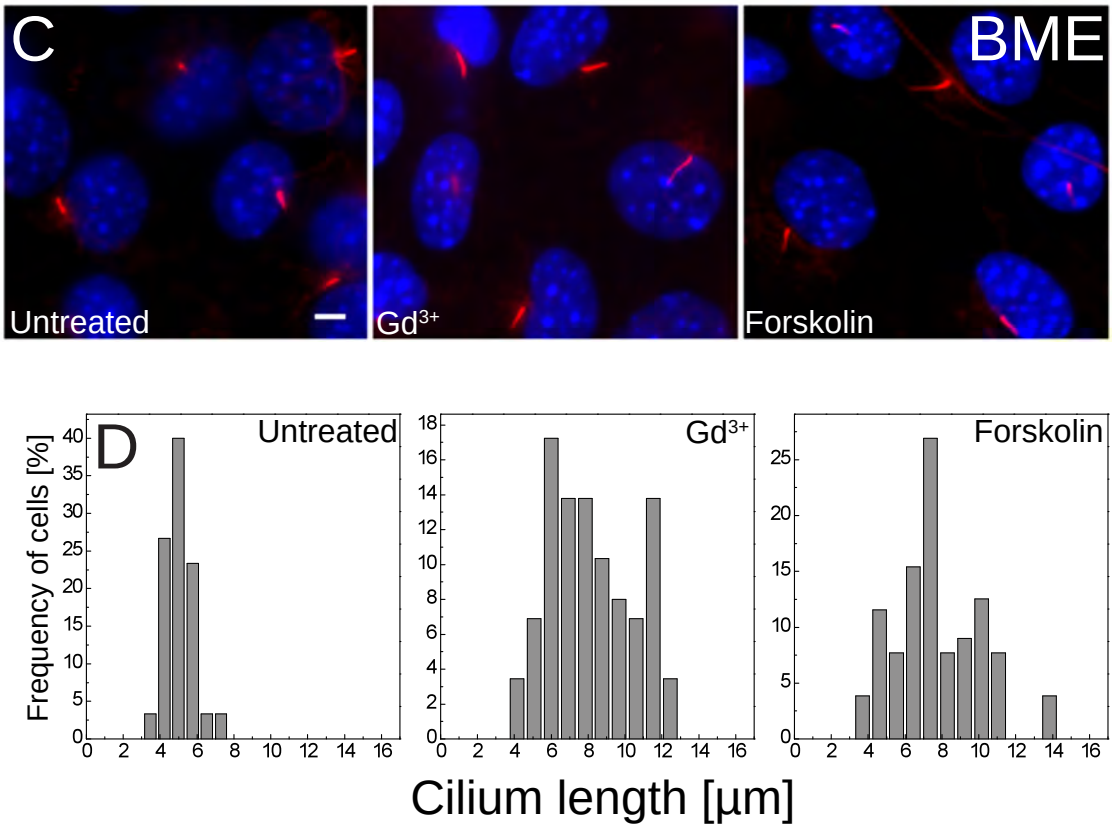
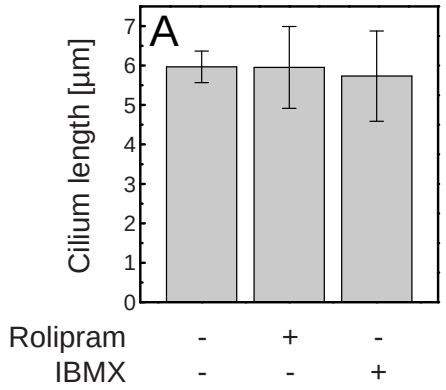
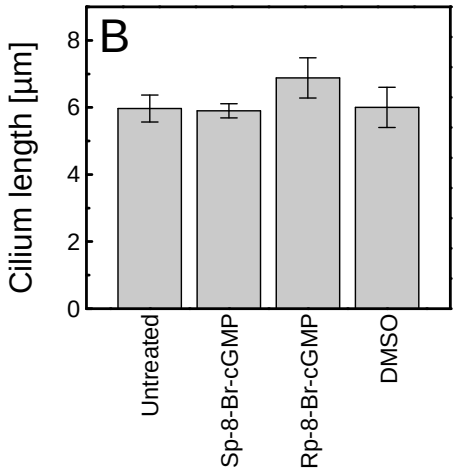
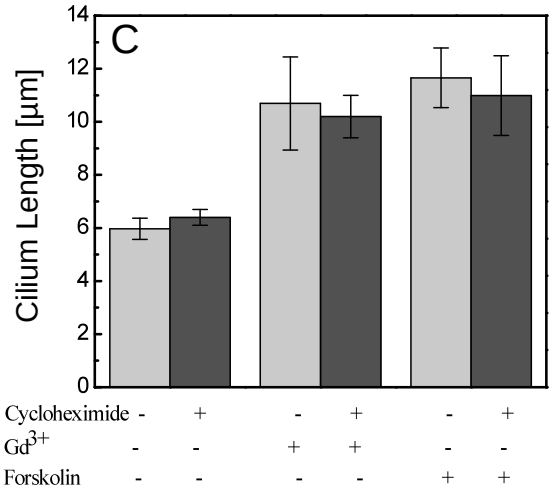
Gd3+
Forskolin
B
Untreated
Gd3+
Forskolin
Frequency of cells [%]
Cilium length [µm]

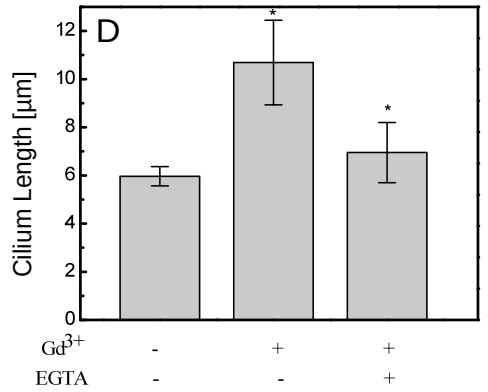

C
BME
Untreated
Gd3+
Forskolin
D
Untreated
Gd3+
Forskolin
Frequency of cells [%]
Cilium length [µm]

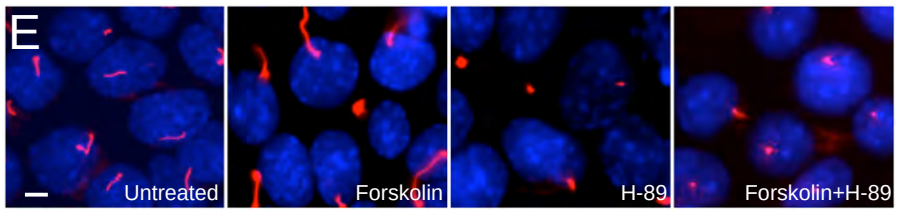
Supplemental Data

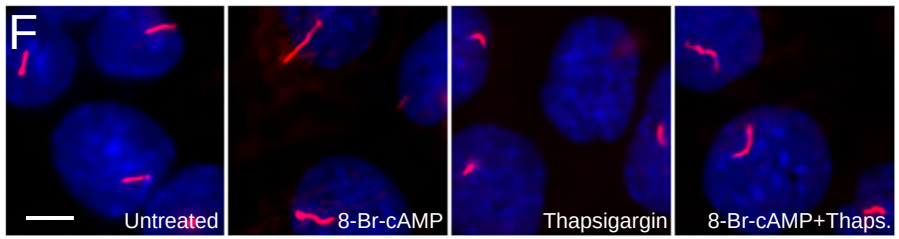


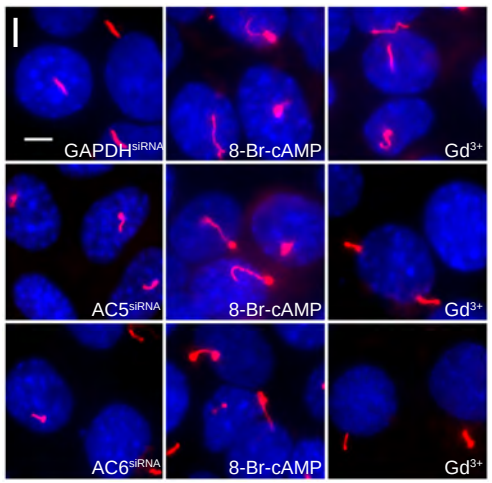


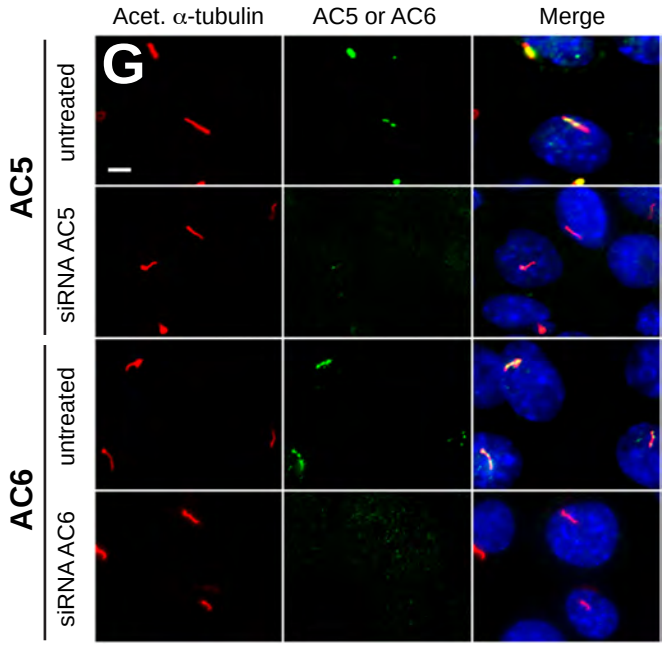


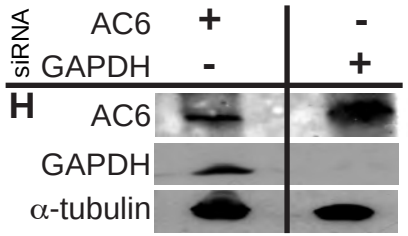












Supplemental Figure 4