Supplemental Materials

Molecular Biology of the Cell

Turn *et al*.

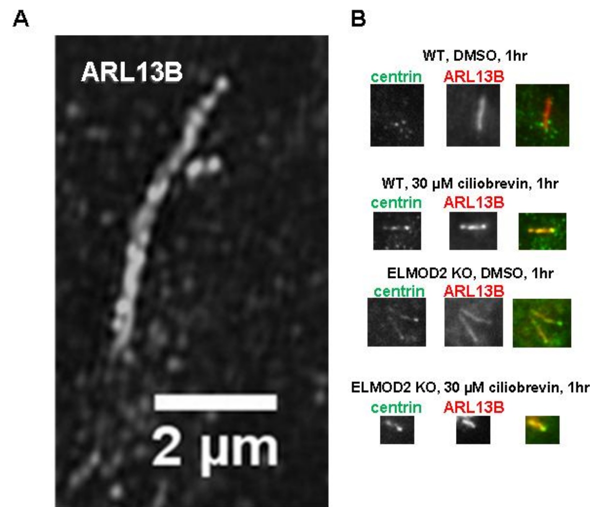


Figure S1: *Loss of ELMOD2 leads to disrupted ciliary morphology and protein localization.* **(A)** Representative image of branching in ELMOD2 KO cilia. SIM images were collected at 100x magnification using cells stained for ARL13B. Scale = 2µm. **(B)** Centrin staining in cilia is increased in both WT and ELMOD2 KO cells after treatment with ciliobrevin. Representative images collected via widefield microscopy (100x magnification) are shown. WT and KO cells were serum starved for 24 hours before being treated with either DMSO or 30µM ciliobrevin for 1hr at 37˚C. Insets highlight individual cilia and the presence or absence of centrin.

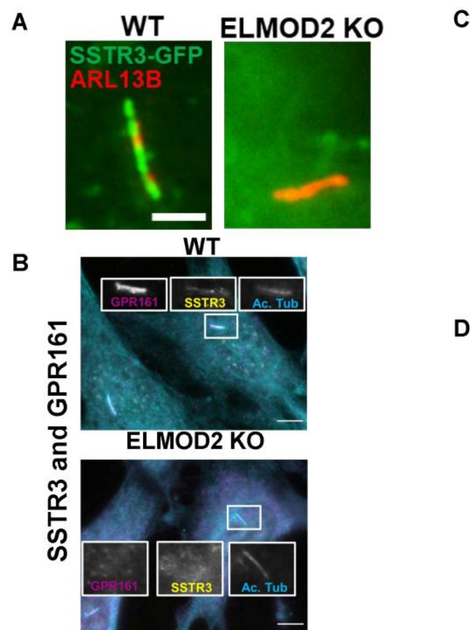


Figure S2: *SSTR3 and GPR161 localization is decreased/lost in ELMOD2 KO cilia.* **(A)** WT or ELMOD2 KO cells were transfected with plasmid directing expression of SSTR3-GFP and the next day were serum starved. KO cells displayed strongly reduced ciliary GFP. Cells were fixed with 4% PFA, permeabilized with 0.1% Triton X-100, and co-stained with ARL13B. Representative images were collected using widefield microscopy (100x magnification). Scale bar = 2 µm. **(B)** ELMOD2 KO cells show decreased recruitment of (endogenous) SSTR3 and GPR161. Serum-starved cells were fixed and stained using protocols required for detecting the appropriate antigen, as described under Materials and Methods. Representative images were collected via widefield microscopy at 100x magnification. Samples were co-stained with either acetylated tubulin or ARL13B to mark cilia. Scale bar = 10 µm. **(C)** Cells were scored for endogenous SSTR3 staining (which is naturally faint, so were either binned as existent or non-existent). The experiment was performed in duplicate, and the average of the duplicates of individual lines are shown here as individual points of interleaved scatterplot. Error bars represent SEM, and statistical significance was assessed via One-Way ANOVA. ***=$p<0.0001$.

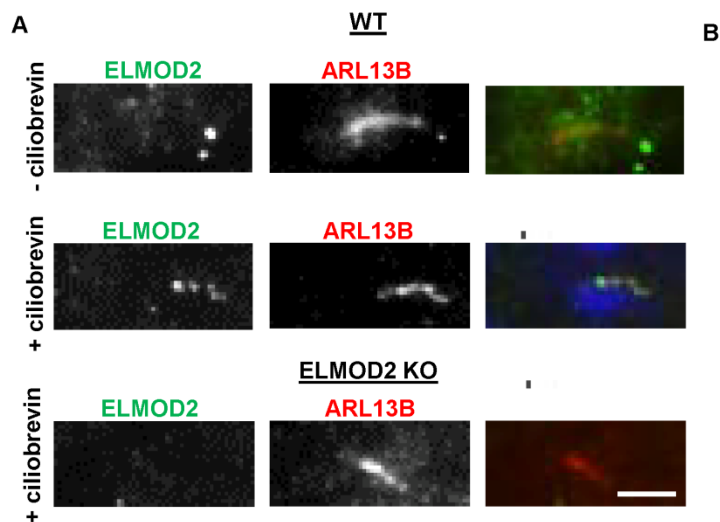


Figure S3: *ELMOD2 localizes to cilia in WT MEFs upon ciliobrevin treatment.* WT MEFs treated either with 0.6% DMSO (top) or 30µM ciliobrevin (bottom) for 1hr were fixed with 4% PFA, permeabilized with 0.1% Triton X-100, and stained for ELMOD2 and ARL13B. Only upon blocking of ciliary retrograde transport via ciliobrevin do we observe ELMOD2 localization to cilia. Images were collected via widefield imaging at 100x magnification.

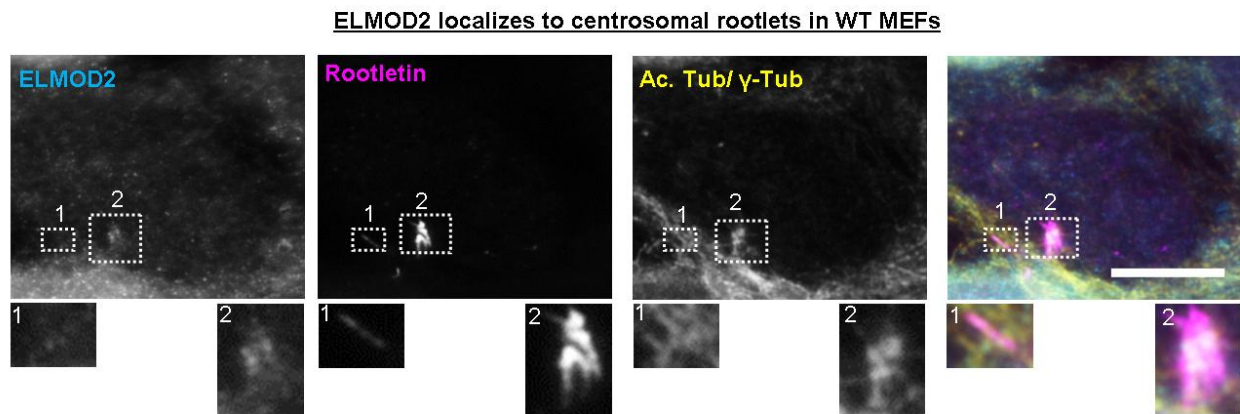


Figure S4: *ELMOD2 does not localize to non-centrosomal rootlets.* ELMOD2 specifically localizes to centrosome-associated rootlets rather than all Rootletin staining. Serum-starved, WT MEFs were fixed with ice-cold methanol and stained for ELMOD2, acetylated tubulin, and Rootletin. Widefield images were collected at 100x magnification. Scale = 10µm.

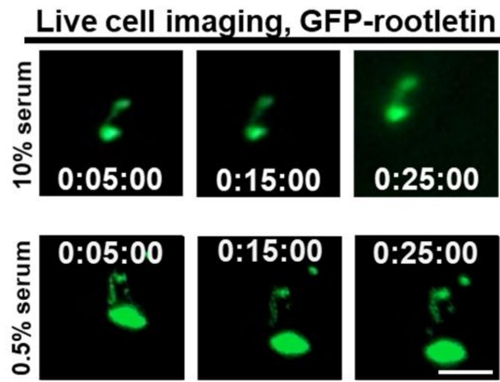
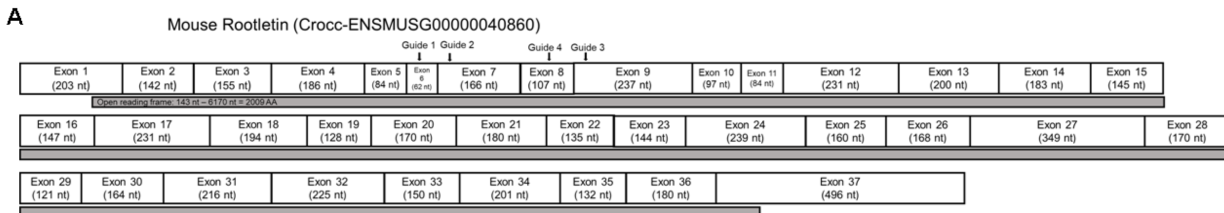


Figure S5: *Live cell imaging of GFP-Rootletin expressing MEFs reveal that serum starvation induces rootlet tendrils to dissociate from the centrosome*. Cells were imaged every 5 minutes over a 1-hour imaging window using widefield microscopy, 20x magnification. Cells were maintained at 37˚C and 5% CO_2 and imaged for 1 hour without (top panels) or with (bottom panels) serum starvation. While no rootlet release was evident without serum starvation, but after serum starvation rootlet release was evident within minutes.

A



B

Clone ID	Guide	Alleles
G1, #12	1	Both alleles: 1 bp deletion (C); **AA sequence:** ...TEHSQDLDSALLR*
G1, #31	1	Both alleles: 1 bp insertion (C); **AA sequence:** ...TEHSQDLDSALLRPRGGTAEVRGWDSPWAPSG*
G2, #17	2	Both alleles: 1 bp insertion (C); **AA sequence:** ...VSKCPLNTPTPPRSASLAQVNAMLREQLDQANLANQALSEDIPQGDQ*
G2, #20	2	Both alleles: 4bp deletion (TACG); **AA sequence:** ...VSKCPLNTPTPPRSASLAQVNAMLREQLDQANLANQALSEDTR*
G4, #2	4	Both alleles: 1 bp insertion (A) ; **AA sequence:** ...SFNAYFSSEHSRLLRLWRQVMGLRQAGQRGEDGHGEVRLLGAQGASCPG*
Rootletin$^{\Delta239}$	1	1: 1 bp insertion (A); **AA sequence: ...**TEHSQDLDSALLRHRGGTAEVRGWDSPWAPSG* 2: 2 bp insertion (CT); **AA sequence: ...** TEHSQDLDSALLRP*

Figure S6: *Summary of Rootletin alleles generated by CRISPR/Cas9.* (**A**) We designed 4 guides to use in CRISPR/Cas9 genome editing and the sites they target are shown above the targeted exons. The mouse *Crocc* gene encodes 37 exons, with the open reading frame shown below the spliced exons. (**B**) A total of 5 Rootletin KO lines were generated, along with the Rootletin$^{\Delta 239}$ line (G1, #21). Two clones were generated using guide 1, two others from guide 2, and 1 from guide 4. Rootletin$^{\Delta 239}$ was generated from guide 1. Genomic DNA sequencing was performed on genomic DNA surrounding each targeted region and the indels are listed under Alleles, along with the resulting protein sequences. The black font indicates WT protein sequence while the red font indicates nonsense protein sequence resulting from a frame shift and an asterisk indicates a stop codon. Each of the knockout clones led to frameshifting mutations which were predicted to generate non-functional protein products, as later confirmed by Western blot. In contrast, the line termed Rootletin$^{\Delta 239}$ displays very strong staining of Rootletin, despite having both alleles frameshifted at the targeted site. The use of a downstream methionine to initiate protein translation is proposed as an explanation of the shorter protein product seen in immunoblots.

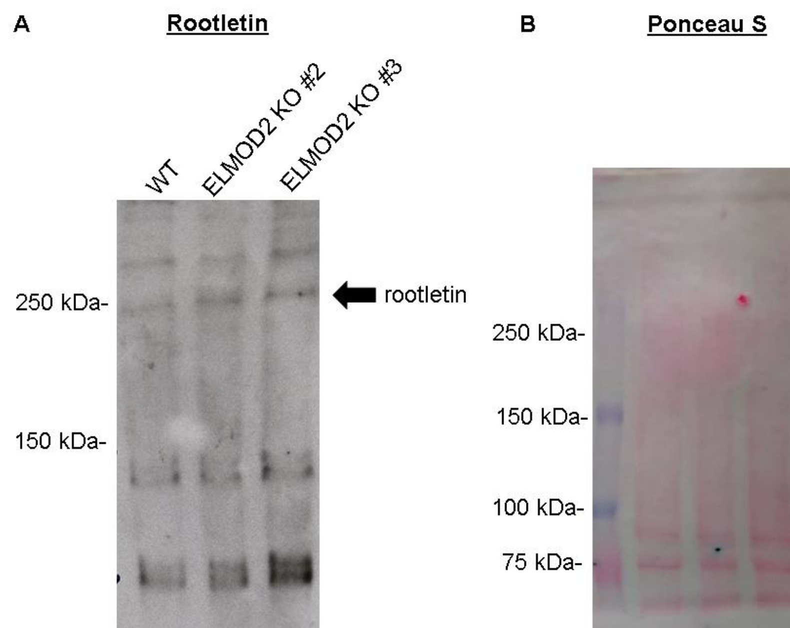


Figure S7: *ELMOD2 KO cells show no change in Rootletin protein expression.* (**A**) Cell pellets from confluent wells of a 6-well plate were thawed and immediately immersed in 1x sample buffer + BME, as in our hands rootletin is unstable after cell lysis. Equal volumes of protein were loaded into a 7.5% acrylamide gel and transferred onto nitrocellulose membrane. Membranes were blotted for chicken-anti-rootletin (1:1000 dilution) to check for Rootletin expression in WT versus ELMOD2 KO cells. (**B**) Ponceau staining of the membrane was performed to check for equal protein loading.

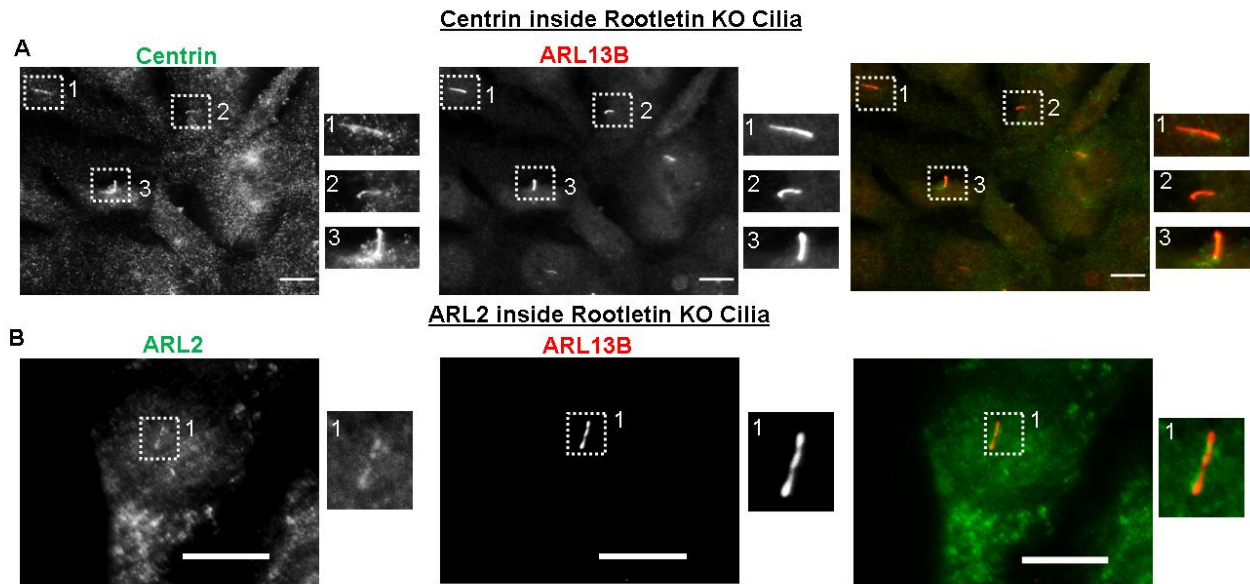


Figure S8: *Centrin and ARL2 localize to Rootletin KO cilia.* Serum-starved Rootletin KO cells have increased recruitment of both ARL2 and centrin. Cells were stained for ARL13B as a marker of cilia and either centrin or ARL2. Wide-field mages were collected at 100x magnification. Scale = 10μm.

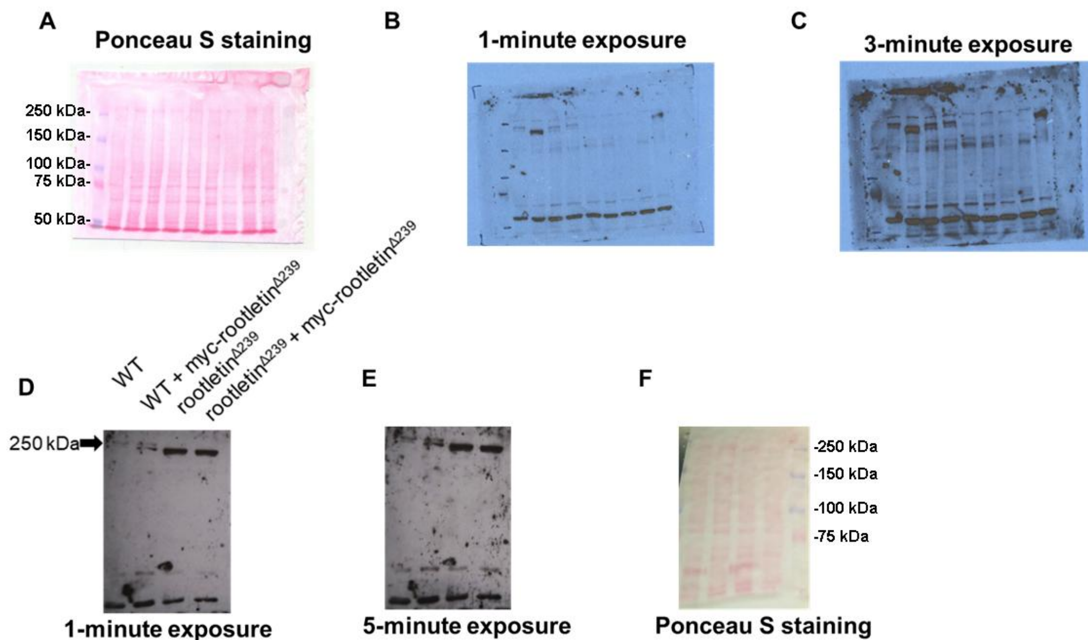


Figure S9: *Western blotting confirms the loss of Rootletin in KO lines and increased expression of an N-terminal truncation in Rootletin^{Δ239} MEFs.* Raw data of the Western shown in Figure 5A are shown, including (**A**) Ponceau S staining of the nitrocellulose membrane to confirm equal

protein loading, and (**B-C**) uncropped images of the films collected at 1 min and 3 min exposures respectively. Membranes were stained with chicken-anti-Rootletin at 1:1000 dilution in 5% Blotto. (**D-F**) Western blot and Ponceau of WT, WT + myc-Rootletin$^{\Delta 239}$, *Rootletin*$^{\Delta 239}$ cells, and *Rootletin*$^{\Delta 239}$ + myc-Rootletin$^{\Delta 239}$, using the same conditions as described for (A).

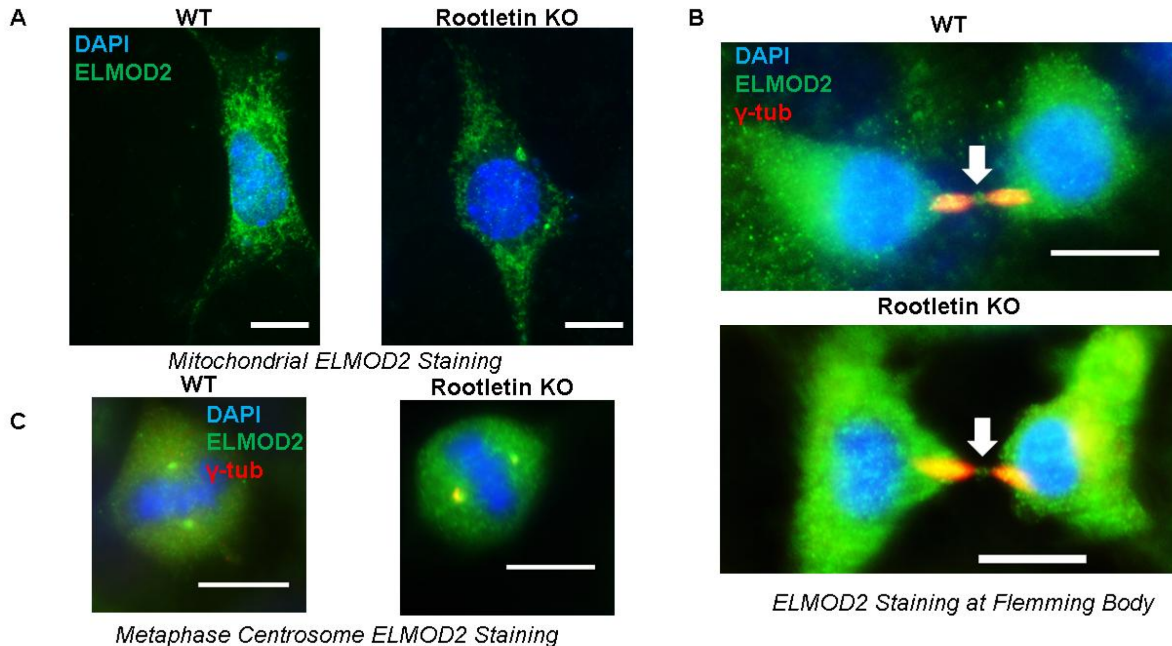


Figure S10: *ELMOD2 still localizes to mitochondria, Flemming bodies, and centrosomes in Rootletin KO cells.* To test whether the deletion of Rootletin alters ELMOD2 staining at sites other than rootlets, we used a number of fixation conditions to stain Rootletin KO cells for ELMOD2. Loss of Rootletin does not alter ELMOD2 staining at mitochondria (**A**), at Flemming bodies (**B**), or at metaphase centrosomes (**C**). Cells were fixed and stained for ELMOD2 and γ-tubulin (to mark both midbodies and centrosomes). Widefield images at 100x magnification are shown. Scale = 10μm.

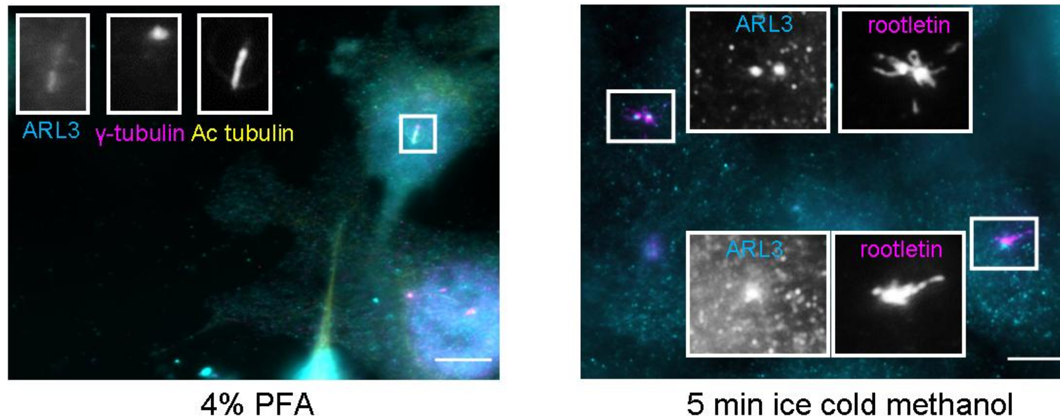


Figure S11: *ARL3 localizes to cilia and centrosomes but not rootlets in WT MEFs.* Representative widefield images (100x magnification) of ARL3 localization in WT MEFs are shown. With 4% PFA fixation, ARL3 staining at cilia is observed, as seen by co-staining with γ-tubulin and acetylated tubulin. With ice-cold methanol fixation, centrosomal staining of ARL3 is evident, but it does not extend to rootlets (using conditions in which one can readily detect ELMOD2 and ARL2 at rootlets). Scale = 10μm.

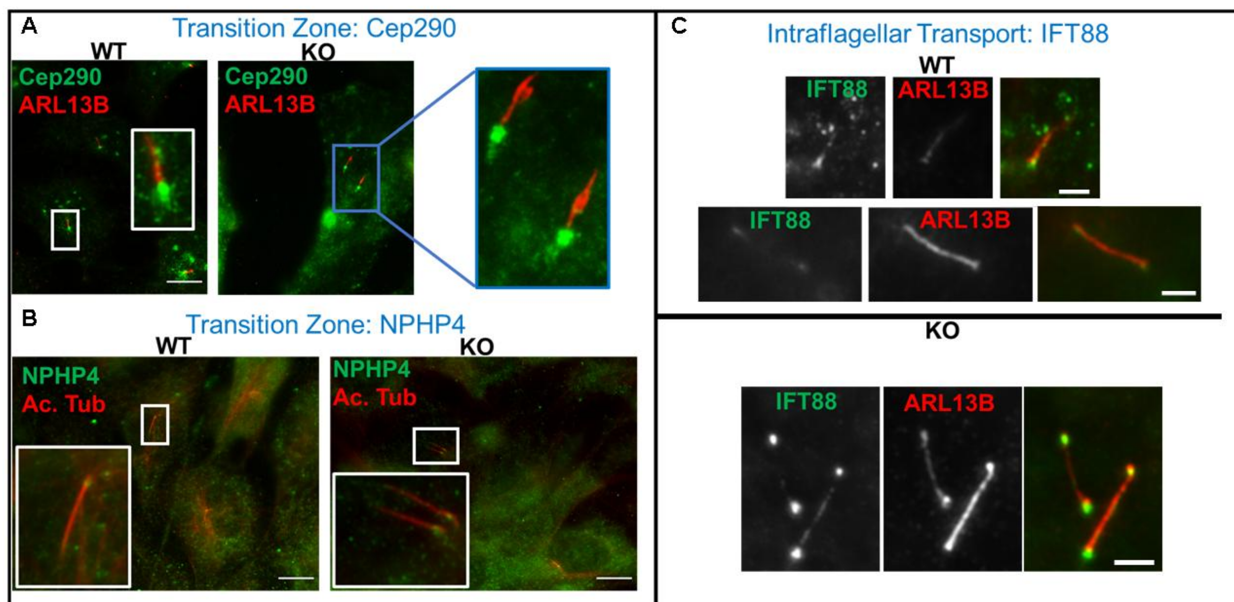


Figure S12: *ELMOD2 KO does not alter the localization of IFT or transition zone (TZ) markers.* Cells were fixed and stained for either markers of transition zone (**A-B**) or intraflagellar transport (**C**) to determine if there are overt defects in these compartments in ELMOD2 KO cells. For

transition zone, cells were fixed for 10 min with ice-cold methanol, blocked with 10% FBS, and stained for either NPHP4 or Cep290, along with a ciliary marker (ARL13B or acetylated tubulin). To look at IFT, cells were fixed with 4% PFA, permeabilized with 0.1% Triton X-100, and stained for IFT88. Representative images were collected via widefield microscopy at 100x magnification. Scale = 10µm.

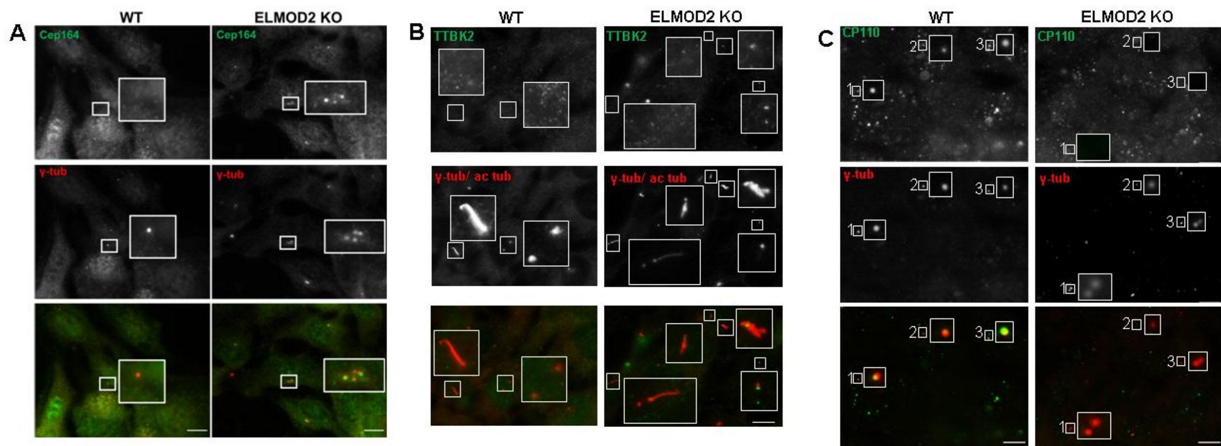


Figure S13: *Representative images demonstrating that ELMOD2 KO leads to misregulation of specific markers of ciliogenesis*. Widefield images were collected at 100x magnification of (**A**) Cep164, (**B**) TTBK2, and (**C**) CP110 staining at centrosomes (γ-tubulin) in WT versus ELMOD2 KO cells. Scale = 10 µm.

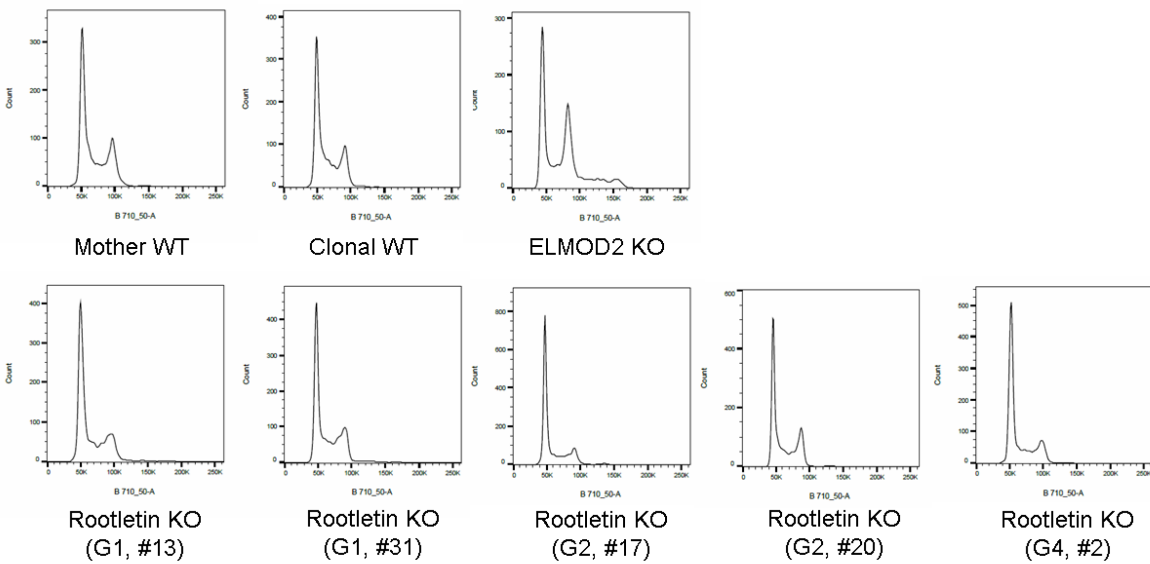


Figure S14: *Loss of Rootletin has no obvious effects on cell cycle of unsynchronized cycling cells.* WT, ELMOD2 KO, and Rootletin KO cells were harvested, fixed, and stained for propidium iodide staining to check the DNA content of cells via flow cytometry. 10,000 events were counted per cell line, and these experiments were performed in duplicate for each of the lines shown. Data were processed and plotted via FlowJo software, ensuring to use the same gating parameters for each sample. Representative graphs are shown here.

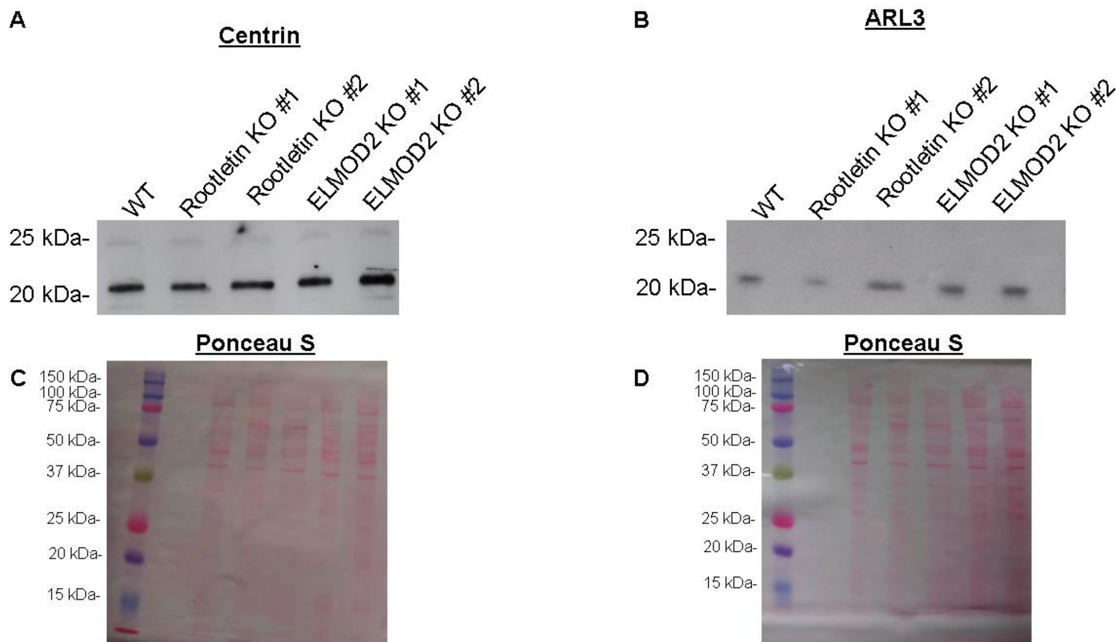


Figure S15: *Loss of either ELMOD2 or Rootletin does not affect ARL3 or centrin expression in MEFs.* Equal protein from whole cell lysates was loaded onto 15% acrylamide gels and

transferred onto nitrocellulose membrane. Membranes were blotted for either mouse-anti-centrin (1:500 dilution) (**A**) or rabbit-anti-ARL3 (1:500 dilution) (**B**) to assess changes in protein expression that may be a product of increases in ploidy. Ponceau staining of each respective membrane (**C-D**) was performed to check for equal loading.

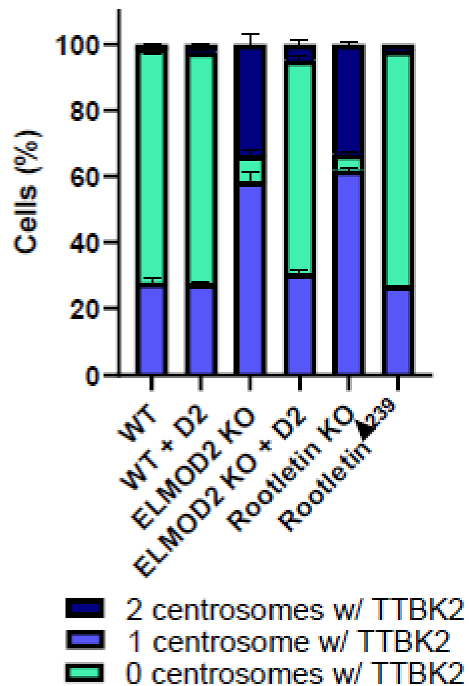
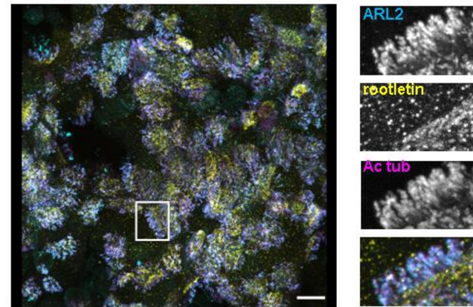


Figure S16: *TTBK2 is increased at centrosomes of ELMOD2 KO and Rootletin KO mononucleated cells having no more than two centrosomes*. Cells were scored as described in Figure 8B, but with scoring only mononucleated cells with 1-2 centrosomes. This was a control to ensure that increased ciliogenesis is not simply a side effect of cells with obvious cell cycle defects. Data are shown as a stacked bar graph, representing the average of duplicates of multiple cell lines. Error bars indicate SEM.



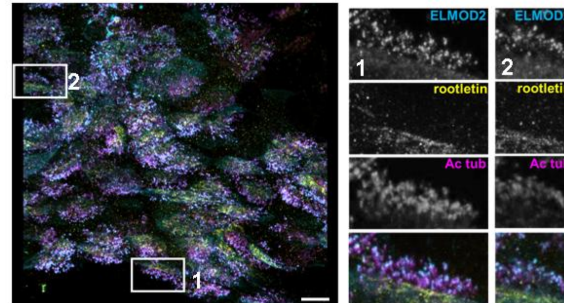


Figure S17: *ARL2 localizes along the length of cilia in human (multiciliated) bronchial epithelial cells, while ELMOD2 localizes to the tips of cilia and rootlets.* Primary cultures of human bronchial cells were grown on transwell plates before being fixed with ice-cold methanol and stained for Rootletin, acetylated tubulin and either (**A**) ARL2 or (**B**) ELMOD2. Confocal images were collected at 100x magnification, and z-projections were generated. Representative images of fields of bronchial cells are shown on the left. On the right, insets that highlight cells in which one can readily distinguish cilia from plasma membrane from rootlets. ARL2 is found almost exclusively at cilia in these cells, while ELMOD2 stains both the tip of cilia and (more faintly) the rootlets.