Figure S1

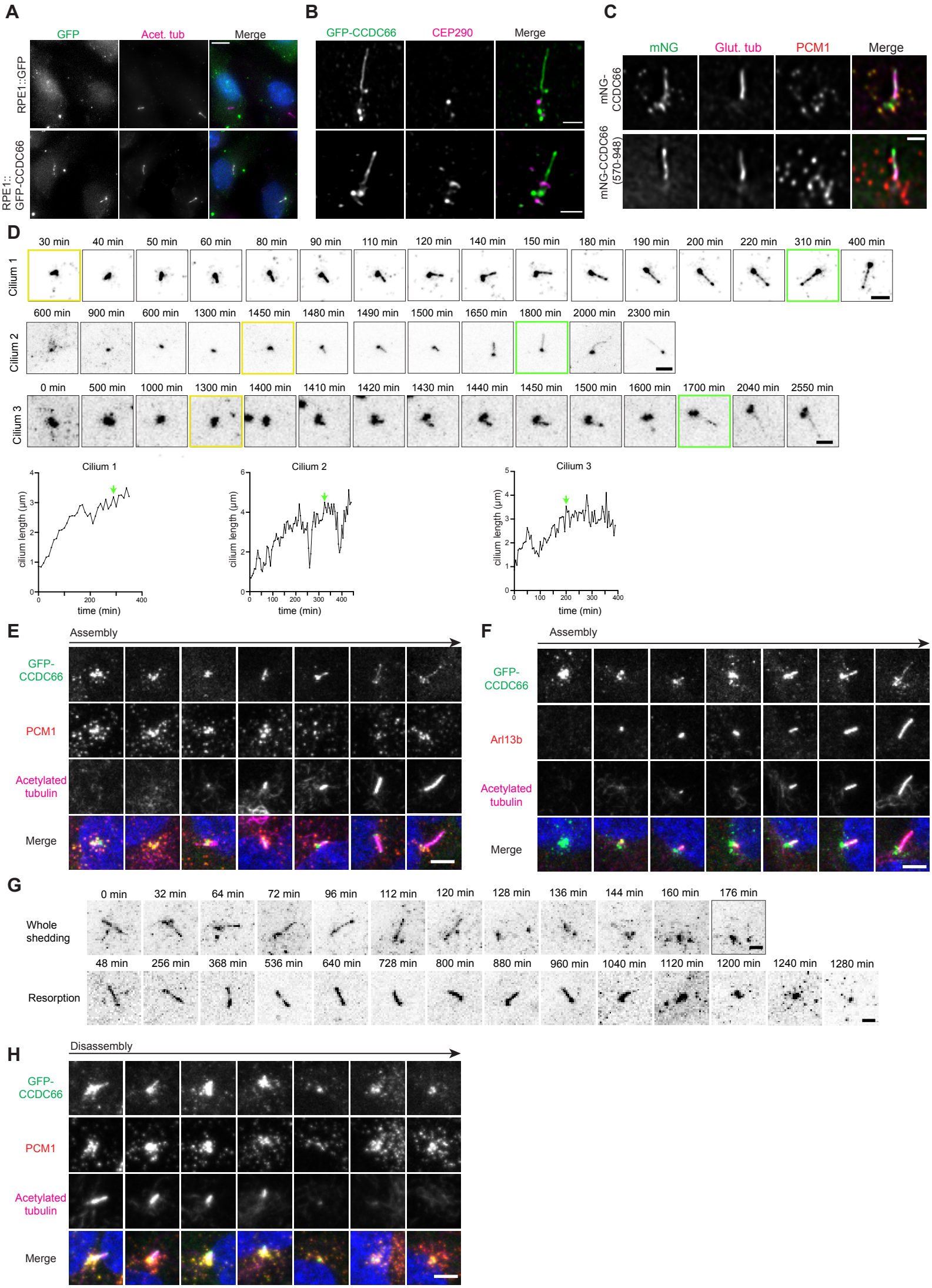


Fig. S1. (A) Ciliary localization of GFP-CCDC66 in RPE1 cells is specific
RPE1::GFP and RPE1::GFP-CCDC66 cells were serum starved for 24 hours and fixed with methanol and stained for GFP and acetylated tubulin antibodies along with DAPI in order to visualize DNA. Scale bar: 10μm

(B) Superresolution imaging of ciliary GFP-CCDC66 with CEP290
RPE1::GFP-CCDC66 cells were serum starved for 24 hours and fixed with methanol and stained for GFP and CEP290 antibodies. Images were acquired with SIM. Scale bar: 3μm.

(C) C-terminal microtubule binding fragment (570-948 a.a.) of CCDC66 localizes to the basal body and the axoneme but not centriolar satellites. RPE1::mNeonGreen (mNG)-CCDC66 and RPE1::mNG-CCDC66 (570-948) cells were serum starved for 48 hours, fixed and stained for mNeonGreen, glutamylated tubulin and PCM1 or acetylated tubulin and gamma tubulin antibodies. Scale bar: 2μm

(D) Spatiotemporal localization dynamics of CCDC66 during cilium assembly. RPE1::GFP-CCDC66 cells were plated onto Lab-Tek imaging dish at 100% confluency and started to be imaged with confocal microscopy every 10 min immediately after serum starvation. Representative images are from three different cells that form primary cilia. GFP signal is inverted and represented as black onto white background for ciliary and centriolar satellite pools of CCDC66 to be distinguished. Below graphs represent the cilium length over the course of imaging from these three cilia. Cilium length was measured starting from the yellow framed time points. Green framed time points and the green arrow represent reaching the steady state cilium. Scale bar: 2μm

(E and F) CCDC66 localization with respect to centriolar satellite, centrosome and ciliary markers during cilium assembly. RPE1::GFP-CCDC66 cells were serum starved for 24 hours and fixed with methanol and stained for **(E)** GFP, PCM1, acetylated tubulin or **(F)** GFP, ARL13B and acetylated tubulin antibodies along with DAPI in order to visualize DNA. Images from left to right represent CCDC66 localization during the initiation and elongation phases of primary cilium formation. Scale bar: 5μm

(G) Spatiotemporal localization dynamics of CCDC66 during cilium disassembly.
RPE1::GFP-CCDC66 cells were plated onto Lab-Tek imaging dish at 100% confluency, serum starved for 48 hours to induce cilium formation, and started to be imaged with confocal microscopy every 10 min immediately after serum addition. Representative images are from two cilia that undergo cilium disassembly by whole cilium shedding and resorption. GFP signal is inverted and represented as black onto white background for ciliary and centriolar satellite pools of CCDC66 to be distinguished. Scale bar: 2μm

(H) CCDC66 localization with respect to centriolar satellite, centrosome and ciliary markers during cilium disassembly After 24h serum starvation**,** RPE1::GFP-CCDC66 cells were incubated with complete media for 24 hours and fixed with methanol and stained for GFP, PCM1, acetylated tubulin along with DAPI in order to visualize DNA. Images from left to right represent CCDC66 localization during the shortening and disassembly of phases of primary cilium formation. Scale bar: 5μm

Figure S2

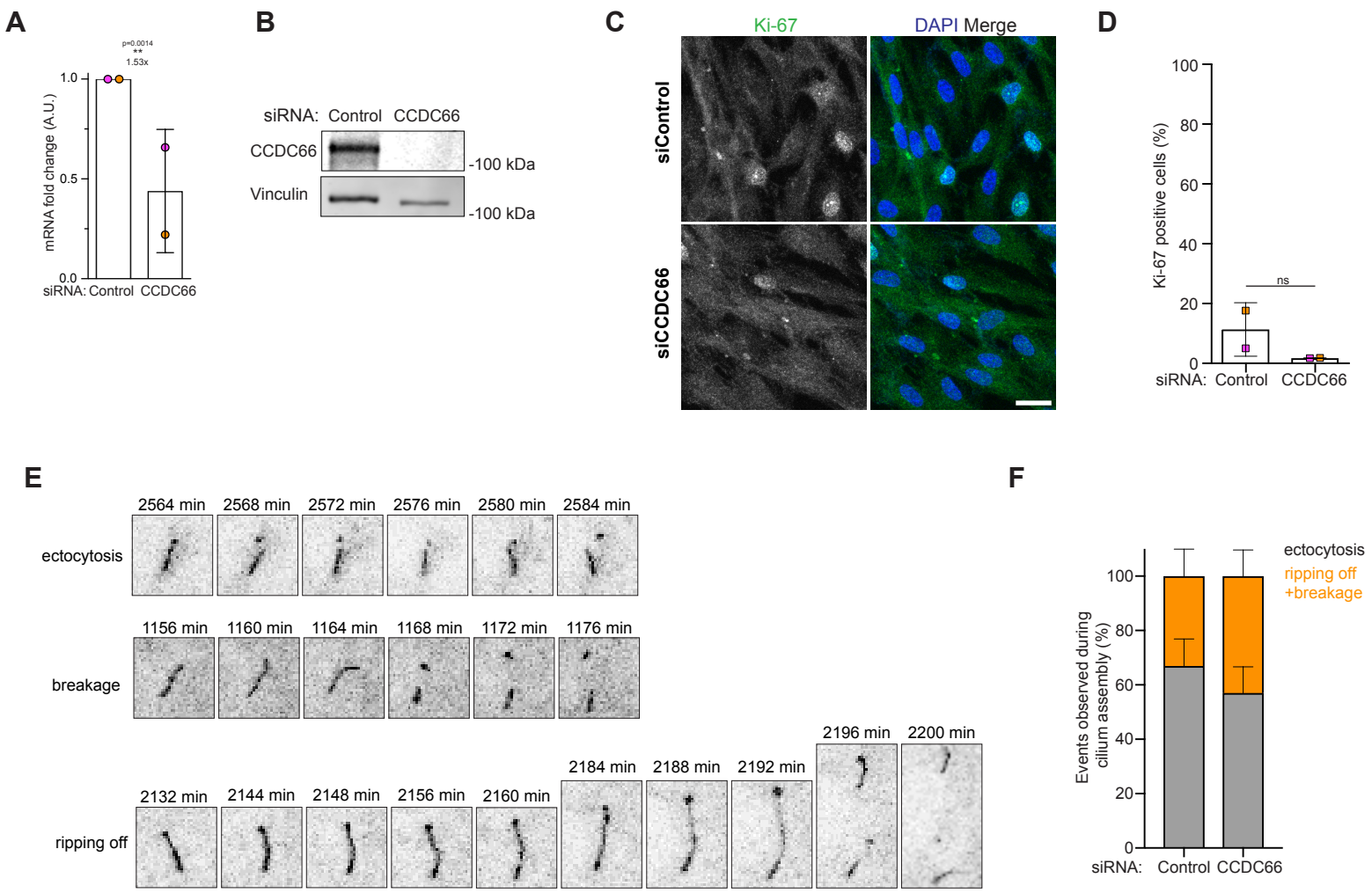


Fig. S2. (A-B) Validation of CCDC66 depletion. RPE1 cells were transfected with two rounds control or CCDC66 siRNA. 48 hours later, they were collected and processed for **(A)** qPCR analysis and **(B)** immunoblotting with CCDC66 and vinculin as control loading control. Graph represents CCDC66 mRNA fold change relative to control depletion condition. (*P < 0.5, t-test)

(C and D) Effects of CCDC66 depletion on the percentage of quiescent cells. RPE1 cells were transfected with two rounds of control or CCDC66 siRNA and serum starved for 24 hours. **(C)** Following fixation with methanol, cells were stained for anti-Ki67 and DAPI for visualization of DNA. **(D)** Graph indicates percentage of Ki-67 positive cells. Data represent the mean ±SD. Scale bar: 15μm.

(E-F) Effects of CCDC66 depletion on steady-state cilia. (C) Control or CCDC66 depleted RPE1::mCitrine-Smoothened cells were imaged after serum starvation. Cilia from both conditions were observed to undergo ectocytosis, breakage and ripping off events during cilium assembly. Images representing these events are given. **(D)** Graph indicates the percentage of ectocytosis (grey) and breakage+ripping off (orange) events in control and CCDC66 depleted cells. Data represents the mean ±SD. (100 cells/experiment, *P < 0.5, t-test)

Figure S3

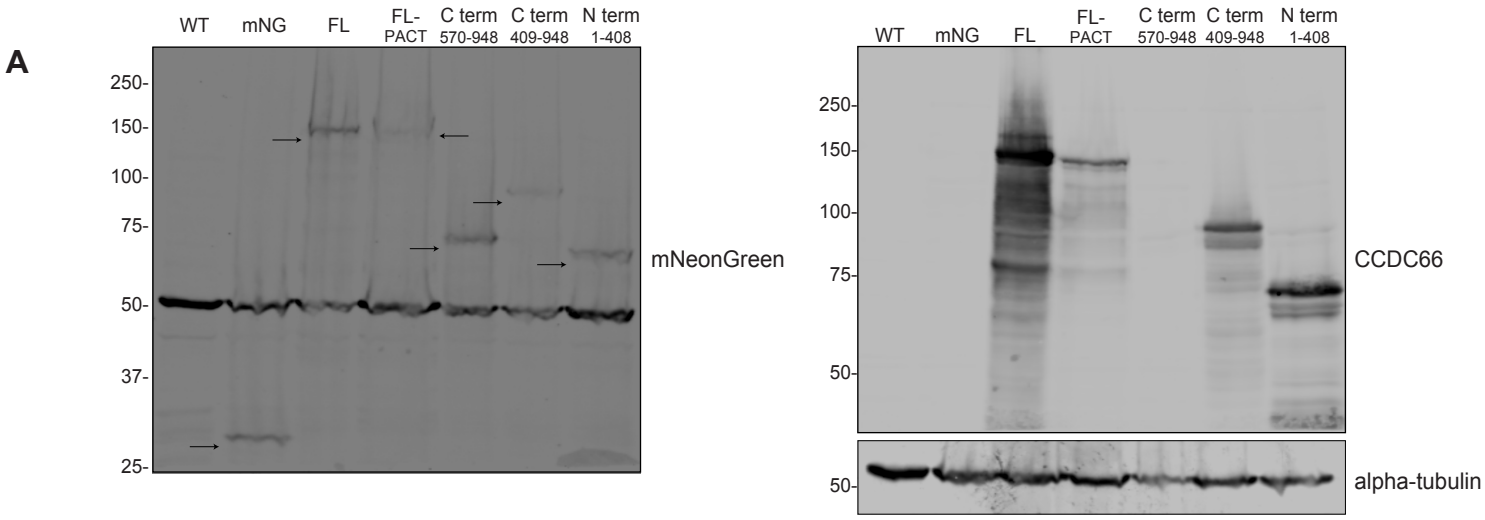
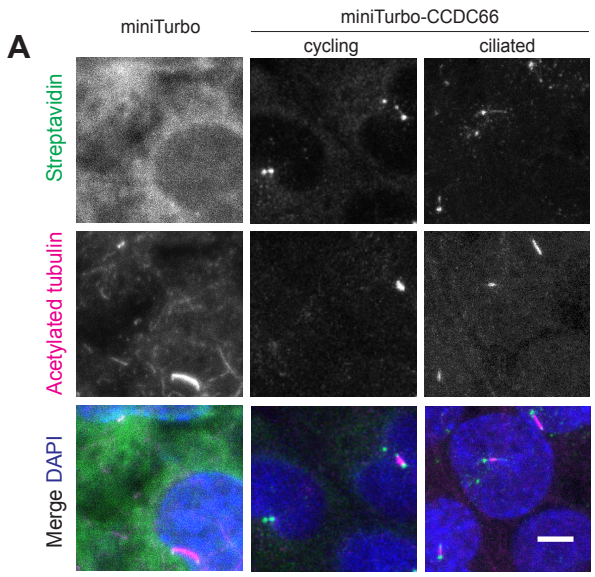


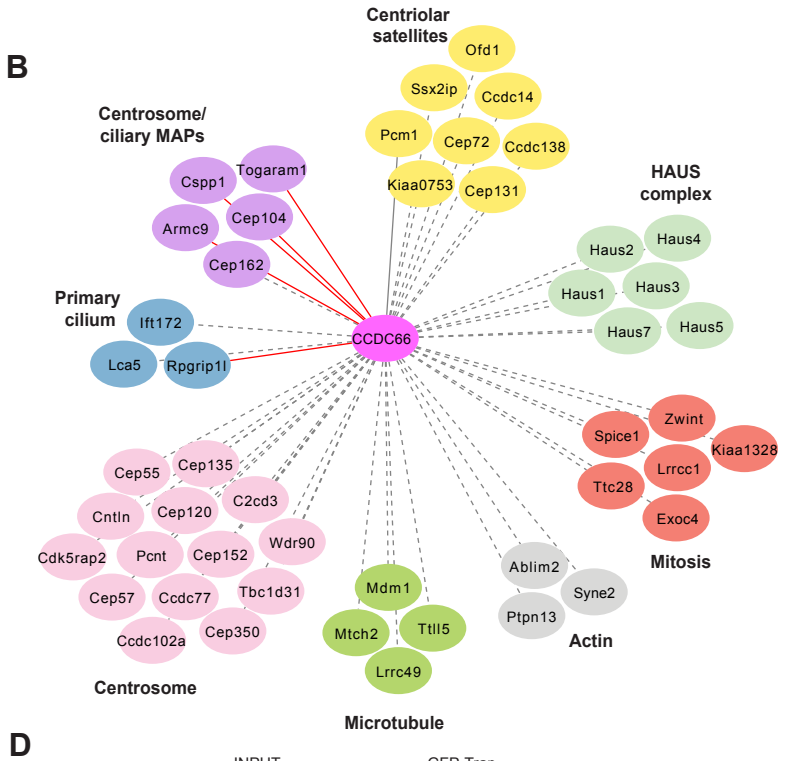
Fig. S3. (A) Validation of stable protein expression. RPE1 cell lines expressing mNeonGreen, mNeonGreen tagged CCDC66 full length, CCDC66-PACT and CCDC66 C terminal (570-948), CCDC66 (409-948) and CCDC66 (1-408) were processed and immunoblotted for mNeonGreen, CCDC66 and alpha tubulin antibodies. Black arrows indicate the corresponding band for NG tagged protein.

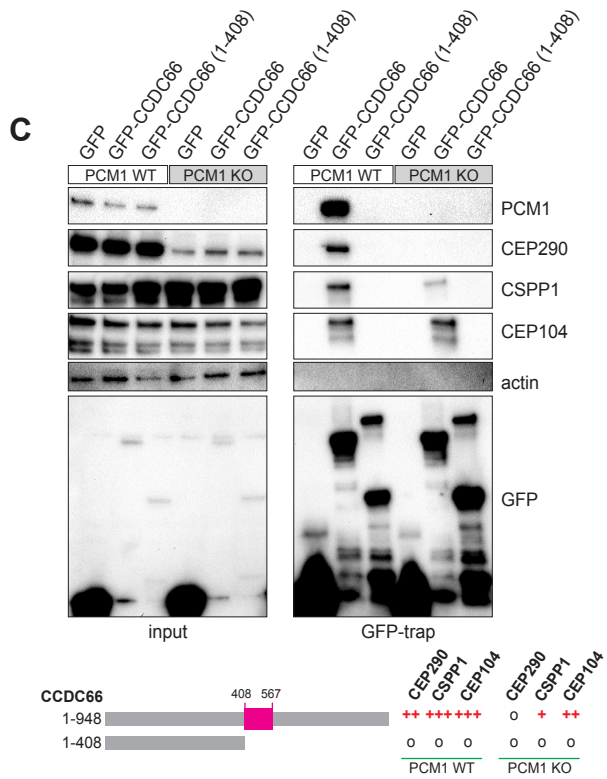
(B) mNG-CCDC66-PACT fusion protein localization is restricted to the basal body. RPE1 cells stably expressing mNeonGreen-CCDC66-PACT were serum starved for 48 hours. Following fixation, cells were stained for mNeonGreen, acetylated tubulin, gamma tubulin antibodies or mNeonGreen, glutamylated tubulin, PCM1 and DAPI for visualization of DNA. Scale bar: 2μm.

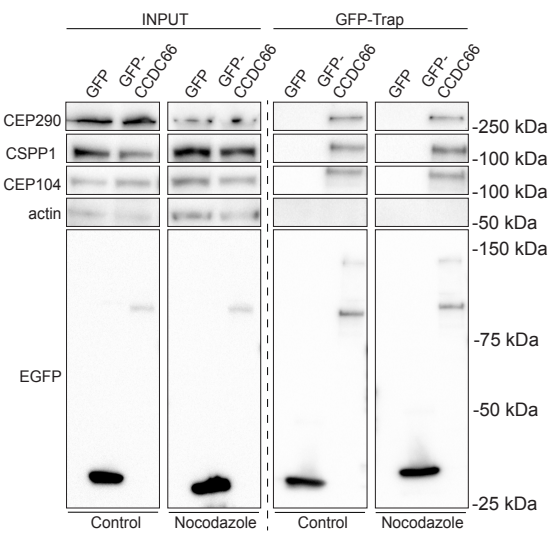
(C) mNG-CCDC66-PACT expression change centrosomal levels of centrosome and satellite proteins. RPE1::mNG-CCDC66 and RPE1::mNG-CCDC66-PACT cells were fixed and stained with CEP290, CEP63, PCM1, CEP131 and centrin3 along with centrosomal marker gamma tubulin. Gamma tubulin is taken as reference to quantify centrosomal levels of indicated proteins. Data represent the mean ±SD. Magenta and orange represent individual values from two independent experiments. (35 cell/ experiment, ns: not significant, t-test) Scale bar: 10μm

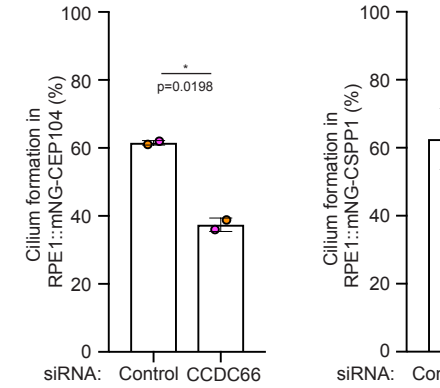
Figure S4











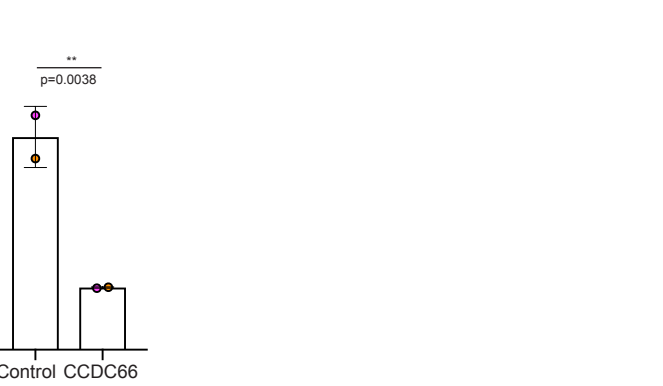


Fig. S4. (A) miniTurbo-CCDC66 induces localized biotinylation at the centrosome, centriolar satellites and primary cilium. 100% confluency IMCD3 cells stably expressing miniTurboID or miniTurboID-CCDC66 were serum starved for 48 hours. Both cell populations were treated with 500 µM biotin for 30 minutes and fixed with methanol. Cells were stained for anti-acetylated tubulin, Streptavidin and DAPI. Scale bar: 5µm

(B) CCDC66 proximity interaction map in ciliated cells. Final ciliated CCDC66 proximity interactome list is generated after applying several filtering steps to remove unspecific proteins using NSAF values. Remaining proteins are categorized based on their function and localization information**.** Dashed line: proximity interaction, solid red line: proximity and direct interactor.

(C) Centriolar satellites regulate interaction between CCDC66 and CEP290, CEP104 and CSPP1. PCM1 wild type (WT) and knockout (KO) HEK293T cells were transfected with EGFP, EGFP-CCDC66 full length and EGFP-CCDC66 (1-408) constructs. 2 days after transfection, cells were collected, lysed, and subjected to pull down with GFP Trap beads. Input and pellet were immunoblotted with anti GFP, PCM1, CEP290, CSPP1, CEP104 and actin as a control. The schematics summarizes the results of the pull down (o: no interaction, +: weak interaction, + +: moderate interaction, +++: strong interaction).

(D) CCDC66 interacts CEP290, CSPP1 and CEP104 independent of microtubules. HEK293T cells were transfected with EGFP or EGFP-CCDC66 full length. 2 days after transfection, cells were treated with 5 ug/ml nocodazole or 0.02% DMSO as a control. Following incubation, cells were collected, lysed, and subjected to pull down with GFP Trap beads. Input and pellet were immunoblotted with anti GFP, CEP290, CSPP1, CEP104 and actin as a control.

(E) Validation of CEP104 and CSPP1 depletion with Western Blotting. RPE1 cells were transfected with two rounds of control, CEP104 or CSPP1 siRNAs. 72 hours after first transfection, cells were collected, lysed and blotted for CSPP1, CEP104 and alpha tubulin antibodies.

(F-H) Validation of CEP104 and CSPP1 depletion by functional assays. RPE1 cells were transfected with two rounds of control, CCDC66, CSPP1 or CEP104 siRNAs and serum starved for 48 hours. Following fixation with methanol, cells were stained for acetylated tubulin and DAPI for visualization of DNA. **(F)** Percentage of cilium formation and **(G)** ciliary length were plotted. (100 cell or cilia /experiment, *P < 0.5, **P < 0.01, ***P < 0.001, ****P < 0.0001, ns: not significant, two way ANOVA) Scale bar: 10µm

(I-J) CCDC66 depletion causes cilium formation defect in RPE1::mNG-CEP104 and RPE1::mNG-CSPP1 cell lines. (I) RPE1::mNG-CEP104 or (J) RPE1::mNG-CSPP1 cells were transfected with two rounds of control or CCDC66 siRNA and serum starved for 48 hours. Percentage of cilium formation was quantified by dividing the cilium number determined by counting ARL13b by total cell number determined by counting nuclei and plotted. Magenta and orange represent individual values from two independent experiments. Error bars represent ±SD. (100 cells/serum starvation time point for each experiment, *P < 0.05, **P < 0.01, t-test

Table S1. Mass spectrometry results of proximity interactors of miniTurboID and miniTurboID-CCDC66 in ciliated IMCD3 cells, related to Supplementary Figure 4A and B. Column explanations of raw data are placed to sheet 2.

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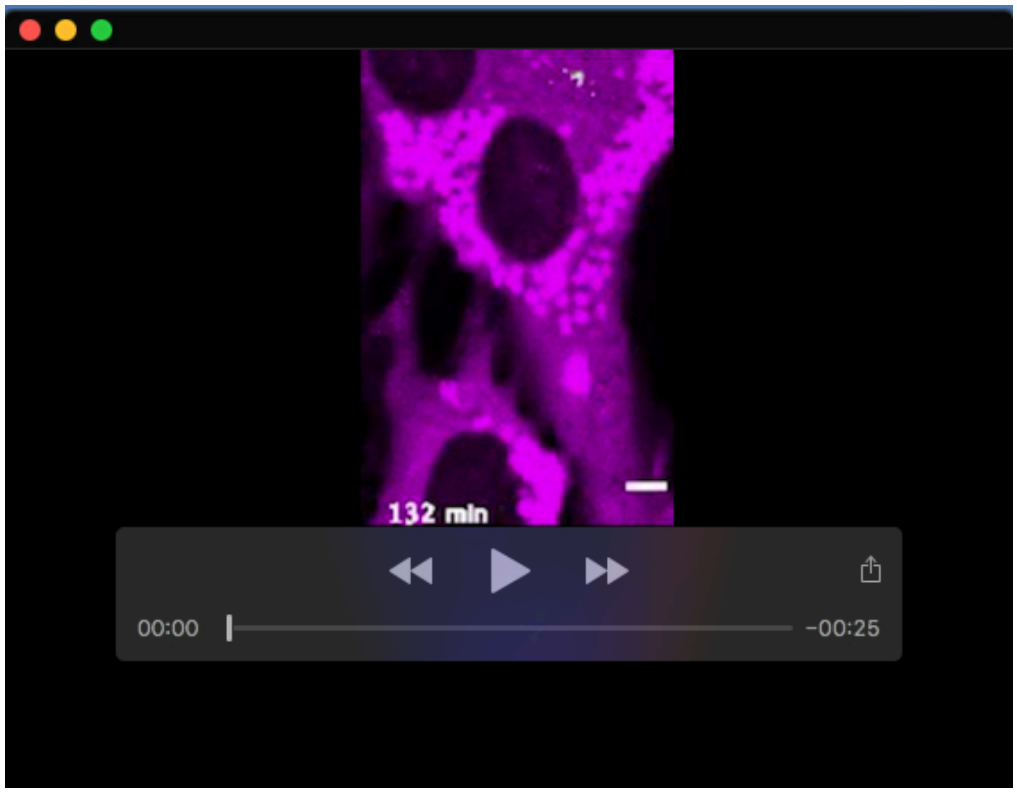
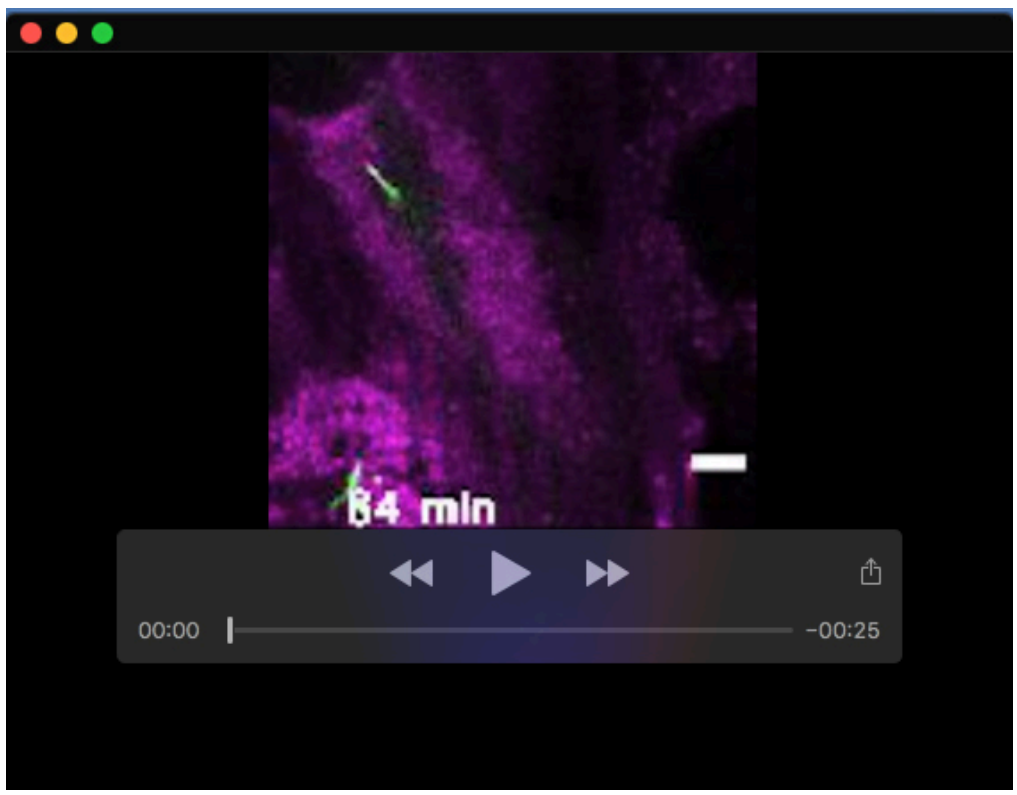


Movie 1. CCDC66 dynamic localization during cilium assembly.
RPE1::mNeonGreen-CCDC66, mScarlet-ARL13B cells were imaged with confocal microscopy every 8 minutes immediately after serum starvation. Scale bar: 5μm**.**



Movie 2. CCDC66 dynamic localization during cilium disassembly. After 48 hours serum starvation, RPE1::mNeonGreen-CCDC66, mScarlet-ARL13B cells were imaged with confocal microscopy every 6 minutes immediately upon serum addition. Scale bar: 5-μm.