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Supplementary Materials for

Cross-talk between CDK4/6 and SMYD2 regulates gene transcription, tubulin methylation, and ciliogenesis

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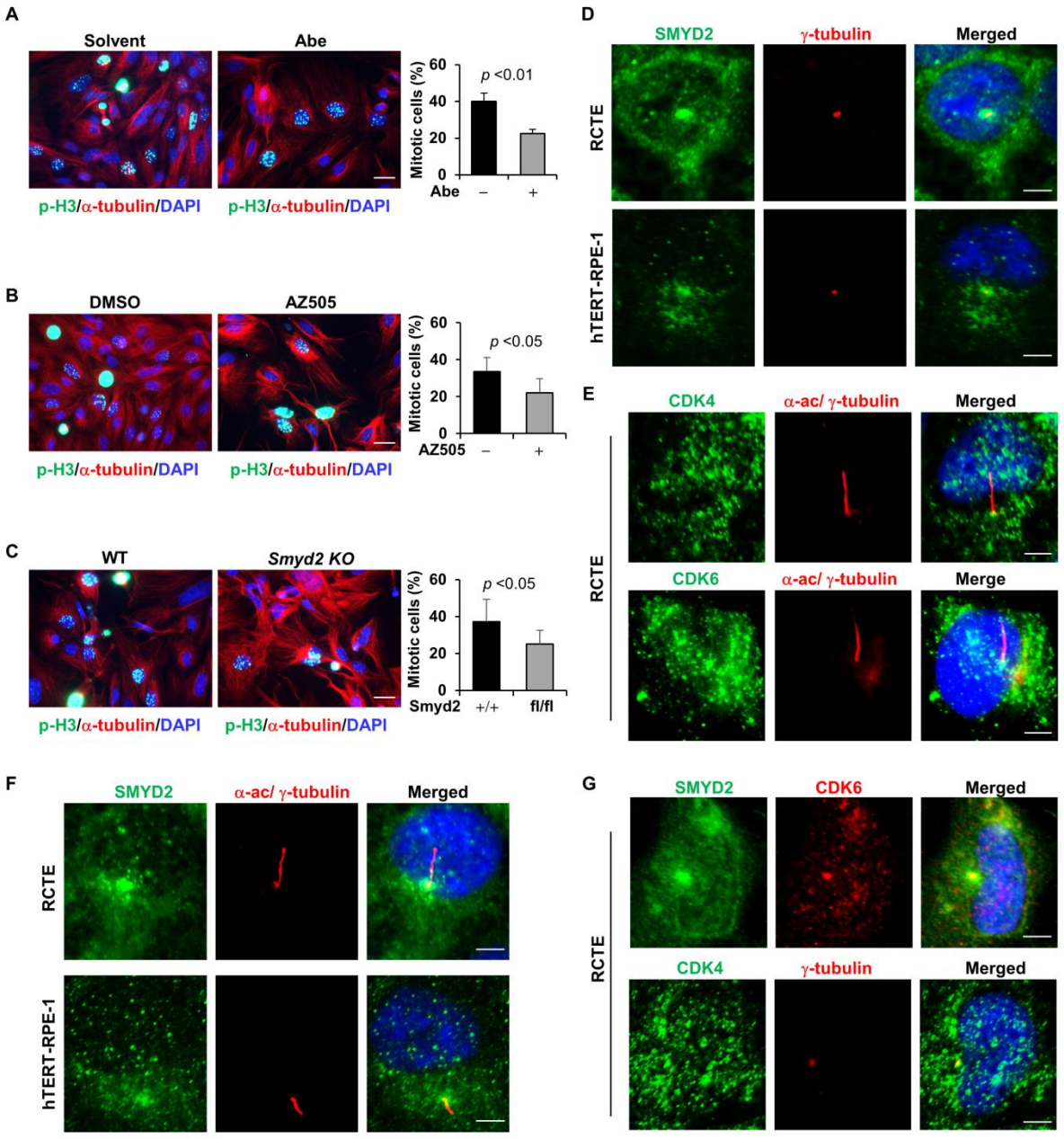

Fig. S1. Smyd2 and CDK4/6 are localized on the basal body but not on cilia and inhibition of CDK4/6 and Smyd2 delay mitotic entry. (**A** and **B**) Inhibition of CDK4/CDK6 with Abe (**A**) and inhibition of SMYD2 with AZ505 (**B**) delayed mitotic entry of mIMCD3 cells as stained with phospho-H3, a mitotic marker (*green*), and α-tubulin (*red*) antibodies as well as DAPI. Scale bar, 10 μm. More than 200 cells were analyzed in right graphs. (**C**) Cell mitosis was suppressed in Smyd2 knockout cells. Knockout of Smyd2 suppressed mitotic entry of primary renal epithelia cells compared to wild type primary renal epithelia cells as examined by immunofluorescence staining with phospho-H3 (*green*) and α-tubulin (*red*) antibodies as well as DAPI. Scale bar, 10 μm. More than 200 cells were analyzed in right graph. (**D**) Representative

images of RCTE cells (*top*) and hTERT-RPE1 cells (*bottom*) stained with SMYD2 (*green*) and γ-tubulin (*red*) antibodies as well as DAPI (*blue*). (**E**) Representative images of RCTE cells stained with CDK4 (*green, top*) or CDK6 (*green, bottom*) and α-ac-tubulin/γ-tubulin (*red*) antibodies as well as DAPI (*blue*). (n = 50 cilia for each group). (**F**) Representative images of RCTE cells (*top*) and hTERT-RPE1 cells (*bottom*) stained with SMYD2 (*green*) and α-ac-tubulin/γ-tubulin (*red*) antibodies as well as DAPI (*blue*). (**G**) Representative images of RCTE cells stained with SMYD2 (*green*) and CDK6 (*red*) antibodies (*top panel*), CDK4 (*green*) and γ-tubulin (*red*) antibodies (*bottom panel*), as well as DAPI (*blue*).

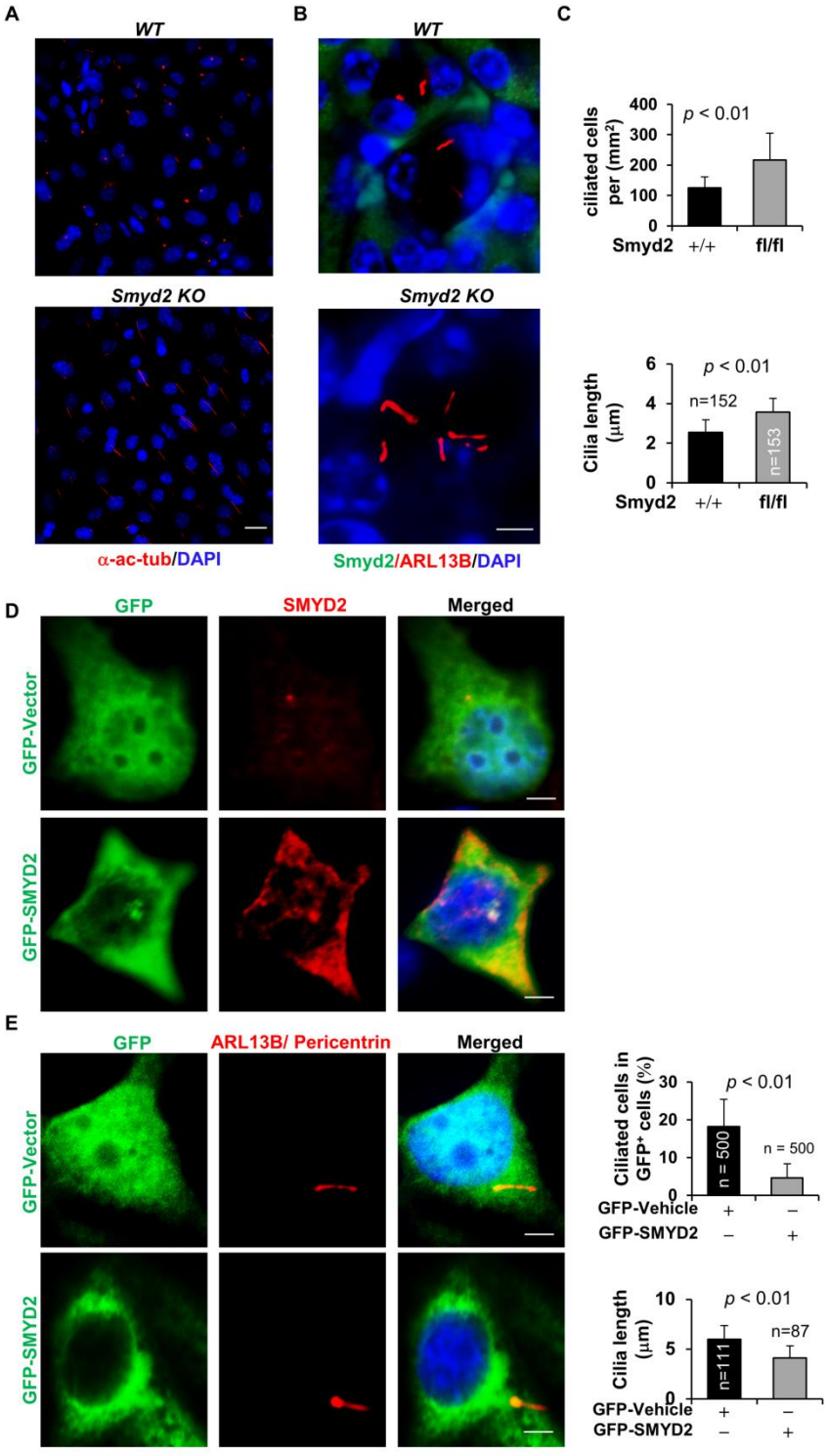

Fig. S2. Depletion of Smyd2 results in more and longer cilia in primary renal epithelia cells and mouse kidneys, and overexpression of SMYD2 suppresses ciliogenesis in HEK293T cells. (**A**) Representative images of cultured primary renal epithelia cells show more

and longer cilia in Smyd2 knockout cells as examined with α-ac-tubulin (*red*) antibody and co-stained with DAPI (*blue*). Scale bar, 10 μm. (**B**) Representative images of mouse kidneys show more and longer cilia in Smyd2 knockout mouse kidneys as examined with Smyd2 (*green*) and ARL13B (*red*) antibodies and co-stained with DAPI (*blue*). Scale bar, 5 μm. (**C**) Statistical analysis of percentages of ciliated cells and cilia length in Smyd2 wildtype and knockout kidneys. N values represent the number of cilia that were analyzed (*bottom panel*). Error bars represent the s.d. (**D**) Validation of GFP-tagged SMYD2 expression in HEK293T cells as examined with SMYD2 (*red*) antibody. (**E**) Representative images and statistical analysis of HEK293T cells show less and shorter cilia in SMYD2 overexpression cells compared to those cells transfected with GFP-Vector as examined with ARL13B/Pericentrin (*red*) antibody and co-stained with DAPI (*blue*). Scale bar, 5 μm. N values represent the numbers of cells (*right top graph*) and cilia (*right bottom graph*), respectively, for each group. Error bars represent the s.d.

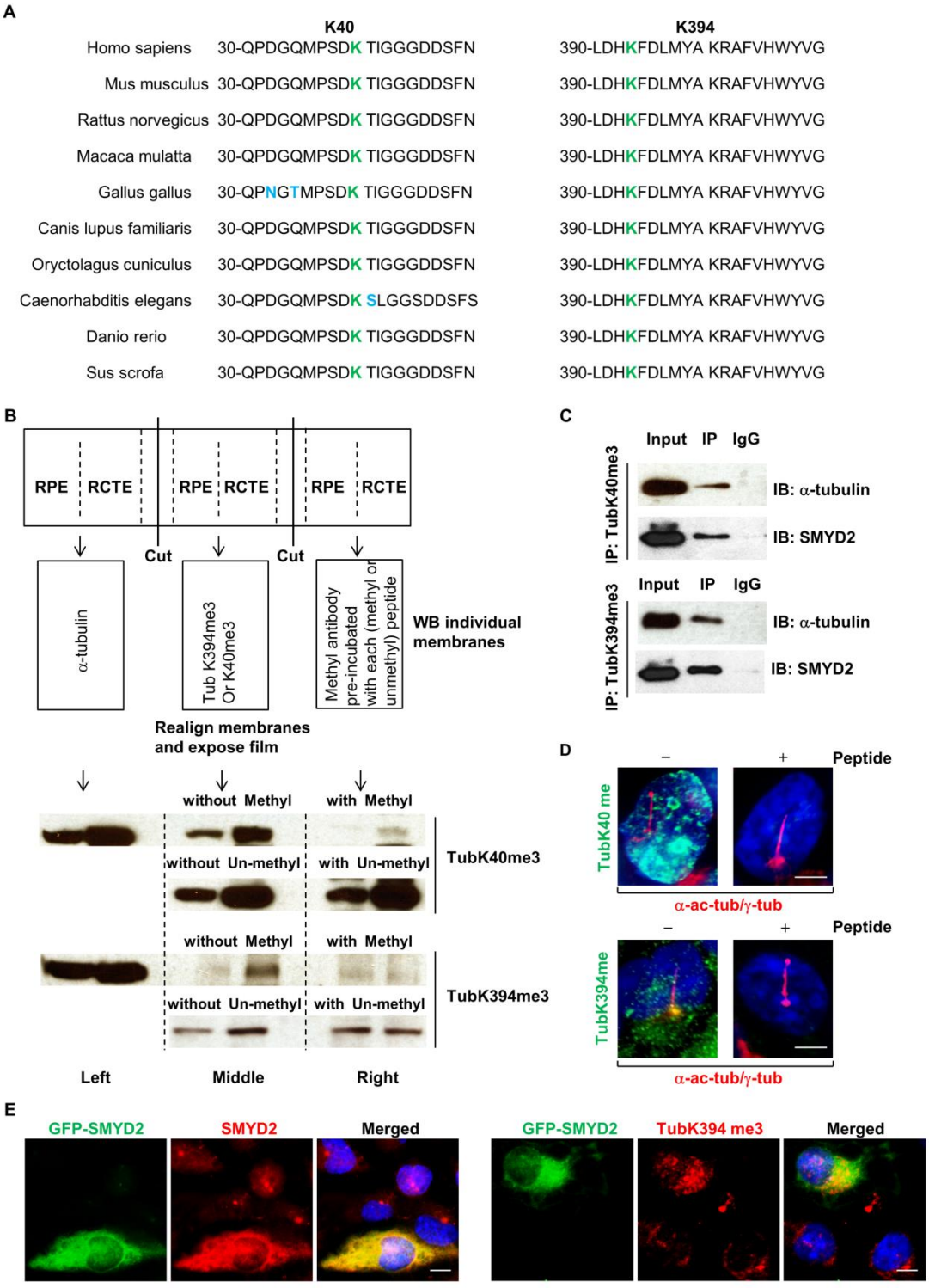

Fig. S3. Validation of the newly generated TubK40me3 and TubK394me3 antibodies. (**A**) The amino acid sequences surrounding lysine 40 (K40) and lysine 394 (K394) of α-tubulin are conserved in different species. The variable amino acids are marked in blue. (**B**) Peptide

competition assay of the TubK40me3 and TubK394 me3 antibodies. Cell extracts (50 μg) of RCTE cells and hTERT-RPE1 cells were run on a 12% SDS-PAGE gel and transferred onto PVDF membranes, and then immunoblotted with either the TubK40me3 or TubK394me3 antibodies pre-incubated with K40 and K394 un-methylated peptides, or K40me3 and K394me3 peptides for 60 min at room temperature. The membranes were realigned and exposed to the same films. (**C**) Co-immunoprecipitation assays showed that TubK40me3 and TubK394me3 pulled down α-tubulin and SMYD2 in RCTE cells. (**D**) Representative images of RCTE cells stained with TubK40me3 and TubK394 me3 antibodies with or without K40me3 and K394me3 peptides. Scale bar, 5 μm. (**E**) Representative images of HEK293T cells stained with SMYD2 (*red, top*) and TubK394me3 (*red, bottom*) antibodies. Overexpressed GFP-SMYD2 (*green*) and DAPI staining (*blue*) are also shown in these images. Scale bar, 5 μm.

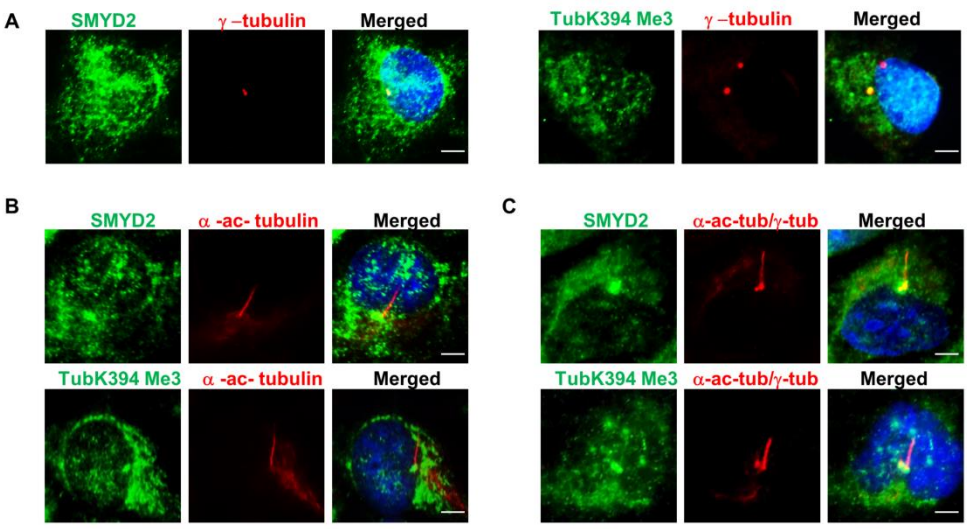

Fig. S4. SMYD2 and methylated K394 α-tubulin localized to basal body and surrounding area. (**A**) Representative images of RCTE cells stained with SMYD2 (green) and γ-tubulin (red) antibodies as well as DAPI (*blue*) (left panels) and stained with TubK394me3 (green) and γ-tubulin (red) antibodies as well as DAPI (*blue*) (*right panels*). Scale bar, 5 μm. (**B**) Representative images of RCTE cells stained with SMYD2 (*top*) or TubK394me3 (*bottom*) antibodies, co-stained with α-ac-tubulin (*red*) antibody and DAPI (*blue*). RCTE cells were serum starved for 48-72 hours before subjected to staining. Scale bar, 5 μm. (**C**) Representative images of RCTE cells stained with SMYD2 (*top*) or TubK394me3 (*bottom*) antibodies, co-stained with α-ac-tubulin/γ-tubulin (*red*) antibodies and DAPI (*blue*). Scale bar, 5 μm.

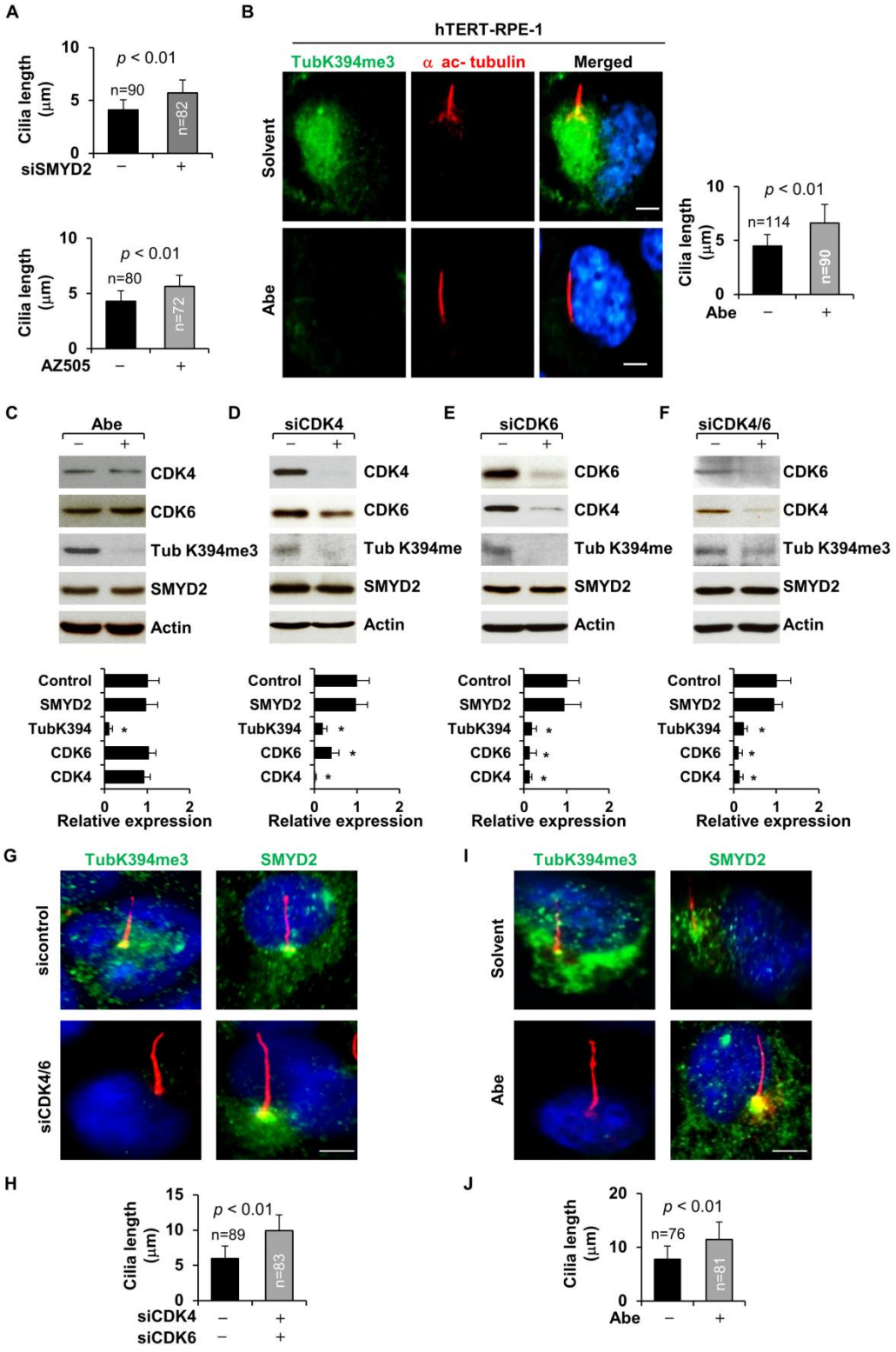

Fig. S5. Inhibition of SMYD2 and CDK4/6 decreased the methylation of α-tubulin at K394 and resulted in longer cilia in hTERT-RPE-1 and RCTE cells. (**A**) Statistical analysis of cilia

length in hTERT-RPE-1 cells transfected with SMYD2 siRNA (*top*) or treated with SMYD2 inhibitor AZ505 (*bottom*). Error bars represent the s.d. N values represent the numbers of cilia for each group. (**B**) Representative images of hTERT-RPE-1 cells stained with TubK394me3 (*green*) and α-ac-tubulin (*red*) antibodies, and co-stained with DAPI (*blue*) in vehicle (*top*) or Abe-treated hTERT-RPE-1 cells. Statistical analysis of cilia length in hTERT-RPE-1 cells treated with vehicle and Abe. Scale bar, 5 μm. Error bars represent the s.d. N values represent cilia numbers. (**C**) Inhibition of CDK4/6 with Abe for 6 hours decreased the methylation of α-tubulin at K394, but did not affect the expression of SMYD2, CDK4 or CDK6 at this time point as examined by Western blotting analysis. (**D** - **F**) Knockdown of CDK4 (**D**), CDK6 (**E**) or both (**F**) decreased expression of CDK4, CDK6 and the methylation of α-tubulin at K394, but did not affect the expression of SMYD2 as examined by Western blotting analysis. The quantification and statistical analysis (n = 3) were shown in the graphs (*bottom panel*s). * represents $p < 0.01$ as compared to controls. (**G**) Representative images of RCTE cells stained with TubK394me3 (*left, green*) and SMYD2 (*right, green*) and co-stained with α-ac-tubulin (*red*) and DAPI (*blue*). Knockdown of both CDK4/CDK6 with siRNAs decreased the basal body staining of TubK394me3 and resulted in longer cilia in RCTE cells. Scale bar, 5 μm. (**H**) Statistical analysis of cilia length in RCTE cells treated with or without siRNAs of CDK4 and CDK6. Error bars represent the s.d. N values represent cilia numbers. (**I** and **J**) Representative images (**I**) and statistical analysis (**J**) of RCTE cells stained with TubK394me3 (*left, green*) or SMYD2 (*right, green*) and co-stained with α-ac-tubulin (*red*) and DAPI (*blue*). Inhibition of CDK4/6 with Abe decreased the basal body staining of TubK394me3 and resulted in longer cilia in these cells. Scale bar, 5 μm. Error bars represent the s.d. N values represent cilia numbers.

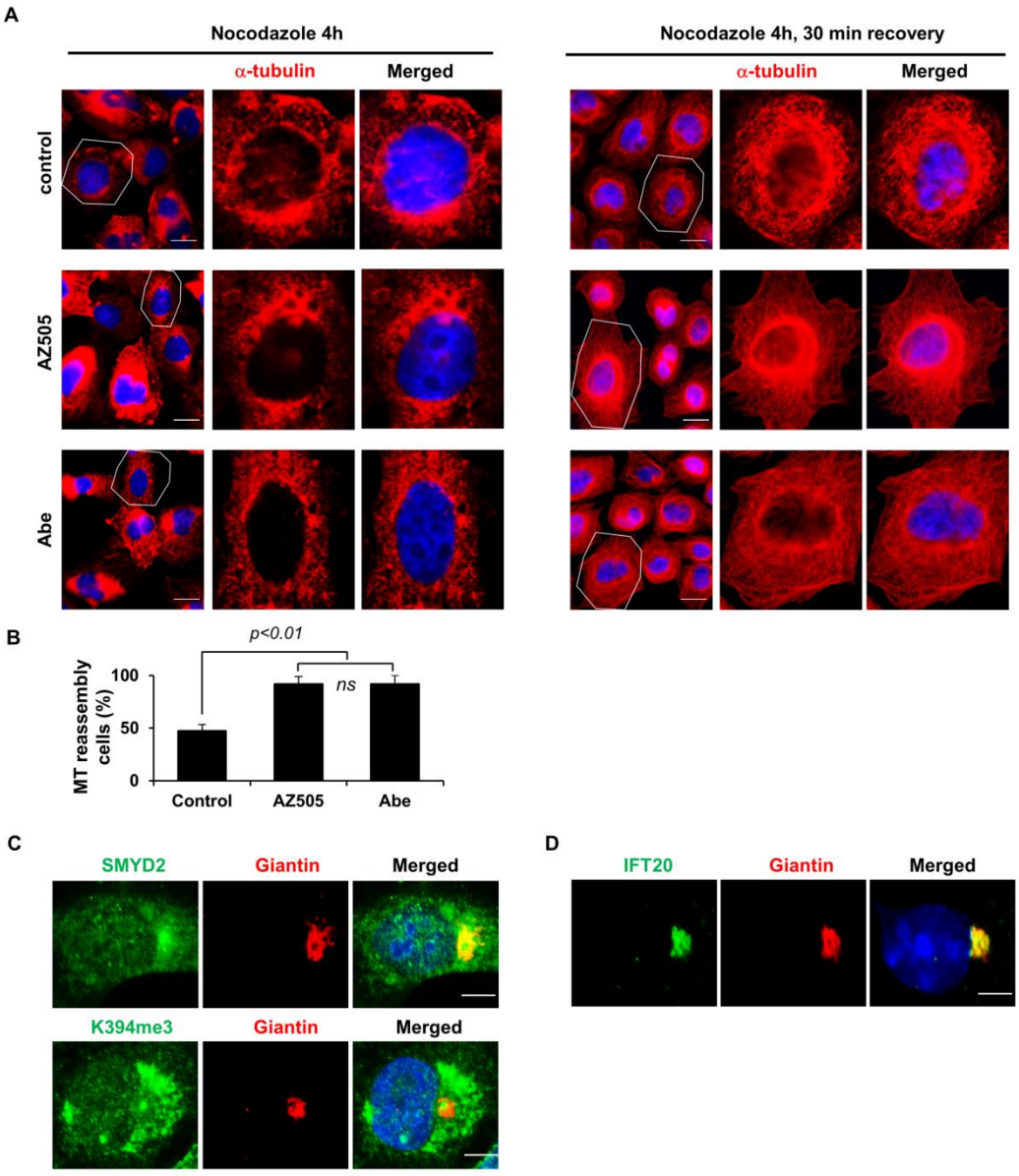

Fig. S6. Inhibition of SMYD2 and CDK4/6 affects the stability of microtubules, and methylated α-tubulin at K394 as well as IFT20 are accumulated at the Golgi and surrounding area. (**A**) Immunofluorescence images of RCTE cells showing the depolymerization of microtubules induced by Nocodazole (0.2 μg/ml, 4 hours, *left*) in the presence of vehicle (*top*), SMYD2 inhibitor AZ505 (*middle*) or CDK4/6 inhibitor Abe (*bottom*), and the repolymerization with fresh complete medium for 30 min (*right*) in the presence of vehicle (*top*), SMYD2 inhibitor AZ505 (*middle*) or CDK4/6 inhibitor Abe (*bottom*) after those cell were incubated with Nocodazole for 4 hours. All the treated cells were stained with α-tubulin antibody (red) and co-stained with DAPI (*blue*). Scale bar, 20 μm. (**B**) The statistical analysis of cells (n = 200) with microtubule re-assembled by 30 minutes in RCTE cells treated with AZ505 or Abe compared to control cells. (**C**) Representative images of RCTE cells stained with SMYD2 (*green, top*) or TubK394me3 (*green, bottom*) and co-stained with the Golgi marker Giantin (*red*)

and DAPI (*blue*). Scale bar, 5 μm. (**D**) Representative images of RCTE cells stained with IFT20 (*green*) and Giantin (*red*) and co-stained with DAPI (*blue*). Scale bar, 5 μm.

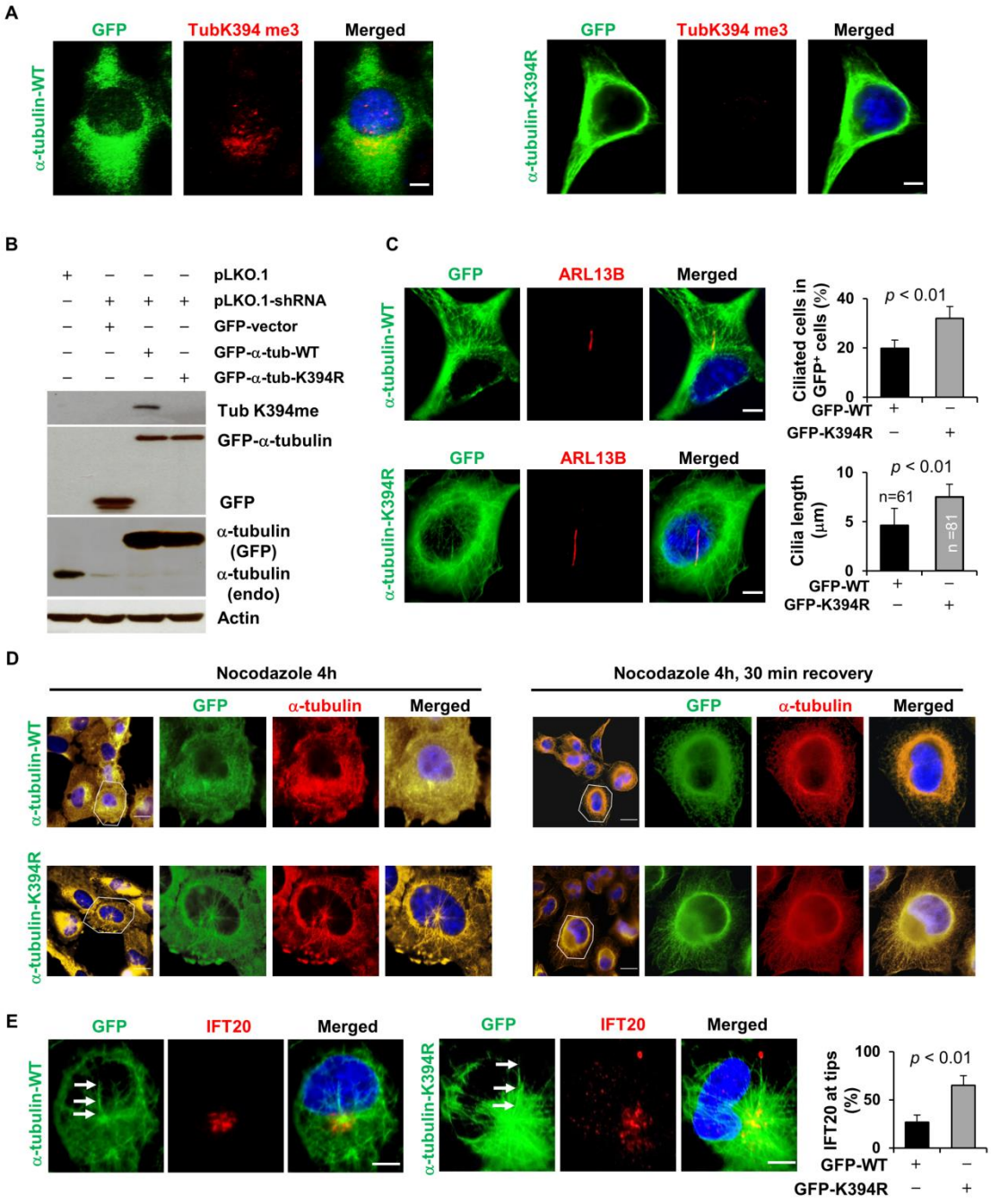

Fig. S7. Mutation of α-tubulin at K394 affects microtubule stability and cilia assembly. (A) Representative images of RCTE cells expressing of GFP-tagged wild type α-tubulin and mutant α-tubulin (K394R) (*green*) stained with TubK394me3 (*red*) antibody and DAPI (*blue*). Scale bar, 5 μm. (B) Western blot analysis of GFP-tagged wild type and mutant α-tubulin (K394R) and methylated α-tubulin (TubK394me) in RCTE cells. (C) Representative images of RCTE cells expressing of GFP-tagged wild type α-tubulin and mutant α-tubulin (K394R) stained with ARL13B. Scale bar, 5 μm. Error bars represent the s.d. N values represent the numbers of cells

(*right top graph*) and cilia (*right bottom graph*), respectively, for each group. (**D**) Immunofluorescence images of RCTE cells showing the depolymerization of microtubules induced by Nocodazole (0.2 μg/ml, 4 hours, *left panels*) in RCTE cells harboring GFP-α-tubulin-WT (*top panels*) and GFP-α-tubulin-K394R (*bottom panels*), and the repolymerization of microtubules in those cells that were cultured with fresh complete medium for 30 min (*right panels*) after those cells were incubated with Nocodazole for 4 hours. All the treated cells were stained with α-tubulin antibody (*red*) and co-stained with DAPI (*blue*). Scale bar, 20 μm. (E) Representative images of RCTE cells stained with IFT20 (*red, middle*) and GFP, and DAPI (*blue*). Scale bar, 5 μm. In the graph, 50 cilia were analyzed for each group.

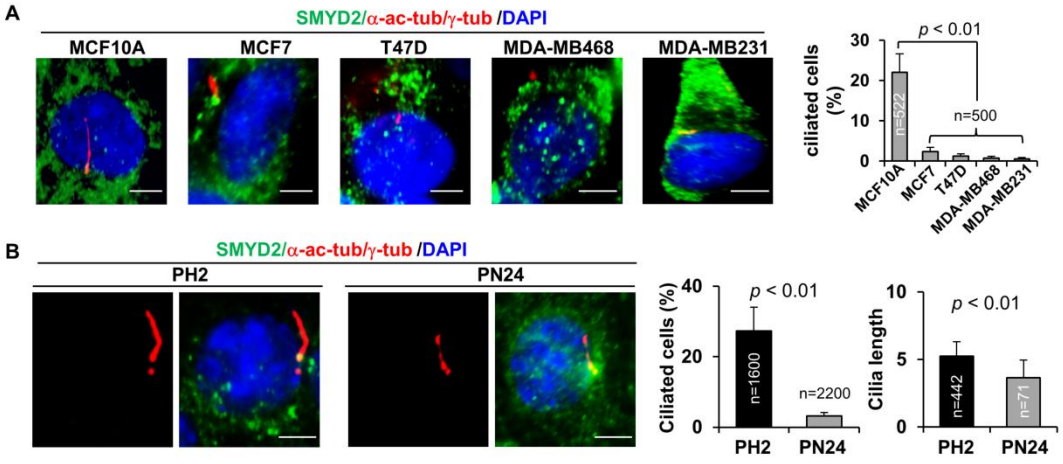

Fig. S8. Primary cilia assembly was decreased in CDK4/6 and SMYD2 upregulated breast cancer cells and cystic renal epithelial cells. (**A**) IF of MCF10A cells and breast cancer cells stained with SMYD2 (*green*) and α-ac-tubulin/γ-tubulin (*red*) antibodies and co-stained with DAPI (*blue*). Scale bar, 5 μm. The percentage of ciliated cells is shown in the graph (*right*). $p < 0.01$. Error bars represent the s.d. N values represent cell numbers analyzed in each cell line. (**B**) IF of *Pkd1* heterozygous PH2 cells and *Pkd1* homozygous PN24 cells stained with Smyd2 (green) and α-ac-tubulin/γ-tubulin (*red*) antibodies and co-stained with DAPI (*blue*). Scale bar, 5 μm. The percentage of ciliated cells and cilia length of PH2 and PN24 cells were statistically analyzed (*right panel*). Error bars represent the s.d. N values represent the numbers of cells (*left graph*) and cilia (*right graph*), respectively, for each cell line.

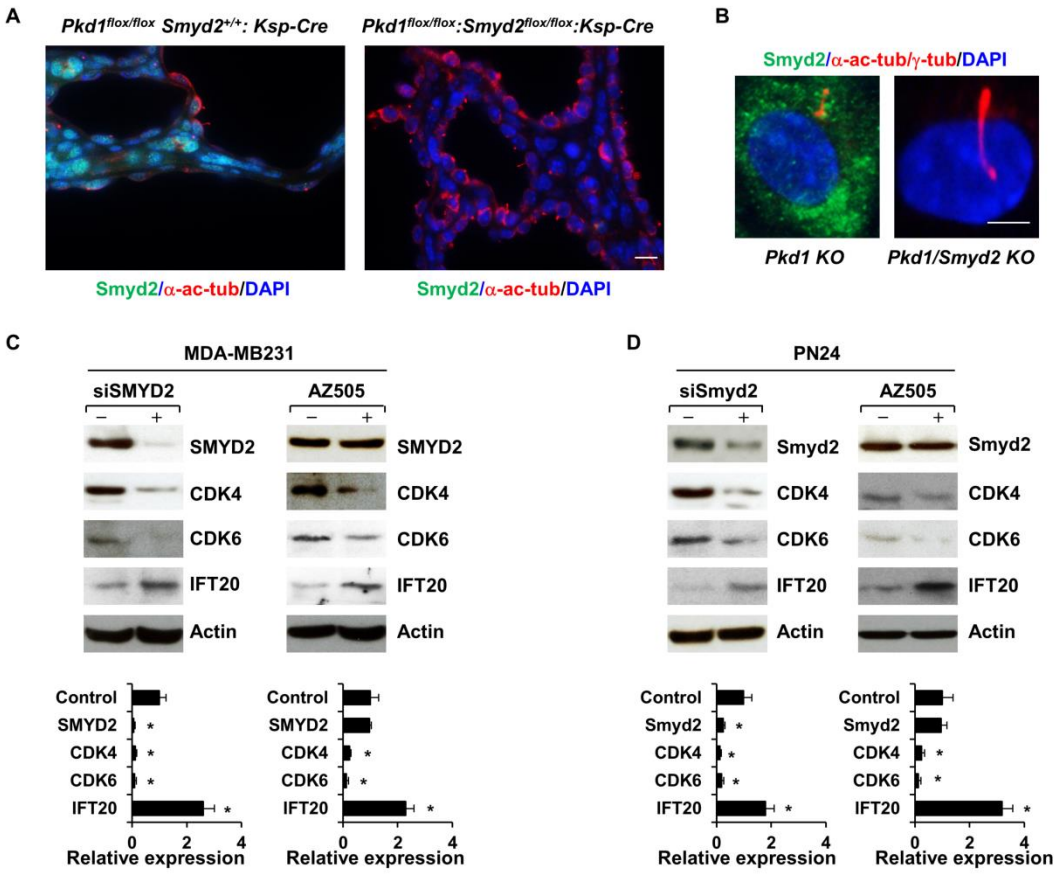

Fig. S9. Inhibition and knockout of SMYD2 decrease CDK4/6 expression and result in the increase of cilia length. (**A**) Representative images of kidneys from *Pkd1* single knockout and *Pkd1/Smyd2* double knockout mice stained with Smyd2 (*green*) and α-ac-tubulin (*red*) antibodies, and co-stained with DAPI (*blue*). Scale bar, 10 μm. (**B**) Immunofluorescence images of primary renal epithelia cells isolated from *Pkd1* single knockout kidneys and *Pkd1/Smyd2* double knockout kidneys stained with Smyd2 (*green*) and α-ac-tubulin (*red*), and co-stained with DAPI (*blue*). Scale bar, 5 μm. (**C**) Western blot analysis of SMYD2, CDK4, CDK6 and IFT20 in MDA-MB231 cells treated with SMYD2 siRNA and SMYD2 inhibitor AZ505. The quantification and statistical analysis of three independent experiments were showed in the graphs (*bottom panel*), as is also shown in **D**. * represents $p < 0.01$ as compared to controls. (**D**) Western blot analysis of Smyd2, CDK4, CDK6 and IFT20 in PN24 cells treated with Smyd2 siRNA and SMYD2 inhibitor AZ505.

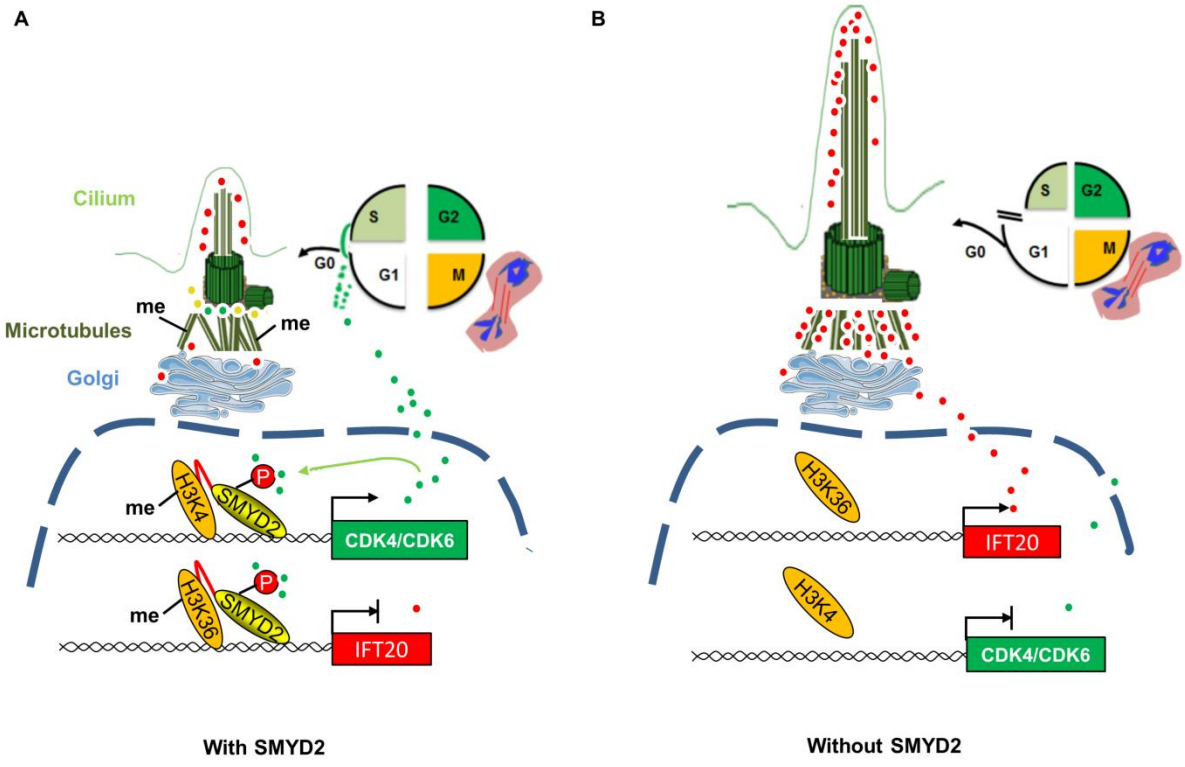

Fig. S10. Working model of CDK4/6 and SMYD2 in the regulation of ciliary gene transcription and ciliogenesis during the cell cycle. (**A**) In the presence of SMYD2, CDK4 and CDK6 phosphorylate and activate SMYD2, leading to the methylation of histone H3K4 and H3K36 as well as α-tubulin during the cell cycle. Methylation of H3K4 positively regulates the transcription of CDK4 and CDK6 to form a positive feedback loop as CDK4/6-SMYD2-H3K4-CDK4/6, which promotes the CDK4/6-mediated G1 to S phase transition. Methylation of H3K36 negatively regulates the transcription of IFT20 to decrease the transport of ciliary proteins from the Golgi to cilia, which facilitate cilia disassembly and resorption. Methylation of α-tubulin at lysine 394 decreases the stability of microtubules, particularly at the region surrounding the Golgi and cilia base, to further decrease the transport of ciliary proteins from the Golgi to cilia, which facilitate cilia disassembly and resorption to promote cell cycle progression. In breast cancer cells and cystic renal epithelial cells, these three mechanisms mediated by the upregulation of CDK4/6 and SMYD2 increase cell proliferation and cilia disassembly and resorption to promote breast cancer and renal cyst progression. (**B**) In the absence of SMYD2, targeting CDK4/6 and SMYD2 decreases the activity of SMYD2 and then the methylation of H3K4 and H3K36 and microtubules to decrease the expression of CDK4/6 but increase the expression of IFT20 and the stability of microtubules surrounding the Golgi and the cilia base. This delays CDK4/6 mediated G1-S transition but increases the transport of ciliary proteins from the Golgi to cilia, leading to decreased cell proliferation and increased cilia assembly and elongation.