Cell Reports
Supplemental Information

The Microtubule-Depolymerizing Activity of a Mitotic Kinesin Protein KIF2A Drives Primary Cilia Disassembly Coupled with Cell Proliferation

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Supplemental information

Extended Experimental Procedures

TALEN-mediated gene targeting. hTERT-RPE1 cells (1×10^6 ; American Type Culture Collection; CRL-4000) were plated in six-well plates at 24 h before transfection. The cells were transfected with 10 ng of targeting vector (pTV-TK-2A-puro or pTV-TK-2A-neo) and 300 ng each of TALEN expression vectors using Lipofectamine LTX (Life Technologies) according to the manufacturer's instructions. After 24 h, the cells were reseeded to 15 cm plates, incubated for 72 h, and then subjected to puromycin selection (5 μg / mL) or neomycin (2 μg / mL). The drug-resistant cell colonies were picked on days 17–20 after transfection. Colonies were divided into two aliquots: one half was transferred into one well of a 96-well plate for clonal expansion, and the other half was lysed and used for PCR genotyping. PCR-positive clones were further expanded and gene targeting verified by Southern blotting. See Supplemental information for more details.

Antibodies. The primary antibodies used were: mouse anti-acetylated tubulin monoclonal antibody mAb (Sigma Aldrich : T7451); rabbit anti-KIF2A polyclonal antibody pAb (Abnova : PAB12407); rabbit anti-Arl13b pAb (Proteintech : 17711-1-AP); rabbit anti-CP110 pAb (Proteintech : 12780-1-AP); rabbit anti-pericentrin pAb (Bethyl Laboratories : A301-348A); rat anti-α-tubulin mAb (Novus : NB600-506); mouse anti-ninein mAb (Merk Millipore : MABT29); mouse anti-γ-tubulin mAb (Sigma Aldrich : T6557); mouse anti-β-tubulin mAb (Sigma Aldrich : T8328); mouse anti-GAPDH mAb (Santa Cruz Biotechnology : sc-32233); rabbit anti-GFP pAb (MBL :

598); rabbit anti-PLK1 pAb (Santa Cruz Biotechnology : sc-5585); rabbit anti-phospho-T210 PLK1 pAb (BioLegend : 618602); mouse anti-GFP mAb (Roche : 11-814-460-001); mouse anti-CDC27 mAb (BD Transduction Laboratories : 610454); rabbit anti-hemagglutinin (HA) pAb (Santa Cruz Biotechnology : sc-805) and mouse anti-DYKDDDDK (FLAG) tag mAb (Wako : 018-22381). The rabbit anti-KIF24 pAb and rabbit anti- BUBR1 pAb antibodies were raised and characterized previously (Kobayashi et al., 2011; Matsuura et al., 2006).

Anti-phosphorylated T554-KIF2A pAbs were raised in two rabbits using keyhole limpet hemocyanin (KLH)-conjugated RVKEL(pT)VDPTC peptide. Antibodies were affinity purified on phosphorylated- and/or nonphosphorylated-epitope-bound columns.

RNA interference. The following Stealth siRNAs (Life Technologies) were used: human *KIF2A* (5'-CCCUGACCUUGUUCCUGAUGAAGAA-3'); human *KIF2A-3'UTR* (5'-GGUCAGCUGUAAGGGCCAUUUGAA-3'); and human *PLK1* (5'-UUAGGAGUCCCACACAGGGUCUUCU-3'). Stealth negative control duplexes (Cat. No. 12935-300 and 12935-112; Life Technologies) were also used.

Plasmids. *AcGFP1*-tagged human *KIF2A*, *KIF2B*, *KIF2C*, and *KIF24* expression vectors for mammalian cells and a *GST*-tagged human *KIF2A* plasmid for *E. coli* were constructed by PCR and standard cloning techniques. We used site-directed mutagenesis to insert mutations into KIF2A. All mutations were verified by automated sequencing. To construct transcription activator-like effector

nuclease (TALEN) expression vectors, the assembled repeats of the DNA binding domain of transcription activator-like effectors (TALEs) were inserted into pTALEN5 as previously described (Ochiai et al., 2014). The FokI domains with ELD:KKR mutations used were obligate heterodimers to decrease off-target cleavage by TALENs. The target sequences and amino acid sequences of TALENs for human *KIF2A* gene are shown in Figure S3 A. The functionality of assembled TALENs was assessed by the single-strand annealing assay as previously described (Ochiai et al., 2014).

PCR genotyping. PCR genotyping to screen for homologous recombinants was performed using KOD-FX neo DNA polymerase (TOYOBO) with a pair of PCR-F (5'-GCCTTTGATGACTCAGCTCCTAATG-3') and PCR-R (5'-AAAGTTCCCACTTACCTTTCCAC-3') primers designed on the regions outside the targeting vector arms and using extracted genomic DNA as a template. PCR products were run on a 0.7 % agarose gel.

Southern blot analysis. To confirm targeting in candidate clones, genomic DNA was extracted from cells, and 20 μg aliquots were digested overnight with EcoRV at 37°C and run on a 0.8 % agarose gel. DNA transfer to positively charged nylon membranes (Hybond-N+; Amersham), UV cross-linking (70 mJ / cm^2), pre-hybridization, and hybridization were performed according to the instructions for DIG Easy Hyb Granules (Roche). PCR DNA fragments were labeled with the PCR

DIG Probe Synthesis Kit (Roche) using primers for 3'-external probe (Forward: 5'-CTGGTAATTTGGGGTTATATCATGTGC-3'; Reverse: 5'-GAAGGCACTAGAAAACAATCAAACTCCAGC-3') and genomic DNA of cell clones as templates. To evaluate random integration of the targeting vectors, genomic DNA from candidate cells was cut with PstI, and DIG-labelled *TK* gene probe synthesized using the primers (Forward: 5'-CAGAGCTGGTTTAGTGAACCGT-3'; Reverse: 5'-TCATGCTGCCCATAAGGTATCG-3') and pTK-2A-puro targeting vector as template DNA. The membrane was developed using the DIG Luminescent Detection Kit (Roche) following the manufacturer's instructions.

RT-PCR. Total RNA was extracted from *KIF2A*+/+, *KIF2A*+/−, and *KIF2A* −/− hTERT-RPE1 cells using TRIzol reagent (Life Technologies) according to the manufacturer's protocol. First-strand cDNAs were generated with random hexamers using M-MLV reverse transcriptase (Life Technologies). To examine the expression of *KIF2A* mRNA, primers spanning the initiation codon (5'-ATGGCAACGGCCAACTTCGGCAAG-3') and the stop codon (5'-TTAAAGGGCACGGGGTCTCTTCG-3') were designed. *HPRT* was assayed as an internal control, (Forward: 5'-GAAGAGCTATTGTAATGACC-3'; Reverse: 5'-GCGACCTTGACCATCTTTG-3'). PCR amplification was carried out with KOD-Fx neo DNA polymerase (TOYOBO).

Fractionation of soluble and polymerized tubulin. Soluble tubulin extraction buffer A (137 mM

NaCl, 20 mM Tris-HCl (pH7.5), 1 % Triton X-100, and 10 % glycerol) was supplied to cells at 4°C for 3 min and the buffer was collected and saved as soluble fraction. Immediately after, polymerized tubulin extraction buffer B (buffer A + 1 % SDS) was added for 1 min, cells were collected, and the polymerized fraction was sonicated and incubated on ice for 30 min.

Cell-cycle synchronization. Cells were synchronized at the G1/S phase boundary by double-thymidine block, and at the G0 phase by serum starvation. For thymidine block, cells were incubated with 2 mM thymidine for 16 h, washed extensively with DMEM supplemented with 10% FBS, released for 8 h, and subjected to a second thymidine block for 18 h and released.

Immunoprecipitation and western blot analyses. Cells were transfected with siRNAs or plasmid DNA, and cultured in serum-free D-MEM for 24 h. The cells were lysed in lysis buffer (0.5% Triton X-100, 150 mM NaCl, 20 mM Tris-HCl pH 7.5, 1 mM EDTA, 0.5 mM PMSF, 2 mg/ml pepstatin A, 10 mg/ml leupeptin, 5 mg/ml aprotinin). The lysates were sheared with a 21-gauge needle, incubated on ice for 15 min and clarified by centrifugation at 20,817 g for 15 min at 4°C. The supernatants were precleared with Protein A/G-conjugated magnetic beads (Merck Millipore) and incubated with anti-FLAG, anti-KIF2A or anti-GFP antibodies for 2 h at 4°C with constant rotation. Protein A/G-conjugated magnetic beads was then added to the lysates and the mixtures were rotated for a further 16 h at 4°C. The magnetic beads were washed three times with wash buffer (1 % Nonidet P-40, 0.1 % SDS, 0.5 % deoxycholate, 150 mM NaCl, 50 mM Tris-HCl pH 7.5,

1 mM EDTA, 0.5 mM PMSF, 2 mg / ml pepstatin A, 10 mg / ml leupeptin, 5 mg / ml aprotinin) before elution with sample buffer. The immunoprecipitated proteins were analyzed by 10 % SDS-PAGE and transferred to PVDF membranes for western blot analyses as described previously (Miyamoto et al., 2011).

Supplementary Figure legend

Supplementary Figure 1. KIF2A polyubiquitination by APC/C inhibits primary cilia disassembly, Related to Figure 1

(A) Western blot analysis of whole cell lysates from hTERT-RPE1 cells grown for 24 h without serum (0 hr), or at the indicated times post serum stimulation. The levels of phospho-KIF2A (T554) and total KIF2A were normalized by total KIF2A and GAPDH, respectively.

(B) Immunoprecipitation and western blot analysis of KIF2A in hTERT-RPE1 cells grown with serum (asynchronous) (serum (+)), without serum for 24 h (serum (-)) or at 12 hours post serum stimulation. Endogenous KIF2A was immunoprecipitated using an anti-KIF2A polyclonal antibody and analyzed by western blotting-detection of HA tagged-ubiquitin (HA-Ub). KIF2A was polyubiquitinated in a serum dependent manner.

(C) HA-tagged ubiquitin and 3×FLAG-tagged KIF2A, KIF2B, KIF2C or KIF24 were co-transfected into HEK293T cells. Whole lysates were immunoprecipitated with anti-FLAG antibody and analyzed for the physical interaction between Kinesin-13s and APC/C by western

blotting with anti-CDC27 antibody.

(D) Whole cell lysates from HEK293T cells expressing EGFP-tagged CP110 and 3 tandem FLAG tagged-KIF2A fragments were immunoprecipitated with anti-GFP antibody and immunoblotted with anti-GFP or anti-FLAG antibody. Endogenous CDC27 in the IP fraction and inputs were also detected by western blotting.

(E) KIF2A contained three D-box (RXXL) consensus sequences. HA-tagged ubiquitin (HA-Ub) and 3×FLAG-tagged KIF2A D-box mutants (AXXA) were co-transfected in to HEK293T cells. Whole lysates were immunoprecipitated with anti-FLAG antibody and analyzed for their ubiquitination state by western blotting with anti-HA antibody. Mutation of D-box 3 (685-688 a.a.) profoundly inhibited polyubiquitination of KIF2A.

(F) Quiescent hTERT-RPE1 cells were transfected with AcGFP1-tagged KIF2A D-box 3 mutant or AcGFP1-tagged KIF2A triple mutant of D-box, KEC, and KVD, and immunostained with anti-GFP (green), and anti-acetylated-tubulin (red) antibodies. DNA was stained with DAPI (blue). Arrow indicates the centrosome / basal body. Scale bar, 10 μm.

(G) Columns indicate percentage of ciliogenesis in GFP-positive cells from (F). The D-box 3 mutation of KIF2A significantly increased the negative effect on ciliogenesis by KIF2A. However, KEC/KVD mutations of KIF2A abrogated the enhancement of KIF2A activity by the D-box 3 mutation (**$p < 0.01$, *** $p < 0.001$: *t*-test, n=3: > 200 cells per experiment).

Supplementary Figure 2. Loss of the human *KIF2A* gene impairs primary cilia disassembly in the proliferative phase, Related to Figure 4.

(A) The targeting strategy for the human *KIF2A* gene in hTERT-RPE1 cells. KIF2A-TALENs recognized the specific sequence on exon 10 to induce DNA double-strand breaks. Black bars indicate Southern blot probes. E, EcoRV; P, PstI.

(B) Southern blot analysis of EcoRV-digested genomic DNA from wild-type (+/+), heterozygous (+/−), and homozygous (−/−) hTERT-RPE1 cells for the mutant *KIF2A* allele. Southern blotting with the 3'-probe yielded a 16.8 kb and a 7 kb band from the wild-type and targeted allele, respectively.

(C) Loss of *KIF2A* mRNA in the KIF2A knockout hTERT-RPE1 cells examined by RT-PCR. The *HPRT* transcript was amplified in all samples as a positive control.

(D) Western blot analysis showing depletion of KIF2A in the homozygous clones. GAPDH and β-tubulin served as loading controls. The levels of KIF2A and β-tubulin were normalized by GAPDH.

(E) Immunostaining with anti-KIF2A (green), anti-ninein (red), and anti-acetylated tubulin (blue) in *KIF2A* +/+ and *KIF2A* −/− hTERT-RPE1 cells in quiescent G0 phase. Scale bar, 2 μm.

(F) After 24 h serum starvation, *KIF2A* +/+ and *KIF2A* −/− hTERT-RPE1 cells were stimulated with 10 % serum for 12 hours and then immunostained with anti-Arl13b (red) and anti-γ-tubulin (green) antibodies. DNA was stained with DAPI (blue). Arrows indicate primary cilia. Scale bar, 10 μm.

(G) Quantification of the percentage of ciliated cells from (F). *KIF2A* −/− hTERT-RPE1 cell clones did not effectively disassemble primary cilia after serum stimulation (The fractions of ciliated KIF2A−/− cells were compared to those of ciliated *KIF2A* +/+ cells. **p <0.05, *** p <0.01 : *t*-test, n=3: > 200 cells per experiment).

Supplementary Figure 3. TALEN-mediated *KIF2A* depletion induces ectopic ciliogenesis in the proliferating phase, Related to Figure 4.

(A) The target sequences and TALEN repeat variable diresidue (RVD) sequences used for human *KIF2A* gene targeting in this study.

(B) Southern blot analysis of PstI-digested genomic DNA from wild-type (+/+), heterozygous (+/−), and homozygous (−/−) hTERT-RPE1 cells. Southern blotting with the *hsvTK*-probe in Figure 4A yielded a 6.8 kb band from the targeted allele and did not detect random integration of the targeting vector.

(C) Immunostaining with anti-KIF2A (red) and anti-α-tubulin (green) antibodies of *KIF2A* +/+ and *KIF2A* −/− hTERT-RPE1 cells during mitosis. DNA was stained with DAPI (blue). Scale bar, 2 μm.

(D) After 24h serum starvation, *KIF2A* +/+ and *KIF2A* −/− hTERT-RPE1 cells were stimulated with 10 % serum for 12 hours and then immunostained with anti-acetylated tubulin (red) and anti-pericentrin (green) antibodies. DNA was stained with DAPI (blue). Arrows indicate primary cilia. Scale bar, 5 μm.

(E) Quantification of the length of primary cilia from (D). Primary cilia on *KIF2A* −/− hTERT-RPE1 cell clones did not significantly shorten after serum stimulation. (*** $p < 0.001$: *t*-test, > 100 primary cilia per sample were measured)

(F) *KIF2A* +/+ and *KIF2A* −/− hTERT-RPE1 cells were asynchronously cultured with 10 % serum and then immunostained with anti-acetylated tubulin (red) and anti-pericentrin (green) antibodies.

DNA was stained with DAPI (blue). Arrows indicate primary cilia. Scale bar, 10 μm.

(G) Quantification of the percentage of ciliated cells cultured in the presence of 10 % serum from (F). *KIF2A* −/− cell clones significantly exhibited impaired suppression of ciliogenesis during the cycling phase (*** $p < 0.001$: *t*-test, n=3: > 100 cells per experiment).

(H, I) Immunostaining with anti-acetylated-tubulin (blue), anti-ninein (red) and anti-CP110 (H) or anti-KIF24 (I) (green) antibodies in serum-starved *KIF2A* +/+ and *KIF2A* −/− hTERT-RPE1 cells. During the quiescent G0 phase, CP110 localized only to the daughter centriole, while KIF24 were detected in the distal portion of mother centriole and the daughter centriole. CP110 and KIF24 localizations in basal bodies did not depend on KIF2A. Scale bar, 1μm.

Supplementary Figure 4. Loss of *KIF2A* affects cytosolic microtubule polymerization but not bipolar spindle formation and the cell cycle progression, Related to Figure 4.

(A) Western blot analysis of whole cell lysates from cycling hTERT-RPE1 cells grown for 24 h in the absence or presence of 100 nM BI2536, and cycling *KIF2A* deficient cells. BI2536 treatment diminished the signal of phosphor-KIF2A (T554), but not total KIF2A. Both the bands were completely depleted in *KIF2A* deficient cells. GAPDH served as a loading control.

(B) Western blot analysis of soluble and polymerized tubulin fractions extracted from *KIF2A* +/+ and *KIF2A* -/- cells. Relative amount of tubulin contained in soluble and polymerized fractions is represented by percentage of band intensity. GAPDH served as a loading control.

(C, D) Immunostaining with anti-γ-tubulin (cyan), anti- α -tubulin (green) and anti-phosphor-KIF2A (T554) (C) or anti-total-KIF2A (D) (red) antibodies in asynchronous *KIF2A* +/+ and *KIF2A* −/− hTERT-RPE1 cells. The major signals of KIF2A and phosphor-KIF2A (T554) were detected at nucleus and centrosome in *KIF2A* +/+ hTERT-RPE1 cells, while they were depleted in *KIF2A* −/− cells. Arrows indicate centrosomes. Scale bar, 20μm.

(E) Immunostaining with anti-Pericentrin (PCNT) (green) and anti-α-tubulin (red) antibodies of *KIF2A* +/+ and *KIF2A* −/− hTERT-RPE1 cells during mitosis. DNA was stained with DAPI (cyan). Scale bar, 2 μm.

(F) Quantification of the percentage of normal bipolar formation from (E). A part of *KIF2A* −/− hTERT-RPE1 cells significantly exhibited impaired spindle formation characterized by multipolar spindle formation (E) (* $p < 0.05$: *t*-test, n=3: > 100 cells per experiment).

(G) FACS analysis of asynchronous *KIF2A* +/+ and *KIF2A* −/− hTERT-RPE1 cells.