

Figure S1. KIF7 localization does not correlate with level of expression. (A) Left,

representative image of COS-7 cells expressing KIF7(1-1114). Scale bars, 10 µm. Right, plot of average KIF7(1-1114) fluorescence intensity per cell for 30 randomly chosen cells. Expression was defined as high, medium, or low expression and only cells with medium or low expression were included in the analysis. (B) Criteria for microtubule (MT) localization. Left, representative images of MT localization phenotypes in COS-7 cells. Scale bars, 10 μm. If the expressed protein exhibited a cytoplasmic/diffuse localization, this was scored as 'no MT binding'. If the expressed protein could be seen both in the cytoplasm and on microtubules, this was scored as 'weak MT binding'. If the exogenous protein could only be seen on MTs, this was scored as 'strong MT binding'. Right, plot of average KIF7 fluorescence intensity across MT binding phenotypes. Each spot represents the average KIF7 fluorescence intensity of one cell across a total of 70 randomly selected cells. (C) Criteria for cilium localization. Left, representative images of cilium localization phenotypes in NIH-3T3 cells. Scale bars, 1 um, If the expressed protein could not be detected in the cilium, this was scored as 'absent from cilium'. If the expressed protein was observed at the base of the cilium (defined by presence of pericentrin staining), this was scored as 'at base' (not shown). If the expressed protein was observed at the tip of the cilium (opposite end as pericentrin staining), the localization was scored as 'at tip'. If the expressed protein was distributed uniformly along the cilium, the localization was scored as 'along shaft'. Right, plot of average KIF7 fluorescence intensity across cilium localization phenotypes. Each spot represents the average KIF7 fluorescence intensity of one cell across a total of 60 randomly selected cells.



**Figure S2. KIF7 is not a processive motor.** Neuronal CAD cells expressing mCit-tagged versions of (A) full-length or (B) truncated (1-558) KIF7. The cells were fixed and stained with an antibody to  $\beta$ -tubulin to mark microtubules and with DAPI to mark nuclei. Scale bar, 20  $\mu$ m.





Figure S3. Coiled-coil predictions for human and mouse KIF7 using the indicated software

human monkey mouse	MGLEAQRLPGAEEAPVRVALRVRPLLPKELLHGHQSCLQVEPGLGRVTLGRDRHFGFHVVLAEDAGQEAVYQACVQPLLEAFFEGFNATVFAYGQTGSGK MGLEAQRLPGPEEAPVRVALRVRPLLPKELLHGHQSCLQVEPGLGRITLGRDRHFGFHVVLAEDAGQEAVYQACVQPLLEAFFEGFNATVFAYGQTGSGK MGLEAQRLPGAEEAPVRVALRVRPLLPKELLHGHQSCLRVEPERGRITLGRDRHFGFHVVLGEDTGQEAVYQACVQPLLEAFFEGFNATVFAYGQTGSGK	100 100 100
human	TYTNCFACVASTI EDEOCTVPRAMAFAEVI TOENDI I DCI VHVSVI EVYVEEERDI I EVCTASRDTOL REDERONVU COVVEVOVECI DEVI STI ENON	288
monkey	TYTMGEASWASIL EDGGTVPRAWAEAFKI TOENDILDCLVHVSYLEV NEEFRDLLEVGTASRDTQLEEDERGMVVLCGWEVDVEGLEVISILEMGN	200
mouse	TYTMGEASVASLHEDEQGIIPRAMAEAFKLIDENDLLDCLVHVSYLELYKEEFRDLLEVGTASRDIQLREDDRGNVVLCGVKEVDVEGLDEVLSLLEMGN	200
human	AARHTGATHLNHLSSRSHTVFTVTLEORGRAPSRLPRPAPGOLLVSKFHFVDLAGSERVLKTGSTGERLKESIDINSSLLALGNVISALGDPORRGSHIP	300
monkey	AARHTGATHLNRLSSRSHTVFTVTLEQRGRIPSRLPRPAQQQLLVSKFHFVDLAGSERVLKTGSTGERLKESIQINSSLLALGNVISALGDPQRRGSHIP	300
mouse	AARHTGATHFNRLSSRSHTVFTVTLEQRGRTPSRLPRPAAGHLLVSKFHFVDLAGSERVLKTGSTGERLKESIQINSTLLALGNVISALGDPQRRGSHIP	300
human	YRDSKITRILKDSLGGNAKTVMIACVSPSSSDFDETLNTLNYASRAQNIRNRATVNMRPEAERPPEETASGARGPPRHRSETRIIHRGRRAPGPATASAA	400
monkey	YRDSKITRILKDSLGGNAKTVMIACVSPSSSDFDETLNTLNYASRAQNIRNRATVNWRPEAERPPEEAVSGARGPPRHRSETRIIHRGRRAPGPATASAG	400
mouse	YRDSKITRILKDSLGGNAKTVMIACVSPSSSDFDETLNTLNYASRAQNIRNRATVNWRPEAERVPEEQAAGARGPPRHRSETRIIHRGRRVPCPAVGSAA	400
human	AAMRUGAECARYRACTDAAYSLLRELQAEPGLPGAAARKVRDWLCAVEGERSALSSASGPDSGIESASVEDQAAQGAGGRKEDEGAQQLLTLQNQVARLE	500
monkey	AAMRLDAECARYRACTDAAYNLLRELQAEPGLPGATARKVRDMLCVVEGERSALSSASGPDSGIESASVEGQAAQGAGGRKEDEGAQQLLTLQNQVARLE	588
mouse	VAAGLGAECARCRARTSAAYSLLRELQAEPGLPGAAARKVRDWLCAVEGERSTLSSASGPDSGIESAPAEDQAAQGTSGRKGDEGTQQLLTLQSQVARLE	500
human	EENRDFLAALEDAMEQYKLQSDRLREQQEEMVELRLRLELVRPGWGGPRLLNGLPPGSFVPRPHTAPLGGAHAHVLGNVPPACLPGDEVGSEQRGEQVTN	600
monkey	EENRDFLAALEDAMEQYKLQSDRLREQQEEMVELRLRLELVRPGWGCPGLLNGLPPRSFVPRPHTAPLGGAHDHVLGMVPPACFPGDEVGSEQRGEQVTN	699
mouse	EENRDFLAALEDAMEQYKLQSDRLREQQEEMVELRLRLELAQPGWGAPGLLQGLPPGSFVPRPHTAPLGGAHTHMLGMMPSTCLPGEEVSSEQQVVS	597
human	GREAGAELLTEVNRUGSGSSAASEEEEEEEEPPRRTUHURRNRISNCSQRAGARPGSUPERKGPEUCLEELDAAIPGSRAVGGSKARVQARQ	<del>6</del> 92
monkey	GREAGVELLTEVNRLGSGSSAASEEEEEEELPRRTLHLRRNGISNCSQRAGARPGSLPERKGPELCLEELDAAIPGSRAVGGSKARVQARQ	692
mouse	GKEVKAEVLAQADKURSASSTTSEEEGEEEEEEEEEEEPPRRTLYLRRNGISN#SQRAGLSPGSPPDRKGPEVCPEEPAAAIPAPQAVGSGKVPVQTRQ	697
human	VPPÄTÄSEWRLAQAQQKIRELAINIRMKEELIGELVRTGKAAQALNRQHSQRIRELEQEAEQVRAELSEGQRQLRELEGKELQDAGERSRLQEFRRVAA	79Z
monkey	VPSATASEWRLAQAQQKIRELAINIRMKEELIGELVRTGKAAQALNRQHSQRIRELEQEAERVRAELSEGQRQLQELEGKEPQDAGERSRLQEFRRRVAA	79Z
mouse	APAAMASEWRLAQAQQKIRELAINIRMKEELIGELVRTGKAAQALNRQHSQRIRELEQEAERVRAELCEGQRQLRELEGREPQDASERSRLQEFRKRVAA	797
human	AQSQVQVLKEKKQATERLVSLSAQSEKRLQELERNVQLMRQQQGQLQRRLREETEQKRRLEAEMSKRQHRVKELELKHEQQQKILKIKTEEIAAFQRKRR	89Z
monkey	AQSQVQVL KEKKQATERLVSL SAQSEKRLQELERNVQLMRQQQQQLQRRLREETEQKRRLEAEMSKRQHRVKELELKHEQQQKILKIKTETAAFQRKR	89Z
mouse	AQSQVQVLKEKKQATERLYSLSAQSETRLQELERNYQLMKRQQGQLQRKLREETEQRKRLETEMNKQHKYKELELKHEQQQKILKIKTEETAAFQRKRK	897
human	SGSNGSVVSLEQQQKIEEQKKWLDQENEKVLQQRRALEELGEELHKREAILAKKEALMQEKTGLESKRLRSSQALNEDIVRVSSRLEHLEKELSEKSQQL	992
monkey	<u>SGSNGSVVSLEQQQKIEEQKKWLDQEMEKVLQQRRALEELGEELHKREAILAKKEALMQEKTGLESKRLRSSQALNEDIVRVSSRLEHLEKELSEKSGQL</u>	992
mouse	SGSNGSVVSLEQQQ-IEEQKKWLDQEMEKVLQQRRALEELGEELKKREVILAKKEALMQEKTGLESKRLRSSQALNEDIVRVSSRLEHLEKELSEKSGQL	996
human	RQGSAQSQQQIRGEIDSLRQEKDSLLKQRLEIDGKLRQGSLLSPEEERTLFQLDEAIEALDAAIEYKNEAITCRQRVLRASASLLSQCEMNLMAKLSYLS	1892
monkey	RQGSAQSQQQIRREIDSLRQEKDSLLKQRLEIDSKLRQGSLLSPEEERTLFQLDEAIEALDAAIEYKNEAITCRQRVLRASASLLSQCEMNLMAKLSYLS	1892
mouse	RQGSAQNQQQIRGEIDTLRQEKDSLLKQRLEIDSKLRQGSLLSPEEERTLFQLDEAIEALDAAIEYKNEAITCRQRVLRASASLLSQCEMNLMAKLSYLS	1896
human	SSETRALLCKYFDKVVTLREE0H000IAFSELEM0LEE00RLVYWLEVALERORLEMDROLTLOOKEHE0NMOLLLOOSRDHLGEGLADSROYEARTOA	1192
monkey	SSETRALLCKYFDKVVTLREEQHQQQIAFSELEMQLEEQQRLVYWLEVALERQRLEMDRQLTLQQKEHEQNMQLLLQQSRDHLSEGLADSRRQYEARIQA	1192
mouse	SSETRALLCKYFDKVVTLREEQHQQQIAFSELEMQLEEQQRLVYWLEVALERQRLEMDRQLTLQQKEHEQNVQLLLQQGRDHLGEGLADSKRQYEARIHA	1196
human	LEKELGRYHWINDELKOKLGGVNAVGHSRGGEKRSLCSEGROAPGNEDELHL-APELLWLSPLTEGAPRTREETRDLVHAPLPLTWKRSSLCGEEOGSPE	1291
monkey	LEKOLGRYMWINDELKOKLGSVNTVGHSRGGEKRSLCSEGRQAPGNEDELHP-APELLWLYPLTEGAPRTREETRDLVHAPLPLTWKRSSLCGEEQGSPE	1291
mouse	LEKELGRHMWINQELKQKLSAGSTAQQSRGCERRSLCLENRQCLGNEDGLHPANPEPLWQSSLLEQVSRVWDESRDLVHAPLPLTWKRSSLCS-EQGSSE	1295
human	EL ROREAAEPL VGRVL PVGEAGL PWNFGPL SKPRREL RRASPGMI DVRKNPL	1343
monkey	ELROREAAEPLVGRVLPVGEAGLPWNFGPLSKPRRELRRASPGMIDVRKNPL	1343
mouse	ESRVRETTEPPVGRVLPMGEVGLSINNEGPLPKPRWEPRRTSPGMIDVRKNPL	1347

Figure S4. Alignment of the primary sequences of human (Homo sapiens NP\_940927), green monkey (Chlorocebus sabaeus XP\_007988548), and mouse (Mus musculus NP\_034756) KIF7 proteins. Purple boxed resides indicate residues with high conservation across these mammalian species. Red text indicates disease-associated mutations introduced into mouse KIF7 in this manuscript. Pink overline indicates the kinesin motor domain, black overlines indicate predicted coiled-coil regions. Sequence alignments were carried out using T-COFFEE (version 11.00) and edited in JalView (2.11.1.4).



## Figure S5. Sequence alignment of the rCC region across kinesin-4 family members

**KIF21A, KIF21B, and KIF7.** Red text indicates mutations in *KIF21A* associated with CFEOM1 (Cheng et al., 2014; van der Vaart et al., 2013), in *KIF21B* associated with neurodevelopmental disorders associated with brain malformations including corpus callosum agenesis and microcephaly (Asselin et al., 2020), and in *KIF7* associated with AI-Gazali-Bakalinova and Bardet-Biedl syndromes (Ali et al., 2012; Putoux et al., 2012). Gray arrows indicate residues mutated in concert that relieve autoinhibition of full-length KIF21A (Bianchi et al., 2016). Red arrows indicate residues mutated individually in this study that do not relieve autoinhibition (Figure 5).



**Figure S6. KIF7 does not track the plus ends of microtubules in cells.** (A-D) NIH-3T3 cells were transfected with plasmids for co-expression of EB3-mCherry with KIF7-mCit. (A,C) Representative images in the (A) absence or (C) presence of 500 nM SAG treatment for 3-4 h. The periphery of the transfected cells is indicated by white dotted lines. Scale bar, 10  $\mu$ m. (B,D) Representative kymographs of KIF7 and EB3-mCherry on the microtubule plus end indicated by the white boxed regions in (A) and (C). Time is on the y axis (scale bar, 5 sec); distance is on the x axis (scale bar, 2  $\mu$ m). (E,F) Verification of Hedgehog pathway activation by SAG treatment. (E) Representative images of NIH-3T3 cells expressing KIF7-mCit in the absence (left) or presence of 500 nM SAG treatment for 3 h (right). The cells were fixed and stained with antibodies against acetylated-tubulin to mark the ciliary axoneme (red) and with DAPI to mark the nucleus (blue). Scale bar, 10  $\mu$ m. Inserts show magnification of the area outlined with the white box with the fluorescence signals offset by 6 pixels for clarity. Yellow arrowheads indicate the tip of the cilium. (F) Quantification of the percent of cells exhibiting ciliary tip localization of KIF7 in the absence or presence of 500 nM SAG treatment for 3 h. Each spot indicates the result of one independent experiment and the bar indicates the average of the two experiments.



Figure S7. Comparison of KIF7(1-558)-Halo-Flag localization on growing microtubules *in vitro* under various buffer conditions. (A) Coomassie-stained gel of KIF7(1-558)-Halo-Flag protein purified from COS-7 cells. (B-G) Representative kymographs of microtubule dynamics in the presence of (B,C) 70 nM, (D) 80 nM or (E-G) 8 nM purified KIF7(1-558)-Halo-Flag protein. All assays were carried out in BRB80 buffer containing 18.8  $\mu$ M tubulin (10% Hilyte488-labeled tubulin), 3 mM MgCl<sub>2</sub>, 1 mM GTP, 1 mM ATP, 0.1 % methylcellulose, and 1 ul oxygen scavenger mix. Supplemental reagents that differ between the assays are indicated below each kymograph. Magenta= GMPCPP-containing microtubule seeds; green= growing microtubules; red= purified KIF7(1-558)-Halo-Flag protein (JF552 ligand). Time is on the y axis (scale bar, 5 min); distance is on the x axis (scale bar, 5  $\mu$ m). Yellow brackets indicate events where KIF7(1-558)-Halo-Flag protein showed a slight enrichment at the plus end of a growing microtubule.