The kinesin-4 protein Kif7 regulates mammalian Hedgehog signalling by organizing the cilium tip compartment

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Mammalian Hedgehog (Hh) signal transduction requires a primary cilium, a microtubule-based organelle, and the Gli–Sufu complexes that mediate Hh signalling, which are enriched at cilia tips. Kif7, a kinesin-4 family protein, is a conserved regulator of the Hh signalling pathway and a human ciliopathy protein. Here we show that Kif7 localizes to the cilium tip, the site of microtubule plus ends, where it limits cilium length and controls cilium structure. Purified recombinant Kif7 binds the plus ends of growing microtubules in vitro, where it reduces the rate of microtubule growth and increases the frequency of microtubule catastrophe. Kif7 is not required for normal intraflagellar transport or for trafficking of Hh pathway proteins into cilia. Instead, a central function of Kif7 in the mammalian Hh pathway is to control cilium architecture and to create a single cilium tip compartment, where Gli–Sufu activity can be correctly regulated.

Although kinesin motor proteins are best known for their roles in intracellular transport, some kinesins can shape the microtubule cytoskeleton by regulating the dynamics of tubulin polymerization. For example, KIF4A, a kinesin-4 protein, controls microtubule length during cell division14, and another kinesin-4 protein, KIF21A, inhibits microtubule growth at the cell cortex5. Kif7, a conserved regulator of Hedgehog (Hh) signalling6–9, is also a member of the kinesin-4 family but its relationship to microtubules has not been defined.

The Hedgehog signalling pathway is an evolutionarily conserved pathway responsible for many aspects of embryonic development and stem cell maintenance, and is disrupted in a spectrum of tumours10,11. Drosophila Costa (COS) and its mammalian homologue Kif7 are required to relay the signals from the membrane protein Smoothened (Smo) to the transcription factors of the Gli family. Genetic inactivation of either cos or Kif7 causes a relatively mild ectopic activation of Hh signalling due to their roles in production of both activator and repressor forms of Gli proteins11,12. Recessive mutations in human Kif7 are associated with fetal hydrolethalus, and acrocallosal and Joubert syndromes; affected individuals exhibit polydactyly, brain abnormalities and cleft palate, consistent with a role for Kif7 in human Hh signalling13,14.

A fundamental difference between the Drosophila and vertebrate Hh pathways is the dependence of vertebrate Hh signalling on a microtubule-based organelle, the primary cilium15. Mutations that block formation of primary cilia prevent cellular responses of cells to Hedgehog ligands, and all of the proteins required for vertebrate Hh signal transduction are highly enriched in cilia and change localization in response to ligand16. The activity of Kif7 in the mouse Hh pathway depends on the presence of the primary cilium6.

Despite the conserved role for COS/Kif7 in the Hh pathway, the motor domain of COS lacks residues critical for motility17 and is considered as a microtubule-associated scaffold for Hh signalling complexes18,19. In contrast, mouse Kif7 has the sequence motifs necessary for ATP and microtubule binding, and the crystal structure of its motor domain is superimposable on that of a conventional kinesin20. How Kif7 acts within cilia and whether its motor activity is important for its function is not known.

Here we show that, unlike other core components of the mammalian Hh pathway, Kif7 is required for the normal structure of primary cilia. Kif7 localizes to the distal tips of primary cilia, the site of the plus ends of axonemal microtubules. In the absence of Kif7, cilia are

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Figure 1 Kif7 constrains the length of primary cilia. (a,b) Kif7 localizes to the tips of primary cilia in e10.5 wild-type embryos. Arl13b marks cilia in a and acetylated α-tubulin marks microtubule axonemes in b. γ-tubulin marks basal bodies. 4,6-diamidino-2-phenylindole (DAPI) labels nuclei. Scale bars, 5 μm. (c) In wild-type MEFs, Kif7 (green) is enriched in tips of primary cilia on treatment with Shh-N recombinant protein (2 μg ml−1) or the small-molecule agonist SAG (100 nM) for 24 h. Scale bar, 2 μm. (d) Quantitation of Kif7 fluorescence intensity shown in c. n = 150 cilia pooled from three independent experiments were counted for each condition. The error bars represent the s.d. P < 0.0001 between control and SAG or Shh treatments by one-way analysis of variance (ANOVA). (e) Kif7 is associated with one of the two centrioles before ciliogenesis and localizes to the tips of primary cilia throughout cilium elongation induced by serum starvation in wild-type MEFs. (f) Kif7 is not detected at the tips of Kif7L130P or Kif7mutant cilia. Acetylated α-tubulin marks primary cilia and γ-tubulin marks basal bodies in c,e and f. Scale bars, 1 μm in c,e and f. (g) Primary cilia of wild-type and Kif7L130P MEFs stained with Arl13b, acetylated α-tubulin and γ-tubulin. Scale bar, 2 μm. (h) Measurements of MEF cilium length between wild-type and Kif7 mutants using Arl13b as a cilium marker. More than 100 cilia pooled from four independent experiments were counted for each genotype (n = 108 cilia for wild type, n = 133 cilia for Kif7L130P mutant, and n = 111 cilia for Kif7mutant). P < 0.0001 between wild-type cilia and Kif7 mutant cilia by one-way ANOVA. The error bars represent the s.d. (i) Scanning electron microscopy shows neural tube cilia of e10.5 wild-type, Kif7L130P and Kif7mutant embryos. Wild-type neural cilia were 0.97 ± 0.17 μm and Kif7L130P neural cilia were approximately 1.2 ± 0.28 μm long (n = 50 for each genotype). Scale bar, 0.5 μm. (j) Transmission electron microscopy images of longitudinal sections of wild-type, Kif7L130P and Kif7mutant neural tube cilia. Arrowheads point to twisted tips of mutant cilia. Scale bar, 0.5 μm.

RESULTS
Kif7 localizes to cilia tips
We found that endogenous Kif7 was enriched in primary cilia tips in Sonic hedgehog (Shh)-responsive cells in wild-type embryos (Fig. 1a,b) and cultured fibroblasts and was further enriched at tips in response to activation of the pathway (Fig. 1c,d and Supplementary Fig. 1b), similar to the core Shh pathway proteins Gli2, Gli3 and Sufu. Kif7 was also present at cilia tips in mouse embryonic fibroblasts (MEFs) derived from mutant embryos that lack Smo or Gli2 and Gli3, indicating that Kif7 is targeted to cilia independently of Shh pathway proteins (Supplementary Fig. 1c,d). In wild-type MEFs, Kif7 was present at cilia tips at all stages of ciliogenesis (Fig. 1e).

Kif7 mutant cilia are long and twisted
Cilia were present on about 70% of Kif7L130P and Kif7mutant MEFs, as in wild type, but the cilia seemed to be longer than wild
Kif7 stabilizes primary cilia. (a,b) Tubulin acetylation (a) and glutamylation (b) are reduced at the distal segment of cilia in Kif7L130P MEFs when compared with wild type. Arl13b labels the entire cilia in a,b, and γ-tubulin marks basal bodies in a. Scale bars, 1 μm. (c) Fluorescence intensities of acetylation and glutamylation, as shown in a,b, normalized for cillum length. n = 50 cilia were measured for each genotype pooled from three independent experiments. The error bars represent the s.d. For tubulin acetylation, P < 0.001 for the distal half of the cilia. For tubulin glutamylation, P < 0.0001 for the distal 80% of the cilia. (d) Time course of cillum shortening in response to nocodazole (Noc) treatment in wild-type and Kif7L130P MEFs. IFT88 accumulated distally in Kif7L130P MEF cilia during cillum retraction. γ-tubulin marks basal bodies. Scale bar, 2 μm. (e) Cillum length was measured using acetylated α-tubulin from the time-course experiment shown in d. n = 30 for each condition and each time pooled from three independent experiments. The error bars represent the s.d. (P < 0.001 between wild type and Kif7L130P at 0 and 10 min; not significant between two genotypes at 10 min). (f) Time course of cillum retraction at 4°C in wild-type and Kif7L130P MEFs. Cilia are marked with acetylated α-tubulin and IFT88. Scale bar, 2 μm.

Kif7 mutant axonemes are under-modified and unstable

Axonemal microtubules are acetylated and glutamylated, modifications associated with stable microtubules. Line-scan analysis of fluorescence intensity revealed that the levels of tubulin acetylation and glutamylation were comparable in wild type and Kif7L130P at the base of the ciliary axoneme (Fig. 2a–c). However, both tubulin modifications faded towards the ciliary tip, and tubulin glutamylation was often undetectable distally in Kif7L130P and Kif7−/− mutant cilia (Fig. 2b,c and Supplementary Fig. 3).

To test whether changes in tubulin modification were associated with decreased ciliary stability, we treated cells with nocodazole to induce microtubule depolymerization. In wild-type MEFs, the length of acetylated α-tubulin-labelled axonemal microtubules was reduced by about 20% at 30 min after addition of nocodazole. In contrast, the Kif7L130P axoneme was half as long 10 min after addition of nocodazole as at time zero, and was barely detectable at 30 min (Fig. 2d,e). Cillum disassembly is associated with accumulation of intraflagellar transport (IFT) particles, which were first detected in wild-type cells 30 min after addition of nocodazole. Strong IFT88 staining was apparent in...
Kif7 does not affect the rates of IFT

It has been proposed that cilium length can be regulated by the balance between the rates of anterograde and retrograde IFT (ref. 28). Kinesin-2 is the evolutionarily conserved anterograde motor required for carrying the IFT complex from the base of the cilium to the tip\(^2\); other kinesins can also promote anterograde ciliary trafficking and modify the overall rate of IFT (refs 30–32). To test whether the long cilia in Kif7 mutants arise because Kif7 alters the rates of IFT, we expressed two fusion proteins, IFT88–GFP (green fluorescent protein) to label IFT particles and Arl13b–mCherry (a red fluorescent protein) to label the ciliary membrane, in MEFs (Fig. 3a), and documented the movements of IFT using time-lapse imaging (Supplementary Videos 1 and 2).

We analysed the speed of IFT trains of wild-type and Kif7 mutant MEF cilia by measuring the slopes of each IFT trajectory on the kymograph (Fig. 3b). The rates of anterograde IFT were 1.09 ± 0.24 \(\mu\)m s\(^{-1}\) and 1.06 ± 0.19 \(\mu\)m s\(^{-1}\) in wild-type and Kif7\(^{L130P}\) cilia, respectively. The rates of retrograde IFT were 0.72 ± 0.26 \(\mu\)m s\(^{-1}\) and 0.76 ± 0.29 \(\mu\)m s\(^{-1}\) in wild type and Kif7\(^{L130P}\), respectively. Thus there was no significant difference in the rates of IFT between wild-type and Kif7\(^{L130P}\) cilia, indicating that Kif7 does not control cilium length by affecting the rates of IFT.

Kif7 can interact directly with microtubules but lacks detectable motility

Sequence analysis places Kif7 in the kinesin-4 family\(^3\) (Supplementary Fig. 4a), which includes the homodimeric kinesins KIF4A/Xklp1 and KIF21A (refs 1,5,34). To test whether Kif7 acts as a dimer, we expressed full-length carboxy-terminal Flag- and GFP-tagged Kif7 constructs in HEK293T cells. Kif7–GFP co-immunoprecipitated with Kif7–Flag, and vice versa (Supplementary Fig. 4b), indicating that...
Figure 5  Kif7 alters microtubule dynamics in vitro. (a) Schematic representation of the experimental setup of microtubule dynamics assay: X-rhodamine- and biotin-labelled microtubule seeds, which were polymerized in the presence of GMP-CPP, were immobilized on a glass coverslip, and then incubated with a mixture of X-rhodamine-labelled tubulin (1.8 µM) and non-fluorescent tubulin (18 µM; 1 mM MgGTP). (b) Representative kymographs of X-rhodamine-labelled microtubules (shown in grey scale) polymerizing in the absence or presence of Kif7-GFP at the indicated concentrations in the presence of either 1 mM MgATP or 2 mM AMP-PNP. The frame rate was one frame per 5 s. Microtubule seed and polarity are indicated above each kymograph. Horizontal scale bar, 2 µm. Vertical scale bar, 180 s. (c) The frequencies of microtubule catastrophe in the absence of Kif7 (n = 272), with 35 nM KIF560-GFP (n = 263; P < 0.0001 when compared with control) and 70 nM KIF560-GFP (n = 492; P < 0.0001) when compared with control and P < 0.01 when compared with 35 nM KIF560-GFP in the presence of 1 mM MgATP, and with 70 nM KIF560-GFP (n = 179; n.s. when compared with control) in the presence of 2 mM AMP-PNP, were plotted against the Kif7-GFP concentration. The catastrophe frequencies of microtubule minus ends were not affected by KIF560-GFP (n = 82 for control, n = 51 for 35 nM KIF560-GFP with MgATP, n = 94 for 70 nM KIF560-GFP with MgATP and n = 53 for 70 nM KIF560-GFP with AMP-PNP). Data sets pooled from three independent experiments were analysed. Error bars represent the s.d. P values were calculated by non-parametric test for multiple comparisons.

Kif7 can form a homodimer. When expressed in cell lines, Kif7–GFP seemed to co-localize with microtubule bundles, suggesting that Kif7 can associate with microtubules in vivo (Supplementary Fig. 4c). Domain mapping of Kif7 showed that microtubule association and Kif7 dimerization depended on both the motor domain (amino acids 13–357) and the first coiled coil of Kif7 (amino acids 487–539; Supplementary Fig. 4c,d).

To define the biochemical functions of the Kif7 motor, we expressed and purified a recombinant construct in Escherichia coli that included the amino-terminal motor domain and the first coiled coil of Kif7, with a C-terminal GFP tag (Kif7560-GFP; Fig. 4a; Supplementary Fig. 5a). We used a TIRF microscopy-based assay to examine the association of Kif7560-GFP with microtubules (Fig. 4b). In the presence of MgATP (1 mM), Kif7560-GFP uniformly decorated the entire microtubule (Supplementary Fig. 5b) and binding was proportional to motor protein concentration (Fig. 4c). Addition of 2 mM β-γ-imidoadenosine 5′-triphosphate (AMP-PNP), a non-hydrolysable ATP analogue that typically promotes tight kinesin–microtubule binding, resulted in an approximately twofold increase in filament-bound Kif7 (Fig. 4c and Supplementary Fig. 5b).

We tested whether Kif7 can move along microtubules by analysing the behaviour of single molecules of Kif7560-GFP in our TIRF microscopy assay (Fig. 4b,d). Analysis of kymographs revealed that, in the presence of 1 mM MgATP, Kif7560-GFP molecules were stationary when bound to microtubules, exhibiting neither unidirectional
Kif7 tracks microtubule plus ends. (a,b) Representative images showing Kif7<sub>560</sub>–GFP (70 nM) associated with dynamic microtubules in the presence of 1 mM MgATP (a) or 2 mM AMP-PNP (b). Arrows indicate microtubules used for line-scan-based fluorescence intensity analysis. X-rh-MT, X-rhodamine-labelled microtubules. Scale bar, 2 μm. Also see Supplementary Video 3. (c,d) Representative kymographs of Kif7<sub>560</sub>–GFP on X-rhodamine-labelled dynamic microtubules in the presence of 1 mM MgATP (n = 253; two examples shown; c) and 2 mM AMP-PNP (n = 101; d). Plus ends of microtubules are to the right on the kymographs in c and d. Horizontal scale bar, 2 μm. Vertical scale bar, 180 s. The frame rate was one frame per 5 s. Data sets from two or three independent experiments were analysed.

The Kif7 motor domain inhibits the growth of microtubule plus ends in vitro

KIF4A/Xklp1 and KIF21A can regulate microtubule length by altering tubulin polymerization dynamics<sup>15,35</sup>. We tested whether Kif7<sub>560</sub>–GFP could regulate microtubule dynamics using a modified TIRF microscopy-based assay to visualize microtubule polymerization in vitro, with stochastic transitions between periods of elongation (growth) and shrinkage (catastrophe) at the ends of the microtubules (Fig. 5a; refs 4,38). When Kif7<sub>560</sub>–GFP (1 mM MgATP) was added to the dynamic microtubule assay, there was an increase in the frequency of catastrophe at microtubule plus ends. The catastrophe frequency (0.25 ± 0.10 catastrophes per minute in the absence of Kif7) increased by 36% in the presence of 35 nM KIF<sub>560</sub>–GFP (0.34 ± 0.10 catastrophes per minute) and 60% in the presence of 70 nM KIF<sub>560</sub>–GFP (0.40 ± 0.12 catastrophes per minute; Fig. 5b,c). In the presence of AMP-PNP (2 mM), 70 nM KIF<sub>560</sub>–GFP had no detectable effect on the catastrophe frequency (0.26 ± 0.11 catastrophes per minute; Fig. 5b,c). When guanylyl α, β-methylene-diphosphonate (GMP-CPP)-polymerized X-rhodamine-labelled microtubules were incubated in solution with relatively high concentrations of Kif7<sub>560</sub>–GFP (500 nM) and 2 mM MgATP for 2–5 min and then allowed to bind to a glass coverslip, the microtubules seemed fragmented, as if they had been severed in the middle (Supplementary Fig. 6), suggesting that high concentrations of Kif7 might have a destabilizing effect on microtubules.

In addition to promoting catastrophe, Kif7 decreased the rate of microtubule growth in a dose-dependent manner (Fig. 5b). In the presence of 500 nM Kif7<sub>560</sub>–GFP and 2 mM MgATP, microtubule growth was reduced by 50% (17 ± 8 μm/min in the absence of Kif7 to 9 ± 5 μm/min in the presence of Kif7<sub>560</sub>–GFP; Fig. 5b,c). Increasing the ionic strength of the assay buffer reduced the binding lifetime of Kif7<sub>560</sub>–GFP associated with microtubules (Fig. 4d), similar to other kinesins<sup>35,37</sup>.

The movement of microtubules is not significantly different along the microtubule lattice (Fig. 4d), unlike most kinesins. Increasing the ionic strength of the assay buffer reduces the binding lifetime of Kif7<sub>560</sub>–GFP associated with microtubules (Fig. 4d), similar to other kinesins<sup>35,37</sup>.
Figure 7  Multiple cilium tip-like compartments form in the absence of Kif7. (a) IFT81 localization in the wild-type and Kif7<sup>L130P</sup> cilia. (b) Cilium localization of Smo, Gli2 and Sufu in wild-type and Kif7<sup>−/−</sup> MEFs in response to pathway activation by 100 nM SAG treatment for 24 h. (c) Gli2 localizes to puncta along Kif7<sup>−/−</sup> ciliary axonemes independent of pathway activation. Acetylated α-tubulin marks axonemal microtubules and γ-tubulin marks basal bodies in a–c. (d) Distribution of fluorescence intensity of Gli2 in SAG-treated wild-type and Kif7<sup>L130P</sup> cilia, normalized to cilium length. n = 50 cilia were analysed for each condition, pooled from three independent experiments. P < 0.0001. The error bars represent the s.e.m. (e) Sufu and Gli2 co-localization in the tip of wild-type cilia. In Kif7<sup>L130P</sup> mutant cilia, Sufu and Gli2 co-localize to the same puncta along the axoneme. (f) IFT81 and Gli2 co-localization in the tip of wild-type cilia. In Kif7<sup>L130P</sup> mutant cilia, IFT81 and Gli2 co-localize to the same puncta along the axoneme. Acetylated α-tubulin shown in blue in merged panels and in grey scale in separated channels. Scale bars, 1 μm.

The presence of 70 nM Kif7<sub>560</sub>–GFP (1 mM MgATP), the microtubule growth rate (0.52 ± 0.17 μm min<sup>−1</sup>) was 35% less than the rate in the absence of Kif7<sub>560</sub>–GFP (0.81 ± 0.24 μm min<sup>−1</sup>; Fig. 5d). An intermediate growth rate (0.66 ± 0.16 μm min<sup>−1</sup>) was observed with 35 nM Kif7<sub>560</sub>–GFP. In contrast, 70 nM Kif7<sub>560</sub>–GFP in the presence of AMP-PNP (2 mM) only mildly suppressed microtubule growth (0.71 ± 0.20 μm min<sup>−1</sup>), suggesting that ATP hydrolysis contributes to the attenuation of tubulin polymerization by Kif7<sub>560</sub>–GFP. At microtubule minus ends, neither the catastrophe frequency nor the growth rate was significantly affected by Kif7<sub>560</sub>–GFP (Fig. 5c,d), indicating that Kif7<sub>560</sub>–GFP preferentially regulates microtubule dynamics at the plus ends.
Kif7 acts at growing microtubule plus ends

To determine the localization of Kif7 on growing and shrinking microtubules, we analysed snapshots from near-simultaneous imaging of Kif7-GFP (70 nM; 1 mM MgATP) and X-rhodamine-labelled microtubules. Line-scan analysis showed that Kif7-GFP associated preferentially with the GMP-CPP-tubulin seed and with the tip of the microtubule, whereas it was barely detected in the region of the newly polymerized microtubule proximal to the tip (Fig. 6a,c and Supplementary Video 3). Time-lapse imaging of Kif7-GFP localization on dynamic microtubules showed that the motor protein tracked the plus ends of microtubules during the growth phase (Fig. 6c). During the depolymerization/catastrophe phase of the cycle, Kif7-GFP was not detected at microtubule plus ends (Fig. 6c), suggesting that Kif7 dissociated from the filament plus end at the onset of catastrophe. Microtubule tip-associated Kif7-GFP was also observed at growing minus ends, but at less than 30% of the frequency for plus ends. In the presence of AMP-PNP (2 mM), Kif7-GFP (70 nM) was observed all along the lattice of dynamic microtubules and there was no preferential association with growing microtubule tips (Fig. 6b,d). As microtubule plus ends are capped by GTP-tubulin and the conformation of GMP-CPP-tubulin is similar to that of GTP–tubulin, the data suggest that Kif7 associates preferentially with GTP-bound tubulin. Together, our in vitro analysis suggests that the plus-end-associated Kif7 promotes catastrophe and inhibits microtubule growth in an ATP-hydrolysis-dependent manner.

Kif7 is required for organization of the cilium tip compartment in vivo

The ability of Kif7 to associate directly with microtubule plus ends and limit microtubule growth in vitro, together with the localization of Kif7 to cilia tips, suggested that the Kif7 might control the dynamics of the distal ends of axonemal microtubules. Close examination of the IFT kymographs revealed that, whereas moving IFT88–GFP appeared as diagonal lines in the kymographs, there were also vertical lines of IFT88–GFP in the kymographs of Kif7 mutant cilias (Fig. 3b and Supplementary Fig. 7c–e), which represented stationary IFT88 particles. Retrograde tracks almost always began at the distal tip in wild-type cilia, but the Kif7 kymographs showed that retrograde trafficking frequently initiated from the stationary IFT88 puncta in the middle of the axoneme (Supplementary Fig. 7a,b). Thus the stationary IFT88 puncta in Kif7 mutant cilia mark compartments that have properties of the normal distal tip.

IFT81, an IFT-B complex protein, binds directly to tubulin dimers and facilitates their incorporation into the microtubule axoneme. In wild-type MEFs, IFT81 was enriched in the distal tip of primary cilium and was barely detected along the axoneme (Fig. 7a), consistent with the expression pattern of GFP-tagged IFT81 in ciliated cells. In contrast, multiple IFT81 puncta were present in Kif7L130P cilia, suggesting that the tip structure of the Kif7 mutant cilia is defective. These results suggest that some axonemal microtubules in mutant cilia do not extend to the distal tip and that ectopic microtubule plus ends can organize cillum-like compartments (Fig. 7a).

Gli2 and Sufu co-localize with distal tip markers along the Kif7 mutant axoneme

To understand how these defects in cillum structure affected Shh signalling, we assayed the localization of Shh pathway proteins in mutant cilia. Smo moved into cilia normally in response to the small-molecule agonist SAG (Smoothened agonist) in both wild-type and Kif7L130P mutant MEFs (Fig. 7b), indicating that Kif7 is not required for trafficking of Smo, consistent with the finding that Kif7 acts genetically downstream of Smo.
In wild-type cilia, Gli2 is present in cilia tips and is further enriched by pathway activation. In contrast, Gli2 was present in puncta along the ciliary axoneme in the absence of pathway activation in both Kif7<sup>−/−</sup> and Kif7<sup>L130P</sup> MEFS (Fig. 7b,c). When treated with SAG, extra Gli2 accumulated along the length of mutant cilia (Fig. 7b,c). Despite an elevated level of Gli2 along the mutant axoneme, less Gli2 was present at the distal tip of Kif7<sup>L130P</sup> cilia than in wild type (Fig. 7d). Thus Kif7 is not required for trafficking of Gli2 into cilia, but it does control proper localization of Gli2 within the cilium. Sufu, a negative regulator of the Shh pathway, forms a complex with Gli proteins at cilia tips and is further enriched in response to pathway activation<sup>24,42</sup> (Fig. 7b,c). In Kif7<sup>L130P</sup> cilia, Sufu was present in multiple puncta in the distal half of the ciliary axoneme, where it co-localized with Gli2 (Fig. 7b,e), indicating that Kif7 is required to restrict Gli–Sufu complexes to the distal end of the cilium. Double staining showed that IFT81 and Gli2 were enriched at the distal cilium tip in wild type and frequently co-localized in ectopic puncta along the axoneme in Kif7<sup>L130P</sup> cilia (Fig. 7f). Thus, in the absence of Kif7, the Gli–Sufu complex and IFT81 localize to ectopic tip-like compartments along the axoneme.

**DISCUSSION**

Mammalian Kif7 and *Drosophila* COS are both required for the switch of the Gli/Ci from transcriptional repressors to activators in the presence of Hh ligand, but Kif7 acts through the primary cilium, which is not required for *Drosophila* HH signalling. Our data show that Kif7 has an essential role in cilium structure: mouse Kif7 localizes to the tips of primary cilia, where it controls the length and structure of primary cilia and the localization of Hh signalling complexes through its ability to regulate the dynamics of microtubule plus ends.

Mouse Kif7<sup>L130P</sup> mutant cilia tend to be long, and are unstable and twisted. Cilia in human Kif7<sup>L130P</sup> mutant fibroblasts are longer than controls<sup>43</sup>, indicating that Kif7 has a conserved role in the control of axoneme length. Two other classes of kinesin are known to limit cilium length. *Chlamydomonas* kinesin-13 moves into flagella through IFT and depolymerizes microtubules from the flagellar tip<sup>43</sup>. Mouse KIF19A, a member of the kinesin-8 family, is thought to use its motor activity to move along axonemes and depolymerize microtubules at the tips of motile cilia<sup>44</sup>. Thus each of these kinesins can control cilium length, but they act through distinct mechanisms.

Our *in vitro* reconstitution studies indicate that Kif7 associates with microtubule plus ends, where it limits microtubule length. Whereas other kinesins arrive at microtubule plus ends either by one-dimensional diffusion (for example Xklp1; ref. 45) or by directional motility (for example Xklp1; ref. 1), Kif7 neither diffuses nor moves directionally on microtubules under our assay conditions. Instead, our data suggest that Kif7 tracks the plus ends of growing microtubules by preferential association with GTP-bound tubulin at microtubule tips (Fig. 8a). This tip-tracking behaviour is ATP-hydrolysis dependent, which distinguishes it from end-binding proteins such as EB1 (ref. 46). At the microtubule plus ends, Kif7 inhibits the rate of microtubule growth, an activity shared with other kinesin-4 proteins. However, unlike KIF4A/Xklp1 and KIF21A, Kif7 is a catastrophe-promoting factor.

The primary cilium is built on a scaffold of parallel microtubule bundles of nine doublet protofilaments, with the plus ends at the distal cilium tip. Because Kif7 localizes to cilia tips throughout ciliogenesis and purified Kif7 motor protein can autonomously associate with the plus ends of growing microtubules, we propose that Kif7 binds directly to the distal ends of axonemal microtubules, where it limits the growth of the individual microtubules during cilium assembly. Because tip-associated proteins are mislocalized along the axoneme in the absence of Kif7, Kif7 also seems to help synchronize the growth of the nine doublet microtubules (Fig. 8b).

The data show that Kif7 is critical for organization of the compartment at the tip of the cilium, a topic that has long been of interest<sup>47</sup>. The IFT complexes that control cilium elongation must disassemble and reorganize at the tip as they convert from kinesin-based to dynein-based motility in the process called tip turnaround<sup>48</sup>. In *Chlamydomonas*, the mating signalling cascade is initiated from the flagellar tip<sup>49</sup>. In mammals, key regulators of Hedgehog signalling are highly enriched at tips of primary cilia, and it has been inferred that their activity is regulated in this compartment<sup>24,42</sup>. We propose that the ectopic activation of the pathway seen in Kif7 mutants is due to the ectopic Gli–Sufu complexes away from the cilium tip, where they become inappropriately activated in the absence of ligand (Fig. 8b). Because of its specific actions on microtubules, we propose that the ancestral function of Kif7 was to sculpt the structure of the primary cilium, and that it acted in this context in the Hedgehog pathway in the metazoan ancestor of both *Drosophila* and mammals.

**METHODS**

Methods and any associated references are available in the online version of the paper.

*Note: Supplementary Information is available in the online version of the paper.*

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**AUTHOR CONTRIBUTIONS**

M.H. designed, carried out and analysed experiments and assembled figures. T.O. helped with TIRF microscopy. K.F.L. did the TEM. T.M.K. and M.H. carried out and analysed the microtubule dynamics assays. F.B. carried mass spectrometry. This work was supported by National Institutes of Health grants NS044385 to K.V. Anderson and GM65933 to T.M. Kapoor and the MSKCC Cancer Center Support Grant P30 CA008748.

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METHODS

Mouse strains and mouse embryonic fibroblasts. Kif7<sup>−/−</sup> (the maki allele) has been described<sup>6</sup>. Kif7<sup>+/−</sup>, which we refer to as Kif7<sup>+/−</sup>, was a gift from C. C. Hui (The Hospital for Sick Children, Canada; ref. 8). Snai<sup>−/−</sup> (ref. 50), Gilz<sup>−/−</sup> (ref. 51) and Gli2<sup>−/−</sup> have been described. MEFs were derived from e10.5 mouse embryos using standard protocols, as previously described<sup>21</sup>.

Cell cultures and antibodies. MEFs, NIH3T3, U2OS and HEK293T cells were grown in DME supplemented with 10% bovine calf serum, penicillin and streptomycin. To induce cilia, confluent MEFS and NIH3T3 cultures were serum starved for 24 h followed by treatment with either Shh-N recombinant protein (R & D Systems (464-SE)) or SAG (EMD) for 24 h to activate the Shh pathway. To induce cili disassembly, cells were either treated with 10 μM nocodazole (Sigma) or incubated in 4 °C culture medium. Rabbit Kif7 polyclonal antibody (1:1,000 dilution; Pocono Rabbit Farm and Laboratory) was raised against a Kif7 peptide (amino acids 362–600) fused to a His tag expressed in bacteria. Other antibodies used were guinea-pig anti-Gli2<sup>25</sup> (1:1,000 dilution; a gift from A. Salic, Harvard University, USA), mouse anti-acetylated α-tubulin (1:2,000 dilution; 6-11B-1; Sigma-Aldrich T6793), mouse anti-γ-tubulin (1:2,000 dilution; GTU-88; Sigma-Aldrich T6557), mouse anti-glutamylated tubulin (1:1,000 dilution; GT335; Enzo ALX804-885-C100), mouse anti-GM130 (1:500 dilution; BD Sciences 610822), rabbit anti-IFT88 (1:1,000 dilution; Proteintech 11744-1-AP), rabbit anti-IFT18 (1:1,000 dilution; Proteintech 11744-1-AP), mouse anti-Flag M2 (1:5,000 dilution; Invitrogen F1804), rabbit anti-GFP (1:2,000 dilution; Invitrogen A-11212), goat anti-rabbit IgG (1:1,500 dilution; Sigma A0545) and Alexa-Fluor 488-, 594- and 633-conjugated secondary antibodies (Invitrogen).

Immunostaining and electron microscopy. Cells were fixed with 2% paraformaldehyde for 20 min at room temperature followed with cold methanol for 5 min. Immunostaining on cells and embryos was carried out as described<sup>6</sup>. Fluorescent microscopy of cilia was carried out using an image restoration system (DeltaVision, Applied Precision) with a wide-field microscope (IX70, Olympus). Images were processed by iterative constrained deconvolution (softWoRx, Applied Precision) and corrected for chromatic aberration. Constructions of full-length Kif7 and Kif7 fragments for deletion analysis were generated by cloning corresponding complementary DNAs into pEGFP-N3 vector and transfected into cells with Lipofectamine 2000 (Invitrogen). Confocal microscopy of GFP expression in cell lines was carried out using an upright Leica TCS SP2 AOBs laser scanning microscope. The intensity of fluorescent signals was measured using ImageJ. Images of the neural tube were acquired with a wide-field monochrome camera (AxioCam MR) using AxioVision software (v4.6, Zeiss). For electron microscopy, semi-quantitative mass spectrometry analysis. Tubulin was purified and sparsely labelled with biotin or X-rhodamine according to published protocols<sup>34</sup>.

In vitro fluorescent microscopy. In vitro microscopy was carried out as described<sup>21</sup>. To test if the Kif7 motor fragment alters microtubule dynamics, X-rhodamine- and biotin-labelled microtubules, polymerized in the presence of the non-hydrolysable GTP analogue GMP-CPP, were immobilized on a coverslip by first coating the surface with neutravidin. After a brief incubation with casein to block non-specific binding to the surface, 18.8 μM tubulin (1X-rhodamine-labelled tubulin:10 unlabelled tubulin), 1 mM GTP, 20 μM ATP, 0.1% methylcellulose, 1X BRB80 (80 mM K-PIPES at pH 6.8, 2 mM MgCl<sub>2</sub> and 1 mM EGTA at pH 6.8), 20 mM KCl, 3 mM MgCl<sub>2</sub>, 0.2 mg ml<sup>−1</sup> κ-casein and 3% sucrose were added to the chamber in the absence or presence of Kif7<sub>△1–560</sub>-GFP (35 nM and 70 nM) and MgATP (1 mM) or AMP-PNP (2 mM). Time-lapse images were acquired at a rate of 5 s per frame for 15 min. To test whether the Kif7 motor fragment has motility, a similar assay was carried out except that immobilized microtubules were further stabilized with taxol. Proteins were diluted 1:1,000 to 1:10,000 in 0.5% or 1X BRB80 with 1 mM MgCl<sub>2</sub>, 5% sucrose and 0.2 mg ml<sup>−1</sup> κ-casein and 3% sucrose were added to the chamber in the absence or presence of Kif7<sub>△1–560</sub>-GFP (35 nM and 70 nM) and MgATP (1 mM) or AMP-PNP (2 mM). Time-lapse images were acquired at a rate of 0.5 s per frame for 2 min or 5 s per frame for 15 min. Fresh oxygen scavenger mix (25 mM glucose, 40 mg ml<sup>−1</sup> glucose oxidase, 35 mg ml<sup>−1</sup> catalase and 0.5% β-mercaptoethanol) was included in all buffers for imaging. Experiments with dynamic and non-dynamic microtubules were carried out at 30 °C and room temperature respectively.

Data analysis for microtubule dynamics assays. The fluorescent intensity of Kif7<sub>△1–560</sub>-GFP was analysed using line-scan-based analysis in ImageJ; the average intensities over a five-pixel-wide line along the entire length of dynamic microtubules were measured. Values for microtubule growth rate and catastrophe frequency were obtained using kymograph analysis. For microtubule growth rate, events with a slope over a three-pixel window were analysed. Catastrophe frequencies were obtained by dividing the total number of catastrophes observed by the total time microtubules spent in growth.

Statistical tests. Methods for statistical analysis and numbers of samples measured in this study are specified in figure legends. The error bars indicate standard deviation or standard error.

Protein expression and purification. An N-terminal fragment of Kif7 (amino acids 1–560) including the N-terminal motor domain and the first coiled-coiled domain was cloned into a modified pDEST21b (Novagen) that includes a C-terminal GFP tag and tobacco etch virus protease cleavable 6× His tag. Kif7 fusion protein was expressed in BL21(DE3) Rosetta (Novagen) E. coli at 18 °C with 0.25 mM isopropylthiogalactoside for 16–18 h. Bacterial pellets were lysed by a short sonication in buffer A (50 mM phosphate at pH 8, 30 mM imidazole, 10% glycerol, 100 μM ATP and 0.1% Tween-20), 300 mM NaCl and protease inhibitor cocktail (Roche). The lysate was clarified by centrifugation and the supernatant was incubated with Ni-NTA for 1 h. The resin was washed with buffer A and protein was eluted with 30 mM phosphate at pH 7, 150 mM NaCl, 400 mM imidazole and 100 μM ATP. Peak protein fractions were pooled and incubated with tobacco etch virus protease (1:50 w/w) overnight at 4 °C. The protein was purified further by size exclusion chromatography (Superose 6; Amersham Pharmacia Biotech) in buffer A without Tween-20. After concentration, the protein was dialyzed against buffer A supplemented with 200 mM KCl and 30% w/v sucrose before flash freezing in liquid nitrogen. All proteins were dialyzed or diluted into the appropriate buffer before the experiments. Owing to the very low level of expression, the maximum protein concentration included in the assays with dynamic microtubules was 70 nM. Non-specific protein of a lower molecular weight was identified as E. coli ArnA using semi-quantitative mass spectrometry analysis. Tubulin was purified and sparsely labelled with biotin or X-rhodamine according to published protocols<sup>34</sup>.

Mouse strains and mouse embryonic fibroblasts.

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Supplementary Figure 1  

*Kif7*<sup>L130P</sup> encodes a stable protein that does not localize to cilia tips. (a) Immunoblot with KIF7 antibody in cell lysates of wild-type, *Kif7*<sup>L130P</sup> and *Kif7*/- MEFs. (b) KIF7 (green) is absent from *Kif7*<sup>L130P</sup> cilia regardless of pathway activation. Acetylated-a-tubulin (red) stains cilia and g-tubulin (blue) stains basal body. Arrows indicate KIF7 at the transition zone. Scale bar = 1 µm. (c) KIF7 localizes to cilia tip in Smo<sup>-/-</sup> and (d) Gli2<sup>-/-</sup> Gli3<sup>-/-</sup> double mutant MEFs. Acetylated-a-tubulin (red) stains cilia and g-tubulin (blue) in (c) and magenta in (d) stains basal body. Scale bar = 5 µm. (e) GM130 (green), a Golgi membrane protein, appears normal in *Kif7*<sup>L130P</sup> MEFs. DAPI in blue. Scale bar = 17 µm. (f) In contrast to *Kif7* knockdown cells 14, normal numbers of centrosomes and cilia are present in wild-type and *Kif7*<sup>L130P</sup> MEFs. Acetylated-a-tubulin (red) marks axonemal microtubules and g-tubulin (magenta) marks basal bodies. DAPI is in blue. n = 165 cilia were analyzed and were pooled from 4 independent experiments. Error bars represent the SD, p>0.0001 by student t-test. Scale bar = 22 µm.
Supplementary Figure 2  

*Kif7L130P* mutant cells have long cilia and abnormal axonemal structure. (a) The diameter of proximal and distal region was measured from neural tube cilia from e10.5 wild-type and *Kif7L130P* embryos. The width of proximal cilia was 0.21±0.02 μm in wild-type and 0.20±0.02μm in *Kif7L130P*. The width of distal cilia was 0.12±0.02μm in wild-type and 0.08±0.01 μm in *Kif7L130P* (n=50 cilia were measured for each genotype; p<0.001 by one-way ANOVA analysis). Error bars represent the SD. (b) Low power SEM of the node of an e8.0 wild-type embryo (black square). (c) Node cilia from e8.0 wild-type and *Kif7L130P* mutant embryos. Scale bar = 2μm. (d) TEM images of transverse sections of *Kif7L130P* neural tubule cilia (4 examples). *Arrows* indicate singlet microtubules. Scale bars = 50nm.
Supplementary Figure 3  Defects of tubulin modification in Kif7−/− mutant MEFs. Tubulin acetylation (red) and glutamylation (green) are reduced at the distal segment of cilia in Kif7−/− MEFs compared to wild type. Scale bar = 1μm.
Supplementary Figure 4 KIF7 associates with microtubule in vivo. (a) Protein domain analysis of mouse KIF7 and KIF4. Motor domain is in green, neck in pink and coiled-coil in purple. Note the long linker between the neck and the first coiled-coil of KIF7, which may inhibit its ability to act as a processive motor. Arrows and numbers indicate the positions of respective domains in amino acid. (b) Co-immunoprecipitation of Flag- and GFP-tagged full-length KIF7 in 293T cells. (c) Expression of C-terminal GFP-tagged KIF7-full length and KIF7560 constructs in HEK293T cells visualized by confocal fluorescent microscope. DAPI in blue. Scale bar = 8μm. (d) Deletion analysis of KIF7 for in vivo microtubule bundling activity and dimerization.
Supplementary Figure 5 purified KIF7560-GFP directly binds to microtubules. (a) Purified KIF7560-GFP for *in vitro* analysis. Asterisk indicates bacterial protein Arna (Bifunctional polymyxin resistance protein Arna). (b) 70 nM KIF7560-GFP binds to X-rhodamine-labeled microtubules in the presence of 1 mM MgATP or 2 mM AMP-PNP. Scale bar = 5μm.
Figure S6 Induced microtubule destabilization by purified KIF7-GFP. Examples of fragmented microtubules observed when X-rhodamine-labeled microtubules were incubated with 500nM of KIF7-GFP in the presence of 1 mM MgATP.
**Figure S7** *Kif7^L130P* mutant cilia have defects in IFT dynamics. (a-b) Representative kymographs generated from time lapse imaging of wild-type and (C-D) *Kif7^L130P* primary cilium (Supplementary Movie. S1-2). (a) and (c) are taken from Figure 3. The base and tip of the cilium are indicated with arrowheads. Asterisks indicate stationary IFT88 puncta associated with the initiation of retrograde trafficking. Horizontal scale bar (distance) = 1μm. Vertical scale bar (time) = 7.5 seconds. Enlarged portion of kymographs are highlighted (yellow rectangles). Asterisks show the position of stationary IFT88 puncta. Our kymograph analysis shows IFT88-GFP (4/200 retrograde trains) switches tracks in the wild-type cilia very rarely, whereas significantly more trains (79/214 retrograde trains) switch tracks in *Kif7* mutants. (e) Numbers of stationary IFT88-GFP puncta per cilium in wild-type and *Kif7^L130P* (n = 50 cilia were imaged for each condition and were pooled from 5 independent experiments; *p*<0.0001 by student t-test). The error bars represent SD.
Supplementary Figure 8 Uncropped western blots referring to Figure S1a and Figure S4b. Figure S1a KIF7 protein level in wild-type, Kif7\textsuperscript{L130F} and Kif7\textsuperscript{-/-} MEFS. g-tubulin (asterisk) was used as loading control. Figure S4b co-immunoprecipitation of Flag- and GFP-tagged full-length KIF7 in 293T cells. Membranes were blotted with anti-Flag and anti-GFP antibodies, respectively.
Supplementary Video legends

Video S1. Live imaging of IFT88-GFP in wild-type cilia. IFT visualized by time-lapse imaging of a wild-type primary cilium expressing IFT88-GFP. Images are taken every 250ms for 30 seconds. Movie shows 10 frames/second.

Video S2. Live imaging of IFT88-GFP in Kif7L130P mutant cilia. IFT visualized by time-lapse imaging of a Kif7L130P primary cilium expressing IFT88-GFP. Images are taken every 250ms for 30 seconds. Movie shows 10 frames/second.

Video S3. Effect of KIF7560-GFP on dynamic microtubules. KIF7560-GFP (70nM; 1mM MgATP) and dynamic X-rhodamine-microtubule visualized by time-lapse TIRF microscopy. Images are taken every 5 seconds for 15 minutes. Movie shows 15 frames/second.