

Roles of kinesin-2 motor proteins
involved in intraciliary protein trafficking

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GENERAL INTRODUCTION

Cilia are microtubule-based organelles protruding from the surface of most eukaryotic cells and serve as cellular antennae by mechanosensation of extracellular stimuli, such as fluid flow, and chemosensation of developmental signals, such as the Hedgehog (Hh) signal (Mukhopadhyay and Rohatgi, 2014; Bangs and Anderson, 2017). To perform these functions, cilia contain a large number of unique proteins, including a variety of G protein-coupled receptors (GPCRs) and ion channels. The crucial roles of cilia as cellular sensory antennae have been highlighted by findings that defects in ciliary assembly and functions result in a variety of hereditary disorders, collectively called the ciliopathies, with a broad spectrum of symptoms, including retinal degeneration, polycystic kidney, morbid obesity, and polydactyly (Schwartz *et al.*, 2011; Madhivanan and Aguilar, 2014; Braun and Hildebrandt, 2017). Although the ciliary membrane is continuous with the plasma membrane, protein and lipid compositions of cilia differ greatly from those of the cell body, because the ciliary transition zone (TZ), which is located at the ciliary base, serves as a diffusion/permeability barrier (Sung and Leroux, 2013; Wei *et al.*, 2015). Proteins required for the assembly and functions of cilia are transported from the cell body. The transport process includes entry of the proteins into cilia across the TZ and trafficking of proteins along axonemal microtubules within cilia.

The ciliary TZ is structurally characterized by the presence of transition fibers and Y-linked structures, which have been proposed to constitute a barrier similar to the nuclear pore complex (NPC; Takao and Verhey, 2016). Therefore, some ciliary proteins, such as KIF17 and RP2, contain a nuclear localization signal (NLS) and enter the cilium by a mechanism dependent on the Ran GTPase and importin β 2 (also known as transportin 1 [TNPO1]; Dishinger *et al.*, 2010; Hurd *et al.*, 2011).

On the other hand, bidirectional trafficking of ciliary proteins along the axoneme is mediated by intraflagellar transport (IFT) particles containing two large multisubunit complexes, IFT-A and IFT-B, powered by the kinesin-2 and dynein-2 motor proteins. At the ciliary base, the IFT-A and IFT-B complexes assemble to form IFT particles, to which kinesin-2 and dynein-2 bind via the IFT-B and IFT-A complexes, respectively (Fig. 0-1) (Taschner *et al.*, 2012; Lechtreck, 2015; Katoh *et al.*, 2016; Hirano *et al.*, 2017; Nakayama and Katoh, 2018). Subsequently, ciliary soluble cargo proteins, including α/β -tubulin dimers, are loaded onto the assembled particles, and membrane

cargo proteins, such as GPCRs, are connected to the IFT-A complex through TULP3, which interacts with the IFT-A complex (also see Fig. 0-2A). The IFT particles then enter the cilium across the TZ and undergo anterograde trafficking along the axoneme powered by the kinesin-2 motor. At the ciliary tip, IFT particles are thought to disassemble to release cargo proteins, and kinesin-2 is inactivated. IFT particles are then reassembled and cargoes are loaded, and the assembled particles undergo retrograde trafficking powered by the dynein-2 motor. The particles exit the cilium across the TZ and are disassembled.

Our laboratory recently demonstrated the overall architecture of the IFT-A and IFT-B complexes. The IFT-A complex can be divided into the core subcomplex composed three subunits (IFT122/140/144), which interacts with TULP3, and the peripheral subcomplex also composed of three subunits (IFT43/121/139) (Fig.0-2A) (Hirano *et al.*, 2017; Nakayama and Katoh, 2018). The IFT-B complex can also be divided into the core and peripheral subcomplexes, which are composed of 10 subunits (IFT22/25/27/46/52/56/70/74/81/88) and 6 subunits (IFT20/38/54/57/80/172), respectively; these subcomplexes are linked by the connecting tetramer involving composite interactions of IFT38/52/57/88 (Fig. 0-2B) (Katoh *et al.*, 2016; Nakayama and Katoh, 2018). Deficiency in any of the IFT-B subunits often leads to extremely short or no cilia, indicating a crucial role of the trafficking of ciliary proteins in assembly of cilia.

Two different types of kinesin-2 motors have been implicated in ciliary anterograde protein trafficking; heterotrimeric kinesin-II composed of motor subunits, KIF3A and KIF3B, and an accessory protein KAP3 (KLP-20, KLP-11, and KAP-1 in *Caenorhabditis elegans*), and homodimeric KIF17 (OSM-3 in *C. elegans*) (Scholey, 2013). In *C. elegans*, heterotrimeric kinesin-II and the OSM-3 homodimer have been shown to participate in IFT within the axoneme proximal/middle segment and the distal segment, respectively (Pan *et al.*, 2006; Scholey, 2013; Prevo *et al.*, 2015). However, in vertebrates, differential roles of the two types of the kinesin-2 motors in ciliary anterograde protein trafficking are unclear. Furthermore, although previous studies suggested that these kinesin-2 motors interact with the IFT-B complex (Baker *et al.*, 2003; Snow *et al.*, 2004; Insinna *et al.*, 2008; Howard *et al.*, 2013; Liang *et al.*, 2014), it remains uncertain which IFT-B subunit(s) directly participate in the interaction with these kinesin-2 motor proteins.

In the present study, I set out to determine the subunit(s) of the IFT-B complex involved in its interaction with kinesin-2 motor proteins and elucidate the differential roles of these kinesin-2 motors in ciliary protein trafficking. I revealed that KIF17 interacts with the IFT-B complex through the IFT46–IFT56 dimer and is a cargo of, rather than serving as an anterograde motor for, the IFT-B complex (Chapter 1). On the other hand, I found that heterotrimeric kinesin-II interacts with the IFT-B complex through the connecting tetramer and showed that the interaction between heterotrimeric kinesin-II and the IFT-B-connecting tetramer is crucial for ciliogenesis (Chapter2).

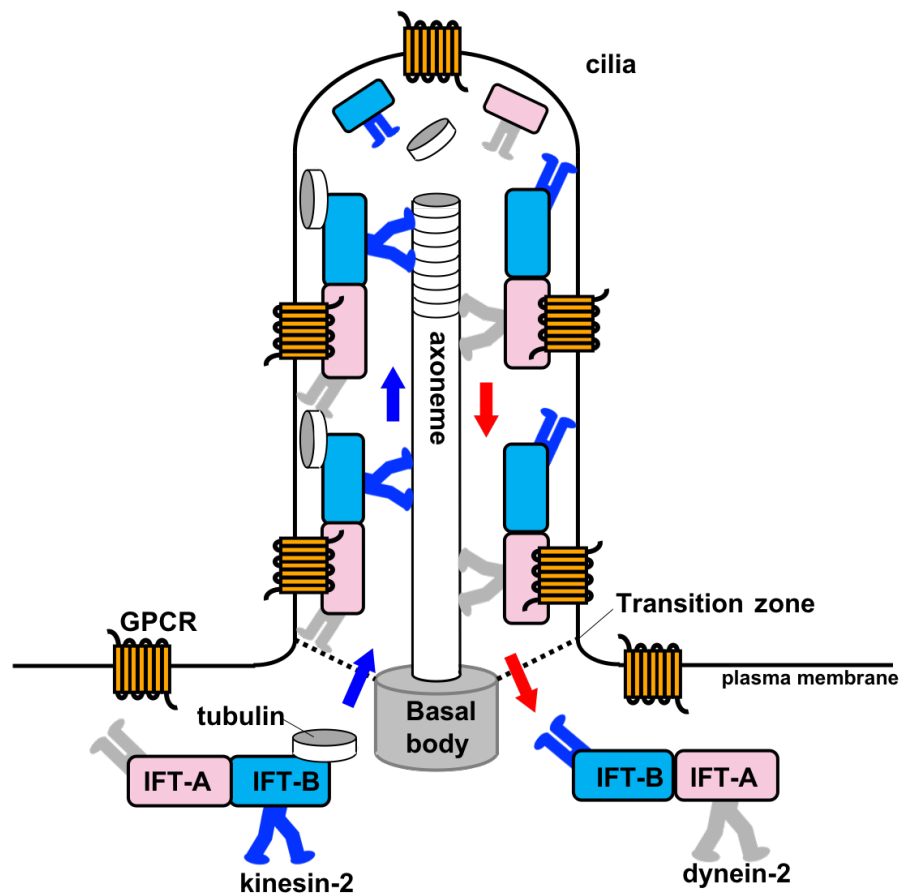


Fig. 0-1. Schematic illustration of a cilium and IFT.

Cilia are the microtubule-based structures projecting from the cell surface of most eukaryotic cells. Bidirectional trafficking of ciliary proteins along the axoneme is mediated by IFT particles, which contain the IFT-A and IFT-B complexes. At the base of cilia, the IFT-A and IFT-B complexes assemble to form IFT particles. Kinesin-2 and dynein-2 bind to IFT-B and IFT-A, respectively. Subsequently, ciliary soluble cargo proteins, such as α/β -tubulin dimers, are loaded onto the assembled particles, and membrane cargo proteins, such as GPCRs, are connected to the IFT-A complex via TULP3, which interacts with the IFT-A core subcomplex (also see Fig. 0-2). The particles then enter the cilium across the TZ and undergo processive anterograde trafficking along the axoneme powered by heterotrimeric kinesin-2. At the ciliary tip, IFT particles are thought to disassemble to release cargo proteins, and kinesin-2 undergoes inactivation. IFT particles are then reassembled and cargos are loaded, and the assembled particles undergo retrograde trafficking powered by dynein-2. The particles exit the cilium across the TZ.

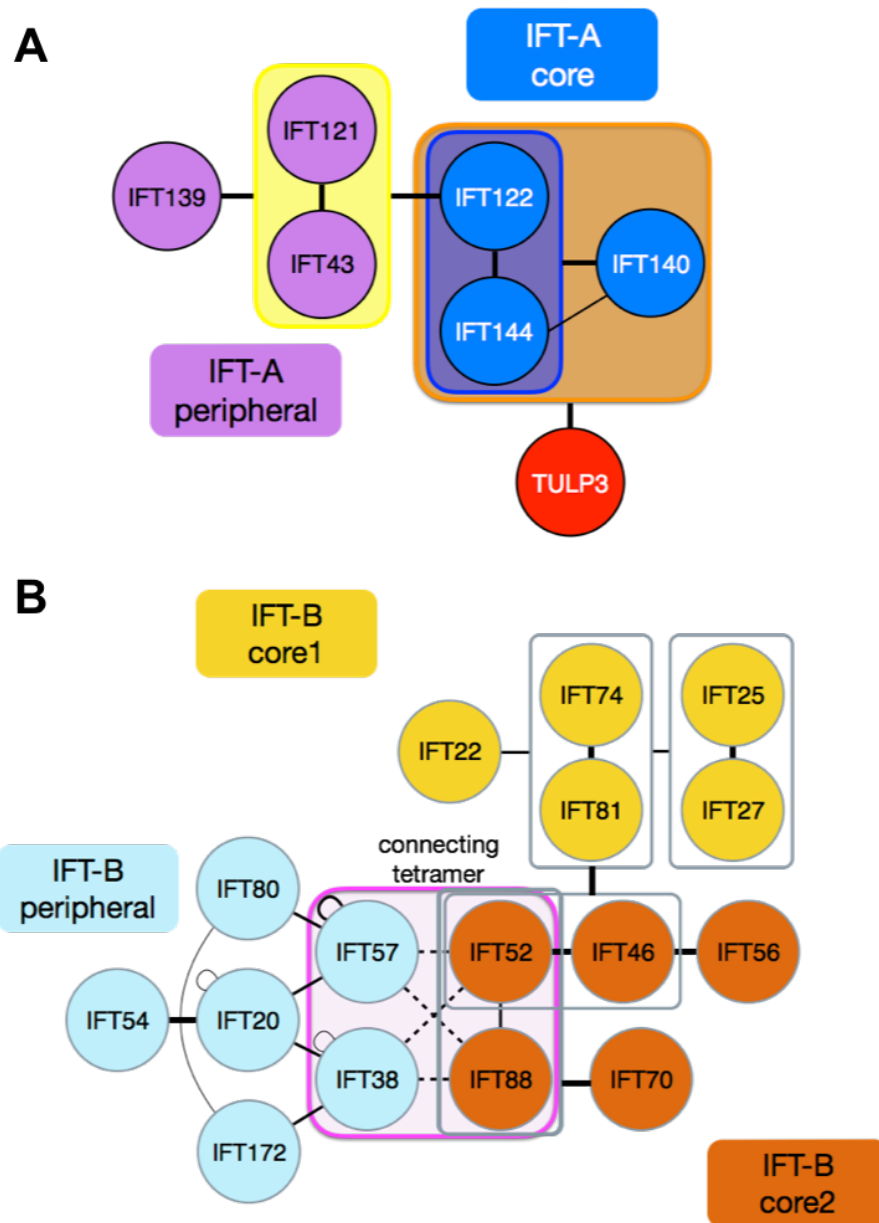


Fig. 0-2 Architectures of the IFT-A and IFT-B complexes

Architectures of the IFT-A and IFT-B complexes predicted from our previous studies (Katoh et al., 2015; Hirano et al., 2017; Nakayama and Katoh, 2018). (A) The IFT-A complex can be divided into the core subcomplex composed of IFT122/140/144, which interacts with TULP3, and the peripheral subcomplex composed of IFT43/121/139. (B) The IFT-B complex are also composed of the core and peripheral subcomplexes. The former is composed of 10 subunits (IFT22/25/27/46/52/56/70/74/81/88) and the latter is composed by six subunits (IFT20/38/54/57/80/172); these subcomplexes are linked by composite interactions involving the connecting tetramer composed of IFT38/52/57/88.

ABBREVIATIONS

Ac- α -Tubulin	acetylated α -tubulin
Cas	CRISPR-associated protein
CC	coiled-coil
cDNA	complementary DNA
<i>C. elegans</i>	<i>Caenorhabditis elegans</i>
CRISPR	clustered regularly interspaced short palindromic repeat
DNA	deoxyribonucleic acid
DRD1	dopamine receptor D1
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EGFP	enhanced green fluorescent protein
FBS	fetal bovine serum
GFP	green fluorescent protein
GPCR	G protein–coupled receptor
GST	glutathione <i>S</i> -transferase
GTP	guanosine triphosphate
HEK	human embryonic kidney
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
Hh	Hedgehog
hTERT	human telomerase reverse transcriptase
IFT	intraflagellar transport
KAP	kinesin associated protein
KIF	kinesin family
KO	knockout
KPN	karyopherin
mChe	mCherry
Nb	Nanobody

NLS	nuclear localization signal
NPC	nuclear pore complex
PAM	proto-spacer adaptor motif
PCR	polymerase chain reaction
PY-NLS	proline-tyrosine nuclear localization signal
RFP	red fluorescent protein
RPE	retinal pigment epithelial
RNA	ribonucleic acid
SAG	Smoothened Agonist
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
sgRNA	single guide RNA
Smo	Smoothened
tBFP	TagBFP
TNPO	transportin
tRFP	TagRFP
TULP	Tubby-like protein
TZ	transition zone
VIP	visible immunoprecipitation
WT	wild type

Chapter 1: KIF17 is a cargo of the IFT-B complex rather than being a motor for the IFT machinery

ABSTRACT

Cilia function as cellular antennae to sense and transduce extracellular signals. A number of proteins are specifically localized in cilia. Anterograde and retrograde ciliary protein trafficking are mediated by the IFT-B and IFT-A complexes in concert with kinesin-2 and dynein-2 motors, respectively. However, the role of KIF17, a homodimeric kinesin-2 protein, in protein trafficking has not been fully understood in vertebrate cilia. In this chapter, I first demonstrated by taking advantage of the visible immunoprecipitation assay, that KIF17 interacts with the IFT46–IFT56 dimer in the IFT-B complex through its C-terminal sequence located immediately upstream of the NLS. I then showed that KIF17 requires IFT-B binding for its entry into cilia, rather than for its intraciliary trafficking. I further showed that KIF17 ciliary entry is dependent not only on its binding to IFT-B but also on its NLS, to which importin α proteins bind. Taken together, I conclude that in mammalian cells, KIF17 is dispensable for ciliogenesis and IFT-B trafficking, but requires IFT-B as well as its NLS for its ciliary entry across the TZ located at the ciliary base.

INTRODUCTION

Cilia are microtubule-based structures projecting from the surface of most eukaryotic cells and perform a variety of biological functions, such as perception and transduction of physiological and developmental signals, such as the Hh signaling pathway. A number of unique proteins, including GPCRs and ion channels, are specifically localized in cilia. Proteins required for the assembly and functions of cilia are transported from the cell body into cilia across the TZ and trafficked along axonemal microtubules within cilia.

The ciliary TZ is structurally characterized by the presence of transition fibers and Y-linked structures, which have been proposed to constitute a barrier similar to the NPC (Takao and Verhey, 2016). Therefore, some ciliary proteins, such as KIF17 and RP2, contain an NLS and enter the cilium by a mechanism dependent on the Ran GTPase and importin β 2 (also known as transportin 1 [TNPO1]; Dishinger *et al.*, 2010; Hurd *et al.*, 2011).

Anterograde and retrograde ciliary protein trafficking are mediated by the IFT-B and IFT-A complexes in concert with kinesin-2 and dynein-2 motors, respectively. Two different types of kinesin-2 motors, heterotrimeric kinesin-II and homodimeric KIF17, have been implicated in ciliary anterograde protein trafficking (Scholey, 2013). In *C. elegans*, heterotrimeric kinesin-II and the OSM-3 (*C. elegans* homologue of KIF17) homodimer have been shown to participate in IFT within the axoneme core (proximal/middle) segment and the distal segment, respectively (Pan *et al.*, 2006; Scholey, 2013; Prevo *et al.*, 2015). In contrast, in vertebrates, the contribution of KIF17 to ciliary assembly is unclear, as ciliogenesis appeared to be normal in *KIF17*-deficient mice and zebrafish (Yin *et al.*, 2011; Pooranachandran and Malicki, 2016), although its involvement in the trafficking of some ciliary proteins has been implicated (Jenkins *et al.*, 2006; Leaf and von Zastrow, 2015).

KIF17 accumulates at the ciliary tip of mammalian cells (Fig. 1-1A) (Dishinger *et al.*, 2010; Kee *et al.*, 2012; Takao and Verhey, 2016). Namely, it is translocated into cilia through the TZ by a mechanism dependent on its NLS and on the Ran GTPase and TNPO1. On the other hand, it was unknown as to how KIF17 is transported to the ciliary tip along the axoneme in mammalian cells, because a construct of KIF17 that lacked its motor domain was able to reach the ciliary tip (see Fig. 1-1C) (Williams *et al.*, 2014;

Jiang *et al.*, 2015). Previous studies suggested that the IFT-B complex interacts with KIF17/OSM-3 (Snow *et al.*, 2004; Ou *et al.*, 2005; Insinna *et al.*, 2008; Howard *et al.*, 2013), although it was not clear as to which IFT-B subunit(s) are directly involved in the interaction.

Our laboratory recently developed a novel and versatile strategy for studying protein–protein interactions, called the visible immunoprecipitation (VIP) assay (see Fig. 1-2) (Katoh *et al.*, 2015; Katoh *et al.*, 2016); the VIP assay can visually detect binary protein interactions by fluorescence microscopy without performing electrophoresis and immunoblotting, and can determine interactions between more than two proteins at a time. By taking advantage of this strategy, our laboratory determined the architecture of the IFT-B complex (Katoh *et al.*, 2016). In the present study, I applied our flexible VIP assay system to detect the interaction of KIF17 with the IFT-B complex and found that KIF17 interacts with the IFT46–IFT56 dimer in the IFT-B complex. I also found that a sequence conserved between vertebrate KIF17 and *C. elegans* OSM-3, located immediately upstream of the NLS, is responsible for the interaction of KIF17 with IFT46–IFT56. Furthermore, I showed that both the NLS and the IFT-B–interacting sequence of KIF17 are required for its ciliary entry.

I further established *IFT56*-KO and *KIF17*-KO lines of hTERT-RPE1 (human telomerase reverse transcriptase-immortalized retinal pigmented epithelial) cells using a modified CRISPR/Cas9 system (Katoh, *et al.*, 2017), and showed that *IFT56*-KO and *KIF17*-KO cells are normal with respect to ciliogenesis. However, I found that KIF17 was unable to enter cilia in *IFT56*-KO cells whereas IFT-B localization was not affected by the KIF17 deficiency. These results unequivocally demonstrate that KIF17 requires IFT-B binding as well as its NLS for entry into cilia.

RESULTS

KIF17 interacts with the IFT46–IFT56 dimer of the IFT-B complex

Although previous studies suggested that KIF17/OSM-3 interacts with the IFT-B complex (Snow *et al.*, 2004; Ou *et al.*, 2005; Insinna *et al.*, 2008; Howard *et al.*, 2013), it was uncertain as to which subunit(s) were directly involved in the interaction between IFT-B and KIF17. To address this issue, I applied the VIP assay, which our laboratory recently developed as a simple and flexible strategy for visually detecting protein–protein interactions (Katoh *et al.*, 2015; Katoh *et al.*, 2016). Using this assay, our laboratory recently determined the architectures of the IFT-B complex composed of 16 subunits. Our study unequivocally showed that the IFT-B complex comprises two parts, the core subcomplex composed of 10 subunits (IFT22/25/27/46/52/56/70/74/81/88) and the peripheral subcomplex composed of 6 subunits (IFT20/38/54/57/80/172) (Katoh *et al.*, 2016); these subcomplexes are linked by composite interactions involving the connecting tetramer, IFT38/52/57/88 (see Fig. 0-2B) (Katoh *et al.*, 2016; Taschner *et al.*, 2016).

I first examined whether KIF17 can interact with individual IFT-B subunits. When EGFP-KIF17 was co-expressed with any one of the 16 IFT-B subunits fused to mCherry (mChe) or TagRFP (tRFP) in HEK293T cells, and lysates prepared from the transfected cells were subjected to immunoprecipitation with glutathione *S*-transferase (GST)-tagged anti-GFP nanobody (Nb) prebound to glutathione-Sepharose beads. However, I was unable to detect the interaction of KIF17 with any of the IFT-B subunits (Fig. 1-3). I then made use of one of the advantages of our flexible VIP assay; our previous study successfully found not only one-to-many but also many-to-many subunit interactions in the IFT-B complexes (Katoh *et al.*, 2016). When EGFP-KIF17 was co-expressed with all of the IFT-B subunits, or with all of the core or peripheral subunits as mChe/tRFP fusions, I was able to detect red-fluorescent signals when all IFT-B subunits and all core subunits, but not all peripheral subunits, were co-expressed with EGFP-KIF17 (Fig. 1-4A). Therefore, data from the VIP assay indicate that the core subcomplex of IFT-B contains subunits responsible for its interaction with KIF17.

I then applied another advantage of our flexible assay to find the core subunit(s) of IFT-B responsible for its interaction with KIF17; namely, the subtractive VIP assay, in which omitting one or more of the core subunits is expected to abolish red-fluorescent signals if those core subunit(s) are crucial for the interaction of KIF17 with the IFT-B

complex. The red-fluorescent signals were attenuated considerably by omitting IFT46 or IFT56 from the 10 core subunits, and weakly by omitting IFT52, but not by omitting any of the other core subunits (Fig. 1-4B). To examine whether IFT46, IFT52, and/or IFT56 are indeed involved in the interaction of the IFT-B complex with KIF17, I then performed VIP assays for one-to-many protein interactions. As shown in Fig. 1-4C, red signals were detected robustly when mChe-IFT46+IFT56, or mChe-IFT46+IFT52+IFT56 were co-expressed with EGFP-KIF17, and detected weakly when mChe-IFT46+IFT52 were co-expressed. I confirmed the VIP data by subjecting the immunoprecipitates to conventional immunoblotting analysis (Fig. 1-4D); bands for mChe-tagged proteins were detected when mChe-IFT46+IFT56 (lane 5) or mChe-IFT46+IFT52+IFT56 (lane 6) were co-expressed with EGFP-KIF17, and weak bands were detected when mChe-IFT46+IFT52 were co-expressed (lane 4). I therefore concluded that the IFT46–IFT56 dimer mainly participates in interaction of the IFT-B complex with KIF17 as schematically shown in Fig. 1-4E, although IFT52 may play an auxiliary role in the interaction. The substantial attenuation of the red fluorescent signal observed when IFT52 was omitted in the subtractive VIP assay (Fig. 1-4C) was likely due to the critical role of IFT52 in connecting IFT46–IFT56 to the other seven core subunits (see Fig. 1-4E).

KIF17 interacts with IFT46–IFT56 via a conserved sequence immediately upstream of its NLS

I then set out to determine the region of the KIF17 protein that is responsible for its interaction with the IFT46–IFT56 dimer. To this end, I constructed a series of C-terminal truncation mutants of KIF17, as its NLS is located in the C-terminal region (see Fig. 1-5A) and because KIF17 lacking its motor domain was reported to retain its ability to reach the ciliary tip (Williams *et al.*, 2014; Jiang *et al.*, 2015) (also see Fig. 1-1C). As seen in the VIP data shown in Fig. 1-5B, not only KIF17(1-1,018) (second row), which contains the NLS (see Fig. 1-5A), but also KIF17(1-1,014) (third row), which lacks the NLS, interacted with the IFT46–IFT56 dimer. Supporting these results was that substitution of all the basic residues in the NLS (^{1,015}KRKK^{1,018}) to Ala did not affect the interaction of KIF17 with IFT46–IFT56 (AAAA, fifth row). A further truncation up to residue 999, however, abolished the interaction of KIF17 with IFT46–IFT56 (fourth row).

By comparing the sequences of various vertebrate KIF17s, I noticed that the sequence (RPxRLxSL; residues 1,000-1,007 in human KIF17) immediately upstream of

the NLS is highly conserved (Fig. 1-5A). A similar sequence is also found at the C-terminus of *C. elegans* OSM-3, although it lacks the NLS. I therefore substituted the conserved Arg residues at amino acids 1,000 and 1,003 to Ala (R1000/1003A), and found that KIF17(R1000/1003A) did not interact with the IFT46–IFT56 dimer in the VIP assay (Fig. 1-5B, bottom row). The VIP data were confirmed by subjecting the immunoprecipitates to conventional immunoblotting analysis (Fig. 1-5C); EGFP-KIF17(1-999) and EGFP-KIF17(R1000/1003A) had very low ability to co-immunoprecipitate mChe-IFT46 and mChe-IFT56 (lanes 6 and 2, respectively). Note that I constantly observed another band between IFT46 and IFT56 after co-immunoprecipitation of EGFP-KIF17, and mChe-IFT46 and mChe-IFT56 (Fig. 1-4D and Fig. 1-5C, top panels), although the extra band was not detected when a reciprocal combination of fluorescent fusion proteins was used (see Fig. 1-17D, lane 1). The extra band may represent mChe-IFT46 molecules with slow electrophoretic mobility upon SDS-PAGE due to its weak interaction with mChe-IFT56 even under denaturing conditions.

Both the NLS and IFT-B-binding sequence are required for the ciliary entry of KIF17

The long-standing question regarding vertebrate KIF17 is whether this motor protein functions as a motor or is a cargo of the IFT machinery. To obtain an answer to this question, I examined the localization of the KIF17 constructs used in the experiments shown in Fig. 1-5. After transfection of an expression vector for the EGFP-KIF17 construct into hTERT-RPE1 cells, the cells were cultured under serum-starvation conditions for 24 h to induce ciliogenesis (see MATERIALS AND METHODS) and processed for immunostaining for acetylated α -tubulin (Ac- α -tubulin; a marker for the ciliary axoneme) and γ -tubulin (a marker for the basal body). As shown in Fig. 1-6A, EGFP-KIF17(WT) accumulated at the ciliary tips. In addition, a weak EGFP signal was also observed in the nucleus. Essentially the same localization pattern was observed for EGFP-KIF17(1-1,018) (Fig. 1-6B), which contains both the NLS and the IFT-B-binding sequence. In striking contrast, no signal was observed at the ciliary tip or in the nucleus for EGFP-KIF17(1-1,014) (Fig. 1-6C), indicating that the NLS is required for entry into both the cilium and nucleus. This is supported by the observation that EGFP-KIF17(AAAA), an NLS mutant, failed to enter the cilium and nucleus (Fig. 1-6F; also

see Fig. 1-6G).

EGFP-KIF17(1-999), which lacks both the NLS and the IFT-B-binding sequence, also failed to enter the cilium and nucleus (Fig. 1-6D). By contrast, EGFP-KIF17(R1000/1003A), an IFT-B-binding defective mutant, retained the ability to enter the nucleus, although it could not enter the cilium (Fig. 1-6E; also see Fig. 1-6G). These observations together indicate that the NLS serves as a signal for entry into the cilium as well as the nucleus, consistent with previous reports (Dishinger *et al.*, 2010; Jiang *et al.*, 2015). On the other hand, IFT-B binding is required for KIF17 entry into the cilium. This is compatible with the notion that KIF17 is a cargo of the IFT-B complex. However, the observation that KIF17(R1000/1003A) cannot enter the cilium was somewhat unexpected as the IFT-B complex mediates anterograde ciliary protein trafficking, but there has been no report suggesting the involvement of IFT-B in the ciliary entry of proteins (see DISCUSSION).

IFT56 is essential for the entry of KIF17 into cilia

To support the idea that the ciliary entry of KIF17 is mediated by the IFT-B complex, I then attempted to establish hTERT-RPE1 cell lines defective in IFT56. I expected that the lack of IFT56 would inhibit the binding of IFT-B to KIF17, but would not severely affect IFT-B assembly or ciliogenesis in human cells for the following reasons: (i) our model of the architecture of IFT-B (Katoh *et al.*, 2016; also see Fig. 1-4E) predicts that IFT56 has an auxiliary role in the assembly of the IFT-B complex; (ii) Lorentzen and colleagues purified the recombinant IFT-B complex in the absence of IFT56 (Taschner *et al.*, 2016); and (iii) mutations of DYF13/TTC26/IFT56 in *Chlamydomonas* and in mice were reported to marginally affect IFT-B assembly and ciliogenesis (Ishikawa *et al.*, 2014; Swiderski *et al.*, 2014).

Because hTERT-RPE1 cells retain the characteristics of normal primary cells and are thought to have low homologous recombination activity, the establishment of bi-allelic KO lines from hTERT-RPE1 cells was reported to be difficult, although not impossible; Miyamoto *et al.* established bi-allelic *KIF2A*-KO lines from hTERT-RPE1 cells through sequential gene targeting with two distinct targeting vectors containing neomycin-resistance and puromycin-resistance genes (Miyamoto *et al.*, 2015). To overcome this problem, our laboratory recently developed an efficient genome editing strategy applicable to hTERT-RPE1 cells by modification of the CRISPR/Cas9 system

(see MATERIALS AND METHODS, and also see Fig. 1-7A), and established *IFT88*-KO and *IFT20*-KO cell lines, which are both defective in ciliogenesis (Katoh *et al.*, 2017). Using two different target sequences (see Table 2), I established a total of five independent *IFT56*-KO cell lines. Among them, I selected two cell lines (#56-1-7 and #56-2-6) established using different target sequences for the following detailed analyses. PCR analysis of genomic DNAs demonstrated that clones #56-1-7 and #56-2-6 have mono-allelic reverse and forward integration, respectively, of the knock-in donor vector in the *IFT56* gene (Fig. 1-7B). Direct sequencing of the PCR products demonstrated that, in the other allele, clones #56-1-7 and #56-2-6 have a 1-bp insertion and a 7-bp deletion, respectively (Fig. 1-7, C and D), probably resulting from error-prone non-homologous end joining after CRISPR/Cas9-mediated DNA cleavage.

I then examined the phenotype of the *IFT56*-KO cell lines. After incubation of control hTERT-RPE1 cells and the two *IFT56*-KO cell lines under serum-starvation conditions to induce ciliogenesis, the cells were triple immunostained with antibodies against Arl13b, Ac- α -tubulin, and γ -tubulin (Fig. 1-8, A–C). In control RPE1 cells, Arl13b and Ac- α -tubulin mark the ciliary membrane and axoneme, respectively, and γ -tubulin is localized to the basal body (Fig. 1-8, A–A'''). The staining pattern for these marker proteins in the *IFT56*-KO cell lines (B–B''' and C–C''') was similar to that in control cells, indicating that ciliogenesis or the integrity of cilia is not affected by the absence of *IFT56*. This was in striking contrast to the phenotypes of *IFT88*-KO and *IFT20*-KO cell lines, in which ciliogenesis is abolished (Katoh *et al.*, 2017).

I then examined the localization of Smoothened (Smo), which is a GPCR involved in Hh signaling. Smo is known to enter cilia when the Hh signaling pathway is activated by Smoothened Agonist (SAG), as observed in control RPE1 cells (Fig. 1-9, compare A and B). As in control cells, Smo was not found within cilia under basal conditions (Fig. 1-9, C and E), whereas it entered cilia when stimulated with SAG (D and F) in the *IFT56*-KO cell lines #56-1-7 and #56-2-6. The phenotype of *IFT56*-KO cells is different to that of cells derived from *IFT25*-KO and *IFT27*-KO mice, in which a substantial fraction of Smo accumulates within cilia even under basal conditions, resulting in aberrant Hh signaling, although ciliogenesis appears to be unaffected (Keady *et al.*, 2012; Eguether *et al.*, 2014).

Staining for IFT88, an IFT-B core subunit, was observed mainly around the basal body, and faintly along the cilium and at the tip in both control RPE1 cells and the

IFT56-KO cell lines (Fig. 1-10, A–C). Taken together with the fact that ciliogenesis or Smo trafficking is not affected in the *IFT56*-KO cell lines, I conclude that IFT56 is not necessary for the assembly or major functions of the IFT-B complex.

I then set out to compare KIF17 localization between control and *IFT56*-KO cells. When stably expressed in control RPE1 cells, EGFP-KIF17 was found at the ciliary tip (Fig. 1-11A). In striking contrast to control cells, no ciliary signals of EGFP-KIF17 were observed in the *IFT56*-KO cell lines (Fig. 1-11, B and C; also see Fig. 1-11D). These observations together unequivocally demonstrate that, for its entry into cilia, KIF17 requires its binding to the IFT-B complex through the IFT46-IFT56 dimer.

KIF17 is dispensable for ciliogenesis and IFT-B trafficking

I then established *KIF17*-KO lines of hTERT-RPE1 cells to examine whether KIF17 is required for ciliogenesis and IFT-B trafficking. Using two different target sequences (one is in exon 2 and the other is in exon 3; see Table 2), I established two independent *KIF17*-KO cell lines, #17-1-20 and #17-2-31, both of which have mono-allelic forward integration of the knock-in donor vector and a 1-bp insertion in the other allele (Fig. 1-12, A–C).

When cilia were immunostained with antibodies against Arl13b and Ac- α -tubulin after induction of ciliogenesis (Fig. 1-13, A–C), no significant difference was observed in the proportion of ciliated cells (Fig. 1-13D) or ciliary length (Fig. 1-13E) between control RPE1 cells and the two *KIF17*-KO cell lines. When immunostained for IFT88, the majority of both control RPE1 cells and the two *KIF17*-KO cell lines exhibited IFT88 staining at the ciliary base, and minor populations at both the base and tip (Fig. 1-14, A–C, and D). Taken into account previous reports showing that tubulins and outer dynein arm proteins are trafficked through binding to the IFT-B complex (Hou *et al.*, 2007; Ahmed *et al.*, 2008; Bhogaraju *et al.*, 2013; Kubo *et al.*, 2016), the normal ciliary assembly and normal IFT88 localization in *KIF17*-KO cells indicate that KIF17 is not essential for the IFT-B function.

Staining for Smo was rarely observed within cilia under basal conditions in both control RPE1 cells and the two *KIF17*-KO cell lines (–SAG), whereas the majority of control and KIF17-KO cells exhibited ciliary Smo staining after SAG treatment (+SAG) (Fig. 1-15). Leaf and von Zastrow recently reported that ciliary localization of dopamine receptor D1 (DRD1) was greatly reduced by exogenous expression of a KIF17

mutant lacking motor activity, although ciliary localization of another GPCR, SSTR3, was not affected by expression of the KIF17 mutant (Leaf and von Zastrow, 2015). I therefore examined the localization of DRD1-EGFP stably expressed in control RPE1 cells and the two *KIF17*-KO cell lines. As shown in Fig. 1-16A, DRD1-EGFP was uniformly distributed along cilia in control cells. The uniform ciliary distribution of DRD1-EGFP was not changed in the two *KIF17*-KO cell lines (Fig. 1-16, B and C). Although I do not know the exact reason for the apparent discrepancy between the result obtained using cells with exogenous expression of a motor-defective KIF17 mutant (Leaf and von Zastrow, 2015) and the presented result using *KIF17*-KO cell lines, expression of such a dominant-negative KIF17 mutant could indirectly affect the DRD1 trafficking.

Anyway, these results indicate that, at least in mammalian cells, KIF17 is dispensable for ciliogenesis and IFT-B trafficking. This is compatible with previous studies showing that *KIF17*-deficient mice and zebrafish are normal with respect to ciliary assembly (Yin *et al.*, 2011; Pooranachandran and Malicki, 2016).

Interaction of KIF17 NLS with importin α

A previous study indicated that KIF17 interacts with importin β 2 (TNPO1; because the nomenclature of importins is confusing as it varies among species (Miyamoto *et al.*, 2016), I have also included the official symbols of the corresponding importins) and mutation of its NLS (KRKK) to AAAA abolished the interaction (Dishinger *et al.*, 2010). I therefore attempted to confirm the potential interaction between KIF17 and TNPO1 by the VIP assay. However, I was unable to detect an interaction between EGFP-TNPO1 and mChe-KIF17 (Fig. 1-17A, second column). Furthermore, I did not detect an interaction between EGFP-KPNB1 (importin β 1) and mChe-KIF17 (third column). As the sequence KRKK is typical of a classical monopartite-type NLS, which is recognized by importin α family proteins (Miyamoto *et al.*, 2016), I then examined whether some importin α proteins can bind to KIF17, by the VIP assay. Data shown in Fig. 1-17A suggest that KIF17 interacts with KPNA1/importin α 1 (fourth column) and KPNA6/importin α 6 (eighth column); KPNA1 and KPNA6 belong to the same importin α 1 subfamily and show 82% amino acid identity in their primary structure (Miyamoto *et al.*, 2016). The VIP data were confirmed by immunoblotting; as shown in Fig. 1-17B, KIF17 interacted robustly with KPNA1 (lane 4) and weakly with KPNA6 (lane 8), but not with any other KPNA examined.

I then addressed whether KPNA1 and KPNA6 interact with the NLS (^{1,015}KRKK^{1,018}) of KIF17 by the VIP assay and conventional immunoblotting. As shown in Fig. 1-17, C and D, the interaction of KIF17 with KPNA1 or KPNA6 was abolished by the AAAA mutation. In contrast, mutation of the IFT-B binding sequence (R1000/1003A) did not affect the KIF17–KPNA interaction.

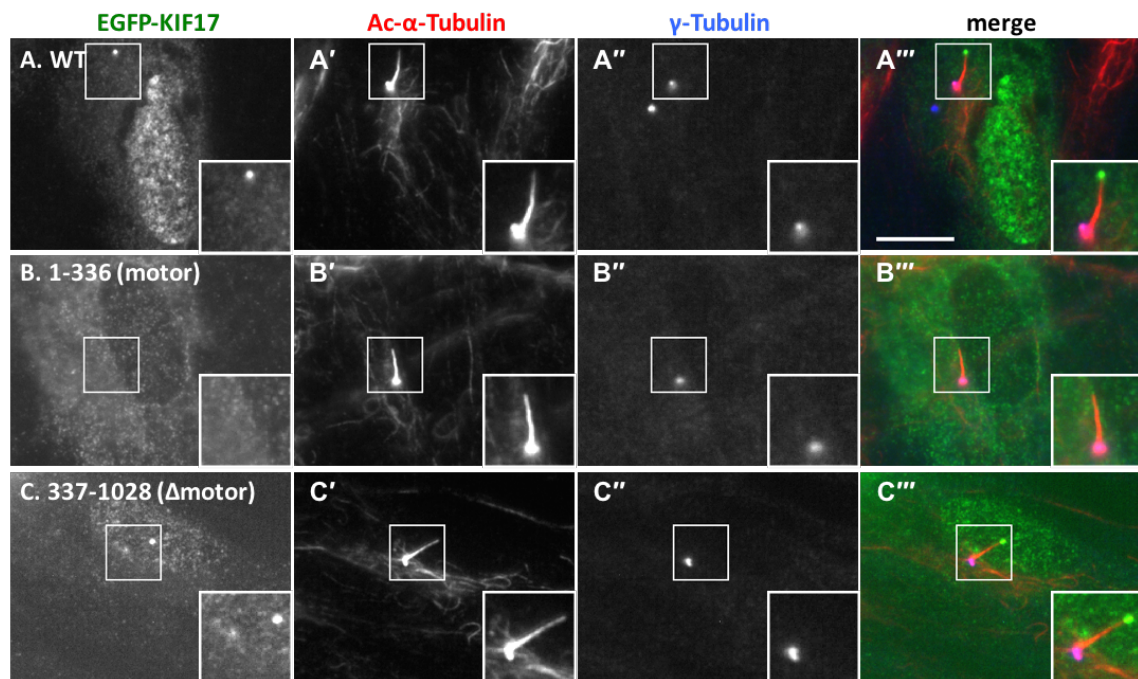


Fig. 1-1. Localization of motor-less KIF17 at the ciliary tip.

hTERT-RPE1 cells transfected with an expression vector for EGFP-KIF17(WT) (A), EGFP-KIF17(1-336) (B), or EGFP-KIF17(337-1,028) (C). The transfected cells were cultured for 24 h under normal conditions and for a further 24 h under serum-starvation conditions, as described in MATERIALS AND METHODS. The cells were immunostained for EGFP (A–C), Ac- α -tubulin (A'–C'), and γ -tubulin (A''–C''). Merged images are shown in A'''–C'''. Insets, enlarged images of the boxed regions. Scale bar, 10 μ m. The motor-less construct, KIF17(337-1,028), but not the motor-domain construct, KIF17(1-336), was found at the ciliary tip.

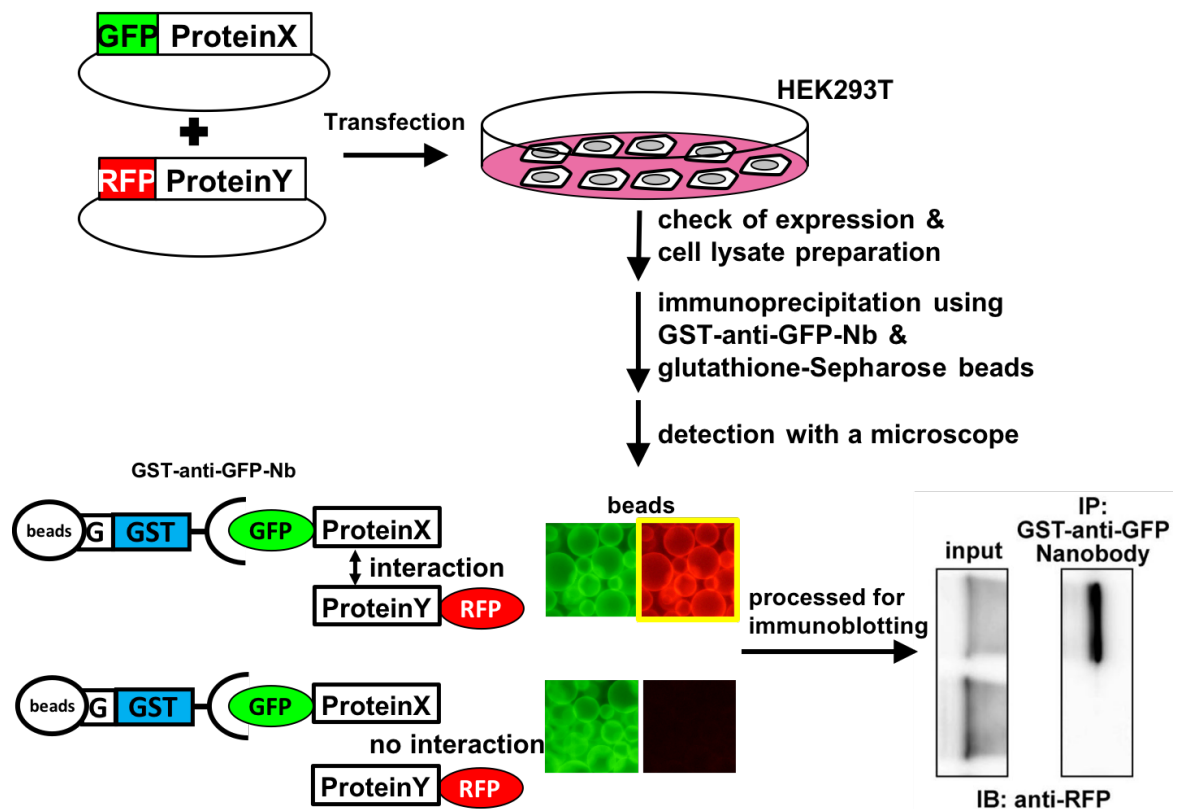


Fig. 1-2. Outline of the visible immunoprecipitation (VIP) assay.

HEK293T cells were transfected with expression vectors for proteins X fused to GFP and protein Y fused to RFP. After expression of the fluorescent fusion proteins was confirmed under a fluorescence microscope, cell lysates were prepared and processed for immunoprecipitation with GST-tagged anti-GFP nanobody (Nb) pre-bound to glutathione-Sepharose-4B beads. Beads bearing immunoprecipitates were directly observed with a fluorescent microscope. If protein X interacts with protein Y, both the green and red signals are detected on the surface or perimeter of the beads. If protein X does not interact with protein Y, only the green signal is detected. Proteins bound to the precipitated beads can be then processed for conventional immunoblotting.

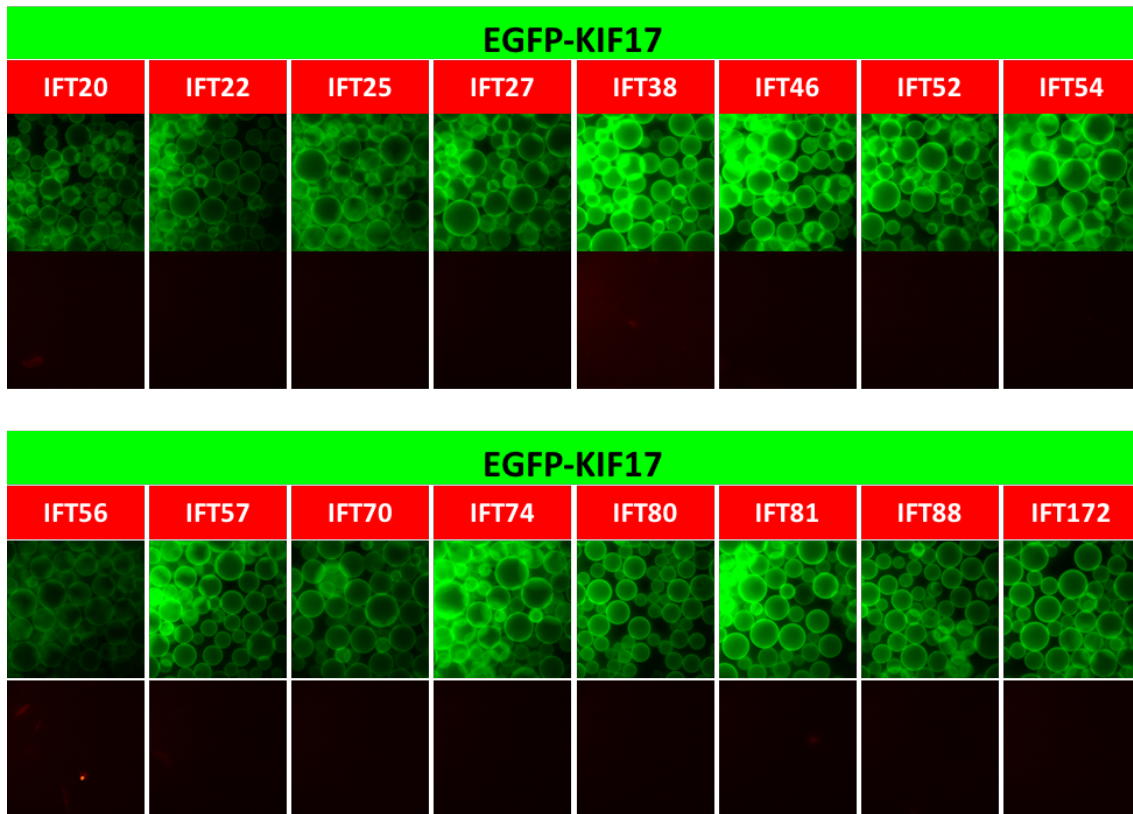


Fig. 1-3. None of the IFT-B subunits interacts with KIF17 by itself.

Lysates were prepared from HEK293T cells coexpressing EGFP-KIF17 and any of IFT-B subunit fused to mCherry/tRFP and processed for the VIP assay using GST-tagged anti-GFP-Nb.

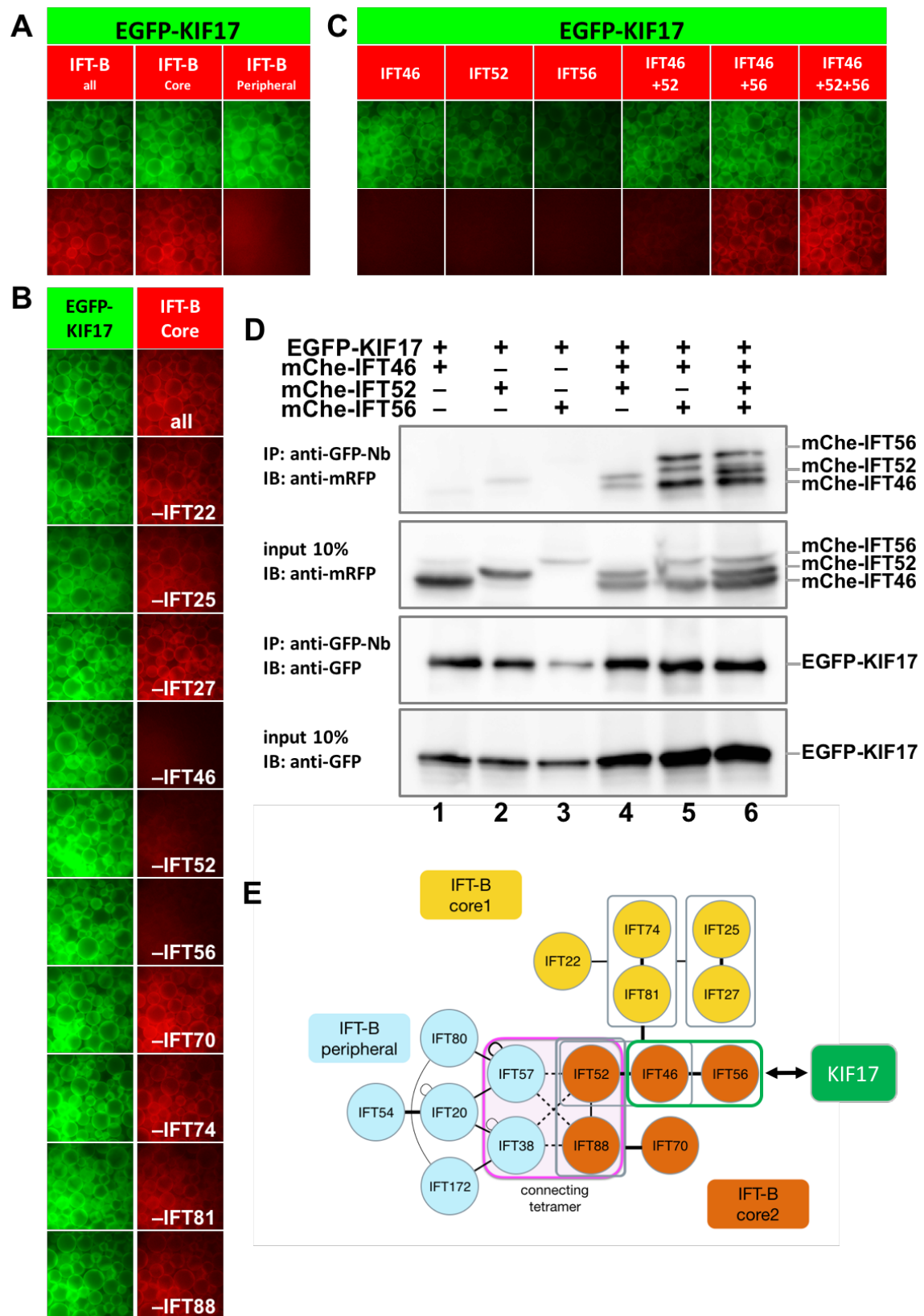


Fig. 1-4. Interaction of KIF17 with the IFT46–IFT56 dimer demonstrated by the VIP assay.

(A) Interaction of KIF17 with the IFT-B core subcomplex. Lysates were prepared from HEK293T cells coexpressing EGFP-KIF17 and all IFT-B subunits, all core subunits, or all peripheral subunits fused to mChe/tRFP, and processed for the VIP assay using GST-tagged anti-GFP Nb. (B) A subtractive VIP assay was performed to determine the IFT-B core subunits involved in its interaction with KIF17. Lysates prepared from HEK293T cells coexpressing EGFP-KIF17 and all but one (as indicated) of the core subunits fused to mChe/tRFP were processed for the VIP assay. (C, D) Confirmation of the involvement of the IFT46–IFT56 dimer in the KIF17–IFT-B interaction. Lysates prepared from HEK293T cells coexpressing EGFP-KIF17 and mChe-tagged IFT46 (lane 1), IFT52 (lane 2), IFT56 (lane 3), IFT46 + IFT52 (lane 4), IFT46 + IFT56 (lane 5), or IFT46 + IFT52 + IFT56 (lane 6) were processed for the VIP assay (C) or immunoblotting analysis (D). (E) Schematic representation of the interaction of KIF17 with the IFT46–IFT56 dimer in the IFT-B complex.

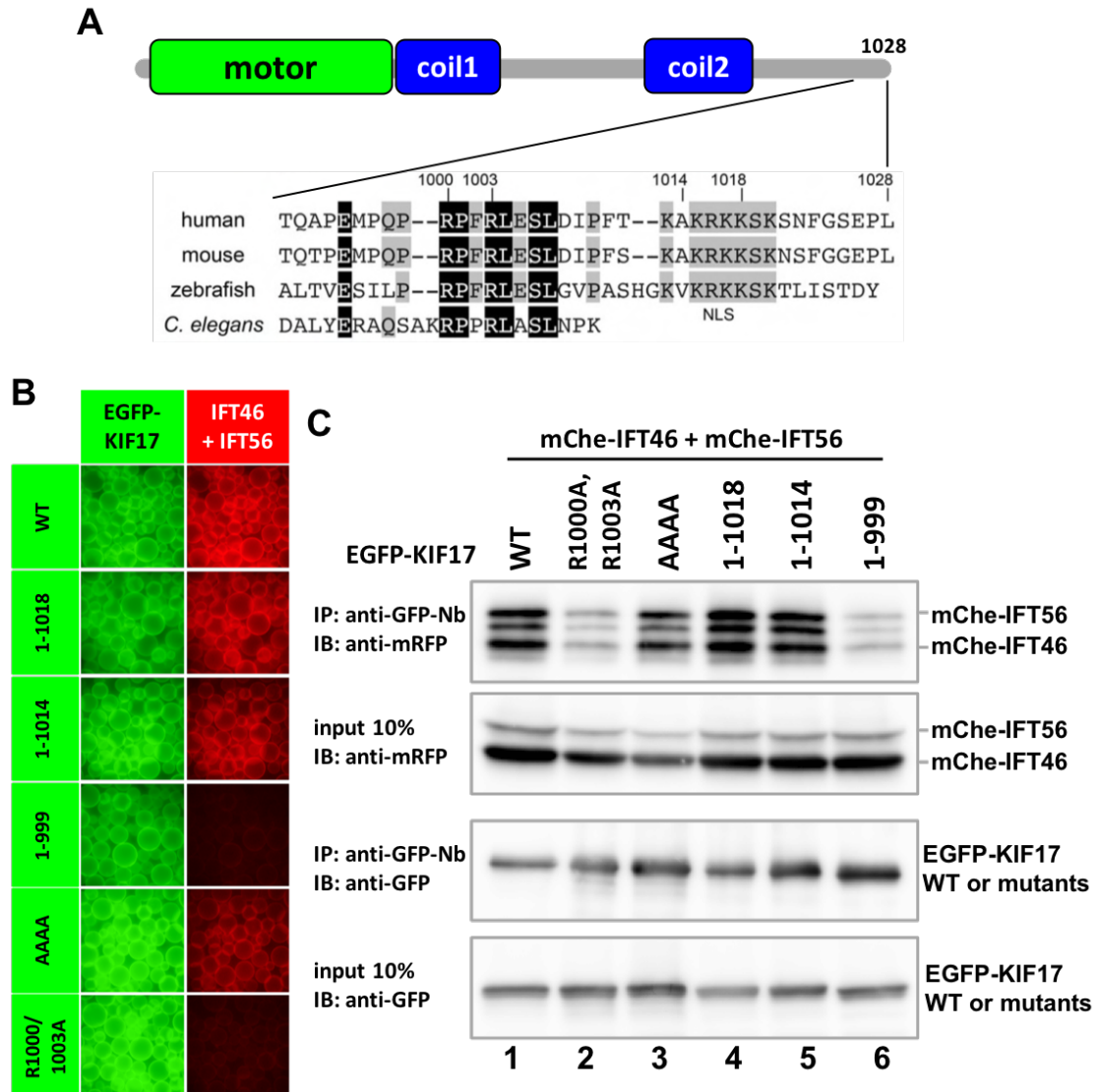


Fig 1-5. Interaction of KIF17 with the IFT46–IFT56 dimer through its conserved C-terminal sequence.

(A) Schematic representation of the overall structure and domain organization of KIF17 and alignment of the C-terminal sequences of human, mouse, and zebrafish KIF17, and *C. elegans* OSM-3. Coil1 and Coil2 are coiled-coil regions. Residues conserved in all and three members are shown in black and gray boxes, respectively. (B, C) Involvement of the conserved C-terminal sequence of KIF17 in its interaction with the IFT46–IFT56 dimer. Lysates prepared from HEK293T cells coexpressing mChe-IFT46 and mChe-IFT56 and EGFP-KIF17(WT), EGFP-KIF17(1-1018), EGFP-KIF17(1-1014), EGFP-KIF17(1-999), EGFP-KIF17(AAAA), or EGFP-KIF17(R1000/1003A) were subjected to the VIP assay (B) or immunoblotting analysis (C).

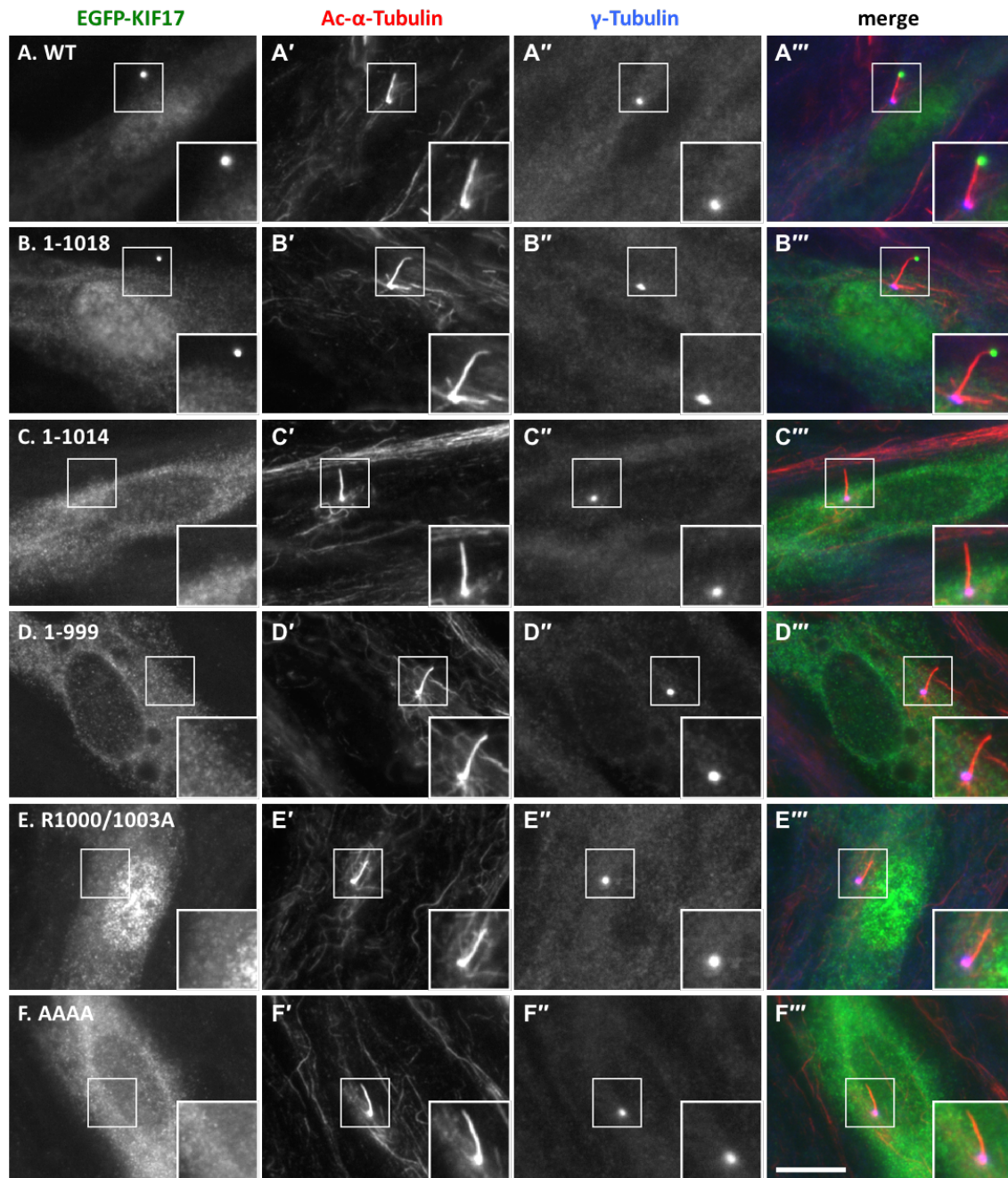
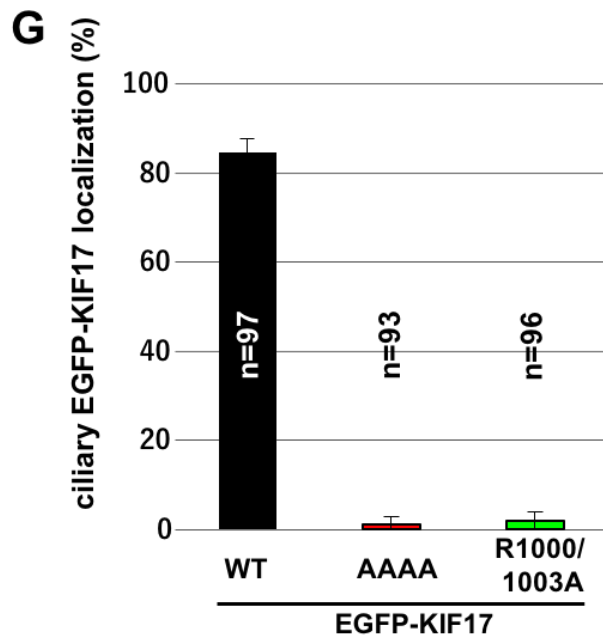


Fig. 1-6. Ciliary entry of KIF17 is dependent on both its binding to IFT-B and its NLS.

hTERT-RPE1 cells were transfected with an expression vector for (A) EGFP-KIF17(WT), (B) EGFP-KIF17(1-1018), (C) EGFP-KIF17(1-1014), (D) EGFP-KIF17(1-999), (E) EGFP-KIF17(R1000/1003A), or (F) EGFP-KIF17(AAAA) were processed as described in the legend for Fig.1-1. Insets, enlarged images of the boxed regions. Scale bar, 10 μ m.



(G) Cells positive for ciliary signals of EGFP-KIF17(WT), EGFP-KIF17(AAAA), or EGFP-KIF17(R1000/1003A) were counted; percentages of positive ciliated cells. Values are means $\pm$ SE (error bars) of three independent experiments. In each set of experiments, 30–34 ciliated cells were observed, and the total numbers of ciliated cells observed (n) are shown.

A

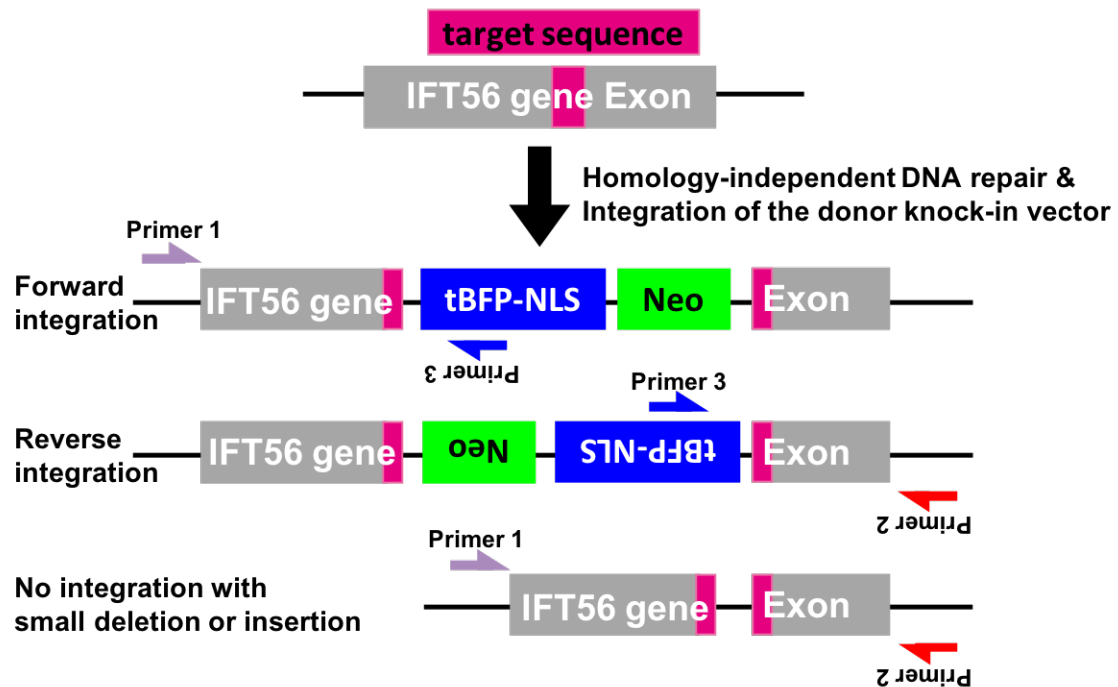
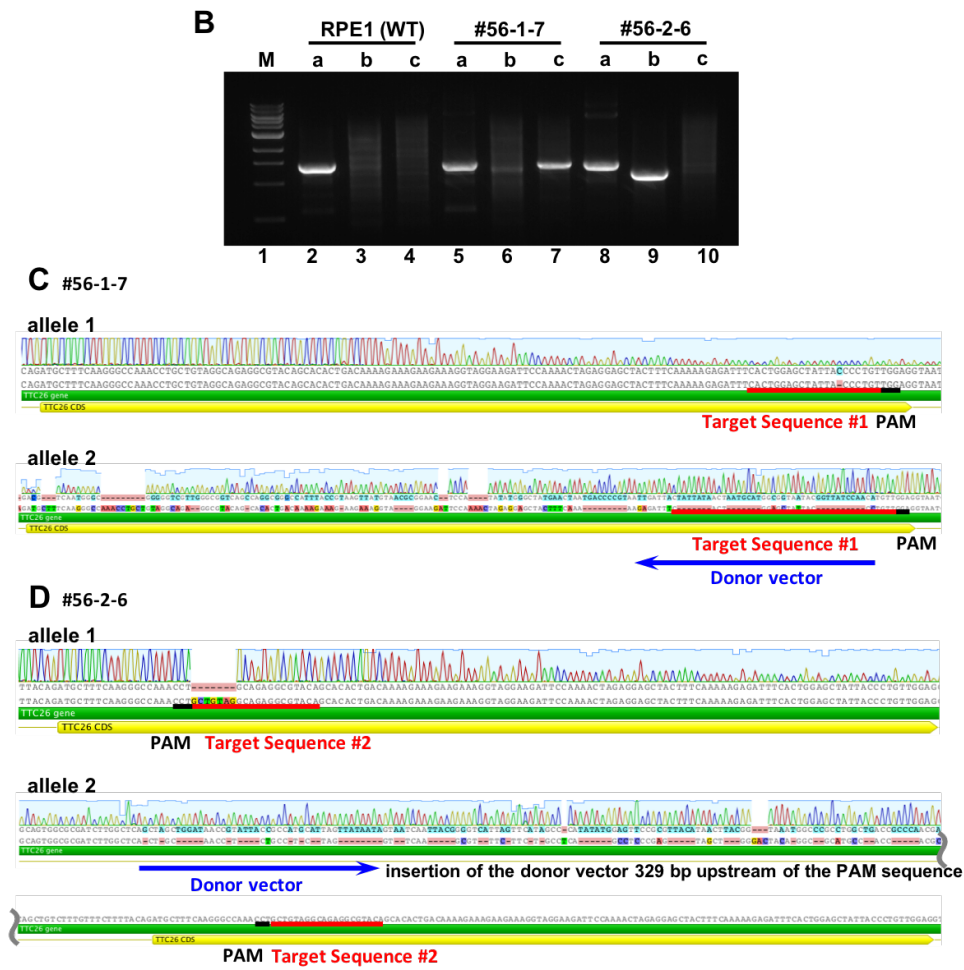


Fig. 1-7. Strategy for the establishment of KO cell lines and confirmation of knock-in donor vector integration or a small deletion/insertion in the genome of selected *IFT56*-KO cell lines.

(A) Schematic outline of the establishment of KO cell lines. Upon homology-independent DNA repair of the target sequence in the target gene, two types of integration of the donor knock-in vector containing *tBFP*-3×*NLS* (blue) and *Neo* (green) can occur; forward integration and reverse integration. In other cases, the target gene often undergoes error-prone repair with small insertions or deletions, causing a frameshift. Half-headed arrows indicate primers used for genomic PCR, with the same color representing identical primers. The target sequence is shown in magenta.



(B) Genomic DNAs extracted from control hTERT-RPE1 cells (lanes 2–4), and from cell lines (#56-1-7, lanes 5–7; and #56-2-6, lanes 8–10) established using a knock-in vector containing target sequences 1 and 2, respectively (see Table 2), both of which target the coding region within exon 2 of the human IFT56 gene, were subjected to PCR using the primer pair a (primers 1 + 2; lanes 2, 5, and 8), pair b (primers 1 + 3; lanes 3, 6, and 9), or pair c (primers 2 + 3; lanes 4, 7, and 10) (see Table 2) to detect alleles with a small deletion or insertion, with donor vector forward integration, and with reverse integration, respectively (see Table 2). (C and D) Alignments of allele sequences of the #56-1-7 (C) and #56-2-6 (D) cell lines determined by direct sequencing of the genomic PCR products. Red and black lines indicate the target sequence and PAM sequence, respectively, and blue arrows indicate the direction of donor vector integration. Note that in the #56-2-6 cell line, donor vector integration occurred in a region considerably upstream of the PAM sequence (329-bp upstream).

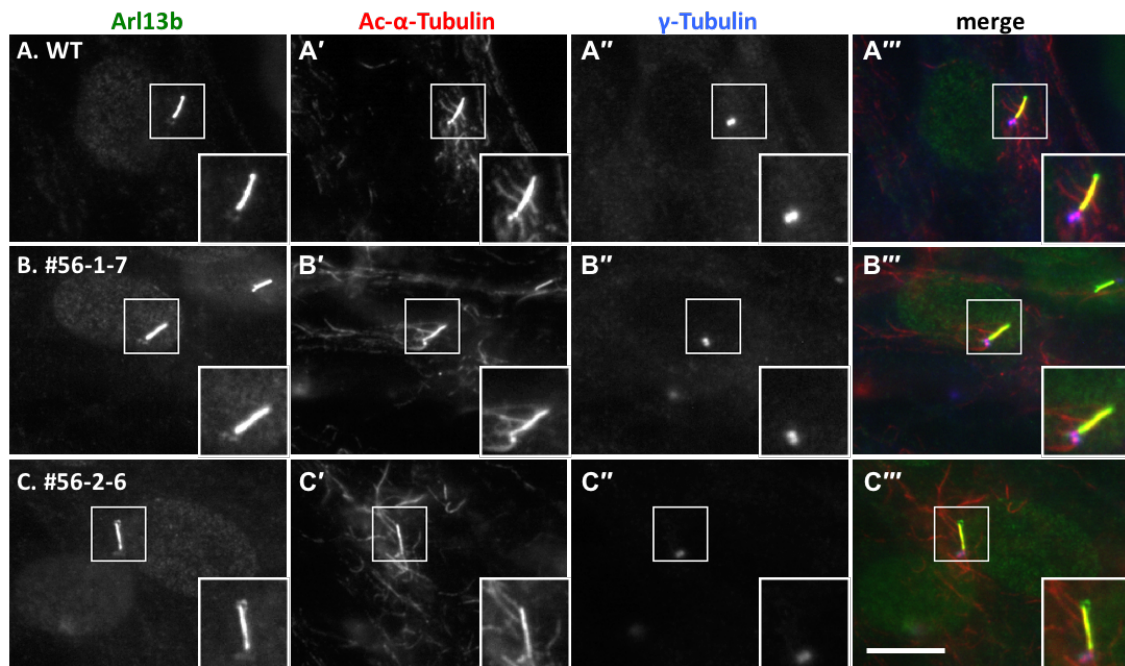


Fig. 1-8. Localization of Arl13b in *IFT56*-KO cells.

Control RPE1 cells (A) and *IFT56*-KO cell lines #56-1-7 (B) and #56-2-6 (C) were cultured under serum starvation conditions for 24 h to induce ciliogenesis and triple immunostained for Arl13b (A–C), Ac- α -tubulin (A'–C'), and γ -tubulin (A''–C''). Merged images are shown in A'''–C'''. Insets, enlarged images of the boxed regions. Scale bar, 10 μ m.

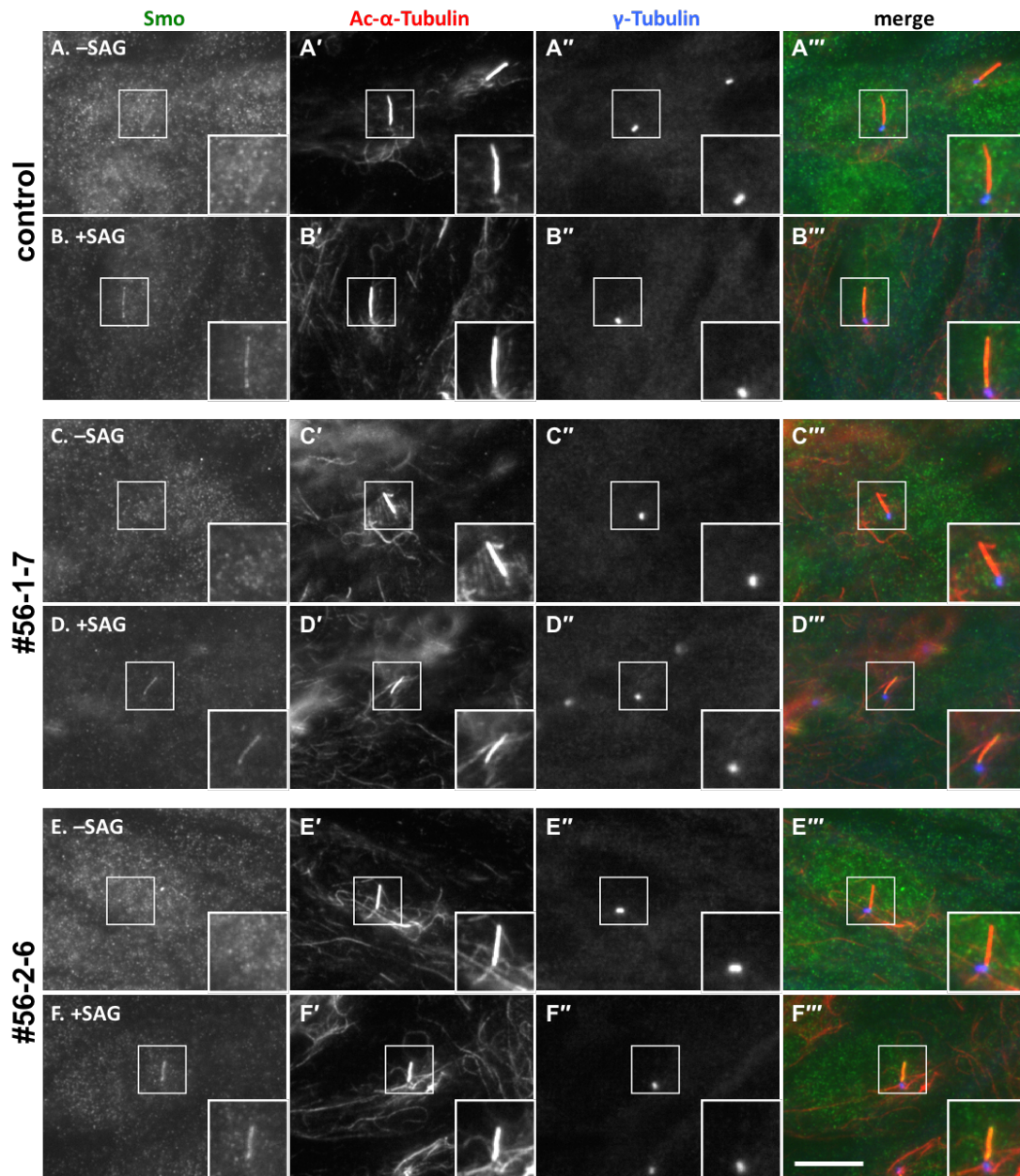


Fig. 1-9. Localization of Smo in *IFT56*-KO cells.

(A)–(F) Control hTERT-RPE1 cells (A and B), and the *IFT56*-KO cell lines #56-1-7 (C and D) and #56-2-6 (E and F) were cultured under serum starvation conditions for 24 h to induce ciliogenesis. The cells were cultured for a further 24 h in Opti-MEM containing 0.2% bovine serum albumin with (B, D, and F) or without (A, C, and E) 200 nM SAG. The cells were then triple immunostained for Smo (A–F), Ac- α -tubulin (A'–F'), and γ -tubulin (A''–F''). Merged images are shown in A'''–F'''. Enlarged images of the boxed regions are shown in the insets. Scale bar, 10 μ m.

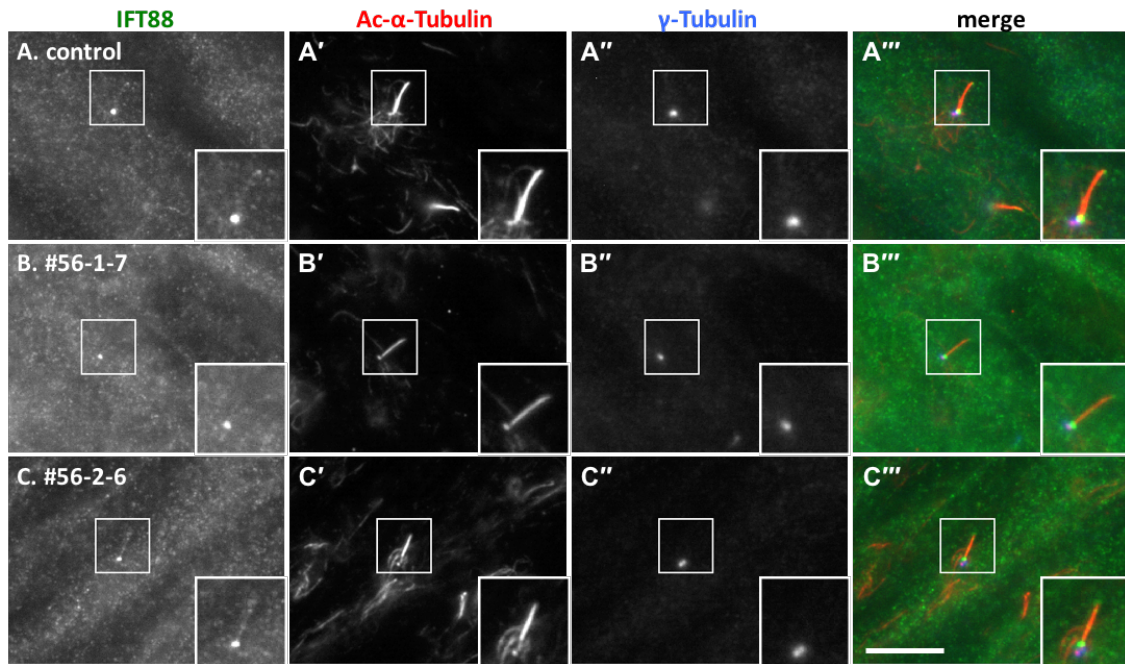


Fig. 1-10. Localization of IFT88 in *IFT56*-KO cells.

Control RPE1 cells (A) and *IFT56*-KO cell lines #56-1-7 (B) and #56-2-6 (C) were cultured under serum starvation conditions for 24 h to induce ciliogenesis and triple immunostained for IFT88 (A–C), Ac- α -tubulin (A'–C'), and γ -tubulin (A''–C''). Merged images are shown in A'''–C'''. Insets, enlarged images of the boxed regions. Scale bar, 10 μ m.

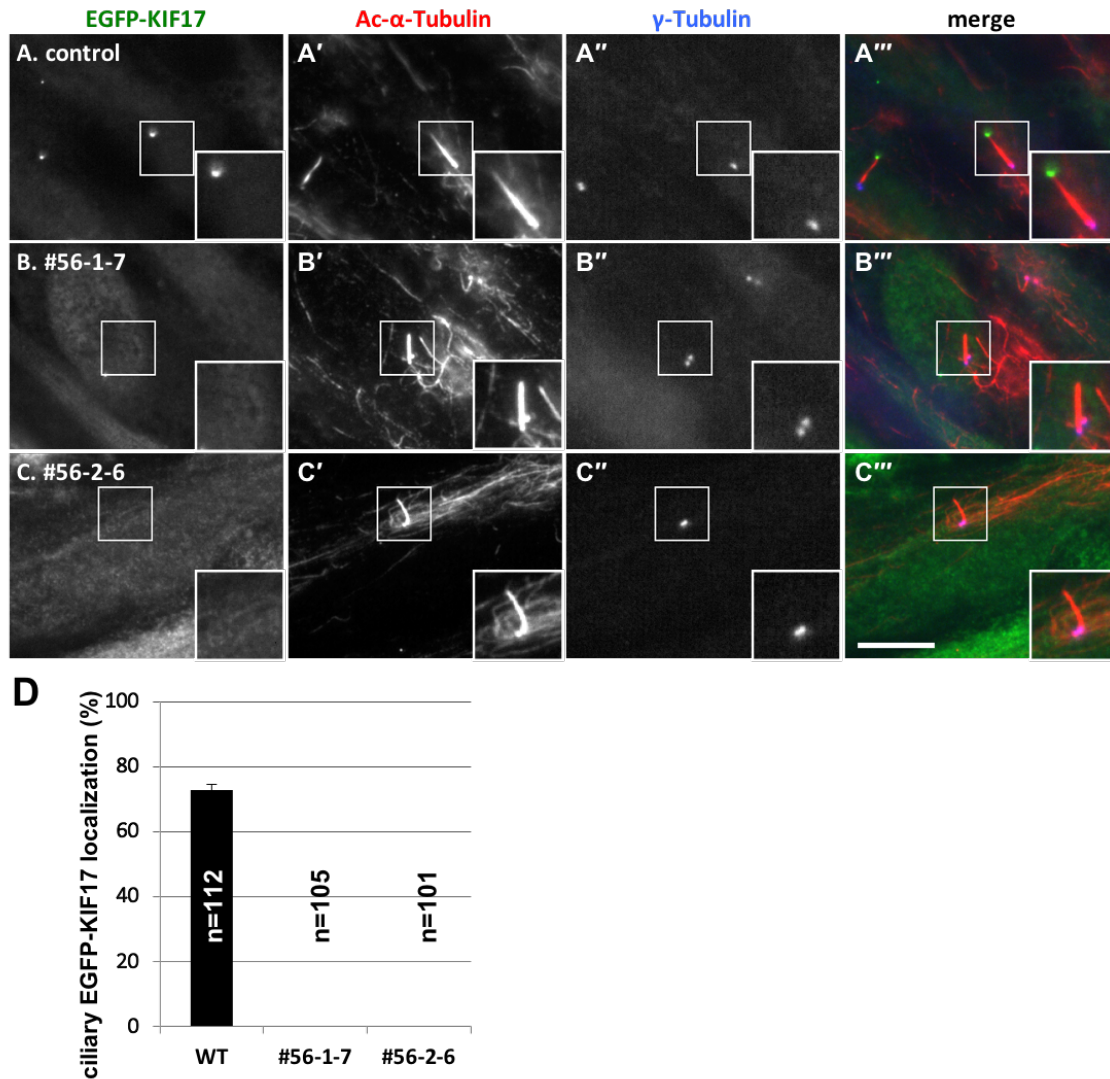


Fig. 1-11. Localization of KIF17 in *IFT56*-KO cells.

Control RPE1 cells (A) and the *IFT56*-KO cell lines #56-1-7 (B) and #56-2-6 (C), which stably express EGFP-KIF17 (A-C) and were established by infection of a lentiviral expression vector, were cultured under serum starvation conditions and immunostained for Ac- α -tubulin (A'-C') and γ -tubulin (A''-C''). Merged images are shown in A'''-C'''. Insets, enlarged images of the boxed regions. Scale bar, 10 μ m. (D) Control, #56-1-7, and #56-2-6 cells positive for ciliary EGFP-KIF17 signals were counted; percentages of positive ciliated cells. Values are means $\pm$ SE (error bars) of three independent experiments. In each set of experiments, 50-55 ciliated cells were observed, and the total numbers of ciliated cells observed (*n*) are shown. Note that no ciliary localization of EGFP-KIF17 was observed in the two *IFT56*-KO cell lines.

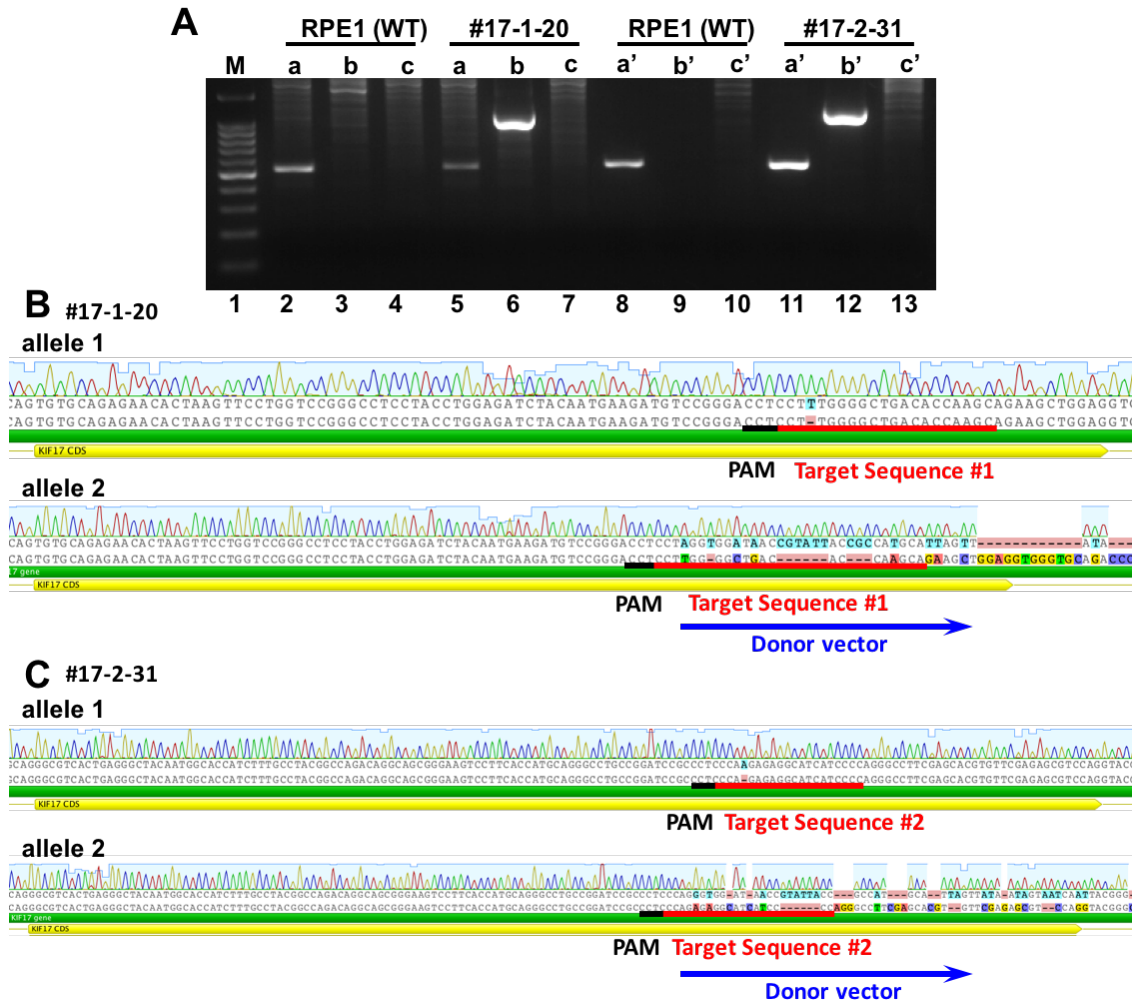


Fig. 1-12. Genomic PCR and sequence analyses of the *KIF17*-KO cell lines.

(A) Genomic DNA was extracted from control hTERT-RPE1 cells and from the *KIF17*-KO cell lines #17-1-20 and #17-2-31, which were established using a donor knock-in vector containing the target sequence and subjected to PCR using the primer sets to detect alleles with a small indel or no insertion (a or a'), or with forward (b or b') or reverse (c or c') integration of the donor knock-in vector. (B and C) Alignments of allele sequences of the #17-1-20 (B) and #17-2-31 (C) cell lines determined by direct sequencing of the genomic PCR products. Red and black lines indicate the target sequence and PAM sequence, respectively, and blue arrows indicate the direction of donor vector integration.

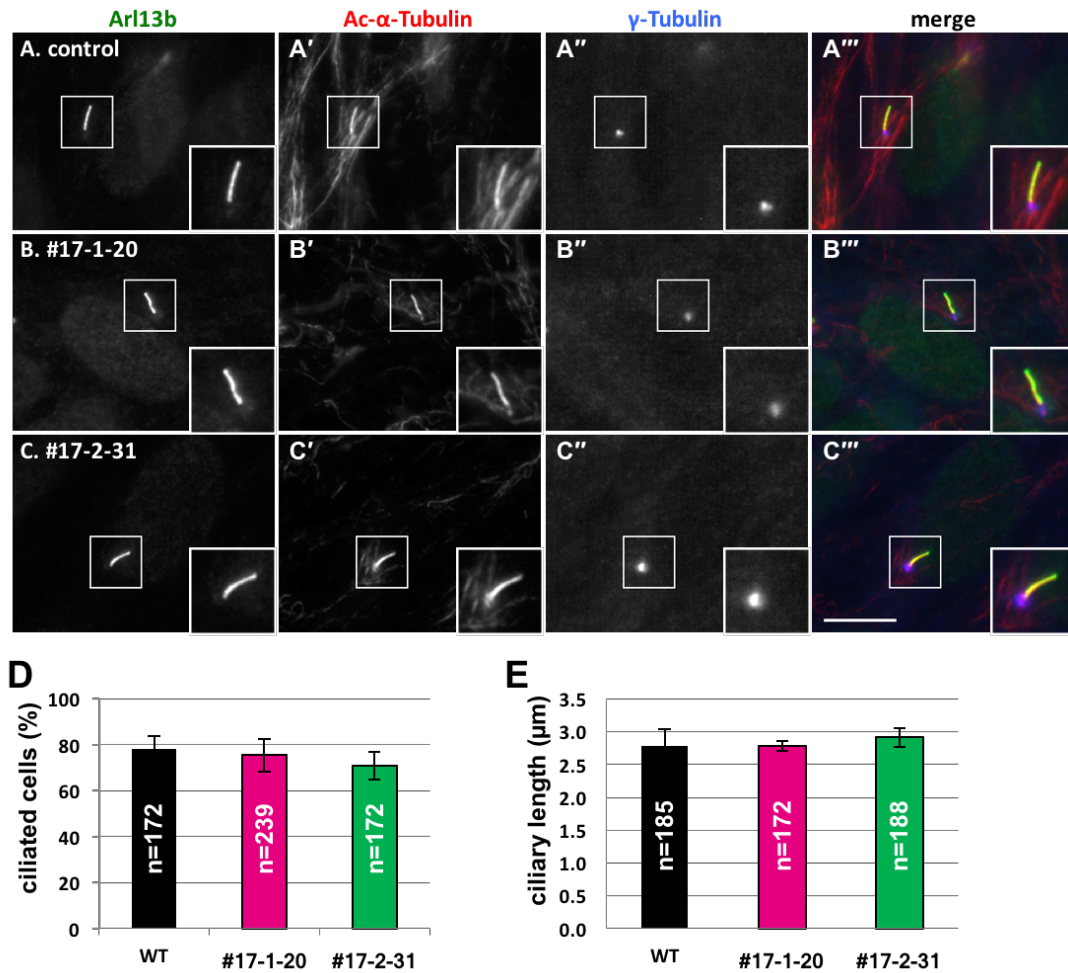


Fig. 1-13. Localization of Arl13b in *KIF17*-KO cells.

Control RPE1 cells (A) and *KIF17*-KO cell lines #17-1-20 (B) and #17-2-31 (C) were cultured under serum starvation conditions for 24 h to induce ciliogenesis and triple immunostained for Arl13b (A–C), Ac- α -tubulin (A'–C'), and γ -tubulin (A''–C''). Merged images are shown in A'''–C'''. Insets, enlarged images of the boxed regions. Scale bar, 10 μ m. (D) Ciliated control and *KIF17*-KO cells were counted; percentages of ciliated cells. Values are means $\pm$ SE (error bars) of three independent experiments. In each experiment, 55–81 cells were observed, and the total numbers of cells observed (n) are shown. (E) Ciliary lengths (lengths of Ac- α -tubulin staining) of individual control cells and *KIF17*-KO cells were measured. Values are means $\pm$ SE (error bars) of three independent experiments. In each set of experiments, 54–69 ciliated cells were observed, and the total numbers of ciliated cells observed (n) are shown.

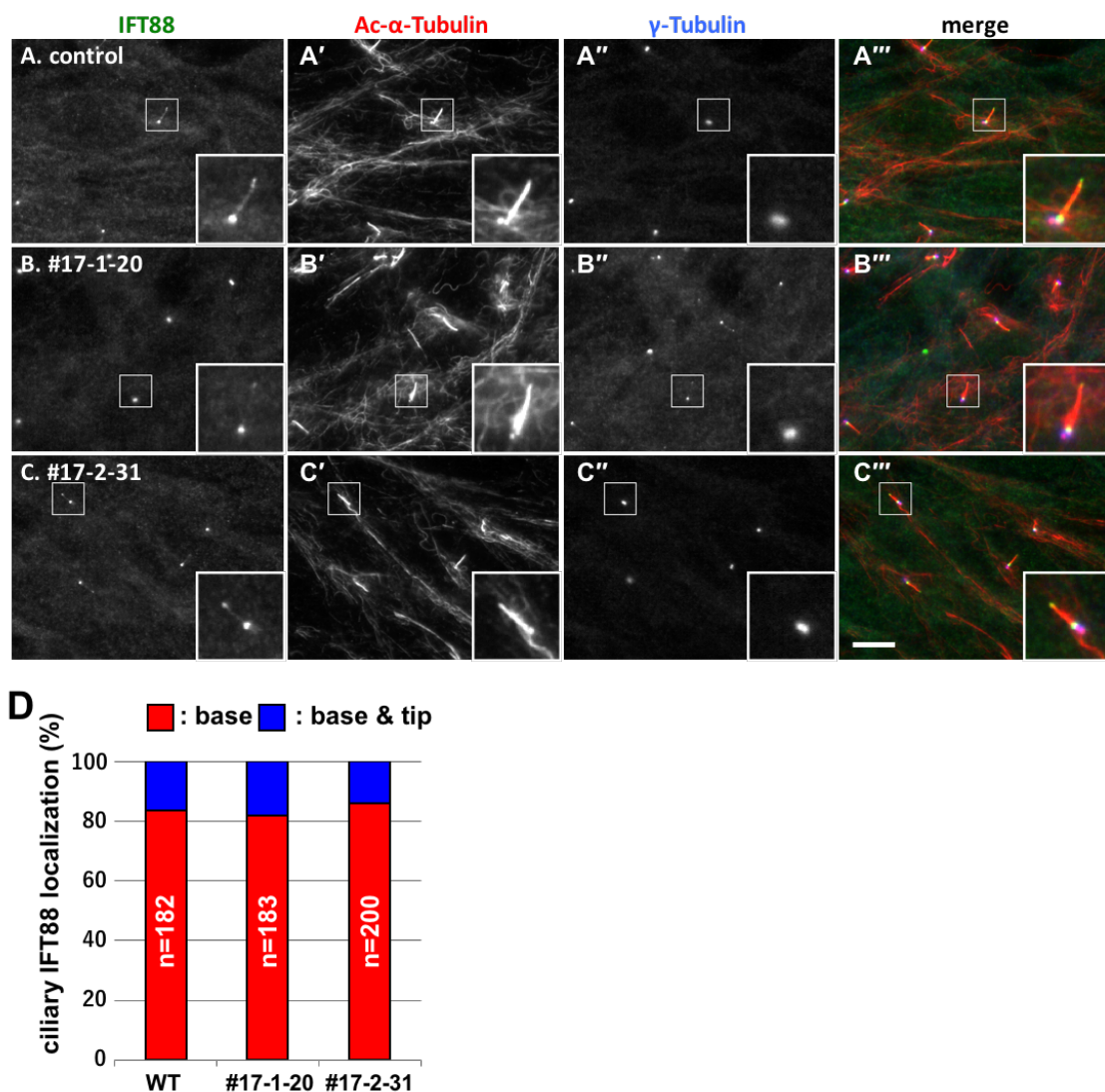


Fig. 1-14. Localization of IFT88 in *KIF17*-KO cells.

Control RPE1 cells (A) and *KIF17*-KO cell lines #17-1-20 (B) and #17-2-31 (C) were cultured under serum starvation conditions for 24 h to induce ciliogenesis and triple immunostained for IFT88 (A–C), Ac- α -tubulin (A'–C'), and γ -tubulin (A''–C''). Merged images are shown in A'''–C'''. Insets, enlarged images of the boxed regions. Scale bar, 10 μ m. (D) Localization of IFT88 in individual control and *KIF17*-KO cells was classified as “ciliary base,” “ciliary tip,” and “base and tip” and counted. The percentages of these populations are expressed as stacked bar graphs. In each set of experiments, 55–71 ciliated cells were observed, and the total numbers of ciliated cells observed (n) are shown. Note that cells with IFT88 signals at only the ciliary tip were not observed.

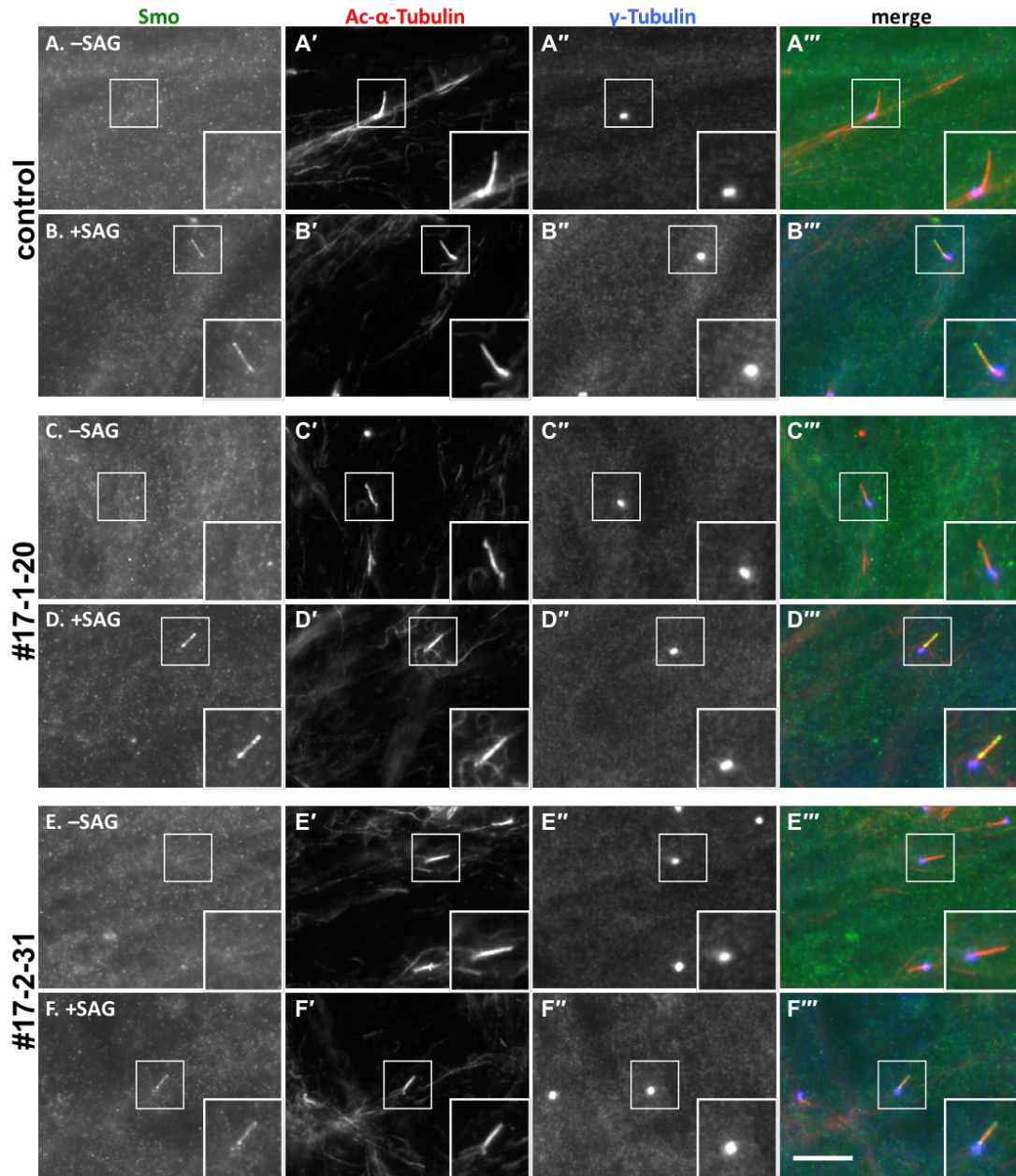


Fig. 1-15. Localization of Smo in *KIF17*-KO cells.

(A)–(F) Control hTERT-RPE1 cells (A and B), and the *KIF17*-KO cell lines #17-1-20 (C and D) and #17-2-31 (E and F) were cultured under serum starvation conditions for 24 h to induce ciliogenesis. The cells were cultured for a further 24 h in Opti-MEM containing 0.2% bovine serum albumin with (B, D, and F) or without (A, C, and E) 200 nM SAG. The cells were then triple immunostained for Smo (A–F), Ac- α -tubulin (A'–F'), and γ -tubulin (A''–F''). Merged images are shown in A'''–F'''. Enlarged images of the boxed regions are shown in the insets. Scale bar, 10 μ m.

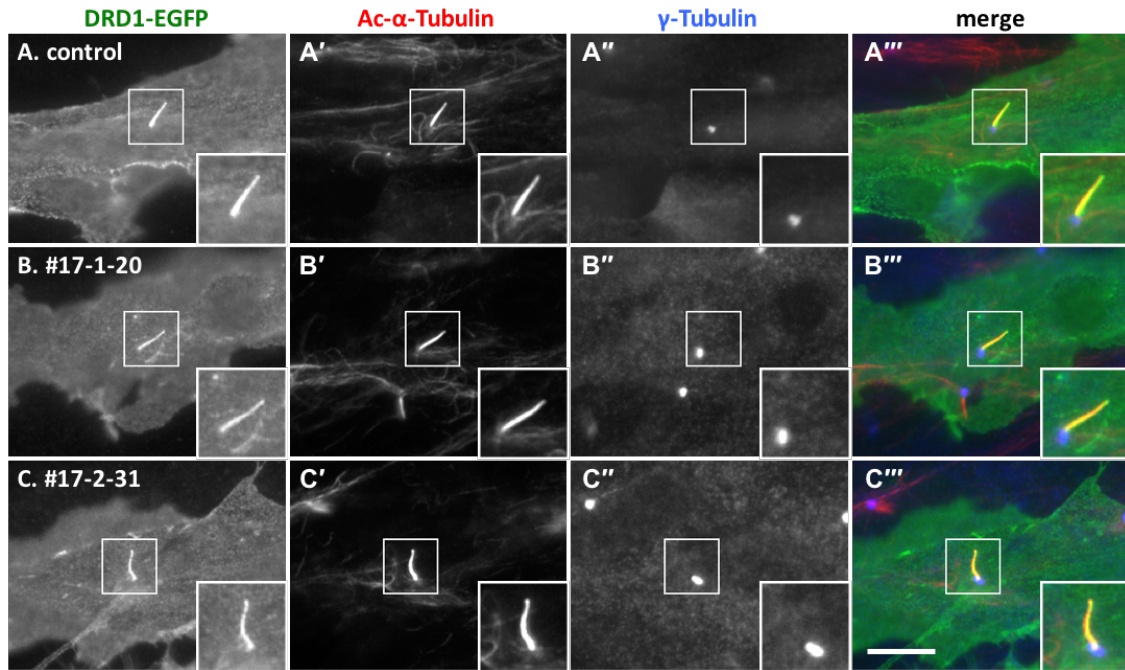


Fig. 1-16. Localization of DRD1 in *KIF17*-KO cells.

Control RPE1 cells (A) and *KIF17*-KO cell lines #17-1-20 (B) and #17-2-31 (C), which stably express DRD1-EGFP (A–C) and were established by infection of a lentiviral expression vector, were cultured under serum starvation conditions and immunostained for Ac- α -tubulin (A'–C') and γ -tubulin (A''–C''). Merged images are shown in A'''–C'''. Insets, enlarged images of the boxed regions. Scale bar, 10 μ m.

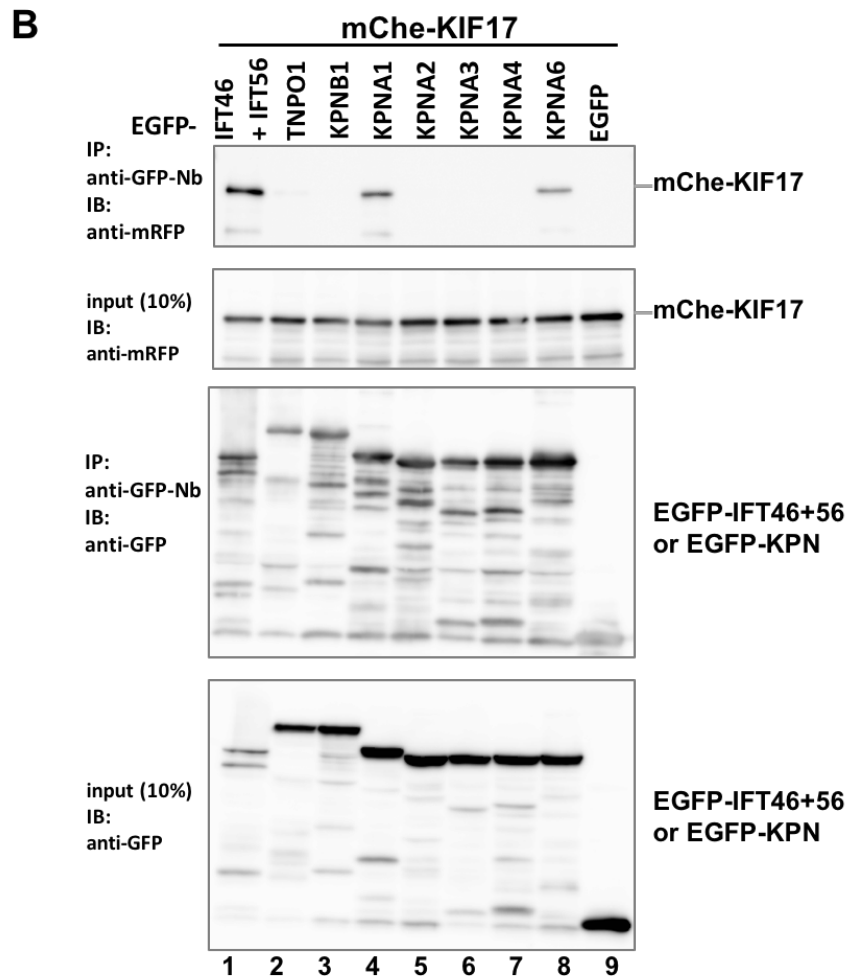
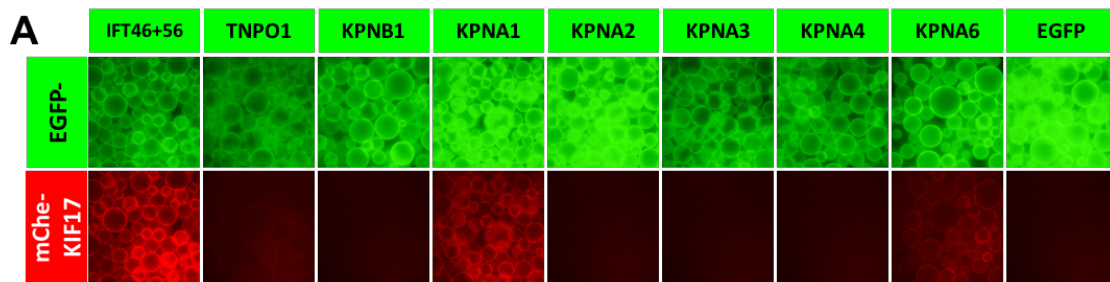
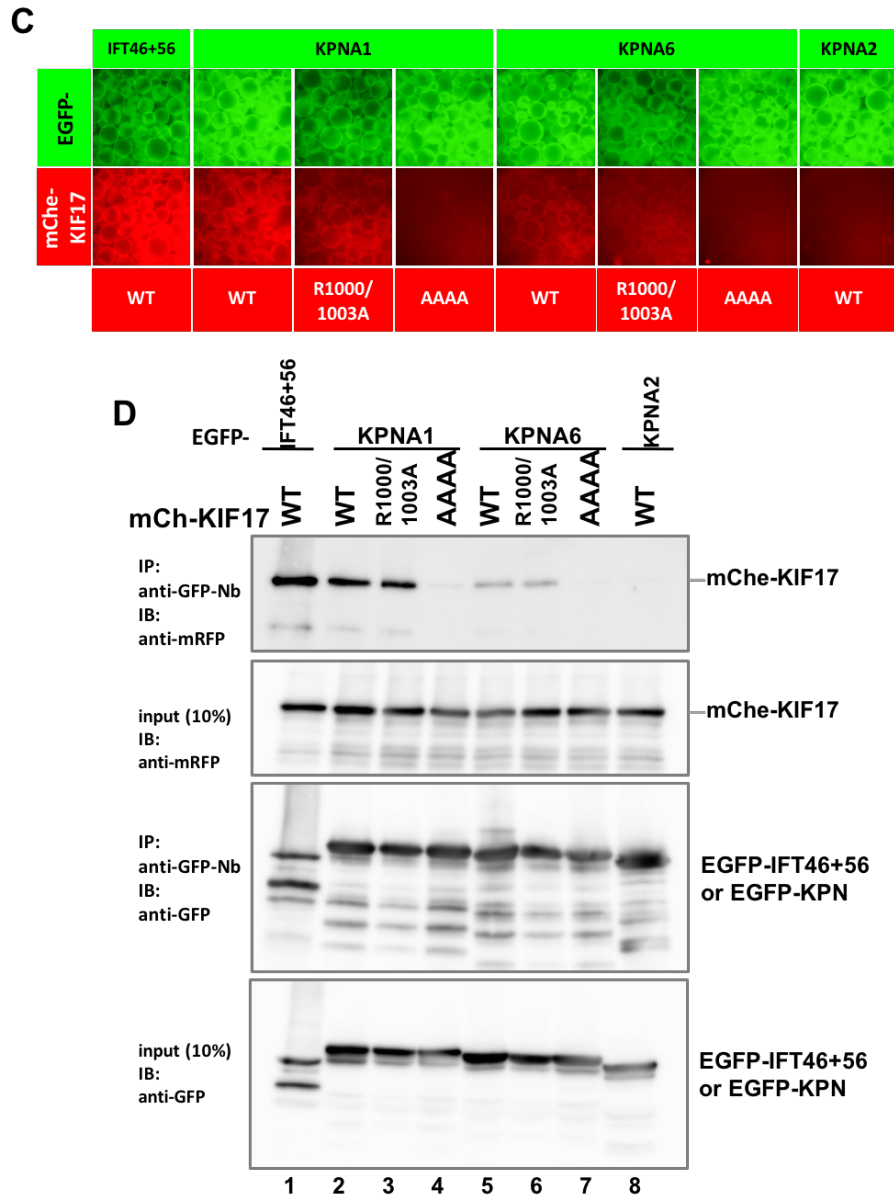


Fig.1-17. KIF17 NLS interacts with importin α proteins.

(A, B) KIF17 interacts with KPNA1 and KPNA6. Lysates prepared from HEK293T cells coexpressing mChe-KIF17 and EGFP-IFT46 + GFP-IFT56, EGFP-TNPO1, EGFP-KPNB1, EGFP-KPNA1, EGFP-KPNA2, EGFP-KPNA3, EGFP-KPNA4, EGFP-KPNA6, or EGFP were processed for the VIP assay (A) or immunoblotting analysis (B).



(C, D) Binding of KPNA1 and KPNA6 to the KIF17 NLS. Lysates prepared from HEK293T cells coexpressing mChe-KIF17(WT), mChe-KIF17(R1000/1003A), or mChe-KIF17(AAAA) and EGFP-IFT46 + EGFP-IFT56, EGFP-KPNA1, EGFP-KPNA6, or EGFP-KPNA2 as indicated were processed for the VIP assay (C) or immunoblotting analysis (D).

DISCUSSION

Prior to this study, it had been controversial whether KIF17 is a motor for or a cargo of the IFT-B complex in vertebrate cilia, since KIF17-deficient mice and zebrafish are normal with respect to ciliary assembly (Yin *et al.*, 2011; Pooranachandran and Malicki, 2016), and since motor-less KIF17 can reach the ciliary tips (Williams *et al.*, 2014; Jiang *et al.*, 2015; also see Fig. 1-1C). By taking advantage of the VIP assay, I first demonstrated the interaction of KIF17 with the IFT-B complex via the IFT46–IFT56 dimer, and identified a C-terminal IFT-B-binding sequence, which is conserved in vertebrate KIF17 and likely also in *C. elegans* OSM-3. Subsequent analyses unequivocally showed that KIF17 entry into the cilium depends on its binding to IFT-B, in addition to the NLS. Further experiments using *IFT56*-KO and *KIF17*-KO cells revealed that KIF17 cannot localize within cilia in *IFT56*-KO cells, whereas ciliary assembly, and localization of the IFT-B complex and ciliary GPCRs examined were unaffected by the KIF17 deficiency. The most plausible explanation for these results is that KIF17 enters the cilium, most likely across the TZ, by binding to the IFT-B complex via the IFT46–IFT56 dimer and is then transported along the axoneme toward the ciliary tip by continuous binding to IFT-B.

How can one explain the apparent discrepancy of the roles of vertebrate KIF17 and *C. elegans* OSM-3? The axoneme of the sensory cilia of *C. elegans* has a bipartite structure with the proximal segment composed of nine microtubule doublets and with the distal segment composed of nine singlets (Scholey, 2013; Mijalkovic *et al.*, 2016); the proximal segment corresponds to the typical axoneme of vertebrate cilia, whereas the distal segment structure is not generally found in vertebrate cilia. Recently, by a set of experiments, Peterman and colleagues provided data demonstrating the model of the concerted actions of heterotrimeric kinesin-II and OSM-3 in *C. elegans* cilia, which was proposed by Scholey and colleagues (Scholey, 2013); heterotrimeric kinesin-II transports IFT particles along the proximal segment toward a ‘handover zone’, where OSM-3 gradually replaces kinesin-II and drives the transport of IFT particles along the distal segment toward the ciliary tip (Prevo *et al.*, 2015; Mijalkovic *et al.*, 2016). Thus, one can speculate that the transport powered by heterotrimeric kinesin-II is a common event in both vertebrate and *C. elegans* cilia, whereas OSM-3-mediated transport along the distal segment is specific for *C. elegans* cilia. If this is the case, OSM-3 needs to be trafficked

toward the handover zone as a cargo of IFT particles along the proximal segment of *C. elegans* cilia. From another perspective, a future issue that should be addressed is the role of KIF17 at the ciliary tip of vertebrate cells.

How does KIF17 gain entry into cilia in an IFT-B–dependent manner? At the ciliary base, there is a permeability barrier with properties resembling those of the NPC (Takao and Verhey, 2016; Verhey and Yang, 2016); similar to the NPC, the permeability barrier of the TZ contains nucleoporin proteins (Kee *et al.*, 2012). A study by Verhey and colleagues showed that the crosslinking of a nucleoporin blocked the ciliary entry of various soluble proteins, including KIF17 and IFT88, whereas it did not affect the entry of membrane proteins (Takao *et al.*, 2014). On the other hand, Hu and colleagues showed that FBF1, a protein located at the transition fiber and the distal appendage of the basal body, interacts directly with IFT54 and is required for transit of assembled IFT particles across the barrier (Wei *et al.*, 2013). Furthermore, a recent super-resolution microscopic study of Liao and colleagues suggested the assembly of IFT particles around the TZ prior to entry into the cilium (Yang *et al.*, 2015). Overall, it is most likely that ciliary entry of KIF17 requires its binding to assembled IFT particles as well as to an NPC-like machinery around the TZ.

The study in this chapter showed that the NLS of KIF17 is essential for its entry into both the nucleus and cilium, although OSM-3 lacks an NLS in the corresponding region. I showed binding of the KIF17 NLS to KPNA1 (importin α 1) and KPNA6 (importin α 6), although I was unable to confirm the previously proposed binding to TNPO1 (importin β 2); a recent study suggested that binding of KIF17 to TNPO1 is enhanced by regulatory proteins, such as Rab23 (Lim and Tang, 2015), but our attempts to show the involvement of Rab23 in KIF17–TNPO1 binding have been unsuccessful so far. However, our results are compatible with the fact that the KIF17 NLS is typical of classical monopartite-type NLSs, which are recognized by importin α proteins (Miyamoto *et al.*, 2015) but does not fit PY-NLSs, which are recognized by transportin/importin β 2 proteins (Twyffels *et al.*, 2014). In any case, further studies are required towards understanding the roles of specific importins in the ciliary entry of proteins.

Chapter2: Heterotrimeric kinesin-II is essential for ciliogenesis serving as an anterograde motor for the IFT-B complex

ABSTRACT

In the previous chapter, I demonstrated that KIF17 interacts with the IFT-B complex through the IFT46–IFT56 dimer and is trafficked toward the ciliary tip along the axoneme as a cargo of the IFT-B complex, rather than serving as an anterograde motor for IFT-B. I therefore predicted that heterotrimeric kinesin-II serves as an anterograde motor for the IFT-B complex, although there has been no direct evidence showing that heterotrimeric kinesin-II interacts with the IFT-B complex.

In this chapter, using the VIP assay, I identified a three-to-four protein interaction involving the kinesin-II trimer KIF3A/KIF3B/KAP3 and the IFT-B-connecting tetramer IFT38/IFT52/IFT57/IFT88; among the kinesin-II subunits, KIF3B contributed mainly to IFT-B binding. Furthermore, I showed that the ciliogenesis defect of *KIF3B*-KO cells can be rescued by the exogenous expression of wild-type KIF3B, but not by that of its mutant compromised with respect to IFT-B binding. Thus, interaction between heterotrimeric kinesin-II and the IFT-B-connecting tetramer is crucial for ciliogenesis, via the powering of IFT particles to move in the anterograde direction.

INTRODUCTION

In the previous chapter, I revealed that KIF17 interacts with the IFT-B complex through the IFT46–IFT56 dimer. Moreover, I showed that ciliogenesis and the trafficking of most ciliary proteins appear normal in *KIF17*-KO cells, and that KIF17 is a cargo of, rather than a motor for, the IFT-B complex; namely, it is imported into cilia by binding to the IFT-B complex through the IFT46–IFT56 dimer.

In addition to KIF17, heterotrimeric kinesin-II motor composed of KIF3A, KIF3B, KAP3 has been implicated in ciliary anterograde protein trafficking (Scholey, 2013). Given that KIF17 is a cargo of the IFT-B complex, I predicted that heterotrimeric kinesin-II is the motor for the IFT-B complex. Knockout of the *Kif3a* or *Kif3b* gene in mice leads to a ‘no cilia’ phenotype (Nonaka *et al.*, 1998; Marszalek *et al.*, 1999), indicating that heterotrimeric kinesin-II is crucial for the protein trafficking essential for the ciliogenesis. However, the molecular basis for the interaction between the IFT-B complex and heterotrimeric kinesin-II has been poorly understood to date, although *Chlamydomonas* kinesin-II was reported to coimmunoprecipitate with components of the IFT-B complex (Liang *et al.*, 2014).

In this chapter, I therefore aimed to determine how heterotrimeric kinesin-II regulates IFT-B trafficking. Using the VIP assay, I here identified a composite interaction involving the kinesin-II trimer KIF3A/KIF3B/KAP3 and the IFT-B-connecting tetramer IFT38/IFT52/IFT57/IFT88; among the kinesin-II subunits, KIF3B contributed mainly to IFT-B binding among the kinesin-II subunit.

I further established *KIF3B*-KO cells using the CRISPR/Cas9 system and found that *KIF3B*-KO cells exhibited severe defect in ciliogenesis indicating that heterotrimeric kinesin-II is indispensable for the trafficking of proteins essential for ciliogenesis. Moreover, the ciliogenesis defect observed in *KIF3B*-KO cells can be rescued by the exogenous expression of KIF3B(WT) but not its C-terminally truncated mutant, which retains the ability to form heterotrimer with KIF3A and KAP3, but is compromised with respect to IFT-B binding.

RESULTS

KIF3B is an essential component of the kinesin-II trimer

I first aimed to clarify how the subunits of heterotrimeric kinesin-II are bound to each other using the VIP assay, which we recently developed as a simple and versatile assay for protein–protein interactions, in which not only binary but also one-to-many and many-to-many protein interactions can be visually detected without the need of electrophoresis and immunoblotting (Katoh *et al.*, 2015; Katoh *et al.*, 2016).

Lysates prepared from cells coexpressing EGFP-fused KIF3A, KIF3B, or KAP3 and its mCherry (mChe)-fused constructs in all possible combinations, and processed them for immunoprecipitation with GST-tagged anti-GFP Nb prebound to glutathione-Sepharose beads. As depicted by red signals on the precipitated beads under a conventional fluorescence microscope, KIF3A interacted with all proteins (KIF3A, KIF3B, and KAP3) (Fig. 2-1B). On the other hand, KIF3B interacted with KIF3A and KAP3, but not with itself. These results demonstrate that in addition to the heterotrimer composed of KIF3A, KIF3B, and KAP3 (Fig. 2-1C, right), the kinesin-II trimer can also comprise two KIF3A molecules and one KAP3 molecule, without the need of KIF3B (left).

To address whether KIF3B is an essential component of the kinesin-II trimer at the cellular level, I established hTERT-RPE1 cell lines defective in KIF3B, although a previous study showed that *Kif3b*-KO mice are embryonic lethal (Nonaka *et al.*, 1998). Among the several independent *KIF3B*-KO cell lines established using a CRISPR/Cas9 system with our original modifications (Katoh *et al.*, 2017), I used two KO cell lines in the following experiments: the #3B-2-1 cell line has a one-nucleotide deletion in one KIF3B allele and a forward integration of the donor knock-in vector in the other allele (Fig. 2-2A, lanes 5–7, and B); and #3B-2-4 has an eight-nucleotide deletion in one allele and a forward integration of the donor vector in the other allele (Fig. 2-2A, lanes 8–10, and C).

As expected from the phenotype of KO mice (Nonaka *et al.*, 1998), no cilia were observed in these *KIF3B*-KO cell lines (Fig. 2-3, A–F; compare B', C', E', and F', with A' and D'). Thus, KIF3A is unable to functionally compensate for the absence of KIF3B, at least with respect to ciliary protein trafficking. The phenotype of *KIF3B*-KO

cells was in striking contrast to that of *KIF17*-KO cells, in which ciliogenesis did not appear to be impaired (see Fig. 1-13D).

When the *KIF3B*-KO cells were immunostained for IFT88 and IFT140, which are subunits of the IFT-B and IFT-A complexes, respectively, both IFT88 (Fig. 2-3, B and C) and IFT140 (E and F) were localized on one of two γ -tubulin-positive centrioles; note that, as described previously (Hirano *et al.*, 2017; Takahara *et al.*, 2018), the available anti-IFT140 antibody also stained undetermined nuclear structures in RPE1 cells (see the manufacturer's website: <http://www.ptglab.com/Products/IFT140-Antibody-17460-1-AP.htm>). These observations suggest that both the IFT-A and IFT-B complexes, and thereby the IFT particles, can be assembled around the basal body in the absence of cilia due to the lack of functional kinesin-II. The observations are also in line with a recent report indicating that in the case of *Chlamydomonas* flagella, IFT proteins are recruited to the basal body prior to recruitment of motor proteins (Wingfield *et al.*, 2017).

Heterotrimeric kinesin-II interacts with the IFT-B complex via the IFT-B-connecting tetramer

I then addressed whether heterotrimeric kinesin-II can interact with the IFT-B complex, as there is virtually no direct data regarding their mode of interaction, although *Chlamydomonas* kinesin-II was reported to coimmunoprecipitate with components of the IFT-B complex (Liang *et al.*, 2014). Furthermore, a yeast two-hybrid study performed more than 10 years previously suggested an interaction between KIF3B and IFT20 (Baker *et al.*, 2003), but my preliminary VIP-based analysis did not detect this interaction. I therefore took advantage of the VIP assay for many-to-many protein interactions (Katoh *et al.*, 2016) to reveal the interaction mode between the kinesin-II heterotrimer and the IFT-B complex. When lysates of HEK293T cells coexpressing all kinesin-II subunits fused to EGFP and all IFT-B subunits (16 subunits) fused to mCh or tRFP were immunoprecipitated with GST-anti-GFP Nb, weak but substantial red signals were observed on the precipitated beads (Fig. 2-4A, leftmost row), indicating that kinesin-II interacts with IFT-B. When the IFT-B subunits were grouped into those of the core subcomplex, peripheral subcomplex, and the connecting tetramer (IFT38/IFT52/IFT57/IFT88) that constitutes the interface between the core and peripheral subcomplexes (see Fig. 2-4F) (Katoh *et al.*, 2016), and expressed as mCh/tRFP fusions, relatively strong red signals were detected when subunits of the

connecting tetramer were coexpressed with EGFP-fused kinesin-II subunits (Fig. 2-4A, rightmost row). I then used the subtractive VIP assay to determine which subunits in the tetramer are important for its interaction with kinesin-II. As shown in Fig. 2-4B, omitting any one subunit of the tetramer reduced the red signals, indicating that all the tetramer components make substantial contributions to the kinesin-II interaction. The VIP results were confirmed by conventional immunoblotting analysis (Fig. 2-4C). The band intensities were moderately reduced by omitting any one of the tetrameric subunits (top and middle panels, compare lanes 2–5 with lane 1), most prominently by omitting IFT88 (lane 5); note that the bands for tRFP-IFT38 and tRFP-IFT57 overlapped with each other, as described previously (Kato *et al.*, 2016).

I then aimed to identify which subunits of the kinesin-II trimer are important for its interaction with the IFT-B-connecting tetramer. As shown in Fig. 2-4D, although the interaction was strongest when all the kinesin-II subunits were coexpressed, a substantial interaction was detected when KIF3B was expressed on its own or in combination with KIF3A or KAP3. Immunoblotting analysis confirmed the VIP results (Fig. 2-4E); intense bands for the IFT-B tetrameric subunits were detected when all kinesin-II subunits were present (top and middle panels, lane 1), and relatively weak bands were detected when KIF3B was included (lanes 2, 4, and 6). Thus, a maximal interaction between the kinesin-II and IFT-B complexes requires the three-to-four protein interaction involving all the kinesin-II subunits and all the connecting IFT-B subunits (Fig. 2-4F); however, among the kinesin-II trimer, KIF3B appears to make a major contribution to the IFT-B binding. This is also consistent with the fact that KIF3A cannot compensate for the absence of KIF3B.

I also analyzed whether kinesin-II can interact with the IFT-A complex, as it was proposed that in the sensory cilia of *C. elegans*, the IFT-A complex moves along the axonemal middle segment together with kinesin-II (Ou *et al.*, 2005), although there was no direct evidence for the IFT-A–kinesin-II interaction. However, I could not detect an interaction of the kinesin-II trimer with all, core, or peripheral subunits of the IFT-A complex (Fig. 2-4G).

Regions of the KIF3B protein required for its interactions with KIF3A, KAP3, and IFT-B can be dissected

In an attempt to discriminate between the region of KIF3B responsible for the formation of the kinesin-II heterotrimer and that required for the kinesin-II–IFT-B interaction, I constructed various deletion and truncation mutants of KIF3B.

When the coiled-coil (CC) region was deleted (Δ CC) (see Fig. 2-5A), KIF3B lost its ability to interact with both KIF3A and KAP3 (Fig. 2-5, B and C, and Fig. 2-6 A and B, lane 2), confirming that the CC region is essential for formation of the kinesin-II trimer. When truncated from the C-terminus, KIF3B(1–704) and KIF3B(1–662) retained the ability to interact with both KIF3A and KAP3 (lanes 3 and 4). On the other hand, KIF3B(1–625) lost the ability to interact with KAP3, but not KIF3A (lane 5), whereas KIF3B(1–579), which lacks the entire globular domain (see Fig. 2-5A), lost the ability to interact with both KIF3A and KAP3 (lane 6). Thus, the interaction of KIF3B with both KIF3A and KAP3 requires its CC region and at least the N-terminal part of the globular domain, although overlapping but distinct regions of KIF3B are required for its interaction with KIF3A and KAP3. These results are compatible with pioneering studies of Hirokawa and colleagues based on single particle electron microscopic analysis of recombinant KIF3A–KIF3B and KIF3A–KIF3B–KAP3, suggesting that the KIF3A–KIF3B heterodimer appears to have two heads, a middle rod, and a globular tail, and KAP3 appears to wrap around the latter (Yamazaki *et al.*, 1995; Yamazaki *et al.*, 1996).

I then analyzed whether any of the KIF3B truncation constructs together with KIF3A and KAP3 can interact with the IFT-B-connecting tetramer (Fig. 2-7). KIF3B(1–704) (lane 2) interacted with the IFT-B tetramer, comparably to KIF3B(WT) (lane 1). In striking contrast, KIF3B(1–579), which cannot interact with KIF3A or KAP3, no longer interacted with the IFT-B tetramer (lane 5). In the case of KIF3B(1–662), which retained its ability to interact with both KIF3A and KAP3, and KIF3B(1–625), which retained its ability to interact with only KIF3A, the interaction with the IFT-B tetramer was moderately attenuated (lanes 3 and 4); in particular, there was a prominent reduction in the amount of coimmunoprecipitated IFT38/IFT57. Thus, even though KIF3B(1–662) appears to retain the ability to form the kinesin-II heterotrimer, it is partially compromised with respect to its interaction with the IFT-B complex.

Phenotype of KIF3B-KO cells exogenously expressing wild-type or mutant KIF3B

I then analyzed whether the exogenous expression of KIF3B(WT) or its truncation mutants characterized above (Fig. 2-5 to 2-7) can rescue the no-cilia phenotype

of the *KIF3B*-KO cell lines. As shown in Fig. 2-8A, the exogenous expression of EGFP-fused KIF3B(WT) in the KIF3B-KO cell line #3B-2-1 restored ciliogenesis, although the localization of EGFP-KIF3B(WT) around the ciliary base was unclear due to the relatively high background signal throughout the cytoplasm (Fig. 2-8A'). Similarly, EGFP-KIF3B(1-704), which retains the ability to form the kinesin-II heterotrimer and interact with the IFT-B complex, also rescued the ciliogenesis defect (Fig. 2-8B). In striking contrast, not only EGFP-fused KIF3B(1-579), which completely lost the ability to interact with IFT-B, but also KIF3B(1-662) and KIF3B(1-625), which demonstrated a partially compromised IFT-B binding, failed to rescue the ciliogenesis defect of the *KIF3B*-KO cell line (Fig. 2-8, C-E). Essentially the same results were obtained using the other KO cell line, #3B-2-4 (Fig. 2-8G). Taken together, these results indicate that the binding of kinesin-II to the IFT-B complex via the connecting tetramer is crucial for ciliogenesis.

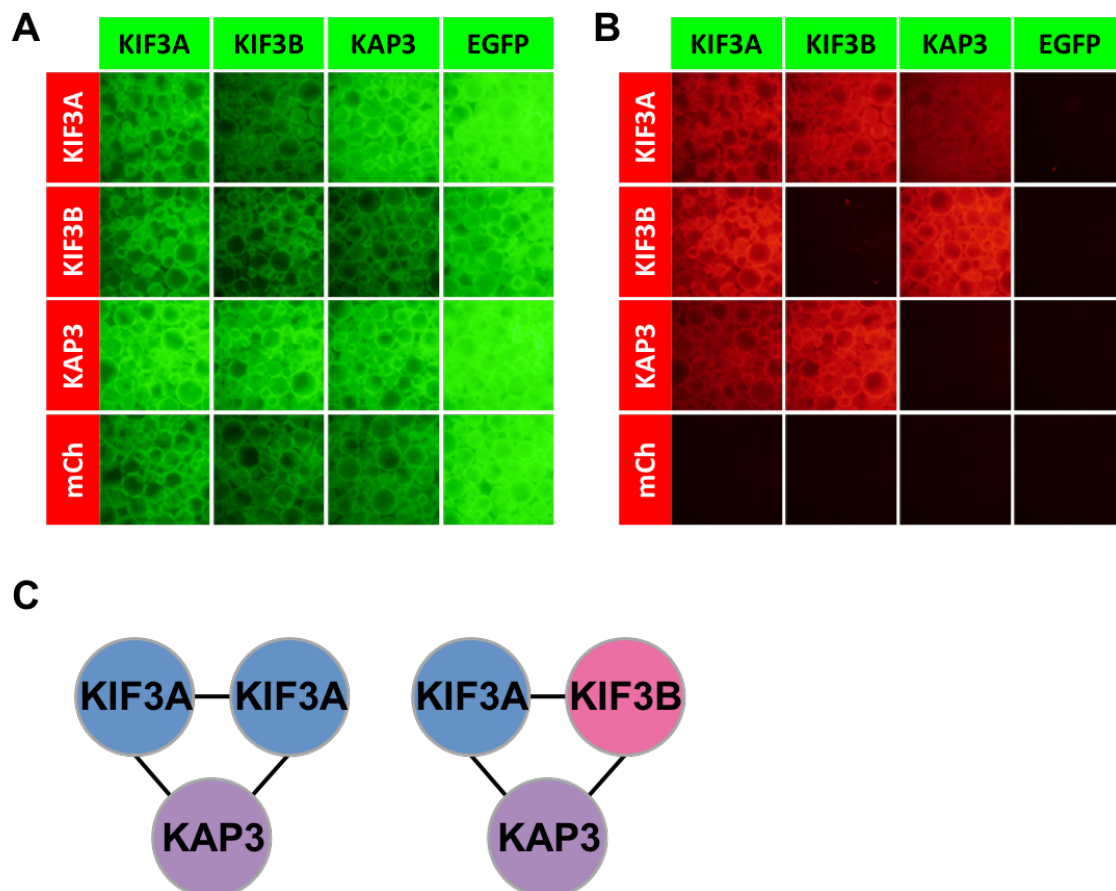


Fig. 2-1. Architecture of kinesin-II trimer demonstrated by all-by-all VIP assay.

(A, B) Lysates prepared from HEK293T cells coexpressing the indicated combination of EGFP-fused and mCh-fused kinesin-II subunits were processed for the VIP assay using GST–anti-GFP Nb prebound to glutathione–Sepharose beads. EGFP (A) and mCh (B) signals on the precipitated beads were observed under a microscope. (C) Kinesin-II models predicted from the binary interaction data.

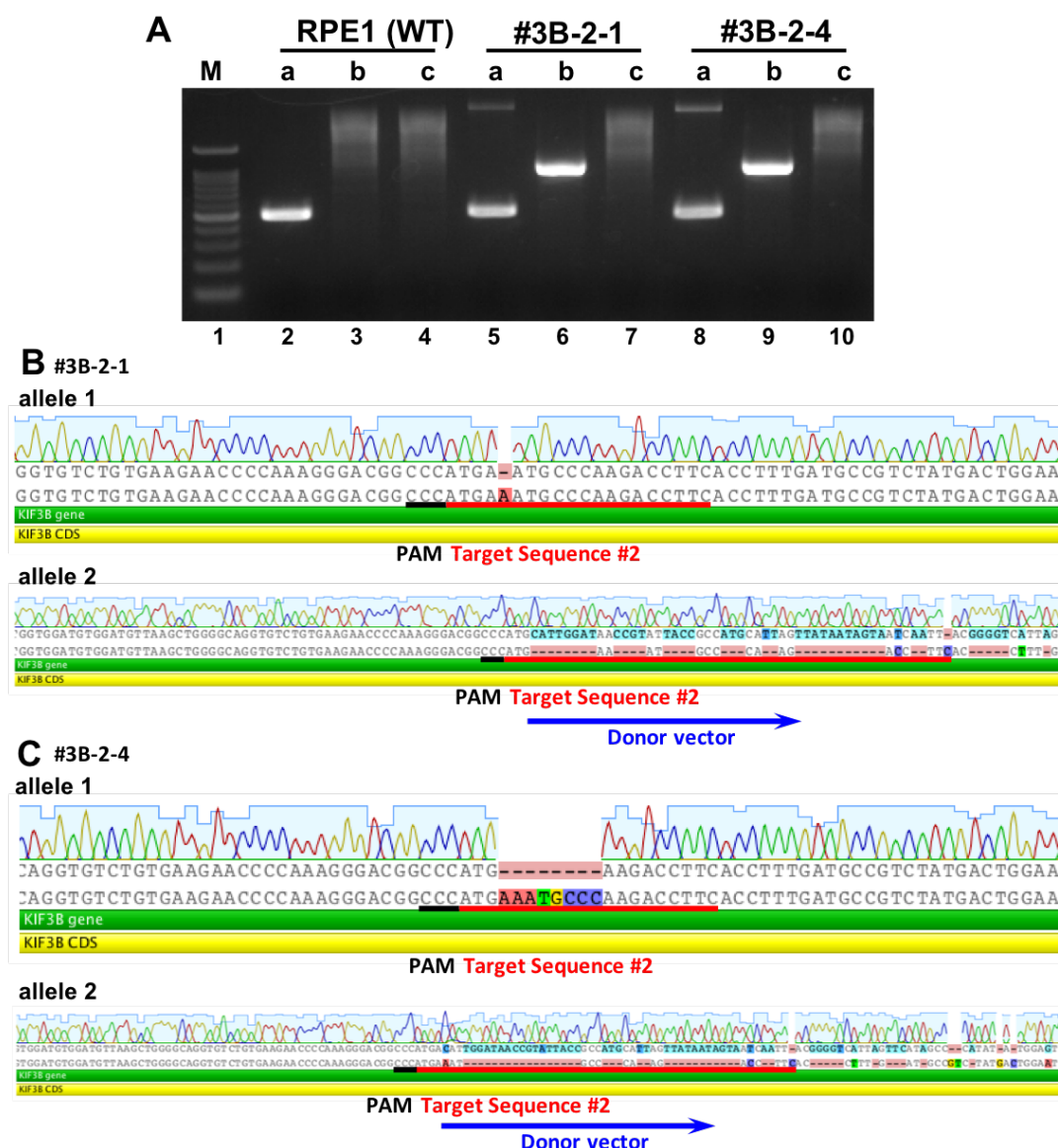


Fig. 2-2. Genomic PCR and sequence analyses of the *KIF3B*-KO cell lines.

(A) Genomic DNA was extracted from control hTERT-RPE1 cells and from the *KIF3B*-KO cell lines #3B-2-1 and #3B-2-4, which were established using a donor knock-in vector containing the target sequence, and subjected to PCR using the indicated primer sets (see Table 2) to detect alleles with a small indel or no insertion (a), or with forward (b) or reverse (c) integration of the donor knock-in vector. (B and C) Alignments of allele sequences of the #3B-2-1 (B) and #3B-2-4 (C) cell lines determined by direct sequencing of the genomic PCR products. Red and black lines indicate the target sequence and PAM sequence, respectively, and blue arrows indicate the direction of donor vector integration.

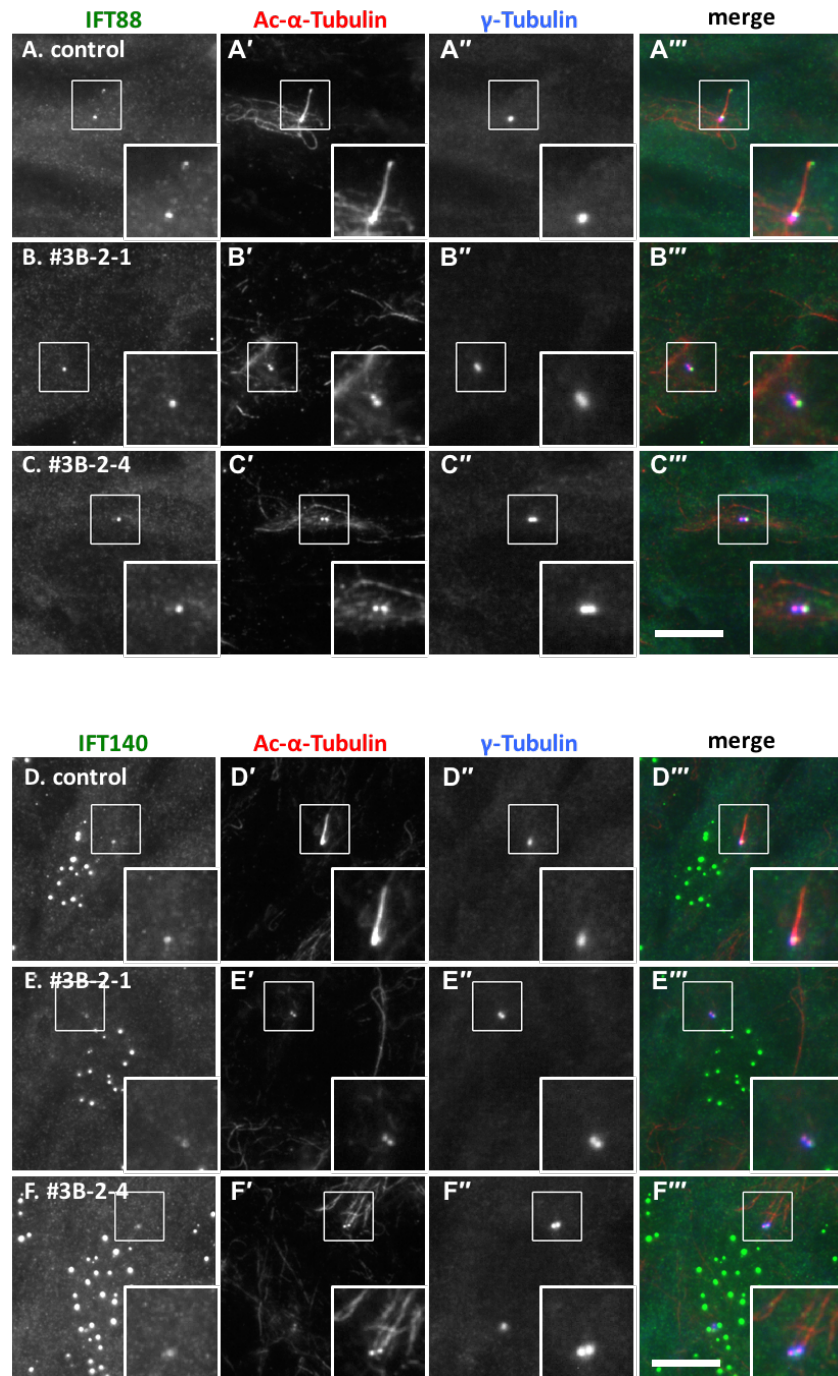


Fig. 2-3. KIF3B is an essential component of the kinesin-II trimer.

Control RPE1 cells (A, D), and *KIF3B*-KO cell lines #3B-2-1 (B, E) and #3B2-4 (C, F) were serum-starved to induce ciliogenesis and triple immunostained for either IFT88 (A–C) or IFT140 (D–F), together with Ac- α -tubulin (D'–I') and γ -tubulin (D''–I''). Merged images are shown in A'''–F'''. Insets, enlarged images of the boxed regions.

Scale bar, 10 μ m.

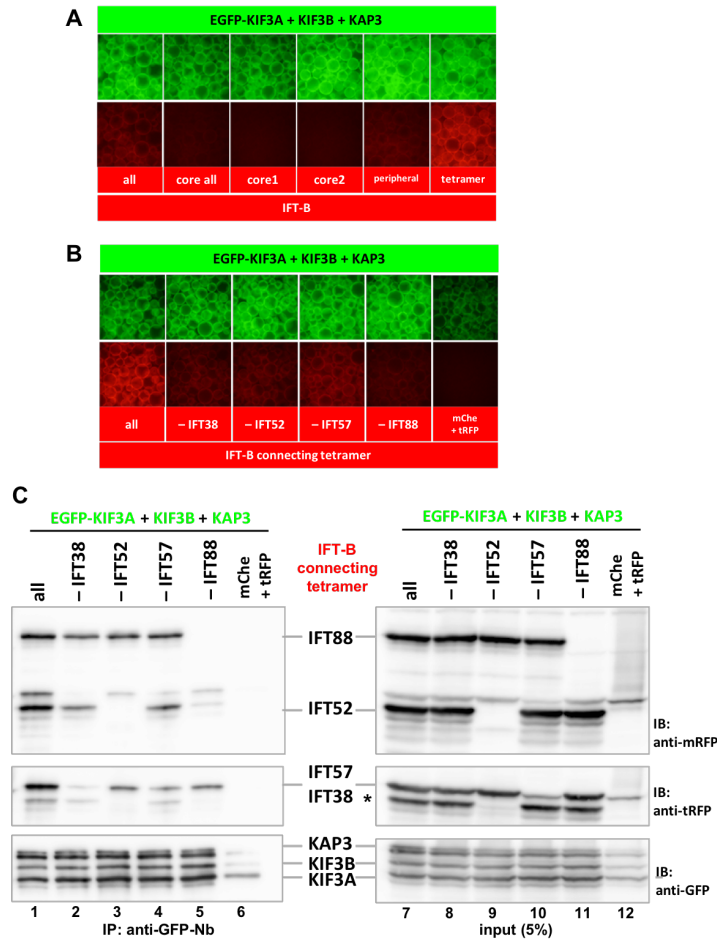
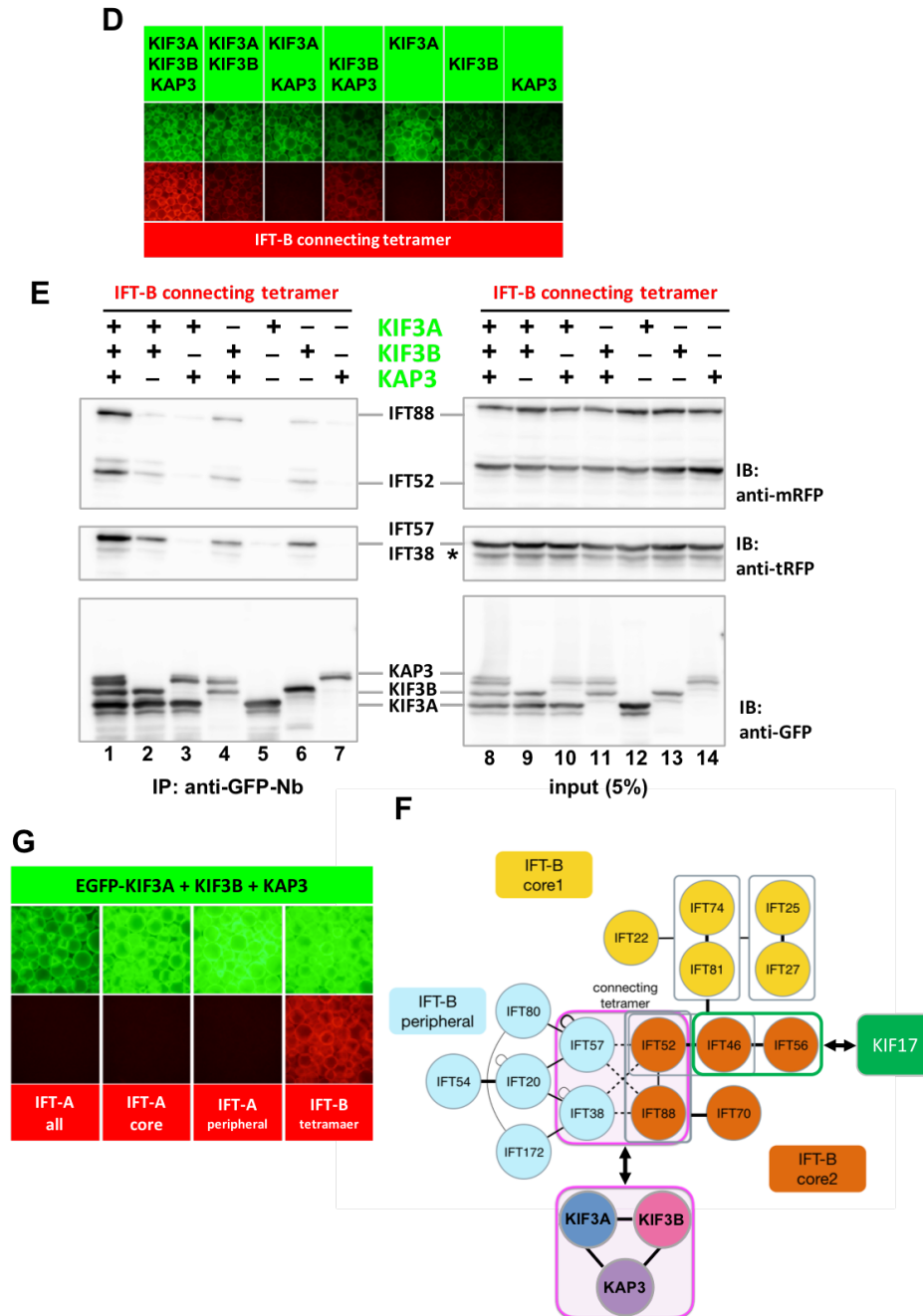


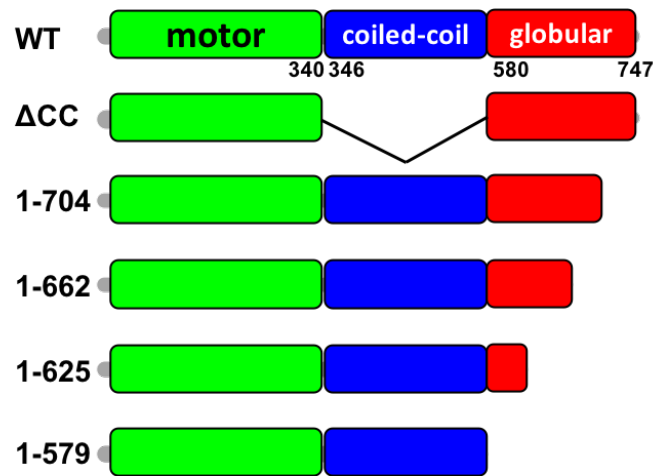
Fig. 2-4. Interaction of heterotrimeric kinesin-II with the IFT-B-connecting tetramer.

(A) Identification of an interaction between heterotrimeric kinesin-II and the IFT-B-connecting tetramer. Lysates from cells coexpressing EGFP-fused kinesin-II subunits and all IFT-B subunits, subunits of all core, core-1, core-2, or peripheral subcomplexes, or connecting tetramer, fused to mChe/tRFP were processed for the VIP assay. (B, C) Subtractive VIP assay and immunoblotting analysis to determine subunits of the IFT-B-connecting tetramer required for its interaction with kinesin-II. Lysates from cells coexpressing EGFP-fused kinesin-II subunits and all but one (as indicated) subunits of the IFT-B tetramer fused to mChe/tRFP were processed for the VIP assay (B) or immunoblotting analysis (C) using an anti-RFP antibody (top panels), which reacts with mChe, an anti-tRFP antibody (middle panels), which reacts with tRFP and weakly with mChe (indicated by an asterisk), or an anti-GFP antibody (bottom panel). As a negative control, a mixture of mChe-fused and tRFP-fused IFT56 was used in place of mChe/tRFP-fused tetrameric subunits (labeled as mChe+tRFP).

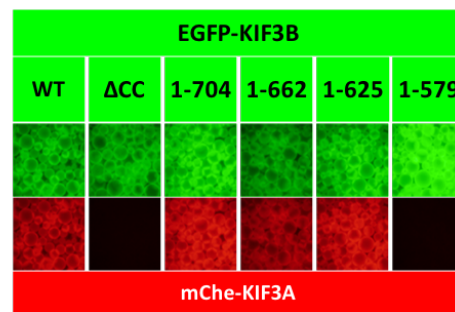


(D, E) Subtractive VIP assay and immunoblotting analysis to determine subunits of kinesin-II required for its interaction with the IFT-B tetramer. Details are essentially the same as described in the legend for (B, C). (F) A model for the interaction of IFT-B with homodimeric KIF17 and heterotrimeric kinesin-II. (G) Lack of an interaction between the IFT-A complex and heterotrimeric kinesin-II. Lysates from cells coexpressing EGFP-fused kinesin-II subunits and mCh-e-fused IFT-A subunits (all, core, or peripheral) were subjected to the VIP assay.

A



B



C

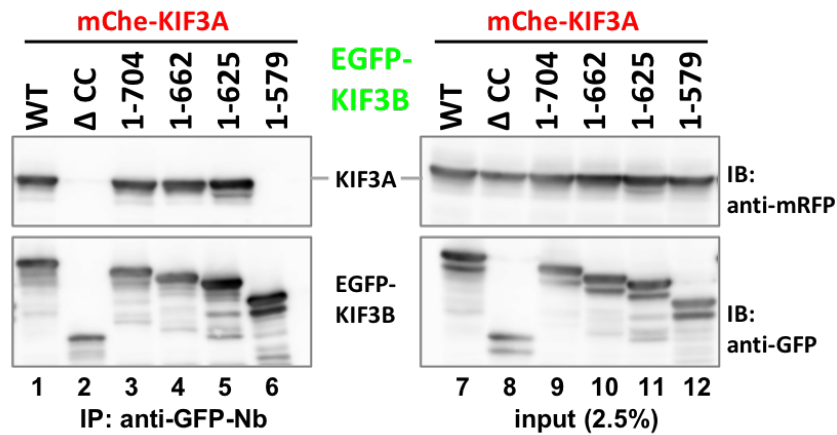


Fig. 2-5. Regions of the KIF3B protein responsible for its interactions with KIF3A.
 (A) Schematic representation of the KIF3B constructs used and their domain organizations. (B, C) VIP assay (B) and immunoblotting analysis (C) to determine the regions of KIF3B responsible for its interaction with KIF3A.

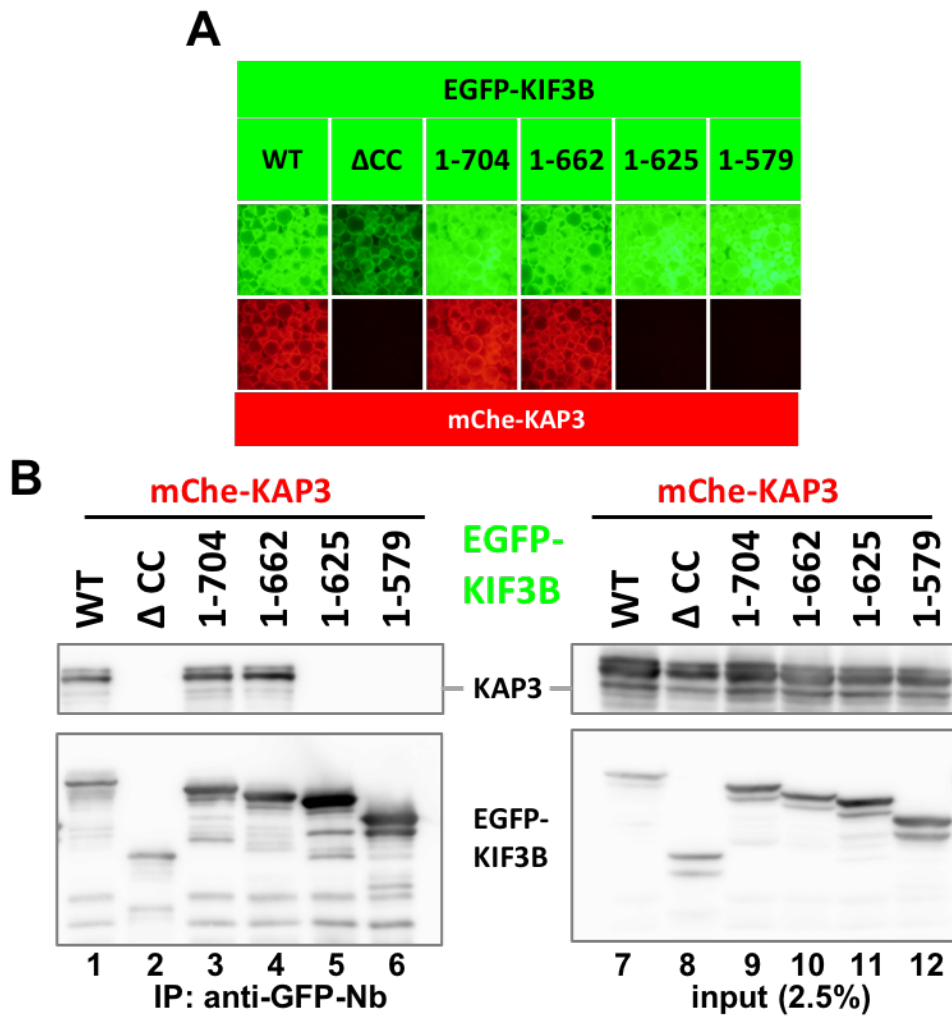


Fig. 2-6. Regions of the KIF3B protein responsible for its interactions with KAP3. VIP assay (A) and immunoblotting analysis (B) to determine the regions of KIF3B responsible for its interaction with KAP3.

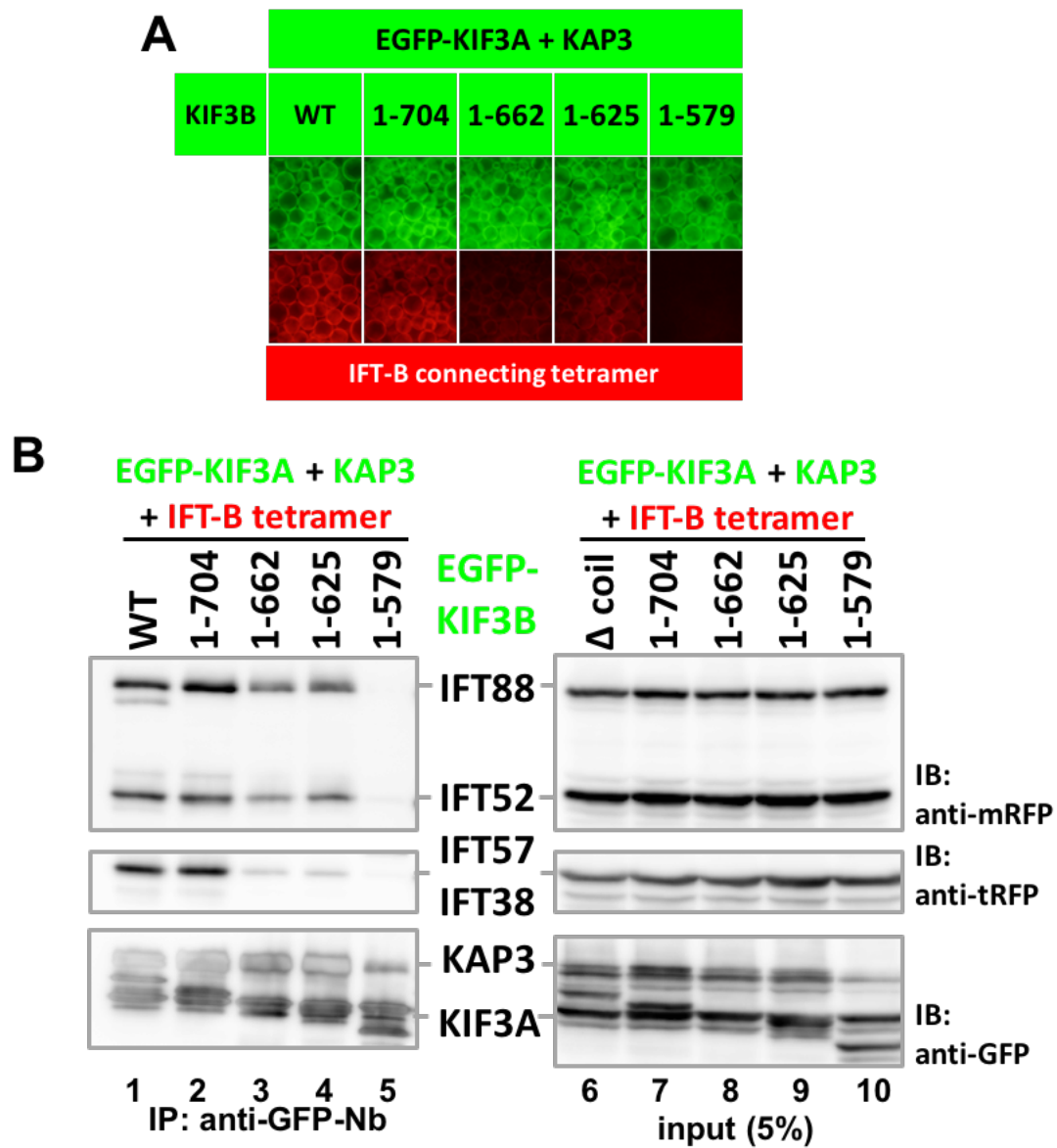
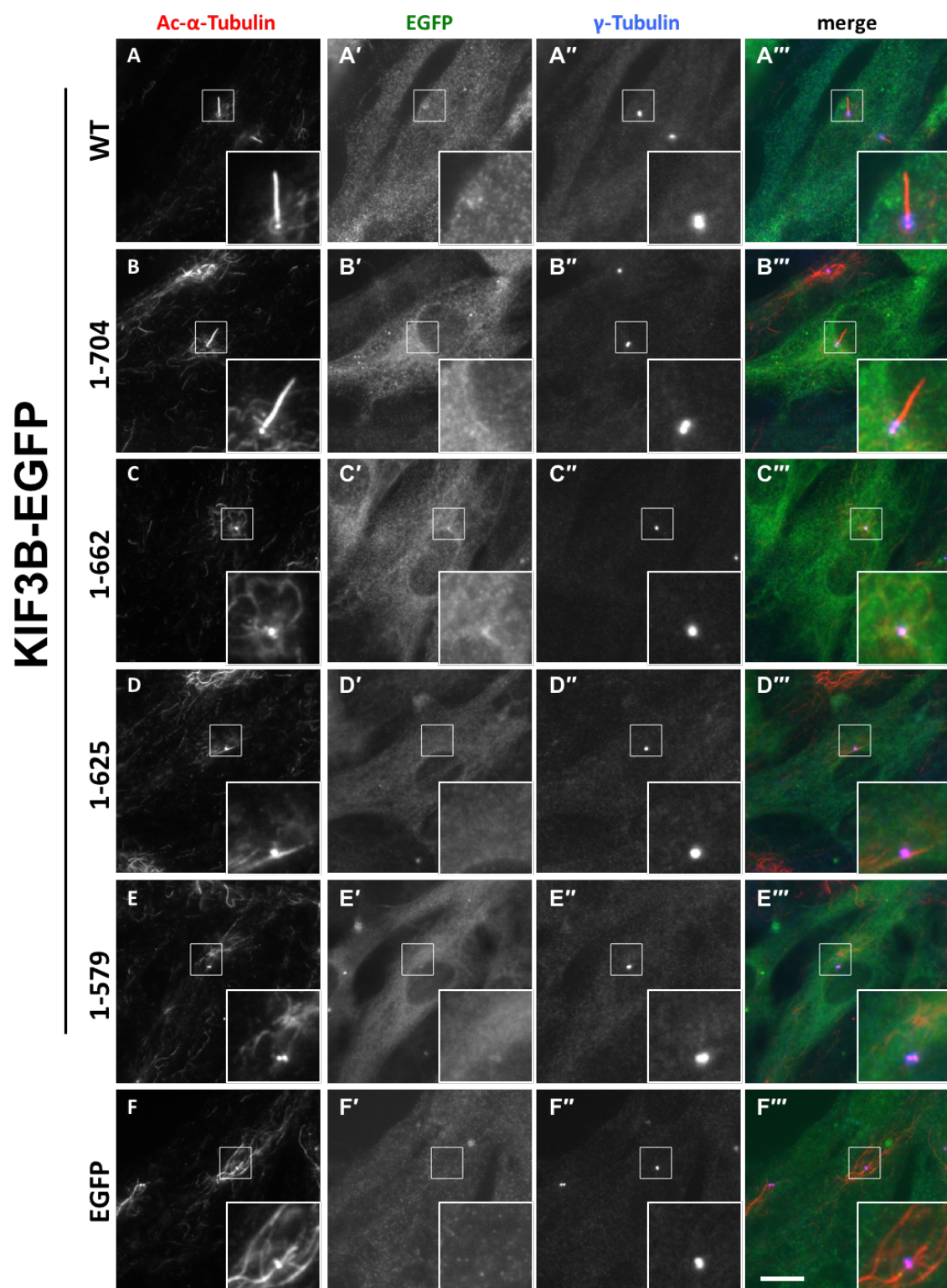


Fig. 2-7. Regions of the KIF3B protein responsible for its interactions with the IFT-B-connecting tetramer.

VIP assay (A) and immunoblotting analysis (B) to determine the regions of KIF3B responsible for its interaction with the IFT-B-connecting tetramer.



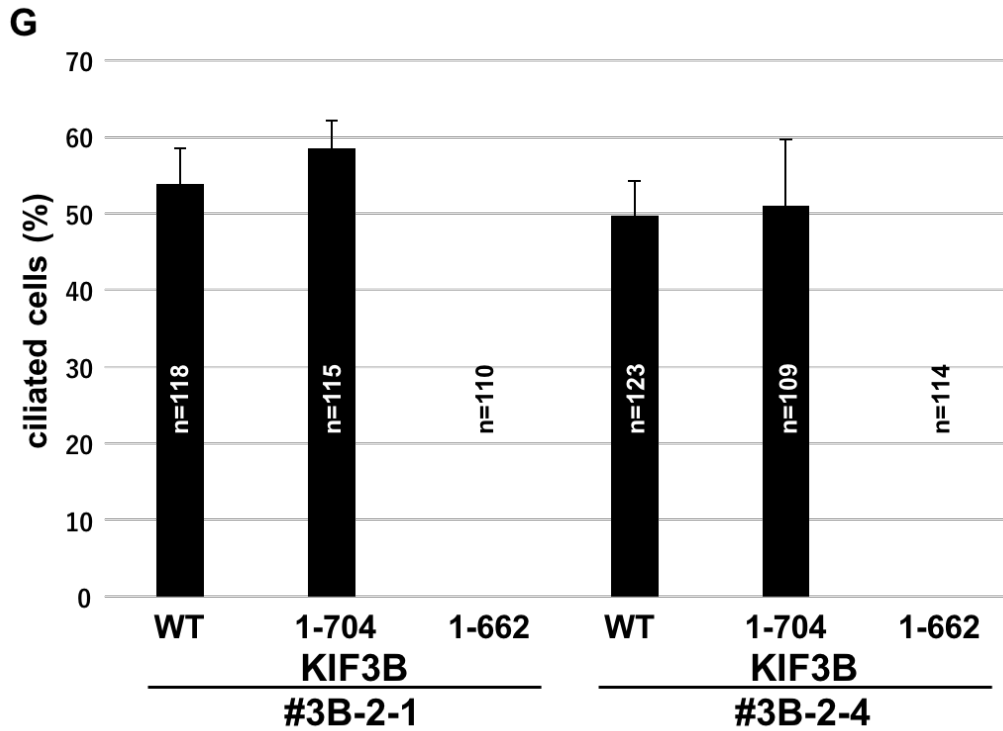


Fig. 2-8. KIF3B–IFT-B interaction is essential for ciliogenesis.

(A–F) The *KIF3B*-KO cell line #3B-2-1 stably expressing EGFP-fused KIF3B(WT) (A), KIF3B(1–704) (B), KIF3B(1–662) (C), KIF3B(1–625) (D), or KIF3B(1–579) (E), or EGFP (F) were triple immunostained for Ac- α -tubulin (A–F), GFP (A'–F'), and γ -tubulin (A''–F''). Scale bar, 10 μ m. (G) Ciliated cells of the *KIF3B*-KO cell lines, #3B-2-1 and #3B-2-4, stably expressing EGFP-fused KIF3B(WT), KIF3B(1–704), or KIF3B(1–662) were counted, and percentages of ciliated cells are represented as bar graphs. Values are shown as means $\pm$ SE of three independent experiments. In each set of experiments, 31–50 cells with EGFP signals were analyzed, and the total number of cells analyzed (n) for each sample is shown.

DISCUSSION

Anterograde ciliary protein trafficking is believed to be mediated by the IFT-B complex powered by the kinesin-2 motor, whereas retrograde trafficking is thought to be mediated by the IFT-A complex powered by dynein-2. I here answered the long-standing question since the discovery that *Kif3*-KO mice demonstrate ciliary morphogenesis defects (Nonaka *et al.*, 1998; Marszalek *et al.*, 1999); namely, the molecular basis for the trafficking of IFT particles driven by kinesin-2. By taking advantage of the flexible VIP-based method, I unraveled the interaction between heterotrimeric kinesin-II and the IFT-B complex. The most robust interaction was observed between the kinesin-II trimer and the IFT-B connecting tetramer composed of IFT38/IFT52/IFT57/IFT88 (see Fig. 2-4F). The subtractive VIP assay demonstrated that omitting any one of the kinesin-II subunits or any of the IFT-B-connecting subunits moderately attenuates the interaction (Fig. 2-4, B–E). In the previous chapter, I showed that KIF17 interacts with the IFT-B complex via the IFT46–IFT56 dimer (see Fig. 2-4F), and does not act as a motor for IFT-B, but rather is a cargo of IFT-B. Thus, the studies in the previous and this chapters unequivocally show that homodimeric KIF17 and heterotrimeric kinesin-II interact with distinct parts of the IFT-B complex (see Fig. 2-4F) and play distinct roles within cilia.

Analysis of KIF3B truncation mutants expressed in *KIF3B*-KO cells demonstrated that the exogenous expression of KIF3B(1–704), which retains its ability to form the kinesin-II trimer and to interact with the IFT-B tetramer, but not the expression of KIF3B(1–662), which can also form a trimer but demonstrates a compromised IFT-B interaction, is able to rescue the ciliogenesis defect caused by the loss of KIF3B. Taking into account that the ciliogenesis defect reflects a lack of the trafficking of tubulins, which are building blocks of axonemal microtubules, and that the IFT-B complex is responsible for tubulin trafficking (Lechtreck, 2015; Taschner and Lorentzen, 2016), a straightforward explanation for the observations in the KIF3B(1–662) rescue experiment would be that the ‘no-cilia’ phenotype was caused by uncoupling of the IFT-B complex from the KIF3B(1–662)-containing kinesin-II trimer. Of course, it remains possible that an unidentified protein that can interact with the C-terminal region of KIF3B is implicated in ciliogenesis, because the loss of the IFT-B-binding ability of KIF3B(1–662) was not complete. However, I believe it unlikely that some unknown protein binds to the KIF3B C-terminal region, as previous single particle analyses of recombinant KIF3A–KIF3B

and KIF3A–KIF3B–KAP3 by electron microscopy suggested that KAP3 covers the globular tail region of the KIF3A–KIF3B dimer (Yamazaki *et al.*, 1995; Yamazaki *et al.*, 1996).

The IFT-B-connecting tetramer constitutes the interface between the core and peripheral subcomplexes (Taschner and Lorentzen, 2016; Nakayama and Katoh, 2018); IFT52 and IFT88 from the core subcomplex, and IFT38 and IFT57 from the peripheral subcomplex (see Fig. 2-4F). Given that the kinesin-II trimer interacts with the connecting tetramer, the hypothesis that kinesin-II triggers the complete IFT-B assembly by clamping the core and peripheral subcomplexes and thereby serves as a cue for the anterograde trafficking of IFT particles at the ciliary base, is somewhat intriguing. However, our preliminary analyses did not detect an enhancement of the interaction between the IFT-B core and peripheral subcomplexes in the presence of kinesin-II. In view of the recent observations indicating that IFT proteins are first recruited to the basal body and in turn kinesin-II is recruited (Wingfield *et al.*, 2017), it is likely that the binding of kinesin-II to preassembled IFT particles triggers entry into cilia and the subsequent anterograde trafficking of IFT particles.

A previous study on *Chlamydomonas* FLA8/KIF3B indicated that calcium-dependent phosphorylation of FLA8 at a serine residue in the globular domain disrupts the interaction between kinesin-II and IFT-B, and inhibits IFT entry (Liang *et al.*, 2014). However, my preliminary analysis using phosphomimetic mutants of human KIF3B has so far failed to show a decrease in the kinesin-II–IFT-B interaction. Although components involved in IFT are highly conserved between *Chlamydomonas* and vertebrates, the detailed mechanism underlying regulation of the kinesin-II–IFT-B interaction might be different.

CONCLUSIONS

The summary of the results presented in this study is as follows:

Chapter 1

1. KIF17 accumulates at the ciliary tip regardless of presence or absence of its motor domain.
2. KIF17 interacts with the IFT46–IFT56 dimer of the IFT-B complex via its conserved sequence located immediately upstream of the NLS in its C-terminal region.
3. In addition to its NLS, KIF17 requires its binding to the IFT-B complex to enter the cilium across the TZ.
4. *KIF17*-KO cells are normal with respect to ciliogenesis, and anterograde trafficking of the IFT-B complex and ciliary proteins, such as Arl13b, Smo, and DRD1.

These results indicate that KIF17 is a cargo of the IFT-B complex and transported into the cilium across the TZ to the ciliary tip, rather than serving as an anterograde motor for the IFT-B complex to transport the proteins required for the ciliogenesis or ciliary functions.

Chapter 2

1. Heterotrimeric kinesin-II, composed of KIF3A, KIF3B, and KAP3, interacts with the IFT-B complex through the IFT-B connecting tetramer composed of IFT38, IFT52, IFT57, and IFT88; among the kinesin-II subunits, KIF3B contributed mainly to the IFT-B binding.
2. *KIF3B*-KO cells exhibit the severe defects in ciliogenesis, although the IFT-B complex localizes at the basal body.
3. The ciliogenesis defect observed in *KIF3B*-KO cells can be rescued by the exogenous expression of wild type KIF3B but not its C-terminally truncated mutant that retains the ability to form heterotrimer with KIF3A and KAP3, but is compromised with respect to IFT-B binding.

These results indicate that the interaction between heterotrimeric kinesin-II and the IFT-B complex through the IFT-B connecting tetramer is essential for the anterograde trafficking of the IFT-B complex, by which proteins essential for the ciliogenesis, such as α/β -tubulin dimers, are transported from the basal body toward the ciliary tip.

Together, the results presented in this study unequivocally showed that, among two types of kinesin-2 motors, heterotrimeric kinesin-II serves as an anterograde motor for the IFT-B complex, and thereby plays essential roles in ciliary anterograde trafficking of proteins required for ciliogenesis. On the other hand, KIF17 is transported into the cilium across the TZ to the ciliary tip as a cargo of the IFT-B complex along the axoneme, rather than as an anterograde motor for the IFT-B complex, although the roles of KIF17 at the ciliary tip remain unclear.

The present study will provide new insight into understanding the molecular basis of ciliogenesis regulated by the IFT-B complex and the pathogenic mechanisms of the ciliopathies.

MATERIALS AND METHODS

Plasmids, antibodies, and reagents

cDNAs for human KIF17 (clone ID: IRAK140P09), KIF3A (clone ID: IRAK106P17), and KAP3 (clone ID: IRAK034F02) were obtained from the RIKEN Bio-Resource Center through the National Bio-Resource Project of the MEXT, Japan; cDNAs for human KIF3B (clone ID: ORK00515) and DRD1 (product ID: FXC00007) were from Kazusa DNA Research Institute, Japan; and Mouse importin and Human transportin cDNAs were kindly provided by Yoshihiro Yoneda, Noriko Yasuhara, Masahiro Oka, and Tetsuji Moriyama (National Institutes of Biomedical Innovation, Health and Nutrition, Japan; Tsuji *et al.*, 1997). Expression vectors for kinesin-2 and IFT proteins, and their deletion/point-mutated constructs used in this study are listed in Table 1. Plasmids for production of the replication-defective, self-inactivating lentiviral vector pRRLsinPPT-EGFP and packaging plasmids (pRSV-REV, pMD2.g, and pMDLg/pRRE) were kindly provided by Peter McPherson (McGill University, Montreal, Canada; Thomas *et al.*, 2009). Antibodies used in this study are as follows: monoclonal mouse anti-Ac- α -tubulin (6-11-B1) and anti- γ -tubulin (GTU88), Sigma-Aldrich; polyclonal rabbit anti-Arl13B (17711-1-AP), polyclonal rabbit anti-IFT88 (13967-1-AP), and anti-IFT140 (17460-1-AP), Proteintech; polyclonal rabbit anti-Smo (ab38686), Abcam; polyclonal rabbit anti-GFP (A11122), Molecular Probes; monoclonal mouse anti-GFP (JL-8), BD Biosciences; polyclonal rabbit anti-RFP (PM005), MBL Life Science; polyclonal rabbit anti-tRFP (AB233), Evrogen; AlexaFluor-conjugated secondary antibodies (A11034, A21240, A21147), Molecular Probes; and peroxidase-conjugated secondary antibodies (115-035-166, 111-035-144), Jackson ImmunoResearch Laboratories. SAG and Polyethylenimine Max were purchased from Enzo Life Sciences and Polysciences, respectively. GST-tagged anti-GFP Nb prebound to glutathione-Sepharose 4B beads were prepared as described previously (Katoh *et al.*, 2015).

VIP assay and immunoblotting analysis

The VIP assay was performed as described previously (Katoh *et al.*, 2015). Briefly, HEK293T cells grown to $\sim 1.6 \times 10^6$ cells on a six-well plate in DMEM with high glucose (Nacalai Tesque) supplemented with 5% fetal bovine serum (FBS) were transfected with expression vectors for EGFP and mChe/tRFP fusions (2 μ g each) using

Polyethylenimine Max (20 µg). After 24 h, the cells were lysed in 250 µl of lysis buffer (that used in chapter 1 was consisted of 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.4, 150 mM NaCl, 0.1% Triton X-100, and 10% glycerol, and that used in chapter 2 was consisted of 10 mM HEPES, pH 7.4, 5 mM MgSO₄, 1 mM DTT, 0.5 mM EDTA, 25 mM KCl, and 0.5% NP-40 (Nishijima *et al.*, 2017)) containing a protease inhibitor cocktail (EDTA-free; Nacalai Tesque). The lysates were mixed with 5 µl of GST–anti-GFP Nb beads at 4°C for 1 h. After washing three times with lysis buffer, the precipitated beads bearing fluorescent proteins were observed using an all-in-one-type fluorescence microscope (Biozero BZ-8000, Keyence) with a 20×/0.75 objective lens under constant conditions (sensitivity ISO 400, exposure 1/30 s for green; and sensitivity ISO 800, exposure 1/10 s for red) unless otherwise noted.

Establishment of KO cell lines using the CRISPR/Cas9 system

The strategy for knockout of the *IFT56*, *KIF17*, or *KIF3B* gene in hTERT-RPE1 cells (American Type Culture Collection CRL-4000) using the CRISPR/Cas9 system using homology-independent DNA repair (version 1 method) was previously described in detail (Kato et al., 2017). The single guide RNA (sgRNA) sequence targeting the human *IFT56*, *KIF17*, or *KIF3B* gene (see Table 2) was designed using CRISPRscan (<http://www.crisprscan.org/>) (Moreno-Mateos *et al.*, 2015). Double-stranded oligonucleotides for these sequences were inserted into the knock-in donor vector pDonor-tBFP-NLS-Neo (Addgene ID 80766) and the all-in-one sgRNA expression vector, pSpCas9(BB)-2A-Puro (PX459; Addgene ID 48139). hTERT-RPE1 cells were grown on a 12-well plate to approximately 3.0×10^5 cells, and transfected with 1 µg of the sgRNA vector and 0.25 µg of the donor knock-in vector using X-tremeGENE9 DNA Transfection Reagent (Roche Applied Science). After selection in the presence of G418 (600 µg/mL), cells with nuclear tBFP signals were isolated. To confirm KO of the target gene, genomic DNA was extracted from the isolated cells and subjected to PCR using KOD FX Neo DNA polymerase (Toyobo). Three sets of primers (Table 2) were used for PCR to distinguish the following three states of integration of the donor vector: forward integration, reverse integration, and no integration with a small indel (see Fig. 1-7A). Direct sequencing of the PCR products ensured the KO of both alleles of the *IFT56*, *KIF17*, or *KIF3B* gene, with integration of the donor vector and/or small deletion/insertion causing a frameshift.

Preparation of cells stably expressing EGFP-KIF17, DRD1-EGFP, or KIF3B-EGFP

Lentiviral vectors were prepared as described previously (Takahashi et al., 2012). Briefly, pRRLsinPPT-EGFP-KIF17, pRRLsinPPT-DRD1-EGFP, or pRRLsinPPT-KIF3B-EGFP or its deletion construct was transfected into HEK293T cells with packaging plasmids (pRSV-REV, pMD2.g, and pMDL/pRRE). Culture media were replaced 8 h after transfection. Culture media containing lentiviral particles were collected at 24, 36, and 48 h after transfection, passed through a 0.45- μ m filter (Sartorius) and centrifuged at 32,000 g at 4 °C for 4 h using an R15A rotor and Himac CR22G centrifuge (Hitachi Koki, Japan). Precipitated viral particles were resuspended in Opti-MEM (Invitrogen). *IFT56*-KO cells expressing EGFP-KIF17, *KIF17*-KO cells expressing DRD1-EGFP, and *KIF3B*-KO cells expressing KIF3B-EGFP were prepared by the addition of a lentiviral suspension into the culture medium, followed by a 24-h incubation. These cells were used for immunofluorescence analysis.

Immunofluorescence analysis

hTERT-RPE1 cells were cultured in DMEM/F-12 (Nacalai Tesque) supplemented with 10% FBS and 0.348% sodium bicarbonate. To induce ciliogenesis, cells were grown on coverslips up to 100% confluence, and starved for 24 h in Opti-MEM containing 0.2% bovine serum albumin. For immunofluorescence analysis, cells were fixed and permeabilized with 100% methanol for 5 min at -20°C , and washed three times with phosphate-buffered saline. The fixed/permeabilized cells were blocked with 10% FBS, stained with antibodies diluted in 5% FBS, and observed using an Axiovert 200M microscope (Carl Zeiss).

Table 1. Plasmid vectors used in this study

No.	Vector	Insert	Reference
1	pCAG2-EGFP-C	Human KIF17	This study
2	pCAG2-mCherry-C	Human KIF17	This study
3	pCAG2-EGFP-C	Human KIF17 (1-1018)	This study
4	pCAG2-EGFP-C	Human KIF17 (1-1014)	This study
5	pCAG2-EGFP-C	Human KIF17 (1-999)	This study
6	pCAG2-EGFP-C	Human KIF17 (R1000/1003A)	This study
7	pCAG2-mCherry-C	Human KIF17 (R1000/1003A)	This study
8	pCAG2-EGFP-C	Human KIF17 (AAAA)	This study
9	pCAG2-mCherry-C	Human KIF17 (AAAA)	This study
10	pRRLsinPPT-EGFP-C	Human KIF17	This study
11	pRRLsinPPT-EGFP-N	Human DRD1	This study
12	pCAG2-EGFP-C	Human TNPO1	This study
13	pCAG2-EGFP-C	Human KPNB1	This study
14	pEGFP-C1	Mouse KPNA1	Tsuji et al. (1997)
15	pEGFP-C1	Mouse KPNA2	Tsuji et al. (1997)
16	pEGFP-C1	Mouse KPNA3	Tsuji et al. (1997)
17	pEGFP-C1	Mouse KPNA4	Tsuji et al. (1997)
18	pEGFP-C1	Mouse KPNA6	Tsuji et al. (1997)
19	pCAG2-EGFP-C	Human KIF3A	This study
20	pCAG2-mCherry-C	Human KIF3A	This study
21	pCAG2-EGFP-C	Human KIF3B	This study
22	pCAG2-mCherry-C	Human KIF3B	This study
23	pCAG2-EGFP-C	Human KAP3	This study
24	pCAG2-mCherry-C	Human KAP3	This study
25	pCAG2-EGFP-C	Human KIF3B (1–704)	This study
26	pCAG2-EGFP-C	Human KIF3B (1–662)	This study
27	pCAG2-EGFP-C	Human KIF3B (1–625)	This study
28	pCAG2-EGFP-C	Human KIF3B (1–579)	This study
29	pCAG2-EGFP-C	Human KIF3B (Δ Coil: Δ 346–579)	This study
30	pRRLsinPPT-EGFP-N	Human KIF3B	This study

31	pRRLsinPPT-EGFP-N	Human KIF3B (1–704)	This study
32	pRRLsinPPT-EGFP-N	Human KIF3B (1–662)	This study
33	pRRLsinPPT-EGFP-N	Human KIF3B (1–625)	This study
34	pRRLsinPPT-EGFP-N	Human KIF3B (1–579)	This study
35	pTagRFP-T-N	Human IFT20	Katoh et al. (2016)
36	pTagRFP-T-C	Human IFT22	Katoh et al. (2016)
37	pTagRFP-T-C	Human IFT25	Katoh et al. (2016)
38	pTagRFP-T-C	Human IFT27	Katoh et al. (2016)
39	pTagRFP-T-C	Human IFT38	Katoh et al. (2016)
40	pCAG-EGFP-C	Human IFT46	Katoh et al. (2016)
41	pCAG-mCherry-C	Human IFT46	Katoh et al. (2016)
42	pCAG-mCherry-C	Human IFT52	Katoh et al. (2016)
43	pTagRFP-T-C	Human IFT54	Katoh et al. (2016)
44	pCAG-EGFP-C	Human IFT56	Katoh et al. (2016)
45	pCAG-mCherry-C	Human IFT56	This study
46	pCAG-TagRFP-T-C	Human IFT56	Katoh et al. (2016)
47	pTagRFP-T-C	Human IFT57	Katoh et al. (2016)
48	pCAG2-mCherry-C	Human IFT70B	Katoh et al. (2016)
49	pCAG-mCherry-C	Human IFT74	Katoh et al. (2016)
50	pCAG-mCherry-N	Human IFT80	Katoh et al. (2016)
51	pCAG-mCherry-C	Human IFT81	Katoh et al. (2016)
52	pCAG-mCherry-C	Human IFT88	Katoh et al. (2016)
53	pCAG2-mCherry-C	Human IFT172	Katoh et al. (2016)
54	pmCherry-C1	Human FT43	Hirano et al. (2017)
55	pCAG2-mCherry-C	Human IFT121	Hirano et al. (2017)
56	pCAG2-mCherry-C	Human IFT122	Hirano et al. (2017)
57	pCAG2-mCherry-C	Human IFT139	Hirano et al. (2017)
58	pCAG2-mCherry-C	Human IFT140	Hirano et al. (2017)
59	pCAG2-mCherry-C	Human IFT144	Hirano et al. (2017)
60	pmCherry-C1	Human TULP3	Hirano et al. (2017)

Table 2. Oligo DNAs used in this study

No.	Name	Sequence
1	pTagBFP-N-RV2	5'-CGTAGAGGAAGCTAGTAGCCAGG-3'
2	IFT56-gRNA-#1-S	5'-CACCGCACTGGAGCTATTACCCTGT-3'
3	IFT56-gRNA-#1-AS	5'-AAACACAGGGTAATAGCTCCAGTGC-3'
4	IFT56-Donor#1-AS	5'-TCCAACAGGGTAATAGCTCCAGTGC-3'
5	IFT56-gRNA-#2-S	5'-CACCGTGTACGCCTCTGCCTACAGC-3'
6	IFT56-gRNA-#2-AS	5'-AAACGCTGTAGGCAGAGGCGTACAC-3'
7	IFT56-Donor#2-AS	5'-TCCAGCTGTAGGCAGAGGCGTACAC-3'
8	IFT56-genome-FW	5'-GTCTAGGTGTGTCCGCAGAGAG-3'
9	IFT56-genome-RV	5'-GGATAAGCAATGTGTGAAATGAC-3'
10	KIF17-gRNA-#1-S	5'-CACCGCTTGGTGTGACCCCCAAGG-3'
11	KIF17-gRNA-#1-AS	5'-AAACCTTGGGGCTGACACCAAGC-3'
12	KIF17-Donor#1-AS	5'-TCCACCTTGGGGCTGACACCAAGC-3'
13	KIF17-gRNA-#2-S	5'-CACCGGGGATGATGCCTCTCTGGG-3'
14	KIF17-gRNA-#2-AS	5'-AAACCCCAGAGAGGCATCATCCCC-3'
15	KIF17-Donor#2-AS	5'-TCCACCCAGAGAGGCATCATCCCC-3'
16	KIF17-genome-#1-FW	5'-GAGAGAGCTTGCCAGGAAGG-3'
17	KIF17-genome-#1-RV	5'-CTCCTGACCTCAAGTGATCTG-3'
18	KIF17-genome-#2-FW	5'-CAGAGAGTCAACTCCTTGAGGAC-3'
19	KIF17-genome-#2-RV	5'-GTTAGAAGGCAAAAATGGTGAGAG-3'
20	KIF3B-gRNA#2-S	5'-CACCGAAGGTCTTGGGCATTTTCAT-3'
21	KIF3B-gRNA#2-AS	5'-AAACATGAAATGCCCAAGACCTTC-3'
22	KIF3B-Donor#2-AS	5'-TCCAATGAAATGCCCAAGACCTTC-3'
23	KIF3B-genome-FW	5'-GCTGCAATGACAACGGTACTG-3'
24	KIF3B-genome-RV	5'-GATGTGGGTGAAGATATGGTC-3'

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