Intraflagellar transport protein IFT52 recruits IFT46 to the basal body and flagella

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Summary statement

IFT46-C1 is the basal body and ciliary localization sequence of IFT46. IFT52 interacts with IFT46 in the cytoplasm and recruits it to the basal body.

Abbreviations used in this paper

IFT, intraflagellar transport
YFP, yellow fluorescent protein
TIRFM, total internal reflection fluorescence microscopy
NLS, nuclear localization signals or sequences
WT, wild-type
CoIP, co-immunoprecipitation
TGN, trans-Golgi network
Abstract

Cilia are microtubule based organelles and perform motile, sensing and signaling functions. The assembly and maintenance of cilia depend on intraflagellar transport. Besides ciliary localization, most IFT proteins accumulate at basal bodies. However, little is known about the molecular mechanism of basal body targeting of IFT proteins. We first identified the possible basal body targeting sequence of IFT46 by expressing IFT46 truncations in *ift46-1* mutant. The C-terminal sequence 246-321 aa, termed BBTS3, was sufficient to target YFPs to basal bodies in *ift46-1*. Interestingly, BBTS3 is also responsible for the ciliary targeting of IFT46. BBTS3::YFP moves bidirectionally in flagella and interacts with other IFT-B proteins. Using IFT and motor mutants, we show that the basal body localization of IFT46 depends on IFT52, but not on IFT81, IFT88, IFT122, FLA10 or DHC1b. IFT52 interacts with IFT46 through L285/L286 of IFT46 and recruits it to basal bodies. Ectopic expression of IFT52C in the nucleus resulted in accumulation of IFT46 in nuclei. These data suggest IFT52 and IFT46 can preassemble as a complex in the cytoplasm, which is then targeted to basal bodies.
Introduction

Eukaryotic cells evolved different types of organelles carrying out specialized functions. One of them is the cilium/flagellum (interchangeable terms), which is found on most eukaryotic cells. Cilia are hair-like microtubule-based organelles that protrude from the cell surface and are composed of axoneme, ciliary matrix, and ciliary membrane (Mizuno et al., 2012; Satir and Christensen, 2007). Cilia primarily play two vital roles (Satir and Christensen, 2007). One is to function as a cellular motor to move either the cell itself or surrounding liquids/particles. The other function of cilia is to serve as a hub for cellular signaling (Ishikawa and Marshall, 2011; Mourao et al., 2016; Singla and Reiter, 2006; Wood and Rosenbaum, 2015). Defects in ciliary structures and functions in human lead to diseases commonly referred to as ‘ciliopathies’ such as Polycystic Kidney Disease, blindness, heterotaxy, Bardet-Biedl Syndrome, and skeletal abnormalities (Fliegauf et al., 2007; Gerdes et al., 2009; Hildebrandt et al., 2011; Hildebrandt and Zhou, 2007).

The formation, and maintenance of cilia, as well as ciliary signaling, depend on intraflagellar transport (IFT) (Ishikawa and Marshall, 2011; Mourao et al., 2016; Pedersen and Rosenbaum, 2008; Scholey, 2003), a bidirectional movement of granular particles between the outer doublet microtubules and the flagellar membrane along the cilium (Kozminski et al., 1993). The anterograde (base to tip) movement of IFT-particles is powered by kinesin-2 (Kozminski et al., 1995; Miller et al., 2005; Ou et al., 2005; Pan et al., 2006; Snow et al., 2004; Walther et al., 1994), while cytoplasmic dynein 2/1b drives the retrograde IFT (tip to base) (Hou et al., 2004; Pazour et al., 1999). More than 22 IFT proteins form at least three biochemically distinct complexes, namely IFT-A, IFT-B1 and IFT-B2 (Taschner and Lorentzen, 2016; Taschner et al., 2016). Since cilia lack the machinery of protein synthesis, proteins including structural components and signaling molecules are synthesized in the cell body and some are delivered into cilia via IFT. IFT and cargoes may assemble into periodic IFT trains (Pigino et al., 2009; Stepanek and Pigno, 2016; Vannuccini et al., 2016). Anterograde IFT is about 233 nm and moves along B-microtubules. Retrograde IFT is about 209 nm and moves along A-microtubules. In addition, IFT proteins also function in other cellular processes including vesicle exocytosis, cell division, and the formation of
immunological synapses and microtubule-based nanotubes (Baldari and Rosenbaum, 2010; Borovina and Ciruna, 2013; Griffiths et al., 2010; Inaba et al., 2015; Wood et al., 2012).

In addition to the dynamic movement of IFT in cilia, nearly all IFT and motor proteins are concentrated at the basal body. The IFT-B proteins and anterograde motor appear as a semi-circular tri-lobed arc, and the IFT-A proteins and retrograde motor appear as a semi-circular di-lobed arc (Brown et al., 2015; Deane et al., 2001). The two anterior lobes (pool) are co-localized (Brown et al., 2015). But the mechanism of the basal body localization of IFT subunits, which is one of the key steps in the initiation of ciliogenesis, remains largely unknown. Since the IFT proteins such as IFT46 are localized in trans-Golgi network (TGN) derived vesicles during flagellar regeneration. Woods et al. suggested that IFT may associate with the vesicle first, recruit the cargos, and then be targeted to the basal body (Wood and Rosenbaum, 2014). Many ciliary proteins, especially ciliary membrane proteins, contain ciliary targeting sequences (CTSs) (Bhogaraju et al., 2013; Malicki and Avidor-Reiss, 2014). These CTSs mediate the basal body and ciliary localization of target proteins. However, CTSs are diverse, which suggests different transporting systems participate in these processes (Bhogaraju et al., 2013; Malicki and Avidor-Reiss, 2014). Well-identified cases are motifs found in the third intracellular loop of different GPCRs, the Ax(S/A)xQ and RVxP motifs, nuclear localization signal and SUMOylation (Bhogaraju et al., 2013; Dishinger et al., 2010; Malicki and Avidor-Reiss, 2014; McIntyre et al., 2015). Nonetheless, none of them has been found in IFT proteins.

Some factors such as BBS7, BBS8, C2cd3, CCDC41, OFD1, Rsg1, and TTBK2 affect the basal body recruitment of certain IFT subunits (Blacque et al., 2004; Brooks and Wallingford, 2013; Goetz et al., 2012; Joo et al., 2013; Ye et al., 2014), but their functions are unspecific (Toriyama et al., 2016). Recently, ciliogenesis and planar polarity effectors (CPLANE) including Jbts17, Intu, Fuz, Wdpcp, and Rsg1 were shown to recruit peripheral IFT-A proteins to basal bodies (Brooks and Wallingford, 2012; Toriyama et al., 2016). This finding represents a real breakthrough although the CPLANE proteins may be required for the stability and assembly of peripheral IFT-A complexes rather than direct basal body recruitment (Toriyama et al., 2016).
In this paper, we explored the molecular mechanism of the basal body localization of IFT46 and found that the C-terminus of IFT46 mediates its basal body localization. The targeting sequence can assemble into the IFT complex and move along the flagellum. Biochemical and genetic studies showed that IFT52 binds and recruits IFT46 to basal bodies. These new findings suggest that IFT52 and IFT46 preassemble in the cytoplasm or the TGN, but not in the basal body.

Results

YFP-tagged IFT46 functions as the endogenous form and localizes at the basal body and flagella

To study the molecular mechanism of IFT accumulation at the basal body, we focused on one pivotal subunit of IFT-B1, IFT46, since both the null mutant and a specific antibody are available (Hou et al., 2007). We firstly fused Citrine yellow fluorescent protein (YFP) to the C-terminus of IFT46 with a linker peptide in between (Fig. 1A) (Griesbeck et al., 2001; Long and Huang, 2012). YFP-tagged IFT46 was then expressed in ift46-1 (Hou et al., 2007). The null mutant has stumpy, paralyzed flagella and can transcribe the 5’ end of the IFT46 gene (Hou et al., 2007). In theory, rescued strains will thus have full length flagella and swim normally. Indeed, we identified 52 rescued strains among 132 transformants (Movies 1, 2, 3). The middle part and C-terminus of IFT46 were only expressed in WT and ift46-1 IFT46::YFP. The YFP was only expressed in ift46-1 IFT46::YFP (Fig. 1B). IFT46 was transcribed equally in WT and in ift46-1 IFT46::YFP (Fig. 1C). Subsequently, four strains were chosen randomly for immunoblotting analysis. A 75 kD protein was only detected in ift46-1 IFT46::YFP, not in WT (Fig. 1D), suggesting it is the fusion protein IFT46::YFP. The expression level of IFT46 in WT and of IFT46::YFP in rescued strains are nearly equal (P>0.05, Fig. 1D). These data demonstrated that the endogenous promoter of IFT46 drove the expression of IFT46::YFP efficiently.

To evaluate the influence of the YFP tag on the function of IFT46, we compared flagella-related phenotypes of ift46-1 IFT46::YFP with WT. The average ciliary lengths and the percentages of ciliated cells of the rescued strains were akin to those of WT cells (Fig.
S1A,B). The anterograde and retrograde velocities and frequencies of IFT46::YFP were similar to results published previously (Fig. S1C,D,E, Movie 4) (Brown et al., 2015). In summary, these data demonstrated that the 28 kDa YFP tag does not affect the motility and function of IFT46.

Due to the stumpy, paralyzed flagella, the daughter cells of *ift46-1* are more likely to be restricted in the mother cell wall (Movies 1, 2, 3) (Hou et al., 2007). When rescued, the cells grew flagella with normal length (Figs 1E, S1A). IFT46::YFP accumulated at basal bodies and localized as a dotted pattern along the flagella (Fig. 1E). We also expressed only YFP in *ift46-1* as a negative control. Free YFPs accumulate around the nuclei in *ift46-1 YFP* (Fig. 1E). These data confirmed that the basal body localization of IFT46::YFP is specific. Moreover, the punctate pattern of IFT46::YFP along the flagella is more obvious when examined by total internal reflection fluorescence microscope (TIRFM) (Fig. 1F). IFT46::YFP also moves along the axoneme bidirectionally just as other IFT subunits (Movie 4). Collectively, these data suggest that the rescued strains can be used to study the molecular mechanism of IFT accumulation at basal bodies.

**BBTS3 targets IFT46 to the basal body**

IFT46 is mainly composed of alpha helices and random coils (Fig. S2A). The N-terminus and glycine-rich tail of IFT46 are predicted to be intrinsically unstructured and disordered (Fig. S2B). To identify the targeting sequence in IFT46 responsible for its basal body localization, we generated and expressed a series of truncated IFT46 fused with YFP in *ift46-1* according to its secondary structure and potential domain boundaries (Buchan et al., 2013; Jones, 1999) (Figs 2A,C, S2). These truncated IFT46 constructs were named IFT46-N1, IFT46ΔN1, IFT46-N, IFT46-C, IFT46ΔC1, and IFT46-C1, respectively (Fig. 2A). Since none of these truncated forms can fully or partially rescue the null mutant, positive transformants were screened and identified using immunoblotting with GFP antibody (Fig. 2C). All fusion proteins were expressed with predicted molecular weights (Fig. 2C). For example, the predicted molecular weight of IFT46-N::YFP is about 46.3 kDa and it migrated at 50 kDa on SDS-PAGE in lane 4 (Fig. 2C). Then, the localization of truncated IFT46 in cell bodies was determined by confocal imaging. The fusion proteins, IFT46-N1::YFP,
IFT46-N::YFP, and IFT46ΔC1::YFP, were evenly distributed across cell bodies and did not accumulate at the basal body (Fig. 2E). On the other hand, the fusion proteins, IFT46ΔN1::YFP, IFT46-C::YFP, and IFT46-C1::YFP, were localized at basal bodies (Fig. 2E). Since all these fusion proteins localized at the basal body contain the C1 domain, this suggests that the C1 domain is necessary and sufficient for basal body targeting of IFT46. In other words, IFT46-C1 may be the basal body targeting sequence of IFT46.

As most known ciliary targeting sequences are shorter than IFT46-C1 (99 amino acids) (Berbari et al., 2008; Bhogaraju et al., 2013; Dishinger et al., 2010; Hurd et al., 2011; Malicki and Avidor-Reiss, 2014; McIntyre et al., 2015; Santos and Reiter, 2014), we further truncated IFT46-C1 and expressed them in *ift46-1* (Fig. 2B,D). These constructs were named as BBTS1 to BBTS6 sequentially (Fig. 2B). Positive transformants expressing the fusion proteins were identified by immunoblotting. These fusion proteins were expressed nearly equally with predicted molecular weight in the null mutant *ift46-1* (Fig. 2D). We found that only BBTS3::YFP localized at the basal body (Fig. 2F). Therefore, BBTS3 may be the shortest basal body localization sequence (86 amino acids) of IFT46 we were able to identify.

**BBTS3 targets IFT46 to cilia and moves along the ciliary axoneme**

We have demonstrated above that the IFT46-C1 and BBTS3 are necessary and sufficient for the basal body targeting of IFT46. Next question is whether they can also target IFT46 to cilia. To address this, the full length and truncated IFT46 were expressed in WT cells which have normal flagella. Positive transformants expressing the fusion proteins were identified by immunoblotting with GFP antibody. All fusion proteins were expressed with predicted molecular weights (Fig. 3A,B). The localization of the fusion proteins was determined by confocal microscopy. IFT46::YFP, IFT46ΔN1::YFP, IFT46-C::YFP, and IFT46-C1::YFP localized predominantly in basal bodies (Fig. 3D) and flagella (Fig. 3C,D). In addition, these four proteins moved along the axoneme in live cell imaging (Fig. 3F, Movies 5, 6, 7, 8). However, IFT46-N1::YFP, IFT46-N::YFP, and IFT46ΔC1::YFP did not accumulate at basal bodies (Fig. 3D). Interestingly, we could detect these proteins in flagella by western blotting analysis (Fig. 3C). Using TIRFM imaging, we also observed
evenly, weak and immobile fluorescence signals of YFPs along the entire length of flagella (Fig. 3F), which is completely different to the dotted localization patterns of the full-length IFT46::YFP, IFT46ΔN1::YFP, IFT46-C::YFP, and IFT46-C1::YFP in flagella. As the molecular weights of these soluble proteins such as IFT46-N1::YFP, IFT46-N::YFP, and IFT46ΔC1::YFP, are all less than 100 kDa, it is reasonable to speculate that they enter cilia by diffusion through the transition zone (Breslow et al., 2013). These results not only confirmed the previous conclusion that IFT46-C1 can target IFT46 to basal bodies, but also demonstrated that IFT46-C1 can also target IFT46 to cilia.

As BBTS3 may be the shortest basal body localization sequence of IFT46 we identified so far, we were wondering whether BBTS3 is also the ciliary localization sequence of IFT46. When the fusion construct was transformed into WT cells, positive clones could not be obtained in 200 transformants through immunoblotting analysis. That may be caused by the low expression level or instability of BBTS3 in WT cells in the presence of full-length IFT46. As we were able to express BBTS3 in ift46-1 (Fig. 2D), we then co-transformed IFT46 and a selection marker pHyg3 into strain ift46-1 BBTS3::YFP, resulting in ift46-1 BBTS3::YFP IFT46, which is comparable to CC-125 BBTS3 (Fig. 3C). Confocal and TIRFM imaging revealed that BBTS3 also localized at flagella in dotted pattern and moved along the axoneme (Fig. 3E,F, Movie 9). In summary, BBTS3 can also target IFT46 to cilia. Compared with IFT46-C1, BBTS3 lacks the glycine-rich tail (Fig. 2B, S3) which may contribute to its stability. For this reason, IFT46-C1 was chosen for subsequent studies.

**IFT46-C1 is assembled into the IFT machinery**

IFT46-C1 and BBTS3 are able to move along the axoneme and are likely incorporated into IFT-B complexes as IFT46. To prove this, we isolated and analyzed the flagellar membrane-plus-matrix fraction of CC-125 IFT46-C1::YFP and performed co-immunoprecipitation (CoIP) experiments. We found that IFT46-C1 forms a complex with IFT81, IFT74/72, and IFT52 just as the full-length IFT46 (Fig. 4A). Results of sucrose density gradient centrifugation also showed that IFT46-C1 forms a complex with IFT-B complex protein IFT81 (Fig. 4B). The overall data suggest that IFT46-C1 is assembled into the IFT machinery.
The basal body region localization of IFT46 depends on IFT52, but not vice versa

As IFT46 interacts at least with ODA16, IFT88, IFT81, IFT74, IFT70 and IFT52 (Ahmed et al., 2008; Fan et al., 2010; Lucker et al., 2005; Lucker et al., 2010; Taschner et al., 2011; Taschner et al., 2014), we next determined whether the basal body localization of IFT46 is dependent on other IFT components. If so, this typical accumulation will be abolished in corresponding null mutants. Accordingly, we expressed full-length IFT46::YFP and IFT46-C1::YFP in bld1, ift88, fla10-2, and dhc1b (Figs 5A,B, S4A,B,D,E,G,H). These strains are null mutants of IFT52, IFT88, FLA10, and DHC1b, respectively. Positive transformants expressing IFT46::YFP or IFT46-C1::YFP were screened using western blotting with GFP antibody (Fig. 5A,B). We also examined the subcellular localization of IFT46 in ift81-1 and ift122-1 using immunostaining (Fig. S4J,K). IFT46 or IFT46-C1 still localized at the basal body region in ift88, fla10-2 or dhc1b (Fig. S4C,F,I). These results demonstrated that the basal body localization of IFT46 is independent of IFT88, FLA10 or DHC1b. In ift81-1 or ift122-1, IFT46 colocalized with acetylated α-tubulin which predominantly located at the basal body and the stumpy flagella (Fig. S4J,K). It implies that the basal body localization of IFT46 is not disturbed. These data demonstrate that the basal body localization of IFT46 is independent of IFT81 or IFT122. Surprisingly, IFT46 or IFT46-C1 was not targeted to the basal body region in bld1 (Fig. 5C). IFT52 is at the center of the IFT-B complex, and makes several interactions with both IFT-B1 and IFT-B2 subunits (Lucker et al., 2005; Lucker et al., 2010; Taschner et al., 2011; Taschner et al., 2014; Taschner and Lorentzen, 2016; Taschner et al., 2016). The failure of basal body localization of IFT46 or IFT46-C1 in bld1 is not likely to be caused by their decreased expression level as we used IFT46 or IFT46-C1 highly expressed algal strains (Fig. 5A,B). In summary, the basal body localization of IFT46 depends upon IFT52.

The knock-out of IFT52 disrupts the basal body localization of IFT46. In theory, IFT46 will restore its basal body localization when bld1 is rescued by IFT52. Under that scenario, we expressed IFT52::3HA in bld1 IFT46::YFP or bld1 IFT46-C1::YFP (Fig. 5D,E). Indeed, IFT46::YFP or IFT46-C1::YFP was targeted to the basal body region in bld1 IFT46::YFP.
IFT52::3HA or bld1 IFT46-C1::YFP IFT52::3HA (Fig. 5F). This result further demonstrates that the basal body localization of IFT46 relies on IFT52. However, there are two possibilities. One is that the dependence is mutual and IFT52/46 work together to complete basal body targeting. The other is that IFT52 works upstream of IFT46 and recruits IFT46 to basal bodies. To exclude one of these possibilities, we expressed IFT52::YFP in ift46-1. Positive transformants were screened using western blotting with GFP antibody (Fig. 5G). Live cell imaging showed that IFT52 accumulated around the basal body region (Fig. 5H). This indicates that the basal body localization of IFT46 depends on IFT52, but not vice versa.

IFT52 binds and recruits IFT46 to the basal body region

The direct interaction between IFT46 and IFT52 has been demonstrated in vitro (Fan et al., 2010; Lucker et al., 2005; Lucker et al., 2010; Taschner et al., 2011; Taschner et al., 2014). We showed above that IFT46 relies on IFT52 for its basal body localization in vivo (Fig. 5). It is reasonable to speculate that the protein-protein interaction between IFT46 and IFT52 may mediate its basal body localization. Indeed, CoIP with whole-cell lysates of bld1 IFT46-C1::YFP IFT52::3HA and HA antibody showed that IFT46 and IFT46-C1 were enriched in precipitates. Thus, IFT46-C1 interacts with IFT52 as the full-length IFT46 (Fig. 6A). To further test whether the interaction is necessary for IFT46 basal body localization, we mutated two critical hydrophobic leucines to glutamic acids in IFT46-C1 (Figs 6B, S3). These two sites are evolutionarily conserved (Fig. S3) and located in the binding interface between CrIFT52C and CrIFT46C (Fig. 6C), as highlighted in the three dimensional structure modeled based on the TtIFT52C/46C crystal structure (Taschner et al., 2014). The L285E/L286E mutant of IFT46 no longer bound to the tagged C-terminal domain of IFT52 when co-expressed in E. coli, even though it was expressed at the same level as the WT IFT46 protein (Fig. 6D). When expressed in ift46-1, the expression level of IFT46-C1L285E/L286E declined to 32% on average compared with that of the non-mutated form of IFT46-C1 (Fig. 6E,F). This is in accordance with the fact that IFT46L285E/L286E does not form a subcomplex with IFT52 in vitro (Fig. 6D). The decreased expression level may be in large part due to degradation of free IFT46-C1L285E/L286E. As shown in Fig. 6G, mutations of
residues 285 and 286 in IFT46-C1 abolished its basal body localization. As IFT46-C1 and BBTS3 still localized at the basal body when their expression level decreased to about 30% after the presence of full-length IFT46 (Figs 3D,E), the aborted basal body localization of IFT46-C1_L285E/L286E cannot be ascribed to its low expression level. These results indicate that the interaction between IFT46-C1 and IFT52 is of vital importance for basal body localization of IFT46. IFT52 binds and recruits IFT46 to the basal body region.

NLS-tagged IFT52C recruits IFT46 to nuclei

IFT46-C1 is necessary and sufficient for the basal body and ciliary targeting of IFT46 (Figs 2, 3, 4). IFT52 binds and recruits IFT46-C1 to the basal body (Figs 5, 6). It is also known that some IFT46 associates with cytoplasmic vesicles and is then delivered to the basal body region in *Chlamydomonas* (Wood and Rosenbaum, 2014). However, there are still two possibilities regarding the molecular mechanism of the basal body targeting of IFT46. One is that IFT52 is delivered to the basal body first and recruits the free IFT46 to form a complex at the basal body. The interaction between IFT46 and IFT52 only occurs at the basal body region. The other is that newly synthesized IFT46 and IFT52 preassemble as subcomplexes and that these subcomplexes are delivered to the basal body region. The key difference between these two scenarios is the location where IFT46 assembles with IFT52. One is around the basal body and the other is in the TGN or cytoplasm. To rule out one of these possibilities, we aimed to express IFT52 in nuclei and examine whether IFT46 follows its localization.

There are various nuclear localization signals or sequences (NLSs) in animal and plant cells (Kropat et al., 2005; Lange et al., 2007; Rasala et al., 2014). However, only a few have been reported in *Chlamydomonas* (Kropat et al., 2005; Rasala et al., 2014). We attached simian virus 40 (SV40), 2×SV40, 4×SV40, copper response regulator 1 and nucleoplasmin NLSs to the C-terminus of YFP and expressed them in WT cells. Notably, only 4×SV40 NLS worked efficiently to bring the YFP into nuclei (Fig. 7B). For the sake of simplicity, NLS hereafter refers to 4×SV40 NLS. As previously reported, the C-terminus of IFT52 interacts with IFT46 (Lucker et al., 2010; Taschner et al., 2011; Taschner et al., 2014). Furthermore, overexpression of the C-terminus of MmIFT52 has a strong dominant-
negative effect on ciliogenesis in MDCK cells, which may be caused by the mislocalization of other IFT-B subunits (Taschner et al., 2014). These results make IFT52C a perfect target to be fused with the NLS. In WT cells, positive transformants expressing IFT52C::YFP::NLS were identified using immunoblotting with GFP antibody (Fig. 7A). Using confocal imaging, we found that the fusion protein IFT52C::YFP::NLS enriched in nuclei successfully just as YFP::NLS did (Fig. 7B), which was also apparent from immunoblotting against nuclear lysates (Fig. 7C). Likewise, IFT46 was detected in nuclear lysates of CC-125 IFT52C::YFP::NLS (Fig. 7C). This was further verified by immunostaining in which IFT46 colocalized with IFT52C::YFP::NLS in nuclei of CC-125 IFT52C::YFP::NLS (Fig. 7D). We also found that IFT52C immunoprecipitated with IFT46 in nuclear lysates of CC-125 IFT52C::YFP::NLS (Fig. 7E). These data demonstrate that IFT46 can be recruited ectopically to nuclei by NLS-fused IFT52C, which indicates that they have been preassembled in the cytoplasm, not at the basal body.
Discussion

Cilia are evolutionarily conserved organelles. Ciliogenesis and ciliary sensory functions rely on IFT. Although IFT was described more than 20 years ago (Kozminski et al., 1993), the mechanism of basal body localization for IFT complex has remained elusive. Our data demonstrated that the C-terminus of IFT46 is indispensable for its basal body targeting. IFT52 binds and recruits IFT46 to the basal body. The assembly of IFT52 and IFT46 can occur in the nucleus ectopically. These results suggested that they are preassembled in the cytoplasm or in the TGN, not at the basal body (Fig. 8). During the flagellar regeneration in *Chlamydomonas*, IFT46 was observed in the TGN-derived vesicle (Wood and Rosenbaum, 2014), it is possible that IFT52 and IFT46 are preassembled in the TGN and are delivered to the basal body through vesicular transport (Fig. 8). However, only IFT20 has been show to localized in Golgi and most IFT proteins are not membrane-associated (Follit et al., 2006; Richey and Qin, 2012). Thus, the possibility that IFT52/IFT46 preassembled in the cytoplasm and then targeted to the basal body through non-vesicle-mediated means cannot be ruled out (Fig. 8).

IFT46-C1 cannot be regarded as the basal body and ciliary targeting sequence of IFT46 because IFT46-C1 directly interacts with IFT52, which is the putative targeting carrier of IFT46 (Figs 4, 5). IFT46-C1 can no longer accumulate in basal bodies when this interaction is disrupted with its two important consensus residues mutated to glutamic acid (Fig. 5). However, the interaction partner of IFT46 is still an IFT subunit, IFT52 (Fig. 6). This makes IFT46-C1 not a general or "classical" basal body targeting sequence such as the third intracellular loop of different GPCRs, the Ax(S/A)xQ and RVxP motifs, nuclear localization signal or SUMOylation (Bhogaraju et al., 2013; Dishinger et al., 2010; Malicki and Avidor-Reiss, 2014; McIntyre et al., 2015). Future work should be carried out to identify the "real" targeting sequence in IFT52 or other upstream IFT proteins.

The IFT machinery has at least 22 members (Taschner and Lorentzen, 2016; Taschner et al., 2016). They all accumulate at the basal body. One simple model would be that all IFT proteins are translated at ribosomes and assembled at the basal body. However, IFT46 and IFT52 can assemble in the nucleus when the C-terminus of IFT52 was ectopically expressed in nuclei. These data did not support this model. Another possibility is that IFT
proteins preassemble as small subcomplex before their translocation to and localization at the basal body. Our results here support the latter case. In fact, Brown J. M. et al. found that IFT-A co-localized with IFT-B within the basal body IFT pool in WT, but not in *ift74-1* (Brown et al., 2015). These results implied that IFT-A and IFT-B were targeted to basal bodies independently. The speculation here matches observations that IFT-A localized normally to the basal body in null mutants of IFT-B and IFT-B proteins localized normally in null mutants of IFT-A components (Behal et al., 2012; Brown et al., 2015; Hou et al., 2007; Richey and Qin, 2012). Furthermore, CPLANE proteins can regulate the basal body recruitment of peripheral IFT-A, but not IFT-A core or IFT-B (Toriyama et al., 2016). The future work is to determine how many sub-complexes of IFT are assembled before targeting to the basal body and whether IFT cargoes are associated before these sub-complexes reach the basal body. These studies will pave the way for the deciphering of the mechanism of interaction between the IFT proteins, motors and cargoes at the basal body.
Materials and methods
Algal strains and culture conditions

The *Chlamydomonas reinhardtii* strains used in this study were listed in Table S1. The strains CC-125, *ift46-1*, *bld1*, *ift88*, *fla10-2* and *dhc1b* were obtained from the *Chlamydomonas* Resource Center, University of Minnesota. Strains *ift81-1* and *ift122-1* were gifted by Professor Junmin Pan at Tsinghua University in China.

The *Chlamydomonas* cells were grown in liquid Tris-acetate-phosphate (TAP) (Harris et al., 1989) or minimal (MI) (Sager and Granick, 1953) media at 22°C with constant agitation and illumination (about 25 μE.M⁻².S⁻¹) in 96-well plates, 250 mL Erlenmeyer flasks or 5 liter aerated glass container (Hu et al., 2014). For long term storage, the *Chlamydomonas* cells were maintained on solid TAP media supplemented with 1.5% agar at 20°C with a cycle of fourteen hours light and ten hours dark.

Primers and plasmid construction

To rescue the null mutant of *IFT46*, *ift46-1*, two plasmids pHK214 and pHK266 expressing IFT46::YFP were constructed as described previously (Cheng et al., in press). To create a vector expressing only YFP, a fragment was amplified from pHK214 using primers ACE-F and Pro-R. This fragment was cleaved with *NdeI* and *EcoRV*. The resulted fragment was cloned into *NdeI*/*EcoRV*-digested pHK214, creating pHK281.

To rescue the null mutant of *IFT52*, *bld1*, three plasmids pHK250 (expressing IFT52::YFP), pHK268 (expressing IFT52::YFP) and pHK409 (expressing IFT52::3HA) were created. The genomic DNA of *IFT52* was amplified using primers IFT52A-F/IFT52B-R with the genomic DNA of CC503 as the template. After cleaved by *NdeI*/*EcoRV*, the genomic DNA fragment was ligated into *NdeI*/*EcoRV*-digested pHK86 or pHK266, yielding pHK250 and PHK268, respectively. To fuse 3×HA tag on the C-terminus of IFT52, four oligonucleotides (HA1, HA2, HA3, and HA4) were annealed and the resulted fragment was inserted in frame into *EcoRV*/*EcoRI*-digested pHK250, resulting pHK409.

In order to express truncated versions of IFT46, pHK231, pHK232, pHK233, pHK243, pHK244 and pHK245 were constructed. These vectors can express IFT46-N1, IFT46-N, IFT46ΔC1, IFT46ΔN1, IFT46-C, and IFT46-C1 in *Chlamydomonas*, respectively. *IFT46-
N1 was amplified with primers ACE-F and A-R using pGEM-T Easy-IFT46 as the template. The PCR products were trimmed with NdeI and EcoRV and inserted into NdeI/EcoRV-digested pHK86, resulting pHK231. The expression vectors of IFT46-N (pHK232) and IFT46ΔC1 (pHK233) were constructed just as pHK231 except using primer sets ACE-F/C-R and ACE-F/E-R, respectively. To obtain the coding sequence of IFT46ΔN1, two elements, the promoter of IFT46 and the coding sequence of IFT46ΔN1, were needed. The promoter of IFT46 was amplified with primers ACE-F and B-R using pGEM-T Easy-IFT46 as the template. The coding fragment of IFT46ΔN1 was also amplified using pGEM-T Easy-IFT46 as the template with primers B-F and IFT46-R. These two elements were overlapped together and then cut with NdeI and EcoRV. The digested fragment was ligated into NdeI/EcoRV-cleaved pHK86, giving rise to pHK243. The construction procedures of pHK244 (expressing IFT46-C) and pHK245 (expressing IFT46-C1) were similar to those of pHK243 using different primers. Primer pairs ACE-F/D-R and ACE-F/F-R were used to amplify the promoter of IFT46 for pHK244 and pHK245, respectively. Their coding fragments were obtained using D-F/IFT46-R and F-F/IFT46-R, respectively.

To express truncated versions of IFT46-C1, pHK308, pHK310, pHK309, pHK311, pHK312 and pHK313 were constructed. These vectors can express BBTS1, BBTS3, BBTS2, BBTS4, BBTS5, and BBTS6, respectively. BBTS1 was amplified using primers BBTS-F and BBTS1-R with pHK245 as the template. The PCR products were cut with NdeI/EcoRV and then cloned back into NdeI/EcoRV-digested pHK245, yielding pHK308. The plasmid expressing BBTS3, pHK310, was constructed in the similar way using primers BBTS-F and BBTS3-R. To create pHK309 expressing BBTS2, the promoter sequence of IFT46 (amplified from pHK245 using primers BBTS-F and BBTS2-R) and the coding region of BBTS2 (amplified from pHK245 using primers BBTS2-F and IFT46-R) were overlapped together. Then, the overlapped PCR products were digested with NdeI/EcoRV and subcloned into pHK245. The cloning procedures of pHK311 (expressing BBTS4), pHK312 (expressing BBTS5) and pHK313 (expressing BBTS6) were identical to pHK309. To amplify the promoter sequence of IFT46 for pHK311, pHK312, and pHK313, primer sets BBTS-F/BHTS4-R, BBTS-F/BHTS5-R1, and BBTS-F/BHTS6-R were used, respectively. To obtain the coding sequence of BBTS4, five oligonucleotides (BBTS4-1, BBTS4-2,
BBTS4-3, BBTS4-4 and BBTS4-5) were annealed together. To amplify the coding region of BBTS5, primers BBTS5-F/BBTS5-R2 were used. The coding DNA of BBTS6 was generated using similar procedures to that of BBTS4. Thus, it was created by annealing BBTS6-6, BBTS6-7, BBTS4-2, BBTS4-3 and BBTS4-5.

To construct pHK464 expressing YFP::NLS, the second yfp gene in pHK469 was removed by digestion with EcoRV/EcoRI and replaced by 4×SV40 NLS (4×DPKKKRKV). The 4×SV40 NLS was obtained by annealing five oligonucleotides, namely 4×NLS-1F, 4×NLS-2R, 4×NLS-3F, 4×NLS-4R, and 4×NLS-5R. The yfp-NLS fragment in pHK464 was amplified with primers NLS-F/NLS-R. The PCR products were trimmed with SmaI/EcoRI and cloned into EcoRV/EcoRI-digested pHK409, giving rise to pHK470. To further create pHK473 expressing IFT52C::YFP::NLS, a fragment was amplified reversely from pHK470 with primers IFT52C-F and IFT52C-R. The purified reverse PCR products were assembled seamlessly via In-Fusion HD Cloning method (Clontech, #639648, Japan).

All plasmids and primers used in this work are listed in Table S2 and S3, respectively. All constructs were verified by sequencing (Shenggong Inc., China).

Site-directed mutagenesis

The fragment of IFT46-C1 was cut out from pHK245 with NdeI/EcoRV and cloned into NdeI/EcoRV-digested pHK266, resulting pHK242. In order to disrupt the protein-protein interaction between IFT46-C1 and IFT52, pHK242 which expresses IFT46-C1 was mutated using Fast Mutagenesis System (TRANS, #FM111-01) with primers SDM-F and SDM-R, generating pHK267. When expressed, the critical binding sites of IFT46-C1 with IFT52, namely leucine 285 and 286, were replaced by glutamic acid residues.

Measurement of flagellar length

The cells were fixed with 0.5% Lugol’s solution for 2 minutes at room temperature. Flagellar lengths were measured under a phase microscope (Nikon Eclipse Ti) equipped with an electron multiplying charge-coupled device. For each sample, at least fifty cells and one hundred flagella were measured.
**Bioinformatic analysis**

The genomic DNA and protein sequences of *IFT46* and *IFT52* were retrieved from *Chlamydomonas reinhardtii* v5.5 database in Joint Genome Institute Phytozome 11. The protein sequences of *IFT46* of *Volvox carteri f. nagariensis* (XP_002950030), *Homo sapiens* (NP_001162089), *Mus musculus* (NP_076320), *Caenorhabditis elegans* (NP_001076770), *Xenopus laevis* (NP_001090393), *Bos taurus* (NP_001068677), *Canis lupus familiaris* (XP_536553), *Rattus norvegicus* (NP_001019931), *Gallus gallus* (XP_417918), *Danio rerio* (XP_003199413), *Strongylocentrotus purpuratus* (XP_795443), *Schistosoma japonicum* (Q5DHJ5), *Emiliania huxleyi* (XP_005763582), *Tetrahymena thermophila* (XP_001017111) and *Apis mellifera* (XP_006565024) were obtained from National Center for Biotechnology Information (NCBI). Multiple sequence alignment was performed by Clustal X 2.0 (Larkin et al., 2007).

The secondary structures of *IFT46* were predicted by PSIPRED (Buchan et al., 2013). The protein disorder of *IFT46* was analyzed using GeneSilico Metadisorder (Kozlowski and Bujnicki, 2012). The crystal structure data of *Tetrahymena thermophila* TtIFT52C/46C was downloaded from RCSB Protein Data Bank (ID: 4UZZ). The three dimensional structure of CrIFT52C/46C was modeled based on TtIFT52C/46C using the server Phyre2 (Kelley et al., 2015; Taschner et al., 2014).

**Electro-transformation of Chlamydomonas reinhardtii**

Plasmids were linearized by *NdeI* or *ScaI* before electro-transformation. Detailed procedures of electroporation were carried out as described previously (Brown et al., 1991; Hu et al., 2014; Shimogawara et al., 1998).
Protein extraction and quantification

Whole cell soluble proteins were extracted as described previously (Fowkes and Mitchell, 1998; Hu et al., 2014). Nuclear proteins were extracted with CellLytic PN Plant Nuclei Isolation/Extraction Kit (Sigma-Aldrich, #CELLYTPN1, U.S.) according to manufacturer’s guidelines. The protein concentration was determined by Amido black 10B binding method using BSA as a standard (Hu et al., 2014; Schaffner and Weissmann, 1973).

Flagellar isolation and fractionation

Flagella were isolated by pH shock as previously described (Cole et al., 1998; Witman et al., 1972). For membrane-plus-matrix and axonemal fractionation, the flagellar pellets were dissolved in HMDEK buffer containing 0.5% NP-40. The suspension was rotated for 20 minutes at 4°C and centrifuged at 10000 g for 10 minutes at 4°C. The supernatant and pellet were membrane-plus-matrix and axonemal fractions, respectively.

SDS-PAGE and immunoblotting assay

SDS-PAGE and immunoblotting assay were performed as previously described (Hu et al., 2014). Primary and secondary antibodies used in this study are listed in Table S4. For quantification of the signals of immunoblotting, the developed films were scanned and analyzed using Adobe Photoshop CS6.

Pull-down assay

CrIFT46 (wt or mutant) and CrIFT52C constructs were transformed either alone or in combination in E. coli BL21(DE3). Proteins were expressed and purified as reported previously (Taschner et al., 2016). GST-pulldown assays were done as previously described (Taschner et al., 2016).

Sucrose density gradient centrifugation

Sucrose density gradient centrifugation of flagellar membrane-plus-matrix fraction was performed as described previously (Behal and Cole, 2013). Firstly, insoluble materials in
flagellar membrane-plus-matrix fraction were removed by centrifugation twice at 100000 g for 10 minutes at 4°C. Then, 150 μL supernatant was loaded on top of 10%-25% sucrose density gradients. This 5 mL ultracentrifuge tube was centrifuged at 200000 g for 4.5 hours at 4°C in the MLS-50 rotor (Optima-XP Ultracentrifuge, Beckman Coulter). The resulting gradients were fractioned into 200 μL aliquots. Aliquots were analyzed by SDS-PAGE and immunoblotting.

**Immunofluorescence staining**

Immunofluorescence staining of Chlamydomonas cells were performed as detailed previously (Engel et al., 2012). If needed, 5 μg/mL DAPI was added in PBS for followed washing steps. Antibodies used for immunofluorescence staining are given in Table S4.

**Immunoprecipitation**

Immunoprecipitation was done as described by Richey et al. (Richey and Qin, 2013).

**Nucleus isolation**

Nuclei of *Chlamydomonas* were isolated using an optimized method as described previously (Winck et al., 2011) with one additional step. Specifically, the cell pellets were digested with autolysin for 1 hour at room temperature before cryogenic grinding with liquid nitrogen.

**RNA preparation and quantitative real-time PCR**

Total RNA was isolated using TRIzol reagent (Invitrogen). cDNA was generated through reverse transcription using First Strand cDNA Synthesis Kit with oligo(dT)₁₈ primers (Thermo Scientific). Quantitative real-time PCR was performed with Applied Biosystems 7900HT Fast Real-Time PCR System according to the manufacturer's instructions using SYBR Green Realtime PCR Master Mix (TOYOBO). *CBLP* served as the control. For *IFT46*, primer pair 2 was used. For *CBLP*, CBLP-F and CBLP-R were used.
Microscopy

Live cell imaging of *Chlamydomonas* was performed on an NLS-LSM710 confocal laser-scanning microscope (Carl Zeiss, Germany) using a 63× oil objective lens (NA 1.40). The excitation and emission parameters were listed as follows: chlorophyll, 488 nm/560-580 nm; YFP, 512 nm/515-560 nm; CFP, 458 nm/459-530 nm. Images of immunostaining samples were obtained on a Leica TCS SP8 confocal microscope equipped with a 63× oil-immersion lens (NA 1.40). The excitation and emission parameters were listed as follows: DAPI, 405 nm/423-488 nm; FITC, 488 nm/495-545 nm; Alexa Fluor 594, 552 nm/612-671 nm. Brightness and contrast were adjusted using Carl Zeiss Zen 2009 Light Edition, Leica LAS AF 2.6.3, or ImageJ 1.46r.

IFT measurement

Visualization and recording of IFT were done according to published protocols (Engel et al., 2012; Lechtreck, 2013; Lechtreck, 2016). Videos were recorded with Nikon Eclipse Ti total internal reflection fluorescence microscope. This microscope was equipped with a cooled electron multiplying charge-coupled device, a 100× oil objective lens (NA 1.49) and a 514 nm laser with a 540 nm bandpass (30 nm) filter.

Statistical analysis

Statistical results were obtained from at least three independent experiments. All data were reported as mean ± s.e.m. or mean ± s.d. Differences between groups were tested by analysis of variance using Student’s *t* test or one-way ANOVA followed by *Turkey* test using Prism 5.01 (GraphPad Software, U.S.). Differences were considered significant when *P* was less than 0.05. Asterisks represent *P* value of < 0.05 (*), <0.01 (**), and < 0.001 (***)}, respectively.
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We appreciate Junmin Pan for kindly offering us the null mutants of *ift81* and *ift122*. We thank Gai Liu for help with the amplification of the genomic DNA of *IFT52*. We thank Kangsup Yoon, Jinliang Liu, Keke Zhang, and Wei Li for their comments and suggestions that improved the manuscript.

Competing interests

No competing interests declared.

Author contributions

BL designed and performed research, analysed data and wrote the manuscript; LW, MT and XC designed and performed research; LW, MT and XC contributed to design and editing of the manuscript; EL and KYH supervised and designed research, analysed data and wrote the manuscript.

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Fig. 1. YFP-tagged IFT46 localizes at the basal body and flagella.

(A) Schematic diagram of the construct used to rescue *ift46-1*. Its predicted transcript and corresponding protein in *Chlamydomonas* are also shown below. L: protein linker sequence. Red triangles represent primer pairs used to detect mRNA of *IFT46* and *IFT46-L-YFP*.

(B) RT-PCR with cDNA templates from WT, *ift46-1*, and rescued cells *ift46-1 IFT46::YFP* using primers designed to amplify transcripts from the 5' (primer pair 1), middle (primer pair 2), 3' (primer pair 3) regions of *IFT46* gene and *yfp* gene (primer pair 4). Expression of CBLP was used as the internal control.

(C) Quantification the expression level of *IFT46* in *ift46-1 IFT46::YFP* using real-time PCR (five samples for each group). Values were normalized to the expression level of *IFT46* in WT cells and expressed as mean ± s.e.m. Statistical analysis was performed using two-
tailed unpaired Student's t test. n.s.: not significant.

(D) Western blot analysis of whole cell lysates (5 μg protein per lane) of \textit{ift46-1}, CC-125, and \textit{ift46-1 IFT46::YFP} probed with antibodies raised against IFT46, GFP or α-tubulin. The asterisk indicates nonspecific bands. IB: immunoblot.

(E) Live cell imaging of non-transformed \textit{ift46-1} cells and \textit{ift46-1} expressing YFP or IFT46::YFP. White dashed circles mark the nucleus area. White arrowhead: eyespot; CHL: chlorophyll; Scale bar: 5 μm.

(F) TIRFM imaging of the flagella of \textit{ift46-1 IFT46::YFP}. White arrowheads mark IFT trains. Scale bar: 5 μm.
Fig. 2. IFT46-C1 and BBTS3 target IFT46 to the basal body.

(A) Diagram of the full-length and the truncated IFT46 produced in this study. YFPs are fused to the C-termini of full-length and the truncated IFT46.

(B) Diagram of the truncation constructs of IFT46-C1.

(C, D) Immunoblots of whole-cell lysates (5 μg protein per lane) of ift46-1 expressing indicated proteins probed with anti-GFP antibody. IB: immunoblot.

(E) Confocal imaging of ift46-1 expressing the indicated fusion proteins. White arrowheads:
eyespots; CHL: chlorophyll; Scale bar: 5 μm.

(F) Confocal imaging of *ilt46-1* expressing the indicated fusion proteins. BB in A and B represents the basal body localization results of the fusion proteins. “+” means yes. “-” indicates not. In E and F, arrowheads indicate eyespots. CHL represents chlorophyll and scale bars are 5 μm.
Fig. 3. IFT46-C1 and BBTS3 target IFT46 to cilia and move along axoneme.

(A, B) Immunoblots of whole-cell lysates (5 μg protein per lane) of CC-125 expressing indicated proteins probed with the anti-GFP antibody. “+” means proteins localize at the basal body (BB), “-” indicates not. IB: immunoblot.

(C) Immunoblot of flagellar membrane-plus-matrix fractions of CC-125 expressing indicated proteins probed with the indicated antibodies. “+” means proteins localize in cilia (CI) or assembled in IFT particles (AIIP). “-” indicates not. IB: immunoblot.

(D) Confocal imaging of CC-125 expressing the indicated fusion proteins. White arrowheads: eyespots; CHL: chlorophyll; Scale bar: 5 μm.
(E) Confocal imaging of \textit{itt46-1} expressing BBTS3::YFP and IFT46. White arrowheads: eyespots; CHL: chlorophyll; Scale bar: 5 μm.

(F) TIRFM imaging of the flagella of the strains listed in D and E. Differential interference contrast images are shown on the left. CHL: chlorophyll. Scale bar: 5 μm.
Fig. 4. IFT46-C1 is assembled into IFT machinery.

(A) Anti-GFP antibody was used to immunoprecipitate YFP-tagged IFT46-C1 from the flagellar membrane-plus-matrix of *ift46-1 IFT46::YFP* and CC-125 IFT46-C1::YFP. Immunoblots of the precipitate were probed with antibodies against IFT81, IFT74/72 and IFT52. mIgG: mouse IgG; IP: immunoprecipitation.

(B) Flagellar membrane-plus-matrix of CC-125 IFT46-C1::YFP was separated by sucrose density gradient centrifugation and analyzed by immunoblots probed with antibodies as indicated. IFT46 migrates as doublet bands due to phosphorylation. IB: immunoblot. Numbers at the bottom indicate lanes.
Fig. 5. The basal body localization of IFT46 depends on IFT52, but not vice versa.

(A) Western blots of whole-cell lysates (5 μg protein per lane) of bld1, CC-125, and bld1 IFT46::YFP probed with the anti-GFP antibody and anti-α-tubulin antibody. IB: immunoblot.

(B) Western blots of whole-cell lysates (5 μg protein per lane) of ift46-1 IFT46-C1::YFP, bld1, and bld1 IFT46-C1::YFP probed with the indicated antibodies. IB: immunoblot.

(C) Confocal imaging of bld1 expressing IFT46::YFP or IFT46-C1::YFP. CHL: chlorophyll; Scale bar: 5 μm.

(D) Western blots of whole-cell lysates (5 μg protein per lane) of bld1 IFT52::3HA, bld1, and bld1 IFT46::YFP IFT52::3HA probed with the indicated antibodies. IB: immunoblot.

(E) Western blots of whole-cell lysates (5 μg protein per lane) of bld1 IFT52::3HA, bld1, and bld1 IFT46-C1::YFP IFT52::3HA probed with the indicated antibodies. IB: immunoblot.
(F) Confocal imaging of *bld1* expressing IFT46::YFP IFT52::3HA or IFT46-C1::YFP IFT52::3HA. CHL: chlorophyll; Scale bar: 5 μm.

(G) Western blots of whole-cell lysates (5 μg protein per lane) of *ift46-1 IFT46::YFP, ift46-1*, and *ift46-1 IFT52::YFP* probed with the indicated antibodies. IB: immunoblot.

(H) Confocal imaging of *ift46-1* expressing IFT52::YFP. CHL: chlorophyll; Scale bar: 5 μm.
Fig. 6. IFT52 binds and recruits IFT46 to the basal body.

(A) Anti-HA antibody was used to immunoprecipitate HA-tagged IFT52 from whole-cell lysates of CC-125, bld1 IFT52::3HA and bld1 IFT46-C1 IFT52::3HA. Immunoblots of the precipitate were probed with antibodies as indicated. IP: immunoprecipitation; IB: immunoblot.

(B) Schematic diagram of site-directed mutagenesis of IFT46 gene. The upper panel shows the original DNA and protein sequence while the lower pane displays mutated one.

(C) Model of CrIFT52C/46C based on the TtIFT52C/46C crystal structure (PDB ID: 4UZZ). The two residues we chose for mutagenesis were highlighted as grey sticks.

(D) Pull-down of His-tagged GST-IFT52(366-454) with untagged IFT46WT or IFT46L285E/L286E coexpressed in E. coli demonstrates that double point mutations are
sufficient to disrupt the interaction between IFT46 and IFT52. The weak band in the area where IFT46 WT,MUT ran (Fig. 6D, lane 8) was due to non-specific binding of the IFT46 protein to the beads (as it was also present in the control, lane 7). Numbers at the bottom indicate lanes.

(E) Western blots of whole-cell lysates (3 μg protein per lane) of ift46-1 IFT46::YFP, ift46-1 and ift46-1 IFT46-C1 L285E/L286E probed with the indicated antibodies. IB: immunoblot.

(F) Relative expression level of IFT46-C1 L285E/L286E in three transformants compared to the expression level of IFT46-C1. Values are shown as the mean ± s.e.m. of five independent experiments. Statistical analysis was performed using one-way ANOVA followed by Turkey test. ***P < 0.001.

(G) Confocal imaging of non-transformed cells ift46-1 and ift46-1 expressing IFT46-C1::YFP or IFT46-C1 L285E/L286E::YFP. White arrowheads: eyespots; CHL: chlorophyll; Scale bar: 10 μm.
Fig. 7. NLS-tagged IFT52C recruits IFT46 to nuclei.

(A) Western blots of whole-cell lysates (5 μg protein per lane) of CC-125, CC-125 YFP, CC-125 YFP::NLS, bld1 IFT52::YFP and CC-125 IFT52C::YFP::NLS probed with the indicated antibodies. IB: immunoblot.

(B) Live cell imaging of WT cells and WT cells expressing YFP, YFP::NLS and IFT52C::YFP::NLS. White dashed circles mark the nucleus area. CHL: chlorophyll; Scale bar: 5 μm.

(C) Western blots of nuclear lysates (2 μg protein per lane) of CC-125, CC-125 YFP, CC-125 YFP::NLS, bld1 IFT52::YFP and CC-125 IFT52C::YFP::NLS probed with the indicated antibodies. IB: immunoblot.
(D) Immunofluorescence microscopy of CC-125 YFP::NLS and CC-125 IFT52C::YFP::NLS using anti-GFP antibody (red), anti-IFT46 antibody (green) and 4, 6-diamidino-2-phenylindole (DAPI, blue). Scale bar: 5 μm.

(E) Anti-GFP antibody was used to immunoprecipitate IFT52C::YFP::NLS from the nuclei of CC-125 IFT52C::YFP::NLS. mlgG: mouse IgG.
Fig. 8. Model for basal body localization of IFT46.

Newly synthesized IFT52 and IFT46 from the ribosome are preassembled in the cytoplasm and are delivered to the basal body in a non-vesicle-mediated way. A second scenario is that IFT52 and IFT46 are preassembled in the TGN and are targeted to the basal body through the vesicle-mediated eay. BB: basal body.
Fig. S1. YFP-tagged IFT46 rescued the flagellar defects in *ift46-1*.

(A) Flagella length of rescued strain *ift46-1 IFT46::YFP* is nearly identical to that of WT cells. At least 100 flagella per experiment (n=6) were measured. Scatter plots show the median, the upper and the lower quartiles. Dots represent individual data points.

(B) Percentages of ciliated cells of WT and the rescued strains *ift46-1 IFT46::YFP*. At least 100 cells per experiment (n=6) were measured. Values are shown as the mean ± s.e.m. Statistical analysis was performed using one-way ANOVA followed by Turkey test. n.s.: not significant.

(C) A typical kymograph generated from an image stack of flagella of *ift46-1 IFT46::YFP* showed anterograde and retrograde tracks. The horizontal axis matches the length of one flagellum while the vertical axis corresponds to the passed time. Horizontal scale bar: 2 μm; vertical scale bar: 2 s.

(D) Frequency distribution of anterograde IFT velocity (green bar, n=149) and retrograde IFT velocity (blue bar, n=145) of *ift46-1 IFT46::YFP*.

(E) IFT frequencies obtained from kymographs of image stacks (for anterograde frequency, n = 21; for retrograde frequency, n = 20).
Fig. S2. Advanced structure predictions of IFT46.

(A) Secondary structure prediction of IFT46. Meanings of symbols were given below.

(B) Protein disorder prediction indicates that the N-terminus and the C-terminus tail of IFT46 are largely disordered. The line at 0.5 (vertical axis) is the cutoff for disorder (> 0.5) and order (< 0.5) predictions. Curved lines with different colors represent results returned by four different meta method as shown.

(C) Domain prediction of IFT46 using PSIPRED DomPred. Potential domain boundaries were shown in colored boxes.
Fig. S3. IFT46-C1 is highly conserved in evolution.

The amino acid sequences of IFT46-C1 from indicated species were aligned. The black triangles mark the two critical hydrophobic leucines we chosen for mutagenesis. The black squares mark the glycine-rich tail of CrIFT46. The consensus sequence and the graph showing percentages of conservation are given at the bottom.
Fig. S4. The basal body localization of IFT46 is independent of IFT122, IFT88, IFT81, FLA10 or DHC1b.

(A) Western blots of whole-cell lysates (5 μg protein per lane) of *ift46-1 IFT46::YFP, ift88 null, and ift88 IFT46::YFP probed with the indicated antibodies. IB: immunoblot.

(B) Western blots of whole-cell lysates (5 μg protein per lane) of *ift46-1 IFT46-C1::YFP, ift88 null, and ift88 IFT46-C1::YFP probed with the indicated antibodies. IB: immunoblot.

(C) Confocal imaging of *ift88 null mutant expressing IFT46::YFP or IFT46-C1::YFP. White arrows mark the eyespots. CHL: chlorophyll.

(D) Western blots of whole-cell lysates (5 μg protein per lane) of *ift46-1 IFT46::YFP, dhc1b null, and dhc1b IFT46::YFP probed with the indicated antibodies. IB: immunoblot.

(E) Western blots of whole-cell lysates (5 μg protein per lane) of *ift46-1 IFT46-C1::YFP,
dhc1b null, and dhc1b IFT46-C1::YFP probed with the indicated antibodies. IB: immunoblot.

(F) Confocal imaging of dhc1b null mutant expressing IFT46::YFP or IFT46-C1::YFP. CHL: chlorophyll.

(G) Western blots of whole-cell lysates (5 μg protein per lane) of ift46-1 IFT46::YFP, fla10-2, and fla10-2 IFT46::YFP probed with the indicated antibodies. IB: immunoblot.

(H) Western blots of whole-cell lysates (5 μg protein per lane) of ift46-1 IFT46-C1::YFP, fla10-2, and fla10-2 IFT46-C1::YFP probed with the indicated antibodies. IB: immunoblot.

(I) Confocal imaging of fla10-2 expressing IFT46::YFP or IFT46-C1::YFP. CHL: chlorophyll.

(J) Immunostaining of ift81-1 using anti-acetylated α-tubulin antibody (red), anti-IFT46 antibody (green) and DAPI (blue).

(K) Immunostaining of ift122-1 using anti-acetylated α-tubulin antibody (red), anti-IFT46 antibody (green) and DAPI (blue).

In panel C, F, I and J, scale bars represent 5 μm.

Tables

Table S1

Click here to Download Table S1
Table S2. Plasmids used in this study.

<table>
<thead>
<tr>
<th>Plasmid Number</th>
<th>Relevant Genotype or usage&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Source</th>
</tr>
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<tbody>
<tr>
<td>pGEM-T Easy-IFT46</td>
<td>plIFT46, AMP&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Dr. Joel Rosenbaum</td>
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<td>Dr. Wolfgang Mages</td>
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<td>for TA cloning, AMP&lt;sup&gt;R&lt;/sup&gt;</td>
<td>TaKaRa Bio Inc.</td>
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<sup>*</sup>AMP<sup>R</sup>, ampicillin resistance; PRM<sup>R</sup>, paromomycin resistance; HYG<sup>R</sup>, hygromycin B resistance

Table S3

Click here to Download Table S3
Table S4. Antibodies used in this study.

<table>
<thead>
<tr>
<th>Immunogen</th>
<th>Biological source</th>
<th>Dilution Factor</th>
<th>Note and Source</th>
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<td>1: 1000</td>
<td>Sigma, #T6793</td>
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<td>mouse</td>
<td>1: 1000</td>
<td>Roche, #11814460001</td>
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<td>mouse</td>
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<td>HA</td>
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<td>histone H3</td>
<td>rabbit</td>
<td>1: 5000</td>
<td>Agrisera, #AS10710</td>
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<td>IFT46</td>
<td>rabbit</td>
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<td>Prepared by Genscript using the N-terminal 20 amino-acids peptide and purified using the same peptide</td>
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<td>(Deane et al., 2001)</td>
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<td>rabbit</td>
<td>1: 2500</td>
<td>(Qin et al., 2004)</td>
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<td>IFT81</td>
<td>mouse</td>
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<td>(Lucker et al., 2005)</td>
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<td>mIgG</td>
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<td>1: 500</td>
<td>Alexa Fluor 488 conjugated, Life technologies, A-11029</td>
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<td>HRP conjugated, Sigma-Aldrich, #A4416</td>
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Movies

Movie 1 Locomotion of WT cells.

A representative movie taken for WT cells. Scale bar: 10 μm. Images were observed under a DIC microscope with a 100x objective, recorded at one frame per 0.13 sec and displayed as it is.
Movie 2 Immobility of *ift46-1*.

A representative movie taken for the null mutant of *IFT46, ift46-1*. Scale bar: 10 μm. Images were observed under a DIC microscope with a 100x objective, recorded at one frame per 0.13 sec and displayed as it is.
Movie 3 Locomotion of *ift46-1 IFT46::YFP*.

A representative movie taken for the rescued strain *ift46-1 IFT46::YFP*. Scale bar: 10 μm. Images were observed under a DIC microscope with a 100x objective, recorded at one frame per 0.13 sec and displayed as it is.
Movie 4 Dynamics of IFT46::YFP in the flagella of the rescued strain *ift46-1 IFT46::YFP*.

A representative movie shows IFT46::YFP in the flagella of the rescued strain *ift46-1 IFT46::YFP*. Scale bar: 10 μm. Images were observed under a total internal reflection fluorescence microscope with a 100x objective, recorded at one frame per 0.13 sec and displayed as it is.
Movie 5 Dynamics of IFT46::YFP in the flagella of WT cells.

A representative movie shows IFT46::YFP in the flagella of the WT cells. Scale bar: 10 μm. Images were observed under a total internal reflection fluorescence microscope with a 100x objective, recorded at one frame per 0.13 sec and displayed as it is.
Movie 6 Dynamics of IFT46ΔN1::YFP in the flagella of WT cells.
A representative movie shows IFT46ΔN1::YFP in the flagella of the WT cells. Scale bar: 10 μm. Images were observed under a total internal reflection fluorescence microscope with a 100x objective, recorded at one frame per 0.13 sec and displayed as it is.
Movie 7 Dynamics of IFT46-C::YFP in the flagella of WT cells.

A representative movie shows IFT46-C::YFP in the flagella of the WT cells. Scale bar: 10 μm. Images were observed under a total internal reflection fluorescence microscope with a 100x objective, recorded at one frame per 0.13 sec and displayed as it is.
Movie 8 Dynamics of IFT46-C1::YFP in the flagella of WT cells.

A representative movie shows IFT46-C1::YFP in the flagella of the WT cells. Scale bar: 10 μm. Images were observed under a total internal reflection fluorescence microscope with a 100x objective, recorded at one frame per 0.13 sec and displayed as it is.
Movie 9 Dynamics of BBTS3::YFP in the flagella of strain *ift46-1 BBTS3::YFP IFT46*.

A representative movie shows BBTS3::YFP in the flagella of strain *ift46-1 BBTS3::YFP IFT46*. Scale bar: 10 μm. Images were observed under a total internal reflection fluorescence microscope with a 100x objective, recorded at one frame per 0.13 sec and displayed as it is.