Tetrahymena IFT122A is not essential for cilia assembly but plays a role in returning IFT proteins from the ciliary tip to the cell body

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Summary
Intraflagellar transport (IFT) moves multiple protein particles composed of two biochemically distinct complexes, IFT-A and IFT-B, bi-directionally within cilia and is essential for cilia assembly and maintenance. We identified an ORF from the Tetrahymena macronuclear genome sequence, encoding IFT122A, an ortholog of an IFT-A complex protein. Tetrahymena IFT122A is induced during cilia regeneration, and epitope-tagged Ift122Ap could be detected in isolated cilia. IFT122A knockout cells still assembled cilia, albeit with lower efficiency, and could regenerate amputated cilia. Ift172p and Ift88p, two IFT-B complex proteins that localized mainly to basal bodies and along the cilia in wild-type cells, became preferentially enriched at the ciliary tips in IFT122A knockout cells. Our results indicate that Tetrahymena IFT122A is not required for anterograde transport-dependent ciliary assembly but plays a role in returning IFT proteins from the ciliary tip to the cell body.

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Key words: Intraflagellar transport, Cilia/flagella, Ciliary assembly, WD domain protein, Gene targeting

Introduction
Cilia/flagella are present in most eukaryotic organisms, including protozoans and humans, and share a highly organized and conserved microtubule-based structure, the axoneme (Sleigh, 1974). Intraflagellar transport (IFT) is a bi-directional process that moves particles along the axonemal microtubule tracks within the cilia/flagella and plays an essential role in cilia assembly and length maintenance (Kozminski et al., 1993; Marshall and Rosenbaum, 2001; Rosenbaum and Witman, 2002). Anterograde IFT, mediated by kinesin-2 motor proteins, moves IFT particles and their cargos from the cell body to the ciliary tip where the motor-cargo-IFT complexes dissociate from the microtubules, interact with ciliary tip proteins, change their configuration and probably exchange their cargo (Iomini et al., 2001; Kozminski et al., 1995; Pedersen et al., 2006; Pedersen et al., 2005). Retrograde IFT, mediated by cytoplasmic dynein 1b, returns the modified complex from the ciliary tip back to the cell body (Pazour et al., 1999; Porter et al., 1999; Signor et al., 1999). When the turnaround step or retrograde transport is impeded, ciliary proteins fail to return to the cell body and accumulate at the distal region (Hou et al., 2004; Pazour et al., 1998; Schafer et al., 2003).

IFT particles in the green alga Chlamydomonas reinhardtii contain multiple proteins that can be separated into two complexes using sucrose gradient centrifugation (Cole, 2003; Cole et al., 1998). IFT complex A (IFT-A) contains five proteins including IFT140, 122A and 122B. IFT complex B (IFT-B) is composed of at least 11 proteins including IFT172, 88, 57 and 52. The physiological significance of the two complexes is not fully understood, but recent data suggest that they have distinct roles (Scholey, 2003). Numerous loss-of-function mutant strains of IFT-B genes have been obtained in Chlamydomonas, Tetrahymena, Caenorhabditis elegans, Drosophila, zebrafish and mouse by forward genetic screening or reverse genetic manipulation (Beales et al., 2007; Brazelton et al., 2001; Brown et al., 2003; Deane et al., 2001; Follit et al., 2006; Han et al., 2003; Haycraft et al., 2003; Hou et al., 2007; Huangfu and Anderson, 2005; Pazour et al., 2000; Qin et al., 2001; Qin et al., 2007; Sun et al., 2004). The common phenotype of these IFT-B mutants is loss or abnormal shortening of cilia/flagella, arguing that IFT-B proteins function in anterograde IFT and play an essential role in cilia assembly (Scholey, 2003). Fewer mutant phenotypes of the IFT-A genes have been described. For example, morpholino knockdown of zebrafish IFT140 does not lead to the sensory neuronal defect observed in IFT-B gene knockout strains (Tsujikawa and Malicki, 2004). The C. elegans mutations in CHE-11/IFT140 and DAF-10/IFT122A cause the diameter of neuronal cilia to become irregular and electron-dense material to accumulate (Perkins et al., 1986). The Chlamydomonas retrograde IFT mutant strains fla15, fla16 and fla17-1 all lack the same two IFT-A proteins in the flagella (Iomini et al., 2001), but the actual mutated genes are not yet identified. These observations suggest a correlation between retrograde IFT and IFT-A complex proteins, but this relationship has not yet been directly demonstrated.

The ciliated protozoan Tetrahymena thermophila possesses hundreds of motile oral and somatic cilia with the canonical ciliary structure found in most organisms (Gaertig, 2000). With the recently available macronuclear genome sequence (Eisen et al., 2006) and facile gene knockout and replacement methods (Hai et al., 2000), Tetrahymena is an ideal model system to elucidate the function of conserved ciliary genes. In this paper, we report characterization of an IFT-A gene, IFT122A, in this organism. We
identified an IFT122A ortholog from the *Tetrahymena* genome database and generated IFT122A knockout strains. Phenotypic studies revealed that, while IFT122A is not essential for cilia assembly, it is required for the efficient return of IFT proteins from the ciliary tip to the cell body. Our results strongly support the hypothesis that IFT-A is involved in retrograde IFT.

**Results**

*Tetrahymena IFT122A* is a conserved gene

Compared with studies on IFT-B proteins, little is known about the physiological role of IFT-A proteins. To explore the functional implication of an IFT-A protein in typical motile cilia, we used reverse genetic techniques to study IFT122A in *Tetrahymena*. IFT122A is the smallest IFT-A protein whose sequence was available in the GenBank database, allowing us to search for possible orthologs encoded in the recently sequenced *Tetrahymena* macronuclear genome. Using the *C. elegans* DAF-10/IFT122A as the query sequence, we found a predicted open reading frame (ORF), 107.m00117, showing strong sequence homology (E-value 6.9e-100). Although the putative *Tetrahymena IFT122A* homolog appears to be a single copy gene, the *Tetrahymena* genome contains other genes with weak homology to it. Furthermore, several IFT proteins, as well as OSEG proteins identified by a computational genomic comparison and shown to be involved in the formation of the neuronal cilium in *Drosophila* (Avidor-Reiss et al., 2004), share similar domain features with IFT122A. To exclude the possibility that the gene we identified is not the ortholog of IFT122A but one of the other IFT or OSEG genes, we searched the *Tetrahymena* genome database to find other similar sequences and performed a phylogenetic analysis. The gene encoding 107.m00117 was the only one that clearly grouped with the IFT122A orthologs from other species on the un-rooted tree (Fig. 1A), arguing that it is the *Tetrahymena IFT122A* and we have named it accordingly. Single *Tetrahymena* genes from the other IFT/OSEG clades (Avidor-Reiss et al., 2004; Cole, 2003) were also identified.

Further determination of *Tetrahymena IFT122A* coding sequence by sequencing the cDNA and 5’ rapid amplification of the cDNA end revealed that the actual first exon and intron were missing in the predicted ORF and several splicing sites were also wrongly predicted, causing in-frame insertions. Based on the cDNA sequence, we deduced a revised ORF containing 1230 amino acid residues and encoding a 142 kDa protein with a pI of ~7.6. It shows 25% identity and 38% similarity to DAF-10, 32% identity and 54% similarity to *Chlamydomonas* predicted protein C510064, and 33% identity and 55% similarity to WDR10, the closest human ortholog (Gross et al., 2001). As illustrated in Fig. 1B, a protein secondary structure analysis predicts that the N-terminal half of the protein forms β strands while the rest of the protein contains mainly α helical structures and short loops. Domain analysis identified six WD40 motifs in the β strand-rich N-terminal region (Fig. 1B, yellow boxes) and two highly degenerate repeat units (Fig. 1B, green boxes; also see alignment in Fig. 1C) in the C-terminal region. This two-domain organization is similar to that of several other IFT proteins and OSEG genes.

*Tetrahymena IFT122A* is induced during cilia regeneration

To determine whether the IFT122A gene was involved in ciliary function, we first investigated whether its expression is induced during regeneration after cilia are amputated, which is a property of previously described *Tetrahymena* ciliary genes (Guttman and Gorovsky, 1979). Northern blotting showed that in growing and starved cells, IFT122A was expressed at a very low level. After 1 hour of cilia regeneration, however, the IFT122A RNA level was highly elevated (Fig. 2A, upper panel). Thus, similar to tubulin (Guttman and Gorovsky, 1979) and to other *Tetrahymena* IFT genes such as IFT52 (Brown et al., 2003) and IFT172 (Tsao and Gorovsky, 2008), the expression of IFT122A can be induced during cilia regeneration, suggesting that IFT122A is a ciliary gene.

**HA-tagged Ift122Ap localizes in isolated cilia**

To determine if IFT122A indeed encodes a ciliary protein, we constructed an IFT122A-HA strain in which Ift122Ap fused with a hemagglutinin (HA) epitope at the C-terminus was expressed from its endogenous locus. Unfortunately, we could not detect any signal by immunofluorescence staining using anti-HA antibody with various fixation methods. As an alternative approach to examine whether Ift122Ap-HA was present in the cilia, we extracted proteins from isolated cilia and cell body fractions and analyzed them by immunoblotting using anti-HA antibody. We observed a 140 kDa band from the cilia fractions prepared from the IFT122A-HA cells, but no band was detected in the cell body fraction (Fig. 2B, upper panel). As controls, the cilia and cell body

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*Fig. 1. Sequence analysis of the *Tetrahymena IFT122A* gene. (A) An un-rooted neighbor-joining tree of IFT122A and other IFT and OSEG proteins, which share similar domain organization. Ce, Caenorhabditis elegans; Dm, Drosophila melanogaster; Hs, Homo sapiens; Ti, Tetrahymena thermophila. The number at the branches indicates the value of 1000 bootstraps. (B) Primary and secondary structure analysis of Ift122Ap. Yellow boxes indicate six WD motifs, and green boxes show two degenerate TPR-like repeat units. Secondary structure prediction shows two distinct regions of Ift122Ap: an N-terminal β-strand-rich region (yellow) and an α-helix-rich region (green). (C) Sequence alignment of the degenerate repeat units on Ift122Ap. Identical residues are shaded.*
fractions isolated from cells expressing Ift172p-HA and wild-type CU428 cells were also probed with anti-HA antibodies. A 190 kDa band was detected in the cilia fraction from IFT122A-HA cells, and no specific band was observed in the either fraction in CU428 cells (Fig. 2B, upper panel). These results indicate that the 140 kDa band we observed in the cilia fraction from the IFT122A-HA cells is specific and co-fractionates with Ift172p, a protein previously demonstrated by immunofluorescence to localize to cilia (Tsao and Gorovsky, 2008) (also see Fig. 5). To assess the purity of the two fractions, we re-probed the same blot with a control antibody previously shown to detect Pgp1p, a cytoplasmic protein that localizes to the endoplasmic reticulum (Xie et al., 2007). Pgp1p appeared as multiple bands and could only be detected in the cell body fraction but not in the cilia fraction (Fig. 2B, lower panel). These results argue strongly that Ift122Ap is a ciliary protein. The failure to detect a ciliary signal by fluorescence microscopy is probably because the protein concentration is too low or the epitope tag on Ift122Ap-HA is masked so that it is not accessible to the antibody.

Ciliary assembly in the IFT122A knockout cells is affected but not abolished

To explore the in vivo function of IFT122A, we created Tetrahymena IFT122A germine knockout strains (Hai et al., 2000). In these cells, the IFT122A coding sequence was disrupted with a neo3 cassette in both the germline micronucleus and somatic macronucleus. The genotype of the knockout cells was examined by PCR using a locus-specific primer outside the targeting construct and an allele-specific primer to either the wild-type IFT122A allele or the knockout construct (Fig. 3A, primer sites shown as arrow heads). In the wild-type cells, a predicted 1.8 kb band was obtained, while in the IFT122A knockout (KO), only the 2.4 kb band corresponding to the knockout locus could be amplified. (C) Ink uptake assay to test the function of oral cilia. IFT122A knockout cells can ingest ink particles to form black food vacuoles. Scale bar: 10 μm. (D) The proportion of cells that formed black food vacuoles at different time points. Open squares, CU428 cell; filled (red) circles, IFT122A knockout cells. (E-G) Immunostaining of IFT122A knockout (E), IFT122A knockout (F) and CU428 (G) cells using anti-tubulin antibody. The arrowhead indicates the anterior end of the cell. Scale bars, 10 μm.
completely paralyzed. By contrast, the \textit{IFT122A} knockout cells could still swim. Close comparison with the wild-type cells revealed that \textit{IFT122A} knockout cells swam more slowly than the wild-type cells (data not shown), suggesting that the somatic cilia were affected. To test oral cilia function, we compared the ability of the wild-type and \textit{IFT122A} knockout cells to ingest ink particles, which requires motile oral cilia to generate flow currents. Like the wild-type cells, \textit{IFT122A} knockout cells could ingest ink particles and formed black food vacuoles (Fig. 3C) but did so more slowly than the CU428 wild-type cells (Fig. 3D). This observation suggests that the oral cilia can be assembled but function less efficiently than wild-type oral cilia.

To compare the morphological differences between wild type, \textit{IFT122A} knockout and \textit{IFT172} knockout cells, we performed immunostaining using anti-tubulin antibodies to visualize cilia. While \textit{IFT172} knockout cells lacked or had extremely short cilia (Fig. 3E), \textit{IFT122A} knockout cells still possessed long oral and somatic cilia (Fig. 3F). The numbers of the cilia in \textit{IFT122A} knockout cells, however, were fewer than those in the wild-type cells (Fig. 3G). In addition, cilia length and distribution were less homogeneous in \textit{IFT122A} knockout cells than in wild-type cells, with the density of cilia in the \textit{IFT122A} knockout cells being higher at the anterior (Fig. 3F, arrowhead) than in the rest of the cell. All of these observations show that, while \textit{IFT122A} clearly affects cilia, unlike \textit{IFT52} and \textit{IFT172}, it is not essential for cilia assembly in \textit{Tetrahymena}.

Deletion of \textit{IFT122A} causes preferential localization of Ift88p-GFP at the ciliary tips

Cilia assembly is dependent on anterograde IFT transport, indicating that \textit{IFT122A} is not required for this process. To determine whether deletion of \textit{IFT122A} affected retrograde transport, we compared the distribution of another IFT protein in cells with or without Ift122Ap. We created an \textit{IFT88-GFP} targeting construct carrying a cycloheximide-resistant selectable marker and used it to transform both wild type and \textit{IFT122A} knockout cells. When the tagged strains were cultured with cycloheximide, a number of the endogenous \textit{IFT88} genes in the polyploid macronuclear genome were replaced by the introduced GFP-tagged version, enabling the Ift88–GFP fusion protein to be detected (Fig. 4A).

In wild-type cells (Fig. 4B,C), the Ift88p-GFP mainly localized to basal bodies (Fig. 4C, arrow) and along the cilia (Fig. 4C, arrowhead), especially in the oral cilia (Fig. 4B, OA). In the \textit{IFT122A} knockout cells (Fig. 4D-G), however, Ift88p-GFP was rarely observed in basal bodies or along the cilia. Instead, strong fluorescence was detected at the distal tip region of cilia (Fig. 4F,G, arrowhead). The tip localization of Ift88p-GFP was not detected in every cilium but was especially prominent at the tips of oral cilia. (Fig. 4F, OA). These observations suggest that when \textit{IFT122A} is deleted, Ift88p (an IFT-B component) is retained at the distal tip of the cilia, either because it is released from the complex and/or because the entire complex inefficiently interacts with the retrograde IFT machinery. Therefore, although \textit{IFT122A} is not essential for anterograde transport, it plays a role in returning Ift88p from the tip to the base of the cilia.

Deletion of \textit{IFT122A} causes a preferential accumulation of full-length Ift172p-HA at the ciliary tips

In our strategy to introduce \textit{IFT88-GFP} into \textit{IFT122A} knockout cells, both the native and GFP-fusion Ift88p were present in the same cells, and we could not rule out the possibility that Ift88p-GFP might behave differently from the untagged, endogenous forms. To determine the effect of \textit{IFT122A} on a complex B protein but avoid this concern, we used an alternative strategy to investigate the localization of another IFT protein, Ift172p, in the \textit{IFT122A} knockout cells (Fig. 5A). We generated \textit{IFT122A, IFT172} double knockout cells by crossing the two single knockout heterokaryon strains and made the cells homozygous for both loci in both nuclei (Hai et al., 2000). The double
cells, indicating that the HA-tagged Ift172p was functional and which mediates anterograde transport, a step that precedes retrograde transport in IFT172 knockout cells. Instead, deletion of IFT122A, IFT172 double knockout cells were transferred into regeneration buffer, the cells did regenerate cilia (Fig. 6). Although the heterogeneity in cilia length and numbers in IFT122A knockout cells makes it difficult to compare with the regeneration kinetics of the wild-type cells, we observed short nascent cilia in IFT122A knockout cells at 20 minutes (Fig. 6A-C, arrowhead), and the regeneration proceeded (Fig. 6D-F) until after 60 minutes (Fig. 6G-I). Similar to the wild-type cells, newly formed long cilia could be observed in the IFT122A knockout cells and the cells regained motility.

We also examined the localization of HA-tagged Ift172p during cilia regeneration in the IFT122A knockout cells. Ift172p-HA became localized in the nascent cilia after 20 minutes of regeneration (Fig. 6B,C, arrow). Since the nascent cilia are still short, it is difficult to discriminate any uneven distribution of Ift172p along the cilia if it occurred at this time. However, by 40 minutes, when cells had longer cilia, we observed Ift172p accumulation in the distal regions of the cilia in IFT122A knockout cells (Fig. 6E,F, arrow). After 60 minutes of regeneration, the preferential distal localization of Ift172p in the IFT122A knockout cells became more prominent and could be easily observed (Fig. 6H,I, arrow). This experiment confirms that the IFT122A knockout cells can assemble new cilia after cilia amputation and that the preferential localization of IFT proteins at the tips occurs in the newly formed cilia. Collectively, these results argue that deletion of IFT122A does not abolish the anterograde IFT process required for transporting building blocks into the cilia. Instead, deletion of IFT122A affects the return of proteins from the ciliary tip to the cell body.

IFT122A knockout cells can regenerate cilia after cilia amputation and accumulate IFT proteins at the ciliary tip

To analyze how IFT122A affects IFT, we deciliated IFT122A knockout cells carrying HA-tagged IFT172. If IFT122A is not essential for anterograde IFT, one prediction is that, after cilia are amputated, cilia regeneration should occur and the progress of cilia regeneration should not be dramatically affected. As predicted, when the deciliated IFT122A knockout cells were transferred into regeneration buffer, the cells did regenerate cilia (Fig. 6). Although the heterogeneity in cilia length and numbers in IFT122A knockout cells makes it difficult to compare with the regeneration kinetics of the wild-type cells, we observed short nascent cilia in IFT122A knockout cells at 20 minutes (Fig. 6A-C, arrowhead), and the regeneration proceeded (Fig. 6D-F) until after 60 minutes (Fig. 6G-I). Similar to the wild-type cells, newly formed long cilia could be observed in the IFT122A knockout cells and the cells regained motility.

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IFT-A and IFT-B proteins have distinct functions

Although IFT proteins can be biochemically separated into two complexes, IFT-A and IFT-B, they move coordinately in the same particle in vivo (Ou et al., 2005; Qin et al., 2001). This observation raises two questions: are IFT-A and IFT-B functionally distinct complexes? What are their respective roles in vivo?

Comparing the loss-of-function phenotypes of IFT-A and IFT-B mutants in the same system addresses the first question. Using Tetrahymena as the model, we and others performed three knockout studies on IFT genes, including one IFT-A gene, IFT122A (this study), and two IFT-B genes, IFT52 (Brown et al., 2003) and IFT172 (C.-C. Tsao and M. A. Gorovsky, submitted), enabling a direct comparison of IFT-A and IFT-B gene functions in typical motile cilia. We found that disruption of an IFT-A gene shows a distinct phenotype from disruption of IFT-B genes in Tetrahymena. Both IFT52 and IFT172 knockout cells cannot assemble cilia or assemble only very short cilia, and the knockout cells become virtually paralyzed (Brown et al., 2003). The IFT122A knockout cells, however, still assemble oral and somatic cilia during growth and regenerate cilia after cilia amputation. The cells remain motile although they swim more slowly. These results clearly demonstrate that IFT-A and IFT-B proteins are differentially involved in the assembly of motile cilia.

Other evidence suggests that IFT-A and IFT-B proteins also play different roles in non-motile neuronal sensory cilia. The cilia in C. elegans, IFT-B mutants osm-1/IFT172, osm-5/IFT88, osm-6/IFT52 and che-13/IFT57 do not extend beyond the transition zones and have shortened axonemes, while the IFT-A mutants che-11/IFT140 and daf-10/IFT122A have irregular sized axonemes and their cilia are filled with material in the middle (Perkins et al., 1986). In zebrafish, mutated IFT88 or morpholinon knockdown of IFT52 and IFT57, three IFT-B genes, cause defects in the maintenance of sensory cilia and lead to the degeneration of photoreceptors, olfactory sensory cells and auditory hair cells. When IFT140, an IFT-A gene, is knocked down, however, only the cilia in the anterior nasal pit are significantly affected and the phenotype is weaker and infrequent (Tsujikawa and Malicki, 2004). Together, these studies and our results argue that IFT-A and IFT-B proteins have distinct functions in both canonical motile cilia and in non-motile sensory cilia derivatives.

An IFT-A protein is important for returning IFT proteins from the ciliary tip to the cell body

The different function of IFT-A or IFT-B proteins was initially inferred by comparative phenotypic studies among IFT mutants in different models. IFT-B proteins have been shown to be important for cilia formation in several organisms, indicating that they are essential for the anterograde process (for a review, see Scholey, 2003). Chlamydomonas fla15, fla16 and fla17, three temperature-sensitive mutant strains in retrograde IFT, are deficient for the same two IFT-A proteins in the flagella at the restrictive temperature (Piperno et al., 1998). This correlation between the absence of some IFT-A proteins and a defect in the retrograde transport led Piperno et al. (Piperno et al., 1998) to hypothesize that IFT-A is responsible for retrograde transport, although other interpretations were also possible. Here we deleted
one of the IFT-A genes in *Tetrahymena* and demonstrated an effect on retrograde transport of two other IFT proteins to provide a direct verification of this hypothesis.

In *Tetrahymena* IFT122A knockout cells, Ift88p and Ift172p became enriched at the distal region of the ciliary tip. The accumulation of IFT proteins could be due to a defect in retrograde transport, since the phenotype is similar to loss-of-function of a heavy chain or of a light intermediate chain of the cytoplasmic dynein retrograde motor in *Chlamydomonas* and *C. elegans* (Pazour et al., 1999; Schafer et al., 2003; Signor et al., 1999). In these mutants, dense materials accumulated and a bulge appeared at the flagellar/ciliary tip. Accumulations of IFT proteins in cilia were also reported in *C. elegans* IFT-A mutants che-11/IFT140 and daf-10/IFT122A (Perkins et al., 1986). Another gene, ifta-1, which phenocopied retrograde IFT defects when it was mutated, is a strong candidate for an uncharacterized IFT-A gene, IFT122B (Blacque et al., 2006). These results from *Tetrahymena* and *C. elegans* collectively argue strongly that the IFT-A complex plays an important role in retrograde IFT.

Is IFT122A also involved in other IFT processes? IFT122A knockout cells still assemble cilia, but in fewer numbers and with less length homogeneity. One explanation is that disruption of IFT122A causes accumulation of IFT-B proteins at the ciliary tips, which eventually perturbs anterograde IFT particle assembly. IFT122A could also have additional, non-essential roles in anterograde transport and/or other IFT processes.

Recent studies using *C. elegans* as a model showed that IFT-A and IFT-B are moved in the same particle in anterograde transport (Ou et al., 2005). In the sensory cilium, anterograde IFT is dependent on two kinesin-2 motors, heterotrimeric kinesin-II and homodimeric kinesin Osm-3, whose functions are partially redundant in some sensory neurons but are completely redundant in others (Evans et al., 2006; Ou et al., 2005; Snow et al., 2004). Interestingly, IFT-A is moved by kinesin-II and the IFT-B is carried by Osm-3, respectively, and the concerted movement of the whole particle also requires the non-kinesin proteins BBS-7/8 (Ou et al., 2005). *Tetrahymena* kinesin-II and Osm-3 orthologs have been cloned (Awan et al., 2004; Brown et al., 1999), and knockout of the kinesin-II alone is sufficient to abolish cilia assembly (Brown et al., 1999), indicating that the two types of kinesin-2 are not functionally redundant in *Tetrahymena*. If this two-kinesin mechanism is conserved in other species, such as *Tetrahymena*, one prediction is that IFT-A also has a role in the anterograde transport since it mediates the kinesin-II movement of the whole IFT particle. Our knockout results imply that a complete IFT-A complex is not essential for kinesin-II to move the IFT particles, but the kinesin-II–IFT interaction could still be partially compromised when IFT122A is deleted so that ciliary assembly is not as efficient as in the wild-type cells.

The phenotype of *Tetrahymena* IFT122A knockout cells is similar to *Tetrahymena* IFT172 mutants with a partial truncation of the Ift172p C-terminal repeat domain, in which Ift88p and the truncated Ift172p itself preferentially accumulated at the tips of the cilia (C.-C.T. and M.A.G., submitted). *Chlamydomonas* IFT172 has been implicated in anterograde/retrograde IFT turnaround at the flagellar tip, probably through interaction with the flagellar tip protein EB1 (Pedersen et al., 2006; Pedersen et al., 2003; Pedersen et al., 2005). Given similarities between IFT122A knockout and IFT172 partial truncation mutant cells, it is possible that IFT122A also mediates the anterograde/retrograde transition at the tip region. The very distal tip of *Tetrahymena* cilia only contains the A-tubule of axonemal microtubule doublet (Sale and Satir, 1976). This configuration is similar to the outer segment of *C. elegans* sensory cilia, and Osm-3 motors are essential to assemble this portion (Snow et al., 2004). In IFT122A knockout cells, in some cases we observed the accumulation of Ift172p in proximity to the ciliary tip, probably at the transition to the region containing the A-tubule singlet. This suggests a possible functional interaction between Ift122Ap, Ift172p, Osm-3 motors and/or ciliary tip proteins at the distal ciliary region where the cilia assembly and IFT particle remodeling take place. The utilization of two anterograde motors may contribute to neuronal cilia diversity in *C. elegans* (Evans et al., 2006), and the IFT machinery was proposed to utilize a combination of different 'functional modules' such as IFT-A/B, BBS complex and motor proteins (Ou et al., 2007). Whether a similar mechanism is also used in other species and the physiological significance of using different complexes to transport cilia/flagella proteins in the same direction remains to be further studied.

### Materials and Methods

**Tetrahymena** strains, culture growth, and conjugation

Wild-type CU428 and B2086 strains of *Tetrahymena thermophila* were provided by Dr Peter Bruns (Cornell University). Cells were grown in 1× SPP medium (Gorovsky et al., 1975) containing 1% peptone at 30°C, except that IFT122 knockout and IFT122/172 double knockout cells were grown in MEPP medium (Orias and Rasmussen, 1976). For starvation, mid-log-phase cells were washed and resuspended in 10 mM Tris-Ci (pH 7.5) for 16–20 hours at 30°C. For conjugation, equal numbers of the starred cells from two different mating types were mixed together and incubated at 30°C without shaking.

**Deciliation and cilia regeneration**

Cells were grown to mid-log-phase and starved before deciliation. For cilia regeneration analysis, the cells were deciliated by pH shock and immediately incubated in Tris-Ci buffer (pH 7.5) containing 10 mM CaCl₂ as previously described (Calzone and Gorovsky, 1982).

**Gene cloning and sequence analysis**

The Tetrahymena IFT122A gene was identified by a BLAST search against the *Tetrahymena* Genome Database (TGD, http://www.ciliate.org). A gene encoding predicted protein, 107.m00117, was identified using the *C. elegans* DAF-10 sequence (GI 133930820) as the query sequence. To verify the predicted coding sequence, total RNA was extracted from the cells at 1 hour after deciliation using Trizol reagent (Invitrogen). The cDNA was reverse transcribed by both the random hexamer and d(T)₁₇ primer using SuperScript II (Invitrogen) following manufacturer’s instructions and amplified by PCR using specific primers based on the 107.m00117 sequence. The 5' end of the cDNA was determined by an RLM-RACE kit (Ambion) following manufacturer’s instructions. The IFT122A ORF (Genbank EF601830) was deduced from the cDNA sequence.

The consensus secondary structure was predicted by NPS@PBIL-IBCP (http://npsa-pbil.ibcp.fr). The protein motif and repeat identification were analyzed by SMART (Schultz et al., 1998) and REP v1.1 (Andrade et al., 2000), respectively. To construct the phylogenetic tree, full-length protein sequences were aligned by CLUSTALX (Thompson et al., 1997) using default parameters (see supplementary material Tables S1 and S2 for the accession numbers of the sequences analyzed). After the initial alignment, large gaps and un-aligned terminal sequences were removed as described (Hall, 2001) to refine the alignment, and the distance was calculated by the neighbor-joining method. The bootstrap values were calculated by 1000 re-samplings.

**Germline gene knockout**

The 5' and 3' flanking regions for homologous recombination were amplified from CU428 genomic DNA. Overlapping PCR (Kuwayama et al., 2002) was used to join the flanking regions and the neo3 cassette (Shang et al., 2002) to make the knockout construct. The crude PCR products, mixed with EcoRI linearized pBluescript DNA as carriers, were introduced into the conjugating CU428 and B2086 cells by biolistic particle bombardment 3 hours after mixing as described (Cassidy-Hanley et al., 1997). The germline transformants were selected, assorted and made homozygous for the knockout allele in the micronucleus as described (Hai et al., 2000). After the initial alignment, large gaps and un-aligned terminal sequences were removed as described (Hall, 2001) to refine the alignment, and the distance was calculated by the neighbor-joining method. The bootstrap values were calculated by 1000 re-samplings.
dish. These progeny were examined with an Olympus SZH-ILLD dissecting microscope.

To generate IFT122A, IFT127 double knockout cells, the IFT122A and IFT127 single knockout homoyzogous heterokaryons (Tsao and Gorovsky, 2008) were crossed, and the progeny cells were selected, assorted and made homoyzogous for the micrornuclear genome as previously described (Haa et al., 2000). To test the genotype after round 3 genomic excision, the double knockout exconjugants were test crossed with IFT122A and IFT127 single knockout cells. A total of 48 individual conjugating pairs from each cross were isolated into 1× SSP medium 6 hours after mixing, and the phenotypes of the test-cross progeny cells were examined using a dissecting microscope.

Genotyping by PCR
To analyze the genotype, genomic DNAs from CU428 and IFT122A knockout cells were extracted as described (Liu et al., 2004) and used as templates for PCR analysis. A common forward primer sitting outside the construct and two locus-specific reverse primers (Fig. 3A, arrowhead) were added in the same PCR reaction (93°C, 30 seconds; 50°C, 30 seconds; 66°C, 3 minutes; 30 cycles).

Northern analysis
Total RNAs were extracted with Trizol reagent (Invitrogen) from mid-log-phase and from deciliated CU428 cells incubated in cilia regeneration buffer for 0, 1 or 2 hours. 20 μg total RNA was resolved on 2.2 M formaldehyde-1% agarose gels and blotted to MagnaGraph membranes (Osmonics). Hybridization was performed at 42°C overnight in hybridization buffers containing 50% formamide as previously described (Dou et al., 2005). The probe was labeled by random priming with α-[32P]dATP using IFT122A cDNA as the template.

Ink particle uptake assay
Cells were washed and re-suspended in 10 mM Tris (pH 7.5). A drop of Higgins India black ink was added to the cell suspension (~1 μl ink per ml of cells), and the cells were kept at 30°C with gentle shaking for at least 30 minutes. Cells were fixed with 10% formalin and examined using a bright field Olympus BH-2 microscope.

Epitope tagging
To make IFT122A-HA strains, the HA-tagged IFT122A construct was created by overlapping PCR to introduce an HA-epitope at the C-terminus of Ift122Ap. The PCR products mixed with the pBluescript II plasmid linearized by EcoRI were introduced into nontransformed IFT122A knockout cells by biolistic transformation (Cassidy-Hanley et al., 1997). The cells were recovered in SPP medium for 4 hours, distributed into 100 5 minutes, resuspended in phosphate-buffered saline (PBS) and examined using an Olympus BH-2 fluorescence microscope.

Immunofluorescent staining
Cells were doubly fixed with 0.2% paraformaldehyde-0.5% Triton-X100 in PHEM buffer before transformation. The fixed cells were incubated with poly-L-lysine coated glass slides and blocked with 10% normal goat serum and 3% bovine serum albumin in PBS with 0.1% Tween-20 at 37°C for 30 minutes. After blocking, the cells were incubated with rabbit anti-tubulin antisum (Van De Water, 3rd et al., 1982) (1:500) and mouse anti-HA monoclonal antibody (1:500) at room temperature for 1 hour. The samples were washed in PBS with 0.1% Tween-20 for 15 minutes, three times, followed by incubation with goat anti-rabbit IgG FITC (1:500) and goat anti-mouse IgG+M Alexa 595 conjugate (1:500) at room temperature for 1 hour. The slides were washed in PBS with 0.1% Tween-20 for 15 minutes, three times, and mounted with DABCO and observed with a Leica TCS SP confocal microscope or Olympus BH-2 fluorescence microscope. The images were pseudo-colored and merged by Adobe Photoshop 7.0.

Western blot analysis
IFT122A-HA, IFT127-HA and CU428 cells were grown to 2×106 cells ml–1, washed with 10 mM Tris buffer (pH 7.5) and deciliated with dibucaine-HCl as previously described (Johnson, 1986). The cell body fraction was pelleted by low speed centrifugation at 1500 g for 10 minutes and immediately dissolved in SDS-sample buffer (5% beta-mercaptoethanol, 10% glycerol, 2% SDS, 60 mM Tris-HCl, pH 6.8). The supernatant containing the cilia was centrifuged again at the same speed, and the supernatant was collected by centrifugation at 15,000 g for 20 minutes. The cilia pellet was washed, re-centrifuged at the same speed for 10 minutes and dissolved in SDS-sample buffer.

Proteins from ~5×105 cells were loaded onto 8% SDS-PAGE and transferred onto an Immobilon-P membrane (Millipore) using a semidy electromorphic transfer unit (Bio-Rad). The membrane was blocked in 5% dry skimmed milk in PBS for 30 minutes at room temperature, incubated with anti-HA primary antibody (1:5000) at 4°C overnight and a rabbit anti-mouse IgG-HRP-conjugant secondary antibody (1:5000) at room temperature for 1 hour. The protein signal was detected using Western Blot Chemiluminescence Reagent (NEN). For reprobing the blot, the membrane was incubated with 62.5 mM Tris-HCl (pH 6.8), 2% SDS and 100 mM β-mercaptoethanol at 55°C for 4 hours and washed several times with PBS. The membrane was blocked at room temperature for 1 hour, incubated with rabbit anti-poly-glycine glutamine antisum (Van De Water, 3rd et al., 1982) (1:500) and washed with 0.1% Tween-20 for 15 minutes, three times, followed by incubation with goat anti-rabbit IgG HRP (1:5000) and goat anti-mouse IgG+M Alexa 595 conjugate (1:500) at room temperature for 1 hour. The samples were loaded in PBS with 0.1% Tween-20 for 15 minutes, three times, and mounted with DABCO and observed with a Leica TCS SP confocal microscope or Olympus BH-2 fluorescence microscope. The images were pseudo-colored and merged by Adobe Photoshop 7.0.

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References


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