Chlamydomonas IFT70/CrDYF-1 Is a Core Component of IFT Particle Complex B and Is Required for Flagellar Assembly

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DYF-1 is a highly conserved protein essential for ciliogenesis in several model organisms. In Caenorhabditis elegans, DYF-1 serves as an essential activator for an anterograde motor OSM-3 of intraflagellar transport (IFT), the ciliogenesis-required motility that mediates the transport of flagellar precursors and removal of turnover products. In zebrafish and Tetrahymena DYF-1 influences the cilia tubulin posttranslational modification and may have more ubiquitous function in ciliogenesis than OSM-3. Here we address how DYF-1 biochemically interacts with the IFT machinery by using the model organism Chlamydomonas reinhardtii, in which the anterograde IFT does not depend on OSM-3. Our results show that this protein is a stoichiometric component of the IFT particle complex B and interacts directly with complex B subunit IFT46. In concurrence with the established IFT protein nomenclature, DYF-1 is also named IFT70 after the apparent size of the protein. IFT70/CrDYF-1 is essential for the function of IFT in building the flagellum because the flagella of IFT70/CrDYF-1–depleted cells were greatly shortened. Together, these results demonstrate that IFT70/CrDYF-1 is a canonical subunit of IFT particle complex B and strongly support the hypothesis that the IFT machinery has species- and tissue-specific variations with functional ramifications.

INTRODUCTION

The assembly and maintenance of flagella and cilia depend on the microtubule-based transport system known as intraflagellar transport (IFT; Rosenbaum and Witman, 2002), a process characterized as a bidirectional movement of large protein particles between the flagellar base and tip (Kozminski et al., 1993). The IFT machinery includes three key components: the IFT particle, the anterograde motors, and the retrograde motor. IFT particles are composed of at least 18 polypeptides that are organized into complex A and complex B. These particles are also called IFT trains because they appear as repetitive arrays of variable numbers of the same unit (Pigino et al., 2009). In this report, these two terms are used interchangeably. IFT trains serve as adaptors to bridge the axonemal precursors required for flagellar assembly with the motors (Qin et al., 2004; Hou et al., 2007; Ahmed et al., 2008). In the anterograde direction from the flagellar base to the tip, IFT particles are transported by either the heterotrimeric kinesin-II motor alone or kinesin-II together with the homodimer OSM-3. In the retrograde direction, IFT is powered by the cytoplasmic dynein 1b. Presently, little is known concerning how the motor activity is choreographed with the directional movement of IFT particles or how IFT carries cargo. To gain insight into the regulation of IFT, we chose to understand the function of DYF-1, the only protein to have shown a clear role in regulating IFT by activating the motor OSM-3 in Caenorhabditis elegans (Ou et al., 2005).

In C. elegans, two sequential IFT pathways are essential for full assembly of the cilia. Kinesin-II and OSM-3 function collaboratively to assemble the proximal part of the cilium, whereas OSM-3 alone is responsible for building the remainder of the distal segment (Snow et al., 2004). The osm-3 mutant has shortened cilia missing the distal segment, and this partially shortened ciliary defect is also seen in the dyf-1 mutant. In the dyf-1 mutant, the OSM-3 kinesin is capable of entering the ciliary compartment, but cannot bind to the microtubule or move and thus is inactive. Therefore, DYF-1 was postulated to be an OSM-3 positive regulator, required for either mediating OSM-3 binding with the IFT particle or docking onto microtubules (Ou et al., 2005). The interaction of DYF-1 with the IFT particle is not mediated through OSM-3 because in the osm-3 mutant DYF-1 moves together with IFT particles along the remaining proximal part of the cilium (Ou et al., 2005). DYF-1 is predicted to be a complex B–associated protein because it moves together with IFT complex B in bbs7 and bbs8 mutants in which complex A and B move separately with different speeds (Ou et al., 2005, 2007).

Evidence obtained from zebrafish and Tetrahymena revealed that DYF-1 must have additional roles in ciliogenesis in addition to regulating the activity of OSM-3. The zebrafish DYF-1 homologue Fleer is required for systemic ciliogenesis

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MATERIALS AND METHODS

Strains and Cultures

*C. reinhardtii* wild-type (wt) strain c1215, cell wall–deficient strain cu92, and the temperature-sensitive flagella assembly mutant fla10 (fla10–1 allele, cc1919) were obtained from the *Chlamydomonas* center (http://www.chlamy .org). Cells were grown on Tris-acetate–phosphate (TAP) solid plates or in M1 liquid medium with constant aeration in a Conviron environmental cabinet (http://www.conviron .com). Cells were grown on TAP plates supplemented with 0.1% (w/v) glucose. The temperature-sensitive fla10 strain was incubated at 35°C for 24 h and then shifted to 22°C for 24 h. The cells were further grown for 2 days at 22°C.

Phylogenetic Analysis

The sequences of IFT70/CrDYF-1 homologues were obtained from the National Center for Biotechnology Information database. Gene accession numbers are listed in the legend to Figure 1 and Table S1. The sequences were aligned with ClustalX 1.81 (UCD Conway Institute, University College Dublin, Dublin, Ireland). A neighbor-joining tree was calculated using the Treeview 1.6.6 (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html).

Antibodies

Polyclonal anti-IFT70/CrDYF-1 antisera were raised against the IFT70/CrDYF-1 terminus of amino acids 1–380 of IFT70/CrDYF-1 cloned into the pMALc-2 expression vector (New England Biolabs, Beverly, MA) for generation of a maltose-binding protein (MBP)-tagged fusion protein. The subcutaneous injection of the fusion protein into the rabbits was performed by Bethyl Laboratories (Montgomery, TX). The collected antisera were affinity-purified using nicotine-cellulose-bound MBP-IFT70/CrDYF-1 fusion protein as bait.

This study also used antibodies against α-tubulin (clone B-5-1-2, ascites fluid; Sigma, St. Louis, MO), IFT75, IFT81, IFT139 (Cole et al., 1998), IFT74 (Qin et al., 2004), IFT16 (Hou et al., 2007), IFT27 (Qin et al., 2007), FLA10 (Cole et al., 1998), DbILIC (Hou et al., 2004), and FMG1 (Bloodgood et al., 1986).

Sucrose-Density Gradients

The method for flagella isolation has been detailed elsewhere (Qin et al., 2004). The soluble flagellar proteins were fractionated through 12–19% sucrose density gradients in HDMDEK (10 mM HEPES, pH 7.4, 5 mM MgSO4, 1 mM DTT, 0.5 mM EDTA, and 25 mM KCl) in a SW41Ti rotor (Beckman Coulter, Fullerton, CA) for 14 h at 38,000 rpm. The gradients were typically fractionated into 24–26 0.5-ml aliquots. The standards used to calculate S values were bovine serum albumin (BSA; 4.4 S), aldolase (7.3 S), catalase (11.3 S), and thyroglobulin (19.4 S).

Immunoprecipitation

Before the immunoprecipitation experiments, protein A-Sepharose beads (GE Healthcare, Piscataway, NJ) were washed three times with HDMDEK buffer plus 3% BSA. Flagellar soluble proteins (protein concentration approximately 3 mg/ml) were clarified by centrifugation at 100,000 g for 10 min. The prepared cells were then incubated with antibodies overnight at 4°C. Immune complexes were recovered by incubation with pretreated protein A-Sepharose beads for 2–8 h at 4°C. After washing three times with 1 ml of HDMDEK plus 0.05% NP-40 and then once with HDMDEK plus 300 mM NaCl (each wash was for 10 min at room temperature), proteins were eluted from the resin by boiling in SDS-PAGE loading buffer and analyzed by SDS-PAGE, followed by immunoblotting.

Copurification of IFT70/CrDYF-1 and IFT46

IFT70/CrDYF-1 was expressed as a C-terminal fusion to MBP in pMalc-2X (New England Biolabs). IFT46 was expressed with the N-terminal epitope Strep-II-Tag (W-S-H-P-Q-F-E-K; IBA Go¨ttingen, Germany) in a vector derived from pRSFDuet-1 (Novagen, Madison, WI). The two expression plasmids were sequentially introduced into the NE537 strain (Qin et al., 2004) and transformed into the temperature-sensitive flagella assembly mutant *C. reinhardtii*. The expression strain was transformed with the miRNA construct pchlamymiRNA3-INT-IFT70/CrDYF-1 was created as described previously (Mohar et al., 2009). The target sequence of IFT70/CrDYF-1 was created as described previously (Mohar et al., 2009). The target sequence of IFT70/CrDYF-1 was created as described previously (Mohar et al., 2009).
targeting the IFT70/CrDYF-1 3'-untranslated region (UTR) was chosen for further study. In detail, two 90-mer oligonucleotides (IFT70/CrDYF-1-ami-forward: CTAGTCTCCTAGGATATTGCTTCTAATCTCGCTGATCGGCACCATGGGGTGGTGGTGATCAGCGCTATTAGTAGCAATATCCTAGGAGG and IFT70/CrDYF-1-ami-reverse: CTAGCCTCCTAGGATATTGCTACTAATAGCGCTGATCACCACCACCCCCATGGTGCCGATCAGCGAGATTAGAAGCAATATCCTAGGAGA, Invitrogen, Carlsbad, CA), containing the targeting sequence in opposite directions separated by a 42-base pair spacer sequence, were annealed in vitro and treated with T4 polynucleotide kinase (PNK, Fermentas, Hanover, MD) for phosphorylation. The miRNA vector pchlamymiRNA3int was digested with SpeI followed with CIAP (Fermentas) treatment for dephosphorylation. Thereafter, the 90-base pair DNA oligo was digested with SpeI and inserted into the pchlamymiRNA3int vector. The correct miRNA construct was confirmed by direct nucleotide sequencing.

Transformation of C. reinhardtii cells with DNA was performed with glass beads as described previously (Kindle, 1990). Before the transformations, the cell wall of the wt cc125 cells was removed by autolysin treatment. The clones harboring the transgene IFT70/CrDYF-1 RNAi or miRNA constructs were selected in accordance with the previously described method (Rohr et al., 2004; Molnar et al., 2009).

Measurement of the Flagellar Length
Cells were fixed with 1% polyglutaraldehyde, mounted to slides, and viewed with an Olympus IX-70 inverted fluorescence microscope at 1000 magnification. The phase-contrast images of the cells were captured with an ImagePoint CCD camera (Photometrics), and the flagellar length was measured with the software ImageJ 1.42 (http://rsb.info.nih.gov/ij/). The histogram showing the percentile distributions of flagellar length was created with Prism 5 (GraphPad Software, La Jolla, CA).

Transmission Electron Microscopy
IFT70/CrDYF-1 knockdown cells (strain miRNA-4) grown in TAP growth medium were fixed for 20 min at 20°C in TAP containing 2.5% glutaraldehyde and 1% paraformaldehyde. The cells were rinsed three times in 100 mM Na-cacodylate, pH 7.2, and osmicated in 1% OsO4 in distilled water for 1 h at 4°C. The cells were washed three times in distilled water, embedded in 1% agar, stained en bloc with 1% aqueous uranyl acetate, rinsed three times in distilled water, and then dehydrated and embedded in Epon 812 (Serva, Heidelberg, Germany) as described by McFadden and Melkonian (1986). Ultrathin sections (~50–60 nm) were cut with a diamond knife (type ultra 45°; Diatome, Biel, Switzerland) on an EM UC6 ultramicrotome (Leica, Wetzlar, Germany), and then stained with uranyl acetate and lead citrate (Reynolds, 1963) and viewed with a JEM-2100 transmission electron microscope (JEOL, Tokyo, Japan) operated at 80 kV. Micrographs were taken using a 4000 × 4000 charge-coupled device camera (UltraScan 4000; Gatan, Pleasanton, CA) and Gatan Digital Micrograph software (version 1.7.0.16.).

RESULTS

DYF-1 is the Most Conserved Protein among All the IFT Particle Subunits
By homologous sequence blast, a C. reinhardtii DYF-1 homologue was identified in the Joint Genome Institute (JGI) database and named as IFT70/CrDYF-1 (Figure 1A, also see Figure 3 for why the protein is named as IFT70). IFT70/CrDYF-1 is encoded by a single gene 128801 (JGI version 3, http://genome.jgi-psf.org/Chlre3/Chlre3.home.html). The phylogenetic tree of IFT70/DYF-1 proteins. Branch lengths represent evolutionary relatedness.

Z.-C. Fan et al.
of the amino acids of DYF-1 are conserved from the green alga C. reinhardtii to humans.

**IFT70/CrDYF-1 Has a Typical IFT Distribution Pattern and Its Flagellar Localization Is FLA10 Kinesin-II–dependent**

To confirm the flagellar localization of IFT70/CrDYF-1, a polyclonal antibody was raised against a recombinant MBP-IFT70/CrDYF-1 (1–380) and affinity-purified (see Materials and Methods). Immunoblot analysis of the flagellar proteins prepared from wt ccl25 cells revealed that the antibody α-IFT70/CrDYF-1 specifically recognized a single protein band migrating at M₅₀ ~70,000 (Figure 2A). Immunofluorescent microscopy analysis with this affinity-purified antibody showed that IFT70/CrDYF-1 is concentrated at peri-basal body regions and is localized along the entire length of the flagellum as punctuated dots (Figure 2B), a cellular distribution pattern typical for IFT particle proteins (Cole et al., 1998; Deane et al., 2001).

The entrance of the IFT particle proteins into the flagella of C. reinhardtii is dependent on heterotrimERIC FLA10 kinesin (Kozminski et al., 1995), the sole anterograde IFT motor (Huang et al., 1977; Walther et al., 1994; Kozminski et al., 1995; Cole et al., 1998). The temperature-sensitive mutant fla10ts harbors a mutation in the fla10 gene that encodes a motor subunit of FLA10 kinesin-II (Cole et al., 1998). The fla10ts cells have normal IFT at permissive temperature (22°C), whereas the anterograde IFT movement ceases within 1–2 h after the cells are shifted to the restrictive temperature (32°C; Kozminski et al., 1995; Iomini et al., 2001). Once anterograde IFT ceases, the IFT particle proteins are soon depleted from the fla10ts flagella, and thereafter the flagella gradually shorten. We found that, similarly to the known IFT particle proteins, IFT70/CrDYF-1 was dramatically reduced in the flagella of the fla10ts cells within 50 min after the cells were shifted to the restrictive temperature (Figure 2C). At the 50-min time point, flagella shortening was not observed, which was consistent with the previous observation that the disappearance of IFT precedes the shortening of flagella (Iomini et al., 2001). This result confirmed that the flagellar localization of IFT70/CrDYF-1 is FLA10 kinesin-II–dependent.

**IFT70/CrDYF-1 Is a Subunit of the IFT Complex B Core**

To determine whether IFT70/CrDYF-1 is an IFT particle subunit, sucrose density gradient analysis was utilized to determine its sedimentation pattern. The results clearly showed that IFT70/CrDYF-1 peaked in the 16S fractions with other IFT particle subunits (Figure 3, A and C). Based on the results of Coomassie Blue staining and Western blotting, the antibody α-IFT70/CrDYF-1 recognized a band between IFT74/72 and IFT57/55. After the bands corresponding to IFT74/72, IFT57/55 and IFT70/CrDYF-1 were cut off from the Coomassie Blue–stained gel, proteolytically digested and subjected to mass spectrometry, the identities of these proteins were confirmed. Compared with the staining intensity of IFT74/72 and IFT57 on the Coomassie Blue–stained gel (Figure 3A), IFT70/CrDYF-1 was present at a molar ratio of ~1 relative to other IFT particle subunits, as expected for a bona fide IFT particle subunit (Cole et al., 1998). In concurrence with the IFT particle protein nomenclature, we named the C. reinhardtii DYF-1 homologue as IFT70/CrDYF-1 after the apparent size of the purified protein from isolated flagella. For clarity, in the rest of the report, IFT70 is used to represents orthologues in different organisms and IFT70/CrDYF-1 is used specifically for the C. reinhardtii protein.

Coimmunoprecipitation of flagellar soluble proteins was performed to determine if IFT70/CrDYF-1 belonged to IFT complex A or B. The immunoprecipitation assay with α-IFT70/CrDYF-1 showed that IFT70/CrDYF-1 and IFT81 (IFT complex B subunit) but not IFT139 (IFT complex A subunit) were enriched in the precipitates (Figure 3B), suggesting that IFT70/CrDYF-1 is an IFT complex B protein. Similar results were also previously observed when FLAG-tagged DYF-1 protein was immunoprecipitated from mouse IMCD3 cell extracts (Follit et al., 2009). Additionally, IFT70/CrDYF-1 was enriched in the precipitates when the assay was performed with α-IFT46 and α-IFT72 antibodies, both of which are against complex B subunits. In contrast, α-IFT139 (against the complex A protein IFT139) antibody which effectively precipitated complex A proteins (Cole et al., 1998; Qin et al., 2004) was unable to precipitate IFT70/CrDYF-1 (Figure 3B). Together, these data support that IFT70/CrDYF-1 is associated more strongly with IFT complex B than complex A.

At low ionic strength, both IFT complexes A and B sediment at approximately 16S on sucrose density gradient (Piper and Mead, 1997; Cole et al., 1998). At higher ionic conditions, complex A remains intact, whereas complex B dissociates into an 11S core and a few free subunits (Lucker et al., 2005). Applying a similar analysis, we found that

### Table 1. IFT70/DYF-1 is the most conserved protein among IFT particle complex B subunits

| C. reinhardtii | T. brucei | C. elegans | D. melanogaster | Homo sapiens |
|---------------|----------|-----------|----------------|--------------|
| IFT70 XP_001692406 | XP_844139 (75) | AAY55187 (63) | NP.491494 (65) | NP.689488 (73) |
| IFT46 ABH06907 | XP_845431 (62) | AAL48848 (49) | NP.001076767 (66) | NP.684538 (72) |
| IFT52 AAL12162 | XP_827974 (56) | NP.609045 (54) | NP.741633 (58) | NP.057088 (68) |
| IFT80 ABQ96217 | XP_827975 (60) | NP.610064 (54) | NP.508106 (58) | NP.065851 (67) |
| IFT172 XP_001691740 | XP_822375 (60) | NP.647700 (58) | NP.510681 (55) | NP.056477 (65) |
| IFT20 AAM75748 | XP_845450 (58) | NP.724409 (51) | NP.740843 (55) | AAP50265 (64) |
| IFT88 AAG32228 | XP_828263 (60) | ABG02143 (45) | NP.508511 (56) | NP.783195 (58) |
| IFT25 ABU90455 | XP_828937 (45) | — | — | NP.057210 (57) |
| IFT27 XP_001689745 | XP_844145 (51) | — | — | AAP36177 (57) |
| IFT57 XP_001696648 | XP_823340 (52) | NP.608792 (49) | NP.492749 (53) | NP.060480 (57) |
| IFT72 AAQ92260 | XP_845960 (49) | — | NP.495359 (47) | NP.001092693 (49) |
| IFT81 AAT99262 | XP_822517 (48) | — | — | NP.508900 (46) |
| IFT22 XP_001689669 | XP_829740 (52) | — | NP.503073 (47) | NP.07614 (50) |

The percentages of positive conserved amino acids of IFT-B subunits between C. reinhardtii and other organisms are listed in parentheses after the GenBank accession numbers. —, there is no homologue of the IFT particle protein in the genome sequence of the organism.
IFT70/CrDYF-1 cosedimented with IFT complex B proteins IFT81 and IFT57 on the low-salt sucrose density gradient and comigrated with IFT81 at 11S on the high-salt sucrose density gradient (Figure 3C), thus confirming that IFT70/CrDYF-1 is one of the core subunits of the IFT complex B.

IFT70/CrDYF-1 binds directly to IFT46

Until 2006, all the identified IFT complex B mutants in C. elegans shared a characteristic and distinct ciliary morphology: the ciliary axoneme of amphid cilia are highly stunted (Perkins et al., 1986; Haycraft et al., 2001, 2003; Qin et al., 2001). However the complex B mutant ift46 (Bell et al., 2006) was shown to have a different ciliary morphology from any of the previous identified complex B mutant. Similar to dyf-1, the mutant ift46 can assemble the middle segment, but fail to form the distal segment of the cilia. These observations prompted us to test if IFT70/CrDYF-1 and IFT46 interact directly. We therefore initiated a heterologous bacterial expression system (see Materials and Methods), which allowed coexpression of two proteins in a single host bacterium. Full-length MBP tagged IFT70/CrDYF-1 and Strep-II-Tag IFT46 were expressed simultaneously and then purified by tandem affinity chromatography. IFT70/CrDYF-1 and IFT46 were co-purified with a 1:1 stoichiometric ratio (Figure 4), demonstrating that these two subunits interact directly. This result also strongly suggests that these two subunits should be capable forming a heterodimer in vivo.

IFT particle proteins are partially cosedimented with the axoneme

The zebrafish dyf-1/fleer mutant is missing a structural component of the axoneme, which leads to the hypothesis that DYF-1/Fleer is an integral axonemal protein (Pathak et al., 2007). Furthermore, Chlamydomonas flagellar proteomics analysis identified three IFT70/CrDYF-1-specific micropeptides with two in the detergent-soluble fraction and one in the axonemal fraction (Pazour et al., 2005). To investigate whether IFT70/CrDYF-1 is associated with the axoneme, the flagella isolated from wt cells was fractionated into the soluble flagellar matrix fraction and the insoluble membrane plus axoneme fraction by the freeze-thaw method (Figure 5A). Immunoblot assay showed that the majority of IFT70/CrDYF-1 pro-
tein was detected in the soluble matrix fraction; however, a small amount of IFT70/CrDYF-1 was also found in the insoluble membrane plus axoneme fraction (Figure 5B). To determine if this pool of IFT70/CrDYF-1 was associated with membrane or axoneme, the membrane was further separated from the axoneme fraction by applying the non-ionic detergent NP-40 to the insoluble membrane plus axoneme fraction (Figure 5A). After this treatment, the flagellar transmembrane protein FMG-1 stayed in the membrane fraction, whereas the anterograde and retrograde motor proteins FLA10 and cytoplasmic dynein D1bLIC were exclusively detected in the axoneme fraction (Figure 5B). Therefore, the application of NP-40 had effectively stripped the membrane away from the axoneme, and this treatment did not dissolve IFT motor proteins. IFT70/CrDYF-1 together with other components of the IFT particle, including IFT139, IFT81, IFT57, and IFT27, were detected in both the flagellar matrix and the axonemal fractions but not the membrane fraction. The association of the IFT complex proteins with the axoneme was probably bridged through the IFT motors FLA10 kinesin-II and/or cytoplasmic dynein. The detailed mechanism, however, remains unknown.

**IFT70/CrDYF-1 Is Essential for Flagella Assembly**

To investigate the role of IFT70/CrDYF-1 in flagellar assembly, vector-based RNAi was performed in wt cells. A total of 70 transformed colonies were obtained in two independent experiments. Among the 70 transformants, nine showed detectable reduction of the cellular IFT70/CrDYF-1 level as determined by immunoblot assay performed on the whole cell extracts (data not shown). For further phenotype analysis, we focused on two knockdown strains, Ri-6 and Ri-41, which both showed different levels of reduced IFT70/CrDYF-1 expression. Strain Ri-6 had a much lower IFT70/CrDYF-1 expression level than strain Ri-41 (Figure 6A). Corresponding to the dramatic reduction of IFT70/CrDYF-1, most of the Ri-6 cells had extremely short flagella, indicated by α-tubulin staining (Figure 6B) and phase-contrast microscopy (data not shown). It was determined that the majority of the cells have a flagella length of ~3–4 μm, although cells with full-length flagella (2~5%) and flagella-less (bald) cells (<10%) also could be observed in the population. Immuno-
fluorescence microscopy assay was performed on Ri-6 cells with /H9251-IFT72 and /H9251-FLA10 antibodies, which showed that both IFT72 (data not shown) and FLA10 were localized to the basal body region and flagella (Figure 6B). Therefore, the IFT70/CrDYF-1 depletion had no effect on the cellular localization of other IFT proteins or FLA10.

To confirm that the short flagella were caused by the lack of IFT70/CrDYF-1 rather than accidental insertional mutagenesis or off-targeting of the RNAi vector, artificial miRNA that targeted the IFT70/CrDYF-1 3’-UTR region was used to knock down the expression of IFT70/CrDYF-1. The construct was transformed into the cell wall–deficient but otherwise normal cw92 cells and a total of 40 transformants were obtained in two independent experiments. Determined by immunoblot assays, two transformed strains, miRNA-1 and miRNA-4, showed reduced cellular levels of IFT70/CrDYF-1 (Figure 7A).

Examination by phase-contrast microscopy showed that miRNA-1 and miRNA-4 cells had shorter flagella than the control cw92 cells. miRNA-1 and miRNA-4 cells showed a average flagella length of 6.63 and 4.23 μm, respectively, whereas cw92 cells had a average flagella length of 10.19 μm (Figure 7B). These results confirmed that the depletion of IFT70/CrDYF-1 resulted in the formation of short flagella.

Ultrastructural analysis by transmission electron microscopy (EM) did not reveal any abnormal structure in the basal bodies and flagella of miRNA-4 cells (Figure 8). The doublet axonemal microtubules, along with the attached dynein arms, and radial spokes appeared normal. The structure of the very distal tip of the flagellum, which contains only A subfibers also appeared to be normal (Figure 8G). In addition, the IFT-trains in the miRNA-4 flagella were observable along the axoneme in both cross and longitudinal sections, and at the distal tip (Figure 8, B–G). Taken together, these data strongly support that the remaining IFT particle complex B in the miRNA-4 cells were still functional, but insufficient to assemble full-length flagella.

Partial Depletion of IFT70/CrDYF-1 Causes the Reduced Cellular Levels of the IFT Complex B Proteins

As previously reported, complex B mutants /H46 (Hou et al., 2007) and bld1/ift52 (Qin et al., 2007) have elevated cellular levels of the complex A proteins but substantially reduced levels of the complex B subunits. Therefore, some complex B

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**Figure 4.** Coexpression and tandem purification of recombinant MBP-IFT70/CrDYF-1 and SIIT-IFT46. Shown here is the Coomassie Blue stained gel of samples from each step of the tandem purification; the first two lanes following the markers contain the insoluble and soluble fractions of bacterial cell lysates. Note that both proteins copurify after tandem purification with amylose and StrepTactin affinity resin. F/T stands for the flow-through proteins that did not bind to the resistive resins.

**Figure 5.** IFT70/CrDYF-1 is partially associated with the axoneme. (A) Procedure for the preparation of the flagellar fractions. This procedure is essentially the same as described in Huang et al. (2007). (B) Stoichiometrically equivalent levels of whole flagella, matrix, membrane plus axoneme, membrane proteins, and bare axoneme (labeled on the top) were isolated according to the procedure described in A. Note that, like other IFT particle proteins, a portion of IFT70/CrDYF-1 remains associated with the axoneme. The samples were separated by 10% SDS-PAGE and analyzed by Western blotting.
subunits are critical in maintaining the stability of the B complex. Immunoblot assays of whole cell extracts showed that the complex A protein IFT139 was increased, whereas complex B proteins were dramatically decreased in all the examined IFT70/CrDYF-1 knockdown cell lines including Ri-6, Ri-41 (Figure 6A), miRNA-1 and miRNA-4 (Figure 7A). This result is consistent with IFT70/CrDYF-1 serving as an integral component of the B complex and its loss results in the destabilization of the other B subunits in *C. reinhardtii*.

**DISCUSSION**

Through rigorous biochemical analysis, this study firmly establishes that IFT70/CrDYF-1, the DYF-1 homologue in *C. reinhardtii*, is an essential, stoichiometric component of IFT complex B. Previously, based on the motility profile of green fluorescent protein (GFP)-tagged proteins in wt and *bbs* mutants in *C. elegans*, DYF-1 was predicted to be a complex B–associated protein (Ou et al., 2007). BBS proteins are important in maintaining an intact IFT particle (Blacque et al., 2004) because in *bbs-7* and *bbs-8* mutants complex A and B are moved separately at different speeds by kinesin-II and OSM-3, respectively (Ou et al., 2005, 2007). In these mutants, DYF-1 moves at the same velocities as complex B, but not complex A; thus DYF-1 must be a protein associated with complex B, not complex A. The current study confirms this prediction and further shows unequivocally that IFT70/CrDYF-1 is a core component of complex B.

The addition of IFT70 as an integral IFT complex B subunit also indicates that the current inventory of IFT particle subunits is unlikely to be complete. The identification of IFT particle proteins has relied primarily on biochemical purifications (Piperno and Mead, 1997; Cole et al., 1998). Although powerful, these approaches have their own imperfections. In *C. reinhardtii* for example, putative IFT particle proteins have to be visible on the stained gels (Cole et al., 1998). We believe that IFT 70 CrDYF-1 evaded early detection because it was disguised by other proteins that co-migrated on early pro-
Figure 8. The flagella of IFT70/CrDYF-1 knockdown cells display a normal ultrastructure. (A) Cross section of a flagellum of a C. reinhardtii cu92 cell showing the 9 + 2 microtubule architecture of the axoneme. (B–G) Electron micrographs of flagella of IFT70/CrDYF-1 knockdown cells (strain miRNA-4). (B–D) Cross sections of flagella. IFT-trains (arrowheads) attached to the B-subfiber of outer-doublets are visible. (E) Longitudinal section of a flagellum with a visible IFT-train (arrowhead). (F) Cross section through a transition zone showing a normal ultrastructure. (G) Cross section through the distal tip of a flagellum. An IFT-train is visible (arrowhead). Scale bar, (A–G), 100 nm.

tein gels. Since the electrophoretic mobility of the IFT proteins can be affected substantially by the various reagents used in both the SDS-PAGE gel and the buffer, such as SDS (Wang et al., 2009), we changed conditions to maximize separation of IFT70/CrDYF-1 from other proteins such as IFT 74/72 (Figure 3A). In addition, we also show that yield of individual IFT complex B subunits extracted from flagella varies (Figure 5B), indicating that the strength of which different IFT complex B subunits interact with the axoneme is variable. It is thus possible that IFT70/CrDYF-1 was not extracted effectively from the flagella in the previous purifications.

Accumulating evidence in recent years clearly demonstrates that IFT directly transports flagellar precursors to balance the continuous turnover at the flagellar tip (Qin et al., 2004; Hou et al., 2007; Ahmed et al., 2008). Because no ultrastructural defects were identified, the inability to assemble full-length flagella likely results from insufficient amount of IFT particles in IFT70/CrDYF-1 knockdown cells. However, neither the RNAi nor miRNA methods could completely deplete IFT70/CrDYF-1, thus we were unable to address if IFT70/CrDYF-1 is important for a particular flagellar structure or carries a specific precursor. On the other hand, interfering with precursor transport alone may not solely account for the shorter flagella phenotype in IFT70/CrDYF-1–depleted cells. The fact that IFT70, an IFT particle subunit, functions specifically in regulating OSM-3 activity in C. elegans (Ou et al., 2005) and affects tubulin polyglutamylation in both zebrafish (Pathak et al., 2007) and Tetrahymena (Dave et al., 2009), raises a tantalizing possibility that the role of this IFT particle protein in ciliogenesis could be multifaceted. Almost certainly, IFT particle proteins are used as more than just a scaffold to bridge flagellar precursors to the motors. They might facilitate transport of other nonaxonemal structural proteins, such as tubulin polyglutamylation, to indirectly impact flagellar assembly or stability.

Clearly, the IFT machinery likely has species- and tissue-specific variations with functional ramifications. In C. reinhardtii (this report), zebrafish (Pathak et al., 2007), and Tetrahymena (Dave et al., 2009), IFT70/CrDYF-1 is essential for maintaining the entire axoneme structure of cilia and flagella. In zebrafish, Fleer/DYF-1 is an essential regulator of cilia tubulin polyglutamylation, which is important in stabilizing the axonemes. The cause for the dyf-1 mutant of Tetrahymena failing to assemble axonemes or only assemble extremely short remnants is still unresolved. In the axoneme remnants of the DYF1p knockout Tetrahymena, in contrast to the results with the fleer zebrafish, the level of tubulin glutamylation was increased (Dave et al., 2009). However, further investigation is needed to address whether the increased level of tubulin glutamylation is the cause or the consequence of the absence of DYF-1 protein. In C. reinhardtii IFT70/CrDYF-1 appears to be involved in the stability of IFT complex B, as the cellular levels of IFT complex B proteins were reduced proportionally to that of IFT70/CrDYF-1 in the IFT70/CrDYF-1 knockdown cells (Figures 6 and 7). This result is consistent with IFT70/CrDYF-1 being an IFT particle complex B subunit. Therefore, in this study, results from both biochemical purifications and functional analysis all point to an unambiguous conclusion: IFT70/CrDYF-1 is a canonical subunit of IFT particle complex B.

In contrast, DYF-1 is essential only for assembly of the distal segment of sensory cilia in C. elegans (Ou et al., 2005). In this organism, DYF-1 clearly has unique functions that are not possessed by many complex B proteins. Mutations affecting existing known proteins that function as part of IFT particle complex A or B in C. elegans display characteristic and distinct morphologies. The ciliary axoneme of most complex B mutants is much shorter compared with that of complex A mutants (Perkins et al., 1986; Haycraft et al., 2001, 2003; Qin et al., 2005). Mutant dyf-1 nematodes, however, possess an intact ciliary middle segment, which is different from the complete loss of cilia observed in most complex B mutants. In addition, many complex B proteins are essential for the stability or intraflagellar transport of complex B (Haycraft et al., 2003; Hou et al., 2007; Qin et al., 2007). This is not the case in the dyf-1 mutant in which IFT movement persists along the remaining middle segment (Ou et al., 2005); therefore, the DYF-1 protein is not essential for nematode IFT complex B formation and function. Furthermore, DYF-1 serves as an essential OSM-3 regulator (Ou et al., 2005), a function that has not been revealed for any complex B subunit yet. Based on these observations, it appears that in C. elegans DYF-1 is no longer an essential component of complex B, but has gained specialized roles to sustain the species specific ciliary structures.

It is well established that IFT particles serve as scaffolds to bridge flagellar precursors to IFT motors (Qin et al., 2004; Hou et al., 2007; Ahmed et al., 2008). However, subunits within IFT particles clearly function beyond that spectrum.
At least one IFT particle complex B protein, DYF-1, regulates the activity of the IFT motor OSM-3 (Ou et al., 2005). Moreover, recent studies in C. elegans showed that similar to dyf-1 mutant, the cilia of complex B mutants ift46 (Bell et al., 2006), ift81 and ift72 (Kobayashi et al., 2007) assemble the middle segment, but fail to form the complete distal segment. Furthermore, here we showed that IFT70/CrDYF-1 directly interacts with IFT46 (Figure 4); thus, these two subunits may function cooperatively to regulate the activity of OSM-3. On the basis of these observations, we speculate that the activity of OSM-3, and possibly all of the IFT motors, is subject to regulation by the subunits within the IFT complexes. Presently, little is known about how IFT motor activity is activated and deactivated. Future efforts in understanding the role of IFT particle proteins in regulating the activity of IFT motors will provide insight into this important problem.

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Z.-C. Fan et al.

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