CHAPTER TEN

Analysis of Interactions Between Intraflagellar Transport Proteins

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Contents

Abstract

Intraflagellar transport (IFT) involves the movement of large proteinaceous particles or trains along the length of ciliary and flagellar axonemal microtubules. The particles contain multiple copies of two protein complexes. As isolated from the flagellated model organism, *Chlamydomonas reinhardtii*, IFT A contains 6 distinct gene products while IFT B contains at least 13 distinct gene products. To better understand the architecture of these two complexes, a multifaceted approach has been employed to identify subcomplexes and specific protein–protein interactions. The high biochemical yields afforded with *Chlamydomonas* preparations have allowed traditional biochemical approaches including chemical cross-linking and disruption of native complexes, which,

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in the case of IFT B, have revealed a core subcomplex retaining nine of the B subunits. Complementing these results are molecular approaches including two-hybrid screenings and heterologous expression that have identified specific protein–protein interactions. Lastly, genetic approaches utilizing *Chlamydomonas* IFT mutants have shown how the loss of specific subunits perturb the complexes and, in the case of IFT A, they have revealed a core subcomplex containing half of the A subunits.

1. INTRODUCTION

Intraflagellar transport or IFT is a bidirectional movement of proteinaceous particles along the axonemal microtubules of cilia found in many eukaryotic organisms (Rosenbaum & Witman, 2002; Scholey, 2003). IFT was first discovered when video-enhanced differential interference contrast microscopy was used to visualize the biflagellate green alga, *Chlamydomonas reinhardtii* (Kozminski, Johnson, Forscher, & Rosenbaum, 1993). In green algae, this robust movement of particles consists of the anterograde transport of ~100 particles a minute moving at ~2 μm/s, and the retrograde transport of a greater number of smaller particles moving at more than 3 μm/s (Dentler, 2005; Iomini, Babaev-Khaimov, Sassaroli, & Piperno, 2001; Kozminski, Beech, & Rosenbaum, 1995). Strong evidence supports the identity of the algal anterograde IFT motor as the heterotrimeric kinesin-2 complex consisting of the FLA10 and FLA8 motor subunits and the non-motor accessory protein, FLA3 (Cole et al., 1998; Kozminski et al., 1995; Miller et al., 2005; Mueller, Perrone, Bower, Cole, & Porter, 2005; Walther, Vashishtha, & Hall, 1994). The retrograde IFT motor is widely believed to be cytoplasmic dynein 1b/2 with the algal components identified as two copies each of the DHC1b motor subunit, the FAP133 intermediate chain, the D1bLIC light chain, and the LC8 light chain (Hou, Pazour, & Witman, 2004; Pazour, Dickert, & Witman, 1999; Pazour, Wilkerson, & Witman, 1998; Perrone et al., 2003; Porter, Bower, Knott, Byrd, & Dentler, 1999; Rompolas, Pedersen, Patel-King, & King, 2007).

The protein scaffolding that comprises the IFT particles was first isolated from the flagella of *Chlamydomonas* and found to be associated with one of two complexes, A and B (Cole et al., 1998; Piperno & Mead, 1997; Piperno et al., 1998). As isolated from *Chlamydomonas*, IFT B contains protein subunits expressed by at least 13 different genes while IFT A contains a separate set of 6 protein subunits (Table 10.1; Behal et al., 2012; Brazelton, Amundsen, Silflow, & Lefebvre, 2001; Deane, Cole, Seeley, Diener, & Rosenbaum, 2001; Fan et al., 2010; Hou et al., 2007; Lechtreck, Luro,

Table 10.1 Intraflagellar transport proteins of *Chlamydomonas reinhardtii*

IFT protein	GenBank	Predicted size	IFT protein	GenBank	Predicted size
IFT A core subunits			*IFT A peripheral subunits*		
IFT144	ABU95019	1367 aa/ 150.8 kDa	IFT139	ABU95018	1355 aa/ 152.0 kDa
IFT140	AAT95430	1384 aa/ 154.6 kDa	IFT121	ABU89876	1224 aa/ 136.1 kDa
IFT122	AFC88886	1239 aa/ 139.2 kDa	IFT43	ABU93234	272 aa/ 28.8 kDa
IFT B core subunits			*IFT B peripheral subunits*		
IFT88	AAG37228	782 aa/86.3 kDa	IFT172	AAT99263	1755 aa/ 197.6 kDa
IFT81	AAT99262	683 aa/77.1 kDa	IFT80	ABQ96217	765 aa/ 85.7 kDa
IFT74/72	AAO92260	641 aa/71.4 kDa	IFT57	ABB72789	469 aa/ 51.3 kDa
IFT70	XP_001692406	647 aa/74.2 kDa	IFT20	AAM75748	135 aa/ 15.6 kDa
IFT52	AAL12162	454 aa/50.4 kDa			
IFT46	ABH06907	343 aa/37.9 kDa			
IFT27	EDP09483	204 aa/22.8 kDa			
IFT25	ABU90455.1	189 aa/20.4 kDa			
IFT22	XP_001689669	192 aa/21.3 kDa			

Awata, & Witman, 2009; Lucker et al., 2005; Pazour et al., 2000; Pedersen et al., 2005; Qin, Diener, Geimer, Cole, & Rosenbaum, 2004; Qin, Rosenbaum, & Barr, 2001; Qin, Wang, Diener, & Rosenbaum, 2007; Wang, Fan, Williamson, & Qin, 2009). Sensitive to increasing ionic strength, the B complex contains four peripheral subunits (IFT172, IFT80, IFT57, and IFT20) that are easily removed, revealing an IFT B core containing the remaining nine B proteins (IFT88, IFT81, IFT74/72, IFT70, IFT52, IFT46, IFT27, IFT25, and IFT22; Lucker et al., 2005). The algal IFT A is stable to increasing ionic strength, but mutant analysis reveals that, in the absence of IFT121, there exists a stable IFT A core consisting of IFT144, IFT140, and IFT122 (Behal et al., 2012). The model that

IFT139, IFT121, and IFT43 are peripheral IFT A subunits was first suggested when reduced expression of the IFT139 or IFT121 genes in mammalian cells resulted in an IFT144/140/122 core (Mukhopadhyay et al., 2010).

When IFT is completely disrupted, assembly of the organelle (ciliogenesis) is severely inhibited making the study of specific functions such as the selective transport of specific cargos difficult to analyze (Blacque, Cevik, & Kaplan, 2008; Cole & Snell, 2009). With less severe disruptions that allow for at least partial ciliogenesis, information can be collected regarding specific ciliary cargos such as the microtubule end-binding protein, EB1 (Pedersen et al., 2005), the axonemal outer dynein arm binding factor, ODA16 (Ahmed, Gao, Lucker, Cole, & Mitchell, 2008), and a negative regulator of Sonic hedgehog signaling, TULP3 (Mukhopadhyay et al., 2010). The importance of IFT and ciliary function in human health has also been demonstrated with human ciliopathies being linked directly to mutations affecting several IFT genes, including *IFT80* and *IFT121* (Beales et al., 2007; Gilissen et al., 2010). Thus, it has become increasingly important to understand the molecular mechanism of IFT function. Achieving this understanding, however, requires an intimate knowledge of how the IFT machinery is assembled.

2. ISOLATION OF IFT COMPLEXES FROM ISOLATED FLAGELLA

2.1. Flagellar isolation

Flagella are isolated from liquid *Chlamydomonas* cultures by pH shock as described by Witman, Carlson, Berliner, and Rosenbaum (1972) with minor modifications as follows:

1. Grow *Chlamydomonas* liquid cultures to a stationary phase ($1–3 \times 10^7$ cells/ml) in 8 l bottles (32–64 l total) under a 14:10 light: dark cycle in TAP medium (Gorman & Levine, 1965) at 23 °C as described previously (Behal, Betleja, & Cole, 2009). Harvest the cells during the first half of the light cycle.
2. Concentrate cells 10–15-fold using a Pellicon tangential flow cell concentrator (0.45 μm HVMP, Millipore, Bedford, MA) to a final volume of 1–4 l. The cells can be maintained at room temperature until immediately following deflagellation.
3. Concentrate cells further by centrifugation at $900 \times g$ for 3 min in a swinging bucket rotor (Allegra 6R, Beckman Coulter). Resuspend cell pellets with 4–12 l of fresh TAP medium and place under light with

continuous air bubbling until cells are optimally flagellated. This is an optional step employed when cells are slow to hatch and/or flagellate.

4. Concentrate flagellated cells by centrifugation at $900 \times g$ for 3 min in a swinging bucket rotor (Allegra 6R, Beckman Coulter). Resuspend cells in 1/80 vol. of the original culture (100–800 ml) in 10 m*M* HEPES, pH 7.2. To enhance the yield of IFT proteins in the isolated flagella, add 1/10 vol. of 50% sucrose to a final concentration of 5% 10 min prior to deflagellation; continuously stir cells.
5. With constant monitoring of the cell culture pH, add 0.50 *M* acetic acid rapidly to lower the pH of the vigorously stirred cells to ~4.6.
6. After 30 s, confirm deflagellation using phase contrast microscopy. Quickly neutralize the cell suspension to a pH of 7.2 using 0.50 *M* KOH; place the cell suspension immediately on ice with continuous stirring to keep cells suspended and to efficiently dissipate heat. All subsequent steps are performed on ice or at 4 °C.
7. While the cell suspension cools on ice, add protease inhibitors to the following final concentrations: 1 m*M* PMSF, 2 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, and 5 μg/ml soybean trypsin inhibitor.
8. After 10 min of cooling, remove the majority of the cell bodies by centrifugation at $900 \times g$ for 2 min in a swinging bucket rotor (Allegra 6R, Beckman Coulter). Remove the supernatant carefully with a pipettor to minimize contamination with cell bodies.
9. To remove additional cell bodies, layer the flagellar supernatant over 10 ml of 25% sucrose cushions in 50-ml conical tubes prior to centrifugation at $800 \times g$ for 10 min in a swinging bucket rotor (Allegra 6R, Beckman Coulter).
10. Pool the upper layer flagellar supernatants and concentrate by high-speed centrifugation at 10,000 rpm for 15 min with a fixed angle SS-34 or SLA-1500 rotor (RC-5B, Thermo Scientific Sorvall).
11. If desired, the flagellar pellet can be stratified by resuspension in 2–8 ml of 10 m*M* HEPES, pH 7.2 prior to a 10-min centrifugation (10,000 rpm) over a 6-ml cushion of 15% sucrose in 10 m*M* HEPES, pH 7.2 in a conical tube in the SS-34 rotor (RC-5B, Thermo Scientific Sorvall). Discard the supernatant and upper flocculent precipitate and avoid any dark green cell bodies during resuspension.
12. Resuspend flagellar pellets in minimal (100–800 μl) HMDEK buffer (10 m*M* HEPES, 5 m*M* $MgSO_4$, 1 m*M* DTT, 0.5 m*M* EDTA, 25 m*M* KCl, pH 7.2) with the protease inhibitor cocktail described above. The flagellar suspension can be immediately frozen (−80 °C)

in aliquots or concentrated in a microcentrifuge for 10 min (16,100 × *g*) in order to remove excess buffer prior to cold storage at −80 °C.

The final buffer used for flagellar resuspension prior to cold storage can vary depending on intended subsequent analysis. The reductant DTT, for example, can interfere with chemical cross-linking and metal chelate chromatography.

2.2. Protein extraction and sucrose density centrifugation

1. Thaw aliquots of previously frozen flagella on ice; resuspend thawed flagella in 0.1–0.6 ml of HMDEK buffer (10 m*M* HEPES, 5 m*M* $MgSO_4$, 1 m*M* DTT, 0.5 m*M* EDTA, 25 m*M* KCl, pH 7.2) containing protease inhibitors (1 m*M* PMSF, 2 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, and 5 μg/ml soybean trypsin inhibitor). Add 0.1–0.5% NP-40 to HMDEK to extract membrane-associated proteins; omit the NP-40 to extract only matrix proteins. Perform all subsequent steps on ice or at 4 °C.
2. Extract soluble proteins using 80 cycles of trituration (100–150 μl each cycle using a 200 μl-pipettor) and incubate 10–15 min on ice.
3. Remove most of the insoluble material by 15 min of centrifugation at 13,000 rpm in a microcentrifuge (16,100 × *g*). For additional clarification, centrifuge the supernatant for 10–15 min in an Airfuge A-18/100 rotor (Beckman Coulter) run at 15 psi. The resulting soluble protein fraction is known as the flagellar matrix (HMDEK only extraction) or the membrane plus matrix (M + M; HMDEK + NP-40 extraction).
4. Prepare 5–20% or 10–25% sucrose density gradients in HMDEK buffer or in 10 m*M* HEPES, pH 7.2 as described by Behal et al. (2009). The 5-ml ultracentrifuge tubes that fit the SW55Ti swinging bucket rotor (Beckman Coulter) work well for samples that are ≤250 μl while the 13-ml ultracentrifuge tubes that fit the SW41Ti swinging bucket rotor (Beckman Coulter) work well with samples up to 600 μl. If desired, prepare an additional gradient for sedimentation standards.
5. Load the clarified flagellar matrix or M + M (200–600 μl) on top of the gradient and centrifuge for 10 h at 35,000 rpm in the SW55Ti rotor or 12 h at 38,000 rpm in the SW41Ti rotor (Optima L-100 XP, Beckman Coulter). Spinning the SW55Ti rotor at full speed (55,000 rpm) will result in separation of IFT172 from complex B as described previously (Behal et al., 2009). If desired, load an identical sucrose density gradient with sedimentation standards as a mixture of 200 μl containing 100–200 μg each of equine cytochrome *c* (1.86 S), ovalbumin (3.5 S), BSA (4.65 S), porcine catalase (11.3 S), and bovine thyroglobulin (19.3 S).
6. Soon after centrifugation, fractionate the gradient(s) into 16 (SW55Ti) to 28 (SW41Ti) aliquots using capillary tubing and a peristaltic pump.

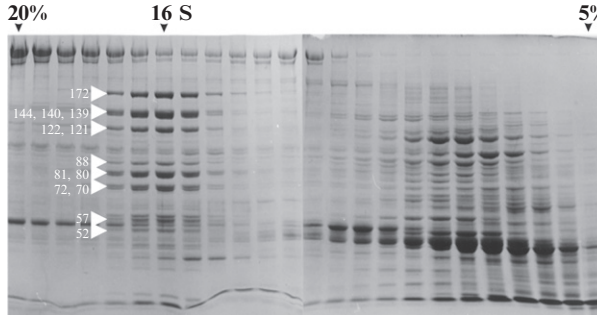

Figure 10.1 Sucrose density gradient profile of *Chlamydomonas* flagellar membrane plus matrix. The membrane plus matrix extract from flagella of wild-type cells (CC-125) was fractionated on an 11-ml (SW41 rotor; Beckman) of 5–20% sucrose gradient in 10 m*M* HEPES, pH 7.2. The Coomassie-stained SDS-PAGE gel (7.5% acrylamide) of the gradient profile shows many of the IFT particle polypeptides that cosediment at ~16 S (highlighted by white arrowheads with apparent electrophoretic mobilities listed on the left). *Reprinted with labeling modification from Cole et al. (1998) with permission from Rockefeller University Press, New York, NY, USA.*

7. Analyze the gradient fractions by running aliquots on SDS-PAGE (7.5% acrylamide). IFT complexes A and B will sediment at approximately 16 S and will display a characteristic pattern of protein bands as shown in Fig. 10.1. If the protein load is too dilute to visually identify the IFT proteins using Coomassie Blue staining, silver staining or western transfers probed with anti-IFT antibodies can be employed.

3. ISOLATION OF IFT COMPLEX A FROM WHOLE CELL EXTRACTS

Soluble fractions containing *Chlamydomonas* IFT proteins can be extracted from whole cells or from cell bodies following flagellar excision or from aflagellate mutant cells. If the strains are cell wall-deficient (e.g., CC-503), growth of large quantities of *Chlamydomonas* in aerated liquid culture is prohibitive because of cell lysis. Cell wall-deficient strains can, therefore, be grown on solid medium using 4–10 plates of 100 mm diameter. Strains with normal cell walls can be grown on solid or in aerated liquid medium.

3.1. Harvesting cells from solid medium

1. Inoculate 4–10 TAP-agar plates of 100 mm diameter (Gorman & Levine, 1965) with the desired *Chlamydomonas* strain and grow under constant light (2300 lux average) at room temperature for 2–3 days.
2. Flood each plate with 10–13 ml of TAP liquid medium.

3. Incubate under light for 2 h at room temperature (1200 lux average). The flooding incubation allows cells to form flagella and enhances the ability to easily remove cells from the agar.
4. Harvest *Chlamydomonas* cells from the plates by trituration using a 10-ml pipette; aflagellates and short flagella cells can be loosened from the agar by gentle brushing with a 10- or 50-μl disposable loop. Wash the plates with an additional 5–10 ml of liquid TAP medium to fully harvest the cells; transfer the cells to 125-ml flasks.
5. Allow the cells to recover for 1–2 h with gentle shaking (100–150 rpm) under light (1200 lux average) at room temperature. If desired, cells can be monitored with phase microscopy to ensure that cells are flagellated.
6. Collect cells with a 3–5-min centrifugation at 2300 rpm ($1500 \times g$) at room temperature (Allegra 6R, Beckman Coulter); add 1/2000 vol. of 10% Tween-20 to enhance cell yield. Carefully remove the supernatant by aspiration and discard; the cells can be immediately frozen and stored at −80 °C indefinitely or kept on ice for a short time before proceeding immediately to extraction by sonication or mechanical disruption.

3.2. Preparation of protein extracts from whole cells

Two different methods for the extraction of proteins from cells and cell bodies are utilized in these studies. Sonication, while quick and efficient, can lead to the release of significant quantities of chloroplast components, which can have a deleterious impact on subsequent analyses. Cellular disruption by shearing (ESGE Biohomogenizer) tends to leave the chloroplasts more intact but sometimes results in less than optimal protein recovery. In general, sonication can be performed on a smaller total volume and is utilized when a higher concentration of soluble protein is required.

3.2.1 Sonication

1. Suspend cells or cell bodies in 1–5 ml of HMDEK (10 m*M* HEPES, 5 m*M* $MgSO_4$, 1 m*M* DTT, 0.5 m*M* EDTA, 25 m*M* KCl, pH 7.2) with protease inhibitors (1 m*M* PMSF, 2 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, and 5 μg/ml soybean trypsin inhibitor). Maintain samples on ice; thaw previously frozen cells in a cold-water bath.
2. Disrupt cell suspensions by sonication using a Branson microtip connected to a Branson 450 power unit. Sonicate cells for a total of 2 min with a power level of 1–3 (based on volume) and a duty cycle of 50%. Maintain the cell suspensions in an ice-water bath; pause during sonication to prevent excessive heating of the sample.

3. Remove insoluble material using two or three sequential centrifugations for 15 min each at 13,000 rpm (microcentrifuge, 4 °C). Divide the disrupted cell suspension into several 1.5- or 2.0-ml microcentrifuge tubes in order to accommodate the total volume; the supernatant containing the soluble protein fraction is carefully transferred between each centrifugation to clean tubes.
4. To further clarify the protein extract (optional), centrifuge the supernatant for 10–15 min in an A-18/100 rotor (Airfuge, Beckman Coulter) run at 15 psi or centrifuge for 25 min at 50,000 rpm in an SW55Ti rotor (Optima L-100 XP, Beckman Coulter). This step will remove the dark green protein-rich thylakoid membranes.

3.2.2 Mechanical cell disruption

The Bio Spec Biohomogenizer fits into a 15-ml centrifuge tube, allowing for a minimal volume of buffer for cell suspension and extraction.

1. Suspend cells in 3–7 ml of HMDEK (10 m*M* HEPES, 5 m*M* $MgSO_4$, 1 m*M* DTT, 0.5 m*M* EDTA, 25 m*M* KCl, pH 7.2) with protease inhibitors (1 m*M* PMSF, 2 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, and 5 μg/ml soybean trypsin inhibitor) and transfer to a 15-ml glass centrifuge tube (Corex or Kimax) to dissipate heat; maintain samples on ice throughout the extraction (if previously frozen, the stored cells are thawed in cold water).
2. Disrupt cells with two sequential high-speed disruptions with 1–2 min on ice between the two cycles.
3. Clarify the disrupted cell suspension by two sequential 15-min centrifugations at 9500 rpm and 4 °C (SS-34 rotor, Sorvall Thermo Scientific).
4. To further clarify the protein extract (optional), centrifuge the supernatant for 10–15 min in an A-18/100 rotor (Airfuge, Beckman Coulter) run at 15 psi or centrifuge for 25 min at 50,000 rpm in an SW55Ti rotor (Optima L-100 XP, Beckman Coulter).

3.3. Immunoaffinity purification of IFT A proteins

With specific antibodies raised against the IFT A subunit proteins, antibody–resins can be utilized to pull IFT A complexes and subcomplexes out of crude cell extracts.

3.3.1 Preparation of anti-IFT A immunoresins

1. Dialyze 4 ml clarified (16,100 × *g*, 10 min at 4 °C) polyclonal antiserum against 2 l of 20 m*M* potassium phosphate, pH 8.0, overnight at 4 °C.

2. Using a Buchner funnel, wash 10–20 ml DEAE Affi-Gel® Blue resin (Bio-Rad) with 5–10 vol. of 1.4 *M* NaCl in 0.1 *M* acetic acid, pH 3 containing 40% isopropanol followed by 10 or more volumes of Affi-Gel® Blue (AGB) buffer (50 m*M* NaCl in 20 m*M* Tris, pH 7.2). Transfer ~8 ml (bed volume; BV) of the washed resin to an Econo-Pac® chromatography column (Bio-Rad) and wash again with 3–5 vol. of AGB buffer.
3. Load the clarified antiserum on the column and elute the immunoglobulin-enriched fractions with AGB buffer; collect 0.25 BV aliquots (~2 ml) for a total of 3 BV. Identify and pool the peak fractions using absorbance at 280 nm or analyze the fractions using SDS-PAGE.
4. Dialyze the AGB-purified antibody against 2 l coupling buffer (0.5 *M* NaCl in 0.1 *M* $NaHCO_3$, pH 8.3) overnight at 4 °C.
5. Prepare cyanogen bromide-activated Sepharose® 4B (Sigma-Aldrich) according to the supplier's instructions. In a small Buchner funnel, wash 0.25 g of resin (~1 ml hydrated volume) with five 10 ml aliquots of 1 m*M* HCl, followed by 10–20 ml water. Wash the resin with 5 ml coupling buffer (0.5 *M* NaCl in 0.1 *M* $NaHCO_3$, pH 8.3), air dry briefly, and use a nickel spatula to immediately transfer the resin from the Buchner funnel to the dialyzed immunoglobulin solution. Incubate the resin–antibody suspension overnight at 4 °C with gentle mixing on an end-over-end rotator.
6. Transfer the coupled antibody–resin to a small fritted Poly-Prep® column (Bio-Rad) and wash with 5 BV (~5 ml) of coupling buffer.
7. Transfer the antibody–resin to a 15-ml conical tube using 10 ml blocking buffer (1 *M* ethanolamine, pH 8.0); mix by gentle rotation for 2 h at room temperature. Any primary amine should function as a blocking reagent for unreacted resin, but the use of ethanolamine in place of other reagents such as Tris avoids coating the resin with a charged molecule.
8. Transfer the blocked resin to a 2-ml microcentrifuge tube and wash alternately with five 2 BV (2 ml) aliquots each of coupling buffer and 0.5 *M* NaCl in 0.1 *M* sodium acetate, pH 4.0. The resin can be spun down briefly (20–30 s) in a microcentrifuge at 10,000 × *g*.
9. Resuspend the resin in 1 ml of 1 *M* NaCl, 0.02% NaN_3 and store at 4–6 °C.

3.3.2 Immunoadsorption of IFT A proteins

Although the immunoadsorption of IFT A is typically performed with cell extracts, it works well with flagellar extracts and IFT A-enriched fractions from sucrose density gradients.

1. Mix aliquots of clarified *C. reinhardtii* cell extracts (0.2–0.5 ml) with subunit-specific antibody–resin (0.05–0.1 ml packed resin); add 5 *M* NaCl to a final concentration of 0.15 *M* to reduce nonspecific binding to the resin. A 0.5-ml microcentrifuge tube works well for the incubation and subsequent steps to maximize recovery of the resin.
2. Incubate the suspension overnight at 4 °C with gentle mixing on a slow end-over-end rotator (<10 rpm).
3. Consolidate the resin using brief centrifugation (10,000 × *g*, 15 s); carefully remove the unbound protein supernatant for electrophoretic analysis.
4. Wash the resin thoroughly with a minimum of four washes of 0.5 ml of 0.15 *M* NaCl in HMDEK buffer (10 m*M* HEPES, 5 m*M* $MgSO_4$, 1 m*M* DTT, 0.5 m*M* EDTA, 25 m*M* KCl, pH 7.2) at room temperature.
5. Elute antibody-bound proteins with the incubation of two resin volumes of 1% SDS at room temperature for 10 min, followed by brief centrifugation (16,100 × *g*, 15 s). Remove the supernatant eluate and save for subsequent analysis.
6. Release any residually bound proteins from the resin by the addition of one resin volume of 2 × SDS sample buffer, followed by heating at 95 °C for 5 min. Following centrifugation (16,100 × *g*, 2 min), remove the supernatant for electrophoretic analysis.
7. Characterize protein samples using SDS-PAGE and immunoblots following standard procedures as described previously (Behal et al., 2012). To increase sensitivity of immunoblot detection, utilize infrared secondary antibodies and the Odyssey scanner (LI-COR Biosciences); this system also allows for simultaneous visualization of two unique secondary antibodies (e.g., anti-rabbit and anti-mouse) that carry unique fluorescent tags. The Odyssey scanner can also be used to enhance the visualization of Coomassie-stained SDS-PAGE gels.

Immunoadsorption of IFT A proteins from cellular extracts reveals that all six IFT A subunits are simultaneously pulled down from wild-type (CC-503) extract while only a subset of IFT A subunits are pulled down from *ift121* mutant extract (Fig. 10.2).

4. ARCHITECTURAL ANALYSIS OF IFT COMPLEX B

4.1. Partial dissociation of IFT B reveals a stable core

The analysis of protein complexes by sucrose density gradient centrifugation is commonly used to characterize protein complex size and composition. This procedure is used to separate the IFT B core from peripheral subunits

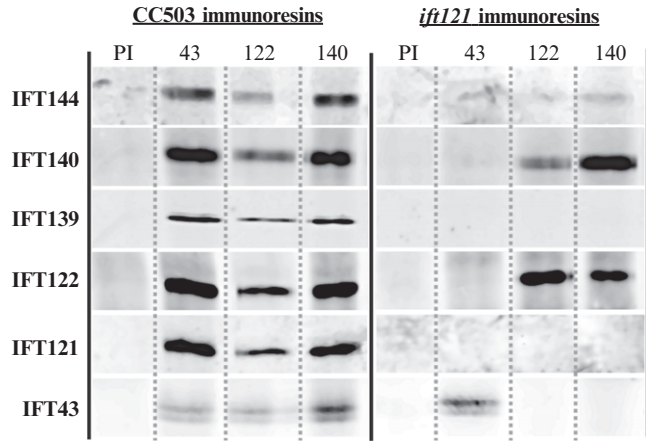

Figure 10.2 Antibody pull-downs of IFT A reveal formation of a subcomplex in *ift121*. Aliquots of soluble protein from whole-cell extracts of CC-503 or *ift121* were incubated with preimmune- (PI) or subunit-specific antibody–resins as indicated above each column. Immunoadsorbed proteins were resolved by SDS-PAGE and transferred to nitrocellulose and probed with anti-IFT A antibodies indicated to the left of each row. In the absence of IFT121, a core subset of the IFT A subunits (IFT144, IFT140, and IFT122) is immunoadsorbed; IFT43 is present but not associated with this core. *Reprinted from Behal et al. (2012) with modification by permission of American Society for Biochemistry and Molecular Biology.*

by taking advantage of the fact that increasing the concentration of NaCl leads to the dissociation of the peripheral IFT B subunits (Lucker et al., 2005; Lucker, Miller, Dziedzic, Blackmarr, & Cole, 2010). Contingent on subsequent procedures, the reductant DTT can be omitted from the gradient.

1. Prepare flagellar matrix samples by resuspending frozen flagellar pellets in HMDEK buffer (10 m*M* HEPES, 5 m*M* $MgSO_4$, 1 m*M* DTT, 0.5 m*M* EDTA, 25 m*M* KCl, pH 7.2) + 300 m*M* NaCl (HMDEK-300) with protease inhibitors (2 m*M* PMSF, 5 μg/ml soybean trypsin inhibitor, 1 μg/ml pepstatin A, 2 μg/ml aprotinin, 1 μg/ml leupeptin). To reduce axonemal protein extraction, the 300-m*M* NaCl can be added after flagellar HMDEK extraction but prior to loading the gradient.
2. Prepare soluble flagellar extract and sucrose density gradients (10–25%) as described in Section 2.2. Prepare 12 ml gradients in SW41Ti centrifuge tubes (Beckman Coulter) with solutions containing HMDEK-300. Apply the flagellar extract (0.2–0.6 ml) to the top of the gradient and centrifuge for 16 h at 37,000 rpm (169,000 × *g*) at 4 °C.

3. Collect gradient fractions (~525 μl) from the bottom of the tube using capillary tubing and a peristaltic pump with a flow rate of 1.05 ml/min (30-s fractions).
4. Characterize gradient fractions using SDS-PAGE; visualize proteins using Coomassie Blue staining and sensitive imaging with an infrared scanner (Odyssey, LI-COR Biosciences); individual IFT subunits can be identified or verified using immunoblot analysis. IFT A will remain intact and will sediment at ~16 S. Four IFT B subunits will dissociate from the 11 S B core; IFT172 and IFT80 cosediment at 6.5 S while IFT57 and IFT20 sediment at 7.5 and 3.5 S, respectively, under these conditions.
5. To estimate sedimentation values for the IFT proteins, centrifuge and fractionate a separate gradient loaded with sedimentation standards: thyroglobulin (19.3 S), catalase (11.3 S), bovine serum albumin (4.65 S), ovalbumin (3.5 S), and equine heart cytochrome *c* (1.86 S).

The above preparation generates a fraction that is enriched with 11 S IFT B core but contains significant levels of other flagellar proteins. For a more pure preparation of the IFT B core (and peripheral subunits), prepare 16 S IFT B using the protocol described in Section 2.2. After pooling the IFT B-enriched fractions, it is necessary to remove most of the sucrose by dialysis, rapid-gel filtration (Penefsky, 1977), or repeated concentration in an Amicon Ultra-4 or Ultra-15 centrifugal concentrator (EMD Millipore) where the concentrated sample is repeatedly brought up in HMDEK with no sucrose; NaCl is added to a final concentration of 0.300 *M* prior to loading on a second sucrose density gradient containing HMDEK-300. If the sucrose from the original gradient remains present, the sample will sink into the second gradient when loading the sample.

4.2. Chemical cross-linking identifies neighboring subunits

Numerous cross-linking agents are available to covalently link nearby protein subunits within a complex. These reactive agents interact with the functional groups on specific amino acid side chains, and care must be taken to avoid any buffer components that may interfere. The following protocol requires the absence of reductants (e.g., DTT) and primary amines (e.g., Tris buffer). This type of cross-linking can be performed with any protein complex such as IFT A or intact IFT B, but the protocol described here focuses on the IFT B core.

1. Pool sucrose gradient fractions enriched in the complex B core and divide into equal aliquots; five aliquots work well for this protocol but less protein can be used. If the gradient solution does not contain

reductant or primary amines, proceed to cross-linking; if either is present, it must be quantitatively removed with extensive dialysis or rapid-gel filtration.

2. Treat the IFT B core aliquots with the chemical cross-linkers 1-5-diflouro-2,4-dinitrobenzene (DFDNB; Pierce Thermo Scientific), or dimethyl adipimidate (DMA; Pierce Thermo Scientific) at final concentrations of 0.0, 0.03, 0.1, 0.3, and 1.0 m*M* for 10 min on ice before being quenched with 10 m*M* Tris–HCl, pH 8.5.
3. Prepare anti-IFT81 resin as described in Section 3.3.1. A mixture of AGB-purified monoclonal antibodies raised against *Chlamydomonas* IFT81 (81.1, 81.3, and 81.4) is more effective than a single antibody (Cole et al., 1998).
4. Incubate 100 μl anti-IFT81 resin with each cross-linking aliquot for 1 h at room temperature with gentle mixing.
5. Centrifuge the resin briefly (16,100 × *g*, 15 s) before removing the supernatant. Wash the resin three times (5 min each) with 15 BV (1.5 ml) of HMEK-300.
6. Elute the proteins from the resin by boiling in an equal volume of 2 × SDS sample buffer. Remove the eluate from the resin following a 10-min centrifugation at 16,100 × *g*. To reduce contamination by IgG protein, a two-stage elution using 2% SDS followed by 2 × SDS sample buffer can be used with a majority of the IFT B protein eluting with the first treatment.
7. Fractionate each IFT B eluate on 4.0%, 5.0%, 6.0%, or 7.5% SDS-PAGE gels and visualize with Coomassie Blue. Lower percentage acrylamide gels will allow for better separation of cross-linked products with a total mass of 100–200 kDa.
8. Excise cross-linked protein bands that appear in the aliquots containing cross-linker. Digest the protein bands with trypsin and determine peptide masses present using matrix-assisted laser desorption ionization time-of-flight mass spectrometry; other modes of mass spectrometry are acceptable.
9. Compare the resulting peptide masses with expected tryptic peptides that would result from digestion of the IFT B subunits (Protein Prospector; Clauser, Baker, & Burlingame, 1999). Following this protocol using either DFDNB or DMA should result in easily visualized cross-linked products that contain only IFT81 and IFT74/72 (Lucker et al., 2005).

5. BACTERIALLY BASED INTERACTION ANALYSIS OF IFT B PROTEINS

5.1. Construction of unique expression vectors

Multiple commercially available vectors have been modified to create new expression vectors for these studies. For bacterial coexpression, we have modified the Duet series of vectors (Novagen), which are a derivative of the pET plasmid that allow for the expression of two different proteins from each vector. These vectors are available from the supplier with several different origins of replication (ORIs) and unique selectable antibiotic resistance markers, which allow for the transformation of an individual bacterial strain with up to four unique plasmids. The following vectors have been utilized: pCDFDuet (CloDF13 ORI, spectinomycin resistance); pETDuet (pBR322-derived ColE1 ORI, ampicillin resistance); and pRSFDuet (RSF1030 ORI, kanamycin resistance).

Vectors have been modified by replacing the epitope tags and altering the multiple cloning sites (MCSs). For each vector, MCS-1 was changed to (*Eco*RI–*Bam*HI–*Sal*I–STOP) while MCS-2 was changed to (*Mfe*I–*Bgl*II–*Xho*I–STOP). The restriction endonuclease sites of *Eco*RI and *Mfe*I, and of *Sal*I and *Xho*I, generate compatible cohesive ends upon digestion, thereby facilitating the cloning of the various subunits by reducing the number of unique PCR primers required for cDNA amplification. Four unique epitope tags were chosen based on the availability of epitope-specific affinity chromatography resins.

A. MBP—maltose binding protein—369 AA, 40621 Da
  i. Binds to amylose resin; eluted with maltose (Sachdev & Chirgwin, 2000).

B. SIIT—StrepIITag—9 AA, 1190 Da
  i. Binds to StrepTactin™ resin; eluted with desthiobiotin (Skerra & Schmidt, 2000).

C. GST—glutathione *S*-transferase—225 AA, 26226 Da
  i. Binds to glutathione–agarose; eluted with glutathione (Smith, 2000).

D. XPR—XPress + 6 Histidine—25 AA, 3000 Da
  i. Binds to Ni^{2+}- or Co^{2+}-resin; eluted with imidazole (Bornhorst & Falke, 2000).

Each of these unique epitope tags is created as a cassette and inserted just upstream of and in-frame with the first restriction site of either MCS,

creating N-terminal chimeric recombinant proteins. The ability to incorporate two of the possible four epitope tags (or no tag) in any given expression vector allows us to create a variety of unique protein expression constructs. Extensive use is also made of the pMalc2X protein expression plasmid (New England BioLabs) in its supplied state; the relatively large MBP epitope tag helps to solubilize the larger IFT A proteins.

5.2. Cloning of candidate IFT cDNAs

Fragments of cDNA encoding either the entire IFT subunit or smaller putative domains are amplified by PCR from cDNA clones utilizing the high-fidelity Phusion® DNA Polymerase (New England BioLabs). The specific PCR primers are designed to incorporate the appropriate endonuclease restriction sites for subsequent cloning, which follows standard methods. The *E. coli* protein expression strain BL21(DE3) (Novagen) is used for all expression experiments. The Duet vectors require this strain for protein expression; while the pMalc2X plasmid does not, the use of only one strain simplifies cloning. Initial bacterial transformation is performed using chemically competent BL21(DE3) cells; the incorporation of additional plasmid(s) into a previously transformed strain utilizes electroporation. All of the cloning techniques follow standard protocols. Following the creation and verification of each protein expression strain, cell samples are frozen in the presence of 15% glycerol and stored at −80 °C for subsequent growth and analysis.

5.3. Affinity purification of recombinant proteins

5.3.1 Protein expression and extraction of soluble proteins

1. Inoculate LB medium, supplemented with the appropriate antibiotic, with 1:100 vol. of overnight culture; a typical experiment utilizes 100 ml of liquid media in a 500-ml flask shaken at 200–250 rpm at 37 °C for 2–3 h until OD_{600} reaches 0.4–0.6.
2. Induce protein expression with 0.5 m*M* IPTG and continue shaking incubation at 37 °C for an additional 2 h.
3. Place cultures in an ice bath for 10 min before harvesting cells with a 10-min centrifugation at 10,000 rpm in an SLA-1500 rotor (RC-5B, Thermo Scientific Sorvall) at 4 °C; discard the supernatant and place the cell pellet on ice. Immediately resuspend by trituration the bacterial cell pellet in 5–10 ml of 20 m*M* Tris, 200 m*M* NaCl, pH 7.5.
4. Add protease inhibitors to the following final concentrations: 2 m*M* PMSF, 1 μg leupeptin, 1 μg/ml pepstatin, 2 μg/ml aprotinin, 5 μg/ml soybean trypsin inhibitor.

5. Transfer the bacterial cell suspension to a 15-ml screw-cap polypropylene tube and proceed to sonication or store indefinitely at −20 °C.
6. Thaw frozen bacterial cell suspensions quickly in cold water; add fresh PMSF to 2 m*M*. Disrupt cells by sonication with a Branson microtip connected to a Branson 450 power unit. Subject cells to a total of 2.5 min of sonication, with a power level of 3 and a duty cycle of 50%. The cell suspensions are kept in an ice-water bath, and the temperature during sonication is monitored carefully. Cell lysis can be confirmed using phase microscopy.
7. Remove insoluble material with 10 min of centrifugation at 10,000 rpm in an SS-34 rotor (Thermo Scientific Sorvall) at 4 °C. Carefully decant the supernatant and store on ice; resuspend the insoluble pellet in an equal volume of 200 m*M* NaCl in 20 m*M* Tris, pH 7.5, for subsequent SDS-PAGE analysis.

5.3.2 Affinity chromatography

Purification of recombinant protein(s) is performed using different affinity chromatography resins that correspond to the appropriate affinity tags fused to each expressed protein. Initial studies sometimes utilize a single affinity resin; two or more affinity tags, however, allow for tandem chromatographic purifications. The combination of epitope tags present in a given bacterial strain determines the identity and often the order of resins used. A common scheme utilized in our approaches combines the MBP and SIIT epitope tags; a detailed protocol for the copurification of MBP:IFT70 and SIIT:IFT46 is given below (Fig. 10.3) (Fan et al., 2010).

1. To purify MBP-tagged protein (i.e., MBP:IFT70), prepare a 0.5 ml (packed BV) amylose resin (New England BioLabs) in a 0.8 cm × 4 cm conical polypropylene column (Bio-Rad); the binding capacity of amylose resin is 6–8 mg MBP fusion/ml resin. The resin is prepared for loading by washing with 2.5 ml (5 BV) amylose column buffer (ACB; 200 m*M* NaCl in 10 m*M* Tris, pH 7.5).
2. Load the clarified cell lysate (from Section 5.3.1) containing soluble recombinant protein onto the amylose resin; collect the flow-through and reload onto the column two more times to maximize binding.
3. Remove unbound and nonspecifically associated protein with extensive washing (≥20 BV) with ACB.
4. Elute specifically bound protein from the amylose resin with ≥2 ml ACB containing 10 m*M* maltose; collect 0.5-ml fractions. Monitor

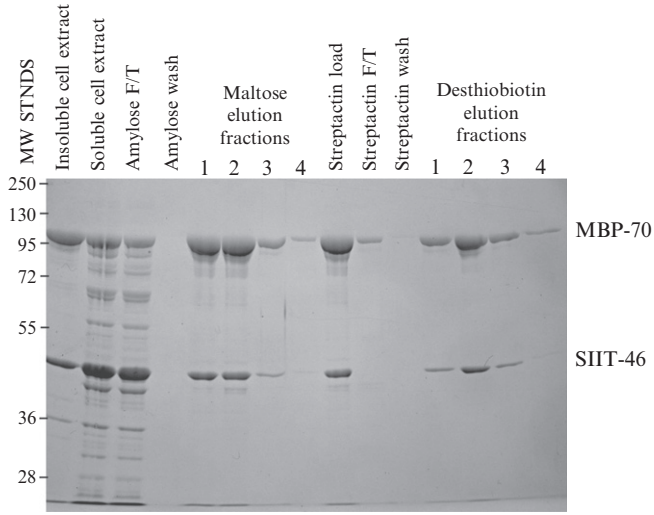

Figure 10.3 Coexpression and tandem purification of recombinant MBP:IFT70 and SIIT:IFT46. Shown here is the Coomassie Blue-stained SDS-PAGE gel (10% acrylamide) of samples from each step of the tandem purification of MBP:IFT70 (MBP-70) and SIIT:IFT46 (SIIT-46). The first two lanes following the mobility markers (MW STNDS) contain the insoluble and soluble fractions of bacterial cell lysates. F/T represents the flow-through proteins that did not bind to the respective resins. Note that both proteins copurify after tandem purification with both types of chromatography. *Reprinted from Fan et al. (2010) with modification by permission of the American Society for Cell Biology.*

absorbance at 280 nm to identify peak elution; further analyze fractions using SDS-PAGE.

5. For tandem chromatography, prepare a 0.5 ml column of Strep-Tactin™ Sepharose™ (GE Healthcare) using a 0.8 cm × 4 cm conical polypropylene column (Bio-Rad). Prepare the StrepTactin™ resin for loading by washing with 2.5 ml StrepTactin™ column buffer (SCB; 150 m*M* NaCl in 10 m*M* Tris, pH 8.0).
6. Pool and load the peak amylose fractions onto the StrepTactin™ resin; collect the flow-through and reload one to two more times to maximize binding.
7. Wash the nonspecifically bound proteins off the StrepTactin™ resin with 2.5–5 ml SCB.
8. Elute the Strep-II-tagged protein (i.e., SIIT:IFT46) from the resin with ≥2 ml SCB containing 2.5 m*M* desthiobiotin. Collect 0.5-ml fractions for subsequent electrophoretic analysis.

6. YEAST-BASED INTERACTION ANALYSIS OF IFT A PROTEINS

Yeast-based two-hybrid analysis is commonly used to identify putative protein–protein interactions. The conventional approach utilizes a single bait protein and a library of potentially interacting proteins; typically, this library consists of most or all the cDNAs generated from the total mRNA of a given organism. Alternatively, every possible pair of interacting proteins from a defined list (such as the IFT proteins) can be evaluated by this method, but this can be time-consuming. In order to accelerate this process, a pooled library approach was designed to quickly screen for interacting IFT A subunits.

6.1. Two-hybrid analysis using a pooled library approach

6.1.1 Construction and screening of pooled libraries

With all six of the *Chlamydomonas* genes identified that encode the IFT A proteins, it was possible to amplify the full-length cDNA for each gene for use in these studies (Behal et al., 2012).

1. Using cDNA as template, clone full-length copies of each of the six IFT A genes, following standard protocols, into pAD-MCS (a derivative of pAD-GAL4; Stratagene) and into pGBKT7 (Clontech Laboratories); these vectors express fusion proteins incorporating the GAL4-activation domain and the GAL4 DNA-binding domain as N-terminal fusions to the cloned exogenous proteins.
2. Transform competent AH109 yeast cells with each individual activation domain (AD) plasmid construct, utilizing a standard lithium acetate/PEG-8000/sheared salmon sperm DNA protocol as described by the manufacturer (Stratagene); select transformed yeast on solid medium (SD, -Leu).
3. Transform competent Y187 yeast cells with each individual binding domain (BD) plasmid construct as above; select transformed yeast colonies on solid medium (SD, -Trp).
4. Generate an IFT A AD library by actively growing and mixing equal numbers of yeast cells expressing each subunit in the AD vector. Generate an analogous IFT A BD library by actively growing and mixing equal numbers of yeast cells expressing each subunit in the BD vector.
5. Combine the AD and BD libraries and allow the yeast to mate following instructions in the BD Matchmaker Kit (BD Biosciences, Clontech Laboratories); select colonies on minimal medium that is deficient in Leu, Trp,

His, and adenine (SD, -Leu, -Trp, -His, -Ade). Only cells carrying fusion proteins that interact should be able to grow well in the absence of His.

6.1.2 Analysis of potential interacting protein pairs

Yeast colonies that grow on selective (SD, -Leu, -Trp, -His, -Ade) media are analyzed by PCR to determine the identity of the interacting proteins.

1. Suspend individual colonies in 20 μl of water and heat at 95 °C for 5 min.
2. Remove insoluble material with a 5-min centrifugation at 13,000 rpm (16,100 × *g*) at room temperature.
3. Set up two PCRs, each specific for either the AD or BD vector. Using 1.0 μl of the clarified yeast extract as template in an 11 μl PCR; in addition, each PCR contains a specific vector primer (AD or BD) and six unique subunit-specific primers, and all the other reagents necessary for amplification. Each vector/subunit primer pair generates a different-sized DNA fragment in the presence of its corresponding template DNA.
4. Agarose gel electrophoresis of the resulting DNA fragments results in identification of the two recombinant proteins in each yeast colony.

For any putative pairs of interacting proteins, cotransform competent yeast with both combinations of AD and BD fusion plasmids. Test all of the plasmids in question against the appropriate AD and BD control plasmids to verify the specificity of the interaction as described previously; when this approach was applied to the *Chlamydomonas* IFT A proteins, the only pairwise interaction that resulted was between IFT121 and IFT43 (Behal et al., 2012).

7. SUMMARY

The process of IFT requires multiple interactions of ~20 IFT proteins. These interactions range in complexity from simple pairs of specific IFT components, through the IFT A and B cores, to the larger assemblies of intact IFT A and B complexes, and finally up to the large IFT particles or trains visualized by electron microscopy (Kozminski et al., 1995, 1993; Pigino et al., 2009). As expected, no single approach has been successful in clarifying all of the interactions within these complexes and particles. The combination of diverse approaches, however, has yielded the identity of direct interactions as well as information about the larger architectural arrangement of these proteins. As a model system, *C. reinhardtii* has provided several important advantages to pursue these studies. The relative ease with which mutants can be generated and characterized allows the researcher to

isolate *Chlamydomonas* strains displaying a specific phenotype; for IFT studies, these mutations typically reveal themselves as alterations in flagellar physiology. Equally important, large biochemical quantities of *Chlamydomonas* cultures can be grown and harvested allowing for routine biochemical isolation of the IFT proteins.

We present here a group of protocols that we have utilized to study the interactions of IFT proteins. A number of these approaches have exploited the biochemical preparation of *Chlamydomonas* protein extracts which furnish the raw material which can be further analyzed. Chemical cross-linking, for example, is an approach that requires biochemical quantities of protein complexes to identify neighboring subunits. Immunoaffinity pull-downs also take advantage of the algal model organism because of the availability of antibodies raised against many of the *Chlamydomonas* IFT proteins. Other approaches are less specific to the model organism. Bacterial coexpression, for example, is a valuable tool for identifying IFT protein–protein interactions and could be employed to examine such interactions in any ciliated organism. Interpretation of these recombinant results, though, must always take into consideration the heterologous aspect of eukaryotic protein expression in a prokaryotic system; both false positives and false negatives are to be expected. Hydrodynamic methods such as sucrose density gradient centrifugation and size-exclusion chromatography allow the researcher to determine the size and shape (and infer the stoichiometry) of various IFT protein assemblies, whether the source is a simple two-component interaction from bacterial coexpression, or a large subcomplex or complex derived from native cells or tissues. In sum, this collection of protocols provides the researcher with the tools to generate IFT complexes and to characterize the interactions among their components.

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REFERENCES

Ahmed, N. T., Gao, C., Lucker, B. F., Cole, D. G., & Mitchell, D. R. (2008). ODA16 aids axonemal outer row dynein assembly through an interaction with the intraflagellar transport machinery. *The Journal of Cell Biology*, *2008*(183), 313–322.

Beales, P. L., Bland, E., Tobin, J. L., Bacchelli, C., Tuysuz, B., Hill, J., et al. (2007). *IFT80*, which encodes a conserved intraflagellar transport protein, is mutated in Jeune asphyxiating thoracic dystrophy. *Nature Genetics*, *39*, 727–729.

Behal, R. H., Betleja, E., & Cole, D. G. (2009). Purification of IFT particle proteins and preparation of recombinant proteins for structural and functional analysis. In S. King & G. Pazour (Eds.), *Cilia: Motors and regulation: Vol. 92. Methods in cell biology* (pp. 179–196). San Diego: Academic Press (chapter 10).

Behal, R. H., Miller, M. S., Qin, H., Lucker, B. F., Jones, A., & Cole, D. G. (2012). Subunit interactions and organization of the *Chlamydomonas reinhardtii* intraflagellar transport complex A proteins. *The Journal of Biological Chemistry*, *287*, 11689–11703.

Blacque, O. E., Cevik, S., & Kaplan, O. I. (2008). Intraflagellar transport: From molecular characterisation to mechanism. *Frontiers in Bioscience*, *13*, 2633–2652.

Bornhorst, J. A., & Falke, J. J. (2000). Purification of proteins using polyhistidine affinity tags. *Methods in Enzymology*, *326*, 245–254.

Brazelton, W. J., Amundsen, C. D., Silflow, C. D., & Lefebvre, P. A. (2001). The *bld1* mutation identifies the *Chlamydomonas osm-6* homolog as a gene required for flagellar assembly. *Current Biology*, *11*, 1591–1594.

Clauser, K. R., Baker, P., & Burlingame, A. L. (1999). Role of accurate mass measurement (+/−10 ppm) in protein identification strategies employing MS or MS/MS and database searching. *Analytical Chemistry*, *71*, 2871–2882.

Cole, D. G., Diener, D. R., Himelblau, A. L., Beech, P. L., Fuster, J. C., & Rosenbaum, J. L. (1998). *Chlamydomonas* kinesin-II-dependent intraflagellar transport (IFT): IFT particles contain proteins required for ciliary assembly in *Caenorhabditis elegans* sensory neurons. *The Journal of Cell Biology*, *141*, 993–1008.

Cole, D. G., & Snell, W. J. (2009). SnapShot: Intraflagellar transport. *Cell*, *137*, 784–784.e1.

Deane, J. A., Cole, D. G., Seeley, E. S., Diener, D. R., & Rosenbaum, J. L. (2001). Localization of intraflagellar transport protein IFT52 identifies basal body transitional fibers as the docking site for IFT particles. *Current Biology*, *11*, 1586–1590.

Dentler, W. (2005). Intraflagellar transport (IFT) during assembly and disassembly of *Chlamydomonas* flagella. *The Journal of Cell Biology*, *170*, 649–659.

Fan, Z. C., Behal, R. H., Geimer, S., Wang, Z., Williamson, S. M., Zhang, H., et al. (2010). *Chlamydomonas* IFT70/CrDYF-1 is a core component of IFT particle complex B and is required for flagellar assembly. *Molecular Biology of the Cell*, *21*, 2696–2706.

Gilissen, C., Arts, H. H., Hoischen, A., Spruijt, L., Mans, D. A., Arts, P., et al. (2010). Exome sequencing identifies WDR35 variants involved in Sensenbrenner syndrome. *The American Journal of Human Genetics*, *87*, 418–423.

Gorman, D. S., & Levine, R. P. (1965). Cytochrome f and plastocyanin: Their sequence in the photosynthetic electron transport chain of *Chlamydomonas reinhardi*. *Proceedings of the National Academy of Sciences of the United States of America*, *54*, 1665–1669.

Hou, Y., Pazour, G. J., & Witman, G. B. (2004). A dynein light intermediate chain, D1bLIC, is required for retrograde intraflagellar transport. *Molecular Biology of the Cell*, *15*, 4382–4394.

Hou, Y., Qin, H., Follit, J. A., Pazour, G. J., Rosenbaum, J. L., & Witman, G. B. (2007). Functional analysis of an individual IFT protein: IFT46 is required for transport of outer dynein arms into flagella. *The Journal of Cell Biology*, *176*, 653–665.

Iomini, C., Babaev-Khaimov, V., Sassaroli, M., & Piperno, G. (2001). Protein particles in *Chlamydomonas* flagella undergo a transport cycle consisting of four phases. *The Journal of Cell Biology*, *153*, 13–24.

Kozminski, K. G., Beech, P. L., & Rosenbaum, J. L. (1995). The *Chlamydomonas* kinesin-like protein FLA10 is involved in motility associated with the flagellar membrane. *The Journal of Cell Biology*, *131*, 1517–1527.

Kozminski, K. G., Johnson, K. A., Forscher, P., & Rosenbaum, J. L. (1993). A motility in the eukaryotic flagellum unrelated to flagellar beating. *Proceedings of the National Academy of Sciences of the United States of America*, *90*, 5519–5523.

Lechtreck, K. F., Luro, S., Awata, J., & Witman, G. B. (2009). HA-tagging of putative flagellar proteins in *Chlamydomonas reinhardtii* identifies a novel protein of intraflagellar transport complex B. *Cell Motility and the Cytoskeleton*, *66*, 469–482.

Lucker, B. F., Behal, R. H., Qin, H., Siron, L. C., Taggart, W. D., Rosenbaum, J. L., et al. (2005). Characterization of the intraflagellar transport complex B core: Direct interaction of the IFT81 and IFT74/72 subunits. *The Journal of Biological Chemistry*, *280*, 27688–27696.

Lucker, B. F., Miller, M. S., Dziedzic, S. A., Blackmarr, P. T., & Cole, D. G. (2010). Direct interactions of intraflagellar transport complex B proteins IFT88, IFT52, and IFT46. *The Journal of Biological Chemistry*, *285*, 21508–21518.

Miller, M. S., Esparza, J. M., Lippa, A. M., Lux, F. G., III, Cole, D. G., & Dutcher, S. K. (2005). Mutant kinesin-2 motor subunits increase chromosome loss. *Molecular Biology of the Cell*, *16*, 3810–3820.

Mueller, J., Perrone, C. A., Bower, R., Cole, D. G., & Porter, M. E. (2005). The FLA3 KAP subunit is required for localization of kinesin-2 to the site of flagellar assembly and processive anterograde intraflagellar transport. *Molecular Biology of the Cell*, *16*, 1341–1354.

Mukhopadhyay, S., Wen, X., Chih, B., Nelson, C. D., Lane, W. S., Scales, S. J., et al. (2010). TULP3 bridges the IFT-A complex and membrane phosphoinositides to promote trafficking of G protein-coupled receptors into primary cilia. *Genes & Development*, *24*, 2180–2193.

Pazour, G. J., Dickert, B. L., Vucica, Y., Seeley, E. S., Rosenbaum, J. L., Witman, G. B., et al. (2000). *Chlamydomonas IFT88* and its mouse homologue, polycystic kidney disease gene *tg737*, are required for assembly of cilia and flagella. *The Journal of Cell Biology*, *151*, 709–718.

Pazour, G. J., Dickert, B. L., & Witman, G. B. (1999). The DHC1b (DHC2) isoform of cytoplasmic dynein is required for flagellar assembly. *The Journal of Cell Biology*, *144*, 473–481.

Pazour, G. J., Wilkerson, C. G., & Witman, G. B. (1998). A dynein light chain is essential for the retrograde particle movement of intraflagellar transport (IFT). *The Journal of Cell Biology*, *141*, 979–992.

Pedersen, L. B., Miller, M. S., Geimer, S., Leitch, J. M., Rosenbaum, J. L., & Cole, D. G. (2005). *Chlamydomonas* IFT172 is encoded by *FLA11*, interacts with CrEB1, and regulates IFT at the flagellar tip. *Current Biology*, *15*, 262–266.

Penefsky, H. S. (1977). Reversible binding of Pi by beef heart mitochondrial adenosine triphosphatase. *The Journal of Biological Chemistry*, *252*, 2891–2899.

Perrone, C. A., Tritschler, D., Taulman, P., Bower, R., Yoder, B. K., & Porter, M. E. (2003). A novel dynein light intermediate chain colocalizes with the retrograde motor for intraflagellar transport at sites of axoneme assembly in *Chlamydomonas* and mammalian cells. *Molecular Biology of the Cell*, *14*, 2041–2056.

Pigino, G., Geimer, S., Lanzavecchia, S., Paccagnini, E., Cantele, F., Diener, D. R., et al. (2009). Electron-tomographic analysis of intraflagellar transport particle trains *in situ*. *The Journal of Cell Biology*, *187*, 135–148.

Piperno, G., & Mead, K. (1997). Transport of a novel complex in the cytoplasmic matrix of *Chlamydomonas* flagella. *Proceedings of the National Academy of Sciences of the United States of America*, *94*, 4457–4462.

Piperno, G., Siuda, E., Henderson, S., Segil, M., Vaananen, H., & Sassaroli, M. (1998). Distinct mutants of retrograde intraflagellar transport (IFT) share similar morphological and molecular defects. *The Journal of Cell Biology*, *143*, 1591–1601.

Porter, M. E., Bower, R., Knott, J. A., Byrd, P., & Dentler, W. (1999). Cytoplasmic dynein heavy chain 1b is required for flagellar assembly in *Chlamydomonas*. *Molecular Biology of the Cell*, *10*, 693–712.

Qin, H., Diener, D. R., Geimer, S., Cole, D. G., & Rosenbaum, J. L. (2004). Intraflagellar transport (IFT) cargo: IFT transports flagellar precursors to the tip and turnover products to the cell body. *The Journal of Cell Biology*, *164*, 255–266.

Qin, H., Rosenbaum, J. L., & Barr, M. M. (2001). An autosomal recessive polycystic kidney disease gene homolog is involved in intraflagellar transport in *C. elegans* ciliated sensory neurons. *Current Biology*, *11*, 457–461.

Qin, H., Wang, Z., Diener, D., & Rosenbaum, J. (2007). Intraflagellar transport protein 27 is a small G protein involved in cell-cycle control. *Current Biology*, *17*, 193–202.

Rompolas, P., Pedersen, L. B., Patel-King, R. S., & King, S. M. (2007). *Chlamydomonas* FAP133 is a dynein intermediate chain associated with the retrograde intraflagellar transport motor. *Journal of Cell Science*, *120*, 3653–3665.

Rosenbaum, J. L., & Witman, G. B. (2002). Intraflagellar transport. *Nature Reviews. Molecular Cell Biology*, *3*, 813–825.

Sachdev, D., & Chirgwin, J. M. (2000). Fusions to maltose-binding protein: Control of folding and solubility in protein purification. *Methods in Enzymology*, *326*, 312–321.

Scholey, J. M. (2003). Intraflagellar transport. *Annual Review of Cell and Developmental Biology*, *19*, 423–443.

Skerra, A., & Schmidt, T. G. (2000). Use of the Strep-Tag and streptavidin for detection and purification of recombinant proteins. *Methods in Enzymology*, *326*, 271–304.

Smith, D. B. (2000). Generating fusions to glutathione S-transferase for protein studies. *Methods in Enzymology*, *326*, 254–270.

Walther, Z., Vashishtha, M., & Hall, J. L. (1994). The *Chlamydomonas FLA10* gene encodes a novel kinesin-homologous protein. *The Journal of Cell Biology*, *126*, 175–188.

Wang, Z., Fan, Z. C., Williamson, S. M., & Qin, H. (2009). Intraflagellar transport (IFT) protein IFT25 is a phosphoprotein component of IFT complex B and physically interacts with IFT27 in *Chlamydomonas*. *PLoS One*, *4*, e5384.

Witman, G. B., Carlson, K., Berliner, J., & Rosenbaum, J. L. (1972). *Chlamydomonas* flagella. I. Isolation and electrophoretic analysis of microtubules, matrix, membranes, and mastigonemes. *The Journal of Cell Biology*, *54*, 507–539.