WD60/FAP163 is a dynein intermediate chain required for retrograde intraflagellar transport in cilia

Ramila S. Patel-King⁎, Renée M. Gilbert⁎⁎, Erik F. Y. Hom⁎, and Stephen M. King⁎

⁎Department of Molecular, Microbial and Structural Biology, University of Connecticut Health Center, Farmington, CT 06030; ⁎Department of Molecular and Cellular Biology, Harvard University, Cambridge, MA 02138

ABSTRACT Retrograde intraflagellar transport (IFT) is required for assembly of cilia. We identify a Chlamydomonas flagellar protein (flagellar-associated protein 163 [FAP163]) as being closely related to the D1bIC(FAP133) intermediate chain (IC) of the dynein that powers this movement. Biochemical analysis revealed that FAP163 is present in the flagellar matrix and is actively trafficked by IFT. Furthermore, FAP163 copurified with D1bIC(FAP133) and the LC8 dynein light chain, indicating that it is an integral component of the retrograde IFT dynein. To assess the functional role of FAP163, we generated an RNA interference knockdown of the orthologous protein (WD60) in planaria. The Smed-wd60(RNAi) animals had a severe ciliary assembly defect that dramatically compromised whole-organism motility. Most cilia were present as short stubs that had accumulated large quantities of IFT particle–like material between the doublet microtubules and the membrane. The few remaining approximately full-length cilia had a chaotic beat with a frequency reduced from 24 to ~10 Hz. Thus WD60/FAP163 is a dynein IC that is absolutely required for retrograde IFT and ciliary assembly.

INTRODUCTION Intraflagellar transport (IFT) is a complex bidirectional motility system that is required for the assembly and maintenance of both motile and primary/sensory cilia (Rosenbaum and Witman, 2002). In general, the components of this system have been broadly conserved from protists to vertebrates, and defects impact a wide array of motility, signaling, and sensory functions. In vertebrates, this leads to many different developmental defects and complex syndromes (e.g., pazour et al., 2000; li et al., 2004; hildebrandt et al., 2011).

The core of the IFT system consists of particles (or trains) comprising multiple copies of two subcomplexes (termed A and B), which have very distinct protein compositions (cole, 2003). A combination of genetics, biochemistry, and fluorescence imaging suggests that IFT-B is necessary for anterograde transport, whereas IFT-A functions in the retrograde direction (reviewed in cole, 2009). Cargoes required for assembly and maintenance of cilia are thought to be loaded onto the IFT trains at the basal body region of the cell. After gated entry into the ciliary compartment, these IFT particles are then transported toward the ciliary tip by one or more kinesin motors (snow et al., 2004). After reorganization at the tip, IFT particles are returned to the ciliary base by a dynein that is more closely related to canonical cytoplasmic dynein than it is to those that power the axoneme. Mutants defective in known components of this retrograde motor are able only to assemble short ciliary stubs in which the region between the doublet microtubules and the membrane is swollen due to the accumulation of IFT particles that are sent into the organelle but cannot be returned to the cell body (pazour et al., 1999; signor et al., 1999).

Two different kinesins have been implicated in anterograde IFT in several organisms (pan et al., 2006; wickstead and gull, 2006), but it has been generally assumed that there is a single dynein holoenzyme responsible for retrograde transport (here termed the IFT dynein for clarity, although it may have additional functions outside of cilia). There has been a suggestion, however, that two different dyneins might participate in this process in Caenorhabditis elegans (hao et al., 2011). In Chlamydomonas, the IFT dynein is believed to contain two heavy chain (HC) motor units (D1bHC, encoded at
DHC16; Pazour et al., 1999; Porter et al., 1999) that are tightly associated with light intermediate chains (D1bIC, encoded at DLI1; Perrone et al., 2003) and an intermediate chain/light chain (IC/LC) complex that consists of a WD-repeat IC (D1bIC (flagellar-associated protein 133 [FAP133]), encoded at DIC5; Rompolas et al., 2007) and the LC8 dynein light chain dimer (encoded at DLI1, also known as FLA14; Pazour et al., 1998). Canonical cytoplasmic dynein also contains LC dimers of the DYNLT (Tctex1) and DYNLRB (roadblock) families; although these have not yet been identified biochemically within the Chlamydomonas IFT dynein (Witman, 2012), there is evidence previously demonstrated was an IC of the D1bIC (FAP133), which we propose that the FAP163 protein (FAP163) is closely related to D1bIC (FAP133), and its orthologue WD60 after two iterations, 9 x 10^-103; Figure 1A). Sequence analysis of human WD60 (1066 residues; molecular weight, 122,570 Da; pI = 6.58) revealed that it contains an N-terminal segment that is predicted to be almost completely α-helical, containing two short stretches that have a very high probability of forming coiled coils (Figure 1B) and a repetitive region (residues 24–328) containing many charged residues (Figure 1C), followed by a C-terminal region that is predicted to consist entirely of a β-strand with a single short helical segment near the very terminus. Three canonical WD repeats were found within this β-strand segment using the SMART algorithm (Figure 1D). At least three additional putative

![Diagram](image)

**FIGURE 1:** Phylogeny and domain organization of WD60/FAP163. (A) The sequences of dynein-associated WD-repeat components, as well as mammalian, planarian, and algal WD60/FAP163 proteins, were aligned using CLUSTALW, and a neighboring-join tree was generated. All WD60/FAP163 sequences cluster together and are most closely related to the Chlamydomonas retrograde dynein IC D1bIC (FAP133) and its human orthologue (WD60). Sequences used were H. sapiens (HS) WD60 (NP.060521), Rattus norvegicus (Rn) WD60 (NP.001178702), Bos taurus (Bt) WD60 (XP.001788990), S. mediterranea (Sm) WD60 (mk4.000489.09.01), C. reinhardtii (Cr) FAP163 (XP.0001702532), Volvox carteri (Vc) FAP163 (XP.002954132), H. sapiens WD34 (AAH01614), C. reinhardtii D1bIC/FAP133 (BC133909), C. reinhardtii IC2 (P27766), Strongylocentrotus purpuratus (Sp) IC3 (XP.796667), C. reinhardtii IC10 (EDP0123), C. reinhardtii Lis1 (ABG33844), Mus musculus (Mm) Lis1 (NP.038653), C. reinhardtii IC1 (Q39578), and St. purpuratus IC2 (XP.003724308). (B) The propensity for coiled-coil formation within human WD60 was analyzed using the COILS algorithm. Two segments have a very high prediction for coiled-coil formation. (C) Comparison of human WD60 against itself using DOTLET (matrix = BLOSUM62; window = 15) revealed that the N-terminal region is repetitive, consisting of multiple charged segments with high similarity. (D) Domain organization of human WD60. The N-terminal region contains two coiled-coil segments embedded within a region predicted to be almost entirely α-helical. The C-terminal domain consists of a series of predicted β-strands; three canonical WD repeats were identified within this region using SMART (residues 686–725, 901–938, and 941–983). WD repeats are often highly degenerate and difficult to identify; visual inspection of the predicted secondary structure, however, suggests that there may be two additional, poorly conserved repeats between the first and second canonical WD repeats and a third before the first canonical repeat. An IQ motif is located between residues 432 and 450.

As we demonstrate that FAP163 is a dynein IC involved in retrograde IFT, we propose that the Chlamydomonas gene name be reassigned as D1bIC in accordance with the unified nomenclature for ciliary dyneins (Hom et al., 2011). Previously the FAP133 protein was assigned as D1bIC (Hom et al., 2011). Because it is now clear that this dynein contains two different ICs, we suggest that FAP133 and FAP133 be renamed as D1bIC1 and D1bIC2, respectively.
canonical repeats. In addition, a single IQ motif (residues 432–450) was identified by the Eukaryotic Linear Motif Resource (http://elm.eu.org), suggesting that this protein may bind calmodulin.

Biochemical analysis of FAP163 in Chlamydomonas flagella

Analysis of the Chlamydomonas flagellar proteome revealed the presence of FAP163 migrating at $M_r = 90,000$ (Pazour et al., 2005), although the calculated mass is only 76,134 Da. When blot-purified antibody CT295 raised against the FAP163 C-terminal domain was used to probe samples of wild-type flagella, a single major band of $M_r 89,000$ was detected, which is completely consistent with the electrophoretic migration of FAP163 observed in the proteomic study (Figure 2A). FAP163 was present in flagella from mutants that lack outer arms (oda1-6), inner arms (ida1, ida4), radial spokes (pf14), or the central pair microtubule complex (pf18) (Figure 2B), although levels did appear reduced in ida1 and possibly ida4. To determine where FAP163 is located within the flagellum, we solubilized membrane/matrix components by detergent treatment. FAP163 was observed almost exclusively in the soluble fraction. Similarly, most FAP163 was present in the extract obtained from flagella after multiple rounds of freeze/thaw, which release the flagellar matrix but leave the membrane associated with the axoneme (Figure 2C). Thus FAP163 is a component of the flagellar matrix and is not tightly bound to the microtubular axoneme or the membrane.

To assess the oligomeric state of FAP163, we fractionated freeze/thaw extracts by chromatography in a Superose 6 gel filtration column. FAP163 eluted in a single broad peak with an estimated mass of $>350$ kDa and precisely copurified with one peak of the LC8 dynein light chain, which is present in multiple flagellar complexes (Figure 3A). Furthermore, we observed that under these solution conditions, FAP163 also coeluted with D1bIC(FAP133), suggesting that both WD-repeat proteins might be part of the same complex. To further test this putative association, we fractionated a wild-type freeze/thaw flagella extract by ion exchange chromatography in a Mono Q column (Figure 3B). D1bIC(FAP133) was found in two distinct peaks, as described previously (Rompolas et al., 2007). We also observed that FAP163 precisely copurified with both D1bIC(FAP133) peaks. These data strongly suggest that FAP163 and D1bIC(FAP133) are associated within the same IC/LC complex.

degenerate WD repeats were tentatively identified by visual inspection of the predicted secondary structure; one is located before the first canonical WD repeat and two more as the first and second...
To determine whether FAP163 is transported by the IFT machinery, we incubated wild-type (cc125) and fla10 cells at 22 or 32°C for 3 h and then deflagellated them. As expected, analysis of the resulting flagella revealed that D1bIC(FAP133), a component of the retrograde IFT motor, was highly depleted in flagella from fla10 cells held at the restrictive temperature compared with wild type. Similarly, FAP163 levels also were reduced, whereas those of the axoneme-associated outer arm dynein proteins LC1 and IC1 were not (Figure 4). This result indicates that flagellar FAP163 is actively trafficked by IFT.

RNAi-mediated knockdown of WD60 in planaria

No Chlamydomonas FAP163 mutant currently exists. Therefore, to test the hypothesis that WD60/FAP163 might represent another dynein IC that plays a key role in retrograde IFT, we performed an RNAi-mediated knockdown in the planarian S. mediterranea. Of interest, this organism lacks a recognizable D1bIC(FAP133). Accordingly, we obtained a 325-base pair region of the WD60 coding region by PCR from planarian first-strand cDNA and subcloned this into the L4440 vector that has opposing T7 promoters and
expressed double-stranded RNA (dsRNA) in Escherichia coli strain HT115(DE3). Planaria were fed every 2–3 d for several weeks with this dsRNA or with bacteria containing an empty vector.

Analysis of mRNA by RT (reverse transcription)-PCR from first-strand cDNA prepared using a random hexamer as the primer revealed that two bands for WD60 were present. One product obtained was the originally targeted 325–base pair fragment that encodes the central portion of the WD60 protein, whereas the second product of 502 base pairs consisted of this same exonic segment but included two unspliced introns (Figure 5, A and B). The 502–base pair product was not observed when first-strand cDNA was prepared using oligo-dT as the primer for reverse transcriptase, nor was it obtained when the original RNA sample was treated with DNasel. Consequently, this second product derives from genomic DNA. Of interest, when first-strand cDNA was prepared using an oligo-dT primer, we obtained the 325–base pair product and two minor additional products that included one or the other of the two introns (Figure 5C). Thus planaria maintain a detectable pool of partially spliced polyadenylated mRNA for WD60. RT-PCR using total RNA derived from Smed-wd60(RNAi) planaria further revealed that ingestion of WD60 dsRNA resulted in greatly decreased amounts of the 325–base pair product compared with empty vector or no vector controls but had essentially no effect on transcript levels for actin, IFT88, or IC2 (Figure 5A).

Phenotypic analysis of Smed-wd60(RNAi) planaria

We next monitored organismal gliding locomotion and found that Smed-wd60(RNAi) animals moved at 0.55 ± 0.19 mm/s (mean ± SD; n = 13) compared with 1.48 ± 0.21 mm/s (n = 7) for empty vector controls (Figure 5D and E, and Supplemental Movie S1). This was taken from the decompiled combined video (see Supplemental Movie S2).
decrease in gliding velocity is similar to that observed for planaria that lack functional outer dynein arms (Smed-ic2(RNAi)) or indeed are unable to assemble cilia at all (Smed-ift88(RNAi); Rompolas et al., 2010). Furthermore, Smed-wd60(RNAi) animals exhibited the alteration in the mode of motility, from cilia-driven gliding to peristaltic waves of muscular contraction, that was found previously (Rompolas et al., 2010) to be a defining characteristic of defects in ciliary assembly and motility (Figure 5F and Supplemental Movie S2). After prolonged feeding with dsRNA for >4 wk, Smed-wd60(RNAi) planaria became essentially stationary and had no discernible cilia at the dorsal/ventral margin when examined by differential interference contrast microscopy. Furthermore, these planaria were unable to use the musculature in the rear of the body and eventually split approximately halfway along their length, leading to the partial disintegration of the animal.

Examination of the ventral surface by scanning electron microscopy (EM) of animals fed the RNAi vector for ~3 wk revealed a dramatic reduction in both number and length of cilia assembled by Smed-wd60(RNAi) planaria compared with controls (Figure 6). In control animals, the ventral epithelium bore thousands of ~10-μm-long cilia, especially toward the anterior and in the central region surrounding the pharyngeal pore (Figure 6, A and C). In contrast, only relatively few almost-full-length cilia and many short ciliary stubs (<0.5 μm in length) were evident on Smed-wd60(RNAi) planaria (Figure 6, B, D, and E). These observations suggested that WD60 is required for ciliary assembly. To further assess this hypothesis, we examined cilia and ciliary stubs from Smed-wd60(RNAi) planaria by thin-section transmission EM (Figure 7). We found that the ciliary stubs contained doublet microtubules and associated structures but had an enlarged diameter due to the presence of large quantities of amorphous electron-dense material located between the axonemal doublet microtubules and the ciliary membrane. Indeed, even in longer cilia, accumulations of this material were readily evident.

High-speed video microscopy and kymograph analysis revealed that the few longer cilia remaining on the ventral surface of Smed-wd60(RNAi) planaria were motile (Figure 8A and Supplemental Movie S3). Their beat appeared uncoordinated, however, and occurred at rates of ~10–14 Hz; some cilia were observed to protrude essentially vertically from the epithelium and to move only at the base, leading to a twitching motion. In contrast, the cilia of control animals exhibited a high degree of metachronal synchrony and beat at ~24 Hz with both power and recovery strokes (Figure 8B and Supplemental Movie S4).

**DISCUSSION**

In this article we examine the functional role of WD60/FAP163, a WD-repeat protein that has been broadly conserved among ciliated organisms. Sequence analysis suggests that this protein is an IC of...
Biochemical analysis reveals that FAP163 is almost exclusively present in the flagellar matrix, as are core IFT components and associated kinesin and dynein motors. Using the fla10 temperature-sensitive anterograde IFT mutant, we found that FAP163 was reduced in Chlamydomonas flagella when cells were held at the restrictive temperature, suggesting that this protein is indeed trafficked by IFT. In contrast, the flagellar levels of FAP163 were not affected by the presence or absence of outer arms, radial spokes, or the central pair complex, although they did appear decreased in the ida1 and perhaps also ida4 inner-arm dynein mutants. When expression of WD60/FAP163 was disrupted in planaria, we observed severe disruption of ciliary structures such that, in general, only short ciliary stubs were retained. These stubs contained accumulated material located between the outer doublet microtubules and the membrane, as is commonly seen in Chlamydomonas strains unable to undergo retrograde IFT (Pazour et al., 1998, 1999; Porter et al., 1999). Thus it appears that WD60/FAP163 is an integral dynein IC that in planaria is absolutely required for ciliary assembly.

FAP163/WD60 is a component of the IFT machinery and required for retrograde IFT
Phylogenetic analysis reveals that WD60/FAP163 groups with the dynein IC subfamily of WD-repeat proteins, and indeed these proteins are most closely related to D1bIC(FAP133 or WD34), which we previously demonstrated to be part of the IFT dynein in Chlamydomonas (Rompolas et al., 2007). Mammalian WD60 has an N-terminal region that is highly repetitive and contains two segments of coiled coil followed by a C-terminal domain consisting of WD-repeat motifs. Furthermore, there is a single IQ motif located within the N-terminal region. Although this motif does not appear to be present in Chlamydomonas FAP163, it does raise the possibility that WD60 in vertebrates binds calmodulin and thus may be responsive to alterations in Ca\textsuperscript{2+} levels. In contrast, D1bIC(FAP133) consists of a short N-terminal domain containing two degenerate LC8 binding sites followed by a C-terminal segment consisting of six WD repeats (Rompolas et al., 2007).

Organization of the dynein motor that powers retrograde intraflagellar transport
Dyneins with multiple HC motor units (outer arm, inner-arm I1/f, and canonical cytoplasmic dynein) all contain an IC/LC complex that at a minimum consists of two WD-repeat ICs associated with dimers of three distinct LC species and is absolutely required for assembly of
the dynein particle (Tang et al., 1982; Mitchell and Kang, 1991). The ICs within these complexes associate directly (Mitchell and Rosenbaum, 1986; King et al., 1991; Lo et al., 2006), whereas LC binding apparently brings structure to inherently disordered regions of the ICs (Nyarko et al., 2004). In axonemal dyneins, the two ICs in the complex are different gene products and form obligate heterodimers (reviewed in King, 2012), whereas in canonical mammalian cytoplasmic dynein the various alternative spliced forms of the two very closely related IC genes are capable of forming both homodimers and heterodimers (Lo et al., 2006), although whether all of the possible combinations actually occur in vivo is uncertain.

Previously the dynein that powers retrograde IFT in Chlamydomonas was known to contain a distinct HC and associated light IC (LIC), as well as D1bIC (also known as FAP133 or WD34) with bound LC8 dimer. Our biochemical analysis using two different chromatographic procedures to fractionate the IC/LC complex demonstrated that FAP163 is also part of this complex, and thus FAP163 and D1bIC(FAP133) presumably form a heterodimer that is further stabilized by LC8. The similar reductions in flagellar levels of both FAP163 and D1bIC(FAP133) in the fla10 mutant shifted to the restrictive temperature further supports this suggestion. We also observed that FAP163 and D1bIC(FAP133) elute together in two distinct complexes after anion exchange, and we demonstrated previously that both these peaks of D1bIC(FAP133) subsequently migrate at ~7 S in sucrose density gradients (Rompolas et al., 2007) and are thus not associated with the D1bHC. This raises the possibility that there are two forms of the IC/LC complex. Further biochemical analysis will be required to determine whether these two IC/LC complex forms differ in protein content or whether the change in elution properties is the result of posttranslational modifications.

Intriguingly, although platyhelminths such as Ca. elegans and S. mediterranea express most components of the IFT dynein and exhibit retrograde IFT (e.g., Signor et al., 1999), they do not encode a recognizable orthologue of D1bIC (Wickstead and Gull, 2012). Given that ICs are believed to be essential for the assembly and function of dyneins with multiple motor domains, this observation was rather puzzling, as it implied that the dynein mediating retrograde IFT in platyhelminths might have an organization distinct from that of all other multimotor dyneins. Our present results suggest an alternative; namely, that some organisms (such as Chlamydomonas and vertebrates) actually employ two distinct dynein ICs for retrograde IFT, whereas planarians appear to have secondarily lost the orthologue of D1bIC(FAP133/WD34) and only use WD60/FAP163. Thus in Schmidtea the IC/LC complex of the IFT dynein may be composed of a FAP163/WD60 homodimer; alternatively, it is possible that an additional IC awaits identification in this organism. Surprisingly, Ca. elegans also appears to lack a WD60/FAP163 orthologue; thus there may be even more diversity in IFT dynein components. Indeed, it has been reported that a second IFT dynein based on a different HC may exist in Ca. elegans (Hao et al., 2011), further suggesting that the mechanisms of retrograde IFT are more complex than originally envisaged.

**Retrograde IFT and human ciliopathies**

Defects in the HC that powers retrograde IFT (DYNC2H1) have been demonstrated to cause ciliopathies in humans and have been particularly associated with short-rib polydactyly and asphyxiating thoracic dystrophy (Jeune’s syndrome; Dagneau et al., 2009; Merrill et al., 2009). Furthermore, HC mutations that result in premature termination occur only in the heterozygous state, suggesting that the phenotype is hypomorphic (Schmidt et al., 2013). Mutations in the NEK1 kinase and the core IFT component IFT80 also result in the failure of ciliary assembly and lead to similar phenotypes (Beales et al., 2007; Thiel et al., 2011), although DYNC2H1 mutations are considered to be the major cause (Bajaj et al., 2013). Our demonstration that lack of the WD60/FAP163 IFT dynein IC results in a severe ciliary assembly defect in planaria due to the failure of retrograde IFT thus raises the clear possibility that in vertebrates, mutations in this gene might cause phenotypes similar to those observed for DYNC2H1, NEK1, and IFT80. Indeed, a very recent report indicates that point mutations that result in truncation of WDR60 before the WD-repeat domain lead to both short-rib polydactyly and Jeune’s syndrome (McInerney-Leo et al., 2013).

**MATERIALS AND METHODS**

**Bioinformatics**

Chlamydomonas FAP163 was classified as a close relative of D1bIC(FAP133) (WD34 in vertebrates) by the Protein Analysis Through Evolutionary Relationships (PANTHER) clustering algorithm (www.pantherdb.org). Orthologous sequences from other organisms...
closely related to Chlamydomonas FAP163 were identified using BLAST. Alignments with known dynein-associated WD-repeat proteins were generated using CLUSTALW (www.clustal.org) and the output used to construct a neighbor-joining phylogenetic tree. The secondary structure of WD60 was assessed using PREDICTPROTEIN (www.predictprotein.org). Identification of WD-repeat domains, coiled-coil regions, and the repetitive N-terminal segment was performed using SMART (http://smart.embl-heidelberg.de), COILS (http://embnet.vital-it.ch/software/COILS_form.html), and DOTLET (www.iwk.unibe.ch/dotlet/dotlet.html), respectively. The IQ motif was identified using the Eukaryotic Linear Motif Resource.

*Chlamydomonas strains and culture*

*Chlamydomonas reinhardtii* wild-type strains cc124/cc125 and mutants fla10, oda1, oda2, oda3, oda4, oda5, oda6, ida1, ida4, pf14, and pf18 were grown in R medium bubbled with 95% air/5% CO₂ on 15:9 h light/dark cycle.

*Preparation of α-FAP163 antibody*

Sequence encoding the C-terminal 264 residues of FAP163 was obtained by PCR from first-strand cDNA and subcloned into the pMAL-c2 vector across the Xmal/XbaI restriction sites, resulting in a C-terminal fusion to maltose-binding protein (MBP). Expression of the MBP fusion protein in *E. coli* BL21(DE3) was induced with 1 mM isopropylthigalactoside and the protein subsequently purified by chromatography on an amylose affinity column. The purified fusion protein was then used as the immunogen for antibody production in rabbit CT295 (Covance Immunology Services, Denver, PA). Specific antibody was obtained from the resulting immune serum by blot affinity using the FAP163 C-terminal domain after removal of the MBP fusion partner.

Other antibodies used were rabbit polyclonals CT248, R4058, and R5932 against D1bic(FAP133), LC8, and LC1, respectively (King and Patel-King, 1995; Benashski et al., 1999; Rompolas et al., 2007), and mouse monoclonal 1878A, which reacts specifically with IC1 (King et al., 1985).

*Flagella preparation and biochemical analysis of Chlamydomonas FAP163*

*Chlamydomonas* strains were deflagellated with dibucaine and flagella purified by standard methods (King, 1995). Flagella (~3 mg/ml) were demembranated with 1% Igepal CA 630 in HMEK buffer (30 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.5, 5 mM MgSO₄, 0.5 mM EDTA, and 25 mM KCl). Alternatively, matrix components were released by three rounds of freeze/thaw as described previously (Rompolas et al., 2007). Chromatographic separations were performed using an AKTApurifier-10 workstation (GE Healthcare Biosciences, Piscataway, NJ). Flagellar matrix samples were fractionated by gel filtration in a Superose 6 10/300 column equilibrated in HMEK or were applied to a HR5/5 Mono Q ion exchange column equilibrated with 20 mM Tris-Cl, pH 8.0, and then eluted using a 0–1 M NaCl gradient. For the fla10 temperature-shift experiment, prewarmed sucrose-containing buffers and dibucaine were added to the cell suspensions so that deflagellation occurred at the appropriate temperature. Subsequent flagella purification steps were performed at 4°C. Samples were electrophoresed in 10 or 12.5% SDS–polyacrylamide gels and either stained with Coomassie brilliant blue or transferred to nitrocellulose, stained with reactive brown 10, and subsequently probed with various antibodies (as described), followed by horse-radish peroxidase–conjugated secondary antibodies. Chemiluminescence was detected using ECL reagent and a LAS4000 cooled charge-coupled device (CCD) digital imaging system (both from GE Healthcare Biosciences).

*RNAi in planaria*

Colonies of the planarian *S. mediterranea* were maintained and expanded as detailed previously (Rompolas and King, 2009). RNAi was performed essentially as described previously (Newmark et al., 2003). In brief, to generate the RNAi construct, a 325-base pair region of the WD60 coding sequence was obtained from first-strand cDNA by PCR and then subcloned across the XbaI/XhoI sites in plasmid L4440, which contains opposing T7 promoters. dsRNA production was induced after transformation into *E. coli* strain HT115(DE3), which lacks RNase III and thus cannot degrade dsRNA. Bacteria containing dsRNA or the empty vector control were fed to groups of planaria every 2–3 d for several weeks. The mRNA levels of the target and control genes were assessed by RT-PCR using standard methods.

*Video microscopy*

Videos of planarian movement in 9-cm Petri dishes were taken with a CCD color camera (DFK 31BU03; The Imaging Source, Charlotte, NC) using IC-capture, version 2.2, software at 30 fps and decomilled using Blaze Media Pro (Mystik Media, Hampstead, NC). Images illustrating the paths taken by individual animals were constructed by overlaying every 100th frame from the decomilled video using Photoshop CS4 (Adobe, San Jose, CA). The 20x real-time movie illustrating this motion was generated by decimating the video frames by 20 while keeping the frame rate at 30 fps using VirtualDub (www.virtualdub.org/). Individual movies were then cropped, combined, and labeled using ImageJ (National Institutes of Health, Bethesda, MD). Higher-magnification real-time videos were taken using a Canon PowerShot SD880 (Canon, Melville, NY), converted with Super C, and processed, cropped, and combined with VirtualDub and ImageJ.

To visualize ciliary beating, individual planaria were confined on a microscope slide by Parafilm spacers or held immobile using a Commodore Compressor (VU e-innovations, Nashville, TN). Cilia at the anterior dorsal–ventral junction were imaged using an Olympus BX51 microscope (Tokyo, Japan) equipped with differential interference contrast optics and either a Plan Apo 60x/1.40 numerical aperture (NA) or 100x/1.35 NA objective lens. Videos of ciliary motility were captured using an X-PRI F1 digital camera (AOS Technologies, Baden Daetwyl, Switzerland) at 250 fps. The video frame rate, compression, cropping, and brightness/contrast were adjusted using VirtualDub and ImageJ. Videos were decomilled with Blaze Media Pro.

*Scanning and transmission electron microscopy*

For scanning EM, planaria were treated overnight with relaxant solution (1% HNO₃, 0.85% formaldehyde, 50 mM MgSO₄), rinsed with buffer, and then fixed with 2.5% glutaraldehyde in 50 mM Na cacodylate, pH 7.4. Subsequently, samples were postfixed with 1% OsO₄ and dehydrated through an ethanol series. After critical-point drying, planaria were mounted, sputter coated with gold, and imaged in a JSM5900 scanning EM (JEOL, Peabody, MA).

To visualize internal ciliary structure, the anterior region of individual planarians was excised and fixed with 1% glutaraldehyde in phosphate-buffered saline for 15 min and then with 1% glutaraldehyde in 50 mM Na cacodylate for 1 h. The fixative solution was changed every 10–15 min to avoid it becoming exhausted due to the large quantity of mucus on the planarian ventral surface. Samples were postfixed with 1% OsO₄, stained en bloc with 1% methanolic
uranyl acetate, dehydrated through ethanol, and embedded in Epon. Thin sections were poststained with uranyl acetate and lead citrate and imaged using a Hitachi H-7650 transmission EM (Hitachi, Tokyo, Japan) operating at 80 kV.

ACKNOWLEDGMENTS

We thank Panteleimon Rompolas (Yale University School of Medicine, New Haven, CT) for many helpful discussions and Maya Yankova (University of Connecticut Health Center, Farmington, CT) for her assistance with electron microscopy. We are also grateful to Carol Wicking (University of Queensland, Brisbane, Australia) for providing a preprint describing human WDR60 mutations before publication. This study was supported by Grant GM051293 from the National Institutes of Health (to S.M.K). E.F.Y.H. was supported in part by the Jane Coffin Childs Memorial Research Fund and the National Institute of General Medical Sciences Center for Systems Biology (GM068763).

REFERENCES

Benashski SE, Patel-King RS, King SM (1999). Light chain 1 from the Chlamydomonas outer dynein arm is a leucine-rich repeat protein associated with the motor domain of the γ heavy chain. Biochemistry 38, 7253–7264.