Dynein-2 Affects the Regulation of Ciliary Length but Is Not Required for Ciliogenesis in *Tetrahymena thermophila*

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Submitted July 21, 2008; Revised October 28, 2008; Accepted November 7, 2008
Monitoring Editor: Paul Forscher

Eukaryotic cilia and flagella are assembled and maintained by the bidirectional intraflagellar transport (IFT). Studies in alga, nematode, and mouse have shown that the heavy chain (Dyh2) and the light intermediate chain (D2LIC) of the cytoplasmic dynein-2 complex are essential for retrograde intraflagellar transport. In these organisms, disruption of either dynein-2 component results in short cilia/flagella with bulbous tips in which excess IFT particles have accumulated. In *Tetrahymena*, the expression of the *DYH2* and *D2LIC* genes increases during reciliation, consistent with their roles in IFT. However, the targeted elimination of either *DYH2* or *D2LIC* gene resulted in only a mild phenotype. Both knockout cell lines assembled motile cilia, but the cilia were of more variable lengths and less numerous than wild-type controls. Electron microscopy revealed normally shaped cilia with no swelling and no obvious accumulations of material in the distal ciliary tip. These results demonstrate that dynein-2 contributes to the regulation of ciliary length but is not required for ciliogenesis in *Tetrahymena*.

INTRODUCTION

Cilia and flagella are important organelles in eukaryotes (reviewed in Gibbons, 1981; Zariwala *et al.*, 2007). For example, cilia are essential for *Tetrahymena* swimming, feeding, and cell division. The cytoskeleton of the cilium/flagellum is the axoneme comprising more than 600 proteins that assemble into the “9 + 2” arrangement of microtubules, outer and inner rows of dynein arms, and the radial spokes (Piperno *et al.*, 1977; Pazour *et al.*, 2005; Smith *et al.*, 2005).

Because of the essential roles of cilia and flagella, mechanisms have evolved to ensure the proper assembly and disassembly of the axoneme. Ciliary components are synthesized in the cell body but are incorporated at the tip of the axoneme; this requires a transport mechanism to carry components from base to tip. Intraflagellar transport (IFT; reviewed in Rosenbaum and Witman, 2002; Scholey, 2003; Blacque *et al.*, 2008) is this transport mechanism producing bidirectional movement of material along the axoneme (Kozminski *et al.*, 1993). IFT can be divided into two distinct stages. Anterograde IFT, mediated by complex B components, carries axonemal components from the cell body to the growing tip. Retrograde IFT, mediated by complex A components, carries the anterograde IFT machinery back from the tip to the cell body in order to recycle the IFT components. Both stages of IFT are powered by microtubule

motors: kinesin-II is the anterograde motor and dynein-2 is the retrograde motor.

Dynein-2 is one of the several dynein complexes present in cells with cilia and flagella. The dynein-2 heavy chain, originally called DHC1b, was first discovered in a search of the dynein heavy chain genes expressed in sea urchin embryos (Gibbons *et al.*, 1994). The dynein-2 motor complex is composed of four subunits: the heavy chain, Dyh2, contains the microtubule motor activity; the dynein-2 light intermediate chain, D2LIC, is thought to stabilize the dynein-2 complex; and the dynein-2 IC, D2IC, and light chain 8, LC8, are hypothesized to mediate the connection between the motor and the retrograde complex A components (Rompolas *et al.*, 2007). In *Tetrahymena*, the dynein-2 components are encoded by the *DYH2* (heavy chain), *D2LIC* (light intermediate chain), *D2IC* (intermediate chain), and *LC8* (light chain) genes (Lee *et al.*, 1999; Wilkes *et al.*, 2007).

In several organisms, dynein-2 is required for the formation of normal cilia/flagella. In *Chlamydomonas*, the individual disruption of the heavy chain, D2LIC or light chain results in two striking effects: 1) the flagella are stumpy and swollen and 2) the flagella are filled with electron-dense material due to the inability of the system to recycle the IFT components (Pazour *et al.*, 1998, 1999; Porter *et al.*, 1999; Hou *et al.*, 2004). In *Caenorhabditis*, the individual disruption of the heavy chain or the D2LIC results in shortened and swollen sensory cilia (Wicks *et al.*, 2000; Schafer *et al.*, 2003). Both dynein-2 heavy chain and D2LIC mutations produce bulbous primary cilia in mice, consequently affecting body patterning during development (Rana *et al.*, 2004; Huangfu and Anderson, 2005; May *et al.*, 2005). In *Leishmania*, deletion of the nonessential isoform of the two dynein-2 heavy chains, LmxDHC2.2, results in rounded cells with no apparent flagella (Adhiambo *et al.*, 2005). Taken together, these observations demonstrate a well-conserved requirement of dynein-2 in normal cilia formation, and that loss of any of

This article was published online ahead of print in *MBC in Press* (<http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E08-07-0746>) on November 19, 2008.

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Abbreviations used: IFT, intraflagellar transport; DYH2, cytoplasmic dynein-2 heavy chain; D2LIC, cytoplasmic dynein-2 light intermediate chain.

the dynein-2 components results in shortened and bulbous flagella or cilia.

The ciliated protozoan *Tetrahymena thermophila* is an important system in which to study IFT because its cilia are essential and are frequently generated. The >1000 surface cilia propel the cell through the medium; cilia draw nutrients into the oral apparatus; and cilia power rotokinesis, which is the final stage of cell division (Brown *et al.*, 1999a). In the laboratory, *Tetrahymena* divide rapidly, thus forming new arrays of cilia every 3 h (Frankel, 2000). *Tetrahymena* anterograde IFT appears to be the same as what has been found in other systems. Disruption of the kinesin-II genes *KIN-1* and *KIN-2* results in the complete absence of cilia (Brown *et al.*, 1999b). As expected, individual disruptions of the *Tetrahymena* complex B genes, *IFT52*, *IFT80*, and *IFT172*, result in extremely short or no cilia (Brown *et al.*, 2003; Beales *et al.*, 2007; Tsao and Gorovsky, 2008a).

Although *Tetrahymena* anterograde IFT is the same as that observed in other species, the role of *Tetrahymena* retrograde IFT appears to be different. In contrast to what has been found in other model organisms, the knockdown of *DYH2* in *Tetrahymena* did not result in the absence of functional cilia (Lee *et al.*, 1999). These *DYH2* knockdown cells continued to produce cilia and were motile, but the transformants were often mis-sized and mis-shaped. This earlier study used a macronuclear knockdown of *DYH2*, and, because of the high copy number of structural genes in the *Tetrahymena* somatic macronucleus, one interpretation of our previous results is that there were sufficient copies of the wild-type *DYH2* gene remaining to permit ciliogenesis to proceed.

To understand more clearly the role of dynein-2 in *Tetrahymena* ciliogenesis, we produced separate knockout heterokaryons for both the *DYH2* and *D2LIC* genes. Knockout heterokaryons have both copies of the targeted gene disrupted in their transcriptionally silent diploid germline micronuclei and are wild-type in their somatic macronuclei (Hai *et al.*, 2000). When two heterokaryons are mated, the gametic micronuclei undergo syngamy and produce new macronuclei; thus, the macronuclei of the progeny of such a mating completely lack the targeted gene. In this way, we have created knockout cell lines of *DYH2*, called KO-DYH2, and *D2LIC*, called KO-D2LIC. As expected, the macronuclei of the knockout cell lines are completely devoid of the targeted genes. Both KO-DYH2 and KO-D2LIC cells produce motile cilia, though the cilia are fewer in number and of more variable lengths than wild-type cilia. The cilia do not have bulbous tips nor do they appear to collect aggregated IFT particles. Thus, dynein-2 is not essential for ciliogenesis in *Tetrahymena*.

MATERIALS AND METHODS

Cell Strains and Culture Media

T. thermophila wild-type strains B2086.1, CU428.1, and CU427, and star strains A*III and B*VII were obtained from the Stock Center for *Tetrahymena thermophila* at Cornell University (Ithaca, NY) or from Jacek Gaertig (University of Georgia, Athens, GA). *Tetrahymena* cell cultures were grown in modified Neffs medium (0.5% proteose peptone, 0.5% yeast extract, 1.1% glucose, 100 μ M FeCl₃) supplemented with 100 U/ml penicillin, 100 μ g/ml streptomycin, and 0.25 μ g/ml amphotericin B at 30°C.

Gene and Protein Sequence Analyses

The full length *T. thermophila* *D2LIC*, *D2IC*, and *D1LIC* gene sequences and 4986 base pairs (bp) of the *DYH2* gene encoding the region past the AAA-4 domain (including the microtubule-binding domain) were determined initially by a tBLASTn search of the *T. thermophila* genome (Eisen *et al.*, 2006; <http://tigrblast.tigr.org/er-blast/index.cgi?project=ttg>). Introns were confirmed by RT-PCR and sequencing at the Rancho Santa Ana Botanical Garden (Claremont, CA) sequencing facility. Total RNA was isolated from B2086.1

Table 1. Dynein intermediate chain sequences used in phylogenetic analyses

Organism	Gene	Accession no.
<i>Chlamydomonas reinhardtii</i>	CrIC69	CAA39053
	CrD2IC	C_20188
<i>Danio rerio</i>	DrD2IC	AAI33910
<i>Dictyostelium discoideum</i>	DdIC74	P54703
<i>Drosophila melanogaster</i>	DmIC74	AAC70937
<i>Gallus gallus</i>	GgIC3	XP_415701
	GgIC74	NP_001006519
<i>Homo sapiens</i>	HsIC3	CAC17464
	HsIC74	AAP97254
	HsD2IC	NP_443076
<i>Strongylocentrotus purpuratus</i>	SpIC3	XP_796667
	SpD2IC	XM_001197794
<i>Tetrahymena thermophila</i>	TtIC3	XP_001015764
	TtIC4	XP_001031628
	TtD2IC	ABX82716

cells by the method of Chirgwin *et al.* (1979). The RNA was treated with amplification grade DNaseI to remove any contaminating genomic DNA (Invitrogen, Carlsbad, CA). Reverse transcription was performed with Superscript III RNaseH⁻ reverse transcriptase according to the manufacturer's protocol (Invitrogen, Carlsbad, CA).

Sequence analyses were done by DNASTAR (DNASTar Lasergene, Madison, WI). Domain analyses of D2LIC and D2IC sequences were done with the Protean program (DNASTar Lasergene) and SMART (<http://dylan.embl-heidelberg.de/>) programs, respectively. Multiple sequences were aligned by ClustalW (ebi.ac.uk/clustalw/index.html). Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 2.1 (www.megasoftware.net) (Kumar *et al.*, 2001). UPGMA, neighbor-joining, and maximum parsimony trees were constructed with known D2LIC and D1LIC sequences or known dynein-2 IC, dynein-1 IC, and outer arm dynein IC3/IC69 sequences. Only the neighbor-joining trees are shown in Figure 1. Similar results were obtained with all three methods. Accession numbers for the sequences compared are shown in Tables 1 and 2.

Gene Regulation after Deciliation

B2086.1 cells grown to $3-4 \times 10^5$ cells/ml were washed once and starved in 10 mM Tris (pH 7.5) overnight for ~18 h at a density of 3×10^5 cells/ml. For deciliation the method of Calzone and Gorovsky (1982) was followed. At 2 h after the first deciliation the cells were deciliated for a second time. Total RNA was isolated 30 min after the second deciliation (Chirgwin *et al.*, 1979). As a baseline, total RNA was isolated from B2086.1 cells that were not deciliated (time 0). RNA was DNaseI-treated and reverse-transcribed as described above. Quantitative real-time PCR was performed using Platinum SYBR

Table 2. Dynein light intermediate chain sequences used in phylogenetic analyses

Organism	Gene	Accession no.
<i>Caenorhabditis elegans</i>	CeDLI-1	CAC42262
	CeXBX-1	CAB01645
<i>Chlamydomonas reinhardtii</i>	CrD1bLIC	AAT37069
<i>Gallus gallus</i>	GgD1LIC1	CAA55698
<i>Homo sapiens</i>	HsD1LIC1	AAD44481
	HsD1LIC2	AAB88513
	HsD2LIC	AAH06969
<i>Mus musculus</i>	MmD2LIC	AAH39070
<i>Rattus norvegicus</i>	RnD1LIC1	AAF22294
	RnD1LIC2	AAA80334
<i>Strongylocentrotus purpuratus</i>	SpD1LIC	XP_795494
	SpD2LIC	XP_794270
<i>Tetrahymena thermophila</i>	TtD1LIC	ABV90642
	TtD2LIC	AAW31507
<i>Xenopus laevis</i>	XID1LIC1	AF317841

Green qPCR SuperMix UDG according to the manufacturer's directions (Invitrogen). Real-time reactions were done in the GeneAmp 5700 Sequence Detector (Applied Biosystems, Foster City, CA). The expression levels of *DYH2*, *D2LIC*, *D2IC*, and *LC8* genes in nondeciliated and deciliated cells were determined and compared with *DYH1* (noninduced control) and *DYH4* (induced control). Gene-specific primers that spanned introns were used for all genes examined in this study. Real-time reactions were done with two different RNA preparations and in duplicate for each. The cDNAs were used over a 100-fold range of dilutions. Regulation relative to *DYH1* was determined by the method of Pfaffl (2001) as described in Wilkes *et al.* (2007).

Knockout Constructs

The *DYH2* and *D2LIC* knockout constructs were built using the *NEO3* cassette kindly provided by Martin Gorovsky (University of Rochester, Rochester, NY). The *NEO3* cassette has a metallothionein promoter that drives the expression of the neomycin resistance gene when induced with cadmium (Shang *et al.*, 2002). In the *DYH2* knockout construct, the upstream flanking region is 1623 bp and corresponds to +6938 to +8561 in the *DYH2* gene sequence, where the A of the start codon ATG is at +1. The downstream flanking region is 516 bp and corresponds to +8694 to +9210 in the *DYH2* gene sequence. The upstream and the downstream flanking regions were ligated into either side of the *NEO3* gene using *SacI*/*SpeI* and *XhoI*/*ApaI* restriction sites, respectively. For the *D2LIC* knockout construct, the upstream flanking region is 1070 bp and corresponds to −1094 to −24 of the *D2LIC* gene sequence (the A of the start codon ATG is at +1). The downstream flanking region is 2026 bp and corresponds to +574 and +2600 in the *D2LIC* gene sequence. The upstream and the downstream flanking regions of the *D2LIC* knockout construct were ligated into either side of *NEO3* using *NotI*/*BamHI* and *XhoI*/*ApaI* restriction sites, respectively. Before biolistic transformation, both plasmids were purified using QIAGEN Plasmid Maxi Kit (QIAGEN, Valencia, CA). The *DYH2* and *D2LIC* knockout plasmids were linearized by digestion with *SacI*/*ApaI* and *NotI*/*ApaI* enzymes, respectively. The digested plasmids were purified by organic extraction. For each bombardment, ~10 µg of DNA was used.

Germline Knockouts Using Biolistic Bombardment

Tetrahymena strains B2086.1 (mating type II) and CU428.1 (mating type VII) were grown to midlogarithmic phase (~4 × 10⁵ cells/ml), washed once, and starved in 10 mM Tris (pH 7.5) at 30°C. After 18–20 h, the starved cells were mixed and left unshaken at 30°C. Biolistic bombardment was performed using Model PDS 1000/He Biolistic Particle Delivery System (Bio-Rad Laboratories, Hercules, CA) at 3.0, 3.5, 4.0, and 4.5 h after initiating mating (Bruns and Cassidy-Hanley, 2000). Bombarded cells were left at 30°C unshaken overnight to complete mating. About 18 h later, they were refed and 1 µg/ml final concentration of CdCl₂ was added to induce the MTT promoter. Five hours after MTT induction, transformants were selected by adding paromomycin (neomycin analog) to a final concentration of 120 µg/ml. Homozygous heterokaryons were produced and screened by the method of Hai *et al.* (2000).

Homozygous heterokaryons of different mating types carrying the targeted deletion in their germline micronuclei were grown to ~4 × 10⁵ cells/ml, washed, and starved overnight at 30°C in 10 mM Tris (pH 7.5). Equal volumes of parental cell lines were mixed and allowed to mate at 30°C. Single-pair isolation (SPI) was done as described by Hamilton and Orias (2000). Each pair was isolated into separate wells of modified Neffs medium, and each pair was allowed to grow at 30°C. As a control, wild-type B2086.1 and CU428.1 cells were mated with one another, and SPI was used to establish clones. After the individual pairs grew up, one clone each of wild-type, KO-DYH2, and KO-D2LIC cell lines was used for genotypic and phenotypic analyses.

Verification of Targeted Gene Disruptions

Genomic DNA (gDNA) was isolated from wild-type, KO-DYH2, and KO-D2LIC cell lines (Gaertig *et al.*, 1994). About 200 ng of gDNA of each cell line was used for each of the PCR reactions. For each knockout cell line, two sets of primers were designed to test the deletion of the targeted gene sequence and the integration of *NEO3* in the targeted locus. The presence of wild-type copies of *DYH2* and *D2LIC* genes in the knockout cell lines was tested with gene-specific primers. The *DYH2* gene-specific primers were as follows: A: 5' CTGAGCATAATCTAATGGCAG 3', B: 5' TACTCTTGAATCCAATCCTC 3', C: 5' CAGAGTCAGTCAAGGCAC 3', and D: 5' GAGGTTTATCTCAGACAAAG 3'. The *D2LIC* gene-specific primers were as follows: F: 5' GACAGCATTCACACCTG 3', G: 5' CGAGTGAGCCATTACG 3', H: 5' CCACTCAAAAGTATCTTCTTCAG 3', and I: 5' GCTCTCTTCTTTTACTGCCTC 3'. The integration of the *NEO3* cassette into the targeted gene loci was tested with the following *NEO3*-specific primers: N1: 5' CGCCTTC-TATCGCCTTC 3' and N2: 5' CTACAAAGAATCAAGAGCGTTGC 3'. The PCR products were separated on a 1.0% (wt/vol) agarose gel and visualized with ethidium bromide using the Bio-Rad Gel Doc EQ gel documentation system (Bio-Rad Laboratories).

RNA was isolated from wild-type, KO-DYH2, and KO-D2LIC cell lines grown to low log-phase densities using Tri-reagent according to the manufacturer's directions (Sigma-Aldrich, St. Louis, MO). About 5 µg of each RNA was used for reverse-transcription as described earlier. For each knockout cell

line, a pair of gene-specific primers, one within the deleted region, was used to test for the lack of expression of the gene in the deleted region. The *DYH2* gene-specific primers were as follows: C: 5' CAGAGTCAGTCAAGGCAC 3' and E: 5' CAGAGTAGAGAAGAGTTTCAG 3'. The *D2LIC* gene-specific primers were as follows: H: 5' CCACTCAAAAGTATCTTCTTCAG 3' and J: 5' AATGCTCTAAGTAGGTC 3'. Equal volumes (1 µl) of each cDNA were used as template for PCR reactions. The PCR products were separated on a 2.0–2.5% (wt/vol) agarose gel and visualized as described above.

For *DYH2* tail expression tests, total RNA was isolated from B2086.1 (control), KO-DYH2, and KO-D2LIC cells by the method of Chirgwin *et al.* (1979). A total of 10 µg of RNA from each cell line were DNaseI-treated and reverse-transcribed as described before. Equal volumes (1 µl) of each cDNA were used as template for PCR reactions. The primers a: 5' CAAGAACACACTGCCCTTC 3' and b: 5' CGGTTGAGCAAGCCATTCCAGC 3' in the tail region of the *DYH2* gene were used for PCR reactions. The PCR products were separated on a 1.0% (wt/vol) agarose gel and visualized as described before.

Swimming Characteristics

For determination of swimming speeds, wild-type, KO-DYH2, and KO-D2LIC cultures grown to ~5 × 10⁴ cells/ml were used. Each cell type was further diluted in modified Neffs medium so that the field of view contained only a few cells. The tracks of moving cells were viewed by dark-field microscopy using a 2.5× objective and 6-s time-lapse images were captured using a CCD camera. A micrometer scale was also photographed and the lengths of the tracks were measured. The swimming path linearity coefficient was defined as the ratio of the linear distance from the starting point to the end point divided by the total path length (perfect linearity = 1.00). *p* values were determined by unpaired one-tailed *t* tests.

Scanning Electron Microscopy

Wild-type and knockout cells for scanning electron microscopy (SEM) were grown in modified Neffs medium to 4 × 10⁵ cells/ml. They were fixed with a final concentration of 1.25% glutaraldehyde, 2% paraformaldehyde in 0.05 M cacodylate buffer (pH 7.0) for 25 min at room temperature. A drop of cells was placed on precoated poly-L-lysine (0.01%) coverslips and incubated for 20 min at room temperature, washed with 0.05 M cacodylate buffer (pH 7.0) and ddH₂O, and dehydrated using a series of ethanol concentrations. Coverslips were critical-point dried using Tousimis SAMDRI-780A critical-point dryer and sputter-coated with 90-nm gold particles using Anatec Hummer VI Sputter Coater. Samples were observed using JEOL 840A (Peabody, MA) or Zeiss Supra 35 scanning electron microscope (Thornwood, NY) using an accelerating voltage of 12 KeV.

Transmission Electron Microscopy

Tetrahymena cells were fixed and prepared for transmission electron microscopy (TEM) similar to the methods of Allen (1967) and Jerka-Dziadosz (1981). Cultures were grown to midlog phase, centrifuged at 750 × *g*, and the supernatant was aspirated. The remaining pellets were fixed with 3% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for 30 min at room temperature and washed with 0.1 M cacodylate buffer. Cells were then transferred to microfuge tubes, pelleted at 325 × *g* and postfixed in 1% osmium tetroxide (OsO₄) for 1 h at 4°C. After the excess OsO₄ was removed, 1% liquid agar was added and pelleted at 325 × *g* for 1 min at room temperature. The microfuge tubes were placed in the freezer for 10 min to harden the agar. The agar was then diced into 1-mm³ pieces, washed with 0.1 M cacodylate buffer, and en bloc-stained with 0.1% tannic acid for 30 min at room temperature. Samples were dehydrated in an acetone series and embedded in Firm Spurr's resin. Ultrathin sections of 70-nm thickness were cut and stained with 2% uranyl acetate and 2% lead citrate. Sections were examined with a JEOL 100S TEM using a high voltage of 80 KeV.

Cilia Density and Length Analyses

Wild-type, KO-DYH2, and KO-D2LIC cells were grown to ~5 × 10⁴ cells/ml, washed once in 1× PHEM buffer (60 mM PIPES, 25 mM HEPES, 10 mM EGTA, and 2 mM MgCl₂, pH 6.9), and fixed with 2% paraformaldehyde, 0.4% Triton-X 100 in 1× PHEM buffer. Cells were stained with mAb 1–6.1, an antibody specific for acetylated α-tubulin (Asai *et al.*, 1982), at 1:50 dilution in 0.1% BSA in PBS. Goat anti-mouse IgG antibody was used as the secondary antibody (Kirkegaard & Perry Laboratories, Gaithersburg, MD). The stained cells were examined by confocal fluorescence microscopy, using a Zeiss LSM510 system. An image of the widest confocal section of each cell was captured, and the ciliary densities and lengths were determined using the LSM510 software. A total of 12 cells for the control and 36 cells for each of the knockout cell lines were evaluated. The density of cilia was defined as the number of measurable cilia per micrometer of the cell circumference. *p* values were determined by unpaired, one-tailed *t* tests.

Sequential Cell Division Analyses

Growth curves were determined by isolating a single cell to begin the culture. For each cell type, triplicate cultures were analyzed by measuring the cell

Figure 1. *Tetrahymena* dynein-2 subunits. *Tetrahymena* expresses one each of the dynein-2 IC, *D2IC* (A) and dynein-2 light intermediate chain, *D2LIC* (B) genes. Neighbor-joining trees of intermediate chains and light intermediate chains were generated based on ClustalW alignment of protein sequences. Ce, *Caenorhabditis elegans*; Cr, *Chlamydomonas reinhardtii*; Dd, *Dictyostelium discoideum*; Dm, *Drosophila melanogaster*; Gg, *Gallus gallus*; Hs, *Homo sapiens*; Mm, *Mus musculus*; Sp, *Strongylocentrotus purpuratus*; Rn, *Rattus norvegicus*; Tt, *Tetrahymena thermophila*; Xl, *Xenopus laevis*. Bar, 0.2 substitutions/amino acid. The bootstrap percentages (500 iterations) are shown at the branch points. (C) Increased expression of *Tetrahymena* dynein-2 subunit genes in response to deciliation. Quantitative real-time PCR was used to measure transcript levels of each gene before and after deciliation. The data were normalized to the expression of cytoplasmic *DYH1* because this gene is not induced. Outer arm β (*DYH4*) was used as the positive control. A relative level of 2.0 indicates a

density every day. Cells were fixed with formaldehyde (1% final concentration) and counted using a hemacytometer. After 6 d, a single cell from each of the initial cultures was picked and placed in fresh medium, and the second round of cell growth measurements was determined. After 6 d, single cells were again picked, and a third round of cell densities was determined. The generation times of the different cell types were calculated from the initial slopes of their growth curves.

Phagocytosis

For phagocytosis experiments, freshly seeded cultures of cells at $\sim 1.2 \times 10^5$ cells/ml were used. Red fluorescent latex beads of 2- μ m diameter (Sigma-Aldrich) were added to cultures, and the cells were incubated at 30°C with shaking at 100 rpm. At 1.5 and 24 h, cells were washed three times in 10 mM Tris (pH 7.5) and then fixed with 2% paraformaldehyde, 0.4% Triton X-100 in 1 $\times$ PHEM buffer. The percentage of cells that contained beads was determined ($n = 100$). Cells that contained ≤ 5 beads were considered as having no beads because it is not unusual to have small numbers of beads adhere to the outside of cells.

RESULTS

Tetrahymena Expresses All Four Components of the Dynein-2 Complex

Dynein-2 comprises a homodimer of the Dyh2 heavy chain associated with the D2IC, D2LIC, and LC8 accessory subunits (Perrone *et al.*, 2003; Rompolas *et al.*, 2007). The *Tetrahymena* *DYH2* gene encodes a protein of 4236 amino acids with a molecular weight of 487,769 Da (GENBANK accession no. AAV37189). The single *Tetrahymena* *D2IC* gene (accession no. ABX82716) encodes a protein of 594 amino acids with a molecular weight of 68,231 Da. *Tetrahymena* *D2IC* includes four WD40 repeats, which are characteristic of dynein ICs. A comparison of several dynein intermediate chains is shown in Figure 1A. It is clear that the *Tetrahymena* *D2IC* belongs to the cytoplasmic dynein-2 IC group and not the cytoplasmic dynein-1 IC group or an axonemal dynein IC group. *Tetrahymena* expresses a conventional LC8 gene and five LC8-like genes (accession nos. ABF38950, ABF38951, ABF38952, ABF38953, ABF38954, and ABF38955; Wilkes *et al.*, 2007). The conventional *Tetrahymena* LC8 shares 76–90% sequence identity with the LC8 proteins from other organisms; the *Tetrahymena* LC8-like proteins are 33–56% identical to conventional LC8s across species.

The *Tetrahymena* *D2LIC* gene includes a 1607-bp coding region interrupted by a single intron of 185 bp; the location of this intron was verified by RNA-directed PCR and sequencing of the amplified product (data not shown). The deduced *D2LIC* protein is 474 residues in length with a molecular weight of 54,711 Da (accession no. AAW31507). Comparison of the sequences of several light intermediate chains of dyneins-1 and -2 is shown in Figure 1B. The *Tetrahymena* *D2LIC* sequence groups well with the *D2LIC* proteins from other organisms. The *Tetrahymena* *D2LIC* is 20–27% identical to other *D2LIC* proteins and is 11–16% identical to *D1LIC* orthologues. *Tetrahymena* *D2LIC* has a P-loop motif beginning 50 residues from the amino terminus, a feature found in most other dynein light intermediate chain sequences. The *D2LIC* orthologues in human, *Chlamydomonas*, and *Caenorhabditis elegans* all contain coiled-coil domains that are thought to participate in protein–protein interactions (Grissom *et al.*, 2002; Perrone *et al.*, 2003; Schafer *et al.*, 2003). However, the *Tetrahymena* *D2LIC* sequence does not contain the same coiled-coil domain.

doubling of expression after deciliation. RNA from two different preparations was used in this analysis. Bars indicate range of values from duplicate experiments with each RNA.

Figure 2. Knockout of *DYH2* and *D2LIC* genes. (A) *DYH2* gene knockout strategy and location of primers (A–E, N1, and N2) used for PCR verification of knockout genotype. The essential catalytic P-loop (P1) was deleted, and the *NEO3* gene was inserted. (B) *NEO3* is correctly integrated into the *DYH2* locus and no wild-type copies of the *DYH2* gene are present in the KO-DYH2 cells. From wild-type cells (WT), the expected PCR products using the primer pairs A/B and C/D were 1827 and 1917 bp, respectively. These primer pairs should produce no product for the KO-DYH2 template (KO). From the knockout cells (KO) the expected products using the primer pairs A/N1 and N2/D were 2037 and 1999 bp, respectively. These primer pairs should produce no product from wild-type template (WT). (C) The disrupted region of the *DYH2* gene is not expressed in the KO-DYH2 cells. Expression was determined by PCR using primer pair C/E. cDNA was prepared from wild-type (WT), KO-DYH2, and KO-D2LIC RNA and used as template. A 408-bp band indicating the expression of the deleted region of *DYH2* was observed only with wild-type and KO-D2LIC cDNA. A nonspecific band larger than the 408-bp band was seen in the wild-type cDNA sample. (D) *D2LIC* gene knockout strategy and location of primers (F–J, N1, and N2) used for PCR verification of knockout genotype. Approximately one-third of the *D2LIC* coding region (574 bp) including the start codon was deleted, and *NEO3* was inserted. (E) *NEO3* is correctly integrated into the *D2LIC* locus and no wild-type *D2LIC* copies exist in the KO-D2LIC cells. The expected PCR products from the wild-type (WT) copies of *D2LIC* gene using primer pairs F/G and H/I were 1513- and 2277-bp, respectively; these primers should produce no product from KO-D2LIC gDNA (KO). The expected products from the KO-D2LIC gDNA (KO) using primer pairs F/N1 and N2/I were 1735- and 2364 bp, respectively; these primer pairs should not produce a product with wild-type gDNA (WT). A nonspecific band of ~1700 bp is seen in both wild-type and KO-D2LIC PCR reactions with H/I primer pair. (F) The disrupted region of the *D2LIC* gene is not expressed in the KO-D2LIC cells. Expression was determined by PCR using primer pair H/J. cDNA prepared from wild type (WT), KO-DYH2, and KO-D2LIC RNA was used as template. A 422-bp band indicating the expression of the deleted region of *D2LIC* was seen only with wild-type and KO-DYH2 cDNA.

Expression of *Tetrahymena* Dynein-2 Genes Increases after Deciliation

To determine if the dynein-2 gene products may be involved in IFT in *Tetrahymena*, the expression of each gene after deciliation was measured by quantitative real-time PCR. An increase in the expression of a gene after deciliation is an indication that the gene product is either a component of the cilia or is involved in the process of ciliogenesis (Lefebvre *et al.*, 1980; Schloss *et al.*, 1984; Soares *et al.*, 1993). For example, the expression levels of *Chlamydomonas* dynein-2 heavy chain, *D2LIC*, and *IC* genes are up-regulated after deflagellation (Pazour *et al.*, 1999; Porter *et al.*, 1999; Perrone *et al.*, 2003; Rompolas *et al.*, 2007). *Tetrahymena* were twice deciliated with a calcium shock, and the cells were allowed to regenerate their cilia. Total RNA was isolated before deciliation and 30 min after the second deciliation and reverse-transcribed into cDNA. The levels of expression of all four of the *Tetrahymena* dynein-2 component genes significantly increased 30 min after deciliation (Figure 1C). In this experiment, the change in expression of each gene was normalized to that of *DYH1*, which should not be affected by deciliation. The positive control was the ciliary outer arm β dynein gene *DYH4*.

Complete Disruption of *Tetrahymena* *DYH2* and *D2LIC*

To examine the function of dynein-2 in *Tetrahymena*, germline knockouts of the *DYH2* and *D2LIC* genes were produced. The *DYH2* gene was disrupted by a deletion of 132 bp that includes the region encoding the essential catalytic P1 loop (Lee-Eiford *et al.*, 1986; Figure 2A). The *D2LIC* gene was disrupted by a deletion of 598 bp that begins 24 bp upstream of the ATG initiation codon and extends 574 bp into the coding region (Figure 2D). For both genes the deletions were replaced with the 3.6 kb *NEO3* gene placed in opposite orientation to the direction of transcription of the dynein gene. Homozygous heterokaryons were produced in which both copies of the targeted gene were disrupted in the diploid micronuclei (Hai and Gorovsky, 1997). Mating of the homozygous heterokaryons produced progeny that completely lacked wild-type copies of the targeted gene in both the transcriptionally silent germline micronucleus and the somatic macronucleus. These knockout progeny are called KO-DYH2 and KO-D2LIC.

Disruption of the targeted genes and the insertion of *NEO3* in the appropriate loci were verified by PCR (Figure 2, B and E). Primer pairs from outside the region used for the disruption construct and within the deleted region showed that no wild-type gene copies were present in the KO cells (Figure 2B; primers A/B [1827 bp] and C/D [1917 bp];

Figure 3. KO-DYH2 and KO-D2LIC cells swim more slowly than control cells. Swimming tracks of wild-type (A), KO-DYH2 (B), and KO-D2LIC (C) cells were photographed with dark-field microscopy using a 6-s exposure. (D) The KO-DYH2 and KO-D2LIC cells swam more slowly than wild-type cells. $**p < 0.01$ versus wild type. (E) The swimming paths of KO-DYH2 but not KO-D2LIC cells were statistically less straight than the wild-type controls. Perfect linearity = 1.00. $*p < 0.05$ versus wild type. Unpaired, one-tail *t* tests were performed to determine statistical differences. Bar, SD.

Figure 2E, primers F/G [1513 bp] and H/I [2277 bp]). Additionally, primers from outside the region used for the disruption construct and within *NEO3* verified that the disruptions were in the correct loci (Figure 2B, primers A/N1 [2037 bp] and N2/D [1999 bp]; Figure 2E, primers F/N1 [1735 bp] and N2/I [2364 bp]). RNA-directed PCR demonstrated the elimination of expression of the deleted region of the targeted gene in the two knockout cell lines (Figure 2, C and F). As expected, disruption of one gene did not affect the expression of the other gene.

KO-DYH2 and KO-D2LIC Cells Are Motile But Swim More Slowly and Less Straight

In other organisms, dynein-2 disruptions result in short, bulbous cilia that do not support motility. However, both the KO-DYH2 and KO-D2LIC cells were motile indicating that they had functioning cilia. Time-lapse dark-field microscopy of 27 wild-type, 36 KO-DYH2, and 41 KO-D2LIC cells was used for determining swimming velocities and the relative linearity of the swimming path. The swimming speed of wild-type cells was $181.8 \pm 34.5 \mu\text{m}/\text{s}$. The two knockout cell lines swam ~ 3.5 -fold slower than control cells; the average velocities of the KO-DYH2 and KO-D2LIC cell lines were 52.8 ± 21.2 and $51.3 \pm 19.9 \mu\text{m}/\text{s}$, respectively (Figure 3D, Table 3). Both knockout cell lines also showed a reduction in straight swimming; the difference in straightness was statistically significant between KO-DYH2 and wild-type cells (Figure 3E, Table 3). Many of the KO-DYH2 and KO-D2LIC cells exhibited a corkscrew swimming pattern or frequent turns (Figure 3, A–C). Thus, *Dyh2* and *D2LIC* contribute in similar ways to cell motility.

KO-DYH2 and KO-D2LIC Cells form Normal Cilia

The cilia of the dynein-2 knockout cells were examined by electron microscopy (EM). The flagella of dynein-2 mutants of *Chlamydomonas* are bulbous and accumulate electron-dense material between the axoneme and flagellar membrane (Pazour *et al.*, 1998, 1999; Porter *et al.*, 1999; Hou *et al.*, 2004). Both SEM and TEM examinations of the *Tetrahymena*

Table 3. Swimming characteristics of *Tetrahymena* KO-DYH2 and KO-D2LIC cells compared with wild-type controls

Cell type	No. of cells	Velocity $\pm$ SD ($\mu\text{m}/\text{s}$)	Swimming linearity $\pm$ SD ^a
Wild-type	27	181.8 $\pm$ 34.5	0.84 $\pm$ 0.17
KO-DYH2	36	52.8 $\pm$ 21.2	0.74 $\pm$ 0.23
KO-D2LIC	41	51.3 $\pm$ 19.9	0.78 $\pm$ 0.19

p values determined by unpaired, one-tail *t* tests for swimming velocities were 8.6×10^{-27} (wild type vs. KO-DYH2), 9.4×10^{-30} (wild type vs. KO-D2LIC), and 0.37 (KO-DYH2 vs. KO-D2LIC). *p* values determined by unpaired, one-tail *t* tests for swimming linearity were 0.03 (wild type vs. KO-DYH2), 0.11 (wild type vs. KO-D2LIC), and 0.18 (KO-DYH2 vs. KO-D2LIC).

^a Swimming linearity = ratio of the linear distance from the starting point to the end point divided by the total path length (perfect linearity = 1.00).

cilia were conducted using a blinded protocol. One of us (A.S.) was provided coded cultures that she prepared for EM analyses and evaluated without knowing the coding. SEM showed that the cilia on *Tetrahymena* KO-DYH2 and KO-D2LIC cells were of varying lengths, including some very short cilia (Figure 4, A–C). However, none of the cilia had bulbous tips (Figure 4, D–F). Examination of the thin-section TEM images found no differences among the three samples and no evidence of the accumulation of material in the cilia of the *Tetrahymena* knockouts (Figure 4, G–L). The axonemal organization was normal in the knockouts with the 9 + 2 pattern of microtubules, radial spokes, and inner and outer arm dyneins (Figure 4, G–I).

KO-DYH2 and KO-D2LIC Cells Have Fewer Cilia of Varying Lengths

The cilia of the dynein-2 knockout cells were examined by confocal immunofluorescence microscopy using antibodies to tubulin. A total of 12 wild-type, and 36 each of the KO-DYH2 and KO-D2LIC cells were examined. For each cell the widest optical section was imaged, and the ciliary density and ciliary lengths in that section were measured (Figure 5, A and B; Table 4). The average cilium length on wild-type cells was $4.17 \pm 0.28 \mu\text{m}$. The average length of cilia on KO-DYH2 and KO-D2LIC cells was 3.41 ± 0.63 and $3.40 \pm 0.75 \mu\text{m}$, respectively. The density of cilia in KO-DYH2 and KO-D2LIC cells was 0.14 ± 0.04 and 0.13 ± 0.05 cilia per μm of cell circumference, respectively, whereas wild-type cells had a higher cilia density of 0.19 ± 0.03 cilia per μm of cell circumference. The cilia length distributions for wild-type, KO-DYH2, and KO-D2LIC cell lines were evaluated (Figure 5, C–E). The average cilium length of the knockout cells was less than that of wild-type cells, but the knockout cells also showed a greater variation in cilia lengths, ranging from 0.5 to $>10.0 \mu\text{m}$. This cilia length variation was present on individual cells as well as the population of cells. Thus, disruption of *DYH2* and *D2LIC* genes does not impair the ability of the cells to generate cilia, although the regulation of length appears to be compromised.

KO-DYH2 and KO-D2LIC Cells Divide More Slowly than Wild-Type Cells

Motility defects in *Tetrahymena* have been shown to result in cytokinesis defects (Brown *et al.*, 1999a,b, 2003; Williams *et*

Figure 4. KO-DYH2 and KO-D2LIC cells have normal cilia. (A–F) Scanning electron micrographs. (G–L) Transmission electron micrographs of thin sections. (A, D, G, and J) wild-type; (B, E, H, and K) KO-DYH2; (C, F, I, and L) KO-D2LIC. (D–F) High magnification of boxed area of A–C. The cilia on KO-DYH2 (B) and KO-D2LIC (C) cells appear to be like wild-type cilia (A). Short cilia (arrows) on KO-DYH2 (E) and KO-D2LIC (F) cells did not have bulbous tips. Cross-sections of KO-DYH2 (H) and KO-D2LIC (I) cilia showed the presence of the 9 + 2 arrangement of axonemal microtubules, radial spokes, and dynein arms similar to wild-type (G). No accumulation of IFT particles was observed between the axonemes and ciliary membranes. Longitudinal sections of KO-DYH2 (K) and KO-D2LIC (L) cilia showed no accumulation of IFT particles. Bars, (A–C) 10 μm ; (D–F) 5 μm ; (G–L) 0.1 μm .

al., 2006). To examine the ability of the dynein-2 knockouts to divide, single cells were isolated and grown to high density; then a single cell from the first culture was isolated to start a second clone of cells, and then the process was repeated a third time. Results of such experiments are shown in Figure 6. Over this long protocol, the KO-DYH2 and KO-D2LIC cells continued to divide, albeit more slowly. At 30°C without shaking, wild-type *Tetrahymena* cells divided every 2.9 h and grew to a density of $1\text{--}1.5 \times 10^6$ cells/ml. The KO-DYH2 and KO-D2LIC grew with longer generation times of 4.6 and 4.2 h, respectively, and achieved densities of $0.4\text{--}0.5 \times 10^6$ cells/ml.

The KO-DYH2 and KO-D2LIC cell morphologies varied significantly from those of the wild-type controls. The

variety of cell shapes and sizes for both knockout cell lines ranged from relatively normal to large multilobed “monsters.” When single monsters were isolated into individual wells with medium, they did not divide, indicating that the extreme monsters were a terminal phenotype. However, knockout cells that were normally or near-normally shaped did survive and grew into moderately dense cultures. Cultures started from a single KO-DYH2 or KO-D2LIC “normal” or “near-normal” cell eventually always contained a mixture of normal, near-normal, and monster cells. Many of the normal- and near-normal-shaped cells contained multiple nuclei, presumably because of failed cytokinesis. The large monster cells were likely the result of consecutive failures in cytokinesis as

Figure 5. KO-DYH2 and KO-D2LIC cells have fewer cilia and a wider range of cilia lengths than wild-type cells. Wild-type, KO-DYH2, and KO-D2LIC cells were stained using an anti-tubulin

Table 4. Ciliary characteristics of *Tetrahymena* KO-DYH2 and KO-D2LIC cells compared with wild-type controls

Cell type	No. of cells	No. of cilia	Cilia length $\pm$ SD (μ m)	Cilia density $\pm$ SD ^a
Wild-type	12	261	4.17 $\pm$ 0.28	0.19 $\pm$ 0.03
KO-DYH2	36	595	3.41 $\pm$ 0.63	0.14 $\pm$ 0.04
KO-D2LIC	36	535	3.40 $\pm$ 0.75	0.13 $\pm$ 0.05

p values determined by unpaired, one-tail *t* tests for lengths of cilia were 8.86×10^{-5} (wild type vs. KO-DYH2), 5.6×10^{-4} (wild type vs. KO-D2LIC), and 0.48 (KO-DYH2 vs. KO-D2LIC). p values determined by unpaired, one-tail *t* tests for cilia densities were 1.95×10^{-4} (wild type vs. KO-DYH2), 2.86×10^{-4} (wild type vs. KO-D2LIC), and 0.23 (KO-DYH2 vs. KO-D2LIC).

^a Cilia density = number of measurable cilia per micrometer of the cell circumference at the widest confocal section of a cell.

seen with other *Tetrahymena* motility mutants (Brown *et al.*, 1999b, 2003; Williams *et al.*, 2006). Examples of knock-out cells are shown in Figure 7. We conclude that the impaired cell motility of the dynein-2 knockout cells resulted in inefficient cell division that underlies the longer generation times and lower cell densities.

KO-DYH2 and KO-D2LIC Cells Are Defective in Phagocytosis

Tetrahymena use cilia to sweep food particles into their oral apparatus for feeding by phagocytosis (Frankel, 2000). A simple assay for phagocytosis is to incubate cells with latex beads and then to evaluate ingestion of the beads by light microscopy. Both KO-DYH2 and KO-D2LIC cells had oral apparatuses as visualized by immunostaining with anti-tubulin antibody and SEM, but neither phagocytosed beads normally. After 1.5 h, 71% of wild-type cells contained beads, whereas none of the KO-DYH2 and only 4% of the KO-D2LIC cells contained beads. After 24 h, neither KO-DYH2 nor KO-D2LIC cells contained significant numbers of beads (Figure 8).

DISCUSSION

A previous study suggested that, in contrast to what has been described in other organisms, dynein-2 is not required for ciliogenesis in *Tetrahymena*. To unambiguously understand the role of dynein-2 in *Tetrahymena* ciliogenesis, the present study produced knockout heterokaryons targeting two dynein-2 component genes in *Tetrahymena*. These disruptions resulted in cells completely missing the catalytic portion of the Dyh2 heavy chain, and the first 192

antibody and visualized by confocal immunofluorescence microscopy. (A) Ciliary density (i.e., the number of cilia per μ m of the cell circumference at the widest confocal section) was determined for all three cell types. The densities of cilia on KO-DYH2 and KO-D2LIC cells were lower than wild-type cells. (B) The length of measurable cilia at the widest confocal section for each cell type was measured. The lengths of KO-DYH2 and KO-D2LIC cilia were shorter than control. ***p* < 0.01 versus wild-type. Bar, SD. (C-E) Length distributions of cilia. The median ciliary length was less on KO-DYH2 (D) and KO-D2LIC (E) cells compared with wild-type (C) cells. The two dynein-2 mutants also showed a wider range of ciliary lengths compared with wild-type cilia.

Figure 6. KO-DYH2 and KO-D2LIC divide more slowly than wild-type cells. Cultures were started from single cells and cell densities were measured daily for 6 d. New cultures were then started with single cells from the previous cultures. Three rounds of cell density measurements were obtained. Shown here are the averages and SDs from three experiments. □, wild-type; ●, KO-DYH2; ▲, KO-D2LIC.

residues of the D2LIC accessory subunit. We have characterized the knockout cells for the structure and activity of their cilia.

Dynein-2 Is Not Required for Ciliogenesis in Tetrahymena

In other organisms, the loss of dynein-2 results in the failure to produce normal and motile cilia or flagella. In *Chlamydomonas*, disruptions of the dynein-2 genes result in very short and swollen flagella containing massive accumulations of electron-dense IFT material in the bulbous distal tips (Pazour *et al.*, 1998, 1999; Porter *et al.*, 1999; Hou *et al.*, 2004). In *Leishmania*, disruption of one of the DYH2 isoforms results in the complete loss of flagella (Adhiambo *et al.*, 2005). Dynein-2 mutants in *C. elegans* have short, stumpy sensory cilia (Signor *et al.*, 1999; Wicks *et al.*, 2000; Schafer *et al.*, 2003), and in mouse, dynein-2 mutations result in bloated and bulbous embryonic nodal cilia that are shorter than normal or completely absent (Rana *et al.*, 2004; Huangfu and Anderson, 2005; May *et al.*, 2005). In striking contrast to what has been observed in these other model systems, the disruption of dynein-2 components in *Tetrahymena* did not prevent the formation of motile, normal-appearing cilia.

Because the parental heterokaryon cell lines expressed wild-type levels of the Dyh2 and D2LIC proteins, some dynein-2 may remain in the progeny soon after mating even though the knockout cells do not have the capacity to express the genes. Therefore, it is important to consider the possibility that this residual dynein-2 is sufficient to drive ciliogenesis. The growth rates experiment shown in Figure 6

Figure 7. Dynein-2 mutants have varied morphologies. (A and B) DIC microscopy of dynein-2 mutants: normal sized and shaped cells (arrows); multilobed "monster" cells (arrowheads). Bar, 100 μ m. (C and D) Fluorescence microscopy of dynein-2 mutants: normal sized and shaped cells (arrows); multilobed "monster" cells (arrowheads). These cells have disorganized cortical microtubules (red). (E and F) Multinucleated "normal"-shaped KO-D2LIC cells. Bar, (C) 20 μ m; (D) 10 μ m. E and F are not shown at the same scale as C and D.

Figure 8. KO-DYH2 and KO-D2LIC cells are defective in feeding. Wild-type (A), KO-DYH2 (B), and KO-D2LIC (C) cells were incubated with beads to test oral cilia function. Cells and beads were visualized by DIC and fluorescence microscopy, respectively. In these images, the beads appear as white spots. Bar, 100 μ m.

provides the basis for evaluating this possibility. In this experiment, each culture was begun with a single cell, the culture was allowed to grow up for 6 d, and then a single cell from the 6-d culture was used to begin a new culture. This protocol was repeated a third time. From the cell densities

measured at the end of every expansion, we estimate that the contents of the single dynein-2 mutant cell used to start the experiment were effectively diluted more than 10^{16} -fold. Of course, this is a gross underestimation because it does not consider protein degradation. We conclude that the fact that the dynein-2 knockout cells continue to produce motile cilia is not due to residual carry over of dynein-2 proteins.

In light of our result with the retrograde motor, it is interesting to ask whether loss of function of the retrograde complex A results in a more severe phenotype. The disruption of the *Tetrahymena* complex A *IFT122A* gene resulted in cells that continued to assemble cilia similar to our KO-DYH2 and KO-D2LIC cells (Tsao and Gorovsky, 2008b). Our lab also has produced a knockdown of another *Tetrahymena* complex A gene, *IFT140*, again resulting in only a mild effect on ciliogenesis (J. DuMond, V. Rajagopalan, D. Wilkes, unpublished results). Together, these results demonstrate that retrograde IFT—at least as it is powered by dynein-2—is not required for ciliogenesis in *Tetrahymena*.

Dynein-2 Mutants Have Lost the Ability to Regulate the Lengths of Their Cilia

The dynein-2 knockout cells have impaired motility, as measured by several parameters. The mutants swim more slowly and less straight, fail to consume fluorescent beads, and exhibit inefficient cell division. A close examination of the cilia on individual cells reveals that the principal effect of the gene knockouts was on ciliary length. Although average lengths of cilia on knockout cells were less than that of wild-type cells, there was an abnormally wide range of ciliary lengths on the knockout cells, including some unusually long cilia (Figure 5). This loss of ciliary length homogeneity is reminiscent of what was observed when the IFT complex A component 122A was knocked out (Tsao and Gorovsky, 2008b).

The metachronous coordination of ciliary beating is expected to be sensitive to variations in lengths among individual cilia (Nelsen and DeBault, 1978; Frankel, 2000). Thus, the impaired motility exhibited by the knockout cells is interpreted to be a secondary effect caused by the loss of ciliary length regulation. These results suggest that in *Tetrahymena* retrograde IFT is responsible for the close control of cilia length.

Retrograde IFT and Ciliogenesis

The intraflagellar transport model is an important framework in which to understand ciliogenesis. Anterograde transport, which depends on kinesin-II and complex B components, carries materials to the tip, which is the site of incorporation of new ciliary proteins. Retrograde transport, which depends on dynein-2 and complex A components, is responsible for returning the anterograde machinery to the base of the cilium so that it can be recycled. The IFT model predicts that loss of function of the anterograde motor or complex B will result in no ciliary growth and that loss of function of the retrograde apparatus will result in accumulation of IFT material at the ciliary tip, producing short, stumpy, and bulbous cilia. Despite some variations in different organisms—e.g., two different anterograde motors in *C. elegans* versus one in *Chlamydomonas* (Kozminski *et al.*, 1995; Snow *et al.*, 2004; Mukhopadhyay *et al.*, 2007)—the general model holds well for anterograde IFT. The disruption of the kinesin or individual complex B components results in no cilia or flagella in all of the organisms examined, including *Tetrahymena* (Kozminski *et al.*, 1995; Collet *et al.*, 1998; Nonaka *et al.*, 1998; Brown *et al.*, 1999b; Snow *et al.*,

Figure 9. KO-DYH2 cells express a truncated *DYH2* transcript. (A) Maps of the *DYH2* gene in wild-type (top) and KO-DYH2 (bottom) cells. The KO-DYH2 cells lack the catalytic P1 P-loop and have the *NEO3* gene. The locations of the PCR primers, a and b, are indicated. (B) Results from RNA-directed PCR; the source of each template is included above each lane of the gel. Wild-type gDNA, which includes introns, produced a 1581-bp product (arrow). Amplification of cDNA produced a 1124-bp PCR product (no introns). The 1124-bp product (arrowhead) was found in cDNAs from wild-type, KO-DYH2, and KO-D2LIC. PCR with primers a or b, alone, did not produce products of these sizes.

2004; Pedersen *et al.*, 2005; Beales *et al.*, 2007; Absalon *et al.*, 2008; Tsao and Gorovsky, 2008a).

In contrast to the highly conserved requirement for anterograde IFT in ciliogenesis in all organisms, the requirement for retrograde IFT is not universal. As this study shows, the disruption of dynein-2 components does not prevent ciliogenesis in *Tetrahymena*, and the cilia on the knockout cells do not have accumulations of IFT material as seen in other systems. Other exceptions to the retrograde IFT model include *Toxoplasma gondii* and the marine diatom *Thalassiosira pseudonana*. Both organisms lack the genes for dynein-2 heavy chain and D2LIC even though they form flagella (Wickstead and Gull, 2007).

Why Is *Tetrahymena* Different?

Clearly, dynein-2 and IFT complex A (Tsao and Gorovsky, 2008b) do not make the same contributions to *Tetrahymena* ciliogenesis as they do in other model organisms, notably *Chlamydomonas*. Why is *Tetrahymena* different? Here we consider two possibilities.

One possibility is that retrograde IFT is required for *Tetrahymena* ciliogenesis, but that motors other than dynein-2 can power retrograde IFT. The KO-DYH2 cells were produced by the deletion of the region of the *DYH2* gene encoding the catalytic P-loop, which is essential for dynein motor activity (Lee-Eiford *et al.*, 1986). Because the deleted portion of the *DYH2* gene is several kilobasepairs away from the initiation codon, a large portion of the 5' end of the *DYH2* gene remains in the KO-DYH2 cells. As expected, the deleted portion of the *DYH2* gene was not expressed in the KO-DYH2 cell line (Figure 2C). However, RNA-directed PCR revealed that the portion of the *DYH2* gene located upstream of the catalytic P-loop was transcribed (Figure 9).

This is reminiscent of earlier work in which transcripts corresponding to truncated versions of *DYH* genes were found to persist in *Tetrahymena* dynein transformants (Liu *et al.*, 2004).

The 5' end of the dynein heavy chain gene encodes the tail domain that tethers the motor to other dynein-2 subunits, thus mediating the connection to the cargo. It is intriguing to consider the possibility that the motorless truncated Dyh2 can serve to anchor a different motor to the remainder of the dynein-2 complex and that this other motor can substitute for Dyh2. There are many potential alternative motors in *Tetrahymena* including four kinesin-like genes predicted to encode minus-end-directed motors (V. Rajagopalan, unpublished results) and the 25 different dynein heavy chains (Wilkes *et al.*, 2008). This possible redundancy of IFT motors may extend to the *Tetrahymena* anterograde machinery (see Awan *et al.*, 2004). Future experiments—currently not feasible because of the lack of specific antibody reagents—aimed at localizing dynein-2 subunits in the knockout cells may provide evidence for the formation of complexes of dynein-2 subunits with different motor proteins.

A second possibility is that retrograde IFT is simply not important in *Tetrahymena*. The cilia on *Tetrahymena* are shorter and less dynamic than *Chlamydomonas* flagella which undergo cycles of lengthening and resorption (Johnson and Porter, 1968; Marshall and Rosenbaum, 2001). The static nature of *Tetrahymena* cilia is underscored by our preliminary experiments in which we have measured the rate of ciliary growth in nondividing cells. Even after 22 h of reciliation, the dynein-2 mutants continue to exhibit motile cilia of varying lengths (V. Rajagopalan, D. Wilkes, and D. Asai, unpublished results). Thus, whereas anterograde IFT is essential for *Tetrahymena* cilia formation, as is the case in all organisms examined, *Tetrahymena* retrograde IFT may only contribute to the regulation of ciliary length. If this is the case, then we would expect to find that dynein-2 mutants will be unable to resorb their cilia, for example, by the overexpression of NIMA-related kinases (Wloga *et al.*, 2006). An earlier study reported that partial deciliation of cells causes the remaining cilia to stiffen and then to undergo resorption (Rannestad, 1974). Although we have not purposely attempted to obtain partial deciliation, we have not observed unresorbed cilia on dynein-2 knockout cells after their deciliation.

In conclusion, the elimination of dynein-2 subunits in *Tetrahymena* resulted in cells that continue to produce motile cilia. The cilia are normally shaped and show no signs of accumulation of unrecycled IFT material. Unlike what is observed on wild-type cells, there is a significant variation of the lengths of cilia on individual dynein-2 mutant cells. We conclude that in *Tetrahymena* dynein-2 is not required for ciliogenesis, but that retrograde IFT plays an important role in regulating cilia length.

ACKNOWLEDGMENTS

We thank Donna Cassidy-Hanley (Cornell *Tetrahymena* Stock Center) for providing us with cell strains for this study. We also thank Steve Adolph, Charles Brokaw, Jim Forney, and Jacek Gaertig for helpful discussions. Julianna Erickson and Jonathan Hetzel contributed to the *Tetrahymena* *DYH2*, *D1LIC*, and *D2IC* sequence determinations. This work was supported by grants from the National Science Foundation to D.J.A. and the National Institutes of Health to D.G.P.

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