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, D2IC, 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), D2IC (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, D2IC 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 D2IC results in shortened and swollen sensory cilia (Wicks et al., 2000; Schafer et al., 2003). Both dynein-2 heavy chain and D2IC 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...
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 antherograde 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 IFT72, result in extremely short or no cilia (Brown et al., 2003; Beales et al., 2007; Tsao and Gorovsky, 2008a).

Although Tetrahymena antherograde 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 transfectants 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 dynactin-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 macronucleus of the progeny of such a mating completely lacks 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, dynactin-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 μM FeCl₃) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/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 BLASTin search of the *T. thermophila* genome (Eisen et al., 2006; http://tigrblast.tigr.org/er-blast/index.cgi?project = _tg_). 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 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 (DNASStar Lasergene, Madison, WI). Domain analyses of D2LIC and D2IC sequences were done with the Protein program (DNASStar Lasergene) and SMART (http://smart.embl-heidelberg.de/) programs, respectively. Multiple sequences were aligned by ClustalW (eib.ac.uk/cluster/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 D2IC sequences or known dynactin-2 IC, dynactin-1 IC, and outer arm dynactin 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 × 10⁶ cells/ml were washed once and starved in 10 mM Tris (pH 7.5) overnight for ~18 h at a density of 3 × 10⁵ 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
green PCR Master Mix (Invitrogen) and performed using a LightCycler 2.0 (Roche Applied Science) according to the manufacturer’s protocol. The primers used in the real-time PCR reactions were designed with Primer3 (Rozen and Skaletsky, 2000) and: Han et al. (2009).

#### Phylogenetic Analyses

The DNA sequences of the known D2LIC and D2IC sequences or known *T. thermophila* D2LIC, D2IC, and D1LIC gene sequences were used in phylogenetic analyses. 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 D2IC sequences or known dynactin-2 IC, dynactin-1 IC, and outer arm dynactin 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.

### Table 1. Dynein intermediate chain sequences used in phylogenetic analyses

<table>
<thead>
<tr>
<th>Organism</th>
<th>Gene</th>
<th>Accession no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlamydomonas reinhardtii</td>
<td>CrIC69</td>
<td>CA39053</td>
</tr>
<tr>
<td>Danio rerio</td>
<td>DrD2IC</td>
<td>20188</td>
</tr>
<tr>
<td>Dicyostelium discoideum</td>
<td>DdIC74</td>
<td>PS4703</td>
</tr>
<tr>
<td>Drosophila melanogaster</td>
<td>DmIC74</td>
<td>AAC70937</td>
</tr>
<tr>
<td>Gallus gallus</td>
<td>GgIC3</td>
<td>XP_415701</td>
</tr>
<tr>
<td>Homo sapiens</td>
<td>HsIC3</td>
<td>CAC71464</td>
</tr>
<tr>
<td>Strongylocentrotus purpuratus</td>
<td>SpIC3</td>
<td>XP_796667</td>
</tr>
<tr>
<td>Tetrahymena thermophila</td>
<td>SpD2IC</td>
<td>XM_001197794</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Organism</th>
<th>Gene</th>
<th>Accession no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caenorhabditis elegans</td>
<td>CeD1L1</td>
<td>CAC42262</td>
</tr>
<tr>
<td>Chlamydomonas reinhardtii</td>
<td>CrD1bLIC</td>
<td>AA137069</td>
</tr>
<tr>
<td>Gallus gallus</td>
<td>GgD1LIC1</td>
<td>CA55698</td>
</tr>
<tr>
<td>Homo sapiens</td>
<td>HsD1LIC1</td>
<td>AAD44881</td>
</tr>
<tr>
<td>Mus musculus</td>
<td>MmD2LIC</td>
<td>AAH39070</td>
</tr>
<tr>
<td>Rattus norvegicus</td>
<td>RnD1LIC1</td>
<td>AAF22294</td>
</tr>
<tr>
<td>Strongylocentrotus purpuratus</td>
<td>SpD1LIC</td>
<td>XP_795494</td>
</tr>
<tr>
<td>Tetrahymena thermophila</td>
<td>TtD1LIC</td>
<td>ABV90642</td>
</tr>
<tr>
<td>Xenopus laevis</td>
<td>XiD1LIC1</td>
<td>AF317841</td>
</tr>
</tbody>
</table>
Valencia, CA). The both plasmids were purified using QIAGEN Plasmid Maxi Kit (QIAGEN, and XhoI/ApaI restriction sites, respectively. Before biolistic transformation, sequence (the A of the start codon ATG is at restriction sites, respectively. For the ligated into either side of the digestion with SacI/ApaI and NotI/ApaI enzymes, respectively. The digested NEO3/H11001 flanking region is 516 bp and corresponds to NEO3/H11001-DYH1 region is 1623 bp and corresponds to NEO3/H11001-DYH2. Regulation relative to genes examined in this study. Real-time reactions were done with two different primer sets. The presence of wild-type gene-specific primers were as follows: A: 5'-CAGATGGCAGTCAGGACAGCC3' and B: 5'-CTACACAAAGATGGTCAGT3'. The D2LIC gene-specific primers were as follows: H: 5'-CCACTCAAAATCTCTTCAG3' and J: 5'-AATGCTCTTAAGTGGTTC3'. Equal values (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.

**Swimming Characteristics**

For determination of swimming speeds, wild-type, KO-DYH2, and KO-D2LIC cultures grown to ~5 × 10^6 cells/ml were used. Each cell type was further diluted in modified Nefsf 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 calculated for each cell type by finding 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 Nefsf medium for 24 h 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 0.1 M cadbylate buffer (pH 7.0) and dehydrated using a series of ethanol concentrations. Coverslips were critical-point dried using Tousimis SAMDR-780A critical-point dryer and sputter-coated with 90-nm gold particles using Anatec Hammer V1 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 kV.

**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 1 M cadbylate buffer (pH 7.0) for 4 h, postfixed in 2% osmium tetroxide, and washed with 0.1 M cadbylate 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 cadbylate buffer, and embloc-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 kV.

**Cilia Density and Length Analyses**

Wild-type, KO-DYH2, and KO-D2LIC cultures grown to ~5 × 10^6 cells/ml were used. Each cell type was grown and 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, at a 1:50 dilution in PHEM buffer. Cells were then washed in 0.1 M cadbylate buffer and dehydrated using a series of ethanol concentrations. Coverslips were critical-point dried using Tousimis SAMDR-780A critical-point dryer and sputter-coated with 90-nm gold particles using Anatec Hammer V1 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 kV.

**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'-CTACACAAAGATGGTCAGT3' and B: 5'-CTACACAAAGATGGTCAGT3'. The D2LIC gene-specific primers were as follows: H: 5'-CCACTCAAAATCTCTTCAG3' and J: 5'-AATGCTCTTAAGTGGTTC3'. Equal values (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.

**Cilia Density and Length Analyses**

Wild-type, KO-DYH2, and KO-D2LIC cultures grown to ~5 × 10^6 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, at a 1:50 dilution in PHEM buffer. Cells were then washed in 0.1 M cadbylate buffer and dehydrated using a series of ethanol concentrations. Coverslips were critical-point dried using Tousimis SAMDR-780A critical-point dryer and sputter-coated with 90-nm gold particles using Anatec Hammer V1 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 kV.
Tetrahymena Dynein-2 and Ciliogenesis

**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 (Griscom et al., 2002; Perrone et al., 2003; Schafer et al., 2003). However, the Tetrahymena D2LIC sequence does not contain the same coiled-coil domain.

**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-μm 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× PHEM buffer. The percentage of cells that contained beads was determined (n = 100). Cells that contained \(\leq 5\) beads were considered as having no beads because it is not unusual to have small numbers of beads adhere to the outside of cells.

**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; 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 \(b\) (*DYH4*) was used as the positive control. A relative level of 2.0 indicates a 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.

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.

doubling of expression after deciliation. The data were normalized to the expression of cytoplasmic *DYH1* because this gene is not induced. Outer arm \(b\) (*DYH4*) was used as the positive control. A relative level of 2.0 indicates a
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, germ-line 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 germine 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];
KO-DYH2 and KO-D2LICCells 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 $181.8 \pm 34.5 \, \text{μm/s}$. 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 ± 19.9 μm/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 3E, Table 3). 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* 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 ± 0.78 μm. The average length of cilia on KO-DYH2 and KO-D2LIC cells was 3.41 ± 0.63 and 3.40 ± 0.75 μm, 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 cillum length of the knockout cells was less than that of wild-type cells, but the knockout cells also showed a greater variation in cillum lengths, ranging from 0.5 to >10.0 μm. This cillum 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; Williams et
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 1–1.5 × 10⁶ 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 0.4–0.5 × 10⁶ 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 grow 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
seen with other Tetrahymena motility mutants (Brown et al., 1999b, 2003; Williams et al., 2006). Examples of knockout 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 antitubulin 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

| Cell type       | No. of cells | No. of cilia | Cilia length ± SD (μm) | Cilia density ± SD  
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>12</td>
<td>261</td>
<td>4.17 ± 0.28</td>
<td>0.19 ± 0.03</td>
</tr>
<tr>
<td>KO-DYH2</td>
<td>36</td>
<td>595</td>
<td>3.41 ± 0.63</td>
<td>0.14 ± 0.04</td>
</tr>
<tr>
<td>KO-D2LIC</td>
<td>36</td>
<td>535</td>
<td>3.40 ± 0.75</td>
<td>0.13 ± 0.05</td>
</tr>
</tbody>
</table>

*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.
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...
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 indicated. Amplification of cDNA produced a 1124-bp PCR product (no introns). The 1124-bp product (arrow) 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 kilobases 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 (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.

REFERENCES
from molecular characterisation to mechanism. Front. Biosci.
preferentially targeted to assembling cilia and is required for ciliogenesis and
normal cytokinesis in
Cytoplasmic dynein heavy chains at ultraviolet irradiation in the presence of ATP and

Lee, S., Wisniewski, J. C., Dentieler, W. L., and Asai, D. J. (1999). Gene knock-
outs reveal separate functions for two cytoplasmic dyneins in Tetrahymena

Increased levels of mRNAs for tubulin and other flagellar proteins after
amputation or shortening of Chlamydomonas flagella. Cell 20, 469–477.

Disruption of genes encoding predicted inner arm dynein heavy chains
214.

continuous turnover of outer doublet microtubules: implications for flagellar

disrupts localization of Smo to cilia and prevents the expression of both

Mukhopadhyay, S., Lu, Y., Qin, H., Lanjuin, A., Shaham, S., and Sengupta, P.
(2007). Distinct IFT mechanisms contribute to the generation of ciliary struc-
tural diversity in C. elegans. EMBO J. 26, 2966–2980.


Nomaka, S., Tanaka, Y., Okada, Y., Takeda, S., Harada, A., Kanai, Y., Kido, M.,
and Hirokawa, N. (1998). Randomization of left-right asymmetry due to loss
of nodal cilia generating leftward flow of extraembryonic fluid in mice lacking


Pazour, G. J., Dickert, B. L., and Witman, G. B. (1999). The DHCIb (DHC2)
144, 473–481.

is essential for the retrograde particle movement of intraflagellar transport

Pedersen, L. B., Miller, M. S., Geimer, S., Leitch, J. M., Rosenbaum, J. L., and
Cole, D. G. (2005). Chlamydomonas IFT172 is encoded by FLA11, interacts with
C9EB1, and regulates IFT at the flagellar tip. Curr. Biol. 15, 262–266.

Perrone, C. A., Tittscher, D., Taulman, P., Bower, R., Yoder, B., and Porter,
M. E. (2003). A novel dynein light intermediate chain colocalizes with the
retrograde motor for intraflagellar transport at sites of axoneme assembly in

real-time RT-PCR. Nucleic Acids Res. 29, e5.

Piperno, G., Huang, B., and Luck, D. J. (1977). Two-dimensional analysis of
flagellar proteins from wild-type and paralyzed mutants of Chlamydomonas

Cytoplasmic dynein heavy chain 1b is required for flagellar assembly in

Rana, A. A., Barbera, J. P., Rodriguez, T. A., Lynch, D., Hirst, E., Smith, J. C.,
and Beddington, R. S. (2004). Targeted deletion of the novel cytoplasmic
dynein m2DLC disrupts the embryonic organiser, formation of the body axes

Rannestad, J. (1974). The regeneration of cilia in partially deciliated Tetrahym-


