Targeted gene disruption of dynein heavy chain 7 of *Tetrahymena thermophila* results in altered ciliary waveform and reduced swim speed

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Accepted 6 July 2007
Journal of Cell Science 120, 3075-3085 Published by The Company of Biologists 2007
doi:10.1242/jcs.007369

Summary

*Tetrahymena thermophila* swims by the coordinated beating of hundreds of cilia that cover its body. It has been proposed that the outer arm dyneins of the ciliary axoneme control beat frequency, whereas the inner arm dyneins control waveform. To test the role of one of these inner arms, dynein heavy chain 7 protein (Dyh7p), a knockout mutant was generated by targeted biolistic transformation of *Tetrahymena thermophila* and show ciliary reversal in response to depolarizing stimuli. High-speed video microscopy of intact, free-swimming DYH7neo3 mutants revealed an irregular pattern of ciliary beat and waveform. The mutant cilia appeared to be engaging in less coordinated, swiveling movements in which the typical shape, periodicity and coordination seen in wild-type cilia were absent or disturbed. We propose that the axonemal inner arm dynein heavy chain 7 proteins contribute to the formation of normal ciliary waveform, which in turn governs the forward swimming velocity of these cells.

Supplementary material available online at http://jcs.biologists.org/cgi/content/full/120/17/3075/DC1

Key words: Axonemal dynein, Ciliary waveform, *Tetrahymena thermophila*

Introduction

Dyneins are force-generating molecular motors that utilize the chemical energy of adenosine triphosphate (ATP) binding and hydrolysis to power movement toward the minus ends of microtubules (Sale and Satir, 1977; Paschal et al., 1987). Dyneins can be divided into two structurally and functionally distinct classes, axonemal dyneins and cytoplasmic dyneins. Axonemal dyneins direct the oscillatory bending observed in beating of motile cilia and flagella. Cytoplasmic dyneins participate in a variety of intracellular transport processes, including mitotic spindle organization, retrograde intraflagellar transport, and the trafficking of various organelles such as nuclei in migrating neurons of the mammalian brain (reviewed by Vale, 2003). Thus, uncovering of the nature and function of dynein motors is important for understanding a wide spectrum of cellular processes.

Dynein heavy chains are classified as members of the AAA family (ATPases associated with cellular activities) of ATPases. Six AAA domains have been identified in the C-terminal globular head region as well as a seventh unrelated unit (King, 2000). These AAA domains contain P-loop ATP-binding motifs that are conserved among currently determined dynein sequences. The first P-loop, designated P1, is considered the ATP hydrolysis site that initiates movement (Asai and Koonce, 2001; Gibbons et al., 1987). The following three P-loops (P2-P4) are thought to have lower affinity for nucleotide binding, but may be necessary for functional regulation (Mocz and Gibbons, 2001). The remaining two domains appear to lack an intact nucleotide-binding site (King, 2000; Neuwald et al., 1999). Between P4 and P5 is located a protruding coiled-coil stalk structure termed the B-Link, which may make physical contact with an adjacent microtubule (Goodenough and Heuser, 1984). The N-terminal tail domain is the most highly divergent portion of the dynein heavy chain. Protein interactions with this region govern heavy chain dimerization and association of various accessory molecules that determine its cellular location. Studies with mutants of the flagellated alga, *Chlamydomonas*, that contain truncated axonemal dynein heavy chains suggest that the N-terminal tail is sufficient to direct assembly and localization of the remaining dynein motor complex (Perrone et al., 2000).

Researchers previously reported the expression of 14 distinct...
axonemal dynein heavy chain genes in *Tetrahymena* (Asai and Wilkes, 2004). At present, the new *Tetrahymena* genome database suggests the possibility of up to 23 different axonemal dynein heavy chain genes in the organism (see http://www.ciliate.org/).

The axonemal family of dynein motors can be further subdivided into two groupings, outer arms and inner arms. At least three of the known heavy chain gene products are found in the outer arms. At least two of the heavy chains form the inner arm dynein I1 complex. Several or all of the remaining isoforms are thought to assemble into single-headed inner arm dynein complexes (Piperno and Luck, 1979; Pfister and Witman, 1984; Asai and Wilkes, 2004).

Several different axonemal models describing the structural arrangement of outer and inner arm dyneins have been proposed (Goodenough and Heuser, 1985; Piperno et al., 1990; Muto et al., 1991; Mastronarde et al., 1992; Taylor et al., 1999). The outer arm dyneins appear on the axoneme at regular 24 nm periods along the outer microtubule doublet (Goodenough and Heuser, 1982; Goodenough and Heuser, 1985). The arrangement of inner arm dyneins on the axoneme appears to be more complex. All of the models of inner arm superstructure share the concept of a 96 nm periodicity, with each repeating unit consisting of three regions, termed II1, I2 and I3. It is generally thought that the I1 region contains one two-headed dynein, whereas the I2 and I3 regions each contain two or more single-headed dyneins. Furthermore, it has been suggested that the I2 and I3 regions may have different compositions of heavy chains depending on their position along the axoneme (Piperno and Ramanis, 1991).

Studies of dynein mutants in *Chlamydomonas* suggest that outer arms have a different function than inner arms. Mutants with outer arm defects display reduced beat frequency, whereas mutants with inner arm defects show reductions in shear amplitude with only small reductions in beat frequency (Brokaw and Kamiya, 1987). Although it is generally thought that inner arm dyneins determine waveform, it remains unclear whether the many individual inner arm dynein heavy chains of *Tetrahymena* contribute separate functions to the orchestration and shape of this waveform. The multi-dynein hypothesis (Asai, 1995) suggests that different dynein heavy chains of *Tetrahymena* are capable of producing different forces and are therefore functionally specialized.

Some previous studies have begun to gather data pertaining to this important aspect of the multi-dynein hypothesis by making targeted axonemal dynein gene knockouts and analyzing their effects on cilary functions. A *Tetrahymena* mutant containing a disrupted gene corresponding to inner arm dynein heavy chain 6 (*DYH6*) displayed slow swimming and abnormal waveform, but retained normal beat frequencies (Angus et al., 2001; Hennessy et al., 2002). This mutant, called KO6, was also defective in its ability to produce ciliary reversals and consequent backward swimming in response to depolarizing stimuli (Hennessy et al., 2002). Similar studies in which gene disruptions were introduced into the inner arm heavy chain genes *DYH8*, *DYH9* and *DYH12* of *Tetrahymena* suggested that *DYH8* may actually play a role in determining ciliary beat frequency, whereas *DYH9* and *DYH12* may play roles in generating waveform (Liu et al., 2004). The following study describes the gene disruption of inner arm dynein heavy chain 7 (*DYH7*) of the *Tetrahymena* axoneme. The resulting mutants, termed DYH7neo3, displayed defective ciliary waveforms, which impair normal forward swimming behavior.

**Results**

**Incorporation and fixation of the gene disruption construct is confirmed**

Biolistic transformation of the DYH7neo3 gene disruption construct resulted in hundreds of cell lines resistant to paromomycin in the presence of the metallothionein promoter inducer, cadmium chloride, at an initial concentration of 1 µg/ml. A few of these lines were selected and cultured such that the concentration of paromomycin was increased upon each feeding to create selective pressure toward fixation (complete replacement of all wild-type copies of this gene) of the mutant allele (Fig. 1). One particular cell line, which initially displayed resistance to 90 µg/ml paromomycin following transformation, was able to grow in a paromomycin concentration of 20 mg/ml after approximately 7 weeks of phenotypic assortment in super proteose peptone (SPP) media with 0.5 µg/ml cadmium chloride. A single-cell isolate of these highly resistant *Tetrahymena* was obtained and expanded to produce the mutant cell line, DYH7neo3, studied in the remainder of these experiments.

To confirm that the paromomycin resistance phenotype observed in culture was in fact because of incorporation of the gene disruption construct into the macronuclear genome by

![Fig. 1. Disruption of the *DYH7* gene. A 2.4 kb DNA fragment near the 5’ end of the 14.5 kb *DYH7* open reading frame (ORF) was cloned to pcR4-TOPO vector. The neo3 gene disruption cassette was inserted into *ClaI* and *Xmal* restriction sites that were introduced to this *DYH7* fragment by site-directed mutagenesis. This disruption interrupts the predicted N-terminal tethering domain of *DYH7* and is upstream of its P-loop domains. The resulting plasmid was linearized and used in biolistic transformation of vegetative CU427.4 *Tetrahymena*. The annealing sites for the *DYH7* gene-specific primers (gsp) and neo3 cassette-specific primer (neo3BTU) are indicated.](image-url)
homologous recombination, polymerase chain reaction (PCR) analysis was performed with genomic DNA isolated from mutant and wild-type cells. Both of these cell lines were cultured in the absence of selective agents for more than 2 months to eliminate possible side effects of the drug treatments. A primer specific for the 5’ DYH7 sequence that was upstream of the neo3 insertion site (gsp1-5) was paired with a primer specific for the DYH7 region that was downstream of the neo3 insertion site (gsp1-3). This primer set amplified a band size of 2.4 kb from wild-type genomic DNA template (Fig. 2A, panel 1, lane 3). When mutant genomic DNA was used as template for these primers, two bands were amplified, a lower 2.4 kb band and an upper 3.8 kb band (Fig. 2A, panel 1, lane 1). The amplification of the lower band is expected because of the presence of the micronuclear wild-type DYH7 gene in the mutant genomic DNA template. The upper band represents the larger PCR product expected if the neo3 cassette has been successfully incorporated into the macronuclear genome.

PCR was also performed on genomic DNA samples with gsp1-5 and a primer specific for the 3’ β-tubulin-2 (BTU) region of the neo3 cassette (neo3BTU). This primer set amplified a band size of 2.8 kb when mutant genomic DNA was used as template (Fig. 2A, panel 1, lane 2). This confirmed the incorporation of neo3 into the macronuclear genome of mutant cells. As expected, this primer set failed to amplify a band from wild-type genomic DNA, because the exogenous neo3 cassette sequence is not present in wild type (Fig. 2A, panel 1, lane 4).

Reverse transcription-PCR analysis (RT-PCR) was used to assess DYH7 expression in wild-type and mutant cells. Following DNAse I treatment of this RNA, first-strand cDNA synthesis was performed using gene-specific primer complimentary to a region of the DYH7 gene fragment that was approximately 2400 bp from its 5’ end (gsp1-3). PCR analysis was performed on the resulting cDNA using the gsp1-5/gsp1-3 primer set, which flanks the neo3 insertion region. When wild-type cDNA is used as template, a strong DYH7 signal is observed (Fig. 2A, panel 2, lane 6). The wild-type DYH7 gene signal is not detected when the cDNA template prepared from mutant is used (Fig. 2A, panel 2, lane 5). This result demonstrates that the mutant cell line has lost its ability to express the full-length DYH7 gene at a level detectable by PCR, and indicates that the wild-type signal observed in genomic DNA PCR experiments (Fig. 2A, panel 1, lane 1) is because of the presence of DNA from the transcriptionally silent micronucleus.

Another PCR primer set, gsp2-5/gsp2-3, was designed to amplify a small (~300 bp) region of the DYH7 sequence upstream of the neo3 insertion site. As expected, a band is observed when wild-type cDNA is used as template for this primer set (Fig. 2A, panel 3, lane 9). No band was seen with these primers when mutant cDNA was used as template (Fig. 2A, panel 3, lane 7). As a positive control for expressed cDNA sequence, a primer set specific for the ribosomal protein gene RPL21 was used in reactions with both wild-type and mutant cDNA templates generated from the same total RNA preparation using a universal oligo (dT) primer. The RPL21 primers flank the first two introns of the RPL21 gene and amplify a region of approximately 350 bp from cDNA or a region of approximately 850 bp from genomic DNA template.

**Fig. 2.** Genotypic analysis of wild-type (CU427.4) and mutant (DYH7neo3) cell lines. (A) (Panel 1) Total genomic DNA was isolated and used as template for PCR with DYH7 gene-specific and neo3-specific primers. A 3.8 kb band and a 2.4 kb band were amplified from the mutant genomic DNA template using the gsp1 primer set (gsp1-5 and gsp1-3, see Fig. 1) specific for the 5’ and 3’ ends of the DYH7 fragment (lane 1). The 3.8 kb band, representing the presence of neo3 insertion, was absent when wild-type genomic DNA template was used (lane 3). A 2.8 kb band was amplified from the mutant genomic DNA template when a 5’ gene-specific primer, gsp5-1, was paired with a neo3-specific primer, neo3BTU (lane 2). This band was absent when wild-type genomic DNA template was used (lane 4). (Panel 2) Total RNA was isolated and used as template for first-strand synthesis of cDNA with the gsp1-3 primer used above. The same DYH7 gene-specific primer set used in panel 1, gsp1-5/gsp1-3, amplified a 2.2 kb band from wild-type cDNA template (lane 6). This band was absent when mutant cDNA template was used (lane 5). (Panel 3) Primers specific for a DYH7 gene region upstream of the neo3 insertion site (gsp2-5 and gsp2-3) amplified a 300 bp band from the wild-type cDNA template (lane 9). This band was absent when mutant cDNA template was used (lane 7). As a positive control, primers specific for ribosomal protein gene RPL21 amplified a 370 bp band from both mutant and wild-type cDNA templates prepared using an oligo (dT) primer (lanes 8 and 10).

(B) Real-time PCR analysis of mutant and wild-type cDNA. The same templates and primers from A (panel 3) were used in real-time PCR amplification with SYBR green-fluorescent probe. The fluorescent signal specific for expression of the endogenous DYH7 gene was observed with wild-type template (closed squares), but was absent with the mutant template (closed circles). The RPL21 control reactions, performed in separate tubes, produced a signal of similar threshold cycle with both mutant (open circles) and wild-type templates (open squares).
The control primers amplified the cDNA-specific product when used with both wild-type (Fig. 2A, panel 3, lane 10) and mutant (Fig. 2A, panel 3, lane 8) cDNA templates. The product of approximately 850 bp was not observed, indicating the absence of genomic DNA contamination in this experiment.

Although the negative results displayed in Fig. 2A suggest that DYH7 is absent in the expressed sequences of DYH7neo3 mutants, it is possible that trace amounts of this sequence are present at levels below that which can be detected by the standard PCR methods used. With this in mind, the gsp2 primer set was also used as a probe in a more sensitive SYBR green fluorescence-detection-based real-time, quantitative PCR analysis of the cDNA templates. The expression of DYH7 is readily observed in the cDNA of wild-type cells (Fig. 2B, closed squares). No signal is detected in the cDNA of mutant, further supporting that this gene is not expressed in the transformant (Fig. 2B, closed circles). Because no DYH7 signal is amplified from the mutant DNA template, primers specific for the RPL21 gene were used as a positive control to verify that expressed sequences are present in the cDNA preparations used. The control experiments, which were performed in separate tubes with the same cDNA templates as above, indicate the presence of RPL21 gene expression in both wild-type and mutant cDNAs.

These data indicate that the neo3 gene disruption construct has successfully integrated into the targeted position within the DYH7 gene. The wild-type DYH7 signal is not detected when cDNA from mutant is used as template for both standard PCR amplification and SYBR green fluorescence-detection-based real-time PCR. This observation is consistent with the idea that all endogenous, wild-type macronuclear loci have been disrupted. The templates used were prepared from mutant cell lines that had been cultured in the absence of selective agents for more than 2 months. Previous studies, as well as our own observations, have shown that Tetrahymena dynein mutant genotypes that have not been fully assorted to fixation tend to rapidly revert back to wild type in the absence of selective agents (Angus et al., 2001; Lee et al., 1999). No genotypic reversion is observed in this DYH7neo3 mutant cell line. Taken together, these data support that the mutant genotype has phenotypically assorted to fixation.

Intact mutant cells display a motility defect
To investigate whether the targeted gene disruption of DYH7 results in phenotypic defects in ciliary function, the swimming speeds of mutant and wild-type cells were assayed and compared. The mutant cell line, cultured in the presence of selective agents, displayed a strong and consistent phenotype in which swim speeds measured approximately half that of wild type (Fig. 3; Fig. 4A, first two columns). Phenotypically assorted mutant cells were cultured, both shaken and standing, in the absence of selective agents for 1 month. The phenotype remained stable (Fig. 4A, third column; Fig. 4B). The DYH7 gene product appears to play a role in normal cell motility, and the mutation does not revert in the absence of paromomycin-selective pressure following phenotypic assortment.

Mutants retain their depolarizing and hyperpolarizing stimulus-response mechanisms
Wild-type and mutant cells were assayed for their ability to modulate swim speed when exposed to depolarizing and hyperpolarizing concentrations of KCl stimulus. It has been previously shown in the free swimming, freshwater ciliate Paramecium that depolarizing stimuli can decrease both beat frequency and forward swimming velocity, whereas hyperpolarization may result in increased beat frequency and faster forward swimming (Sugino and Macheiner, 1988). This observation was also made with Tetrahymena in recent behavioral studies (Hennessey et al., 2002). 8 mM KCl is a depolarizing stimulus that is below the threshold necessary for ciliary reversal. When wild-type cells are exposed to this stimulus, they decrease their speed of forward swimming (Fig. 5A). Although the mutant cells have a reduced basal swim speed relative to wild type, they are able to further decrease their swim speed under the 8 mM KCl depolarizing stimulus in a way that mirrors that of wild type (Fig. 5A). Both wild-type and mutant cells are able to increase their swim speeds when the KCl stimulus is removed (Fig. 5A). These data suggest that although the mutant cells appear to have a defect in their ability to swim at normal speed, they retain the machinery necessary to modulate their rate of movement under electrophysiological stimulus. When the KCl stimulus is increased beyond a threshold concentration of approximately 10 mM, both wild-type and mutant cells begin to display prolonged durations of backward swimming behavior because of continuous ciliary reversal (CCR). Individual cells of wild type and mutant were exposed to increasing concentrations of KCl stimulus and assayed for the resulting durations of backward swimming (Fig. 5B). The mutant cells retain their ability to show ciliary reversals in response to high KCl.

![Fig. 3. Swim paths of Tetrahymena. Examples of wild-type (CU427.4) and mutant (DYH7neo3) swim paths prepared by digital video microscopy of 2-second time intervals are shown.](image-url)
Dynein knockout affects ciliary waveform

Membranes were extracted from wild-type and mutant Tetrahymena to create motile cell models using a Nonidet P40 (NP-40) detergent treatment method similar to that previously reported (Goodenough, 1983). Cell models are devoid of all membrane systems present in intact cells. This procedure effectively removes all electrophysiological properties of the cell, allowing for observation of the behavioral phenomena that are specific to the axonemal structure. Fig. 6 displays differential interference contrast (DIC) microscopic images representing detergent-extracted cells (bottom) and intact cells (top). Although cell membranes are removed in the

stimulus in this assay, and they do so in a way that is similar to the behavior of wild-type cells.

The mutant phenotype is stable in detergent-extracted models

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models, their general integrity is provided by the remaining fibrous epiplasmic structure and intact cortical microtubular system.

Both the mutant and wild-type Tetrahymena models were totally immotile unless provided with exogenous MgATP. After testing a range of MgATP concentrations, 1 mM was determined to be the optimal for reactivation of cell motility. When detergent-extracted cells were transferred to media supplemented with 1 mM MgATP, reactivation of ciliary beating is observed. Both mutant and wild type resume
swimming behavior with very similar characteristics to that of intact cells. Approximately 90% of extracted cells were observed to possess the ability to be reactivated by this method. Once exposure to MgATP is initiated, individual cells would typically resume swimming for up to 15-30 minutes, after which motility would cease.

Swim speeds of wild-type and mutant demembranated models were determined by mixing extracted cells with 1 mM MgATP and recording their movement over 5-second intervals with digital video microscopy, as described above for motility assays of intact cells. The basal swim speeds of both wild-type and mutant models were lower than that observed with intact cells. Extracted models of mutant cells cultured both in the presence and absence of selective agents possessed consistent swim speeds of almost half that of wild type (Fig. 7). This observation reveals that the mutant phenotype is present when the plasma membranes, and hence electrophysiological properties of these cells, are removed. The data therefore support the conclusion that this motility phenotype is because of an axonemal defect rather than an electrophysiological artifact.

Cilia of mutant cells display an aberrant waveform

*Tetrahymena* were analyzed with high-speed video microscopy to directly examine the behavior of cilia in motion. Digital recordings of cilia on the aboral surfaces of free-swimming wild-type and mutant cells were obtained at 500 frames per second. In these assays, the cilia of wild type were observed to beat in a highly coordinated manner, with obvious and defined metachronal waves. By contrast, the cilia of mutant cells showed loss of metachrony, and often displayed highly anomalous patterns of beat characterized by asynchrony, aberrant periodicity and erratic propagation of bends. The cilia of mutant were frequently observed to entangle and impede neighboring cilia. Fig. 8 displays an illustration that represents an example of the waveforms observed. Individual frames were extracted from digitized videos of wild-type and mutant *Tetrahymena*. Frames from one beat cycle of three adjacent cilia on the aboral surface of each cell were chosen and digitally traced for clear visual representation. A sequence of six frames depicting wild-type cilia (top) and mutant cilia (bottom) are shown for comparison (Fig. 8). Whereas the wild-type sequence displays an organized, coordinated series of movements, the mutant sequence displays the characteristic out-of-phase beating that was observed. In addition, a comparison of frames 1, 5 and 6 of wild type with those of mutant shows that this mutant cell does not produce the typical out-of-plane recovery stroke, made visible by the apparent shortening of cilia in wild-type frames 1, 5 and 6. These data demonstrate that gene disruption of *DYH7* results in an altered ciliary waveform, and supports the notion that the *DYH7* gene product is an inner arm dynein that functions in the ciliary axoneme. Examples of digital videos can be seen as Movie 1 and Movie 2 in the supplementary material. Additional movies and animations can be accessed at http://www.rhiscarts.com/science/dyh7/. Although the waveforms were different, repetitive beat cycles could be estimated in both mutant and wild-type beat patterns.

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**Fig. 6.** *Tetrahymena* retain their body shape after detergent extraction of membrane systems. Differential-interference contrast microscopy of an intact wild-type cell (top) and a detergent-extracted cell model (bottom).

**Fig. 7.** Swim speed of reactivated models. Mutant cells were harvested for analysis from growth media containing paromomycin and cadmium (+ drug) or from growth media in which these selective agents were absent (− drug). Detergent-extracted models of wild type (CU427.4) and mutant (DYH7neo3) were prepared. Forward swimming resumed when cell models of wild type and mutant were reactivated in the presence of 1 mM MgATP. Reactivated mutant models displayed a slower swim speed than that observed for wild-type models. Each bar represents the mean ± s.d. of three trials of 50-cell measurements.
Mutant cilia generated an average beat of 16.1±4.6 Hz, whereas wild-type cilia generated a beat of 15.9±3.7 Hz (\(n=15\) for each trial).

**A growth defect is observed in mutant cell cultures**

Cell densities of wild-type and mutant SPP cultures were observed over time in both standing tube and shaken flask methods. A reduction in both growth rate and stationary phase cell density is observed in standing tube cultures of mutant when compared with those of wild type (Fig. 9A). The reduced growth rate and densities observed in mutant cultures increase to levels similar to those observed in wild type when the shaking culture method is employed (Fig. 9B). These data suggest that the disruption of \(DYH7\) may confer a defect in feeding, energy consumption or cell division that is reversed by shaking.

**\(DYH7\) groups with other I1 dyneins**

Previous phylogenetic analysis had grouped \(DYH7\) with the inner arm dynein beta group (Asai and Wilkes, 2004), based on the database sequence information available at that time. We have expanded this analysis by including the new database information currently available and have confirmed this grouping with a posterior probability of 1.0 or 100% (Fig. 10).

**Discussion**

The cilia of *Tetrahymena* employ a variety of axonemal dynein isoforms to drive the orchestration of movements that produce motility. Although each of the many inner arm dynein motors appear to possess highly conserved structural domains, their functional effects on the propagation of axonemal bending may differ (Brokaw, 1994; Asai, 1995). The inner arm dyneins initiate and perpetuate bending by generating shear forces between adjacent microtubules sufficient to overcome resistances to sliding (Piperno and Ramanis, 1991; Asai, 1995). These resistances and degrees of friction are likely to vary as a function of position along the axoneme, and therefore the force-producing requirements for inner arm dyneins located in the proximal region of the axoneme may be different from those of dyneins located in the medial and distal regions of the axoneme. In this case, a complex spatial arrangement of diverse inner arm dynein isoforms may be necessary in order to allow the series of movements observed in this organelle. The genetic complexity of dynein heavy chain genes presently apparent in the *Tetrahymena* genome database seems to reflect this notion of functional multiplicity (Asai and Wilkes, 2004).

The *Tetrahymena* genome database (http://www.ciliate.org) currently displays 25 different annotated dynein heavy chain genes. Of these presently identified sequences, only two represent heavy chain subunits of non-axonemal dynein complexes (\(DYH1\) and \(DYH2\)), which function in intracellular trafficking, phagocytosis, chromosome distribution and maintenance of the cortical microtubule cytoskeleton (Asai and Wilkes, 2004). Three of the remaining 23 dynein heavy chain genes, \(DYH3\), \(DYH4\) and \(DYH5\) are characterized as outer arm dyneins. The outer arm dyneins are structurally distinct from the inner arm dyneins. These isoforms are responsible for an increased sliding velocity between adjacent outer double microtubules, which is reflected in an increased beat frequency of the axoneme (Brokaw and Kamiya, 1987).

**Fig. 8.** Cilia on the aboral surface of wild-type and mutant cells observed using high-speed video microscopy techniques. Displayed left to right are six digitally traced frames extracted from video footage of one beat cycle of free-swimming wild type (CU427.4) and mutant (DYH7neo3). Each frame shows three adjacent cilia. Wild-type cilia displayed a consistent and highly organized metachronous waveform, whereas mutant cilia displayed aberrant waveforms and distorted metachrony.

**Fig. 9.** Cell densities of wild-type (CU427.4) and mutant (DYH7neo3) cultures observed over time. (A) Mutant standing tube cultures (broken line) displayed a reduced growth rate when compared with wild-type CU427.4 standing tube cultures (solid line). (B) This growth-defect phenotype is rescued when the shaken flask culturing method is employed. Each bar and data point represents the mean ± s.d. of three trials of 100-cell measurements.
This leaves a remaining 20 different genes, which are all tentatively annotated as inner arm heavy chains of the axoneme.

Dyh7p, the focus of this study, is grouped among the 20 presently identified inner arms of the Tetrahymena axoneme (Asai and Wilkes, 2004). Database sequence analysis predicts an open reading frame (ORF) of approximately 14.5 kb for the DYH7 gene, which confers a large (greater than 550 kDa) protein of approximately 4830 amino acids. This predicted amino acid sequence shows high homology to the dynein-1-beta heavy chain of the flagellar inner arm I1 complex (1-beta DHC) of Chlamydomonas reinhardtii, a well-studied algal model system for axonemal assembly and flagellar-based cell motility (Perrone et al., 2000). Our phylogenetic analysis also groups the Tetrahymena DYH7 with this Chlamydomonas I1-beta with a posterior probability of 1.0 (Fig. 10).

Previous studies of the 1-beta dynein heavy chain of the Chlamydomonas axoneme revealed insights into the structural organization of the I1 inner arm complex. A flagellar motility mutant lacking the 1-beta peptides associated with the I1 inner arm complex was transformed with constructs containing truncated versions of the 1-beta gene. Transformation with constructs encoding less than 20% of the dynein heavy chain were observed to partially rescue the motility defects by reassembly of an I1 complex containing an N-terminal 1-beta dynein heavy chain fragment and a full-length 1-alpha heavy chain (Perrone et al., 2000). In light of this data, a 5' region near the beginning of the DYH7 coding sequence was cloned and targeted for gene disruption, in order to minimize the possibility of expression of functional truncated fragments in the DYH7 mutant. This approach is in contrast to previous inner arm dynein studies in Tetrahymena, in which targeted insertion of gene disruption constructs took place in the central regions of the large dynein ORF (Angus et al., 2001; Hennessey et al., 2002; Liu et al., 2004).

The gene disruption constructs were introduced, in this study, by biolistic transformation of vegetative CU427.4 wild-type Tetrahymena strains to produce macronuclear knockouts from a single parental line. This method differs from previous studies of inner arm dynein mutants in Tetrahymena which involved conjugal electrottransformation (CET) of mating cells during macronuclear development (Angus et al., 2001; Hennessey et al., 2002; Liu et al., 2004). We have recently found that two wild-type strains commonly used for CET, CU427 and CU428, differ considerably in their behavioral responses to depolarizing stimuli (our observations). In light of this observation, the biolistic method is preferred for behavioral mutant studies because the resulting transfectants have more consistent genetic backgrounds and more clearly interpretable phenotypes. The mating and genetic exchange that is necessary for the CET method results in a more complex genetic background for resulting transfectants, which may have a less predictable phenotypic character in the case of certain cell properties.

The set of genomic DNA-, cDNA- and real-time PCR-based genotyping methods employed in this study indicate that the gene disruption cassette has stably incorporated into the intended target and that no full-length DYH7 message is expressed in mutant cells. Previous studies in which gene disruptions were made in cytoplasmic dyneins of Tetrahymena resulted in a mutant in which the disrupted allele could not be brought to fixation even after prolonged selection in paromomycin (Lee et al., 1999). This incomplete dynein knockout displayed reversion to the wild-type genotype within a span of days if the selective agent was removed from cell culture. With this in mind, the genotype of the DYH7neo3 mutant cell line was monitored for more than 2 months in the absence of paromomycin. No genetic or phenotypic reversion was observed, which suggests that the mutant allele was driven to fixation in these cells and that there were no complete remaining wild-type copies of this gene.

At the onset of this project, it was hypothesized that introducing a gene disruption in DYH7 would result in a ciliary waveform defect that would manifest itself as an aberrant
Dynein knockout affects ciliary waveform

Motility phenotype. The homology of **DYH7** to the I1-beta inner arm dynein heavy chain of *Chlamydomonas* (approximately 40% amino acid sequence identity), along with the data presented in Fig. 10, provide evidence supporting the idea that **DYH7** is likely to encode an inner arm dynein localized to the I1 complex of the *Tetrahymena* axoneme. The *Chlamydomonas* I-beta dynein, thought to be orthologous to **DYH7**, has been shown to be required for maintenance of proper flagellar waveform and swimming behavior (Perrone et al., 2000). Motility assays of both intact and reactivated models of **DYH7**neo3 mutants revealed a stable, slow-swimming phenotype in which mutants displayed velocities of almost half that of wild-type cells (Figs 4 and 7). Although the mutant cells displayed a slower basal swim rate than wild type, they were able to decrease and increase their swim speed in response to depolarizing and hyperpolarizing stimuli (Fig. 5). These data, taken together with the observation that the slow-swimming phenotype persists following membrane extraction, suggests that the motility deficiency is because of an axonemal defect rather than a problem with regulation of electrophysiological state.

When cilia of the **DYH7**neo3 mutants are examined directly using high-speed video microscopy techniques, an obvious deformity in ciliary waveform is observed. The consistently coordinated, metachronous ciliary movements observed in the wild-type cells appeared to be generally disrupted in those of mutant (Fig. 8). The majority of cilia observed on the aboral surfaces of mutant cells appeared to be engaging in less coordinated, swiveling movements in which the typical shape and periodicity seen in wild-type cilia were absent or disturbed. Often, adjacent cilia of mutant cells would interfere with one another, thereby disrupting the beat cycles of their neighbors. Although the beat cycles of mutant cilia appeared misshapen, their periodicity displayed a beat frequency similar to that of wild type. These data suggest that gene disruption of the **DYH7** gene may not affect beat frequency, although it should be emphasized that the waveform defect causes an obvious change in the shape of the ciliary beat cycle, which may confound beat frequency observations. Given these direct observations of cilia, it is surprising that these mutants can produce even the greatly reduced level of forward propulsion observed in the swim speed assays. Mutant cells showed a slower growth rate when compared with wild-type cells in standing tube cultures. Interestingly, the growth rate and stationary phase cell densities of mutant cells were increased to wild-type levels when mutants were adjusted to pH 7.5 with HCl.

Materials and Methods

**Cell cultures**

CU427.4 *Tetrahymena* cells (provided by Donna Cassidy-Hanley, Cornell University Tetrahymena Stock Center) were used as the wild-type background for creating the **DYH7**neo3 mutant. Cells were cultured with SPP media (1% proteose peptone, 0.2% dextrose, 0.1% yeast extract, 0.001% EDTA ferric-sodium salt) in either 5 ml standing tubes or 50 ml shaken flasks at 30°C (VWR orbital shaker). In order to establish mutant cell lines, bioptic transfomants were initially cultured in SPP supplemented with 90 μg/ml paromomycin and 1 μg/ml cadmium chloride. Cadmium chloride concentration was lowered to 0.5 μg/ml and paromomycin concentration was slowly increased to 20 mg/ml during the phenotypic assortment process. For starvation conditions, *Tetrahymena* were incubated in 10 mM Tris-base, adjusted to pH 7.5 with HCl.

**Engineering of gene disruption constructs**

The *Tetrahymena* genome database (http://www.ciliate.org/) (Eisen et al., 2006) was used to locate a 2.4 kb region near the 5’ end of the **DYH7** ORF. This gene fragment was amplified by PCR and cloned into a pCR4-TOPO sequencing vector (Invitrogen). The QuikChange kit by Stratagene was used to introduce *ClaI* and *Xmal* restriction sites into the fragment by site-directed mutagensis. Using these restriction sites, the **neo3** cassette (provided by Martin Gorovsky, University of Rochester, NY) was inserted to create the gene disruption construct. The **neo3** cassette includes the neomycin-resistance gene from bacterial transposon Tn5 flanked by the Cd2+ inducible metallothionein promoter (MTT) at the 5’ end, and the BTU polyadenylation termination signal at the 3’ end (Gaertig et al., 1994; Shang et al., 2002). The final **DYH7** gene disruption construct consisted of the pCR4-TOPO plasmid background containing a 2.5 kb, 5’ region of the **DYH7** ORF interrupted by the **neo3** cassette (Fig. 1). The **neo3** cassette was inserted in the same transcriptional orientation as the flanking **DYH7** gene sequence.
Biotic transformation of CU427.4

The DNA of the plasmid construct was purified using the Qiagen Plasmid Maxi kit (Qiagen) and linearized by sequential restriction digestion with SpeI and NotI enzymes. The resulting linearized DNA was extracted with phenol:chloroform: isoamyl alcohol (25:24:1, pH 6.9) and ethanol precipitated. The purified construct was used to coat 1.0 micron gold microcarrier beads, which were introduced to the macronuclear models (starved, vegetative) CU427.4 Tetrahymena by biotic bombardment as described previously (Brüns and Cassidy-Hanley, 1996). Following bombardment, cells were recovered in beakers of SPP media for approximately 4 hours. Cells were slowly shaken at 30°C during the recovery phase. First, paromomycin was added to a concentration of 90 μg/ml and cadmium chloride added to a concentration of 1.0 mg/ml. The entire volume of cells was transferred to 96-well microtiter plates, using a multichannel pipette to deliver 150 μl per well. Log-phase proliferation of paromomycin resistant, macronuclear transformant cell lines was observed after approximately 96 hours of standing incubation at 30°C.

Phenotypic assortment of transformed Tetrahymena

Following recovery after biotic bombardment, cells were cultured in 96-well depression plates containing 150 μl of SPP media per well supplemented with 90 μg/ml paromomycin and 1 μg/ml CdCl2. After 4-7 days, resistant lines were transferred to 1 ml cultures in 24-well depression plates. This initial low-level resistant media indicated that each dissected mutant allele has replaced at least one of the endogenous DH7 genes in the polymidy macronuclei. In order to drive this initial mutant allele to replace all of the estimated 45 endogenous copies in the macronuclei, the CdCl2 concentration was lowered to 0.5 μg/ml and the paromomycin concentration was increased gradually as cells were transferred to fresh media every 4-7 days. The anotic nature of macronuclear division in Tetrahymena leads to an imperfect segregation of the macronuclear genome during vegetative growth. As a consequence, alleles are unequally partitioned to daughter cells following division. In this case, steady increasing the paromomycin concentration in cell culture results in survival of only those cells that receive more copies of the mutant DH7neo3 allele, biasing the culture toward fixation of the mutant allele over time. The result is a phenomenon termed phenotypic assortment (Sonnewen, 1974). Mutant cell lines showed resistance to paromomycin as high as 20 mg/ml. Cells were passed for at least 100 generations at the peak paromomycin concentration and single-cell isolates were prepared from these resistant lines and expanded to 5 ml standing tube cultures.

Confirmation of transformation and fixation

Mutant genotype was verified by PCR examination of both genomic DNA and cDNA. Genomic DNA isolated from wild-type (CU427.4) and mutant (DYH7neo3) cells was used as template for PCR amplification with primer sets specific for the neo3 cassette (neo3F: 5’-CGATGTTGTTAATTAAATTGTTAG-3’; neo3R: 5’-CAAGTATTTTCTACAATACGCAC-3’) and DH7 sequences flanking the neo3 insertion site (gsp1-5: 5’-CTGGGTAAGAA-AAGATGAGAC-3’; gsp1-3: 5’-CTCTTCTTCAATTAGCCGAC-3’). The annealing sites for these primers are illustrated in Fig. 1. Total RNA was isolated from wild-type and mutant cells with the TRIzol reagent (Invitrogen), and used as template for reverse transcription. First-strand cDNA synthesis was performed with Superscript II RT (Invitrogen) using a gene-specific primer (gsp1-3) designed to be the 3’ end of the DH7 gene fragments. The resulting cDNA was examined by PCR using the gsp1-5/gsp1-3 primer (Fig. 2A). The DNA template was also examined by real-time PCR analysis with a BioRad MiniOpticon thermal cycler. The iQ SYBR Green Supermix reaction using the gsp1-5/gsp1-3 primer (Fig. 2A).

Analysis of Tetrahymena swim speed

CU427.4 and mutant cells of mid-log phase culture (3-5x103 cells/ml) were transferred from growth media to standard buffer (10 mM Tris, 0.5 mM MOPS, 50 μM CaCl2, pH adjustment to 7.2), using a pipette, and incubated for 30 minutes prior to examination or testing in stimuli. The swim speed analysis was performed in a total volume of 100 μl on a glass slide, with circular raised ring wells with an inner diameter of 13 mm, outer diameter of 16 mm and depth of approximately 0.5 mm. The buffered media was placed in the well such that it was spread out and in contact with the entire inner circumference of the ring. This created a thin, consistent layer of medium, which minimized artifacts in measurement owing to cells swimming out of the horizontal plane during recordings. Visualization was performed using a Boreal binocular research microscope with a 40X magnification objective, and avi-format video recordings were made using the Moticam480 system. For typical experiments under these conditions, 15 frames/second recordings of 5-second intervals were taken. Digital videos were subsequently processed using ImageJ software (http://rsb.info.nih.gov/iij/) to determine swim path length of individual cells, per unit of time (see Fig. 3).

Preparation and reactivation of cell models

Detergent-extracted Tetrahymena cell models were prepared by a procedure similar to that previously described by Goodenough (Goodenough, 1983). CU427.4 and mutant cells were harvested from mid-log phase cultures by centrifugation (IEC HN-II centrifuge) at ~200 g for 1.5 minutes. Pellets were resuspended in 25 ml of HMDES (10 mM HEPES, 5 mM MgSO4, 1 mM DTT, 2 mM EGTA, 4% glucose, pH 7.4) by gentle inversion and swirled and centrifuged again at ~200 g for 1.5 minutes. This HMDES wash was repeated twice. Pellets were then resuspended in 4 ml of HMDEK (30 mM HEPES, 5 mM MgSO4, 1 mM DTT, 2 mM EGTA, 25 mM KC1, pH 7.4). An additional 4 ml of HMDEK supplemented with 1.0% NP-40 detergent was then added and the suspension was gently mixed by inversion and swirled for 1.5 minutes. Next, 25 ml of HMDEKP [HMDEK plus 2% polyethylene glycol (PEG), 20,000 molecular weight] was added, followed by gentle mixing and centrifugation at ~200 g for 1.5 minutes (IEC HN-IIII centrifuge). The resulting supernatant was carefully removed and 25 ml of HMDEKP was added. Finally, the cell suspension was pelleted at ~200 g for 1.5 minutes (IEC HN-IIII centrifuge) and the supernatant was gently removed to leave approximately 1 ml of concentrated detergent-extracted cell models. To initiate reactivation, the detergent-extracted models suspended in HMDEKP were mixed with an equal volume of HMDEK supplemented with MgATP. A final concentration of 1 mM MgATP achieved optimal reactivation in these experiments.

Video microscopy of Tetrahymena cilia

Digital videos of cilia on the aboral surfaces (anterior regions of the cell membrane opposite the oral groove) of mutant and wild-type Tetrahymena cells were recorded at 500 frames/sec (Zeiss Axioshot microscope, differential-interference optics, Redlake high-speed digital camera). During recordings, cells were allowed to swim freely. All videos were obtained at room temperature. Although DH7neo3 and wild type displayed different waveforms, a ciliary beat cycle could still be determined in both. Beat frequencies were estimated by measuring the time it took each cilium to go through one complete beat cycle.

Phylogenetic analysis

Clade reliability was assessed using posterior probabilities from Bayesian analysis as implemented in MrBayes 3.1.2 (Huelsenbeck and Ronquist, 2001). A mixed amino acid model was used as a prior and an among-site rate variation parameter was also used. The analysis involved 120,000 generations, with tree sampling every 10 generations. Convergence was assessed with a plot of likelihood scores and 90,000 trees were conservatively assigned to the burnin phase. Posterior probabilities were visualized by plotting an unrooted phylogram (based on the tree file from MrBayes using the 100 top-scoring trees) showing the dynein heavy chain sequences used for this figure is provided at http://www.rhiscarts.com/science/dyh7/.

We are especially grateful to David Pennock for sharing advice, procedures and encouragement for this project. We thank Jody Bowen and Alan Siegel for their advice and technical assistance, Derek Taylor in helping us with the phylogenetic analysis and James Berry for the use of his gene gun. We also thank Joel Rosenbaum for his comments and suggestions. This work was supported by NSF grant MCB-0445362 to T.M.H.

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