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Silencing of a putative inner arm dynein heavy chain results in flagellar immotility in *Trypanosoma brucei*

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Abstract

The *Trypanosoma brucei* flagellum controls motility and is crucial for cell polarity and division. Unique features of trypanosome motility suggest that flagellar beat regulation in this organism is unusual and worthy of study. The flagellar axoneme, required for motility, has a structure that is highly conserved among eukaryotes. Of the several dyneins in the axonemal inner arm complex, dynein f is thought to control flagellar waveform shape. A *T. brucei* gene predicted to encode the dynein f alpha heavy chain, TbDNAH10, was silenced using RNA interference in procyclic *T. brucei* cells. This resulted in immotile flagella, showing no movement except for occasional slight twitches at the tips. Cell growth slowed dramatically and cells were found in large clusters. Microscopic analysis of silenced cultures showed many cells with detached flagella, sometimes entangled between multiple cells. DAPI staining showed an increased frequency of mis-positioned kinetoplasts and multinucleate cells, suggesting that these cells experience disruption at an early cell cycle stage, probably secondary to the motility defect. TEM images showed apparently normal axonemes and no discernable defects in inner arm structure. This study demonstrates use of RNAi as an effective method to study very large genes such as dynein heavy chains (HCs), and the immotility phenotype of these dynein knockdowns suggests that an intact inner arm is necessary for flagellar beating in *T. brucei*. Since analogous mutants in *Chlamydomonas reinhardtii* retain motility, this phenotype likely reflects differences in requirements for motility and/or dynein assembly between the two organisms and these comparative studies will help elucidate the mechanisms of flagellar beat regulation.

Keywords

Trypanosoma; flagellar motility; axoneme; inner arm dynein; dynein heavy chain

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Supplementary Material: Videos S1-S3, motility of wild type and induced DNAH10^{RNAi} cells (48 hours post-induction).

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1. Introduction

Trypanosoma brucei causes bovine wasting disease (called Nagana) and human African sleeping sickness, and new treatments for these diseases are urgently needed [40]. These parasites possess a single flagellum that is essential for motility and has roles in cell morphogenesis, polarity and cell division [14,20,32]. For these reasons, the flagellum is of interest as a source of potential drug targets. The overall structure of the axoneme that provides motility is highly conserved among eukaryotes [33], it consists of microtubules in a cylindrical 9+2 arrangement where nine doublet microtubules encircle a pair of singlet microtubules (Figure 1A). Flagellar bending occurs when the doublet microtubules slide against each other in a process driven by multiple sets of dynein motors [30]. Dyneins are made up of several polypeptides, including the heavy chains that contain motor domains. Axonemal dyneins are arranged in complexes along the inner and outer face of the axoneme, referred to as inner and outer arms, respectively (Figure 1A). Fine structures of these complexes have been determined for the axoneme of *Chlamydomonas reinhardtii*, a widely-utilized model for eukaryotic flagella [9,16,27]. The outer arms of *C. reinhardtii* contain sets of three dynein HCs (alpha, beta and gamma) in repeating arrays along the microtubule. Inner arms are more heterogeneous in structure and distribution along the axoneme, in *C. reinhardtii* there are seven different dynein species a-g (Figure 1B), comprised of eight different dynein heavy chains, named DHC1-3, 6,7, 9-11 [30,44,49]. Three additional minor dynein isoforms have recently been identified [49], but are not included in Figure 1. Dynein f is a “two-headed” dynein, i.e. contains two heavy chains (DHC1 and 10), while the other inner arm dyneins are single-headed dyneins, with one dynein HC each. In *C. reinhardtii*, the inner and outer arms have been shown to have different roles in flagellar movement. The dyneins of the outer arm are thought to be important for force generation but not beat formation [16], while those of the inner arm are proposed to determine the size and shape of the flagellar bend [21], and the precise coordination of all these dynein HCs produces the regular flagellar beat [30,46]. The functions of the individual proteins within the inner arm complex are not known, but it seems likely that each of the eight major dynein HCs has a distinct role in flagellar movement [9,19,48], and that they work together in a coordinated fashion to produce rapid beat frequencies and establish the proper amount of bend to enable propulsion [19,48]. Despite the observed structural conservation, the *T. brucei* flagellum has unique features, such as attachment along the cell body, paraflagellar rod (PFR) that runs along its length [3], tip-to-base direction of movement [15] and bihelical beat pattern [36] that suggest different roles of dynein motors in *T. brucei* relative to those in *C. reinhardtii* and other eukaryotic flagella [33,36]. Furthermore, recent studies of three separate *T. brucei* outer arm dynein genes showed that, unlike in *C. reinhardtii*, the outer arms are important for determining beat form in *T. brucei* [2,4]. Hence, while structural comparisons to other organisms provide a foundation for understanding the trypanosome flagellum, *T. brucei* axonemal dyneins should be studied specifically.

Very few studies have been performed with components of inner arm dyneins in *T. brucei*. One light chain component, TAX-1 [50], causes inviability when silenced in bloodstream form parasites, and a motility defect with no growth phenotype when silenced in procyclics [5]. A single headed inner arm dynein HC was silenced in procyclic cells, but mRNA knockdown was not effective [4]. Dynein HCs are challenging to study in any system because of their size: the coding regions of their genes are 12-14 kb in length, and as such are difficult to clone. Such large genes can be studied in *T. brucei* using RNA interference (RNAi) for which the cloning of only a few hundred bases of DNA is required to target a specific gene for silencing. Indeed RNAi knockdown of another dynein HC, homologous to the gamma heavy chain of outer arm dynein of *C. reinhardtii* has recently been reported and results in motility and cytokinesis defects [24]. The availability of this approach, combined with the unique features of flagellar beating in trypanosomes, make *T. brucei* an excellent system for investigating the roles of the inner arm dynein components.

This study describes the silencing of TbDNAH10, predicted to encode the alpha heavy chain of inner arm dynein f [44]. In the *C. reinhardtii* model, this dynein HC is thought to play an important role in regulating flagellar waveform [21] and shows several differences from other inner arm dynein HCs: *in vitro* it exhibits slower gliding velocities and it shows an unusual orientation relative to the microtubule suggesting that it interacts differently with the adjacent doublet microtubule [9]. *In vitro* studies with the *C. reinhardtii* dynein f indicate it has a high affinity for the adjacent outer doublet but low processivity so that it binds the microtubule tightly but does not move it very fast [1,21]. This suggests that dynein f might inhibit the extent of sliding of adjacent microtubules or help determine the amount of curvature of the flagellar bend [21]. We find that silencing of DNAH10 in procyclic *T. brucei* cells caused flagellar immotility as well as growth, cell division and morphological defects. Transmission electron microscopy did not reveal a significant change in ultrastructure of the inner arm relative to control samples, a result that is not unexpected for loss of a single inner dynein subspecies. The immotility phenotype suggests the importance of the inner arm in generating flagellar movement in trypanosomes.

2. Materials and methods

2.1 RNAi knockdown of TbDNAH10

TbDNAH10 (Tb927.4.870) was proposed to be the DNAH10 ortholog in a previous study [44]. For induction of RNAi against TbDNAH10, the region from bases 3-520 in the sequence was PCR-amplified from *T. brucei* Lister 427 genomic DNA with the following primers: (5' CAC CTC GAG GAT CGT ACC GTC GTT GGT TAC 3' and 5' CAC AAG CTT GAA TCG TCG AAG ATA TAA GG 3'), using PCR amplification with Taq DNA polymerase (Promega). These primers were chosen to amplify a region that is unique to this dynein HC and so lacks identity with other dynein HC genes in *T. brucei*. *Xho*I and *Hind*III restriction sites were included, respectively, in the 5' and 3' primers. This amplicon was cloned into *Xho*I and *Hind*III sites of pZJM to allow tetracycline-inducible production of double-stranded RNA [43].

2.2 Cultures and transfection

T. brucei procyclic strain 29-13 [47] was grown in SDM-79 [8] medium with 15% heat inactivated fetal bovine serum (Invitrogen) in a 28°C CO₂ incubator. The following antibiotics (Fisher Scientific) were used, as appropriate: G418 15 µg/ml, hygromycin 50 µg/ml, phleomycin 2.5 µg/ml, tetracycline (for dsRNA induction) 1 µg/ml. Growth was monitored by hemocytometer, viewed using a 10× objective lens in a Nikon TMS inverted microscope. For transfection, 15 µg of DNA was linearized by digestion with *Not*I (Promega) and transfected by electroporation in a Bio-Rad Gene Pulser at 1.6kV and 25 µFarad as described [11]. Stable transfectants were cloned by limiting dilution in conditioned medium [11] to obtain clonal RNAi knockdown lines, referred to as DNAH10^{RNAi}. For growth curves, cell titer was determined for uninduced and tetracycline-induced cultures approximately every 24 hours after time of induction, cultures were split 1/20 when they reached 6-9 × 10⁶ cells/ml. To study effect of mechanical agitation on growth, culture flasks were placed on an orbital shaker at 80 rpm for the duration of the induction analysis.

2.3 RNA isolation and real-time RT-PCR analysis

Total RNA was isolated from 5 × 10⁷ cells using the Masterpure RNA purification kit (Epicentre). RNA samples (2 µg) were treated with DNase I using the DNA-free kit (Ambion), and reverse transcription was performed using the Superscript Reverse Transcriptase (RT) III kit (Invitrogen). Absence of gDNA contamination was verified by comparing amplification of reverse-transcribed and non-reverse-transcribed RNA samples. Real-time PCR was performed using Brilliant II SYBR green QPCR master mix (Stratagene) and gene-specific primers in an

MX3000P QPCR instrument (Stratagene). PCR specificity was verified by melt curve analysis of the resulting PCR products. All primer sets were calibrated against a standard curve of PCR-amplified target DNA. The housekeeping genes GAPDH (Tb927.6.4280/ Tb927.6.4300) and RPS23 (Tb10.70.7020/Tb10.70.7030) were used as normalization controls. Relative gene expression was determined using the $2\Delta\Delta CT$ method [29]. Two independent sets of uninduced and tetracycline-induced cultures were used for RNA preparation and real-time RT-PCR. Standard deviations, indicated by error bars, were calculated using MS Excel. Primers used were as follows: GAPDH, 5' GGC TGA TGT CTC TGT GGT GGA 3' and 5' GGC TGT CGC TGA TGA AGT CG 3'; RPS23 5' AGA TTG GCG TTG GAG CGA AA 3' and 5' GAC CGA AAC CAG AGA CCA GCA 3'; DNAH10, 5' ATC ACT GGG AAC ACC GCA TAT GGA 3' and 5' ACA ATG GTG GTC GCT TTG CTG ATG 3'.

2.4 Sedimentation assay

Sedimentation assays were as described [34]. Uninduced or tetracycline-induced cell cultures were grown to approximately 5×10^6 cells/ml and divided into four cuvettes per sample. Optical density (600nm) was measured every two hours in a Beckman DU640 spectrophotometer (Beckman Coulter). The differences in optical density, averages and standard deviations were calculated using MS Excel.

2.5 Time lapse motility assay

Cells were observed in a hemocytometer using an inverted Nikon Eclipse TE2000-U microscope. Images were taken every second for thirty seconds using a Coolsnap HQ digital camera (Roper Scientific) using MetaVue Imaging software (Molecular Devices), the resulting stack was made into a standard 30 frame per second Apple Quicktime movie. Screenshots were made of the starting frame of these movies, and individual cells were observed for the length of the movie, and analyzed using GIMP (GNU image manipulation program www.gimp.org) and image modifying features in Appleworks. Cells classifications were adapted from [2]: runners had progressive motion for more than five seconds within this period; tumblers moved but did not show progressive motion for at least five seconds; or immotile cells showed no perceptible movement of cell bodies, with or without occasional twitches of the anterior tip of the flagellum.

2.6 DIC and Fluorescent Microscopy

Cells were washed and resuspended in phosphate-buffered saline [38] before being adhered to poly-L-lysine-coated slides for 10 min. Care was taken to disperse cells well before adhering to slides, to minimize number of clusters. Slides were fixed with 4% paraformaldehyde, stained with 2 μ g/ml 4'-6'-diamidino-2-phenylindole (DAPI), and mounted in Vectashield (Vector Laboratories) as described [11]. Slides were viewed in a Nikon Eclipse E600 microscope using an 100 $\times$ objective lens, under differential interference contrast (DIC) optics or epifluorescence with DAPI filters. Images were taken using a Spot digital camera (Diagnostic Instruments).

2.7 Scanning Electron Microscopy

Protocol was adapted from Sherwin and Gull [39]. 1×10^8 cells were spun down, washed in phosphate buffered saline, and fixed in 4% paraformaldehyde (Sigma-Aldrich) at 4°C overnight. Cells were then placed on coverslips coated with poly-L-lysine for 45 minutes in a humid chamber. Coverslips were rinsed with distilled water and mounted on aluminum stubs, and sputter coated with ~15nm of gold. Samples were viewed using a FEI scanning electron microscope.

2.8 Transmission Electron Microscopy

Flagellar preparation was performed using a two-step extraction protocol described previously [28,35]. Briefly, approximately 2×10^8 cells from either induced or uninduced cultures were collected by centrifugation, washed and then extracted with 1% NP-40 (IPEGAL CA-630, Sigma-Aldrich) to remove membranes, followed by 0.5M NaCl extraction on ice for 30 minutes to depolymerize subpellicular microtubules, which are then removed from intact flagellar microtubules by centrifugation. This extraction protocol removes outer arm dyneins as well as some central pair components, thus providing very good visualization of inner arm structures [28]. Extracted flagella were fixed overnight at 4°C in 10mM phosphate buffer containing 2% glutaraldehyde (Sigma-Aldrich) and 1% tannic acid and prepared as described [39]. Sections approximately 70nm in thickness were prepared by Marian Rice, Mount Holyoke College. Images were viewed on a Philips C150 transmission electron microscope (FEI.com), and captured digitally or scanned from Kodak Electron microscope film no. 4489 (Aremac).

3. Results

The gene Tb927.4.870 encodes a predicted protein 4448 amino acids (aa) in length, and shows 43% identity over its entire length to the *C. reinhardtii* DNAH10 homolog *Dhc1* (4499 aa) and 42% identity over 4432 aa to human DNAH10 (4471 aa). Phylogenetic analysis shows that dynein HCs of particular functions cluster together [13,44], and this approach was used to show Tb927.4.870 to be most related to the DNAH10 orthologs and distinct from other dynein HCs [44]. Thus we refer to this gene as TbDNAH10.

To study TbDNAH10 function, the gene was silenced using tetracycline-inducible RNAi as described in Materials and Methods. The reduction in mRNA in induced cultures of DNAH10^{RNAi} was confirmed by real time PCR analysis: by 48 hours post-induction the transcript levels were reduced to 26% of that seen in uninduced cultures (Figure 2A). RNAi knockdown of DNAH10 resulted in a significant reduction in growth rate (Figure 2B) and caused cells to form clusters, visible within 48 hours of induction and becoming very large by 96 hours post-induction (Figure 2C-E). Clusters of cells have been observed upon RNAi knockdown of other flagellar proteins and mechanical agitation reduced clustering [4,34]. Large clusters of induced DNAH10^{RNAi} cells were easily disrupted by brief (3-5 seconds) manual agitation of flasks but this did not completely separate all cells. Care was taken to perform such agitation on flasks before cell counts were recorded. When induced cultures were not agitated in this way, their hemocytometer counts remained the same or decreased over the course of the induction. Mechanical agitation of cultures for the duration of the growth curve analysis was performed as described in Materials and Methods but did not eliminate all clustering behavior nor did it restore growth rates (data not shown). Washing and removal of tetracycline did not completely reverse the phenotype (data not shown).

Sedimentation analysis of induced cells showed that at 48 hours post-induction they settled out of solution more rapidly than uninduced cells, representing a significant defect in motility (Figure 3A). Motility traces of RNAi knockdown strains were recorded based on time lapse microscopy (Figure 3B). Individual trypanosomes (approximately 200 for each time point) were observed for thirty seconds and classified as runners, tumblers or immotile as described in Materials and Methods. When compared to uninduced cells, induced DNAH10^{RNAi} showed a substantially increased percentage of immotile cells as early as 24 hours after induction, and the majority of cells were immotile by 48 hour post induction. The percentage scored as runners decreased accordingly although the percentage of tumblers was relatively constant (Figure 3B). Uninduced *T. brucei* cells moved rapidly (supplemental video S1), but induced DNAH10^{RNAi} cells scored as immotile appeared mostly paralyzed, with no movement of the flagella, except for twitching sometimes observed at the flagellar tip (supplemental videos S2 and S3). Detached flagella were not motile.

Cell morphology was examined with differential interference contrast (DIC), fluorescence and scanning electron microscopy (SEM). Cultures in which DNAH10 was silenced showed many cells with abnormal morphologies as compared to uninduced cultures (Figure 4). Specifically, many cells exhibited detached or partially detached flagella: 30.7 % of cells 24 hours post-induction and 65.2 % of cells 48 hours post-induction (Figure 4A-E).

Additionally, clusters or abnormally large bodies of cells were observed that were difficult to classify, but which appeared to have entangled flagella. Such clusters were very rarely seen with uninduced cells. Nuclear and kinetoplast staining of induced DNAH10^{RNAi} cells revealed that after 48 hours, 48% of cells contained two or more nuclei as compared to 6.7% of uninduced cells with multiple nuclei (Figure 4E). The percentage of cells with greater than two nuclei also increased with time of induction, from 0% of uninduced cultures to 2.4% and 11.2% in cultures 24- and 48- hours post-induction, respectively. The kinetoplasts were often mis-positioned, residing closer to the nuclei than expected and away from the posterior of the cell (Figure 4B-C).

An increased frequency of cells (4.3% at 24 hours post-induction and 25.2% at 48 hours post-induction) had kinetoplast:nucleus ratios of less than one (Figure 4E), suggesting a disruption in cell cycle regulation whereby nuclear division continues but kinetoplast segregation and cytokinesis do not occur at appropriate times. While care was taken to view multiple focal planes when scoring kinetoplasts, the close proximity to the nucleus meant that it is likely some kinetoplasts were not discernible, resulting in a slight overestimation of the percentage of cells with low kinetoplast:nucleus ratios. The morphologies of uninduced and induced DNAH10^{RNAi} cells were observed at greater magnification using SEM. When DNAH10 was silenced, individual cells generally appeared to be intact, but cell bodies and flagella were entangled making it difficult to tell if adjacent cells were completely separated (Figure 4 G-H).

To assess axoneme ultrastructure, transmission electron microscopic (TEM) analysis of axonemes from uninduced and induced DNAH10^{RNAi} cells was performed. We did not detect differences in axoneme ultrastructure in DNAH10^{RNAi} induced versus uninduced cells (Figure 5 and Table 1). This was not unexpected, as TEM analysis does not resolve individual dynein HCs and is thus unlikely to resolve the loss of a single dynein species or dynein heavy chain from an inner arm complex. In both samples, some variation in the sizes of the inner arm structures was observed, inner arms were scored as large if the discernible projection extended to (but did not necessarily contact) the adjacent doublet, small inner arms either had no projection or ones that extended less than half the distance to the adjacent doublet. Whether this variation reflects altered composition or simply the position of the dyneins relative to the section is not known. In either case, quantification provides an objective measure for comparing induced and uninduced samples. The average number of large inner arms per axoneme was comparable between uninduced and induced samples: 8.0 and 7.8 respectively. A relatively harsh (0.5M NaCl) extraction procedure for flagellar preparation was used in order to remove outer arm dynein proteins for improved visualization of the inner arm structures [28]. Because of this, flagellar preparations were expected to contain axonemes lacking outer arm dyneins and some having abnormalities such as disruption of connections between outer doublets or loss of central pair or radial spoke structures [28]. To assess whether DNAH10 knockdown affected global axoneme integrity, we determined whether the abnormalities noted above differed between uninduced and induced samples. The number of “broken” axonemes in which the cylindrical pattern of the outer doublets was flattened or had come apart, was counted (see Table 1). The fraction of broken axonemes in the population was similar between uninduced and induced cultures (0.24 and 0.26, respectively), suggesting that the axonemes of induced cells were not compromised any more than those from uninduced cells. In *T. brucei*, the central pair is oriented such that a plane through the two central pair microtubules is roughly parallel

to the PFR [34]. As a further measure of axoneme integrity, we sought to determine if the central pair microtubules were correctly oriented in the DNAH10 knockdown samples. Many axonemes had lost their central pair microtubules and had separated from the PFR, as reported previously for high-salt extraction in *T. brucei* [28]. Nonetheless, the majority of axonemes that could be scored had the correct central pair orientation: 8/10 in the uninduced sample and 7/9 in the induced sample (Table 1). For all the features reported in Table 1, the differences between uninduced and induced samples were not statistically significant by chi square test. Thus, while TEM analysis cannot confirm whether or not dynein f is altered, it does demonstrate that DNAH10 knockdown does not grossly disrupt axoneme ultrastructure. This result is consistent with a specific requirement for DNAH10 in flagellar motility, rather than an indirect effect of DNAH10 knockdown on axoneme integrity.

4. Discussion

This study presents an analysis of a suspected inner arm dynein HC, DNAH10, in *T. brucei*. Dyneins are multimeric complexes consisting of heavy, intermediate and light chains that transport cargo in the cell or drive flagellar bending. The C-terminal portion of a dynein HC contains the motor domain, made up of 6 AAA+ modules, one of which is a functional ATPase [37]. While the motor domains are common to all dynein HCs, variation in amino acid sequence among different dynein HC classes may be responsible for the different motor properties among these classes [1,45]. Between the fourth and fifth AAA+ modules is a coiled coil domain which forms a protrusion, known as the stalk, whose end binds to the microtubule to be translocated [12]. The N-terminal portion forms the tail domain which binds cargo and is associated with the various light and intermediate chains. Each axonemal dynein has a specific complement of light and intermediate chains which facilitate anchoring to the outer doublet microtubule, and may also have regulatory roles [10,37]. Thus, sequence variations among the motor and tail domains are characteristic of dynein function and phylogenetic classification can be used to predict the function of a dynein HC [1,13,44]. The Tb927.4.870 coding sequence shows strong identity across its entire length with DNAH10 genes of humans and *C. reinhardtii*, groups with these genes by Bayesian phylogenetic analysis, and has been assigned as the DNAH10 ortholog in *T. brucei* [44].

The silencing of DNAH10 has a significant effect on flagellar motility and cell motility in procyclic cells: immotile flagella and immotile cells appear within 24 hours post induction. While many of the knockdown cells had partially detached flagella, in all cases the flagella were immotile indicating that lack of cell movement was due to flagellar immotility. It should be noted that flagellar detachment alone is not sufficient to cause immotility, the FLA1 mutant in *T. brucei* shows extensive flagellar detachment but cells are still motile [22].

The marked immotility reported here was somewhat unexpected, since the DNAH10 mutant in *C. reinhardtii*, *pf9*, shows slower swimming velocities but flagella are not immotile [25, 26]. The *pf9* flagella have nearly normal beat frequencies but reduced amplitudes of flagellar waveform [26]. Axonemes of *C. reinhardtii pf9* mutants lack both the alpha and beta heavy chains of dynein f [25] but flagella retain some motility. It is possible that silencing of TbDNAH10 results in axonemes missing several inner arm components beyond just dynein f and this is what causes flagellar immotility. For example in *C. reinhardtii* the *ida2xida4* mutant lacks five inner arm dyneins (including those of the single headed dyneins a, c, d and the two-headed dynein f), and is nonmotile [17]. However, while the axonemes of nonmotile *ida2xida4* mutant cells show severely reduced inner arm structures by TEM, the axonemes of immotile TbDNAH10 knockdown axonemes do not exhibit gross ultrastructural defects (Figure 5). Thus, we favor the hypothesis that loss of motility in DNAH10 knockdowns reflects specific loss of DNAH10, rather than an indirect disruption of axonemal structures. It should

be noted that axonemes of *C. reinhardtii* DNAH10 mutant lacking both HCs of dynein f do not exhibit gross disruption of inner arm ultrastructure [17] at the resolution of TEM analysis.

Flagellar beating in *C. reinhardtii* and *T. brucei* flagella differ in several respects: the flagella in *C. reinhardtii* drive forward swimming by generating an asymmetric ciliary beat that propagates base-to-tip, and drive backward swimming by producing a symmetric beat that is also propagated base-to-tip [7]. By contrast the flagellum in *T. brucei* drives forward swimming by a complex bihelical beat that is propagated tip-to-base [36,42]. This tip-to-base beat is the predominant beat, although base-to-tip waves can also be generated and appear to coincide with periods of cell reorientation [4]. In the absence of outer dynein arms, the base-to-tip beat becomes the predominant beat and can drive backward swimming [2,4]. These different beats in *T. brucei* versus *C. reinhardtii* likely have different requirements in terms of timing and application of axonemal dynein motors, and may explain variations in mutant phenotypes between species. Different beat patterns may reflect the environment of the organism: *T. brucei*, needs to move not only through the liquid environments but also between layers of tissue in order to cross the blood-brain barrier of the mammalian host or enter the ectoperitrophic space in the insect host [41] and thus may require different dynein motor properties than those needed to navigate the soil and aquatic environments of the green alga *C. reinhardtii*.

While the phenotype of DNAH10 knockdown strain is very different than that of the DNAH10 mutant in *C. reinhardtii*, this is not the first report of a flagellar mutant in *T. brucei* whose phenotype differs from that of the corresponding *C. reinhardtii* mutant. In *T. brucei*, the loss of the outer arm structure results in reversal of the tip-to-base generated flagellar waveform so that cells swim backwards, while in *C. reinhardtii* loss of the outer arm dyneins affects beat frequency but not beat direction [2,6,18,23]. This suggests there are different ways that orthologous components can exert control over flagellar movement, and these may reflect differences in flagellar motility between these organisms [33].

RNAi knockdown of DNAH10 caused nuclear and kinetoplast abnormalities suggesting defects in cell division or cell cycle timing. In previous reports of knockdowns of flagellar genes in *T. brucei* many, but not all, exhibit cell division defects [4,5,24,31,33,34]. The cell cycle defects observed in the DNAH10 knockdown strain are consistent with silencing of a flagellar protein and, since the onset of motility inhibition occurs rapidly, this strain is informative for analyses of flagellar motility.

This study demonstrates the both the importance of the inner arm structure in *T. brucei* motility, and advantages of *T. brucei* for studies of large genes like those encoding dynein HCs, which can be genetically dissected using RNA interference. Through the use of molecular genetics we can analyze *in vivo* roles of components of the inner arm complex, which help to deepen our understanding of the coordinated functions of axonemal components.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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List of abbreviations used in text

RNAi	RNA interference
HC	Heavy chain
kb	Kilobase
aa	Amino acid
PCR	Polymerase Chain Reaction
PFR	Paraflagellar rod
ATP	Adenosine Triphosphate
RT	Reverse Transcriptase
DAPI	4',6-diamidino-2-phenylindole
DIC	Differential Interference contrast
SEM	Scanning electron microscopy
TEM	Transmission electron microscopy
OD	Optical density
nm	nanometer

Figure 1.

Axoneme structure.

A. Transmission electron micrograph of a *T. brucei* flagellum cross-section with accompanying schematic representation of the axoneme showing the ring of nine outer doublet microtubules and central pair of single microtubules (9+2). Dynein complexes of the outer arm (OA, in blue) and inner arm (IA, in ochre) are indicated, as well as the radial spoke (RS, in green) structures that extend between the central pair and outer doublet microtubules. The micrograph image also shows the electron-dense paraflagellar rod structure underneath the axoneme and the flagellar membrane. Scale bar is 100nm.

B. Schematic diagram showing a longitudinal view model for the structure outer arm (OA) and inner arm (IA) dynein complexes of *C. reinhardtii*, adapted from [9,48]. In this view, the microtubule is represented as a horizontal tube with base- and tip-proximal ends indicated, OA, IA and RS are indicated. Each of the eight major inner arm dynein HCs is shown. DNAH10 encodes the dynein f alpha heavy chain, part of the two-headed dynein f, and is shown in red. Scale bar is indicated. Although no model has been reported for the *T. brucei* axoneme, the genome sequence includes only two outer arm dynein HC genes (equivalent to β and γ shown here) and seven inner arm dynein HC genes including those of dynein f [44].

Figure 2.

Analyses of DNAH10 *RNAi* knockdown cells.

A. Relative transcript levels of DNAH10 RNA by real time RT-PCR analysis. Gene expression was normalized to the housekeeping genes GAPDH and RPS23. The level of gene expression in uninduced cells (-Tet) was set as 1.0, and the relative expression in tetracycline-induced DNAH10^{RNAi} cells (+Tet) is indicated. Data represent averages from two independent sets of uninduced and induced cultures, with standard deviation indicated by error bars.

B. A representative cumulative growth curve of DNAH10^{RNAi} uninduced (solid line) or tetracycline-induced (dashed line), averages of cell densities are shown with standard deviation indicated by error bars.

C, D, E. Phase contrast images of live DNAH10^{RNAi} cultures viewed at 10× magnification, showing clusters in tetracycline-induced cultures: C. Uninduced (-Tet). D. 48 hours post induction (+ Tet). E. 96 hours post-induction (+ Tet). Scale bar is 40 μm.

Figure 3.

Motility is disrupted in DNAH10 *RNAi* knockdown cells.

A. Sedimentation assay showing the motility of DNAH10^{RNAi} cells in the absence (closed diamonds) or presence (open squares) of tetracycline (48 hours post induction).

B. Summaries of traces from time-lapse motility assays on DNAH10^{RNAi}. Pie charts with percentages of cells scored as runners (white), tumblers (gray) or immotile (black) in cultures that were uninduced, 24 hours, or 48 hours post-induction with tetracycline. Raw numbers of cells scored for each sample indicated in parenthesis.

Figure 4.

Microscopic analyses of DNAH10^{RNAi} cultures.

A-C. Paired DIC and fluorescent images of DNAH10RNAi that were uninduced (A), or induced for 48 hours with tetracycline (B-C). Multinucleate cells and cells with detached flagella are visible in the induced cultures. Representative examples of multinucleate cell (white arrowhead) and detached flagellum (white arrow) are indicated. Scale bar is 10 μ m. D. Enlarged image of individual cells from white square in image B, shown to highlight the flagellar detail. Scale bar is 10 μ m. E. Bar graph showing percentages of different morphological classes scored from uninduced, 24 hours post-induction, and 48 hours post-induction DNAH10^{RNAi} cells. Inset shows key to

shading: Black = cells with detached or partially detached flagella. Gray = cells with two or more nuclei (the percent of cells with greater than two nuclei is indicated by the darker gray boxes within the gray bar). Hatched = cells with kinetoplast: nucleus ratio <1. F-H. SEM images of DNAH10^{RNAi} cells that were uninduced (F) or 48 hours post induction (G-H). Scale bar is 5µm.

Figure 5.

TEM analysis of DNAH10 *RNAi* cultures.

Representative axonemes prepared from high salt extraction of flagella from DNAH10 *RNAi* uninduced (A) or induced (B-C) cultures. This extraction protocol removes outer arm dyneins as well as some internal axonemal structures, but yields very good visualization of inner arm structures. Representative inner arm complexes scored as “large” (arrow) or “small” (arrowhead) are indicated, see text for details. Scale bar is 100 nm.

Table 1

Summary of results from TEM analysis of D Δ H10 *RNAi* cultures.

	Mean IA per axoneme ^a			Integrity of axoneme ^b			CP Orientation ^c	
	Large	Small	n	# broken	# intact	Fraction	# correct	# aberrant
Uninduced	8.0	1.0	81	259	755	0.26	8	2
Induced	7.8	1.2	54	78	250	0.24	7	2

^aMean inner arm (IA) per axoneme with clearly discernable inner arms (out of 9 doublets) over number of axonemes counted (n).

^bFraction = Number of broken axonemes per total (broken + intact) axonemes observed in > 10 fields.

^cOrientation of central pair (CP), reported as number of axonemes scored with correct (parallel to PFR) or aberrant (not parallel) orientation.