Establishing *Paramecium tetraurelia* as a new genetic model system for studying ciliary structure and function

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by
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Establishing *Paramecium tetraurelia* as a new genetic model system for studying ciliary structure and function

A thesis presented to the Department of Biochemistry

Graduate School of Arts and Sciences  
Brandeis University  
Waltham, Massachusetts  

By Michael Carlone

*Paramecium tetraurelia* is a ciliate that has long been used for a variety of different scientific studies, but has yet to be established as a model organism for studying ciliary structure and function. *P. tetraurelia* cells are covered with thousands of cilia, which are hair-like projections that are important for sensing the environment and for cell motility, e.g. to propel the cell itself or food into the cell’s mouth. It has also been shown that gene knockdown in *P. tetraurelia* can be induced by RNA interference via bacterial feeding. By using feeding RNA interference, we targeted *pf16*, a gene whose mutant phenotype is characterized by ciliary paralysis in the green algae *Chlamydomonas*. Our study aims at accomplishing two tasks: to establish *P. tetraurelia* as genetic model organism to systematically manipulate and study ciliary structure and function, and to localize the protein Pf16 in the central pair complex of cilia. We found that while *P. tetraurelia* is a promising candidate for genetic studies using RNA interference, some technical aspects have to be further optimized in future studies before this approach can become a general tool for studying ciliary structure and function.
# Table of Contents

Acknowledgements iii  
Abstract iv  
List of Tables vi  
List of Figures vii  
Introduction 1  
Materials and Methods 9  
Results 25  
Discussion 42  
Bibliography 50  
Supplement 53
List of Tables

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 1</td>
<td>Comparison of Knockdown to Wild type, Control per Time Point</td>
<td>34</td>
</tr>
<tr>
<td>Table 2</td>
<td>Comparison of Knockdown to Wild type, Control Over Whole Study</td>
<td>35</td>
</tr>
<tr>
<td>Table 3</td>
<td>Comparison of Samples to Their Respective Rescue</td>
<td>36</td>
</tr>
</tbody>
</table>
List of Figures

Figure 1 – Ciliary Coverage of Control *Paramecium*, Fluorescent Image 25
Figure 2 – Ciliary Coverage of Knockdown *Paramecium*, Fluorescent Image 25
Figure 3 – Z Projection of Swim Distance per 2.5 Seconds, 22 Hours 28
Figure 4 – Z Projection of Swim Distance per 2.5 Seconds, 44 Hours 29
Figure 5 – Z Projection of Swim Distance per 2.5 Seconds, 68 Hours 30
Figure 6 – Z Projections of Swim Distance per 2.5 Seconds, Rescue 31
Figure 7 – Graphical Comparison of Mean Swim Distance per 2.5 Seconds 37
Figure 8 – Graph of Mean Swim Distance per 2.5 Seconds per Sample 38
Figure 9 – Graph of Mean Swim Distance as a Percent of Wild Type 39
Figure 10 – 2 Dimensional Comparison of Structure of Central Pair Complex 41
Figure 11 – 3 Dimensional Comparison of Structure of Central Pair Complex 41
Figure S1 – Plasmid Map of pCR 2.1 TOPO Vector 53
Figure S2 – Plasmid Map of L4440 Vector 54
Introduction

Cilia and flagella are ubiquitous and highly conserved, cellular organelles that are present in virtually all cells at least at some point during the cell’s development. They have important roles in cell motility and/or sensing of environmental cues. Motile cilia and flagella propel cells through their aqueous environment and/or fluid over tissues, e.g., to clear mucus out of mammalian airways (Fliegauf et al., 2007). Studies of motile cilia and flagella, or their microtubule-based core-structure, the so called axoneme, have been performed using on a few model organisms, most notably the unicellular green algae *Chlamydomonas reinhardtii*, the single-celled ciliate *Tetrahymena thermophila*, and various types of sperm cells, e.g., from sea urchins or bovines. Different characteristic traits of these models make them particularly suited for different types of studies, for example, *Chlamydomonas* – which is also called “green yeast” - is well-suited for forward screens to isolate random mutations causing motility defects, and sea urchin sperm can be harvested in large quantities and have thus been used abundantly for biochemical studies (Dutcher, 1995; Linck and Norrander, 2003). Despite of these established organisms, there is a “bottleneck” for fairly easy and fast reverse genetic manipulations that target and eliminate function of specific ciliary genes. *Tetrahymena* and mice are currently the most used organisms for targeted gene knockouts, but generating strains can take more than a year. Therefore, the present study aims at establishing *Paramecium tetraurelia* as model organism for targeted gene knockdown by feeing RNA interference to study cilia.
Introduction to Paramecium – The genus Paramecium contains unicellular ciliates with approximately 100-250 µm length. These predatory organisms live in freshwater environments around the world and feed on bacteria, yeast, or algae. Developed as a model system in the mid-1900s, Paramecia have been considered ideal candidates for genetic studies because of their two forms of reproduction: conjugation (cross-fertilization by temporary union of two organisms and the exchange of genetic material) and autogamy (self-fertilization after production of gametes by the division of a single parent cell) (Beisson et al., 2010). Autogamous reproduction allows for the production of massive, homozygous cultures. By allowing autogamous reproduction to occur, one can add more of the same genotype and sex of Paramecium to the culture without the repercussion of conjugative reproduction (Sonneborn, 1937). Paramecium also contain two nuclei: a macronucleus (MAC) and a micronucleus (MIC). During a process called endomixis the MAC will disappear and two new MAC, arising from the MIC, which will sort themselves into the two daughter cells of a Paramecium undergoing asexual binary fission (Sonneborn, 1937).

Paramecium has been used for a long time as a model organism in a variety of different fields. For example, Paramecium has been used as an ideal model to study DNA recombination, because during both sexual and asexual reproduction, Paramecium undergoes a massive amplification of the MAC to about 800n (Beisson et al., 2010); this amplification and subsequent excision of “internal eliminated sequences” and imprecise elimination of repetitive sequences allows for the duplication of MAC during reproduction (Beisson et al., 2010).

Although ciliary structure and function studies have been few and far between in Paramecium, partly due to the success found in such studies with Chlamydomonas reinhardtii and Tetrahymena thermophila, several characteristics could make Paramecium also a useful
model organism for ciliary studies. *Paramecium* are covered in longitudinal rows with cortical units, termed unit territories, that contain each one (or rarely two) basal body with extending cilium (Sonneborn, 1970). Overall, there are ~4000 unit territories with about 5000 cilia (Sonneborn, 1970) that cover the entire *Paramecium* surface. Functionally, as in most other organism, *Paramecium* uses its cilia for sensing and motility, i.e., for swimming, feeding (due to their localization on the oral groove), and cytokinesis (Beisson et al., 2010). Genetically, ciliary mutations, either experimental or accidental, are carried over throughout multiple generations through cortical inheritance (Beisson et al., 2010). In order to affect these ciliary genes, *Paramecium*, RNA interference (RNAi) can be used (Valentine et al., 2012). As an added benefit, such mutations can be genetically rescued through feeding of normal, unaltered bacteria.

**Genetic Analysis of Paramecium Using RNAi** – RNAi is a powerful genetic tool first used to knockdown specific target genes in *Caenorhabditis elegans* (Fire et al., 1998). However, it can also be used in *Paramecium* to achieve the same effect. RNAi involves the recognition of double stranded RNA (dsRNA) in the cell and its cleavage into small interfering RNA (siRNA) 21-25 nucleotides long by the protein Dicer (Matzke et al., 2001). Antisense siRNA enter the RNA-induced silencing complex (RISC), which then goes on to cleave mRNA paired with the sequence specific siRNA (Matzke et al., 2001). RISC tends to cut in the middle of the complementary siRNA sequence on the mRNA strand, which leads to further degradation of the target mRNA (Matzke et al., 2001). This post translational gene silencing allows for a specific gene to be targeted and silenced within the organism. Timmons and Fire further showed that ingestion of the bacteria *E. coli* containing the target gene in dsRNA form can start the RNAi

Both Galvani et al. (2002) and Beisson et al. (2010) showed that RNAi silencing can occur by feeding Paramecium the same *E. coli* strains used by Timmons and Fire (1998). RNAi can generate large scale knockdowns of Paramecium because it will induce deletion of the target gene in the replicating MAC, creating stable knockdown strains (Beisson et al., 2010). Likewise, ingestion of dsRNA to induce RNAi can allow for knockdowns in large scale cultures, where normally microinjection would have to be used on a single-cell level. Finally, Kamath et al. (2000) showed that ingestion of dsRNA to induce RNAi is as effective and sensitive as microinjection.

**Introduction to Cilia and Flagella** – Cilia and flagella are ubiquitous and highly conserved organelles that are important for both sensing and motility. They are mostly described as hair-like appendages of cells. There are two general types of cilia: motile and non-motile (primary) cilia. Beating of motile cilia is driven by ATP-hydrolyzing motor proteins, called dyneins. Ciliary motility is responsible for general movement of cells or extracellular fluid along tissues (Fliegauf et al., 2007). In humans, this motility helps with mucociliary clearance and sexual reproduction (Fliegauf et al., 2007). Non-motile cilia are generally involved with sensing. The ciliary membrane, which is contiguous with the plasma membrane of the cell, contains different receptors, channels, and signaling molecules, allowing the use of cilia for mechanosensation as well as chemosensation (Fliegauf et al., 2007). In humans, cilia are important for many different functions within the body, including during early development and asymmetrical specification,
and defects in cilia can cause a number of different diseases, termed ciliopathies, such as primary ciliary dyskinesias (PCDs) (Fliegauf et al., 2007).

Cilia can be divided up into three sections: the basal body, the transition zone, and the axoneme, which usually has a 9+2 or 9+0 architecture of nine doublet microtubules (DMTs) forming a cylindrical arrangement (Fliegauf et al., 2007). The present study focuses primarily on the 9+2 axoneme of motile cilia, which have two central singlet microtubules with associated projections, termed the central pair complex (CPC).

There is much known about the 9 DMTs regarding their structure and function. Each DMT is made up of an A- and a B-tubule and a plethora of associated proteins, including the motility-driving dynein motors (Heuser et al., 2009). Dyneins are situated along the A-tubule, but “walk” along the B-tubule of the neighboring doublet in an ATP-sensitive manner, which generates a sliding motion between adjacent doublets and contributes to the bending patterns found in motile cilia and flagella (Heuser et al., 2009). Heuser et al. (2009) also showed that the DMTs are linked by the nexin-dynein regulatory complex (N-DRC) that not only links DMTs together but also acts as a regulator for dynein activity (2009). The radial spokes (RS) protrude from the A-tubule of the DMTs and are oriented towards the CPC. Cilia of most organisms, including humans, contain 3 RS, while only a few organisms, such as Chlamydomonas, contain only 2 full-length RS and a thirst shorter RS (Heuser and Dymek et al., 2011). The positioning of the RS on the A-tubule suggests a signaling pathway between the CPC and the dynein arms and/or the NDRC (Dymek and Heuser et al., 2011).

The main focus of this study, however, is the CPC, which is made up of two singlet microtubules, named the C1 and C2. Both are structurally distinct due to their associated projections that differ in length and periodicity (Smith and Yang, 2004, Carbajal-González et al.,
The CPC can rotate in some organisms, including in *Paramecium*, where it was shown to rotate once per beat like in *C. reinhardtii* (Smith and Yang, 2004; Omoto and Kung, 1979). It has been hypothesized that the projections of the CPC interact with the heads of the RS and are involved in the motility of the cilia (Smith and Yang, 2004).

The C1 microtubule of the CPC has 6 major projections associated, all of which are distinct from the C2 microtubule projections. C1a and C1b are the longest projections with C1e and C1f, respectively, sitting underneath them (Carbajal-González et al., 2013). C1c and C1d make up the rest of the projections on the C1 microtubule, with an approximate size between 11 and 14 MDa (Carbajal-González et al., 2013). It has been hypothesized that the projections are distinct because they have different functions within the axoneme relating to motility regulation (Carbajal-González et al., 2013).

*Ciliary motility and Pf16* — It has been well documented that defects in the CPC affect ciliary motility. The mutation of the flagellar associated (FAP) protein FAP74 cause loss of the C1d projection and subsequent loss of movement in *C. reinhardtii* (Dipetrillo and Smith, 2010).

The gene *pf16* is an Armadillo (ARM)–repeat protein first characterized in *Drosophila melanogaster* as a repeating 42 amino acid motif with a variety of different cellular functions (Riggleman et al., 1989; Peifer et al., 1994; Smith and Lefebvre, 1996). In particular, it is thought that ARM repeats are important in various protein-protein interactions (Peifer et al., 1994). In the ciliary axoneme, Pf16, a 57-60 kDa protein, localizes in the general vicinity of the C1 microtubule(Dutcher et al., 1984; Smith and Lefebvre, 1996), but the precise location remains unknown. Mutation of the *pf16* gene results in massive structural defects in the axoneme, including destabilization and eventual loss of the C1 microtubule (Dutcher et al., 1984;
From the hypothesized 9 polypeptides unique to the C1 microtubule, assembly failures involving three of the polypeptides reduce the stability of the C1 microtubule (Smith and Lefebvre, 1996; Dutcher et al., 1984). Similarly, Smith and Lefebvre (1996) also showed that the pf16 mutant of C. reinhardtii is defective of three polypeptides. Furthermore, they showed that de-membranation of the flagella destabilized the already weakened C1 microtubule, resulting in the loss of C1 (Smith and Lefebvre, 1996). Straschil et al. (2010) showed that pf16 mutations in Plasmodium berghei results in total destabilization of the C1 microtubule, even without prior de-membranation. In both organisms, pf16 mutation causes a characteristic paralyzed phenotype, which led to the belief that destabilization and/or loss of the C1 microtubule negatively affects the flagellar motility of the mutant strains (Smith and Lefebvre, 1996; Straschil et al., 2010).

Summary – As stated above, Paramecium has been widely used as a model organism for a variety of different biological fields, but its use for ciliary studies has been limited. The gene pf16 is a suitable target for this study because of its characteristic phenotype and importance to the structure and function of the CPC and axoneme (Dutcher et al., 1984; Smith and Lefebvre, 1996; Straschil et al., 2010), allowing for good control if the newly established approach is successful. By targeting this gene with RNAi, it is possible to generate a large scale culture of pf16 deficient mutant Paramecium cells both quickly and robustly.

We seek to show that Paramecium is viable for both genetic and ciliary studies. Given that Paramecium are covered in cilia and given that we have a genetic tool to target and knockdown specific genes within the genome, it becomes a strong candidate in theory. In order to prove its viability, we determined that a strong candidate would be easy to maintain, easy to
affect genetically, and would easily show an effect. We also chose pf16 because of its characteristic phenotype and its size, which should allow visualization of the knockdown by cryo-electron tomography (Nicastro et al., 2006). In choosing pf16, we also seek to localize pf16 in the CPC.

To show that Paramecium is a viable reverse genetic model, we used RNAi to induce the knockdown, and then fluorescence microscopy and cryo-electron tomography to compare any phenotypic and structural differences. Furthermore, we used high speed light microscopy and image processing to show a quantifiable difference is swim speeds between wild type, control (empty RNAi vector), and knockdown mutant Paramecium. Paramecium cells were cultured and exposed to RNAi over a course of 68 hours and then rescued by the feeding of Enterobacter aerogenes, which is normally fed to them as food. In performing the rescue, we also sought to show that any phenotypes induced by RNAi were reversible (Timmons and Fire, 1998).

We found that Paramecium proved to be promising candidate, however, more optimization has to be done to successfully establish Paramecium as model organism for combined genetic and ciliary studies. We found that RNAi is a robust tool in Paramecium studies, enabling us to generate large scale knockdown cultures quickly and easily. Cryo-electron tomography showed a possible defect in the C1 microtubule projections of the CPC, further giving evidence to pf16 localizing to the C1. High speed microscopy and image processing showed a reduced swimming speed phenotype in the knockdown mutants, whereas motility could be recovered by genetic rescue with E. aerogenes. In summary, Paramecium has the potential to be a model organism for genetic and ciliary studies, but there is a lot of work that needs to be done in order to cement itself as such.
## Materials and Methods

**Strains** – Below is a list of the strains referenced throughout the experiments.

<table>
<thead>
<tr>
<th>Strain Name</th>
<th>Organism</th>
<th>Genotype / Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>HT115</td>
<td><em>Escherichia coli</em></td>
<td>F-, mcrA, mcrB, IN(rrnD-rrnE)1, rnc14::Tn10(DE3 lysogen:lavUV5 promoter – T7 polymerase) (IPTG inducible T7 polymerase) (RNaseIII–)</td>
</tr>
<tr>
<td>NEB5α</td>
<td><em>Escherichia coli</em></td>
<td>fhuA2Δ(argF-lacZ)U169 phoA glnV44 Φ80Δ (lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17</td>
</tr>
<tr>
<td>WT</td>
<td><em>Enterobacter aerogenes</em></td>
<td>Wild type</td>
</tr>
<tr>
<td>51S</td>
<td><em>Paramecium tetraurelia</em></td>
<td>Wild type stock</td>
</tr>
</tbody>
</table>
**Plasmids and Primers** – Below is a list of the primers and vectors used throughout the experiments.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>L4440For¹</td>
<td>5’ CTGGCGTAATAGCGAAGAG 3’</td>
</tr>
<tr>
<td>L4440Rev¹</td>
<td>5' ATAATACCGCGCCACATAGC 3'</td>
</tr>
<tr>
<td>Pf16For1²</td>
<td>5' ATGAGTCGTGCAGTGTACAACC 3'</td>
</tr>
<tr>
<td>Pf16Rev1²</td>
<td>5' AATCTGCATTATAATCGTCCAATTTC 3'</td>
</tr>
<tr>
<td>Pf16For2³</td>
<td>5' TGCAAGGTAATCCCAAGACC 3’</td>
</tr>
<tr>
<td>Pf16Rev2³</td>
<td>5' CCAAGAGACCAAGCAGAAGC 3’</td>
</tr>
<tr>
<td>2.1 TOPO Vector⁴</td>
<td>See Supplement for Plasmid Map</td>
</tr>
<tr>
<td>L4440 Vector⁵</td>
<td>See Supplement for Plasmid Map</td>
</tr>
</tbody>
</table>

¹: Expected Size: 895 bp  
²: Expected Size: 1645 bp containing full length *pf16* gene  
³: Expected Size: 249 bp containing internal segment of *pf16* gene  
⁴: Invitrogen by Life Technologies  
⁵: L4440 was a gift from Andrew Fire (Addgene plasmid #1654)
Samples in the Experiments – Below is a list of the samples used throughout the experiments.

<table>
<thead>
<tr>
<th>Name</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>Control, empty L4440 plasmid</td>
</tr>
<tr>
<td>F2</td>
<td>Control, empty L4440 plasmid, will control for density (qualitatively)</td>
</tr>
<tr>
<td>F3</td>
<td>Experimental, pf16 knockdown</td>
</tr>
<tr>
<td>F4</td>
<td>Only bacteria, observe bacterial growth</td>
</tr>
<tr>
<td>F5</td>
<td>Wild type, fed <em>E. aerogenes</em> only</td>
</tr>
</tbody>
</table>

Growth and Culturing – *Paramecium* were grown at room temperature without agitation in 10 mL wheat grass medium (WGM), a medium containing wheat grass (Pines, Lawrence, KS), 3 g/L stigmasterol (Note: stigmasterol must be dissolved in 180 proof (or higher) ethanol), and 1 g/L Na$_2$HPO$_4$ x 7H$_2$O (adjusted to pH≈7). Feeding involved inoculating WGM with *Enterobacter aerogenes* and growing overnight at 37°C. Inoculated WGM was allowed to cool to room temperature and 200µL of the previous 10 mL *Paramecium* cell culture were transferred from the old to the fresh culture. Feeding and exchange took place on a 2-week cycle.

Isolation of Genomic *Paramecium* DNA – [Modified from Ray, 2008 (PhD Thesis, Burlington, VT)] An improved wheat grass medium (IWGM) containing wheat grass, 0.333g/L proteose peptone, 3 g/L stigmasterol, 0.787 g/L Trizma hydrochloride, 1.07 g/L Na$_2$HPO$_4$, and 0.21 g/L NaH$_2$PO$_4$ x H$_2$O (adjusted to pH≈7) was inoculated with *E. aerogenes* and grown overnight at 37°C. The inoculated IWGM was allowed to cool to room temperature and 200µL of the previous 10 mL *Paramecium* cell culture. This culture was left at room temperature to grow for
two days without agitation. The culture was then filtered using a custom filter, i.e., a funnel containing the following 3 layers: kimwipe, cheesecloth, and kimwipe, to remove large, unsolvable waste produced by *E. aerogenes* and *Paramecium*. *Paramecium* cells in the culture were passed through the filter, by pouring, while biofilm and waste were collected on the filter surface, resulting in a “clean” culture for isolation. The filtered culture was centrifuged in pear-shaped flasks in an ICN Centrifuge at approximately 500g for 5 minutes. A glass Pasteur pipette was used to collect the resultant pellet. This pellet was transferred to 15 mL of Dryl’s solution (137.99 mg/L NaH$_2$PO$_4$ x H$_2$O, 268.07 mg/L Na$_2$HPO$_4$ x 7H$_2$O, 428.22 mg/L Sodium citrate, and 193.49 mg/L CaCl$_2$ x H$_2$O, adjusted to pH≈6.8-7) and then centrifuged for an additional 3 minutes at approximately 500g, harvested with a glass Pasteur pipette, washed, and centrifuged once more. The pellet was then resuspended in a small volume of Dryl’s solution and transferred to a sterile 1.5 mL centrifuge tube. Then, 600µL of lysis buffer was added (2 g/L NaOH, 13 g/L EDTA, 5 g/L SDS, 1 g/L Proteinase K, 1 mg/L RNase). The mixture was incubated at 65˚C for 1 hour and then 10µL 3M NaCH$_3$COO was added. The mixture was separated into two centrifuge tubes, due to volume restraints, and 500 µL of a 25/24/1 Phenol/Chloroform/Isoamylalcohol solution was added to each tube. The solution was incubated on ice for 10 minutes and then centrifuged at 4˚C for 20 minutes at 16,000 g. The aqueous phase was isolated and transferred into a fresh tube before adding an equal volume of cold isopropanol. The solution was incubated for 30 minutes at -20˚C and then centrifuged at 4˚C for 20 minutes at 16,000 g. The supernatant was discarded and the resulting pellet was subjected to two wash steps involving the addition of 1 mL 70% ethanol and centrifugation at 4˚C for 20 minutes at 16,000 g. After the wash steps the supernatant is discarded and the pellet left to air dry. Pellets were resuspended and combined in a total of 50µL of H$_2$O and stored at -20˚C.
**Isolation of Paramecium Cilia** – [Based on Nelson (1995)] A 1 L culture of IWGM with *E. aerogenes* and *Paramecium* was prepared and filtered as described above. The *Paramecium* cells were centrifuged, transferred to Dryl’s solution and washed as described above. After washing, the resultant pellet of *Paramecium* was resuspended in 10 mL of cold, premixed Dryl’s / STEN with equal parts Dryl’s solution and STEN solution (171.5 g/L sucrose, 3.15 g/L Trizma hydrochloride, 672.4 mg/L Na$_2$EDTA, 350.65 mg/L, adjusted to pH≈7.5) and incubated on ice for 10 minutes (Note: *Paramecium* are very intolerant of cold temperatures and will become immobile quickly, and the cells will lyse if kept cold for too long). After incubation, 10 mL cold Ba-shock solution (4.73 g/L KCl, 4.16 g/L BaCl$_2$) was added and the cells were incubated between 3 to 5 minutes. During this time, aliquots were taken to determine if cells had begun lysing (if so, the incubation would have been stopped and the next steps would have proceeded). The solution was transferred into pear-shaped flasks and centrifuged at approximately 500g for 3 minutes. The resultant pellet, which included trichocysts released as a stress response by the *Paramecium*, was removed with a glass Pasteur pipette and discarded. Centrifugation was repeated and the rest of the cell debris pellet was removed and discarded. The supernatant was transferred into a sterile centrifuge tube and centrifuged at 10,000g for 10 minutes at 4°C to pellet the cilia. The supernatant was discarded and the pellet was resuspended in 20-500µL of HMEEN buffer (7.15 g/L HEPES, 1.46 g/L NaCl, 601.85 mg/L MgSO$_4$, 380.35 mg/L EGTA, 29.22 mg/L EDTA, adjusted to pH≈7.4) with proteinase inhibitors (final concentrations in solution of 1 µg/L Pepstatin A, 1µg/L Leupeptin, 174.2 mg/L PMSF, 18.595 mg/L Iodoacetic acid), depending on the starting amount of culture used. A final concentration of 1% IGPAL CA-630 was added to demembranate cilia for axoneme preparation. The sample was incubated...
at 4°C for 20-30 minutes with gentle rocking. The sample was then centrifuged at 10,000g for 10 minutes at 4°C. The supernatant was discarded and the axoneme-containing pellet was suspended in 20-200μL of HMEEN buffer with proteinase inhibitors, depending on starting volume used.

**Generation and Insertion of Target Gene into 2.1-TOPO Vector** – A polymerase-chain reaction (PCR) was performed using Taq DNA polymerase (New England BioLabs, Inc., Ipswich, MA), primers Pf16For1 and Pf16Rev1, and previously isolated genomic *Paramecium* DNA. Prior to the induction of RNAi in *Paramecium*, it is necessary to multiply the target by PCR and transform it into a stable vector. The following components were used for amplification:

- **Taq Polymerase (10X) Buffer** 5 µL
- 10 mM dNTPs 1 µL
- ddH2O 42.55 µL
- **Taq Polymerase (5000U/ml)** 0.25 µL
- Paramecium DNA 1 µL
- 10 µM Pf16For1 Primer 0.3 µL
- 10 µM Pf16Rev1 Primer 0.3 µL
- 25 mM MgCl2 3 µL

The following protocol was used for the initial amplification:

1. 30s 95°C
2. 20s 95°C
3. 45s 54°C
4. 2min 68°C
5. Go to 2 29X (30X total)
6. 5min 68°C
7. Forever 4°C

Following PCR, the product size was verified with gel electrophoresis, which showed a band with the expected size. Insertion of the target gene into a stable vector was done following the protocol set forth by pCR 2.1-TOPO TA Cloning Kit (Invitrogen by Life Technologies,
Carlsbad, CA), which is based on insertion of the PCR product into the vector by topoisomerase which is attached near the vector’s insertion site.

**Transformation of pCR 2.1-TOPO-pf16 into Competent *E. coli* NEB5α** - *E. coli* cells were mixed with 2µL of the above cloning reaction and incubated on ice for 30 minutes. The mixture was then heat shocked at 42˚C for 30 seconds, without shaking. To recover the cells, 250µL of Super Optimal Broth with Catabolite repression (S.O.C.) media was added to the tube. The culture was incubated at 37˚C, with shaking, for 1 hour. An aliquot of the transformation as plated on Luria Broth (LB) plates containing X-Galactose (X-Gal)+100µg/ml Ampicillin (Amp)+12.5µg/ml Tetracycline (Tet) and incubated overnight at 37˚C. Colonies that contain the target insert appeared white or light blue in color, and were isolated to verify that the insert was correct using PCR and gel electrophoresis.

**Isolation of High Quality Plasmid DNA from *E. coli* NEB5α + pCR 2.1-TOPO-pf16 and from *E. coli* L4440 Vector** – High quality plasmid DNA was isolated from *E. coli* + 2.1-TOPO vector and *E. coli* L4440 using the protocol set forth by Qiagen Plasmid Purification Kits (Qiagen, Venlo, Limburg, Netherlands). In this case, both *E. coli* strains were NEB5α (New England Biolabs, Inc., Ipswich, MA; L4440 was a gift from Andrew Fire, Addgene #1654).

**Cloning of Gene Target into L4440 Plasmid and Transformation into HT115** – Two double restriction digest were performed with the high quality plasmid isolations of the 2.1-TOPO vector with pf16 target gene insert and with the L4440 vector using SacI-HF and NotI-HF (New
Cloning of Gene Target into L4440:

500 ng (2138 ng) purified pf16 antisense insert from 2.1-TOPO Vector
2 µL NEB4 (New England Biolabs, Inc., Ipswich, MA)
1 µL SacI-HF
1 µL NotI-HF
6 µL H₂O
0.2 µL BSA

The restriction digest was incubated at 37°C for approximately 3 hours. Gel electrophoresis was performed and the corresponding bands were cut from the gel and purified following the protocol set forth by Promega Wizard SV Kit (Promega, Madison, WI). A ligation was then prepared to fuse the pf16 antisense insert from the 2.1-TOPO Vector into the L4440 plasmid. T4 ligase and T4 ligation buffer (New England Biolabs, Inc., Ipswich, MA) was used where 0.1 of the total volume was T4 buffer and 0.05 of the total volume was T4 ligase. Ratios of insert:target were used as follows: 1:3, 1:1, and 3:1. The volume was adjusted with ddH₂O. Ligation was performed overnight at 16°C.

[The following is a modified protocol by Timmons, L. (2000)] Competent HT115 E. coli were grown in LB+Tetracycline (Tet) (12.5 mg/L) medium overnight at 37°C. From this culture, 1 mL was transferred into a fresh 100 mL LB+Tet volume and grown at 37°C while shaking. At an OD₆₀₀ of approximately 0.4, the culture was transferred into a centrifuge bottle and incubated on ice for 30 minutes. From here on out, the culture was kept cold. The culture was centrifuged at 8,000g for 10 minutes at 4°C. The supernatant was discarded and the pellet was resuspended in about 25 mL ice cold CaCl₂ (5.55 g/L). The mixture was centrifuged at 8,000g for 10 minutes at 4°C and the supernatant was discarded. The pellet was resuspended in 1 mL ice cold CaCl₂
and aliquoted. A ratio of 1:25 ligation sample:competent HT115 cells was prepared and incubated on ice for 30 minutes. Cells were heat shocked at 37°C for 1 minute and then placed on ice for 2 minutes. Cells were recovered with 1 mL LB and incubated for 1 hour, with shaking, at 37°C. LB+100mg/L Ampicillin (Amp)+12.5mg/L Tetracycline(Tet) plates were prepared and recovered samples were spread and grown at 37°C for 24-48 hours, depending of rate of growth. Single colonies were chosen and streaked on LB+Amp+Tet plates and grown at 37°C for 24-48 hours.

**Preparation of the *Paramecium* Cultures for Knockdown** – IWGM was inoculated with *E. aerogenes* and grown at 37°C overnight. The inoculate was allowed to cool to room temperature and 50 mL of starving *Paramecium* (not fed for at least 7 days, to favor autogamous reproduction) was added. The *Paramecium* culture was allowed to grow for 2 days. Note that for larger volumes, multiple inoculations were used where 50 mL of *Paramecium* was substituted for the previously grown culture. Inoculation with bacteria remained the same. After 2 days, cultures were then exchanged into fresh Dryl’s solution. Cultures passed through a funnel with the same custom filter mentioned above and then centrifuged in pear-shaped flasks for 3 minutes at approximately 500g. *Paramecium* were removed with a glass Pasteur pipette and temporarily placed in a sterile 50 mL conical tube. Once all *Paramecium* were removed from the IWGM culture, the entire volume was washed 3 times with 100 mL Dryl’s solution. Centrifugation and removal followed the same steps above. *Paramecium* were finally resuspended in an equal volume of Dryl’s solution as compared to the previous IWGM.
**Preparation of Bacterial Cultures for Knockdown** – Bacterial cultures were inoculated and grown in either LB (*E. aerogenes*) or LB+Amp+Tet (HT115) at 37°C overnight. A 1:100 dilution of inoculated:fresh LB/LB+Amp+Tet was made and incubated at 37°C with shaking until $OD_{600}$ was between 0.3 and 0.6. IPTG was added (125 mg/L) and the culture was grown for 4 more hours.

**Paramecium Knockdown** – [process of knocking down *Paramecium* genes using RNAi is adapted from a protocol set forth by Beisson et al. (2012) and Valentine et al. (2012)] Induced bacteria expressing the RNAi construct were centrifuged at 3000g for 10 minutes. The supernatant was removed and the pellet was resuspended in 100 mL Dryl’s solution. The solution was centrifuged again and the supernatant was removed. The pellet was resuspended in a final volume of 50 mL Dryl’s solution. $OD_{600}$ was taken and equal amounts of cells were added to the corresponding culture. After feeding the *Paramecium*, IPTG (125 mg/L), stigmasterol (8 mg/L), and Ampicillin (100 mg/L) were added to the cultures. For the wild type culture, only stigmasterol was added.

Before feeding, 1 mL of *Paramecium* culture was added into a plastic cuvette observed under a stereomicroscope (Olympus SZX10, Upper Saucon, PA) at 0.6x under dark field illumination. Recordings were taken using Infinity Capture software and Infinity 1 Camera (Lumenera Corporation, Ottowa, ON). After feeding, new bacterial cultures were inoculated following the protocol above. The steps above were repeated 2 more times for a complete 48-hour knockdown.
**Rescue of the *Paramecium* Knockdown** – An *E. aerogenes* culture was inoculated the night of the final knockdown and grown overnight at 37˚C without shaking. A small aliquot of *Paramecium* culture was isolated and placed into a test tube for rescue. In multiple centrifuge tubes, the *E. aerogenes* culture was pelleted at 10,000 rpm for 1 minute and the supernatant was discarded. This process repeated until the whole culture was centrifuged. The pellet was resuspended in 1 mL Dryl’s solution and centrifuged at 10,000 rpm for 1 minute. The supernatant was discarded and the aliquots were combined after resuspension in a small amount of Dryl’s solution. The culture was washed in 500 µL Dryl’s solution and centrifuged at 10,000 rpm for 1 minute. The supernatant was discarded and the pellet resuspended in 1 mL of Dryl’s solution. *Paramecium* were fed 200 µL aliquots of this culture. The above steps were repeated one more time for a 48-hour (2 days of feeding) rescue (same time as the knockdown). Recordings of the cultures were taken under a stereomicroscope as described above.

**Fluorescence Imaging of *Paramecium*** – A small aliquot of cells were taken from the culture and centrifuged in pear-shaped flasks for 3 minutes at approximately 500g. A glass Pasteur pipette was used to remove the *Paramecium* from the flask and the cells were placed into a microcentrifuge tube. The cells were resuspended in 1 mL PBS (8 g/L NaCl, 0.2 g/L KCl, 1.44 g/L Na₂HPO₄, 0.24 g/L KH₂PO₄, pH=7.4) and centrifuged at 10,000 rpm for 1 minute. The supernatant was aspirated off and 200 µL PBS was added. Microscope slides were washed with ethanol and air dried. One drop of 0.1% Poly-L Lysine was added and incubated at room temperature for 15 minutes. The drop was removed with a pipette and the slide left to dry. The slides were then placed in a kimwipe lined box until samples were ready to be added.
One drop of cells were added to the dry microscope slide and incubated for 10 minutes at room temperature. The rest of the drop was then removed with a pipette. A drop of PBS with 1% IGPAL was added and incubated at room temperature for 5 minutes. The liquid was removed with a pipette and the slide washed with PBS. A drop of PBS with 4% formaldehyde was added and incubated for 10 minutes at room temperature. Then the remaining liquid was removed with a pipette and the slide washed with PBS. One drop of a 1:200 dilution of primary antibody (mouse anti-acetylated α-tubulin) in PBS with 5% BSA (bovine serum albumin) was added and incubated in a covered box for 1 hour. The slide was washed 3 times in PBS and one drop of secondary antibody (anti-mouse conjugated to FITC) at a 1:400 dilution was added and incubated at room temperature for 1 hour. The slide was then washed with PBS and one drop of PermaFluor (Thermo Scientific, Waltham, MA) was added, along with a glass coverslip. The slide was imaged on a Marianas Spinning Disk Confocal Microscope (Intelligent Imaging Innovations, Inc., Denver, CO) with a 488 nm 50 mW laser and SlideBook software (Intelligent Imaging Innovations, Inc., Denver CO).

**High Speed Imaging of Paramecium** – Paramecium cells were prepared for imaging by splitting a small culture into aliquots. To generate a small sample chamber on the glass objective slide for the fairly thick Paramecium cells, double-sided tape was oriented in a parallel fashion on a microscope slide, before placed a cover slip on the tape and gently pressing it down (making a small flow-chamber). A small amount of culture was pipetted under the cover slip and a UV cure resin (Norland Optical Adhesive, Cranbury, NJ) was applied to seal the chamber and avoid evaporation. After the resin cured, cells were imaged and recorded with 400 frames per second using a Phantom v9.0 camera (Vision Research Inc., Wayne, NJ) at 40X magnification with a
phase contrast objective. Recording was done using software provided by Vision Research (Vision Research Inc., Wayne, NJ).

**Analysis of Paramecium Movement** – Movies recorded at the stereomicroscope to document swimming speed were imported into FIJI (Schindelin et al., 2012). The movie was then cropped to focus on the target area, minimizing glare from the outside of the cuvette, and a Z projection was made out of a 2.5 second (25 frame) segment of the movie. Using the segmented line tool provided by FIJI (Schindelin et al., 2012), 20 tracks were measured for length in pixels. Extremely small or smudged tracks were not analyzed in an attempt to separate moving Paramecium from floating stigmasterol particles in the buffer. A 2-Tailed T Test was performed for a statistical comparison of all the samples.

**Preparation of Grids for Electron Microscopy Imaging** – Quantifoil Cu 200 mesh, grids coated with holey (R2/2) carbon film (Quantifoil Micro Tools, Jena, Germany) were soaked in a petri dish filled with ethyl acetate over night. Prior to use, grids were removed with anticapillary tweezers and dipped in fresh ethyl acetate. Grids were then dried by touching the edge of the grid with filter paper. A glass microscope slide was wrapped in Parafilm (Bemis, NA, Neenah, WI) and the grids were placed on the slide after completely drying. Grids were then glow discharged at -40 mA for 30 seconds and placed back in a petri dish. During this process, the carbon side of the grid is always facing up. Grids are then suspended by holding the edge with anticapillary tweezers. A drop of 10 nm colloidal gold (Sigma, St. Louis, MO) solution was added to the carbon side of the grid and the grid-tweezer combination was covered loosely and
left to dry overnight. Then, the grids were washed by dipping them in fresh Milli-Q H$_2$O twice and drying with a piece of filter paper. Grids were covered again and left overnight to dry.

**Freezing Samples on Microscopy Grids** – A 10 nm colloidal gold solution was prepared for freezing by taking a portion of 10 nm colloidal gold stock solution (Sigma, St. Louis, MO) (15 µL per sample) and centrifuging the solution at max speed of a table centrifuge in the cold room (4°C) for 20 minutes. Approximately 90% of the liquid was removed by pipette and the rest was used to resuspend the gold at a 10X concentration.

To prevent shearing, all samples were pipetted using cut tips. Axonemes prepared previously (in Dryl’s solution) were checked for concentration by negative staining with 2% uranyl acetate solution. Axonemes in solution were diluted accordingly so that axonemes would be properly spread throughout the grid and in the holes of the carbon film to best imaging.

While grids were drying, a home-made plunge-freezing device was cooled with liquid nitrogen. A custom made grid box was placed into the liquid nitrogen. It is important to note that liquid nitrogen levels must not fall below 75% to allow for adequate temperature control. Once bubbling ceases, ethane was liquefied in the central cup. A thin layer of frozen ethane was allowed to form within the central cup to signify that ethane was adequately cool. A grid and tweezers were then manually mounted into the plunger and 3 µL of sample was pipetted onto the grid. Then, 1 µL of the previously prepared 10X colloidal gold solution was added. A blotting time of approximately 1.5 to 2 seconds was used by folding filter paper in a bow and gently touching the grid. At the end of the blotting, the grid was plunged into the liquid ethane by the instrument. The tweezers were then detached, making sure the grid always stayed fully submerged in the liquid ethane. The grid was then quickly transferred to the liquid nitrogen and
placed into the grid box before storage in liquid nitrogen until imaging in the cryo-electron microscope.

**Imaging of Paramecium Samples Using Cryo-Electron Tomography** – [Modified from Heuser et al. (2009)] A cryo-transfer holder (Gatan, Inc.) was used to load samples into a Tecnai F30 transmission electron microscope (FEI, Inc., Pleasanton, CA) where it was kept below devitrification temperature at all times. Electron micrographs were recorded using a 2k x 2k charge-coupled device camera (Gatan, Inc.). The microscope was equipped with a field-emission gun, high tilt stage, and post column energy filter (Gatan, Inc.). Axonemes were identified and screened throughout the process based on how intact and compressed the structure looked, as well as orientation on the carbon grid. Single axis tilt series of 60-80 images were recorded over a tilt range of -65° to +65°, in 1-2 degree increments. The instrument was operated at 300 keV in low dose mode in zero loss mode of the energy filter. Acquisition throughout the microscopy process was aided by the control program SerialEM (Mastronarde, 2005).

**Reconstruction of the Paramecium Tomograms** – [Reconstruction followed previously set forth protocols from Nicastro et al. (2006) and Heuser et al. (2009)] Tomograms of Paramecium axonemes were reconstructed, analyzed, and modeled in the IMOD software package (Kremer et. al, 1996). Distortion and X-ray correction were performed using previously created files generated during prior calibration of the microscope and camera, along with manual adjustments allowed within the IMOD software. Fiducial markers (10 nm gold particles) were used for alignment of the 2D images for reconstruction into a raw 3D tomogram by weighted
backprojection. All images were processed according to this protocol and within the IMOD software.

**Averaging of Previously Reconstructed Tomograms** – [Averaging utilized protocols previously set forth by Nicastro et. al (2006)] Following reconstruction of the *Paramecium* axonemes, models of the CPC repeat units were generated by following the path of the C1 and C2 microtubules of the CPC along the longitudinal length of the axoneme. Unique models were generated for each reconstruction for both wild-type and mutant. Using the PEET (Particle Estimation for Electron Tomography) software package (Nicastro et. al, 2006), the subtomographic volumes containing the CPC repeats were aligned and averaged. Averages were run in triplicate with an incremental reduction on the search range, from the range of [-20:2:20] (-20 to 20 in steps of 2) to [-10:1:10] to [-3:1:3]. First, a wild-type average of the CPC was generated using the *Chlamydomonas* CPC as a reference (Carbajal-González et al., 2013). This *Paramecium* wild-type CPC average was then used as a reference for the rest of the averaging.
Results

Fluorescent Images Showed No Difference in Ciliary Coverage between Wildtype and Knockdown Cells – As shown in figures 1 and 2, there seems to be no discernable difference between the amounts of cilia present on the body of Paramecium between wild type/control and knockdown mutant cells. Coverage seemed to be about equal throughout the entire surface of the Paramecia; however, occasionally some “bald spots” are observed in all samples, which are likely sample preparation artifacts. During the imaging process, broken or damaged Paramecium cells could be observed, indicating that the sample preparation conditions could have been slightly too harsh.

Figure 1 and 2: Fluorescence microscopy images using anti-acetylated tubulin antibody to specifically visualize cilia (green) show that Paramecium cells from both the control (1) and the
knockdown (2) sample taken approximately 64 hours into the knockdown experiment have full ciliary coverage along the body of the organism, showing no qualitative difference between control and knockdown.

**High Speed Video Shows Varying Degrees of Paralysis** – High speed video taken of the wild type, control, and knockdown samples showed some differences in the ciliary beating, but overall the observed differences were variable and difficult to quantify. A general observation that was made throughout all the samples was that the cilia located on the posterior end of the cells showed very little movement except when the *Paramecium* attempted to switch direction.

As expected, there was also no discernable difference between the ciliary beating patterns of the wild type and the control cells (with empty RNAi vector). Both *Paramecium* samples seemed to beat with about the same frequency and moved at relatively the same speed throughout the medium.

During observation of the knockdown sample, it was noted that some of the cilia seemed to beat at lower frequencies than the wild type and control cilia. However, this was not an all-encompassing effect, with a majority of the cilia seeming to beat with a similar frequency to both the wild type and control. We observed what seemed to be a gradient of paralysis with regard to the cilia, where some subsamples were less paralyzed than others. This did have an effect on swim speed overall and the knockdown cells were observed to be moving at a slower rate than the wild type or control cells.

**Z Projections From Microscopy Movies Show Slower Swimming Motion for Knockdown Cells** – Z projections of 2.5 second segments of movies showed a difference in movement between the control, wild type, and knockdown *Paramecium* samples. Starting at 22 hours
(Figure 3), and extending to 68 hours (Figure 5), into the experiment, Z projections show that the *Paramecium* in the knockdown samples have motion tracks that appear to be thicker and shorter (Figures 3A, 4A, and 5A) than either of the wild type or the control cells (Figures 3B-C, 4B-C, 5B-C). The shorter the track the less movement occurred in the 2.5 second segment. This lack of movement or local tumbling could also contribute to the thickness of the line, as the control and the wild type *Paramecium* samples show thinner motion tracks. Overall, it seems as if there is a decreased capacity for movement in the knockdown cells when compared to the wild type or control. Both the wild type and control show similar movements throughout the experiments.

Occasionally very short and thick tracks (see Figure 4C) appear throughout all the samples and were not analyzed due to the inability to tell whether or not these were actual *Paramecium* cells or specks of stigmasterol flakes randomly floating throughout the otherwise clear medium.

Upon a genetic rescue using *E. aerogenes* feeding, movement seems to be similar throughout all the different samples (Figure 6), including restored motility in the rescued knockdown cells. Tracks from the knockdown sample appear to be similar in width and length when compared to both the wild type and the control. This suggests a recovered motility for the *Paramecium*. 
Figure 3: The Z-projections show the movement of single *Paramecium* during a 2.5 second segment of movie taken at the 22 hour mark of the experiment. Both 3B (control) and 3C (wild type) have similar motions and population, with many thin and long lines, indicating long swimming distances over the averaged time interval. In contrast in 3A (knockdown) the tracks are shorter and the solution is less densely populated. The lines are thicker, showing less movement over the allotted time.
Figure 4: The Z projections show the movement of a single *Paramecium* over a 2.5 second segment of movie taken at the 44 hour mark of the experiment. Both 4B (control) and 4C (wild type) have similar motions and populations, with many thin and long lines indicating long swimming distances over the averaged time interval. In contrast, in 4A (knockdown), the solution is less densely populated with shorter, thicker tracks, showing a decreased movement over the allotted time.
Figure 5: The Z projections show the movement of a single *Paramecium* over a 2.5 second segment of movie taken at the 68 hour mark of the experiment. Both 5B (control) and 5C (wild type) have similar motions, with many thin and long lines indicating long swimming distances over the averaged time interval. Their population densities seem to differ. In contrast, in 5A (knockdown), the solution shows single *Paramecium* with shorter, thicker tracks, showing a decreased movement over the allotted time. The density appears to be similar.
Figure 6: The Z projections show the movement of a single *Paramecium* over a 2.5 second segment of movie taken at the Rescue mark of the experiment. All samples, 6A, 6B, and 6C, have similar motions and populations, with many thin and long lines indicating long swimming distances over the averaged time interval. In contrast with the above figures, this indicates an apparent recovery of motility in the knockdown *Paramecium*. 
Measurements of Movement Show a Statistical Difference Between Knockdown and Wild Type/Control Samples – Statistical analyses of the microscopy motion tracks show that the differences in swimming between the samples increased with the length of the knockdown experiment. Table 1 shows that the samples are not very statistically different at 22 hours knockdown, with the only difference actually being observed between control and wild type cells. Whereas there seems to be no significant difference between wild type and knockdown or control and knockdown.

In contrast, at 44 and 68 hours, there is a statistical difference between the knockdown samples and the wild type, as well as the control sample. At both time-points, the difference is highly significant and suggests a decrease in motility of the knockdown cells. The wild type and control both remain statistically similar. Table 2 shows the difference in motility when comparing all 60 motion tracks taken, and merging the three time-points (22, 44, and 68 hours). This produces a very clear difference between the knockdown and the wild type/control, while also showing the similarity of the control and the wild type.

At the Rescue time-point, the average distance traveled is not statistically different between the wild type, control, and rescued knockdown. When comparing the difference between the different time-point samples and the Rescue, Table 3 shows that there is a difference in average distance traveled in the 44 and 68 hour knockdown samples, as shown in Tables 1 and 2. There is no difference between the rescued and experimental Paramecium throughout the other time-points, with a small inconsistency in the measurements for the 22 hour wild type sample. This difference between the knockdown before and after rescue suggests a recovery of motility in the rescued cells.
Figures 7 and 8 show the same decrease in average distance traveled in the knockdown. Throughout the samples, there seems to be a high degree of deviation from the mean, but Figure 7 shows that the most noticeable difference is observed for the knockdown sample at the 44 and 68 hour time-points of the experiment. Likewise, Figure 8 shows that the knockdown sample never approaches either the 22 hour or Rescue time-point distances, with a decreasing average distance traveled until the Rescue time-point.

Figure 9 shows the normalized average distance traveled as percentage of the wild type Paramecium. In the 44 and 68 hour time-points, the knockdown sample has its average distance traveled by approximately 60% when compared to the wild type. The control sample swims farther, on average, or very close to the wild type distance throughout the three time points. At the Rescue time-point, it can be seen that wild type and the knockdown swim for approximately the same average distance in 2.5 seconds with the control returning to its baseline 120% of the wild type. The variance, calculated to measure the error, was very low throughout the samples.

As shown above, the statistical analyses show that there is a clear difference in the average distance traveled by the knockdown sample when compared to the wild type and the control. Furthermore, they show that there is a recovery of motility when the knockdown sample is subjected to a genetic rescue through the feeding with E. aerogenes. The data also shows that the knockdown does not show a decreased average distance traveled until after the 22 hour time-point, suggesting a slow onset of the knockdown effect.
Table 1: shows 2 Tail T-Tests performed for each time point of the experiment to correlate the statistical significance of the differences measured in the motion tracks. At 22 hours, the knockdown is statistically similar to all other samples, where the only difference comes from a comparison of the wild type and the control. However, at 44 hours and 68 hours, there is a statistically significant difference between the knockdown and both the wild type and control, where both the wild type and control are highly similar. At the Rescue time-point, all samples seem to be statistically similar, suggesting recovery of motility.
Table 2: shows the statistical difference between the three samples when all 60 samples are considered, averaging all time point for each sample. Statistically, there is a difference between the wild type and the knockdown and the control and the knockdown, suggesting a difference in average distance traveled in a 2.5 second period. Likewise, there seems to be no statistically significant difference between the wild type and the control.

<table>
<thead>
<tr>
<th>Key</th>
<th>Samples (n=60)</th>
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<tbody>
<tr>
<td>Wt = Wild Type</td>
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<tr>
<td>Ctrl = Control</td>
<td>Ctrl-Kd</td>
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</tr>
<tr>
<td>Kd = Knockdown</td>
<td>Wt-Ctrl</td>
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</table>
Table 3: shows the statistical difference at different sample time-points when compared to the Rescue time-point. Overall, there seems to be a statistical difference seen in the average distance traveled in the knockdown 44 and 68 hour samples when compared to the knockdown Rescue time-point. The wild type at 22 hours is barely statistically insignificant but is in accordance to other analysis above. This table suggests a recovery of motility in the knockdown during rescue.

<table>
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<th>Time-point</th>
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<tr>
<td></td>
<td>Ctrl-Rescued Ctrl</td>
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<td></td>
<td>Wt-Rescued Wt</td>
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<td></td>
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Figure 7: The diagrams show the average swim distance in millimeters travelled for *Paramecium* cells within a 2.5 second time interval of the recorded movies. The average track length was calculated and plotted in comparison to the other samples. At 22 hours, there is no difference in track length, but as the experiment progresses a difference appears between the knockdown and the other samples. At the Rescue time-point, motility seems to have been recovered. Asterisks denote the statistical difference of the knockdown compared to the other samples.
Figure 8: The diagrams show the average swim distance in millimeters travelled for *Paramecium* cells within a 2.5 second time interval of the recorded movies and grouped by sample. The average track length was calculated and plotted in comparison to the other samples. At 22 hours, there is no difference in track length, but as the experiment progresses a difference appears between the knockdown and the other samples. At the Rescue time-point, motility seems to have been recovered. Note the descending nature of the knockdown sample as the experiment progresses, before the Rescue time-point.
Figure 9: The diagrams show the average swim distance in pixels travelled for *Paramecium* cells within a 2.5 second time interval of the recorded movies. The average track length was calculated, normalized by the wild type sample swim distance, and plotted in comparison to the other samples. At 22 hours, there is no difference in track length, but as the experiment progresses a difference appears between the knockdown and the other samples. At the Rescue time-point, motility seems to have been recovered. The Control sample seems to normally be swimming at a longer distance, with only the 68 hour mark distances being less than the wild type. Asterisks denote the statistical difference of the knockdown when compared to the other samples, prior to the Rescue time-point.
**Cryo-Electron Tomography Shows a Possible Structural Difference in the C1 projections of the CPC Between Knockdown and Wild Type**– Cryo-EM imaging and preliminary subtomogram averaging of the axonemal CPC showed a possible difference between the wild type and knockdown *Paramecium* cells. After reconstructing the C1 microtubule, a possible defect of the C1a an/or C1c projections of the CPC was observed in the knockdown axonemes. Figures 10A and 10B show that there seems to be a density missing from the middle of the two projections, with C1a marked with a red arrow and the area in question marked with a blue arrow. Overall, the C1a seems less dense in the knockdown than in the wild type and could suggest a structural defect. However, in the surface rendering (Figure 11), the defect is less noticeable. Figure 11A shows the wild type CPC with the C1a projection marked with a red arrow. Compared to Figure 11B, which shows the knockdown CPC with a red arrow indicating the C1a projection and a blue arrow indicating an area with less mass, the defect is not as clearly visible.

Currently, the subtomogram averages are still preliminary and require further improvement and verification to exclude modeling and alignment errors during the averaging and visualization (e.g. threshold and dust removal during isosurface rendering in the Chimera software seems to play a role in the differences in Figure 11).
Figure 10: Tomographic slices of the averaged CPC of the *Paramecium* wild type (A) and knockdown (B) samples. The averages are made up of 335 (A) and 354 (B) separate repeat units, respectively, and focus on the projections of the C1 microtubule. The C1a projection (marked with red arrows) seems to show a reduced density in the knockdown (B), suggesting a structural defect. An additional difference between wild type and knockdown appears to localize to the region of the C1c projection (blue arrow).

Figure 11: Isosurface rendering of the 3D averaged CPC in wild type (A) and knockdown (B) cilia. The C1a projection is marked with red arrows in both images and the area of the missing mass in question is marked with the blue arrow in Figure 4B. The difference in the images is not as striking as in the tomographic slices (Figure 10).
Discussion

**Ciliary Coverage and Motility** – Overall, the knockdown of *Paramecium* proved to be quick and effective. In the span of approximately three days, a knockdown mutant was generated and analyzed in relation to the wild type and control cells.

We expected that due to destabilization of the CPC in the PF16 knockdown, which was previously shown by Smith and Lefebvre (1996) and Straschil et al. (2010), the ciliary structure and function would be affected, possibly with a paralyzed cilia phenotype. We also hypothesized that the ciliary length and/or coverage of the cell surface of *Paramecium* could be affected in the knockdown, with there being less cilia overall.

Fluorescence microscopy showed that there was almost no difference between the ciliary length and coverage on the control and the knockdown cells. Although we had some difficulty with the specimen preservation due to the harshness of the preparation, it seemed as if ciliary length and coverage, overall, did not change for either sample.

Using high-speed video recording we found that the beating patterns varied between different samples, however, the data were also heterogeneous. In one case, we found that the ciliary movement of the knockdown did not differ from the wild type or the control, whereas in other cases, we saw that there were varying amounts of cilia paralyzed in the knockdown when compared against itself and the other two samples.
Due to the differing numbers of cilia paralyzed by the knockdown, we hypothesize that the knockdown has a varying effect on the *Paramecium*. Unlike the results shown by Kamath et al. (2000), it may be that the ingestion of dsRNA to induce RNAi may not be as robust as previously thought or the visible/functional effects of the knockdown of ciliary proteins is delayed because manifestation of the defect requires protein turnover in exciting cilia. One cause for this observation may also be that the *Paramecium* cells that have the most severe knockdowns cannot swim, as per the recognized phenotype (Dutcher et al., 1984). Furthermore, those that are paralyzed may not be able to feed on the bacteria being added to the culture due to oral groove cilia being rendered immotile. Whereas cells that are not as paralyzed or not as affected initially will still be able to feed on the bacteria as they are added in later time points; meanwhile cells with paralyzed cilia may become partially rescued due to a lack of dsRNA intake and thus RNAi induction, as shown in *C. elegans* (Timmons and Fire, 1998). Therefore, we may be seeing *Paramecium* that are in different stages of knockdown in terms of effect but with none, or very few, exhibiting the complete, paralyzed phenotype. Agitating the culture would be a way to control for *Paramecium* that cannot feed and cannot swim but previous (and our) experiments showed that shaking a culture, even at a slow speed, stressed the *Paramecium* to the point of death (unpublished data).

**Swim Distance Analysis Shows Decreased Movement in the Knockdown** – As shown in Tables 1-3 and Figures 3-9, there is a noticeable and quantifiable difference between the swimming distance of the three cultures (wild type, control, and knockdown) measured over specific time intervals. Furthermore, the above figures and tables show that swim distance could be recovered following a genetic rescue. These results show that *pfI6* is important for the motility of
Paramecium, though it does not paralyze the organism as previously shown in other studies with C. reinhardtii and P. berghei (Smith and Lefebvre, 1996; Straschil et al., 2010).

Our data also confirm the rescue result shown by Timmons and Fire (1998) in knockdown samples fed regular bacteria. Figure 8 and Table 3 show that, in the rescued knockdown, the swim distance approaches that of the wild type and is statistically different from that of the knockdown samples before the Rescue. This genetic rescue lends credibility to the effect of a genetic knockdown in Paramecium and its ability to have a quick, lasting effect on the organism.

One thing that we noticed was that the wild type was behaving abnormally in the 22 hour sample when compared with the later 44 and 68 hour samples, the controls and the Rescue. There was a noticeable difference between the swim distance of the wild type and the control, while not with the knockdown. This difference may be due to the fact that the culture of the wild type was not “happy.” In general, the Paramecium may have been stressed at that point when compared to the other samples due to centrifugation. However, this stress seems to disappear in later time points.

**Preliminary Structural Analysis is still Inconclusive Regarding Localization of the defect and PF16 Protein** – In the tomographic slices shown in Figure 10, there seems to be a density missing in the vicinity of the C1a or C1e/C1c on the C1 microtubule of the knockdown cilia. Given that pf16 has not been localized specifically in previous studies, this seems to provide further evidence on the link between pf16 and the C1 microtubule of the CPC.

However, as shown in Figure 11, there is a high degree of symmetry between the two sides of the C1 microtubule. During tomogram reconstruction and the averaging process, a
model of the CPC must be generated for the averaging program. During this process, it is important to have all models of the CPC to be going in the same direction, either proximal to distal or vice versa. This directionality is usually dependent on the orientation of the radial spokes and can be determined through merely looking at their orientation on the axoneme. In the raw tomograms reconstructed of the *Paramecium* axonemes, it was hard to recognize the directionality of the axoneme and this could have contributed to an inadvertent summarization of the CPC average. Because the C1 microtubule has distinct projections coming off of it, averaging particles of different orientation can wash out structures that we would otherwise want to see. This would provide a symmetrical looking C1 microtubule where the C1a and C1b projections look identical, when they otherwise should not. Although we can see a faint difference in Figures 10 and 11 when comparing the knockdown to the wild type, it provides neither definitive nor conclusive evidence towards the location of *pf16* and further analysis will be required.

Furthermore, the structural phenotype is different compared to the phenotypes shown by classical electron microscopy in cilia of other protists (Smith and Lefebvre, 1996; Straschil et al., 2010). Both studies showed that in the majority of the *pf16* mutant cilia the C1 microtubule was missing completely (Smith and Lefebvre, 1996; Straschil et al., 2010). In the majority of our reconstructed tomograms, the C1 microtubule was present; specifically out of 11 tomograms, only one had the C1 microtubule missing, but it was uncertain as to whether or not the C1 microtubule was missing due to the knockdown or because the axoneme was extremely compressed by ice when grid preparations took place.

The presence of the C1 microtubule in *Paramecium pf16* knockdown cilia could be due to a few things. One possibility is that, as stated previously, the effect of the knockdown was not
complete, and therefore, did not affect the *Paramecium* ciliary structure as severely. A weak response to the knockdown could be to blame for the presence of the C1 in the knockdown culture. Another possibility is that there are interactions between the C1/CPC and the outer DMTs. It is known that there are interactions between the radial spokes and the CPC and that the projections coming off of the individual C1 and C2 microtubules may play a role in the beating and signaling of the cilium (Smith and Yang, 2004; Carbajal-González et al., 2013). These interactions could have a stabilizing effect with respect to the CPC and its individual components. While we could not definitively show where the defect is, the C1a and C1b projections, the longest of the C1, are shown to be intact, and these could be important in keeping the C1 in place, even through de-membranation. More work needs to be done in analyzing the structure of the CPC, as well as the rest of the axoneme.

**Future Directions** – In order to determine whether or not *Paramecium* can be considered useful for ciliary studies and genetic manipulation in concurrence with these studies, more work is required.

Because *pf16* affects the motility of *Paramecium* cells, we would like to see if there are any adverse affects in the mutants. In the future, we want to quantify the number of cells in each sample at different time points, before motility analysis, in order to see if cells died during the experiment. This would also be important for determining if the buffer used for the knockdown affects the growth and the stability of the cultures throughout the different time points.

We would also like to perform an analysis on the effect of *pf16* on the *Paramecium’s* ability to feed. Using nigrosin (India Ink), the vacuoles of the *Paramecium* would turn black
upon ingestion of the dye. As stated above, there may be an effect on the oral groove cilia of *Paramecium* and that may be to blame for the varying degrees of knockdown.

Another analysis we would like to perform is to see how *Paramecium* may be able to rescue itself in the absence of bacteria (or the lack of the feeding capability of *Paramecium*). Because it may be possible that those unable to feed on bacteria can partially rescue themselves from the knockdown, we would like to have a culture that goes through the knockdown but, upon the Rescue time point, would be fed no bacteria. Their motility would be analyzed as above and, could shed some light as to what is happening in the culture.

We would like to continue the structural analysis by taking more tilt series for the tomogram reconstruction and averaging. A lot of the data that we had used to analyze the structure was compressed axonemes, which affects the overall composition and structure of the CPC. Also, more particles can be used in the averaging step to provide a higher resolution structure for comparison. We would also like to revisit the averages and models that we had already generated and reconfirm the directionality of the model and the parameters chosen in the averaging process. In reanalyzing the CPC averages, we would also like to visualize the projections of the C2 microtubule and possibly see if there are any interactions that may be present between the two components of the CPC. Also, we also need to average the DMTs of the axoneme to control for possible additional defects and to characterize the interactions between the RS heads and the CPC in more detail. It may be the case that there are interactions stabilizing the C1 microtubule that are not present in other organisms that will cause the C1 microtubule to be present. In generating more tomograms, we may also be able to localize *pf16* accurately in the future.
Ideally, Western Blot analysis would be performed on the axonemal fractions to determine the levels of Pf16 protein in the various samples. We have obtained an antibody from a collaborator (Elizabeth Smith, Dartmouth College) that has previously been used in *C. reinhardtii*. We will test for cross-reactivity in *Paramecium* and then hope to be able to use this for *Paramecium* to show that there are varying levels of PF16 protein present. One possibility to refine our experiment and the Western Blot analysis, might be to let those cells who are not as severely affected by the knockdown disperse (i.e. swim away) after centrifugation and before isolation. By letting those disperse, we are hopeful that those severely affected by the knockdown are not able to swim from the bottom of the flask. Those that cannot disperse may have a more pronounced phenotype and, therefore, are the ones we would like to target. Last but not least, we also want to perform PCR analyses of the samples in order to quantify the levels of mRNA present in the *Paramecium* at the last time point (64 hour of knockdown) before genetic rescue.

Finally, changing the medium that *Paramecium* are cultured in may be a possibility. Using an axenic medium and soaking the *Paramecium* in dsRNA may be a way to overcome their inability to feed or adequately uptake the dsRNA to induce RNAi. However, bacteria would thrive in such a medium and may outcompete the *Paramecium* during both organisms’ respective growths. This medium, however, may not be cost effective and prove too unwieldy, with respect to preparation, to be considered in the long term.

*Viability of Paramecium as a Model Organism for RNAi and Ciliary Studies* – So far, our study is not completely conclusive if *Paramecium* can be a suitable candidate for combined genetic and ciliary studies. We have shown that, while we can show a decreased movement
phenotype in the organism, we cannot reconfirm those results found in more established organisms, such as *C. reinhardtii*, when looking at *pf16* (Smith and Lefebvre, 1996). Furthermore, we cannot reconfirm the structural defects shown in the CPC (Smith and Lefebvre, 1996; Straschil et al., 2010). Though a knockdown can be performed quickly, as compared to other organisms, the effect may not be robust enough for following structural and functional studies of the cilia.

While there is more work to be done regarding this organism, it remains a candidate for ciliary studies. *Paramecium* have been long considered a leading candidate for studies in many different biological fields. From its discovery in the late 1700s until the present, *Paramecium* have been used in a variety of biological and biochemical studies to help us understand cellular function and molecular mechanisms. However, to show that *Paramecium* are viable as a model organism in ciliary studies, a knockdown mechanism needs to be reliable. Our data does not, yet, show that this is the case in *Paramecium*. However, as stated above, the ease of knockdown is something that cannot be ignored, as is its ability to develop into a genetically homogenous culture.

In developing *Paramecium* as a model organism, our hope is that it can become a tool to help provide a better understanding of ciliary structure and function. While we are not at that level yet, we believe that there are only a few more steps left in providing other researchers the information needed to help develop ciliary studies in *Paramecium*. 
Bibliography


Supplement

Figure S1: Plasmid map of the pCR 2.1-TOPO vector used to house pf16 for PCR amplification. The pCR 2.1-TOPO vector was obtained from Invitrogen by Life Technologies (Carlsbad, CA).
Figure S2: Plasmid map of the L4440 vector used as a stable vector to house pf16 for transformation into *E. coli* HT115. L4440 was a gift from Andrew Fire (Addgene #1654).