FUNCTIONAL ANALYSIS OF ENKURIN, A NOVEL FLAGELLAR PROTEIN IMPLICATED IN MOTILITY, USING Chlamydomonas reinhardtii AS A MODEL ORGANISM

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By

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Approved:

______________________________
Professor Destin Heilman, Primary Advisor

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Abstract

Enkurin is a novel protein first detected in mammalian sperm, and is thought to contribute to the maintenance of Ca^{++} ion channels. It was first observed in the acrosome (head) of mammalian sperm cells, but further localization studies found that it is also located in the principle piece (middle section) of flagellum. The purpose of this study was to determine the role of flagellar Enkurin in motility using Chlamydomonas reinhardtii, a unicellular biflagellated alga which expresses a homolog of mammalian Enkurin, as a model organism. Artificial microRNA (amiRNA) was introduced via electroporation to Chlamydomonas organisms normally expressing Enkurin in an attempt to silence the Enkurin gene and knock down protein production. The transformed organisms were then cultured, and phototactic responses were measured. In addition, Enkurin transcript levels were measured via Q-PCR. Though some mutant phenotypes were observed when measuring phototaxis, no significant transcript knockdown was observed in the transformant strains.
Acknowledgements

Foremost, I would like to thank Professor D. Heilman, my Major Advisor, for advising this Major Qualifying Project. This study would not have been possible without his invaluable advice and constant support. I would also like to thank Dr. Harvey Florman (University of Massachusetts Medical School, Dept. of Cell Biology) for allowing me to work in his laboratory, and Keith Sutton, PhD for working closely with me and aiding my understanding of the experimental as well as theoretical portions of the project. Additionally, I would like to thank Melissa Jungnickel, PhD for her insight and guidance in the laboratory. I would also like to express my gratitude to the Witman Lab (University of Massachusetts Medical School, Dept. of Cell Biology) for providing me with Chlamydomonas organisms.

In addition, I am greatly indebted to my mother, my father, and my brother for understanding my erratic schedule and for providing constant encouragement and support as I worked on this project. I would also like to thank my roommates, Marina Varlamova and Giao (Kat) Tran for checking up on me, celebrating with me during my successes, and supporting me through my failures. Finally, I would like to acknowledge all my friends who also completed MQPs during this time; in particular, I would like to acknowledge Molly Congdon and Noah Cohen for constantly being available to brainstorm and commiserate.
Introduction

Eukaryotic flagellar structure

Unicellular organisms possess two major structures, flagella and cilia, to elicit motility. Cilia are multiple small, hairlike protrusions that beat to create movement, flagella are long, whiplike structures that undulate to propel the organism, and are generally smaller in number than cilia. Flagella and cilia are possessed by both eukaryotes and prokaryotes and operate using the same basic principles. While the ion flow and chemical gradients involved in flagellar motion have yet to be completely elucidated, the structure and organization of the proteins comprising eukaryotic flagella are fairly well characterized. It is currently known that flagella are composed of nine microtubule doublets arranged in a circle and held together by dynein arms, with a pair of microtubules in the center. Each microtubule doublet includes two types of microtubules, A and B. From each microtubule A on the outer ring of doublets protrudes a multi-protein structure known as the radial spoke, which are thought to be integral in generating motility. As a whole, the flagellar cytoskeleton is known as the axoneme. In order to fully understand the mechanism in which flagella generate motility, a detailed inspection of each part of the axoneme is required.
Detailed inspection of a flagellar cross section reveals that much of the axoneme is composed of microtubules. Microtubules are rigid, hollow tubular structures comprised of the small globular protein tubulin. In addition to composing much of the flagellar axoneme, microtubules can also be found in several other roles (such as in the axons of neurons) in which they provide structural integrity to the cell. Microtubules have several unique features which allow them incredible versatility; for example, their ability to polymerize and depolymerize very quickly allows rapid changes in the conformation of the cell or organelle. Tubulin, the protein from which microtubules are synthesized, exists

**Figure 1**

Cross section of a eukaryotic flagellum. The above picture depicts the main motor unit of the eukaryotic flagellum, known as the axoneme. The classic “9+2” microtubule structure (nine outer pairs of microtubules surrounding a central doublet can be seen as well as the dynein arms and radial spokes, both of which are thought to contribute to motility.)
in two forms: alpha and beta. Tubulin forms alpha-beta heterodimers, which form the monomer unit of microtubules.⁷ These alpha-beta heterodimers form long strands (known as protofilaments) which then arrange themselves into a sheet-like lattice. This lattice then curves around and closes on itself, forming a hollow tube. While a detailed mechanism for the polymerization of tubulin has not yet been elucidated, is thought that Guanine Triphosphate (GTP) plays a role in regulating the polymerization behavior of tubulin.¹⁰

While microtubules provide structure to the axoneme, additional proteins are required to generate motility in the flagellum. Dynein is one such protein integral to the movement of flagellar microtubules. Of the two existing forms of dynein (axonemal and cytosomal), only axonemal is found in cilia and flagella. Axonemal dynein is comprised of three main components: a coiled portion, (stalk), a long chain that attaches to a neighboring microtubule system (stem), and a heavy chain with an ATPase containing motor unit. The heavy chain is generally located between the stalk and the stem, and arranges itself into a globular head region.¹⁰

![Dynein arm structure](image)

**Figure 2**
ATP binding to the heavy chain elicits a conformational change in the dynein; this causes the microtubules to slide against each other and generates the bending motion needed for flagellar movement\textsuperscript{15}.

Dynein arms, which are the main contributors in providing force of movement to flagella, all have the same basic structure and are found along the outside of the axoneme, as well as within it.\textsuperscript{11} While the outer arm is fairly homogeneous, the inner arm shows more diversity in types of dynein and in spatial distribution of the arms themselves. The two sets of arms also seem to perform different functions in providing the flagella with motility.\textsuperscript{11} Studies of organisms lacking the outer arm have shown that while velocity is greatly reduced, the pattern of beating (known as the waveform) remains fairly constant. Conversely, mutants lacking the inner dynein arm maintained rapid flagellar velocity, but the waveform and pattern of movement was observed to be more erratic.\textsuperscript{11}

While it is known that dynein arms produce and maintain the mechanical force required for the microtubules to slide, what controls dynein activity is yet unclear. Radial spokes are, however, thought to be an important step in activating and controlling dynein arms.\textsuperscript{19} Radial spokes are T-shaped multimers which attach to the A microtubule of each microtubule doublet. Each radial spoke is comprised of a thin “stalk” portion, which attaches to microtubule A and extends inwards towards the central microtubule doublet.\textsuperscript{19} At the end of each stalk is a spoke head; these spoke heads interact with the central pair of microtubules in the axoneme. It is thought that approximately seventeen proteins compose the entire radial spoke, with about approximately five comprising the stalk, and approximately twelve comprising the head. It was also found that in addition to
the seventeen proteins that compose the radial spoke, calmodulin, a calcium channel regulating protein, is also present in the stalk portion of the spoke.\textsuperscript{19} It is thought that calcium ions binding to calmodulin causes a conformational change in the radial spoke, thus changing the spoke’s interaction with the central doublet and allowing a steady pattern of movement. In the study of organisms possessing malformed radial spokes or lacking them altogether, a loss of efficient flagellar waveform was observed. These findings are significant because they indicate the importance of radial spokes and calcium gradients in maintaining flagellar motility.\textsuperscript{19}

To provide the necessary energy to generate motion in the flagellum, organisms utilize multiple molecular motor systems. A molecular rotary motor is utilized in order to generate a spinning motion, which allows the flagellum to reach maximal velocity.\textsuperscript{3} Similar to other biological rotary motor systems (such as the F\textsubscript{1}F\textsubscript{0} ATPase utilized in cellular respiration), the flagellar system is comprised of two main portions: the motor (moving portion) and the stator (static portion). While bacteria and eukaryotic cells rely on different ions (dependent on the environment in which each organism inhabits), it can generally be said that changes in ion gradients drive the hydrolysis of ATP. Eukaryotic flagella possess one main such motor at the base of the flagellum.\textsuperscript{3} In addition to the large rotary motor which provides the flagellum with spinning motion, the heavy chain on each dynein arm in the axoneme contains ATPases, which provide the energy to elicit bending motion. This bending motion is what provides the flagellum its constant waveform.\textsuperscript{10}
The importance of Ca++ channels, Calmodulin, and cAMP to flagellar motility

Calcium++ channels have been known to be very important to mammalian cell function in many instances, such as muscle contraction and fertilization. Calmodulin is a protein employed by the cell to regulate calcium ion movement across the cell membrane; it is found in multiple loci in mammalian sperm, and is thought to be important in multiple facets of fertilization. In order for a mammalian sperm cell to be able to fertilize an oocyte, a process known as capacitation must occur. During capacitation, molecules such as cholesterol and other glycoproteins are removed from the acrosome (tip) of the sperm, resulting in a more permeable membrane. At this stage, calmodulin regulates a Ca++ influx, increasing motility. Thus, the presence of calmodulin in the acrosome of the cell is important in the fertilization process. When guinea pig sperm was tested for calmodulin presence, it was observed in four main loci (the tip of the head, the middle of the head, the base of the tail, and the tip of the tail). While calmodulin presence in the head was expected due to the necessity of a calcium gradient in the process of capacitation, calmodulin presence in the tail of the sperm showed that calcium ions gradients are not only necessary for the acrosome reaction portion of fertilization, but also in sperm motility.

Cyclic AMP (cAMP) has also shown to work in conjunction with Ca++ channels to allow motility in mammalian flagella. When sperm were placed in a calcium ion free environment, it was shown that the flagella curved into a circular shape, prohibiting them from swimming efficiently or creating a discernable waveform. It was seen that when extracellular Ca++ levels were low, intracellular cAMP levels were also low and the
sperm was immotile; however, when treated with only a cAMP analog, the flagella were able to form steady beating patterns and motility was re-established. It was thus shown that Ca ++ levels are essential to motility as calcium ions regulate intracellular concentrations of cAMP, which, in turn, directly affect motility.6

TRP Channels

TRP channels, or transient receptor potential channels, are selectively permeable ion channels that establish an ion gradient. TRP channels can be permeable to several types of ions, including protons, calcium ions, sodium ions, and magnesium ions, among others.1 TRP channels have eight hydrophobic regions in their primary structures which potentially form transmembrane helices. While TRP channels were first discovered when studying phototransduction in Drosophila, it has since been found that several homologs exist in mammalian cells, all involved in signal transduction pathways.17

TRP channels participate in a heterotrimeric G protein pathway known as the phosphoinositide pathway. In this pathway, a heterotrimeric G protein known as Gqα binds to and activates an enzyme known as Phospholipase C. Phospholipase C generates two secondary messengers: inositol triphosphate (IP3), and diacyl glycerol (DAG). The inositol triphosphate pathway leads to a phosphorylation cascade, ending with the binding of calmodulin and subsequent activation of Ca ++ channels, such as the TRP family. 1 There are seven sub-families of TRP channels that exist in mammals; of these, TRPC (canonical transient receptor potential channels) are involved in signal transduction within sperm cells. 17
TRP C and its role in the mammalian fertilization process

TRPC channels have been identified as particular subunits of many Phospholipase C dependent Ca^{++} channels. Several types of TRPC channels have been identified by looking at variants of the TRPC gene. TRPC channels have been identified in a number of species, including lower organisms such as nematodes. While there have been several differing models of TRPC structure, it is generally agreed that TRPC is the closest vertebrate homolog to the original TRP gene found in Drosophila. TRPC channels have six transmembrane regions, with one section that is hypothesized to be the pore through which calcium ions pass. It has also been observed that four TRPC proteins must complex to form a functional TRPC channel. There are seven types of mammalian TRPC proteins (named TRPC 1-7) and can be placed in four distinct subgroups based on structure and function: TRPC1, TRPC2, TRPC 3, 6, 7, and TRPC 4 and 5. TRPC 2 has been implicated not only in the mechanism of fertilization, but also in the olfactory signal transduction process. TRPC 2 has also generated much interest in the research community due to the fact that it may be a store-operated calcium channel. Store operated calcium channels are thought to be calcium channels which open and close in response to intracellular Ca^{++} concentration; however, no conclusive evidence exists to prove that TRPC 2 is one of these channels.

TRP C channels have been seen to be important in the mammalian fertilization process. In order for the head (acrosome) of mammalian sperm to be able to interact with mammalian eggs, a secretory event involving Ca ++ channels must occur for the acrosome to penetrate the zona pellucida, the extracellular matrix of the egg. This
reaction is known as the acrosome reaction. The mechanism for this reaction involves ZP3, a surface glycoprotein found on mammalian eggs. When ZP3 is activated, signal transduction involving heterotrimeric G proteins occurs, leading to a Ca\(^{++}\) influx and an elevation of pH. The Ca\(^{++}\) influx into the sperm is thought to have a direct effect on the occurrence of a secretory event that leads to the acrosome reaction, which allows the sperm to fuse with the egg. It was noted that when TRPC2 was affected with an antibody, it was shown to inhibit acrosome reaction activity. In order to determine this, synthetic antibodies were created corresponding to residues 664-686 (known as RDAS) and 831-852 (known as ADVE) in the amino acid sequence for mouse testis TRPC2. Upon tagging, the distribution of the protein could be determined; both the anti RDAS and the ADVE were seen in the anterior head and to a lesser degree, in the posterior head. Tests in a cell line which did not endogenously produce TRPC2 channels showed that when the anti-RDAS inhibitor was not present, the inward Ca\(^{++}\) ion current was far larger than TRPC2 channels affected with the anti-RDAS inhibitor. These findings lead to the conclusion that that the presence of TRPC2 channel inhibitors greatly affect the inward current and thus affect the Ca\(^{++}\) influx into the cell.

The specific signal transduction pathway leading to the activation of TRPC 2 channels is yet unknown for sure. It is known definitively that TRPC channels are activated by some downstream product of Phospholipase C (an enzyme that hydrolyzes lipids in order to form second messengers) activity, which is part of the phosphoinositide signal transduction pathway. Phospholipase C generally acts on PIP\(_2\) (phosphoinositol 4,5 bisphosphate), which is then cleaved into IP\(_3\) (inositol triphosphate) and DAG (diacyl
While it is not entirely clear which pathway TRPC channels are activated (it is thought that each type of TRPC channel may utilize a different method of activation) preliminary studies have found that some TRPC channels may be activated by internal Ca\(^{++}\) depletion, while others may utilize the DAG second messenger system.\(^1\)

TRPC 2, while a member of the TRPC family of calcium channels, has several important differences from the six other TRPC members.\(^{14}\) Firstly, it has largely been implicated in olfactory signal transduction (though more recently, it has also been implicated in the fertilization process). It has been found almost exclusively in the olfactory microvilli of mammals, and does not oligomerize with TRPC proteins; instead, it complexes as homotetramers to form transmembrane Ca\(^{++}\) channels.\(^{14}\) The primary structure of both the N terminus and the C terminus of TRPC 2 indicates that protein-protein interactions are possible; for example, the protein calmodulin has the ability to interact with TRPC 2 at its N terminus. In addition, the phosphoinositol pathway second messenger IP\(_3\) has been shown to associate with the C terminus of TRPC 2.\(^{14}\) While the majority of Ca\(^{++}\) movement across cell membranes has been attributed to TRPC channels (TRPC 2 in particular), there are several other proteins that may be involved in generating Ca\(^{++}\) ion gradients. One such novel protein found in mammalian sperm cells is Enkulin.

**Enkulin: A novel protein implicated in flagellar motility**

Enkulin is thought to be a calmodulin-binding protein by analysis of its primary sequence. It is a small, lysine-rich, soluble protein, with no predicted transmembrane
regions. Enkurin also contains a proline rich amino terminus that is the probable ligand for an SH3 protein domain, most probably in the p85 regulatory region of the 1-phosphatidylinositol-3 kinase. Upon testing for the presence of Enkurin in various tissue types, very small quantities of the protein were found in somatic tissues and mouse ovary tissues; in contrast, high concentrations of Enkurin were found in the tissues of the mouse testis as well as sperm cells. It was also hypothesized that Enkurin may accumulate in acidic environments; therefore, it is thought that Enkurin participates in a multiprotien complex involved in the acrosome reaction in sperm cells.

**Enkurin and its interactions with TRP C channels**

In studying the function of the TRPC2 channel and its effects on the ZP3- Ca\(^{++}\) influx, it was noted that a heterologous system left a functional end of the TRPC2 channel completely blocked and thus inhibited; however, experimentally, it was found that only 80-85% of the current was diminished in these systems. This showed that TRPC2 did not act alone in establishing a Ca\(^{++}\) gradient. When different members of the TRPC channel family were mapped in mouse sperm, it was found that TRPC1 (in the acrosomal head) TRPC5 (in the acrosomal head), TRPC3 (in the posterior head and principle piece of the flagellum), and Enkurin (both in the acrosomal head and the principal piece of the flagellum) existed as well.

Enkurin has been seen to associate with the N terminus of TRPC 1, 2, and 5, but not of TRPC 3. Enkurin has also been shown to bind calmodulin in manner dependent on the concentration of Ca\(^{++}\). What is still not known about acrosome reaction and TRPC
activity is how Phospholipase C affects Ca\(^{++}\) influx, and what signal transducer elements are involved in converting a Ca\(^{++}\) influx to the secretory event.\(^{17}\)

It has been hypothesized that Enkurin, due to its interactions with TRPC channels, is somehow integral to the motility of sperm and the proper movement of flagella. In order to elucidate the specific purpose of Enkurin, this study will seek to create Enkurin knockdowns via amiRNA, using *Chlamydomonas reinhardtii* as a model organism. By knocking down Enkurin expression levels, we hope to generate and characterize a mutant phenotype. This phenotype will aid us in understanding the purpose of Enkurin in eukaryotic flagella.

**Using RNAi as a tool to characterize protein function**

In order to better understand the function of a gene in an organism, a tool known RNA interference (RNAi) can be implemented. RNAi refers to a method in which the RNA for some gene is modified in order to control its levels of expression. One of the most widespread methods of RNAi gene silencing is the use of artificial microRNA (amiRNA).\(^{2}\) microRNA (miRNA) are small strands of RNA, generally \(~22\) nucleotides long, that can be used to silence genes. miRNAs rise from \(~70\) nucleotide regions in single stranded RNAs of any length. The region that is to become the precursor to miRNA folds over itself, forming a hairpin loop generally containing some mismatched base pairs. At this point, the nuclear protein Pasha recognizes the hairpin, and nuclear enzyme Drosha cleaves hairpin at its base, releasing it from the rest of the longer RNA strand. The hairpin loop is then transferred to the cytoplasm of the cell, where the enzyme
Dicer removes the loop portion of the hairpin, yielding double stranded miRNA known as a miRNA duplex. At this point, one of the now mature miRNA strands (generally the more thermodynamically unstable strand) is incorporated into the RNA-Induced Silencing Complex (RISC), where it is bound by a protein known as Argonaut. Argonaut orients the miRNA such that it is able to bind to the target mRNA. mRNA bound to miRNA is more likely to be targeted for degradation, leading to lower levels of gene expression.

**Chlamydomonas reinhardtii as a model organism**

*Chlamydomonas reinhardtii*, a biflagellated eukaryotic photosynthetic alga, is often used as a model organism in studies of mammalian flagella. Just as other organisms utilize their flagella in to find and move into nutrient-rich areas (known as chemotaxis), Chlamydomonas uses its flagella to move into areas of optimal light (known as phototaxis). While the reason for taxis may differ between mammalian sperm cells and algae, the signal transduction pathway that follows is analogous. The Chlamydomonas flagellum utilizes intraflagellar Ca++ concentrations (via channels such as TRP C channels, as discussed above), in order to change their flagellar beating patterns, which in turn leads to the cell changing its motility pattern. In addition to possessing the same structure as mammalian flagella, Chlamydomonas flagella were also found to contain many of the same proteins (including Enkurin) which are thought to regulate mammalian flagellar motion also regulate Chlamydomonas flagellar motion.
Though they possess the same flagellar structure as mammalian sperm cells, Chlamydomonas organisms prove a far more efficient model for study than do mammalian cells. Firstly, the full genome sequence of Chlamydomonas is readily available, allowing them to be easily characterized. Secondly, Chlamydomonas cells possess cell walls, making them robust and thus allowing several methods of transformation. Finally, the time it takes to produce a new generation of Chlamydomonas cells is vastly shorter than the time it takes to produce a new generation of mice from which to extract sperm. Thus, Chlamydomonas reinhardtii proves itself the optimal model organism when studying eukaryotic flagellar proteins.
Materials and Methods

Screening the existing genomic library for Enkurin mutants.

Genomic PCR was performed on an existing genomic library in order to find pre-existing Enkurin mutants. The primers that were used were as follows:

**Table 1:** Genomic DNA library primers

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sense</th>
<th>Anti-sense</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primer 1</td>
<td>5'CTTATTCTTGCCTCAGTACTCT3'</td>
<td>5'CAAAGTCAAACCTGGGCAGGATG3'</td>
</tr>
<tr>
<td>Primer 2</td>
<td>5'CGTACCGGCGTTCGTCG3'</td>
<td>5'GACATGTGATATGATTTACAC3'</td>
</tr>
<tr>
<td>Primer 3</td>
<td>5'CAGCAGCTGTGGGCGGACCTC3'</td>
<td>5'TTCACAGCACTGCTCCACTCT3'</td>
</tr>
<tr>
<td>Primer 4</td>
<td>5'CCATCGGGCTGCCTCTATCTC3'</td>
<td>5'GCCGGGCCCTCTCTCCATCT3'</td>
</tr>
<tr>
<td>Primer 5</td>
<td>5'TGCCGGCGCTCTCAGCTCGTT3'</td>
<td>5'TCCCCTTCTTGTCGACGACATCACC3'</td>
</tr>
<tr>
<td>Primer 6</td>
<td>5'CCGGCGCCTGGCCAGCAACC3'</td>
<td>5'CCGGCAACGTCAGCACGCAAGCAAGA3'</td>
</tr>
</tbody>
</table>

The PCR protocol was as follows: Initially 95°C for 15 min, then 40 cycles [58°C 30 sec | 72 °C 30 sec] [95 °C 30 sec].

Creation of amiRNA constructs

The RNAi constructs were created by creating artificial microRNA (amiRNA) following the “dsDNA Oligonucleotide cloning for amiRNA Expression” protocol (Molnar et al, 2008). The Enkurin gene was analyzed for miRNA target sites, and three amiRNA hairpins were created by combining oligonucleotides with the following PCR primers:
### Table 2: amiRNA PCR primer sequences

<table>
<thead>
<tr>
<th>Construct</th>
<th>Forward Sequence</th>
<th>Reverse Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Construct A</td>
<td>5’CTAGTGTGCAGCATGAGGCAATT TTAT CTTCGCTGATCGGCACCATGGGGGTGGTG CCGATCAGCGCTATAAATATTGCCTCA TG CGCAC3'</td>
<td>5’CTAGCGTGCGCATGAGGCAATATTTAT AGCGCTGATCACCACCACCCCCCATGGT GGTCATCAGCGAGATAAAAATTTGCTCAT GCAC3'</td>
</tr>
<tr>
<td>Construct B</td>
<td>5’CTAGTGTGCAGAATTACCCCTGAGTAAT CTCGCTGATCGGCCACCATGGGGGTGGTG GGTGATCAGCGCTATAAATATTGCCTCA TG CGCAC3'</td>
<td>5’CTAGCGTGCGAATTACCCCTGAGTAAT AGCGCTGATCACCACCACCCCCCATGGT GGTCATCAGCGAGATAAAAATTTGCTCAT GCAC3'</td>
</tr>
<tr>
<td>Construct C</td>
<td>5’CTAGTAGGCGAATGCGTATAGGTAGAT CTCGCTGATCGGCACCATGGGGGTGGTG GGTGATCAGCGCTATAAATATTGCCTCA TG CGCAC3'</td>
<td>5’CTAGCGTGCGAATGCGTATAGGTAGAT AGCGCTGATCACCACCACCCCCCATGGT GGTCATCAGCGAGATAAAAATTTGCTCAT GCAC3'</td>
</tr>
</tbody>
</table>

(Gene analysis and amiRNA primer design performed by K. Sutton, Florman Lab, University of Massachusetts Medical School Dept. of Cell Biology). The PCR parameters were: 38 cycles [92°C 30 sec| 58 °C 30 sec|72 ° C 30 sec]. PCR products were ligated into a digested, dephosphorylated kanamycin-resistant plasmid. The plasmid was then transfected into competent E. coli cells. Select colonies were cultured, and the DNA was extracted using the Quiagen Plasmid Prep kit.
Transformation of *Chlamydomonas reinhardtii*

amiRNA construct-containing plasmids were transferred to *Chlamydomonas reinhardtii* via electroporation using a BTX Electro Square Porator and following the “Protocol for Electroporating Chlamydomonas Cells” (adapted by Jason Brown, Witman Lab, UMass Medical School from “Chlamydomonas Electroporation Protocol”, Moroney, JV lab, LSU). G1 strain Chlamydomonas cells were cultured in a light room in ~100 mL TAPS + 40 mM sucrose media (TAPS buffer obtained from Witman Lab, University of Massachusetts Medical School Dept. of Cell Biology, TAPS CAS number: [29915-38-6]) for up to a week, or until media appeared medium to dark green. Cells were transferred into 500 mL centrifuge tubes and centrifuged at 7,000 g for 30 min at 19°C. Supernatant was removed and cells were suspended in 1.0 mL TAPS + 40mM sucrose media. 20 µL of cells were placed in a hemocytometer and counted. 0.8 µL cells were then placed in each electroporation cuvet with the desired quantity of plasmid. Cuvets were incubated on ice for 5 minutes, electroporated at the desired voltage and pulse length and incubated on ice again for 15 min. Electroporation parameters were as follows:

**Table 3: Electroporator Settings**

<table>
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<tr>
<th>RNAi construct</th>
<th>Cuvet</th>
<th>Plasmid concentration (µg)</th>
<th>Voltage</th>
<th>Time (ms)</th>
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Electroporated cells were extracted from cuvets by rinsing each cuvet with 1 mL TAPS + 40 mM sucrose media. Re-suspended cells were placed in centrifuge tubes and 4 additional mL media was added. Cells were incubated overnight on a rocker under a light source. After overnight incubation transformed organisms were plated on 0.1% kanamycin/agarose plates. Select colonies were cultured and phototaxis was monitored.

**RNA extraction from transformed cells**

0.5 mL cells were taken from culture and placed in microcentrifuge tubes. Three equivalents of TRIzol reagent (purchased from Invitrogen) were added for every equivalent of cells. Cells were then centrifuged at 13,200 rpm and 4°C for 15 minutes. The aqueous phase was transferred to a fresh tube. One equivalent of a solution of phenol/chloroform/isoamyl alcohol (25:24:1) per original volume of cells was added to the aqueous phase. The solution was incubated on ice for 20 min and allowed to separate. The aqueous phase was then transferred to a fresh tube and one equivalent of isopropyl alcohol per original volume of cells was added. The solution allowed to precipitate at -80°C for one hour. The solution was then centrifuged for 30 min at 13,200
rpm and 4°C. The resulting pellet was washed in 70% ethanol (centrifuged for 10 min at
at 13,200 rpm and 4°C) and allowed to air-dry for two hours. RNA was re-suspended in
50µL RNAse-free water. Extracted RNA was used to perform q-PCR (q-PCR performed
by K. Sutton, Florman Lab, University of Massachusetts Medical School Dept. of Cell
Biology) to determine Enkurin transcript levels.

**Synthesizing Enkurin for study**

In order to synthesize Enkurin, *Chlamydomonas reinhardtii* cDNA was first used
to amplify the Enkurin gene via PCR. An EcoRI site (underlined) was engineered into
each primer (sense primer: 5' AAAGAATTCAGGA TGCAGGAGGAGTCTGGT3',
antisense primer: 5'AAAGAATTCTCAATCGTCCACC ACCAGCAC 3'). PCR
parameters were: 38 cycles [92ºC 30 sec| 58 ºC 30 sec|72 º C 30 sec]. The gene was then
inserted into an EcoRI digested, dephosphorylated pThioHis A plasmid (purchased from
Invitrogen), which was transformed into StrataClone Solo Pack Competent Cells
following the “StrataClone Blunt PCR Cloning Kit”. The competent cells were then
plated on .1% LB/Ampicillin plates, each with 40 microliters 2% X-gal. Select white
colonies were then cultured in .1% LB/Ampicillin medium, and DNA was extracted
using the Qiagen Miniprep Kit, and following the “QIAprep Spin Miniprep Kit Using a
Microcentrifuge”. Currently, extracted DNA is being sequenced to verify the proper
orientation of the ligated insert.
Results

Analysis of Existing Genomic Library for Enkurin mutants

To begin analysis of Enkurin mutants, an existing *Chlamydomonas reinhardtii* genomic library was analyzed to determine whether any existing Enkurin mutants were available for study. The genomic library (as provided by Witman Lab, University of Massachusetts Medical School Dept. of Cell Biology) was analyzed via genomic PCR (genomic PCR and analysis performed by K. Sutton, Florman Lab, University of Massachusetts Medical School Dept. of Cell Biology) with the primers listed in Table 1. The intent of this genomic PCR was to check the genomic library for any existing Enkurin mutants. Gel electrophoresis was performed on those samples whose heating curves appeared abnormal. While some genomic DNA samples tested with primers 4 and 5 showed small disturbances in the heating curve, electrophoresis showed no difference in DNA fragment lengths between those samples whose heating curves seemed disturbed and those whose seemed normal. Therefore, it was determined that there were no existing versions of Enkurin whose sequence lengths deviated from that of the wild type. Sequencing of the DNA samples that showed small disturbances in the heating curve would have shown any existing sequence variants in the genomic library.

Transforming Chlamydomonas reinhardtii

When choosing the most effective method of transforming Chlamydomonas organisms, their cell walls had to be considered. As Chlamydomonas cell walls are thick, rigid, protective structures, techniques such as heat-shocking to induce transformation
would have been significantly difficult to perform; even if attempted, the likelihood of obtaining a sizeable number of cells that had successfully taken up the introduced plasmid was unlikely. Therefore, a method of transformation was needed that would be significantly more forceful than heat shocking, while maintaining precision. Electroporation was decided upon as the preferred method of transforming Chlamydomonas organisms expressing cell walls because it provided a precise enough method to apply voltage to the cells without killing all the organisms intended for transformation, while still being a forceful method of compromising the cells enough to allow the amiRNA containing plasmid to diffuse in.

The electroporator used to transform Chlamydomonas cells was the BTX Electro Square Porator. When using electroporators, it is normal for a certain number of cells to be killed due to the exposure to high levels of electricity. For the BTX Electro Square Porator, it is generally accepted that the optimal percent of non-viable cells is 50%; that is, when about 50% of cells are killed by the electroporation technique, the largest possible number of live cells have taken up the introduced plasmid. In order to determine the number of viable cells at different conditions, a kill curve was made. Cells suspended in media (TAPS +40 mM sucrose) were added to electroporator cuvets, and various voltages were passed through the cells for various pulse durations. After electroporation was completed, the cells were stained with 0.1% Evans Blue dye, and the number of non-viable cells was determined using a hemocytometer by counting the number of cells that had taken up the dye. (Table 3, Figure 3) This assay showed that several of the tested conditions resulted in ~50% of cells killed by the applied voltage. However, when the
amiRNA hairpin containing plasmid was added to cells and the organisms were plated after electroporation, only the 400 V and 500 V conditions resulted in a large quantity of viable colonies. Thus, the optimal conditions for electroporation were determined to be 400 and 500 V at an 11 ms pulse. 0.5 μg, 1 μg, and 5 μg of each of the three amiRNA construct-containing plasmids was added to cuvets containing Chlamydomonas organisms (suspended in TAPS + 40 mM sucrose media) and porated. The porated cells were washed out of the cuvets and plated on .1% kanamycin agar plates, which were stored in a light room. Transformed colonies were then cultured in 2-4 μL of media for several weeks.

**Enkurin transcript level analysis**

While it can be generally said that the purpose of RNAi is to knock down regulate expression of genes, the manner in which this regulation occurs is highly dependent on the target site. Each of the amiRNA target sites that was chosen for Enkurin all existed in the 3’ untranslated region (UTR) of the gene. amiRNA directed towards the 3’UTR can either affect gene expression by degrading the target mRNA and thus knocking down transcription levels or by affecting the translational efficiency of the gene. In order to measure the extent to which the introduced amiRNA caused mRNA degradation, Enkurin mRNA transcript levels were measured in each transformed strain via q-PCR. In order to carry out q-PCR analysis, mRNA was extracted from each transformant strain as well as the G1 wild type strain, and Enkurin primers were added. (Primers provided and q-PCR performed by K. Sutton, Florman Lab, University of Massachusetts Medical School, Dept. of Cell Biology) By comparing the Enkurin mRNA amplification levels in the
transformant strain to the wild type strain, the level of transcript knockdown can be
determined. Enkurin transcript levels were first tested in the A5 and B1 transformant
strains. (Figures 4, 5, 6) The amplification curve shows clearly that the amplification
levels of wild type mRNA are nearly identical to those of the A5 and B1 strains,
suggesting no knockdown of Enkurin transcript in those transformant strains. q-PCR was
then performed on the rest of the available A strains. However, no significant Enkurin
transcript knockdown was seen in any of the A strains. Interestingly, Enkurin transcript
levels were consistently slightly higher in the B1 transformant strain than in the G1 wild
type strain.

Visual Analysis of amiRNA transformants

After the transformed colonies had been cultured in small quantities of media for
approximately one week, their phototactic behaviors were observed. It was hypothesized
that organisms that had successfully been affected with the introduced amiRNA would
exhibit taxis behaviors different from the wild type strain. The available cultures were
placed in a light room where the light source was placed behind the three well plates (that
is, closest to the A5 and B1 strains) for a period of one hour. The cells were monitored at
30 minute intervals to visually observe phototaxis. (Figure 7) The majority of G1 (wild
type) organisms were seen to immediately move towards the light source, and over the
course of the hour, all the cells had clustered in the portion of the well closest to the light
source. It was also seen that transformed strain B1 exhibited an immediate, robust,
positive phototactic response in a manner quite similar to the wild type strain.
Transformant strains B2, B3, and C1 all exhibited a significantly less dramatic phototactic than that of G1 or B; however, over the course of an hour, the cell density was observed to move slightly closer to the light source. It was hypothesized that the reason for this slight movement may have been a decreased level of Enkurin production, which would allow for some movement, but no movement as dramatic as that of the wild type strain. Finally, the A5 strain exhibited no visible taxis response when monitored over the course of the hour. When the cells were visually examined, it was also noted that the G1 wild type strain formed an even suspension in media, while the A5 cells settled to the bottom of the well and formed aggregates.

Due to the abnormal clumping behavior of the A5 strain, the G1 and A5 strain were compared on a cellular level. The cells from each strain were placed on slides and viewed using a compound light microscope (slides prepared and photos obtained by K. Sutton, Florman Lab, University of Massachusetts Medical School Dept. Cell Biology). (Figure 8) When viewed, the G1 strain cells were seen to posses features characteristic to all normal Chlamydomonas cells; that is, they were observed to be biflagellated, and possess cell walls. However, none of the A5 cells were seen to possess flagella; in addition, what appeared to be improper cell divisions were observed. While the inner membrane appeared to have divided completely, new cell walls had failed to form around daughter cells, leading to multiple membrane bound cells trapped within a single cell wall. It is likely that this phenomenon was the cause of the aggregation seen in the culture. The A4 amiRNA affected strain (which was not assayed for phototaxis) also exhibited the same aggregation behavior when suspended in media; when viewed under a
microscope, it exhibited a very similar phenotype to the A5 strain. However, instead of every cell wall encapsulating two membrane-bound cells (as seen in the A5 strain), each cell wall in the A4 strain contained 4 separate, membrane-bound cells.

**Synthesis of Enkurin for study**

The aim of this portion of the experiment was to synthesize wild type protein for antibody production. In order to produce Enkurin, Chlamydomonas cDNA was amplified via PCR using Enkurin primers. These primers were engineered such that EcoRI sites flanked the region desired for expression. These EcoRI sites allowed the insert to be ligated into a EcoRI digested plasmid. The PCR product was EcoRI digested and electrophoresed in a 0.8% agarose gel. The resulting band was approximately 0.7 kb, as expected. *(Figure 9)* The purified DNA was then T4 Polynucleotide Kinase (PNK) treated. A pThioHis A plasmid was then EcoRI digested and dephosphorylated using Calf Intestinal Phosphatase (CIP). The plasmid is now in the process of being transformed into competent E.coli cells. *(Figure 10)*
Table 4: Electroporation kill curve table

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Electroporation kill curve table. The above table shows the conditions used to generate the kill curve for the BTX Electro Square Porator. Cells were placed in the 0.8 mL cells were placed in the electroporator and porated with the above conditions. 0.01 mL cells were taken from each cuvet after the procedure and treated with 0.1% Evan’s Blue dye, and counted using a hemocytometer to determine the percentage of non-viable cells.

Electroporation kill curve. The above table shows the kill curve generated by the BTX Electro Square Porator, using the parameters given in Table 3. The greatest number of cells were killed when cells were exposed to 300 volts for 22 seconds, and the fewest number of cells were killed when the cells were exposed to 100 volts for 22 seconds.

Figure 3
q-PCR of A5 and B1 strains. q-PCR shows amplification levels of Enkurin transcript in the A5 and B1 strains. q-PCR was performed for 41 cycles. The B1 strain showed the highest amplification level (slightly higher than 0.35 ng). There was very little variance in the amplification levels; the A5 strain showed the lowest amplification levels (approximately 0.325 ng).
q-PCR of B1, A1, A2, A4, and A5 strains. q-PCR shows amplification levels of Enkurin transcript in all the existing A transformant strains as well as the B1 strain. q-PCR was performed for 41 cycles. The B1 strain showed the highest amplification level (slightly higher than 0.40 ng). There was very little variance in the amplification levels; the A4 strain showed the lowest amplification levels (slightly higher than 0.35 ng). The control consisted of the G1 (wild type) mRNA without added reverse transcriptase.
Figure 6. Q-PCR of B1, A4, and A5 strains. q-PCR shows amplification levels of Enkurin transcript in the A4, A5, and B1 transformant strains. q-PCR was performed for 41 cycles. The B1 strain showed the highest amplification level (approximately 0.36 ng). There was very little variance in the amplification levels; the A4 strain showed the lowest amplification levels (approximately 0.325 ng). The control consisted of the G1 mRNA without added reverse transcriptase.
Chlamydomonas phototaxis assay. Five strains of transformed Chlamydomonas (A5, B1, B2, B3, and C1) were placed in a 12-well plate along with the G1 wild type strain and placed in a light room for one hour. Cells were placed in every other well to avoid contamination. Figure A shows a photo of the cells immediately after being placed in the light room. Figure B shows the cells after 30 minutes, and figure C shows the cells after 60 minutes had elapsed.
**Figure 8**

**Chlamydomonas Organisms.** Organisms from the G1, A5, and A4 strains were viewed under a light microscope. The organisms from the G1 strain possess two flagella and are seen to be unicellular. It can be seen that the organisms from strain A5 and A4 strains have no flagella and up to 4 individual cells can be seen within one cell wall.
**Synthesis of Enkurin for study.** Figure 9 shows the Enkurin insert electrophoresed through agarose gel. Figure 9 shows a map of the cloning vector containing the Enkurin insert.
Discussion

Electroporation

Enkurin is a small, soluble protein found in the flagella of all *Chlamydomonas reinhardtii* organisms. In order to elucidate the specific function of Enkurin in the flagella, amiRNA gene silencing was utilized to attempt to knock down protein expression levels. In order to transfet wild type organisms with the amiRNA-containing plasmid, a technique known as electroporation (in which a voltage is applied to cells in order allow the desired plasmid to diffuse in) was employed. The electroporator used was the BTX Electro Square Porator. Overall, the electroporation technique proved fairly successful; after a few attempts to transform the organisms at different voltages, it was found that 400 V and 500 V provided the most viable colonies. However, it must be noted that the voltages which provided optimal transformation killed over half the cells in each cuvet. Therefore, while those cells that were transformed were successful, a very large number of cells were wasted. It was thus determined that some changes could be implemented to increase the efficacy of electroporation.

One of the simplest facets of electroporation that could be changed to provide more efficient transformation would be to change the parameters at which the cells were porated. Most of the transformed cells were porated at very high voltages (eg. 500 V) for 11 ms. This high voltage and low pulse lead to a surge in heat in the cuvet, and often caused the cells to appear to “boil”; in some instances, the pressure inside the cuvet became so great that the cuvet cap dislodged itself. Instead of applying this high voltage, a lower voltage and a longer pulse could be applied in the future. This would cause a
more even current and a minimal heat surge. This way, the Chlamydomonas cell walls would be compromised more slowly and in a more even manner. The lower voltage would also lead to a less violent reaction from the cells; thus, fewer of them would be wasted and more cells could take up the introduced plasmid.

Another method to increase the efficacy of electroporation would be to decrease the cell density in the cuvet. When a current is passed through cells, the resistance provided by the cell wall generates heat. Therefore, when a very large quantity of cells (e.g. on the order of $10^8$) was placed inside the cuvet, a large portion may have been killed by the heat generated due to resistance. If the cells were diluted further in an aqueous buffer, the smaller number of cells would result in less heat formation. In addition, the aqueous buffer would act as a heat sink, protecting the cells from further heat damage. This could reduce the number of cells destroyed, and possibly increase the efficiency with which the organisms take up the introduced plasmid.

**amiRNA knockdown**

In order to attempt to knock down Enkurin levels in Chlamydomonas cells, three amiRNA constructs were introduced via short hairpins, each targeting a different location on the 3’ untranslated region (UTR) of the Enkurin gene. q-PCR was then performed on the transformant mRNA to assess levels of Enkurin transcript knockdown. While the q-PCR did not show any significant Enkurin knockdown in the transformed strains, it cannot necessarily be said that the introduced amiRNA did not reach the proper target sequence. When amiRNAs are introduced to the untranslated region, they can affect
protein production by either causing the mRNA to be degraded, or by causing some translational modification, reducing protein production levels. \(^{20}\) It is thus possible that the introduced amiRNA targeted the proper sequence; however, if it caused translational modifications, no change would have been detected in q-PCR.

While it is highly possible that amiRNA introduction could have resulted in a translational modification, the possibility remains that the amiRNA missed the target sequence, resulting in the low levels of transcript knockdown. One reason for this may be due to the local mRNA structure of the Enkurin gene. Previous studies have found that the 3’ UTR of many genes forms T-shaped loops; if the target sequence was located in one of these structures, it would not have been exposed enough to associate with the amiRNA.\(^4\) Other previous studies attempting to knock down the expression of several genes via amiRNA introduction in Chlamydomonas organisms also saw that in general, Chlamydomonas mRNA is resistant to degradation by amiRNA targeting. While the specific reason for this resistance is yet unknown, it is thought that the structure of Chlamydomonas mRNA contribute to its hardiness.\(^{12}\)

**Phototaxis assay**

In order to understand how possible Enkurin protein knockdown affected the flagella of transformed strains, the tactic behavior of both the transformed strains and the wild type strain was observed. While the B2, B3, and C1 strains all exhibited decreased levels of phototaxis, the A5 strain showed absolutely no tactic behavior and when viewed closely, was seen to completely lack flagella. It was also seen that the A4 strain lacked
flagella, and both strains had lost the capability to undergo replication properly. If these phenotypes were indeed generated due to an Enkurin protein knockdown, it could be hypothesized that Enkurin not only serves as a protein important to motility, but also one important to development. It is also important to note that both a lack of flagella and replication defects were seen together in the A4 and A5 cells. These linked phenotypes could suggest that Enkurin may be a regulatory protein involved in both the expression of flagella and the formation of cell walls during replication.

**Synthesis of Enkurin for study**

While the knockdown of Enkurin in Chlamydomonas cells resulted in novel phenotypes, much characterization is still necessary in order to determine the specific function of Enkurin in the cell. In order to do this, it is important to synthesize Enkurin in an attempt to make an antibody to the cell. In order to carry this out, the gene must be ligated into a plasmid, which will then be transformed into E.coli cells. Once the E.coli begins to express Enkurin, antibodies can be obtained, and insight into the cause of the mutant phenotypes in the A4 and A5 strains will be apparent. By purifying protein from the transformed Chlamydomonas strains and performing a Western blot, protein knockdown levels in the transformed strains can be elucidated; it can thus be determined whether the generated phenotypes were due to lower levels of Enkurin expression or whether they should be attributed to other factors, such as endogenous mutations.
Future recommendations

After determining the role of Enkurin protein levels in generating the phenotypes seen in the A4 and A5 strains, several future avenues of study can be pursued to further understand the importance of Enkurin to the flagellum. One possible study would be to make a knockout organism by removing the Enkurin gene, and thus the protein from the flagellum. Motility defects in the mutant organisms could then be recorded, giving possible phenotypes for Enkurin knockouts. In order to confirm these knockouts, a rescue experiment could be performed; if Enkurin was re-introduced to the knockout organisms and normal flagellar function resumed, the mutant phenotypes could be definitively be attributed to a lack of Enkurin.

Currently, very little is known about which, if any, proteins Enkurin interacts with in the flagellum. Because flagellar proteins are so numerous, it is almost impossible to hypothesize which other proteins Enkurin is likely to interact with. However, several other flagellar proteins involved in motility defects have been studied and their mutant phenotypes have been documented. Therefore, once a motility defect for Enkurin is confirmed, it will be possible to make a more educated hypothesis as to which other flagellar proteins may be worthwhile to study in conjunction with Enkurin. By comparing the mutant phenotypes of Enkurin knockout organisms and the knockout organisms of other flagellar proteins, it may be possible to find a phenotypic correlation. This phenotypic correlation may prove valuable in hypothesizing with other proteins interact with Enkurin; the study of Enkurin in conjunction with these other proteins could then elucidate further the signal transduction pathway involved in flagellar motility.
References


12. **Molnar A, Bassett A, Thuenemann E, Schwach F, Karkare S, Ossowski S,**


