MIG-10 and CSN-5 interactions during nervous system development in C. elegans

Major Qualifying Project Report submitted to the Faculty of Worcester Polytechnic Institute in partial fulfillment of requirements for the Degree of Bachelor of Science

Submitted by:

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4/25/2013

Submitted to:

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Abstract

Mutations in genes involved in nervous system development can result in disorders such as Down’s syndrome and autism. MIG-10 is a cytoplasmic adaptor protein in the model system *C. elegans* that functions in neuronal migration and axon guidance during development. To study possible interactions between MIG-10 and candidate protein CSN-5, co-immunoprecipitation and western blotting were performed. An interaction between MIG-10 and CSN-5 was confirmed, suggesting that CSN-5 is needed to stabilize MIG-10.
Acknowledgements

I would like to thank Charu Jain for her assistance with maintaining cells and also additional help with the Co-IP and Westerns throughout my MQP. Also, I would like to thank the Duffy Lab for their cooperation with equipment and supplies.

Thank you, Erin Flaherty for her support while in lab and continued interest in my project. It made lab more enjoyable having a friendly face around.

I would especially like to thank Meagan Sullender and Erin Maloney for giving me a foundation in lab by training me so that I could do it on my own throughout my MQP. I’m especially thankful for the many answered questions and support they provided me throughout the year. And a special thank you to Meagan for the constructs.

A huge thank you goes to Liz Ryder for being such a dedicated advisor. I’m grateful for the time she spent answering my questions, helping me troubleshoot, and preparing me for my future career. She taught me how to be extremely efficient in lab which is an important trait I definitely will need throughout my career. It truly amazes me how she manages to do so much and keep everything straight (of course with some friendly reminders).
Introduction

The nervous system is a complex series of circuits that come together to allow organisms to feel sensations, think, and generate behaviors. A major component of the nervous system is the brain which through its billions of cells allows organisms to function. Within the brain lie neurons that send messages throughout the body. In order to understand how the nervous system functions, it is important to comprehend how it develops. The complicated interconnections of the nervous system cannot function properly if they do not form appropriately during the beginning of development (Sanes et al., 2005). Defects in nerve connections can lead to neurological disorders such as Down’s syndrome and autism. Further research in developmental neuroscience could lead to treatment of neurodegenerative diseases or injury caused by damaged neuronal connections (Quinn and Wadsworth, 2008). Additionally, inappropriate cell migration can lead to mental disorders, cancer, vascular diseases, or dysfunctional homeostatic processes. The study of molecular biology and biotechnology is critical to understanding these problems and developing an approach to solve them (Horwitz and Webb, 2003). Specifically, this project studied the MIG-10 protein, which functions in cell migration, and a potential interactor, CSN-5, which functions in protein stability and degradation among additional areas that continue to be researched.

Cell Migration, Axon Guidance, and Actin Polymerization

Developmental neurobiology is a large area of study. Two specific areas of interest are cell migration and axon guidance. Within the brain, histogenesis is of great importance for a properly functioning nervous system. Neurons and glial cells are formed during embryogenesis in one location and must be moved to their final location within the complex circuitry of the nervous system. Cell migration is a key element in this process (Sanes et al., 2005). Embryogenesis begins the process of forming the cells, and then the cells move to their target location where differentiation occurs into a particular cell type. The process of cell migration occurs as a result of signals which then cause polarization and protrusion of the cell. Phosphatidylinositol (3,4,5) trisphosphate (PIP₃) is an initial component of this pathway. The polarization and accumulation of PIP₃ leads to the activation of Rho GTPases. Rac, a member of the Rho family GTPases, is a regulator of actin polymerization which is important in the
protrusion of the cell during migration. In neurons, an important element in this process is the growth cone at the tip of the axon (Horwitz and Webb, 2003).

The circuitry of the nervous system is very complex, and it is important during the development of the neurons to make the right connections. Axon growth begins with growth cones which are located at the tip of the axon. Guidance cues are needed to activate the growth cone in order to move towards or away from a target location. Examples of guidance cues are netrins, slits, bone morphogenic proteins (BMPs), wnts, ephrins, and semaphorins. Asymmetric axon outgrowth is guided by actin and microtubules as a response to these guidance cues (Quinn and Wadsworth, 2008). Actin polymerization is guided by the extracellular cues functioning as attractants or repellants. This therefore directs the orientation of axon growth due to whether the growth cone is promoted or inhibited by the guidance cues (Quinn et al., 2006). The structure of the growth cone is shown in Figure 1. Lamellipodia are considered to be at the edges with filopodial projections extending outward. These components contain actin which is important in cell movement and guidance. Ena/VASP proteins are actin regulators which function in response to the attractive and repulsive guidance cues (Sanes et al., 2005). These proteins are important in actin polymerization and can be found at the leading edge of filopodia and lamellipodia of the growth cone (Quinn et al., 2006). The extension of actin filaments is promoted by Ena/VASP proteins which prevent capping proteins from terminating growth along the barbed ends of the actin (Chang et al., 2006).
C. elegans as a Model Organism

Model organisms are crucial to scientific research. *Caenorhabditis elegans* is a model organism often used in neurobiology. This nematode worm is easy to work with, reproduces rapidly, and has a small genome (Hart and Bernstein, 2000). *C. elegans* is especially useful within the study of neuroscience because of the animals’ simple nervous system composed of only 302 neurons and 56 glial cells (Sanes et al., 2005). These features allow for an easier study during scientific research. *C. elegans* and humans share similar genes providing a better understanding of humans without the process being time-consuming or unethical.
MIG-10 and its Role in Axon Guidance and Cell Migration

A particular gene of interest found in *C. elegans* that functions in cell migration and axon guidance is *mig-10*. MIG-10 is a member of the MRL adaptor molecules family sharing common features with other MRL member proteins such as Lamellipodin and RIAM (Rap1-GTP-interacting adaptor molecule). Lamellipodin is the human ortholog of *mig-10*. Multiple isoforms of lamellipodin have been identified, but the main form is 1,250 amino acids long (Coló *et al.*, 2012). Within the growth cone, lamellipodin colocalizes with Ena/VASP proteins stimulating F-actin formation (Sanes *et al.*, 2005). RIAM is important in actin polymerization and formation of lamellipodia in non-neuronal cells (Lafuente *et al.*, 2004).

The MRL family has two important domains centrally located within the protein. The first domain is a Ras-association (RA) domain, and the second is a pleckstrin-homology (PH) domain. Additionally, members of the MRL family have a proline-rich region at the C-terminus of the gene (Coló *et al.*, 2012). Specifically, MIG-10 has additional proline-rich regions at the N-terminus. A FPPPP (FP4) motif is found within the proline-rich region. Ena/VASP homology 1 (EVH1) domain of Ena/VASP proteins binds to the FP4 domain of the MRL protein (Chang *et al.*, 2006). RIAM has multiple proline-rich regions and six EVH1 binding motifs allowing interactions with Ena/VASP proteins (Lafuente *et al.*, 2004). Lamellipodin has four EVH1 binding motifs which allow the colocalization of lamellipodin and Ena/VASP proteins at the tip of the growth cone. These proteins work together in the polymerization of actin filaments (Krause *et al.*, 2004). The N-terminus of each protein contains a coiled-coil region. MIG-10 has three isoforms, MIG-10A, MIG-10B, and MIG-10C which differ in the lengths of their N-termini (Figure 2). The remaining portions of the isoforms are the same. MIG-10A is 667 amino acids in length while MIG-10B is 650 amino acids long. MIG-10C is the longest isoform. Research on MIG-10A and MIG-10B has been extensively conducted while research on MIG-10C is more limited (Coló *et al.*, 2012; McShea, 2011).
Research has shown that in *C. elegans*, mig-10 is important during embryogenesis and the cell migration of embryonic neurons. Two mutations of mig-10, ct41 and e2527, have been identified which result in the truncation of the excretory canal of the excretory cell within developing *C. elegans*. Shortened migration of CAN, ALM, and HSN embryonic neurons was also an effect in these two mutants (Manser *et al.*, 1997). In additional experiments, over-expression of MIG-10 in *C. elegans* AVM neuron, without guidance cues, has shown misguided axon outgrowth. Further experiments with the addition of guidance cues such as netrin and slit showed enhanced axon guidance (Quinn and Wadsworth, 2008).

Figure 3 provides a model of the role MIG-10 plays in cell migration and actin polymerization. In this example, netrin, a guidance cue, is recognized by its receptor UNC-40 at the membrane of the leading edge of the growth cone. This binding triggers the phosphorylation of PIP to PIP$_2$, and the activation of RAC (GTPase), which then recruit MIG-10 to the membrane. Once bound, MIG-10 can bind to other proteins which are involved in actin polymerization, axon guidance, or cell migration. Here, ABI-1 is displayed as an interactor of MIG-10 (Sullender, 2012). UNC-34 is the sole *C. elegans* homolog of the Ena/VASP proteins (Quinn *et al.*, 2006). In this signaling cascade, MIG-10 represents an effector protein which binds to RAC. The asymmetric activation of RAC and phosphorylation of PIP is what brings MIG-10 to the membrane and allows binding through the RAPH domain. As a result, lamellipodia and filopodia are formed asymmetrically during actin polymerization, allowing outgrowth in a particular direction (Quinn and Wadsworth, 2008).
Figure 3: Schematic of MIG-10 Pathway (McShea, 2011). The signaling cascade begins with a guidance cue, known to be UNC-6 in axon guidance (Quinn and Wadsworth, 2008). The activation of the UNC-40 receptor results in MIG-10 interacting with other proteins to promote actin polymerization.

ABI-1 and its Role in Actin Polymerization

A yeast two hybrid screen revealed Abl Interactor 1 (ABI-1) as a possible interactor with MIG-10 (Gosselin and O’Toole, 2008). Abi proteins have been shown to work together with adaptor proteins. ABI-1 is involved in cell movement through actin polymerization. Interactions of the WAVE-binding domain of ABI-1 with Wave-1 proteins results in ABI-1 localizing at the leading edge of the growth cone. The WAVE domain as well as the SNARE domain and the homeodomain homologous region (HHR) are located on the N-terminus of ABI-1. The C-terminus of ABI-1 contains proline-rich regions which make up the SH3 binding domains. Proteins binding to these domains are important in the localization of ABI-1 and its role in cell movement (Echarri et al., 2004). Figure 4 displays a visual of the ABI-1 domains.
Figure 4: ABI-1 Domains (McShea, 2011). The SNARE and HHR domains can be seen on the N-terminus of ABI-1. The SH3 binding domain can be seen on the C-terminus. The middle portion of ABI-1 contains serine and proline-rich regions.

Actin polymerization transforms the globular actin protein into a helical structure as shown in Figure 5 (Disanza et al., 2005). The helical filaments have a fast and a slow growing end which are affected by polarization. G-actin begins the process at the growing end (positive end). Then, ATP hydrolysis plays a key role as ATP-actin is converted to ADP-actin. This slow process ends with the depolymerization of the actin filaments. The Arp2/3 complex and the WAVE complex interact and localize at the plasma membrane to begin branching of actin filaments. Capping proteins attach to portions of the actin filaments after branching has begun to prevent further elongation (Disanza et al., 2005). ABI-1 binds to Wave-1 through its wave-binding domain at the amino terminus. Once bound, the complex can localize at the lamellipodium. Mutations in the wave-binding domain within ABI-1 prevent the localization of Wave-1 at the leading edge of the growth cone. Wave-1 is needed to activate Arp2/3 which initiates actin filament branching. If Wave-1 does not localize at the lamellipodium, actin polymerization will be disrupted (Echarri et al., 2004).
Interactions Between ABI-1 and MIG-10

Past research has been conducted displaying that MIG-10 binds ABI-1. Co-immunoprecipitation experiments have isolated specific regions of each protein to target the particular binding domains of the proteins. The SH3 domain of the C-terminus of ABI-1 has been shown to be important in binding MIG-10. As a comparison, the middle portion of ABI-1 lacking the SH3 domain was analyzed and did not bind MIG-10 (McShea et al., 2013). Preliminary co-immunoprecipitation experiments have been conducted to examine the possible interactions between the C-terminus or the middle portion (RAPH domain) of MIG-10 and ABI-1. These preliminary results have shown that the C-terminus as well as the RAPH domain of MIG-10 binds ABI-1. The N-terminus of MIG-10 has not been studied to examine its individual interactions with ABI-1 within axon guidance.
The COP9 signalosome (CSN) is a complex of eight subunits that is involved in protein stability, gene transcription, modulating signal transduction, cell-cycle control, and DNA-damage response (Shackleford and Claret, 2010). One particular subunit of interest is CSN-5 which is highly conserved. CSN-5 is 334 amino acids long and contains a MPN domain (Figure 6) unlike a majority of the subunits which have a PCI domain (Wei and Deng, 2003). The MPN domain contains a JAMM motif (metalloenzyme) which together are important for protein interactions and enzymatic activity such as degradation (Shackleford and Claret, 2010). The primary function of CSN-5 is the degradation of proteins involved in the ubiquitin-mediated proteolysis pathway (Zhou and Wee, 2003). The MPN domain of CSN-5 is important in the removal of Nedd8 from cullin-based E3 ubiquitin ligases (Figure 7). Additionally, CSN-5 acts as a transcriptional co-activator by playing a role in protein stability (Chamovitz, 2009). CSN-5 has been shown to stabilize c-Jun (member of activating protein-1 complex) and hypoxia inducible factor α (HIF-1α) (Shackleford and Claret, 2010). Research in muscle cells of *C. elegans* has shown that CSN-5 both stabilizes and degrades M-line proteins, such as UNC-96 and UNC-98 respectively (Miller et al, 2009).

**Figure 6: CSN-5 domains (Shackleford and Claret, 2010).** The largest and most important domain of CSN-5 is the MPN domain which contains a JAMM motif. This domain plays a role in protein interactions and degradation.
Figure 7: Model of ubiquitin ligases activity (Cope and Deshaies, 2003). CSN-5 is needed to remove Nedd8 from the ubiquitin ligases so CAND1 can be recruited. This cycle is needed for protein degradation.

Project Goals

The first goal of this project was to study a possible interaction between MIG-10 and CSN-5. A yeast-two hybrid screen revealed CSN-5 as a possible interactor of MIG-10 (Gossellin and O’Toole, 2008). Further research was conducted in vitro through western blotting and co-immunoprecipitation to provide more evidence of this protein interaction. A lack of time and repetition of experiments resulted in inconclusive results (Sullender, 2012). In the current project, research was continued to further examine the possible interaction between MIG-10 and CSN-5, and how they play a role in axon guidance or cell migration. Experiments were conducted using constructs from previous research through co-immunoprecipitation experiments and western blotting.

The second goal of this project was to determine which specific domains of MIG-10 are involved in binding CSN-5. The N-termini of MIG-10 A and B were cloned into insect vectors with a V5 tag. Regions shown to be involved in the binding ability of MIG-10 such as proline-rich regions are lacking in the N-terminus of MIG-10B. Additionally, a previously cloned construct of the C-terminus of MIG-10 was investigated. Experiments were conducted to determine whether interactions occur between each MIG-10 domain (N-terminus and C-terminus) and CSN-5.
Methods

Molecular Biology

PCR

Table 1 outlines the components of each 25 μl PCR reaction for MIG-10A and B N-terminus. A list of the forward and reverse primers can be seen in Table 2, and the temperature cycles used are shown in Table 3. After completion of PCR, 4 μl of DNA was run on a 0.8% agarose gel to examine for accuracy. Gel purification was conducted using the Gel Extraction Kit protocol (Qiagen).

Table 1: PCR Reaction Components

<table>
<thead>
<tr>
<th></th>
<th>MIG-10A N-Term</th>
<th>MIG-10B N-Term</th>
</tr>
</thead>
<tbody>
<tr>
<td>10X Thermopol Buffer</td>
<td>2.5 μl</td>
<td>2.5 μl</td>
</tr>
<tr>
<td>10mM dNTPs</td>
<td>0.5 μl</td>
<td>0.5 μl</td>
</tr>
<tr>
<td>Vent</td>
<td>0.5 μl</td>
<td>0.5 μl</td>
</tr>
<tr>
<td>5 μM Forward Primers</td>
<td>1.0 μl</td>
<td>1.0 μl</td>
</tr>
<tr>
<td>5 μM Reverse Primer</td>
<td>1.0 μl</td>
<td>1.0 μl</td>
</tr>
<tr>
<td>dH2O</td>
<td>17.5 μl</td>
<td>17.5 μl</td>
</tr>
<tr>
<td>MIG-10A N-Term DNA</td>
<td>2.0 μl</td>
<td>0.0 μl</td>
</tr>
<tr>
<td>MIG-10B N-Term DNA</td>
<td>0.0 μl</td>
<td>2.0 μl</td>
</tr>
</tbody>
</table>

Table 2: PCR Primers

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Primer Direction</th>
<th>Sequence</th>
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</thead>
<tbody>
<tr>
<td>mig10aforatt</td>
<td>Forward</td>
<td>GGG GAC AAC TTT GTA CAA AAA AGT TGG AAA ATG GAC ACT TAC GAC TTC CCG</td>
</tr>
<tr>
<td>mig10bforatt</td>
<td>Forward</td>
<td>GGG GAC AAC TTT GTA CAA AAA AGT TGG AAA ATG TAT CAC GAT CGA CGG CG</td>
</tr>
<tr>
<td>mig10_Ntermrevatt</td>
<td>Reverse</td>
<td>GGG GAC AAC TTT GTA CAA GAA AGT TGG AAA TGC AGA ATC ACC GTC TGG AGA</td>
</tr>
</tbody>
</table>

Table 3: PCR Temperature Cycle (30 cycles)

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>94°C</td>
<td>5 minutes</td>
</tr>
<tr>
<td>94°C</td>
<td>30 seconds</td>
</tr>
<tr>
<td>62°C</td>
<td>1 minute</td>
</tr>
<tr>
<td>72°C</td>
<td>1 minute</td>
</tr>
<tr>
<td>72°C</td>
<td>10 minute</td>
</tr>
<tr>
<td>4°C</td>
<td>Forever</td>
</tr>
</tbody>
</table>
BP/LR Reactions and Product Analysis

Gel purified PCR products were used in the Gateway Cloning System to produce constructs for co-immunoprecipitation and western blotting. An outline of this cloning system can be seen in Figure 10 in the Results. BP and LR reactions are performed similarly, except for a few minor differences which are highlighted below. A mixture with a total reaction volume of 8 µl containing 15-150 ng of PCR product (BP) or 50-150 ng of Entry clone (LR), 1 µl of Donor vector (BP: 150 ng/µl) or Destination vector (LR: 150 ng/µl), 1-3 µl of TE buffer (pH 8), and 2 µl of BP Clonase II or LR Clonase II enzyme (Invitrogen) was briefly vortexed, microcentrifuged, and incubated at 25°C. After 1 hour, 1 µl of Proteinase K solution (Invitrogen) was added and placed at 37°C for 10 minutes. Then, the DNA was transformed by adding 5 µl of the BP or LR Reaction to 50 µl of maximum efficiency bacterial cells (New England Biolabs) and incubated on ice for 30 minutes. The cells were then heat shocked for 30 seconds in a 42°C water bath followed by the addition of 400 µl of SOC. After 3 hours gently rotating on a nutator at 37°C, 50 µl and 150 µl samples were each spread on a 50 µg/ml Kan (BP) or 50 µg/mL Amp (LR) plate and allowed to incubate at 37°C overnight. The next day, 5 individual colonies were picked from each plate using a pipette tip which was ejected into a conical tube of 5 mL of liquid LB media + 50 µg/mL Kan (BP) or 50 µg/mL Amp (LR). After rotating overnight, DNA was isolated by using a miniprep kit (Qiagen). To verify that the samples were correct, a digest was performed for each reaction for 2 hours at 37°C. The BP entry clone digestions combined 2 µl of isolated DNA with 1 µl of BanII, 15 µl of dH2O, and 2 µl of 10X Buffer 4. LR expression clone digestions contained 2 µl of isolated DNA, 1 µl of BamHI, 13 µl of dH2O, 2 µl of Buffer 3, and 2 µl of BSA. After digestion, ~18 µl of DNA was run on a 0.8% agarose gel and observed for accuracy. Additionally, a sample was sent out for sequencing using GENEWIZ protocol to analyze the accuracy of the sequence of interest.

Growing CSN-5::GFP, MIG-10A::V5, and MIG-10 C-terminus::V5 Expression Clone Cultures

A frozen glycerol stock of CSN-5::GFP, MIG-10A::V5, and MIG-10 C-terminus::V5, each in an insect expression vector from a previous MQP (Sullender, 2012) were individually streaked on a 50 µg/mL Amp plate and left overnight at 37°C. Colonies were picked and a miniprep was performed as stated for the BP/LR Reactions. A restriction digest was performed by mixing 2 µl of isolated DNA, 1 µl of HindIII, 15 µl of dH2O, and 2 µl of Buffer 2 for each expression clone. After 2 hours of digestion at 37°C, ~18 µl of DNA was run on a 0.8% agarose
gel and observed for accuracy. Additionally, a sample of each clone was sent out for sequencing using GENEWIZ protocol to analyze the accuracy of the sequence of interest. Maps of the expression clones can be seen in Appendix B.

**Biochemistry**

**Cell Maintenance**

Two flasks of an S3 cell line of *D. melanogaster* cells were maintained throughout the project. The cells were fed with Schneider’s media + 12.5% FBS and kept at 25°C. Every 3-4 days cells were split using a 1:5 dilution. All cell culture work was conducted in a tissue culture hood using sterile technique.

**Transfections**

In preparation for western blotting, 2 mL of a 1:5 dilution of the parent flask was plated into each well of a six-well plate. After 3 days at 25°C, the plate was transfected with the preferred DNA samples using the Effectene reagent kit (Qiagen). A dilution of the DNA samples with TE buffer was prepared before transfection to reach an ideal final concentration of 66.6 ng/μl. Then, 2 μl of each desired expression clone DNA was mixed with 4 μl of Arm-GAL4 DNA (66.6 ng/μl) and the appropriate amounts of reagents from the transfection kit following the Qiagen protocol. The actual concentrations of DNA were below the desired amount of 66.6 ng/μl for many experiments. Lastly, 600 μl of Schneider’s media + 12.5% FBS was combined with the mixture and added drop-wise to the desired well. The plate was left at 25°C for 4 days at which point it was harvested for a Co-IP and/or western blot.

**Co-immunoprecipitation**

After 4 days at 25°C, the six-well plate was observed under a fluorescent microscope to examine the transfection efficiency. Cells were re-suspended in media, and a 200 μl sample of cells was reserved from each well of the plate, centrifuged at 8,000 rpm for 5 minutes, and re-suspended with 50 μl of 1X Sample Buffer (Appendix A). These samples were used as whole
cell lysates (WCL). WCL samples were frozen at -20°C overnight or used the same day. After reserving 200 μl from each sample in the six-well plate for WCL, the remaining sample was transferred into a 15 mL conical tube and centrifuged at 2,000 rpm for two minutes in a bench-top centrifuge. The supernatant was removed, and the pellet was re-suspended in 1 mL of Lysis Buffer (Appendix A). The tubes were incubated on ice for 15 minutes, transferred into 1.5 mL Eppendorf tubes, and centrifuged in a microfuge at 14,000 rpm for 10 minutes at 4°C. The supernatants were transferred into new 1.5 mL Eppendorf tubes, combined with 2 μl of anti-GFP (Rabbit polyclonal from Clontech), and incubated at 4°C for 30-45 minutes on the rotating nutator. Next, 100 μl of Protein A Magnetic Beads (Miltenyi) was added, and incubated at 4°C for 30-45 minutes on the nutator. 

Columns were placed on a magnetic board with a rack of tubes below to collect the liquids that were run through the columns. First, 200 μl of Lysis Buffer was run through the columns. Once the buffer was completely through the column, the lysate samples were applied to the columns. After the samples had passed entirely through the column, 200 μl of Lysis Buffer was run through the columns four times. Once clear, 100 μl of Buffer X (Appendix A) was run through the columns followed by 100 μl of Final Wash Buffer (Appendix A). Next, 20 μl of preheated 2X Sample Buffer (Appendix A) was added to the columns and allowed to incubate for 5 minutes. Then, an additional 50 μl of 2X Sample Buffer was added to the columns. The collection tubes were frozen overnight or used the same day, and then boiled for 5 minutes before loading them onto the western gels.

**Western Blotting**

The same day or the following day, 2D polyacrylamide gels were prepared. The gels were placed in an electrophoresis apparatus which was then filled with protein electrophoresis buffer. Each well was rinsed by pipetting up and down followed by loading 10 μl of each WCL or Co-IP sample (pre-boiled for approximately 5 minutes) plus one lane with 10 μl of ladder (Novex® Sharp Pre-Stained Protein Standards). Once loaded, the gels were run at 20mA for roughly 50 minutes.

Roughly 15-20 minutes before the run was complete, components needed to transfer the gel onto a nitrocellulose/ECL membrane were soaked in transfer buffer. Once the gels ran
completely, a ‘sandwich’ was created to surround the gel and membrane during transfer. Each ‘sandwich’ cassette was prepared with a fiber pad on the bottom, a piece of Whatman paper, the gel, the membrane (notched top right corner), another piece of Whatman paper, and another fiber pad. The cassettes were placed in a transfer apparatus with an ice pack and filled with transfer buffer. Transfer of the gels was run at 100V for 1 hour. After transfer, the membranes were removed, flipped, and placed in separate containers depending upon the experiment. Each membrane soaked and rotated for 1 hour in blocking solution made of a mixture of 1.25 g of non-fat dry milk (NFDM) and 25 mL of TBST (10X TBS + 0.1% Tween). Next, the membranes incubated in primary antibody overnight at 4°C. Gels testing for V5 tags were incubated in a mixture of 4 μl of anti-V5 antibody (mouse monoclonal, Invitrogen) in 1% NFDM and 10 mL TBST. Experiments testing for GFP tags incubated in a mixture of 10 μl of anti-GFP antibody (mouse monoclonal JL-8, Clontech) in 0.5% NFDM and 10 mL TBST.

The following day, the membrane containers were washed with TBST five times for five minutes each (enough TBST to cover/move the membranes). Then, the membranes incubated in 0.5 μl of secondary antibody (HRP conjugated goat anti-mouse, Jackson ImmunoResearch) in 5% NFDM and 10 mL of TBST for 1 hour. Following the secondary antibody, the membranes were washed again with TBST five times for five minutes each. Once the washes were complete, the membranes were removed and placed on plastic wrap after dabbing off excess liquid. A 1:1 mixture of peroxide:luminol solution was used to cover the membranes and soak for 10 minutes. Finally, the membranes were developed onto film where they could be analyzed for protein expression.
Results

Through this project, interactions of MIG-10 were investigated through co-immunoprecipitation and western blotting. The first part of the project looked at the possible interaction between MIG-10 and a candidate protein, CSN-5. Secondly, the N-terminus and C-terminus of MIG-10 were examined as possible binding domains.

MIG-10 and CSN-5 Interactions

For the first goal of this project, MIG-10::V5 and CSN-5::GFP constructs were co-immunoprecipitated to observe if there was an interaction. The Co-IP used an anti-GFP antibody to pull down the protein of interest. Protein constructs containing a V5 tag will only produce a band on the western blot if there is an interaction with the GFP construct protein. Figure 8 displays a schematic outlining the process and expected outcome of the Co-IP and western blot. Therefore if MIG-10 and CSN-5 interact, a band corresponding to CSN-5::GFP would be expected on the anti-GFP blot, while a band corresponding to MIG-10::V5 would be expected on the anti-V5 blot. All constructs used for this experiment were previously cloned (McShea et al., 2013; Sullender, 2012). MIG-10::V5, CSN-5::GFP, and ABI-1::GFP correspond to full-length wild type proteins, while the ABI-1(174-426) construct encodes only the central portion of the protein.
All samples were co-transfected, harvested as either whole cell lysates or Co-IPs, run on a gel, and blotted. As seen highlighted in the yellow box on the V5 gel of Figure 9, a precipitate was observed indicating the interaction between MIG-10 and CSN-5. ABI-1::GFP and ABI-1(174-426)::GFP were each co-transfected with MIG-10 as a positive and negative control respectively (Lanes 6,7). Research has shown that MIG-10 binds to ABI-1 but not ABI-1(174-426) because the mutant lacks the SH3 domain which is important in binding (McShea et al., 2013). MIG-10 and CSN-5 were individually transfected as additional negative controls. WCL samples were used as additional controls for expression. Overall, the anti-GFP blot serves as a control for the Co-IP. Two additional western blots confirming the interaction between MIG-10 and CSN-5 can be seen in Appendix C which used the same DNA concentrations as Figure 9.
Figure 9: MIG-10 and CSN-5 Interaction. The table above the blots gives a breakdown of what DNA was transfected in each lane. Lanes 1-4 represent WCL samples, while lanes 6-10 show Co-IP samples, with a ladder in lane 5. The yellow box highlights where MIG-10 (66.6 ng/μl) was pulled down by CSN-5 (40.4 ng/μl) on the anti-V5 gel. The green arrow points to the expression of the CSN-5 WCL on the anti-GFP gel, and MIG-10 WCL expression is emphasized by the blue arrow on the anti-V5 gel. Precipitate was observed for the co-transfection of MIG-10 and ABI-1 (174-426) (66.6 ng/μl) on the anti-V5 gel which is expected. The red arrow at ~50kDa shows a background band observed from the Co-IP samples not to be confused with the proteins of interest. Arm-GAL4 used during the transfection had a concentration of 17.55 ng/μl.

Possible MIG-10 Binding Domains

In order to investigate the possible binding domains of MIG-10, constructs of MIG-10A and B N-terminus were cloned to insert a 6XHis tag. This was achieved through the Gateway Cloning System which is outlined in Figure 10.
The N-termini of MIG-10 A and B were cloned with a V5 tag using the Gateway Cloning System. Whole cell lysate samples of these two constructs were prepared through transfection and applied to a western blot, but no expression was observed after three experiments (Figure 11). Thus, it cannot be concluded whether MIG-10 N-terminus is a binding domain. The Co-immunoprecipitation assay was not conducted because expression of the whole cell lysates could not be seen. The N-terminus of MIG-10 is only ~14 kDa, and therefore it was possible the samples ran off the gel in the initial experiment. A shorter run time for the gel was conducted, but still no expression was observed. Expression of ABI-1 and MIG-10A (whole) control whole cell lysates was observed which showed that the transfection was efficient and the western was conducted properly. Additionally, sequence analyses of the N-terminus constructs showed no mistakes from PCR or cloning.
Figure 11: No expression of MIG-10 A and B N-terminus in whole cell lysates. The table above the gels gives a breakdown of what DNA was transfected in each lane. The anti-GFP gel displays the expression of the ABI-1 (66.6 ng/μl) control which is GFP tagged. Expression of MIG-10A (whole: 66.6 ng/μl) can be seen on the anti-V5 gel. No expression was seen in the MIG-10A (66.6 ng/μl) and B N-terminus (66.6 ng/μl) lanes.

Further experiments explored the C-terminus of MIG-10 as a possible binding domain. A MIG-10 C-terminus::V5 construct was previously cloned using the Gateway Cloning System (Sullender, 2012). Preliminary results showed no precipitation where MIG-10 C-terminus was co-transfected with CSN-5 (Figure 12). This experiment was only performed once, and can therefore not be confirmed. The co-transfection of MIG-10 and CSN-5 was used as a positive control where interaction was confirmed. Single transfections of MIG-10 C-terminus, MIG-10, and CSN-5 were used as negative controls. Whole cell lysate samples were used as controls for expression. Overall, the anti-GFP gel serves as a control for the Co-IP.
Figure 12: MIG-10 C-terminus possibly not interacting with CSN-5. The table above the gels gives a breakdown of what DNA was transfected in each lane. Lanes 1-4 represent WCL samples while lanes 6-10 show Co-IP samples, with a ladder in lane 5. No precipitate is seen in lane 7 on the anti-V5 gel indicating no interaction between MIG-10 C-terminus (55 ng/μl) and CSN-5 (88.8 ng/μl). As a control, interaction between MIG-10 (88.8 ng/μl) and CSN-5 is seen in lane 6. The green arrow points to the expression of the CSN-5 WCL on the anti-GFP gel, and MIG-10 C-terminus WCL expression is emphasized by the blue arrow on the anti-V5 gel. The red arrow at ~50kDa shows a background band observed from the Co-IP samples not to be confused with the proteins of interest. Arm-GAL4 used during the transfection had a concentration of 66.6 ng/μl.
Discussion

The overall goal of this project was to research protein interactions with MIG-10 during nervous system development. First, interactions between MIG-10 and a candidate protein, CSN-5, were investigated through co-immunoprecipitation and western blotting. CSN-5 was identified as a possible interactor of MIG-10 in previous yeast-two hybrid experiments. Secondly, specific binding domains of MIG-10 were investigated.

Through this project, the interaction between MIG-10 and CSN-5 was confirmed. Four repetitions of the co-transfection of MIG-10 and CSN-5 experiment displayed the co-immunoprecipitation of MIG-10 by CSN-5, confirming interaction. Since CSN-5 is involved in both protein stability and degradation (Miller et al, 2009), it is possible MIG-10 is regulated by CSN-5. One possible model could be that CSN-5 is needed to degrade MIG-10 once the neuronal migration and axon guidance is complete. Preliminary csn-5(RNAi) experiments within C. elegans appear to disprove this theory (McMasters, 2013). These preliminary results show the truncation of ALML neuron migration, suggesting CSN-5 may be needed to stabilize rather than degrade MIG-10 during neuronal migration. Although these results are surprising, they are not unprecedented, since CSN-5 has been shown to stabilize other proteins (Miller et al., 2009). Additionally, CSN-5 has been shown to regulate transcription by stabilizing transcription factors, which could indirectly affect MIG-10 protein levels (Chamovitz, 2009). Further future research should be conducted to observe interactions between MIG-10 and CSN-5 in vivo. csn-5 null mutants could be crossed into marker strains to observe the effects on ALM migration. If CSN-5 acts to stabilize MIG-10, as predicted by csn-5(RNAi) experiments, then csn-5 mutants would be expected to result in the truncation of the neuronal migration.

Additionally through this project, the C-terminus and N-terminus of MIG-10 were investigated as possible binding domains. Preliminary experiments involving the co-transfection of MIG-10 C-terminus and CSN-5 did not display interaction. These preliminary results suggest that the C-terminus of MIG-10 is not a binding domain. The N-terminus of MIG-10 did not show any expression from the whole cell lysate samples on the western blot. It is possible that since the N-terminus segment of MIG-10 protein is so small that it is not stable for such experiments. Therefore, no conclusion can be made about the N-terminus of MIG-10 being a binding domain.
Throughout the project, a couple of technical issues were encountered. For example, the best results were seen when the Co-IP and western blot were performed in one day (“marathon”). When the procedures were broken up into two days, the WCL and Co-IP samples were either placed at 4°C or frozen at -20°C overnight. This appeared to have a negative effect on the Co-IP samples, especially when left at 4°C. Therefore, future experiments should be performed as a “marathon” to achieve ideal results. Additionally throughout the project, the Arm-GAL4 concentration (17.55 ng/µl) was much lower than the ideal concentration of 66.6 ng/µl. As a result bands appeared fainter and needed a longer exposure time to be seen. For the last experiment (Figure 12), Arm-GAL4 with the ideal concentration was used, and gave a much stronger signal. Based on these findings, it is important to have DNA concentrations of 66.6 ng/µl for the best results.

Future research investigating the different domains of MIG-10 would be beneficial to understand what part of the protein is binding. Continued co-immunoprecipitation experiments with MIG-10 C-terminus and CSN-5 could confirm that the C-terminus is not a binding domain. Further co-immunoprecipitation experiments with the RAPH domain of MIG-10 and CSN-5 would confirm if this region of MIG-10 is a binding domain. Additionally, the N-terminus could further be examined as a possible binding domain by creating expression clones that increase the length of the N-terminus. This might increase the stability of the protein which could be used in Co-IP experiments with CSN-5.

Based on the results of this project, the regulation of MIG-10 can be more thoroughly understood. MIG-10 which is found in C. elegans serves as a model for the human ortholog, lamellipodin. Research of MIG-10 can be used to get a better grasp of the effects of mutations within the development of the nervous system. Overall, such research aids in better understanding neurological disorders such as Down’s syndrome and autism.
References


McMasters J (2013). The role of CSN-5 in neuron migration in C. elegans. WPI MQP
McShea M (2011). Evidence of an interaction between the actin cytoskeletal regulators MIG-10 and ABI-1. WPI Thesis: 1-78


## Appendix

### Appendix A: Buffer Solutions

<table>
<thead>
<tr>
<th>Calculations:</th>
<th>1x Sample Buffer (100µl 1x Sample Buffer needed per WCL sample)-</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Total 1x Sample Buffer needed (µl) / 5x (Sample Buffer Stock Conc.) = Volume 5x Sample Buffer (µl)</td>
</tr>
<tr>
<td>2.</td>
<td>Add BME (5% of the volume of 5x Sample Buffer used (µl)).</td>
</tr>
<tr>
<td>3.</td>
<td>Add TBS to reach total 1x Sample Buffer volume.</td>
</tr>
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</table>

<table>
<thead>
<tr>
<th>Lysis Buffer (2mL Lysis Buffer needed per Co-IP sample)-</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. 2x Protease Inhibitors:</td>
</tr>
<tr>
<td>2x (Final Conc. Protease Inhibitors) × Total Lysis Buffer needed (µl) / 25x (Protease Inhibitor Stock Conc.) = Volume Stock Protease Inhibitor (µl)</td>
</tr>
<tr>
<td>2. 5mM Phosphatase Inhibitors:</td>
</tr>
<tr>
<td>5mM (Final Conc. Phosphatase Inhibitors) × Total Lysis Buffer needed (µl) / 100mM (Phosphatase Inhibitor Stock Conc.) = Volume Stock Phosphatase Inhibitor (µl)</td>
</tr>
<tr>
<td>3. 1mM Sodium Orthovanadate (Na₃VO₄):</td>
</tr>
<tr>
<td>1mM (Final Conc. Na₃VO₄) × Total Lysis Buffer needed (µl) / 200mM (Na₃VO₄ Stock Conc.) = Volume Stock Sodium Orthovanadate (µl)</td>
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<td>4. Add a volume of EBC Buffer to reach the final volume of Lysis Buffer needed for the Co-IP samples.</td>
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<table>
<thead>
<tr>
<th>2x Sample Buffer (90µl per Co-IP sample)-</th>
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<tbody>
<tr>
<td>1. 2x (Final Conc. Sample Buffer) × Total 2x Sample Buffer needed (µl) / 5x Sample Buffer Stock Conc. = Volume 5x Sample Buffer (µl)</td>
</tr>
<tr>
<td>2. Add BME (5% of the volume of 5x Sample Buffer used (µl)).</td>
</tr>
<tr>
<td>3. Add TBS to reach total 2x Sample Buffer volume.</td>
</tr>
<tr>
<td>4. Heat to ~ 95°C using boiling water before using in columns.</td>
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Appendix B: Expression Clone Maps with Restriction Enzyme Cut Sites
Appendix C: Additional Western Blot of MIG-10 and CSN-5 Interactions

<table>
<thead>
<tr>
<th>Sample</th>
<th>Lane 1</th>
<th>Lane 2</th>
<th>Lane 3</th>
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<th>Lane 5</th>
<th>Lane 6</th>
<th>Lane 7</th>
<th>Lane 8</th>
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<td></td>
<td></td>
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<td>X</td>
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Anti-GFP

Anti-V5