Worcester Polytechnic Institute Major Qualifying Project

Do ARX-3 and CSN-5 Protein Subunits Interact with MIG-10 to Promote Axon Outgrowth and Neuronal Migration in *C. elegans*?

Major Qualifying Project Report
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Submitted by:

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Submitted to:

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Abstract

During nervous system development, the cytoplasmic signaling protein MIG-10 activates cellular processes in response to guidance cues which ultimately lead to axonal growth and migration. Interactions between MIG-10 and two candidate downstream proteins, CSN-5 and ARX-3, were studied *in vivo*, using mutant transgenic *C. elegans* strains, and *in vitro*, using a co-immunoprecipitation system. Preliminary results indicate interaction *in vitro* between MIG-10 and both proteins. *In vivo* experiments are ongoing.

Acknowledgements

I would like to thank Liz Ryder for being the best project advisor I could have asked for by showing me what it is to be a scientist. She guided me through the research process, made me a better scientific reader and writer, nurtured my love for all things neuro and proved to be a wonderful role model as a woman scientist. I would also like to thank Molly McShea and Karen Tran for teaching me the techniques of the Ryder Lab and for answering my questions about the co-immunoprecipitation and western blot assays. I would also like to thank Harita Menon for providing the lab with and helping maintain cell cultures. I would like to thank the Duffy, Vidali, and Politz labs for their shared knowledge and equipment. I would also like to thank Andi Reis and Erin Maloney for always being there to make this project fun and unforgettable. Lastly, I would like to thank my parents for their advice and patience.

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Introduction

The nervous system is an amazingly intricate and complex organ system; not only physically, where the interlacing of nerves throughout a body creates a complex informational network, but also mechanistically, where many of the underlying processes of neuron growth and guidance are not yet well understood. Developmental neurobiology considers and examines both the molecular and cellular mechanisms which govern the growth and development of the nervous system during embryonic development, and throughout the life of any given animal (Yaron & Zheng, 2007).

During development, billions of neurons can develop, each with a specific target and route of axon elongation. Neurons rely on sensing attractive and repulsive guidance cues from their local extracellular environment with growth cones in order to travel the great distances necessary to make connections with the correct targets, and produce the wiring of a functional neuronal network (Letourneau, 2003). Failure to make correct and functional networks results in a dysfunctional nervous system, which could result in many neurodevelopmental disorders such as autism and Down's syndrome. Understanding the processes responsible for neuronal connectivity would not only help elucidate the causes of neurodevelopmental disorders, but could also provide various new or improved treatment options for individuals with nerve damage due to injury such as a traumatic brain injury, or neurodegenerative disorders such as Alzheimer's disease (Quinn & Wadsworth, 2008).

Recently, there have been many discoveries concerning the extracellular component of neuron navigation. Many molecular guidance cues have been identified through biochemical

purifications, and many growth cone surface receptors which correspond to these molecules have been identified using genetic screens of vertebrates and invertebrates alike. Less is known about the internal, cytoplasmic signaling pathways that lead to the activation of cellular processes which lead to axonal growth and migration, and this MQP concerns some of the components of such a pathway (Yaron & Zheng, 2007; Quinn & Wadsworth, 2008).

Background

C. elegans as a Genetic Model System

Sydney Brenner first proposed *Caenorhabditis elegans* - a small, translucent nematode - as a genetic model system in the 1960s due to the worm's small size and rapid lifecycle which made genetic manipulations simple. In 1998, *C. elegans* was the first multicellular organism to have its complete genome sequenced. Since then, a physical map of the genome has been produced with ever-improving annotations (Hillier, 2008). The nervous system of *C. elegans* is simple, stereotyped and is comprised of only 302 neurons of which nearly every axon and synapse has been mapped. This makes *C. elegans* an ideal organism to identify and characterize neurological defects, and the molecules that affect axon migration during development (Gabel, 2008).

Axon Guidance

Axon guidance refers to the developmental process in which neurons rely on sensing attractive and repulsive guidance cues from their local extracellular environment in order to initiate axon elongation and make connections with the correct targets, producing the wiring of a functional neuronal network (Letourneau, 2003). Leading the outgrowth and guidance of neuronal axons is the growth cone, a highly motile structure at the tip of the axon which examines the extracellular environment and compiles the information gathered from guidance cues in order to make migratory decisions (Quinn, 2006) Several extracellular guidance biomolecules, such as netrin, slit, ephrin and semaphorin have already been identified as signaling molecules, which are conserved in both vertebrates and invertebrates (Yaron & Zheng,

2007). These ligands bind to receptors on the tip of the growth cone and elicit an intracellular signaling cascade which results in the initiation of cytoskeleton rearrangement in order to alter the direction of axon outgrowth. Specifically, there is an asymmetrical local accumulation of F-actin and microtubule bundles in the filopodia, which are finger-like protrusions of the growth cone that extend beyond the lamellipodium as shown in figure 1 (Quinn, 2006). Unfortunately little is known about the cytoplasmic signaling pathways that regulate axon guidance.

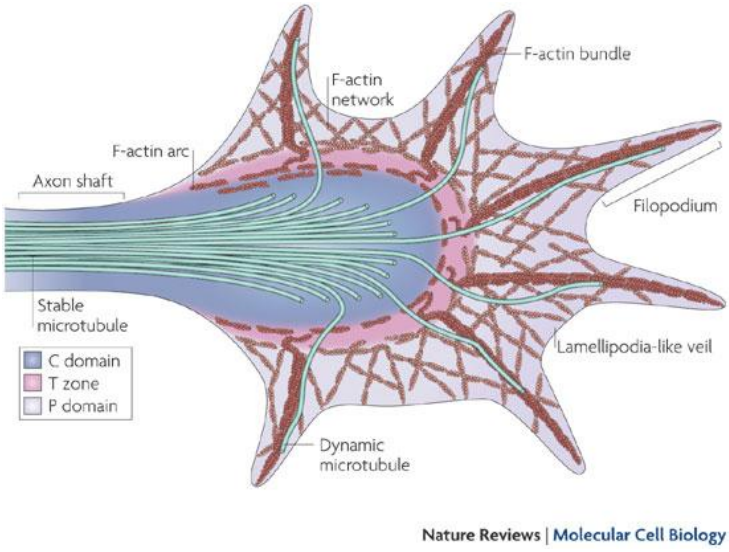

Figure 1. Schematic of Axonal Growth Cone (Lowery & Vactor, 2009). F-actin and microtubule bundles (red) are seen in the filopodia which are finger-like protrusions of the growth cone that extend beyond the lamellipodia-like veil.

A dynamic actin network is necessary for normal cell morphology, cytokinesis, locomotion, and for the outgrowth of filopodia. All of these cellular processes require the polymerization of actin monomers into filaments, and the bundling of those filaments under the control of actin-binding proteins (ABPs). ABPs cross-link filamentous actin into ordered bundles arranged in a parallel fashion. These actin bundles maintain the structural integrity of cells,

filopodia, and microvilli. Little is known about how cells initiate this dynamic assembly of actin in the right place at the right time during development, disease and injury (Zhang et al., 2009).

MIG-10 and Its Role in the Proposed Actin Guidance Mechanism

MIG-10, an ortholog of vertebrate lamellipodin, is a cytoplasmic signaling protein, belonging to the MRL protein family, which localizes in the filopodia of growth cones during axon guidance. A genetic screen for neuronal defects led to the discovery of several recessive *mig-10* mutations, which result in abnormal neuronal migrations and severe excretory cell defects (truncation) (Manser and Wood, 1990; Manser *et al.*, 1997).

The excretory cell, which functions in osmoregulation, has a cell body which lies ventral to the posterior bulb of the pharynx and has parallel tubular processes which extend anteriorly and posteriorly from the cell body. Null mutations in *mig-10* result in severe truncation of the excretory cell processes (Manser and Wood, 1990). The neurons investigated in this project are the Anterior Lateral Microtubule neurons (ALMs) and Anterior Ventral Microtubule neuron (AVM). The ALMs are known to migrate in an anterior to posterior direction to a location approximately half the length of the worm. The AVM is known to send an axon, ventrally, to the nerve cord. Null mutations in *mig-10* result in disruptions in the posterior migrations of the ALMs (Manser and Wood, 1990) and misguidance of the AVM axons (Quinn *et al.*, 2006; Chang *et al.*, 2006).

As with other MRL proteins, MIG-10 has several proline-rich regions, a Ras-interacting (RA) domain and a lipid-binding Plexstrin Homology (PH) domain. The PH domain has been shown to bind to PI(3,4)P2 (phosphotydyl inositol bisphosphate) and the RA domain has been shown to interact with CED-10/Rac GTPase for lamellipodin (Quinn, 2008). In addition, MRL family signaling proteins are known to interact with Ena/VASP proteins - most likely through proline-rich regions - which play a role in actin polymerization (Quinn et al., 2006).

The proposed mechanism of axon migration mediated by MIG-10 is demonstrated by figure 2. This model supplies a possible sequence of events beginning with the activation of the UNC-40 transmembrane receptor by UNC-6 (netrin). This activates phosphoinositide 3-kinase (PI3K) and CED-10/Rac GTPase which in turn recruit MIG-10, resulting in its localization at the cell membrane. MIG-10 binds to the PI(3,4)P2 phospholipids and a Ras-related protein via its PH and RA domains, respectively (Quinn, 2006). The PI(3,4)P2 phospholipids were suggested to be phosphorylated by AGE-1, the *C. elegans* equivalent of PI3K, in a study in which a null mutant for AGE-1 resulted in suppression of cell outgrowth in neurons overexpressing MIG-10 (Chang et al., 2006).

From here MIG-10 recruits complexes that in turn activate F-actin polymerization. The Ena/VASP protein UNC-34 is activated by binding to a proline-rich region on MIG-10 and is thought to interact with a complex which initiates actin anticapping and debranching. A WAVE/ABI-1 complex is also likely to be activated through an association with a proline-rich region of MIG-10, and it is thought that this complex may go on to activate the Arp2/3 complex which is responsible for F-actin polymerization (McShea, 2011).

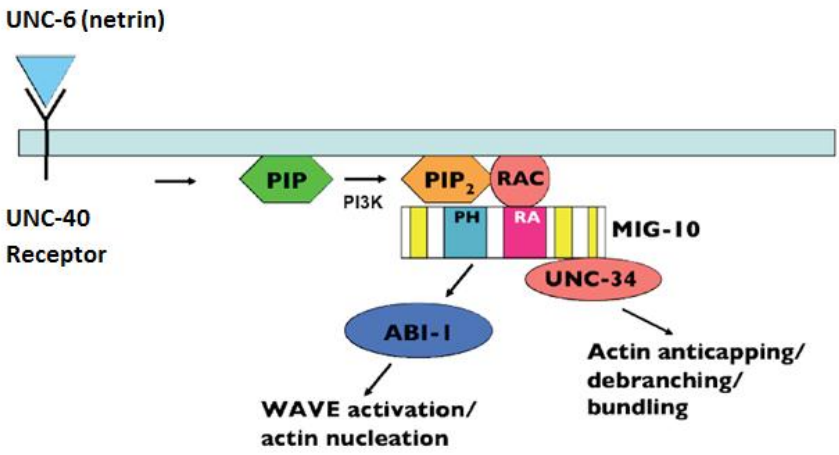

Figure 2. Speculative Model for MIG-10 signaling cascade (Ficociello and Ryder 2007). In the presence of a guidance cue, the UNC-40 transmembrane receptor activates CED-10/Rac GTPase and PI3 kinase which in turn localizes MIG-10 and associated actin polymerization proteins to the cell membrane.

Yeast Two Hybrid Screen Reveals More Potential Interactors of MIG-10

A yeast two hybrid library screen, using MIG-10A as bait, identified both ARX-3 and COP-9 as potential interactors of MIG-10 in the proposed signaling model. ARX-3 and COP-9 were each independently isolated in 1 independent transformation of the *C. elegans* cDNA library. The same screen revealed ABI-1 as a potential interactor of MIG-10, and that interaction has since been confirmed through co-immunoprecipitation (Gossellin and O'Toole, 2008; McShea, 2011).

ARX-3

ARp2/3 compleX component-3 (ARX-3) is one of seven subunits of the Arp2/3 complex, which nucleates and branches F-actin and caps the pointed ends of filaments forming y-branched actin networks (Letourneau, 2003). The mechanism of nucleation and branching is not well understood, but it is thought that the nucleation promoting factors (NPFs), responsible for

ARP2/3 activation are SCAR/WAVE proteins. The ARP2/3 complex is conserved in most eukaryotes, and the severity of the phenotypes that are a result of compromised ARP2/3 function demonstrates its important role in fundamental and conserved cellular processes including cellular migration (Disanza, et al., 2005). During cell migration, the ARP2/3 complex is localized to lamellipodia and pseudopodia within several micrometers of the leading edge, demonstrating that it may be a necessary component of cell migration, and perhaps growth cone elongation (Goley and Welch, 2006). It is interesting to note that a study proposed that UNC-53/NAV2 may act as a scaffold that links ABI-1 (a confirmed member of the MIG-10 signaling pathway) to the ARP2/3 complex to regulate cytoskeleton remodeling (Schmidt et al., 2009). ARX-3 is an ortholog of the mammalian p41Arc subunit, and the p41Arc subunit is considered to be a putative regulatory component of the human ARP2/3 complex that may play an important regulatory role for the complex as a whole during actin remodeling (Welch et al., 1997).

CSN-5

COP-9 SigNalosome Subunit-5 (CSN-5) is one of eight subunits of the COP-9 SigNalosome which has been implicated in a wide variety of biological functions, yet its biochemical and physiological functions are only beginning to be understood (Wei and Deng, 2003). The complex is conserved throughout eukaryotes, and has been found to be an integral part of the proper development of all multicellular organisms in which its function has been investigated. It has been suggested that COP-9 plays a key role in sustaining the activity of cullin-based ubiquintin ligases, which may explain its part in eukaryotic development (Cope and Deshaies, 2003; Miller et al., 2009) . COP-9 may also regulate proteolysis through its association with protein kinases and deubiquitylating enzymes, which accounts for its role in

regulating protein degradation as well as in development (Schwechheimer, 2004). The CSN-5 subunit of COP-9 (also known as Jab-1), specifically, is thought to interact with target proteins and has been implicated in numerous signaling pathways such as cell cycle control, integrin signaling and steroid hormone signaling, among other developmental pathways (Wei and Deng, 2003; Chamovitz and Segal, 2001).

Project Goals

The overall goal for this Major Qualifying Project (MQP) was to determine whether ARX-3, an Arp2/3 subunit or CSN-5, a COP-9 SigNalosome subunit, interact with MIG-10 to promote axon outgrowth and migration. The MIG-10/subunit interactions were identified in a previous MQP using a yeast two hybrid system (Gossellin and O'Toole, 2008). Even though the results were confirmed by retransformation, the positive results could have been caused by self-activation so the interactions need to be confirmed using entirely different systems.

Project Goal A: Confirming the MIG-10/Subunit Interaction *In Vivo*

The first goal of this MQP was to confirm the *in vivo* interaction between MIG-10 and the two protein subunits (ARX-3 or CSN-5). Steps toward this goal were achieved by creating mutant transgenic *C. elegans* strains labeled with *pgp-12*::GFP which marks the excretory cell and with *flp-20*::GFP which marks the migratory neurons ALM and AVM. These strains are currently being quantitatively assessed. If the strains display truncation of the excretory canal or migratory neuron defects these phenotypes will constitute strong support for an *in vivo* interaction.

Project Goal B: Confirming the MIG-10/Subunit Interaction *In Vitro*

The second goal of this MQP was to further confirm the MIG-10/subunit interaction that had been indicated in a previous MQP, this time in vitro. Co-immunoprecipitation (Co-IP) was chosen to be the biochemical system to further confirm the MIG-10/subunit interaction. In order to prepare this system the sequence of the constructs produced by the yeast two hybrid screen

were cloned into fusion-tagged insect vectors via the Gateway System©. Protein expression was achieved and a possible interaction was observed.

Materials and Methods

Genetics

Strain Maintenance

All stock and mutant *C.elegans* strains used were maintained on Nematode Growth Medium (NGM) agar plates spotted with OP50 *E. coli.* Active strains were maintained twice weekly by transferring three L4 hermaphrodites of the correct phenotype to a new plate and were stored in a 20°C incubator. Inactive strains were maintained weekly in the same manner, and were stored in a 15°C incubator. Any starved plates were chunked and allowed to grow through several generations before use. Table 1 contains a list of maintained strains.

Name	Strain
BC06288	*sIs10089 (*denoted pgp-12::GFP in this MQP)
NY2053	*ynIs54* (denoted flp-20::GFP in this MQP)
N2M	N2M
VC3166	arx-3 (ok1122)/mT1[dyp-10(e128))]II;III
VC861	csn-5 (ok1064)/nT1[gIs51]IV;V
RY1162	arx-3 (ok1122)/ mT1[dyp-10(e128))]II;III; pgp-12::GFP (Het)
RY1160	csn-5 (ok1064)/ nT1[gIs51]IV;V; pgp-12::GFP (Het)
RY1163	arx-3 (ok1122)/ mT1[dyp-10(e128))]II;III; flp-20::GFP (Het)
RY1161	csn-5 (ok1064)/ nT1[gIs51]IV;V; flp-20::GFP (Het)

Table 1. *C. elegans* strains maintained during project. The first strain was provided by E. Stringham (UBC, Canada). The next four strains listed were provided by the *C. elegans* Genetics Center (U Minnesota). The last four strains were designed by Meagan Sullender.

Strain Design

The mutant strains of *C.elegans*, RY1160, RY1161, RY1162 and RY1163, were generated through a series of crosses between a mutant strain containing the desired mutation and a transgenic marker strain. *C.elegans* strains RY1160 - RY1164 were crossed in parallel, and are summarized in figure 3. The first step consisted of crossing N2 males with L4

hermaphrodites containing the *pgp-12*::GFP or *flp-20*::GFP marker. The resultant offspring were homozygous for the *pgp-12*::GFP or *flp-20*::GFP marker. These male offspring were crossed with L4 hermaphrodites that were heterozygous for the *csn-5 (ok1064)* or *arx-3 (ok1122)* mutation for the RY1160/RY1161 and RY1162/RY1163 strains, respectively. The resultant male offspring were crossed back to their respective parent strain containing either the *csn-5 (ok1064)* or *arx-3 (ok1122)* mutation. Twelve of the resultant offspring were singled to separate plates and were self-crossed to determine whether worms carried the desired mutation. The plates that expressed the desired markers and phenotype were self-crossed again and checked until the final strains were obtained. The RY1160 and RY1161 strains were considered a success when the strain appeared to be heterozygous for *csn-5 (ok1064)* and 100% homozygous for the marker. The RY1162 and RY1163 strains were considered to be a success when the strain appeared to possess the same lethality (measured by egg : worm ratio) as the parent *arx-3 (ok1122)* and 100% homozygous for the marker.

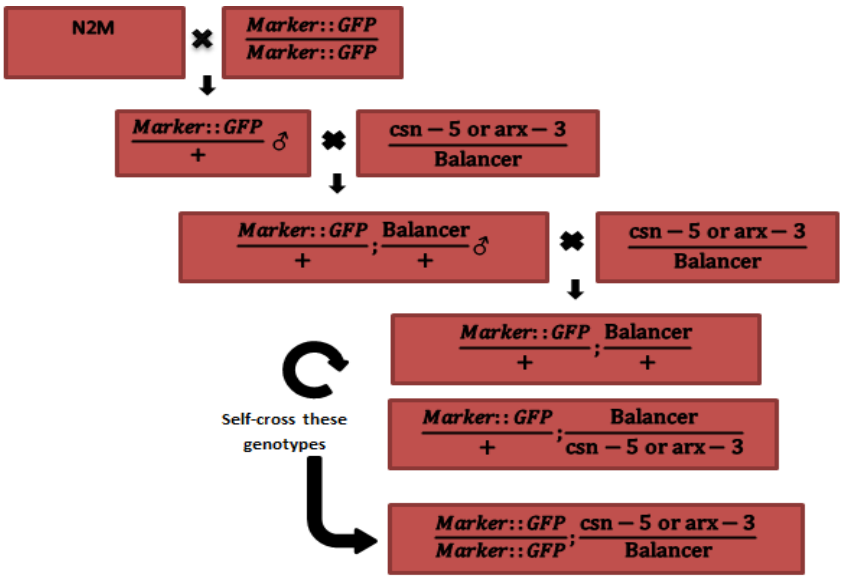

Figure 3: Summary of RY1160 – RY1163 Crosses.

Mutant Phenotype Quantitation

Mutant *C.elegans* were washed from a plate using a glass pipette full of M9 buffer, and then swirling the plate so that the worms were washed from the surface of the plate and suspended into the buffer. The worm containing buffer was then pipetted into a 1.0 mL eppendorf tube, and the worms were allowed to settle to the bottom of the tube while the slides were prepared. Slides for quantitation were prepared by melting a 2.0 mL aliquot of agarose in a beaker of boiling water. Once the agarose was melted, 20 µl of 1M sodium azide was added, and one drop of this mixture was placed on a glass slide using a glass pipette. The drop was immediately covered with a second slide creating an agar pad between the slides. 5.0 µl of the worm containing buffer was added on top of the agar pad once it had solidified. A coverslip was immediately placed on top of the drop of worm containing buffer.

The worm slide was then viewed under fluorescence at either 10x or 20x magnification (depending upon the worm) and a fluorescent and non-fluorescent photo was taken (using pixelfly) of each worm to be quantitated. The two photos were then superimposed and the length of the excretory canal (for *pgp-12*::GFP) or the $ALM_{1 \text{ and } 2}$ and AVM (for *flp-20*::GFP) was measured in pixels. Figure 4 shows where the measurements were taken and what reference points were used for each measurement.

For *pgp-12*::GFP animals, the posterior process of the excretory canal (a) was measured from the middle of the cell body posterior, to the end of the canal. This was then compared to the length of the entire worm. The anterior process of the excretory canal (b) was measured from the

middle of the cell body anterior, to the "beginning" of the canal. This was then compared to the "nose" length of the worm, from the posterior of the pharynx to the "nose".

For *flp-20*::GFP animals, the AVM (c) was measured from the middle of the cell body posterior, to the middle of the vulva. This was then compared to the length of the worm between the posterior of the pharynx and the vulva (length PV). The $ALM_{1 \text{ and } 2}$ (d) were measured from the middle of the cell body posterior, to the middle of the vulva. This was then compared to the length of the worm between the posterior of the pharynx and the vulva (length PV).

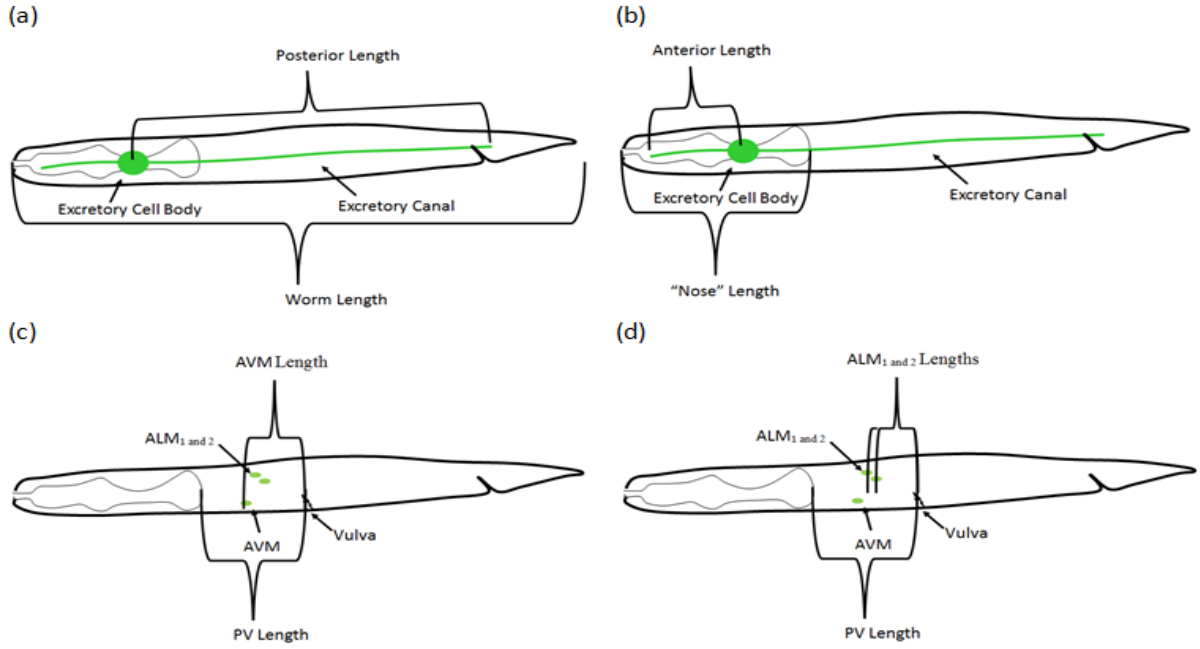

Figure 4. Sketches of Worm Quantitation. For *pgp-12*::GFP animals, the measurements of the posterior canal (a) and the anterior process of the excretory canal (b) were measured according to this schematic. For the *flp-20*::GFP animals, the measurements of the AVM (c) and the ALM $_{1 \text{ and } 2}$ (d) were measured according to this schematic.

Molecular Biology

Vector Design

The vector constructs to be used in the co-immunoprecipitation system were created using the Invitrogen Gateway cloning system, summarized in figure 5. PCR products were generated using 2.5 µl of 10X Thermopol buffer, 0.5 µl of dNTPs (stock concentration 10 mM in each dNTP), 1.0 unit of Vent polymerase (NEB), 5.0 pmol of each primer, and 3300 ng of *C. elegans* DNA library template. The primers shown in table 2 were designed in order to generate PCR products encoding the desired subunit (*arx-3* or *csn-5*) of which the 5' and 3' ends were flanked by modified attB1 and B2 Gateway recombination sites, respectively. The annealing temperatures for *csn-5* and *arx-3* were 62^{o}C and 64^{o}C respectively. In order to confirm the presence of a PCR product of the appropriate size, 5-10% of the total volume of each PCR reaction was run on a 0.8% agarose gel in 1X TAE buffer.

Primer Name	Forward or Reverse Primer	Generated Amplicon	Length (bp)	Sequence
arx-3foratt	Forward	*arx-3*	56	GGG GAC AAC TTT GTA CAA AAA AGT TGG AAA ATG ACC AAC AGA ATC GTC TCA TGT TC
arx-3revatt	Reverse	*arx-3*	49	GGG GAC AAC TTT GTA CAA GAA AGT TGG GAC GAG TGA TGG GTG ATT CTT C
arx-3_N_foratt	Forward	*arx-3*_C	60	GGG GAC AAC TTT GTA CAA AAA AGT TGG AAA ATG TCG TCC GGC GTT CCC GTT CTC CAC TGG
arx-3_C_revatt	Reverse	*arx-3*_C	60	GGG GAC AAC TTT GTA CAA GAA AGT TGG GAC GAG TGA TGG GTG ATT CTT CAA ATC CCA AAG

Table 2. Primers Used for Generation of AttB Flanked PCR Products. Primer names and sequences used to produce the indicated amplicons are shown. Top primer pair designed by Molly McShea and bottom primer pair designed by Meagan Sullender. Primers provided by IDT.

BP reactions were performed for each PCR product by mixing 150 ng of both pDONR vector and PCR product with 2.0 µl of BP Clonase II Enzyme mix (Invitrogen) and incubating the mixtures overnight at 25^{o}C. The reactions were terminated the next day by incubation at 37^{o}C and with the addition of 1.0 µl of Proteinase K. 5.0 µl of each reaction was then transformed into 50 µl of Max-Efficiency DH5α *E. coli* chemically competent cells (Invitrogen). The cells were allowed to incubate on ice for 30 minutes, were heat shocked for 30 seconds at 47^{o}C, and were incubated at 37^{o}C on a nutator for 3 hours after the addition of 400 µl of SOC. The transformed cells were then plated on LB-agar plates containing 50 µg/mL kanamycin and incubated overnight at 37^{o}C and colonies selected the next day were individually picked to 5.0 ml aliquots of liquid LB medium containing 50 µg/mL kanamycin. The liquid cultures were incubated overnight at 37^{o}C. 0.5 ml of each culture was frozen down as a glycerol stock, and then the plasmid DNA of each sample was extracted via the QIAprep Spin Miniprep kit (Qiagen). Potential entry clones were screened by restriction digest using buffer 4 and 10 units Ban II (NEB) per 2 hour reaction at 37^{o}C, and the identities of positively selected entry clones were confirmed by sequence analysis performed on A Plasmid Editor using sequencing data provided by GENEWIZ's Cambridge, MA facility. Samples were prepared for sequencing according to GENEWIZ's pre-mixed guidelines.

LR reactions were then performed for each BP product in order to insert the selected coding sequence into a vector suitable for transfection by mixing 150 ng of BP product with 150 ng of either pUAST GFP or pUAST V5/6XHis (Duffy, WPI) with 2.0 µl of BP Clonase II Enzyme mix (Invitrogen) and incubating the mixtures overnight at 25^{o}C. 5.0 µl of each reaction was then transformed into 50 µl of Max-Efficiency DH5α *E. coli* chemically competent cells

(Invitrogen) and were then plated on LB-agar plates containing 50 µg/mL ampicillin. The plates were incubated overnight at 37°C and colonies selected the next day were individually picked to 5.0 ml aliquots of liquid LB medium containing 50 µg/mL ampicillin. Plasmid DNA was extracted via the QIAprep Spin Miniprep kit (Qiagen) as before, and the identities of the expression clones were confirmed by restriction digest using buffer 2 and 10 units Hind III (NEB) per 2 hour reaction at 37°C, followed by sequencing analysis.

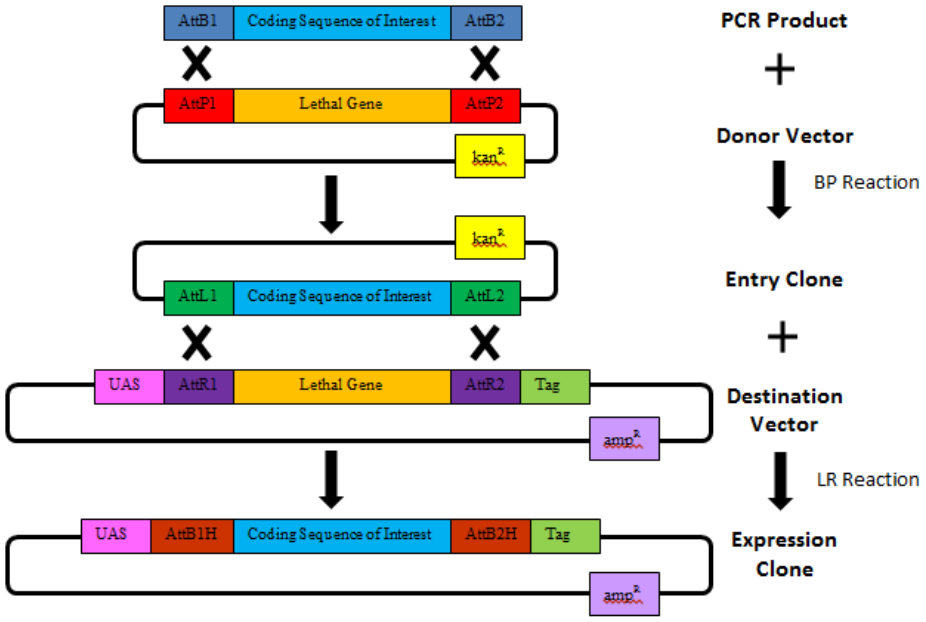

Figure 5. Gateway Cloning Strategy Summary. Each PCR product encoding the gene of interest was flanked with attB1 and attB2 sites. During the BP cloning reaction, these sites were recombined with attP sites in the donor vector, pDONR201. This produced a pDONR201 entry clone with an attL-flanked gene of interest. Any transformed cells possessing an unreacted donor vector were negatively selected by the lethal gene. During the LR cloning reaction, the attL sites of the pDONR201 Entry Clone were recombined with the attR sites of an expression vector containing a UAST element and a GFP or V5/6XHis tag. This produced expression vector with a hybrid attB-flanked gene of interest.

Mutant Genotype Confirmation

The genotype of each mutant strain of *C.elegans* generated was confirmed via 10 worm PCR. Approximately ten worms were picked and added to a mixture of lysis buffer and 60

µg/mL proteinase K enzyme in the cap of a PCR tube. The tubes were centrifuged and stored at -80°C for a minimum of 30 minutes before proceeding to the lysis step. Lysis was performed by running the 10 worm tubes in the PCR machine under the lysis program (1 hour at 60°C, 15 minutes at 95°C and 4°C forever). The lyses were then stored at -80°C for a minimum of 30 minutes before proceeding to the PCR amplification step. The PCR step was performed by running the worm lysis tubes in the PCR machine under the short PCR program (10 minutes at 94°C, 30 cycles with 1 minute at 94°C, 2 minutes at an annealing temperature of 55°C and 10 minutes at 77°C and 4°C forever) after 16.5 µl of dH_2O, 2.5 µl of 10X Thermopol buffer, 2.0 units of Vent polymerase (NEB) and 5.0 pmol of each primer were added to each tube of worm lysis. The primers shown in table 3 were designed in order to generate PCR products encoding the desired subunit (*arx-3* or *csn-5*) from the lysed mutant strains. In order to confirm the presence of a PCR product of the appropriate size, 5-10% of the total volume of each PCR reaction was run on a 0.8% agarose gel in 1X TAE buffer. The mutations were further confirmed by sequence analysis performed on A Plasmid Editor using sequencing data provided by GENEWIZ's Cambridge, MA facility. Samples were prepared for sequencing according to GENEWIZ's pre-mixed guidelines.

Strain	**Primer**	**Primer Name**	**Sequence**
VC3166 (*arx-3*)	External Left	OK1122_external_for	ctt gga aat cgt ttt ggc at
	External Right	OK1122_external_rev	tta aaa ctc cgg cca atc ag
	Internal Left	OK1122_internal_for	aac cac ttt tcc tcg tcc ct
	Internal Right	OK1122_internal_rev	ttt tcg tgg caa att cct tc
VC3166 Amplicons	Wild type	...gatttttaatgttttcgatgt **[1688 BP DELETION]** gtttttgcggttaagccggtc…	
	ok1122	...gatttttaatgttttcgatgt ---------- gtttttgcggttaagccggtc...	
VC861 (*csn-5*)	External Left	OK1064_external_for	cct gaa aca gtc acc aac ga
	External Right	OK1064_external_rev	cag act tcc cct tcc cag tt
	Internal Left	OK1064_internal_for	ttt tct tgc tca gct tgt gg
	Internal Right	OK1064_internal_rev	cag aaa aca ggg gga ctt ga

VC861 Amplicons	Wild type	...tgagaattacttaatttgtt **- [1030 BP DELETION]** gcttgaaattttaaactttt...
	ok1064	...tgagaattacttaatttgtt **G----------** gcttgaaattttaaactttt...

Table 3. Primers Used for 10 Worm PCR. Primer names and sequences used to produce the indicated amplicons are shown. Primer pairs were designed by Meagan Sullender. Primers provided by IDT.

Cell Culture and Protein Expression

S3 Cell Culture Maintenance

Cells from the *D. melanogaster* S3 line (Duffy, WPI) were maintained at room temperature in 1X Schneider's medium (Gibco) supplemented with 12.5% FBS (Valley Biomedical). Cells were split when 80-90% confluent (every 3-4 days) using a 1:10 dilution. Following aseptic technique in the tissue culture hood, the old media was removed from the mother flask, and 4.5 mL and 5.0 mL of fresh media was added to the daughter and mother flask, respectively. The media in the mother flask was pipetted up and down 5-10 times, suspending the semi-adherent cells, and 0.5 mL of the suspension was added to the daughter flask. The media in the daughter flask was pipetted up and down 5-10 times, uniformly distributing the cells.

Transfection

In order to prepare cells for transfection, 2 mL of a 1:10 dilution of confluent cells was used to seed each well of one or more 6-well plates. When cells reached 80-90% confluence (2-3 days post seeding), 133 ng of Arm-GAL4 (Duffy, WPI) and 133 ng of each of the appropriate expression constructs were co-transfected using the Effectene reagent kit and protocol (Qiagen). Cells were harvested 3-5 days post-transfection for use in either a practice western blot or the co-immunoprecipitation system.

Biochemistry

Western Blotting

Transfections intended for practice western blotting were harvested by suspending and transferring 200 µl of cells from each well to correspondingly labeled 1.0 mL eppendorf tubes. The tubes were centrifuged at 8000 rpm for 5 minutes, the supernatant was removed and 20 µl of 5X sample buffer, 80 µl of TBS and 1 µl of BME was added to each tube. These were frozen at -20oC until use and were boiled for 5 minutes before being loaded into a gel.

Gels were prepared by first sandwiching clean glass plates together, clamping them into a casting apparatus, and seal testing them for 5 minutes using dH_2O. Meanwhile, the 10% separating gel solution was prepared in a 50 mL conical tube with 10% APS and TEMED added last. Working quickly, this solution was pipetted between the plates leaving about 3.0 cm of head space for the stacking gel, and the gel was immediately covered with 70% ethanol. After 40-60 minutes, the ethanol was poured off and the stacking gel solution, made with 10% APS and TEMED added last, was quickly pipetted to the top of the plates. The comb was slowly inserted at an angle after the stacking gel was poured and was removed after the gel had polymerized, 20-30 minutes later.

The gel plates were removed from the casting apparatus and placed into the electrophoresis apparatus. The inner chamber was filled about half way with 1X electrophoresis buffer, and the wells were rinsed several times by pipetting electrophoresis buffer into each well.

Then, the inner chamber was filled with additional buffer to cover the wells, and the outer chamber was filled to cover the bottom of the gels. 5.0 µl Fisher EZ-Run Pre-stained Rec Protein Ladder and prepared samples (from -20^{o}C freezer) were pipetted neatly into wells. The gels were run at 20mA (constant current) for 2-2.5 hours.

In preparation for protein transfer, one corner of a nitrocellulose/ECL membrane (GE Healthcare) was notched and transferred with tweezers into 1X transfer buffer to soak for 15-20 minutes. Fiber pads and Whatman paper were also allowed to soak in 1X transfer buffer in the transfer apparatus for 15-20 minutes. The stacking gel and a corner notch of the separating gel were cut off, and the gel was removed from between the glass plates and soaked in 1X transfer buffer in a tip box for 5 minutes. A transfer "sandwich" was then made by placing a fiber pad and a piece of Whatman paper on the black side of a cassette. The gel was carefully laid on the paper, and the membrane was placed over the gel (matching notched corners). The remaining Whatman paper and fiber pad were added over the membrane being careful to remove any air bubbles. Transfer "sandwiches" were then placed into the transfer apparatus (filled with 1X transfer buffer), matching black sides. An ice pack was placed into the transfer tank, and the transfer was run for 1 hour at 100V (constant voltage). Ladder bands appeared on membrane when transfer was complete. To confirm protein transfer, the membrane was stained with Ponceau for about 5 minutes, and the membranes were rinsed with dH_2O until bands were visible.

Immunoblotting began with blocking the nitrocellulose membrane in 20-25mL 5% NFDM in TBST at RT for 30 minutes to 1 hour. The membrane was then rinsed with TBST to

remove excess blocking solution, then incubated in 10 mL of the appropriate primary antibody diluted in TBST+NFDM overnight at 4°C. Invitrogen mouse monoclonal anti-V5 antibody - diluted 1:5,000 in 1% NFDM in TBST – was used for detection of V5-tagged proteins. Clontech mouse monoclonal anti-GFP antibody (JL-8) - diluted 1:1,000 in 0.5% NFDM in TBST – was used for detection of GFP-tagged proteins. The membrane was washed 5 times with 20-25 mL TBST - 5 minutes each wash - after the overnight incubation. The membrane was then incubated with a secondary antibody, Jackson ImmunoResearch Labs HRP conjugated goat anti-mouse - diluted 1:20,000 in 5% NFDM in TBST, for 1 hour at room temperature. The membrane was washed 5 times with 20-25 mL TBST - 5 minutes each wash - after the hour long incubation. During the final wash, 1.5 mL of the substrate working solution (1:1 peroxide:luminol solutions) was made for the gel. After the final wash, the membrane was removed with tweezers, dried slightly on a Kimwipe and placed on plastic wrap. The substrate working solution was applied uniformly to the membrane in a drop-wise fashion, and was allowed to incubate for 5 to 10 minutes. The membrane was then picked up, dried slightly on a Kimwipe, and placed inside a sheet protector in an autoradiography cassette.

The cassette was brought to the darkroom, and (under red light) one piece of film was placed in the cassette over the membrane and the cassette was closed. Exposure times varied between 30 seconds and 3 minutes. After exposure, the film was fed into the X-omat to develop; when it beeped, the lights were turned back on. The ladder bands and the 72 Da band label were drawn on after the film was developed.

Co-Immunoprecipitation (Protein A and His-tagged Magnetic Beads)

For the whole cell lysate (WCL) samples, 200 µl of each well was set aside in a labeled 1.0 mL eppendorf tube. This WCL sample was centrifuged at 8000 rpm for 5 minutes, and the supernatant was poured off. The pellet was resuspended using 100 µl of 1X sample buffer, and the sample was set aside at 4^{o}C until use.

For the immunoprecipitation samples, the remaining cells in each well were transferred to a 15 mL conical, and were pelleted at 2000rpm for 2 minutes at 4^{o}C, and the supernatant was poured off. These cells were lysed in 1.0 mL of ice cold EBC buffer, 2X protease inhibitors and 1X phosphatase inhibitors. Each conical was vortexed in order to completely resuspend the pellets. These lysates were left on ice for 15 minutes to complete the lysis. These lysates were then transferred to labeled 1.0 mL eppendorf tubes, and the insoluble fraction was pelleted at 14,000 rpm for 10 minutes at 4^{o}C. Meanwhile, the water block was preheated to 95^{o}C and 2X sample buffer (90 µl per column to be used) was heated.

The supernatant from each pelleted IP lysate (not WCL!) was transferred to fresh, labeled 1.0 mL eppindorf tubes and the antibody of choice was added to the samples. 2.0 µl of anti-GFP (Rabbit polyclonal from Clontech) was used per tube reaction. These samples were rotated for 30 minutes (min) to 2 hours (max). 100 µl of Protein A magnetic beads were added to each sample and the sample-bead mixes were incubated on a rotator for 30 minutes (min) to 2 hours (max) or even overnight.

Columns were placed onto the magnetic board with a waste container underneath, and each column was rinsed once with 200 µl of Lysis Buffer (EBC+ inhibitors). The cell lysates (for IP, not WCL) were applied to the columns, and were allowed to run through. The flow through was saved in labeled 1.0 mL eppindorf tubes in case the IP did not work. New waste tubes were then used when the columns were rinsed with 200ul of Lysis Buffer 4X, 100ul of Buffer X and then 100ul of Final Wash Buffer.

Fresh, labeled 1.0 mL eppindorf tubes were used for collection. Elution was performed by applying 20 µl of preheated 2X sample buffer to each column, and incubating for 5 minutes at room temperature. An additional 50 µl of preheated 2X sample buffer was added to each column, and the liquid elution was collected. The IP and WCL samples were boiled at 100^{o}C for 5 minutes, and loaded onto a western gel.

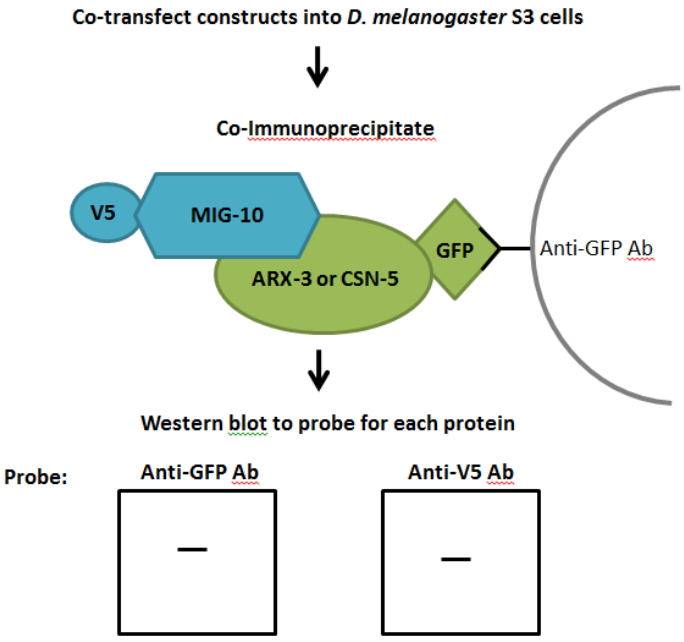

Figure 6: Co-Immunoprecipitation Strategy. DNA constructs were transfected into *D. melanogaster* S3 cells in order to produce proteins of interest. The GFP-tagged protein would be "pulled down" by the protein A magnetic

beads via the PC GFP antibody, and the V5-tagged protein would be “pulled down” by the GFP-tagged protein if there is an interaction. The co-immunoprecipitates were then run on a western blot and probed using anti-GFP or anti-V5 antibodies to detect the GFP or V5-tagged proteins respectively

Results

Interactions between MIG-10 and two candidate downstream proteins, CSN-5 and ARX-3, were studied *in vitro*, using a co-immunoprecipitation system, and *in vivo*, using mutant transgenic *C. elegans* strains. Expression clones containing the sequence of the ARX-3 and CSN-5 proteins were generated for use in an insect cell co-immunoprecipitation system, and western blots were performed in order to probe co-immunoprecipitates for proteins of interest. Mutant transgenic *C. elegans* strains were generated in order to quantitate the excretory canal and migratory neuron truncation phenotypes of worms possessing either *arx-3 (ok1122)* or *csn-5 (ok1064)* mutation.

Expression Clones Used in the CO-IP System

Full-length Att flanked *arx-3* was generated by PCR from *C. elegans* library DNA stock using forward and reverse primers. During the BP cloning reaction, Att sites were recombined producing an entry clone containing full-length *arx-3*. The entry clone DNA was transformed into *E. coli* which was then plated and colonies were cultured in liquid media and plasmid DNA was extracted via Miniprep. Miniprep DNA was then digested with BanII and run on an electrophoresis gel (Figure 7.).

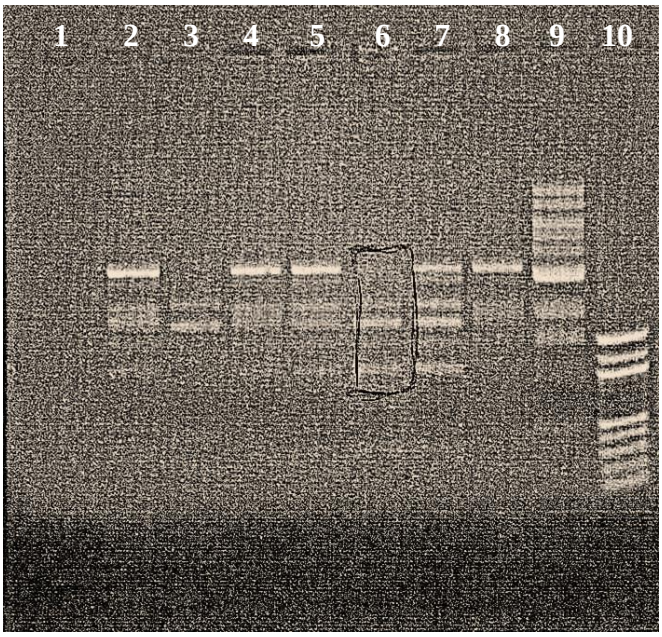

Figure 7. Electrophoresis Gel of Digested Entry Clones. Entry clones generated via BP Clonase reaction were digested with BanII and run on a 0.8% agarose electrophoresis gel.

This gel shows a partial digest of candidate entry clones; DNA corresponding to lanes 2, 4, 5 and 7 had bands of the appropriate size, and therefore underwent sequencing analysis. The clone from lane 2 (entry clone 7a) exhibited the correct sequence, highest DNA concentration and least protein contamination, so a sample of the *E. coli* culture for entry clone 7a was frozen down (1:1 ratio of *E. coli* culture to 50% glycerol) into the -80oC glycerol stocks for future use.

The DNA from entry clone 7a was used in order to generate expression clones tagged with GFP. During the LR cloning reaction, Att sites were recombined producing an expression clone containing full-length *arx-3*. The expression clone DNA was transformed into *E. coli* which was then plated and colonies were cultured in liquid media and plasmid DNA was extracted via Miniprep. Miniprep DNA was then digested with HindIII and run on an electrophoresis gel. This cloning step was repeated four times where *E. coli* yielded no plasmid DNA.

An antibiotic dosage experiment where liquid cultures were grown in media supplemented with 1.0 µg/mL, 2.0 µg/mL or 4.0 µg/mL ampicillin was performed in order to investigate the possibility of weak selection for the growth of only *arx-3*$^{+}$ *E. coli.* Two cultures (at 2.0 µl/mL ampicillin) exhibited growth, and plasmid DNA was extracted via Miniprep after less than 12 hours of incubation. The Miniprep DNA was run on an electrophoresis gel before a restriction digest was attempted (Figure 8.).

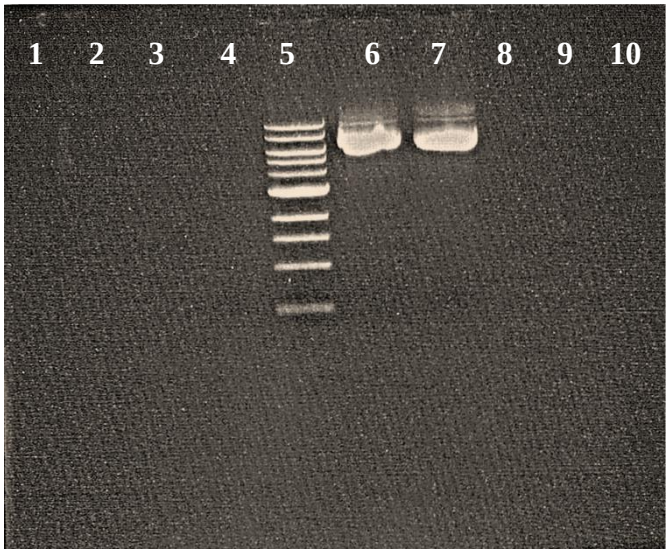

Figure 8. Electrophoresis Gel of Undigested Expression Clones. Entry clones generated via LR Clonase reaction via an antibiotic dosage experiment were run on a 0.8% agarose electrophoresis gel.

This gel shows uncut candidate expression clones generated from the antibiotic dosage experiment. DNA corresponding to lanes 6 and 7 had bands of the appropriate size, and were therefore digested with HindIII and run on an electrophoresis gel (Figure 9.).

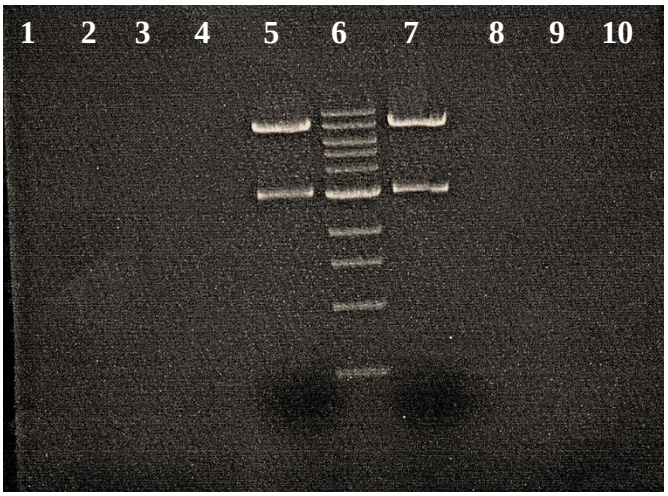

Figure 9. Electrophoresis Gel of Digested Expression Clones. Entry clones generated via LR Clonase reaction were digested with HindIII and run on a 0.8% agarose electrophoresis gel.

This gel shows a complete digest of expression clones generated from the antibiotic dosage experiment; DNA corresponding to lanes 5 and 7 had bands of the appropriate size, and therefore underwent sequence analysis. The clones from both lanes 5 and 7 (expression clone 1 and 2) exhibited the correct sequence, high DNA concentration and low protein contamination, so samples of the *E. coli* culture for expression clones 1 and 2 were frozen down (1:1 ratio of *E. coli* culture to 50% glycerol) into the -80oC glycerol stocks for future use. Entry clone 1 was selected for use in transfections. This GFP-tagged full-length *arx-3* plasmid was then ready for use in the insect cell co-immunoprecipitation system along with the *csn-5* and partial-length *arx-3* GFP-tagged plasmids which were generated in the summer of 2011 by Meagan Sullender.

Do Candidate Proteins Interact with MIG-10 *In Vitro*?

The MIG-10/subunit (ARX-3 and CSN-5) interaction was identified using a yeast two hybrid system (Gossellin and O'Toole, 2008) in a previous MQP. Even though the results were confirmed by retransformation, the positive results could have been caused by self-activation so

the interaction needed to be confirmed using an entirely different system. Co-immunoprecipitation (Co-IP) was chosen to be the biochemical system to further confirm the MIG-10/subunit interaction. The plasmids containing GFP-tagged *csn-5* and *arx-3*, which were created via the Gateway System©, were used in this Co-IP system.

Simple western blots were performed initially, in order to assess the transfection efficiency of the plasmids and stability of the expressed proteins. The plasmids containing GFP-tagged *csn-5* and *arx-3* (one construct per well) were co-transfected into nearly confluent D. melanogaster S3 cells along with Arm::Gal-4 using an effectine based technique resulting in protein expression in about 10% of the cells three to five days after transfection (Figure 10). Cells were harvested on day four post transfection, run on an acrylamide gel, and probed with monoclonal anti-GFP antibodies (JL-8) to detect the GFP-tagged proteins. The blots (results not shown) showed good protein expression, so co-transfection of plasmids for the co-immunoprecipitation system began.

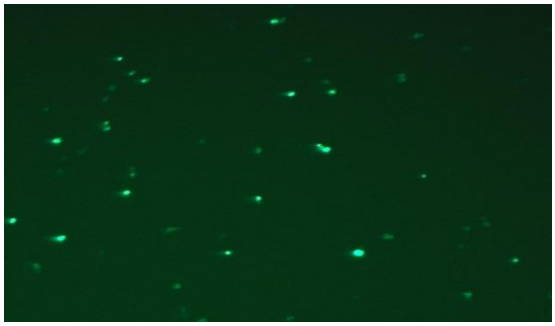

Figure 10. Transfected S3 Cells Expressing GFP-tagged Proteins. . The plasmids containing GFP-tagged *csn-5* and *arx-3* (one construct per well) were co-transfected into nearly confluent D. melanogaster S3 cells along with Arm::Gal-4 using an effectine based technique resulting in protein expression in about 10% of the cells three to five days after transfection. This photo was taken on day 3 post-transfection ("single" construct transfection), and this GFP expression level was considered to be the minimum level acceptable to later run a Co-IP from a co-transfection.

Generation of samples for use in the Co-IP system began when the plasmids containing GFP-tagged *csn-5* and *arx-3* (one construct per well) were co-transfected singly and with *mig-10a*::V5 into nearly confluent *D. melanogaster* S3 cells along with Arm::Gal-4. In addition, GFP-tagged *abi-1* WT and mutant *abi-1* were co-transfected with *mig-10a*::V5 and were used as positive and negative controls respectively. Cells were harvested on day four post transfection, run through the Co-IP system, run on an acrylamide gel, and probed with monoclonal anti-GFP antibodies (JL-8) to detect the GFP-tagged proteins (CSN-5, ARX-3, ABI-1 WT and ABI-1 Mutant) and monoclonal anti-V5 antibodies to detect V5-tagged MIG-10.

Initial Co-IP experiments yielded minimal protein expression, and what small protein concentration was present in the samples, was reduced even more when the samples were run through the Co-IP system.

A plasmid DNA concentration experiment was performed in order to investigate as to whether plasmid DNA concentration could influence the concentration of expressed proteins. The plasmid DNA concentrations were adjusted for transfection as follows; a "control" transfection was performed with the usual 2:2:2 ratio (2.0 µl of 66.6 ng/ µl DNA per plasmid) of Arm::Gal4 to MIG-10::V5 to ARX-3::GFP, then four experimental transfections were performed with a 4:2:2 ratio, a 4:4:2 ratio, a 4:2:4 ratio, and a 4:4:4 ratio. The transfections performed with the 4:2:2 and 4:2:4 ratios demonstrated significantly brighter fluorescence than any of the other transfections, and were therefore run through the Co-IP system along with the 2:2:2 ratio "control" transfection and appropriate Co-IP controls. The samples were then run on an acrylamide gel, and the blots were probed with anti-GFP and anti-V5 antibodies. The 4:2:2 ratio

proved to produce higher apparent protein concentrations (bands in lanes 2 and 9 were broader and darker than those in lane 8) and produced a positive result (see Figure 12), so this ratio was used in all future transfections rather than the previously used 2:2:2 ratio.

								WCL	
Constructs	1	2	3	4	5	6	7	8	9
ARM::Gal-4	2	4	4	2	2	2	2	4	4
ARX-3::GFP	2	2	2	2				2	2
MIG-10::V5	2	2	4		2	2	2	2	4
ABI-1::GFP WT						2			
ABI-1::GFP Mutant							2		

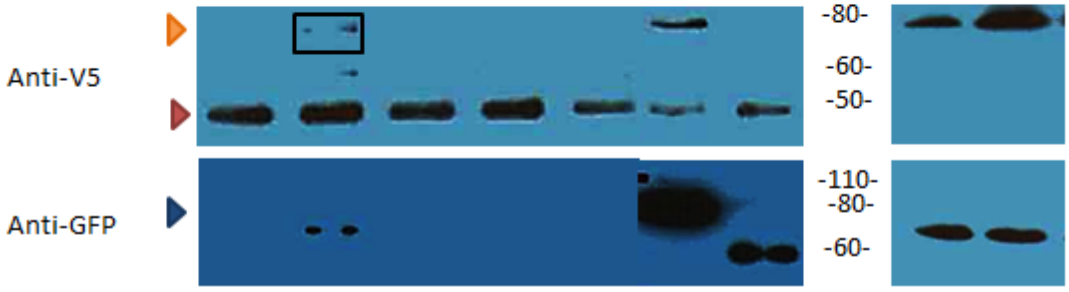

Figure 11. Blot of ARX-3 and MIG-10 Co-Immunoprecipitate. Constructs were transfected as shown in the table. Cell lysates were immunoprecipitated with anti-GFP antibody, and immunoblotted with either an anti-V5 antibody (MIG-10 constructs) or and anti-GFP antibody (ARX-3 or ABI-1 constructs). Molecular weight markers are shown. Yellow arrow shows MIG-10::GFP at 79 kDa; blue arrow shows ARX-3::GFP at 64 kDa and red arrow shows Co-IP background band at about 50 kDa.

This figure shows that there is an interaction between MIG-10 and ARX-3 in the co-immunoprecipitation system. The anti-GFP blot (bottom) showed the presence of ARX-3 in lane 2 (blue arrow) with a band of the appropriate size, while the anti-V5 blot (top) showed the presence of MIG-10 (boxed) with a band of the appropriate size. This result shows that MIG-10 was indeed pulled down by ARX-3 indicating an *in vitro* interaction between the two proteins. WCL (lanes 8 and 9) samples showed the presence of ARX-3 when probed with anti-GFP and the presence of MIG-10 when probed with anti-V5. The positive (ABI-1 WT) and negative (ABI-1 Mutant) controls (lanes 6 and 7) in this experiment showed that co-immunoprecipitation

was possible, and that the pull down was not random or nonspecific, respectively. Also, all Co-IP lanes contained a background band (red arrow). Previous work has shown that this band is due to reaction of the antibodies with Protein A sepharose (McShea, 2011).

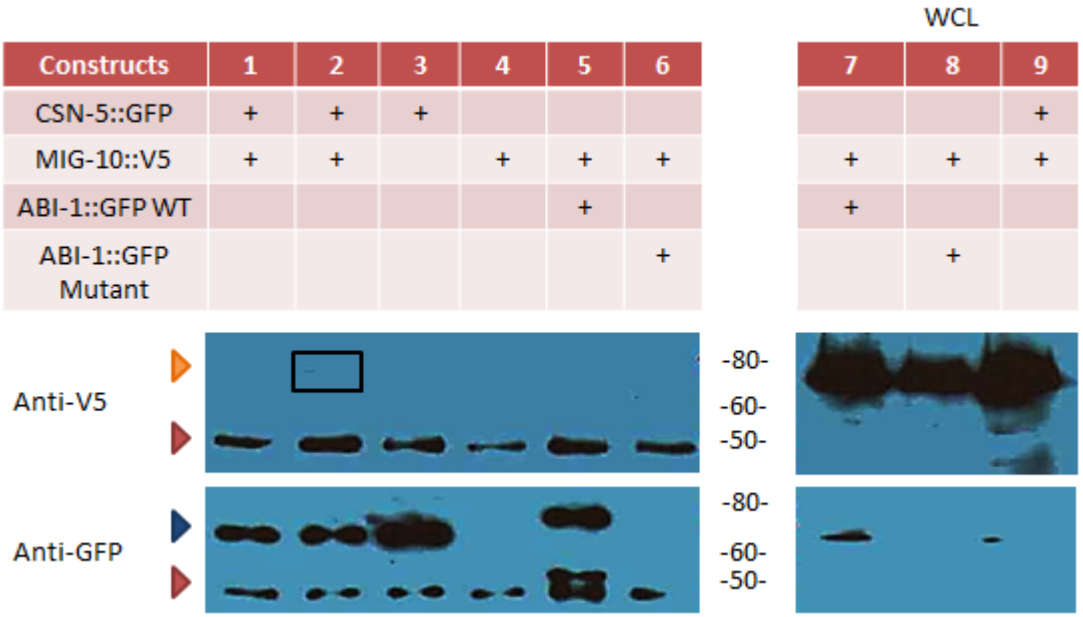

Figure 12. Blot of CSN-5 and MIG-10 Co-Immunoprecipitate. Constructs were transfected as shown in the table according to the 4:2:2 DNA ratio described in the text. Cell lysates were immunoprecipitated with anti-GFP antibody, and immunoblotted with either an anti-V5 antibody (MIG-10 constructs) or and anti-GFP antibody (ARX-3 or ABI-1 constructs). Molecular weight markers are shown. Yellow arrow shows MIG-10::GFP at 79 kDa; blue arrow shows ARX-3::GFP at 70 kDa and red arrow shows Co-IP background band at about 50 kDa.

This figure suggests that there may be an interaction between MIG-10 and CSN-5 in the co-immunoprecipitation system. The anti-GFP blot (bottom) showed the presence of CSN-5 in lane 1-3 with bands of the appropriate size (blue arrow), while the anti-V5 blot (top) showed the presence of MIG-10 (boxed) in lane 2 with a faint band of the appropriate size. This result shows that MIG-10 was indeed pulled down by CSN-5 indicating an *in vitro* interaction between the two proteins. WCL of the lane 2 sample (lane 9) showed the presence of CSN-5 when probed with anti-GFP and the presence of MIG-10 when probed with anti-V5. As for the previous experiment, the positive (ABI-1 WT) and negative (ABI-1 Mutant) controls (lanes 5 and 6) in

this experiment showed that the pull down was not random or unspecific. Also, all Co-IP lanes contained a background band (red arrow).

With these two positive results, it can be said that preliminary results from the co-immunoprecipitation system suggest that there may indeed be an interaction between MIG-10 and the candidate proteins, ARX-3 and CSN-5.

Do Candidate Proteins Interact with MIG-10 *In Vivo*? (Ongoing)

Confirming the *in vivo* interaction between MIG-10 and the two protein subunits (ARX-3 or CSN-5) was achieved by creating mutant transgenic *C. elegans* strains labeled with *pgp-12*::GFP which marks the excretory cell and with *flp-20*::GFP which marks the migratory neurons ALM and AVM. This was done in order to quantitate the excretory canal and migratory neuron truncation phenotypes of worms possessing either *arx-3 (ok1122)* or *csn-5 (ok1064)* mutation.

After the last crossing step, L4 hermaphrodites were picked to single plates in order to determine the homogeneity. The desired strain demonstrated a homozygous *pgp-12*::GFP marker genotype (100% fluorescent excretory canal phenotype across worms) and a heterogeneous *csn-5 (ok1064)* or *arx-3 (ok1122)* with balancer genotype (determined by vulval mass or survival rate phenotype across worms, respectively). Table 4 shows the singled L4s and the egg/worm counts to objectively determine survival rates for the *arx-3* worms. The plates of *csn-5* worms were scanned for the vulval masses, but were not counted.

Strain/Cross Name	Plate Number	Eggs?	Egg Count	Worm Count	Percent Worms
pgp-12;csn-5 X self (Progeny)	1	YES	x	X	
pgp-12;csn-5 X self (Progeny)	2	YES	x	X	
pgp-12;csn-5 X self (Progeny)	3	YES	x	X	
pgp-12;csn-5 X self (Progeny)	4*	YES	x	X	
pgp-12;csn-5 X self (Progeny)	5	YES	x	X	
pgp-12;arx-3 X self (Progeny)	1	YES	78	0	0%
pgp-12;arx-3 X self (Progeny)	2	YES	68	29	43%
pgp-12;arx-3 X self (Progeny)	4	No**			
pgp-12;arx-3 X self (Progeny)	5	YES	46	19	41%
pgp-12;arx-3 X self (Progeny)	9	YES	46	20	43%
pgp-12;arx-3 X self (Progeny)	10*	YES	79	12	15%
pgp-12;arx-3 X self (Progeny)	12	YES	60	26	43%
csn-5 (Parent Strain)	1	YES	x	X	
csn-5 (Parent Strain)	2	YES	x	X	
arx-3 (Parent Strain)	1	YES	40	12	30%
arx-3 (Parent Strain)	2	YES	78	19	24%

Table 4. Strain Verification Experiment for C. elegans Strains Possessing Marked Excretory Cells. *These plates were used in quantitation experiments. **This *arx-3* worm appeared to be an "escaper" as it grew to maturity, but was sterile. The 'Strain/Cross Name' column is not the real genotype of the strain, but rather a shorthand for the mutant/balancer strain produced.

This table shows the singled L4s and the egg/worm counts to objectively determine survival rates for the *arx-3* worms (RY 1162). The plates of csn-5 worms (RY 1160) were scanned for vulval masses, but were not counted. The plate numbers marked with an asterisk were believed to be the sought after strains and were maintained and used in quantitation experiments. The *csn-5* plate chosen had the most obvious and highest incidence of worms exhibiting vulval masses (subjective determination) because this is the phenotype of worms homozygous for *csn-5 (ok 1064).* The *arx-3* plate chosen had the lowest survival rate, which most closely resembled that of the parent strain, where worms homozygous for *arx-3 (ok 1122)* are thought to die early in development due to the "lethality" of the *arx-3 (ok 1122)* mutation.

The "No" marked with a double asterisk was believed to be an *arx-3* worm which appeared to be an "escaper" as it grew to maturity, but was sterile, thus evolutionarily "dead". So perhaps this sterility is a potential phenotype for worms homozygous for *arx-3*.

Strain/Cross Name	Plate Number	Eggs?	Egg Count	Worm Count	Percent Worms
flp-20;csn-5 X self (Progeny)	1	YES	x	X	
flp-20;csn-5 X self (Progeny)	2	YES	x	X	
flp-20;csn-5 X self (Progeny)	3	YES	x	X	
flp-20;csn-5 X self (Progeny)	4*	YES	x	X	
flp-20;csn-5 X self (Progeny)	5	YES	x	X	
flp-20;csn-5 X self (Progeny)	6	YES	x	X	
flp-20;csn-5 X self (Progeny)	7	YES	x	X	
flp-20;csn-5 X self (Progeny)	8	YES	x	X	
flp-20;csn-5 X self (Progeny)	9*	YES	x	X	
flp-20;csn-5 X self (Progeny)	10	YES	x	X	
flp-20;arx-3 X self (Progeny)	1*	YES	62	21	34%
~~**flp-20;arx-3 X self (Progeny)**~~	~~2~~	~~YES~~	~~2~~		
flp-20;arx-3 X self (Progeny)	3	YES	16	7	44%
flp-20;arx-3 X self (Progeny)	4	YES	111	99	89%
flp-20;arx-3 X self (Progeny)	5*	YES	84	30	36%
flp-20;arx-3 X self (Progeny)	6	YES	37	18	49%
flp-20;arx-3 X self (Progeny)	7	YES	142	113	80%
flp-20;arx-3 X self (Progeny)	8	YES	75	32	43%
~~**flp-20;arx-3 X self (Progeny)**~~	~~9~~	~~YES~~			
flp-20;arx-3 X self (Progeny)	10	YES	60	28	47%
flp-20;arx-3 X self (Progeny)	11	YES	116	46	40%
flp-20;arx-3 X self (Progeny)	12	YES	70	28	40%
csn-5 (Parent Strain)	1	YES	x	X	
csn-5 (Parent Strain)	2	YES	x	X	
arx-3 (Parent Strain)	1	YES	77	22	28%
arx-3 (Parent Strain)	2	YES	34	10	29%

Table 5. Strain Verification Experiment for C. elegans Strains Possessing Marked Migratory Neurons. *These plates were maintained and were used in quantitation experiments. The 'Strain/Cross Name' column is not the real genotype of the strain, but rather a shorthand for the mutant/balancer strain produced.

This table shows the singled L4s and the egg/worm counts to objectively determine survival rates for the *arx-3* worms (RY 1163). The plates of *csn-5* worms (RY 1161) were scanned for vulval masses, but were not counted. The plate numbers marked with an asterisk were believed to be the sought after strains and were maintained and used in quantitation experiments. The *csn-5* plate chosen had the most obvious and highest incidence of worms exhibiting vulval masses (subjective determination) because this is the phenotype of worms homozygous for *csn-5 (ok 1064).* The *arx-3* plate chosen had the lowest survival rate, which most closely resembled that of the parent strain where worms homozygous for *arx-3 (ok 1122)* are thought to die early in development.

These counting experiments were performed in order to distinguish between balancer/mutant and balancer / + in the last step of each cross. This was done because both *arx-3* and the balancer used increase lethality, so the desired strain of *arx-3* / balancer was distinguished from +/balancer by looking for animals with fewer progeny.

Ten worm PCRs are currently underway to further confirm that the generated strains for ARX-3 (RY 1162 and RY 1163) and CSN-5 (RY 1160 and RY 1161) are correct. PCR samples are being run on 0.8% acrylamide gels and band sizes are being compared to those of the parent strains. Each strain is currently being quantitated in order to study mutant phenotypes which may indicate an *in vivo* interaction. Some preliminary photos of mutant *pgp-12 C. elegans* strains as compared to wild type are shown below, no obvious excretory cell phenotypes have been seen.

Figure 13. Mutant Transgenic *C. elegans* Strains (a) CSN-5; pgp-12, (b) WT pgp-12 and (c) ARX-3; pgp-12. A sampling of photos being used to quantitate the excretory cell truncation phenotypes of the RY 1162 and RY 1161 *C. elegans* strains as compared to wild type worms.

Discussion

The overall goal for this Major Qualifying Project (MQP) was to determine whether ARX-3, an Arp2/3 subunit or CSN-5, a COP-9 SigNalosome subunit, interact with MIG-10 to promote axon outgrowth and migration. The MIG-10/subunit interaction was previously identified using a yeast two hybrid system (Gosselin and O'Toole, 2008). This MQP further confirms the MIG-10/subunit interaction *in vitro*, by using an insect cell expression system, and *in vivo*, using mutant transgenic *C. elegans* strains. Preliminary results indicate interaction *in vitro* between MIG-10 and both proteins. *In vivo* experiments are ongoing.

The preliminary result that ARX-3 and CSN-5 interact with MIG-10 to promote neuronal outgrowth and migration does support the findings of a prior MQP where a yeast-two hybrid assay suggested both interactions, but there is insufficient data to confidently confirm the interaction as there was only one successful repetition of each protein specific co-immunoprecipitation experiment whereas three repetitions of each would have been significant. In addition, the "successful" experiments yielded weak bands at high exposure times, while darker bands at lower exposure times would have been more desirable. These results were most likely due to technical difficulties with the assays (cell health, wash stringency, incubation times etc.), so ultimately, these experiments should be continued and repeated for more definite results.

There are many aspects of the techniques used in this project that should be noted or changed when experiments are repeated in the future. First, when performing cloning experiments, care should be taken to use antibiotic dosage experiments in the event of difficulties

with plasmid uptake and production as higher antibiotic dosage was found to remedy the problem. Secondly, when co-immunoprecipitation experiments are repeated, special attention should be paid to the health quality of the *D. melanogaster* S3 cells. Over the course of this project the S3 cells deteriorated in condition from fast growing with a round morphology to slow growing displaying a branched morphology. The first two out of six co-immunoprecipitation experiments yielded interpretable results while the last four yielded no reliable results. This was likely due to suboptimal DNA uptake at transfection due to deteriorated health. An investigation into cell culture techniques, perhaps using conditioned media or reducing splitting ratios temporarily, may remedy this problem in the long run. Lastly, co-immunoprecipitations could be improved through alterations in antibody/bead incubation times by lengthening the incubation times, and alterations in the salinity or pH of washes and buffers in order to reduce their stringency.

The *in vivo C.elegans* experiments are ongoing and the mutant strains do appear to be correct, but PCR confirmation and a larger quantitation sample size is required before any remarks can be made about the *in vivo* interaction of ARX-3 and CSN-5 with MIG-10. Through subjective observation, the *csn-5::pgp-12:GFP* strain does appear to have a very slight excretory cell truncation while the *arx-3::pgp-12:GFP* strain appears to demonstrate wild type excretory cells. However, the worms that can be observed as the L4 stage in the *arx-3* strain are likely heterozyogotes, since the *arx-3* mutation is thought to be embryonic lethal.

In the future, the *csn-5* strain quantitation will continue at the L4 stage as usual but it should be advised to quantitate the *arx-3* strains as egg preps so that the embryonic phenotype

can be measured before the lethality of the mutation takes effect. Also, the strains should be frozen down for future use after a sample of each undergoes PCR and perhaps sequencing analysis in order to confirm and preserve the strains for future research.

This interaction or lack thereof should be confirmed in the future, and if such an interaction was to be confirmed with significant data, then an effort should be made in order to investigate any potential interaction domains of ARX-3 and CSN-5 that interact with the various domains of MIG-10. This could be achieved by creating deletion mutants of both ARX-3 and CSN-5 and performing pull down experiments first with the deletion mutants and MIG-10a, and then with the confirmed domain of the candidate proteins with stock deletion mutants of MIG-10a. This way more detail can be understood of the signaling cascade which promotes neuronal outgrowth and migration, and can help researchers better understand MIG-10 orthologs, like lamellipodin, and vertebrate ARX-3 and CSN-5 proteins and how they interact in the vertebrate neuronal guidance system during development. This research could ultimately lead to a much better understanding of many neurodevelopmental disorders such as autism and Down's syndrome. Understanding the processes responsible for neuronal connectivity would not only help elucidate the causes of neurodevelopmental disorders, but could also provide various new or improved treatment options for individuals with nerve damage due to injury such as a traumatic brain injury, or neurodegenerative disorders such as Alzheimer's disease.

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Recipe Appendix

For Protein Gels and Westerns

Stock Solutions:

2M Tris-HCl (pH 8.8; adjust with HCl and autoclave)
1M Tris-HCl (pH 6.8; adjust with HCl and autoclave)
10% SDS
1% bromophenol blue (dissolve as completely as possible, centrifuge and keep supernatant)

Working Solutions (all should be kept at 4°C unless stated otherwise):

Solution A (acrylamide mix) [100 mL]:
29.2% acrylamide [29.2 g]
0.8% bis-acrylamide [0.8 g]

Solution B (4X separating gel buffer) [50 mL]:
1.5M Tris-HCl (pH 8.8) [37.5 mL 2M]
0.4% SDS [2 mL 10%]
dH_2O [10.5 mL]

Solution C (4X stacking gel buffer) [50 mL]:
0.5M Tris-HCl (pH 6.8) [25 mL 1M]
0.4% SDS [2 mL 10%]
dH_2O [23 mL]

*10% APS **(-20°)** [1 mL]:*
10% APS [0.1g]
dH_2O [1 mL]

5X Sample Buffer [5 mL; then make 1mL aliquots]:
60mM Tris-HCl (pH 6.8) [0.3 mL 1M]
25% glycerol [1.25 mL 100%]
2% SDS [1 mL 10%]
14.4mM β-mercaptoethanol [0.25 mL]
0.1% bromphenol blue [0.5 mL 1%]
dH_2O [1.7 mL]

*10X Electrophoresis Buffer **(RT)** [1 L]:*
250mM Tris [30 g]
1.92M glycine [144 g]

1% w/v SDS [10 g]
dH_2O [to 1 L]

*20X Transfer Buffer **(RT)*** *[1 L]*
312mM Tris [38.6 g]
2.4M glycine [180 g]
dH_2O [to 1 L]

Separating Gel (at least 5mL per gel)

	5 mL	**10 mL**	**15 mL**	**20 mL**
dH_2O	2.42	4.84	7.26	9.68
Solution A	1.33	2.66	3.99	5.32
Solution B	1.25	2.5	3.75	5
10% APS	0.025	0.05	0.075	0.1
TEMED	0.0025	0.005	0.0075	0.01

Stacking Gel (5 mL per 2 gels)

	5 mL	**10 mL**
dH_2O	2.3	4.6
Solution A	0.67	1.34
Solution C	1	2
10% APS	0.03	0.06
TEMED	0.005	0.01

For Co-Immunoprecipitations

EBC Buffer (aka NP-40 Buffer):
50 mM Tris, pH 8
150 mM NaCl
2 mM EDTA
0.5% NP-40

Buffer X:
50 mM Tris, pH 8.5
250 mM NaCl
2 mM EDTA
1% NP-40

Final Wash Buffer:
50 mM Tris, pH 8.5

Protease inhibitor cocktail:
Dissolve 1 Roche Complete EDTA-free protease inhibitor cocktail tablet in 2 mL dH_2O to produce a 25X stock. Aliquot and store at -20°C.

Phosphatase inhibitor cocktail:	Stock [C]	Solvent
1 mM Sodium orthovanadate	200 mM	dH_2O
5 mM Sodium pyrophosphate	100 mM	dH_2O
5 mM Sodium fluoride	100 mM	dH_2O