A Thesis

entitled

Rho-Family GTPase Signaling in the Nervous System: An Analysis of the *C. elegans* RhoGEF UNC-73

by

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Submitted to the Graduate Faculty as partial fulfillment of the requirements for the

Master of Science Degree in Biology

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An Abstract of

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Rho-family GTPases regulate various neuronal signaling pathways that are critical for proper nervous system development and function. Defects in these signaling pathways can cause severe behavioral phenotypes including a reduction in cognitive and intellectual abilities in humans. RhoGEFs are critical activators of Rho-family GTPase pathways. UNC-73 is an evolutionarily conserved C. elegans RhoGEF that regulates cytoskeletal rearrangement during axon guidance and modulates neurotransmission to control locomotory behavior. unc-73 encodes multiple differentially expressed isoforms, some of which contain the putative lipid binding domain, Sec14, at their N-termini. This study is an examination of UNC-73 isoform function and localization, focusing on the role of the UNC-73 Sec14 domain in the nervous system. Transgenic UNC-73 Sec14 domain expression, independent of full-length UNC-73, is localized to punctate subcellular regions in the cell bodies that may coincide with the Golgi. The transgenic animals overexpressing the UNC-73 Sec14 domain exhibit uncoordinated locomotion similar to unc-73 RhoGEF1 mutants suggesting UNC-73(Sec14) overexpression alters endogenous UNC-73 localization and/or function. Animals overexpressing UNC-
73(Sec14) also develop more slowly compared to wildtype, but this phenotype may result from interference with the function of other Sec14 domain-containing proteins. These data point to the possible importance of the Sec14 domain in nervous system function and protein localization.

To further characterize UNC-73 function, the *unc-73c1* expression pattern was examined and a potential UNC-73C1 interaction with the Rab11 binding protein FIP-3/4 was characterized *in vitro*. UNC-73C1 localizes to a subset of neurons, including sensory neurons and interneurons, but not motorneurons. UNC-73C1 expression in these neurons rescues the *unc-73* lethargic movement phenotype, indicating UNC-73 activity is required in upstream modulatory neurons to maintain a wild-type locomotion rate. Together, these results give us a better understanding of how the RhoGEF, UNC-73, functions in the nervous system and suggest new avenues for further study of UNC-73 homologs in mammals.
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List of Abbreviations

ACh...........................Acetylcholine
Cadherin....................Calcium dependent CAM
CAM ..........................Cell adhesion molecule
cAMP .........................Cyclic adenosine monophosphate
CGR ..........................CRAL-Trio and GOLD domain
DAG ..........................Diacylglycerol
DCV ..........................Dense core vesicles
DCC ..........................Deleted in colorectal cancer
DEB ..........................Dense body
DH ............................Dbl homology
DYS ..........................Dystrophin
EAT ..........................Eating deficient-abnormal pharyngeal pumping
EGL ..........................Egg-laying defective
FIP ..........................Family interacting protein
FnIII .........................Fibronectin type III
GABA ..........................Gamma-aminobutyric acid
GAP ..........................GTPase activator protein
GEF ..........................Guanine nucleotide exchange factor
GFP ..........................Green fluorescent protein
GPCR ..........................G-Protein coupled receptor
GST ..........................Glutathione S-transferase
GTP ..........................Guanosine-5’-triphosphate
His ..........................Histidine
HRP ..........................Horseradish peroxidase
ICR ..........................Intercistronic region
Ig ............................Immunoglobulin
IP3 ..........................Inositol 1,4,5-triphosphate
LAR ..........................Leukocyte common antigen-related
LIN ..........................Abnormal cell lineage
N2 ............................Wildtype (Bristol)
NGM ..........................Nematode Growth Media
OP50 ..........................Strain of E. coli
PAGE ..........................Polyacrylamide gel electrophoresis
PAM....................Peptidylglycine α-amidating monooxygenase
pDEST..................Gateway® cloning destination vector
pDONR..................Gateway® cloning donor vector
PH........................Pleckstrin homology
PIP2....................Phosphatidylinositol 4,5-bisphosphate
PITP.....................Phosphatidylinositol transfer protein
PKA......................Protein kinase A
PLCβ.....................Phospholipase C β
PSD......................Post-synaptic density

RGA.....................Rho GTPase activating protein
ROCK.....................Rho associated protein kinase

SDS......................Sodium dodecyl Sulfate
SH3......................Src homology 3
SV.........................Synaptic vesicles

Trio......................Triple function domain protein

UNC......................Uncoordinated
Chapter One

1. Introduction

Rho-family GTPase signaling regulates multiple fundamental cellular processes such as differentiation, cell division, vesicle transport, and control of cytoskeletal organization (Lundquist, 2006). In the nervous system, Rho-family GTPases control many processes including neurite outgrowth and the regulation of synaptic connections (van Galen and Ramakers, 2005). These processes are required for nervous system development and function, and the ability of an organism to control its behavior. Studying these processes at the molecular level in Caenorhabditis elegans, can lead to a better understanding of how neurons develop and how neural networks are regulated. The importance of Rho-family GTPase signaling pathways is highlighted by the fact that defects in these pathways in humans result in intellectual disabilities (Ba et al., 2013; Ramakers, 2002). We are especially interested in the conserved RhoGEF (Rho Guanine Nucleotide Exchange Factor) activator of Rho-family signaling pathways, UNC-73, and its role in nervous system development and the modulation of nervous system function.

1.1 Nervous System

The human nervous system is composed of two major cell types, neurons and neuroglia. Neurons are highly specialized for the processing and transmission of cellular signals and are the functional units of the nervous system whereas glial cells are responsible for structural support, insulation, and protection of neurons. The human nervous system is composed of billions of neurons and trillions of synapses, allowing for exceedingly complex patterns of dynamic activity. At their simplest, neurons are composed of three main structural regions: dendrites, cell bodies, and axons and signal on
to one another through both chemical synapses and gap junctions (Cohen, 1973). Dendrites receive and integrate signals from other neurons and propagate those signals to the cell body. In mammals, dendrites also have specialized dynamic structures called dendritic spines that increase the surface area of the dendrite (Ebrahimi and Okabe, 2014). More dendritic spines allows for a higher level of synaptic activity. The plasticity of these structures is associated with memory, learning, and motivation (Roberts et al., 2010). The cell body contains the nucleus and other organelles common to living cells. Neurotransmitters are chemical signals that allow communication between neurons (Snyder and Innis, 1979). Neurotransmitter synthesis can occur in the cell body or peripherally at the synapse, with the axonal transport of both mRNAs and ribosomes. The axon is primarily responsible for conducting signals away from the cell body and synapsing onto one or more other neurons or cells. Neurotransmitters produced in the cell body are sent to the axon for packaging and release at its terminus (Cohen, 1973). The specificity of synapse formation and neuronal circuitry requires the precise execution of developmental events, including cell migration, axon guidance, dendritic growth, synaptic target selection, and synaptogenesis (Salie et al., 2005).

1.2 Axon Guidance during Embryonic Development

Axon guidance is a complex stage in the development of neuronal circuits that is required for proper nervous system function. Axon guidance occurs during embryogenesis when the nascent axons of neurons extend outward towards their designated targets (Tessier-Lavigne and Goodman, 1996). Axons are directed by an actin-supported extension at the tip, which is known as the growth cone. Axonal outgrowth occurs when actin polymerization begins at the growth cone’s leading edge,
causing directional migration (Huber et al., 2003). Many chemical signals, including proteins and other molecules are present in the extracellular matrix to regulate the directionality of the extending growth cone. Directional guidance is typically controlled by gradients of both attractive and repellent signals, including netrins such as netrin-1 (UNC-6) that either attract or repel axons based on the receptors they activate on the growth cone. Netrins are attractive when bound to DCC (UNC-40) and repulsive when bound to netrin-1 receptor (UNC-5) (Hong et al., 1999; Song and Poo, 1999). Similarly, Ephrins binding to Eph receptors can be both attractive and repulsive (Egea and Klein, 2007). Sources of repulsive cues include Slit/Robo signaling and Semaphorins which activate Plexins and Neuropilins (Bagnard et al., 1998; Wong et al., 2002). These guidance cues and receptors mediate actin cytoskeletal reorganization by activating downstream Rho GTPases (Shekarabi and Kennedy, 2002). For example, netrin-1 binds DCC, which activates downstream Rac1. Trio (Triple Function Domain), a RhoGEF, interacts with DCC to promote Rac1 activation, leading to actin cytoskeletal reorganization for directed axon growth (Briancon-Marjollet et al., 2008; Forsthoefel et al., 2005). Rac1 and RhoA often function downstream of the repulsive cues to mediate actin cytoskeletal dynamics (Dickson, 2001). Permissive guidance cues such as cell adhesion molecules (CAMs) are also required for the assembly of neural circuits (Yu and Bargmann, 2001). CAMs are integral membrane proteins that mediate adhesion between growing axons and elicit intracellular signaling within the growth cone. Cadherins (Calcium-dependent CAMs) are a more diverse group of CAMs. Differential cadherin expression at select synapse populations mediates adhesive interactions during axon growth, synaptogenesis, and plasticity (Ranscht, 2000).
1.3 Neurotransmission

Cell to cell communication in the nervous system, called neurotransmission, primarily occurs through chemical transmission across the synapse between adjacent neurons (Lin and Scheller, 2000). The regions of a synapse include a presynaptic and postsynaptic membrane surrounding a synaptic cleft. Biochemical experiments revealed that vesicles that package signaling molecules involved in neurotransmission are of two types, small clear synaptic vesicles (SVs) and dense core vesicles (DCVs) (Vaaga et al., 2014; Wegrzyn et al., 2010). Synaptic vesicles are responsible for rapid signaling at the synapse using small neurotransmitters like acetylcholine (ACh), gamma-aminobutyric acid (GABA), and glutamate. ACh is a major neurotransmitter at neuromuscular junctions while GABA mainly functions at inhibitory synapses (Jorgensen, 2005; Rand, 2007). By far, the most prevalent neurotransmitter in humans is glutamate, which is excitatory at well over 90% of the synapses in the brain (Cartmell and Schoepp, 2000; Nakanishi et al., 1998). DCVs package neuropeptides and monoamines like dopamine, serotonin, and norepinephrine. Monoamines are small molecules whereas neuropeptides are usually larger molecules. Both have a modulatory effect on the nervous system and are released from many areas of the neuron (Lin et al., 2010; Vaaga et al., 2014).

Exocytosis of both vesicle types is calcium dependent, but each uses different mechanisms of release. SVs cluster neurotransmitters at release sites in the axon terminal, and fuse with the presynaptic membrane with the help of docking and priming proteins. SVs are trafficked from the cell body to the axon terminal along microtubules with the help of kinesins. Upon neuronal depolarization by an action potential, neurotransmitters are released from SVs and often bind ionotropic receptors on the postsynaptic neuron
leading to quick regeneration of the electrical signal in the postsynaptic neuron (Brown et al., 2005). DCVs are localized throughout the presynaptic neuron, even at non-synaptic sites. Neuropeptides and monoamines released from DCVs often bind G-protein-coupled receptors (GPCRs) on adjacent or distant neurons, and modulate postsynaptic neuron function by activating heterotrimeric G-protein mediated signaling cascades (Ludwig and Leng, 2006). Both the $G_\alpha$ and $G_{\beta\gamma}$ subunits of the heterotrimeric G-proteins participate in signaling. When GPCRs are activated, the $G_\alpha$ subunit binds to GTP which causes it to dissociate from the $G_{\beta\gamma}$ subunits and activate downstream effectors (Betke et al., 2012).

There are four known G-protein signaling molecules and their role in vesicle priming is conserved from C. elegans to humans. There are four main $G_\alpha$ signaling pathways, $G_\alpha_{12}$ (GPA-12 in C. elegans), $G_\alpha_q$ (EGL-30), $G_\alpha_s$ (GSA-1), and $G_\alpha_o$ (GOA-1) (Bastiani and Mendel, 2006; Brundage et al., 1996; Hiley et al., 2006; Mendel et al., 1995). In C. elegans, EGL-30 has two main effectors, EGL-8 and UNC-73, which function in parallel pathways (Williams et al., 2007). EGL-8 is the C. elegans neuronal phospholipase Cβ (PLCβ), which hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP$_2$) into inositol 1,4,5-triphosphate (IP$_3$) and diacylglycerol (DAG) to promote vesicle priming through activation of UNC-13 (Reynolds et al., 2005; Sternweis and Smrcka, 1993). Similarly, UNC-73 activates RHO-1 (RhoA), which can also regulate DAG levels and UNC-13 priming by negatively regulating DAG kinase (Hiley et al., 2006; Hu et al., 2011; Williams et al., 2007). The EGL-30 pathway is negatively regulated by the GOA-1 pathway, causing a reduction in vesicle priming due to decreased levels of DAG (Miller et al., 1999). Alternatively, the GSA-1 pathway positively regulates the EGL-30 pathway through adenylyl cyclase pathway activation, which produces cyclic AMP (cAMP).
cAMP activates PKA, which can modulate SV release and the properties of ion channels (Baba et al., 2005; Hu et al., 2011)

Exocytosis of SVs and DCVs is dependent on SNARE-mediated vesicle fusion. Three well-conserved proteins, syntaxin, synaptobrevin and SNAP-25 form the central SNARE complex, which is modulated by proteins such as Munc13 (UNC-13), NSF (NSF-1), and CAPS (UNC-31). The SNARE complex mediates the attachment and fusion of vesicles to the plasma membrane in the axon terminal. Both UNC-13 and UNC-31 promote vesicle priming and docking, steps that occur before the vesicle fuses with the plasma membrane and releases its contents (Lin et al., 2010; Richmond et al., 1999). UNC-13 interacts with Syntaxin to promote SV release whereas UNC-31 promotes DCV exocytosis (Madison et al., 2005; Speese et al., 2007). NSF-1 is primarily responsible for the recycling of SNARE complexes after vesicles fuse with the plasma membrane. NSF-1 directly interacts with RIC-4 to disassemble the SNARE complex (Yu et al., 2011).

1.4 Rho-Family GTPases

The Ras superfamily of GTPases modulate an array of physiological processes including mitogenesis, cytoskeletal reorganization, vesicular trafficking, and nuclear transport (Macara et al., 1996). The Ras superfamily contains the Rho family GTPase subgroup. Rho family GTPases are small (~21kDa) signaling G proteins that include 22 members in mammals, including the most well studied members RhoA, Rac1, and CDC42 (Boureux et al., 2007). *C. elegans* has only five Rho GTPase family members: RHO-1, CDC-42, and the Rac-like proteins CED-10, RAC-2, and MIG-2 (Lundquist, 2006). Rho-family GTPases have functional roles in all cell types, however, we are most interested in studying their role in the nervous system where they function to regulate
actin cytoskeletal organization in axon guidance during development and to
physiologically modulate neurotransmission (Linseman and Loucks, 2008).

In the active state, Rho family GTPases, like all Ras superfamily GTPases, are
bound to GTP and activate downstream effectors. GTPase activating proteins (GAPs)
accelerate the intrinsic catalytic activity of the GTPase converting GTP to GDP and
generating a GDP-bound inactive state. Guanine nucleotide exchange factors (GEFs) are
responsible for the exchange of GDP for GTP switching the Rho GTPase to an active
form. These GTPases thus act like a molecular switch. The human genome encodes over
60 RhoGEFs and over 80 RhoGAPs, which have diverse cell-specific roles in modulating
the activity of the ubiquitously expressed Rho family GTPases (Macara et al., 1996;
Rossman et al., 2005; Tcherkezian and Lamarche-Vane, 2007).

Rho GTPases modulate the activity state of many downstream effectors. For
example, Rac1 and CDC42 function in parallel pathways to modulate actin cytoskeleton
dynamics necessary for cell and growth cone movement (Meyer and Feldman, 2002).
Rac1 activation leads to indirect activation of the WAVE-1 complex, which is
responsible for inducing the actin nucleating activity of Arp2/3 (Eden et al., 2002). Rho
GTPase regulation of Arp2/3 and ADF/cofilin is critical to the formation of lamellipodia
found on motile cells (Meyer and Feldman, 2002). Active RhoA tends to be involved in
growth cone collapse/retraction (Hall and Lalli Rev, 2010).

Importantly, many different neurological disorders are associated with mutations
in these regulators of the human Rho family GTPase signaling pathways. Specifically,
mutations in RhoGAPs, such as oligophrenin and SrGAP, cause intellectual disabilities
(Ramakers, 2002). Experiments with oligophrenin-1 null mice show a down regulation of
AMPA receptor endocytosis and decreased synaptic vesicle recycling, leading to an overall decrease in neurotransmission (Khelfaoui et al., 2009). Mutations in RhoGEFs such as ARHGEF9 and Alsin can result in intellectual disabilities and the neurodegenerative disease ALS (Amyotrophic Lateral Sclerosis), respectively (Linseman and Loucks, 2008; Yang et al., 2001). Rac and Rho typically have antagonistic effects on dendritic spine morphology in which Rac promotes spine formation and Rho inhibits their formation (Tashiro et al., 2000). Often, mutations in the Rho GTPase pathway lead to alterations in dendritic spine morphology that result in cognitive or behavioral problems (van Galen and Ramakers, 2005).

1.5 The Model System *Caenorhabditis elegans*

Our lab uses *Caenorhabditis elegans* to study nervous system development and function. *Caenorhabditis elegans* was first introduced as a model organism in 1965 by Sydney Brenner (Brenner, 1974). These organisms are free-living nematodes that exist as males and hermaphrodites, which are capable of reproducing by self-fertilization generating approximately 300 progeny each. Animals grow to be approximately 1mm in length over their 3.5 day life cycle and are found naturally existing in the soil (Kaletta and Hengartner, 2006). Populations of *C. elegans* are easy to maintain in the lab on agar plates containing a simple medium. They have transparent bodies, which allows for whole organism imaging and utilization of fluorescently tagged proteins in live animals.

Using *C. elegans* as a model organism for studying the nervous system is beneficial because, in comparison to mammals, they have a much smaller nervous system consisting of only 302 neurons, in which the neuronal circuitry and synapses have been mapped and the neurons contain a similar set of neurotransmitters, ion channels and other
fundamental neuronal proteins as found in mammals (Seifert et al., 2006). In 1998, the 100MB C. elegans genome was fully sequenced and made available with annotations (Hodgkin, 2005). This has allowed for large-scale genetic screens using classical genetics, RNAi, and genetic markers for identification of new genes, phenotypes, and interacting proteins (Sieburth et al., 2005). Importantly, many C. elegans proteins and molecular mechanisms are conserved in humans (Fraser et al., 2000).

1.6 The C. elegans RhoGEF UNC-73

A forward genetic screen looking for genes involved in cell and growth cone migration identified unc-73. unc-73 encodes an ortholog of the two paralogous mammalian proteins, Trio and Kalirin, which play key roles in regulating synapse morphology, neuron development, and neurotransmission (Hu et al., 2011; Luo, 2000; Steven et al., 2005). The unc-73 gene encodes multiple differentially expressed UNC-73 isoforms containing one or two RhoGEF domains (Figure 1). The UNC-73B isoform specifically encodes only the RhoGEF1 domain and is ubiquitously expressed in the cytoplasm of C. elegans neurons (Steven et al., 1998). Mutations made in the UNC-73B RhoGEF1 domain, like unc-73(rh40), alter actin cytoskeleton reorganization resulting in axon patterning defects that are associated with an uncoordinated movement phenotype and RhoGEF1 domain deletion is embryonic lethal (Steven et al., 1998). This uncoordination phenotype is the reason for the unc-73 gene name (Steven et al., 1998). The UNC-73 RhoGEF1 domain specifically activates C. elegans Rac-like GTPases to regulate the direction of axonal growth during development. RhoGEF1 activity is thought to act downstream of receptors for axon guidance cues to control cytoskeletal polymerization (Steven et al., 1998). The UNC-73 RhoGEF2 domain specifically
activates RHO-1 to regulate locomotion, likely through DCV-mediated signaling in the nervous system (Hu et al., 2011). A point mutation in the RhoGEF2 domain causes animals to have a slow, yet coordinated locomotory phenotype that is distinct from the RhoGEF1 mutant phenotype (Steven et al., 2005; Williams et al., 2007).

UNC-73 encodes several other non-enzymatic domains. The N-terminus contains a Sec14 domain followed by eight spectrin-like repeats. Generally, the Sec14 domain binds lipids and localizes proteins to the plasma membrane by binding phosphatidylinositols (Aravind et al., 1999). Spectrin repeats often are expressed in multiples in tandem within a protein, just like in UNC-73 (Djinovic-Carugo et al., 2002). Spectrin repeats serve as structural support by coordinating cytoskeletal interactions and play regulatory roles by functioning as a “switchboard” for interactions with multiple proteins (Djinovic-Carugo et al., 2002). There is also evidence that spectrin repeats can bind to RhoGEF domains to inhibit the activity of RhoGEF proteins (Chen et al., 2011).

Next is the RhoGEF1 domain (also known as a Dbl homology (DH) domain) in tandem with a Pleckstrin Homology domain (PH). PH domains often interact with phosphatidylinositol 4,5-bisphosphate for intracellular membrane targeting. More importantly, PH domains can both positively and negatively regulate RhoGEF activity (Maffucci and Falasca, 2001). Almost all identified RhoGEF domains are associated in tandem with a PH domain and this is true for UNC-73 (Viaud et al., 2012). The RhoGEF1/PH domain is followed by a Src Homology-3 domain (SH3). SH3 domains bind proline-rich sequences to mediate protein-protein interactions (Li, 2005). The UNC-73 SH3 domain is followed by the RhoGEF2 domain, again in tandem with a PH domain. The C-terminus of some of the UNC-73 isoforms also contains an Immunoglobulin (Ig)
domain, Fibronectin Type III (FnIII) domain, and a potential PDZ-binding motif. Ig and FnIII domains are typically found in the extracellular regions of transmembrane receptors or intracellular muscle proteins, but their role in the UNC-73 isoforms, where they are located at the C-terminus of some of the UNC-73 isoforms is not known (Benian et al., 1996). The specificities of the UNC-73 RhoGEF domain activities have been determined (Steven et al, 1998; Spencer et al, 2001); however, the UNC-73 non-enzymatic domain functions are not well characterized.

As mentioned previously, the locomotion and developmental phenotypes that result from RhoGEF1 domain mutations are not observed in animals with mutations affecting the RhoGEF2 domain. RhoGEF2 domain deletion causes arrested development at the L1 life stage due to defects in pharyngeal pumping that result in starvation (Steven et al., 2005). The lethal phenotype can be rescued by UNC-73D1 or UNC-73D2 expression specifically in pharynx muscle, but the rescued animals still have a lethargic locomotion phenotype (Steven et al., 2005). Neuronally expressed C1, C2, F, and E isoforms can rescue this lethargic phenotype (Steven et al., 2005). Point mutations in the RhoGEF2 domain, as found in unc-73(ce362), also cause a lethargic locomotion phenotype. The UNC-73 RhoGEF2 activity functions downstream of EGL-30 (Goq) to activate RHO-1. Activation of this signaling pathway promotes acetylcholine release at the synapse (Williams et al., 2007). Although the Rho pathway can regulate vesicle priming by inhibiting DGK kinase, this activity does not appear to play a role downstream of UNC-73 (Steven et al., 2005). Instead, acetylcholine release is likely modulated by DCV-mediated neurotransmission controlled by UNC-73 RhoGEF2/Rho signaling, but the exact mechanism is not known (Hu et al, 2011). It is my objective to
further investigate the UNC-73 RhoGEF2 domain’s role in modulating neurotransmission and the possibility that RhoGEF1 activity could also have a role in neurotransmission. Although the *unc-73* RhoGEF1 uncoordinated movement phenotype is 100% penetrant, the axon guidance defects in the RhoGEF1 mutants are not 100% penetrant (Steven et al., 1998). This suggests the possibility that neurotransmission defects in the *unc-73* RhoGEF1 mutants may play a role in the uncoordinated behavior.

![Figure 1](image.png)

**Figure 1.** *Unc-73* encodes multiple differentially expressed isoforms. Isoform lengths are shown in kilodaltons (kDa). UNC-73A is the largest isoform and is the only isoform encoding both RhoGEF domains. UNC-73B encodes the Rac-specific RhoGEF-1 domain, but not the RhoGEF-2 domain. UNC-73C1, C2, F, D1, D2, and E all encode the Rho-specific RhoGEF-2 domain, but not the RhoGEF-1 domain.

1.7 Trio and Kalirin Function in the Nervous System

The UNC-73 orthologs Trio and Kalirin are paralogous mammalian proteins that have redundant, yet multi-functional roles (Bateman and Van Vactor, 2001). There is about 68% nucleotide and 65% amino acid sequence identity between these proteins.
Trio and Kalirin undergo extensive alternative splicing, producing many functionally distinct isoforms, including many that are similar to UNC-73 isoforms. The Trio and Kalirin isoforms range in size from 100kDa to nearly 400kDa (Miller et al., 2013). Trio, Kalirin and their orthologs are the only proteins that contain more than one RhoGEF domain (Ma et al., 2003). Interestingly, the Trio and Kalirin RhoGEF1 domains share over 90% identity whereas the RhoGEF2 domains share only 67% identity (van Rijssel and van Buul, 2012). The RhoGEF1 domain specifically activates Rac1 and RhoG and the RhoGEF2 domain specifically activates RhoA. Rac1 and RhoA are activated sequentially, so it may be efficient to have only one protein with two distinct GEF domains, like Trio or Kalirin, that can activate both GTPases in a spatially and temporally coordinated manner (van Rijssel and van Buul, 2012; Welch et al., 2011). Trio and Kalirin enzymatic activity is well characterized but studies reveal they may also be used as scaffolds by other proteins for correct cellular targeting (Bellanger et al., 2000).

Trio is ubiquitously expressed, whereas Kalirin is primarily expressed in the central nervous system in the mammalian system (Ferraro et al., 2007). Trio is a 341 kDa protein that regulates cytoskeletal rearrangements, cell migrations and growth, and is involved in hormone secretion (Debant et al., 1996; Xin et al., 2004). Trio was identified through its interactions with LAR, a transmembrane protein tyrosine phosphatase that is involved in regulating cell-matrix interactions (Debant et al., 1996). Trio isoforms A, B, and D are expressed in the brain and during development whereas other isoforms are expressed in skeletal muscle (O'Brien et al., 2000; Portales-Casamar et al., 2006). Trio knock-out mice are embryonic lethal due to defects in skeletal muscles as well as in the
nervous system, including hippocampal and olfactory bulb development (Ma et al., 2005; O'Brien et al., 2000).

Kalirin was originally identified as a peptidylglycine α-amidating monooxygenase (PAM) interacting protein. PAM is important for the synthesis of peptides necessary for dense core vesicle (DCV) signaling (Alam et al., 1996). The Kal7 isoform of Kalirin is most closely related to UNC-73B and both activate Rac-family GTPases. Kal7 binds to Rac1 and modulates cytoskeletal organization to promote axon guidance and neuropeptide secretion (Ferraro et al., 2007; Hansel et al., 2001; Steven et al., 1998). Kal7 is typically localized to post-synaptic densities (PSDs), which are electron-dense thickenings of ionotrophic and metabotropic neurotransmitter receptors, cytoskeletal and scaffolding proteins, and adhesion and signaling molecules (Iasevoli et al., 2014). Morphological changes in dendritic spines occur rapidly through Kal7 expression (Kiraly et al., 2011). Kal7 expression and dendritic spine localization are also required for activity-dependent enhancement of AMPA-mediated synaptic transmission and this requires actin cystoskeletal modulation by Kal7 (Xie et al., 2007). UNC-73/Trio/Kalirin RhoGEF signaling is important in regulating key processes that govern overall animal behavior. UNC-73 study in C. elegans will help reveal fundamental roles for Trio and Kalirin in mammals.

1.8 Neuronal Secretory Pathway

Rho GTPases are extensively involved in regulating the neuronal secretory pathway responsible for neurite outgrowth and neurotransmission (Villarroel-Campos et
Specifically, UNC-73/Trio/Kalirin function to promote DCV signaling in the nervous system, possibly in trafficking and/or packaging of DCV (Hu et al., 2011; Miller et al., 1996; Williams et al., 2007). Separate experiments reveal the Rho signaling pathway also regulates neuroendocrine secretion, but the specific RhoGEF(s) involved in this signaling is not known (Momboisse et al., 2011). Activated RhoA associates with secretory granules to stabilize the surrounding actin cytoskeleton network to inhibit exocytosis of secretory granules in chromaffin cells (Gasman et al., 1998). However, RhoA is required for calcium-dependent secretory granule exocytosis from mast cells (Sullivan et al., 1999). RhoA’s regulation of secretory granule release may share a similar mechanism to that of DCV exocytosis in neurons. \( \text{rab-2} \) and \( \text{unc-31} \) mutant animals exhibit a lethargic phenotype that is similar to \( \text{unc-73} \) RhoGEF2 mutants suggesting a role for \( \text{unc-73} \) RhoGEF2 activity in the DCV secretory pathway. Analysis of UNC-73 RhoGEF2 function suggests that the Rho pathway may play a role in the packaging of DCVs in the cell body of neurons or the transport of DCVs down axons (Hu et al., 2011). RhoGEF2 mutants have normal levels of neuropeptides present in neuronal cell bodies, but reduced levels in axons. There are also fewer neuropeptides released from neurons in the RhoGEF2 mutants (Hu et al., 2011). Additionally, \( \text{G}_s \)/GSA-1 pathway activation increases DCV exocytosis and can bypass UNC-73, RAB-2 and UNC-31 based defects in DCV signaling (Charlie et al., 2006; Hu et al., 2011; Zhou et al., 2007). It is important for us to understand how these RhoGEF domains are regulated in the nervous system so that we can further understand their function in neurotransmission.

1.9 Sec14 Domain
The UNC-73B, N-terminal, Sec14 domain (also known as a CRAL-TRIO domain) is the focus of this study. Sec14 domains are a derivative of the Sec14p super family of lipid binding proteins that were originally identified in *Saccharomyces cerevisiae* (Saito et al., 2007). The Sec14 domain is highly conserved in plants, yeast, invertebrates, and mammals however there is no known mammalian ortholog of the yeast Sec14p (Aravind et al., 1999). The *S. cerevisiae* Sec14p crystal structure has been solved and supports the idea that Sec14p functions to transfer phospholipids between membranes. The Sec14p structure consists of an N-terminal α-helical domain and a C-terminal lipid-interacting domain (Kostenko et al., 2005). Sec14p binds phosphatidylinositols and phosphatidylcholines which are primarily found at the cell membrane and functions to mediate their transfer between membranes and coordinate Golgi complex lipid metabolism and protein transport (Ma et al., 2014; Saito et al., 2007). In mammals, the Sec14 domains coordinate protein-lipid interactions essential for protein targeting, signal transduction, lipid transport, and the maintenance of cellular compartments and membranes (Saito et al., 2007).

The Sec14 domain is often encoded along with other protein-protein interaction or catalytic domains and is also found at the N-terminus of three additional Rho GTPase regulators: p50RhoGAP, Dbl, and Dbs (Kostenko et al., 2005; McPherson et al., 2002). Dbs exists in two forms, Dbs-130 and Dbs-80, named based on their molecular weight. Dbs-130, which encodes a Sec14 domain, localizes to the Golgi and regulates the secretory pathways responsible for cell polarization during migration (Fitzpatrick et al., 2014). The Sec14 domain in p50RhoGAP localizes the protein to endosomes, suggesting a role for GTPases in the secretory pathway (Sirokmany et al., 2006). The UNC-73B
Sec14 domain may be involved in both intramolecular and intermolecular protein interactions necessary to either target UNC-73B to the plasma membrane or Golgi apparatus or regulate its enzymatic function. The Sec14 domain may form intramolecular binding sites with the PH domain, which interferes with the PH domain’s phospholipid binding function, as seen in the Neurofibromin RhoGAP (Kubiseski et al., 2003; Welti et al., 2011).

Recently, the role of the Sec14 domain was examined in Kal7 regulation of dendritic spine morphology (Ma et al., 2014). Overexpression of just the Sec14p domain and a few spectrin-like repeats of Kal7 led to an increase in dendritic spine size. Kal7 mutants without the Sec14p domain were able to increase dendritic spines, however they were much shorter than those grown using wild type Kal7 (Ma et al., 2014). Defects in dendritic spine formation are linked to memory problems and psychiatric diseases like schizophrenia in extreme cases (de Bartolomeis et al., 2014). It is also possible that the Sec14p domain modulates receptor-mediated endocytosis (Ma et al., 2014).

There is currently little known about Sec14 domain function in vivo. This work used the C. elegans model system to examine the role of the Sec14 domain in UNC-73B neuronal function and localization.
Figure 2. The Sec14 domain is conserved from yeast to mammals. Proteins encoding a Sec14 domain are shown. There are several mammalian RhoGAPs and RhoGEFs that encode a Sec14 domain. Most often, the Sec14 domain is found at the N-terminus of the protein, except for Patellin from plants and Neurofibromin from mammals. Some proteins contain an N-lobe CRAL-Trio domain which is indicated by a thin blue rectangle immediately preceding the Sec14 domain indicated by the larger blue rectangle.
Chapter Two

2. Materials and Methods

2.1 General C. elegans maintenance

Strains were maintained at 21°C on plates containing standard nematode growth media (NGM) unless otherwise noted. Plates were seeded with OP50, a strain of *Escherichia coli*, which serves as a food source. Strains used in examining Sec14 function were: N2 Bristol (wild type), UNC-73B(Sec14)::GFP, *unc-73(rh40)*, and UNC-73C1(N-Term). Strains used in examining UNC-73C1 localization are: N2 Bristol (wild type), *unc-73(ce362)*, UNC-73C1, UNC-73C1::GFP, UNC-73C1::ICR::GFP, *unc-17p::gfp*.

To generate transgenic animals, DNA was injected into *C. elegans* gonads by Dr. Robert Steven or Amanda Korchnak. A 20μl injection mixed was prepared containing 50ng/μl DNA, 50ng/μl *unc-122::gfp* (coelomocyte co-transformation marker), and 1x Tris EDTA (pH 8). Standard microinjection techniques were used to generate stable transgenic *C. elegans* lines carrying extra-chromosomal DNA arrays.

2.2 Characterization of the UNC-73B Sec14 domain

2.2.1 Cloning and Sequencing. We designed Multisite Directed Gateway® clones to be expressed as transgenes in *C. elegans* to study phenotypes associated with Sec14 domain mutations. The *rab-3* promoter was used to direct expression exclusively in the nervous system and *gfp* was fused to the *unc-73b 3’* end to for visual confirmation of expected protein expression. The 500bp *unc-73b* region that encodes the Sec14 domain was amplified by PCR using primers UT271 (forward) and UT272 (reverse). The NW252 cDNA containing *unc-73b* was used as a template in the PCR reaction. The
reaction contained 10μl 5x iProof High-Fidelity Buffer, 1μl 10mM dNTP mix, 1μl each of UT271 and UT272 primers at 20μM, 1μl 50ng/μl NW252 cDNA template, 35.5μl sterile H₂O, and 0.5μl IProof DNA Polymerase mixed in a 0.2mL Eppendorf tube and run on a programmed cycle in a thermocycler (Table 2). The PCR product was recombined into the Gateway® entry vector pDONR221 according to standard protocols and the resulting entry clone was verified by restriction enzyme mapping and DNA sequencing through Genewiz®, using their universal primers M13F(-21) and M13R. UNC-73B(Sec14) in pDONR221 was recombined with rab-3 pDONR4-1, and a third entry clone, encoding green fluorescence protein (GFP) and the let-858 3’UTR, both gifts from the Jorgensen lab at the University of Utah. This recombination, generated through standard procedures, produced the final destination clone, rab-3p::unc-73b(Sec14)::gfp in pDEST4-R3, which was verified by restriction enzyme mapping. DNA sequencing of the recombination regions using universal primers T7 (forward) and T7 Term (reverse) revealed the correct sequence except for a three base pair insertion at the 5’ end of the rab-3 promoter, which is not expected to affect expression.

**Table 1. PCR primer sequences for the multisite Gateway® cloning of UNC-73B(Sec14).** UT271 is a forward primer targeted to the UNC-73B N-terminal coding region with the Gateway® entry vector attB4r site added at the 5’ end, indicated in capital letters. UT272 is a reverse primer targeted to end of the UNC-73B Sec14 domain with the Gateway® entry vector attB3r site added to the 5’ end.

<table>
<thead>
<tr>
<th>Name</th>
<th>Primer Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>UT271</td>
<td>GGGGACAAGTTTTGTACAAAAAACGAGGCTAAatgggcgattatgatggaaaa</td>
</tr>
<tr>
<td>UT272</td>
<td>GGGGAGGACACTTTTGTAACAAAAAGCTGGGTTGcaacctgagctcaageccatte</td>
</tr>
</tbody>
</table>
Table 2. PCR conditions used to generate the UNC-73B(Sec14) clone. These conditions were used successfully to amplify the unc-73b(Sec14) clone using the iProof™ DNA polymerase.

<table>
<thead>
<tr>
<th>Stage of Thermocycler</th>
<th>Temperature (°C)</th>
<th>Time</th>
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<tbody>
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<td>Initial Denaturation</td>
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<td>30 sec</td>
</tr>
<tr>
<td>Denaturation</td>
<td>98</td>
<td>10 sec</td>
</tr>
<tr>
<td>Anneal</td>
<td>63</td>
<td>20 sec</td>
</tr>
<tr>
<td>Extension</td>
<td>72</td>
<td>65 sec</td>
</tr>
<tr>
<td>Final Extension</td>
<td>72</td>
<td>10 min</td>
</tr>
</tbody>
</table>

Table 3. Sequencing primers. These primers were used in DNA sequencing reactions to establish the sequence of the Gateway recombination products.

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Primer Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>M(13)F-21</td>
<td>5’-d(TGTTAACACGACGGCCAGT)-3’</td>
</tr>
<tr>
<td>M(13)R</td>
<td>5’-d(CAGGAACAGCTGATGAC)-3’</td>
</tr>
<tr>
<td>T7</td>
<td>5’-d(TAATACGACTCAGTGAGA)-3’</td>
</tr>
<tr>
<td>T7 Term</td>
<td>5’-d(GCTAGTTATTGCTGAGCGG)-3’</td>
</tr>
</tbody>
</table>

2.2.2 Cloning rab-3p::unc-73b::gfp and rab-3p::unc-73b(Δsec14)::gfp constructs. Standard ligation reactions, Gateway® cloning, PCR fusion, and Gibson cloning mechanisms have been used in attempts to clone unc-73b::gfp (7.4kb) and unc-73b(Δsec14)::gfp (6.9kb) under the control of the pan-neuronal, rab-3p, promoter. Thorough manipulations to template concentrations, polymerases, thermocycler conditions, etc. have been made in an attempt to generate these clones with no success.
We are still trying to generate these two clones in an effort to examine Sec14 domain function.

2.2.3 Body bends assay. Nine stable transgenic Ex[rab3p::Sec14::gfp] lines were isolated and three lines with the strongest and most consistent GFP fluorescence, as observed under the dissecting microscope were used for further analysis. Pictures were taken of Ex[rab3p::Sec14::gfp] transgenic animals and their tracks in comparison to wild-type and unc-73(rh40) animals and their tracks. Each animal was left on the bacterial lawn for five minutes at which point their tracks were imaged.

Healthy and well-nourished worms are used in the body bend assays. The assay was performed at room temperature (22-23°C). Worms are transferred to an NGM plate without OP50 for 20 seconds then moved to another NGM plate and left for at least one minute before assaying. Each worm is assayed for 20 seconds in which the number of body bends is recorded. The assay is voided and restarted if an animal stops moving forward at any time during the 20 seconds. A body bend is counted if an entire sinusoidal wave is completed by the animal in forward movement. A minimum of ten worms per strain are assayed at a time and the average number of body bends per minute is determined for the set. An unpaired student’s t-test was used to determine statistical significance in comparison to wild type animals.

2.2.4 Spontaneous reversal assay. Healthy and well fed young adult animals were transferred to NGM assay plates without bacteria and left for at least one minute before assaying. Spontaneous reversal is defined as the number of direction changes from forward to backward on the plates without additional stimulus in 3 minutes (Gray et al., 2005). A minimum of ten worms per strain are assayed at a time and the average number
of spontaneous reversals is determined for the set. An unpaired Student’s T-test was performed to determine statistical significance in comparison to wild type animals.

2.2.5 Developmental Assay. First, the developmental timing of progeny from three independent transgenic lines expressing UNC-73B(Sec14)::GFP were examined and compared to N2 and *unc-73*(rh40) animals. For each strain, five young adult animals were left on a seeded NGM plate for 12 hours to lay eggs, and then removed. Plates were examined every few hours for the next three days after the parent animals were removed. Any late larval animals that were observed on the plates were counted and picked off the plate. Late larval animals were picked to avoid having adults lay another generation of eggs on the plates. The animals remaining on the plates after the final examination period were counted and they were all observed to be young larvae. These young larval animals were considered to exhibit “slow development” and the number was plotted as a percentage of the total progeny for each strain in Figure 6A.

A second assay was performed over a longer, eight day, time period. Plates were examined every few hours for the next eight days after the parent animals were removed. Any late larval animals that were observed on the plates were counted and picked off the plate. The animals remaining on the plates after the final examination period were all young larvae. There appeared to be an equal number of green (transgenic) and non-green animals in this population of slowly developing animals. These remaining larvae were counted and the number was added to the total number of late larvae picked from the plates over the eight days to get the total number of progeny examined. The number of late larvae picked each day, expressed as a percentage of the total number of animals examined, was plotted in Figure 6B.
2.2.6 Confocal microscopy. Worms expressing GFP were placed in 10 mM levamisole on a 2% agarose pad for imaging. Images were taken using a Leica TCS SP8 confocal microscope.

2.2.7 UNC-73 Sec14 sequence analysis. MegAlign software was used to obtain an UNC-73B Sec14 domain consensus sequence. A blastx search of the *C. elegans* genome with the Sec14 domain consensus sequence was used to identify additional Sec14 domain containing proteins.

2.3 Confirming UNC-73C1 and Rab-11-FIP3/4 interaction *in vitro*.

2.3.1 Cloning UNC-73C1 protein fragments. The protein fragments were designed based on the positions of the known protein domains. The UNC-73C1(N-Term) protein is from 1-741 base pairs, UNC-73C1(RhoGEF2) is from 711-1677 base pairs, and UNC-73C1(C-Term) is from 1674-2724 base pairs of the *unc-73c1* cDNA. Clones were amplified by PCR from cDNA pools and subsequently recombined into Gateway® vectors, pDEST15 and pDEST17 to add N-terminal GST or His tags respectively. Final clones were verified by restriction enzyme mapping and DNA sequencing.
Table 4: Primers used for the cloning of the UNC-73C1 fragments. UT265 is a forward primer targeted to the UNC-73C1 N-terminal coding region. UT266 is a reverse primer targeted to end of the UNC-73C1 N-terminal fragment. UT267 is a forward primer targeted to the UNC-73C1 RhoGEF2 coding region. UT268 is a reverse primer targeted to end of the UNC-73C1 RhoGEF2 fragment. UT269 is a forward primer targeted to the UNC-73C1 C-terminal coding region with the. UT270 is a reverse primer targeted to end of the UNC-73C1 C-terminal fragment. UT114 is a forward primer targeted to the FIP3/4 coding region. UT115 is a reverse primer targeted to end of the FIP3/4 coding region. All forward primers have the Gateway® entry vector attB1 site added at the 5’ end, indicated in capital letters. All reverse primers have the Gateway® entry vector attB2 site added to the 5’ end, indicated in capital letters.

<table>
<thead>
<tr>
<th>Clone Name</th>
<th>Primer Name</th>
<th>Primer Sequence</th>
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<tbody>
<tr>
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<td>UT265</td>
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<td>UNC-73C1(N-Term)</td>
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<td>GGGGAAAAGTTTTGTAACAAAAAAGCAGGCTG GGGGAAAAGTTTTGTAACAAAAAAGCAGGCTG</td>
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<tr>
<td>UNC-73C1(RhoGEF2)</td>
<td>UT267</td>
<td>GGGGACAAGTTTTGTAACAAAAAAGCAGGCTG GGGGACAAGTTTTGTAACAAAAAAGCAGGCTG</td>
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<tr>
<td>UNC-73C1(C-Term)</td>
<td>UT268</td>
<td>GGGGACAAGTTTTGTAACAAAAAAGCAGGCTG GGGGACAAGTTTTGTAACAAAAAAGCAGGCTG</td>
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<tr>
<td>UNC-73C1(C-Term)</td>
<td>UT269</td>
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<td>UNC-73C1(C-Term)</td>
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<td>FIP3/4</td>
<td>UT115</td>
<td>GGGGACAAGTTTTGTAACAAAAAAGCAGGCTG GGGGACAAGTTTTGTAACAAAAAAGCAGGCTG</td>
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</tbody>
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Table 5: PCR conditions to generate the UNC-73C1 clones. These conditions were used successfully to amplify the three unc-73c1 clones using the iProof™ DNA polymerase kit.

<table>
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<th>Stage of Thermocycler</th>
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2.3.2 UNC-73C1 and Rab-11-FIP3/4 protein expression. Plasmid construction for protein expression was performed in DH5α E. coli cells. The C. elegans genes were cloned from a cDNA pool then inserted into protein expression vectors either by restriction enzyme cut/ligation (Fisher Fermentas) or by Gateway® system (Invitrogen). pDEST15 was used for N-terminus GST tag and pDEST17 was used for N-terminus His tag.

Plasmids were transformed in E. coli BL21 competent cells and a 2mL culture was grown from a single colony at 27°C overnight. 400μl of this culture is used to inoculate a new 2mL LB and ampicillin culture that is incubated at 27°C for three hours. The culture is then induced with 10μl of 0.1M IPTG and continued growing for four hours at 27°C. 1.5mL of the culture is centrifuged and the supernatant is removed. 100 μl of 2x SDS sample buffer was added to the bacterial pellet. The samples were boiled for ten minutes then 7.5μl was loaded on an SDS-PAGE gel for analysis.
2.3.3 UNC-73C1 and Rab-11-FIP-3/4 protein solubility. 2mL bacterial cultures containing plasmid DNA are grown at 27°C overnight. The next morning, 250μl of this culture is used to inoculate a new 5mL LB culture that is grown at 27°C for two hours. 10μl of 0.1M IPTG is added to the culture and allowed to grow for an additional five hours. Bacteria were then harvested by centrifugation at 4,000g for 20 minutes at 4°C. The bacterial pellet was re-suspended in 300μl GST/His lysis buffer with 1.5 μl bacterial protein inhibitors (BPI) and transferred to a 1.5 mL Eppendorf tube. Lysozyme was added to the solution at a concentration of 0.1 mg/mL and the tube was incubated on ice for 30 minutes. Samples were sonicated four times at 30% amplitude with ten seconds pulse and ten seconds pause. 10μl of sonicated solution was transferred into a new Eppendorf tube to be used as the whole cell lysate sample. The remaining sonicated solution was centrifuged at 10,000g for 30 minutes at 4°C. All of the supernatant was transferred into a new Eppendorf tube and a 10μl aliquot is placed in a separate tube to be used to check solubility. 10μl 2x SDS loading buffer was added to samples for electrophoresis then boiled for ten minutes and run on an SDS-PAGE gel.

2.3.4 UNC-73C1 and Rab-11-FIP3/4 GST pull down. DNA constructs were transformed into *E. coli* BL21 cells and 4mL liquid cultures incubated at 27°C to reach O.D. 0.6, then induced with 5μl 1mM IPTG either at 27°C for an additional 4 hours or at 16°C overnight. Bacterial pellets were re-suspended in either GST lysis buffer (50mM Tris pH 7.5, 150mM NaCl, 5% glycerol, 0.1% Triton X-100, 1mM TCEP), or His lysis buffer (50mM Tris pH 7.5, 300mM NaCl, 5% glycerol, 0.1% Triton X-100, 1mM TCEP). The bacterial whole cell lysates containing each exogenous protein were combined in a 1:1 ratio. The cell lysates mixture was incubated and rocked for 2 hours at
4°C. 5μl of prepared Glutathione Sepharose (GE Healthcare) 50% slurry was applied into the mixture, incubated and rocked for an additional 2 hours at 4°C. The beads were collected by centrifugation at 500g for 5 minutes at 4°C, and washed three times with GST-wash buffer (50mM Tris pH 7.5, 300mM NaCl, 5% glycerol, 1mM TCEP). After the final spin the beads were drained carefully and 10μl of 2x SDS sample buffer was applied to the beads. The samples were boiled for ten minutes and loaded on an SDS-PAGE gel for analysis.

2.3.5 Immunoblotting. SDS-PAGE was typically run at 100V for 110 minutes in a Bio-Rad cartridge with running buffer (15.1g Tris, 72g Glycine, 50ml 10% SDS, add H2O up to 5L). Gels were transferred to a PVDF membrane (Millipore) in Western buffer (19g Tris, 90g Glycine, 62.5ml 10% SDS, 1L Methanol, add H2O up to 5L) with the transfer unit at 400mA for 100 minutes. The transferred membrane was blocked in blocking buffer (5% non-fat milk, 1% Tween-20 in 1x TBS) for 1 hour at room temperature. The primary antibody (Santa Cruz) was applied (SC803 for anti-His at 1:500, or SC-459 for anti-GST at 1:2000) by rocking overnight at 4°C. The membrane was washed with 1x TBS-T for 15 minutes, four times. The HRP conjugated goat-anti-rabbit secondary antibody (Pierce) was applied (at 1:5000 for anti-His, or at 1:10,000 for anti-GST) rocking for 1 hour at room temperature, then the membrane was washed three times with 1x TBS-T for 15 minutes. The blot was incubated with 500μl Western Blotting Substrate (Pierce) for 1min and autoradiography film was subsequently applied.

2.4 Determining UNC-73C1 localization and function.

2.4.1 Chemosensory neuron staining. DiD staining was used on animals expressing *unc-73c1::icr::gfp* for co-localization studies. Worms grown on NGM plates
are removed using 300μl M9 and placed in a 1.5mL Eppendorf tube. 3μl DiD stain is added to the Eppendorf tube and incubated for three hours. Worms are pelleted by centrifugation for 1min at maximum speed on a microcentrifuge and the M9 solution was then aspirated. The worms are washed three times with 300μl M9 solution then moved to a fresh, non-seeded, NGM plate. Worms are picked from this plate onto a 2% agar pad containing 10mM levamisole for confocal microscopy imaging.
Chapter Three

3. Results

3.1 UNC-73 Sec14 domain analysis.

The UNC-73 enzymatic domains are well characterized; however, it is likely that the other non-enzymatic domains play a role in regulating UNC-73 RhoGEF activity. For example, the UNC-73B N-terminal Sec14 domain is a presumed lipid-binding domain and may be involved in regulating UNC-73 localization and RhoGEF1 function in the secretory pathway. To examine UNC-73B Sec14 domain’s role in neuronal function, we set out to perform overexpression, deletion, and rescue experiments (Figure 3). We made an UNC-73 Sec14 domain overexpression construct using Multisite Gateway® Cloning and generated transgenic animals which were observed. The construction of UNC-73B(ΔSec14) transgenic animals is still in progress. Gateway® cloning, PCR fusion, and Gibson cloning approaches have all been currently unsuccessful in generating an unc-73b(Δsec14)::gfp DNA construct.
Figure 3. UNC-73B deletions designed to examine Sec14 domain function. DNA constructs were designed to produce variations of the UNC-73B protein as shown. First is full-length UNC-73B. Next is full length UNC-73B with a C-terminal GFP tag to observe protein localization. Third is UNC-73B with an N-terminal truncation that removes the Sec14 domain in order to observe potential loss-of-function phenotypes in transgenic rescue experiments. Last is UNC-73B with a C-terminal truncation that removes everything except for the Sec14 domain. This was used to observe potential phenotypes in transgenic animals overexpressing the construct. In all constructs, unce-73b expression of was driven by the pan-neuronal rab-3 promoter.

3.1.1 UNC-73B(Sec14) exhibits subcellular localization. If the Sec14 domain is required for UNC-73 function and localization, then we might expect to see subcellular UNC-73B(Sec14)::GFP localization. GFP fluorescence in transgenic animals expressing UNC-73B(Sec14)::GFP was observed exclusively in neurons as expected using the rab-3 promoter. The strongest GFP fluorescence in the expressing neurons was localized to subcellular regions in neuronal cell bodies while weaker fluorescence was observed along thick axon bundles such as the ventral cord (Figures 4 & 5).
expressing GFP two puncta flanked the nuclei similar to what is typically observed with Golgi markers (Sumakovic et al, 2009), suggesting UNC-73B(Sec14)::GFP may localize to the Golgi apparatus (Figures 2 & 3). This localization is consistent with the idea that UNC-73 may play a role in the modulation of neuronal secretory pathways.

Figure 4. UNC-73(Sec14)::GFP has a punctate sub-cellular localization in the ventral nerve cord. UNC-73(Sec14)::GFP was expressed in the neurons of wild-type animals using the rab-3 promoter. A. UNC-73B(Sec14)::GFP localizes in a punctate, subcellular manner through the mid-body of a young adult animal. Red arrowheads indicate nuclei in cell bodies. B. Increased magnification of the animal observed in figure 2A reveals distinct puncta on either side of the nucleus within ventral cord motor neuron cell bodies (red arrowheads). This pattern is suggestive of Golgi localization as similar patterning is observed with Golgi markers (Sumakovic et al., 2009). Anterior is to the right, ventral is down in both images. Images were captured using a confocal microscope.
Figure 5. UNC-73(Sec14)::GFP has a punctate sub-cellular localization in neurons near the nerve ring. Image of the head region, which contains the major ganglia and the nerve ring of the animal. The red box indicates the region of the animal corresponding to the top image. GFP fluorescence is not observed in neuronal processes. Anterior is to the left, ventral is down in both images. Ovals overlaid on the top image indicate the location of the two pharynx bulbs. The top image was captured using a confocal microscope. The C. elegans drawing was modified from wormbase.org.

3.1.2 UNC-73B(Sec14) expressing transgenic animals develop slowly. Since locomotory phenotypes associated with UNC-73B(Sec14) expression seemed to be more robust in larval than adult animals (data not shown), we tested if UNC-73B(Sec14) expression was slowing or arresting animal development (Figure 6). Population growth from two UNC-73B(Sec14) transgenic lines was observed in comparison to wild-type and unc-73(rh40) growth over three or eight days in two separate assays. All wild-type eggs grew to fertile adults within three days, while UNC-73(Sec14) expressing lines still had many larval stage animals on the plates representing 35-60% of the population even after eight days (Figure 6A). The animals that did not grow to adulthood appeared to remain longer in early larval stages. Quantification of the average developmental length
and life span for UNC-73(Sec14) transgenic animals in comparison to wild type will be done in future experiments.

**Figure 6. UNC-73(Sec14)::GFP expression inhibits development.** A. The developmental timing of progeny from two independent transgenic lines expressing UNC-73(Sec14)::GFP were examined and compared to N2 (wild type) and unc-73(rh40) animals. Animals that did not develop to adults after three days were considered to exhibit “slow development”. All N2 and unc-73(rh40) animals were able to grow to adults within three days but UNC-73(Sec14)::GFP expression slowed development of nearly half of the transgenic animals. The bars represent the number of animals that were still young larvae after three days, expressed as a percentage of the total number of progeny. B. The graph shows the growth curve of N2 (wild type) animals in comparison to two lines of UNC-73B(Sec14) overexpression animals. Percentage of the total population that grew to late larvae each day was recorded and plotted. UNC-73(Sec14)::GFP transgenic lines contain the unc-122p::gfp co-transformation marker and express unc-73b(sec14) in the nervous system under the control of the rab-3 promoter.
3.1.3 UNC-73B(Sec14) expressing transgenic animals display a slow and uncoordinated locomotory phenotype. While examining the UNC-73B(Sec14) developmental phenotype, we observed that these animals also exhibited a locomotory phenotype. Locomotion was examined in two separate lines of larval and adult wild-type animals expressing the \( \text{rab-3p::unc-73b(sec14)::gfp} \) transgene. Many larval animals and about 1/3 of adult animals exhibited a slow, uncoordinated phenotype reminiscent of \( \text{unc-73(rh40)}, \text{RhoGEF1}, \) mutant animals (Figure 7). All animals displaying the uncoordinated phenotype had visible GFP fluorescence and it appeared that the severity of the uncoordinated locomotion correlated with the intensity of UNC-73B(Sec14)::GFP expression, however, this correlation was not quantified. Importantly, the uncoordinated phenotype of UNC-73B(Sec14)::GFP expressing animals is similar to the uncoordinated phenotype of \( \text{unc-73(rh40)}, \text{RhoGEF1}, \) mutant animals, suggesting UNC-73B(Sec14) might act as a dominant negative by inhibiting wild-type UNC-73 RhoGEF1 activity (Figure 7).

The uncoordinated and slow locomotion phenotype that was observed in UNC-73B(Sec14) animals, was quantified by measuring the frequency of body bends in forward moving animals, (body bend assay), which correlates with locomotory speed (Figure 8). The UNC-73C1 N-terminus (amino acids 1-247) that does not contain a Sec14 domain was pan-neuronally expressed with a C-terminal GFP tag in wild-type animals as a control. UNC-73B(Sec14) animals had significantly slower locomotion both on and off food in comparison to wild-type and control animals (Figure 8). The slowed locomotion resembles that of \( \text{unc-73(rh40)}, \text{RhoGEF1}, \) mutant animals but is not as slow. The locomotion rate in \( \text{unc-73(rh40)} \) animals was difficult to quantify because they fail to
move forward consistently; therefore, a different assay is being developed to better compare locomotion rates in UNC-73(Sec14) and unc-73(rh40) animals.

Figure 7. UNC-73(Sec14)::GFP expression causes uncoordinated locomotion similar to unc-73 RhoGEF1 loss of function mutations. A. An N2 (wild type) C. elegans animal with a characteristic sinusoidal movement pattern observed during locomotion. B. An unc-73(rh40) animal with the characteristic RhoGEF1 loss of function-like uncoordinated locomotion phenotype. C. Ex[rab3p::Sec14::GFP] transgenic C. elegans animal with an unc-73 RhoGEF1 loss of function-like uncoordinated locomotion phenotype. The movement is characterized by kinked body bends and slow speed. Images of animal tracks in the bacterial lawns on standard NGM plates were taken five minutes after the animals were placed on the plates.
Figure 8. UNC-73(Sec14)::GFP expression reduces the speed of locomotion. Both on food (A) and off food (B) expression decreases the rate of locomotion, measured as the number of body bends in a one minute interval. ***p<0.0001 and **p=0.0002 based on un-paired Student’s t-test. n=30 animals per strain.

3.1.4. Spontaneous reversal frequency is altered by UNC-73(Sec14) transgenic expression. To better assess locomotory differences between transgenic lines, additional locomotory criteria were quantified. C. elegans locomotion is sinusoidal and consists of forward crawling, interrupted by spontaneous bouts of backward movement, termed reversals (Zhao et al., 2003). The frequency of spontaneous reversals can be altered depending on genetic and environmental factors (Zhao et al., 2003). Wild-type animals on food typically reverse spontaneously only one or two times per minute, whereas wild-type animals immediately off food spontaneously reverse about six times
per minute (Gray et al., 2005). UNC-73B(Sec14) transgenic expression increases the frequency of spontaneous reversals on food and decreases the frequency of spontaneous reversals when immediately off food in comparison to control UNC-73C1(N-term) and wild-type animals (Figure 9). These data suggest the possibility that when on food, UNC-73B(Sec14) animals receive an inappropriate signal that makes them act as though they are not on food and vice versa. The altered frequency of spontaneous reversals could be due to an alteration in the neuronal circuitry controlling locomotory behavior.

Figure 9. UNC-73(Sec14)::GFP expression alters the frequency of spontaneous reversal on and off food. A. On food, UNC-73(Sec14)::GFP expression significantly increases the frequency of spontaneous reversals. B. Off food, UNC-73B(Sec14) expression reduces the frequency of spontaneous reversals. ***p<0.0001 based on Student’s unpaired t-test. N= 30 animals for all strains.
3.1.5 Fifteen *C. elegans* proteins have a Sec14 domain. A blastx search of the *C. elegans* genome with a consensus Sec14 domain sequence (Ognibene et al., 2014) revealed fifteen genes encoding a putative Sec14 domain. Interestingly, there is an additional RhoGEF, CGEF-1, and a RhoGAP, RGA-1, similar to those that exist in mammals, as well as other proteins with unknown functions. Most of the *C. elegans* Sec14 domain-containing proteins do not have any other identifiable protein domains, however, the Sec14 is almost always located near the N-terminus of each protein (Figure 10). One protein includes a GOLD (Golgi dynamics) domain, which is found in several proteins involved in the regulation of Golgi dynamics and secretion, indicating at least one Sec14 domain protein is likely involved in secretory pathway function. Mutated versions of these proteins will be made to see if expression will result in behavioral or locomotory phenotypes in animals similar to those seen in UNC-73B(Sec14) overexpressing animals.
Figure 10. There are fifteen *C. elegans* Sec14 domain-containing proteins. Blastx searches were used to find fifteen *C. elegans* proteins with a Sec14 domain, which are shown. Each protein contains just one Sec14 domain, which is generally located at the N-terminus. Some proteins have other known domains, which are also shown. The larger blue rectangle indicates a Sec14 domain and the thinner blue rectangle is an N-lobe CRAL-Trio domain.

3.2 In vitro Analysis of Potential UNC-73C1 Interacting Proteins

3.2.1 UNC-73C1 protein expression in bacteria. Previous lab member John Farver performed a yeast-two hybrid screen to identify candidate UNC-73C1 interacting proteins that could further define the mechanisms of neurotransmission modulation. Candidates include Rab-11-FIP3/4 (Rab-11 Family Interacting Protein), DEB-1 (Dense body), DYS-1B (Dystrophin), and LIN-2 (abnormal cell lineage), which are the *C. elegans* Sec14 domain-containing proteins.
*elegans* homologs of Rab11-FIP3/4, vinculin, dystrophin, and CASK respectively. My objective was to confirm the potential interaction between UNC-73C1 and Rab-11-FIP3/4 and characterize a role for UNC-73C1 in vesicle movement in neurons. Mammalian Rab-11-FIP3 was identified through its interaction with Rab11, a GTPase regulator of vesicle fusion and transport that has also been recognized as playing a role in neurite extension (Shirane and Nakayama, 2006). Rab11-FIP3 also binds NSF which is responsible for SNARE recycling after vesicle fusion to the membrane (Vivona et al., 2013). NSF binds other mammalian Ras superfamily GTPases such as Arf6 and the RhoGEF βPIX (Manifava et al., 2001), giving us reason to believe UNC-73C1 could be part of this complex, functioning to regulate vesicular transport during neurotransmission.

Another former lab member, S. Hu, made four expression constructs designed to produce UNC-73C1 and FIP-3/4 with GST and His N-terminal tags, respectively. These proteins were to be used in GST pull down experiments in an attempt to confirm the candidate interactions identified in the yeast-two hybrid screen, however, I was unable to successfully express either version of the N-terminally tagged full-length UNC-73C1 fusion proteins in bacteria (Figure 11). Both GST- and His-tagged FIP3/4 expressed well in bacteria (Figure 11).
Figure 11. **Full length UNC-73 isoforms do not express well in bacteria.** A. Lysates from bacteria expressing GST and His fusion proteins were separated by SDS-PAGE, blotted on PVDF membrane and probed with α-GST (A) and α-HIS (B) antibodies. Full length UNC-73C1 (130 kDa) and UNC-73D1 (110 kDa) fusion proteins and His-DYS1B (32.4 kDa) were not detected. GST-DYS-1B (57.4 kDa), GST-FIP3/4 (81.3 kDa) and His-FIP3/4 (56.3 kDa) were expressed by the bacteria. Expressed proteins are the predicted sizes.

3.2.2 **Structural Design of UNC-73C1 fragments used in GST pull down experiments.** UNC-73C1 is a large, 130 kDa, protein which may be the reason it does not express well in bacteria. Due to the inability to express a full length N-terminally tagged UNC-73C1, constructs expressing three smaller UNC-73C1 fragments were designed to use in the GST pull-down experiments. The locations of the UNC-73C1 structural domains were considered in fragment design in the effort to preserve endogenous protein folding and structure. The three UNC-73C1 fragments were each
made in two N-terminally tagged versions, with GST and His, respectively. The first UNC-73C1 fragment consists of amino acids 1-247 and is referred to as UNC-73C1(N-Term) as it is the N-terminal portion of UNC-73C1 (Figure 12). This section includes the SH3 domain. The second UNC-73C1 fragment consists of amino acids 237-559 and is referred to as UNC-73C1(RhoGEF2). This fragment includes the RhoGEF2 and PH domains (Kubiseski et al., 2003). Finally, the third UNC-73C1 fragment from amino acid 558-928 is referred to as UNC-73C1(C-Term) as it is the C-terminal portion of UNC-73C1. This fragment includes the Ig and FnIII domains, and the potential PDZ-binding motif.

Figure 12. UNC-73C1 fusion proteins used for protein-protein interaction studies. A. Full length UNC-73C1 domain structure. B. Three separate UNC-73C1 fragments were each fused to GST at the C-terminus. UNC-73C1 (N-term) includes amino acids 1-247 of UNC-73C1. UNC-73C1 (RhoGEF2) includes amino acids 237-559. UNC-73C1 (C-Term) includes amino acids 558-928.
3.2.3 GST-UNC-73C1 fragments and His-FIP3/4 are soluble when expressed in bacteria at 27°C. GST-tagged UNC-73C1 fragments and His-tagged FIP-3/4 were expressed in bacteria and examined for solubility. Neither UNC-73C1(C-Term) tagged fragment expressed well in bacteria (Figure 13) so they were not used in subsequent experiments. Initial experiments with a standard protein expression protocol did not yield any soluble proteins (data not shown). Reducing the bacterial culture temperature and increasing sonication time and frequency to more efficiently lyse bacterial cells resulted in all proteins being soluble and expressed at detectable levels by western blot analysis (Figure 14). It is critical that these proteins are soluble for GST pull down experiments.

**Fig.13 FIP-3/4 is the only His-tagged protein that expressed in bacteria.** Western blot analysis shows only His-FIP-3/4 is able to express in bacteria. No other His-tagged UNC-73 fragment or DYS-1B was expressed in bacteria under the conditions tested.
Bacterially expressed GST-UNC-73C1 (N-Term) and GST-UNC-73C1 (RhoGEF2), 65 kDa and 54 kDa respectively, are present in the supernatant from whole cell lysates indicating the fusion proteins are soluble. B. His-FIP3/4 is present in the supernatant from a bacterial cell lysate indicating the fusion protein is soluble. All bands are the expected sizes.

3.2.4 UNC-73C1 and FIP-3/4 N-terminal tagged proteins may not interact in vitro. Western blot analysis of GST pull down experiments revealed that all proteins were expressed; however, neither UNC-73C1 GST-tagged fragment pulled down His-FIP-3/4 in seven separate experiments (Figure 15). These data suggest that FIP-3/4 does not interact with UNC-73C1 in vitro in the N-terminal region through the RhoGEF2 and tandem PH domain region. However, additional experiments are required to determine whether full length UNC-73C1 interacts with FIP3/4 in vitro. The current design of the UNC-C1 fragments could possibly alter the binding site for FIP3/4 so interactions are no longer possible. Also, the GST-UNC-73C1(C-Term) fragment that did not express in bacteria, may contain the FIP-3/4 binding site, which could be why an interaction was not observed. Testing C-terminally tagged versions of the UNC-73C1 fragments would rule out N-terminal tag alterations to UNC-73C1’s binding capabilities to FIP3/4.
Figure 15. An in vitro interaction between UNC-73C1 and FIP3/4 fusion proteins was not detected. A. His-FIP-3/4 is detected in the whole cell lysate control, but it is not pulled down by UNC-73 N-term or RhoGEF2 fragments. GST is loaded as a negative control for protein interaction. B. Whole cell lysates and pull downs probed with an anti-GST antibody reveal that the fusion proteins and GST control are expressed. Bands present were expressed at the expected sizes.

3.3 Analyzing UNC-73C1 in vivo expression.

3.3.1. UNC-73C1 expression analysis by co-expression using an ICR sequence. UNC-73C1 modulates locomotion through RhoGEF2 activation of RHO-1; however, the specific neurons that require UNC-73C1 RhoGEF2 activity for proper locomotion have not been identified. If these neurons can be identified, neuron-specific experiments can be designed to understand RhoGEF2 modulation. A construct expressing
UNC-73C1 without any tags rescues the RhoGEF2 loss of function lethargic phenotype, however, the same construct with a C-terminal GFP tag does not rescue the lethargic phenotype indicating it is no longer functional. The fluorescence observed in UNC-73C1::GFP animals is localized to a subset of neurons. It is possible that UNC-73C1::GFP did not rescue because it was not expressed in the correct neurons, or not functional in these neurons (Steven et al., 2005). To resolve this issue and determine where UNC-73C1 is expressed, without creating a fusion to GFP, we designed a construct with an inter-cistronic region (ICR) sequence, from a C. elegans operon, between the 3’ end of unc-73c1 and gfp (Figure 16) (Blumenthal, 2005; Lee et al., 2010). This allowed for a single mRNA to be transcribed under the control of the unc-73c1 endogenous promoter with UNC-73C1 and GFP translated as separate proteins in the same cells. To determine if the UNC-73C1 protein expressed from this construct was functional, the construct was microinjected into wild-type animals and subsequently crossed into unc-73(ce362) animals to test for rescue. The unc-73(ce362) mutation is a point mutation in the RhoGEF2 domain that causes a lethargic phenotype due to defects in neurotransmission (Hu et al., 2011; Steven et al., 2005; Williams et al., 2007). UNC-73C1 expression from the ICR construct rescued the RhoGEF2 mutant slow locomotion phenotype as Ex[unc-73c1::ICR::gfp]unc-73(ce362) animals had a locomotion rate close to wild-type (Figure 16). These results indicated that the UNC-73C1 protein produced from the unc-73c1::ICR::gfp construct is functional and an analysis of GFP fluorescence from animals containing the construct will reveal which cells express UNC-73C1.
Figure 16. unc-73c1::icr::gfp expression rescues the unc-73 RhoGEF2 slow locomotion phenotype. A. UNC-73C1 GFP constructs for expression in *C. elegans*. *unc-73C1* expresses without a fluorescent tag and was used previously to rescue the *unc-73C1(ce362)* lethargic locomotion phenotype. *unc-73C1::gfp* expresses as a fusion with GFP at the C-terminus but does not rescue the lethargic mutant phenotype. Construct *unc-73c1::icr::gfp* contains an inter-cistronic region (ICR) which allows UNC-73C1 and GFP to be expressed in *C. elegans* as separate proteins in the same cells. B. *unc-73(ce362)* RhoGEF2 mutant animals exhibit a lethargic phenotype. *unc-73c1::icr::gfp* expression in *unc-73(ce362)* animals rescues the slow movement phenotype. ***p<0.0001

3.3.2. GFP expression from *unc-73c1::icr::gfp* is localized to a subset of neurons. The functional *unc-73c1::icr::gfp* construct was used to determine which neurons express UNC-73C1 so UNC-73C1 function in the neuromodulation of locomotion can be studied in more detail in those specific neurons. GFP fluorescence was examined in *Ex[unc-73c1::ICR::gfp]unc-73(ce362)* animals. We observed that UNC-73C1 expression was localized to a subset of neurons and body wall muscle (Figure 17).
Previous rescue experiments revealed that UNC-73 RhoGEF2 activity in neurons, not muscles, is required for wild-type locomotion (Hu et al, 2011). In a first attempt to identify the neurons expressing GFP in the *unc-73c1::ier::gfp* animals fluorescence co-localization was performed using the fluorescent dye DiD to label chemosensory neurons. DiD co-localization revealed that the chemosensory neurons AFD, ASH, ASK, PHA, and PHB likely expressed *unc-73c1::ier::gfp* based on neuron localization and morphology (Figure 17, Table 6). GFP fluorescence was observed in neurons that were not DiD stained meaning some non-chemosensory neurons expressed UNC-73C1. Interestingly, expression was also observed in many interneurons but not detected in motorneurons. More co-localization experiments are required to identify all expressing neurons but a list of candidate interneurons is found in Table 6. Future rescue experiments using neuron specific promoters will test which neurons require UNC-73C1 expression for wild-type locomotion.
Figure 17. *unc-73c1::icr::gfp* is expressed in a subset of neurons. Confocal images of animals expressing *unc-73c1::icr::gfp* (green) stained with DiD to reveal chemosensory neurons (red). In the head region GFP fluorescence is observed in multiple neurons including sensory neurons and interneurons. In the tail region GFP is observed in the phasmid sensory neurons. A list of candidate neurons expressing *unc-73c1::icr::gfp* is given in Table 6.
Table 6. Candidate neurons expressing UNC-73C1 as determined by morphology and location. Using images from WormAtlas, candidate sensory and interneurons expressing \textit{unc-73C1::icr::gfp} were identified based on morphology and location.

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<th>Candidate Sensory Neurons Expressing UNC-73C1</th>
<th>Candidate Interneurons Expressing UNC-73C1</th>
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Chapter Four

4. Discussion

My work was primarily focused on defining the molecular mechanisms of the UNC-73 Sec14 domain function and the roles it plays in regulating nervous system function. In addition, I examined how individual domains contribute to UNC-73 RhoGEF2 regulation of neurotransmission by characterizing a potential UNC-73 interacting protein, Rab-11-FIP-3/4.

4.1 UNC-73B Sec14 domain function.

The *C. elegans* UNC-73A and B isoforms have Sec14 domains at their very N-termini. While the Sec14 domain functions as a lipid binding domain in other proteins, its function in the UNC-73 isoforms is not known. Neuronal overexpression of the UNC-73 Sec14 domain in wild type animals appears to have a dominant-negative effect that suggests the Sec14 domain is important for UNC-73 function. For example, UNC-73B(Sec14) overexpression is characterized by a slow uncoordinated locomotory phenotype, similar to an UNC-73B loss of function phenotype. Point mutations in the UNC-73B RhoGEF1 domain (*rh40*), and other mutations affecting UNC-73B, cause slow uncoordinated locomotion likely due to axon guidance defects during development (Steven et al., 1998). It is currently unknown if mutations in any of the other *C. elegans* Sec14 containing genes result in uncoordinated or slow moving phenotypes.

UNC-73B Sec14 overexpression also slowed development relative to wild-type animals. Currently, there is limited evidence supporting nervous system control of development. Animals with M4 neuron ablation results in L1 arrest due to the resulting pharyngeal pumping defects (Avery and Horvitz, 1987). Also, deletion of *eat-1, -6, -8,*
and *eat-9* result in post-embryonic lethality due to defects in the extrapharyngeal nervous system that slow the pumping rate (Raizen et al., 1995). *unc-73(rh40)* animals are not slow growing, suggesting the UNC-73(Sec14) overexpression phenotype is likely independent of UNC-73 RhoGEF1 pathway function. Interestingly however, *unc-73* RhoGEF2 deletion mutants arrest development at the L1 stage due to pharynx pumping defects (Steven et al., 2005). These defects in pharynx pumping inhibit the animal’s ability to eat, resulting in slowed or arrested development. It is possible that something similar is occurring in the UNC-73B(Sec14) transgenic animals.

UNC-73B Sec14 domain overexpression could be affecting the function of the 14 other genes encoding a Sec14 domain. In fact, one *C. elegans* Sec14 containing gene, *cgr-1*, has an L1 arrest phenotype (Goldstein et al., 2006). CGR-1 (CRAL-Trio and GOLD domain) function is not well characterized, but CGR-1 was identified as a regulator of the Ras signal transduction pathway to determine vulval cell fate (Goldstein et al., 2006). CGEF-1 is a RhoGEF that functions during anterior-posterior polarization of the developing embryo and activates CDC42 (Kumfer et al., 2010).

In addition, UNC-73B(Sec14) neuronal overexpression dramatically alters the nutritional modulation of locomotory behavior. For example, the rate of spontaneous reversal for wild-type animals typically increases from about 1 reversal per 3 minutes to 6 reversals per 3 minutes as animals are moved from agar plates with food to plates without food (Gray et al., 2005). In contrast, UNC-73B(Sec14) neuronal overexpression increases the frequency of spontaneous reversal on food to 8 reversals per 3 minutes and decreases the frequency of spontaneous reversal off food to 1 reversal per 3 minutes, suggesting that UNC-73B(Sec14) overexpression may interfere with the food signal, i.e., animals on
food behave as if they are off food and animals off food behave as if they are on food. Food is perceived by a limited number of chemosensory neurons whose signaling is integrated by interneurons to control motorneurons and determine speed and direction of movement. For example, the two AWC sensory neurons synapse onto the two AIB interneurons, which in turn synapse onto the two RIM interneuron/motorneurons to modulate reversal and backward locomotion (Gray et al., 2005). The inhibition of RIM activity increases spontaneous reversals on food, so that altered signaling in any or all of these neurons could account for the observed UNC-73B(Sec14) over expression reversal phenotypes. It will be interesting to assess if UNC-73B(Sec14) overexpression has similar effects on other nutritionally-dependent behaviors and to identify the individual neurons responsible for the overexpression phenotypes. Nutritional status modulates most key behaviors in C. elegans, including olfaction, aversive responses, and locomotion through neuropeptide and monoamine signaling (Harris et al., 2011). UNC-73 is thought to play a role in neuropeptide signaling, largely through RhoGEF2 activation of RHO-1. It is currently unknown how or if UNC-73 RhoGEF1 activity relates to neuropeptide signaling; however, Trio and Kalirin RhoGEF1 activity is involved in DCV secretion mechanisms (Ferraro et al, 2007; Xin et al, 2004; Mains et al, 1999).

The Sec14 domain in other proteins, including RhoGEFs and RhoGAPs, is responsible for protein localization (Figure 8), so perhaps the UNC-73B Sec14 domain could play a role in protein localization. UNC-73B::GFP expression is quite faint and the only subcellular localization visible is in axons around the nerve ring (Steven et al, 2005). Subcellular localization to other locations could be transient, but still part of UNC-73 function. When expressed in neurons UNC-73B(Sec14)::GFP exhibits a subcellular
punctate fluorescence in the neuronal cell body (Figures 2&3). This sub-cellular localization is similar to that seen with the *C. elegans* RAB-2 protein, for example, which localizes to the Golgi apparatus to promote DCV signaling (Hannemann et al., 2012). This is also consistent with the localization pattern of other Sec14 domain-containing proteins, such as Dbs, that localize to organelles in the secretory pathway (Kostenko et al., 2005). These observations suggest that UNC-73B(Sec14) overexpression might prevent the trafficking of endogenous UNC-73B required for RhoGEF1 function. The UNC-73B Sec14 domain could be acting to interfere with UNC-73B RhoGEF signaling, especially by interfering with proper UNC-73B localization. In mammals, the RhoGAP p50 requires proper localization to the plasma membrane in order to regulate its GAP activity (Krugmann et al., 2002).

**4.1.1 Future aims.** We are trying to transgenically overexpress full length UNC-73B and UNC-73B(Δsec14) to observe any loss of function phenotype and perform rescue experiments. We also want to compare UNC-73B neuronal localization in UNC-73B::GFP and UNC-73B(Δsec14)::GFP transgenic animals. Also, we are working on overexpressing RGA-1(Sec14), CGR-1(Sec14), and CGEF(Sec14) to further characterize neuronal Sec14 domain functions *in vivo*. We will determine if the UNC-73B(Sec14) phenotypes are specific to UNC-73B or if overexpression of other Sec14 domains causes similar phenotypes. An experiment using a heat-shock promoter to drive *unc-73B(Sec14)* expression only in adult animals would determine if this domain is required during development. It would also be interesting to overexpress the Sec14 domain in non-neuronal cells to observe any other cell-specific phenotypes and functions. Co-localization studies will be performed to confirm that UNC-73B(Sec14) is localizing to
the Golgi. We have both Golgi and endosomal markers, gifts from Dr. Bruce Bamber’s lab, to be used in these experiments.

We are also interested in further investigating the other *C. elegans* proteins that have a Sec14 domain and their similarity to mammalian proteins. A *C. elegans* RhoGEF and a RhoGAP each have a Sec14 domain, but we hope to determine the functions of the other identified proteins and if any are mammalian homologs. Conversely, it would also be interesting if any of them are unique to *C. elegans*.

Finally, we want to analyze another UNC-73B non-enzymatic domain. The SH3 domain’s localization and function is not well characterized, but may play a role in negatively regulating RhoGEF enzymatic activity. This has never been studied in regards to UNC-73 neuronal function and we hope to investigate it further.

### 4.2 UNC-73C1 may not interact with protein identified by yeast 2-hybrid screening.

*unc-73C1* encodes the RhoGEF2 domain and functions to regulate neurotransmission through activation of RHO-1. Little is known about how this regulation occurs so we were interested in determining if UNC-73C1 had any other interacting proteins. We wished to confirm the potential UNC-73C1 interacting proteins identified in a yeast two-hybrid screen performed by former lab member, John Farver. One protein identified, RAB-11-FIP3/4, is particularly interesting to us because it binds to Rab11 and NSF. NSF is an ATPase that binds the SNARE complex during neurotransmission and promotes SNARE complex recycling (Vivona et al., 2013).

The full length UNC-73C1, made by Shuang Hu, with N-terminal GST or His tag did not express well in bacteria (Figure 9). This was the basis for expressing UNC-73C1 in three separate fragments, with the added possibility of potentially identifying a specific
binding region on UNC-73C1 for the candidate interacting protein FIP-3/4 (Figure 10). Two out of three GST-UNC-73C1 fragments and full length His-FIP-3/4 expressed well in bacteria. One UNC-73C1 fragment did not express well in bacteria (Figure 11). After confirming protein solubility for candidate proteins (Figure 12), a GST pull down was performed. The experimental conditions including protein concentration, bacterial growth rate, film exposure time, etc. were all extensively optimized and never confirmed any interaction between UNC-73C1 fragments and FIP-3/4 (Figure 13). It is possible that FIP-3/4 could bind the UNC-73C1 C-terminus that was unable to express in bacteria. Another possible explanation for the lack of binding is that the N-terminal tags interfere with protein binding or folding. Tagging the C-termini of the proteins and repeating the pull down experiments may rule out this explanation.

4.2.1 Future aims. We are not fully convinced that UNC-73C1 does not interact with FIP-3/4. First and foremost, we hope to perform an experiment with a positive control. We hope to try different mechanisms of performing IP experiments using full length UNC-73C1 and FIP-3/4. In addition, we are interested in confirming the interactions between UNC-73C1 and the other candidate proteins from the yeast two-hybrid screen such as DYS-1B. If any interactions are found to occur with UNC-73C1 in vitro, then we would like to also confirm these interactions in vivo.

4.3 UNC-73C1::ICR::GFP transgenic expression rescues the RhoGEF2 lethargic movement phenotype and is observed in a subset of neurons.

To further understand how UNC-73C1 is modulating neurotransmission, we wanted to examine its endogenous expression pattern. If UNC-73C1 expression is localized to a subset of neurons, this could provide insight into the molecular mechanisms
of regulation. This will also allow us to perform neuron specific studies and identify which neurons require UNC-73C1 expression.

UNC-73C1::ICR::GFP is expressed in a subset of neurons and body wall muscle when under the control of its endogenous promoter. Confocal images of DiD stained animals shows co-localization in some sensory neurons (Figure 15). GFP fluorescence is also observed in interneurons but not in motorneurons. This expression pattern leads us to believe that UNC-73C1 may play a role in integrating and propagating sensory signals to control locomotion and behavior.

**4.3.1 Future aims.** Future neuron specific co-localization studies will confirm which neurons express UNC-73C1::ICR::GFP. Upon identification of the UNC-73C1 expressing cells, neuron specific rescue experiments will be performed to determine which neurons require UNC-73C1 expression to control locomotion. Electron microscopy can be utilized to look at SVs and DCVs in specific neurons to examine patterns and defects in *unc-73* mutant animals.
References


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