Caenorhabditis elegans RHGF-2 is required for embryonic elongation and co-localizes in vivo with the PDZ-domain containing scaffold protein MPZ-1

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*in vivo* with the PDZ-domain Containing Scaffold Protein MPZ-1

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

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An Abstract of

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Rho GTPases function in important biological processes such as cell proliferation and survival, cell polarity and morphogenesis, migration, transcription and neuronal development. Rho GTPases act as bi-molecular switches and are highly regulated by guanine nucleotide exchange factors (GEFs), GTPases-activating proteins (GAPs) and GDP dissociation inhibitors (GDIs). Currently, Rho GTPase signaling within neurons is not well understood; therefore, identifying essential components of Rho signaling is important. For example, a point mutation in the human RhoA GEF, PLEKHG5, results in an autosomal recessive form of lower motor neuron disease. PLEKHG5 and its homologs Tech and Syx are expressed in neurons and regulate RhoA activity during development in mammalian systems.

This study focuses on characterizing a putative ortholog of Tech/Syx/PLEKHG5, RHGF-2 (Rho guanine nucleotide exchange factor), in the model organism *Caenorhabditis elegans*. RHGF-2 exhibits ~30% identity to Tech/Syx/PLEKHG5 in the RhoGEF domain, and has a similar domain layout with a PH domain immediately following the RhoGEF domain and a C-terminal PDZ binding motif. RHGF-2 is
expressed exclusively in neurons starting around the 1.5-fold embryonic stage and continuing through adulthood. RHGF-2 is expressed in a variety of head, body and tail neurons, including the ALM, AVM, PLM and PVM mechanosensory neurons. However, RHGF-2 does not appear to be expressed in the DiD accessible sensory neurons.

*rhgf-2*(gk216) null animals hatch, but arrest development at the L1 stage, as dumpy immobile animals. By following epidermal seam cell morphology using the AJM-1::GFP apical junction marker, we observed that seam cells in gk216 animals do not properly elongate. *rhgf-2* is encodes at least two isoforms, RHGF-2l and RHGF-2s, and the expression of either isoform rescued the gk216 embryonic elongation phenotype.

The overexpression of *rhgf-2* results in loopy movement with a higher amplitude waveform. This locomotory phenotype is similar to that observed in constitutively active RHO-1 animals, suggesting that RHGF-2 plays an additional role in modulating *C. elegans* locomotion. The results of this study suggest that the expression of RHGF-2, a RHO-1 specific RhoGEF, capable of binding to the MPZ-1 PDZ-domain containing scaffold protein, is essential for the elongation of epidermal seam cells and wild-type embryonic development and may play additional neuronal roles in modulating locomotory behavior.
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Chapter 1

Introduction

Rho GTPase signaling is involved in a variety of biological processes including cell proliferation and survival, cell polarity and morphogenesis, migration, gene transcription vesicular trafficking and deregulation of Rho Pathways is associated with cancer, neurological and developmental disorders (Chelly and Mandel, 2001; Stevens and Der, 2010; Govek et al., 2005). The most extensively studied and characterized Rho GTPases are Rho, Rac and Cdc42. Rho GTPases are low molecular weight proteins (21-28kDa) that act as bi-molecular switches, cycling between two conformational states: GDP-bound (inactive) and GTP-bound (active).

Rho GTPases are highly regulated and have three classes of regulators; guanine nucleotide exchange factors (GEFs) catalyze the exchange of bound GDP for GTP. GEFs for Rho family GTPases contain a RhoGEF domain of ~200 amino acids, which catalyzes the exchange reaction. The RhoGEF domain stabilizes the unbound form of Rho GTPases allowing GTP to bind due to higher cellular GTP levels (Rossman et al., 2005). GTPase-activating proteins (GAPs) stimulate Rho GTPase intrinsic GTP hydrolytic activity and convert active GTP-bound Rho to inactive GDP-bound Rho (Moon et al., 2003). Lastly, GDP dissociation inhibitors (GDIs) block the dissociation of GDP and
stabilize the inactive Rho GTPase (Der Mardirossian et al., 2005). Rho GTPases are activated through GEFs or the inhibition of GAPs and this study will focus on characterizing a particular RhoGEF, called RHGF-2.

Rho signaling in neuronal morphology has not been extensively studied, but additional characterization of key components of Rho signaling pathways may help dissect the etiology of certain disease. For example, some genes in the Rho GTPase pathways are associated with mental disorders such as Lowe syndrome and Fragile X syndrome (Atree et al., 1992; Billuart and Chelly, 2003). Abnormal Rho signaling also leads to motor neuron diseases such as Amyotrophic Lateral Sclerosis (ALS), a progressive degeneration of motor neurons (Hadano et al., 2001; Rosen et al., 1993; Yang et al., 2001). Characterizing Rho GTPases and their regulators will aid in the development of therapies and treatments for these diseases. This study characterizes the function, expression, and interactions of RHGF-2, a RhoGEF that specifically binds and activates RHO-1.

1.1 Rho GTPases in the nervous system

The most extensively studied Rho GTPases are RhoA, Rac1 and Cdc42 and all three are key regulators in reorganizing the actin cytoskeleton (Lundquist, 2006). Activated RhoA stimulates the formation of stress fibers and focal adhesions, Rac1 stimulates lamellipodia formation and Cdc42 stimulates filopodia formation (Ridley and Hall, 1992; Ridley et al., 1992; Nobes and Hall, 1995; Kozma et al., 1995). Actin and microtubule cytoskeleton reorganization is the main tool in neuronal development to assemble neurite extensions, axonal and dendritic migrations and branching and perhaps
the regeneration of neurites (Govek et al., 2005). Figure 1 outlines RhoA, Rac1, Cdc42 and their corresponding effectors involved in actin and microtubule coordination. Rac and Cdc42 activation and the inactivation of Rho stimulates neurite formation, however activation of Rho results in neurite retraction (Negaishi and Katoh, 2002; Nusser et al., 2002; Jalink et al., 1994; Katoh et al., 1996; 1998; Postma et al., 1996). Rac and Cdc42 promote axonal elongation and Rho retracts axonal migration and branching. For example, the Drosophila protein Trio, through Rac activation, is important for axonal growth since null animals develop shortened axons that barely reach to the CNS midline (Hakeda-Suzuki et al., 2002). Inactivation of Rho activity, through the inhibition of the protein Kalirin, reduces axonal length and branching in mouse hippocampal neurons. In contrast, activation of p190 RhoGAP, which inactivates RhoA, stimulates axon growth (Ahnert-Hilger et al., 2004; Penzes et al., 2001; Brouns et al., 2001). Lastly, RhoA is implicated in axonal regeneration since neuronal RhoA activity increases after injury in rats, as demonstrated by increased levels of the RhoA effector, Rhotekin. Therefore, dissecting RhoA GTPase pathways may aid in finding treatments for spinal cord injuries (Dubreuil et al., 2003; Madura et al., 2004). Similarly, Rho GTPases have many important functions yet more research needs to be done on Rho GTPase regulators that ultimately affect Rho GTPase activity.
Figure 1: Rho GTPases effectors in actin and microtubule cytoskeleton dynamics (Govek et al., 2005). Toca-1 (Transducer of Cdc42-dependent acting assembly), WIP (WASP-interacting protein), WASP (Wiskott-Aldrich-syndrome protein), Arp2/3 (actin-related proteins 2 and 3), PAK (p21-activated kinase), LIMK (Lin-11, Isl-1, and Mec-3 kinase), Cdk5 (cyclin-dependent kinase 5), IRSp53 (insulin receptor substrate of 53 kDa), Mena (mammalian Ena), WAVE (WASP family Verprolin-homologous protein), CYFIP (cytoplasmic FMR1-interacting protein), PIR121 (p53-inducible mRNA), Nap125 (Nck-associated protein), Abi2 (Abl interactor 2), HSPC (heat-shock protein C), MLCK (myosin light chain), MLCP (myosin light chain phosphatase), and Dia (Diaphanous-related formins).

1.2 Mammalian RhoA specific GEFs Tech/Syx/PLEKHG5

To investigate Rho GTPase signaling, key proteins are mutated or deleted to observe defects in neuronal morphology. One key set of proteins is the mammalian
RhoGEF family of rat Tech, mouse Syx, and human PLEKHG5, all of which are RhoA specific exchange factors belonging to a new branch of the Dbl family of Rho specific GEFs (De Toledo et al., 2001). Dbl family members contain a Dbl homology (DH) domain, which is also referred as a RhoGEF domain, and a tandem pleckstrin homology (PH) domain (Whitehead et al., 1997; Stam and Collard, 1999). The Tech/Syx/PLEKHG5 RhoGEF family is unique due to the presence of a Type I C-terminal PDZ (PSD-95, Disc-large, ZO-1) binding motif (X-S/T-X-Φ, X=unspecified, Φ=hydrophobic) and each protein has ~80% identity to each other.

PLEKHG5 is expressed in the brain and weakly expressed in heart and skeletal muscle tissues (De Toledo et al., 2001). PLEKHG5 functions in neuronal morphology, via activated RhoA, to inhibit neurite outgrowth stimulated by NGF (Nerve Growth Factor) (Sebok et al., 1999; De Toledo et al., 2001). De Toledo et al (2001) also mapped PLEKHG5 to the chromosomal region 1p36.2-1p36.3, a region associated with high proto-oncogene activity and various cancers, particularly in neuroblastoma (Grenet et al., 1998; Nomoto et al., 2000; Hogarty et al., 2000).

A single point mutation in PLEKHG5 causes a form of autosomal recessive Lower Motor Neuron Disease (LMND) (Maystadt et al., 2007). The point mutation causes a Phe647Ser change in the PH domain; the phenylalanine in the PH consensus sequence is conserved across species. The Rho families of GTPases are known to activate NFκB and it has a neuronal antipoptotic role (Perona et al., 1997; Barger et al., 2005). The mutant PLEKHG5 RhoGEF results in a loss of NFκB activation and promotes apoptosis of neurons. In addition, the mutant PLEKHG5 is misfolded and aggregated in when transfected in HEK293 and MCF10A cell lines. This is likely the cause of toxic
interactions and the loss of function connected with certain types of LMND (Maystadt et al., 2007; Blomberg et al., 1999).

Rat Tech is selectively expressed in neurons localizing to the cell bodies and extending into the dendrites. Tech functions in neurons to decrease the number of dendritic processes by activating RhoA. Decreased number of neurite extensions and lack of focal point formations results in an increased number of rounded cells (Marx et al., 2005).

Mouse/zebrafish Syx functions in lumen formation particularly in angiogenesis and in elongating the anterior-posterior body axis (Garnass et al., 2008). Syx has two splice variants termed Syx 1 and Syx 2. The difference between the two variants is Syx 2 does not have the C-terminal PDZ binding motif (1070-1073, SEV). Syx1 contains the C-terminal type I PDZ ligand motif, SEV, and binds to the single PDZ domain of the scaffold protein synectin. Syx 1 at the plasma membrane activates RhoA in endothelial cells (EC) and stimulates cell migration and tube formation. The absence of Syx during angiogenesis reduces the abundance of secondary blood vessels, such as capillaries, and shortens the anterior-posterior elongation in zebrafish (Garnass et al., 2008). This suggests that cell migration requires the spatial and temporal coordination of the Rho GTPase activity (Van Nieuw Amerongen et al., 2003; Fukata et al., 2003).

1.3 PDZ-domain scaffold proteins MUPP1/MPZ-1 and Synectin

The C-terminal PDZ binding motif, in proteins such as Tech/Syx/PLEKHG5, mediates interactions with PDZ-domain containing protein. PDZ-domain containing scaffold proteins can organize large signaling complexes for efficient spatial and
temporal timing for signal transduction. Scaffold proteins are not freely diffused in the cytoplasm but typically are attached to the cell membrane via transmembrane receptors or through other membrane-associated proteins (Zhang and Wang, 2003). PDZ-domain scaffold proteins contain either catalytic domains or have no intrinsic enzymatic activities. MUPP1/MPZ-1 is the latter. PDZ domains are modular protein interaction domains that associate with specific proteins containing a PDZ binding motif at the C-terminus to assemble large signaling complexes (Lee and Zheng, 2010). The PDZ domain consists of approximately 90 amino acids and is found across species from yeast to humans.

PDZ domains interact with three classes of amino acid sequences found at the C-terminus of proteins. The C-terminus Type I consensus sequence is X-S/T-X-Φ, Type II sequence is X-Φ-X-Φ and the Type III sequence is X-D-X-Φ (X=unspecified, Φ=hydrophobic). Several studies have shown that residues up to position -8 may also be important for the interaction (Zhang et al., 2007; Lee and Zheng, 2010; Tonikian et al., 2008). The C-terminal sequences allow PDZ-domain containing proteins to interact with target proteins without interrupting the structure and function of the protein. PDZ domains are also able to interact with other distinct protein binding motif such as ankyrin and spectrin repeats and LIM domains (Lee and Zheng, 2010).

Multi-PDZ-domain containing proteins can simultaneously bind to multiple targets making the complex efficient for co-localizing all components of a specific cellular process. The C-terminal binding motif of Tech/Syx/PLEKHG5 binds to either MUPP-1 or synectin scaffold proteins. Mammalian MUPP1 (Multi-PDZ-domain Protein 1) has 13 PDZ domains and is highly expressed in the cortex, hippocampus, amygdaloid
nuclei, myelencephalon and choroid plexus of the brain (Ullmer et al., 1998; Manicini et al., 2000; Becamel et al., 2001; Sitek et al., 2003). Serotonin receptors 5-HT\textsubscript{2A}, 5-HT\textsubscript{2B} and 5-HT\textsubscript{2C} function in control of nociception, motor behavior, endocrine secretion, thermoregulation, regulation of appetite and cerebrospinal fluid production (Lucki et al., 1989; Murphy et al., 1991; O’ Brien et al., 1998). 5-HT\textsubscript{2C} co-localizes with MUPP1 in the central nervous system, specifically in the choroid plexus, and MUPP1 functions as the adaptor protein to cluster 5-HT\textsubscript{2C} receptors and/or link the receptor to other proteins (Abramowski et al., 1995; Clemett et al., 2000).

In *Caenorhabditis elegans*, MPZ-1 displays significant homology to MUPP1 from 36\%-60\% identity to each PDZ domain. The *C. elegans* serotonin receptor SER-1 binds to PDZ domain 10 on MPZ-1; however the PDZ domain 10 has little similarity to any of MUPP1 PDZ domains (Xiao et al., 2006). ser-1 and mpz-1 co-localizes in 3 sets of motor neurons: RMHs, RMFs and RMDs. Importantly, the function of SER-1 in 5-HT stimulated egg-laying is dependent on the presence of the C-terminal PDZ binding motif (TFL) (Xiao et al., 2006).

The Rat RhoGEF Tech contains a Type I PDZ binding motif (SEV) that interacts with PDZ domain 10 on MUPP1 to regulate RhoA signaling within neuronal cells (Estévez et al., 2008). MUPP1 and Tech co-localize in distal dendritic processes, in which Tech activates RhoA in the dendritic extensions near the synaptic zones. However, whether MUPP1 co-localizes with Tech at the puncta near the synaptic zones remain inconclusive (Estévez et al., 2008). The MUPP1-Tech interaction allows activation of the RhoA pathway to stimulate actin cytoskeleton reorganization that may influence neurite extensions (Neumann et al., 2002; Ahnert-Hilger et al., 2004; Govek et al., 2005).
Scaffolding proteins like MUPP1 are able to link GEFs and GTPases to other specific effector proteins that can bind to the alternative PDZ domains on MUPP1 (Buschsbaum et al., 2002; 2003; Jaffe et al., 2004).

Synectin is another PDZ-domain containing protein that functions as an adaptor protein for Syx allowing interaction with Myosin VI. Simultaneously, Syx binds to the single PDZ domain, while Myosin VI binds to the C-terminus of synectin (Liu and Horowitz 2006; Reed et al., 2005). As mentioned above, Syx in mouse and zebrafish has two splice variants, Syx1 and Syx2 differing by the presence/absence of the PDZ binding motif. Syx1 localizes to the perinuclear region and plasma membrane when bound to synectin via the PDZ binding motif. However, Syx2 is diffusely distributed in the cytoplasm (Liu and Horowitz, 2006). Interestingly, the ability of Syx1 and Syx2 to activate RhoA is the same level following lysophosphatidic acid (LPA) stimulation in rat fat pad endothelial cells (RFPEC). However, the basal level of RhoA activity level in unstimulated Syx 2-expressing RFPECs was ~ 70% higher than Syx 1-expressing cells. In addition, upon LPA stimulation, Syx 1 increased RhoA activity along the plasma membrane compared to Syx2-expressing cells, which had a global increase of RhoA activity (Liu and Horowitz, 2006). Binding to synectin determines the rate of endothelial cell migration because Syx 2-expressing cells had decreased EC migration (Liu and Horowitz, 2006). Together, this suggests that the PDZ binding motif determines the function of Tech and localization of RhoA activity.
1.4 Caenorhabditis elegans model organism

*Caenorhabditis elegans* is an excellent model organism to examine neuronal development and small in size physically and genomically. Animals are inexpensive and easy to use for genetic analysis (Brenner, 1974). *C. elegans* is a free-living transparent nematode, normally found living in temperate soil or rotting fruit (Kiontke et al., 2011). *C. elegans* has a three day life cycle, therefore in a short period of time labs are able to cultivate several generations of animals. The nematode grows to be about one millimeter and is cultivated on agar plates seeded with OP50 *E. coli*. The genome is fully sequenced (*Caenorhabditis elegans* Genome Consortium, 1998), and the worm is the one of the smallest and simplest organism with a nervous system (White et al., 1986). *C. elegans* has 302 neurons with the connectivity mapped. In addition, about 83% of the *C. elegans* proteome has human homologous proteins (Lai et al., 2000).

In the present study, model organism *Caenorhabditis elegans* is used because neuronal development can be easily studied. In addition, *C. elegans* have a putative ortholog to mammalian MUPP1 and RhoGEF Tech/Syx/PLEKHG5, which are MPZ-1 (Multi-PDZ-domain scaffold protein 1) and RHGF-2 (Rho guanine nucleotide exchange factor 2), respectively. Within *C. elegans*, we can see similar pathways to the mammalian orthologs, as well as, observe different phenotypes that can shed light on other Rho GTPases signaling pathways MPZ-1 and RHGF-2 participate in (Figure 2).
Figure 2: MUPP1/MPZ-1 PDZ-domain containing scaffold proteins interactions are conserved from mammals to *C. elegans* (Lin et al., 2012). Mammalian serotonin receptor, 5-HT$_{2C}$ binds to MUPP1 PDZ domain 10 via C-terminal PDZ binding motif (SSV). RhoA GEF, Tech binds to MUPP1 PDZ domain 13 via C-terminal PDZ binding motif (SEV). Tech activates the downstream effector, RhoA. Likewise, serotonin receptor, SER-1 binds to PDZ domain 10 on MPZ-1 via (TFL) C-terminal PDZ binding motif and RHGF-2 binds to MPZ-1 PDZ domain 8 via (SDV) and activates RHO-1.

1.4.1 *C. elegans* contains conserved homologs of RhoA, Rac and Cdc42 identified as RHO-1, CED-10 and CDC-42, respectively. RHO-1 controls neuronal morphogenesis after the neuron has established axon and dendrite in a similar fashion as mammalian RhoA (Lundquist, 2006). CED-10 controls multiple characteristics of axon pathfinding such as axon growth, axon guidance, and axon branching (Lundquist et al., 2001; Wu et al., 2002). CDC-42 establishes the embryonic polarity, which essential determines the anterior-posterior axis (Gotta et al., 2001; Kay and Hunter, 2001; Lundquist 2006). For neuronal cells, the established polarity determines which initial extensions from the cell body differentiate as the axon and dendrites (Pruyne and Bretscher, 2000; Manneville and Hall, 2002).

1.4.2 Epidermal morphogenesis during embryonic development of *C. elegans*. Embryonic morphogenesis depends on the epidermis tissue, which determines the shape of the animal (Figure 3). *C. elegans* matures into a long cylindrical body ~1mm in size.
The epidermis is synonymous to hypodermis, but here it will be described as the epidermis. The single epidermal layer of cells interacts with underlying neurons and muscle. The epidermis provides the actual force to elongate the anterior-posterior body axis during the embryonic bean-shaped stage to the three-fold stage (Priess and Hirsh, 1986; Chisholm, 2005). The epidermis is formed from 71 major epidermal cells and 11 minor epidermal cells. After the epidermis is produced, three major groups of cells extending along the anterior-posterior axis are defined: dorsal epidermal cells as two rows of ten cells, two lateral rows called seam cells and the ventral epidermal cells (Chisholm, 2005). The extracellular epidermis sheath is formed within a few minutes after the major epidermal cells, and the apical epithelial markers, AJM-1, DLG-1 and CHE-14 begin to localize (McMahon et al., 2001; Chisholm, 2005). The ventral neuroblasts produce the substrate the epidermal cells require to migrate over and enclose the embryo. Additionally, the ventral neuroblasts undergo ventral cleft closure, which must occur before the epidermal closure begins; otherwise embryonic elongation will be arrested (Mango et al., 1994; Chisholm, 2005). However the interaction between the migrating epidermal cells and neuronal substrate has not been extensively studied. Some theories suggest epidermal enclosure requires a uniform substratum of cells, which can be disrupted by a persisting ventral cleft. Specific substratum cells may function to guide the epidermal cell movements (Chisholm, 2005).
Figure 3: Developmental timing of morphogenetic events (Chisholm, 2005).

Developmental landmarks are indicated and represented with Nomarski differential interference contrast images of embryos at the corresponding stages. Developmental times are relative to the division of the zygote ($P_0$) in minutes at 20°C. Between 350 and 400 minutes, the embryo begins turning, therefore, images before turning are either ventral or dorsal view and after turning images are either left or right lateral views. For all lateral embryo views anterior is on the left side of the image.

After ventral cleft closure, epidermal enclosure can begin; otherwise incomplete ventral closure will halt elongation. The two rows of dorsal epidermal cells undergo dorsal intercalation and form a single row, which requires actin microfilaments and
microtubules (Figure 4; Sulston et al., 1983; Williams-Masson et al., 1998). Ventral closure proceeds by which ventral epidermal cells migrate toward the ventral midline to encase the underlying cells (Figure 5). The ventral enclosure happens in two phases; first phase is two pairs of the anterior leading cells extend large protrusions and migrate toward the ventral midline. In the second phase, posterior ventral cells elongate toward the midline. The migrating ventral epidermal cells make bilaterally symmetric contacts, which form new cell junctions at the midline. Enclosure is complete when the extreme anterior epidermis encases the embryo with a single layer of epidermal cells (Chisholm, 2005). Once again actin and microtubule cytoskeletal systems are required for ventral enclosure (Williams-Masson et al., 1997).

Figure 4: Dorsal intercalation (Chisholm, 2005). A-D) Schematics, dorsal cells are teal, seam cells are yellow and deirids are white. E-H) Nomarski DIC images of embryos in the dorsal view. I-L) DLG-1::GFP highlights epidermal cell boundaries in the dorsal view. DLG-1 is an epithelial marker localized to apical junctions. Scale bar is 5µm.
Figure 5: Epidermal enclosure (Chisholm, 2005). A-D) Schematics, Ventral cells are pink, seam cells are yellow and dorsal cells are teal. E-H) Nomarski DIC images of embryos in the ventral view. I-L) DLG-1::GFP images highlights epidermal cell boundaries in the ventral view. DLG-1 is an epithelial marker localized to apical junctions. Scale bar is 5µm. Leading cells are the first two pairs of ventral epidermal cells to reach the ventral midline.

Immediately after the epidermis is enclosed and has encased the underlying cells, elongation ensues (Figure 6). Epidermal elongation begins at ~350 minutes and ends ~600 minutes after the first cleavage. The embryo via epidermal cells reduces the body’s circumference by a factor of three and increase in length by a factor of four during elongation (Priess and Hirsh, 1986; Chisholm, 2005). Early elongation occurs between the comma and two-fold stage. First, the actin and microtubules in the dorsal and ventral epidermal cells become organized into a circumferential pattern, although the mechanisms by which circumferential actin filament bundles (CFBs) form is unknown.
(Costa et al., 1997; Priess and Hirsch, 1986; Williams-Masson et al., 1997). However, actomyosin-based contraction localized to the cytoplasm of lateral seam cells is required for epithelial cells to elongate. The epidermal actin cytoskeleton actively regulates the shape of the epidermis. The lateral seam cells transmit the contractile force to the dorsal and ventral epidermal cells via adherens junctions (Chisholm, 2005). RHO-1 controls embryonic elongation. RHO-1 activates the LET-502 Rho Kinase and results in hypodermal contraction of the CFB (Wissmann et al., 1997). On the other hand, the UNC-73/Trio Rac/Rho GEF activates the Rac-like GTPases MIG-2, which in turns activates the MEL-11 myosin phosphatase to inhibit hypodermal contraction (Steven et al., 1998; Wissmann et al., 1999; Spencer et al., 2001; Wu et al., 2002).

Late elongation occurs beyond the two-fold stage. Muscle plays an important function during the second phase of the elongation. For example, in paralyzed Pat (paralyzed at two-fold) mutants, elongation occurs normally until the two-fold stage. Afterwards, elongation arrests due to paralyzed state of the animal (Williams and Waterston, 1994). The mechanism for the muscle to function in epithelial cell shape is unknown. A more severe Pat mutant is defective in epidermal attachment structures and show normal muscle contractions at the 1.75-fold stage, yet afterwards, the muscles are detached from the epidermis (Chisholm, 2005). Elongation ends after the three-fold stage is complete. The epidermis begins to secret the cuticle for the L1 stage, which now holds the epidermal cells in place.
Figure 6: Embryonic stages of development (Altun and Hall, 2009). Numbers below the horizontal axis show approximate minutes after fertilization at 20-22°C. First cleavage occurs approximately 40 minutes after fertilization. (Yellow-green bars). The first cells that move inwards from the ventral surface are gut precursors (E), followed by mesoderm (MS) and germline (P4) precursors. (Blue bar) Gastrulation is between 270 and 330 minutes (Red bar) Elongation of the embryo occurs between 400 and 640 minutes. The stages, number of nuclei, marker events and DIC images of the embryos and a newly hatched larva are shown above the horizontal axis.

1.5 Characteristics of RHGF-2

1.5.1 RHGF-2 is a specific RHO-1 exchange factor. To determine the GTPase binding specificity of RHGF-2, a GST pull down assay was used with the three
prototypical members of the Rho GTPase: RhoA (RHO-1), Rac (CED10), and Cdc42 (CDC-42). RHGF-2 bound strongly to RHO-1 compared to CED-10 and CDC-42 (Figure 7A) (Lin et al., 2012). Similarly, RHGF-2 co-immunoprecipitated strongly to RHO-1 when both proteins are co-expressed in HEK293 cells (Figure 7B) (Lin et al., 2012). Examination of RHGF-2 RhoGEF activity for each of the three Rho GTPases indicated RHGF-2 significantly activated RHO-1 compared to CED-10 and CDC-42 (Figure 7C).

Figure 7: RHGF-2 binds to and activates RHO-1 (Lin et al., 2012). A) GST::RHO-1, GST::CDC42, and GST::CED-10 fusion proteins were purified on glutathione sepharose beads (lower panel). The purified fusion proteins were incubated with lysates from HEK295T cells expressing FLAG::RHGF-2s. Bound proteins were separated by SDS-
PAGE and analyzed by immunoblotting with anti-FLAG antibody (upper panel). B) FLAG::RHGF-2s was transiently co-transfected with GST::RHO-1, GST::CDC42, and GST::CED-10 fusion proteins. Cell lysates were prepared 48 hrs after transfection and immunoprecipitated with anti-GST antibody, and analyzed with anti-FLAG antibody (upper panel). Cell lysates were immunoblotted with anti-GST to confirm the expression of the appropriate fusion protein (lower panel). C) RHGF-2s activity is specific to RHO-1. Purified GST::RHO-1, GST::CDC42, and GST::CED-10 were preloaded with unlabelled GDP and incubated with (black bars) or without (white bars) FLAG::RHGF-2s. The GEF assay was performed in the presence of 0.1 nM radiolabelled \[^{35}\text{S}]\text{GTP}\gamma\text{S}. Error bars indicate SEM. **p<0.001 and *p<0.01 in comparison to control reactions using Student’s t-test.

1.5.2 RHGF-2 increases cell rounding in N1E-115 Neuroblastoma and NIH3T3 Fibroblast Cells. RHGF-2 inhibits neurite outgrowth similar to the observations with Tech. RHGF-2-expressing N1E-115 cells are more rounded and have reduced neurite extensions compared to the control cells (Figure 8). RHGF-2 transfected cells had a significantly decreased the number of neuritis, similar to the positive control cells containing constitutively active Ga12 (Figure 8). The activated Ga12 pathway stimulates cell rounding and neurite retraction through the RhoA GTPase pathway (Nurnberg et al., 2008; Kranenburg et al., 1999).
Figure 8: N1E-115 neuroblastoma cells expressing FLAG::RHGF-2 reduces neurite extension and increase the amount of cell rounding (Lin et al., 2012). A & B) N1E-115 cells were transiently transfected with FLAG vector, FLAG::RHGF-2s, or constitutively active Gα12 as the positive control. After 4-6 hrs, cells were transferred to serum-free DMEM to induce morphological differentiation. After 48 hrs, the cells were fixed and visualized with anti-FLAG and FITC-coupled secondary antibody. The percentage of rounded, flattened and neurite-bearing transfected cells from three independent experiments is indicated in the graph. N= ~200 transfected cells, error bars represent SEM. **p<0.001 and *p<0.01 in comparison to the control cells using the Student’s t-test.

1.5.3 RHGF-2 binds to MPZ-1 PDZ domains 8 and 9. A yeast two hybrid screen was used to find binding partners for the scaffold protein, MPZ-1. Individual and combinations of PDZ domains: PDZ 8, 9, 10, 8-10 and 9-10 were used as bait in screens of a mixed-staged C. elegans hermaphrodite cDNA library. RHGF-2 was identified and was observed to bind to PDZ domain 8 and to a lesser extent to PDZ domains 9 and 10 (Figure 9A). RHGF-2 interaction with MPZ-1 is dependent on the C-terminal PDZ binding motif, SDV (Figure 9B). RHGF-2 binds to MPZ-1 in a similar fashion to the
Tech/Syx/PLEKHG5 family which may indicate the interaction could determine the localization of RHGF-2.

**Figure 9: RHGF-2 binds strongly to MPZ-1 PDZ domain 8 and is dependent on the C-terminal PDZ-binding motif (Lin et al., 2012).** A) Purified GST::MPZ-1 PDZ domains 8, 9, 10, 9-10, and 8-10 were incubated with lysates from HEK293T cells transfected with FLAG::RHGF-2s. GST fusion proteins were pulled down with glutathione-sepharose beads, separated by SDS-PAGE, and analyzed by immunoblotting with anti-FLAG antibody. B) $[^{35}S]$-labeled FLAG::RHGF-2s, with or without the C-terminal PDZ-binding motif (SDV), was incubated with GST::MPZ-1 PDZ domains 8-10. Glutathione-sepharose beads pulled down the GST fusion protein, analyzed by SDS-PAGE, and visualized by autoradiography.
Chapter 2

Materials and Methods

2.1 Worm Strains

*C. elegans* strains were maintained on agar plates seeded with OP50 *E. coli* at 21°C. Nematode strain N2 Bristol (wild-type) was provided by the *Caenorhabditis* Genetics Center and VC455 *rhgf-2(gk216)/mIn1[mIs14 dpy-10(e128)]* and VC559 *mpz-1(gk273)/mIn1[dpy-10(e128) mIs14(myo-2::GFP)]* were provided by the *C. elegans* Gene Knockout Consortium. *rhgf-2(gk216)* was backcrossed five times and *mpz-1(gk273)* was backcrossed eight times to wild-type and placed over the *mIn1* balancer to produce the strains, XS245 and XS224, respectively. XS245 was used in all the experiments involving *rhgf-2(gk216)* mutant animals. *jcls1[pJS191(ajm-1::GFP);pRF4(rol-69su1006dm)]; C45D3(unc29(+)); him-5(e1490)* was provided by Joe Culotti. General techniques involving the handling of animals are described in (Brenner, 1974).

2.2 cDNA Analysis

An oligo dT-primed library pool was created using Superscript II (Invitrogen) reverse transcriptase and mRNA purified from mixed stage *C. elegans* wild-type
hermaphrodite animals. *rhgf*-2 cDNAs were amplified by PCR from the cDNA library and another mixed stage *C. elegans* cDNA library (Origene Technologies, Rockville, MD) using a forward primer, RS521 (GAGCAGTAGTGGTGGACATGC), to the predicted upstream gene of *rhgf*-2, TO8H4.2 and a reverse primer, RS523 (AAAAGGACAAATTGCAGCTGG), to *rhgf*-2. The sequence of a portion of the new *rhgf*-2 cDNA is printed below. The unshaded portion indicates the 3’end of exon 3 (previously designated as T08H4.2 exon 3 sequence) while the shaded portion indicates the 5’ end of the new exon 4 (the previous designation for *rhgf*-2 exon one from Wormbase.org began with the ATG in white letters).

...GGGAGCAGCTTCTGGCTGAGAATTTCGTTAGAGGACGAGGGG
ATATGAAAAGTGGTGAGACACACGAGAGGCCACACACGTATGGGACGAAAGTA
ACGAGCGAGCAACGAGAAAAATGAGCGCTGAAGCTG...

The intron sequences in the genomic DNA immediately adjacent exon 3 and exon 4 (not shown in the cDNA sequence above) match consensus splice donor and acceptor sequences, respectively, indicating the cDNA sequence likely represents a true mRNA and not a fusion artifact generated in the production of the cDNAs (Figure 11; 12).

**2.3 DNA Constructs**

*rhgf*-2 and *mpz*-1 genomic DNA fragments were made by PCR using either iProof (Bio-Rad, Hercules, CA) or PCR Extender (Fisher Scientific, Pittsburgh, PA) high fidelity DNA polymerases. Genomic DNA fragments were produced in triplicate, confirmed by restriction enzyme mapping and mixed together before injecting into
animals. Overlapping genomic DNA fragments were mixed together in equimolar concentrations. *rhgf-2* and *mpz-1* DNAs tagged with either GFP or tagRFP were produced by the technique of PCR fusion (Hobert, 2002). Each tag was followed by the *let-858* 3’ UTR (approximately 450 bp).

### 2.4 Transgenic Lines

The *rhgf-2* DNA fragments generated in this study are shown in Figure 17 and the primers used are indicated in Table 1. The following is a list of the extrachromosomal arrays, the primers used to generate the DNA fragments in the arrays and the sizes of the 5’ regulatory regions upstream of the ATG included in the fragments in brackets: *Ex73*, RS796/RS797 (3.3 kb); *Ex102* RS796/RS945 (3.4 kb); *Ex105*, RS974/UT19 and UT20/RS945 (6.4 kb); *Ex110*, UT20/UT28 (5.7 kb); *Ex113* UT25/RS945 (3 kb).

Five genomic DNA fragments spanning the *mpz-1* gene, including 3 kb of 5’ regulatory region, were generated with the most 3’ fragment fused to tagRFP (with a *let-858* 3’ UTR) after the last *mpz-1* amino acid codon. The primers used to generate the five *mpz-1* genomic DNA fragments were UT78/UT79, UT80/UT81, UT82/UT83, UT84/UT85 and UT89/UT93. Primer sequence information is in Table 1. Each *mpz-1* fragment is between 7 and 10 kb with 1 to 1.5 kb of overlap between fragments.

**Table 1: DNA Oligonucleotide Primer List.** Forward and reverse primers used in this study to amplify sequences from *T08H4.2*, *rhgf-2* and *mpz-1* genes.

<table>
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<th>Primer</th>
<th>Sequence (5’ to 3’”)</th>
<th>Direction</th>
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<td>Forward</td>
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<tr>
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<td>Direction</td>
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<td>-----------</td>
</tr>
<tr>
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<td>RS523</td>
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<td>Reverse</td>
</tr>
<tr>
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<td>Forward</td>
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<tr>
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<td>RS797</td>
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<td>Reverse</td>
</tr>
<tr>
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</tr>
<tr>
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<td>UT93</td>
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<td>Reverse</td>
</tr>
</tbody>
</table>
2.5 Microinjections

Standard microinjection techniques were used to generate stable transgenic *C. elegans* lines carrying extrachromosomal DNA arrays (Mello et al., 1991). Overlapping genomic DNA fragments in a DNA injection mix were assumed to undergo homologous recombination in the animal (Maryon et al., 1996). From 5 to 75 ng/μl of *rhgf-2* or *mpz-1* DNA was mixed with 50 ng/μl of co-transformation marker *unc-122p::gfp* (coelomocyte specific promoter) and 100 ng/μl of herring sperm DNA for injection. In addition, 50 ng/μl of the co-transformation marker pRF4 [*rol-6(su1006dm)*] was used with the *rhgf-2p::rfp* construct. *rhgf-2* DNA mixes were injected into *rhgf-2(gk216)/mIn1*5 or N2 animals, and the *mpz-1* DNA was only injected into N2. Progeny were screened for stable expression of the extrachromosomal array, and homozygous *rhgf-2(gk216)* lines with the array were established. Extrachromosomal arrays in an N2 background were crossed into the *rhgf-2(gk216)* background. At least three independent transgenic lines for each extrachromosomal array were examined. To examine co-localization of *rhgf-2lp::rfp* and *rhgf-2s::gfp* expression, the *uxEx110[rhgf-2s::gfp]* strain was crossed into the *uxEx73[rhgf-2lp::rfp]* transgenic line.

2.6 Confocal Microscopy

Worms were immobilized with 30 mg/ml BDM (2, 3-butanedione monoxime; Sigma) in M9 and mounted on 2% agarose pads for examination by epifluorescence. Animals were stained with 1, 1’-dioctadecyl-3, 3, 3’, 3’-tetramethylindodicarbocyanine perchlorate (DiD) as described previously (Hedgecock et al., 1985; Shaham, 2006).
Images were obtained with an Olympus Fluoview 300/IX70 confocal microscope using Olympus Fluoview 5.0 software.

### 2.7 Actin Staining

Actin staining of embryos protocol was taken from (Duerr, 2006). Embryos were obtained by an alkaline hypochlorite treatment of gravid adults described in (Shaham, 2006). The embryos were fixed using the methanol/acetone fixation procedure explained in (Duerr, 2006). Mutant *rhgf*-2(*gk216*) embryos actin staining was compared to wild-type animals near the embryonic three-fold stage.

### 2.8 Video Analysis

Animals were picked to standard nematode growth media plates with bacteria and left to lay eggs for about three hours at room temperature. Eggs were mounted on a 5% agarose pad with M9 for imaging at room temperature. All four of the imaged *rhgf*-2(*gk216*) embryos arrested elongation at the 1.5 fold stage of embryogenesis. Video images were captured with a Photometrics CoolSNAP HQ² camera mounted on an Olympus IX81 microscope with a PlanApo N 60×/1.42 oil objective using the Slidebook software (Intelligent Imaging Innovations, CO).
Chapter 3

Results

3.1 RHGF-2 is a new member of the Tech/Syx/PLEKHG5 family

To determine which Dbl-related family RHGF-2 belonged to, the predicted RHGF-2 amino acid sequence (accession number NP_494723.1) was inputted into the SMART program to identify protein domains and unique sequences (Letunic et al., 2009). RHGF-2 contains a RhoGEF135-322 domain, PH380-483 domain, and Type I PDZ859-862 binding motif (Figure 10B). RHGF-2 RhoGEF domain sequence was compared against a non-random database using the DNAStar software from Lasergene. Proteins most similar to the RHGF-2 RhoGEF domain were used to create the phylogenetic tree in Figure 10A (Tamura et al., 2011). The RhoGEF376-563 domain of Tech (NP_958429), RhoGEF410-597 domain of Syx (NP_001004156), and RhoGEF417-604 domain of PLEKHG5 (NP_941374.2) had approximately 54%, 53%, and 53% similarity, respectively to the RHGF-2 RhoGEF domain. Tech/Syx/PLEKHG5 family members have ~80% identity to each other while PLEKHG6 has a ~50% identity the family. RHGF-2 has a high sequence similarity and similar domain arrangements to Tech/Syx/PLEKHG5, but not PLEKHG6; therefore it is not included in the analysis.
Figure 10: RHGF-2 is a member of the Tech/Syx/PLEKHG5 family. A) Phylogenetic tree comparing the RhoGEF domains from selected GEF proteins. The RhoGEF domains were defined by SMART (http://smart.embl-heidelberg.de/) and MEGA 5 software was used for sequence analysis with ClustalW, bootstrapping (500 replicates) and tree
C. elegans protein sequences were obtained from WormBase.org and all other sequences are from NCBI. Ce, Caenorhabditis elegans (ECT-2: NP_496318.1, RHGF-1: NP_509791, RHGF-2: NP_494723.1, UNC-73 (C-terminal RhoGEF): AAC71110.1, VAV-1: NP_001041223.1), Dm, Drosophila melanogaster (Pebble: NP_729306.1, RhoGEF-2: NP_477317.1, Trio (C-terminal RhoGEF): NP_728561.1, Vav: NP_573372.1), Hs, Homo sapiens (ARHGEF-11b: NP_937879.1, Ect-2: NP_060568.3, PLEKHG5: NP_941374.2, PLEKHG6: NP_001138329.1, Kalirin (C-terminal RhoGEF): NP_008995.2, Vav1: AAB34377.1), Mm, Mus musculus (Syx: NP_001004156) and Rn, Rattus norvegicus (Tech: NP_958429). B) Domain structures of RHGF-2 and putative orthologs rat Tech, mouse Syx and human PLEKHG5. The percent similarities between the sequences of RHGF-2 RHOGEF domain to the mammalian orthologs are indicated.

3.2 RHGF-2 has two isoforms: RHGF-2l and RHGF-2s

The predicted RHGF-2 protein sequence (mentioned as rhgf-2s throughout the thesis) according to WormBase is 862 amino acids long which is small compared to the putative orthologs Tech/Syx/PLEKHG5 at 1039, 1073, and 1083 amino acids, respectively (Figure 10B). To determine whether rhgf-2 encodes a longer isoform, cDNA analysis was performed. This analysis identified a 22 nucleotide nematode-specific trans-spliced leader, SL1 sequence (GGTTAATTACCCAAGTTTGAG), which defined the beginning of exon 1 for rhgf-2s (Figure 11). cDNA analysis also revealed the upstream gene, T08H4.2 is actually a part of rhgf-2 (Figure 12; Materials and Methods).
Figure 11: cDNA analysis of *rhgf-2s*. The bold font indicates the trans-spliced leader, SL1 sequence and the underlined nucleotides are the predicted WormBase exon 1 for *rhgf-2s*. The *rhgf-2* cDNA was made by PCR of a cDNA library using the SL1 sequence as the forwards primer and 3′ *rhgf-2* sequence as the reverse primer RS523 and RS524. The alternate blue and black colors indicate exons.
Figure 12: The rhgf-2l cDNA and translated protein sequences. Exons are indicated by alternate blue and black highlighting of nucleotides. The nucleotides deleted in gk216 are indicated in bold. The rhgf-2 exon sequence identified in this study is encased in a red box. The amino acids for the RhoGEF domain are highlighted red and the PH domain is highlighted yellow. The C-terminal PDZ binding motif is in a black box.

The two genes when combined yield a 1007 amino acid protein, which is identified as rhgf-2l and is comparable in length to the other mammalian RHGF-2 orthologs. The additional N-terminal sequence was not crucial for RHGF-2 function, since various constructs spanning smaller parts of the gene could rescue the rhgf-2 null phenotype. However, the sequence may be an autoinhibitory domain, similar to that
proposed for Tech (de Toledo et al., 2001). Further analysis is required to uncover the function of the ~200 amino acids N-terminus of RHGF-2.

### 3.3 RHGF-2 mutants arrest development and do not properly progress through embryo elongation

There is only one rhgf-2 allele available, rhgf-2(gk216)/mIn1 which was obtained from the C. elegans Genes Knockout Consortium. gk216 is a 1381 bp deletion that removes 141 bp of early rhgf-2 coding sequence (Figure 12). The gk216 deletion is expected to put the remaining coding sequence out of frame, which may disrupt RHGF-2 production and function. Homozygous gk216 mutants hatch and arrest development at the L1 stage. The mutants are immobile with a severe Dpy phenotype (dumpy; shorter than wild-type) (Figure 13). Analysis of progeny from the balanced rhgf-2(gk216)/mIn1 strain revealed 25.6% arrested L1 larvae, 73.4% wild-type and 1.0% unhatched eggs (n=312). This suggests homozygous rhgf-2(gk216) animals are arresting at the L1 stage. The mIn1 balancer does not contribute to the phenotype. Time-lapse video analysis revealed that rhgf-2(gk216) mutants arrested in early embryonic elongation at the 1.5-fold stage (Figure 14). Cell differentiation did occur beyond the 1.5 fold stage as the intestine and pharynx pumping was visible after hatching, and the larvae exhibited minor muscle movements.
Figure 13: *rhgf-2(gk216)* deletion mutants arrest development and hatch as short, dpy and immobile animals. A) Wild-type N2 and B) *rhgf-2(gk216)* animals ~two hrs after hatching. Scale bar is 100 μm. C) N2 and D) *rhgf-2(gk216)* animals ~48 hrs after hatching, inset is a differential interference contrast image of another *rhgf-2(gk216)* mutant. Scale bar is 200 μm.

Figure 14: *rhgf-2(gk216)* mutants do not elongate past the 1.5-fold stage of embryogenesis. A time-lapse video of *rhgf-2(gk216)* (top) and *rhgf-2(gk216)/mIn1*
(bottom) embryonic development. The homozygous gk216 embryo arrests development at the 1.5 fold stage, but is still capable of small movements. The control gk216 heterozygous embryo of a comparable age continues through to the three-fold stage. A) Eggs are laid and indicate the image of the initial stage of recording. B) Bean stage. C) Comma stage. D) Heterozygous animal is at the 1.5-fold stage and homozygous animal is at comma stage. E) Heterozygous animal is at the two-fold stage where twitching starts and the homozygous worm is at the comma stage. F) Heterozygous animal has completed elongation to three-fold stage, while homozygous mutant is at comma stage. H) Homozygous animal is comparable to two-fold stage. I) Image captured before mutant worm breaks egg shell and J) Mutant worm twitches and breaks through the egg shell. The heterozygous embryo died just before hatching. Images were captured every minute over 6.5 hours.

Epidermal seam cells provide the force for the initial stages of elongation (Priess JR. and Hirsh DI, 1986). To examine the embryonic elongation process in rhgf-2(gk216), the apical cell junction marker AJM-1::GFP protein fusion was used (Figure 15; Francis R. and Waterston RH. 1991; Mohler et al., 1998; Koppen et al., 2001). Normally, the epidermal lateral seam cells elongate during embryogenesis and appear rectangular at larval stage one (L1). However rhgf-2(gk216) L1 mutants seam cells were more round or square shaped (Figure 15), which suggested the gk216 elongation defect may result from a lack of proper epidermal cell shape changes required during embryogenesis.

C. elegans embryos elongate as a result of circumferential actin filament bundle contraction within the lateral epidermal seam cells. RHGF-2 may target the RHO-1 GTPase, which plays a role in regulating actin contractions as mentioned in the introduction. Actin staining revealed the disorganization of the actin cytoskeleton in rhgf-
2(gk216) mutants seam cells compared to the wild-type pattern of parallel actin filaments (Figure 16).

**Figures 15: Epidermal lateral seam cells in rhgf-2(gk216) mutants do not elongate.**
The apical cell junction marker AJM-1::GFP was used to examine epidermal cell morphology in control and *rhgf-2(gk216)* animals at the indicated development stage. A) Epidermal seam cells in control animals are square or rounded at the 1.5 fold stage. B) The seam cells elongate appearing rectangular at the L1 stage. C) In *rhgf-2(gk216)* mutants the epidermal cells remain round or square shaped. The control strain, which has an embryonic expression pattern similar to wild-type, is *jcIs1[ajm-1::gfp]; him-5*. The *rhgf-2* strain is *rhgf-2(gk216); jcIs1[ajm-1::gfp]*. Scale bars are 25 μm.

**Figure 16: Actin staining of wild-type animals and rhgf-2(gk216) mutants.** A) Early L1 stage wild-type worm with the parallel organization of actin. B) Early L1 rhgf-2(gk216) mutant with a disorganization of actin.
Transgenic DNA constructs spanning various lengths of the \textit{rhgf-2} genomic region rescued the \textit{rhgf-2(gk216)} developmental arrest and severe Dpy phenotype (Figure 17). However, all rescued strains laid fewer eggs than wild-type and some mutants containing the rescuing transgene exhibited partial rescue (Table 2). Partially rescued animals grew to the adult stage, but maintained a moderate Dpy phenotype. Variations in adult Dpys frequencies between the lines may be due to the rate of spontaneous loss of the extrachromosomal array at some cell divisions during development. These transgenic rescue experiments indicated the deletion within the \textit{rhgf-2} gene is most likely responsible for the phenotypes observed in \textit{gk216} animals. Interestingly, RNAi-specific knockdown of RHGF-2 resulted in an elongation phenotype similar to \textit{rhgf-2} null animals. The promoter of a pan-neuronal gene, \textit{unc-119}, was used to expressed RHGF-2 RNAi in a wild-type background using the RhoGEF domain as the target sequence (data not shown).

\textbf{Figure 17: The predicted \textit{rhgf-2} transcripts with \textit{rhgf-2} DNA constructs used in this study.} cDNA regions are represented by boxes (exons) and genomic DNA is represented by straight black lines with the corresponding extrachromosomal array name indicated above. The genomic region deleted in \textit{rhgf-2(gk216)} animals is indicated at the top of the
diagram. The red and green lines represent tagRFP and GFP sequences, respectively, and each is followed by the let-858 3’ UTR (not indicated). The diagram is drawn to scale. The predicted full-length *rhgf*-2 transcript is indicated as *rhgf*-2l and the shorter isoform with a trans-spliced SL1 leader sequence, is indicated as *rhgf*-2s. The ability of a particular array to rescue the *rhgf*-2(gk216) developmental arrest and Dpy phenotypes is indicated in the column on the right.

**Table 2: Categorization of progeny from rescued *rhgf*-2(gk216) transgenic lines.**

*Transgenic lines for each extrachromosomal array are indicated by the letter after the strain names. §Partial rescued animals refer to the transgenic lines. ¥ Complete rescued animals referred to transgenic lines. £Young adults (less than 12 hours beyond the L4 stage; number of animals indicated in brackets) were left to lay eggs over a 24 hour period and the progeny were categorized after developing over four days.

<table>
<thead>
<tr>
<th>Strain*</th>
<th>Transgene Encodes</th>
<th>Unhatched Eggs (%)</th>
<th>L1 Arrested (%)</th>
<th>Dpy Adult (Partial Rescue§) (%)</th>
<th>NonDpy Adult (Rescue¥) (%)</th>
<th>Eggs Laid/Worm/24hrs£ (N)</th>
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<td>0 (0)</td>
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<td><em>rhgf</em>-2l</td>
<td>22 (6)</td>
<td>181 (51)</td>
<td>44 (12)</td>
<td>109 (31)</td>
<td>30 (12)</td>
</tr>
<tr>
<td>gk216;Ex113-A</td>
<td><em>rhgf</em>-2s</td>
<td>19 (2)</td>
<td>378 (46)</td>
<td>59 (7)</td>
<td>373 (45)</td>
<td>69 (12)</td>
</tr>
<tr>
<td>gk216;Ex113-B</td>
<td><em>rhgf</em>-2s</td>
<td>7 (1)</td>
<td>216 (39)</td>
<td>88 (16)</td>
<td>244 (45)</td>
<td>23 (24)</td>
</tr>
</tbody>
</table>

Smaller DNA fragments encoding RHGF-2s were sufficient to rescue the developmental arrest and Dpy phenotypes in most *rhgf*-2(gk216) animals (Figure 17). These rescue experiments support the fact that the *rhgf*-2s transcript predicted by the
cDNA analysis encodes a functional RHGF-2s isoform. The full length rhgf-2 gene therefore encodes at least two mRNA transcripts, a long (l) and a short (s) version (Figure 17).

### 3.4 RHGF-2 is neuronally expressed

To investigate RHGF-2 expression in *C. elegans*, a rhgf-2s::gfp construct was made containing rhgf-2 genomic DNA starting from 5.7 kb upstream of the predicted rhgf-2s start ATG through to the last amino acid codon, which was fused to GFP coding sequence with a 3’ UTR from let-858 (Figure 17). The rhgf-2s::gfp construct was functional since it could rescue the rhgf-2(gk216) mutant phenotype. The rhgf-2s::gfp transgenic animals expressed the GFP in neurons from about the 1.5-fold embryonic stage through adulthood, which is surprising because of the developmental arrest phenotype (Figure 18). No obvious RHGF-2s::GFP fluorescence was visible in earlier embryonic stages and anti-GFP antibody staining further displayed RHGF-2::GFP is expressed neuronally (data not shown).
Figure 18: RHGF-2 expression is restricted to the nervous system. A functional rhgf-2s::gfp transgene was used to examine rhgf-2 expression in a wild-type background. Animals were also stained with DiD (red) to label the ciliated sensory neurons (arrowheads), none of which expressed RHGF-2s::GFP (green). RHGF-2s::GFP localization is shown in neurons in the head and the tail as well as the ALM, AVM, PVM and PLM mechanosensory neurons. The last panel shows RHGF-2lp::RFP fluorescence (red) in a small number of head neurons and in two tail neurons (not shown). Anterior is to the left and ventral is down in all images. The coelomocytes (cc) fluoresce as a result of the co-transformation marker.

Many, but not all of the 302 C. elegans hermaphrodite neurons expressed GFP in the transgenic animals examined (Figure 18). RHGF-2::GFP was expressed in several cell bodies in the head and tail ganglia and many neuronal processes in the nerve ring and ventral cord. The GFP fluorescence was diffusely distributed throughout most neurons, and there were instances of a more punctate fluorescence within some cell bodies and processes, particularly in the ventral cord. Neurons that were positively identified in late larval/adult animals based on their position and morphology were the ALM, AVM, PLM
and PVM mechanosensory neurons and the BDUs (Figure 18). Expression from ciliated sensory neurons, most of the ventral cord motor neurons, and mid-body neurons such as the CANs, PDEs and SDQs, was notably absent.

Expression driven by the 5’ regulatory regions of the full length *rhgf-2l* transcript was examined using a tagRFP transcriptional fusion reporter (*rhgf-2lp::rfp*; Figure 17; 18). *rhgf-2lp::rfp* was also restricted to the nervous system, but to a smaller subset of neurons that partially overlapped with *rhgf-2s::gfp* expression in the head and tail regions (Figure 18).

3.5 RHGF-2 and MPZ-1 co-localize in some neurons

RHGF-2 and MPZ-1 interact based on yeast two hybrid interactions and *in vitro* pull down assays (Figure 9). To determine if RHGF-2 and MPZ-1 interact *in vivo*, co-localization would be expected in some cells. Previous analysis revealed that MPZ-1 is expressed in a large portion of the nervous system, as well as, the body wall and vulval muscles (Xiao *et al*., 2006). MPZ-1 genomic DNA is ~36kb long and encodes ten known isoforms (WormBase; Figure 19A).
Figure 19: MPZ-1 isoforms and the DNA fragments used to create transgenic animals. A) Schematic of the 10 mpz-1 isoforms, A-J. Scale bar is 1.0kb B) Schematic of the number and length of each fragment to form the whole genomic DNA of mpz-1d isoform. The second set of fragments contains fragment 5 C-terminally tagged with tagRFP. Scale bar equals 1.5kb. The schematics of the mpz-1 gene were made using the program found at “http://www/wormweb.org/exonintron”.

Transgenic lines containing the rescuing rhgf-2s::gfp and the mpz-1 neuronal promoter driven mpz-1::tagrfp extrachromosomal array (Xiao et al., 2006) were examined for RHGF-2 and MPZ-1 co-localization (Figure 19B). As expected, RHGF-2s::GFP was expressed in many of the same neurons expressing MPZ-1::tagRFP (Figure 20; data not shown). MPZ-1::tagRFP was most strongly localized to the axons of the nerve ring where RHGF-2::GFP was also highly distributed (Figure 20). RHGF-2 and
MPZ-1 co-localization in the nervous system is consistent with the hypothesis that these proteins function together in a neuronal signaling pathway, however, RHGF-2 was expressed in cells such as the mechanosensory neurons where MPZ-1 is not expressed, indicating this signaling pathway is not active in all neurons.

**Figure 20: RHGF-2 and MPZ-1 co-localize in the nervous system.** RHGF-2s::GFP co-localizes with MPZ-1::tagRFP in the axons of the nerve ring (NR). Anterior is to the left and ventral is down in all images. The coelomocytes (cc) fluoresce as a result of the co-transformation marker.
3.6 RHGF-2 overexpression alters locomotion

Additional observations were consistent with RHGF-2 functioning in *C. elegans* neurons. Constitutive Rho GTPase activity in *C. elegans* cholinergic motor neurons results in hyperactive movement and exaggerated body bend (“loopy”) phenotypes due to Rho-mediated increases in motor neuron acetylcholine release (McMullan *et al.*, 2006). Similarly, transgenic animals that overexpressed Rho-specific RHGF-2s or RHGF-2l from an extrachromosomal array, in either wild-type or *rhgf-2(gk216)* mutant backgrounds moved in a loopy manner in comparison to wild-type (Figure 21). There are two possible categories for hyperactivity: worm travels further (increase number of body bends) and worm moves longer without pausing. A speed assay was done to measure the time the worms kept moving in a specific time period. There was no significant difference between the speeds of movement of the transgenic animals compared to wild-type worms (data not shown). Additional analysis of RHGF-2 function in the nervous system is required to further define its role in regulating *C. elegans* locomotion.

![Figure 21: RHGF-2s overexpression exhibits a loopy locomotion compared to wild-type.](image)

RHGF-2s overexpression from an extrachromosomal array alters *C. elegans* locomotion. The track left in the bacterial lawn by an N2 animal has a characteristic sinusoidal wave pattern (left panel). An animal overexpressing RHGF-2s (XS RHGF-2; Ex115[*rhgf-2s::gfp*] *rhgf-2*(gk216)) moves with exaggerated body bends with a higher amplitude wave compared to N2 (right panel).
Chapter 4

Discussion

*C. elegans* RHO-1 is required at the earliest stages of development for embryonic cytokinesis and cell polarity, and in later stages of development for epidermal P cell migrations, embryo elongation and myosin thick filament organization in muscle cells (Lundquist, 2006). In adult animals, RHO-1 signaling plays non-developmental roles in modulating locomotion, pharyngeal pumping, egg laying, defecation cycling, and cell morphology (McMullan *et al.*, 2006; 2011). Multiple RhoGEF proteins operating in different pathways most likely activate RHO-1, and this study focused on characterizing the *C. elegans* RHGF-2 RhoGEF.

4.1 RHGF-2 functions within embryonic elongation similarly or not to VAB-1

The first step to characterizing RHGF-2 was to determine its function *in vivo*. The null *rhgf-2(gk216)* deletion mutant revealed that RHGF-2 is required early in embryogenesis to elongate the body into the characteristic cylindrical shape. What is interesting is RHGF-2 is not expressed in the epidermal seam cells, similar to other proteins functioning in embryo elongation. *rhgf-2* is expressed exclusively in neuronal
cells from the embryonic 1.5 fold stage and remains expressed through adulthood (Figures 13-16). Neuroblasts play a role early in epidermal morphogenesis by providing the signals for cells to migrate or to provide a substrate for the epidermal cells to migrate on. For example, RHGF-2 may function in a pathway similar to the Eph/Ephrin signaling pathway. VAB-1 (variable abnormal), an Eph tyrosine kinase receptor, is required in neurons for epidermal morphogenesis during embryogenesis (Chin-Sang et al., 1999). VAB-1::GFP is exclusively expressed in neurons similar to RHGF-2 (George et al., 1998). VAB-1 receptor is activated when bound by its ligand, VAB-2/EFN-1 (Chin-Sang et al., 1999). vab-1 mutants have defects in ventral closure, and underlying substrate cells leak out from defective embryos (George et al., 1998). The vab-1 phenotype is distinct from the rhgf-2 elongation defects because no cells spill out in the rhgf-2 mutants and the rhgf-2 elongation defect occurs after enclosure (Figure 14; 15). The specific mechanism of VAB-1 and VAB-2/EFN-1 function in embryo formation is still unclear. However, the two different phenotypes suggest RHGF-2 does not function exactly as VAB-1 in epidermal morphogenesis. Interestingly however, VAB-1 was another positive protein in the yeast two hybrid screening with MPZ-1 (unpublished data).

4.2 RHGF-2 overexpression phenotype may indicate activated RHO-1 GTPase involvement in the C. elegans locomotion pathway

In addition to the role in embryonic elongation, RHGF-2 most likely works with RHO-1 in a post-developmental role. RHGF-2 overexpression in transgenic animals results in exaggerated body bends and loopy locomotion, which is similar to the constitutively activated RHO-1 phenotype (McMullan et al., 2006). RHO-1 alters
cholinergic neurotransmitter release (Hiley et al., 2006; McMullan et al., 2006). RHO-1 function at synapses suggests RHGF-2 may affect locomotion through RHO-1 in neurotransmission (Figure 21; McMullan et al., 2006).

Fluorescently tagged synaptobrevin, a presynaptic protein, could be used to examine if RHGF-2 is localized at presynaptic sites (Nonet, 1999). RHGF-2s::GFP expression is in a variety of neurons, so cell specific knockdown of RHGF-2 in neurons involved in the locomotory pathway, especially presynaptic acetylcholine (Ach)-releasing neurons (unc-17) post-development would show if RHGF-2 function is critical in these cells for locomotion (Alfonso et al., 1993). Body bend assays can quantify a locomotory phenotype. An aldicarb assay can be used to check for neurotransmission defects (Mahoney et al., 2006). Aldicarb is a carbamate pesticide used to mainly kill nematodes. Aldicarb is a cholinesterase inhibitor and prevents ACh degradation at the neuromuscular junction. ACh will accumulate and cause paralysis (Lewis et al., 1980). Animals that have defective ACh release will have a slower paralysis response time than wild-type (Miller et al., 1996; Nguyen et al., 1995; Nonet et al., 1993). If RHGF-2 does activate RHO-1 in neurotransmission to release ACh, then a knockdown of RHGF-2 in ACh-releasing neurons should result in aldicarb resistance. RHO-1 would not be fully activated resulting in a reduced amount of ACh release. Animals expressing C3 transferase, a RHO-1 inhibitor, release reduced levels of Ach and display slowed locomotion (McMullan et al., 2006). In addition, constitutively active RHO-1 causes hypersensitivity of muscles to ACh due to an influence on receptor localization. RHO-1 regulates actin-myosin filaments via Rho-associated kinase, which could localize
nicotinic ACh receptors on muscles through an unknown pathway (McMullan et al., 2006; Weston et al., 2003).

4.3 RHGF-2 neuronal expression suggests a function in neuronal morphology similar to mammalian orthologs Tech/Syx/PLEKHG5

RHGF-2 is expressed in the pair of ALML/R. The pair of mechanosensory neurons have been extensively characterize for morphology, position and function (Chalfie et al., 1985; White et al., 1986; Wood et al., 1988). The ALML/R cell body is located anterior and lateral of the body and extends a single long receptor process into the nerve ring where the synaptic branch connects to the other ALM, AVM and other neurons. A shorter posterior process is also observed. The ALM regulates the backward motion response (reversal) to anterior gentle touch (Chalfie et al., 1985). rhgf-2 hypomorphic mutants or animals with rhgf-2 knocked down in the mechanosensory neurons could be analyzed for the development of the ALM cell body and its axonal processes to determine if RHGF-2 influences neuronal morphology. The diameter, length and number of ALM processes would be examined. The reversal response of the animals to gentle anterior touch can determine the severity of each morphological defect. The mammalian orthologs Tech/Syx/PLEKHG5 are expressed neuronally and do influence neurite extensions and cell morphology.

4.4 RHGF-2 and MPZ-1 interaction depends on the PDZ binding motif

RHGF-2 binds to MPZ-1 based on the yeast two-hybrid screening, in vitro protein-protein interactions, co-immunoprecipitations and in vivo co-localization (Figure 9; 20).
PDZ domain-containing scaffolding proteins like MUPP1/MPZ-1 link GEFs to their GTPases at a specific time and location in the cell, which requires the C-terminal PDZ binding motif (Buchsbaum et al., 2002; 2003; Jaffe et al., 2004). Bioinformatics analysis of human proteins revealed approximately 40% of RhoGEFs and a large number of RhoGAPs contain a putative PDZ binding motif indicating an importance for Rho GTPase signaling in PDZ scaffold-mediated signaling complexes (Garcia-Mata and Burridge, 2007). RHGF-2 and MPZ-1 co-localize in some mechanosensory neurons such as the AVM and PVM (Figure 20; Xiao et al., 2006; Chalfie et al., 1985). The AVM mechanosensory neuron is located laterally on the right side of the anterior half of the body. Some alterations in AVM results in an increased or decreased response time to an Anterior touch (Chalfie et al., 1985).

RHGF-2::GFP is diffusely dispersed throughout the AVM : cell body and neurite extensions and MPZ-1 is also localized to the AVM (Figure 20; Xiao et al., 2006). The mechanosensory neurons in mpz-1 mutants could be analyzed in length and diameter of the processes, number of synapses and other general morphology differences to determine if MPZ-1 is necessary for proper neuronal morphology. Any AVM defects can also be quantified in a behavioral assay by measuring reversal times to anterior touch. mpz-1 has two alleles available, mpz-1(tm1136) and mpz-1(gk273). mpz-1(tm1136) has no noticeable phenotype and appears wild-type like, while mpz-1(gk273) has an embryonic lethal phenotype. It has not yet been confirmed if this phenotype is due to the mpz-1 mutation. Two fosmids must be injected together to span the entire mpz-1 gene and preliminary analysis indicates that these fosmids do not rescue the embryonic lethality of
gk273 mutants. Interestingly, if the mpz-1 embryonic lethality phenotype is confirmed it could mean RHGF-2 and MPZ-1 both play a role in embryo development.

4.5 Conclusion

The function of RHGF-2 was characterized *in vivo*. RHGF-2 plays a role in embryonic elongation since epidermal seam cells of *rhgf-2* null animals do not elongate past the 1.5-fold embryonic stage. Likewise, Syx, the zebrafish ortholog to RHGF-2 has a similar phenotype, where the anterior-posterior body axis is shortened. Interestingly, RHGF-2 is expressed exclusively in neurons and not in epidermal seam cells to cause the elongation defect. One possibility is that RHGF-2 may be weakly expressed directly in epidermal cells during elongation, but RHGF-2::GFP fluorescence is not detectable in the transgenic lines. Another possibility is a C-terminally truncated RHGF-2 isoform we are unaware of. To help determine where RHGF-2 is required, the protein could be expressed in the seam cells using the apical junction marker AJM-1. Rescue of the elongation phenotype in these animals would suggest that RHGF-2 does, in fact, function in epidermal cells.

*C. elegans* allows for defects on the molecular level to be observed as affects on the whole organism. *C. elegans* is an excellent model organism to dissect RHGF-2 and MPZ-1 interaction and further define the role for Rho GTPase signaling in neurons.
Chapter 5

Attribution of Data

cDNA analysis to determine the upstream gene, T08H4.2 is actually a part of rhgf-2 was provided by Dr. Robert Steven. Todd Cramer performed the backcrossed experiments on rhgf-2(gk216)/mIn1 to create the strain XS245. Todd also made the T0H4.2.2p::tagrfp construct to help determine T08H4.2 expression. Shuang Hu made the 25 and 22 kb rhgf-2l gene constructs for the rescuing experiments.
References


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