Identification of Genes Involved in the *C. elegans* VAB-1 Eph Receptor Tyrosine Kinase Signaling Pathway

by

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Abstract

The generation of a functional nervous system requires that neuronal cells and axons navigate precisely to their appropriate targets. The Eph Receptor Tyrosine Kinases (RTKs) and their ephrin ligands have emerged as one of the important guidance cues for neuronal and axon navigation. However, the molecular mechanisms of how Eph RTKs regulate these processes are still incomplete. The purpose of this work was to contribute to the understanding of how Eph receptors regulate axon guidance by identifying and characterizing components of the Caenorhabditis elegans Eph RTK (VAB-1) signaling pathway. To achieve this objective I utilized a hyper active form of the VAB-1 Eph RTK (MYR-VAB-1) that caused penetrant axon guidance defects in the PLM mechanosensory neurons, and screened for suppressors of the MYR-VAB-1 phenotype. Through a candidate gene approach, I identified the adaptor NCK-1 as a downstream effector of VAB-1. Molecular and genetic analysis revealed that the nck-1 gene encodes for two isoforms (NCK-1A and NCK-1B) that share similar expression patterns in parts of the nervous system, but also have independent expression patterns in other tissues. Genetic rescue experiments showed that both NCK-1 isoforms can function in axon guidance, but each isoform also has specific functions. In vitro binding assays showed that NCK-1 binds to VAB-1 in a kinase dependent manner. In addition to NCK-1, WSP-1/N-WASP was also identified as an effector of VAB-1 signaling. Phenotypic analysis showed that nck-1 and wsp-1 mutants had PLM axon over extension defects similar to vab-1 animals. Furthermore, VAB-1, NCK-1 and WSP-1 formed a complex in vitro. Intriguingly, protein binding assays showed that NCK-1 can also bind to the actin regulator UNC-34/Ena, but
genetic experiments suggest that *unc-34* is an inhibitor of *nck-1* function. Through various genetic and biochemical experiments, I provide evidence that VAB-1 can disrupt the NCK-1/UNC-34 complex, and negatively regulate UNC-34. Taken together, my work provides a model of how VAB-1 RTK signaling can inhibit axon extension. I propose that activated VAB-1 can prevent axon extension by inhibiting growth cone filopodia formation. This is accomplished by inhibiting UNC-34/Ena activity, and simultaneously activating Arp2/3 through a VAB-1/NCK-1/WSP-1 complex.
Chapter 4: The *C. elegans* Eph receptor activates NCK and N-WASP, and inhibits Ena/VASP to regulate growth cone dynamics during axon guidance.

Jeffrey Boudreau conducted the protein pull-down assays shown in Figure 4-3B and 4-3C. Jeffrey Boudreau also conducted the yeast two-hybrid analysis that resulted in Figure 4-3D.

Jun Liu developed a technique to enable the simultaneous expression of two vectors in bacteria. Jun Liu also designed and made the pIC582 – His6::VAB-1 vector.
Acknowledgements

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To my committee members, Dr. Paul Young and Dr. Virginia Walker, thank you for your guidance through both my M.Sc. and Ph.D thesis. To Dr. William Bendena, thank you for your advice, encouragement and entertaining conversations.

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<td>Actin binding protein 1</td>
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<td>ADAM10</td>
<td>a disintegrin and metalloprotease 10</td>
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<tr>
<td>Arp</td>
<td>Actin-related proteins</td>
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<tr>
<td>BR</td>
<td>Basic region</td>
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<tr>
<td>C</td>
<td>Cofilin domain</td>
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<tr>
<td>C-domain</td>
<td>Central-domain</td>
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<tr>
<td>C-terminal</td>
<td>Carboxyl-terminal</td>
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<tr>
<td>CAN</td>
<td>Canal-associated neuron</td>
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<tr>
<td>CC</td>
<td>Conserved cytoplasmic motif</td>
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<tr>
<td>Cdc42</td>
<td>Cell division cycle 42</td>
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<tr>
<td>DB</td>
<td>DCC-binding</td>
</tr>
<tr>
<td>DCC</td>
<td>Deleted in colorectal cancer</td>
</tr>
<tr>
<td>DSCAM</td>
<td>Down’s syndrome cell adhesion molecule</td>
</tr>
<tr>
<td>DTC</td>
<td>Distal tip cell</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>Ena</td>
<td>Enabled</td>
</tr>
<tr>
<td>Eph</td>
<td>Erythropoietin producing hepatocellular carcinoma cell line</td>
</tr>
<tr>
<td>Ephexin</td>
<td>Eph-interacting exchange factor</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>EST</td>
<td>Expressed sequence tag</td>
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<tr>
<td>EVH</td>
<td>Ena/VASP homology</td>
</tr>
<tr>
<td>F-actin</td>
<td>Filamentous actin</td>
</tr>
<tr>
<td>FNIII</td>
<td>Fibronectin type III</td>
</tr>
<tr>
<td>G-actin</td>
<td>Monomeric globular actin</td>
</tr>
<tr>
<td>GABA</td>
<td>γ-aminobutyric acid</td>
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<tr>
<td>GAP</td>
<td>GTPase activating protein</td>
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<tr>
<td>GBD</td>
<td>GTPase binding domain</td>
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<tr>
<td>Term</td>
<td>Description</td>
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<td>--------------------------------------------------</td>
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<tr>
<td>GDP</td>
<td>Guanosine diphosphate</td>
</tr>
<tr>
<td>GEF</td>
<td>Guanine exchange factor</td>
</tr>
<tr>
<td>GPI</td>
<td>Glycosylphosphatidylinositol</td>
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<td>GST</td>
<td>Glutathione-S-transferase</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine triphosphate</td>
</tr>
<tr>
<td>GTPases</td>
<td>Guanosine triphosphatases</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>HSN</td>
<td>Hermaphrodite specific motor neuron</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IgSF</td>
<td>Immunoglobulin superfamily</td>
</tr>
<tr>
<td>IRSp53</td>
<td>Insulin-receptor substrate p53</td>
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<tr>
<td>LRR</td>
<td>Leucine-rich repeats</td>
</tr>
<tr>
<td>LAR</td>
<td>Leukocyte common antigen related</td>
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<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MBP</td>
<td>Maltose binding protein</td>
</tr>
<tr>
<td>mDia</td>
<td>Mammalian diaphanous</td>
</tr>
<tr>
<td>MMPs</td>
<td>Matrix metalloproteinases</td>
</tr>
<tr>
<td>MSP</td>
<td>Major sperm protein</td>
</tr>
<tr>
<td>MYR</td>
<td>Myristoylation</td>
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<tr>
<td>N-Terminal</td>
<td>Amino-terminal</td>
</tr>
<tr>
<td>N-WASP</td>
<td>Neuronal-Wiskott-Aldrich syndrome protein</td>
</tr>
<tr>
<td>Nck</td>
<td>Non-Catalytic region of tyrosine Kinase</td>
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<tr>
<td>NMD</td>
<td>Nonsense-mediated mRNA decay</td>
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<tr>
<td>P1-P2</td>
<td>Conserved domains in Dcc</td>
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<tr>
<td>P-domain</td>
<td>Peripheral-domain</td>
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<td>PAK</td>
<td>p21-activated serine/threonine kinases</td>
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<tr>
<td>PDZ</td>
<td>PSD-95 Disc large Zonula occludens-1</td>
</tr>
<tr>
<td>PIP2</td>
<td>Phosphatidylinositol (4,5)-bisphosphate</td>
</tr>
<tr>
<td>PIP5K-Iα</td>
<td>Phosphatidylinositol-4-phosphate 5-kinase Iα</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
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<tr>
<td>PLM</td>
<td>Posterior lateral microtubule</td>
</tr>
<tr>
<td>PRO</td>
<td>Proline rich domain</td>
</tr>
<tr>
<td>Rac</td>
<td>Ras-related C3 botulinum toxin substrate</td>
</tr>
<tr>
<td>Rho</td>
<td>Ras homologous</td>
</tr>
<tr>
<td>RNAi</td>
<td>RNA interference</td>
</tr>
<tr>
<td>Robo</td>
<td>Roundabout</td>
</tr>
<tr>
<td>Rock</td>
<td>Rho kinase</td>
</tr>
<tr>
<td>RPTP</td>
<td>Receptor protein tyrosine phosphatase</td>
</tr>
<tr>
<td>RTK</td>
<td>Receptor tyrosine kinase</td>
</tr>
<tr>
<td>SAM</td>
<td>Sterile-α motif</td>
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<tr>
<td>SDF</td>
<td>Stromal cell-derived factor</td>
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<tr>
<td>Sema</td>
<td>Semaphorin</td>
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<tr>
<td>SH</td>
<td>Src-homology domain</td>
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<tr>
<td>SL</td>
<td>Spliced leader</td>
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<tr>
<td>Slit</td>
<td>Shiga-like toxin</td>
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<tr>
<td>SOCS</td>
<td>Suppressor of cytokine signaling</td>
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<tr>
<td>SPAR</td>
<td>Spine-associated RapGAP</td>
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<tr>
<td>T-zone</td>
<td>Transition-zone</td>
</tr>
<tr>
<td>UNC</td>
<td>Uncoordinated</td>
</tr>
<tr>
<td>V</td>
<td>Verprolin</td>
</tr>
<tr>
<td>VAB-1</td>
<td>Variable abnormal</td>
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<tr>
<td>VAP</td>
<td>VAMP associated protein</td>
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<td>Vasodilator-stimulated phosphoprotein</td>
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<td>VPR</td>
<td>VAP33-related</td>
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<tr>
<td>WH</td>
<td>WASP-homology domain</td>
</tr>
<tr>
<td>WRK</td>
<td>Wrapper, rega-1, klingon-like</td>
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<td>ZU-5</td>
<td>A domain present in the gap junction protein Zona Occludens-1</td>
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Chapter 1

General Introduction

The development of a functional nervous system requires neurons to guide their axons to the appropriate targets. To accomplish this task, axons must correctly interpret signaling cues in their extracellular environment. The part of the axon that is most sensitive to these guiding signals is a dynamic structure at the tip of the axon called the growth cone (Dickson, 2002). These signals, either membrane bound or secreted, bind to receptors located at the tip of the growth cone to activate a signaling cascade that would attract or repel the axon (Geraldo and Gordon-Weeks, 2009). Although details of the guidance receptor signaling mechanisms are still incomplete, it is clear that most, if not all, these signals exert their effect by modulating the actin cytoskeleton of the growth cone (Bashaw and Klein, 2010; O'Donnell et al., 2009). There are several conserved families of guidance molecules that have been shown to regulate the growth cone cytoskeleton, among which are the Netrins, Slits, Semaphorins and Ephrins (Figure 1-1) (Dickson, 2002; O'Donnell et al., 2009).

1.1 Netrins and their receptors

The Netrins are a conserved family of guidance cues that are mainly present as secreted proteins in most organisms, but membrane anchored forms (netrin G1 and G2) have also been identified in vertebrates (Moore et al., 2007; Nakashiba et al., 2000; Nakashiba et al., 2002). All netrins have an amino-terminal (N-terminal) composed of
Figure 2-1: Structure of ligand families and their receptors.

Selected members of conserved ligand families (top) and their receptors (bottom) involved in axon guidance. EGF: epidermal growth factor; GPI: glycosylphosphatidylinositol; Ig: immunoglobulin; FNIII: fibronectin type III; P1-P3: domains specific to Dcc; TSP: thrombospondin type I; ZU-5: domain similar to zona occludens-1; DB: Dcc-binding domain; DD: death domain; LRR: leucine-rich repeats; LamG: laminin-G domain; CC0-CC3: conserved cytoplasmic motif; Sema: semaphorin motif; PSI: plexin, semaphorin, integrin (also called MRS – Met-related sequence); IPT: immunoglobulin-like shared by plexins and transcription factors (also called G-P: glycine-proline rich); SP: sex-plexin domain; CUB: complement-binding (also called a1/a2); CF 5/8: coagulation factor 5/8 (also called b1/b2); MAM: meprin, A5, µ-phosphate; PDZ: PSD-95 disc large zonula; SAM: sterile α-motif. (Idea of figure was adapted and modified from Dickson (2002)).
two domains, VI and V (which has three epidermal growth factor (EGF) repeats), that are homologous to those found in laminins (Figure 1-1) (Moore et al., 2007; Rajasekharan and Kennedy, 2009). The carboxyl-terminal (C-terminal), in secreted netrins possess a conserved region, called the C-domain, that is enriched in basic amino acids, while the membrane anchored netrins have a C-terminal glycosylphosphatidylinositol (GPI) tail that anchors them to the cell surface (Figure 1-1) (Moore et al., 2007; Rajasekharan and Kennedy, 2009). Although netrins were initially identified for their role in midline attraction, they were later found to also function in midline repulsion (Huber et al., 2003; Rajasekharan and Kennedy, 2009). The outcome of the secreted netrin response is in part dependent on the activation of different netrin receptors (Hall and Lalli, 2010). Growth cone attraction is promoted upon the interaction with the transmembrane receptor deleted in colorectal cancer (DCC) family, which are composed of four immunoglobulin (Ig) domains, six fibronectin type III (FNIII) repeats, and cytoplasmic domains with three conserved motifs (P1, P2 and P3) (Figure 1-1) (Chan et al., 1996; Keino-Masu et al., 1996; Rajasekharan and Kennedy, 2009; Vielmetter et al., 1994). Recent work has also shown that the transmembrane protein Down’s syndrome cell adhesion molecule (DSCAM) is a receptor for netrin-1, and is involved in mediating netrin dependent attraction during development (Andrews et al., 2008; Ly et al., 2008). DSCAM receptors are composed of ten Ig domains and six FNIII repeats in its extracellular region, and no identifiable motifs in the intracellular region (Figure 1-1) (Yamakawa et al., 1998). The growth cone repulsion response to netrins, on the other hand, involves the transmembrane
receptor uncoordinated UNC-5 family, which have an extracellular domain composed of two Ig domains followed by two thrombospondin domains, and an intracellular domain made up of three conserved domains: a ZU-5 domain (a domain present in the gap junction protein Zona Occludens-1), a DCC-binding (DB) domain and a death domain (Figure 1-1) (Leonardo et al., 1997; Leung-Hagesteijn et al., 1992; Rajasekharan and Kennedy, 2009). Intriguingly, although the UNC-5 receptor is sufficient on its own for short range repulsion, a complex formation between DCC and UNC-5 is required for netrin mediated long range repulsion (Hong et al., 1999; Moore et al., 2007).

1.2 The Slits and Robos

Slit proteins are highly conserved diffusible glycoproteins that are involved in midline axon repulsion and neuronal migration in various organisms (Brose et al., 1999; Kidd et al., 1999; Wu et al., 1999; Ypsilanti et al., 2010). Slits are composed of four domains containing leucine-rich repeats (LRR), seven to nine EGF repeats, a laminin-G domain and a C-terminal cysteine-rich knot (Figure 1-1) (Rothberg et al., 1990; Ypsilanti et al., 2010). Slits are known to mediate their repulsion signal through the conserved transmembrane protein family roundabout (Robo) that are expressed by axons navigating the midline (Kidd et al., 1999). Robo receptors contain five Ig motifs, three FNIII domains and four conserved cytoplasmic (CC) motifs designated CC0, CC1, CC2 and CC3 (Figure 1-1) (Kidd et al., 1999; Ypsilanti et al., 2010). The cytoplasmic CC domains of Robo are essential for Slit mediated repulsion, but they are also required for the silencing of netrin meditated attraction (Huber et al., 2003; Stein and Tessier-Lavigne, 2004).
2001). Although Robo proteins are known to be the receptors for Slit, work carried out in the nematode *Caenorhabditis elegans* (*C. elegans*) suggest that three other receptors, UNC-40/DCC, VAB-1/Eph and EVA-1, are involved in mediating Slit/Robo signaling through their physical interaction with SAX-3/Robo (Fujisawa et al., Ghenea et al., 2005; 2007; Yu et al., 2002).

1.3 The Semaphorins, Plexins and Neuropilins

The semaphorin family of secreted and membrane bound proteins can promote both repulsive and attractive guidance events through their interaction with the plexin and neuropilin receptors (Koncina et al., 2007 163; Zhou et al., 2008 162). Semaphorins (20 in total) are classified into eight classes depending on their structural similarities and species of origin, but all semaphorins are defined by their semaphorin (Sema) motif in the N-terminal (Figure 1-1) (Zhou et al., 2008). The plexin receptor family, which possess semaphorin domains in their extracellular portion, are the most prominent semaphorin receptors (Chilton, 2006; Zhou et al., 2008). With the exception of Sema1, Sema3E, Seam4, Sema6 and Sema7, the plexin receptors do not directly bind to semaphorins (Chilton, 2006; Koncina et al., 2007; Winberg et al., 1998; Zhou et al., 2008). Instead, most plexin receptors form a co-receptor complex with a second semaphorin receptor, the neuropilins (Koncina et al., 2007). Neuropilins were first identified as receptors required for Sema3s to induce repulsion of axons (He and Tessier-Lavigne, 1997; Kolodkin et al., 1997). The short intracellular domains of neuropilins are not essential for mediating semaphorin repulsive signals and can only convey the signal when associated with
plexins (Nakamura et al., 1998; Takahashi et al., 1999; Tamagnone et al., 1999).

Although most semaphorins cause axon repulsion, some semaphorins are bi-functional and can induce both repulsion and attraction (Koncina et al., 2007; Zhou et al., 2008). Sema3D is an example of a bi-functional semaphorin where it acts as a repellent when associated with receptors containing neuropilin-1A, but acts as an attractant through receptors containing the heterodimer neuropilin-1A/neuropilin-2B (Wolman et al., 2004). Hence, axon repulsion or attraction seems to depend on several variables such as: the type of receptor combination, the combination between ligand and receptor, and the cell-type in which the signaling occurs (Zhou et al., 2008). Additionally, recent work has shown that semaphorins themselves can function as a receptor and induce a reverse signal as a result of its interaction with plexins (Yu et al., 2010).

1.4 The Ephrins and Eph RTKs

The Ephrins are membrane tethered guidance cues that mainly mediate cell-contact repulsion signals through their interaction with the Eph receptor tyrosine kinase (RTK) (Egea and Klein, 2007). The Eph RTK family is the largest family of receptor tyrosine kinases, with the vertebrates containing sixteen Eph receptors and nine ephrin ligands (Lackmann and Boyd, 2008). Eph RTKs and their ephrin ligands are involved in regulating various neurodevelopmental processes such as establishing hindbrain segment boundaries, regulating migration patterns of neural crest cells and establishing neural topographic maps (Reber et al., 2007). In addition to their neuronal role, Eph RTKs and ephrins function in other processes such as vascular development, intestinal cell
positioning and cancer development (Pasquale, 2010; Pitulescu and Adams, 2010). As a result, much work has been put into trying to understand the molecular cascades involved in eliciting Eph receptor responses (Lackmann and Boyd, 2008). Unfortunately, progress in this area has been difficult and the network of molecules involved in Eph/ephrin signaling is still poorly characterized (Reber et al., 2007). In addition, some of the potential Eph RTK interactors identified through proteomics still require conformation of *in vivo* function (Zhang et al., 2008). The difficulty in identifying the Eph RTK signaling pathway is in part due to the functional redundancy and cross talk by the large number of Eph receptors and ephrins, and in addition, bidirectional signaling between Eph receptors and ephrins makes the signaling more complex (Egea and Klein, 2007; Pitulescu and Adams, 2010). Unlike the vertebrate system, the nematode *C. elegans* possesses only a single Eph receptor (VAB-1) (George et al., 1998). The presence of only one Eph RTK would eliminate many of the difficulties faced in studying the vertebrate Eph signaling pathway, as well as provide simpler signal transduction events from the receptor. Furthermore, due to the conservation of guidance molecules across species, observations made from studying the *C. elegans* VAB-1 Eph RTK will be of relevance to the vertebrate system (Tessier-Lavigne and Goodman, 1996). A literature review on the vertebrate and *C. elegans* Eph RTK and their role in axon guidance is provided in Chapter 2.

In addition to having a single Eph receptor, there are other factors that make *C. elegans* a good genetic model for studying the Eph RTK signaling pathways in neuronal
guidance. For example, *C. elegans* has a simple nervous system composed of 302 neurons that have been completely characterized in terms of lineage, morphology, projection patterns and synaptic connectivity (Li, 2001; Sulston et al., 1983; White et al., 1986). Furthermore, the transparent body of *C. elegans* allows us to view individual neurons in the living animal. These characteristics make it easy to study neuronal defects caused by mutations in *C. elegans*. Similarly, the ease and rapidity of applying various genetic techniques, including RNA interference (RNAi) (Ahringer, 1997), makes it possible to examine molecules that could be involved in the Eph signaling pathway. The *C. elegans* VAB-1 Eph RTK has been shown to be required in various aspects of epidermal and neuronal development (Boulin et al., 2006; George et al., 1998; Mohamed and Chin-Sang, 2006; Zallen et al., 1999). Unfortunately, what we are still missing is an understanding of the molecular mechanisms involved in the VAB-1 Eph RTK signaling pathway during neuronal development.
1.5 References


Chapter 2

Literature Review

2.1 The axon growth cone

2.1.1 Structural organization of the growth cone

The growth cone is a highly motile and dynamic structure located at the tip of the growing axon. These growth cones act as the sensory device that guides neurons to their appropriate targets by correctly responding to cues in the environment (Geraldo and Gordon-Weeks, 2009). When stimulated by an attractive cue, growth cones are stabilized and move forward, but when sensing a repellent the growth cone retracts or even collapses (Bashaw and Klein, 2010). The response of the growth cone to a guidance cue depends on the signaling cascades that can regulate the cytoskeleton (Gupton and Gertler, 2007).

The cytoskeleton of the neuronal growth cone is mainly composed of microtubules and filamentous actin (F-actin) that are spatially organized in different regions of the growth cone (Figure 2-1) (Lowery and Van Vactor, 2009). Microtubules are dynamic hollow structures composed of 13 protofilament polymers of α/β tubulin heterodimers (Siegrist and Doe, 2007). In the growth cone, microtubules are predominantly found in the central (C)-domain, which is the region closest to the axon shaft (Figure 2-1) (Geraldo and Gordon-Weeks, 2009). In the axon shaft, microtubules are found in bundles, but as they enter the C-domain they unbundle into single microtubules that can cross the C-domain and invade the actin-rich peripheral (P)-domain.
Figure 2-1: Growth cone structure.

The growth cone is divided into three regions: a central (C)-domain that contains stable and unstable microtubules; a transition (T)-zone that contains actin arcs; and a peripheral (P)-domain that is composed of two types of filamentous (F)-action called filopodia and lamellipodia. The filopodia are finger-like structures at the tip of the growth cone, and are composed of long F-actin bundles. Lamellipodia are made of short networks of branched F-actin that gives the growth cone its veil-like structure. (Adapted and modified from Lowery and Van Vactor (2009)).
(Geraldo and Gordon-Weeks, 2009). The extension of microtubules into the P-domain allows it to interact with actin filaments at the growth cone tip, and has been found to be fundamental for neurite initiation and growth cone turning in response to guidance cues (Dent et al., 2007; Myers et al., 2006; Tanaka and Kirschner, 1995; Williamson et al., 1996).

The other type of cytoskeletal filament, F-actin, is a helical polymer formed by the addition of globular actin to the growing end of the filament (the barbed end), and is primarily found in the P-domain at the most distal part of the growth cone (Figure 2-1) (Bouquet and Nothias, 2007; Geraldo and Gordon-Weeks, 2009). The actin filaments found in the P-domain form two different actin structures that differ in their network organization (Bouquet and Nothias, 2007; Lewis and Bridgman, 1992). The first structure, called lamellipodia, is formed of a densely veil-like branched network of F-actin, whereas the second structure, called filopodia, is formed of long parallel bundles of F-actin that extend in a finger-like manner beyond the cell edge (Figure 2-1) (Gupton and Gertler, 2007; Lewis and Bridgman, 1992). It is these finger-like filopodia structures that are responsible for sensing the guidance molecules in the surrounding environment, and hence play a pivotal role in axon guidance by responding accordingly to the received guidance signal (Bentley and Toroian-Raymond, 1986; Geraldo and Gordon-Weeks, 2009).

In addition to the P-domain, actin filaments are also found in the transition (T)-zone that is located between the P-domain and the C-domain (Figure 2-1). Actin in the T-
zone is oriented parallel to the growth, and forms arc-shaped structures that are thought to function as a physical barrier to control microtubule extensions (Schaefer et al., 2002).

2.1.2 Regulators of the growth cone actin cytoskeleton

The change in growth cone motility behavior induced by a guidance cue is associated with changes in extension or retraction of growth cone filopodia and lamellipodia. Work in the past decades have identified several molecules that are involved in regulating these changes, some of which include Ena/VASP, the Arp2/3 complex, Rho GTPases, N-WASP and Nck (Gupton and Gertler, 2007; Li et al., 2001; Pollard and Borisy, 2003).

**Ena/VASP:**

The Enabled (Ena)/vasodilator-stimulated phosphoprotein (VASP) family is a conserved family of proteins characterized by the presence of a N-terminal Ena/VASP homology 1 (EVH1) domain, a central proline (PRO) rich domain and a C-terminal EVH2 domain (Krause et al., 2003). The Ena/VASP proteins have been shown to be required in axon guidance and growth cone filopodia formation in vertebrate and invertebrate systems (Gertler et al., 1995; Lanier et al., 1999; McIntire et al., 1992). Additionally, Ena/VASP proteins were reported to function as effectors in repulsive cues such as Eph RTK, SAX-3/Robo and UNC-5, as well as in attractive cues such as UNC-40/Dcc (Bashaw et al., 2000; Colavita and Culotti, 1998; Evans et al., 2007; Forsthoefer et al., 2005; Gitai et al., 2003; Lebrand et al., 2004; Yu et al., 2002). These results provided a strong connection between guidance signals and the regulation of the actin
cytoskeleton. Ena/VASP is involved in promoting filopodia formation by: (1) protecting the growing barbed end of actin filaments from capping proteins that bind to these barbed ends to inhibit their elongation (Bear et al., 2002; Mejillano et al., 2004), (2) elongating actin filaments and clustering them together to form filopodia structures (Applewhite et al., 2007; Chereau and Dominguez, 2006; Ferron et al., 2007; Mejillano et al., 2004), and (3) inhibiting actin-filament branching caused by the Arp2/3 complex actin nucleating factor (Bear et al., 2002; Skoble et al., 2001). It is still unknown if Ena/VASP has similar effects on the actin cytoskeleton in both attractive and repulsive signaling, or if these signals cause Ena/VASP to have a differential effect on the actin cytoskeleton.

The actin nucleator Arp2/3 complex:

The forward movement of axons and growth cones depend on the ability of actin filaments to elongate at their fast growing barbed ends. This process of filament elongation and assembly requires the help of molecules that can nucleate actin, so that actin monomers (G-actin) can be polymerized to form F-actin. One of these nucleating factors is the conserved eukaryotic actin-related protein 2 and 3 (Arp2/3) complex that is composed of Arp2, Arp3 and five other subunits (ARPC1-5) (Campellone and Welch, 2010). The Arp2/3 complex is intrinsically inactive, but when activated by members of the WASP/Scar family the Arp2/3 complex initiates the formation of new filaments as a 70° branch from the side of preexisting filaments (Insall and Machesky, 2009; Mullins et al., 1998). The net effect of the Arp2/3 complex activity on actin filaments is the formation of an elaborate network of short, highly branched actin filaments similar to
those found in lamellipodia (Blanchoin et al., 2000; Mullins et al., 1998; Svitkina and Borisy, 1999).

As for its role in axon guidance, reports indicate that the Arp2/3 complex is a negative regulator of axon extension, and is also required for axons to respond to inhibitory guidance signals (Pinyol et al., 2007; Strasser et al., 2004). Furthermore, high concentrations of active Arp2/3 seem to prevent filopodia formation, which is most likely due to the highly branched short filaments that make conditions for filopodia formation unfavourable (Ideses et al., 2008; Mejillano et al., 2004). It has been suggested that the extensive branching actin network produced by Arp2/3 is also important in regulating microtubules during axon guidance, by acting as a barrier to control the direction of microtubule invasion during axon guidance (Kakimoto et al., 2006; Strasser et al., 2004).

While many reports suggest the Arp2/3 complex is dispensable and not required for filopodia formation, it is still controversial as there have been reports that indicate that the Arp2/3 complex is required for filopodia formation (Goncalves-Pimentel et al., 2011; Korobova and Svitkina, 2008; Norris et al., 2009; Steffen et al., 2006; Strasser et al., 2004).

Unlike the Arp2/3 complex, other known actin nucleating factors produce linear unbranched actin filaments (Campellone and Welch, 2010). Three of these actin nucleators, formins, spire and cordon-bleu, are widely expressed in the nervous system (Ahuja et al., 2007; Pleiser et al., 2010; Schumacher et al., 2004). The best characterized of these are the formins, and have been reported to be required for filopodia formation in
neuronal growth cones (Goncalves-Pimentel et al., 2011; Matusek et al., 2008). Although the second type of actin nucleator, spire, was found to localize with formins in neurons, its exact role in the growth cones is unknown, but it would most likely involve filopodia regulation due its association with formins (Kessels et al., 2010; Pechlivanis et al., 2009; Schumacher et al., 2004). The last of the three nucleators, cordon-bleu, is expressed throughout hippocampal neurons, as well as in the growth cone of axons and dendrites, and its over-expression results in increased branching in axons and dendrites in an actin polymerization-dependent manner (Ahuja et al., 2007). Overall, the role of formins, spire and cordon-bleu in axon guidance has not been well characterized, and the upstream signaling pathways that can regulate these actin nucleators are yet to be fully explored (Campellone and Welch, 2010; Kessels et al., 2010).

**The Rho GTPases Rac1, Cdc42 and RhoA:**

In neurons, the small guanosine triphosphatases (GTPases) of the Rho subfamily have a major role in growth cone remodelling and axon guidance. These proteins act as molecular switches that alternate between an active guanosine triphosphate (GTP)-bound state and an inactive guanosine diphosphate (GDP)-bound state (Hall and Lalli, 2010). The switch from their inactive state to an active one is facilitated by the guanine exchange factors (GEFs), while the switch from an active to an inactive state is facilitated by the GTPase activating proteins (GAPs) (O'Donnell et al., 2009). The best studied members of the Rho-family GTPases are RhoA (Ras homologous member A), Rac1 (Ras-related C3 botullinum toxin substrate 1) and Cdc42 (cell division cycle 42) (Heasman and Ridley,
2008). These three Rho GTPases regulate different aspects of the growth cone structure, where Cdc42 and Rac1 regulate the formation of filopodia and lamellipodia, respectively, and Rho regulates the collapse of the growth cone structure (O'Donnell et al., 2009). Cdc42 is able to regulate the actin cytoskeleton during filopodia extension in several ways including: 1) the direct activation of actin nucleators such as the Arp2/3 complex (via the Arp2/3 activators N-WASP and WASP) and the formin member mDia2 (mammalian diaphanous) (Miki et al., 1998a; Peng et al., 2003; Symons et al., 1996), 2) the enhancement of actin filament elongation and bundling by recruiting the Ena/VASP member Mena through the protein IRSp53 (insulin-receptor substrate p53) (Krugmann et al., 2001), and 3) the inhibition of actin retrograde flow and filament turnover by activating the p21-activated serine/threonine kinases (PAK) (Edwards et al., 1999; Manser et al., 1994; Sanders et al., 1999). Similarly, Rac can also regulate the actin cytoskeleton during lamellipodia formation in several ways. First, Rac can induce actin nucleation by activating the Arp2/3 complex through the Arp2/3 activators WAVE and N-WASP (Eden et al., 2002; Miki et al., 1998b; Tomasevic et al., 2007). Second, Rac can affect the availability of free barbed ends for actin nucleation by increasing the levels of phosphatidylinositol (4,5)-bisphosphate (PIP2), through phosphatidylinositol-4-phosphate 5-kinase 1α (PIP5K-1α) activation, which can uncap actin filament barbed ends (Hartwig et al., 1995; Tolias et al., 2000). Third, similar to Cdc42, Rac can also affect actin retrograde flow and filament cycling by activating PAK (Edwards et al., 1999; Manser et al., 1994; Sanders et al., 1999). Rho, on the other hand, causes growth cone collapse and
retraction by inducing contractions in the arc actin filament and actin bundles in the C-domain due to myosin activation by the Rho effector ROCK (Rho kinase), which in turn leads to P domain retraction (Amano et al., 1996; Kimura et al., 1996; Lin et al., 1996; Matsui et al., 1996; Zhang et al., 2003). However, Rho has also been shown to mediate axon elongation through the actin nucleator mDia1 in response to low level of the neural chemokine stromal cell-derived factor (SDF)-1α (Arakawa et al., 2003; Watanabe et al., 1997).

As for their role in axon guidance, the common model is that Rho is activated by repulsive cues to induce growth cone collapse, while Rac1 and Cdc42 are activated by attractive cues to promote growth cone protrusions (Bashaw and Klein, 2010; Heasman and Ridley, 2008). However, the analysis of the signalling pathways of several guidance cues indicates a more complex picture. For example, the repulsion induced by Slit/Robo and Sema3A/Plexin-A signaling require Rac activation, meanwhile Eph/Ephrin reverse signalling requires the activation of both Rac and Cdc42 to induce repulsion (Fan et al., 2003; Turner et al., 2004; Vastrik et al., 1999; Xu and Henkemeyer, 2009; Yang and Bashaw, 2006). As a result, it has been suggested that Rho GTPases affect the growth cone in response to guidance signals based on the combination of activated GEFs and GAPs upstream of the Rho GTPases, as well as which effectors are activated by the Rho GTPase (Lowery and Van Vactor, 2009).
The Neuronal-Wiskott-Aldrich syndrome protein (N-WASP) is a member of the WASP scaffold protein family that is abundant in neuronal tissue (Takenawa and Suetsugu, 2007). N-WASP is characterized by a N-terminal containing an Ena/VASP homology-1 domain (EVH1; also called WASP-homology-1 domain (WH1)), a central region composed of a basic region (BR), a GTPase binding domain (GBD) and a PRO rich domain, and a C-terminal with two verprolin (V) homology domains (also called WH2), a hydrophobic cofilin domain (C) and an acidic (A) region (Padrick and Rosen, 2010; Takenawa and Suetsugu, 2007). Under resting conditions, N-WASP is autoinhibited by folding on itself due to the intramolecular interaction between the N-terminal GBD domain and VCA region (Kim et al., 2000). This autoinhibition can be released by the interaction of N-WASP with molecules such as Cdc42, Nck, PIP2 and F-actin binding protein 1 (Abp1) (Pinyol et al., 2007; Rohatgi et al., 1999; Rohatgi et al., 2001). Upon its release from the autoinhibited state, N-WASP can direct actin polymerization to form branched actin filaments through the Arp2/3 (Takenawa and Suetsugu, 2007; Yamaguchi et al., 2002). Among the activators of the Arp2/3 complex, the N-WASP VCA domain is found to be the most effective activator of the Arp2/3 complex (Yamaguchi et al., 2002). Despite the fact N-WASP induced actin filaments are branched, there has been suggestions that N-WASP is required for the formation of filopodia which contain straight bundles of actin filaments (Miki et al., 1998a; Shekarabi et al., 2005). However, these suggestions were challenged by the finding that N-WASP
deficient cells are still able to form filopodia, which indicates that N-WASP is not essential for filopodia formation (Snapper et al., 2001; Takenawa and Suetsugu, 2007).

Considering its role in actin regulation, it would not be surprising if N-WASP had a role in axon guidance. Unfortunately, our knowledge in this area is limited to a few reports. Initial work using a dominant negative form of N-WASP suggested that N-WASP is required for neurite extension (Banzai et al., 2000). In contrast to this conclusion, a reduction in the levels of N-WASP in hippocampal neurons resulted in enhancement of axon lengths, indicating that N-WASP acts as a negative regulator of axon elongation in hippocampal neurons (Kakimoto et al., 2006; Pinyol et al., 2007). Also, work carried out in C. elegans showed that the absence of WSP-1, the N-WASP homolog, resulted in defects in neuronal cell migration and axon guidance (Shakir et al., 2008; Withee et al., 2004). Currently, what is lacking is the identification of the receptors that function upstream of N-WASP during axon guidance.

The Nck adaptor proteins:

The mammalian Nck (Non-Catalytic region of tyrosine Kinase) adaptor protein family has two members (Nck1 and Nck2) and are composed entirely of three SH3 domains followed by a single SH2 domain, and have been shown to play a role in axon guidance in different organisms such as mouse, Drosophila and C. elegans (Chen et al., 1998; Fawcett et al., 2007; Garrity et al., 1996; Lehmann et al., 1990; Mohamed and Chin-Sang, 2011). Furthermore, Nck has been reported to be an effector of both attractive guidance receptors such as DCC and DSCAM, as well as repulsive receptors such as the
Eph RTK and Robo (Fan et al., 2003; Fawcett et al., 2007; Holland et al., 1997; Li et al., 2002; Schmucker et al., 2000; Stein et al., 1998). The Nck adaptor is thought to function downstream of these receptors as a protein bridge in signal transduction pathways. The NCK SH2 domain can bind phosphorylated tyrosine residues of receptors, or cytoplasmic molecules, while the SH3 domains bind proline rich regions to provide the protein-protein interaction required for transducing signals intracellularly (Buday et al., 2002; Li, 2005; Schlessinger and Lemmon, 2003).

Once recruited to the membrane, Nck is proposed to regulate the neuronal actin cytoskeleton through several pathways. The first is by binding and activating N-WASP and WAVE, which in turn activate the Arp2/3 complex to form branched actin networks (Eden et al., 2002; Rohatgi et al., 2001; Shekarabi et al., 2005; Tomasevic et al., 2007). The second method involves regulating the activity of Rac GTPase by directly interacting with Rac GEFs such as Dock180 and Sos, as well as Rac GAPs like α-chimaerin (Wegmeyer et al., 2007; Xu and Henkemeyer, 2009; Yang and Bashaw, 2006). Finally, Nck is predicted to affect the retrograde flow and recycling of actin filaments by binding to PAK (Fan et al., 2003; Hing et al., 1999).
2.2 The vertebrate Eph receptor tyrosine kinase

As illustrated in Chapter 1, there are several conserved families of guidance molecules that have been shown to regulate the growth cone cytoskeleton during axon guidance. Of these molecules, I am interested in studying the Eph receptor tyrosine kinases and understanding how they regulate the actin cytoskeleton for axon guidance.

2.2.1 The Eph RTK structure and its activation

The Eph receptor tyrosine kinase (RTK) gets its name from the erythropoietin-producing hepatocellular carcinoma cell line, where it was first isolated (Eph Nomenclature Committee, 1997; Hirai et al., 1987). Although initially isolated from carcinogenic cells, implicating its role in tumorigenesis, the Eph receptors are involved in diverse biological roles including early aspects of embryogenesis, segmental boundary formation, cell migration, neurogenesis, axon guidance, dendritic spine formation, vascular development, postnatal and tumor angiogenesis (Klein, 2009; Pasquale, 2010; Pitulescu and Adams, 2010). What is intriguing about Eph RTKs is that they do not act like other known RTKs and differ from them in several ways: 1) most activated RTKs form dimers, while activated Eph receptors form higher order complexes, 2) RTKs tend to regulate cell growth and differentiation, while Eph receptors mainly regulate cell shape and migration, and 3) Eph receptor interaction with its ligand can induce forward and reverse signaling (Egea and Klein, 2007).

Structural analyses of Eph receptors show that all Eph RTKs have a highly conserved molecular structure. They possess an extracellular region containing a globular
ligand-binding domain, a cysteine rich region, and two fibronectin type III (FNIII) repeats (Figure 2-2) (Pitulescu and Adams, 2010; Truitt and Freywald, 2011). The intracellular region of Eph receptors is composed of a juxtamembrane region with two highly conserved tyrosine residues, a tyrosine kinase domain, a sterile-α motif (SAM) and a PSD-95 Disc large Zonula occludens-1 (PDZ)-binding motif (Figure 2-2) (Pitulescu and Adams, 2010). Although all Eph receptors are structurally similar, they are classified into two subclasses, A and B, based on their amino acid sequence and ligand binding preference to the two ephrin (Eph receptor interacting molecules) ligands, ephrinAs and ephrinBs (Egea and Klein, 2007). EphA receptors bind to the glycosylphosphatidylinositol (GPI) anchored ephrinAs, while EphB receptors bind to transmembrane anchored ephrinBs (Figure 2-2) (Egea and Klein, 2007). Exceptions to the binding rule include EphA4 that can also bind to ephrinB3, and EphB2 that can bind to ephrinA5 (Gale et al., 1996; Himanen et al., 2004).

Structural data also show that the initial Eph-ephrin interaction occurs at a 1:1 stoichiometry, but then results in a tetrameric complex composed of two Eph receptors and two ephrin ligands that can further aggregate into larger order clusters (Himanen et al., 2001; Vearing and Lackmann, 2005; Wimmer-Kleikamp et al., 2004). The dimerization and oligomerization of the Eph-ephrin complexes result in the auto- and transphosphorylation of three conserved tyrosine residues (two in the juxtamembrane and one in the kinase activation loop) in the Eph receptors that are required for full receptor activity (Binns et al., 2000; Fang et al., 2008; Kalo and Pasquale, 1999; Shi et al., 2010).
Figure 2-2: General features of Eph receptors and their ephrin ligands.

The Eph receptor interacts with the ephrin ligand via its globular domain. EphrinA ligands are bound to the membrane via a GPI anchor (top left), while ephrinB ligands are inserted via a transmembrane domain followed by a cytoplasmic tail (top right). GPI: glycosylphosphatidylinositol; PDZ: PSD-95 disc large zonula occludens-1; SAM: sterile α-motif. (Adapted and modified from Kullander and Klein (2002)).
The phosphorylation of the two tyrosine residues in the juxtamembrane is particularly important, as they result in relieving the inhibitory intramolecular interactions between the juxtamembrane region and the kinase domain and promotes the kinase activity of Eph receptors, resulting in a forward signal transduction to occur (Binns et al., 2000; Reber et al., 2007; Wybenga-Groot et al., 2001). In addition to the signal being conveyed by the Eph receptor, the ephrin ligands can also convey signals into the ephrin-expressing cell, a phenomenon called reverse signaling (Adams et al., 2001; Henkemeyer et al., 1996; Holland et al., 1996; Huai and Drescher, 2001). Once the Eph-ephrin interaction occurs, the destabilization of the cell-cell contact to allow repulsion is thought to occur through two mechanisms. The first mechanism involves the trans-endocytosis of the Eph-ephrin complex and requires the activation of Rac1, which is proposed to occur through the function of the Rac GEFs Vav2 and Tiam1 (Cowan et al., 2005; Marston et al., 2003; Yoo et al., 2011; Zimmer et al., 2003). The second method involves the proteolytic shedding of Ephs and ephrins by metalloproteases such as ADAM10 (a disintegrin and metalloprotease 10) and MMPs (matrix metalloproteinases) (Georgakopoulos et al., 2006; Hattori et al., 2000; Janes et al., 2005; Lin et al., 2008; Litterst et al., 2007; Tomita et al., 2006). In some cases both mechanisms have been shown to be utilized at the same time. For example, the binding of ephrinA2/A5 to EphA3 results in the cleavage of ephrinA2/5 by ADAM10 followed by the internalization of the EphA3-ephrinA2/5 complex into the EphA expressing cell (Janes et al., 2005). Additionally, endocytosis was found to be necessary for the ectodomain shedding of EphB2 after its interaction with the ephrinB2
ligand, where inhibition of endocytosis also inhibited the cleavage of EphB2 (Litterst et al., 2007).

Activation of the Eph receptor generally results in the repulsion of neighbouring cells or cellular processes, such as growth cones, and down-regulation of adhesion molecules (Lackmann and Boyd, 2008; Pasquale, 2005). However, in some situations Eph activation can also lead to increased adhesion or attraction (Lackmann and Boyd, 2008; Pasquale, 2005). Nonetheless, both outcomes of Eph signaling depend on downstream effectors involved in regulating cytoskeletal organization and cell adhesion (Egea and Klein, 2007; Pasquale, 2005).

2.2.2 The vertebrate Eph RTK in axon guidance

Within the nervous system, Eph signaling is involved in regulating many processes like neural crest migration, axon guidance, dendritic spine formation, synaptic plasticity, hindbrain segmentation and the formation of neural topographic maps (Henkemeyer et al., 1996; Irie and Yamaguchi, 2002; Klein, 2009; Reber et al., 2007). The Eph RTK family regulate these processes by utilizing molecules that can affect the actin cytoskeleton (Egea and Klein, 2007). One of the first reports to confirm this showed that activation of EphA resulted in retinal ganglion cell growth cone collapse by activating Rho and downregulating Rac (Wahl et al., 2000). This connection was further strengthened by identifying the Rho GEF ephexin1 (Eph-interacting exchange factor) and Rac GAP α2-chimaerin as direct interactors with the EphA receptor, and both molecules are required to mediate growth cone collapse induced by Eph RTK activation (Sahin et
al., 2005; Shamah et al., 2001; Shi et al., 2007; Wegmeyer et al., 2007). The activation of EphA promotes the Src-dependent phosphorylation of ephexin1, which results in shifting ephexin1’s GEF activity toward RhoA thereby causing growth cone collapse (Knoll and Drescher, 2004; Sahin et al., 2005). At the same time, EphA signaling inactivates Rac1 through activation of the Rac GAP α2-chimaerin (Shi et al., 2007; Wegmeyer et al., 2007). Interestingly, the inactivation of Rac1 is found to be only transient, and growth cone collapse requires the resumption of Rac1 activity to induce endocytosis of the Eph/ephrin complex (Cowan et al., 2005; Jurney et al., 2002). Indeed, EphA was found to recruit and phosphorylate the Rac GEF Vav2, which results in Rac1 activation leading to Rac1-dependent endocytosis of the Eph-ephrin complex and growth cone collapse (Cowan et al., 2005; Jurney et al., 2002). In addition to Vav2, EphA has also been shown to require the Rac GEF Tiam1 for endocytosis of the Eph/ephrin complex and repulsion in nasal axons (Yoo et al., 2011). Thus, it seems that Eph signaling results in growth cone retraction by upregulating RhoA via ephexin1 and downregulating Rac1 via α-chimaerin, and then activating Vav2, and/or Tiam-1, to locally activate Rac1-dependent endocytosis to allow cell detachment.

Another mechanism by which Eph RTK induces the retraction of neurites and growth cone collapse is by downregulating levels of the GTP-bound Ras proteins R/H-Ras and Rap1 (Dail et al., 2006; Elowe et al., 2001; Miao et al., 2001; Richter et al., 2007). Eph activation has been shown to inhibit GTP-bound R/H-Ras in a p120-RasGAP-dependent manner, while GTP-bound Rap1 is inhibited through the activation of the
spine-associated RapGAP (SPAR) (Dail et al., 2006; Elowe et al., 2001; Richter et al., 2007). In addition to inhibiting Ras proteins, Eph activation can consequently inactivate the extracellular signal-regulated kinase/mitogen-activated protein kinase (ERK/MAPK) (Elowe et al., 2001; Miao et al., 2001; Yue et al., 2008). Surprisingly, the inhibition of ERK/MAPK activity was found to be not required for Eph RTK induced growth cone collapse, at least in hippocampal neurons (Yue et al., 2008).

Eph signaling is also involved in dendritic spine morphogenesis and neurite outgrowth (Irie and Yamaguchi, 2002; Penzes et al., 2003; Tanaka et al., 2004; Tolias et al., 2007). EphB receptors are able to regulate dendritic spine morphology by binding and activating different GEFs such as kalirin-7, Tiam1 and intersectin (Irie and Yamaguchi, 2002; Penzes et al., 2003; Tolias et al., 2007). The activation of kalirin-1 and Tiam1 by EphB receptors induces local activation of Rac and its effector PAK to cause the necessary actin rearrangements required for spine morphogenesis (Penzes et al., 2003; Tolias et al., 2007). At the same time, EphB receptors can also physically bind and activate intersectin-1, which in turn recruits N-WASP that further enhances the GEF activity of interasectin-1 to induce the local activation of Cdc42 (Irie and Yamaguchi, 2002). The recruitment of N-WASP by EphB would result in the formation of branched actin networks mediated by the Arp2/3 complex, which is consistent with the cytoskeletal organization of dendritic spines (Landis and Reese, 1983). Similar to EphB, EphA was also found to recruit Tiam1 to activate Rac1, but this interaction was required for the induction of neurite outgrowth in primary cortical neurons (Tanaka et al., 2004).
2.2.3 Eph repulsion versus adhesion

In general, activation of Eph receptors results in contraction of the cytoskeleton, loss of adhesion and cell-cell repulsion or detachment (Lackmann and Boyd, 2008). However, some in vivo neuronal processes require Eph-ephrin mediated adhesion rather than repulsion, which suggests that the function of Eph signaling can be adjusted to induce adhesion or repulsion (Cooke et al., 2005; Eberhart et al., 2004; Halloran and Wolman, 2006; McLaughlin et al., 2003). It is believed that the strength of the Eph signal determines the repulsion versus adhesion decision, where adhesion signals occur only when the Eph signal is below a certain threshold (Halloran and Wolman, 2006; Lackmann and Boyd, 2008; Shintani et al., 2006). Indeed, there are several mechanisms that have already been identified that can regulate the strength of Eph RTK forward signaling. For example, one such mechanism involves reducing Eph receptor activation through the co-expression of Eph splice variants that lack kinase activity, but can compete in ephrin binding and hence sequester the ligand away from the full length Eph receptor (Holmberg et al., 2000). A second mechanism involves controlling the level of Eph signaling by modulating the concentration of ephrin ligands, where low concentrations of ephrins are known to promote adhesion, while high concentrations cause repulsion (Hansen et al., 2004; Matsuoka et al., 2005; McLaughlin et al., 2003). Finally, Eph repulsive signaling is found to be silenced through the expression of ephrin ligands on the same membrane as the Eph receptor, such that ephrinA expressed in cis can bind to EphA to reduce its sensitivity to ephrin ligands presented in trans (Carvalho et al., 2006; Hornberger et al., 1999; Yin et al., 2004). Nonetheless, taking into account all these mechanisms, it seems
that the bias towards repulsion or adhesion is, in many cases, cell context dependent and the means of their regulation is yet to be understood (Halloran and Wolman, 2006).

2.3 The *C. elegans* VAB-1/Eph RTK and its function in axon guidance

The *C. elegans* VAB-1 represents the only Eph RTK homolog, and was originally named based on its variable abnormal (Vab) phenotype, where *vab-1* animals had notched heads, epidermal tail defects, and variable embryonic and larval lethality (Brenner, 1974). The VAB-1 protein sequence was found to have similarities to both vertebrate EphA and EphB subclasses, and contained all the features of an Eph receptor, including an extracellular region with an N-terminal globular domain, a cysteine rich domain, and two fibronectin type III repeats, while the intracellular domain contained a juxtamembrane domain, a tyrosine kinase, a SAM like motif and a PDZ-binding consensus (George et al., 1998). In terms of its expression, VAB-1 is expressed in neuroblasts during embryogenesis, and in later stages VAB-1 is widely expressed in the nervous system. Initial work addressing the role of VAB-1 in embryogenesis showed that *vab-1* mutants had disorganized neuroblasts, which consequently resulted in embryonic lethality due to the inability of epidermal cells to properly migrate over the disorganized neuroblast substrate (George et al., 1998). As a result, embryos do not enclose properly and usually rupture at the midline, and internal cells leak out from the ventral midline (George et al., 1998). The defects in neural cell organization in *C. elegans* Eph signaling mutants resembles cell sorting defects observed in the vertebrates when Eph signaling is disrupted (Mellitzer et al., 1999). Surprisingly, a *vab-1* mutation that is predicted to abolish the
tyrosine kinase activity, \textit{vab-1(e2)}, displayed only a weak phenotype when compared to a null allele \textit{vab-1(dx31)}, suggesting that the kinase activity is not the only function of the VAB-1 receptor and therefore kinase independent functions must exist, perhaps through reverse signaling by the ephrin ligands (Chin-Sang et al., 1999; George et al., 1998).

Similar to the vertebrate Eph RTKs, VAB-1 is activated by ephrin ligands (Chin-Sang et al., 1999; Wang et al., 1999). The \textit{C. elegans} genome encodes four GPI-linked ephrins (EFN-1 through EFN-4) that share similar features to both vertebrate ephrinA and ephrinB subfamilies (Chin-Sang et al., 1999; Wang et al., 1999). Of the four ephrins, EFN-1, EFN-2 and EFN-3 are closely related and are involved in the VAB-1 signaling pathway during embryogenesis, where it is suggested that these three ephrins might account for all signaling functions of VAB-1 during embryogenesis based on the \textit{efn-1 efn-2 enf-3} triple mutant phenotype (Chin-Sang et al., 1999; Chin-Sang et al., 2002; Wang et al., 1999). On the other hand, EFN-4 has been found to be more divergent and functions in a VAB-1-independent pathway (Chin-Sang et al., 1999; Chin-Sang et al., 2002; Wang et al., 1999). In addition to the EFNs, two other molecules have been identified as VAB-1 ligands. The first molecule is the cell-surface-anchored immunoglobulin superfamily (IgSF) member WRK-1 (\textit{wrapper, rega-1, klingon-like}) (Boulin et al., 2006). WRK-1 is composed of three Ig domains, one FNIII domain and a GPI anchor, and was found to bind to VAB-1 and EFN-1 suggesting the potential formation of a heteromeric complex between all three molecules (Boulin et al., 2006). The second molecule, VPR-1 (\textit{VAP33-related}), is a member of the VAP (VAMP
(synaptobrevin)-associated protein) protein family composed of an N-terminal major sperm protein (MSP) domain, a central coiled-coil motif and a hydrophobic C-terminal transmembrane domain (Tsuda et al., 2008). Although VPR-1 and its homologs are membrane bound, it is proposed that a secreted form of VPR-1 is what binds to the extracellular domain of VAB-1/Eph RTKs (Tsuda et al., 2008).

Beyond its role in embryogenesis, VAB-1 is also found to be required for the proper guidance of various neuronal cell bodies and axon projections. Analysis of vab-1 mutants revealed the presence of neuronal defects that include aberrant axon crossing in the ventral midline, disruption in the ventral guidance of amphid axons, anterior axon outgrowth in the nerve ring, and displacement of the CAN (canal-associated neurons) and PLM (posterior lateral microtubule) cell bodies (Boulin et al., 2006; Hao et al., 2001; Mohamed and Chin-Sang, 2006; Zallen et al., 1999). To further understand the role of VAB-1 in axon guidance, a comparison between the effect of loss-of-function and gain-of-function of VAB-1 on the PLM axons was conducted (Mohamed and Chin-Sang, 2006). While wild-type animals have PLM axons that normally terminate near the middle region (vulva), vab-1 mutants had PLM axons that overextended beyond the normal termination region (Figure 2-3) (Mohamed and Chin-Sang, 2006). In contrast, a constitutively active VAB-1, generated by targeting the intracellular region of VAB-1 to the plasma membrane by N-terminal myristoylation (MYR-VAB-1), caused the PLM axons to terminate prematurely (Figure 2-3) (Mohamed and Chin-Sang, 2006). The combination of these results suggest that the VAB-1 Eph RTK is required for limiting
axons to specific regions, and is probably inducing its effect by transducing a stop signal. However, the identities of the molecules that are involved in the VAB-1 Eph RTK signaling pathway during axon guidance are still unknown.
Figure 2.3: Phenotype of the PLM axons in vab-1(dx31) mutants and MYR-VAB-1 gain-of-function transgenic animals.

A line diagram corresponding to the PLM morphology of each genotype is shown to the left of the gfp panels. PLM axons were visualized with zdIs5 (mec-4::gfp). Arrow indicates the tip of the PLM axon. (A) In adult wild-type animals the PLM axons terminate around the vulva (triangle). (B) vab-1(dx31) animals have PLM axons that overextend past the vulva and the ALM neuron (asterisk). (C) Constitutively active MYR-VAB-1 caused PLM axons to terminate prematurely (opposite to the loss-of-function vab-1 mutant). (Adapted and modified from Mohamed and Chin-Sang (2006)).
2.4 Thesis Objectives and Overview

The objective of this study was to identify and characterize molecules involved in the *C. elegans* VAB-1/Eph RTK signaling pathway for axon guidance. In this work I focused mainly on the posterior lateral microtubule (PLM) mechanosensory neurons, which have stereotypical cell body positions and axon projections, making it ideal for scoring neuronal defects. Other neurons that were briefly analyzed include the hermaphrodite specific motor neurons (HSNs) and command interneurons.

I identified the adaptor protein NCK-1 as an effector of VAB-1/Eph signaling in axon guidance. The Nck protein family are known regulators of the actin cytoskeleton, and have been reported to be an effector for several axon guidance receptors (Buday et al., 2002; Fan et al., 2003; Li et al., 2002; Stein et al., 1998). I provided the first genetic and molecular characterization of the *C. elegans* nck-1, and provide a role for NCK-1 in neuronal development. The findings of this work are presented in Chapter 3 of this thesis.

In addition to NCK-1, I also identified two other actin regulators, WSP-1/N-WASP and UNC-34/Enabled, as molecules associated with VAB-1/Eph signaling in axon guidance. Details of how VAB-1 activates NCK-1, WSP-1 and inhibits UNC-34 to regulate actin dynamics during axon guidance is presented in Chapter 4 of this thesis.

Overall, results from this work present a detailed analysis on the role of the VAB-1/Eph RTK effector NCK-1 in neuronal development, as well as provide an insight into some of the molecular events that allow VAB-1/Eph to elicit its effect on the axon growth cone.
2.5 References


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Chapter 3

The *C. elegans nck-1* gene encodes two isoforms and is required for neuronal guidance
3.1 Abstract

The NCK adaptor proteins are composed entirely of SH3 and SH2 domains and serve as protein interaction bridges for several receptors during signal transduction events. Here we report the molecular and genetic analysis of the *C. elegans nck-1* gene. *C. elegans nck-1* encodes two isoforms: NCK-1A and a shorter isoform that lacks the first SH3 domain, NCK-1B. *C. elegans nck-1* mutants exhibit defects in axon guidance and neuronal cell position, as well as defects in the excretory canal cell, gonad, and male mating. NCK-1 is broadly expressed in neurons and epithelial cells with NCK-1B being the most abundant isoform. NCK-1A and NCK-1B share a similar expression pattern in parts of the nervous system, but also have independent expression patterns in other tissues. Interestingly, NCK-1B is localized to the nuclei of many cells. Genetic rescue experiments show that NCK-1 functions cell autonomously and, in general, either NCK-1A or NCK-1B is sufficient to function in axon guidance. However, there appears to be specific roles for each isoform, for example NCK-1B is required for HSN cell migration while NCK-1A is required for efficient male mating. Genetic epistasis experiments show that NCK-1 functions redundantly with the LAR Receptor Tyrosine Phosphatase, PTP-3, and the Netrin receptor UNC-40.

**Key words:** NCK; axon guidance; cell movement; *C. elegans*; adaptor protein; SH2 domain; SH3 domain, NCK isoforms
3.2 Introduction

Cells can sense signals in the extracellular environment, which are then relayed through the cell to elicit specific cellular responses. The formation of intracellular protein-protein interactions provides an underlying framework through which signal transduction cascades are organized. In many cases, the proteins involved in signal transduction pathways are adaptor proteins that have src homology 2 and 3 (SH2 and SH3) domains (Pawson and Nash, 2003). Adaptor proteins, such as Grb2, Nck and Crk, do not have catalytic activity, and their main function appears to mediate protein-protein complexes at the plasma membrane, or in the cytoplasm, in response to a signal stimulation (Buday, 1999). SH2 domains bind to phosphorylated tyrosine residues, and play a central role in mediating intracellular signaling by receptor tyrosine kinases and cytoplasmic tyrosine kinases (Schlessinger and Lemmon, 2003). On the other hand, the SH3 domains bind to proline rich regions and help provide the protein-protein interactions required for transducing signals inside of the cell (Li, 2005). The NCK (Non-Catalytic region of tyrosine Kinase adaptor protein) family is composed of three SH3 domains followed by a single SH2 domain, and have been implicated in the regulation of the actin cytoskeleton (Buday et al., 2002). Mammals have two Nck genes (Nck1, also known as Nckα, and Nck2, also known as Nckβ or Grb4), which are thought to function redundantly as well as having distinct roles in signal transduction (Chen et al., 1998; Li et al., 2001). In addition, both Nck1 and Nck2 have also been found to be expressed in the
same cells, which makes investigating their individual roles in development difficult (Buday, 1999; Chen et al., 1998).

Here, we report the characterization of the C. elegans nck-1 gene, its products and mutant phenotypes. We show that the nck-1 gene encodes two NCK-1 isoforms. The larger isoform, NCK-1A, is similar to the standard NCK adaptor proteins, having three SH3 domains followed by a single SH2 domain, while the second isoform, NCK-1B, lacks the first SH3 domain. Analysis of the NCK-1 expression pattern revealed that NCK-1 is expressed strongly in the nervous system and various non-neuronal tissues. Loss of function mutations in the nck-1 gene causes defects in axon guidance, neuronal cell migration and male mating. Non-neuronal defects include excretory cell defects as well as smaller gonads with a reduced brood size. Our results suggest that the C. elegans NCK-1 plays a role in axon guidance and neuronal cell positioning, but these roles can be isoform dependent. Both NCK-1 isoforms function in axon guidance, while the shorter NCK-1B has cell migration specific roles, and the larger NCK-1A is involved in male mating.

The C. elegans PTP-3/LAR Receptor tyrosine phosphatase was previously reported to function in embryogenesis and axon guidance (Ackley et al., 2005; Harrington et al., 2002). Here we provide evidence that nck-1 functions in the same pathway with ptp-3 in PLM axon guidance, but functions in parallel pathways with ptp-3 in embryogenesis and AVM cell migrations. We also provide evidence to show that NCK-1 functions in a parallel pathway with the UNC-40 Netrin receptor in AVM/PVM axon guidance.
3.3 Materials and Methods

3.3.1 Genetics and culture conditions

All *C. elegans* strains were manipulated as described by Brenner (Brenner, 1974). All alleles were isolated in the standard wild-type Bristol strain N2. All experiments were performed at 20°C unless otherwise indicated. The following alleles were used in this study: N2 *wild-type* (var. Bristol) (Brenner, 1974); LGI: *unc-40* (e271) (Brenner, 1974), *(zdIs5)* [mec-4::gfp] (Clark and Chiu, 2003); LGII: *ptp-3*(op147) (Harrington et al., 2002); LGIII: *(rhIs4)* [glr-1::gfp] (Lim et al., 1999), *(juIs73)* [unc-25::gfp] (Hallam et al., 2000); LG IV: *(zdIs13)* [tph-1::gfp] (Clark and Chiu, 2003); LGX: *nck-1*(ok694, ok383; tm1645; tm2561) (this study), syDf1 (Ahnn and Fire, 1994); Extrachromosomal arrays (this study): *(quEx134-136; quEx138; quEx190)* [nck-1A-gfp]; *(quEx151; quEx154; quEx181)* [nck-1 genomic]; *(quEx158-161)* [nck-1 genomic::gfp]; *(quEx185–186)* [mec-4::nck-1A]; *(quEx129; quEx193; quEx195-196)* [nck-1B genomic]; *(quEx200)* [mec-4::nck-1B]; *(quEx216)* [daam-1::gfp]; *(quEx275)* [nck-1B-gfp]; *(quEx283–286)* [mec-4::nck-1A-gfp]; *(quEx287-290, quEx314)* [mec-4::nck-1B-gfp]. Unless noted, all *C. elegans* strains were obtained from the *C. elegans* Genetics Stock Center, care of T. Stiernagle (U. of Minnesota), or the Mitani lab (Tokyo Women’s Medical University).

3.3.2 Molecular Biology

*Plasmid constructs*: pIC313 (mec-4::nck-1A::unc-54 3’UTR) was created by PCR using primers for the full-length (1188bp) isoform nck-1A, and the RB2 cDNA as
template (Library a gift from R. Barstead). The *nck-1A* PCR product was cloned into pIC62 (Mohamed and Chin-Sang, 2006) replacing the *myr-vab-1* 1.6kbp fragment behind the *mec-4* promoter. pIC474 (*mec-4::nck-1B::unc-54 3'UTR*) was made by PCR using primers for the full length *nck-1B* cDNA (861bp) in a similar manner to pIC313. pIC666 (*mec-4::nck-1A-gfp::unc-54 3'UTR*) is similar to pIC313, but has a *gfp* sequence fused to the *nck-1A* cDNA. pIC725 (*mec-4::nck-1B-gfp::unc-54 3'UTR*) is similar to pIC474, but has a *gfp* sequence fused to the *nck-1B* cDNA.

**PCR products:** The *nck-1A-GFP* translational reporter was created by a PCR fusion approach (Hobert, 2002) with four different fragments: 1. a ~2kbp region upstream of the ATG start sequence of *nck-1A* amplified from wild-type genomic DNA, 2. a 1188bp *nck-1A* cDNA fragment using RB2 cDNA library as template, 3. a 868bp *GFP* fragment amplified from pPD95.75 (Dr. Andy Fire’s Lab) and 4. a 774bp *nck-1* 3’UTR fragment amplified from wild-type genomic DNA. The *nck-1B-GFP* reporter was created using the same PCR fusion approach, with four fragments: 1. a 1929 bp upstream of the *nck-1b* ATG (corresponding to the second intron and exon 3 of *nck-1a*), 2. a 861bp *nck-1B* isoform amplified from RB2 cDNA library, 3. a 868bp *GFP* fragment amplified from pPD95.75 (Dr. Andy Fire’s Lab) and 4. a 774bp *nck-1* 3’UTR fragment amplified from genomic DNA. The *nck-1 genomic* rescuing fragment was generated by PCR fusion of two fragments from wild type animals: 1. a ~2kbp region upstream of the ATG start sequence of *nck-1* and 2. a ~5.2kbp *nck-1* fragment including 3’UTR. The *nck-1 genomic::gfp* reporter was generated in a similar manner to the *nck-1 genomic* fragment,
except that it had a GFP fragment fused in frame followed by the nck-1 3’UTR. The nck-1B(genomic) fragment was created by PCR fusion using the same region of the nck-1(genomic above), but included two site directed point mutations to change tryptophan amino acids W37 W38 to stop codons. Details of plasmid/PCR products and primer sequences are available upon request.

3.3.3 Transgenic animals

Transgenic animals were generated by germ-line transformation as previously described (Mello et al., 1991). The PCR product nck-1A-GFP translational GFP reporter was injected at 30 ng/μL into N2 (quEx134-136), zdIs5; nck-1(ok694) (quEx138) and zdIs13; nck-1(ok694) (quEx190). quEx138 was later crossed into zdIs13; nck-1(ok694). The nck-1 genomic::gfp PCR fragment was injected into N2 at 30 ng/μL (quEx158-161). The nck-1 (genomic) PCR rescuing fragment was injected at 15 ng/μL into N2 (quEx154), nck-1(ok694) (quEx181), zdIs5; nck-1(ok694) (quEx151) animals. quEx154 was later crossed into zdIs13; nck-1(ok694). The nck-1B(genomic) PCR rescuing fragment was injected at 30 ng/μL into nck-1(ok694) (quEx129), zdIs5; nck-1(ok694) (quEx195 – 196) and zdIs13; nck-1(ok694) (quEx193). The nck-1B-GFP reporter was injected at 15ng/μL into N2 (quEx275). The pIC313 (mec-4::nck-1A) construct was injected at 30 ng/μL into zdIs5; nck-1(ok694) (quEx185 – 186). The pIC474 (mec-4::nck-1B) construct was injected at 30 ng/μL into zdIs5; nck-1(ok694) (quEx200). The pIC666 (mec-4::nck-1A-gfp) construct was injected at 30 ng/μL into N2 animals (quEx283-286). The pIC667 (mec-4::nck-1B-gfp without IQ) and pIC726 (mec-4::nck-1B-gfp with IQ) constructs were
injected at 30 ng/μL into N2 (quEx287-290, and quEx314 respectively). Transgenic animals were identified by the co-injection marker pRF4/rol-6 (30 ng/μl) (Mello et al., 1991) or odr-1::rfp (30 ng/μl) (L'Etoile and Bargmann, 2000).

### 3.3.4 Immunoblotting

Whole worm protein lysates were prepared in standard SDS-PAGE sample buffer. Samples were analyzed by SDS-PAGE followed by immunoblotting. NCK-1 was detected with Rabbit anti-NCK-1 antibodies (antigen GST-NCK-1 produced by Covance Inc.) at 1:500 dilutions, and secondary HRP-anti-rabbit antibodies (Upstate).

### 3.3.5 RT-PCR

RNA was extracted using the Roche High Pure RNA isolation kit according to the manufacturer’s protocol. A 100μl of mixed stage worm pellet was used for each strain. Reverse transcription was carried out using the New England Biolabs Protoscript RT-PCR kit. The region 1-813 nt was used for nck-1 cDNA amplification followed by direct sequencing of the PCR products. Relative band intensities of RT-PCR product were calculated using the NIH Image J program.

### 3.3.6 Phenotypic analysis

Neurons were visualized using cell-specific gfp reporters. The mechanosensory neurons were visualized using the mec-4::gfp (zdIs5) reporter (Clark and Chiu, 2003), and PLM axon overextension defects were quantified as described previously (Mohamed and Chin-Sang, 2006). The AVM was scored as posteriorly displaced if it was located
posterior to the vulva. The AVM and PVM axons were scored as having ventral guidance defects if they migrated at least 2 µm laterally before migrating ventrally or not migrating ventrally at all. The HSN neurons were visualized using the \textit{tph-1::gfp(zdIs13)} reporter (Clark and Chiu, 2003), and scored as having a midline crossover defect if the HSNL or HSNR migrated along the wrong path. HSN cell bodies were considered posteriorly displaced if they were greater than 25µm past the vulva. The command interneurons were visualized using the \textit{glr-1::gfp(rhIs4)} reporter (Lim et al., 1999), and defects were scored if any axons were found on the left side of the ventral midline. L4 animals were picked and allowed to develop to young adults (less than12 hours), and scored for neuronal defects. The wild-type morphology for all neurons was defined by analysis of neuronal GFP reporters, which are consistent with the electron microscopic reconstruction of the \textit{C. elegans} nervous system (White et al., 1986) and immunocytochemical staining of wild-type animals (Desai et al., 1988; McIntire et al., 1992). Animals were anesthetized using 0.2% tricaine and 0.02% tetramisole in M9 buffer.

The excretory canal cell was visualized using a \textit{daam-1::gfp} reporter construct (Chin-Sang lab unpublished).

To analyze the gonad arms diameter, young adults (less than 12 hours) were dissected in M9 buffer to extract the gonad arms. The gonad arm width was measured 55µm from the distal tip cell. Animals or dissected gonads were mounted on 3% agarose pads and analyzed using a Zeiss Axioplan microscope, Axiocam and Axiovision software unless otherwise noted.
Brood size and lethality were quantified by placing L4 hermaphrodites on individual plates at 20°C and transferring to a fresh plate every 8-16 hours until they ceased to lay eggs. The number of L1s and eggs on the plate from the previous day were counted, and an embryo was scored as being dead if it did not hatch after 24 hours.

Male mating efficiency was tested using the quantitative mating test described by Jonathan Hodgkin, (Hodgkin, 1983). We used unc-34(e566) hermaphrodites in our mating tests, and used the same mating efficiency scale used by Hodgkin (1983) [4 = very efficient mating (30 – 100% of wild-type); 3 = efficient mating (10 – 30% of wild-type); 2 = poor mating (1 – 10% of wild-type); 1 = very poor mating (less than 1% of wild-type); 0 = no mating] (Hodgkin, 1983). Since nck-1 is on the X chromosome, nck-1 males were generated by crossing wild-type males into nck-1 hermaphrodites and picking male cross progeny.
3.4 Results

3.4.1 The *C. elegans* genome has a single *nck-1* gene that encodes for two isoforms

The larger NCK-1 isoform in *C. elegans*, NCK-1A, is most similar to the human NCK2 (58% similarity; 42% identity) and *Drosophila* DOCK (53% similarity; 37% identity) (Figure 3-1A). NCK-1A has all the domain features of the NCK2 and Dock adaptor proteins, which are three SH3 domains followed by a single SH2 domain (Figure 3-2A). Expressed sequence tags (ESTs) revealed the presence of a second isoform, NCK-1B (www.wormbase.org and AceView). Both NCK-1 isoforms are detectable by immunoblot analysis (Figure 3-2E). The longer NCK-1A isoform, contains either 395 or 397 amino acids, while the shorter isoform, designated NCK-1B, contains either 284 or 286 amino acids (Figure 3-2). The two extra amino acids are caused by alternative splicing, as the *nck-1* genomic sequence has an alternate splice acceptor for intron 4, which would result in the two extra amino acids (IQ) (Figure 3-2D). In our study, plasmid constructs used for NCK-1A or NCK-1B rescue analysis were derived from the IQ splice forms. Transgenic animals created by injecting PCR products amplified from genomic DNA or cDNA library produced a mixture of the IQ (with and without) NCK-1 splice forms (see methods).

An NCK-1B protein BLAST search against the vertebrates revealed the presence of similar Nck isoforms with two SH3 domains followed by a single SH2 domain in several organisms including human, mouse, rat, dog and zebra fish (Figure 3-1B, not shown). Alignment analysis shows that the *C. elegans* NCK-1B isoform is most similar to
C. elegans: MGED---------VVLYIYLAERGIELIARYKRGREIKLLYKIDNDNEKDNMDG
D. melanogaster: MARGNDNKHGSDQVCCIVEYATRRQGGELELDRENREKLLLYKIDNDNEKDNMDG
H. sapiens: MTEDB----------TVAAARCTYQQPDELTKRIRLLEKNDNMDG
* * * * * * * * * * ; * * * * * * * * * ; * * * * * * * * * ; * * * * * * * * * ;
C. elegans: VESNVRKSE----STVDKCGSTK--------GLARGNRSRS--DPEPERLAVIRL
D. melanogaster: VESNVRKSE----STVDKCGSTK--------GLARGNRSRS--DPEPERLAVIRL
H. sapiens: VESNVRKSE----STVDKCGSTK--------GLARGNRSRS--DPEPERLAVIRL
* * * * * * * * * * ; * * * * * * * * * ;
C. elegans: AFSLLSINCATVPSNKNIKMNASKTSVKDVKVQYQYTVDSGDNRK
D. melanogaster: AFSLLSINCATVPSNKNIKMNASKTSVKDVKVQYQYTVDSGDNRK
H. sapiens: AFSLLSINCATVPSNKNIKMNASKTSVKDVKVQYQYTVDSGDNRK
* * * * * * * * * * ; * * * * * * * * * ; * * * * * * * * * ;
C. elegans: GEAGPMQKGGEPNSQLENVEGSTNSQSISDITRIIYNKNAAFAPMDNK--APKPKQASRS
D. melanogaster: GEAGPMQKGGEPNSQLENVEGSTNSQSISDITRIIYNKNAAFAPMDNK--APKPKQASRS
H. sapiens: GEAGPMQKGGEPNSQLENVEGSTNSQSISDITRIIYNKNAAFAPMDNK--APKPKQASRS
* * * * * * * * * * ; * * * * * * * * * ;
C. elegans: FFVVPAMYFDASSEEKFKEKSGELEIVDDFFAEDDIANNASNRTGTILFVQNPYIDV
D. melanogaster: FFVVPAMYFDASSEEKFKEKSGELEIVDDFFAEDDIANNASNRTGTILFVQNPYIDV
H. sapiens: FFVVPAMYFDASSEEKFKEKSGELEIVDDFFAEDDIANNASNRTGTILFVQNPYIDV
* * * * * * * * * * ; * * * * * * * * * ;
C. elegans: NDSSSDSSKAHODQFAOSYOQENG---PMQPQ
D. melanogaster: NDSSSDSSKAHODQFAOSYOQENG---PMQPQ
H. sapiens: NDSSSDSSKAHODQFAOSYOQENG---PMQPQ
* * * * * * * * * * ; * * * * * * * * * ;
C. elegans: ---QFWYFRIGRLVELEDDL-LHHHQQEELTVESEESOOQDELLSHEIHDNHEKRYQOV
D. melanogaster: ---QFWYFRIGRLVELEDDL-LHHHQQEELTVESEESOOQDELLSHEIHDNHEKRYQOV
H. sapiens: ---QFWYFRIGRLVELEDDL-LHHHQQEELTVESEESOOQDELLSHEIHDNHEKRYQOV
* * * * * * * * * * ; * * * * * * * * * ;
C. elegans: DGLKLXGRTFQVXLNDITTVISSPTT-EKLXISLFLK------
D. melanogaster: DGLKLXGRTFQVXLNDITTVISSPTT-EKLXISLFLK------
H. sapiens: DGLKLXGRTFQVXLNDITTVISSPTT-EKLXISLFLK------
* * * * * * * * * * ; * * * * * * * * * ;
C. elegans: NYDCTGQCFDRHFLDVLQDHYQRAFETYNNQGKELLYVLVPSLKAQ--
D. melanogaster: NYDCTGQCFDRHFLDVLQDHYQRAFETYNNQGKELLYVLVPSLKAQ--
H. sapiens: NYDCTGQCFDRHFLDVLQDHYQRAFETYNNQGKELLYVLVPSLKAQ--
* * * * * * * * * * ; * * * * * * * * * ;

B
C. elegans: MWS------------------------
H. sapiens: M------------------------
D. rerio: MIGTSHDQREGYSNSEMNDEDELPHNTTIPSITQGAEQEVTVTSLPQITALSASFG
* * * * * * * * * * ;
C. elegans: -------------SKTK--
H. sapiens: -------------SKTK--
D. rerio: -------------SKTK--
* * * * * * * * * * ;
C. elegans: AVAXAVYEBELEDGULTRGDFYVVEKSTODWGKREAPMGGNSWSNFY
H. sapiens: AVAXAVYEBELEDGULTRGDFYVVEKSTODWGKREAPMGGNSWSNFY
D. rerio: AVAXAVYEBELEDGULTRGDFYVVEKSTODWGKREAPMGGNSWSNFY
* * * * * * * * * * ;
C. elegans: VEEVAASTNGQCSHIENVFAAAAPFASIMQYAEKFKQASRSAPVYPPASSE
H. sapiens: VEEVAASTNGQCSHIENVFAAAAPFASIMQYAEKFKQASRSAPVYPPASSE
D. rerio: VEEVAASTNGQCSHIENVFAAAAPFASIMQYAEKFKQASRSAPVYPPASSE
* * * * * * * * * * ;
C. elegans: ELNFKGRKELIVCDHEBDIRIDNSSGTSGLVPRNXIYVWNDE----SSKASHQ
H. sapiens: ELNFKGRKELIVCDHEBDIRIDNSSGTSGLVPRNXIYVWNDE----SSKASHQ
D. rerio: ELNFKGRKELIVCDHEBDIRIDNSSGTSGLVPRNXIYVWNDE----SSKASHQ
* * * * * * * * * * ;
C. elegans: DFAPFQRSGINERQEQWYFRIGRLVEELRDLSEDSPPGGSIKSRG
H. sapiens: DFAPFQRSGINERQEQWYFRIGRLVEELRDLSEDSPPGGSIKSRG
D. rerio: DFAPFQRSGINERQEQWYFRIGRLVEELRDLSEDSPPGGSIKSRG
* * * * * * * * * * ;
C. elegans: IERNHHKHEQVYQGK YEKSTIFMELETVHYYKAEIPTSEQQGKLYWHL-S
D. rerio: IERNHHKHEQVYQGK YEKSTIFMELETVHYYKAEIPTSEQQGKLYWHL-S
* * * * * * * * * * ;
**Figure 3-1: Sequence alignment of \textit{C. elegans} NCK-1.**

(A) Alignment of the \textit{C. elegans} NCK-1A sequence with the \textit{D. melanogaster} DOCK and Human NCK2. (B) Alignment of the \textit{C. elegans} NCK-1B sequence with its homologs from humans (Genbank: AK301460) and zebrafish \textit{D. rerio} (Genbank: XM_687914). Alignment was obtained using the MAFFT (v5.860) (Katoh et al., 2002) multiple sequence alignment program. \textit{Dashes} denote gaps, and amino acid conservations are denoted by \textit{asterisks}, whereas \textit{colons} and \textit{periods} represent a high and low degree of similarity, respectively.
Figure 3-2: Structure and sequence analysis of the *C. elegans* NCK-1.

(A) Sequence of the *C. elegans* NCK-1A protein. The NCK-1A isoform represents the official NCK-1 adaptor form, with three SH3 domains (Italics underlined) and single SH2 domain (Bold underlined). (B) Sequence of the NCK-1B protein. The NCK-1B isoform lacks the first SH3 domain. (C) Structure of the *nck-1* genomic region and location of the *ok694* deletion. The *nck-1* transcripts code for two NCK-1 isoforms: NCK-1A and NCK-1B. Both transcripts have an SL1 splice site sequence. Sequence analysis of *ok694* mRNA shows exon 2 splicing into a cryptic splice acceptor located 19nt after the deletion in exon 4. (D) Both isoforms can carry or lack two amino acids (I, Q in red) due to alternative splice acceptors. (E) Immunoblot of wild-type (WT) animals shows the presence of two NCK-1 isoforms. The *nck-1(ok694)* allele does not produce either NCK-1 isoform, consistent with a null allele. The NCK-1A and NCK-1B isoforms can be selectively expressed in the *nck-1(ok694)* allele background. The arrows point at either the NCK-1A, NCK-1B or NCK-1A-GFP bands. (F) *nck-1(ok694)* produces a transcript smaller in size and lower in levels than wild-type *nck-1* (top). Total RNA levels and 28s and 18s rRNA subunits (bottom).
an Nck isoform in *H. sapiens* (57% similarity; 41% identity), and a predicted Nck isoform in *D. rerio* (zebra fish) (56% similarity; 40% identity) (Figure 3-1B).

The *C. elegans* nck-1 gene is likely to be regulated as a two promoter system because it is SL1 (spliced leader 1) spliced at two locations: one at the beginning of nck-1A and one at an internal SL1 acceptor before exon-3. The second intron in nck-1 is significantly longer (1785bp) than the average intron length (321bp), and suggests that this intron could act as a promoter for the nck-1B transcript as has been described for other genes in *C. elegans* (Choi and Newman, 2006; Choi et al., 2006). Taken together, these characteristics suggest that the shorter NCK-1B isoform is generated through an independent internal promoter, rather than alternative splicing or a post translational processing such as protein cleavage.

### 3.4.2 The NCK-1 isoforms have overlapping as well as independent expression patterns

To examine the nck-1 expression pattern, we generated a functional translational reporter in which a genomic nck-1 DNA, including a ~2kb promoter sequence was fused in frame to a GFP sequence, followed by the nck-1 3’UTR. In transgenic embryos, nck-1 genomic::gfp was expressed in many tissues including epidermal cells, nervous system, intestine and pharynx (Figure 3-3A, B). In the adults, GFP was observed in the intestine, pharynx, vulva, uterus, head neurons, ventral nerve cord, excretory canal cell, seam cells, gonads, ray sub lineages and the adult male tail (Figure 3-3C, not shown). As mentioned above, it is likely that the NCK-1B isoform is produced through an internal promoter. To
Figure 3-3: Expression pattern of NCK-1A.

(A-C) General NCK-1 expression pattern (Both isoforms). In embryos, NCK-1 expression is detected in the hypodermis (A), intestine, nervous system and pharynx (B). In adult animals, NCK-1 expression was observed in various cells such as the intestine, pharynx, excretory canal, vulva (arrow head), nervous system, gonads and male tail (C, and not shown). (D-L) NCK1-A-GFP specific expression: In embryos NCK-1A is expressed in neuroblasts in the anterior, lateral and posterior regions of the embryo (D). NCK-1A is also expressed in the hypodermal cells of the developing embryo (E). During the Larval stages, animals show NCK-1A expression in the head neurons, dorsal nerve cord (DNC), motor neurons in the ventral nerve cord and some tail neurons (F). In adult animals (G-J) NCK-1A is expressed in the VC neurons, commissure neurons, head and tail neurons (G, not shown), SDQ neurons, mechanosensory neurons (PLM, PVM, and AVM) (H, not shown), CAN neurons, HSN neurons, uterine-seam cell (utse), spermathecal-uterine junction (sujn) (I), and excretory canal cell (J). NCK-1A is also expressed in uv2 and uv3 cells of the developing uterus (K). In males, NCK-1A is expressed in all the ray sub lineages and the adult male tail (L). In panels D-L, yellow labels indicate shared expression patterns between NCK-1A and NCK-1B, while white labels indicate expression patterns exclusive to the NCK-1A isoform. Scale bars = 20μm in all panels.
test this, we generated transgenic animals expressing GFP translation reporters that were either regulated by the predicted NCK-1A promoter or the NCK-1B internal promoter (see materials and methods). Analysis of the expression patterns revealed that NCK-1A and NCK-1B have similar expression patterns, but they are also expressed independently in different tissues. Both isoforms were expressed in head neurons, ventral and dorsal nerve cords, CANs, HSNs, the Q neuroblast descendants SDQs, mechanosensory neurons, hypodermal cells and the spermathecal-uterine junction (Figure 3-3; Figure 3-4, yellow labels). The NCK-1A isoform was expressed exclusively in the motor neurons and VC neuronal cell bodies, the excretory canal cell, uterus (utse, uv2 and uv3 cells), the ray sub lineage and male tail (Figure 3-3D-L, white labels, not shown). On the other hand, the NCK-1B isoform was expressed exclusively in the vulva cells of the developing vulva, pharynx, intestine, distal tip cells (DTCs), gonad arms and spermatheca (Figure 3-4A-F, white labels). It is interesting to note that the overlapping expression pattern between NCK-1A and NCK-1B occurred mainly in the nervous system. We also noticed a difference in sub-cellular localization between NCK-1A and NCK-1B. The NCK-1B isoform was localized to the cytoplasm and nucleus, while NCK-1A was predominantly localized to the cytoplasm. To further confirm that this difference in localization was inherent to the NCK-1B isoform, we expressed both isoforms under the mec-4 promoter to visualize their localization in the mechanosensory neurons. We found that NCK-1B was still able to localize to the cytoplasm and nucleus of the cell, while NCK-1A was mostly localized to the cytoplasm (Figure 3-4G, H). Although NCK-1A expression was
Figure 3-4: NCK-1B expression pattern.

In embryos, NCK-1B-GFP is expressed in the intestine, nervous system, epidermal cells and pharynx (A, not shown). (B-D) In adult animals, NCK-1B-GFP is expressed in the nerve ring, amphid neurons, ventral nerve cord (VNC) and dorsal nerve cord (DNC), SDQ neurons, mechanosensory neurons (PVM and AVM), as well as in the CAN and HSN neurons (not shown). (E-F) NCK-1B is also expressed in non-neuronal cells. (E) NCK-1B is expressed in vulB1, vulB2, vulA, vulF and vulD cells, as well as in the leading edge (arrows) of the migrating vulva cells. The upper panel is a DIC image of a L4 developing vulva. (F) NCK-1B is expressed in distal tip cells (DTC), gonad arms, spermatheca and the spermathecal-uterine junction (sujn). In all panels, yellow labels indicate shared expression patterns between NCK-1A and NCK-1B, while white labels indicate expression patterns exclusive to the NCK-1B isoform. For panels B-D, and F, mosaic animals that lost NCK-1B-GFP in the E cell lineage were used to observe the various cells, as the strong GFP expression from the intestine and pharynx made it difficult to observe them. (G-H) The NCK-1B is localized to the nucleus. We expressed either NCK-1A-GFP (G) or NCK-1B-GFP (H) under the control of the mec-4 promoter (touch neurons, PLM shown). NCK-1B-GFP displayed strong nuclear localization. Images in panels G and H were taken using a Zeiss LSM710 confocal microscope, while all other panels were taken using a Zeiss Axioplan microscope. Scale bars = 20μm in all panels.
primarily found in the cytoplasm, we cannot eliminate the possibility of low levels of NCK-1A in the nucleus. Since the only difference between NCK-1A and NCK-1B is the absence of the first SH3 domain in NCK-1B, this suggests that the first SH3 domain might be required to keep NCK-1A out of the nucleus.

In summary, we found that the NCK-1A and NCK-1B isoforms have overlapping as well as independent expression patterns, and that their combined expression pattern is similar to that of the nck-1 genomic translational reporter. Furthermore, we found that NCK-1B, but not NCK-1A, is often abundant in nuclei.

### 3.4.3 nck-1(ok694) likely encodes a null allele

To determine a developmental role for nck-1 we characterized a putative null allele ok694. The nck-1(ok694) allele is a deletion of 1814 bp, and eliminates exon 3 and almost all of exon 4 (Figure 3-2C). We provide three lines of evidence indicating that the ok694 deletion allele is likely a null allele. First, analysis of the mRNA sequence of ok694, produced from RT-PCR, showed that the end of exon 2 is spliced into a region located 19nt after the deleted lesion in exon 4 (Figure 3-2C, 3-2F). This causes a frame shift in the coding sequence leading to two stop codons located 24nt from the cryptic splice acceptor in exon 4. Consequently, the ok694 deletion results in the absence of any NCK-1 protein C-terminal of the first SH3 domain. Furthermore, after normalizing the mRNA levels of wild-type and ok694 to that of total RNA used, we found that the level of nck-1(ok694) RT-PCR produced was approximately 17% of the wild-type level (Figure 3-2F). The lower level of ok694 mRNA is most likely due to nonsense-mediated mRNA
decay (NMD), which degrades mRNAs with premature termination codons, and frame shifts, in *C. elegans* and other eukaryotes (Chang et al., 2007; Longman et al., 2007; Pulak and Anderson, 1993). Second, genetic data showed that the number of PLM axon defects observed in homozygous *ok694* was not different from that of *ok694* over the deficiency strain *syDf1* (Table 3-1). Finally, immunoblots showed that both NCK-1 protein isoforms are absent, which is consistent with *nck-1(ok694)* encoding a molecular null (Figure 3-2E).

Other *nck-1* deletion alleles include *ok383* and *tm1645*, both of which are deletions in introns, are not null alleles since immunoblots show that the NCK-1 proteins are still made (data not shown). A fourth allele *nck-1(tm2561)* has a small (186bp) deletion in part of exon 5 and no protein was detected on immunoblots (data not shown). Since *nck-1(ok694)* has the largest deletion and our analysis suggests that it is likely to be a null allele, we carried out most of our genetic analysis using the *nck-1(ok694)* allele.

### 3.4.4 nck-1 mutants display axon guidance and neuronal cell migration defects

Previous work on the mouse and *Drosophila* Nck/Dock proteins focused on its role in nervous system development (Fawcett et al., 2007; Garrity et al., 1996). Based on the expression pattern of *C. elegans* NCK-1, and that the Nck proteins have roles in axon guidance in other organisms, we chose first to investigate the role of NCK-1 in the *C. elegans* nervous system. To examine several classes of neurons, we used *C. elegans* strains carrying neuron-specific *gfp* reporters. The *mec-4::gfp* reporter was used to visualize the mechanosensory neurons (Clark and Chiu, 2003). The mechanosensory
neurons are composed of six neurons, a pair of ALMs (Right/Left) and AVM in the anterior region, and a pair of PLMs (Right/Left) and PVM in the posterior region (Chalfie and Sulston, 1981). The AVM and PVM, located laterally, are descendants of the QR and QL neuroblasts respectively, and migrate anteriorly and posteriorly respectively (Chalfie and Sulston, 1981). Once in their final position, the AVM and PVM cells extend axons that migrate ventrally from their cell bodies to the ventral cord (Chalfie and Sulston, 1981). The PLM neuron cell bodies are located in the tail region and send axons anteriorly that terminate near the centre of the animal (Figure 3-5A). In nck-1(ok694) mutants, all six mechanosensory neurons were located in their proper position, and most of the axons migrated in their proper path. The PLM axons, however, overextended towards the anterior (Figure 3-5A, Table 3-1).

To examine the Hermaphrodite Specific Neurons (HSNs) we used the tph-1::gfp reporter (Clark and Chiu, 2003). In wild-type animals, the HSN neuronal cells migrate anteriorly from the tail until they reach the vulva, and extend an axon ventrally to the midline and then anteriorly to the head without crossing the midline (Sulston et al., 1983; White et al., 1986) (Figure 3-5B). Two types of HSN defects were observed in nck-1 mutants. The first was in the HSN cell positioning, whereby nck-1 (ok694) animals had HSN cells that did not migrate at all, or they stopped before reaching their normal position near the vulva (Figure 3-5B, Table 3-1). The second defect was an HSN axon guidance defect, where HSN axons inappropriately crossed the ventral midline (Figure 3-5B, Table 3-1).
Figure 3-5: nck-1 is required for proper axon guidance.

(A–C) Neuron specific gfp reporters reveal the role of nck-1 in neuronal development. A line diagram corresponding to the morphology of each neuron class is shown below the gfp panels. (A) In adult wild-type (WT) animals the PLM axons, visualized with zdIs5 (mec-4::gfp), terminate around the vulva (triangle). nck-1(ok694) animals (bottom gfp panel) have PLM axons that overshoot past the vulva and ALM neuron (asterisk). Arrow indicates the tip of the PLM axon. (B) nck-1 (ok694) mutants have HSN axons, visualized with zdIs13 (tph-1::gfp), that inappropriately cross the midline (arrow head; middle gfp panel). nck-1 mutants also have HSN cell bodies that fail to migrate anteriorly and remain in the tail region (arrow, bottom gfp panel). (C) In wild-type animals, the command interneuron axons, visualized with rhIs4 (glr-1::gfp), are only found on the right side of the midline. nck-1 mutants have command interneuron axons that cross the midline and migrate along the left side of the animal (arrow head).
### Table 3-1: *nck-1* neuronal defects

<table>
<thead>
<tr>
<th>Axons Scored</th>
<th>Genotype</th>
<th>% Defects</th>
<th>N&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLML/R overextension (zdIs5)</td>
<td>Wild-type</td>
<td>3</td>
<td>200</td>
</tr>
<tr>
<td></td>
<td><em>nck-1</em>(ok694)</td>
<td>16***</td>
<td>556</td>
</tr>
<tr>
<td></td>
<td><em>nck-1</em>(ok694)/syDf1</td>
<td>17**</td>
<td>148</td>
</tr>
<tr>
<td></td>
<td><em>nck-1</em>(tm2561)</td>
<td>6*</td>
<td>200</td>
</tr>
<tr>
<td></td>
<td><em>ptp-3</em>(op147)</td>
<td>6*</td>
<td>210</td>
</tr>
<tr>
<td></td>
<td><em>ptp-3</em>(op147);<em>nck-1</em>(ok694)</td>
<td>16**</td>
<td>280</td>
</tr>
<tr>
<td>HSNL/R midline axon crossover (zdIs13)</td>
<td>Wild-type</td>
<td>6</td>
<td>140</td>
</tr>
<tr>
<td></td>
<td><em>nck-1</em>(ok694)</td>
<td>32**</td>
<td>105</td>
</tr>
<tr>
<td>HSNL/R posterior cell body displacement (zdIs13)</td>
<td>Wild-type</td>
<td>6</td>
<td>140</td>
</tr>
<tr>
<td></td>
<td><em>nck-1</em>(ok694)</td>
<td>23***</td>
<td>105</td>
</tr>
<tr>
<td>Command interneurons midline axon crossover (rhIs4)</td>
<td>Wild-type</td>
<td>4</td>
<td>105</td>
</tr>
<tr>
<td></td>
<td><em>nck-1</em>(ok694)</td>
<td>20**</td>
<td>184</td>
</tr>
<tr>
<td></td>
<td><em>nck-1</em>(tm2561)</td>
<td>18***</td>
<td>85</td>
</tr>
</tbody>
</table>

<sup>a</sup> The N values refers to the number of animals scored, except for the PLM overextension defects where it refers to the number of PLM axons scored.

*P < 0.05, **P < 0.01, *** P < 0.001 (Student’s t-test) indicates significantly higher than wild-type.
To examine other neurons in the ventral nerve cord we looked at the command interneurons using a \textit{glr-1::gfp} reporter (Lim et al., 1999; Maricq et al., 1995). In wild-type animals, the command interneurons project axons along the right side of the ventral nerve cord (Figure 3-5C) (Hart et al., 1995; Maricq et al., 1995). In \textit{nck-1} mutants, the command interneuron had axons that inappropriately crossed into the left side of the ventral nerve cord (Figure 3-5C, Table 3-1). We also examined the GABA (\textit{γ}-aminobutyric acid) DD/VD neurons (using an \textit{unc-25::gfp} reporter), but found that these neurons were not significantly affected by the \textit{nck-1} mutation (not shown).

In summary, our results indicate that NCK-1 is required in a signaling pathway(s) that guide axons of the PLMs, HSNs and command interneurons. In addition, NCK-1 is required for progression of the HSN cells to their normal location.

\textbf{3.4.5 \textit{nck-1} mutants display non-neuronal defects}

Since NCK-1 is expressed in the excretory canal cell, we decided to analyze the excretory canal cell in the \textit{nck-1} mutant background. The excretory canal cell is a large H-shaped ectodermal cell that regulates the osmolarity of the animal (Hedgecock et al., 1987). In adult wild-type animals, the excretory canal extends from the anterior tip of the animal to the tip of the tail, and has a posterior diameter of approximately 2\textmu{}m (Buechner et al., 1999). To visualize the excretory canal we used a \textit{daam-1::gfp} transcriptional reporter construct (Chin-Sang Lab, unpublished). The \textit{nck-1} mutant animals had excretory canal cells that generally looked wild-type. The only exception was the presence of a low frequency (5\%) of \textit{nck-1} mutants that had branched excretory canals (Figure 3-6A).
Figure 3-6: *nck-1* is required in excretory canal cell and gonad development.

(A) Photomicrograph shows a lateral view of the canal excretory cell visualized by a *daam-1::gfp* reporter. Wild-type excretory canals run as a single tube along each side of the animal, while some branching of the tube is seen in *nck-1 (ok694)* animals (arrow). (B) DIC image of dissected gonads, with the distal tip cell to the left. The *nck-1(ok694)* and *tm2561* mutants have gonad arms that are on average ~7μm smaller than the wild-type. *P < 0.05* (Student’s t-test) indicates significantly higher than wild-type. n.s. refers to not statistically significant. ‘N’ refers to number of animals scored, and scale bars = 20 μm in all panels.

<table>
<thead>
<tr>
<th>Genotype (N)</th>
<th>Branching</th>
<th>Posterior truncation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type (140)</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>*nck-1(ok694) (105)</td>
<td>5%*</td>
<td>2% n.s.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Genotype (N)</th>
<th>Average gonad width (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type (31)</td>
<td>30.3 ± 3.5</td>
</tr>
<tr>
<td>*nck-1(ok694) (33)</td>
<td>22.5 ± 2.4*</td>
</tr>
<tr>
<td>*nck-1(tm2561) (31)</td>
<td>24.1 ± 3.9*</td>
</tr>
</tbody>
</table>
To our knowledge, this is the first report of this type of defect in the excretory canal cell and suggests that NCK-1 has a role in preventing branching during excretory canal development. *nck-1* mutants have been reported to show a weak truncated excretory canal cell defect (Schmidt et al., 2009), however we did not observe this defect at a level that is significantly different from wild-type (Figure 3-6A).

While injecting DNA into the *nck-1(ok694)* animals, we noticed that the gonad arms seemed narrower than the wild-type animals. To confirm this, we dissected and measured the width of the gonad arms of wild-type and *nck-1* animals. We found that *nck-1(ok694)* and *nck-1(tm2561)* mutants had gonads that were significantly narrower than the wild-type animals (Figure 3-6B), suggesting that NCK-1 could either be involved in regulating the diameter of the gonad arms directly, or indirectly such as through osmoregulation.

During the construction of *nck-1* strains we found that *nck-1(ok694)* males do not mate well and had a mating efficiency score of 1 (1= poor mating <1% of wild-type) (Hodgkin, 1983). Finally, we found that *nck-1* animals had a lower brood size than wild-type animals and also exhibited some larval lethality (3.6% ± 2.1), suggesting that NCK-1 has a minor role in early development (Table 3-2).

In summary, our results suggest that NCK-1 might be required in excretory canal, gonad arms and larval development as well as in male mating efficiency.
3.4.6 NCK-1A and NCK-1B act redundantly but also have independent functions

To show that NCK-1 is required for proper PLM axon guidance, we rescued the nck-1(ok694) PLM defects by expressing a genomic nck-1 fragment (Table 3-3). Since the nck-1 gene codes for two different isoforms, we wanted to determine if there is a functional difference between the two isoforms in neuronal development. To address this, we performed isoform specific rescue experiments on nck-1(ok694) neuronal defects. A gfp-tagged nck-1A cDNA under the control of its own promoter was able to rescue the PLM overextension defect (Table 3-3). Similarly, expressing nck-1A cDNA under the mec-4 promoter, fully rescued the PLM axon overextension defect (Table 3-3). This shows that NCK-1A can function cell autonomously in the mechanosensory neurons. Furthermore, we found that expressing NCK-1A from its own promoter can partially suppress the HSN midline crossover defect (32% reduced to 20% and 13%) (Table 3-3). However, the NCK-1A in the same transgenic animals was unable to rescue the HSN posterior cell body displacement defect (Table 3-3). This suggests that NCK-1A has a role in HSN axon guidance that can be separated from the HSN cell body migration.

To test the ability of the NCK-1B isoform to rescue the PLM axon overextension defect, we looked at nck-1(ok694) animals carrying the nck-1B cDNA regulated by the mec-4 promoter. NCK-1B, like NCK-1A, can function cell autonomously in the mechanosensory neurons to rescue the PLM axon overextension phenotype (Table 3-3).
Table 3-2: Brood size and Embryonic Lethality

<table>
<thead>
<tr>
<th>Genotype (N)</th>
<th>Average brood size (±)</th>
<th>Embryonic lethality (±)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type (17)</td>
<td>278 (35)</td>
<td>1.6% (1.3)</td>
</tr>
<tr>
<td>*nck-l(ok694) (16)</td>
<td>201 (45)</td>
<td>1.9% (1.2)</td>
</tr>
<tr>
<td>*ptp-3(op147) (6)</td>
<td>209 (41)</td>
<td>5.2% (1.2)</td>
</tr>
<tr>
<td>*ptp-3(op147); nck-l(ok694) (7)</td>
<td>133 (28)</td>
<td>10% (3.1)</td>
</tr>
</tbody>
</table>

N refers to the number of animals scored.

*P < 0.01 Student’s t-test.
Table 3-3: NCK-1 isoform rescue of nck-1(ok694) neuronal defects

<table>
<thead>
<tr>
<th>Construct</th>
<th>Transgenic Lines</th>
<th>Rescue</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PLM axon overextension</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mec-4::nck-1A</td>
<td>quEx185;186</td>
<td>+; +</td>
</tr>
<tr>
<td></td>
<td>(N = 210; 210)</td>
<td></td>
</tr>
<tr>
<td>mec-4::nck-1B</td>
<td>quEx200</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>(N = 210)</td>
<td></td>
</tr>
<tr>
<td>nck-1(genomic)</td>
<td>quEx151</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>(N = 201)</td>
<td></td>
</tr>
<tr>
<td>nck-1A-GFP</td>
<td>quEx138</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>(N = 210)</td>
<td></td>
</tr>
<tr>
<td>nck-1B(genomic)</td>
<td>quEx195;196</td>
<td>+; +</td>
</tr>
<tr>
<td></td>
<td>(N = 210; 210)</td>
<td></td>
</tr>
<tr>
<td><strong>HSN midline crossover</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>nck-1(genomic)</td>
<td>quEx154</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>(N= 105)</td>
<td></td>
</tr>
<tr>
<td>nck-1A-GFP</td>
<td>quEx138;190</td>
<td>+/-; +/-</td>
</tr>
<tr>
<td></td>
<td>(N = 90; 105)</td>
<td></td>
</tr>
<tr>
<td>nck-1B(genomic)</td>
<td>quEx193</td>
<td>+/-</td>
</tr>
<tr>
<td></td>
<td>(n = 140)</td>
<td></td>
</tr>
<tr>
<td><strong>HSN cell posterior displacement</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>nck-1(genomic)</td>
<td>quEx154</td>
<td>+/-</td>
</tr>
<tr>
<td></td>
<td>(N = 105)</td>
<td></td>
</tr>
<tr>
<td>nck-1A-GFP</td>
<td>quEx138;190</td>
<td>-; -</td>
</tr>
<tr>
<td></td>
<td>(N = 90; 105)</td>
<td></td>
</tr>
<tr>
<td>nck-1B(genomic)</td>
<td>quEx193</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>(N = 140)</td>
<td></td>
</tr>
</tbody>
</table>

+ = Full rescue, not significantly different from wild-type (wt).
+/- = Partial rescue, significantly less defects than nck-1(ok694) but higher than wt.
- = No rescue, not significantly different from nck-1(ok694).
Student’s t-test P<0.05.
To produce the NCK-1B isoform in its genomic context, we introduced two stop codons in the first SH3 domain, in place of W37 and W38, of the genomic rescuing fragment. These mutations specifically affected the NCK-1A isoform, but not NCK-1B, and when placed in the *nck-1(ok694)* background only the NCK-1B isoform was made (Figure 3-2E). This genomic NCK-1B was able to completely rescue the PLM axon overextension defect (Table 3-3). Like the NCK-1A, NCK-1B only partially rescued the HSN crossover defect (32% reduced to 14%). However, unlike NCK-1A, the NCK-1B was able to completely rescue the HSN cell migration defect (Table 3-3).

In summary, it appears that both NCK-1A and NCK-1B can function redundantly in PLM axon guidance, as either isoform is sufficient for this task. NCK-1A or NCK-1B alone can partially rescue the HSN midline crossover defect and suggests that both NCK-1 isoforms are necessary for full HSN midline rescue. Interestingly only NCK-1B was able to rescue the HSN cell migration defect. Hence, our results reveal a functional difference between NCK-1A and NCK-1B isoforms in HSN cell migration.

We also wanted to determine which NCK-1 isoform is required for male mating efficiency. We tested each NCK-1 isoform individually for rescue and only the larger NCK-1A isoform was able to rescue the male mating defects, raising the mating efficiency score from 1 to 4. This provides an NCK-1A specific role in male mating and is also consistent with NCK-1A, and not NCK-1B, being expressed in the male tail (Figure 3-3L).
3.4.7 *nck-1* genetically interacts with *ptp-3/LAR* in the mechanosensory neurons and early development

Previous work in *Drosophila* showed the importance of DOCK/Nck in regulating the growth cones of R1-R6 axons in the developing optic lobe (Garrity et al., 1996). Garrity et al. (1999) also identified a mutation in the receptor tyrosine phosphatase PTP69D, which had similar defects in the R1-R6 axons as the *dock* mutation (Garrity et al., 1999). PTP69D is a member of the Type IIa receptor protein tyrosine phosphatase (RPTP) family which also includes LAR (Leukocyte Common Antigen Related), PTPδ, PTPσ and HmLAR2 (Ensslen-Craig and Brady-Kalnay, 2004). The *C. elegans ptp-3* gene codes for the only type IIa RPTP and has been shown to be required in axon guidance, embryogenesis and, like NCK-1, is found to be expressed in the nervous system, epidermal cells and the developing uterus (Ackley et al., 2005; Harrington et al., 2002).

To determine if there was a genetic interaction between *nck-1* and *ptp-3*, we looked at the effect of the single and double mutations on the mechanosensory neurons. The *ptp-3(op147)* mutants show a low frequency of PLM overextension defects (Table 3-1). The *ptp-3;nck-1* double mutation did not enhance the PLM overextension defect compared to the single *nck-1* mutation (Table 3-1). Genetically, this suggests that *nck-1* and *ptp-3* are functioning in the same pathway to prevent the PLM axon from overshooting its target.

Although the single *nck-1* and *ptp-3* mutation did not result in any cell body positioning defects in the mechanosensory neurons, the *ptp-3;nck-1* double mutation did cause the AVM cell body to be displaced posterior to the vulva at a frequency of 6%
(P<0.001). In wild-type animals, the AVM normally migrates anteriorly, past the vulva, to the mid-anterior region of the animal (Chalfie and Sulston, 1981). This indicates that $ptp-3$ and $nck-1$ function redundantly in AVM cell positioning. Furthermore, the $ptp-3;nck-1$ double showed a low ventral guidance defect in the PVM axon that was not previously seen in the single mutants (Table 3-4). This suggests that $nck-1$ and $ptp-3$ also function redundantly in the ventral guidance of the PVM axon.

The $ptp-3$ mutation was initially reported to cause some embryonic lethality (Harrington et al., 2002). Our $ptp-3;nck-1$ double mutation analysis revealed an increased level of embryonic lethality, suggesting that $ptp-3$ and $nck-1$ act redundantly in embryogenesis (Table 3-2).

In summary, our results show that NCK-1 and PTP-3 are involved in the same pathway to prevent PLM axon overextension, but function in parallel and partially redundantly to regulate AVM migration, ventral guidance of the PVM axon, and embryonic development.

### 3.4.8 NCK-1 functions with UNC-40/DCC in AVM/PVM axon guidance

UNC-40 is the *C. elegans* homolog of the Netrin receptor DCC (Deleted in Colorectal Cancer), and has been shown to be required for the migration of the PVM cell, and the ventral guidance of the AVM and PVM axons (Chan et al., 1996; Hedgecock et al., 1990). In addition, the mammalian DCC has been reported to induce neurite outgrowth via its interaction with the mammalian Nck-1 adaptor molecule (Li et al., 2002). These results, combined with our finding of the involvement of NCK-1 in PVM
Table 3-4: *nck-1* enhances *unc-40* AVM/PVM axon guidance Defects

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th><em>nck-1</em></th>
<th><em>ptp-3</em></th>
<th><em>ptp-3; nck-1</em></th>
<th><em>unc-40</em></th>
<th><em>unc-40; nck-1</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>AVM ventral</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td>1%</td>
<td>20%</td>
<td>39%</td>
</tr>
<tr>
<td>guidance defect</td>
<td></td>
<td></td>
<td></td>
<td>n.s.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PVM ventral</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td>4%</td>
<td>19%</td>
<td>34%</td>
</tr>
<tr>
<td>guidance defect</td>
<td></td>
<td></td>
<td></td>
<td>*</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N</td>
<td>100</td>
<td>209</td>
<td>105</td>
<td>140</td>
<td>120</td>
<td>121</td>
</tr>
</tbody>
</table>

AVM and PVMs were analyzed in young adults. Schematic drawing represents the mid-lateral region of the animal. The vulva location is indicated by black triangle. N = number of animals scored for each genotype. n.s. = not significant. * P < 0.05 Student’s t-test.
axon guidance, prompted us to ask if there was a genetic interaction between the C. elegans nck-1 and unc-40. We found that the unc-40(e271);nck-1(ok694) double mutation had a significantly higher level of AVM and PVM ventral guidance defects compared to the unc-40 single mutant (Table 3-4). This indicates that nck-1 and unc-40 are functioning in different pathways in AVM/PVM axon ventral guidance.
3.5 Discussion

The NCK adaptor molecules are conserved proteins that aid in several signal transduction pathways. Here we show that the *C. elegans* nck-1 encodes two isoforms that have overlapping expression patterns, primarily in the nervous system, but each NCK-1 isoform is expressed exclusively in other cells/tissues. We provide evidence that the shorter isoform, NCK-1B, has a functional role in HSN cell migration, while the longer NCK-1A isoform is necessary and sufficient for male mating. Double mutant analyses suggest that nck-1 functions in the same pathway with *ptp-3* (a LAR RPTP) in PLM axon guidance, but in a parallel pathway in AVM cell migration, PVM axon guidance and embryonic development. We also show that nck-1 functions in a parallel pathway with *unc-40/DCC* in AVM and PVM axon guidance.

3.5.1 A role for the *C. elegans* NCK-1 in neuronal development

The Nck adaptor molecules are implicated in regulating processes that regulate the actin cytoskeleton (Buday et al., 2002; Li et al., 2001). We show that the *C. elegans* NCK-1 is required for proper neuronal development. The *nck-1(ok694)* is likely to encode a null allele and results in PLM guidance defects, whereby the PLM axons overshoot their target. We show that either NCK-1A or NCK-1B can function cell autonomously in the mechanosensory neurons. The PLM overextension phenotype is not completely penetrant which suggests that other genes function redundantly in this process. Indeed, *rpm-1*, *unc-53*, *sax-1*, *sax-2*, *clec-38* and *vab-1* are all implicated in PLM targeting (Gallegos and Bargmann, 2004; Kulkarni et al., 2008; Mohamed and Chin-Sang, 2006; Schaefer et al., 2006).
2000; Schmidt et al., 2009). Future work will address the roles of NCK-1 in these pathways. Intriguingly, the PLM axon overshooting phenotype is similar to the defects reported in the Drosophila dock mutants, where many R1-R6 axons bypassed the lamina, their appropriate target layer, and overextend into the medulla (Garrity et al., 1996). We also report that NCK-1 has a role in preventing midline crossing of the HSNs and command interneurons. Similar results have been reported in the mammalian system, where mice deficient in Nck showed aberrant midline crossover in corticospinal axons and spinal interneurons (Fawcett et al., 2007). In addition to its role in axon guidance, NCK-1 is required for the HSN cells to properly migrate from the posterior end to the midsection of the animal. It is noteworthy to mention that several homologous genes required for HSN cell migration in C. elegans are also expressed, and function, in the vertebrate neural crest cell migration (Kee et al., 2007). Therefore, the mammalian Nck proteins may also be involved in neural crest migrations.

### 3.5.2 NCK-1, PTP-3 and UNC-40 control AVM/PVM cell migration and axon guidance

Although both dock and Ptp69D are expressed and required in R cell growth cones, a genetic interaction between DOCK/Nck and the Drosophila receptor tyrosine phosphatase PTP69D has not been established (Garrity et al., 1999; Garrity et al., 1996). Our genetic analyses suggest that nck-1 and ptp-3 act redundantly in positioning the AVM cell. However, nck-1 and ptp-3 function in the same pathway in PLM axon guidance. Thus, the interaction between NCK-1 and PTP-3 differs depending on the cellular context. This is also reminiscent of DPTP69D which functions differentially in
the photoreceptor neurons (R cells) where it is required to stop the R1-R6 axons once reaching their targets, but it is also required in the R7 axons to promote axon extension (Garrity et al., 1999; Newsome et al., 2000). A similar tissue dependent context has also been observed in the vertebrate PTPδ, where it functions as a growth cone chemoattractant in forebrain neurons, but as a repulsive guidance molecule in thalamocortical axons (Sun et al., 2000; Tuttle et al., 1999).

Our results indicate that unc-40 and nck-1 function in parallel pathways in AVM and PVM ventral axon guidance. These parallel pathways may include the Robo and Eph receptor pathways, as both receptors have been shown to bind to Nck (Fan et al., 2003; Stein et al., 1998). This is further complicated, as a combinatorial set of receptor interactions between Netrin, Robo and Eph receptors can dictate signaling function in C. elegans (Ghenea et al., 2005; Yu et al., 2002). Our results do not rule out the possibility that unc-40 and nck-1 function in the same pathway and it is very probable that unc-40 and nck-1 work in the same pathway as the mammalian DCC/UNC40 and NCK-1 physically interact and are required for neurite outgrowth (Li et al., 2002; Stein et al., 1998).

3.5.3 A role for a truncated NCK adaptor

All published reports on NCK adaptor proteins have been based on the canonical protein with three SH3 domains followed by a single SH2 domain. We report an alternative isoform that lacks the first SH3 domain. Our NCK-1 rescue data suggests, for the most part, that NCK-1A and NCK-1B are functionally interchangeable for axon
guidance. This is consistent with reports that show that the majority of Nck targets implicated in cytoskeleton regulation are associated with the second and third SH3 domains of Nck (Blasutig et al., 2008; Buday et al., 2002). For example, the second SH3 domain of Nck, and Dock are necessary and sufficient to bind to Pak (Galisteo et al., 1996; Hing et al., 1999; Lu et al., 1997). Surprisingly, only the second SH3 domain of Dock was sufficient for the restoration of normal connectivity in the optic lobe. However, the SH2 domain of Dock and the first and third SH3 domains are required to rescue lethality suggesting other roles for these domains (Rao and Zipursky, 1998). Similarly, we show that the NCK-1A isoform, but not NCK-1B, is required for efficient male mating. The underlying cause for inefficient male mating is likely to be abnormal male tail morphology and or behavior. Future work should clarify NCK-1A’s role in male mating.

We also provide functional evidence for the shorter NCK-1B isoform in C. elegans. Protein BLAST searches revealed several other species, including humans, which have NCK adaptor proteins similar to the C. elegans NCK-1B (shorter 2SH3 and 1SH2) isoform. What is the significance of the two isoforms? We show that NCK-1B can rescue the HSN cell migration defect, but surprisingly the NCK-1A does not, which suggests that the absence or presence of the first SH3 provides novel functions. An auto-inhibitory region between the first and the second SH3 domains in the full length human Nck2 is suggested to provide specificity and functionality (Takeuchi et al., 2010). The auto-inhibitory domain is conserved in C. elegans NCK-1A and may be regulated in a
similar way to that proposed for Nck2. However, given that NCK-1B lacks the first SH3 domain and the putative auto-inhibitory domain, it is likely that the NCK-1B isoform escapes this auto-inhibitory interaction to give new function/regulation to the NCK-1B protein. Indeed, NCK-1A and NCK-1B have different functions which are apparent by their independent expression patterns in various tissues, and their different sub-cellular localizations. Our expression studies revealed that NCK-1B is localized to the nucleus and the cytoplasm, while NCK-1A is generally localized to the cytoplasm. While we still do not know the role of the NCK-1B nuclear localization, we show that the first SH3 domain is important in determining the sub-cellular localization of the NCK-1 isoforms. It is interesting to point out that the mammalian NCK has been found in the nucleus during DNA damage cell cycle arrest (Kremer et al., 2007). NCK shuttles between the cytoplasmic and nuclear compartments, and the NCK nuclear localization is mediated by SOCS7 (suppressor of cytokine signaling 7) (Kremer et al., 2007). Future research on C. elegans NCK-1 signaling will help clarify the roles for the specific isoforms and role of NCK-1B in the nucleus.
3.6 Acknowledgments

We thank members of the Chin-sang lab for critical reading of this manuscript. Special thanks to Jeffrey Boudreau for assistance with experiments, and Tony Papanicolaou for microinjections. Some strains used in this work were provided by the Caenorhabditis Genetic Center, which is supported by the National Institutes of Health - National Center for Research Resources, and Dr. S. Mitani from the National BioResource Project of Japan. We thank Drs. A. Fire, S. Clark and Y. Jin for strains and reagents. This work was supported by research grants from the Canadian Institutes of Health Research (CIHR), and Canadian Cancer Society Research Institute (CCSRI).
3.7 References


Chapter 4

The *C. elegans* Eph receptor activates NCK and N-WASP, and inhibits Ena/VASP to regulate growth cone dynamics during axon guidance
4.1 Summary

The Eph receptor tyrosine kinases (RTKs) are regulators of cell migration and axon guidance. However, the molecular mechanisms of how Eph RTKs regulate these processes are still incomplete. To understand how Eph receptors regulate axon guidance in *C. elegans* we screened for suppressors of axon guidance defects caused by a hyperactive VAB-1/Eph RTK. We identified NCK-1 and WSP-1/N-WASP as downstream effectors of VAB-1. Furthermore, VAB-1, NCK-1 and WSP-1 can form a complex *in vitro*. We also report that NCK-1 can physically bind to UNC-34/Enabled (Ena), and suggest that VAB-1 inhibits the NCK-1/UNC-34 complex and negatively regulates UNC-34. Our results provide a model of the molecular events that allow the VAB-1 RTK to regulate actin dynamics for axon guidance. We suggest that VAB-1/Eph RTK can stop axonal outgrowth by inhibiting filopodia formation at the growth cone by activating Arp2/3 through a VAB-1/NCK-1/WSP-1 complex, and inhibiting UNC-34/Ena activity.
4.2 Introduction

An important part of neuronal development is the intricate process of guiding axons to their correct location to form a precise network of neuronal connections. For an axon to navigate from its origin to its final destination, it has to correctly interpret the extracellular signals it receives from its environment through the growth cone. Several axon guidance receptors, and their ligands, involved in this process have been identified and are found to be conserved in various organisms (Chilton, 2006; Dickson, 2002; Wen and Zheng, 2006). Examples of these ligand/receptors include Ephrin/Eph, Semaphorins/plexins, Netrin/DCC/UNC-5, Slit/Robo and Wnts/Frizzled/Ryk/Ror (Chilton, 2006; Endo and Rubin, 2007; Lu et al., 2004). The study of the vertebrate Eph receptor tyrosine kinases (RTKs) has identified a number of downstream signaling pathways in axon guidance (Pasquale, 2005). Unfortunately, the signaling pathways that regulate the cytoskeleton and direct axon growth and guidance are still incomplete. This is in part due to the large number of Ephrins and Eph RTKs that can engage in crosstalk (Egea and Klein, 2007; Pasquale, 2005). The presence of a single Eph RTK, VAB-1, in Caenorhabditis elegans can simplify the analysis of the signal transduction events from the receptor. The C. elegans VAB-1 Eph RTK is involved in various aspects of neuronal development, as vab-1 null mutations result in abnormal neuroblast movements, and have various axon guidance defects (Boulin et al., 2006; George et al., 1998; Mohamed and Chin-Sang, 2006; Zallen et al., 1999). To date, we do not know what molecules are involved in the VAB-1 Eph RTK signaling pathway in axon guidance. In an attempt to resolve this issue, we used a genetic suppressor approach as well as a physical interaction...
approach and identified NCK-1, WSP-1/N-WASP and UNC-34/Enabled (Ena) as molecules regulated by VAB-1/Eph RTK signaling in axon guidance.

The Nck (Non-Catalytic region of tyrosine Kinase) adaptor protein family is implicated in organizing the actin cytoskeleton through various proteins such as the Pak serine/threonine kinases, N-WASP and WAVE (Buday et al., 2002; Eden et al., 2002). Furthermore, Nck has been shown to function downstream of several axon guidance receptors including Robo, low-density lipoprotein receptors, Dcc and the Eph RTKs (Bisson et al., 2007; Fan et al., 2003; Holland et al., 1997; Li et al., 2002; Pramatarova et al., 2003; Stein et al., 1998). Although the function of NCK has been studied in various organisms, the biological function of NCK-1 in *C. elegans* has only been recently explored (Mohamed and Chin-Sang, 2011). Furthermore, what molecules interact with the *C. elegans* NCK-1 is still unknown.

The WASP protein family (WASP and N-WASP) are scaffolds that integrate multiple signaling pathways, leading to the formation of branched actin filaments through the activation of the Arp2/3 complex (Miki and Takenawa, 2003; Takenawa and Suetsugu, 2007). WSP-1 is the *C. elegans* homolog of the mammalian N-WASP and although the *C. elegans* WSP-1/N-WASP has been shown to be involved in neuronal cell migration and axon guidance, a connection between WSP-1 and a guidance receptor has not yet been established (Shakir et al., 2008; Withee et al., 2004).

The Ena/VASP proteins are involved in actin-dependent movements including neuronal migration and axon guidance, and are known for their role in filopodia
formation and elongation (Chereau and Dominguez, 2006; Drees and Gertler, 2008). In *C. elegans*, the Ena/VASP homolog UNC-34 has been shown to be involved in neuronal cell migration, axon guidance and filopodia formation (Chang et al., 2006; Desai et al., 1988; McIntire et al., 1992; Norris et al., 2009; Withee et al., 2004). Previous work has shown that Ena/VASP proteins can function as effectors in repulsive cues downstream of receptors such as SAX-3/Robo, UNC-5/Netrin receptor and EphB4, as well as effectors for attractive cues downstream of receptors such as UNC-40/DCC (Bashaw et al., 2000; Colavita and Culotti, 1998; Evans et al., 2007; Gitai et al., 2003; Yu et al., 2002). How Ena/VASP is able to function as an effector for both repulsive and attractive cues is still unclear.

In this paper, we describe some of the molecular events that allow the VAB-1 Eph RTK to regulate actin dynamics for axon guidance. We provide genetic and biochemical evidence to show that VAB-1 signals through NCK-1 and WSP-1/N-WASP, and negatively regulates UNC-34/Ena. We propose a model for PLM (Posterior lateral microtubule) axon termination whereby the VAB-1 Eph RTK is able to prevent axon extension via two different methods. First: by preventing further actin filament extension by negatively regulating UNC-34/Ena. Second: we propose that the Arp2/3 complex is activated as a result of the formation of a VAB-1/NCK-1/WSP-1 complex, and prevents filopodia formation by forming branched actin filaments.
4.3 Results

4.3.1 VAB-1 signals through the C. elegans NCK-1 SH3/SH2 adaptor protein

To identify VAB-1 Eph RTK effectors, we utilized transgenic animals carrying mec-4::myr-vab-1 (quIs5) which encodes a constitutively active VAB-1 tyrosine kinase (myristoylated-VAB-1 (MYR-VAB-1)) in the mechanosensory neurons (Mohamed and Chin-Sang, 2006). In wild type animals, PLM neuron cell bodies are located in the tail region and send axons anteriorly that stop at the centre of the animal (Figure 4-1A). We previously showed that myr-vab-1 caused neuronal defects in the mechanosensory neurons, in particular the premature termination of PLM axons (Figure 4-1A, 4-1B) (Mohamed and Chin-Sang, 2006). Since the MYR-VAB-1 behaves as a constitutively active VAB-1 RTK, we reasoned that mutations in effectors of the VAB-1 signal may suppress the neuronal defects. We used a candidate gene approach to examine genes with known roles in axon guidance and tested whether loss-of-function mutations could suppress the myr-vab-1 PLM premature termination phenotype. We identified nck-1 as a candidate effector of VAB-1 Eph RTK signaling. The nck-1(ok694) mutation partially suppressed the PLM axon premature termination (Figure 4-1B), indicating that other effectors are involved in the MYR-VAB-1 signaling. The C. elegans genome encodes for only one nck-1 adaptor protein, and is most similar to the human Nck2 and Drosophila DOCK (Mohamed and Chin-Sang, 2011). NCK-1 has all the domain features of the NCK adaptor proteins, including three SH3 domains followed by a single SH2 domain. We previously reported that the deletion allele nck-1(ok694) is predicted to be a null allele,
**Figure 4-1: PLM defects in various backgrounds and their genetic interactions.**

(A) All panels show young adults visualized with *zdIs5 (mec-4::gfp)*. Anterior is to the left. Solid arrow points to where the PLM axon ends. A line diagram that corresponds to the morphology of the neuron is shown below. In wild-type animals (top *gfp* panel) the PLM axons terminate at the middle (vulva region triangle). MYR-VAB-1 (middle *gfp* panel) causes PLM axons to terminate before reaching their target. *wsp-1(gm324)* animals (bottom *gfp* panel) have PLM axons that overshoot past the vulva (triangle) and ALM neuron (dashed arrow). (B) The *nck-1(ok694)* and *wsp-1(gm324)* alleles significantly reduced the early termination defects caused by MYR-VAB-1. Over expressing *unc-34* in the PLMs also reduced the MYR-VAB-1 termination defect. (C) *vab-1, nck-1* and *wsp-1* animals have PLM overextension defects. Reducing the levels of *UNC-34* via tissue specific RNAi suppressed the PLM overextension defects of *vab-1(dx31)* and *wsp-1(gm324)*. (D) *unc-34(e566)* loss-of-function and tissue specific *unc-34* RNAi exhibit PLM axon termination defects. Over expression of NCK-1 in the mechanosensory neurons (*mec-4::nck-1*) caused low levels PLM early termination defects, but synergized in the *unc-34(e566)* background. Activating the Arp2/3 complex via the WSP-1 VCA domain (*mec-4::vca*) caused PLM axon termination defects. Error bars indicated the SEM, and significant differences between some of the strains were compared (using student’s t-test), *P < 0.05; **P < 0.01; ***P < 0.001; n.s. = not statistically significant. ‘N’ refers to the number of axons scored.
thus all of our genetic work was carried out using the *ok694* allele (Mohamed and Chin-Sang, 2011).

If NCK-1 is an effector of VAB-1 signaling then we predicted that *nck-1* loss-of-function mutations would display *vab-1* phenotypes. Indeed, previous work showed that both *vab-1* and *nck-1* mutants have similar neuronal defects, including an overextension in PLM axons (Figure 4-1C) (Boulin et al., 2006; Mohamed and Chin-Sang, 2006; Mohamed and Chin-Sang, 2011). To further confirm that *nck-1* and *vab-1* are in the same pathway in the PLM neurons, we analyzed the effect of the double mutation on the PLM axons. The *vab-1; nck-1* double mutation did not enhance the PLM over extension phenotype (Figure 4-1C), indicating that NCK-1 and the VAB-1 Eph receptor function in the same pathway to guide the PLM axons.

4.3.2 *NCK-1 is expressed in the nervous system and co-localizes with VAB-1*

We previously showed that NCK-1 is expressed in various tissues including the nervous system (Mohamed and Chin-Sang, 2011). In addition, like VAB-1, NCK-1 can function cell autonomously in the mechanosensory neurons for PLM axon guidance (Mohamed and Chin-Sang, 2006; Mohamed and Chin-Sang, 2011). If NCK-1 and VAB-1 function in the same pathway during neuronal development, then they should be localized in the same cells. Indeed, NCK-1 and VAB-1 were co-localized in some of the neurons, consistent with the role of NCK-1 as an effector of VAB-1 (Figure 4-2). However, the expression pattern of VAB-1 and NCK-1 did not completely overlap suggesting that both NCK-1 and VAB-1 also have independent roles (Figure 4-2).
Figure 4-2: NCK-1 co-localizes with VAB-1.

Anterior is to the left in all panels. (A-C) NCK-1 (Green) and VAB-1 (Red) co-localized in some cells. Arrow head points to cells where both NCK-1 and VAB-1 are co-localized, and the arrow points to cells that only express NCK-1. The *nck-1::gfp* transgene used encodes the NCK-1A isoform and was detected using anti-GFP antibodies. Endogenous VAB-1 was detected using anti-VAB-1 antibodies (see experimental procedures).
4.3.3 The NCK-1 SH2 domain interacts with VAB-1 phosphotyrosine Y673

In a parallel approach we used yeast two-hybrid screens to identify effectors of VAB-1/Eph RTK signaling and identified the full length NCK-1 as a binding partner of the VAB-1 intracellular kinase region. Yeast two-hybrid analysis showed that the NCK-1 SH2 domain is sufficient to bind VAB-1 and that VAB-1 tyrosine Y673 (YEDP) is crucial for the interaction with the NCK-1 SH2 domain (Figure 4-3A).

To further confirm the NCK-1/VAB-1 interaction we used GST-pull down assays. Deletion analyses confirmed that the SH2 domain is necessary and sufficient to bind VAB-1 (Figure 4-3B). Furthermore, the NCK-1 interaction required an active tyrosine VAB-1 kinase (Figure 4-3C). Since SH2 domains are known to bind phosphotyrosines we wanted to test how specific the NCK-1 SH2 domain is for VAB-1. We found that four other SH2 domains (MIG-10, SEM-5, ABL-1, VAV-1) were unable to bind to VAB-1 (Figure 4-3D). In summary, NCK-1 interacts with VAB-1 in a kinase dependent manner, the interaction is mediated via the NCK-1 SH2 domain and the VAB-1 Y673 juxtamembrane tyrosine, and VAB-1 has high specificity for the NCK-1 SH2 domain.

4.3.4 The Ena/VASP homolog UNC-34 can bind and inhibit NCK-1

How does VAB-1 cause the PLM to stop once the VAB-1 Eph RTK is activated and adaptor proteins such as NCK-1 bind to the receptor? A previous report indicated that Ena/VASP was required for repulsion caused by EphB4 signaling in fibroblasts, but it was unclear how the signal was conveyed (Evans et al., 2007). The Ena/VASP family are composed of an N-terminal EVH1 domain, a central PRO region and a C-terminal
Figure 4-3: NCK-1 physically interacts with VAB-1 and UNC-34.

(A) The NCK-1 SH2 domain binds to the Y673 of VAB-1. Yeast-two hybrid assays shows that the NCK-1 SH2 domain (Prey) can bind to wild-type (WT) VAB-1 (669aa-985aa), but fails to interact when Y673 of VAB-1 is changed to glutamic acid (Y673E). (B-C) GST pull-down assays. L = Load; U = unbound fraction; B = bound fraction. (B) Different NCK-1 domains fused to GST show that the SH2 domain is required for VAB-1 binding. The three SH3 domains alone do not bind to VAB-1. (C) The binding of SH2 domain of NCK-1 to VAB-1 is kinase dependent. The kinase inactive VAB-1(G912E) failed to interact with the SH2 domain of NCK-1. (D) The NCK-1 SH2 domain shows high specificity for VAB-1. Yeast-two hybrid assays of other SH2 domains failed to interact with VAB-1, (E-F) GST-NCK-1 pull down assays with MBP-UNC-34. (E) UNC-34 bind to NCK-1, both the proline rich (PRO) and EVH2 domains are required to bind to NCK-1 (not shown). (F) Different GST-NCK-1 domains show that all SH3 domains can independently interact with full length UNC-34. The asterisks marks the correct protein fragment expected, and all other fragments below the marked are break down products. The dashed lines in E and F indicate a cropped region from the same blot.
Ena/VASP homology II domain (EVH2) (Drees and Gertler, 2008). We asked if NCK-1 could be the link between the Eph RTK and Ena/VASP. We first tested if NCK-1 and UNC-34 can directly interact. *In vitro* binding assays with bacterially expressed NCK-1 and UNC-34 confirmed that both molecules can indeed physically interact (Figure 4-3E, 4-3F). Furthermore, we found that the PRO-EVH2 domains are required together to bind to NCK-1 (data not shown). We also showed that all three NCK-1 SH3 domains were able to bind UNC-34 (Figure 4-3F).

While *nck-1* and *vab-1* animals have overextended PLM axons, *unc-34* animals have the opposite phenotype and have PLM axons that terminate prematurely (Figure 4-1D). This suggests that UNC-34 is involved in PLM axon extension, and reflects a known role of Ena/VASP in actin filament formation and elongation (Chereau and Dominguez, 2006; Drees and Gertler, 2008). To understand the genetic nature of the interaction between *nck-1* and *unc-34*, we analyzed the *nck-1(ok694); unc-34(e566)* double mutant and found that *nck-1* partially suppressed the *unc-34* PLM termination defect (Figure 4-1D). This suggests that, in PLM axon outgrowth, *unc-34* negatively regulates *nck-1*. To provide further evidence for this genetic interaction we over expressed NCK-1 (*mec-4::nck-1*) in the PLM neurons in *unc-34(e566)* animals which resulted in a synergistic enhancement of the PLM termination phenotype (Figure 4-1D). Overall, these results suggest that UNC-34 can inhibit the function of NCK-1 and may do so by physically binding to it.
4.3.5 VAB-1 disrupts the NCK-1 / UNC-34 interaction and negatively regulates UNC-34

After the identification of UNC-34 as a NCK-1 binding partner, we wanted to examine whether VAB-1, NCK-1 and UNC-34 could form a complex *in vitro*. Surprisingly, although UNC-34 can bind strongly to NCK-1, the introduction of VAB-1 abolished the binding between UNC-34 and NCK-1 (Figure 4-A Lane 4, 5). This result suggests that VAB-1 might be inducing its effect at the growth cone membrane by relieving the inhibition of NCK-1 that is caused by UNC-34. To provide *in vivo* support of this we over expressed UNC-34 in the mechanosensory neurons (*mec-4::unc-34*) and it significantly reduced the MYR-VAB-1 PLM premature termination phenotype (Figure 4-B).

To gain more insight into the interaction between VAB-1 and UNC-34, we sought to analyze the effect of the *vab-1;unc-34* double on PLM axons. We found that the *vab-1;unc-34* double mutant is synthetic lethal (data not shown), so we used a mechanosensory specific *unc-34* RNAi approach (see experimental procedures). The *unc-34(RNAi)* strain had PLM termination defects that were similar to *unc-34(e566)* (Figure 4-D). Analysis of the *vab-1;unc-34(RNAi)* double showed that reducing the levels of *unc-34* can rescue the PLM overextension defects seen in *vab-1(dx31)* (Figure 4-C), which is consistent with *vab-1* inhibiting *unc-34* function. Since the genetic data suggested that *vab-1* negatively regulates *unc-34*, we questioned if the activation of VAB-1 could affect the expression and/or localization of UNC-34. Induction of MYR-VAB-1 did not change the localization of UNC-34, but instead resulted in the reduction of
Figure 4-4: VAB-1 inhibits the UNC-34/NCK-1 complex and negatively regulates UNC-34 protein levels.

(A) NCK-1 (GST) pull-down experiments. NCK-1 can pull-down UNC-34 (lane 3). Adding VAB-1 (either co-expressed, lane 4 or mixing lane 5) inhibits the interaction between NCK-1 and UNC-34. VAB-1, NCK-1 and GST protein levels shown below, the dashed line indicates a cropped region from the blot. Tagged protein fusions used: MBP-WSP-1, GST-NCK-1, HIS-VAB-1, and GST-UNC-34. Proteins were detected with antibodies to GST, MBP or VAB-1. (B) Inducing hyper active MYR-VAB-1 via a heat shock promoter reduces the levels of UNC-34::GFP. unc-34::gfp transgenic animals had a GFP relative mean intensity of 1±0.07 under heat shock conditions, while hs:myr-vab-1;unc-34::gfp animals had a GFP relative mean intensity of 0.56±04 (p < 0.01; student’s t-test) under the same conditions. All panels show UNC-34::GFP image of the CAN neuron.
UNC-34 levels compared to wild-type animals (Figure 4-4B). This suggests that the activation of VAB-1 can reduce UNC-34 protein levels.

In summary, our binding assays and genetic analyses show that VAB-1 activation results in binding NCK-1 which in turn blocks the UNC-34 binding to NCK-1, freeing NCK-1 from the negative influence of UNC-34 and in addition VAB-1 negatively regulates UNC-34 protein levels.

4.3.6 WSP-1 is an effector for VAB-1/NCK-1

Since mammalian Nck is known to physically bind and activate N-WASP to regulate actin filaments through the Arp2/3 complex (Buday et al., 2002; Rivero-Lezcano et al., 1995; Rohatgi et al., 2001), we questioned whether VAB-1 is linked to the cytoskeleton through WSP-1/N-WASP. If WSP-1 acts downstream of VAB-1, then the \textit{wsp-1} mutants should suppress the PLM termination defect caused by MYR-VAB-1. Indeed, we found that \textit{wsp-1(gm324)} partially suppressed the PLM termination defect caused by MYR-VAB-1 (Figure 4-1B).

If WSP-1 is an effector of VAB-1 signaling then we would expect to see some neuronal defects in \textit{wsp-1} mutants that are similar to \textit{vab-1} animals. It has previously been reported that the \textit{wsp-1} had only very weak axon guidance defects, such as in the PDE and VD/DD neurons (Shakir et al., 2008). We report here that approximately 50% of \textit{wsp-1(gm324)} animals have overextended PLM defects (Figure 4-1C). Since the \textit{wsp-1} PLM overextension frequency is much greater than \textit{vab-1} (Figure 4-1C), this implies that WSP-1 also functions in other pathways in addition to the VAB-1 pathway for PLM axon
guidance. We also found that the *vab-1(dx31);wsp-1(gm324)* double mutants are synthetic lethal (data not shown), which is consistent with WSP-1 functioning in parallel pathways with VAB-1.

The presence of WSP-1 in the VAB-1 signaling pathway points to the possibility that the PLM termination phenotype caused by MYR-VAB-1 could be due to the activation of the Arp2/3 complex. WSP-1, like its mammalian counterpart, is composed of an N-terminal Ena/VASP homology I domain (EVH1; also known as WASP-homology-1 domain (WH1)), a central section containing a basic region (BR), a GTPase binding domain (GBD) and a proline-rich region (PRO), and a C-terminal with two verprolin homology domains (V; also known as WH2), a cofilin homology domain (C) and an acidic domain (A) (Sawa et al., 2003; Takenawa and Suetsugu, 2007; Withee et al., 2004) collectively known as the VCA region. The C-terminal VCA regions of both WSP-1 and N-WASP have been shown to be effective in activating the Arp2/3 complex *in vitro* (Sawa et al., 2003; Yamaguchi et al., 2002). We utilized the C-terminal VCA region of WSP-1 to selectively activate the Arp2/3 complex in the mechanosensory neurons (*mec-4::wsp-1\textsuperscript{vca})*. The WSP-1\textsuperscript{VCA} caused PLM premature termination defects that were very similar to MYR-VAB-1 (Figure 4-1D).

The activation of high levels of the Arp2/3 complex produces extensive short branched actin networks that prevent the formation of filopodia, and hence can inhibit axon extension (Ideses et al., 2008; Strasser et al., 2004). Ena/VASP, on the other hand, promotes axon extension through filopodia formation and elongation (Chereau and
Dominguez, 2006; Drees and Gertler, 2008; McIntire et al., 1992; Norris et al., 2009). Thus, activation of Arp2/3 complex and UNC-34/Ena have opposite roles in the axon growth cone, and perhaps Arp2/3 complex activation can antagonize the function of UNC-34/Ena. Since WSP-1/N-WASP is an activator of the Arp2/3 complex, we wanted to genetically test if wsp-1 can antagonize unc-34 function. Due to the inviability of wsp-1; unc-34 double mutants (Sheffield et al., 2007; Withee et al., 2004), we analyzed the PLM axons in wsp-1; unc-34(RNAi) animals. The reduction of unc-34 resulted in the partial suppression of PLM overextension defects caused by wsp-1(gm324) (Figure 4-1C), consistent with WSP-1/ Arp2/3 activity antagonizing UNC-34 function.

In summary, we show that WSP-1 functions in PLM axon termination, and is possibly doing this in various signaling pathways, among which includes the VAB-1 receptor. Our results suggest that MYR-VAB-1 is exerting its effect by activating the Arp2/3 complex through WSP-1. We also suggest that WSP-1 can antagonize UNC-34 function by activating the Arp2/3 complex.

4.3.7 VAB-1 enables WSP-1 to outcompete UNC-34 for NCK-1 binding

We used in vitro binding assays to ask whether VAB-1, NCK-1 and WSP-1 could form a complex. WSP-1 was able to bind to NCK-1 (Figure 4-5A, Lane 6), but not VAB-1 (Figure 4-5A, Lane 5). However, WSP-1 was able to pull down VAB-1 in the presence of NCK-1, indicating that a VAB-1/NCK-1/WSP-1 complex can occur (Figure 4-5A, Lane 7).
Figure 4-5: VAB-1, NCK-1 and WSP-1 interact in a complex.

(A-B) WSP-1 (MBP) pull-down experiments. (A) VAB-1/NCK-1/WSP-1 can form a complex. WSP-1 does not pull-down VAB-1 (lane 5), WSP-1 can pull down NCK-1 (lane 6), and WSP-1 can pull down VAB-1 only in the presence of NCK-1 (compare lane 5 and lane 7). (B) VAB-1 enables WSP-1 to outcompete UNC-34 for NCK-1 binding. WSP-1 does not pull down UNC-34 (lane 6). WSP-1 pulls down NCK-1 (lane 7, NCK-1 relative intensity 1.0) and adding UNC-34 reduces the level of interaction between NCK-1 and WSP-1 (lane 8 (asterisks), NCK-1 relative intensity 0.5). UNC-34 is not detected in lane 8 suggesting that NCK-1/UNC-34/WSP-1 do not form a complex. Adding VAB-1 prevents UNC-34 from reducing the interaction level between NCK-1 and WSP-1 (lane 9, NCK-1 relative intensity 1.2). Protein levels shown below, dashed line indicates a cropped region from the blot. Tagged protein fusions used: MBP-WSP-1, GST-NCK-1, HIS-VAB-1 and GST-UNC-34. Proteins were detected with antibodies to GST, MBP or VAB-1.
Since NCK-1 is able to bind both UNC-34 and WSP-1, we wanted to determine whether all three molecules can form a complex, or do UNC-34 and WSP-1 compete for NCK-1 binding. We first confirmed that WSP-1 was unable to bind to UNC-34 (Figure 4-5B, Lane 6). We found that although WSP-1 binds to NCK-1, the presence of UNC-34 resulted in the reduction of the NCK-1/WSP-1 complex by 2-fold (Figure 4-5B, Lane 8). This shows that UNC-34 can effectively compete with WSP-1 for NCK-1 binding. Furthermore we could not detect NCK-1/UNC-34/WSP-1 in a complex (Figure 4-5B, Lane 8). Interestingly, adding VAB-1 to the binding interaction increased the level of NCK-1 binding to WSP-1, indicating that VAB-1 eliminated UNC-34’s ability to compete for NCK-1 binding (Figure 4-5B, Lane 9). In summary, our binding assays show that VAB-1, NCK-1 and WSP-1 form a complex, that UNC-34 competes with WSP-1 for NCK-1 binding, and that VAB-1 enables WSP-1 to outcompete UNC-34 for binding to NCK-1.

4.3.8 VAB-1 signaling inhibits filopodia on the PLM growth cone

Identification of the actin regulating molecules NCK-1 and WSP-1 as MYR-VAB-1 suppressors indicates that VAB-1 can affect the actin cytoskeleton during axon migration. To confirm this, we monitored the PLM growth cone of wild-type and myr-vab-1 transgenic animals at the time of hatching. In wild-type animals, the PLM growth cone exhibited dynamic changes and had many filopodia protrusions (Figure 4-6A). Transgenic myr-vab-1 animals, on the other hand, had growth cones that were less dynamic and were usually void of filopodia like structures (Figure 4-6B). This shows that
Figure 4-6: VAB-1 activation affects PLM growth cone dynamics

(A-B) Series of time-lapse images of PLM growth cones in newly hatched L1 as they migrate anteriorly. Growth cones were visualized with zdIs5 (mec-4::gfp). Scale bar represents 2 µm. (A) Wild-type PLM growth cones exhibit dynamic changes and display multiple filopodia protrusions. (B) Transgenic myr-vab-1 animals have PLM growth cones that are less dynamic and mostly void of any protrusions. (C) A model of how VAB-1 induces its termination effect during PLM axon guidance. In the absence of VAB-1 activation, UNC-34/Ena promotes axon extension through the polymerization of actin filaments and forming filopodia. UNC-34/Ena also physically binds to and inhibits NCK-1. Activation of VAB-1 results in binding NCK-1, disrupts the interaction between NCK-1 and UNC-34, and negatively regulates UNC-34. Furthermore, VAB-1, NCK-1 and WSP-1/N-WASP form a complex and induce high levels of Arp2/3 activation to form an extensive network of short, branched actin filaments. The short branched networks stop the formation of new filopodia structures. The net result is the termination of axon extension in response to VAB-1 signaling.
VAB-1 can affect the growth cone morphology and effectively inhibit growth cone filopodia formation. We propose that the change in PLM growth cone morphology in *myr-vab-1* transgenic animals is a direct result of changes in the actin cytoskeleton, which causes the PLM axon to stop further outgrowth.
4.4 Discussion

We previously reported a functional role for VAB-1 as a receptor for a repellent or stop signal in PLM axon guidance. Here we describe some of the molecular events involved in VAB-1 signaling that allow the regulation of actin dynamics for PLM axon guidance. Our genetic and in vitro interaction analysis identified NCK-1, WSP-1 and UNC-34 as molecules regulated by VAB-1 Eph RTK signaling. Our data supports a model in which VAB-1 suppresses axon extension by negatively regulating UNC-34, and activating the Arp2/3 complex through a VAB-1/NCK-1/WSP-1 complex. Furthermore, using time-lapse analysis we show that activation of VAB-1 inhibits filopodia formation in the PLM growth cone.

4.4.1 NCK-1 and Eph RTK signaling

Our results show that the C. elegans NCK-1 adaptor protein is an effector of the VAB-1 RTK signal in vivo. Several lines of evidence indicate that VAB-1 and NCK-1 act together to regulate axon guidance. First, nck-1 and vab-1 animals have similar neuronal defects. Second, NCK-1 and VAB-1 physically interact and co-localize in similar neuronal cells and axons. Finally, the nck-1 loss-of-function suppresses the defects caused by the constitutively active VAB-1. We found that NCK-1 binds to VAB-1 via its SH2 domain, and that it specifically binds to the Y673 (YEDP) of the VAB-1 juxtamembrane in a kinase dependent manner. This is consistent with the published binding specificity of the Nck SH2 domain, as well as reports of Nck1 binding to the second juxtamembrane tyrosine residue (YEDP) in EphA3 and EphA2 (Y602 and Y594 respectively) (Frese et
Interestingly, Nck adaptors have been reported to function downstream of Eph RTKs but it appears that the activated EphA RTKs are direct targets of Nck adaptors (Bisson et al., 2007; Fawcett et al., 2007; Hu et al., 2009; Miura et al., 2009), whereas Nck may indirectly interact with EphBs (Becker et al., 2000; Hock et al., 1998; Holland et al., 1997). Considering that the intracellular region of VAB-1 is more similar to EphA receptors (George et al., 1998), our results in C. elegans provides relevant insight into how mammalian EphA receptors could regulate the actin cytoskeleton for axon guidance.

### 4.4.2 Ena/VASP in Eph RTK signaling

The Ena/VASP protein family is required in processes that involve dynamic actin remodeling such as platelet shape change, axon guidance and Jurkat T cell polarization (Bear et al., 2000; Krause et al., 2003). The ability of Ena/VASP proteins to remodel actin stems from their ability to polymerize actin, which is required for filopodia formation and elongation (Drees and Gertler, 2008; Huttelmaier et al., 1999; Lebrand et al., 2004). In C. elegans, UNC-34/Ena functions in neuronal cell migration, axon guidance and filopodia formation (Chang et al., 2006; Desai et al., 1988; Gitai et al., 2003; McIntire et al., 1992; Withee et al., 2004). Our results further confirm the role of UNC-34 in axon extension, where we show that the *unc-34* PLM axons terminated prematurely. The cause of early termination is likely due to a reduction of filopodia elongation in the growth cone, resulting in the persistence of more densely branched filaments that can slow axon migration. This is supported by the finding that *unc-34* loss-of-function results in the
reduction in the number of filopodia structures on growth cones, and a reduction in the
rate of growth cone migration (Norris et al., 2009) (and our unpublished observations). In
addition, mammalian studies show that depletion of Ena/VASP generates shorter and
more densely branched filaments (Bear et al., 2000).

We propose that VAB-1 negatively regulates UNC-34 for PLM termination. This
is supported by our observations that: 1. the loss-of-function unc-34 resulted in PLM axon
defects similar to the hyper active MYR-VAB-1; 2. over expressing UNC-34 in the PLM
partially suppressed the MYR-VAB-1 phenotype; 3. the hyperactivation of VAB-1
reduced the levels of UNC-34 and 4. the reduction of UNC-34 levels by RNAi suppressed
the vab-1 PLM overextension defects. The VAB-1 negative regulation of UNC-34 would
prevent further filopodia elongation or formation, and as a result inhibit, or slow down,
growth cone translocation.

Our finding, that VAB-1 negatively regulates UNC-34, is different from the
previous observation that the mammalian Ena/VASP mediates the EphB4 fibroblast cell
repulsion induced by ephrin-B2 (Evans et al., 2007). In fibroblast cells, the activation of
Ena/VASP by EphB4 results in destabilizing lamellipodia and the internalization of
EphB4 which is needed for cell repulsion (Bear et al., 2002; Evans et al., 2007). In C.
elegans axons, on the other hand, the VAB-1 negative regulation of UNC-34/Ena
prevents actin filament elongation and bundling that is required for efficient growth cone
filopodia, hence preventing further growth cone migration. This difference could be
attributed to species differences, or to a difference in Eph RTK function that is dependent
on cellular context. Furthermore, the intracellular region of VAB-1 is more related to EphA than EphB, which can contribute to the difference in results (George et al., 1998).

4.4.3 A VAB-1/NCK-1/WSP-1 complex regulates the actin cytoskeleton

Our results provide evidence that the VAB-1/Eph RTK can be connected to the cytoskeleton through its interaction with NCK-1 and WSP-1. This is based on our observation that vab-1, nck-1 and wsp-1 mutants share the same phenotype of PLM axon overextension, that both nck-1 and wsp-1 were able to partially suppress the MYR-VAB-1 PLM termination defect, that VAB-1, NCK-1 and WSP-1 are able to form a complex in vitro, and that the activation of the Arp2/3 complex via the WSP-1 VCA domain resulted in PLM termination defects similar to MYR-VAB-1. The role of N-WASP as a negative regulator of axon elongation has been shown by two separate reports, where the reduction of the levels of N-WASP resulted in the enhancement of axon elongation (Kakimoto et al., 2006; Pinyol et al., 2007). This phenotype is very similar to the PLM overextension defects we observed in wsp-1 animals. There have been conflicting reports on the role of the Arp2/3 complex in axon elongation, where some reports indicate that the Arp2/3 complex acts as a negative regulator of axon elongation (Pinyol et al., 2007; Strasser et al., 2004), while other reports indicate that the Arp2/3 complex is required for axon elongation (Korobova and Svitkina, 2008; Norris et al., 2009). A paper by Ideses et al (2008) provided a potential resolution to this paradox by looking at the characteristics of actin assembly in the presence of variable amounts of Arp2/3 complex in vitro (Ideses et al., 2008). It is proposed that high levels of the Arp2/3 complex prevents the formation of
filopodia bundles due to the extensive branching networks of actin that produces very short, closely located tips, and prevents any G-actin to be free to elongate. On the other hand, at low concentrations of Arp2/3 the filaments produced have longer tips and are further apart making it easier to form filopodia bundles (Ideses et al., 2008). Hence, it would be expected that the complete elimination of Arp2/3 would prevent any neurite elongation. Similarly, the excessive activation of Arp2/3 would also prevent neurite elongation due to the increased levels of short, branched networks of actin filaments.

N-WASP has been shown to interact in a complex with the mammalian EphB2, but with intersectin as the link between N-WASP and EphB2 (Irie and Yamaguchi, 2002). Furthermore, this interaction between EphB2, intersectin and N-WASP is required for dendritic spine formation, which consists mainly of a meshwork of branched filaments caused by the activation of the Arp2/3 complex (Irie and Yamaguchi, 2002). We provide evidence that the VAB-1 Eph RTK can be connected to WSP-1/N-WASP through another molecule, the NCK-1 adaptor protein. We further propose that this connection also results in the activation of Arp2/3, and negatively regulates growth cone migration.

4.4.4 Model for VAB-1 signaling in the PLM to stop axon growth

In this study we propose a model of how the molecules NCK-1, UNC-34, VAB-1, WSP-1 and Arp2/3 are functioning in axon growth cones for extension and termination (Figure 4-6C). During PLM axon outgrowth, the growth cone is stimulated by an attractive cue that results in the accumulation of UNC-34/Ena at the growth cone. The result is a net forward movement due to the role of UNC-34/Ena in inhibiting actin
capping proteins, and allowing filopodia elongation by polymerizing F-actin at the leading edge. In addition, UNC-34/Ena binds to NCK-1 to prevent it from signaling in a pathway(s) that would otherwise inhibit axon extension. Once the VAB-1/Eph RTK receives the signal to inhibit axon extension, VAB-1 becomes autophosphorylated and provides a docking site (Y673) for NCK-1 to bind. The binding of NCK-1 to VAB-1 disrupts the interaction between NCK-1 and UNC-34 to release the inhibitory effect of UNC-34 on NCK-1, and makes NCK-1 available to form a complex with WSP-1.

Through an unknown mechanism, we also show that VAB-1 negatively regulates the UNC-34/Ena protein levels. The VAB-1/NCK-1/WSP-1 complex results in high levels of Arp2/3 activation, which causes the formation of an extensive meshwork of actin filaments. The combined actions of VAB-1/Eph blocking UNC-34/Ena activity, while activating Arp2/3 through NCK-1/WSP-1 contributes to the molecular events required to stop forward movement of the growth cone.
4.5 Experimental Procedure

4.5.1 Strains

All *C. elegans* strains were manipulated as described by Brenner (Brenner, 1974). All alleles were isolated in the standard wild type Bristol strain N2. All experiments were performed at 20°C unless otherwise indicated. The following strains were used in this study: N2 (var. Bristol) (Brenner, 1974); LGI: *zdIs5*:mek-4::gfp; LGII: vab-1(dx31), *quIs5*:mek-4::myr-vab-1; LG IV: wsp-1(gm324), LG V: unc-34(e566); LGX:, *quIs6*:unc-34::unc-34::gfp; Unmapped: *quIs16*:hs::myr-vab-1 (Brisbin et al., 2009); Extrachromosomonal arrays (this study): *quEx131*:mek-4::nck-1A, *quEx190*:nck-1::nck-1A-gfp] (Mohamed and Chin-Sang, 2011), *quEx281*:mek-4::unc-34, *quEx321*:mek-4::vca, *quEx338*:mek-4::unc-34 RNAi] (see tissue specific RNAi). Unless noted otherwise, all *C. elegans* strains were obtained from the *C. elegans* Genetics Stock Center, care of T. Stiernagle (U. of Minnesota).

4.5.2 Tissue specific RNAi

To produce double stranded RNA (dsRNA) only in the mechanosensory neurons, we constructed a cloning vector (pIC659) with head to head Pmec-4 promoters on each side of a Multiple Cloning Sites (MCS) such that the sense and antisense strands of an inserted cDNA would be transcribed. The mek-4::unc-34 RNAi construct (pIC727) was created by cloning an unc-34 cDNA fragment (ATG start to the first SalI site, 388bp) into the pIC659 dual Pmec-4 RNAi cloning vector.

4.5.3 Molecular Biology
The *mec-4::nck-1A* construct (pIC313) was made as previously described in Mohamed and Chin-Sang (2011). The *mec-4::unc-34* construct (pIC624) was generated by amplifying *unc-34* cDNA and sub-cloning behind the *mec-4* promoter. To create the *mec-4::vca* construct (pIC673), the VCA region of WSP-1 (9108-9606 of the *wsp-1* gene; *C07G1.4a* in Wormbase) was amplified from genomic DNA and cloned behind the *mec-4* promoter. The *unc-34::unc-34::gfp* translation reporter was generated by a PCR fusion approach (Hobert, 2002) using the following pieces: 1. A ~5kb genomic region that includes 2kb of 5’UTR and the first two exons of *unc-34*, 2. Exons 2-7 were amplified from RB2 cDNA library, and 3. a 868bp GFP fragment amplified from pPD95.75 (gift from Dr. Andre Fire). Details of plasmid/PCR constructs and primer sequences are available upon request.

### 4.5.4 Transgenic Animals

Transgenic animals were generated by germ-line transformation as previously described (Mello et al., 1991). The *unc-34::unc-34::gfp* translational reporter was injected at a concentration of 20 ng/μL, and one of the *unc-34* rescuing lines (quEx61) was integrated to create quIs6. The *mec-4::unc-34* construct was injected at a concentration of 30 ng/μL into *mec-4::gfp(zdIs5); mec-4::myr-vab-1(quIs5)*. *mec-4::unc-34RNAi, mec-4::vca* and *mec-4::nck-1* were injected into *mec-4::gfp(zdIs5)* at 30 ng/μL. *mec-4::nck-1(quEx131)* was later crossed into *unc-34(e566)*, and *mec-4::unc-34RNAi (quEx338)* was crossed into *vab-1(dx31)* and *wsp-1(gm324)*. Transgenic animals were identified by the co-injection marker pRF4/rol-6 (30 ng/μl), or odr-1::rfp (30 ng/μl)
(Mello et al., 1991). At least two independent lines were isolated and analyzed. The data shown are from one representative line.

4.5.5 Antibodies

Mixed stage animals were fixed and stained as described in (Chin-Sang et al., 1999). Rabbit anti-VAB-1 antibodies (antigen VAB-1-HIS6) and chicken polyclonal antibodies against GFP (Chemicon) were used at 1:100 dilutions. Texas Red-conjugated goat anti-rabbit (Jackson’s Lab) were used at a 1:100 dilution. For Western blot analysis, antibodies were used at the following dilutions: anti-NCK-1 at 1:500, anti-VAB-1 at 1:2500, anti-MBP-HRP at 1:8000, anti-GST-HRP at 1:4000. Goat-anti-rabbit-HRP and goat-anti-mouse-HRP were used as at 1:10000 dilutions on western blots. Relative band intensities in Figure 5B were calculated using the National Institutes of Health Image J program.

4.5.6 Phenotypic Analysis

The mechanosensory neurons were visualized using the mec-4::gfp (zdIs5) reporter, and PLM axon defects were quantified as described previously (Mohamed and Chin-Sang, 2006). All animals scored for neuronal defects were young adults. The wild-type neuron morphology was defined by analysis of neuronal GFP reporters and is consistent with the electron microscopic reconstruction of the C. elegans nervous system (White et al., 1986). Animals were anesthetized using 0.2% tricaine and 0.02% tetramisole in M9, and mounted on 3% agarose pads. Fluorescent animals were analyzed using a Zeiss Axioplan microscope, Axiocam and Axiovision software.
4.5.7 Time Lapse Imaging of PLM Growth Cones

PLM growth cones were visualized using a mec-4::gfp (zdIs5) reporter. Eggs were allowed to hatch for 5 minutes, and the newly hatched L1 animals were examined immediately on 3% agarose pads with a drop of 0.2% tricaine and 0.02% tetramisole in M9. PLM growth cones were imaged with a Zeiss LSM710 confocal microscope at intervals of 20-30s.

4.5.8 Yeast Two-Hybrid Assays

Yeast cells were grown on standard and selective media as required (Sherman, 1991). The desired plasmids were transformed into yeast cells using the lithium acetate method (Schiestl and Gietz, 1989). For binding and deletion analysis, the pGBK7 vector was used as bait and the pGADT7 vector (Clontech) as prey, and β-galactosidase activity was measured qualitatively by X-GAL overlay assays (Serebriiskii and Golemis, 2000). To identify interactors with VAB-1, the Kinase Region (669aa-985aa) of vab-1 was cloned into pGBK7 (pIC187) and used in a screen against the RB2 cDNA library (gift from Bob Barstead), and about 600,000 colonies were screened. Site directed mutagenesis (QuickChange, Stratagene) of pIC187 was used to change the juxtamembrane tyrosine 673 changed to glutamic acid (Y673E). The SH2 domains of NCK-1, MIG-10, SEM-5, ABL-1 and VAV-1 were cloned into the activation domain of the pGADT7 vector. Primer sequences and details of plasmid constructs are available upon request.
4.5.9 Pull Down and Co-purification Assays

The following constructs were created by cloning the desired cDNA fragment into glutathione-S-transferase (pGEX4T-2, Amersham): pIC282 – NCK-1 SH2 domain (298aa-397aa), pIC297 – all three NCK-1 SH3 domains (1aa-308aa), pIC308 – 1st NCK-1 SH3 domain (1aa-72aa), pIC593 – 2nd NCK-1 SH3 domain (112aa-186aa), pIC309 – 3rd NCK-1 SH3 domain (198aa-308aa), pIC324 – full length (F.L.) NCK-1 (1aa-397aa), and pIC606 – F.L. UNC-34 (1aa-454). The following constructs were created by cloning the desired cDNA fragment into maltose-binding protein (pMAL\textsuperscript{im}-p2X, New England Biolabs): pIC225 – F. L. intracellular region of wild-type VAB-1 (581aa-1117aa), pIC119 – F. L. intracellular kinase dead/deficient VAB-1 (G912E), pIC603 – UNC-34 RPO-EVH2 domain (128aa-454aa), pIC605 – F.L. UNC-34 (1aa-454aa), pIC671 – UNC-34 PRO domain (128aa-274), pIC674 – UNC-34 EVH2 domain (246aa-454aa), pIC670 – WSP-1 VCA domain (334aa-607aa). pIC582 – His-6::VAB-1 (581aa-1117aa) was described in Brisbin et al (2009). All fusion constructs were expressed in E. coli Tuner (DE3). For Figures 4-3(B-C, E-F) and 4-4A, a GST ‘pull-down’ assay was used to confirm the VAB-1, NCK-1 and UNC-34 interactions. Soluble/purified (Load) MBP-VAB-1, MBP-VAB-1(G912E), MBP-UNC-34 F.L., MBP-UNC-34-PRO-EVH2, MBP-UNC-34-PRO or MBP-UNC-34-EVH2 were incubated for 2-3 hrs at 4°C with soluble extracts containing either GST, GST-NCK-1 F.L., GST-NCK-1-all SH3 domains, GST-NCK-1(1\textsuperscript{st}SH3), GST-NCK-1(2\textsuperscript{nd}SH3), GST-NCK-1(3\textsuperscript{rd}SH3), GST-NCK-1(SH2), His-6::VAB-1(581aa-1117aa) (pIC582) or GST-NCK-1 F.L. coexpressed with pIC582 bound to 50µl glutathione sepharose beads (GE healthcare). Unbound fractions were collected,
protein bound to GST beads were washed four times (25mM Hepes, 10% Glycerol, 0.1% Triton-X, 285mM NaCl), and a proportional loading of each sample was analyzed by standard SDS polyacrylamide gel, followed by western blotting. All loads fused to MBP were detected using anti-MBP conjugated to HRP (New England Biolabs). His6-VAB-1 was detected using Rabbit anti-VAB-1 antibodies (antigen VAB-1-His6) (Figure 4-4A). GST and GST-NCK-1 F.L., and GST-NCK-1 deletion domains were detected either by Ponceau S or anti-GST conjugated to HRP. For Figure 4-5, MBP ‘pull-down’ was used to confirm VAB-1, NCK-1, WSP-1 and UNC-34 interactions. Soluble extracts (Load) of GST-NCK-1 F.L., His-VAB-1 (pIC582), GST-NCK-1 F. L. coexpressed with pIC582, or GST-UNC-34 F.L. were incubated for 2-3 hours at 4°C with soluble extracts containing either MBP or MBP-WSP-1(334aa-608aa) bound to 100µl amylose resin beads (New England Biolabs). Unbound fractions were collected, protein bound to amylose beads were washed four times (20mM Tris-Cl [pH7.5], 200mM NaCl, 1mM EDTA, 1mM DTT), and a proportional loading of each sample was analyzed by standard SDS polyacrylamide gel, followed by western blotting. VAB-1 was detected by Rabbit anti-VAB-1, GST fused proteins were detected by anti-GST conjugated to HRP, MBP and MBP-WSP were detected by anti-MBP conjugated to HRP.
4.6 Acknowledgements

We are grateful to Theresa Stiernagle from the *Caenorhabditis elegans* Genetic Center (CGC) for providing us with strains, Dr. S. Clark for the *mec-4::gfp (zdIs5)* strain, Dr. A. Fire for the GFP vectors and Dr. Robert Barstead for the Y2H cDNA library. Thanks to Tony Papanicolaou for microinjections. We thank members of the Chin-Sang lab for reviewing and commenting on the manuscripts. This work was funded by the Canadian Institutes of Health Research (CIHR), and Natural Science and Engineering Research Council of Canada (NSERC) operating grants to I.C.-S., and the R. S. McLaughlin Fellowship to A.M.M.
4.7 References


Chapter 5
General Discussion

5.1 Concluding remarks and future directions

Over the past decade, the Eph RTKs have received a lot of attention due to their role in many developmental processes. The Eph RTK research has been fruitful and led to the identification of some of the molecules involved in the vertebrate Eph RTK signaling. However, Eph RTK research has also indicated the presence of a complex signaling network that is still not completely understood (Jorgensen et al., 2009; Lackmann and Boyd, 2008). Part of the difficulty is due to the large number of vertebrate Eph receptors, and the lack of an adequate number of in vivo studies. These problems can be eliminated by studying Eph RTK signaling in simpler organisms such as C. elegans, which possess a single Eph RTK homolog called VAB-1 (George et al., 1998). Results from such studies will likely present a simpler signal transduction pathway that will be relevant to the vertebrate system due to the conservation of guidance molecules across species (Tessier-Lavigne and Goodman, 1996). Work carried out on the C. elegans VAB-1 Eph RTK already showed that VAB-1 is required in epidermal and neuronal development, and that it is likely functioning by transducing a stop signal (Boulin et al., 2006; George et al., 1998; Mohamed and Chin-Sang, 2006; Zallen et al., 1999). The overall goal of this thesis was to further advance our understanding of Eph receptor signaling by identifying components of the C. elegans VAB-1 Eph RTK signaling pathway, and further characterize these components by molecular and genetic analysis.
Using the PLM mechanosensory neurons as a model, along with a series of genetic approaches and protein-protein interaction assays, I identified NCK-1, WSP-1 and UNC-34 as molecules regulated by the VAB-1 Eph RTK. My results provide the first demonstration that the membrane receptor VAB-1 can be directly connected to the cytoskeleton through an NCK-1/WSP-1 complex. I further present evidence that VAB-1 can negatively regulate UNC-34 during PLM axon guidance. The combination of these findings provided a mechanism for the regulation of PLM growth cone dynamics whereby the VAB-1/NCK-1/WSP-1 complex activates Arp2/3 to increase branched actin filaments, and the inhibition of UNC-34/Ena by VAB-1 prevents further filopodia formation. The net result of these interactions is the inhibition of axon extension. Thus, the results presented in this thesis makes a significant contribution to the molecular mechanisms of axon guidance, Eph RTK signal transduction, and understanding of how receptor signaling regulates the actin cytoskeleton during neuronal cell movement.

One question that remains to be answered is which of the VAB-1 ligands is required to activate VAB-1 in the PLM axon? As mentioned in Chapter 2.3, there are five ligands (EFN-1, EFN-2, EFN-3, WRK-1 and VPR-1) that can activate VAB-1. Previous work has already shown that EFN-1 is required in PLM axon guidance, however the efn-1 null mutants have PLM over extension defects that are lower in frequency than the vab-1 null (Figure 5-1) (Mohamed and Chin-Sang, 2006). This suggests that EFN-1 is not the only ligand involved in PLM axon guidance. Furthermore, the triple efn-1 efn-2;efn-3 mutant have PLM defects that are not different from the efn-1 mutant alone (Figure 5-1).
5-1: The VAB-1 receptor and EFN ligands show PLM overextension defects.

Mutants carrying the *efn-1(ju1)* null allele have PLM overextension defects that are lower in frequency than the *vab-1(dx31)* null animals. This suggests that there are other ligands involved in activation of the VAB-1 receptor in PLM axon guidance. Animals with the triple *efn-1 efn-2;efn-3* had PLM overextension defects similar to the *enf-1* null alone suggesting that there are other ligands that are required to activate VAB-1 in the PLM axon. The PLM axons were scored using a *zds5(mec-4::gfp)* marker. Error bars indicate the SEM, and significant differences between some of the strains as well as wild-type were compared (using student’s t-test), *P < 0.05; **P < 0.01; n.s. = not statistically significant. ‘N’ refers to the number of axons scored. (Figure was taken and modified from Mohamed et al., 2006).
This indicates that it is likely one of the remaining ligands, or both, that is required in PLM axon guidance. Considering the region where the PLM stops in the animal, I favor a model where VPR-1 is secreted from the head region to activate VAB-1. One important experiment involves analyzing the PLM axons in vpr-1 and wrk-1 mutant backgrounds, which will provide insight into which gene is required for PLM guidance. Genetic analyses should be extended to look at double mutations with enf-1 to determine if we have identified all the ligands that would account for VAB-1 signaling. Another experiment that would provide further evidence for VAB-1 ligands for PLM axon guidance is to analyze the effect of ectopically expressing EFN-1, VPR-1 and WRK-1. These genes can be ectopically expressed in epidermal cells using an ajm-1 promoter, in muscle cells using a him-4 promoter (Vogel and Hedgecoch, 2001), or in the ventral nerve cord through a hmr-1b promoter (Broadbent and Pettitt, 2002). Ectopic expression of a ligand that can activate VAB-1 will likely result in PLM axon guidance defects, which can further be verified by showing that the defects are suppressed in the vab-1 mutant background.

Another question that arises from this thesis is how does VAB-1 negatively regulate the levels of UNC-34? At this point one can only speculate. One interesting hypothesis involves the adaptor NCK-1. This is based on preliminary results showing that the levels of UNC-34 are reduced in nck-1 mutants, suggesting that NCK-1 is required to maintain the levels of UNC-34 in the PLM (Figure 5-2). It is possible that the physical interaction between NCK-1 and UNC-34 is required for stabilization of the UNC-34
protein, and the inhibition of this interaction by VAB-1 activation would result in the degradation of UNC-34. A more elaborate hypothesis is based on the role of the mammalian Nck in increasing protein translation. Nck was reported to form a complex with the initiation factor eIF2β, and this interaction requires Nck’s first and third SH3 domains (Kebache et al., 2002). Hence, one can hypothesize that NCK-1 is required for the translation of UNC-34 by activating the C. elegans eIF2β homolog IFTB-1. The activation of VAB-1 could possibly disrupt this process by inhibiting the ability of NCK-1 to interact with IFTB-1. Alternatively, VAB-1 could also possibly modulate the C. elegans eIF2β (IFTB-1) by phosphorylating it at Y89 (TYEEA), as this region fits the consensus for an Eph kinase substrate (Warner et al., 2008). All of these are testable hypotheses that can explain the reduced levels of UNC-34 caused by the activation of VAB-1 or loss of NCK-1.

As for the VAB-1 signaling pathway, although both NCK-1 and WSP-1 were identified as effectors of VAB-1, the fact that both molecules did not completely suppress the PLM defects caused by the hyperactive MYR-VAB-1 indicates that there are still other unidentified effectors. Thus, future work can be directed towards continuing the candidate gene suppressor screen to identify these other effectors. Candidate genes would include molecules that have been already identified as effectors of the vertebrate Eph receptor, as well as known interactors with the adaptor NCK. Additionally, a yeast two-hybrid screen with the intracellular region of VAB-1, or NCK-1, can also be utilized to further identify potential VAB-1 effectors. The candidate effectors isolated from the yeast
5-2: NCK-1 is required to maintain the levels of UNC-34.

A unc-34::gfp translational reporter was expressed in the PLM neuron of wild-type and nck-1(ok694) animals via the mec-4 promoter. The top two panels show an image of UNC-34 levels in the PLM of wild-type and nck-1 background. The absence of NCK-1 results in a reduction in the levels of UNC-34, indicating that NCK-1 is required to maintain UNC-34 levels. We do not yet know why in wild-type animals UNC-34 shows up as bright spots. The cell bodies in the top panels are outlined by the dotted line. The bottom table shows a quantification of the level of UNC-34::GFP. High level refers to PLM neuron with strong spots as shown in wild-type. nck-1 mutants a significantly lower number of animals with high UNC-34::GFP (*P < 0.05; student’s t-test). ‘N’ refers to the number of animals scored.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>High GFP (%)</th>
<th>Low GFP (%)</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>95</td>
<td>5</td>
<td>39</td>
</tr>
<tr>
<td>nck-1(ok694)</td>
<td>82</td>
<td>18</td>
<td>50</td>
</tr>
</tbody>
</table>

- "*" indicates statistically significant difference compared to wild-type.
two-hybrid screen can be genetically tested for their relationship with \textit{vab-1}.

Future investigation should also test whether the identified VAB-1 signaling pathway from the PLM neurons works in a similar way in other neurons. Considering that the vertebrate Eph RTK has already shown a degree of cell-context dependency (Lackmann and Boyd, 2008; Pitulescu and Adams, 2010), there is a possibility that VAB-1 might also signal in different pathways depending on the cell context. Preliminary work on verifying the relationship between VAB-1 and NCK-1 in the other neurons indicates that VAB-1 and NCK-1 are functioning in the same pathway to guide HSN cells and axons, as well as command interneurons along their proper route (Appendix A).

However, it also seems that NCK-1 may also be signaling in parallel to VAB-1 to guide the command interneuron axons to the ventral side of the animal (Appendix A). Additionally, preliminary results also suggest that NCK-1 and UNC-34 have a cell context-dependent interaction (Appendix A). Further genetic and molecular analyses will help explain the reasons, or mechanisms, that are responsible for this cell context behavior.

The results obtained from these future experiments will help identify the complete signaling network of the VAB-1 Eph RTK signaling pathway, as well as clarify how these molecules are integrated to regulate axon guidance. Furthermore, results obtained from analyzing other neurons will aid in identifying different cell context VAB-1 signaling pathways. The results obtained will also complement the approaches taken by
other researchers studying more complex organisms, and will be of wide interest to the basic sciences and medical research fields.

5.2 Summary

1) I identified NCK-1 as an effector of the VAB-1 Eph RTK signaling pathway.

2) The *C. elegans* genome contains a single *nck-1* gene that codes for two different NCK-1 isoforms, NCK-1A and NCK-1B. The *nck-1(ok480)* allele behaves as a genetic and molecular null.

3) NCK-1A is composed of three SH3 domains followed by a single SH2 domain, while NCK-1B is composed of two SH3 domains followed by a single SH2 domain.

4) Expression pattern analysis showed that NCK-1A and NCK-1B have overlapping expression patterns in the nervous system, but are also differentially expressed in other tissues.

5) NCK-1A is mainly found in the cytoplasm, while NCK-1B is localized to the cytoplasm and the nucleus.

6) I provided genetic evidence that NCK-1 is required for proper axon targeting and neuronal cell migration in *C. elegans*.

7) Genetic rescue analysis showed that NCK-1A and NCK-1B have different functional roles in the HSN neurons.

8) Protein–protein binding assays showed that NCK-1 binds to the Y673 (YEDP) residue of VAB-1 in a kinase dependent manner.
9) Protein-protein binding assays and genetic analysis indicated that UNC-34 inhibits NCK-1 by binding to it.

10) I provided evidence that VAB-1 can relieve the inhibition of NCK-1 caused by UNC-34, and negatively regulates UNC-34 protein levels.

11) WSP-1/N-WASP was identified as an effector of the VAB-1 Eph RTK signaling pathway.

12) VAB-1, NCK-1 and WSP-1 form a complex in vitro.

13) I proposed that the VAB-1/NCK-1/WSP-1 complex results in the activation of the actin nucleators Arp2/3.

14) In vitro binding assays suggested that VAB-1 allows WSP-1 to outcompete UNC-34 for the binding of NCK-1.

15) Time-lapse analysis showed that VAB-1 activation inhibits filopodia formation in the PLM growth cone.

16) I proposed a model of how VAB-1 regulates the PLM growth cone, whereby the activation of VAB-1 induces high levels of Arp2/3 activation, through an NCK-1/WSP-1 complex, to from an extensive meshwork of short actin filaments. At the same time, VAB-1 activation results in the negative regulation of UNC-34/Ena. The end result of this combined action is the inhibition of filopodia formation and prevention of axon extension.
5.3 References


Appendix A

Genetic analysis of the NCK-1/VAB-1 and NCK-1/UNC-34 relationship in the HSN and Command interneurons

*nck-1* and *vab-1* function in the same pathway in the HSNs and command interneurons

The work presented in Chapter 4 proposed a signaling model where VAB-1 and NCK-1 act in the same pathway during PLM axon guidance. To determine if VAB-1 and NCK-1 act in a similar manner in other neurons, I analyzed two other neurons (the HSNs and command interneurons) that are also affected by the loss-of-function of VAB-1 and NCK-1 (Boulin et al., 2006; Mohamed and Chin-Sang, 2011; Zallen et al., 1999).

**HSN Motor neurons:** The HSN neurons were examined using the *tph-1::gfp(zdIs13)* reporter (Clark and Chiu, 2003). The *nck-1* and *vab-1* mutants exhibited two types of HSN neuronal defects. The first defect was a migration defect in the HSN cell bodies. *vab-1(dx31)* and *nck-1(ok694)* animals had HSN cells that either did not migrate at all, or they stopped prematurely before reaching their normal position near the vulva (Table A-1; Figure A-1A). This phenotype has not been reported previously for *vab-1*. The *vab-1*; *nck-1* double mutation did not result in an enhancement of the HSN neuronal cell displacement phenotype and was very similar to the *vab-1* null (Table A-1), suggesting that *nck-1* and *vab-1* are functioning in the same pathway during HSN migration. The second type of HSN defect was an axon migration defect. Instead of having axons that
<table>
<thead>
<tr>
<th>Axons Scored (Reporter Strains)</th>
<th>Genotype</th>
<th>Defects (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSNL/R midline axon cross over (<em>ph-1::gfp</em>)</td>
<td>Wild-type</td>
<td>6%</td>
</tr>
<tr>
<td></td>
<td>vab-1(dx31)</td>
<td>36%</td>
</tr>
<tr>
<td></td>
<td>nck-1(ok694)</td>
<td>32%</td>
</tr>
<tr>
<td></td>
<td>vab-1(dx31);nck-1(ok694)</td>
<td>38%</td>
</tr>
<tr>
<td></td>
<td>unc-34(e566)</td>
<td>82%</td>
</tr>
<tr>
<td></td>
<td>unc-34(e566);nck-1(ok694)</td>
<td>82%</td>
</tr>
<tr>
<td>HSNL/R posterior cell body displacement (<em>ph-1::gfp</em>)</td>
<td>Wild-type</td>
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</tr>
<tr>
<td></td>
<td>vab-1(dx31)</td>
<td>34%</td>
</tr>
<tr>
<td></td>
<td>nck-1(ok694)</td>
<td>23%</td>
</tr>
<tr>
<td></td>
<td>vab-1(dx31);nck-1(ok694)</td>
<td>36%</td>
</tr>
<tr>
<td></td>
<td>unc-34(e566)</td>
<td>61%</td>
</tr>
<tr>
<td></td>
<td>unc-34(e566);nck-1(ok694)</td>
<td>55%</td>
</tr>
<tr>
<td>Command interneurons midline axon cross over (<em>glr-1::gfp</em>)</td>
<td>Wild-type</td>
<td>4%</td>
</tr>
<tr>
<td></td>
<td>vab-1(dx31)</td>
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</tr>
<tr>
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<td>nck-1(ok694)</td>
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</tr>
<tr>
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<td>vab-1(dx31);nck-1(ok694)</td>
<td>41%</td>
</tr>
<tr>
<td></td>
<td>unc-34(e566)</td>
<td>45%</td>
</tr>
<tr>
<td></td>
<td>unc-34(e566);nck-1(ok694)</td>
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</tr>
<tr>
<td>Command interneurons ventral guidance defects (<em>glr-1::gfp</em>)</td>
<td>Wild-type</td>
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</tr>
<tr>
<td></td>
<td>vab-1(dx31)</td>
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</tr>
<tr>
<td></td>
<td>nck-1(ok694)</td>
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<tr>
<td></td>
<td>vab-1(dx31);nck-1(ok694)</td>
<td>17%</td>
</tr>
<tr>
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<td>unc-34(e566)</td>
<td>61%</td>
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<tr>
<td></td>
<td>unc-34(e566);nck-1(ok694)</td>
<td>56%</td>
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Significant differences between some genotypes have been conducted to determine the significances between them, *P < 0.05, ***P < 0.001, n.s. (not significant). N > 100.
Figure 5-3: Neuronal defects observed in nck-1 and vab-1 mutants.

(A-B) Neuron specific gfp reporters reveal a role for nck-1 and vab-1 in neuronal development. A line diagram corresponding to the morphology of each neuron class is shown below the gfp panel. (A) Upper gfp panel show wild-type (WT) HSNs visualized using zdIs13(tpi1::gfp). The two lower gfp panels show HSN defects observed in nck-1 null animals, where HSN axons aberrantly cross the midline (arrow head; middle gfp panel) and have HSN cell bodies displaced posteriorly (arrow, bottom gfp panel). (B) The command interneurons, visualized using rhIs4(glr-1::gfp), of wild-type animals are only found on the right side of the midline (first gfp panel) in the ventral region of the body (third gfp panel). nck-1 mutants have command interneuron axons that cross the midline to the left side of the animal (second gfp panel; arrow head). The vab-1; nck-1 double show an increased level of ventral guidance defects (fourth gfp panel; arrow pointing to lateral command interneuron axons).
travel ipsilaterally along the right and left side of the midline, both \textit{vab-1} and \textit{nck-1} animals had HSN axons that inappropriately crossed the ventral midline (Figure A-1A, Table A-1). The \textit{vab-1}; \textit{nck-1} double mutation also exhibited midline cross over defects at a similar frequency to both single mutations, indicating that both genes are also function in the same pathway to guide HSN axons along the ventral midline (Table A-1). Thus, \textit{nck-1} and \textit{vab-1} function in the anterior migration of HSN neuronal cells and help prevent the HSN axons from inappropriately crossing the ventral midline.

\textbf{Command interneurons}: The command interneurons were visualized using a \textit{glr-1::gfp(rhIs4)} reporter (Lim et al., 1999; Maricq et al., 1995). In \textit{vab-1} animals, two types of defects were observed. First, 5\% of the \textit{vab-1} animals had one or more of the head neurons extend their axons at a lateral position instead of along the ventral nerve cord (Table A-1; Figure A-1B). In contrast, \textit{nck-1} mutants did not exhibit the lateral guidance defect observed in \textit{vab-1} animals (Table A-1). Surprisingly, the \textit{vab-1}; \textit{nck-1} double mutant had command interneurons axons along the lateral side at a higher frequency than the \textit{vab-1} null alone (Figure A-1B; Table A-1). This suggests that both \textit{nck-1} and \textit{vab-1} are involved in guiding axons ventrally to the ventral nerve cord, and that \textit{nck-1} might be signaling in the \textit{vab-1} pathway as well as in parallel pathways. The second type of command interneuron defect observed in \textit{vab-1} and \textit{nck-1} mutants was that axons inappropriately crossed the midline (Figure A-1B, Table A-1). The \textit{vab-1}; \textit{nck-1} double exhibited an increase in the level of axon midline crossing in the command interneurons (Table A-1). The increase in level of midline crossing is most likely due to an additive
effect of the double mutation. Nonetheless, our results show that both \textit{vab-1} and \textit{nck-1} are required to guide the command interneurons to the ventral midline, as well as keeping the command interneurons restricted to the right side of the ventral midline.

In summary, \textit{nck-1} and \textit{vab-1} mutants have overlapping neuronal defects, and both genes are signaling in the same pathway to guide axons and neuronal cells to their proper location. NCK-1 works with VAB-1 to guide the PLM axons, HSN cells and axons, and command interneuron axons along their proper route. At the same time NCK-1 may signal in parallel to VAB-1 to guide the command interneuron axons to the ventral side of the animal.

\textbf{The relationship between \textit{nck-1} and \textit{unc-34} is cell context dependent}

Unlike the PLM axons phenotype, \textit{unc-34} mutants exhibited HSN and command interneuron defects that were similar to the ones observed in \textit{vab-1} and \textit{nck-1} (Table A-1). The \textit{unc-34}; \textit{nck-1} double mutant had HSN axon midline cross over and cell displacement defects at a similar frequency to the \textit{unc-34}(\textit{e566}) single mutation (Table A-1). This indicates that both \textit{nck-1} and \textit{unc-34} are functioning in the same pathway to guide HSN axons and cell bodies to their appropriate location. In the command interneurons, \textit{unc-34}; \textit{nck-1} double mutants had a higher frequency in midline crossing over than the single mutations on their own, indicating that UNC-34 and NCK-1 are functioning in parallel to limit the axons to the right side of the midline. On the other hand, \textit{unc-34}; \textit{nck-1} double was not significantly different from the \textit{unc-34} single mutation command interneuron ventral guidance which suggests that UNC-34 and NCK-1 are working in the same
pathway to guide the command interneurons to the ventral region of the animal. In
general, the genetic analysis carried out on the PLMs, HSNs and command inteurneurons
imply that NCK-1 and UNC-34 have a cell context-dependent interaction. Work carried
out in the future will help explain the reasons or mechanisms that are responsible for this
cell context behavior.
References:


Appendix B

MYR-Eph versus Y667E and Y673E Eph gain-of-function

Considering the relevance of the two juxtamembrane tyrosine residues in the VAB-1/Eph RTKs, it is interesting to note that one way to create a gain-of-function constitutively active Eph receptor in the vertebrate system is by changing the two juxtamembrane tyrosines to glutamic acids (Y667E and Y673E; referred to as YY-EE) (Zisch et al., 2000). We made GST-VAB-1 intracellular (YY-EE) fusion proteins and this version still retained tyrosine autophosphorylation (data not shown). When a version of the VAB-1 RTK (YY-EE) was expressed in the touch neurons under the mec-4, the animals displayed premature PLM termination phenotypes but not as penetrant as the MYR-VAB-1 gain of function (Figure B-1) (Mohamed and Chin-Sang, 2006). Since the YY-EE affects the crucial Y673 needed for NCK-1 binding, it would suggest that the gain-of-function phenotype is independent of NCK-1. To test this we asked whether nck-1 mutants could suppress the VAB-1 (YY-EE) phenotypes. The nck-1(ok694) mutation did not suppress the VAB-1 (YY-EE) gain-of-function phenotype, consistent with NCK-1 binding to and functioning through the phosphorylated VAB-1 juxtamembrane residues (Figure B-1).
Figure 5-1: Comparison of defects between myr-vab-1 and vab-1(YYEE).

The MYR-VAB-1 form of gain-of-function is causes a higher frequency of PLM axon defects than the VAB-1(Y667E and Y673E) form of gain-of-function. Error bars indicated the SEM, and significant differences between some of the strains were compared (using student’s t-test), ***P < 0.001; n.s. = not statistically significant.
References:
