ZAG-1, A ZN-FINGER HOMEODOMAIN TRANSCRIPTION FACTOR, AFFECTS NEURONAL OUTGROWTH PATTERNING BY REGULATING UNC-40(DCC)-MEDIATED OUTGROWTH ACTIVITY

by

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ABSTRACT OF THE DISSERTATION

ZAG-1, A ZN-FINGER HOMEODOMAIN TRANSCRIPTION FACTOR, AFFECTS NEURONAL OUTGROWTH PATTERNING AND GUIDANCE BY REGULATING UNC-40(DCC)-MEDIATED OUTGROWTH ACTIVITY

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Axon guidance is a fundamental process during animal nervous system development. For neurons to function correctly, they must be wired into their target areas. Several neurodevelopment disorders result from wiring defects. The guidance process is continuous, and outgrowth direction activity may fluctuate across the plasma membrane. We propose this process is statistically dependent. If the probability of axon outgrowth at one site changes then the probability at another site must change. The plasma membrane movement can be modeled by a random walk.

Axon guidance process is regulated by many conserved extracellular guidance cues that can act as attractants or repellants, but how these guidance cues are regulated is still being researched. In *Caenorhabditis elegans*, the UNC-6/Netrin extracellular cue directs neuronal outgrowth. Neuronal response to UNC-6/Netrin is mediated by UNC-40/DCC and UNC-5/UNC5 receptors and the UNC-53/NAV2 cytoplasmic protein. My study

provides evidence that links stochasticity with transcriptional regulation of axon guidance to regulate axon orientation at the transcriptional level. ZAG-1, a member of the ZEB family of transcription factors, affects neuronal outgrowth patterning by regulating UNC-40-mediated neuronal outgrowth activity. Understanding the functional relationship between transcription factors and the cell-surface and cytoskeletal proteins that mediate their effects is a current challenge in neurobiology.

Here I present evidence that ZAG-1 and UNC-5 regulate UNC-40::GFP localization, axon outgrowth patterning, and the directional bias of axons by regulating the UNC-6/Netrin signaling pathway and UNC-53 activities. *unc-5* mutations alter UNC-40::GFP asymmetric localization and axon outgrowth patterning that neurons develop. Genetic interactions suggest that UNC-5 and ZAG-1 act with UNC-53 to regulate axon outgrowth patterning and UNC-40::GFP asymmetric localization in response to extracellular guidance cues.

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INTRODUCTION

Background of axon guidance

The nervous system is a complex of specialized cells that includes neurons for communicating activity and glial cells that support of neuron function. These cells are important for animals to interact with their environment. The nervous system is divided into two parts: central nervous system (CNS) and peripheral nervous system (PNS). The central nervous system includes brain, nerves, and spinal cord, whereas the peripheral nervous system includes ganglia, sensory neurons and nerves.

Axon guidance is a highly regulated process that guides the axons to navigate toward their specific targets and serves as the major process in neuronal network formation. During neuronal development, neurons extend axonal processes in specific directions and direct the outgrowth activity to their final destination. The axon is guided by a sensory and highly dynamic structure at the leading edge of the growing axon named the growth cone. The neuronal cell migration and axon outgrowth are steps to establish functional neuronal networks (Zhang and Cai, 2010). The main point in the construction of neuronal networks is the direction of the migration of axons, which depend on their ability to respond to a variety of chemoattractants and chemorepellents cues in the extracellular environment (Chilton, 2006). The growth cone has specific transmembrane receptors that interact with guidance cues (Dickson, 2002) resulting in cytoskeleton organization, actin filament polymerization and depolarization to decide which way the growth cone will turn toward an attractant (Dent et al., 2011). Multiple conserved extracellular guidance molecules and their receptors that direct axons such as Netrins, Slits, Ephrin and Semaphorins have been discovered (Dickson, 2002).

Different nervous system disorders and dysfunction, mental retardation and

neurodegenerative diseases, such as autism, Down's syndrome (Quinn and Wadsworth, 2008; Yaron and Zheng, 2007) and Alzheimer's disease (Zhang and Cai, 2010), are related to aberrant proliferation, axon migration and axon outgrowth. Thus, studying axon guidance allows us to understand nervous system function and connections to develop treatments for these neuronal diseases.

It was known that the transcription factors cascade could be involved during early steps of neuronal differentiation (Schmid et al., 2006). Understanding transcriptional regulation of the neuronal outgrowth pattern is crucial for the understanding of nervous system development and the neuronal cell fate. Therefore, this study can suggest novel strategies for promoting axon regeneration after injury and disease.

Recently, it was found that transcription factors can control the axon guidance process by regulating the expression and function of the genes that directly determine the responses of neurons to axon guidance cues and neurotransmitters in the vertebrate and invertebrate nervous systems (Polleux et al., 2007), indicating that the transcription factors might influence axon guidance decisions. Nevertheless, how transcription factors regulate axonal guidance cues remains a critical question in neurobiology.

Neuronal polarization in nervous system development

There are three steps during axon development: axon polarization, axon outgrowth and guidance, and finally axon branching (Lewis et al., 2013). Neuronal polarization into axon and multiple dendrites is the main step in normal nervous system development critical for functioning and essential for axon guidance to create proper neuronal connections.

The polarization process consists of five stages. In the first stage, during early

development, the neurons are born, and they display the lamellipodia and filopodia activity. Then, the neurons begin to extend several immature, short neurites in the second stage. The third stage represents a critical step; one of these neurites extends more rapidly to form a future axon that plays a role in the polarization step. In the fourth stage, the minor neurites mature into dendrites, and the axon migrates over a long distant to make contacts with the target tissue. Finally, in the fifth stage, the axon and dendrites continue to grow, including spine formation and the formation of functional synapses (Lalli, 2012).

Recently, it has been demonstrated that transcription factors can control the neuronal polarization. For example, FOXO transcription factors regulate neuronal polarity. FOXO proteins are expressed in the brain, including in cerebellar granule and hippocampal neurons when they undergo neuronal polarization. FOXO knockdown in these neurons leads to neuronal polarity defects (de la Torre-Ubieta et al., 2010), suggesting that FOXO transcription factors have roles in neuronal polarity regulation. Understanding the normal functions of transcription factors in neuronal polarization, axon guidance, and synapse formation might be essential steps to understanding neuronal disorders pathogenesis.

Growth cone morphology and mechanics of axon guidance

A critical part of axon guidance is changing neuronal morphology, including growth cone extension or retraction (Dent and Gertler, 2003; Lowery and Van Vactor, 2009). The detection of guidance cues occurs at the structure located at the leading edge of the growing axons, the growth cone, which was visualized first by Raymon Y. Cajal (Cajal, 1909). Growth cones can migrate along the anterior-posterior (AP) axis and the dorsal-ventral (DV) axis by extracellular guidance cues during *C. elegans* development (Hedgecock et

al., 1990). The growth cone structure has become a significant interest in the current developmental neuroscience field. Also, studying the pathfinding of the growth cone is fundamental to our understanding of axon guidance. Therefore, to gain a deeper understanding of axon guidance activity, I first summarize the growth cone structure.

The growth cone consists of different cytoplasmic structures, including lamellipodia, a thin region containing actin filaments at the leading edge of the cell, and filopodia, an extension of the growth cone, which is composed of bundles of F-actin. Together, Lamellipodia and filopodia control the growth cone movement (Huber et al., 2003). Functionally, the growth cone can be divided into three regions: the peripheral domain (P-domain), the transition domain (T-domain) and the central domain (C-domain) (Bouquet and Nothias, 2007). Both growth cones and migrating cells extend different numbers of filopodia and lamellipodia (Dickson, 2002). The growth cone has three cytoskeleton filaments: F-actin localized in the P and T-domains, neurofilaments, and finally microtubules localized in the C-domain (Bouquet and Nothias, 2007).

Previous studies demonstrated that axon guidance responds to either attractive (axon growth promoting) or repulsive (axon growth inhibiting) guidance cues depending on the receptors present in the leading edge of growth cones. Attractive cues cause growth cones to turn towards the guidance gradient, whereas repulsive cues cause growth cones to migrate away from guidance gradient (Dickson, 2002; Knobel et al., 1999). For example, UNC-6/Netrin guidance cue mediates the attraction by UNC-40/DCC receptor and it mediates the repulsion by UNC-5/UNC5 receptor (Norris and Lundquist, 2011), indicating that the UNC-6/Netrin acts bifunctionally in controlling filopodia protrusion polarity from growth cones and directing axon outgrowth.

Semaphorins and Ephrins can also act to attract or repel growth cones. Ephrin-B attracts forebrain commissural axons to EphA4 expression (Kullander et al., 2001), while Ephrin-B repels them away from EphB expression (Henkemeyer et al., 1996). SLT-1 acts to repel growth cones of different axons (Huber et al., 2003) through the receptor SAX-3/Robo (Hao et al., 2001).

Understanding the molecular mechanisms of axon guidance and how axons migrate to their final destinations is an essential theme of nervous system development (Alto et al., 2009), including formation proper neuronal connections. As mentioned above, extracellular guidance cues and their receptors can perform this function (Dickson, 2002).

The interaction between guidance cues and their receptors engage a specific downstream signaling cascade that regulates microtubule dynamics during axon outgrowth and guidance (Huber et al., 2003). The important pathways are Rho family of GTPases (Huber et al., 2003; Lowery and Van Vactor, 2009), and their regulators, including Cdc-42, Rac and RhoA, that regulate many aspects of neuronal morphology including axonal outgrowth and guidance by regulating actin cytoskeleton dynamics to steer growth cones (Rohatgi et al., 1999). Rho GTPases cycle between active GTP-bound state and inactive GDP-bound state to regulate actin cytoskeleton through the association with GAPs and GEFs respectively (Kolodkin and Tessier-Lavigne, 2011). Netrins, Slit, Ephrins, and Semaphorins influence Rho-GTPase activity. Attractive cues activate Rho GTPases leading to the extension of actin filament of the growth cone. In contrast, repulsive cues deactivate the Rho GTPases resulting in a retraction of growth cones (Zhang et al., 2003; Meyer and Feldman, 2002).

Furthermore, calcium signaling plays a role in growth cone steering. Calcium levels

can affect axon outgrowth and axon guidance decisions. Moderate levels of calcium promote the growth cone attraction, whereas the very low or very high levels of calcium promote growth cone repulsion (Gomez and Zheng, 2006). Additionally, cAMP (cyclic adenosylmonophosphate) regulates growth cone turning by altering the dynamics of cytoskeletal components. Neurons with high levels of cAMP are attracted to protein gradients, whereas neurons with a low level of cAMP repel from protein gradient (Song et al., 1998).

Finally, the *C. elegans* homolog, UNC-34, controls axon outgrowth activity in response to the following extracellular guidance cues: Wnt (CWN-1, CWN-2 and EGL-20), UNC-6 and SLT-1 (Gitai et al., 2003; Yu et al., 2002; Quinn et al., 2006; Fleming et al., 2010). UNC-34 functions with other proteins (UNC-115(abLIM) and Arp2/3) in controlling actin dynamics to regulate the formation of the growth cone filopodia (Struckhoff and Lundquist, 2003; Norris et al., 2009). The Arp2/3 complex is activated through WASP and WAVE/SCAR to enhance actin polymerization and axon outgrowth and organize epithelial cell during morphogenesis (Rohatgi et al., 1999; Patel et al., 2008).

Structure of the *C. elegans* nervous system

The nervous system of the *C. elegans* is an ideal model to study the mechanisms of neuronal function, axon guidance and outgrowth. *C. elegans* has a simple nervous system that is well characterized, with that exhibit a high functional and structural diversity. The *C. elegans* hermaphrodite nervous system is composed of 302 neurons including motor neurons, interneurons and sensory neurons (White et al., 1986; Sulston et al., 1983), as compared to the human brain that consists of about 100 billion neurons (Drachman, 2005).

Each of the 302 neurons has unique characters such as neurochemistry, morphology and synaptic network, and mediate the variety of neurons behaviors, including feeding, locomotion and egg laying (Brenner, 1974).

The *C. elegans* neurons can be divided into 118 different classes of neurons based on their function and morphology (White et al., 1986). These include sensory neurons, which are similar to somatosensory neurons in morphology such as having dendritic neurites (Albeg et al., 2011) that transfer the messages from sensory receptor to the spinal cord and brain; motor neurons that receive the messages from brain and spinal cord to outside in order to control muscle movement and gland activities; and the interneurons, which represent the large group of neurons that make connections with other neurons, not with sensory receptors, muscles, or glands.

The *C. elegans* HSNs and mechanosensory neurons in studying axon guidance

In this study, I focused on HSN, AVM, ALM, and PLM neurons to achieve a better understanding of axon outgrowth patterning and the guidance process. The HSN neuron is a hermaphrodite-specific motorneuron. HSNs are a pair of motorneurons that drive egg laying. HSN is helpful for our study of the role of molecules in axon guidance pathway because the HSN cell body, which is located in mid-body of the worm, is large enough to visualize with transgenic markers using the florescence microscope (Desai et al., 1988).

The development of HSN morphology has been characterized (Adler et al., 2006). After the egg hatching, the HSN cell body extends multiple short neurites near the vulva in random directions (White et al., 1986). These neurites will extend to the ventral side of the neuron, and one of these neurites develops into a single axon that extends toward the

ventral nerve cord and then turns anteriorly towards the nerve ring. HSN axon development can be visualized by *kyIs262 [unc-86::myr-GFP]* (Adler et al., 2006) and *zdIs13 [Ptph-1::gfp]* transgenic markers.

Ventral HSN axon outgrowth is induced by UNC-6/Netrin guidance cue through the localization of UNC-40/DCC to the ventral side of the HSN cell body (Adler et al., 2006). In *unc-6* and *unc-40* mutants, the HSN axon can grow normally, demonstrating that the HSN neuron is regulated independently of the UNC-6/Netrin pathway, but it is misguided in these mutants (Adler et al., 2006). Additionally, abnormal HSN neuron development and function cause many egg laying defects in hermaphrodite animals (Trent et al., 1983).

There are six mechanosensory neurons: ALMR, ALML, PLMR, PLML, AVM, and PVM found in *C. elegans*. One pair, ALML and ALMR, sends axons that migrate in an anterior direction toward the nerve ring. The other pair is located at the end of the worm tail; this pair includes PLML and PLMR neurons. PLM axons extend anteriorly near the vulva in wild-type animals. The third pair of neurons, located in different positions, is the AVM neuron at the mid anterior and the PVM neuron at the mid posterior of the worm. These two neurons, AVM and PVM, send axons that migrate ventrally to reach the ventral nerve cord and then migrate anteriorly toward the nerve ring (Chalfie and Sulston, 1981). ALM, AVM, and PVM axon development can be visualized by the *zdIs5 [Pmec-4::GFP]* transgenic marker.

Genetic screens in *C. elegans* discovered several genes required for mechanosensory neuron development. These genes are called *mec* (mechanosensory defective); *mec* genes include transcription factors (Chalfie et al., 1981; Chang et al., 1996). The first set of genes includes *mig-2*, a small GTPase (Zipkin et al., 1997), and *mig-13* (Sym et al., 1999). The

second set of genes code for the UNC-6/Netrin and its receptors that control a different axonal migration (Kim et al., 1999). Finally, the third set of genes includes *unc-2* (Schafer and Kenyon, 1995), *egl-19* (Lee et al., 1997), and *unc-43*, a caMII kinase (Reiner et al., 1999) that affect the migration of AVM cell bodies.

Several guidance molecules control axon guidance

In the past few years, an extensive body of research in molecular biology and genetics has led to the characterization of four families of canonical guidance molecules and their receptors that are involved in axon guidance, including UNC-6/Netrin and its receptors, UNC-5/UNC5 and UNC-40/DCC, SLT-1 and its receptor, Robo, semaphorins/Plexin (PLX) and Ephrins. These guidance cues are members of highly conserved families, and they have been studied in invertebrate and vertebrate systems in the process of axon migration (Tessier-Lavigne and Goodman, 1996; Yaron and Zheng, 2007; Dickson, 2002).

It was found that other molecules can act as axon guidance cues; these include Shh, BMPs (Ishii, et al., 1992; Harris et al., 1996; Mitchell et al., 1996; Serafini et al., 1994; Brose et al., 1999), and WNTs and their FZ receptors (Pan et al., 2006). Recently, studies demonstrated that some of the growth factors and cell adhesion molecules could also act as guidance cues (Kolodkin and Tessier-Lavigne, 2011).

Most of axon guidance proteins have conserved peptide domains that are important for protein-protein interactions (Dickson, 2002), and polarize protrusive activity within HSN neurons during axon generation (Adler et al., 2006; Quinn et al., 2006). The different functions of guidance cues, their ability to bind with their receptors at the membrane, and

their combination with alternative mRNA splicing result in various signaling pathways (Dickson, 2002).

The SLT-1 guidance proteins are large secreted proteins that play an important role in dorsal-ventral and anterior-posterior guidance (Hao et al., 2001). SLT-1 s are expressed in dorsal muscles, and they promote ventral guidance through their receptor SAX-3/Robo, which is required for ventral guidance of HSN neurons, whereas *sax-3* mutants cause defects in HSN migration (Zallen et al., 1998). It was also found that AVM axons require SLT-1 to mediate repulsive activity (Hao et al., 2001).

The Semaphorins are a large conserved family of guidance proteins that were first identified in grasshoppers for their crucial role in axon guidance. Semaphorins play roles in both epithelial morphogenetic cell movement and axon guidance (Kolodkin et al., 1992). They are expressed at the midline and in the ventral tissue, and they are required for midline guidance. Therefore, the lacks of a Semaphorin receptor, a neuropilin-2, causes defects in midline guidance in mice (Zou et al., 1999). There are several classes of Semaphorins: classes 1 and 2 were found in invertebrates; classes 3, 4, 5, and 6 were found in vertebrates; and finally there is a class that is encoded by viruses (Raper, 2000).

The Ephrins guidance cues are members of Eph family of receptor tyrosin kinase that play roles in neuronal development and morphogenesis; they regulate axon guidance and mediate either attraction or repulsion processes (Wilkinson, 2001).

WNT/EGL-20 signaling

EGL-20/Wnt proteins are members of a secreted cysteine-rich glycoprotein family that controls different biological processes of development, including tissue development,

proliferation, cell polarity, division, migration, and differentiation (Zecca et al., 1996; Angers and Moon, 2009). In *C. elegans*, Wnt signaling has a role in embryonic and neuronal migration during nervous system formation. Wnt controls HSN, CAN, ALM, and BDU neuron migration during embryogenesis and QL and QR migration in early larval stage development (Sulston and Horvitz, 1977; Hedgecock et al., 1987).

Wnt signaling is essential for axon guidance along the anterior-posterior axis in *Drosophila* and mice. Wnt 4 can bind to the Fz3 receptor to guide the axons by the attraction process (Lyuksyutova et al., 2003; Yoshikawa et al., 2003). There are several homologs of Wnt genes and their receptors in different model systems. For example, *C. elegans* genome has five Wnt genes and four Frizzled (Fz) receptors (Korswagen et al., 2002); the *Drosophila* genome encodes four Wnt molecules; and the human genome encodes 17 Wnt molecules (Ruvkun and Hobert, 1998). Wnts signal through their receptors (Frizzled) by binding to the cysteine-rich domain (Bhanot et al., 1996; Dann et al., 2001).

Mutations in Wnt signaling lead to a different number of human development abnormalities and diseases, including breast cancer, colon cancer, skeletal and human disorders, such as neural tube closure birth (Logan and Nusse, 2004). In addition, Wnt overexpression leads to a variety of cancers (Polakis, 2000).

There are three known Wnt signaling pathways; the canonical Wnt pathway, the planar cell polarity pathway, and the non-canonical Wnt pathway (Wnt/Ca2+). The canonical Wnt pathway was identified in *Drosophila.* In this signaling pathway, the adherence junction associated-protein β-catenin accumulates and translocates into the nucleus (He et al., 2004; Gordon and Nusse, 2006). The function of the canonical Wnt pathway prevents cytoplasmic β-catenin degradation by the GSK3β/Axin/APC complex (MacDonald et al.,

2009). Also, the Wnt and transmembrane receptors interact in the Frizzled protein family (Logan and Nusse, 2004). Planar cell polarity (PCP) is the second Wnt signaling pathway. The term planar cell polarity refers to the coordinated orientation of cell and cellular structures along an axis within a single plane of epithelial surface (Vladar et al., 2009). Finally, the non-canonical pathway does not require β-catenin transcription activity and plays a major role in ventral signaling during Xenopus embryonic development (Saneyoshi et al., 2002). In this pathway, Wnt signaling results in the release of intracellular calcium (Veeman et al., 2003).

UNC-6/Netrin signaling

Netrin is a laminin-related extracellular protein expressed in glia, neurons, and muscle cells; Netrin is secreted from the ventral nerve cord that runs between the basement membranes of the hypodermis and body wall muscles on the ventral side of the worm (Wadsworth et al., 1996). Since this work is more relevant to UNC-6/Netrin and its receptors, this signaling pathway will be reviewed in more details in this section.

The Netrins are a small family of conserved axon guidance proteins that regulate axon guidance toward the ventral midline (Huber et al., 2003). The exploration of Netrins came as result of the search for a chemoattractant for vertebrate axons. During more than six hundred million years of evolution, the UNC-6/Netrin signaling pathways are conserved across many species from nematodes to vertebrates (Hedgecock et al., 1990). *C. elegans* has one Netrin only which is UNC-6, whereas *Drosophila* has two Netrins, which are Netrin-A and Netrin-B, and mammals have five Netrin homologs (Lai Wing Sun et al., 2011). Netrin-A and Netrin-B are expressed in the ventral midline and they guide the axon

(Harris et al., 1996).

UNC-6/Netrin is divided into multiple domains, which are N-terminus signal peptide, followed by the laminin domains VI, V-1, V-2, and V-3 as well as C-terminal domain. VI, V-2, and V-3 domains are required for dorsal migration, whereas V-1 and V-3 domains are required for ventral migration. The domain VI is necessary for axon attraction and repulsion. The C-domain regulates Netrin-mediated axonal branching. In the absence of C-domain (UNC-6ΔC), the UNC-6 can express and induce ectopic axonal branching in ventral cord motorneurons (Lim et al., 1999).

The Netrin homolog was first identified in the nematode *C. elegans* in genetic screens. Later, vertebrate homologs Netrins-1 and Netrins-2 were isolated (Harris et al., 1996; Ishii et al., 1992; Meyerhardt et al., 1999; Mitchell et al., 1996). Netrins were first purified from chicken brain (Serafini et al., 1994). Netrin-1 is expressed by the floor plate of the spinal cord, whereas Netrin-2 is expressed in the lower two thirds of the spinal cord (Kennedy et al., 1994). Netrin-1 and Netrin-2 play significant roles as axonal guidance cues in central nervous system regions such as hippocampal, spinal commissural, retinal, and dopaminergic axon guidance (Kennedy et al., 1994; Colamarino and Tessier-Lavigne 1995; Braisted et al., 2000; Shewan et al., 2002; Lin et al., 2005).

In addition, Netrin-3, Netrin-4, Netrin-G-1, and Netrin-G-2 were identified in human and mice (Barallobre et al., 2005). Netrin-3 is expressed in the peripheral nervous system during development, and is less expressed in the central nervous system, whereas Netrins-G-1 and Netrin-G-2 are expressed in the central nervous system in vertebrates only (Barallobre et al., 2005).

UNC-6/Netrin is required for dorsal and ventral axon migration (Wadsworth et al.,

1996).UNC-6/Netrin is arranged along cellular surfaces to interact with cell surface receptors to direct axons migration during neural development (Wadsworth and Hedgecock, 1992). UNC-6/Netrin has been observed that some neurons that express UNC-40/DCC receptor extend ventrally towards the UNC-6/Netrin source; while some neurons expressing both UNC-5/UNC5 and UNC-40/DCC receptors extend dorsally away from UNC-6/Netrin (Lim and Wadsworth, 2002; Andrews et al., 2008).

Moreover, UNC-6/Netrin promotes ventral axon formation by localizing the UNC-40/DCC receptor ventrally (Adler et al., 2006). *unc-6* mutants have axon guidance defects in neurons causing animals with uncoordinated (unc) phenotype (Killeen and Sybingco, 2008; Hedgecock et al., 1990). Two other mutants were reported; these are *unc-40* with ventral and dorsal axon guidance defects and *unc-5* with dorsal axon guidance defects (Hedgecock et al., 1990). These mutants have severe uncoordinated phenotypes during dorsal migration of motor neurons. For example, the DA, DB, DD, VD, and AS motor neurons exhibit defects in migration and do not reach their targets in *unc-6, unc-40* and *unc-5* null mutants (Hedgecock et al., 1990). There is evidence that these three mutants also cause defects in the migration of non-neutral cells (Hedgecock et al., 1987).

Additionally, the UNC-6/Netrin pathway has important roles in the development and regulation of tissue outside the nervous system. A recent study demonstrated that the Netrins act as cancer detection and diseases markers. Netrin and its receptors have a role in controlling colorectal tumorigenesis by regulating the apoptosis (Mazelin et al., 2004). The absence of Netrin-1 leads to the promotion of the apoptosis pathway (Arakawa, 2004), whereas the overexpression of Netrin-1 leads to the reduction of apoptosis and the formation of spontaneous hyperplasic lesion and adenomas in the intestinal tract in mice

(Forcet et al., 2002). Also, in different human cancers, there is an overexpression of Netrin. For instance, metastatic breast cancer shows a high level of Netrin-1 (Fitamant et al., 2008).

Other studies demonstrated that the Netrin pathway has a role during branching morphogenesis of the lung and mammary gland tissue by controlling cell migration and cell adhesion development. It controls the development of complex tubular organs (Srinivasan et al., 2003; Liu et al., 2004; Lu et al., 2004; Navankasattusas et al., 2008).

Structure of UNC-40/DCC receptor and function

UNC-40/DCC encodes a nematode homolog of the vertebrate, Deleted in Colorectal Cancer (DCC), and Neogenin (Hedrick et al., 1994; Vielmetter et al., 1994). The *DCC* gene, which identified within the18q chromosome region, acts as a tumor suppressor in the colorectal carcinomas of human (Fearon et al., 1990; Cho and Fearon, 1995).

UNC-40/DCC has extracellular domains, which are immunoglobulin domains, containing four immunoglobulin (Ig) repeats, and fibronectin type III domains, containing six fibronectin type III (FN3) repeats that play a major role to bind to UNC-6/Netrin. The cytoplasmic domains of UNC-40/DCC are composed of three conserved motifs, P1, P2, and P3 (Wang et al., 2014). The P1 domain is a highly conserved seventeen amino acid motif, and the P2 domain contains a high level of proline residues and four putative SH3 domain-binding motifs. The P3 domain contains putative phosphorylation sites (Kolodziej et al., 1996). It was found that the UNC-40/DCC requires UNC-34 (that acts through the P1 cytoplasmic motif), and UNC-115, and CED-10 (that act through the P2 motif (Gitai et al., 2003)). UNC-40/DCC polarizes within the plasma membrane toward UNC-6/Netrin and forms F-actin at the cell cortex (Wang et al., 2014). In a recent study, it was found that

the intracellular domain of the UNC-40/DCC receptor acts as a transcription activator for axon guidance (Neuhaus-Follini and Bashaw, 2015).

The *unc-40* mutant was first found in *C. elegans* because of its effect on behavior (Brenner, 1974). UNC-40/DCC was identified to guide mesodermal cells and pioneer axons (Hedgecock et al., 1987; Hedgecock et al., 1990). The ventral extension of HSN axon requires UNC-40/DCC. In *unc-40* mutants, the HSN axon grows anteriorly instead of ventrally (Adler et al., 2006). UNC-40/DCC guides the ventral axon extension of mechanosensory neurons, including AVM neuron in response to UNC-6/Netrin guidance cues (Chan et al., 1996). As a conclusion, the UNC-40/DCC is required for cell migration and axon guidance.

UNC-40/DCC also has a role in controlling muscle differentiation, and is required for muscle arms extension. In the absence of UNC-40/DCC, many of muscle arms fail to extend (Alexander et al., 2009). Also, it was found that the UNC-40/DCC is required during the invasion of the anchor cell. *unc-40* mutants cause delays in anchor cell invasion (Manitt et al., 2001; Ziel et al., 2009). UNC-40/DCC polarization to the invasive cell membrane depends on UNC-6/Netrin.

Additionally, in the absence of UNC-6/Netrin, the UNC-40/DCC may respond to other guidance cues to guide the SDQR neuron, a sister neuron of AVM. The SDQR axon, born in the first larval stage, grows dorsally to the lateral edge of the body muscles and then anteriorly toward the nerve ring. SDQR migrates away from ventral UNC-6/Netrin expression; this indicates that the UNC-6/Netrin repels SDQR axon. However, in the background of an *unc-6* mutant, the SDQR axon grows ventrally. In *unc-40* mutants, the SDQR axon has defects in migration, but they are less severe than the *unc-5* and *unc-6*

mutants (Kim et al., 1999; Kim and Wadsworth, 2000).

Structure of UNC-5/UNC5 receptor and function

UNC-5/UNC5 is a transmembrane UNC-6/Netrin receptor that is expressed on the cell surface and mediates repulsion by the UNC-6/Netrin gradient. In vertebrates, UNC-5 has four homologs, which are UNC5H1, UNC5H2, UNC5H3, and UNC-5H4 (Ackerman et al., 1997; Leonardo et al., 1997; Engelkamp, 2002). The extracellular domain of these receptors is composed of two immunoglobulin (Ig) domains that bind to Netrin and two thrombospondin (TSP) domains. The intracellular domain of Ig and TSP includes three conserved domains, which are the ZU-5 domain (important for UNC-40 dependent functions), the Z-D region (important for UNC-40 independent function), and the C-terminal Death Domain (DD), (role unclear (Leonardo et al., 1997; Killeen et al., 2002; Aravind et al., 2001)). *unc-5* plays an important role in axon migration; specifically, *unc-5* is required for axon and mesodermal cell migration along dorsoventral axis. In contrast, *unc-5* null mutations cause defects in axon pathfinding in the dorsal direction (Hedgecock et al., 1990; Leung-Hagesteijn et al., 1992).

UNC-53/NAV2 adaptor protein

UNC-53/NAV2 is a cytoskeletal binding protein that controls cell migration and growth cone extension during embryogenesis in *C. elegans*. UNC-53 associates with different actin binding proteins such as α-actinin and dystrophin, which play a role in crosslinking actin filaments to bundles (Stringham et al., 2002). Additionally, UNC-53 was found associated with microtubules and actin filaments as a linker actin filament to

microtubule (Stringham and Schmidt, 2009). Three mammalian UNC-53 homologs have been identified, which are NAV1, NAV2, and NAV3. These were named as Neuron Navigators based on their function in cell migration and axon outgrowth in mammals and *C. elegans*. NAV1 is expressed in the nervous system, whereas the NAV2 is expressed in placenta, kidney, heart, and in the nerve tissues. Finally, NAV3 is expressed in the nervous system and it decreases after brain injury in nerve cells (Stringham and Schmidt, 2009).

unc-53 maps between *bli-1* and *rol-1*, to the right of the center of chromosome II (Brenner, 1974). *unc-53* comprises 23 exons. UNC-53 is a 1654 amino acid long protein (Stringham, 2002). UNC-53/NAVs have multiple domains, a calponin homology (CH) domain at the N-terminus, LKK actin binding motif or actin binding domain (ABD), polyproline-rich domain (PRD), and (AAA) domain at the C-terminus, which is in the actin binding proteins. UNC-53/NAV2 interacts with ABI-1 through calponin homology (CH) domain (Stringham and Schmidt, 2009). The *unc-53* expression pattern has been using green fluorescent protein (GFP). *unc-53*::GFP expression is detected in head neurons, tail neurons and body wall muscles in the embryo. Furthermore, the *unc-53*::GFP expression in males is detected in diagonal and spicule retractor muscles (Stringham et al., 2002).

unc-53 is required for axon migration along the anteroposterior axis. UNC-53 is needed for neuronal outgrowth in mechanosensory neurons including ALM, PLM, AVM, and PVM neurons. UNC-53 plays a role in remodeling actin cytoskeleton at the leading edge of the cell (Stringham and Schmidt, 2009) and is also involved in receptor trafficking in ventral axon guidance of AVM and HSN neurons (Hekimi and Kershaw, 1993; Stringham et al., 2002). Several studies demonstrated that the mutations in the *unc-53* gene cause multiple guidance phenotype defects in axon pathfinding (Hekimi and Kershaw,

1993; Schmidt et al., 2009; Stringham et al., 2002; Stringham and Schmidt, 2009), whereas the *unc-53* overexpression increases the growth in muscle cells during embryogenesis (Stringham and Schmidt, 2009).

unc-53 has a role in the UNC-40 localization. Whereas the UNC-40::GFP transgene is localized asymmetrically to the ventral side of the HSN neuron in wild type (Xu et al., 2009), in *unc-53* mutants, the UNC-40::GFP localization distributes randomly.

The role of transcription factors during neuronal development

Transcription factors are specific DNA binding proteins that often are involved in regulate gene expression and tissue patterning. Transcription factors are highly involved in animal development (Lemons and McGinnis, 2006), pluripotency and differentiation processes (Takahashi and Yamanaka, 2006).

Transcription factors are divided into multiple families depending on the DNA binding motif structure, which is usually composed of positively charged amino acid residues. Several families of transcription factors have been known to play crucial roles in different aspects of nervous system development. In particular, these transcription factors control the genes that regulate neuronal differentiation during the animal development process, survival, proliferation, and axon migration and guidance (Thiel, 2006). These processes occur by the binding of transcription factors to gene promoter DNA (Kumar et al., 2004).

The transcription factors expressed in neurons are necessary for the identity of neurons, as well as for neuron differentiation. Transcription factor expression increases after axon injury (Moore and Goldberg, 2011). Transcription factors are often detectable

in the nucleus, however some transcription factors have been detected in axons (Otis et al., 2006). Transcription factors can be detectable in axons, like axonal proteins, as a result of anterograde transport from the cell body of neurons. Most transcription factors have NLS sequences that play roles in transporting them to the nucleus (Hanz et al., 2003; Perlson et al., 2005).

The transcriptional control of axon guidance has been studied recently (Polleux et al., 2007). Studies have well documented the correlations between transcription factors and the correct target areas of their axons in zebrafish, mice, chicks, and *C. elegans* (Tsuchida et al., 1994; Apple et al., 1995; Thor and Thomas, 2002), indicating that transcription factors are required for motor axon trajectory.

It is important to identify the role of transcription factors directly in neuronal development. In mice and chicks, the Isl1, Lhx3, and Nkx6 transcription factors regulate motor neuron development, and the *Drosophila* orthologs of these transcription factors regulate axon guidance (Santiago and Bashaw, 2014). Understanding how transcription factors control axon guidance may lead to new approaches to understanding neuronal development.

Recent studies have reported different transcription factors that regulate axon outgrowth ability and regeneration *in vitro* and *in vivo*; these include p53, SnoN, E47, CREB, STAT3, NFAT, c-Jun, ATF3, Sox11, NFκB, and Kruppel-like factors (KLFs) (Moore and Goldberg, 2011). As for axon guidance, increasing evidence suggests that axonal guidance is controlled by the transcriptional regulation of genes. Additionally, studies have shown that the POU transcription factor regulates RGC axon pathfinding in the vertebrate visual system, suggesting that this transcription factor controls molecules

that mediate growth cone responsiveness to guidance cues for correct axon pathfinding (Erkman et al., 2000). A more recent study has identified that the Arnt2 and Sim1a transcription factors control lateral position through downregulating Robo3 receptors in zebrafish hypothalamic neurons (Schweitzer et al., 2013). In the absence of either transcription factor, the Robo3 receptor was misexpressed, and the axon pathfinding shifted, resulting in altering phenotype (Santiago and Bashaw, 2014). It has also been found that the GATA transcription factor Grn controls axonal projections by regulating the *unc-5* guidance receptors expression (Zarin et al., 2012).

Basic structure of ZEB family of transcription factors

ZEB family proteins are complex transcription factors that control diverse aspects of neuronal development. ZEB transcription factors are responsible for many human diseases. They consist of two members, ZEB1 and ZEB2. ZEB1 includes δ-EF1, Tcf8, Nil-2-a, Bzp, Meb1, Zfhep, Zfhx1a, and Areb6. ZEB2 includes Zfhx1b and Smad-interacting protein1 (SIP1). Structurally, ZEB proteins have many functional domains, which are two zing finger clusters (N-terminal cluster and C-terminal cluster), and with a homeodomain in the middle. The N-terminal cluster has four zing-fingers, whereas the C-terminal cluster has three zinc–fingers (Vandewalle et al., 2009).

Since ZEB1 and ZEB2 are able to bind to the same nucleotide sequence, they can control the expression of the same gene (Remacle et al., 1999). The ZEB protein domains that are in the middle regions play crucial roles in transcriptional activation or repression. These domains include the C-terminal binding protein (CTBP) interacting domain (CID), Smad-binding domain (SBD), and homeodomain (HD) (Vandewalle et al., 2009; Browne

et al., 2010).

ZEB1 was first discovered in chicken embryos as a nuclear factor that represses the lens specific delta-crystalline (Funahashi et al., 1991). ZEB2 was first identified by a two-hybrid screen in yeast (Verschueren et al., 1999). ZEB1 and ZEB2 proteins can bind to R-Smad activator through the SBD. The ZEB1-Smad complex activates transcription, whereas the ZEB2-Smad complex represses transcription. It was found that ZEB1 and ZEB2 proteins can act as transcriptional activators or transcriptional repressors through the transcriptional co-activators, p300 and p/CAF, and co-repressors, CTBP (Postigo et al., 2003).

In *Drosophila*, the Zfh1 is expressed in muscles and motor neurons (Lai et al., 1991). In mice, the δEF1 is expressed in neural crest as skeletal muscles and notochord (Higashi et al., 1997). Knockout mice have shown that ZEB proteins function in neuronal development. For instance, ZEB2 is important for central nervous system myelination by the modulation of Wnt-β-catenin and BMP-Smad pathways (Weng et al., 2012). Moreover, ZEB1 and ZEB2 can regulate cellular processes, including apoptosis and proliferation (Vandewalle et al., 2009).

ZEB protein family as transcriptional activators

There is much interest in the biological roles of the ZEB proteins family. It has been shown that the ZEB family proteins can have dual roles as transcriptional activators and as transcriptional repressors (Gheldof et al., 2012). ZEB proteins activate transcription mechanisms by regulating their target genes. For instance, ZEB1 can activate transcription of *ser-7b* and *ceh-28* (Ramakrishnan and Okkema, 2014).

In mammals, ZEB1 functions as the transcription activator of the ovalbumin gene in response to estrogen signaling (Dillner and Sanders, 2004). The capacity of ZEB1 to control genes involved in differentiation, migration, and proliferation makes this transcription factor necessary for normal homeostasis and development. Also, the ZEB1 protein plays a significant role in a smooth muscle differentiation by activating the SM α-actin promoter during A404 SM cell line differentiation (Nishimura et al., 2006). In *Drosophila*, Zfh1 activates FMRF gene expression in neurons (Vogler and Urban, 2008).

ZEB protein family as transcriptional repressors

As with transcription activation, transcription repression is a fundamental process that regulates gene expression during differentiation and development events in eukaryotic (Ogbourne and Antalis, 1998). ZEB1 and ZEB2 can repress the transcription of many genes involved in development (Postigo and Dean, 1999). ZEB can repress transcription through repressor motifs located between the N-terminal and C-terminal domains of these proteins, including the corepressors CtBP (C-terminal binding protein) (Zhang et al., 2002). CtBP, a transcription corepressor, is a NAD-dependent that interacts with many DNA binding proteins to repress transcription (Chinnadurai, 2002). CtBP is a 48 kDa cellular phosphoprotein that binds to the C-terminal domain of the E1A human adenovirus protein (Boyd et al., 1993; Schaeper et al., 1995). Ct BP can interact with PLDLS sequence located in the C-terminal region of the E1A oncoprotein. The deletion of PLDLS sequence increases the tumorigenesis and metastasis, and also increases the ability of E1A protein to interact with *ras* in cell transformation, demonstrating that CtBP plays a role in regulating cell proliferation; also, CtBP is a corepressor because it does not bind directly to DNA, but

it binds to a promoter through a Gal4 DNA binding domain to repress transcription (Postigo and Dean, 1999). It was shown that Zfh-1, a ZEB homolog in *Drosophila* that regulates somatic and cardiac myogenesis during embryogenesis and synapse generation during motoneuron development (Vogler and Urban, 2008), can bind to E-box sequences and interact with CtBP-1 and CtBP-2 to repress the transcription (Postigo and Dean, 1999).

A study demonstrated that ZEB is a strong suppressor of E-cadherin expression which are an essential molecules that maintains epithelial cell characteristics (Peinado et al., 2007). In contrast, the inhibition of ZEB1 and ZEB2 by miR200 restores the expression of E-cadherin (Kajita et al., 2004). Also, ZEB can repress *mef2* expression during the development of muscles (Postigo and Dean, 1999). Likewise, *zag-1* can repress the transcription of several aspects of neuron characteristics such as axonal development and gene expression, axon structure and expression of cell-surface proteins and transmembrane receptors during neuronal differentiation (Clark and Chiu, 2003; Wacker et al., 2003).

Additionally, the ZEB protein acts as a transcription repressor in regulating the differentiation of muscles and lymphoid in vertebrates. It was found that ZEB1 can repress a collagen gene, *Col1a2*, and collagen type II synthesis in mice (Kato et al., 2007). Thus, depending on the target tissue and gene, the ZEB proteins can repress gene expression through active transcriptional repression by identifying many repressor domains in the central regions of the ZEB proteins.

ZEB protein family transcription factors have functions in development and disease

As mentioned earlier, the ZEB transcription factor is the protein that controls gene expression by binding to DNA to make the genes more or less active. The ZEB family of

transcription factors plays critical roles in embryonic development and cell cycle. Many kinds of human diseases are related to transcription factor mutations, such as different types of cancer (Gregg, 1999).

The ZEB family of zinc finger transcription factors are important regulators during embryonic development. ZEB1/dEF1 knockout mice exhibit severe respiratory defects, many skeletal defects that include limbs and malformed ribs, and T-cell deficiency of the thymus (Vandewalle et al., 2009). These phenotypes demonstrate that the δEF1/ZEB1 has a major role during mice development.

ZEB protein family induces tumor metastasis

ZEB1 and ZEB2 proteins are involved in regulating a great number of pathological and physiological processes (Vandewalle et al., 2009). ZEB1/ZEB2 play a role in tumorigenesis, tumor invasion and metastasis, and chemotherapy resistance. Several target genes are activated or repressed by ZEB proteins (Vandewalle et al., 2009). Early evidence showed that the ZEB1 and ZEB2 proteins regulate tumor progression by inducing epithelial to mesenchymal transition (EMT) (Comijn et al., 2001; Shi et al., 2003; Eger et al., 2005). EMT is an essential process during embryonic development in different tissue formation such as heart, peripheral nervous system, neural crest, and musculoskeletal (Thiery, 2003). ZEB/2 was also found to be a repressor of E-cadherin (Vandewalle et al., 2009), which is a cell adhesion molecule in the plasma membrane that can repress tumor and invasion. E-cadherin gene mutations are found in breast cancer (Berx et al., 1995). ZEB1 and ZEB2 can also repress P-cadherins, R-cadherins, and epithelial cell polarity markers in desmosome, gap junctions, and tight junctions (Vandewalle et al., 2005; Aigner et al.,

2007).

Additionally, ZEB1 has a role in colon cancer metastasis; ZEB 2 has a role in gastric, ovarian, and pancreatic cancers (Peinado et al., 2007). ZEB1 can regulate by Wnt signaling pathway and induce invasive colorectal cancer (Sanchez-Tillo et al., 2011). Studies demonstrated that the ZEB1 knockdown in breast and colorectal cancer models lead to partial restoration of differentiation of epithelial with the reexpression of E-cadherin and epithelial cancer suppressor genes (Eger et al., 2005; Spaderna et al., 2006). These results indicate that ZEB proteins may act as diagnostic and as therapeutic cancer targets in future work.

Mutations in ZEB proteins cause Mowat Wilson Syndrome

Mowat Wilson Syndrome (MWS) is a congenital disorder that causes abnormal facial and severe congenital abnormalities, including heart diseases and Hirschsprung disease (Mowat et al., 1998). Mutations in the *ZEB2* gene are related to Mowat Wilson Syndrome. The relationship between MWS and ZAG-1 proteins result in the deletion or heterozygous mutation of the *Zfhx1b (ZEB2/SIP1)* gene located on chromosome II (Mowat et al., 2003).

The homeodomain transcription factor ZAG-1: a member of ZEB family

ZAG-1 in *C. elegans* is a Zn-finger-homeodomain protein. It belongs to the vertebrate ZEB family of transcription factor C2H2 Zn-finger/homeodomain proteins. ZAG-1 is a highly conserved transcription factor. ZAG-1 was discovered in screens for mutations that cause defects during development of PVQ (a pair of interneurons located in the lumbar ganglia in the tail of *C. elegans*). *zag-1::*GFP is expressed in most or all neurons

during embryogenesis and in some muscles (Clark and Chiu, 2003; Wacker et al., 2003).

Structurally, ZAG-1 has five C2H2-type zinc finger domains: two domains at the N-terminal domain and three domains at the C-terminal domain, as well as a single homeodomain in the middle, followed by CtBP (Clark and Chiu, 2003). The N-terminal and C-terminal zinc finger domains are the same. The ZAG-1 homeodomain is similar to homeodomains in LIM homeodomain proteins, like LIM-4 and MEC-3 (Chinnadurai, 2002).

zag-1 maps between *vab-2* and *dpy-13* on chromosome IV. Expression of the F28F9 cosmid can rescue the uncoordinated phenotype of *zag-1* worms (Clark and Chiu, 2003). *zag-1* has seven exons, and it encodes 596 amino acids; its alleles, *zd85* and *zd86,* are not null and each allele contains a single point mutation within the *F28F9.1* gene. Both of these mutants exhibit an uncoordinated behavioral phenotype. Both alleles lack the C-terminal Zn-finger domains, whereas they retain the N-terminal Zn-finger domains, homeodomain, and CtBP interaction motif. Therefore, they keep a partial function of ZAG-1 (Clark and Chiu, 2003).

The animals in *zag-1* mutants can move forward, but they have difficulty moving backward. *zag-1* was identified in genetic screens for mutants with ventral cord axon outgrowth defects (Vandewalle et al., 2009). *zag-1* acts as a transcriptional repressor to regulate multiple neuron characteristics, including ion channel expression, cell position, biosynthetic enzyme, and axonal development (Wacker et al., 2003).

While *C. elegans* and *Drosophila* have only one homolog, *zag-1* and *Zfh1*, respectively (Clark and Chiu, 2003), vertebrates have two homologs which are ZEB1/ δEF1and ZEB2/SIP1 (Verschueren et al., 1999). *zag-1* is described as a repressor that

regulates its expression negatively by interacting with conserved sequences in its promoter and probably introns (Clark and Chiu, 2003; Wacker et al., 2003). *zag-1* can also act as a transcriptional switch and control the fate of neuronal cells in sensory neurons (Smith et al., 2013).

ZAG-1 acts as an upstream regulator of CEH-28 transcription factor and regulates many aspects of M4 pharyngeal neuron differentiation, but *zag-1* null mutations cause defects in M4 differentiation, and the M4 cell cannot respond to stimulation by serotonin to make isthmus peristalsis (Ramakrishnan and Okkema, 2014). Therefore, null mutants of *zag-1* are lethal because the larva is unable to eat food and die during the first larva stage; this death results from defects in M4 pharyngeal muscle development. This demonstrates that the *zag-1* has another important role in the development of pharynx (Wacker et al., 2003).

Statistically dependent asymmetric localization (SDAL) for axon outgrowth patterning

The neurons extend one or several processes from the neuron cell body. How a neuron becomes polarized is unknown. Previous studies have shown that the extracellular guidance cues, such as UNC-6 /Netrin and Wnt/EGL-20, have a role in inducing neuronal polarity and directing axons to the appropriate locations in *C. elegans* (Adler et al., 2006; Prasad and Clark 2006; Hilliard and Bargmann 2006; Montcouquiol et al., 2006). Furthermore, it was found that the UNC-40::GFP asymmetric localization is associated with neuronal polarity within the neuron cell membrane. Genetic evidence indicates that UNC-40/DCC, a central regulator of polarization in multicellular animals (Ziel and

Sherwood, 2010), mediates several signals that regulate the polarization and orientation in response to UNC-6/Netrin. UNC-40::GFP clusters randomly along the cell surface in response to polarization signal, whereas the orientation signal causes the UNC-40::GFP to cluster in a specific direction (Xu et al., 2009; Kulkarni et al., 2013; Tang and Wadsworth, 2014).

During HSN development, UNC-40/DCC receptor clusters asymmetrically at the ventral side of neuron in response to UNC-6/Netrin extracellular guidance cue. In *unc-6* mutants, the UNC-40 is uniformly distributed, whereas in double mutants such as *unc-6(ev400);unc-53(n152)* and *unc-6(ev400);unc-40(ur304)*, the UNC-40::GFP is asymmetrically clustered either at anterior, posterior or ventral directions of HSN neuron cell body (Adler et al., 2006; Kulkarni et al., 2013), suggests that the UNC-40::GFP can cluster in the absence of UNC-6/Netrin guidance cues, but UNC-40::GFP clusters in different sites of the neuron's cell body, indicating that the neuron can randomly determine the direction of axon outgrowth activity and then the axon grows in various directions (Xu et al., 2009; Kulkarni et al., 2013; Tang and Wadsworth, 2014). These results provide evidence that the axon outgrowth activity is a stochastic process in response to UNC-6/Netrin guidance cue.

We refer to this process as 'Statistically dependent asymmetric localization' (SDAL). According to stochastic model, UNC-40::GFP clusters to promote axon outgrowth activity in different directions of neuron by different signals. For instance, the UNC-6/Netrin signal enhances the clustering of UNC-40::GFP to the ventral side of neuron toward UNC-6 source. The EGL-20 and Muscle/Epidermis matrix signals inhibit the anterior and posterior directions and induce the ventral direction of UNC-40::GFP clustering (Kulkarni et al.,

2013; Yang et al., 2014).

The extension development is regarded as a stochastic process. At one any instance of time, UNC-40 interacts with UNC-6 at the neuron's surface to mediate UNC-40 asymmetric localization and outgrowth activity. At the next instance of time, other UNC-40 receptors may interact with UNC-6 guidance cues. The cell membrane is fluid, therefore the movements that occur by the outgrowth activity are not always in parallel and the outward movement direction fluctuate. Normally, at the leading edge, the molecular mechanism produces axon outgrowth activity and moves plasma membrane extension of HSN cell body in the one direction in wild type (Adler et al., 2006). Mathematically, in mutants, the different directions (anterior, posterior, ventral and dorsal) of axon outgrowth from the HSN cell body scored to create a probability distribution for axon outgrowth activity directions, diffusion, and displacement (Tang and Wadsworth, 2014). Therefore, the axon outgrowth movement patterning changes. During HSN development, the probability of UNC-40-mediated outgrowth activity is oriented ventrally and then the ventral axon outgrowth develops, whereas in mutants, it is oriented randomly in different directions.

To understand the random movements, we used the random walks, a mathematical model of a path consisting of a succession of random steps in which the direction of each move is randomly determined (Yang et al., 2014). Random walk models describe different types of behavior. They are used to model random movement of membrane mass to understand how gene activity affects the membrane outward displacement. The probability distribution derived for axon outgrowth direction from the HSN cell body gives the fluctuating force. The more direction of movement fluctuates, the distance of axon

extension in a given amount of time will be shorter. So the diffusive movement promotes results in reducing the displacement of the movement of the membrane and that influences the axon outgrowth rates compared with wild type. This diffusive movement occurs at the microscale in the neurons. An increase in the diffusive movement at this scale at the plasma membrane may be observed at the macro scale as a decrease in the outgrowth rates. Using the probability distribution, we compared the probabilities in wild type and mutants. For example, UNC-6/Netrin extracellular guidance cues can decrease or increase the probability of UNC-40 activity at each side of the neuron. Thus, this probability is directed as a stochastic process. To elaborate more, the probability of axon outgrowth activity in ventral direction decreases, whereas the probability of axon outgrowth in other directions increases in mutants (Xu et al., 2009; Kulkarni et al., 2013; Yang et al., 2014; Tang and Wadsworth, 2014). Therefore, the guidance cues control of the movement direction fluctuates over time. We have reported that these probabilities can cause different regions of the neuron's plasma membrane to move in different directions. Thus, the axon outgrowth extension morphology is abnormal, and it causes a new axon outgrowth patterning. As a conclusion, the random walk could be used to describe the changing pattern of axon outgrowth in response to axon guidance cues

This study used ZAG-1 transcription factor to regulate the asymmetric localization of UNC-40 polarization and axon guidance direction, suggesting that the signals involved are affected by the transcriptional mechanisms. Here I provide experimental evidence that the axon outgrowth activity regulated by ZAG-1 can be explained by the random walk that is caused by the stochastic process of UNC-40::GFP polarization. It is useful to study axon guidance directions as a stochastic process to treat all axon outgrowth activities that is

important for many biological systems, including axons polarization to do their functions. So it will be interesting to study the ZAG-1 transcription factor and its interplay with UNC-40 function in regulating neuronal polarity and axon outgrowth as a stochastic process.

RATIONALE

Caenorhabditis elegans as an excellent model system for studying axon guidance

C. elegans is found free-living in soil. WormBase, a database that includes the genetics of worm and WormAtlas, describes the worm's anatomy in great detail.

Since the vertebrate and cell culture are not ideal systems for genetic screen, the nematode *C. elegans* has many advantages as a model organism in genetics, neurophysiology and developmental biology with experimental and biological advantages. Furthermore, *C. elegans* has been used as a less complex organism for studying the interaction of the proteins that are chromatin-associated and their modifications with transcription factors to control gene expression through transcriptional regulation (Reinke et al., 2013).

There are powerful features that make *C. elegans* an ideal model organism for studying neuronal functions, cell migration, and axon guidance and patterning. First, it has a simple nervous system (White et al., 1986). The nervous system in vertebrates feature a more complex morphology than that of the invertebrate, and their individual neurons are highly branched. Second, *C. elegans* is a multicellular eukaryote feeding on bacteria. It has a small size, about 2-3 mm, body length and a limited number of cells; it has 959 somatic cells. Also, it has a short generation cycle, about three days to grow from egg hatching to adult. Its body is cylindrical, non-segmented tube, surrounded by hypodermis covered with a cuticle. It can grow at 20 °C (Altun and Hall, 2008). *C. elegans* is transparent; which allows us to visualize the neurons using green fluorescent protein (GFP) while the worms are alive (Chalfie et al., 1994).

C. elegans is the first multicellular organism to have a full sequence of the genome determined (Consortium, 1998). The *C. elegans* genome size is small, about 97 Mb,

containing 19000 genes (Consortium, 1998). The *C. elegans* genome encodes about 934 transcription factors in comparison to the human genome which has 1,962 transcription factors (Messina et al., 2004).

Hermaphrodites of *C. elegans* are self-fertilizing animals and produce oocytes and sperm. A single hermaphrodite can produce about 300 progeny. The other important feature of *C. elegans* is that its development lineage from the embryo to adult is determined. The worm embryo passes through multiple different phases in growth and development within three days life cycle, including cell proliferation, organogenesis, and morphogenesis. Cell division, movement, and death occur in the cell proliferation and organogenesis processes; the neural outgrowth begins in the morphogenesis process (Sulston et al., 1983).

Since many of the *C. elegans* proteins have orthologues in humans, it is possible to use this animal as an ideal disease model to examine different neurodegenerative diseases and improve people's understanding of life (Kaletta and Hengartner, 2006). Thus, by using less complex organisms related to humans, the medical and biological concepts can be more easily achieved.

Transcriptional regulation of axon guidance decisions

How is axonal projection regulated by transcription activity? Axon guidance, a process that is regulated by environment cues and their receptors, became an active field of neuroscience research. The trajectory of axons to their target is the crucial issue for the normal function of the nervous system. Over the past few decades, many guidance molecules and transcription factors have been identified that regulate neuron differentiation

and development.

Now, it is important to expand our study to close the gap and find the guidance molecules that cooperate with the transcription factor to regulate axon guidance directly. A study on transcription factors and guidance cue cooperation would be useful to understanding transcriptional control of axon patterning. Possibly, identifying the transcription factor gene targets that direct axon pathfinding will lead us to understand how an axon is directed correctly to its particular target.

The mutation in ZEB family of transcription factors has been implicated in human disorder such as Mowat Wilson syndrome (Mowat et al., 2003). These transcription factors are likely involved in neural development. Several studies have investigated their roles in neuron differentiation.

MATERIALS AND METHODS

Experimental strains

All *C. elegans* strains were incubated and handled at 20°C by general techniques as described by Brenner, 1974. *Bristol* strain N2 was used as a wild type and the worms were grown on the *OP50* strain of *Escherichia coli* as food. To examine genetic interactions between *zag-1* and other genes, we looked at animal phenotype of the single, double and triple mutants. If the double and triple mutants phenotypes show the same single mutants phenotype, these two or three genes work in the same pathway. In contrast, if the double and triple mutants phenotype show the different single mutants phenotype, these two or three genes work in different pathways.

The following mutant strains and transgenes were used in this thesis:

LGI, *unc-40 (e1430), unc-40(ur304), zdIs5 [mec-4::GFP]*; **LGII,** *unc-53(n152)*; **LGIV,** *unc-5(e53), unc-5(e152), unc-5(ev480), unc-5(ev585), egl-20(n585), zag-1(zd85), zag-1(zd86), kyIs262 [unc-86::myr GFP;odr-1::dsRed], zdIs13 [Ptph-1::gfp]*; **LGV**, *madd-2(ky592), madd-2(tr103);* **LGX,** *unc-6(ev400), mig-15(rh148), sax-3(ky123), sax-3(ky200).*

Transgenes maintained as extrachromosomal arrays included *kyEx1212 [unc-86::unc-40-GFP;odr-1::dsRed]*. Some strains were received from *Caenorhabditis Genetics Center*.

Genetic crosses of *C. elegans*

All crosses were carried out by putting young adult hermaphrodites together with young adult N2 males on a mating plates to increase the mating opportunity. Always, we put 10 young adult males and 5 adult hermaphrodites in one mating plate. All crosses were

performed at 20ºC. 10 of the resulting F1 heterozygous hermaphrodite were transferred into single plates. These hermaphrodites were allowed to self-fertilize. The presence of transgenes in cross progeny was detected by using an epifluorescence microscope.

Analysis of the HSN axon outgrowth patterning phenotypes and cell body position

For analysis of the HSN axon outgrowth phenotypes, HSN neurons morphology was visualized by using the expression of *kyIs262 [unc-86::myr-GFP]* and *zdIs13 [Ptph-1::gfp]* transgenic strains. After eggs hatching, the L1 larvae were fed and grew to a specific larvae stage.

Axon migration defects were scored in the L4 larval stage. The larvae stage was determined by examining the gonad cell number and gonad size by using differential interference contrast (DIC) microscopy. The L4 larvae were mounted in M9 buffer with 10 mM levamisole on a 5% agarose pad. The direction of HSN axon protrusion was regarded as anterior if the axon extended anteriorly for a distance at least three cell body lengths. The HSN dorsal or posterior protrusion was scored if the axon extended dorsally or posteriorly for a distance at least two cell body lengths. The HSN was regarded multipolar if more than one process extending from cell body at least one cell body length were observed. The HSN cell body in L2 stage larvae was scored as dorsal if the cell body had failed to migrate ventrally and was not positioned near the PLM axon. In L4 stage larvae, a multiple ventral processes phenotype was scored if more than one major extension protruded from the ventral side of cell body.

The worm numbers with specific phenotypes were counted and the percentage of them was calculated from the total measured sample population. The standard error of the

mean was used. By using epifluorescent microscopy with a Zeiss 40X objective, the images were taken.

Analysis of the ALM, AVM, and PLM axons outgrowth patterning phenotypes

The mechanosensory neurons, ALM, AVM, and PLM, were visualized by using the expression of the *zdIs5 [Pmec-4::GFP]* transgenic strain. To analyze the ALM, AVM, and PLM axons protrusion phenotypes, the L4 stage larvae were mounted on a 5% agarose pad with 10 mM levamisole buffer. The ALM and AVM axons were regarded as a dorsal or posterior protrusion if the axon extended from the cell body dorsally or posteriorly for a distance at least two cell body lengths. Cells were regarded multipolar if more than one neurite extended from the cell body at least one cell body length. AVM axons were scored as anterior protrusion if the axon extended from the cell body laterally at least three cell body lengths. PLM axons are considered as displaying a short extension if they failed to reach the vulva position. The images were taken by using epifluorescent microscopy with a Zeiss 63X water immersion objective.

Analysis of HSN axon outgrowth and development

HSN neuron morphology was visualized by using the expression of *kyIs262 [unc-86::myr-GFP]* transgenic strains. The larval stages were mounted on a 5% agarose pad with 10 mM levamisole buffer. They were fed and grown at 20°C. The larvae stage was determined by examining the gonad cell number and gonad size by using the differential interference contrast (DIC) microscopy. The ventral neurites were scored by observing the neurites that extended at least one cell body length from the ventral side of cell body. The

number of processes during early L1 larval stage was scored by counting the number of processes that extended for a distance greater than the length of one cell body. We report instances in which there were no such processes, one process or more than one processes. In the L2 larval stage, a single early process was scored if there was only one major extension from the ventral leading edge of cell body of HSN neuron. The number of processes during L3 larval stage was scored by counting the number of processes that extended ventrally for a distance greater than the length of one cell body. The images were taken by using epifluorescent microscopy with a Zeiss 63X water immersion objective. The student t-test was used to determine if there were significant differences between strains.

Analysis of ALM and AVM branching at the nerve ring and anteriorly

To analyze ALM and AVM axons branching, the L4 stage worms were mounted on a 5% agarose pad with 10 mM levamisole buffer. The larvae stage was determined by examining the gonad cell number and gonad size by using the differential interference contrast (DIC) microscopy. The ALM and AVM mechanosensory neurons were visualized by using the expression of the *zdIs5 [Pmec-4::GFP]* transgenic strain under epifluorescent microscopy. The anterior extension defects were scored if the ALM and AVM axons did not extend anteriorly past the nerve ring. The nerve ring branching defects were scored if the ALM and AVM axons did not grow from dorsal or ventral end to midline respectively. The images were taken by using epifluorescent microscopy with a Zeiss 40X objective. The student t-test was used to determine if there were significant differences between strains.

Analysis of UNC-40::GFP localization in L2 stage animals

The larva stage was determined by estimating the gonad cell number and gonad size under the differential interference contrast (DIC) microscopy. The UNC-40::GFP localization was visualized by using the expression of the *kyEx1212[unc-86::unc-40::GFP; odr-1::dsRed]* transgenic strain. The L2 stage larvae were mounted on a 5% agarose pad with 10 mM levamisole buffer.

The UNC-40::GFP Dorsal/Ventral distribution was determined by measuring the ratio of average GFP intensity. Using ImageJ (htt://rsb.inf.nih.gov/ij/) software, we drew lines along the dorsal and ventral edges of each one of the HSN cell body. The images were taken by using the epifluorescent microscopy with a Zeiss 63X water immersion objective. To analyze of the anterior–posterior orientation of UNC-40::GFP, the dorsal segment of HSN cell surface was divided into three equal regions by length: dorsal anterior, dorsal central and dorsal posterior segments. The line-scan intensity plots of each of the above-mentioned segments was recorded. To determine if there was a significant differences between the intensities of three segments, an ANOVA test was used. The UNC-40 was regarded uniform if $p \geq 0.05$ and asymmetrical if $p \leq 0.05$ - from an asymmetric population if the intensity of one region is the higher percentage than the other two, it was considered as either anterior, posterior or central localization of the dorsal surface. Additionally, UNC-40::GFP was regarded as anterior-posterior localization if the intensity was significantly higher in anterior and posterior regions than the central region.

Computations

A program to simulate a two-dimensional lattice random walk based on the

probability of dorsal, ventral, anterior, and posterior outgrowth for a mutant (Table 1 and Table 2) was created using MATLAB (The directions of the axons from multipolar neurons were not scored). These axons appear to behave in the same manner as the axons from monopolar neurons, but this has not yet been tested). The probability of dorsal, ventral, anterior, or posterior outgrowth was assigned for the direction of each step of a random walk moving up, down, left or right, respectively. Each variable is considered independent and identically distributed. Simulations of 500 equal size steps (size =1) were plotted for 50 tracks (Figures 1B, 5B, 6B inserts, 30A, 30B and 30 C). A Gaussian distribution for the final positions of the tracks was generated using Matlab's random function (Figure 6).

The mean squared displacement (MSD) is used to provide a quantitative characteristic of the motion that would be created by the outgrowth activity undergoing the random walk. Using the random walks generated for a mutant the MSD can be calculated:

$msd(\tau) = < 〚[r(t+\tau)-r(t)]〛 ^2>$

Here, r(t) is the position at time t and τ is the lag time between two positions used to calculate the displacement, $\Delta r(\tau) = r(t+\tau) - r(t)$. The time-average over t and the ensemble-average over the 50 trajectories were calculated. This yields the MSD as a function of the lag time. A coefficient giving the relative rate of diffusion was derived from a linear fit of the curve. The first two lag time points were not considered, as the paths often approximate a straight line at short intervals.

RESULTS

CHAPTER 1

UNC-5 (UNC5) mediates neuronal outgrowth patterning in *Caenorhabditis elegans* by regulating UNC-40(DCC) asymmetric localization

Manuscript under revision:

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Contributions

In this chapter, I describe the research that I and other members of the Wadsworth laboratory did. We provide evidence that UNC-5 (UNC5) receptor activity regulates UNC-40 asymmetric localization and the outgrowth patterning. We show that *unc-5* mutations alter UNC-40 asymmetric localization and the patterns of outgrowth. We find that UNC-5(UNC5) receptor and cytoskeletal binding protein UNC-53 (NAV2) regulate UNC-40 asymmetric localization in response to both the UNC-6 and EGL-20 extracellular cues.

UNC-5 regulates the patterning of outgrowth extensions from HSN (Figure 7A-7E):

During the L1 stage in wild type animals, the HSN neurons extend more than one short neurite. I observed that in *unc-5(e53)* mutants, half of HSN neurons do not extend a neurite (7A and 7B). During the L2 stage, the HSN neurons extend a single process and the cell body migrates ventrally. However, in *unc-5* mutants, I noticed that the cell body fails to migrate ventrally and remains in dorsal position (Figures 7A, 7C and 7E). This indicates that the loss of *unc-5* function affects the patterning of HSN outgrowth.

UNC-5 regulates the patterning of extension from ALM, AVM, and PLM (Figure 8A-8E):

I performed the PLM, ALM, and AVM neurons outgrowth extensions at the L4 stage in wild type and in *unc-5* and *unc-40* mutants (8A- 8E). I found that in *unc-40* mutants, the AVM and ALM neurons often fail to extend into the nerve ring (8C and 8D). Also, I found that the AVM neuron extends anteriorly in *unc-5* and *unc-40* mutants like the wild type (8E). In addition to the contributions mentioned above, I had a role in discussing the results we arrived at.

ABSTRACT

Neurons extend processes that vary in number, length, and direction of outgrowth. Extracellular cues help determine outgrowth patterns. In *Caenorhabditis elegans,* neurons respond to the extracellular UNC-6(netrin) cue via UNC-40(DCC) and UNC-5(UNC5) receptors. Previously we presented evidence that UNC-40 asymmetric localization at the plasma membrane is self-organizing and that UNC-40 can localize and mediate outgrowth at randomly selected sites. We also postulate that the process is statistically dependent, *i.e.* if the probability of outgrowth at one site changes then the probability at another site(s) must also change. Over time, the direction of outgrowth activity fluctuates across the membrane. A probability distribution describes the likelihood of outgrowth in each direction. Random walk modeling predicts that the degree to which the direction of outgrowth fluctuations affects the outward displacement of the membrane. We predict that extracellular cues create patterns of outgrowth by differentially affecting the degree to which the direction of outgrowth activity fluctuates along the membrane. This produces different rates of outgrowth along the surface and creates patterns of extension. Here we present evidence that UNC-5(UNC5) receptor activity regulates UNC-40 asymmetric localization and the patterning of outgrowth. We show that *unc-5* mutations alter UNC-40 asymmetric localization and the patterns of outgrowth that neurons develop. Genetic interactions suggest UNC-5 acts through the UNC-53/NAV2 cytoplasmic protein to regulate UNC-40 asymmetric localization in response to both the UNC-6 and EGL-20 (wnt) extracellular cues.

INTRODUCTION

During development, an intricate network of neuronal connections is established. As processes extend from the neuronal cell bodies, distinct extension patterns emerge. Some extensions remain as a single process, whereas others branch and form multiple processes. If they branch, the extensions can travel in the same or in different directions. Extensions vary in length. Extracellular cues are known to influence this patterning, but the underlying logic that governs the formation of patterns remains a mystery.

The secreted extracellular UNC-6(netrin) molecule and its receptors, UNC-5(UNC5) and UNC-40(DCC) are highly conserved in invertebrates and vertebrates, and are known to play key roles in cell and axon migrations. In *Caenorhabditis elegans*, UNC-6 is produced by ventral cells in the midbody and by glia cells at the nerve ring in the head (Asakura, Ogura, and Goshima, 2007; Wadsworth, Bhatt, and Hedgecock, 1996; Wadsworth and Hedgecock, 1996). It has been observed that neurons that express the receptor UNC-40(DCC) extend axons ventrally, towards the UNC-6 sources; whereas neurons that express the receptor UNC-5/UNC5 alone or in combination with UNC-40 extend axons dorsally, away from the UNC-6 sources (Chan et al., 1996; Hedgecock, Culotti, and Hall, 1990; Leung-Hagesteijn et al., 1992; Wadsworth et al., 1996).

It is commonly proposed that axons are guided by attractive and repulsive mechanisms (Tessier-Lavigne and Goodman, 1996). According to this model, an extracellular cue acts as an attractant or repellant to direct neuronal outgrowth towards or away from the source of a cue. UNC-5(UNC5) has been described as a “repulsive” netrin receptor because it mediates guidance away from netrin sources (Hong et al., 1999; Keleman and Dickson, 2001; Leung-Hagesteijn et al., 1992; Moore et al., 2007). The attraction and repulsion

model is deterministic. That is, given the same conditions, the response of the neuron, attractive or repulsive, will always be the same. This idea forms the bases of the analysis and interpretation of experimental results. Axonal growth cone movement towards or away from the source of a cue is considered to be mediated by attractive or repulsive responses to the cue. In genetic studies, a mutation that disrupts movement towards the cue source denotes gene function within an attractive pathway, whereas mutations that disrupt movement away from a source denotes gene function within a repulsive pathway. If an axonal growth cone is observed to move towards and then away from the source of a cue, the responsiveness of a neuron is thought to switch from attractive to repulsive. However, it is important to note that attraction or repulsion is not an intrinsic property of the interaction between the receptor and ligand. In fact, the interaction only promotes or inhibits outward movement of the membrane. Attraction and repulsion refers to a direction, which is an extrinsic property of the cellular response that varies depending on the physical positions of the ligands. Movement towards or away from a cue source is caused by attractive and repulsive effects, such as chemoattraction and chemorepulsion, which is movement that is directed by chemical gradients of ligands. We argue that classifying cellular mechanisms as attractive or repulsive is problematic since attraction and repulsion are not intrinsic properties of cellular mechanisms.

We have proposed an alternative model in which the movement of neuronal outgrowth is not considered in terms of attraction and repulsion. This model comprises three concepts. The first concept is that receptors along the surface of the membrane change position. This is important since the spatial distribution of receptors can influence the movement that a neuron has in response to the extracellular ligands (Nguyen et al., 2014; Nguyen et al.,

2015). We hypothesize that the spatial distribution of UNC-40 can influence the manner though which force is applied to the membrane and thereby affect the outward movement of the membrane. It's known that the surface localization of the UNC-40 receptor undergoes dramatic changes during the development of the HSN axon (Adler et al., 2006; Kulkarni et al., 2013; Xu et al., 2009). As HSN axon formation begins, UNC-40 becomes asymmetrically localized to the ventral surface of the cell body, which is nearest the ventral sources of the secreted UNC-6 ligand. Live imaging of the developing leading edge reveals a dynamic pattern of UNC-40 localization with areas of concentrated UNC-40 localization shifting positions along the surface (Kulkarni et al., 2013). Dynamic UNC-40::GFP localization patterns have also been reported during anchor cell extension (Wang et al., 2014). Similar to axon extension, the anchor cell also sends an extension through the extracellular matrix and this extension is also regulated by UNC-40 and UNC-6 (Hagedorn et al., 2013; Ziel et al., 2009). Live imaging of the anchor cell reveals that UNC-40::GFP "clusters" form, disassemble, and reform along the anchor cell's plasma membrane (Wang et al., 2014).

The second concept is that the asymmetric localization of the receptor, and the subsequent outgrowth activity it mediates, is stochastically oriented. It was observed that UNC-40 can asymmetrically localize to a randomly selected surface if the UNC-6 ligand is not present to provide a pre-established asymmetric cue (Xu et al., 2009). We noted that the self-organizing nature of UNC-40 localization is reminiscent of a self-organizing process observed in single-cell yeast, *Dictyostelium discoideum*, and neutrophils, where cell movement will occur in a random direction if the chemotactic cue is absent or is uniformly presented (Arrieumerlou and Meyer, 2005; Fraser et al., 2000; Mortimer et al.,

2008). The process through which outgrowth activity becomes asymmetrically organized is thought to utilize positive- and negative-feedback loops (Bourne and Weiner, 2002; Graziano and Weiner, 2014). Such loops might also drive the asymmetric localization of UNC-40 (Wang et al., 2014; Xu et al., 2009). Positive and negative feedback are considered complementary mechanisms; positive feedback amplifies the polarized response to an extracellular cue, while negative feedback limits the response and can confine the positive feedback to the leading edge (Bourne and Weiner, 2002). The biological nature of feedback loops controlling UNC-40 activity is unclear. However, they may involve the differential transport of receptors and effectors to the plasma membrane surface. Imaging experiments of cells in culture suggest that netrin-1 (UNC-6) regulates the distribution of DCC (UNC-40) and UNC5B (UNC-5) at the plasma membrane (Gopal et al., 2016). In these studies, netrin-1 (UNC-6) was shown to stimulate translocation of DCC (UNC-40) and UNC5B (UNC-5) receptors from intracellular vesicles to the plasma membrane and, further, the transported receptors were shown to localize at the plasma membrane (Gopal et al., 2016).

We argue that the process that localizes UNC-40 to a site on the plasma membrane possesses inherent randomness (Figure 1). Evidence suggests that the conformation of the UNC-40 molecule controls whether the process will cause UNC-40 localization to the site of UNC-6 interaction or to another site (Xu et al., 2009). We observed that a single amino acid substitution in UNC-40 will allow UNC-40 to asymmetrically localize to different surfaces in the absence of UNC-6. The binding of UNC-6 to this UNC-40 molecule causes localization to the surface nearest the UNC-6 source. However, the binding of UNC-6 with a single amino acid substitution will enhance the asymmetric localization to different surfaces. A second-site UNC-6 amino acid substitution will suppress this enhancement and

increase UNC-40 asymmetric localization at the surface towards the UNC-6 source. These results indicate that UNC-40 conformational changes differentially influence each activity. In the context of feedback loops, UNC-40 activity regulates both the positive and negative loops that control the asymmetric localization of UNC-40 to the plasma membrane. Because the system is controlled by the conformation of the molecule, randomness will be introduced in the system by stochastic fluctuations in ligand-receptor binding and by stochastic conformational changes.

The outcome of an UNC-40 receptor's activity is to either cause an UNC-40 receptor to localize to the site of UNC-6 interaction or to a different site (Figure 1). This is an important discovery because it means that the asymmetric localizations events are mutually exclusive and, therefore, there is statistical dependence. We refer to this process as 'statistically dependent asymmetric localization' (SDAL). This model states that the probability of UNC-40 localizing and mediating outgrowth at the site of UNC-6 interaction affects the probability of UNC-40 localizing and mediating outgrowth at another site, and vice versa. We have found that other extracellular cues can also affect UNC-40 asymmetric localization, and thus can influence the probability of UNC-40-mediated outgrowth from different sites (Tang and Wadsworth, 2014; Yang et al., 2014).

The development of an extension can be considered as a stochastic process. At any one instance of time at innumerable sites along the neuron's surface, UNC-40 interacts with UNC-6 to mediate UNC-40 asymmetric localization and the outgrowth response. At the next instance of time, other UNC-40 receptors, including any just transported to the surface, may interact with UNC-6. Because the plasma membrane is a fluid, the forces generated by the outgrowth response are not always acting in parallel and the direction of

outward force can fluctuate (Figure 2A). It is the collective impact of all outgrowth events over a period of time that allows the extension to form. The outward movement of an extension could be precisely described if the effect of each outgrowth event were known. However, it is extremely difficult to measure the effect of each single event since there are innumerable events happening at each instance of time. We also argue that UNC-40 localization is a random process that evolves over time. Therefore, the outgrowth events can only be described probabilistically, and as such the time evolution of extension is also probabilistic in nature.

Understanding the role a gene plays in controlling outgrowth movement might require knowledge of its role in regulating this stochastic process. To do this, we use the direction of HSN extension. We reason that the collective impact of all the outgrowth events over a period of time cause the development of the HSN axon. The direction of extension from the cell body has a probability of being orientated in one direction (Kulkarni et al., 2013; Tang and Wadsworth, 2014; Yang et al., 2014). Mathematically, the direction of HSN extension is a variable that takes on different values; “anterior”, “posterior”, “ventral”, and “dorsal”. A probability is associated with each outcome, thus creating a probability distribution. This distribution describes the effect that all the outgrowth events had over a period of time. During normal development, the probability of each UNC-40-mediated outgrowth event being ventrally oriented is very high and a ventral extension develops. We have shown that certain gene mutations affect the probability distribution, thus revealing that a gene plays a role in the stochastic process. We can compare wildtype and mutants to gage the degree to which a mutation causes the direction of extension to fluctuate. This reflects the degree to which the mutation has caused the direction of outgrowth activity,

and the outward force it creates, to fluctuate over the course of extension development.

We argue that understanding the function of a gene in terms of a stochastic model of membrane movement is useful. Often the goal of a genetic analysis of axon guidance is to uncover a molecular mechanism. Frequently, a deterministic model is made which describe some molecular event that the gene affects. Because the mutation affects axon guidance, the molecular event plays a role in causing directed movement. However, these models tend to reduce a complex biological process to an isolated component. In reality, understanding how a molecular event is able to cause directed movement requires knowledge of all the many ways in which the event influences, and is influenced by, the other molecular events of directed movement. A stochastic model is a useful tool of exploring how a gene affects the overall behavior of the system. To make an analogy, a roulette wheel can be described deterministically; if every force acting on the ball at every instance of time is known than the number on which the ball stops can be precisely determined. The role of a component of the roulette wheel could be described by the effect that it has the forces which act on the ball at every instance of time. However, understanding how the effect of this component causes a particular outcome require understanding the effects of all the other components. Because this is so complex, studying the roulette wheel using a stochastic model is useful. For example, a roulette wheel must be exactly levelled to have an equal probability for each number. Removing a component of the wheel can cause the wheel to tilt in a particular manner. This will result in a new outcome, i.e. the ball will have a higher probability of stopping on certain numbers. Although this does not reveal the precise event that occurs between the component and the ball, it will reveal the effect that the component has in determining an outcome. Further,

by studying the effect of removing multiple components, relationships that lead to particular outcomes can be revealed.

The third concept of our model is that neuronal membrane outgrowth is a mass transport phenomena can be described as advection and diffusion (Figure 2). Signaling by UNC-40 receptors along a surface of the neuron can lead to cytoskeletal changes which create force and membrane movement (Figure 2A). As a result, there is a mean flow of membrane mass in an outward direction (Figure 2B). This motion is advection, which is mass transport by a mean velocity field. In addition to advection, membrane mass transport also occurs through random movement, i.e. diffusion. Because the cell membrane is fluid, membrane mass will move in different directions as the membrane is subjected to forces which change its shape (Figure 2C). The degree to which the membrane mass undergoes random movement is important because diffusion processes and advection processes have different effects on the extent to which mass will be displaced outward in a given amount of time. The random movements can be mathematically described using random walks. A random walk is a succession of randomly directed steps (Figure 2D). Random walk models are used to describe many diverse types of behavior, including the movement of a particle through fluid, the search pattern of a forging animal, and the fluctuating price of a stock. The behavior of neuronal growth cone movement during chemotaxis has also been modeled using random walks (Buettner et al., 1994; Katz et al., 1984; Maskery et al., 2004; Odde and Buettner, 1995; Wang et al., 2003). However, rather than using a random walk model to describe the gross morphological changes observed during growth cone movement, in this study the random walk is used to model the random movement of membrane mass in order to understand how gene activity influences the outward

displacement of the membrane. A property of random motion is that the mean square displacement grows proportionate to the time traveled. This means that the more the direction of movement fluctuates, the shorter the distance of travel in a given amount of time (Figure 2E). The model predicts that if force is applied to the membrane in a manner that increases random movement then the outward displacement of the membrane's mass will decrease.

Because of the effect random movement has on displacement, the SDAL model makes predictions about how UNC-40 activity affects the rate of extension. In a deterministic model, outgrowth activity causes straight-line outward motion from the site of interaction. The SDAL model predicts that the interaction between UNC-40 and UNC-6 increases the probability that UNC-40 asymmetric localization and UNC-40-mediated outgrowth will be oriented at the site of interaction. It also decreases the probability that localization and outgrowth will be oriented elsewhere. Therefore, the interaction influences the spatial distribution of UNC-40 along the surface and, in doing so, will change the way forces are applied to the membrane. As this process continues over time, the direction of the forces acting on the fluid membrane fluctuates. This will alter the advective and diffusive transport of membrane mass. As an example, if the probability of ventral outgrowth is 0.33, of anterior outgrowth is 0.33, and posterior outgrowth is 0.33 then there will be a high degree of random movement. Interactions between UNC-40 and UNC-6 at the leading ventral surface could shift the probabilities for ventral outgrowth to 0.8, for anterior outgrowth to 0.1, and for posterior outgrowth to 0.1. This change will decrease the degree to which the direction of outgrowth fluctuates. As modeled in Figure 2E, this will increase displacement, meaning that the membrane mass now will be able to travel further outwards

over a given amount of time. It is worth noting that fluctuations in the direction of outgrowth activity could occur as very rapid minute movements of membrane mass. When observed at the macro-scale, these micro-scale fluctuations might not be seen. Instead, the outward movement of an extension will appear as linear, straight-line, movement. In this paper, "fluctuation" refers to variation in the direction of outgrowth activity. "Outgrowth" refers to the movement of membrane mass at the micro-scale, whereas "extension" refers to the movement of the axon that is observed at the macro-scale.

The SDAL model makes distinctive predictions about UNC-40-mediated outgrowth activity in vivo and the direction of extension. In a deterministic model, the direction of extension is determined by the outward movement of the membrane from the site where UNC-6 and UNC-40 interact. Positional information is encoded by gradients so that UNC-6 guides extension towards the UNC-6 source. In the SDAL model, UNC-6 and other extracellular cues govern the probability of UNC-40 asymmetric localization, and subsequent UNC-40-mediated outgrowth, at each surface of the membrane (Kulkarni et al., 2013; Xu et al., 2009). The direction of outgrowth is determined by a directional bias that is created over time by the combined effect of extracellular cues. If, for example, the probability of outgrowth towards a ventral UNC-6 source is 0.3 and the probability of dorsal outgrowth is 0.3 and of anterior outgrowth is 0.4, the direction of outgrowth will be in the anterior direction. That is, at any instance of time there is a chance that outgrowth movement will be directed ventrally towards the UNC-6 source, however over a longer period of time the outgrowth will travel anteriorly because there is always a greater likelihood that outgrowth will be anterior instead of ventral or dorsal. To reiterate, the direction of extension is a product of a stochastic process, in which the outcome evolves

over time. The probability of ventral outgrowth created by the UNC-40-mediated response to UNC-6 is required for the anterior bias. Without the ventrally directed outgrowth in response to UNC-6, the probability of ventral outgrowth would decrease, shifting the directional bias. Thus, when considered as a stochastic process, the observed directional response to the interactions between UNC-40 and UNC-6 is not necessarily extension towards the UNC-6 source. Because of SDAL, positional information is encoded by the location and level of the extracellular cues along the surface of the neuron.

The SDAL model suggested that UNC-40 activity could affect extension movement in ways that had not been obvious. First, the forward movement of an extension could be inhibited as it moves towards a source of a cue that promotes outgrowth (Figure 3A). A strong directional basis for movement towards an UNC-6 source occurs only as long as the probability of UNC-40 localization at the leading edge of an outgrowth is greater than the probability of UNC-40 localization at flanking sites. As an extension moves towards an UNC-6 source a higher proportion of the UNC-40 receptors that flank the leading edge can become ligated (Figure 3B). Because of the SDAL process, this will increase the probability of localization and outgrowth at the flanking sites while decreasing the probability of localization and outgrowth at the leading edge. The result will be an increase in random movement and a decrease in the outward displacement of the membrane's mass. Paradoxically, the rate of extension will decrease as the extension moves towards the UNC-6 source (Figure 3C). It is worth noting that even if the probability of outgrowth in each direction becomes equal, there will still be a directional bias. For example, if the probability of outgrowth towards a ventral UNC-6 source is 0.33 and of anterior outgrowth is 0.33 and of posterior outgrowth is 0.33, the directional bias is still ventral. (The probability of

movement in the direction of the axon shaft (backwards) is low.)

A second insight is that an extension could move towards the source of a cue that inhibits outgrowth (Figure 3A). For example, if together the extracellular cues create a probability for ventral outgrowth of 0.7, for anterior outgrowth of 0.15, and for posterior outgrowth of 0.15, a directional bias for ventral outgrowth is created (Figure 3B). This can occur even if there is a ventral source of an inhibitory cue. The extension can move ventrally towards this inhibitory cue source. Eventually the probability for ventral outgrowth might change to 0.33, anterior to 0.33, and posterior to 0.33 (Figure 3C). But even in this case, there is still a directional bias for ventral outgrowth and extension will continue to move towards the source of the inhibitory cue.

The model predicts that movement towards a source of a cue causes the system to trend towards a state where the probabilities of outgrowth in different directions become equal. Axons often change their trajectory near the source of a cue. It is possible that the state is important because the equilibrium might allow cues to be more effectual at reorienting outgrowth (Figure 4).

The third insight is that multiple extensions from a neuron could move in the same direction without having to follow prepatterned extracellular pathways. Some neurons send out multiple extensions that run in parallel towards a target. It is commonly proposed that these patterns form because extensions follow parallel pathways that were previously formed by extracellular guidance cues. The SDAL model suggests that multiple UNC-40-mediated outgrowths can be initiated at a leading surface and that multiple extensions can maintain their positions without having to follow prepatterned extracellular pathways. In this model, a separate extension begins to form at the leading edge because the directional

bias at one site becomes greater than that at flanking sites. We propose that along the leading edge the self-organizing UNC-40 localization process can create multiple sites that have a greater directional bias (Figure 5A). The positive and negative feedback loops of the SDAL process can allow spatial patterns of outgrowth to develop autonomously. Once these sites are established, outgrowth can proceed from each site in the same direction (Figure 5B). The strongest directional bias is created when the probabilities for outgrowth are equal in the directions perpendicular to the direction of extension. The actual value of the perpendicular probabilities is not crucial for establishing a directional bias. Even though the value of the perpendicular probabilities may vary depending on the position of outgrowth along the perpendicular axis, the direction of outgrowth will be the same. If a perpendicular equilibrium is maintained, then cues that affect UNC-40 localization and outgrowth and which are distributed along the perpendicular axis will have little effect on the direction of outgrowth. Such an equilibrium can be established if the inhibiting and promoting effects of the cues distributed along the perpendicular axis balance out each other. Even if cues are distributed in a gradient an equilibrium can exist. Studies indicate that gradient steepness, rather than the concentration of cues, is important for growth cone turning and guidance (Baier and Bonhoeffer, 1992; Mortimer et al., 2010; Rosoff et al., 2004; Sloan et al., 2015). Therefore, cues may create a perpendicular equilibrium if they are distributed in a shallow gradient along the perpendicular axis.

A fourth insight is that cues can direct movement without being in a concentration gradient. The SDAL activity within the cell initiates random walk movement. As long as an equilibrium along the perpendicular axis exists, a directional bias along the other axis can be created. In Figure 5B outgrowth is towards a ventral cue source, and as outgrowth

moves up the concentration gradient of this cue the probability of outgrowth in each direction changes. However, movement towards the source would still occur if the concentration of extracellular cues remains constant and the probabilities nerve change. Because of the SDAL process, a directional bias can be maintained along a track of a uniformly distributed cue.

The last insight is that extracellular cues could affect UNC-40 localization and outgrowth, but not affect the direction of outgrowth. However, such cues may still have an effect on the morphology and patterning of an extension. A candidate for such a cue is EGL-20 (wnt). The *egl-20* gene is one of several Wnt genes in *C. elegans*. These genes are expressed in a series of partially overlapping domains along the anteroposterior axis of the animal (Sawa and Korswagen, 2013). EGL-20 is expressed in cells posterior to HSN (Harterink et al., 2011; Pan et al., 2006; Whangbo and Kenyon, 1999). The sources of UNC-6 and EGL-20 are roughly perpendicular to each other. We have observed that loss of EGL-20 function causes UNC-40 asymmetrical localization to orient to randomly selected surfaces of HSN and causes the axon to initially extend from the HSN cell body in different directions (Kulkarni et al., 2013; Tang and Wadsworth, 2014). UNC-6 and EGL-20 signaling could both impinge on the feedback loops that regulate UNC-40 SDAL. In doing so, these cues would act together to influence the pattern of extension. In this paper, we provide further genetic evidence that the downstream signals from both cues converge to regulate the UNC-40 SDAL process.

We suggest that UNC-5 plays an important role in coordinating the UNC-40 SDAL process with non-UNC-40-mediated responses that affect outgrowth. Previously we reported that loss of UNC-5 causes UNC-40 asymmetrical localization to orient to

randomly selected surfaces of HSN, causing the axon to initially extend in different directions (Kulkarni et al., 2013). This suggests that UNC-5 functions to increase the probability of UNC-40 asymmetric localization being oriented to the site of UNC-6 and UNC-40 interaction. That is, UNC-5 promotes straight-line motion by inhibiting the degree to which the direction of UNC-40-mediated outgrowth fluctuates. UNC-5 has other functions as well. UNC-5 is primarily known for its role in mediating movement away from UNC-6 sources. For example, UNC-5 is required for the dorsal migration of DA and DB motor neuron axons away from ventral UNC-6 sources (Hedgecock et al., 1990). DA and DB guidance utilizes both UNC-40-dependent and UNC-40-independent pathways, although guidance is significantly less disruptive by loss of UNC-40 than by loss of UNC-5 (Hedgecock et al., 1990; MacNeil et al., 2009). In keeping with the SDAL model, we predict that UNC-5 increases the probability of non-UNC-40-mediated outgrowth being oriented towards sites where there are not interactions with UNC-6. This increases the probability of outgrowth movement in directions not towards UNC-6 sources. At the surface where UNC-6 and UNC-5 interact, random movement increases and the outward displacement of membrane's mass decreases. Finally, we predict that UNC-5 function can also increase the probability that non-UNC-40-mediated outgrowth will orient to the site of interaction between non-UNC-40 receptors and non-UNC-6 extracellular cues. Evidence for this comes from the observation that in *rpm-1* mutants the overextension of the PLM axon can be suppressed by loss of UNC-5, but not by the loss of UNC-40 or UNC-6 (Li et al., 2008). In summary, we believe UNC-5 can: 1) increase the probability of UNC-40-mediated outgrowth at the sites of UNC-6 and UNC-40 interactions; 2) decrease the probability of UNC-40-mediated outgrowth at sites where UNC-6 is not present; 3)

decrease the probability of non-UNC-40-mediated outgrowth at the site of UNC-6 and UNC-40 interactions; and 4) increase the probability of non-UNC-40-mediated outgrowth at sites where there are not UNC-6 interactions. These functions can be considered in terms of the positive and negative feedback loops of the SDAL model (Figure 6), where UNC-5 helps regulated the feedback loops associated with UNC-40 activity.

Because of these ideas, we reasoned that UNC-5 activity could affect extension movement in ways that had not been obvious to us. First, UNC-5 might affect the patterning of extension that travels towards an UNC-6 source. As discussed above, previous evidence suggests UNC-5 regulates the asymmetric localization of UNC-40. UNC-5 interactions with UNC-6 and UNC-40 could influence the feedback loops (Figure 1 and 6). By regulating the degree to which the direction of UNC-40-mediated outgrowth fluctuates, UNC-5 could affect random movement and the outward displacement of membrane mass. This could affect the rate of extension towards an UNC-6 source or whether extension can occur. In cases where multiple extensions form from a surface, the effect UNC-5 has on the loops could influence whether sites with a predominate directional bias can be established.

Second, UNC-5 could play a role in determining whether an extension changes direction. As discussed earlier, if the UNC-40 receptors become saturated near an UNC-6 source then the probability of UNC-40-mediated outgrowth towards the source and along the perpendicular axis tends to become equal. At this point, even a small increase in the probability of non-UNC-40-mediated outgrowth to the site of non-UNC-6 and non-UNC-40 interactions could alter the directional bias (Figure 4). A change in UNC-5 activity could promote a shift from a directional bias determined by UNC-40 and UNC-6 interactions, to

one determined by non-UNC-40 and non-UNC-6 interaction.

Because of the predictions that the UNC-40 SDAL model makes, we decided to reexamine the *unc-5* loss-of-function phenotypes and to investigate genetic interactions among *unc-5, unc-6, unc-40* and *egl-20* that regulated the asymmetric localization of UNC-40. We find evidence that UNC-5 regulates the length and number of processes that extend towards an UNC-6 source and that UNC-5 helps control the ability of axons to extend in different directions. In addition, we find genetic interactions that suggest UNC-5, together with UNC-53/NAV2, functions to regulate UNC-40 SDAL in response to the UNC-6 and EGL-20 (wnt) extracellular cues. We suggest that the SDAL model is useful for understanding how genes regulate the patterning of axon extensions.

RESULTS

UNC-5 regulates the pattern of outgrowth from the HSN neuron

To investigate whether UNC-5 activity can regulate the length or number of processes that a neuron can develop when outgrowth is towards an UNC-6 source, we examined the development of the HSN axon in *unc-5* mutations. The HSN neuron sends a single axon to the ventral nerve cord, which is a source of the UNC-6 cue (Adler et al., 2006; Asakura et al., 2007; Wadsworth et al., 1996). Axon formation is dynamic (Adler et al., 2006). Shortly after hatching, HSN extends short neurites in different directions. These neurites, which dynamically extend and retract filopodia, become restricted to the ventral side of the neuron where a leading edge forms. Multiple neurites extend from this surface until one develops into a single axon extending to the ventral nerve cord. Measurements of growth cone size, maximal length, and duration of growth cone filopodia indicate that UNC-6, UNC-40, and

UNC-5 control the dynamics of protrusion (Norris and Lundquist, 2011).

We observe that in *unc-5* mutants, the patterns of extension are altered. In wild-type animals at the L1 stage of development most HSN neurons extend more than one short neurite, however in *unc-5(e53)* mutants nearly half the neurons do not extend a process (Figures 7A and 7B). During the L2 stage in wild-type animals a prominent ventral leading edge forms and the cell body undergoes a short ventral migration that is completed by the L3 stage. By comparison, in *unc-5* mutants the cell body may fail to migrate and instead a single large ventral process may form early during the L2 stage (Figures 7A, 7C and 7E). It may be that the ventral migration of the HSN cell body requires the development of a large leading edge with multiple extensions. Together the observations indicate that loss of *unc-5* function affects the patterning of outgrowth, *i.e.* the timing, length, and number of extensions that form. Loss of *unc-5* function does not prevent movement, in fact, a single large ventral extension can form in the mutant at a time that is even earlier than when a single ventral extension can be observed in wildtype. The earlier appearance of a single ventral extension in *unc-5* mutants appears to be the result of a difference in morphology, rather than of developmental timing. The failure of the HSN cell body to migrate ventrally and the different pattern of outgrowth at the leading edge causes an earlier discernable single extension.

We tested four different *unc-5* alleles in these experiments. The *unc-5(e53)* allele is a putative molecular null allele, *unc-5(ev480)* is predicted to truncate UNC-5 after the cytoplasmic ZU-5 domain and before the Death Domain, *unc-5(e152)* is predicted to truncate UNC-5 before the ZU-5 domain and Death Domain, and *unc-5(ev585)* is a missense allele that affects a predicted disulfide bond in the extracellular Ig(C) domain

(Killeen et al., 2002). Although both the *unc-5(ev480)* and *unc-5(e152)* are predicted to cause premature termination of protein translation in the cytodomain, the *unc-5(e152)* product retains the signaling activity that prevents these phenotypes. Based on other phenotypes, previous studies reported that the *unc-5(e152)* allele retains UNC-40-dependent signaling functions (Killeen et al., 2002; Merz et al., 2001).

UNC-5 is required for the induction of multiple HSN axons by UNC-6ΔC and a *mig-15* mutation

The results above suggest that UNC-5 activity can regulate the number of HSN extensions that form. To further test this hypothesis, we checked whether loss of UNC-5 function can suppress the development of additional processes that can be induced. Previously we reported that expression of the N-terminal fragment of UNC-6, UNC-6ΔC, induces excessive branching of ventral nerve cord motor neurons and that UNC-5 can suppress this branching (Lim et al., 1999). We now report that HSN develops an extra process in response to UNC-6ΔC and that UNC-5 suppresses the development of this extra process (Figures 7D and 7F).

To investigate whether this UNC-5 activity might involve known effectors of asymmetric neuronal outgrowth, we tested for genetic interactions between *unc-5* and both *mig-10* and *mig-15*. MIG-10 (lamellipodin) is a cytoplasmic adaptor protein that can act cell-autonomously to promote UNC-40-mediated asymmetric outgrowth (Adler et al., 2006; Chang et al., 2006; McShea et al., 2013; Quinn et al., 2006; Quinn et al., 2008). MIG-15 (NIK kinase) is a cytoplasmic protein and evidence indicates that *mig-15* functions cell-autonomously to mediate a response to UNC-6 (Poinat et al., 2002; Teulière et al.,

2011). It's proposed that *mig-15* acts with *unc-5* to polarize the growth cone's response and that it controls the asymmetric localization of MIG-10 and UNC-40 (Teulière et al., 2011; Yang et al., 2014). We previously noted that HSN neurons often become bipolar in *mig-15* mutants and frequently UNC-40::GFP is localized to multiple surfaces in a single neuron, suggesting that loss of MIG-15 enhances the ability of UNC-40::GFP to cluster (Yang et al., 2014). In our experiments we used the *mig-10 (ct141)* loss-of-function allele (Manser et al., 1997; Manser and Wood, 1990) and the *mig-15(rh148)* allele, which causes a missense mutation in the ATP-binding pocket of the kinase domain and is a weak allele of *mig-15* (Chapman et al., 2008; Shakir et al., 2006).

We find that the extra processes induced by UNC-6ΔC expression are suppressed by *mig-10(ct141)* (Figures 7F). We also find that the *mig-15* mutation causes extra HSN processes and that the loss of UNC-5 function suppresses these extra HSN processes (Figures 7F and 7G). These results support the hypothesis that the ability of UNC-5 to regulate the development of multiple protrusions involves the molecular machinery that controls UNC-40-mediated asymmetric neuronal outgrowth.

UNC-5 is required for PLM overextension

The SDAL model predicts that the ability of UNC-5 to regulate the length and number of neural protrusions is independent of the direction of outgrowth. HSN sends a single axon ventrally, while PLM sends an axon anteriorly from a posteriorly positioned cell body. The HSN axon travels towards UNC-6 sources, whereas the PLM axon pathway is perpendicular to UNC-6 sources. To investigate whether UNC-5 activity can regulate the length or number of processes that develop perpendicular to UNC-6 sources we examined

the development of the PLM axon. We also chose PLM because UNC-5 was already known to affect the length of the PLM axon (Li et al., 2008).

Given that UNC-5 activity is involved in the overextension of the PLM axon, and that the *mig-15* mutation affects HSN outgrowth in an UNC-40 dependent fashion, we decided to test whether PLM overextension might be induced by the *mig-15* mutation in an UNC-40-dependent fashion. The HSN results suggest that altering *mig-15* function creates a sensitized genetic background. That is, the *unc-5(ev480)* mutation suppresses HSN outgrowth extension in both the wild-type and *mig-15(rh148)* backgrounds, but the *mig-15* mutation creates a stronger patterning phenotype. This idea is supported by the evidence that the *mig-15* mutation enhances the ability of UNC-40 to localize at surfaces (Yang et al., 2014).

We find that in *mig-15(rh148)* mutants the PLM axon often fails to terminate at its normal position and instead extends beyond the ALM cell body. This overextension is suppressed in *unc-5(e53);mig-15(rh148)* and *unc-40(e1430);mig-15(rh148)* mutants (Figures 8A and 8B). The results are consistent with the idea that UNC-5 is required for the UNC-40-mediated outgrowth activity that causes overextension in *mig-15(rh148)* mutants.

UNC-5 is required for ALM and AVM branching and extension

We also investigated the effect of UNC-5 activity on patterning where sources of UNC-6 and other cues are in a more complex arrangement. Specifically, we examined whether UNC-5 plays a role in the outgrowth of AVM and ALM processes at the nerve ring. During larval development, processes from the AVM neuron and the two ALM

neurons (one on each side of the animal) migrate anteriorly to the nerve ring at dorsal and ventral positions respectively (Figure 8C). At the nerve ring each axon branches; one branch extends further anteriorly and the other extends into the nerve ring. Evidence suggests that at the midbody of the animal the positioning of these axons along the dorsal-ventral axis requires UNC-6, UNC-40, and UNC-5 activity. In *unc-6, unc-40,* and *unc-5* null mutants, or when the UNC-6 expression pattern is altered, the longitudinal nerves are mispositioned (Ren et al., 1999). Glia cells and neurons at the nerve ring are sources of UNC-6 (Wadsworth et al., 1996). The guidance of some axons in the nerve ring are disrupted in *unc-6* and *unc-40* mutants (Hao et al., 2001; Yoshimura et al., 2008). The precise spatial and temporal arrangement of the UNC-6 cue in relationship to the position of the migrating growth cones is not fully understood. Nevertheless, the anteriorly migrating growth cones appear to use the UNC-6 cue from the ventral sources to help maintain the correct dorsal-ventral position, even while moving towards the nerve ring, which is a new source of UNC-6 that is perpendicular to the ventral source. At the nerve ring the axons branch. One process continues anteriorly, moving past the new UNC-6 source, whereas the other projects at a right angle and moves parallel to the new source.

We find genetic interactions involving *unc-5, unc-40,* and *mig-15* that affect outgrowth patterning of the ALM and AVM extensions at the nerve ring (Figures 8C, 8D, and 8E). In *mig-15(rh148);unc-5(e53)* mutants, the AVM axon often fails to extend anteriorly from the branch point and only extends into the nerve ring, or it fails to extend into the nerve ring and only extends anteriorly, or it fails to do both and terminates at this point. In *unc-40(e1430)* mutants, the axon often fails to branch into the nerve ring, although it extends anteriorly. In comparison, in *unc-40(e1430);mig-15(rh148)* mutants more axons extend

into the nerve ring. These results suggest that UNC-5 helps regulate UNC-40-mediated outgrowth to pattern the outgrowth at the nerve ring.

Interactions between *unc-5* and other genes affect a probability distribution for the direction of extension

We hypothesize that there are interactions between *unc-5* and other genes that control the degree to which the direction of outgrowth fluctuates. Probability distributions for the direction of extension are used to study how genes affect the fluctuation of outgrowth activity. By comparing the distributions created from wild-type and mutant animals, the relative effect that genes have on the fluctuation can be determined. To accomplish this, the direction that the HSN axon initially extends from the cell body is scored (Figure 9A). Using this assay, we examined genetic interactions between *unc-5* and four other genes; *elg-20, sax-3, madd-2,* or *unc-6.* We have chosen these particular genes because previous observations suggest interactions. 1) EGL-20 (Wnt) is a secreted cue expressed from posterior sources (Pan et al., 2006) and it affects to which surface of the HSN neuron the UNC-40 receptor localizes and mediates outgrowth (Kulkarni et al., 2013). Based on a directional phenotype, a synergistic interaction between *unc-5* and *egl-20* has been observed. In either *unc-5* or *egl-20* mutants the ventral extension of AVM and PVM axons is only slightly impaired, whereas in the double mutants there is much greater penetrance (Levy-Strumpf and Culotti, 2014). 2) SAX-3(Robo) is a receptor that regulates axon guidance and is required for the asymmetric localization of UNC-40 in HSN (Tang & Wadsworth, 2014). Based on a directional phenotype, SAX-3 and UNC-40 appear to act in parallel to guide the HSN towards the ventral nerve cord (Xu et al., 2015). 3) MADD-2

is a cytoplasmic protein of the tripartite motif (TRIM) family that potentiates UNC-40 activity in response to UNC-6 (Alexander et al., 2009; Alexander et al., 2010; Hao et al., 2010; Morikawa, Kanamori et al., 2011; Song et al., 2011; Wang et al., 2014). MADD-2::GFP and F-actin colocalize with UNC-40::GFP clusters in the anchor cell (Wang et al., 2014). 4) Of course, UNC-6 is an UNC-5 ligand. DCC (UNC-40) and UNC5 (UNC-5) are thought to act independently or in a complex to mediate responses to netrin (UNC-6) (Colavita and Culotti, 1998; Hong et al., 1999; Lai Wing Sun et al., 2011; MacNeil et al., 2009).

In a test for interaction with *egl-20*, we find that in comparison to *unc-5(e53)* or *egl-20(n585)* mutants, the *unc-5(e53);egl-20(n585)* double mutant have a lower probability for ventral outgrowth and higher probability for outgrowth in other directions (Table 1). This suggests that *unc-5* and *egl-20* may act in parallel to achieve the highest probability for HSN ventral outgrowth, *i.e.* they act to prevent UNC-40-mediated outgrowth from fluctuating in other directions.

In a test for interaction with *sax-3,* we find that the probability of outgrowth in each direction in *unc-5(e53);sax-3(ky200)* mutants is similar to the probabilities in *sax-3(ky200)*or *sax-3(ky123)* mutants (Table 1). Given the results with *unc-5* and *egl-20*, we further tested the probability of outgrowth in each direction in *egl-20(n585);sax-3(ky123)* mutants. We find that it is similar to the probabilities in *sax-3(ky200)* or *sax-3(ky123)* mutants (Table 1). The *sax-3(ky123)* allele results in a deletion of the signal sequence and first exon of the gene, whereas *sax-3(ky200)* contains a missense mutation which is thought to cause protein misfolding and mislocalization at the restrictive temperature (25°C) (Wang et al., 2013; Zallen, Yi, & Bargmann, 1998). The *egl-20(n585);sax-3(ky123)* mutants do

not grow well and so it is easier to use the temperature sensitive *sax-3* allele. Together, the results suggest that *sax-3* may be required for both the *unc-5*- and the *egl-20*-mediated activities that allow the highest probability for HSN ventral outgrowth.

In a test for interaction with *madd-2*, we find that the probability of outgrowth in each direction in *unc-5(e53);madd-2(tr103)* mutants is similar to the probabilities in *madd-2(tr103)* mutants (Table 1). There is a higher probability for anterior HSN outgrowth, similar to what is observed in *unc-40(e1430)* mutants. These results suggest that *madd-2* might be required for the *unc-40* outgrowth activity. The probability of outgrowth in each direction in *madd-2(tr103);sax-3(ky123)* mutants is similar to the probabilities in *sax-3(ky200)*or *sax-3(ky123)* mutants (Table 1). The *madd-2(tr103)* allele appears to act as a genetic null (Alexander et al., 2010).

In a test for interaction with *unc-6*, we find that the probability of outgrowth in each direction in *unc-5(e53);unc-6(ev400)* and *unc-40(e1430);unc-5(e53)* mutants is similar to the probabilities in *unc-6(ev400)* mutants insofar as there is a lower probability for ventral outgrowth and a higher probability for anterior outgrowth (Table 1). However, the probabilities in each direction are closer to those obtained from the *unc-40(e1430)* mutants because the probability of anterior outgrowth is lower in these mutants than in *unc-6* mutants. This suggest that UNC-5 and UNC-40 might help increase the probability of anterior outgrowth in the absence of UNC-6.

unc-5 is a member of a class of genes that has a similar effect on the spatial extent of movement

The results above show that *unc-5* and its interactions with other genes affect the

degree to which the direction of outgrowth fluctuates. The degree of fluctuation differs depending on the genes involved. A property of random movement is that the more the direction of movement fluctuates, the shorter the distance of travel is in a given amount of time (Figure 2E). To depict how *unc-5* and other genes differentially regulate the spatial extent of movement, we use random walk modeling. Random walks describe movement that occurs as a series of steps in which the angles and the distances between each step is decided according to some probability distribution. By using the probability distribution obtained from a mutant for each step of a random walk, and by keeping the distance of each step equal, a random walk can be constructed (Figure 9A). In effect, this method applies the probability distribution to discrete particles having idealized random walk movement on a lattice. By plotting random walks derived from wild-type animals and different mutants, the relative effect that mutations have on random walk movement can be visualized. For example, Figure 9B shows 50 tracks of 500 steps for wildtype and two mutants (mutant A is *unc-5(e53)* and mutant B is *egl-20(n585);sax-3(ky123)*). This reveals the effect that a mutation has on the displacement of movement. After 500 steps the displacement from the origin (0, 0) is on average less for mutant A than for wildtype, and less for mutant B than for wildtype or mutant A.

The random walk models show the relative effect that a mutation has on a property of outgrowth movement. It is worth noting that this is not modeling the actual trajectory of migrating axons. Neuronal outgrowth is essentially a mass transport process in which mass (the molecular species of the membrane) is sustained at the leading edge and moves outward. Our assay compares the effect that different mutations would have on the movement of mass at the leading edge of an extension if the conditions of the system were

kept constant. Of course, *in vivo* the conditions are not constant. For one, as an extension moves it will encounter new environments where the cues may be new or at different concentrations, all of which affect the probability distribution. The actual patterns of outgrowth observed are the result of all the probabilities for outgrowth that occur at each instance of time. It has recently been suggested that our description might be more accurately described as neuro-percolation, a superposition of random-walks (Aiello, 2016).

Our random walk analysis compares the effect that different mutations have on the properties of movement. In wild-type animals, there is a high probability for outgrowth in the ventral direction. The analysis shows that conditions in wildtype create nearly straight-line movement, *i.e.* if the same random walk is repeatedly done for the same number of steps, starting at the same origin, the final position of the walk along the x axis does not vary a great amount. In comparison, we find that a mutation can create random walk movement in which the final position is more varied. This variation occurs because the mutation increases the probability of outgrowth in other directions. For each mutation, we simulate 50 random walks of 500 steps and derive the mean and standard deviation of the final position along the X-axis. To compare strains, we plot the normal distribution, setting the mean at the same value for each. The difference between the curve for a mutant and wildtype shows the degree to which the mutation caused the direction of outgrowth to fluctuate (Figure 9C).

The results reveal four different distribution patterns (Figure 10). The first class is the wild-type distribution, which has the distribution curve with the highest peak. The second class comprises *unc-5, egl-20, unc-53,* and *unc-6* in which the distribution curve is flatter than the wild-type curve. We included *unc-53* because our previous study showed that it

has genetic interactions with *unc-5* and *unc-6* (Kulkarni et al., 2013). The *unc-53* gene encodes a cytoskeletal regulator related to the mammalian NAV proteins and *unc-53* mutations cause guidance defects (Maes et al., 2002; Stringham et al., 2002; Stringham and Schmidt, 2009). The third class has a distribution curve which is flatter than the second and comprises *sax-3, mig-15*, and several double mutation combinations (Figure 10). The fourth class has the flattest distribution curve and comprises *egl-20;sax-3, unc-40;sax-3,* and *unc-53;sax-3;unc-6*. This class indicates the greatest degree of fluctuation. The ability to cause the direction of movement to fluctuate is not associated with a specific direction of HSN movement. For example, *unc-5;sax-3, unc-53;unc-6, unc-40;egl-20,* and *madd-2;sax-3* each show a widely dispersed pattern, but the direction is ventral, dorsal, anterior, and posterior, respectively (Figure 10).

The distribution patterns indicate that genes have different effects on the extent that outgrowth movement can travel through the environment. Mean squared displacement (MSD) is a measure of the spatial extent of random motion. The MSD can be calculated from the random walk data. Plotting MSD as a function of the time interval shows how much an object displaces, on average, in a given interval of time, squared (Figure 11A). For normal molecular diffusion, the slope of the MSD curve is directly related to the diffusion coefficient. In cell migration models this value is referred to as the random motility coefficient. Coefficients are experimentally determined; they describe how long it takes a particular substance to move through a particular medium. We determine this value in order to numerically and graphically compare how mutations can alter displacement relative to wildtype (Figure 11B). The four classes of genes are apparent by comparing the height of the bars in Figure 11B. Results for the *unc-40* mutation are also

show. The random walk pattern is published (Tang and Wadsworth, 2014).

The results of this modeling suggest that the activities of certain genes, and combinations of genes, have distinct effects on the rate of outgrowth movement. In theory, these differences could be an important means by which genes cause different outgrowth patterns.

UNC-40 receptor clustering is coupled to the SDAL process

We investigated the relationship between UNC-40::GFP localization and outgrowth movement. Beginning in the early L2 stage, UNC-40::GFP becomes localized to the ventral side of HSN in wildtype (Adler et al., 2006; Kulkarni et al., 2013). Reflecting the dynamic morphological changes that occur as the HSN axon forms, the site of asymmetric UNC-40::GFP localization alternates in the neurites and along the ventral surface of the neuron (Kulkarni et al., 2013). Dynamic UNC-40::GFP localization patterns have also been reported for the anchor cell, in which UNC-40 and UNC-6 are also key regulators of extension (Hagedorn et al., 2013; Ziel et al., 2009). Live imaging of the anchor cell reveals that UNC-40::GFP "clusters" form, disassemble, and reform along the membrane (Wang et al., 2014). However, live imaging can't directly ascertain whether the position of a cluster is randomly determined since a movement event cannot be repeatedly observed to determine a probability distribution. Mathematical modeling of cluster movement as a stochastic process has not been done.

The UNC-40::GFP clustering phenomena raises questions about the relationship between robust UNC-40 clustering (*i.e.,* sites of distinct UNC-40 localization observable by UNC-40::GFP) and UNC-40-mediated outgrowth activity. Two models are presented

in Figure 12. In the first model, the output of the SDAL process is receptor clustering (Figure 12A). After a cluster becomes stabilized at a site, the machinery required for outgrowth is recruited and outgrowth occurs. In our model, the SDAL process and UNC-40-mediated outgrowth activity are coupled and are part of the same stochastic process that occurs at the micro-scale (Figure 12B). UNC-40::GFP clustering is a macro-scale event which can be observed. It is a consequence of the micro-scale events.

The models make specific predictions that can be tested. In the first model, UNC-40-mediated outgrowth will not happen if UNC-40 does not cluster. In our model, the loss of UNC-40 clustering does not lead to a loss of UNC-40-mediated outgrowth. In the *sax-3* mutant there is a large fluctuation in the direction of outgrowth; it is in the third class of mutants (Figures 10 and11). We previously reported that *sax-3* is required for robust UNC-40::GFP asymmetric localization; in *sax-3* mutants UNC-40::GFP remains uniformly dispersed around the periphery of HSN ((Tang and Wadsworth, 2014) and Figure13). Whereas in the *sax-3* mutant there is a ventral bias for outgrowth, in the *unc-40;sax-3* mutant there is not (Figure10). This suggests that in the *sax-3* mutant there is UNC-40-mediated outgrowth activity that helps create a ventral bias. This is consistent with our model because UNC-40-mediated outgrowth activity is occurring even when robust UNC-40::GFP is not observed.

We hypothesize that a consequence of the micro-scale SDAL process over time is macro-scale UNC-40 clustering. If so, then *unc-5* activity should affect UNC-40::GFP clustering because it affects the degree to which the direction of UNC-40 receptor localization fluctuates. However, even though there is a higher probability that localization occurs at surfaces other than at the ventral surface, we observe robust asymmetrically

localized UNC-40::GFP clustering in *unc-5(e53)* mutants (Kulkarni et al., 2013). We speculate that *unc-5(e53),* as well as other gene mutations, does not cause the direction of UNC-40 localization to fluctuate enough to prevent observable UNC-40::GFP clustering. We therefore decided to examine UNC-40::GFP clustering in double mutants to determine whether the ability to observe UNC-40::GFP clustering is correlated with the degree of fluctuation.

We made double mutant combinations between *unc-5*, and *egl-20* or *unc-53.* In *egl-20* and *unc-53* single mutants there is fluctuation in the direction of outgrowth (Figures 10 and 11) and robust asymmetrical UNC-40::GFP localization (Figure 13, *unc-53* results were previously reported (Kulkarni et al., 2013)). In comparison to the single mutants, the double mutants all show an increase in the degree to which the direction of outgrowth fluctuates (Figures 10 and 11). Further, in contrast to the single mutants, UNC-40::GFP remains uniformly dispersed around the periphery of HSN in the double mutants (Figure 13). The results suggest a correlation between the degree to which UNC-40-mediated outgrowth activity fluctuates and the ability to detect UNC-40::GFP clustering. This is consistent with our model (Figure 12B). We also observe that in *madd-2(tr103)* mutants the direction of outgrowth fluctuates (Table 1), but unlike *egl-20* and *unc-53* single mutants, there is not robust asymmetrical UNC-40::GFP localization and UNC-40::GFP remains uniformly dispersed (Figure 13). The double mutants, *unc-5;madd-2*, are similar to the single *madd-2* mutant. Similar results are observed with *sax-3* and *unc-5;sax-3* mutants (Figure 13). We hypothesize that in the *madd-2* and *sax-3* mutations the degree to which the direction of UNC-40 localization fluctuates is so great that the *unc-5* mutation makes no difference on the UNC-40::GFP clustering phenotype.

DISCUSSION

We have proposed a model of neuronal outgrowth movement that is based on statistically dependent asymmetric localization (SDAL). This model states that the probability of UNC-40 localizing and mediating outgrowth at one site affects the probability of localization and outgrowth at other sites as well. By regulating this process, genes control the degree to which the direction of outgrowth fluctuates and, consequently, the outward movement of the plasma membrane. UNC-5 is a ligand for UNC-6 and can form a complex with UNC-40. UNC-5 is commonly proposed to direct outgrowth by mediating a repulsive response to UNC-6. In contrast, our model is not based on the concept of repulsion and it predicts that UNC-5 can control the rate of outward movement that is directed towards, away from, or perpendicular to UNC-6 sources. We report that *unc-5* loss-of-function mutations affect the development of multiple neurites that develop from HSN and extend towards UNC-6 sources. They also suppress the development of extra HSN processes which are induced by a *mig-15* mutation or by expression of the N-terminal fragment of UNC-6 and which extend towards UNC-6 sources. We also observe that *unc-5* mutations suppress the anterior overextension of the PLM axon that occurs in the *mig-15* mutant. This axon extends perpendicular to UNC-6 sources. Finally, *unc-5* loss-of-function mutations affect the branching and extension of ALM and AVM axons at the nerve ring where the sources of UNC-6 are in a more complex arrangement. Below we discuss how the SDAL model can be used to interpret *unc-5* mutant phenotypes. We argue that in each case, phenotypes can be explained by the ability of UNC-5 to affect UNC-40 asymmetric localization, which in turn controls the degree to which the direction of outgrowth activity fluctuates and the extent of outward movement. Our model also suggests

genes that were previously classified as regulating attraction or repulsion might act with *unc-5* to regulated neuronal outgrowth by controlling the degree to which the direction of UNC-40-mediated outgrowth fluctuates. We show that UNC-5 acts together with the cytoplasmic protein UNC-53 to regulate UNC-40 asymmetric localization in response to the UNC-6 and EGL-20 extracellular cues.

PLM extension phenotype: We hypothesize that cue(s) present around the PLM cell body create a strong bias for anterior outgrowth activity (Figure 14A). These include UNC-6 and other cues that flank the longitudinal pathway and cause an equal probability of outgrowth in the dorsal and ventral directions. UNC-40 SDAL activity acts to suppress nonUNC-40 SDAL activity (Figure 6). As the extension moves towards more anterior positions (Figure 14A, positions 2 and 3), it encounters higher levels of a cue(s) that promotes outgrowth through nonUNC-40 receptors. As a result, the probability of nonUNC-40 SDAL activity at the dorsal and ventral surfaces of the leading edge increases. Because the asymmetric localization of a receptor is statistically dependent, the probability of nonUNC-40 SDAL activity at the anterior surface of the leading edge must decrease as the localization elsewhere increases. While this effect does not necessarily change the anterior bias for outgrowth, it does significantly increase the degree to which the direction of nonUNC-40 outgrowth activity fluctuates, which consequently decreases the extent of outward movement (Figure 14C). This effect stalls outward movement.

We hypothesize that mutations affect the degree to which the direction of nonUNC-40 outgrowth activity fluctuates at position 3 (Figure 15). MIG-15 appears to promote nonUNC-40 SDAL activity, whereas UNC-5 promotes UNC-40 SDAL activity. Because

each activity can suppress the other, different domains of nonUNC-40 and UNC-40 SDAL activity can be established along the surface of the leading edge. As the extension moves towards position 3, there is higher nonUNC-40 activity at the more anterior surface and higher UNC-40 activity at the dorsal and ventral surfaces. By suppressing nonUNC-40 activity, the *mig-15* mutation increases, relative to wildtype, the UNC-40 activity at the dorsal and ventral surfaces at position 3. This UNC-40 activity decreases the probability of nonUNC-40 activity at these surfaces and increases the probability of nonUNC-40 activity at the anterior surface. By reducing the degree to which nonUNC-40 outgrowth fluctuates, a greater anterior directional bias is created in the *mig-15* mutants. This results in overextension. The *unc-5* mutation represses the UNC-40 activity at the dorsal and ventral surfaces, increasing the degree to which the direction of the nonUNC-40 outgrowth activity fluctuates. This suppresses the overextension caused by the *mig-15* mutation.

AVM nerve ring branching and extension phenotype: Similar to what is proposed for PLM at position 3 in Figure 14, all the surfaces of the leading edge of AVM become exposed to high levels of a cue(s) (Figure 16A, position 1). The degree to which the direction of outgrowth activity fluctuates greatly increases and outward movement stalls. For AVM, this occurs at the nerve ring, which is a source of UNC-6. However, for AVM there are cues at the nerve ring which are arranged perpendicular to one another. We propose that the high level of UNC-6 at all surfaces allows UNC-40 SDAL activity to become more uniformly distributed along all surfaces of the leading edge (Figure 16B) and creates a state where the probabilities of outgrowth in every direction become equal. Both anterior and dorsal outward movement stalls (Figure 16C). This state allows any new cues

encountered to effectively create a directional bias (Figure 4). UNC-6 and other cues are arranged along the nerve ring, whereas nonUNC-6 cues are arranged anterior of the nerve ring. As some outgrowth ventures anteriorly and dorsally, these cues stimulate the development of nonUNC-40 and UNC-40 SDAL activity domains (Figure 16B, position 2). Even slight outward movement in the anterior or dorsal directions may reinforce movement in that direction if cues arranged along the axis perpendicular to the direction bias suppress the UNC-40 or nonUNC-40 SDAL activity along the surfaces perpendicular to the directional bias (as depicted in Figure 14B, position 1).

We hypothesize that mutations affect the degree to which the direction of UNC-40 and nonUNC-40 outgrowth activity fluctuates at position 2 (Figure 17). In *unc-40* mutants, the lack of dorsal UNC-40 activity allows the direction of nonUNC-40 outgrowth to fluctuate more. However, the anterior cues increase the probability of anteriorly directed nonUNC-40 outgrowth and, thereby, decrease the probability of dorsally directed activity. This suppresses dorsal extension, while still allowing anterior extension. Loss of MIG-15 activity in the *unc-40* mutant background suppresses the nonUNC-40 SDAL. In comparison to the single *unc-40* mutant, in *unc-40;mig-15* mutants the probability of anterior outgrowth in response to the anterior cues is lower. Consequently, the probability of dorsal nonUNC-40 outgrowth is higher. We speculate that this allows some dorsal extension. In *unc-5* mutants, UNC-40 SDAL activity is reduced, but the activity is still sufficient to allow dorsal extension. Loss of both UNC-5 and MIG-15 function most severely hampers the ability to direct the receptors specifically to one surface. The *unc-5;mig-15* mutants have the most abnormal outgrowth patterns.

HSN extension phenotypes: We hypothesize that there is high probability for ventrally directed outgrowth from the HSN cell body because of the strong outgrowth-promoting effect of the UNC-40-mediated response to the UNC-6 cue, which is in a higher concentration ventral of the cell body (Figure 18A). We hypothesize that the same process takes place in the HSN neuron as in the PLM neuron, except the movement is towards the UNC-6 source. We depict in Figure 8B that at position 1 there is some nonUNC-40 SDAL activity at the ventral surface of the leading edge. By position 2, higher levels of UNC-6 increase UNC-40 SDAL and suppress ventral nonUNC-40 SDAL activity. At positon 3, UNC-6 is present at high levels along all surfaces and the direction of UNC-40 outgrowth greatly fluctuates. Possibly, the degree to which the direction of UNC-40 and nonUNC-40 outgrowth activity fluctuates is greater at position 1, than at position 2 (Figure 18C). However, at position 3 the fluctuation is greatest.

We hypothesize that the interplay between UNC-40 and nonUNC-40 SDAL activity allows multiple extensions to develop in the same direction. In fact, UNC-40, UNC-5, MIG-15, and UNC-6 (netrin) activities may function as a type of reaction-diffusion system (Gierer and Meinhardt, 1972; Goehring and Grill, 2013; Kondoand Miura, 2010; Meinhardt and Gierer, 2000; Turing, 1952). Along the ventral surface there is a competition between UNC-40 receptors to direct further UNC-40 localization to that site and to inhibit flanking receptors from doing the same. Overtime, the SDAL activity that began at one site predominates, leading to an area of higher outgrowth activity (Figure 19). We speculate that by helping to suppress UNC-40 SDAL activity, nonUNC-40 activity increases the threshold by which the SDAL activity at one site can begin to predominate. The *mig-15* mutation suppresses the nonUNC-40 SDAL activity and decreases the threshold. This may

allow more sites along the membrane where UNC-40 SDAL activity can predominate. The sites do not overlap because of the long-range negative feedback that inhibits neighboring UNC-40 activity. The *unc-5* mutation suppresses UNC-40 SDAL activity, both the positive and negative feedback loops. This retards the ability to enhance and localize the process to one area of the surface. This causes greater fluctuation in the direction of outgrowth activity across the entire ventral surface of the neuron. As a result, the rate of initial outgrowth is even less than that which occurs in wildtype and the area of outward growth is more broad.

A genetic pathway for UNC-40 asymmetric localization

We present a genetic pathway for the asymmetrical localization of UNC-40 based on the phenotype of robust UNC-40::GFP clustering in HSN. A full understanding of the molecular mechanisms underlying the SDAL process is an important long-term goal. Since we believe that UNC-40::GFP clustering is a readout of that process, constructing genetic pathways for the clustering of UNC-40::GFP is a step toward this goal. We wish to know how UNC-5 mediates signaling within HSN that controls the UNC-40 asymmetric localization process. However, a role for UNC-5 in HSN is paradoxical given the widespread idea that UNC-5 mediates a repulsive response to UNC-6 and that HSN outgrowth is towards the source of UNC-6. All the same, we suggest a cell-autonomous role for UNC-5 in HSN is the most parsimonious model. First, UNC-5 is an UNC-6 receptor that can mediate neuronal responses when in complex with UNC-40 (Finci et al., 2014; Geisbrecht et al., 2003; Hong et al., 1999; Kruger et al., 2004). We previously showed that UNC-40 conformational changes regulate HSN asymmetric localization in HSN (Xu et al., 2009) and we now show that UNC-5 regulates UNC-40 asymmetric

localization in HSN. It is therefore plausible that UNC-5 affects UNC-40 conformational changes that regulate UNC-40 asymmetric localization. Second, UNC-5 can alter the number of HSN outgrowths in response to UNC-6 and to the UNC-6ΔC ligand. Directional guidance by UNC-6 and UNC-6ΔC is generally normal in an *unc-5* mutant, suggesting that the ability of UNC-5 to regulate the number of outgrowths is not due to an alteration in the extracellular distribution of its UNC-6 ligand. Further, the UNC-6ΔC ligand and the *mig-15* mutation create the same outgrowth phenotype, which can be suppressed by loss of UNC-5 function, and we have shown that MIG-15 acts cell autonomously in HSN to regulate UNC-40 asymmetric localization (Yang et al., 2014). Further, we have shown that the UNC-5-mediated response that regulates UNC-40 asymmetric localization also depends on UNC-53 (NAV2) (Kulkarni et al., 2013), a cytoplasmic protein that functions cell-autonomously for cell migration and axon guidance (Stringham et al., 2002). Together, these observations strongly suggest that UNC-5 directly regulates signaling within HSN. Third, a role for UNC-5 in the guidance of AVM and PVM axons towards UNC-6 sources has also been suggested. A synergistic interaction between *unc-5* and *egl-20* is observed; in either *unc-5* or *egl-20* mutants the ventral extension of AVM and PVM axons is only slightly impaired, whereas in the double mutants there is a much greater penetrance (Levy-Strumpf and Culotti, 2014). The expression of an *unc-5* transgene in AVM and PVM can rescue the AVM and PVM axon guidance defects of the *unc-5;egl-20* double mutant (Levy-Strumpf and Culotti, 2014). We note that for HSN, transgenic rescue using *unc-5* constructs have not been successful and in wild-type animals UNC-5 expression in HSN has not been reported. As well, expression has not been reported in AVM, PVM, and PLM wild-type neurons. We suspect there may be technical difficulties or that UNC-5 expression might

be low in these cells. UNC-5 is detected in PLM in *rpm-1* mutants, which is consistent with evidence that UNC-5 activity is required for PLM overextension in these mutants (Li et al., 2008).

To construct genetic pathways, we use the readout of whether UNC-40::GFP is clearly and consistently localized to any side of the HSN neuron in different mutants (Figure13). A summary of the results is presented (Figure 20A). UNC-6 is required for robust asymmetric UNC-40 localization; in the absence of UNC-6 function UNC-40 remains uniformly distributed along the surface of the plasma membrane. The loss of both UNC-53 and UNC-5 function also results in a uniform distribution, however loss of either one alone does not. This suggests that UNC-53 and UNC-5 pathways act redundantly downstream of UNC-6 (Figure 20B). Moreover, we observe there is robust asymmetric UNC-40 localization when there is a loss of UNC-6 activity in addition to the loss of UNC-53 and UNC-5. This suggests a third pathway that is suppressed by UNC-6 when UNC-53 and UNC-5 activity are missing. Loss of both UNC-5 and UNC-6 does not allow UNC-40 localization, whereas loss of both UNC-53 and UNC-6 does, therefore UNC-53, rather than UNC-5, acts with UNC-6 to suppress the third pathway.

UNC-40 becomes localized when EGL-20 activity is lost. As well, UNC-40 becomes localized when both EGL-20 and UNC-53 activities are lost. This is consistent with UNC-6 promoting UNC-40 localization via the UNC-5 pathway. Loss of EGL-20 and UNC-5 prevents UNC-40 localization. In these animals, the UNC-5 pathway is absent and UNC-6 is present to blocks the third pathway, therefore the UNC-53 pathway that leads to UNC-40 localization must require EGL-20, as well as UNC-6.

Loss of UNC-6 activity or loss of both UNC-6 and EGL-20 activity prevents

localization, whereas loss of only EGL-20 does not. To explain this, we propose that when UNC-6 is lost, the third pathway, which would otherwise be activated by the loss of UNC-6, remains suppressed because EGL-20 activity promotes suppression via UNC-53 activity. This suppression also explains why loss of UNC-6 and UNC-5 activity does not cause localization.

The genetic pathways are consistent with the models proposed in Figures 1 and 6. In the models, positive feedback loops amplify the polarized responses to extracellular cues, whereas negative feedback limits the responses and confines the positive feedback to the sites of interaction. We hypothesize that the UNC-5 and UNC-53 genetic pathways shown at the top and bottom of figure 20B correspond to the positive feedback loops depicted in Figures 1 and 6 by the arrows. The "?" genetic pathway corresponds to UNC-40 asymmetric localization and outgrowth activity in the absence of UNC-6. The UNC-53 genetic pathway in Figure 20B that block the "?" pathway corresponds to the negative feedback loops (lines) in Figures 1 and 6 which prevent UNC-40 asymmetric localization and outgrowth in the absences of UNC-6. Loss of both UNC-6 and EGL-20 prevents robust asymmetric UNC-40 localization because both UNC-6 and EGL-20-mediated positive feedback loops are disrupted. A positive feedback loop may be necessary to establish negative feedback loop. Therefore, the "?" pathway is not active when both UNC-6 and EGL-20 are absent.

Importantly, this genetic analysis indicates that netrin (UNC-6) and wnt (EGL-20) signaling are integrated to regulate self-organizing UNC-40 asymmetric localization. An implication of this result is that the extracellular concentrations of UNC-6 and EGL-20 could control the activation or inhibition of UNC-40-mediated outgrowth. This could be

important for generating patterns of outgrowth when neurons move to new locations within the animal. The picture is complicated by the evidence that both UNC-6 and EGL-20 affect the SDAL of both UNC-40-mediated and nonUNC-40-mediated outgrowth activity. It is possible that overlapping sets of extracellular cues and their receptors are involved in setting the probability of outgrowth for each activity. SAX-3 and MADD-2 are required for UNC-40::GFP localization, but also affect nonUNC-40-mediated outgrowth. The *egl-20;sax-3* and *unc-40;sax-3* double mutations have the greatest effect on restricting the extent of outgrowth movement in any direction (Figures 6 and 7). Moreover, the number of HSN neurites is reduced in *unc-5* mutants, whereas the number in *unc-5;sax-3* double mutants appears normal (Figure 7B). Understanding the interdependence of these outgrowth activities could provide a better understanding of how extracellular cues affect the patterns of outgrowth *in vivo*.

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TABLES AND FIGURES

Table 1. Direction of Axon Formation from the HSN Cell Body

	direction of axon protrusion						
	dorsal	ventral	anterior	posterior	multipolar		
	%	%	%	%	%	*n*	reference
wildtype	0	96±2	3±2	0	1±1	221	(Kulkarni et al., 2013)
unc-6(ev400)	2±2	3±2	81±2	8±2	6±1	218	(Kulkarni et al., 2013)
unc-40(e1430)	2±1	6±2	67±2	19±1	6±1	183	(Kulkarni et al., 2013)
unc-5(e53)	0	75±3	19±2	1±1	5±1	245	(Yang et al., 2014)
unc-53(n152)	0	67±3	22±2	5±1	6±1	238	(Kulkarni et al., 2013)
sax-3(ky123)	2±1	31±1	21±1	37±2	9±2	232	(Tang & Wadsworth, 2014)
*sax-3(ky200)**	2±1	32±1	19±2	42±3	5±2	198	(Tang & Wadsworth, 2014)
unc-5(e53);sax-3(ky200)	2±1	40±3	24±2	28±2	6±1	120	
unc-5(e53);unc-6(ev400)	4±2	5±3	59±4	22±4	9±1	201	
unc-5(e53);egl-20(n585)	3±1	28±4	22±4	35±5	11±2	114	
unc-53(n152);unc-5(e53)	0	19±1	62±2	17±1	3±1	224	(Kulkarni et al., 2013)
unc-53(n152);unc-6(ev400)	24±2	0	19±2	22±2	34±3	144	(Kulkarni et al., 2013)
unc-53(n152);sax-3(ky123)	1±1	47±3	24±2	23±5	6±3	207	(Tang & Wadsworth, 2014)
unc-40(e1430);unc-5(e53)	5±1	6±1	55±2	19±2	14±1	196	(Kulkarni et al., 2013)
*unc-40(e1430);sax-3(ky200)**	14±3	2±1	40±2	35±3	9±4	191	(Tang & Wadsworth, 2014)
sax-3(ky200); unc-6(ev400)*	8±1	8±2	49±3	20±5	14±2	211	(Tang & Wadsworth, 2014)
unc-53(n152);unc-5(e53);unc-6(ev400)	23±2	0	34±2	15±2	28±2	148	(Kulkarni et al., 2013)
unc-53(n152);sax-3(ky200);unc-6(ev400)*	11±2	2±1	33±4	30±3	25±5	189	
egl-20(n585)	0	64±2	21±2	7±1	8±1	304	(Tang & Wadsworth, 2014)
egl-20(n585); unc-6(ev400)	18±2	0	43±2	15±2	24±2	205	(Tang & Wadsworth, 2014)
unc-40(e1430); egl-20(n585)	6±2	17±2	45±5	15±2	16±2	173	(Tang & Wadsworth, 2014)
egl-20(n585);sax-3(ky123)	1±1	12±2	39±2	39±1	8±3	177	(Tang & Wadsworth, 2014)
madd-2(tr103)	0	19±2	55±5	17±4	8±2	179	
madd-2(ky592)	0	52±2	43±2	5±1	0	95	
unc-5(e53);madd-2(tr103)	3±1	15±2	52±4	17±4	13±1	197	
madd-2(tr103);sax-3(ky123)	2	24±3	19±4	47±1	7±2	171	
unc-53(n152);madd-2(tr103)	1±1	15±2	43±2	17±1	24±4	148	
mig-15(rh326)	2±1	15±1	24±3	11±3	48±8	131	(Yang et al., 2014)

Numbers represent percentage value ± SEM.

*Animals grown at the *sax-3(ky200)* restrictive temperature (25°C).

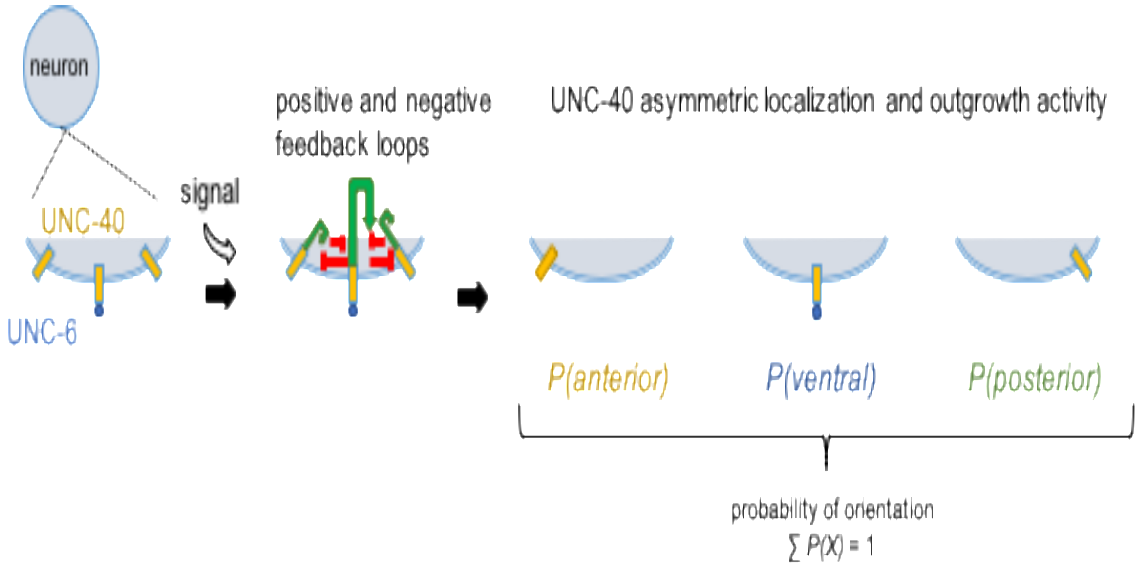

Figure 1. Statistically dependent asymmetric localization (SDAL). At sites along the plasma membrane, UNC-40 interacts with the UNC-6 extracellular cue. A self-organizing process is triggered that utilizes positive- and negative-feedback loops. Positive feedback (green arrows) amplifies the polarized response to an extracellular cue, while negative feedback (red lines) limits the response and can confine the positive feedback to the site of UNC-40 and UNC-6 interaction. The outcome of an UNC-40 receptor's activity is to either cause an UNC-40 receptor to localize and mediate outgrowth at the site of UNC-6 interaction or at a different site. Randomness is considered inherent in this process and each localization event is mutually exclusive. This statistical dependence means that the probability of UNC-40 localizing and mediating outgrowth at the site of UNC-6 interaction affects the probability of UNC-40 localizing and mediating outgrowth at another site, and vice versa. As time passes, this process causes randomly directed outgrowth activity (force) that drives the outward movement of the membrane.

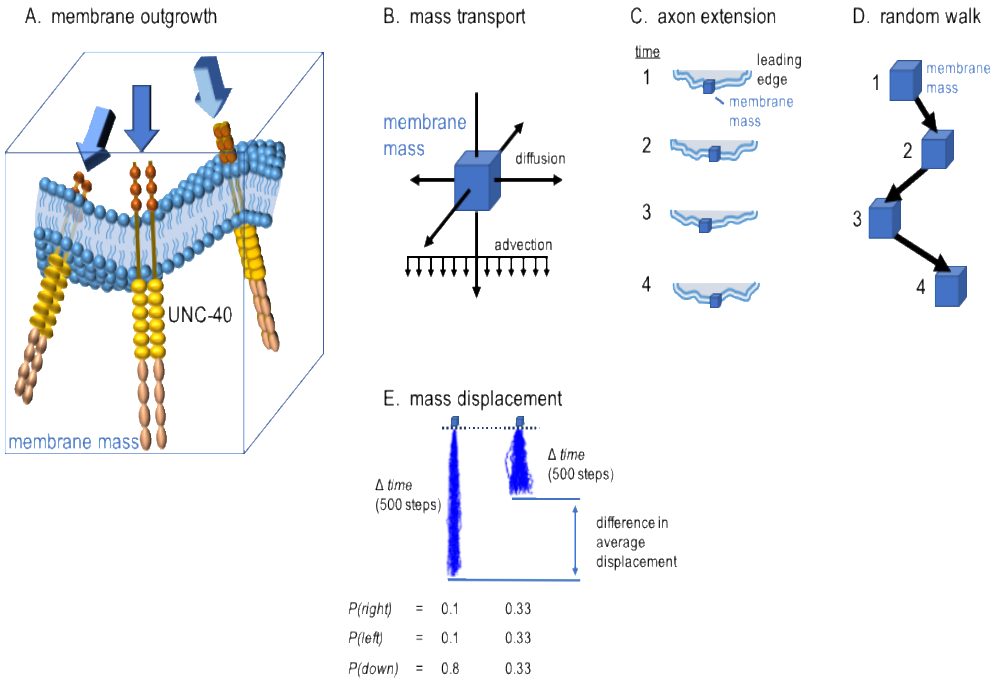

Figure 2. Model for outgrowth movement. (A) The outward movement of the neuronal membrane is depicted as a mass transport phenomena. The cell membrane is fluid and membrane mass will move in different directions as the membrane is subjected to forces (arrows) which change its shape. Receptors mediate cellular responses that creates the outward force. The force causes movement of the lipids and proteins of the plasma membrane. A unit of this mass is shown within a box. Membrane mass is represented by a box in subsequent schematic diagrams. (B) The mean flow of membrane mass (box) can be described as advection and diffusion. The probability density function of the position of mass as a function of space and time is described mathematically by an advection-diffusion equation. Mass transport by a mean velocity field is advection. Because of the SDAL process and the fluid nature of the membrane, mass transport also occurs through random movement, which is diffusion. (C) During outward movement of the leading edge (times 1-4), membrane molecules move in the direction of advection as well as randomly in other directions. (D) The path that the membrane molecules take during outgrowth can be described as a random walk, which is a succession of randomly directed steps. Depicted are the position of mass after each step of a succession of four steps as shown in C. Each step corresponds to a time point. (E) For two examples, 50 simulated random walks of 500 steps were plotted from an origin (0, 0). For each step the probability of moving to the right, left, or down is given below the plots. The plots illustrate that increasing the degree to which the direction of movement fluctuates, decreases the outward distance that mass can travel. We predict that the SDAL process influences the degree of random membrane movement and, consequently, the outward displacement of the membrane.

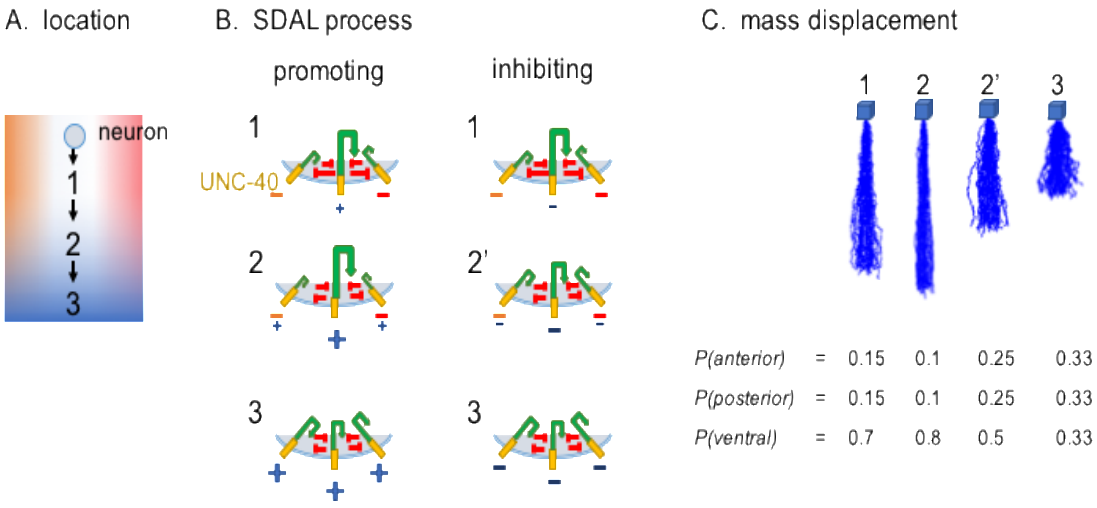

Figure 3. Model for outgrowth movement towards extracellular cues that promote or inhibit outgrowth activity. (A) Schematic diagram of the outgrowth of a neuron through an environment of multiple extracellular cues. These cues may be molecules present at the surfaces of surrounding cells and extracellular matrix, or they may be physical interactions that influence outgrowth activity. The extracellular cues are represented as color gradients of blue, orange, and red. The neuron's response to cues arranged along the anterior/posterior axis (orange and red), create an equal probability for UNC-40 asymmetric localization and outgrowth in the anterior and posterior directions. The extension transverses three different positions (1-3) as it develops towards a ventral source of a cue (blue). (B) The SDAL process is illustrated as in Figure 1 for the three positions shown in A. Shown are scenarios for movement towards a cue (A, blue) that promotes outgrowth (blue +) or that inhibits outgrowth (blue -). At positions 1 and 2 cues along the anterior/posterior axis (orange - and red -) prevent outgrowth in the anterior or posterior directions. At position 3, the cue from the ventral source predominates. (C) Random walk modeling as described in Figure 2E. At each position (A, 1-3), cues alter the probability distribution for the direction of localization and outgrowth. Below each plot is the probability distribution used to create the random walk (see Materials and Methods). Probability distributions were selected to represent how different levels of the ventral cue might change the probability distribution at each position. The plots illustrate the probability density function of the position of mass as a function of space and time if movement occurred according to that probability distribution. For both scenarios, an equal probability of anterior and posterior outgrowth can allow a ventral directional bias at position 1. Movement towards a promoting cue source can allow a greater probability for ventral outgrowth and, correspondingly, a lower probability for anterior and posterior outgrowth (position 2). Movement towards an inhibiting cue source can allow a lower probability for ventral outgrowth and, correspondingly, a greater probability for anterior and posterior outgrowth (position 2'). The ventral direction bias is maintained.

In either scenario, an equal probability for outgrowth in all directions may occur as the receptors become saturated because of the high level of the cue from the ventral source (position 3). The modeling predicts that in both scenarios changing levels of the ventral cue will not alter the direction of outward movement, although it may alter the outward displacement of the membrane's mass. See text for details.

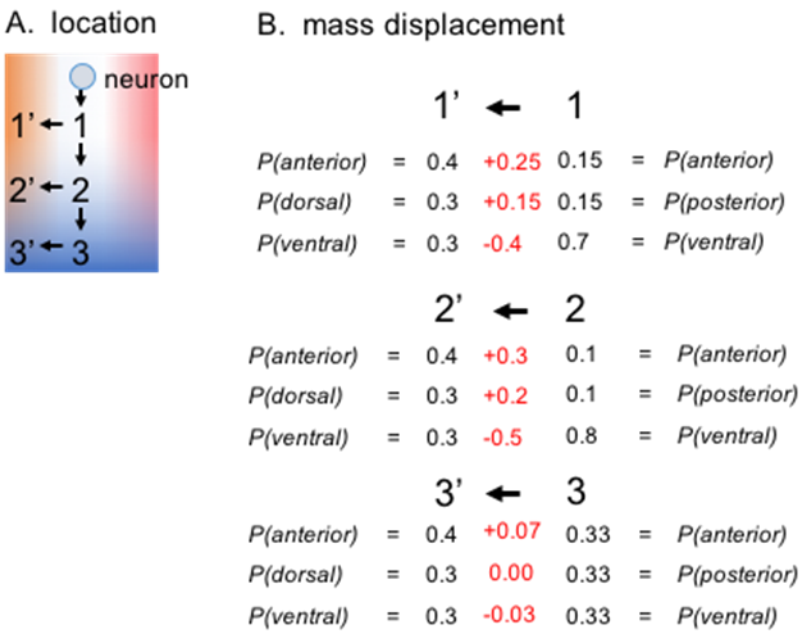

Figure 4. Model for outgrowth movement that changes direction. (A) Schematic diagram of the outgrowth of a neuron through an environment of multiple extracellular cues as described in Figure 3A. Positions 1'-3' represent the position after a change from ventral to anterior outgrowth. (B) At each position (A, 1-3), the probability distribution for the direction of localization and outgrowth is given as in Figure 3C. In order for the direction of outgrowth to shift anteriorly at each position, the probability distribution must shift to create a bias for anteriorly directed outgrowth. This is depicted by the probability distribution of 0.4 anterior, 0.3 dorsal, and 0.3 ventral for positions 1'-3'. Numbers in red indicate the degree to which the probabilities must change between the positions. The model predicts that as the system trends towards a state where the probabilities of outgrowth in different directions become equal, cues that could shift the direction bias become more effectual.

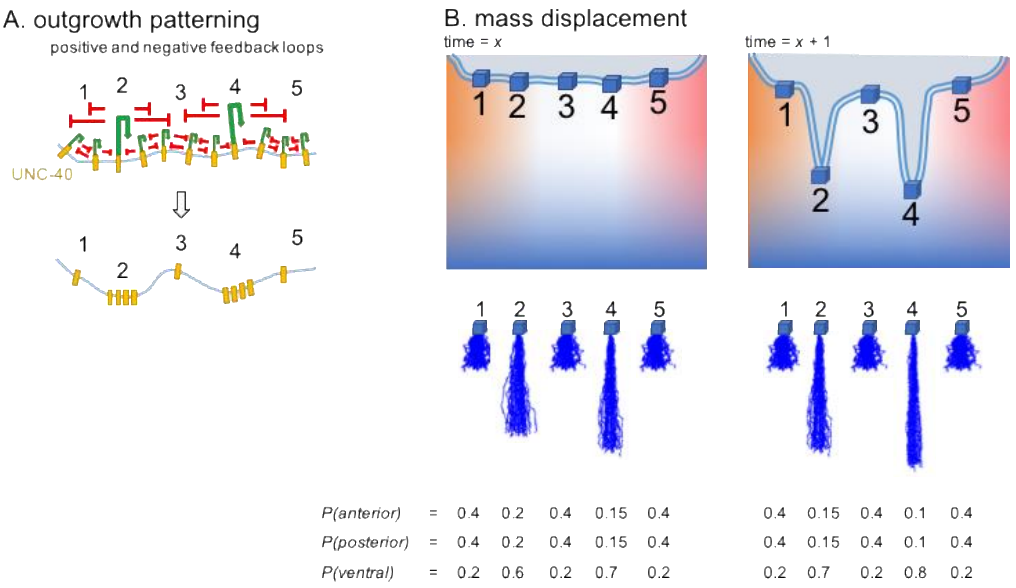

Figure 5. Model for the development of multiple outgrowths that extend in the same direction. (A) The SDAL process is illustrated for sites along a surface of a neuron as in Figure 1. The positive and negative feedback loops of the SDAL process allow spatial patterns of outgrowth to develop autonomously. The number of sites where a strong directional bias is ultimately created is dictated by the relative effectiveness of the positive and negative feedback loops. (B) Schematic diagram of the outgrowth of a neuron through an environment of multiple extracellular cues as described in Figure 3A. The flow of membrane mass (box) at different sites depends on the probability distribution for the direction of outgrowth created at regions along the surface. Random walk modeling as described in Figure 2E is shown below the schematic diagram. At time X, two sites which have a greater directional bias (2 and 4) are established by the SDAL process as depicted in A. Cues may not be present in steep gradients along the axis perpendicular to the direction of extension. The response to these cues creates probabilities for outgrowth that are equal in the perpendicular directions. The greatest directional bias is created when there is an equilibrium for the probability of outgrowth in perpendicular directions. Because cue levels may vary gradually along the perpendicular axis, the strength of the directional bias at sites may differ, however the bias will be oriented in the same direction. At time $x + 1$, positions 2 and 4 have proceeded further outward because of greater membrane displacement. This effect is magnified by increasing levels of the outgrowth promoting cue (blue). This activity together, when averaged over time across a surface, is predicted to cause the dynamic development of multiple extensions.

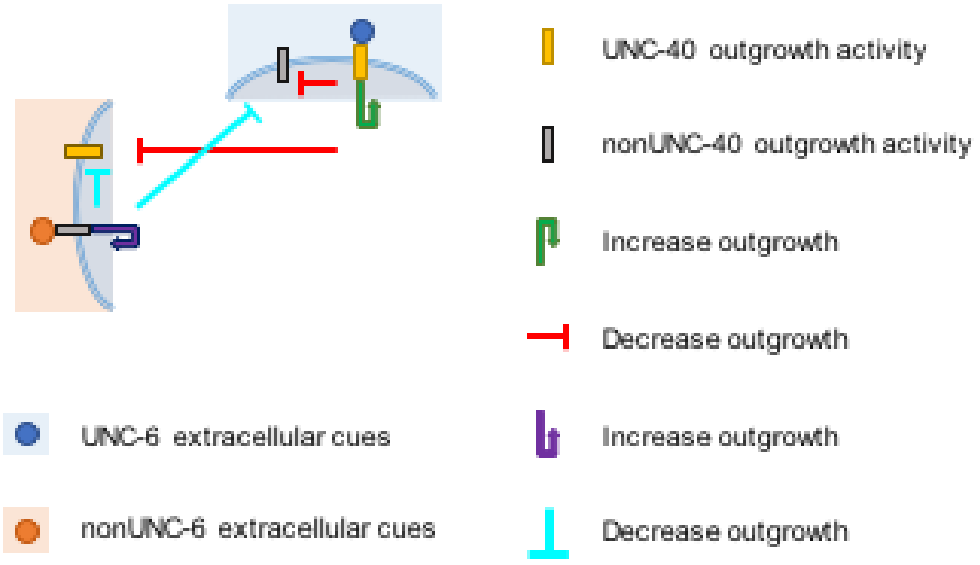

Figure 6. Model for the control of outgrowth activity by SDAL. Schematic diagram of the control of UNC-40- and nonUNC-40-mediated outgrowth activity. Neuronal surfaces of the neuron are subjected to different levels of UNC-6 (blue) as well as nonUNC-6 extracellular cues (orange). The SDAL process regulates both UNC-40 and nonUNC-40-mediated outgrowth activity. Positive feedback (arrows) amplifies the polarized response to an extracellular cue, while negative feedback (lines) limits the response and can confine the positive feedback to the site of ligand interaction. The long-range negative feedback mediated by UNC-40 inhibits the UNC-40 response to UNC-6, as well as nonUNC-40 activity. Similarly, long-range negative feedback mediated by nonUNC-40 activity inhibits the UNC-40 response to UNC-6.

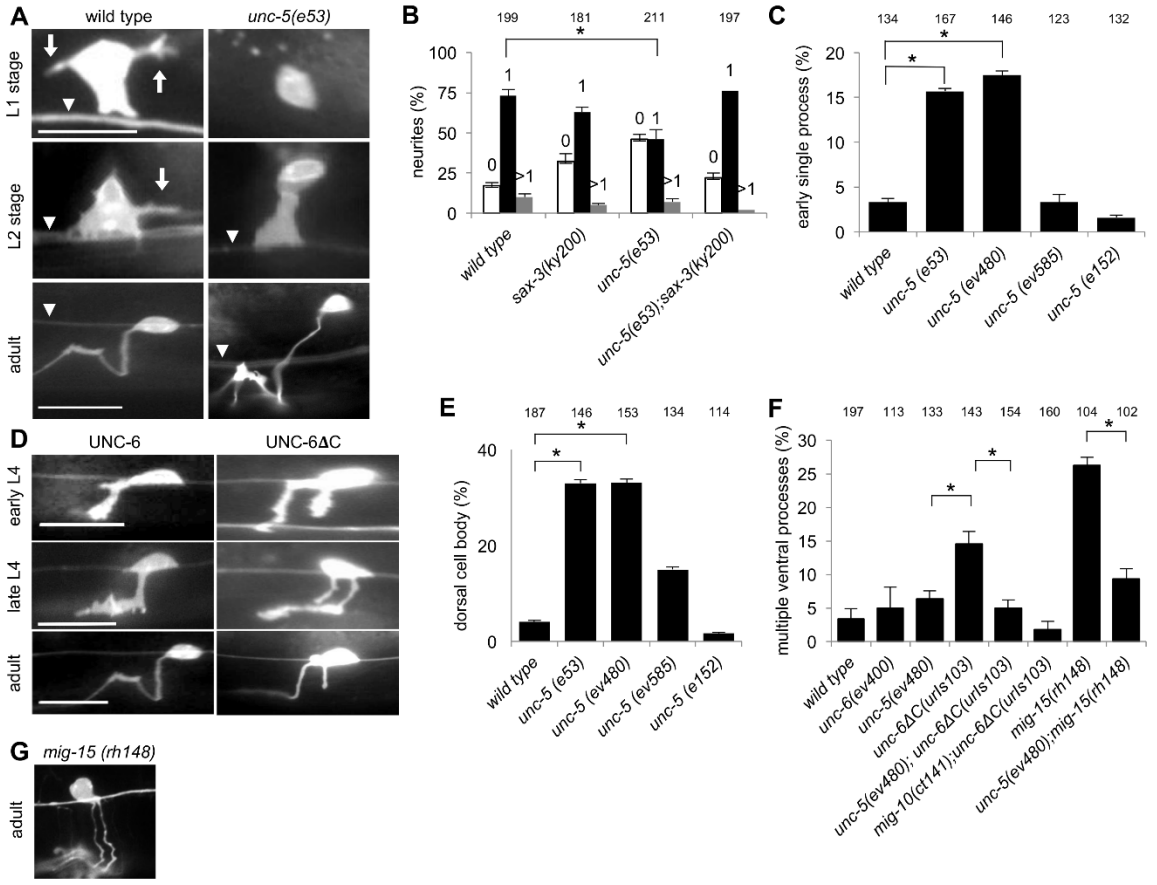

Figure 7. UNC-5 regulates the patterning of outgrowth extensions from HSN. (**A**) Photomicrographs of HSN at the L1, L2, and adult stages in wildtype and *unc-5(e53)* mutants. In L1 and L2 animals neurite extensions (arrows) are often observed in wild-type animals but are more rare in *unc-5* mutants. The short ventral migration of the cell body that occurs in wild-type animal sometimes fails in *unc-5* mutants, leaving the cell body farther from the PLM axon (arrowhead) with a single longer ventral extension. The position of the cell body remains dorsal. Scale bar: 10 μm. (B) The percentage of HSN neuron with 0, 1, or more than 1 neurite extension at the L1 stage. In *unc-5* mutants nearly half of the neurons do not extend a process. Error bars indicated the standard error mean; n values are indicated above each column. Significant differences (two-tailed t-test), *P<0.001. (C) The percentage of HSN neurons with a single long extension at the L2 stage. Several *unc-5* alleles were tested as described in the text. In mutants with loss-of-function there is more often a single extension from the cell body and the cell body is dorsally mispositioned. (D) Photomicrographs of HSN at the early L4, late L4, and adult stages in wildtype and in animals expressing UNC-6ΔC. The expression of UNC-6ΔC induces multiple processes, most often two major extensions that are guided ventrally. (E) The percentage of HSN neurons with a cell body mispositioned dorsally at the L2 stage. In loss-of-function mutants the cell body often fails to undertake a short ventral migration during the L2 stage. The migration is not delayed, but rather it remains dorsal. (F) The percentage of HSN neurons

with multiple ventral extensions at the L4 stage. The additional processes induced by UNC-6ΔC can be suppressed by *unc-5* and *mig-10* mutations. Additional processes induced by *mig-15(rh148)* can also be suppressed by the *unc-5* mutation. (G) Photomicrographs of HSN at adult stages in a *mig-15* mutant. Similar to UNC-6ΔC expression, *mig-15* mutations can also cause additional processes that are guided ventrally (YANG *et al.* 2014).

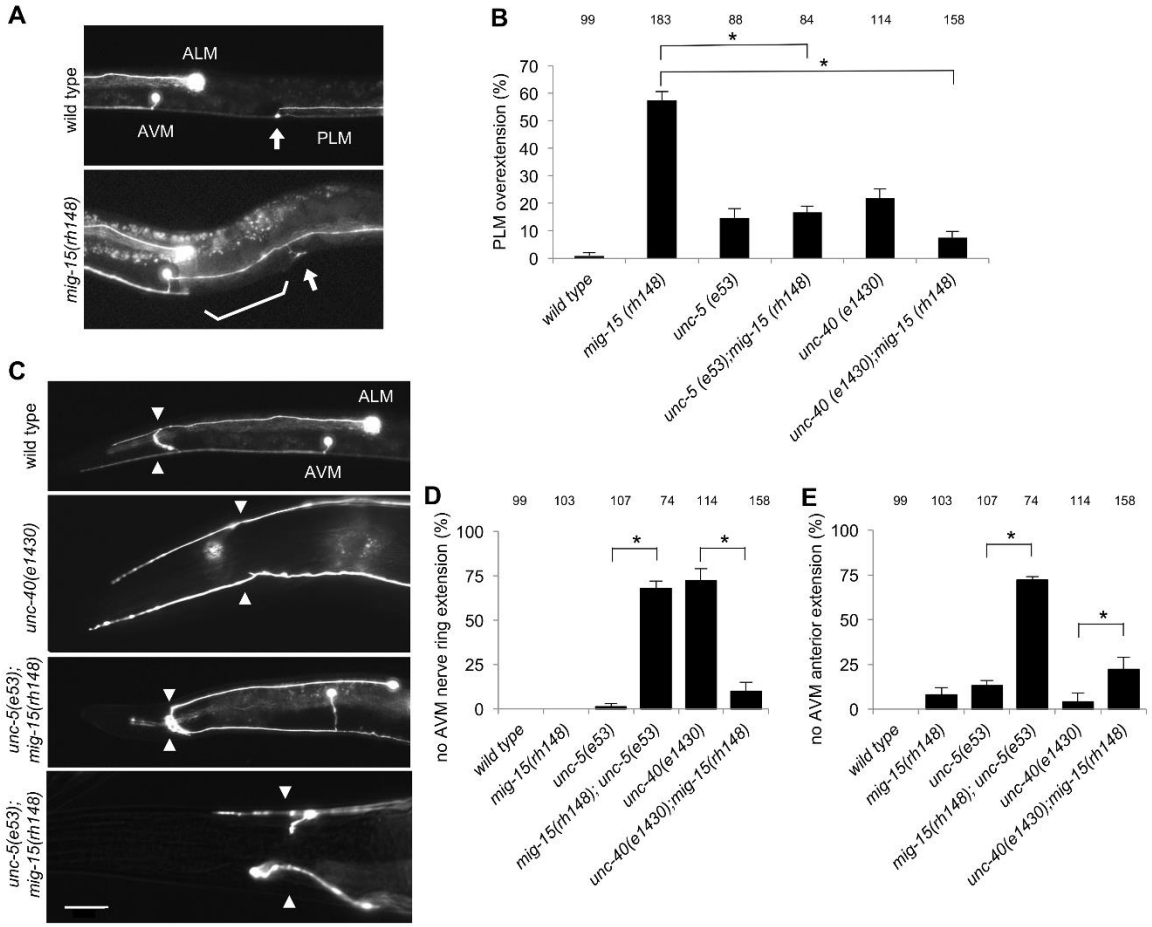

Figure 8. UNC-5 regulates the patterning of extension from ALM, AVM, and PLM. (A) Photomicrographs of the ALM, AVM, and PLM neurons at the L4 stage in wild-type animals and *mig-15* mutants. In wildtype (top) a single PLM axon travels anteriorly from the posterior cell body (not shown). Near the vulva (arrow) the axon branches; one branch extends to the ventral nerve chord and another extends anteriorly. The anterior extension terminates before reaching the area of the ALM cell body. In *mig-15* mutants the PLM can extend anteriorly past the ALM cell body (bottom). (B) The percentage of PLM neurons where the PLM neuron extends anteriorly past the ALM cell body. The anterior extension often over-extends in *mig-15* mutants. Loss of *unc-5* or *unc-40* function can suppress this phenotype. (C) Photomicrographs of the ALM and AVM neurons at the L4 stage in wild-type animals and mutants showing different patterns of outgrowth extension. In wildtype (top) a single axon travels anteriorly to the nerve ring (arrowheads). At the nerve ring the axon branches; one branch extends further anteriorly and the other extends into the nerve ring. In mutants, one or both axons may only extend anteriorly and will not extend into the nerve ring (second from top). Or one or both axons will only extend into the nerve ring and will not extend anteriorly (third from top). Or one or both axons will fail to extend into either the nerve ring or anteriorly (bottom). Scale bar: 20 μm. (D) The percentage of AVM neurons where the AVM neuron failed to extend into the nerve ring. The

neuron often fails to extend in the *unc-40* and *mig-15;unc-5* mutants, whereas it does extend in the *mig-15*, *unc-5*, and *mig-15;unc-40* mutants. Error bars indicated the standard error mean; n values are indicated above each column. Significant differences (two-tailed t-test), *P<0.001. (E) The percentage of AVM neurons where the AVM neuron failed to extend anteriorly, past the nerve ring. The neuron often fails to extend anteriorly in the *mig-15;unc-5* mutants, whereas it does extend in the *mig-15*, *unc-5*, *unc-40*, and *unc-40;mig-15* mutants. There is a significant difference between the *unc-40* and *unc-40;mig-15* mutants.

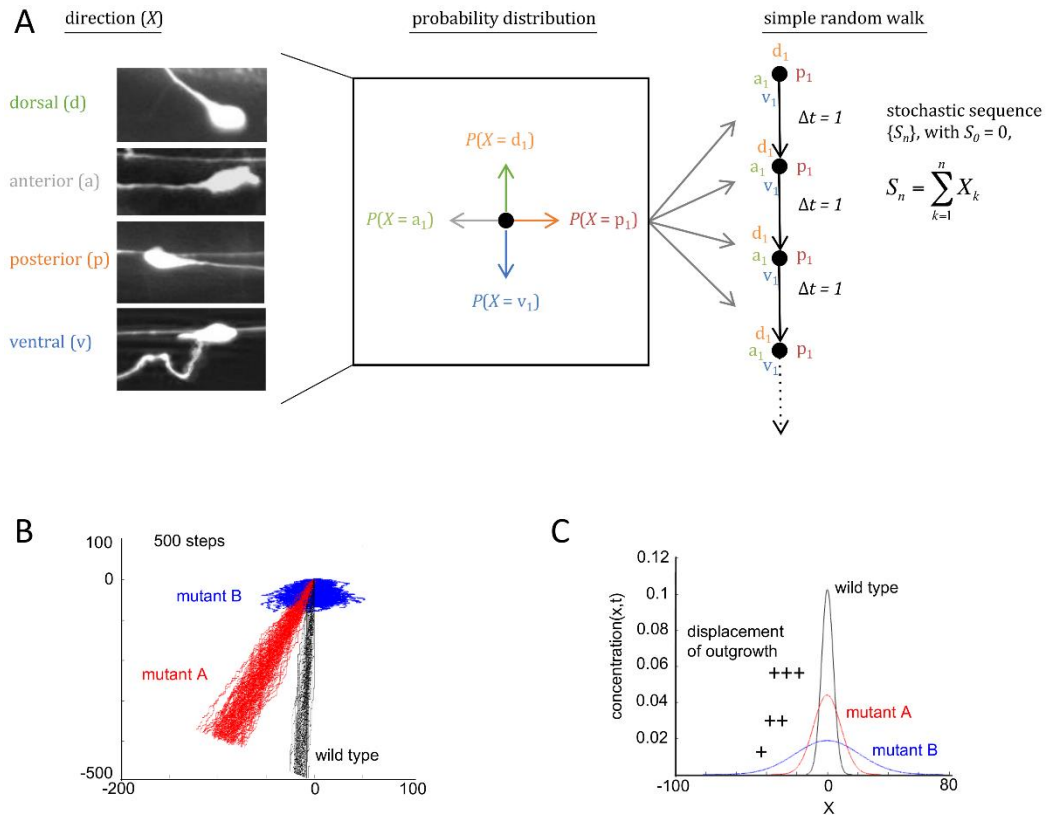

Figure 9. Assay to measure the effects a mutation has on movement. (A) The direction of outgrowth extension from the HSN cell body can vary and whether the axon developed in the dorsal, anterior, posterior, or ventral direction in L4 stage animals is scored (left panel). This creates a probability distribution in which the direction (*X*) is a random variable (center panel). A simple random walk is generated by using the same probability distribution for a succession of steps with an equal time interval (right panel). (B) For wildtype and two mutants, 50 simulated random walks of 500 steps were plotted from an origin (0, 0). The results graphically indicate the directional bias for movement. For random walk movement created in mutant A (red, results from *unc-5(e53)*), the directional bias is shifted anteriorly (left) relative to wildtype. The results also graphically show the displacement of movement. For random walk movement created in mutant B (blue, results from *egl-20(n585);sax-3(ky123)*), the average of the final position (displacement) from the origin is a much shorter distance than wildtype. (C) Plots of the normal distribution of the final position along the x axis of the random walk tracks shown in B. The mean position for each is set at 0. The plots graphically illustrate how random walks constructed from the probability distribution for the direction of outgrowth extensions can reveal a diffusion process.

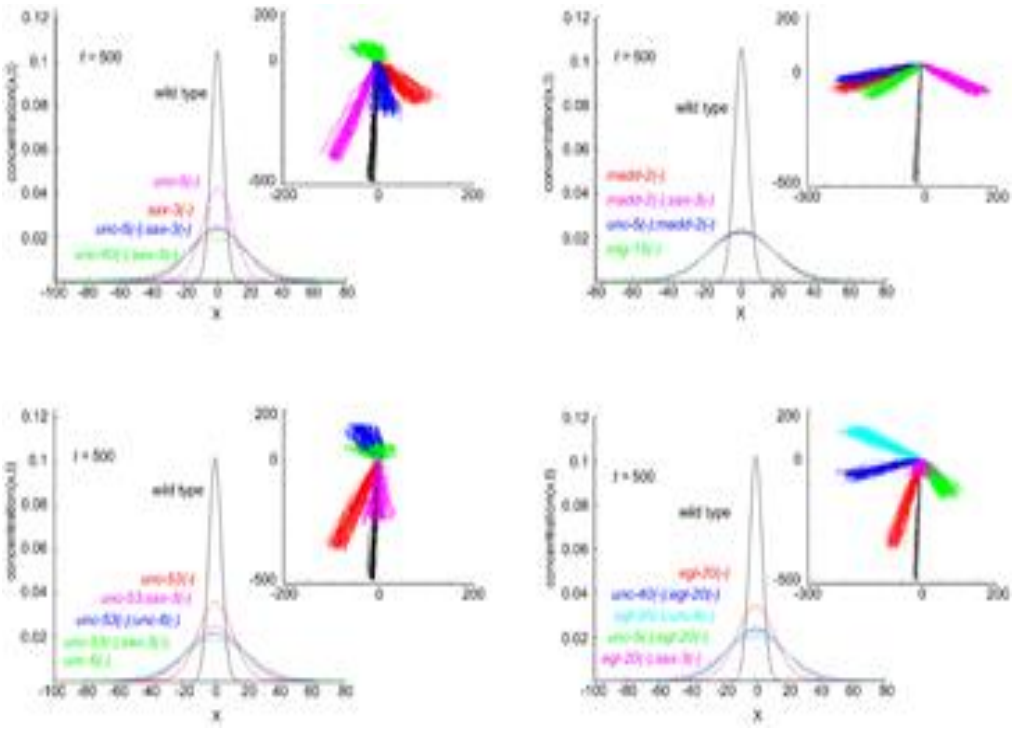

Figure 10. Mutations have different effects on movement. Examples of random walk analyses using the direction of axon development from the HSN neuron in different mutants (Table 1). The graphs were created as described in the figure legend of Figure 9. For each panel, plots are shown for the normal distribution of the final position along the x axis for the random walk tracks plotted in the inserts. The inserts depict the random walk movement that would be produced by the probability distribution for the direction of outgrowth in the mutant. Plots derived from the same data are colored alike. Each panel depicts the analyses of four different mutants and wildtype. Three different distribution patterns are observed: (1) the wild-type distribution, which has the distribution curve with the highest peak; (2) the *unc-5*, *egl-20*, *unc-53*, and *unc-6* (not shown) distribution, which is flatter than the wild-type curve; (3) the *madd-2*, *sax-3*, *mig-15*, and double combinations, which have the flattest distribution curve.

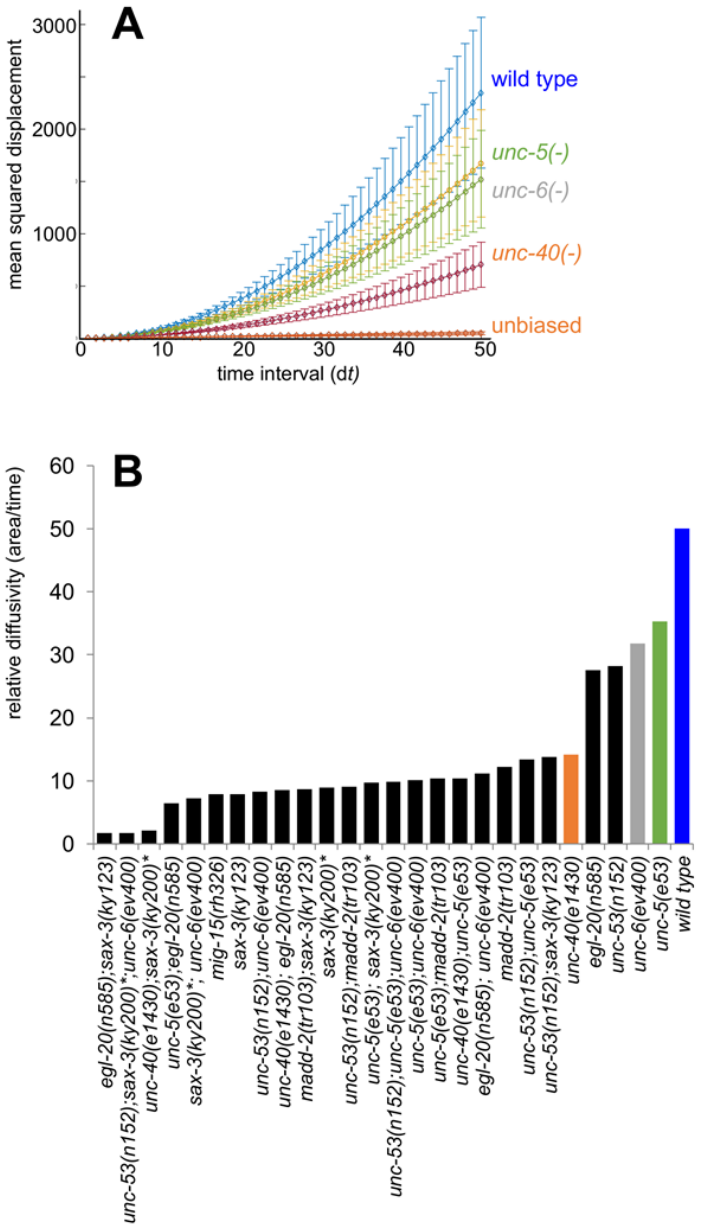

Figure 11. Mutations alter the spatial extent of movement. (A) Plotted are the mean squared displacement (MSD) curves as a function of time interval (d*t*). The values are in arbitrary units, since the time scale was arbitrarily set at 1. The curves show the extent that different mutations can alter the MSD relative to wildtype and the MSD caused by an unbiased random walk. For each time interval, mean and s.e.m. are plotted. (B) From the slope of MSD curves a coefficient can be derived that gives the relative rate of diffusion. Colored bars correspond to the like-colored curves given in panel A. The coefficients for *unc-5*, *egl-20*, *unc-53*, and *unc-6* form a class that is distinct from that derived from wildtype and from the double mutants.

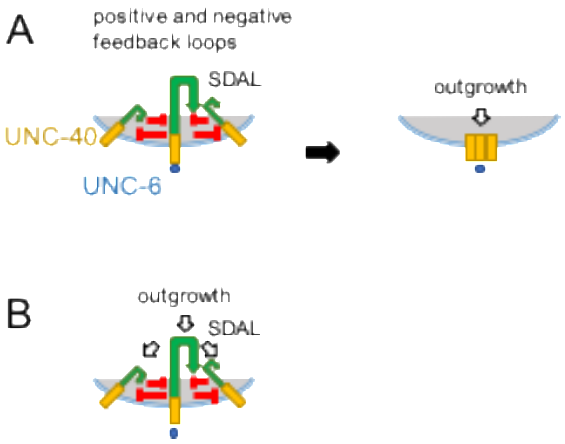

Figure 12. Models for the relationship between UNC-40-mediated outgrowth activity and UNC-40 receptor clustering. (A) The SDAL process is illustrated as in Figure 1. In this model, the self-organizing UNC-40 SDAL process causes observable UNC-40 receptor clustering. UNC-6 stabilizes receptor clustering at a site and the outgrowth machinery is then recruited to cause outgrowth at the site. Although the initial direction of asymmetric receptor localization is determined stochastically, the direction of outgrowth is determined by the site of stabilization. (B) In this model, the self-organizing UNC-40 SDAL process is coupled to the outgrowth machinery. The direction of both asymmetric receptor localization and outgrowth activity are stochastically determined. Observable receptor clustering arises as the result of the process because receptor localization can become successively concentrated to a smaller area over time. Cluster formation is an observable phenomenon of the process, not a prerequisite for outgrowth activity. This model postulates innumerable fluctuating sites that generate force in various directions along the membrane.

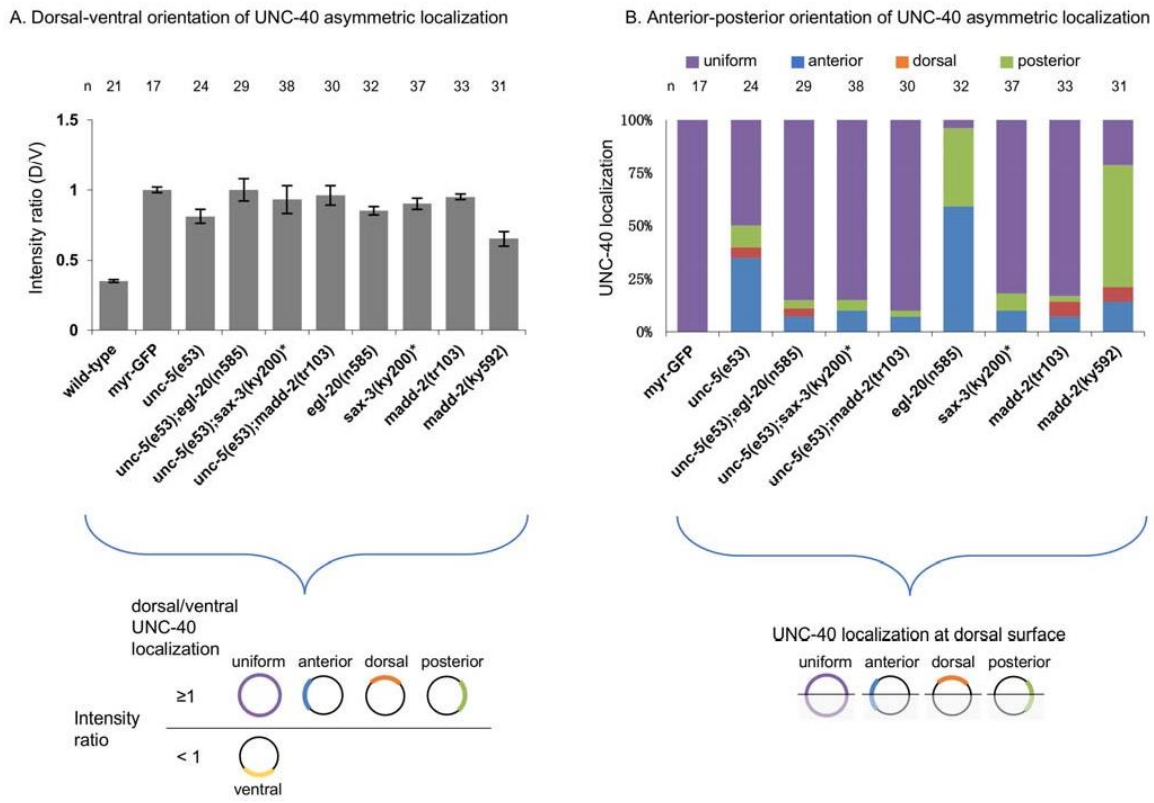

Figure 13. Mutations affect asymmetric intracellular UNC-40::GFP localization. (A) Graph indicating the dorsal-ventral localization of UNC-40::GFP in HSN. The graph shows the average ratio of dorsal-to-ventral intensity from linescan intensity plots of the UNC-40::GFP signal around the periphery of the HSN cell. UNC-40::GFP is ventrally localized in wildtype, but the ratio is different in the mutants. Error bars represent standard error of mean. Below is a graphic representation of the possible UNC-40 localization patterns when the intensity ratio is ≥ 1 or is <1. (B) Graph indicating the anterior-posterior localization of UNC-40::GFP. To determine orientation, line-scan intensity plots of the UNC-40::GFP signal across the dorsal periphery of the HSN cell were taken, the dorsal surface was geometrically divided into three equal segments, and the total intensity of each was recorded. The percent intensity was calculated for each segment and ANOVA was used to determine if there is a significant difference between the three segments (see Material and Methods). Whereas in the *unc-5* and *egl-20* mutants there is a bias for anterior or posterior localization, there is a uniform distribution in *unc-5;egl-20* double mutants. Uniform distribution is also observed in strong loss-of-function *sax-3* and *madd-2* mutants. (*) Animals grown at the *sax-3(ky200)* restrictive temperature (25°C). Below is a graphic representation of the possible UNC-40 localization patterns.

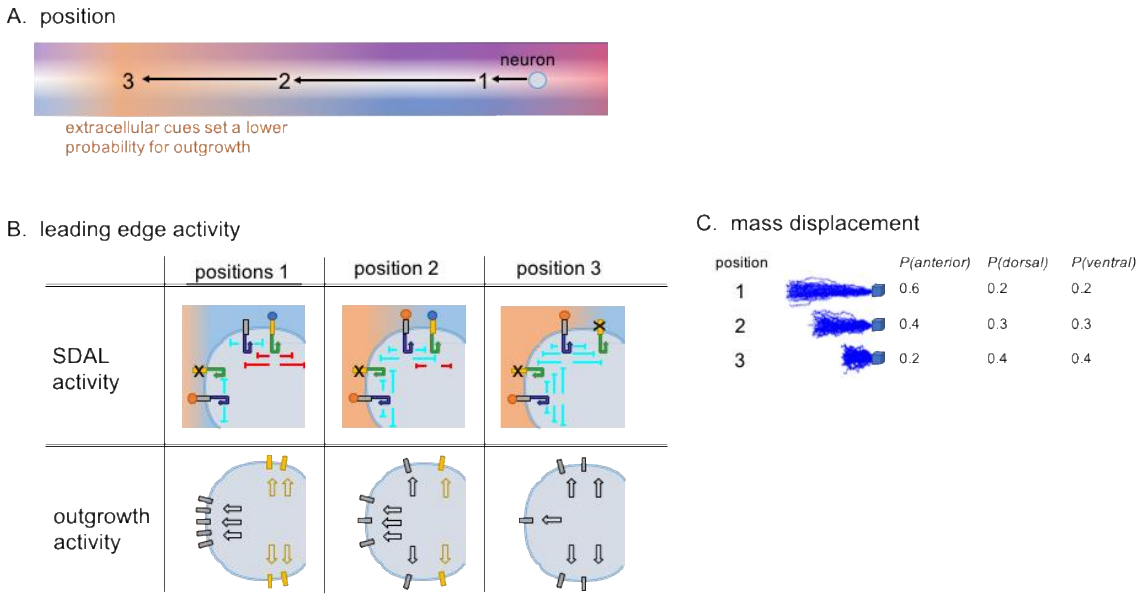

Figure 14. Model for the outgrowth movement of PLM. Schematic diagrams of the anteriorly directed outgrowth of PLM. The features of the schematic are presented in Figure 3. (A) An extension encounters different levels of extracellular cues at each of three positions (1-3). Cues dorsal and ventral of the pathway maintain an equal probability of outgrowth in both directions. The extension encounters increasing levels of an extracellular cue(s) as it moves towards position 3. At position 3, a cue(s) prevent further anterior extension in wildtype. (B) Table showing for each of the positions depicted in A the positive feedback (arrows) and negative feedback (lines) associated with SDAL activity (see Figure 6) and the predicted effect the SDAL activity has on outgrowth activity. At position 1, strong UNC-40 activity along the dorsal and ventral (not shown) facing surfaces of the leading edge inhibit nonUNC-40 activity. Because of the SDAL process, this inhibition increased nonUNC-40 activity at the anterior surface of the leading edge. At positions 2 and 3, increasing levels of the nonUNC-40 activity at the dorsal and ventral surfaces inhibit UNC-40 activity. The increase in nonUNC-40 activity at the dorsal and ventral surfaces cause a decrease in nonUNC-40 activity at the anterior surface. As a result, the degree to which the direction of nonUNC-40-mediated outgrowth activity fluctuates is greatest at position 3. (C) For each position in A, random walk modeling is shown as described in Figure 2E. The response to the extracellular cues progressively increases the degree to which the direction of outgrowth activity fluctuates. By position 3, the degree of fluctuation causes a much lower displacement of membrane mass. The low rate of outward movement causes extension to stall.

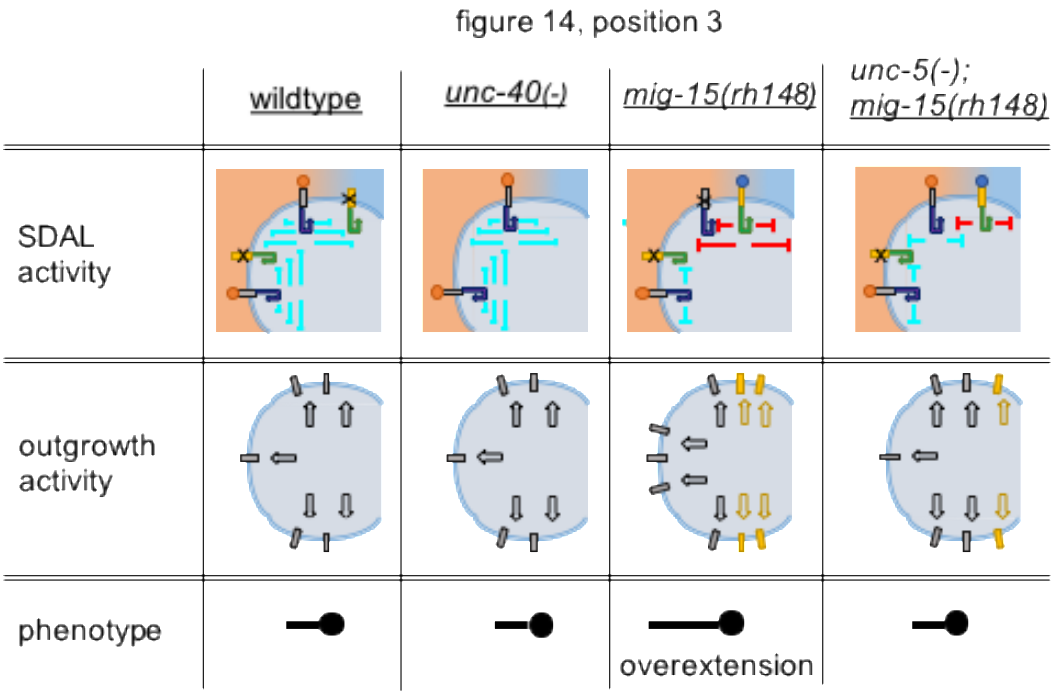

Figure 15. Model for the effects that mutations have on the outgrowth movement of PLM. Table showing the effects of different mutations on the outgrowth of PLM at position 3, Figure 14. PLM outgrowth stalls in wildtype, *unc-40*, and *unc-5;mig-15* mutants at position 3, but overextends in *mig-15* mutants. In this model, the *mig-15* mutation represses the ability of nonUNC-40 SDAL activity to suppress UNC-40 activity at the dorsal and ventral surfaces. Increased UNC-40 SDAL activity suppresses nonUNC-40 SDAL activity at these surfaces. Because of the statistical dependence of the localization process, decreasing nonUNC-40 SDAL activity at the dorsal and ventral surfaces increases nonUNC-40 SDAL activity at the anterior surface. As compared to wildtype, the degree to which the direction of nonUNC-40 outgrowth activity fluctuates is less and, therefore, outward displacement is greater. This allow further anterior outgrowth at position 3. Loss of UNC-5 function in the *mig-15* mutant decreases the ability of UNC-40 SDAL activity to suppress nonUNC-40 SDAL activity at the dorsal and ventral surfaces, thereby increasing the degree to which the direction of nonUNC-40 outgrowth activity fluctuates. This reduces outward displacement and suppresses the overextension phenotype.

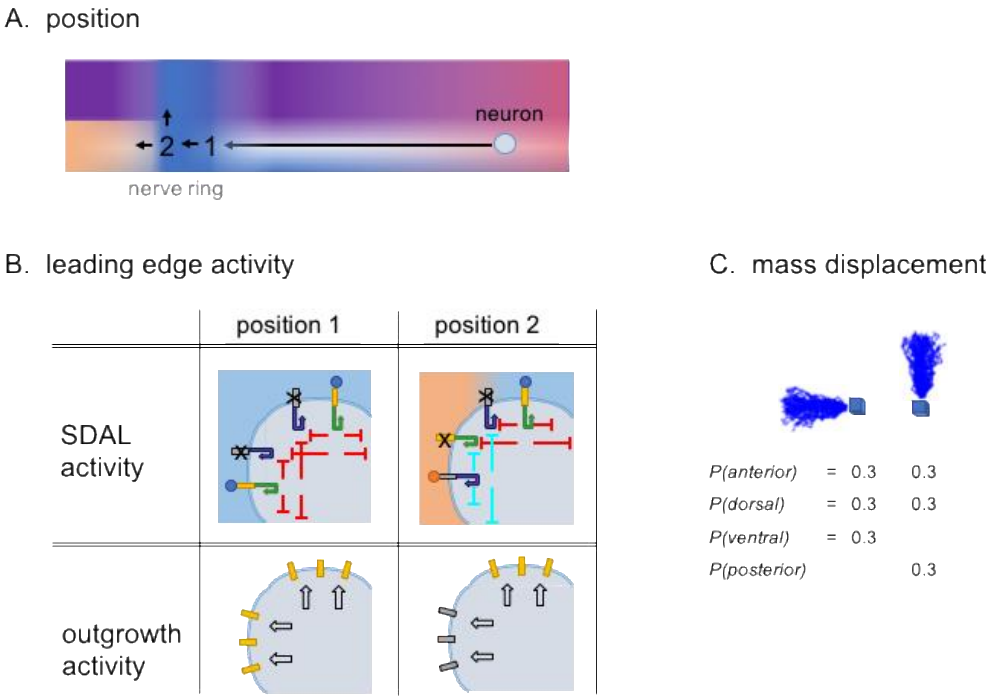

Figure 16. Model for the outgrowth movement of AVM at the nerve ring. Schematic diagrams of the outgrowth of AVM at the nerve ring. The features of the schematic are described in Figure 3. (A) At position 1, all surfaces experience high levels of UNC-6. At position 2, the extension encounters new cue(s) at the anterior surface. (B) Table showing for each of the positions depicted in A the positive feedback (arrows) and negative feedback (lines) associated with SDAL activity (see Figure 6) and the predicted effect that the SDAL process has on outgrowth activity. At position 1, strong UNC-40 SDAL activity along anterior and dorsal facing surfaces of the leading edge inhibit nonUNC-40 SDAL activity. There is strong UNC-40 mediated outgrowth from all surfaces. At position 2, nonUNC-40 SDAL activity at the anterior surface inhibits UNC-40 SDAL activity, whereas UNC-40 SDAL activity at the dorsal surface inhibits nonUNC-40 SDAL activity. As a result, UNC-40 outgrowth activity is limited to the dorsal surface and nonUNC-40 outgrowth activity is limited to the anterior surface. (C) Random walk modeling is shown as described in Figure 2E. Because of the large degree to which the direction of outgrowth fluctuates, outward movement stalls. This allows cues that are arranged dorsal and anterior to the projection to effectively create a bias for outgrowth in the respective direction (see Figure 4). A dorsal directional bias will develop because UNC-40 and other receptors mediates an outgrowth response to UNC-6 and other cues along the nerve ring. An anterior directional bias will develop because nonUNC-40 mediates an outgrowth response to cues along an anterior pathway.

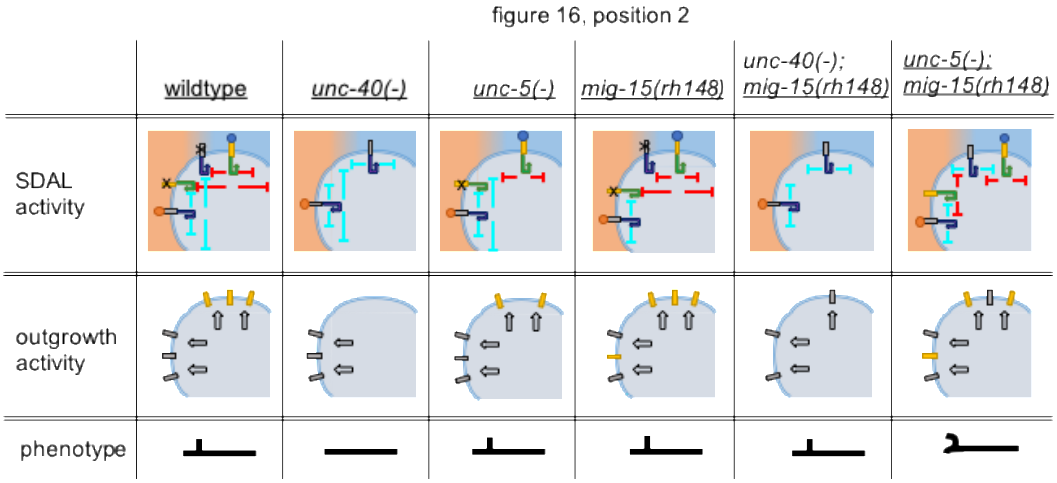

Figure 17. Model for the effects that mutations have on the outgrowth movement of AVM at the nerve ring. Table showing the effects of different mutations on the outgrowth of AVM at position 2, Figure 16. Whereas in wildtype, UNC-40 SDAL activity suppresses nonUNC-40 SDAL activity at the dorsal surface, in *unc-40* mutants there is no suppression and nonUNC-40 activity may occur. However, nonUNC-40 activity is greater at the anterior surface because of the response to anterior cues and this depresses nonUNC-40 activity at the dorsal surface because of the SDAL process. As a result, there is often anterior extension from the nerve ring area but no dorsal extension into the nerve ring. In *unc-5* mutants, UNC-40 SDAL activity is reduced but it is still dorsally oriented. This allows dorsal extension in the mutants. In *mig-15* mutants, nonUNC-40 SDAL activity is repressed. This results in lower nonUNC-40 activity at the anterior surface. However, anterior extension still occur, as does dorsal extension because of UNC-40 activity. Loss of UNC-40 in the *mig-10* background allows extension in both directions. In comparison to the single *unc-40* mutant, the reduced nonUNC-40 SDAL activity at anterior surface in the double *unc-40;mig-15* mutant doesn't depress dorsal nonUNC-40 outgrowth activity as much, allowing more extension into the nerve ring. Loss of UNC-5 in the *mig-10* background causes the most abnormal outgrowth morphologies, presumably because the repression of both UNC-40 and nonUNC-40 SDAL activities does not allow UNC-40 and nonUNC-40 outgrowth activities to be well sorted to different surfaces.

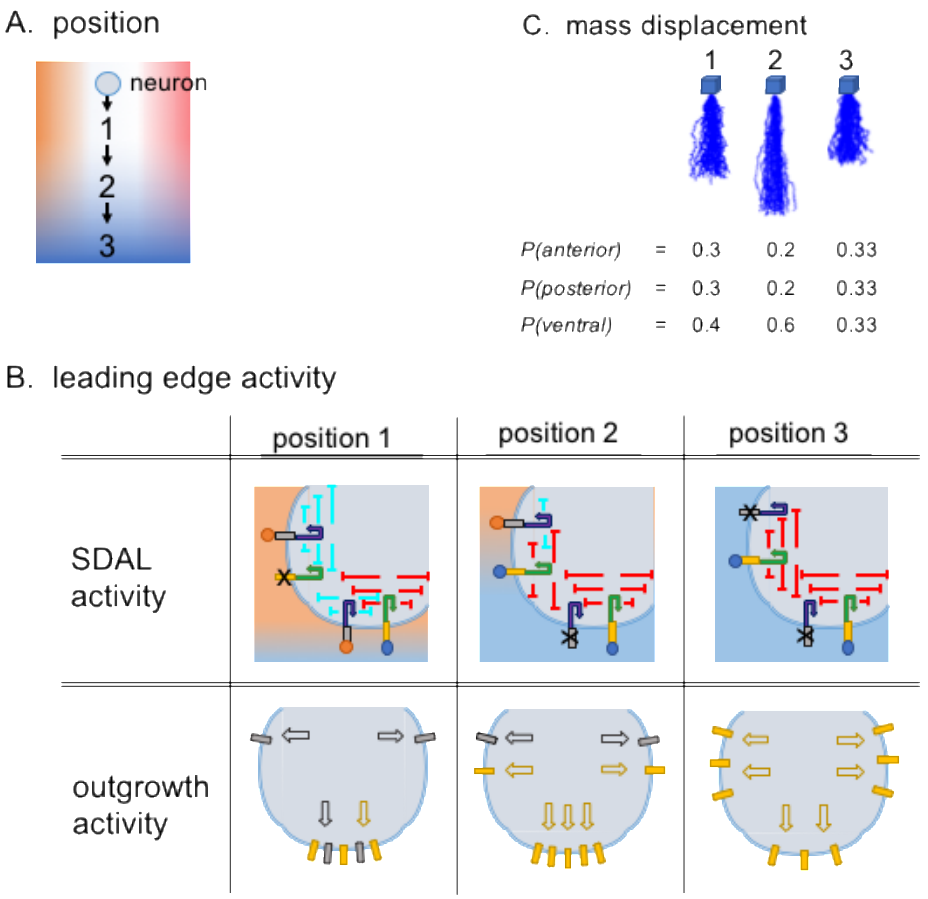

Figure 18. Model for the outgrowth movement of HSN. Schematic diagrams of the ventral outgrowth of HSN. The features of the schematic are described in Figure 3. (A) As the leading edge of the extension moves ventrally it encounters higher levels of UNC-6. At position 3, all surfaces experience high levels of UNC-6. Cues anterior and posterior of the pathway maintain an equal probability of outgrowth in both directions. (B) Table showing for each of the positions depicted in A the positive feedback (arrows) and negative feedback (lines) associated with SDAL activity (see Figure 6) and the predicted effect that the SDAL process has on outgrowth activity. At position 1, strong UNC-40 SDAL activity along the ventral facing surfaces of the leading edge inhibit nonUNC-40 SDAL activity. Because of the SDAL process, this inhibition increased nonUNC-40 activity at the anterior and posterior (not shown) surfaces of the leading edge. NonUNC-40 SDAL activity along the anterior and posterior surfaces suppress UNC-40 SDAL activity at these surfaces. At positions 2 and 3, increasing levels of the UNC-40 SDAL activity at the anterior and posterior surfaces inhibit nonUNC-40 SDAL activity. The increase in UNC-40 activity at the anterior and posterior surfaces cause a decrease in nonUNC-40 SDAL activity. As a result, the degree to which the direction of UNC-40-mediated outgrowth activity fluctuates is greatest at position 3. (C) For each position in A, random walk modeling is shown as described in Figure 2E. At first, the response to the extracellular

cues may progressively decreases the degree to which the direction of UNC-40 and nonUNC-40 outgrowth activity fluctuates. However, as UNC-40 SDAL activity predominates at all surfaces, the degree by which the direction of UNC-40 outgrowth fluctuates increases. By position 3, the degree of fluctuation causes a much lower displacement of membrane mass.

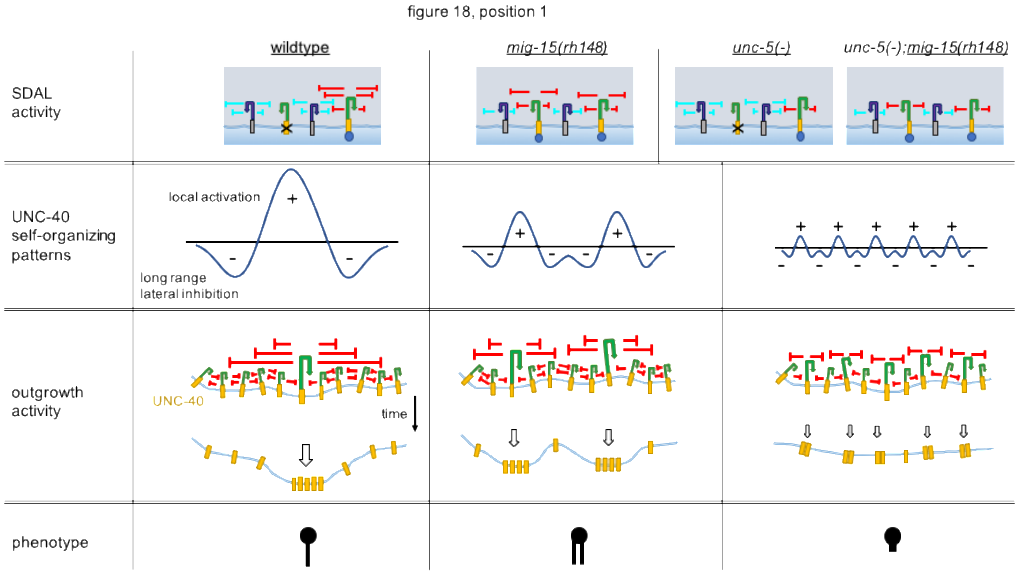

Figure 19. Model for the effects that mutations have on the outgrowth movement of HSN. Table showing the effects of different mutations on the outgrowth of HSN at position 1, Figure 18. Mutations can alter the rate of outgrowth and the number of extensions. In this model, the relative levels of UNC-40 and nonUNC-40 SDAL activity controls this patterning. In wildtype, the ability of UNC-40 SDAL activity to predominate at one site along the membrane is enhanced by nonUNC-40 SDAL activity, which may increase the threshold at which UNC-40 positive feedback becomes effective. Overtime, the area where UNC-40 SDAL predominates causes greater UNC-40 outgrowth activity. As outward movement occurs, higher levels of UNC-6 are encountered. This enhances and localizes the process to that area. In *mig-15* mutants the suppression of nonUNC-40 SDAL activity reduces the threshold at which UNC-40 SDAL activity may predominate at a site. This allows multiple sites along the membrane where UNC-40 SDAL activity may predominate. Multiple extensions may develop as shown in Figure 5. Loss of UNC-5, which suppresses UNC-40 SDAL activity, retards the ability to enhance and localize the process. This causes greater fluctuation in the direction of outgrowth activity across the entire ventral surface of the neuron, which uniformly decreases the displacement of membrane.

A

loss-of-function			robust asymmetric UNC-40 localization	reference
unc-6			no	(Kulkarni et al., 2013)
unc-5			yes	(Kulkarni et al., 2013)
unc-5	*unc-6*		no	(Kulkarni et al., 2013)
unc-5	*egl-20*		no	
unc-5	*sax-3*		no	
unc-5	*unc-53*		no	(Kulkarni et al., 2013)
unc-5	*unc-53*	*unc-6*	yes	(Kulkarni et al., 2013)
unc-5	*unc-53*	*sax-3*	no	
unc-5	*madd-2*		no	
unc-6	*unc-53*		yes	(Kulkarni et al., 2013)
unc-6	*sax-3*		no	(Tang and Wadsworth, 2014)
unc-6	*unc-53*	*sax-3*	no	(Tang and Wadsworth, 2014)
egl-20			yes	(Kulkarni et al., 2013)
egl-20	*unc-53*		yes	(Kulkarni et al., 2013)
egl-20	*unc-6*		no	(Kulkarni et al., 2013)
egl-20	*sax-3*		no	(Tang and Wadsworth, 2014)
sax-3			no	(Tang and Wadsworth, 2014)
sax-3	*unc-53*		no	(Tang and Wadsworth, 2014)
unc-53			yes	(Kulkarni et al., 2013)
madd-2			no	
mig-15			yes	(Yang et al., 2014)

B

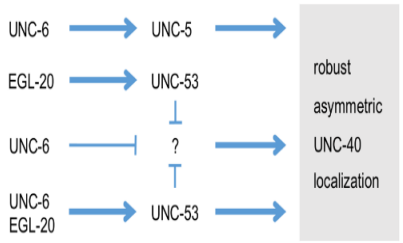

Figure 20. Genetic pathways for self-organizing UNC-40 asymmetric localization. (A) Table summarizing the results of experiments previously reported and described in Figure 13 of this paper. (B) The genetic data support a model whereby the UNC-6 and EGL-20 extracellular cues regulate at least three pathways leading to robust asymmetric UNC-40 localization. Robust asymmetric UNC-40 localization refers to the ability to observe UNC-40::GFP clustering at the surface of the neuron. Arrows represent activation; bars represent repression. See text for the logic used to construct the pathways.

CHAPTER 2

ZAG-1 regulates neuronal outgrowth patterning

INTRODUCTION

Neuronal polarity is an essential process for the correct functioning of nervous system and neuronal wiring, resulting in axon extensions. However, a major challenge for development is understanding how neuronal polarity forms and how the neuron develops. This process is regulated by different signaling pathways in response to attractive or repulsive guidance cues. These guidance cues interact with transmembrane receptors that are linked to the actin cytoskeleton (Engle, 2010), which is an important key in neuronal polarization. Thus, the axon outgrowth direction is directly linked to guidance cues gradients.

Axon guidance is thought to be determined by the attractive and repulsive activity model. Both attraction and repulsion are equally important for the growth cone guidance and neuronal connections shaping formation (Dickson, 2002). According to this model, it has been shown that on the surface of HSN neurons, the UNC-6/Netrin guidance cue, which is expressed in the ventral nerve cord and is part of established dorsal-ventral gradient, interacts with the UNC-40/DCC receptor, a member of the Deleted in Colorectal Cancer family of plasma membrane receptor proteins, to mediate an attraction activity and cause asymmetric UNC-40::GFP localization to the ventral side of HSN neuron cell body (Adler et al., 2006); therefore, UNC-40::GFP localization determines where the axon will grow out regardless of the axon outgrowth direction (Kulkarni et al., 2013). In *unc-6* mutations, the UNC-40::GFP is present uniformly at each side of neuron and thus the neurites extend in different directions (Adler et al., 2006).

Furthermore, the sensory neurons, AVM and PVM, require extracellular guidance cues, UNC-6/Netrin and SLT-1/slit, to grow ventrally; however, in the absence of these

guidance cues, these neurons fail to grow ventrally and grow anteriorly instead (Quinn et al., 2006; Hao et al., 2001). This chapter is a study of genetic interaction between ZAG-1 and UNC-6/Netrin, its receptors (UNC-5 and UNC-40), and UNC-53 adapter protein to control axon outgrowth activity (Figure 21).

RESULTS

ZAG-1 regulates HSN axon protrusion from the cell body

I studied the role of *zag-1* mutants *(zd85, zd86)* in axon protrusion from HSN neuron cell body. In wild type, the HSN axon extends ventrally from the cell body toward the ventral nerve cord and then turns anteriorly towards the nerve ring (Adler et al., 2006) (Figure 22A and 22B). In mutants, for example in *zag-1(zd86);unc-53(n152), zag-1(zd86);unc-5(e53), zag-1(zd86);unc-6(ev400),* and *zag-1(zd86);unc-40(e1430),* the HSN axon migrates anteriorly, posteriorly, dorsally or have bipolar phenotypes (Figure 22C, 22D, 22E, and 22F) respectively.

In L4 larvae, different patterns of HSN axon protrusion from the cell body in mutations were found. To support the idea that *zag-1* functions in the UNC-6/Netrin pathway, double and triple mutants were made with *unc-6* and its receptors, *unc-5* and *unc-40*, and with the *unc-53* cytoskeletal protein gene. Although the *zag-1, unc-6* and *unc-40* single mutants have axon outgrowth defects (Table 2), I found that the *zag-1(zd86);unc-6(ev400)* and *zag-1(zd86);unc-40(e1430)* double mutants had similar axon outgrowth defects to *zag-1(zd86), unc-6(ev400)* and *unc-40(1430)* single mutants. Since there is no significant differences between single and double mutants, these genes act in the same pathway.

unc-5(e53) and *unc-53(n152)* single mutants had few HSN axon outgrowth defects, while in *zag-1(zd86);unc-5(e53)* and *zag-1(zd86);unc-53(n152)* double mutants, the HSN axons displayed a much higher frequency of axon outgrowth defects relative to wild type.

The percentage of the axon outgrowth defects in *unc-5(e53)* and *unc-53(n152)* single mutants is low, and the *zag-1* mutants were shown to enhance the axon outgrowth defects of *unc-5(e53)* and *unc-53(n152)* mutants. These double mutants had an approximately double percentage of overall axon outgrowth defects compared to the *unc-5(e53)* and *unc-53(n152)* single mutants. In addition to the above observation, the *unc-5(-)* and *unc-53(-)* mutants suppress the *zag-1(zd86)* mutant multipolar process phenotype. Since *zag-1(zd86)* mutants enhanced the outgrowth defects of *unc-5(e53)* and *unc-53(n152)* mutants, the idea that these genes function in different pathway to regulate axon outgrowth patterning is supported.

Interestingly, the triple mutants *zag-1(zd86);unc-53(n152);unc-5(e53)* had more HSN ventral axon outgrowth defects than *zag-1(zd86);unc-5(e53)* double mutants, suggesting that the UNC-53 regulates the axon outgrowth activity (Table 2). Thus, the effect of UNC-5 and UNC-53 on the axon outgrowth requires the presence of ZAG-1, but ZAG-1 also has UNC-5 and UNC-53 independent axon outgrowth defects. Taken together, these data indicate that ZAG-1 is a novel effector of UNC-5 and UNC-53 activity.

These observations suggest that there could be genetic interactions between *zag-1(zd86)* and *unc-5(e53)* and *unc-53(n152)* to form a complex to control axon pathfinding and neuronal migration.

Genetic interaction between *zag-1* and *unc-53* regulate AVM axon protrusion from the cell body

I was interested in the role ZAG-1 might play with UNC-53 in AVM neuron. To test that ZAG-1 functions with UNC-53, a cross was made to generate double mutants. The AVM axons migrate ventrally towards the source of UNC-6/Netrin gradients in the wild type pattern (Chalfie and Sulston, 1981), and the percentage of ventral AVM axon direction is 100% (Figure 23A and 23B). Alone, the *unc-53(n152)* mutant does not have axon guidance defects, and all AVM axons are able to migrate to the ventral side like the wild type (Kulkarni et al., 2013), but when *unc-53(n152)* is made as a double mutants with *zag-1(zd86)*, axon guidance defects were observed and AVM axons protrude anteriorly, posteriorly, or have extra extensions (Table3, Figure 23C, 23D, and 23E). The percentage of ventral axon protrusion from AVM in *zag-1(zd86); unc-53(n152)* double mutants is 33%, and the anterior axon protrusion is 52%. This suggests that *zag-1* is involved in axon guidance defects of the AVM axon, and that *zag-1* regulates *unc-53* activity. Thus, the effect of UNC-53 on axon outgrowth patterning requires the presence of ZAG-1. These results indicate that ZAG-1 regulates the axon protrusive activity on its genetic interaction with UNC-53 and they are similar to our observations on HSN neuron.

In contrast to the combined activity of the *zag-1(zd86)* and *unc-53(n152)* on migration regulation, the *zag-1(zd86);unc-6(ev400), zag-1(zd86);unc-40(e1430)* and *zag-1(zd86); unc-5(e53)* double mutants did not enhance the axon guidance defects and did not induce significant differences in AVM axon outgrowth defects between single and double mutants. This suggests that the *zag-1* has less genetic influence in AVM ventral migration with *unc-6(ev400), unc-40(e1430) and unc-5(e53)* mutants.

Next, I tested whether the *zag-1(zd86);unc-6(ev400);unc-53(n152)* triple mutants altered the axon outgrowth defects and found these mutants increased the ventral axon guidance defects significantly compared with *zag-1(zd86);unc-6(ev400)* double mutants (from 49% to 19%) (Table 3). The results of these experiments show that *zag-1* plays a role in axon guidance defects, and it likely functions in the same pathway as UNC-6, UNC-5, and UNC-40, and in a different pathway as UNC-53. UNC-53 requires the presence of ZAG-1 and UNC-6 to enhance AVM axon outgrowth defects.

Additionally, *zag-1(zd86);unc-5(e53);unc-53(n152)* triple mutants act synergistically to cause the most mixed axon outgrowth phenotype, suggesting that they are more severe than the other mutants.

ALM axon guidance is defective in *zag-1* mutants

In wild type, the ALM axon migrates anteriorly towards the nerve ring (Figure 24A and 24B). *zag-1* single mutants have an obvious bipolar phenotype (Table 4 and Figure 24C and 24D). To test the idea that ZAG-1 functions with UNC-53 in ALM neuron, double mutants and triple mutants were made. I found that the penetrance of axon outgrowth defects in *zag-1(zd86);unc-53(n152)* double mutants were less than the *zag-1(zd86)* mutants alone, suggesting that the *zag-1(zd86)* mutants can be rescued when they are doubled with *unc-53(n152)* mutants and they enhance anterior ALM axon migrations. *unc-5(e53)* and *unc-53(n152)* mutants suppress the *zag-1(zd86)* mutants multipolar phenotype. This provides further evidence that the ZAG-1 transcription factor can influence ALM axon outgrowth activity by regulating UNC-5 and UNC-53 adaptor protein activity.

zag-1 mutants affect HSN axon development

In this experiment, the HSN morphology during the L3 larval stage was tested in two different *zag-1* alleles *(zd86* and *zd85), unc-5(e53), unc-40(1430)* single mutants, *zag-1(zd86);unc-5(e53)* and *zag-1(zd86);unc-40(e1430)* double mutants. I found that all these mutants altered the HSN axon outgrowth development patterns relative to wild type background, and that the ability of the neurons to extend ventrally is suppressed by *zag-1*, *unc-5* and *unc-40* mutants during L3 stages (Figure 25B and 25C). The evidence suggests that the ZAG-1 activity is required for early ventral neurites formation in HSN neuron during development; the same is true in the case of UNC-5 and UNC-40.

ZAG-1 regulates the ALM and AVM axons extension patterning

It was interesting to show the different patterns of AVM and ALM anterior outgrowth extension and at the nerve ring in L4 stage in wild type animals and mutants. In wild-type animals, a single axon migrates anteriorly to the nerve ring. At the nerve ring, one axon extends anteriorly, and the other extends into the nerve ring (Figure 26A and 26B). In mutants, one or both axons might extend anteriorly only and not into the nerve ring (Figure 26C), or both extend into the nerve ring, but not anteriorly (Figure 26D). To identify axon guidance cues affected in *zag-1* mutants, the double mutants with UNC-6/Netrin receptors (*unc-5* and *unc-40*), and with *unc-53(n152)* were generated. ALM and AVM extend anteriorly and there are no significant differences between *zag-1(zd86)* single and *zag-1(zd86); unc-5(e53), zag-1(zd86);unc-40(e1430) and zag-1(zd86);unc-53(n152)* doubles and they have a wild type phenotype (Figure 26G).

The dorsoventral branche formation of ALM and AVM is not suppressed, and the

neurons extend into the nerve ring in *zag-1(zd86)* and *zag-1(zd85)* single mutants; double mutants between *zag-1(zd86)* and *unc-5(e53)* show that the dorsoventral branches are not significantly different from the *zag-1(zd86)* single mutants. In *unc-40(e1430)* single mutants, the dorsoventral branche formation is suppressed (Figure 26H), whereas in *zag-1(zd86);unc-40(e1430)* double mutants, the defects of formation of these two branches is inhibited. This demonstrated that the genetic interactions involving *zag-1(zd86)* and *unc-40(e1430)* affect dorsoventral outgrowth patterning at the nerve ring.

ZAG-1 regulates the PLM axon extension patterning

In wild type, the PLM axon migrates anteriorly from the posterior cell body and terminates before reaching the ALM cell body position (Figure 26 A and 26 E). I have examined whether genetic interactions between *zag-1(zd86)* and *unc-5(e53)*, *unc-40(e1430)*, or *unc-53(n152)* respectively affect PLM axon extension. I have found that ZAG-1 influences PLM axon extension. In *zag-1(zd86);unc-5(e53), zag-1(zd86);unc-40(e1430)* and *zag-1(zd86);unc-53(n152)* double mutants, the percentage of PLM short extension increase compared with the animals with the *unc-5*, *unc-40* and *unc-53* alone, suggesting that ZAG-1 regulates UNC-5, UNC-40 and UNC-53 activity to affect PLM axon extension. PLM axon is regarded as a short extension if it failed to reach the vulva position (Figure 26F and 26I).

DISCUSSION

Role of ZAG-1 in neuronal polarization and axon outgrowth activity

It is important to study the neuron polarization pathway toward guidance cues to

understand different biological processes such as cell and growth cone migration. Molecular gradients provide signals to guide growth cones into their proper targets. UNC-6/Netrin and UNC-40/DCC play important roles during HSN axon polarization to direct axonal outgrowth protrusion (Adler et al., 2006). A few years ago, it was found that the UNC-40/DCC receptor guides the growth cone migration toward a high level of UNC-6/Netrin in the second step of axon development, axon guidance (Wadsworth et al., 1996). In response to UNC-6/Netrin, UNC-40/DCC molecules cluster asymmetrically to the surface of the neuron cell where the axon outgrowth occurs, whereas when UNC-40/DCC is mutated, the neurons send out axons in different directions (Kulkarni et al., 2013; Xu et al., 2009). Thus, the UNC-6/Netrin is a major regulator of axon outgrowth activity.

It is of interest to identify the functional relationship between transcription factors and axon guidance to explain how alterations in their activity produce axon outgrowth defects and how the axon is directed to its correct location at transcriptional mechanisms. Several reports have established the links between transcription factors regulating polarization and axon guidance. Recently, studies in vertebrates indicate that the transcription factors exert an important influence on axonal projections by regulating axon guidance cues and their receptors in developing neurons (Polleux et al., 2007).

zag-1 acts as a transcription activator and repressor to regulate several aspects of neuronal development and *zag-1* mutants disrupt HSN differentiation (Clark and Chiu, 2003). Taken together, based on our data, the *zag-1(zd58)* and *zag-1 (zd68)* single mutants enhance the migration defects of neurons significantly; the percentage of the axon outgrowth defects is high.

zag-1 causes defects in axon guidance of the HSN, in *zag-1(zd86);unc-5(e53)* and *zag-*

1(zd86);unc-53(n152) double mutants, the HSN axons have a higher percentage of axon outgrowth defects relative to wild type. These defects include the axons that migrate anteriorly, posteriorly, dorsally or have bipolar phenotypes (Figure 22). This result suggests that the ZAG-1 regulates UNC-5 and UNC-53 activity. UNC-5 and UNC-53 require the presence of ZAG-1 to alter axon outgrowth patterning. These results indicate that the genetic interaction between ZAG-1 and UNC-5 or UNC-53 regulates the HSN axon protrusive activity as well as AVM axon outgrowth activity (Table 2 and Table 3). Furthermore, ZAG-1 transcription affects ALM axon outgrowth activity by regulating activity of UNC-5 and UNC-53 (Table 4). More importantly, *zag-1(zd86);unc-53(n152)* double mutants reduce ALM migration defects compared to *zag-1* single mutants, and *zag-1(zd86);unc-53(n152)* double mutants have very few axon outgrowth defects, suggesting that *unc-53* mutant suppresses the ALM axon outgrowth defects when it is in combination with *zag-1* mutant. In other words, the *zag-1(zd86)* requires *unc-53(n152)* to function properly in ALM neuron, and it rescues the axon migration defects. From these results, I can conclude that the anterior outgrowth of ALM neuron is promoted in *zag-1(zd86);unc-53(n152)* double mutants, and these genes likely act in the different pathway. Thus, the presence of these genes seems to be the enhancer of axon migration to the anterior direction (Table 4).

Additionally, ZAG-1 regulates PLM axon outgrowth patterning. In *zag-1(zd86);unc-5(e53), zag-1(zd86);unc-40(e1430)* and *zag-1(zd86);unc-53(n152)* double mutants, the short extension of PLM axon is enhanced. The PLM axon failed to reach the vulva position (Figure 26). It is helpful to find the role of *zag-1* transcription factor within axon guidance molecules and receptors in axon pathfinding. While different guidance cues that affect axon

migration have been identified, it is not understood how transcription factor affect axons pathfinding within these signaling molecules. Therefore, I tried to gain a better understanding of the role of transcription factor activity through axon polarity and guidance during neuronal development.

zag-1 regulates the HSN axon protrusion

HSN axon development is dynamic in wild-type animals. During the L2 larvae stage, the HSN axon is polarized ventrally with neurites in the ventral side of the cell body. In the mid-L3 larvae stage, the HSN extends multiple short neurites from the cell body in different directions (Figure 25A). These neurites grow into the ventral nerve cord; one neurite increases in size developing into an axon and the others disappear in L4 larvae stage (Adler et al., 2006). However, in *unc-6* and *unc-40* mutants, the HSN axon development is delayed, and the axon does not extend to the neuron ventral side during the L3 larvae stage as in the wild type (Adler et al., 2006). These results indicate that the UNC-6 and UNC-40 are required for leading edge formation in HSN neuron, ventral cell migration, and for ventral axon guidance. In *zag-1* mutants, the ability of HSN polarization is delayed and ventral neurites are not observed during mid-L3 larvae stage. Similarly, *unc-5* and *unc-40* single, *zag-1;unc-5*, *zag-1;unc-40* double mutants suppressed the ventral neurite formation during mid-L3 stage. These results suggest that the ZAG-1 activity is required for early ventral neurites formation in HSN neuron during development.

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FIGURES

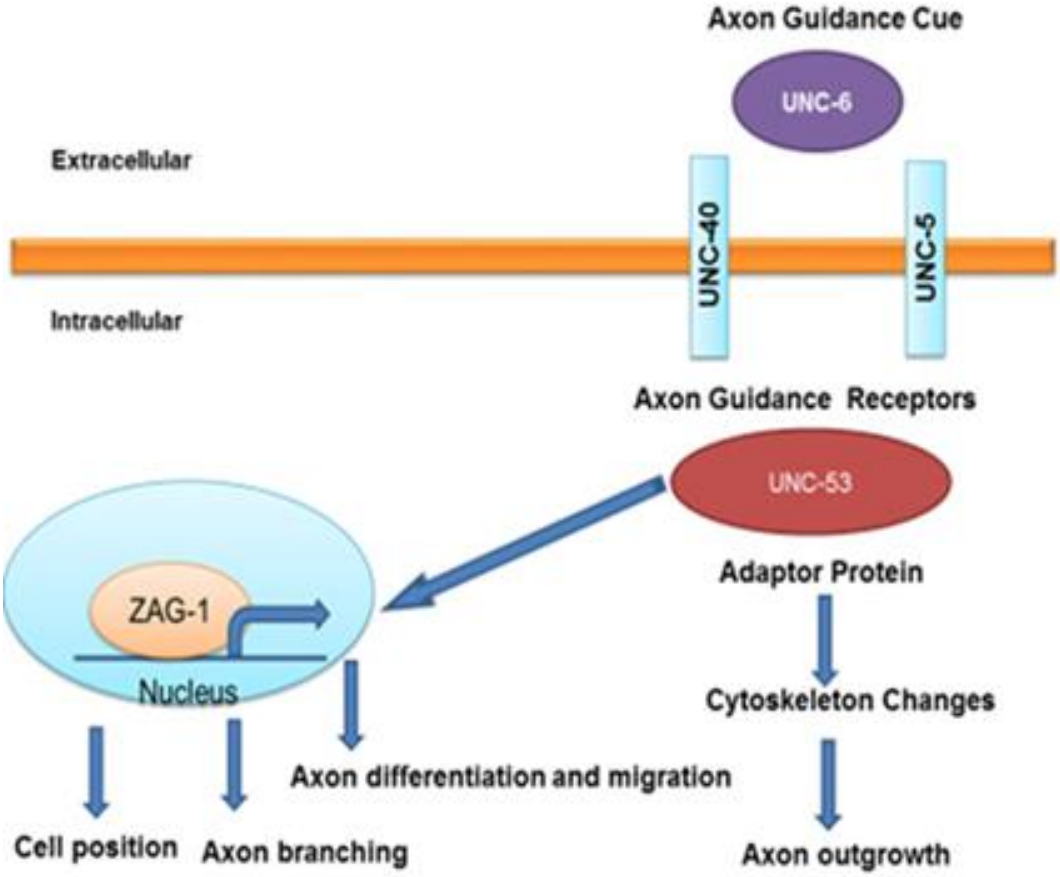


Figure 21. Schematic diagram of model working: UNC-5/UNC5 and UNC-40/DCC are the receptors in the UNC-6/Netrin signaling pathway. Genetic interaction between ZAG-1 and UNC-6/Netrin, its receptors, and UNC-53 to control axon outgrowth activity is shown here.

A

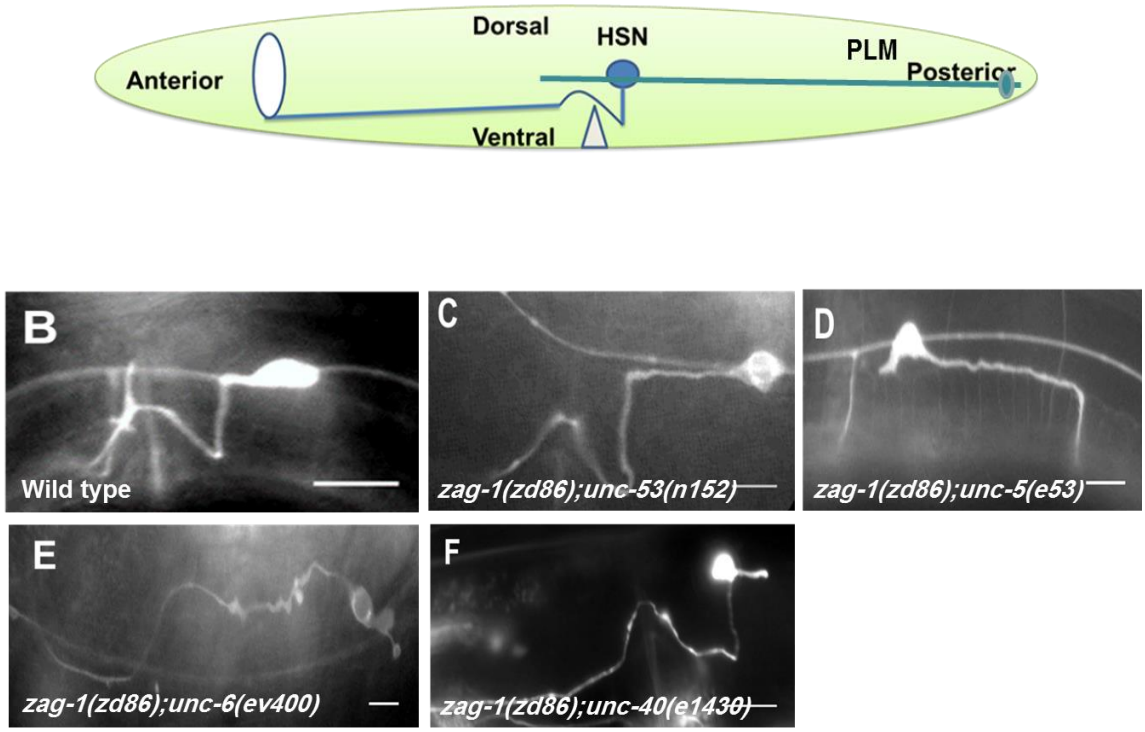


Figure 22. *zag-1* mutants showing HSN axon outgrowth defects. A schematic diagram of wild type HSN axon protrusion phenotype (A). Graphs show axon protrusion from HSN in L4 larvae stage animals. The ventral direction is down, and the anterior direction is left; the scale bars represent 10 µm. In wild type, HSN axons migrate ventrally and then turn anteriorly towards the nerve ring (B). In mutants, for example in *zag-1(zd86);unc-53(n152), zag-1(zd86);unc-5(e53), zag-1(zd86);unc-6(ev400),* and *zag-1(zd86);unc-40(e1430),* the defects include axons that migrate anteriorly (C), posteriorly (D), dorsally (E) or have an extra extension (F). The HSN axons were visualized with *kyIs262 [unc-86::myr-GFP]* and *zdIs13 [Ptph-1::gfp]*.

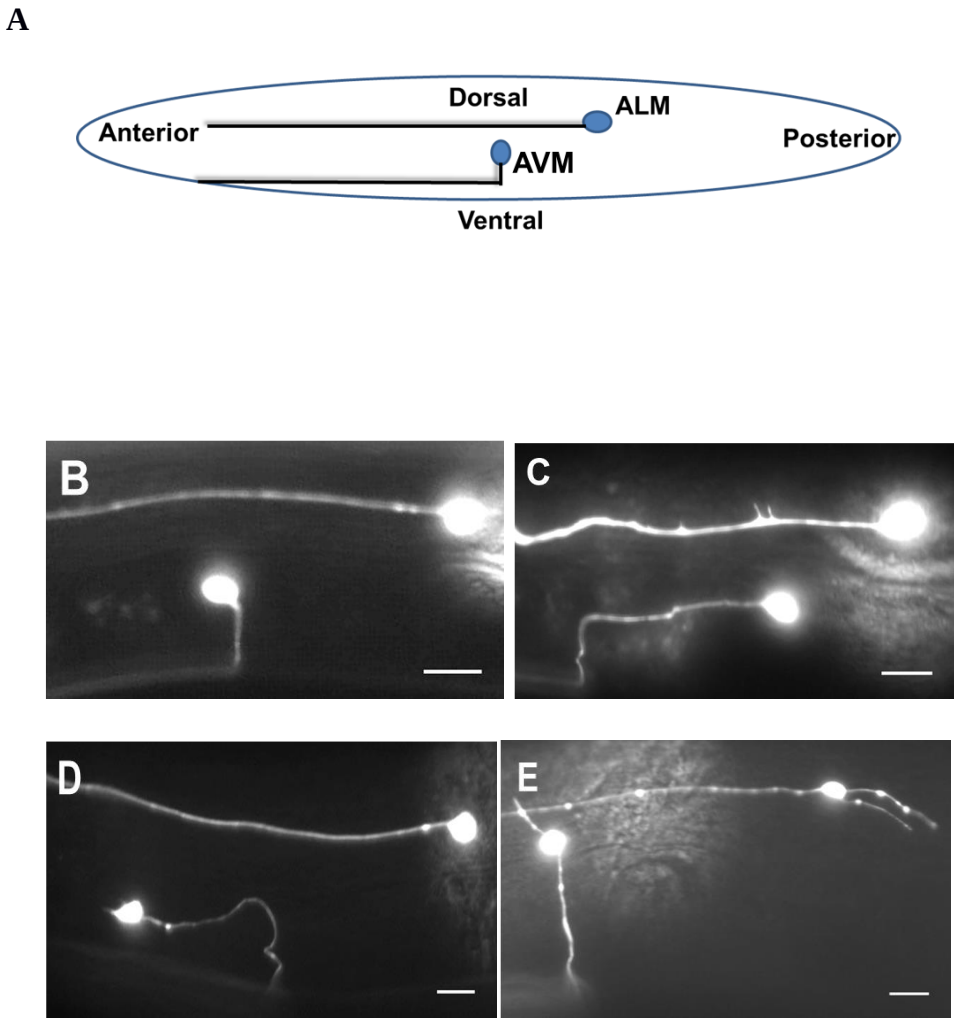


Figure 23. *zag-1* mutants cause AVM axon outgrowth defects. A schematic diagram of wild type AVM axon phenotype (A). Graphs show axon protrusion from AVM in L4 larvae stage animals. The anterior direction is left, and ventral direction is down; the scale bars represent 10 µm. In wild type, AVM axons migrate to the ventral nerve cord and then turn anteriorly towards the nerve ring (B). In *zag-1* mutants, the defects include axons that migrate anteriorly (C), posteriorly (D) or have bipolar phenotypes (E). The AVM axons were visualized by expression of the *zdIs5 [Pmec-4::GFP]*.

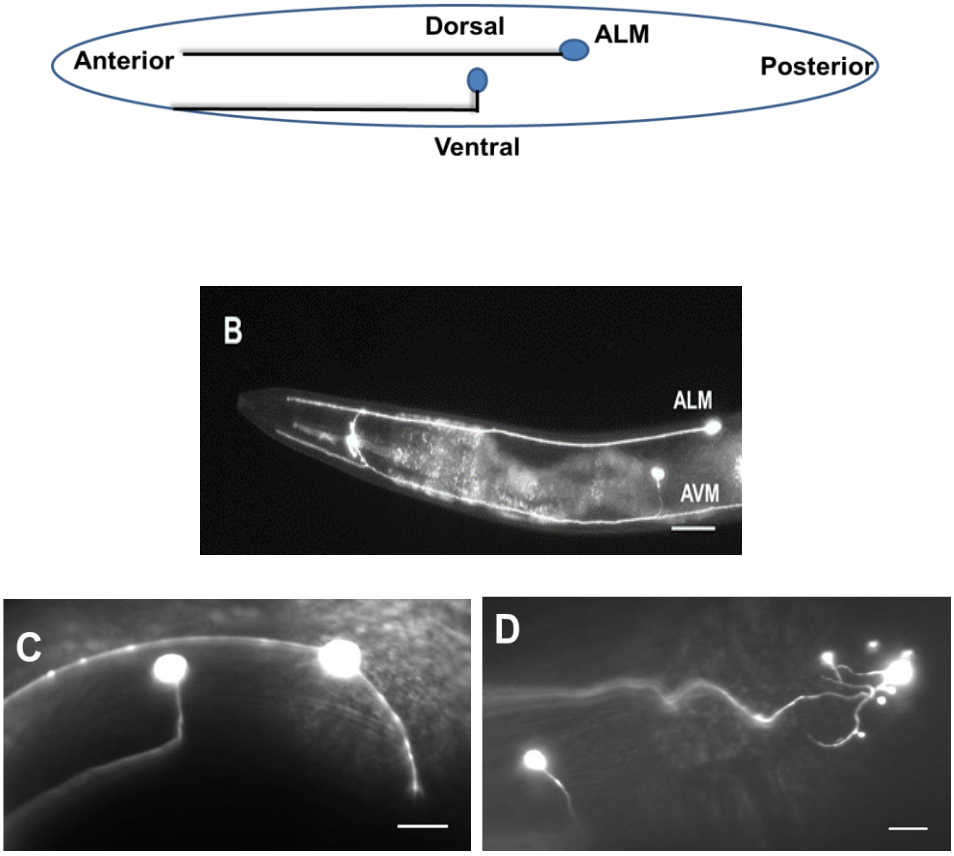
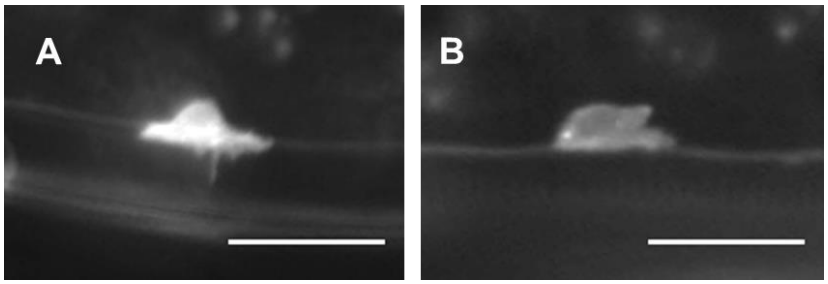


Figure 24. ALM axon protrusion phenotypes in wild type and mutations. A schematic diagram of wild type ALM axon phenotype (A). Graphs show axon protrusion from ALM in L4 larvae stage animals. The anterior direction is left, and ventral direction is down; the scale bars represent 10 µm. In wild type, ALM axons migrate anteriorly towards the nerve ring (B). In *zag-1* mutatnts, ALM axons have bipolar phenotypes (C, D). The ALM axons were visualized by the expression of the *zdIs5 [Pmec-4::GFP]*.



C

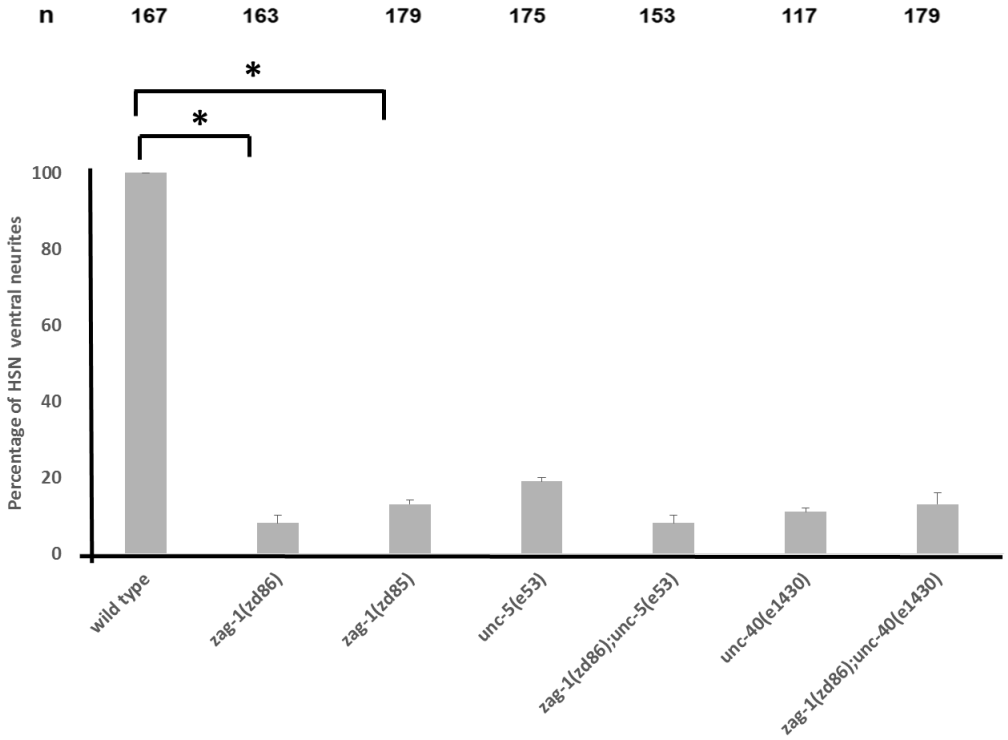
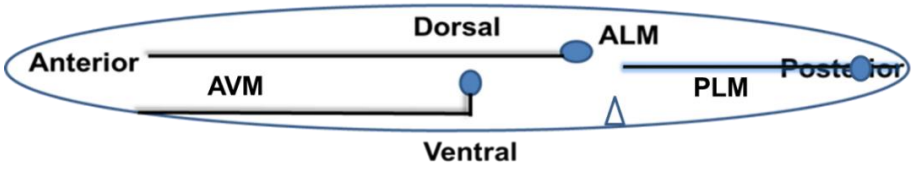
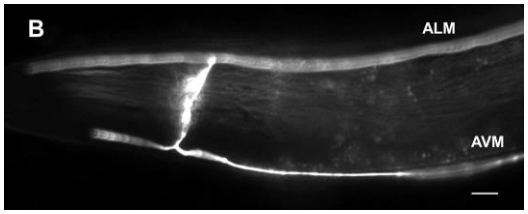
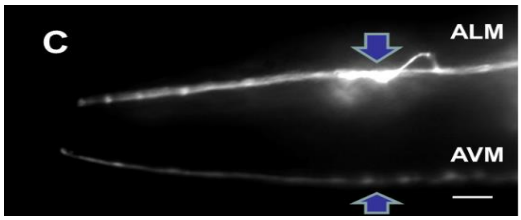


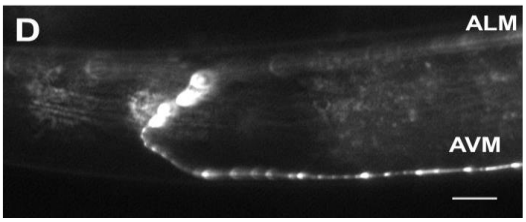
Figure 25. *zag-1* mutants delay the early HSN neurites development. Graphs show axon protrusion from HSN in L3 larvae stage animals in wild type (A) and mutants (B). The ventral direction is down, and anterior direction is left; the scale bars represent 10 µm. The figure also show the percentage of HSN neurons with ventral neurites(C). In the wild type animals, there are many ventral neurites. In mutants there is a delay. HSN was visualized with *kyIs262 [unc-86::myr-GFP]*. Error bars indicate the standard error mean; n values are indicated above each column. The student t-test was used to determine if there are significant differences between strains, *$p<0.05$.

A


Dorsal
ALM
Anterior
AVM
PLM
Posterior
Ventral


B
ALM
AVM


C
ALM
AVM


D
ALM
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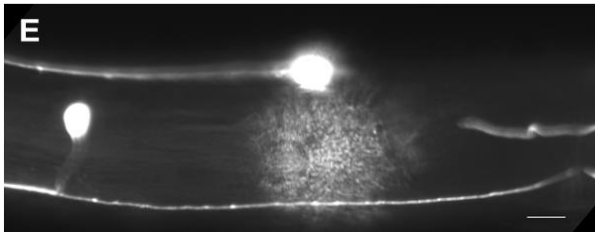
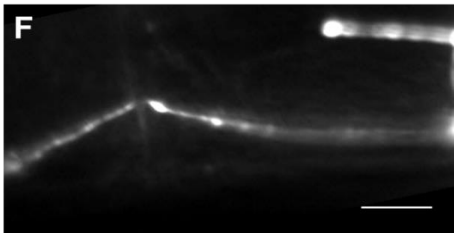
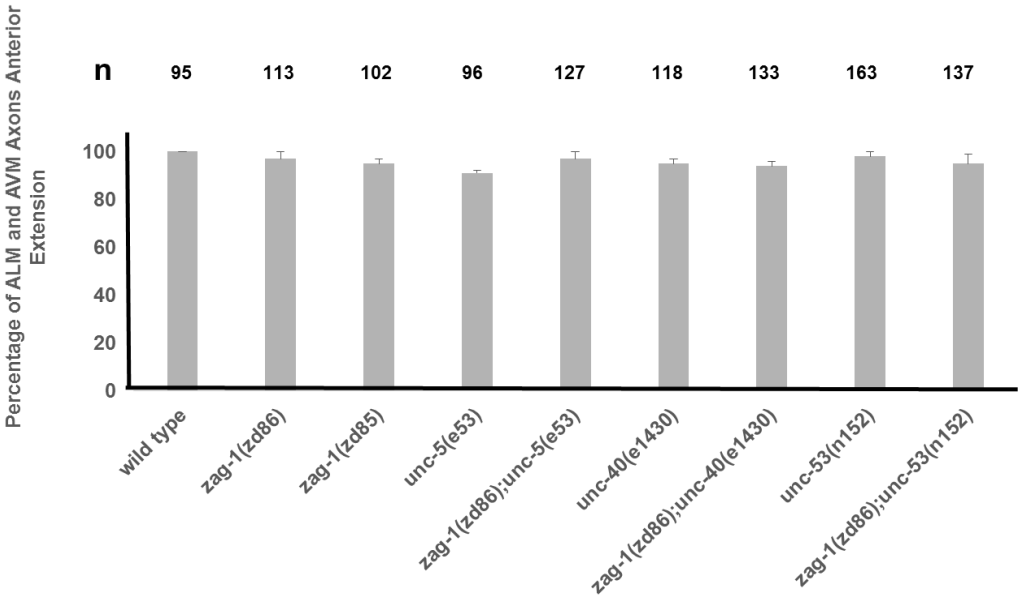
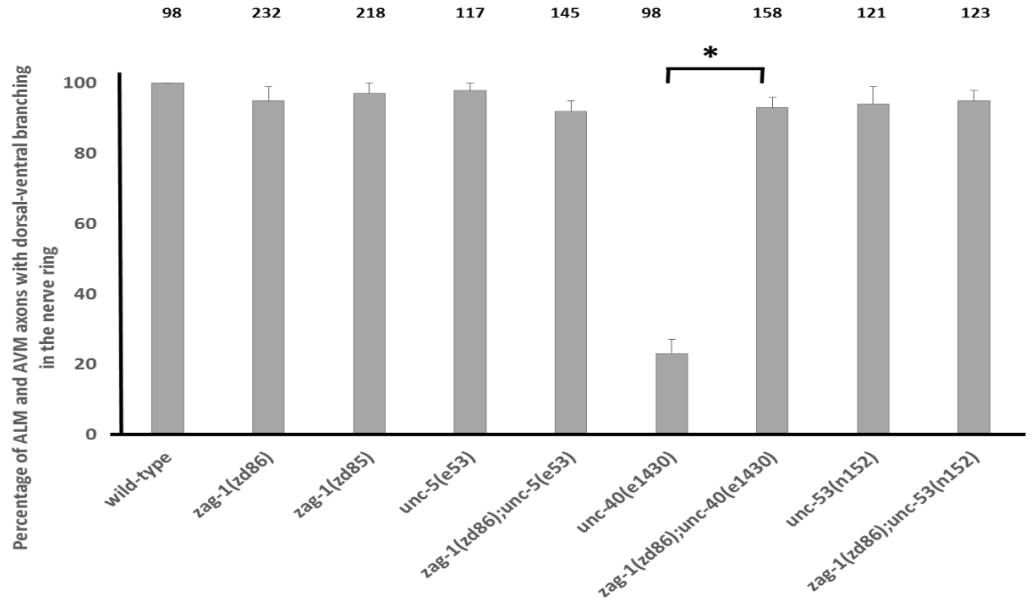

E


F

G


n
95
113
102
96
127
118
133
163
137
Percentage of ALM and AVM Axons Anterior Extension
100
80
60
40
20
0
wild type
zag-1(zd86)
zag-1(zd85)
unc-5(e53)
zag-1(zd86);unc-5(e53)
unc-40(e1430)
zag-1(zd86);unc-40(e1430)
unc-53(n152)
zag-1(zd86);unc-53(n152)

H


98
232
218
117
145
98
158
121
123
*
Percentage of ALM and AVM axons with dorsal-ventral branching in the nerve ring
100
80
60
40
20
0
wild-type
zag-1(zd86)
zag-1(zd85)
unc-5(e53)
zag-1(zd86);unc-5(e53)
unc-40(e1430)
zag-1(zd86);unc-40(e1430)
unc-53(n152)
zag-1(zd86);unc-53(n152)

I

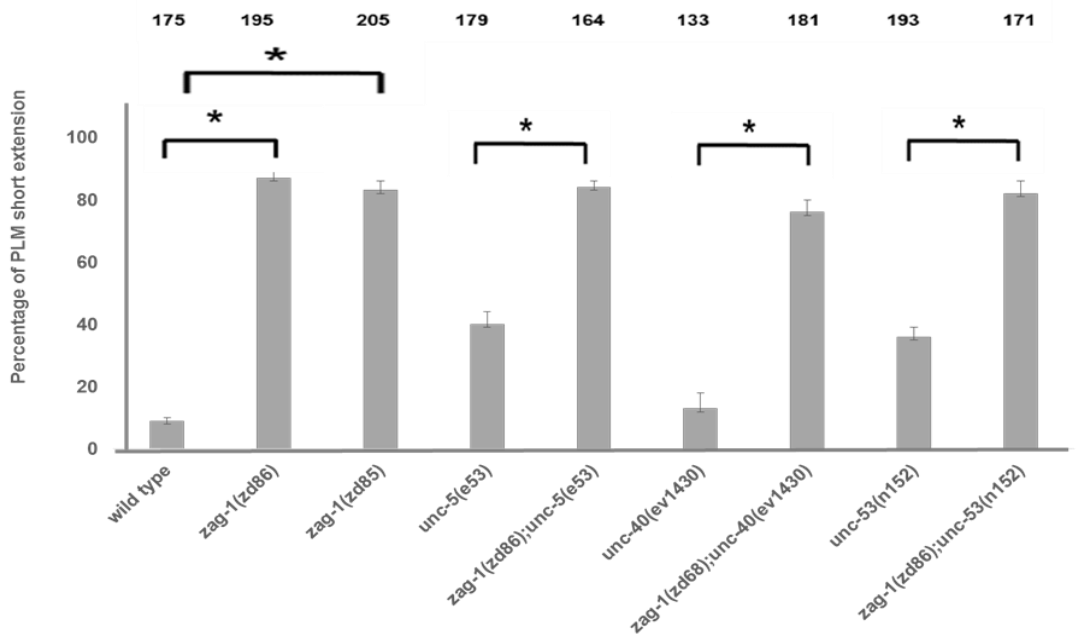


Figure 26. Role of *zag-1* in regulating ALM, AVM, and PLM extension patterning. Schematic diagram of the wild type of the nerve ring in adult worms (A). Graphs show axon extension patterning in L4 larvae stage animals. The ventral direction is down, and anterior direction is left; and the scale bars represent 10 µm. In wild type, the ALM and AVM axons extend anteriorly into the nerve ring. At the nerve ring, the ALM and AVM axons branch dorsoventrally (B). In mutants, the ALM and AVM axons extend anteriorly and not into the nerve ring (C), or they extend into the nerve ring and not anteriorly (D). In wild type, the PLM axon migrates anteriorly from the posterior position of the cell body, and its extension stops before reaching ALM neuron cell body (E). In *zag-1* mutants, the PLM displays a short axon extension (F). The figure shows the percentage of ALM and AVM axons that extend anteriorly (G). It also shows the quantification of the branch suppression phenotype at the nerve ring. In *unc-40* mutants, the branch formation is suppressed. *zag-1* single mutants and *zag-1;unc-40* double mutants do not suppress the dorsoventral branch formation (H). The PLM short extension quantification in different mutants is shown (I). The ALM, AVM, and PLM axons extension were visualized by expression of the *zdIs5 [Pmec-4::GFP]*. ZAG-1 and UNC-40 cooperate to regulate AVM and ALM axons branching in the nerve ring and regulate *unc-5*, *unc-40,* and *unc-53* activity to affect PLM axon extension. The student t-test was used to determine if there are significant differences between strains, *$p<0.05$.

CHAPTER 3

ZAG-1 regulates the asymmetric localization of UNC-40/DCC and outgrowth activity

INTRODUCTION

The axon outgrowth direction process is not well understood. The HSN, AVM, ALM, and PLM neurons of *C. elegans* display different axon outgrowth patterns (Figures 24A, 25A, and 26A). The HSN neuron extends several neurites in different directions from the cell body randomly. These neurites grow to the ventral side to develop into a single axon that extends to the ventral nerve cord near the vulva (Adler et al., 2006; Wadsworth et al., 1996). The AVM mechanosensory neuron extends ventrally into the ventral cord, whereas the ALM neuron extends anteriorly into the nerve ring. The PLM axon extends anteriorly from the posterior position of the cell body and terminates before ALM neuron cell body position (Li et al., 2008).

Evidence suggests that different extracellular axon guidance cues such as UNC-6/Netrin and SLT-1 and their receptors UNC-40 and SAX-3 respectively play roles in determining the direction of axon outgrowth extension as mentioned earlier. The UNC-6/Netrin forms a ventral-dorsal gradient (Wadsworth et al., 1996), whereas the SLT-1 forms dorsal-ventral and anterior-posterior gradients (Hao et al., 2001). Normally, UNC-40::GFP locates asymmetrically at the ventral side of the HSN neuron cell body in response to UNC-6/Netrin guidance cue to form the ventral leading edge (Kulkarni et al., 2013).

We hypothesize that UNC-40 asymmetric localization is a self-regulating, and we have recently suggested that the asymmetric UNC-40::GFP localization follows a " biased random walk model," suggesting that the UNC-40::GFP localization and axon outgrowth activity occur through a stochastic process. Thus, the random fluctuation of UNC-40::GFP outgrowth activity determines the HSN axon outgrowth direction (Kulkarni et al., 2013).

Unlike wild type, the probability of UNC-40::GFP localization at the ventral side decreases in mutants, whereas the probability of UNC-40::GFP localization at the posterior or anterior side increases. Therefore, the axon outgrowth patterning changes based on these probabilities (Kulkarni et al., 2013). UNC-40::GFP localization at the plasma membrane can fluctuate randomly in different directions along ventral HSN neuron's surface (Kulkarni et al., 2013). The random walk model describes the axon outgrowth activity in different directions mathematically.

HSN axon development occurs in accordance with random walk movement characteristics (Kulkarni et al., 2013; Yang et al., 2014). For instance, the axon formation is delayed in mutants compared to wild-type animals (Kulkarni et al., 2013). The mean square displacement (msd) tends to increase only linearly with time in the mutants, whereas the msd increases quadratically with time for straight line motion in wild-type animals. Therefore, in mutants, the axon outgrowth takes longer to grow out, and the extension cannot move that far in the same span of time (Kulkarni et al., 2013).

UNC-6/Netrin is not the only axon guidance cue that regulates the direction of UNC-40::GFP localization, and UNC-40::GFP localization at the anterior or posterior direction may be inhibited by various proteins. For instance, in *egl-20* and *mig-1* mutants, the UNC-40::GFP is localized asymmetrically to the anterior or posterior side of HSN neuron; in *egle-20* mutants, the probability of anterior UNC-40::GFP localization is 0.59 and the probability of posterior UNC-40::GFP localization is 0.37, whereas in *egl-20;unc-6* and *mig-1; unc-6* double mutants, the UNC-40::GFP is distributed uniformly (Kulkarni et al., 2013). Furthermore, *unc-6(ev400);unc-40(ur304)* and *unc-6(ev400);unc-53(n152)* doubles cause UNC-40::GFP to cluster at different asymmetric positions of the HSN neuron (Xu et

al., 2009; Kulkarni et al., 2013). The random fluctuations are essential in influencing the behavior of cell, organisms, and ecology (Kulkarni et al., 2013). This chapter is a study of the role of ZAG-1 transcription factor in UNC-40::GFP localization, how a *zag-1* mutation affects the probability of axon outgrowth direction from the HSN neuron cell body, and how the combination of *zag-1*, *unc-6, unc-5*, *unc-40,* and *unc-53* mutants alter the axon direction bias to help understand how genetic interactions affect this process.

RESULTS

zag-1 mutants disrupt ventral UNC-40::GFP localization

At each surface of the neuron, extracellular guidance cues determine the probability of axon outgrowth activity by regulating the probability of UNC-40::GFP distribution to the neuron plasma membrane (Kulkarni et al., 2013; Yang et al., 2014; Tang and Wadsworth, 2014). During HSN axon leading edge formation, the UNC-40::GFP becomes localized asymmetrically to the ventral side of neuron in response to UNC-6/Netrin axon guidance cue to induce axon outgrowth activity (Adler et al., 2006). We can visualize this process using a *kyEx1212 [unc-86::unc-40::GFP; odr-1::dsRed]* transgenic strain. In the *unc-6* loss-of-function, the UNC-40::GFP becomes uniformly dispersed around the periphery of the HSN cell surface in most cases (Adler et al., 2006; Kulkarni et al., 2013).

In these experiments, to determine whether ZAG-1 affects UNC-40::GFP asymmetric localization during the formation of the HSN axon, the UNC-40::GFP localization in the mutants was analyzed. First I conducted an experiment for UNC-40::GFP distribution along the dorsal-ventral axis. After that, I conducted an experiment for bias along the anterior-posterior axis by determining the UNC-40::GFP localization at

the dorsal surface of HSN neuron.

In mutants, the probability of UNC-40::GFP localization at the ventral side decreases, whereas the probability of UNC-40::GFP localization at the anterior and posterior directions increases compared to wild-type animals (Kulkarni et al., 2013). I found that in *zag-1(zd85), zag-1(zd86)* and *unc-40(e1430)* single mutants, UNC-40::GFP is distributed anteriorly or posteriorly; however, *in zag-1(zd86);unc-40(e1430)* doubles, it is distributed uniformly around the periphery of HSN cell surface (Figures 27 and 28). In *zag-1* mutants, the UNC-40::GFP clusters in both anterior and posterior sides of HSN neuron cell body (Figures 27 and 29B). In *unc-6(ev400)* mutants, the UNC-40::GFP is distributed uniformly around the periphery of HSN cell surface. However, in *zag-1(zd86);unc-6(ev400)* double mutants, the UNC-40::GFP is clustering in the anterior or posterior direction (Figure 9A). UNC-53 and ZAG-1 play different roles in regulating UNC-40 asymmetric localization. In *unc-40* and *zag-1* single mutants UNC-40::GFP does localize, but is not localized in *zag-1;unc-40* doubles mutants. However, UNC-40::GFP does localize in *zag-1;unc-53;unc-40,* and *unc-40;unc-53;unc-6* triples (Kulkarni et al., 2013).

In *unc-5(e53);unc-53(n152)* double mutants, the UNC-40::GFP localizes uniformly, whereas in *zag-1(zd86);unc-5(e53)* and *zag-1(zd86);unc-53(n152)* doubles, the UNC-40::GFP localization is localized into posterior or anterior (Figures 27, 28, and 29). Loss of both (*zag-1* and *unc-53*) allows UNC-40 asymmetric localization. Additionally, in *zag-1(zd86);unc-53(n152);unc-5(e53)* triple mutants, UNC-40::GFP remains clustering anteriorly or posteriorly (Figure 29A). Similarly, in *unc-52(e444), unc-52(e998), unc-112(r367), unc-97(su110), and vab-19(e1036),* the probability of UNC-40::GFP

asymmetrical localization in the anterior and posterior sides increases in HSN neuron compared to a low probability in ventral side of HSN neuron cell body (Yang et al., 2014).

ZAG-1 controls axon outgrowth directional bias differently

We calculated the axon outgrowth probability in the dorsal, ventral, anterior and posterior based on the axon outgrowth direction. HSN axon extends ventrally and then anteriorly (Adler et al., 2006). We found that the HSN axon can grow in different directions in a stochastic manner from the cell body in L4 larval stages in mutants. To explain how *zag-1* functions in the direction of axon outgrowth distribution within the UNC-6/Netrin pathway and with the UNC-53 adaptor protein, double and triple mutants were made with *unc-6* and its receptors (*unc-5* and *unc-40*) and with *unc-53*. To test the effect of these mutants on the direction of axon outgrowth, we used a MATLAB program to simulate a two-dimensional random walk based on the probability of ventral, dorsal, anterior and posterior axon outgrowth directions. The probability distribution of anterior, posterior, dorsal and ventral axon outgrowth direction was assigned for each random walk step direction moving left, right, up and down respectively. The simulations help us to understand how the transcription factor and axon guidance cues act to control the directional bias of axon outgrowth.

In *zag-1* mutants, the probability of anterior axon outgrowth of HSN is higher than the ventral and posterior probability (Table 2, Figure 30 A and 30 B). In *zag-1(zd86);unc-6(ev400)* and *zag-1(zd86);unc-40(e1430)* double mutants, the axon directional bias is also anterior (Figure 30 B). Since there is no significant difference between single and double mutants, the directional bias induced by *zag-1* mutants does not require *unc-6* or *unc-40*.

In *unc-53(n152)* single mutants, the probability of ventral bias is more than the anterior and posterior bias, whereas in *zag-1(zd86);unc-53(n152)* double mutants, the HSN axons have a strong bias to migrate anteriorly instead of ventrally (Table 2, Figure 30 A and 30 C).

The AVM axons grow ventrally towards the source of UNC-6/Netrin gradients and then migrate anteriorly into the nerve ring (Chalfie and Sulston, 1981). *In zag-1* mutants, the probability of ventral axon outgrowth bias is high, while the probability of anterior and posterior bias is low. *unc-53(n152)* mutants cause a strong ventral bias, like what happens in wild type animals (Kulkarni et al., 2013), but when *unc-53(n152)* was made as double mutants with *zag-1(zd86)* mutants, the AVM axons exhibit a strong anterior bias (Table3). This suggests that the presence of *zag-1* is required to regulate axon directional bias with *unc-53(n152).*

In contrast to the *zag-1(zd86)* mutant activity on axon directional regulation combined with *unc-53(n152),* the *zag-1(zd86);unc-6(ev400), zag-1(zd86);unc-40(e1430)* and *zag-1(zd86);unc-5(e53)* double mutants do not show any significant differences in altering axon directional bias between single and double mutants; they still show an anterior bias (Table 3). This indicates that *zag-1* has less effect with these mutants in AVM ventral directional bias. In addition, *zag-1(zd86);unc-6(ev400); unc-53(n152)* triple mutants cause an anterior directional bias, instead of a ventral (Table 3).

The ALM axons grow anteriorly into the nerve ring (Chalfie and Sulston, 1981). In *zag-1* mutants, the probability of anterior axon outgrowth bias is lower than the probability of *unc-6, unc-40, unc-5* and *unc-53* single mutants. In double and triple mutants, there is an increase the anterior directional bias compared to *zag-1* single mutants (Table 4).

DISSCUSION

ZAG-1 affects UNC-40::GFP asymmetric localization

UNC-40-mediated axon outgrowth activity directs axons. UNC-40 can cluster along the neuron cell surface and direct axons to specific direction in response to guidance cues. In this chapter, I analyze how zag-*1* mutants affect the probability of axon outgrowth in different directions. ZAG-1 plays a role in regulation of UNC-40 asymmetric localization (Figure 29). Because the UNC-40::GFP localization is stochastically determined, there is a probability of orientation at each surface of the HSN neuron. In wild type, UNC-40::GFP clusters ventrally. In *unc-6* mutants UNC-40::GFP is distributed uniformly around HSN neuron cell body (Yang et al., 2014), but in *zag-1;unc-6* doubles it localizes in anterior or posterior side. This is similar to the *unc-53;unc-6* double mutant result. In *unc-6* mutants UNC-40::GFP does not localize, but in *unc-53;unc-6* doubles it localizes (Figure 29).

In addition, ZAG-1 and UNC-53 play different roles in regulating UNC-40 asymmetric localization. In *unc-40* and *zag-1* single mutants UNC-40::GFP localizes, but in *zag-1;unc-40* doubles mutants does not localize. However, UNC-40::GFP localizes in *zag-1;unc-53;unc-40* and in *unc-40;unc-53;unc-6* triples. The asymmetric localization activity of the UNC-40::GFP molecule is effective with the loss of both UNC-53 and UNC-40 function, even if ZAG-1 activity is reduced. Loss of UNC-53 can compensate for loss of ZAG-1 activity to allow asymmetric localization activity.

ZAG-1 regulates the directional bias of axon outgrowth from the cell body

In *zag-1* mutants, the probability of anterior axon outgrowth of HSN is higher than the ventral and posterior probability (Table 2, Figure 30 A and 30 B). ZAG-1 and UNC-53

play different roles in UNC-40 asymmetric localization regulation. In *unc-53(n152)* single mutants, the probability of ventral bias is more than the anterior and posterior bias, whereas in *zag-1(zd86);unc-53(n152)* double mutants, the HSN axons have a strong bias to migrate anteriorly instead of ventrally (Table 2, Figure 30 A and 30 C). During HSN axon guidance, the reduction of ZAG-1 function and loss of UNC-53 function cause a different probability distribution for the direction of axon outgrowth. They affect the directional bias differently. Loss of both results in different directional bias. Data suggest must act on primarily on different outgrowth activity. Altering both outgrowth activities results in a new outgrowth pattern.

As mentioned earlier, *zag-1* alleles (*zd85* and *zd86*) are not null and they keep some function of ZAG-1. However, the phenotypes for *unc-53* and *zag-1* seem different (Figure 30). The different directional bias suggests that each is affecting the response to different sets of guidance cues, rather than the reduction of *zag-1* activity just further altering the response to the same cues that are altered due to the loss of *unc-53* activity. This conclusion is supported by the result that reduction of ZAG-1 appears to prevent UNC-40 asymmetric localization, whereas loss of UNC-53 activity allows UNC-40 activity even in the absence of UNC-6.

The genetic evidence presented here suggests that the probability of outgrowth direction is different in the double mutants compared to single mutants (Table 2). Based on the genetic interactions, I suggest *zag-1* functions together with UNC-6/Netrin signaling pathway and UNC-53 in neuronal migration and axon directional bias in each direction of neurons in the nematode *C. elegans* during development, demonstrating that *zag-1* regulates the UNC-6/Netrin pathway components and UNC-53 activities (Figure 30).

FIGURES AND TABLES

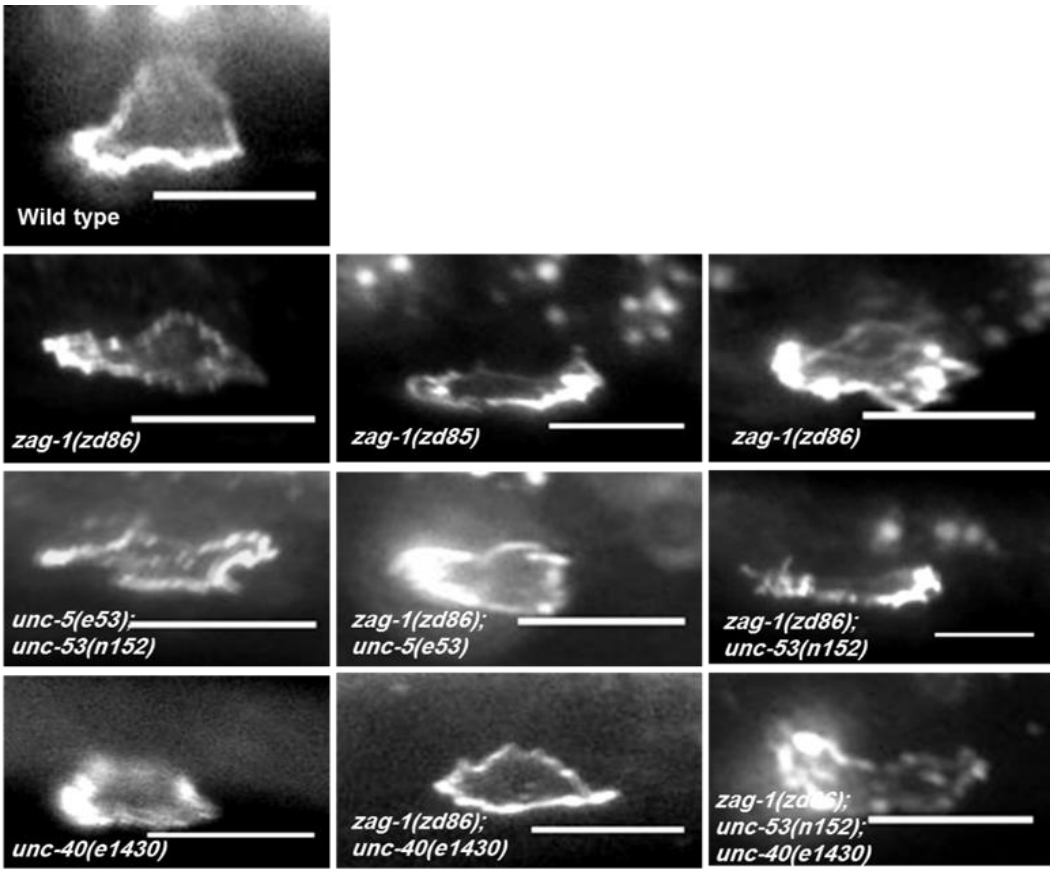


Figure 27. *zag-1* mutants affect intracellular UNC-40::GFP localization. A Photomicrograph showing UNC-40::GFP localization in L2 larvae stage of HSN neuron. The ventral is down, and the anterior is left; the scale bars represent 10 µm. In wild type animals, UNC-40::GFP is ventrally localized, but in *zag-1(zd85)* and *zag-1(zd86)* mutants, UNC-40::GFP localization is polarized to different directions of the neuron, including anterior or posterior, or in both regions. In *unc-5(e53);unc-53(n152)* doubles, UNC-40::GFP is uniformly distributed, whereas in *zag-1(zd86);unc-5(e53)* and *zag-1(zd86);unc-53(n152)* double mutants, UNC-40::GFP localization is polarized to different directions of the neuron, including anterior or posterior respectively. In *unc-40(e1430),* UNC-40::GFP localization is polarized to different directions of the neuron, including anterior, whereas in *zag-1(zd86);unc-40(e1430),* the UNC-40::GFP localization is uniform and in *zag-1(zd86); unc-53(n152);unc-40(e1430)* triple mutants, UNC-40::GFP localization polarized back to different directions of the neuron.

Dorsal-ventral direction of UNC-40::GFP localization

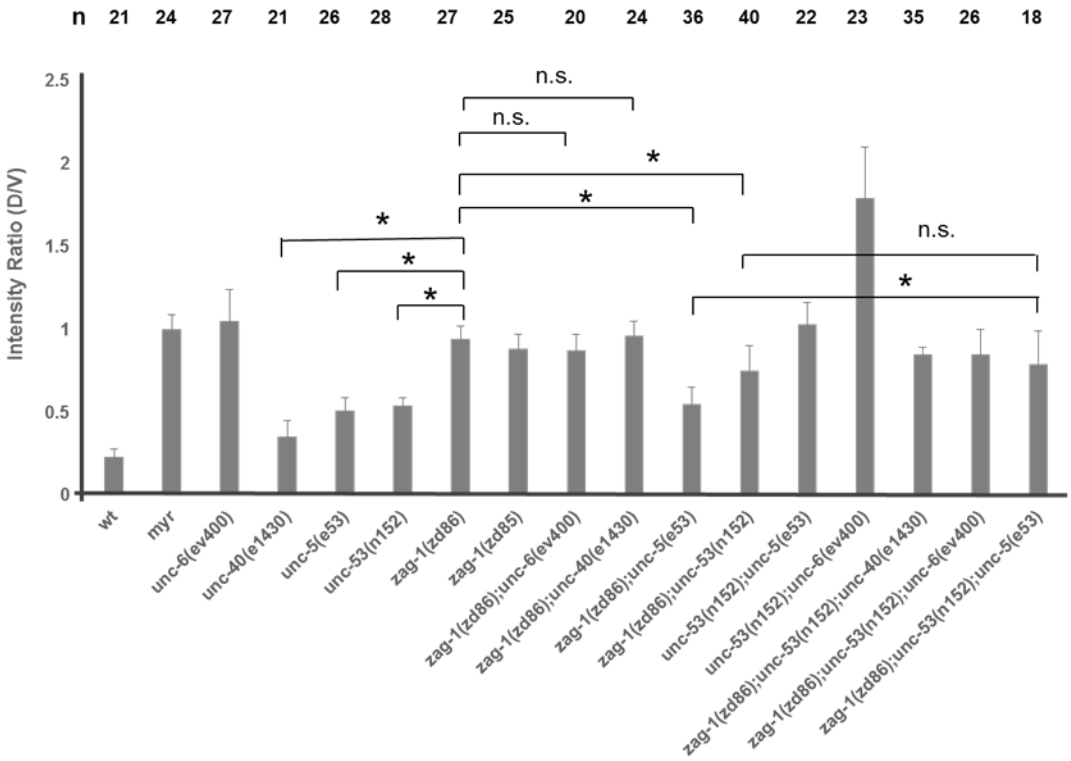
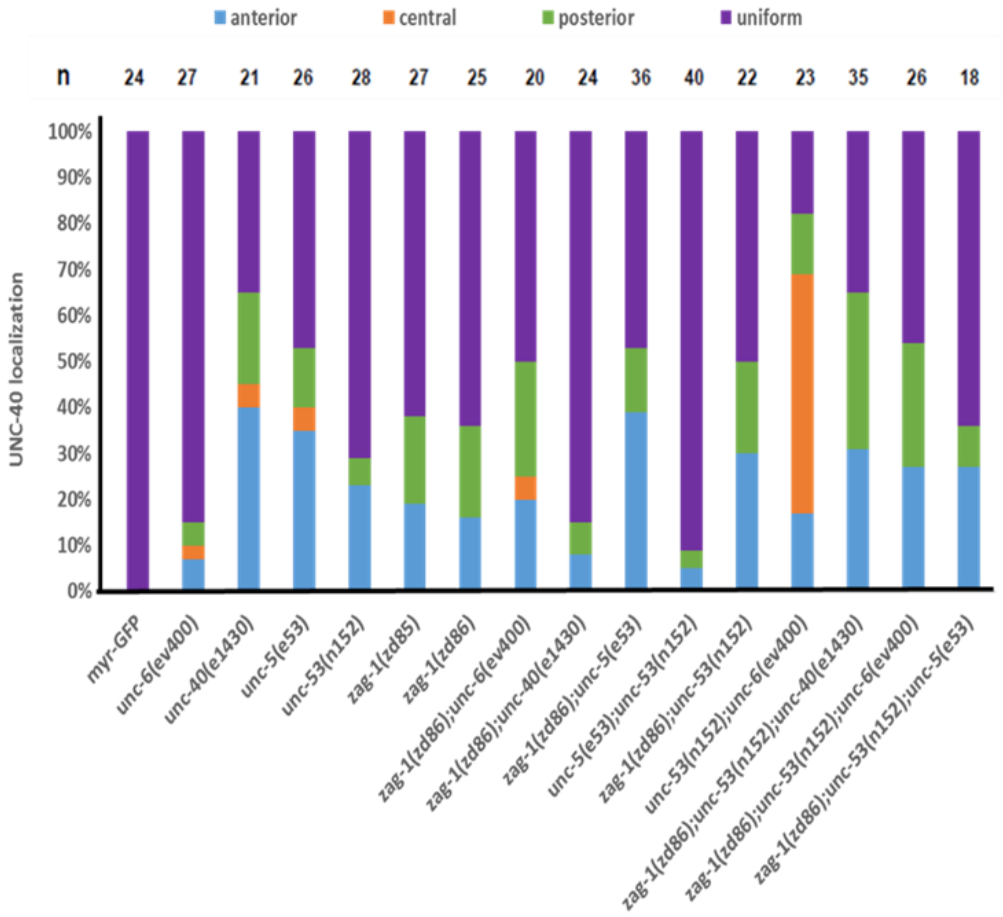


Figure 28. Graph shows the dorsal-ventral intensity average ratio of UNC-40::GFP localization around the periphery of the HSN cell body. A strong ventral bias is shown in wild-type animals; however, there is a uniform distribution in *zag-1(zd86)* and *zag-1(zd85)* single mutants, whereas the *zag-1(zd86);unc-5(e53)* and *zag-1(zd86);unc53(n152)* double mutants show ventral bias phenotypes. In *unc-6(ev400);unc-53(n152),* there is an increase in the dorsal bias. However, introducing *zag-1* mutants to these mutants is back to uniform. The error bar represents the standard error mean, and n represents the number of counted animals for each column. The student t-test was used to determine if there are significant differences between strains, *$p<0.05$. n. s. is statistically not significant.

A

Anterior-posterior direction of UNC-40::GFP localization



B

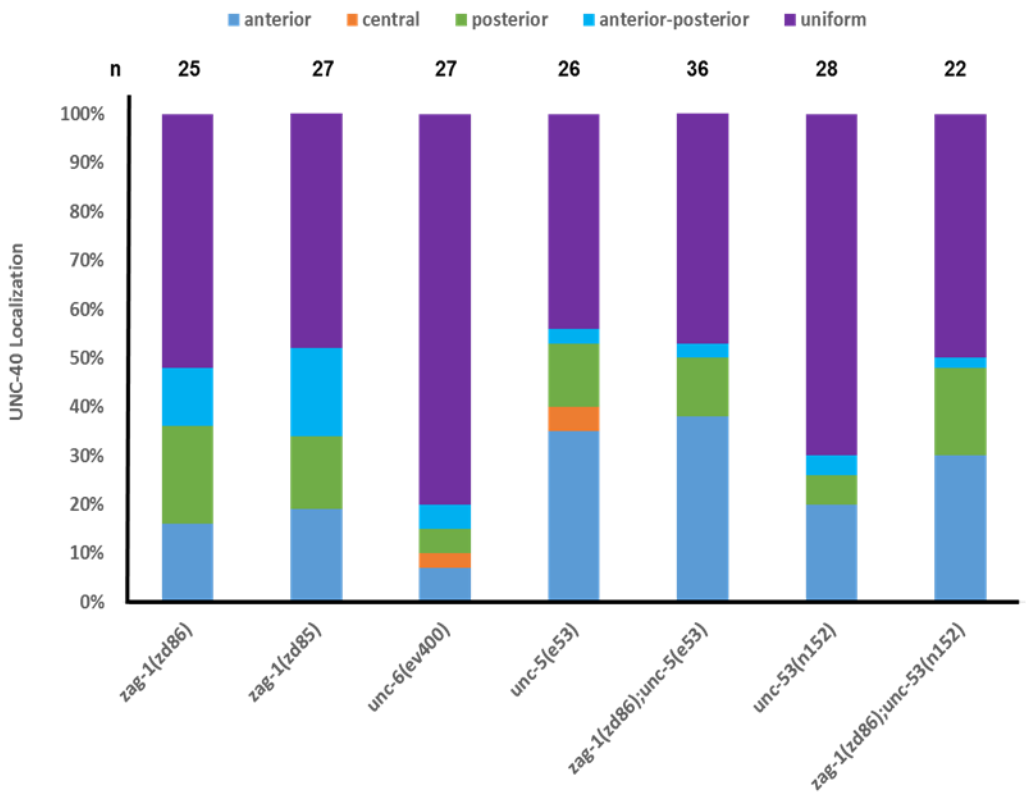


Figure 29. Graphs indicate the anterior-posterior orientation of UNC-40::GFP localization at the dorsal surface of HSN neuron cell body. Here is the anterior or posterior distribution in *zag-1(zd86)* and *zag-1(zd85)* single mutants. In *unc-6(ev400)*, there is a uniform distribution, while *zag-1(zd86);unc-6(ev400)* double mutants has a random distribution of UNC-40::GFP, including anterior, dorsal or posterior. However, in *zag-1(zd86); unc-53(n152);unc-6(ev400)* triple mutants, the distribution of UNC-40::GFP is anterior or posterior. The UNC-40::GFP localization is uniform in *unc-53(n152);unc-5(e53)*, whereas the UNC-40::GFP localization is polarized in different directions when the *zag-1(zd86)* mutant is introduced into these double and triple mutants. The UNC-40::GFP localization stays in different directions when *zag-1* mutants is input in double and triple mutants at the dorsal surface of HSN neuron cell body. n represents the number of counted animals for each column (A). Graph B includes the anterior-posterior direction of UNC-40::GFP localization in mutants.

Table 2. ZAG-1 regulates the directional bias of HSN axon outgrowth from the cell body

	direction of axon protrusion					
	dorsal	ventral	anterior	posterior	multipolar	
	%	%	%	%	%	n
wild-type	0	95±1	4±1	0	1±1	188
unc-6(ev400)	1±1	2±1	78±2	11±1	8±1	198
unc40(e1430)	1±1	4±1	71±2	15±2	9±1	179
unc-5(e53)	0	72±1	23±1	1±1	4±1	211
unc-53(n152)	0	61±3	25±1	6±1	8±1	199
zag-1(zd85)	0	14±1	36±2	1±1	49±1	159
zag-1(zd86)	0	11±1	41±3	4±1	44±2	182
zag-1(zd85);unc-6(ev400)	0	8±1	34±2	11±1	47±2	180
zag-1(zd86);unc-6(ev400)	1±1	7±1	28±1	13±1	51±2	164
zag-1(zd86);unc40(e1430)	0	11±1	29±1	10±1	50±2	187
zag-1(zd86);unc-5(e53)	0	37±2	49±2	12±1	2±1	245
zag-1(zd86);unc-53(n152)	0	25±1	55±2	15±1	5±1	185
unc-53(n152);unc-6(ev400)	17±2	1±1	29±3	21±2	32±3	154
zag-1(zd86);unc-53(n152); unc-40(e1430)	0	8±1	44±2	20±1	27±1	163
zag-1(zd86);unc-53(n152);unc-6(ev400)	0	13±1	47±3	15±1	25±2	220
zag-1(zd86);unc-53(n152); unc-5(e53)	0	16±1	38±3	18±1	28±1	154

Most of the wild-type animals project axon from the ventral. The *zag-1* mutants enhance the anterior HSN axon outgrowth directional bias when doubled with *unc-5* and *unc-53* mutants, of the instead ventral bias. Strains are indicated in the first column. The percentage of animals with axon migration defects is shown. n is the number of animals scored. The anterior is left and the ventral is down. The numbers represent percentage value ±SEM.

Table 3. ZAG-1 regulates the directional bias of AVM axon outgrowth from the cell body

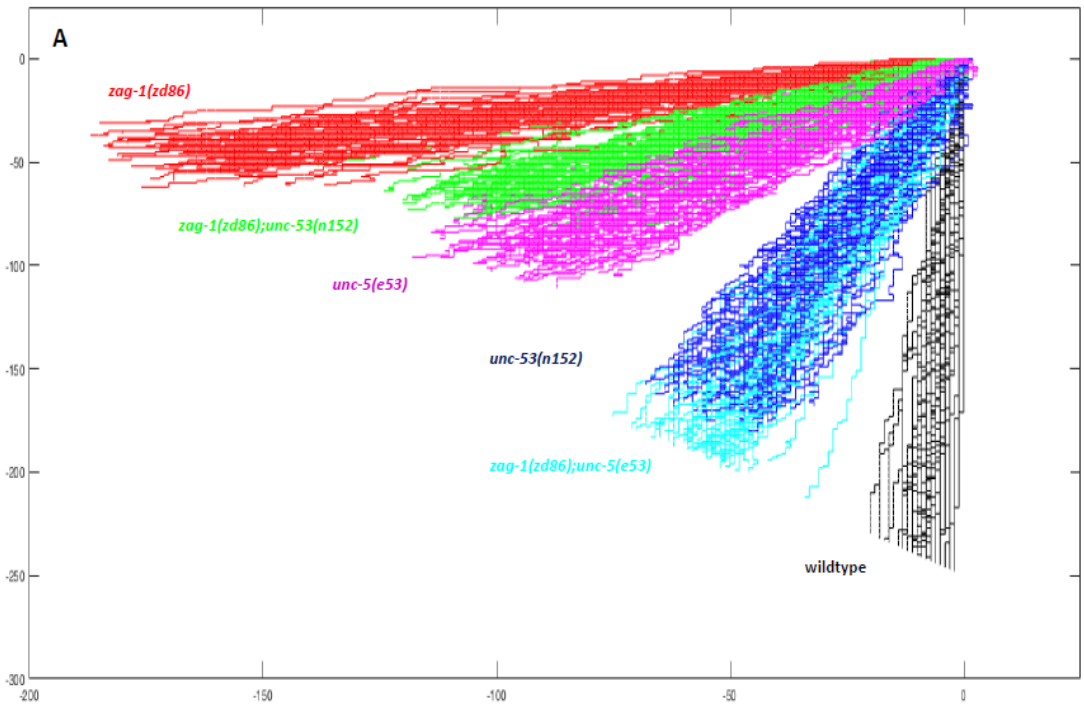
	direction of axon protrusion					
	dorsal	ventral	anterior	posterior	multipolar	
						
	%	%	%	%	%	n
wild-type	0	100	0	0	0	185
unc-6(ev400)	0	67±3	33±1	0	0	122
unc40(e1430)	0	79±2	21±1	0	0	164
unc-5(e53)	0	62±2	21±2	11±1	6±1	136
unc-53(n152)	0	100	0	0	0	187
zag-1(zd85)	0	66±1	17±1	6±1	11±1	139
zag-1(zd86)	0	57±3	25±1	5±1	13±1	166
zag-1(zd86);unc-6(ev400)	0	49±1	32±1	7±1	12±1	116
zag-1(zd86);unc-5(e53)	0	58±2	16±2	14±1	12±1	133
zag-1(zd85);unc-40(e1430)	0	65±1	29±1	0	6±1	192
zag-1(zd86);unc-40(e1430)	0	49±3	36±2	2±1	13±1	158
zag-1(zd86);unc-53(n152)	0	33±1	52±1	10±1	5±1	190
unc-53(n152);unc-6(ev400)	9±1	29±2	49±3	8±1	5±1	158
zag-1(zd86);unc-53(n152); unc-40(e1430)	0	28±2	38±2	26±2	8±1	175
zag-1(zd86);unc-53(n152);unc-6(ev400)	1±1	19±1	55±2	16±1	9±1	216
zag-1(zd86);unc-53(n152); unc-5(e53)	0	22±1	32±1	19±1	27±2	155

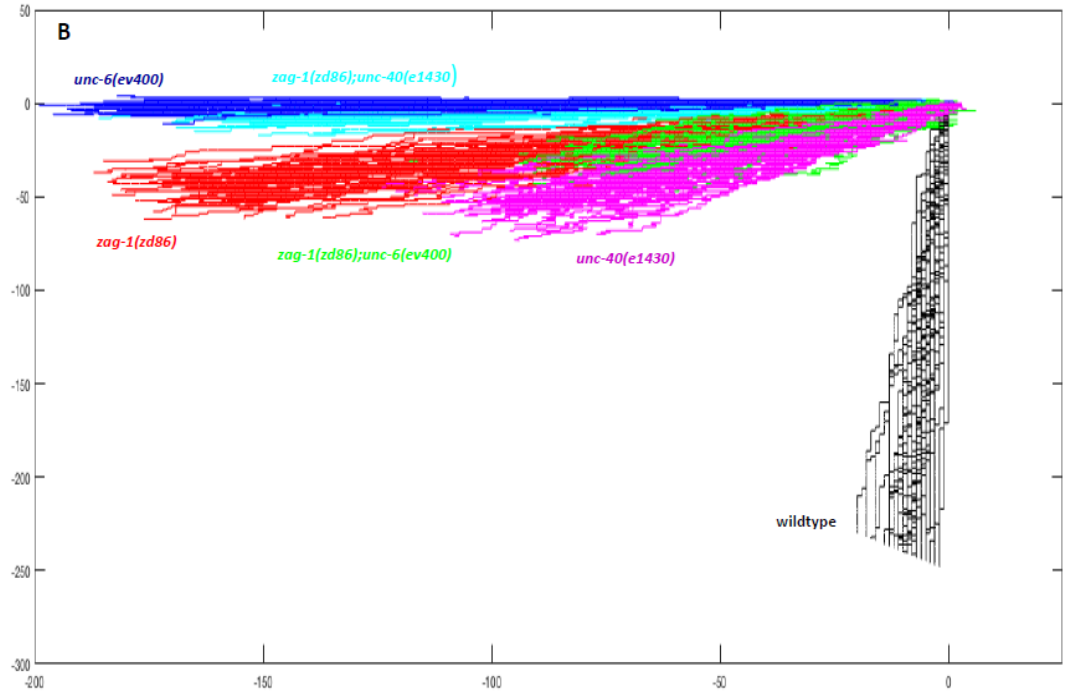
The *zag-1* mutants play a role in AVM axon directional bias. All of the wild-type animals project an axon from the ventral. The *zag-1* mutants enhance the anterior axon outgrowth bias when combined with *unc-53* mutants. Strains are indicated in the first column. The percentage of animals with axon migration defects is shown. n is the number of animals scored. The anterior is left and the ventral is down. The numbers represent percentage value ±SEM.

Table 4. Axon protrusion direction from the ALM cell body

	direction of axon protrusion					
	dorsal	ventral	anterior	posterior	multipolar	
						
	%	%	%	%	%	n
wild-type	0	0	100	0	0	158
unc-6(ev400)	0	0	71±3	0	29±2	170
unc-40(e1430)	0	0	68±1	0	32±1	139
unc-5(e53)	0	0	74±3	0	26±2	175
unc-53(n152)	0	0	68±2	0	32±2	195
zag-1(zd85)	0	0	39±1	0	61±1	165
zag-1(zd86)	0	0	33±1	0	67±1	221
zag-1(zd86);unc-6(ev400)	0	0	65±2	0	35±2	173
zag-1(zd86);unc-5(e53)	0	0	67±3	0	33±2	137
zag-1(zd85);unc-40(e1430)	0	0	76±2	0	24±1	181
zag-1(zd86);unc-40(e1430)	0	0	64±2	0	36±1	175
zag-1(zd86);unc-53(n152)	0	0	87±2	0	13±1	238
unc-53(n152);unc-6(ev400)	0	0	73±3	0	27±3	193
zag-1(zd86);unc-53(n152); unc-40(e1430)	0	0	71±3	0	29±4	169
zag-1(zd86);unc-53(n152);unc-6(ev400)	1±1	0	67±1	1±1	31±1	207
zag-1(zd86);unc-53(n152); unc-5(e53)	0	0	65±2	0	35±3	113

The *zag-1* mutants decrease ALM axon migration defects when in combination with *unc-53* mutants. All of the wild-type animals project axon from the anterior, but in *zag-1* mutants, the direction of the axon changes. Strains are indicated in the first column. The percentage of animals with axon migration defects is shown. n is the number of animals scored. The anterior is left and the ventral is down. The numbers represent percentage value ±SEM.


A
zag-1(zd86)
zag-1(zd86);unc-53(n152)
unc-5(e53)
unc-53(n152)
zag-1(zd86);unc-5(e53)
wildtype
0
-50
-100
-150
-200
-250
-300
-200
-150
-100
-50
0


B
unc-6(ev400)
zag-1(zd86);unc-40(e1430)
zag-1(zd86)
zag-1(zd86);unc-6(ev400)
unc-40(e1430)
wildtype
50
0
-50
-100
-150
-200
-250
-300
-200
-150
-100
-50
0

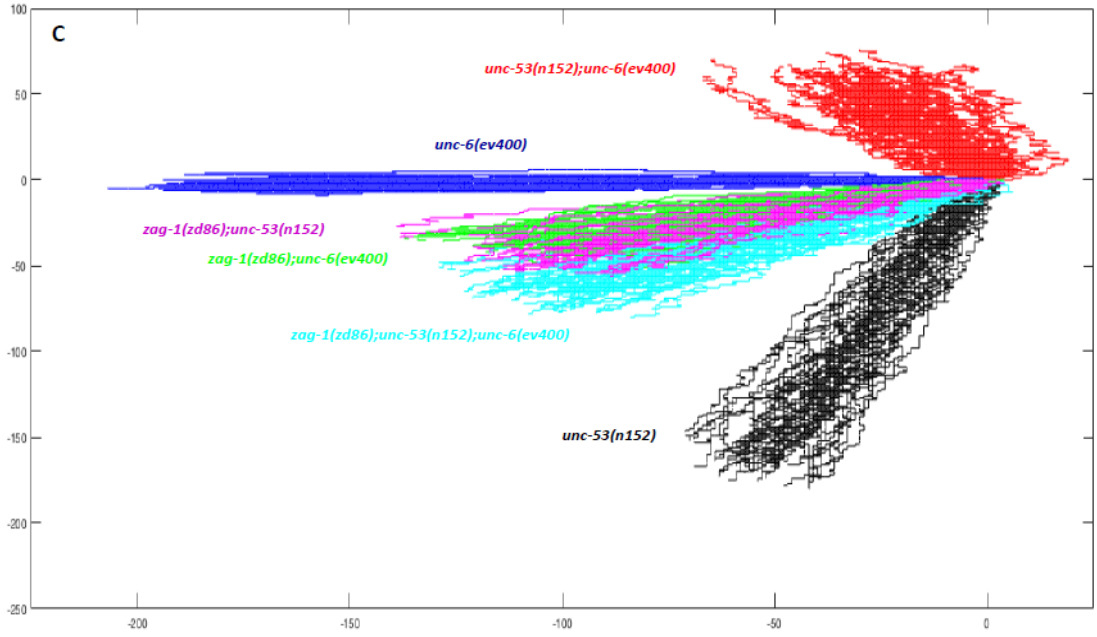


Figure 30. The directional bias of axon outgrowth extension from cell body of HSN neuron. From an origin (0, 0) 50 simulated of 500 steps of random walks were plotted for each mutant upon the probabilities of axon outgrowth in the anterior, posterior, dorsal, and ventral directions as in table 1. The plots display the directional bias that occur by mutants to compare the directional bias caused by *zag-1* mutants relative to wild type for HSN. The directional bias changes anteriorly (A, B, and C).

DISCUSSION

The stochastic model and the regulation of axon outgrowth patterning

Do axon guidance cues decisions depend on the ZAG-1 transcription factor or not? How do the genetic interactions affect the axon outgrowth during the neuronal development? What is the role of *zag-1* in UNC-6/Netrin signaling pathway? And what is the role of *zag-1* in UNC-40::GFP asymmetric localization? I have attempted to answer these questions through studying the potential role of the ZAG-1 transcription factor with axon guidance cues and its receptors in axon pathfinding using a genetic approach in *C. elegans*. This may lead to new approaches to therapeutic treatment for multiple types of human disorders.

The correct axon outgrowth direction is a crucial process for the proper functioning of the nervous system. A better understanding of axon guidance regulation has a clinical importance to develop therapeutic techniques. Recent studies in our lab provide evidence that the HSN neuron outgrowth activity is directed through a stochastic process in response to external guidance cue, UNC-6/Netrin, to increase or decrease HSN axon outgrowth in different directions. UNC-6/Netrin directs UNC-40-mediated axon outgrowth into ventral side of the neuron. However, the neuron can polarize without cues to a specific site randomly (Kulkarni et al., 2013; Xu et al., 2009; Yang et al., 2014; Tang and Wadsworth, 2014).

We provide evidence that the movement of axon outgrowth activity is directed stochastically to any side of the neuron in response to guidance cues (Kulkarni et al., 2013), suggesting that the stochastic model is useful for understanding how genes regulate the patterning of axon extensions. There are two ways to measure the probability of orientation: the probability of UNC-40::GFP localization at each surface of HSN neuron

and the probability of axon outgrowth from each surface of HSN neuron. In wild-type animals, the probability of axon outgrowth activity in the downward direction is high. In mutants, the probability of UNC-40::GFP localization and axon outgrowth changes at each surface and the probability of axon outgrowth is low in the downward direction and is high in the left and right directions (Kulkarni et al., 2013; Tang and Wadsworth, 2014). Even though the axon outgrowth movement is the same in wild type and mutants, there is a difference in the displacement, resulting in different patterns of axon outgrowth and a shorter distance of axon migration in a given amount of time. At each instance of time, the probability of axon outgrowth in different directions is high, whereas over time the fluctuation in axon outgrowth directions is great. In statistical physics, the axon outgrowth net movement is diffusive. Diffusive motions occur at the micro-scale at the plasma membrane. A decrease in the axon outgrowth at the macro scale relates to an increase in the diffusive motion, resulting in a low-rate outgrowth that prevents neurites from developing and a change in the axon outgrowth patterning. It is known that the dorsal axon outgrowth bias is induced in the *unc-53(n152);unc-6(ev400)* double mutants (Kulkarni et al., 2013); however, in *zag-1(zd86);unc-53(n152);unc-6(ev400)* triple mutants, the anterior axon outgrowth bias is induced, indicating that the presence of ZAG-1 transcription factor regulates the directional bias of the growing axon randomly.

The AVM axons grow ventrally towards the source of UNC-6/Netrin gradients and then migrate anteriorly into the nerve ring (Chalfie and Sulston, 1981). *unc-53(n152)* mutants cause a strong ventral bias, like what happens in wild type animals (Kulkarni et al., 2013). ZAG-1 creates a weaker ventral bias. We observed that *zag-1(zd86);unc-53(n152)* double mutants create a strong anterior bias (Table3), suggesting that the anterior

directional bias requires UNC-53 and ZAG-1 activity. In HSN and AVM, the *zag-1* mutants enhance the anterior axon outgrowth directional bias when in combination with *unc-53* mutants, of the instead ventral bias (Table 2 and Table 3).

The identification of the target genes controlled by *zag-1* might directly identify genes that control axon outgrowth bias in future work. However, how the *zag-1* transcription factor regulates axon guidance direction remains unclear. Studies such as these could lead to new strategies to treat diseases in the vertebrate nervous system.

As a conclusion, the differences in axon outgrowth directions, which occur by mutation, can be modeled by the random walk model that can describe the axon outgrowth movement mathematically in response extracellular guidance cues.

ZAG-1 controls neuronal differentiation

The nervous system development is controlled by different transcription factors. The *C. elegans zag-1* gene has roles in regulating multiple aspects of neuronal differentiation and nervous system development such as cell position, gene expression, axon outgrowth and guidance, and axon branching of many interneurons and motor neurons. ZAG-1 is required for differentiation of HSN, and it acts as the first transcription factor that regulates neurons of development (Clark and Chiu, 2003).

zag-1 mutants cause severe axon pathfinding defects in HSNs morphology, including the axon trajectory. Furthermore, *zag-1* mutants cause several defects in sensory neurons (Clark and Chiu, 2003). ALMs neurons require *zag-1* for cell migration, axon development, and expression of *mec-4* (mechanosensory abnormal) (Clark and Chiu, 2003; Wacker et al., 2003), which is one of *C. elegans* genes family that can mutate to promote neuronal

degeneration (Driscoll and Chalfie, 1991). Also, the expression of different motor neurons markers like *unc-4::gfp*, *unc-47::gfp* and *glr::gfp* is misregulated in a *zag-1* mutants, suggesting that ZAG-1 is required for differentiation of these neurons (Clark and Chiu, 2003; Wacker et al., 2003). Additionally, *zag-1* mutants do not cause defects in muscle development and differentiation (Clark and Chiu, 2003).

A number of studies have shown that the transcription factors can interact with other transcription factors to regulate neuronal differentiation and neuronal cell fates. For instance, *zag-1* interacts genetically with Ast-1, a novel transcription factor that is required for axon and pharynx development in *C. elegans*, demonstrating that it is a part of a transcription network for regulating axon outgrowth (Schmid et al., 2006). A recent study demonstrated that the ZAG-1, AHR, and MEC-3 transcription factors act together in determining sensory neuron fates (Smith et al., 2013). In *C. elegans*, three homeodomain transcription factors, which are *ceh-10*, *ceh-23*, and *ttx-3*, function in regulating AIY interneuron differentiation (Altun-Gultekin et al., 2001). *zag-1* may act as the regulator of genes that are important in guidance cues.

It was found that transcription factors provide extrinsic and intrinsic information to guide axons into their particular targets to make the correct pattern of axon extensions. In *C. elegans*, there are many transcription factors that regulate axon guidance molecules. For instance, *fax-1*, a nuclear hormone receptor gene, is required for axonal guidance in the ventral nerve cord and the nerve ring to regulate the transcription of genes that play a role in axonal guidance (Much et al., 2000). Further, the *unc-3*, a member of O/E (olf-1/EBF) family of transcription factor, is identified as a regulator of the cholinergic identity of motor neurons and motor axon guidance (Prasad et al., 1998).

In *Drosophila*, the Lola (longitudinal lacking), a transcriptional regulator of axon guidance of many numbers of neurons, regulates the SLT-1 receptor, Robo (Roundabout). In the absence of *Lola*, the levels of Robo and slit decrease, demonstrating that the axon guidance defects result from the role of *Lola* to activate both Robo and SLT-1 transcription (Crowner et al., 2002).

Although I have focused on a transcription factor that regulates axon guidance patterning in *C. elegans*, there is a substantial evidence that transcription factors control axon guidance in vertebrate nervous system. A recent study found that the LIM homeodomain transcription factor (Isl-1), which plays important roles in survival, differentiation and neuronal development, including rat striatum during development (Wang and Liu, 2001), regulates axon guidance cues in the developing limb by expression of Lim1 and IsI1 respectively in LMC neurons (Kania et al., 2000). Indeed, Sp8, a zinc-finger transcription factor expressed in the nervous system during development tail buds and limbs in mice, is required for forebrain patterning and controls the survival of the telencephalon ventral cells (Zembrzycki et al., 2007). Moreover, the homeodomain gene *unc-42*, required for axon outgrowth, regulates neuron development of multiple chemosensory and glutamate receptors, whereas *unc-42* mutants show multiple axonal pathfinding defects (Baran et al., 1999; Brockie et al., 2001). Also, the transcription cascade regulates motor neurons in chicks and mice embryos (Dasen, 2009). Other studies have identified that the axon guidance decision by Retinal Ganglion Cell (RGC) axons at the optic chiasm is regulated by transcription factors expression (Herrera et al., 2003; Pak et al., 2004). The zinc finger transcription factor expressed in the ventral part of the retina has a role in the RGC axon responsiveness to repulsive cues at the optic chiasm (Pollex et

al., 2007).

This study is the first to determine the ZAG-1 transcription factor role through UNC-6/Netrin signaling pathway and its receptors, UNC-5/UNC5, UNC-40/DCC, and with UNC-53/NAV2 adaptor protein on HSN, AVM, ALM, and PLM axon outgrowth patterning, neuronal polarity, controlling neuronal circuits and axon direction to its right position during the neuronal development, and its effect on UNC-40 asymmetric localization in HSN neuron cell body. The axon guidance, in response to extracellular cue and transcriptional regulation, may provide an excellent mechanism to achieve polarization process and neuronal outgrowth activity.

zag-1 is involved in axon guidance defects of the HSN axon, in *zag-1(zd86);unc-5(e53) and zag-1(zd86);unc-53(n152)* double mutant; these defects include the axons that migrate anteriorly, posteriorly, dorsally or have bipolar phenotypes (Figure 22), indicating that *zag-1* regulates *unc-5* and *unc-53* activity. Thus, the effect of UNC-5 and UNC-53 on axon outgrowth patterning requires the presence of ZAG-1. These results indicate that ZAG-1 regulates the axon outgrowth activity on its genetic interaction with UNC-5 and UNC-53 and they are similar to our data on AVM neurons (Table 3). Further evidence that ZAG-1 transcription factors affects ALM axon outgrowth activity by regulating UNC-5 and UNC-53 adaptor protein activity (Figure24). Additionally, ZAG-1 affects PLM axon extension. *zag-1(zd86);unc-5(e53), zag-1(zd86);unc-40(e1430)* and *zag-1(zd86);unc-53(n152)* double mutants enhance the PLM short extension. PLM axon failed to reach the vulva position (Figure 26).

FUTURE WORK AND DIRECTIONS

The identification of the transcriptional mechanisms that regulate axon outgrowth activity

Although studying axon guidance is the main event in neuronal circuit formation and is challenging, it is important to study transcriptional regulation mechanism to complete this process. Most of the families of axon guidance cues have been discovered, but how the axon guidance is regulated is still an open question. In addition, different transcription factor families that regulate axon guidance, neuron differentiation, generation, and development have been identified. It is interesting to determine this mechanism, which is likely to be a major goal to increase our understanding of the transcriptional control of the axon pathfinding. Studies in the last few years have demonstrated that the transcription factors regulate the guidance molecules expression in specific neurons (Zarin et al., 2012). However, knowledge about the mechanisms and how these possesses regulate the axon guidance receptors expression is still limited.

The identification of the role of ZAG-1 transcription factor in regulating outgrowth in response to SLT-1 and Wnt extracellular guidance cues.

It will be interesting to determine whether the ZAG-1 transcription factor is required for the regulation of other extracellular guidance cues such as SLIT-1/ Robo and Wnt/EGL-20. Determining how *zag-1* regulates these guidance cues will be the key to achieve a better understanding of how this transcription factor controls the changes in axon outgrowth patterning during nervous system development.

CONCLUSIONS

We have proposed a model of neuronal outgrowth activity that is based on statistically dependent asymmetric localization (SDAL). According to this model, the probability of UNC-40 localization and outgrowth at one site affects the probability of localization and outgrowth at other sites.

The link between stochastic process and transcriptional regulation of axon guidance became a big goal in understanding axon outgrowth activity. In addition, defining the relationship between transcription factor and neuron guidance molecules is a major goal to understand the role of the transcription factor in regulating neuronal outgrowth patterning and UNC-40 asymmetric localization. It is important to study the critical roles of transcription factor and the interplay with UNC-6/Netrin guidance cue and its receptors to direct circuit formation. The ZEB family of zinc finger homeodomain transcription factors are expressed in neurons and muscles, and they function in neuronal development and disease. They also have important roles in embryonic development and they regulate epithelial to mesenchymal transitions (Vandewalle et al., 2005).

Therefore, this study focuses on the identification of the role of the ZAG-1 transcription factor in regulating axon guidance proteins activity in influencing the movement in HSN, AVM, ALM, and PLM axons outgrowth patterning and UNC-40::GFP asymmetric localization as novel strategies in *C. elegans*.

Since *zag-1*, which encodes the EF1/ZFH-1 Zn-finger-homeodomain protein, has been shown previously to regulate axonal pathfinding (Clark and Chiu, 2003; Wacker et al., 2003), it is possible that *zag-1* mutants cause different defects in various aspects of

neuronal development.

I have presented evidence that the ZAG-1 transcription factor has unique roles in neuronal development and the regulation of important processes such as axon migration, protrusion, branching and UNC-40::GFP asymmetric localization, in response to guidance cue and cell surface guidance receptors. Using the mathematical model, random walk, to determine the effects of mutants on axon movement, I have shown that *zag-1* regulates the UNC-6/Netrin signal activity especially, the *unc-5, unc-40* receptors and *unc-53* cytoplasmic protein activity. Also, *zag-1* mutants alter the UNC-40::GFP asymmetric localization in HSN neuron. Specifically, these mutants increase the probability of UNC-40::GFP asymmetry localization in the anterior, posterior, or both positions (anterior and posterior), whereas the probability of UNC-40::GFP asymmetry localization in the ventral position decreases (Figures 27 and 29). These data will allow us to understand the transcriptional regulation of axon guidance systems. However, it remains unclear how the combination of guidance cue and its receptors and the ZAG-1 transcription factor control the axon pathfinding decisions or how they regulate at transcriptional level. Also, we show that UNC-5 acts together with the UNC-53 to regulate UNC-40 asymmetric localization in response to the UNC-6 and EGL-20 extracellular cues. This understanding of the role of ZAG-1 transcription factor and UNC-5 in regulating of UNC-40 asymmetric localization will surely lead to develop new therapies for treating neuronal disorder or injuries within the vertebrate nervous system as well as understanding how axons start in the correct position and how the transcription factor directs the axons into the target area.

ZAG-1 MODEL FIGURES

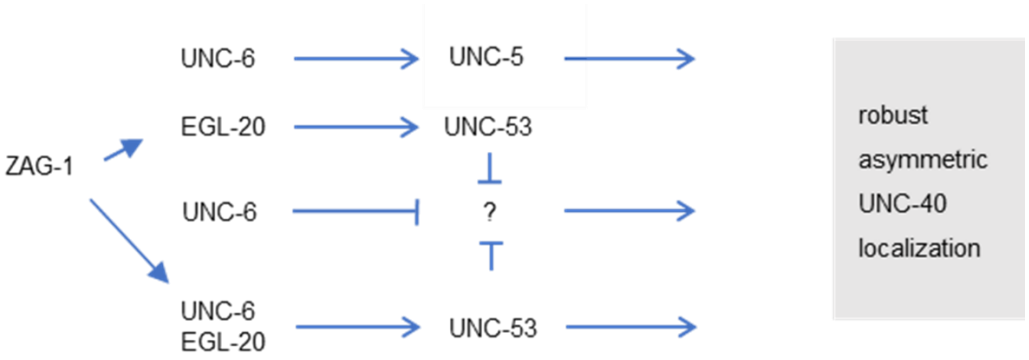


Figure 31. ZAG-1 function is required for UNC-53 function. Loss of both UNC-53 and UNC-6 allows asymmetric UNC-40 localization. Loss of ZAG-1 and UNC-6 also allows asymmetric UNC-40 localization. This pathway for localization is UNC-6 independent is represented by the "?" pathway. Loss of UNC-53 allows this localization even if ZAG-1 is defective. (UNC-40::GFP does localize in *zag-1;unc-53;unc-40* mutants. Therefore, losing UNC-53 allows the "?" pathway even if ZAG-1 is disrupted.) ZAG-1 is not in the UNC-6, UNC-40, and UNC-5 pathway. UNC-40::GFP does localize in *zag-1;unc-6* double mutants, but not in *zag-1;unc-40* double mutants. So we place them in parallel pathways.

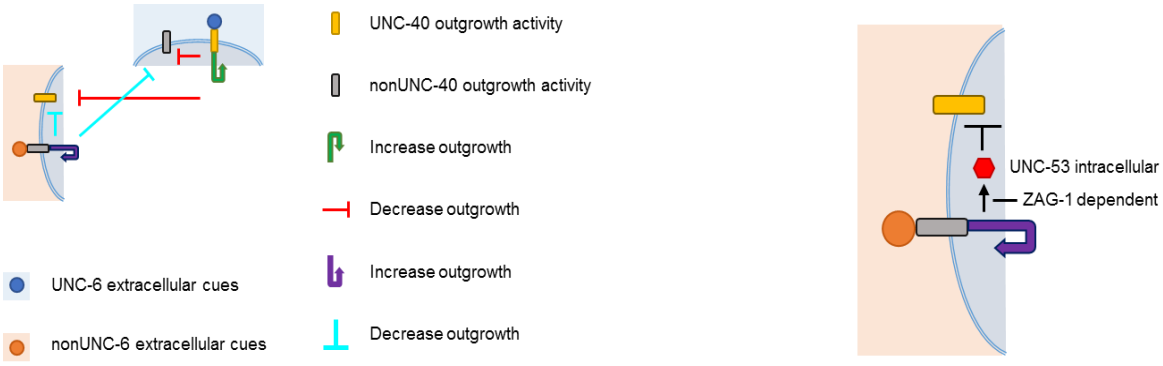


Figure 32. UNC-40 SDAL and nonUNC-40 SDAL. Genetics suggests that ZAG-1 is required for UNC-53 function. UNC-53 is required to inhibit UNC-40 outgrowth in the absence of UNC-6.

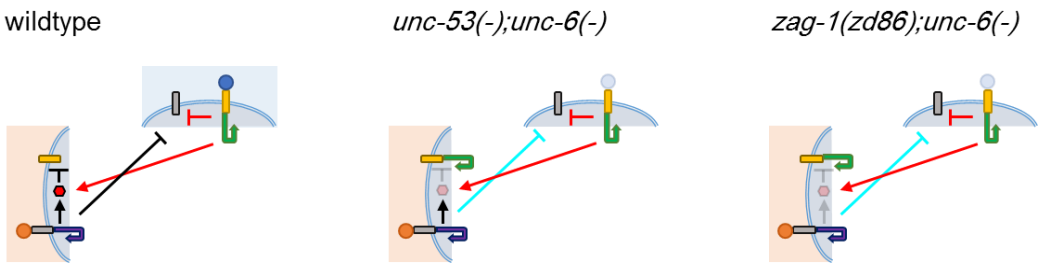


Figure 33. Loss of UNC-53 or ZAG-1 allows UNC-40 outgrowth activity in the absence of UNC-6.

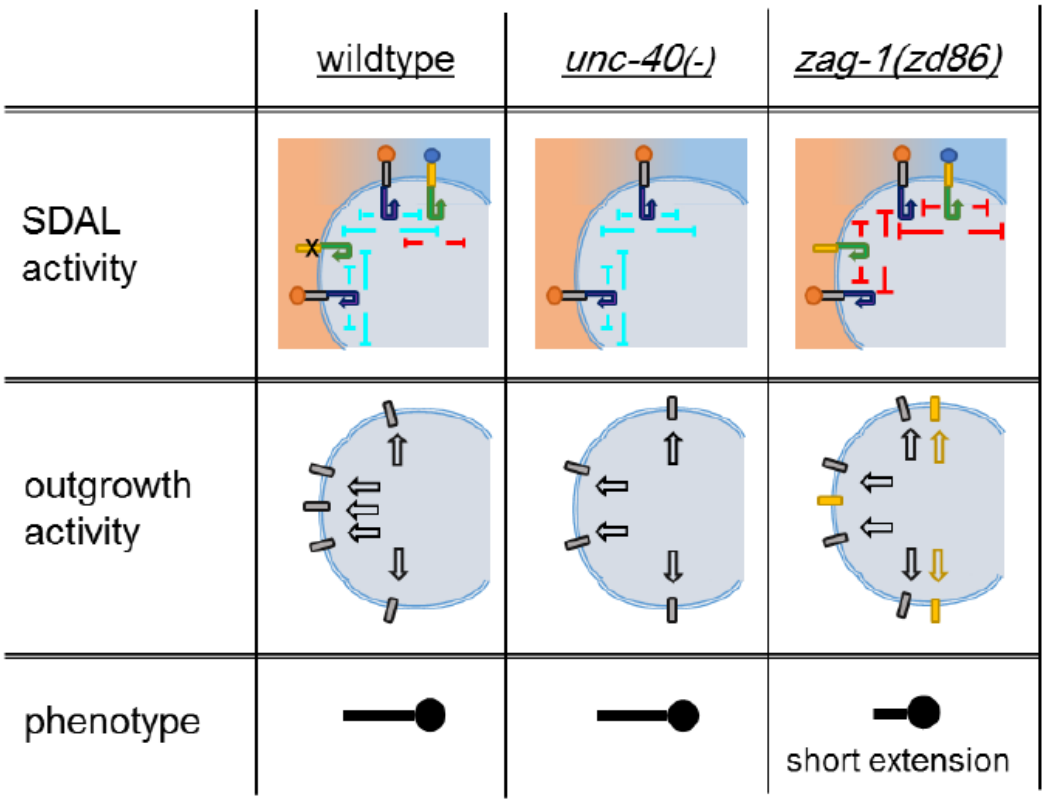


Figure 34. Model for PLM outgrowth movement. Reduction of ZAG-1 functions allows UNC-40 outgrowth activity where in wildtype it would be repressed by nonUNC-40. This increases the degree to which outgrowth activity fluctuates, thereby reducing outward displacement. This causes a short PLM extension.

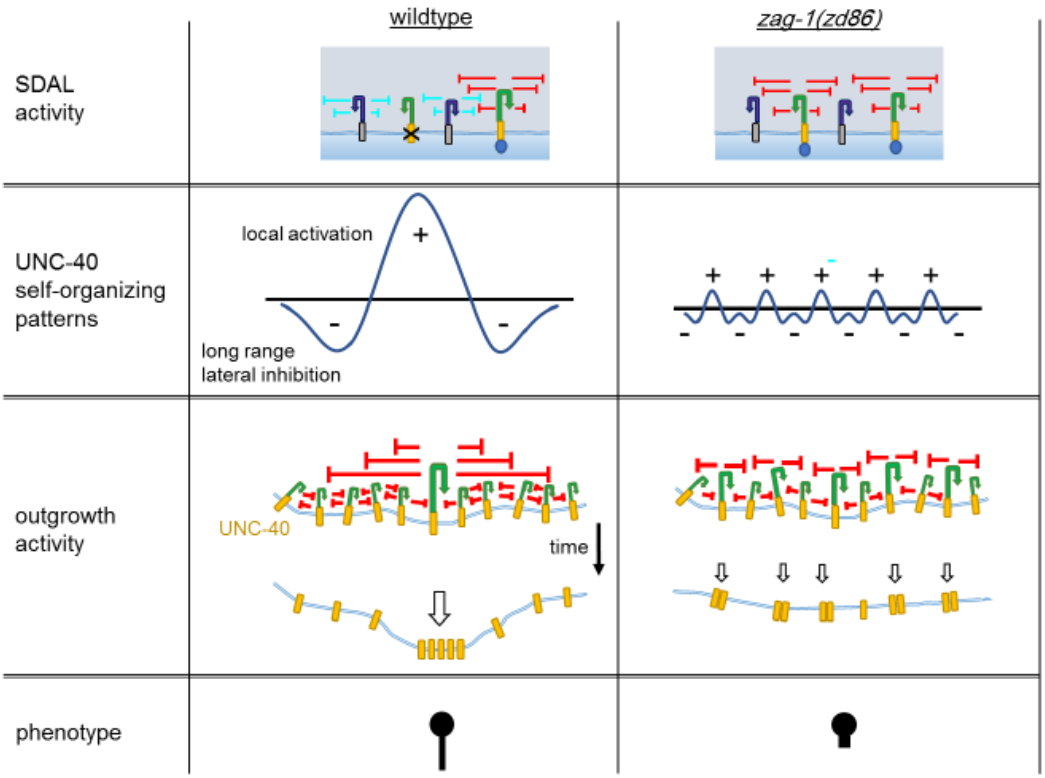


Figure 35. Model for HSN outgrowth movement. The nonUNC-40 activity causes outgrowth towards the anterior. There is still a strong anterior bias in *unc-40* mutants. In comparison, reduction of ZAG-1 activity may further decrease the probability of anterior outgrowth and thereby increase the probability of dorsal outgrowth. NonUNC-40 mediated outgrowth may allow extension into the nerve ring where cues that are anterior and posterior create an equal probability for outgrowth in those directions. This allows nonUNC-40 mediated outgrowth to maintain a dorsally directed bias.

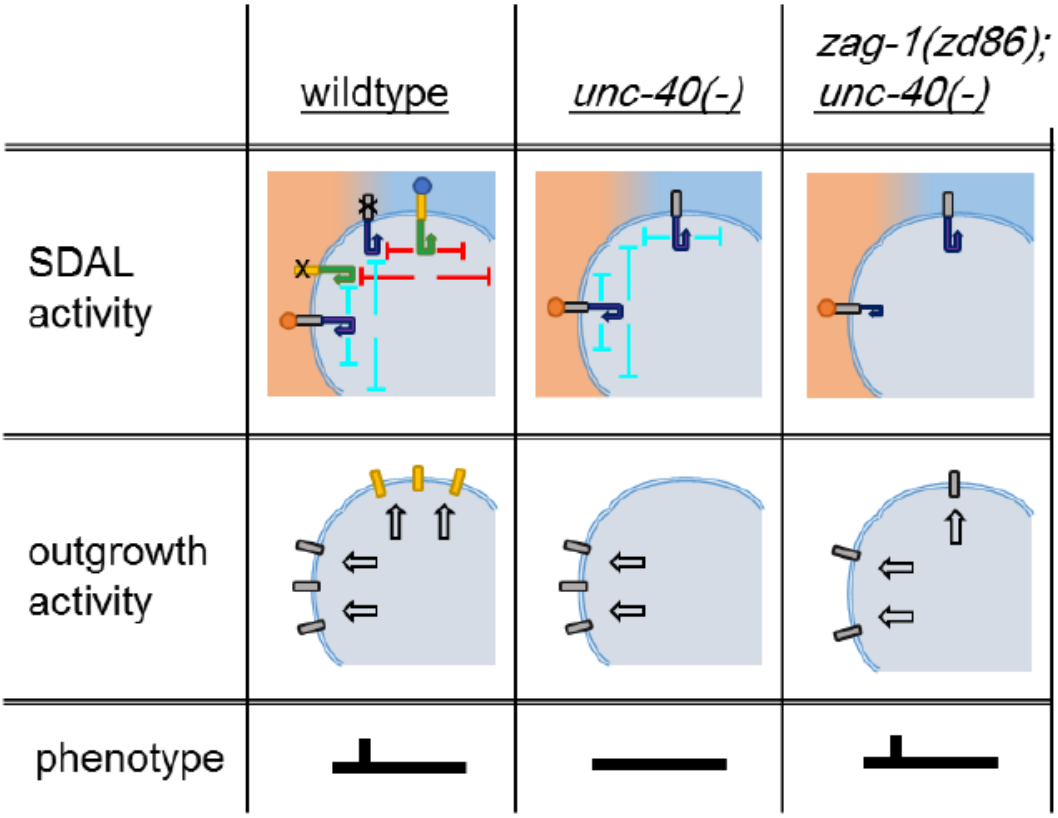


Figure 36. Model for AVM outgrowth movement at the nerve ring. In wildtype, nonUNC-40 activity may reduce the threshold for the UNC-40 SDAL at one site to dominate. After this threshold is met, UNC-40 begins to clusters at this area over time. ZAG-1 may reduce this threshold and allow outgrowth at sites that are not UNC-6 ligated. This reduces the ability of one site to predominate. This causes a broad outward movement with decreased displacement since the degree to which UNC-40 outgrowth activity fluctuates is greater over a larger area.

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APPENDIX

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