Investigation of PHR Signaling in *C. elegans*

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Abstract

The nervous system consists of billions of neurons interconnected through specialized connections known as synapses. When and where these synapses form determines the functionality of the nervous system, and abnormal synaptic development has been linked to a variety of disorders such as autism and epilepsy. In addition, understanding how synapses are formed could lead to treatments for a variety of conditions characterized by a loss of synaptic connectivity, including Alzheimer's disease and stroke. Therefore, understanding the molecular processes that regulate the formation of synapses has become a major goal of modern neuroscience research.

To form a synapse, a neuron must grow an axon, guide that axon to its proper target, and then form a synaptic connection. All of these processes must be coordinated to ensure synapses form at the correct time and in the correct location. One family of proteins that has become viewed as candidates to mediate this coordination is the Pam/Highwire/Rpm-1 (PHR) protein family. Over the past decade, the PHR proteins have emerged as key regulators of axon outgrowth and guidance, synapse formation, and degeneration. Given their central role in neurodevelopment, the discovery of any additional components of PHR signaling has the potential to increase our understanding of how a synapse is made, as well as identify therapeutic targets for pharmaceutical intervention. In the following dissertation, I outline two studies focused on the *C. elegans* PHR protein RPM-1 and the identification of novel mediators of RPM-1 signaling. In

the first study, I provide evidence that the PP2C phosphatase PPM-1 functions as a second regulatory mechanism to RPM-1 to negatively regulate a MAP kinase cascade. This study provides insight into the regulation of MAP kinase signaling in neurons, as well as a new role for PP2C phosphatases in neural development. In the second study, I identify the nuclear anchorage protein ANC-1 as a novel binding partner of RPM-1. My genetic data also indicates that ANC-1 functions through the β-catenin BAR-1 to regulate neurodevelopment. Our study highlights a new mechanism by which RPM-1 functions, as well as the first genetic link between RPM-1 and a pathway that is regulated by extracellular signals, such as Wnts.

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1   Introduction

1.1   Overview of synapse formation

The complexity of the human nervous system is staggering. The brain contains trillions of neurons, each forming as many as a thousand connections with other neurons in a precise pattern that is essential for proper function. Understanding how these connections, known as synapses, form is of great importance clinically, as abnormal circuit development has been linked to a variety of disorders including autism, anxiety, and epilepsy (Leonardo and Hen, 2008; Betancur et al., 2009). In addition to its role during development, synapse formation also appears to be important in the action of some antidepressants (Li et al., 2010), as well as memory storage (Kleim et al., 2002). Furthermore, understanding what molecules can promote the formation of synapses could lead to treatments for a number of neurodegenerative diseases such as Alzheimer's disease, in which a great number of synapses are lost. These are just a few examples of why a major goal of neuroscience research is to discover the cellular and molecular processes that govern and guide the creation of a nervous system.

A functioning nervous system is dependent on the creation of synapses. Synapses are structures that permit a neuron to pass an electrical or chemical signal to another cell (neuron, muscle, or gland). Chemical synapses are composed of both a pre- and postsynaptic terminal that is separated by a

synaptic cleft. The presynaptic terminal is composed of two main regions: the electron dense "active zone" and a pool of synaptic vesicles that surrounds the active zone.

The active zone is the region of synaptic vesicle fusion and neurotransmitter release (Couteaux and Pecot-Dechavassine, 1970). Proteins such as RIM, Munc13, RIM-BP, α-liprin and ELKS are some of the many proteins which function to dock synaptic vesicles and initiate the release of neurotransmitters into the synaptic cleft (reviewed in Sudhof, 2012). In the synaptic vesicle pool, vesicles are loaded with neurotransmitters and await docking and fusion (reviewed in Rizzoli and Betz, 2005). The postsynaptic terminal contains receptors for the neurotransmitters, as well as other molecules such as ion channels that are required for a response in the postsynaptic cell.

In order to form a synapse, a neuron must extend an axon out towards its target. As the axon migrates, it is directed by extracellular guidance cues. These cues can either be secreted or membrane bound and include molecules such as Netrins (Kennedy, 2000), Slits (Brose and Tessier-Lavigne, 2000), semaphorins (Pasterkamp and Kolodkin, 2003) and Ephrins (Kullander and Klein, 2002). The guidance cues are detected by a specialized structure at the tip of the developing axon called the growth cone, a filamentous (F) actin rich structure coated in receptors for the guidance cues. Guidance cues can either be attractive or repulsive, and when guidance cues interact with their specific receptor they initiate localized intracellular signaling cascades. It is the asymmetric activation of

these signaling cascades that leads to localized cytoskeleton modifications, thereby navigating the axon towards its specific target (Dickson, 2002).

Once the axon reaches its target, a synapse can then be formed. While numerous molecules are known to be required for synapse formation, the mechanisms by which many of them function are just beginning to be understood. Various cell adhesion molecules have been proposed to act as major players in triggering synapse formation. For example, neuroligins and their binding partners, the neurexins, have been shown to induce the formation of presynaptic terminals on non-neuronal cells (Scheiffele et al., 2000; Fu et al., 2003). It was further established that each partner can form a hemisynapse; neuroligins trigger presynaptic differentiation and neurexins trigger postsynaptic differentiation (reviewed in Craig and Kang, 2007). In addition, N-cadherins are found in both pre- and postsynaptic terminals and are found to accumulate prior to differentiation of the terminal, as well as function in the recruitment of synaptic components (Fannon and Colman, 1996; Togashi et al., 2002; Takeichi, 2007). Disruption of cadherin based contact also inhibits the formation of synapses in hippocampal cell cultures (Togashi et al., 2002; Bozdagi et al., 2004).

Creating proper synaptic connections requires precise axon navigation, as well a coordinated switch between axon growth and the formation of synapses. While they are often studied independently, axon guidance, outgrowth, and synapse formation are all interrelated processes that occur during the development of the nervous system. This is supported by evidence that guidance

cues, adhesion molecules, and various morphogens can function in both axon outgrowth as well as synapse formation (Shen and Cowan, 2010). The focus of this dissertation is the characterization of two molecules and their novel role in regulating this developmental coordination.

1.2   *C. elegans* as a model to study neurodevelopment

C. elegans is a free-living microscopic nematode found in temperate soil environments. Its small size, short lifespan, transparent body, and the availability of numerous genetic tools has made it a popular genetic system (Brenner, 1974). Detailed studies have precisely mapped the nervous system of *C. elegans*, revealing that it is relatively simple at only 302 neurons, and that the positioning of these neurons is invariant (White, 1986). In addition, the vast majority of the known molecules that regulate *C. elegans* neurodevelopment are conserved in higher organisms (Margeta et al., 2008). Taken together, these features have made *C. elegans* an excellent model system in which to study the development of the nervous system. In this dissertation, I will highlight two specific areas of the *C. elegans* nervous system that will be used to study synapse formation as well as axon outgrowth: the GABAergic neuromuscular junctions and the soft-touch mechanosensory neurons.

1.3   *C. elegans* **GABAergic synapses**

In *C. elegans* the GABAergic nervous system consists of 26 neurons. Of these

neurons, the VD and DD subsets of motorneurons are required for movement

(McIntire et al., 1993). The VD and DD motor neurons innervate the ventral and

dorsal body wall muscles, respectively, and form multiple synapses along the

length of their axons. These synapses are referred to as *synapse en passant* and

are morphologically similar to GABAergic and glutamatergic synapses found in

the vertebrate CNS. Activation of the VD or DD motorneurons leads to the

release of GABA, causing relaxation of the body wall muscles. The release of

GABA is coordinated with excitatory output on the opposing side of the worm,

resulting in the sinusoidal movement of *C. elegans*.

With the development of Green Fluorescent Protein (GFP) markers, it has

become possible to observe synaptic vesicle proteins in living animals (Chalfie et

al., 1994). Specifically, the construction of synaptobrevin::GFP (SNB-1::GFP) by

M. Nonet allowed for the visualization of synaptic terminals in live *C. elegans*

(Nonet, 1999). By driving SNB-1::GFP expression with a GABAergic motor

neuron specific promoter (*unc-25*), the presynaptic terminals of the VD and DD

motorneurons can be observed. This transgene, P_{unc-25}SNB-1::GFP, also known

as *juIs1*, exhibits uniformly shaped puncta evenly distributed along the length of

the ventral and dorsal nerve cords (Jin et al., 1999) (Figure 1-1). In forward

genetic screens, *juIs1* has been a useful tool in identifying mutants with abnormal

presynaptic terminals, such as *syd-2* (Zhen and Jin, 1999) and *rpm-1* (Zhen et

al., 2000). *juIs1* has also been used to characterize new regulators of synapse

formation such as *fsn-1* (Liao et al., 2004*)* and *glo-4* (Grill et al., 2007).
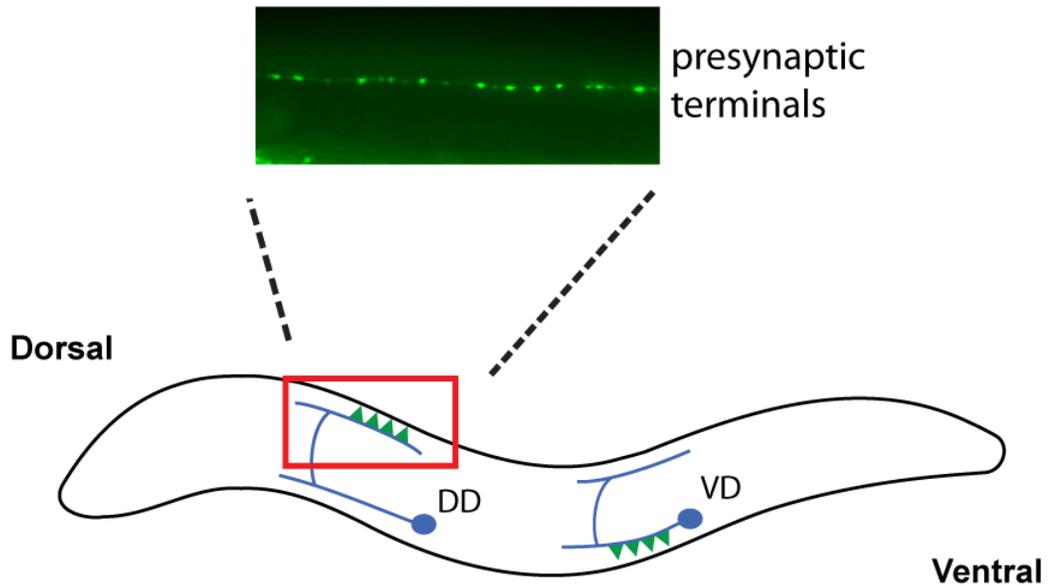
Figure 1-1 *C. elegans* **VD and DD Motorneurons.**
Schematic of D-motor neuron wiring. VD motor neurons receive input from the dorsal
excitatory neurons and release GABA onto ventral body wall muscles. DD motor neurons
receive input from ventral excitatory neurons and release GABA onto dorsal body wall
muscles. The presynaptic terminals of these neurons can be visualized using the transgene
juIs1(P_{unc-25}SNB-1::GFP) (upper image).

1.4 *C. elegans* mechanosensory neurons

In order to sense soft touch, *C. elegans* relies on six touch receptor neurons.

These neurons, known as the mechanosensory neurons, extend long

microtubule filled processes that span a large portion of the animal (Chalfie and

Thomson, 1979). Upon activation, the mechanosensory neurons function in a

variety of behaviors, including locomotion, feeding, and mating.

Due to their morphology and location, the mechanosensory neurons make an excellent system in which to study axon outgrowth and targeting. Specifically, two sets of neurons, the ALM and PLM neurons, extend a long single process that terminates its growth at a precise anatomical location (Figure 1-2). By using a transgenic marker that specifically labels the worm mechanosensory neurons, *muIs32* (P_{mec-7}GFP), mutants can easily be examined for any ALM or PLM targeting or termination abnormalities.
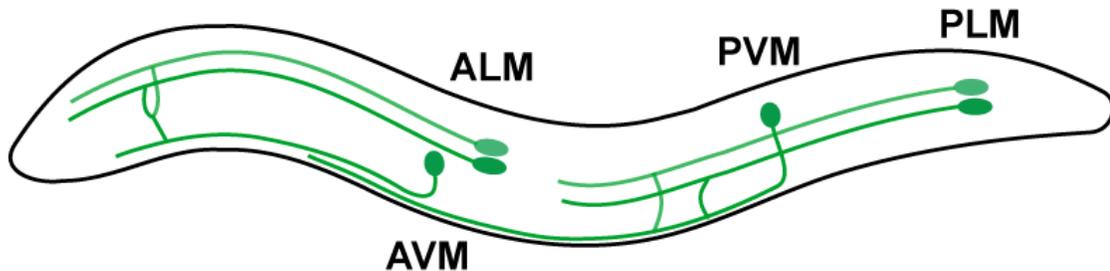
Figure 1-2 *C. elegans* mechanosensory system.
The soft touch response is mediated by six touch receptor neurons (2ALM, 2PLM, AVM, PVM). Each neuron extends a long, microtubule containing process along the length of the animal

1.5 The PHR proteins

Recently, one family of proteins that has emerged as key regulators of axon guidance and outgrowth as well as synapse formation is the Pam/Highwire/RPM-1 (PHR) protein family. The PHR protein family was initially founded with the discovery of human Protein Associated with Myc (Pam) (Guo et al., 1998). Since then, the family has been expanded to include members such as mouse Phr1,

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zebrafish *esrom*, *Drosophila* Highwire, and *C. elegans* RPM-1. A role for PHR proteins in neurodevelopment first emerged from genetic screens in *C. elegans* (Schaefer et al., 2000; Zhen et al., 2000) and *Drosophila* (Wan et al., 2000) looking for mutants with abnormal synapse morphology. In *C. elegans rpm-1* mutants, some neurons overgrow while others display atypical synapse morphology. RPM-1 has also been shown to affect axon regeneration. *rpm-1* mutants show enhanced regeneration of motorneuron axons after axotomy, while overexpression of RPM-1 inhibits axon regeneration (Hammarlund et al., 2009; Nix et al., 2011). *Drosophila* Highwire mutants display an increased number of axon branches with higher numbers of presynaptic terminals. Highwire mutants also show improved regeneration and inhibition of axonal degradation following injury (Xiong et al., 2010; Xiong et al., 2012).

In vertebrates, PHR proteins have also been shown to play a large role in axon guidance. In Zebrafish *esrom* mutants, axons in the retina fail to target to the posterior and instead arborize ectopically (D'Souza et al., 2005). In *Phr1* knockout mice, the phrenic nerve no longer completely innervates the diaphragm (Burgess et al., 2004). Phr1 knockouts also display axon guidance defects with a reduction of axon tracts in the CNS and a reduction of neurites in the cerebral cortex (Bloom et al., 2007). Similar to *Highwire*, loss of Phr1 also results in prolonged survival of injured axons (Babetto et al., 2013).

In invertebrates, PHR expression is limited to the nervous system. In vertebrates, however, PHRs are not as restricted. Pam transcripts are found in

many tissues but are most prevalent in the brain and thymus (Guo et al., 1998).

Zebrafish *esrom* is also widely expressed in both neuronal and non-neuronal

cells (D'Souza et al., 2005). Unlike their invertebrate counterparts, Phr1 and

esrom are required for viability (Karlstrom et al., 1996; Burgess et al., 2004).

While PHR mutants exhibit a diverse range of phenotypes; axon guidance,

axon outgrowth, and synapse formation are all interrelated events that must be

coordinated during development. Thus, a common theme in PHR mutants may

be that neurons fail to transition properly between these various stages of growth

(Po et al., 2010). This could lead to a variety of phenotypes, such as axon

overgrowth or undergrowth, axon oversprouting, and underdeveloped or

overgrown synapses, all depending on the cell type and the surrounding

extracellular cues.

1.6   PHR protein signaling

The involvement of PHR proteins in so many different processes is likely due to

their large size and the presence of multiple conserved domains (reviewed in Po

et al., 2010)). PHR proteins contain an RCC1 like domain with inferred guanine

exchange activity, dual PHR protein specific repeats, a Myc binding domain (in

vertebrates), a RING H2 zinc finger domain (a motif indicative of E3 ubiquitin

ligase activity) (Guo et al., 1998; Wan et al., 2000; Zhen et al., 2000), and a

recently identified RAE-1 binding domain (Grill et al., 2012) (Figure 1-3A).

Through these domains, the PHRs function in multiple signaling pathways.

Studies in *C. elegans, Drosophila,* and mice have shown PHR proteins function as E3 ubiquitin ligases that negatively regulate p38 and JNK MAP kinase cascades (Nakata et al., 2005; Collins et al., 2006; Lewcock et al., 2007). Mammalian PHRs also regulate mTOR signaling by ubiquitinating the Tuberous Sclerosis Complex (Han et al., 2008; Han et al., 2012b). Besides acting as an ubiquitin ligase, other PHR protein signaling partners include adenylate cyclase (Scholich et al., 2001), myc (Guo et al., 1998), a Rab GEF GLO-4 (Grill et al., 2007), microtubule binding protein RAE-1 (Grill et al., 2012) and a neuronal K+ Cl- transporter KCC2 (Garbarini and Delpire, 2008).
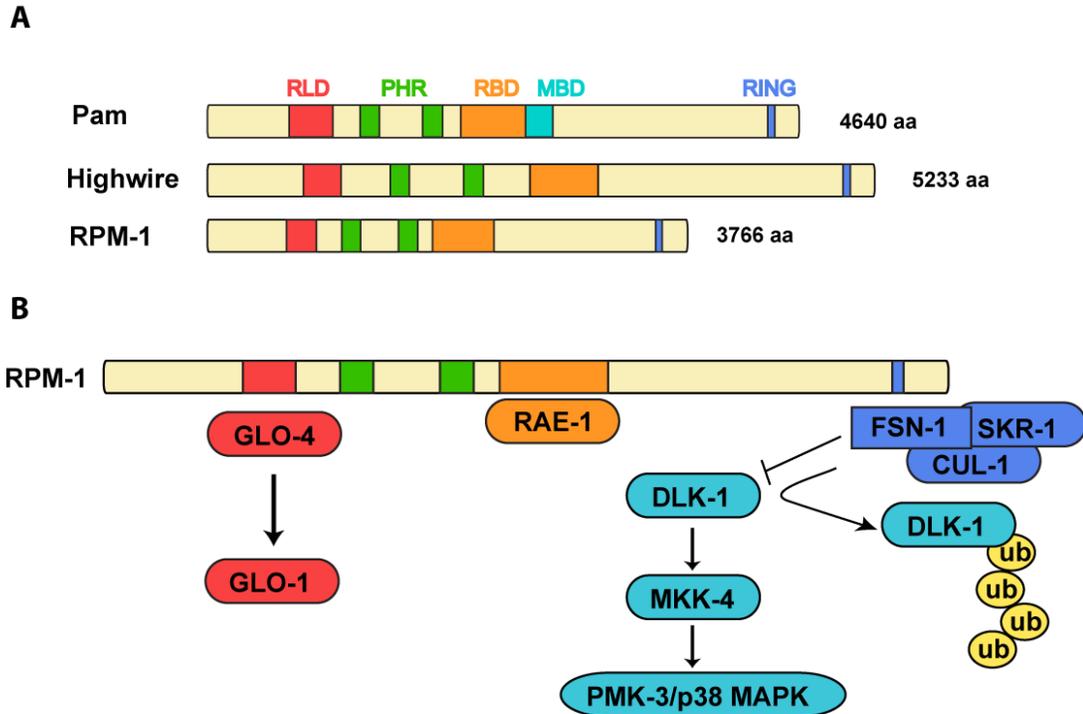
Figure 1-3 The PHR Proteins
(A) Diagram of conserved domains in the PHR family members Pam, Highwire, and RPM-1.
RLD: RCC1 like domain. PHR: PHR repeats. RBD: Rae-1 binding region. MBD: Myc binding
domain. RING: RING-H2 finger domain. (B) RPM-1 functions through multiple signaling
pathways to regulate neurodevelopment. It is a positive regulator of the glo-4 pathway,
consisting of the Guanine Nucleotide Exchange Factor (GEF) GLO-4 and the Rab GTPase
GLO-1. RPM-1 also forms an Skip-Cullin-F-box (SCF) ubiquitin ligase complex with the F-box
protein FSN-1, which negatively regulates a p38 MAPK cascade.

Based on the importance PHR proteins play in regulating

neurodevelopment, finding additional molecules that function in their signaling

pathways is an important step in learning how synapses are made. While the list

of interacting proteins is already large, biochemical and genetic evidence

suggests that there are still more molecules to be discovered and characterized.

To find these additional players, I have focused my work on the *C. elegans* PHR protein RPM-1.

1.7   C. elegans RPM-1

The *C. elegans* PHR protein, Regulator of Presynaptic Morphology (RPM)-1, was first identified in screens for mutants with synaptic and axon morphology defects (Schaefer et al., 2000; Zhen et al., 2000). In *rpm-1* loss of function (lf) mutants, synapses viewed with the transgene *juIs1* showed a disorganized appearance, with some regions of the dorsal cord containing no visible transgene, while others possess large accumulations of synapses (Zhen et al., 2000). Schaefer et. al. concurrently showed that mechanosensory neuron synapses were also abnormally patterned. Additionally, using the *muIs32* transgene, they showed that the morphology of the mechanosensory neurons was abnormal, with the axons of the ALM and PLM neurons routinely growing past their normal termination points.

RPM-1 is known to regulate neurodevelopment through a number of signaling pathways (Figure 1-3B). In one of the most studied pathways, RPM-1 forms an SCF-like ubiquitin ligase complex with the F-box protein FSN-1 (Liao et al., 2004). A major target of this complex is DLK-1, a component of a MAPK cascade (Nakata et al., 2005). RPM-1 also binds to GLO-4, a guanine exchange factor that regulates GLO-1, a Rab GTPase (Grill et al., 2007). Finally, RPM-1

was shown to bind to and function upstream of the microtubule binding protein RAE-1 (Grill et al., 2012).

In order to identify novel molecules involved in *rpm-1* signaling, I used a genetic as well as a biochemical approach. First, genetic and transgenic work suggested the existence of additional regulators of the *dlk-1* MAP kinase cascade. Here I provide evidence that *ppm-1*, a serine/threonine phosphatase homologous to human PP2Ca(PPM1A) and PP2Cb(PPM1B) acts as a second negative regulatory mechanism to control the *dlk-1* pathway. I show that *ppm-1* functions through its phosphatase activity in a parallel genetic pathway with *glo-4* and *fsn-1* to regulate both synapse formation in the GABAergic motorneurons, and axon termination in the mechanosensory neurons. My transgenic analysis shows that *ppm-1* acts downstream of *rpm-1* to negatively regulate the *dlk-1* pathway, with *ppm-1* most likely acting at the level of *pmk-3*. This study provides insight into the negative regulatory mechanisms that control the *dlk-1* pathway in neurons, and demonstrates a new role for the PP2C/PPM phosphatases as regulators of neuronal development.

In addition, this dissertation describes my work to characterize the novel RPM-1 binding protein ANC-1. I show that *anc-1*, like *rpm-1*, regulates axon termination and synapse formation. My genetic analysis also indicates that *anc-1* functions through the β-catenin *bar-1*, and that the *anc-1/bar-1* pathway functions cell autonomously, downstream of *rpm-1* to regulate neuronal development. This study highlights a new role for ANC-1 in neuronal development and unveils a

new mechanism by which RPM-1 functions. Furthermore, my findings represent the first genetic link between RPM-1 and a pathway that is regulated by extracellular signals, such as Wnts.

2 PPM-1, a PP2Cα/β phosphatase, regulates axon termination and synapse formation in *C. elegans*

Content adapted from published article: **Tulgren ED**, Baker ST, Rapp L, Gurney AM, Grill B (2011) PPM-1, a PP2Calpha/beta phosphatase, regulates axon termination and synapse formation in Caenorhabditis elegans. Genetics 189:1297-1307.

2.1   Introduction

The Pam/Highwire/RPM-1 (PHR) proteins are key regulators of neuronal development that function in synapse formation, axon termination and guidance, axon regeneration, and glutamate receptor trafficking (Schaefer et al., 2000; Wan et al., 2000; Zhen et al., 2000; Burgess et al., 2004; D'Souza et al., 2005; Lewcock et al., 2007; Li et al., 2008a; Hammarlund et al., 2009; Park et al., 2009; Po et al.). The PHR protein family includes: human Pam, mouse Phr1, zebra fish esrom/Phr1, *Drosophila* Highwire and *C. elegans* Regulator of Presynaptic Morphology (RPM)-1.

PHR proteins function through multiple downstream signaling pathways. In *C. elegans*, RPM-1 functions as part of an ubiquitin ligase complex that includes the F-box protein, F-box Synaptic Protein (FSN)-1 (Liao et al., 2004). This complex negatively regulates a MAP kinase cascade that includes *dual leucine zipper-bearing kinase (dlk-1)*, *map kinase kinase (mkk)-4*, *p38 map kinase (pmk)-3*, *map kinase activated protein kinase (mak)-2*, and the transcription factor *cebp-1* (Nakata et al., 2005; Yan et al., 2009). *Drosophila* Highwire and mouse Phr1 negatively regulate the ortholog of DLK-1 through a similar mechanism (Wu et al., 2007; Saiga et al., 2009; Tada et al., 2009; Nakata et al., 2005; Collins et al., 2006; Lewcock et al., 2007). Phr1 also ubiquitinates and negatively regulates the Tuberin Sclerosis Complex (Murthy et al., 2004; D'Souza et al., 2005; Han et al., 2008). RPM-1 positively regulates signaling through a Rab GTPase pathway by binding to Gut Granule Loss (GLO)-4 (Grill et al., 2007).

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While RPM-1 negatively regulates the DLK-1 pathway, there are a number of reasons to suspect that the DLK-1 pathway may also be controlled by other negative regulatory mechanisms. First, overexpression of *dlk-1* causes more dramatic phenotypes than *rpm-1* loss of function (lf), including uncoordinated movement and small body size (Nakata et al., 2005; Abrams et al., 2008). Second, the *dlk-1* pathway consists of 5 signaling molecules providing numerous points where regulation might occur. Third, ubiquitination is a relatively slow acting mechanism to restrict DLK-1 signaling. The observation that UEV-3 is a possible positive regulator of PMK-3 (Trujillo et al., 2010) further supports the idea that multiple mechanisms may control the DLK-1 pathway.

There is a large body of evidence that MAP kinases are negatively regulated by phosphatases including MAP kinase-specific phosphatases, and broad acting PP2C/PPM family phosphatases (Lu and Wang, 2008; Shi, 2009; Bermudez et al.). While MAP kinases are known to function in neurons (Ji et al., 2009; Samuels et al., 2009), the negative regulatory phosphatases that control MAP kinase signaling in neurons remain relatively poorly understood.

Here we provide evidence that neuronal development is regulated by a PP2C/PPM family phosphatase from *C. elegans* that we call *protein phosphatase* mg^{2+}/mn^{2+} *dependent (ppm)-1*. We have found that *ppm-1* acts through its phosphatase activity to regulate axon termination and synapse formation by acting in a parallel genetic pathway to *fsn-1* and *glo-4*. Loss of function in *ppm-1* is suppressed by loss of function in *pmk-3* (p38 MAPK), suggesting that *ppm-1*

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negatively regulates *pmk-3* activity. This finding is consistent with our observation that *ppm-1* functions downstream of *rpm-1*. Overall, our observations demonstrate that the DLK-1 pathway is negatively regulated by at least two mechanisms in neurons: the action of an SCF complex that includes RPM-1 and FSN-1, and the activity of a serine/threonine phosphatase, PPM-1.

2.2   Results

2.2.1   A PP2Cα/β phosphatase, PPM-1, regulates axon termination in the mechanosensory neurons of *C. elegans*.

While the DLK-1 pathway is negatively regulated by RPM-1, several observations suggest that phosphatases of the PP2C/PPM family may also inhibit the DLK-1 pathway. Biochemical experiments *in vitro* and in mammalian cell culture have shown that PP2Cα and β (also called PPM1A and B) can dephosphorylate and negatively regulate MKKKs, MKKs, and MAPKs (Takekawa et al., 1998; Hanada et al., 2001). In yeast, the homologs of PP2Cα negatively regulate the activity of High-osmolarity glycerol (Hog)1, the homolog of p38 MAPK (Maeda et al., 1994; Jacoby et al., 1997; Nguyen and Shiozaki, 1999; Saito and Tatebayashi, 2004). Further, PP2Cα functions in mammalian neurons to control calcium flux (Li et al., 2005) suggesting that PP2C phosphatases may have undiscovered roles in neuronal development.

The *C. elegans* genome contains a single gene, *temporarily assigned gene name (tag)-93 (F25D1.1)*, whose protein product is conserved with two

PP2C phosphatases in humans, PP2Cα/PPM1A and PP2Cβ/PPM1B (49% identity and 69% conservation with PPM1A; 54% identity and 72% conservation with PPM1B) (Stern et al., 2007) (Figure 2-1A and B). PP2C/PPM phosphatases are single subunit enzymes that require magnesium/manganese for activity, and consist of a catalytic domain and a regulatory domain (Lu and Wang, 2008; Shi, 2009) (Figure 2-1B). Based on this homology, we have renamed *tag-93* as *protein phosphatase magnesium^{2+}/manganese^{2+} dependent (ppm)-1*.

There are three open reading frames of *ppm-1* that are predicted in the *C. elegans* genome (*F25D1.1a, F25D1.1b*, and *F25D1.1c*) (www.wormbase.org). One of these open reading frames, *F25D1.1c*, encodes a 385 amino acid protein that has a conserved start site with mammalian PP2Cα/PPM1A (data not shown and Figure 2-1A and B). Using RT-PCR, we confirmed the coding sequence of *F25D1.1c*, and we used this transcript as our frame of reference for analysis of two alleles of *ppm-1*, *ok578* and *tm653*. We sequenced the lesion in *ok578* and found that it deletes 984 base pairs and inserts two thymidine bases, which causes a frameshift and leads to loss of wild-type sequence after amino acid 69. Importantly, *ok578* deletes a residue (D246) that when mutated in mammalian PP2Cα/PPM1A results in a 4000-fold drop in phosphatase activity (Jackson et al., 2003) (Figure 2-1B). Sequencing of *tm653* confirmed it has a 1089 base pair deletion in the *ppm-1* gene that deletes a portion of the promoter, the start codon, and the first 156 amino acids of PPM-1 including a key catalytic residue (Figure 2-1B). These observations show that *ok578* and *tm653* are molecular null alleles.

A

F25D1.1a
F25D1.1b
F25D1.1c
ok578
tm653

1 Kb

B

human PP2Calpha — D60, D239, Catalytic domain, regulatory domain, 382 aa
F25D1.1a — D145, D329, 468 aa
F25D1.1b — 367 aa
F25D1.1c — D62, D246, 385 aa
ok578 — stop
tm653 — stop

C

wt

rpm-1 (big hook)

rpm-1 (short hook)

ppm-1(ok578)

ALM

% total mutant ALM

% mutant ALM

D

% total mutant ALM

wt, ppm-1 (tm653), ppm-1 (ok578), ok578/tm653, glo-1, glo-4, fsn-1, glo-1; fsn-1, fsn-1; glo-4, glo-1; ppm-1, glo-4, ppm-1 (ok578), fsn-1; ppm-1 (tm653), fsn-1; ppm-1 (ok578), fsn-1; ok578/ tm653, glo-1; fsn-1; ppm-1, rpm-1, ppm-1, rpm-1, rpm-1 25°C

E

short hook
big hook

% mutant ALM

wt, ppm-1 (tm653), ppm-1 (ok578), ok578/tm653, glo-1, glo-4, fsn-1, glo-1; fsn-1, fsn-1; glo-4, glo-1; ppm-1, glo-4, ppm-1 (ok578), fsn-1; ppm-1 (tm653), fsn-1; ppm-1 (ok578), fsn-1; ok578/ tm653, glo-1; fsn-1; ppm-1, rpm-1, ppm-1, rpm-1, rpm-1 25°C

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Figure 2-1. *ppm-1* regulates axon termination in the ALM neurons.
A) Shown is a schematic of the exons (hatched boxes) and introns of three open reading frames predicted for *ppm-1* by wormbase. The deletions caused by *ok578* and *tm653* are shown below. *B)* A schematic of the human PP2Ca protein, and predicted PPM-1 proteins with the catalytic phosphatase domain (grey), and the regulatory domain (hatched design) highlighted. Shown below are segments of PPM-1 deleted by *ok578* and *tm653*. Mutagenesis studies on human PP2Ca showed that mutation of residue D60 and D239 lead to 900 and 4000-fold decreases in phosphatase activity, respectively (Jackson et al., 2003). The corresponding conserved residues in PPM-1, D62 and D246 (F25D1.1c), are shown. *C)* Axon termination defects in the ALM mechanosensory neuron were visualized using muIs32[P$_{mec7}$GFP] for wild-type or the indicated mutant genotypes. Images correspond to the boxed region of the diagram. *D)* Quantitation of the total axon termination defects in ALM neurons. *E)* Quantitation of specific, short hook (white) or big hook (grey), axon termination defects in the ALM neurons of the indicated genotypes. Note that the percentage of ALM axons that are normal/wild-type for each genotype is not shown. Scale bar is 10 mm. Error bars represent the standard error of the mean. Significance was determined using an unpaired t test . *p<0.05, ** p<0.01

Defects in *rpm-1* (lf) mutants are due, in part, to excess signaling through the *dlk-1* pathway (Nakata et al., 2005; Grill et al., 2007; Trujillo et al., 2010). We hypothesized that *ppm-1* (lf) might also increase signaling through the *dlk-1* pathway, and result in phenotypes that are similar to *rpm-1* (lf) mutants. To test this hypothesis, we first analyzed the mechanosensory neurons of *ppm-1* (lf) mutants.

In wild-type animals, the two ALM mechanosensory neurons each have a single axon that terminates at a precise location (Figure 2-1C). In contrast, axon termination is defective in the ALM neurons of *rpm-1* (lf) mutants (Figure 2-1C and D). *rpm-1* (lf) mutants display two types of ALM axon termination defects: less severe short hooks, and more severe big hooks where the axon overextends and hooks towards the posterior of the animal (Figure 2-1C and E). In *rpm-1* (lf) mutants, big hooks in the ALM are the predominant phenotype, and this phenotype is temperature sensitive for the *ju44* allele of *rpm-1* (Figure 2-1E). In

ppm-1(ok578) and *ppm-1(tm653)* mutants, we observed small hook defects in the ALM neurons that occurred with low penetrance (Figure 2-1 C, D and E).

Previous studies showed that *rpm-1* (lf) mutants also have defects in the PLM neurons which fall into two main phenotypic categories: 1) axon termination defects (Figure 2-2A) and 2) synaptic branch extension/stabilization defects (Figure 2-3A) (Schaefer et al., 2000; Grill et al., 2007). A given PLM neuron can display one or both of these phenotypes. In *rpm-1* (lf) mutants, a small percentage of PLM axons (8%) display a milder axon termination phenotype in which the PLM axon only overextends beyond the ALM cell body (Figure 2-2A ,overextension). The majority of PLM neurons in *rpm-1* (lf) mutants (90%) display a more severe phenotype in which the PLM axon overextends beyond the ALM cell body and also hooks ventrally, which we will refer to as hooking for ease of discussion (Figure 2-2A hook). Both the hooking and synaptic branch defects in *rpm-1* (lf) mutants are highly penetrant (Figure 2-2B and 2-3B). In *ppm-1(ok578)* mutants, we observed both axon termination phenotypes with a larger percentage of neurons showing the milder overextension phenotype, and a small percentage of neurons showing the more severe hooking phenotype (Figure 2-2A and B). Similar results were obtained for *ppm-1(tm653)* (Figure 2-2B). With regard to synaptic branch extension, *ppm-1(lf)* animals had defects in synaptic branch extension that were very low penetrance (Figure 2-3A and B). These results show that *ppm-1* regulates axon termination in the mechanosensory neurons.
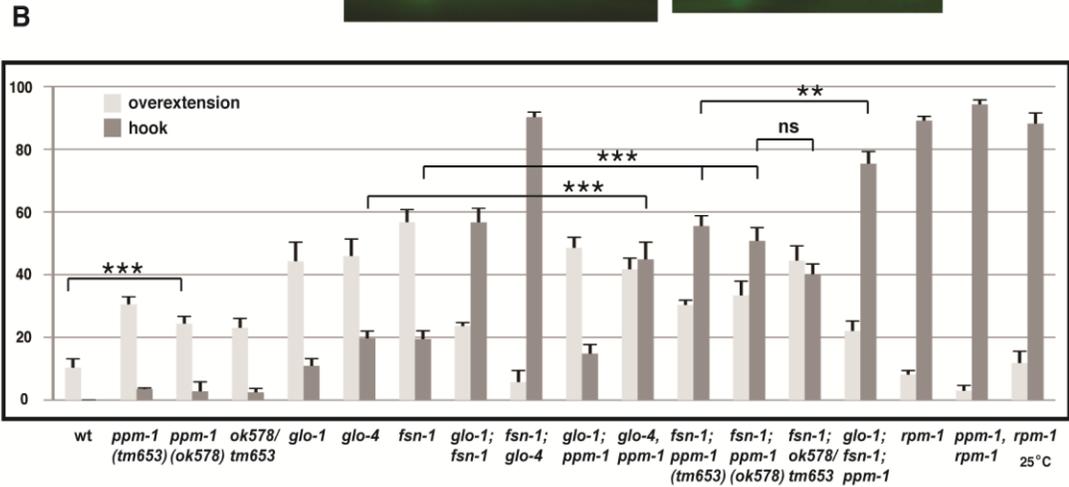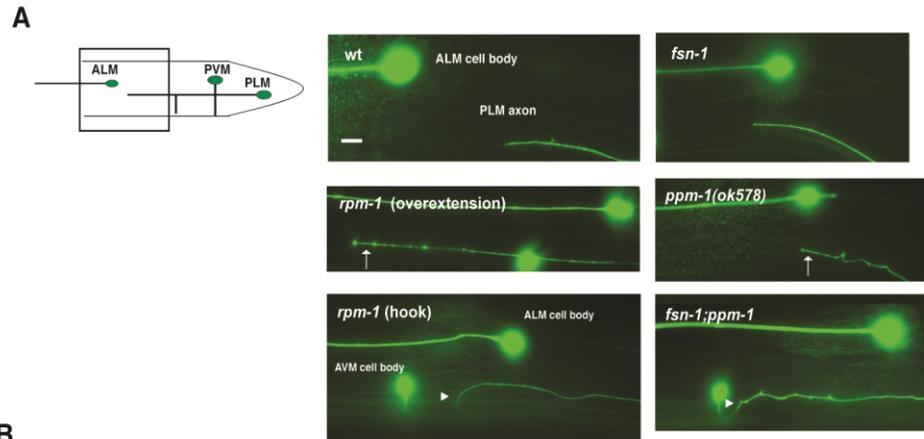
Figure 2-2. *ppm-1* **regulates axon termination in the PLM neurons.**
A) Axon termination defects in the PLM neurons were visualized using muIs32[P$_{mec7}$GFP] for wild-type or the indicated mutant genotypes. Images correspond to the boxed region of the diagram. Arrows mark overextension of the PLM axon beyond the ALM cell body, and arrowheads mark the more severe phenotype of overextension and hooking of the PLM axon. *B)* Quantitation of the percentage of PLM neurons that only overextend (white), or overextend and hook (grey) for the indicated genotypes. Note that the percentage of PLM axons that are wild-type for each genotype is not shown. Analysis was performed on young adults grown at 23°C, unless otherwise specified. Scale bar is 10 mm. Error bars represent the standard error of the mean. Significance was determined using an unpaired t test where n represents the number of independent counts of 10-30 worms for a given genotype. *** p<0.001, ns=not significant

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Figure 2-3. ***ppm-1* regulates synaptic branch extension/stabilization in PLM neurons.**
A) Defects in synaptic branch extension in the PLM neurons were visualized using
muIs32[P$_{mec7}$GFP] in wild-type or the indicated mutant genotypes. Images correspond to the
boxed region of the diagram. The arrowhead highlights the absence of the synaptic branch. *B)*
Quantitation of the defects in synaptic branch extension in the PLM neurons for the indicated
genotypes. Note that the percentage of PLM axons that are wild-type for each genotype is not
shown. Analysis was performed on young adults grown at 23°C, unless otherwise specified.
Scale bar is 10 mm. Error bars represent the standard error of the mean. Significance was
determined using an unpaired t test where n represents the number of independent counts of 10-
30 worms for a given genotype. * p<0.05, *** p<0.001, ns=not significant

2.2.2   *ppm-1* functions in parallel to *fsn-1* and *glo-4* to regulate axon termination.

rpm-1 has two major downstream signaling activities that are known. *rpm-1*

functions with *fsn-1* to negatively regulate the *dlk-1* pathway (Liao et al., 2004),

and binds to GLO-4 to positively regulate the *glo* pathway which includes: *glo-4*,

glo-1 (a Rab GTPase), and *apm-3* (Grill et al., 2007). To determine if *ppm-1*

functions in either of these pathways or as part of an independent pathway, we

constructed double mutants between *ppm-1* and *fsn-1*, *glo-4*, and *glo-1*. When

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total mutant neurons were analyzed, *fsn-1;ppm-1* double mutants had an additive phenotype in the ALM neurons (Figure 2-1B). However, an enhanced penetrance of big hooks was observed in *fsn-1;ppm-1* double mutants compared to single mutants (Figure 2-1E). Both *ppm-1(ok578)* and *ppm-1(tm653)* had similar enhancer effects with *fsn-1* (lf) (Figure 2-1E). While the level of big hooks was mildly increased in both *glo-4,ppm-1* and *glo-1;ppm-1* double mutants, these differences were not statistically significant demonstrating that *ppm-1* does not enhance the *glo* pathway in the ALM neurons (Figure 2-1E).

With regard to the PLM neurons, *fsn-1;ppm-1* double mutants had an enhanced percentage of neurons that show the hooking phenotype (Figure 2-2B). The penetrance of synaptic branch defects was also strongly enhanced in *fsn-1;ppm-1* double mutants (Figure 2-3B). Both alleles of *ppm-1* (*ok578* or *tm653*) gave similar levels of enhancement with *fsn-1*. The axon termination (hooking) and synaptic branch extension defects in the PLM neurons of *glo-4,ppm-1* double mutants were also enhanced (Figure 2-2B and 2-3B). In contrast, axon termination and branch extension defects in the PLM neurons of *glo-1*;*ppm-1* double mutants and *glo-1;fsn-1;ppm-1* triple mutants were increased, but not enhanced (Figure 2-2B and 2-3B). *fsn-1;glo-4,ppm-1* triple mutants were not analyzed as *fsn-1;glo-4* double mutants have maximal phenotypes (Figure 2-2B and 2-3B) (Grill et al., 2007).

To test if the two alleles of *ppm-1* analyzed were null mutants, we performed transheterozygous analysis. With regard to the PLM neurons, *fsn-*

1;ok578/tm653 mutants showed enhanced penetrance of defects in axon termination and synaptic branch extension (Figure 2-2B and 2-3B) that were not significantly different from *fsn-1;ppm-1(ok578)* and *fsn-1;ppm-1(tm653)* double mutants. These results are consistent with *ok578* and *tm653* acting as null alleles of *ppm-1* in the PLM neurons. In the ALM neurons, *fsn-1;ok578/tm653* had an increased percentage of neurons showing big hooks compared to *fsn-1* single mutants, however levels were not increased to the same extent as *fsn-1;ppm-1(ok578)* and *fsn-1;ppm-1(tm653)*. Thus, in the ALM neurons the genes used as visible markers to generate *fsn-1;ok578/tm653* animals (*dpy-11* and *unc-42*) may affect *ppm-1*, or the ALM neurons may be less sensitive to *ppm-1* loss of function.

Overall, our data are consistent with a model in which *ppm-1* functions in a parallel pathway to *fsn-1* and *glo-4* to regulate axon termination and synaptic branch extension/stabilization in the PLM mechanosensory neurons.

2.2.3 *ppm-1* regulates synapse formation in GABAergic motor neurons.

Previous studies have shown that *rpm-1* regulates synapse formation in the motor neurons (Zhen et al., 2000; Nakata et al., 2005). The presynaptic terminals formed by the GABAergic DD motor neurons can be visualized with Synaptobrevin (SNB)-1 fused to GFP (GFP::SNB-1). In wild-type animals, GFP::SNB-1 puncta of the DD neurons are evenly distributed along the dorsal nerve cord (Figure 2-4A). In contrast, *rpm-1* (lf) mutants have disorganized

GFP::SNB-1 puncta with gaps and aggregation (Figure 2-4A). While *ppm-1* (lf) animals are normal, *fsn-1;ppm-1* and *glo-4,ppm-1* double mutants are enhanced with significant disorganization of GFP::SNB-1 puncta (Figure 2-4A) and reduced numbers of SNB-1::GFP puncta (Figure 2-4B). *glo-1;ppm-1* double mutants had reduced numbers of SNB-1::GFP puncta, but defects were too mild to constitute enhancement (Figure 2-4B, p<0.05). We also observed that *ppm-1,rpm-1* double mutants have similar defects as those in *rpm-1* (lf) mutants. Importantly, synapse formation defects in *rpm-1* (lf) mutants are not saturated, as defects become significantly worse in *rpm-1;syd-2* double mutants (Liao et al., 2004). Therefore, our observations are consistent with *ppm-1* regulating synapse formation by functioning in the same genetic pathway as *rpm-1*, and a parallel genetic pathway to *fsn-1* and *glo-4*.
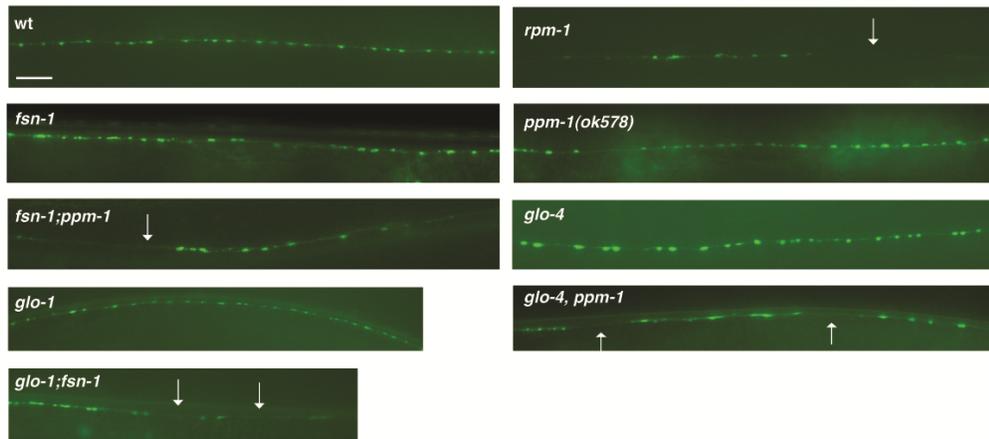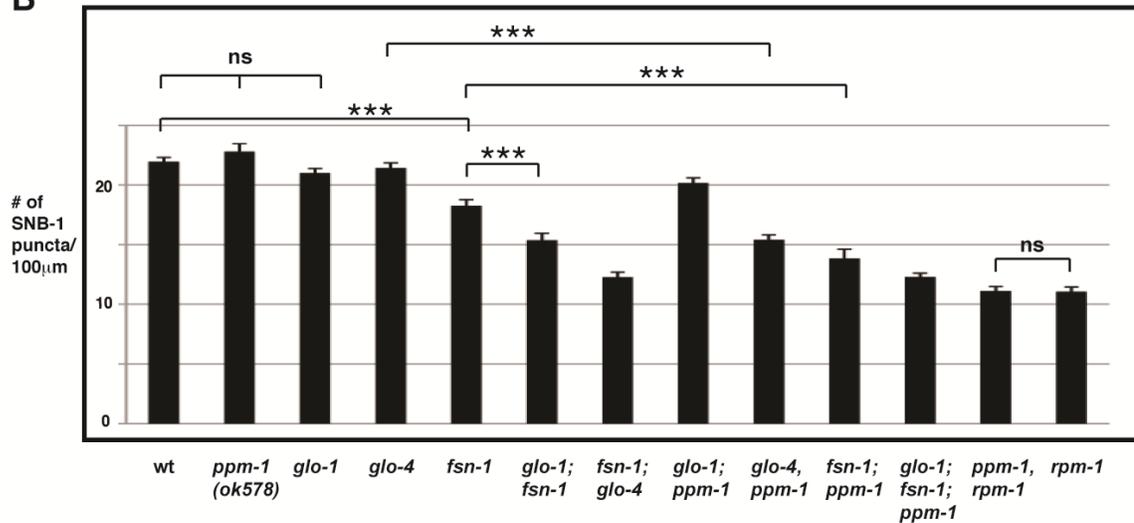
Figure 2-4. *ppm-1* regulates synapse formation in GABAergic motor neurons.
A) Presynaptic terminals of DD neurons were visualized using *juIs1*[P_{unc-25}SNB-1::GFP] in wild-type or mutant genotypes. Arrows highlight the gaps between presynaptic puncta in the dorsal cord. *B)* Quantitation of the average number of SNB-1::GFP puncta per 100μm of dorsal cord. Analysis was performed on young adults grown at 25°C. Scale bar is 10 mm. Significance was determined using a Student's t test, and error bars represent the standard error of the mean. *** p<0.001, ns = not significant.

2.2.4 *ppm-1* **functions in mechanosensory neurons downstream of** *rpm-1*.

rpm-1 and its downstream signaling molecules, *glo-4* and *glo-1*, act cell

autonomously in mechanosensory neurons (Schaefer et al., 2000; Grill et al.,

2007). To determine if *ppm-1* functions cell autonomously, we performed

transgenic rescue experiments in which *ppm-1* expression was driven by

different promoters. Transgenic expression of *ppm-1* using either a fosmid

(native *ppm-1* promoter), P*rgef-1* (a pan-neuronal promoter) or P*mec-7* (a

mechanosensory neuron promoter) rescued PLM axon termination defects in

both *ppm-1* single mutants and *fsn-1;ppm-1* double mutants (Figure 2-5A).

Previous studies identified a single point mutation (D239N) that results in a 4000-

fold decrease in phosphatase activity in human PP2Cα (Jackson et al., 2003)

(Figure 2-1A). *ppm-1* that is mutated at the corresponding residue (D246N), and

presumably catalytically inactive, did not rescue the enhanced axon termination

defects in *fsn-1;ppm-1* double mutants (Figure 2-5A). These results demonstrate

that *ppm-1* regulates axon termination through its phosphatase activity, and that

the lesion in *ok578* causes the enhanced penetrance of axon termination defects

observed in *ppm-1;fsn-1* double mutants.

Our genetic analysis described earlier indicates that *ppm-1* functions in

the same genetic pathway as *rpm-1*. To determine if *ppm-1* functions up or

downstream of *rpm-1*, we generated transgenic animals that overexpress PPM-1

in the mechanosensory neurons of *rpm-1* (lf) mutants. Overexpression of PPM-1

strongly, but partially, reduced the axon termination defects in *rpm-1* (lf) mutants

(Figure 2-5B). In contrast, phosphatase-dead PPM-1 did not rescue axon

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termination defects in *rpm-1* (lf) mutants (Figure 2-5B). These results are

consistent with *ppm-1* functioning as a phosphatase that acts downstream of

rpm-1.
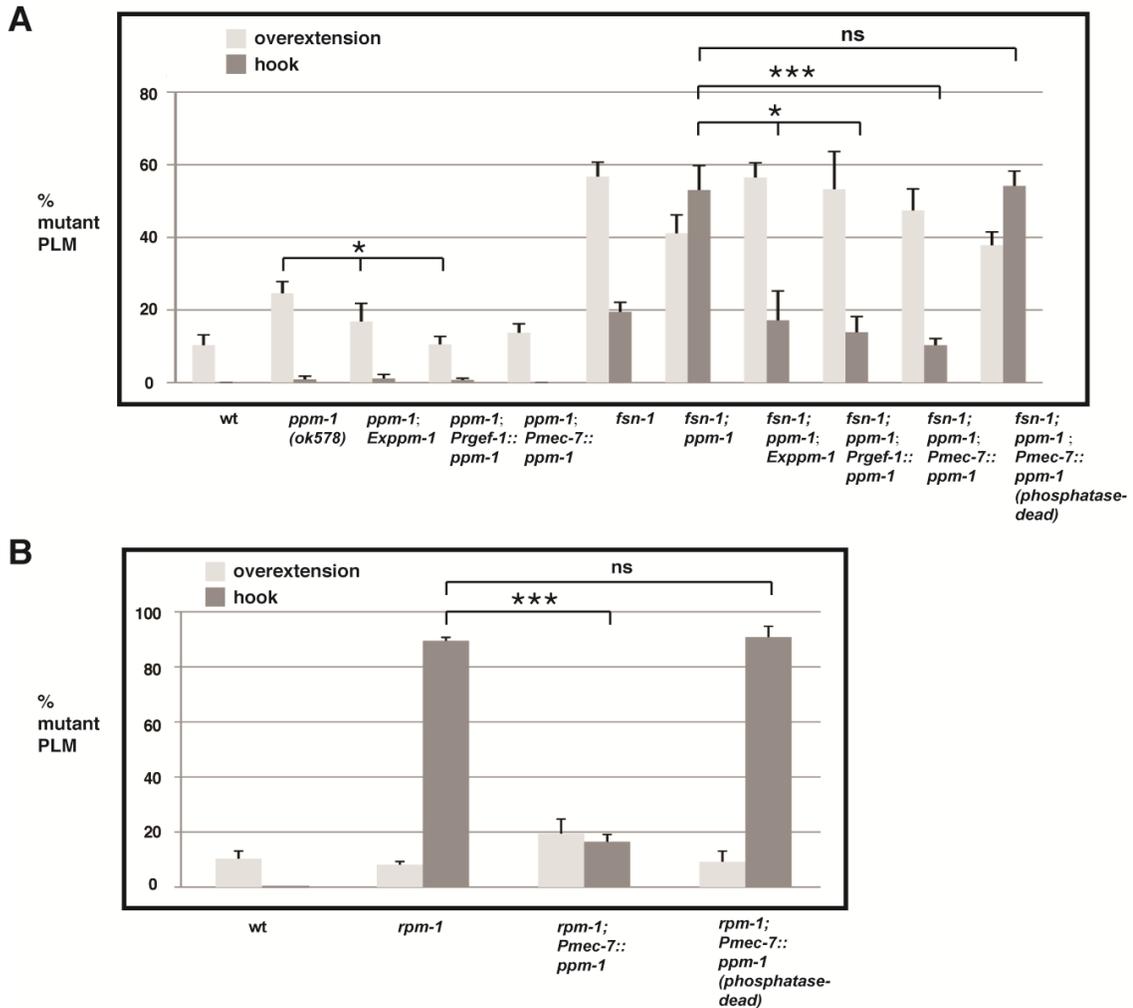
Figure 2-5 Transgenic expression of *ppm-1* in the mechanosensory neurons rescues *ppm-1* (lf) defects in axon termination.
A) Recue of axon termination defects in the PLM neurons of *ppm-1* or *fsn-1;ppm-1* mutants by transgenic expression of PPM-1 using the indicated promoters. The percentage of PLM axons that overextend only (white), or overextend and hook (grey) are shown. The percentage of PLM neurons with normal axon termination is not shown. For all transgenes, the data shown is from two to three transgenic lines, except for the fosmid wrm613bH10 (*Exppm-1*) in which only one transgenic line was analyzed. *B)* Axon termination defects in *rpm-1* (lf) mutants are partially rescued by transgenic expression of PPM-1, but not by transgenic expression of phosphatase-dead PPM-1 (D246N). The data shown is from 2-3 transgenic lines for each genotype. Analysis was performed on young adults grown at 23°C. Error bars represent the standard error of the mean. Significance was determined using an unpaired t test where n represents the number of independent counts of 10-30 worms for a given genotype. *p<0.05, *** p<0.001

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2.2.5   PPM-1 negatively regulates the DLK-1 pathway.

Previous studies have shown that the orthologs of PPM-1 can act at the level of

MKKK, MKK or MAPK to negatively regulate a kinase cascade (Takekawa et al.,

1998; Hanada et al., 2001). These observations suggested that phenotypes in

ppm-1 (lf) mutants might be due to excess activation of *dlk-1*, *mkk-4* and/or *pmk-3*. To address this question, we employed two experimental strategies. First, we

used a transgenic approach to test if *ppm-1* negatively regulates the DLK-1

pathway. Overexpression of DLK-1 resulted in similar severity and penetrance of

phenotypes as those seen in *rpm-1* (lf) mutants including axon termination

defects in the ALM (Figure 2-6A) and PLM neurons (Figure 2-6B), and defects in

synaptic branch extension in the PLM neurons (data not shown). Coexpression

of PPM-1, but not phosphatase-dead PPM-1, partially rescued the defects

caused by overexpression of DLK-1 (Figure 2-6A and B). This observation

suggests that PPM-1 acts as a phosphatase to negatively regulate the DLK-1

pathway.

   Next, we used a genetic approach to determine which kinase in the DLK-1

pathway might be a target of PPM-1's phosphatase activity. To do so, we

constructed double mutants of *ppm-1* with *dlk-1*, *mkk-4*, or *pmk-3*. Axon

termination defects (overextension) in the PLM neurons were analyzed for

double mutants and compared to *ppm-1* (lf) single mutants. Defects in axon

termination were not rescued in *dlk-1;ppm-1* and *mkk-4;ppm-1* double mutants

(Figure 2-6C). In contrast, axon termination defects in *pmk-3;ppm-1* double

mutants were significantly reduced compared to *ppm-1* (lf) single mutants (Figure

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2-6C). These observations are consistent with excess *pmk-3* function leading to axon termination defects in *ppm-1* (lf) mutants.

A
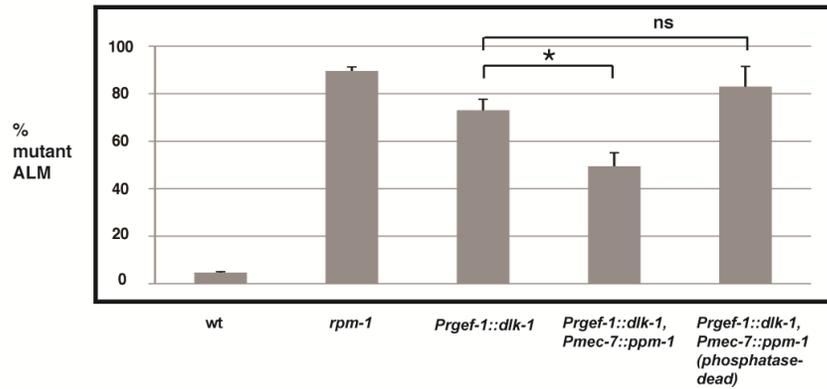
B
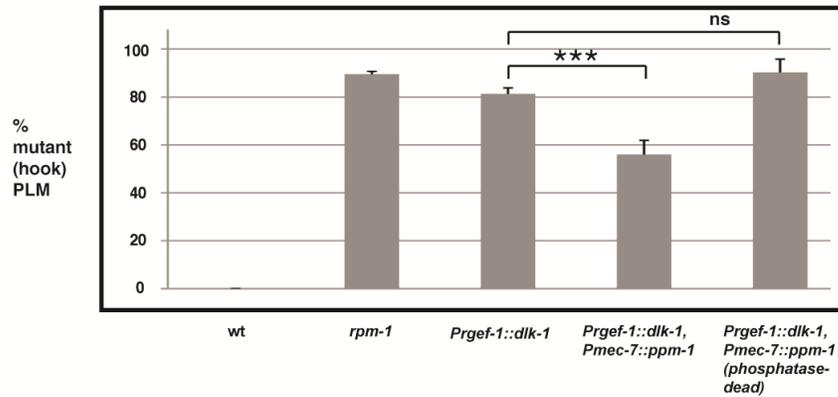
C
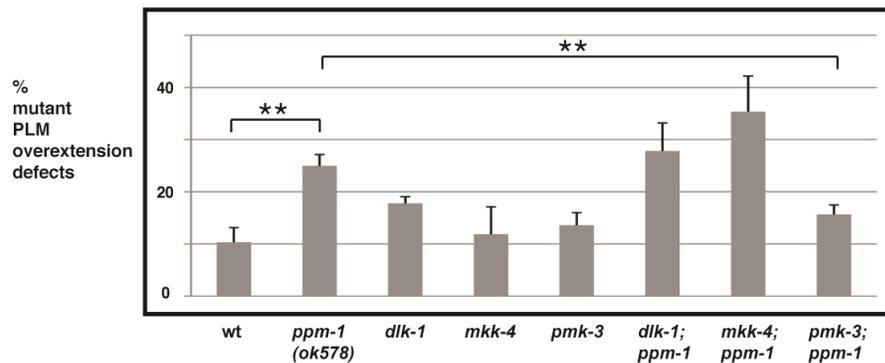
Figure 2-6. *ppm-1* negatively regulates the *dlk-1* pathway.
Transgenic expression of DLK-1 causes axon termination defects in *A)* ALM neurons, and *B)*
PLM neurons, which is suppressed by coexpression of PPM-1, but not phosphatase-dead
PPM-1 (D246N). The data shown is pooled from three or more transgenic lines, except the
data for phosphatase-dead PPM-1, which is from a single transgenic line. The percentage of
PLM axons that hook are shown. The percentage of PLM neurons that overextend or are wild-
type are not shown. *C)* Analysis of suppression of *ppm-1* mutant phenotypes by loss of
function in *dlk-1*, *mkk-4* and *pmk-3*. Quantitation of axon termination defects (overextension
only) in the indicated mutant genotypes. Note that only *pmk-3-/-;ppm-1-/-* mutant animals
show a significant suppression of defects in axon termination. Analysis was performed on
young adults grown at 23°C. Error bars represent the standard error of the mean. Significance
was determined using an unpaired t test where n represents the number of independent
counts of 10-30 worms for a given genotype. *p<0.05, **<0.005, and *** p<0.001

2.2.6   PPM-1 localizes to the presynaptic terminals of motor neurons.

Given that *ppm-1* functions in neurons, we wanted to test if it is expressed in

neurons. To address this question, we generated transgenic animals in which the

3.7 kb promoter of *ppm-1* drives expression of GFP. P*ppm-1*::GFP is expressed

in neurons of the nerve ring, and motor neurons of the ventral nerve cord (Figure

2-7A). Since *ppm-1* is expressed in motor neurons, we used a transgenic

approach to study the subcellular distribution of PPM-1 in the GABAergic DD and

VD motor neurons. We generated transgenic animals that express a fusion

protein of mCherry and PPM-1, and a fusion protein of GFP and SNB-1, a

synaptic vesicle membrane protein. SNB-1::GFP localizes to presynaptic puncta

in the dorsal cord where the DD neurons innervate muscle (Figure 2-7B). While

mCherry::PPM-1 was not always punctate, mCherry::PPM-1 puncta were

observed in the dorsal cord and colocalized with GFP::SNB-1 (Figure 2-7B).

These results demonstrate that PPM-1 can localize to the presynaptic terminals

of GABAergic motor neurons.
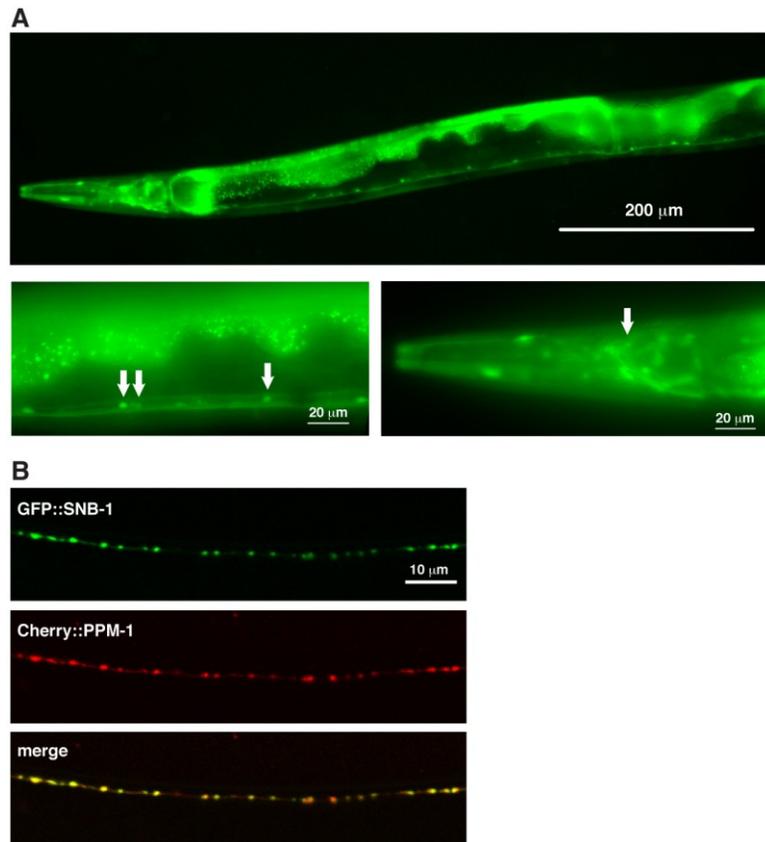
Figure 2-7. PPM-1 is expressed in neurons and localizes to presynaptic terminals.
A) Transgenic worms that use the 3.7 kb promoter of *ppm-1* (*F25D1.1*) to express GFP were analyzed by epifluorescent microscopy. GFP is expressed broadly by the *ppm-1* promoter (upper image), and is present in neurons of the ventral cord (arrows, lower left image), and the nerve ring (arrow, lower right image). *B)* Confocal microscopy was used to analyze transgenic worms that express mCherry::PPM-1 (red) and GFP::SNB-1 (green) specifically in the GABAergic motor neurons using the *unc-25* promoter. mCherry::PPM-1 colocalizes with GFP::SNB-1 at presynaptic terminals in the dorsal nerve cord.

2.3   Discussion

RPM-1 functions as part of an E3 ubiquitin ligase/SCF complex that includes

FSN-1. This complex ubiquitinates and destroys DLK-1 to negatively regulate a

MAP kinase pathway (Nakata et al., 2005). RPM-1 is part of a conserved protein

family called Pam/Highwire/RPM-1 (PHR) proteins, and PHR proteins in flies and

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mice also function as part of SCF complexes to regulate synapse formation and neuronal development (Burgess et al., 2004; Collins et al., 2006; Lewcock et al., 2007; Wu et al., 2007; Saiga et al., 2009; Tada et al., 2009). Thus, negative regulation of the DLK-1/Dlk pathway represents an essential, evolutionarily conserved function of the PHR proteins. While PHR proteins represent one mechanism for negatively regulating the DLK-1 pathway, it remains uncertain if other, complementary mechanisms also restrain the activity of this pathway. In the second chapter of this dissertation we provide evidence of a conserved PP2Cα/β phosphatase, PPM-1, that also negatively regulates the DLK-1 pathway.

Our genetic analysis shows that loss of function in *ppm-1* results in relatively mild phenotypes compared to *rpm-1*. This finding explains why *ppm-1* mutants were not isolated in previous genetic screens for mutants with defective axon termination or synapse formation. We also show that *ppm-1* (lf) enhances *fsn-1* and *glo-4* (lf). Our observations are consistent with *ppm-1* functioning in a genetic pathway that is parallel to both *fsn-1* and *glo-4*.

With regard to axon termination in the PLM neurons, and synapse formation in the GABAergic motor neurons, we observed that *glo-4,ppm-1* double mutants were enhanced, and *glo-1;ppm-1* double mutants were not enhanced. This observation suggests that *glo-4* plays a greater role in axon termination and synapse formation than *glo-1*. This interpretation is consistent with our observations that *glo-4* has stronger enhancer effects than *glo-1* with *fsn-1* (Grill

et al., 2007) (Figure 2-4). Presumably a certain level of reduced GLO pathway function is needed to enhance *ppm-1* (lf). While *glo-4* (lf) achieves this level of inactivation of the GLO pathway, *glo-1* does not. This model suggests that an unidentified small GTPase or signaling molecule, besides GLO-1, functions downstream of GLO-4. Presumably loss of function in both *glo-1* and this other molecule(s) are required to enhance *ppm-1* (lf).

Our observation that *ppm-1* (lf) does not enhance *rpm-1* (lf) demonstrates that *ppm-1* functions in the same genetic pathway as *rpm-1*. This is consistent with our transgenic experiments showing that *ppm-1* functions downstream of *rpm-1* to negatively regulate the DLK-1 pathway.

Previous studies have suggested that the orthologs of PPM-1 may act at the level of MAPKKK, MAPKK, or MAPK to negatively regulate the kinase pathway (Takekawa et al., 1998; Hanada et al., 2001). Our data showing suppression of *ppm-1* axon termination defects by *pmk-3* (lf) suggests that PPM-1 may negatively regulate PMK-3 directly by dephosphorylation. If this is the case, we may be able to detect this interaction using a biochemical approach. Studies have shown that mutations in a conserved residue of PP2C phosphatases reduce catalytic activity and cause the phosphatase to bind its target with increased affinity (Chin-Sang and Spence, 1996; Takekawa et al., 1998). If when coimmunoprecipitated with PMK-3, mutant PPM-1 showed stronger binding than wild type PPM-1, it would suggest PPM-1 directly binds to PMK-3. This technique was previously used successfully for another PP2C

phosphatase in *C. elegans* (Baker and Grill, unpublished). Another experimental option would be to test for an interaction using a yeast two-hybrid assay. Alternatively, PPM-1 may negatively regulate a positive regulator of PMK-3, such as UEV-3. Genetic analysis of *ppm-1;uev-3* mutants may reveal if this is the case.

It is not immediately clear to us why only *pmk-3* (lf) suppresses *ppm-1* (lf) defects. We anticipated that loss of function in any component of the DLK-1 pathway would prevent activation of this pathway, and suppress phenotypes caused by *ppm-1* (lf). One explanation for our results is that kinases other than DLK-1 and MKK-4 also function upstream of PMK-3, and suppression only occurs with loss of function in the target of PPM-1, presumably PMK-3. Mixed Lineage Kinase (MLK)-1, a MAP3K, was recently shown to function upstream of PMK-3 in the context of axon regeneration (Nix et al., 2011), and is a likely candidate as an alternative mechanism for activation of PMK-3 in the context of development. MLK-1 functions as part of another MAPK pathway consisting of the MAP2K MEK-1 and the JNK kinase KGB-1 (Nix et al., 2011), While the role of the MLK-1 pathway in development remains unknown, it is possible that PPM-1 may also regulate this pathway. A genetic analysis of these kinases with *ppm-1* mutants may be informative.

Importantly, the *dlk-1* pathway is required not just in a developmental context, but also for axon regeneration in the mechanosensory neurons (Yan et al., 2009) and in the motor neurons of adult *C. elegans* (Hammarlund et al.,

2009). Overexpression of *dlk-1*, or loss of function in *rpm-1* or *fsn-1* leads to improved axon regeneration (Hammarlund et al., 2009). Our discovery that *ppm-1* is a negative regulator of the *dlk-1* pathway, similar to *rpm-1* and *fsn-1*, suggests that *ppm-1* may also function in axon regeneration. Given our finding that *ppm-1* enhances *fsn-1* with regard to defects in both axon termination and synapse formation, it is plausible that *fsn-1;ppm-1* double mutants may also show enhanced increases in axon regeneration. Future experiments aimed at addressing this possibility will be informative, as PP2C phosphatase inhibitors could be a possible tool for improving neuron regeneration. While the PP2C phosphatases are resistant to traditional phosphatase inhibitors, a recent study has identified several compounds that appear to selectively inhibit PP2Cα (McConnell and Wadzinski, 2009).

In summary, our study provides new insight into the molecular mechanisms of axon termination and synapse formation by showing that PPM-1 constitutes a new regulatory mechanism to control signaling through the DLK-1 pathway. Our study highlights the potential importance of the PP2C/PPM phosphatases in neuronal development. Addressing whether other members of the PP2C/PPM family function in axon termination and/or synapse formation remains an important goal for the future.

2.4   Materials and Methods

Genetics

C. elegans strains were maintained as described (Brenner, 1974). Alleles used in this study include: *rpm-1(ju44), glo-1(zu391), fsn-1(hp1), ppm-1/tag-93(ok578)*, *ppm-1/tag-93(tm653)*, *dlk-1(ju476)*, *mkk-4(ju91)*, and *pmk-3(ok169)*. All double mutants were constructed following standard procedures, and were confirmed by the associated phenotypes or by PCR genotyping. *glo-4,ppm-1* double mutants were constructed by recombination without using visible markers. Primers and PCR conditions are available upon request. *fsn-1;ok578/tm653* animals were constructed using *dpy-11* linked to *ok578*, and *unc-42* linked to *tm653*. Non-dpy, non-unc animals with the genotype *fsn-1;ok578,dpy-11/unc-42,tm653* were scored for trans-heterozygous analysis. The transgenic strains used in this study are: *muIs32*[P$_{mec-7}$GFP] (Ch'ng et al., 2003), *juIs1*[P$_{unc-25}$SNB-1::GFP] (Hallam and Jin, 1998), *bggEx35* [wrm613bH10], *bggEx33* [P$_{rgef-1}$::ppm-1 (cDNA F25D1.1c)], *bggEx34, 40,* and *41* [P$_{mec-7}$::ppm-1 (cDNA F25D1.1c)], *bggEX58,*

59, 60, 61, and *62* [P$_{mec-7}$::ppm-1 (D246N) (cDNA F25D1.1c)], *bggEx55, 56 and 57* [P$_{ppm-1}$::GFP].

Transgene Constructs
To construct cell specific expression vectors of *ppm-1*, a *ppm-1* cDNA (corresponding to the coding sequence of F25D1.1c.1) was amplified by RT-PCR from *C. elegans* RNA and cloned into the pCR8-Topo gateway entry vector (Invitrogen) to create pBG-GY146. pBG-GY146 was recombined into destination vectors containing the *rgef-1* promoter, the *mec-7* promoter or the *myo-3* promoter to generate pBG-GY153 (P$_{rgef-1}$*ppm-1*), pBG-GY163 (P$_{mec-7}$*ppm-1*), and pBG-GY116 (P$_{myo-3}$*ppm-1*). The D246N point mutant of PPM-1 was generated by site directed mutagenesis to create pBG-GY200 (pCR8 TopoGY *ppm-1* (D246A). pBG-GY200 was recombined into destination vectors containing the *mec-7* promoter to generate pBG-GY202 (P$_{mec-7}$*ppm-1* (D246N)). The fosmid wrm613bH10 and pBA183 (P$_{myo-2}$mCherry) were gifts from Drs. David Greenstein (University of Minnesota) and Brian Ackley (University of Kansas), respectively.

Transgenic animals were generated as described previously (Mello et al., 1991). Plasmid DNA of interest was injected at 1-25ng/μL along with P$_{ttx-3}$RFP (50ng/μL) or (P$_{myo-2}$mCherry 1.5-2.5ng/μL) and pBluescript (50ng/μL). Initially all transgenic animals were generated on *ppm-1-/-* backgrounds. To create transgenic animals that were *fsn-1;ppm-1* double mutants, *fsn-1;ppm-1* mutants were heat shocked and males were mated to array positive *ppm-1* mutants. For DLK-1 overexpression experiments, P*rgef-1*::dlk-1 was amplified by long PCR

using pBG-57 as a template and injected at 5-10ng/μL. For rescue experiments with PPM-1, pBG-GY163 (P*mec-7*::ppm-1) or pBG-GY202 (P*mec-7*::ppm-1 (D246N)) were coinjected at 2ng/μL with P*rgef-1*::dlk-1. For analysis of PPM-1 subcellular distribution, pBG-GY208 (P*unc-25*::mCherry-PPM-1) was injected at 5ng/μL into *ppm-1(tm653);juIs1* animals.

Axon termination and Synapse Formation Analysis
Analysis was carried out using a Nikon epifluorescent microscope and a Q-imaging camera at 40x magnification. Live animals were anesthetized using 1% (v/v) 1-phenoxy-2-propanol in M9 buffer. Axon termination defects were quantified by scoring 1-3 pools of worms consisting of 7-20 animals from 3 or more independent experiments for each genotype. The mean for a given phenotype was calculated and is shown in all histograms. All error bars represent the standard error of the mean. Statistical significance was calculated using an unpaired t test. For synapse formation defects, data was averaged from 20-30 animals from a minimum of three independent experiments. The error bars represent the standard error of the mean, and statistical significance was determined using an unpaired t test. Results were considered significant for axon termination defects or synapse formation defects if a p value <0.05 was obtained. All analysis was done without blinding for genotypes.

3   The Nesprin family member ANC-1 regulates synapse formation and axon termination by functioning in a signaling pathway with RPM-1 and β-Catenin

Content adapted from: **Tulgren ED,** Turgeon SM, Opperman KJ, Grill B. The Nesprin family member ANC-1 regulates synapse formation and axon termination by functioning in a signaling pathway with RPM-1 and β-Catenin. (PLOS Genetics, under revision).

3.1 Introduction

The mammalian Nuclear Envelope Spectrin repeat proteins (Nesprins) (also called Syne-1/Enaptin and Syne-2/NUANCE) mediate the anchorage of nuclei in multinucleated cells such as muscle (Zhang et al., 2007b; Puckelwartz et al., 2009; Starr and Fridolfsson, 2010; Zhang et al., 2010; Mellad et al., 2011), and mediate nuclear movement and positioning in mononuclear cells (Zhang et al., 2009; Luxton et al., 2010). The orthologs of Nesprin-1 and 2 are called MSP-300 in *Drosophila* and abnormal nuclear Anchorage (ANC)-1 in *C. elegans*. MSP-300 and ANC-1 also function in nuclear anchorage (Hedgecock and Thomson, 1982; Starr and Han, 2002; Zhang et al., 2002; Elhanany-Tamir et al., 2012), and regulate positioning of organelles including mitochondria and the endoplasmic reticulum (Starr and Han, 2002; Elhanany-Tamir et al., 2012).

Nesprin family members are attached to the nuclear envelope by the SUN proteins (SUN 1 and 2), which together compose the Linker of the Nucleoskeleton and Cytoskeleton (LINC) complex (Starr and Han, 2002; Zhen et al., 2002; Padmakumar et al., 2005; Lei et al., 2009; Starr and Fridolfsson, 2010; Mellad et al., 2011). A C-terminal Klarsicht/ANC-1/Nesprin homology (KASH) domain anchors Nesprin-1 and 2 in the outer nuclear membrane by binding to SUN1 and 2, which are localized to the inner nuclear membrane. *C. elegans* has two SUN family proteins: UNC-84 which is expressed in most somatic cells and retains ANC-1 in the nuclear membrane, and SUN-1 which functions in the germ line and early embryo. Tandem calponin homology domains at the N-terminus of the Nesprins mediate binding to the actin cytoskeleton. Nesprin-1, Nesprin-2 and

MSP-300 also anchor the nucleus to microtubules (Fan and Beck, 2004; Zhang et al., 2009; Schneider et al., 2011; Elhanany-Tamir et al., 2012).

Aside from their role in nuclear anchorage, Nesprin-1 and 2 have several other functions. Nesprin-1 and 2 regulate centrosome orientation in migrating cells and ciliogenesis (Dawe et al., 2009; Zhang et al., 2009; Luxton et al., 2010), regulate cytokinesis (Fan and Beck, 2004), and regulate formation and trafficking of the Golgi (Gough et al., 2003; Gough and Beck, 2004). Importantly, mutations in Nesprin-1 and 2 are associated with numerous diseases including: autism (O'Roak et al., 2011; Yu et al., 2013), cerebellar ataxia (Gros-Louis et al., 2007), Emery Dreifuss muscular dystrophy (Wheeler et al., 2007; Zhang et al., 2007a; Puckelwartz et al., 2009), cancer (Sjoblom et al., 2006; Tessema et al., 2008; Doherty et al., 2010), arthrogryposis (Attali et al., 2009), and cardiomyopathy (Puckelwartz et al., 2010). Genome-wide association studies have also identified single nucleotide polymorphisms in Nesprin-1 that are associated with schizophrenia (Lindholm et al., 2001; Andreassen et al., 2013) and bipolar disorder (Sklar, 2011; Andreassen et al., 2013; Green et al., 2013; Smoller et al., 2013).

Nesprin-1 and 2 perform several functions at the neuromuscular junction (NMJ) and in the central nervous system (CNS). At the NMJ, multiple nuclei are anchored in clusters directly adjacent to the postsynaptic terminal. Nesprin-1 is enriched on these postsynaptic nuclei (Apel et al., 2000), and required for their clustering (Grady et al., 2005; Zhang et al., 2007b; Puckelwartz et al., 2009;

Zhang et al., 2010). Nesprin-1 is required for axon termination of motor neurons that innervate the diaphragm (Zhang et al., 2007b). Nesprin-1 and 2 are also expressed in neurons of the CNS (Apel et al., 2000; Padmakumar et al., 2004; Gros-Louis et al., 2007; Lein et al., 2007; Zhang et al., 2009), where they function in neuronal migration and neurogenesis by mediating connections between the nucleus and the cytoskeleton (Del Bene et al., 2008; Zhang et al., 2009). Nesprin-2 regulates nuclear positioning in photoreceptor cells (Tsujikawa et al., 2007). Of particular note, Nesprin-1 shows extremely strong, broad expression in the adult murine CNS (Allen Brain Atlas: http://mouse.brain-map.org) (Lein et al., 2007), which suggests that Nesprin-1 has an important function in neurons beyond its role in neural precursor migration and neurogenesis. This is consistent with the observation that a splice variant of Nesprin-1 called CPG2 regulates synaptic plasticity (Cottrell et al., 2004). A recent study in *C. elegans* showed that ANC-1 functions to maintain neural cell body position (Johnson and Kramer, 2012). At present, it remains unclear if full length Nesprin-1 or its orthologs play broader roles in neuronal function and development outside of their roles in neural migration, neurogenesis, and maintenance of neuronal cell body position.

The role of Nesprin-1 and 2 in signal transduction has begun to be explored, but remains relatively poorly understood. In vascular smooth muscle cells, small isoforms of Nesprin-2 interact with Erk MAP kinase and regulate its signaling activity (Warren et al., 2010). Studies using a keratinocyte cell line showed that Nesprin-2 binds to α- and ß-catenin, and regulates the nuclear

localization of ß-catenin (Neumann et al., 2010). While these studies demonstrate that Nesprin-2 has the potential to regulate signal transduction, the broader functional consequences of these activities remain unclear. Further, it remains unknown if Nesprin-1 and/or Nesprin-2 mediate signal transduction in neurons.

Members of the Pam/Highwire/RPM-1 (PHR) protein family are large signaling proteins that include: human Pam (also called MYCBP2), murine Phr1, zebrafish Phr1 (Esrom), *Drosophila* Highwire, and *C. elegans* Regulator of Presynaptic Morphology (RPM)-1 (Fulga and Van Vactor, 2008; Po et al., 2010). The PHR proteins are important regulators of neuronal development that function in axon outgrowth and termination (Schaefer et al., 2000; Wan et al., 2000; Grill et al., 2007; Lewcock et al., 2007; Kim et al., 2013; Wang et al., 2013), axon guidance (D'Souza et al., 2005; Bloom et al., 2007; Li et al., 2008a; Shin and DiAntonio, 2011; Vo et al., 2011; James et al., 2013), and synapse formation (Wan et al., 2000; Zhen et al., 2000; Burgess et al., 2004; Bloom et al., 2007; Grill et al., 2007). *C. elegans* RPM-1 and *Drosophila* Highwire also function in axon regeneration (Hammarlund et al., 2009; Xiong et al., 2010; Nix et al., 2011) and axon degeneration following damage (Xiong et al., 2012). A recent study demonstrated an intriguing role for Highwire in aversive long-term memory (Huang et al., 2012).

The PHR proteins function through multiple mechanisms. Studies using worms, flies, fish and mice have shown that the PHR proteins act as E3 ubiquitin

ligases that negatively regulate p38 and JNK MAP kinase signaling by ubiquitinating the MAP3K <u>D</u>uel <u>L</u>eucine Zipper-bearing <u>K</u>inase (Dlk) (called Wallenda in *Drosophila* and DLK-1 in *C. elegans*) (Nakata et al., 2005; Collins et al., 2006; Lewcock et al., 2007; Wang et al., 2013). Further, PHR proteins regulate vertebrate TOR signaling by ubiquitinating and negatively regulating the Tuberous Sclerosis Complex (Murthy et al., 2004; D'Souza et al., 2005; Han et al., 2008; Hendricks et al., 2008; Han et al., 2012).

The PHR proteins also have functions that are independent of their ubiquitin ligase activity. Studies in *C. elegans* have shown that RPM-1 positively regulates both a Rab GTPase pathway (Grill et al., 2007) and the mitotic regulatory protein RAE-1 (Grill et al., 2012b). Work in flies has also supported a role for Rae1 in Highwire function (Tian et al., 2011). Biochemical studies have shown that PHR proteins negatively regulate adenylate cyclase activity (Scholich et al., 2001), and bind to the transcription factor myc (Guo et al., 1998).

While the PHR proteins regulate several signal transduction pathways, it is poorly understood if their activities are linked to signaling by extracellular guidance cues, morphogens, or adhesion molecules. Work in *Drosophila* and *C. elegans* has shown that Highwire negatively regulates BMP signaling (McCabe et al., 2004), and RPM-1 negatively regulates Slit and Netrin signaling (Li et al., 2008a). However, it is remains unclear if PHR protein activity converges with extracellular cues on common signaling targets. It is also uncertain if the PHR proteins have the ability to positively regulate, modify or enhance signals

generated by extracellular cues. The discovery of further molecular links between the PHR proteins and extracellular signals are likely to be helpful in understanding how PHR proteins facilitate axon extension, axon guidance, and synapse formation.

Using a proteomic approach in *C. elegans*, we have identified ANC-1 as an RPM-1 binding protein. Similar to *rpm-1*, *anc-1* functions in both axon termination and synapse formation. Our analysis indicates that *anc-1* functions in a genetic pathway with *beta-catenin/armadillo related protein (bar)-1* downstream of *rpm-1*. Because BAR-1 is a component of canonical Wnt signaling pathways, our observations provide the first evidence of a link between RPM-1 signaling and morphogens that regulate neuronal development.

3.2   Results

3.2.1   Identification of ANC-1 as an RPM-1 binding protein.

To better understand the mechanism of how RPM-1 functions in neuronal development, we previously performed a proteomic screen to identify RPM-1 binding proteins (Grill et al., 2007). Briefly, RPM-1 fused with GFP was transgenically expressed using the native *rpm-1* promoter. This construct was purified from whole worm lysate using an anti-GFP antibody, and RPM-1 binding proteins were identified using mass spectrometry and *de novo* peptide sequencing. To date, our screen has successfully identified three functional RPM-1 binding proteins: GLO-4 (a putative Rab GEF) (Grill et al., 2007), RAE-1 (a microtubule binding protein) (Grill et al., 2007), and PPM-2 (a PP2C

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phosphatase (Baker and Grill, unpublished observation). Our screen also identified the F-box protein FSN-1 (Grill et al., 2007), which was previously discovered using a genetic approach (Liao et al., 2004). Importantly, GLO-4, RAE-1 and PPM-2 are not targets of RPM-1's ubiquitin ligase activity. Thus, our proteomic screen preferentially identifies RPM-1 binding proteins that are not degraded by RPM-1, and are stable interaction partners.

Another RPM-1 binding protein identified in our proteomic screen was ANC-1, a gigantic protein that is composed of 8545 amino acids and has an approximate molecular weight of 956 kDa. ANC-1 consists mostly of predicted coiled regions, including six repeats that are nearly identical at the nucleotide level (Figure 3-1A). The N-terminus contains dual calponin-homology (CH) domains which bind to actin (Korenbaum and Rivero, 2002; Starr and Han, 2002), and the C-terminus consists of a KASH domain which targets ANC-1 to the nuclear envelope (Apel et al., 2000; Zhang et al., 2001; Starr and Han, 2002; Zhen et al., 2002). Previous work showed that ANC-1 is present at the nuclear envelope and in the cytoplasm of all post-embryonic somatic cells (Starr and Han, 2002).
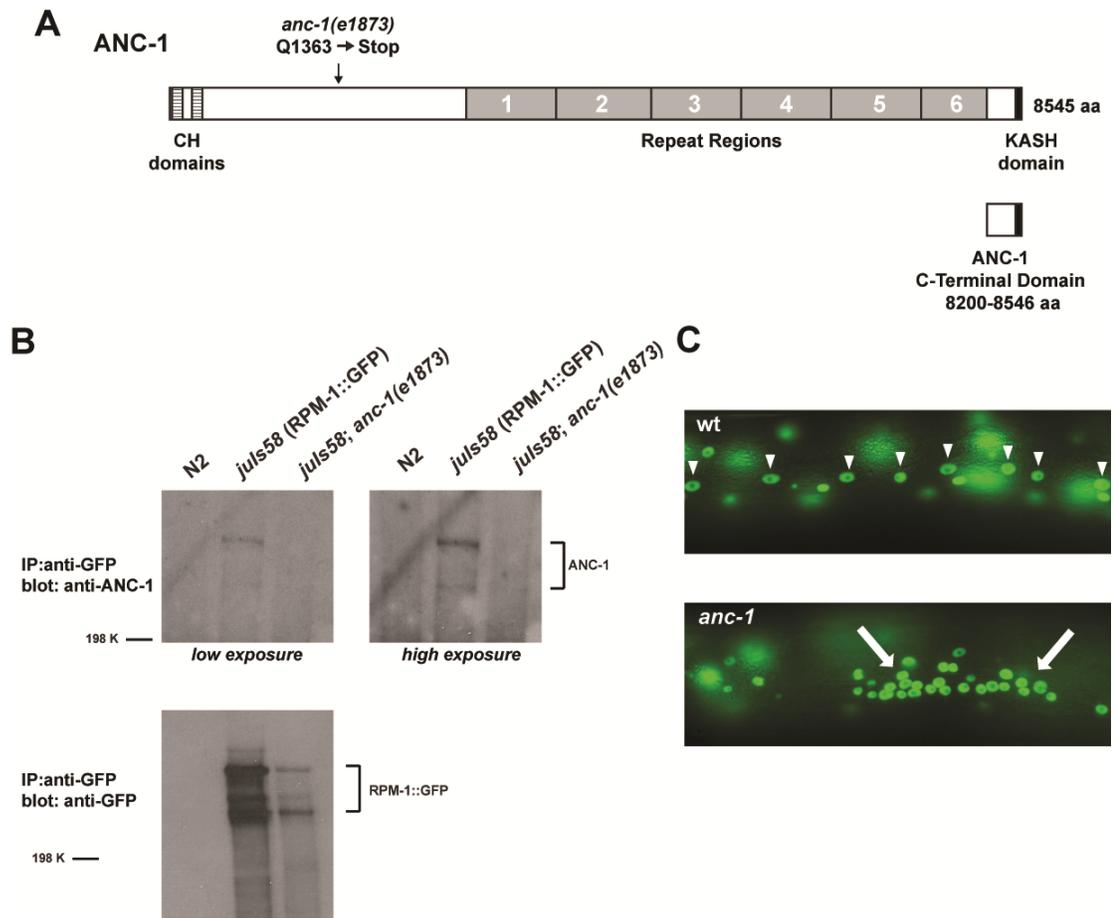
Figure 3-1 ANC-1 binds to RPM-1.
(A) Schematic of ANC-1 protein structure which consists of two calponin homology (CH) domains that bind F-actin (dashed boxes), 6 repeat regions (grey), and a KASH domain (black) that mediates binding to the nucleus. Also shown is the C-terminal domain of ANC-1 that functions as a dominant negative. (B) CoIP of endogenous ANC-1 with RPM-1::GFP. CoIPs were performed from whole worm lysates prepared from transgenic animals (*juIs58*) or non-transgenic animals (N2). (C) Epifluorescent microscopy was used to visualize SUR-5::GFP in the multinucleated hypodermal cells of *C. elegans*. In wild-type animals, nuclei are anchored to the actin cytoskeleton and evenly spaced throughout the syncytium (arrowheads). In *anc-1-/-* mutants, impaired nuclear anchorage leads to aggregation of nuclei (arrows). Scale bar is 20mm.

Our proteomic analysis identified 10 peptides that covered 6.3% of the total ANC-1 protein sequence (Data not shown). The majority of peptide sequence identified was from the ANC-1 specific repeats, presumably because repeat sequence is present at 6-fold molar excess over other regions of ANC-1.

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To confirm the biochemical interaction between ANC-1 and RPM-1 we utilized coimmunoprecipitation from whole worm lysates generated from transgenic animals. A prior study developed polyclonal anti-ANC-1 antibodies that recognize endogenous ANC-1, which was detected as multiple high molecular weight bands in immunoblots (Starr and Han, 2002). We used these anti-ANC-1 antibodies in coimmunoprecipitation (coIP) experiments with transgenic animals expressing RPM-1::GFP (*juIs58*). When GFP::RPM-1 was immunoprecipitated using an anti-GFP antibody, coprecipitating ANC-1 was detected as multiple high molecular weight bands (Figure 3-1B, *juIs58*). Further examples of this coIP are shown in Figure 3-2. Coprecipitating bands were absent or strongly reduced in intensity in *juIs58;anc-1-/-* animals demonstrating that these bands represent endogenous ANC-1 (Figure 3-1B). Coprecipitating ANC-1 was not detected in precipitates from non-transgenic animals (Figure 3-1B, N2). Thus, ANC-1 did not bind to the agarose beads or the anti-GFP antibody demonstrating that the interaction between ANC-1 and RPM-1 was specific. These biochemical results confirm that RPM-1 binds to ANC-1 or a protein complex that contains ANC-1.
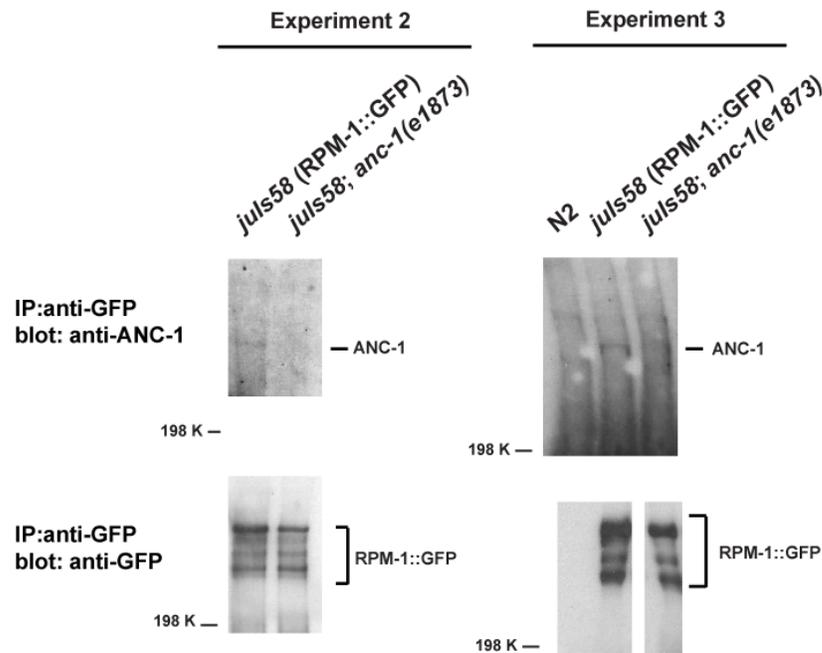
Figure 3-2 Further examples of ANC-1 coIP with RPM-1
Shown are two independent examples of endogenous ANC-1 coprecipitating with RPM-1::GFP from lysates of transgenic *C. elegans.*

3.2.2 *anc-1* regulates synapse formation in the GABAergic motor neurons.

Previous studies have shown that *rpm-1* regulates synapse formation in the

GABAergic DD motor neurons (Zhen et al., 2000). The DD motor neurons

innervate, and inhibit the dorsal muscles of the worm (Figure 3-3A schematic).

The presynaptic terminals of DD neurons can be visualized in living animals

using the transgene *juIs1*, which uses a cell specific promoter (*Punc-25*) to

express a fusion protein of GFP and Synaptobrevin-1 (SNB-1::GFP) (Hallam and

Jin, 1998). In wild type animals, SNB-1::GFP localized to evenly sized puncta

that were uniformly positioned along the dorsal nerve cord (Figure 3-3A). In *rpm-*

1-/- mutants, SNB-1::GFP puncta in the dorsal nerve cord were abnormally

aggregated (Figure 3-3A, arrowheads), and there were regions of the cord

lacking any puncta (Figure 3-3A, arrows). Quantitation showed that the number

of SNB-1::GFP puncta in *rpm-1-/-* mutants was significantly lower than wild-type

animals (compare 11.9 +/- 0.4 SNB-1::GFP puncta/100μm for *rpm-1-/-* to 21.9 +/-

0.4 puncta/100μm for wild type) (Figure 3-3B). These findings are consistent with

results from previous studies (Zhen et al., 2000; Nakata et al., 2005; Tulgren et

al., 2011; Grill et al., 2012). Importantly, previous electron microscopy studies

showed that the defects in SNB-1::GFP puncta localization in *rpm-1-/-* mutants

reflect defects in synapse formation, rather than defects in the formation of

presynaptic terminals or the trafficking of synaptic vesicles (Zhen et al., 2000;

Nakata et al., 2005). Subsequent studies have also shown that milder defects in

organization of SNB-1::GFP puncta, such as those that occur in *fsn-1-/-* mutants,

are also due to defects in both pre and postsynaptic terminals (Hung et al.,

2013).

Our observation that ANC-1 binds to RPM-1 led us to hypothesize that

anc-1 might function in synapse formation similar to *rpm-1*. To test this

hypothesis, we analyzed two alleles of *anc-1*, *e1873* and *e1753*. DNA

sequencing confirmed that *e1873* is a nonsense mutation that results in a

severely truncated protein (Figure 3-1A) (Starr and Han, 2002). The molecular

nature of the lesion in *e1753* remains unknown. However, a previous study

showed that antibodies raised against the repeat region of ANC-1 fail to detect all

isoforms of ANC-1 greater than 175kDa in both *anc-1(e1873)* and *anc-1(e1753)* mutants (Starr and Han, 2002). These results suggest that *e1873* and *e1753* are likely to be molecular null alleles of *anc-1*.
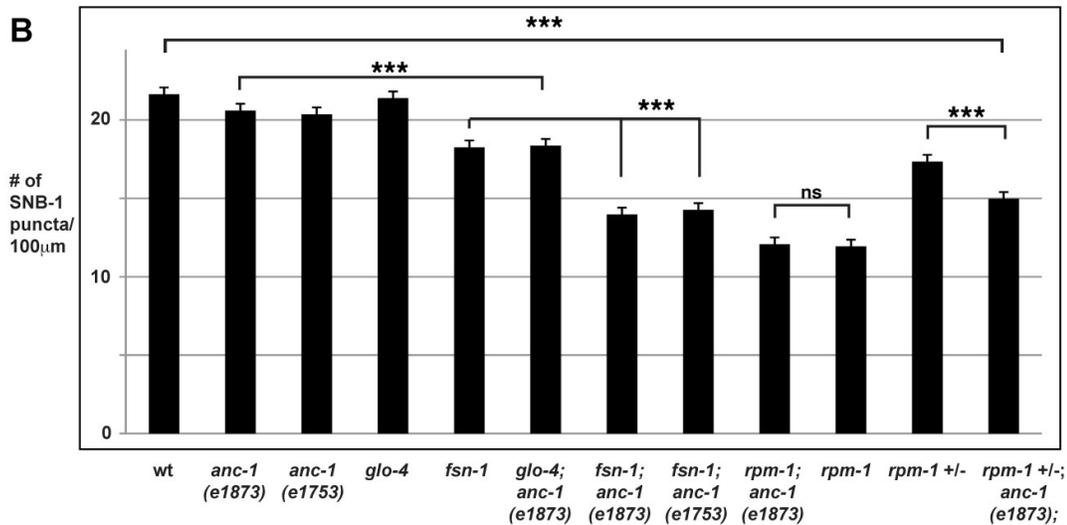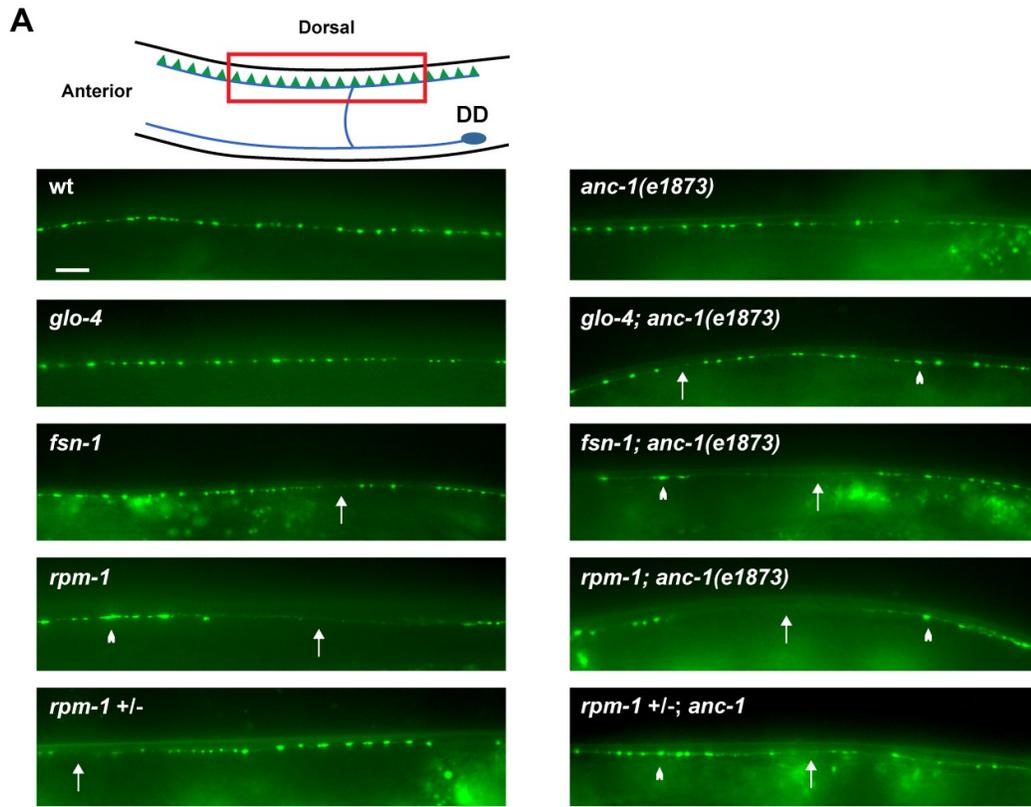
Figure 3-3 *anc-1* regulates synapse formation in the GABAergic motor neurons.
(A) Upper panel diagrams the GABAergic DD neurons (blue) that innervate dorsal muscle.
DD Presynaptic terminals are shown in green (modified from Worm Atlas). The red box
highlights the region of the dorsal cord that was visualized by epifluorescent microscopy. The
transgene *juIs1* [Punc-25::SNB-1::GFP] was used to visualize the presynaptic terminals for
the indicated genotypes. Arrows highlight regions lacking presynaptic terminals represented
by SNB-1::GFP puncta. Arrowheads note abnormal aggregation of presynaptic terminals.
Scale bar is 10μm. (B) Quantitation of the average number of SNB-1::GFP puncta per 100μm
of dorsal cord. Analysis was done on young adults grown at 25°C. Significance was
determined using an unpaired Student's t test; error bars represent the standard error of the
mean. ***$P < 0.001$, ns = not significant.

Previous studies have shown that *anc-1* loss of function (lf) results in abnormal nuclear <u>anc</u>horage (Anc) of the nuclei in the syncytial cells that form the hypodermis of *C. elegans* (Hedgecock and Thomson, 1982; Starr and Han, 2002). The hypodermal nuclei can be visualized using a transgene, *kuIs54*, which expresses SUR-5::GFP (Yochem et al., 1998). In wild-type animals, the hypodermal nuclei are anchored to the cytoskeleton, and distributed in a well organized, even pattern (Figure 3-1C, arrowheads). Consistent with previous results, we observed that *anc-1-/-* animals display an Anc phenotype, in which the nuclei are no longer anchored and aggregate dramatically (Figure 3-1C, arrows).

With regard to synapse formation, *anc-1-/-* mutants had normal spatial distribution and number of SNB-1::GFP puncta when analyzed qualitatively (Figure 3-3A) and quantitatively (Figure 3-3B). These results suggested that if *anc-1* functions in synapse formation, it is likely to do so as a component of the *rpm-1* pathway. To test this hypothesis, we constructed double mutants of *anc-1* with two *rpm-1* pathway members, *fsn-1* and *glo-4*. FSN-1 is an F-box protein that binds to RPM-1 and mediates RPM-1's ubiquitin ligase activity (Liao et al., 2004). GLO-4 binds to RPM-1, and is a putative guanine nucleotide exchange factor for a Rab pathway (Grill et al., 2007). We found that *fsn-1-/-;anc-1-/-* and *glo-4-/-;anc-1-/-* double mutants had enhanced defects in synapse formation compared to single mutants (compare 14.0 +/- 0.5 puncta/100μm for *fsn-1;anc-1(e1873)* to 18.3 +/- 0.3 for *fsn-1*) (Figure 3-3A and B). *rpm-1-/-;anc-1-/-* double

mutants had similar to defects to those observed in *rpm-1-/-* single mutants

(Figure 3-3A and B). Additionally, we constructed *rpm-1+/-;anc-1-/-* mutants and

found enhanced defects in synapse formation compared to *rpm-1+/-* animals

(Figure 3-3A and B). These results are consistent with several conclusions. First,

because we used null alleles, we conclude that *anc-1* functions in a parallel

genetic pathway to both *fsn-1* and *glo-4* to regulate synapse formation. Second,

our observation that *rpm-1-/-;anc-1-/-* double mutants were not enhanced, in a

phenotypic assay that is not saturated (Liao et al., 2004), suggests that *anc-1*

functions in the same genetic pathway as *rpm-1.* This conclusion is further

supported by our finding that *rpm-1+/-;anc-1-/-* double mutants had enhanced

defects in synapse formation. Third, these results also support the conclusion

that ANC-1 is not a target of RPM-1's ubiquitin ligase activity. If this were the

case, we would expect to see suppression of synapse formation defects in *anc-1-*

/-;rpm-1-/- double mutants similar to what was shown for DLK, a known target of

RPM-1's ubiquitin ligase activity (Nakata et al., 2005).

3.2.3   The β-catenin *bar-1* functions in the same genetic pathway as *anc-1* to regulate synapse formation

We next sought to dissect the mechanism of how *anc-1* regulates synapse

formation. A previous study found that Nesprin-2 (a mammalian ortholog of ANC-

1) regulates nuclear localization of β-catenin, thereby potentially regulating Wnt

signaling (Neumann et al., 2010). When Wnt signaling is not active, β-catenin is

degraded. When Wnt signaling is activated, β-catenin accumulates, enters the nucleus, and interacts with TCF/LEF family transcription factors to promote gene expression (Mosimann et al., 2009). Previous work in *C. elegans* has shown that Wnt signaling plays a prominent role in neuronal development (Forrester et al., 2004; Pan et al., 2006; Prasad and Clark, 2006; Maro et al., 2009; Kirszenblat et al., 2011). In addition, *lin-23* (an F-box protein that negatively regulates β-catenin in *C. elegans*) regulates the abundance of postsynaptic glutamate receptors in the ventral nerve cord (Dreier et al., 2005), as well as axon termination in the ALM mechanosensory neurons (Mehta et al., 2004). Both of these developmental events are also regulated by *rpm-1* (Schaefer et al., 2000; Grill et al., 2007; Park et al., 2009). Based on these studies, we hypothesized that *anc-1* may function as a genetic link between *rpm-1* and the β-catenin signaling pathway.

To test this hypothesis, we started by determining if β-catenin regulates synapse formation in the DD motor neurons. In *C. elegans,* there are four β-catenins that have diverged to perform separate functions (Korswagen et al., 2000; Natarajan et al., 2001; Liu et al., 2008; Phillips and Kimble, 2009; Jackson and Eisenmann, 2012). The canonical Wnt pathway operates through the β-catenin homolog BAR-1 (Korswagen et al., 2000; Natarajan et al., 2001) and a single TCF homolog, POP-1 (Lin et al., 1995; Thorpe et al., 1997). To test the role of *bar-1* in synapse formation, we analyzed a null allele of *bar-1, ga80* (Eisenmann et al., 1998). While small, consistent sections of the dorsal cord

were absent in *bar-1-/-* mutants (data not shown), we were able to analyze

synapse formation in sections of the dorsal cord that formed normally. *bar-1-/-*

mutants showed a distribution and number of SNB-1::GFP puncta that were

similar to wild-type animals (Figure 3-4A and B). However, *fsn-1-/-;bar-1-/-*

double mutants showed enhanced defects in synapse formation compared to

single mutants (compare 14.1 +/- 0.4 SNB-1::GFP puncta/100μm for *fsn-1;bar-1*

to 18.3 +/- 0.3 puncta/100μm for *fsn-1*) (Figure 3-4A and B). The enhanced

phenotype in *fsn-1-/-;bar-1-/-* double mutants was similar to what was observed

for *fsn-1-/-;anc-1-/-* double mutants (Figure 3-3B and 3-4B), which suggested that

anc-1 and *bar-1* may function in the same genetic pathway. To test this

possibility, we constructed double and triple mutants between *bar-1*, *anc-1* and

fsn-1. *anc-1-/-;bar-1-/-* double mutants were not enhanced compared to *bar-1-/-*

and *anc-1-/-* single mutants (Figure 3-4A and B). Likewise, *fsn-1-/-;anc-1-/-;bar-1-*

/- triple mutants were not enhanced compared to *fsn-1-/-;bar-1-/-* or *fsn-1-/-;anc-*

1-/- double mutants (Figure 3-4A and B).
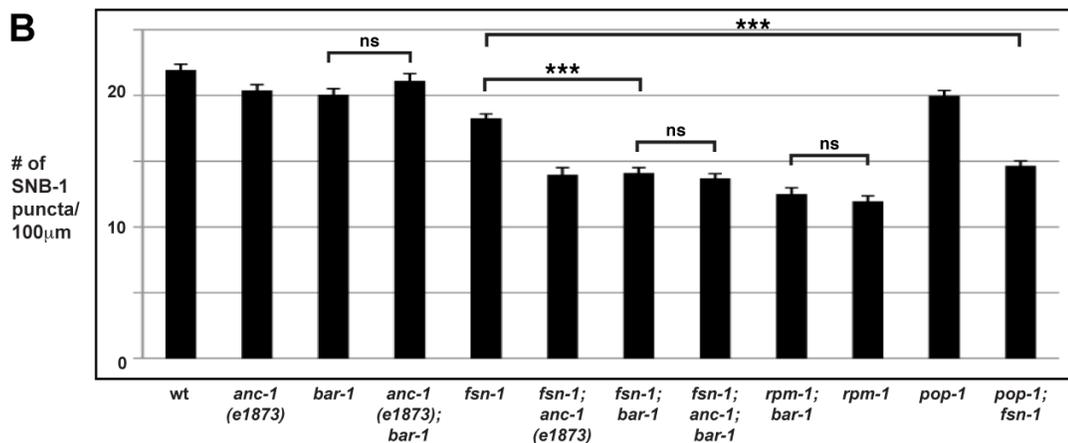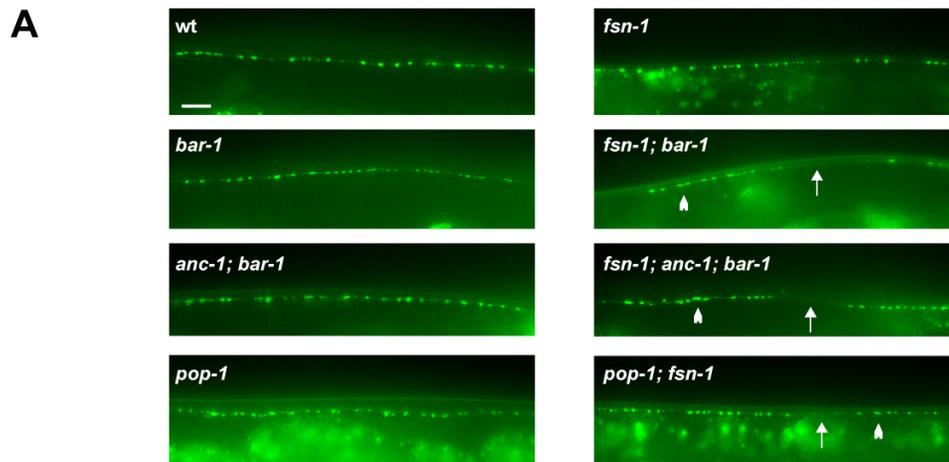
Figure 3-4 *anc-1* and *bar-1* function in the same genetic pathway to regulate synapse formation.
(A) Epifluorescent microscopy was used to visualize presynaptic terminals labeled using the transgene *juIs1* [Punc-25::SNB-1::GFP] for the indicated genotypes. Arrows highlight regions lacking presynaptic terminals represented by SNB-1::GFP puncta. Arrowheads note abnormal aggregation of presynaptic terminals. Scale bar is 10µm. (B) Quantitation of the average number of SNB-1::GFP puncta per 100µm of dorsal cord. Analysis was done on young adults grown at 25°C. Significance was determined using an unpaired Student's t test; error bars represent the standard error of the mean. ***P < 0.001, ns = not significant.

To determine if BAR-1 regulates synapse formation by acting through a canonical signaling pathway that includes the TCF transcriptional factor POP-1, we analyzed the role of *pop-1* in synapse formation. Because null alleles of *pop-1* are lethal, we opted to analyze a hypomorphic allele of *pop-1, q645*. The POP-1

protein produced by *q645* shows reduced interaction with BAR-1 due to a

mutation in the β−catenin binding domain (Siegfried and Kimble, 2002). Similar to

our findings with *bar-1*, *pop-1-/-* animals were largely wild-type, but *pop-1-/-;fsn-1-/-* double mutants had enhanced defects in synapse formation that were of

similar severity to *fsn-1-/-;bar-1-/-* double mutants (compare 14.6 +/- 0.4 SNB-1::GFP puncta/100μm for *pop-1;fsn-1* to 18.2 +/- 0.3 puncta/100μm for *fsn-1*)

(Figure 3-4A and B).

　　　To determine if *bar-1* functions in the same pathway as *rpm-1*, we

constructed *bar-1-/-;rpm-1-/-* double mutants. We observed no change in the

severity of synapse formation defects in *bar-1-/-;rpm-1-/-* double mutants

compared to *rpm-1-/-* single mutants (Figure 3-4B).

　　　As a whole, our results support several conclusions. First, *anc-1*, *bar-1*

and *rpm-1* function in the same genetic pathway to regulate synapse formation.

Second, the *anc-1/bar-1* pathway acts in parallel to *fsn-1* to regulate synapse

formation. Finally, *bar-1* is likely to regulate synapse formation by functioning

through a canonical Wnt signaling pathway that includes the TCF transcription

factor *pop-1*.

3.2.4　*anc-1* regulates axon termination in the PLM mechanosensory neurons

Previous work showed that *rpm-1* regulates axon termination in the Posterior

Lateral Microtubule (PLM) mechanosensory neurons (Schaefer et al., 2000). The

PLM neurons are an excellent system in which to study axon termination, as each PLM neuron extends a single axon that terminates extension at a precise anatomical location (Chalfie and Thomson, 1979; Du and Chalfie, 2001). In addition, these neurons are easily visualized using a transgene (*muIs32*) that expresses GFP specifically in the mechanosensory neurons (Ch'ng et al., 2003).

      C. elegans contains two PLM neurons whose axons terminate extension prior to the cell body of another mechanosensory neuron, the Anterior Lateral Microtubule (ALM) neuron (Figure 3-5A, schematic). In *rpm-1-/-* mutants, the PLM axons fail to terminate extension properly and overgrow past the ALM cell body and hook towards the ventral side of the animal (Figure 3-5A) (Schaefer et al., 2000). This defect, which we refer to as "hooking", is highly penetrant in *rpm-1-/-* mutants (87.6 +/- 1.5%) (Figure 3-5B).

      To determine whether *anc-1* also functions in axon termination, we analyzed *anc-1-/-* animals as well as double mutants of *anc-1* and members of the *rpm-1* signaling pathway. Both *anc-1(e1873)-/-* and *anc-1(e1753)-/-* showed hook defects (3.4 +/- 0.8% for *anc-1(e1873)* and 1.9 +/- 0.8% for *anc-1(e1753)*) that were significant compared to wild-type animals, but occurred with extremely low penetrance compared *rpm-1-/-* mutants (Figure 3-5B). *fsn-1-/-;anc-1-/-* double mutants showed enhanced penetrance of defects compared to single mutants (compare 33.2 +/- 2.4 for *fsn-1;anc-1(e1873)* and 27.5 +/- 4.6% for *fsn-1;anc-1(e1753)* to 9.1 +/- 1.1% for *fsn-1*) (Figure 3-5B). Likewise, *glo-4-/-; anc-1-/-* double mutants also showed enhanced penetrance of defects compared to

single mutants (compare 36.9 +/- 1.9% for *glo-4;anc-1(e1873)* to 17.7 +/- 4.3%

for *glo-4*) (Figure 3-5B). These results demonstrate that *anc-1* regulates axon

termination by functioning in a parallel pathway to both *fsn-1* and *glo-4*.

We also analyzed *rpm-1-/-;anc-1-/-* double mutants, which had the same

penetrance of defects as *rpm-1-/-* single mutants. This result suggests that *anc-1*

functions in the same genetic pathway as *rpm-1* (Figure 3-5B). Consistent with

this conclusion, we observed enhanced axon termination defects in *rpm-1+/-;anc-*

1-/- heterozygous animals (Figure 3-5B).
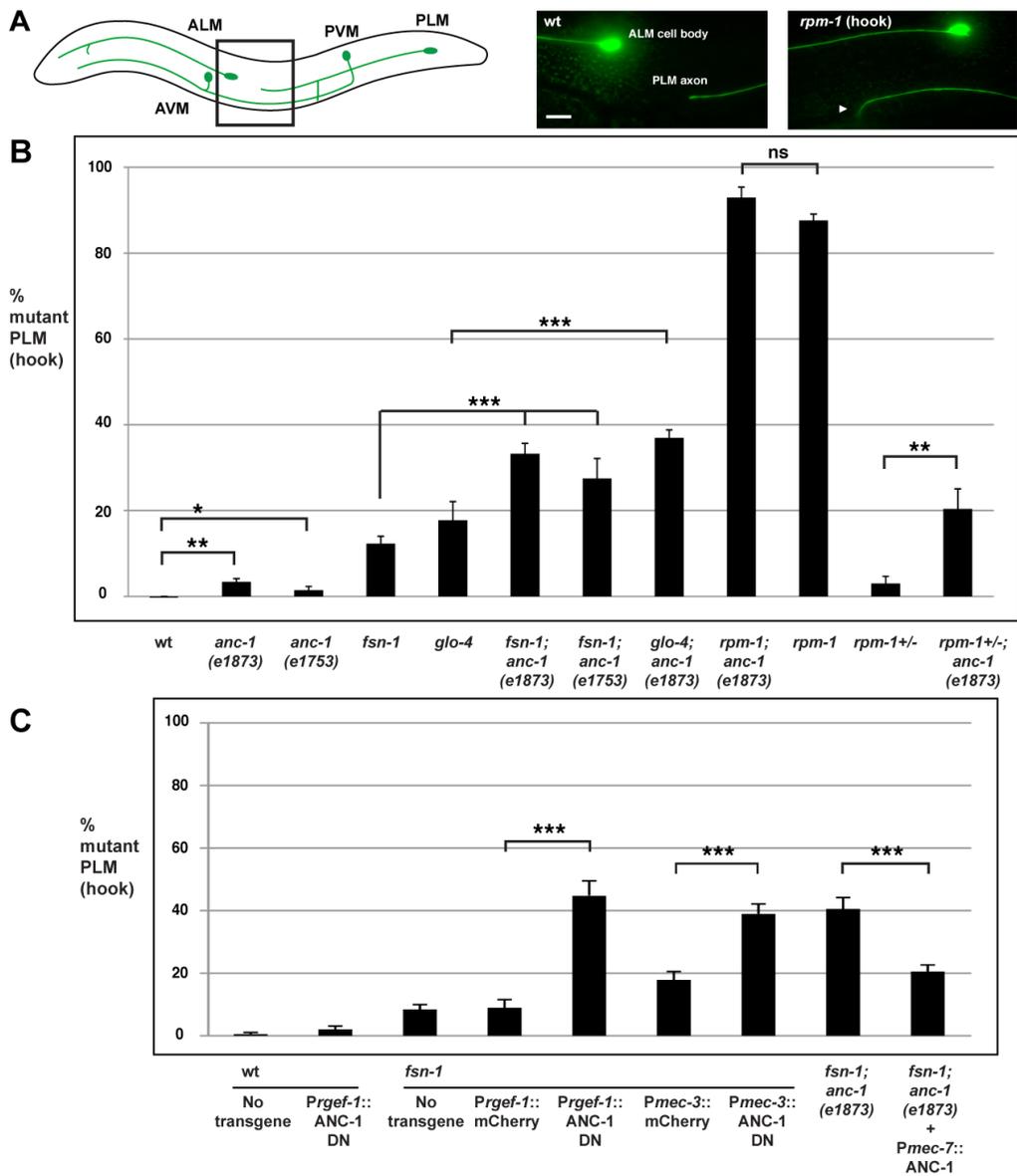
Figure 3-5 *anc-1* functions cell autonomously to regulate axon termination in the PLM mechanosensory neurons.

(A) Upper panel diagrams the mechanosensory neurons of *C. el*egans (modified from Worm Atlas). PLM neurons were visualized using *muIs32* [Pmec7::GFP]. The black box indicates the region of the animal that is visualized by epifluorescent microscopy and shown on the right. Shown for the *rpm-1-/-* mutant is the PLM axon termination phenotype that we refer to as a hook defect (arrowhead). Scale bar is 10μm. (B) Quantitation of axon termination (hook) defects in PLM neurons for the indicated genotypes. (C) An ANC-1 dominant negative construct (ANC-1 DN) was expressed using a pan-neuronal promoter (P*rgef-1*) or a mechanosensory neuron specific promoter (P*mec-3*) with the indicated genotypes. A full length ANC-1 rescue construct (P*mec-7*::ANC-1) was expressed in *anc-1-/-;fsn-1-/-* double mutants. The data shown is an average of 4 or more transgenic lines for each genotype. Analysis was done on young adults grown at 23°C. Error bars represent standard error of the mean. Significance was determined using an unpaired Student's t test. * P<0.05, **P < 0.01, ***P < 0.001, ns = not significant

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Having established that *anc-1* regulates axon termination by functioning in the same pathway as *rpm-1*, we wanted to test if *anc-1* functions cell autonomously in PLM neurons to regulate axon termination. Our first approach was to transgenically overexpress a dominant negative fragment of ANC-1. Previous work by Starr and Han showed that a C-terminal fragment of ANC-1 that contains the KASH domain (see Figure 3-1A, C-terminal domain aa 8200-8546) acts as a dominant negative by blocking binding of endogenous ANC-1 to the SUN domain protein UNC-84, thereby preventing ANC-1 localization to the nuclear envelope (Starr and Han, 2002). We engineered *fsn-1-/-* mutants to transgenically express this dominant negative ANC-1 fragment using a pan-neuronal promoter (P*rgef-1*), and observed increased penetrance of axon termination defects compared to transgenic animals that expressed only the *rgef-1* promoter (compare 44.9 +/- 4.6% for *fsn-1* + P*rgef-1*::ANC-1 dominant negative to 8.9 +/- 2.6 % for *fsn-1* + P*rgef-1*) (Figure 3-5C). Transgenic expression of dominant negative ANC-1 in wild-type animals did not give a significant phenotype (Figure 3-5C). Next, we generated transgenic *fsn-1-/-* mutants in which a promoter that is specifically expressed in the mechanosensory neurons (P*mec-3*) was used to drive expression of dominant negative ANC-1. *fsn-1-/-* mutants carrying transgenic arrays expressing dominant negative ANC-1 showed increased penetrance of axon termination defects (38.8 +/- 3.3%) compared to *fsn-1-/-* mutants that transgenically expressed mCherry (17.9 +/- 2.5%) (Figure 3-5C). Thus, transgenic expression of dominant negative ANC-1, specifically in the

mechanosensory neurons, enhances *fsn-1* (lf). We also transgenically expressed

an *anc-1* mini-gene using another mechanosensory neuron specific promoter

(P*mec-7*) and observed a strong, partial rescue of the enhanced axon

termination defects in *fsn-1-/-;anc-1-/-* double mutants (compare 20.5 +/- 1.9 %

for *fsn-1;anc-1* + P*mec-7*::ANC-1 to 40.4 +/- 3.8 % for *fsn-1;anc-1*) (Figure 3-

5C). These results demonstrate several conclusions. First, *anc-1* functions cell

autonomously in the mechanosensory neurons to regulate axon termination.

Second, ANC-1 needs to be associated with the nuclear envelope via its C-

terminal KASH domain in order to regulate axon termination. Finally, the lesion in

anc-1 causes the enhanced axon termination defects observed in *fsn-1-/-;anc-1-

/-* double mutants .

3.2.5   *bar-1* regulates axon termination in the PLM mechanosensory neurons

Our genetic analysis indicated a role for *bar-1* in synapse formation, and we next

sought to determine if *bar-1* also functions in axon termination of the PLM

neurons, similar to *anc-1*. *bar-1-/-* mutants showed no defects in axon termination

(Figure 3-6A). However, *bar-1-/-;fsn-1-/-* double mutants had enhanced

penetrance of axon termination defects compared to *fsn-1-/-* single mutants

(compare 25.8 +/- 2.5% for *fsn-1;bar-1* to 9.1 +/- 1.1% for *fsn-1*) (Figure 3-6A).

The level of enhancement was similar to what was observed in *fsn-1-/-;anc-1-/-*

double mutants, which suggested that *bar-1* might function in the same pathway

as *anc-1*. To test this, we generated *anc-1-/-;bar-1-/-* double mutants and *fsn-1-/-*

;anc-1-/-;bar-1-/- triple mutants. As shown in Figure 3-6A, *anc-1-/-;bar-1-/-* double mutants were not enhanced. Similarly, *fsn-1-/-;anc-1-/-;bar-1-/-* triple mutants were not enhanced compared to *fsn-1-/-;bar-1-/-* double mutants and *fsn-1-/- ;anc-1-/-* double mutants (Figure 3-6A). Thus, *bar-1* and *anc-1* regulate axon termination by functioning in the same genetic pathway. Given that BAR-1 regulates the transcription factor POP-1, we also tested if *pop-1* regulated axon termination. Similar to *bar-1*, *pop-1-/-* animals had axon termination defects that occurred with very low penetrance, and *pop-1-/-;fsn-1-/-* double mutants were enhanced compared to single mutants (compare 47.7 +/- 4.3% for *pop-1;bar-1* to 9.1 +/- 1.1% for *fsn-1*). Importantly, *pop-1-/-;bar-/-* double mutants were not enhanced consistent with *pop-1* and *bar-1* functioning in the same genetic pathway (Figure 3-6A). Thus, BAR-1 functions through POP-1 to regulate axon termination.

To address if *bar-1* and *rpm-1* function in the same genetic pathway to regulate axon termination, we generated *rpm-1-/-;bar-1-/-* double mutants. As shown in Figure 3-6A, *rpm-1-/-;bar-1-/-* double mutants had similar penetrance of hook defects to *rpm-1-/-* single mutants.

Given that ANC-1 functions cell autonomously in the mechanosensory neurons, we also wanted to determine if BAR-1 functions cell autonomously. To do this, we generated *fsn-1-/-;bar-1-/-* double mutants that transgenically express BAR-1 using a cell specific promoter. We performed our analysis on *fsn-1-/-;bar-1-/-* double mutants because the penetrance of axon termination defects was

much higher than in *bar-1-/-* single mutants. When BAR-1 was expressed using a mechanosensory neuron specific promoter (P*mec-7*), the axon termination defects in *fsn-1-/-;bar-1-/-* double mutants were significantly reduced (compare 29.6 +/- 3.1 % for *fsn-1;bar-1* to 12.1 +/- 2.0 % for *fsn-1;bar-1* + P*mec-7*::BAR-1) (Figure 3-6C).

Overall, these findings support several conclusions. First, *bar-1* regulates axon termination in the PLM mechanosensory neurons by functioning in the same genetic pathway as *rpm-1*, *anc-1*, and *pop-1*. Second, *bar-1* functions in a parallel genetic pathway to *fsn-1*. Finally, *bar-1* regulates axon termination by functioning cell autonomously in the PLM mechanosensory neurons.

3.2.6 *bar-1* functions downstream of *anc-1* and *rpm-1* to regulate axon termination

Our genetic analysis indicated that *rpm-1*, *anc-1* and *bar-1* function in the same pathway. We next sought to determine whether *bar-1* functions up or downstream of *anc-1* and *rpm-1.* We chose to perform our epistasis analysis using *bar-1* for two reasons. First, the existing knowledge of how BAR-1 functions allowed the design and implementation of highly informative experiments. Second, a previous study in mammalian cells on Nesprin-2 and β-catenin (the orthologs of ANC-1 and BAR-1, respectively) suggested that *bar-1* might function downstream of *anc-1* (Neumann et al., 2010). Therefore, axon termination defects caused by loss of function in *rpm-1* and enhanced axon termination defects in *fsn-1-/-;anc-1-/-* double mutants might be due, in part, to

decreased *bar-1* activity. If this model was correct, we anticipated that overexpression of *bar-1* might suppress the axon termination defects caused by loss of function in *rpm-1* and the enhanced axon termination defects in *fsn-1-/- ;anc-1-/-* double mutants. We choose to address this question using a genetic approach that utilized a loss of function mutation in *apr-1*, as toxicity prevented us from transgenically overexpressing BAR-1 at high levels.

APR-1 is the *C. elegans* ortholog of human Adenomatous Polyposis Coli (APC) (Rocheleau et al., 1997; Hoier et al., 2000). In the vertebrate canonical Wnt signaling pathway, APC forms a complex with the scaffold protein Axin and the kinase GSK3β to phosphorylate β-catenin and target it for destruction (Mosimann et al., 2009; Clevers and Nusse, 2012). APR-1 interacts with the functional axin ortholog PRY-1, and has been shown to negatively regulate the β-catenin BAR-1 during vulva development and neuroblast (Q cell) migration (Gleason et al., 2002; Korswagen et al., 2002). Thus, APR-1 can negatively regulate BAR-1 signaling, and *apr-1* mutants are likely to have increased levels of BAR-1 which is consistent with findings from other organisms on Wnt signaling and APC function.

Because the axon termination defects in *anc-1-/-* mutants occurred with relatively low penetrance, we chose to analyze *fsn-1-/-;anc-1-/-* double mutants, which have enhanced defects. As shown in Figure 3-6B, *fsn-1-/-;anc-1-/-;apr-1-/-* triple mutants had significantly reduced penetrance of hook defects (21.4 +/- 1.9%) compared to *fsn-1-/-; anc-1-/-* double mutants (33.2 +/- 2.4%). Importantly,

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fsn-1-/-;apr-1-/- double mutants did not show reduced penetrance of defects

(Figure 3-6B). These results show that increased BAR-1 activity suppresses *anc-1* (lf), which is consistent with *bar-1* functioning downstream of *anc-1*.

To test if *bar-1* functions downstream of *rpm-1* we took a similar genetic approach using *apr-1*. As shown in Figure 3-6B, *rpm-1-/-;apr-1-/-* double mutants showed suppressed penetrance of axon termination defects when compared to *rpm-1-/-* single mutants (compare 65.1 +/- 3.9% hook for *rpm-1;apr-1* to 87.6 +/- 1.5% for *rpm-1*). These results support the conclusion that *bar-1* functions downstream of *rpm-1*.
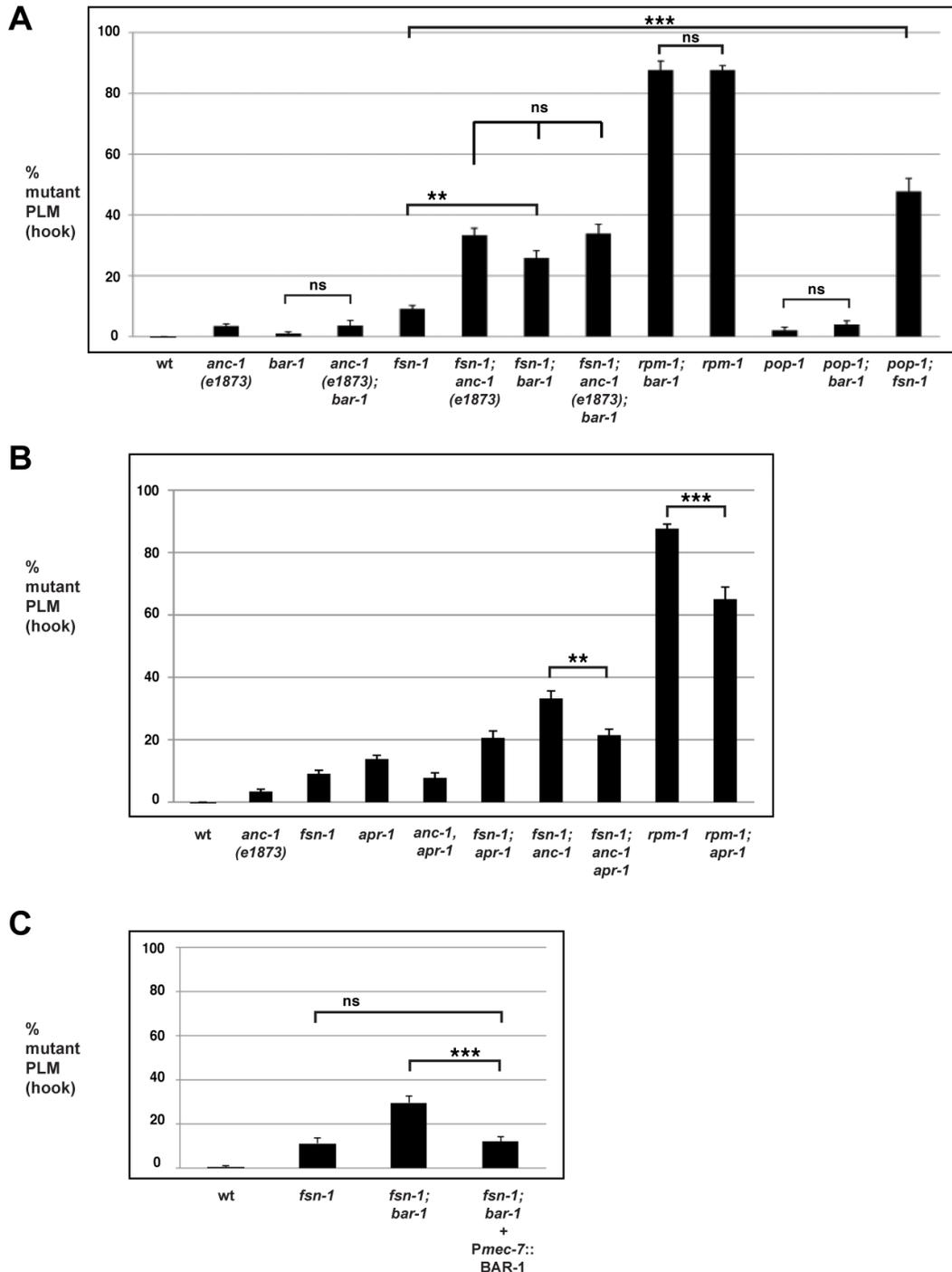
Figure 3-6 *bar-1* functions downstream of *anc-1* and *rpm-1* to regulate axon termination. Quantitation of axon termination defects (hook) in PLM neurons for the indicated genotypes using *muIs32*. (A) *bar-1* and *pop-1* analysis. (B) *apr-1* analysis. (C) A cell specific promoter (P*mec*-7) was used to transgenically express BAR-1. Analysis was done on young adults grown at 23°C. Error bars represent standard error of the mean. Significance was determined using an unpaired Student's t test. **P < 0.01, ***P < 0.001, ns = not significant

3.2.7 *unc-84* regulates axon termination

Our observation that transgenic overexpression of the ANC-1 KASH domain in

fsn-1-/- mutants results in enhanced axon termination defects suggested that

ANC-1 needs to localize to the nuclear envelope in order to regulate axon

termination (Figure 3-5C). To further support this concept, we examined the role

of UNC-84 in axon termination. UNC-84 is a conserved SUN domain protein that

is localized to the inner nuclear membrane. UNC-84 binds to ANC-1, thereby

tethering ANC-1 in the nuclear envelope and mediating formation of the LINC

complex (Malone et al., 1999; Starr and Han, 2002; Starr and Fridolfsson, 2010).

Therefore, we hypothesized that if ANC-1 needs to be localized to the nuclear

membrane to function in axon termination, then *unc-84-/-* mutants would have

axon termination defects that were similar to *anc-1-/-* mutants.
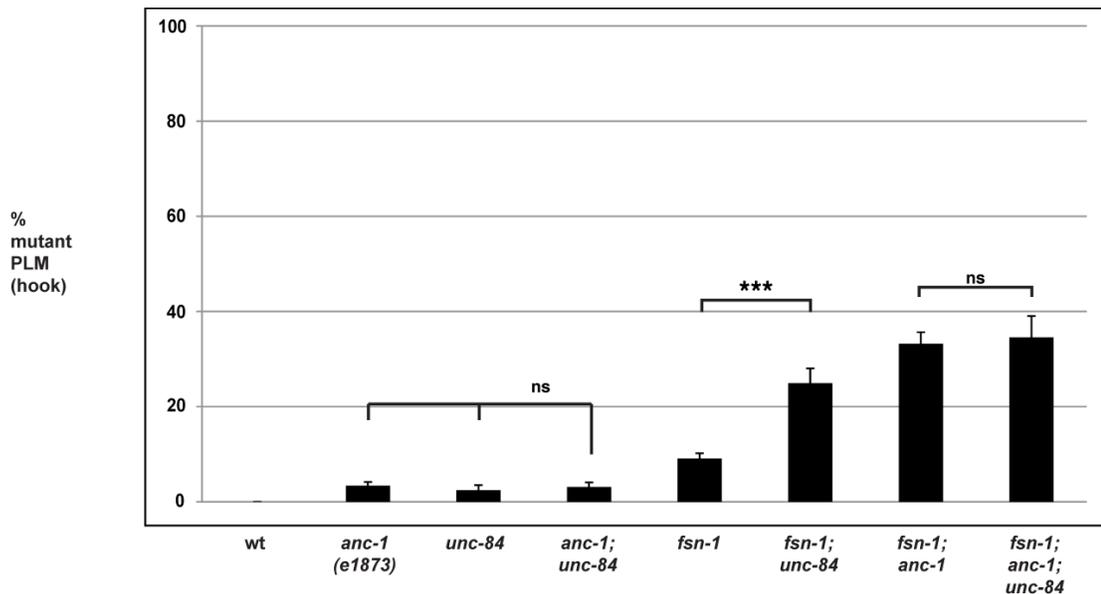
Figure 3-7 *anc-1* and *unc-84* function in the same genetic pathway to regulate axon termination.
Quantitation of axon termination defects (hook) in PLM neurons for the indicated genotypes using *muIs32*. Analysis was done on young adults grown at 23°C. Significance was determined using a Student's t test; error bars represent the standard error of the mean. ***P < 0.001, ns = not significant

In *unc-84-/-* mutants we observed a very mild penetrance of hook defects, similar to *anc-1-/-* animals (Figure 3-7). *fsn-1-/-;unc-84-/-* double mutants had enhanced penetrance of defects compared to *fsn-1-/-* single mutants (compare 24.9 +/- 3.1% hook for *fsn-1;unc-84* to 9.1 +/- 1.1% for *fsn-1*) (Figure 3-7). In contrast, *anc-1-/-;unc-84-/-* double mutants did not show a significant increase in penetrance compared to single mutants (Figure 3-7). Likewise, the penetrance of defects in *fsn-1-/-;anc-1-/-;unc-84-/-* triple mutants was not increased compared to *fsn-1-/-anc-1-/-* and *fsn-1-/-;unc-84-/-* double mutants (Figure 3-7). While the *unc-84* allele we used, *e1410*, is not a full null, it results in loss of function in the SUN domain of UNC-84, and has nuclear anchorage defects (Malone et al.,

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1999). Previous studies have shown that the SUN domain of UNC-84 is required for recruitment of ANC-1 to the nuclear envelope (Starr and Han, 2002). Thus, *unc-84(e1410)* is predicted to lack nuclear localization of ANC-1. This is consistent with our genetic data showing that loss of function in *unc-84* does not enhance loss of function in *anc-1*. Thus, we conclude that *unc-84* regulates axon termination by functioning in the same genetic pathway as *anc-1*, and in a parallel pathway to *fsn-1*. Importantly, these findings also support the conclusion that ANC-1 regulates axon termination by functioning at the nuclear envelope.

3.3   Discussion

While the role of the Nesprins in nuclear positioning and movement is well established, mounting evidence has suggested additional roles for this protein family. High expression of Nesprin-1 in the adult brain, links to synaptic plasticity, and an association with neurological conditions suggest that the Nesprins may function in neuronal development. Here, we show that ANC-1 binds to the PHR protein RPM-1, a large signaling protein involved in numerous developmental events in neurons. Our genetic analysis indicates that ANC-1 functions cell autonomously to regulate axon termination in the mechanosensory neurons, and synapse formation in the GABAergic motor neurons. Importantly, we also show that ANC-1 functions by positively regulating BAR-1, the β-catenin isoform that functions in canonical Wnt signaling (Figure 3-8). Our study reveals a novel role for ANC-1 in intracellular signaling and neuronal development. Importantly, we

have also identified the ANC-1/BAR-1 pathway as a new mechanism by which RPM-1, and possibly other PHR proteins, regulate axon termination and synapse formation.
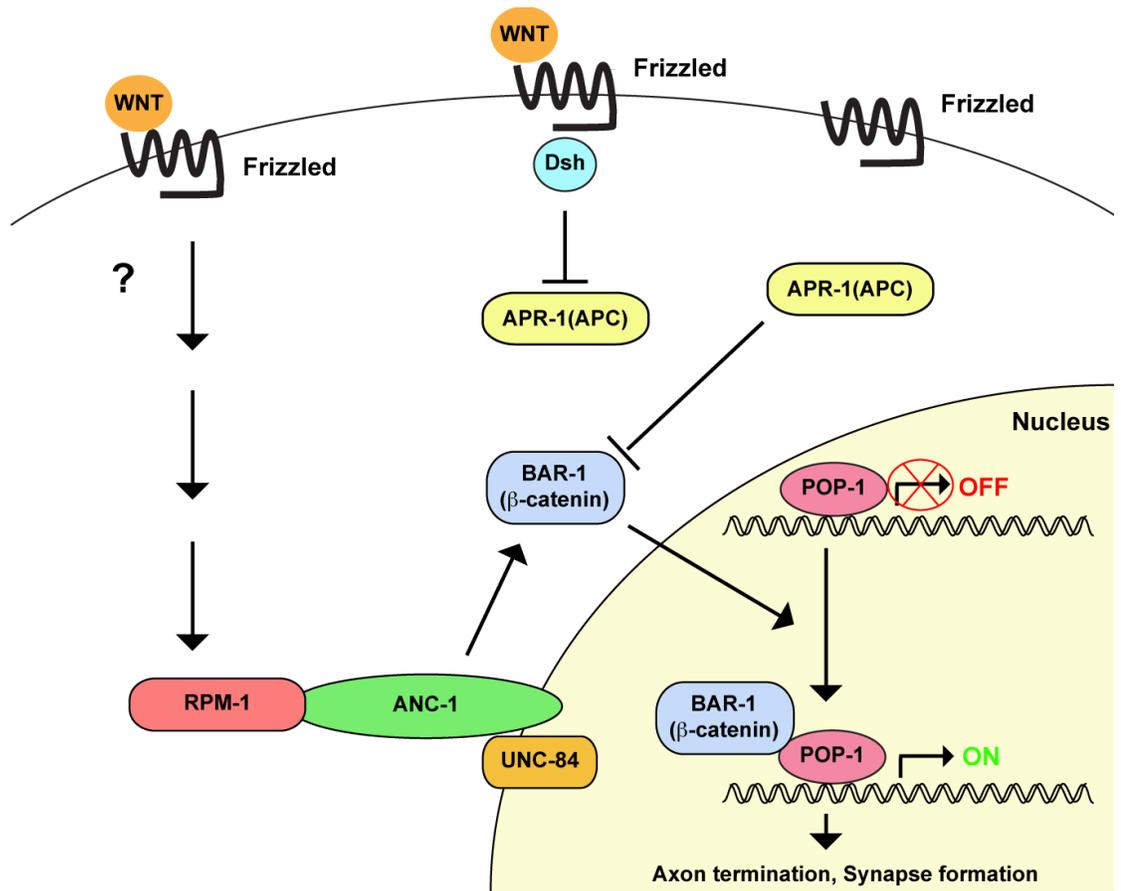
Figure 3-8 Summary of RPM-1 signaling through the ANC-1/BAR-1 pathway.
Canonical Wnt signaling through Disheveled and APR-1, an APC ortholog, regulates the β-catenin BAR-1. In the absence of Wnt, APR-1 is active and inhibits BAR-1. In the presence of Wnt, APR-1 is inhibited and BAR-1 activity is increased. Higher levels of BAR-1 lead to increased activation of the transcription factor POP-1 (TCF/LEF). We have shown that RPM-1 binds to ANC-1, and RPM-1 and ANC-1 function in the same pathway to positively regulate BAR-1 activity. RPM-1 and ANC-1 are likely to function in a protein complex at the nuclear envelope to regulate BAR-1. We propose two models of how RPM-1 and Wnt signaling may regulate BAR-1 activity. 1) Canonical Wnt signaling may act coordinately with RPM-1 and ANC-1 to regulate BAR-1. 2) Wnt may function through RPM-1 and ANC-1 as a novel, non-canonical mechanism to regulate BAR-1.

ANC-1 mediates RPM-1's function

Our proteomic screen for RPM-1 binding proteins identified the nuclear anchorage protein ANC-1, which was confirmed using coimmunoprecipitation. Consistent with these findings, our genetic analysis indicated that *anc-1* and *rpm-1* function in the same genetic pathway to regulate synapse formation in the GABAergic motor neurons and axon termination in the PLM mechanosensory neurons. Importantly, transgenic analysis indicated that *anc-1* functions cell autonomously in the mechanosensory neurons to regulate axon termination, similar to *rpm-1*. Our observation that defects in *anc-1-/-;rpm-1-/-* double mutants were not suppressed also suggests that RPM-1 positively regulates ANC-1.

It is likely that *anc-1* functions downstream of *rpm-1* for two reasons. First, all other RPM-1 binding proteins identified in our proteomic screen function downstream of RPM-1 (Grill et al., 2007; Grill et al., 2012b) (Baker and Grill, unpublished observation). This is presumably because potential upstream regulators of RPM-1 signaling engage in more transient interactions that are difficult to detect using proteomics. Second, our genetic analysis showed that *bar-1* functions downstream of *anc-1* and *rpm-1*, and these three genes function in the same genetic pathway. Because *bar-1* and *anc-1* (lf) result in similar enhancer effects with *fsn-1* (lf), and defects caused by *rpm-1* (lf) are more penetrant, it is probable that *anc-1*, like *bar-1*, functions downstream of *rpm-1*.

The PHR protein family is highly conserved with orthologs in *Drosophila*, zebrafish and mice (Po et al., 2010). To date, all of the proteins identified in our proteomic screen for RPM-1 binding proteins (including FSN-1, GLO-4, and RAE-1) are evolutionarily conserved (Grill et al., 2007; Grill et al., 2012b). Therefore, RPM-1 and its downstream signaling pathways are likely to function through conserved mechanisms. ANC-1 is also conserved with orthologs in *Drosophila* (Msp-300) and mammals (Nesprin-1 and 2). Thus, ANC-1's function in axon termination and synapse formation during development is also likely to be conserved. This is supported by the observation that the branches of phrenic nerves in Nesprin-1-/- knockout mice are overgrown, suggesting possible termination defects (Zhang et al., 2007b). Further addressing if ANC-1's role in axon and synapse development is evolutionarily conserved remains an important goal.

ANC-1 functions through the β-catenin BAR-1

Our genetic results showed that the β-catenin *bar-1* functions in synapse formation in the GABAergic motor neurons, and axon termination in the mechanosensory neurons. While prior studies have shown that BAR-1 regulates axon extension (Maro et al., 2009) and glutamate receptor trafficking in motor neurons (Dreier et al., 2005), our results show that BAR-1 plays a more expansive role in neuronal development than originally thought. We also provide significant insight into the mechanism of how BAR-1 is regulated by showing that

a novel pathway containing RPM-1 and ANC-1 functions upstream of BAR-1. Our functional genetic and transgenic findings are consistent with a prior study on a keratinocyte cell line, which showed that Nesprin-2 regulates the nuclear localization of β-catenin (Neumann et al., 2010). Our results do not rule out the possibility that ANC-1 regulates the ubiquitination or phosphorylation of BAR-1. We used epifluorescent microscopy to investigate if BAR-1::GFP localization to the nucleus was altered in *anc-1-/-* mutants, but found no obvious changes (data not shown). However, this was done using multicopy arrays, thus more sensitive methods may be required to detect such changes. One possibility would be to engineer a strain with a single copy insertion of BAR-1::GFP. The lower expression of BAR-1::GFP may allow us to visualize minor changes in nuclear BAR-1 levels. Also, a reporter construct has been developed which allows for the visualization of TCF/POP-1 activity *in vivo*. This reporter, known as POPTOP, contains several copies of a POP-1 binding site which drives the expression of the fluorescent protein *mCherry* (Green et al., 2008). If when expressed in neurons the level of fluorescence was diminished in *anc-1* mutants, it would suggest POP-1/BAR-1 activity was being affected.

In the canonical Wnt signaling pathway, BAR-1 activates POP-1 (the sole *C. elegans* TCF/LEF transcription factor) (Jackson and Eisenmann, 2012). Our observation that defects in axon termination and synapse formation were enhanced in both *fsn-1-/-;pop-1-/-* and *fsn-1-/-;bar-1-/-* double mutants, and that defects were not enhanced in *pop-1-/-;bar-1-/-* double mutants is consistent with

bar-1 and *pop-1* functioning in the same genetic pathway. Because *fsn-1-/-;pop-1-/-* double mutants displayed more penetrant defects than *fsn-1-/-;bar-1-/-* double mutants, it is possible that another β-catenin may also function through POP-1 to regulate axon termination. HMP-2 is an unlikely candidate as it functions in a cadherin-catenin complex and does not act through POP-1 (Costa et al., 1998; Korswagen et al., 2000). SYS-1 or WRM-1 are more likely as they regulate transcriptional activation and nuclear export of POP-1, respectively (Rocheleau et al., 1997; Lo et al., 2004; Kidd et al., 2005). In the case of synapse formation in the GABAergic motor neurons the situation is simpler. Phenotypes were similar in *fsn-1-/-;pop-1-/-* and *fsn-1-/-; bar-1-/-* double mutants suggesting that only *bar-1* is likely to regulate *pop-1*'s activity in synapse formation in GABAergic motor neurons.

In vertebrate neurons, β-catenin plays a broad and important role in neurons by regulating a range of processes including: neuronal differentiation (Zechner et al., 2003; Hirabayashi et al., 2004; Fang et al., 2013), synaptic vesicle assembly (Bamji et al., 2003; Sun et al., 2009; Sun and Bamji, 2011), dendrite morphogenesis and plasticity (Murase et al., 2002; Yu and Malenka, 2003), axon extension (Votin et al., 2005; David et al., 2008), and axon arborization and targeting in retinal ganglion cells (RGC) (Elul et al., 2003). β-catenin also regulates the morphology of neuromuscular junctions (NMJ) and phrenic nerve growth (Li et al., 2008b). Notably similar to β-catenin's function, Phr1 regulates RGC axon arborization and targeting (D'Souza et al., 2005) and

NMJ morphology (Burgess et al., 2004; Bloom et al., 2007), and both Phr1 and

Nesprin-1 regulate axon growth in phrenic nerves (Burgess et al., 2004; Zhang et

al., 2007b). While these studies hinted at a possible functional link between PHR

proteins, Nesprins and β-catenin, our finding that RPM-1, ANC-1 and BAR-1

function in the same pathway provides the first mechanistic explanation for these

phenotypic relationships in mammals. Thus, the functional relationship between

RPM-1, ANC-1 and BAR-1 is likely to be evolutionarily conserved. Further, our

study outlines a signaling network that links two central and important regulators

of neuronal development, the PHR proteins and β-catenin, through the function

of ANC-1.

ANC-1 regulates axon termination by functioning at the nuclear envelope

ANC-1 regulates nuclear anchorage by binding to the nuclear envelope via its C-

terminal KASH domain, and binding to the actin cytoskeleton via its N-terminal

calponin homology domains (Starr and Han, 2002). The SUN domain protein

UNC-84 mediates binding of ANC-1 to the nuclear envelope. Several of our

findings demonstrate that ANC-1 needs to be anchored to the nuclear envelope

in order to regulate axon termination. First, we found that transgenic

overexpression of dominant negative ANC-1 which acts by inhibiting recruitment

of endogenous ANC-1 to the nuclear envelope (Starr and Han, 2002), enhances

the axon termination defects caused by *fsn-1* (lf). Second, loss of function in

either *anc-1* or *unc-84* enhances the axon termination defects caused by *fsn-1*

(lf). Finally, we observed that *anc-1* and *unc-84* function in the same genetic pathway to regulate axon termination. Collectively, these findings support the conclusion that ANC-1 regulates axon termination by functioning at the nuclear envelope.

While our genetic results indicate that ANC-1 positively regulates BAR-1, how this occurs is not clear. In mammals, Nesprin-2 binds to a complex of a- and β-catenin, thereby facilitating nuclear localization or inhibiting nuclear export of β-catenin (Neumann et al., 2010). Thus, RPM-1 may mediate formation of an ANC-1/BAR-1 complex, thereby facilitating nuclear localization of BAR-1. Alternatively, binding of RPM-1 to ANC-1 (or an ANC-1 containing protein complex) may regulate ANC-1 mediated export of BAR-1 from the nucleus. Isoforms of Nesprin-1 and Nesprin-2 bind to the nuclear membrane protein Emerin (Mislow et al., 2002; Libotte et al., 2005; Wheeler et al., 2007), and Emerin regulates nuclear export of β-catenin (Markiewcz et al., 2006). It is possible that ANC-1 may interact with the *C. elegans* Emerin, EMR-1, to decrease nuclear export of β-catenin, and thus positively regulate β-catenin signaling. Future genetic experiments analyzing *anc-1; emr-1* and *emr-1;fsn-1* double mutants may be helpful in addressing this possibility. If it EMR-1 is functioning with ANC-1, it would be expected to also function in a parallel pathway to *fsn-1*, and in the same genetic pathway as *anc-1*.

Previous studies showed that RPM-1 is localized to the perisynaptic zone of presynaptic terminals (Zhen et al., 2000; Grill et al., 2007; Abrams et al.,

2008). However, a transgenically expressed fusion protein of GFP and RPM-1 is also found at low levels in the cell body and excluded from the nucleus of motor neurons (Zhen et al., 2000) and the mechanosensory neurons (Opperman and Grill, unpublished). In addition, RPM-1 binds to RAE-1, a protein that localizes to the nuclear envelope as well as presynaptic terminals (Grill et al., 2012b). Our finding that ANC-1 binds to RPM-1, that both genes function in the same pathway, and that ANC-1 regulates axon termination by functioning at the nucleus suggest that RPM-1 may play a novel signaling role in the neuronal cell body, possibly at the nuclear envelope. This model is supported by immunohistochemistry in mammals, which showed that the rat ortholog of RPM-1 (called MYCBP2 or Pam) is present in the cell bodies of neurons in the spinal cord and throughout the brain (Ehnert et al., 2004; Santos et al., 2006).

Implications of RPM-1 signaling on Wnt function

An emerging question is whether the PHR proteins, or the signaling pathways they control, are regulated or integrated with signals originating from outside the cell. Extracellular guidance cues, adhesion molecules and morphogens, such as Wnts, play roles in both axon guidance and synapse formation (Shen and Cowan, 2010). By showing that the β-catenin BAR-1 functions downstream of RPM-1, we have provided the first link between RPM-1 and the canonical Wnt signaling pathway (Figure 3-8). It seems intuitive that RPM-1 may act coordinately with canonical Wnt signaling to regulate the activity of BAR-1,

possibly in a spatially or temporally sensitive manner. However, an alternative possibility is that RPM-1 itself may be regulated by components of a Wnt pathway, thereby acting as a novel secondary mechanism by which Wnts regulate β-catenin signaling. Future genetic experiments on different Wnts and Wnt receptors are likely to be helpful in differentiating between these models.

Wnts are expressed as shallow gradients in the CNS (Lyuksyutova et al., 2003; Yoshikawa et al., 2003), and function in both axon guidance and synapse formation (Shen and Cowan, 2010; Budnik and Salinas, 2011; Hollis and Zou, 2012b). At present, it is uncertain how shallow gradients of Wnts lead to strong effects on neuronal development. Our results suggest that the PHR proteins may potentiate β-catenin activity, thereby sensitizing neurons to Wnt signaling and shallow gradients.

Outside of their role in development, Wnts and PHR proteins also regulate axon regeneration (Hammarlund et al., 2009; Xiong et al., 2010; Nix et al., 2011; Hollis and Zou, 2012a). The PHR proteins regulate regeneration primarily by acting on the MAP3K Dlk. Our developmental findings suggest that PHR protein signaling may also converge with Wnt signaling on β-catenin to control axon regeneration.

3.4   Materials and Methods

Genetics

The N2 isolate of *C. elegans* was propagated using standard procedures

(Brenner, 1974). Alleles used in this study included; *anc-1(e1873)*, *anc-1(e1753)*,

apr-1(ok2970), *pop-1(q645)*; *fsn-1(gk429)*; *glo-4(ok623)*, *rpm-1(ju44)*; *bar-1(ga80)*, *unc-84(e1410)*. The strain MH1870, which contains the transgene

kuIs54, and *anc-1(e1873)* were kind gifts from Dan Starr. All mutants were

constructed using standard procedures, and were confirmed using PCR or by the

associated phenotypes. All heterozygous analysis with *rpm-1* was done by

linking *rpm-1(ju44)* with *dpy-11(e224)* and balancing with *unc-42(e270)*. Other

homozygous alleles were isolated as necessary and non-dpy non-unc (*rpm-1, dpy-11/unc-42*) animals were scored. The transgenic strains used in this study

were: *muIs32* [P$_{mec-7}$GFP] and *juIs1* [P$_{unc-25}$SNB-1::GFP].

Axon termination and synapse formation

Analysis was carried out on live animals at 40x magnification using a Nikon

epifluorescent microscope and a Q-imaging camera. Animals were anesthetized

using 1% (v/v) 1-phenoxy-2-propanol in M9 buffer. Synapse formation defects

were quantified by collecting images of *juIs1* (Punc-25::SNB-1::GFP) and

manually scoring puncta numbers in Adobe Photoshop. Dorsal cord lengths were determined in μmeters using Q-imaging software. Both *bar-1-/-* and *pop-1-/-* animals displayed reproducible gaps in their dorsal cord at stereotypical locations. Care was taken to avoid collecting images at these areas. Axon termination defects were visualized using *muIs32* (Pmec-7::GFP) and manually scored.

Transgenics

Transgenic animals were generated using standard microinjection procedures. Transgenes were constructed by injection of plasmid DNA or DNA generated by PCR with P_{ttx-3}RFP (50 ng/μL) and pBluescript (50 ng/μL). Dominant negative ANC-1 was cloned as a genomic fragment (36523-37778). For *rgef-1* promoter lines, the plasmid pBG-GY360 was amplified by PCR and injected at 10 ng/μL. For the *mec-3* promoter lines, the plasmid pBG-GY370 was amplified by PCR and injected at 5 ng/μL. The *anc-1* mini-gene was constructed by ligating together 4 fragments: a cDNA fragment from 1-4154 bp using an engineered ApaI site and BamHI, a BamHI to NotI genomic fragment (14,808-24,000), a NotI to KpnI genomic fragment (24,001-36,849), and a KpnI to 3' UTR fragment containing an engineered SacII site (36,850-38,921). Plasmid encoding the P_{mec-7}ANC-1 (mini-gene, pBG161) was injected at 40 ng/μL for *anc-1-/-* rescue. Plasmid encoding P_{mec-7}BAR-1 (genomic clone, pBG-GY318) was injected at 1 ng/μL for *bar-1-/-* rescue.

Biochemistry.

Proteomic analysis of RPM-1 binding proteins, including ANC-1, was described previously (Grill et al., 2007). For biochemical analysis of RPM-1 binding to ANC-1, worms were grown in liquid culture, harvested by centrifugation, frozen in liquid N2, ground with a mortar and pestle under liquid N2, and extracted using 0.1% NP-40 lysis buffer as described previously (Grill et al., 2007). CoIPs were performed from 40 mg of total protein extract. RPM-1::GFP was precipitated using a mouse monoclonal antibody (3E6, MP Biomedical) and protein G agarose beads (Roche). For immunoblotting, precipitates were run on an SDS-PAGE gel (3-8% Tris Acetate, Invitrogen), and proteins were wet transferred to PVDF membrane in Tris acetate transfer buffer (30 volts for 24-30 hours). Blots were blocked with part-skim milk in TBST, and probed with an anti-GFP antibody (mouse monoclonal, Roche) or purified anti-ANC-1 polyclonal antibodies that were used previously (Starr and Han, 2002). Primary antibodies were detected with secondary antibodies coupled to HRP, Supersignal FemtoWest enhanced chemiluminescent reagent (Pierce), and autoradiography.

4   Conclusion

4.1   Future Directions

4.1.1   *emr-1*

While I have shown a genetic link between *anc-1* and *bar*-1, the question still remains as to the mechanism of how ANC-1 actually regulates BAR-1 signaling. Recent studies have shown that the inner nuclear membrane protein emerin negatively regulates β-catenin by restricting its accumulation in the nucleus. (Markiewicz et al., 2006). Cells transfected with emerin show lower levels of β-catenin in the nucleus as well as decreased TCF target gene activation. In addition, treatment with the CRM1 dependent nuclear export inhibitor leptomycin lead to increases in the amount of nuclear β-catenin in emerin transfected cells, suggesting emerin participates in the nuclear export of β-catenin. Isoforms of Nesprin-1 (Nesprin-1α) and Nesprin-2 (Nesprin-2β) have also been shown to bind emerin (Mislow et al., 2002; Libotte et al., 2005). Furthermore, the Nesprins bind to emerin in the same region known to bind β-catenin (Wheeler et al., 2007). Thus *anc-1* may antagonize the ability of emerin to export β-catenin. This could be by competing with BAR-1 for binding to emerin, or perhaps by preventing emerin from forming a protein complex required for its β-catenin export activity. This model is summarized in Figure 4-1.
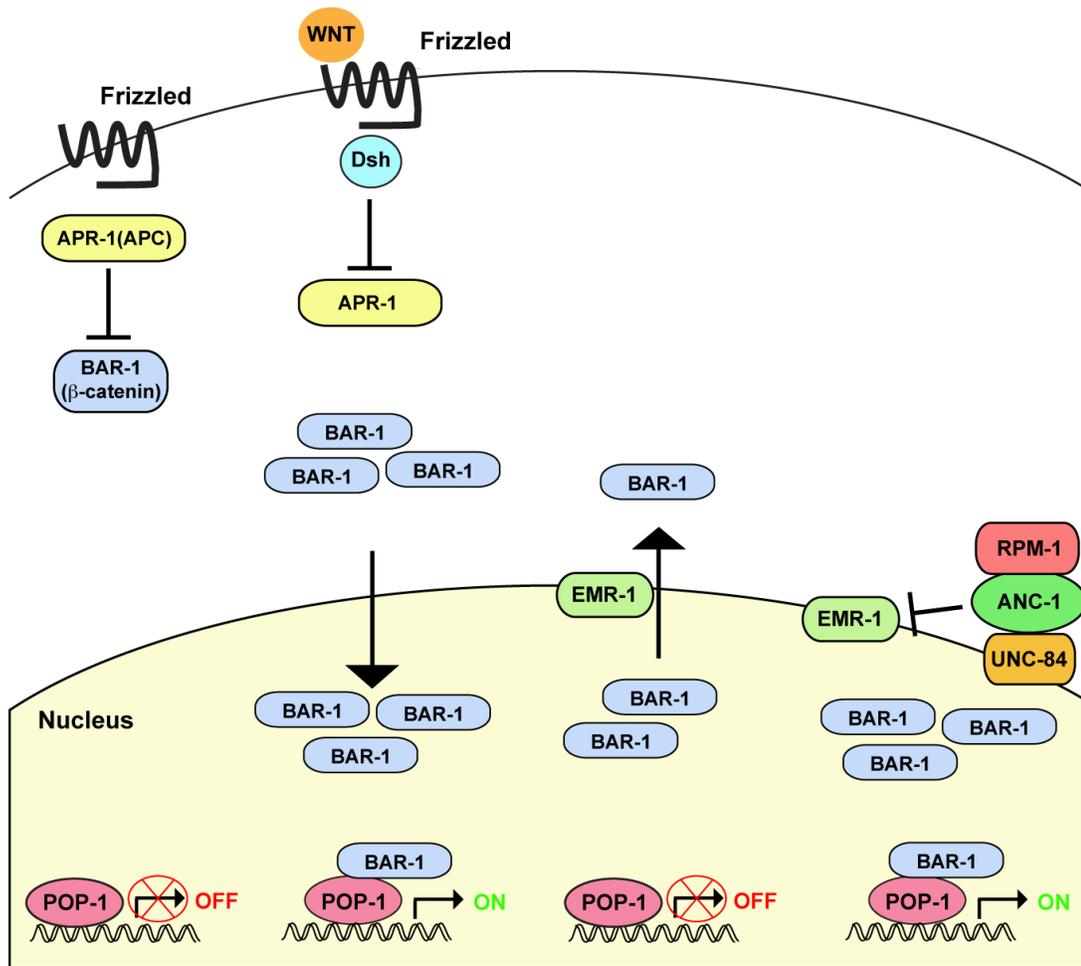
Figure 4-1 *anc-1* regulates the nuclear export of *bar-1* via *emr-1*.
Canonical Wnt signaling through Disheveled and APR-1, an APC ortholog, regulates the β-catenin BAR-1. In the absence of Wnt, APR-1 is active and inhibits BAR-1. In the presence of Wnt, APR-1 is inhibited and BAR-1 activity is increased. Higher levels of BAR-1 lead to increased activation of the transcription factor POP-1 (TCF/LEF). I have shown that RPM-1 binds to ANC-1, and RPM-1 and ANC-1 function in the same pathway to positively regulate BAR-1 activity. I propose that the nuclear envelope protein EMR-1 functions to increase the export of BAR-1 from the nucleus, thereby decreasing nuclear levels of BAR-1 and decreasing POP-1 activation. RPM-1 and ANC-1 function as a complex to negatively regulate the nuclear export activity of EMR-1, resulting in decreased BAR-1 export and increased POP-1 activation.

To test this model, I have begun constructing and analyzing mutants for the *C. elegans* emerin *emr-1*. My model predicts that ANC-1 functions as a negative regulator of EMR-1; thus the phenotype observed in *anc-1* mutants may

be due in part to over activation of *emr-1*. Therefore mutations in *emr-1* may

suppress the axon termination defects found in *anc-1* mutants. *anc-1* mutants

alone have a very low penetrance of termination defects; thus observing

suppression is unlikely. However I do observe suppression of axon termination

defects in the enhanced *anc-1;fsn-1* double mutants when constructed with *emr-1*,

consistent with my model (Figure 4-2).
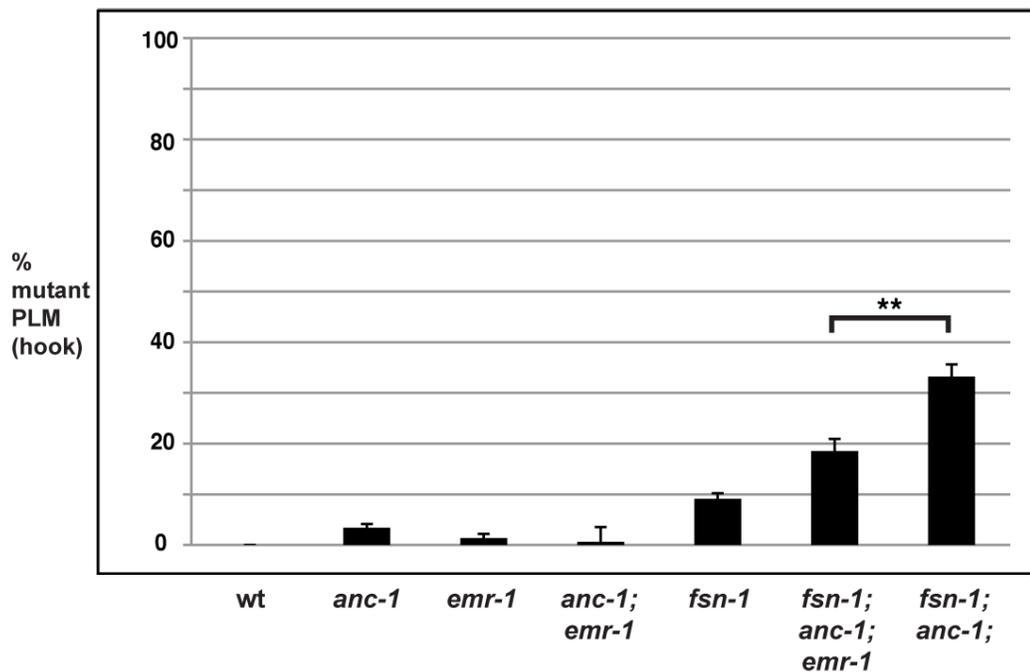
Figure 4-2 *emr-1* regulates axon termination in the PLM mechanosensory neurons.
Quantitation of axon termination (hook) defects in PLM neurons for the indicated genotypes.
Analysis was done on young adults grown at 23°C. Error bars represent standard error of the
mean. Significance was determined using an unpaired Student's t test. $**P < 0.01$,

To further examine the role of *emr-1* in the *rpm-1/anc-1* pathway, I also

plan to construct *emr-1;rpm-1* double mutants and analyze them for defects in

axon termination. If my model is correct, I would expect to see a decreased

penetrance of PLM hooks when compared to *rpm-1* mutants alone, similar to what I have shown in *apr-1;rpm-1* double mutants. Also, co-immunoprecipitation could be used to determine whether ANC-1 and EMR-1 bind. If they are found to bind, using varying genetic backgrounds may indicate whether ANC-1 and BAR-1 compete for binding to EMR-1. If ANC-1 and BAR-1 do compete for binding, I may see increased ANC-1 EMR-1 binding in a *bar-1* mutant due to the loss of *bar-1*, while an *apr-1* mutant background may show decreased ANC-1 EMR-1 binding due to an overabundance of BAR-1.

4.1.2   Role of Wnts in axon termination

My findings suggest that RPM-1/ANC-1 may act coordinately with the canonical Wnt signaling pathway to regulate the activity of BAR-1 and POP-1. Thus RPM-1 may fine tune or potentiate Wnt signals to regulate neurodevelopment. To investigate this possibility, I plan to construct mutants of the Wnt ligands and analyze them for PLM axon termination defects as well as look for genetic interactions with the *rpm-1* pathway. Because the PLM projects its axon towards the anterior of the worm, a possible model of Wnt signaling in PLM axon termination is that the PLM axon grows until it reaches a repulsive Wnt cue, which signals the axon to terminate growth. This model is supported by the finding that Wnts expressed in the tail of *C. elegans* are required to orient the growth of the PLM axon towards the anterior of the worm (Hilliard and Bargmann, 2006; Prasad and Clark, 2006).

The *C. elegans* genome encodes five Wnt ligands: *lin-44, egl-20, mom-2, cwn-1,* and *cwn-2* (Shackleford et al., 1993; Herman et al., 1995; Rocheleau et al., 1997; Thorpe et al., 1997; Maloof et al., 1999). Of these Wnts, only *cwn-2* is expressed highly in the anterior of the worm, making it the most likely candidate to mediate axon termination in the PLM neuron (Herman et al., 1995; Coudreuse et al., 2006; Song et al., 2010). My model predicts that as the PLM process grows, it encounters a gradient of CWN-2 that eventually leads to activation of the *bar-1/pop-1* pathway and subsequent termination of growth. If my model is correct, I hypothesize that mutants lacking *cwn-2* should display axon termination defects. In addition, my *anc-1* data suggests that *anc-1* and *rpm-1* may function in parallel to *cwn-2* to regulate activation of *bar-1/pop-1*, therefore *cwn-2* may enhance axon termination defects of *rpm-1* and *anc-1* mutants.

Besides *cwn-2*, *cwn-1* and *egl-20* are also possible regulators of PLM axon termination. Both *cwn-1* and *egl-20* function in establishing axon growth polarity in the ALM mechanosensory neuron (Prasad and Clark, 2006). The cell body of the ALM neuron is located in close proximity to the PLM axon termination point, indicating that these Wnts are present and functional in this area of the worm.

4.1.3　Upstream regulators of *rpm-1*

While genetic and proteomic approaches have been successful in identifying new molecules that function downstream of RPM-1, little is known about how the PHR

proteins themselves are regulated. If the PHR proteins do indeed act as a molecular switch to transition neurons from one stage of growth to the next, it would be predicted that they are under precise spatial and temporal regulation. What molecules mediate this regulation is unknown. Thus an important goal for future research is to identify the molecules/pathway that regulate PHR protein activity. In an attempt to answer this question, I have begun a biased genetic screen to identify molecules that function upstream of *rpm-1*. Although I am still in the early stages, several promising molecules have already been identified.

4.2   Summary

The formation of the nervous system depends on the creation of synapses. While it is clear that a number of events must be coordinated to form functional synapses, the molecular mechanism of how this occurs is unclear. The PHR proteins function in axon guidance (D'Souza et al., 2005; Lewcock et al., 2007; Li et al., 2008a), axon termination (Schaefer et al., 2000; Lewcock et al., 2007), and synapse formation (Schaefer et al., 2000; Wan et al., 2000; Zhen et al., 2000; Bloom et al., 2007). Thus the PHR proteins may function as molecules that can coordinate and integrate events during neurodevelopment. Understanding how the PHR proteins function and how their signaling pathways are regulated brings us one step close to understanding how a synapse is created. In this dissertation, my studies provide evidence that two molecules, PPM-1 and ANC-1, function in the RPM signaling pathway (Figure 4-3).
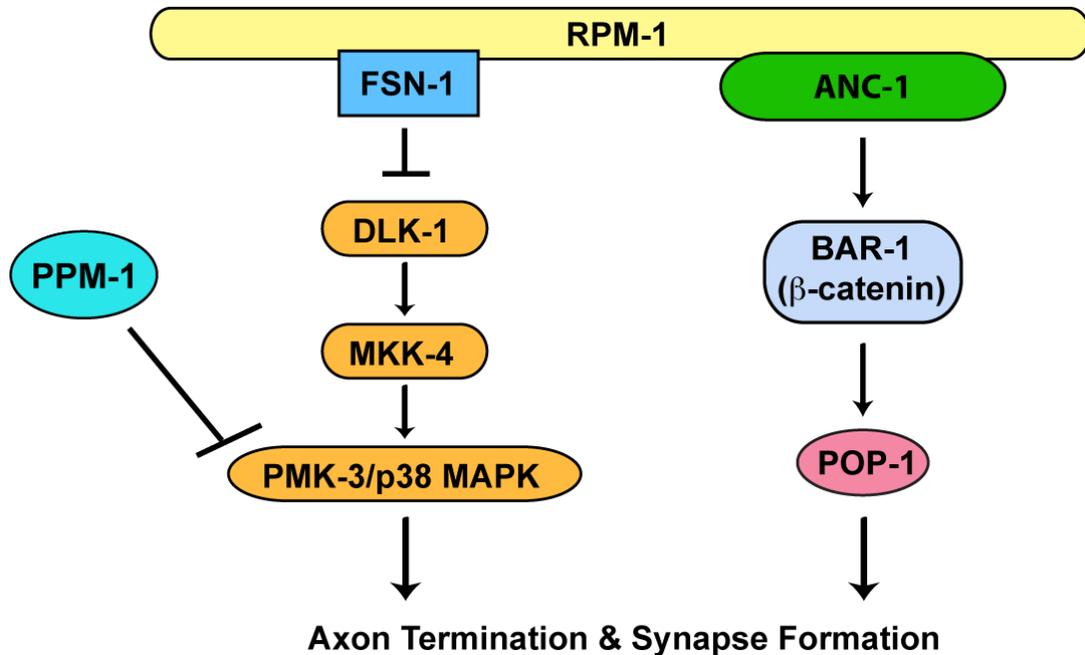
Figure 4-3 *ppm-1* and *anc-1*, two novel *rpm-1* pathway components.
The serine/threonine phosphatase *ppm-1* functions parallel to *fsn-1* and downstream of *rpm-1* to regulate the *dlk-1* pathway. *ppm-1* likely functions at the level of PMK-3. The nuclear anchorage protein ANC-1 binds to RPM-1 and functions in the regulation of synapse formation and axon termination. My genetic data also suggests that *anc-1* functions in the same genetic pathway as the β-catenin *bar-1*.

In chapter 2, I provide evidence that the serine/threonine phosphatase *ppm-1* functions as an additional regulatory mechanism for the *dlk-1* pathway. I show that the phosphatase activity of *ppm-1* is required for this regulation and that the most likely target is the p38 MAPK PMK-3. My work demonstrates a novel role for this family of proteins in neurodevelopment as well as a new mechanism for regulating the *dlk-1* MAPK cascade in neurons.

In chapter 3, I show that the nuclear anchorage proteins ANC-1 binds to RPM-1. I find that *anc-1* functions in the *rpm-1* pathway to regulate axon

termination as well as synapse formation. Furthermore, my genetic analysis

indicates *anc-1* functions through the β-catenin *bar-1*. This study provides a new

mechanism by which RPM-1 functions, as well as describing a novel role for

ANC-1 in neurodevelopment.

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