THE ROLE OF RNA-BINDING PROTEINS IN DENDRITE MORPHOGENESIS

A Senior Thesis Presented to

The Faculty of the Department of Biology,

The Colorado College

By

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Bachelors of Arts Degree in Biology

20th day of May, 2013

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Abstract

Neurons have highly asymmetric cellular morphologies and polarized cellular functions that are necessary for establishing neural circuitry and for proper functioning of the nervous system. Specialized processes, called dendrites, are used by neurons for reception of stimuli, while axons function in the transmission of signals. In neurons, mRNA localization and translational repression are used to change the protein composition of various regions of the cell, allowing for distinct axonal and dendritic morphologies and environments that are equipped for their various cellular tasks. A significant portion of the eukaryotic genome encodes for RNA-binding proteins (RBPs), which play important roles in localizing and translationally regulating RNAs. Since studies have shown that a large number of mRNAs are localized within dendrites, this suggests that the RBPs contribute broadly to neuronal development and function by localizing and regulating mRNAs. Based on a previous screen of RBP-encoding genes that affect dendrite morphogenesis in dendritic arborization neurons (da neurons) in *Drosophila* that identified 89 genes (Olesnicky, Killian, and Gavis; in preparation), I extended this screen to determine if any of these evolutionarily conserved RBP genes are important for dendrite morphogenesis in *C. elegans* PVD neurons as well. A significant decrease in dendritic arborization was found in *dcr-1* mutants and preliminary results suggest that *sup-26* and *mtr-4* mutants may have decreased 3rd and 4th order dendritic branching. In addition, several other candidate genes are currently being investigated. Thus far, the results suggest that DCR-1/Dicer, an RBP involved in the microRNA pathway, SUP-26/Alan Shepard, an RBP implicated in translational control of mRNAs,

and MTR-4/L(2)35Df, a component of the eukaryotic RNA exosome play an evolutionarily conserved role in dendrite development in flies and worms.

Chapter 1-Introduction

The Role of Dendrites

Neurons are highly polarized cells that communicate with one another through electrical signals called action potentials. Communication occurs across small gaps between neurons referred to as synapses, where the post-synaptic components of these connections are generally dendrites (Jan & Jan, 2003). Dendrites are delicate processes originating from the soma of most neurons. They can form elaborate branching networks connecting many neurons. Their role is to carry action potentials between neuronal cells to send sensory and motor signals to and from processing centers such as the brain and spinal cord (Häusser et al., 2000). Dendrite growth is a highly dynamic process in which connections between cells frequently changes depending on sensory input (Bestman & Cline, 2008). There are many, possibly thousands of types of neurons and each has its own distinct dendritic arborization pattern. Axons and dendrites form from neurites, which are small projections out of the soma, to create these elaborate connections. However, it is not very well understood how dendrite development is controlled. Many of the factors affecting growth have yet to be identified (Jan & Jan, 2003). What is known is that synaptic plasticity requires new protein synthesis (Darnell et al., 2011).

The role of RNA binding proteins

Dendrite morphogenesis is an energy consuming process and therefore neurons need orderly mechanisms to maintain efficiency. Post-transcriptional regulation is one effective way to ensure that cellular components are present when and where they should be (Figure 1). RNA-binding proteins (RBPs) are an important component of post-transcriptional regulation. They allow for the fine-tuning of gene expression by stabilizing mRNAs, repressing translation, activating translation, and localizing mRNAs within the cell. RNAs associated with their RBPs are called ribonucleoprotein (RNP) complexes. Eukaryotic cells encode many RBPs, with up to thousands of different varieties in vertebrates. There is so much diversity among RBPs that there may be a unique RNP for each RNA (Glisovic et al., 2008). Because RBPs control when and where mRNA is translated, they allow cells to respond more quickly to changing environmental conditions than *de novo* transcription (Siomi & Dreyfuss, 1997). This is especially important to proper neuron development, when synapses frequently change depending on sensory stimuli (Bestman & Cline, 2008).

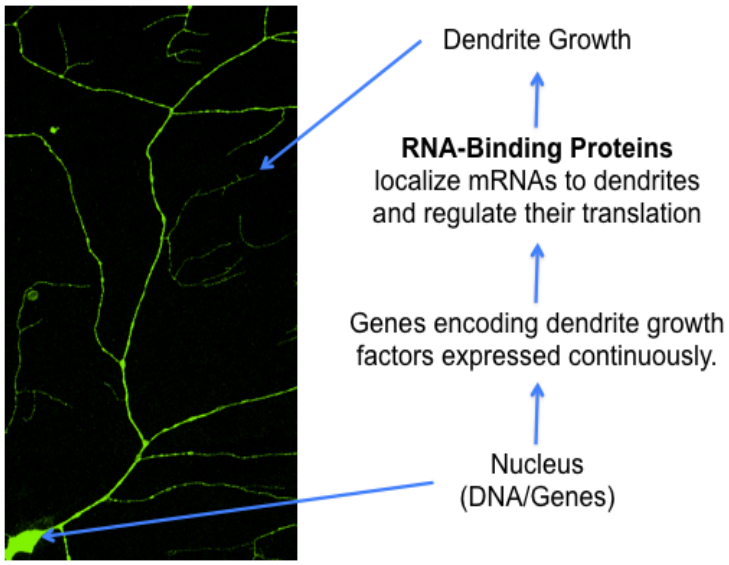

Figure 1. Hypothesis for the role of RNA-binding proteins in dendrite morphogenesis.

All RNA-binding proteins contain at least one RNA-binding domain. These domains have many basic residues that serve to interact with the negatively charged RNA and are rich in β sheets because both the major and minor grooves of RNA are too small to interact with α helices, unlike DNA and DNA-binding proteins. The binding of RBPs to RNA serves many functions; it may alter the RNA structure to facilitate or hinder interactions with proteins or complementary RNAs, prevent higher-order RNA structures, and provide localization or targeting signals for transport of RNA molecules to distinct intracellular locations. The spatial regulation of protein synthesis is important to cellular organization, especially in polarized cells such as neurons. The localization of proteins is an efficient way to establish correct protein positioning and prevent harmful protein-protein interactions from occurring elsewhere in the cell. It has been found that the signals for mRNA localization lie in the 3'-UTR, and that the timing of translation can be regulated by the modification of the 3'-UTR. The idea of post-transcriptional localization came from Joahchim Hämmerling's observation in the 1940s in the algae *Acetabularia* that nucleus-derived information for cap formation traveled several centimeters from the nucleus at the base of the stalk to the apical tip of the stock. He also demonstrated that this information can be stored in the apical tip for several weeks (Siomi & Dreyfuss, 1997). This algae shares many similarities with neurons, in which the rhizoid containing the nucleus, the stalk, and the cap can be compared to the cell body, axon or dendrite, and the growth cone, respectively. However, the regulation of neuron growth is a bit more complex than that in *Acetabularia*, as neurons are complicated by the number of branches. In response to sensory stimulus, neurons need to strengthen or change the

connections of only some dendrites to ensure proper adaptation to a changing environment. The idea of local protein synthesis is not a new one, as it was first proposed by David Bodian in 1965 when he discovered ribosomes isolated within dendrites. The goal of this study is build upon the existing body of knowledge regarding dendrite growth through the identification of the mechanisms by which local translation may be achieved.

Da and PVD neurons as a model

Both *Caenorhabditis elegans* and *Drosophila melanogaster* serve as excellent model organisms for higher eukaryotes due to long histories of research, relatively complex eukaryotic body systems, and short life cycles. Because these animals are well understood by the scientific community, they are easily handled and there is a wealth of research tools such as preexisting mutants and RNAi libraries. The body of knowledge and implements surrounding these organisms offers an excellent platform for further research.

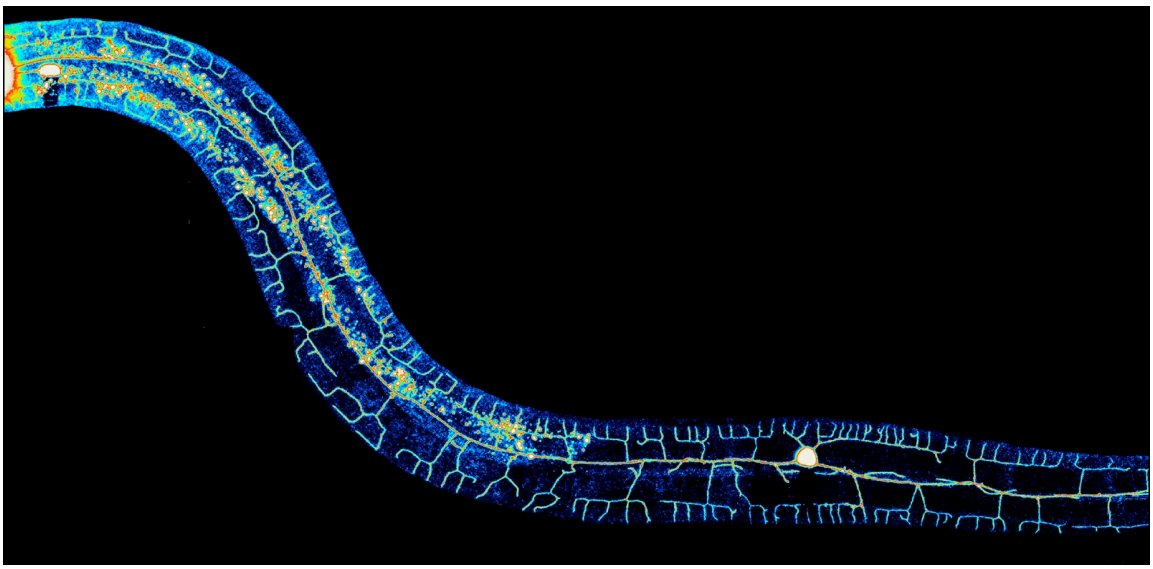

Figure 2. Fluorescently labeled *C. elegans* PVD neuron (Oren-Suissa et al., 2010).

C. elegans is an especially good model due to a number of characteristics. It is a small transparent nematode that is about a millimeter in length and is very easy to culture in the laboratory on a diet of *Escherichia coli*. It also has a short life cycle, reaching adulthood within three days of hatching from the egg. Each self-fertilizing adult hermaphrodite has about 300 progeny in a short period of a few days. Due to its transparency, internal structures are easily viewed in the living organism using light and epifluorescence microscopy. Despite its small size, it is a sophisticated multicellular animal with many similar structures to more advanced animal organisms including muscle tissue, a hypodermis (skin), intestine, nervous system, and reproductive system (Kaletta & Hengartner, 2006).

The nervous system is the most complex tissue in *C. elegans*, contributing 37% of the somatic cells in hermaphrodites. There are 302 neurons and 57 glial cells comprising 118 morphologically distinct neuron classes. Despite their simple appearance, most *C. elegans* neurons use highly evolved information processing mechanisms that use a variety of neurotransmitters and neuropeptides. Due to this diversity in function and cell types, these nematodes are a good model for how the nervous system develops (Hobert, 2010).

While most *C. elegans* neurons are relatively simple in structure, the PVD neuron is atypical, in that it has a complex dendritic branching pattern, making this sensory neuron an especially excellent model (Albeg et al., 2011). The PVD neuron forms net-like dendrites that cover the body surface directly beneath the hypodermis, creating a non-overlapping sensory field. There are two of these neurons in each organism (PVDL

and PVDR) that span the entire length of the central body region (Figure 2). Two neurites, an anterior and a posterior, branch from the PVD soma located in the mid-region of the body (Goodman, 2006). PVD neurons are polymodal nociceptors, which detect noxious stimuli including toxic chemicals, harsh touch/painful stimuli, and the extremes of heat and cold. PVD neurons have been shown to use DEG/ENac ion channels to sense harsh touch and TPRA-1 channels to detect cold temperatures (Chatzigeorgiou, 2010). It has been suggested that the application of harsh mechanical stimulus to the body wall stretches tertiary branches by displacing quaternary processes. This observation is reenforced by the finding that quaternary processes lack the DEG/ENaC mechanosensory ion channels that open locally when a dendrite is stretched (WormAtlas). In addition to nociception and thermoreception, PVD neurons have also been found to be involved in proprioception, as ablation of PVD neurons leads to defective body posture. Fourth-order dendrites branch across muscle quadrants and monitor muscle tension, thereby relaying body-positioning information to the central nervous system. Defective PVD neurons have been shown to cause a reduced bending angle when *C. elegans* movement is analyzed (Albeg et al., 2011).

Like the PVD neuron, dendritic arborization (da) neurons in *D. melanogaster* are used for nociception and locomotion. They are believed to be an evolutionary adaptation to parasitoid wasp stings, helping larva to avoid the painful stimuli (Hwang et al., 2007). There are four different classes of da neurons that vary slightly in function. Of the four subtypes, Class IV neurons have the most complex arbors, in which the dendrites create a non-overlapping field that completely tiles the larval body wall (Grueber et al., 2003,

Olesnicky et al., 2012). GFP labeled Class IV da neurons are easily visualized through the larval cuticle, making these neurons ideal models to observe dendrite development (Olesnicky et al., 2012). Because nociceptors are typically highly branched in most organisms (Albeg et al., 2011), both PVD and da neurons serve as excellent models for examining dendrite morphogenesis.

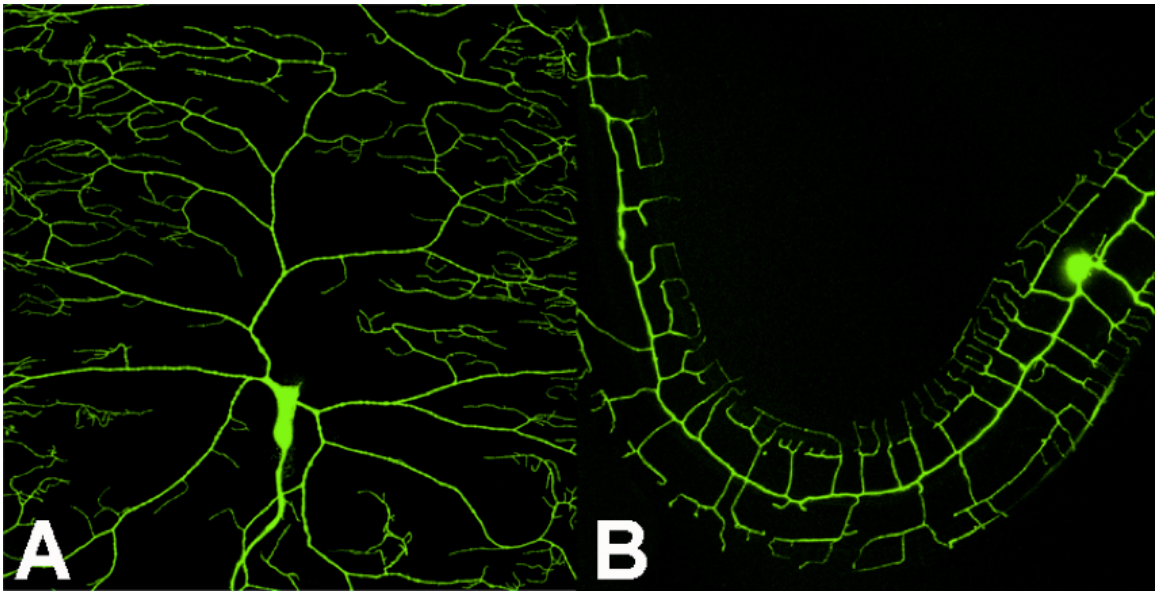

Figure 3. (A) *Drosophila* da neuron. (B) *C. elegans* PVD neuron.

A study to identify evolutionarily conserved RBPs that are required for dendrite morphogenesis

In an effort to identify all of the RNA-binding proteins that are important for dendrite morphogenesis, *Drosophila* RBP-encoding genes were screened in larva by Olesnicky, Killian and Gavis (unpublished), which revealed 89 RBPs that affect dendrite morphogenesis in Class IV da neurons. The screen was conducted using cell type-specific RNA interference (RNAi), in which the expression of the majority of the RBPs examined was knocked down specifically in Class IV da neurons. This was accomplished with the

use of *UAS-RNAi* transgenes, expressed using *GAL4*^{477} (Grueber et al., 2003), which drives expression specifically within differentiated Class IV da neurons late in embryogenesis and during larval development. This study screened 302 of the 403 RBP genes known in *Drosphila* (Olesnicky, Killian, and Gavis in preparation). Changes in average dendritic termini, length, or branching patterns constituted positive results. For example, both *dcr-1* and *yu* exhibited patchy branching and field coverage.

Table 1: A list of candidate gene RBP genes in *C. elegans* for the proposed screen.

The *Drosophila* RBPs listed are those that give dendrite morphogenesis defects in da neurons as per Olesnicky, Killian, and Gavis (in preparation). The *C. elegans* homologs were found by a BLAST search (BLAST score and *C. elegans* RBP are listed). *C. elegans* RBPs in **bold** are enriched in PVD neurons compared to other cell types (Smith et al, 2010). Mutations are available for many (38/53) of the *C. elegans* RBP genes: 23 of the mutants are homozygous viable, 5 are sterile, 10 are lethal, and there are no

***Drosophila* RBP**	**BLAST Score**	***C. elegans* Homologs**
Homozygous viable null/deletion mutants.		
Ssx	1E-47	**EXC-7**
Spoon/Yu	3E-30	**C56G2.1**
CG4119	8E-28	**W04D2.6**
L(2)35Df	0	**MTR-4**
Snf	5E-70	**RNP-3**
CG5168	2E-98	WDFY-2
CG5439	2E-08	**F13E9.1**
Nos	1E-07	NOS-1
Stau	1E-42	**STAU-1**
Loq	6E-09	D1037.1
Glo	3E-32	**SYM-2**
Pum	6E-128	**PUF-9**
X16	2E-25	**RSP-6**
Brat	2E-172	**NCL-1**
Orb	8E-69	CPB-3
CG11726	8E-06	**HRPF-1**
Bl	2E-39	F26B1.2
CG14718	2E-08	FUST-1
Mib2	7E-23	T28D6.4
Shep	5E-60	SUP-26
MASK	2E-154	**R11A8.7**
Mbl	3E-57	MBLI-1
CG34354	1E-77	TIAR-1
Homozygous sterile null/deletion mutants.		
Dcr-1	3E-124	DCR-1
Gem3	2E-67	CGH-1
SF2	1E-70	RSP-3

***Drosophila* RBP**	**BLAST Score**	***C. elegans* Homologs**
Homozygous lethal null/deletion mutants.		
RpS3	6E-124	RPS-3
Set1	2E-71	**SET-2**
U2AF38	9E-90	UAF-2
Srp54	8E-45	**RSP-7**
CG4887	2E-23	**T08B2.5**
Pit	1E-175	**B0511.6**
Hel25E	0	HEL-1
Swm	3E-16	**B0336.3**
CG10466	2E-48	**C30B5.4**
Sqd	3E-41	SQD-1
No mutants available.		
Cyp33	5E-124	CYN-13
Smg	7E-17	**ZC190.4**
CG5589	6E-112	**R05D11.4**
CG5800	1E-172	Y23H5B.6
CG6418	0	**C46F11.4**
Ddx1	0	Y55F3BR.1
CG9107	1E-11	ZC434.3
Sm	3E-69	C44B7.2
Rin	3E-10	**K08F4.2**
Dbp73D	4E-59	ZK686.2
CG11266	6E-84	**Y55F3AM.3**
CG11454	5E-10	**SAP-49**
CG11505	1E-33	LARP-5
Aret/Bru	9E-118	**ETR-1**
CG32706	3E-11	**F57B10.8**

Since all eukaryotic genomes encode for many RBPs, it is possible that there is a conserved set of RBPs that regulate dendrite morphogenesis in diverse animals. To test this hypothesis and to identify these conserved RBPs involved in dendrite regulation, a BLAST search of amino acid sequences was conducted to identify the *C. elegans* homologs of the RBP genes that are important for dendrite development in fly (Table 1). Of these 53 genes, 11 are currently being evaluated. Previous analysis of *ncl-1* mutants (performed by Genevieve Kerr of Colorado College) showed reduced fourth-order branching (Figure 4). Here I show that *dcr-1* mutants have decreased PVD dendrite branching and show that *sup-26* and *mtr-4* mutants may have defects in PVD dendrite development, but additional tests are needed. *rnp-3* mutants along with the RNAi-mediated knockdown of *C56G2.1* and *larp-5* showed no significant PVD dendrite defects. Future research will include screening more RBP genes and functional assays to assess whether there is a diminished behavioral response to harsh touch or altered movement in RBP mutants and RNAi treated worms.

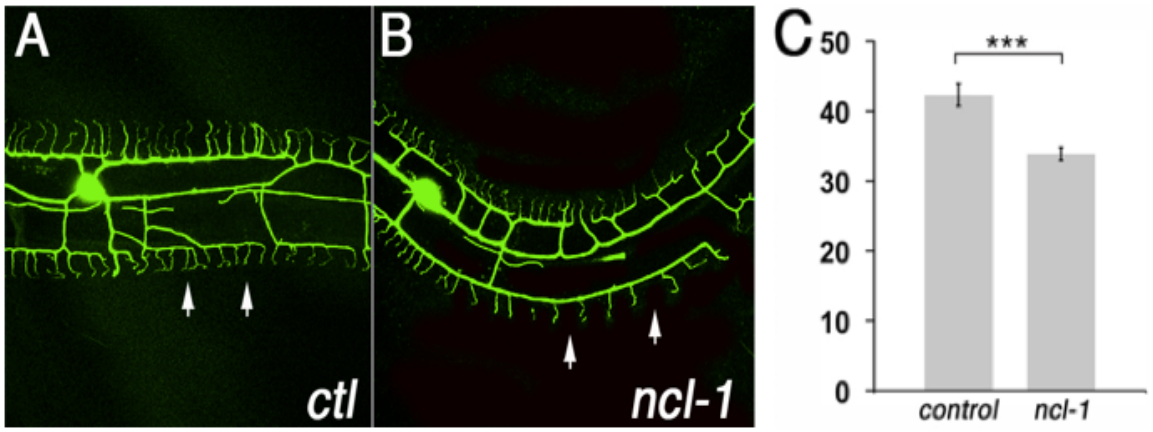

Figure 4. Previous research on the effects of diminished RBP gene function in *ncl-1* mutants. (A) Wild type PVDs have dendritic branches that tile the hypodermis of the worm, while *ncl-1* mutants (B) display fewer 4th order dendritic branches and have gaps in hypodermal coverage (compare arrows in A and B). (C) Bar graph showing significantly fewer dendrites in *ncl-1* mutants (mean ± SE). *** P<0.001. Images provided by Eugenia Olesnicky, University of Colorado Colorado Springs and Genevieve Kerr, Colorado College.

In addition to screening RBP genes for PVD dendrite function in *C. elegans*, I also contributed to follow-up studies on the *Drosophila* RBPs to determine how these proteins function together. Brat and Nos, two RBPs, were analyzed to identify whether they are cofactors of the RBP Cg11505 by examining *Drosophila* Class IV da neurons. *cg11505* was overexpressed which causes a decrease in dendritic termini (Olesnicky and Gravis, unpublished). If either Nos or Brat are required for Cg11505 function, lowering the dosage of these other two RBPs using heterozygous mutants should increase the number of dendrites to be closer to wild type numbers. Preliminary results suggest that Brat, but not Nos is a cofactor of Cg11505.

Chapter 2-Methods

C. elegans handling

C. elegans strains were raised on 10 mL LB agar plates (2.5 g tryptone, 3 g NaCl, 17 g agar, 5 mg cholesterol, 1 mL 1 M $CaCl_2$, 1 mL 1M $MgSO_4$, 25 mL KP Buffer (pH 6.0), ddH_2O to 1L) seeded with *E. coli* OP50. The plates were stored at 20 °C in an incubator. Individuals were moved from plates using sterile platinum wire picks.

Strains

The following *C. elegans* strains were used to observed dendrite morphogenesis: NC1841 (*wdIs52 [*$P_{F49H12.4}$*::*GFP] *rwIs1* [$P_{mec\text{-}7}$*::*RFP] II)CB1339 (*mec-4(e1339)* X), DJK62 (*wdIs52* II; *sup-26(gk462)* III), DJK63 (*wdIs52* II; *dcr-1(ok247)/hT2[GFP]*

(I;III)), DJK66 (*wdIs52* II; *rnp-3(ok1424)* IV), DJK67 (*wdIs52* II; *mtr-4(ok2642)* IV), DJK68 (*wdIs52* II; *sid-1(pk3321)* V; *uIs69 [$P_{unc-119}$::sid-1, P_{myo-2}::Cherry]* V), RB1308 (*rnp-3(ok1424)* IV), PD8753 (*dcr-1(ok247)* III/*hT2[bli-4(e937) let-?(q782) qIs48]* (I;III)), RB1997 (*mtr-4(ok2642)* IV), VC901 (*sup-26(gk426)* III).

Mutant constructions

The mutant strain NC1841, which carries GFP and RFP reporters associated with the PVD neuron, was crossed into RBP mutant strains by isolating males from either the NC1841 strain or the RBP strain of interest. NC1841 worms carry the PVD promoter F49H12.4 driving expression of the *GFP* gene. A Leica Stereo-Fluorescence microscope was used to identify individuals carrying the PVD GFP reporter. Because the *dcr-1* mutants cannot be maintained as homozygotes since the mutation causes sterility, it was balanced as a heterozygote using the translocation balancer *hT2* (chromosomes I and III). This balancer is useful because it causes lethality in homozygous individuals and contains a GFP insertion that identifies heterozygous individuals (Edgley et al., 2006). Visual markers such as *hT2* and *unc* genes were used to positively select for individuals carrying the RBP gene of interest for some strain constructions by using markers located on the same chromosome as the RBP gene.

RNAi

RNAi feeding procedures were adapted from Fraser et al., 2000. *larp-5* and *C56G2.1 E. coli* samples were taken from an RNAi library (Fraser et al., 2000) and

streaked on LB agar ampicillin tetracycline plates (2.5 g tryptone, 3 g NaCl, 17 g agar, 5 mg cholesterol, 1 mL 1 M $CaCl_2$, 1 mL 1M $MgSO_4$, 25 mL KP Buffer (pH 6.0), 100 mg ampicillin, 15 mg tetracycline, ddH_2O to 1L). The plates were incubated at 37 °C overnight until colonies were visible. Then three individual colonies from each plate were separately inoculated in 5 mL LB ampicillin broth and grown for 18 hrs at 37 °C. LB ampicillin 10 mL RNAi plates (2.5 g tryptone, 3 g NaCl, 17 g agar, 5 mg cholesterol, 1 mL 1 M $CaCl_2$, 1 mL 1M $MgSO_4$, 25 mL KP Buffer (pH 6.0), 10 mL 20% lactose, 100 mg ampicillin, ddH_2O to 1L) were then each seeded with 0.5 mL of the cloudy bacterial solution. The plates were allowed to dry before allowing worms to feed on the *E. coli*. All hatch-off procedural steps were carried-out on RNAi plates for RNAi-treated worms.

Hatch-off

To synchronize hermaphrodite worms' ages for scoring, a hatch-off was performed. First ~60 gravid adult worms were placed on two plates (~30 per plate), and allowed to lay eggs overnight. Once L1 stage worms were visible, a few millimeters of M9 buffer (5.8 g Na_2HPO_4, 3.0 g KH_2PO_4, 5.0 g NaCl, 1 mL of 1M $MgSO_4$, ddH_2O to 1L) were poured onto the plates and swirled to dislodge adult and young worms into the liquid, while the eggs remained stuck to the bacteria. The M9 was poured-off with the worms and the plates were inspected under a microscope to ensure only eggs remained. After one hour, the newly hatched worms that were now within one hour of age of one another, were collected into a 15 mL tube using a few milliliters of M9 buffer. The tube was spun down in a clinical centrifuge at 3000 rpm for 5 minutes. Using a Pasteur

pipette, the supernatant was removed and discarded. The pellet containing the worms was then rinsed with 1.5 mL of M9 by spinning the tube down again at 3000 rpm for 5 minutes and discarding the supernatant. The pellet was aspirated from the tube using a Pasteur pipette and the worms were squirted onto a fresh plate. The transfer of worms onto the fresh plate was considered time zero. This process was repeated several times to collect several batches of synchronized worms for each strain. Every hatch-off procedure included using either the NC1841 or DJK68 stain as a control, in addition to the RBP strain of interest.

Scoring of PVD dendrites

C. elegans hermaphrodites were selected for scoring at the young adult stage, just after transitioning out of the L1 stage and before egg laying, identified by an "M" shape associated with the developing vulva. This occurred typically between 46 hours and 55 hours of development, depending on how the mutations affected the rate of development. Approximately 20 of these individuals were mounted onto 2% agarose pads on glass microscope slides, ~5-8 worms per slide. They were paralyzed using 5 µL of levamisole solution (3 µL 1 M levamisole in 5 mL M9 buffer). A Zeiss Axioimager Fluorescent microscope was used to observe the PVD neuron at 40X using the GFP filter. Dendritic termini were counted posterior to the PVD cell body and recorded. Statistical significance was measured by performing the Student's *t*-test.

***Drosophila* da neuron tracing and analysis**

Drosophila larval Class IV da neurons from wandering larval stages were imaged using a confocal microscope at 108-120 hours after egg laying. Larval fillet preparations (Ye et al., 2004) were immunostained with 1:350 Alexa Fluor 488 rabbit anti-GFP (Invitrogen), mounted in 70% glycerol, and imaged on a Leica SP5 confocal microscope using a 40x/1.25 NA oil objective. ddaC neurons from the second through fifth abdominal segment were imaged and scored. The total number of dendrites, mean branch length, and sum branch length were quantified in Z series projections of a single ddaC neuron. All dendritic termini visible within the field of view were analyzed from neuronal tracings generated with NeuronJ (Meijering et al., 2004) and statistical significance was measured by performing the Student's *t*-test. The following fly strains were used to drive dsRNAi hairpins within Class IV da neurons and to illuminate Class IV da neuron morphology: *GAL4^{477}, UAS-mCD8::GFP* (Grueber et al., 2003), *ppk-GAL4, UAS-mCD8::GFP* (Grueber et al., 2007), *UAScg11505, brat^{11},* and *nos^{rc}*.

Chapter 3-Analysis of CG11505, an RBP that is important for dendrite morphogenesis in *Drosophila* da Neurons

In an effort to identify post-transcriptional regulators of the *Drosophila* RNA-binding protein gene *cg11505*, genetic epistasis analysis was conducted to identify

Cg11505 cofactors. Preliminary data conducted by E. C. Olesnicky et al. (unpublished) shows that *cg11505* functions in dendrite elaboration of *Drosophila* Class IV da neurons, and that *cg11505* is maternally expressed and localized in the posterior pole of developing fly embryos. The candidate regulators examined were *brat* and *nos.* The RBP Brat was chosen for exploration because a genome-wide yeast two-hybrid experiment identified it as a potential regulator of Cg11505 (Murali et al., 2010). Nos was identified to be a candidate regulator of Cg11505 function because Brat and Nos function together in multiple developmental events, including dendrite elaboration (Olesnicky et al., 2012). Additionally, *nos* mutant sensory neurons display similar dendritic phenotypes to *cg11505* deficient neurons and maternal *nos* mRNA is localized in a similar pattern to *cg11505* mRNA in the posterior pole of early *Drosophila* larva. Previous research has also shown that *nos* mRNA is localized within the dendrites of Class IV da neurons (Brechbiel & Gavis, 2008).

A *UAScg11505* construct containing the endogenous *cg11505 ORF and* 5' and 3'-UTRs, which likely contain contain the elements important for *cg11505* post-transcriptional regulation, was used to overexpress *cg11505. UAScg11505* was overexpressed using the Class IV da neuron specific driver *ppkGal4 or GAL4*^{477}. Overexpression of *cg11505* has been previously shown to cause a decrease in dendritic arborization in Class IV da neurons (Olesnicky et al, unpublished). The dosage of candidate regulators was then lowered using genetic mutants heterozygous for *nos* and *brat,* with the idea being that a lower dosage of a cofactor necessary for Cg11505

function will suppress the overexpressed *cg11505* phenotype, increasing the number of dendrites to more closely reflect the wild type.

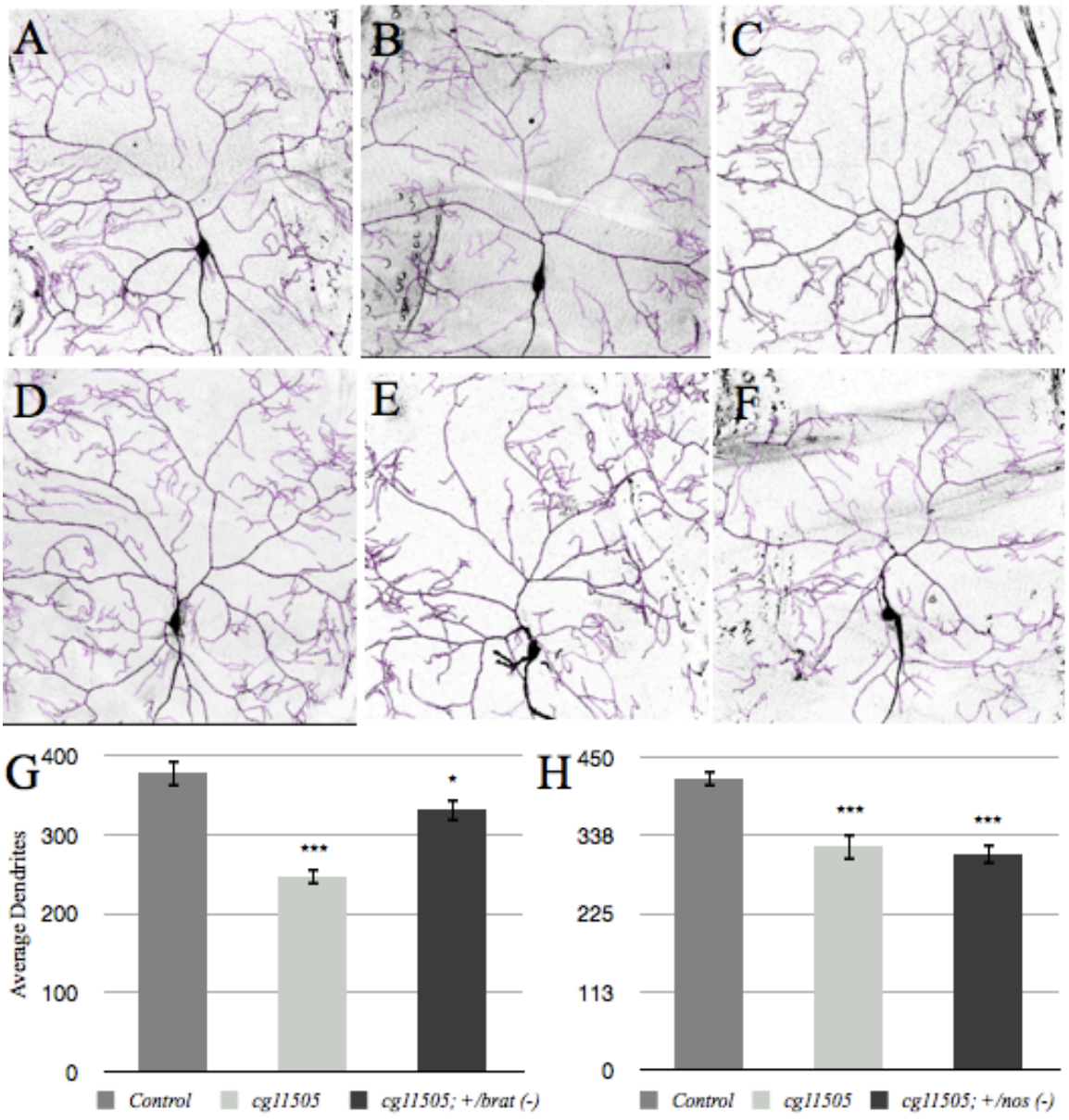

Figure 5. Neuron epistasis. (A) Tracing of *brat* control Class IV da neuron. (B) Tracing of *cg11505* overexpression. (C) Tracing of *cg11505* overexpression in *+/brat (-)* mutant heterozygote. (D) Tracing of *nos* control. (E) Tracing of *cg11505* overexpression. (F) Tracing of *cg11505* overexpression in *+/nos (-)* mutant heterozygote. (G) Bar graphs of average dendrites in Brat Cg11505 cofacator experiment (mean ± SE). (H) Bar graphs of average dendrites in Nos Cg11505 cofactor experiment. *** P<0.001, * P>0.05.

In this epistasis study, overexpression of *cg11505* in *brat* mutant heterozygotes displayed no significant difference from the control. Because of this finding and the discovery that larva with *UAScg11505* overexpression had significantly fewer dendritic termini than the control, it appears that Brat interacts with Cg11505 to influence dendrite morphogenesis in Class IV da neurons (Figure 5 A-C, G). The lower dosage of Brat in *brat* mutant heterozygotes apparently decreased the affects of the overexpression of *cg11505,* thus demonstrating an interaction between the two RBPs. Animals with *cg11505* overexpression averaged 247 dendrites with a standard deviation of 46.6, *brat* heterozygotes with *cg11505* overexpression averaged 331 dendrites with a standard deviation of 69.0, and the controls averaged 377 dendrites with a standard deviation of 73.6.

Nos does not appear to be a regulator of Cg11505 as *nos* mutant heterozygotes still had significantly fewer dendritic termini than the control (Figure 5 D-F, H). In this portion of the study, larva with overexpressed *cg11505* averaged 320.8 dendrites with a standard deviation of 87.4, *nos* heterozygotes with *cg11505* overexpression averaged 309.4 dendrites with a standard deviation of 65.9, and the controls averaged 419 dendrites with a standard deviation of 54.0.

Chapter 4-Screening candidate RBP genes for a role in *C. elegans* PVD morphogenesis

In an effort to identify RNA-binding proteins that are conserved in their evolutionary function in dendrite morphogenesis, *C. elegans* candidate genes identified through a screen of *Drosophila* RBPs were analyzed phenotypically in PVD neurons.

DCR-1/Dicer

DCR-1 is a *C. elegans* RNA-binding protein involved in small-RNA-mediated gene-silencing pathways. The Dicer family of proteins are endoribonucleases that contain dsRNA binding motifs and RNase III domains that are used to cut dsRNA into small single-stranded fragments of ~21 nucleotides called small interfering RNAs (siRNAs) or microRNAs (miRNAs). miRNA is used by the cell to target RNA complementary to the miRNA for destruction using RNA-induced silencing complexes (RISC) in a process called RNA interference (RNAi). In addition to being a vital component of the pathway involved in the destruction of foreign viral dsRNA, it has been suggested that DCR-1 is required for proper chromosome segregation, production of endogenous small RNAs of unknown function, silencing endogenous genes, and a temperature-dependent process required for sperm function (Duchaine *et al*., 2006). DCR-1 may be involved in dendrite morphogenesis because Dicer is enriched in the dendrites of mice brains (Lugli et al., 2005). Several other studies have identified certain miRNAs that are localized specifically within dendrites and are important for regulating gene expression in response

to neuronal activity (Khudayberdiev et al., 2009, Wayman et al., 2008). In fact, a significant fraction of miRNAs have been found to be enriched or specifically expressed in the nervous system where they play a role in development and may be important to neuronal plasticity (Kye et al., 2007, Wayman et al., 2008).

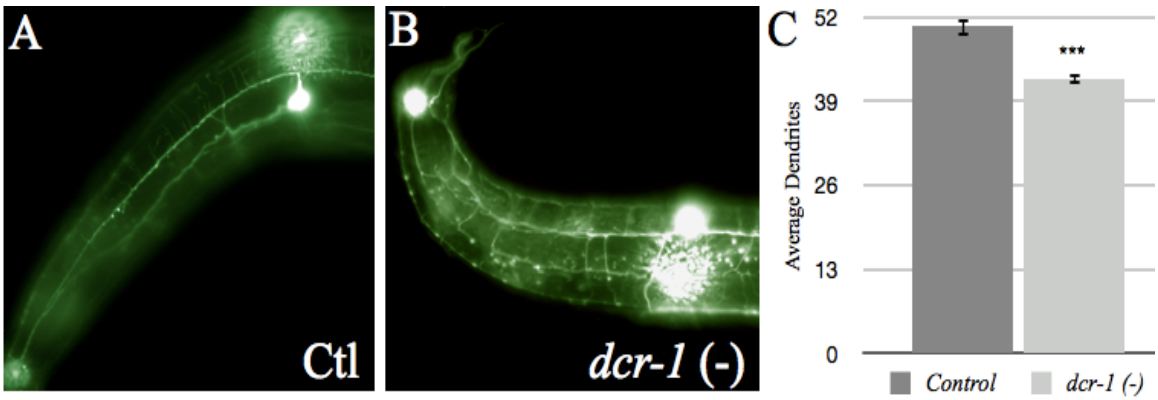

Figure 6. Analysis of DCR-1's role in dendrite morphogenesis. (A) Wild type PVD neuron. (B) *dcr-1* mutants show fewer branches on average and more irregular branching than the control. (C) *dcr-1* mutants have significantly fewer dendrites than the control (mean ± SE). *** P<0.001.

Scoring of *dcr-1; wdIs52* mutants revealed a 16% reduction in dendrites as compared to the NC1841 control (Figure 6). The control averaged 50.57 dendrites with a standard deviation of 6.27 for 21 individuals, while the DCR-1 constructs averaged 42.50 dendrites with a standard deviation of 3.71 for 18 individuals. Some *dcr-1* mutants demonstrated more aberrant branching of PVD neurons than the control.

SUP-26/Alan Shepard

The *sup-26* gene in *C. elegans* is homologous to the *shep* gene in *Drosophila.* This gene has been shown to function in somatic cell sex determination in *C. elegans* by negatively regulating *tra-2* translation. SUP-26 specifically binds to the TRA-2/GLI element (TGE), located in the *tra-2* 3' untranslated region. SUP-26 may repress *tra-2*

expression by associating with poly(A)-binding protein 1 (PAB-1) and thereby repressing PAB-1's translation-stimulating activity (Mapes *et al.,* 2010).

The results of *sup-26* experiments proved to be somewhat inconclusive, but indicate that the activity of SUP-26 may be involved in dendrite morphogenesis. Mutants were scored on three separate occasions; the first occasion demonstrated a 15% reduction in dendrites, while the second only demonstrated an 8% decrease, and the third a 12% reduction compared with the NC1841 control strain (Figure 7). On the earlier date, the average number of dendrites was 39.68 with a standard deviation of 5.12 for 25 *sup-26* mutants compared with 46.58 and 6.20 for 26 control animals. Later data, while still

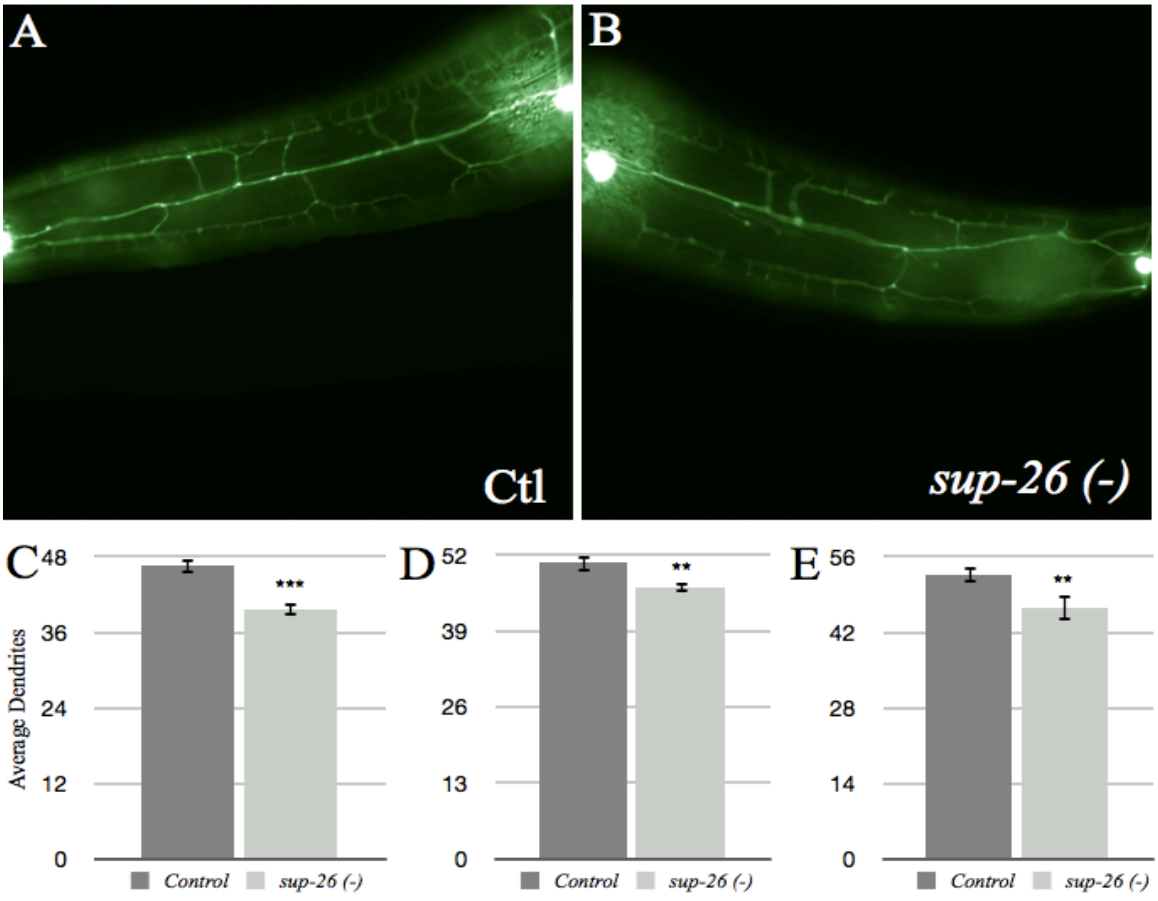

Figure 7. Analysis of the role of SUP-26 in PVD dendrite morphogenesis. (A) Wild type PVD neuron. (B) *sup-26* mutant PVD neuron displays fewer dendrites on average than the control. (C) Graph of first scoring event (mean ± SE). (D) Graph of second scoring event. (E) Graph of third scoring event. *** P<0.001, ** P<0.05.

significantly different, showed a smaller decrease with a mean of 46.50 and a standard deviation of 4.39 for 24 *sup-26* mutants compared to 50.57 and 6.27 for 21 controls**.** The most recent scoring event when analyzed gave values of 46.55, 11.04, 52.57, and 7.34 for the average dendrite counts and standard deviations of 20 *sup-26* mutants and 23 control animals respectively. *sup-26* mutants demonstrated regular branching patterns in all experiments.

MTR-4/L(2)35Df

MTR-4 is an RNA helicase that has been found to be located in the nucleolus and nucleoplasm of eukaryotic cells. It functions as an RNA-dependent ATPase that unwinds RNA duplexes in the 3' to 5' direction and is a necessary part of the eukaryotic RNA exosome. It has been shown to be important to ribosome biogenesis due to its activity in trimming 7S rRNA precursors to mature 5.8S rRNA. *mtr-4* mutations have been shown to cause defective ribosomes. In addition to its ribosomal activity, MTR-4 associates with the TRAMP (Trf4-Air2-Mtr4 Polyadenylation) complex, which adds a poly(A) overhang to the 3' end of aberrant or unstable transcripts to mark them for degradation. MTR-4 appears to function in this complex by unwinding secondary RNA structures. The amino acid sequence of MTR-4 is highly conserved, with a 50% sequence identity shared between yeast and human proteins (Weir et al., 2010).

Analysis of PVD neurons showed a small decrease in dendrites of approximately 9% in *mtr-4* mutants compared to the control (Figure 8). Mutants averaged 47.91 dendritic termini with a standard deviation of 5.58 compared to 52.57 dendrites and a

standard deviation of 7.34 for the NC1841 control. *mtr-4* mutants displayed regular PVD branching patterns.

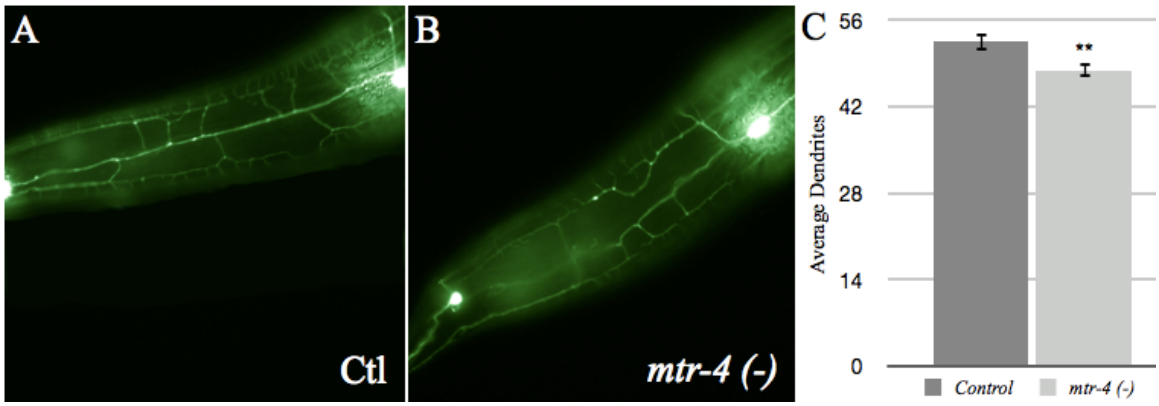

Figure 8. Analysis of MTR-4's role in dendrite morphogenesis. (A) Wild type PVD neuron. (B) *mtr-4* mutants show fewer branches on average than the control. (C) *mtr-4* mutants have significantly fewer dendrites than the control (mean ± SE). ** $P<0.05$.

Negative results

RNAi-mediated knockdown of *larp-5/cg11505 and C56G2.1/Spoon/Yu*

Neither *larp-5* nor *C56G2.1* have a known function in *C. elegans* and there is little understanding of their homologs in other organisms other than knowledge of the existence of RNA-binding motifs (WormBase a, b). RNAi-mediated knockdown of *larp-5* and *C56G2.1* expression yielded no significant difference from the control. *larp-5* RNAi was conducted using the strain DJK68, which is sensitized to RNAi specifically in neurons by overexpressing *sid-1* (See Materials and Methods). Analysis of dendritic termini resulted in an average of 44.38 branches with a standard deviation of 3.89 for *larp-5*-treated individuals compared with the DJK68 control values of 47.60 and 3.97 (Figure 9). The strain NC1841 was used to conduct *C56G2.1* RNAi analysis, resulting in an average of 36.50 dendrites and a standard deviation of 5.88 compared to the NC1841

control, which averaged 38.63 with a standard deviation of 6.03 (Figure 10). Branching patterns appeared to be very regular in both sets of RNAi-treated animals.

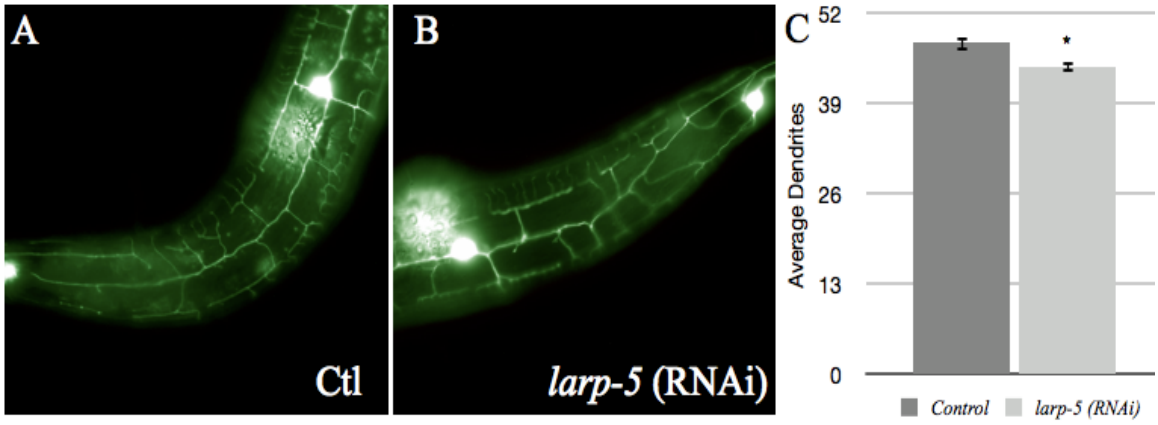

Figure 9. Analysis of *larp-5* RNAi. (A) Wild type PVD neuron. (B) *larp-5* RNAi-treated PVD neuron. (C) Graph shows no significant differenece in number of dendrites (mean ± SE). * P>0.05.

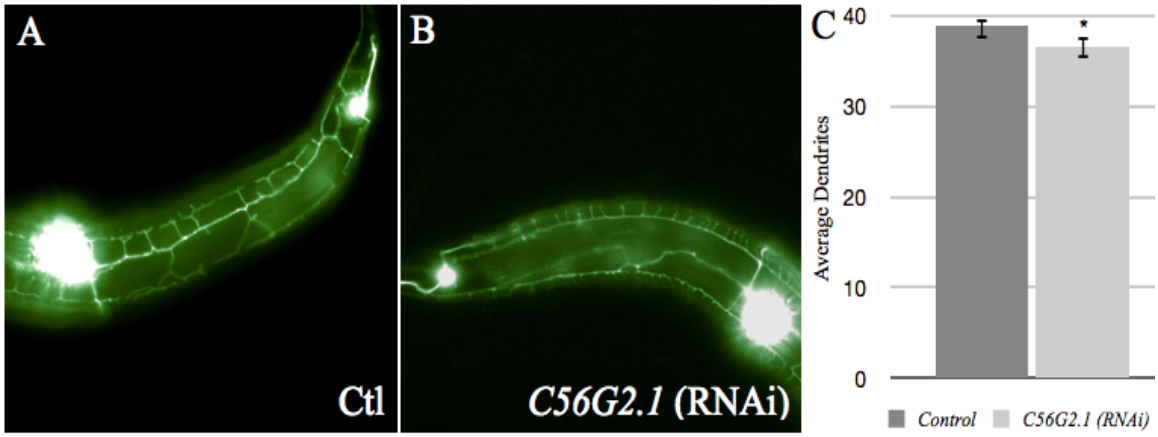

Figure 10. Analysis of *C56G2.1* RNAi. (A) Wild type PVD neuron. (B) *C56G2.1* RNAi-treated PVD neuron. (C) Graph shows no significant differenece in number of dendrites (mean ± SE). * P>0.05.

RNP-3/Snf

rnp-3 encodes for the small nuclear ribonucleoprotein (snRNP)-associated protein RNP-3/U2B", where U2B" is the human homologous snRNP. There is little information about it's known function other than mutations to this gene cause low levels of embryonic lethality and it is likely involved in proper splicing of pre-mRNA by associating with the

branchpoint (Zanetti et al., 2011). RNP-3/U2B" has been found to act redundantly with RNP-2/U1A, as double *rnp-3*; *rnp-2*/U1A mutants have high rates of embryonic lethality (Saldi et al., 2007). RNP-3 also appears to have an interaction with SAP-1/U2A' because double mutants demonstrate a much more severe phenotype than either single mutation (WormBase c).

Analysis of the PVD neuron revealed no significant difference in dendritic termini between *rnp-3* mutants and the NC1841 control (Figure 11). The average dendrite counts were 45.44 and 46.54 and the standard deviations were 1.09 and 1.22 for the *rnp-3* mutants and the control animals respectively. No aberrant branching patterns were observed.

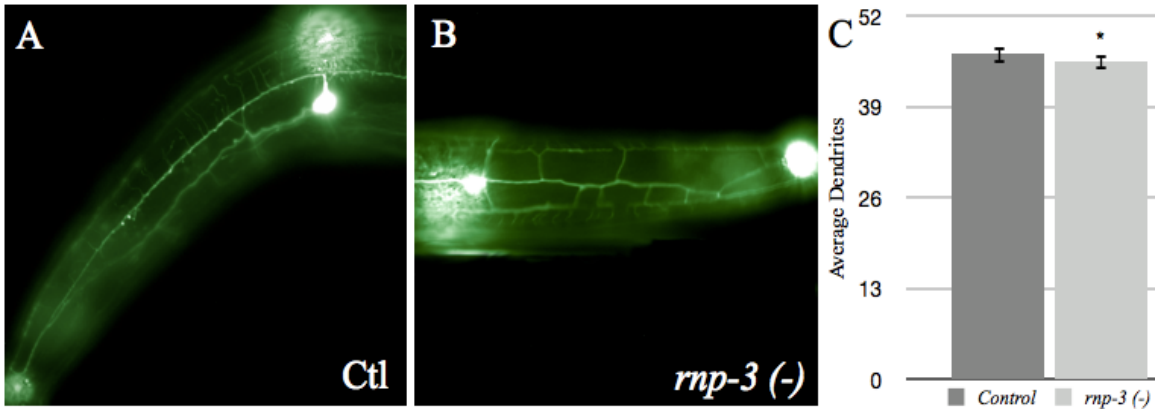

Figure 11. Analysis of RNP-3's role in dendrite morphogenesis. (A) Wild type PVD neuron. (B) *rnp-3* mutants show fewer branches on average than the control. (C) Graph shows no significant difference in number of dendrites (mean ± SE). * P>0.05.

Chapter 5-Discussion

The object of this study was to identify RNA-binding proteins that demonstrate an evolutionarily conserved role in dendrite morphogenesis. Of the 53 RBPs with

homologous structures between *Drosophila* and *C. elegans* that have been shown to affect dendrite formation in *Drosophila*, 11 have been evaluated in the Killian Lab. Four of these RBPs in *C. elegans* appear the have an affect on PVD neuron formation. *ncl-1* and the fly homolog *brat* both demonstrated phenotypes different from the control (Olesnicky et al 2012; G Kerr senior thesis). Here I gathered evidence that *dcr-1*/*dicer* is evolutionarily conserved in its function of dendrite morphogenesis. Both *sup-26*/*alan shep* and *mtr-4*/*L(2)35Df* have weak roles in dendrite morphogenesis in PVD neurons that warrant further investigation. However, *larp-5*/*cg11505, C56G2.1*/*spoon*/*yu,* and *rnp-3*/*snf* do not seem to have an evolutionarily conserved role in dendrite development.

The DCR-1 result of a 16% decrease in dendritic termini in PVD neurons is intriguing because there is a body of research indicating that *dcr-1* has some function in neuron development. Dicer has been reported to form a complex with Argonaute, Fragile X mental retardation protein (FMRP), and miRNAs in neurons to regulate synaptogenesis (Tai & Schuman, 2006). Mutations within the *FMR1* gene, which encodes FMRP, result in Fragile X Syndrome and have been linked with Autism Spectrum Disorder. Like DCR-1, FMRP is an RBP and has been shown to regulate dendritic mRNA translation and function in mRNA localization to dendrites (Darnell et al., 2011). Because Dicer and FMRP form a complex, it is possible that loss of function mutations to *dicer* could cause similar neuronal defects to those seen in *FMR1* mutants. The discoveries that certain miRNAs are localized specifically within dendrites (Khudayberdiev et al., 2009, Wayman et al., 2008) further serves to emphasize *dcr-1*'s likely role in synaptic plasticity. Based on the result of fewer PVD dendritic termini in *dcr-1* mutants in this study, it is very

possible that DCR-1 may be involved in a pathway that silences the expression of genes that inhibit dendrite morphogenesis.

Although analysis of both *sup-26* and *mtr-4* mutants yielded significantly fewer dendrites than the NC1841 control, there were numerous overlapping dendrite counts to the control, indicating that both genes may only have a weak influence on dendrite morphogenesis in *C. elegans*. Since SUP-26 works to interfere with the function of poly (A)-binding proteins, thereby preventing mRNA translation of *tra-2* (Mapes *et al.,* 2010), it is possible that it may work to prevent the translation of other genes that inhibit dendrite formation. The slight decrease in dendrites seen in *mtr-4* mutants may be due to a reduction in ribosomal biogenesis (Weir et al., 2010) causing a general decline in protein translation. Additionally, because previous studies have found MTR-4 to be localized within the nucleus (Weir et al., 2010), it is possible that it does not function at all to localize and translate proteins specifically within dendrites. It is clear from these results that further analysis will need to be done to more clearly define the roles of SUP-26 and MTR-4.

Based on the negative analytical results for *larp-5, C56G2.1,* and *rnp-3,* it can be concluded that these genes likely do not have an evolutionarily conserved role in dendrite morphogenesis. Because so little is known about the function of LARP-5 or C56G2.1, it is difficult to speculate about their roles in neuronal formation and to identify why they do not appear to cause a phenotype in PVD neurons when their expression is knocked-down with RNAi. The negative result in *rnp-3* mutants, however, may be due to RNP-3's redundant function with RNP-2 (Saldi et al., 2007). It is likely that a phenotype in PVD

neurons will only be observed when both proteins are mutated if RNP-3 in fact plays a role in dendrite development. However, mutating both genes poses other problems, as double mutants exhibit high rates of embryonic lethality (Saldi et al., 2007). RNAi may be preferable to using mutants in this instance as the effect is less detrimental to the organism.

Chapter 6-Future Directions

Currently, only a small portion of the 53 candidate *C. elegans* RNA-binding proteins have been screened. The remaining 42 will be scored for their effects on dendrite growth in PVD neurons. The RBPs that have been found to have an evolutionarily conserved role in dendrite morphogenesis will be further analyzed to discover the exact nature of the protein interaction within *C. elegans.* A RBP::GFP fusion will be created through molecular cloning and injected into the syncytium of adult hermaphrodite worms to create transgenic progeny with extrachromosomal arrays. The transgenes will use either a *F49H12.4* promoter, which is specific to the PVD neuron and useful for determining subcellular localization within the PVD, or the native promoter to verify that the genes are expressed in PVD neurons. This will hopefully allow for the visual localization of the candidate RBP within PVD neurons to identify whether it is expressed specifically in dendrites as hypothesized. The biochemical properties of the RBPs will also be examined by determining the protein and RNA components of the PVD dendrite RNPs using co-immunoprecipitation and RNA sequencing studies.

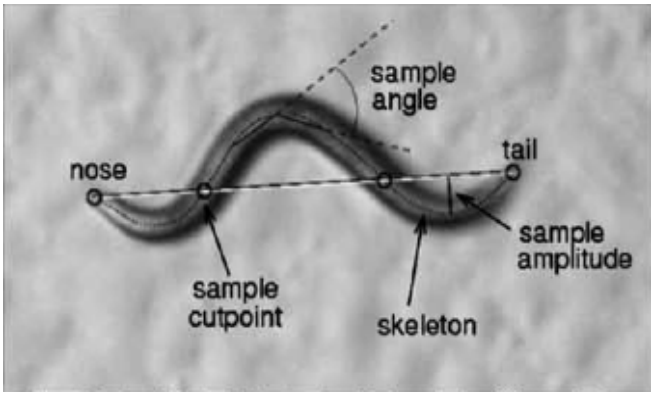

Figure 12. Diagrammatic representation of parameters used for postural analysis (Albeg et al., 2010).

In addition to RBP localization analysis, functional assays will be conducted, adapted from Albeg et al. 2010. When *C. elegans* nematodes crawl through plates seeded with *E. coli*, they leave tracks in the bacteria. Because PVD neurons have been shown to have a role in proprioception (Albeg et al., 2010), these tracks will be analyzed to observe whether there is a difference in amplitude, bending angle, and cut-point number in animals with RBP defects compared with a control (Figure 12). The strain CB1339, which carries a *mec-4* mutation, will be used to conduct this portion of the study. The *mec-4* mutation causes cell death in neurons involved in soft touch, thus removing confounding sensory information which may interfere with PVD function.

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