CHARACTERIZATION OF RAB-10

IN PLM DENDRITE TERMINATION OF *C.ELEGANS*

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Abstract

To better understand how the brain works, it is necessary to study how axons and dendrites (neurites) regulate their outgrowth and maintain their shape. Directed membrane trafficking is crucial to neural development and morphogenesis. RAB-10, a small GTPase, has been shown to mediate polarized secretion and recycling of transport vesicles at the plasma membrane in both neuronal and non-neuronal cells. However, little is known about RAB-10 function in neural morphogenesis.

Using the *in vivo* model, *C. elegans*, we focus on dendrite termination of the posterior mechanosensory neuron, PLM (posterior lateral microtubule). The conserved molecular pathway involved in termination includes two conserved genes, *sax-1* and *sax-2*. *sax-2* encodes a large scaffold-like protein that functions with *sax-1,* a protein kinase. *sax-1* and *sax-2* single mutants, as well as the *sax-1*/*sax-2* double mutant, lead to similar levels of PLM overextension, suggesting they function together to terminate dendrite outgrowth. Moreover, single mutant analysis of F09A5.4, a known SAX-1 cofactor, also leads to PLM overextension.

Few downstream effectors of the *sax-1*/*sax-2* pathway have been identified. In yeast, genetic and biochemical studies with Cbk1p, an ortholog of SAX-1, displays an interaction with Sec2p, an exchange factor of Sec4p, the founding member of the Rab family of GTPases. The closest animal homologs of Sec4p include RAB-8 and RAB-10. We find that loss of *rab-10*, but not *rab-8*, disrupts PLM dendrite termination comparably to *sax-1*. Rab GTPases function primarily in vesicle trafficking. Our finding that RAB-10

functions in PLM dendrite termination would be consistent with the observation that SAX-2 localizes to small puncta resembling transport vesicles.

To determine if *rab-10* functions in the same pathway as *sax-1* and *sax-2*, double mutant strains, involving *sax-1* and *sax-2* were examined. Findings suggest that RAB-10 may utilize the SAX-2 scaffolding protein, but does not function in the same pathway as SAX-1. Next, transgenic animals expressing a *rab-10::mCherry* fusion in select worm neurons including PLM were built. Importantly, *rab-10(+)::mCherry* rescued the *rab-10* loss-of-function mutant indicating that RAB-10::mCherry is functional. Moreover, this rescuing *rab-10*::mCherry fusion was used to observe the subcellular localization of RAB-10 in the PLM mechanosensory neuron, where RAB-10 was seen at the growth cone and various points along the PLM dendrite in L1 larval stage animals.

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Introduction

A tiling pattern of dendrite growth is observed in neurons that process spatial information (i.e. visual and tactile), where dendrites occupy a distinct region with little or no overlap with neighboring neurons of the same type (Wassle et al., 1981; Wassle & Rieman, 1978; Grueber et al., 2001, 2003). How do these dendrite morphologies form? Specific signaling molecules released from the target tissue to establish and maintain this tiling behavior may trigger the termination of dendrite outgrowth. Few neurite termination cues have been identified and the intracellular signaling proteins that respond to these cues are mostly unknown.

The Gallegos lab has taken a candidate gene approach to identify genes involved in PLM dendrite termination in our model system of choice, *C. elegans*. In this method, one chooses a gene to study based on previous work found in the literature. This candidate gene exhibits a similar mutant phenotype to *sax-1*/*sax-2* of an overextended PLM dendrite, including *rab-10*, a homolog of yeast Sec4P.

Rab proteins are an ubiquitous family of Ras-like GTPases that regulate vesicle trafficking and are likely to play a role in neurite polarization and outgrowth. Rab-10, in particular, has demonstrated involvement in membrane transport in many polarized cell types such as *Drosophila* follicle cells (Lerner et al., 2012), rat hippocampal neurons (Wang et al., 2011), and *C.elegans* intestinal epithelial cells (Chen et al., 2006, 2014; Shi

et al., 2010, 2012) and neurons (Glodowski et al., 2007; Taylor et al., 2015; Zou et al., 2015). However, it is unclear whether Rab-10 is required for dendrite termination and tiling.

Dendritic Tiling

Numerous studies of neuronal systems in both vertebrates and invertebrates have noted the tiling behavior of dendrites (Wassle et al., 1981, Gallegos & Bargmann 2004, Grueber et al., 2003, Hitchcock, 1989, Kramer & Kuwada 1983, Lin et al., 2004). During development, sensory dendrites of a similar neuron type grow and cover a wide area without redundancy (Figure 1). Tiling was first observed in mammalian retinal ganglian neurons where little or no overlap of similar dendrites covered the retina, while dendrites of different types displayed overlap (Wassle et al., 1981). Tiling has also been observed in mechanosensory neurons of *Drosophila*, where similar neurons repel each other and the tiling boundary is set when dendrites initially meet (Grueber & Sagasti, 2010).

Several mechanisms leading to tiling have been proposed. Homotypic repulsion describes dendrites that touch and retract when in contact with similar dendrites, as observed in sensory neuron development of *Drosophila* (Grueber et al., 2003). Specifically, when da neurons were ablated, neighboring da neurons encroached in the territory of the ablated neuron (Grueber et al., 2003). By contrast, Lin et al. (2004) describes a contact-independent mechanism in mutant mice, whereby a lack of neural contact during development did not increase dendrite size.

Caenorhabditis elegans mechanosensory neurons

To study neural development, our lab focuses on the mechanosensory circuit of *C.elegans*. It's responsible for common behaviors such as egg-laying, locomotion, and touch. The circuit is comprised of six neurons: anterior lateral microtubule left (ALML), anterior lateral microtubule right (ALMR), anterior ventral microtubule (AVM), posterior ventral microtubule (PVM), posterior lateral microtubule left (PLML), and posterior lateral microtubule right (PLMR) (Figure 2). The two sets of bilaterally symmetric sensory neurons, ALML/R and PLML/R, transduce touch stimuli where a gentle touch sensation at the anterior initiates a backward motion, and a posterior touch stimulus generates a forward motion (Chalfie et al., 1985, Girard et al., 2007).

In the mature sensory circuit, the posterior lateral microtubule (PLM) cell bodies lie in the tail region, each with a short process directed posteriorly and a longer process extending anteriorly, termination just posterior to the anterior lateral microtubule (ALM) cell body, at the worm's midsection (Figure 2). Importantly, the ALM and PLM dendrites extend without overlap, exhibiting a tiling pattern simpler but reminiscent of other mechanosensory and visual systems including da mechanosensory neurons in *Drosophila* (Gallegos & Bargmann, 2004). However, unlike *Drosophila*, homotypic repulsion is not responsible for this pattern of dendrite growth as ablation of the anterior mechanosensory neuron, ALM, does not cause significant overextension of PLM (Gallegos & Bargmann, 2004; unpublished data). This observation suggests that ALM has little to no influence on PLM termination. Interestingly, this process may be regulated instead by a heterotypic

repulsion mechanism. The interneuron, BDU, is the lineal sister cell of ALM. This cell extends a posterior neurite that terminates at the PLM dendrite tip. Moreover, genetic ablation of the BDU neuron in worms displayed significantly longer PLM neurite lengths compared to animals that retain BDU (Alejandro Escalante, undergraduate, unpublished).

To gain a better understanding of how this tiling pattern is established, PLM neurite length was measured from hatching to adulthood. Interestingly, during development, the PLM dendrite overlaps with ALM for a brief time, then the overlap disappears during a period of slowed PLM neurite growth compared to body growth (Gallegos & Bargmann, 2004). Thereafter, a period of maintenance growth ensues where PLM neurite growth matches the growth of the worm (Gallegos & Bargmann, 2004). This observation of overgrowth followed by retraction is very similar to what has been observed in mouse retinal ganglion cells, which display growth and retraction. Specifically, dendrites extend more rapidly than the retina during early development, and later exhibit a period of slow growth, in comparison to the retina, until non-overlapping dendritic fields are achieved (Ren et al., 2010).

Mechanosensory neuron morphology is regulated by SAX-1 and SAX-2

Several genes have been identified that play a role in mechanosensory dendrite tiling including *sax-1, sax-2*. *sax-1* and *sax-2* were first shown to be required in stabilization and maintenance of *C. elegans* neuron morphology (Zallen et al., 1999, 2000). Specifically, *sax-1* or *sax-2* loss-of-function single mutants have defects in

neuronal cell shape and abnormal growth of secondary neurites that increase in number throughout the larval stages and into adulthood (Zallen et al. 1999). *sax-1* and *sax-2* also play a role in PLM dendrite termination. Mutants of *sax-1* or *sax-2* exhibit sustained overlap of the primary PLM dendrite with the ALM soma, suggesting a role in dendrite termination that ultimately disrupts tiling of ALM and PLM (Figure 3) (Gallegos & Bargmann, 2004). Moreover, the *sax-1*/*sax-2* double mutant phenotype was comparable to that of single mutants, implying *sax-1* and *sax-2* work in the same molecular pathway (Gallegos & Bargmann, 2004).

sax-1 encodes a conserved serine/threonine kinase of the nuclear Dbf-2 related (NDR) family. *sax-2* encodes a novel conserved protein. Both genes are conserved from yeast to humans (Table 1). Not only are the individual gene sequences conserved but the *sax-1*/*sax-2* signaling pathway may also be conserved (Chihara & Luo, 2004). Cbk1 and Tao3/Pag1 in budding yeast, orthologs of *sax-1* and *sax-2,* respectively, also function together in a single pathway or protein complex. Single and double mutant *Tao3*/*Pag1* displayed defects similar to *Cbk1* single mutants (Du & Novick, 2002), Moreover, two-hybrid and coimmunoprecipitation experiments have demonstrated that Cbk1, interacts with both Tao3/Pag1, and Mob2 (Figure 4) (Nelson et al., 2003). Thus, both genetic and molecular evidence indicates that Cbk1, Tao3/Pag1 and Mob2 form a complex, with Tao3/Pag1 acting as a scaffold (Nelson et al., 2003).

In yeast, this pathway is necessary for polarized growth during budding and mating, differential gene expression and maintenance of cell integrity (Kurishcko et al.,

2008). Thus it has been named the RAM pathway for Regulation of Ace2 and Morphogenesis. RAM pathway members include the kinases Cbk1 (the SAX-1 ortholog) and Kic1, along with several associated proteins Tao3/Pag1 (the SAX-2 ortholog), Mob2 (a Cbk1 cofactor), Hym1, and Sog2 (Figure 4) (Nelson et al., 2003).

Consistent with its role in polarized growth during budding and mating, a process that requires post-Golgi secretion, Cbk1 binds and phosphorylates Sec2, a guanyl nucleotide exchange factor (GEF) for Rab GTPases (Kurischko et al., 2008). Sec2 is known to activate Sec4, a Rab GTPase, required for post-Golgi vesicle trafficking to the plasma membrane. Wang et al. (2015) found that the kinase ERK1/2 directly phosphorylates Rabin 8, a GEF for Rab-8, in a pathway regulating vesicular trafficking to the membrane. It is not clear if the link between RAM pathway proteins and vesicle trafficking regulators are conserved, however knockdown of Cbk1-orthologs have resulted in stunted cell growth and developmental defects (Hirata et al., 2002, Zallen et al., 2000, Emoto et al., 2004, Gallegos & Bargmann, 2004). These mutant phenotypes may be associated with compromised Rab GTPase thereby disrupting polarized growth and development in numerous cell types and species (Stenmark & Olkkonen, 2001).

Rab proteins and recycling endosomes

Rab proteins are a conserved family of small, monomeric Ras-like GTPases that play important and diverse roles in vesicle trafficking. Through effector recruitment, Rab proteins are involved in the regulation of each step of membrane trafficking including

cargo selection, vesicle formation, vesicle delivery, tethering to the target membrane, and vesicle fusion (Grosshans et al., 2006). Rab GTPases act as molecular switches, alternating between a GTP-bound and GDP-bound state. GEFs catalyze the exchange of GDP with GTP, causing the Rab GTPase to switch on. In this conformation, Rab GTPases are able to activate and recruit effector molecules, such as kinases, motors, and sorting adaptors, which carry out the different tasks of vesicle trafficking. They may also reversibly bind to specific lipid membranes via C-terminal prenylation motifs, essential to its role in regulating membrane traffic. GTP hydrolysis to GDP switches the Rab GTPase off, catalyzed by GTPase activating proteins (GAPs) and the intrinsic GTPase activity of the Rab protein. As Rab GTPases regulate membrane identification and vesicle trafficking, they may be important in neurite polarization and outgrowth.

RAB-10, an ortholog to yeast Sec4p that controls post-Golgi vesicle trafficking, has been shown to mediate trafficking in numerous polarized cell types including neurons (Lerner et al., 2012; Glowdowski et al., 2007; Sasidharan et al., 2012, Shi et al., 2010, 2012; Chen et al., 2006, 2014) and has an important role in endosomal sorting and endocytic recycling in *C.elegans*. RAB-10 was found to be a key regulator of endocytic recycling, as *rab-10* null mutants displayed abnormally large early endosomes in intestinal cells. Moreover, GFP-tagged RAB-10 localized to the endosomes and Golgi of *C.elegans* wild-type intestinal cells (Chen et al., 2006).

Recycling endosomes have been implicated as crucial players in neurite development. Neurite outgrowth and guidance depends upon membrane secretion in the

dendrite tip, as well as receptor trafficking to the growth cone surfaces (Sann et al., 2009). Consistent with this observation, RAB-10 localizes in rat hippocampal neurons to neurite growth cones and the soma of Stage 2 neurons, and at the distal shaft region of the axon in Stage 3 neurons (Wang et al., 2011). Rab-10 activation stimulated membrane trafficking of precursor vesicles, which went on to fuse with the plasmalemma causing axonal growth in both rat hippocampal neurons (Wang et al., 2011) and cortical neurons (Xu et al., *2014*). Similarly, inhibition of *rab-8,* a *rab-10* paralog, disrupts neuronal outgrowth in developing embryonic rat hippocampal neurons (Huber et al. 1995). In *C.elegans,* dendritic morphogenesis of the PVD sensory neuron required RAB-10 and the exocyst secretory complex (Taylor et al., 2015, Zou et al., 2015). Specifically, loss of *rab-10* in PVD leads to reduced dendritic branches within the posterior region of the cell but excessive dendritic branches in the anterior region of the cell. These results suggest that *rab-10* is required for outgrowth where the membrane is delivered from a distinct portion of the cell. However, it is unclear whether *rab-10* is required for dendritic termination during development.

Preliminary Evidence

Previous work in the Gallegos Lab involving *rab-10* null mutant PLM neurite analyses displayed significant overlap of PLM and ALM, contrasting with the wild-type phenotype. Relative to WT, a 15% increase in PLM neurite length was observed in *rab-10* null mutants suggesting their possible affiliation in the *sax-1*/*sax-2* pathway while *rab-*

8 null mutant PLM displayed no significant overextension (Pranti Das & Catherine Ndungu-Case, Gallegos Lab, data not shown).

Further investigation of *rab-10,* in *C.elegans,* will provide greater insight into the extent of conservation of the RAM pathway between yeast and worms. Specifically, double mutant analysis would determine if RAB-10 functions in the same pathway as SAX-1 and SAX-2. Moreover, the creation of a fusion protein with RAB-10 and mCherry will help determine where RAB-10 is localized during PLM development and help illuminate its role in dendrite development and dendritic tiling.

Research objectives

Characterize the role of RAB-10 in PLM mechanosensory dendrite development in *C.elegans*.

1.) To determine if *rab-10* acts within the *sax-1*/*sax-2* pathway, phenotypic analysis of the double mutant of *rab-10* with *sax-1* and *sax-2*, respectively, was completed. *sax-2; rab10* mutants displayed similar levels of PLM overextension. However, PLM neurite lengths were longer on average in *rab-10; sax*-1 mutants compared to single mutant controls.

2.) To perform *in vivo rab-10* gene function studies, a set of gene fusions were created. Specifically mCherry was fused in-frame to *rab-10* wild-type (+), constitutively-active (CA) and dominant-negative (DN) forms fused to mCherry. These gene expression clones were designed to be expressed in a

subset of C.elegans neurons including the mechanosensory neurons, ALM and PLM.

3.) Transgenic animals expressing *rab-10(+)::mCherry* were characterized *in vivo*. Specifically, phenotypic analysis of *rab-10(+)::mCherry; rab-10(lf)* animals demonstrate the functionality of the WT *rab-10* expression clone.

4.) Confocal microscopy was used to observe and attempt to characterize the in the PLM neurite at the L1 larval stage of the animal. RAB-10 was predominantly observed in the cell body but also localized at various points along the PLM dendrite and at the growth cone.

Materials and Methods

Experimental Strains

Nematodes were cultivated according to standard protocols (Brenner, 1974), and maintained at 15°C unless otherwise noted. Strains were obtained from the Caenorhabditis Genetics Center (CGC) except where indicated. Genes and alleles studied:

Bristol N2: wild-type strain

sax-1 (ky491) X: The ky491 null allele contains a 2184 base-pair deletion. This deletion removes conserved domains of the SAX-1 kinase.

Molecular Details of *ky491:*

```
...aattttattctcttttagcg----------tcttggagcagaaagactgtt... -- Wild type
...aattttattctcttttagcg[2184 BP DEL]tcttggagcagaaagactgtt... -- ky491
```

sax-2 (ot10) III: The ot10 allele contains an early stop codon at amino acid residue 809. It is believed to be a null mutant as the SAX-2 protein is 2886 amino acids long with conserved domains distributed throughout the protein from the N-terminus to the C-terminus.

rab-10 I (ok1494): The ok1494 allele contains a 663 bp deletion and "T" insertion. The small GTPase protein motif is completely deleted thus this allele is expected to be a null.

Molecular details of *ok1494:*

```
...atttcgaattttcacatcctt - ----------------- gtcatacggtcggcgagccat... --Wild-type
...atttcgaattttcacatcctt T [663 BP DELETION] gtcatacggtcggcgagccat... -- ok1494
```

kyIs262 IV (Bargmann lab)*:* This integrated transgenic line contains *unc-86p::*myr GFP + *odr-1::RFP* GFP that expresses membrane associated GFP in a variety of neurons including HSN (the egg-laying neuron) and mechanosensory neurons. RFP expressed in a sensory neuron pair in the head.

zdIs4 IV (Clark Lab): This integrated transgenic line contains *mec-4p::GFP* that expresses soluble GFP in all 6 mechanosensory neurons.

Strains analyzed (made by others in the lab):

Wild-type and single mutant strains for phenotypic analysis: 1) *zdIs4 IV*, 2) *rab-10(ok1494) I; zdIs4 IV, 3) zdIs4 IV; sax-1(ky491) X, 4) sax-2(ot10) III; zdIs4 IV*. Double mutant strains for phenotypic analysis: 1) *rab-10(ok1494) I; zdIs4 IV; sax-1(ky491) X* and 2) *rab-10(ok1494) I; sax-2(ot10)III; zdIs4 IV*. Unless otherwise noted in the text, single mutant controls were siblings of double mutants.

For transgenic experiments, the *rab-10* extrachromosomal array could not be successfully injected into Rab-10 null animals, so WT animals were injected with the array and crossed into *rab-10(ok1494)* mutants.

Genotyping Strains by PCR, enzyme digestion and gel electrophoresis

PCR was used to genotype the following strains: (1) *zdIs4 IV; sax-1(ky491) X*, (2) *rab-10(ok1494) I; zdIs4 IV; sax-1(ky491) X*, (3) *sax-2(ot10) III; zdIs4 IV*, (4) *rab-10(ok1494) I; sax-2(ot10)III; zdIs4 IV*. Genomic DNA was extracted from animals using a popular worm protocol. Specifically, 100uL DNA-grade water was added onto a starved worm plate. 25uL of the water/worm mixture was transferred to a PCR tube and mixed with 25uL of 2x proteinase K buffer (2.5mL – 1M KCL (final concentration: 50mM), 500uL – 1M TRIS pH 8.3 (final concentration:10mM), 125uL – 1M $MgCl_2$ (final concentration: 2.5mM), 2.25mL - 10% TWEEN-20 (final concentration: 0.45%), 2.25mL – 10% IGEDAL (final concentration: 0.45%), 250uL - 2% gelatin (final concentration: 0.01%), 500uL – 20mg/mL proteinase K (final concentration: 200ug/mL), water was added to bring up the solution volume to 50mL). PCR tubes were placed at -80°C for >30 minutes, then placed in a thermocycler with the following settings: 65°C for 4 hours, 95°C for 15 minutes, and 4°C forever. The mixture was flicked once after thawing before being returned to the thermocycler. Genomic extracts were stored at -20°C indefinitely.

Touchdown PCR was used to genotype strains. The general PCR reaction mix is described in Table 2. General Touchdown PCR settings were as follows: (1) 94°C for 2

minutes, (2) 94°C for 30 seconds, (3) 64°C for 30 seconds (-1°C per cycle), (4) 72°C for 1 minute (extend time for 1 minute / kb), (5) go to Step 2, 9 times, (6) 94°C for 30 seconds, (7) 55°C for 30 seconds, (8) 72°C for 1 minute, (9) go to Step 6, 40 times, (10) 72°C for 15 minutes, (11) 4°C forever. Step 4 of the Touchdown PCR settings was adjusted for detecting different genes: *sax-1* – 1:45 minutes, *sax-2* – 2:15 minutes, *rab-10* – 1:10 minutes.

To detect *sax-1,* the following three primers were included in the PCR reaction: forward primer MG1401 sequence GTTCGCGCTGAAAGAGACAT, reverse primer #1 MG1400 sequence GAGCATACGCCATGTCAGAT, reverse primer #2 MG1399 sequence TCAGGGATGAACGACTGTTC. MG1401 and MG1400 were used to amplify the *sax-1* mutant, with an expected band size of 320 bp. MG1401 and MG1399 were used to amplify wild-type *sax-1*, with an expected band size of 384 bp.

To detect *rab-10,* the following three primers were included in the PCR reaction: forward primer MG1133 sequence TCGCCCGTGCAAAGAATGTA, reverse primer #1 MG1134 sequence CGCGTTAATAGCTCCGTTCA, and reverse primer #2 MG1135 sequence GTGATAGACGAGTTGTGAGC. MG1133 and MG1134 were used to detect mutant *rab-10(lf),* with an expected band size of 346 bp, and wild-type *rab-10,* with an expected band size of 1008 bp. MG1133 and MG1135 were used to detect wild-type *rab-10* with an expected band size of 299 bp.

To detect *sax-2,* the following primer pair was used: forward primer MG313 with sequence CAAGGTGCTCCATCAACATC, reverse primer MG315 with sequence GCTTTGGAGGAATGTAGGCA. After touchdown PCR, the Hpy188I restriction enzyme was used to digest the amplicon and identify isolates that have the mutant or WT allele as the *sax2(ot10)* mutant allele possessed a snip-SNP, a single nucleotide polymorphism that causes a loss or gain of a restriction enzyme site. The following fragment sizes were expected from the restriction enzyme digest: uncut DNA – 692 bp, wild-type *sax-2* – 405 bp, 189 bp, 70 bp, and 28 bp, mutant *sax-2* – 475 bp, 189 bp, and 28 bp. The PCR reaction mix is described in Table 2 with the following changes: 0.25uL of forward primer MG313, 0.25uL of reverse primer MG315, and 16.3uL of DNA-grade water. The Hpy188I reaction mix contained: 16.5 uL DNA-grade water, 2.5 uL NEBuffer 4 (10x) (NEB Cat# B7004S), and 1uL Hpy188I restriction enzyme (NEB Cat# R0617S) and 5uL of DNA template (PCR amplicon. The mixture was then incubated at 37°C for 1 hour, and then placed at -20°C until it was analyzed by gel electrophoresis.

Site-directed mutagenesis (SDM)

The pDONR *rab-10* entry clone (M.Vidal Lab, Harvard, MA) was first verified by sequence, then modified by site-directed mutagenesis to create the dominant negative (DN) and constitutively active (CA) mutant forms (Completed in Biology 6141, Instructor: Dr. Gallegos). The ORFs in the Vidal ORFeome library contain neither start nor stop codons, to achieve flexibility for a wide array of uses. Since most Rab proteins are post-translationally modified at their C-termini. This post-translational modification is

required for function and proper localization of Rab proteins to the membrane. Since entry clones are used with Gateway Recombinational Cloning, the stop codon would fall after the 25 bp attB sequence scar. This would add a peptide to the end of a RAB-10 protein rendering it nonfunctional. Thus, a stop codon needed to be added to each of the *rab-10* entry clones (WT, CA and DN) prior to use in recombinatorial cloning to create expression clones.

To add a stop codon to the end of each *rab-10* ORF, Site Directed Mutagenesis (SDM) was used (Figure 5). Specifically, QuikChange II XL- Site-Directed Mutagenesis (Agilent) was used to create a TAG stop codon, by inserting nucleotides AG to the 3' end of the ORF. Selection of the TAG codon was based on its use in the NM_059456.3 mRNA sequence. Mutagenic primers were designed using the following parameters: between 25-45 bases in length, melting temperature of ≥ 78°C, ~10-15 bases of correct sequence on both sides, and primers contain a minimum GC content of 40% and terminates in one or more C or G bases.

The mutagenic forward primer MG1411:

5′- GTG GAG GAT GCT GCT **AG** ACC CAG CTT TCT TGT AC -3′

The mutagenic reverse primer MG1412:

5′ GTA CAA GAA AGC TGG GT **CT** AGC AGC ATC CTC CAC -3′

The added sequence is highlighted in bold. The reaction mix is described in Table 3. Thermocycling parameters were as follows: (1) 95°C for 1:50 minutes (2) 95°C for 50 seconds, 60°C for 50 seconds, 68°C for 2:51 minutes, for 18 cycles (3) 68°C for 7 minutes, (4) 4°C forever. XL10-Gold Ultracompetent Cells (Agilent) were used for transformation, and plated onto LB-KAN (50 ug/mL) plates. Individual colonies were cultured overnight in LB-KAN (50 ug/mL). Plasmids were isolated using QIAprep Miniprep (Qiagen). UV spectrophotometery (Genesys5 UV) was used to measure DNA concentrations and SDM products were confirmed by DNA sequencing (Elim Biopharmaceuticals, Hayward, CA). The following entry clones were created:

- DVD 63-2: rab-10 + stop
- DVD 45-2: rab-10(DN) + stop
- DVD 45-3: rab-10(CA) + stop

Recombinational Cloning into an mCherry Gateway destination vector

An optimized mCherry Gateway Destination vector (Shen Lab, Stanford, CA) was used for Gateway recombinational cloning. The vector was designed to fuse an N-terminal mCherry fusion to a protein of interest. This destination vector also contains the *unc-54* 3'UTR, a standard UTR used in *C.elegans* transgenes and the *unc-86* promoter that drives expression in 55 neurons including the mechanosensory neurons ALM and PLM (Finney & Ruvkun, 1990).

The destination vector contains chloramphenicol (CmR) and ampicillin resistance

(AmpR) in addition to a *ccdB* suicide gene. Thus to grow large amounts of the destination vector, it was transformed into One Shot *ccdB* Survival 2 T1-phage resistant competent cells (Invitrogen). Next it was plated onto LB-Chloramphenicol (30 ug/uL) - Ampicillin (50 ug/uL) agar plates. These cells are slow growing and carry a low yield of plasmid. Overnight cultures of selected colonies were propagated in LB-Chloro (30ug/uL), harvested by microcentrifuge, and plasmid purified by Qiagen Tip-20 (Qiagen), to increase yield of DNA. DNA concentration was measured by UV spectrophotometry. To verify successful purification of the destination vector, a HindIII (New England Biolabs) digest of the destination vector isolates was performed. DNA fragments were then separated by 1.5% agarose gel electrophoresis for verification (data not shown).

Gateway recombination

The WT/DN/CA-rab-10 entry clones containing the stop codon were recombined into the optimized mCherry Gateway destination vector described above. Half-reaction amounts were used for the LR Clonase reaction (Table 4) and transformation. Transformation used MAX efficiency DH5-alpha competent cells (Invitrogen), and plated on LB-Carbenicillin (Carb) (50 ug/uL). Carbenicillin, an analog of Ampicillin, was selected for its stability. The desired expression clone was screened by Colony PCR. The reaction mix is described on Table 5. TD Primers for the destination vector, flanking the gene of interest, amplified a 671bp region. Primers used: MG1084 sequence GCTAGCACAAGTTTGTACAAA and MG1085 sequence GGTACCTTACACCACTTTGTA. Touchdown colony PCR parameters were: (1) 94°C

for 3 minutes, (2) 94°C for 30 seconds, (3) 65°C for 30 seconds, -1.0°C every cycle, (4) 72°C for 1 minute, (5) go to Step 2, 11 times, (6) 94°C for 30 seconds, (7) 52°C for 30 seconds, (8) 72°C for 1 minute, (9) go to Step 6, 30 times, (10) 72°C for 15 minutes, (11) 4°C forever.

Individual colonies were cultured overnight using LB-Carbenecillin (50ug/uL). Plasmid purification of transfection-grade DNA was performed by the Qiagen Tip-20 kit then DNA concentration was measured by UV spectrophotometry. A HinfI digest of the putative expression clones (Figure 6) was performed then separated by 1.5% agarose gel electrophoresis to verify successful recombination (Figure 7).

Transgenic strain

Dr. Gallegos injected the *unc-86p::mCherry::rab-10(+)* construct, along with a visual co-injector marker (*odr-1p::RFP*), into *zdIs4 IV* wild type animals. *odr-1p:: RFP*, expressed in the sensory neurons of the head, was exploited to identify animals containing the *unc-86::mCherry::rab-10(+)* transgene. Transgenic worms usually carry large extrachromosomal arrays that have many copies of the injected DNA. The repetitive arrays are unstable and often fail to segregate properly at each cell division, however they can be inherited (Evans, 2006). Typically, a small number of first generation (F1) progeny that contain the transgene are able to pass the array through an infinite number of successive generations, with limited changes in expression. L4 stage F1 progeny, identified by the presence of the ODR-1::RFP transgene and possibly RAB-

10::mCherry, were picked onto nematode growth media (NGM) plates (1 - F1 animal per plate). Plates were observed for transgenic F2 progeny, with an expected transmittance of 2-15% (Evans, 2006). Transgenic animals were picked at each generation for strain maintenance. Once a transgene line had been established, animals were crossed into *rab-10(ok1494) I; zdIs4 IV* mutant animals for phenotypic analysis and *kyIs262* for confocal analysis.

Phenotypic analysis of PLM neurite length

To determine if the *mCherry::rab-10(+)* transgene was functional, PLM neurite lengths were measured in *rab-10(ok1494)I; zdIs4* with and without the *mCherry::rab-10(+)* extrachromosomal array. Specifically, L4 animals were mounted on 3% agar pads containing 10mM sodium azide (an anesthetic) and M9 buffer (6 g/L Na2HPO4, 3 g/L KH2PO4, 5 g/L NaCl, 0.25 g/L of MgSO47H2O). Worms were analyzed using an Axiovert 100 Zeiss compound fluorescence microscope at 200x magnification, and bright field and fluorescence images were captured. Fluorescent images were used to measure the GFP-tagged PLM dendrite, while bright field images were used to measure total body length. PLM dendrite length and total body length of 35-40 worms per strain were measured using ImageJ (http://rsbweb.nih.gov/ij/). The PLM dendrite was measured from the center of the PLM soma to the tip of the dendrite. The total body length of the worm was measured from the PLM soma to the posterior end of the pharynx. The normalized PLM neurite length was determined by dividing "PLM Length" by "Total Body Length."

Normalized PLM length data was subjected to statistical analysis (mean, standard deviation and standard error of the means).

Mounting L1 worms

To collect worms at the L1 stage, adult hermaphrodites carrying eggs were treated with a 6% bleach solution (0.8g NaOH, 1.2mL commercial 5% bleach, 8.8mL sterile deionized water) on unseeded NGM agar plates. The treated worms were placed in the 20° C incubator for 2 hours. The adult hermaphrodites worms die due to bleach exposure while the eggs remain intact due to their hardy chitin covering. Hatched L1 progeny were collected and placed on a 3% agar pad containing 10 mM sodium azide and M9 buffer. L1 worms were observed with a Zeiss SP8 Confocal microscope and images processed using FIJI software (NIH). Confocal settings were 0.61 gamma emission and the numerical aperture of the objective was 1.4.

Results

Aim 1: Double mutant analyses of *rab-10* combined with *sax-1* and *sax-2*

Preliminary studies suggest *rab-10* might function in the *sax-1*/*sax-2* pathway during PLM development. Using a candidate gene approach, Pranti Das (former Gallegos Lab graduate student) found that the *rab-10* deletion lead to significant PLM overextension, similar to the phenotype displayed by *sax-1*/*sax-2 (lf)* mutants.

To test the hypothesis that *rab-10* functions in the *sax-1*/*sax-2* pathway, double mutant strains were analyzed and compared to single mutant controls. A double mutant analysis is a useful way to address this question as one may infer how two or more genes interact with each other, by studying the phenotype displayed from combining gene mutations (Girard et al., 2007). If two genes function in the same pathway, the double mutant phenotype should not have a significant difference from the most severe single mutant phenotype.

Confirmation of strains created by Dr. Gallegos was performed, and they indeed contained the expected mutations. This was done by PCR amplification of *rab-10, sax-1* and *sax-2* loci then visualization of the amplicons by gel electrophoresis (Figures 8, 9). Prior to analysis of the *sax-2* amplicon, the DNA fragments were digested first with Hpy 188I as the *ot10* point mutation disrupted a Hpy 188I recognition site. Once the genotype of each double and single mutant was confirmed, the PLM dendrites of animals were measured and compared with the single mutant sibling controls.

sax-2 single mutant controls and *sax-2; rab-10* double mutants displayed similar levels of PLM overextension (Figure 10). Specifically, the *sax-2; rab-10* double mutant PLM average was 0.7002 +/- 0.004 (N = 88), and the *sax-2* single mutant PLM average was 0.6972 +/- 0.005 (N = 102). The difference between the *sax-2* and *sax-2;rab-10* was not significant (P-value = 0.6540). These results suggest that *rab-10* functions in the same pathway as *sax-2*.

Surprisingly, PLM dendrite lengths were significantly longer in the *sax-1;rab-10* double mutant compared to the single mutant control (P-value = 0.0001) (Figure 11). Specifically, the *sax-1;rab-10* double mutant PLM average was 0.6925 +/- 0.006 (N = 84), and the *sax-1* single mutant PLM average was 0.6544 +/- 0.008 (N = 91). These results suggest that *rab-10* does not function in the same pathway as *sax-1*. See the discussion for speculation about why our results suggest that *rab-10* might function in the same pathway as *sax-2* but not *sax-1*.

Aim 2: Create a *rab-10* toolset

A Rab-10 toolset was created containing a wild-type (WT), constitutively-active (CA), and dominant negative (DN) version of Rab-10. The CA and DN mutant forms of Rab-10 ORF clones were created by students in Biology 6141 (Instructor: Dr. Gallegos) using site-directed mutagenesis. The ORFs came from the Marc Vidal lab. The Marc Vidal ORfeome library consists of *C.elegans* open reading frame (ORF) clones representing over 10,000 worm genes (Reboul et al. 2003). The ORFs of the entry clones

contain neither start nor stop codons, to achieve flexibility for a wide array of manipulations. Thus, a stop codon was inserted at the end of the ORF using site-directed mutagenesis (Figure 5). Afterwards, each of the entry clones was added to an optimized mCherry Gateway Destination vector (Figure 6) that contained a start codon and fused an N-terminal mCherry to the gene of interest. The *unc-86* promoter in the destination vector expressed the mCherry fusion in neurons. Confirmation was done by HindIII (NEB Cat# R0104S) digest and gel electrophoresis (results not shown). The expected bands 1826, 881, 722, 687, 573, 546, 517, 444, 427, 426, and 396 bp confirmed the expression clones. The negative control was the empty destination vector. In order to determine the functionality of the Rab-10 toolset, phenotypic rescue analysis was performed using the WT expression clone.

Aim 3: *rab-10* mutant rescue analysis

To determine the functionality of the *rab-10* toolset, quantitative analysis of *rab-10 (lf)* animals containing the transgene was done and compared to sibling control animals that lost the transgene through segregation defects. The WT*rab-10*::mCherry fusion was injected into wild-type animals and then crossed with *rab-10 (lf*) animals, since injection of the extrachromosomal array directly into *rab-10 (lf)* animals was not successful. Animals that were *rab-10 (lf*) and contained the extrachromosomal *mCherry::rab10 (+)* were confirmed visually through fluorescent microscopy. The mCherry extrachromosomal array was seen as red puncta in the head and body. *rab-10*

(lf) animals also contained an *odr-1::*RFP (red fluorescent protein) marker, phenotypically expressed as a red pharynx when excited. *rab-10 (lf)* animals also had clear vesicles throughout their bodies, visible thru a brightfield microscope, as a result of an endocytic recycling defect.

Wild-type and *rab-10 (lf)* animals containing the *rab-10* extrachromosomal array displayed overexpression of RAB-10 (Figure 12), where a high level of activity lead to premature termination (Figure 12) and an underdeveloped PLM dendrite. Wild-type animals containing the extrachromosomal array had a PLM average of 0.5663 +/- 0.006 (N=63). *rab-10 (lf)* mutants with the extrachromosomal array had a PLM average of 0.5404 +/- 0.01 (N=56) *rab-10 (lf)* mutants without the *rab-10* extrachromosomal array exhibited PLM overextension with a PLM average of 0.6176 +/- 0.008 (N = 63) (Figure 12). *rab-10 (lf)* animals with the *rab-10* extrachromosomal array and *rab-10 (lf)* animals without the array were significantly different (P-value < 0.0001).

Aim 4: Phenotypic characterization of RAB-10 in the PLM neurite

The mCherry*::rab10* transgene was expressed in *unc86*::myristyolated GFP worms to observe where RAB-10 was expressed in the PLM neurite. *unc-86* drives the expression of GFP in mechanosenory neurons, and it's myristoylated to target the cellular membrane so neurite structure is easily visible. *unc-86* also drives the expression of mCherry in neurons.

The L1 larval stage of *C.elegans* was selected based on Gallegos' previous work (2004) studying ALM and PLM neurite development. The animal undergoes three stages. In Phase 1, the PLM neurite grows at a faster pace than the body, occurring 0-3 hours post-hatching. Phase 2 occurs 5-11 hours post-hatching, where the body grows at a faster rate than PLM. Phase 3 occurs at L2-Adult stage of the worm, where the PLM neurite grows at the same rate as the body. Gallegos (2004) found that SAX-2 appeared to be required for Phase 2 of neurite development, the refinement stage, and that SAX-2 mutants may jump from Phase 1 to Phase 3, resulting in PLM overextension.

Images, taken using confocal microscopy, show mCherry::RAB-10 in the growth cone region and at points along the growing axon in L1 stage animals (Figure 13). Default settings for the microscope were used, and pixel size was under sampled. There is more information left to be observed, as these images do not fully represent all the detail that is available from this transgenic experiment.

Discussion

Aim 1: Double Mutant Analysis

Double mutant analyses were performed to determine whether *rab-10* participates in the *sax-1*/*sax-2* pathway. In yeast, Cbk1, an ortholog of SAX-1, was shown to interact with Sec2p, an exchange factor of Sec4p. The closest animal homolog of Sec4p includes both RAB-8 and RAB-10. RAB-10 was identified as a possible participant through a candidate gene approach (Pranti Das, former Gallegos Lab graduate student). Pranti found that loss of *rab-10*, but not *rab-8*, shows PLM overextension levels similar to *sax-1* mutants. As Rab GTPases are known to regulate vesicle trafficking and has been observed to be involved in trafficking within neurons (Glodowski et al., 2007; Sasidharan et al., 2012; Zou et al., 2015; Taylor et al., 2015), it is plausible that RAB-10 may function in PLM neurite termination. Moreover, our lab has observed that SAX-2 localized to small puncta resembling transport vesicles.

Double mutant analysis helps one to understand how two or more genes interact with each other, by observing the phenotype resulting from combining gene mutations (Girard et al., 2007). When the double mutant phenotype is not significantly different from the most severe single mutant phenotype, one could conclude that the two genes function in the same pathway. Double mutant experimental animals and single mutant sibling controls were created in lab and confirmed by PCR genotyping (Figure 8, 9). PLM dendrite images of animals were taken and quantitatively analyzed.

Findings revealed that *sax-2;rab-10* mutants displayed similar levels of mutant defect as *sax-2* (Figure 10). This suggests RAB-10 may function in the same pathway as SAX-2. However, the *rab-10;sax-1* double mutant did not have similar levels of PLM overextension as the single mutant control (Figures 11). An additive effect was not observed, as one would expect for genes involved in parallel pathways. Perhaps RAB-10 interacts with a kinase other than SAX-1, but utilizes the scaffolding protein of SAX-2. One could argue that the *sax-2* mutant has the most severe phenotype of PLM overextension, thus blocking any possible insight about the relationship between *rab-10* and *sax-2*. However, our lab has observed a more severe phenotype with *sax-2; unc-11* mutants, where PLM terminated near the pharynx of animals. Moreover, our lab has repeated double mutant phenotypic analysis experiments multiple times, and the results consistently show that *rab-10* functions in a pathway with *sax-2*.

Aim 2: *rab-10* toolset creation

A *rab-10* toolset consisting of WT, CA, and DN versions of *rab-10* fused to mCherry, and driven by an *unc-86* promoter has been successfully created. CA RAB-10 has the GTPase attached to GTP and hydrolysis activity of GTP to GDP is suspended, leaving the Rab GTPase in the on conformation. The DN RAB-10 has a greater affinity for GEF than normal, causing the GTPase to become nonfunctional, even when attached to GTP, and limiting the amount of free GEF (Feig 1999). Thus DN RAB-10 actually interferes normal function with the GTPase, and acts as if it is switched in the OFF

position. The *unc-86* promoter drives expression in 55 neurons including the mechanosensory neuron of interest PLM (Finney & Ruvkun 1990).

Marc Vidal entry clones contain neither start nor stop codons. While the mCherry at the N-terminus provided a start codon, a stop codon for each of the 3 variants was needed in order to have a fully functioning *rab-10* gene. The stop codon was added using site-directed mutagenesis and confirmed by DNA sequencing. While CA and DN versions of *rab-10* were not used in this project, the WT version was used to show the functionality of the toolset, by phenotypic rescue.

Aim 3: RAB-10 Phenotype Rescue

The wild-type *rab-10* extrachromosomal array was injected into wild-type worms and then crossed into *rab-10 (lf)* mutants to determine whether the extrachromosomal array was functional. Initially, the extrachromosomal array was injected into *rab-10 (lf)* mutants, but was not successful. However, the genetic crossing of wild-type animals with the array and *rab-10 (lf)* animals achieved the desired strain of *rab-10 (lf)* with the mCherry::*rab-10* extrachromosomal array. The strain was confirmed visually. The mCherry extrachromosomal array was seen as red puncta in the head and body. *rab-10 (lf)* animals also contained an *odr-1::RFP* (red fluorescent protein) marker, phenotypically expressed as a red pharynx when excited. *rab-10 (lf)* animals also had clear vesicles throughout their bodies, visible thru brightfield microscopy, as a result of

an endocytic recycling defect. *rab-10 (lf)* siblings without the extrachromosomal array acted as the control.

The PLM dendrite phenotype was measured and analyzed, and overexpression of the mCherry::*rab-10(+)* extrachromosomal array caused the PLM dendrite to be underdeveloped compared to *rab-10 (lf)* mutants and wild-type animals. This shows that the *rab-10* extrachromosomal array is functional and viable for experimentation.

Aim 4: Characterize RAB-10 in L1 Larval Stage Worms

Wild-type *rab-10::*mCherry worms were crossed with unc86::myristoylated GFP worms in order to qualitatively observe where RAB-10 is located during neural development. Myristoylated GFP is ideal for visualizing neurons for characterization studies. RAB-10 was visible at the soma as well as the growth cone and various points along the PLM neurite. In a paper by Wang et al., (2011), RAB-10 functions in polarized fusion or secretion of vesicles to and from the plasma membrane along the neurite. However, my images were not at optimal pixilation so much more information has yet to be revealed from this worm strain.

Future Directions:

Possible directions to further this study would be to look into other candidate genes in other model organisms that utilize RAB-10 and see what other kinases associate

with it, as well as SAX-1/SAX-2. The toolset could also be used to study colocalization of SAX-2 and RAB-10, since double mutant analyses highly suggests they work together in a pathway. Also, one could continue the characterization studies of RAB-10 with confocal microscopy, ensuring the settings are ideal to get the full spectrum of information that was not apparent in the images taken initially.

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Figures

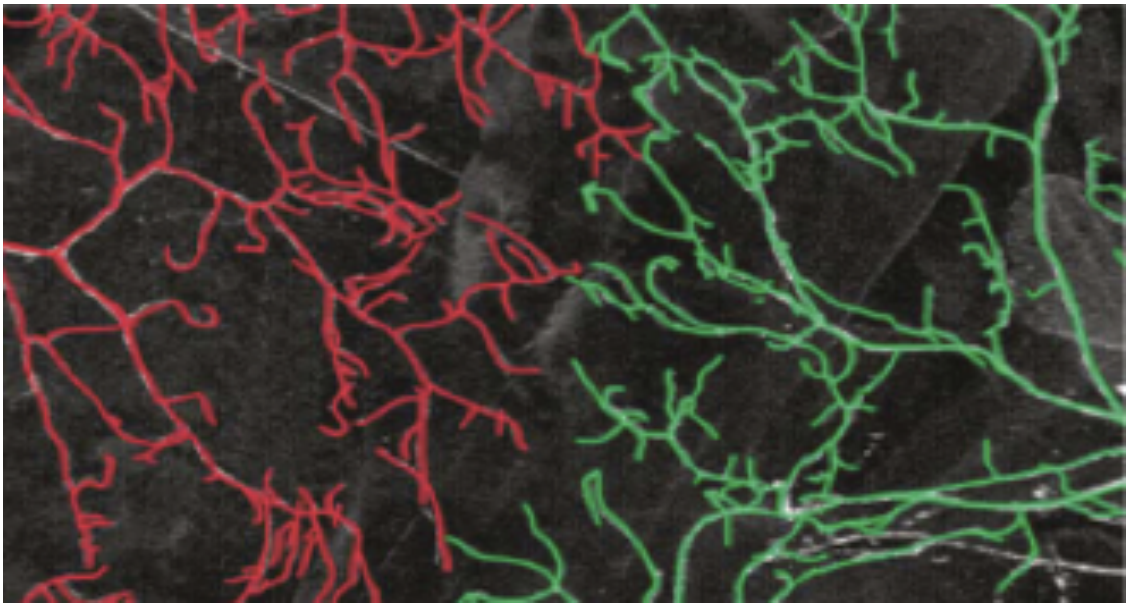

Figure 1. Neurites of two Class IV dendritic arborization (da) neurons (red and green) exhibit non-overlapping, non-redundant field coverage (Jan & Jan, 2003).

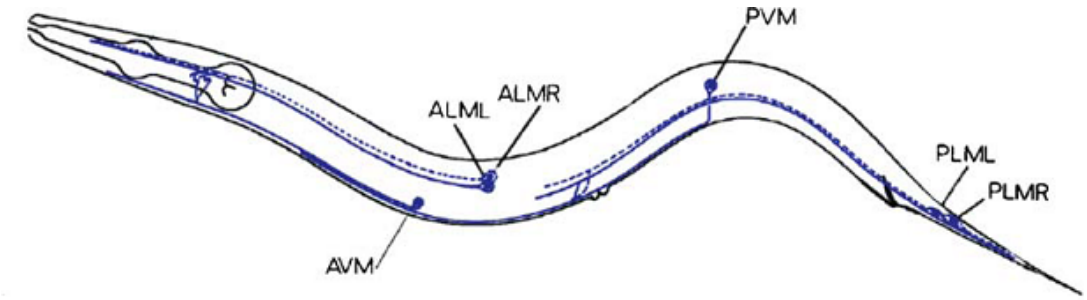

Figure 2. Mechanosensory touch receptor neurons in *C. elegans*
The mechanosensory circuit is responsible for egg-laying, locomotion, and touch. It is comprised of six neurons: anterior lateral microtubule left (ALML), anterior lateral microtubule right (ALMR), anterior ventral microtubule (AVM), posterior ventral microtubule (PVM), posterior lateral microtubule left (PLML), and posterior lateral microtubule right (PLMR). (Bounoutas & Chalfie, 2007)

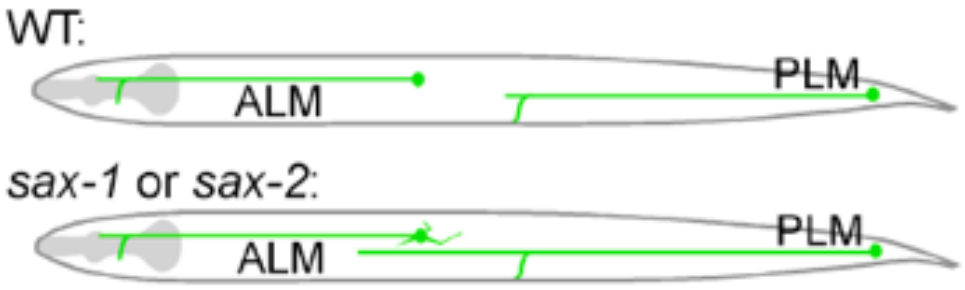

Figure 3. Comparison of ALM and PLM position and morphology on one side in wild-type (WT) and typical *sax-1* or *sax-2* mutant animals. In *sax-1* or *sax-2* mutants, the posterior lateral microtubule (PLM) displays a defective overextension past the anterior lateral microtubule (ALM), and secondary dendrite development from the ALM soma. (Gallegos & Bargmann 2004)

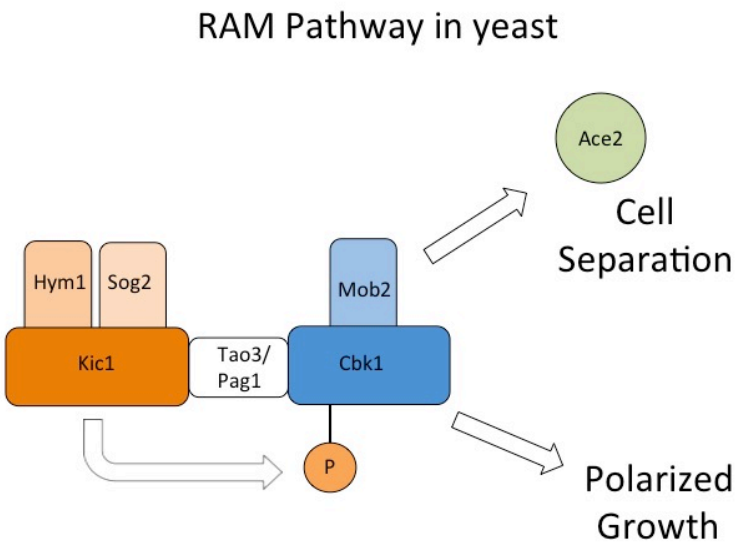

Figure 4. RAM pathway in yeast
RAM stands for Regulation of Ace2 and Morphogenesis. The Mob2-Cbk1 complex is likely to form later in the pathway, as Cbk1 is dependent on all known RAM proteins. F09A5.4 is the worm ortholog of Mob2. SAX-1 is the worm ortholog of Cbk1. Formation of a Hym1/Kic1/ Sog2 complex is suggested, as Hym1 is able to binds to both Kic1 and Sog2, and depends on them for localization. The Tao3 (the SAX-2 ortholog) interacts with Cbk1 and Kic1, and may act as a protein scaffold to facilitate Mob2-Cbk1 kinase activation. (Modified from Nelson et al., 2003)

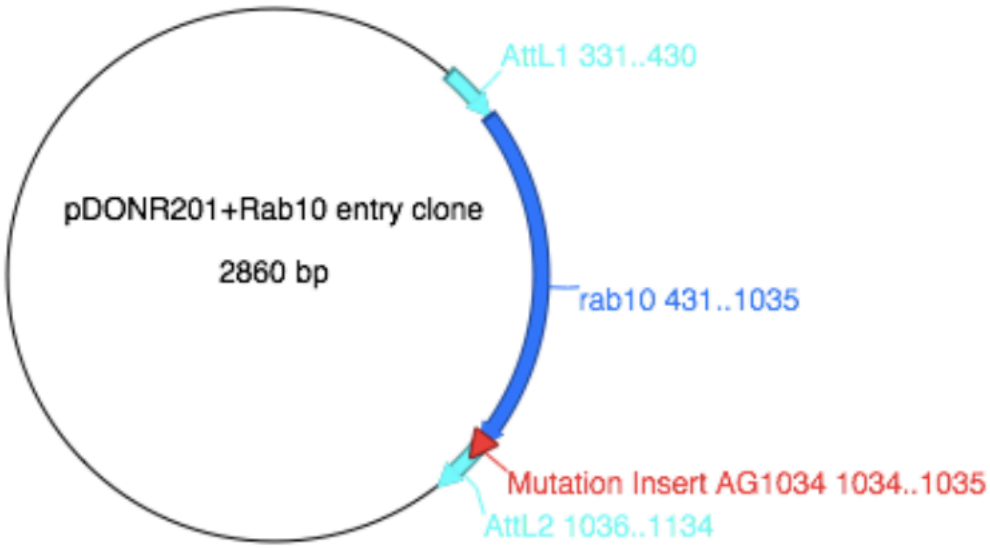

Figure 5. Site of STOP Codon Mutation on Marc Vidal pDONR201 + *rab10* entry clone
Marc Vidal entry clones contain neither start nor stop codons, allowing flexibility for a wide array of manipulations. A stop codon was inserted using site-directed mutagenesis. The start codon was present in the Gateway destination vector.

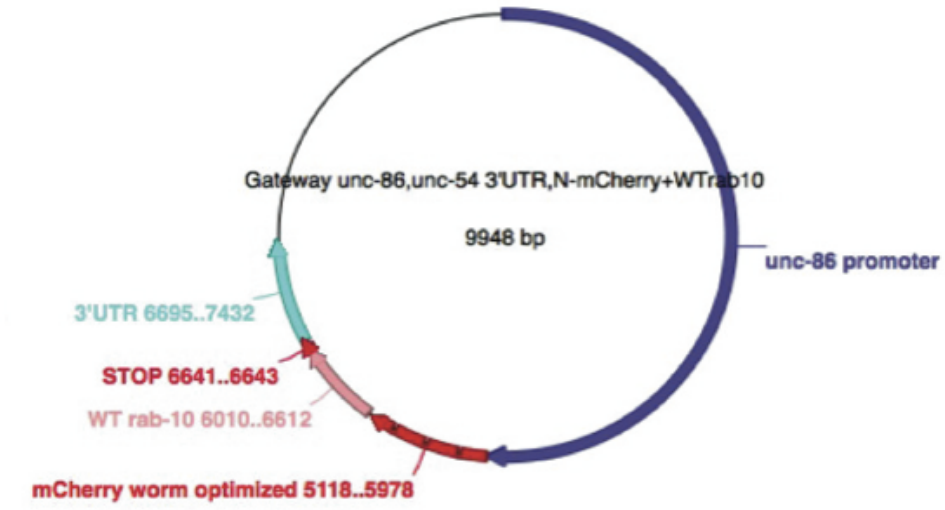

Figure 6. The mCherry::WT*rab-10* Transgene Expression Clone
The wild-type (WT) expression clone contains an *unc-86* promoter that drives expression in 55 neurons including the mechanosensory neurons. The *unc-54* gene encodes the protein myosin-4. N-terminus mCherry is a red fluorescent protein that fuses to the gene of interest, WT *rab-10*. A stop codon is also present as well as a 3'UTR (untranslated region).

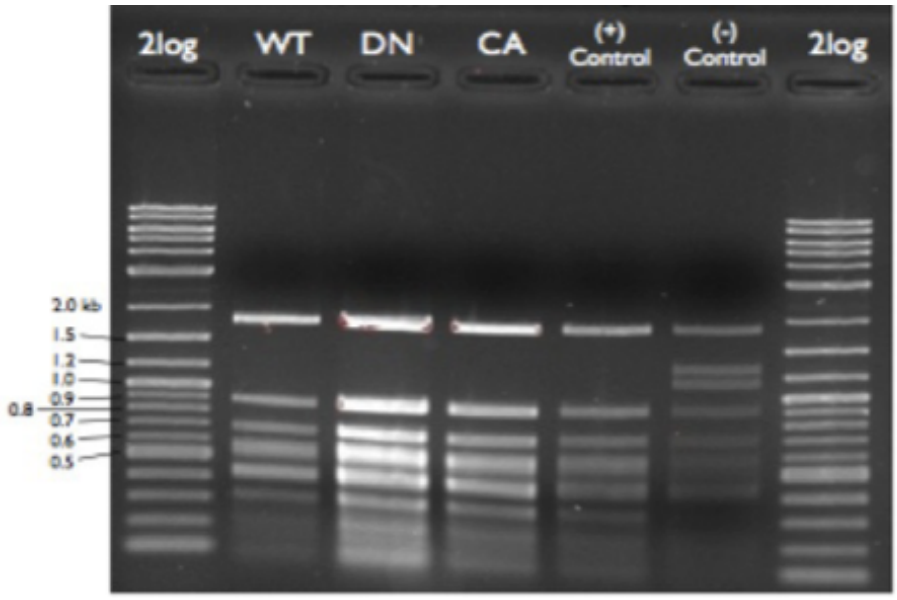

Figure 7. HinfI Digest of unc86::mCherry::rab-10 expression clones
The expression clones comprised of the wild-type (WT), constitutively active (CA) and the dominant negative (DN) versions of *rab-10*. The positive control was a known mCherry::WT*rab-10* expression clone without the stop codon. The negative control was the Gateway destination vector. Expression clone isolates displayed the expected bands: 1826, 881, 722, 687, 573, 546, 517, 444, 427, 416, 396 bp. 2log ladder (New England Biolabs (NEB) Cat# N3200S) was used. HinfI enzyme was from New England Biolabs (NEB Cat # R0155S)

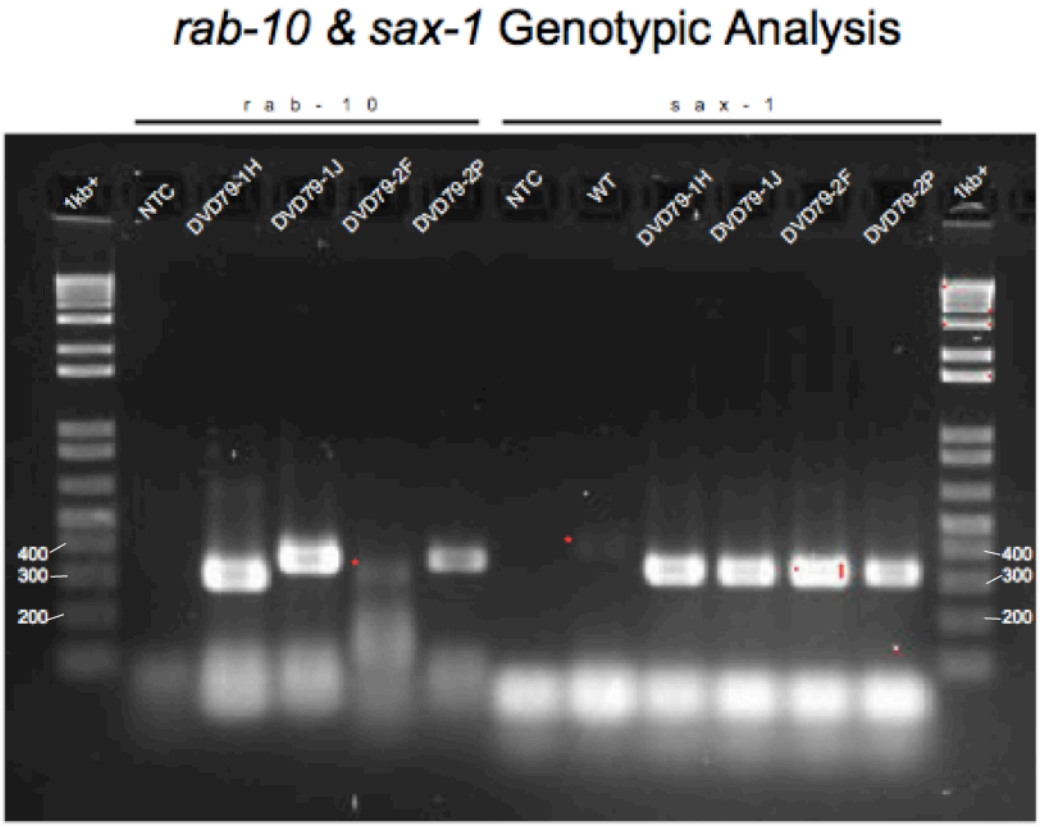

Figure 8. *sax-1* Single and Double Mutant Genotypic Analysis
Putative single and double mutant isolates were confirmed by PCR. Confirmed *sax-1; rab-10* double mutant strains were DVD 79-1J, DVD 79-2P. Confirmed *sax-1* single mutant strains were DVD 79-1H, DVD 79-2F. The primers MG1133 and MG1134 were used to detect *rab-10 (lf)* mutants, with an expected band size of 346 bp. The primers MG1133 and MG1135 were used to detect wild-type *rab-10* with an expected band size of 299 bp. *rab-10 (lf)* isolates are on the left side (346bp). WT-*rab-10* isolates have the 299 bp band. NTC (no template control) was used as a negative control. The primers MG1400 and MG1401 were used to amplify the *sax-1* mutant with an expected band size of 320 bp. The primers MG1401 and MG1399 were used to amplify wild-type *sax-1*, with an expected band size of 384 bp. A wild-type *sax-1* control was used as a positive control, and NTC as a negative control. A 1kb+ ladder (ThermoFisher Cat# 10787018) was used.

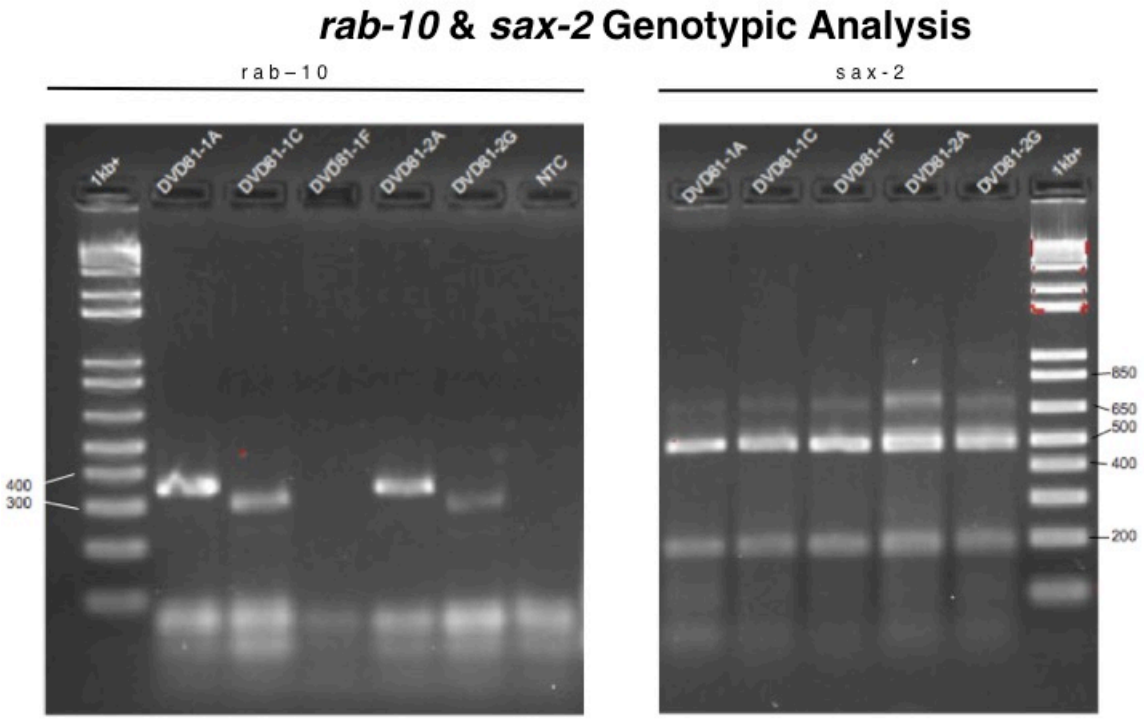

Figure 9. *sax-2* Single and Double Mutant Genotypic Analysis
Putative single and double mutant isolates were confirmed by PCR. Confirmed *sax-2; rab-10* double mutant strains were DVD 81-1A, DVD 81-2A. Confirmed *sax-2* single mutant strains were DVD 81-1C, DVD 81-2G. The primers MG1133 and MG1134 were used to detect *rab-10 (lf)* mutants, with an expected band size of 346 bp. The primers MG1133 and MG1135 were used to detect wild-type (WT) *rab-10* with an expected band size of 299 bp. *rab-10 (lf)* isolates are on the left side (346bp). WT-*rab-10* isolates have the 299 bp band. NTC (no template control) was used as a negative control. The following primers were used to amplify *sax*-2: MG313 and MG315. After touchdown PCR, a Hpy188I restriction enzyme digest was used to digest the amplicon and identify isolates that have the mutant allele. The expected bands for WT-*sax-2* were: 405bp, 189bp, 70bp, 28bp. The expected bands for mutant *sax-2* were 475 bp, 189 bp, and 28bp. The uncut amplicon had an expected band size of 692 bp. A 1kb+ ladder (ThermoFisher Cat# 10787018) was used.

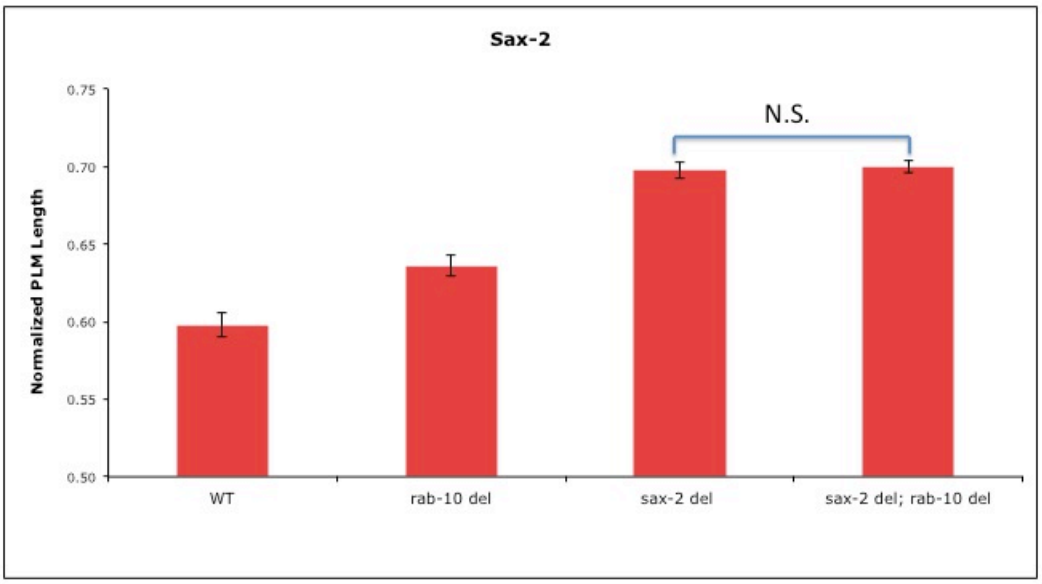

Figure 10. *sax-2; rab-10* Double Mutant Phenotypic Analysis
The *sax-2; rab-10* double mutant (average = 0.700 +/- 0.004) displayed a similar level of PLM overextension defect as the *sax-2* single mutant control (average = 0.697 +/- 0.005) (P-value = 0.6540). The *rab-10* single mutant (average = 0.636 +/- 0.007) also displayed PLM overextension but not nearly to the extent of the *sax-2* single and double mutants. The wild type PLM average was 0.598 +/-0.008. N.S. = not significant

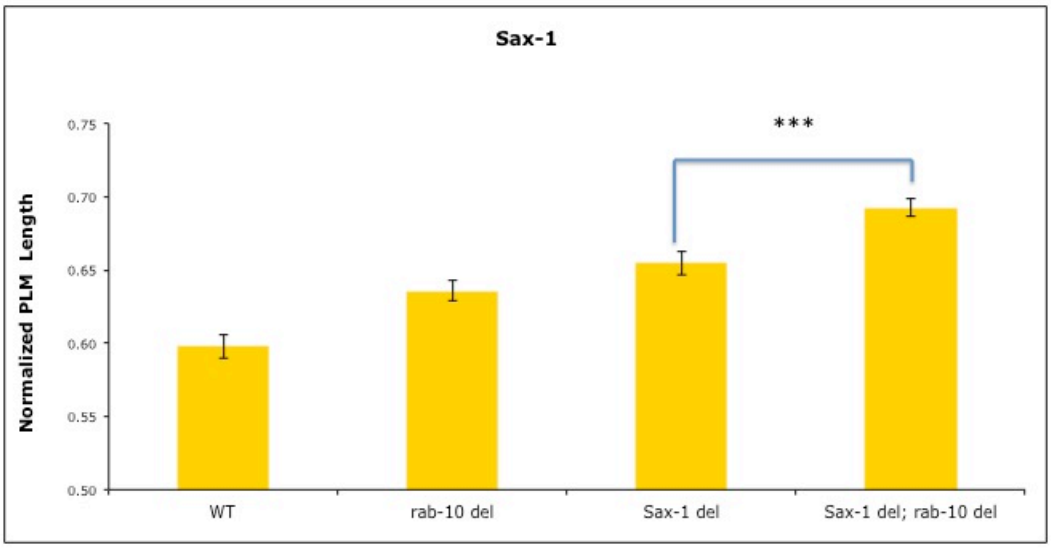

Figure 11. ***sax-1; rab-10*** **Double Mutant Phenotypic Analysis**
The *sax-1; rab-10* double mutant (average = 0.693 +/- 0.006) displayed significantly different levels of PLM overextension defect as the *sax-1* single mutant control (average = 0.654 +/- 0.0058 (P-value = 0.0001). The *rab-10* single mutant (average = 0.636 +/- 0.007) also displayed PLM overextension but not nearly to the extent of the *sax-2* single and double mutants. The wild type PLM average was 0.598 +/-0.008. *** = P-value ≤=0.001

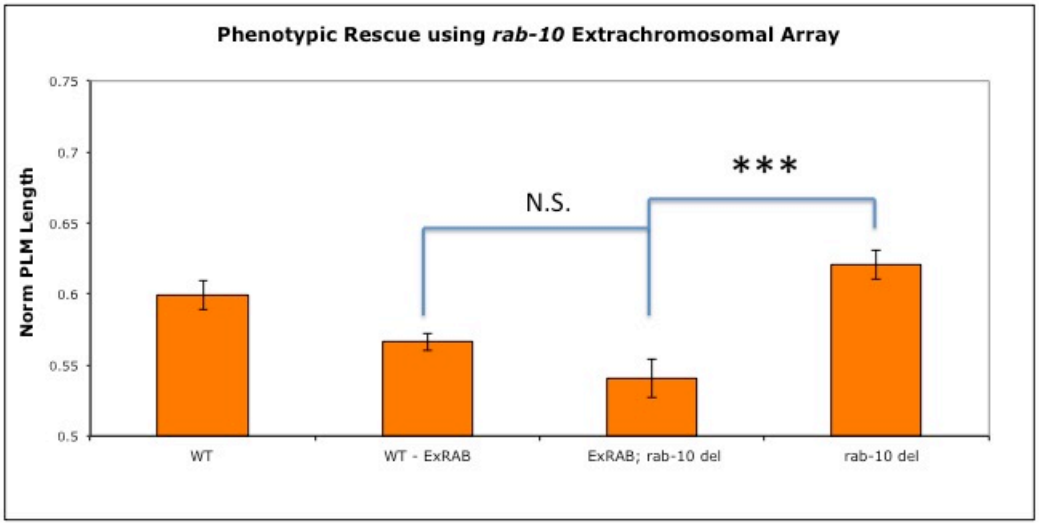

Figure 12. Phenotypic Rescue of *rab-10* mutant with *rab-10::mCherry* extrachromosomal array (ExRAB)
Animals with ExRAB displayed overexpression of Rab-10 with a high level of activity leading to premature termination. ExRab; *rab-10* animals had a PLM average of 0.540 +/- 0.01. Wild-type (WT) animals with ExRAB had a PLM average of 0.566 +/- 0.006. The difference between both types of ExRAB animals was not significant (N.S.) (P-value = 0.069). In comparison, *rab-10* deletion (del) mutants displayed PLM overextension with an average of 0.62 +/- 0.01. There was a very significant different between *rab-10 del* mutants and *rab-10 del* mutants with ExRAB (P-value = 0.0001). *** = P-value ≤*0.001

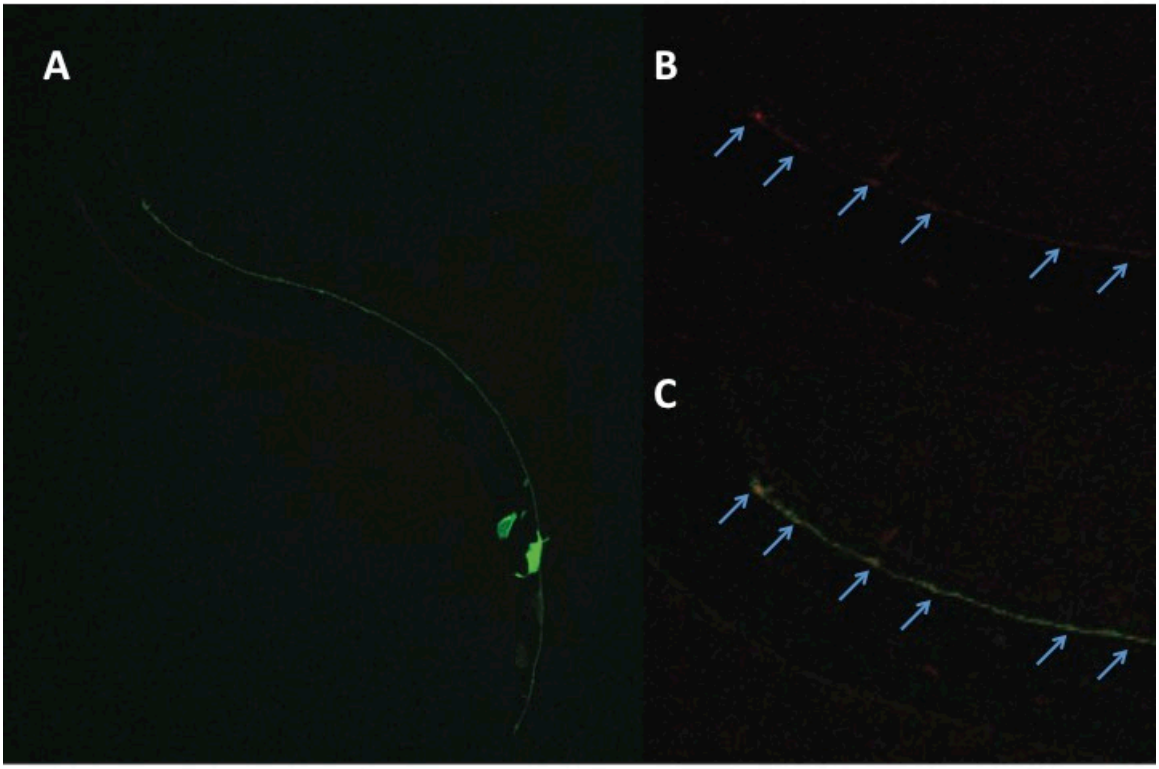

Figure 13. Phenotypic characterization of RAB-10 in the PLM dendrite.
A shows the full image of the PLM dendrite of the L1 larval stage animal in the GFP channel. **B** shows the mCherry signal for Rab-10. **C** shows the composite of the two channels. **B and C** have arrows that highlight areas where RAB-10 is expressed in the PLM dendrite at the L1 larval stage. In this animal, we observe expression in the growth cone and at several points along the dendrite.

Tables

Table 1. Orthologs

C.elegans	*Drosophila*	*S.cerevisiae*	*S.pombe*	*N.crassa*
sax-1	Tricornered	Cbk1	Orb6	Cot-1
sax-2	Furry	Tao3/Pag1	Mor2	--
F09A5.4	Mob2a	Mob2	Mob2	--
rab-8	--	Sec4	Sec4	--

Table 2. General Genotyping Touchdown PCR Reaction Mix

Reagent	Amount (uL)	Final Conc
Template	0.5	--
MG1401(100uM)	0.5	2uM
MG1399 (100uM)	0.25	1uM
MG1400 (100uM)	0.25	1uM
GoTAQ (5U/uL)	0.2	1 U
GoTAQ Buffer (5x)	5	1x
MgCl2 (25mM)	2.5	2.5mM
dNTPs (10mM)	0.5	0.2mM
DNA-grade water	15.3	--

Table 3. Site-directed Mutagenesis Reaction Mix

Reagent	Concentration	Amount (uL)
Reaction Buffer	10x	2.5
DNA	5 ng/uL	1.0
MG1411	7 pmol/uL	1.0
MG1412	7 pmol/uL	1.0
dNTP mix	proprietary	0.5
Quik solution	proprietary	1.5
DNA-grade water	--	17.5
Pfu Ultra DNA pol	2.5 U/uL	0.5

Table 4. Sample LR Clonase Reaction Mix

Reagent	Concentration	Working Conc	Amount
WT-2 Entry Clone	216 ng/uL	75 ng	0.70uL of 2x dilution
EB Buffer	--	--	2.09 uL
Isolate 4 Dest Vector	238 ug/uL	288.75 ng	1.21 uL
LR Clonase II Enz Mix	--	--	1.00uL

Table 5. Touchdown Colony PCR Reaction Mix

Single Rxn (uL)	Reagent (conc)	Final Conc
--	DNA Template	--
2.5	MG1084 (10uM)	25 pmol
2.5	MG1085 (10uM)	25 pmol
0.5	dNTPs (10mM)	0.2 mM
5	GoTaq Buffer (5x)	1x
2.5	MgCl2 (25mM)	2.5 mM
12uL	DNA-grade water	--

Appendix - Sequencing of SDM Isolates

Mutation highlighted in red. AttL sites highlighted in blue.

SDM WT-*rab10* Isolate 2

```
   1 ACGTCAAATCCGCTCCCGGCGGATTNGTCNTANTCAGGAGAGCGTTCACC 50
  51 GACAAACAACAGATAAAACGAAAGGCCCAGTCTTCCGACTGAGCCTTTCG 100
 101 TTTTATTTGATGCCTGGCAGTTCCCTACTCTCGCGTTAACGCTAGCATGG 150
 151 ATCTCGGGCCCcaaataatgattttattttgactgatagtgacctgttcg 200
 201 ttgcaacaaattgatgagcaatgctttttataatgccaactttgtacaa 250
 251 aaaagcaggctTGGCTCGCCGACCGTATGACATGCTCTTCAAATTGCTGC 300
 301 TAATAGGCGACTCAGGAGTTGGAAAAACTTGCATTCTGTACAGATTTTCG 350
 351 GATGATGCCTTTAACACCACATTCATCTCAACTATTGGAATCGACTTCAA 400
 401 AATTAAAACTATCGAATTAAAAGGAAAAAAGATTAAATTACAAATCTGGG 450
 451 ACACAGCTGGACAAGAACGATTCCACACAATCACAACATCATATTACCGC 500
 501 GGAGCAATGGGAATTATGCTGGTTTACGATATCACAAATGCGAAAAGTTT 550
 551 TGACAATATTGCAAAGTGGTTGCGCAATATTGACGAACACGCATCGGAAG 600
 601 ATGTTGTTAAAATGATATTGGGAAATAAATGTGATATGAGTGATAGACGA 650
 651 GTTGTGAGCAGAGAACGAGGAGAGAAGATCGCACAAGATCATGGTATTAG 700
 701 CTTCCACGAGACTTCAGCTAAATTGAATGTTCACGTCGATACAGCGTTTT 750
 751 ACGATTTAGCAGAAGCAATCCTGGCTAAGATGCCTGATTCCACTGACGAG 800
 801 CAATCCCGCGATACGGTGAATCCAGTGCAACCACAGAGGCAGAGTAGCAG 850
 851 TGGAGGATGCTGCTAGacccagctttcttgtacaaagttggcattataag 900
 901 aaagcattgcttatcaatttgttgcaacgaacaggtcactatcagtcaaa 950
 951 ataaaatcattatttgCCATCCAGCTGCAGCTCTGGCCCGTGTCTCAAAA 1000
1001 TCTCTGATGTTACATTGCACAAGATAAAAATATATCATCNGANCAATAAA 1050
1051 AC 1052
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SDM DN-*rab10* Isolate 1

```
   1 CCANNCNTCAGGNTNNNNNTGNTAGTTNATGCCNGNCAGTTTANNNGGNN 50
  51 NCNNCCNNCCNCCTTNCGGGCCGTGCTTCACAACGTCAAATCNGCTCCCG 100
 101 GCGGATTTNNNCTACTCAGGAGAGCGTCACCGACAAACNACAGATNAAAN 150
 151 GAAAGGCCCAGTCTTCCGACTGAGCCTTTNGTTTTATTTGATGCCTGGCA 200
 201 GTTCCNTACTCTCGCGTTAACGCTAGCATGGATCTNNGGCCCcaaataat 250
 251 gattttattttgactgatagtgacctgttcgttgcaacaaattgatgagc 300
 301 aatgctttttataatgccaactttgtacaaaaaagcaggctTGGCTCGC 350
 351 CGACCGTATGACATGCTCTTCAAATTGCTGCTAATAGGCGACTCAGGAGT 400
 401 TGGAAAAAATTGCATTCTGTACAGATTTTCGGATGATGCCTTTAACACCA 450
 451 CATTCATCTCAACTATTGGAATCGACTTCAAAATTAAAACTATCGAATTA 500
 501 AAAGGAAAAAAGATTAAATTACAAATCTGGGACACAGCTGGACAAGAACG 550
 551 ATTCCACACAATCACAACATCATATTACCGCGGAGCAATGGGAATTATGC 600
 601 TGGTTTACGATATCACAAATGCGAAAAGTTTTGACAATATTGCAAAGTGG 650
 651 TTGCGCAATATTGACGAACACGCATCGGAAGATGTTGTTAAAATGATATT 700
 701 GGGAAATAAATGTGATATGAGTGATAGACGAGTTGTGAGCAGAGAACGAG 750
 751 GAGAGAAGATCGCACAAGATCATGGTATTAGCTTCCACGAGACTTCAGCT 800
 801 AAATTGAATGTTCACGTCGATACAGCGTTTTACGATTTAGCAGAAGCAAT 850
 851 CCTGGCTAAGATGCCTGATTCCACTGACGAGCAATCCCGCGATACGGTGA 900
 901 ATCCAGTGCAACCACAGAGGCAGAGTAGCAGTGGAGGATGCTGCTAGacc 950
 951 cagctttcttgtacaaagttggcattataagaaagcattgcttatcaatt 1000
1001 tgttgcaacgaacaggtcactatcagtcaaaataaaatcattatttGCCA 1050
1051 TCCAGCTGCAGCTCTGGCCCGTGTCTCAAAATCTCTGATGTTACATTGCA 1100
1101 CAAGATAAAAATATATCATCANGAACAATAAAA 1133
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SDM CA-*rab10* Isolate 1

```
   1 GCCNCCNTCNGNNNGTGTTCACNACNTCAAANNGNNCCCGCGGNNTTNNT 50
  51 NCTANTCAGNNAGAGCGTCNCCGNCNANCAACAGNTAAAACGAAAGGCCC 100
 101 AGTNTTCCGACTGANCCTTTCGTTTTATTTGATGCNTGNCAGTTCCCTAC 150
 151 TNTCGCGTTAACGCTAGCATGGATNTCGGGCCCcaaataatgattttatt 200
 201 ttgactgatagtgacctgttcgtngcaacaaantgatgagcaatgctttt 250
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 401 CAACTATTGGAATCGACTTCAAAATTAAAACTATCGAATTAAAAGGAAAA 450
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 501 AATCACAACATCATATTACCGCGGAGCAATGGGAATTATGCTGGTTTACG 550
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1051 AATATATCATCANGAACAATAAA 1073
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