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

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Running Title: ppm-1 regulates axon termination

keywords: PPM-1, RPM-1, synapse, axon termination, and p38 MAPK

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Abstract

The PHR (Pam/Highwire/RPM-1) proteins are evolutionarily conserved ubiquitin ligases that regulate axon guidance and synapse formation in *C. elegans*, *Drosophila*, zebrafish and mice. In *C. elegans*, RPM-1 (Regulator of Presynaptic Morphology-1) functions in synapse formation, axon guidance, axon termination, and postsynaptic GLR-1 trafficking. Acting as an E3 ubiquitin ligase, RPM-1 negatively regulates a MAP kinase pathway that includes: *dlk-1*, *mkk-4* and the p38 MAPK, *pmk-3*. Here we provide evidence that *ppm-1*, a serine/threonine phosphatase homologous to human PP2Cα (PPM1A) and PP2Cβ (PPM1B) acts as a second negative regulatory mechanism to control the *dlk-1* pathway. We show that *ppm-1* functions through its phosphatase activity in a parallel genetic pathway with *glo-4* and *fsn-1* to regulate both synapse formation in the GABAergic motorneurons, and axon termination in the mechanosensory neurons. Our transgenic analysis shows that *ppm-1* acts downstream of *rpm-1* to negatively regulate the DLK-1 pathway, with PPM-1 most likely acting at the level of *pmk-3*. Our study provides insight into the negative regulatory mechanisms that control the *dlk-1* pathway in neurons, and demonstrates a new role for the PP2C/PPM phosphatases as regulators of neuronal development.
Introduction


PHR proteins function through multiple downstream signaling pathways. In C. elegans, RPM-1 functions as part of an ubiquitin ligase complex that includes the F-box protein, F-box Synaptic Protein (FSN)-1 (LIAO et al. 2004). This complex negatively regulates a MAP kinase cascade that includes dual leucine zipper-bearing kinase (dlk-1), map kinase kinase (mkk)-4, p38 map kinase (pmk)-3, map kinase activated protein kinase (mak)-2, and the transcription factor cebp-1 (NAKATA et al. 2005; YAN et al. 2009). Drosophila Highwire and mouse Phr1 negatively regulate the ortholog of DLK-1 through a similar mechanism (SAIGA et al. 2009; TADA et al. 2009; WU et al. 2007) COLLINS et al. 2006; LEWCOCK et al. 2007). Phr1 also ubiquitinates and negatively regulates the Tuberin Sclerosis Complex (D’SOUZA et al. 2005; HAN et al. 2008; MURTHY et al. 2004). RPM-1 positively regulates signaling through a Rab GTPase pathway by binding to Gut Granule Loss (GLO)-4 (GRILL et al. 2007).
While RPM-1 negatively regulates the DLK-1 pathway, there are a number of reasons to suspect that the DLK-1 pathway may also be controlled by other negative regulatory mechanisms. First, overexpression of *dlk-1* causes more dramatic phenotypes than *rpm-1* loss of function (lf), including uncoordinated movement and small body size (Abrams et al. 2008; Nakata et al. 2005). Second, the *dlk-1* pathway consists of 5 signaling molecules providing numerous points where regulation might occur. Third, ubiquitination is a relatively slow acting mechanism to restrict DLK-1 signaling. The observation that UEV-3 is a possible positive regulator of PMK-3 (Trujillo et al. 2010) further supports the idea that multiple mechanisms may control the DLK-1 pathway.

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

Here we provide evidence that neuronal development is regulated by a PP2C/PPM family phosphatase from *C. elegans* that we call *protein phosphatase mg²⁺/mn²⁺ dependent (ppm)-1*. We have found that *ppm-1* acts through its phosphatase activity to regulate axon termination and synapse formation by acting in a parallel genetic pathway to *fsn-1* and *glo-4*. Loss of function in *ppm-1* is suppressed by loss of function in *pmk-3* (p38 MAPK), suggesting that *ppm-1* negatively regulates *pmk-3* activity. This finding is consistent with our observation
that ppm-1 functions downstream of rpm-1. Overall, our observations
demonstrate that the DLK-1 pathway is negatively regulated by at least two
mechanisms in neurons: the action of an SCF complex that includes RPM-1 and
FSN-1, and the activity of a serine/threonine phosphatase, PPM-1.

Materials and Methods

Genetics

C. elegans strains were maintained as described (BRENNER 1974). Alleles used
in this study include: rpm-1(ju44), glo-1(zu391), fsn-1(hp1), ppm-1/tag-93(ok578),
ppm-1/tag-93(tm653), dlk-1(ju476), mkk-4(ju91), and pmk-3(ok169). All double
mutants were constructed following standard procedures, and were confirmed by
the associated phenotypes or by PCR genotyping. glo-4,ppm-1 double mutants
were constructed by recombination without using visible markers. Primers and
PCR conditions are available upon request. fsn-1;ok578/tm653 animals were
constructed using dpy-11 linked to ok578, and unc-42 linked to tm653. Non-dpy,
non-unc animals with the genotype fsn-1;ok578,dpy-11/unc-42,tm653 were
scored for trans-heterozygous analysis. The transgenic strains used in this study
are: mul32[P_mec-7::GFP] (CH’NG et al. 2003), juls1[P_unc-25::SNB-1::GFP] (HALLAM
and JIN 1998), bggEx35 [wrm613bH10], bggEx33 [P_gef-1::ppm-1 (cDNA
F25D1.1c)], bggEx34, 40, and 41 [P_mec-7::ppm-1 (cDNA F25D1.1c)], bggEX58,
59, 60, 61, and 62 [P_mec-7::ppm-1 (D246N) (cDNA F25D1.1c)], bggEx55, 56 and
57 [P_ppm-1::GFP].
Transgene Constructs

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

Transgenic animals were generated as described previously (MELLO et al. 1991). Plasmid DNA of interest was injected at 1-25ng/µL along with *P*$_{ttx-3}$RFP (50ng/µL) or (*P*$_{myo-2}$mCherry 1.5-2.5ng/µL) and pBluescript (50ng/µL). Initially all transgenic animals were generated on *ppm-1*-/ backgrounds. To create transgenic animals that were *fsn-1;ppm-1* double mutants, *fsn-1;ppm-1* mutants were heat shocked and males were mated to array positive *ppm-1* mutants. For DLK-1 overexpression experiments, *Prgef-1::dlk-1* was amplified by long PCR using pBG-57 as a template and injected at 5-10ng/µL. For rescue experiments with PPM-1, pBG-GY163 (*P*$_{mec-7}$::*ppm-1*) or pBG-GY202 (*P*$_{mec-7}$::*ppm-1* (D246N)) were coinjected at 2ng/µL with *Prgef-1::dlk-1*. For analysis of PPM-1
subcellular distribution, pBG-GY208 (Punc-25::mCherry-PPM-1) was injected at 5ng/µL into ppm-1(tm653);juls1 animals.

**Axon termination and Synapse Formation Analysis**

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

**Results**

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

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

The C. elegans genome contains a single gene, temporarily assigned gene name (tag)-93 (F25D1.1), whose protein product is conserved with two PP2C phosphatases in humans, PP2Cα/PPM1A and PP2Cβ/PPM1B (49% identity and 69% conservation with PPM1A; 54% identity and 72% conservation with PPM1B) (Stern et al. 2007) (Figure 1A and B). PP2C/PPM phosphatases are single subunit enzymes that require magnesium/manganese for activity, and consist of a catalytic domain and a regulatory domain (Lu and Wang 2008; Shi 2009) (Figure 1B). Based on this homology, we have renamed tag-93 as protein phosphatase magnesium²+/manganese²+ dependent (ppm)-1.

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

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

In wild-type animals, the two ALM mechanosensory neurons each have a 
single axon that terminates at a precise location (Figure 1C). In contrast, axon 
termination is defective in the ALM neurons of rpm-1 (If) mutants (Figure 1C and 
D). rpm-1 (If) mutants display two types of ALM axon termination defects: less 
severe short hooks, and more severe big hooks where the axon overextends and 
hooks towards the posterior of the animal (Figure 1C and E). In rpm-1 (If) 
mutants, big hooks in the ALM are the predominant phenotype, and this
phenotype is temperature sensitive for the \textit{ju44} allele of \textit{rpm-1} (Figure 1E). In \textit{ppm-1(ok578)} and \textit{ppm-1(tm653)} mutants, we observed small hook defects in the ALM neurons that occurred with low penetrance (Figure 1 C, D and E).

Previous studies showed that \textit{rpm-1} (\textit{lf}) mutants also have defects in the PLM neurons which fall into two main phenotypic categories: 1) axon termination defects (Figure 2A) and 2) synaptic branch extension/stabilization defects (Figure 3A) \cite{Grill2007, Schaefer2000}. A given PLM neuron can display one or both of these phenotypes. In \textit{rpm-1} (\textit{lf}) mutants, a small percentage of PLM axons (8\%) display a milder axon termination phenotype in which the PLM axon only overextends beyond the ALM cell body (Figure 2A, overextension). The majority of PLM neurons in \textit{rpm-1} (\textit{lf}) mutants (90\%) display a more severe phenotype in which the PLM axon overextends beyond the ALM cell body and also hooks ventrally, which we will refer to as hooking for ease of discussion (Figure 2A hook). Both the hooking and synaptic branch defects in \textit{rpm-1} (\textit{lf}) mutants are highly penetrant (Figure 2B and 3B). In \textit{ppm-1(ok578)} mutants, we observed both axon termination phenotypes with a larger percentage of neurons showing the milder overextension phenotype, and a small percentage of neurons showing the more severe hooking phenotype (Figure 2A and B). Similar results were obtained for \textit{ppm-1(tm653)} (Figure 2B). With regard to synaptic branch extension, \textit{ppm-1(lf)} animals had defects in synaptic branch extension that were very low penetrance (Figure 3A and B). These results show that \textit{ppm-1} regulates axon termination in the mechanosensory neurons.
**ppm-1 functions in parallel to fsn-1 and glo-4 to regulate axon termination.**

*rpm-1* has two major downstream signaling activities that are known. *rpm-1* functions with *fsn-1* to negatively regulate the *dlk-1* pathway (Liao et al. 2004; Nakata et al. 2005), and binds to GLO-4 to positively regulate the *glo* pathway which includes: *glo-4, glo-1* (a Rab GTPase), and *apm-3* (Grill et al. 2007). To determine if *ppm-1* functions in either of these pathways or as part of an independent pathway, we constructed double mutants between *ppm-1* and *fsn-1, glo-4, and glo-1*. When total mutant neurons were analyzed, *fsn-1;ppm-1* double mutants had an additive phenotype in the ALM neurons (Figure 1B). However, an enhanced penetrance of big hooks was observed in *fsn-1;ppm-1* double mutants compared to single mutants (Figure 1E). Both *ppm-1(ok578)* and *ppm-1(tm653)* had similar enhancer effects with *fsn-1* (lf) (Figure 1E). While the level of big hooks was mildly increased in both *glo-4,ppm-1* and *glo-1;ppm-1* double mutants, these differences were not statistically significant demonstrating that *ppm-1* does not enhance the *glo* pathway in the ALM neurons (Figure 1E).

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

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

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

*ppm-1 regulates synapse formation in GABAergic motor neurons.*
Previous studies have shown that \textit{rpm-1} regulates synapse formation in the motor neurons (\textit{NAKATA et al.} 2005; \textit{ZHEN et al.} 2000). The presynaptic terminals formed by the GABAergic DD motor neurons can be visualized with \text{Synaptobrevin (SNB)}-1 fused to GFP (GFP::SNB-1). In wild-type animals, GFP::SNB-1 puncta of the DD neurons are evenly distributed along the dorsal nerve cord (Figure 4A). In contrast, \textit{rpm-1} (lf) mutants have disorganized GFP::SNB-1 puncta with gaps and aggregation (Figure 4A). While \textit{ppm-1} (lf) animals are normal, \textit{fsn-1;ppm-1} and \textit{glo-4,ppm-1} double mutants are enhanced with significant disorganization of GFP::SNB-1 puncta (Figure 4A) and reduced numbers of SNB-1::GFP puncta (Figure 4B). \textit{glo-1;ppm-1} double mutants had reduced numbers of SNB-1::GFP puncta, but defects were too mild to constitute enhancement (Figure 4B, p<0.05). We also observed that \textit{ppm-1,rpm-1} double mutants have similar defects as those in \textit{rpm-1} (lf) mutants. Importantly, synapse formation defects in \textit{rpm-1} (lf) mutants are not saturated, as defects become significantly worse in \textit{rpm-1;syd-2} double mutants (\textit{LIAO et al.} 2004). Therefore, our observations are consistent with \textit{ppm-1} regulating synapse formation by functioning in the same genetic pathway as \textit{rpm-1}, and a parallel genetic pathway to \textit{fsn-1} and \textit{glo-4}.

\textbf{ppm-1 functions in mechanosensory neurons downstream of rpm-1.}

\textit{rpm-1} and its downstream signaling molecules, \textit{glo-4} and \textit{glo-1}, act cell autonomously in mechanosensory neurons (\textit{GRILL et al.} 2007; \textit{SCHAEFER et al.} 2000). To determine if \textit{ppm-1} functions cell autonomously, we performed
transgenic rescue experiments in which \textit{ppm-1} expression was driven by different promoters. Transgenic expression of \textit{ppm-1} using either a fosmid (native \textit{ppm-1} promoter), \textit{Prgef-1} (a pan-neuronal promoter) or \textit{Pmec-7} (a mechanosensory neuron promoter) rescued PLM axon termination defects in both \textit{ppm-1} single mutants and \textit{fsn-1;ppm-1} double mutants (Figure 5A). Previous studies identified a single point mutation (D239N) that results in a 4000-fold decrease in phosphatase activity in human PP2C\(\alpha\) (\textit{Jackson et al}. 2003) (Figure 1A). \textit{ppm-1} that is mutated at the corresponding residue (D246N), and presumably catalytically inactive, did not rescue the enhanced axon termination defects in \textit{fsn-1;ppm-1} double mutants (Figure 5A). These results demonstrate that \textit{ppm-1} regulates axon termination through its phosphatase activity, and that the lesion in \textit{ok578} causes the enhanced penetrance of axon termination defects observed in \textit{ppm-1;fsn-1} double mutants.

Our genetic analysis described earlier indicates that \textit{ppm-1} functions in the same genetic pathway as \textit{rpm-1}. To determine if \textit{ppm-1} functions up or downstream of \textit{rpm-1}, we generated transgenic animals that overexpress PPM-1 in the mechanosensory neurons of \textit{rpm-1} (lf) mutants. Overexpression of PPM-1 strongly, but partially, reduced the axon termination defects in \textit{rpm-1} (lf) mutants (Figure 5B). In contrast, phosphatase-dead PPM-1 did not rescue axon termination defects in \textit{rpm-1} (lf) mutants (Figure 5B). These results are consistent with \textit{ppm-1} functioning as a phosphatase that acts downstream of \textit{rpm-1}. 
**PPM-1 negatively regulates the DLK-1 pathway.**

Previous studies have shown that the orthologs of PPM-1 can act at the level of MKKK, MKK or MAPK to negatively regulate a kinase cascade (Hanada et al. 2001; Takekawa et al. 1998). These observations suggested that phenotypes in \textit{ppm-1} (lf) mutants might be due to excess activation of \textit{dlk-1}, \textit{mkk-4} and/or \textit{pmk-3}. To address this question, we employed two experimental strategies. First, we used a transgenic approach to test if \textit{ppm-1} negatively regulates the DLK-1 pathway. Overexpression of DLK-1 resulted in similar severity and penetrance of phenotypes as those seen in \textit{rpm-1} (lf) mutants including axon termination defects in the ALM (Figure 6A) and PLM neurons (Figure 6B), and defects in synaptic branch extension in the PLM neurons (data not shown). Coexpression of PPM-1, but not phosphatase-dead PPM-1, partially rescued the defects caused by overexpression of DLK-1 (Figure 6A and B). This observation suggests that PPM-1 acts as a phosphatase to negatively regulate the DLK-1 pathway.

Next, we used a genetic approach to determine which kinase in the DLK-1 pathway might be a target of PPM-1’s phosphatase activity. To do so, we constructed double mutants of \textit{ppm-1} with \textit{dlk-1}, \textit{mkk-4}, or \textit{pmk-3}. Axon termination defects (overextension) in the PLM neurons were analyzed for double mutants and compared to \textit{ppm-1} (lf) single mutants. Defects in axon termination were not rescued in \textit{dlk-1};\textit{ppm-1} and \textit{mkk-4};\textit{ppm-1} double mutants (Figure 6C). In contrast, axon termination defects in \textit{pmk-3};\textit{ppm-1} double mutants were significantly reduced compared to \textit{ppm-1} (lf) single mutants (Figure
6C). These observations are consistent with excess pmk-3 function leading to axon termination defects in ppm-1 (If) mutants.

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

Given that ppm-1 functions in neurons, we wanted to test if it is expressed in neurons. To address this question, we generated transgenic animals in which the 3.7 kb promoter of ppm-1 drives expression of GFP. Ppm-1::GFP is expressed in neurons of the nerve ring, and motor neurons of the ventral nerve cord (Figure 7A). Since ppm-1 is expressed in motor neurons, we used a transgenic approach to study the subcellular distribution of PPM-1 in the GABAergic DD and VD motor neurons. We generated transgenic animals that express a fusion protein of mCherry and PPM-1, and a fusion protein of GFP and SNB-1, a synaptic vesicle membrane protein. SNB-1::GFP localizes to presynaptic puncta in the dorsal cord where the DD neurons innervate muscle (Figure 7B). While mCherry::PPM-1 was not always punctate, mCherry::PPM-1 puncta were observed in the dorsal cord and colocalized with GFP::SNB-1 (Figure 7B). These results demonstrate that PPM-1 can localize to the presynaptic terminals of GABAergic motor neurons.

**Discussion**

RPM-1 functions as part of an E3 ubiquitin ligase/SCF complex that includes FSN-1. This complex ubiquitinates and destroys DLK-1 to negatively regulate a MAP kinase pathway (NAKATA et al. 2005). RPM-1 is part of a conserved protein
family called Pam/Highwire/RPM-1 (PHR) proteins, and PHR proteins in flies and mice also function as part of SCF complexes to regulate synapse formation and neuronal development (Burgess et al. 2004; Collins et al. 2006; Lewcock et al. 2007; Saiga et al. 2009; Tada et al. 2009; Wu et al. 2007). Thus, negative regulation of the DLK-1/Dlk pathway represents an essential, evolutionarily conserved function of the PHR proteins. While PHR proteins represent one mechanism for negatively regulating the DLK-1 pathway, it remains uncertain if other, complementary mechanisms also restrain the activity of this pathway. Here we provide evidence of a conserved PP2Cα/β phosphatase, PPM-1, that also negatively regulates the DLK-1 pathway.

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

With regard to axon termination in the PLM neurons, and synapse formation in the GABAergic motor neurons, we observed that glo-4,ppm-1 double mutants were enhanced, and glo-1;ppm-1 double mutants were not enhanced. This observation suggests that glo-4 plays a greater role in axon termination and synapse formation than glo-1. This interpretation is consistent with our observations that glo-4 has stronger enhancer effects than glo-1 with fsn-1 (Grill et al. 2007) (Figure 1-4). Presumably a certain level of reduced GLO pathway
function is needed to enhance \textit{ppm-1} (lf). While \textit{glo-4} (lf) achieves this level of inactivation of the GLO pathway, \textit{glo-1} does not. This model suggests that an unidentified small GTPase or signaling molecule, besides GLO-1, functions downstream of GLO-4. Presumably loss of function in both \textit{glo-1} and this other molecule(s) are required to enhance \textit{ppm-1} (lf).

Our observation that \textit{ppm-1} (lf) does not enhance \textit{rpm-1} (lf) demonstrates that \textit{ppm-1} functions in the same genetic pathway as \textit{rpm-1}. This is consistent with our transgenic experiments showing that \textit{ppm-1} functions downstream of \textit{rpm-1} to negatively regulate the DLK-1 pathway. Suppression of \textit{ppm-1} axon termination defects by \textit{pmk-3} (lf) suggests that PPM-1 may negatively regulate PMK-3 directly by dephosphorylation. Alternatively, PPM-1 may negatively regulate a positive regulator of PMK-3, such as UEV-3. Future biochemical experiments aimed at testing if PPM-1 regulates the phosphorylation of PMK-3 should provide a definitive answer to this question.

It is not immediately clear to us why only \textit{pmk-3} (lf) suppresses \textit{ppm-1} (lf) defects. We anticipated that loss of function in any component of the DLK-1 pathway would prevent activation of this pathway, and suppress phenotypes caused by \textit{ppm-1} (lf). One explanation for our results is that kinases other than DLK-1 and MKK-4 also function upstream of PMK-3, and suppression only occurs with loss of function in the target of PPM-1, presumably PMK-3.

Importantly, the \textit{dlk-1} pathway is required not just in a developmental context, but also for axon regeneration in the mechanosensory neurons (\textit{Yan et al.} 2009) and in the motor neurons of adult \textit{C. elegans} (\textit{Hammarlund et al.} 2009).
Overexpression of \textit{dlk-1}, or loss of function in \textit{rpm-1} or \textit{fsn-1} leads to improved axon regeneration (Hammarlund et al. 2009). Our discovery that \textit{ppm-1} is a negative regulator of the \textit{dlk-1} pathway, similar to \textit{rpm-1} and \textit{fsn-1}, suggests that \textit{ppm-1} may also function in axon regeneration. Given our finding that \textit{ppm-1} enhances \textit{fsn-1} with regard to defects in both axon termination and synapse formation, it is plausible that \textit{fsn-1};\textit{ppm-1} double mutants may also show enhanced increases in axon regeneration. Future experiments aimed at addressing this possibility will be informative.

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

\textbf{Acknowledgements}

We wish to thank Drs David Greenstein, Lihsia Chen, and Yishi Jin for helpful discussions. We are grateful to the \textit{C. elegans} Genetics Center and the \textit{C. elegans} knockout consortium for providing strains and deletion mutants, respectively. Finally, we wish to acknowledge Shane Turgeon's technical contributions. This work was supported by a grant from the Minnesota Medical Foundation and a Grant-in-aid from the University of Minnesota.
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**Figure Legends**
**FIGURE 1.** *ppm-1* regulates axon termination in the ALM neurons. **A)** Shown is a schematic of the exons (hatched boxes) and introns of three open reading frames predicted for *ppm-1* by wormbase. The deletions caused by *ok578* and *tm653* are shown below. **B)** A schematic of the human PP2Cα protein, and predicted PPM-1 proteins with the catalytic phosphatase domain (grey), and the regulatory domain (hatched design) highlighted. Shown below are segments of PPM-1 deleted by *ok578* and *tm653*. Mutagenesis studies on human PP2Cα showed that mutation of residue D60 and D239 lead to 900 and 4000-fold decreases in phosphatase activity, respectively (Jackson *et al.* 2003). The corresponding conserved residues in PPM-1, D62 and D246 (F25D1.1c), are shown. **C)** Axon termination defects in the ALM mechanosensory neuron were visualized using muls32[P*mec7*GFP] for wild-type or the indicated mutant genotypes. Images correspond to the boxed region of the diagram. **D)** Quantitation of the total axon termination defects in ALM neurons. **E)** Quantitation of specific, short hook (white) or big hook (grey), axon termination defects in the ALM neurons of the indicated genotypes. Note that the percentage of ALM axons that are normal/wild-type for each genotype is not shown. Analysis was performed on young adults grown at 23°C, unless otherwise specified. Scale bar is 10 µm. Error bars represent the standard error of the mean. Significance was determined using an unpaired t test where n represents the number of independent counts of 10-30 worms for a given genotype. *p<0.05, ** p<0.01
**FIGURE 2.** *ppm-1* regulates axon termination in the PLM neurons. **A)** Axon termination defects in the PLM neurons were visualized using *muIs32[P_mec7GFP]* for wild-type or the indicated mutant genotypes. Images correspond to the boxed region of the diagram. Arrows mark overextension of the PLM axon beyond the ALM cell body, and arrowheads mark the more severe phenotype of overextension and hooking of the PLM axon. **B)** Quantitation of the percentage of PLM neurons that only overextend (white), or overextend and hook (grey) for the indicated genotypes. Note that the percentage of PLM axons that are wild-type for each genotype is not shown. Analysis was performed on young adults grown at 23°C, unless otherwise specified. Scale bar is 10 µm. Error bars represent the standard error of the mean. Significance was determined using an unpaired t test where *n* represents the number of independent counts of 10-30 worms for a given genotype. *** *p*<0.001, ns=not significant

**FIGURE 3.** *ppm-1* regulates synaptic branch extension/stabilization in PLM neurons. **A)** Defects in synaptic branch extension in the PLM neurons were visualized using *muIs32[P_mec7GFP]* in wild-type or the indicated mutant genotypes. Images correspond to the boxed region of the diagram. The arrowhead highlights the absence of the synaptic branch. **B)** Quantitation of the defects in synaptic branch extension in the PLM neurons for the indicated genotypes. Note that the percentage of PLM axons that are wild-type for each genotype is not shown. Analysis was performed on young adults grown at 23°C, unless otherwise specified. Scale bar is 10 µm. Error bars represent the standard
error of the mean. Significance was determined using an unpaired t test where n represents the number of independent counts of 10-30 worms for a given genotype. * p<0.05, *** p<0.001, ns=not significant

**FIGURE 4.** *ppm-1* regulates synapse formation in GABAergic motor neurons. 
*A* Presynaptic terminals of DD neurons were visualized using *juls1[Punc-25SNB-1::GFP]* in wild-type or mutant genotypes. Arrows highlight the gaps between presynaptic puncta in the dorsal cord. 
*B* Quantitation of the average number of SNB-1::GFP puncta per 100µm of dorsal cord. Analysis was performed on young adults grown at 25°C. Scale bar is 10 µm. Significance was determined using a Student's t test, and error bars represent the standard error of the mean. *** p<0.001, ns = not significant.

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

FIGURE 6. ppm-1 negatively regulates the dlk-1 pathway. Transgenic expression of DLK-1 causes axon termination defects in A) ALM neurons, and B) PLM neurons, which is suppressed by coexpression of PPM-1, but not phosphatase-dead PPM-1 (D246N). The data shown is pooled from three or more transgenic lines, except the data for phosphatase-dead PPM-1, which is from a single transgenic line. The percentage of PLM axons that hook are shown. The percentage of PLM neurons that overextend or are wild-type are not shown. C) Analysis of suppression of ppm-1 mutant phenotypes by loss of function in dlk-1, mkk-4 and pmk-3. Quantitation of axon termination defects (overextension only) in the indicated mutant genotypes. Note that only pmk-3-/-;ppm-1-/- mutant animals show a significant suppression of defects in axon termination. Analysis was performed on young adults grown at 23°C. Error bars represent the standard error of the mean. Significance was determined using an unpaired t test where n represents the number of independent counts of 10-30 worms for a given genotype. *p<0.05, **<0.005, and *** p<0.001
FIGURE 7. PPM-1 is expressed in neurons and localizes to presynaptic terminals. A) Transgenic worms that use the 3.7 kb promoter of ppm-1 (F25D1.1) to express GFP were analyzed by epifluorescent microscopy. GFP is expressed broadly by the ppm-1 promoter (upper image), and is present in neurons of the ventral cord (arrows, lower left image), and the nerve ring (arrow, lower right image). B) Confocal microscopy was used to analyze transgenic worms that express mCherry::PPM-1 (red) and GFP::SNB-1 (green) specifically in the GABAergic motor neurons using the unc-25 promoter. mCherry::PPM-1 colocalizes with GFP::SNB-1 at presynaptic terminals in the dorsal nerve cord.
Figure 7

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