Neuronal Development in *Caenorhabditis elegans* Is Regulated by Inhibition of an MLK MAP Kinase Pathway

Scott T. Baker,*,† Shane M. Turgeon,* Erik D. Tulgren,† Jeanne Wigant,† Omeed Rahimi,† Karla J. Opperman,* and Brock Grill*,[1]

*Department of Neuroscience, The Scripps Research Institute, Scripps Florida, Jupiter, Florida 33458, and †Department of Pharmacology, University of Minnesota, Minneapolis, Minnesota 55455

ABSTRACT We show that loss-of-function mutations in kinases of the MLK-1 pathway (*mlk-1*, *mek-1*, and *kgb-1/jnk*) function cell-autonomously in neurons to suppress defects in synapse formation and axon termination caused by *rpm-1* loss of function. Our genetic analysis also suggests that the phosphatase PPM-1, like RPM-1, is a potential inhibitor of kinases in the MLK-1 pathway.

IN *Caenorhabditis elegans*, the ubiquitin ligase **R**egulator of **P**resynaptic **M**orphology 1 (RPM-1) regulates neuronal development by inhibiting the DLK-1 pathway (composed of DLK-1, MKK-4, and PMK-3) (Nakata *et al.* 2005). RPM-1 and the DLK-1 pathway regulate axon regeneration postdevelopmentally (Hammarlund *et al.* 2009; Yan *et al.* 2009). The MLK-1 pathway, which includes the kinases MLK-1, MEK-1, and KGB-1/JNK, also regulates axon regeneration (Nix *et al.* 2011). It remains unclear if RPM-1 functions through the MLK-1 pathway to regulate development.

Protein **P**hosphatase **M**g/Mn2+-dependent 1 (PPM-1) and PPM-2 negatively regulate the DLK-1 pathway (Tulgren *et al.* 2011; Baker *et al.* 2014). PPM-2 is regulated by RPM-1 and dephosphorylates full-length DLK-1 (DLK-1L). PPM-1 is likely to function lower in the DLK-1 pathway. It remains unclear whether PPM-1 and PPM-2 regulate other signaling pathways in the neurons of *C. elegans*.

Here, we show that mutations in *mlk-1*, *mek-1*, and *kgb-1/jnk* suppress defects in synapse formation and axon termination caused by *rpm-1* loss of function (lf). These results suggest that RPM-1 might negatively regulate the MLK-1 pathway, which is consistent with our observation that transgenic overexpression of MLK-1 or KGB-1 caused axon termination defects. Furthermore, our results are consistent with PPM-1 negatively regulating the MLK-1 pathway, in addition to inhibiting the DLK-1 pathway. In contrast, our findings suggest that PPM-2 acts only on DLK-1L.

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[1]Corresponding author: The Scripps Research Institute, 130 Scripps Way, Jupiter, FL 33460. E-mail: bgrill@scripps.edu

Results

Loss-of-function mutations in kinases of the MLK-1 pathway suppress defects in synapse formation caused by rpm-1 (lf)

The fly ortholog of RPM-1, called Highwire, functions through JNK to regulate synapse formation (Collins *et al.* 2006). It is unclear if RPM-1 functions through JNK to regulate synapse formation in worms, but studies on axon regeneration suggested that this might be a possibility (Nix *et al.* 2011). Hence, we assessed the genetic relationship between kinases of the MLK-1 pathway, including *kgb-1/jnk* and *rpm-1* in the context of synapse formation.

Consistent with previous studies, the GABAergic motor neurons of *rpm-1−/−* mutants had synapse formation defects that were strongly, but incompletely, suppressed in *rpm-1−/−*; *dlk-1−/−* double mutants (Figure 1, A and B) (Nakata *et al.* 2005). In double mutants of *rpm-1* with *mlk-1*, *mek-1*, or *kgb-1*, significant but modest suppression occurred (Figure 1, A and B). *rpm-1−/− mlk-1−/−*; *kgb-1−/−* triple mutants did not show increased suppression, demonstrating that *mlk-1* and *kgb-1* function in the same genetic pathway (Figure

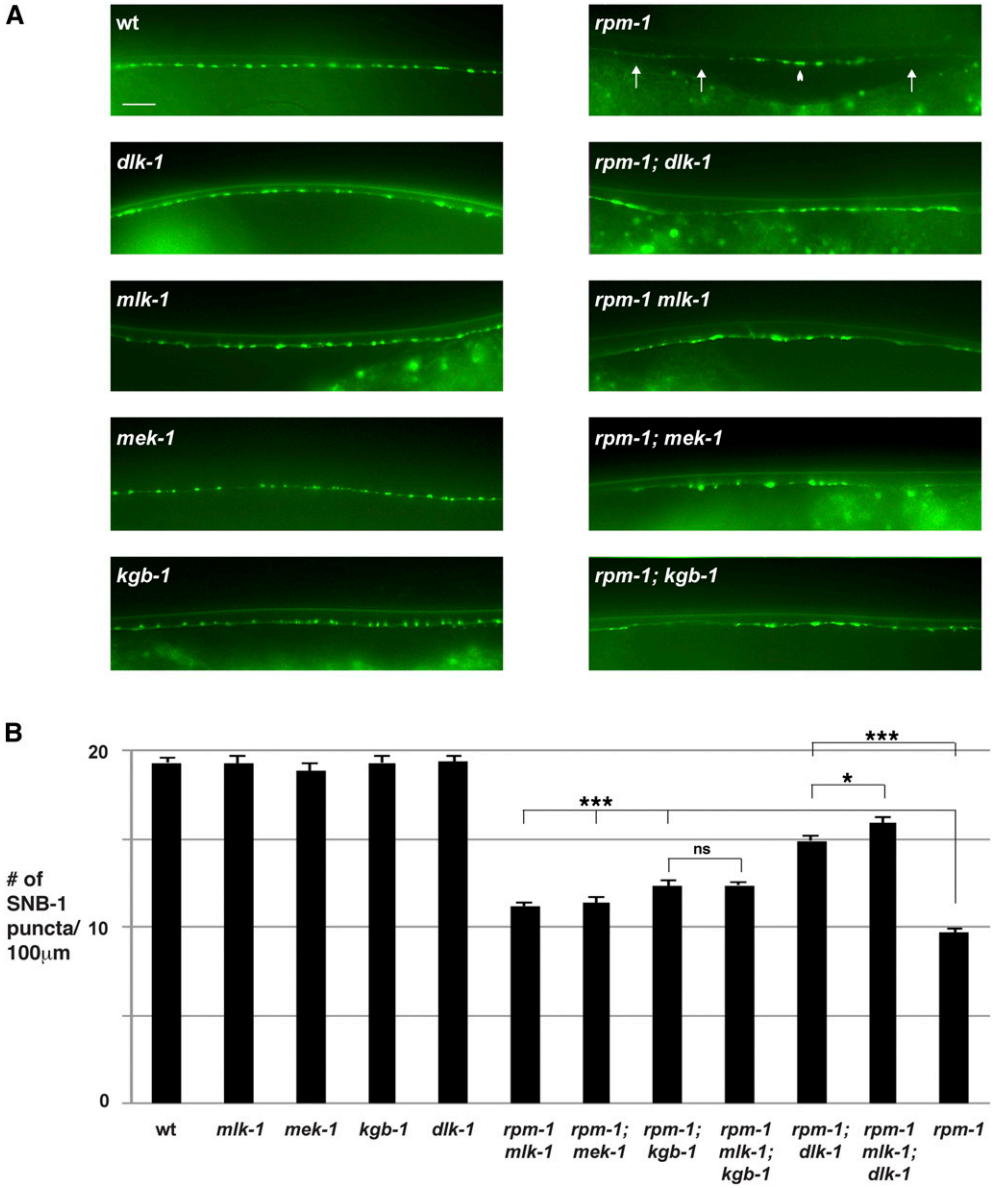

Figure 1 Loss-of-function mutations in kinases of the MLK-1 pathway suppress synapse formation defects in *rpm-1* mutants. (A) Defects in synapse formation in the GABAergic motor neurons were analyzed using a transgene, *juIs1* ($P_{unc\text{-}25}$SNB-1::GFP), and epifluorescent microscopy under ×40 magnification. *rpm-1* mutants had abnormal synapse formation with aggregated synapses (arrowhead) and gaps in the dorsal cord (arrows). Defects caused by *rpm-1* (lf) were partially suppressed by loss of function in *mlk-1*, *mek-1*, or *kgb-1*. Bar, 10 μm. (B) Quantitation of synapse formation defects in GABAergic motor neurons for the indicated genotypes. Alleles used included *rpm-1 (ju44)*, *dlk-1 (ju476)*, *mlk-1 (ok2471)*, *mek-1 (ks54)*, and *kgb-1 (um3)*. Shown are averages for data collected from three or more independent experiments performed at 25° in which 15–20 synchronized, young adult worms were analyzed. Error bars represent the standard error of the mean, and significance was determined using an unpaired Student's *t*-test. ***$P <$ 0.001; *$P < 0.05$; ns, not significant.

1B). *rpm-1−/−* *mlk-1−/−*; *dlk-1* triple mutants showed a small, but significant, increase in suppression, consistent with *mlk-1* and *dlk-1* functioning in partially redundant pathways (Figure 1B).

These results are consistent with RPM-1 regulating synapse formation by inhibiting both the DLK-1 and the MLK-1 pathways. Notably, these findings do not rule out the possibility that kinases in the MLK-1 pathway function in a parallel genetic pathway to *rpm-1*.

Mutations in kinases of the MLK-1 pathway suppress axon termination defects caused by rpm-1 (lf)

Two types of axon termination defects are present in the PLM mechanosensory neurons of *rpm-1−/−* mutants (Schaefer *et al.* 2000; Grill *et al.* 2007; Tulgren *et al.* 2011): (1) severe, highly penetrant defects in which an axon overextends and hooks toward the ventral cord, referred to as "hook defects" (Figure 2, A and B) and (2) rarely observed, milder defects in which an axon overextends but fails to hook ventrally, referred to as "overextension defects." Hook defects were strongly suppressed in *rpm-1−/−*; *dlk-1−/−* double mutants (Figure 2B). In double mutants of *rpm-1* with *mlk-1*, *mek-1*, or *kgb-1*, the frequency of hook defects was moderately suppressed, while the expressivity of less severe overextension defects was increased (Figure 2, A and B). These effects were not increased in *rpm-1−/−* *mlk-1−/−*; *kgb-1−/−* triple mutants (Figure 2B). Transgenic expression of MLK-1 specifically in the mechanosensory neurons rescued the suppression in *rpm-1−/−* *mlk-1−/−* double mutants (Figure 2C).

These results show that *mlk-1*, *mek-1*, and *kgb-1* function cell-autonomously in the same genetic pathway to suppress *rpm-1* (lf). These findings are also consistent with RPM-1 negatively regulating the MLK-1 pathway.

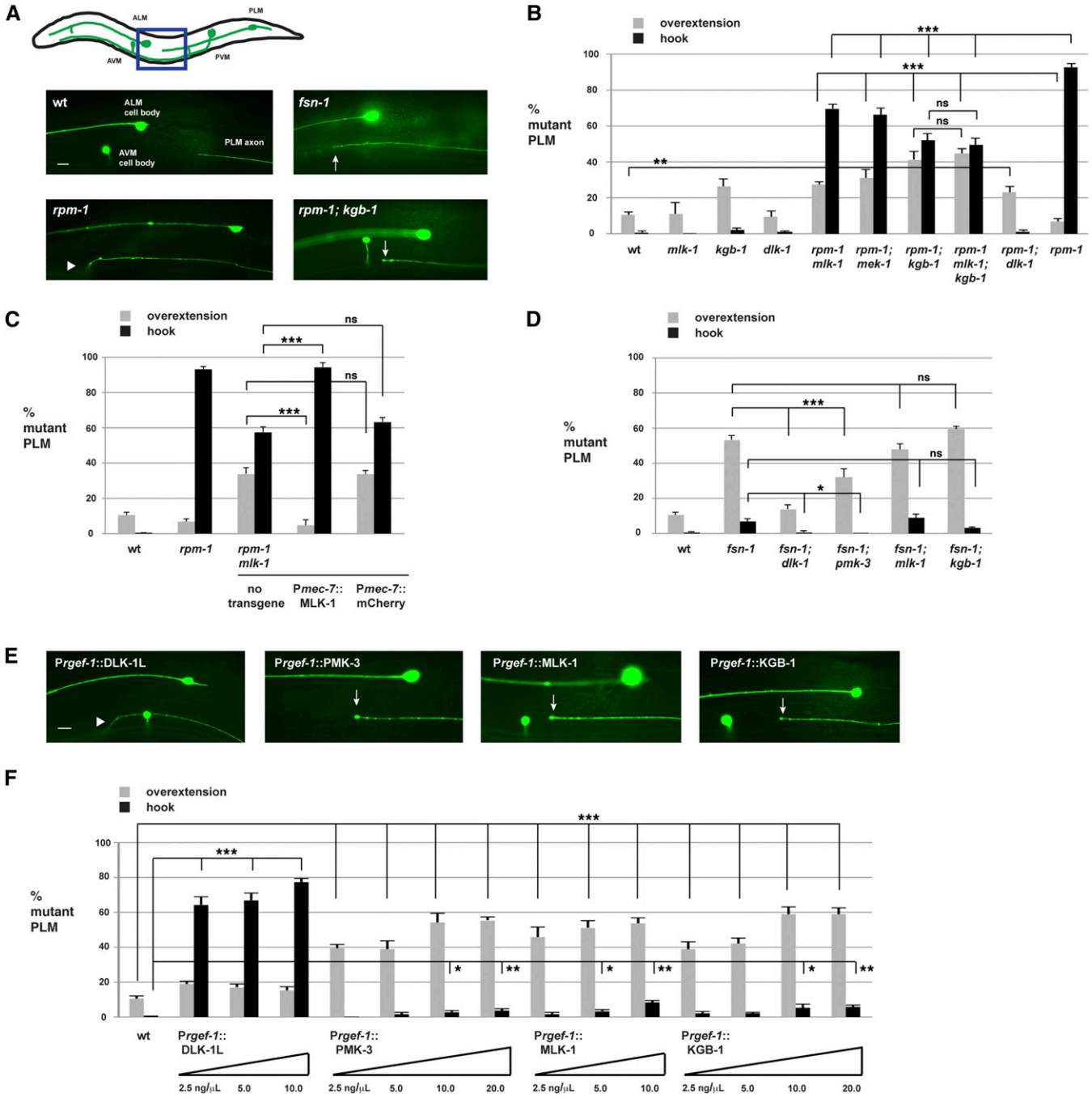

Figure 2 Axon termination defects in *rpm-1* mutants are suppressed by loss of function in *mlk-1*, *mek-1*, or *kgb-1*. (A) A schematic of the mechanosensory neurons of *C. elegans* (adapted from Baker *et al.* 2014). Axon termination of the PLM mechanosensory neurons was analyzed using a transgene, *muIs32* ($P_{mec\text{-}7}$GFP), and epifluorescent microscopy under ×40 magnification. Shown are representative images of a more severe axon termination defect in a *rpm-1* mutant in which the PLM neuron overextends beyond the ALM cell body and hooks toward the ventral cord (hook, arrowhead) and a less severe axon termination defect in a *fsn-1* mutant and in a *rpm-1*; *kgb-1* double mutant in which the PLM axon only overextends beyond the ALM cell body (overextension, arrow). Note that the AVM cell body is present on only one side of the animal and is not always shown. Bar, 10 μm. (B) Quantitation of PLM axon termination defects for the indicated genotypes. Note that double mutants of *rpm-1* and kinases in the MLK-1 pathway result in a reduction in hook defects and increased expressivity of less severe overextension defects. (C) Transgenic expression of MLK-1 using a promoter that is specifically expressed in mechanosensory neurons (P*mec-7*) rescues suppression of severe hook defects and rescues increased expressivity of less severe overextension defects in *rpm-1 mlk-1* double mutants. Rescue does not occur with transgenic expression of a control protein, mCherry. (D) Quantitation of PLM axon termination defects for the indicated genotypes. Note that *fsn-1* (lf) is not suppressed by *mlk-1* or *kgb-1* (lf). (E) Representative images are shown for a PLM neuron from transgenic animals overexpressing the indicated kinases using a pan-neuronal promoter (P*rgef-1*). A more severe hook defect is highlighted with an arrowhead, and less severe overextension defects are highlighted with arrows. (F) Quantitation of the PLM axon termination defects caused by transgenic overexpression of the indicated kinases. Shown are different concentrations of the indicated PCR products that were injected to generate extrachromosomal arrays. Notably, the molar ratios of constructs were similar with the exception of *mlk-1*, which was 1.6

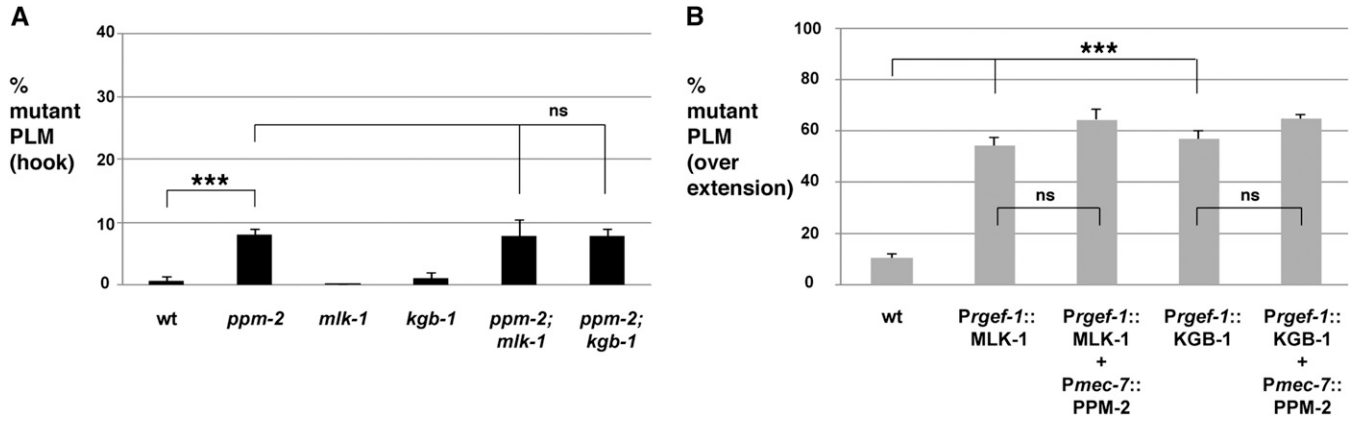

Figure 3 The PP2C phosphatase PPM-2 does not regulate the MLK-1 pathway. (A) Quantitation of PLM axon termination defects (hook) for the indicated genotypes. Alleles used included *mlk-1 (ok2471)*, *kgb-1 (um3)*, and *ppm-2 (ok2186)*. Shown are averages for data collected from five to eight independent counts of 20–30 PLM neurons. (B) Quantitation of the PLM axon termination defects (overextension) caused by transgenic overexpression of MLK-1 (5 ng/μl PCR product) or KGB-1 (10 ng/μl PCR product) using the pan-neuronal *rgef-1* promoter. Note that transgenic coexpression of PPM-2 (5 ng/μl plasmid) using the *mec-7* promoter, which is specifically expressed in mechanosensory neurons, fails to rescue defects caused by expression of MLK-1 or KGB-1. Shown are averages for data pooled from four or more transgenic lines for the indicated genotypes. In all cases, young adult worms grown at 23° were analyzed. Error bars represent the standard error of the mean, and significance was determined using an unpaired Student's *t*-test. ***$P < 0.001$; ns, not significant.

fsn-1 (lf) is suppressed by mutations in kinases of the DLK-1 pathway, but not in kinases of the MLK-1 pathway

RPM-1 functions as a complex with the F-box protein FSN-1 to regulate PLM axon termination (Liao *et al.* 2004; Grill *et al.* 2007). Axon termination defects in *fsn-1−/−* mutants were suppressed in *fsn-1−/−*; *dlk-1−/−* and *fsn-1−/−*; *pmk-3−/−* double mutants (Figure 2D) (Baker *et al.* 2014). In contrast, the frequency of axon termination defects remained unchanged in *fsn-1−/−*; *mlk-1−/−* or *fsn-1−/−*; *kgb-1−/−* double mutants (Figure 2D). These findings are consistent with FSN-1 inhibiting the DLK-1 pathway, but not the MLK-1 pathway.

Excess MLK-1 pathway function impairs axon termination

One explanation for why *rpm-1* (lf) is suppressed by mutations in the MLK-1 pathway is that *rpm-1* mutants have excess, unchecked MLK-1 pathway function. To test this hypothesis, we generated transgenic animals with extrachromsomal arrays that expressed different kinases in the DLK-1 and MLK-1 pathways. To assess the range of defects that might be caused by kinase overexpression, we generated arrays with varying levels of DNA encoding different kinases. For MLK-1 and KGB-1, we observed primarily less severe overextension defects and very low, but significant, levels of hook defects at higher concentrations (Figure 2, E and F). Similar results were observed with overexpression of PMK-3 (Figure 2, E and F). In contrast, overexpression of DLK-1L caused more severe hook defects, which occurred with increasing frequency as the concentration of DLK-1L increased (Figure 2, E and F) (Tulgren *et al.* 2011; Baker *et al.* 2014). We did not analyze MKK-4, but previous work showed that MKK-4 overexpression causes an intermediate frequency of hooks (Baker *et al.* 2014). These results provide further support for the model that RPM-1 negatively regulates the MLK-1 pathway.

Analysis of PPM-1 and PPM-2 function on the MLK-1 pathway

The phosphatases PPM-1 and PPM-2 negatively regulate the DLK-1 pathway (Tulgren *et al.* 2011; Baker *et al.* 2014). Using a combination of suppressor genetics and transgenics, we tested whether PPM-1 and/or PPM-2 affect the function of the MLK-1 pathway.

Previously, we found that *ppm-2−/−* mutants had very low penetrance hook defects, which were completely suppressed in *ppm-2−/−*; *dlk-1−/−* double mutants (Baker *et al.* 2014). In contrast, hook defects were not suppressed in *ppm-2−/−*; *mlk-1−/−* or *ppm-2−/−*; *kgb-1−/−* double mutants (Figure 3A). In the case of *ppm-1−/−* mutants that lack hook defects, we utilized a *glo-4* (lf) sensitizing background that is enhanced by *ppm-1* (lf) (Figure 4A) (Tulgren *et al.* 2011). The enhanced frequency of hooks present in *ppm-1−/− glo-4−/−* double mutants was suppressed in

times larger than other constructs. Transgenic animals were generated by microinjecting a mixture of PCR product encoding the indicated construct, 50 ng/μl of P*ttx-3*::RFP (coinjection marker) and 50 ng/μl of pBluescript. Injection conditions and genotypes for all transgenes are annotated in supporting information, Table S1. Averages are shown for data collected from five to eight independent counts of 20–30 PLM neurons from young adult worms grown at 23°. For transgenic genotypes, averages shown are data-pooled from four or more independent lines. Alleles used included *rpm-1 (ju44)*, *fsn-1 (gk429)*, *dlk-1 (ju476)*, *pmk-3 (ok169)*, *mlk-1 (ok2471)*, *mek-1 (ks54)*, and *kgb-1 (um3)*. Error bars represent the standard error of the mean, and significance was determined using an unpaired Student's *t*-test. ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$; ns, not significant.

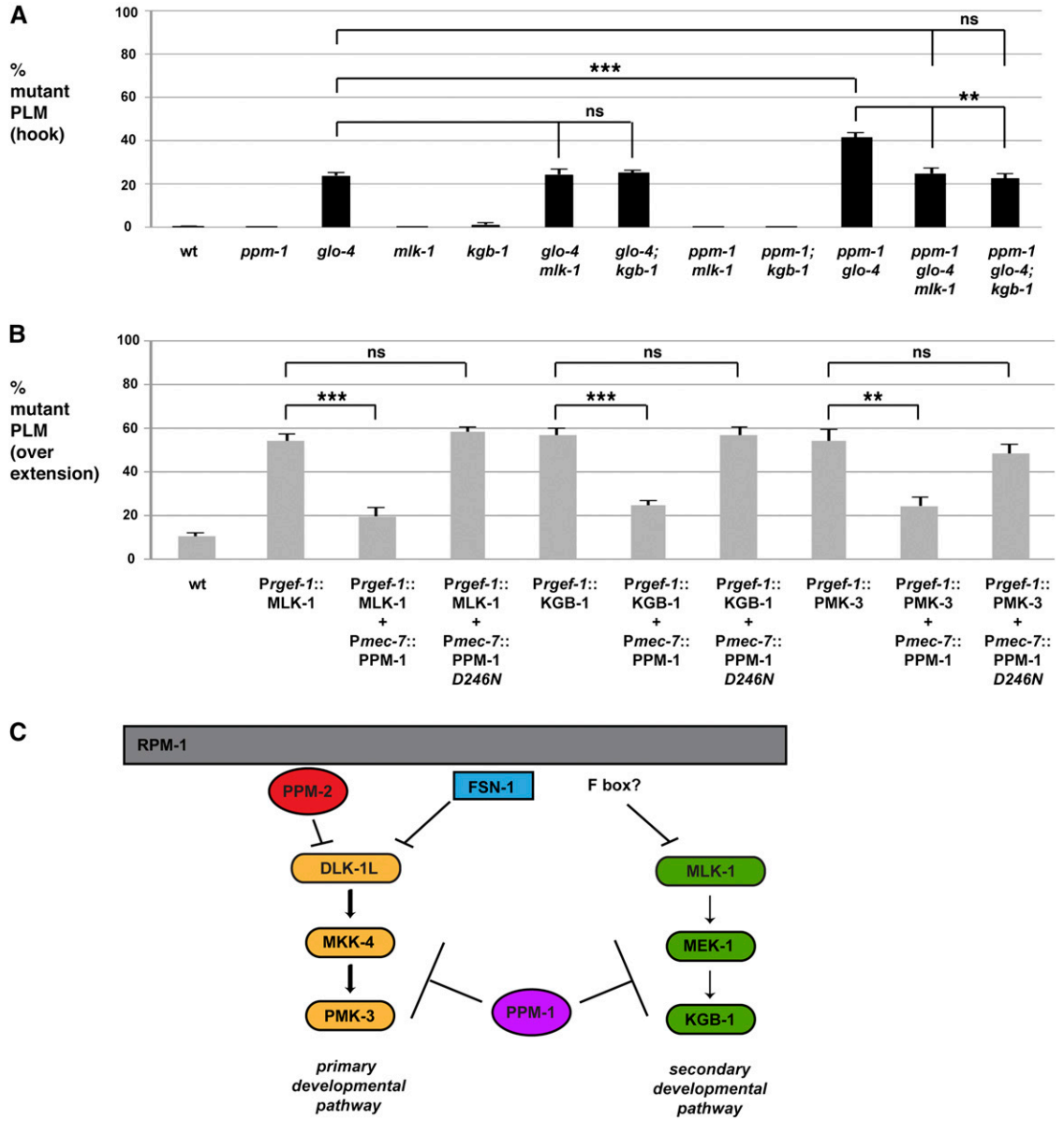

Figure 4 PPM-1 inhibits the MLK-1 and DLK-1 pathways. (A) Quantitation of PLM axon termination defects (hook) for the indicated genotypes. Alleles used included *mlk-1 (ok2471)*, *kgb-1 (um3)*, *ppm-1 (ok578)*, and *glo-4 (ok623)*. Shown are averages for data collected from five to eight independent counts of 20–30 PLM neurons. (B) Quantitation of the PLM axon termination defects (overextension) caused by transgenic overexpression of MLK-1 (5 ng/μl PCR product), KGB-1 (10 ng/μl PCR product), or PMK-3 (10 ng/μl PCR product) using the pan-neuronal *rgef-1* promoter. Note that transgenic coexpression of PPM-1 (2 ng/μl plasmid) using the *mec-7* promoter rescues defects caused by expression of all kinases. (C) Signaling model of the DLK-1 and MLK-1 pathways with regulatory mechanisms that function during neuronal development. Because *rpm-1* (lf), but not *fsn-1* (lf), is suppressed by kinases in the MLK-1 pathway, we speculate that should RPM-1 ubiquitinate and inhibit MLK-1, it would be likely to do so through a presently unknown F-box protein. ***$P < 0.001$; **$P < 0.01$; ns, not significant.

ppm-1−/− glo-4−/− mlk-1−/− and *ppm-1−/− glo-4−/−*; *kgb-1−/−* triple mutants (Figure 4A). In contrast, hook defects in *glo-4−/−* mutants were not suppressed in *glo-4−/− mlk-1−/−* and *glo-4−/−*; *kgb-1−/−* double mutants.

Next, we analyzed whether PPM-1 and PPM-2 regulate the MLK-1 pathway in the context of transgenic overexpression experiments. As shown in Figure 3B, transgenic overexpression of MLK-1 or KGB-1 resulted in axon termination defects, and coexpression of PPM-2 in the same transgenic arrays did not affect MLK-1 or KGB-1 functional efficacy. In contrast, transgenic coexpression of PPM-1 significantly reduced the defects caused by overexpression of MLK-1 and KGB-1 (Figure 4B). Consistent with the previous findings on DLK-1L (Tulgren *et al.* 2011), defects caused by transgenic overexpression of PMK-3 were reduced by transgenic coexpression of PPM-1 (Figure 4B). Importantly, catalytically inactive PPM-1 *D246N* did not reduce defects caused by overexpression of MLK-1 or KGB-1. Thus, PPM-1 phosphatase activity regulates excess MLK-1 pathway function, and the reduction caused by coexpression of PPM-1 is not an indirect

consequence of incorporating a second gene into extrachromosomal arrays (Figure 4B).

Collectively, these results are consistent with PPM-1 phosphatase activity inhibiting both the DLK-1 and the MLK-1 pathways. In contrast, these results suggest that PPM-2 is more specific for DLK-1L and not capable of regulating the MLK-1 pathway.

Discussion

RPM-1 is an important signaling molecule that regulates neuronal development through multiple mechanisms (Grill *et al.* 2007, 2012; Baker *et al.* 2014; Tulgren *et al.* 2014), including ubiquitination and inhibition of DLK-1L (Nakata *et al.* 2005). Our genetic suppressor analysis and transgenic results suggest that RPM-1 also negatively regulates the MLK-1 pathway during development. Our findings are consistent with a previous study, which showed that MLK-1 levels are increased in the neurons of *rpm-1* (lf) mutants (Nix *et al.* 2011). One simple explanation for these findings is that RPM-1 ubiquitinates MLK-1, which results in MLK-1 degradation and inhibition of the MLK-1 pathway (Figure 4C). However, an alternative explanation for our results is that the MLK-1 pathway functions in parallel to RPM-1. Because mutations in kinases of the DLK-1 pathway are stronger suppressors of *rpm-1* (lf) than mutations in kinases of the MLK-1 pathway, it is likely that RPM-1 functions primarily through the DLK-1 pathway and secondarily through the MLK-1 pathway (Figure 1 and Figure 2).

Our results are consistent with the PPM-1 phosphatase representing a further, conserved negative regulatory mechanism imposed on the DLK-1 and MLK-1 pathways (Figure 4C). In contrast, we found no evidence that PPM-2 regulates the MLK-1 pathway. Therefore, taking prior work into account, PPM-2 is likely to be a relatively specific mechanism for restraining DLK-1L activity (Baker *et al.* 2014).

During neuronal development, JNK and p38 MAP kinases mediate the function of *Drosophila* Highwire and mammalian Phr1 (Collins *et al.* 2006; Lewcock *et al.* 2007; Huntwork-Rodriguez *et al.* 2013; Klinedinst *et al.* 2013). Given prior work and our findings here, it is increasingly likely that the Pam/Highwire/RPM-1 protein family generally regulates two MAP kinase pathways exemplified by the DLK-1 and MLK-1 pathways in *C. elegans*.

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Table S1

Figure #	Injection Conditions^+	Genotype*
2C	P_{mec-7}MLK-1 (pBG-GY554) (5ng/µL)	*muIs32; mlk-1 rpm-1*
2C	P_{mec-7}mCherry (pBG-GY258) (5ng/µL)	*muIs32; mlk-1 rpm-1*
2F	P_{rgef-1}DLK-1 cDNA (pBG-57) (2.5ng/µL PCR Product)	*muIs32*
2F	P_{rgef-1}DLK-1 cDNA (pBG-57) (5ng/µL PCR Product)	*muIs32*
2F	P_{rgef-1}DLK-1 cDNA (pBG-57) (10ng/µL PCR Product)	*muIs32*
2F	P_{rgef-1}PMK-3 (pBG-GY449) (2.5ng/µL PCR Product)	*muIs32*
2F	P_{rgef-1}PMK-3 (pBG-GY449) (5ng/µL PCR Product)	*muIs32*
2F, 4B	P_{rgef-1}PMK-3 (pBG-GY449) (10ng/µL PCR Product)	*muIs32*
2F	P_{rgef-1}PMK-3 (pBG-GY449) (20ng/µL PCR Product)	*muIs32*
2F, 3B, 4B	P_{rgef-1}MLK-1 (pBG-GY356) (5ng/µL PCR Product)	*muIs32*
2F	P_{rgef-1}MLK-1 (pBG-GY356) (10ng/µL PCR Product)	*muIs32*
2F	P_{rgef-1}MLK-1 (pBG-GY356) (20ng/µL PCR Product)	*muIs32*
2F	P_{rgef-1}KGB-1 (pBG-GY357) (2.5ng/µL PCR Product)	*muIs32*
2F, 3B, 4B	P_{rgef-1}KGB-1 (pBG-GY357) (5ng/µL PCR Product)	*muIs32*
2F	P_{rgef-1}KGB-1 (pBG-GY357) (10ng/µL PCR Product)	*muIs32*
2F	P_{rgef-1}KGB-1 (pBG-GY357) (20ng/µL PCR Product)	*muIs32*
3B	P_{rgef-1}MLK-1 (pBG-356) (5ng/µL PCR Product) P_{mec-7}PPM-2 (pBG-GY121) (5ng/µL)	*muIs32*
3B	P_{rgef-1}KGB-1 (pBG-357) (10ng/µL PCR Product) P_{mec-7}PPM-2 (pBG-GY121) (5ng/µL)	*muIs32*
4B	P_{rgef-1}MLK-1 (pBG-356) (5ng/µL PCR Product) P_{mec-7}PPM-1 cDNA (pBG-GY163) (2ng/µL)	*muIs32*
4B	P_{rgef-1}MLK-1 (pBG-356) (5ng/µL PCR Product) P_{mec-7}PPM-1 *D246N* (pBG-GY202) (2ng/µL)	*muIs32*
4B	P_{rgef-1}KGB-1 (pBG-357) (10ng/µL PCR Product) P_{mec-7}PPM-1 cDNA (pBG-GY163) (2ng/µL)	*muIs32*
4B	P_{rgef-1}KGB-1 (pBG-357) (10ng/µL PCR Product) P_{mec-7}PPM-1 *D246N* cDNA (pBG-GY202) (2ng/µL)	*muIs32*
4B	P_{rgef-1}PMK-3 (pBG-GY449) (10ng/µL PCR Product) P_{mec-7}PPM-1 cDNA (pBG-GY163) (2ng/µL)	*muIs32*
4B	P_{rgef-1}PMK-3 (pBG-GY449) (10ng/µL PCR Product) P_{mec-7}PPM-1 *D246N* cDNA (pBG-GY202) (2ng/µL)	*muIs32*

* Alleles used are *mlk-1(ok2471)* and *rpm-1(ju44)*
^ Plasmids were injected, unless noted otherwise
+ Genomic DNA was used, unless noted otherwise