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A Neuronal piRNA Pathway Inhibits Axon Regeneration in *C. elegans*

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SUMMARY

The PIWI-interacting RNA (piRNA) pathway has long been thought to function solely in the germline, but evidence for its functions in somatic cells is emerging. Here we report an unexpected role for the piRNA pathway in *Caenorhabditis elegans* sensory axon regeneration after injury. Loss of function in a subset of components of the piRNA pathway results in enhanced axon regrowth. Two essential piRNA factors, PRDE-1 and PRG-1/PIWI, inhibit axon regeneration in a gonad-independent and cell-autonomous manner. By smFISH analysis we find that *prde-1* transcripts are present in neurons, as well as germ cells. The piRNA pathway inhibits axon regrowth independent of nuclear transcriptional silencing but dependent on the slicer domain of PRG-1/PIWI, suggesting post-transcriptional gene silencing is involved. Our results reveal the neuronal piRNA pathway as a novel intrinsic repressor of axon regeneration.

eTOC Blurb

Kim et al. discovered that specific factors in the PIWI-interacting small RNA (piRNA) pathway inhibit axon regeneration in *C. elegans.* Transcripts of the piRNA factor *prde-1* are present in neurons and piRNA factors function cell-autonomously to limit axon regeneration.

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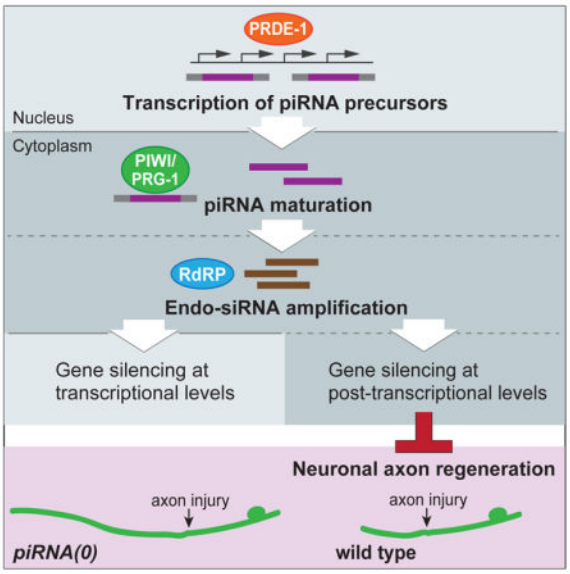
DECLARATION OF INTEREST

The authors declare no competing interests.

AUTHOR CONTRIBUTIONS

Conceptualization and methodology, K.W.K., A.D.C. and Y.J.; Investigation, K.W.K. and Z.W; Writing–Original Draft, K.W.K.; Writing–Review & Editing, K.W.K., N.H.T., M.G.A., A.D.C. and Y.J.; Funding Acquisition, A.D.C. and Y.J.; Resources, K.W.K, N.H.T. and M.G.A.; Supervision, K.W.K., A.D.C. and Y.J.



INTRODUCTION

Small non-coding RNAs are key regulators in the transcriptional and epigenetic control of gene expression in the nervous system (Briggs et al., 2015). piRNAs bind PIWI proteins, which are conserved Argonaute family protein that contains a PIWI domain with endonuclease or 'slicer' activity (Bagijn et al., 2012; Parker et al., 2004; Song et al., 2004). piRNA-PIWI interactions are required for piRNA expression and function (Iwasaki et al., 2015). Mature piRNA-PIWI complexes regulate the expression of transposable elements, protein-coding genes, and foreign nucleic acids, through diverse modes of action. Target repression by piRNAs involves both transcriptional gene silencing (TGS) and post-transcriptional gene silencing (PTGS) in flies and mice (Czech and Hannon, 2016). Typically, piRNA mediated-PTGS involves endonucleolytic cleavage of the mRNA targets by PIWI after complementary base-pair recognition (Czech and Hannon, 2016). By contrast, piRNA mediated-TGS does not rely on target RNA cleavage, but represses production of targets through chromatin modifications by formation of repressive histone or DNA methylation ('nuclear silencing') (Aravin et al., 2008; Sienski et al., 2012).

piRNA expression and function have generally been thought to be germline-specific, and mutants lacking PIWI in diverse animal species display sterility (Batista et al., 2008; Lin and Spradling, 1997). However, PIWI/piRNAs are reportedly expressed in the nervous system of flies, *Aplysia,* and mice (Lee et al., 2011; Nandi et al., 2016; Perrat et al., 2013; Rajasethupathy et al., 2012). In *Aplysia* sensory neurons neuronal piRNAs function in long-term facilitation (Rajasethupathy et al., 2012).

C. elegans piRNAs are 21 nucleotides (nt) in length with a 5′ uridine, known as 21U-RNAs (Das et al., 2008; Ruby et al., 2006). Most piRNA loci contain an upstream Ruby motif and encode type I piRNAs (Gu et al., 2012; Ruby et al., 2006). The nuclear factor PRDE-1 (piRNA-dependent silencing defective) promotes transcription of type I piRNA precursors

(Weick et al., 2014), whereas the PIWI family protein PRG-1 (PIWI-related gene) is essential for the expression of mature piRNAs (Bagijn et al., 2012; Das et al., 2008; Lee et al., 2012). In *C. elegans*, a designated nuclear RNAi pathway executes piRNA-mediated TGS by histone modifications (Ashe et al., 2012; Shirayama et al., 2012). It is not yet known if piRNA-mediated PTGS occurs in *C. elegans*.

Here, we report that the piRNA pathway represses *C. elegans* axonal regeneration after injury, based on four lines of evidence. First, loss of function in select genes of the piRNA pathway results in enhanced axon regrowth, independent of piRNA-mediated nuclear silencing. Second, two essential piRNA factors, PRDE-1 and PRG-1/PIWI, act in a cell-autonomous and gonad-independent manner to inhibit axonal regrowth after injury. Third, *prde-1* and *prg-1* transcripts are expressed in multiple somatic cells including neurons. Fourth, the slicer domain of PRG-1/PIWI is required for its inhibitory effect on axon regrowth. We conclude that a neuronal piRNA-based mechanism inhibits axon regeneration after injury.

RESULTS

The piRNA pathway inhibits axon regeneration

We tested whether piRNA pathway genes were involved in *C. elegans* adult sensory axon regeneration. We screened genetic null mutants for factors in piRNA expression and function: (1) piRNA transcription, (2) piRNA maturation (3′ and 5′ end processing), (3) secondary amplification that generates 22G-endo siRNAs, and (4) nuclear RNAi and target gene silencing (Figure 1) (Weick and Miska, 2014). By comparing regrowth length of the posterior lateral microtubule (PLM) mechanosensory neuron (or called Touch Receptor Neuron (TRN)) 24 hours after laser axotomy, we found that a subset of components in the piRNA pathway, specifically those regulating piRNA transcription, maturation and secondary amplification, inhibited PLM axon regeneration (Figures 1A and 1B). We observed increased PLM regrowth in homozygous mutants propagated for several generations, such as animals lacking PRDE-1, TOFU-3, and PRG-1, as well as in homozygous mutants produced from heterozygous mothers (maternal+ zygotic−), such as animals lacking TOFU-5, TOFU-7, EKL-1, DRH-3, and EGO-1 (Figure 1A and Table S1). These mutants were grossly normal in growth rate and body size, arguing that the enhanced axon regrowth is not due to altered organismal growth (Figure S1A and data not shown). The RNA dependent RNA polymerases (RdRPs), EGO-1 and RRF-1 function redundantly in the generation of secondary 22G-RNAs and the piRNA sensor silencing (Bagijn et al., 2012; Sapetschnig et al., 2015); interestingly, EGO-1, but not RRF-1, affects axon regeneration (Figure 1A).

Some, but not all, factors involved in piRNA transcription and maturation also affect axon regeneration. For example, at the piRNA maturation step, PRG-1 and TOFU-7 repressed axon regrowth, while HENN-1 and PARN-1 showed no effect. We tested whether genes acting at the same step might function redundantly. We found that double mutants of *parn-1* and *henn-1* showed increased axon regrowth, compared to wild type or either single mutant (Figure 1A). These data suggest that regulators of piRNA transcription and maturation generally inhibit PLM axon regeneration.

Strikingly, animals deficient in nuclear transcriptional silencing, such as mutants lacking the nuclear Argonaute HRDE-1, the putative histone methyltransferases SET-25 and SET-32, and the HP1 ortholog HPL-2 (Ashe et al., 2012; Shirayama et al., 2012), showed normal or marginally decreased axon regeneration (Figure 1A). Moreover, double mutants of *hrde-1* and *nrde-3*, which encode Argonaute proteins acting in germline and somatic nuclear RNAi (Bagijn et al., 2012; Guang et al., 2008), respectively, also showed normal axon regrowth (Figure 1A).

Together, these results reveal a previously unknown role for the piRNA pathway in axon regeneration, likely independent of nuclear transcriptional silencing. We next focused on PRDE-1 and PRG-1, the two essential components for piRNA expression.

PRDE-1 inhibits PLM axon regeneration independently of the gonad

PRDE-1 acts in the nucleus and is essential for piRNA biogenesis, as mutants lacking PRDE-1 do not express canonical type I piRNAs (Weick et al., 2014). We tested two independent alleles of *prde-1*: the missense mutant *mj207* (Weick et al., 2014) and a 5′ deletion mutant *ju1509* (Figure S1B). Both mutants displayed normal morphology of PLM neurons, but showed enhanced axon regrowth of injured PLM over 24 hours, compared to wild type controls (Figure 2A). Under our laser axotomy conditions, PLM axons initiate regrowth approximately 6 hours post injury, showing filopodia and growth cones (Wu et al., 2007). *prde-1(mj207)* mutants displayed axon regrowth at 6 hours, with regenerative growth cones formed at frequencies similar to wild type controls (Figures S1C and S1D). This analysis suggests that the piRNA pathway likely acts at later phases of axon regeneration.

A neuronal role for PRDE-1 was unexpected, as PRDE-1 function has been thought to be restricted to the germline, where it is required for fertility (Weick et al., 2014). We next asked whether the enhanced regeneration of *prde-1* mutants reflected a non-autonomous effect of the gonad by ablating the somatic gonad and germline precursor cells. Specifically, we ablated all four gonadal precursor cells (Z1–Z4) in L1 stage larvae (Figure 2B; left panel), then cut PLM axons in the same animals at the L4 stage and measured regrowth at 24 hours post-axotomy. We found that gonad-ablation in either wild-type animals or *prde-1(0)* mutants had no effect on axon regrowth, when compared to the mock or unablated controls (Figure 2B; middle and right panels). We conclude that PRDE-1 inhibits axon regeneration independent of its function in the gonad.

PRDE-1 is expressed in PLM neurons and acts cell-autonomously

Transcriptional profiling of adult PLM neurons has detected expression of several genes in the piRNA pathway, including *prde-1* and *prg-1* (Kaletsky et al., 2016; Lockhead et al., 2016). Thus, we asked whether *prde-1* mRNAs could be visualized in PLM neurons using smFISH (single-molecule fluorescence *in situ* hybridization), which allows detection of single mRNAs in cells (Raj et al., 2008). We detected *prde-1* mRNAs in PLM neurons (Figure 3A) as well as in germ cells. Intriguingly, *prde-1* expression in PLM neurons was detectable primarily in adult animals.

We next examined endogenous PRDE-1 protein expression by generating a *gfp* knock-in (KI) allele (*ju1534*) at the *prde-1* locus using CRISPR-Cas9 genome editing (Figures 3B and

S2A). *prde-1(ju1534)* KI animals produced a full-length PRDE-1 protein tagged at its N-terminus (GFP-PRDE-1) (Figure S2B), and were indistinguishable from wild type in development, fertility, and PLM axon regrowth length (Figure S2C). We observed GFP fluorescence as puncta in the germline nuclei of adult hermaphrodites (Figure 3C), consistent with reported antibody staining for PRDE-1 (Weick et al., 2014). We were not able to detect GFP-PRDE-1 fluorescence in somatic tissues by live imaging or by antibody staining with anti-GFP antibodies. In regenerating PLM neurons after axonal injury, GFP-PRDE-1 fluorescence remained below visible detection. When we overexpressed PRDE-1 in mechanosensory neurons, we detected PRDE-1-GFP predominantly in the nuclei of mechanosensory neurons (Figure S2D), consistent with its function in piRNA precursor transcription; the pattern and levels of overexpressed PRDE-1-GFP were also not discernably different in regenerating PLM.

We hypothesized that even low levels of PRDE-1 expression in neurons could be functionally significant. To test this, we took advantage of a tissue-specific protein knockdown method that relies on GFP-DEGRON (GFP nanobody-ZIF-1 fusion) to target and degrade GFP-tagged proteins (Armenti et al., 2014; Wang et al., 2017) (Figure 3D). We expressed the GFP-DEGRON in mechanosensory neurons (trnDEGRON), and confirmed that it eliminated GFP fluorescence (Figure S3), and also did not alter axon morphology. We then tested the effect of trnDEGRON in the *prde-1 gfp* KI (*ju1534)* animals. We found that compared to control animals, depletion of GFP-PRDE-1 in mechanosensory neurons resulted in enhanced axon regrowth (Figure 3E). This analysis indicates that PRDE-1 functions cell-autonomously in axonal regrowth. To further support this conclusion, we transgenically expressed PRDE-1 in *prde-1(0)* mutants. We observed that the enhanced axon regrowth was rescued by cell-type specific transgenic expression of PRDE-1 from a mechanosensory neuron-specific promoter (*Pmec-4*), but not from a motor neuron-specific promoter (*Punc-25*) or a muscle-specific promoter (*Pmyo-3*) (Figure 3F). Together, these data support a specific role of PRDE-1 in mechanosensory neurons to inhibit axon regrowth.

PRG-1/PIWI inhibits PLM axon regeneration cell-autonomously

Once piRNA precursors are generated, PRG-1/PIWI is required for normal expression of mature piRNAs (Bagijn et al., 2012; Das et al., 2008; Lee et al., 2012). We found that both *prg-1*(*n4357)* and *prg-1*(*tm872)* null mutants displayed enhanced PLM regrowth (Figures 4A and 4B) resembling that of *prde-1* mutants. Moreover, PLM regeneration defects in *prg-1* mutants were rescued by transgenic expression of PRG-1 with its own promoter, as well as by expression of PRG-1 from a mechanosensory neuron-specific promoter (*Pmec-4*) but not from a motor neuron-specific promoter (*Punc-25*) (Figure 4C). These data support a conclusion that PRG-1 can also act cell-autonomously to inhibit axonal regrowth.

In the germline PRG-1 is localized to the cytoplasm (Batista et al., 2008). We found that in neurons, overexpressed GFP-PRG-1 showed predominantly cytoplasmic localization (Figure 4D), consistent with its function in cytosolic piRNA maturation. We then asked whether *prg-1* mRNAs are detected in somatic cells using germline-less animals caused by a temperature-sensitive mutation of *glp-1* grown at restrictive temperature (Austin and Kimble, 1987). Using quantitative RT-PCR, we detected a significant amount of *prg-1*

mRNA levels in germline-less animals, although the total levels were lower than those in wild type animals (Figure S4A; 30% compared to wild type at 20°C). Interestingly, we detected little *prde-1* mRNA expression in somatic cells in the same germline-less animals (Figure S4A), consistent with its selective expression in PLM neurons (Figure 3A). We further performed *prg-1* smFISH and found specific fluorescent signals outside of the germ cells, comparing to control (Figure S4B). However, the seemingly widespread smFISH signals precluded us to discern specific expression of *prg-1* in PLM neurons. All together, these data indicate that *prg-1* mRNA is present in somatic cells and its endogenous steady state expression is much lower than in germ cells.

The slicer domain of PRG-1/PIWI is required for axon regeneration

Our findings above suggest that inhibition of axon regeneration by piRNAs does not involve nuclear silencing (Figure 1). We next hypothesized that the piRNA pathway might repress axon regeneration via PTGS rather than TGS. PTGS requires the slicer activity of PIWI (De Fazio et al., 2011; Li et al., 2009; Reuter et al., 2011). To test whether the slicer activity of PRG-1/PIWI is required for axon regeneration, we performed genome editing at the slicer catalytic site in the endogenous *prg-1* locus. In the resulting *prg-1(ju1574)* animals the first conserved aspartate of the catalytic triad (D-D-H motif) was mutated to alanine (D583A), creating an A-D-H motif (Figure 4A). Mutations in the corresponding aspartate in other Argonaute proteins abolish slicer activity (Liu et al., 2004; Steiner et al., 2009). The *prg-1(ju1574)* A-D-H mutants expressed mRNA levels of *prg-1* comparable to wild type (Figure S4C). *prg-1(ju1574)* mutant animals became sterile at the 5th generation grown at 25°C (Figure 4E), a progressive sterility phenotype seen in other *prg-1* loss-of-function mutants (Bagijn et al., 2012; Simon et al., 2014). Following laser axotomy, we found that *prg-1(ju1574)* mutants displayed increased axon regeneration, resembling the *prg-1* null mutants (Figure 4F). These results indicate that the slicer activity of PRG-1/PIWI is required to inhibit axon regrowth after injury, as well as maintaining germline fertility.

DISCUSSION

Although the piRNA pathway has long been thought to function solely in the germline, evidence for somatic roles is emerging (Ross et al., 2014). For example, in *Aplysia* neurons, PIWI and piRNAs are implicated in long-term synaptic facilitation (Rajasethupathy et al., 2012). Here, we reveal that a set of components in the piRNA pathway inhibits adult axon regeneration of mechanosensory neurons in *C. elegans*. We provide evidence that this role of PRDE-1 and PRG-1/PIWI in neurons is cell autonomous and independent of the gonad. Furthermore, our smFISH analysis reveals somatic expression of *prde-1* and *prg-1* transcripts, with *prde-1* mRNA prominently detected in PLM neurons, in addition to their strong expression in germ cells.

Our data show that selected genes regulating piRNA and secondary siRNA biogenesis, but not the genes regulating nuclear transcriptional silencing, affect axon regeneration. These findings suggest somatic cells may use other piRNA-mediated gene silencing mechanisms. One possibility is piRNA-mediated PTGS, which is not yet reported in *C. elegans*. In support of this idea, we find that the slicer domain of PRG-1/PIWI is required for its roles in

axon regeneration, as well as germline fertility. Previously it is reported that PRG-1/PIWI slicer activity is not required for the accumulation of the majority of 22G-RNAs (Lee et al., 2012). Together, these results suggest that the function of PRG-1/PIWI in eliminating target mRNAs by endonucleolytic cleavage could be responsible for target gene silencing in somatic cells. By mining published TRN transcriptomes (Kaletsky et al., 2016; Lockhead et al., 2016) and piRNA target database (Bagijn et al., 2012), we identified numerous candidate target mRNAs for piRNA in TRN neurons (see STAR methods and Table S2). Further studies will be required to elucidate specific targets of the neuronal piRNA pathway critical for axon regeneration.

Our data also show that some, but not all, of the genes in the previously identified germline piRNA pathway affect axon regeneration. This likely reflects redundancy among the piRNA regulators. Both PARN-1 and HENN-1 have key roles in piRNA maturation, and we found that they act redundantly to inhibit PLM regeneration. Alternatively, the neuronal piRNA pathway may involve different players from the germline piRNA pathway. Supporting this idea, while both RdRPs EGO-1 and RRF-1 affect germline fertility, we found that only EGO-1 has an effect on PLM axon regeneration.

In summary, we find that the piRNA pathway inhibits *C. elegans* axon regeneration after injury. A recent study reported that reduction of a PIWI-like protein using RNAi in cultured rat sensory neurons increases axonal regrowth after injury (Phay et al., 2016), suggesting that the inhibitory role of PIWI/piRNAs in axon regeneration may be evolutionarily conserved. Our data imply that the piRNA pathway inhibits axon regeneration by repressing target genes likely via PTGS, but not nuclear transcriptional silencing. Further understanding of the piRNA pathway based mechanisms in axon regeneration should be relevant to strategies to repair damaged neurons.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- piRNA pathway inhibits axon regeneration in *C. elegans*
- piRNA factors act cell-autonomously in neurons to limit axon regeneration
- Transcripts of the piRNA factor *prde-1* are present in neurons
- The slicer activity of PRG-1/PIWI is required to inhibit axon regrowth

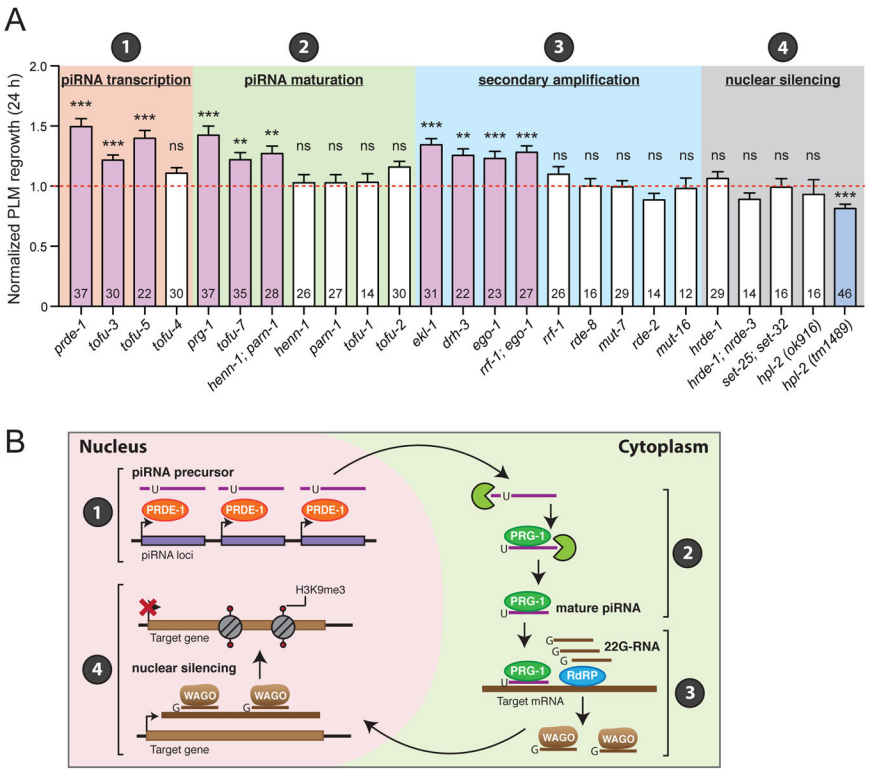

Figure 1. Specific components in the piRNA pathway inhibit PLM axon regeneration

(A) Normalized PLM axon regrowth 24 hours (h) post-axotomy in mutants affecting piRNA transcription and maturation, secondary amplification, and nuclear silencing. Student's *t*-test with the same day controls. Data are represented as mean ± SEM. n, number of animals shown within columns. Absolute values of axon regrowth are provided in Table S1.

(B) Overview of *C. elegans* type 1 piRNA biogenesis from Ruby motif-containing loci and piRNA-mediated gene silencing. Briefly, piRNA genomic loci contain > 16,000 piRNA-producing genes, each of which produces a short transcript of 28 to 29 nt, requiring the function of PRDE-1, TOFU-3, TOFU-4, and TOFU-5 (Goh et al., 2014; Weick et al., 2014) (black circled 1). piRNA maturation entails processing at 5′ and 3′ ends of precursors into 21 nt mature piRNAs with a 5′ monophosphorylated uridine and a 2′–O–methylated 3′ residue; this multi-step event involves PRG-1/PIWI, PARN-1/RNase, HENN-1/RNA methyltransferase, TOFU-1, TOFU-2, and TOFU-7 (Batista et al., 2008; Billi et al., 2012; Goh et al., 2014) (black circled 2). Target recognition by PIWI/piRNA is followed by the generation of secondary endo-siRNAs (also known as 22G-RNAs) (Bagijn et al., 2012) (black circled 3). TGS of piRNA targets occurs through endogenous nuclear silencing mechanisms, thought to interfere with transcription by causing repressive histone modifications (Ashe et al., 2012; Bagijn et al., 2012; Shirayama et al., 2012) (black circled 4).

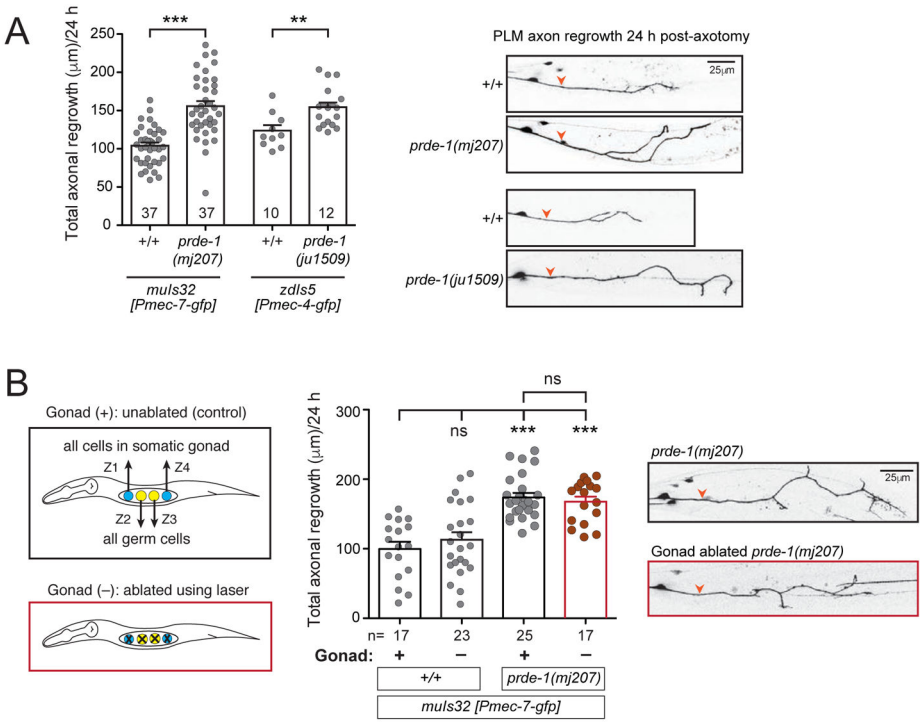

Figure 2. PRDE-1 inhibits PLM axon regeneration independently of the gonad

(A) Enhanced PLM axon regrowth in *prde-1* mutants. Student's *t*-test. Right: representative inverted grayscale images of PLM 24 h post-axotomy.

(B) Ablation of the gonad does not abolish enhanced PLM axon regrowth in *prde-1(mj207)* mutants. Left: schematic of laser ablation of all four gonadal precursor cells in early L1 stage. Middle: PLM axon regrowth 24 h post-axotomy. Right: representative inverted grayscale images of PLM 24 h post-axotomy. One-way ANOVA followed by Tukey's multiple comparison tests.

Data are represented as mean ± SEM. Orange arrowheads, site of axotomy.

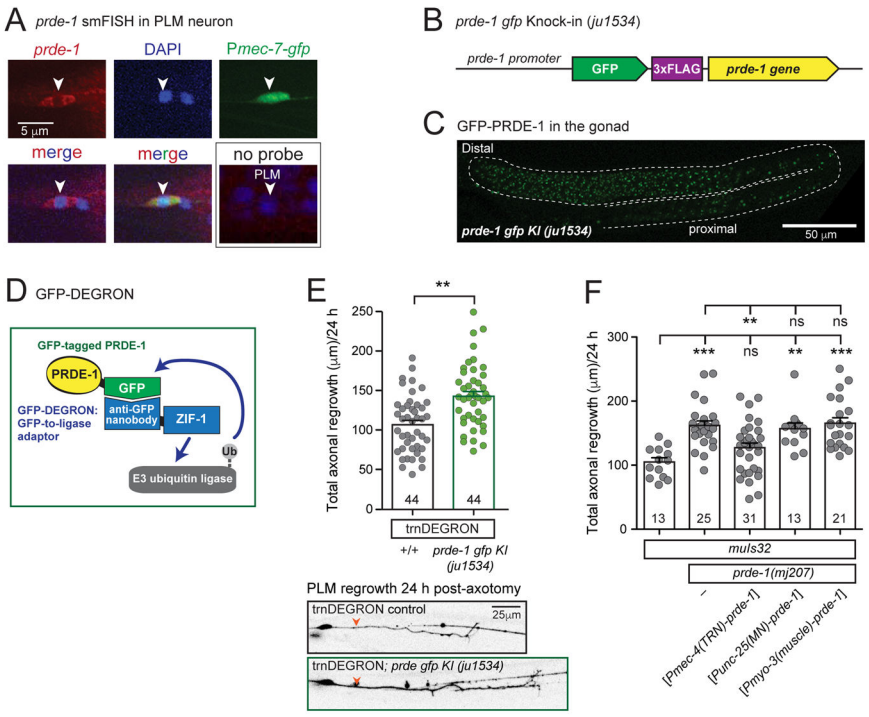

Figure 3. *prde-1* mRNA is present in PLM, and *prde-1* inhibits axon regeneration cell-autonomously

(A) smFISH shows *prde-1* mRNAs in the cytoplasm of PLM neuron labeled with *Pmec-7-gfp* (*muIs32*). Right-bottom shows no probe control.

(B) Schematic of *gfp* KI into *prde-1* genomic locus (*ju1534*). See also Figure S2A.

(C) Confocal image of endogenous GFP::PRDE-1 fluorescence in the germline nuclei in the *prde-1* (*ju1534*) *gfp KI* animals.

(D) Schematic of depletion of GFP-tagged PRDE-1 protein using GFP-DEGRON.

(E) PLM axon regrowth 24 h post-axotomy. Data are represented as mean ± SEM. Student's *t*-test. Bottom: representative images of PLM neurons 24 h post-axotomy. Orange arrowhead, site of axotomy.

(F) PLM regrowth 24 h post-axotomy in transgenic animals expressing *prde-1* driven by tissue-specific promoters for mechanosensory neurons (*Pmec-4*), motor neurons (*Punc-25*), or muscle (*Pmyo-3)* in a *prde-1(mj207)* background. Data are represented as mean ± SEM. One-way ANOVA followed by Tukey's multiple comparison tests.

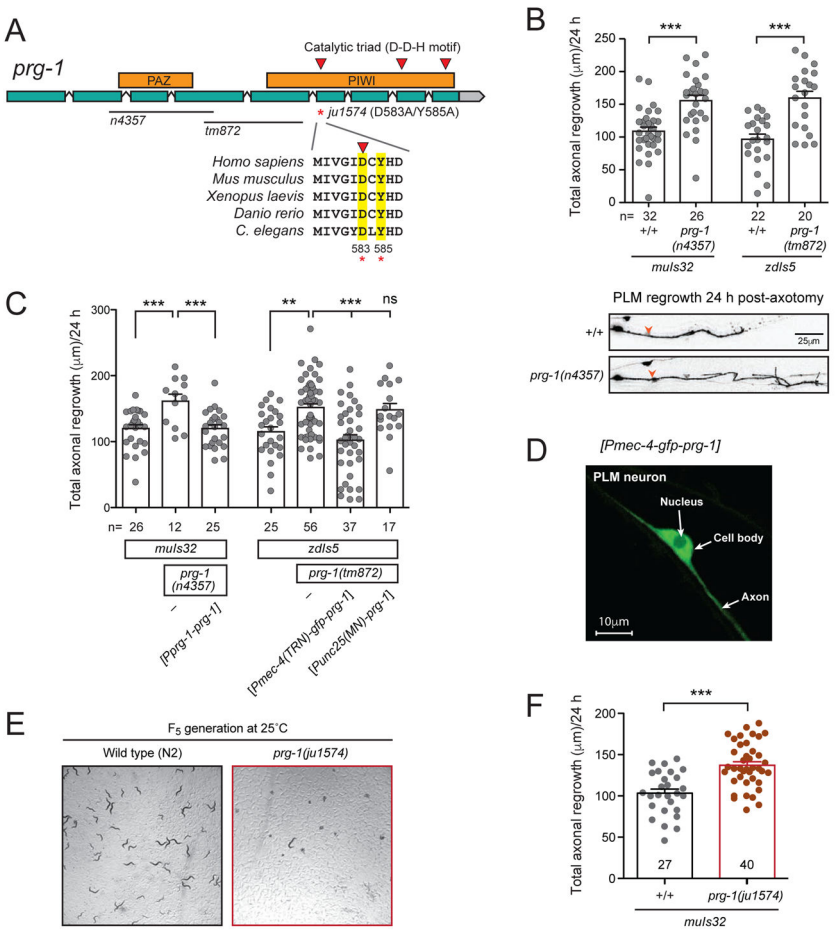

Figure 4. PRG-1/PIWI inhibits PLM axon regeneration cell-autonomously, dependent on its slicer domain

(A) Schematic of the *prg-1* gene (top) and sequence conservation (bottom). PRG-1 contains PAZ and PIWI domains. The PIWI domain contains a catalytic triads D-D-H motif (red arrowheads). Sequence alignment of the first slicer active site of *C. elegans* PRG-1/PIWI (amino acids 578–587) with human (accession number NP_004755), mouse (NP_067286), frog (XP_018117962), and zebrafish (NP_899181) Sequences were analyzed using Clustal Omega. Asterisks mark *prg-1(ju1574)* mutation.

(B) Enhanced PLM axon regrowth in *prg-1* mutants. Student's *t*-test. Bottom: representative images of PLM 24 h post-axotomy. Orange arrowhead, site of axotomy. Data are represented as mean ± SEM.

(C) PLM axon regrowth 24 h post-axotomy in transgenic animals expressing *prg-1(+)* driven by its own promoter in a *prg-1(n4357)* background, or by tissue-specific promoters for mechanosensory neurons (*Pmec-4*) or motor neurons (*Punc-25*) promoters in a *prg-1(tm872)* background. One-way ANOVA followed by Tukey's multiple comparison tests. Data are represented as mean ± SEM.

(D) Fluorescence confocal image of GFP-PRG-1 in PLM (P*mec-4*) in late L4 animal.

(E) Images show that *prg-1(ju1574)* slicer mutants exhibited progressive sterility when strains were passaged at 25°C.

(F) Enhanced PLM axon regrowth 24 h post-axotomy in *prg-1(ju1574)* animals. Data are represented as mean ± SEM. Student's *t*-test.