PLP-1 is essential for germ cell development and germline gene silencing in *Caenorhabditis elegans*

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MS TITLE: PLP-1 is essential for germ cell development and germline gene silencing in C. elegans

AUTHORS: Rajaram Vishnupriya, Linitha Thomas, Lamia Wahba, Andrew Fire, and Kuppuswamy Subramaniam

I have now received reviews of your manuscript from 2 experts. The reviewers' comments are appended below, or you can access them online: please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

As you will see, both reviewers express interest in your study but have some concerns. They offer excellent suggestions to address those concerns and improve your study and manuscript. Reviewer 1 requests additional analysis of small RNAs which are specifically lost in the *plp-1* mutants, additional analysis of the mRNAs which may be affected in the *plp-1* mutant and cross analysis with the existing literature from genes regulated by *plg-1* (Knutson et al., 2017), *set-2* mutant (Robert et al., 2014) and *mut-16* mutants (Rogers and Phillips, 2020). Reviewer 2 raises a very critical point with respect to the specificity of the PLP-1 antibody (which is not presented in the current manuscript) and the differences from the published expression of PLP-1 in the field (Witze et al.,). The discrepancies in the localization of PLP-1 between the current study and the published work needs to be addressed. Reviewer 2 makes excellent suggestions on generation of a V5 tagged PLP-1 to detect any artifacts generated by a GFP tag. Reviewer 2 also suggests additional analysis and recommendations for data presentation to clarify the figures. I realize that accessing the lab may be impossible or slow during the pandemic, but strengthening your paper along the experimental lines suggested is critical for your paper to meet the criteria for publication in Development.

I invite you to consider the reviewers' suggestions and submit a revised manuscript that addresses their concerns. Your revised manuscript would be re-reviewed, and acceptance would depend on your satisfactorily addressing the reviewers' concerns. Please note that Development normally permits only one round of 'major revision' and that because of the pandemic we are happy to extend revision time frames as necessary.

In your revised manuscript, please clearly HIGHLIGHT all changes made in the revised version. You should avoid using 'Tracked Changes' in Word files as these are lost in PDF conversion. I also request a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. If you do not agree with any of the reviewers' criticisms or suggestions, please explain why.

Reviewer 1

Advance summary and potential significance to field

In this work, Vishnupriya et al examine the role of PLP-1 in C. elegans germ cells. plp-1 mutants are synthetic sterile with puf-8 mutants and temperature-sensitive sterile on their own. PLP-1 localizes to embryonic and germline P granules, as well as to perinuclear granules in somatic cells. Additionally, plp-1 mutants fail to silence a piRNA sensor or transgene but cannot desilence a sensor or transgene already silenced by the mutator pathway, suggesting a role in initiation rather than maintenance of silencing, Finally, through small RNA and mRNA sequencing, they show that the majority of small RNAs are unchanged in plp-1 mutants, but that plp-1 mutants misexpress many genes that are also misexpressed in prg-1 and mut-16 mutants, suggesting they may act in the same or parallel pathways.

This paper provides a nice characterization of the germline phenotypes associated with plp-1, however I have a number of suggestions regarding the presentation clarity, or interpretation of the data (see specific comments). Also, some additional analysis of the small RNA and mRNA data would be beneficial to help determine how plp-1 fits into the RNA silencing pathway. For example, are there specific classes of small RNA targets that gain or lose small RNAs in the plp-1 mutants? And are any of the upregulated somatic genes in common with the somatic genes upregulated in pgl-1 mutants (Knutson et al, Genetics, 2017), set-2 mutants (Robert et al, Cell Report 2014), or mut-16 mutants (Rogers and Phillips, NAR 2020)?

Comments for the author

Major comments –
How old are the animals imaged in Figure 1? Are they age-matched? And does the proportion of animals with sperm at the proximal end of the germline change as the animals age?

On line 150, the authors claim that there is no defect observed in puf-8; plp-1 male germlines. However, in the images shown in Fig S2, the puf-8; plp-1 male germline appears to have significantly fewer germ cells compared to the wild-type germline, and the single mutant germlines also appear to have somewhat fewer germ cells. Is this a correct observation and is consistently observed in the mutant animals? Quantifying the germ cell number in the male as well as in the hermaphrodite single and double mutant animals would address this.

Line 161 – "The number of metaphase nuclei, detected by immunostaining with anti-phosphohistone H3 antibodies, was slightly reduced in the mitotic zone of puf-8; plp-1 germ lines" – quantification? Based on the single images, could be as much as 50% reduced. Could this be due to reduced numbers of mitotic germ cells? Also the glp-1; puf-8; plp-1 and gld-2; gld-1; puf-8; plp-1 data should be shown. Either as images or by quantifying the number of germ cells per gonad.

Line 269 – "both these transgenes remained active in plp-1 germ lines for at least 100 generations at 20°C, whereas they became silent within three generations in wild-type germ lines". How are transgenes in the wild-type background desilenced to allow for scoring of resilencing? Same question is applicable to silencing assay for other transgene and sensor lines. In following section (starting line 301), how are transgenes crossed to these mutants while maintaining in the silenced state? A diagram explaining the crosses could be helpful.

Fig 7 – it looks like there are several hundred genes with differentially expressed small RNAs. Why is this shown in the figure but not discussed in the text? Are these particular classes of genes? ERGO-1, CSR-1, WAGO-1 target genes? Also, it seems an important point that piRNAs are not reduced. This demonstrates that plp-1 is not required for the piRNA biogenesis step of silencing the piRNA sensor,

and also not required for the maintenance of silencing step. This should be at least mentioned in the text.

Line 364 – "Since plp-1 mutants as well displayed gene-silencing defects, we expected a significant overlap among the sets of genes upregulated in prg-1, mut-16 and plp-1 mutants. This indeed was the case". How is significance defined? Need to clarify and provide p values for significance of overlap between gene lists.

Line 379 – are the up-regulated somatic genes misexpressed in somatic or germline tissue. This experiment could be done by qPCR on dissected gonads or with RNA FISH. A number of papers have shown recently that loss of pgl-1, csr-1, mut-16 (at 25C), or a number of chromatin factors can cause mis-expression of both spermatogenic genes and somatic genes in the germ cells. Thus the upregulation of somatic genes here may not be distinct from the germline gene silencing pathways.

Minor comments –
The fertility data described starting on line 120, line 181, and 202 and RNAi data on line 291 would be easier to follow if also included in a table or bar graph.

Line 186 – "a few oocytes could be seen in 63% of plp-1 germ lines a day later (n = 162)". What day of adulthood is this, day 2? In the following sentence, what is n=68 referring to? Endomitotic reduplication or embryo production?

Line 260 - Images of gfp::H2B::pal-1 3′ UTR transgene should be included in the manuscript.

Reviewer 2

Advance summary and potential significance to field

PLP-1 in C. elegans is homologous to the human Pur-alpha protein, which is thought to bind nucleic acids to regulate transcription. The authors initially identified PLP-1 as an enhancer of sterility in puf-8 mutants, which disrupts oogenesis. Here they ascribe a function for PLP-1 in germline silencing pathways downstream of small RNA biogenesis. A somatic role as a transcriptional activator in the soma was previously described by Witze et al. 2009. The authors note an additional function in the soma, specifically the mating defect of plp-1 males, but this is not thoroughly explored. Instead they focus on a novel role for PLP-1 in the germline with PUF-8. Like PUF-8 PLP-1 localizes to P granules. P granule proteins mediate the silencing of transposons, foreign genetic, and repetitive elements. plp-1 mutants appear to initiate the expression of both repetitive transgenes and piRNA sensor strains but is not itself essential for small RNA biogenesis, suggesting PLP-1 functions downstream in these silencing pathways.

The plp-1 de-silencing phenotype, enhancement of puf-8 sterility, and function within P granules are substantial findings that could provide novel insight into the role of Pur-alpha in human white blood cell disorders. While the results are primarily descriptive and fall short of showing a mechanism, they represent new and novel functions for this protein in small-RNA mediated silencing pathways. The images are convincing; the writing is clear and concise, but the following two significant concerns need to be addressed:

Comments for the author

The localization of PLP-1 was initially reported in the Witze el al paper, and there are some discrepancies with PLP-1 expression in this report. The subcellular location of PLP-1 should be resolved. For example, Witze shows that PLP-1 is primarily nuclear, whereas this report focuses on the perinuclear and P granule association – with good reason given PUF-8 localization. A new PLP-1 antibody was generated for this study, but no data is demonstrating its specificity to PLP-1, either with a western or immunostaining plp-1 mutants, is included. These controls should be done. While GFP-tagged PLP-1 doesn't seem to be nuclear, this could be attributed to the tag, which increases the predicted 25kD size of PLP-1 to ~50kD. 25kD proteins can diffuse freely into the nucleus, giving weight to the nuclear localization in the Witze paper. At the same time, a 50kD GFP-tagged PLP-1 would require active transport, which would look like the fusion was excluded from nuclei. The

quality and specificity of the antibody in detecting untagged PLP-1 could resolve this matter by addressing whether PLP-1 is nuclear, cytoplasmic, or both. If the antibody is not good enough to make this distinction, adding a small molecule tag with CRISPR (something like a V5 or even a GFP11 in a split GFP background) that keeps the protein under 40kD could also resolve the localization matter. Given that PLP-1 seems to function downstream of small RNA biogenesis, a function in the nucleus may be more relevant than a function in the cytoplasm.

The second concern is that the data presented in figures 8 and 9 could be much more informative. Venn diagrams do a poor job of showing enrichment.
Including enrichment statistics for 8A, comparing averages for all spermatogenic genes in 8B with significance, and proportional Venn diagrams with statistics in Figure 9 would substantially improve the manuscript.

First revision

Author response to reviewers' comments

We thank both reviewers for taking the time to critically evaluate our data and providing suggestions. Our point-point responses are provided below:

Reviewer 1

Comment:
How old are the animals imaged in Figure 1? Are they age-matched? And does the proportion of animals with sperm at the proximal end of the germline change as the animals age?

Response:
Germ lines shown in Fig. 1 were from 1-day-old adults, age-matched for the different genotypes. This information has now been included in the figure legend. Proportion of animals with sperm does not change as the animals age.

Comment:
On line 150, the authors claim that there is no defect observed in *puf-8; plp-1* male germlines. However, in the images shown in Fig S2, the *puf-8; plp-1* male germline appears to have significantly fewer germ cells compared to the wild-type germline, and the single mutant germlines also appear to have somewhat fewer germ cells. Is this a correct observation and is consistently observed in the mutant animals? Quantifying the germ cell number in the male as well as in the hermaphrodite single and double mutant animals would address this.

Response:
The *plp-1* single mutant males do not show any noticeable change in the number of germ cells. The *puf-8* single mutant germ lines have fewer germ cells, and this result has been published earlier (Subramaniam and Seydoux, 2003). Therefore, it is best to compare the *puf-8; plp-1* germ lines with the *puf-8* single mutant. We have now revised the manuscript to clarify this point.

Comment:
Line 161 – "The number of metaphase nuclei, detected by immunostaining with antiphosphohistone H3 antibodies, was slightly reduced in the mitotic zone of *puf-8; plp-1* germ lines" – quantification? Based on the single images, could be as much as 50% reduced. Could this be due to reduced numbers of mitotic germ cells? Also, the *glp-1; puf-8; plp-1* and *gld-2; gld-1; puf-8; plp-1* data should be shown. Either as images or by quantifying the number of germ cells per gonad.

Response:
Numbers for the PH3-positive nuclei have been included in the revised legend of Fig. S4. Images of

DAPI-stained germ lines of *glp-1; puf-8; plp-1* and *gld-2; gld-1; puf-8; plp-1* genotypes have been included as **Fig. S5** in the revised Supplemental material.

Comment:
Line 269 – "both these transgenes remained active in *plp-1* germ lines for at least 100 generations at 20°C, whereas they became silent within three generations in wild-type germ lines". How are transgenes in the wild-type background desilenced to allow for scoring of resilencing? Same question is applicable to silencing assay for other transgene and sensor lines. In following section (starting line 301), how are transgenes crossed to these mutants while maintaining in the silenced state? A diagram explaining the crosses could be helpful.

Response:
1. Strains carrying the integrated transgenes, GFP::H2B::*pal-1* 3′ UTR, GFP::PGL-1 and GFP::GLD-1, and the ccEx7271 extrachromosomal array were maintained in active state at 25°C. They were mated with *plp-1(+/-)* males at 25°C, the progeny were shifted to 20°C and cloned. The transgenes and the *ok2155* allele *of plp-1* were detected by single-worm PCRs, and animals homozygous for both the transgene and ok2155 were obtained by further cloning and single-worm PCRs. Similar strategy was employed for the piRNA sensor 21UR-1349 as well. The piRNA sensor 21UR-1 was transferred from the *prg-1(-)* background, in which it is active, into the *plp-1* mutant.
2. Maintenance at 20°C of strains carrying the transgenes, extrachromosomal array and piRNA sensors mentioned above for several generations led to complete silencing. Such silenced transgenes—their presence was detected by PCR—were then introduced into *plp-1* and *rde-3* mutants through crosses setup at 20°C.
The above description is included in the Materials and methods section of the revised manuscript and a diagram is included in the Supplemental material.

Comment:
Fig 7 – it looks like there are several hundred genes with differentially expressed small RNAs. Why is this shown in the figure but not discussed in the text? Are these particular classes of genes? ERGO-1, CSR-1, WAGO-1 target genes? Also, it seems an important point that piRNAs are not reduced. This demonstrates that plp-1 is not required for the piRNA biogenesis step of silencing the piRNA sensor, and also not required for the maintenance of silencing step. This should be at least mentioned in the text.

Response:
We have analyzed the subset of small RNAs that are differentially expressed in the *plp-1* mutant, and included the results in two additional sheets in the **revised Supplementary dataset-1**. These sheets list 312 mRNAs whose small RNAs are differentially expressed in *plp-1* mutants relative to wild type based on a cutoff of 4-fold. We have indicated the classes for each of these 312 based on the annotations available in the literature. Essentially, the differentially-expressed small RNAs are not enriched for any specific classes of small RNAs. These observations have been included in the Results section of the revised text. We have included an additional sentence which states "Despite the involvement of *plp-1* in the silencing of piRNA sensors (Fig. 5), levels of piRNAs are not reduced in *plp-1* mutants." Furthermore, the last sentence of the previous paragraph (lines 310-313) explicitly comments on the dispensability of *plp-1* for the maintenance step of silencing.

Comment:
Line 364 – "Since *plp-1* mutants as well displayed gene-silencing defects, we expected a significant overlap among the sets of genes upregulated in *prg-1*, *mut-16* and *plp-1* mutants. This indeed was the case". How is significance defined? Need to clarify and provide p values for significance of overlap between gene lists.

Response:
We have included the *p* values for the overlaps in the revised text, and present the data using proportional Venn diagrams in the **revised Fig. 9**. The tools used for *p*-value calculation and drawing proportional Venn diagrams are mentioned in Fig. 9 legend.

Comment:
Line 379 – are the up-regulated somatic genes misexpressed in somatic or germline tissue? This

experiment could be done by qPCR on dissected gonads or with RNA FISH. A number of papers have shown recently that loss of *pgl-1*, *csr-1*, *mut-16* (at 25C), or a number of chromatin factors can cause mis-expression of both spermatogenic genes and somatic genes in the germ cells.
Thus the upregulation of somatic genes here may not be distinct from the germline gene silencing pathways.

Response:
1. We have now compared the list of genes upregulated in *plp-1* mutants with the sets of soma-enriched genes upregulated in germ lines lacking P granules [pgl(-)], *set-2* and *mut- 16* (at 25°C) mutants, and included the results in **Tables S12-S14** in the **Supplementary dataset-2**. The soma-enriched genes upregulated in *plp-1* mutants are listed in **Table S15**. Essentially, *plp-1* does not appear to be involved in the suppression of soma- enriched genes in the germ line: 1) only 22 of the 780 genes upregulated in *plp-1* belong to this category, and 2) there are no significant overlaps between these 780 and the soma- enriched genes upregulated in *pgl(-)*, *set-2* or *mut-16* mutants. We have included a brief discussion on this in the revised text.
2. Additionally, we have now performed qRT-PCR for 5 genes selected from the 306 non-germline-enriched genes (part of the 780 genes upregulated in *plp-1* mutants) and included the results in Fig. S8. Two of them—*col-2* and *dpy-8*—are upregulated in the *plp-1* germ lines. We have used the *glp-1(q175)* genetic background to determine specific upregulation in the germ line.

Thus, our data suggest that *plp-1* may not play a major role in suppressing soma-enriched genes in the germ line, although it may be involved in the suppression of a few non-germline-enriched genes.
3. We have removed the sentence, "…which suggests that *plp-1* affects some of the somatic genes, notably collagen and other cuticle-related genes, through a pathway that is distinct from the germline gene-silencing pathways" in the revised text, which, based on the reviewer's comment, we realized is misleading.

Comment:
The fertility data described starting on line 120, line 181, and 202 and RNAi data on line 291 would be easier to follow if also included in a table or bar graph.
Response:
These data are presented in **Tables S1-S4** included in the revised Supplemental material.

Comment:
Line 186 – "a few oocytes could be seen in 63% of *plp-1* germ lines a day later (n = 162)". What day of adulthood is this, day 2? In the following sentence, what is n=68 referring to? Endomitotic reduplication or embryo production?

Response:
Yes, these are 2-day-old adults. None of the 68 animals examined produced embryos. We have clarified both these ambiguities in the revised text.

Comment:
Line 260 – Images of gfp::H2B::pal-1 3′ UTR transgene should be included in the manuscript.

Response:
We have included these images in the **revised Fig.5**.

Reviewer 2

Comment:
A new PLP-1 antibody was generated for this study, but no data is demonstrating its specificity to PLP-1, either with a western or immunostaining plp-1 mutants, is included. These controls should be done. While GFP-tagged PLP-1 doesn't seem to be nuclear, this could be attributed to the tag, which increases the predicted 25kD size of PLP-1 to ~50kD. 25kD proteins can diffuse freely into the nucleus, giving weight to the nuclear localization in the Witze paper. At the same time, a 50kD GFP-tagged PLP-1 would require active transport, which would look like the fusion was excluded from nuclei. The quality and specificity of the antibody in detecting untagged PLP-1 could resolve this matter by addressing whether PLP-1 is nuclear, cytoplasmic, or both.

Response:
We have now immunostained wild-type and *plp-1* mutant embryos with the affinity-purified anti-PLP-1 antibodies generated by us (described in the Materials and methods). The results are presented in **Fig. S6**. As shown in this figure, this antibody is specific, and the PLP-1 distribution pattern detected by this antibody is similar to that detected by the GFP tag. Results of these immunostaining experiments reveal the presence of PLP-1 in the cytoplasm of all blastomeres throughout embryogenesis. We did not see any immunofluorescence in nuclei during the course of embryonic development that could be attributed to PLP-1. In our experience, nuclei and P granules of poorly freeze-cracked *C. elegans* embryos readily non- specifically stain with various antibodies. This and the absence of a null allele for *plp-1* at the time of Witze at al. work might explain the difference between their and our results.

Additionally, we wish to draw the reviewer's attention to our observation that PLP1::GFP fully rescues all the phenotypic defects observed in animals homozygous for the null allele (*ok2155*) of *plp-1*.

Comment:
The second concern is that the data presented in figures 8 and 9 could be much more informative. Venn diagrams do a poor job of showing enrichment. Including enrichment statistics for 8A, comparing averages for all spermatogenic genes in 8B with significance, and proportional Venn diagrams with statistics in Figure 9 would substantially improve the manuscript.

Response:
1. We have now performed gene cluster enrichment analysis using the DAVID tool, and presented the results in **revised Fig. 8A** in the form of bar graphs, along with the *p*-values for the statistical significance of the enrichment. The lists of genes in different classes are presented in **Supplementary dataset-3 and -4**.
2. The gene-set overlaps are now presented using proportional Venn diagrams, along with the corresponding *p*-values (**revised Fig. 9**).

Second decision letter

MS ID#: DEVELOP/2020/195578

MS TITLE: PLP-1 is essential for germ cell development and germline gene silencing in C. elegans

AUTHORS: Rajaram Vishnupriya, Linitha Thomas, Lamia Wahba, Andrew Fire, and Kuppuswamy Subramaniam
ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Development, pending our standard ethics checks.

Reviewer 1

Advance summary and potential significance to field

In this work, Vishnupriya et al examine the role of PLP-1 in C. elegans germ cells. plp-1 mutants are synthetic sterile with puf-8 mutants and temperature-sensitive sterile on their own. PLP-1 localizes to embryonic and germline P granules, as well as to perinuclear granules in somatic cells. Additionally, plp-1 mutants fail to silence a piRNA sensor or transgene but cannot desilence a sensor or transgene already silenced by the mutator pathway, suggesting a role in initiation rather than maintenance of silencing, Finally, through small RNA and mRNA sequencing, they show that the majority of small RNAs are unchanged in plp-1 mutants, but that plp-1 mutants misexpress

many genes that are also misexpressed in prg-1 and mut-16 mutants, suggesting they may act in the same or parallel pathways.

Comments for the author

The authors have addressed all of my concerns and the manuscript is now appropriate for publication in Development.

Reviewer 2

Advance summary and potential significance to field

PLP-1 in C. elegans is homologous to the human Pur-alpha protein, which is thought to bind nucleic acids to regulate transcription. The authors initially identified PLP-1 as an enhancer of sterility in puf-8 mutants, which disrupts oogenesis. Here they ascribe a function for PLP-1 in germline silencing pathways downstream of small RNA biogenesis. A somatic role as a transcriptional activator in the soma was previously described by Witze et al. 2009. The authors note an additional function in the soma, specifically the mating defect of plp-1 males, but this is not thoroughly explored. Instead they focus on a novel role for PLP-1 in the germline with PUF-8. Like PUF-8 PLP-1 localizes to P granules. P granule proteins mediate the silencing of transposons, foreign genetic, and repetitive elements. plp-1 mutants appear to initiate the expression of both repetitive transgenes and piRNA sensor strains but is not itself essential for small RNA biogenesis, suggesting PLP-1 functions downstream in these silencing pathways.

The plp-1 de-silencing phenotype, enhancement of puf-8 sterility, and function within P granules are substantial findings that could provide novel insight into the role of Pur-alpha in human white blood cell disorders.

Comments for the author

Issues brought up concerning the antibody have been sufficiently addressed with the inclusion of Sup Fig 6. Figures 8 and 9 have been updated and improved as requested. These changes, and points addressed by the other reviewer, have improved this manuscript.