Orb-dependent polyadenylation contributes to PLP expression and centrosome scaffold assembly

Junnan Fang and Dorothy A. Lerit
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First decision letter

MS ID#: DEVELOP/2021/200426

MS TITLE: Orb-dependent polyadenylation contributes to PLP expression and centrosome scaffold assembly

AUTHORS: Junnan Fang and Dorothy A. Lerit

I apologize for the long delay before coming back to you. One of the reviewers conveys their apologies for being late due to difficulties in the current pandemic situation. I have now received all the referees' reports on the above manuscript, and have reached a decision. The referees' 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, two of the referees express strong interest in your work, one questions the extent of advance, and all have some criticisms and recommend a revision of your manuscript before we can consider publication. If you are able to revise the manuscript along the lines suggested, which may involve further experiments, I will be happy receive a revised version of the manuscript. Your revised paper will be re-reviewed by one or more of the original referees, and acceptance of your manuscript will depend on your addressing satisfactorily the reviewers' major concerns. Please also note that Development will normally permit only one round of major revision.

We are aware that you may be experiencing disruption to the normal running of your lab that make experimental revisions challenging. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Please attend to all of the reviewers' comments and ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion. I should be grateful if you would also provide 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 their criticisms or suggestions please explain clearly why this is so.

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Reviewer 1

Advance summary and potential significance to field

This manuscript examines the role of the Drosophila CPEB protein orthologue Orb in the regulation of pericentrin-like (plp) mRNA localisation and translation. The authors find that orb hypomorphs have little effect on plp mRNA localisation to the centrosome but reduce the levels of Plp protein, suggesting that Orb regulates Plp translation. The observed reduction in Plp levels leads to an increased frequency of mitotic defects and nuclear fall out in the blastoderm embryo.

Comments for the author

Although the regulation of mRNA localisation and translation at the centrosome is an interesting topic, this manuscript falls short in several ways. 1) The study does not provide any mechanistic insights and instead relies on earlier studies from Xenopus laevis to propose a model for the role of Orb. 2) The evidence that Orb binds to CPE elements in plp mRNA to regulate its polyadenylation and translation is neither complete nor convincing. 3) The maternal-effect phenotype of orb is relatively low penetrance and only partially due to effects on Plp expression, reducing the significance of the authors' observations. I therefore think that this manuscript is unsuitable for Development although a revised version might be appropriate for Biology Open.

Major points:

1) The localisation of plp mRNA to the centrosome is at best partial and is not convincing. Their previous publication on centrosomal mRNA localisation (Ryder et al, 2020) showed that 11% of plp mRNA signal localised at centrosomes, but they have now changed their criterion from mRNAs at the centrosome (i.e 0µm) to mRNAs within 1µm of the centrosome. This gives them higher numbers of "localised" mRNAs, particularly as the number of centrosomes increases during the syncytial blastoderm divisions, but is an artefact of including an increasing amount of the embryo cytoplasm in the area defined as localised. I don't think that an mRNA 1µm way from the centrosome can count as centrosomally localised. Instead, the distribution of smFISH signal seems more likely to me to correspond to an association with microtubule, which are obviously more frequent closer to the centrosomes.

2) The evidence that Orb binds to CPEs within plp mRNA is based on pull-downs of GFP-Orb using an anti-GFP antibody that pulls down at least five endogenous proteins in addition to GFP (Fig S2 A and B), several of which are more abundant than GFP-Orb. Indeed, plp mRNA is pulled down by the anti-GFP antibody from wild-type extracts that do not express GFP-Orb, albeit to a lesser extent than the GFP-Orb expressing extracts. More importantly, we are not shown the distribution of Orb protein to see if it co-localises with plp mRNA at or near centrosomes, and no evidence is provided to show that the pull-down depends on the CPE elements within plp mRNA.

3) The authors claim that Plp protein levels are reduced by 50% in embryos from orbF343/orbmel mothers is based on quantifying the levels of the largest isoform of Orb protein on western blots, but does not take into account the effects on the other two major isoforms, which are more abundant in some lanes. While there probably is an effect on Plp levels, no evidence is provided to show that this is a direct effect of reduced Orb levels on Plp translation and not an indirect effect of changes in one of the many other Orb targets that have been reported. The authors' case would be strengthened by showing that mutations in the putative Orb binding sites in plp mRNA has a similar effect on Plp protein levels.

4) Figure 4 does not provide convincing evidence that the poly-A tail length is reduced in orbF343/orbmel embryos. The authors focus on lanes 9 and 10 but do not explain what the lower band and smear below 293bp are. They then analyse the part of the gel above 293bp and conclude that the poly-A tail length is reduced, although the only clear difference between the wild-type and mutant samples is the strength of the band at ~300bp. Given that this gel has spurious background bands, how do they know that this band represents the short isoform of plp mRNA. More worryingly, they fail to mention the data in lanes 11 and 12, which seem more clear cut because the Bmr1 digestion has reduced the size of the plp signal to the region of the gel where the size resolution is better. In these lanes, there appears to be no difference between the wild-type and mutant samples.

5) The mitotic defects in embryos derived from orb mutant mothers are quantified on a per embryo basis, when they should measure the proportion of spindles with lagging chromosomes. By using embryos as the denominator, they are hugely amplifying what is a rather weak effect,

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because each embryo that is scored as containing a spindle with a lagging chromosome, presumably has thousands of spindles that are normal.

Minor points:
1) The scale bar in Fig1 F is not defined.
2) The plots in Figure 1G are meaningless, because the smFISH signal corresponds to single molecules of plp mRNA and the intensity distribution is therefore that of a diffraction limited spot.
3) Figure S1B shows the DNA sequence whereas the text refers to the RNA sequence.
4) It is not stated whether the Orb-GFP protein trap flies used for the pull-downs are homozygous or heterozygous, although Figure S2B suggests that the females are heterozygous. Is this because the line is homozygous lethal, and if so, how do they know that the trapped protein is functional?
5) "Consistent with a requirement for orb in PLP localization, we noted a significant reduction (~40% less than WT; $p<0.001$ by one-way ANOVA) in recombinant plp/+, orb mutants (Figure 5A,B), suggesting PLP dosage at centrosomes is regulated by orb activity." Any conclusions about the function of Orb should be based upon comparing plp/+, orb with plp/+, not with wild-type.
6) What is the y-axis in Fig 6D?
7) The references are listed in order of citation but are not numbered.

Reviewer 2

Advance summary and potential significance to field

In the manuscript entitled "Orb-dependent polyadenylation contributes to PLP expression and centrosome scaffold assembly", Fang and Lerit provide convincing evidence that the RNA binding protein Orb regulates PLP expression post-transcriptionally. This work contributes to the field of Developmental Biology and RNA biology by describing evidence for the post-transcriptional regulation of centrosomally localized RNAs, an area that has been historically understudied. The manuscript is appropriately suited to the audience of development. It is well written and the experiments are done with rigor, using both biochemical and genetic approaches. There are a handful of concerns that are easily addressed that would make the manuscript suitable for publication.

Comments for the author

Major comments:
"Next, we aligned the plp 3'-UTR across multiple Drosophila species using the conservation insect track on the UCSC genome browser and found these CPE motifs were conserved across millions of years of evolutionary 168 distance (data not shown)(Kent et al., 2002)."
This data should be shown and alignments between fly and human should be shown within the main text, instead of as supplemental data.
In Figure 3, the authors should quantify each of the PLP isoforms. It is not clear why they chose to only quantify one isoform (and which one this is). It remains possible that only expression of specific PLP isoforms is affected. Or in contrast it is possible that the overall expression (when taking into account all protein isoforms) remains unchanged.
Furthermore, what are the differences in these 12 protein isoforms? Could they possibly have different functions? Is there any evidence that only a specific isoform is localized to the centrosome?
Embryonic viability using hatch rate is not ideal. There are multiple reasons that embryos do not hatch, including embryonic lethality and unsuccessful fertilization of eggs. Since orb is a critical regulator of oogenesis and embryogenesis, the authors should rule out the presenceof unfertilized eggs by performing DAPI stain on aged embryos. This will also be useful to determine the stage at which they die, in addition to knowing if they are unfertilized. Otherwise, they cannot conclude that this is a result of embryonic lethality.
Finally, although certain statistical analyses are not significant, it is important to state the actual p values either in the text of the manuscript or in the figure descriptions for the sake of accuracy and transparency.

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Minor comment:
In the introduction, it is not accurate to say that PCNT dysregulation results in Trisomy 21. While PCNT may be affected in Trisomy 21 is there evidence that PCNT dysregulation alone recapitulates the phenotypes of Trisomy 21?

Reviewer 3

Advance summary and potential significance to field

This interesting paper explores the role of Drosophila Orb (not Orb2) in Pericentrin (PCNT)-like protein (PLP) function in centrosomes. They identified a specific biochemical association between Orb and pop mRNA and showed that Orb promotes polyadenylation of the short form of pop mRNA, containing a shorter 3'UTR. They show that Orb is required for correct Plp protein levels through translational regulation in centrosomes and pricentriolar organisation and genome stability.

These are important findings of great general interest suitable for Development and interesting to its readers.
Localised production of centrosome components is an emerging theme of great interest to the centrosome field.

Comments for the author

I only have one specific major issue that I think should be addressed in a revision before the manuscript can be accepted for publication.
In the manuscript the authors state that plp mRNA is localised to the centrosome. e.g. in line 107: "...plp mRNA localization to centrosomes...". When I look at the images presented in Fig1. plp mRNA is not particularly convincingly localised in centrosomes. The quality of the smFISH images is not in question, they are outstanding. But I am referring to the interpretation of the distribution of the single molecules of mRNA relative to the centrosomes labelled by GFP-gamma-Tubulin. For example, if you look at panel A, there is plp mRNA evident around one of the centrosomes that is highlighted, but the other centrosome has only one plp RNA molecules co-localised with it. Given that pop mRNA is also distributed all over the cytoplasm, as is very evident in these images, it is not really clear whether plp is enriched at centrosomes and present everywhere too at lower levels, or in fact just everywhere and in a few centrosomes enriched by chance. This problem is compounded by the fact that the control mRNA used in Fig. 1C (GAPDH) is so abundant that it too is present in the centrosomes shown, perhaps by chance. This issue should be resolved definitevely by the authors if they are to make the statement that plp mRNA is localised. There are many ways they could do this by image analysis and some statistics. The authors have attempted to do this in Fig. 1E by plotting the % RNA present within <1micron to centrosomes for plp and gaped mRNA. I do not find this indirect measure very convincing at all, and a much more direct metric for localisation is needed in my view.

Some suggestions are:
A) Make an identical image of centrosomes without mRNA. paint in the same number of mRNAs but in randomly chosen coordinates. Then compare how many mRNAs are localised near the centrosomes (with a specific cutoff of distance) in the experiment verses the randomly distributed single mRNA spot controls. This is similar to a traditional way that co-localisation of gold spots with organelles is assessed by electron microscopists.
B) There are more complex and sophisticated alternative methods such as K mean clustering (distribution of distances between spots) or Montecarlo statistical analysis that could also be used. But I think the suggestion in 1, will not take long to do with common image analysis tools.

Minor issues to correct:
1) There is a minor problem with the PDF, which may not be due to the authors. Some of the text is thin and some fatter. I believe it is because the pdf is made of an image of the text (it cannot be infinitely zoomed into for example), rather than a normal pdf where the text is infinitely zoomable. This seems very minor, but is a problem, as one cannot zoom into the images in the figures to look at them at sufficient enlargement.

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2) there is some kind of corruption of the formatting of some references - where instructions for the formatting of the text are present as text characters *Drosophila* for example on line 1170. Again, this maybe a problem with the journal site, not the authors files, but there are at least 7 instances of this error.

First revision

Author response to reviewers' comments

Thank you for providing feedback on our summitted manuscript DEVELOP/2021/200426, titled “Orb-dependent polyadenylation contributes to PLP expression and centrosome scaffold assembly.” We also thank Dr. Lecuit for evaluating our revision plan to address reviewer comments. We appreciate the overall positive feedback on our submission, and we appreciate the opportunity to address the reviewer comments in our revised manuscript.

In the following point-by-point response letter, we address editorial and reviewer comments using blue font. Our revised text also uses blue text to highlight revisions; although, a standard black-and-white document is also attached to our submission as a supplemental file.

We thank you for your time and thoughtful comments. We agree the work is now stronger as a result. We hope it is now suitable for publication in ***Development***.

I apologize for the long delay before coming back to you. One of the reviewers conveys their apologies for being late due to difficulties in the current pandemic situation. I have now received all the referees' reports on the above manuscript, and have reached a decision. The referees' 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.

We recognize these are difficult and unprecedented times, especially for those dealing with dependent care, illness, or loss.

As you will see, two of the referees express strong interest in your work, one questions the extent of advance, and all have some criticisms and recommend a revision of your manuscript before we can consider publication. If you are able to revise the manuscript along the lines suggested, which may involve further experiments, I will be happy receive a revised version of the manuscript. Your revised paper will be re-reviewed by one or more of the original referees, and acceptance of your manuscript will depend on your addressing satisfactorily the reviewers' major concerns. Please also note that Development will normally permit only one round of major revision.

We are pleased the reviewers are overall positive about the work and appreciate their thoughtful comments to strengthen our submission.

We are aware that you may be experiencing disruption to the normal running of your lab that make experimental revisions challenging. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Please attend to all of the reviewers' comments and ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion. I should be grateful if you would also provide 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 their criticisms or suggestions, please explain clearly why this is so.

Thank you for reviewing our revision plan, which helped us focus our efforts on the major concerns. In this response letter, we directly address each reviewer comment using blue font. We also highlight in blue font major changes made to the main text. A black-and-white file is also included as a supplemental text file.

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Reviewer 1 *Advance Summary and Potential Significance to Field:*
This manuscript examines the role of the *Drosophila* CPEB protein orthologue Orb in the regulation of pericentrin-like (plp) mRNA localisation and translation. The authors find that orb hypomorphs have little effect on plp mRNA localisation to the centrosome but reduce the levels of Plp protein, suggesting that Orb regulates Plp translation. The observed reduction in Plp levels leads to an increased frequency of mitotic defects and nuclear fall out in the blastoderm embryo.

Reviewer 1 Comments for the Author:
Although the regulation of mRNA localisation and translation at the centrosome is an interesting topic, this manuscript falls short in several ways. 1) The study does not provide any mechanistic insights and instead relies on earlier studies from Xenopus laevis to propose a model for the role of Orb. 2) The evidence that Orb binds to CPE elements in plp mRNA to regulate its polyadenylation and translation is neither complete nor convincing. 3) The maternal-effect phenotype of orb is relatively low penetrance and only partially due to effects on Plp expression, reducing the significance of the authors' observations. I therefore think that this manuscript is unsuitable for Development although a revised version might be appropriate for Biology Open.

We appreciate the reviewer's interest in our examination of RNA localization and local translation at the centrosome. Three weaknesses are raised: 1) mechanism, 2) inadequate evidence for direct binding of *plp* CPE motifs by Orb, and 3) low penetrance.

Regarding the topic of mechanism, in our study, we define Orb as the first known regulator of a critical centrosome factor, PLP, and show PLP protein levels and RNA polyadenylation are altered upon *orb* depletion. As Orb regulates the translation of its targets in a polyadenylation-dependent manner, these two points suggest Orb likely promotes translation. We also identify, for the first time, mitotic defects in *orb* hypomorphs, largely rescued by increasing PLP dosage. Together, our data provide mechanistic insight into how Orb regulates PLP dosage to permit normal cell division. Regarding reliance on the *Xenopus* data, the majority of CPEB work was done by the Richter lab in this model, and we believe it is critical to cite and give credit where credit is due. Nonetheless, we do cite several *Drosophila* papers likewise showing the primary mechanism for Orb to regulate translation is via a poly(A)-tail length-dependent mechanism, arguing aspects of CPEB regulation are conserved. In our revised work, we also specifically discuss on line 487 the recruitment of PAP to an Orb target mRNA, *cortex*, further showcasing conservation.

Regarding direct binding, the reviewer is correct we do not attempt to show direct binding of Orb to the conserved CPE sites within the *plp* 3'UTR. In our text, we are careful to indicate our findings "suggest" Orb regulates *plp* via these sites; moreover, we revised the legend and model Figure 8E by adding a question mark next to our diagram showing Orb binding the *plp* CPE and note "Orb associates with *plp* mRNA, and this interaction may be direct through CPE motifs in the 3'UTR or indirect." We also added line 463: "Future work is needed to determine whether Orb binds *plp* mRNA directly to stimulate local translation of *plp* mRNA at centrosomes."

Regarding the penetrance of *orb* related phenotypes, it is unclear which specific phenotype is under question; however, we use robust quantifiable approaches and statistical comparisons to show depletion of *orb* results in impaired centrosome scaffolding (increased Cnn fragments), reduced PLP protein levels, increased CIN and NUF. In addition to being reproducible and statistically significant, we further show genetic rescue by expression of a *PLP-GFP* transgene. We also are careful to note these phenotypes are likely <u>under-representative</u>, as some functional Orb protein remains in the hypomorphic genetic backgrounds. In our revised work, we further emphasize how redundant mechanisms likely safeguard embryos from mitotic catastrophe in the absence of Orb (e.g., lines 499-503).

Major points:
1)The localisation of plp mRNA to the centrosome is at best partial and is not convincing. Their previous publication on centrosomal mRNA localisation (Ryder et al, 2020) showed that 11% of plp mRNA signal localised at centrosomes, but they have now changed their criterion from mRNAs at the centrosome (i.e 0μm) to mRNAs within 1μm of the centrosome. This gives them higher numbers of "localised" mRNAs, particularly as the number of centrosomes increases during the syncytial blastoderm divisions, but is an artefact of including an increasing amount of the embryo cytoplasm in the area defined as localised. I don't think that an mRNA 1μm way from the centrosome can count as centrosomally localised. Instead, the distribution of smFISH signal seems more likely to me to correspond to an association with microtubule, which are obviously more frequent closer to the centrosomes.

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Our prior live imaging and super-resolution imaging indicates the Cnn flares, which define the outer margin of the PCM, are dynamic structures which extend and retract and on average sample an area in excess of 1.5 microns from the centrosome center, sometimes much more (Lerit et al. 2015, JCB). We recognize sampling a static snapshot in fixed images and limiting our analysis only to RNA in direct contact with Cnn at a given time is an under-representation of centrosome proximal RNAs. Indeed, localization of the RNA is itself likely a dynamic process; thus, a static image necessarily under-samples RNA residence at a structure per unit of time.

For genetic reasons, the current study uses GFP-gTub to label centrosomes (our GFP-Cnn bac line is on the same chromosome as *orb*). We and others previously showed gTub occupies a smaller volume than Cnn (e.g., by quantifying intensity measurements and via SIM microscopy; Lerit et al 2015, *JCB*). Based on our Python pipeline and prior work, we know the smaller the volume of the centrosome 'object,' the lesser the absolute number of mRNAs will overlap. In our prior work, we noted the same observation for *cen* mRNA at Cnn versus gTub (Ryder et al, 2020, *JCB*).

Nevertheless, localization of *plp* mRNA at the centrosome is markedly robust. In the revised manuscript, we now report RNA overlapping the gTub surface (i.e., 0 mm away) – across syncytial and cell cycle stages, *plp* mRNA remains significantly enriched at centrosomes over the much higher expressed *gapdh* mRNA. These data are presented in revised Figures 1 and 3. Further, based on suggestions by Reviewer 3, we now include a new Figure 1F showing the specificity of pixel overlap. Namely, we took the oldest NC syncytial stage (NC 13), which has the smallest pseudo-cells and might be assumed to artefactually 'enrich' mRNAs to centrosomes. We rotated the red (RNA) channel for *plp* and *gapdh* images and remeasured RNA enrichment using our Python code. These data show rotating the *plp* mRNA channel abolishes localization of *plp* mRNA signals at the centrosome, demonstrating the enrichment of *plp* mRNA to centrosomes in normal, unrotated images is not a result of spurious overlap. In contrast, rotating *gapdh* mRNA does not alter *gapdh* distributions, further demonstrating the *gapdh* distribution is effectively random.

2) The evidence that Orb binds to CPEs within plp mRNA is based on pull-downs of GFP-Orb using an anti-GFP antibody that pulls down at least five endogenous proteins in addition to GFP (Fig S2 A and B), several of which are more abundant than GFP-Orb. Indeed, plp mRNA is pulled down by the anti-GFP antibody from wild-type extracts that do not express GFP-Orb, albeit to a lesser extent than the GFP-Orb expressing extracts. More importantly, we are not shown the distribution of Orb protein to see if it co-localises with plp mRNA at or near centrosomes, and no evidence is provided to show that the pull-down depends on the CPE elements within plp mRNA.

We regret it was not clear Fig S2A-B are not pull-down experiments but are western blots showing expression of GFP-Orb vs. endogenous Orb proteins in whole lysate from ovaries and embryos, using anti-GFP and anti-Orb antibodies. We now label the upper part of each of these blots to more clearly note this. It is true, some non-specific signals are detected in the immunoblots, but we label the expected band (based on MW) for GFP-Orb versus endogenous Orb.

In contrast, Figure 2C shows the RNA-IP. There is a faint *plp* band also detected in the WT control; however, based on this reviewer comment, we now include quantification of the fold-enrichment in the GFP-Orb samples in the main text (it is >7-fold enriched).

Regarding Orb protein distribution in embryos, we now include a new Figure S4D showing Orb localization in the embryonic mid-region (somatic nuclei) in control versus *orb* hypomorphs. As expected and based on prior work (and the low level of Orb protein detected by western blot using embryo extracts), relative abundance of Orb is low. Nonetheless, some Orb protein is detected in the cytoplasm. Moreover, some Orb is proximal to centrosomes (insets). Finally, Orb signals are reduced in the hypomorphic mutants, demonstrating specificity of the signals, which we display using the same parameters (i.e., LUT, lookup table). We describe these findings in the revised text starting on line 346. Thank you for suggesting this experiment.

Regarding evidence for direct binding, the reviewer is correct we do not test for this, as we further elaborate in this response letter on lines 76–82.

3)The authors claim that Plp protein levels are reduced by 50% in embryos from orbF343/orbmel mothers is based on quantifying the levels of the largest isoform of Orb protein on western blots, but does not take into account the effects on the other two major isoforms, which are more abundant in some lanes. While there probably is an effect on Plp levels, no evidence is provided to show that this is a direct effect of reduced Orb levels on Plp translation and not an indirect effect of changes in one of the many other Orb targets that have been reported. The authors' case would be strengthened by showing that mutations in the putative Orb binding sites in plp mRNA has a similar effect on Plp protein levels.

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We value the suggestion to examine levels of the other major isoforms detected on the PLP immunoblots. First, to confirm which bands are PLP isoforms, we generated null *plp* germline clone embryos and ran a western blot using these plus control extracts (1–2 hr) and probed the blot with anti-PLP antibodies. Our results are presented in a new Figure S3A and show the lower band (closest to ~250 kDa) is present in the *plp* null sample. We conclude that band is non-specific. In contrast, the upper-MW and mid-MW bands are not present in the *plp* null extracts; we conclude those are true PLP products. Next, to address the reviewer's point, we quantified relative amounts of the mid-MW PLP product. The revised Figure 4 shows two quantifications. In Fig 4E, we show the upper-MW product (as in the original submission, but more clearly labeled as such). In Fig 4F, we show the mid-MW product quantification. Similar trends of reduced PLP protein levels were observed, which we also note in the main text on lines 218–37.

Regarding the idea other Orb targets may influence PLP levels, we are careful to propose our results are "consistent with a model" where Orb regulates PLP translation. Indeed, this motivated our analysis of the CPSF binding site and *plp* polyadenylation status. We agree identifying specific CPE sites important for translational regulation is an interesting avenue for future work. In our revised discussion, on line 499, we we also added text indicating Orb may regulate other mRNAs.

4)Figure 4 does not provide convincing evidence that the poly-A tail length is reduced in orbF343/orbmel embryos. The authors focus on lanes 9 and 10 but do not explain what the lower band and smear below 293bp are. They then analyse the part of the gel above 293bp and conclude that the poly-A tail length is reduced, although the only clear difference between the wild-type and mutant samples is the strength of the band at ~300bp. Given that this gel has spurious background bands, how do they know that this band represents the short isoform of plp mRNA. More worryingly, they fail to mention the data in lanes 11 and 12, which seem more clear cut because the Bmr1 digestion has reduced the size of the plp signal to the region of the gel where the size resolution is better. In these lanes, there appears to be no difference between the wild-type and mutant samples.

In this experiment, lanes 9 and 10 show the undigested PAT products; a line scan is provided in Fig 5D to show the band intensity profiles, which are standard for the field. We add new text on line 284–5 explaining the non-specific signals observed in all PAT lanes: "Based on the position of our plpRev1 primer, products below 293 bp (i.e., below 0 nt poly(A) length for the short 3'UTR) likely represent non-specific bands; these were detected in all PAT samples (Fig 5B, lanes 9–14)."

We remain confident we are detecting *plp* polyadenylation products, as we included restriction enzyme digestion controls. If the PAT products at the top of the gel in lanes 9 and 10 were themselves non-specific, they would not be expected to be BmrI sensitive. Our data in lanes 11 and 12 confirm the specificity of the PAT products as *plp* products. Further, we argue that the majority of PAT bands are from the *plp* isoforms contain the short 3'UTR for three reasons: 1) We used the long-3'UTR-specific enzyme, EcoRI to digest the PAT products (lanes 13 and 14) and found they are not EcoRI-sensitive, indicating the majority of PAT products are from the short 3'UTR; 2) We performed isoform-specific qRT-PCR and found plp^{RM} (the only variant utilizing the long 3'-UTR) represents only about 5% of total *plp* mRNA, which also argues most PAT products would have the short 3'UTR (Figure 5C). These data independently verify the published modENCODE transcriptomics data. 3) Finally, in the revised text, we now include additional line profiles of the EcoR1-digested PAT products (dashed lines in Fig 5D). Because EcoR1 is specific to the long isoform, those products that remain following EcoR1 digestion should have the short *plp* 3'UTR. Our line scans show a similar leftward shift as the undigested PAT lanes. These results are described on lines 293–5 and argue the short 3'UTR is subject to reduced polyadenylation in *orb* depleted samples. We expect the left shift may be even more pronounced in the null background. Unfortunately, *orb* null embryos cannot be recovered.

5)The mitotic defects in embryos derived from orb mutant mothers are quantified on a per embryo basis, when they should measure the proportion of spindles with lagging chromosomes. By using embryos as the denominator, they are hugely amplifying what is a rather weak effect, because each embryo that is scored as containing a spindle with a lagging chromosome, presumably has thousands of spindles that are normal.

We now also include a per embryo quantification of CIN in a new Figure 7C. These results, described in new text beginning on line 369, are consistent with our prior analysis of the frequency of CIN across a population of embryos.

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Minor points:

1) The scale bar in Fig1 F is not defined.

All scale bars are now defined in the figure legends.

2) The plots in Figure 1G are meaningless, because the smFISH signal corresponds to single molecules of plp mRNA and the intensity distribution is therefore that of a diffraction limited spot.
Our intent was to use the line scan to show proximity of RNA and protein; however, we now omit Figure 1G.

3) Figure S1B shows the DNA sequence whereas the text refers to the RNA sequence.
We revised the sequence alignments by replacing T with U, now displayed in revised Figure 2.

4)It is not stated whether the Orb-GFP protein trap flies used for the pull-downs are homozygous or heterozygous, although Figure S2B suggests that the females are heterozygous. Is this because the line is homozygous lethal, and if so, how do they know that the trapped protein is functional?
The reviewer is correct the Orb-GFP protein trap is heterozygous. We did not directly test if the GFP-trap line is homozygous lethal. However, we do include a positive control in our RNA-immunoprecipitation experiments, which show GFP-Orb pulls down *orb* mRNA, as previously described. Further, a negative control mRNA (*gapdh*) is not pulled down. These results suggest the tagged protein is at least partially functional, as noted on revised lines 198–202.

5)"Consistent with a requirement for orb in PLP localization, we noted a significant reduction (~40% less than WT; p<0.001 by one-way ANOVA) in recombinant plp/+, orb mutants (Figure 5A,B), suggesting PLP dosage at centrosomes is regulated by orb activity." Any conclusions about the function of Orb should be based upon comparing plp/+, orb with plp/+, not with wild-type.

We revised the analysis and corresponding text accordingly.

6)What is the y-axis in Fig 6D?

We revised Figure 7E (former Fig 6D).

7)The references are listed in order of citation but are not numbered.

For the revised text, we use the Harvard output style and arranged it alphabetically, as recommended under the 'for authors' section of the *Development* webpage. We will also work with the *Development* production team to make sure any formatting issues are resolved.

Reviewer 2 *Advance Summary and Potential Significance to Field:*
In the manuscript entitled "Orb-dependent polyadenylation contributes to PLP expression and centrosome scaffold assembly", Fang and Lerit provide convincing evidence that the RNA binding protein Orb regulates PLP expression post-transcriptionally. This work contributes to the field of Developmental Biology and RNA biology by describing evidence for the post-transcriptional regulation of centrosomally localized RNAs, an area that has been historically understudied. The manuscript is appropriately suited to the audience of development. It is well written and the experiments are done with rigor, using both biochemical and genetic approaches. There are a handful of concerns that are easily addressed that would make the manuscript suitable for publication.

We appreciate the overall enthusiasm and positive comments from the reviewer – thank you.

Reviewer 2 Comments for the Author:
Major comments:

"Next, we aligned the plp 3'-UTR across multiple Drosophila species using the conservation insect track on the UCSC genome browser and found these CPE motifs were conserved across millions of years of evolutionary 168 distance (data not shown)(Kent et al., 2002)."

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This data should be shown and alignments between fly and human should be shown within the main text, instead of as supplemental data.

These data are now presented in revised Figure 2.

In Figure 3, the authors should quantify each of the PLP isoforms. It is not clear why they chose to only quantify one isoform (and which one this is). It remains possible that only expression of specific PLP isoforms is affected. Or in contrast it is possible that the overall expression (when taking into account all protein isoforms) remains unchanged.

Thank you for this suggestion, which was also requested by Reviewer 1. We now include quantification of the upper and mid-MW bands (please see our full response on lines 164–74 of this letter, above). We did not quantify the lower band, as immunoblotting controls versus null *plp* germline clones indicates this is in fact a non-specific band (new Fig S3A). Results from this work are presented in revised Figure 4 E & F and show Orb similarly downregulates both the upper and mid-MW PLP bands. Addressing the identity of the isoforms is more challenging. As we describe in our revised text (e.g., line 226), many of the 12 PLP isoforms are predicted to migrate near the same MW. However, given this information, we do know which PLP isoforms are likely to co-migrate in the upper versus mid-MW bands, and we now list these in the revised text (lines 225–7 and lines 236–7). We concur investigating individual isoforms would be an exciting line of future investigation.

Furthermore, what are the differences in these 12 protein isoforms? Could they possibly have different functions? Is there any evidence that only a specific isoform is localized to the centrosome?

We would love to know the answers to these questions! At present, the answers are unknown. We include revised discussion on line 450: "Whether the various PLP isoforms have different functions or regulatory paradigms is an interesting topic for future study."

Embryonic viability using hatch rate is not ideal. There are multiple reasons that embryos do not hatch, including embryonic lethality and unsuccessful fertilization of eggs. Since orb is a critical regulator of oogenesis and embryogenesis, the authors should rule out the presence of unfertilized eggs by performing DAPI stain on aged embryos. This will also be useful to determine the stage at which they die, in addition to knowing if they are unfertilized. Otherwise, they cannot conclude that this is a result of embryonic lethality.

The reviewer is correct unhatched embryos represent a population of dead plus unfertilized embryos. In prior work, we attempted to incorporate DAPI staining to our protocol by harvesting unhatched embryos and staining them; however, we invariably lost several embryos throughout the process.

In this study, we also examined numerous slides of *orb* mutant embryos to visualize nuclei and centrosomes, for example. During these studies, we did not note an apparent elevated rate of unfertilized embryos. Thus, we do not believe unfertilized embryos overrepresent the unhatched population. In our revised text, we now add the following information to our hatch rate Methods (lines 547–52): "Unhatched embryos were counted from each plate as a proxy for embryonic lethality, and three independent replicates were performed. Although not directly quantified, we did not observe elevated rates of unfertilized embryos in our *orb* mutant samples during centrosome structure or mitotic fidelity analyses." In the main text, we also acknowledge on line 401, "We conducted hatch-rate analysis to approximate embryonic viability in *orb* mutants versus controls."

Finally, although certain statistical analyses are not significant, it is important to state the actual p values either in the text of the manuscript or in the figure descriptions for the sake of accuracy and transparency.

Our revised figures now include the p-values over the "n.s." symbol.

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Minor comment:
In the introduction, it is not accurate to say that PCNT dysregulation results in Trisomy 21. While PCNT may be affected in Trisomy 21 is there evidence that PCNT dysregulation alone recapitulates the phenotypes of Trisomy 21?

In the revised introduction, we specify on line 55 elevated levels of PCNT underly the ciliary defects in Trisomy-21 derived fibroblasts.

Reviewer 3 *Advance Summary and Potential Significance to Field:*
This interesting paper explores the role of Drosophila Orb (not Orb2) in Pericentrin (PCNT)-like protein (PLP) function in centrosomes. They identified a specific biochemical association between Orb and plp mRNA and showed that Orb promotes polyadenylation of the short form of plp mRNA, containing a shorter 3'UTR. They show that Orb is required for correct Plp protein levels through translational regulation in centrosomes and pericentriolar organisation and genome stability.

These are important findings of great general interest suitable for Development and interesting to its readers. Localised production of centrosome components is an emerging theme of great interest to the centrosome field.

We appreciate the overall enthusiasm and positive comments from the reviewer – thank you.

Reviewer 3 Comments for the Author:
I only have one specific major issue that I think should be addressed in a revision before the manuscript can be accepted for publication.

In the manuscript the authors state that plp mRNA is localised to the centrosome. e.g. in line 107: "...plp mRNA localization to centrosomes...". When I look at the images presented in Fig1. plp mRNA is not particularly convincingly localised in centrosomes. The quality of the smFISH images is not in question, they are outstanding. But I am referring to the interpretation of the distribution of the single molecules of mRNA relative to the centrosomes labelled by GFP-gamma-Tubulin. For example, if you look at panel A, there is plp mRNA evident around one of the centrosomes that is highlighted, but the other centrosome has only one plp RNA molecules co-localised with it. Given that plp mRNA is also distributed all over the cytoplasm, as is very evident in these images, it is not really clear whether plp is enriched at centrosomes and present everywhere too at lower levels, or in fact just everywhere and in a few centrosomes enriched by chance. This problem is compounded by the fact that the control mRNA used in Fig. 1C (GAPDH) is so abundant that it too is present in the centrosomes shown, perhaps by chance. This issue should be resolved definitively by the authors if they are to make the statement that plp mRNA is localised. There are many ways they could do this by image analysis and some statistics.

The authors have attempted to do this in Fig. 1E by plotting the % RNA present within <1micron to centrosomes for plp and gaped mRNA. I do not find this indirect measure very convincing at all, and a much more direct metric for localisation is needed in my view.

Some suggestions are:
A) Make an identical image of centrosomes without mRNA. paint in the same number of mRNAs but in randomly chosen coordinates. Then compare how many mRNAs are localised near the centrosomes (with a specific cutoff of distance) in the experiment verses the randomly distributed single mRNA spot controls. This is similar to a traditional way that co-localisation of gold spots with organelles is assessed by electron microscopists.

B) There are more complex and sophisticated alternative methods such as K mean clustering (distribution of distances between spots) or Montecarlo statistical analysis that could also be used. But I think the suggestion in 1, will not take long to do with common image analysis tools.

To address an earlier comment by R1, we revised our measurements to display data relative to 0 mm from the centrosome surface – a most conservative measure. Strikingly, limiting our analysis to only those pixels of RNA overlapping the centrosome surface maintains a significant enrichment of *plp* mRNA at centrosomes throughout syncytial stages.

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The suggestion to control for overlapping signals based on chance is a good one. In the revised manuscript, we include a new Figure 1F to address this point. Here, we took all NC 13 images showing either *plp* or *gapdh* mRNA in control embryos. Then, using our Python code, we compared the RNA enrichment at centrosomes (0 mm distance away; RNA overlapping the centrosome surface) of *plp* and *gapdh* mRNA before versus after rotating the (red) RNA channel from the raw images by 90˚. This test allows us to determine if localization changes following rotation of the RNA channel – if the localization remains the same, we conclude the enrichment was spurious and by chance. By contrast, a significant drop in localization would argue the enrichment is specific. Figure 1F shows the percentage of *plp* mRNA at the centrosome is significantly decreased after rotating the RNA channel. Indeed, upon rotation, *plp* becomes (de-)localized to a similar extent as dispersed *gapdh* mRNA. In contrast, relative enrichments of *gapdh* mRNA remain unchanged upon rotating the RNA channel. We conclude *plp* mRNA does significantly localize to centrosomes. This experiment also demonstrates the non-specific distribution of the highly expressed *gapdh* mRNA. We thank the reviewer for their suggestion.

Minor issues to correct:

1) There is a minor problem with the PDF, which may not be due to the authors. Some of the text is thin and some fatter. I believe it is because the pdf is made of an image of the text (it cannot be infinitely zoomed into for example), rather than a normal pdf where the text is infinitely zoomable. This seems very minor, but is a problem, as one cannot zoom into the images in the figures to look at them at sufficient enlargement.

We regret this issue, which likely resulted from compression of the .pdf. We will attempt to preserve the quality of the .pdf and also include a supplemental text file of a black-and-white document of the entire text.

2) there is some kind of corruption of the formatting of some references - where instructions for the formatting of the text are present as text characters *Drosophila* for example on line 1170. Again, this maybe a problem with the journal site, not the authors files, but there are at least 7 instances of this error.

We regret the references were not displaying correctly. For the revised text, we use the Harvard output style, as recommended under the 'for authors' section of the *Development* webpage. We will also work with the *Development* production and proofing teams to make sure any formatting issues are resolved.

We once again thank the reviewers for their time and these thoughtful comments and suggestions. We appreciate the opportunity to improve the quality of our manuscript. We hope the revised manuscript is now suitable for publication in *Development*.

Second decision letter

MS ID#: DEVELOP/2021/200426

MS TITLE: Orb-dependent polyadenylation contributes to PLP expression and centrosome scaffold assembly

AUTHORS: Junnan Fang and Dorothy A. Lerit
ARTICLE TYPE: Research Article

I apologize for the delay before being able to come back to you with a decision. I have looked at the response from reviewers, two of whom support strongly publication, though one is less enthusiastic, and in light of all the comments I am happy to tell you that your manuscript has been accepted for publication in Development, pending our standard ethics checks.

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Reviewer 1

Advance summary and potential significance to field

See previous review

Comments for the author

The revised version of this manuscript by Fang and Lerit has improved in several ways. They now measure plp mRNA localisation at the centrosome rather than around the centrosome and have more accurately quantified the effect of orb mutants on Plp expression. However, I remain unconvinced that the PAT assay shows a clear shortening of the plp mRNA poly-A tail in orb mutants. The effect of orb mutants on Plp expression and the centrosomal size and chromosomal instability phenotypes are also not very striking. Although the analysis of the data is generally good, I feel that the authors are pushing too hard to make a mild phenotype seem significant and am sceptical about whether the manuscript is appropriate for Development.
1) The authors have now quantified three sets of PAT assays treated with EcoR1, which cuts the products from the long plp isoform, but they continue to ignore the lanes treated with Bmr1, which should cut the short isoform and give smaller poly-adenylated products that are better resolved by the gel. These lanes appear to show no obvious effect of orb mutants on plp mRNA poly-A tail length.
2) The improved quantification of Plp protein isoforms shows that the orb mutant combination used reduced Plp levels by 50%. Although this is not a null condition, it is still a relatively weak effect, and the partially penetrant mitotic defects observed in cleavage stage embryos are consistent with this result. These are only partially rescued by Plp-GFP, so the effect of orb on Plp levels is even weaker. Have they compared these phenotypes with embryos from heterozygous plp mutant mothers, which should show a similar reduction in protein levels?

Reviewer 2

Advance summary and potential significance to field

The revised manuscript has satisified all of my initial concerns and is now ready for publication in Development.

Comments for the author

None

Reviewer 3

Advance summary and potential significance to field

Many thanks for tackling the issues I had with the manuscript so well. I find Fig. 1F (rotation of the image for

both localised and control unlocalised) very convincing. I also find the other responses to my comments and

those of the other referee comments very convincing.

Comments for the author

no suggestions

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