The 3′UTR of the *Drosophila* CPEB translation factor gene *orb2* plays a crucial role in spermatogenesis

Rudolf Gilmutdinov, Eugene N. Kozlov, Konstantin V. Yakovlev, Ludmila V. Olenina, Alexei A. Kotov, Justinn Barr, Mariya Zhukova, Paul Schedl and Yulii Shidlovskii
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Original submission

First decision letter

MS ID#: DEVELOP/2020/198788

MS TITLE: The 3’UTR of the orb2 gene encoding the Drosophila CPEB translation factor plays a critical role in spermatogenesis

AUTHORS: Rudolf A Gilmutdinov, Eugene N Kozlov, Ludmila V Olenina, Alexei A Kotov, Justinn Barr, Mariya V Zhukova, Paul Schedl, and Yulii Shidlovskii

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, the referees express considerable interest in your work, but have some significant criticisms and recommend a substantial 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.

Reviewer 1

Advance summary and potential significance to field

Gilmutdinov and colleagues report a characterization of the CPE motif-rich 3'UTR of Orb2. As a CPEB protein Orb2 has the ability to control the translation of many targets, including itself, through binding to CPE motif containing mRNAs. Interestingly, the five isoforms of Orb2 have drastically differing numbers of CPE motifs ranging from 0 to 37. Using CRISPR mutagenesis, they created a mutant that lacks most of the CPE motifs as well as the polyadenylation sequences of three of the five RNA isoforms. They show that this mutant is viable but mostly sterile, likely due to mislocalization of mRNA and protein during spermatid elongation, along with a few other proteins controlled by Orb2 that localize to the elongating spermatids, leading to spermiogenesis defects. This mutant provides an intriguing tool to investigate how spermatid elongation is correctly polarized through mRNA localization. Overall, this is a well-conducted study that provides important insights into the molecular mechanisms of mRNA localization (and likely localized translation) during cell morphogenesis. I have a few minor comments that need to be addressed prior to publication.

Comments for the author

- The manuscript is unnecessarily wordy in places to the point that will distract many readers. For example around line 160, they first explain overall reduction of fertility in orb2R mutant, then develop a logic asking whether all males have reduced fertility or there is a mixture of males that are near-wt fertile and others that are completely sterile. Nothing is wrong with the logic, but such detailed discussion and argument seems unnecessary and does not add to the manuscript (after all, they reveal that orb2R mutant males are mixture of completely sterile males and subfertile males, and it is more like everyone's fertility is reduced with varying extents). Can they go through the manuscript to remove this kind of unnecessarily lengthy writing?

-similarly, starting line 190, their explanation of moderate mRNA/protein reduction not being able to explain orb2R's sterility is again unnecessarily lengthy. Especially given that their motivation of generating 3'UTR mutant (orb2R) must have been to see its possible effect on localization, the explanation starting line 190 feels really excessive (they go through expansive 'detective work' as if mislocalization of orb2R mutant transcript was a surprise).

Reviewer 2

Advance summary and potential significance to field

The manuscript of Rudolf A. Gilmutdinov and coauthors characterizes the role of the Orb2 protein, a CPEB protein, in Drosophila spermatogenesis. The authors report that deletion of the greater portion of the 3'UTR of orb2 transcript disrupts spermatid differentiation causing defects in the polarization of the 64-cell spermatid cysts, altered morphology of the elongating spermatid tails, and failure to assemble the actin-rich individualization complex. Moreover the authors demonstrate that orb2 mRNAs and proteins fail to correctly localize within the 64-cell spermatid cyst.

Comments for the author

Most of the observations in the manuscript are novel and can be suitable for publication in Development.

The authors need to address the following points in the revised manuscript.
1) The authors should demonstrate that Orb2 protein can bind the greater portion of the 3'UTR of orb2 transcript (by RNA pull-down from testes or other approaches)

2) A more accurate documentation of male meiotic divisions in orb2R mutants is necessary.
3) The differentiation defects are intriguing. It would be important to identify spermiogenesis specific-mRNA that depend on Orb2 protein for localization and translation. This would link the differentiation defects to specific proteins.

Minor points
The following sentences are not clear and should be reformulated:
1) Line 193: “This finding suggests that the deletion mRNAs are less stable. “
2) Line 204: “These findings indicate that the reduction in orb2 mRNA and protein in homozygous orb2R testes is if anything less than that in orb236 heterozygotes, while the fertility of these flies is significantly different. “

Reviewer 3

Advance summary and potential significance to field

Orb2 is a translational regulator of the CPEB protein family that has previously been found to regulate meiosis and spermatid differentiation during spermatogenesis in Drosophila. Here, the authors investigate the function of the 3’UTR of the orb2 transcript, which contains multiple CPE motifs, suggesting that it regulates itself. Such auto-regulation has been previously shown by the authors for Orb in oogenesis. The authors used CRISPR to delete the region of the endogenous orb2 gene that generates the 3’UTR of several transcripts encoding the larger protein isoform of Orb2. The authors then discovered that this 3’UTR is important for the polarized distribution of the Orb2 protein and other proteins during late stages of spermatogenesis, which disrupts spermatid differentiation and leads to severely reduced fertility.

Comments for the author

The molecular and phenotypic data are carefully presented and support the conclusions of the authors.
The manuscript is clearly but not concisely written, and often repetitive. The text could be strongly shortened.
This is a solid piece of research but I believe that the manuscript does not provide sufficient conceptual progress to be suitable for Development.

Minor points:
- Actual breakpoints of the deletion should be determined and described.
- I am concerned about the fertility assay. If this was done at 25°C, after 21 days (7+14) the authors most likely looked not only at the F1 but also at the F2 generation, at least in the wt control.
- Replace stock center numbers with actual genotypes in M&M.
- The authors sometimes use the term ‘distal’, which is confusing. I suggest to consistently use the term ‘basal’ (which I assume the authors refer to).
- Lines 248-249; sentence sounds contradictory to me. Please, check.
- There are small grammar errors here and there.
- Graphs in Fig4: change colour scheme as grey and purple are hard to distinguish.

First revision

Author response to reviewers' comments

Reviewer 1

- The manuscript is unnecessarily wordy in places to the point that will distract many readers.

We made the Results section more concise.

Reviewer 2

The authors need to address the following points in the revised manuscript.
1) The authors should demonstrate that Orb2 protein can bind the greater portion of the 3'UTR of orb2 transcript (by RNA pull-down from testes or other approaches)
Experiments of this type have already been done by us and others. As shown in our previous study (Xu et al., 2014), Orb2 binds to orb2 mRNAs in testis extracts. We have also found that a mutant version of the Orb2 protein, Orb2deltaQ, binds to the orb2 mRNAs in testis extracts. Similar results were obtained in tissue culture cells by Stepien et al. (2016) using a completely different procedure for identifying mRNAs bound by Orb2 protein. These authors also identified multiple motifs—which are found in the orb2 3' UTRs—that serve as recognition sequences for Orb2 binding to its own mRNA and to many other mRNAs. We note that the remaining 3' UTR in the orb2R mRNA still contains five sequences that match the motifs identified by Stepien et al.

2) A more accurate documentation of male meiotic divisions in orb2R mutants is necessary.
This was a good suggestion. To address this question we probed testes for meiotic markers cyclin A and B (Figs 4C, 4D). Unlike with orb2 protein mutants (Xu et al., 2012) we detected a WT distribution of these proteins in orb2R. In addition to WT morphology observed during meiosis I and II, this would provide further support to what appears to be a complete normal meiotic progression.

3) The differentiation defects are intriguing. It would be important to identify spermiogenesis specific-mRNA that depend on Orb2 protein for localization and translation. This would link the differentiation defects to specific proteins.
Our results show that, in addition to orb2 mRNA and protein, orb mRNA and protein are mislocalized during spermatid tail elongation and that the Bol translation factor is not accumulated at the tip of elongating spermatid tails, unlike in wild type.
In other experiments we have found that the relative accumulation of yuri mRNA (encoding a factor important for spermatid cyst differentiation) is altered in orb2R testes. Thus, in addition to altering the spatial distribution of mRNAs and proteins in elongating spermatid tails, an imbalance in the relative abundance of proteins produced by some mRNAs may impact the differentiation process. In this context, there is evidence that an imbalance in the abundance of cytoskeletal proteins in dfmr1 mutant embryos is likely to contribute to some of the abnormalities observed during the early stages of embryogenesis (Deshpande et al., 2006).

The following sentences are not clear and should be reformulated:
1) Line 193: "This finding suggests that the deletion mRNAs are less stable. "
This sentence was removed.

2) Line 204: "These findings indicate that the reduction in orb2 mRNA and protein in homozygous orb2R testes is if anything less than that in orb236 heterozygotes, while the fertility of these flies is significantly different. "
This sentence was reformulated.

Reviewer 3 Advance summary and potential significance to field...

The manuscript is clearly but not concisely written, and often repetitive. The text could be strongly shortened.
We made the Results section more concise.

This is a solid piece of research but I believe that the manuscript does not provide sufficient conceptual progress to be suitable for Development.
Our paper provides a number of significant advances:
- The orb2 3'UTR is important for the proper functioning of Orb2 during spermatogenesis.
- The orb2 3' UTR deletion we generated differentially affects Orb2 activity.
i) Although Orb2 is required for meiosis, the 3' UTR deletion has no apparent effect on meiosis.
ii) The 3' UTR is critical for the spermatid differentiation.

- The deletion disrupts spermatid cyst polarization. Previous studies on the orb2 null allele, orb236, hinted that orb2 is required for the initial polarization of the spermatid cyst. However, this was only a hint. Like mutations in other genes that fail to undergo meiosis, orb236 cysts begin to differentiate. One of the first steps in sperm maturation is the polarization of the cysts. In orb236 cysts, polarization fails. The nuclei are scattered throughout the cyst instead of clustering on its basal side, and pseudo-flagellar axonemes appear to elongate in completely random orientations. We suspected this meant that orb2 was required for cyst polarization in WT, but this was at best a speculation, since orb236 failed to undergo meiosis. It was equally possible that 16-cell cysts can polarize properly for reasons unrelated to the lack of orb2 function when the cysts begin differentiating. The results presented in this manuscript directly implicate orb2 in cyst polarization. In addition, they argue that the 3' UTR is required for orb2 function in this process.
--Spermatid tail elongation is abnormal in orb2R. Defects include abnormal localization of proteins and mRNAs (Bol, orb2 mRNA, Orb2 protein, orb mRNA, Orb protein) that normally accumulate in a band just below the tip of the elongating tail. In in orb2R, however, these proteins and mRNAs accumulate closer the basal end of the tail. Instead of having a uniform thickness and ending in a bulb, the tail is tapered at the end, suggesting that the 64 flagellar axonemes do not elongate at an uniform rate as they do in WT. These results implicate orb2 in the process of tail elongation. Moreover, they show that the orb2 function in spermatid tail elongation requires the 3' UTR of the orb2 mRNA.
- Accumulation of mRNA that encodes a factor important for the initial polarization of the spermatid cyst (yuri) is altered in orb2R testes.
- The 3' UTR is also required for the individualization process, together with the accumulation of mRNAs.
- While we have not directly demonstrated that orb2 mRNA localization/translation during spermatid differentiation depends upon a 3' UTR-dependent orb2 autoregulatory activity, our results provide strong support for this idea.

Minor points:

- Actual breakpoints of the deletion should be determined and described.
Exact positions of Cas9 cut are given in Supplement

- I am concerned about the fertility assay. If this was done at 25°C, after 21 days (7+14) the authors most likely looked not only at the F1 but also at the F2 generation, at least in the wt control.
The description of the fertility assays was incorrectly phrased. It has been corrected.

- Replace stock center numbers with actual genotypes in M&M.
Genotypes were indicated.

- The authors sometimes use the term 'distal', which is confusing. I suggest to consistently use the term 'basal' (which I assume the authors refer to).
The term was replaced.

- Lines 248-249; sentence sounds contradictory to me. Please, check.
The sentence was changed.

- There are small grammar errors here and there.
The errors were corrected.

- Graphs in Fig4: change colour scheme as grey and purple are hard to distinguish.
Colors were corrected to provide higher contrast.

Second decision letter

MS ID#: DEVELOP/2020/198788

MS TITLE: The 3’UTR of the orb2 gene encoding the Drosophila CPEB translation factor plays a critical role in spermatogenesis

AUTHORS: Rudolf A Gilmutdinov, Eugene N Kozlov, Ludmila V Olenina, Alexei A Kotov, Justinn Barr, Mariya V Zhukova, Paul Schedl, and Yulii Shidlovskii

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.

The overall evaluation is positive and we would like to publish a revised manuscript in Development, provided that the referees' comments can be satisfactorily addressed. Please attend to all of the reviewers' comments in your revised manuscript and detail them in your point-by-point response. Please consider seriously the concerns of Reviewer #3 regarding a number of potential alternative interpretations for some of the observations you report, and the conceptual and mechanistic overlap with some of your previous work - please consider revising the manuscript to make it very clear to the reader the things that distinguish this work from previous work, and to acknowledge the potential for alternative explanations for the data where appropriate. If you do not agree with any of their criticisms or suggestions explain clearly why this is so.

We are aware that you may currently be unable to access the lab to undertake experimental revisions. 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.

Reviewer 1

Advance summary and potential significance to field

Gilmutdinov and colleagues report a characterization of the CPE motif-rich 3’UTR of Orb2. As a CPEB protein, Orb2 has the ability to control the translation of many targets, including itself, through binding to CPE motif containing mRNAs. Interestingly, the five isoforms of Orb2 have drastically differing numbers of CPE motifs ranging from 0 to 37.
Using CRISPR mutagenesis, they created a mutant that lacks most of the CPE motifs as well as the polyadenylation sequences of three of the five RNA isoforms. They show that this mutant is viable but mostly sterile, likely due to mislocalization of mRNA and protein during spermatid elongation, along with a few other proteins controlled by Orb2 that localize to the elongating spermatids, leading to spermiogenesis defects. This mutant provides an intriguing tool to investigate how spermatid elongation is correctly polarized through mRNA localization. Overall, this is a well-conducted study that provides important insights into the molecular mechanisms of mRNA localization (and likely localized translation) during cell morphogenesis. I have a few minor comments that need to be addressed prior to publication.

Comments for the author

The authors have addressed major concerns raised previously by reviewers. This is a nice piece of work demonstrating the role of orb2 3’UTR, revealing their spermatogenesis specific function, and contribute to our understanding of cyst polarization during spermatogenesis.
There are a few minor issues to be taken care of.
Fig2 is a bit confusing. Not sure why they have to separate fertile vs. sterile males, and only record fertile males’ progeny #. Isn’t it simple to merge all data into Fig2B (and sterile males can be plotted as ‘zero’ for their progeny number).
Line 273: subheading ‘Individualization complex was not properly assembled in mutants’ ==> which mutant? (please be specific, even though one can tell which mutant by the context)

Reviewer 2

Advance summary and potential significance to field

Rudolf A. Gilmutdinov and coauthors characterize the role of the Orb2 protein, a CPEB protein, in Drosophila spermatogenesis. The authors report that deletion of the greater portion of the 3'UTR of orb2 transcript disrupts spermatid differentiation causing defects in the polarization of the 64-cell spermatid cysts, altered morphology of the elongating spermatid tails, and failure to assemble the actin-rich individualization complex. Moreover the authors demonstrate that orb2 mRNAs and proteins fail to correctly localize within the 64-cell spermatid cyst.

Comments for the author

The revised version of the paper is now suitable for publication in Development.

Reviewer 3

Advance summary and potential significance to field

The authors generated a new orb2 mutation (R) that deletes most but not all of the CPE motifs of the 3'UTR. R mutants have strongly reduced male fertility.
Defects in spermatogenesis in R homozygous mutants are compared to testes having R in trans to a null mutation (R/36). Consistently, stronger defects were found with R/36 than with R/R, suggesting a normal phenotypic series. The phenotypic analysis revealed defects in spermatid differentiation, including defects in cyst polarization (scattered cell nuclei and abnormal distribution of orb2, orb and boule transcripts/proteins), and defects in tail elongation, and sperm individualization.

Although the manuscript is now much more succinct, I am not convinced that the manuscript contains sufficient novelty for publication in Development. The phenotypes described here have already been described by Dr. Schedl's group in Plos Genetics in 2012 and 2014. Already those publications distinguish between an earlier function during meiosis and a later function in spermatid differentiation (cyst polarization, spermatid elongation) with the help of different alleles, and show the mislocalization of molecules during spermatid elongation. These publications already show the autoregulation of orb2. In addition, those publications have in part higher resolution images, and very nice and helpful schematic drawings.

The manuscript here aims towards showing that the 3'UTR of orb2 is important for cyst polarization and the normal distribution of orb2 and other factors during spermatid differentiation. Although this is a reasonable and interesting hypothesis, the manuscript falls short of showing this. The manuscript only shows that removal of the 3'UTR leads to reduced mRNA/protein levels and is associated with a mutant phenotype.

Comments for the author

Here are my concerns:
Much of the argumentation is based on the finding that R/R produces more orb2 transcript/protein than the heterozygous null (36/+) but shows substantially lower male fertility. However, the authors did not rule out the possibility that the increased sterility is due to a second-site mutation on the R chromosome. The best way to address this issue would be to use two independent 3'UTR deletion mutations in trans.
Second, the data show that R/R has spermatogenesis defects and mislocalization of RNAs and proteins, and the authors seem to assume that the heterozygous null does not have any of those defects but they do not show this. It would be important to analyze the phenotype of 36/+ testes.
Third, it remains unclear whether the mislocalization of molecules, which the authors hypothesize to be a result of the lack of the orb2 3'UTR is the cause or the effect of abnormal cyst polarization and tail elongation.
I believe it would be important to conduct rescue experiments, using an orb2 cDNA transgenic construct containing all CPEs and a construct without any CPEs to test how much each of them can

rescue male sterility, mislocalization of molecules and spermatogenesis defects in R/R and R/36 or null/null males.
The manuscript would benefit from schematic drawings.
Minor points:
- The authors still only provide the hypothetical/expected breakpoints in Supplemental. Please, provide the actual breakpoints of the deletion based on sequencing.
- Please, use always the terms 'apical' and basal' -> remove 'proximal' in figure 7 legend.
- Fig 2A legend: mentions twice orb2[R]/+; should this not be orb2[36]/+ ?
- M&M: 3 day males -> change to '3 day-old males' throughout M&M.
- fix °C signs in M&M
- Exchange all 'portions' in M&M. For example, change to: ... was washed three times with PBST.
- line 457. The level of the transcripts -> The amount of the transcripts
- Fig 3B: change 20ug to: 20 µg
(make similar change for the other lanes)
- Fig 5: indicate location of the measured areas 1-3 in the images (panels A B). Although this information is provided in Supplemental it is important to show this in relation to the actual stainings.
- Figures: I am not always certain about the orientation of the testes in some of the images, especially in Figure 4. Please, show testes in the same orientation in all figures and describe the orientation in the figure legends.
- line 179 and 185: Figure 4 (not Figure 3!)
- I might have missed this but did not notice a description of the unspliced orb2 transcripts (shown in Fig 3A) in the main text.
- line 88: 'spermatid' -> spermatids
- line 162: ', as was true for orb2R mRNA' -> similar to orb2R mRNA
- line 283: 'contain no functional sperm': was the sperm tested ? - otherwise delete 'functional'
- lines 286-294: several typos/grammar errors

Second revision

Author response to reviewers' comments

Reviewer 1
Fig2 is a bit confusing. Not sure why they have to separate fertile vs. sterile males, and only record fertile males' progeny #. Isn't it simple to merge all data into Fig2B (and sterile males can be plotted as 'zero' for their progeny number).
The experiment in Fig 2A presents different information from that in 2B. It shows the number of males that are fertile. Note, for example, that all orb236/+ males produce progeny, while only 25% of the orb2R males have progeny. By contrast, as shown in a subsequent Fig., there is a greater reduction in the levels of orb2 mRNA and protein in orb236/+ than in orb2R. It also shows that orb2R and orb236 do not complement each other (see reviewer 3) and that as expected for a hypomorph over a null, the phenotype is enhanced. Fig 2B shows that the few orb2R males that are fertile produce far fewer progeny than either WT or orb236/+ males. Moreover, the average number of progeny produced by orb236/+ males is not too much different from WT.

Line 273: subheading 'Individualization complex was not properly assembled in mutants' ==> which mutant? (please be specific, even though one can tell which mutant by the context)
The title was corrected.

Reviewer 3
Although the manuscript is now much more succinct, I am not convinced that the manuscript contains sufficient novelty for publication in Development. The phenotypes described here have already been described by Dr. Schedl's group in Plos Genetics in 2012 and 2014. Already those publications distinguish between an earlier function during meiosis and a later function in spermatid differentiation (cyst polarization, spermatid elongation) with the help of different

alleles, and show the mislocalization of molecules during spermatid elongation. These publications already show the autoregulation of orb2.
The present manuscript represents a significant advance over previous studies. The two earlier papers described phenotypes of a null allele and a deletion of polyQ domain, Orb2dQ. The null allele did not undergo meiosis, indicating that Orb2 protein is required for this step in spermatogenesis. By contrast, the polyQ domain of Orb2, and as we show here, the orb2 mRNA 3' UTR, are not required for meiosis. The orb2 null also exhibited a range of defects in spermatid differentiation, a process that only occurs in WT after meiosis is complete. While this observation raised the possibility that orb2 is required at different steps during differentiation, this was at best only a tentative suggestion as some or even all of the defects in differentiation could be the indirect consequence of failing to complete meiosis. This nagging problem was explicitly discussed in the discussion of Xu et al., 2012. Note also that differentiation defects in Orb2dQ were only rather modest.
The current paper provides much more direct evidence that orb2 is required after meiosis is finished for spermatid differentiation. Moreover, it implicates the orb2 3' UTR at potentially multiple steps in differentiation process. While 3'UTRs are known to have an important role in regulating gene activity (locally and/or temporally) during, for example, fly oogenesis and early embryogenesis, there are few if any papers documenting equivalents functions in fly spermatogenesis. Of the findings reported here, perhaps the most interesting are the defects in the initial polarization of the cyst. While this intriguing phenotype was observed in orb2 null testes, one's excitement was necessarily tempered because the cysts didn't enter into meiosis. For example, one could argue that proper cyst polarization can only take place after the completion of the 2nd meiotic division to form a 64 cell cyst.

In addition, those publications have in part higher resolution images, and very nice and helpful schematic drawings.
The current resolution of images corresponds to requirements of journal and is enough to see the described effects. One more schematic drawing was added to Fig. 4.

The manuscript here aims towards showing that the 3'UTR of orb2 is important for cyst polarization and the normal distribution of orb2 and other factors during spermatid differentiation. Although this is a reasonable and interesting hypothesis, the manuscript falls short of showing this. The manuscript only shows that removal of the 3'UTR leads to reduced mRNA/protein levels and is associated with a mutant phenotype.
It's not clear to us how the manuscript falls short of implicating the orb2 3'UTR in either spermatid differentiation or in the localization of orb2 mRNAs and proteins. Contrary to the reviewer's assertion, the reductions in mRNA and protein levels in orb2R do not account for the defects in spermatid differentiation. In particular, trans-heterozygotes for the orb2 null allele, orb236, and a WT orb2 allele exhibit even greater reductions in both orb2 mRNA and protein levels than orb2R homozygotes. However, all orb236/+ males are fertile, and the average number of progeny produced by these males is only marginally less than +/+ males. If the reviewer were actually correct in thinking that reductions in orb2 mRNA and protein in orb2R are the cause of the spermatid differentiation defects, then there should be an even greater reduction in male fertility in orb236/+ males than in orb2R homozygotes. Clearly the opposite is the case.

The authors did not rule out the possibility that the increased sterility is due to a second-site mutation on the R chromosome. The best way to address this issue would be to use two independent 3'UTR deletion mutations in trans.
In our CRISPR/Cas9 mutagenesis we recovered 5 independent lines. All 5 had similar phenotypes including: reduce male fertility, misorganized spermatid cysts, and partially filled seminal vesicles. Since all 5 were the same we kept one line for further study and discarded the remaining lines. Also given that all 5 lines had similar phenotypes, there was no reason to do complementation tests between the lines as they would not be expected to complement.
Also, as suggested by the reviewer, we have done complementation tests between orb2R and the orb2 null allele, orb236. Note that the orb236 deletion is derived from two fly stocks that are completely unrelated to the stock used for the CRISPR/Cas9 mutagenesis and it was one of several isolates which also had the same set of phenotypes.
As described for multiple phenotypes in the text, the orb2R lesion is not complemented by orb236. Moreover, as expected the spermatogenesis phenotypes observed for orb2R/orb236 are in all cases more severe and penetrant than those observed for orb2R/orb2R flies. This is classically what is

observed when hypomorphs are trans to null alleles in the same gene. In addition, the failure to complement indicates the phenotypes observed in orb2R are due to defects in the functioning of the orb2R gene and not some 2nd site mutation on the chromosome.

Second, the data show that R/R has spermatogenesis defects and mislocalization of RNAs and proteins, and the authors seem to assume that the heterozygous null does not have any of those defects but they do not show this. It would be important to analyze the phenotype of 36/+ testes.
We don't understand why the reviewer thinks that such an analysis would make a critical contribute to the studies reported here. This paper is about orb2R not flies heterozygous for an orb2 deletion. In addition, all orb236/+ males are fertile and produce nearly the same number of progeny as WT. Thus, there is no reason to think a) that orb2 is haploinsufficient, or b) that we would, with any frequently, observe obvious spermatogenesis phenotypes. Even if we were lucky and observed spermatogenesis phenotypes, this would add little to the current manuscript.

It remains unclear whether the mislocalization of molecules, which the authors hypothesize to be a result of the lack of the orb2 3'UTR is the cause or the effect of abnormal cyst polarization and tail elongation.
We used a number of markers to investigate i) cyst polarization, ii) the extension and organization of the spermatid tails, iii) the organization of spermatid nuclei, and iv) the IC. The fact that ßNACtes, Bol or orb mRNAs/proteins, etc are not properly localized indicates that these steps are defective in orb2R. However, we never suggested that the mislocalization of any these markers is the cause of abnormal cyst polarization, tail elongation defects, etc. Since Keleman's lab has shown that Orb2 protein interacts with hundreds of mRNAs, each of the phenotypes could be the cumulative result of defects in the expression of multiple targets. It is also possible that there are only a few targets that are important for, for example, cyst polarization.

I believe it would be important to conduct rescue experiments, using an orb2 cDNA transgenic construct containing all CPEs and a construct without any CPEs to test how much each of them can rescue male sterility, mislocalization of molecules and spermatogenesis defects in R/R and R/36 or null/null males.
Manipulations of this type are being considered for future studies.

The manuscript would benefit from schematic drawings.
As suggested by the reviewer, the manuscript now contains diagrams of orb2 locus (Fig 1) and spermatid cysts (Fig 5) and testes (Fig. 4).

-The authors still only provide the hypothetical/expected breakpoints in Supplemental. Please, provide the actual breakpoints of the deletion based on sequencing.
The sequence coincides completely with that expected from the design of the CASPR/Cas9 deletion. As mentioned in the text, the deletion is 4522 bp in length.

-Please, use always the terms 'apical' and basal' -> remove 'proximal' in figure 7 legend.
The term was substituted.

-Fig 2A legend: mentions twice orb2[R]/+; should this not be orb2[36]/+ ?
Corrected.

- M&M: 3 day males -> change to '3 day-old males' throughout M&M.
The phrase was corrected.

- fix °C signs in M&M
The symbol was replaced.

- Exchange all 'portions' in M&M. For example, change to: ... was washed three times with PBST.
The phrases were corrected.

- line 457. The level of the transcripts -> The amount of the transcripts
The phrase was corrected.

- Fig 3B: change 20ug to: 20 µg (make similar change for the other lanes)

The symbol was replaced.

- Fig 5: indicate location of the measured areas 1-3 in the images (panels A, B). Although this information is provided in Supplemental it is important to show this in relation to the actual stainings.
Figures were fused, now complete information about the experiments is given on Fig 5.

- Figures: I am not always certain about the orientation of the testes in some of the images, especially in Figure 4. Please, show testes in the same orientation in all figures and describe the orientation in the figure legends.
Orientation on Fig 4 is given. Other Figs contain indication of basal ends of cysts.

- line 179 and 185: Figure 4 (not Figure 3!)
Corrected

- I might have missed this but did not notice a description of the unspliced orb2 transcripts (shown in Fig 3A) in the main text.
Primers pair covers the last intron-exon junction, indicated in line 165

- line 88: 'spermatid' -> spermatids
Corrected

- line 162: ', as was true for orb2R mRNA' -> similar to orb2R mRNA
Corrected

- line 283: 'contain no functional sperm': was the sperm tested ? - otherwise, delete 'functional'
Corrected

- lines 286-294: several typos/grammar errors
Corrected

Third decision letter

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MS TITLE: The 3’UTR of the orb2 gene encoding the Drosophila CPEB translation factor plays a critical role in spermatogenesis

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