Mouse EWSR1 is crucial for spermatid post-meiotic transcription and spermiogenesis

Hui Tian and Petko Petkov
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MS TITLE: Mouse EWSR1 is critical for spermatid post-meiotic transcription and spermiogenesis

AUTHORS: Hui Tian and Petko Petkov

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 concerns and recommend a substantial revision of your manuscript. The reviewers provide excellent suggestions to improve the manuscript. 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.

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

Here Tian and Petkov report the characterization of the post-meiotic function of EWSR1 in differentiating male germ cells, following the generation of conditional ko of Ewsr1in late meiotic and post-meiotic cells.
They show that the post-meiotic activities of EWSR1 is a prerequisite to the generation of condensing spermatids. In particular, transcriptomic analyses demonstrate that EWSR1 is required for the expression of several genes mediating the process of histone-to-protamine replacement such as transition proteins (TPs) and protamines (PRMs). Indeed, the lack of TPs and PRMs observed by the authors in the absence of EWSR1 is sufficient here to explain the impairment of spermiogenesis during the genome compaction stages and the lack of condensed spermatids. Although the molecular basis of the EWSR1-dependent control of expression of a specific set of mRNAs is not unraveled here, this report remains of interest to investigators interested in the molecular basis of spermiogenesis and hence could be published.

Comments for the author

However, before publication, it is important that the authors pay attention to the following points.

1 – Because of the observed involvement of EWSR1 in the integrity of the chromocenter and heterochromatin organization, it would have been interesting to focus on meiotic sex chromosome inactivation (MSCI) phenomenon in spermatocytes and in round spermatids in the Ewsr1 cko cells. Indeed by specifically considering the expression of the sex chromosome linked gene expression, the authors could see if MSCI is fully operational in meiotic cells and whether the partial escape from MSCI in round spermatids is also occurring in the absence of EWSR1.

2 – There could be at least two reasons for the observed down-regulations of the reported late stage-specific genes expression, i. e., down-regulation of mRNAs encoding TPs and PRMs.
First, the lack of EWSR1 would specifically down-regulate these mRNA in the cells normally expressing them but in the same cells other stage-specific genes remain normally expressed.
Second, these cells die and their absence within the heterogenous cell population studied here would lead to an apparent down-regulation of the corresponding set of genes.
It is important that the authors try to consider this issue to highlight the specific relationship between EWSR1 and a particular set of mRNAs.
One way to consider this issue would be to use the transcriptome and compare the expression levels of all the known stage-specific genes together. An example of such an approach can be found in pmid 30257209 (Fig. S4 - related to Fig. 6). The approach is based on the principle that a change in the transcriptome due to cell loss should be associated with the downregulation of all the corresponding specific transcripts. If only some of the stag-specific transcripts are down regulated, while others remain unchanged, then it is possible to conclude that these changes are due to specific gene deregulations.

3 – The authors report the down-regulation of TPs and PRMs without considering the fact that recent investigations revealed that TPs' activity depends on the co-expression of a specific histone variant H2A.L.2, which is required for the invasion of nucleosomes by TPs, which themselves allow a controlled assembly of PRM (pmid: 28366643). Additionally, NUT and BRDT (see the reference cited in point "2") are also involved upstream of histone displacement, when a histone hyperacetylation occurs. BRDT is a multifunctional factor expressed at the initiation of meiotic differentiation, which has a role in meiosis itself as well as in post-meiotic cells, in the removal of acetylated histones (pmid: 22922464).
With respect to the point discussed in "2" and in order to better understand the role of EWSR1 in the coordination of genes that would be specifically involved in histone-to-PRM replacement, it would be very interesting if the authors would integrate the current knowledge and specifically consider this set of genes.
Among these genes the authors have already considered BRDT which does not seem to be affected by EWSR1. But it would be interesting to also consider the other actors, including NUT, H2A.L.2, TPs and PRMs (these two later are shown).

4 – Minor points:
A - As mentioned above, the reference Rathke et al. 2014 in support of "replacement of histone by protamines" does not consider our recent knowledge on this process. Please update the references to give an updated information on the topic.
B- Page 7, line 234, the authors state "Specifically, we show that EWSR1 is expressed exclusively in heterochromatin regions in pachytene/diplotene spermatocytes and round spermatids".
This is very confusing since earlier in the text they say the contrary: in page 3, line 78-9, they state "EWSR1 signal is absent in heterochromatin regions….".
C- Page 7, line 211, a reference is missing.
D - Page 8, line 246, the authors state "This misregulation most likely leads to cell-cycle arrest..". Spermatids do not replicate their genome therefore "cell cycle arrest" is a misleading term here.
E - Page 7, line 257, the authors state "Mutation of Brdt leads to chromocenter fragmentation". This sentence needs clarification. Indeed, Brdt knock-out leads to a complete arrest during meiosis (late pachytene stage), therefore, there is no round spermatids. In Brdt lacking its first bromodomain, an arrest of spermiogenesis is observed at stage 9 affecting acetylated histone removal (pmid: 22922464).
Additionally, the effect of Brdt on the chromocenters seems to be dependent in the mouse genetic background (Berkovitz et al., cited).
It is therefore very important that the authors clearly set the basis of our knowledge of Brdt's functions to avoid sending wrong messages to the readers.

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Advance summary and potential significance to field

Review of DEVELOP-2021-199414v1-Petkov In this manuscript Tian and Petkov use Hspa2-Cre to conditionally knockout the gene encoding RNA binding protein EWSR1 to show that this protein is important for post-meiotic spermiogenesis processes. They show that spermatozoa maturation is incomplete and gene expression during the transition from meiosis to spermiogenesis is aberrant.

Comments for the author

While this manuscript is interesting and a nice contribution to determining gene functions required for differentiation processes to form mature sperm there are some level of assessment missing:

Major comments:
1. The authors need to take more time on the text. Particularly in the abstract, intro, and discussion. There are sentence structure issues. For example:
- Line 12 to 14 – loss of EWSR1….. conditional knockout in male mice.

2. More detailed assessment of EWSR1 is needed. In Paranov et al., 2017 and here they do not show a comprehensive characterization localization during different stages of spermatogenesis is needed (i.e. there needs to be more than just chromatin spreads at a few substages and low magnified tubule sections). Also, the data in Fig. S1 should really be more detailed, higher resolution, and in the main figures.

3. Hspa2-Cre is expressed in other tissues beyond the testis when assessed for cleavage of loxP-stop-loxP upstream of LacZ expression. The authors should report if they observed any adverse effects on due to the Cre (i.e. lethality or adverse health issues).

4. The authors did not assess chromosome segregation - - Are there signs of chromosome missegregation/microtubule defects during meiotic division?
cytokinesis failures? In Fig. 2b there seems to be an accumulation of metaphase cells. Are the round spermatids normal in the context of chromosome number? - they isolate spermatids via FACS and there appear to be less in the profile compared to the het control. There were much more in the intermediate zone between the pachytene/diplotene 4C population and the RS population.

5. The authors mention that the manchette was not observed in the CKO. he authors should assess the centrioles/centrosome. The sperm has two major types of microtubule base systems: (1) the centriole–cilium complex that forms the sperm tail, and (2) the manchette that shapes the sperm nucleus. Many genes that are essential for the centriole–cilium complex are also essential for the manchette (Lehti and Sironen, 2016 [46]). Therefore, a mutation in a gene affecting the manchette may affect the centriole and vice versa.

6. The authors assess chromocenters in spermatids and argue that there is chromocenter fragmentation. The authors should assess chromocenter characteristics in diplonema. They can also not decipher between chromocenter fragmentation or an inability for chromocenters to coalesce in the first place.

7. The authors assess mRNA levels that increase (i.e. Mlh1, Cntd1 Mov101, and Sycp3) and ones that decrease (Prm1, Prm2, Tnp1, and Tnp2). Does this translate to increases and decreases in protein levels in these isolated populations?

Minor comments:
Check for typos – there are spaces and brackets missing and paragraphs where there needn't be: e.g. line 25, line 76, line 105

Reviewer 3

Advance summary and potential significance to field

EWSR1 has been traditionally studied as a molecular marker of round cell sarcoma, however, little is known about its role in the germline development. The authors have previously demonstrated that this protein is regulator of meiotic recombination through interactions with PRDM9 and REC8 during early prophase I. However, no gene regulation mechanism or RNA binding was associated with this protein during these stages. The work presented here by Tian and Petkov focuses on the post meiotic function of EWSR1 as a gene regulator during spermiogenesis. In this sense, their research is original and brings relevant information to the field. Using a Ewsr1 pachytene stage-specific knockout mouse model, authors showed that EWSR1 expression is specifically abolished from Pachytene stage onward. This manuscript shows evidence of the role of EWSR1 in gene regulation during the transition from meiotic cells to spermatids through RNA seq analysis, where they show how the lack of EWSR1 leads to an upregulation of meiotic genes and a downregulation of spermiogenesis genes. In particular, the work presented here shows evidence of a role of EWSR1 in chromocenter organization in round spermatids possibly via H1fnt. However, the authors do not expand on this phenotype which could provide a more mechanistic insight on the role of EWSR1 in spermiogenesis. Overall, the data presented here do not support the claims of paper well. In the discussion section, authors conclude there is no EWSR1 involvement in chromatoid body function by MVH staining. However, a possible role of EWSR1 in gene silencing via small RNA can't be excluded given the evidence showing that EWSR1 regulates miRNA levels via DROSHA in other cell types:

Uvrag targeting by Mir125a and Mir351 modulates autophagy associated with Ewsr1 deficiency. Autophagy. 2015;11(5):796-811. doi: 10.1080/15548627.2015.1035503. PMID: 25946189

EWS promotes cell proliferation and inhibits cell apoptosis by regulating miR-199a-5p/Sox2 axis in osteosarcoma. Peng He & Junjie Ding. Biotechnol Lett. 2020 Jul;42(7):1263-1274. doi: 10.1007/s10529-020-02859-4. Epub 2020 Apr 1. PMID: 32236759

The RNA binding protein EWS is broadly involved in the regulation of pri-miRNA processing in mammalian cells. Nucleic Acids Res. 2017 Dec 1;45(21):12481-12495. doi: 10.1093/nar/gkx912. PMID: 30053258

Moreover, the authors found that mRNA transcripts of Mov10l1, a gene involved in piRNA biogenesis that is predominantly expressed in pachytene spermatocytes and its mRNA normally disappears in post-meiotic cells remain present in stage-specific EWSR1 KO. This result suggests that dysregulation of piRNAs could be involved in the phenotype observed in these mice and should be analyzed further.

Comments for the author

The following are main comments regarding this research:

1) In Fig 1.
a. Panel A does a poor job of demonstrating the specificity of expression of both Stra8 and EWSR1. At this magnification, it is very difficult to visualize distinct cell types. An additional image at higher magnification may be more appropriate for this purpose. Furthermore, there appears to be substantial unspecific staining with respect to Stra8 and this is addressed. This staining pattern is present in supplemental figure 1A as well. It is difficult to tell whether the staining limited to mature spermatozoa or if it may also be present in round spermatids.

b. Within the text, the authors refer to VII-VIII stage tubes. In the figure however, this tubule is covered by text and difficult to see fully.

c. Panel B is problematic. It is not clear what cell type is being shown here and where in relation to the rest of the tubule these cells are located. The text references both spermatids and pachytene spermatocytes with respect to this panel. Furthermore, it is very difficult to see that there is a lack of staining in the sex body/chromocenter of these cells and that this protein is truly absent. Why not do a series of meiotic spreads to demonstrate that EWSR1 is not present in the sex body? Also, the figure legend does not mention the arrow.

d. Another concern is that the quality of this images in Figure 1 is very low.

2) Fig 2.
a. The major concern with Fig2 is that the authors use a heterozygous mouse as a control. While this is not necessarily a problem, there is no evidence that demonstrates the protein levels in heterozygous animals is comparable to wild type animals and that they can therefore be appropriately used as a control in these studies. The generation of heterozygous control mice is mentioned in the methods, however an additional figure depicting the breeding scheme used to generate all animals in the study would be helpful.

b. For panel C and D it would be helpful if the authors included sperm counts and or mating plugs from these mice to fully analyze sterility. Sterility can be due to many factors. Furthermore, it is not specified how many males or mating pairs were used to generate the data in panel D specifically.

c. In panel E, there are cell present in the tubules of the epididymis in the CKO mouse but there is supposed to be an arrest and loss of spermatids. The presence if these cells is not addressed and this is another instance where sperm counts would help explain whether there is a decrease or absence of sperm.

3) In figure 3, as in figure 2, comparisons are made only to heterozygous animals without any validation that the heterozygote is effectively wild type.

4) In supplemental figure 1A- they state co-staining with STRA8 and EWSR1 is depicted, however the figure does not show co-staining but rather only depicts STRA8 expression in control and CKO mice.

5) Results shown in figures 3 and 4 correspond only to two mice. It is expected to have at least three replicates.

6) The authors use FACS to separate pachytene and diplotene spermatocytes from round spermatids. This method needs to be validated by microscopy or western blot to show that these populations were effectively separated and how efficient was the method to enrich for these populations (percentage of each cell type in each fraction). Or, if the method was used before, reference previous papers from the group in which they validate the gating of the FACS.
7) In Fig. 5 c and d, comparisons between FPKM of a gene in different cell populations are compared. For review purposes and to facilitate the readers interpretation of data, results table from the differential expression analysis with the list of genes with log2(fold-change) and p-values

could be provided. Also, heat map of differentially expressed genes between cell populations (Supplementary Fig. 5) could be included in the main paper.

8) Fig. 5. For panel C and D the legend should be more prominent, or common to all the graphics.

9) Since H1fnt is involved in chromocenter formation, one of the observed phenotypes in KO mice round spermatids, Supplementary Figure 5c showing down regulation of this gene in CKO testis could be in the main body of the paper.

10) Fig 6. B. Same comments than point 6 above. Considering a possible role of EWSR1 in gene silencing via small non coding RNA to count with the log 2 (fold change) values of other genes associated with these pathways (besides the already shown in figure 6) would be interesting.

The following are minor comments regarding this research:
- Line 25, extra left margin space
- Line 211 error in cites? “Taken together, these data suggest that the transition of a meiotic to a post-meiotic gene-expression211 program is impaired in Ewsr1 CKO testes[?]”
- Materials and Methods: Line 302. Histology, TUNEL assay, and immunostaining. There is no TUNEL assay in the manuscript.
- Line 376, there is a lack of space between the words spermatids and from

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First revision

Author response to reviewers' comments

We appreciate the thoughtful reviews and useful suggestions made by all three reviewers. We have addressed all their points that do not require additional experiments. Although some of the experimental suggestions are valid, we had to close all our mouse colonies due to the Covid crisis. Their recovery is not possible at this time and will require at least two or three years. In addition, the first author moved to China before the crisis and our communications were limited in both time and means. We hope that the Editor and the Reviewers will appreciate these difficulties and consider the paper for publication in its current shape.

Reviewer 1 Advance summary and potential significance to field

Here Tian and Petkov report the characterization of the post-meiotic function of EWSR1 in differentiating male germ cells, following the generation of conditional ko of Ewsr1in late meiotic and post-meiotic cells.
They show that the post-meiotic activities of EWSR1 is a prerequisite to the generation of condensing spermatids. In particular, transcriptomic analyses demonstrate that EWSR1 is required for the expression of several genes mediating the process of histone-to-protamine replacement such as transition proteins (TPs) and protamines (PRMs). Indeed, the lack of TPs and PRMs observed by the authors in the absence of EWSR1 is sufficient here to explain the impairment of spermiogenesis during the genome compaction stages and the lack of condensed spermatids. Although the molecular basis of the EWSR1-dependent control of expression of a specific set of mRNAs is not unraveled here, this report remains of interest to investigators interested in the molecular basis of spermiogenesis and hence could be published.

Reviewer 1 Comments for the author

However, before publication, it is important that the authors pay attention to the following points.

1 – Because of the observed involvement of EWSR1 in the integrity of the chromocenter and heterochromatin organization, it would have been interesting to focus on meiotic sex chromosome inactivation (MSCI) phenomenon in spermatocytes and in round spermatids in the Ewsr1 cko cells. Indeed, by specifically considering the expression of the sex chromosome linked gene expression,

the authors could see if MSCI is fully operational in meiotic cells and whether the partial escape from MSCI in round spermatids is also occurring in the absence of EWSR1.

We delete EWSR1 in pachytene, after MSCI occurs normally. However, we do analyze the changes in meiotic genes expression in round spermatids by RNA-seq and report the disregulation of spermatocyte and spermatid gene expression.

2 – There could be at least two reasons for the observed down-regulations of the reported late stage-specific genes expression, i. e., down-regulation of mRNAs encoding TPs and PRMs.

First, the lack of EWSR1 would specifically down-regulate these mRNA in the cells normally expressing them but in the same cells other stage-specific genes remain normally expressed.
Second, these cells die and their absence within the heterogenous cell population studied here would lead to an apparent down-regulation of the corresponding set of genes.
It is important that the authors try to consider this issue to highlight the specific relationship between EWSR1 and a particular set of mRNAs.

One way to consider this issue would be to use the transcriptome and compare the expression levels of all the known stage-specific genes together. An example of such an approach can be found in pmid 30257209 (Fig. S4 - related to Fig. 6). The approach is based on the principle that a change in the transcriptome due to cell loss should be associated with the downregulation of all the corresponding specific transcripts. If only some of the stag-specific transcripts are down regulated, while others remain unchanged, then it is possible to conclude that these changes are due to specific gene deregulations.

We do analyze the transcriptome and report all genes whose expression levels are changed. Our results do not suggest spermatocyte cell loss before the disregulation at the stage of round spermatids.

3 – The authors report the down-regulation of TPs and PRMs without considering the fact that recent investigations revealed that TPs' activity depends on the co-expression of a specific histone variant, H2A.L.2, which is required for the invasion of nucleosomes by TPs, which themselves allow a controlled assembly of PRM (pmid: 28366643). Additionally, NUT and BRDT (see the reference cited in point "2") are also involved upstream of histone displacement, when a histone hyperacetylation occurs. BRDT is a multifunctional factor expressed at the initiation of meiotic differentiation, which has a role in meiosis itself as well as in post-meiotic cells, in the removal of acetylated histones (pmid: 22922464).
With respect to the point discussed in "2" and in order to better understand the role of EWSR1 in the coordination of genes that would be specifically involved in histone-to-PRM replacement, it would be very interesting if the authors would integrate the current knowledge and specifically consider this set of genes.
Among these genes the authors have already considered BRDT which does not seem to be affected by EWSR1. But it would be interesting to also consider the other actors, including NUT, H2A.L.2, TPs and PRMs (these two later are shown).

As mentioned above, we have reported all genes whose expression is dysregulated, and followed this up with qPCR analysis confirming the expression changes.

4 – Minor points:

A - As mentioned above, the reference Rathke et al. 2014 in support of "replacement of histone by protamines" does not consider our recent knowledge on this process. Please update the references to give an updated information on the topic.

The correct reference is now added.

B- Page 7, line 234, the authors state "Specifically, we show that EWSR1 is expressed exclusively in heterochromatin regions in pachytene/diplotene spermatocytes and round spermatids".

This is very confusing since earlier in the text they say the contrary: in page 3, line 78-9, they state “
EWSR1 signal is absent in heterochromatin regions….”.

Sorry about the confusion. The earlier statement is indeed correct. We have changed the Discussion accordingly.

C- Page 7, line 211, a reference is missing.

Fixed. No reference was meant to be there.

D - Page 8, line 246, the authors state “This misregulation most likely leads to cell-cycle arrest..”. Spermatids do not replicate their genome therefore “cell cycle arrest” is a misleading term here.

The reference to cell-cycle arrest is removed.

E - Page 7, line 257, the authors state “Mutation of Brdt leads to chromocenter fragmentation”. This sentence needs clarification. Indeed, Brdt knock-out leads to a complete arrest during meiosis (late pachytene stage), therefore, there is no round spermatids. In Brdt lacking its first bromodomain, an arrest of spermiogenesis is observed at stage 9 affecting acetylated histone removal (pmid: 22922464).
Additionally, the effect of Brdt on the chromocenters seems to be dependent in the mouse genetic background (Berkovitz et al., cited).
It is therefore very important that the authors clearly set the basis of our knowledge of Brdt’s functions to avoid sending wrong messages to the readers.

The discussion is amended to reflect the reviewer’s points. It now reads:
“A mutation of the bromodomain of Brdt leads to arrest of spermiogenesis at stage 9 affecting acetylated histone removal (Gaucher et al., 2012). Other mutations cause chromocenter fragmentation (Berkovits and Wolgemuth, 2011), although this effect is dependent on the genetic background.”

Reviewer 2 Advance summary and potential significance to field

Review of DEVELOP-2021-199414v1-Petkov

In this manuscript Tian and Petkov use Hspa2-Cre to conditionally knockout the gene encoding RNA binding protein EWSR1 to show that this protein is important for post-meiotic spermiogenesis processes. They show that spermatozoa maturation is incomplete and gene expression during the transition from meiosis to spermiogenesis is aberrant.

Reviewer 2 Comments for the author
While this manuscript is interesting and a nice contribution to determining gene functions required for differentiation processes to form mature sperm, there are some level of assessment missing:

Major comments:
1.The authors need to take more time on the text. Particularly in the abstract, intro, and discussion. There are sentence structure issues.

For example:
•Line 12 to 14 – loss of EWSR1….. conditional knockout in male mice.

We have revised the manuscript thoroughly and we hope the current version fixes the text problems.

2.More detailed assessment of EWSR1 is needed. In Paranov et al., 2017
and here they do not show a comprehensive characterization localization during different stages of spermatogenesis is needed (i.e. there needs to be more than just chromatin spreads at a few

substages and low magnified tubule sections). Also, the data in Fig. S1 should really be more detailed, higher resolution, and in the main figures.

All figures were updated and of the highest possible quality. Comprehensive EWSR1 localization has already been reported in Tian et al, 2020 (PMID: 33175657).

3.Hspa2-Cre is expressed in other tissues beyond the testis when assessed for cleavage of loxP-stop-loxP upstream of LacZ expression. The authors should report if they observed any adverse effects on due to the Cre (i.e. lethality or adverse health issues).

A sentence reporting lack of any effects of Cre expression is now added.

4.The authors did not assess chromosome segregation - - Are there signs of chromosome missegregation/microtubule defects during meiotic division? cytokinesis failures? In Fig. 2b there seems to be an accumulation of metaphase cells. Are the round spermatids normal in the context of chromosome number? - they isolate spermatids via FACS and there appear to be less in the profile compared to the het control. There were much more in the intermediate zone between the pachytene/diplotene 4C population and the RS population.

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We appreciate the reviewer's suggestions regarding adding more experimental analyses. However, we are unable to do any additional experiments due to reasons outlined in our general responses.

Minor comments:
Check for typos – there are spaces and brackets missing and paragraphs where there needn't be: e.g. line 25, line 76, line 105

Fixed wherever found.

Reviewer 3

EWSR1 has been traditionally studied as a molecular marker of round cell sarcoma, however, little is known about its role in the germline development. The authors have previously demonstrated that this protein is regulator of meiotic recombination through interactions with PRDM9 and REC8 during early prophase I. However, no gene regulation mechanism or RNA binding was associated with this protein during these stages. The work presented here by Tian and Petkov focuses on the post meiotic function of EWSR1 as a gene regulator during spermiogenesis. In this sense, their research is original and brings relevant information to the field. Using a Ewsr1 pachytene stage-specific knockout mouse model, authors showed that EWSR1 expression is specifically abolished from Pachytene stage onward. This manuscript shows evidence of the role of EWSR1 in gene regulation during the transition from meiotic cells to spermatids through RNA seq analysis, where they show how the lack of EWSR1 leads to an upregulation of meiotic genes and a downregulation of spermiogenesis genes. In particular, the work presented here shows evidence of a role of EWSR1 in chromocenter organization in round spermatids possibly via H1fnt. However, the authors do not expand on this phenotype which could provide a more mechanistic insight on the role of EWSR1 in

spermiogenesis. Overall, the data presented here do not support the claims of paper well. In the discussion section, authors conclude there is no EWSR1 involvement in chromatoid body function by MVH staining. However, a possible role of EWSR1 in gene silencing via small RNA can't be excluded given the evidence showing that EWSR1 regulates miRNA levels via DROSHA in other cell types:

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EWS promotes cell proliferation and inhibits cell apoptosis by regulating miR-199a-5p/Sox2 axis in osteosarcoma. Peng He & Junjie Ding. Biotechnol Lett. 2020 Jul;42(7):1263-1274. doi: 10.1007/s10529-020-02859-4. Epub 2020 Apr 1. PMID: 32236759

The RNA binding protein EWS is broadly involved in the regulation of pri-miRNA processing in mammalian cells. Nucleic Acids Res. 2017 Dec 1;45(21):12481-12495. doi: 10.1093/nar/gkx912. PMID: 30053258

We discuss this possible role of EWSR1 via small RNA. All three papers suggested here are now properly cited.

Moreover, the authors found that mRNA transcripts of Mov10l1, a gene involved in piRNA biogenesis that is predominantly expressed in pachytene spermatocytes and its mRNA normally disappears in post-meiotic cells remain present in stage-specific EWSR1 KO. This result suggests that dysregulation of piRNAs could be involved in the phenotype observed in these mice and should be analyzed further.

This is an interesting point but we are unable to further it in any experimental way currently.

The following are main comments regarding this research:

1) In Fig 1.
a. Panel A does a poor job of demonstrating the specificity of expression of both Stra8 and EWSR1. At this magnification, it is very difficult to visualize distinct cell types. An additional image at higher magnification may be more appropriate for this purpose. Furthermore, there appears to be substantial unspecific staining with respect to Stra8 and this is addressed. This staining pattern is present in supplemental figure 1A as well. It is difficult to tell whether the staining limited to mature spermatozoa or if it may also be present in round spermatids.

The purpose of this panel was not to show the co-staining of the STRA8 and EWSR1. Using of STRA8 here is to indicate the stage of each seminiferous tubule.

b. Within the text, the authors refer to VII-VIII stage tubes. In the figure however, this tubule is covered by text and difficult to see fully.

Updated.

c. Panel B is problematic. It is not clear what cell type is being shown here and where in relation to the rest of the tubule these cells are located. The text references both spermatids and pachytene spermatocytes with respect to this panel. Furthermore, it is very difficult to see that there is a lack of staining in the sex body/chromocenter of these cells and that this protein is truly absent. Why not do a series of meiotic spreads to demonstrate that EWSR1 is not present in the sex body? Also, the figure legend does not mention the arrow.

We have done a thorough determination of EWSR1 expression in our previous paper on the subject (PMID 33175657).

The legend is updated to
Fig. 1. EWSR1 is highly expressed in euchromatin regions of late spermatocytes and round spermatids.
(A) Co-staining of EWSR1 (magenta) and STRA8 (green) on adult B6 testicular sections. Scale bar, 100 μm. The Roman numerals indicate the stage of each seminiferous tubule. (B) Co-staining of

EWSR1 (magenta) and the heterochromatin marker H3K9me3 (green). White arrow, round spermatid. Scale bar, 10 μm.

d. Another concern is that the quality of this images in Figure 1 is very low.

All figures are now of the highest possible quality.

2) Fig 2.
a. The major concern with Fig2 is that the authors use a heterozygous mouse as a control. While this is not necessarily a problem, there is no evidence that demonstrates the protein levels in heterozygous animals is comparable to wild type animals and that they can therefore be appropriately used as a control in these studies. The generation of heterozygous control mice is mentioned in the methods, however an additional figure depicting the breeding scheme used to generate all animals in the study would be helpful.

The description of the breeding scheme in the methods section is thorough, and we do not believe a graphic representation will help substantially. Moreover, it is identical to the one we describe in Tian et al., 2020 (PMID: 33175657), except for the Cre deleter.

b. For panel C and D it would be helpful if the authors included sperm counts and or mating plugs from these mice to fully analyze sterility. Sterility can be due to many factors. Furthermore, it is not specified how many males or mating pairs were used to generate the data in panel D specifically.

We did not do the sperm count, because, as shown in the testis and epididymis staining, there was no mature sperm in the CKO male mice.

c. In panel E, there are cell present in the tubules of the epididymis in the CKO mouse but there is supposed to be an arrest and loss of spermatids. The presence if these cells is not addressed and this is another instance where sperm counts would help explain whether there is a decrease or absence of sperm.

See the response to the previous remark.

3) In figure 3, as in figure 2, comparisons are made only to heterozygous animals without any validation that the heterozygote is effectively wild type.

Comparison to heterozygous controls produced in the same mating is the standard way of evaluation of the mutant phenotype, as the genetic background is removed as a variable. We believe this is the right control in this and most other cases.

4) In supplemental figure 1A- they state co-staining with STRA8 and EWSR1 is depicted, however the figure does not show co-staining but rather only depicts STRA8 expression in control and CKO mice.

The purpose of this panel was not to show the co-staining of the STRA8 and EWSR1. Using of STRA8 here is to indicate the stage of each seminiferous tubule.

5) Results shown in figures 3 and 4 correspond only to two mice. It is expected to have at least three replicates.

Sorry, we cannot add another replicate at this time.

6) The authors use FACS to separate pachytene and diplotene spermatocytes from round spermatids. This method needs to be validated by microscopy or western blot to show that these populations were effectively separated and how efficient was the method to enrich for these populations (percentage of each cell type in each fraction). Or, if the method was used before, reference previous papers from the group in which they validate the gating of the FACS.

A reference is now added to Material and Methods.

7) In Fig. 5 c and d, comparisons between FPKM of a gene in different cell populations are compared. For review purposes and to facilitate the readers interpretation of data, results table from the differential expression analysis with the list of genes with log2(fold-change) and p-values could be provided. Also, heat map of differentially expressed genes between cell populations (Supplementary Fig. 5) could be included in the main paper.

8) Fig. 5. For panel C and D the legend should be more prominent, or common to all the graphics.

9) Since H1fnt is involved in chromocenter formation, one of the observed phenotypes in KO mice round spermatids, Supplementary Figure 5c showing down regulation of this gene in CKO testis could be in the main body of the paper.

Figure 5 is now updated according to the suggestion.

10) Fig 6. B. Same comments than point 6 above. Considering a possible role of EWSR1 in gene silencing via small non coding RNA to count with the log 2 (fold change) values of other genes associated with these pathways (besides the already shown in figure 6) would be interesting.

A reference is now added to Material and Methods.

The following are minor comments regarding this research:

- Line 25, extra left margin space
  Fixed.
- Line 211 error in cites? “Taken together, these data suggest that the transition of a meiotic to a post-meiotic gene-expression211 program is impaired in Ewsr1 CKO testes[?]”
  Fixed.
- Materials and Methods: Line 302. Histology, TUNEL assay, and immunostaining. There is no TUNEL assay in the manuscript.
  Fixed.
- Line 376, there is a lack of space between the words spermatids and from
  Fixed.

Second decision letter

MS ID#: DEVELOP/2021/199414

MS TITLE: Mouse EWSR1 is critical for spermatid post-meiotic transcription and spermiogenesis

AUTHORS: Hui Tian and Petko Petkov

I have now received 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 have taken your request of Covid-19 related lack of access to the lab and resources into consideration in their review and are supportive of the revised manuscript. However, as reviewer 1 points out, there are some considerations which need to be addressed, which are of textual in nature, and can be incorporated. These are important for the rigor of the study. If you are able to incorporate these recommendations I will be happy to receive a revision. I do not expect to send the manuscript back to the reviewers, but please do highlight all the changes in the text and in response to reviewers to allow me to assess the incorporation of the changes.

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

please see my previous report

Comments for the author

Some of the concerns raised previously have been taken into account and the manuscript has now been improved.
The author's response to minor point "E" is not appropriate.

1 - In the text, line 267, the authors state: “Brdt codes for a testis-specific double bromodomain protein that is required for chromocenter organization (Berkovits and Wolgemuth, 2011)”.
To correctly reflect the information provided in the cited publication, the authors should add "depending on the genetic background."

2 -Lines 331-334: the information on Brdt is not correct.
The authors state: "BRDT is also known to be associated with spermiogenesis and chromocenter formation. A mutation of the first bromodomain of Brdt leads to arrest of spermiogenesis at stage 9 affecting acetylated histone removal (Gaucher et al., 2012). Other mutations cause chromocenter fragmentation (Berkovits and Wolgemuth, 2011), although this effect is dependent on the genetic background".
This sentence should be changed into what follows:
The deletion of the first bromodomain of Brdt affects acetylated histone removal in elongating spermatids
(Gaucher et al., 2012). This mutant also causes chromocenter fragmentation, depending on the genetic background (Berkovits and Wolgemuth, 2011).

Reviewer 2

Advance summary and potential significance to field

In this manuscript Tian and Petkov use Hspa2-Cre to conditionally knockout the gene encoding RNA binding protein EWSR1 to show that this protein is important for post-meiotic spermiogenesis processes. They show that spermatozoa maturation is incomplete and gene expression during the transition from meiosis to spermiogenesis is aberrant. This manuscript is interesting and a nice contribution to determining gene functions required for differentiation processes to form mature sperm.

Comments for the author

All comments addressed as best possible considering the issues with the COVID-19 pandemic.

Second revision

Author response to reviewers' comments

Reviewer 1 Comments for the author

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Changed as requested.

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Changed as requested.

Third decision letter

MS ID#: DEVELOP/2021/199414

MS TITLE: Mouse EWSR1 is critical for spermatid post-meiotic transcription and spermiogenesis

AUTHORS: Hui Tian and Petko Petkov
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.