Loss of perinuclear theca ACTRT1 causes acrosome detachment and severe male subfertility in mice

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MS TITLE: Loss of perinuclear theca Actrt1 causes acrosome detachment and severe male subfertility

AUTHORS: Xiao-Zhen Zhang, Lin-Lin Wei, Xiao-Hui Zhang, Hui-Juan Jin, and Su-Ren Chen

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 also raise many 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

Detachment of acrosome from sperm nucleus was observed due to the loosened acroplaxome structure in Actrt1-KO males, indicating the potential role of Actrt1 in anchoring the developing acrosomes to nuclei. These authors showed different results and drew a different conclusion on the function of Actrt1 in mice from a published article. Thus, this paper provides new explanation for the functional role of Actrt1in spermiogenesis.

Comments for the author

The manuscript, by Zhang et al., entitled “Loss of perinuclear theca Actrt1 causes acrosome detachment and severe male subfertility” reported the KO mice of Actrt1, a sperm PT-specific actin-related protein. The Actrt1-KO males were subfertile due to a deficiency of fertilization with malformed heads.
Furthermore, detachment of acrosome from sperm nucleus was observed due to the loosened acroplaxome structure. The interaction between inner acrosomal membrane protein Spaca1 and nuclear envelope protein Parp11 was detected, showing the potential role of Actrt1 in anchoring the developing acrosomes to nuclei The manuscript is well organized and the data generally support their conclusion. It’s worth noting that these authors showed different results and drew a different conclusion on the function of Actrt1 in mice from a published article.
However, I have some concerns as follows.

Major comments:
1. To better support the point ‘Actrt1 forms a complex with Actrt2 Actl7a, and Actl9’, the localization of these proteins especially for Actrt1 should be shown by immunofluorescence.
2. For Fig. 5A, how these proteins are selected? Mainly based on the phenotypes of Actrt1-KO mice or any other reasons? It’s not only for the positive ones like Spaca1 and Parp11, but also for the negative ones like Fam209 and so on.
3. The authors gave indirect evidence that Actrt1 localize at the PT, it’s better to give a direct evidence using immunofluorescence. Due to the predicted localization of Actrt1, my advice is to try immunofluorescence with no Triton X-100, it may give a better result for some acrosome/PT localized protein.
4. The authors mentioned the potential functional redundancy of Actrt1 and Actrt2. Can Actrt2 also interact with Actl7a/Actl9/Spaca1/Parp11/…? If the interaction can be detected, there will be more evidence for this potential redundancy.

Other comments:
Fig. 1A, the expression of flag-tagged Actrt1 in membrane is almost the same as that in cytoskeleton, a gray level difference analysis is suggested to give a more obvious result.

Reviewer 2

Advance summary and potential significance to field

The authors found that loss of Actrt1 caused a significantly high incidence of malformed heads and especially the detachment of acrosome from sperm nucleus. This is different from another recently reported data of Actrt1-KO mice. Overall, this manuscript presented here is comprehensive, and is well-referenced and the phenotypic data in this manuscript are solid. At the same time, this article also has some problems, which need to be address.

Comments for the author

The authors found that loss of Actrt1 caused a significantly high incidence of malformed heads and especially the detachment of acrosome from sperm nucleus. This is different from another recently reported data of Actrt1-KO mice. Overall, this manuscript presented here is comprehensive, and is well-referenced and the phenotypic data in this manuscript are solid. At the same time, this article also has some problems, which need to be address.

Specfic points for consideration:

1. Figure 1C why not use KO mice as control.
2. It is recommended to provide WB to verify whether ACTRT1 is completely absent or truncated mutants appear in the testis of KO mice. This is very important to evaluate the phenotypic differences of different mouse models.
3. Page 6 Fig.S2D 'Actrt1 could interact with Sun4' This conclusion seems to have some conflict with the final model in Figure 5C. The localization of SUN4 does not overlap with acroplaxome. It is difficult to imagine that ACTRT1 is a component of acroplaxome and also covers other regions of sperm nucleus. In fact, it is understandable that acrosome anomaly leads to manchette anomaly, because manchette assembly depends on the stability of acrosome structure. In fact, it is best to provide ACTRT1 localization data, especially when KO mice are used as negative controls. In this way, the proposed model for the role of ACTRT1 in Figure 5C is more convincing.
4. Page 2: one more 'at the end of the abstract.
5. The whole protein should be capitalized, such as ACTRT1, rather than Actrt1.
6. The sentence 'The assembly of such sperm-specific ARPs into a protein complex suggests that they may play coordinated roles in spermiogenesis.' of page 4 results should not start with another paragraph.

Reviewer 3

Advance summary and potential significance to field

There is no significant advance in this paper to the field

Comments for the author

In the present work Actrt1-KO mice were generated to define the function/s of Actrt1 in sperm. The authors report that Actrt1-KO males are severely sub-fertile exhibiting sperm with detached acrosome and malformed heads. Moreover, the Authors have found that Actr1 anchors developing acrosome to the nucleus by interacting with inner acrosomal membrane proteins Spaca1 and Tmco2 and nuclear envelope protein Parp11; Actrt1 interacts also with Actr2, Actl7a, and Actl9 to form a multimeric protein complex of the perinuclear theca (PT).

The work deals with more experimental approaches (generation of a mouse model, fertility test light and electron microscopy, in vitro fertilization, statistical analysis), but the results obtained are rather confused and do not justify often the conclusions drawn, the experimental procedures are not reported in detail and lack in fundamental information, and the work in general is not so original considering that an Actrt1-KO mouse model and Actrt1 variants have already been described and published (for instance, Sha et al., Front Cell Dev Biol 2021).
Major concerns First. Introduction. The Authors have to specify better the question of the perinuclear theca. PT although structurally continuous, is distinguished in different segments, i.e., 1) the subacrosomal region (SAR), intercalated between the inner acrosomal membrane (IAM) and the nuclear envelope (NE), 2) PT overlying the equatorial segment (ES), between plasma membrane and outer acrosomal membrane (OAM), SAR-PT + ES-PT give rise to the subacrosomal layer (SAL) of the PT, and 3) PT of the post-acrosomal sheath (PAS). SAL-PT and PAS-PT are different in both protein constituents and timing of formation, i.e., emergence of SAL occurs early in concert with the acrosome development, whereas PAS appears to be assembled during caudal descent of the manchette concomitantly with spermatid elongation. It follows that SAL (consequently, its constituents) is involved in acrosome assembly whereas PAS (consequently, its constituents) is involved in sperm-egg interactions at fertilization. And this has been demonstrated and is known since 2000s (see among others, the works of Oko's group, Franke's group, Kierszenbaum's group). Sentences repeated more times throughout the present manuscript, like that recalled here below, are therefore misleading:
"although the protein constituents of PT are well-studied during the last twenty years (Aul and Oko, 2002; Boeda et al., 2011; Hamilton et al., 2017; Heid et al., 2002; Herrada and Wolgemuth 1997; Hess et al., 1993; Longo et al., 1987; Mujica et al., 2003; Oko and Morales, 1994; Olson and Winfrey, 1988; von Bulow et al., 1997), the physiological roles of PT remain largely unclear due to a lack of knockout/mutated mouse models".

As to the above raised question about specification of PT segments, the Authors never demonstrate where Actrt1 is located. According to the authors, reading the text, Actrt1 anchors the developing acrosome to the nucleus (so, Actrt1 is a SAL component), but it is also responsible for reduced Plcζ content and fertility (so, Actrt1 is a PAS component). Curiously, there is no datum immunolocalization, or figure of Actrt1 localization along the entire text. Nothing. This is mandatory. In the work of Sha et co-workers (where the generation and characterization of Actrt1-KO mice is described for the first time), here heavily censured by the present Authors (Discussion, the entire last paragraph, page 9-11), Actrt1 localizes in the peri-centriolar region (human control spermatozoa); on the other hand, Actrt1 was found in the PAS/calyx region and at centriolar level in bull spermatozoa (see the original work by Heid et al. 2002). Here, as said, there is no experimental evidence about Actrt1 localization, while the cartoon of Fig. 5C, illustrating a hypothetical model, depicts Actrt1 at the acroplaxome/SAR-PT level.
Secondly. The insufficiency of Materials and Methods. It is not very clear how the experiments were performed. Few examples are listed below.
Mouse model. The targeting strategy has to be described. Which is the genomic region deleted? How was the CAS9 mRNA obtained? Which is the sequence (and the purpose) of the ssODNs ? Which is the method used for generating the gRNAs? The only anonymous cartoon of Fig. 2A is insufficient. Provide with Table S1 the nucleotide sequence of mouse Actrt1 and sign where the designed primers match. At a rapid control, it seems that the gRNAs used for deletion are not inside the coding sequence of ACTRT1 gene, but 1258 bp upstream and 5521 downstream. The protocol used for generation of mutant mice is lacking at all. Describe the used method or at least give a reference.
Expression plasmids and transfection. Give some information about Actrt1 cDNA synthesis. How were the various plasmids generated? Plasmid pUC57 is bacterial, from where were the Flag and Myc tags obtained? For the expression of cloned mouse cDNAs, a mammalian promoter is required; nothing is reported about this. How were HEK293T cells cultured and Lipofectamine-treated?
Fertility test of Actrt1-KO mice. If I have read well, only two females were used (Page 12: “Briefly the sexually mature male mice (8 to 12 weeks old) were individually caged with two C57BL/6J females (6 to 8 weeks old) for 2 months”). Do these two females include also the female/s for control male mice? Were the females previously stimulated (for instance with PMSG)? Under the grounds of what reported above, what does mean: “The pregnancy rate was calculated as the ratio of the number of females with pregnancy to the number of females with successful mating” page 12 and, repeated exactly, in legend of the graphic of Fig. 2B, page 17?
IVF in mice. Please, include the composition of TYH medium used for capacitation. Why were so young (4 weeks) females used? Describe better the induction of superovulation and how COCs were obtained and managed (including washing steps). Include the composition of HTF (?) and KSOM (?) liquid drops.
IIF. How has been the “cell suspension containing spermatids” obtained? Specify, please. Why has not been Actrt1-immunolabeling performed? The antibody used in the present study (Table S4 Invitrogen PA5-31691) is recommended for immunohistochemistry/immunocytochemistry and WB by the firm.
TEM. Page 13 “Sperm from cauda epididymis were used for investigating sperm ultrastructure.” Indeed, in addition to the unique TEM image of a single section of a spermatozoon (Fig. 3 E), Fig 4A shows TEM images of spermatids at cap/acrosome phase, i.e., from testis samples. However no mention about how these TEM testis sections were obtained is made (pages 13-14). I suggest to the Authors to control better the sizes assigned to the bars in Fig. 3 E and Fig. 4 A, because these are in evident contrast. Regard to Fig. 4A and related Fig. 4C graphic, how has been the thickness of acroplaxome measured?
IP. Page 14. Indicate the composition of both IP lysis buffer and the successive washing buffer/s in particular the detergents present and the salt/s molarity. Mention how the input was recovered and its protein concentration. The same for each immunoprecipitate. Provide how many protein micrograms were loaded/lane/gel.
Statistical analysis. I do not understand how statistical analyses were performed and the data obtained to do the relative graphics since the number of samples compared/experiment and/or how many times the experiments were repeated, and similar information, are specified neither in Mat & Meth nor in the related Fig. legends.
Additional concerns 1. I do not agree with some general sentences in the Discussion, as that “one of the major causes of male infertility is the abnormal development of spermatids into mature sperm”. At least it is one of the causes. Globozoospermia remains a rare condition, with an

incidence of less than 0.1% (Kuentz et al. Hum. Reprod., 2013). Another sentence: “Recent studies, including ours provide solid animal evidence to suggest that PT is critical for the attachment of acrosome to the nucleus (Xin et al., 2020; Dai et al., 2021)”. As already said, PT is known to be critical since the early 2000.
Another: “The partial fertilization failure of Actrt1-KO mice may be explained by the reduced protein content of Plc zeta and Actl7a in Actrt1-KO sperm because i) Plc is a well-known sperm PT protein to induce Ca2+ oscillations after entering into oocytes”. Please, explain how it can be possible that Actrt1 regulates the content of PLC zeta, considering that according to the Authors Actrt1 is a SAR-PT protein (cartoon Fig. 5C) whereas PLC zeta is in the PAS-PT.
2. I have some doubts that all the proteins here reported to interact directly with Actrt1 (Actrt2 Actl7a, Actl9, Spaca1, Tmco2, Parp11, Sun 4 and others) effectively interact. The experiments of co-immunoprecipitation do not report the assay conditions (see concerns to IP, above). Usually protein interactions are demonstrated by assays as GST-pull down, yeast two-hybrid, etc. (see for instance, the cited Chen et al., Hum. Reprod. 36, 2021).
3. It is a little arduous to conciliate “the manchette of Actrt1-KO spermatids was largely disorganized as a potential cause of abnormal head shapes (Fig. S2C)” (page 6), with “ Double immunofluorescence (IF) staining of sperm using PNA-FITC (an acrosome dye) and mitotracker red (a mitochondria dye) further revealed normal assemble of mitochondrial sheath”, bottom page 5 considering also the unique TEM image provided for a spermatozoon (Fig. 3 E) showing disorganization, if not absence, of a mitochondria tail sheath. By the way, PNA-FITC and mitotracker red are not immunolabeling.
4. Figure legends are not clear and exhaustive, and sometimes legends do not correspond to what is shown (example, Legend of Fig. 1C and the Fig. 1C).

In conclusion, this reviewer does not find the manuscript suitable for publication in Development

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First revision

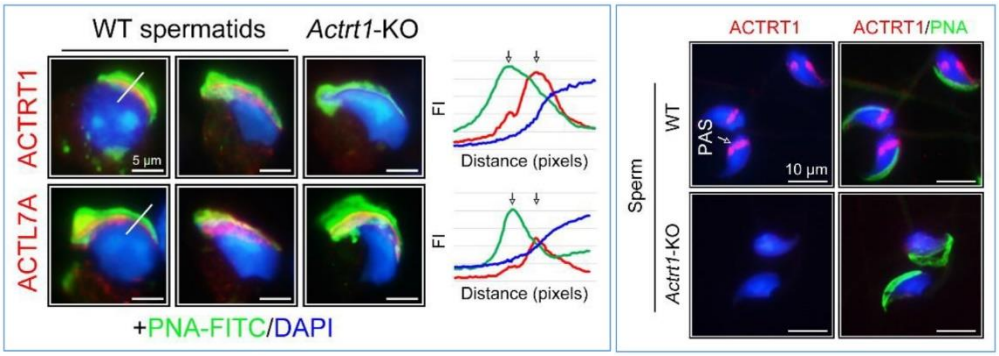
Author response to reviewers' comments

Reviewer 1
Detachment of acrosome from sperm nucleus was observed due to the loosened acroplaxome structure in *Actrt1*-KO males, indicating the potential role of ACTRT1 in anchoring the developing acrosomes to nuclei. These authors showed different results and drew a different conclusion on the function of ACTRT1 in mice from a published article. Thus, this paper provides new explanation for the functional role of ACTRT1 in spermiogenesis. The manuscript, by Zhang et al., entitled “Loss of perinuclear theca ACTRT1 causes acrosome detachment and severe male subfertility” reported the KO mice of ACTRT1, a sperm PT-specific actin-related protein. The *Actrt1*-KO males were subfertile due to a deficiency of fertilization with malformed heads. Furthermore, detachment of acrosome from sperm nucleus was observed due to the loosened acroplaxome structure. The interaction between inner acrosomal membrane protein SPACA1 and nuclear envelope protein PARP11 was detected, showing the potential role of ACTRT1 in anchoring the developing acrosomes to nuclei. The manuscript is well organized and the data generally support their conclusion. It’s worth noting that these authors showed different results and drew a different conclusion on the function of ACTRT1 in mice from a published article. However, I have some concerns as follows.

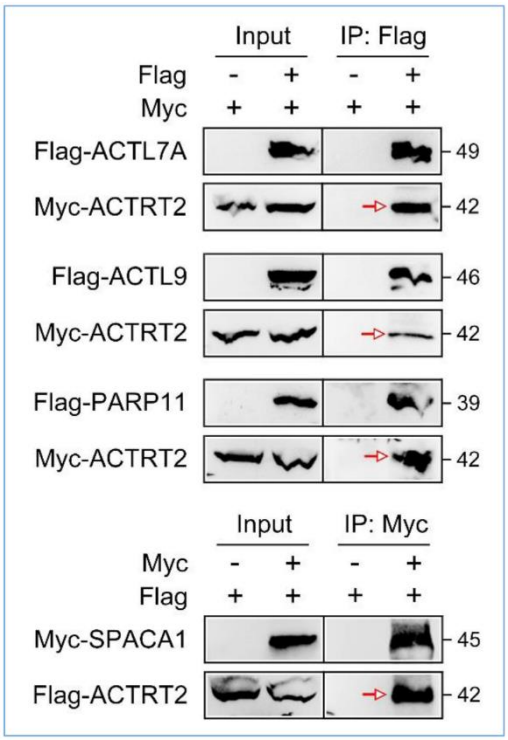
Major comments:

1. To better support the point ‘ACTRT1 forms a complex with ACTRT2, ACTL7A, and ACTL9’, the localization of these proteins especially for ACTRT1 should be shown by immunofluorescence.
We truly appreciate this referee’s constructive suggestion. As suggested, we performed IF staining of ACTRT1 and ACTL7A (please see revised Fig. 1B). In round and elongated spermatids, ACTRT1 signals were detected in the subacrosomal region (SAR), and a certain distance existed between ACTRT1 signals and PNA-FITC (an acrosome dye) signals. The ACTRT1 signals in wild-type spermatids were specific because no staining was present using *Actrt1*-KO spermatids. Unfortunately, we could not perform the co-IF staining of ACTRT1 and ACTL7A because both are rabbit polyclonal antibodies. ACTL7A also exhibited SAR distribution in round

and elongated spermatids. Intriguingly, ACTRT1 was translocated to the postacrosomal sheath (PAS) of mature sperm in the epididymis (please see revised Fig. S1). The ACTRT2 antibody could be used for WB but not IF, and the ACTL9 antibody was unavailable to us.



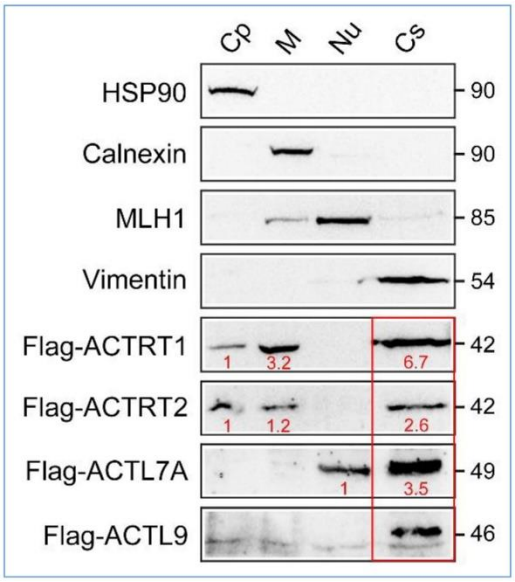
2. For Fig. 5A, how these proteins are selected? Mainly based on the phenotypes of *Actrt1*-KO mice or any other reasons? It's not only for the positive ones like SPACA1 and PARP11, but also for the negative ones like FAM209 and so on.
   Thank you for your question, and we would like to explain why we selected these proteins. As detachment of developing acrosomes from nuclei was identified in *Actrt1*-KO mice, we hypothesized that acroplaxome-expressed ACTRT1 may interact with the above IAM (inner acrosomal membrane) and below NE (nucleus envelope) proteins to form an adhesive structure. We planned to identify ACTRT1-interacting proteins within the testis using immunoprecipitation-mass spectrometry (IP-MS) analysis; however, we found that commercial ACTRT1 antibodies could not be applied for IP experiments. Accordingly, we chose a candidate-based approach by searching the literature for IAM and NE proteins whose knockout mice exhibit a deficiency in the IAM-acroplaxome-NE structure. Based on the similar phenotype as *Actrt1*-KO sperm, SPACA1, DPY19L2, FAM209, PARP11, and SPATA46 are candidate partners of ACTRT1. 1) No close association of the IAM with NE is formed in *Spaca1*-deficient spermatids (Fujihara et al., 2012), and importantly, the IAM protein SPACA1 could interact with ACTL7A to anchor the acrosome to the acroplaxome (Chen et al., 2021). 2) DPY19L2 is the first NE protein shown to be essential for anchoring the acrosome to the nucleus (Pierre et al., 2012). 3) FAM209 interacts with DPY19L2 at the NE to maintain the developing acrosome (Castaneda et al., 2021). 4) Both PARP11 and SPATA46 are NE proteins, and their deficient spermatids exhibit structural defects in the NE associated with an abnormal nuclear shape (Meyer-Ficca et al., 2015; Chen et al., 2016). We added this explanation to the revised manuscript and thank you for your constructive suggestion.
3. The authors gave indirect evidence that ACTRT1 localize at the PT, it's better to give a direct evidence using immunofluorescence. Due to the predicted localization of ACTRT1, my advice is to try immunofluorescence with no Triton X-100, it may give a better result for some acrosome/PT localized protein.
   As suggested, we performed IF staining of ACTRT1. Please see the figure in our answer to your first question. Furthermore, we found that there was no obvious difference with or without Triton X-100 treatment for ACTRT1 and ACTL7A staining.
4. The authors mentioned the potential functional redundancy of ACTRT1 and ACTRT2. Can ACTRT2 also interact with ACTL7A/ACTL9/SPACA1/PARP11/…? If the interaction can be detected, there will be more evidence for this potential redundancy.
   We truly appreciate this reviewer's constructive suggestion. We suggest a partial functional redundancy between ACTRT1 and ACTRT2 because i) these two proteins share over 70% similarity in nucleotide sequence and ii) ACTRT2 expression was increased in sperm samples from *Actrt1*-KO mice compared with those from control mice (Fig. 4D). However, the evidence is not adequate. As suggested, we performed Co-IP experiments and found that ACTRT2 could be immunoprecipitated with ACTL7A, ACTL9, SPACA1, and PARP11 (*please see the figure below*).



Other comments:

Fig. 1A, the expression of Flag-tagged ACTRT1 in membrane is almost the same as that in cytoskeleton, a gray level difference analysis is suggested to give a more obvious result.

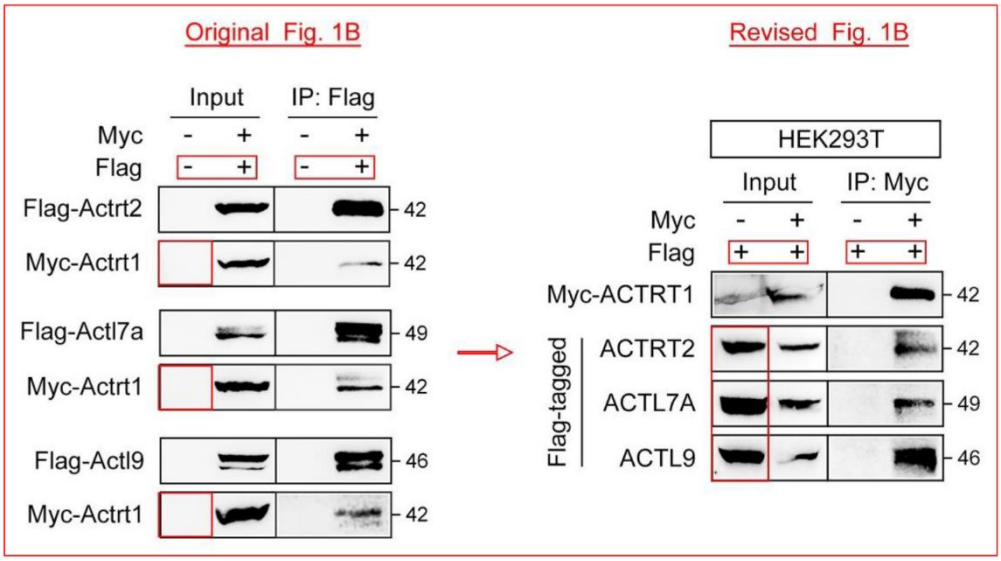
Thank you for your suggestion. We analysed the grey value of the protein bands using ImageJ software (*please see the figure below*). ACTRT1, ACTRT2, ACTL7A and ACTL9 were all mainly distributed in the cytoskeletal (Cs) components.

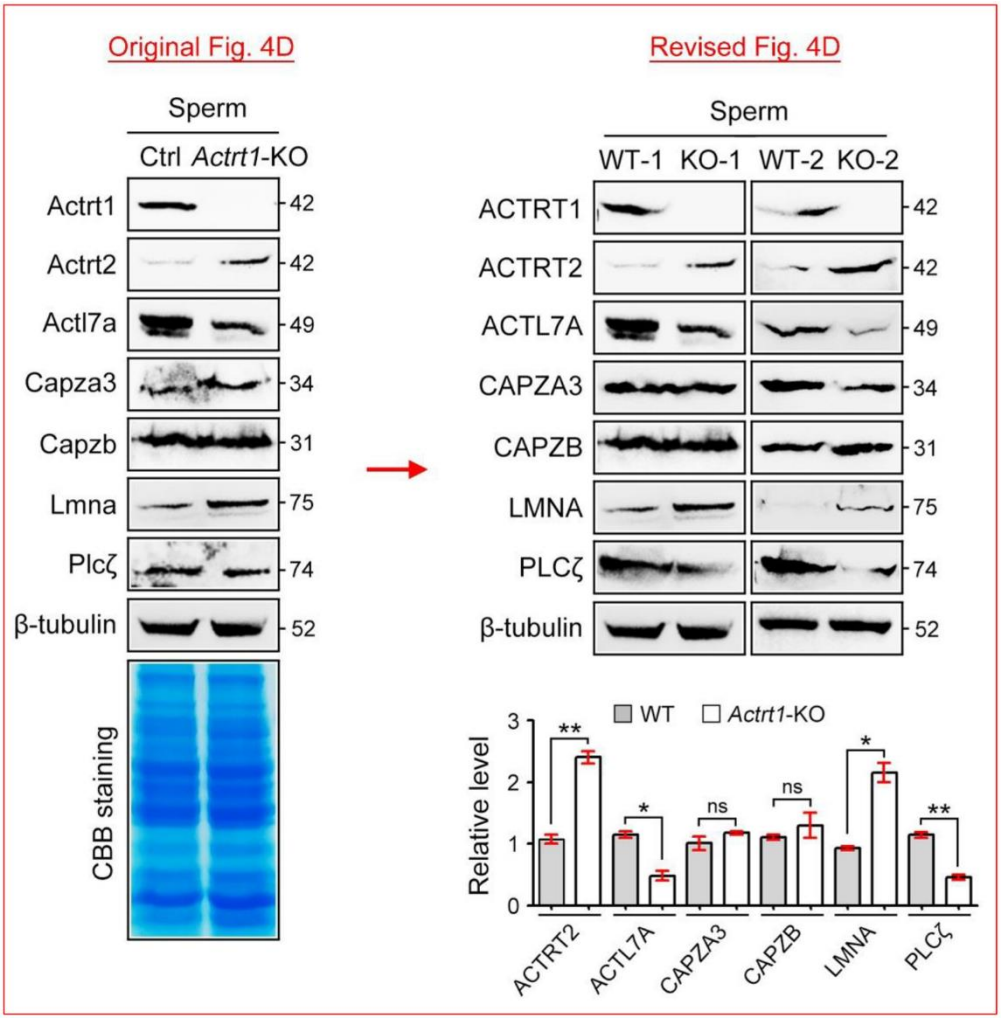


We really appreciate this reviewer's constructive comments. Our manuscript was much improved after careful consideration of your suggestions.

Taking the opportunity for revision, the whole manuscript was carefully modified by us and polished using the English editing services of AJE. In addition to the reviewers' constructive suggestions, we repeated some of the experiments and made some changes as follows. 1) We realize the control group (empty vectors) of Co-IP experiments in HEK293T cells was not proper. In the revised version, we repeated the experiments shown in Fig. 1B and Fig. 5A. If Flag antibody was utilized to

immunoprecipitate Flag-tagged protein A, Myc-tagged protein B was transfected into both the control and IP groups. Then, we determined the co-immunoprecipitation of protein B was specific (*please see the figure below*). 2) The WB study of PT proteins between wild-type sperm and *Actrt1*-KO sperm was repeated, and statistical analysis was performed (*please see revised Fig. 4D*).





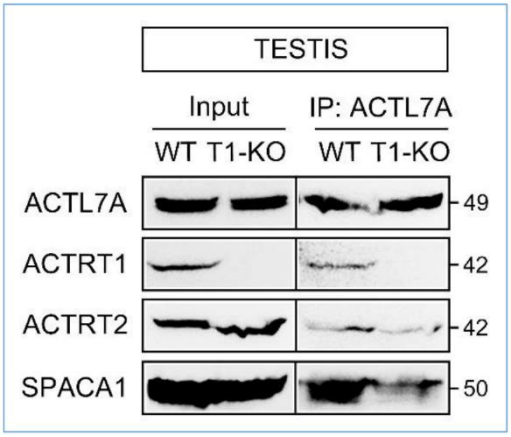
Reviewer 2

The authors found that loss of ACTRT1 caused a significantly high incidence of malformed heads and especially the detachment of acrosome from sperm nucleus. This is different from another recently reported data of *Actrt1*-KO mice. Overall, this manuscript presented here is comprehensive, and is well-referenced and the phenotypic data in this manuscript are solid. At the same time, this article also has some problems, which need to be address.

Specific points for consideration:

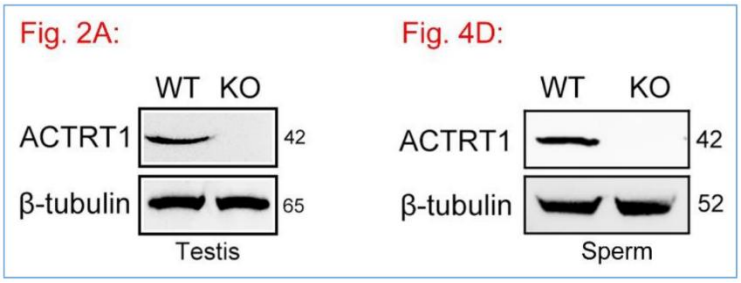
1. Figure 1C why not use KO mice as control.

   Thank you for your suggestion. In Fig. 1C, we showed that endogenous ACTL7A could be immunoprecipitated with ACTRT1 and ACTRT2 using WT testis extracts. As we start to introduce the results of KO mice from Fig. 2, KO testes were not used as a control in Fig. 1C. As suggested by this reviewer, we have redone the endogenous Co-IP experiment using KO testis extracts as a control (*please see Fig. 5C*). Loss of ACTRT1 (*Actrt1*-KO) did not disrupt the ACTL7A-ACTRT2 interaction but reduced the connection between ACTL7A and the IAM protein SPACA1.



2. It is recommended to provide WB to verify whether ACTRT1 is completely absent or truncated mutants appear in the testis of KO mice. This is very important to evaluate the phenotypic differences of different mouse models.

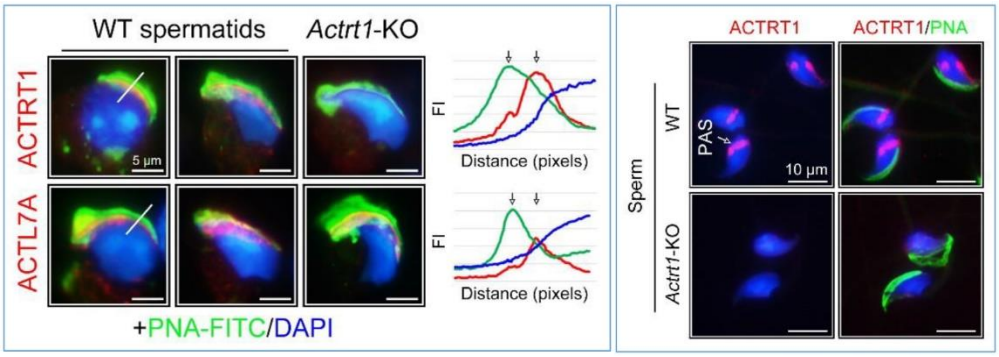
   We appreciate your constructive suggestion. In our initial submission, we showed that ACTRT1 is completely absent in the sperm sample of *Actrt1*-KO mice (Fig. 4D). As suggested, we performed WB analysis of ACTRT1 in control and *Actrt1*-KO testes and showed that ACTRT1 is completely absent in the testis of *Actrt1*-KO mice (*please see revised Fig. 2A*).



3. Page 6 Fig.S2D 'ACTRT1 could interact with SUN4'. This conclusion seems to have some conflict with the final model in Figure 5C. The localization of SUN4 does not overlap with acroplaxome. It is difficult to imagine that ACTRT1 is a component of acroplaxome and also covers other regions of sperm nucleus. In fact, it is understandable that acrosome anomaly leads to manchette anomaly, because manchette assembly depends on the stability of acrosome structure. In fact, it is best to provide ACTRT1 localization data, especially when KO mice are

used as negative controls. In this way, the proposed model for the role of ACTRT1 in Figure 5C is more convincing.

We agree with this reviewer that the interaction between ACTRT1 and SUN4 is somewhat misleading and thus chose to delete this supplementary data (Fig. S2D). We should thank this reviewer to advise us an explanation of abnormal manchette formation in *Actrt1*-KO spermatids. We wrote 'The manchette anomaly observed in *Actrt1*-KO spermatids (Fig. S2C) may be a secondary effect of acrosome anomaly after the loss of ACTRT1, because manchette assembly depends on the stability of acrosome structure (Kierszenbaum et al., 2004)'. As suggested, we also provided IF staining of ACTRT1 in both control and *Actrt1*-KO spermatids/sperm. In round and elongated spermatids, ACTRT1 signals were detected in the subacrosomal region (SAR), and a certain distance existed between ACTRT1 signals and PNA-FITC (an acrosome dye) signals. The ACTRT1 signals in wild-type spermatids were specific because no staining were present using *Actrt1*-KO spermatids. Unfortunately, we could not perform the co-IF staining of ACTRT1 and ACTL7A because both are rabbit polyclonal antibodies. ACTL7A also exhibited SAR distribution in round and elongated spermatids. Intriguingly, ACTRT1 was translocated to the post-acrosomal sheath (PAS) of mature sperm in the epididymis (please see revised Fig. S1). The ACTRT2 antibody could be used for WB but not IF, and the ACTL9 antibody is unavailable to us.



4. Page 2: one more 'at the end of the abstract.

   Thank you. We corrected the mistake.

5. The whole protein should be capitalized, such as ACTRT1, rather than Actrt1.

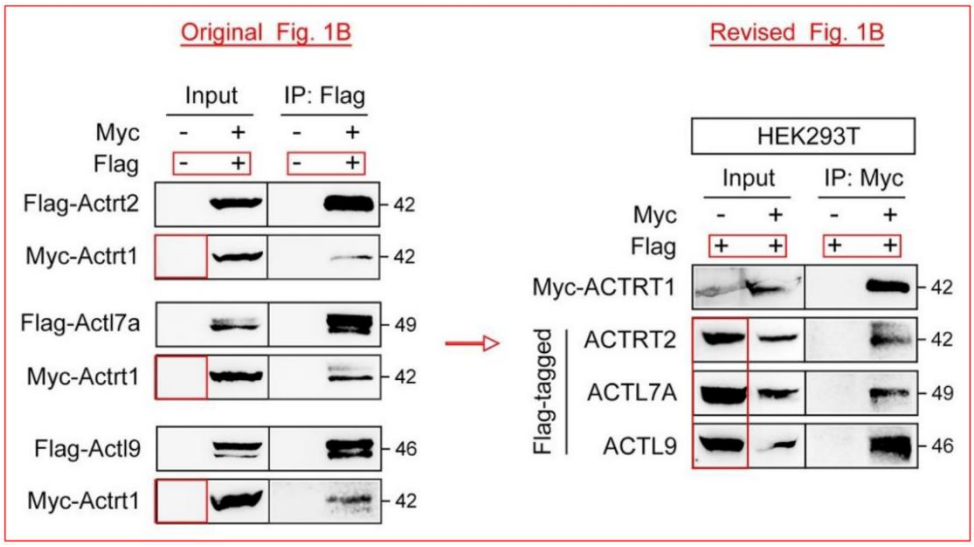
   Thank you. We corrected all protein names throughout the manuscript.

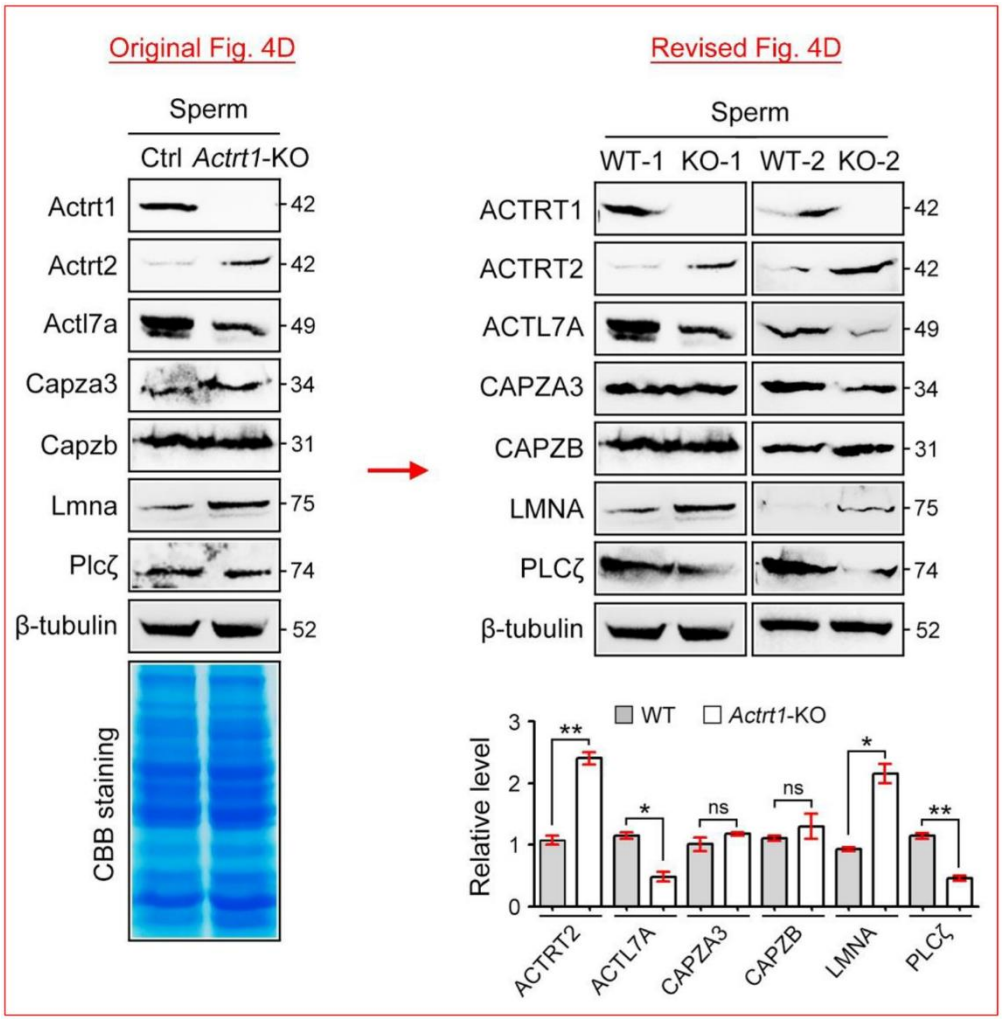
6. The sentence 'The assembly of such sperm-specific ARPs into a protein complex suggests that they may play coordinated roles in spermiogenesis.' of page 4 results should not start with another paragraph.

   Thank you. We corrected the mistake.

We truly appreciate this reviewer's constructive comments. Our manuscript was much improved after careful consideration of your suggestions.

Taking the opportunity for revision, the whole manuscript was carefully modified by us and polished using the English editing services of AJE. In addition to the reviewers' constructive suggestions, we repeated some of the experiments and made some changes as follows. 1) We realize the control group (empty vectors) of Co-IP experiments in HEK293T cells was not proper. In the revised version, we repeated the experiments shown in Fig. 1B and Fig. 5A. If Flag antibody was utilized to immunoprecipitate Flag-tagged protein A, Myc-tagged protein B was transfected into both the control and IP groups. Then, we determined the co-immunoprecipitation of protein B was specific (*please see the figure below*). 2) The WB study of PT proteins between wild-type sperm and *Actrt1*-KO sperm was repeated, and statistical analysis was performed (*please see revised Fig. 4D*).





Reviewer 3

Comments for the Author

In the present work *Actrt1*-KO mice were generated to define the function of ACTRT1 in sperm. The authors report that *Actrt1*-KO males are severely subfertile exhibiting sperm with detached acrosome and malformed heads. Moreover, the authors have found that ACTRT1 anchors developing

acrosome to the nucleus by interacting with inner acrosomal membrane proteins SPACA1 and TMCO2 and nuclear envelope protein PARP11; ACTRT1 interacts also with ACTRT2, ACTL7A, and ACTL9 to form a multimeric protein complex of the perinuclear theca (PT). The work deals with more experimental approaches (generation of a mouse model, fertility test, light and electron microscopy, in vitro fertilization, statistical analysis), but the results obtained are rather confused and do not justify often the conclusions drawn, the experimental procedures are not reported in detail and lack in fundamental information, and the work in general is not so original considering that an *Actrt1*-KO mouse model and ACTRT1 variants have already been described and published (Sha *et al.*, *Front Cell Dev Biol*, 2021).

Thank you for all your significant concerns and suggestions below. While we were preparing our manuscript, Sha et al. reported two point mutations (c.95G>A and c.662A>G) of the *ACTRT1* gene in two acephalic spermatozoa syndrome (ASS) patients and approximately 60% of sperm from *Actrt1*-KO mice are headless (Sha *et al.*, *Front Cell Dev Biol*, 2021). Surprisingly, the phenotype observed in our *Actrt1*-KO mice was inconsistent with that in Sha's *Actrt1*-KO mice. We did not observe the ASS phenomenon in our *Actrt1*-KO mice; instead, detachment of acrosome from the nucleus was identified in our mouse model. Data from independent laboratories (Chen *et al.*, 2021; Dai *et al.*, 2021), including ours, suggest that ACTRT1, ACTL7A, and ACTL9 form a large ARP complex and interact with the IAM protein SPACA1. The phenotype of our *Actrt1*-KO males is identical to *Actl7a*-(Xin *et al.*, 2020) and *Actl9*-mutated mice (Dai *et al.*, 2021), showing a phenotype of acrosome detachment but not ASS. We did not censure the work of Sha et al. and merely showed different results and drew a different conclusion for the function of ACTRT1 in mice. We think our study is novel, solid, and essential because if *ACTRT1* is truly an ASS-associated gene, screening for *ACTRT1* mutations may be recommended to ASS patients for their diagnosis.

Major concerns

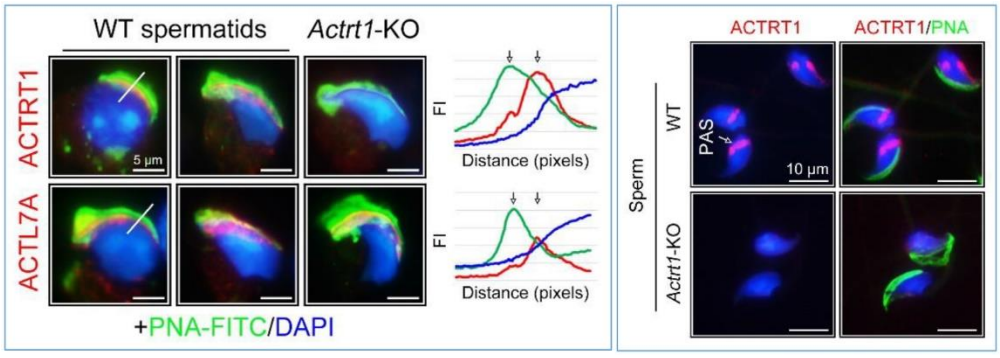
1. **Introduction.** The authors have to specify better the question of the perinuclear theca. PT, although structurally continuous, is distinguished in different segments, i.e., 1) the subacrosomal region (SAR), intercalated between the inner acrosomal membrane (IAM) and the nuclear envelope (NE), 2) PT overlying the equatorial segment (ES), between plasma membrane and outer acrosomal membrane (OAM), SAR-PT + ES-PT give rise to the subacrosomal layer (SAL) of the PT, and 3) PT of the post-acrosomal sheath (PAS). SAL-PT and PAS-PT are different in both protein constituents and timing of formation, i.e., emergence of SAL occurs early in concert with the acrosome development, whereas PAS appears to be assembled during caudal descent of the manchette, concomitantly with spermatid elongation. It follows that SAL (consequently, its constituents) is involved in acrosome assembly whereas PAS (consequently, its constituents) is involved in sperm-egg interactions at fertilization. And this has been demonstrated and is known since 2000s (the works of Oko's group, Franke's group, Kierszenbaum's group). Sentences, repeated more times throughout the present manuscript, like that recalled here below, are therefore misleading: "although the protein constituents of PT are well-studied during the last twenty years, the physiological roles of PT remain largely unclear due to a lack of knockout/mutated mouse models". As to the above raised question about specification of PT segments, the authors never demonstrate where ACTRT1 is located. According to the authors, reading the text, ACTRT1 anchors the developing acrosome to the nucleus (so, ACTRT1 is a SAL component), but it is also responsible for reduced PLCζ content and fertility (so, ACTRT1 is a PAS component). Curiously, there is no datum, immunolocalization, or figure of ACTRT1 localization along the entire text. Nothing. This is mandatory. In the work of Sha et co-workers (where the generation and characterization of *Actrt1*-KO mice is described for the first time), here heavily censured by the present Authors (Discussion, the entire last paragraph, page 9-11), ACTRT1 localizes in the peri-centriolar region (human control spermatozoa); on the other hand, ACTRT1 was found in the PAS/calyx region and at centriolar level in bull spermatozoa (see the original work by Heid *et al*. 2002). Here, as said, there is no experimental evidence about ACTRT1 localization, while the cartoon of Fig. 5C, illustrating a hypothetical model, depicts ACTRT1 at the acroplaxome/SAR-PT level.

   First, we appreciate this referee's suggestions and have revised some parts of the Introduction. 1st, we wrote: "PT, although structurally continuous, is distinguished in different segments: i) the subacrosomal region (SAR), intercalated between the inner acrosomal membrane (IAM) and the nuclear envelope (NE), ii) PT overlying the equatorial segment (ES), between plasma

membrane and outer acrosomal membrane (OAM), and iii) PT of the postacrosomal sheath (PAS). SAR-PT and ES-PT give rise to the subacrosomal layer (SAL) of the PT (Oko and Sutovsky, 2009)". 2nd, we wrote "SAL-PT and PAS-PT are different in both protein constituents and timing of formation. It has been suggested that SAL proteins originate from acrosomal vesicles during acrosome biogenesis, whereas PAR proteins are translated in the cytoplasmic lobe and then transport via the manchette, concomitantly with spermatid elongation (Oko and Sutovsky, 2009)". 3rd, we wrote: "It has been suggested that SAL-PT is involved in acrosome assembly, whereas PAS-PT is involved in sperm-egg interactions at fertilization (Oko and Sutovsky, 2009)".

Second, we appreciate the original works from Oko's group, Franke's group and Kierszenbaum's group, but the physiological roles of many PT proteins have not been studied by gene knockout mouse models. Furthermore, great efforts are still needed to explore i) the profile of PT proteins, ii) the organization of PT structure, iii) the dynamics of PT proteins during spermiogenesis and so on. Therefore, I am afraid to say that the conclusion 'SAL-PT is involved in acrosome assembly whereas PAS-PT is involved in sperm-egg interactions at fertilization the conclusion' can not be drawn. **2)** We did not validate the work of Sha et al. (Sha Y et al., Front Cell Dev Biol, 2021) and showed different results and drew a different conclusion on the function of ACTRT1 in mice. We provided evidence to suggest that ACTRT1, ACTL7a, and ACTL9 form a sperm PT-specific actin-related protein (ARP) complex. Importantly, *Actl7a*- (Xin A et al., Sci Adv, 2020), *Actl9*- (Dai J et al., Am J Hum Genet, 2021), and *Actrt1*-KO (this study) sperm exhibited quite a similar phenotype—acrosome detachment from the nucleus. We did not observe any evidence indicating that ACTRT1 plays a role in the head-tail connection. To avoid any unnecessary controversy, we deleted most of our comments about Sha's paper from the Discussion.

Third, we provided the immunostaining of ACTRT1 in the revised manuscript. In round and elongated spermatids, ACTRT1 signals were detected in the subacrosomal region (SAR), and a certain distance existed between ACTRT1 signals and PNA-FITC (an acrosome dye) signals. The ACTRT1 signals in wild-type spermatids were specific because no staining were present using *Actrt1*-KO spermatids. Unfortunately, we could not perform co-IF staining of ACTRT1 and ACTL7A because both are rabbit polyclonal antibodies. ACTL7A also exhibited SAR distribution in round and elongated spermatids. Intriguingly, ACTRT1 was translocated to the postacrosomal sheath (PAS) of mature sperm in the epididymis (please see revised Fig. S1). The ACTRT2 antibody could be used for WB but not IF, and the ACTL9 antibody is unavailable to us.



2. The insufficiency of Materials and Methods. It is not very clear how the experiments were performed.
Mouse model. The targeting strategy has to be described. Which is the genomic region deleted? How was the CAS9 mRNA obtained? Which is the sequence (and the purpose) of the ssODNs? Which is the method used for generating the gRNAs? The only anonymous cartoon of Fig. 2A is insufficient. Provide with Table S1 the nucleotide sequence of mouse *Actrt1* and sign where the designed primers match. At a rapid control, it seems that the gRNAs used for deletion are not inside the coding sequence of Actrt1 gene, but 1258 bp upstream and 5521 downstream. The protocol used for generation of mutant mice is lacking at all. Describe the used method or at least give a reference.

We appreciate this referee's suggestion. As suggested, we provided the experimental details to generate *Actrt1*-KO mice by CRISPR/Cas9 technology. "The mouse *Actrt1* gene has 1 transcript (ENSMUST00000059466.2) and is located on chromosome X. The *Actrt1* gene has only one exon, and the whole *Actrt1* gene was selected as the knockout region. In brief, we selected two sgRNA primers with high scores (CRISPR finder: https://www.sanger.ac.uk/htgt/wge/find_crisprs) to generate a deletion of the whole *Actrt1* gene in mice (GRCm38). The wild-type genomic sequence is provided in Table S1, in which the *Actrt1* gene and the position of gRNAs are marked in red and blue, respectively. The sgRNAs (5'-CCATTGGTTGCTCAGTTCAA-3' and 5'-CTGGATAAGTAAAGTAACTC-3') were synthesized by Sangon Biotech (Shanghai, China). The two complementary DNA oligos of each sgRNA target were annealed (95 °C for 5 min and then naturally cooled to room temperature) and ligated to the pUC57-sgRNA plasmid (Cat# 51132, Addgene, USA) for cloning. The recombinant plasmid was transformed into DH5α competent cells, and the positive clone was screened based on kanamycin resistance and sequencing. The recombinant plasmid was linearized and purified by phenol chloroform extraction. Transcriptions of the sgRNAs in vitro were performed using the MEGAshortscript Kit (Cat# AM1354, Ambion, USA) and purified using the MEGAclear Kit (Cat# AM1908, Ambion, USA). Cas9 mRNA was purchased from TriLink BioTechnologies (Cat# L-7206). Mouse zygotes were coinjected with an RNA mixture of Cas9 mRNA (50 ng/μl) and sgRNA (30 ng/μl). The injected zygotes were transferred into pseudopregnant recipients to obtain the F0 generation. DNA was extracted from tail tissues from 7-day-old offspring, and PCR amplification was carried out with genotyping primers (Table S2) using the Mouse Tissue Direct PCR Kit (Tiangen Biotech, Beijing, China) under the following conditions: 94 °C for 5 min; 35 cycles of 94 °C for 30 s, 61 °C for 30 s, and 72 °C for 30 s; and a final step of 72 °C for 5 min. PCR products were run on a 1% agarose gel in 1×TBE buffer and then subjected to sequencing (Sangon Biotech). A stable F1 generation (heterozygous mice) was obtained by mating positive F0 generation mice with wild-type C57BL/6JGpt mice".

Expression plasmids and transfection. Give some information about *Actrt1* cDNA synthesis. How were the various plasmids generated? Plasmid pUC57 is bacterial, from where were the Flag and Myc tags obtained? For the expression of cloned mouse cDNAs, a mammalian promoter is required; nothing is reported about this. How were HEK293T cells cultured and Lipofectamine-treated?

We appreciate this referee's suggestion. As suggested, we provided the experimental details of expression plasmids and transfection. "Mouse *Actrt1* cDNA was chemically synthesized by GenScript Biotech Corporation (Suzhou, China) and inserted into Flag- or Myc-tagged pCMV vectors (Cat# D2632, D2672, Beyotime, Shanghai, China). Full-length cDNA encoding ACTRT2, ACTL7A, ACTL9, SPACA1, DPY19L2, FAM209, SPATA46, and PARP11 was amplified by PCR using mouse testis cDNA as the template and cloned into Flag- or Myc-tagged pCMV vectors. To obtain mouse testis cDNA, total RNA was extracted from mouse testes using an RNA Easy Fast Tissue/Cell Kit (Tiangen Biotech, Cat# DP451). First-strand cDNA synthesis and RT-PCR were performed using a FastKing One-Step RT-PCR Kit (Tiangen Biotech, Cat# KR123) according to the manufacturer's instructions. The primers for plasmid construction are listed in the Table S4. The construction of expression plasmids in this study was confirmed by sequencing (Sango Biotech, Shanghai, China). All sequencing results (.ab1 files) were provided in Supplementary Materials and can be viewed by Chromas software (http://technelysium.com.au/wp/chromas/). HEK293T cells (ATCC, Cat# CRL-11268) were cultured at 37 °C in a 5% CO_2 incubator (Panasonic/Sanyo CO_2 incubator MCO-18AIC) with Dulbecco's modified Eagle's medium (DMEM) (Thermo Fisher Scientific, Gibco™, Cat# 10569-044)+10% foetal bovine serum (FBS) (HyClone, Cat# 10099-141)+1% penicillin-streptomycin (Thermo Fisher Scientific, Gibco™, Cat# 15140-163). The transient transfection of HEK293T cells was performed by using Lipofectamine 3000 transfection reagent (Thermo Fisher Scientific, Invitrogen™, Cat# 11668-019) following the manufacturer's protocol. Cells were then harvested 48 h after transfection."

Fertility test of *Actrt1*-KO mice. If I have read well, only two females were used (Page 12: "Briefly, the sexually mature male mice (8 to 12 weeks old) were individually caged with two C57BL/6J females (6 to 8 weeks old) for 2 months"). Do these two females include also the female/s for control male mice? Were the females previously stimulated (for instance with PMSG)? Under the grounds of what reported above, what does mean: "The pregnancy rate was

calculated as the ratio of the number of females with pregnancy to the number of females with successful mating" page 12 and, repeated exactly, in legend of the graphic of Fig. 2B, page 17?

We appreciate this referee's suggestion. As suggested, we rewrote the method of fertility test. "Briefly, three *Actrt1*-KO and three littermate wild-type sexually mature male mice (8 to 12 weeks old) were paired with 6~8-week-old C57BL/6J females (each male was mated with two female mice) for 2 months. The vaginal plugs of the mice were examined every morning. Then, the female mice with vaginal plugs were separately fed, and the number of pups per litter was recorded. Of the 30 female mice mated with control males, 27 were pregnant and gave rise to 231 offspring. In contrast, of the 27 female mice mated with *Actrt1*-KO males, only 6 were pregnant and gave rise to 11 offspring."

IVF in mice. Please, include the composition of TYH medium used for capacitation. Why were so young (4 weeks) females used? Describe better the induction of superovulation and how COCs were obtained and managed (including washing steps). Include the composition of HTF (?) and KSOM (?) liquid drops.

We appreciate this referee's suggestion. As suggested, we provided the experimental details of IVF. "Eight -week-old C57BL/6J female mice were superovulated by injecting 5 IU (0.1 ml) of pregnant mare serum gonadotropin (PMSG) (Nanjing Aibei Biotechnology, Cat#M2620), followed by 5 IU (0.1 ml) of human chorionic gonadotropin (hCG) (Nanjing Aibei Biotechnology, Cat# M2520) 48 h later. The sperm was released from the cauda epididymis of 10-week-old male mice, and sperm capacitation was performed for 50 min using TYH solution (Nanjing Aibei Biotechnology, Cat# M2030). Cumulus-oocyte complexes (COCs) were obtained from the ampulla of the uterine tube at 14 hours after hCG injection. The ampulla was torn with syringe needle, and the COCs were gently squeezed onto the liquid drops of HTF medium (Nanjing Aibei Biotechnology, Cat# M1130). COCs were then incubated with 5 to 10 μl sperm suspension (sperm concentration: 1~5×10^6) in HTF liquid drops at 37 °C under 5% CO_2. After 6 hours, the eggs were washed several times using HTF medium to remove the cumulus cells and then transferred to liquid drops of KSOM medium (Nanjing Aibei Biotechnology, Cat# M1430). Two-cell embryos were counted at 24 hours postfertilization. All lipid drops were covered with mineral oil (Nanjing Aibei Biotechnology, Cat# ART-4008P) and equilibrated overnight at 37 °C under 5% CO_2."

TEM. Page 13 "Sperm from cauda epididymis were used for investigating sperm ultrastructure." Indeed, in addition to the unique TEM image of a single section of a spermatozoon (Fig. 3 E), Fig 4A shows TEM images of spermatids at cap/acrosome phase, i.e., from testis samples. However, no mention about how these TEM testis sections were obtained is made (pages 13-14). I suggest to the authors to control better the sizes assigned to the bars in Fig. 3 E and Fig. 4 A, because these are in evident contrast. Regard to Fig. 4A and related Fig. 4C graphic, how has been the thickness of acroplaxome measured?

We appreciate this referee's suggestion. As suggested, we provided the experimental details of TEM. "Precipitation of mouse sperm and testis tissues (~1 mm^3) were fixed with 2.5% (vol/vol) glutaraldehyde in 0.1 M phosphate buffer (PB) (pH 7.4) for 24 hours at 4 °C. The samples were washed four time in PB and first immersed in 1% (wt/vol) OsO4 and 1.5% (wt/vol) potassium ferricyanide aqueous solution at 4 °C for 2 h. After washing, the samples were dehydrated through graded alcohol (30%, 50%, 70%, 80%, 90%, 100%, 100%, 10 min each) into pure acetone (10 min for two times). Samples were infiltrated in a graded mixture (3:1, 1:1, 1:3) of acetone and SPI-PON812 resin (21 ml SPO-PON812, 13 ml DDSA and 11 ml NMA), and then changed pure resin. The specimens were embedded in pure resin with 1.5% BDMA and polymerized for 12 h at 45 °C, 48 h at 60 °C, cut into ultrathin sections (70 nm thick), and then stained with uranyl acetate and lead citrate for subsequent observation and photography with a Tecnai G2 Spirit 120 kV (FEI, Lausanne, Netherlands) electron microscope. All reagents were purchased from Zhongjingkeyi Technology (Beijing, China)." Moreover, the bars in Fig. 3 E and Fig. 4 A were corrected. We have described the methods to measure the thickness of acroplaxome in Fig. 4 legend: "Five sites of the acroplaxome in a spermatid were randomly selected (stars indicated in A). The thickness of the acroplaxome structure in the TEM images at a magnification of 30,000x was measured and averaged. Data in B and C are presented as the means±SEM. *n*=3 mice for each group. Student's *t test*. ***p<0.001. At least 50 spermatids were counted in each experiment."

IP. Page 14. Indicate the composition of both IP lysis buffer and the successive washing buffer/s, in particular the detergents present and the salt/s molarity. Mention how the input

was recovered and its protein concentration. The same for each immunoprecipitate. Provide how many protein micrograms were loaded/lane/gel.
We appreciate this referee's suggestion. As suggested, we provided the experimental details of IP. "Forty-eight hours after transfection, HEK293T cells were lysed with Pierce™ IP Lysis Buffer (Thermo Fisher, Cat# 87787) with protease inhibitor cocktail (MedChemExpress, Cat# HY-K0010) for 30 min at 4 °C and then centrifuged at 12,000 g for 10 min. Pierce IP Lysis Buffer was composed of 25 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% NP-40 and 5% glycerol. To prepare Input samples, 30 µl protein lysates (~25 µg) were collected and boiled for 5 min in 1.2×SDS loading buffer (Beyotime, Cat# P0015). The lysates were precleared with 10 µl Pierce™ Protein A/G-conjugated Agarose (Thermo Fisher, Cat# 20422) for 1 h at 4 °C. Precleared lysates were incubated overnight with 2 µg anti-Myc antibody (Abmart, Cat#M20002) or anti-Flag antibody (Abmart, Cat# M20018) at 4 °C. The lysates were then incubated with 20 µl Pierce™ Protein A/G-conjugated Agarose for 2 h at 4 °C. The agarose beads were washed four times with Pierce™ IP Lysis Buffer and boiled for 5 min in 1.2×SDS loading buffer. Input and IP samples were analysed by Western blotting by using anti-Flag or anti-Myc antibodies. For endogenous co-IP, adult mouse testis tissues were lysed with Pierce™ IP Lysis Buffer. Precleared lysates were separated into two groups: one group was treated with 2 µg anti-ACTL7A antibody (Proteintech, Cat# 17355-1-AP) or anti-SPACA1 antibody (Abcam, Cat# ab191843), and another group (negative control) was treated with 2 µg rabbit IgG (Beyotime, Cat# A7016). Other endogenous co-IP procedures were similar to the co-IP assay in HEK293T cells."

Statistical analysis. I do not understand how statistical analyses were performed and the data obtained to do the relative graphics since the number of samples compared/experiment and/or how many times the experiments were repeated, and similar information, are specified neither in Mat & Meth nor in the related Fig. legends.
We appreciate this referee's suggestion. As suggested, we provided the experimental details of statistical analysis in both each figure legend and Methods.

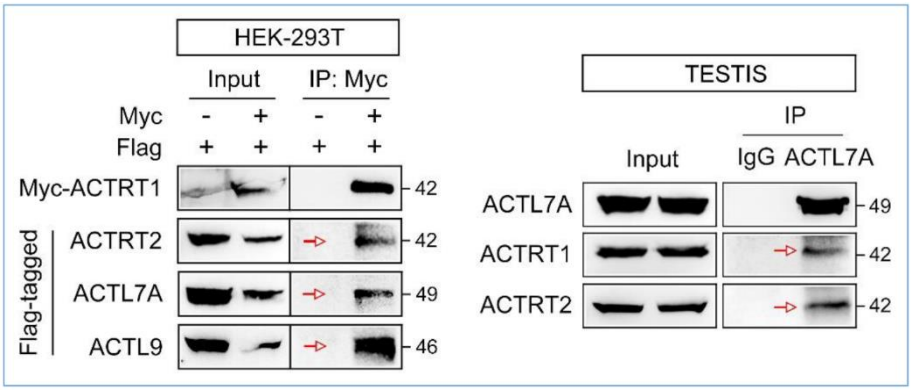
Additional concerns

1. I do not agree with some general sentences in the Discussion, as that "one of the major causes of male infertility is the abnormal development of spermatids into mature sperm". At least, it is one of the causes. Globozoospermia remains a rare condition, with an incidence of less than 0.1% (Kuentz *et al*. *Hum. Reprod*, 2013). Another sentence: "Recent studies, including ours, provide solid animal evidence to suggest that PT is critical for the attachment of acrosome to the nucleus (Xin *et al*., 2020; Dai *et al*., 2021)". As already said, PT is known to be critical since the early 2000. Another: "The partial fertilization failure of *Actrt1*-KO mice may be explained by the reduced protein content of PLC zeta and ACTL7a in *Actrt1*-KO sperm because i) PLC zeta is a well-known sperm PT protein to induce Ca^{2+} oscillations after entering into oocytes". Please, explain how it can be possible that Actrt1 regulates the content of PLC zeta, considering that according to the authors ACTRT1 is a SAR-PT protein (cartoon Fig. 5C) whereas PLC zeta is in the PAS-PT.

   We appreciate your concerns and have made the following modifications. 1) one of the major causes of male infertility → one of the causes of male infertility. 2) are two major abnormalities of astheno-teratozoospermia → are two types of astheno-teratozoospermia. 3) The sentence 'Recent studies, including ours, provide solid animal evidence to suggest that PT is critical for the attachment of acrosome to the nucleus' was deleted in the revised MS. 4) Although reduced expression and/or abnormal localization of PLCζ are observed in *Actl7a*-, *Actl9*-, and *Actrt1*-deficient sperm, the mechanisms underlying the regulation of PLCζ by ACTL7A, ACTL9, and ACTRT1 are still unknown. These ARPs may regulate the content and localization of PLCζ in an indirect way.

2. I have some doubts that all the proteins here reported to interact directly with ACTRT1 (ACTRT2, Actl7a, Actl9, Spaca1, Tmco2, Parp11, Sun 4 and others) effectively interact. The experiments of co-immunoprecipitation do not report the assay conditions (see concerns to IP, above). Usually, protein interactions are demonstrated by assays as GST-pull down, yeast two-hybrid, etc. (see for instance, the cited Chen *et al*., *Hum. Reprod*, 2021).
   Thank you for your comment. In the revised manuscript, we provided the step-by-step experimental details of the co-IP (please see Methods). It is well accepted that co-IP is a

commonly used method to study protein-protein interactions. We repeated the co-IP experiments in HEK293T cells using the proper control (if Myc-ACTRT1 was immunoprecipitated by using anti-Myc antibody, Flag-tagged ACTRT2/ACTL7A/ACTL9 were also transfected into the control group to determine the specific interaction). The endogenous co-IP had already been provided in the original MS.

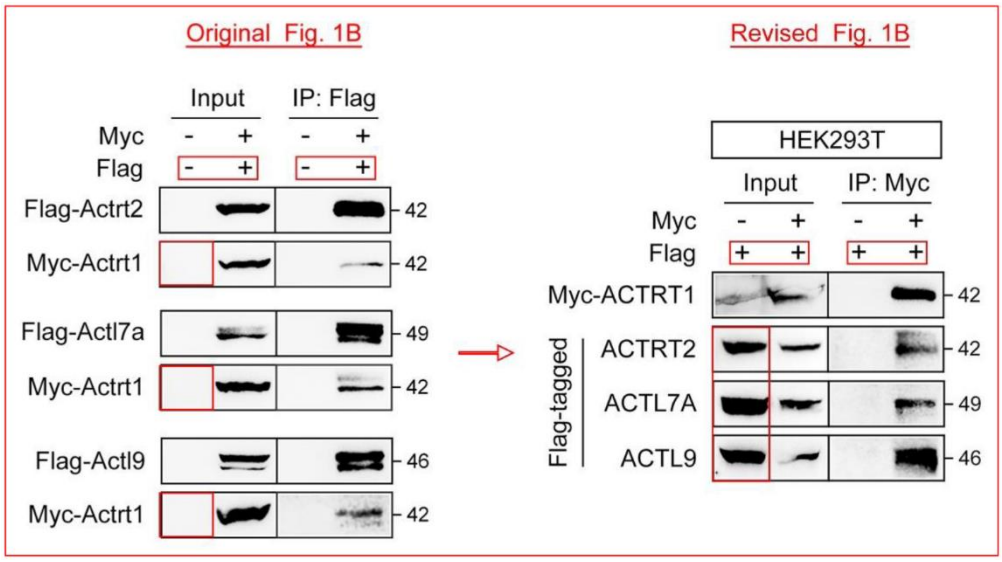


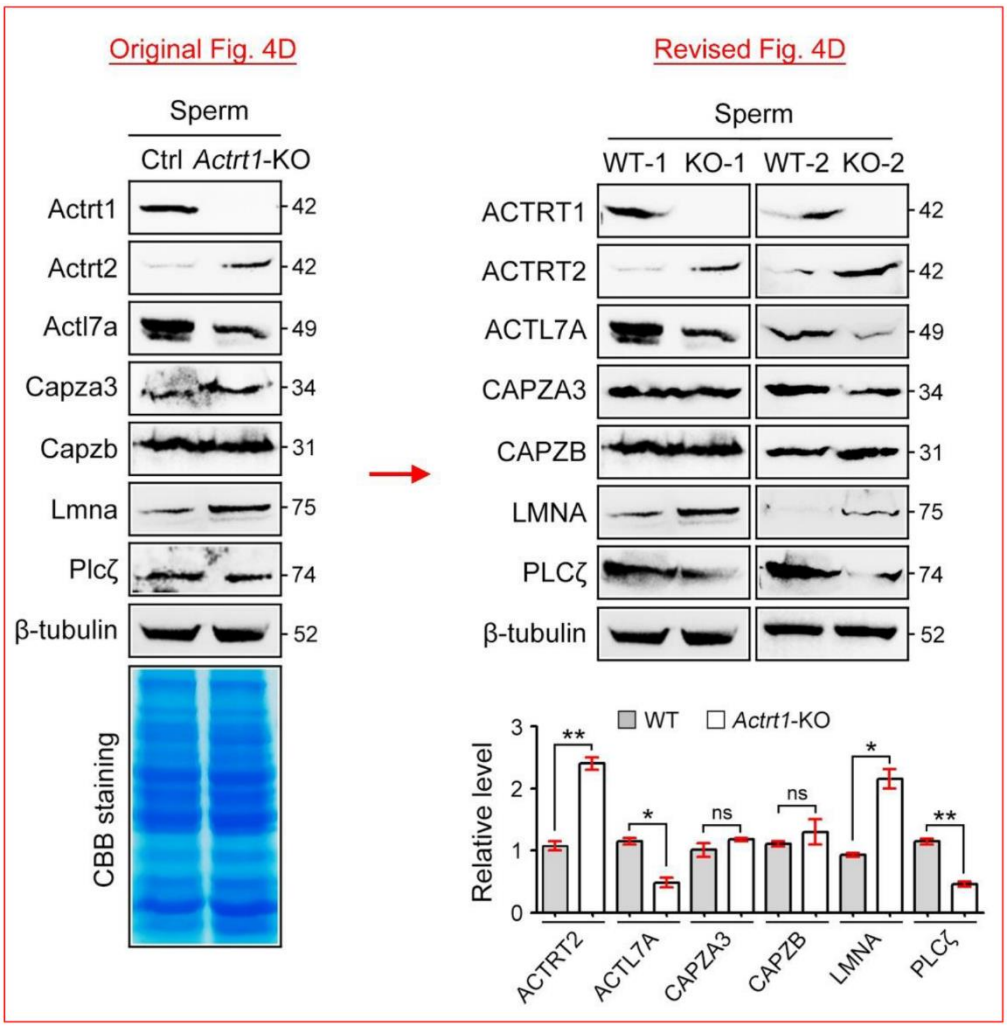
3. It is a little arduous to conciliate "the manchette of *Actrt1*-KO spermatids was largely disorganized as a potential cause of abnormal head shapes (Fig. S2C)" (page 6), with "Double immunofluorescence (IF) staining of sperm using PNA-FITC (an acrosome dye) and mitotracker red (a mitochondria dye) further revealed normal assemble of mitochondrial sheath", bottom page 5, considering also the unique TEM image provided for a spermatozoon (Fig. 3 E) showing disorganization, if not absence, of a mitochondria tail sheath. By the way, PNA-FITC and mitotracker red are not immunolabeling.
We appreciate your concerns and have made the following modifications. 1) the manchette of *Actrt1*-KO spermatids was largely disorganized as a potential cause of abnormal head shapes (Fig. S2C) → the manchette of *Actrt1*-KO spermatids was largely disorganized (Fig. S2C). 2) double immunofluorescence (IF) staining of sperm using PNA-FITC (an acrosome dye) and mitotracker red (a mitochondria dye) further revealed normal assemble of mitochondrial sheath but a disturbance of typical crescent moon shape of acrosome in *Actrt1*-KO mice (Fig. 3C) → double staining of sperm using PNA-FITC (an acrosome dye) and MitoTracker red (a mitochondria dye) further revealed a disturbance of the typical crescent moon shape of the acrosome in *Actrt1*-KO mice (Fig. 3C).

4. Figure legends are not clear and exhaustive, and sometimes legends do not correspond to what is shown (example, Legend of Fig. 1C and the Fig. 1C).
As suggested, we modified all figure legends and added more detail information.

We truly appreciate this reviewer's constructive comments. Our manuscript was much improved after careful consideration of your suggestions.

Taking the opportunity for revision, the whole manuscript was carefully modified by us and polished using the English editing services of AJE. In addition to the reviewers' constructive suggestions, we repeated some of the experiments and made some changes as follows. 1) We realize the control group (empty vectors) of Co-IP experiments in HEK293T cells was not proper. In the revised version, we repeated the experiments shown in Fig. 1B and Fig. 5A. If Flag antibody was utilized to immunoprecipitate Flag-tagged protein A, Myc-tagged protein B was transfected into both the control and IP groups. Then, we determined the co-immunoprecipitation of protein B was specific (*please see the figure below*). 2) The WB study of PT proteins between wild-type sperm and *Actrt1*-KO sperm was repeated, and statistical analysis was performed (*please see revised Fig. 4D*).





Second decision letter

MS ID#: DEVELOP/2021/200489

MS TITLE: Loss of perinuclear theca ACTRT1 causes acrosome detachment and severe male subfertility

AUTHORS: Xiao-Zhen Zhang, Lin-Lin Wei, Xiao-Hui Zhang, Hui-Juan Jin, and Su-Ren Chen

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. If you do not agree with any of their criticisms or suggestions explain clearly why this is so. If it would be helpful, you are welcome to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating your plans for addressing the referee’s comments, and we will look over this and provide further guidance.

Reviewer 1

Advance summary and potential significance to field

This paper provides new evidence for the functional role of ACTRT1 in spermiogenesis. The Actrt1 KO males showed subfertility due to malformed sperm heads, and detachment of acrosome from sperm nucleus probably because of the loosened acroplaxome structure. The interaction between inner acrosomal membrane protein SPACA1 and nuclear envelope protein PARP11 was observed, indicating a potential role of ACTRT1 in anchoring the developing acrosomes to nuclei. This paper reported different results and drew a different conclusion on the function of ACTRT1 in mouse spermiogenesis from a published paper.

Comments for the author

All my concerns are answered, and basically I am satisfied with their answers.
Several experiments, such as like Co-IP, were repeated properly, which has improved this manuscript.
However, I have a new question about the localization of ACTRT1 in mature sperm.
Since other sperm-specific ARP complex proteins like ACTL7A localized to the acrosomes in mature sperm, why ACTRT1 is translocated to the postacrosomal sheath?
It would be better to discuss this in the revision.

Reviewer 2

Advance summary and potential significance to field

The authors found that loss of Actrt1 caused a significantly high incidence of malformed heads and especially the detachment of acrosome from sperm nucleus. Although this is different from another recently reported data of Actrt1-KO mice. Overall, this manuscript presented here is comprehensive, and is well-referenced and the phenotypic data in this manuscript are solid.

Comments for the author

There are no additional suggestions.

Reviewer 3

Advance summary and potential significance to field

The authors have provided sufficiently satisfactory replies to the raised points with the revised version of the manuscript, although some perplexities (see below some exemplification) still remain.
Materials and Methods section has been enriched with the due information and, in general, the entire revised manuscript has been improved consistently.
The potential significance of the paper to the filed is moderate

Comments for the author

Few examples of perplexities:
Fig.1B and Fig. S1, IF. I am not able to explain to me why Fig. 1B shows elongated testis spermatids of Actrt1-KO mice (Fig. 1B, right panel) so morphologically similar to the wild type elongated spermatids (left panel), with their acrosomes anchored to the nucleus. Even epididymal sperm of Actrt1-KO mice (Fig. S1) are very similar to the wild type ones. But are not Actrt1-KO mice severely subfertile because they have the acrosome detached and malformed heads?
Moreover, I think that an explanation, or at least a suggestion, has to be given about the finding that ACTRT1 is reported to be subacrosomal in elongated mature spermatids whereas it is found suddenly at the postacrosomal sheath in epididymal (not acrosome-reacted) sperm.
Fig 1D (and the figures of immunoprecipitates in general). The heavy chain (around the 50 kDa) of antibodies in both the preimmune serum (IgG) and immune sera is never present. The lanes loaded with IgG are completely white. This is apparently strange, if not explained, considering also that the blots show just the portion near the 50 kDa.
Table S1. It is possible to realize now that the region used to delete Actrt1 is very large, well upstream and downstream to the coding sequence, in line with what I noticed already in my previous revision.
Expression plasmids. The pUC57 plasmid previously reported has been deleted in the revised ms. The due TEM procedure, previously neglected, is reported now.
I do not find how many protein micrograms were loaded/lane/gel also in the revised version.
About my previous observation on Authors' sentences like "although the protein constituents of PT are well-studied during the last twenty years, the physiological roles of PT remain largely unclear due to a lack of knockout/mutated mouse models" , I keep on thinking that the physiological roles of PT proteins cannot be established by gene knockout mouse models only, like strongly reaffirmed by the authors in their reply letters. It is sufficient and explanatory the case here reported. The knockouts of the same gene, Actr1, in the same animal model, mouse, give rise to two different phenotypes (Sha et al., 2021 and the submitted manuscript).
As to another previous observation (I have some doubts that all the proteins here reported to interact directly with Actrt1 (Actrt2, Actl7a, Actl9, Spaca1, Tmco2, Parp11, Sun 4 and others) effectively interact), I remain with such an opinion notwithstanding the authors' sentence (It is well accepted that co-IP is a commonly used method to study protein–protein interactions). Co-IP carried out as it is now reported here (mild detergent conditions) can show protein complexes, i.e., indirect protein-protein interactions. A direct protein-protein interaction is provided as suggested in my previous revision

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Second revision

Author response to reviewers' comments

Reviewer 1

Advance summary and potential significance to field

This paper provides new evidence for the functional role of ACTRT1 in spermiogenesis. The *Actrt1*-KO males showed subfertility due to malformed sperm heads, and detachment of acrosome from sperm nucleus probably because of the loosened acroplaxome structure. The interaction between

inner acrosomal membrane protein SPACA1 and nuclear envelope protein PARP11 was observed, indicating a potential role of ACTRT1 in anchoring the developing acrosomes to nuclei. This paper reported different results and drew a different conclusion on the function of ACTRT1 in mouse spermiogenesis from a published paper.

Comments for the author

All my concerns are answered, and basically I am satisfied with their answers. Several experiments, such as like Co-IP, were repeated properly, which has improved this manuscript.

We would like to thank the reviewer for the positive comments.

1. However, I have a new question about the localization of ACTRT1 in mature sperm. Since other sperm-specific ARP complex proteins like ACTL7A localized to the acrosomes in mature sperm, why ACTRT1 is translocated to the postacrosomal sheath? It would be better to discuss this in the revision.
   In Fig. 1B, we showed that both ACTRT1 and ACTL7A were localized to the subacrosomal region (SAR) of testicular spermatids. In Fig. S1B, we showed that both ACTRT1 and ACTL7A were translocated to the postacrosomal sheath (PAS) of spermatozoa. Although PAS proteins are proposed to be synthesized in the cytoplasmic lobe of elongating spermatids and transported up the manchette for final deposition and assembly in the postacrosomal region (Oko and Sutovsky, 2009), some PAS proteins, such as CCIN (Longo et al., 1987; Lecuyer et al., 2000), CAPZA3 (Geyer et al., 2009), ACTRT1 (Heid et al., 2002; this study), and ACTL7A (Boeda et al., 2011; this study), are present in the SAL of spermatids and then translocated to the PAS of spermatozoa (P5, line 4~10).

Reviewer 2

Advance summary and potential significance to field

The authors found that loss of Actrt1 caused a significantly high incidence of malformed heads and especially the detachment of acrosome from sperm nucleus. Although this is different from another recently reported data of *Actrt1*-KO mice. Overall, this manuscript presented here is comprehensive, and is well-referenced and the phenotypic data in this manuscript are solid.

Comments for the author

There are no additional suggestions.

We would like to thank the reviewer for the positive comments.

Reviewer 3

Advance summary and potential significance to field
The authors have provided sufficiently satisfactory replies to the raised points with the revised version of the manuscript, although some perplexities (see below some exemplification) still remain. Materials and Methods section has been enriched with the due information and, in general, the entire revised manuscript has been improved consistently. The potential significance of the paper to the field is moderate.

We would like to thank the reviewer for the positive comments.

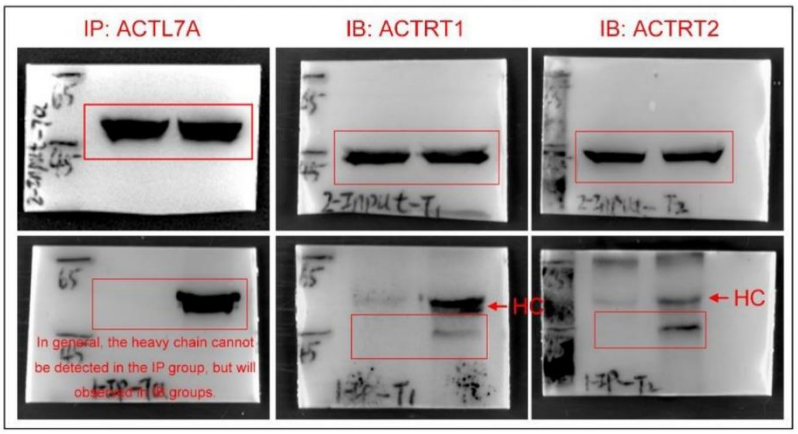
Comments for the author

1. Fig.1B and Fig. S1, IF. I am not able to explain to me why Fig. 1B shows elongated testis spermatids of *Actrt1*-KO mice so morphologically similar to the wild type elongated spermatids, with their acrosomes anchored to the nucleus. Even epididymal sperm of *Actrt1*-KO mice are very similar to the wild type ones. But are not *Actrt1*-KO mice severely subfertile because they have the acrosome detached and malformed heads? Moreover, I think that an explanation, or at least a suggestion, has to be given about the finding that ACTRT1 is reported to be subacrosomal in elongated mature spermatids whereas it is found suddenly at the postacrosomal sheath in epididymal sperm.

We appreciate your concerns. First, not all spermatozoa of *Actrt1*-KO mice showed detached acrosome and we took photos randomly. The ratio of detached acrosomes in *Actrt1*-KO sperm by PNA-FITC staining was ~40% (Fig. 3C). The number of sperm with detached acrosomes in *Actrt1*-KO males was ~75%, owing to the high resolution of TEM (Fig. 3E). For differentiating spermatids, a loosened acroplaxome structure was observed only by TEM (Fig. 4A). Second, we provided an explanation "PAS proteins are suggested to be synthesized in the cytoplasmic lobe of elongating spermatids and transported up the manchette for final deposition and assembly in the PAR (Oko and Sutovsky, 2009); however, some PAS proteins, such as Calicin (Longo et al., 1987; Lecuyer et al., 2000), CAPZA3 (Geyer et al., 2009), ACTRT1 (Heid et al., 2002; this study), and ACTL7A (Boeda et al., 2011; this study), are present in the acroplaxome of spermatids and then translocated to the PAS of spermatozoa (P5, line 4~10)". Third, we moved the negative controls (*Actrt1*-KO) from Fig. 1B to Fig. S1A because we started to describe the *Actrt1*-KO mice from Fig. 2.

2. Fig 1D (and the figures of immunoprecipitates in general). The heavy chain (around the 50 kDa) of antibodies in both the preimmune serum (IgG) and immune sera is never present. The lanes loaded with IgG are completely white. This is apparently strange, if not explained, considering also that the blots show just the portion near the 50 kDa.
Thank you for your concern. We have provided the original blots in Supplementary Material in the previous revision.

Fig. 1D original blots that have provided in Supplementary Material.



3. Table S1. It is possible to realize now that the region used to delete *Actrt1* is very large, well upstream and downstream to the coding sequence, in line with what I noticed already in my previous revision. Expression plasmids. The pUC57 plasmid previously reported has been deleted in the revised MS. The due TEM procedure, previously neglected, is reported now. I do not find how many protein micrograms were loaded/lane/gel also in the revised version.
Flag- or Myc-tagged pCMV vectors (Beyotime, China) were utilized to construct expression plasmids. We feel sorry that we provided wrong information of plasmid (pUC57) in original submission. For WB and input of IP, we loaded ~25 μg protein per lane. For IP experiments, we added 30 μl 1.2×SDS loading buffer to beads and loaded all protein solution after boiling for denaturation.
4. About my previous observation on authors' sentences like "although the protein constituents of PT are well-studied during the last twenty years, the physiological roles of PT remain largely unclear due to a lack of knockout/mutated mouse models", I keep on thinking that the physiological roles of PT proteins cannot be established by gene knockout mouse models only, like strongly reaffirmed by the authors in their reply letters. It is sufficient and explanatory the case here reported. The knockouts of the same gene, Actrt1, in the same animal model, mouse, give rise to two different phenotypes (Sha et al., 2021 and the submitted manuscript).
We agree with the reviewer that the physiological roles of PT proteins cannot be established by gene knockout mouse models ONLY.
5. As to another previous observation (I have some doubts that all the proteins here reported to interact directly with Actrt1 (Actrt2, Actl7a, Actl9, Spaca1, Tmco2, Parp11, Sun 4 and others) effectively interact), I remain with such an opinion notwithstanding the authors' sentence (It is well accepted that co-IP is a commonly used method to study protein-protein interactions).

Co-IP carried out as it is now reported here (mild detergent conditions) can show protein complexes, i.e., indirect protein-protein interactions. A direct protein-protein interaction is provided as suggested in my previous revision.
We feel sorry that GST-pull down and yeast two-hybrid can't be formed in our lab at the current stage. In this study, we have performed the *in vitro* and endogenous co-IP to study ACTRT1-interacting proteins. We wrote 'whether the protein-protein interaction is direct or indirect needs further investigations' in revised MS (P10, line 19~20).

Third decision letter

MS ID#: DEVELOP/2021/200489

MS TITLE: Loss of perinuclear theca ACTRT1 causes acrosome detachment and severe male subfertility

AUTHORS: Xiao-Zhen Zhang, Lin-Lin Wei, Xiao-Hui Zhang, Hui-Juan Jin, and Su-Ren Chen
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.