

Spermatozoa lacking TEX51 display hypofertility and defects in morphology

Corresponding Author: Dr Sandrine Barbaux

Parts of this Peer Review File have been redacted as indicated.

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

Summary of the manuscript

The manuscript by Dupuis et al., titled "Spermatozoa lacking TEX51 display hypofertility and defects in morphology", identified the gene TEX51 encoding a protein with structural similarity to the previously defined IST (Izumo1-Spaca6-Tmem95) superfamily proteins. They hypothesized that this testis-specific gene might play a role in fertilization like other IST proteins. They characterized the fertility phenotypes in the TEX51 knockout (KO) mice. KO males show reduced overall fertility, measured by reduced average litter sizes. In vivo and in vitro fertilization assay show that KO spermatozoa are less capable of fertilizing the oocytes. Testis morphology, sperm count, and sperm motility seemed normal in KO males. Using light and electron microscopy, the authors identified abnormal morphology of KO sperm, including disjunctions between midpiece and principal piece, cytoplasmic remnants on the flagella, and asymmetrically positioned acrosomes. They concluded that the TEX51 gene functions in sperm formation, which implies a broader role of the IST family in fertility.

Overall impression

The predicted structure of a previously uncharacterized protein TEX51, its structural comparison to other IST proteins, and the analyses on the gene function together provide new and useful information for the research community who study fertilization or gamete development. Data presented in this paper support the conclusions well.

Specific comments

1. The authors showed fertility defects and morphological defects in sperm in KO mice. The two KO strains have deletions in slightly different regions and show consistent phenotypes. This provides support that the defects are unlikely due to non-specific targeting effect by Crispr-Cas9. However, given that the deleted regions are around 4500 bp, a transgenic rescue experiment will help strengthen the argument that the defects shown here are due to the loss of function of the TEX51 gene, not due to loss of any other elements (like ncRNAs, if any) in the region.
2. Figure 3a showed the individual data points for KO male fertility, measured by litter sizes. The data points fall into two groups, one within the WT range, and one completely at 0, suggesting sterility. First, based on the distribution, a non-parametric statistical test may be more appropriate than the t-test mentioned in methods. Second, does this distribution reflect variation among individual KO males, or variation among mating trials? Whichever the possibility is, does the same variation exist in in vivo and in vitro fertilization assays presented in Figure 3b-d? It would be helpful to add more details on the observations, and an interpretation of the variation.
3. Figure 6b shows the quantification of abnormal morphology in mutant sperm. According to the main text, page 7 line 138, cytoplasmic remnants are seen in 49% of WT sperm. However, the bar in the graph in 6b looks more like 60%. Moreover, the top right image in 6a seems to show only flagella without cytoplasmic remnants, which is not representative of a 49% (or 60%) with remnants.
4. In supplemental Figure 3, the DNA gel image in the middle panel (TEX51 Ex6F-Ex8R) did not show the band from WT gDNA, which, according to supplemental Table 2, should be 597bp. The DNA gel image on the right (Sdha F-R) shows band sizes (between 75bp and 200bp) that are inconsistent with expected sizes as in supplemental Table 2 (106bp for cDNA, 1458bp for gDNA).

Reviewer #3

(Remarks to the Author)

The authors of this paper utilized gene-editing technology to discover that Tex51-KO mice exhibited reduced fertility, along with sperm head and flagellar abnormalities. IVF results indicated a decreased fertilization rate. However, the work presented here appears to be largely limited to phenotypic studies, making it difficult to discern how TEX51 functions mechanistically based on the current experimental results.

Specific Points:

1. The first and second paragraphs should be combined for better flow and coherence.
2. More logical support is needed to substantiate this prediction. Key points to address include:
 - o What functional domains characterize IST-superfamily members?
 - o What has been reported regarding the roles of these domains?
 - o Which structural features of TEX51 are similar to other IST-superfamily members, and which are distinct?
 - o What are the potential implications of these similarities or differences?
3. Provide more detailed information on the precise localization of TEX51 within sperm cells. How is TEX51 distributed during spermatogenesis?
4. How does TEX51 participate in sperm fertilization activities? What experimental evidence supports this?
5. How can the tail defects observed in Tex51-KO sperm be explained? A mechanistic discussion would strengthen the interpretation of these findings.
6. Figure 5 – The sperm images of Tex51-KO and WT appear to be at different magnifications, yet the scale bars are identical. This discrepancy should be clarified to ensure accurate representation and comparison.

Reviewer #4

(Remarks to the Author)

The manuscript entitled "Spermatozoa lacking TEX51 display hypofertility and defects in morphology" explores the role of TEX51 as a newly identified member of the IST-superfamily proteins. Using a Tex51-knockout (KO) mouse model, the authors demonstrate that TEX51 is essential for optimal male fertility, as KO males exhibit hypofertility despite normal sperm motility. Morphological abnormalities, including defects in acrosome anchoring and flagellar structure, suggest a broader role for TEX51 in sperm development. The study is well-written and provides valuable insights without making over-conclusive statements. However, additional functional assays, such as evaluating the acrosome reaction and sperm hyperactivation, would enhance the mechanistic understanding of TEX51's contribution to fertilization.

Comments:

- 1) Given the observed morphological abnormalities in Tex51-KO sperm, particularly in the acrosome and flagellum, additional functional assays could help clarify the mechanistic role of TEX51 in fertilization. Specifically, assessing the acrosome reaction in Tex51-KO sperm would provide insights into whether the acrosomal defects impact its exocytosis and function. Similarly, since CASA data is available, reporting whether hyperactivation (or its parameters like ALH, VCL, LIN and STR) is affected would be valuable to determine if the flagellar anomalies translate into functional motility defects. Including these analyses could strengthen the understanding of TEX51's role in sperm function and fertilization.
- 2) For clarity and reproducibility, it would be beneficial to include as supplementary information the complete list of candidate proteins identified in the Dali Server AlphaFold database search. This would help readers assess the selection criteria and explore other potential functional insights.
- 3) The manuscript mentions that TEX51 was identified through manual inspection of candidate proteins. Could the authors elaborate on the specific criteria used during this process? Understanding how TEX51 was distinguished from other Ig-containing proteins would provide valuable insight into the selection rationale.

Minor:

- 1) Figure 3 Figure Legend: please replace in (d) males for sperm. Please move "Numbers in brackets represent the number of observed oocytes." to the end of the whole figure legend as this info goes for (b), (c) and (d), to make it clearer.
- 2) It would be more informative to show Figure 3 (b), (c) and (d) with the values for each experiment, as it is shown in (a).
- 3) Discussion, line 157: regarding the morphology defects found in the Tex51-KO sperm, as they probably appear at the spermiogenesis step, and that could be considered a developmental issue, I suggest clarifying this.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The changes made in the revisions have strengthened the paper. One specific comment on the possible lncRNA in the region:

Mammalian testis expresses high levels of long non-coding RNAs. A small number of lncRNAs have been shown to function in the testis, for example, by affecting spermatogonial stem cell renewal or sperm count. (examples: PMID: 29044429, 26962690, review PMID: 39940895). If the lncRNA Gm44577 is indeed close to Tex51, both KO alleles would have deleted Gm44577 promoter. It may be helpful to include discussions about the specificity of the phenotype and possible location of the lncRNA in the text, so the reader has all the information available to them. Additionally, RT-PCR from KO and control samples may help rule out that the lncRNA is affected.

Reviewer #3

(Remarks to the Author)

The authors have not conducted supplementary experiments addressing the core issues I raised, including in vitro fertilization, protein localization, and further exploration of the mechanisms. I do not believe this revision has substantially improved the manuscript.

Reviewer #4

(Remarks to the Author)

I believe that this manuscript has been improved from the original version. The authors did a good job addressing the concerns we raised in the first round. I do have one minor comment: in Supplementary Table 2 the values are expressed in mm, which I believe is an error—they should be in micrometers.

In particular, regarding the question of whether the authors have adequately addressed the identity of the causal element within their deleted region, I think that although they did not perform the transgenic rescue experiment, they provide a rationale to support that the observed phenotype is due to the absence of Tex51.

Version 2:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The authors have addressed previous concerns thoroughly. No additional comments.

Reviewer #4

(Remarks to the Author)

I believe that my concerns have been adequately addressed, and the manuscript has improved as a result. The authors have provided satisfactory clarifications and additional data where necessary.

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The authors are grateful to all reviewers for their work and remarks that will help to improve the manuscript.

Reviewer #1 (Remarks to the Author):

Summary of the manuscript

The manuscript by Dupuis et al., titled "Spermatozoa lacking TEX51 display hypofertility and defects in morphology", identified the gene TEX51 encoding a protein with structural similarity to the previously defined IST (Izumo1-Spaca6-Tmem95) superfamily proteins. They hypothesized that this testis-specific gene might play a role in fertilization like other IST proteins. They characterized the fertility phenotypes in the TEX51 knockout (KO) mice. KO males show reduced overall fertility, measured by reduced average litter sizes. In vivo and in vitro fertilization assay show that KO spermatozoa are less capable of fertilizing the oocytes. Testis morphology, sperm count, and sperm motility seemed normal in KO males. Using light and electron microscopy, the authors identified abnormal morphology of KO sperm, including disjunctions between midpiece and principal piece, cytoplasmic remnants on the flagella, and asymmetrically positioned acrosomes. They concluded that the TEX51 gene functions in sperm formation, which implies a broader role of the IST family in fertility.

Overall impression

The predicted structure of a previously uncharacterized protein TEX51, its structural comparison to other IST proteins, and the analyses on the gene function together provide new and useful information for the research community who study fertilization or gamete development. Data presented in this paper support the conclusions well.

Specific comments

1. The authors showed fertility defects and morphological defects in sperm in KO mice. The two KO strains have deletions in slightly different regions and show consistent phenotypes. This provides support that the defects are unlikely due to non-specific targeting effect by Crispr-Cas9. However, given that the deleted regions are around 4500 bp, a transgenic rescue experiment will help strengthen the argument that the defects shown here are due to the loss of function of the TEX51 gene, not due to loss of any other elements (like ncRNAs, if any) in the region.

We agree with Reviewer #1 that a transgenic rescue would be the most elegant way to confirm that the gene under study is solely responsible for the phenotype. However, this experiment is time-consuming and additional transgenesis is not an easy procedure.

Near the *Tex51* gene, a lncRNA is present, *Gm44577*, transcribed in the opposite direction. There seems to be discrepancies between databases about their relative positions. They are very close in Ensembl, with one of our deletions removing the first 18 nt of *Gm44577* while the other does not affect its exons.

[redacted]

In NCBI, both genes seem to be more distant with more than 40 kb between them.

[redacted]

Regardless of the deletion and the position of both genes, the phenotypes of both KO were identical.

In addition, available expression data about Gm44577 describes a gene expressed in testis but also in other organs. However, our KO mice display only a testicular phenotype. Based on this, we are confident that the observed phenotype is due to the absence of Tex51.

2. Figure 3a showed the individual data points for KO male fertility, measured by litter sizes. The data points fall into two groups, one within the WT range, and one completely at 0, suggesting sterility. First, based on the distribution, a non-parametric statistical test may be more appropriate than the t-test mentioned in methods. Second, does this distribution reflect variation among individual KO males, or variation among mating trials? Whichever the possibility is, does the same variation exist in in vivo and in vitro fertilization assays presented in Figure 3b-d? It would be helpful to add more details on the observations, and an interpretation of the variation.

We thank the reviewer for the suggestion to use a non-parametric statistical test. As recommended, the statistical test has been revised, and the data now includes results from new litters that arrived after the submission. In both KO lines, some males exhibited variability in mating outcomes during successive matings, with some litters having pups and others showing no pups, despite a confirmed vaginal plug. This information has been added to the text.

The intensity of the phenotype could be different depending on successive spermatogenesis waves.

It is difficult to compare for a given male, some in vivo fertility results (corresponding to successive matings taking place during different spermatogenesis waves) with one single in vitro fertilization result (occurring inevitably only once and at a later spermatogenesis wave, also inevitably different from the previous waves used for in vivo fertility).

The most significant impact is observed in cumulus-intact IVF where the environment is artificial and sub-optimal, requiring sperm to cross all natural barriers (e.g. cumulus, ZP and oolemma).

3. Figure 6b shows the quantification of abnormal morphology in mutant sperm. According to the main text, page 7 line 138, cytoplasmic remnants are seen in 49% of WT sperm. However, the bar in the graph in 6b looks more like 60%. Moreover, the top right image in 6a seems to show only flagella without cytoplasmic remnants, which is not representative of a 49% (or 60%) with remnants.

Reviewer #1 is correct: we apologize for the typo. Cytoplasmic remnants are present in 60% (59.18%) after the observed sperm (n=49).

Regarding the absence of cytoplasmic remnants in upper panel of Figure 6a, we apologize for the confusion. The 3 sperm flagella cut longitudinally and located on the right of the image do not contain cytoplasmic remnants. However, the 3 sperm flagella on the top left, cut transversally, do show pockets of cytoplasm around the axoneme. We have added red arrows to the panel for clarity.

4. In supplemental Figure 3, the DNA gel image in the middle panel (TEX51 Ex6F-Ex8R) did not show the band from WT gDNA, which, according to supplemental Table 2, should be 597bp. The DNA gel image on the right (Sdha F-R) shows band sizes (between 75bp and 200bp) that are inconsistent with expected sizes as in supplemental Table 2 (106bp for cDNA, 1458bp for gDNA).

The gDNA wells were tested as an additional control to confirm which bands they produce and ensure that these bands were absent from the cDNA wells, thus verifying an absence of gDNA contamination in the cDNA samples. While this precaution is somewhat redundant since: 1) RNA was DNase-treated before reverse transcription, and 2) PCR primers for cDNA amplification are always located in different exons, we nonetheless repeated the PCR amplification at higher temperatures and with longer elongation times to minimize non-specific bands and to amplify longer fragments. An updated figure is included (Supplementary Fig 3). The 1.5 kb genomic Sdha band is present, though weak, in the KO samples.

Reviewer #3 (Remarks to the Author):

The authors of this paper utilized gene-editing technology to discover that Tex51-KO mice exhibited reduced fertility, along with sperm head and flagellar abnormalities. IVF results indicated a decreased fertilization rate. However, the work presented here appears to be largely limited to phenotypic studies, making it difficult to discern how TEX51 functions mechanistically based on the current experimental results.

Specific Points:

1. The first and second paragraphs should be combined for better flow and coherence.

We tried to combine the first and second paragraphs, however the flow and coherence did not improve. A combined paragraph was too long. We wanted the first paragraph to introduce some of the critical protein players involved in fertilization and the second paragraph to describe the IST superfamily of proteins. Thus, we have left the structure of the introduction the same. However, based on comments from other reviewers, we have included additional details. We hope that our rewritten introduction now has better flow and coherence for Reviewer #3.

2. More logical support is needed to substantiate this prediction. Key points to address include:

We thank Reviewer #3 for this comment and agree that additional support would be helpful. We have now expanded the Discussion describing the features of the IST superfamily. Table 1 also summarizes the IST superfamily members and their roles in reproductive biology.

o What functional domains characterize IST-superfamily members?

Members of the IST superfamily have the following features:

- 4-helix bundle;
- β -hinge;
- two CXXC motifs;
- Ig-like domain with a rare 2+5 stranded topology (strand I- *b,e*; strand II- *a,g,f,c,c'*)

o What has been reported regarding the roles of these domains?

There have been little reported regarding these domains in fertilization. However, it has been reported that the 4-helix bundle shares structural similarity with SPECT1, a Plasmodium protein involved in host cell traversal and possibly for pore formation by highly motile sporozoites prior to invading hepatocytes. The β -hinge was reported to be similar to another Plasmodium protein (TRAP) that is involved in host cell invasion. CXXC motifs are known redox sensors and have been implicated in viral membrane fusion.

o Which structural features of TEX51 are similar to other IST-superfamily members, and which are distinct?

TEX51 is most similar to TMEM95, IZUMO2, IZUMO3 and IZUMO4. These proteins all have the 4-helix bundle with the two pairs of CXXC motifs, but they lack the Ig-like domain that is observed in IZUMO1, SPACA6 and TMEM81.

o What are the potential implications of these similarities or differences?

We initially suspected that TEX51 might have a similar function to IZUMO1, TMEM95 and SPACA6 and have an involvement in gamete interaction or fusion. It appears TEX51's function is more closely related to IZUMO3, which is also expressed in early and late spermatids and is critical in spermatogenesis (acrosome biogenesis).

3. Provide more detailed information on the precise localization of TEX51 within sperm cells. How is TEX51 distributed during spermatogenesis?

Unfortunately, there is no available antibody to analyze this precisely. However, numerous transcriptomic and proteomic studies on isolated cells from different stages of spermatogenesis consistently show an increase in expression level (with concordance between RNA and protein levels) during sperm differentiation (references added in text). To complement Supplementary Figure 1, we have included panel 1d from ProteinAtlas, which demonstrates high mRNA expression in both early and late spermatids.

4. How does TEX51 participate in sperm fertilization activities? What experimental evidence supports this?

The effect on fertilization appears to be indirect. *Tex51*-KO sperm exhibit structural defects that impair certain sperm functions, which will require further analysis.

5. How can the tail defects observed in *Tex51*-KO sperm be explained? A mechanistic discussion would strengthen the interpretation of these findings.

We observed a small fraction of sperm with bent flagellum in the KO, both in capacitated and non-capacitated sperm. Unfortunately, our CASA equipment is not designed to detect hyperactivated sperm. Our hypothesis is that this fragility may be more pronounced in hyperactivated sperm, which exhibit intense beating. Since hyperactivation is a necessary step before fertilization, this anomaly could severely affect motility just prior to fertilization. In addition, sperm that have not completely eliminated their cytoplasm may be less capable of achieving the proper beating pattern required for optimal fertilization.

As suggested by Reviewer #4, we also conducted acrosome reaction experiments, which confirm that, as seen in our EM studies, the acrosomes are poorly formed and/or poorly attached. A premature and excessive spontaneous acrosome reaction seems to occur in KO sperm, which could further compromise fertilization rates. We have added 2 panels in Figure 6 and expanded the discussion in the text. These cumulative defects may sometimes be compatible with fertilization, but other times, they may reach a threshold that compromises fertilization, depending on the timing of spermatogenesis waves.

6. Figure 5 – The sperm images of *Tex51*-KO and WT appear to be at different magnifications, yet the scale bars are identical. This discrepancy should be clarified to ensure accurate representation and comparison.

We agree with Reviewer #2. We apologize for this mistake and have now corrected the figure.

Reviewer #4 (Remarks to the Author):

The manuscript entitled "Spermatozoa lacking TEX51 display hypofertility and defects in morphology" explores the role of TEX51 as a newly identified member of the IST-superfamily proteins. Using a Tex51-knockout (KO) mouse model, the authors demonstrate that TEX51 is essential for optimal male fertility, as KO males exhibit hypofertility despite normal sperm motility. Morphological abnormalities, including defects in acrosome anchoring and flagellar structure, suggest a broader role for TEX51 in sperm development. The study is well-written and provides valuable insights without making over-conclusive statements. However, additional functional assays, such as evaluating the acrosome reaction and sperm hyperactivation, would enhance the mechanistic understanding of TEX51's contribution to fertilization.

Comments:

1) Given the observed morphological abnormalities in Tex51-KO sperm, particularly in the acrosome and flagellum, additional functional assays could help clarify the mechanistic role of TEX51 in fertilization. Specifically, assessing the acrosome reaction in Tex51-KO sperm would provide insights into whether the acrosomal defects impact its exocytosis and function. Similarly, since CASA data is available, reporting whether hyperactivation (or its parameters like ALH, VCL, LIN and STR) is affected would be valuable to determine if the flagellar anomalies translate into functional motility defects. Including these analyses could strengthen the understanding of TEX51's role in sperm function and fertilization.

A Supplementary Table 2 reporting all CASA measurements is now included.

We evaluated the acrosome reaction in WT and KO sperm using IZUMO1 immunolabeling and observed a highly significant difference. This result is discussed in the text and presented in an additional Figure, (now Fig 6c and d), which includes representative images and a count of intact, reacted and abnormal sperm. We are grateful to Reviewer #4 for suggesting this experiment. The results provide a more precise understanding of the phenotype, offering clues into the potential causes of infertility in *Tex51*-KO mice

2) For clarity and reproducibility, it would be beneficial to include as supplementary information the complete list of candidate proteins identified in the Dali Server AlphaFold database search. This would help readers assess the selection criteria and explore other potential functional insights.

We have now generated a spreadsheet with the complete list of candidate proteins identified from the Dali Server AlphaFold database search (submitted as Supplementary Data File 1). Proteins belonging to the proposed IST-superfamily are highlighted in yellow within the spreadsheet.

3) The manuscript mentions that TEX51 was identified through manual inspection of candidate proteins. Could the authors elaborate on the specific criteria used during this process? Understanding how TEX51 was distinguished from other Ig-containing proteins would provide valuable insight into the selection rationale.

Reviewer #3 also had comments regarding how we identified new proteins in the IST superfamily.

Members of the IST superfamily have the following features:

- 4-helix bundle;
- β -hinge;
- two pairs of CXXC motifs;
- Ig-like domain with a rare 2+5 stranded topology (strand I- *b,e*; strand II- *a,g,f,c,c'*)

Given that AlphaFold predictions may not always accurately predict the β -stranded topology, we focused on identifying proteins with an anti-parallel 4HB, β -hinge and CXXC motifs. This allowed us to identify TEX51. We have now expanded our explanation of manual inspection in the Results and Methods section of the manuscript.

Minor:

1) Figure 3 Figure Legend: please replace in (d) males for sperm. Please move "Numbers in brackets represent the number of observed oocytes." to the end of the whole figure legend as this info goes for (b), (c) and (d), to make it clearer.

The legend was corrected. The sentence "*Numbers in brackets represent the number of observed oocytes*" was moved to the end of the figure text.

2) It would be more informative to show Figure 3 (b), (c) and (d) with the values for each experiment, as it is shown in (a).

Fig 3b and c report fertilization rates, which represent the percentage of oocytes that are fertilized in each condition. This will be a binary setting where the oocyte is either fertilized or not. Thus, if we were to show all the values for each experiment, it will show on the Y axis a cloud of dots at 0 (unfertilized, 0 sperm) and a cloud at 1 (fertilized by one sperm), with 50-200 dots per cloud. In Fig 3d, the fertilization index reflects the mean number of sperm heads counted that have penetrated the oocyte. Thus, we would also have clouds of dots corresponding to

oocytes with various sperm head numbers. We believe that these alternative representations would be challenging to interpret effectively and that the bar graphs for Figure 3 b,c,d are best shown as is.

3) Discussion, line 157: regarding the morphology defects found in the *Tex51*-KO sperm, as they probably appear at the spermiogenesis step, and that could be considered a developmental issue, I suggest clarifying this.

We thank Reviewer #4 for this suggestion and have now modified the sentence to now read:

Analysis of *in vivo* fertilization and the presence of live births confirmed that the observed defect in *Tex51*-KO mice stemmed from a fertilization issue, not a defect of embryo development.

Reviewers' comments:

We thank all reviewers for their comments and insights.

Reviewer #1 (Remarks to the Author):

The changes made in the revisions have strengthened the paper. One specific comment on the possible lncRNA in the region:

Mammalian testis expresses high levels of long non-coding RNAs. A small number of lncRNAs have been shown to function in the testis, for example, by affecting spermatogonial stem cell renewal or sperm count. (examples: PMID: 29044429, 26962690, review PMID: 39940895). If the lncRNA Gm44577 is indeed close to Tex51, both KO alleles would have deleted Gm44577 promoter. It may be helpful to include discussions about the specificity of the phenotype and possible location of the lncRNA in the text, so the reader has all the information available to them. Additionally, RT-PCR from KO and control samples may help rule out that the lncRNA is affected.

We have included in the text (Results section) the existence and position of this lncRNA. A new additional Supplemental Figure 3 describes the different isoforms found in genomic databases, the proximity between both genes. We have also performed the suggested RT-PCR experiment and we also show the results in this new figure. The Gm44577 isoforms that present exons close to Tex51 and that do express in a WT testis sample, are lost in both KO lines. The deletions likely remove this proximal promoter. Another isoform, that apparently uses a more distal and different promoter, is present in both WT and KO samples. Our conclusion is therefore a partial loss of expression of Gm44577 that could possibly participate to the observed phenotype. We note these results, this limitation and comment it in the Discussion section.

Reviewer #3 (Remarks to the Author):

The authors have not conducted supplementary experiments addressing the core issues I raised, including in vitro fertilization, protein localization, and further exploration of the mechanisms. I do not believe this revision has substantially improved the manuscript.

N/A

Reviewer #4 (Remarks to the Author):

I believe that this manuscript has been improved from the original version. The authors did a good job addressing the concerns we raised in the first round. I do have one minor comment: in Supplementary Table 2 the values are expressed in mm, which I believe is an error—they should be in micrometers.

Yes indeed. We corrected this typing mistake.

In particular, regarding the question of whether the authors have adequately addressed the identity of the causal element within their deleted region, I think that although they did not perform the transgenic rescue experiment, they provide a rationale to support that the observed phenotype is due to the absence of Tex51.