

Peer Review File

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Reviewer A

The study reports the functional characterization of Asb12 in male reproductive tract. While the findings report that Asb12 is dispensable for spermatogenesis and male fertility, a detailed characterisation based on the expression profile is missing. In addition, the writing was not clear and a number of errors were identified.

1. The authors should provide further information on the Asb protein family in the introduction. Is the testis-specific expression limited to several members of the family? What is the function of the Asb protein family in other tissues?

Response: Thanks for this suggestion. In the new revision, we have added a description about the testis-specific expression of Asb and the function of the Asb in other tissues. (see, Page 6, line 117-126)

2. What would be the effect of 1-bp insertion on the protein sequence of ASB12? How does that affect the two conserved domains?

Response: Thanks for this comment. In the new revision, we describe the effects of 1-bp insertion on ASB12 sequence and domains. (see, Page 11, line 219-221 and figure 1B)

3. The results of immunofluorescence staining are not convincing (Fig 1C). Although the authors claim that the signals in more primitive germ cells were non-specific, the overall intensity was weaker than that in the wild type. Therefore, the signals observed in the acrosome could be a false positive result due to the stronger signal intensity but not the knockout of Asb12.

Response: Thanks for the good question. In order to avoid this confusion, we replace the image of KO with a more suitable one. (see, figure 1D)

4. Fig 1D, a low power image is required to show the signals in a representative population of sperm instead of an isolated sperm.

Response: Thanks for this suggestion. In our new revision, we provide a low power image instead of an isolated sperm. (see, figure 1E)

5. Since the authors claimed that ASB12 was expressed in the acrosome, it is surprising that they did not test the capability of the sperm to undergo acrosome reaction in WT and KO mice. It is plausible that the AR is altered in the KO but the fertility is preserved due to the redundancy of a large number of sperm.

Response: We agree with the reviewer's constructive advice. However, no significant change of progesterone-induced acrosome reaction between WT and KO was observed.

See Figure S3.

6. Fig 2I, the axoneme structure in WT and KO are not comparable as the authors have captured a different part of the sperm.

Response: Thanks for the comment. In order to avoid this confusion, we change with a more suitable one.

(see, Fig 2I)

7. Fig 2D, define “M” in the y axis.

Response: We are sorry for this confusion. “M” in the y axis means million. In our new revision, we add the full name in figure legends.

(see, Page 19, line 439)

8. Fig 4J, a discrepancy of gH2Ax signals was observed between WT and KO in leptotene and zygotene stages.

Response: We agree with the reviewer’s advice. In the new revision, we have modified the figure.

(see, figure 4J)

9. Fig 5, among the other Asb family members, which of them is/are specifically expressed in the testis and localised at the acrosome? This would help to identify the family member(s) that compensate for the function of Asb12.

Response: Thanks for the good question. As we mentioned in NEW introduction section, Asb3, Asb4, Asb8, Asb9, and Asb17 were reported to be abundantly expressed in mouse testis. Among them, Asb17 seemed to be located in spermatogonia, since deficiency of Asb17 in mice can prevent spermatogonial apoptosis (Biology (Basel). 2021 Mar 18;10(3):234. PMID: 33803505). However, the distributions of other members (such as Asb3, Asb4, Asb8 and Asb9) in mouse testes are unclear, which need to be explored in the further.

10. A number of grammatical errors warrant English editing.

Response: We agree with the reviewer’s advice. In the new revision, the manuscript has been edited by the Charlesworth company (<https://www.cwauthors.com>). The EDITORIAL CERTIFICATE has also been submitted.

Reviewer B

This manuscript aimed to investigate a testis-enriched gene Asb12, which is a member of the ankyrin repeat and SOCS box (Asb) family. Mouse ASB12 protein was found to be specifically located in the acrosome of elongated spermatids. Asb12 knockout male mice did not display defects in male fertility, sperm morphology, spermatogenesis, and integrity of testis and epididymis. The presented data clearly demonstrate that mouse Asb12 gene is

dispensable for animal development and male reproduction.

These novel findings are very informative to other researchers. Data presentation and analysis are comprehensive.

This reviewer recommends publishing this manuscript after some typo errors have been corrected.

Line 71-72: “several members of the Asb family were significantly elevated in Asb12-KO testes”; rewrite this sentence, such as “gene expression of several Asb family members was significantly elevated in Asb12-KO testes”.

Response: Thanks for the good suggestion. The sentence has been changes as “Gene expression of several Asb family members was increased significantly in the testes of *Asb12*-KO mice when compared with that in *Asb12*-WT mice.....”

(see, Page 3, line 71-73)

Line 110: change “In contrast” to “On the other hand”.

Response: We follow the reviewer’s advice, and the sentence has been corrected as “On the other hand, we have also shown that several genes (*Rai14*, *Tex33*, *Fank1*, and *Eef2k*).....”

(see, Page 5, line 110)

Line 111-112: change “turn out to be dispensable” to “are dispensable”.

Response: We follow the reviewer’s advice, and the sentence has been corrected as “are dispensable for spermatogenesis and male fertility”.

(see, Page 5, line 111-112)

Line 210: change “Taken together, we first constructed” to “Thus, we first successfully constructed”.

Response: We follow the reviewer’s advice, and the sentence has been corrected as “Thus, we first successfully constructed an *Asb12*-KO (-/Y) mouse strain.”

(see, Page 11, line 229-230)

Line 211: change “in mouse testis” to “in mouse sperm”.

Response: We follow the reviewer’s advice, and the sentence has been revised as “The specific location of ASB12 in mouse sperm suggested that ASB12 may be associated with acrosome maturation during spermatogenesis.”

(see, Page 11, line 230-232)

Line 223: change “both Asb12-WT and KO” to “Asb12-KO”.

Response: We follow the reviewer’s advice, and the sentence has been revised as “Ultrastructurally, *Asb12*- KO sperm displayed normal morphology with a well-formed acrosome.....”

(see, Page 12, line 242-243)

Line 265: change “spermatogenesis, using KO models” to “spermatogenesis in KO animal models”.

Response: We follow the reviewer's advice, and the sentence has been revised as "several testis-enriched genes that had been postulated previously to be essential factors for spermatogenesis have been shown to be unnecessary for spermatogenesis in KO animal models....."

(see, Page 14, line 287-289)

Line 276: cross out "individually".

Response: We follow the reviewer's advice, and "individually" has been removed from the context.

(see, Page 14, line 301)

Line 292: cross out "In conclusion".

Response: We follow the reviewer's advice, and "In conclusion" has been removed from the context.

(see, Page 15, line 316)

Reviewer C

The manuscript by Zhang et al reports the knock-out of the *Asb12* gene in mouse. This work was motivated by a testis-enriched expression of the gene and the homology to other *Asb* genes involved in spermatogenesis. Though an extensive exploration of fertility phenotypes, no effect of the absence of the ASB12 protein could be detected. Although the study did not bring the expected results, the dispensable role of *Asb12* deserves to be published.

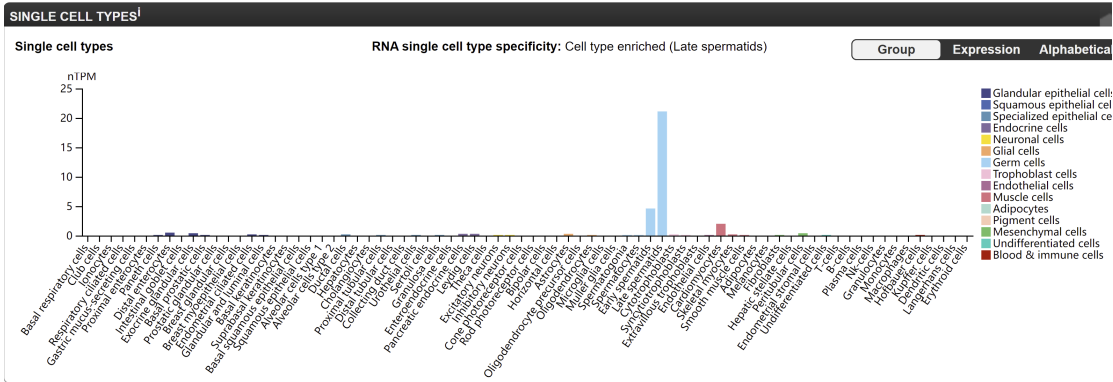
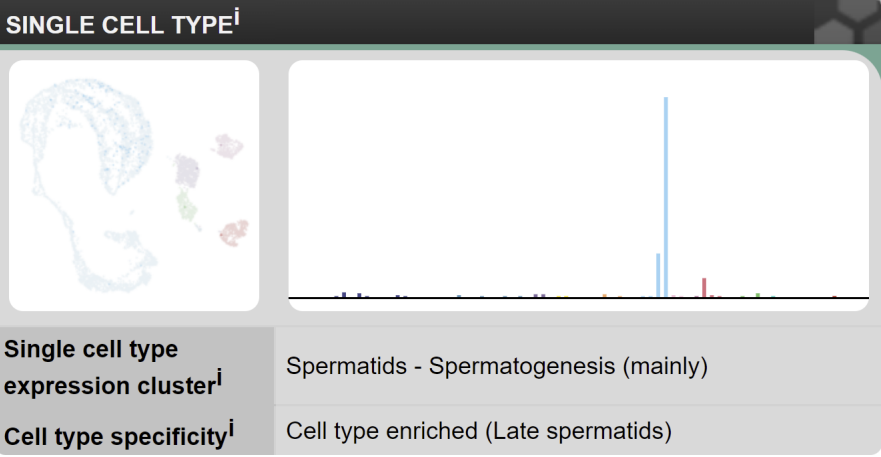
However, some important concerns should be corrected first.

1-To prove the absence of the product of the invalidated gene in a KO, a Western blot is usually produced. Here, the authors only provide immunostaining figures of testis slides and sperm. These data are less convincing than a Western because:

- It is less quantitative.
- There is a low labeling that is still visible in the KO, considered as not specific.
- The identity of the targeted protein is not proven by its molecular weight.
- Cells transfected with a construct expressing the protein could unambiguously validate the specificity of the antibody.

Response: Thanks for the good question. We have used nearly all of the commercial anti-ASB12 antibodies, but none of them is suitable for Western blot. In our study, only one ASB12 antibody (Invitrogen, Cat: PA5-107068, RRID:AB_2817784) is suitable for IF staining, since it showed specific distribution in sperm acrosome, and this signal was totally undetectable in KO group (see, Fig 1D,E). Moreover, according to the Human Protein Atlas, the single cell RNA-seq database shows that *Asb12* is enriched in late spermatids (see Figures below), which is highly consistent with our findings (see, Fig 1D,E):

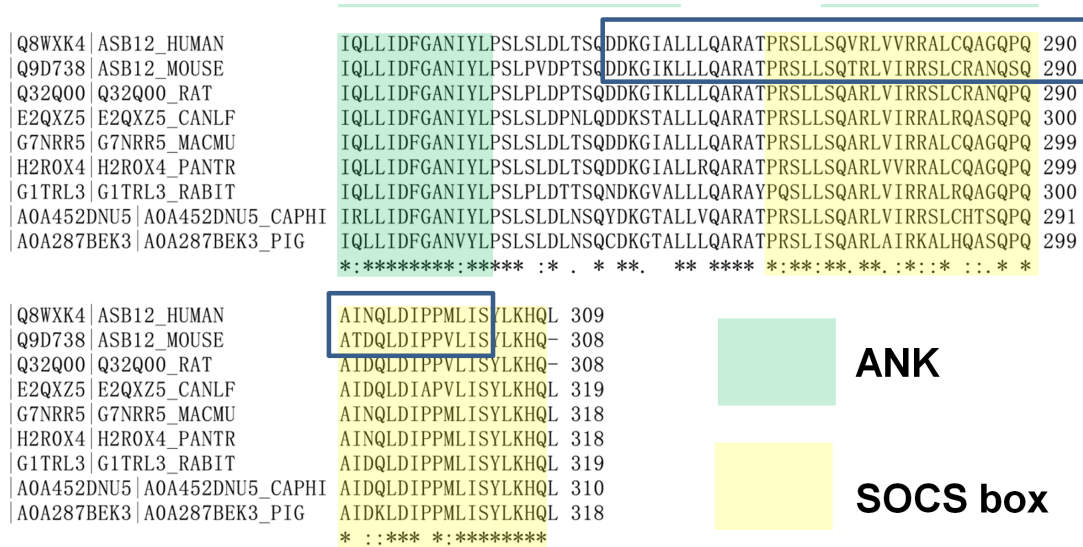
HUMAN PROTEIN ATLAS SUMMARY ⁱ	
Protein ⁱ	Ankyrin repeat and SOCS box containing 12
Gene name ⁱ	ASB12 (FLJ39577)
Tissue specificity ⁱ	Group enriched (skeletal muscle, tongue)
Tissue expression cluster ⁱ	Striated muscle - Muscle contraction (mainly)
Single cell type specificity ⁱ	Cell type enriched (Late spermatids)
Single cell type expression cluster ⁱ	Spermatids - Spermatogenesis (mainly)
Immune cell specificity ⁱ	Immune cell enhanced (basophil)
Brain specificity ⁱ	Not detected in human brain
Cancer prognostic summary	Gene product is not prognostic



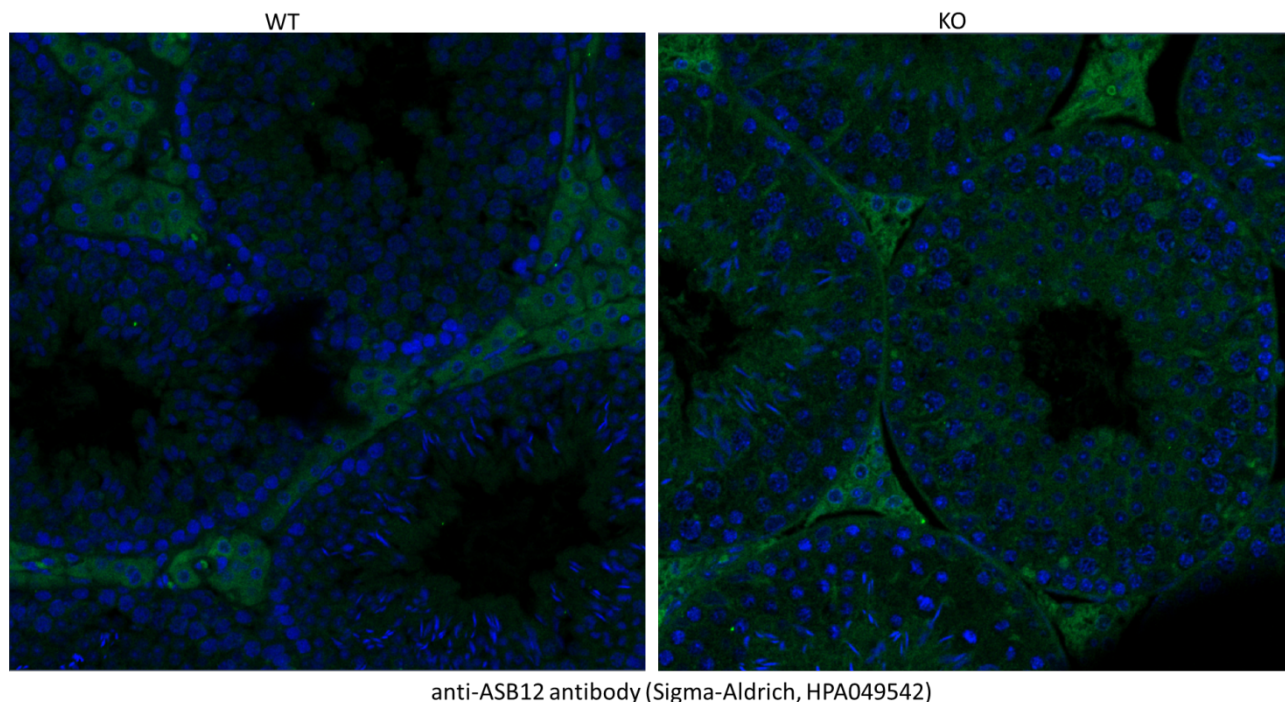
1.1-Another concern is the identity of the anti Asb12 antibody. It is reported by the manufacturer as reacting with the human protein. The immunogen is not precisely described, located within the C term region that is not the most conserved but homologous with the mouse... Validation is done on muscle proteins. In addition, in the ProteinAtlas site, the ASB12 protein is mainly expressed in muscle, weakly in testis, only in Leydig cells. This point should be unequivocally solved to clarify the specificity of the antibody.

Response: Thanks for this good comment. In our study, we used an anti-ASB12 antibody (Invitrogen, Cat: PA5-107068, RRID:AB_2817784) for IF staining. According to the manufacturer's information, the immunogen located within the C-term region of human ASB12: DDKGIALLLQ ARATPRSLLS QVRLVRRAL CQAGQPQAIN QLDIPPMLIS. The sequence homology between human and mouse is 78% (see Figure below), according to our calculation.

In addition, as we mentioned above, this antibody showed specific distribution of ASB12 in sperm acrosome.



On the other hand, the reviewer also found that ASB12 was located in testicular Leydig cells, according to the Human Protein Atlas. We notice that the above immunostaining was based on another anti-ASB12 antibody (Sigma-Aldrich, HPA049542). Actually, in our previous study, we used this antibody for Western blot and immunostaining in WT and KO testis. However, this antibody did not work. Leydig cells could also be labeled using this antibody in KO group (see figure below). Given that the results from single cell RNA-seq database (see above figures) are highly consistent with our findings (see, Fig 1D, E), we therefore concluded that ASB12 is enriched in spermatids, but not in Leydig cells.



1.3bis-Immunostaining figures should include a control with only the secondary antibody.

Response: Thanks for the comment. In the new revision, we provided the negative control for immunostaining (see, Figure S1 in the new revision).

2-Figure 1B is a bit strange. Chromatogram softwares usually don't produce "holes" in the reading of sequences. WT and mutant sequences should look different one compared to the other from the insertion point.

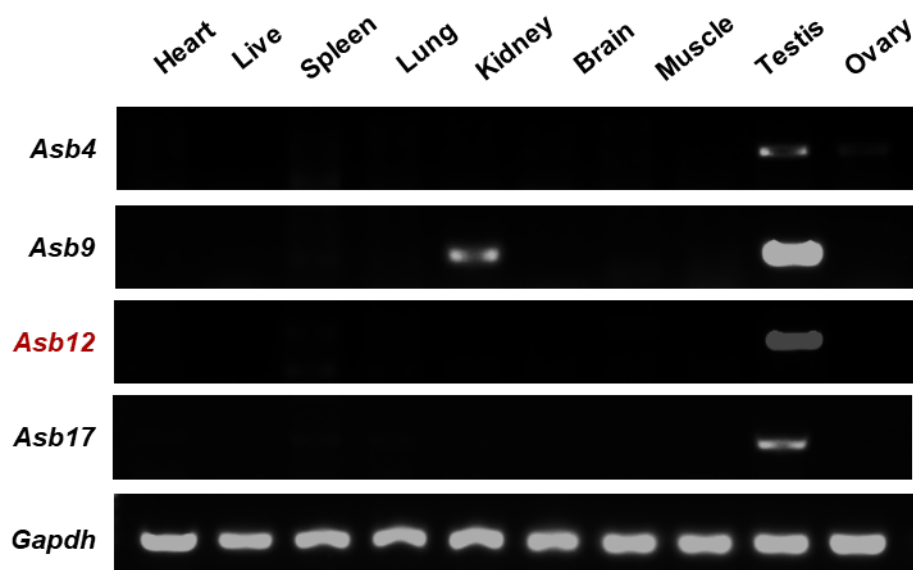
It would also help the reader if the consequence of the 1-bp insertion on protein sequence was presented. Frameshift for sure, premature stop codon? Length of the mutant protein?

Response: Thanks for the suggestions. In the new revision, we corrected Figure 1B and C, and give a description of the consequence of the 1-bp insertion. (see, Page 11, line 219-221)

3-It is surprising that the RT-PCR on various tissues is positive only for testis samples. In human, muscle and heart are expressing high levels of ASB12. In the Encode data, for mouse, lung and heart are also positive for RNA expression. By BLASTing the primers used for the 18s rRNA, I found an obsolete accession number but could locate them in Ensembl Rn18s-201 ENSMUST00000240377.1. However, this non-coding gene is encoded by a single exon. Therefore, this amplification cannot be considered as a good control for RT-PCR, whether regular or real-time. These experiments should be done again.

Response: Thanks for the suggestions. The primers used for 18s rRNA was designed from NCBI-Primer BLAST, and were considered to be specific for the amplification of 18s rRNA. To further verify the RT-PCR data, we repeat for several times (see, figure below) using different samples. And the results also showed a testis-enriched expression of *Asb12* gene. In addition to detect *Asb12*, we also detect the known testis-enriched *Asb* genes (such as

Asb4,9,17) with *Gapdh* as an internal control. The expressions of *Asb4,9,17* in various tissues are highly consistent with previous reports (Mol Cells 2008;25:317-21, PMID: 18414003; Mol Cells 2008;26:621-4, PMID: 18776735; Zygote 2004;12:151-6, PMID: 15460110).



3.2bis- In Figure 5, the analysis is not quantitative but rather semi-quantitative or relative. It should be stated in the text and/or legend that there was a normalization to a housekeeping gene expression. Ideally more than one.

Response: Thanks for the comments. In the new revision, we add a statement in figure legends. (see, Page 20, line 468)

Additionally, although our present study only used 18s rRNA as an internal control, we believe that more than one internal control will further improve our results in future studies.

4-For replicates, it should be notified what was repeated, experiments, fields, etc.

Response: Thanks for the comments. In the new revision, we add the sentence in Methods sections and figure legends.

(see, Page 10, line 204; Page 19-20, line 435-469)

5-The figures including text (1B and S1A) are of poor quality and could be improved.

Response: Thanks for the advice. In our new revision, we improve the figure quality.

(see, figure 1C and S2A in the new revision)

Reviewer D

In the manuscript, the authors developed mice deficient for testis-specific gene ASB12 and employed a variety of methods (sperm count, morphology, histological analyses of the testes, etc.) to assess relevant reproductive parameters of the mice. The authors observed no significant differences between the knockout and wild-type (WT) mice in them and therefore the authors concluded that ASB12 is dispensable for spermatogenesis and fertility in mouse. Because this paper consists of negative data and because there is very little useful information

in it, personally, I don't find this research interesting at all. However, the experiments were almost well performed. I find the manuscript has several flaws and errors that should be addressed by the authors. Specific comments are as follows.

Major points:

1. Immunostaining of the ASB12 protein in mouse testicular tissue revealed that there was strong fluorescence in the basal compartment of the seminiferous, not only sperm center. The authors claim that this was probably non-specific, since it also existed in *Asb12* mutant testes. However, if it was non-specific signal, the fluorescence in testes of *Asb12* mutant mice should be observed at the same extent as those of WT mice. What part of ASB12 is the epitope of the antibody you used? Since a single nucleotide insertion was made in the *Asb12* gene, it is possible that “a small amount of incomplete product of ASB12” is created as a protein, which is recognized by the antibody. This point should be clarified.

Response: Thanks for this good comment. In our study, we used an anti-ASB12 antibody (Invitrogen, Cat: PA5-107068, RRID:AB_2817784) for IF staining. According to the manufacturer's information, the immunogen located within the C-term region of ASB12. However, as showed in Figure 1B of the new revision, a single nucleotide insertion is predicted to result in a truncated protein of 123 amino acids within the N-term region of ASB12. We therefore conclude that fluorescence in the basal compartment of the seminiferous was probably non-specific. To avoid any confusion, we correct Figure 1D with a more representative image in KO group.
(see, figure 1D in the new revision)

2. The reviewer recommends detecting a specific band of ASB12 by western blotting in order to verify the deletion of the *Asb12* gene in the testis (needs uncropped image with molecular weight marker). This is a minimum request.

Response: We know western blotting is a “gold standard” method to verify gene knock out. However, we have used nearly all of the commercial anti-ASB12 antibodies, but none of them is suitable for western blot. In our study, only one ASB12 antibody (Invitrogen, Cat: PA5-107068, RRID:AB_2817784) is suitable for IF staining, since it showed specific distribution in sperm acrosome, and this signal was totally undetectable in KO group (see, Fig 1D,E). Moreover, according to the Human Protein Atlas, the single cell RNA-seq database shows that *Asb12* is enriched in late spermatids (see Figures below), which is highly consistent with our findings (see, Fig 1D,E):

background? If so, they should be clearly stated. Line 143: There is lack of information on fertility test (e.g. litter numbers, pair numbers, ages of both sexes).

Response: Thanks for this good comment. In our new revision, we state the age of mice, sequence of sgRNA and Cas9 mRNA, mouse background, and pair numbers for fertility test in Methods section. In addition, litter size can be found in Figure 2A. (see, Methods section, line 141,143, 145, 153,154, 164 and Figure 2A in the new revision)

4. Overall, this paper lacks information in figure legend. For example, the exact number of mice or samples used for each test should be indicated in the legend of every figure.

Response: Thanks for this suggestion. In the new revision, we provide the sample number and technical replicates in Methods and Figure legends sections.

(see, Methods and Figure legends sections in the new revision, line 204, 436, 438, 439, 441. 453, 454, 456, 458, 460, 468)

Minor points:

1. Figure 5: The expression of *Asb12* should be also analyzed to show its deletion.

Response: Thanks for this suggestion. In our new revision, we detect *Asb12* expression in WT and KO testes, and the result show that *Asb12* was dramatically reduced in *Asb12*-KO testes (Figure 5). This finding suggests that *Asb12* mutation could cause the decay of the encoded mRNA.

(see, Figure 5)

2. Line 234: “SSCs” should be spelled out.

Response: Thanks for this suggestion. We revised SSCs as “SSCs (spermatogonial stem cells)”.

(see, page 12, line 254)

3. English should be carefully revised by a native English speaker or a professional editing service.

Response: We agree with the reviewer’s advice. In the new revision, the manuscript has been edited by the Charlesworth company (<https://www.cwauthors.com>). The EDITORIAL CERTIFICATE has also been submitted.