EMBO reports

# *The intramembrane protease SPPL2c promotes male germ cell development by cleaving phospholamban*

Johannes Niemeyer, Torben Mentrup, Ronny Heidasch, Stephan A. Müller, Uddipta Biswas, Rieke Meyer, Alkmini Papadopoulou, Verena Dederer, Martina Haug-Kröper, Vivian Adamski, Renate Lüllmann-Rauch, Martin Bergmann, Artur Mayerhofer, Paul Saftig, Gunther Wennemuth, Rolf Jessberger, Regina Fluhrer, Stefan F. Lichtenthaler, Marius K. Lemberg, and Bernd Schröder

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## Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 25 June 2018

Thank you for the submission of your research manuscript to our journal. We have now received the full set of referee reports that is copied below.

Your manuscript has been evaluated by three referees. Referee 1 and 2 are experts in intramembrane proteolysis, while referee 3 is an expert in spermatogenesis. After reading the referee reports, I conclude that the manuscript reports interesting findings for the proteolysis field (referee 1 and 2) but that the spermatogenesis phenotype is less compelling (referee 3). It appears that SPPL2c has a non-essential role in spermatogenesis since the male mice are fertile. Yet, given the potential interest of the findings for the proteolysis field, and the positive evaluation from both referee 1 and 2, I would like to invite you to revise your manuscript for EMBO reports, provided that all concerns are addressed and that the effect of SPPL2c on spermatogenesis and fertility is not overstated.

Please address all referee concerns (as detailed above and in their reports) and take their suggestions taken on board. Please provide a complete point-by-point response. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions. Please contact us if a 3-months time frame is not sufficient for the revisions so that we can discuss the revisions further.

You can submit the revision either as a Scientific Report or as a Research Article. For Scientific Reports, the revised manuscript can contain up to 5 main figures and 5 Expanded View figures. If the revision leads to a manuscript with more than 5 main figures it will be published as a Research Article. In this case the Results and Discussion section can stay as it is now. If a Scientific Report is

submitted, which I recommend, these sections have to be combined. This will help to shorten the manuscript text by eliminating some redundancy that is inevitable when discussing the same experiments twice. In either case, all materials and methods should be included in the main manuscript file.

Supplementary/additional data: The Expanded View format, which will be displayed in the main HTML of the paper in a collapsible format, has replaced the Supplementary information. You can submit up to 5 images as Expanded View. Please follow the nomenclature Figure EV1, Figure EV2 etc. The figure legend for these should be included in the main manuscript document file in a section called Expanded View Figure Legends after the main Figure Legends section. Additional Supplementary material should be supplied as a single pdf labeled Appendix. The Appendix includes a table of content on the first page with page numbers, all figures and their legends. Please follow the nomenclature Appendix Figure Sx throughout the text and also label the figures according to this nomenclature. For more details please refer to our guide to authors.

Regarding data quantification, please ensure to specify the name of the statistical test used to generate error bars and P values, the number (n) of independent experiments underlying each data point (not replicate measures of one sample), and the test used to calculate p-values in each figure legend. Discussion of statistical methodology can be reported in the materials and methods section, but figure legends should contain a basic description of n, P and the test applied. Please also include scale bars in all microscopy images.

We now strongly encourage the publication of original source data with the aim of making primary data more accessible and transparent to the reader. The source data will be published in a separate source data file online along with the accepted manuscript and will be linked to the relevant figure. If you would like to use this opportunity, please submit the source data (for example scans of entire gels or blots, data points of graphs in an excel sheet, additional images, etc.) of your key experiments together with the revised manuscript. Please include size markers for scans of entire gels, label the scans with figure and panel number, and send one PDF file per figure.

When submitting your revised manuscript, we will require:

- a complete author checklist, which you can download from our author guidelines (http://embor.embopress.org/authorguide#revision). Please insert page numbers in the checklist to indicate where the requested information can be found.
- a letter detailing your responses to the referee comments in Word format (.doc)
- a Microsoft Word file (.doc) of the revised manuscript text
- editable TIFF or EPS-formatted figure files in high resolution
(In order to avoid delays later in the publication process please check our figure guidelines before preparing the figures for your manuscript:
http://www.embopress.org/sites/default/files/EMBOPress_Figure_Guidelines_061115.pdf)
- a separate PDF file of any Supplementary information (in its final format)
- all corresponding authors are required to provide an ORCID ID for their name. Please find instructions on how to link your ORCID ID to your account in our manuscript tracking system in our Author guidelines (http://embor.embopress.org/authorguide).

We would also welcome the submission of cover suggestions, or motifs to be used by our Graphics Illustrator in designing a cover.

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I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

REFEREE REPORTS
**********

**Referee #1:**

The manuscript by Niemeyer et al is focused on characterizing the function of the aspartyl intramembrane protease SPPL2c, which has remained perhaps the most mysterious of the SPP proteases (even being at one time thought to be a pseudogene). The authors made isotype-specific antibodies, verified its subcellular localization using multiple independent approaches, defined its endogenous tissue-specific expression patterns, found it has a largely distinct substrate and inhibitor profile relative to other aspartyl proteases, discovered a novel phenotype in a knockout mouse model, and identified at least one physiological substrate.

This is a rigorous, true tour-de-force work that leaves very little to the imagination. If it sounds like I'm impressed, it's because I am. I would only offer two thoughts to the authors. First, they paradoxically find that, despite a spermatogenesis phenotype, there is little or no effect on litter size when knockout male mice are crossed with wild type female mice. The authors do not mention whether they conversely tried crossing knockout female mice to wild type males, or tested the unlikely possibility of any maternal (grandmother) contribution to masking the phenotype.

Second, if the phenotype does indeed come from cleavage of PLN alone, the authors should test whether an uncleavable mutant form of PLN phenocopies a SPPL2c knockout. Arguably this could even be tested transgenically once an uncleavable transmembrane mutant is engineered. Although this could be a perfect capstone to this beautiful work, given the many achievements already made in this single manuscript I do not consider it a requirement for publication.

**Referee #2:**

The manuscript of Niemeyer, Mentrup, Heidasch and colleagues is a complementary study along with a co-submitted manuscript. The body of work in this paper focuses on understanding the role of the SPPL2c protease in vivo. While this enzyme has been found in some tissues, its activity has not previously been reported *in vivo, *and physiological substrates are unknown. They identified that SPPL2c, an ER localized intramembrane protease, is found in the testis (both message and protein), and specifically localized to the elongated spermatids. They demonstrate some cleavage of substrates known to be cleaved by related SPPs (aspartyl proteases), and interesting show a unique inhibition profile demonstrating SPPL2c substrate specificity is distinct from related aspartyl intramembrane proteases, such as gamma secretase, SPPL2a/c and SPP. Both SPP and SPPL2c appear to be part of distinct complexes. Due to its localization in the seminal tubules and elongated cell, fertility mating revealed subfertility, which was also detected when SPPL2c-/- females were mated with WT males (very interesting!), but the mechanism for this latter discovery in unknown. Lastly the group determines physiological substrates using label-free quantitative proteomics and identity syntaxin (the subject of the supporting manuscript) and phospholambin as substrates. Phospholambin is a single pass TM regulates Ca+2 uptake in the ER by negatively regulating the SERCA transporter. Subsequently, decrease in Ca+2 was measured in in SPPL2c deficient spermatids.

Overall this is a very detailed study on the role of SPPL2c *in vivo*. Physiological substrates were identified. The paper is well-written, figures are clear and appropriate references are cited.

 

Major points concerning the data:

1. While a few tissues were analysed for SPPL2c expression, importantly muscle and heart is missing. This is quite important given the finding of PLN as a substrate. Addition of this data would strengthen the manuscript.

2. Page 8. It is mentioned that SPPL2c did not result in cleavage of SPPL2a/b substrate TNF, however Supplemental Fig. 2a shows both an NTF and CTF for the protein. This needs clarification since it appears to be cleaved.

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1. Supp Fig 1B. What does the asterisk indicate? Controls are missing. Are non-specific bands related to the secondary antibody? This is important, considering the validation for IF experiments.
2. Please show the full un-cropped WB in order to analyze antibody specificity in Supp Fig1
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11. I could not find in the methodology section how were data regarding Fluo4 signals normalized, in order to compare calcium concentration between null and WT cells. This is very important in order to make measurements independent of cell size and Fluo4 loading differences among other effects.

1st Revision - authors' response 19 October 2018

**Point-by-point reply to the editor's and reviewer's comments:**

*Thank you for the submission of your research manuscript to our journal. We have now received the full set of referee reports that is copied below.*
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*and that the effect of SPPL2c on spermatogenesis and fertility is not overstated.*

We thank the editors and reviewers for their time and effort in evaluating our manuscript and for their constructive feedback. A detailed point by point reply to the individual reviewer's comments is provided below. We have taken care not to overstate the phenotypic impact of SPPL2c deficiency on fertility and to rather stick to a descriptive terminology when presenting our findings on spermatogenesis, sperm function and litter size statistics in *SPPL2c-/-* mice.

**Referee #1:**

*The manuscript by Niemeyer et al is focused on characterizing the function of the aspartyl intramembrane protease SPPL2c, which has remained perhaps the most mysterious of the SPP proteases (even being at one time thought to be a pseudogene). The authors made isotype-specific antibodies, verified its subcellular localization using multiple independent approaches, defined its endogenous tissue-specific expression patterns, found it has a largely distinct substrate and inhibitor profile relative to other aspartyl proteases, discovered a novel phenotype in a knockout mouse model, and identified at least one physiological substrate.*

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Thank you very much for the positive feedback. As suggested, we have analyzed the litter size upon breeding of SPPL2c knockout females with wild type males. This data has been added to the already existing diagram, which is shown in Fig. 3L in the revised version. Similar to the results from KO males X WT Females, also in this breeding constellation the mean litter size was not significantly different from that of WT X WT breedings. Therefore, we can conclude that lack of SPPL2c in just one of the partners, either male or female, does not lead to a detectable subfertility. However, when both partners are SPPL2c-deficient this results in a significant reduction of the mean litter size. This argues for a yet to be analysed role of SPPL2c in the female reproductive system and with regard to fertility additive effects of SPPL2c deficiency in males and females.

Finding entirely uncleavable mutants for substrates of SPP/SPPL proteases has so far not been successful. Previous studies analysing substrates of SPPL2a and SPPL2b (e.g. Hüttl et al., Biochem J 2016, Fluhrer et al., J Biol Chem 2012) identified certain determinants supporting cleavage, like helix-destabilizing residues. However, these features only impaired, but not abolished substrate cleavage.

 

In order to gain initial insights into the substrate determinants recognized and required by SPPL2c we created several mutants of the Phospholamban transmembrane domain. The corresponding data is depicted in Fig. 4 G-I. We systematically mutated blocks of amino acids in the transmembrane domain of phospholamban to alanines. Two of these mutations apparently interfered with folding of the protein since the corresponding constructs were not expressed. Cleavage of the other two mutants was not significantly impaired. In addition, we replaced three cysteine residues by alanines, since they caught our attention as hydrophilic residues within the transmembrane segment. Interestingly, processing of this CallA Phospholamban mutant by coexpressed SPPL2c was significantly impaired, but similar to the mentioned studies on SPPL2a/b not entirely blocked.

Therefore, these findings may represent a good starting point to further analyse the cleavage determinants of SPPL2c. Whether such efforts in the end result in an absolutely uncleavable mutant which at the same time preserves folding and functions of the phospholamban protein is not sure. However, having such a well-characterized mutant would certainly be a prerequisite to move this *in vivo* by creating knock-in mice with this mutant. Furthermore, it would need be confirmed that the introduced mutation does not influence or even abolish the interaction of phospholamban with the SERCA Ca2+ ATPase. Provided that these prerequisites could be fulfilled, generating and analyzing the phenotype of such mice would certainly be of great interest.

**Referee #2:**

*The manuscript of Niemeyer, Mentrup, Heidasch and colleagues is a complementary study along with*

*a co-submitted manuscript. The body of work in this paper focuses on understanding the role of the SPPL2c protease in vivo. While this enzyme has been found in some tissues, its activity has not previously been reported *in vivo, *and physiological substrates are unknown. They identified that SPPL2c, an ER localized intramembrane protease, is found in the testis (both message and protein), and specifically localized to the elongated spermatids. They demonstrate some cleavage of substrates*

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*Overall this is a very detailed study on the role of SPPL2c *in vivo*. Physiological substrates were identified. The paper is well-written, figures are clear and appropriate references are cited.*

*Major points concerning the data:*

*1. While a few tissues were analyzed for SPPL2c expression, importantly muscle and heart is missing.*

*This is quite important given the finding of PLN as a substrate. Addition of this data would strengthen*

*the manuscript.*

We have analyzed a potential expression of SPPL2c in murine heart and muscle by RT-PCR (Fig. 1C revised version) and Western blotting (Expanded View Fig. 1D revised version). Similar to other organs, we failed to detect any SPPL2c expression in these tissues. Therefore, at least under physiological conditions we can exclude a

 

role of SPPL2c in phospholamban homeostasis in these tissues.

*2. Page 8. It is mentioned that SPPL2c did not result in cleavage of SPPL2a/b substrate TNF, however*
*Supplemental Fig. 2a shows both an NTF and CTF for the protein. This needs clarification since it appears to be cleaved.*

Indeed, TNF is processed into a membrane-bound NTF and soluble CTF by ectodomain shedding. The latter is the soluble cytokine sTNFα and ADAM17 has a well-established leading role in this process. Therefore, release of sTNFα and the concurrent generation of the TNFα NTF represents the first step of the Regulated Intramembrane Proteolysis cascade and is not affected by any of the intramembrane proteases - including SPPL2c. In agreement with previous reports, we observe two effects upon co-expression of SPPL2a or SPPL2b (Fluhrer et al., Nat Cell Bio. 2006; Friedmann et al., Nat Cell Biol 2006): the membrane-bound TNFα NTF, which is the direct substrate of these proteases, is depleted and at the same time the rather instable cleavage product, the released intracellular domain (ICD) is detected. Both effects only occur upon co-expression of either SPPL2a or SPPL2b with TNFα, but do not occur when SPPL2c is co-expressed. Therefore, we conclude that TNFα is not a substrate of SPPL2c.
Since TNFa NTF accumulation is slightly variable in different cell types due to technical reasons, we repeated the experiment (n=5) and consistently observed TNFa NTF accumulation similar to control cells and complete lack of ICD generation in SPPL2c coexpressing cells. We now show a representative experiment that convincingly depicts these findings.

*3. Page 10. BN PAGE of complexes should be described as digitonin-resistant. Since these proteins are*
*housed in large complexes it is possible some epitopes are masked by other proteins and this should be mentioned in the results. Therefore it should not be conclusively states that they are not found in the same complex. Perhaps reword to say "suggests" or run and SDS-PAGE of complexes and repeat*
*western with the antibodies.*

We have reworded the respective partin the results section as suggested. It now reads:
"SPP has been described to be part of high molecular weight complexes in the ER (Chen et al., 2014, Schrul et al., 2010). Upon blue-native PAGE separation of murine testis lysates, we predominantly observed a digitonin-resistant SPP complex with an apparent molecular weight of ~250 kDa (Fig 2F). Very minor amounts of SPP were present in assemblies of ~500 kDa and ~900 kDa, respectively. In contrast, the bulk of SPPL2c was part of a digitonin-resistant complex with a size of ~500 kDa. This indicates that SPPL2c like SPP has the intrinsic ability to form high molecular weight complexes in the membrane of the ER. However, the complex organisation we observed for both proteases in murine testis was distinct. Though it remains possible, that antibody epitopes in these native complexes have been masked preventing protease detection, our findings strongly suggest that SPP and SPPL2c are not part of the same high molecular weight assemblies."

**Referee #3:**

*The manuscript presented by Johannes Niemeyer et al, presents evidence for the expression of the protease SPPL2c, previously considered non-expressed pseudogene, identifying also its proteolytic activity. The manuscript shows a large amount of work and is well written. However, the work is mostly*
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*failed to show striking defects, where males are fertile. Overall, I consider that the manuscript is not appropriate for a wide audience as the case of EMBO.R readers, suggesting a more specialized journal,*
*after addressing these concerns:*

*1. Supp Fig 1B. What does the asterisk indicate? Controls are missing. Are non-specific bands related*
*to the secondary antibody? This is important, considering the validation for IF experiments.*
The asterisks mark unspecific bands not related to SPPL2c, but apparently recognized by our primary anti-SPPL2c antibodies. As requested, we performed an additional control with just secondary antibody (Expanded View Fig. 1B, revised version). There, no bands at all were detected clearly indicating that these non-specific bands do not reflect unspecific binding of the secondary antibody used, but instead of the primary antibody.
We consider these bands as unspecific since they are also detected in the vector/control-transfected cells. As these bands do not get stronger by SPPL2c overexpression they are apparently not related to SPPL2c. Since overexpressed SPPL2c is at the same time detected based on the appended Myc epitope tag, which clearly identifies bands related to SPPL2c, we consider this as a well-controlled system and are confident about the assignment of bands.
Similarly, when analyzing endogenous SPPL2c, we consider bands as unspecific which are also observed in samples from SPPL2c knockout mice, where the entire SPPL2c coding region has been deleted from the genome and where we confirmed absence of SPPL2c transcripts by RT-PCR. According to our experience, a certain degree of additional unspecific binding of anti-peptide antibodies is not uncommon. However, when comparing SPPL2c-transfected with control-transfected cells or wild type and knockout tissues, respectively, we consider experiments as suitably controlled.
Also for visualization of murine SPPL2c by immunohistochemistry we always performed stainings in sections from wild type and SPPL2c knockout mice in parallel. Thus any staining being detected in the latter would need to be considered as unspecific. However, there is very little background staining and the difference to the signal in the wild type sections, which represents SPPL2c, is easily discernible. Therefore, we think that also these histological analyses are quite well controlled.

*2. Please show the full un-cropped WB in order to analyze antibody specificity in Supp Fig1*
The uncropped blots including the control exposure with just secondary antibody are depicted in Expanded View Figure 1B.

*3. Again, in Fig 1D and E, show the full WB*
The uncropped blots are shown in Expanded View Figure 1C.

*4. The authors say nothing related to the non specific staining of SPPL2c -/- when using the N-term antibody (Fig 1E), being this a critical issue.*
For Western blot analysis the N-term antibody was validated in the very same way as the C-terminal antibody with the controls described above (SPPL2c-transfected vs. control-transfected cells; wild type vs. SPPL2c-deficient tissue). All this data is depicted in Expanded View Figure 1B and C. With these controls at hand, use of this antibody in Western blot experiments is sufficiently controlled and blots can be unambiguously interpreted, though the signal to noise ration with this N-terminal antibody was higher than with the C-terminal which we therefore preferentially used.
For *in situ* detection of SPPL2c (immunohistochemistry) the N-terminal antibody was not very well suited. Signals in testis sections from wild type mice were rather weak, on the other hand background in knockout tissue was rather high. This may reflect that this antibody was generated against an internal epitope of the protein which is probably not so well accessible in the native protein and is recognized with higher efficiency when the protein is denatured for SDS-PAGE. Therefore, we refrained from using this antibody for immunohistochemical stainings and all stainings of murine SPPL2c which are depicted throughout the manuscript were performed with the Cterminal

 

antibody.

*5. Why did the C-term Ab fail to detect Iso B in Fig 2D?*
As can be seen in scheme in Fig. 1B of the revised version, the two SPPL2c isoforms differ in their C-terminus. Isoform B is shorter so that the C-terminal epitope from isoform A which was used for generation of the C-term antibody is not present in isoform B. Therefore, this particular antibody is not expected to recognize isoform B.

*6. Overall, figures are to difficult to follow without going to Figure legends.*
Without further details, we were unsure how to exactly improve labelling of the figures.

*7. Please perform periodic acid Schiff staining either on the gel or a subsequent Blot membrane in order to confirm deglycosylation on exps shown in Fig 1G-H, in fully deglycosylated samples*
We do not consider PAS staining as a suitable control to confirm deglycosylation experiments with Endo H and PNGase F. Endo H only removes mannose-rich Nlinked glycans. And also PNGase F which should remove all N-linked sugars would not cleave O-linked sugars or remove glycosaminoglycans. Therefore, even with complete and perfect performance of both enzymes residual PAS-reactive bands can be expected to remain. In order to provide further confirmation for the successful deglycosylation in these samples we visualized a number of additional glycoproteins and demonstrate their molecular weight shifts upon treatment with PNGase F or Endo H. These include the protease SPPL2b, tetraspanin 3 (TSPAN 3) or the transferrin receptor 1 (TfR1). These additional controls are depicted in Figs. EV1E and EV1H. We are convinced that these glycoproteins exhibiting the expected behavior confirm that the established deglycosylation protocols using commercially available and frequently used enzymes have worked also in our hands. Apparently, the observed molecular weight shift of SPPL2c upon deglycosylation is small. However, with just one glycosylation site being in use in a protein of more than 70 kDa this is expected.

*8. Speculation related to Sppl2c expression during spermatogenesis needs to be tuned down by performing WB analysis in different isolated cell stages during spermatogenesis*
We have FACS-sorted a testicular cell suspension based on their DNA content (1C, 2C, 4C). The obtained fractions were analyzed by Western blotting for SPPL2c. The Western blot depicted in Fig. 3B is representative of n=3 independent experiments. In agreement with the immunohistochemical data, the observed the highest abundance of SPPL2c in the 1C fraction, which contains all cells with completed meiosis, the spermatids. However, we could also detect minor levels (about 20% of the 1C population) of the protease in the 2C and 4C fractions which is not so easily appreciated in the immunohistochemical stainings, presumably reflecting a narrow linear detection range for this method, and indicates that SPPL2c expression starts early in developing germ cells before reaching a maximum in spermatids. This observation is also in agreement with the findings in the accompanying manuscript by Papadopoulou *et al.* and we have clarified this throughout the manuscript accordingly.

*9. Do sppl2c -/- mature cauda sperm show any morphological defects? This is not shown*
We thank reviewer 3 for this question, since information on this aspect was indeed missing in the first version of the manuscript. We have isolated sperm from the caudae epididymides of n=6 mice per genotype which were subsequently analysed for potential morphological defects by phase contrast microscopy in a blinded manner. Representative pictures of mature spermatozoa are included in Figure 3I of the revised manuscript. In summary, we were not able to detect any clear differences between sperms from wild type or SPPL2c-deficient animals. A certain degree of dysmorphic sperms was observed in mice from both genotypes. However, their abundance appeared to be similar in all samples by observation. Therefore, we did not perform a precise quantification of their frequency since this was very unlikely to reveal relevant differences. Based on this we conclude, that SPPL2c-deficiency does not affect sperm morphology at the microscopic level and that morphological defects are not able to

 

account for the reduced motility of the SPPL2c-deficient spermatozoa.

*10. Authors state that sppl2c -/- are subfertile, but this is not supported by the data. KO males crossed* with WT females are completely normal, ruling out this possibility.
We totally agree that SPPL2c-deficient males and females *per se* cannot be regarded as subfertile since they produce normal litter sizes when bred with their wild type counterparts. However, our initial statement that *SPPL2c-/-* mice are subfertile refers to the notion that pure knockout breedings result in significantly reduced pup numbers (Figure 3L of the revised manuscript) if compared to wild type controls. We consider this as a physiologically relevant observation. This finding remains surprising since we were unable to detect SPPL2c in any organ of the female reproductive system so far (data not shown). Nevertheless, this does not exclude transient expression of SPPL2c in certain stages of oocyte maturation, which are not detected even by sensitive RT-PCRs that might add up to the functional deficits, which we have uncovered for SPPL2c-deficient sperm. Further investigations that are beyond the scope of the present manuscript will be needed to deepen our insights into the mechanistic aspects of the observed subfertility of *SPPL2c-/-* breedings.

To avoid any misunderstandings, we have reworded the respective passage to:

"To define the patho-physiological consequences, we analysed litter sizes from different mating schemes. The mean litter size of homozygous breedings of *SPPL2c-/-* mice (-/- x -/-) was significantly reduced versus that of heterozygous (+/- x +/-) or wild type mice (+/+ x +/+) (Fig 3L), indicating a physiologically relevant subfertility of SPPL2c Ko x Ko matings. However, when mating male *SPPL2c-/-* mice with wild type females the resulting litter sizes did not differ significantly from that of wild type. The same was observed when crossing SPPL2c-deficient females with wild type males. This may indicate that in addition to the described impact of SPPL2c-deficiency on spermatogenesis and sperm function, which on its own does not impair fertility, other not yet discovered mechanisms, possibly in the female reproductive tract, may contribute to the subfertility of the -/- X -/- matings."

We hope that these modifications help to better discriminate between an isolated subfertility of male *SPPL2c-/-* mice, which is not evident in these mice, and that of homozygous *SPPL2c-/-* breedings, which we indeed observed.

*11. I could not find in the methodology section how were data regarding Fluo4 signals normalized, in order to compare calcium concentration between null and WT cells. This is very important in order to make measurements independent of cell size and Fluo4 loading differences among other effects.*

To increase the transparency of the normalization procedure for Fluo4-based Ca2+-measurements in testis suspensions, we expanded the description of the normalisation process in the Material and Methods section with the following sentences:

"Median Fluo4-AM signals of each population were normalised to those observed for the respective wild type control. In addition, median forward scatter of the 1C-co population was compared between wild type controls or SPPL2c-deficient animals to ensure that differences in Fluo4-AM staining were not caused by differences in cell size."

We also transferred the comparison of mean forward scatters observed for the 1C-co population from Suppl. Fig. 6E of the old manuscript to the main Figure 5G to allow a direct visual comparison when comparing Fluo-4 staining intensities presented in Figure 5F of the revised manuscript.

 

2nd Editorial Decision 30 November 2018

Thank you for the submission of your revised manuscript to EMBO reports. We have now received the full set of referee reports that is copied below.

As you will see, all referees are very positive about the study and support publication without further revision.

Browsing through the manuscript myself, I noticed a few minor editorial changes that we need before we can proceed with the official acceptance of your study.

We look forward to seeing a final version of your manuscript as soon as possible.

REFEREE REPORTS
**********

**Referee #1:**

The revised manuscript by Niemeyer et al has addressed all of the questions that I asked during the first round of review. Moreover, the manuscript has done a valiant job of addressing the concerns raised by the other two Reviewers with new experimental data. The end result is a stronger manuscript with significant new data and additional points of clarification. I support publication of the revised manuscript without any further delay.

**Referee #2:**

The authors have addressed the points raised in the review and conducted appropriate experimentation where requested. All edits have improved the clarity of the manuscript, which is suitable for publication in EMBO Reports.

**Referee #3:**

I thank the Authors for their comments to my concerns, and the corrections made to the manuscript. It has been a pleasure to read them.
I found the manuscript suitable for publication as it is.

2nd Revision - authors' response 7 December 2018

The authors performed all minor editorial changes.

 

EMBO PRESS

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PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

| |
|---|
| Corresponding Author Name: Prof. Dr. Bernd Schröder |
| Journal Submitted to: EMBO Reports |
| Manuscript Number: EMBOR-2018-46449 |

**Reporting Checklist For Life Sciences Articles (Rev. June 2017)**

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

## A- Figures

### 1. Data

**The data shown in figures should satisfy the following conditions:**

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

### 2. Captions

**Each figure caption should contain the following information, for each panel where they are relevant:**

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

**In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.**

## B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

| | |
|---|---|
| 1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size? | For most experiments effects of SPPL2c depletion were unknown before performing the experiment so that typical sample sizes of at least n=3 up to n= 6 were chosen. |
| 1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used. | Depending on the expected difference between wild type or SPPL2c-deficient animals at least 3 animals per genotype were analyzed. For breeding statistics, about 10 breedings per condition were estimated to result in statistically significant changes. Also for histological analyses, which were not quantified, at least n=3 biological replicates were analysed. |
| 2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established? | No samples or animals were excluded from analysis. |
| 3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe. | Mice were chosen for experiments exclusively due to their genotype, gender (due to studies of the male reproductive system) and were age-matched with corresponding control animals. All animals in one experiment received the same treatment making randomization impossible. |
| For animal studies, include a statement about randomization even if no randomization was used. | No randomization was necessary for animal studies. Mice were allocated to the experimental groups based on their genotype (wild type, SPPL2c-/-), which were compared. Animals in these groups were age- and sex-matched and housed under identical conditions. |
| 4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe. | All studies based on cells and tissues directly isolated from mice were performed in a blinded manner if possible. This mainly refers to the analysis of sperm morphology and quantification of elongated spermatids in testicular cross sections. |
| 4.b. For animal studies, include a statement about blinding even if no blinding was done | Blinding was done for analysis of sperm morphology analysis and spermatid counting. |
| 5. For every figure, are statistical tests justified as appropriate? | For all quantitative datasets appropiate statistical tests were performed as applicable. The respective information has been added to the figure legends. |
| Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. | Where applicable, one-way-ANOVA with Tukey or Dunett's post hoc testing was performed. This assay tolerates deviation from the normality assumption and therefore was chosen whereever possible. In other cases, t-test was performed in the assumption of a normal distribution. |
| Is there an estimate of variation within each group of data? | For all data sets standard deviation is depicted to visualize variation within each group of data. |
| Is the variance similar between the groups that are being statistically compared? | Statistical variance was in general comparable. |

## C- Reagents

## USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repo

http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title

http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tun

http://datadryad.org

http://figshare.com

http://www.ncbi.nlm.nih.gov/gap

http://www.ebi.ac.uk/ega

http://biomodels.net/

http://biomodels.net/miriam/
http://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

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|---|---|
| 6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right). | All commercially available antibodies are listed in the Material and Methods section of the paper including the providing company, clone and catalogue numbers. The newly generated SPPL2c-antibodies were validated as depicted in Figure 1 of the paper using knockout controls. |
| 7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination. | Sources of cell lines are listed in the Material and Methods section. Cells were used at low passage numbers. All cell lines used were tested for mycoplasm contamination prior to aliquoting and freezing. |

* for all hyperlinks, please see the table at the top right of the document

## D- Animal Models

| | |
|---|---|
| 8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals. | Wild type as well as SPPL2c-deficient mice were on a C57BL/6N Crl background. Mice used for the same experiment were age- and sex-matched. Typically, age of mice varied from 10 to 25 weeks if not depicted diretly in the Figure legends. Mice were housed in individually ventilated cages (IVCs) according to the legal requirements. Typically, heterozygous breedings were chosen to compare direct littermates, if possible. Detailed information about generation of SPPL2c-deficient mice can be found in the Material and Methods section of the manuscript. |
| 9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments. | All animals used for this study were kept under specific pathogen-free conditions in individually ventilated cages (IVC) at room temperature (19-20 °C) and 45-60% relative humidity with a circadian rhythm of 12 h lightness and 12 h darkness in the Victor-Hensen-Haus of Christian-Ulbrichts-University of Kiel (CAU Kiel). Animal care and handling were strictly performed according to the guidelines of the CAU Kiel and authorized by the Ministerium für Energiewende, Landwirtschaft, Umwelt und ländliche Räume of Schleswig-Holstein. |
| 10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance. | We confirm compliance to ARRIVE guidelines. |

## E- Human Subjects

| | |
|---|---|
| 11. Identify the committee(s) approving the study protocol. | The Ethical Committee (Ethikkommission, Technische Universität München, Fakultät für Medizin, München, project number 5158/11) has approved the study. |
| 12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report. | For the scientific use of the samples, all patients had granted written Informed Consent. The experiments were performed in accordance with relevant guidelines and regulations. |
| 13. For publication of patient photos, include a statement confirming that consent to publish was obtained. | NA |
| 14. Report any restrictions on the availability (and/or on the use) of human data or samples. | NA |
| 15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable. | NA |
| 16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list. | NA |
| 17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines. | NA |

## F- Data Accessibility

| | |
|---|---|
| 18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'.<br><br>Data deposition in a public repository is mandatory for:<br>a. Protein, DNA and RNA sequences<br>b. Macromolecular structures<br>c. Crystallographic data for small molecules<br>d. Functional genomics data<br>e. Proteomics and molecular interactions | All data obtained from the mass spectrometric analysis of membrane preparations of either wild type or SPPL2c-deficient testis are presented in an additional source data table associated with the manuscript. |
| 19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right). | All data obtained from the mass spectrometric analysis of membrane preparations of either wild type or SPPL2c-deficient testis are presented in an additional source data associated with the manuscript. |
| 20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right). | NA |
| 21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information. | NA |

## G- Dual use research of concern

| | |
|---|---|
| 22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could. | NA |