EMBO reports

Signal Peptide-Peptidase-Like 2c (SPPL2c) impairs vesicular transport and cleavage of SNARE proteins

Alkmini Papadopoulou, Stephan A. Müller, Torben Mentrup, Merav D. Shmueli, Johannes Niemeyer, Martina Haug-Kröper, Julia von Blume, Artur Mayerhofer, Regina Feederle, Bernd Schröder, Stefan F. Lichtenthaler and Regina Fluhrer

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Editor: Martina Rembold

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 26 June 2018

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

Your manuscript has been evaluated by two experts in intramembrane proteolysis (referee 1 and 2) and one expert in trafficking and SNARE proteins (referee 3). As you will see, while the referees acknowledge that the identification of potential SPPL2c substrates is interesting, they also point out that the study relies a lot on the ectopic overexpression of SPPL2c and that the physiological significance of the findings remains unclear.

Importantly, we all notice that your manuscript depends to quite some extent on the co-submitted manuscript from the Schroeder lab. This was also recognized by referee 2 during our further discussion of the referee reports. Referee 3 emphasized again that s/he considers "... the making of a full article without demonstrating which substrate is causally related to the phenotype preliminary". Since each manuscript from a back-to-back submission has to stand on its own, I want to emphasize that it will be crucial to strengthen the independent aspect of this study and to strengthen the functional insights and physiological significance. Referee 2 suggests analyzing if SPPL2c is important for spermatid maturation by disrupting intracellular compartments. Referee 3 suggests studying the effect of SPPL2c-resistant SNAREs.

On balance, I have decided to give you the opportunity to address the concerns and would be willing to consider a revised manuscript with the understanding that the referee concerns must be fully addressed and their suggestions taken on board. If the physiological significance cannot be strengthened you might also consider merging both manuscripts. Should you decide to embark on such a revision, 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, 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
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Referee #1:

The authors provide a manuscript that focuses on the substrate(s) and role of the signal peptide SPPL2c, an intramembrane protease. Using a label-free proteomics approach in HEK293 cells, several candidate membrane proteins (Type II and TypeIV) were identified. SDS-PAGE confirmed the decrease (and loss, degradation) in several candidates: vesicle-associated membrane proteins (VAPA A and B), VAMP8, syntaxin9 and membrin; while for syntaxin 18, a cleavage product was observed. During this ectopic expression (notably SPPL3 is not expressed in HEK293 cells), the protein expression levels of a homologs intramembrane protease SSPL3 were decreased. Furthermore, the transport of glycosyl transferases, membrane associated enzymes, were influenced by the presence of SPPL2c with a concomitant hypogycosylation of targets. Analysis of cells after siRNA decrease of these SNARE substrates leads to an impaired cargo transport, similar to the SPPL2c expression. Overall the group demonstrates that SPPL2c disturbs the integrity of the subcellular compartmentalization in the cell from disruptions in vesicular trafficking, membrane fusion and glycosylation. Interestingly, disruption of subcellular compartmentalization is essential in certain cell types, such as the elongated cell in the testis, which is where SPPL2c is predominantly found.

The proteomics study is detailed and rigorously conducted. The paper is well written; Figures are clear. Analysis of membrane protein:protein interaction is complex, since these proteins tend to self-association non-specifically. This paper will be of interest to a broad audience.
While this manuscript is strong, I have some considerations for the authors:
1. One small concern was that while SPPL2c is not expressed in the HEK cells, upon its expression there is a decrease of the homolog SPPL3. The authors however do a good job of explaining the results and taking this into consideration (appropriately discussed). Of note, however, the expression of SPP3 is drastically enhanced in the presence of the inactive (D to A mutation) SPPL2c. This almost suggests that a physical interaction between the two proteases. Does SPPL2c cleave SPP3? Is there any evidence for this? If so, perhaps discuss.
2. Where does SPPL2c cleave its substrates? Is there a substrate recognition motif? This is important since cleavage products may be subject to N-end rule cleavage pathways while other not, explaining the differences in degradation of substrates post cleavage.
Minor considerations:
1. The protease is called pseudo protease in the abstract. Later in the introduction this also appears, however with a reference to other work (page 4). In the discussion it is described as an orphan protease. For the abstract, perhaps orphan protease may be more appropriate.

Referee #2:

Expansion of intramembrane-cleaving proteases, or iCLiPs, in the human genome has presented major challenges to defining their individual functions. Papadopoulou et al studied SPPL2c, which until now had been considered to be inactive. By ectopic expression in TRex HEK293 cells, they show that SPPL2c is catalytically active, cleaves a whole network of vesicle trafficking proteins, and impacts on Golgi glycosyltransferase functions, perhaps by retaining them in the ER and interfering with SPPL3 expression. They conclude that the compartment disruption that high levels of SPPL2c causes could be important for spermatid maturation.

This study establishes that SPPL2c is an active iCLiP, has a distinct substrate repertoire, and presents an interesting model for SPPL2c function in maturing spermatids. The main limitation is that it relies on SPPL2c expression in cells that normally do not express SPPL2c, and then comes to a contradictory conclusion from what's been observed in knockout mouse studies. This contradiction needs to be resolved. If SPPL2c is important for spermatid maturation by disrupting intracellular compartments, evidence should be seen in knockout testes. Without this information, it remains possible that the observations made by the authors are not physiologically relevant.

Referee #3:

In this article the authors identify susbtrates of Signal Peptide-Peptidase-Like 2c, a peptidase targeting type II, tail-anchored proteins. Among the susbtrates that the authors identified, were several SNARE proteins and also cargo proteins. Because membrane compartments such as the Golgi apparatus are impaired when the peptidase is expressed, the authors tried to make a link between cleavage of SNAREs like Syntaxin 5 and impairment of the secretory pathway. While this study is technically sound and rigourous, the interpretation of the data and the title are clear overstatements and the discussion fails to deliver a clear-cut message. To convincingly make their point, the authors should show the effect of expressing Signal Peptide-Peptidase-Like 2c-resistant SNAREs including Syntaxin 5 and cargoes.

In addition, the article refers many times to the SPPL2c KO article (which this reviewer could not find whereas it was written by the authors to accompany the present article) and the results presented here appear more as an extension of Niemayer et al. than a fully independent study.

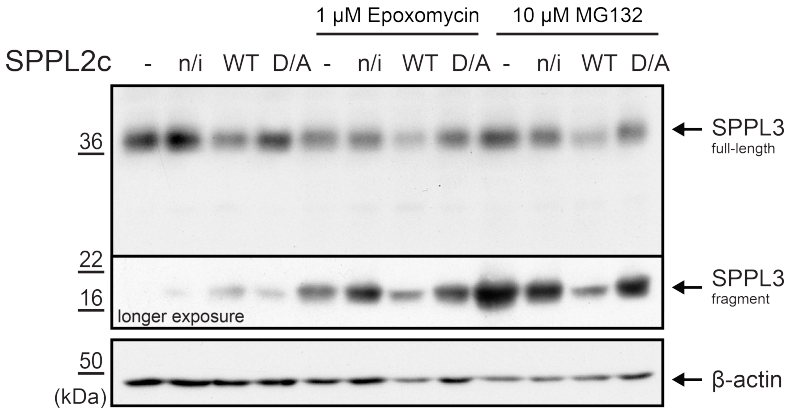
Minor points:
VAPs A and B are not components of SNARE complexes (page 3).

1st Revision - authors' response 19 October 2018

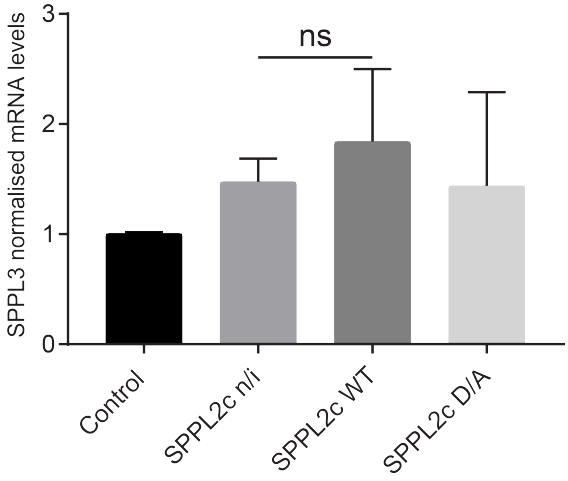
Comments of referee #1:

1. *One small concern was that while SPPL2c is not expressed in the HEK cells, upon its expression there is a decrease of the homolog SPPL3. The authors however do a good job of explaining the results and taking this into consideration (appropriately discussed). Of note, however, the expression of SPP3 is drastically enhanced in the presence of the inactive (D to A mutation) SPPL2c. This almost suggests that a physical interaction between the two proteases. Does SPPL2c cleave SPP3? Is there any evidence for this? If so, perhaps discuss.*

We are not sure where Referee 1 found the drastic enhancement of SPPL3 expression in presence of SPPL2c D/A. In Figure 2A, were we analyzed this effect, expression of endogenous SPPL3 in cells expressing SPPL2c D/A is similar to that observed in control cells. None of the other figures shows SPPL3 expression in the context of SPPL2c D/A expression. It might be that our figure ordering is somewhat misleading, since Figure 2B shows overexpression of SPPL3 wt in the last lane and thus very high SPPL3 protein levels, while Figure 2A showed overexpression of SPPL2c D/A in the last lane and Western Blot of SPPL3 which in this case only reflects the endogenous level.
Nonetheless, we found the idea of the reviewer that SPPL3 and SPPL2c might interact very interesting, in particular, since we sometimes observe smaller SPPL3-fragments that may result from degradation. To address the question we analyzed protein levels of endogenous SPPL3 and the corresponding SPPL3-fragment in cells expressing SPPL2c and control cells. In addition, we also added proteasome inhibitors to test whether the proteasome is involved in degradation of SPPL3.



In all conditions, exogenous expression of SPPL2c wt resulted in reduction of SPPL3, but to a similar level also in reduction of the SPPL3 fragment. Proteasome inhibition did neither effect levels of SPPL3 nor those of the SPPL3 fragment. From that we conclude that downregulation of SPPL3 in the presence of SPPL2c is not a direct effect, and proteasomal degradation seems not to be involved in this process. We then also checked whether SPPL2c affects transcription of the SPPL3 gene.



However, changes in mRNA levels of SPPL3 were not significant in any of the tested conditions. Consequently, downregulation of SPPL3 in the presence of SPPL2c is more likely an indirect effect as discussed in the manuscript. Although these data are interesting, we think they do not further strengthen the major points of our manuscript and, therefore, decided not to integrate them into the manuscript.

2. *Where does SPPL2c cleave its substrates? Is there a substrate recognition motif? This is important since cleavage products may be subject to N-end rule cleavage pathways while other not, explaining the differences in degradation of substrates post cleavage.*

These are certainly very valid questions but would, however, require a complete new study with a different focus. Analysis of cleavage sites and substrate requirements in the context of intramembrane proteolysis is very complicated since cleavage takes place in the hydrophobic environment of cellular membranes. Addressing these questions for other SPP/SPPL-family members required multiple independent studies and today the understanding of substrate recognition and cleavage by intramembrane proteases is still not complete.

Minor considerations:

1. The protease is called pseudo protease in the abstract. Later in the introduction this also appears, however with a reference to other work (page 4). In the discussion it is described as an orphan protease. For the abstract, perhaps orphan protease may be more appropriate.

We agree with the reviewer and changed the wording in the abstract accordingly.

Comments of referee #2:

This study establishes that SPPL2c is an active iCLiP, has a distinct substrate repertoire, and presents an interesting model for SPPL2c function in maturing spermatids. The main limitation is that it relies on SPPL2c expression in cells that normally do not express SPPL2c, and then comes to a contradictory conclusion from what's been observed in knockout mouse studies. This contradiction needs to be resolved.

It's not entirely clear to us why reviewer 2 thinks that the two manuscripts come to contradictory conclusions, but agree that we failed to appropriately discuss the common sense of the two manuscripts. Thus, we not only added new data sets that strengthen our hypothesis (see next point), but also rewrote the discussion. Since SPPL2c has the capability to not only cleave SNARE proteins, as shown by our study, but also other substrates like phospholamban, as shown by the accompanying study, it is expected that it affects multiple cellular pathways. In case of SPPL2c, the two pathways analysed, vesicular trafficking and Ca^{2+}- signalling, both impact on proper sperm maturation and contribute to the observed phenotype in the SPPL2 $^{-/-}$ mice. We hope our manuscript is now much clearer on this point and convinces the reader that the two studies, although coming from different angles, do not contradict but support each other.

If SPPL2c is important for spermatid maturation by disrupting intracellular compartments, evidence should be seen in knockout testes. Without this information, it remains possible that the observations made by the authors are not physiologically relevant.

We agree with the reviewer that an impact of SPPL2c on the compartment integrity should be seen in seminiferous tubules of SPPL2c $^{-/-}$ mice. We performed immunohistological stainings of Cab45, a Golgi marker protein, in seminiferous tubular cross sections of SPPL2c $^{-/-}$ mice and littermate controls. The new data are added in Figure 6F and Figure EV4. While in wt spermatids the Golgi is dissolved, a prerequisite to proper acrosome formation SPPL2c $^{-/-}$ spermatids depict reduced capability in this process. Quantification revealed a roughly 50% reduction of compact, dense structures, which most likely represent pre-acrosomal structures in SPPL2c $^{-/-}$ mice compared to wt controls. This supports our hypothesis that active SPPL2c is required to allow effective compartmental reorganization during spermatogenesis, as observed in SPPL2c overexpressing HEK293 cells.
In our HEK293 model system SPPL2c expression also impacts on the sorting of glycan-modifying enzymes. Mature spermatozoa are characterized by a very special glycan composition that can be achieved by altered sorting of the responsible glycosyltransferases as observed in HEK293 cells overexpressing SPPL2c, which reflect the wt situation in elongated spermatids. To test if the lack of SPPL2c protein expression affects glycosylation during spermatogenesis, we performed lectin chip microarray (LecChip) analysis on mature spermatozoa isolated from the cauda epididymis of SPPL2c -/- and control mice. The new data are added in Figure 6G and Figure EV5. Reactivity with 10 out of 45 lectins was significantly reduced, while one was increased in sperms from SPPL2c $^{-/-}$ compared to wt controls, indicating that SPPL2c deficiency significantly affects the glycan fingerprint of mature sperms. This provides in vivo evidence for SPPL2c causing miss-sorting of cargo-proteins due to its impact on vesicular trafficking. Of note, one lectin that depicted reduced reactivity in SPPL2c -/- spermatozoa had been previously reported as an acrosome-content marker, providing further evidence that expression of SPPL2c is required for the extraordinary sorting processes during sperm maturation. We believe that these new data sets provide profound evidence that our observations made in HEK293 cells are of physiological relevance and hope the reviewer agrees with us.

In order to integrate these new data sets and ensure a proper flow of the manuscript, we put the data on compartment reorganisation in HEK293 cells (former manuscript version Figure 5F) in a separate

figure (new Figure 5) and summarized all in vivo data in the new Figure 6. Panels 6A-E represent former Figures 5A-E. Consequently our model is now labelled as Figure 7.

Comments of referee #3:

While this study is technically sound and rigourous, the interpretation of the data and the title are clear overstatements and the discussion fails to deliver a clear-cut message.

We apologize for not being clear in interpretation and discussion of our data. To improve this, we rewrote the discussion and also added new in vivo data to further support our hypothesis (details see referee #2). Furthermore, we would like to suggest to change the title of our manuscript to "*Signal Peptide-Peptidase-Like 2c (SPPL2c) impacts on vesicular transport in elongated spermatids by cleavage of SNARE proteins*". However, this title would exceed the word count allowed. So it is up to editorial decision.

To convincingly make their point, the authors should show the effect of expressing Signal Peptide-Peptidase-Like 2c-resistant SNAREs including Syntaxin 5 and cargoes.

One aspect that distinguishes intramembrane proteolysis from substrate processing by soluble proteases is that hardly any non-substrates are known, for instance only one single non-substrate has been identified for SPPL2b, one out of five SPP/SPPL family members [1, 2]. In addition, the cleavage sites of intramembrane proteases are not precise and many of them apply a consecutive cleavage within the transmembrane domain of their substrates. Consequently, single point mutations at or close to the cleavage site do not abolish the cleavage, as normally observed for soluble proteases, but only shift the cleavage sites within the substrates transmembrane domain. Moreover, we do not know yet, where exactly SPPL2c cleaves Syntaxin 5 or its other substrates, since determination of cleavage sites that are located in highly hydrophobic amino acid sequences is complicated and requires a separate study (see referee #1).
Nonetheless, we established Syntaxin 5 chimeras that harbour the transmembrane domain of Syntaxin 6, which we found not be processed by SPPL2c. However, this chimera is still processed by SPPL2c, because besides the architecture of the transmembrane domain most likely other determinants within the substrate account for cleavability as observed before for other intramembrane aspartyl proteases [1, 3]. We then exchanged the annotated transmembrane domain of Syntaxin 5 by a poly-Leucine stretch, which is expected to form a perfect helix with cellular membranes and is supposed to reduce cleavage efficiency by intramembrane aspartyl-proteases. However, also this mutant substrate is still processed by SPPL2c. Based on this, we think it is not possible to provide experimental evidence to answer the reviewers question at this level of knowledge on intramembrane proteolysis.

In addition, the article refers many times to the SPPL2c KO article (which this reviewer could not find whereas it was written by the authors to accompany the present article) and the results presented here appear more as an extension of Niemayer et al. than a fully independent study.

We disagree on our study not being independent. The only mutuality between the two studies is the mouse model that was used. Since this is the only mouse model for SPPL2c-deficiency that is currently available and the accompanying manuscript is the first study describing the phenotype, we have to refer to this manuscript. However, identification of SNARE-proteins as SPPL2c substrates and the consequence of their processing in spermatids is a fully independent and novel finding that is not covered by the accompanying paper. Given the accompanying paper would have been published earlier, our paper would still be novel and we would have simply cited the manuscript accordingly. In the discussion we put the two papers in context to form an even larger and more in depth picture of SPPL2c-function in sperm maturation. We believe that it is now apparent to readers of both papers that the two studies are related to each other, but are based on two independent novel findings.

Minor points:
VAPs A and B are not components of SNARE complexes (page 3).

We apologize for this mistake and corrected it accordingly.

Literature:

1. Martin, L., R. Fluhrer, and C. Haass, *Substrate requirements for SPPL2b-dependent regulated intramembrane proteolysis.* J Biol Chem, 2009. **284**(9): p. 5662-70.
2. Martin, L., et al., *Regulated intramembrane proteolysis of Bri2 (Itm2b) by ADAM10 and SPPL2a/SPPL2b.* J Biol Chem, 2008. **283**(3): p. 1644-52.
3. Hemming, M.L., et al., *Proteomic profiling of gamma-secretase substrates and mapping of substrate requirements.* PLoS Biol, 2008. **6**(10): p. e257.

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 request only minor changes to clarify text and statistics.

From the editorial side, there are also a few things 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
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Referee #1:

The authors have addressed all concerns. The manuscript adds to the growing literature on intramembrane proteases, and importantly provides information on substrate for the signal peptidase family.

Referee #2:

The authors have revised their manuscript to address the concerns of the Reviewers. Particularly strong are the new data concerning the spermatid Golgi compaction in Figures 6F and EV Fig 4. Although these new analyses appear to have been conducted quite rigorously, no statistical significance values are reported for the differences that are observed. P-values should be reported for these analysis.

Referee #3:

The authors have satisfactorily answered the reviewers' requests.

Regarding the title, one suggestion could be:

Signal Peptide-Peptidase-Like 2c (SPPL2c) cleaves a subset of SNARE proteins and impairs vesicular transport.

I think it is still important to tone down the strong causal relationship that the 'by' in the original title would convey. This is why I propose a 'and' here.

2nd Revision - authors' response 7 December 2018

Comments of referee #1:

The authors have addressed all concerns. The manuscript adds to the growing literature on intramembrane proteases, and importantly provides information on substrate for the signal peptidase family.

We again thank the referee for his/her thoughtful comments that improved our manuscript and forsupporting publication.

Comments of referee #2:

The authors have revised their manuscript to address the concerns of the Reviewers. Particularly strong are the new data concerning the spermatid Golgi compaction in Figures 6F and EV Fig 4. Although these new analyses appear to have been conducted quite rigorously, no statistical significance values are reported for the differences that are observed.
P-values should be reported for these analysis.

We are delighted that referee 2considers our in vivo data strong and supports publication of the manuscript. We added statistical significance values and the test applied to the legend of Fig. EV4. It now reads:
"Unpaired, two-sided Welch's t test ***$p<0.001$ ($p=0.0004$)." Statistical significance has been also implemented in the respective figure, accordingly.

Comments of referee #3:

The authors have satisfactorily answered the reviewers' requests.
Regarding the title, one suggestion could be:
Signal Peptide-Peptidase-Like 2c (SPPL2c) cleaves a subset of SNARE proteins and impairs vesicular transport.
I think it is still important to tone down the strong causal relationship
that the 'by' in the original title would convey.
This is why I propose a 'and' here.

We thank referee 3 for his/her thoughtful comments and for supporting publication of our manuscript. We have changed the title as suggested.

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YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Regina Fluhrer
Journal Submitted to: EMBO Reports
Manuscript Number: EMBOR-2018-46451

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 χ2 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?	The effect of SPPL2c was unknown at the beginning of the study. Each experiment was performed at least three times to ensure an unbiased judging. Every cell culture experiment was independetly performed at least three times (3 biological replicates). For quantification three technical replicates were performed for each biological replicate. Every mouse experiment included a minimum of three individual mice per genotype, for histological analysis (Fig. 6F) and quantifiaction of the maturation phenotype in spermatids (EV4) the indicated number of microscopic pictures from six individual mice per genotype were used. For the Mass Spectrometry, six biological replicates per cell type were used .
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	Six aged and sex matched C57BL/6J were used for organ analysis. For the quantification of Stx8, testis samples of three animals per genotype were used. For the lectin microarray sperms from four animals per genotype were used. For the CAB45 stainings, testis samples from six animals per genotype were analyzed.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	In cell culture and animal studies no samples or animals were excluded from analysis. One SPPL2c wt sample was excluded from the proteomic analysis due to technical problems.
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.	No treatment was performed on animals. Animals were analysed purely based on their genotype. All cell lines were treated with both control and experimental conditions and were randomly allocated to the respective group.
For animal studies, include a statement about randomization even if no randomization was used.	Animals were untreated and only analysed based on their genotype.
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.	There was no treatment allocation performed. For the picture assessment, the pictures were assigned random numbering and even that numbering was hidden during the allocation and only used after the separation in two groups to evaluate the resutls
4.b. For animal studies, include a statement about blinding even if no blinding was done	Blinding was done for the assessment of the CAB45 stainings.
5. For every figure, are statistical tests justified as appropriate?	Yes
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Normal distribution was expected in all experimental procedures. Correlations were calculated were applicable and two-sided, unpaired ttest was used on all occasions.
Is there an estimate of variation within each group of data?	Standard deviation of the samples was calculated and depicted on all quantifications.

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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/

Is the variance similar between the groups that are being statistically compared?	Statistical variance was comparable

C- Reagents

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 commercial antibodies are listed in the Materials and Methods including the company and catalog and/or clone number. All in-house produced antibodies have either been published before and are cited accordingly in the materials and methods section, or are validated by reduced reactivity on knock out or knock down tissues/cells.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Source of all cell lines is listed in materials and methods and are tested for mycoplasma on a regular bases.

* 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.	All animal experiments were performed in accordance to local animal handling laws. Housing conditions included standard pellet food and water provided ad libitum, 12-hour light-dark cycle at temperature of 22 °C with cage replacement once per week and regular health monitoring. Mice were housed in individually ventilated cages (IVCs) in the animal facility of the Center for Stroke and Dementia Research in Munich. Six male C57BL/6J littermate mice at ages of 16, 28 or 36 weeks were used in pairs for organ analysis. Tissue samples and organs from SPPL2c KO and WT mice were provided to use by our collaborator Prof. Dr. Bernd Schröder (Niemeyer et al).
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	C57BL/6J mice were bred and sacrificed for organ collection according to legal requirements. All other mouse samples used in this project were provided to us post mortem.
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 that all animal realted studies complied to ARRIVE guidelines.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	N/A
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.	N/A
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	N/A
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	N/A
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	N/A
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.	N/A
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.	N/A

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'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	All data obtained for the Mass Spectrometry analysis, the lectin Microarray analysis and the pathway analysis are included either in the tables of the manuscript or as supplementary material.
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).	Extended Excel tables are submitted with the manuscript including all details regarding the Mass Spectometry and Lectin Microarray.
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).	N/A
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.	N/A

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.	N/A