

## SPINK2 deficiency causes infertility by inducing sperm defects in heterozygotes to azoospermia in homozygotes

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### Review timeline:

Submission date:	13 December 2016
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Editorial Decision:	10 April 2017
Revision received:	14 April 2017
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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.)

*Editor: Céline Carret*

1st Editorial Decision

17 January 2017

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Thank you for the submission of your manuscript to EMBO Molecular Medicine. We have now heard back from the two referees whom we asked to evaluate your manuscript.

You will see that the referees find your study well conducted and interesting. While referee 2 is supportive of publication in its current form, referee 1 suggests adding some mechanism, increasing clinical relevance by making the same point mutation in the mouse as in patients, and eventually back-crossing the new mouse to acrosin-KO animals. In principle, we agree that all these experiments would greatly improve the paper, however we also believe that this might be too much to ask for a revision. From our point of view, we would like to encourage you to address the CRISPR/Cas9 experiment to increase clinical relevance or to add molecular mechanism to better understand the data.

Given the balance of these evaluations, we feel that we can consider a revision of your manuscript if you can address the issues that have been raised within the time constraints outlined below. Please note that it is EMBO Molecular Medicine policy to allow only a single round of revision and that, as acceptance or rejection of the manuscript will depend on another round of review, your responses should be as complete as possible. EMBO Molecular Medicine has a "scooping protection" policy, whereby similar findings that are published by others during review or revision are not a criterion

for rejection. Should you decide to submit a revised version, I do ask that you get in touch after three months if you have not completed it, to update us on the status.

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions, except under exceptional circumstances in which a short extension is obtained from the editor.

Please read below and consult our guidelines for important editorial formatting. I would also encourage you to limit the number of main figures by pooling figures that are not already too crowded. Figures should be provided in portrait format. Please remember that you can add up to 5 EV figures, the rest can go into Appendix supplementary figures (see guidelines).

I look forward to receiving your revised manuscript.

\*\*\*\*\* Reviewer's comments \*\*\*\*\*

Referee #1 (Remarks):

Kherraf et al., report non-obstructive azoospermia and male infertility in two brothers of a consanguineous family. Each brother has the same c.56-C>G mutation in the SPINK2 gene that encodes an 84 aa Kazal type 2 serine protease inhibitor. Two abnormal splice variants were predicted and confirmed by RT-PCR and DNA sequence in one of the brothers. By screening 611 non-consanguineous patients with a- or oligospermia, an additional SPINK2 variant (c.1A>T) which mutates the start codon was identified. The patient, heterozygous for the mutation, was oligospermic with defects in the acrosome and sperm head-neck morphology. One son was born to this patient who sought medical attention for subsequent infertility.

The association of defective SPINK2 and fertility was further explored in a mouse model system in which a gene trap (tm1.1) mutated the Spink2 gene. Male, but not female mice, were infertile with loss of mature sperm in their epididymides. Using immunofluorescence and electron microscopy, the authors defined a defect in acrosome biogenesis, Golgi apparatus fragmentation and early arrest of round spermatid differentiation.

Comments:

Overall, this is a careful study focused on documenting that the association of a male infertility with a genetic mutation in SPINK2 is causal using KO mice as a model system.

1. Lee et al., (JBC 286:29108, 2011) previously reported a gene-trap mutation of Spink2 lacking exon 1 with decreased levels of protein and oligospermia, but a rather modest decrease in litter size (5.2 vs. 8.6 pups/litter). The discussion of this earlier study should be expanded and integrated with the current observations.
2. Although the phenotype of Spink2<sup>-/-</sup> mice support causality of human phenotype, the same point mutation observed in humans made by CRISPR/Cas9 in mice would make the case more compelling.
3. It would be of interest to cross the Spink2<sup>-/-</sup> mice with Acrosin<sup>-/-</sup> mice which should rescue the Spink2<sup>-/-</sup> phenotype if SPINK2's sole physiologic function is to inhibit acrosin during spermatogenesis.
4. The descriptive morphology of abnormalities of the Golgi and acrosome do not provide molecular mechanistic insight as to why/how the absence of SPINK2, an inhibitor acrosin, results in the observed phenotype.
5. Fig. 1B should have age-matched fertile controls.
6. The hypothetical transcript in Fig. 3B should be confirmed by RT-PCR and sequence.

## Referee #2 (Remarks):

By using exome sequencing in two azoospermic brothers, born from related parents, the authors found a homozygous mutation in SPINK2 gene encoding a serine protease inhibitor. Mutations of this gene seem to be rare since only one oligozoospermic patient carrying a heterozygous mutation was subsequently found among 611 infertile men.

Then, they obtained and studied Spink2 KO mice and observed that males were completely infertile whereas fertility in females was not impaired. Histological analysis of testis in Spink2  $-/-$  male mice revealed a post-meiotic blockage at the early round spermatid stage. Immunohistochemical studies, in human and mouse testis, showed that the protein SPINK2 was present within the acrosomal vesicle in round spermatids and then in the acrosome in mature spermatozoa. In Spink2  $-/-$  male mice, electron microscopy revealed that proacrosomal vesicle fusion was impaired and that Golgi apparatus was fragmented. The authors showed also that the Golgi was localized randomly around the spermatid nucleus instead of being face to a particular area of the nuclear membrane where acrosome is supposed to be linked to the nucleus. The observation of multivesicular bodies within the cytoplasm of Spink2  $-/-$  spermatids led the authors to suggest that the lack of SPINK2 protein could activate a self-degradation process, named microautophagy, independent from apoptosis. Although Spink2  $+/-$  male mice are fertile, the authors analyzed in details the sperm parameters of these mice and found that the rate of teratozoospermia was significantly increased whereas mobility was decreased. These abnormal parameters were similar to those observed in the oligozoospermic patient carrying a heterozygous mutation which led the authors to conclude that haploinsufficiency of SPINK2 can lead to oligoteratozoospermia in man.

The discussion deals with the role of SPINK family proteins in protecting secretory cells against damages due to enzymes they produce. Particularly, the role of SPINK2 in protecting spermatids against acrosomal enzymes is treated and its relationships with the phenotype of the patients who were studied is convincing.

In conclusion, this is an interesting paper dealing with a rare event, the discovery of a new gene involved in human spermatogenesis. There is no major modification to make and it can be published in state.

## Minor comments:

- At the end of results, it is written that "SPINK2 haploinsufficiency is not be deleterious". Please, correct.

- At the beginning of the discussion, the sentence "chronic pancreatitis or Netherton syndrome" suggests that this syndrome corresponds to the pancreatitis when it's a dermatologic disease, as defined a few lines later. Please modify by "chronic pancreatitis and Netherton syndrome"

- In the next paragraph, acrosomal activity is released just before fertilization, not at fertilization. Two lines later, a space is missing before "among".

1st Revision - authors' response

06 April 2017

## Point by point answer to the reviewers:

## Referee #1 (Remarks):

*Comments:*

*Overall, this is a careful study focused on documenting that the association of a male infertility with a genetic mutation in SPINK2 is causal using KO mice as a model system.*

- Lee et al., (JBC 286:29108, 2011) previously reported a gene-trap mutation of Spink2 lacking exon 1 with decreased levels of protein and oligospermia, but a rather modest decrease in litter size (5.2 vs. 8.6 pups/litter). The discussion of this earlier study should be expanded and integrated with the current observations.*

This paper was already cited page 12. We have now expanded the description of this paper in the discussion section.

2. *Although the phenotype of Spink2<sup>-/-</sup> mice support causality of human phenotype, the same point mutation observed in humans made by CRISPR/Cas9 in mice would make the case more compelling.*

In our manuscript we report two variants: c.56-3C>G which was homozygous in the two brothers and c.1A>T which was heterozygous in a patient with oligoteratospermia. We agree that the replication of the exact mutations in mice would be ideal but these are expensive and time-consuming experiments, which are not compatible with the timeframe of this revision.

Regarding the first mutation we demonstrated that it induces two abnormal splices, which are not expected to produce any functional proteins. The effect of this mutation is therefore perfectly comparable to the null mutation created in the KO animal.

Regarding the second mutation we have added some additional results. We introduced this mutation (c.1A>T) in a plasmid and tested the production of a putative mutant protein in a heterologous expression system (HEK293 cells). Using two antibodies, no signal corresponding to a mutant SPINK2 protein was observed. This experiment is now shown in Fig 3C and demonstrates that this mutation also corresponds to a null mutation and thus that patient 105 is SPINK2 haploinsufficient.

3. *It would be of interest to cross the Spink2<sup>-/-</sup> mice with Acrosin<sup>-/-</sup> mice which should rescue the Spink2<sup>-/-</sup> phenotype if SPINK2's sole physiologic function is to inhibit acrosin during spermatogenesis.*

Although this experiment would be very informative, such an experiment is not compatible within the time windows of this revision and was thus not possible.

To address this issue we however carried out some extensive cell work. We demonstrate that Acrosin (ACR) overexpression in HEK cells stops cell proliferation, and that this anomaly is overruled by SPINK2 coexpression. These experiments therefore clearly demonstrate that SPINK2 inhibits ACR and prevents the cellular stress initiated by unchallenged ACR expression. These results are discussed throughout the manuscript and presented in (a new) figure 9.

4. *The descriptive morphology of abnormalities of the Golgi and acrosome do not provide molecular mechanistic insight as to why/how the absence of SPINK2, an inhibitor acrosin, results in the observed phenotype.*

The experiment described in the previous section also permit to answer this question. We studied the impact of proacrosin expression on HEK293 cell proliferation and adherence by real time cell analysis (RTCA). We showed first that proacrosin can autoactivate (by Western blot) and induces a cellular stress leading to cell proliferation arrest and cell detachment (by RTCA), a phenotype similar to that observed in round spermatids from *Spink2* KO males. Moreover, Western blot results demonstrate that co-expression of SPINK2 with proacrosin prevents its auto-activation and rescues acrosin-dependent cell proliferation defects. These experiments demonstrate that proacrosin autoactivates within the cell and we therefore believe that SPINK2, which transits through the same cellular compartments, quenches this premature protease activity and prevents the described cascade of events leading to azoospermia.

5. *Fig. 1B should have age-matched fertile controls.*

Done, see new Fig 1B

6. *The hypothetical transcript in Fig. 3B should be confirmed by RT-PCR and sequence.*  
See our response to point two. We now indicate that this hypothetical transcript is actually not transcribed.

Referee #2 (Remarks):

*Minor comments:*

- *At the end of results, it is written "SPINK2 haploinsufficiency is not be deleterious". Please, correct.*

This was corrected

- *At the beginning of the discussion, the sentence "chronic pancreatitis or Netherton syndrome" suggests that this syndrome corresponds to the pancreatitis when it's a dermatologic disease, as defined a few lines later. Please modify by "chronic pancreatitis and Netherton syndrome"*

This was corrected

- *In the next paragraph, acrosomal activity is released just before fertilization, not at fertilization.*

This was corrected

*Two lines later, a space is missing before "among".*

This was corrected

2nd Editorial Decision

10 April 2017

Thank you for the submission of your revised manuscript to EMBO Molecular Medicine. We have now received the enclosed report from the referee who was asked to re-assess it. As you will see the reviewer is now supportive and I am pleased to inform you that we will be able to accept your manuscript pending the following final editorial amendments:

1) Figures

- Your article currently has 10 figures and 4 EV figures. However, we feel like this could be shortened as follows: Figures 4 and 5 could be made into a single figure as they are described within the same paragraph in the results section, and the same goes for figures 7 and 8. We also feel that EV3 and EV4 could be put together within the same EV figure (--> 8 figures and 3 EV figures). Further, as this would make 3 EV figures, 2 main figures could be moved to EV figures if possible. This would then result in 6 figures and 5 EV figures, shortening the length of the article, which is more attractive to the readers.

2) The author checklist:

- in section F-18, we do not fully agree with the written comment. You have genotyped the 2 brothers and the sequences should be supplied to EGA or a similar database. Please see <https://www.ebi.ac.uk/ega/home> for details

Please submit your revised manuscript within two weeks. I look forward to seeing a revised form of your manuscript as soon as possible.

\*\*\*\*\* Reviewer's comments \*\*\*\*\*

Referee #1 (Remarks):

The authors have addressed my concerns.

**YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓**

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Pierre F. Ray

Journal Submitted to: EMBO MOLECULAR MEDICINE

Manuscript Number: EMM-2016-07461-V2

### Reporting Checklist for Life Sciences Articles (Rev. July 2015)

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.

Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where the information can be located. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable).

#### 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?	Sample size were not predefined. In human we analysed as many patients as available. In mice we analysed 5 mice of each genotype for each experiment.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	For each condition for all experiments, at least 5 animals were tested to achieve statistical significance.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No inclusion/exclusion criteria were used for animal selection.
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. Littermates of different genotypes were compared.
For animal studies, include a statement about randomization even if no randomization was used.	No randomization were used.
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.	No
4.b. For animal studies, include a statement about blinding even if no blinding was done	No blinding was done.
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.	Our low number of sample did not allow us to verify that but T-test and non parametric test like Mann Whitney provide similar statistical significance on our results.
Is there an estimate of variation within each group of data?	Yes we measured the standard deviation.
Is the variance similar between the groups that are being statistically compared?	Due to the size of the samples (<30), we made the assumption that variances were similar and a common variance was calculated from the variances of sample1 and 2

#### 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-tur>

<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://jij.biochem.sun.ac.za>

[http://oba.od.nih.gov/biosecurity/biosecurity\\_documents.html](http://oba.od.nih.gov/biosecurity/biosecurity_documents.html)

<http://www.selectagents.gov/>

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).	This was in manuscript text p.15
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	This was indicated p. 19

\* 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.	This was specified p.16
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	This was specified p.16
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.	OK

#### E- Human Subjects

11. Identify the committee(s) approving the study protocol.	This was specified p.13.
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.	This was specified p.13.
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	We have no photos of patients
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	We had no restrictions.
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	The study was undertaken as a diagnostic study and is not a clinical trial.
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.	Not applicable
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.	Not applicable

#### F- Data Accessibility

18. Provide accession codes for deposited data. See author guidelines, under '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	We did not identify any new molecule, DNA or protein.
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 relevant data is provided.
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).	All relevant data is provided.
21. As far as possible, primary and referenced data should be formally cited in a Data Availability section. Please state whether you have included this section.  Examples: <b>Primary Data</b> Wetmore KM, Deuschbauer AM, Price MN, Arkin AP (2012). Comparison of gene expression and mutant fitness in <i>Shewanella oneidensis</i> MR-1. Gene Expression Omnibus GSE39462 <b>Referenced Data</b> Huang J, Brown AF, Lei M (2012). Crystal structure of the TRBD domain of TERT and the CR4/5 of TR. Protein Data Bank 4O26 AP-MS analysis of human histone deacetylase interactions in CEM-T cells (2013). PRIDE PXD000208	We have not included this section.
22. 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.	Not applicable

#### G- Dual use research of concern

23. 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.	Not applicable
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