EMBO Molecular Medicine

PSMC3 variants cause neurosensory syndrome combining deafness and cataract due to proteotoxic stress

Ariane Kroell Hermi, Frederic Ebstein, Corinne Stoetzel, Veronique Geoffroy, Elise Schaefer, Sophie Scheidecker, Severine Bar, Masanari Takamiya, Koichi Kawakami, Barbara Zieba, Fouzia Studer, Valerie Pelletier, Carine Eyermann, Claude Speeg-Schatz, Vincent Laugel, Dan Lipsker, Florian Sandron, Steven McGinn, Anne Boland, Jean-François DELEUZE, Lauriane Kuhn, Johana Chicher, Philippe Hammann, Sylvie Friant, Christelle Etard, Elke Kruger, Jean Muller, Uwe Strähle, and Helene Dollfus
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Corresponding author(s): Jean Muller (jeanmuller@unistra.fr) , Uwe Strähle (uwe.straehle@kit.edu), Elke Kruger (elke.krueger@uni-greifswald.de), Helene Dollfus (dollfus@unistra.fr)

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(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. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

1st Editorial Decision 10th Jan 2020

9th Jan 2020

Dear Dr. Muller,

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 from the set of reports pasted below that both referees are enthusiastic about the paper while still requesting additional supporting experiments to be performed along with some clarifications and details. Of interest, both referees request a rescue experiment, ref. #1 on the fish model, ref. #2 on the cellular model. We would leave it to you whether to perform the rescue experiment in cells or in the fish model. Further, ref. #1 would like to see a more thorough characterisation of the deafness phenotype of patients and ref. #2 would like data to be shown from more than one patient. We believe that following this line of revision would improve the clinical relevance of the findings as well as strengthen its conclusiveness.

We would therefore welcome the submission of a revised version within three months for further consideration and would like to encourage you to address all the criticisms raised as suggested to improve conclusiveness and clarity. Please note that EMBO Molecular Medicine strongly supports 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.

Please also contact us as soon as possible if similar work is published elsewhere. If other work is published we may not be able to extend the revision period beyond three months.

Please read below for important editorial formatting and consult our author's guidelines for proper formatting of your revised article for EMBO Molecular Medicine.

I look forward to receiving your revised manuscript.

Yours sincerely,

Celine Carret

Celine Carret, PhD
Senior Editor
EMBO Molecular Medicine

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2) separate figure files*

3) supplemental information as Expanded View and/or Appendix. Please carefully check the authors guidelines for formatting Expanded view and Appendix figures and tables at https://www.embopress.org/page/journal/17574684/authorguide#expandedview

4) a letter INCLUDING the reviewers' reports and your detailed responses to their comments (as Word file)

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8) EMBO Molecular Medicine now requires a complete author checklist (https://www.embopress.org/page/journal/17574684/authorguide) to be submitted with all revised manuscripts. Please use the checklist as a guideline for the sort of information we need WITHIN the manuscript as well as in the checklist. This is particularly important for animal reporting, antibody dilutions (missing) and exact p-values and n that should be indicated instead of a range.

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10) A Conflict of Interest statement should be provided in the main text

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

Referee #1 (Comments on Novelty/Model System for Author):

The patients' cells were essential to confirm in the human genomic context the outcome of the pathogenic variant, and dissect its molecular and cellular effects. The zebtafish model enabled illustration in vivo of Psmc3 knockdown.

Referee #1 (Remarks for Author):

In this paper, upon WGS of 3 patients & 2 healthy controls, Kröll-Hermi et al report the first biallelic pathogenic variant occurring in one of the ATPase Rpt subunits of the base of the 19S regulatory particle in human. Using patients' cells, carrying the deep intronic homozygous PSMC3 pathogenic variant, the authors show that proteasome from patients while in greater number, are ineffective. Based on these data & other experiments performed in cellular and zebrafish PSMC3/Psmc3 models, the authors conclude that the origin of the observed phenotypes could be linked to proteasome deficit, due to an haploinsufficiency mechanism.

Substantial amounts of genetic, molecular and some functional data are reported supporting the key involvement of PSM3 mutation in a new syndrome, associating severe deafness and early-onset cataracts, plus some neurological and cutaneous symptoms.
There are some issues regarding the correlations versus causality between the data in the PSMC3/Psmc3 models, and clinical features in patients that the authors could answer to improve further the manuscript.

Major comments:

1 - The expression profile of PSMC3 in the eye, ear, and brain is not clear. The authors refer to ubiquitous expression, but high magnification images in studied tissues showing cellular expression (epithelial, neuronal, support, mesenchymal cells ...) are necessary.

2- About zebrafish models, the authors describe the presence of 2 zebrafish psmc3 isoforms that share 83% sequence identity with the human orthologue. It was not clear if the MO used target one, or both isoforms.
Could the authors try a rescue using the human PSCM3 gene?

3- The link between proteasome abnormalities observed in patients' cells and the morphological abnormalities observed in zebrafish is missing?

4- Deafness is one major phenotype common to all 3 PSMC3-affected patients, It would be interesting to add a detailed phenotypic description (symmetry, age of onset, progression, &

severity of the hearing loss) of the hearing loss. The fact that patients have cochlear implant may indicate profound hearing loss, but the presentation of patient audiogram (& age) could highlight some genotype-phenotype correlations that would be great to discuss and will add more depth to the paper. For instance, do the patients present (or have presented) signs of balance problems? Are there CT scans of MRI data from patients that support defects in circular canal formation? The origin of deafness is not clear. In the paper, the authors have extensively characterized the defects in semicircular canal morphogenesis, which has nothing to do with hearing. More information on the morphology and function of the hair cells would be more informative. Besides showing reduction of kinocilia, what about hair cells (& support cells) morphology? hair bundle architecture? The authors could use FM1-43 that provide indication of hair cells activity.

Minor comments:

2- Patients present some variability in phenotype; what's the authors explanation for those differences?

4- What about cell death (TUNEL, caspase ...) in the eye and ear of zebrafish models?

3- It's not clear if haploinsufficiency is the sole mechanism taking place in patients. Heterozygotes (50% of protein) are normal. Patients cells show no difference in protein localization, but it's not clear how much wild-type protein (or transcript) is present despite the PSMC3 pathogenic variant. Is the truncated protein still present or not? If the truncated form might have some gain (or semi-dominant) function is not clear.

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e.g. Page 9: last line:
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Referee #2 (Comments on Novelty/Model System for Author):

This is an interesting paper. If the authors follow my (easy) suggestions the technical quality would move to high. This is a new example of another proteasomal subunit that -when mutated- would cause neurological pathologies.

Referee #2 (Remarks for Author):

This is an interesting manuscript where the authors identified a mutation in the 19S proteasomal subunit PSMC3 that relates to malformation and a range of neurological disorders. They report that this mutation results in an accumulation of ubiquitinated species in patient fibroblasts, which is -as expected- not associated to the activity of the proteasome but likely the transfer into the proteasomal proteolytic cage for degradation. As a result, the patient fibroblasts do not really respond to proteotoxic stress. Importantly, the authors could mimic the patient's phenotypes in zebrafish where the PSMC3 was eliminated by morpholinos.
This is important work and reveals another proteasome related case for neurological diseases.
I have only a few points.
1. The data are shown with fibroblasts from only one (out of three) patients and it would be better to show at least one other patient's fibroblast as well.

2. The experiments would be even better when normal psmc3 is overexpressed in the fibroblast to compete mutant psmc3 away and then show that the phenotypes (ubiquitin accumulation, responses to proteolytic stress) are corrected.
3. PA28 is highly expressed in the patient fibroblasts. What is the reason for this effect? And why does it decrease following proteotoxic stress?

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There are some issues regarding the correlations versus causality between the data in the PSMC3/Psmc3 models, and clinical features in patients that the authors could answer to improve further the manuscript.

We thank the reviewer for the summary and the comments. Please find our comments below in return.

Major comments:
1 - The expression profile of PSMC3 in the eye, ear, and brain is not clear. The authors refer to ubiquitous expression, but high magnification images in studied tissues showing cellular expression (epithelial, neuronal, support, mesenchymal cells ...) are necessary.

These data are just verification data, which have been previously published by Thisse *et al* 2004 as part of an unbiased genome-wide screen of expression patterns (Thisse, B., Thisse, C. (2004), Fast Release Clones: A High Throughput Expression Analysis). These data were deposited in a publicly accessible database (Zebrafish Information Network (ZFIN), Direct Data Submission: https://zfin.org/ZDB-PUB-040907-1). Our *in situ* staining experiments confirmed the deposited data. This expression pattern is also consistent with that reported for the mouse orthologue in particular within the visual system, the auditory system and in the nervous system among other tissues (MGI source: 1098754). In human, the situation is very similar with a very large RNA and protein expression according to the human protein atlas (https://www.proteinatlas.org/ENSG00000165916-PSMC3/). For these reasons, we thus believe that a thorough assessment of PSMC3 expression would not bring any new insights in this regard. We hope the referee understands our choice and considers this concern as being addressed.

2- About zebrafish models, the authors describe the presence of 2 zebrafish psmc3 isoforms that share 83% sequence identity with the human orthologue. It was not clear if the MO used target one, or both isoforms.

This is indeed major information and we might have not been clear enough. The morpholino targets both psmc3 isoforms as indicated in the supplementary Figure S14A (splice morpholino).

Could the authors try a rescue using the human PSCM3 gene?

We appreciate the reviewer's comment; however rescuing the zebrafish KO using the human gene is only done when the zebrafish orthologous proteins cannot be obtained. Both zebrafish psmc3 isoforms share 83% amino acid sequence identity with the human orthologue. To our point of view, it is not clear what could be learned from repeating this experiment with the human gene. Indeed, such cross-species experiments are extremely difficult to control. Different expression levels from the artificial expression systems, different codon usage etc or evolutionary adaptations in proteasome interactions may complicate the interpretation, thereby not telling us a lot about the function of the protein.
Thus, in this study, we have performed the rescue with the wildtype zebrafish gene demonstrating specific phenotypes for our morpholino. Rescue was properly performed with a rescue rate of ~58% of the cases (n=60) for the cataract phenotype and 76% (n=60) for the ear phenotype (Figure 5B'+D'). For these reasons and we have followed the Editor's suggestion and rescued the patient's cells phenotype instead of the zebrafish's one.

3- The link between proteasome abnormalities observed in patients' cells and the morphological abnormalities observed in zebrafish is missing?

The point raised by the reviewer is valid and although we did not assess the proteasome activity here as in the patient's cells and concentrated on the morphological aspects in the zebrafish; we provide relevant information in the discussion to give more background on those aspects. See below:
"Interestingly, a reduction of proteasome activity has previously been associated with lens defects in zebrafish. The knock out of the zebrafish gene psmd6 and the knock down of psmd6 and psmc2, both encoding proteins of the proteasome, resulted in severe impairment of lens fibre development. Cataract was also noted as a consequence of disrupted lens fibre differentiation (Richardson *et al*, 2017). The ear phenotype of the psmd6 mutant and both psmd6 morphants were not assessed (Imai et al., 2010). A direct link between the UPS and auditory hair cell death or impaired semicircular canal morphogenesis has not been described in zebrafish yet. However, knock down of atoh1, a gene regulated by the UPS, has been shown to severely affect hair cell development in the inner ear of zebrafish (Millimaki et al., 2007). The malformations of canal pillars observed in zebrafish *psmc3* morphants and crispants might be also a secondary effect, as abnormal sensory cristae with few hair cells have been previously assumed to lead to an abnormal development of semicircular canals (Cruz et al., 2009; Haddon and Lewis, 1991)."

4- Deafness is one major phenotype common to all 3 PSMC3-affected patients, It would be interesting to add a detailed phenotypic description (symmhair etry, age of onset, progression, & severity of the hearing loss) of the hearing loss. The fact that patients have cochlear implant may indicate profound hearing loss, but the presentation of patient audiogram (& age) could highlight some genotype-phenotype correlations that would be great to discuss and will add more depth to the paper. For instance, do the patients present (or have presented) signs of balance problems? Are there CT scans of MRI data from patients that support defects in circular canal formation? The origin of deafness is not clear. In the paper, the authors have extensively characterized the defects in semicircular canal morphogenesis, which has nothing to do with hearing.

We agree with the reviewer's comment and we provide additional information below. However, it is to notice that given the time of the initial analysis and the early cochlear implantation little data is available. For example, no audiogram could be retrieved before the cochlear implantation particularly because of the patients care outside of the hospitals (private medical doctors) and difficulty to establish full audiogram for very young children.

It is also remarkable that none of the family branches were connected to each other prior to their accidental meeting in the patients' waiting room in our reference center and after our examination and discovery of such rare combined conditions. This prevented any anticipation for the health care of the older children. All 3 patients had very early (in the first year of life) severe to profound bilateral hearing loss that required early cochlear implantation. Implantation was done for patients II.4 at 3 years and 3 months old, II.2 at 1 year and 10 months old and patient II.7 at 2 years and 4 months old.

In summary, OtoAcoustic Emissions (OAE) were positive at birth for each affected patients. However, deafness was suspected for all of them within the early months of life, respectively 8 months (II.4, II.2) and 1 year and 4 months (II.7). Auditory Brainstem Response (ABR) was in favor of profound deafness (no response at 110dB). MRI did not reveal any anomalies of the cochleovestibular nerves or labyrinthitis. However, temporal bone CT scan analysis of patient II.7 revealed lateral semicircular canal malformation (absence of the bony island in the right ear and small bony island in the left ear).
It is hard to say whether the patients had any balance problem given the rest of the symptoms and if this could be linked to their neuropathy or the inner ear trouble. For sure, this was not obvious.
Vestibular testing was impossible for patient II.4 and II.7. Patient II.2 had a preserved vestibular function: after cochlear implantation, vestibular testing showed presence and symmetrical responses for the lateral semicircular canals at middle (rotary-chair test) and low frequencies (caloric test).

In conclusion, deafness presented by the 3 affected children is either a progressive endocochlear deafness or an auditory neuropathy (cochlear response but no signal transmission) or more likely a combination of both. The manuscript and Figure 1 have been modified to reflect this data. We hope that we have clarified the deafness aspects for the reviewer.

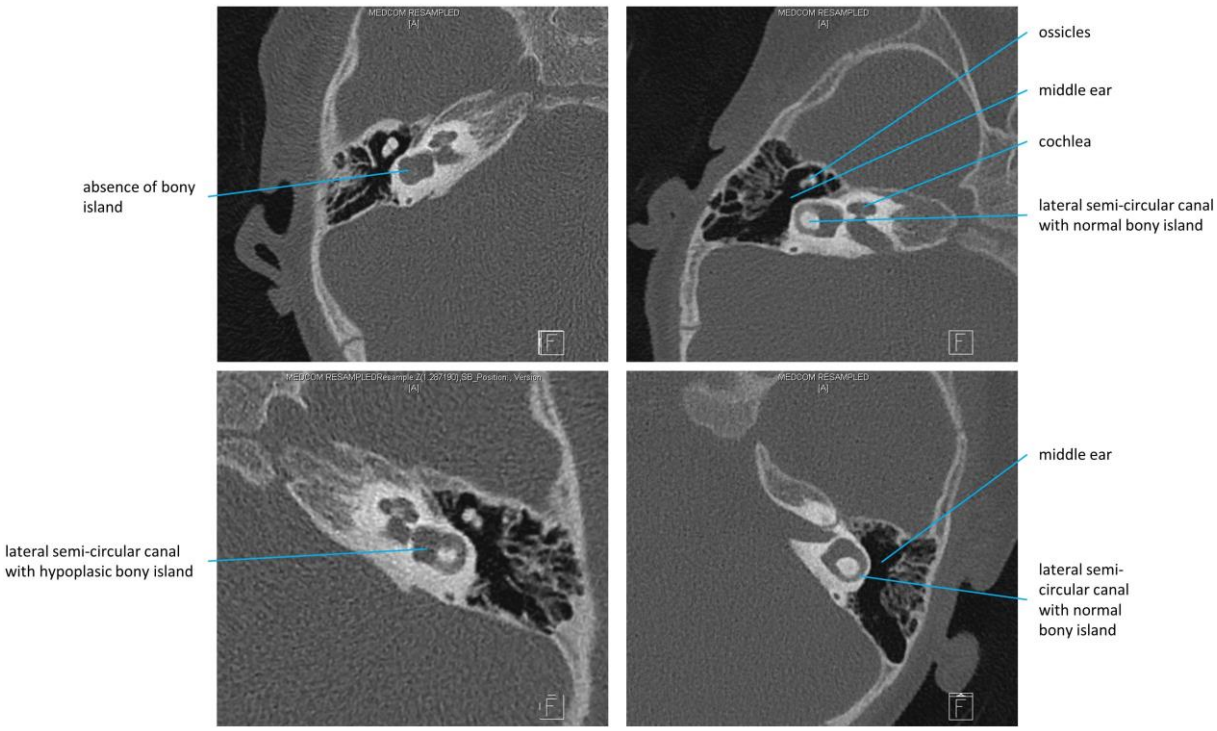
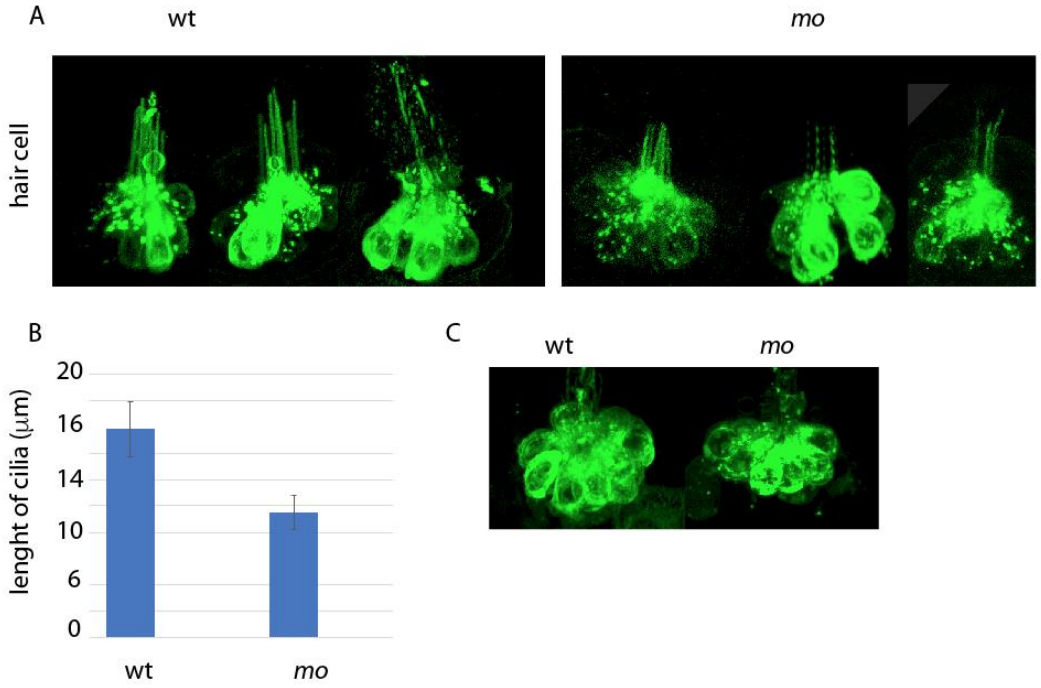


Figure legend: Temporal bone CT-scan from patient II.7 (left column) and a normal scan (right column). The left ear is shown on the upper panels while de right ear on the lower panels

More information on the morphology and function of the hair cells would be more informative. Besides showing reduction of kinocilia, what about hair cells (& support cells) morphology? hair bundle architecture? The authors could use FM1-43 that provide indication of hair cells activity.

Following the reviewer's suggestion, we stained 5 dpf wild-type and morphant zebrafish embryos with FM1-43 and observed hair cells. The hair cell morphology did not appear affected in morphants. However, we observed a decrease of hair cell length in morphant embryos, which may contribute to ear deficiency.



(A) Hair cell and stereo/kinocilia of 5 dpf wild-type (wt) and morphants (mo) embryos after incubation in FM1-43.
(B) Graph showing cilia length in µm. P-value: 0.022.
(C) Shape of hair cell body. No differences are noted.

In line with these results, we added and modified the following paragraph in the manuscript:
"The inner ear possesses hair cells to sense both vestibular and auditory stimuli. These apical structures consist of a bundle of villi-like structures called stereocilia and kinocilia, collectively referred to as a hair bundle. Because these cilia have been shown to play a key role at least in mechanosensation during development (Kindt et al., 2012), we immunostained crispants (sgRNA2 injected with Cas9) and control injected embryos (sgRNA2 without Cas9) at 5 dpf using anti-acetylated tubulin antibody. In 40% of crispants (n=15), a reduced number of cilia was observed while their number in control injected embryos (n=10) was similar to that of uninjected embryos (n=10) (Figure 6E+E'). In order to examine the morphology of hair cells themselves, we used FM1-43. This did not reveal any obvious difference between wild-type and morphant embryos. However, we observed that the length of the cilia was decreased when compared to wild-type embryos (data not shown)."

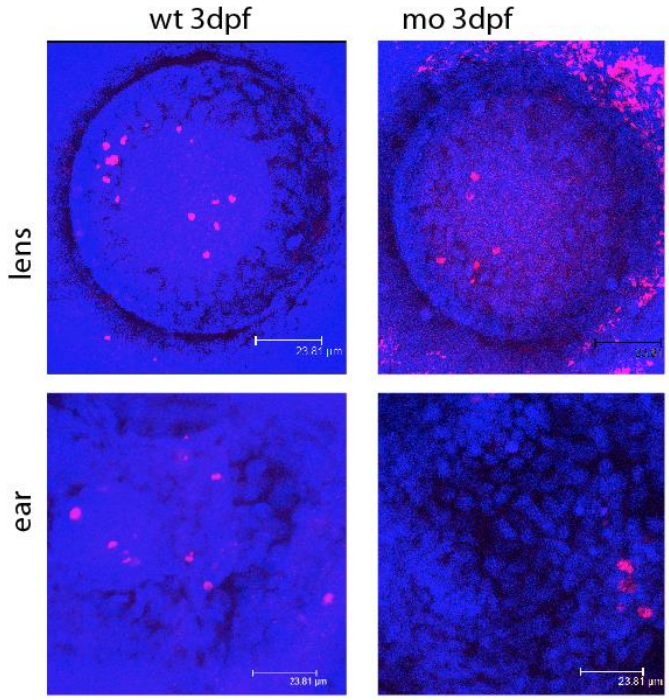
Minor comments:
2- Patients present some variability in phenotype; what's the authors explanation for those differences?

We thank the reviewer for pointing this out. This is always very intriguing to observe subtle or major phenotypic variability while having the same gene mutated and in the present case even the same

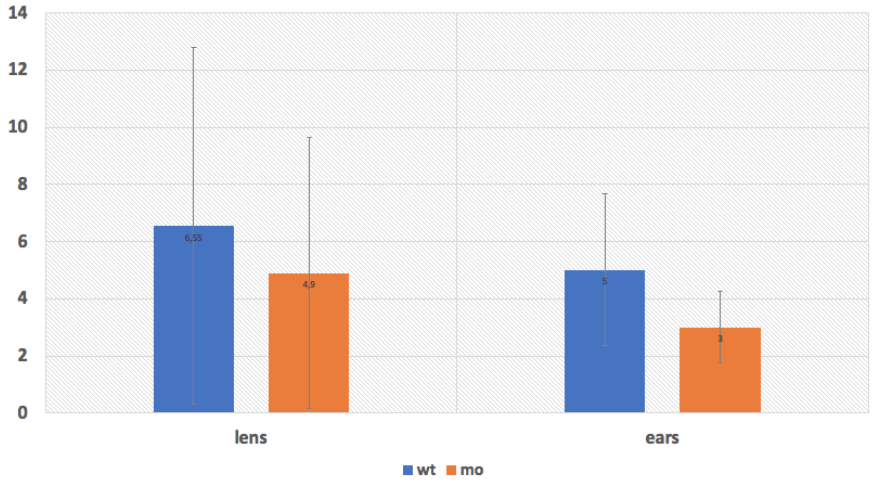
pathogenic variation. However, this is not an isolated case. For instance, having worked in the ciliopathy field for many years, especially on the Bardet-Biedl syndrome (BBS; MIM 209900), there is a single pathogenic variation (founder European effect, c.1169T>G, p.M390R) in *BBS1* that can lead to the full spectrum of the disease (*retinitis pigmentosa*, postaxial polydactyly, obesity, hypogonadism, cognitive impairment and kidney dysfunction) or "only" an isolated *retinitis pigmentosa.* Even in the same family with multiple affected siblings, there is variability in their phenotype.
The observed variability can be linked to the genetic background of each individual. In this large family of consanguineous origin only a few shared homozygous regions were observed. In fact, the region of 2.12 Mb encompassing *PSMC3* on chromosome 11 is the largest observed (Figure S1). In line with that, one cannot exclude modifier genes that would enhance or moderate some phenotypic aspects although nothing was obvious could be observed and would require more families and individuals to be explored. Among the other possible explanations, differences in their respective gene expression patterns or their respective environment can possibly influence their conditions.

4- What about cell death (TUNEL, caspase ...) in the eye and ear of zebrafish models?

The number of apoptotic cells detected with the TUNEL assay in the lens of morphants appears to be slightly smaller than that in wild-type embryos at 3 dpf. However these results are not significant (see p-value). The same tendency is observed in the ear. In conclusion, cataract or deafness cannot be explained by an increased apoptosis.



Number of TUNEL-positive cells in lenses. N=9 for each conditions.



p-value for lens: 0.52
p-value for ears: 0.32

In line with these results, we added the following sentence to the manuscript: “The observed cataract was not due to increased apoptosis, as TUNEL staining did not reveal more positive nuclei in the morphant compared to wild-type (data not shown). “

3- It's not clear if haploinsufficiency is the sole mechanism taking place in patients. Heterozygotes (50% of protein) are normal. Patients cells show no difference in protein localization, but it's not clear how much wild-type protein (or transcript) is present despite the PSMC3 pathogenic variant. Is the truncated protein still present or not? If the truncated form might have some gain (or semi-dominant) function is not clear.

We agree with the reviewer as we do not have a definitive answer on this but only hypothesis. Further families and patients with additional pathogenic alleles would be necessary for sorting this out. We have tried to clarify this in the updated version of the manuscript. Situation is complex given the effect on the proteasome regulation.
As we have pointed out in the discussion, *PSMC3* is predicted to be extremely intolerant to loss of function (LoF) variations as the other reported proteasome genes causing diseases. In other words, only *de novo* LoF would be expected for such gene.

The effect of the homozygous variation is to incorporate a novel cryptic exon leading to a frameshift in the coding sequence of the transcript. There are three facts that indicate an additional (semi-)dominant negative effect (although only minor) of the altered version missing the C-terminus of the protein, which is important for proteasome assembly and function:

1. We observe minor expression of the transcript with the cryptic exon (Figure 1E).
2. In Figure 3C (native PAGE analysis) there is a clear accumulation of 19S precursor complexes in the patient’s cells indicating assembly problems (lower bands).
3. In Figure 3D in the immunoblots for Rpt5 there is faint band coming up in the patient’s sample only, which runs below of the correct size of Rpt5 (i.e. 49,203.54 Da) and corresponds to the truncated form in its size ((i.e. 43,458.95 Da using longer exposure time).

It is well known that haploinsufficiency describes a single allele (by extension 50% of the protein) not sufficient to maintain its function in a given cellular process. In our situation, we hypothesize that each allele is maybe contributing only to 25% of damaging allele which when heterozygous does not lead to defects in the cell and thus no phenotype. However at the homozygous state, we have 50% (combined) of damaging allele that is enough to cause the phenotype. This would also fit with a so called

hypomorphic allele. The difficulty to analyze such cases has been reported very recently (Misra *et al*, 2020; Monies *et al*, 2017).

1- There are many typographical errors (missing spaces; most references positioned after the "."; ref TANAKA (not in capitals),...)
e.g. Page 9: last line:
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This is important work and reveals another proteasome related case for neurological diseases.
I have only a few points.

We thank the reviewer for the kind words and for the comments. Please find our comments in return.

1. The data are shown with fibroblasts from only one (out of three) patients and it would be better to show at least one other patient's fibroblast as well.

We appreciate the reviewer's comment and we also wished we had access to further samples of affected patients. Unfortunately, we had one ethical agreement for taking a skin biopsy for only patient II.4, while the others branches did not want to do so. Nevertheless, we have tried again after the reviewer's comment and they did not change their mind.

2. The experiments would be even better when normal psmc3 is overexpressed in the fibroblast to compete mutant psmc3 away and then show that the phenotypes (ubiquitin accumulation, responses to proteolytic stress) are corrected.

We have followed the referee's excellent suggestion and performed a series of rescue experiments in which patient fibroblasts were transfected with constructs expressing wild-type PSMC3 driven by CMV promoter. These results are now integrated in a novel Figure 5. Indeed, PSMC3/Rpt5 is highly expressed upon transfection. Our data show that PSMC3/Rpt5 overexpression resulted in decreased accumulation of K48-linked ubiquitin-protein conjugates in mutant cells, as determined by western-blotting (Figure 5A). Densitometry analysis of four independent experiments revealed that the intracellular concentration of ubiquitin-modified proteins was reduced by about 20% in patient cells following PSMC3 transfection (Figure 5B), thereby confirming the decisive role of the *PSMC3* homozygous mutation in perturbing protein homeostasis. In addition, restoring wild-type PSMC3 in PSMC3 mutant fibroblasts led to a decreased overload of the TCF11/Nrf1 pathway, as evidenced by decreased processing/activation of the TCF11/Nrf1 transcription factor under these conditions (Figure 5C). Most importantly, this was further accompanied by the capacity of the patient cells to rescue proteasome subunit expression in response to proteotoxic stress initiated by carfilzomib (Figure 5C). Altogether, these data clearly identify the deep intronic homozygous *PSMC3* variation as the genetic cause for the failure of the cells to preserve protein homeostasis under proteotoxic stress. This point is now addressed in the revised version of the manuscript (Figure 5, page 6, lines 237-250 and page 9, lines 385-390).

3. PA28 is highly expressed in the patient fibroblasts. What is the reason for this effect? And why does it decrease following proteotoxic stress?

The reviewer raises a valid point here, as the PA28 expression profile observed in patient's cells clearly differs from that detected in control fibroblasts. Noteworthy and apart from PA28, patient fibroblasts carrying the deep intronic homozygous *PSMC3* variation are also endowed with higher amounts of immunoproteasome subunits (i.e. β5i and β1i), as determined by western blotting (Figure 4). The upregulation of PA28 and immunoproteasome subunits in these cells might reflect a mechanism destined to compensate the inefficiency of mutant proteasomes at eliminating damaged proteins. It is indeed understood that 26S immunoproteasomes are more effective than their standard counterparts in clearing ubiquitin-marked proteins (Seifert *et al*, 2010; Ebstein *et al*, 2013; St-Pierre *et al*, 2017). Likewise, it has been shown that PA28-20S complexes are more efficient than free 20S proteasomes at removing oxidant-damaged proteins (Pickering *et al*, 2010; Li *et al*, 2010). It is therefore seductively easy to imagine that patient cells upregulate PA28 and immunoproteasomes in order to assist their mutant proteasomes to cope with protein aggregates. The process leading to the upregulation of both PA28 and immunoproteasome subunits in patient fibroblasts remains unclear but may conceivably rely on a type I interferon (IFN) autocrine loop which is frequently detected in cells suffering from proteasome loss-of-function mutations (Brehm *et al*, 2015; Poli *et al*, 2018). The reason why the steady-state expression level of PA28-α drops following proteasome inhibition in patient fibroblasts is not addressed in this manuscript but may be explained by the overall decrease of proteasome subunits observed in these cells under these conditions. Because PA28 physically associates with 20S core particles to form PA28-20 proteasome complexes, it is highly likely that PA28-α undergoes degradation together with the 20S proteasome subunits. These points are now clarified in the revised version of the manuscript (page 6, lines 227-229 and 238-239 as well as page 9, lines 405-407).
In this context it is also interesting to note that type interferon impacts stem cell function (Eggenberger *et al*, 2019; Yu *et al*, 2015) .

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1st Revision - Editorial Decision 15th Apr 2020

20th Apr 2020

Dear Dr. Muller,

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 this reviewer is now supportive and I am pleased to inform you that we will be able to accept your manuscript pending the following final amendments:

1) Please address the minor comments of referee 1 and expand the discussion.

Please provide a point-by-point letter INCLUDING my comments as well as the reviewer's reports and your detailed responses to their comments (as Word file).

2) Please carefully check the authors guidelines for formatting your supplemental information: Expanded view and Appendix (see: https://www.embopress.org/page/journal/17574684/authorguide#expandedview)
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-For the movie, legend should be removed from appendix and zipped together with the movie file. Nomenclature should be corrected to "Movie EV1"
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-fig s10D is missing magnification scale bar

3) Source Data:

We encourage the publication of source data, particularly for electrophoretic gels, blots, but also microscopy images with the aim of making primary data more accessible and transparent to the reader. Would you be willing to provide a PDF file per figure that contains the original, uncropped and unprocessed scans of all or key gels used in the figure (including molecular weight markers)? The PDF files should be labeled with the appropriate figure/panel number (1 file/figure), and should have molecular weight markers; further annotation may be useful but is not essential. The PDF files will be published online with the article as supplementary "Source Data" files. If you have any questions regarding this just contact me.

4) In the main manuscript file, please do the following:
- correct/answer the track changes suggested by our data editors by working from the attached document
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- revise the cal out for figure 4A to figure 4
- move Table 1 before figure legends
- spell out "ENT" line 488
- remove "data not shown" line 284. As per our guidelines, on "Unpublished Data" the journal does

not permit citation of "Data not shown". All data referred to in the paper should be displayed in the main or Expanded View figures. "Unpublished observations" may be referred to in exceptional cases, where these are data peripheral to the major message of the paper and are intended to form part of a future or separate study, the names of the persons that reported the observation should be listed in brackets. Personal communications (Author name(s), personal communications) must be authorised in writing by those involved, and the authorisation sent to the editorial office at time of submission.

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6) Authors' contribution: the contribution of every author must be detailed in a separate section. Make sure to differentiate the contribution of Fouzia Studer and Florian Sandron

7) The Paper Explained should b emoted in the main article, after For More Information. Please label the subsections as Problem, Results, Impact

8) Synopsis.

I have slightly modified the text, would the following be of for you?

Synopsis
Whole genome sequencing in a large consanguineous family with neurosensory syndrome including revealed a unique homozygous deep intronic pathogenic variant in PSMC3, encoding one of the proteasome subunit. Further in vitro and in vivo analyses confirmed the pathogenicity of the PSMC3 mutation.

Bullet points
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• PSMC3 plays a major role in inner ear, lens and central nervous system development
• These results expand our knowledge on the genetic background of the emerging proteasomopathy.

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I look forward to reading a new revised version of your manuscript as soon as possible.

Yours sincerely,

Celine Carret

Celine Carret, PhD
Senior Editor
EMBO Molecular Medicine

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4) a letter INCLUDING the reviewer's reports and your detailed responses to their comments (as Word
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***** Reviewer's comments *****

Referee #1 (Remarks for Author):

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The precise causal mechanisms of some reported patient phenotypes still warrant further studies, but the authors provide a substantial amount of new data that deserve publication in the EMM journal.

Minor points:
The discussion of inner ear related data can be further improved.
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20th Apr 2020

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Dear editor, this is very good news and we hope that we have now replied to all of the points. See our comments (in red) below.

1) Please address the minor comments of referee 1 and expand the discussion.
This has been done. See our replies in the corresponding section.

Please provide a point-by-point letter INCLUDING my comments as well as the reviewer's reports and your detailed responses to their comments (as Word file).

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Ok this has been done.

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Ok this has been done.

-appendix figures are of rather poor quality, please try to improve the resolution of the figures.
We are surprised by this and it must be linked to some default Word document settings (automatic image compression) that has been changed now. Figures have also been regenerated with a higher resolution and incorporated again.

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Ok this has been added and corrected in the corresponding figure legend.

3) Source Data:

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We fully agree on this point. This is a very important part of science and we have now assembled all source data that were required.

4) In the main manuscript file, please do the following:
- correct/answer the track changes suggested by our data editors by working from the attached document
Ok this has been done.

- add up to 5 keywords
Ok this has been done.

- in M&M, a statistical paragraph is needed and it should reflect all information that you have filled in the Authors checklist, especially regarding randomisation, blinding, replication.
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Ok this has been done in the manuscript and in the Appendix Data. We provide now an Appendix Table S8 including the exact *p*-values when not available already in the figures.

- revise the cal out for figure 4A to figure 4
Ok this has been done.

- move Table 1 before figure legends
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- spell out "ENT" line 488
Ok ENT mean “Ear-Nose-Throat”. This has been spelled out in the updated version of the manuscript.

- remove "data not shown" line 284. As per our guidelines, on "Unpublished Data" the journal does not permit citation of "Data not shown". All data referred to in the paper should be displayed in the main or Expanded View figures. "Unpublished observations" may be referred to in exceptional cases, where these are data peripheral to the major message of the paper and are intended to form part of a future or separate study, the names of the persons that reported the observation should be listed in brackets. Personal communications (Author name(s), personal communications) must be authorised in writing by those involved, and the authorisation sent to the editorial office at time of submission.
We understand this. The “data not shown” was referring to the additional data provided during the reviewing process. This has been now included as Appendix Figure S10 and S15.

5) Funding:

Please make sure to indicate in our submission system all sources of funding including grant numbers and to whom they are allocated.
We will take make sure all will be done properly.

6) Authors' contribution: the contribution of every author must be detailed in a separate section. Make sure to differentiate the contribution of Fouzia Studer and Florian Sandron
Ok this has been done. Fouzia Studer is abbreviated as F.S. while Florian Sandron as F.Sa.

7) The Paper Explained should b emoted in the main article, after For More Information. Please label the subsections as Problem, Results, Impact
Ok this has been done.

8) Synopsis.

I have slightly modified the text, would the following be of for you?
This is ok for us. We would simply remove the word “including” highlighted in red below as it does not fit anymore. We have now removed the file from the online data deposit as it is included in this file in an updated version.

Synopsis
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Please note that the Authors checklist will be published at the end of the RPF.
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10) Please note that we now mandate that all corresponding authors list an ORCID digital identifier. This takes less than 90 seconds to complete. We encourage all authors to supply an ORCID identifier, which will be linked to their name for unambiguous name identification.
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Please relabel the section and format as following:

The datasets (and computer code) produced in this study are available in the following databases:

- [data type]: [full name of the resource] [accession number/identifier] ([doi or URL or identifiers.org/DATABASE:ACCESSION])

examples:
* RNA-Seq data: Gene Expression Omnibus GSExxxxx (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSExxxxx)
* Chip-Seq data: Gene Expression Omnibus GSEyyyyy (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSEyyyyy)
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* Protein interaction AP-MS data: PRIDE PXD000xxx (http://www.ebi.ac.uk/pride/archive/projects/PXD000xxx)
* Imaging dataset: Image Data Resource doi:10.17867/10000xxx (http://doi.org/10.17867/10000xxx)
Ok this has been done. All datasets will be made or are already public. The ClinVar data will be released upon publication and availability in PubMed.

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

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Yours sincerely,

Celine Carret

Celine Carret, PhD
Senior Editor
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Indeed, OtoAcoustic Emissions (OAE) were present at birth, indicating that outer hair cells were initially functional. But at the time deafness was diagnosed (8 month and 1 year and 3 month old), otoacoustic emissions were no longer recorded: transitory-evoked OAE were not present for patient II.2 and II.7 and Distortion-Product OAE (DP-OAE) were not present for patient II.4 who underwent full auditory examination under general anesthesia in Belgium in July 2005. During this examination, a neuropathic component was evoked after recording a cochlear microphonic on both sides (during Auditory Brainstem Response, using separate runs of condensation and rarefaction polarity clicks), even though DP-OAE were absent (with the assumption that the DP-OAE were absent due to the presence of grommets). However, lack of additional information did not help to either confirm or infirm this hypothesis and discordance between tone and vocal audiometry cannot be established due to autistic features and severe language delay.
According to your remarks, we have now exposed the cochlear implant outcome performances in the discussion.

2nd Revision - Editorial Decision 7th May 2020

7th May 2020

Dear Dr. Muller,

We are pleased to inform you that your manuscript is accepted for publication and will be soon sent to our publisher to be included in the next available issue of EMBO Molecular Medicine.

Before we proceed however, could you please clarify the following: in the figure 3, the molecular weight for PSMC1 is indicated at ~ 40kDa but it is higher in the source data file. Please amend the figure 3 accordingly and send the new one to us by email.

Can you please also encourage your co-corresponding author Dr. Strähle to add his ORCID number? We won't be able to move forward without these. Thank you for your cooperation and understanding.

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Corresponding Author Name: Elke Krüger Jean Muller, Uwe Strähle, Hélène Dollfus
Journal Submitted to: EMBO Molecular Medicine
Manuscript Number: EMM-2019-11861

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?	Human sample size was limited by the patients available. For animal studies, significancy was achieved using known guidelines for these type of studies.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	This is done.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	NA
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.	Yes, the samples selected were randomly selected from the larger population considered.
For animal studies, include a statement about randomization even if no randomization was used.	This is done.
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	Yes done.
5. For every figure, are statistical tests justified as appropriate?	Yes done.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	NA
Is there an estimate of variation within each group of data?	Yes

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

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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?	Yes

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).	Yes done.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	NA

* 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.	Yes done.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	Yes done.
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.	Yes done.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	Yes done.
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.	Yes done.
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	Yes done.
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	Yes done.
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'. 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	Yes done.
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).	Yes
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).	Yes done.
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