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Rassf7a promotes spinal cord regeneration and controls spindle orientation in neural progenitor cells

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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 **1st Apr 2022**

Dear Dr. Zhao,

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

As you will see, the referees acknowledge that the findings are potentially interesting. However, they also together raise a number of concerns, and your manuscript is thus a borderline case. I think all points raised are reasonable and important, and all therefore need to be successfully addressed for us to proceed with the handling of your manuscript here. Please let me know in case you disagree, and we can discuss the revisions further.

I would thus like to invite you to revise your manuscript with the understanding that the referee concerns must be fully addressed and their suggestions taken on board. Please address all referee concerns in a complete point-by-point response. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of major 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.

We realize that it is difficult to revise to a specific deadline. In the interest of protecting the conceptual advance provided by the work, we recommend a revision within 3 months (1st Jul 2022). Please discuss the revision progress ahead of this time with the editor if you require more time to complete the revisions.

IMPORTANT NOTE: we perform an initial quality control of all revised manuscripts before re-review. Your manuscript will FAIL this control and the handling will be DELAYED if the following APPLIES:
1) A data availability section providing access to data deposited in public databases is missing. If you have not deposited any data, please add a sentence to the data availability section that explains that.
2) Your manuscript contains statistics and error bars based on n=2. Please use scatter blots in these cases. No statistics should be calculated if n=2.

When submitting your revised manuscript, please carefully review the instructions that follow below. Failure to include requested items will delay the evaluation of your revision.

1) a .docx formatted version of the manuscript text (including legends for main figures, EV figures and tables). Please make sure that the changes are highlighted to be clearly visible.

2) individual production quality figure files as .eps, .tif, .jpg (one file per figure). See https://wol-prod-cdn.literatumonline.com/pb-assets/embo-site/EMBOPress_Figure_Guidelines_061115-1561436025777.pdf for more info on how to prepare your figures.

3) We replaced Supplementary Information with Expanded View (EV) Figures and Tables that are collapsible/expandable online. A maximum of 5 EV Figures can be typeset. EV Figures should be cited as 'Figure EV1, Figure EV2" etc... in the text and their respective legends should be included in the main text after the legends of regular figures.

- For the figures that you do NOT wish to display as Expanded View figures, they should be bundled together with their legends in a single PDF file called *Appendix*, which should start with a short Table of Content. Appendix figures should be referred to in the main text as: "Appendix Figure S1, Appendix Figure S2" etc. See detailed instructions regarding expanded view here: <https://www.embopress.org/page/journal/14693178/authorguide#expandedview>

- Additional Tables/Datasets should be labeled and referred to as Table EV1, Dataset EV1, etc. Legends have to be provided in a separate tab in case of .xls files. Alternatively, the legend can be supplied as a separate text file (README) and zipped together with the Table/Dataset file.

4) a .docx formatted letter INCLUDING the reviewers' reports and your detailed point-by-point responses to their comments. As part of the EMBO Press transparent editorial process, the point-by-point response is part of the Review Process File (RPF), which will be published alongside your paper.

5) a complete author checklist, which you can download from our author guidelines <https://www.embopress.org/page/journal/14693178/authorguide>. Please insert information in the checklist that is also reflected in the manuscript. The completed author checklist will also be part of the RPF.

6) Please note that all corresponding authors are required to supply an ORCID ID for their name upon submission of a revised manuscript (<https://orcid.org/>). Please find instructions on how to link your ORCID ID to your account in our manuscript tracking system in our Author guidelines <https://www.embopress.org/page/journal/14693178/authorguide#authorshipguidelines>

7) Before submitting your revision, primary datasets produced in this study need to be deposited in an appropriate public database (see https://www.embopress.org/page/journal/14693178/authorguide#datadeposition). Please remember to provide a reviewer password if the datasets are not yet public. The accession numbers and database should be listed in a formal "Data Availability" section placed after Materials & Method (see also https://www.embopress.org/page/journal/14693178/authorguide#datadeposition). Please note that the Data Availability Section is restricted to new primary data that are part of this study. * Note - All links should resolve to a page where the data can be accessed. *
If your study has not produced novel datasets, please mention this fact in the Data Availability Section.

8) We would also encourage you to include the source data for figure panels that show essential data. Numerical data should be provided as individual .xls or .csv files (including a tab describing the data). For blots or microscopy, uncropped images should be submitted (using a zip archive if multiple images need to be supplied for one panel). Additional information on source data and instruction on how to label the files are available at <https://www.embopress.org/page/journal/14693178/authorguide#sourcedata>.

9) Our journal also encourages inclusion of *data citations in the reference list* to directly cite datasets that were re-used and obtained from public databases. Data citations in the article text are distinct from normal bibliographical citations and should directly link to the database records from which the data can be accessed. In the main text, data citations are formatted as follows: "Data ref: Smith et al, 2001" or "Data ref: NCBI Sequence Read Archive PRJNA342805, 2017". In the Reference list, data citations must be labeled with "[DATASET]". A data reference must provide the database name, accession number/identifiers and a resolvable link to the landing page from which the data can be accessed at the end of the reference. Further instructions are available at https://www.embopress.org/page/journal/14693178/authorguide#referencesformat

10) Regarding data quantification (see Figure Legends: https://www.embopress.org/page/journal/14693178/authorguide#figureformat)

The following points must be specified in each figure legend:

- the name of the statistical test used to generate error bars and P values,

- the number (n) of independent experiments (please specify technical or biological replicates) underlying each data point,

- the nature of the bars and error bars (s.d., s.e.m.),

- If the data are obtained from n {less than or equal to} 2, use scatter blots showing the individual data points.

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.

11) The journal requires a statement specifying whether or not authors have competing interests (defined as all potential or actual interests that could be perceived to influence the presentation or interpretation of an article). In case of competing interests, this must be specified in your disclosure statement. Further information: https://www.embopress.org/competing-interests

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

As part of the EMBO publication's Transparent Editorial Process, EMBO reports publishes online a Review Process File (RPF) to accompany accepted manuscripts. This File will be published in conjunction with your paper and will include the referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript.

You are able to opt out of this by letting the editorial office know (emboreports@embo.org). If you do opt out, the Review Process File link will point to the following statement: "No Review Process File is available with this article, as the authors have chosen not to make the review process public in this case."

I look forward to seeing a revised form of your manuscript when it is ready. Please use this link to submit your revision: https://embor.msubmit.net/cgi-bin/main.plex

Yours sincerely,

Esther Schnapp, PhD
Senior Editor

Referee #1:

The present study investigates the role of Rassf7a in spindle orientation during regenerative neurogenesis. A large number of overall logical in vivo and in vitro experiments are presented. The described role of Rassf7a is new and as such, this is a study could find some interest amongst colleagues investigating neurogenesis.
As presented, however, there are a number of significant shortcomings. The figures are of insufficient quality (see detail below), and the concepts are not always clearly explained. Some sections are really clear and correctly describe the literature/interpret own results, e.g. lines 100 to 200. Some are not clear at all, e.g. the section on "neuronal proliferation" starting from line 267. Behaviour experiments are inappropriate where manipulations cause gross anatomical abnormalities. It is not clear how cell counts, one key metric in this paper, have been performed. Careful revision could address these issues. It could be one option to "declutter" the paper and focus mainly on the role of Rassf7a in the division axis and regenerative neurogenesis.

comments in order the appear in the manuscript:

Line 29: local neurogenesis defects are not a prominent problem after spinal cord injury, therefore, the authors are framing their findings from the wrong starting point. Interestingly, in the introduction, the scene setting is appropriate and the abstract should be adapted accordingly.
Line 36: neurons are by definition post-mitotic and no longer proliferate (multiple times in the manuscript, e.g. also line 267)
Line 75: "spinal injury scar" is an unusual term in the field. In fact, that phrase is not found on Pubmed at all.
Line 76: it is not clear what is meant by "intracellular and extracomponents" when the authors name ECM and cell types.
Line 79: the sentence is not clear.
Line 97: "lesion-induced paralysis" is not something you perform. It is a consequence of a spinal lesion.
Line 98: Bhatt and colleagues performed lesions at 5-6 days post fertilisation, not 3.
Line 105: Wehner et al 2017 show that inhibition of wnt signalling in glial cells does not impair axonal regeneration.
In intro, give some background how you found rassf7(a) and why you decided to investigate it.
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Fig S2: were cilia of normal length?
Line 227: time line of injury closure is much longer than described by the Dorsky, Becker, and Wehner labs. Why is that?
Line 233ff: The mutant larvae are grossly malformed and the lack of swimming capacity might as well be a consequence of that. Have the authors done a sub-analysis of larvae without dorsal bending, or does successful axonal regeneration co-segregate with straightness?
Fig 2: The resolution of the figures is not good enough to distinguish "cells" from "processes" and the figure legend and references to the data in the figure should be changed accordingly. The same holds true for fig 4S.
Also: the y-axes should start at 0.
Why is the fluorescence in the injury site not at 0 directly after the injury?
Finally, the fish in the top right gfap:GFP wild type has not been fully lesioned.
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Line 260: do you mean there was a rescue of the number of HuC:GFP postive cells after overexpression of Rassf7a?
The whole section starting in line 267 (also Fig 3), speaks of neuronal proliferation or proliferating neurons and this is fundamentally wrong "neuronal cells" are by definition post-mitotic. Neurons that are positive for BrdU will have arisen from division of a glial progenitor after injury, they are not actively proliferating.
Line 299ff: Goldshmit et al 2012 have already shown that neurons react to Fgf8 and should be referenced here.
Line 320ff: it is known that progenitor cells sit at the central canal and make up the ventricular lining.
Line 421: say what specifically Rassf7a regulates.
Line 483ff: regenerating axons are not entirely derived from new neurons.
Line 457: CNS development was not analysed in sufficient detail to say that is was unaltered in mutants.
Line 469: this sentence does not make sense. Can the authors rephrase the relationship between neurogenesis and axonal regeneration?
Line 521: what was the survival rate of the larvae?
M&M: cell counting methods are insufficiently specified. The region of interest choice is not clearly explained and methods to reduce observer bias have not been laid out.

Referee #2:

In this study, Zhu et al. uncover a new role of Rassf7a during zebrafish spinal cord regeneration. After mapping rassf7a and rassf7b expression in developing zebrafish using in situ hybridization, the authors employ newly generated zebrafish with mutation in both rassf7 paralogues to define Rassf7 roles during growth and stress-induced conditions. First, as rassf7b expression resembles the one of genes involved in ciliogenesis, the authors investigate cilia morphology, which result to be

unaffected by rassf7a and/or rassf7b expression. To define potential roles of Rassf7 during stress-induced conditions, the authors perform a series of experiments after subjecting zebrafish larvae to a spinal cord transection injury. In this condition, rassf7 mutant larvae displayed dorsal bending phenotype and reduced motility compared to wild types. Assays with larvae allowing fluorescent labeling of glial cells and neurons revealed that whereas loss of rassf7a impairs regeneration of neurons, rassf7a overexpression improves it. Effects on neuronal cells mostly derived from impaired proliferation of rassf7a-expressing neural progenitors, which could be rescued with FGF8 injections in mutant fish. To characterize the cellular basis of the observed proliferation defects, the authors imaged larvae carrying fluorescently labeled sox2-expressing progenitors. Rotation angles of spindles were increased in rassf7a mutant versus wild type cells, as also validated in cultured cells. The involvement of rassf7a in spindle orientation was further confirmed by transcriptomic analyses on rassf7a mutants, which showed reduction of spindle polarity components.

The involvement of rassf7a in spinal cord regeneration is novel and of potential interest. However, the rationale behind investigating the role of this gene versus other factors is unclear. The introduction could explain in a more concise way the importance of the question being asked and address the focus of the study more clearly. Despite the potential interest for the spinal cord regeneration community, some of the experiments are not performed and explained in the most rigorous manner.

Specific comments:

Variability in larval regeneration assays is extremely high and previous studies used high numbers of larvae to measure glial/axonal bridges (i.e. PMID: 28743881). In figure 2C only 7 larvae are assessed, which is a small number to make solid conclusions. Also, in the images shown in 2A the glial bridge appears to be thicker in mutants than in wild types. Are the images shown representative?

I feel the experiments performed, although informative, are not enough to conclude that there is "axonal regeneration". The lesion site might be filled up with neurons, positive for HuC, rather than new axons growing and crossing the lesion. Quantification of axonal bridge thickness assessed using an anti-acetylated alpha tubulin staining would further strengthen the results.

Is there a difference in the percentage of larvae with axonal and glial bridge between wild type and rassf7a mutant larvae?

Images shown in Fig. 3A make it hard to visualize cells that were counted. There seems to be a lot of noise, higher magnification images and separate channels are needed to convince the reader about potential differences in proliferation. Also, the images shown don't seem to be representative: in the 3 dpi high-magnification view shown in Fig. 3A, HuC positive cells appear to proliferate more in mutants than in wild types, which is the opposite of what shown in the quantification.

In Fig. 3F it is unclear if the curve representing the mutants is missing or the two curves completely overlap.

From the ISH shown in Fig. 4 it is difficult to tell if sox2 and rassf7a co-localize. Ideally, one would want to see sections of a rassf7a reporter line crossed with a sox2 line (or sections stained with antibodies), as well as a DAPI signal, to clearly see both proteins in the same cell. The yellow dots in the figure might be signals from 2 cells overlapping. As an alternative, one could FACS purify sox2+ cells from the sox2 reporter line used in the study and perform qPCR to show increase in rassf7a in this cell population after injury.

Inhibition of Fgf signaling reduces HuC signal in wild types but not in rassf7a mutants, suggesting that rassf7 effect might be independent from Fgf signaling. Conversely, administration of Fgf has effects in rassf7a mutants but not wild types. This makes it hard to understand if rassf7a effect is dependent or independent from Fgf and needs a better explanation.

For the imaging experiments in Tg(sox2:sox2-2a-sfGFP) fish shown in Fig.5 the authors employed morpholinos to reduce rassf7a levels. Morpholinos should be used with caution (Stainier et al., PLoS Genetics, 2017) and mutants are generally preferred when available. These results would be more accurate using available rassf7a mutants crossed with the sox2 reporter line.

Ideally, markers to track cell membranes and histone tagged nuclei should be used to visualize and measure angles of division, in rassf7a wild type and mutant fish. Is not clear how spindles were identified to perform quantifications. Also, how many cells were quantified and from how many larvae?

In the discussion the authors write: "fewer neurons were generated in the mutants, leading to final axonal regeneration defects". This is an overstatement, as axon growth has not been measured (see comment above).

For all assays shown, it is unclear how many times experiments were repeated.

There are multiple mistakes in the text, which needs careful editing. For example:
Fig. 5D y-axis: edit "oritation" with "orientation"
Line 256: "control vivo Mos" remove "vivo".

Line 314: "singals" replace with "signals"
Line 375: Rassf7 has 3s
Fig.S10 legend: edit "spina" with "spinal"
Line 425: "in consistent with this" edit "in agreement with this"
Line 433: "asymmetri "edit with "asymmetric"
Line 447: "were" with "are"

Referee #3:

In their study « Rassf7a regulates spinal cord regeneration through modulating spindle orientation in neural progenitor cells » the authors characterise the function of rass7a & b in zebrafish development. While the initial working hypothesis of the study is that rassf7 proteins may be required for ciliogenesis, the authors show that rassf7 is dispensable for cilia formation and animal viability under normal conditions.
In contrast, rassf7a appears promotes regeneration upon spinal cord injury by regulating the division orientation of neural progenitor cells. In the absence of rassf7a, cells switch from a perpendicular asymmetric mode of division (that would generate one neuronal progenitor and one differentiating neuron) to a planar symmetric orientation that generates two progenitors and thereby impairs the generation of neurons allowing spinal cord regeneration. While implications of Rassf7 in cell division have already been reported (e.g. Sherwood & al 2008, Recino & al 2010, Gulsen & al 2016), the identification of rassf7a as a new regulator of spinal cord regeneration is interesting, even if mechanistic insights into how rassf7 might regulate division orientation are unfortunately lacking. While the findings of this work are potentially interesting, I do however believe that a number of question still need to be addressed.

Major questions :

- I am not convinced by the authors statement (line 232) that « In most cases, regenerated larvae with severe dorsal bending failed to react to tail touch stimulus ». In Movie S2 with severely bent embryos, most embryos DO react to the stimulus, they just fail to elicit a proper escape reponse as they are unable to swim straight due to their curvature. The same applies to Etho Vision experiment displayed in Fig.1G,H. This is an important point, as it raises the question of the actual success (or not) of functional spinal cord regeneration.

- The authors demonstrate convincingly that rassf7a mutants present a reduction in the proliferation of huC-positive cells, while gfap-positive radial glia appears unaffected. A major question is why rassf7b would specifically affect the division of a given cell type? Is rassf7b actually only expressed in one of the two cell types? Right now, the resolution of the RNA in situs shown in Fig.4 is not sufficient to really address this question. Better data are needed to substantiate the point that « rassf7a was mainly enriched in the neural progenitor cells » (as opposed to radial glia). Another important experiment would be to use Rassf7a-GFP to follow protein localization in symmetric as well as asymmetric divisions.

- The major finding of the paper is the observation that rassf7a controls cell division orientation. In Fig.6, the authors present evidence for a change in spindle rotation that appears however relatively minor with most cells still rotating by the normal 0-45{degree sign} (Fig.6B). In Fig.7, they do however show a very dramatic switch from a mostly perpendicular to a mostly planar division orientation (Fig.7B). It is not clear to me whether and how these two aspects of division orientation are linked? One caveat of the current experiments is that they are solely based on the use of rassf7a morphants. As these experiments concern the main finding of the paper, they need to be confirmed using the genetic mutants that the authors have generated.

- A further important issue is that while it appears indeed plausible that the switch from perpendicular to planar divisions promotes the production of neuronal progenitors at the expanse of differentiating neurons, there is presently no direct evidence for this hypothesis. More clearcut evidence would be required here as it concerns the main finding of the study.

Additional points :

- The authors describe the alterations of rassf7a & b mutations at the DNA level (-4 & -7 bp deletions) but do not provide evidence whether these changes indeed induce the expected changes at the level of the mutant transcripts, or cause unexpected changes, e.g. due to exon skipping. Mutant transcripts should be entirely sequenced and their NMD-induced change in expression levels confirmed by qPCR. As both mutants are homozygous viable, these experiments should be straightforward.

- The authors mention that rassf7a or b single mutants are viable and fertile. What about double mutants?

- The authors repeatedly talk about « proliferating neuron cells » (e.g. line 273), a terminology that is incorrect as neurons are postmitotic. The term « proliferating neural progenitors » should be used instead.

- Fluorescent rassf7a in situ hybridization should be perfomed in WT vs rassf7a mutant embryos to provide a specificity control for the data shown in Fig.4 & S8.

- For many experiments the authors indicate individual data points rather than just mean values which is definitely a good thing. However, numerical values rather then */**/*** should be use to indicate p-values. Have the authors systematically test for data normality and variance? For example, the data in Fig.1I,J are mentioned as having been analyzed using t-tests, but I wonder whether there is really a normal data distribution that allows this?

- The photos in Fig3A are highly pixelated, making it impossible to distinguish the GFP and BrdU signals. Seperate channels should be shown as in Fig.3G.

- Changes in sox2 & msi1 expression upon SU5402 should be quantified by qPCR.

Referee #1:

The present study investigates the role of Rassf7a in spindle orientation during regenerative neurogenesis. A large number of overall logical in vivo and in vitro experiments are presented. The described role of Rassf7a is new and as such, this is a study could find some interest amongst colleagues investigating neurogenesis.
As presented, however, there are a number of significant shortcomings. The figures are of insufficient quality (see detail below), and the concepts are not always clearly explained. Some sections are really clear and correctly describe the literature/interpret own results, e.g. lines 100 to 200. Some are not clear at all, e.g. the section on "neuronal proliferation" starting from line 267. Behaviour experiments are inappropriate where manipulations cause gross anatomical abnormalities. It is not clear how cell counts, one key metric in this paper, have been performed. Careful revision could address these issues. It could be one option to "declutter" the paper and focus mainly on the role of Rassf7a in the division axis and regenerative neurogenesis.

Thanks for these great suggestions. We have modified the description and also added all the necessary experiments pointed by this reviewer. Moreover, follow this reviewer's suggestion, we have removed those experiments about FGF signaling as the role of FGF during spinal cord regeneration has been reported previously. In the revised version, we will mainly discuss the role of Rassf7a during mitotic division of neural progenitor cells.

comments in order the appear in the manuscript:

Line 29: local neurogenesis defects are not a prominent problem after spinal cord injury, therefore, the authors are framing their findings from the wrong starting point. Interestingly, in the introduction, the scene setting is appropriate and the abstract should be adapted accordingly.

Thanks. We have changed the description.

Line 36: neurons are by definition post-mitotic and no longer proliferate (multiple times in the manuscript, e.g. also line 267)

Thanks for pointing out this issue. We have rechecked the manuscript and changed all the description.

Line 75: "spinal injury scar" is an unusual term in the field. In fact, that phrase is not found on Pubmed at all.

We adapted this term according to a recent review about glial scar formation after spinal cord injury. They used this term to encompass both cellular and extracellular components across the lesion core, lesion border and surrounding penumbra (Bradbury & Burnside, 2019). In the revised version, we have changed this phrase.

Line 76: it is not clear what is meant by "intracellular and extracomponents" when the authors name ECM and cell types.

After SCI, both glial scar and fibrotic scar are formed in the lesion sites. The glial scar mainly consists of reactive astrocytes, while fibrotic scar contains both fibroblasts and several other cell types, as well as extracellular ECM proteins. In the revised version, we have modified these sentences to make it clear.

Line 79: the sentence is not clear.

We have changed it.

Line 97: "lesion-induced paralysis" is not something you perform. It is a consequence of a spinal lesion.

Thanks, we have changed it.

Line 98: Bhatt and colleagues performed lesions at 5-6 days post fertilisation, not 3.

Yes, thank you for pointing out this mistake. We have changed it.

Line 105: Wehner et al 2017 show that inhibition of wnt signalling in glial cells does not impair axonal regeneration.

Yes, we have included this information in the revised version.

In intro, give some background how you found rassf7(a) and why you decided to investigate it.

Initially, we aimed to identify cilia-related genes via whole mount *in situ* hybridization assay. Both *rassf7a* and *rassf7b* exhibit specific expression pattern in those cilia-enriched tissues including Kupffer's vesicle, pronephric duct and spinal cord. We have mentioned this in the first paragraph of results.

Line 200: but what does the spinal cord development look like?

We measured the thickness of spinal cord in the mutants via Tg(*huc:GFP*) or Tg(*foxj1a:HA-tdTomato*) transgene, which labels neuronal cells and ependymal precursors respectively. There is no significant difference in the thickness of spinal cord between mutant and control larvae. Considering the normal development of the homozygous mutants, we hypothesize that spinal cords are grossly normal in the absence of Rassf7a, which may due to the redundancy

functions of other proteins in the RASSF family as we mentioned in the discussion. We have included these data in Fig S3 of the revised version.

Fig S2: were cilia of normal length?

Yes, we measured the length of cilia in the pronephric duct, Kupffer's vesicle and spinal canal. The length of cilia were comparable in these tissues between wild-type and mutant larvae. We have included these data in Fig S2G-I.

Line 227: time line of injury closure is much longer than described by the Dorsky, Becker, and Wehner labs. Why is that?

Thanks for pointing out this issue. The recovery time of injury sites was similar between ours and those published by other groups. We noticed the injury closure at around 48 hpi, while the injured larvae displayed fully recovery of spinal cord, as well as muscle and skin at around 7 days after injury (10 dpf). The images of the wound sites were also similar between ours (left images) and those published by other groups (Ohnmacht *et al*, 2016; Wehner *et al*, 2017)(see below Figure 1).

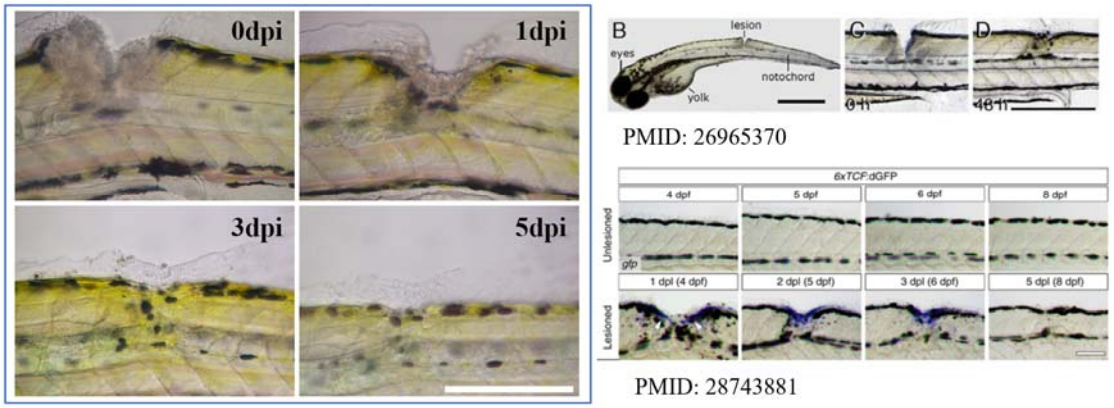

Figure 1 The recovery of lesion sites at different time after injury.

Line 233ff: The mutant larvae are grossly malformed and the lack of swimming capacity might as well be a consequence of that. Have the authors done a sub-analysis of larvae without dorsal bending, or does successful axonal regeneration co-segregate with straightness?

Thanks for these great suggestions. We have repeated this experiment and performed sub-analysis on zebrafish larvae with or without curved body axis at different time points after injury. Zebrafish larvae with straight body axis did have better swimming capacity than those with body curvature defects in both wild type and mutant larvae at 6 dpi. Of note, even in straight groups, wild type larvae also exhibited better swimming activity than those mutants

with straight body axis (Fig EV1E-G). Furthermore, we also compared axonal regeneration in both categories after SCI. Mutant larvae displayed compromised axonal regeneration in both straight and curly mutants at 5 dpi compared with wild type control (Fig EV2E, F). We have included these results in Fig EV1 and EV2 in the revised version.

Fig 2: The resolution of the figures is not good enough to distinguish "cells" from "processes" and the figure legend and references to the data in the figure should be changed accordingly. The same holds true for fig 4S.
Also: the y-axes should start at 0.
Why is the fluorescence in the injury site not at 0 directly after the injury?
Finally, the fish in the top right gfap:GFP wild type has not been fully lesioned.

To quantify the recovery efficiency, we selected a fixed size of box (837 μm x 417 μm) to cover the entire lesion site based on the bright field images of the spinal cord (Fig 2A). The ratio of GFP expression area to the size of the box was used to evaluate the recovery ratio of mutant and control larvae. This is the reason why the initial ratio of GFP fluorescence is not 0. We have also added description about statistical analysis in the revised manuscript. We agree with this reviewer that these fluorescent signals cannot be labeled as cells. We have changed to “Relative GFP expression area” in the revised text.

Line 258: how was efficiency of the morpholino verified?

This morpholino was designed to block the translation of *rassf7a* mRNA through binding to its translation start site. To further verify the blocking efficiency, we designed a reporter construct by fusion of the second exon of *rassf7a* containing ATG site, as well as morpholino binding site, to the N-term of GFP gene. Injection of this construct together with Rassf7a MO reduced GFP expression to background level, suggesting that the MO can bind to the ATG site and block translation with high efficiency. We showed this results in Fig S4A-C.

Line 260: do you mean there was a rescue of the number of HuC:GFP postive cells after overexpression of Rassf7a?
The whole section starting in line 267 (also Fig 3), speaks of neuronal proliferation or proliferating neurons and this is fundamentally wrong "neuronal cells" are by definition post-mitotic. Neurons that are positive for BrdU will have arisen from division of a glial progenitor after injury, they are not actively proliferating.

Thanks for pointing out these mistakes. Yes, we totally agree with this reviewer’s suggestion. These neurons are differentiated from progenitor cells, not self-proliferation. We have rechecked the whole manuscript and corrected all the mistakes. Thanks again.

Line 299ff: Goldshmit et al 2012 have already shown that neurons react to Fgf8 and should be referenced here.

Thanks. Following this reviewer's suggestion, we have removed this part as the role of FGF signaling has been extensively studied in zebrafish during spinal cord regeneration. We have quoted this reference in the introduction.

Line 320ff: it is known that progenitor cells sit at the central canal and make up the ventricular lining.

Yes, these progenitor cells usually localize to the central canal of the spinal cord. Our double fluorescence in situ results also suggested that *rassf7a* showed high expression in the central canal (Fig 4).

Line 421: say what specifically Rassf7a regulates.

We have changed it.

Line 483ff: regenerating axons are not entirely derived from new neurons.

Yes, the regenerating axons are derived from both original and differentiated neurons. We have changed this sentence.

Line 457: CNS development was not analysed in sufficient detail to say that is was unaltered in mutants.

Thanks. We have measured the width of the spinal cord and did not find any difference between mutant and wild type control larvae. Both the survival rate and body size are comparable between adult mutants and siblings. Although we cannot rule out the possibility of subtle defects, we think CNS development is grossly normal in the absence of Rassf7a proteins.

Line 469: this sentence does not make sense. Can the authors rephrase the relationship between neurogenesis and axonal regeneration?

Thanks. We have changed this sentence and also revised all the incorrect description in the whole manuscript.

Line 521: what was the survival rate of the larvae?

We performed the spinal cord injury carefully to keep the notochord and ventral tissues intact. In all the experiments, the average survival rate of the injured larvae was ~ 95% at 5dpi (Figure 2). We have mentioned this in the revised text.

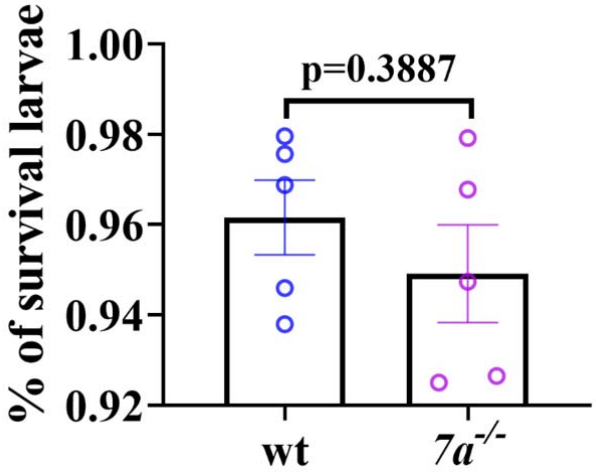

Figure 2 Survival rates of the injured larvae at 5dpi.
Each datapoint corresponds to an independent experiment.

M&M: cell counting methods are insufficiently specified. The region of interest choice is not clearly explained and methods to reduce observer bias have not been laid out.

We have added detailed description of the methods used for cell counting in the revised version. All the experiments were repeated at least three times and counted by different persons to reduce observer bias.

Referee #2:

In this study, Zhu et al. uncover a new role of Rassf7a during zebrafish spinal cord regeneration. After mapping rassf7a and rassf7b expression in developing zebrafish using in situ hybridization, the authors employ newly generated zebrafish with mutation in both rassf7 paralogues to define Rassf7 roles during growth and stress-induced conditions. First, as rassf7b expression resembles the one of genes involved in ciliogenesis, the authors investigate cilia morphology, which result to be unaffected by rassf7a and/or rassf7b expression. To define potential roles of Rassf7 during stress-induced conditions, the authors perform a series of experiments after subjecting zebrafish larvae to a spinal cord transection injury. In this condition, rassf7 mutant larvae displayed dorsal bending phenotype and reduced motility compared to wild types. Assays with larvae allowing fluorescent labeling of glial cells and neurons revealed that whereas loss of rassf7a impairs regeneration of neurons, rassf7a overexpression improves it. Effects on neuronal cells mostly derived from impaired proliferation of rassf7a-expressing neural progenitors, which could be rescued with FGF8 injections in mutant fish. To characterize the cellular basis of the observed proliferation defects, the authors imaged larvae carrying fluorescently labeled sox2-expressing progenitors. Rotation angles of spindles were increased in rassf7a mutant versus wild type cells, as also validated in cultured cells. The involvement of rassf7a in spindle orientation was further confirmed by transcriptomic analyses on rassf7a mutants, which showed reduction of spindle polarity components.

The involvement of rassf7a in spinal cord regeneration is novel and of potential interest. However, the rationale behind investigating the role of this gene versus other factors is unclear. The introduction could explain in a more concise way the importance of the question being asked and address the focus of the study more clearly. Despite the potential interest for the spinal cord regeneration community, some of the experiments are not performed and explained in the most rigorous manner.

Specific comments:

Variability in larval regeneration assays is extremely high and previous studies used high numbers of larvae to measure glial/axonal bridges (i.e. PMID: 28743881). In figure 2C only 7 larvae are assessed, which is a small number to make solid conclusions. Also, in the images shown in 2A the glial bridge appears to be thicker in mutants than in wild types. Are the images shown representative?

Thanks for pointing this out. All the experiments of these manuscript were repeated at least three times. The original panel in Fig 2C was generated through the results of one experiment (7 larvae were investigated until 7 dpi). In the revised version, we included all the data from 3 independent experiments (including 24 mutant larvae and 26 control larvae) and the results were shown in Fig 2C in the revised version.

The average fluorescence of glial cells at the lesion sites was also measured as illustrated in Fig 2B. We didn’t find significant difference in the rescue efficiency between mutant and control siblings (by Two-way ANOVA with Bonferroni's multiple comparisons test,

P=0.5986). As the reviewer suggest, we further measured the thickness of the glial bridge in the mutants at 5 and 7 dpi when the bridges were largely recovered. Surprisingly, we did find that mutant larvae exhibited slightly increased thickness at 5dpi, while no difference was observed at 7 dpi (Figure 3). In contrast, the axon bridges were obviously thinner in the mutant larvae at both 5 and 7 dpi (Fig EV1H-J, EV2 E, F). We hypothesize that the thicker glial bridge may be due to some feedback mechanisms in the mutants, which generated more glial bridges to balance the loss of axon bridges in the mutants. This need to be further investigated.

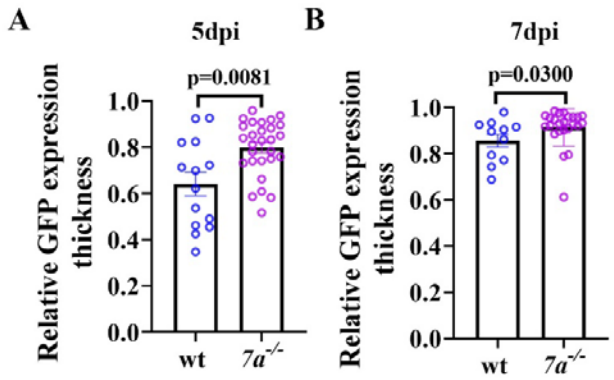

Figure 3 Relative thickness of glial bridges at 5 and 7 dpi as illustrated by GFP fluorescence.

I feel the experiments performed, although informative, are not enough to conclude that there is "axonal regeneration". The lesion site might be filled up with neurons, positive for HuC, rather than new axons growing and crossing the lesion. Quantification of axonal bridge thickness assessed using an anti-acetylated alpha tubulin staining would further strengthen the results. Is there a difference in the percentage of larvae with axonal and glial bridge between wild type and rassf7a mutant larvae?

Thanks. We totally agree with this reviewer's comments. To further confirm whether the regeneration of axons was defective in the mutants after SCI, and also following this reviewer's suggestion, we have quantified the thickness of axons at the injury site with anti-acetylated alpha tubulin antibody staining. The axonal bundles were obviously thinner in the mutants at 5 dpi in both straight and curved groups (Fig EV2E, F). Moreover, we also measured the thickness of the GFP fluorescence in Tg(*huc*:GFP) background. The thickness of GFP fluorescence was significantly reduced in the mutants at both 5 and 7 dpi (Fig EV1H-J). This data suggest that axonal regeneration is compromised in the mutants.

We also compared the initial axonal and glial processes at 1dpi. Both axonal and astroglia-like processes were present in the mutant and control larvae at 1dpi. By using anti-acetylated alpha tubulin antibody and GFAP labeling, we further examined the composition of axonal

and glial processes in the fascicles at the lesion sites. Similar to previous study(Wehner *et al.*, 2017), the majority of fascicles were composed of axonal-only bridges. The relative percentages of axonal-only, glial-only and mixed fascicles were similar between mutant and control larvae at 1dpi (Fig EV2A, B). In addition, we also measured the length of regrowing axons at the injury site, and did not find difference between wild-type and mutant larvae (Fig EV2D). These data suggest that the initial axonal and glial processes were not affected in *rassf7a* mutants after injury. However, we did find thinner axonal bundles at 5 dpi as previously mentioned (Fig EV2E, F). We think such regeneration defects of mutants are mainly caused by failure of the production of neurons from progenitor cells.

Images shown in Fig. 3A make it hard to visualize cells that were counted. There seems to be a lot of noise, higher magnification images and separate channels are needed to convince the reader about potential differences in proliferation. Also, the images shown don't seem to be representative: in the 3 dpi high-magnification view shown in Fig. 3A, HuC positive cells appear to proliferate more in mutants than in wild types, which is the opposite of what shown in the quantification.

Thanks for these suggestions. We have replaced Figure 3 with higher resolution images and also provided separate channels to better display the differences of BrdU labeling. These new data are now included in Fig 3, and the separate channels are now included in Fig EV3.

In Fig. 3F it is unclear if the curve representing the mutants is missing or the two curves completely overlap.

They are not completely overlap, however, these two sets of data are very close. We have changed the line color to make it easier to distinguish in the revised version.

From the ISH shown in Fig. 4 it is difficult to tell if sox2 and rassf7a co-localize. Ideally, one would want to see sections of a rassf7a reporter line crossed with a sox2 line (or sections stained with antibodies), as well as a DAPI signal, to clearly see both proteins in the same cell. The yellow dots in the figure might be signals from 2 cells overlapping. As an alternative, one could FACS purify sox2+ cells from the sox2 reporter line used in the study and perform qPCR to show increase in rassf7a in this cell population after injury.

Thanks for pointing out this issue. We agree with this reviewer that double FISH was not enough to confirm the colocalization between *rass7a* and *sox2* expression. We did generate multiple Rassf7a antibodies, unfortunately, none of these antibodies worked. Following this reviewer's suggestion, we have sorted the $Sox2^{+}$ and Huc^{+} cells from the Tg(*huc*:GFP) or Tg(*sox2*:sox2-2a-sfGFP) transgenic lines. qPCR results showed that expression of *rassf7a*

was significantly enriched in the Sox2^{+} expressing cells, while very low level was detected in Huc^{+} expressing cells (Fig 4P).

Second, we sorted Sox2^{+} cells at 10 hour and 3 days after injury. The expression of *rassf7a* in these Sox2^{+} cells was also increased significantly at 3 dpi, while no difference was observed at 10hpi (Fig 4Q, R). These results further suggested that Rassf7a is involved in the regeneration of Sox2^{+} cells at later stages.

Finally, we plotted the expression of *rassf7a* using two published single-cell transcriptomes (Cavone *et al*, 2021; Scott *et al*, 2021). The expression of *rassf7a*, albeit lower, is mainly colocalized with those clusters enriched with Sox2 or GFAP, i.e. the ERG progenitor cells (Fig EV4).

Altogether, we feel confident that Rassf7a is mainly expressed in the neural progenitor cells in zebrafish. These new data are now included in Fig 4P-R and Fig EV4.

Inhibition of Fgf signaling reduces HuC signal in wild types but not in rassf7a mutants, suggesting that rassf7 effect might be independent from Fgf signaling. Conversely, administration of Fgf has effects in rassf7a mutants but not wild types. This makes it hard to understand if rassf7a effect is dependent or independent from Fgf and needs a better explanation.

This is a good point. It has been shown earlier that Rassf7 may regulate Fgf signaling through binding to RAS protein (Wang *et al*, 2016; Zhang *et al*, 2018). In the absence of Rassf7a, activation of Fgf signaling might be weakened upon injury and ectopic Fgf proteins may somehow compensate such defects and promote spinal cord regeneration. In wild-type larvae, Fgf signaling has already been activated and overexpression of Fgf proteins may not have extra effects due to some feedback mechanisms. Moreover, the amount of proteins we injected was also much lower than previous reported (0.1 μg/injection/larvae vs 0.14 mg/injection/adult) (Yan *et al*, 2000) (Goldshmit *et al*, 2012), which may not sufficient to promote further regenerative process. In contrast, since spinal cord regeneration has already been significantly delayed in the mutants, further inhibition of Fgf signaling may not have additional effects in the mutants.

Considering the role of FGF signaling during spinal cord regeneration has been extensively studied, and also following the first reviewer's suggestion, we have removed this part to focus on the description of Rassf7a during mitotic division in the revised text.

For the imaging experiments in Tg(sox2:sox2-2a-sfGFP) fish shown in Fig.5 the authors employed morpholinos to reduce rassf7a levels. Morpholinos should be used with caution (Stainier et al., PLoS Genetics, 2017) and mutants are generally preferred when available. These results would be more accurate using available rassf7a mutants crossed with the sox2 reporter line.

Thanks. In the revised version, we have confirmed the efficiency of *rassf7a* MO via a reporter line (Fig S4A-C). Moreover, we have repeated these experiments in the mutant background as the reviewer suggested. We obtained 38 and 49 cell division events in the wild and mutant background respectively. Further analysis of these divisions showed that, similar to those of morphants, the division plane and division time was substantially changed in the mutants. We have included these data in Fig 5 in the revised manuscript.

Ideally, markers to track cell membranes and histone tagged nuclei should be used to visualize and measure angles of division, in rassf7a wild type and mutant fish. Is not clear how spindles were identified to perform quantifications. Also, how many cells were quantified and from how many larvae?

Yes, cell membrane and nuclei labeling is a better way to visualize cell division. We have tried to inject mRNA or even generated Tg(*sox2:memb-Dendra2-2A-H2B-cerulean*) transgenic line to imaging this process. Unfortunately, due to the fact that these neural progenitor cells localize to the center of the spinal cord and we need to image them at 4 or 5 dpf larvae, the signals were too weak to be constantly detectable in our hands. In contrast, the GFP fluorescence is very strong in the Tg(*sox2:sox2-2a-sfGFP*) transgene so that we can continue tracing cell division of these neural progenitor cells. We have repeated these experiments in the mutant background and exhibited better images to show cell division. Moreover, we also mentioned the number of cells and larvae investigated in the revised text. These data are included in Fig 5 and Fig 6 in the revised manuscript.

In the discussion the authors write: "fewer neurons were generated in the mutants, leading to final axonal regeneration defects". This is an overstatement, as axon growth has not been measured (see comment above).

Thanks, we have measured the thickness of axonal bundles in the lesion sites. As we mentioned previously, although the initial axonal and glial bridge formation were not affected, mutant larvae displayed thinner axonal bundles at 5 dpi.

For all assays shown, it is unclear how many times experiments were repeated.

All the experiments mentioned in this manuscript were repeated at least three times. We have mentioned this in the methods and also added the number of embryos investigated in each figure.

There are multiple mistakes in the text, which needs careful editing. For example:
Fig. 5D y-axis: edit "oritation" with "orientation"
Thank you for your correction, we have changed it in the revised manuscript.
Line 256: "control vivo Mos" remove "vivo".
Thank you for your correction, we have changed it in the revised manuscript.
Line 314: "singals" replace with "signals"
Thank you for your correction, we have changed it in the revised manuscript.
Line 375: Rassf7 has 3s
Thank you for your correction, we have changed it in the revised manuscript.
Fig.S10 legend: edit "spina" with "spinal"
Thank you for your correction, we have changed it in the revised manuscript.
Line 425: "in consistent with this" edit "in agreement with this"
Thank you for your correction, we have changed it in the revised manuscript.
Line 433: "asymmetri "edit with "asymmetric"
Thank you for your correction, we have changed it in the revised manuscript.
Line 447: "were" with "are"
Thank you for your correction, we have changed it in the revised manuscript.

Thanks. We have changed all these mistakes.

Referee #3:

In their study « Rassf7a regulates spinal cord regeneration through modulating spindle orientation in neural progenitor cells » the authors characterise the function of rass7a & b in zebrafish development. While the initial working hypothesis of the study is that rassf7 proteins may be required for ciliogenesis, the authors show that rassf7 is dispensable for cilia formation and animal viability under normal conditions.
In contrast, rassf7a appears promotes regeneration upon spinal cord injury by regulating the division orientation of neural progenitor cells. In the absence of rassf7a, cells switch from a perpendicular asymmetric mode of division (that would generate one neuronal progenitor and one differentiating neuron) to a planar symmetric orientation that generates two progenitors and thereby impairs the generation of neurons allowing spinal cord regeneration. While implications of Rassf7 in cell division have already been reported (e.g. Sherwood & al 2008, Recino & al 2010, Gulsen & al 2016), the identification of rassf7a as a new regulator of spinal cord regeneration is interesting, even if mechanistic insights into how rassf7 might regulate division orientation are unfortunately lacking. While the findings of this work are potentially interesting, I do however believe that a number of question still need to be addressed.

Major questions :

- I am not convinced by the authors statement (line 232) that « In most cases, regenerated larvae with severe dorsal bending failed to react to tail touch stimulus ». In Movie S2 with severely bent embryos, most embryos DO react to the stimulus, they just fail to elicit a proper escape response as they are unable to swim straight due to their curvature. The same applies to Etho Vision experiment displayed in Fig.1G,H. This is an important point, as it raises the question of the actual success (or not) of functional spinal cord regeneration.

Thanks for pointing out this issue. We do notice that the swimming distance is corelated with the body curvature defects. To better explain this, we performed a sub-analysis of larvae with or without dorsal bending phenotype. Those larvae with straight body axis did have a better swimming capability than those of curly larvae in both wild-type and mutant groups at 6 dpi. Noticeably, the swimming capability was significantly inhibited in both straight and curly mutant larvae than those of wild-type control, suggesting that even the body axis was recovered, the swimming capability was still affected in the mutant groups (Fig EV1E-G).

Moreover, we further compared the thickness of GFP fluorescence between wild-type and mutant larvae carrying Tg(*huc*:GFP) transgene at 5 and 7 dpi. The width of GFP signals at the lesion site were also significantly thinner in both straight and curly groups of the mutant larvae (Fig EV1H-J). These results were also further confirmed via anti-acetylated tubulin antibody staining to visualize the axons at the lesion sites (Fig EV2E, F).

Altogether, these data suggested that mutant larvae may recover the ability of touch response, while the swimming capability was significantly affected. In the revised version, we have changed the description about touch stimulus and included these data in Fig EV1 and Fig EV2.

- The authors demonstrate convincingly that rassf7a mutants present a reduction in the proliferation of huC-positive cells, while gfap-positive radial glia appears unaffected. A major question is why rassf7b would specifically affect the division of a given cell type? Is rassf7b actually only expressed in one of the two cell types?

We think expression of *rassf7a* is mainly enriched in the neural progenitor cells or radial glial cells that are positive for Sox2 and GFAP. Rassf7a deficiency causes abnormal proliferation of these cells and leads to the number of HuC-positive neurons decreased during regeneration. The GFAP cells we counted include both radial glia and glia cells at the lesion sites. We hypothesize that glial cells may be differentiated from multiple cell types, which can compensate the abnormal proliferation of radial glia cells.

Right now, the resolution of the RNA in situs shown in Fig.4 is not sufficient to really address this question. Better data are needed to substantiate the point that « rassf7a was mainly enriched in the neural progenitor cells » (as opposed to radial glia). Another important experiment would be to use Rassf7a-GFP to follow protein localization in symmetric as well as asymmetric divisions.

Thanks. This question has also been mentioned by reviewer 2. To further confirm this, we have performed FACs experiments to sort $Sox2^+$ and Huc^+ cells and qPCR results suggested that expression of *rassf7a* was enriched in $Sox2^+$, but not Huc^+ expressing cells. Second, the expression of *rass7a* was significantly increased in $Sox2^+$ cells after injury. Finally, we plotted the expression of *rassf7a* using two published single-cell transcriptomes (Cavone *et al.*, 2021; Scott *et al.*, 2021). The expression of *rassf7a*, albeit lower, is mainly colocalized with those clusters enriched with Sox2 or GFAP, i.e. the neural progenitor cells. Altogether, we feel confident that Rassf7a is mainly expressed in the neural progenitor cells in zebrafish. These new data are now included in Fig 4P-R.

As for the localization of Rassf7a, we injected Rassf7a-mCherry mRNA into zebrafish embryos and checked the localization of this protein during cell division of neural progenitor cells. Our results showed that Rassf7a protein was enriched at both ends of the spindle during cell division. The new data are now shown in Movie EV9.

- The major finding of the paper is the observation that rassf7a controls cell division orientation. In Fig.6, the authors present evidence for a change in spindle rotation that appears however relatively minor with most cells still rotating by the normal 0-45(Yan *et al.*) (Fig.6B). In Fig.7, they do however show a very dramatic switch from a mostly perpendicular to a mostly planar division orientation (Fig.7B). It is not clear to me whether and how these two aspects of division orientation are linked? One caveat of the current experiments is that they are solely based on the use of rassf7a morphants. As these experiments concern the main finding of the paper, they need to be confirmed using the genetic mutants that the authors have generated.

In zebrafish, rotation of the mitotic spindle is a common phenomenon that is observed during the division of neuroepithelial cells (Kimmel *et al*, 1994) (Concha & Adams, 1998; Geldmacher-Voss *et al*, 2003). The rotation angles, i.e. the angles between the initial position of the spindle axes at the metaphase and final dividing position at the anaphase, are relatively smaller (mostly between 0 and 45 degree) in wild-type larvae. In contrast, we constantly observed dramatic rotations of the spindle axes in the mutants after SCI (Fig 5).

In Fig 6, the final spindle division angles were measured between the spindle axes and the anterior-posterior axis at the anaphase stage to distinguish planar and perpendicular division. We found that the perpendicular division events were significantly inhibited, concomitant with high incidence of planar division in the absence of Rassf7a after SCI.

Finally, to further confirm the specificity of Rass7a, we repeated these experiments in *rassf7a* mutant background. Similar to those of *rassf7a* morphants, the division plane was also significantly affected in the mutants. In the revised manuscript, we provided these data in Fig 5 and 6.

- A further important issue is that while it appears indeed plausible that the switch from perpendicular to planar divisions promotes the production of neuronal progenitors at the expanse of differentiating neurons, there is presently no direct evidence for this hypothesis. More clearcut evidence would be required here as it concerns the main finding of the study.

We agree with this reviewer. This is one of the key hypothesis to explain the regeneration defects in *rassf7a* mutants. During the development of CNS, it is well known that asymmetric cell division is essential to generate neurons while also maintaining the pool of neural progenitors. Several studies have also confirmed that neurons can be derived from the more apical daughter cells from the perpendicular division in zebrafish (Alexandre *et al*, 2010; Bultje *et al*, 2009; Dong *et al*, 2012). To further confirm this, we microinjected *H2B-GFP*

mRNA into the Tg(*huc:gal4;uas-mcherry*) double transgenic embryos at one cell stage. Of the eight cells undergoing planar division, we did not find any red fluorescence signals in the daughter cells. In contrast, red fluorescence signals were detected in the daughter cells of 3 out of 8 cells undergoing perpendicular division. Moreover, in one case, we also found that mCherry proteins are expressed in both daughter cells differentiated from a vertically divided cell. These data showed that neurons are prone to be generated from asymmetric perpendicular division. We have showed these data in Movie EV6, EV7 and EV8.

Additional points:

- The authors describe the alterations of rassf7a & b mutations at the DNA level (-4 & -7 bp deletions) but do not provide evidence whether these changes indeed induce the expected changes at the level of the mutant transcripts, or cause unexpected changes, e.g. due to exon skipping. Mutant transcripts should be entirely sequenced and their NMD-induced change in expression levels confirmed by qPCR. As both mutants are homozygous viable, these experiments should be straightforward.

Thanks. Following this reviewer's suggestion, we have sequenced the mutant transcripts and confirmed the changes of the mRNA sequence, same as the predicted sequence from genomic DNA. Moreover, we also performed PCR analysis to compare the expression *rassf7a* and *rassf7b* between mutant and wild-type larvae. We have included these data in Fig S2B-C.

- The authors mention that rassf7a or b single mutants are viable and fertile. What about double mutants?

Of a total of 263 adults crossed between double heterozygote mutants, we identified 15 double homozygous mutants, including 7 females and 8 males. The percentages of double homozygous mutants matched the Mendelian genetic laws. Moreover, we did not find any fertility defects of these mutants. We think it is very likely that loss of these Rassf7 proteins does not affect the viability and fertility of zebrafish.

- The authors repeatedly talk about « proliferating neuron cells » (e.g. line 273), a terminology that is incorrect as neurons are postmitotic. The term « proliferating neural progenitors » should be used instead.

Thanks for pointing out this issue. We have checked the whole manuscript and changed these incorrect descriptions.

- Fluorescent rassf7a in situ hybridization should be perfomed in WT vs rassf7a mutant embryos to provide a specificity control for the data shown in Fig.4 & S8.

We have repeated the fluorescent *in situ* hybridization experiments in both wild-type and *rassf7a* mutants. Clearly, the fluorescent signals of *rassf7a* gene were substantially decreased in the mutants due to NMD. These new data are now shown in Fig S5.

- For many experiments the authors indicate individual data points rather than just mean values which is definitely a good thing. However, numerical values rather then */**/*** should be use to indicate p-values. Have the authors systematically test for data normality and variance? For example, the data in Fig.1I,J are mentioned as having been analyzed using t-tests, but I wonder whether there is really a normal data distribution that allows this?

Thanks for these suggestions. In most figures, we performed unpaired t-tests to compare the difference between two samples. In cases of 3 or more samples, we used ANOVA tests for analysis. We checked the normality of the data distribution in Fig 1I and J using Shapiro-Wilk tests. As the reviewer concerned, these data were not following normal distribution and we analyzed it by nonparametric Mann-Whitney test. We rechecked all the statistical analysis in the whole manuscript and added the detailed methods used for statistical analysis in each figure legend. We summarized all the statistical analysis for each experiment in supplementary Table S2. Finally, we have added *P*-values to replace the asterisks in the revised version,.

- The photos in Fig3A are highly pixelated, making it impossible to distinguish the GFP and BrdU signals. Seperate channels should be shown as in Fig.3G.

We have replaced these figure panels with higher resolution images. We have also provided separate channels to show potential differences in proliferation. These new data are now shown in Fig 3A and Fig EV3.

- Changes in sox2 & msi1 expression upon SU5402 should be quantified by qPCR.

Thanks. As we previously mentioned, we have removed these experiments regarding FGF signals in the revised text.

Reference

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Dear Dr. Zhao,

Thank you for the submission of your revised manuscript. We have now received the enclosed reports from the referees and I am happy to say that all support its publication now. We can therefore in principle accept it. Please address the last comments from referee 1, and a few editorial requests will also need to be addressed:

- Please add up to 5 keywords to the ms file.

- Please correct the conflict of interest subheading to "Disclosure and Competing Interest Statement"

- Please remove the author credits from the ms file. We now use CRediT to specify the contributions of each author in the journal submission system. CRediT replaces the author contribution section. Please use the free text box to provide more detailed descriptions, if you wish. See also our guide to authors https://www.embopress.org/page/journal/14693178/authorguide#authorshipguidelines.

- Please correct the reference format to the EMBO reports (Harvard) style. Not more than 10 authors may be listed.

- Figure panel callouts are missing for Fig EV4 and Appendix Fig S3, please add.

- The suppl. Materials and suppl. Tables files need to be merged into one single PDF file, with a table of content (with page numbers) added, and the nomenclature corrected to "Appendix Figure S1" etc. and "Appendix Table S1".

- There is only 1 panel in Fig EV3 and Fig S6 so "A" can be removed in the figures, legends and callouts.

- I attach to this email a related ms file with comments by our data editors. Please address all comments in the final manuscript.

I would like to suggest some minor changes to the title and the abstract that needs to be written in present tense. Please let me know whether you agree with the following and whether all claims are correct:

Rassf7a promotes spinal cord regeneration and controls spindle orientation in neural progenitor cells

Spinal cord injury (SCI) can cause long-lasting disability in mammals due to the lack of axonal regrowth together with the inability to reinitiate spinal neurogenesis at the injury site. Deciphering the mechanisms that regulate the proliferation and differentiation of neural progenitor cells is critical for understanding spinal neurogenesis after injury. Compared with mammals, zebrafish show a remarkable capability of spinal cord regeneration. Here we show that Rassf7a, a member of the Ras-association domain family, promotes spinal cord regeneration after injury. Zebrafish larvae harboring a rassf7a mutation show spinal cord regeneration and spinal neurogenesis defects [OK?]. Live imaging shows abnormal asymmetric neurogenic divisions and spindle orientation defects in mutant neural progenitor cells. In line with this, the expression of rassf7a is enriched in neural progenitor cells. Subcellular analysis shows that Rassf7a localizes to the centrosome and is essential for cell cycle progression. Our data indicate a role for Rassf7a in modulating spindle orientation and the proliferation of neural progenitor cells after spinal cord injury.

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I look forward to seeing a final version of your manuscript as soon as possible. Please use this link to submit your revision: https://embor.msubmit.net/cgi-bin/main.plex

Best regards,
Esther

Esther Schnapp, PhD
Senior Editor
EMBO reports

Referee #1:

The authors are to be congratulated on very thorough and thoughtful revisions. As far as I can see, all of my points have been

satisfactorily addressed. I have only one important point and a few language suggestions:

Important: In figure 2A, the authors should carefully examine the 1 and 3 day post injury panels of the HuC:GFP/7a-/- larva. The two panels show an unlikely similarity, given that the 3dpf time point is 48 hours later than the 1 dpf time point.

Line 82: growth (instead of migration) process
Line 240: could not (instead of can not)
Line 273: with a reporter construct (not line)
Line 702: to trace (not tracing)
Line 730: otherwise were (not will be)

Referee #2:

The authors have properly revised the paper and addressed all my concern. I therefore support publication of the manuscript in EMBO reports.

Referee #3:

In the revised version of their manuscript the authors have addressed the majority of my concerns and very clearly inmproved the quality of the presented study. I am therefore supportive of the publication of this study in its present form.

The authors have addressed all minor editorial requests.

Chengtian Zhao
Ocean University of China
Institute of Evolution and Marine Biodiversity and College of Marine Biology
5 Yushan Road
Qingdao 266003
China

Dear Dr. Zhao,

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

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Esther Schnapp, PhD
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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.
- ideally, figure panels should include only measurements that are directly comparable to each other and obtained with the same assay.
- plots 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. Any statistical test employed should be justified.
- Source Data should be included to report the data underlying figures according to the guidelines set out in the authorship guidelines on Data Presentation.

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

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- 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.).
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